

GlaxoSmithKline Pharmaceuticals, King of Prussia, PA 19406-0939.

Abbreviations: Ach, acetylcholine; Cch, carbachol; mAChR, CHO, chinese hamster ovary; COPD, chronic obstructive pulmonary disease; CRC, concentration-response curve; DMEM/F12, Dulbecco’s Modified Eagle Medium nutrient mixture F-12; DMSO, dimethylsulfoxide; DR, dose ratio; FLIPR, fluorometric imaging plate reader; hERG, human ether-a-go-go-related gene; KH, Krebs’ Henseleit; KRH, Krebs’ Ringers Henseleit; MEM, mimimum essential medium; muscarinic acetylcholine receptor; M1-M5, muscarinic subtype 1-5; ON t1/2, time to 50% of maximal relaxation; OFF t1/2, offset half-time of tension recovery; Penh, enhanced pause; USAN, United States adopted name; WHO, world health organization.
GSK573719: A novel inhaled muscarinic cholinergic antagonist

*To whom correspondence should be addressed:

William L. Rumsey, Ph.D.,

709 Swedeland Road,

King of Prussia, PA 19406-0935

Tel: 610 270 4474

Email: Bill.2.Rumsey@gsk.com

Number of text pages = 18

Number of Tables = 2

Number of figures = 8

Number of references = 36

Number of words in Abstract = 250

Number of words in Introduction = 516

Number of words in Discussion = 1960

Preferred Section: Pulmonary
Abstract

Activation of M3 muscarinic cholinergic receptors (mAChR) 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) of GSK573719 for the cloned human M1-M5 mAChRs ranged from 0.05-0.16 nM. Dissociation of [3H]-GSK573719 from the M3 mAChR was slower than that for the M2 mAChR; t1/2 values = 82 and 9 min, respectively. In CHO cells transfected with recombinant human M3 mAChRs, GSK573719 demonstrated pM potency (–log pA2 = 23.9 pM) in an acetylcholine (Ach)-mediated Ca2+ mobilization assay. Concentration-response curves indicate competitive antagonism with partial reversibility after drug wash-out. Using isolated human bronchial strips, GSK573719 was also potent and showed competitive antagonism (–log pA2 = 316 pM) vs 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 min (vs 413 min for tiotropium bromide). In mice, the ED50 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 h. 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 24 h duration of bronchodilation in vivo which suggested umeclidinium will be a once-daily inhaled treatment for 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 provide sufficient oxygenation of extra-pulmonary 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 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 post-ganglionic fibers innervating smaller diameter bronchioles that are largely responsible for establishing resistance for airflow from the atmosphere to the gas exchange units (for review refer to Canning and Fisher, 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 non-adrenergic non-cholinergic fibers affect 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 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 (Coulson and Fryer, 2003, Eglen et al., 1996). Modulation of the release of Ach is also influenced by M2 mACHRs located on the pre-junctional 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 rise in intracellular calcium and enhancement of airway smooth muscle contraction (see for example Chilvers et al., 1990).
Bronchodilator therapy has become the mainstay for controlling symptoms brought about by enhanced parasympathetic nerve activity in patients with bronchoconstrictive pulmonary diseases like 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, anti-muscarinic cholinergic antagonists have been sought for the management of disease to maintain airway caliber for adequate gas exchange (Busch-Petersen et al., 2011). To promote adherence to therapy, those agents with long duration of action are more desirable and to avoid systemic effects of these medicines, administration has benefitted these patients by inhalation of the compound. This study was conducted to characterize the pre-clinical pharmacology of GSK573719 (WHO and USAN 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.
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 prepared according to previous methods (Allard et al., 1987; Bonner et al., 1988; Chapman and Browne, 1990; Peralta et al., 1987). The cells were grown to confluence at 37°C in a humidified incubator gassed with 5% CO₂/95% O₂. The M2 and M4 mAChRs were co-expressed with the chimeric G protein, Gqi5 (Dong et al., 1995). CHO cells containing M1, M3, or M5 mAChR were cultured in Alpha MEM with nucleosides, L-glutamine and 10% fetal calf serum whereas those expressing M2 and M4 mAChRs were cultured in DMEM/F12 media supplemented with 200 mg/L G418 (geneticin) and 10% fetal calf serum. Membranes were prepared by centrifugation (1,000 g for 10 min at 4°C), washing the cell pellet with Phosphate Buffered Saline followed by rapid freezing using liquid N₂ and storage at -80°C (Rominger et al., 2009). The frozen pellet was thawed, resuspended in cold hypotonic medium (40 mM Tris, pH 7.5, 1 mM MgSO₄, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2.5 mg/l leupeptin, 0.1 mg/ml aprotinin) and incubated on ice for 5 min. The suspension was homogenized, centrifuged (2,000 rpm X 6 min at 4°C) and the pellet resuspended. This procedure was repeated twice and the supernatants were collected for centrifugation (100,000 g X 60 min 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 (SPA) for M1, M2 and M3 mAChRs and a filtration assay for M4 and M5 mAChR. For the SPA assay, membranes were incubated with wheatgerm agglutinin beads in 50 mM N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid (HEPES) buffer, pH 7.4 at 4°C for 30 min and then with the radioligand in a 96-well Optiplate (PerkinElmer, Waltham, MA) for 2 hr at room temperature in the presence of vehicle (1% DMSO) or GSK573719 (0.01-300 nM). At the end of the incubation, the plates were centrifuged (Beckman CS-6R; for 5 min at 2,000 RPM) and radioactivity was counted (Top Count, Model A9912 Packard, Meriden CT). For the filtration assay, membranes (M4 and M5) were similarly
incubated in HEPES buffer containing the radioligand for 2 hr at room temperature in the presence of vehicle (1% DMSO) or GSK573719 (0.03-300 nM). Atropine was used a reference agent. Reactions were terminated by rapid filtration (Brandel Cell Harvester, Gaithersburg, MD) through GF/C filters. Membranes were washed with ice cold 50 mM HEPES and transferred to scintillation vials. Radioactivity was counted in a Scintillation Counter (Beckman model LS6500, Fullerton, CA). Reactions were terminated by rapid filtration as described above. Data were obtained from three independent experiments. Specific binding was determined by subtracting non-specific 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_d + 1; K_d = 0.17, 0.28, 0.16, 0.07 and 0.2 nM for M1-M5 mAChR, respectively and [L] is the assay concentration of radioligand. Membranes containing M3 mACHRs were also incubated for 2 hr 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. Non-specific binding was determined using 10 μM atropine. The saturation data was 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-deep well plates 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 non-specific binding and specific binding. After varying incubation periods, binding was terminated by rapid vacuum filtration (Brandel Inc. Gaithersburg, MD, USA). The filtrate was collected on GF/B filter papers pre-soaked in 0.3% v/v poly-ethylenimine, washed three times with ice cold medium and the filters were transferred into vials containing Ultima-Flo™ M (PerkinElmer, Beaconsfield, UK) and analysed using a TriCarb 2900 TR LS counter (PerkinElmer LAS UK Ltd., Beaconsfield, UK). Specific binding of [3H]-GSK573719 or [3H]-tiotropium was not found on cells lacking muscarinic receptors (data not shown). 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 h. 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 h. Rates of dissociation of radioligand ([3H]-GSK573719 ~0.1 nM or [3H]-tiotropium ~0.2 nM) were determined after
incubation membranes for 1 h, diluted (1:20) with medium containing 10 µM atropine and then incubated for varying times up to 24 h 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 $k_{off}$ values and estimates of dissociation half-lives ($t_{1/2}$). $K_D$ values were also calculated from $k_{on}$ and $k_{off}$ values using the equation $K_D = k_{off}/k_{on}$.

Potency measurements of GSK573719 for receptors or sites other than mAChRs were conducted by CEREP laboratories (Poitiers, FR).

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 FLIPR assay (Fluorometric Imaging Plate Reader, Molecular Devices, Sunnyvale CA, Schroeder and Neagle, 1996). CHO cells were placed in 96 well plates (40,000 cells/well, Packard View) for 24 hrs and then the media was replaced with Eagle's Minimal Essential Medium (EMEM) containing Earl's salts, L-Glutamine, 0.1% bovine serum albumin (Sigma A-788), 4 µM Fluo-4-acetoxyethyl ester fluorescent indicator dye (Fluo-4 AM, Molecular Probes, Grand Island NY), and 2.5 mM probenecid for 1 hr at 37°C. This medium was replaced with a similar one minus the dye and 0.1% gelatin instead of albumin. After 10 min, the cells are washed (3X) with Krebs' Ringers Henseleit (KRH) buffer (in mM) NaCl (120), KCl (4.6), KH$_2$PO$_4$ (1.0), NaHCO$_3$ (25), CaCl$_2$ (1.0), MgCl$_2$ (1.1), glucose (11), HEPES (20), 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 sec and analyzed using commercially available software (Prism 5.0, GraphPad Software, San Diego, CA). Potency and mode of receptor interaction were determined by classical Schild analysis (Schild, 1949) using calculations of $EC_{50}$ (half-maximal calcium response) or pA2 values where; pA2= log (DR-1) –log[B], DR was the dose ratio defined as the ratio of equi-active concentration ($EC_{50}$) of agonist in 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 they were exposed to the compound and then washed (3X in 30 min intervals) with KRH 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 rise in intracellular calcium. The antagonist exposure period was 30 min in all cases whereas the duration of clearance was 180 min; the exception was for tiotropium (90 min in most experiments and 180 min 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 (NDRI, Philadelphia, PA). The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents. Sections of bronchus were removed from the lung and cleaned of adherent parenchyma. Bronchial strips (paired; 3-4 mm in width) were suspended (resting tension = 1.5 g) in 10-ml water-jacketed organ baths (37°C) containing Krebs-Henseleit (KH) solution containing (in mM): NaCl (113.0), KCl (4.8), CaCl₂ (2.5), KH₂PO₄ (1.2), MgSO₄ (1.2), NaHCO₃ (25.0) and dextrose (11.0) gassed with 95% O₂: 5% CO₂ and connected to Grass FT03C force-displacement transducers. Meclofenamic acid (1 μM) was added to block endogenous cycloxygenase activity. Isometric responses were recorded using a commercially available data acquisition system (MP100WS/Acknowledge; BIOPAC Systems, Goleta, CA) interfaced with a Macintosh G4 computer (Apple, Cupertino, CA). After a one hr equilibration period pulmonary tissues were contracted with Ach (1 mM) until reaching a sustained response to examine tissue viability. The average Ach-induced rise in tension was 0.84 ± 0.09 g (n = 33). Paired tissues were then rinsed at 15 min intervals to re-establish baseline tone. Tissues were then exposed to mAChR antagonists or vehicle for 120 min before cumulative addition of carbachol to obtain concentration-response curves, CRCs (Muccitelli et al., 2000; Van Rossum, (1963). In the absence of antagonist, the average cumulative tension attained was 1.58 ± 0.14 g (n = 33) with no significant difference among the treatment groups. Agonist-induced responses were expressed as a percentage of the response to the reference agonist, histamine (1 mM), obtained at the end of the experiment. Mean pEC₅₀ and geometric mean EC₅₀ values were calculated from nonlinear regression analyses (Motulsky and Christopoulos, 2003). When appropriate, potency values of the antagonists were expressed as pK₈.
and \( pA_2 \) (Arunlakshana and Schild, 1959) where; \( pK_B = -\log [\text{antagonist}]/X-1 \), \( X \) is the ratio of agonist concentration required to elicit 50% of the maximal contraction in the presence of the antagonist compared with that in its absence and \( pA_2 = -\log \) of the antagonist dissociation constant. Analysis of \( pA_2 \) values was accomplished using global fitting of the data (Motulsky and Christopoulus, 2003).

In separate experiments, human bronchial strips were similarly suspended in superfusion chambers (Coleman and Nials, 1989; Harvard Apparatus, Inc., Holliston, MA connected to BIOPAC TSD125C transducers). These tissues, however, were continuously superfused (2 ml/min) with KH solution throughout the experiment while agonists and antagonists were infused (0.02 ml/min) via a 22-gauge needle inserted into the superfusate in contact with the tissue. After a one hr equilibration period, the tissues were continuously exposed to carbachol (1 \( \mu M \)) until the conclusion of the study when reference data were collected. Upon reaching a sustained contraction, isoproterenol (10 \( \mu M \)) was administered to reference maximal relaxation and stopped to allow restoration of the carbachol-induced tension. Muscarinic antagonists were infused at a single concentration per tissue until reaching a sustained relaxation, about 6 hr to ensure a state of equilibrium at the lower antagonist concentrations, and tissues were rinsed of drug for 10 hr. At this time, carbachol infusion was stopped to permit recovery of basal tone and then a carbachol CRC was obtained, followed by a 1 mM histamine-induced reference contraction. Figure 1 shows the experimental paradigm used in these studies to measure the time to 50% of maximal relaxation produced in the presence of the mAChR antagonists, referred to as ON \( t_{1/2} \), and upon removal of drug, i.e., their \textit{ex vivo} duration of effect or the offset halftime 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, tracheal strips (2 cartilage rings) were prepared and similarly evaluated.

Airway Mechanics: Mice were pretreated intranasally (50 \( \mu l/mouse \)) with vehicle (0.9% saline) or GSK573719 at intervals (0.25–48 hrs) 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 (Penh) values were collected, the mice received methacholine (30 mg/ml or \( EC_{80} \)) by aerosol delivery (flow = 1.6 ml/min X 2 min; DeVilbiss Model 5500D, Ultrasonic
An average Penh was then calculated for 5 min. Enhanced pause (Penh) = [(expiratory time / relaxation time)-1] x (peak expiratory flow / peak inspiratory flow) and relaxation time is the amount of time required for 70% of the tidal volume to be expired. In some cases, animals were treated on multiple, consecutive days as described in the figure legends. The data were expressed as the mean ± SEM percent inhibition of Penh or (mean Penh value of vehicle treated group - Penh each drug treated animal) divided by (mean Penh value of vehicle treated group) x 100%. Data were analyzed using commercially available software (GraphPad Prism 3.0, San Diego, CA).

In Guinea pigs (600-800 g), mAChR antagonists or vehicle (0.5% Tween) were administered intratracheally by instillation (200 μl) under anesthesia (5% isofluorane). The animals were then monitored using plethysmographic methods as described above for 10 min (area under the curve) after receiving aerosol administration of ACh (3.5 mg/ml X 36 sec). In other experiments, the animals were re-anaesthetized (ketamine 90 mg/kg, xylazine 15 mg/kg intramuscular) after 4 hr for cannulation of the jugular vein, carotid artery and trachea for drug delivery, blood pressure recording and ventilation of the animal (pressure = 8 cm H₂O, 60 breaths/min, Harvard Apparatus, Model 683, South Natick, MA). Succinylcholine was administered (2.0 mg/kg iv) to paralyze the animal. Resistance and dynamic compliance were measured throughout the experiment (Diamond and O'Donnell, 1977; Palecek, 1969). Once the animal was stable, Ach (10-100 μg/kg iv) was administered. Alternate studies utilized whole body plethysmography (Buxco XA, Buxco Electronics, Wilmington, NC) 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 X 36 sec followed by a 2 minute drying time, Delvibiss Pulmosonic 5000D, Somerset, PA). 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 ANOVA or Student’s t test using commercially available software (GraphPad Prism, San Diego, CA) with a minimum level of significance established at p < 0.05.

Animals: All studies were conducted in accordance with the GSK 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, St. Constance, Canada) were allowed free access to food and water.

Chemicals: The mAChR antagonist, GSK573719 4-[hydroxy(diphenyl)methyl]-1-{2-[(phenylmethyl)oxy]ethyl}-1-azoniabicyclo[2.2.2]octane (Figure 2) was prepared as the crystalline bromine salt. This compound and other muscarinic antagonists, 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. $[^3]H$-GSK573719 and $[^3]H$-tiotropium (specific activity 43 and 82 Ci/mM respectively) were synthesized by Quotient Bioresearch (Radiochemicals) Ltd. (Cardiff, UK). Scopolamine methyl chloride, [N-methyl-$^3$H], NET-636 was purchased from Perkin Elmer. Chinese hamster ovary cells (CHO) were purchased from American Type Culture Collection. Eagles 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 (K\textsubscript{i}) for GSK573719 obtained using membranes prepared from CHO cells stably expressing the individual five recombinant human mAChRs receptors are displayed in Table 1. The values, (in pM and corresponding negative log value pKi) obtained under steady state conditions using [\textsuperscript{3}H]-N-methyl-scopolamine as the competing ligand are: M1 = 159 (9.8), M2 = 151 (9.8), M3 = 62 (10.2), M4 = 50 (10.3) and M5 = 131 (9.9). Saturation binding of [\textsuperscript{3}H]-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\textsubscript{max} = 5.75 pmol/ml). The dissociation constant, K\textsubscript{D}, rose with each incremental change of [GSK573719].

Additional saturation, association, and dissociation binding studies were performed using [\textsuperscript{3}H]-GSK573719, and for comparison [\textsuperscript{3}H]-tiotropium, to determine receptor binding kinetics at the M2 and M3 mAChRs. The values (equilibrium dissociation constant, K\textsubscript{D}, total number of receptors, B\textsubscript{max}, association rate (k\textsubscript{on}), and dissociation rate, k\textsubscript{off}) are also shown in Table 1. The data indicate that association was rapid for both compounds at each receptor type. [\textsuperscript{3}H]-GSK573719 dissociates from the M3 mAChR more slowly, about 8-fold, than from the M2 mAChR (82 vs 9 min, respectively). This difference in dissociation between receptor sub-type was also found for [\textsuperscript{3}H]-tiotropium, although the time to reach 50% receptor occupancy, t\textsubscript{1/2}, was longer than that for GSK573719. Of note is the finding that [\textsuperscript{3}H]-GSK573719 dissociates from the M2 mAChR more readily (about 4-fold) than does [\textsuperscript{3}H]-tiotropium. The affinity values, pK\textsubscript{D}, for the M3 and M2 mAChRs in these saturation studies showed marginally wider separation for [\textsuperscript{3}H]-GSK573719 (10.5 vs 9.8) than for [\textsuperscript{3}H]-tiotropium (10.7 vs 10.3), respectively.

Evaluation of the selectivity of GSK573719 using a standardized panel of receptors and channels suggested that it is not likely to produce biological effects unrelated to acetylcholine or mAChR activity. Affinity values (K\textsubscript{i}) for those proteins with the most avid binding are: \kappa and \sigma opioid receptors (69 and 220 nM, respectively), L-type Ca\textsuperscript{++} channel (330 nM), site 2 Na\textsuperscript{+} channel (170 nM) and dopamine transporter (780 nM). In HEK-293 cells, GSK573719 inhibited the hERG channel tail current in a concentration-dependent manner, IC\textsubscript{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 concentration-response curve (CRC) obtained with Ach was shifted rightward in the presence of GSK573719 (1-1000 nM) in a concentration-dependent manner (see Figure 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-M3 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 Figure 3) as well as for the M1 and M2 mACHRs (slope = 0.83 and 0.93, respectively, data not shown).

Additional cellular studies showed that the effects of GSK573719 on the M3 mACHR-mediated activity were slowly reversible. In Figure 4, it can be seen that pre-treatment of cells with GSK573719 (3.3-330 nM for 30 min) produced rightward displacement of the Ach CRC comparable to that obtained previously and when followed by extensive washing for 180 min 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 to 1 μM) inhibited the Ach-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.

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 above 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 to those obtained for ipratropium, tiotropium and atropine by using paired bronchial strips excised from the same donors (Figure 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 rise in tension was 1.59 ± 0.14 g). The changes in contractile activity were concentration-dependently blocked by pre-incubation (120 min) 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 (1nM) 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 vs time and
this was compared to ipratropium and tiotropium (see Figure 6 for data using 10 nM) and atropine as an additional reference (data not shown). The time to 50% of maximal relaxation (termed ON \( t_{1/2} \)) for GSK573719 was concentration-dependent and decreased in value as the concentration of antagonist was increased. At 1 nM, the measured ON \( t_{1/2} \) (mean \( \pm \) S.E.M.) was 102 \( \pm \) 10 (n = 3) min whereas at 10 or 100 nM, it decreased to 43 \( \pm \) 5 (n = 5) and 20 \( \pm \) 2 (n = 3) min, respectively. Similarly, concentration-dependent ON \( t_{1/2} \) values were obtained for ipratropium; 19 \( \pm \) 1 (n = 3), 14 \( \pm \) 3 (n = 5), and 6 \( \pm \) 0.4 (n = 3) min, and tiotropium; 24 \( \pm \) 2 (n = 3), 10 \( \pm \) 1 (n = 7), 6 \( \pm \) 0.1 (n = 3) min, respectively (10 nM atropine = 20 min, n=2 for paired tissues, historical reference = 9 \( \pm \) 1 min, n = 17). When these tissues were washed free of GSK573719, the OFF \( t_{1/2} \) (with confidence interval range) was also concentration-dependent. At 1 nM, the OFF \( t_{1/2} \) = 71 min (25-170 min, n = 3) and recovery was fully restored, similar to atropine. However, at the mid and high concentrations, tension recovery was more prolonged; OFF \( t_{1/2} \) = 381 min (241->600 min, n = 7) and >600 min (n = 3), and only returned at 10 hrs to 67% (an average derived from those tissues recovering by 50% or more at 10 hrs, some tissues did not reach this level) and 6%, respectively. By contrast, contractile tension essentially recovered at 10 hrs at all concentrations of ipratropium; OFF \( t_{1/2} \) (min) = 6 (4-9, n = 3), 63 (28-118, n = 5) and 224 (143-310, n = 3), respectively. Tiotropium was slow to recover contractile tension; OFF \( t_{1/2} \) (min) = 176 (62->600, n = 3), 434 (331->600, n=7) and >600, respectively, and had negligible recovery of tension at 100 nM after 10 hrs, about 1% (10 nM atropine OFF\( t_{1/2} \) = 55 min, n = 2, historical reference = 34 min, range = 23-49 min, 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 non-muscarinic 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: GSK573719 = 34 min and >600 min, ipratropium = 12 min and 35 min and tiotropium = 9 min and >600 min, 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 ED₅₀ value for maximal bronchoconstriction) in a dose-dependent manner; ED₅₀ = 0.02 μg/mouse measured 5 hr after instillation. The inhibitory effect following a single administration was sustained for a prolonged period of time, e.g., at 0.05 μg/mouse, inhibition was about 50% or greater for up to 72 hrs (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 hr) than that of GSK573719 (24 hrs post-dose). When GSK573719 was given once daily to mice for five consecutive days (0.025 μg intranasally), the level of inhibition on the 5th day was modestly increased above that obtained after a single administration to the same mice (60% vs 35%, respectively). After the 5th day of dosing, the mice were rested for five additional days allowing bronchomotor tone to return to baseline levels. On the 6th day, the mice received one last dose of antagonist and 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 ED₅₀ value (intranasal), there was no observable protection against a Mch challenge (monitored for 24 hrs post-dose, data not shown).

In similar studies using Guinea pigs rather than mice, intratracheal instillation of GSK573719 (0.25, 2.5 and 25 μg/Guinea pig) dose-dependently blocked the rise in Penh elicited by aerosolized Ach (Figure 7A). Inhibitory effects were sustained for long periods of time and the duration of the protection was more protracted with each increase of GSK573719. For example, the level of blockade corresponding to 50% inhibition was maintained for more than 2 days at 2.5 μg/Guinea pig and for more than 5 days at 25 μg/Guinea pig. These increments were not, however, dose-proportional. When the inhibitory effects of GSK573719 were compared to those obtained with tiotropium (animals were given both compounds at 2.5 μg/Guinea pig), the findings were comparable (Figure 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 (Figure 8A). At the highest dose of Ach (100 μg iv), significant inhibition, i.e., 74.4%, p< 0.05, was obtained 4 hrs after instillation of 0.25 μg/Guinea pig of GSK573719. Comparable data were obtained
using tiotropium over the same dose range (Figure 8B). Complete blockade resulted when the animals received the high dose of either agent. In these same animals, a dose-related fall 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 of heart rate (data not shown). For example, the average fall in heart rate in the absence of antagonist was 148 ± 8 bpm after administration of 100 μg of Ach and at the high dose of GSK573719 (2.5 μg), heart rate declined by 136 ± 14 bpm.
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 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 Choi, 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 pKᵦ values for the M3 mAChR were 10.2 and 10.5 using ['H]-N-methyl-scopolamine or ['H]-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 rise 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., for M3 mAChR = 0.963. The temporal limits imposed by the FLIPR technology i.e., the duration of the antagonist exposure (30 min) with the receptor was markedly greater than that for the agonist (peak Ach response occurs within 4-5 sec 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 equipotent 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 non-competitive, in complex human biological systems. Taken together, the data suggest that the mode of antagonism provided by GSK573719 is competitive although it cannot be ruled out that there are possible insurmountable characteristics.

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 min. Dissociation from the M3 mAChR occurred more slowly than from M2 mAChR, i.e., half-life was 9 min. In our hands, [³H]-tiotropium also dissociated slowly from the two receptors, however, the half-lives were longer lived, i.e., 273 vs 39 min, respectively. 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 and coworkers (2009) reported that tiotropium dissociates more slowly from the M3 mAChR than the M2 mAChR, however, the half-lives were 27 and 2.6 hrs, 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 classical methods used in the present one). 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 most rapid. Recently, Sykes and coworkers (2012) suggested that “physiological” assay conditions, in particular sodium ion concentration of the incubation medium can markedly reduce M3 mAChR residence times by as much as 10-fold, e.g., half-life of tiotropium decreased from 462 to 46 min in the absence and presence of sodium, respectively. Nonetheless, the trends for M3 vs M2 mAChR dissociation seem to be a characteristic that is common to this group of pharmacophores.

Insight on 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 the G_{q/11} family and M2 and M4 with the G_{i/o} type proteins (see for example, Caufield 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 AChR 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 and coworkers (2012) have suggested that as 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 rise 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, data not shown). At all concentrations (1-100 nM) of ipratropium, tension development returned during the wash-out period. On the other hand, removal of the mid (10 nM) and particularly the high (100 nM) concentrations of GSK573719 and tiotropium continued to suppress the tissue response to carbachol even after 10 hrs 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 (Couslon and Fryer, 2003). Although M2 mAChRs are located on pre-junctional 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 and colleagues (2004) showed that in mice deficient of the M2 mAChR, vagal stimulation or methacholine challenge produced enhanced bronchoconstrictor 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., \( \text{ED}_{50} = 0.02 \mu g/\text{mouse} \), is consistent with the levels of potency obtained \textit{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 disease 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.

Lastly, 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 the diffusion of the compound from the lung parenchyma to the large plasma volume occupied by the systemic circulation dilutes the drug concentration several fold, thereby diminishing any potential for M2 mAChR-mediated cardiac effects. Altered cardiac and pulmonary vagal tone is found in many pathophysiological conditions, including COPD. The clinical use of mAChR antagonists such as tiotropium is a currently accepted 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\(_1\)) 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, Laine, 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: Laine, Palovich, Luttmann.

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

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


Cheng Y-C, and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which cause 50 percent inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 22, 3099-3108.


Legends for Figures

Figure 1. Superfusion experimental protocol used for duration of onset and offset of contractile activity of isolated human bronchus in response to mAChR antagonists. The tissues were suspended in a chamber as described in Methods. In brief, Cch was used to contract the tissue (developed tension, g, is noted by the vertical bar at the far right) in the presence or absence of mAChR antagonist, noted in the figure as “test cpd on” and “test cpd off”, respectively. The tissues were exposed to the antagonists for a period of 6 hrs and then cleared for an additional 10 hrs (referred to as wash-out time) to allow the tissue to resume its enhanced contractile tone induced by Cch alone.

Figure 2. Chemical structure of GSK573719. GSK573719 or 4-[hydroxy(diphenyl)methyl]-1-{2-[(phenylmethyl)oxy]ethyl}-1-azoniabicyclo[2.2.2]octane was prepared as the crystalline bromine salt (Lainé et al., 2009).

Figure 3. Blockade of Ach-induced calcium mobilization by GSK573719. CHO cells stably expressing recombinant human M3 mAChRs were pre-treated with GSK573719 at concentrations (1-1000 nM) listed in the insert shown in panel A for 30 min prior to application of Ach. In panel B, a Schild plot shows the Ach response ratio in the presence versus absence of antagonist, Y-axis, plotted against the antagonist concentration, X-axis. The shift value obtained using 1nM of GSK573719 was not included in the Schild analysis as this concentration does not reach equilibrium with the receptor during the 30 min preincubation time. The data represent mean ± S.E.M. (n = 6-7).

Figure 4. Reversal of mAChR antagonism of calcium mobilization in CHO cells. CHO cells were pre-incubated for 30 min 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 or GSK573719 were washed for a period of 180 min. For tiotropium, data were similar for 90 min of cell washing (shown above) and for 180 min (n = 1; data not shown). For reference, some cells were treated with antagonists and Ach without washing. Studies were conducted independently and shown here for comparison. The data represent mean ± S.E.M. (n = 4).
Figure 5. Effects of mAChR antagonists on Cch-induced contraction of isolated strips of human bronchus. Segments of human bronchus were prepared as described in Methods and incubated with mAChR antagonists (or vehicle) for 120 min prior to cumulative addition of carbachol in conventional tissue baths heated at 37° C. Atropine (10 nM) served as a reference. Values represent mean ± S.E.M. (n = 3-5 tissues).

Figure 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 Methods and exposed to agents in accordance with the superfusion protocol shown in Figure 1. All tissues were contracted with Cch (1 μM) and continued to receive the agonist until the end of the experiment. Afterwards, tissues were evaluated for viability using histamine (1 mM). The data represent means ± S.E.M. (n = 5-7) and the line of best fit.

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

Figure 8. Dose-dependent blockade of Ach-induced bronchoconstriction: comparison of GSK573719 (A) and tiotropium (B). Guinea pigs were treated similarly to those in Figure 7 except that they were ventilated (pressure = 8 cm H₂O at 60 breaths/min) and cannulated for recordings of heart rate and blood pressure as well as for cumulative bolus injections of ACh. The data represent means ± S.E.M. (n = 6, asterisks, * to *** refer to p < 0.05-0.001).
### Table 1. Receptor binding data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>mAChR subtype</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</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>
</tr>
<tr>
<td>$pK_i$</td>
<td>9.8</td>
<td>9.8</td>
<td>10.2</td>
<td>10.3</td>
<td>9.9</td>
</tr>
<tr>
<td>$[^3]$H-GSK573719</td>
<td></td>
<td></td>
<td>9.79 ± 0.08</td>
<td>10.5 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>2.22 ± 0.11 X10⁹</td>
<td>5.67 ± 0.45 X10⁹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{off}$</td>
<td>0.074 ± 0.004</td>
<td>0.0089 ± 0.0012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>9.4 ± 0.5</td>
<td>82.2 ± 0.0012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[^3]$H-tiotropium</td>
<td></td>
<td></td>
<td>10.3 ± 0.08</td>
<td>10.7 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>1.26 ± 0.10 X10⁹</td>
<td>4.09 ± 0.55 X10⁹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{off}$</td>
<td>0.023 ± 0.008</td>
<td>0.0026 ± 0.0003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>39.2 ± 9.7</td>
<td>272.8 ± 27.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

This article has not been copyedited and formatted. The final version may differ from this version.
Table 2. Functional potency values in CHO cells

<table>
<thead>
<tr>
<th>mACHR subtype</th>
<th>GA573719 pA2</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK573719</td>
<td>M1 9.6</td>
<td>9.5-9.7</td>
</tr>
<tr>
<td></td>
<td>M2 10.1</td>
<td>10.0-10.2</td>
</tr>
<tr>
<td></td>
<td>M3 10.6</td>
<td>10.5-10.8</td>
</tr>
</tbody>
</table>

Data represent mean and confidence intervals (C.I.) for N=6 experiments. GSK573719 was incubated with CHO cells for 30 min prior to generation of ACh concentration-response curves.
Fig. 2
Fig. 4

A. Ipratropium

B. GSK573719

C. Tiotropium

% Vehicle Max

% Max. Response

[Acetylcholine] (log M)
Fig. 5

A. Ipratropium

- Control
- Atropine 10 nM
- Ipratropium 100 nM
- Ipratropium 1 nM
- Ipratropium 10 nM

B. GSK573719

- Control
- Atropine 10 nM
- GSK573719 10 nM
- GSK573719 100 nM
- GSK573719 1 nM

C. Tiotropium

- Control
- Atropine 10 nM
- Tiotropium 0.1 nM
- Tiotropium 1 nM
- Tiotropium 10 nM

Graphs show the contraction (% 1 mM Post-Histamine) against [Carbachol] (log M). The pA2 values are indicated for each compound.
Fig. 6

Recovery of Tension (% initial inhibition) vs. Time (hours post-compound removal)

- Ipratropium (○)
- Tiotropium (□)
- GSK573719 (△)

Error bars indicate standard deviation.