Pharmacological Characterization of GSK-961081 (TD-5959), a First-in-Class Inhaled Bifunctional Bronchodilator Possessing Muscarinic Receptor Antagonist and β2 Adrenoceptor Agonist (MABA) Properties


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Abbreviations: COPD, chronic obstructive pulmonary disease; MA, muscarinic receptor antagonist; BA, β2 adrenoceptor agonist; MABA, muscarinic receptor antagonist β2 adrenoceptor agonist; ICS, inhaled corticosteroids; CHO-K1, Chinese hamster ovary-K1; EMR, equipotent molar ratio; MCh, methacholine; PILO, pilocarpine; HIS, histamine; VP, ventilation pressure; IA, intrinsic activity; EFS, electrical field stimulation; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; IV, intravenous; IH, inhalation; IM, intramuscular; SC, subcutaneous

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ABSTRACT

The objective of the present studies was to characterize the pharmacological properties of GSK-961081 (TD-5959), a novel first-in-class inhaled bifunctional compound possessing both muscarinic antagonist (MA) and β₂-adrenoceptor agonist (BA) properties (MABA). In competition radioligand binding studies at human recombinant receptors, GSK-961081 displayed high affinity for hM₂ (Kᵢ = 1.4 nM), hM₃ muscarinic receptors (Kᵢ = 1.3 nM) and hβ₂ adrenoceptors (Kᵢ = 3.7 nM). GSK-961081 behaved as a potent hβ₂ adrenoceptor agonist (EC₅₀ = 0.29 nM for stimulation of cAMP levels) with 440- and 320-fold functional selectivity over hβ₁ and hβ₃ adrenoceptors, respectively. In guinea pig isolated tracheal tissues, GSK-961081 produced smooth muscle relaxation through MA (EC₅₀ = 50.2 nM), BA (EC₅₀ = 24.6 nM) and MABA (EC₅₀ = 11 nM) mechanisms. In the guinea pig bronchoprotection assay, inhaled GSK-961081 produced potent, dose-dependent inhibition of bronchoconstrictor responses via MA (ED₅₀ = 33.9 µg/mL), BA (ED₅₀ = 14.1 µg/mL) and MABA (ED₅₀ = 6.4 µg/mL) mechanisms. Significant bronchoprotective effects of GSK-961081 were evident in guinea pigs, via MA, BA and MABA mechanisms, for up to seven days after dosing. The lung selectivity index of GSK-961081 in guinea pigs was 55 to 110-fold greater than that of tiotropium with respect to systemic antimuscarinic antisialagogue effects and was 10-fold greater than that of salmeterol with respect to systemic β₂ adrenoceptor hypotensive effects. These preclinical findings studies suggest that GSK-961081 has the potential to be a promising next-generation inhaled lung-selective bronchodilator for the treatment of airway diseases, including chronic obstructive pulmonary disease (COPD).
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory condition that is characterized by progressive airflow limitation caused by a mixture of small airway disease and parenchymal destruction (Decramer et al., 2012). Bronchodilators form the cornerstone of symptomatic pharmacological management for patients with COPD (Vestbo et al., 2013). These medications, which are usually dosed by inhalation to reduce systemic adverse effects, are administered on an as-needed basis for persistent or worsening symptoms or on a regular basis to prevent or reduce symptoms. Two classes of inhaled bronchodilators, β2-adrenoceptor agonists (BAs) and muscarinic receptor antagonists (MAs), are currently available for use in the treatment of COPD. MAs and BAs cause bronchodilation through distinct but complementary mechanisms that potentially synergize with each other given the functional ‘cross-talk’ between M2/M3 muscarinic and β2 adrenoceptors in airway smooth muscles (Yoshikawa and Kanazawa, 2008). MAs relax airways by reversing cholinergically-mediated bronchoconstriction via antagonism of M2 and M3 muscarinic receptors on airway smooth muscle (Barnes, 1998; Proskocil and Fryer, 2005). BAs cause direct relaxation of airway smooth muscle tissue (Barnes, 1998; Proskocil and Fryer, 2005). Both MAs and BAs improve lung function and health-related quality of life while reducing symptoms including COPD exacerbations (Vestbo et al., 2013). In clinical practice, patients with moderate to severe COPD are usually prescribed more than one bronchodilator. This usually includes a long-acting muscarinic antagonist (LAMA), such as tiotropium, and a long-acting bronchodilator (LABA) such as salmeterol or formoterol (Vestbo et al., 2013; Tashkin and Ferguson, 2013). Several studies have demonstrated that the combination of a MA and BA, in separate inhalers, provides greater improvement in lung function than either single agent alone (see Tashkin and Ferguson, 2013 for review). In patients
with severe to very severe COPD who have repeated exacerbations, inhaled corticosteroids (ICS) are frequently added to the treatment regimen of a MA and BA, to achieve triple therapy (Gaebel et al., 2011).

GSK-961081 (TD-5959, \((R)-1-(3-((2-chloro-4-(((2-hydroxy-2-(8-hydroxy-2-oxo-1,2-dihydroquinolin-5-yl)ethyl)amino)methyl)-5-methoxyphenyl)amino)-3-oxopropyl) piperidin-4-yl [1,1'-biphenyl]-2-ylcarbamate) (Fig.1) is a novel inhaled bifunctional compound that was designed to exhibit dual muscarinic antagonist and \(\beta_2\) adrenoceptor agonist (MABA) pharmacology with the potential to deliver optimal bronchodilation, following inhalation dosing via two validated mechanisms in one molecule. Compounds with MABA activity offer a single pharmacokinetic profile for both pharmacological activities, thereby maximizing the potential synergy between the two mechanisms, which would otherwise be difficult to achieve with co-administration of two separate compounds with distinct pharmacokinetic-pharmacodynamic profiles (Jacobsen et al., 2010). In addition, given the technical challenges associated with co-formulation of two or more compounds in a single device, a MABA offers a conceptually simpler technical path towards achieving triple therapy in one device (Jacobsen et al., 2010). A potential limitation of the MABA approach is that the fixed MA/BA potency ratio in a single dual pharmacology compound precludes one from varying the relative activity at the two targets, a goal that is more readily achievable with a combination of two separate drugs.

The objective of the present studies was to characterize the \textit{in vitro} and \textit{in vivo} preclinical pharmacological properties of GSK-961081. The binding affinity and functional antagonist potency of GSK-961081 were investigated at human recombinant receptors (muscarinic receptors and \(\beta\) adrenoceptors expressed in CHO-K1 or HEK293 cells, respectively) and guinea-pig tracheal tissues expressing native muscarinic receptors and \(\beta_2\) adrenoceptors. The \textit{in vivo}
bronchoprotective potency of inhaled GSK-961081 was assessed using the guinea-pig Einthoven
assay whereas the systemic antimuscarinic and β2 adrenoceptor effects were evaluated using
antisialagogue and hypotensive endpoints in guinea-pigs.

MATERIALS & METHODS

Materials

[3H]-N-methyl scopolamine methyl chloride ([3H]NMS) was purchased from GE Healthcare (Piscataway, NJ). [3H]-dihydroalprenolol ([3H]DHA), [125I]-cyanopindolol ([125I]CYP) and [35S]-
Guanosine 5′-(gamma-thio)triphosphate ([35S]GTPyS) were obtained from PerkinElmer (Waltham, MA). GSK-961081 (TD-5959, (R)-1-3-((2-chloro-4-((2-hydroxy-2-(8-hydroxy-2-
oxo-1,2-dihydroquinolin-5-yl)ethyl)amino)methyl)-5-methoxyphenyl)amino)-3-oxopropyl)
piperidin-4-yl [1,1′-biphenyl]-2-ylcarbamate) was synthesized at Theravance, Inc. (South San
Francisco, CA). Ipratropium bromide, atropine, scopolamine, oxotremorine sesquifumarate,
isoproterenol, salmeterol, albuterol (salbutamol) were purchased from Sigma. Formoterol
hemifumarate was obtained from Tocris. FLUO-4AM ((N-[4-6-((acetyloxy)methoxy]-2,7-
difluoro-3-oxo-3H-xanthen-9-yl]-2-[2-[2-bis[2-((acetyloxy)methoxy]-2-oxoethyl]amino]-5-
methylphenoxy]ethoxy]phenyl]-N-[2-((acetyloxy)methoxy]-2-oxoethyl]-, (acetyloxy)methyl
ester)) was purchased from Molecular Probes/Invitrogen (Carlsbad, CA). Tiotropium was
synthesized at Theravance, Inc. (South San Francisco, CA). Sterile water was used as vehicle for
all in vivo studies.

Animal Studies

Studies were approved by the Institutional Animal Care and Use Committee (IACUC) at
Theravance and conducted in an AAALAC-accredited facility. The animals were housed in a
climate controlled environment (12-h light, 12-h dark cycle) with access to food and water *ad libitum*.

**Radioligand Binding Studies**

**Recombinant Human Muscarinic Receptors.** Muscarinic receptor binding assays were performed on membrane preparations from CHO-K1 cells stably expressing the human muscarinic receptor subtypes, hM<sub>1</sub>, hM<sub>2</sub>, hM<sub>3</sub>, hM<sub>4</sub> or hM<sub>5</sub>, using [³H]NMS (1 nM) as the radioligand. The assay plates containing membranes were incubated in buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, pH 7.4 at 37°C) with radioligand and the appropriate concentration of the competing ligand at 37°C for 60 minutes (except for tiotropium, which was incubated for 16 hours). Bound radioactivity was captured on GF/B glass fiber filter plates pre-treated with 1% BSA using a 96 well harvester and measured using a TopCount scintillation counter (PerkinElmer, Waltham, MA). Atropine (10 µM) was used to define non-specific binding.

**Recombinant Human β adrenoceptors.** β adrenoceptor binding was conducted using membranes prepared from HEK293 cells stably expressing the hβ<sub>1</sub> or hβ<sub>2</sub> adrenoceptors using [³H]-DHA as the radioligand, or with hβ<sub>3</sub> expressed in CHO cells using [¹²⁵I]-CYP as the radioligand. The assay plates containing membranes were incubated in buffer (75 mM Tris/HCl, 12.5 mM MgCl₂, 1 mM EDTA, pH 7.4 at 37°C) with radioligand and appropriate concentration of the competing ligand at 37°C for 60 minutes. Bound radioactivity was captured on GF/B glass fiber filter plates pre-treated with 1% BSA using a 96 well harvester and measured using a TopCount scintillation counter (PerkinElmer, Waltham, MA). Propranolol (10 µM) and
alprenolol (1 mM) were used to define non-specific binding for hβ₁/hβ₂ and hβ₃ adrenoceptors, respectively.

**Functional Studies**

**Recombinant Human Muscarinic Receptors.** Functional antagonism assays were performed in CHO-K1 cells expressing recombinant muscarinic receptors by measuring inhibition of oxotremorine-induced calcium mobilization in FLIPR® using FLUO-4AM as the calcium-sensitive dye as described previously. CHO-K1 cells stably transfected with each receptor subtype were plated in 96 well plates and grown overnight at 37°C. The following day, the cells were gently washed and treated for 40 minutes at 37°C with FLUO-4AM. The cells were gently washed a second time to remove the excess dye. Cells were then incubated with increasing concentrations of GSK-961081 for 20 minutes at 37°C. The cells were stimulated with an EC₉₀ concentration of the muscarinic agonist oxotremorine. EC₉₀ concentration was determined by generating an agonist concentration-response curve with the same batch of cells prior to the experiment. Oxotremorine elicited a Gq mediated calcium release event, which in turn caused the calcium sensitive dye to bind to calcium and fluoresce upon stimulation with a 488 nm laser light source. Change in fluorescence was measured by the FLIPR® for three minutes and the peak height in fluorescence was taken as maximal response to generate the concentration-response curve for GSK-961081.

Functional antagonism assays for hM₂ were performed by measuring inhibition of oxotremorine-induced [³⁵S]GTPγS binding in cells expressing hM₂ receptors. Membranes prepared from CHOK1 cells expressing the hM₂ receptor were treated with GSK-961081. The membranes were subsequently treated with the muscarinic receptor agonist oxotremorine for one
hour to activate the receptors, enhancing Ga\textsubscript{i}/Ga\textsubscript{o} protein binding to exogenous \[^{35}\text{S}]\text{GTP}\gamma\text{S}. Decreases in agonist mediated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding by GSK-961081 were indicative of functional antagonism of the receptor. To measure the intrinsic activity of GSK-961081 toward the hM\textsubscript{2} receptor, the membranes described above were treated with GSK-961081 for one hour in the absence of agonist and binding of \[^{35}\text{S}]\text{GTP}\gamma\text{S} to the Ga\textsubscript{i}/Ga\textsubscript{o} proteins in the membrane was then measured.

**Recombinant Human \(\beta\)-adrenoceptors.** Functional potency was measured in HEK or CHO-K1 cells stably expressing human \(\beta_1\), \(\beta_2\) or \(\beta_3\) adrenoceptors that were stimulated with serially diluted agonists at 37\(^{\circ}\)C for 10 min. Whole cell cAMP was measured using the Flashplate Adenylyl Cyclase Activation Assay System with \[^{125}\text{I}]\text{cAMP} (PerkinElmer, Waltham, MA), according to the manufacturer’s instructions. To measure intrinsic activity of \(\beta_2\) adrenoceptor agonists, the cAMP assay mentioned above was performed in human bronchial epithelial cells (BEAS-2B, ATCC, licensed from NIH) expressing endogenous levels of \(\beta_2\) adrenoceptors (January et al., 1998). Cells were grown to 75-90\% confluency in complete, serum free medium (LHC 9 MEDIUM containing epinephrine and retinoic acid, Biosource International, Camarillo, CA). The day before the assay, medium was switched to LHC 8 containing no epinephrine or retinoic acid.

**Guinea-Pig Isolated Trachea.** Airway smooth muscle relaxation mediated by MA, BA and MABA mechanisms was measured in trachea isolated from Harlan Dunkin-Hartley guinea pigs (male, 200-500 g). Animals were euthanized by CO\(_2\) asphyxiation followed by thoracotomy. The trachea was dissected, cleaned of excess tissues and cut into rings that were 0.5 cm in width. Each segment of tissue was mounted in an organ bath filled with Krebs buffer (10 mM D-glucose, 1.64 mM MgSO\(_4\), 1.18 mM KHPO\(_4\), 4.7 mM KCl, 118 mM NaCl, 24.88 mM NaHCO\(_3\),}
2.52 mM CaCl₂) supplemented with indomethacin (10 µM) to inhibit cyclo-oxygenase activity, and guanethidine (3 µM) to block postganglionic sympathetic nerve transmission. The buffer was constantly aerated with carbogen (95% O₂ and 5% CO₂) and maintained at 37°C. Changes in tracheal isometric force were measured by a force transducer (Model Fort 100, World Precision Instruments, Sarasota, FL) that was connected to a 4-channel amplifier (Model S48, Astro-Med, West Warwick, RI). Data were recorded by an electronic data acquisition system (Model MP 100/UIM 100A, Biopac). The tracheal rings were initially tensioned to 1 g and allowed to equilibrate for 1 hr before evoking contraction with a sub-maximal concentration of either methylcholine (MCh, 10 µM), in the presence of propranolol (10 µM), or histamine (HIS, 30 µM) to assess relaxant effects via MA and BA mechanisms, respectively. Relaxation through the MABA mechanism was evaluated in tissues pre-contracted with MCh in the absence of propranolol. After the contractile tone attained a plateau, the test compound (0.1 nM to 100 µM) was added cumulatively in half log increments, with each concentration being added after achieving a steady-state relaxation response to the previous concentration. After the last concentration of test compound, theophylline (2.2 mM) was added to establish maximum relaxation. In separate studies to obtain equilibrium MA affinity estimates (Kₐ), two MCh concentration-response curves, with and without the test compound, were constructed in the same tissue. After the first curve, the tissue was washed every 15 min for 1 hr and was allowed to return to baseline. The tissues were then equilibrated with GSK-961081 (10, 30, and 100 nM) or vehicle (water) for 60 min, followed by construction of a second MCh curve.

Duration of MABA-induced relaxation was evaluated using electric field stimulated (EFS) guinea pig trachea. Trachea was dissected and isolated as described earlier. For this study, Krebs buffer was also supplemented with choline (1 µM) in addition to indomethacin (10 µM) and
guanethidine (3 µM). Tracheal rings were opened by a transverse cut before mounting under 1 g tension between two platinum electrodes in a tissue bath. Electric field stimulation was applied under the following conditions: 9V, 1 ms pulse duration, 10 Hz for 10 sec every 100 sec (Grass Stimulator (S88), Grass Technologies, West Warwick, RI). After the equilibration period, tissues were exposed to a submaximal concentration (~EC₉₀) of test compound. When steady relaxation was observed, tissues were perfused continuously with buffer (2 mL/min) for up to 20 hours. Response was normalized to the initial maximum relaxation response before the start of perfusion.

**In vivo Bronchoprotection Studies**

Bronchoprotection studies were conducted as described previously using the modified Einthoven assay (McNamara et al., 2011). Male Duncan Hartley guinea pigs (Harlan, Indianapolis, IN), weighing between 250 – 400 g were used for all studies. The test compound or vehicle (sterile water) was nebulized over a 10 minute time period in a pie shaped inhalation chamber (R+S Molds, San Carlos, CA) using 5 mL of dosing solution. Animals were exposed to an aerosol, which was generated from an LC Star Nebulizer Set (Model 22F51, PARI Respiratory Equipment, Inc. Midlothian, VA) driven by Bioblend (5% CO₂/ 95% atmospheric air) at a pressure of 22 psi. Pulmonary function was evaluated at various time points (1.5, 8, 24, 48, 72 and 168 hr) after inhalation dosing using separate cohorts of animals for each pretreatment period.

Forty five minutes prior to the start of pulmonary function evaluation, animals were anesthetized with an intramuscular (IM) injection of ketamine (43.7 mg/kg)/xylazine (3.5 mg/kg)/acepromazine (1.05 mg/kg) cocktail. A supplemental dose of cocktail (50% of initial
dose) was administered when needed for maintenance of a stable plane of anesthesia. The jugular vein was isolated and catheterized with a saline filled polyethylene catheter (PE 50) to allow intravenous (IV) injection of either MCh or HIS. The trachea was dissected free and cannulated with a 14G needle (#NE 014, Small Parts, Miami Lakes, FL). Once the cannulations were complete, the guinea pigs were ventilated using a respirator (Model 683, Harvard Apparatus, Inc., MA) set at a stroke volume of 1mL/100 g body weight but not exceeding 2.5 mL volume, and at a rate of 100 strokes per minute. A T-connector was attached to the respirator expiratory tubing to measure changes in ventilation pressure (VP) using a Biopac transducer that was connected to a Biopac (TSD 137C) pre-amplifier. Body temperature was maintained at 37°C using a heating pad. Prior to initiating data collection, pentobarbital (25 mg/kg, IP) was administered to suppress spontaneous breathing to enable a stable baseline. The changes in VP were recorded on a Biopac Windows data collection interface. Baseline parameters were collected for at least 5 minutes, after which time guinea pigs were challenged with ascending, non-cumulative IV doses of the bronchoconstrictor (MCh or HIS) in two-fold increments. The MA, BA and MABA bronchoprotective potencies were estimated by obtaining bronchoconstrictor dose-response curves to IV MCh (after pretreatment with propranolol, 5 mg/kg, IV), HIS and MCh (in the absence of propranolol), respectively. Changes in VP were recorded using the Acknowledge Data Collection Software (Santa Barbara, CA). After the completion of study, the animals were humanely euthanized by CO₂ asphyxiation followed by a thoracotomy.

**Systemic Muscarinic Antagonist and β₂ adrenoceptor Agonist Effects**

**Anti-sialagogue Activity.** Male guinea pigs (Harlan, Indianapolis, IN) weighing 200-350 g were employed for these studies. Test compounds or vehicle were dosed via IH as described
above. At varying times after IH dosing, guinea pigs were anesthetized with a cocktail mixture of ketamine (43.7 mg/kg, IM), xylazine (3.5 mg/kg, IM) and acepromazine (1.05 mg/kg, IM). Animals were placed on their dorsal side on a heated (37°C) blanket at a 20 degree incline with their head in a downward slope. A pre-weighed gauze pad (Nu Gauze general use sponges, Johnson and Johnson, Arlington, TX) was inserted in the animals mouth and the muscarinic agonist pilocarpine (PILO, 3 mg/kg, subcutaneous (SC)) was administered. Saliva produced during 10 min post PILO was measured gravimetrically by determining the weight of the gauze pad before and after PILO. Upon completion of this assay, animals were humanely euthanized.

Cardiovascular Effects. Under isoflurane anesthesia, guinea pigs were instrumented with a common carotid artery and a jugular vein catheter (PE-50 tubing). The catheters were exteriorized utilizing a subcutaneous tunnel to the subscapular area. All surgical incisions were sutured with 4-0 Ethicon Silk and the catheters locked with heparin (1000 units/mL). Each animal was administered saline (3 mL, SC) and buprenorphine (0.05 mg/kg, IM) at the end of surgery. Animals were allowed to recover on a heating pad before being returned to their holding rooms.

On the day of the study (18-20 hr post-surgery), animals were placed in the whole body dosing chamber and the carotid artery catheter was connected to a transducer to measure arterial pressure and heart rate. Animals were allowed to acclimate to the chamber for 20 min. Following the acclimation period, baseline hemodynamics was recorded for 5-10 min. Three control depressor responses to MCh (0.3 µg/kg, IV) were obtained at 15 min intervals. The animals were subsequently dosed by inhalation with nebulized drug or vehicle using a whole body dosing chamber (as described above). The intrinsic hemodynamic effects of the drug were assessed during and 60 min after inhalation. At 5 min, 1.5 hr and 24 hr post-dosing, the animals were re-
challenged with MCh. At the end of the study, the animals were humanely euthanized by CO₂ asphyxiation followed by a thoracotomy.

**Data Analysis**

**Radioligand Binding Competition Studies.** The binding data were analyzed by nonlinear regression analysis using GraphPad Prism 3.0 software (GraphPad Software, Inc., San Diego, CA). \( K_I \) values for GSK-961081 and comparator compounds were calculated from observed IC\(_{50}\) values according to Cheng and Prusoff (1973).

**Functional Studies.** The FLIPR® and \([^{35}S]GTP_\gamma S\) binding data were analyzed by nonlinear regression analysis using GraphPad Prism 3.0. Antagonist \( K_{i,App} \) values were determined by GraphPad Prism 3.0 using the oxotremorine EC\(_{50}\) value as the \( K_D \) and the oxotremorine EC\(_{90}\) for the ligand concentration according to the Cheng Prusoff equation (Cheng & Prusoff, 1973). \( K_{i,App} \) is reported instead of \( K_i \) since the data were not generated under equilibrium conditions.

For the \( \beta_2 \)-cAMP studies, data were analyzed by nonlinear regression analysis with the GraphPad Prism 3.0 using the 4-parameter logistic model with variable slope. Potency data are reported as EC\(_{50}\) values. Equipotent molar ratios (EMR) for test compounds were calculated relative to isoproterenol as:

\[
EMR = \frac{EC_{50}(\text{test compound})}{EC_{50}(\text{isoproterenol})}
\]

Selectiveties of compounds between receptor subtypes were then calculated from the respective EMR values:

\[
\text{Sel}(\beta_2/\beta_1) = \frac{\text{EMR}(\beta_1)}{\text{EMR}(\beta_2)}
\]

\[
\text{Sel}(\beta_2/\beta_3) = \frac{\text{EMR}(\beta_3)}{\text{EMR}(\beta_2)}
\]
In guinea pig isolated trachea studies conducted to determine potency for inhibition of MCh or electrical-field stimulated (EFS) pre-contracted tissues, inhibition curves were fitted using a non-linear regression analysis with GraphPad Prism 3.0 to estimate EC$_{50}$s. In studies where the MCh curve was shifted dextrally with increasing concentrations of the muscarinic antagonist, concentration-ratios (CR) were calculated as ratio of agonist EC$_{50}$ in the presence and absence of antagonist and pA$_2$ affinity estimates were estimated using the method described by Arunlakshana and Schild, H.O. (1959).

**In vivo Bronchoprotection Studies**

For all the studies where compound was administered by inhalation, doses were expressed as the concentration of the nebulized solution. Change in VP was measured in cm of H$_2$O. The dose response curve to MCh or HIS was fitted to a four parameter logistic equation using GraphPad Prism 3.0. The average bronchoconstrictor response to MCh (16 µg/kg, IV) or HIS (32 µg/kg, IV) in vehicle-treated animals was calculated and used to compute % inhibition of MCh/HIS induced bronchoconstriction in drug treated animals. Inhibition curves were fitted using the four parameter logistic equation with GraphPad Prism 3.0. ID$_{50}$ was defined as the dose required to produce 50% inhibition of the bronchoconstrictor response.

**Systemic Muscarinic Antagonist and β$_2$ adrenoceptor Agonist Effects**

**Salivation Assay:** The mean weight of saliva in vehicle treated animals was calculated and used to compute % inhibition of salivation in each of the drug treated animals. Antisialagogue ID$_{50}$ (dose required to inhibit PILO induced salivation by 50%) was also estimated.
Cardiovascular Assay: Waveform pressure data from the carotid artery was used to measure systolic/diastolic blood pressures (SBP/DBP). Mean Arterial Pressure (MAP) was calculated using the formula: \( MAP = \frac{1}{3}(SBP - DBP) + DBP \). Peak depressor responses to MCh, before (average of three controls) and after treatment, were calculated. The % inhibition of MCh depressor response was calculated at various times post treatment. In addition, % changes in baseline MAP and HR was calculated during and after (60 min) inhalation dosing. A one-way ANOVA with Bonferroni post-test was used to test for statistically significant differences between groups. The No Effect Dose (NED, the highest dose of the test drug that produced no significant change in MAP or HR, compared to vehicle controls) was obtained from the data.
Results

Activity at Human Muscarinic Receptor Subtypes

In competition binding studies, GSK-961081 displayed high affinity for hM₁, hM₂, hM₃ and hM₄ receptors and moderate affinity for hM₅ subtypes (Table 1). At the hM₂ and hM₃ receptor, GSK-961081 displayed approximately 39-65 fold lower affinity than tiotropium and less than 2-fold lower affinity than ipratropium.

In functional studies, GSK-961081 potently antagonized oxotremorine-induced intracellular Ca²⁺ flux at the hM₃ receptors (Kᵢ,app = 0.10 ± 0.05 nM) and oxotremorine-evoked stimulation of [³⁵S]GTPγS binding at the hM₂ receptor (Kᵢ,app = 1.1 ± 0.3 nM). GSK-961081 displayed no agonist activity at the hM₂ and hM₃ receptor subtypes (data not shown).

Activity at Human β Adrenoceptor Subtypes

GSK-961081 was a high-affinity ligand at hβ₂ adrenoceptors, with Kᵢ of 3.7 ± 1.4 nM. GSK-961081’s binding affinity at hβ₂ adrenoceptors was similar to that of salmeterol but approximately 10 and 450-fold greater than that of formoterol and albuterol, respectively (Table 2). GSK-961081 displayed approximately 120-fold and 300-fold selectivity for hβ₂ over hβ₁ and hβ₃ adrenoceptors, respectively, in this assay.

In functional studies, we evaluated the potency of GSK-961081 to stimulate Gs and elicit accumulation of cAMP in cells expressing human recombinant hβ₂, hβ₁ and hβ₃ adrenoceptors. As shown in Table 3, GSK-961081 was a potent agonist at hβ₂ adrenoceptors, with an EC₅₀ value of 0.29 nM. The functional potency of GSK-961081 in this assay was similar to that of salmeterol and formoterol but 100-fold higher than that of albuterol. To evaluate the relative
functional selectivity of GSK-961081 and comparator drugs, we adopted a normalization method (EMR) to account for differences across assay for the 3 receptors subtypes (see Methods section). Using such EMR calculations, we determined that GSK-961081 was greater than 400- and 300-fold selective for hβ2 over hβ1 and hβ3 adrenoceptors, respectively (Table 3).

To determine the relative intrinsic activity of GSK-961081 and comparator drugs at hβ2 adrenoceptors, we evaluated their potency and maximal functional activity for stimulation of cAMP in BEAS-2B cells, which express low levels of endogenous hβ2 adrenoceptors (January et al., 1998). The functional potency of GSK-961081 in this assay was approximately 10-fold less than that of salmeterol, similar to that of formoterol and 65-fold greater than that of albuterol. Intrinsic activities (IA) were expressed relative to a maximal isoproterenol response. As shown in Table 3 and Fig. 2, GSK-961081 had an IA of 0.8, significantly greater than that of salmeterol (0.35) and albuterol (0.40), but lower than that of formoterol (0.95).

Activity at Muscarinic and β2 adrenoceptors in Isolated Guinea-pig Trachea

In tracheal tissues pre-contracted with MCh (in the presence of propranolol to isolate the MA component), GSK-961081 and tiotropium produced concentration-dependent relaxation with EC₅₀’s of 50.2 and 24.6 nM, respectively (Table 4, Fig. 3). The antimuscarinic activity of GSK-961081 was further studied by determining its potency to displace the MCh contractile concentration-response curve. GSK-961081 (10 - 100 nM) produced concentration-dependent dextral shifts of the MCh curve (Fig. 4). Linear regression analysis of the Schild plot yielded a slope (mean with 95% CI) of 1.4 (1.0 – 1.7) and a pA₂ estimate of 8.6 ± 0.1. A pKB estimate of 9.1 ± 0.1 was obtained when the Schild slope was constrained to unity.
In tissues pre-contracted with HIS (to isolate the BA component), GSK-961081 and salmeterol produced concentration-dependent relaxation with EC$_{50}$’s of 25 and 100 nM, respectively (Table 4, Fig 3). GSK-961081 was a full agonist (IA = 0.98) whereas salmeterol behaved as a partial agonist (IA = 0.68). In the presence of propranolol (10 µM), the concentration response curve to GSK-961081 was shifted 200 fold to the right (EC$_{50}$ = 5 µM).

In tracheal tissues pre-contracted with MCh (in the absence of propranolol to allow both MA and BA components to be operative), GSK-961081 produced potent and concentration-dependent relaxation with an EC$_{50}$ of 10 nM that was significantly lower than the IC$_{50}$s for MA and BA components.

In EFS washout studies, GSK-961081 (0.1 µM), tiotropium (3nM) and atropine (0.1 µM) produced approximately 90% inhibition of contractile responses. During the 20 hr washout period, there was < 25% recovery of the inhibitory response with both GSK-961081 and tiotropium whereas the inhibitory effects of atropine recovered by 80% within 3 hr after washout was initiated (Fig. 5).

**Activity at Off-targets**

GSK-961081 was tested in binding assays against 83 cellular targets including receptors, transporters, channels, and enzymes at a single saturating concentration of 1 µM (>700 fold above the M$_3$ receptor Ki value). At the vast majority of the targets, GSK-961081 produced < 80% inhibition of specific binding. GSK-961081 had moderate to low affinity at the human D$_3$ (Ki = 61 nM), human H$_1$ (Ki = 98nM), human I$_2$ (Ki = 320nM) receptors and weak agonist activity at the human 5-HT$_{4C}$ (intrinsic activity 18% of 5-HT). These off-target activities were
considered biologically insignificant given the significantly greater potency at the three principal 
on-targets (M₂, M₃ and β₂).

**Bronchoprotective Activity, via Muscarinic Antagonism and β₂ Adrenoceptor Agonism, in** 
**Anesthetized Guinea-Pigs**

MCh and HIS produced dose dependent increases in VP. The ED₅₀ (µg/kg, IV) of MCh was 7.6 
(6.6 – 8.8) and 7.3 (6.3 – 8.4) in the presence and absence of propranolol, respectively and that of 
HIS was 14.4 (12.3 – 16.7). At 1.5 hr post-dosing, inhaled GSK-961081 produced dose 
dependent dextral shifts of the HIS dose-response curve and MCh dose-response curve in the 
presence and absence of propranolol. The bronchoconstrictor response to 16 µg/kg of MCh and 
32 µg/kg of HIS were also inhibited dose-dependently (Fig. 6). The estimated potencies (ID₅₀) of 
GSK-961081 were 33.9, 14.1 and 6.4 µg/mL, IH for the MA, BA and MABA components, 
respectively. At the doses tested, the maximal observed inhibition was 74.7%, 72.5% and 91.7% 
via the MA, BA and MABA components, respectively. At each of the doses tested, the inhibitory 
effects via the MABA mechanism was greater than via MA and/or BA mechanism alone. In 
duration studies, significant bronchoprotective effects of GSK-961081 via MA, BA and MABA 
mechanism were observed for up to 7 days following inhalation of (Fig. 7). At all time-points, 
the MABA effects were numerically greater than that of MA and BA alone. At 24 hr post- 
dosing, both the MA and BA effects were significantly lower than the MABA effect. At 1.5, 8 
and 48 hr, either the MA or BA effects were significantly lower than the MABA effect. The 
duration studies were not designed to demonstrate synergy of MA and BA effects given that a 
early-maximal dose was studied and the experiment did not have adequate statistical power.
Systemic Antimuscarinic (anti-depressor and anti-sialagogue) and $\beta_2$ Adrenoceptor Hypotensive Effects in Guinea Pigs

**Anti-depressor Antimuscarinic Effects.** In vehicle-treated animals, MCh decreased mean blood pressure by 26.1 ± 1.3 mmHg. Tiotropium, at 1.5 hr post dosing, produced dose dependent inhibition of MCh induced depressor responses ($\text{ID}_{50} = 68.7 \, \mu g/mL$, IH) (Table 5). GSK-961081, at 1.5 hr post dose, also produced significant inhibition of MCh induced depressor responses ($\text{ID}_{50} = > 4500 \, \mu g/mL$, IH) but was at least 66 fold less potent than tiotropium (Table 5).

**Antisialagogue Antimuscarinic Effects.** In vehicle treated animals, PILO increased saliva production by approximately 1.2 (0.6 – 1.8) g. In pilot time-course studies, we showed that inhaled tiotropium produces inhibition of PILO-induced salivation with a slow onset that peaks at 6 hr. In contrast, GSK-961081’s inhibitory effects on salivation have a faster onset which peaks at 1.5 hr post-inhalation. We subsequently conducted a dose-response study with tiotropium and GSK-961081 at 1.5 and 6 hr post-inhalation. Tiotropium produced dose-dependent inhibition of salivation with an $\text{ID}_{50}$ of 38.8 $\mu g/mL$, IH) (Table 5). GSK-961081 at 1.5 hr post dose, also produced significant inhibition of PILO induced salivation ($\text{ID}_{50} = 5083 \, \mu g/ml$, IH), but was 130 fold less potent than tiotropium (Table 5).

**$\beta_2$ Adrenoceptor Hypotensive Effects** Baseline MAP and HR were 60.0 ± 0.7 mmHg and 383.8 ± 5.0 beats/min, respectively. Inhalation of vehicle produced no consistent effects on MAP but evoked an immediate and transient fall in HR (data not shown). Salmeterol produced dose-dependent decreases in MAP (Table 5) with no consistent effects on HR (data not shown). The NED for hypotension was 10 $\mu g/mL$, IH. GSK-961081 also produced falls in MAP with a NED of 500 $\mu g/mL$, IH (Table 5).
Lung Selectivity Index

The ratio of the antidepressor ID_{50} to the bronchoprotective ID_{50} and the ratio of antisialagogue ID_{50} to the bronchoprotective ID_{50} were used to estimate the lung selectivity index with respect to systemic antimuscarinic effects. The lung selectivity index with respect to the antidepressor and antisialagogue effects was 12.7 and 7.2 for tiotropium and >703 and 794 for GSK-961081, respectively.

The ratio of the hypotensive NED to bronchoprotective ID_{50} was used to estimate the lung selectivity index with respect to β_2 hypotensive effects. The lung selectivity of salmeterol and GSK-961081 was 7.7 and 78, respectively.
DISCUSSION

The discovery of GSK-961081 emerged from a research program aimed at identifying a compound that possessed both MA and BA pharmacological properties in order to provide bronchodilation superior to existing monotherapies and simplifying the attainment of triple therapy through co-formulation with ICS (Jacobsen et al., 2010, Hughes et al., 2012). Using MABA prototypic compounds, THRX-198321 and THRX-200495, we have previously demonstrated that this molecular class binds in a multivalent bimodal orientation in the orthosteric and allosteric binding pockets of muscarinic receptor and β₂ adrenoceptor and produces potent bronchodilation in animal models (Steinfeld et al., 2011, McNamara et al., 2012). In the present study, we characterized the *in vitro* and *in vivo* pharmacological profile of GSK-961081, an optimized MABA compound currently in clinical development for COPD.

Competition binding and functional studies demonstrated that GSK-961081 behaves as a high affinity neutral antagonist of hM₂ and hM₃ muscarinic receptors. GSK-961081’s potency for hM₂ and hM₃ receptors was lower than that of tiotropium but comparable to that of ipratropium. M₂ and M₃ are the two postjunctional targets that principally mediate cholinergically evoked airway smooth muscle contraction (Jacobsen et al., 2008). M₂ receptors also mediate proliferation of airway smooth muscle cells and fibroblasts (Matthiesen et al., 2006; Oenema et al., 2013). It has been argued that blockade of prejunctional M₂ muscarinic inhibitory autoreceptors has the potential to augment ACh-release and this may compromise the bronchodilatory actions of non-selective anticholinergics (Barnes, 1993). This is unlikely to be the case if the antagonist adequately blocks postjunctional M₂ and M₃ receptors and assuming that there are no functionally important co-transmitters released from postganglionic parasympathetic nerves.
The muscarinic antagonist properties of GSK-961081 were confirmed in the guinea-pig isolated trachea that expresses native muscarinic M₂ and M₃ receptors mediating smooth muscle relaxation. In a study that was designed to isolate the MA mechanism by inclusion of the β-blocker propranolol in the buffer, GSK-961081 produced potent and concentration-dependent relaxation of MCh-precontracted tissues. In a separate study conducted to further characterize the nature of antagonism, GSK-961081 was shown to produce parallel, dextral shifts of the MCh concentration-response curve without affecting the maximal response and yielding a Schild slope not different from unity, consistent with a competitive mode of action. As observed in human receptors, GSK-961081’s potency in the guinea-pig trachea as a MA was lower than that of tiotropium, albeit by a smaller margin. GSK-961081 has also been shown to relax human isolated bronchial strips with nanomolar potency (data not shown).

At the hβ₂ adrenoceptor, GSK-961081 behaved as a high affinity agonist in stimulating cAMP accumulation with potency comparable to that of salmeterol and formoterol, two widely used LABAs. In order to gain better understanding of the efficacy of GSK-961081 relative to the comparator LABAs, functional activity was assessed in BEAS-2B cells which, owing to their low reserve of hβ₂ adrenoceptors, provide a sensitive system to discern differences in efficacy between compounds (January et al., 1998). In this assay, GSK-961081’s potency was comparable to that of salmeterol and formoterol while the compound’s intrinsic activity was intermediate between the two standards. The clinical significance of intrinsic activity assessed in cellular assays in vitro is poorly understood. Nevertheless a level of intrinsic activity equal to or greater than salmeterol, a LABA that produces robust bronchodilation in patients, is desirable.

Additional corroboration of the BA effects of GSK-961081 was achieved by demonstrating that the compound produced potent relaxation of histamine precontracted guinea-pig trachea through
a propranolol sensitive mechanism. Consistent with observations made in BEAS-2B cells, GSK-961081 had greater intrinsic activity than salmeterol for relaxation of guinea-pig trachea. However, contrary to the findings in BEAS-2B cells, GSK-961081 was 13-fold more potent than salmeterol in the guinea-pig tracheal assay, a finding that may be a reflection of tissue-dependent differences in receptor reserve or coupling efficiency.

Selective agonism of the $\beta_2$ adrenoceptor subtype is a critical prerequisite of inhaled LABAs to avoid safety/tolerability issues associated with activity at the $\beta_1$ and/or $\beta_3$ adrenoceptors. Based on affinity and agonist potency values, GSK-961081 displayed $>$100 fold selectivity for the $\beta_2$ over the $\beta_1$ and $\beta_3$ adrenoceptors. While the absolute $\beta_2$ selectivity margin of GSK-961081 was lower than that of salmeterol, it is comparable to that of formoterol, a LABA that is safe and well tolerated in patients.

We used the Einthoven model of bronchoconstriction in anesthetized guinea-pigs to assess the bronchoprotective effects of GSK-961081 in vivo (McNamara et al., 2011). Similar to the approach adopted in the isolated tracheal tissue studies, the MA and BA mechanisms of GSK-961081 were dissected in the in vivo model by determining potency to protect against the bronchoconstrictor effects of MCh (in the presence of propranolol) and HIS, respectively. We showed that GSK-961081 produced potent and dose-dependent bronchoprotective effects via both MA and BA mechanisms with the latter being more potent by a factor of two-fold. The MA and BA bronchoprotective potencies of GSK-961081 were roughly 6-fold and 8-fold lower than those previously reported for tiotropium and salmeterol (McNamara et al., 2011), respectively.

Since GSK-961081 was designed to be a dual MABA bronchodilator, we were interested in determining the composite MA plus BA airway relaxant effects of the compound in guinea-pigs.
To achieve this, we studied the potency of GSK-961081 to relax MCh-induced isolated tracheal contraction in vitro, and inhibit MCh-induced bronchoconstriction in vivo, each in the absence of propranolol to allow both MA and BA mechanisms to be operative. In both assay systems, GSK-961081 produced effects that were more potent compared to each individual mechanism. While the overall leftward shift in potency in vitro was consistent with additive effects of MA plus BA mechanisms, we observed some evidence of synergy in the in vivo studies. By example, at the lowest dose tested (3 µg/mL) in the Einthoven model, GSK-961081 had no bronchoprotective effects via MA and BA mechanisms when assessed in complementary experiments in which one, or the other, mechanism is not functionally operative. In contrast, it produced a significant leftward displacement of the MCh bronchoconstrictor dose response relationship when both mechanisms are operational. While the clinical relevance of this finding is unclear, it is pertinent to note that evidence for functional ‘cross-talk’ between muscarinic and β₂-adrenoceptors has been reported in the literature (see Pera and Penn, 2014 for review).

Once-daily dosing is a highly desired property for an inhaled bronchodilator given that this feature improves patient compliance and potentially leads to greater overall efficacy (Cazzola and Matera, 2008). We evaluated duration of activity of GSK-961081 in both in vitro and in vivo assays. In the guinea-pig trachea, assessing the recovery of relaxation, following drug washout, is often used to gain confidence in the duration of airway relaxation. This measure is generally predictive of in vivo duration for several bronchodilators including tiotropium and salmeterol. In our studies, we measured the rate of recovery of relaxation of EFS contracted trachea to evaluate the duration of MABA relaxant effects. Similar to tiotropium, there was minimal recovery of GSK-961081-induced relaxation following 20 hr of drug-washout. In the guinea-pig Einthoven assay, there was less than 50% recovery of the GSK-961081’s bronchoprotective effect in 24 hr,
via MA, BA and MABA mechanisms with significant effects still observed seven days after a single inhaled dose. The mechanism underlying the sustained airway relaxant effects of GSK-961081 is unlikely to be slow receptor dissociation kinetics, as has been reported for tiotropium (Barnes et al., 1995), since we have shown that the dissociation half-life of the compound at both M₂ muscarinic, M₃ muscarinic and β₂-adrenoceptors is relatively short (t₁/₂ of 0.9, 3.2 and 17 mins, respectively). An alternate explanation for the sustained biological effects of GSK-961081 may be that its lipophilic nature enables it to associate with lipid membranes leading to slow elimination from smooth muscle tissue. This is one of the postulated mechanisms for the persistent smooth muscle relaxant effects of salmeterol (Coleman, 2009).

One of our key chemical design objectives during the research phase of the GSK-961081 discovery program was to incorporate physicochemical properties that would enable MABA compounds to be preferentially retained in the lung and slowly absorbed into the systemic circulation. This was intended to minimize the potential for systemic MA (e.g. dry mouth) or BA mediated adverse effects (e.g. heart rate increase, tremors). In guinea-pigs, muscarinic receptor agonism stimulates salivation and produces a depressor response (Powell et al., 1994; Aisaka et al., 1989) and β₂ adrenoceptor agonism produces a hypotensive response (Kobayashi et al., 1994). Inhaled GSK-961081 was 130–fold less potent than tiotropium in inhibiting systemic sialagogue responses to pilocarpine and 66–fold less potent than tiotropium in inhibiting depressor responses to MCh. GSK-961081 was approximately 50-fold less potent than salmeterol in evoking hypotensive effects in conscious guinea-pigs. The lung selectivity index of GSK-961081 was 55 to 110-fold greater than that of tiotropium with respect to systemic MA effects and was 10-fold greater than that of salmeterol with respect to systemic BA effects. These
observations are consistent with blunted systemic exposure of GSK-961081 with no active metabolites after inhalation dosing (pharmacokinetic data not shown).

The findings from these preclinical studies collectively demonstrate that GSK-961081 displays dual MABA pharmacological properties and produces potent and long-lasting bronchoprotection in guinea-pigs accompanied by minimal systemic MA and BA effects. These findings have been borne out in the clinic as inhaled GSK-961081 produced robust 24 hr bronchodilation without clinically relevant MA or BA systemic effects in COPD patients (Bateman et al., 2013). To the best of our knowledge, GSK-961081 is the first-in-class MABA and it has the potential to be a promising next-generation bronchodilator for the treatment of COPD.
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Authorship Contributions

Participated in research design: Hegde, Steinfeld, Jasper, Lee, McNamara, Martin, Pulido-Rios, Mammen.

Conducted experiments: Steinfeld, Lee, McNamara, Pulido-Rios.

Contributed new reagents or analytic tools: Hughes, Chen.

Performed data analysis: Hegde, Steinfeld, Jasper, Lee, McNamara, Martin, Pulido-Rios.

Wrote or contributed to the writing of the manuscript: Hegde, Hughes, Chen, Steinfeld, Jasper, Lee, McNamara, Martin, Pulido-Rios, Mammen.
REFERENCES


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor, which causes 50 per cent inhibition (IC$_{50}$) of an enzymatic reaction. *Biochemical Pharmacol* 22:3099-3108.


Footnotes

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Reprint requests: Sharath S. Hegde. 901 Gateway Blvd. South San Francisco, CA-94080. Email: shegde@theravance.com
Legends to figures:

**Fig. 1:** Chemical structure of GSK-961081

**Fig. 2:** Dose-response curves of GSK-961081 and comparator drugs for stimulation of cAMP in human lung epithelial (BEAS-2B) cells. Graphs are representative from n= 6-8 experiments.

**Fig. 3:** Dose-response for relaxant effects of GSK-961081 in isolated pre-contracted tracheal tissue of guinea pigs. The muscarinic antagonist (MA) and β₂ adrenoceptor agonist (BA) concentration-effects were obtained by determining relaxation of MCh pre-contracted tissue (in the presence of propranolol) and histamine pre-contracted tissue, respectively. The composite MABA potencies were obtained by determining relaxation of MCh pre-contracted tissue (in the absence of propranolol). Data showing antagonism of BA effects by propranolol (10 µM) is also shown. Data are expressed as mean±SEM (n = 4-14).

**Fig. 4:** Competitive antagonism by GSK-961081 of MCh contractile responses in the isolated guinea pig trachea. Data are expressed as mean±SEM (n = 4-7).

**Fig. 5:** Reversibility of the inhibitory effects of GSK-961081 on EFS contractions in guinea-pig isolated trachea. Tissues were exposed to vehicle, tiotropium (0.1 nM), atropine (0.1 µM) or GSK-961081 (100nM) until steady-state relaxation was achieved (~ 90%) after which the tissues were washed with drug-free Krebs for 20 hr. Data are expressed as mean±SEM (n = 4-5)

**Fig. 6:** Bronchoprotective effects of GSK-961081 in guinea pigs. Anesthetized guinea-pigs were challenged intravenously with the bronchoconstrictor agent at 1.5 hr after inhalation dosing with vehicle or drug. The muscarinic antagonist (MA) and β₂ adrenoceptor agonist (BA) effects were obtained by determining inhibition of MCh (in the presence of propranolol) and HIST
bronchoconstrictor responses, respectively. The MABA effects were obtained by determining inhibition of MCh bronchoconstrictor responses in the absence of propranolol. Data are expressed as mean±SEM (n = 8)

**Fig. 7:** Duration of bronchoprotective effects of GSK-961081 via MA, BA and MABA mechanisms. Anesthetized guinea-pigs were challenged intravenously with the bronchoconstrictor agent at various time-periods, in separate cohorts, after inhalation dosing with vehicle or GSK-961081 (100 µg/mL). Data are expressed as mean±SEM (n = 8-10). * p<0.05 compared to vehicle-treated group. # p<0.01 compared to MABA group.
Table 1  Radioligand binding affinity estimates (Ki) of GSK-961081 and comparator drugs determined by displacement of [³H]NMS at M₁-M₅ muscarinic receptor subtypes expressed in CHO cell membranes (n= 4-28). Data are expressed as ± S.D.

<table>
<thead>
<tr>
<th>Compound</th>
<th>hM₁</th>
<th>hM₂</th>
<th>hM₃</th>
<th>hM₄</th>
<th>hM₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK-961081</td>
<td>6.4 ± 1.8</td>
<td>1.4 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>5.4 ± 1.3</td>
<td>65 ± 23</td>
</tr>
<tr>
<td>Tiotropium</td>
<td>0.030 ± 0.014</td>
<td>0.036 ± 0.009</td>
<td>0.020 ± 0.005</td>
<td>0.021 ± 0.005</td>
<td>0.084 ± 0.016</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>1.4 ± 0.5</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>1.0 ± 0.4</td>
<td>3.0 ± 1.1</td>
</tr>
</tbody>
</table>
Table 2  Radioligand binding affinity estimates (Ki) of GSK-961081 and comparator drugs determined by displacement of $[^3]$H DHA at $\beta_1$ or $\beta_2$ adrenoceptors expressed in HEK293 cell membranes (n=28-60) or displacement of $[^125]$I-CYP at $\beta_3$ expressed in CHO cells membranes (n=15-45). Data shown are mean ± S.D.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\beta_1$ (nM ± S.D)</th>
<th>$\beta_2$ (nM ± S.D)</th>
<th>$\beta_3$ (nM ± S.D)</th>
<th>$\beta_1/\beta_2$</th>
<th>$\beta_3/\beta_2$</th>
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<tr>
<td>GSK-961081</td>
<td>456 ± 246</td>
<td>3.7 ± 1.4</td>
<td>1215 ± 1108</td>
<td>123</td>
<td>328</td>
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<tr>
<td>Salmeterol</td>
<td>2585 ± 1047</td>
<td>2.7 ± 0.8</td>
<td>&gt;10,000</td>
<td>957</td>
<td>&gt;3700</td>
</tr>
<tr>
<td>Formoterol</td>
<td>2160 ± 563</td>
<td>36.9 ± 15.3</td>
<td>1511 ± 1097</td>
<td>59</td>
<td>41</td>
</tr>
<tr>
<td>Albuterol</td>
<td>7160 ± 1746</td>
<td>1663 ± 682</td>
<td>&gt;10,000</td>
<td>4.3</td>
<td>&gt;6</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>370 ± 152</td>
<td>453 ± 118</td>
<td>445 ± 421</td>
<td>0.8</td>
<td>1</td>
</tr>
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</table>
Table 3  Agonist potency estimates (nM) of GSK-961081 and comparator drugs for cAMP stimulation in whole HEK-293 cells expressing hβ₁ and hβ₂ or CHO K1 cells expressing hβ₃ adrenoceptors (n=16-25). Selectivity was calculated based on ‘equipotent molar ratio (EMR)’ compared to isoproterenol as described in Methods. Intrinsic activity (I.A.) was determined in BEAS-2B cells (n=16-37). Data are expressed as mean ± S.D.

<table>
<thead>
<tr>
<th>Compound</th>
<th>β₁</th>
<th>β₂</th>
<th>β₃</th>
<th>β₁/β₂</th>
<th>β₃/β₂</th>
<th>BEAS-2B</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (nM ± S.D)</td>
<td>Selectivity</td>
<td>EC₅₀ (nM)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>GSK-961081</td>
<td>29 ± 10</td>
<td>0.29 ± 0.09</td>
<td>380 ± 240</td>
<td>440</td>
<td>320</td>
<td>3.6 ± 2.6</td>
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<tr>
<td>Salmeterol</td>
<td>410 ± 160</td>
<td>0.35 ± 0.12</td>
<td>1600 ± 1300</td>
<td>5500</td>
<td>850</td>
<td>0.44 ± 0.18</td>
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<tr>
<td>Formoterol</td>
<td>18 ± 9.6</td>
<td>0.23 ± 0.12</td>
<td>32 ± 23</td>
<td>310</td>
<td>25</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>Albuterol</td>
<td>1100 ± 500</td>
<td>30 ± 17</td>
<td>2100 ± 1000</td>
<td>160</td>
<td>17</td>
<td>237 ± 89</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>0.5 ± 0.2</td>
<td>2.0 ± 1.0</td>
<td>15 ± 11</td>
<td>-</td>
<td>-</td>
<td>35 ± 10</td>
</tr>
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Table 4  Potency estimates (nM) of GSK-961081 and comparator drugs for relaxation of pre-contracted guinea-pig isolated trachea. For GSK-961081, the muscarinic antagonist (MA) and β₂ adrenoceptor agonist (BA) potencies were obtained by determining relaxation of MCh pre-contracted tissue (in the presence of propranolol) and histamine pre-contracted tissue, respectively. The composite MABA potencies were obtained by determining relaxation of MCh pre-contracted tissue in the absence of propranolol. Data shown are mean (95% confidence interval), n=6-14.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MA</th>
<th>BA</th>
<th>MABA</th>
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<tr>
<td>GSK-961081</td>
<td>50.2 (38.5 – 65.5)</td>
<td>24.6 (19.4 – 31.3)</td>
<td>11.0 (8.7 – 13.9)</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>NA</td>
<td>98.4 (44.0 – 219.7)</td>
<td>NA</td>
</tr>
<tr>
<td>Tiotropium</td>
<td>1.1 (0.9 – 1.2)</td>
<td>NA</td>
<td>NA</td>
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Table 5  Systemic antimuscarinic and $\beta_2$ adrenoceptor effects of GSK-961081 and comparator drugs in guinea-pigs. Systemic antimuscarinic effects were evaluated by determining the potency of drugs to inhibit PILO-induced salivation (anti-sialogogue effects) and MCh-induced depressor response (anti-depressor effects). Systemic $\beta_2$ effects were evaluated by determining the magnitude of blood-pressure lowering produced by the drugs (hypotensive effects). Data shown are mean ± S.E.M. n=6

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/mL, IH)</th>
<th>Systemic Antimuscarinic Effects (% Inhibition)</th>
<th>$\beta_2$ Adrenoceptor Hypotensive Effects (% decrease in MAP)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-depressor Effects</td>
<td>Anti-sialogogue Effects</td>
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<tr>
<td>GSK-961081</td>
<td>0.5</td>
<td>3.07 ± 1.10</td>
<td>3.74 ± 12.3</td>
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<td></td>
<td>1.5</td>
<td>5.32 ± 2.01</td>
<td>35.0 ± 5.25</td>
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<td>4.5</td>
<td>9.28 ± 1.76</td>
<td>44.2 ± 7.05</td>
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<tr>
<td>Tiotropium</td>
<td>0.01</td>
<td>15.7 ± 4.99</td>
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<tr>
<td></td>
<td>0.03</td>
<td>24.9 ± 4.11</td>
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<tr>
<td></td>
<td>0.1</td>
<td>63.4 ± 12.8</td>
<td>73.3 ± 12.44</td>
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<tr>
<td></td>
<td>0.3</td>
<td>80.2 ± 6.99</td>
<td>99.1 ± 0.28</td>
</tr>
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<td>Salmeterol</td>
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<tr>
<td></td>
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<td>NA</td>
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<td></td>
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</table>
Fig. 1

![Chemical structure](attachment:chemical_structure.png)
Fig. 4

- Control
- GSK-961081 (10 nM)
- GSK-961081 (30 nM)
- GSK-961081 (100 nM)

% Max. Contraction vs. Log [MCh] (M)
Fig. 6

MA

BA

MABA

- Vehicle
- GSK-961081 (3 μg/ml, IH)
- GSK-961081 (10 μg/ml, IH)
- GSK-961081 (30 μg/ml, IH)
- GSK-961081 (100 μg/ml, IH)