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


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ABSTRACT

The objective of the present studies was to characterize the pharmacologic properties of GSK-961081 (TD-5959; (R)-1-[(2-chloro-4-[(2-hydroxy-2-oxo-1,2-dihydroquinolin-5-yl)ethyl]amino)methyl]-5-methoxyphenyl)amino)-3-oxopropyl)piperidin-4-yl [1,1-diastereomer] (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 broncho-protective effects of GSK-961081 were evident in guinea pigs via MA, BA, and MABA mechanisms for up to 7 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.

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 diseases, including chronic obstructive pulmonary disease. Two classes of inhaled bronchodilators, β2-adrenoceptor agonists (BAs) and muscarinic receptor antagonists (MAs), are currently available for use in the treatment of COPD. BAs and MAs 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 (see Perac and Penn, 2014, for review). MAs relax airways by reversing and parenchymal destruction (Decramer et al., 2012). Bronchodilators form the cornerstone of symptomatic pharmacologic 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. BAs and MAs cause bronchodilation through distinct but complementary mechanisms that potentially synergize with each other.
cholinergically-mediated bronchoconstriction via antagonism of M$_2$ and M$_3$ 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, such as tiotropium, and a long-acting bronchodilator (LABA), such as salmeterol or formoterol (Tashkin and Ferguson, 2013; Vestbo et al., 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 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 after inhalation dosing via two validated mechanisms in one molecule. Compounds with MABA activity offer a single pharmacokinetic profile for both pharmacologic activities, thereby maximizing the potential synergy between the two mechanisms, which would otherwise be difficult to achieve with coadministration of two separate compounds with distinct pharmacokinetic-pharmacodynamic profiles (Jacobsen et al., 2010). In addition, given the technical challenges associated with coformulation of two or more compounds in a single device, a MABA offers a conceptually simpler technical path toward 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 our studies was to characterize the in vitro and in vivo preclinical pharmacologic properties of GSK-961081. The binding affinity and functional potency of GSK-961081 were investigated at human recombinant receptors (muscarinic receptors and $\beta_2$-adrenoceptors expressed in Chinese hamster ovary-K1 [CHO-K1] or human embryonic kidney 293 [HEK293] cells, respectively), human BEAS-2B cells expressing native $\beta_2$-adrenoceptors, and guinea pig tracheal tissues expressing native muscarinic receptors and $\beta_2$-adrenoceptors. The in vivo bronchoprotective potency of inhaled GSK-961081 was assessed using the guinea pig Einthoven assay whereas the systemic antimuscarinic and $\beta_2$-adrenoceptor effects were evaluated using antisialogogue and hypotensive endpoints in guinea pigs.

**Materials and Methods**

$[^3]$H]-Methyl scopolamine methyl chloride was purchased from GE Healthcare (Piscataway, NJ). $[^3]$HDihydroalprenolol, $[^125]$I cyanopindolol, and guanosine 5’-O-3’[35S]thiotriphosphate (GTP$\gamma$S) were obtained from PerkinElmer (Waltham, MA). GSK-961081 was synthesized at Theravance, Inc. (South San Francisco, CA). Ipratropium bromide, atropine, scopolamine, oxotremorine sesquifumarate, iso-proteranol, salmeterol, and albuterol (salbutamol) were purchased from Sigma-Aldrich (St. Louis, MO). Formoterol fumarate was obtained from Tocris Bioscience (Bristol, UK). FLUO-4 AM was purchased from Molecular Probes/Invitrogen (Carlsbad, CA). Ipratropium was synthesized at Theravance, Inc. Sterile water was used as the vehicle for all in vivo studies.

**Animal Studies**

Studies were approved by the Institutional Animal Care and Use Committee at Theravance and conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility. The animals were housed in a climate controlled environment (12-hour light/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$_1$, hM$_2$, hM$_3$, hM$_4$, or hM$_5$ using $[^3]$H-methyl scopolamine methyl chloride (1 nM) as the radioligand. The assay plates containing membranes were incubated in buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl$_2$, 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 grade GF/B glass fiber filter plates pretreated with 1% bovine serum albumin using a 96-well harvester and measured using a TopCount scintillation counter (PerkinElmer). Atropine (10 $\mu$M) was used to define nonspecific binding.

**Recombinant Human $\beta_2$-Adrenoceptors.** The $\beta_2$-adrenoceptor binding was conducted using membranes prepared from HEK293 cells stably expressing the h$\beta_1$, or h$\beta_2$-adrenoceptors using $[^3]$H-dihydroalprenolol as the radioligand, or with h$\beta_2$ expressed in CHO cells using $[^125]$I cyanopindolol as the radioligand. The assay plates containing membranes were incubated in buffer (75 mM Tris/HCl, 12.5 mM MgCl$_2$, 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 pretreated with 1% bovine serum albumin using a 96-well harvester and measured using a TopCount scintillation counter. Propranolol (10 $\mu$M) and alprenolol (1 mM) were used to define nonspecific binding for h$\beta_1$-, h$\beta_2$- and h$\beta_3$-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 (Molecular Devices, Sunnyvale, CA) using FLUO-4 AM as the calcium-sensitive dye. CHO-K1 cells stably transfected with each receptor subtype were plated in 96-well plates and grown

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**Fig. 1.** Chemical structure of GSK-961081.
overnight at 37°C. The next day, the cells were gently washed and treated for 40 minutes at 37°C with FLUO-4 AM. 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. The EC90 concentration was determined by generating an agonist concentration-response curve with the same batch of cells before 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. The change in fluorescence was measured by the FLIPR for 3 minutes, and the peak height in fluorescence was taken as the 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 CHO-K1 cells expressing the hM2 receptor were treated with GSK-961081. The membranes were subsequently treated with the muscarinic receptor agonist oxotremorine for 1 hour to activate the receptors, enhancing Gαi/Gαo protein binding to exogenous [35S]GTPγS. Decreases in agonist-mediated [35S]GTPγS binding by GSK-961081 were indicative of functional antagonism of the receptor. To measure the intrinsic activity (IA) of GSK-961081 toward the hM2 receptor, the membranes described here were treated with GSK-961081 for 1 hour in the absence of agonist, and binding of [35S]GTPγS to the Gαi/Gαo proteins in the membrane was then measured.

Recombinant Human β-Adrenoceptors. Functional potency was measured in HEK or CHO-K1 cells stably expressing human β1-, β2-, or β3-adrenoceptors that were stimulated with serially diluted agonists at 37°C for 10 minutes. Whole-cell cAMP was measured using the Flashplate Adenylyl Cyclase Activation Assay System with [3H]cAMP (PerkinElmer), according to the manufacturer’s instructions. To measure the IA of β2-adrenoceptor agonists, the cAMP assay was performed in human bronchial epithelial cells (BEAS-2B; American Type Culture Collection, Manassas, VA, licensed from the National Institutes of Health) expressing endogenous levels of β2-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, the medium was switched to LHC 8 containing no epinephrine or retinoic acid.

Guinea Pig Isolated Trachea. Airway smooth muscle relaxation mediated by MA, βA, and MABA mechanisms was measured in trachea isolated from Harlan Dunkin-Hartley guinea pigs (male, 200–500 g; Harlan, Indianapolis, IN). Animals were euthanized by CO2 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 Na-glucose, 1.64 mM MgSO4, 1.18 mM KH2PO4, 4.7 mM KCl, 118 mM NaCl, 24.88 mM NaHCO3, 2.52 mM CaCl2) supplemented with choline (1 M) to block ganglionic sympathetic nerve transmission. The buffer was constantly aerated with carbogen (95% O2 and 5% CO2). The isolation of the guinea pig trachea was done using a force transducer (Model Fort 100; World Precision Instruments, Sarasota, FL) that was connected to a four-channel amplifier (Model S48; Astro-Med, West Warwick, RI). Data were recorded by an electronic data acquisition system (Model MP 100/UiM 100A; Biopac Systems, Goleta, CA).

The tracheal rings were initially tensioned to 1 g and allowed to equilibrate for 1 hour before evoking contraction with a submaximal concentration of either methacholine (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 precontracted 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 (KbA), 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 minutes for 1 hour 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 minutes, followed by construction of a second MCh curve.

Duration of MABA-induced relaxation was evaluated using electric field–stimulated (EFS) guinea pig trachea. The 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 gualenethidine (3 μM). Tracheal rings were opened by a transverse cut before mounting under 1 g tension between two platinum electrodes in a tissue bath. EFS was applied under the following conditions: 9 V, 1-millisecond pulse duration, 10 Hz for 10 seconds every 100 seconds (Grass Stimulator [S88]; Grass Technologies, West Warwick, RI). After the equilibration period, the 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. The 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 elsewhere by using the modified Eithenough assay (McNamara et al., 2011). Male Duncan Hartley guinea pigs (Harlan), 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 22FS1; PARI Respiratory Equipment, 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 hours) after inhalation dosing using separate cohorts of animals for each pretreatment period.

Forty-five minutes before the start of pulmonary function evaluation, animals were anesthetized with an intramuscular injection of ketamine (43.7 mg/kg) and xylazine (3.5 mg/kg) intraperitoneal (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 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 663; Harvard Apparatus, Holliston, MA) set at a stroke volume of 1 ml/100 g body weight but not exceeding 2.5 ml volume, and at a rate of 100 strokes per minute. A T-connector was attached to the respiratory expiratory tubing to measure changes in ventilation pressure (VP) using a Biopac transducer that was connected to a Biopac (TSD 137C) preamplifier. Body temperature was maintained at 37°C using a heating pad. Before we initiated data collection, we administered pentobarbital (25 mg/kg i.p.) 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, noncumulative intravenous doses of the bronchoconstrictor (MCh or HIS) in 2-fold increments. The MA, BA, and MABA bronchoprotective potentials were estimated by obtaining bronchoconstrictor dose–response curves to intravenous MCh (after pretreatment with propranolol, 5 mg/kg i.v.) or 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

Antisialogogue Activity. Male guinea pigs (Harlan) weighing 200–350 g were employed for these studies. Test compounds or vehicle were dosed via inhalation (IH) as described earlier. At varying times after IH dosing, guinea pigs were anesthetized with a cocktail mixture of ketamine (43.7 mg/kg i.m.), xylazine (3.5 mg/kg i.m.), and acepromazine (1.05 mg/kg i.m.). 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 preweighed gauze pad (Nu Gauze general use sponges; Johnson & Johnson, Arlington, TX) was inserted in the animal’s mouth, and the muscarinic agonist pilocarpine (PILO; 3 mg/kg s.c.) was administered. Saliva produced during the 10 minutes after 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 polyethylene catheter (PE-50 tubing). The catheters were exteriorized using a subcutaneous tunnel to the subscapular area. All surgical incisions were sutured with 4-0 Ethicon silk (Johnson & Johnson), and the catheters were locked with heparin (1000 units/ml). Each animal was administered saline (3 ml s.c.) and buprenorphine (0.05 mg/kg i.m.) 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 hours after 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 minutes. After the acclimation period, baseline hemodynamic recordings were recorded for 5–10 minutes. Three control depressor responses to MCh (0.3 µg/kg i.v.) were obtained at 15-minute intervals. The animals were subsequently dosed by inhalation with nebulized drug or vehicle using a whole-body dosing chamber (as described earlier). The intrinsic hemodynamic effects of the drug were assessed during and 60 minutes after inhalation. At 5 minutes, 1.5 hours, and 24 hours after dosing, the animals were rechallenged with MCh. At the end of the study, the animals were humanely euthanized by CO₂ asphyxiation followed by a thoracotomy.

Data Analysis

Radioisotopic Binding Competition Studies. The binding data were analyzed by nonlinear regression analysis using GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA). The Kᵦ values for GSX-961081 and comparator compounds were calculated from observed IC₅₀ values according to Cheng and Prusoff (1973).

Functional Studies. The FLPER and [³²P]GTPγS binding data were analyzed by nonlinear regression analysis using GraphPad Prism 3.0. Antagonist Kᵦ values were determined by GraphPad Prism 3.0 using the oxotremorine EC₅₀ value as the Kᵦ and the oxotremorine EC₅₀ for the ligand concentration according to the Cheng Prusoff equation (Cheng and Prusoff, 1973). Kᵦ was reported instead of Kᵦ because the data were not generated under equilibrium conditions.

For the β₂-cAMP studies, data were analyzed by nonlinear regression analysis with the GraphPad Prism 3.0 using the four-parameter logistic model with variable slope. Potency data are reported as EC₅₀ values. Equiconttent molar ratios (EMR) for test compounds were calculated relative to isoproterenol as:

EMR = EC₅₀ (test compound)/EC₅₀ (isoproterenol)

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

Sel(β₂/β₁) = EMR(β₁)/EMR(β₂)

In guinea pig isolated trachea studies conducted to determine potency for inhibition of MCh or EFS precontracted tissues, inhibition curves were fitted using a nonlinear regression analysis with GraphPad Prism 3.0 to estimate EC₅₀. In studies where the MCh curve was shifted dextrally with increasing concentrations of the muscarinic antagonist, concentration ratios were calculated as the ratio of agonist EC₅₀ in the presence and absence of antagonist, and pA₂ affinity estimates were estimated using the method described by Arunlakshana and Schild (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. A change in VP was measured in centimeters of H₂O. The dose response curve to MCh or HIS was fitted to a four-parameter logistic equation using GraphPad Prism 3.0. The average bronchococontractor response to MCh (16 µg/kg i.v.) or HIS (52 µg/kg i.v.) in vehicle-treated animals was calculated and used to compute the percentage 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. ID₅₀ was defined as the dose required to produce 50% inhibition of the bronchoconstrictor response.

Systemic Muscarinic Antagonist and β₂-Adrenoceptor Agonist Effects

Salivation Assay. The mean weight of saliva in vehicle-treated animals was calculated and used to compute the percentage of inhibition of saliva in each of the drug-treated animals. Antisialogogue ID₅₀ (the 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), and the mean arterial pressure (MAP) was calculated using the
GSK-961081 displayed approximately 39- to 65-fold lower affinity for hM1, hM2, hM3, and hM4 receptors and moderate affinity for hM2 and hM3 receptor subtypes (data not shown). In functional studies, GSK-961081 potently antagonized 2-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 three receptors subtypes (see Materials and Methods). 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 2). 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 three receptors subtypes (see Materials and Methods). 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 IA 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. IAIs 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).

### 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- to 65-fold lower affinity than tiotropium and 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-961081s 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- 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.

### Table 2

Radioligand binding affinity estimates (Ki) of GSK-961081 and comparator drugs determined by displacement of [3H]dihydroalprenolol at β1- or β2-adrenoceptors expressed in HEK293 cell membranes (n = 28–60) or displacement of [3H]cyanopindolol at β3 expressed in CHO-K1 cell membranes (n = 15–45).

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<th>Compound</th>
<th>Ki (μM)</th>
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<td>β2</td>
<td>β3</td>
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<td>0.95 ± 0.07</td>
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<tr>
<td>Albuterol</td>
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<td>2100 ± 1000</td>
<td>237 ± 89</td>
<td>0.41 ± 0.01</td>
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<tr>
<td>Isoproterenol</td>
<td>0.07 ± 0.02</td>
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<td>35 ± 10</td>
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### Table 3

Agonist potency estimates of GSK-961081 and comparator drugs for cAMP stimulation in whole HEK293 cells expressing hβ1 and hβ2 or CHO-K1 cells expressing hβ3-adrenoceptors (n = 16–25).

Selectivity was calculated based on EMR compared with isoproterenol as described in Materials and Methods. IA was determined in BEAS-2B cells (n = 16–37). Data are expressed as mean ± S.D.

<table>
<thead>
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<th>Compound</th>
<th>EC50 (nM)</th>
<th>Selectivity</th>
<th>EC50 (nM)</th>
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<td>15 ± 11</td>
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</table>
Activity at Muscarinic and β2-Adrenoceptors in Guinea Pig Isolated Trachea

In tracheal tissues precontracted with MCh (in the presence of propranolol to isolate the MA component), GSK-961081 and tiotropium produced concentration-dependent relaxation, with an EC₅₀ of 50.2 and 24.6 nM, respectively (Fig. 3; Table 4). 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 Schid 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 pKᵢ estimate of 9.1 ± 0.1 was obtained when the Schid slope was constrained to unity.

In tissues precontracted with HIS (to isolate the BA component), GSK-961081 and salmeterol produced concentration-dependent relaxation with an EC₅₀ of 25 and 100 nM, respectively (Fig. 3; Table 4). 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 (EC₅₀ = 5 μM).

In tracheal tissues precontracted 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 EC₅₀ of 10 nM that was significantly lower than the IC₅₀s for MA and BA components.

In EFS washout studies, GSK-961081 (0.1 μM), tiotropium (3 nM), and atropine (0.1 μM) produced approximately 90% inhibition of contractile responses. During the 20-hour 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 hours 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 M₃ 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 D₁ (Kᵢ = 61 nM), human H₁ (Kᵢ = 98 nM), and human I₂ (Kᵢ = 320 nM) receptors and weak agonist activity at the human 5-HT₄ (IA 18% of 5-HT [serotonin]). 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 i.v.) 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 hours after 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

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**Table 4**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MA EC₅₀ (μM)</th>
<th>BA EC₅₀ (μM)</th>
<th>MABA EC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<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>
</tr>
</tbody>
</table>

NA, not applicable.

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**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 precontracted tracheal tissue of guinea pigs. The MA and BA concentration effects were obtained by determining the relaxation of MCh precontracted tissue (in the presence of propranolol) and histamine precontracted tissue, respectively. The composite MABA potencies were obtained by determining the relaxation of MCh precontracted tissue (in the absence of propranolol). Data showing antagonism of BA effects by propranolol (10 μM) is also shown. Data are expressed as mean ± S.E.M. (n = 4–14).

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**Fig. 4.** Linear regression analysis of the Schid 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 pKᵢ estimate of 9.1 ± 0.1 was obtained when the Schid slope was constrained to unity.

---

**Fig. 5.** In EFS washout studies, GSK-961081 (0.1 μM), tiotropium (3 nM), and atropine (0.1 μM) produced approximately 90% inhibition of contractile responses. During the 20-hour 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 hours after washout was initiated (Fig. 5).
observed for up to 7 days after inhalation of 100 \( \mu g/ml \) dose (Fig. 7). At all time points, the MABA effects were numerically greater than that of MA and BA alone. At 24 hours after dosing, both the MA and BA effects were significantly lower than the MABA effect. At 1.5, 8, and 48 hours, 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 near-maximal dose was studied and the experiment did not have adequate statistical power.

**Systemic Antimuscarinic (Antidepressor and Antisialagogue) and \( \beta_2 \)-Adrenoceptor Hypotensive Effects in Guinea Pigs**

**Antidepressor Antimuscarinic Effects.** In vehicle-treated animals, MCh decreased mean blood pressure by 26.1 ± 1.3 mm Hg. Tiotropium, at 1.5 hours after dosing, produced dose-dependent inhibition of MCh-induced depressor responses (ID\(_{50} \) = 68.7 \( \mu g/ml \) IH) (Table 5). GSK-961081, at 1.5 hours after the dose, also produced significant inhibition of MCh-induced depressor responses (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 hours. In contrast, GSK-961081 inhibitory effects on salivation have a faster onset, which peaks at 1.5 hours after inhalation. We subsequently conducted a dose-response study with tiotropium and GSK-961081 at 1.5 and 6.0 hours after inhalation. Tiotropium produced dose-dependent inhibition of salivation with an ID\(_{50} \) of 38.8 \( \mu g/ml \) IH (Table 5). GSK-961081 at 1.5 hours after the dose, also produced significant inhibition of PILO-induced salivation (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 mm Hg 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 heart rate (data not shown). Salmeterol produced dose-dependent decreases in MAP (Table 5) with no consistent effects on heart rate (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 \( \beta_2 \) 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 pharmacologic properties to provide bronchodilation superior to existing monotherapies and simplifying the attainment of triple therapy through coformulation with inhaled corticosteroids (Jacobsen et al., 2010; Hughes et al., 2012). Using MABA prototypic compounds, THRX-198321 [biphenyl-2-yl-carbamic acid 1-\([\{(R)-2-hydroxy-2-(8-hydroxy-2-oxo-1,2-dihydro-quinolin-5-yl)-ethylamino\}-nonyl\]-piperidin-4-yl ester] and THRX-200495 [biphenyl-2-yl-carbamic acid 1-\([\{(4-[2-\{(R)-2-hydroxy-2-(8-hydroxy-2-oxo-1,2-dihydro-quinolin-5-yl)ethylamino\}-ethyl\}-phenoxyl]-phenyl\}-propyl\]-piperidin-4-yl ester], 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 \( \beta_2 \)-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 pharmacologic 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. The potency of GSK-961081 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 (Barnes, 1993). 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 that this may compromise the bronchodilatory actions of nonselective 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 cotransmitters 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, the potency of GSK-961081 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. 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, the potency of GSK-961081 was comparable to that of salmeterol and formoterol while the compound’s IA was intermediate between the two standards. The clinical significance of IA assessed in cellular assays in vitro is poorly understood. Nevertheless, a level of IA equal to or greater than salmeterol, a LABA that produces robust bronchodilation in patients, is desirable.

**Fig. 6.** Bronchoprotective effects of GSK-961081 in guinea pigs. Anesthetized guinea pigs were challenged intravenously with the bronchoconstrictor agent at 1.5 hours after inhalation dosing with vehicle or drug. The MA and BA effects were obtained by determining inhibition of MCh (in the presence of propranolol) and HIS 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 ± S.E.M. (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 ± S.E.M. (n = 8–10). *P < 0.05 compared with vehicle-treated group; #P < 0.01 compared with MABA group.
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 IA 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 $\beta_2$-adrenoceptors. Based on affinity and agonist potency values, GSK-961081 displayed $>100$-fold selectivity for the $\beta_2$- over the $\beta_1$- and $\beta_2$-adrenoceptors. Although 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 2-fold. The MA and BA bronchoprotective potencies of GSK-961081 were roughly 6- and 8-fold lower than those previously reported for tiotropium and salmeterol (McNamara et al., 2011), respectively.

Because 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 to 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 with each individual mechanism. Although 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 $\mu$g/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 bronchoprotective dose-response relationship when both mechanisms are operational. The clinical relevance of this finding is unclear, but it is pertinent to note that evidence for functional cross-talk between muscarinic and $\beta_2$-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 after 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 after 20 hours of drug washout. In the guinea pig Einthoven assay, there was less than 50% recovery of the bronchoprotective effect of GSK-961081 in 24 hours, via MA, BA, and MABA mechanisms with significant effects still observed 7 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) because we have shown that the dissociation half-life of the compound at both M2 muscarinic, M3 muscarinic, and \( \beta_2 \)-adrenoceptors is relatively short (half-life of 0.9, 3.2, and 17 minutes, respectively). An alternate explanation for the sustained biologic 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-mediated (e.g., dry mouth) or BA-mediated (e.g., heart rate increase, tremors) adverse effects. In guinea pigs, muscarinic receptor agonism stimulates salivation and produces a depressor response (Aisaka et al., 1989; Howell et al., 1994), and \( \beta_2 \)-adrenoceptor agonism produces a hypotensive response (Kobayashi et al., 1994). Inhaled GSK-961081 was 130-fold less potent than tiotropium in inhibiting systemic dialogue 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 pharmacologic 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-hour 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

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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


