In Vitro Pharmacological Characterization of Vilanterol, a Novel Long-Acting \( \beta_2 \)-Adrenoceptor Agonist with 24-Hour Duration of Action


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ABSTRACT

Vilanterol trifenatate (vilanterol) is a novel, long-acting \( \beta_2 \)-adrenoceptor (\( \beta_2 \)-AR) agonist with 24 h activity. In this study, we describe the preclinical pharmacological profile of vilanterol using radioligand binding and cAMP studies in recombinant assays as well as human and guinea pig tissue systems to characterize \( \beta_2 \)-AR binding and functional properties. Vilanterol displayed a subnanomolar affinity for the \( \beta_2 \)-AR that was comparable with that of salmeterol but higher than olopatadine, formoterol, and indacaterol. In cAMP functional activity studies, vilanterol demonstrated similar selectivity as salmeterol for \( \beta_2 \)- over \( \beta_1 \)-AR and \( \beta_2 \)-AR, but a significantly improved selectivity profile than formoterol and indacaterol. Vilanterol also showed a level of intrinsic efficacy that was comparable to indacaterol but significantly greater than that of salmeterol. In cellular cAMP production and tissue-based studies measuring persistence and reassertion, vilanterol had a persistence of action comparable with indacaterol and longer than formoterol. In addition, vilanterol demonstrated reassertion activity in both cell and tissue systems that was comparable with salmeterol and indacaterol but longer than formoterol. In human airways, vilanterol was shown to have a faster onset and longer duration of action than salmeterol, exhibiting a significant level of bronchodilation 22 h after treatment. From these investigations, the data for vilanterol are consistent, showing that it is a novel, potent, and selective \( \beta_2 \)-AR receptor agonist with a long duration of action. This pharmacological profile combined with clinical data is consistent with once a day dosing of vilanterol in the treatment of both asthma and chronic obstructive pulmonary disease (COPD).

Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are both chronic diseases of the airways imposing significant burdens on healthcare systems worldwide. The major characteristics of asthma include airflow obstruction, bronchial hyper-responsiveness, and inflammation (Busse and Lemanske, 2001). It involves a number of inflammatory cells and mediators (including histamine, leukotrienes, and cytokines) that cause a reversible airflow obstruction. In contrast, COPD is characterized by a progressive reduction in airflow that cannot be fully reversed. This is linked with an abnormal inflammatory response with thickening and inflammation of the airways due to an uncontrolled infiltration of inflammatory cells into the lungs, primarily as a result of the exposure to noxious particles from cigarette smoking (Celli et al., 2004; Kardos and Keenan, 2006). \( \beta_2 \)-Adrenoceptor (\( \beta_2 \)-AR) agonists play a major role in the management of both asthma and COPD by increasing airflow into the lungs via relaxation of the smooth muscle in the bronchial airway by acting directly on the \( \beta_2 \)-AR G-protein coupled receptor (GPCR). Stimulation of the \( \beta_2 \)-AR activates G-protein \( \alpha \) subunit that stimulates adenylyl cyclase to increase the intracellular levels of 3'-5'-cyclic adenosine monophosphate (cAMP). This in turn activates protein kinase A (PKA) that can cause smooth-muscle relaxation either via a reduction in myosin-regulatory light chain kinase activity or phosphorylation of Ca\(^{2+}\)-dependent \( K \) channels, though

ABBRVIATIONS: ANOVA, one-way analysis of variance; \( \beta_2 \)-AR, \( \beta_2 \)-adrenoceptor; CGP12177, (\(-\)-[3-[1,1-Dimethylethyl]amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one hydrochloride; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; CR, concentration ratio; CRC, concentration response curve; DMSO, dimethyl sulfoxide; EFS, electrical field stimulation; FCS, fetal calf serum; FP, fluorescence polarization; GPCR, G-protein-coupled receptor; Gpp(NH)p, guanosine 5'-[\( \beta \),\( \gamma \)-imido]triphosphate; HBSS, Hanks’ balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; ICI 118551, (\(-\)-[3-Dihydro-7-methyl-1H-inden-4-yl]oxy)-3-[1-methylethyl]amino]-2-butanol hydrochloride; ICS, inhaled corticosteroid; LABA, long-acting \( \beta_2 \)-AR agonist; LAMA, long-acting muscarinic antagonist; LS, liquid scintillation; NSB, nonspecific binding; PBS, phosphate-buffered saline; PCLS, precision-cut lung slice; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; t1/2, half-life.
stimulation of the latter with β₂-AR agonists has also been shown to be PKA independent (Johnson and Druey, 2002).

Since the development of short-acting β₂-AR agonists such as salbutamol in the 1960s (Cullum et al., 1969), continued research in this area has endeavored to develop molecules with a longer duration of action and provide better control of symptoms and lung function. As a consequence, salmeterol (Johnson et al., 1993) and formoterol (Anderson, 1993) were developed, providing relief for at least 12 h. These long-acting β₂-AR agonists (LABA) are used as twice daily bronchodilators in both asthma and COPD. Over recent years, there have been concerns over the use of LABAs as monotherapies since the publication of the Salmeterol Multicenter Asthma Research Trial (SMART) (Nelson et al., 2006). However, despite these concerns, LABAs remain a key therapy for the management of asthma with a low to medium dose of an inhaled corticosteroid (ICS) (Bateman et al., 2008). This combination has been shown to give improved control of asthma with good evidence showing that asthmatic patients treated with a single inhaler containing both LABA and ICS experience fewer exacerbations than similar patients on ICS alone (Hirst et al., 2010). Since the late 1990s, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (Malerba et al., 2012) has recommended the use of LABAs in the treatment of COPD patients in stages II to IV of the disease. As with asthma, the use of combination therapy has been extended further for COPD patients with the alliance of a LABA and a long-acting muscarinic antagonist (LAMA). This combination has been shown to produce significant improvements in lung function and other outcomes compared with the use of the LABA or LAMA alone (Cazzola and Molinard, 2010). In addition, a major problem in the management of both asthma and COPD continues to be poor patient compliance and adherence to treatment plans due to the frequency of dosing regimens and the complexities of delivery. Therefore, development of the next generation of LABAs, which are fast acting and have 24-h duration of action on airflow, could improve patient convenience and, therefore, compliance within both these disease populations (Cazzola et al., 2011).

Vilanterol trifenatate (vilanterol) (Fig. 1) is a novel LABA with 24 h activity in development for inhaled once daily administration in combination with an ICS for both COPD and asthma and once daily treatment in combination with a LAMA for COPD (Hanania et al., 2012; Lotvall et al., 2012). In this study, we describe the preclinical pharmacological profile of vilanterol using radioligand binding and cAMP production studies in recombinant cell and membrane fragment systems as well as human and guinea pig tissue systems to characterize β₂-AR binding and functional properties.

Materials and Methods

Indacaterol, isoprenaline, salbutamol, salmeterol, and vilanterol were synthesized by the Respiratory TAU Medicinal Chemistry Department at GlaxoSmithKline Medicines Research Centre (Stevenage, UK). The chemical synthesis of vilanterol is detailed in Procopiou et al. (2010). CGP12177 (1-4-[3-[1,1-Dimethylamino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazo[2-one hydrochloride), formoterol, ICI118551 ([1-2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[1-methylthyl(aminio)]-2-butanol hydrochloride), propranolol, sotalol, and all other chemicals were purchased from Sigma-Aldrich Co. Ltd. (Gillingham, UK) unless otherwise stated. All cell culture media and reagents were obtained from Invitrogen Ltd. (Paisley, UK). [3H]Vilanterol (specific activity 92 Ci/mmol) was synthesized by Quotient Bioresearch (Radiochemicals) Ltd. (Cardiff, UK). [3H]CGP12177 (specific activity 41 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences UK Ltd. (Beaconsfield, UK). All studies were completed with a final dimethyl sulfoxide (DMSO) concentration of 1% unless otherwise stated.

Cell Culture Techniques

Chinese hamster ovary (CHO) cells stably expressing recombinant human adrenoceptors (β₁, β₂, or β₃; GlaxoSmithKline) were maintained in culture in Dulbecco’s modified Eagle’s medium/F12, supplemented with 5% fetal calf serum (FCS), 2 mM l-glutamine, 200 µg/ml Geneticin (G418; Life Technologies, Grand Island, NY), and 100 µg/ml hygromycin B in 95%:5% air:CO₂ at 37°C. Cells were harvested by treatment with Hanks’ balanced salt solution (HBSS; Ca²⁺ and Mg²⁺ free) and 0.6 mM EDTA for 10 min, washed in phosphate-buffered saline (PBS), and then frozen in 80% dialyzed FCS:20% DMSO. One-milliliter aliquots were then stored at −140°C until required for use in the LANCE cAMP assay.

Membrane Preparation

CHO cells stably expressing recombinant human β₂-AR were harvested by treatment with HBSS and 0.6 mM EDTA for 10 min. The cells were then centrifuged at 300g at 4°C for 10 min, and the cell pellet was resuspended in assay buffer [50 mM HEPES containing 0.1 mM leupeptin, 25 µg/ml bacitracin, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 µM peptatin A]. The cell pellet was homogenized using a glass Waring blender (Waring, CT), and the cell suspension was spun at 500g for 20 min at 4°C. The resulting supernatant was spun for a further 30 min at 48,000g at 4°C. The pellet was resuspended in assay buffer (without PMSF and peptatin A), and the protein concentration was determined using the bicinchoninic acid method described by Smith et al. (1985) using bovine serum albumin as a standard. The membrane suspensions were frozen in aliquots at −80°C until required.

Radioligand Binding Studies

General Protocols for β₂-AR Binding Assays. All radioligand binding experiments were performed in 96-deep well plates at ambient temperature (20–22°C) (unless otherwise stated) in binding buffer (50 mM HEPES, 100 mM NaCl and 10 mM MgCl₂, pH 7.4) with [3H]Vilanterol or [3H] CGP12177, β₂-AR membranes (µg/well dependent on the B_max of individual membrane fragment preparations), and either vehicle or unlabeled β₂-AR agonists/antagonists at varying concentrations. Nonspecific binding (NSB) values determined by 10

![Fig. 1. Chemical structure of the novel, long-acting β₂-AR agonist vilanterol.](image-url)
μM ICI118551 were used to calculate specific binding of [3H]vilanterol or [3H]CGP12177. Plates were incubated with gentle agitation for the time periods indicated and binding terminated by rapid vacuum filtration through a 48-well Brandel harvester (Brandel Inc. Gaithersburg, MD) onto GF/B filter papers presoaked in 0.3% v/v polyethyleneimine. Samples were washed rapidly three times with ice-cold binding buffer and filters transferred into liquid scintillation (LS) vials containing 4 ml of LS fluid (Ultima-Flo M; PerkinElmer Life and Analytical Sciences). The amount of radioligand bound to receptor was measured by LS spectroscopy using a TriCarb 2000 TR LS counter (PerkinElmer Life and Analytical Sciences). By the same method the concentration of total radioligand added to each well was calculated for data analysis and also to ensure that <10% of radioligand was bound thus preventing issues associated with depletion of free radioligand in the system. To determine binding parameters at the low- and high-affinity agonist states of the receptor i.e., G-protein uncoupled and coupled form of the receptor, respectively, certain radioligand experiments (as indicated in Results) were completed in the presence and absence of 100 μM guanosine 5′-β,γ-imido[triphosphate [Gpp(NH)p], a nonhydrolyzable analog of the nucleotide guanosine triphosphate. To confirm the observations in the β2-AR CHO membranes were due to binding to the β2-AR receptor alone, [3H]vilanterol binding in control membranes generated from untransfected CHO cells was tested with no specific [3H]vilanterol binding observed (data not shown).

Radioligand Characterization. Saturation, association, and dissociation binding studies were performed for [3H]vilanterol to determine receptor binding kinetics at the β2-AR (equilibrium dissociation constant (K_D), total number of receptors (B_max), association rate (k_on), and dissociation rate (k_off) were calculated as described under “Data Analysis”). For saturation binding, membranes (in a volume of 1.4 ml to avoid ligand depletion) were incubated with increasing concentrations of [3H]vilanterol (~0.01–1.3 nM) for 5 h before filtration. For association binding, membranes were incubated with different concentrations of [3H]vilanterol (~0.1–1.9 nM) for varying incubation times up to 1 h before filtration. For dissociation binding, membranes were preincubated for 1 h with a fixed concentration of [3H]vilanterol (~1.1 nM) before dissociation was initiated by a 1:20 dilution in binding buffer (containing 10 μM cold vilanterol) and then incubated for varying times up to 8 h before filtration. Saturation binding was also completed for [3H]CGP12177 (increasing concentrations of ~0.01–2.8 nM) in the same format as described above for [3H]vilanterol.

Determination of β2-AR Agonist and Antagonist Affinity. To determine the affinity of β2-AR agonists and antagonists, competition binding displacement studies were completed in which membranes were incubated with a fixed concentration of [3H]vilanterol (~0.2 nM) and increasing concentrations of unlabeled agonist/antagonist for 5 h before filtration. All competition binding displacement studies were completed in the presence of 100 μM Gpp(NH)p to ensure that binding curves were monophonographic.

LANCE cAMP Assays

General Protocols for LANCE cAMP Assays. Increases in cAMP were determined using a LANCE cAMP assay kit (PerkinElmer Life and Analytical Sciences) as per the kit instructions for either 96- or 384-well plate formats. In brief, CHO cells expressing either β1, β2, or β2-AR were thawed at 37°C; diluted in PBS; and centrifuged at 300g for 5 min. Cells were then resuspended in stimulation buffer (HBSS containing 0.1% (v/v) bovine serum albumin, 500 μM 3-isobutyl-1-methylxanthine (IBMX), and 5 mM HEPES, pH 7.4) to the required concentration.

β-Adrenoceptor Subtype Selectivity. Ten thousand cells per well of β1, β2, or β2-AR CHO cells were added to white 384-well plates containing β2-AR agonist concentration response curves (CRC), followed by the addition of antibody solution (stimulation buffer containing Alexia Fluor 647). Plates were incubated for 30 min at ambient temperature (20–22°C) before detection mixture was added (kit components; detection buffer, europium W8044-labeled streptavidin and biotin-cAMP). Plates were covered and incubated at ambient temperature (20–22°C) for 4 h before reading on a ViewLux Microplate Imager (PerkinElmer Life and Analytical Sciences).

β2-AR Agonist Persistence and Reassertion. Methodology to determine the persistence (duration of effect) and reassertion behavior (blockade of agonist receptor activation with an antagonist followed by the restoration of an agonist effect postwashout of all agents with no subsequent agonist addition) of β2-AR agonists were adapted from procedures previously described (Summerhill et al., 2008; Patel et al., 2011). In brief, 55,000 cells/well of β2-AR CHO cells were added to clear, V-bottomed, 96-well plates and then treated with one of the conditions detailed below. β2-AR agonist CRCs were tested with all incubations carried out at 37°C unless otherwise stated. Cell washing was achieved by spinning plates at 300g for 5 min, followed by aspiration of supernatant and resuspension in stimulation buffer.

Condition 1 (control): Cells were incubated with vehicle (1% DMSO) for 30 min, washed three times, and β2-AR agonist was added after additional 30-min incubation.

Condition 2 (agonist washout): Cells incubated with β2-AR agonist for 30 min, washed three times, and incubated for another 30 min.

Condition 3 (control + sotalol): Cells were incubated with 1% DMSO for 30 min, washed three times, and 100 μM sotalol (β2-AR adrenergic receptor antagonist) β2-AR agonist was added after additional 30-min incubation.

Condition 4 (agonist washout + sotalol): Cells incubated with 100 μM sotalol/β2-AR agonist for 30 min, washed three times, and incubated for another 30 min. Plates were then spun at 300g for 5 min, supernatant were aspirated, and cells were resuspended in stimulation buffer. Cell suspension containing 20,000 cells from each well was transferred to a white, flat-bottomed, 96-well plate containing antibody solution (stimulation buffer containing Alexia Fluor 647). Plates were incubated for 45 min at ambient temperature (20–22°C) before detection mixture was added (kit components: detection buffer, europium W8044-labeled streptavidin and biotin-cAMP). Plates were then covered and incubated at ambient temperature (20–22°C) for 4 h before reading on an EnVision Multilabel Reader (PerkinElmer Life and Analytical Sciences). A concentration ratio (CR) for β2-AR agonist persistence was calculated as binding agonist washout:EC50 (condition 2) by the control agonist EC50 (condition 1) with a low CR value suggesting a long persistence of agonist activity. A CR for β2-AR agonist reassertion was calculated by dividing the agonist washout + sotalol EC50 (condition 4) by the control agonist EC50 (condition 1) with a low CR value suggesting agonist reassertion postantagonist washout.

Fluorescent Polarization cAMP Assay

Intrinsic efficacy was measured by increases in cAMP levels in CHO β2-AR membranes using a [FP]2 cAMP assay kit (PerkinElmer Life and Analytical Sciences) as per the kit instructions. In brief, membranes were thawed and diluted in assay buffer (50 mM HEPES, 10 μM IBMX, 10 mM MgCl2, and 100 mM NaCl, pH 7.4) to give a concentration of 3 μg/well. Fluo-cAMP mix was made up by addition of DMSO and tracer (Fluo-cAMP) to stimulation buffer (assay buffer containing 0.3 mM adenosine triphosphate). Fluo-cAMP mix and membranes were added to black 384-well plates containing β2-AR agonist CRCs. Plates were incubated for 45 min at ambient temperature (20–22°C) before the addition of detection mix (detection buffer (50 mM NaOAc, 12 mM CaCl2, and 0.1% w/v pluronic acid, pH 6.2) containing cAMP antibody). Plates were further incubated for 1 h at ambient temperature (20–22°C) and then read on Molecular Devices Analyst GT (Molecular Devices Ltd., Wokingham, UK) to determine fluorescence polarization.
Isolated Guinea Pig Trachea Studies

Guinea Pig Trachea Preparation. All animal studies were ethically reviewed and carried out in accordance with the Animals (Scientific Procedures) Act 1986 and the GSK policy on the care, welfare, and treatment of animals. Tracheal strips obtained from male and female Dunkin-Hartley guinea pigs weighing 300–700 g (as described by Ball et al. (1991)) were mounted under 1 g of tension in either immersion or superfusion tissue baths. Tissue baths were immersed/perfused with oxygenated Krebs solution (118 mM NaCl, 25 mM NaHCO₃, 11.1 mM glucose, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, and 1.2 mM Na₂HPO₄) containing indomethacin (2.8 μM to inhibit endogenous prostaglandin synthesis) at 37°C (superfusion baths perfused at a rate of 2 ml per min) and gassed with 95% O₂/5% CO₂. Platinum electrodes were placed either side of the tissue to allow electrical field stimulation (EFS) of the tissue. To measure the relaxant effect of β₂-AR agonists on guinea pig trachea, phasic contractile responses were induced by EFS with 10 s trains of square wave pulses of 5 Hz frequency, 0.1 mA duration, and submaximal voltage every 2 min. For each preparation, constant responses to isoprenaline were obtained by addition or infusion of increasing concentrations of isoprenaline until a maximum response was obtained. If tissues failed to relax to isoprenaline across an appropriate concentration range, they were rejected. Data were collected using the NOTOCORD-hem Evolution acquisition software (NOTOCORD Systems, Croissy Sur Seine, France).

Potency, Persistence, and Onset Time. Potency, persistence, and onset time were measured using EFS-stimulated tracheal strips in immersion tissue baths. β₂-AR agonists were added to tissue baths (final DMSO concentration of 0.1%) at increasing concentrations to generate CRCs and determine pEC₅₀ values. Tissues were then washed with Krebs’ solution to remove agonist and allowed to recover contractile response for 1 h. The magnitude of the EFS contraction was measured pre- and postagonist addition and 1 h after removal to determine persistence. The onset time was measured as the time taken to reach half-maximal inhibition obtained for an approximate EC₅₀ concentration. In persistence studies in the guinea pig trachea, the CR was calculated by dividing the EC₅₀ of the agonist at 60 min by the EC₅₀ for the agonist obtained at time 0 with a low CR value suggesting a long persistence of agonist activity.

Vilanterol Reassertion. The reassertion of vilanterol with the β₂-AR was measured using EFS-stimulated tracheal strips in superfusion tissue baths. Tissues were superfused with vilanterol until the maximum inhibition of EFS was achieved. Vilanterol was then removed from the perfusate, and the tissue was allowed to recover for 1 h. Tissues were then perfused with sotalol (10 μM) until the blocking effect of the vilanterol inhibition of EFS had equilibrated. Sotalol was then removed from the perfusate, and the tissue was superfused for a further 2 h.

Human Precision-Cut Lung Slices Studies

Preparation. Human precision-cut lung slices (PCLS) were prepared, and studies were completed as previously described by Sturton et al. (2008). The human biological samples were sourced ethically, and their research use was in accord with the terms of informed consents. In brief, macroscopically normal human lung (3–10 g) obtained from subjects undergoing resection was inflated with 2.5% (w/v) ultralow melting point agarose (Type IIA). Cooled tissue was cut into 1 cm slices and then into 8 mm diameter cylindrical cores containing features oriented longitudinally along the core. The cores were processed to 260–280 μm thick slices using a Krumdieck tissue slicer (TSE Systems GmbH, Bad Homburg, Germany). Slices were transferred to a 12-well tissue culture plate containing incubation buffer (RPMI 1640 medium containing 100 units/ml penicillin, 0.1 mg/ ml streptomycin, and 4 mM l-glutamine) and warmed to 37°C. Only slices containing circular airways (30–80 μm diameters) with intact airway walls and beating cilia were used for these studies. Slices were washed with incubation buffer for 2–3 h and left overnight in an incubator in humidified air containing 5% CO₂ at 37°C on a rotating platform (1 rpm). The next day, slices were washed and returned to the incubator for 1 h before commencement of the experiment.

PCLS Potency, Efficacy, and Kinetic Studies. PCLS were positioned under a Nikon SMZ-U dissecting microscope (magnification 75×) mounted with a JVC TK-1280E video camera. The video camera was connected to a Matrox Meteor 2 frame-grabber card with image capture, and analysis was performed using KS 300 image analysis software (Image Associates Ltd, Chorley, UK). In all experiments, baseline images were captured before commencement of either a single or cumulative concentration response to carbachol or histamine where images were collected 5 to 10 min after each dose. Once the majority of airways had contracted to the required baseline area (50% for potency and efficacy, 70% for onset), a single or cumulative concentration response to the β₂-AR agonists (final DMSO concentration of 0.1%) or vehicle was commenced, with images collected 5–20 min after each dose.

For determination of onset time, a single PCLS was positioned in a lung slice chamber (Harvard Apparatus, Edenbridge, UK) and maintained at 37°C in incubation buffer containing 20 mM HEPES at pH 7.4. The slice was held in place by a U-shaped platinum weight with 50 μm diameter attached nylon threads. Baseline images were collected before commencement of a histamine CRC to a concentration required to induce 70% closure. Once 70% closure of the airway was obtained, either 1 nM vilanterol or 1 nM salmeterol was added, with images captured every 30 s for 10 min in the case of vilanterol, and every 30 s for 20 min for salmeterol.

Duration of action studies were carried out by determining baseline response followed by the contractile responses to 0.3 μM carbachol. The slice was then washed with incubation buffer, and either 1 nM vilanterol or 1 nM salmeterol was added before a 1-h incubation at 37°C. Each slice was then washed with incubation buffer and returned to the incubator. At 2, 4, 22, and 28 h after vehicle or β₂-AR agonist removal, two baseline images were collected followed by the images of the contractile response to 0.3 μM carbachol at 5 and 10 min after addition. Carbachol was then removed, and each slice washed with incubation buffer and maintained at 37°C between time points.

PCLS airway areas at baseline and after treatments were calculated using a computer algorithm written within the KS 300 environment. The percentage bronchodilation was calculated as the increase in area as a percentage of the decrease in area caused by the final spasmogen concentration. For the PCLS duration of action experiments, the average percentage contraction to carbachol at the 5- and 10-min time points after the addition of 0.3 μM carbachol was calculated for baseline and all post-β₂-AR agonist/vehicle contractile responses. The post-β₂-AR agonist/vehicle responses for each airway were then expressed as a percentage of the corresponding pre-β₂-AR agonist/vehicle contractions. During all of the studies with the PCLS, the epithelium remained intact and the cilia continued to beat, indicating a healthy airway.

Data Analysis

Analysis of all radioligand binding experiments was completed using Prism 5.0 (GraphPad Software, San Diego, CA). Specific binding data from saturation experiments were fitted to a one- or two-affinity site model to determine Kᵦ and B_max values. Specific binding data from association binding experiments were globally fitted to the association kinetic model to determine kₐ values. Dissociation binding data were fitted to a one- or two-phase dissociation model to determine kₐ values that were subsequently used to calculate dissociation half-life (t₁₂) values using the equation dissociation t₁₂ = 0.693/ kₐ. For comparison of model fitting, the extra sum-of-squares F test was used with a threshold P < 0.05. All other data analysis was carried out using the Microsoft Excel add-ins XCS50 module or Robosage (GlaxoSmithKline in-house programs). Unless otherwise indicated, data shown graphically are mean ± S.E.M. All CRCs and
competition binding displacement curves were fitted using nonlinear regression analysis [four-parameter logistic equation with variable slope (Hill, 1909)] with EC50 and IC50 values, respectively, calculated from the fits. In addition, saturation binding of [3H]CGP12177 to the human β2-AR was carried out in the presence of Gpp(NH)p at ambient temperature (20–22°C) (C) or the absence of Gpp(NH)p at 37°C (D). For [3H]vilanterol saturation studies, specific binding data were best fitted to a one-site (in the presence of Gpp(NH)p and the absence of Gpp(NH)p at 37°C) or two-site (presence of Gpp(NH)p) affinity model (extra-sum-of-square F test, P < 0.05). For [3H]CGP12177 saturation studies, specific binding data in the presence of Gpp(NH)p were best fitted to a one-site affinity model (extra-sum-of-square F test, P < 0.05). Plates were filtered after a 5-h incubation at the temperature detailed above, and the amount of radioligand bound to receptor was measured by LS spectroscopy. NSB values were measured in the presence of 10 μM ICI118551 and used to calculate the amount of specific radioligand bound. Data shown are the mean ± S.D. of duplicate points and are representative of four individual experiments with similar results. Insets: Scatchard transformation of specific binding.

**TABLE 1**
The receptor binding kinetic parameters for [3H]vilanterol at the human β2-AR in the presence and absence of Gpp(NH)p
Data are presented as mean ± S.E.M. for four separate determinations.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Parameters</th>
<th>Fast</th>
<th>Slow</th>
<th>Low Affinity</th>
<th>High Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>k_on M⁻¹min⁻¹</td>
<td>k_off min⁻¹</td>
<td>pKd</td>
<td>pKd</td>
</tr>
<tr>
<td>+ Gpp(NH)p</td>
<td>3.8 ± 0.5 × 10⁸</td>
<td>0.20 ± 0.02</td>
<td>N.D.</td>
<td>9.44 ± 0.07</td>
<td>N.D.</td>
</tr>
<tr>
<td>− Gpp(NH)p</td>
<td>3.2 ± 0.7 × 10⁸</td>
<td>0.27 ± 0.03</td>
<td>0.015 ± 0.001</td>
<td>9.47 ± 0.17</td>
<td>10.82 ± 0.12</td>
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<tr>
<td>− Gpp(NH)p (37°C)</td>
<td>N.D.</td>
<td>0.23 ± 0.04</td>
<td>N.D.</td>
<td>9.52 ± 0.24</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

Results

Radioligand Binding Studies

**Radioligand Saturation Binding.** [3H]Vilanterol and [3H]CGP12177 saturation binding studies were carried out to determine binding affinity and compare the receptor populations labeled in the CHO β2-AR membranes [unless...
otherwise indicated, data corresponds to studies carried out at ambient temperature (20–22°C). Specific binding data from saturation experiments were best fitted to a one-affinity site model ([3H]vilanterol in the presence of Gpp(NH)p, [3H]vilanterol in absence of Gpp(NH)p (37°C), and [3H]CGP12177 in the presence and absence of Gpp(NH)p) or a two-affinity site model ([3H]vilanterol in the absence of Gpp(NH)p). This analysis resulted in a low-affinity pKD for [3H]vilanterol of 9.44 ± 0.07 (n = 4) in the presence of Gpp(NH)p and a high-affinity pKD of 10.82 ± 0.12 (n = 4) and a low-affinity pKD of 9.47 ± 0.17 (n = 4) in the absence of Gpp(NH)p (Fig. 2, A and B; Table 1). In addition, a low-affinity pKD for [3H]vilanterol of 9.52 ± 0.24 (n = 4) in the absence of Gpp(NH)p (37°C) was observed. No significant difference was observed between low-affinity pKD values determined for [3H]vilanterol under all conditions tested (ANOVA, Bonferroni’s post-test). A pKD value of 9.66 ± 0.14 (n = 4) was calculated for [3H]CGP12177 (β2-AR antagonist) at the β2-AR in the presence of Gpp(NH)p (Fig. 2D), which is in good agreement with literature values (Baker, 2005). The Bmax values for [3H]vilanterol saturation binding were 0.33 ± 0.02 and 0.25 ± 0.05 pmol/mg (n = 4) in the presence of Gpp(NH)p and absence of Gpp(NH)p (37°C), respectively, with no significant difference

**TABLE 2**

<table>
<thead>
<tr>
<th>β2-AR Agonist/Antagonist</th>
<th>pKi</th>
<th>Hill Slope</th>
<th>Literature pKi</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vilanterol</td>
<td>9.42 ± 0.02</td>
<td>0.85 (0.64–1.06)</td>
<td>9.44 ± 0.07a</td>
<td>N.A.</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>9.61 ± 0.03</td>
<td>0.74 (0.64–0.84)</td>
<td>9.26 ± 0.06</td>
<td>Baker, 2010</td>
</tr>
<tr>
<td>Formoterol</td>
<td>8.05 ± 0.02</td>
<td>1.12 (0.83–1.42)</td>
<td>8.63 ± 0.02</td>
<td>Baker, 2010</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>7.92 ± 0.02</td>
<td>1.17 (0.84–1.47)</td>
<td>7.36 ± 0.06</td>
<td>Bathram et al., 2006</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>6.25 ± 0.04</td>
<td>1.14 (0.75–1.52)</td>
<td>6.01 ± 0.03</td>
<td>Baker, 2010</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>6.54 ± 0.02</td>
<td>0.93 (0.56–1.30)</td>
<td>6.64 ± 0.09</td>
<td>Baker, 2010</td>
</tr>
<tr>
<td>ICI118551</td>
<td>9.43 ± 0.05</td>
<td>1.10 (0.81–1.40)</td>
<td>9.26 ± 0.03</td>
<td>Baker, 2005</td>
</tr>
<tr>
<td>CGP12177</td>
<td>9.59 ± 0.05</td>
<td>0.62 (0.31–0.93)</td>
<td>9.39 ± 0.07</td>
<td>Baker, 2005</td>
</tr>
<tr>
<td>Propranolol</td>
<td>9.35 ± 0.03</td>
<td>1.14 (0.72–1.56)</td>
<td>9.08 ± 0.06</td>
<td>Baker, 2005</td>
</tr>
<tr>
<td>Sotalol</td>
<td>7.14 ± 0.05</td>
<td>0.98 (0.61–1.35)</td>
<td>6.85 ± 0.09</td>
<td>Baker, 2005</td>
</tr>
</tbody>
</table>

N.A.: not applicable.

a The pKD from [3H]vilanterol saturation binding studies (Table 1).
observed between conditions (ANOVA, Bonferroni's post-test). The $B_{\text{max}}$ value for $[^3H]\text{CGP12177}$ saturation binding in the presence of Gpp(NH)p was $0.43 \pm 0.02 \text{ pmol/mg (n = 4), and no significant difference was observed against the value obtained for }[^3H]\text{vilanterol under the same conditions (ANOVA, Bonferroni's post-test). This confirmed that both radioligands were labeling the same population of receptors.}[^3H]\text{CGP12177 saturation was also carried out in the absence of Gpp(NH)p, with no difference observed between }pK_D \text{ and } B_{\text{max}} \text{ values determined for }[^3H]\text{CGP12177 in the presence of Gpp(NH)p (data not shown).}

Radioligand Kinetic and Competition Binding Studies. In association binding studies, no significant difference was observed between $k_{\text{on}}$ values determined for $[^3H]\text{vilanterol in the absence and presence of Gpp(NH)p (Student's unpaired }t\text{ test) (Fig. 3, A and B; Table 1). In dissociation studies, a single phase dissociation of $[^3H]\text{vilanterol binding from the }\beta_2\text{-AR was observed in the presence of Gpp(NH)p, with a dissociation }t_{1/2}\text{ of 3.5 min [Fig. 3C; Table 1 (dissociation }t_{1/2}\text{ calculated from }k_{\text{off}}\text{]. A biphasic dissociation of }[^3H]\text{vilanterol binding from the }\beta_2\text{-AR was observed in absence of Gpp(NH)p with a dissociation }t_{1/2}\text{ of 2.6 and 46.9 min for the fast and slow phases, respectively [Fig. 3C; Table 1 (dissociation }t_{1/2}\text{ calculated from }k_{\text{off}}\text{]. From dissociation studies in the absence of Gpp(NH)p, }\sim 30 \text{ and 70% of receptors were in the high- and low-affinity agonist receptor states, respectively. A single phase dissociation of }[^3H]\text{vilanterol binding from the }\beta_2\text{-AR was observed in the absence of Gpp(NH)p (37°C), with a dissociation }t_{1/2}\text{ of 3.0 min [Fig. 3D; Table 1 (dissociation }t_{1/2}\text{ calculated from }k_{\text{off}}\text{]. The high- and low-affinity }K_D \text{ values determined from saturation in the absence of Gpp(NH)p were determined to be 0.02 and 0.43 nM, respectively. This agrees well with the values calculated from the equation }K_D = k_{\text{off}}k_{\text{on}} \text{ using the kinetic values determined in the absence of Gpp(NH)p, where the high- and low-affinity }K_D \text{ values were calculated to be 0.04 and 0.86 nM, respectively.}

To determine the affinity of unlabeled $\beta_2$-AR agonists and antagonists under equilibrium conditions, competition displacement binding curves were measured against $[^3H]$vilanterol following a 5-h incubation period. All compounds tested caused significant inhibition of radioligand binding to NSB levels. The $pK_I$ values determined were in good agreement with literature values generated against antagonist radioligands (Table 2). Vilanterol had a comparable affinity for the $\beta_2$-AR as salmeterol but a 5-, 23-, and 31-fold higher affinity than olodaterol (Casarosa et al., 2011), formoterol, and indacaterol, respectively.

CAMP Measurements for Determination of Functional Agonist Responses

$\beta$-AR Subtype Selectivity and Intrinsic Efficacy. The selectivity of vilanterol for $\beta_2$-AR over the other $\beta$-AR receptor subtypes ($\beta_1$ and $\beta_3$) was established by testing the ability of vilanterol to elicit concentration-dependent increases in cAMP in CHO cells expressing human $\beta_1$, $\beta_2$, and $\beta_3$-AR. Vilanterol was demonstrated to be highly selective for the $\beta_2$-AR with at least a 1000-fold selectivity over both $\beta_1$- and $\beta_3$-AR subtypes (Table 3). Vilanterol demonstrated similar selectivity as salmeterol for $\beta_2$- over $\beta_1$-AR and $\beta_2$- over $\beta_3$-AR (Table 3). It was shown to be significantly more selective than formoterol, indacaterol, and isoprenaline for $\beta_2$- over $\beta_1$-AR and $\beta_2$ over $\beta_3$ AR (ANOVA, Bonferroni’s post-test) (Table 3). In terms of functional potency at the $\beta_2$-AR measured by stimulation of cAMP production, vilanterol was equipotent with formoterol but showed

\[K_D = k_{\text{off}}k_{\text{on}}\]
a significantly greater $pEC_{50}$ value than that of salmeterol and indacaterol (ANOVA, Bonferroni’s post-test) (Table 3). In cAMP studies measuring the intrinsic efficacy of $\beta_2$-AR agonists, vilanterol showed a level of intrinsic efficacy that was comparable to indacaterol, significantly greater than that obtained by salmeterol but significantly less than that shown for formoterol (ANOVA, Bonferroni’s post-test) (Fig. 4; Table 3).

**$\beta_2$-AR Agonist Persistence and Reassertion.** The persistence and reassertion of $\beta_2$-AR agonists at the $\beta_2$-AR receptor was established by measuring functional potency following washout studies in the presence and absence of the $\beta_2$-AR receptor antagonist sotalol. This enabled persistence to be ascertained by measuring agonist activation of the $\beta_2$-AR following washout of agonist from the receptor. Reassertion behavior was determined by coincubating the $\beta_2$-AR agonist with a competitive $\beta_2$-AR antagonist (sotalol) to antagonize the receptor activation. By testing this condition with and without a washout step allowed a return or reassertion of agonist receptor activation to be exhibited. If an agonist remained in the system by some other mechanism than being bound to the orthosteric receptor binding site, it would be able to rebind to the free orthosteric receptor binding site following sotalol washout and cause reactivation of the receptor and further stimulation of cAMP production. Concentration-dependent increases in cAMP of $\beta_2$-AR agonists in CHO cells expressing human $\beta_2$-AR were measured. Vilanterol, salmeterol, formoterol, and indacaterol all caused a concentration-related increase in cAMP. Vilanterol, salmeterol, and indacaterol showed a very small shift to the right in the CRC following washout when compared with the control (unwashed agonist). CRs for vilanterol, salmeterol, and indacaterol were low, which indicated they were not readily washed out of the $\beta_2$-AR and exhibited a long persistence of action (Fig. 5, A, B, and D; Table 4). After washout, the formoterol CRC shifted ~80-fold in comparison with the control, indicating that formoterol exhibited a shorter persistence of action compared with the other agonists tested (Fig. 5C, Table 4).

The CRC to vilanterol, salmeterol, indacaterol, and formoterol in the presence of 100 $\mu$M sotalol was shifted to the right as expected (Fig. 5). After removal of the sotalol and agonist,

<table>
<thead>
<tr>
<th>CR Condition</th>
<th>Vilanterol</th>
<th>Salmeterol</th>
<th>Formoterol</th>
<th>Indacaterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistence</td>
<td>3.4 (1.8–6.3)</td>
<td>0.5 (0.3–1.1)</td>
<td>280 (44–1800)</td>
<td>2.4 (1.4–6.3)</td>
</tr>
<tr>
<td>Reassertion</td>
<td>5.0 (3.5–7.2)</td>
<td>0.7 (0.3–1.5)</td>
<td>460 (150–1400)</td>
<td>3.9 (2.0–7.4)</td>
</tr>
</tbody>
</table>
the vilanterol, salmeterol, and indacaterol CRCs shifted to the left, yielding CRs similar to those obtained under agonist washout conditions (Fig. 5, A, B, and D; Table 4) indicating reassertion activity for these agonists. In contrast, for formoterol, there was a minimal change in the CRC following the removal of sotalol and agonist, consistent with minimal reassertion (Fig. 5C; Table 4). These data are in good agreement with historical data reported in the literature for salmeterol, formoterol, and indacaterol (Patel et al., 2011).

**Isolated Guinea Pig Trachea Studies**

**Potency, Persistence, and Onset Time.** $\beta_2$-Agonist potency was determined from the concentration-related inhibition of the EFS contraction of guinea pig trachea. All agonists tested caused a concentration-related inhibition of the EFS-contracted guinea pig trachea. Vilanterol was found to have a similar functional potency to that of formoterol but was more potent than salmeterol and indacaterol (Fig. 6, A–D; Table 5). After washout, the concentration-related inhibition of the EFS contraction in response to the $\beta_2$-agonists was measured 1 h post-removal of agonist to determine persistence of response. Vilanterol showed a small shift to the right in the CRC following washout, resulting in a low CR that was similar to that of salmeterol and indacaterol (CRs of 1.3, 0.2, and 1.6 for vilanterol, salmeterol, and indacaterol, respectively) (Fig. 6, A, B, and D). Formoterol demonstrated a ~51-fold shift of the CRC following washout, indicating that formoterol has a shorter persistence of action compared with the other agonists tested (Fig. 6C). Vilanterol along with indacaterol and formoterol showed a rapid onset of action, which was significantly faster than salmeterol ($P < 0.0001$, ANOVA, Bonferroni’s post-test) (Table 5). These data are in good agreement with historical data reported in the literature.

**TABLE 5**

<table>
<thead>
<tr>
<th>$\beta_2$-AR Agonist</th>
<th>Potency</th>
<th>Onset $t_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$EC$_{50}$</td>
<td>min</td>
</tr>
<tr>
<td>Vilanterol</td>
<td>8.62 ± 0.27</td>
<td>5.8 ± 0.5***</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>6.84 ± 0.03</td>
<td>15.2 ± 0.6</td>
</tr>
<tr>
<td>Formoterol</td>
<td>8.56 ± 0.18</td>
<td>4.0 ± 0.1****</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>6.84 ± 0.16</td>
<td>4.0 ± 0.2****</td>
</tr>
</tbody>
</table>

*** $P < 0.0001$ versus salmeterol.

The onsets time was measured as the time taken to reach half-maximal inhibition obtained for an approximate EC$_{50}$ concentration of $\beta_2$-AR agonist. Data are presented as mean ± S.E.M. for at least four separate determinations. $P$ values were determined via ANOVA and were adjusted using the Holm (1979) method.

Fig. 6. Potency, persistence, and reassertion of $\beta_2$-agonists in EFS guinea pig trachea studies. Concentration-response curves for $\beta_2$-AR agonists in EFS stimulated guinea pig trachea in the presence (●, $t = 0$ min) and 60 min after removal (■, $t = 60$ min) for vilanterol (A), salmeterol (B), formoterol (C) and indacaterol (D). Persistence was determined from the shifts of the competition-response curves 60 min after agonist removal. Data shown are the mean ± S.E.M. of at least three individual experiments carried out in singlicate. The reassertion of vilanterol with the $\beta_2$-AR was measured using EFS stimulated tracheal strips in superfusion tissue baths (E). Tissues were superfused with 100 nM vilanterol until maximum inhibition of EFS was achieved before removal from perfusate and the addition of 10 $\mu$M sotalol. The reassertion of vilanterol was then measured after removal of 10 $\mu$M sotalol. (E) shows the EFS % contractile response in the presence of either vehicle (Control $t = 0$ h), vilanterol (Vilanterol $t = 0$ h), vilanterol then 1 h after removal of vilanterol from perfusate (Vilanterol +sotalol $t = 2$ h), vilanterol then 1 h after removal of vilanterol from perfusate in the presence of 10$\mu$M sotalol (Vilanterol + sotalol) or vilanterol then 1 h after removal of vilanterol from perfusate, addition of 10 $\mu$M sotalol and then 2 h after removal of sotalol from perfusate (Vilanterol + sotalol $t = 2$ h). Data are the mean ± S.E.M. from four individual experiments carried out in at least singlicate.
agonists have been the treatment of choice for patients suffering from asthma and COPD since the discovery of the first generation of bronchodilators salbutamol and terbutaline (Waldeck, 2002), through to the LABAs salmeterol and formoterol. The next generation of LABAs, which includes vilanterol, offers the advantage of being once daily treatments that provide relief of the symptoms suffered by asthma and COPD patients for up to 24 h. It has been shown clinically that vilanterol has a rapid onset of action and improves lung function in patients suffering with moderate to severe COPD when administered once daily (Hanania et al., 2012). In addition, it has been shown clinically that when vilanterol is administered once daily, a prolonged duration of bronchodilation is achieved for at least 24 h in asthma patients receiving maintenance ICS therapy (Lotvall et al., 2012). In this study, we describe the preclinical pharmacological profile of vilanterol using radioligand binding and cAMP production studies in recombinant cell and membrane fragment systems as well as human and guinea pig tissue systems to characterize β2-AR binding and functional properties and endeavor to provide further insight into its duration of action.

Initial studies were completed using membrane preparations from a CHO cell line recombinantly expressing the human β2-AR for characterization of the affinity and receptor kinetics of [3H]vilanterol. [3H]Vilanterol radioligand binding studies were completed in the presence and absence of Gpp(NH)p to investigate the role of the β2-AR agonist affinity state (Samama et al., 1993). Temperature effects were also investigated by measuring binding at ambient (20–22°C) and physiological (37°C) temperatures. In the presence of Gpp(NH)p, GPCRs are converted from the high-affinity agonist state (G-protein bound) to the low-affinity agonist state (G-protein unbound) as the nonhydrolyzable nucleotide uncouples the receptor from its G-protein. From saturation binding studies completed with [3H]vilanterol at ambient temperature in the presence or absence of Gpp(NH)p, it was shown that the radioligand bound to either one or two β2-AR agonist affinity states, respectively (Fig. 2, A and B; Table 1). Even at the low-affinity agonist state, [3H]vilanterol bound with subnanomolar affinity showing that, regardless of receptor state, it has a high-affinity interaction with the β2-AR. The affinity of vilanterol for the low-affinity agonist state was shown to be comparable to salmeterol but higher than that of formoterol, indacaterol, and olodaterol. It is noteworthy that when saturation experiments were completed in the absence of Gpp(NH)p at 37°C, only binding to the low-affinity agonist state was observed, suggesting that increasing the temperature had the same effect as the addition of Gpp(NH)p in terms of shifting all receptors in the system into the low-affinity agonist state (Fig. 2C; Table 1). These data agree with

### Table 6

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Histamine</th>
<th>Carbachol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (nM)</td>
<td>Efficacy (max % bronchodilation)</td>
</tr>
<tr>
<td>Vilanterol</td>
<td>0.6 ± 0.2</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>2.7 ± 1.1</td>
<td>84 ± 7</td>
</tr>
</tbody>
</table>

Potency and efficacy values obtained for vilanterol and salmeterol in human PCLS containing Airways precontracted with either carbachol or histamine

Potency and efficacy data from human PCLS studies were derived by performing nonlinear regression analysis on each individual airway. Data are presented as mean ± S.E.M. for at least four replicates using lung tissue from four different patients.

**Discussion**

In the treatment of asthma and COPD, as with other diseases, it is a major advantage to have long-acting therapies to improve patient compliance (Tashkin, 2005). β2-AR

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**Human PCLS Studies**

**Potency and Efficacy of Vilanterol and Salmeterol in Human Small Airways.** Vilanterol and salmeterol both caused concentration related bronchodilatation of human small airways when precontracted with histamine or carbachol and exhibited a comparable EC50 and efficacy (maximum percentage bronchodilatation) within each spasmogen tested (Table 6).

**Kinetics of Vilanterol and Salmeterol in Human Small Airways.** For measuring the onset of action of vilanterol and salmeterol, histamine was selected as the spasmogen, as the efficacy of both compounds was observed to be greater than that for carbachol. Therefore, this potentially increased the sensitivity of measurement when using histamine. One nanomolar vilanterol was shown to have a significantly faster onset time [t1/2] = 3.1 ± 0.3 min, n = 6 (lung tissue from four different patients), P < 0.0001, Student’s unpaired t test] when compared with 1 nM salmeterol [t1/2 = 8.3 ± 0.8 min, n = 5 (lung tissue from four different patients)] (Fig. 7A). The data obtained in the present studies for salmeterol agrees with historical work comparing the characteristics of salmeterol, formoterol, and indacaterol in this test system (Sturton et al., 2008).

The duration of action of vilanterol and salmeterol in PCLS was demonstrated by repeated determination of the contractile response of a small airway to 0.3 μM carbachol, elicited at different times after a 1-h preincubation with vilanterol, salmeterol, or vehicle (Fig. 7B). Vilanterol and salmeterol were both shown to exert a significant duration of action when compared with vehicle up to 4 h (P < 0.0001, Student’s unpaired t test), but only vilanterol was shown to be significantly different from vehicle-treated airways at 22 h (P < 0.01) (Fig. 7, B and C). No significant duration was observed at 28 h for vilanterol or salmeterol.

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**Vilanterol Reassertion.** One hundred nanomolar vilanterol caused a maximal inhibition of the EFS guinea pig contraction that was still present 1 h after removal of vilanterol from the superfused perfusate. Upon addition of 10 μM sotalol, a β2-antagonist, the EFS contraction was reestablished. After removal of the sotalol, vilanterol reasserted its relaxant effect, despite the absence of vilanterol in the superfusing fluid, indicative of a reassertion effect (Fig. 6E).
Duration of action of vilanterol and salmeterol in human small airways (A) were determined after contraction with histamine (contracted to 70% maximal precontracted human small airways PCLS. The onset of action of vilanterol at the high-affinity agonist state and slow phase (high-affinity agonist state). In the presence of Gpp(NH)\textsubscript{p}, a single phase dissociation profile was observed with a dissociation $t_{1/2}$ value that correlated well with the value determined for the low-affinity agonist state in the presence of Gpp(NH)\textsubscript{p} (Table 1). Dissociation experiments carried out in the absence of Gpp(NH)\textsubscript{p} and at 37°C also exhibited a single phase dissociation, once more suggesting that, at physiological temperature, the $\beta_2$-AR is switched to the low-affinity agonist state. Consequently, these data agree with the saturation binding experiments carried out in the absence of Gpp(NH)\textsubscript{p} and at 37°C. It is clear from the dissociation experiments that $[^{3}H]$vilanterol demonstrates a fast $k_{off}$ from the $\beta_2$-AR low-affinity agonist state and a moderately slow $k_{off}$ from the $\beta_2$-AR high-affinity agonist state at ambient temperature. However, even this slower off rate is too fast to explain the functional duration of action of vilanterol observed in isolated human airways (Fig. 7, B and C) and clinically. Although it has been hypothesized for some LABAs that the duration of action observed in tissue studies is attributed to a slow off rate from the $\beta_2$-AR high-affinity agonist state (Casarosa et al., 2011), these studies were carried out under nonphysiological temperature conditions. Therefore, a significant reduction in the off rate would be expected by increasing the temperature in the system, as observed with other GPCRs (Wallace and Young, 1983), and also, there is the potential for there to be a limited number of receptors in the high-affinity agonist state to contribute to an agonist’s duration of action via this mechanism.

The functional activity and selectivity of vilanterol at the $\beta_1$, $\beta_2$, and $\beta_3$-AR was investigated by measuring stimulation of cAMP production in human recombinant CHO cell lines. Vilanterol was shown to have a superior selectivity profile for the $\beta_2$-AR over both the $\beta_1$- and $\beta_2$-AR subtypes (Table 3) compared with all agonists tested except salmeterol. Vilanterol also had a significantly higher functional potency at the $\beta_2$-AR compared with all agonists tested except formoterol. This trend in potency was also observed in EFS-stimulated guinea pig trachea. The level of $\beta$-AR subtype selectivity observed for vilanterol negates any risk associated with cardiac actions and the cautionary use of historical $\beta_2$-AR agonist therapies in patients suffering from hyperthyroidism and cardiovascular disease (Broadley, 2006). Other safety concerns regarding metabolites, metabolism rates, and systemic exposure of historical $\beta_2$-AR agonists were addressed for vilanterol with studies that determined that any absorbed fraction would be rapidly metabolized and a swallowed fraction of an inhaled dose would be unlikely to contribute to systemic exposure (Procopiou et al., 2010). $\beta_2$-AR intrinsic efficacy data were generated in a fluorescence polarization cAMP assay using membranes generated from $\beta_2$-AR CHO
cells to counteract the issues observed in the highly coupled receptor/G-protein system that existed in the β2-AR LANCE cAMP cellular assay. The intrinsic activity of vilanterol was shown to be significantly greater than salmeterol, comparable with indacaterol, and significantly lower than formoterol and isoprenaline (Table 3). These data may explain why vilanterol has a faster onset of action in human tissue studies than salmeterol (Fig. 7A), as it has been hypothesized that increased agonist efficacy at the β2-AR influences the rate of cAMP accumulation and onset of action (Rosethorne et al., 2010).

To aid in further characterization of the duration of action of vilanterol in vitro, persistence and reassertion studies were completed in cellular cAMP assays and EFS-stimulated guinea pig trachea. In the cellular assay, vilanterol was shown to have a comparable persistence of action with indacaterol, longer persistence of action than formoterol, but a shorter persistence of action than salmeterol. In reassertion studies, vilanterol, salmeterol, and indacaterol all demonstrated reassertion activity following removal of sotolol and agonist, whereas formoterol demonstrated minimal reassertion (Fig. 7; Table 4). The behavior exhibited by vilanterol in the cellular system was also observed in the EFS-stimulated guinea pig trachea studies. This indicates that in both cellular and tissue systems vilanterol exhibits a persistence action at the β2-AR. It also shows that its agonist effect reasserts following blockade by a competitive β2-AR antagonist and subsequent washout of both drugs. To frame these observations in a human system, the airway PCLS studies were investigated and encompassed an extended time period. In human airways, vilanterol was shown to have a longer duration of action than salmeterol and indacaterol either in this study or from historical literature data (Sturton et al., 2008), still exhibiting a significant level of bronchodilation 22 h after treatment.

The radioligand binding studies with [3H]vilanterol have shown that receptor kinetics at the orthosteric agonist site do not contribute to the observations in the persistence and reassertion studies. Therefore, the hypothesis for the persistence and reassertion profile of vilanterol is more than likely the same as that arrived at in previous studies in terms of the "microkinetic" model (Anderson et al., 1994) describing a highly lipophilic molecule partitioning into cell membrane and forming depots of drug [the calculated log P value for vilanterol (3.2) is comparable to that of salmeterol (3.1), calculated by Daylight Chemical Information Systems Inc., Laguna Niguel, CA]. The debate around the mechanism for the long clinical duration of action exhibited by inhaled β2-AR agonists has been ongoing since the discovery and clinical use of salmeterol and formoterol in the early 1990s. As a recent review of the theories put forward for salmeterol's duration of action suggests (Szczucka et al., 2009), there is still not enough conclusive evidence to rule out the "exosite" theory (Coleman et al., 1996) and fully endorse the "microkinetic" theory alone. This study only goes as far as showing that β2-AR receptor dissociation kinetics in a recombinant expression system are insufficiently slow to explain the duration of action of vilanterol. As a consequence, it is clear that further in-depth studies combining radioligand binding, mutagenesis/receptor modeling (based on the crystal structure determined for the β2-AR-G protein αs complex in the presence of a range of structurally distinct LABAs), and functional studies in both basic and more complex physiological systems will be required to fully elucidate the exact mechanism of action that accounts for the duration of action of vilanterol and other LABAs.

In conclusion, the in vitro binding functional and selectivity data for vilanterol are consistent, showing that it is a novel, potent, and selective β2-AR receptor agonist. Moreover, vilanterol displays persistent agonist action as well as reassertion behavior at the β2-AR in recombinant cells and guinea pig trachea, as well as a fast onset and long duration of action in human small airways. This pharmacological profile combined with clinical data is consistent with once a day dosing of vilanterol in the treatment of both asthma and COPD.

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