In vitro pharmacological characterization of vilanterol, a novel long acting β₂–adrenoceptor agonist with 24 hour duration of action.


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Non-standard abbreviations: β₂-AR, β₂-adrenoceptor; $k_{on}$, association rate; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; CR, concentration ratio; CRCs, concentration response curves; cAMP, 3'-5'-cyclic adenosine monophosphate; DMSO, dimethyl sulfoxide; $t_{1/2}$, half-life; $k_{off}$, dissociation rate; EFS, electrical field stimulation; EDTA, ethylenediaminetetraacetic acid; $K_D$, equilibrium dissociation constant; FCS, fetal calf serum; FP, fluorescence...
polarisation; GPCR, G-protein coupled receptor; Gpp(NH)p, guanosine 5'-[β,γ-imido]triphosphate; HBSS, Hank’s balanced Salt Solution; ICS, inhaled corticosteroid; $K_i$, inhibition constant; IBMX, 3-isobutyl-1-methylxanthine; LS, liquid scintillation; LABAs, long acting β2-AR agonists; LAMA, long acting muscarinic antagonist; HEPES, N-2-hydroxyethylpiperazine-N’-ethanesulphonic acid; NSB, non-specific binding; ANOVA, one-way analysis of variance; PMSF, phenylmethylsulphonyl fluoride; PBS, phosphate buffered saline; PCLS, precision cut lung slice; PKA, protein kinase A; S.E.M., standard error of the mean; $B_{max}$, total number of receptors; vilanterol, vilanterol trifenate.

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ABSTRACT

Vilanterol trifenate (vilanterol) is a novel long acting β2–adrenoceptor (β2-AR) agonist with 24 hour activity. In this study we describe the pre-clinical pharmacological profile of vilanterol using radioligand binding and 3'-5'-cyclic adenosine monophosphate (cAMP) studies in recombinant assays as well as human and guinea pig tissue systems to characterise β2-AR binding and functional properties. Vilanterol displayed a sub-nanomolar affinity for the β2-AR that was comparable with that of salmeterol but higher than olodaterol, formoterol and indacaterol. In cAMP functional activity studies vilanterol demonstrated similar selectivity as salmeterol for β2- over β1-AR and β3-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 post-treatment. From these investigations the data for vilanterol are consistent, showing that it is a novel, potent and selective β2-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 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 hyperresponsiveness 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 characterised 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 (Kardos and Keenan, 2006; Celli et al., 2004). β2-Adrenoceptor (β2-AR) agonists play a major role in the management of both asthma and COPD by increasing airflow into the lungs via relaxation of the airways. β2-AR agonists achieve relaxation of the smooth muscle in the bronchial airway by acting directly on the β2-AR G-protein coupled receptor (GPCR). Stimulation of the β2-AR activates G-protein αs 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 Ca2+-dependent K+ channels, though 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 like salbutamol in the 1960s (Cullum et al., 1969), continued research in this area has endeavoured 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 hours. These long acting β₂-AR agonists (LABAs) are used as twice daily bronchodilators in both asthma and COPD. Over recent years there have been concerns over the use of LABAs as mono-therapies 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 and are now recommended for use in combination 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 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. 2011) 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 when compared with the use of the LABA or LAMA alone (Cazzola and Molimard, 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 hour duration of action on airflow, could improve patient convenience and therefore compliance within both these disease populations (Cazzola et al., 2011).
Vilanterol trifenate (vilanterol) (Figure 1) is a novel LABA with 24 hour 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 pre-clinical 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 characterise β₂-AR binding and functional properties.
MATERIALS AND METHODS

Materials. Indacaterol, isoprenaline, salbutamol, salmeterol and vilanterol were synthesised 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. (2011). CGP12177, formoterol, ICI118551, 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 Gibco (Invitrogen Ltd., Paisley, UK). [3H]vilanterol (specific activity 92 Ci/mmol) was synthesised by Quotient Bioresearch (Radiochemicals) Ltd. (Cardiff, UK). [3H]CGP-12177 (specific activity 41 Ci/mmol) was purchased from PerkinElmer LAS UK Ltd. (Beaconsfield, UK). All studies were completed with a final dimethyl sulphoxide (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 Medium/F12, supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 200 μg/ml geneticin and 100 μg/ml hygromycin B in 95%:5% air:CO2 at 37°C. Cells were harvested by treatment with Hank’s balanced Salt Solution (HBSS; Ca2+ and Mg2+ free) and 0.6 mM ethylenediaminetetraacetic acid (EDTA) for 10 min, washed in phosphate buffered saline (PBS) and then frozen in 80% dialysed FCS:20% DMSO. 1 ml 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 300 g at 4°C for 10 min and the cell pellet was re-suspended
in assay buffer (50 mM N-2-hydroxyethylpiperazine-N’-ethanesulphonic acid (HEPES) containing 0.1 mM leupeptin, 25 μg/mL bacitracin, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 μM pepstatin A). The cell pellet was homogenised using a glass Waring blender and the cell suspension spun at 500 g for 20 min at 4°C. The resulting supernatant was spun for a further 30 min at 48,000 g at 4°C. The pellet was re-suspended in assay buffer (without PMSF and pepstatin A) and the protein concentration determined using the bicinchoninic acid method as 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]CGP-12177, β2-AR membranes (μg/well dependent on the Bmax of individual membrane fragment preparations), and either vehicle or unlabelled β2-AR agonists/antagonists at varying concentrations. Non-specific binding (NSB) values determined by 10 μM ICI118551 were used to calculate specific binding of [³H]vilanterol or [³H]CGP-12177. 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, USA) onto GF/B filter papers pre-soaked 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 LS fluid (Ultima-Flo™ M, PerkinElmer LAS UK Ltd., Beaconsfield, UK). The amount of radioligand bound to receptor was measured by LS spectroscopy using a TriCarb 2900 TR LS counter (PerkinElmer LAS UK Ltd., Beaconsfield, UK). By the same
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 of guanosine 5’-[β,γ-imido]triphosphate (Gpp(NH)p), a non-hydrolysable analogue of the nucleotide guanosine triphosphate. In order 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 characterisation. 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 prior to 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 prior to filtration. For dissociation binding, membranes were pre-incubated 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 prior to filtration.
Saturation binding was also completed for \[^3\text{H}\]CGP12177 (increasing concentrations of ~0.01-2.8 nM) in the same format as described above for \[^3\text{H}\]vilanterol.

**Determination of \(\beta_2\)-AR agonist and antagonist affinity.** In order to determine the affinity of \(\beta_2\)-AR agonists and antagonists, competition binding displacement studies were completed where membranes were incubated with a fixed concentration of \[^3\text{H}\]vilanterol (~0.2 nM) and increasing concentrations of unlabelled agonist/antagonist for 5 h prior to filtration. All competition binding displacement studies were completed in the presence of 100 µM Gpp(NH)p to ensure that binding curves were mono-phasic.

**LANCE™ cAMP Assays.** General protocols for LANCE™ cAMP assays. Increases in cAMP were determined using a LANCE™ cAMP assay kit (Perkin Elmer LAS UK Ltd., Beaconsfield, UK) as per the kit instructions for either 96 or 384-well plate formats. Briefly, CHO cells expressing either \(\beta_1\), \(\beta_2\) or \(\beta_3\)-AR were thawed at 37°C, diluted in PBS and centrifuged at 300 g for 5 min. Cells were then re-suspended in stimulation buffer (HBSS containing 0.01% (selectivity studies) or 0.1% (persistence and reassertion studies) w/v bovine serum albumen, 500µM 3-isobutyl-1-methylxanthine (IBMX) and 5 mM HEPES, pH 7.4) to the required concentration.

**\(\beta\)-Adrenoceptor subtype selectivity.** 10,000 cells/well of \(\beta_1\)-, \(\beta_2\)-, or \(\beta_3\)-AR CHO cells were added to white 384-well plates containing \(\beta_2\)-AR agonist concentration response curves (CRCs), 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-labelled 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 (Perkin Elmer LAS UK Ltd., Beaconsfield, UK).
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 post-washout 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). Briefly, 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 300 g for 5 min followed by aspiration of supernatant and re-suspension in stimulation buffer.

Condition 1 (control):- cells incubated with vehicle (1% DMSO) for 30 min, washed three times and β₂-AR agonist added prior to further 30 min.

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

Condition 3 (control + sotalol):- cells incubated with vehicle (1% DMSO) for 30 min, washed three times and 100 μM sotalol (β₂-adrenergic receptor antagonist)/β₂-AR agonist added prior to further 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 further 30 min.

Plates were then spun at 300 g for 5 min, supernatant aspirated and cells re-suspended 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-labelled 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 (Perkin Elmer LAS UK Ltd., Beaconsfield, UK). A concentration ratio (CR) for β2-AR agonist persistence was calculated by dividing the agonist washout EC_{50} (condition 2) by the control agonist EC_{50} (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 EC_{50} (condition 4) by the control agonist EC_{50} (condition 1) with a low CR value suggesting agonist reassertion post-antagonist washout.

Fluorescent Polarisation cAMP Assay. Intrinsic efficacy was measured by increases in cAMP levels in CHO β2-AR membranes using a [FP]² cAMP assay kit (Perkin Elmer LAS UK Ltd., Beaconsfield, UK) as per the kit instructions. Briefly, 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 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 polarisation.

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 Kreb’s buffered salt 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 prostanoid synthesis) at 37°C (superfusion baths perfused at a rate of 2 mL per minute) 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 ms duration and sub-maximal voltage every 2 min. For each preparation constant responses to isoprenaline was 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 Kreb’s solution to remove agonist and allowed to recover contractile response for 1 h. The magnitude of the EFS–contraction was measured pre- and post-agonist addition and 1 h post removal to determine persistence. The onset time was measured as the time taken to reach half-maximal inhibition obtained for an
approximate $EC_{50}$ concentration. In persistence studies in the guinea pig trachea the CR was calculated by dividing the $EC_{50}$ of the agonist at 60 min by the $EC_{50}$ 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 $\beta_2$-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 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 superfused for a further 2 h.

**Human precision cut lung slices (PCLS) studies.**

**PCLS preparation.** Human PCLS were prepared and studies 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 the informed consents. Briefly, macroscopically normal human lung (3-10 g) obtained from subjects undergoing resection was inflated with 2.5% (w/v) ultra low melting point agarose (Type IXA). 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 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 (30-800 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 75x) 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 performed using KS 300 image analysis software (Image Associates Ltd, 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 5-10 min after each dose. Once the majority of airways had contracted to the required baseline area (50% for potency and efficacy, 70% for onset) then 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 Instruments, 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 were 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 added prior to 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 post-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, 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 points after addition of 0.3 µM carbachol were 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 agonists/vehicle contractions. During all 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, USA). Specific binding data from saturation experiments were either fitted to a one or two affinity site model to determine $K_D$ and $B_{\text{max}}$ values. Specific binding data from association binding experiments were globally fitted to the association kinetic model to determine $k_{\text{on}}$ values. Dissociation binding data were fitted to a one or two-phase dissociation model to determine $k_{\text{off}}$ values that were subsequently used to calculate dissociation half-life ($t_{1/2}$) values using the equation $t_{1/2} = 0.693/k_{\text{off}}$. 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 XC50 module or
Robosage (GlaxoSmithKline in-house programs). Unless otherwise indicated, data shown graphically are mean ± standard error of the mean (S.E.M.). All CRCs and competition binding displacement curves were fitted using non-linear regression analysis (four-parameter logistic equation with variable slope (Hill, 1909)) with $EC_{50}$ and $IC_{50}$ values respectively, calculated from the fits. In addition, for agonist CRCs a maximum asymptote response was also calculated. $IC_{50}$ values from competition binding displacement curves were converted to inhibition constant ($K_i$) values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

All statistical analyses were completed using SAS® (SAS Institute Inc., NC, USA) and differences of $p < 0.05$ were considered to be statistically significant. Statistical significance between two data sets was tested using a Student’s unpaired $t$-test. One-way analysis of variance (ANOVA) was used for comparison of more than two datasets to highlight specific inter-group $p$-values, with Holm’s method (Holm S, 1979) used to adjust $p$-values for multiple comparisons and so lessen the occurrence of false positive results.
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 labelled in the CHO $\beta_2$-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 Gpp(NH)p, $[^3H]$vilanterol in absence 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 $pK_D$ for $[^3H]$vilanterol of 9.44 ± 0.07 (n=4) in the presence Gpp(NH)p and a high affinity $pK_D$ of 10.82 ± 0.12 (n=4) and a low affinity $pK_D$ 9.47 ± 0.17 (n=4) in the absence of Gpp(NH)p (Figure 2A and B, Table 1). In addition, a low affinity $pK_D$ 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 $pK_D$ values determined for $[^3H]$vilanterol under all conditions tested (ANOVA, Bonferroni post-test). A $pK_D$ value of 9.66 ± 0.14 (n=4) was calculated for $[^3H]$CGP12177 ($\beta_2$-AR antagonist) at the $\beta_2$-AR in the presence of Gpp(NH)p (Figure 2D) which is in good agreement with literature values (Baker, 2005). The $B_{max}$ 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 observed between conditions (ANOVA, Bonferroni post-test). The $B_{max}$ value for $[^3H]$CGP12177 saturation binding in the presence of Gpp(NH)p was 0.43 ± 0.02 pmol/mg (n=4) and no significant difference was observed against the value obtained for $[^3H]$vilanterol under the same conditions (ANOVA, Bonferroni post-test). This confirmed that both radioligands were labelling...
the same population of receptors. [³H]CGP12177 saturation was also carried out in
the absence of Gpp(NH)p with no difference observed between pKD and Bmax values
determined for [³H]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 kon values determined for [³H]vilanterol
in the absence and presence of Gpp(NH)p (Student’s unpaired t-test) (Figure 3A and
B, Table 1). In dissociation studies, a single phase dissociation of [³H]vilanterol
binding from the β2-AR was observed in the presence of Gpp(NH)p with a
dissociation t1/2 of 3.5 min (Figure 3C, Table 1 (dissociation t1/2 calculated from koff)).
A bi-phasic dissociation of [³H]vilanterol binding from the β2-AR was observed in
absence of Gpp(NH)p with a dissociation t1/2 of 2.6 and 46.9 min for the fast and slow
phases respectively (Figure 3C, Table 1 (dissociation t1/2 calculated from koff)). From
dissociation studies in the absence of Gpp(NH)p ~30 and 70% of receptors were in
the high and low agonist affinity receptor states respectively. A single phase
dissociation of [³H]vilanterol binding from the β2-AR was observed in the absence of
Gpp(NH)p (37°C) with a dissociation t1/2 of 3.0 min (Figure 3D, Table 1 (dissociation

In order to determine the affinity of unlabelled β2-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
inhibition of radioligand binding to NSB levels. The pKi values determined were in
good agreement with literature values generated against antagonist radioligands (Table 2). Vilanterol had a comparable affinity for the β2-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.** β-AR subtype selectivity and intrinsic efficacy. The selectivity of vilanterol for β2-AR over the other β-AR receptor sub-types (β1 and β3) was established by testing the ability of vilanterol to elicit concentration-dependent increases in cAMP in CHO cells expressing human β1-, β2- and β3-ARs. Vilanterol was demonstrated to be highly selective for the β2-AR with at least a 1,000-fold selectivity over both β1- and β3-AR subtypes (Table 3). Vilanterol demonstrated similar selectivity as salmeterol for β2-over β1-AR and β2-over β3-AR (Table 3). It was shown to be significantly more selective than formoterol, indacaterol, and isoprenaline for β2-over β1-AR and β2-over β3 AR (ANOVA, Bonferroni post-test) (Table 3). In terms of functional potency at the β2-AR measured by stimulation of cAMP production, vilanterol was equipotent with formoterol but showed a significantly greater pEC50 value than that of salmeterol and indacaterol (ANOVA, Bonferroni post-test) (Table 3). In cAMP studies measuring the intrinsic efficacy of β2-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 post-test) (Figure 4, Table 3).

**β2-AR agonist persistence and reassertion.** The persistence and reassertion of β2-AR agonists at the β2-AR receptor was established by measuring functional potency following washout studies in the presence and absence of the β2-AR receptor antagonist sotalol. This enabled persistence to be ascertained by measuring agonist
activation of the β₂-AR following washout of agonist from the receptor. Reassertion behavior was determined by co-incubating the β₂-AR agonist with a competitive β₂-AR antagonist (sotalol) to antagonise 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 then be able to rebind to the free orthosteric receptor binding site following sotalol washout and cause re-activation of the receptor and further stimulation of cAMP production. Concentration-dependent increases in cAMP of β₂-AR agonists in CHO cells expressing human β₂-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 to the control (unwashed agonist). CRs for vilanterol, salmeterol and indacaterol were low that indicating they were not readily washed out of the β₂-AR and exhibited a long persistence of action (Figure 5A, B and D, Table 4). Following washout the formoterol CRC shifted ~80 fold in comparison to the control indicating that formoterol exhibited a shorter persistence of action compared with the other agonists tested (Figure 5C, Table 4).

The CRC to vilanterol, salmeterol, indacaterol and formoterol in the presence of 100μM sotalol was shifted to the right as expected (Figure 5). After removal of the sotalol and agonist, vilanterol, salmeterol and indacaterol CRCs shifted to the left yielding a CRs similar to that obtained under agonist washout conditions (Figure 5A, 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 (Figure 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 (Figure 6A-D, Table 5). Following 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 which was similar to that of salmeterol and indacaterol (CRs of 1.3, 0.2 and 1.6 for vilanterol, salmeterol and indacaterol respectively) (Figure 6A, B and D). Formoterol demonstrated a $\sim$51 fold shift of the CRC following washout indicating that formoterol has a shorter persistence of action compared with the other agonists tested (Figure 6C). Vilanterol along with indacaterol and formoterol all showed a rapid onset of action, which was significantly faster than salmeterol ($p < 0.0001$, ANOVA, Bonferroni post-test) (Table 5). These data are in good agreement with historical data reported in the literature for salmeterol, formoterol and indacaterol (Casarosa et al., 2011).

**Vilanterol reassertion.** 100 nM 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 $\mu$M sotalol, a $\beta_2$-antagonist, the EFS contraction was re-established. After removal of the sotalol, vilanterol reasserted its
relaxant effect, despite the absence of vilanterol in the superfusing fluid, indicative of a reassertion effect (Figure 6E).

**Human PCLS studies.** Potency and efficacy of vilanterol and salmeterol in human small airways. Vilanterol and salmeterol both caused concentration related bronchodilation of human small airways when pre-contracted with histamine or carbachol and exhibited a comparable EC$_{50}$ and efficacy (maximum percentage bronchodilation) 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. 1 nM vilanterol was shown to have a significantly faster onset time (t$_{1/2}$ = 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 (t$_{1/2}$ = 8.3 ± 0.8 min, n=5 (lung tissue from four different patients)) (Figure 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 pre-incubation with vilanterol, salmeterol or vehicle (Figure 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) (Figure 7B and C). No significant duration was observed at 28 h for vilanterol or salmeterol.
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 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, that include vilanterol, offer the advantage of being once daily treatments that provide relief of the symptoms suffered by asthma and COPD patients for up to 24 hours. 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 also been shown clinically that when vilanterol is administered once daily a prolonged duration of bronchodilation is achieved for at least 24 hours in asthma patients receiving maintenance ICS therapy (Lotvall et al., 2012). In this study we describe the pre-clinical 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 characterise β₂-AR binding and functional properties and endeavour 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 characterisation 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)
temperature. 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 non-hydrolysable 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 (Figure 2A and B, Table 1). Even at the low affinity agonist state [3H]vilanterol bound with sub-nanomolar 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. Interestingly 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 (Figure 2C, Table 1). This data agrees with previous studies on β-ARs where increasing temperatures were observed to reduce high affinity agonist binding (Toews et al., 1983; Contreras et al., 1986). The consequence of this was the inability to measure the binding properties of [3H]vilanterol at the high affinity agonist state of the β2-AR under physiological temperature.

Dissociation binding studies for [3H]vilanterol from the β2-AR were again carried out in the absence and presence of Gpp(NH)p and at different temperatures to observe high and low agonist affinity state kinetics (Table 1). A bi-phasic dissociation profile was observed in the absence of Gpp(NH)p at ambient temperature with a fast phase (low affinity agonist state) and slow phase (high affinity agonist state). In the presence of Gpp(NH)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)p (Table 1). Dissociation experiments carried out in the absence of Gpp(NH)p and at 37°C also exhibited a single phase dissociation once more suggesting that at physiological temperature the β2-AR is switched into the low affinity agonist state. Consequently, this data agrees with the saturation binding experiments carried out in the absence of Gpp(NH)p and at 37°C. It is clear from the dissociation experiments that [3H]vilanterol demonstrates a fast $k_{off}$ from the β2-AR low affinity agonist state and a moderately slow $k_{off}$ from the β2-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 (Figure 7B and C) and clinically. Although it has been hypothesised for some LABAs that the duration of action observed in tissue studies is due to a slow off rate from the β2-AR high affinity agonist state (Casarosa et al. 2011), these studies were carried out under non-physiological 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, 1982), but 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 β1, β2 and β3-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 β2-AR over both the β1 and β3-AR subtypes (Table 3) compared with all agonists tested except salmeterol. It also had a significantly higher functional potency at the β2-AR compared with all agonists tested except formoterol. This trend in potency was also observed in EFS stimulated guinea pig trachea. The level of β-AR subtype selectivity
observed for vilanterol negates any risk associated with cardiac actions and the cautionary use of historical β2-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 β2-AR agonists were addressed for vilanterol with studies that determined any absorbed fraction would be rapidly metabolised and a swallowed fraction of an inhaled dose would be unlikely to contribute to systemic exposure (Procopiou et al., 2010). β2-AR intrinsic efficacy data were generated in a fluorescence polarisation cAMP assay using membranes generated from β2-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). This data may explain why vilanterol has a faster onset of action in human tissue studies than salmeterol (Figure 7A) as it has been hypothesised 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 characterisation 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 sotalol and agonist whilst formoterol demonstrated minimal reassertion (Figure 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 $\beta_2$-AR. It also shows that its agonist effect reasserts following blockade by a competitive $\beta_2$-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 post-treatment. The radioligand binding studies with $[^{3}H]$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 (Daylight Chemical Information Systems Inc., Laguna Niguel, CA, USA)). The debate around the mechanism for the long clinical duration of action exhibited by inhaled $\beta_2$-AR agonists has been on-going 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 (Szczuka 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 $\beta_2$-AR receptor dissociation kinetics in a recombinant expression system are insufficiently slow to explain the duration of action of vilanterol. Consequently, it is clear that further in-depth studies combining
radioligand binding, mutagenesis/receptor modelling (based on the crystal structure determined for the $\beta_2$-AR-G protein $\alpha_s$ 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 $\beta_2$-AR receptor agonist.

Moreover, vilanterol displays persistent agonist action as well as reassertion behavior at the $\beta_2$-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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AUTHORSHIP CONTRIBUTIONS


Wrote or contributed to the writing of the manuscript: Robert J. Slack, Victoria J. Barrett, Alison J. Ford, Richard G. Knowles.

STATEMENT OF CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.
REFERENCES


Broadley KJ (2006) β-adrenoceptor responses of the airways: For better or worse? 


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099-3108.


Figure 1  Chemical structure of the novel long acting $\beta_2$-AR agonist vilanterol.

Figure 2  Saturation binding of $[^3\text{H}]$vilanterol and $[^3\text{H}]$CGP12177 to the human $\beta_2$-AR. Saturation binding of $[^3\text{H}]$vilanterol to the human $\beta_2$-AR was carried out in the presence (A) and absence (B) of 100 $\mu$M Gpp(NH)p at ambient temperature (20-22°C) or in the absence of Gpp(NH)p at 37°C (C). In addition, saturation binding of $[^3\text{H}]$CGP12177 to the human $\beta_2$-AR was carried out in the presence of Gpp(NH)p at ambient temperature (20-22°C) (D). For $[^3\text{H}]$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 $[^3\text{H}]$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 $\mu$M ICI118551 and used to calculate the amount of specific radioligand bound. Data shown are the mean ± standard deviation of duplicate points and are representative of four individual experiments with similar results. Inset: Scatchard transformation of specific binding.

Figure 3  Receptor binding kinetics of $[^3\text{H}]$vilanterol with the human $\beta_2$-AR. Association binding kinetics of $[^3\text{H}]$vilanterol with the human $\beta_2$-AR in the presence (A) and absence (B) of Gpp(NH)p. Dissociation binding kinetics of $[^3\text{H}]$vilanterol from the human $\beta_2$-AR in the presence and absence of Gpp(NH)p at ambient temperature (20-22°C) (C) or in the absence of Gpp(NH)p at ambient temperature (20-22°C) and 37°C (D). Plates were filtered after the time indicated and the amount
of radioligand bound to receptor was measured by LS spectroscopy. In association binding studies NSB values were measured in the presence of 10 μM ICI118551 and used to calculate the amount of specific radioligand bound. Association binding data generated at the radioligand concentrations indicated were globally fitted using the association kinetic model to derive a single best-fit estimate for $k_{on}$. In dissociation binding studies CHO β2-AR membranes were pre-incubated for 1 h with a fixed concentration of radioligand before dissociation was initiated by a 1:20 dilution in binding buffer (containing 10 μM cold vilanterol). Dissociation binding was determined in the presence of ~1nM radioligand and data fitted to a one-phase (in the presence of Gpp(NH)p and the absence of Gpp(NH)p at 37°C) or two-phase (in the presence of Gpp(NH)p) dissociation model. Data shown are the mean ± S.E.M. of triplicate points and are representative of four individual experiments with similar results.

**Figure 4** Competition-response curves for β2-AR agonists in a fluorescence polarisation (FP) cAMP assay. The potency and intrinsic efficacy of β2-AR agonists was measured by measuring increases in cAMP levels in CHO β2-AR membranes using a [FP]² cAMP assay kit. Plates were incubated for 1 h at ambient temperature (20-22°C) prior to measurement of cAMP by FP. Data shown are the mean ± S.E.M. of at least four individual experiments carried out in singlicate.

**Figure 5** Persistence of action and reassertion profiles for vilanterol (A), salmeterol (B), formoterol (C) and indacaterol (D) in a LANCE™ cAMP assay kit. β2-AR agonist competition-response curves were tested under differing conditions (as detailed in Materials and Methods) to determine their persistence of action and reassertion characteristics at the β2-AR.
Figure 6  Potency, persistence and reassertion of β2-agonists in EFS guinea pig trachea studies. Concentration-response curves for β2-AR agonists in EFS stimulated guinea pig trachea in the presence (t=0 min) and 60 min post-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 post-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 β2-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 μM sotalol. The reassertion of vilanterol was then measured post-removal of 10 μM sotalol. Panel 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 post-removal of vilanterol from perfusate (Vilanterol t=1 h), vilanterol then 1 h post-removal of vilanterol from perfusate in the presence of 10μM sotalol (Vilanterol + sotalol) or vilanterol then 1 h post-removal of vilanterol from perfusate, addition of 10μM sotalol and then 2 h after removal of sotalol from perfusate (Vilanterol – sotalol t=2 h). Data is the mean ± S.E.M. from four individual experiments carried out in at least singlicate.

Figure 7  Onset and duration of action of vilanterol and salmeterol in pre-contracted human small airways PCLS. The onset of action of vilanterol and salmeterol in human small airways (A) were determined post-contraction with histamine (contracted to 70% maximal closure). Duration of action of vilanterol and salmeterol in human small airways (B) with percentage pre-β2-AR agonist 0.3μM carbachol contraction in comparison to baseline contraction taken at 2, 4, 22 and 28 h post removal of β2-AR agonist or vehicle. PCLS airway images at 75x magnification.
(C) obtained from a representative experiment investigating the duration of vilanterol in comparison with salmeterol and vehicle. The top panel shows the airways prior to addition of β₂-AR agonist or vehicle and after contraction to 0.3 μM carbachol at t=0 h. The middle panel shows the airways at baseline at t=22 h post removal of β₂-AR agonist or vehicle. The bottom panel shows the airway after contraction with 0.3μM carbachol at t= 22 h post removal of β₂-AR agonist or vehicle. Percentage closure obtained with 0.3μM carbachol for each airway in relation to baseline response is shown at t=0 h and t = 22 h post-removal of β₂-AR agonist or vehicle. Data shown are mean values ± S.E.M. for at least four replicates using lung tissue from four different patients. Statistical significance is denoted for vilanterol and salmeterol at each time point in comparison to corresponding values obtained for vehicle treated airway (*** p < 0.0001 for vilanterol and salmeterol at 2 and 4 h and ** p < 0.01 for vilanterol at 22 h, Student’s unpaired t-test).
Table 1 The receptor binding kinetic parameters for [3H]vilanterol at the human β₂-AR in the presence and absence of Gpp(NH)p.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$k_{on} M^{-1} min^{-1}$</th>
<th>Fast $k_{off} min^{-1}$</th>
<th>Slow $k_{off} min^{-1}$</th>
<th>Low Affinity pK₆</th>
<th>High Affinity pK₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Gpp(NH)p</td>
<td>$3.8 \pm 0.5 \times 10^8$</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>$3.2 \pm 0.7 \times 10^8$</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>
</tr>
<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>

Data shown are mean values ± S.E.M. for four separate determinations. N.D., not determined.
Table 2 The pKᵢ and Hill slope values at the human β₂-AR from competition binding between [³H]vilanterol and a range of β₂-AR agonist and antagonist.

<table>
<thead>
<tr>
<th>β₂-AR Agonist/Antagonist</th>
<th>pKᵢ</th>
<th>Hill Slope</th>
<th>Literature pKᵢ</th>
<th>Literature 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 ± 0.07</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 (0.48, 1.87)</td>
<td>7.36 ± 0.06</td>
<td>Battram 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>
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<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>
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</tbody>
</table>

Data shown are mean values ± S.E.M. or 95% confidence limits (shown in parentheses) for at least six separate determinations. *pKᵢ from [³H]vilanterol saturation binding studies (Table 1).
N.A., not applicable.
Table 3 β₁-, β₂-, or β₃-AR functional potency values, selectivity ratios and β₂-AR intrinsic efficacy values for a range of β₂-AR agonists determined in cAMP detection assays.

<table>
<thead>
<tr>
<th>β₂-AR Agonist</th>
<th>β₂ pEC₅₀ ± S.E.M.</th>
<th>β₁ pEC₅₀ ± S.E.M.</th>
<th>Selectivity Ratio β₂ over β₁</th>
<th>Selectivity Ratio β₂ over β₃</th>
<th>β₂ Intrinsic Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vilanterol</td>
<td>10.37 ± 0.05</td>
<td>6.98 ± 0.03</td>
<td>2400</td>
<td>7.36 ± 0.03</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.70 ± 0.03</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>9.80 ± 0.10*</td>
<td>6.32 ± 0.12</td>
<td>3000</td>
<td>6.48 ± 0.13</td>
<td>2100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>***0.41 ± 0.01</td>
</tr>
<tr>
<td>Formoterol</td>
<td>10.14 ± 0.08</td>
<td>7.96 ± 0.03</td>
<td>***150</td>
<td>8.36 ± 0.03</td>
<td>***59</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>***0.95 ± 0.04</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>9.48 ± 0.08*</td>
<td>8.28 ± 0.04</td>
<td>***16</td>
<td>8.16 ± 0.03</td>
<td>***20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.86 ± 0.02</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>7.31 ± 0.03*</td>
<td>5.87 ± 0.03</td>
<td>***27</td>
<td>6.33 ± 0.06</td>
<td>***9.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.D.</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>8.43 ± 0.02*</td>
<td>9.04 ± 0.02</td>
<td>***0.24</td>
<td>8.56 ± 0.02</td>
<td>***0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>***1</td>
</tr>
</tbody>
</table>

All pEC₅₀ data shown were generated in the LANCE™ cAMP assay and used to determine selectivity ratios between the β-AR subtypes. β₂-AR intrinsic efficacy data were generated in the fluorescence polarisation cAMP assay. To determine the intrinsic efficacy the fitted maximum asymptote for the test drug was divided by that obtained for isoprenaline. Data shown are mean values ± S.E.M. for at least four separate determinations. Statistical difference (*** p < 0.0001) vs vilanterol (ANOVA with p-values adjusted using Holm’s method (Holm, 1979)).

N.D., not determined.
Table 4 Persistence and reassertion concentration ratios for β2-AR agonists at the β2-AR determined in a LANCE™ cAMP detection assay.

<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>

In cAMP persistence and reassertion studies, a concentration ratio (CR) was calculated by dividing either the agonist washout EC$_{50}$ (for the persistence CR) or agonist washout + sotalol EC$_{50}$ (for the reassertion CR) by the control agonist EC$_{50}$. Data shown are the geometric mean values with 95% confidence limits shown in parentheses for four separate determinations.
Table 5 Potency and onset times obtained for vilanterol, salmeterol, formoterol and indacaterol determined in EFS stimulated guinea pig trachea studies.

<table>
<thead>
<tr>
<th>β₂-AR Agonist</th>
<th>Potency (pEC(_{50}))</th>
<th>Onset t(_{1/2}) (min)</th>
</tr>
</thead>
<tbody>
<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>

The onset time was measured as the time taken to reach half-maximal inhibition obtained for an approximate EC\(_{50}\) concentration of β₂-AR agonist. Data shown are mean values ± S.E.M. for at least four separate determinations. Statistical difference (*** p < 0.0001) vs salmeterol (ANOVA with p-values adjusted using Holm’s method (Holm, 1979)).
Table 6 Potency and efficacy values obtained for vilanterol and salmeterol in human PCLS containing airways pre-contracted with either carbachol or histamine.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Histamine</th>
<th>Carbachol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$EC_{50}$ (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 data from human PCLS studies were derived by performing non-linear regression analysis on each individual airway. Data shown are mean values ± S.E.M. for at least four replicates using lung tissue from four different patients.
FIGURE 1
FIGURE 2

A - Gpp(NH)p

B + Gpp(NH)p

C - Gpp(NH)p (37°C)

D + Gpp(NH)p
FIGURE 5

A

Log [vilarterol] M

% isoprenaline maximum response

Control
Control + sotalol
Agonist washout
Agonist washout + sotalol

B

Log [salmeterol] M

% isoprenaline maximum response

Control
Control + sotalol
Agonist washout
Agonist washout + sotalol

C

Log [formoterol] M

% isoprenaline maximum response

Control
Control + sotalol
Agonist washout
Agonist washout + sotalol

D

Log [indacaterol] M

% isoprenaline maximum response

Control
Control + sotalol
Agonist washout
Agonist washout + sotalol

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FIGURE 6

A. Log [vilaanterol] M
B. Log [salmeterol] M
C. Log [formoterol] M
D. Log [indacaterol] M

E. % contractile response

- Control
- Vilanterol t=0 h
- Vilanterol t=1 h
- Vilanterol + sotalol t=2 h
- Vilanterol - sotalol t=2 h
FIGURE 7