Pharmacological Characterization of 6-Hydroxy-8-[(1R)-1-hydroxy-2-[(2-(4-methoxyphenyl)-1,1-dimethylethyl]amino]ethyl]-2H-1,4-benzoxazin-3(4H)-one monohydrochloride, (Olodaterol), a Novel Inhaled β₂-Adrenoceptor Agonist exerting a 24-hour long Duration of Action in Preclinical Models

T. Bouyssou, P. Casarosa, E. Naline, S. Pestel, I. Konetzki, P. Devillier and A. Schnapp

Boehringer Ingelheim Pharma GmbH & Co. KG, 88397 Biberach, Germany (T.B., P.C., S.P., I.K., A.S.)

2- UPRES EA 220, Hôpital Foch, Université Versailles – Saint Quentin, 11, rue Guillaume Lenoir, 92150 Suresnes (E.N., P.D.)
Running Title Page

Running Title: preclinical pharmacology of olodaterol, a once-daily β2 agonist

Corresponding author: Dr. Andreas Schnapp

Contact Information: Dept. of Pulmonary Diseases Research, BI Pharma GmbH & Co. KG, Birkendorferstrasse 65, Biberach an der Riss, Germany

E-mail: andreas.schnapp@boehringer-ingelheim.com

Phone: +49-7351-54-5240

Fax: +49-7351-83-5240

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Non-standard abbreviations: human beta adrenoceptor, hß-AR; acetylcholine, ACh; long acting beta agonist, LABA; chronic obstructive pulmonary disease, COPD; Chinese hamster ovary, CHO; smooth muscle cells, SMCs.

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Abstract
The preclinical pharmacological profile of 6-hydroxy-8-[(1R)-1-hydroxy-2-[[2-(4-methoxyphenyl)-1,1-dimethylethyl]amino]ethyl]-2H-1,4-benzoxazin-3(4H)-one monohydrochloride, (olodaterol, previously known as BI 1744 CL), a novel, enantiomeric pure, inhaled human β2 adrenoceptor (hβ2-AR) agonist, was compared to marketed drugs, like salmeterol and formoterol. In vitro, olodaterol showed a potent, nearly full agonistic response at the hβ2-AR (EC50 = 0.1 nM; intrinsic activity = 88% compared to isoprenaline) and a significant selectivity profile (219-fold and 1622-fold against the hβ1- and hβ3-ARs, respectively). Similarly, olodaterol was able to potently reverse contraction induced by different stimuli in isolated human bronchi. In vivo, antagonistic effects of single doses of olodaterol and formoterol were measured against acetylcholine challenges in anaesthetized guinea pigs and dogs for up to 24 hours using the Respimat® Soft Mist™ Inhaler. Heart rate and metabolic parameters (serum potassium, lactate and glucose) were monitored to evaluate systemic pharmacodynamic effects in the dog model. In both models, olodaterol provided bronchoprotection over 24 hours. Formoterol applied at an equally effective dose did not retain efficacy over 24 hours. In both models olodaterol showed a rapid onset of action comparable to formoterol. Taken together, the preclinical behaviour of olodaterol suggests that this novel β2 adrenoceptor agonist has the profile for once-daily dosing in man concomitant with a fast onset of action and a favourable systemic pharmacodynamic profile.
Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are conditions characterized by airway obstruction, which is variable and reversible in asthma but is progressive in COPD (Guerra, 2009). Both diseases are very common and their incidence is increasing globally, placing a growing burden on patients and on health services in industrialized and developing countries (Braman, 2006; Pauwels and Rabe, 2004). Beta (ß) adrenoceptor agonists are among the most potent and rapidly acting bronchodilators currently available for clinical use. In asthma, rapid-acting inhaled ß2-adrenoceptor agonists are the therapy of choice as a reliever therapy for episodes of dyspnea and for the pretreatment of exercise-induced bronchoconstriction (Bateman, et al., 2008). In asthma patients with persistent symptoms long-acting ß2 adrenoceptor agonists (LABAs), like salmeterol and formoterol, are administered as an add on controller therapy when the first line treatment of medium dose inhaled corticosteroids alone fails to achieve control of asthma (Bateman, et al., 2008). Recently, formoterol has gained some recognition as a p.r.n. controller therapy because of its fast onset of action, too. In addition, inhaled ß2 adrenoceptor agonists provide major therapeutic benefits in the treatment of COPD, such as reduction in symptoms and exacerbations, increases in exercise capacity and improvements of health-related quality of life (Gold, 2009).

ß2 adrenoceptor agonists exert a bronchodilatory effect through activation of ß2 adrenoceptors (ß2-ARs) expressed on airway smooth muscle cells (SMCs). Additionally, evidence exists that ß2-AR mediated increases in cAMP have an anti-inflammatory effect in immune cells, e.g. neutrophils and mast cells, providing an additional rationale for the use of ß2 adrenoceptor agonists in chronic inflammatory diseases as asthma and COPD.
However, the utility, convenience and persistence of airflow improvement with short-acting $\beta_2$ adrenoceptor agonists, like salbutamol, is limited by the need of repeated administration. Furthermore, there is an appreciable increase in efficacy in terms of patient reported outcomes with long acting bronchodilators (i.e., b.i.d. LABAs and q.d. anticholinergics) (Jenkins, et al., 2009; Tashkin, et al., 2008), steroids and combinations (Jenkins, et al., 2009).

Currently, two $\beta_2$ adrenoceptor agonists with a twice daily dosing regimen are marketed, namely formoterol -a full agonist- and salmeterol -a partial agonist. The clinical significance of the different intrinsic activities between these two agonists is unclear. However, despite the decrease in dosing frequency with formoterol and salmeterol, patient compliance is an issue (Ying et al., 1999).

Thus, a once-a-day LABA may have several advantages compared with short-acting bronchodilators and twice-daily LABAs, including: i) improved convenience and compliance (COPD and asthma), ii) improved airflow over a complete 24-hour period (COPD and asthma), iii) a more convenient and stable once-a-day combination option with a long acting muscarinic antagonist (LAMA), like tiotropium, for patients for whom more than one bronchodilator is indicated (COPD), iv) a more convenient and sustained once-a-day free combination option with inhaled steroids (moderate to severe asthma). Additionally, a higher therapeutic index would be desirable for the new generation of inhaled $\beta_2$ adrenoceptor agonists, as doubling the dose of currently marketed drugs, e.g. salmeterol and formoterol, causes a significant increase in the incidence of adverse effects including headache, tremor, palpitations, muscle cramps and a fall in serum potassium concentration (Sovani, et al., 2004).

To achieve this, improving ligand selectivity for the h$\beta_2$-AR versus the other family subtypes h$\beta_1$-AR (expressed prevalently on cardiac smooth muscle and responsible for inotropic effects) and h$\beta_3$-AR (on adipose tissue) is central.
6-Hydroxy-8-[(1R)-1-hydroxy-2-[[2-(4-methoxyphenyl)-1,1-dimethylethyl]amino]ethyl]-2H-1,4-benzoxazin-3(4H)-one monohydrochloride, (olodaterol, previously known as BI 1744 CL), is a novel, enantiopure inhaled β₂ adrenoceptor agonist that was discovered in a program to identify compounds with a duration of action compatible with once-daily dosing in humans, a fast onset of action and an increased therapeutic index compared to the available inhaled β₂ adrenoceptor agonists. Here we describe the preclinical pharmacological profile of olodaterol, compared to the marketed drugs formoterol and salmeterol. To understand the behaviour of olodaterol at the molecular level, interaction with the different β-AR subtypes was analyzed in binding and functional assays. Efficacy and duration of bronchoprotection were tested in pharmacological models of acetylcholine-induced bronchoconstriction in anaesthetized guinea pigs and dogs over a time period of 24 hours. We report that olodaterol has an optimized profile of an inhaled LABA combining a high β₂-selectivity, a rapid onset of action and at least a 24-hour duration of action after a single once-daily administration with minimal systemic pharmacodynamic effects.
Materials and Methods

Compounds:
Olodaterol hydrochloride (CL), (R,R)/(S,S)-salmeterol xinafoate and (R,R)/(S,S)-formoterol fumarate were synthesized by the Department of Chemical Research (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany). Acetylcholine (Acetylcholine ophthalmicum Dispers) was from Dispersa GmbH (Germering, Germany). Propofol (Propofol-Lipuro 2 %) was obtained from B Braun Melsungen AG, (D-34209 Melsungen, Germany). Propranolol hydrochloride (Obsidan) was from Alpharma-Isis GmbH & Co. KG (D-40764 Langenfeld, Germany).

Cell culture techniques
Chinese hamster ovary (CHO) cells transfected with the cDNAs encoding the human $\beta_1$, $\beta_2$, or $\beta_3$-adrenoeceptors were purchased from Perkin Elmer (Waltham, MD). CHO cells were grown in Ham’s F12 without glycine, hypoxanthine and thymidine; 10% dialysed FBS; 100 U/ml penicillin; 100 µg/ml streptomycin. Due to their high level of receptor expression (see Table 1), these cells were used for performing the affinity binding studies. A second set of CHO cells stably transfected with human $\beta_1$, $\beta_2$, and $\beta_3$-ARs was generated in house and clones harboring low receptor expression were further selected and used in the functional assays, to avoid high receptor spare numbers and potential overestimation of agonist potency and intrinsic activity (IA). This second set of cells, referred to as CHO-h$\beta_{1,3}$LOW (see Table 1) were grown in DMEM supplemented with 1x NEAA and 10% fetal calf serum in the presence of the selection agent Geneticin (500 µg/ml). Cells were maintained at 37°C in humidified air containing 5% CO$_2$.

Equilibrium binding experiments
Membrane isolation and purification from CHO-cells stably expressing the human $\beta_{1,3}$-ARs (high expressing clones) was performed as described previously (Casarosa, et
al., 2005). In all radioligand experiments the binding buffer consisted of Tris-HCl 50 mM, MgCl₂ 2 mM, EGTA 1 mM, pH 7.3. After the indicated incubation period, bound and free [³H]-CGP 12,177 were separated by vacuum filtration using a Brandel Harvester (Gaithersburg, MD) on GF/B filters presoaked in 0.5% polyethyleneimine, and washed three times with ice cold binding buffer. Filter disks were added to 3 ml of scintillation fluid (Ultima Gold from Perkin Elmer) in pony-vials and radioactivity was quantified using liquid scintillation spectrometry on a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer). In all experiments, total binding did not reach 10% of the amount added, limiting complications associated with depletion of the free radioligand concentration. Saturation binding experiments were performed by incubating membranes (usually 5 to 20 µg/ sample, adjusted according to the B_max of the individual cell line) with a range of concentrations of [³H]-CGP 12,177 in a total volume of 4 ml, to avoid significant ligand depletion at the lower concentrations. Samples were incubated at room temperature for at least 2 hours under gentle agitation, before filtration. To obtain affinity estimates of unlabelled agonists, heterologous competition experiments against [³H]-CGP 12,177 were performed at equilibrium. Membranes were incubated in the presence of [³H]-CGP 12,177 (final concentration approximately 0.2 nM), 10 µM GppNHp to ensure an homogeneous receptor population and different concentrations of agonists, at room temperature with gentle agitation for at least 2 hours before filtration. Competition displacement binding data were fitted to the Hill equation and IC₅₀ values obtained from the inhibition curves were converted to K_i values (Cheng and Prusoff, 1973).

**cAMP assay**

To determine the functional potency of the different agonists against the different human β-ARs, changes in intracellular cAMP levels were determined with CHO-hβ₁-₃_LOW cells in suspension (15,000 cells/well) using Alphascreen technology (Perkin
Elmer) and a 384 well-plate format (Optiplate, Perkin Elmer), according to the manufacturer’s protocol. Briefly, cells were stimulated with the respective agonists at different concentrations in Hanks' buffered saline solution supplemented with 5 mM HEPES, 0.1% BSA and 500 mM IBMX for 30 minutes at room temperature. Cells were lysed using Alphascreen reagents. After 2 hours, plates were read on an Envision plate reader (Perkin Elmer). The concentration of cAMP in the samples was calculated from a standard curve.

**In vitro human bronchial tissue assays:**

Human bronchial tissue sampling and tissue preparation was done as described before (Naline, et al., 1994). The use of human lung tissue for in vitro experiments was approved by the Regional Ethics Committee. Lung tissue was obtained from 15 patients (12 men, 3 women, mean age = 66 ± 2 years) undergoing surgery for lung carcinoma. None of the patients had a history of asthma. After the resection a piece of macroscopically normal tissue obtained at a distance of at least 20 mm from the malignancy was supplied by the hospital pathologist. Subsegmental bronchi were dissected free from adhering lung parenchyma and connective tissue, cut in rings and suspended in parallel on tissue hooks in 10 ml organ baths under an initial load of 3 g and were equilibrated for 60-90 min with changes in PSS (NaCl 118 mM, KCl 4.7 mM, CaCl$_2$ 2.5 mM, MgSO$_4$ 0.6 mM, KH$_2$PO$_4$ 1.1 mM, NaHCO$_3$ 25.0 mM, glucose 11.7 mM) every 15-20 min prior to any pharmacological intervention. At the end of the equilibration period, the resting load was stable at 2–4 g. Under these conditions, responses were optimal and reproducible (Naline, et al., 1994). The total number of rings used was 157.

**Potency and efficacy**

A total of 82 rings obtained from 13 patients were used and one concentration–response curve was recorded with a single ring for each compound. Concentration-
response curves for olodaterol and formoterol were produced by cumulative addition of the compounds at intervals of 5-10 min to bronchi at resting tone (to obtain a relaxation plateau) and to bronchi pre-contracted with either histamine (10 µM, representing 51% of the maximal contraction induced by 3 mM ACh), or ACh (0.1 mM, representing 80% of ACh max). After the end of the experiment, theophylline 3 mM was added to determine the maximal relaxation.

**Electrical field stimulation (EFS)**

Experiments were performed as previously described (Naline et al., 2007). A total of 96 rings obtained from 6 patients were used for these experiments. Only one compound and one concentration were studied in each ring. Each organ bath was fitted with two platinum plate electrodes (1 cm²) placed alongside the tissue (10 mm apart) to cause neural release of ACh by transmural EFS (biphasic pulse width 1 ms, constant current of 320 mA for 10 s at 5 Hz). To obtain the plateau of maximal contraction, a control response was determined for all bronchi preparations by adding 3 mM ACh, first. After washing, bronchi were allowed to equilibrate for 60 minutes with a change of the medium every 15 min. For the subsequent duration of the experiment, 1 µM montelukast and 1 µM indomethacin were present in the buffer to avoid the influence of leukotrienes and prostaglandins on the neuronal responses, respectively. After tension had returned to the baseline tone, the preparation was stimulated every 10 minutes at 5 Hz, pulse width 1 ms and 320 mA current for 10 s using a stimulator (EMKA Technologies, Mitry Mory, France) where the voltage output was adjusted to give a constant current and biphasic rectangular pulse of alternating polarity. These contractions represent 20–50% of the maximal contraction induced by 3 mM ACh. Compounds (tested at one dose for each ring) or vehicle were added to the bath for 1 hour in order to reach the relaxation plateau. Magnitude of the relaxation was expressed as percent of inhibition of EFS-induced contraction.
recorded before drug administration to the organ bath. To determine their respective potency in preventing EFS-induced contraction (\(-\log IC_{50}\)), olodaterol and formoterol were tested at different concentrations (\(3 \times 10^{-11} \text{M}\) to \(3 \times 10^{-8}\text{M}\)).

**Animal Studies:**

All animal studies were performed with the approval by the Veterinary Authorities (Regierungspräsidum Tübingen, Germany). For inhaled administration olodaterol and formoterol were dissolved in a mixture of distilled water and ethanol (40/60, v/v) at concentrations permitting the administration of the desired dose with three actuations of the Respimat® Soft Mist™ Inhaler connected to the endotracheal tube. For intraduodenal administration the compounds were dissolved in Natrosol 1% and applied at a volume of 1 ml/kg.

*Bronchoprotection in Guinea pigs*

Male Dunkin-Harley guinea pigs (350-400 g, obtained from Harlan Winkelmann, Germany) fasted over night were used. Anaesthesia was induced by intraperitoneal injection of 50 mg/kg pentobarbital followed by intravenous infusion of pentobarbital (15 mg/kg/h) via the jugular vein. A tracheal cannula was introduced after tracheotomy for artificial ventilation and the internal jugular vein was cannulated for ACh injection. The animals were ventilated (starling ventilator, Hugo Sachs Elektronik, Germany) at a stroke volume of 10 ml / 1 kg at a rate of 60 strokes per minute. A branch of the tracheal cannula was connected to a pressure transducer (bronchospasm transducer 7020, Ugo Basile, Italy). Bronchospasm (cm H₂O) was recorded using a modified version of the method of Konzett-Roessler (Walland, et al., 1997). Blood pressure and heart rate were monitored from a carotid artery. All signals were amplified and measured using a lung and cardiovascular function recording system (Notocord-hem, Notocord, France). After three stable ACh-induced
bronchospasms, compounds were administered via the tracheal tube using a Respimat® Soft Mist™ Inhaler. To address the efficacy and duration of action of the compounds over 5 hours, ACh (10 µg/kg i.v.) was injected every 10 minutes for the entire study period. To address the onset of action of the compounds, bronchoconstrictions were induced by ACh (10 µg/kg i.v.) 1, 3, 5, 7 and 20 minutes after drug inhalation. To address the duration of action, increasing doses of ACh (2-20 µg/kg i.v.) were injected 6 hours or 24 hours after drug inhalation.

Bronchoprotection in dogs

The bronchoprotective effect of olodaterol and formoterol were investigated in a model of ACh-induced bronchoconstriction in anesthetized, ventilated beagle dogs over a period of 3 hours and 24 hours, respectively. The model was essentially performed as described before (Casarosa, et al., 2009). In the 3 hour setting bronchoprotection, cardiovascular and metabolic parameters were evaluated immediately before and 5, 10, 30, 60, 90, 120, 150 and 180 minutes after administration of the compounds. To address the duration of action and the systemic pharmacodynamic effect profile over 24 hours, cardiovascular, metabolic parameters and bronchospasms were recorded 5 minutes, 30 minutes, 6 hours, 12 hours, and 24 hours after administration of the compounds. In this setting dogs were anaesthetized 30 minutes before ACh-challenge (10 µg/kg i.v.) and regained consciousness 1 hour later. Concentrations of potassium, glucose and lactate in heparinized venous blood samples were determined with an ABL 605 analyzer (Radiometer Copenhagen, Denmark).

Data were analyzed using commercially available software (GraphPad Prism®, version 5.02, GraphPad Software Inc., San Diego, CA, USA). All results are expressed as mean ± S.E.M. For the duration of action studies, a two-way ANOVA
with repeated measures was calculated followed by a Bonferroni multiple comparison test versus the time-matched vehicle control.

**Intraduodenal administration**

Beagle dogs of both genders were used (3-5 animals per dose). Animals were anaesthetized with pentobarbital (30 mg/kg; i.v. bolus) for intubation followed by a pancuronium bolus (0.5 mg) for muscle relaxation. Maintenance of anaesthesia was done by i.v. infusion of pentobarbital (10 mg/kg*h) and pancuronium (0.03 mg/kg*h) into the saphenous vein. While placing the devices, piritramid (10 mg i.v.) and fentanyl (0.05 mg i.v.) bolii were applied, too. Artificial respiration was maintained with a gas mixture of 70% nitrous oxide and 30% oxygen using a Vivolec respirator (MEGAMED AG, 6330 Cham, Switzerland). The respiratory parameters were monitored continuously using a POET (model II, CSI-Europe, Bad Homburg, Germany).

After the instrumentation was complete, animals were allowed to stabilize for 20-30 min prior to the start of the experiments. Compound administration was done via a beforehand placed catheter into the duodenum. Blood pressure was measured with a catheter in the femoral artery, heart rate was derived from blood pressure. Blood pressure and heart rate were continuously recorded on a computer system after A/D conversion for further analysis using the NOTOCORD-hem and EXCEL software. At the end of subsequent 10-minute periods, mean values were calculated from data over 1 minute. Data were expressed as mean ± S.E.M and were normalized to the time point just before compound administration (time 0) for graphical presentation.
Results

In vitro Characterisation of olodaterol (BI 1744 CL)

The in vitro pharmacology of olodaterol (Figure 1) was determined in CHO-K1 cell-lines selectively and stably expressing either of the human β₁-, β₂- or β₃-ARs, to ensure that measurements were made at a single receptor subtype. Different clones bearing high or low levels of receptors were selected (Table 1) and used in binding and functional assays, respectively.

The agonists’ affinities for the different β-adrenoceptor subtypes were determined in heterologous competitive binding experiments against [³H]-CGP 12177 in the presence of 5′-Guanylyl-imidodiphosphate (Gpp(NH)p), a non-hydrolyzable analog of GTP, to ensure monophasic binding curves. Results are summarized in Table 2. Olodaterol had a subnanomolar affinity for the β₂-AR (pKi of 9.14) and was selective for this receptor in comparison to the β₁-AR and β₃-AR subtypes.

Given the Gαₛ coupling of β-ARs, the agonist-induced accumulation of cAMP was used as a functional readout (Figure 2). CHO cell lines stably expressing low levels of β-ARs were selected (Table 1), to avoid high receptor spare numbers and potential overestimation of agonist potency and intrinsic activity (IA). The agonists’ potencies (pEC₅₀) and intrinsic activities (reported as percentage of the maximal effect of isoprenaline) are summarized in Table 3. In line with the binding data, olodaterol shows the highest potency for the hβ₂-AR among the tested drugs (EC₅₀ = 0.1 nM), and the profile of an almost full agonist with an IA of 88%, not statistically different from the reference full agonist isoprenaline and formoterol. However, in contrast to formoterol, olodaterol is only a partial agonist for the hβ₁ adrenoceptor (IA at hβ₁AR are 52% and 91% for olodaterol and formoterol, respectively) and shows an increased functional selectivity versus the β₁ and β₃ adrenoceptors (Table 3).
In vitro pharmacological profile of olodaterol on human bronchi: Potency and efficacy

The pharmacological behaviour of olodaterol, in comparison to formoterol, was next assessed in human bronchial strips in the presence of different contractile agents (Table 4). On basal tone preparations, olodaterol and formoterol potently relaxed the bronchi with non-significant differences in potency and efficacy (Table 4, Figure 3A) (two-tailed t test). Similarly, the potency and efficacy of olodaterol and formoterol were not statistically different when histamine was used as a contractile agent (Table 4, Figure 3B). To mimic the cholinergic tone typical of COPD, ACh and electric field stimulation (EFS; to induce neural-mediated release of ACh) were used, too. EFS-induced contractions were potently inhibited in a concentration-dependent manner by olodaterol (pIC$_{50}$ = 9.49) and formoterol (pIC$_{50}$ = 9.73) (Table 4, Figure 3D), with formoterol causing a slightly higher maximal inhibition of EFS-induced contraction (97%) compared to BI 1744 CL (86%).

Conversely, pre-contraction with ACh (100 µM) decreased the potencies and maximal relaxant effects of BI 1744 CL and formoterol (p<0.05), with no significant difference between the two β2 adrenoceptor agonists (Table 4, Figure 3D).

In vivo profile of olodaterol

The in vivo efficacy and systemic pharmacodynamic profile of olodaterol and formoterol were determined in bronchoconstriction models in guinea pigs and dogs. In these models, the compounds were applied intra-tracheally to anesthetized animals using the Respimat® Soft Mist™ Inhaler and bronchoconstriction was induced by intravenous application of acetylcholine at various time-points after administration of the compounds.

Dose Response in Guinea Pigs. After administration of different doses of each compound, bronchoprotection, heart rate and blood pressure were recorded over a
period of 5 hours. As shown in Figure 4A, olodaterol induced a dose-dependent bronchoprotection when applied at doses from 0.1 µg/kg to 3 µg/kg. A full bronchoprotection of 100% was achieved at the dose of 3 µg/kg. Olodaterol demonstrated at all efficacious doses a bronchoprotection lasting over the entire study period of 5 hours. For formoterol the maximal bronchoprotection of 100% was achieved at a dose of 1 µg/kg and 3 µg/kg (Figure 4B). In contrast to olodaterol, formoterol demonstrated an increased duration of action with increased doses. A decline in bronchoprotection was observed after 30 minutes and 150 minutes at doses of 0.3 µg/kg and 1 µg/kg, respectively. Formoterol retained a full bronchoprotection over 5 hours at a dose of 3 µg/kg (Figure 4B). Both compounds did not show an increase in heart rate and blood pressure over the entire study period at all doses tested (data not shown).

**Duration of Action in Guinea Pigs.** To address the duration of action of olodaterol and formoterol, both compounds were applied via intra-tracheal instillation to guinea pigs and bronchoconstrictions were induced by increasing ACh doses from 2 µg/kg to 20 µg/kg after 6 or 24 hours, respectively. Olodaterol and formoterol were applied at a dose that achieved equivalent bronchoprotective efficacy over 5 hours (3 µg/kg). In addition, two lower doses of olodaterol (1 µg/kg and 0.1 µg/kg) were tested in this setting. As shown in Figure 5A, olodaterol and formoterol administered at a dose of 3 µg/kg retained a strong efficacy after 6 hours. However, only olodaterol still protected the animals against ACh-induced bronchospasms when administered at a dose of 3 µg/kg and a lower dose (1 µg/kg) after 24 hours. In contrast, formoterol applied at the initially equal effective dose, retained no activity after 24 hours (Figure 5B).

**Onset of Action in Guinea Pigs.** The onset of action of olodaterol in comparison to formoterol was determined in the guinea pig model described above. Both compounds were administered at three different doses using the Respimat® Soft
Inhaler and bronchospasms were induced by ACh 1, 3, 5, 7 and 20 minutes after drug inhalation. As shown in Figure 6A and 6B, both compounds exerted a rapid onset of action and achieved a full bronchoprotection within 3-6 minutes after inhalation.

_Dose Response and Systemic Pharmacodynamic Effect Profile in Dogs._ The efficacy and duration of bronchoprotection induced by olodaterol was investigated in a second species, namely anesthetized ventilated beagle dogs. Again, test compounds were administered by inhalation using the Respimat® Soft Mist™ Inhaler and bronchoconstriction was induced by repeated intravenous injections of acetylcholine at different time points after compound administration. This model was also used to study the systemic pharmacodynamic effects of the compounds in further detail, since beagle dogs are very sensitive to the cardiovascular (e.g. heart rate increase) and metabolic (e.g. increase in serum potassium, glucose and lactate) effects mediated by systemic stimulation of β-adrenoceptors (Greaves, 1998).

Olodaterol inhibited the ACh-induced bronchoconstriction in dogs in a dose-dependent manner (Figure 7A). A maximal bronchoprotective effect of about 60% was reached after 10 minutes at a dose of 0.3 µg/kg olodaterol. At this dose, bronchoprotection was approximately 20% after 3 hours. At the inhaled dose of 0.6 µg/kg olodaterol exerted the same maximal efficacy but maintained a bronchoprotection of about 40% after 3 hours. At the highest dose tested (1.2 µg/kg), a profile comparable to the 0.6 µg/kg dose was observed (data not shown). However, at this dose cardiovascular effects (e.g. increase in heart rate above 50%, see Figure 7B) were observed. Administration of olodaterol did not result in changes in serum potassium (Figure 7C), or serum lactate (Figure 7D) and serum glucose (data not shown) at any dose tested. From this experiment, the maximum-effective dose of olodaterol in the dog was determined as 0.6 µg/kg. The maximum-effective dose of
formoterol was determined as 0.6 µg/kg, too (see Figure 7A). At this dose, formoterol showed - compared to olodaterol - a more pronounced and longer lasting tachycardia (Figure 7B). In addition and in contrast to olodaterol a long lasting decrease in serum potassium levels (Figure 7C) and a significant increase in serum lactate (Figure 7D) was observed for formoterol at both doses used. Formoterol was devoid of effects on serum glucose (data not shown).

**Duration of Action in Dogs.** The bronchoprotection of olodaterol and formoterol was determined 0.1, 0.5, 6, 12 and 24 hours after inhalation of a single dose of each compound. Both compounds were administered at the respective maximum-effective dose (0.6 µg/kg). Olodaterol was also used at a 2-fold lower dose. As shown in Figure 8, olodaterol (0.6 µg/kg) induced a maximal bronchoprotection of about 60% after 0.5 hour, consistent with the 3 hour study described above. 24 hours after administration, animals treated with 0.6 µg/kg olodaterol retained a bronchoprotection of approximately 20%. In contrast, formoterol tested at its maximum-effective dose was completely inactive after 12 hours (Figure 8). When administered at a 2-fold lower dose olodaterol exerted no bronchoprotection after 12 hours. The two doses of olodaterol tested were devoid of heart rate effects and metabolic effects (data not shown).

**Systemic Pharmacodynamic Effects of Olodaterol after intra-duodenal Administration.** In man a significant proportion of the dose inhaled via the Respimat® Soft Mist™ Inhaler is swallowed (Dalby, et al., 2004). In the animal experiments described above, swallowing did not occur, since the compounds were applied either by intra-tracheal (i.t.) administration or by the Respimat® Soft Mist™ inhaler connected to the endotracheal tube. To mimic the systemic pharmacodynamic effects after complete swallowing of the entire dose, olodaterol
was applied intraduodenally (i.d.) to anesthetized dogs at 1.2 µg/kg and 2.4 µg/kg corresponding to doses 2-fold and 4-fold above its maximum-effective dose. Cardiovascular and metabolic parameters were recorded over a period of 3 hours. For comparison, formoterol was applied in the same experimental setting at its maximum-effective dose (0.6 µg/kg) and 2-fold above (1.2 µg/kg). As shown in Figure 9, i.d. administration of olodaterol induced only a small increase in heart rate of maximally 10% and 20% when given 2-fold and 4-fold above the maximum-effective dose, respectively. Systolic and diastolic blood pressure were decreased by maximally 10% up to 40 minutes post olodaterol administration and returned to normal (data not shown). Formoterol administered i.d. at its maximum-effective dose and 2-fold above induced, compared to olodaterol, more pronounced and stronger dose-dependent effects on heart rate (Figure 9) and systolic blood pressure (data not shown). Blood pressure was initially decreased up to 25% with the 2-fold fully effective dose. This decrease persisted for diastolic blood pressure, while systolic blood pressure increased by about 10% starting at about 40 min post administration.
Discussion

With chronic diseases such as COPD and asthma, patient adherence to medication plans is a major obstacle to successful management (Bender, 2002). One factor contributing to poor adherence is a complicated or a multiple treatment regimen, and simplified dosing regimens are known to improve compliance (Bender, 2002). Therefore, long duration of action (preferably 24 hours) is an important feature of drugs intended to treat chronic diseases, enabling both prolonged efficacy and a simple, once-daily dosing regime that improves patient compliance (Tamura and Ohta, 2007). This strategy, which has been proven successful with the long acting muscarinic antagonist (LAMA) tiotropium (Spiriva®) (Tashkin, et al., 2008), is currently being pursued also within a second class of bronchodilators, namely the β₂ adrenoceptor agonists (Cazzola and Matera, 2008).

Here, we describe a comprehensive preclinical characterization of olodaterol (previously known as BI 1744 CL), which was identified as part of a program aimed at the discovery of selective β₂ adrenoceptor agonists with potential for once-daily administration. *In vitro* data indicate that olodaterol possesses a high, subnanomolar affinity for the hβ₂-AR and an excellent selectivity against the other adrenoceptor subtypes. In line with the binding data, olodaterol was the most potent agonist for the β₂-AR mediated stimulation of cAMP and exerted an excellent selectivity profile. In the evaluation of new β₂ adrenoceptor agonists under development, their intrinsic activity needs to be taken into consideration, as partial agonists may act as a β₂ antagonist in the presence of a full β-agonist (Lipworth and Grove, 1997). In fact, a partial β adrenoceptor agonist exhibits opposite agonist and antagonist activity depending on the prevailing degree of adrenergic tone or the presence of a β adrenoceptor agonist with higher intrinsic activity (e.g. rescue therapies). To this end, we took particular care in testing the functional response of olodaterol in a cell line...
with moderate levels of \( \beta_2 \)-AR expression (Table 1), similar to airway SMCs (expression levels reported to be 100 fmol/mg, (Mak, et al., 1994) to avoid an overestimation of the agonist efficacy, as it is known for systems with high receptor spare numbers (Kenakin, 2004). In this setting, olodaterol offered the profile of an almost full agonist, with an intrinsic activity of 88%. These results were further translated into a more physiologically relevant model, i.e. human lung parenchyma. Here, olodaterol dose-dependently reversed the constriction induced by different stimuli, like histamine, ACh and EFS, with an efficacy not statistically different from the full agonist formoterol under all conditions. Taken together, the \textit{in vitro} data indicate that olodaterol, similarly to formoterol and salmeterol, shows high selectivity for the h\( \beta_2 \)-AR in terms of affinity and potency. However, differently from the currently marketed \( \beta_2 \) adrenoceptor agonists, olodaterol has a differential efficacy profile towards the different \( \beta \)-ARs, with a full agonist-like profile on the h\( \beta_2 \)-AR and a partial agonism against the h\( \beta_1 \)-AR, whereas formoterol and salmeterol exert either a full-agonistic or a partial agonistic profile for all \( \beta \)-ARs, respectively. This profile could translate in an efficacious bronchodilatory effect with reduced cardiovascular side effects.

In order to obtain information regarding the functional \textit{in vivo} bronchoprotective profile, taking into account both pharmacodynamic and pharmacokinetic properties, olodaterol was tested in pharmacological models of acetylcholine (ACh)-induced bronchoconstriction in anaesthetized guinea pigs and dogs. ACh-induced bronchoconstriction models are widely used to test the \textit{in vivo} efficacy, potency, and duration of action of bronchodilators -such as \( \beta \)-agonists and anticholinergics- and are a good predictor for the efficacy of compounds in human airway diseases such as COPD, since an increase in the vagal cholinergic tone is discussed as the major reversible component in COPD (Barnes, 2004).
To mimic the clinical situation further, the Respimat® Soft Mist™ Inhaler was used for the administration of olodaterol, and for better comparison, for formoterol, too. The Respimat® Inhaler is a novel device which creates a soft mist aerosol without the use of propellants. In our in vivo studies the drugs were provided in water ethanol (40/60, v/v) solutions dissolved at concentrations permitting the administration of the desired dose with three actuations.

In both models, olodaterol provided bronchoprotection over 24 hours, whereas formoterol applied at an equally effective dose did not retain efficacy over 24 hours. It is noteworthy to mention that according to our observations the bronchoprotection mediated by β2 adrenoceptor agonists in the ACh-induced bronchoconstriction model in dogs is significantly less efficacious than in guinea pigs. Whereas most β2-adrenoceptor agonists studied during our research project easily exerted a 100% bronchoprotection in guinea pigs we did not identify a β adrenoceptor agonist capable of a 100% bronchoprotection in the dog model. The reason behind this discrepancy is not understood by us, but the different sensitivities of the two models may explain why in the dog model-in contrast to guinea pigs- olodaterol showed only at its maximum-effective dose a duration of action over 24 hours.

Formoterol is known as a β2 adrenoceptor agonist with a fast onset of action in humans and has gained recognition as a p.r.n. controller therapy because of this fast onset of action. Most interestingly, in the two species studied, also olodaterol offered a quick onset of action, similar to formoterol. The maximal bronchoprotection after inhalation of a single dose of olodaterol or formoterol was reached in guinea pigs within 3-6 minutes and 10 minutes in dogs, suggesting that olodaterol may have an rapid onset of action in humans, too.
Since beagle dogs are very sensitive to the cardiovascular (e.g. heart rate increase) and metabolic (e.g. increase in serum potassium, glucose and lactate) effects mediated by the systemic stimulation of β adrenoceptors (Greaves, 2000), the systemic pharmacodynamic effects of olodaterol were studied in this species, too. In the first experimental setting we determined the systemic effects after inhaled (intratracheal) administration of the compound in the same animals used for the efficacy studies. In the second setting we applied the compounds by intra-duodenal administration to mimic swallowing of the entire dose. Our preclinical data obtained in the two settings show that, for a given degree of bronchodilator activity, olodaterol has a greater cardiovascular (as assessed by heart rate) and metabolic (as assessed serum potassium, serum, glucose and serum lactate) safety margin than formoterol. Furthermore, in the two preclinical species analyzed, olodaterol was devoid of systemic pharmacodynamic effects at doses achieving a duration of action of 24 hours, suggesting a sufficient therapeutic window for its use in man. Since the systemic effects of beta agonists on serum potassium, serum lactate, or serum glucose are caused by the activation of β2 adrenoceptors in skeletal muscle and liver, we speculate that the larger safety margin we observed for olodaterol in comparison to formoterol in the dog model reflects differences in the pharmacokinetic profile and thus the systemic exposure of the two compounds.

The preclinical data presented here were confirmed in clinical studies both in asthma (O’Byrne, 2008) and COPD (van Noord, 2008, van Noord, 2009) patients. In all studies olodaterol showed a 24-hour duration of action after once-a day dosing concomitant with a good safety profile. The 24-hour bronchodilator efficacy of once daily dosing with olodaterol in patients with COPD was confirmed in a 4 week study, with all doses of olodaterol showing statistically significant increases in the primary endpoint, trough FEV₁, compared to placebo after 28 days of treatment, again with
an excellent safety profile (van Noord, 2009). Furthermore, in the 4 week study no differences in the FEV₁ profile after the first dose (day 1) and after 4 weeks treatment (day 29) were observed, implying the absence of clinical desensitization noted in some clinical studies after regular use of β₂ adrenoceptor agonists (Larj and Bleecker, 2002).

Therefore, a once-daily β₂ adrenoceptor agonist, like olodaterol, offers - compared with short-acting bronchodilators and b.i.d. LABAs - an improved convenience and compliance for asthma and COPD patients and has the potential to be combined with either a once-daily anticholinergic, like tiotropium (Tashkin, et al., 2008; Casarosa, et al., 2009) or upcoming new compounds within this class (Cazzola and Matera, 2008), once daily corticosteroids, or both, presented to the patients either as free or fixed-dose combinations. Besides the improved convenience, these combinations may offer beneficial long-term outcomes for the patients.

In summary, our preclinical data demonstate that olodaterol is an enantiomeric pure, selective and potent agonist of the human β₂ adrenoceptor. This molecule combines a novel efficacy profile towards the different β-ARs - by exerting almost full intrinsic activity at β₂-AR and a weak partial agonism at β₁-AR - together with a long duration of action, allowing a once-daily administration in humans, a rapid onset of action and an improved systemic pharmacodynamic effect profile.


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.


Legends for Figures

Fig. 1. Chemical structures of olodaterol (A), formoterol (B) and salmeterol (C).

Fig. 2. Functional selectivity of β₂-adrenoceptor agonists against the different β-AR subtypes. A, B, C: CHO cells selectively expressing the hβ₁ (A), hβ₂ (B) or hβ₃ (C) adrenoceptors were stimulated with increasing concentrations of agonists and cAMP levels were quantified. Data are presented as percentage of maximal isoprenaline-induced cAMP accumulation and the mean of three independent experiments (± S.E.M.) is shown.

Fig. 3. Effect of olodaterol and formoterol on isolated human bronchi. A) at resting tone and on bronchi pre-contracted with B) 10 µM histamine (51% acetylcholine (ACh) maximum (max)), C) with 0.1 mM ACh (80% ACh max). and D) electrical field stimulation (EFS). Data are shown as % of theophylline-induced relaxation and are expressed as mean ± S.E.M of a number of experiments indicated in table 4 for each condition.

Fig. 4. Bronchoprotective efficacy of olodaterol and formoterol in guinea pigs. The bronchoprotection of olodaterol (A) and formoterol (B) was determined in a model of ACh-induced bronchoconstriction in anaesthetized guinea pigs. Submaximal bronchospasms were induced by i.v. injections of ACh (10 - 12 µg/kg) every 10 minutes. Bronchoprotection is expressed as the percentage of inhibition of the increase in pulmonary resistance induced by ACh. Both compounds were administered with the Respimat® Soft Mist™ Inhaler. The time course of the bronchoprotection was recorded for 5 hours (N = 2 animals per dose).
Fig. 5. Duration of action of olodaterol and formoterol in guinea pigs. The bronchoprotective efficacy of olodaterol and formoterol was determined 6 hours (A) and 24 hours (B) after intra-tracheal instillation. Bronchospasms were induced by increasing ACh doses from 2 µg/kg to 20 µg/kg with a progression of 2 µg/kg per i.v. injection. N = 5–6 animals per dose. Data are shown as mean ± S.E.M. Significance, p < 0.05, indicated by * is against the respective control values.

Fig. 6. Onset of action of olodaterol and formoterol in guinea pigs. Olodaterol (A) or formoterol (B) were administered with the Respimat® Soft Mist™ Inhaler at three different doses. Bronchospasms were induced by ACh (10 µg/kg) 1, 3, 5, 7 and 20 minutes after drug inhalation. Bronchoprotection is expressed as the percentage of inhibition of the increase in pulmonary resistance induced by ACh. (N = 6 animals per dose).

Fig. 7. Bronchoprotective efficacy and systemic pharmacodynamic effects of olodaterol and formoterol in dogs over 3 hours. A, B, C, D: Both compounds were administered to anaesthetized ventilated dogs at doses of 0.15 µg/kg, 0.3 µg/kg, 0.6 µg/kg and 1.2 µg/kg using the Respimat® Soft Mist™ Inhaler. Bronchospasms were induced by i.v. injection of ACh (10 µg/kg i.v.) after 10, 30, 60, 120 and 180 minutes. Bronchoprotection (A) is expressed as the percentage of inhibition of the increase in pulmonary resistance induced by ACh. Systemic pharmacodynamic effects including heart rate (B), serum potassium (C) and serum lactate (D) were recorded at the same time points. (N = 6 animals per dose).
Fig. 8. Bronchoprotective efficacy and duration of action of olodaterol and formoterol in dogs over 24 hours. Both compounds were administered using the Respimat® Soft Mist™ Inhaler. Olodaterol and formoterol were applied at a dose that achieved equivalent bronchoprotective efficacy over 5 hours (0.6 µg/kg) and a lower dose of olodaterol (0.3 µg/kg). Bronchospasms were induced by i.v. injection of ACh (10 µg/kg i.v.) 0.5, 1, 6, 12 and 24 hours after administration of the compounds. (N = 6 animals per dose). Data are shown as mean ± S.E.M. Significance, p < 0.05, indicated by * is against the respective control values.

Fig. 9. Effect of olodaterol and formoterol on heart rate after intraduodenal (i.d.) administration to anesthetized beagle dogs. Olodaterol was applied at 1.2 µg/kg and 2.4 µg/kg i.d., formoterol was applied i.d. at 0.6 µg/kg and 1.2 µg/kg. Heart rate is expressed as a percentage of heart rate at time 0 (beats/min) recorded 1 minute before i.d. drug application as means ± SEM (N = 3 - 5 animals per dose).
Table 1. Pharmacological characterization of the different CHO cell lines stably expressing the human $\beta_{1,3}$ adrenoceptors. Levels of receptor expression (expressed in pmol/mg of protein content in membrane preparations) were determined in saturation binding assays, as described in the material and methods section. The average of at least three independent experiments performed in triplicate is shown.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$B_{\text{max}}$ (pmol/mg)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-h$\beta_{1,\text{HIGH}}$</td>
<td>12.4 ± 0.3</td>
<td>Binding experiments</td>
</tr>
<tr>
<td>CHO-h$\beta_{2,\text{HIGH}}$</td>
<td>1.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>CHO-h$\beta_{3,\text{HIGH}}$</td>
<td>22.0 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>CHO-h$\beta_{1,\text{LOW}}$</td>
<td>0.52 ± 0.04</td>
<td>Functional experiments (cAMP)</td>
</tr>
<tr>
<td>CHO-h$\beta_{2,\text{LOW}}$</td>
<td>0.056 ± 0.009</td>
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<tr>
<td>CHO-h$\beta_{3,\text{LOW}}$</td>
<td>0.23 ± 0.06</td>
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Table 2. Binding affinities of different β₂-AR agonists against the three human β-adrenoceptor subtypes. The equilibrium dissociation constants (pKᵢ) of the different ligands were determined in heterologous competition experiments against [³H]-CGP 12,177 in the presence of a GTP analog. The pKᵢ values (± SEM) shown are the average of at least three independent experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>pKᵢ hβ₁</th>
<th>pKᵢ hβ₂</th>
<th>pKᵢ hβ₃</th>
<th>Ratio β₁/β₂</th>
<th>Ratio β₃/β₂</th>
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</thead>
<tbody>
<tr>
<td>Isoprenaline</td>
<td>6.49 ± 0.01</td>
<td>6.54 ± 0.03</td>
<td>5.57 ± 0.07</td>
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<td>9</td>
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<tr>
<td>Olodaterol</td>
<td>7.33 ± 0.05</td>
<td>9.14 ± 0.04</td>
<td>5.26 ± 0.14</td>
<td>65</td>
<td>7586</td>
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<tr>
<td>Formoterol</td>
<td>6.07 ± 0.04</td>
<td>8.29 ± 0.03</td>
<td>5.58 ± 0.12</td>
<td>166</td>
<td>513</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>6.14 ± 0.02</td>
<td>9.24 ± 0.08</td>
<td>5.43 ± 0.01</td>
<td>1259</td>
<td>6457</td>
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</table>
Table 3. Functional properties of different β2-AR agonists against the three human β-adrenoceptor subtypes. Potency (pEC_{50} values) and intrinsic activity (I.A., expressed as percentage of isoprenaline-induced maximal response) of the different β2-adrenoceptor agonists were determined in CHO cell lines selectively expressing the β-AR subtype of interest, with the cAMP accumulation assay. Values shown are the average (± SEM) of at least 3 independent experiments, with each point determined in triplicate. The statistical significance of differences in intrinsic activity among the agonists for each receptor subtype were determined using one-way analysis of variance with Dunnett's post test for multiple comparisons. Statistical significance is denoted compared with the reference agonist isoprenaline (*: p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>hβ₁</th>
<th>hβ₂</th>
<th>hβ₃</th>
<th>Ratio</th>
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<tr>
<td></td>
<td>pEC_{50}</td>
<td>I.A.</td>
<td>pEC_{50}</td>
<td>I.A.</td>
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<tr>
<td>Isoprenaline</td>
<td>9.27 ± 0.08</td>
<td>100%</td>
<td>8.58 ± 0.08</td>
<td>100%</td>
<td>7.86 ± 0.07</td>
</tr>
<tr>
<td>Olodaterol</td>
<td>7.55 ± 0.08</td>
<td>52 ± 8% *</td>
<td>9.93 ± 0.07</td>
<td>88 ± 2%</td>
<td>6.57 ± 0.08</td>
</tr>
<tr>
<td>Formoterol</td>
<td>7.83 ± 0.06</td>
<td>91 ± 4%</td>
<td>9.73 ± 0.10</td>
<td>97 ± 3%</td>
<td>7.60 ± 0.07</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>6.08 ± 0.07</td>
<td>40 ± 6% *</td>
<td>9.90 ± 0.04</td>
<td>54 ± 7% *</td>
<td>6.15 ± 0.17</td>
</tr>
</tbody>
</table>

*: p < 0.05
Table 4. Potency and maximal efficacy (Emax) of olodaterol and formoterol on human isolated bronchi at resting tone or after a pre-contraction with 10 µM histamine or 0.1 mM acetylcholine. Potency values (pEC₅₀, mean ± S.E.M.) represent the average of n independent experiments. Maximal efficacy (Emax, mean ± S.E.M.) is reported as percentage of the maximal effect of theophilline. The maximal efficacies obtained with either olodaterol or formoterol in the presence of the different stimuli were analyzed by using one-way ANOVA followed by Dunnet’s multiple-comparison test. Statistical significance is denoted compared versus resting tone (*: p < 0.05).

<table>
<thead>
<tr>
<th>Stimulus:</th>
<th>Olodaterol</th>
<th>Formoterol</th>
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<tr>
<td></td>
<td>pEC₅₀</td>
<td>E_max (%)</td>
</tr>
<tr>
<td>Resting tone</td>
<td>9.97±0.10 (11)</td>
<td>87±3</td>
</tr>
<tr>
<td>Histamine [10 µM]</td>
<td>9.70±0.13 (7)</td>
<td>80±6</td>
</tr>
<tr>
<td>ACh[100 µM]</td>
<td>9.44±0.08 (13)</td>
<td>65±4*</td>
</tr>
<tr>
<td>EFS</td>
<td>9.49±0.24 (5)</td>
<td>86±5</td>
</tr>
</tbody>
</table>
Figure 4

A

B

Time [min]

Bronchoprotection [%]

- Olodaterol 0.1 μg/kg
- Olodaterol 0.3 μg/kg
- Olodaterol 1 μg/kg
- Olodaterol 3 μg/kg

- Formoterol 0.1 μg/kg
- Formoterol 0.3 μg/kg
- Formoterol 1 μg/kg
- Formoterol 3 μg/kg
**Figure 5**

**A**

[Graph showing the relationship between bronchospasm (%) and ACh doses (μg/kg). The graph compares different treatments: vehicle, olodaterol 3 μg/kg, and formoterol 3 μg/kg.]

**B**

[Graph showing the relationship between bronchospasm (%) and ACh doses (μg/kg). The graph compares different treatments: vehicle, olodaterol 0.3 μg/kg, olodaterol 1 μg/kg, olodaterol 3 μg/kg, and formoterol 3 μg/kg.]
Figure 6

A

![Graph showing the effect of different doses of olodaterol on bronchoprotection over time. The graph includes three lines representing 1 µg/kg, 3 µg/kg, and 10 µg/kg of olodaterol, with the 10 µg/kg line showing the highest bronchoprotection at all time points.]

B

![Graph showing the effect of different doses of formoterol on bronchoprotection over time. The graph includes four lines representing 0.3 µg/kg, 1 µg/kg, and 3 µg/kg of formoterol, with the 3 µg/kg line showing the highest bronchoprotection at all time points.]

Bronchoprotection [%]

Time [min]
Figure 8

- Black circles: vehicle
- Blue triangles: olodaterol 0.3 μg/kg
- Red triangles: olodaterol 0.6 μg/kg
- Orange triangles: formoterol 0.6 μg/kg

Y-axis: bronchoprotection [%]
X-axis: time [h]

The graph shows the bronchoprotection percentage over time for different treatments: vehicle, olodaterol 0.3 μg/kg, olodaterol 0.6 μg/kg, and formoterol 0.6 μg/kg. The data points are marked with asterisks (*) indicating statistical significance.
Figure 9

The graph depicts the heart rate (%) over time (min) for different treatments. The treatments include:

- Vehicle
- Olodaterol 1.2 µg/kg
- Olodaterol 2.4 µg/kg
- Formoterol 0.6 µg/kg
- Formoterol 1.2 µg/kg

The data points are represented with error bars to indicate variability.