Title page:

Pharmacological Characterization of Abediterol, a Novel Inhaled β2 Adrenoceptor Agonist with Long Duration of Action and a Favorable Safety Profile in Preclinical Models

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Running Title: *In vitro* and *in vivo* characterization of abediterol

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Abbreviations: $^{125}$I-CYP, (-)-3-$^{[125]}$Iodocyanopindolol; Ach, acetylcholine; ANOVA, analysis of variance; β, beta-adrenoceptor; ATPase, adenosine triphosphatase; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CRC, concentration-response curve; EC$_{50}$, half maximum effect; ECG, electrocardiogram; ED5, dose increasing heart rate by 5%; EDTA, ethylenediaminetetraacetic acid; EFS, electrical field stimulation; E$_{max}$, maximum effect; FDA, US Food and Drug Administration; FEV$_1$, forced expiratory volume in 1 second; HBSS, Hank’s Buffered Salt Solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HTRF, homogenous time resolved fluorescence; IBMX, 3-isobutyl-1-methylxanthine; IC$_{50}$, half maximal inhibitory concentration; ICS, inhaled corticosteroids; ID$_{50}$, dose inhibiting bronchospasm by 50%; i.m., intramuscular; i.v., intravenous; LABA, long-acting beta-adrenergic agonists; LAMA, long-acting muscarinic antagonists; abediterol, 5-((1R)-2-[[6-(2, 2-difluoro-2-phenylethoxy) hexyl] amino]-1-hydroxyethyl)-8-hydroxyquinolin-2(1H)-one; M, muscarinic receptor; PBS, phosphate-buffered saline; PEI, polyethyleneimine; PSS, physiological salt solution; t$_{1/2}$, terminal elimination half-life; SABA, short-acting β-agonist; ST, spontaneous tone.
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Abstract

Abediterol is a novel potent, long-acting inhaled β₂-adrenoceptor agonist in development for the treatment of asthma and COPD. Abediterol shows subnanomolar affinity for the human β₂-adrenoceptor and a functional selectivity over β₁-adrenoceptors higher than that of formoterol and indacaterol both in a cellular model with overexpressed human receptors and in isolated guinea pig tissue. Abediterol is a full agonist at the human β₂-adrenoceptor (Eₘₐₓ = 91 ± 5% of the maximum effect of isoprenaline). The potency and onset of action that abediterol shows in isolated human bronchi (EC₅₀ = 1.9 ± 0.4 nM, t₀½ onset = 7-10 minutes) is not significantly different to that of formoterol, but its duration of action (t₀½ ~ 690 minutes) is similar to that of indacaterol. Nebulized abediterol inhibits acetylcholine-induced bronchoconstriction in guinea pigs in a concentration-dependent manner, with higher potency and longer duration of action (t₀½ = 36 h) than salmeterol (t₀½ = 6 h) and formoterol (t₀½ = 4 h), and similar duration of action to indacaterol up to 48 h. In dogs, the bronchoprotective effect of abediterol is more sustained than that of salmeterol and indacaterol at doses without effects on heart rate, thus showing a greater safety margin (defined as the ratio of ED₅₀ increasing heart rate and ID₅₀ inhibiting bronchospasm) than salmeterol, formoterol, and indacaterol (5.6 versus 3.3, 2.2, and 0.3, respectively). In conclusion, results suggest that abediterol has a preclinical profile for once-daily dosing in humans together with a fast onset of action and a favorable cardiovascular safety profile.
Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are two of the most common chronic inflammatory diseases, affecting about 300 million and 80 million people worldwide, respectively. Recent projections from the World Health Organization estimate that the incidence of both diseases will increase globally in the near future, placing an enormous burden upon patients, families, and society (Masoli et al., 2004; Lokke et al., 2006). Both diseases are characterized by airflow obstruction, and the use of bronchodilator therapy is recommended in current international guidelines (www.ginasthma.org; www.goldcopd.org). In COPD, bronchodilators are the cornerstone of pharmacologic therapy, with inhaled β2-adrenoceptor agonists as one of the first-line therapies, together with anticholinergic drugs (Hanania and Donohue, 2007; Tashkin and Fabbri, 2010). In asthma, the use of long-acting β2-adrenoceptor agonists (LABAs) has been recently reviewed by the U.S. Food and Drug Administration (FDA), which announced that LABAs should never be used as a monotherapy in children or adults. However, when used in combination with inhaled corticosteroids (ICSs), LABAs remain critical in the symptomatic management of the disease (Lipworth, 2007; Robinson, 2010).

β-adrenoceptors have been subdivided into three distinct subtypes: β1, β2, and β3, classically identified in cardiac, airway smooth muscle and adipose tissues, respectively (Johnson, 2006). β2-adrenoceptor agonists act by binding to the β2-adrenoceptor which activates adenylate cyclase via the signal-transducing Gs protein, thereby enhancing cellular cyclic adenosine monophosphate (cAMP) levels and activating protein kinase A. One of the consequences of this signaling is a fall in intracellular calcium and the promotion of smooth muscle relaxation (Giembycz and Newton, 2006). Apart from the
affinity for \( \beta_2 \) receptors, potency of agonists also depends on the intrinsic efficacy of each compound. Intrinsic efficacy refers to the ability of a drug to activate its receptor, regardless of the drug concentration. Based on their intrinsic efficacy, \( \beta_2 \)-agonists are classified as full or partial agonists. Full agonists are defined as compounds that completely activate a receptor, whereas partial agonists are defined as compounds that only partially activate a receptor (Hanania et al., 2002; Kurose, 2004).

The first \( \beta_2 \)-selective adrenoceptor agonists used in respiratory diseases were the short-acting agonists salbutamol and terbutaline, with 4–6 h duration of bronchodilator action (Waldeck, 2002). Although salbutamol is widely used for the relief of acute bronchoconstriction, its short duration of action makes it less appropriate for regular use as maintenance medication (Beeh and Beier, 2010; Cazzola et al., 2010). The concept of LABAs was introduced with the development of twice-daily drugs like formoterol and salmeterol, with a bronchodilator effect lasting for 12 h. Both compounds have been widely used as maintenance medication in asthma and COPD, demonstrating better efficacy than salbutamol in both diseases (Waldeck, 2002; Beeh and Beier, 2010).

Because of the central role of bronchodilators in the treatment of both diseases, there is an interest in developing once-daily LABAs as an attempt to improve patient compliance and disease control (Cazzola et al., 2010). Indeed, the development of new ICSs, which can be used as a once-daily regimen, has provided additional rationale for the development of new ultra-LABAs that can be used in once-a-day combinations (Fitzgerald and Fox, 2007).

The most common dose-related side effects associated with \( \beta_2 \)-adrenoceptor agonists are tachycardia, muscle tremor, hypokalemia, and hyperglycemia (Rabe, 2003). Mild tachycardia is a common side effect observed in patients treated with \( \beta_2 \)-adrenoceptor...
agonists, which in part may result from the activation of β2-adrenoceptors present in peripheral vasculature, leading to vasodilation and reflex tachycardia, and may also be the consequence of the activation of cardiac β1-adrenoceptors, increasing heart rate directly (Sears, 2002). The stimulation of β2-adrenoceptors linked to the membrane-bound Na, K-adenosine triphosphatase (ATPase) on skeletal muscle cells leads to an influx of potassium into the cells, which may cause skeletal muscle tremor and indirectly cardiac effects (Sears, 2002). β-agonists also may affect glucose homeostasis by modulating insulin secretion, liver metabolism, and uptake of glucose (Philipson, 2002). To reduce the potential of systemic adverse events, a new generation of β2-adrenoceptor agonists has been designed to have a higher therapeutic margin than marketed drugs, by reducing systemic compound exposure and/or having higher selectivity versus the β1-adrenoceptor.

Abediterol, 5-((1R)-2-[(6-(2,2-difluoro-2-phenylethoxy)hexyl] amino}-1-hydroxyethyl)-8-hydroxyquinolin-2(1H)-one (Fig. 1), previously known as LAS100977, is a potent and selective inhaled β2-adrenoceptor agonist with a duration of action compatible with once-daily dosing in humans, a fast onset of action and a favorable safety margin in preclinical models. In this article, we report the pharmacological characterization of abediterol in in vitro and in vivo functional models compared with other marketed inhaled LABAs such as salmeterol, formoterol, and indacaterol.
Methods

Materials and Drug Preparation

Abediterol, salmeterol alpha-hydroxynaphthoate, formoterol fumarate, indacaterol maleate and propranolol hydrochloride were synthesized by the Department of Medicinal Chemistry (Almirall, Barcelona, Spain); CGP20712A methanesulfonate, 3-isobutyl-1-methylxanthine (IBMX), isoprenaline hemisulphate, acetylcholine (Ach) hydrochloride, carbachol chloride, salbutamol, zileuton, fexofenadine, theophylline and phosphate-buffered saline (PBS) with calcium and magnesium were purchased from Sigma-Aldrich (Tres Cantos, Spain); alprenolol was purchased from Sigma-RBI (Natick, MA, USA); Hank’s Buffered Salt Solution (HBSS) was obtained from Gibco (Carlsbad, CA, USA); ketamine chlorhydrate (Imalgene®) was purchased from Merial (Barcelona, Spain); xylazine hydrochloride (Rompun®) was purchased from Bayer (Barcelona, Spain); acepromazine maleate (Calmoneosan®) was purchased from Pfizer Salud Animal (Alcobendas, Spain) and propofol (Lipuro®) was obtained from B. Braun Surgical (Rubí, Spain).

Membrane preparations expressing human β₁ and β₂ adrenergic receptors (obtained from SF9 cells) were purchased from Membrane Target Systems, Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). Membranes were prepared from HTB-10 cells (human SK-N-MC neurotumor cells) obtained from the American Type Culture Collection (LGC Standards, Teddington, UK) and used as a source of β₃ adrenergic receptor. U937 cells (human leukemic monocyte lymphoma cell line), from the American Type Culture Collection, were used as a source of β₂ receptor. 3H-CGP12177 was obtained from Perkin Elmer Life and Analytical Sciences. 125I-CYP (-)-3-[125I]Iodocyanopindolol was obtained from GE Healthcare UK Ltd (Slough, UK).
Human lung tissue was obtained from patients undergoing surgery for lung carcinoma. None of the patients had a history of asthma. The protocol was approved by the Ethics Committee of University Clinic Hospital (Valencia, Spain) and informed consent was obtained from all patients.

Agonists were dissolved in dimethyl sulfoxide for binding and cellular studies. For in vitro isolated organ and in vivo studies, compounds were dissolved in a maximum of 2% (v/v) HCl or 2% (v/v) NaOH, and when required, in the presence of polyethylene glycol 300 (the maximum percentage was 5%). Krebs-Henseleit solution (guinea pig trachea and left atria studies) was composed of NaCl 118 mM; KCl 4.7 mM; MgSO4 1.2 mM; NaHCO3 25 mM; KH2PO4 1.2 mM; glucose 5.5 mM; CaCl2 2.6 mM. Test compounds were kept under dry conditions and prepared daily.

Animals

Male Dunkin-Hartley guinea pigs (450–600 g at the time of experimental procedures) were obtained from Charles River (Lyon, France). Guinea pigs were housed in groups of four or five at 20–24°C and 40–70% humidity; room air was changed 10 times per hour and the light/dark cycle was 12 h. Food (maintenance diet for guinea pigs with vitamin C [SAFE 114, SAFE, Augy, France]) and water were available ad libitum.

Male Beagle dogs (9–20 kg at the time of experimental procedures) were supplied by guaranteed commercial suppliers. Dogs were housed at 15–21°C and 40–70% humidity, under a 12 h light/dark cycle and fed a maintenance diet (Harlan TEKLAD 2021, Madison, WI, USA) with free access to water. All experiments were carried out with the approval of the Animal Ethics Committee of Almirall (Barcelona, Spain), and conducted in accordance with guidelines approved by the European Community (Directive 86/609/CEE) and by the Catalan Parliament (Decret 214/1997).
Radioligand Displacement Studies

Affinity for Human $\beta_1$ and $\beta_2$ Adrenergic Receptors

The affinity of abediterol, salmeterol, formoterol, and indacaterol for human $\beta_1$ and $\beta_2$ adrenergic receptors was determined by measuring their ability to displace the binding of $^3$H-CGP12177 to cell membrane preparations expressing one of the receptor subtypes. Protein concentrations were 16 and 5 $\mu$g/well for $\beta_1$ and $\beta_2$ receptor membrane preparations, respectively. Radioligand concentration was 0.14 and 0.6 nM for the $\beta_1$ and $\beta_2$ assays, respectively. A range of agonist concentrations was tested in duplicate to generate competition curves. Non-specific binding was measured in the presence of 1 $\mu$M propranolol. Assay reagents were dissolved in assay binding buffer (25 mM MgCl$_2$ and 2 mM EDTA pH=7.4) in a total volume of 250 $\mu$L. After 60 minutes of incubation at room temperature with gentle shaking in GF/C Multiscreen 96-well plates (Millipore, Barcelona, Spain), previously treated with assay buffer containing 0.3% PEI (Sigma-Aldrich), binding reactions were terminated by filtration and washing with 2.5 volumes of Tris/HCl 50 mM pH=7.4. Filters were then dried for 30 minutes before addition of 30 $\mu$L OptiPhase Hifase II® (PerkinElmer Life and Analytical Sciences) and radioactivity was quantified using a MicroBeta Trilux microplate scintillation counter (PerkinElmer Life and Analytical Sciences).

Affinity for Human $\beta_3$ Adrenergic Receptors

The affinity for human $\beta_3$ adrenergic receptors was determined by measuring ability to displace the binding of $^{125}$I-CYP to cell membrane preparations expressing this receptor. Human SK-N-MC neurotumor cells were grown, and the membranes prepared following methods previously described (Curran and Fishman, 1996). Membranes were
incubated with 1 nM of radioligand and 0.3 µM of the β₁ antagonist CGP20712A. Assay buffer was 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 mM MgCl₂ and 0.4% bovine serum albumin (BSA), pH=7.5. The final volume of each reaction was 250 µl. Non-specific binding was determined in the presence of 100 µM alprenolol. Samples were incubated for 90 minutes at 30°C with shaking. Binding reactions were terminated by filtration through Whatman GF/C membranes (BRANDEL, Gaithersburg, MA, USA), pre-wet in assay buffer at 4°C, using an M-24 harvester (BRANDEL). Filters were washed three times with 4 ml each of 50 mM Tris/HCl and 4 mM MgCl₂, pH 7.4. Filters were then dried for 30 minutes before addition of 5 ml OptiPhase Hifase 2® and radioactivity was quantified using the LKB 1219 RackBeta counter (PerkinElmer Life and Analytical Sciences).

**Data Analysis**

The affinity of each test compound for a given β adrenoceptor was determined by using at least six different concentrations ran in duplicate. Half maximal inhibitory concentration (IC₅₀) values were obtained by non-linear regression using Activity Base (IDBS Ltd, Guildford, UK) and the four parameters-log equation.

**Intrinsic Efficacy**

Intrinsic efficacy was determined by measuring the intracellular accumulation of cAMP induced by the interaction of agonists with the human β₂ adrenoceptors present in U937 cells. Cells were recovered, washed and the pellet dissolved in Assay Buffer with the following composition: HBSS with 10 mM HEPES, 1 mM ascorbic acid, 0.1% BSA and 500 µM IBMX. After counting and making the appropriate dilution, 50,000 cells were dispensed in each well of a 96-well microtiter plate. cAMP was measured as an indicator of the receptor function after stimulation with 1 µM isoprenaline (100%
stimulation) or the appropriate serial dilutions of the tested compounds, using the cAMP dynamic homogeneous time resolved fluorescence (HTRF) kit from Cisbio International (Bagnols/Ceze, France).

Data Analysis

The efficacy of each test compound on the receptor was determined by using at least seven different concentrations ran in duplicate. Maximum (Emax) and half maximum (EC50) stimulation of each compound relative to 1 µM isoprenaline were calculated using Activity Base.

Selectivity Studies

*cAMP Production in β1, β2, and β3 Overexpressing Cells*

Assays to determine selectivity for human β-adrenergic receptors were performed at Euroscreen S.A. (Brussels, Belgium). Briefly, CHO-K1 cells expressing the human adrenergic β1, β2, or β3 receptor were detached by gentle flushing with PBS-ethylenediaminetetraacetic acid (EDTA) (5 mM EDTA), recovered by centrifugation and resuspended in assay buffer (Krebs-Ringer-HEPES, 1 mM IBMX) at a concentration of 5,000 cells per well. The reference compounds were epinephrine, formoterol hemifumarate and ZD7114 for β1, β2, and β3 receptors, respectively.

Cells were incubated with test compounds in 96-well plates for 30 minutes at room temperature. During the incubation, the anti-cAMP cryptate antibody and the cAMP-D2 were added to each well according to the kit specifications (HTRF kit from CisBio International). The plate was covered and incubated for at least 1 h at room temperature. The plate was then read on the RUBYstar – High-Performance Time-Resolved Fluorescence Microplate Reader (BMG Labtech, Ortenburg, Germany). Data were
analyzed by non-linear regression using a single-site model and EC$_{50}$ for each β-
adrenergic receptor subtype were reported.

**Effect on Spontaneous Tone Guinea Pig Trachea**

The β$_2$ functional activity of compounds was assessed in guinea pig tracheal rings by
measuring their ability to relax tracheal smooth muscle, as described previously (Cortijo
et al., 1991). Briefly, after the equilibration period, isoprenaline was added at a
concentration of 0.1 µM to test tracheal ring relaxation. Preparations were then washed
twice with Krebs-Henseleit solution and left to recover for 15–30 minutes. Cumulative
concentration-response curves (CRCs) were built for each compound (0.01 nM to
0.1 µM). Incubation of each concentration did not exceed 30 minutes. At the end of the
experiment, 0.1 µM of isoprenaline was added to each preparation to obtain maximum
relaxation.

**Data Analysis**

β$_2$-adrenergic functional activity was determined through the quantification of the
relaxation produced by each concentration of compound with respect to the response
evoked by the initial administration of 0.1 µM isoprenaline, which was considered as
maximum and therefore equal to 100%. Potency values were expressed as the
concentration required to induce a 50% of maximum relaxation (EC$_{50}$), calculated using
Activity Base and the four parameters-log equation. IC$_{50}$ values were compared using
t test.
Effect on Guinea Pig Left Atria

β1-adrenergic functional activity of each compound was assessed in guinea pig left heart atria by measuring their ability to increase force of contraction. Briefly, atria were dissected and suspended in water jacketed organ baths containing 30 ml of Krebs-Henseleit solution at 37ºC and bubbled with 5% CO2 in oxygen. Isolated atria were connected with cotton thread to an isometric transducer TRI 201,202 (Panlab, Cornellà del Llobregat, Spain) under a resting tension of 1 g. This transducer was connected to a PowerLab system 8/30 (ADInstruments, Oxford, UK) to measure changes in tension and then paced with a field stimulator Hugo Sachs Electronic type D7806 (Harvard Apparatus, March-Hugstetten, Germany) at a frequency of 1 Hz (supra-maximum voltage, 0.1 ms). Tissues were left for 45 minutes to stabilize for basal contractions. Initially, 0.1 µM isoprenaline was added to the bath twice to test atria responsiveness. Atria were then washed twice with Krebs-Henseleit solution and left to recover for approximately 15 minutes. For each compound, a range of increasing and cumulative concentrations (1 nM to 10 µM) was added every 10–15 minutes to allow the reading of a stable effect. After the last administration, atria were washed with Krebs-Henseleit solution and 0.1 µM isoprenaline was administered to confirm that the maximum contraction was still achieved.

Data Analysis

β1-adrenergic functional activity was determined through the quantification of the contraction produced by each concentration of compound with respect to the response evoked by 0.1 µM isoprenaline, which was considered the maximal response and therefore equal to 100%. Potency values were expressed as the concentration required to
induce a 50% of maximum contraction (EC₅₀), calculated using Activity Base and the four parameters-log equation.

**Relaxant Effect on Spontaneous Tone Isolated Human Bronchi**

After the resection of one or more lung lobes, a piece of macroscopically normal tissue at a distance from the malignancy was supplied by the hospital pathologist, submerged in physiological salt solution (PSS, composition in mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.1, NaHCO₃ 25.0, glucose 11.7) at 4°C, and transported to the laboratory where subsegmental bronchi were dissected free from parenchymal lung tissue and preparations cut (3-4 mm length x 2-4 mm internal diameter) as previously reported (Cortijo et al., 2003). Bronchial rings were suspended in parallel on tissue hooks in 10 ml organ baths containing PSS and gassed with 5% CO₂ in O₂ at 37°C (pH 7.4). Each preparation was connected to UFI (Harvard Apparatus) or Grass FTO3 (Grass Instruments, MA, USA) force displacement transducers and isometric tension changes recorded using a standard software (Proto5; Letica, Barcelona or PowerLab™).

The preparations were suspended under an initial load of 2 g and equilibrated for 60–90 minutes with changes in bath PSS every 15–20 minutes before any pharmacological intervention occurred; at the end of equilibration period, the resting load was stable between 1 and 2 g.

To assess potency, human isolated bronchial rings were first contracted with 10 μM Ach to test viability. After washing and re-equilibration, relaxation-response curves were obtained in preparations at spontaneous tone (ST). Cumulative CRCs to tested compounds were obtained. After the maximum effect of each relaxant drug was obtained, theophylline (3 mM) was added to the bath to determine the maximum
relaxation of the preparation. Only one CRC to a relaxant agonist was recorded in each ring.

To assess onset and duration of action, preparation’s viability and responsiveness was tested by adding Ach (10 µM). After washing and re-equilibration, a single concentration that produced approximately 40–60% of relaxation was applied. Then, after 30 minutes, the preparation was washed and continuously superfused, and the changes in tone were followed up to 14–15 h. Control preparations from the same donor received the vehicle used in each case and were followed during the same period of time to check preparation’s stability.

**Onset and Duration of Action in EFS Human Bronchi**

In separate experiments, onset and duration of action of agonists was assessed using electrical stimulation of superfused bronchial strips. Tissue was prepared using superfusion apparatus (Coleman System, HSE-HA, Harvard, MA, USA) at a rate of 2 ml/min with oxygenated (5% CO₂, 95% O₂) PSS at 37°C. Spontaneous tone, induced by endogenous leukotrienes and histamine, was inhibited by zileuton (10 µM) and fexofenadine (10 µM), respectively. Electrical stimulation was delivered by bipolar electrodes placed parallel to the superfused tissue as 10-s trains of square-wave pulses (8 Hz; 0.5 ms duration; 40–50 V) generated every 2 min by a Grass stimulator. After the baseline contractile responses to the electrical stimulation were consistent, a concentration of agonist was added to inhibit around 60% of the stable baseline contractions induced by the electrical stimulation. The preparation was washed free of agonist after 30 min and the recovery of tone was recorded for 14–15 h. Control preparations from the same donor received the vehicle used in each case and were followed during the same period of time to check preparation’s stability.
**Data Analysis**

Agonist potency was calculated from cumulative CRCs and expressed as the concentration required for inducing a 50% of the relaxation caused by each compound (EC$_{50}$), considering 100% relaxation that produced by the agonists at the highest concentration tested. In order to report maximum efficacy of each compound, the effect of the highest concentration tested in ST preparations was referred to the maximum relaxation induced by 3 mM theophylline.

Onset was defined as the time spanning from agonist addition to the attainment of 50% (t$_{50}$) of the maximum relaxation produced by a single concentration. Duration of action was defined as the time spanning from agonist washout to the attainment of 50% (t$_{50}$) recovery from the relaxation produced by the agonist concentration added (Coleman and Nials, 1989). Statistical analysis of results was carried out by analysis of variance (ANOVA) followed by Bonferroni post-test. All calculations and analysis were performed using Prism (GraphPad Software, Inc., San Diego, CA, USA). Significance was accepted when $p < 0.05$.

**Potency and Duration of Action in Anesthetized Guinea Pigs**

To assess anti-bronchoconstrictor effects, animals were anesthetized with an intramuscular (i.m.) injection of ketamine (69.8 mg/kg), xylazine (5.6 mg/kg) and acepromazine (1.6 mg/kg). If required, anesthesia was extended by additional i.m. injections of the aforementioned anesthetic mixture. Animals were then tracheotomized, placed inside single plethysmograph chambers, and connected to a ventilator. An esophageal tube was inserted to measure transpulmonary pressure changes. The carotid artery and jugular vein were cannulated for blood pressure monitoring and intravenous (i.v.) administration, respectively. During the experiment, animals were artificially
ventilated at the rate of 60 strokes/minute and a tidal volume of 10 ml/kg. Temperature was maintained at 37°C.

A FinePointe™ RC System (Buxco Research Systems, NC, USA) was used to register the airway resistance (Raw) and dynamic compliance (Cdyn) in the anesthetized animals. This system measures the pressure changes that are driving respiration, and the resultant flows in and out of the airways. These variations in flow, transpulmonary pressure and blood pressure were registered with the BioSystem XA software (version 2.10 for Windows; NC, USA). Prior to starting the pulmonary dynamics evaluation, animals were connected to the ventilator until their breath pattern was stabilized. Once baseline values were in the range of 0.1 to 0.3 cmH₂O/ml/s of airway resistance and within 0.8–0.3 mL/cmH₂O of dynamic pulmonary compliance, the pulmonary measurement was initiated. After the stabilization period, bronchoconstriction was induced by two consecutive i.v. injections of Ach: a first dose of 10 µg/kg Ach and a second dose of 15 µg/kg Ach once the basal resistance level was recovered. The bronchoconstriction response achieved with the 15 µg/kg Ach bolus, that caused a bronchoconstriction representing a 3- to 5-fold increase of the baseline lung resistance, was the one used to calculate the inhibitory effect of each treated group, compared with the response of its respective control group.

Airway resistance (cmH₂O/ml/s) was calculated as the ratio of the changes in pressure and flow between isovolumetric points on inspiration and expiration. The airway resistance response to the Ach challenge was calculated for the vehicle and agonists from the formula: Airway resistance = \((R_M - R_B) \times 100/R_B\), where \(R_M\) is the peak resistance after challenge (maximum value) and \(R_B\) is the baseline resistance (10 breaths
before challenge). The inhibitory effect of each agonist was compared with its respective control group (vehicle only).

For compounds administered by inhalation route, guinea pigs were placed in a methacrylate box (17x17x25 cm) over a period of 10 minutes. On the 1st and the 5th minutes of this 10-minute period, vehicle or compound was aerosolized for 1 minute using an ultrasonic nebulizer (Devilbiss Ultraneb 2000, Somerset, PA, USA). The nebulization was driven by a mixture of 5% CO₂, 21% O₂ and 74% N₂ at a flow of 3 l/minute. Aerosols were generated from aqueous solutions, using different concentrations for each compound (0.01–3 µg/ml for abediterol, 0.1–30 µg/ml for indacaterol, 0.3–10 µg/ml for salmeterol, 0.1–30 µg/ml for formoterol, and 10–300 µg/ml for salbutamol).

The evaluation of the bronchoprotective potency was assessed 1 h after compound administration. For the duration of action study, the concentration inhibiting ≥85% of the bronchoconstriction at 1 hour post-administration was selected for each compound, and the anti-bronchoconstrictor effect was followed at different time points after compound exposure (2 h, 4 h, 6 h, 18 h, 24 h, 48 h, 72 h, or 96 h).

In preliminary experiments, plasma levels of aerosolized abediterol at 4 µg/ml, measured by HPLC after 1 h of exposure, were close to the limit of quantification (0.02 ng/ml). Plasma levels at the aerosol concentrations used in the experiments (0.01–3 µg/ml) were not detectable and did not allow us to establish PK/PD assessment in the core experiments.
Data Analysis

Potency was calculated from concentration-response values obtained at 1 h after treatment, which were fitted in a sigmoidal CRC using GraphPad Prism software to estimate bronchoprotective IC$_{50}$. IC$_{50}$ values were compared using t test.

Duration of action ($t_{1/2}$) was defined as the time taken to recover 50% of the maximum inhibitory effect achieved by the agonist, derived from time-course bronchoconstriction inhibition curves and calculated using a one-phase exponential decay formula. A one-way ANOVA followed by Dunnett’s post-test was used to determine statistical differences in bronchoconstriction. These analyses were performed using GraphPad Prism software.

Safety Margin and Duration of Action in Anesthetized Beagle dogs

The method of Konzett and Rössler (Konzett and Rössler, 1940) modified according to Misawa and colleagues (Misawa et al., 1986) was adapted. The animals were anesthetized with propofol (6 mg/kg i.v. plus a constant infusion of 0.6–0.7 mg/kg/minute i.v.) with a perfusor (Beckton Dickinson, Program 2, Brèzin, France) through one of the cephalic veins. A cuffed endotracheal tube was inserted through the fauces. Then, the animal was connected to a respirator and mechanically ventilated with room air under a constant pressure of 15 cm of water according to the following conditions: respiratory rate of 15 breaths/minute and tidal volume of 10 ml/kg, by means of a pump (Model 607-A, Harvard Apparatus). Electrocardiograph (ECG) leads were attached to the animal to record the lead II derivative of the ECGs. Responses of the bronchial musculature were measured considering the ventilation overflow, which was continuously measured by means of a pneumotachograph (TSD127, Biopac Systems Inc., Santa Barbara, CA, USA) attached to a High Sensitive Differential
Pressure Transducer (TSD160A, Biopac Systems) as an index of airway resistance. Bronchospasms were induced by a dose of Ach (5 µg/kg, i.v.), administered as a bolus, that produced a bronchial response that returned rapidly to baseline levels, did not affect physiological parameters such as heart rate and blood pressure and was high enough to be dose dependently inhibited by bronchodilators agents. The compounds to be tested were administered by inhalation and aerosolized in solution by means of an ultrasonic nebulizer from Aerogen Ltd. (Galway, Ireland) or from MuMed Ltd (London, UK) for safety margin and duration of action experiments, respectively, which was attached between the respirator pump and the endotracheal tube. Lead II of the ECG, heart rate, and pulmonary resistance were continuously acquired, analyzed and stored by means of a computer-based data acquisition system (Biopac Systems Inc.) and the Acknowledge III program.

Once two similar Ach basal responses at 10-minute intervals were obtained, the compound to be tested was administered by inhalation. Ach was administered again at 10 and 20 minutes after compound aerosolization. The inhibition of the bronchospasm was calculated at 20 minutes. Then, the i.v. infusion of the anesthetic agent was stopped and the animal was allowed to recover. To measure the response to Ach at 3, 6, and 24 h after compounds aerosolization, the same procedure was followed and two Ach injections were performed at 10-minute intervals. The inhibition elicited at these time points was considered as the mean of both bronchospasms.

Duration of action experiments were performed with a dose that produced a submaximum inhibitory effect at 20 minutes after compound inhalation.
The dose of the compound inhaled by each dog was calculated by measuring the amount of liquid delivered by the aerosol in the conditions aforementioned divided by the dog’s body weight in kg.

In preliminary experiments, plasma levels of abediterol at 2.4 µg/kg did not render detectable plasma levels (limit of quantification 0.02 ng/ml). The low plasma levels observed following the nebulization protocol did not allow us to establish PK/PD assessment in the core experiments.

**Data Analysis**

The dose inhibiting bronchospasm by 50% (ID$_{50}$) and the dose increasing heart rate by 5% (ED$_{5}$) were calculated by sigmoidal dose-response and linear regression analysis, respectively. The safety margin was defined as the quotient between the dose increasing heart rate by 5% and the dose inhibiting bronchospasm by 50%.

Statistical analysis of bronchospasm inhibition and heart rate increase data was performed by one-way ANOVA, followed by Dunnett’s post-test in the case of the percentages of inhibition. ID$_{50}$ values were compared using t test. In the duration of action experiments, the statistical analysis of bronchospasm inhibition data at various time points compared with the vehicle treated group was done by two-way ANOVA followed by Bonferroni post-test. These analyses were performed using GraphPad Prism software.
Results

In Vitro Binding, Functional Activity, and Selectivity at the Human Adrenergic Receptors

The affinity of abediterol, salmeterol, formoterol, and indacaterol for the human β-adrenergic receptors was assessed using membranes of Sf9 cells expressing the β1 or β2 receptor subtype and membranes of HTB-10 cells as a source of the β3 receptor subtype, and determined in radioligand binding displacement experiments. The affinities for the different β-adrenoceptor subtypes are summarized in Table 1. Abediterol affinity for the human β2 adrenoceptor was in the subnanomolar range, being the compound that showed the greatest affinity for the β2 receptor (abediterol > salmeterol > formoterol > indacaterol) of those studied.

Selectivity for β2 over the other β-adrenoceptor subtypes was also determined by calculating β1/β2 and β3/β2 selectivity ratios using the IC50 values, as shown in Table 1. Salmeterol was the most selective compound over the human β1 receptor, followed by abediterol, formoterol and indacaterol. Both salmeterol and abediterol showed high selectivity over the human β3 receptor, with higher ratios than formoterol and indacaterol (Table 1).

Functional selectivity for β2- over β1- and β3-adrenoceptors was determined by measuring agonist concentrations required to achieve 50% of maximum cAMP production (EC50) versus reference agonists in CHO-K1 cell lines stably expressing the human β1-, β2-, and β3-adrenergic receptors. β1/β2 and β3/β2 selectivity ratios were then calculated with the EC50 values obtained in the different cell lines, as shown in Table 2.
Salmeterol was the most selective compound for human $\beta_2$ receptor over the human $\beta_1$ and $\beta_3$ receptor, followed by abediterol, formoterol and indacaterol (Table 2).

**Intrinsic Efficacy in U937 Human Monocytic Cells**

The intrinsic efficacy of the compounds was studied by measuring the stimulation of cAMP production in U937 human monocytic cells. This cellular model was first characterized by using selective $\beta_1$- and $\beta_2$-adrenoceptor antagonists. The $\beta_2$ antagonist ICI8551 was able to completely block the isoprenaline-induced cAMP production, whereas no effect was observed when the cells were incubated with the $\beta_1$-adrenoceptor antagonist CGP20712 (data not shown), indicating that in U937 cells the $\beta_2$ receptor is the subtype responsible for cAMP production induced by $\beta$ agonists.

Fig. 2 shows the efficacy of abediterol, formoterol, salmeterol, and indacaterol, expressed as the percentage of the maximum effect of each compound on cAMP production with respect to 1 $\mu$M isoprenaline. Abediterol, formoterol and indacaterol behaved as full agonists with maximum efficacies of 91 ± 5%, 100 ± 3%, and 99 ± 5% at the highest concentration tested, respectively, whereas salmeterol behaved as partial agonist, with a maximum efficacy of 41 ± 3% (Fig. 2).

**Functional $\beta_1/\beta_2$ Selectivity in Guinea Pig Tissue**

All agonists (0.001–30 nM) produced concentration-dependent relaxations of the spontaneous tone in isolated guinea pig trachea (a $\beta_2$ adrenoceptor-containing preparation, Fig. 3A). Maximum efficacy, expressed as percentage of relaxation versus 0.1 $\mu$M isoprenaline, was achieved at 0.3, 1, 10 and 30 nM for formoterol, abediterol, indacaterol and salmeterol, respectively, corresponding to the highest concentration tested for each compound, as shown in Fig. 3A. The potency (EC$_{50}$) of abediterol
(0.02 nM) was comparable to that of formoterol and significantly higher than that of indacaterol and salmeterol \((p < 0.01, \text{Table 3})\).

\(\beta_1\)-adrenergic activity was assessed in electrically-stimulated guinea pig left atria. All compounds (0.001–10 \(\mu\)M) produced concentration-dependent contractions, as shown in Fig. 3B. The maximum efficacy observed at the highest concentration tested (10 \(\mu\)M) in percentage of contraction versus 0.1 \(\mu\)M isoprenaline was 103.0 ± 5.9, 57.8 ± 3.6, 32.5 ± 5.2, and 4.4 ± 5.6\% for formoterol, indacaterol, abediterol, and salmeterol, respectively. Due to methodological reasons it was not possible to test concentrations higher than 10 \(\mu\)M. When potency was expressed as \(\text{EC}_{50}\), compounds followed the potency rank of formoterol > indacaterol > abediterol ~ salmeterol (Table 3).

\(\beta_1/\beta_2\) functional selectivity was calculated as the ratio between the \(\beta_1\) and \(\beta_2\) potency in both guinea pig assays. Abediterol showed a high functional selectivity versus \(\beta_1\) atria activity, showing higher ratio than formoterol and indacaterol (Table 3).

**In Vitro Pharmacological Profile in Human Bronchi: Potency, Onset, and Duration of Action**

The pharmacological behavior of abediterol, salmeterol, formoterol and indacaterol was assessed in isolated human bronchi at spontaneous tone. All of the agonists (0.0001–10 \(\mu\)M) produced concentration-dependent relaxation. The rank of potency (\(\text{EC}_{50}\)) was formoterol > abediterol > indacaterol >> salmeterol, as shown in Table 4. When efficacy was expressed as the maximum effect versus the relaxation achieved by 3 mM theophylline, formoterol was the compound that showed the highest efficacy \((92 \pm 2\%)\), followed by abediterol \((82 \pm 4\%)\), indacaterol \((76 \pm 4\%)\) and salmeterol \((68 \pm 5\%)\).
The onset and duration of action were also studied in preparations at spontaneous tone, using agonist concentrations that produced between 40 and 60% of relaxation with respect to theophylline. As shown in Fig. 4 and Table 4, formoterol was the compound with the fastest onset of action, followed by abediterol, whereas indacaterol and salmeterol showed significantly slower onset of relaxant activity than formoterol. Abediterol was the compound that demonstrated the most sustained duration of action (t½ = 699 minutes), followed by indacaterol (t½ = 449 minutes), whereas salmeterol (t½ = 230 minutes) and formoterol (t½ = 76 minutes) showed shorter duration of the relaxant effect (Fig. 4 and Table 4).

**Onset and Duration of Action in EFS Human Bronchi**

The onset and duration of action of abediterol and reference compounds were studied in preparations contracted by electrical stimuli by using agonist concentrations that caused around 60% inhibition. Formoterol was the compound with the fastest onset of action (t½ = 3.1 minutes), followed by indacaterol (t½ = 6.5 minutes) and abediterol (t½ = 7.4 minutes), whereas salmeterol (t½ = 15.7) showed significantly slower onset, as shown in Table 5. Abediterol, salmeterol and indacaterol showed similar duration of action, significantly longer than that of formoterol (Table 5).

**Potency and Duration of Bronchoprotection in Guinea Pigs**

To evaluate the *in vivo* bronchoprotective potency of inhaled abediterol and comparators, four to five concentrations of each agonist were administered to different groups of guinea pigs (4–12 animals per group) 1 h before Ach-induced bronchoconstriction. All compounds inhibited the bronchoconstriction in a concentration-response manner (Fig. 5), achieving an inhibitory effect of 85–94% at the highest concentration tested. Abediterol showed the highest potency with an IC₅₀ value...
of 0.12 μg/ml, as shown in Table 6. Formoterol, indacaterol and salmeterol showed significantly lower potency with IC₅₀ values of 1.1, 2.2, and 2.7 μg/ml, respectively.

To assess the duration of bronchoprotective effects, submaximum doses of inhaled agonists were studied up to 96 h (Fig. 6). At the concentrations selected, an equieffective inhibition of Ach-induced bronchoconstriction (of 85–94%) was observed in concentration response curve experiments at 1 h after compound inhalation (Fig. 5). Abediterol and indacaterol demonstrated sustained duration of action, showing 57–60% of bronchospasm inhibition at 24 h after treatment, maintained up to 48 h for both compounds, whereas in the case of formoterol and salmeterol there was a significant loss of the bronchoprotective effect at 6 h, with complete loss of effect by 24 h. When the duration of action was expressed as the time taken to reduce the maximum bronchoconstriction achieved at 1 h by 50% (t½), abediterol and indacaterol showed values of 36 h (28–51) and 51 h (39–73), expressed as mean (95% confidence interval [CI]), respectively. This duration of action was considerably longer than that of salmeterol and formoterol, with a t½ of 6 h (4–13) and 4 h (3–7), expressed as mean (95% CI), respectively (Fig. 6). The model was validated with the short-acting β-agonist (SABA) salbutamol, showing a t½ of 1 h (1–4), expressed as mean (95% CI).

**Safety Margin in Anesthetized Beagle Dogs**

These experiments were performed to assess the efficacy and safety ratio of abediterol with respect to salmeterol, formoterol, and indacaterol when administered by inhalation. Efficacy was determined as the ability to revert the acetylcholine-induced bronchospasm in Beagle dogs, and safety was assessed as the effect on heart rate in the same animals.
Compounds inhibited the acetylcholine-induced bronchospasm in a concentration-dependent manner (Fig. 7). At the concentration range tested for each compound, the range of calculated doses was 0.04–0.6, 1.7–13, 0.2–5 and 0.5–11 µg/kg for abediterol, salmeterol, formoterol and indacaterol, respectively. Abediterol and formoterol were the most potent compounds with ID50 values of 0.16 and 0.24 µg/kg, respectively, followed by indacaterol and salmeterol, with significantly lower potency than abediterol (ID50 values of 1.2 and 4.4 µg/kg, respectively), as shown in Table 6.

All tested compounds demonstrated increases in heart rate, with salmeterol and indacaterol as the compounds that showed the highest percentages of increase at the concentrations tested, as shown in Fig. 7. The dose increasing heart rate by 5% (ED5) and the safety margin, expressed as the ratio between ED5 increasing heart rate and the ID50 inhibiting acetylcholine-induced bronchospasm were calculated. Abediterol was the compound with the highest safety margin (5.6), followed by formoterol (3.3), indacaterol (2.2), and salmeterol (0.3).

**Duration of Action in Anesthetized Beagle Dogs**

To assess the duration of bronchoprotection in Beagle dogs, the effect of single concentrations of inhaled agonists were studied up to 24 h in the acetylcholine-induced bronchoconstriction model. The effect on heart rate limited the agonist dose to be tested, thus in the case of salmeterol and indacaterol this effect prevented achievement of submaximum inhibitions (~80%). At the concentrations tested (Fig. 8), the calculated doses of abediterol, salmeterol and indacaterol were 1, 9 and 18 µg/kg, respectively and achieved a peak effect inhibiting the acetylcholine-induced bronchoconstriction by 76 ± 7%, 56 ± 5%, and 45 ± 2%, respectively, at 20 minutes after inhalation (Fig. 8). The effect on heart rate was also recorded just before the first Ach challenge, with
percentages of heart rate increasing by 4 ± 2%, 26 ± 2%, and 17 ± 2% for abediterol, salmeterol, and indacaterol, respectively.

When the bronchoprotective effect of agonists was assessed at different time points after inhalation, abediterol showed a sustained effect up to 6 h with percentages of inhibition of the acetylcholine-induced bronchospasm of 83 ± 2% and 71 ± 4% at 3 and 6 h, respectively, whereas the bronchoprotective effect of the other agonists was markedly decreased after 3 h of administration, with percentages of inhibition of 28 ± 12% and 20 ± 10% for salmeterol at 3 and 6 h, respectively, and percentages of inhibition of 30 ± 6% and 21 ± 5% for indacaterol at 3 and 6 h, respectively (Fig. 8). When the 24-h effects were compared, abediterol was the only compound showing some trend of inhibition of the acetylcholine-induced bronchospasm (38 ± 19%), whereas the effect of salmeterol and indacaterol was negligible, as shown in Fig. 8.
Discussion

The aim of this study was to establish the pharmacological profile of abediterol, a novel long-acting inhaled $\beta_2$-adrenoceptor agonist in development as a once-daily bronchodilator for maintenance treatment of asthma and COPD.

In radioligand binding displacement studies, abediterol demonstrated high affinity for the human $\beta_2$-adrenoceptor and great selectivity over the other human $\beta$-adrenoceptor subtypes. These data agree with the cAMP production data obtained in CHO transfected cells, where abediterol and salmeterol showed higher $\beta_1/\beta_2$ and $\beta_3/\beta_2$ selectivity ratios than formoterol and indacaterol. However, when $\beta_1/\beta_2$ selectivity was calculated with the activity observed in functional models using relevant tissues (atria and trachea) the selectivity was not an issue for any of the agonists tested. There was a trend towards higher selectivity for abediterol and salmeterol than for formoterol and indacaterol; the comparison between abediterol and salmeterol selectivity ratios was difficult due to the low $\beta_1$ efficacy observed for both compounds in guinea pig atria. Our results are consistent with those reported previously, indicating that the $\beta_1/\beta_2$ and $\beta_3/\beta_2$ selectivity ratios of formoterol and indacaterol are inferior to those of salmeterol (Ball et al., 1991; Battram et al., 2006; Bouyssou et al., 2010). In humans, it has been reported that activity at the $\beta_1$ adrenoceptor might be responsible for some of the cardiac side effects associated with $\beta$-adrenergic agonism (Levine and Leenen, 1989). For this reason, discovery programs of new-generation once-daily $\beta_2$ adrenergic agonists have sought compounds with $\beta_2$ selectivity over $\beta_1$. The relevance of the high $\beta_3/\beta_2$ selectivity is less clear; although it has been suggested that the $\beta_3$ adrenoceptor might have a role in lipolysis (Philipson, 2002).
The intrinsic efficacy of β-adrenergic agonists is also an important parameter to consider because partial agonists may act as β₂ antagonists in the presence of an agonist with higher efficacy at the same receptor (Källström et al., 1994). Abediterol behaved as a full or near-full agonist in both U937 cells and human bronchi. The results obtained with salmeterol and formoterol were consistent with those previously reported (Jeppsson et al., 1992; Naline et al., 1994) where the compounds behaved as partial and full agonists, respectively. Interestingly, the different intrinsic efficacies of β₂ agonists measured in preclinical models have not translated in man, where formoterol and salmeterol have reached comparable bronchodilator efficacies in both COPD and asthma (Kottakis et al., 2002). It has been suggested that β₂ intrinsic efficacy, together with physicochemical properties, contribute to the clinical onset of bronchodilation of β₂-adrenoceptor agonists, with full agonists more prone to exhibit faster onset of action (Rosethorne et al., 2010). Accordingly, abediterol showed a fast onset of action relaxing human bronchi, not significantly different to that of formoterol. The results obtained with the reference compounds were consistent with the rates of onset of action in humans; formoterol was an effective bronchodilator within 5 minutes, salmeterol took 15–30 minutes to achieve significant bronchodilation over baseline; and indacaterol showed an onset of action faster than salmeterol (Kottakis et al., 2002; Sugihara et al., 2010). The onset of action of abediterol in preclinical models was in line with first results in humans, where abediterol (5, 10, and 25 µg), at 5 minutes post-dose, significantly improved lung function compared with placebo \( p < 0.0001 \) and salmeterol \( p < 0.05 \) in patients with persistent asthma, suggesting a rapid onset of action (Beier et al., 2010). Although a fast-acting profile is not essential for inhaled drugs used as maintenance medication, a rapid relief of symptoms could increase patient’s confidence and reduce the risk of overdosing.
Potency of abediterol and reference compounds was assessed in isolated human bronchi and in anesthetized guinea pigs. In human bronchi, abediterol demonstrated high potency in relaxing preparations at spontaneous tone, with an effect comparable to that of formoterol and higher than that of salmeterol and indacaterol. These results are in-line with published data of β₂-adrenoceptor agonists in human bronchi, where formoterol was more potent than salmeterol and indacaterol (Naline et al., 2007).

The guinea pig has been widely used to assess the bronchodilator activity of β₂-agonists (Battram et al., 2006; Bouyssou et al., 2010). In the acetylcholine-induced bronchoconstriction model in anesthetized guinea pigs, abediterol exhibited the highest potency in comparison to reference compounds, in agreement with the potency observed in guinea pig tracheal preparations and in human bronchi. The high potency in preclinical models was confirmed in patients with mild to moderate asthma, where single inhaled doses of abediterol (5, 10 and 25 µg) induced significant increases in trough forced expiratory volume in 1 second (FEV₁) compared with placebo (Beier et al., 2010). These results suggest that the therapeutic dose of abediterol in humans will be lower than that of salmeterol, formoterol and indacaterol.

A number of factors may drive the duration of action of β₂-adrenoceptor agonists, for example, affinity for the β₂-adrenoceptor or long dissociation half-life, local binding near the receptor, metabolic stability or low clearance, and membrane diffusion kinetics. These hypotheses have been proposed to explain different duration of action of SABAs, twice-daily and once-daily LABAs (Casarosa et al., 2011). In order to compare the duration of action of abediterol with that of other β₂ agonists, compounds were tested in human bronchial preparations and in anesthetized guinea pigs and dogs. In human bronchi, our results showed that abediterol had similar duration to indacaterol, both in
spontaneous tone and in electrical field stimulated preparations. Similar models in human bronchi and guinea pig trachea have been reported to study the duration of action of LABAs (Nials et al., 1993; Naline et al., 1994). In line with our results, some discrepancies have been reported for the duration of action of formoterol and salmeterol in isolated tissue, showing shorter and longer duration of relaxant activity, respectively, based on what was expected according to their clinical profile (Naline et al., 2007). In our model, the duration of action of abediterol was similar to that of indacaterol, a compound that has demonstrated a profile compatible with once-daily dosing in patients with asthma and COPD (LaForce et al., 2008; Vogelmeier et al., 2010).

Abediterol also demonstrated long duration of action in anesthetized guinea pigs, showing a half-life of 36 h, which was 6-fold and 9-fold longer than that of salmeterol and formoterol, respectively. Moreover, the bronchoprotective effect of abediterol was similar to that of indacaterol up to 48 h, with a slight difference in the effect at 72 h. The effects of abediterol in anesthetized dogs also suggest that the compound has a long-lasting bronchoprotective activity, showing longer duration of action compared with salmeterol and indacaterol at doses without any impact on heart rate. Overall, this preclinical evidence is in line with the results obtained in asthma patients treated with abediterol, indicating that abediterol is suitable for once-daily dosing in man (Beier et al., 2010).

Tachycardia is a side effect typically associated with β2-adrenoceptor agonists, which may result from the activation of both β1- and β2-adrenoceptors; dose-response effects on heart rate have been reported for SABAs and LABAs (Rabe, 2003). In anesthetized dogs, inhaled abediterol had a lesser effect on heart rate compared with the other reference compounds at the doses needed to produce equivalent bronchodilation. Under
these experimental conditions, abediterol showed a safety margin higher than that of the comparators. Indeed, in duration of action experiments in dog, abediterol showed the lowest effect on heart rate at the concentration tested, although it was not possible to test equipotent bronchoprotective doses of salmeterol and indacaterol due to marked effects on heart rate at the highest dose tested. The modest effects of abediterol on heart rate might be partially explained by its selectivity for $\beta_2$ over $\beta_1$ receptors, shown in both binding and functional studies, however, the differences observed between the four agonists could also be explained by other factors such as a direct effect on the $\beta_2$ receptor in the peripheral vasculature and in the cardiac muscle itself. In the clinic, formoterol, salmeterol and indacaterol show a therapeutic margin of 2- to 4-fold, with tachycardia being a transient side effect in most cases (Sears, 2002; LaForce et al., 2008). This might represent a concern in case of overdosing or in susceptible patients with COPD that have concomitant cardiac diseases like chronic heart failure (Tashkin and Fabbri, 2010). In healthy subjects and asthmatic patients, abediterol was well tolerated and did not demonstrate clinically relevant effects on heart rate at doses $<10 \mu g$, suggesting a safety margin at least equivalent to that of currently licensed LABAs (Timmer et al., 2010).

In summary, the preclinical data reported in this study show that abediterol is a potent, selective and full $\beta_2$-adrenoceptor agonist with a rapid onset of action and long-lasting effects. Furthermore, abediterol has a favorable preclinical safety margin, showing reduced effects on heart rate. Hence, these data suggest that abediterol may provide a valuable treatment option for patients with asthma or COPD, with the potential to be combined with inhaled corticosteroids, long-acting anticholinergics or novel anti-inflammatory therapies.
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Participated in research design: Aparici, Gómez-Angelats, Vilella, Gavaldà, De Alba, Gras, Cortijo, Morcillo, Ryder, Beleta, and Miralpeix.

Conducted experiments: Vilella, Otal, Carcasona, Viñals, Ramos, Cortijo, and Morcillo.

Contributed to new reagents or analytic tools: Puig.

Performed data analysis: Aparici, Gómez-Angelats, Vilella, Otal, Carcasona, Viñals, Ramos, Gavalda, Cortijo, and Morcillo.

Wrote or contributed to the writing of the manuscript: Aparici, and Miralpeix.
References


Footnotes

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Figure Legends

**Fig. 1.** Chemical structure of abediterol.

**Fig. 2.** Intrinsic efficacy of β₂ adrenergic receptor agonists. Effect of agonists on intracellular cAMP levels were quantified in U937 cells. Data represent the percentages of cAMP production with respect to the maximum production induced by isoprenaline. Maximum efficacies of abediterol, salmeterol, formoterol, and indacaterol were 91 ± 5% \((n = 6)\), 41 ± 0.3% \((n = 6)\), 100 ± 3% \((n = 6)\), and 99 ± 5% \((n = 4)\), respectively. Data are shown as mean ± S.E.M.

**Fig. 3.** Functional β₁/β₂ selectivity in guinea pig tissues. Cumulative concentration-response curves for the β₂ adrenergic receptor agonists were performed in the guinea pig trachea model with preparations at spontaneous tone (A) and in guinea pig left atria (B) to assess relaxant and inotropic effects, respectively. Data are shown as percentages of relaxation (A) and percentages of contraction (B) relative to 0.1 µM isoprenaline, expressed as mean ± S.E.M., of at least four independent experiments \((n = 4–12)\).

**Fig. 4.** Onset and duration of action of β₂ adrenergic receptor agonists in isolated human bronchi. Onset and duration of action were determined in preparations at spontaneous tone by applying a concentration of agonist that produced a 40–60% of maximum relaxation induced by 3 mM theophylline. Onset was defined as the time spanning from agonist addition to the attainment of 50% of the maximum relaxation produced by the concentration added. Duration of action was defined by the time spanning from agonist washout to the attainment of 50% recovery from the relaxation produced by the agonist concentration added. Data are reported as mean ± S.E.M. of 5–12 experiments from 3–5 patient samples.
**Fig. 5.** Concentration response curves of inhaled abediterol, salmeterol, formoterol and indacaterol in the Ach-induced bronchoconstriction model in guinea pigs. Aerosols were generated from aqueous solutions using different concentrations from each compound. Then animals were anesthetized and bronchoconstriction was induced by Ach (15 µg/kg i.v.) 1 h after aerosol administration. Data are shown as mean ± S.E.M. of 4–12 experiments per dose; *p < 0.05, **p < 0.01, ***p < 0.001 versus baseline bronchospasm (one-way ANOVA, followed by Dunnett’s post-test).

**Fig. 6.** Duration of action of inhaled abediterol, salmeterol, formoterol and indacaterol in the Ach-induced bronchoconstriction model in guinea pigs. Conscious animals were exposed to aerosol solutions of agonists and then, at various time points they were anesthetized and bronchoconstriction was induced by Ach (15 µg/kg i.v.). The bronchoprotective effect of each agonist was assessed and expressed as inhibition percentage of control (vehicle) response. Data are represented as mean ± S.E.M.; n = 5 to 12; *p < 0.05; **p < 0.01; ***p < 0.005 compared with first observational time point (one-way ANOVA followed by Dunnett’s post-test).

**Fig. 7.** Effect of abediterol, salmeterol, formoterol and indacaterol on acetylcholine-induced bronchoconstriction and heart rate in anesthetized Beagle dogs. Nebulized compounds were administered to anesthetized animals, and bronchoconstriction was induced by Ach (5 µg/kg) at 20 minutes. Inhibition of bronchoconstriction was recorded as a percentage of the baseline response to Ach (straight line). Effect on heart rate was recorded as a percentage of increase from baseline values (dotted line). Data are shown as mean ± S.E.M. of 2–4 experiments per dose; *p < 0.05, **p < 0.01 versus baseline bronchospasm (one-way ANOVA, followed by Dunnett’s post-test).
Fig. 8. Duration of action of aerosolized abediterol, salmeterol and indacaterol in acetylcholine-induced bronchoconstriction in anesthetized Beagle dogs. Nebulized compounds or vehicle were administered to anesthetized animals, and bronchoconstriction was induced by Ach (5 µg/kg) at 20 minutes and 3, 6 and 24 h. Inhibition of bronchoconstriction was recorded as percentage of the baseline response to acetylcholine. Data are reported as mean ± S.E.M.; n = 2–5. **p < 0.01, ***p < 0.001 versus the vehicle treated group (two-way ANOVA followed by Bonferroni post-test).
TABLE 1. Binding affinity and selectivity ratios of abediterol, salmeterol, formoterol and indacaterol for the human $\beta_1$, $\beta_2$ and $\beta_3$ adrenergic receptors. Affinities are expressed as IC$_{50}$ (inhibitory concentration at which 50% of the total binding was inhibited). Each value represents the mean ± S.E.M. of at least 3 independent experiments ($n = 3–7$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Affinity</th>
<th>Binding Selectivity</th>
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<td></td>
<td>IC$_{50}$, nM</td>
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<td>Abediterol</td>
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<tr>
<td>Salmeterol</td>
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<td>Indacaterol</td>
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</tbody>
</table>

TABLE 2. Functional $\beta_2$ selectivity of agonists against the different human $\beta$-adrenergic receptor subtypes. CHO cells selectively expressing the human adrenergic $\beta_1$, $\beta_2$, and $\beta_3$ receptors were stimulated with increasing concentrations of agonists (0.03–10000 nM) and cAMP levels were
quantified as described in Materials and Methods. Data are reported as mean ± S.E.M. of three independent experiments. EC₅₀: concentration required to do the 50% of the maximum effect (versus the reference agonists epinephrine, formoterol hemifurate and ZD7114 for the β₁, β₂, and β₃ assays, respectively).

<table>
<thead>
<tr>
<th>Compound</th>
<th>cAMP Production</th>
<th>Functional Cellular Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀, nM</td>
<td>β₁</td>
</tr>
<tr>
<td>Abediterol</td>
<td>6.0 ± 0.7</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>84.0 ± 10.5</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Formoterol</td>
<td>2.5 ± 0.5</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>1.8 ± 0.3</td>
<td>0.96 ± 0.21</td>
</tr>
</tbody>
</table>
**TABLE 3.** Functional $\beta_1/\beta_2$ selectivity in guinea pig tissues. $\beta_1$ and $\beta_2$ activities of abediterol, salmeterol, formoterol and indacaterol were assessed in guinea pig left atria and trachea, respectively. Values are expressed as EC$_{50}$ and represent the mean of 4–12 independent preparations. Functional $\beta_1/\beta_2$ selectivity ratio was calculated.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Guine Pig</th>
<th>Functional Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$ (95% CI), nM</td>
<td>$\beta_1/\beta_2$</td>
</tr>
<tr>
<td></td>
<td>Trachea</td>
<td>Atria</td>
</tr>
<tr>
<td>Abediterol</td>
<td>0.02 (0.01, 0.06)</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>1.12 (0.84, 1.50) **</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Formoterol</td>
<td>0.07 (0.06, 0.11)</td>
<td>462 (314, 680)</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>0.7 (0.66, 1.10) **</td>
<td>6311 (4499, 8852)</td>
</tr>
</tbody>
</table>

CI, confidence interval; EC$_{50}$, concentration required to induce 50% of the maximum effect relative to 0.1 µM isoprenaline; **, $p < 0.01$ vs abediterol (t test).
**TABLE 4.** Potency, onset and duration of action of β-adrenergic receptor agonists in isolated ST human bronchi. EC₅₀ values were calculated from percentages of relaxation obtained from cumulative concentration-response curves in preparations at spontaneous tone. Onset and duration of action were determined by applying a single concentration of agonist that produced a 40–60% of effect with respect to theophylline. Onset of action was defined as the time spanning from agonist addition to the attainment of 50% of the maximum relaxation produced by the concentration added. Duration of action was defined as the time spanning from agonist washout to the attainment of 50% recovery from the relaxation produced by the concentration added.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Potency EC₅₀ (95% CI), nM</th>
<th>Onset Concentration tested, nM</th>
<th>Onset t½, minutes</th>
<th>Duration of Action t½, minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abediterol</td>
<td>1.9 (1.1, 2.8)</td>
<td>18/8</td>
<td>10/5</td>
<td>699 ± 77*</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>14.7 (8.0, 21.1)*</td>
<td>12/5</td>
<td>5/3</td>
<td>230 ± 55</td>
</tr>
<tr>
<td></td>
<td>Potency (95% CI)</td>
<td>Onset</td>
<td>Duration</td>
<td>Mean ± S.E.M.</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------</td>
<td>-------</td>
<td>----------</td>
<td>---------------</td>
</tr>
<tr>
<td><strong>Formoterol</strong></td>
<td>0.9 (0.4, 1.4)</td>
<td>18/9</td>
<td>30</td>
<td>12/4</td>
</tr>
<tr>
<td><strong>Indacaterol</strong></td>
<td>5.1 (1.0, 9.2)*</td>
<td>10/6</td>
<td>30</td>
<td>7/4</td>
</tr>
</tbody>
</table>

Potency data are reported as mean and confidence interval (95% CI). Onset and duration of action data are reported as mean ± S.E.M. *n/p experiments (‘n’, number of rings; ‘p’, number of patients). *p < 0.05 versus the corresponding formoterol values (ANOVA followed by Bonferroni).
TABLE 5. Onset and duration of action of β-adrenergic receptor agonists in EFS isolated human bronchi. Onset and duration of action were determined by applying a single concentration of agonist that produced around 60% inhibition of the EFS-induced contraction. Onset of action was defined as the time spanning from agonist addition to the attainment of 50% of the maximum inhibition produced by the concentration added. Duration of action was defined as the time spanning from agonist washout to the attainment of 50% recovery from the inhibition produced by the concentration added.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration tested, nM</th>
<th>Onset ( t_{1/2} ), minutes</th>
<th>Duration of Action ( t_{1/2} ), minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abediterol</td>
<td>30</td>
<td>5/5 7.4 ± 1.4</td>
<td>691 ± 120*</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>300</td>
<td>3/3 15.7 ± 6.7*</td>
<td>860 ± 20*</td>
</tr>
<tr>
<td>Formoterol</td>
<td>30</td>
<td>8/7 3.1 ± 0.6</td>
<td>117 ± 50</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>30</td>
<td>4/4 6.5 ± 1.4</td>
<td>616 ± 159*</td>
</tr>
</tbody>
</table>

Onset and duration of action data are reported as mean ± S.E.M. \( n/p \) experiments (‘n’, number of rings; ‘p’, number of patients). *\( p < 0.05 \) versus the corresponding formoterol values (ANOVA followed by Bonferroni).
TABLE 6. Potency of inhaled abediterol, salmeterol, formoterol, and indacaterol inhibiting the Ach-induced bronchoconstriction in anesthetized guinea pigs and in anesthetized dogs.

Nebulized agonists were administered to conscious guinea pigs and, 1 h later, maximum bronchoconstriction was induced by Ach (15 µg/kg i.v.) in anesthetized animals. Anti-bronchoconstrictor effects were determined as a percentage of inhibition of response to Ach versus the vehicle group. In anesthetized dogs, maximum bronchoconstriction was induced with Ach (5 µg/kg i.v.). Then, compounds were administered by aerosol, and 20 minutes later animals received a further Ach challenge. Anti-bronchoconstrictor effects were determined as a percentage of inhibition of response to Ach versus baseline values. Potency was expressed as IC$_{50}$ and ID$_{50}$ in the guinea pig and dog model, respectively ($n = 4–12$ guinea pigs, $n = 2–4$ dogs).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Anesthetized Guinea Pig</th>
<th>Anesthetized Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (95% CI), µg/ml</td>
<td>ID$_{50}$ (95% CI), µg/kg</td>
</tr>
<tr>
<td>Abediterol</td>
<td>0.12 (0.08, 0.19)</td>
<td>0.16 (0.07, 0.35)</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>2.7 (1.4, 5.2)***</td>
<td>4.4 (0.3, 71.4)*</td>
</tr>
<tr>
<td>Formoterol</td>
<td>1.1 (0.6, 1.9)***</td>
<td>0.24 (0.12, 0.44)</td>
</tr>
<tr>
<td>Indacaterol</td>
<td>2.2 (0.8, 6.1)***</td>
<td>1.2 (0.2, 8.7)*</td>
</tr>
</tbody>
</table>

CI, confidence interval; IC$_{50}$, concentration required to induce 50% inhibition; ID$_{50}$, dose required to induce 50% inhibition. *$p < 0.05$; ***$p < 0.001$ versus the corresponding abediterol values (t test).
Figure 3

A) Relaxation in trachea (% vs isoprenaline)

-12 -11 -10 -9 -8
-log [agonist] (M)

○ Abediterol
■ Salmeterol
◆ Formoterol
▲ Indacaterol

B) Contraction in atria (% vs isoprenaline)

-9 -8 -7 -6 -5
-log [agonist] (M)
Figure 4

The graph shows the relaxation (%) vs. theophylline over time (min) for different drugs: Abediterol 30nM, Salmeterol 300nM, Formoterol 30nM, and Indacaterol 30nM. As time increases, the relaxation also increases, with the highest relaxation seen in Indacaterol 30nM and the lowest in Salmeterol 300nM.
Figure 7

Abediterol

Salmeterol

Formoterol

Indacaterol

Inhibition of bronchoconstriction (%) vs. Heart rate increase (%)

Aerosol concentration (mg/ml)

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