Pharmacological Profile of AZD8871 (LAS191351), a Novel Inhaled Dual M₃ Receptor Antagonist/ β_2 -Adrenoceptor Agonist Molecule with Long-Lasting Effects and Favorable Safety Profile

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ABSTRACT

AZD8871 is a novel muscarinic antagonist and β_2 -adrenoceptor agonist in development for chronic obstructive pulmonary disease. This study describes the pharmacological profile of AZD8871 in vitro and in vivo assays. AZD8871 is potent at the human M₃ receptor (pIC₅₀ in binding assays: 9.5) and shows kinetic selectivity for the M₃ (half-life: 4.97 hours) over the M₂ receptor (half-life: 0.46 hour). It is selective for the β_2 -adrenoceptor over the β_1 and β_3 subtypes (3- and 6-fold, respectively) and shows dual anti-muscarinic and β_2 -adrenoceptor functional activity in isolated guinea pig tissue (pIC₅₀ in electrically stimulated trachea: 8.6; pEC₅₀ in spontaneous tone isolated trachea: 8.8, respectively), which are sustained over time. AZD8871 exhibits a higher muscarinic component than batenfenterol in human bronchi, with a shift in

potency under propranolol blockade of 2- and 6-fold, respectively, together with a persisting relaxation (5.3% recovery at 8 hours). Nebulized AZD8871 prevents acetylcholine-induced bronchoconstriction in both guinea pig and dog with minimal effects on salivation and heart rate at doses with bronchoprotective activity. Moreover, AZD8871 shows long-lasting effects in dog, with a bronchoprotective half-life longer than 24 hours. In conclusion, these studies demonstrate that AZD8871 is a dual-acting molecule with a high muscarinic component and a long residence time at the M₃ receptor; moreover, its preclinical profile in animal models suggests a once-daily dosing in humans and a favorable safety profile. Thus, AZD8871 has the potential to be a next generation of inhaled bronchodilators in respiratory diseases.

Introduction

Chronic obstructive pulmonary disease (COPD) is a common, preventable, and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation due to airway and/or alveolar abnormalities caused by exposure

to noxious particles or gases (<http://www.goldcopd.org>). The World Health Organization estimates that COPD affects 65 million people worldwide, and it is projected to be the third cause of death by 2030 (Cohen et al., 2016; Benton et al., 2018).

Dual bronchodilator therapy of long-acting muscarinic antagonists (LAMA) and long-acting β_2 -adrenoceptor agonists (LABA) is a cornerstone of COPD treatment, and combinations of both therapies show superior bronchodilation to the individual therapies and are recommended for patients who remain symptomatic despite bronchodilator monotherapy (Thomas et al., 2017; Celli, 2018).

LAMAs and LABAs elicit bronchorelaxant effects by acting on different mechanisms, which are complementary and provide rationale of combining the two classes (Calzetta et al., 2018). LAMAs inhibit the action of acetylcholine (ACh) by binding to the muscarinic ACh receptors in the airway smooth muscle (Barnes, 2004; Belmonte, 2005) and show kinetic selectivity for the M₃ receptor (Gavalda et al., 2009; Sykes et al., 2012; Salmon et al., 2013). On the contrary, LABAs induce direct bronchodilation by activating the β_2 -adrenoceptors in the airways, leading to an increase in cellular cAMP levels

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ABBREVIATIONS: ACh, acetylcholine; CID, compound identifier; COPD, chronic obstructive pulmonary disease; EFS, electrical field stimulation; ID₄₀, dose required for 40% inhibition of ACh-induced bronchoconstriction; LABA, long-acting β_2 -adrenoceptor agonist; LAMA, long-acting muscarinic antagonist; MABA, muscarinic antagonist and β_2 -adrenoceptor agonist; [³H]-NMS, [N-methyl-³H] scopolamine methyl chloride; pEC₅₀, concentration required to induce 50% of maximum effect; pIC₅₀, concentration required for 50% inhibition.

that cause smooth muscle relaxation (Giembycz and Newton, 2006; Cazzola et al., 2012); and, although they primarily activate the β_2 -adrenoceptors, they also have some activity at the β_1 -adrenoceptor level (Aparici et al., 2016). Some authors have proposed a cross-talk between the M_3 and the β_2 -adrenoceptor, and they have suggested that the combination of both mechanisms may lead to synergistic effects (Calzetta et al., 2015, 2018). Furthermore, the blockade of the M_2 receptor on the airways' smooth muscle may provide additional bronchodilation by enhancing the relaxation induced by the β_2 -adrenoceptor agonism (Sarria et al., 2002; Proskocil and Fryer, 2005; Brown et al., 2013).

Several LAMA/LABA fixed-dose combinations have been approved for maintenance therapy of COPD and have demonstrated a good efficacy and tolerability profile (Tashkin and Ferguson, 2013; Singh, 2015; Cohen et al., 2016). However, the addition of inhaled corticosteroids to the dual bronchodilator therapy is recommended in patients who have clinically significant symptoms despite dual-therapy treatment and are at high risk for frequent or severe exacerbations (Lipson et al., 2018). In this regard, bifunctional molecules with both muscarinic ACh receptor antagonist and β_2 -adrenoceptor agonist activity (MABA) represent an alternative to use LAMA/LABA fixed-dose combinations and have the potential to be a useful platform for the development of triple therapy in one inhaler (Cazzola et al., 2013; de Miguel-Diez and Jimenez-Garcia, 2014).

Although a variety of MABA molecules have been disclosed (Hughes and Jones, 2011; Aparici et al., 2017), few drugs of this class are currently in active development. Batefenterol (GSK961081) (Hughes et al., 2015) is the compound that reached the most advanced phase of development (Wielders et al., 2013), which showed effective bronchodilation and long-lasting effects and appeared to be safe and well tolerated in COPD patients after several days of treatment (Bateman et al., 2013; Norris and Ambery, 2013; Wielders et al., 2013); however, the compound does not appear to be in active development. AZD2115 and AZD8999 (LAS190792) (Norman, 2012; Aparici et al., 2017) are MABA molecules that reached phase 2 and phase 1 clinical development, respectively (<https://clinicaltrials.gov/ct2/show/NCT02109406>, <https://clinicaltrials.gov/ct2/show/NCT02059434>), but no further development has been reported.

AZD8871 (*trans*-4-[[[3-[5-(((2*R*)-2-hydroxy-2-(8-hydroxy-2-oxo-1,2-dihydroquinolin-5-yl)ethyl)amino)methyl]-1*H*-1,2,3-benzotriazol-1-yl]propyl](methyl)amino)cyclohexyl hydroxy(di-2-thienyl)acetate ((*trans*)-4-((2-(((2-chloro-4-(((*R*)-2-hydroxy-2-(8-hydroxy-2-oxo-1,2-dihydroquinolin-5-yl)ethyl)amino)methyl)-5-methoxyphenyl)carbamoyloxy)ethyl)(methyl)amino)cyclohexyl 2-hydroxy-2,2-di(thiophen-2-yl)acetate (LAS191351) (Fig. 1) is a novel, potent, selective, and long-lasting MABA compound currently in phase 2 clinical development as a maintenance inhaled therapy for the treatment of COPD (<http://clinicaltrials.gov/ct2/show/NCT02971293>). Together with CHF6366, currently in phase 1 (<https://clinicaltrials.gov/ct2/show/NCT03378648>), they are the only MABA molecules that appear to be in active development at present. In a phase IIa trial, 100 or 600 μ g AZD8871 showed significant and sustained bronchodilation in patients with moderate to severe reversible COPD after 14 days of dosing, with significant symptom improvements and no emerging safety concerns (Psallidas et al., 2018). In this work, we report the preclinical in vitro and in vivo pharmacological profile of AZD8871 in comparison with batefenterol; the muscarinic ACh receptor antagonists tiotropium, aclidinium, glycopyrrolate,

Fig. 1. Chemical structure of AZD8871.

and ipratropium; and the β_2 -adrenoceptor agonists olodaterol, indacaterol, salmeterol, formoterol, and salbutamol.

Materials and Methods

Materials and Drug Preparation

AZD8871 acetate, aclidinium bromide [PubChem compound identifier (CID) 11519741], batefenterol ethane disulfonate (PubChem CID 11629376), formoterol fumarate (PubChem CID 9832292), indacaterol maleate (PubChem CID 6918554), olodaterol free base (PubChem CID 11504295), propranolol hydrochloride (PubChem CID 62882), salmeterol free base (PubChem CID 5152), and tiotropium bromide (PubChem CID 5487426) were synthesized by the Department of Medicinal Chemistry of Almirall (Barcelona, Spain); ACh chloride (PubChem CID 6060), ipratropium bromide (PubChem CID 657308), isoprenaline hemisulfate (PubChem CID 8239), phosphate-buffered saline with calcium and magnesium, pilocarpine hydrochloride (PubChem CID 5909), salbutamol free base (PubChem CID 2083), and urethane were purchased from Sigma-Aldrich (Tres Cantos, Spain). Membrane preparations expressing human M_1 , M_2 , M_3 , M_4 , or M_5 receptors (obtained from CHO cells) were purchased from Membrane Target Systems, PerkinElmer Life, and Analytical Sciences (Boston, MA). [N-methyl- ^{3} H] scopolamine methyl chloride was obtained from PerkinElmer Life and Analytical Sciences. For binding studies, compounds were dissolved in DMSO. 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 studies) was composed of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 , 5.5 mM glucose, and 2.6 mM $CaCl_2$. Test compounds were kept under dry conditions and prepared daily.

Animals

Male Dunkin Hartley guinea pigs (body weight 340–600 g) were obtained from Harlan (Sant Feliu de Codines, Spain) and housed in groups of four or five at 20–24°C and 45%–65% humidity, under a 12-hour light cycle for at least 5 days before use. Standard maintenance diet supplemented with vitamin C (SAFE114, SAFE, France) and water were available ad libitum.

Male Beagle dogs (12 $\pm$ 0.3 kg at the time of experimental procedures) were supplied by Harlan. Dogs were housed at 15–21°C, 40%–70% humidity, under a 12-hour light/dark cycle and fed on a maintenance diet (Harlan Teklad, Madison, WI), with free access to water. On the day of the experimental procedure, dogs were each fasted for 18 hours with water ad libitum prior to anesthesia.

Ethics Approval

In vivo experiments were approved and monitored by the Animal Ethical Committee of Almirall (Barcelona, Spain) following the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and in accordance with European Union Directive 2010/63/EU for animal experiments. Human lung tissue was obtained from patients who were 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) by Act 234 of 28/01/2009, and informed consent was obtained from all patients.

N-Methyl-[³H] Scopolamine Methyl Chloride Radioligand Displacement Studies

Affinity for the Human Muscarinic ACh Receptors. The affinity for the human muscarinic ACh receptors was determined by measuring ability to displace the binding of N-methyl-[³H] scopolamine methyl chloride ([³H]-NMS) to cell membrane preparations expressing the different receptor subtypes, as previously described (Gavalda et al., 2014). At least six different concentrations of each test compound were run in duplicate. IC₅₀ (concentration required for 50% inhibition of binding) values were obtained by nonlinear regression using Activity Base (IDBS, Guildford, Surrey, UK) and the four-parameters log equation, and reported as negative logarithm (pIC₅₀). Values are reported as mean ± S.E.M. of at least three independent experiments. Affinities at equilibrium were determined as equilibrium antagonist dissociation constant values by correcting the experimental IC₅₀ values obtained for each compound, according to Cheng and Prusoff (1973).

Dissociation from Human M₂ and M₃ Muscarinic Receptors. The dissociation of test compounds from the human M₂ and M₃ receptors was assessed by incubating compounds at 10-fold their dissociation constant values with membranes expressing the respective receptor (PerkinElmer Life and Analytical Sciences) (33 µg/ml) in phosphate-buffered saline with calcium and magnesium (Sigma-Aldrich) for 3 hours. Then dissociation was initiated by the addition of [³H]-NMS (PerkinElmer Life and Analytical Sciences) (15 nM). Bound and free [³H]-NMS were separated at different times (between 5 minutes and 22 hours), filtered in Millipore GF/C plates (Millipore, Barcelona, Spain) pretreated 1 hour with polyethylenimine 0.05% (Sigma-Aldrich), washed six times with ice-cold wash buffer (50 mM Tris, 100 mM NaCl, pH 7.4), and counted in a MicroBeta Trilux microplate scintillation counter (PerkinElmer Life and Analytical Sciences). The percentage of inhibition of [³H]-NMS binding at each time point reflected the percentage of occupied receptors by the compounds. Dissociation was expressed as half-life, calculated using Activity Base (IDBS) and the one-phase exponential decay equation.

Activity at Human β -Adrenoceptors. Activity at the human β -adrenoceptors was assessed in CHO-K1 cells expressing the human β_1 -, β_2 -, or β_3 -adrenoceptors by measuring intracellular cAMP production, as previously described (Aparici et al., 2012). Potency of each test compound was determined by using different concentrations run in duplicate. Data from at least two independent experiments were analyzed with XLfit software (IDBS), and concentration required to induce 50% of maximum relaxation (pEC₅₀) values (negative logarithm of the concentration required to achieve 50% of the maximal effect) were reported. Maximal effect was established with the agonists epinephrine, formoterol hemifurate, and ZD7114 for the β_1 , β_2 , and β_3 assays, respectively.

Off-Target Activities. AZD8871 was tested in a panel of 55 targets including receptors, transporters, and channels, at a single concentration of 1 µM, and binding inhibition was measured. For those targets in which the compound showed an effect >50%, IC₅₀ values were obtained by testing eight different concentrations. Experiments were conducted at Eurofins (Celle L'Evescault, France).

Functional Studies in Isolated Tissue

Potency and Duration of Action in Guinea Pig Trachea. Potency was assessed in electrical field-stimulated (EFS) guinea pig tracheal strips, and antimuscarinic activity was dissected in the presence of 1 µM of the β -adrenoceptor antagonist propranolol, as previously described (Aparici et al., 2017). Briefly, potency was determined by the infusion of cumulative increasing concentrations (0.01–1000 nM) of test compounds and expressed as the negative

logarithm of the concentration required for 50% inhibition of the basal electrically stimulated induced contraction (pIC₅₀).

In independent experiments, duration of action was assessed in EFS tracheal strips in presence of 1 µM propranolol. Briefly, preparations were incubated with single concentrations of test compounds producing 60%–80% inhibition for 1 hour. Then preparations were washed, and the percentage of tone recovery was assessed after 15 hours. At least three preparations were used in each experiment.

Potency at the β_2 -adrenoceptors was assessed in guinea pig tracheal rings by measuring their ability to relax spontaneous tone tracheal smooth muscle, as described previously (Aparici et al., 2012). Cumulative concentration–response curves were built for each compound (0.01–100 nM). At the end of the experiment, 0.1 µM isoprenaline was added to each bath to obtain maximum relaxation. Potency was expressed as the negative logarithm of the concentration required to induce a 50% of maximum relaxation (pEC₅₀) evoked by isoprenaline. At the end of the concentration–response curves, preparations were washed twice and tension was followed for 1 hour. Duration of action was expressed as percentage of tone recovery after 1 hour.

Calculations were performed with Activity Base and the four-parameters log equation by using Prism (GraphPad Software, La Jolla, CA). pIC₅₀ and pEC₅₀ of each compound with respect to AZD8871 were compared by *F* test using Prism.

Potency and Duration of Action in Human Bronchi. Isolated human bronchi were used fresh, immediately after surgery, to assure good responses to EFS, as previously described (Aparici et al., 2012). MABA and antimuscarinic potencies were calculated from cumulative concentration–response curves in absence and presence of 1 µM propranolol, respectively. Theophylline (3 mM) was added at the end of the experiment to determine the maximal relaxation. Potency was expressed as the negative logarithm of the concentration required for 50% inhibition of the maximal electrically stimulated induced contraction (pIC₅₀) considering 100% of effect that produced by 3 mM theophylline. Calculations were done using Prism (GraphPad Software) and sigmoidal dose–response equation (variable slope). pIC₅₀ values of each compound in absence and in presence of propranolol were compared by *F* test using Prism.

Duration of action was assessed in independent experiments, as previously described (Aparici et al., 2017), and expressed as percentage of recovery at 8 hours from the end of drug administration.

In Vivo Bronchoprotection Studies

Potency in Guinea Pigs. The bronchoprotective potency of test compounds was assessed in a guinea pig bronchoconstriction model, as previously described (Aparici et al., 2016). Briefly, conscious guinea pigs were placed in a methacrylate box for 10 minutes. On the first and fifth minute of this 10-minute period, vehicle or compound was aerosolized for 1 minute at a flow rate of 3 l/min by using an ultrasonic nebulizer (Devilbiss Ultraneb 2000; Devilbiss Healthcare, Somerset, PA). Study compounds were AZD8871 (0.3, 1, 3, and 10 µg/ml), batesfenterol (0.3, 1, 3, and 10 µg/ml), tiotropium (1, 3, and 10 µg/ml), or vehicle. Each treatment was administered to 4–10 animals. Bronchoconstriction was induced with 15 µg/kg ACh administered by an intravenous bolus 1 hour after compounds administration, as described previously (Aparici et al., 2016). The bronchoprotective potency of each compound was calculated from concentration–response values and expressed as the concentration of each compound required for 50% inhibition of bronchoconstriction exhibited by the vehicle-treated group (IC₅₀). Calculations were performed with GraphPad Prism software using a sigmoidal fitting and one-way ANOVA analysis versus baseline, followed by application of Dunnett's post-test.

Potency and Duration of Action in Dogs. AZD8871 and batesfenterol were characterized in anesthetized Beagle dogs following the method described in Aparici et al. (2016, 2017). Briefly, dogs were anesthetized with propofol (Lipuro; B. Braun Surgical, Rubí, Spain) (7 mg/kg i.v. bolus and constant infusion of 0.25 mg/kg per minute)

and medetomidine (Domptor; Pfizer, Alcobendas, Spain) (2 $\mu\text{g/kg}$ i.v. bolus and constant infusion at 0.02 $\mu\text{g/kg}$ per minute) plus 1% isoflurane (Aerrane; Baxter, Valencia, Spain) as maintenance gas anesthesia. Then compounds were administered as nebulized liquid aerosols using a Cirrus Jet nebulizer (Intersurgical, Mostoles, Spain). The administration volume was 3 ml, and each dog received AZD8871 (0.3, 1, 3, or 10 $\mu\text{g/kg}$), batenfenterol (1, 10, 30, or 100 $\mu\text{g/kg}$), or vehicle. The highest dose of batenfenterol tested was limited by its solubility at physiologic pH. Airway resistance was assessed in response to ACh i.v. challenges of 10 $\mu\text{g/kg}$ given prior to administration of the study compounds, and at 10, 20, and 30 minutes and 1, 3, 6, and 24 hours after administration. Bronchoprotective potency was determined as the dose required for 40% inhibition of ACh-induced bronchoconstriction (ID_{40}) at 30 minutes after compound administration, calculated by linear regression by using GraphPad Prism software. Duration of action was expressed as the bronchoprotective half-life at the highest dose devoid of effects on heart rate. Statistical analysis was performed using a two-way ANOVA plus a Bonferroni multiple comparison test versus vehicle by using Prism GraphPad software.

Systemic Muscarinic Antagonist and β_2 -Adrenoceptor Agonist Side Effects

Antisialagogue Effects in Guinea Pigs. Antisialagogue effects were assessed in guinea pigs. Compounds were administered to conscious animals by aerosol, following the protocol described in the current study to assess bronchoprotective effects in the same species. Test compounds were AZD8871 (10, 30, 100, and 300 $\mu\text{g/ml}$), batenfenterol (10, 100, and 300 $\mu\text{g/ml}$), tiotropium (10, 30, and 100 $\mu\text{g/ml}$), or vehicle. Then animals were anesthetized by an intraperitoneal injection of urethane (1.5 g/kg), and pilocarpine (0.3 mg/kg) was administered subcutaneously 40 minutes postadministration of the compounds. Afterward, the animals were placed with their heads facing downward on an inclined surface that was covered with filter paper for 30 minutes. The area on the filter paper that contained the secreted saliva was cut out and dried prior to being weighed, as previously described (Proctor, 2006).

The antisialagogue effect of each compound was calculated from concentration–response values and expressed as the concentration of each compound required for 50% inhibition of pilocarpine-induced salivation exhibited by the vehicle-treated group (IC_{50}). Calculations were performed with GraphPad Prism software using a sigmoidal fitting and one-way ANOVA analysis versus baseline, followed by application of Dunnett's post-test.

Safety margins were expressed as the ratio between the antisialagogue IC_{50} and the bronchoprotective IC_{50} values against ACh-induced bronchoconstriction in guinea pig.

Cardiovascular Effects in Dogs. Heart rate effects of nebulized AZD8871 (0.3, 1, 3, and 10 $\mu\text{g/kg}$) and batenfenterol (1, 10, 30, and 100 $\mu\text{g/kg}$) were studied in anesthetized Beagle dogs. The assessment was carried out in the same animals in whom bronchoprotective effects were studied and described in the current study. Effects on

heart rate were recorded throughout using electrocardiograms, and the maximal dose without an effect on heart rate ($\leq 10\%$ increase) was calculated. Safety margins were expressed as the ratio between the maximal dose without effects on heart rate and the ID_{40} against ACh-induced bronchoconstriction.

Results

Affinity for the Human Muscarinic ACh Receptors.

The affinity of AZD8871, batenfenterol, tiotropium, acridinium, glycopyrrolate, and ipratropium for the human muscarinic ACh receptors was assessed using membranes of CHO cells expressing M_1 – M_5 receptors and determined in radioligand-binding displacement experiments. The affinities for the different subtypes are summarized in Table 1. AZD8871 IC_{50} for the human M_3 receptor was subnanomolar and in a similar range to that of tiotropium and acridinium, whereas the IC_{50} values of batenfenterol, glycopyrrolate, and ipratropium were five, three, and eight times lower, respectively. When the IC_{50} values for the rest of the muscarinic ACh receptors were compared, none of the compounds was selective for the M_3 receptor over the rest of the muscarinic subtypes, except for the M_5 receptor (5-fold selectivity for AZD8871).

Dissociation from Human M_2 and M_3 Muscarinic Receptors. The dissociation from the human M_2 and M_3 receptors was performed with unlabeled compounds, and off-rates were estimated by measuring the association of [^3H]-NMS to the receptor binding sites. The dissociation of AZD8871 from the M_3 receptor, expressed as half-life, was approximately 30 times longer than that of batenfenterol and ipratropium, twice that of glycopyrrolate, and three times shorter than that of tiotropium (Fig. 2; Table 2). Test compounds showed kinetic selectivity over the M_2 receptor; the kinetic selectivity of the compounds expressed as M_3/M_2 half-life ratios was in the following rank (from highest to lowest selective): glycopyrrolate > tiotropium > AZD8871 > ipratropium > batenfenterol (Table 2).

Activity at Human β -Adrenoceptors. The activity of test compounds at the human β -adrenoceptors was determined by measuring cAMP production in CHO-K1 cell lines stably expressing the human β -adrenoceptors. AZD8871 showed a potency at the β_2 -adrenoceptor in the same range as other LABAs, such as indacaterol, formoterol, and salmeterol, whereas batenfenterol and olodaterol were one order of magnitude more potent (Table 3). All test compounds were more potent at the β_2 -adrenoceptor than at the β_1 - and β_3 -adrenoceptors. Salmeterol and batenfenterol were the most selective compounds over the β_1 -adrenoceptor, followed by formoterol, salbutamol, AZD8871, and indacaterol (Table 3).

TABLE 1

Binding affinity of AZD8871, batenfenterol, tiotropium, acridinium, glycopyrrolate, and ipratropium for the human M_1 , M_2 , M_3 , M_4 , and M_5 receptors

Affinities are expressed as pIC_{50} . Each value represents the mean $\pm$ S.E.M. of 3–10 independent experiments.

Compound	Binding Affinity, pIC_{50}				
	M_1	M_2	M_3	M_4	M_5
AZD8871	9.9 $\pm$ 0.0	9.9 $\pm$ 0.2	9.5 $\pm$ 0.1	10.4 $\pm$ 0.1	8.8 $\pm$ 0.0
Batenfenterol	8.4 $\pm$ 0.1	8.7 $\pm$ 0.1	8.8 $\pm$ 0.0	8.4 $\pm$ 0.1	7.3 $\pm$ 0.0
Tiotropium	9.7 $\pm$ 0.0	9.6 $\pm$ 0.0	9.5 $\pm$ 0.1	9.9 $\pm$ 0.1	9.1 $\pm$ 0.1
Acridinium	9.9 $\pm$ 0.1	9.7 $\pm$ 0.1	9.8 $\pm$ 0.2	9.6 $\pm$ 0.1	9.7 $\pm$ 0.0
Glycopyrrolate	9.1 $\pm$ 0.0	8.5 $\pm$ 0.1	9.0 $\pm$ 0.1	8.8 $\pm$ 0.1	8.6 $\pm$ 0.2
Ipratropium	8.4 $\pm$ 0.1	8.5 $\pm$ 0.0	8.6 $\pm$ 0.0	8.6 $\pm$ 0.1	8.3 $\pm$ 0.0

Fig. 2. Dissociation of AZD8871, batefenterol, tiotropium, and ipratropium from human M_3 receptors. Dissociation profile of unlabeled compounds was assessed from 0 to 22 hours. Plotted data correspond to mean $\pm$ S.E.M. of two independent assays.

Off-Target Activities. When tested in a panel 55 human targets, AZD8871 (1 μ M) produced an inhibition of specific binding $<50\%$ in the majority of the targets, except for the H_1 , 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} receptors, and for the norepinephrine and dopamine transporters. In functional cellular assays, AZD8871 showed moderate antagonism for those targets (IC_{50} : 85, 344, 880, and 600 nM for the H_1 , 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} receptors, respectively). Similarly, the affinity of AZD8871 for the norepinephrine and dopamine transporter was also moderate (IC_{50} : 170 and 720 nM, respectively). Given the high potency of AZD8871 at M_3 and β_2 -adrenoceptors, these off-target activities were considered not biologically relevant. The antagonism of AZD8871 for the human H_1 receptor was the most remarkable off-target activity, which may not be an issue either in the efficacy or the safety profile of the compound after inhalation, but prevented the use of histamine, as smooth muscle contraction inducer, in pre-clinical models to assess the β_2 -adrenergic activity of the compound.

Functional Studies in Isolated Guinea Pig Trachea.

The ability of test compound to relax guinea pig tracheal preparations was assessed in samples contracted with EFS. AZD8871 exhibited a potency not significantly different from that of batefenterol, tiotropium, ipratropium, and indacaterol, whereas formoterol was the most potent compound (Table 4).

The muscarinic antagonism was assessed in electrically stimulated samples in presence of propranolol. Tiotropium, ipratropium, and indacaterol were tested as positive and negative controls, respectively. AZD8871 showed a potency (pIC_{50} : 8.6) significantly higher than batefenterol and not different from that of tiotropium or ipratropium (Table 4). On the contrary, indacaterol exhibited very low inhibitory activity, with a maximum effect of 37% at 1000 nM, which validates that the concentration of propranolol (1 μ M) used was able to block the β_2 -adrenoceptor activity of the test compounds. When the potency of the two MABA molecules in presence of propranolol was compared with their potency in absence of propranolol, AZD8871 exhibited a minimum shift in potency, whereas batefenterol showed a significant shift in potency of 5-fold (Table 5).

The duration of action of the antimuscarinic activity was followed for 15 hours of washout after compounds incubation at equipotent concentrations that produced 60%–80% relaxation. Preparations incubated with AZD8871 (5 nM) or tiotropium (2 nM) did not recover their tone after 15 hours of washout (recovery $<1\%$), whereas the recovery of the samples incubated with batefenterol (20 nM) or ipratropium (5 nM) partially recovered their tone ($25.6\% \pm 5.7\%$ and $40.3\% \pm 8.3\%$ for batefenterol and ipratropium, respectively) (data not shown).

The functional β_2 -adrenoceptor agonist activity was assessed in spontaneous tone guinea pig trachea, and the LABA compounds indacaterol and formoterol were used as positive controls. All test compounds produced a concentration-dependent relaxation. The potency of AZD8871 was in the nanomolar range (pEC_{50} : 8.8) (Table 4), and, when compared with the other test compounds, potency followed the rank, as follows: formoterol $>$ batefenterol $>$ indacaterol $>$ AZD8871 (Table 4). The LAMA tiotropium was used as negative control, with no effect at concentrations up to 1000 nM (data not shown). After a washout period of 1 hour, preparations incubated with test compounds showed minimal recovery ($8.6\% \pm 1.8\%$, $1.0\% \pm 0.7\%$, and $0.0\% \pm 0.0\%$, for AZD8871, batefenterol, and indacaterol, respectively), except for the samples incubated with formoterol, which recovered their tone in $79.0\% \pm 3.7\%$ (data not shown).

Functional Studies in Isolated Human Bronchi. The MABA global activity of AZD8871 and batefenterol was

TABLE 2

Residence time at the human M_2 and M_3 receptors, expressed as half-life ($t_{1/2}$)

Results are expressed as the mean $\pm$ S.E.M. of two independent experiments.

Compound	$t_{1/2}$, h		$t_{1/2}$ Ratio
	M_2	M_3	M_3/M_2
AZD8871	0.46 ± 0.06	4.97 ± 0.36	10.8
Batefenterol	0.07 ± 0.03	0.16 ± 0.02	2.3
Tiotropium	1.31 ± 0.47	16.04 ± 1.58	12.2
Glycopyrrolate	0.07 ± 0.01	2.6 ± 0.14	37.1
Ipratropium	0.04 ± 0.01	0.18 ± 0.04	4.5

TABLE 3

Activity at human β -adrenoceptors

CHO cells selectively expressing the human adrenergic β_1 , β_2 , and β_3 -adrenoceptors were stimulated with increasing concentrations of test compounds (0.03–10,000 nM), and cAMP levels were quantified, as described in *Materials and Methods*. Data are reported as mean $\pm$ S.E.M. of at least two independent experiments; pEC₅₀: negative logarithm of the concentration required to do 50% of the maximum effect (vs. the reference agonists epinephrine, formoterol hemifurcate, and ZD7114 for the β_1 , β_2 , and β_3 assays, respectively).

Compound	cAMP Production, pEC ₅₀		
	β_1	β_2	β_3
AZD8871	9.0 $\pm$ 0.1	9.5 $\pm$ 0.3	8.7 $\pm$ 0.0
Batefenterol	8.5 $\pm$ 0.2	10.5 $\pm$ 0.1	8.7 $\pm$ 0.1
Olodaterol	8.9 $\pm$ 0.0	10.8 $\pm$ 0.0	8.5 $\pm$ 0.0
Indacaterol	8.7 $\pm$ 0.1	9.0 $\pm$ 0.2	8.8 $\pm$ 0.0
Salmeterol	7.1 $\pm$ 0.1	9.8 $\pm$ 0.1	7.6 $\pm$ 0.0
Formoterol	8.6 $\pm$ 0.1	9.7 $\pm$ 0.0	9.2 $\pm$ 0.0
Salbutamol	6.6 $\pm$ 0.0	7.3 $\pm$ 0.0	7.0 $\pm$ 0.0

assessed in EFS human bronchi. Compounds (0.001–1000 nM) produced a concentration-dependent relaxation, with a maximal effect with respect to 3 mM theophylline of 98% $\pm$ 16% and 86% $\pm$ 13% for AZD8871 and batefenterol, respectively. The potency of both compounds, expressed as pIC₅₀, was similar and in the nanomolar range (Table 5). When the antimuscarinic activity of both compounds was assessed in presence of propranolol, they showed a concentration-dependent effect with a maximal effect at 1000 nM of 90% $\pm$ 16% and 78% $\pm$ 3% with respect to 3 mM theophylline, for AZD8871 and batefenterol, respectively. The blockade with propranolol caused a decrease in the potency of AZD8871 about 2-fold, with a pIC₅₀ of 8.7, whereas the drop in the potency of batefenterol was higher (6-fold), with a significant shift in its pIC₅₀, from 8.7 to 7.9 (Table 5).

The duration of action of AZD8871 and batefenterol was studied in preparations contracted by EFS by using single drug concentrations (10 nM) that caused about 40%–50% inhibition. The effect of AZD8871 was more sustained than that of batefenterol, with percentages of recovery at 8 hours after compound washout of 5.3% $\pm$ 5.3% and 41.4% $\pm$ 2.0% for AZD8871 and batefenterol, respectively (number of rings: seven; number of patients: six) (data not shown).

Bronchoprotective and Antisialagogue Effects in Guinea Pigs. The bronchoprotective effect of inhaled AZD8871, batefenterol, and tiotropium was assessed in guinea pigs. Three to four concentrations of each compound were administered to different groups of animals (4–10 per group) 1 hour before the

administration of ACh. All compounds inhibited the bronchoconstriction in a concentration–response manner (Fig. 3), achieving an inhibitory effect of 85%–93% at the highest concentration tested. The potency of the test compounds was in a similar range, with IC₅₀ values between 2.1 and 2.6 μ g/ml (Table 6).

The antisialagogue effect of test compounds was assessed in guinea pigs following the same nebulization protocol. Three to four concentrations of each compound were administered to different groups of animals (four to six per group) 1 hour before the salivation induction with pilocarpine. Tiotropium, used as positive control, was the compound with more antisialagogue effect, showing a concentration-dependent inhibition of induced sialorrhea that reached 95% $\pm$ 5% at the highest concentration tested (Fig. 3C), and an IC₅₀ of 37.6 μ g/ml (Table 6). AZD8871 exhibited a lower effect than tiotropium with a maximal inhibition of sialorrhea of 65% $\pm$ 11% at 300 μ g/ml (Fig. 3A) and an estimated IC₅₀ of 138.4. Batefenterol was the compound with lower effect, with a maximal salivation inhibition of 20% $\pm$ 18% at the higher concentration tested (Fig. 3B).

When the bronchoprotective and antisialagogue effects were compared by calculating the ratio between the IC₅₀ values in each assay, tiotropium was the compound with narrower margin (14.5), followed by AZD8871 (62.9) and batefenterol (>143.0) (Table 6).

Bronchoprotective and Cardiovascular Effects in Dogs. The bronchoprotective potency and cardiovascular effects of inhaled AZD8871 and batefenterol were studied in anesthetized Beagle dogs. Potency was assessed as the ability to inhibit ACh-induced bronchoconstriction after 30 minutes of compound administration, and the effect on heart rate was assessed in the same animals at the same time point. Both compounds showed dose-proportional bronchoprotective effect (Fig. 4), with a nonsignificantly different potency (ID₅₀ of 0.40 and 0.34 μ g/kg, respectively; Table 6). The highest dose tested of each compound (AZD8871, 10 μ g/kg; batefenterol, 100 μ g/kg) was devoid of tachycardia; thus, there was a wide margin between the bronchoprotective effect and the cardiovascular effect for both compounds (Table 7). No relevant changes in glucose or potassium plasma levels were observed (data not shown).

The bronchoprotective effect of different doses of AZD8871 and batefenterol was followed up to 24 hours. AZD8871 showed significant effects over 24 hours at all the doses tested (0.3–10 μ g/kg) (Fig. 4A), whereas in the case of batefenterol a significant effect over this period was observed at higher doses

TABLE 4

Functional MABA activity was assessed in EFS guinea pig trachea, and muscarinic antagonism (MA) was dissected in the same assay in the presence of 1 μ M propranolol. β_2 -adrenoceptor agonism (BA) was assessed in guinea pig preparations at spontaneous tone (ST).

n = 3–15 preparations (AZD8871 EFS: *n* = 4; AZD8871 EFS + propranolol: *n* = 5; AZD8871 ST: *n* = 7; batefenterol EFS: *n* = 6; batefenterol EFS + propranolol: *n* = 5; batefenterol ST: *n* = 6).

	MABA (EFS) pIC ₅₀ (95% CI)	MA (EFS + Propranolol) pIC ₅₀ (95% CI)	BA (ST) pEC ₅₀ (95% CI)
AZD8871	8.6 (8.7–8.6)	8.6 (8.6–8.4)	8.8 (8.9–8.7)
Batefenterol	8.6 (8.8–8.3)	7.9 (8.1–7.7)*+	9.4 (9.6–9.1)**
Tiotropium	8.9 (9.0–8.8)	8.7 (9.0–8.6)	>6
Ipratropium	8.6 (8.7–8.5)	8.6 (8.7–8.4)	nt
Indacaterol	9.1 (9.6–8.6)	>6	9.1 (9.2–9.0)*
Formoterol	9.5 (9.9–9.2)*	nt	10.1 (10.2–10.0)**

CI, confidence interval; nt, not tested; pEC₅₀, negative logarithm of the concentration required to induce 50% of the maximum effect relative to 0.1 μ M isoprenaline; pIC₅₀, negative logarithm of the concentration required for 50% inhibition of the electrically-induced contraction.

*P < 0.05 vs. AZD8871; **P < 0.01 vs. AZD8871 (*F* test); *P < 0.05 vs. MABA potency in EFS (*F* test).

TABLE 5

Potency of AZD8871 and batenfenterol in isolated human bronchi

MABA global activity was assessed in EFS preparations. Dissection of antimuscarinic activity (MA) was assessed in EFS-contracted preparations in presence of 1 μ M propranolol.

	MABA (EFS)		MA (EFS + Propranolol)	
	pIC ₅₀ (95% CI)	n/p	pIC ₅₀ (95% CI)	n/p
AZD8871	9.0 (9.6–8.5)	9/6	8.7 (9.3–8.0)	9/8
Batenfenterol	8.7 (9.2–8.1)	9/7	7.9 (8.4–7.3) ⁺	8/6

CI, confidence interval; n, number of rings; p, number of patients; pIC₅₀, negative logarithm of the concentration required for 50% inhibition of the electrically-induced contraction.⁺*P* < 0.05 vs. MABA potency in EFS (*F* test).

(10–100 μ g/kg) (Fig. 4B). The duration of the bronchoprotective effect, expressed as the time taken to reduce half of maximal inhibition of airway constriction, was calculated for the highest dose devoid of tachycardia. At that dose, AZD8871 (10 μ g/kg) showed long-lasting effects, with a 79% $\pm$ 3.6% of bronchoprotection at 24 hours and a calculated half-life longer than 24 hours (Fig. 4A). In the case of batenfenterol (100 μ g/kg), the bronchoprotection at 24 hours was much lower (31% $\pm$ 3.6%), with a calculated half-life of 18.6 $\pm$ 0.9 hours (Fig. 4B).

Discussion

The aim of this study was to demonstrate that AZD8871 is a compound with dual activity on muscarinic ACh receptors and β_2 -adrenoceptors, long-lasting bronchodilatory effect, and wide safety margins in preclinical animal models.

The dual-acting behavior of AZD8871 was first confirmed in in vitro assays. In radioligand-binding displacement studies, the compound exhibited a subnanomolar affinity for the human M₃ receptor, which was similar to that of antimuscarinic agents such as tiotropium and aclidinium, and five times higher than that of batenfenterol. The muscarinic antagonism was

corroborated in EFS guinea pig trachea, where the potency of AZD8871 in the presence of propranolol was similar to that of tiotropium and five times higher than that of batenfenterol. On the other side, AZD8871 demonstrated a potency at the β_2 -adrenoceptor by measuring cAMP production in cells that was between that of indacaterol and formoterol, and 10 times lower than that of batenfenterol. The β_2 -adrenoceptor activity was confirmed in spontaneous tone guinea pig trachea, where the relaxant potency of AZD8871 was in the nanomolar range but significantly lower than that of batenfenterol. These data demonstrate that AZD8871 is a bifunctional molecule with a potency at both functionalities similar to antimuscarinic and β_2 -adrenoceptor agonists in clinical use. However, it shows a balance of both activities different from batenfenterol, with a predominant antimuscarinic component. This was confirmed in human bronchi, where propranolol only produced a decrease in the potency of AZD8871 of 2-fold, whereas batenfenterol showed a significant shift in potency of 6-fold. Our results are in agreement with published data, in which batenfenterol showed about 5-fold higher potency for the β_2 -adrenoceptor than for the muscarinic receptors in in vitro studies (Hegde et al., 2014) and a noteworthy reduction in the bronchodilator effect under propranolol blockade in clinical trials (Norris and Ambery, 2014). The profile of LAS190792, a MABA compound that also reached clinical stages, is not very different from that of batenfenterol, with a drop in potency in human bronchi under propranolol blockade of 5-fold (Aparici et al., 2017). The structural differences between AZD8871 and LAS190792 lay in the linker region, being a N-phenylcarbamate in the former and a benzotriazole in the later. Important to say, the linker moiety per se is not responsible for either the β_2 -adrenoceptor agonism or the muscarinic antagonism, but is able to modulate both when linked to the β_2 and M₃ motif structures. The linker present in AZD8871, contrarily to that present in LAS190792, unbalances the activities toward M₃, which turns to be more favorable to get a bronchodilator with less

Fig. 3. Bronchoprotective and antisialagogue effects of AZD8871 (A), batenfenterol (B), and tiotropium (C) in anesthetized guinea pigs. Aerosols generated from aqueous solutions of different concentrations from each compound were administered to conscious guinea pigs. Then animals were anesthetized, and inhibition of bronchoconstriction induced by ACh (15 μ g/kg i.v.) was assessed 1 hour after aerosol administration (straight line). In independent experiments, the potency of drugs to inhibit pilocarpine-induced salivation was assessed 1 hour after the administration of test compounds (dotted line). Data are shown as mean $\pm$ S.E.M. of 4–10 animals per dose; *P < 0.05; **P < 0.01; ***P < 0.001 vs. baseline (one-way ANOVA, followed by Dunnett's post-test).

TABLE 6

Bronchoprotective and antisialagogue effects of inhaled AZD8871, batenfenterol, and tiotropium in guinea pigs

Nebulized compounds were administered to guinea pigs, and bronchoprotective potency (IC_{50} : concentration required for 50% inhibition of the ACh-induced bronchoconstriction) and antisialagogue potency (IC_{50} : concentration required for 50% inhibition of the pilocarpine-induced salivation) were assessed 1 hour later. Safety margin was calculated as the ratio between the antisialagogue IC_{50} and the bronchoprotective IC_{50} . Data are mean ($n = 4-10$) and confidence intervals (CI 95%).

	Bronchoprotective Effect IC_{50} (95% CI) (μ g/ml)	Antisialagogue Effect IC_{50} (95% CI) (μ g/ml)	Safety Margin Antisialagogue IC_{50} /Bronchoprotective IC_{50}
AZD8871	2.2 (1.3–3.7)	132.1 (35.5–492.3)	60
Batefenterol	2.1 (1.2–4.0)	>300	>143
Tiotropium	2.6 (1.0–7.0)	38.7 (27.7–53.9)	15

secondary effects associated to the β -adrenoceptor agonism. Both compounds show low β_1 -adrenoceptor activity in functional assays, whereas the β_2 -adrenoceptor activity of LAS190792 is markedly higher than that of AZD8871 (Miralpeix et al., 2014; Aparici et al., 2017). Additionally, AZD8871 shows less potency for the H_1 receptor than LAS190792, which is the main off-target activity of both molecules (Aparici et al., 2017).

The number of daily administrations is a key factor that affects patient compliance (Sanduzzi et al., 2014). In this sense, experiments in anesthetized dogs confirmed the long-lasting effects of AZD8871, with a bronchoprotective half-life longer than 24 hours. This effect is similar to that reported for tiotropium in the same model, and longer than the effect of LAS190792 and olodaterol at maximal doses without cardiac effects ($35\% \pm 8\%$ and $17\% \pm 5\%$ inhibition at 24 hours, respectively) (Aparici et al., 2017). When compared with batenfenterol, a high dose of this compound (100μ g/kg) was required to achieve sustained effects up to 24 hours in dogs. These results are in line with the shorter duration of action of batenfenterol compared with AZD8871 in human bronchi.

When the contribution of the individual pharmacologies to the long-lasting effects was assessed in guinea pig trachea, the duration of the relaxation caused by the β_2 -adrenoceptor agonism of AZD8871 was similar to that of the once-daily indacaterol, whereas the twice-daily formoterol showed much shorter effect. In contrast, the duration of action of the antimuscarinic component of AZD8871 in the electrically stimulated trachea assay was sustained up to 15 hours, similarly to the long-acting tiotropium, whereas the effect of the short-acting ipratropium was not sustained over that period of time. Our results are in agreement with the behavior of AZD8871 in humans. In a phase 2a trial, it demonstrated sustained bronchodilation over 24 hours in COPD patients receiving 100 or 600 μ g for 14 days (Psallidas et al., 2018); and, although the contribution of each individual activity in humans has not been studied, single doses of AZD8871 (1800 μ g) showed greater bronchodilation than both indacaterol (150 μ g) and tiotropium (18 μ g) for both peak and trough forced expiratory volume in 1 second in COPD patients (Singh et al., 2017), suggesting that both components seem to be responsible of the duration of action of the compound. The mechanism underlying the sustained effects of AZD8871 is likely to be a combination of several factors. Slow dissociation kinetics from the M_3 receptor is one of the main mechanisms of the duration of action of once-a-day antimuscarinic drugs (Disse et al., 1993; Barnes et al., 1995). In our experiments, AZD8871 showed a residence time at the human M_3 receptor of approximately 5 hours, which is about three times shorter than that of tiotropium and in a similar range of that of other long-acting antimuscarinics such as glycopyrrolate and aclidinium, which

exhibit a half-life about two to six times shorter than tiotropium, as shown in the present and previous studies reported by our group (Gavalda et al., 2009), and also similar to umeclidinium, with a residence time at the M_3 receptor three times shorter

Fig. 4. Duration of action of AZD8871 (A) and batenfenterol (B) in Beagle dogs. Nebulized compounds or vehicle were administered once to anesthetized animals, and bronchoconstriction was induced by ACh (10μ g/kg) at 10, 20, and 30 minutes, and at 1, 3, 6, and 24 hours. Inhibition of bronchoconstriction was recorded as percentage of the response to ACh before treatment with test compounds. Data are reported as mean $\pm$ S.E.M.; $n = 3-9$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-way ANOVA, followed by a Bonferroni multiple comparison test vs. vehicle).

TABLE 7

Bronchoprotective potency, heart rate effects, and safety margin of inhaled AZD8871 and batenfenterol in anesthetized Beagle dogs

Nebulized compounds were administered once to anesthetized dogs, and bronchoprotective potency (ID₄₀: dose required for 40% inhibition of the ACh-induced bronchoconstriction) was assessed 30 minutes later. Safety margin was calculated as the ratio between the maximal dose devoid of effects on heart rate and the ID₄₀ value of bronchoprotective effect. Data are mean ($n = 3$ – 12) and confidence intervals (CI 95%). Predrug heart rate values of the vehicle, AZD8871, and batenfenterol groups were 112 ± 4 ($n = 12$), 104 ± 2 ($n = 13$), and 101 ± 5 ($n = 19$) beats per minute, respectively, expressed as mean $\pm$ S.E.M.

	Bronchoprotection, ID ₄₀ (95% CI) ($\mu\text{g/kg}$)	Maximal Dose Devoid of Effects on Heart Rate, $\mu\text{g/kg}$	Safety Margin, Maximal Dose without Effects on Heart Rate/ID ₄₀
AZD8871	0.40 (0.20–0.66)	>10	>25
Batenfenterol	0.34 (0.08–0.84)	>100	>294

than tiotropium (Salmon et al., 2013). In contrast, batenfenterol dissociates very fast from the M₃ receptor, with a residence time of 0.16 ± 0.02 hour, in agreement with previous publications (Hegde et al., 2014). Other MABAs such as LAS190792 have also exhibited a short residence time at the M₃ receptor (0.2 ± 0.1 hour, data not shown). These data suggest that, unlike batenfenterol and LAS190792, binding kinetics is probably one of the mechanisms responsible for the duration of action of AZD8871. Some authors have suggested that the residence time at the receptor may be involved in the duration of action of β_2 -adrenoceptor agonists (Casarosa et al., 2011), but no clear relationship between receptor dissociation kinetics and functional effects has been demonstrated (Hegde et al., 2014; Ramos et al., 2018). Another factor that may contribute to the long duration of action of AZD8871 is the character of the two basic centers in its structure. Taking into account that the degree of basicity has been directly related to the lung retention (Cooper et al., 2012), we can assume that the *in silico* predicted pK_a (ChemAxon) for AZD8871 (8.38 and 10.82) compared with that of batenfenterol (7.89 and 9.02, respectively) may partially explain the differences in the duration of action between both compounds.

Mild tachycardia is a common side effect of β -adrenoceptor agonists, which in part may result from the activation of β_2 -adrenoceptors present in peripheral vasculature, but it is mainly a consequence of the activation of cardiac β_1 -adrenoceptors, increasing heart rate directly (Sears, 2002). In this sense, AZD8871 showed some selectivity over the β_1 -adrenoceptors, which was in the same range as that of indacaterol. The M₂ receptor is also expressed in heart and regulates chronotropism and inotropism (Brodde and Michel, 1999); however, combination of antimuscarinic and β_2 -adrenoceptor activities has not led to an increase in tachycardia in COPD patients at doses with long-lasting bronchodilation effects (Wielders et al., 2013; Nardini et al., 2014; Calzetta et al., 2016; Matera et al., 2016), suggesting low potential of cardiovascular effects at therapeutic doses. When tested in the dog model, AZD8871 did not increase heart rate at efficacious and long-acting doses, which confirmed the favorable cardiovascular profile of the compound. Our results are in agreement with clinical data obtained with the combinations of antimuscarinic and β_2 -adrenoceptor, and MABA compounds, in contrast to LABA molecules, which in part may be due to the high kinetic selectivity of the compounds over the M₂ receptors, but also by a potential compensation between the effect of β_1 -adrenoceptors and M₂ receptors in the heart (Myslivecek and Trojan, 2003).

A common side effect of antimuscarinic drugs is the inhibition of salivation (Gavalda et al., 2014). AZD8871 showed significantly less antisialagogue effect than tiotropium when given by inhalation to guinea pigs, with margin between that effect and bronchoprotection in the same species. Our findings

are in agreement with human data, in which single doses of AZD8871 showed good tolerability and safety profile in both asthma and COPD patients (Jimenez et al., 2017; Singh et al., 2017).

In summary, the data reported in this study show that AZD8871 is a potent dual-acting MABA balanced toward M₃ activity, with a slow dissociation from the M₃ receptor, long-lasting effects compatible with once-a-day dosing, and a favorable preliminary safety profile in preclinical models that explain the efficacy, duration of action, and safety observed in COPD patients.

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Authorship Contributions

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Conducted experiments: Carcasona, Ramos, Montero, Otal, Ortiz.

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

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