

Characterization of Acclidinium Bromide, a Novel Inhaled Muscarinic Antagonist, with Long Duration of Action and a Favorable Pharmacological Profile

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ABSTRACT

Acclidinium bromide is a novel potent, long-acting inhaled muscarinic antagonist in development for the treatment of chronic obstructive pulmonary disease. Acclidinium showed subnanomolar affinity for the five human muscarinic receptors (M_1 – M_5). [3H]Acclidinium dissociated slightly faster from M_2 and M_3 receptors than [3H]tiotropium but much more slowly than [3H]ipratropium. Its association rate for the M_3 receptor was similar to [3H]ipratropium and 2.6 times faster than [3H]tiotropium. Residence half-life of [3H]acclidinium at the M_2 receptor was shorter than at the M_3 receptor, demonstrating kinetic selectivity for the M_3 receptor. In isolated guinea pig trachea, acclidinium showed comparable potency to ipratropium and tiotropium, faster onset of action than tiotropium, and duration

of action similar to tiotropium and significantly longer than ipratropium. Nebulized acclidinium inhibited bronchoconstriction induced by acetylcholine in guinea pigs in a concentration-dependent manner with an onset of action faster than tiotropium. Duration of action of acclidinium ($t_{1/2} = 29$ h) was much longer than ipratropium (8 h) but shorter than tiotropium (64 h). In dogs, acclidinium induced a smaller and more transient increase in heart rate than tiotropium at comparable supratherapeutic doses. Therefore, under these conditions, acclidinium showed a greater therapeutic index than tiotropium (4.2 versus 1.6). These results indicate that acclidinium is a potent muscarinic antagonist with a fast onset of action, a long duration of effect, and a favorable cardiovascular safety profile.

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory disease characterized by chronic airflow obstruction attributed to long-term exposure to inhaled

noxious gases and particles, most often related to cigarette smoking that is not fully reversible after bronchodilator therapy (www.goldcopd.org) (Rabe et al., 2007). Recent projections from the World Health Organization predict that COPD will become the fourth most common cause of death by 2030 and the third most common cause of chronic disability by 2020 (Lopez et al., 2006; Mathers and Loncar, 2006). Acetylcholine released by parasympathetic nerves regulates airway constriction, mucus secretion, and vasodilation through its interaction with muscarinic receptors localized in smooth muscle, mucosal glands, pulmonary vasculature, and nerve endings of the lungs (Belmonte, 2005).

There are five subtypes of the muscarinic receptors, M_1 to M_5 , that are members of the superfamily of G-protein-cou-

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ABBREVIATIONS: [3H]NMS, 1-[*N*-methyl- 3H]scopolamine methyl chloride; COPD, chronic obstructive pulmonary disease; M, muscarinic receptor; ANOVA, analysis of variance; AUC, area under the curve; LAS34273, acclidinium bromide, 3*R*-(2-hydroxy-2,2-di-thiophen-2-yl-acetoxy)-1-(3-phenoxy-propyl)-1-azonia-bicyclo[2.2.2] octane bromide.

pled receptors (Eglen, 2005). Different physiological functions have been ascribed to each subtype according to its tissue localization and expression pattern (Abrams et al., 2006). In the human lung expression of the M_1 , M_2 , and M_3 , but not the M_4 and M_5 , muscarinic receptor subtypes have been described previously (Belmonte, 2005). M_3 receptors, localized in airway smooth muscle, are responsible for mediating the bronchoconstrictor response to cholinergic nerve stimulation (Roffel et al., 1990). M_3 and M_1 receptors are also expressed in mucosal glands where their stimulation is believed to promote mucus secretion (Gwilt et al., 2007). M_2 receptors localized presynaptically in cholinergic nerve endings act as autoreceptors, mediating feedback inhibition of acetylcholine release from the nerve (Minette and Barnes, 1988).

Long-acting bronchodilators are the first-line treatment for COPD patients (Hanania and Donohue, 2007; Cazzola and Matera, 2008). Muscarinic antagonists are particularly effective because parasympathetic cholinergic tone seems to be the major reversible component of airway obstruction in COPD (Gross, 2006; Cazzola and Matera, 2008). The inhaled muscarinic antagonists available for the symptomatic treatment of COPD have very different duration of action and, hence, dosing regimes. For instance, ipratropium bromide is a short-acting agent requiring several doses per day, whereas tiotropium bromide is a long-acting agent dosed once daily (Breekveldt-Postma et al., 2007). Both compounds have similar affinities for all muscarinic receptors, but tiotropium has a much longer residence time at the M_3 receptor than ipratropium (Disse et al., 1993; Gross, 2006), explaining the different dosing regimens in humans (Haddad et al., 1994). This long duration of action of tiotropium in humans has also been observed in isolated airways preparations and in animals (Disse et al., 1993, Takahashi et al., 1994).

Given the distribution of muscarinic receptors, systemically available agents that bind to muscarinic receptors outside of the respiratory tract have the potential to produce unwanted physiological effects, such as tachycardia, dry mouth, urinary retention, and constipation (Lieberman, 2004). Whereas dry mouth is the most common systemic anticholinergic side effect associated with the use of ipratropium and tiotropium (Casaburi et al., 2002; Vincken et al., 2002), the potentially most serious systemic effect is tachycardia, which results from blockade of cardiac M_2 receptors (Lieberman, 2004; Lee et al., 2008).

To reduce the potential for systemic side effects, muscarinic antagonists to treat respiratory diseases have been designed as quaternary ammonium salts administered by inhalation to minimize oral bioavailability and penetration of the blood-brain barrier (Cereda et al., 1990). Both local delivery to the lungs and low gastrointestinal absorption help to reduce systemic exposure and, therefore, lower the potential for side effects.

An additional but less exploited strategy to further reduce systemic exposure of antimuscarinics is to increase its plasma clearance. Acclidinium bromide, 3*R*-(2-hydroxy-2,2-dithiophen-2-yl-acetoxy)-1-(3-phenoxy-propyl)-1-azonia-bicyclo[2.2.2] octane bromide (Fig. 1), previously known as LAS34273, is a novel inhaled muscarinic antagonist (Prat et al., 2009) currently being studied in phase III clinical trials for the maintenance treatment of COPD (NCT00363896, NCT00358436, www.ClinTrials.gov, 2008). In contrast to

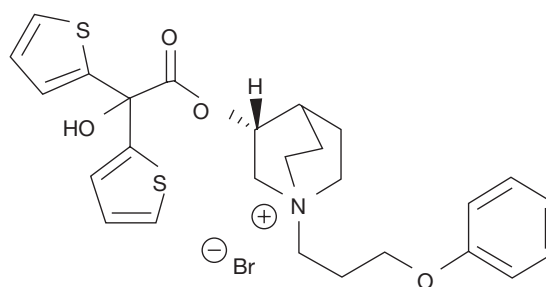


Fig. 1. Chemical structure of acclidinium bromide.

other currently available antimuscarinics including tiotropium, acclidinium has been shown to undergo rapid hydrolysis in human plasma, resulting in very low and transient systemic exposure, suggesting a reduced potential for class-related systemic side effects in the clinical setting (Gavalda et al., 2007, 2008).

In this article, we report the pharmacological characterization of acclidinium in vitro using human muscarinic receptors and isolated guinea pig trachea and in vivo, in different functional animal models, including the assessment of its therapeutic index in dogs.

Materials and Methods

Materials and Drug Preparation

Acclidinium bromide and tiotropium bromide (6*β*,7*β*-epoxy-3*β*-hydroxy-8-methyl-1*αH*,5*αH*-tropanium bromide di-2-thienylglycolate) were synthesized by the Department of Medicinal Chemistry (Laboratorios Almirall, Barcelona, Spain); ipratropium bromide, atropine sulfate, acetylcholine chloride, carbachol chloride, urethane, capsaicin, indomethacin, and phosphate-buffered saline with calcium and magnesium were purchased from Sigma-Aldrich (Tres Cantos, Spain); sodium pentobarbital was obtained from Industrial Kern (Barcelona, Spain); 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 (Rubí, Spain).

Membrane preparations expressing human M_1 , M_2 , M_3 , M_4 , and M_5 receptors (obtained from transfected Chinese hamster ovary K1 cells) were obtained from Membrane Target Systems (PerkinElmer Life and Analytical Sciences, Boston, MA). 1-[*N*-methyl- 3 H]scopolamine methyl chloride (3 H]NMS) was obtained from PerkinElmer Life and Analytical Sciences. 3 H]Acclidinium (2.886 TBq/mmol), 3 H]ipratropium (2.701 TBq/mmol), and 3 H]tiotropium (3.108 TBq/mmol) were custom-synthesized by GE Healthcare UK limited (Slough, Buckinghamshire, UK).

All equilibrium and kinetic binding studies were performed in 96-well plates (Nunc; Thermo Fischer Scientific, Roskilde, Denmark), with all antagonists dissolved in dimethyl sulfoxide. In in vitro isolated organ and in vivo studies, acclidinium was dissolved in 0.2 to 1% (v/v) HCl and, when required, in the presence of polyethylene glycol 300. Ipratropium and tiotropium were dissolved in distilled water, with the exception of the beagle dog study where saline was used. 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 $CaCl_2$.

Animals

Male Dunkin-Hartley guinea pigs (400–600 g at the time of experimental procedures) were obtained from Harlan (Interfauna Ibérica, Sant Feliu de Codines, Spain). Guinea pigs were housed in

groups of four or five at 20–24°C under a 12-h light/dark cycle. 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, 40 to 70% humidity, under a 12-h light/dark cycle and fed on a maintenance diet (Harlan Teklad, Madison, WI), with free access to water. All experiments were carried out with the approval of the Animal Ethical Committee of Almirall (Barcelona, Spain).

[³H]NMS Radioligand Displacement Studies

Affinity for the Human M₁ to M₅ Muscarinic Receptor Subtypes. The affinity of the muscarinic antagonists acridinium, tiotropium, and ipratropium for the different human muscarinic receptor subtypes at equilibrium was determined by measuring their ability to displace the binding of [³H]NMS to cell membrane preparations expressing one of the human muscarinic receptor subtypes.

Protein concentrations were 8.1, 10.0, 4.9, 4.5, and 5.0 μg/well for M₁, M₂, M₃, M₄, and M₅ receptor membrane preparations, respectively. The assays were conducted at [³H]NMS concentrations approximately equal to the radioligand equilibrium dissociation constant (K_d) for the different muscarinic receptors subtypes. The [³H]NMS concentration was 0.3 nM for the M₁ and M₄ assays and 1 nM for the M₂, M₃, and M₅ assays. A range of antagonist concentrations (10^{-14} to 10^{-5} M) were tested in duplicate to generate competition curves. Nonspecific binding was determined in the presence of atropine (1 μM). Assay reagents were dissolved in assay binding buffer (phosphate-buffered saline with calcium and magnesium) to a total volume of 200 μl. After a 2- or 6-h incubation period (M₁–M₄ and M₅, respectively) at room temperature in 96-well microtiter plates to ensure that equilibrium was achieved for all compounds tested, 150-μl aliquots of the reaction were transferred to GF/C filter plates (Millipore, Barcelona, Spain) pretreated for 1 h with wash buffer (50 mM Tris, 100 mM NaCl, pH 7.4) containing 0.05% polyethylenimine. Bound and free [³H]NMS were then separated by rapid vacuum filtration followed by four washes with ice-cold wash buffer. Filters were then dried for 30 min before addition of 30 μl of OptiPhase Supermix (PerkinElmer Life and Analytical Sciences), and radioactivity was quantified using a MicroBeta Trilux microplate scintillation counter (PerkinElmer Life and Analytical Sciences).

Data Analysis. Affinities at equilibrium were determined as equilibrium antagonist dissociation constant (K_i) values by correcting the experimental IC₅₀ values obtained for each compound according to Cheng and Prusoff (1973) and using the experimentally derived values for the K_d of [³H]NMS for each receptor subtype and the concentration of radioligand used in the assays. K_i values were obtained from at least three independent curves of 10 antagonist concentrations run in duplicate. All adjustments were performed using Prism (GraphPad Software, Inc., San Diego, CA).

Studies with Radiolabeled Muscarinic Antagonists

Saturation Studies in Human M₂ and M₃ Receptors. Radioligand binding experiments were conducted in standard polypropylene 96-well plates in a total volume of 204 μl. Membranes were diluted in binding buffer (Tris 25 mM, pH 7.4) to a final assay protein concentration of 15 μg/ml. All of the assays were started by adding 200 μl of the corresponding membrane solution to 4-μl mixtures of radioligand (diluted to obtain final concentrations of 6.25–0.012 nM for [³H]acridinium and [³H]tiotropium and 25–0.012 nM for [³H]ipratropium) and either atropine to achieve a final concentration of 10 μM (nonspecific binding) or vehicle (total binding). All radioligands and atropine were dissolved in 100% dimethyl sulfoxide from a stock solution.

After incubation for 4 h to achieve equilibrium, 150 μl of the assay mixture was transferred at the indicated times to 96-well GF/B filter plates previously treated with 200 μl of the prewet solution (0.5%

polyvinylpyrrolidone, 0.1% Tween 20) over 2 h. Bound and free radioligand were separated by rapid vacuum filtration and washed six times with 200 μl of ice-cold wash buffer, and radioactivity was quantified as indicated previously.

Association with Human M₂ and M₃ Muscarinic Receptors. Association assays were conducted at three radioligand concentrations corresponding to approximately 3-, 1-, and 0.33-fold their K_d . Membranes were diluted in binding buffer to get a final assay protein concentration of 15 μg/ml for M₂ and M₃ receptors. Compounds were incubated for different time intervals (from 2 to 360 min) using the standard reaction mixture, and bound radioactivity was determined for each time point as described previously.

Dissociation from Human M₂ and M₃ Muscarinic Receptors. In dissociation experiments, association of the radioligands was first carried out at a final assay concentration of 2.5 nM for [³H]acridinium and [³H]tiotropium and 10 nM for [³H]ipratropium to ensure approximately 90% occupancy of the binding sites. As in the saturation and association studies, the membrane protein concentration was 15 μg/ml for M₂ and M₃ receptors in the final assay. The assay mixture (202 μl) was incubated for 135 min to allow the radioligands to reach equilibrium. At this time point, 2 μl of atropine was added to obtain a final assay concentration of 10 μM to occupy binding sites as they became available, thereby preventing reassociation. The amount of radioligand that remained bound at different time points was determined by taking 150-μl samples and processing them as described previously.

Data Analysis. K_d and B_{max} values for [³H]acridinium, [³H]tiotropium, and [³H]ipratropium at M₂ and M₃ receptors were calculated by adjusting the specific binding data to a one-site binding hyperbola. For each radioligand concentration, a one-phase exponential association equation was used to calculate the corresponding observed association rate constant (K_{ob}), and a one-phase exponential decay equation was used to calculate K_{off} .

The rate constant of association (K_{on}) for each antagonist was calculated using the equation $K_{on} = (K_{ob} - K_{off})/[antagonist]$, where K_{off} and K_{ob} were obtained from dissociation and association experiments. Dissociation half-lives ($t_{1/2}$) were calculated according to the equation $t_{1/2} = 0.693/K_{off}$ (Dowling and Charlton, 2006).

All reported values represent the mean ± S.E.M. from three independent experiments. In the case of the K_{on} determination, each experiment comprised three different radioligand concentrations, and the reported K_{on} was the mean of the values obtained for each concentration. All calculations were performed using GraphPad Prism software.

Guinea Pig Isolated Trachea—Carbachol Stimulation Assays

Carbachol-induced contraction studies were performed essentially as described previously (Cortijo et al., 1994). After the equilibration period, two control concentration-response curves were generated for carbachol (0.1 nM–1 mM) to demonstrate the reproducibility of the contractile responses to these drugs. The agonist was then washed out, and the tissue was re-equilibrated. Antagonists were then added 30 min before new concentration-response curves were generated for carbachol. The concentration ranges of the antagonists in the carbachol-induced contraction experiments were as follows: 0.1 to 100 nM acridinium, 1 to 100 nM ipratropium, and 1 to 10 nM tiotropium. The potency of each antagonist was determined as a pA_2 value by Schild plot analysis.

The onset of action of the three antagonists was determined in the carbachol-induced contraction study using 10 μM carbachol to obtain a contraction plateau. Onset of action was assessed as the time from addition of the antagonist to achieve 50 ($t_{1/2}$) and 100% (t_{max}) inhibition of the carbachol contraction. The concentrations of antagonists used were 2 nM acridinium, 3 nM ipratropium, and 6 nM tiotropium, which correspond to those that produced around 70 to 80% relaxation.

The duration of action (offset) of the antagonists, defined as the time from antagonist washout to recover 50% ($t_{1/2}$) or maximal recovery (t_{max}) of the cholinergic tone, was determined in the carbachol-induced contraction studies using the method described previously (Nials et al., 1993), with minor modifications. Stabilized preparations were contracted with 10 μ M carbachol and allowed to plateau. Antagonists were then added to relax the tissue (2, 3, and 6 nM for acclidinium, ipratropium and tiotropium, respectively, which correspond to concentrations that produce around 70–80% relaxation). After the inhibition of tone reached a maximum, the tissue was washed in fresh Krebs-Henseleit solution containing 10 μ M carbachol, and the recovery of tone was recorded over time.

Data Analysis. The pA_2 values obtained in the carbachol-induced contraction studies were compared using a one-way analysis of variance (ANOVA) followed by Bonferroni-Dunn post-test where appropriate or a Kruskal-Wallis test. Onset and offset $t_{1/2}$ values obtained in the carbachol-induced contraction studies were determined by interpolation of each concentration-response curve. Differences between onset and offset times were analyzed by a Kruskal-Wallis test. All analyses were performed using GraphPad Prism software.

Potency and Onset of Action in Anesthetized Guinea Pigs

Guinea pigs were anesthetized with an intraperitoneal injection of 1 g/kg urethane and 20 mg/kg sodium pentobarbital. An additional anesthetic was administered after 60 min as required. The trachea was cannulated, and the lungs were artificially ventilated with a small rodent ventilator (Ugo Basile, Biological Research Apparatus, Comerio-Varese, Italy) at a rate of 60 strokes/min and a tidal volume of 10 ml/kg. Animals were maintained at 37°C with a homeothermic blanket throughout the experiment.

Blood pressure was measured in a cannulated carotid artery, and acetylcholine was administered via a cannulated jugular vein. Intrapulmonary pressure and blood pressure were measured by blood pressure transducers (MLT0699; ADInstruments-Panlab, Barcelona, Spain) connected to a bridge amplifier (PowerLab/8sp; ADInstruments-Panlab). The data were recorded using Chart 5 software (ADInstruments-Panlab).

After induction of anesthesia and preparation, animals were allowed to stabilize for 10 min before bronchoconstriction was induced by an intravenous bolus of acetylcholine. Acetylcholine was administered at a dose (10–60 μ g/kg) that approximately doubled the basal intrapulmonary pressure. Repeated bolus injections of acetylcholine at the selected dose were administered until two reproducible responses were obtained; the mean of the two final responses before the addition of the antagonists corresponded to the baseline response to acetylcholine and was used to evaluate the antibronchoconstrictor effect of the antagonists.

Five minutes after the last administration of acetylcholine used to calculate maximal bronchoconstriction, the test antagonist was administered via a nebulizer (5-s duration, ultrasonic nebulizer; Mumed Systems Ltd., London, UK) to investigate the reversal of the bronchoconstriction. Antagonists were delivered in the following concentration ranges: 0.1 to 1 mg/ml acclidinium, 0.03 to 0.3 mg/ml ipratropium, and 0.03 to 0.3 mg/ml tiotropium. Acetylcholine doses were then administered 5, 10, 20, 30, 40, 60, 80, 100, and 120 min after the administration of the antagonist to evaluate their antibronchoconstrictor effects. The effect was expressed as a percentage of the baseline response to acetylcholine. t_{max} was defined as the time taken for the antagonist to achieve maximal inhibition of the acetylcholine-induced bronchoconstriction and indicates the onset of action of the compounds. Potency of the antagonists was determined as an IC_{50} value (the concentration required to produce 50% inhibition) measured at the t_{max} .

Data Analysis. Potency (IC_{50}) was determined from a sigmoidal dose-response curve constructed using inhibition values at t_{max} and calculated using GraphPad Prism software. IC_{50} values were compared using an ANOVA or Kruskal-Wallis test.

Duration of Bronchoprotection in Guinea Pigs

Guinea pigs were placed in a methacrylate box and exposed to a nebulized aerosol of antagonist solution. Antagonists were administered for 1 min at a flow rate of 3 liters/min, and animals were allowed to breathe freely for a 5-min period. This procedure was then repeated. Aerosols were generated via an ultrasonic nebulizer (Devilbiss Ultraneb 2000; Sunrise Medical, Somerset, PA) from solutions of 100 μ g/ml acclidinium, 30 μ g/ml ipratropium, and 10 μ g/ml tiotropium. The concentrations of antagonists used in this study were obtained from a previous pilot study (data not shown), which were found to produce near-maximal inhibition of bronchoconstriction.

After exposure to the nebulized antagonists, animals were anesthetized at various time points with an intramuscular injection of ketamine (43.8 mg/kg), xylazine (3.5 mg/kg), and acepromazine (1.1 mg/kg). Additional anesthetic was administered as needed during the experiment. After induction of anesthesia, animals were artificially ventilated with a small rodent ventilator (Ugo Basile, Biological Research Apparatus, Comerio-Varese, Italy) at 60 strokes/min and a tidal volume of 10 ml/kg. The animals were maintained at 37°C throughout the experiment with a homeothermic blanket. The trachea was cannulated with a polythene tube (0.5/1.0 mm) and connected to a pneumotachograph (Fleisch, Zabona, Switzerland) to record variations in airflow. The esophagus was cannulated with a PE-60 tube to the level of the thorax to measure transpulmonary pressure. The carotid artery and jugular vein were cannulated for blood pressure monitoring and acetylcholine administration, respectively. Blood pressure, transpulmonary pressure, and changes in volume were determined using pressure transducers (Statham P23XL; Spectramed, Oxnard, CA). By integrating changes in volume over time, a measurement of flow was calculated for each breath. The variations in flow, transpulmonary pressure, and blood pressure were registered with Lfr Record software version 7.1 (Mumed Systems Ltd).

After preparation, the animals were allowed to stabilize for approximately 5 min. When the baseline values were within the range 0.1 to 0.2 cm H₂O/ml per second for airway resistance and 0.3 to 0.9 ml/cm H₂O for dynamic compliance, the pulmonary dynamic measurements were initiated. Bronchoconstriction was then induced with a single-bolus dose of acetylcholine (30 μ g/kg i.v.) and the inhibitory effects of antagonists were tested in comparison to vehicle.

Airway resistance (centimeter per milliliter of H₂O per second) was calculated as the quotient of the changes in pressure and flow between isovolumetric points on inspiration and expiration. The airway resistance response to the acetylcholine challenge was calculated for the vehicle and antagonists from the formula: airway resistance = $(R_M - R_B) \times 100/R_B$, where R_M is the peak resistance after challenge (maximal value) and R_B is the baseline resistance (10 breaths before challenge). The inhibitory effect of each antagonist was compared with its respective control group (vehicle only).

The antibronchoconstrictor effect of the three antagonists was studied at the following time points after treatment: 1, 2, 4, 18, 24, and 48 h. Additional times of 6 h for ipratropium, 36 h for acclidinium, and 72 and 96 h for tiotropium were also assessed.

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

Inhibition of Bronchoconstriction in Beagle Dogs

Inhibition of bronchoconstriction was assessed by the method of Konzett and Rossler (1940) modified according to Misawa et al. (1986). Dogs fasted for 18 h were anesthetized with propofol with an initial dose of 6 to 8 mg/kg i.v. followed by a maintenance infusion of 0.6 to 0.8 mg/kg per min in the cephalic vein via an infusion pump (Becton Dickinson Program 2; BD Biosciences, Brézins, France). The trachea

was cannulated with a cuffed endotracheal tube connected to a respirator (Ugo Basile 5025, Comerio, Italy). The animals were artificially ventilated with room air at a constant pressure of 10 cm of H₂O with a respiratory rate of 14 strokes/min and a tidal volume of 15 to 20 ml/kg. Responses of the bronchial musculature in terms of ventilation overflow were continuously measured by a pneumotachograph (TSD127; Biopac Systems, Inc., Goleta, CA) as an index of airway resistance.

After induction of anesthesia and preparation, animals received acetylcholine bolus (5 µg/kg i.v.) at 10-min intervals to induce bronchoconstriction. After two consecutive similar baseline responses to acetylcholine had been obtained, aerosol solutions of 5 µg/kg acclidinium or 0.25 µg/kg tiotropium were administered by using a nebulizer (AG-AL1100; Aerogen, Galway, Ireland), which was attached between the respirator and the endotracheal tube. These doses of acclidinium and tiotropium were selected based on results from a previous pilot study (data not shown) and represent doses that produce near-maximal inhibition of bronchoconstriction. Acetylcholine was administered again at 10 and 20 min and 3 and 6 h after compound administration, and the inhibition of bronchoconstriction was then calculated. Animals regained consciousness after the bronchospasms at 20 min and after the 3- and 6-h measurements. Pulmonary resistance was recorded continuously and analyzed by means of a data acquisition system (AcqKnowledge 3.8.1; Biopac Systems, Inc.).

Heart Rate Assessment in Beagle Dogs

Effects on heart rate were assessed in dogs fasted for 18 h by attaching electrocardiographic leads to record the DII derivative of the ECG. Heart rates were continuously recorded throughout the experiment. Baseline readings, recorded for 15 min, were obtained before the animals were anesthetized with propofol (6–11 mg/kg i.v.). An endotracheal tube was inserted into the trachea, and animals

were artificially ventilated, as described for the dog bronchoconstriction experiments above.

After induction of anesthesia and preparation, 500 µg/kg acclidinium or 25 µg/kg tiotropium were administered by a nebulizer as described in the dog bronchoconstriction experiments. The doses of compounds were 100-fold higher than those used in the bronchoconstriction experiments. After administration of the compound, animals were disconnected from the respirator and allowed to regain consciousness. Monitoring of the heart rate was then restored and recorded continuously over the 6-h study period. Each dog received acclidinium and tiotropium with a washout period of at least 2 weeks between treatments; the same animals were used to check both compounds to minimize dispersion. The order of treatments was randomized.

Therapeutic Index Calculation and Data Analysis

The therapeutic index for each compound was defined as the ratio between the area under the curve (AUC) of the bronchoconstriction inhibition (percentage of bronchoconstriction inhibition per hour) and the AUC of heart rate increase (percentage of heart increase per hour) over the 6-h study period. A two-way ANOVA followed by Bonferroni post-test was used to determine statistical differences in bronchoconstriction and heart rate. These analyses were performed using GraphPad Prism software.

Results

Affinity Studies Using Displacement of [³H]NMS at Equilibrium. The affinity of acclidinium, ipratropium, and tiotropium for human muscarinic receptors was assessed

TABLE 1

Binding affinity of acclidinium, ipratropium, and tiotropium for human M₁, M₂, M₃, M₄, and M₅ receptors. Data are reported as mean ± S.E.M. of three independent experiments.

Compound	K _i				
	M ₁	M ₂	M ₃	M ₄	M ₅
	nM				
Acclidinium	0.10 ± 0.00	0.14 ± 0.04	0.14 ± 0.02	0.21 ± 0.04	0.16 ± 0.01
Ipratropium	1.31 ± 0.15	1.12 ± 0.13	1.24 ± 0.08	1.92 ± 0.18	3.22 ± 0.15
Tiotropium	0.13 ± 0.00	0.13 ± 0.04	0.19 ± 0.04	0.30 ± 0.09	0.18 ± 0.06

K_i, binding affinity.

TABLE 2

Saturation studies with [³H]acclidinium, [³H]ipratropium, and [³H]tiotropium at membranes expressing human M₂ and M₃ receptors. Data are reported as mean ± S.E.M. of three independent experiments.

Compound	M ₂		M ₃	
	K _d	B _{max}	K _d	B _{max}
	nM	pmol/mg	nM	pmol/mg
[³ H]Acclidinium	0.34 ± 0.02	3.13 ± 0.48	0.32 ± 0.04	3.53 ± 0.22
[³ H]Ipratropium	1.60 ± 0.22	1.96 ± 0.04	1.34 ± 0.36	2.24 ± 0.09
[³ H]Tiotropium	0.38 ± 0.00	3.66 ± 0.32	0.41 ± 0.01	3.99 ± 0.23

K_d, equilibrium dissociation constant.

TABLE 3

Association rate and dissociation kinetic parameters of [³H]acclidinium, [³H]ipratropium, and [³H]tiotropium to human M₂ and M₃ receptors. Data are reported as mean ± S.E.M. of three independent experiments.

Compound	K _{on}		K _{off}		t _{1/2}		t _{1/2} Ratio
	M ₂ Receptor	M ₃ Receptor	M ₂ Receptor	M ₃ Receptor	M ₂ Receptor	M ₃ Receptor	M ₃ /M ₂
	M ⁻¹ · min ⁻¹		min ⁻¹		h		
[³ H]Acclidinium	NM	1.4 × 10 ⁸ ± 5.8 × 10 ⁶	0.15 ± 0.01	0.02 ± 0.00	4.69 ± 0.29	29.24 ± 0.61	6.2
[³ H]Ipratropium	NM	1.1 × 10 ⁸ ± 9.1 × 10 ⁶	9.72 ± 1.52	1.49 ± 0.06	0.08 ± 0.01	0.47 ± 0.02	5.9
[³ H]Tiotropium	NM	5.5 × 10 ⁷ ± 4.8 × 10 ⁶	0.047 ± 0.00	0.011 ± 0.00	15.11 ± 1.57	62.19 ± 2.96	4.1

K_{on}, association rate constant of radiolabeled antagonist; K_{off}, rate constant of dissociation of radiolabeled antagonist; t_{1/2}, residence half-life; NM, not measurable.

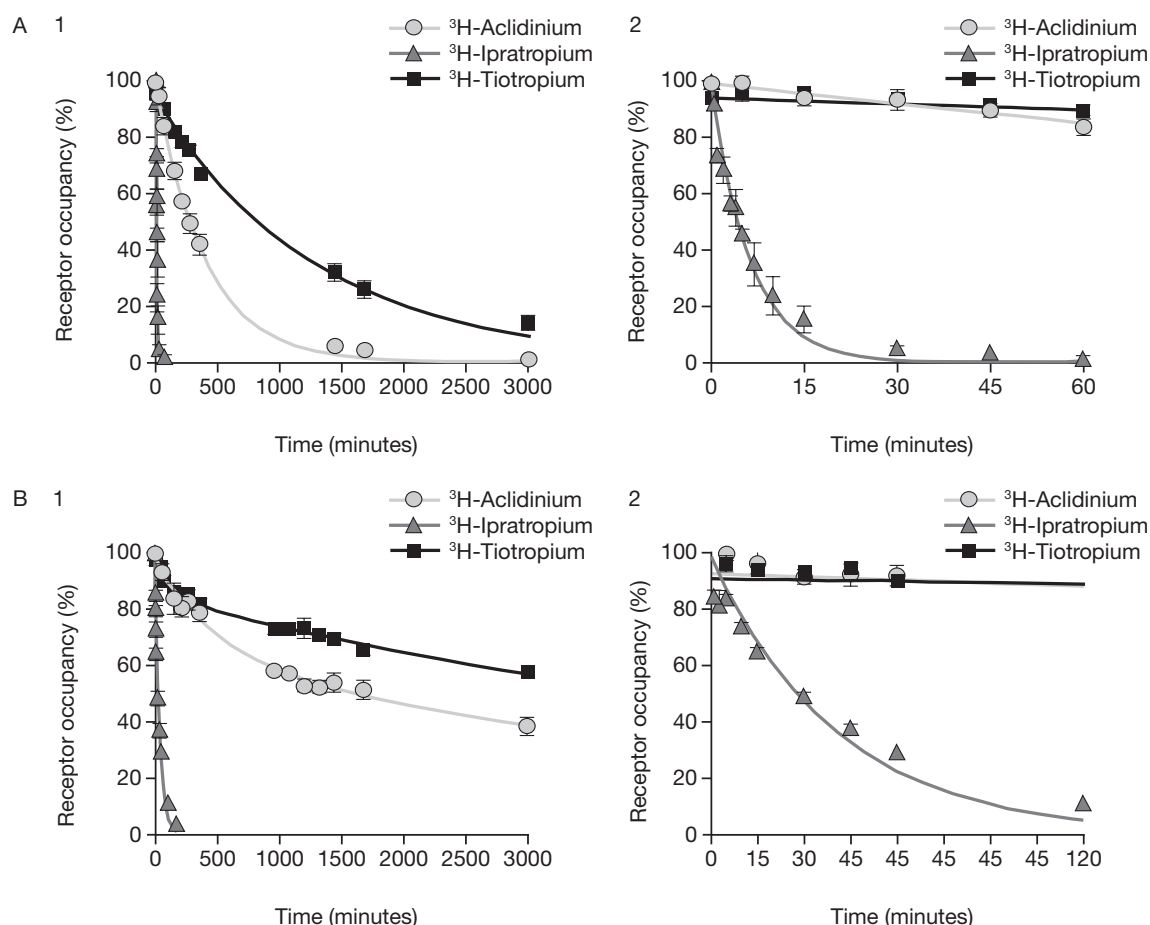


Fig. 2. Dissociation of [³H]acclidinium, [³H]ipratropium, and [³H]tiotropium from human M₂ and M₃ receptors. A, dissociation profile of radiolabeled compounds from human M₂ receptor: dissociation from 0 to 3000 min (1) and dissociation from 0 to 60 min (2). B, dissociation profile of radiolabeled compounds from human M₃ receptor: dissociation from 0 to 3000 min (1) and dissociation from 0 to 120 min (2). Plotted data correspond to the mean ± S.E.M. of three independent experiments.

using membranes of Chinese hamster ovary-KI cells expressing the human M₁ to M₅ receptor subtypes.

B_{max} for the five stably transfected clones and the radioligand K_d were determined using saturation experiments. All M₁, M₂, M₃, M₄, and M₅ receptor membrane preparations demonstrated saturable [³H]NMS binding. Mean (S.E.M.) B_{max} values were 1.19 (0.07), 1.94 (0.28), 2.26 (0.24), 1.28 (0.02), and 2.60 pmol/mg (0.04) for the M₁, M₂, M₃, M₄, and M₅ receptors, respectively. Mean (S.E.M.) K_d values were 0.4 (0.03), 0.81 (0.08), 0.66 (0.01), 0.28 (0.01), and 1.68 nM (0.17) for the M₁, M₂, M₃, M₄, and M₅ receptors, respectively.

Acclidinium, ipratropium, and tiotropium potently blocked the specific binding of [³H]NMS to human M₁ to M₅ receptors in a concentration-dependent manner (Table 1). At the highest concentrations tested (10 μ M), specific binding of [³H]NMS to the receptors was completely blocked by all three compounds. Each antagonist had similar affinity for the M₁ to M₅ receptor subtypes. Acclidinium was approximately equipotent to tiotropium and 8 to 16 times more potent than ipratropium for all five human muscarinic receptor subtypes.

Saturation Studies in Human M₂ and M₃ Receptors. Specific binding of [³H]acclidinium, [³H]tiotropium, and [³H]ipratropium to human muscarinic M₂ and M₃ receptors was found to be saturable in the experimental conditions chosen (data not shown). Analysis of the saturation curves indicated that the three radioligands bound to a homoge-

neous receptor population for both M₂ and M₃ membrane preparations (data not shown). The K_d and B_{max} values are shown in Table 2. The K_d values of [³H]acclidinium for the human M₂ and M₃ receptor subtypes were comparable to those of [³H]tiotropium and approximately four times lower than those of [³H]ipratropium.

Association with Human M₂ and M₃ Receptors. The association rates of [³H]acclidinium, [³H]tiotropium, and [³H]ipratropium with the human M₃ receptor subtype are shown in Table 3. [³H]Acclidinium and [³H]tiotropium were assayed at 0.11, 0.33, and 1 nM, whereas [³H]ipratropium was assayed at 0.33, 1, and 3 nM based on the corresponding K_d values. The K_{on} for [³H]acclidinium was 1.42×10^8 M/min. This association rate was similar to that of [³H]ipratropium and 2.6 times faster than that of [³H]tiotropium. The K_{on} of the three antagonists for the human M₂ receptor subtype was too fast to be reliably measured under the experimental conditions used.

Dissociation from Human M₂ and M₃ Receptors. Binding of radiolabeled antagonist to human muscarinic M₂ and M₃ membrane preparations reached equilibrium before 120 min of incubation and remained stable for at least 52 h. K_{off} values and their derived residence $t_{1/2}$ values are shown in Table 3. [³H]Acclidinium and [³H]tiotropium showed slow dissociation from M₃ receptors (Fig. 2), with residence half-lives of approximately 29 and 62 h, respectively. Conversely,

dissociation of [³H]ipratropium from the same receptor was much faster (Fig. 2), resulting in a residence half-life approximately 60- to 130-fold shorter than that for [³H]acclidinium and [³H]tiotropium, respectively. The [³H]acclidinium half-life for the M₂ receptor was 3.22 times shorter than that obtained for [³H]tiotropium. The kinetic selectivity of the three compounds expressed as M₃/M₂ half-life ratios were comparable (Table 3).

In Vitro Functional Activity in Isolated Guinea Pig Trachea. Carbachol (0.1 nM–1 mM) produced concentration-dependent contractions of the guinea pig trachea, the reproducibility of which was confirmed by control experiments (data not shown). A 60-min pretreatment with acclidinium, ipratropium, or tiotropium shifted the concentration response curves of carbachol (Fig. 3) to the right, demonstrating concentration-dependent antagonism of the contractile responses induced by the cholinergic agonists.

The potency (pA₂) of acclidinium was similar to that of ipratropium and tiotropium. No significant differences between the pA₂ values were observed (Table 4). Acclidinium and ipratropium both demonstrated competitive antagonism, with no suppression of the maximal contraction induced by agonist. In contrast, tiotropium demonstrated a nonsurmountable antagonism with an attenuated maximal contraction of agonist at the greatest concentrations. The nonsurmountable nature of the tiotropium antagonism was reflected in the calculated slope of the Schild plot analysis, which was significantly greater than 1 (Table 4). The Schild plot slopes for acclidinium and ipratropium were not significantly different from unity.

The onset of action of acclidinium was also studied in the carbachol-induced contraction assay, using antagonist concentrations that produce around 70 to 80% relaxation (Fig. 4). Acclidinium showed an onset of action ($t_{1/2} = 6.8 \pm 1.5$ min, $t_{max} = 35.9 \pm 8.2$ min) faster than tiotropium ($t_{1/2} = 13.6 \pm 2.7$ min, $t_{max} = 61.2 \pm 10.6$ min) and similar to ipratropium ($t_{1/2} = 5.1 \pm 1.5$ min, $t_{max} = 24.1 \pm 3.5$ min).

The duration of action of acclidinium, tiotropium, and ipratropium was assessed in the carbachol-contracted guinea pig trachea assays (Table 4). Acclidinium and tiotropium exhibited a significantly longer duration of action compared with ipratropium in terms of $t_{1/2}$ and t_{max} ($p < 0.05$). Neither acclidinium nor tiotropium allowed complete recovery of tone over the washout period (t_{max}), whereas a significant recovery of tone was observed following ipratropium washout (Table 4). Each antagonist produced a similar percentage inhibition of carbachol-induced contraction at the concentrations selected (data not shown).

Potency and Onset in Anesthetized Guinea Pigs. To evaluate the in vivo bronchoprotective effect of inhaled acclidinium and the comparators with respect to potency and onset of action, three doses of each antagonist were administered to different groups of anesthetized guinea pigs before acetylcholine-induced bronchoconstriction. Acclidinium, ipratropium, and tiotropium produced bronchoprotection over the 120-min study period and displayed a dose-dependent effect (Fig. 5). The IC₅₀ values of acclidinium, ipratropium, and tiotropium were determined at the time when maximal bronchoprotective effects were observed (Fig. 5) and are shown in Table 5. No significant differences between IC₅₀ values were observed (Table 5). The maximal inhibition of bronchoconstriction at the highest dose tested was 88, 83,

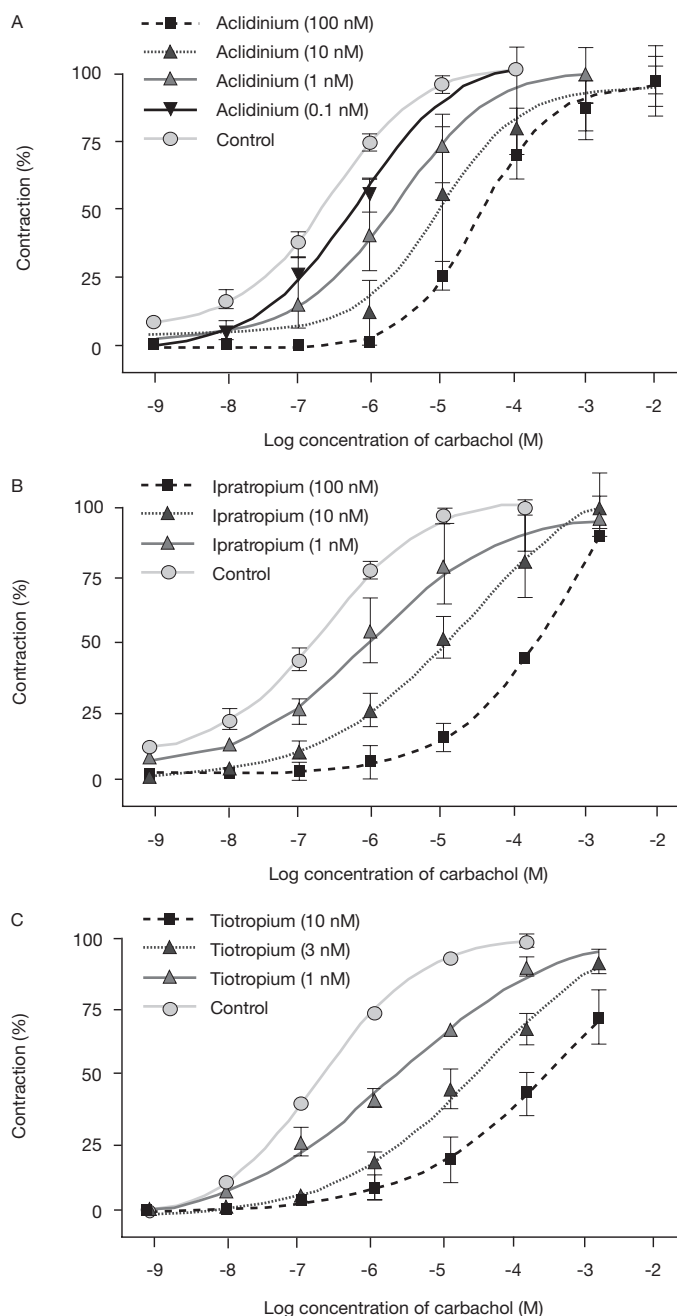


Fig. 3. Effect of acclidinium (A), ipratropium (B), and tiotropium (C) on carbachol-induced contractions in isolated guinea pig trachea. All contraction values are reported as a percentage of the maximal control carbachol contraction. Data are reported as mean \pm S.E.; $n = 3$ to 5.

and 94% for acclidinium, ipratropium, and tiotropium, respectively. The onset of action (defined as time to achieve maximal inhibition of bronchoconstriction) of acclidinium and ipratropium was the same (30 min) but faster than that of tiotropium (80 min) (Table 5; Fig. 5).

Duration of Bronchoprotection in Guinea Pigs. To assess the duration of bronchoprotection of compounds, the effect of a submaximal dose of inhaled antagonists was studied up to 96 h in the acetylcholine-induced bronchoconstriction model in guinea pigs. At the doses selected, acclidinium, ipratropium, and tiotropium achieved a peak inhibitory effect of airway resistance of 97 to 98% at 1 h, showing an equipotent inhibition of acetylcholine-induced bronchoconstriction.

TABLE 4

Potency and duration of action of acclidinium, ipratropium, and tiotropium in isolated guinea pig trachea

pA_2 values were determined by Schild plot analysis using carbachol (0.1 nM–1 mM) as the contractile agent. Antagonists were incubated for 30 min before a concentration-response curve of cholinergic agonists was generated. The concentration ranges of the antagonists used were acclidinium (0.1–100 nM), ipratropium (1–100 nM), and tiotropium (1–10 nM). Data are reported as the mean \pm S.E. One-way analysis of variance of pA_2 values showed no significant differences. In the duration of action studies, contraction of isolated guinea pig trachea was induced by carbachol (10 μ M). Antagonists were added at the concentrations indicated and were washed out after a period of time (30-min carbachol-contraction assay). Duration of action was calculated as the time from antagonist washout to recovery of 50% ($t_{1/2}$) or maximal recovery (t_{max}) of the maximal contraction induced by carbachol. Data are reported as mean \pm S.E.

	Potency			Duration of Action			
	<i>n</i>	pA_2	Slope	<i>n</i>	Dose <i>nM</i>	$t_{1/2}$ <i>min</i>	t_{max}
Acclidinium	13	9.9 \pm 0.1	0.8 \pm 0.1	7	2	139.8 \pm 51.4*	NR ^a
Ipratropium	12	9.6 \pm 0.1	0.7 \pm 0.1	6	3	24.1 \pm 13.3	91.6 \pm 26.7
Tiotropium	9	9.6 \pm 0.1	1.8 \pm 0.2 ^a	5	6	NR ^b	NR ^b

* $P < 0.05$ versus ipratropium; NR, no recovery of tension after washout period.

^a Indicates a slope significantly greater from unity.

^b Washout period = 4 h.

tion (Fig. 6). All tested compounds inhibited acetylcholine-induced bronchoconstriction in a concentration-dependent manner (IC_{50} at 1 h: 5.9, 2.4, and 6.9 μ g/ml for acclidinium, tiotropium, and ipratropium, respectively). The duration of action [defined as the time taken to reduce the maximal bronchoconstriction achieved at 1 h by 50% ($t_{1/2}$)] was 29 h for acclidinium. This duration of action was considerably longer than that of ipratropium ($t_{1/2} = 8$ h) and somewhat shorter than that of tiotropium ($t_{1/2} = 64$ h).

Therapeutic Index in Beagle Dogs. These experiments were performed to assess the efficacy and safety ratio of acclidinium with respect to tiotropium 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 effects on heart rate in beagle dogs. The doses selected for each compound produced a similar and long-lasting bronchoprotective effect that was statistically significant compared with vehicle at all time points over the 6-h study period ($p < 0.001$). The calculated bronchoconstriction inhibition $AUC_{0-6 h}$ values for acclidinium (462%) and tiotropium (540%) were comparable (Fig. 7). When doses of acclidinium and tiotropium 100-fold greater than those used in the efficacy

study were administered by inhalation, an increase in the heart rate was observed for both compounds (Fig. 8). Acclidinium at 500 μ g/kg induced a maximal increase in heart rate of 55% after 1 h, compared with a 99% maximal increase with tiotropium (25 μ g/kg) after 2 h. The effect of acclidinium on heart rate was transient and was not significantly different to that of vehicle from 2.5 h onward. In contrast, the increase in heart rate observed with tiotropium persisted up to 6 h after administration (30% increase) and was significantly greater than that of vehicle at all time points (starting at 1 h) over the 6-h study period. Overall, acclidinium had a considerably smaller effect on heart rate compared with tiotropium over the 6-h study period (acclidinium heart rate increase $AUC_{0-6 h} = 108\%$ compared with 341% for tiotropium). The therapeutic index, calculated as $AUC_{0-6 h}$ bronchoconstriction inhibition (%) / $AUC_{0-6 h}$ heart rate increase (%), was 4.2 for acclidinium and 1.6 for tiotropium.

Discussion

The aim of this study was to establish the pharmacological profile of acclidinium bromide, a novel and long-acting inhaled

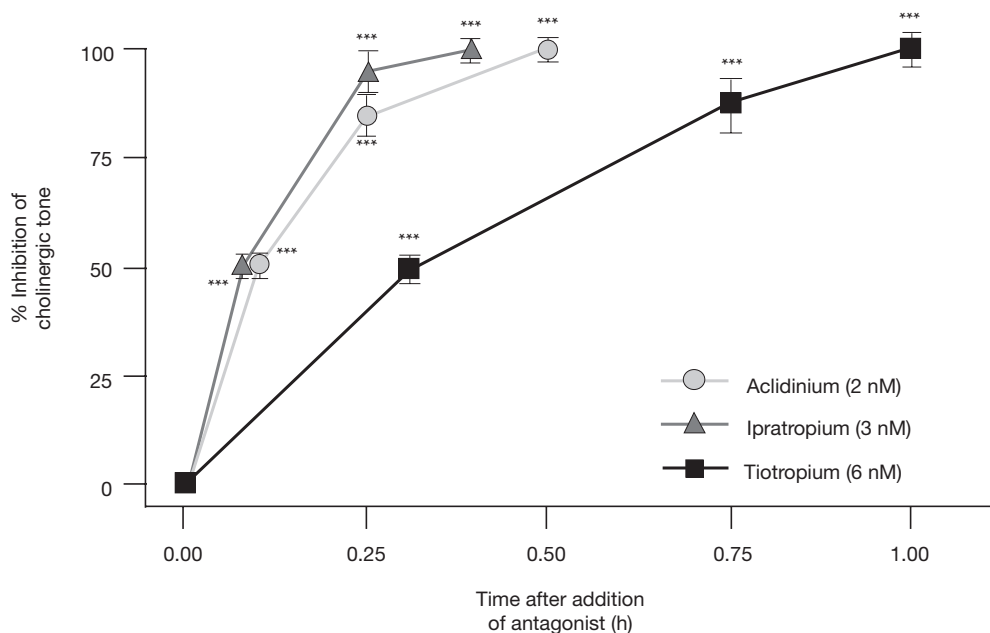


Fig. 4. Onset of action of acclidinium, ipratropium, and tiotropium in isolated guinea pig trachea. Contraction was induced with 10 μ M carbachol and allowed to plateau before the addition of antagonists. Onset was defined as the time from antagonist addition to achieve inhibition of 50 ($t_{1/2}$) or 100% (t_{max}) of the contraction. Data are reported as mean \pm S.E.; $n = 5$ to 7. ***, $p < 0.001$ compared with first observational time point.

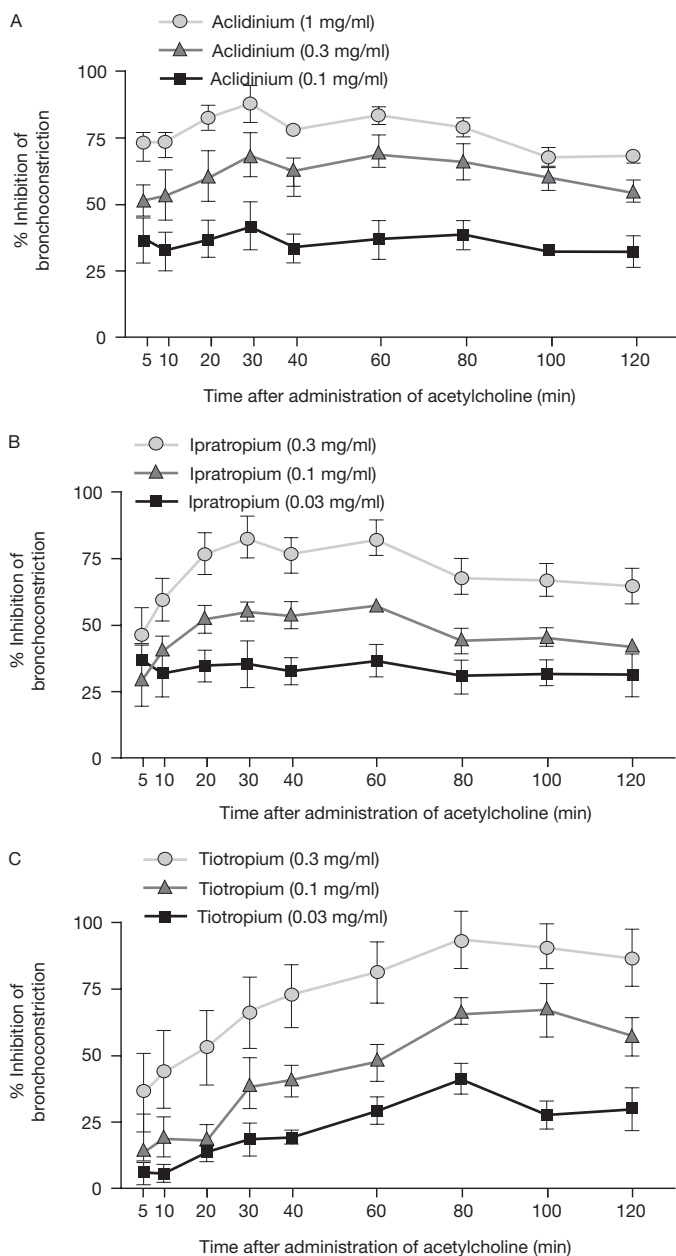


Fig. 5. Potency and onset of action of inhaled acclidinium (A), ipratropium (B), and tiotropium (C) on acetylcholine-induced bronchoconstriction in anesthetized guinea pigs. Nebulized antagonists were administered to anesthetized animals after maximal bronchoconstriction induced by acetylcholine (10–60 $\mu\text{g}/\text{kg}$ i.v.) was established. Inhibition of bronchoconstriction by antagonists was assessed by acetylcholine administration at the indicated time points. Antibronchoconstrictor effects of antagonists were determined as the percentage of inhibition of the maximal bronchoconstriction induced by acetylcholine. Data are reported as mean \pm S.E.; $n = 4$ to 10.

muscarinic antagonist in development for maintenance treatment of COPD.

In radioligand binding displacement studies carried out at equilibrium, acclidinium like tiotropium, demonstrated subnanomolar affinity and no selectivity for the five human muscarinic receptor subtypes analyzed. The affinity of ipratropium is in the low nanomolar range for M_1 to M_5 receptors. The data obtained for ipratropium and tiotropium are consistent with those reported previously (Haddad et al., 1994).

TABLE 5

Potency and onset of action of acclidinium, ipratropium, and tiotropium in the acetylcholine-induced bronchoconstriction model in anesthetized guinea pigs

Nebulized antagonists (acclidinium, 0.1–1 mg/ml; ipratropium and tiotropium, 0.03–0.3 mg/ml) were administered to anesthetized animals after maximal bronchoconstriction induced by acetylcholine (10–60 $\mu\text{g}/\text{kg}$ i.v.) was established. Determination of inhibition of bronchoconstriction induced by acetylcholine (10–60 $\mu\text{g}/\text{kg}$ i.v.) was established. Determination of inhibition of bronchoconstriction by antagonists was assessed by acetylcholine administration up to 2 h. Antibronchoconstrictor effects of the antagonists were determined as a percentage of inhibition of the baseline response to acetylcholine. IC_{50} values were determined at the time when maximal inhibition of bronchoconstriction was achieved (t_{max}). One-way analysis of variance of IC_{50} values showed no significant differences.

	<i>n</i>	IC_{50} (95% CI)	t_{max}
		$\mu\text{g}/\text{ml}$	<i>min</i>
Acclidinium	4–10	140 (76–254)	30
Ipratropium	6–9	68 (43–110)	30
Tiotropium	5–6	45 (29–70)	80

CI, confidence interval; IC_{50} , concentration required to induce 50% inhibition.

The kinetics of the binding of the three radiolabeled compounds to the M_2 and M_3 receptor subtypes were assessed. The interaction of the compounds with the M_2 receptors is interesting beyond its potential implication on their efficacy because inhibition of cardiac M_2 receptors is known to induce tachycardia, which is potentially the most severe side effect associated with systemic antimuscarinic agents (Eglen, 2005). The M_3 receptor, as previously mentioned, is the key receptor subtype through which the therapeutically relevant muscle relaxant and bronchodilatory effects of the antimuscarinic agents are mediated.

The experimental K_{off} values and the corresponding dissociation half-lives should be regarded as a lower limit to the actual effective duration of action of the compounds at the receptor. Under the experimental conditions used, with excess amounts of competitor, reassociation of the antagonist to the receptor is unlikely. The excess competitor present will readily occupy any free binding sites generated through dissociation of the test compound as soon as they became available. This would prevent the “proximity effect,” described by Copeland et al. (2006), which is based on the probability of any ligand molecule leaving a receptor and reassociating with the same receptor molecule. Accordingly, the residence half-life obtained for acclidinium at the M_3 receptor (29.24 h) suggests a long duration of action *in vivo*.

All three radiolabeled antagonists had a faster dissociation from the M_2 receptor than the M_3 receptor, conferring a certain kinetic selectivity for the M_3 versus the M_2 receptor subtype. The residence half-life at the M_3 is 4- to 6-fold longer than that at M_2 receptor varied for all three compounds. These data are consistent with those reported previously for tiotropium and ipratropium (Disse et al., 1993; Gross, 2006).

Association to the M_2 receptors was not measurable under the experimental conditions used due to the rapidity of antagonist association with this receptor. Association to the M_3 receptors was slower, allowing for quantitative determination, and was found to be similar for acclidinium and ipratropium. The association of tiotropium to the M_3 receptors was 2.6-fold slower than acclidinium with respect to K_{on} values. Using a different experimental approach, previous studies have reported K_{on} values for ipratropium and tiotropium in the same range as those reported here (Dowling and Charlton, 2006).

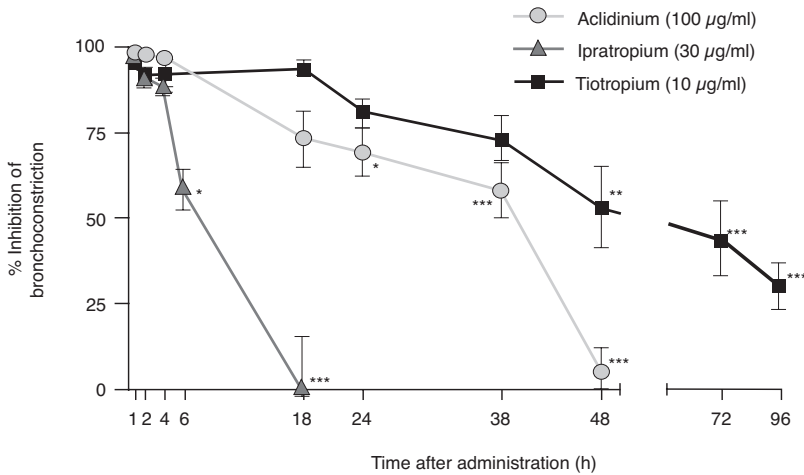


Fig. 6. Duration of action of inhaled acclidinium, ipratropium, and tiotropium in the acetylcholine-induced bronchoconstriction model in guinea pigs. Conscious animals were exposed to aerosol solutions of antagonist and then anesthetized at various time points and bronchoconstriction induced by acetylcholine (30 µg/kg i.v.). The anti-bronchoconstrictor effect of each antagonist was assessed as a percentage of the control (vehicle) response. Data are reported as mean \pm S.E.; $n = 4$ to 9 ; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with first observational time point (1 h).

The bronchoconstrictor response to muscarinic agonists in isolated lung tissue is mainly mediated via M_3 receptors in airway smooth muscle, as demonstrated in tracheal smooth muscle preparations derived from $M3R^{-/-}$ knockout mice (Wess et al., 2007). In the present study, the potency of acclidinium in isolated guinea pig trachea stimulated with carbachol was not significantly different from that of ipratropium and tiotropium. The pA_2 values for ipratropium and tiotropium obtained here are similar to those previously reported in guinea pig trachea stimulated with metacholine (Disse et al., 1993). Similar pIC_{50} values have been reported for these two antagonists in another study of guinea pig trachea stimulated with carbachol (Villetti et al., 2006).

In the carbachol-stimulated guinea pig trachea studies, acclidinium and ipratropium demonstrated competitive antagonism. However, the antagonism produced by tiotropium was nonsurmountable, as indicated by a slope greater than one on the Schild plot adjustment and by attenuation of the maximal cholinergic contraction, suggesting that equilibrium could not be achieved after incubation with tiotropium. A significantly greater Schild plot slope for tiotropium has also been reported in previous studies in isolated guinea pig trachea and human airways (Disse et al., 1993; Villetti et al., 2006).

Acclidinium demonstrated a fast onset of action in the isolated guinea pig trachea precontracted with carbachol, similar to that of ipratropium and twice as fast as tiotropium. Takahashi et al. (1994) also have reported a faster onset of action for ipratropium compared with tiotropium in reverting electrical field stimulation-induced contractions of isolated guinea pig trachea. The duration of action of ipratropium and tiotropium in the carbachol-stimulated guinea pig trachea experiments is in line with their clinical profiles. Acclidinium demonstrated a long duration of action comparable with that of tiotropium in these studies. Unlike acclidinium, tiotropium did not wash out sufficiently to allow recovery of tone in the carbachol experiments, as similarly described by Villetti et al. (2006).

In the acetylcholine-induced bronchoconstriction model in anesthetized guinea pigs, the IC_{50} of acclidinium was two and three times greater than ipratropium and tiotropium, respectively, although the differences were not statistically significant. Differences between the acclidinium IC_{50} values observed from in vitro tracheal assays and in vivo animal models may be explained by the high susceptibility of acclidinium to hydrolysis by esterases. Acclidinium is hydrolyzed significantly faster in human plasma compared with ipratropium and tiotropium, with a half-life in human plasma of 2

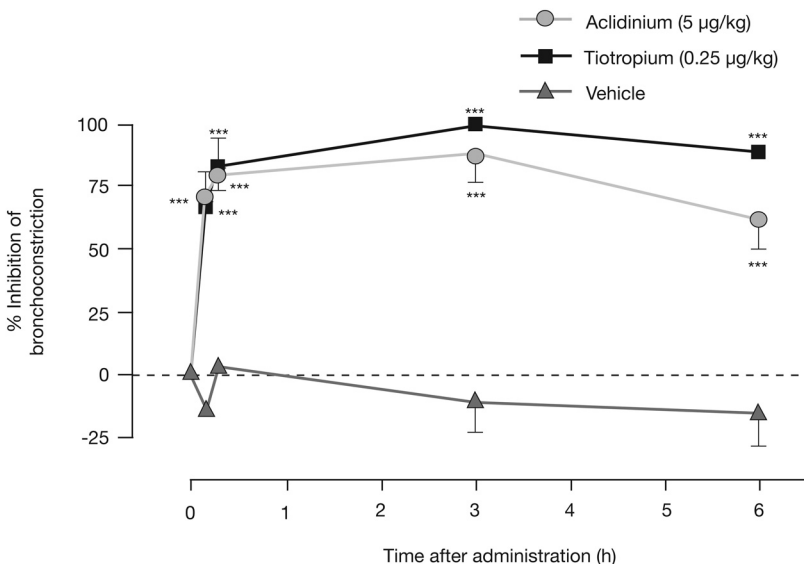


Fig. 7. Effects of acclidinium and tiotropium on acetylcholine-induced bronchoconstriction in anesthetized beagle dogs. Nebulized compounds or vehicle were administered to anesthetized animals, and bronchoconstriction was induced by acetylcholine (5 µg/kg) at 10 and 20 min and 3 and 6 h. Inhibition of bronchoconstriction was recorded as a percentage of the baseline response to acetylcholine. Data are reported as mean \pm S.E.; $n = 3$. ***, $p < 0.001$ compared with vehicle. There are no differences between active treatments.

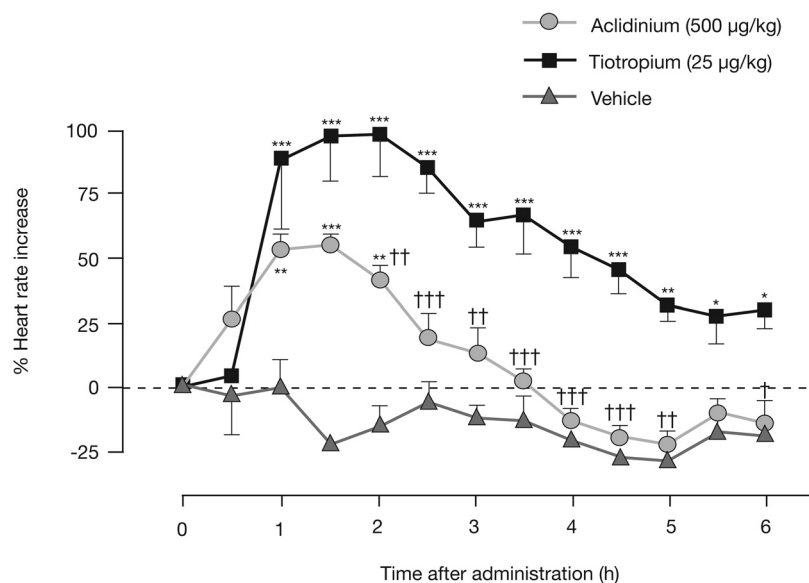


Fig. 8. Effect of acclidinium and tiotropium on heart rate in conscious beagle dogs. Animals were anesthetized in order to deliver the nebulized compounds or vehicle and were allowed to regain consciousness. The effect on heart rate of a dose 100 times higher than that used to achieve submaximal bronchodilation was assessed continuously up to 6 h and expressed as a percentage change from baseline heart rate. Data are reported as mean \pm S.E.; $n = 4$ for acclidinium and tiotropium; $n = 3$ for vehicle. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with vehicle; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ compared with tiotropium.

min for acclidinium and greater than 60 min for reference compounds (Gavaldà et al., 2007). Similar hydrolysis rates have been observed in guinea pig and dog plasma (Gavaldà et al., 2008). The onset of action of acclidinium reverting the acetylcholine-induced bronchoconstriction in guinea pigs was similar to ipratropium and faster than tiotropium, in accordance with the results found in in vitro studies using human M_3 receptors and isolated guinea pig trachea.

Acclidinium also demonstrated a long duration of action in anesthetized guinea pigs ($t_{1/2} = 29$ h), which was approximately 4-fold that of ipratropium ($t_{1/2} = 8$ h) and less than that of tiotropium ($t_{1/2} = 64$ h). Moreover, in anesthetized dogs, acclidinium demonstrated a similar bronchoprotective effect to tiotropium, with both antagonists producing a significant inhibition of bronchoconstriction over the 6-h study period. These in vivo data are in agreement with both the in vitro residence half-life values of [3 H]acclidinium ($t_{1/2} = 29$ h) at the human muscarinic M_3 receptor and the in vitro guinea pig studies reported in this article. Altogether, these results suggest that acclidinium has long-lasting effects in preclinical models. The therapeutic index in dogs was investigated to assess whether the rapid plasma hydrolysis of acclidinium (Gavaldà et al., 2007) and a short M_2 receptor residence time translate into an improved efficacy-safety ratio compared with tiotropium.

In conscious dogs, inhaled acclidinium had a reduced effect on heart rate compared with tiotropium at doses that were 100-fold those needed to produce equivalent antibronchoconstrictor effects in the same species. Under these experimental conditions, acclidinium produced a transient increase in heart rate, which was resolved 2.5 h after administration. In contrast, tiotropium caused a significant increase in heart rate that persisted 6 h after administration. These data concur with a recent preclinical cardiovascular safety study in guinea pigs and dogs that showed that acclidinium has reduced potential for cardiovascular side effects compared with tiotropium (Gras et al., 2008). Acclidinium is rapidly hydrolyzed in plasma (Gavaldà et al., 2008) and has an in vitro K_{off} value for human M_2 receptors four times faster than tiotropium, as presented here. The combination of these two characteristics may explain the lower and more transient effects

seen for acclidinium on heart rate in dogs compared with tiotropium.

Despite the kinetic selectivity ($M_3 > M_2$) profile described for tiotropium, its slow dissociation from the M_3 receptor was also accompanied by an increase in the residence time on the M_2 receptor (Disse et al., 1993). Its longer residence time at the M_2 receptor in comparison with acclidinium demonstrated here may explain the long-lasting effects of tiotropium on the heart rate in the present study. Although tachycardia has not been reported after the regular use of tiotropium (Tashkin et al., 2008), the presence of this drug in blood plasma could compromise the use of tiotropium in combination with other cardioactive drugs. On the contrary, a compound with a rapid plasma hydrolysis, such as acclidinium (Gavaldà et al., 2007), is likely to have a lower potential for cardiovascular effects and would therefore be advantageous.

In summary, the preclinical data reported in this study show that acclidinium is a potent and selective muscarinic antagonist, which interacts rapidly with muscarinic receptors, and provides a sustained blockade of their action. These properties are reflected in the rapid onset and a prolonged duration of action for acclidinium in guinea pig in vitro and in vivo functional models.

Furthermore, acclidinium has a good cardiovascular safety profile, which may be attributed to its reduced residence time at M_2 receptors and rapid hydrolysis in plasma. Together, these data suggest that acclidinium may have a favorable benefit-to-risk ratio in the clinical setting, therefore providing a valuable treatment option for patients with COPD.

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