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

Amadeu Gavalda, Montserrat Miralpeix, Israel Ramos, Raquel Otal, Cristina Carreño, Marisa Viñals, Teresa Doménech, Carla Carcasona, Blanca Reyes, Dolores Vilella, Jordi Gras, Julio Cortijo, Esteban Morcillo, Jesús Llenas, Hamish Ryder, and Jorge Beleta

Almirall, Biology Department, R&D Center, Sant Feliu de Llobregat, Barcelona, Spain (A.G., M.M., I.R., R.O., Cr.C., M.V., T.D., Ca.C., B.R., D.V., J.G., J.L., H.R., J.B.); Department of Pharmacology, Faculty of Medicine, University of Valencia, Valencia, Spain (J.C., E.M.); Ciber Respiratory Diseases (CIBERES), Valencia, Spain (J.C., E.M.); Research Foundation, University General Hospital, Valencia, Spain (J.C.); and Clinical Pharmacology Unit, Research Foundation, University Clinic Hospital, Valencia, Spain (E.M.)

Received January 29, 2009; accepted August 25, 2009

ABSTRACT

Aclidinium bromide is a novel potent, long-acting inhaled muscarinic antagonist in development for the treatment of chronic obstructive pulmonary disease. Aclidinium showed subnanomolar affinity for the five human muscarinic receptors (M1–M5). [3H]Aclidinium dissociated slightly faster from M2 and M3 receptors than [3H]tiotropium but much more slowly than [3H]ipratropium. Its association rate for the M3 receptor was similar to [3H]ipratropium and 2.6 times faster than [3H]tiotropium. Residence half-life of [3H]aclidinium at the M2 receptor was shorter than at the M3 receptor, demonstrating kinetic selectivity for the M3 receptor. In isolated guinea pig trachea, aclidinium 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 aclidinium 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 aclidinium (t1/2 = 29 h) was much longer than ipratropium (8 h) but shorter than tiotropium (64 h). In dogs, aclidinium induced a smaller and more transient increase in heart rate than tiotropium at comparable supratherapeutic doses. Therefore, under these conditions, aclidinium showed a greater therapeutic index than tiotropium (4.2 versus 1.6). These results indicate that aclidinium 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, M1 to M5, that are members of the superfamily of G-protein-coupled receptors.
plied 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 M1, M2, and M3, but not the M4 and M5, muscarinic receptor subtypes have been described previously (Belmonte, 2005). M2 receptors, localized in airway smooth muscle, are responsible for mediating the bronchoconstrictor response to cholinergic nerve stimulation (Roffel et al., 1990). M3 receptors are also expressed in mucosal glands where their stimulation is believed to promote mucus secretion (Gwilt et al., 2007). M2 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 M3 receptor than ipratropium (Biskey 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 M2 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. Aclidinium bromide, 3R-(2-hydroxy-2,2-di-thiophen-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 other currently available antimuscarinics including tiotropium, aclidinium 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 aclidinium 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**

Aclidinium bromide and tiotropium bromide (6β,7β-epoxy-3β-hydroxy-8-methyl-10H,11H-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 pentobarbitol was obtained from Industrial Kern (Barcelona, Spain); ketamine chloride (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 (Rubi, Spain).

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...
grope 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 aclidinium, 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 receptor 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 \((10^{-14} \text{ to } 10^{-6} \text{ M})\) were tested in duplicate to generate competition curves. Non-specific 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 (M₂, M₃, 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/B filter plates (Millipore, Barcelona, Spain) pretreated for 1 h with wash aliquots of the reaction mixture, and bound radioactivity was determined using a MicroBeta Trilux microplate scintillation counter (PerkinElmer Life and Analytical Sciences).

Data Analysis. Affinities at equilibrium were determined as equilibrium antagonist dissociation constant \( K_d \) 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. \( K_d \) 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]aclidinium 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 prewash solution (0.5% polivinylpyrrolidone, 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]aclidinium 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 Bₘₐₓ, values for [3H]aclidinium, [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 \( (K_{on}) \), 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 \( K_{on} = (K_{cb} - K_{off})/\text{[antagonist]} \), where \( K_{off} \) and \( K_{cb} \) 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 equilibrium 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 aclidinium, 1 to 100 nM ipratropium, and 1 to 10 nM tiotropium. The potency of each antagonist was determined as a pA₂ 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% (t₁/₂) and 100% (tₘₐₓ) inhibition of the carbachol contraction. The concentrations of antagonists used were 2 nM aclidinium, 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 (\(t_{\text{max}}\)) 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 \(\mu\)M carbachol and allowed to plateau. Antagonists were then added to relax the tissue (2, 3, and 6 \(nM\) for acridinium, 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 \(\mu\)M carbachol, and the recovery of tone was recorded over time.

**Data Analysis.** The \(P_{A2}\) 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 (ML7069; 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 \(\mu\)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 acridinium, 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. \(t_{\text{max}}\) 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 \(t_{\text{max}}\).

**Data Analysis.** Potency (IC\(_{50}\)) was determined from a sigmoidal dose-response curve constructed using inhibition values at \(t_{\text{max}}\) 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 (Devibis Ultraneb 2000; Sunrise Medical, Somerset, PA) from solutions of 100 \(\mu\)g/ml acridinium, 30 \(\mu\)g/ml ipratropium, and 10 \(\mu\)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 (45.8 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 homothermic 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 H\(_2\)O/ml per second for airway resistance and 0.3 to 0.9 ml/cm H\(_2\)O for dynamic compliance, the pulmonary dynamic measurements were initiated. Bronchoconstriction was then induced with a single-bolus dose of acetylcholine (30 \(\mu\)g/kg i.v.) and the inhibitory effects of antagonists were tested in comparison to vehicle.

Airway resistance (centimeter per milliliter of H\(_2\)O 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\(_{\text{M}}\) - R\(_{B}\)) \(\times\) 100/R\(_{B}\), where R\(_{B}\) is the peak resistance after challenge (maximal value) and R\(_{M}\) 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 acridinium, and 72 and 96 h for tiotropium were also assessed.

**Data Analysis.** Duration of action (\(t_{\text{max}}\)) 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 Rosler (1940) modified according to Misawa et al. (1986). Dogs fasted for 18 h were anesthetized with propofol with an initial dose of 0.6 to 0.8 mg/kg per min in the cephalic vein via an infusion pump (Becton Dickinson Program 2; BD Biosciences, Brezin, 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 acetycholine bolus (5 μg/kg i.v.) at 10-min intervals to induce bronchoconstriction. After two consecutive similar baseline responses to acetylcholine, aerosol solutions of 5 μg/kg aclidinium or 0.25 μg/kg tiotropium were administered by using a nebulizer (AG-ALL100; Aerogen, Galway, Ireland), which was attached between the respirator and the endotracheal tube. These doses of aclidinium 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 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.) at 10-min intervals to induce bronchoconstriction. After induction of anesthesia and preparation, animals received ace

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kd (nM)</th>
<th>Bmax (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Aclidinium</td>
<td>3.13 ± 0.48</td>
<td>3.13 ± 0.48</td>
</tr>
<tr>
<td>[3H]Ipratropium</td>
<td>1.96 ± 0.04</td>
<td>1.96 ± 0.04</td>
</tr>
<tr>
<td>[3H]Tiotropium</td>
<td>3.66 ± 0.32</td>
<td>3.66 ± 0.32</td>
</tr>
</tbody>
</table>

Kd: equilibrium dissociation constant.

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>M2</th>
<th>M3</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Aclidinium</td>
<td>0.34 ± 0.02</td>
<td>3.13 ± 0.48</td>
</tr>
<tr>
<td>[3H]Ipratropium</td>
<td>1.60 ± 0.22</td>
<td>1.96 ± 0.04</td>
</tr>
<tr>
<td>[3H]Tiotropium</td>
<td>0.38 ± 0.00</td>
<td>3.66 ± 0.32</td>
</tr>
</tbody>
</table>

Data are reported as mean ± S.E.M. of three independent experiments.

**Affinity Studies Using Displacement of [3H]NMS from Human Muscarinic Receptor Subtypes M2 to M3 at Equilibrium.** The affinity of aclidinium, ipratropium, and tiotropium for human muscarinic receptors was assessed.

**Results**

**TABLE 3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>M2 Receptor</th>
<th>M3 Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Aclidinium</td>
<td>1.4×10^9 ± 5.5×10^6</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>[3H]Ipratropium</td>
<td>1.1×10^9 ± 4.8×10^6</td>
<td>9.72 ± 1.52</td>
</tr>
<tr>
<td>[3H]Tiotropium</td>
<td>5.5×10^9 ± 4.8×10^6</td>
<td>0.047 ± 0.00</td>
</tr>
</tbody>
</table>

Kd, association rate constant of radiolabeled antagonist; Koff, rate constant of dissociation of radiolabeled antagonist; t1/2, residence half-life; NM, not measurable.
using membranes of Chinese hamster ovary-KI cells expressing the human M1 to M5 receptor subtypes.

$B_{\text{max}}$ 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 $[^3\text{H}]$NMS binding. Mean (S.E.M.) $B_{\text{max}}$ 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.

Aclidinium, ipratropium, and tiotropium potently blocked the specific binding of $[^3\text{H}]$NMS to human M1 to M5 receptors in a concentration-dependent manner (Table 1). At the highest concentrations tested (10 $\mu$M), specific binding of $[^3\text{H}]$NMS to the receptors was completely blocked by all three compounds. Each antagonist had similar affinity for the M1 to M5 receptor subtypes. Aclidinium 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 $[^3\text{H}]$aclidinium, $[^3\text{H}]$tiotropium, and $[^3\text{H}]$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 homogeneous receptor population for both M2 and M3 membrane preparations (data not shown). The $K_d$ and $B_{\text{max}}$ values are shown in Table 2. The $K_d$ values of $[^3\text{H}]$aclidinium for the human M2 and M3 receptor subtypes were comparable to those of $[^3\text{H}]$tiotropium and approximately four times lower than those of $[^3\text{H}]$ipratropium.

**Association with Human M2 and M3 Receptors.**
The association rates of $[^3\text{H}]$aclidinium, $[^3\text{H}]$tiotropium, and $[^3\text{H}]$ipratropium with the human M3 receptor subtype are shown in Table 3. $[^3\text{H}]$Aclidinium and $[^3\text{H}]$tiotropium were assayed at 0.11, 0.33, and 1 nM, whereas $[^3\text{H}]$ipratropium was assayed at 0.33, 1, and 3 nM based on the corresponding $K_d$ values. The $K_{\text{on}}$ for $[^3\text{H}]$aclidinium was $1.42 \times 10^8$/M/min. This association rate was similar to that of $[^3\text{H}]$ipratropium and 2.6 times faster than that of $[^3\text{H}]$tiotropium. The $K_{\text{on}}$ 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. $K_{\text{off}}$ values and their derived residence $t_{1/2}$ values are shown in Table 3. $[^3\text{H}]$Aclidinium and $[^3\text{H}]$tiotropium showed slow dissociation from M2 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]aclidinium and [3H]tiotropium, respectively. The [3H]aclidinium 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 aclidinium, 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 aclidinium was similar to that of ipratropium and tiotropium. No significant differences between the pA2 values were observed (Table 4). Aclidinium and ipratropium showed competitive antagonism, with no suppression of the maximal contraction induced by agonist. In contrast, tiotropium demonstrated a non-saturable antagonism with an attenuated maximal contraction of agonist at the greatest concentrations. The non-saturable 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 aclidinium and ipratropium were not significantly different from unity.

The onset of action of aclidinium was also studied in the carbachol-induced contraction assay, using antagonist concentrations that produce around 70 to 80% relaxation (Fig. 4). Aclidinium showed an onset of action (t1/2 = 6.8 ± 1.5 min, tmax = 35.9 ± 8.2 min) faster than tiotropium (t1/2 = 13.6 ± 2.7 min, tmax = 61.2 ± 10.6 min) and similar to ipratropium (t1/2 = 5.1 ± 1.5 min, tmax = 24.1 ± 3.5 min).

The duration of action of aclidinium, tiotropium, and ipratropium was assessed in the carbachol-contracted guinea pig trachea assays (Table 4). Aclidinium and tiotropium exhibited a significantly longer duration of action compared with ipratropium in terms of t1/2 and tmax (p < 0.05). Neither aclidinium nor tiotropium allowed complete recovery of tone over the washout period (tmax), 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 aclidinium 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. Aclidinium, ipratropium, and tiotropium produced bronchoprotection over the 120-min study period and displayed a dose-dependent effect (Fig. 5). The IC50 values of aclidinium, 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, and 94% for aclidinium, ipratropium, and tiotropium, respectively. The onset of action (defined as time to achieve maximal inhibition of bronchoconstriction) of aclidinium 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, aclidinium, 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 bronchoconstric-
Potential and duration of action of aclidinium, ipratropium, and tiotropium in isolated guinea pig trachea.

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

<table>
<thead>
<tr>
<th>Potency</th>
<th>Duration of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>pA2</td>
</tr>
<tr>
<td>Acclidinium</td>
<td>13</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>12</td>
</tr>
<tr>
<td>Tiotropium</td>
<td>9</td>
</tr>
</tbody>
</table>

*P < 0.05 versus ipratropium; NR, no recovery of tension after washout period.
**Indicates a slope significantly greater from unity.
***Washout period = 4 h.

Therapeutic Index in Beagle Dogs. These experiments were performed to assess the efficacy and safety ratio of aclidinium 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 AUC0–6 h values for aclidinium (462%) and tiotropium (454%) were comparable (Fig. 7). When doses of aclidinium 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). Aclidinium at 500 µg/kg induced a maximal increase in heart rate of 55% after 1 h, compared with a 99% maximal increase with tiotropium (25 µg/kg) after 2 h. The effect of aclidinium 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, aclidinium had a considerably smaller effect on heart rate compared with tiotropium over the 6-h study period (aclidinium heart rate increase AUC0–6 h = 108% compared with 341% for tiotropium). The therapeutic index, calculated as AUC0–6 h bronchoconstriction inhibition (%)/AUC0–6 h heart rate increase (%), was 4.2 for aclidinium and 1.6 for tiotropium.

Discussion

The aim of this study was to establish the pharmacological profile of aclidinium bromide, a novel and long-acting inhaled
muscarinic antagonist in development for maintenance treatment of COPD.

In radioligand binding displacement studies carried out at equilibrium, aclidinium like tiotropium, demonstrated sub-nanomolar affinity and no selectivity for the five human muscarinic receptor subtypes analyzed. The affinity of ipratropium is in the low nanomolar range for M₁ to M₅ receptors. The data obtained for ipratropium and tiotropium are consistent with those reported previously (Haddad et al., 1994).

The kinetics of the binding of the three radiolabeled compounds to the M₂ and M₃ receptor subtypes were assessed. The interaction of the compounds with the M₂ receptors is interesting beyond its potential implication on their efficacy because inhibition of cardiac M₂ receptors is known to induce tachycardia, which is potentially the most severe side effect associated with systemic antimuscarinic agents (Eglen, 2005). The M₃ 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 $K_{\text{off}}$ 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 aclidinium at the M₃ receptor (29.24 h) suggests a long duration of action in vivo.

All three radiolabeled antagonists had a faster dissociation from the M₂ receptor than the M₃ receptor, conferring a certain kinetic selectivity for the M₃ versus the M₂ receptor subtype. The residence half-life at the M₃ is 4- to 6-fold longer than that at M₂ 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 M₂ receptors was not measurable under the experimental conditions used due to the rapidity of antagonist association with this receptor. Association to the M₃ receptors was slower, allowing for quantitative determination, and was found to be similar for aclidinium and ipratropium. The association of tiotropium to the M₃ receptors was 2.6-fold slower than aclidinium with respect to $K_{\text{on}}$ values.

Using a different experimental approach, previous studies have reported $K_{\text{on}}$ values for ipratropium and tiotropium in the same range as those reported here (Dowling and Charlton, 2006).
The bronchoconstrictor response to muscarinic agonists in isolated lung tissue is mainly mediated via M₃ receptors in airway smooth muscle, as demonstrated in tracheal smooth muscle preparations derived from M₃ knockout mice (Wess et al., 2007). In the present study, the potency of aclidinium in isolated guinea pig trachea stimulated with carbachol was not significantly different from that of ipratropium and tiotropium. The pA₂ 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 pIC₅₀ 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, aclidinium and ipratropium demonstrated competitive antagonism. However, the antagonism produced by tiotropium was non-surmountable, 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).

Aclidinium 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. Aclidinium demonstrated a long duration of action comparable with that of tiotropium in these studies. Unlike aclidinium, 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 IC₅₀ of aclidinium was two and three times greater than ipratropium and tiotropium, respectively, although the differences were not statistically significant. Differences between the aclidinium IC₅₀ values observed from in vitro tracheal assays and in vivo animal models may be explained by the high susceptibility of aclidinium to hydrolysis by esterases. Aclidinium is hydrolyzed significantly faster in human plasma compared with ipratropium and tiotropium, with a half-life in human plasma of 2
min for aclidinium and greater than 60 min for reference compounds (Gavalda et al., 2007). Similar hydrolysis rates have been observed in guinea pig and dog plasma (Gavalda et al., 2008). The onset of action of aclidinium 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 M2 receptors and isolated guinea pig trachea.

Aclidinium also demonstrated a long duration of action in anesthetized guinea pigs (t1/2 = 29 h), which was approximately 4-fold that of ipratropium (t1/2 = 8 h) and less than that of tiotropium (t1/2 = 64 h). Moreover, in anesthetized dogs, aclidinium 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 [3H]aclidinium (t1/2 = 29 h) at the human muscarinic M2 receptor and the in vitro guinea pig studies reported in this article. Altogether, these results suggest that aclidinium has long-lasting effects in preclinical models. The therapeutic index in dogs was investigated to assess whether the rapid plasma hydrolysis of aclidinium (Gavalda et al., 2007) and a short M2 receptor residence time translate into an improved efficacy-safety ratio compared with tiotropium.

In conscious dogs, inhaled aclidinium had a reduced effect on heart rate compared with tiotropium at doses that were 100-fold those needed to produce equivalent antibronchoprotective effects in the same species. Under these experimental conditions, aclidinium 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 aclidinium has reduced potential for cardiovascular side effects compared with tiotropium (Gras et al., 2008). Aclidinium is rapidly hydrolyzed in plasma (Gavalda et al., 2008) and has an in vitro K_{off} value for human M2 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 aclidinium on heart rate in dogs compared with tiotropium.

Despite the kinetic selectivity (M3 > M2) profile described for tiotropium, its slow dissociation from the M3 receptor was also accompanied by an increase in the residence time on the M2 receptor (Disse et al., 1993). Its longer residence time at the M2 receptor in comparison with aclidinium 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 aclidinium (Gavalda 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 aclidinium 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 aclidinium in guinea pig in vitro and in vivo functional models.

Furthermore, aclidinium has a good cardiovascular safety profile, which may be attributed to its reduced residence time at M2 receptors and rapid hydrolysis in plasma. Together, these data suggest that aclidinium may have a favorable benefit-to-risk ratio in the clinical setting, therefore providing a valuable treatment option for patients with COPD.

Acknowledgments

We would like to acknowledge the significant technical support of Rosario Cerrato, Manel De Luca, Joan Mañé, Maria Rosa Ortiz, Isabel Pagán, and Núria Torán (Almirall); Max Qian and José Freire (Forest Research Institute, Jersey City, NJ) for review and excellent suggestions on the manuscript; and Dr. Stuart Grant (Complete Medical Communications, Cheshire, UK), who provided medical writing assistance.

References


Address correspondence to: Dr Jorge Beleta, Almirall, S.A., Laureu Miro, 408-410, 08980 Sant Feliu de Llobregat, Barcelona, Spain. E-mail: jorge.beleta@almirall.com