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The *in vitro* and *in vivo* profile of aclidinium bromide in comparison with glycopyrronium bromide

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ABSTRACT

This study characterised the *in vitro* and *in vivo* profiles of two novel long-acting muscarinic antagonists, aclidinium bromide and glycopyrronium bromide, using tiotropium bromide and ipratropium bromide as comparators. All four antagonists had high affinity for the five muscarinic receptor sub-types (M_1–M_5); aclidinium had comparable affinity to tiotropium but higher affinity than glycopyrronium and ipratropium for all receptors. Glycopyrronium dissociated faster from recombinant M_3 receptors than aclidinium and tiotropium but more slowly than ipratropium; all four compounds dissociated more rapidly from M_2 receptors than from M_3 receptors. *In vitro*, aclidinium, glycopyrronium and tiotropium had a long duration of action at native M_3 receptors (>8 h versus 42 min for ipratropium). *In vivo*, all compounds were equi-potent at reversing acetylcholine-induced bronchoconstriction. Aclidinium, glycopyrronium and ipratropium had a faster onset of bronchodilator action than tiotropium. Aclidinium had a longer duration of action than glycopyronnium (time to 50% recovery of effect [$t_{½}$ offset] = 29 h and 13 h, respectively); these compare with a $t_{½}$ offset of 64 h and 8 h for tiotropium and ipratropium, respectively. Aclidinium was less potent than glycopyrronium and tiotropium at inhibiting salivation in conscious rats (dose required to produce half-maximal effect [ED_{50}] = 38, 0.74 and 0.88 μg/kg, respectively) and was more rapidly hydrolysed in rat, guinea pig and human plasma compared with glycopyrronium or tiotropium. These results indicate that while aclidinium and glycopyrronium are both potent antagonists at muscarinic receptors with similar kinetic selectivity for M_3 receptors versus M_2, aclidinium has a longer dissociation half-life at M_3 receptors and a longer duration of bronchodilator action *in vivo* than glycopyrronium. The rapid plasma hydrolysis of aclidinium, coupled to its kinetic selectivity, may confer a reduced propensity for systemic anticholinergic side effects with aclidinium versus glycopyrronium and tiotropium.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by persistent airflow limitation, and an enhanced chronic inflammatory response in airways and lung to noxious particles or gases [1]. Characteristic symptoms of COPD include airway limitation and chronic coughing due to mucus hypersecretion [1]. Acetylcholine is the primary parasympathetic neurotransmitter in the airways [2] and plays an important role in regulating both airway smooth muscle tone [3] and mucus secretion [4,5] via stimulation of airway muscarinic receptors. The primary reversible component of airway limitation is sensitive to muscarinic receptor antagonists [2,6]. Of the five muscarinic receptors identified to date (M_1–M_5), only the M_1–M_3 subtypes are found in the airways [7]. The M_3 receptor

Abbreviations: COPD, chronic obstructive pulmonary disease; EC_{50}, concentration required to produce 50% effect; ED_{50}, dose required to produce 50% effect; K_d, equilibrium dissociation constant; K_i, antagonist dissociation constant; LAMA, long-acting muscarinic antagonist; M_1–M_5, muscarinic receptor subtypes 1–5; [^{3}H]-NMS, 1-[N-*methyl*-^{3}H] scopolamine methyl chloride; Raw, airway resistance; SAMA, short-acting muscarinic antagonist; $t_{½}$, dissociation half-life/hydrolysis half-life; $t_{½}$ offset, time to 50% recovery of effect; t_{max}, time to maximal effect.

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mediates acetylcholine-induced contraction of airway smooth muscle [8,9], and stimulation of M_1 and M_3 receptors on submucosal mucus glands promotes mucus secretions in airways [5,10]. By contrast, M_2 receptors are presynaptic autoreceptors which serve as a negative feedback mechanism to modulate acetylcholine release from parasympathetic nerves [7].

As a consequence of the central role of muscarinic receptors in mediating the underlying pathophysiology of COPD, anticholinergics, specifically muscarinic receptor antagonists, are recommended as a first-line bronchodilator treatment option in patients with COPD [1,11]. Short-acting muscarinic antagonists (SAMAs), such as ipratropium bromide, are recommended for use in Group-A patients who are characterised as having few symptoms and a low risk of exacerbation [1]. By contrast, long-acting muscarinic antagonists (LAMAs), such as aclidinium bromide, glycopyrronium bromide and tiotropium bromide, are preferred for maintenance treatment in patients with more severe airflow limitation, more symptoms or a higher risk of exacerbation (Groups C–D) [1]. However, ipratropium and tiotropium, which have been available for many years, are associated with systemic side effects typical of the anticholinergic class of compounds, such as dry mouth [12,13] and an increased risk of cardiovascular side effects [14–16].

In 2012, two new LAMAs, aclidinium and glycopyrronium, were approved in Europe for maintenance bronchodilator treatment in adult patients with COPD [17,18]; aclidinium has also been approved in the US [19]. In preclinical studies, both aclidinium [20] and glycopyrronium [21] had high affinity for all five muscarinic receptors. Aclidinium was also shown to be rapidly hydrolysed in human plasma to two inactive metabolites [22], suggesting a reduced potential for systemic anticholinergic effects with aclidinium.

Here we compare the *in vitro* pharmacology of aclidinium and glycopyrronium at muscarinic receptors with that of tiotropium and ipratropium. The potency, onset of action and duration of action of each antagonist in *in vitro* and *in vivo* bronchoconstriction models were also assessed. Additional studies were conducted to investigate the potential of all four antagonists to cause systemic side effects in the rat pilocarpine-induced sialorrhea model. Finally, the stability of the four antagonists in guinea pig, rat and human plasma was compared.

2. Materials and methods

2.1. Chemicals and reagents

Aclidinium, glycopyrronium and tiotropium were synthesised by the Department of Medicinal Chemistry (Almirall R&D Centre, Barcelona, Spain). Acetylcholine hydrochloride, atropine sulfate, carbachol chloride, ipratropium and pilocarpine were obtained from Sigma–Aldrich (Madrid, Spain); ketamine chlorhydrate (Imalgene) was from Merial (Barcelona, Spain); xylazine (Rompun 2%) from Bayer (Barcelona, Spain); acepromazine maleate (Calmoneosan) from Pfizer Salud Animal (Alcobendas, Spain); propofol (Lipuro) from B. Braun (Rubí, Spain); acetonitrile from Scharlau (Barcelona, Spain); Milli-Q water from Millipore S.A. (Madrid, Spain); and formic acid, ammonia and hydrochloric acid from Merck (Madrid, Spain).

Membrane preparations expressing recombinant human M_1, M_2, M_3, M_4 and M_5 receptors (prepared from transfected CHO-K1 cells) were obtained from Membrane Target Systems (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). 1-[N-*methyl*-^{3}H] scopolamine methyl chloride ([^{3}H]-NMS) was obtained from Perkin Elmer Life and Analytical Sciences; [^{3}H]aclidinium (2.89 TBq/mmol), [^{3}H]glycopyrronium (2.59 TBq/mmol), [^{3}H]tiotropium (3.11 TBq/mmol) and [^{3}H]ipratropium (2.70 TBq/mmol) were custom synthesised by GE Healthcare UK Ltd (Slough, UK).

All equilibrium binding studies were performed in 96-well plates (NUNC; Thermo Fischer Scientific, Roskilde, Denmark). All assay reagents were dissolved in assay buffer (TRIS 25 mM pH: 7.4) [Sigma–Aldrich, Tres Cantos, Spain]) and test compounds were dissolved in dimethyl sulfoxide. Aclidinium was prepared in 0.2% HCl/20% polyethylene glycol for use in *in vitro* organ bath experiments and *in vivo* studies; carbachol, ipratropium, glycopyrronium and tiotropium were dissolved in distilled water. Krebs-Henseleit solution was composed of: NaCl 118 nM, KCl 4.7 nM, $CaCl_2$ 2.52 nM, $MgSO_4$ 1.2 nM, $NaHCO_3$ 24.9 nM, KH_2PO_4 1.18 nM, glucose 5.55 nM and sodium pyruvate 2 nM. In plasma stability studies, stock solutions (1 mg/mL) of aclidinium, glycopyrronium, tiotropium and ipratropium were prepared in 20:80, v/v 0.1 N HCl/acetonitrile; working solutions were dissolved in Milli-Q water. Rat plasma was obtained from RCC Cida (Barcelona, Spain).

2.2. Animals

Male Dunkin-Hartley guinea pigs (400–600 g) 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 and fed a maintenance diet for guinea pigs, supplemented with vitamin C (SAFE114, SAFE, France); water was *ad libitum*. Guinea pigs were allowed to acclimatise for a minimum of 5 days prior to experimental procedures. Male Wistar rats (180–260 g) were also obtained from Harlan. Rats were housed at 20–24 °C under a 12-h light/dark cycle. Standard chow and water were available *ad libitum*. All experiments were approved and monitored by the Animal Ethical Committee of Almirall (Barcelona, Spain) and in accordance with EU Directive 2010/63/EU for animal experiments.

2.3. Radioligand binding studies

2.3.1. Affinity for the human M_1 to M_5 muscarinic receptors

The affinity (equilibrium antagonist dissociation constant [K_i] values) of muscarinic antagonists at recombinant human muscarinic M_1–M_5 receptors was determined as described previously [20]. Briefly, human M_1, M_2, M_3, M_4 and M_5 receptor membrane preparations (protein concentrations 8.1, 10.0, 4.9, 4.5 and 5.0 μg/well, respectively) were incubated at room temperature for 2 or 6 h (M_1–M_4 and M_5, respectively) with [^{3}H]-NMS concentrations approximately equal to the radioligand equilibrium dissociation constant (K_d) for each receptor subtype (0.3 nM for M_1 and M_4; and 1 nM for M_2, M_3 and M_5). Non-specific binding to membranes was determined in the presence of atropine 1 μM. Antagonist concentrations (10^{-5} to 10^{-14} M) were tested in duplicate. Incubation times were selected to ensure equilibrium binding was achieved. Bound and free [^{3}H]-NMS were separated by rapid vacuum filtration of GF/C filter plates (Millipore, Barcelona, Spain), and radioactivity was quantified using a MicroBeta Trilux microplate scintillation counter (Perkin Elmer Life and Analytical Sciences). K_i values were calculated as described by Cheng and Prusoff for competitive inhibitors [23]. All binding studies were performed in non-physiological assay binding buffer containing 25 mM TRIS pH: 7.4.

2.3.2. Dissociation from M_2 and M_3 muscarinic receptors

Dissociation of radiolabelled muscarinic antagonists was assessed as described previously [20]. Association of radioligands, with approximately 90% binding-site occupancy, was achieved by incubating membranes expressing human M_2 and M_3 receptors (final protein concentration 15 μg/mL) at room temperature with

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[^{3}H]aclidinium (2.5 nM), [^{3}H]glycopyrronium (15 and 5 nM for M_2 and M_3 receptors, respectively), [^{3}H]tiotropium (2.5 nM) or [^{3}H] ipratropium (10 nM) for 135 min. Dissociation from the receptor was initiated by the addition of atropine 10 μM (final concentration). The amount of bound radioligand remaining over time was assessed by separating bound and free radioligand as described in section 2.3.1. Dissociation half-lives ($t_{½}$) were calculated using one-phase exponential decay. All binding studies were performed in non-physiological assay binding buffer containing 25 mM TRIS pH: 7.4.

2.4. In vitro potency and duration of action at native M_2 and M_3 muscarinic receptors

2.4.1. M_2 receptors

Potency and duration of action at M_2 receptors were assessed in the isolated guinea pig left-atria preparation. Briefly, left atria (n = 3–13) were dissected and suspended in an organ bath containing oxygenated Krebs-Henseleit solution at 32 °C. Isolated tissues were connected to a force transducer (Letica TRI201, Barcelona, Spain) and isometric changes recorded using PowerLab software (AD instruments, Panlab, Barcelona, Spain). A stable resting tone was achieved by applying a pre-load of 1 g prior to electrical stimulation (1 Hz, 8 V 5 ms); baseline contractions were assessed during a 60-min stabilization period. Inhibition of electrically induced contractions via the M_2 receptor was achieved by the addition of carbachol 1 μM. Increasing cumulative concentrations of antagonists (0.01–1000 nM) were added every 5–10 min to assess the potency of each compound to reverse carbachol-induced relaxation of electrically stimulated contractions. The EC_{50} (concentration required to produce 50% inhibition of the maximum carbachol-induced relaxation) was determined for each compound using non-linear regression.

Duration of action was assessed as time to 50% recovery of the maximum carbachol-induced relaxation ($t_{½}$ offset). Following the addition of carbachol 10 μM, antagonists were added at a concentration that inhibited 80% of the maximum carbachol-induced relaxation; inhibition of tone was then allowed to stabilise for 20–30 min. The antagonists were washed out and the atria re-incubated with carbachol 10 μM for 4 h. The $t_{½}$ offset was calculated using one-phase (aclidinium, glycopyrronium and tiotropium) or two-phase (ipratropium) exponential decay.

2.4.2. M_3 receptors

The potency and duration of action of antagonists at M_3 receptors were assessed in the isolated guinea pig trachea preparation, as described previously [20,24]. Briefly, trachea were excised and mounted in a superfusion chamber containing oxygenated Krebs-Henseleit solution, supplemented with propranolol 1 μM at 37 °C. Trachea strips (n = 3–13) were connected to a force transducer and isometric changes recorded as in Section 2.4.1. A pre-load of 1 g was applied to obtain a stable resting tone prior to the induction of M_3 receptor-mediated contractions (10 s trains of square wave pulses of 5 Hz and 0.1 ms every 2 min). Baseline was established by stimulating trachea strips for ≥20 min at a voltage of 10–15% above that required for a maximal response. Increasing concentrations of antagonists (0.01–1000 nM) were then added every 30 min to assess the potency of each compound to inhibit electrically stimulated contractions. A cumulative concentration–response curve for inhibition of electrically stimulated contractions was constructed and the EC_{50} determined using non-linear regression.

Duration of action at M_3 receptors was assessed as $t_{½}$ offset (time to 50% recovery of electrically-stimulated contractions). Tissues were incubated for 45 min in antagonist solution, at a concentration that produced sub-maximal (80–90%) inhibition of electrically stimulated contractions. Antagonists were then washed out and $t_{½}$ offset calculated using non-linear regression analysis.

2.5. In vivo potency, onset of action and duration of action in anaesthetised guinea pigs

The *in vivo* potency, onset and duration of bronchodilation were assessed in an anaesthetised guinea pig bronchoconstriction model. Conscious guinea pigs were placed in a methacrylate box and exposed to a nebulised aerosol of antagonist solution. Antagonists were administered for 1 min at a flow rate of 3 L/min and animals were allowed to breathe freely for a 5-min period. This procedure was then repeated. Muscarinic antagonists (1–1000 μg/mL) or vehicle were administered to guinea pigs (n = 4–9 by dose and time point) as nebulised aerosols via an ultrasonic nebuliser (DeVilbiss UltraNeb 2000; Sunrise Medical, Somerset, PA). This nebulisation was driven by a mixture of 5% CO_2, 21% O_2, 74% N_2 at a flow of 3L/min as previously described [20]. At various time points (1, 2, 4, 6, 18 and 24 h) after antagonist exposure, guinea pigs were anaesthetised with an intramuscular injection of ketamine (43.8 mg/kg), xylazine (3.5 mg/kg) and acrepromazine (1.1 mg/kg); additional anaesthetic was administered as necessary. Animals were adequately anesthetised during surgical procedure and during all study time points. Airflow, transpulmonary pressure and blood pressure were monitored throughout the procedure as previously described [20]. During experiments, guinea pigs were artificially ventilated as previously described [20]; body temperature was maintained at 37 °C with a homeothermic blanket. Pulmonary airway resistance (Raw) was assessed as a measure of bronchoconstriction. Raw was calculated as the quotient of the changes in flow and pressure between isovolumetric points on inspiration and expiration. Measurements were initiated once baseline Raw values were in the range 0.1–0.2 cM H_2O/mL per s.

Bronchoconstriction was induced by intravenous administration of a single bolus dose of acetylcholine (30 μg/kg), and the inhibitory effect of each antagonist was assessed relative to vehicle. Potency was defined as the concentration required to produce 50% inhibition of acetylcholine-induced bronchoconstriction (EC_{50}), determined from a sigmoidal dose–response curve constructed using the inhibition values at each of the time points studied. Onset of action for each compound was defined as the time to maximal inhibition of bronchoconstriction (t_{max}) taken from EC_{50} values. The duration of action, defined as the time to 50% recovery of the maximal inhibitory effect achieved by the antagonist ($t_{½}$ offset), was derived from time-course bronchoconstriction inhibition curves using one-phase exponential decay.

2.6. Salivation in conscious rats

The effect of aclidinium, glycopyrronium and tiotropium on salivation in conscious rats was assessed as follows: rats (n = 6–24) were fasted for 18 h (with water *ad libitum*) prior to administration of aclidinium (0.1–1000 μg/kg), glycopyrronium (0.1–10 μg/kg), tiotropium (0.1–100 μg/kg) or vehicle subcutaneously in the intercapular area. After 30 min, pilocarpine (0.5 mg/kg) was administered via the caudal vein. The presence of any sialorrhea (excess saliva) was recorded during the first 15-min post-pilocarpine administration by gently pressing filter paper on the animal's snout. Animals were considered positive for sialorrhea if the filter paper was spotted. The proportions of animals showing salivation following antagonist treatment were compared with vehicle-treated animals using Fisher's exact test. The ED_{50} values (dose required to inhibit pilocarpine-induced salivation in 50% of

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Table 1
Binding affinity of aclidinium, glycopyrronium, tiotropium and ipratropium for human M_1, M_2, M_3, M_4 and M_5 receptors.

	K_i (nM)				
	M_1	M_2	M_3	M_4	M_5
Aclidinium	0.10 ± 0.00	0.14 ± 0.04	0.14 ± 0.02	0.21 ± 0.04	0.16 ± 0.01
Glycopyrronium	0.42 ± 0.02	1.77 ± 0.06	0.52 ± 0.04	0.78 ± 0.04	1.29 ± 0.09
Tiotropium	0.13 ± 0.00	0.13 ± 0.04	0.19 ± 0.04	0.30 ± 0.09	0.18 ± 0.06
Ipratropium	1.31 ± 0.15	1.12 ± 0.13	1.24 ± 0.08	1.92 ± 0.18	3.22 ± 0.15

Data are reported as mean ± standard error of the mean of three independent experiments.
K_i, antagonist dissociation constant.

rats) were calculated by non-linear regression (sigmoidal dose–response curve fit).

2.7. In vitro rat, guinea pig and human plasma stability

In vitro plasma stability was assessed as described previously [22]. Briefly, guinea pig ($n = 10$) plasma samples were prepared using sodium heparin as anticoagulant (25 units/mL; 2000 × *g* at 4 °C). Plasma samples from human volunteers ($n = 6$) were obtained in a similar manner. Rat plasma was commercially available.

Triplicate plasma samples were pre-incubated at 37 °C for 5 min prior to the addition of aclidinium, glycopyrronium, tiotropium or ipratropium at a final concentration of 83 nM, 126 nM, 102 nM and 120 nM, respectively (40 ng/mL, expressed as cation) to initiate the reaction. Following incubation for predetermined time points up to 1 h, 100 μL aliquots of each reaction were combined with 300 μL ice-cold acetonitrile:1 N HCl (90/10, v/v). Samples were centrifuged at 2500 × *g* for approximately 10 min at 4 °C. Control plasma incubations in the absence of antagonist were also performed. Samples were analysed by ultra performance liquid chromatography (Acquity Ultra Performance LC, Waters, Milford, MA, USA) with mass spectrometry detection (Quattro Premier, Micromass Technologies, Waters). For each time point, the percentage of remaining unaltered compound was calculated. The dissociation half-life ($t_{½}$) in plasma was calculated using WinNonlin software (version 5.0.1., Pharsight Corporation, USA).

3. Results

3.1. Determination of affinity of muscarinic antagonist for the human M_1 to M_5 muscarinic receptors

The affinity of aclidinium, glycopyrronium, tiotropium and ipratropium for the human M_1–M_5 receptors was assessed in competitive binding experiments using membranes from transfected CHO-K1 cells, stably expressing each of the recombinant receptors. Prior to determining ligand affinity, the amount of drug required to saturate a population of receptors and the K_d value for each receptor were established in saturation (equilibrium binding) experiments using [^{3}H]-NMS; these data have been reported previously [20]. All of the antagonists tested inhibited the specific binding of [^{3}H]-NMS to human M_1–M_5 receptors in a concentration-dependent manner. Aclidinium and tiotropium had comparable affinity for all of the receptor subtypes and higher affinity compared with glycopyrronium and ipratropium (Table 1). The affinity of glycopyrronium was 4- to 13-fold lower than that of aclidinium across the M_1–M_5 receptors (Table 1). Ipratropium had the lowest affinity for all receptor subtypes with the exception of M_2 (Table 1). Glycopyrronium was the only antagonist that exhibited some degree of preference in terms of affinity for M_3 versus M_2 receptors (approximately 3-fold; Table 1).

3.2. Dissociation from human M_2 and M_3 muscarinic receptors

The dissociation half-lives of [^{3}H]aclidinium, [^{3}H]glycopyrronium, [^{3}H]tiotropium and [^{3}H]ipratropium were determined (Table 2). [^{3}H]aclidinium dissociated more slowly from both M_2 and M_3 receptors than [^{3}H]glycopyrronium; [^{3}H]tiotropium dissociated the most slowly from both receptors (approximately 2- to 3-fold slower than [^{3}H]aclidinium) and [^{3}H]ipratropium dissociated most rapidly (Fig. 1a and b; Table 2). All the antagonists displayed a similar magnitude of kinetic selectivity for M_3 over M_2 receptors (as determined by the $t_{½}$ M_3/M_2 ratio; Table 2).

3.3. Potency and duration of action at endogenous M_2 and M_3 muscarinic receptors

3.3.1. M_2 receptors

To evaluate the potency and duration of action at endogenous M_2 receptors, the ability of muscarinic antagonists to inhibit the effects of carbachol in electrically stimulated guinea pig left-atria preparations was assessed. Each of the antagonists reversed carbachol-mediated inhibition of electrically stimulated contractions in a concentration-dependent manner. Tiotropium displayed the greatest potency at endogenous M_2 receptors, whereas the potencies of aclidinium, glycopyrronium and ipratropium were comparable (Table 3). Glycopyrronium had a $t_{½}$ offset time approximately 3-fold shorter than that of aclidinium and 6-fold shorter than that of tiotropium. By contrast, the $t_{½}$ offset of glycopyrronium was 8-fold longer than that of ipratropium (Table 3).

3.3.2. M_3 receptors

The ability of antagonists to inhibit cholinergic tone in isolated guinea pig trachea was investigated to determine the potency and duration of action of each compound at endogenous M_3 receptors. All four antagonists exhibited comparable, low nanomolar, potency (3.0–5.3 nM) at endogenous M_3 receptors (Table 3). The duration of action of the three LAMAs at M_3 receptors was comparable, whereas the duration of action of the SAMA, ipratropium, was much shorter (>480 min versus 42 min, respectively; Table 3).

3.4. In vivo onset of action, potency and duration of action in anaesthetised guinea pigs

All of the antagonists produced concentration-dependent inhibition of acetylcholine-induced bronchoconstriction *in vivo*; maximal inhibition was 97–99% with all four antagonists. With regard to onset of action, aclidinium, glycopyrronium and ipratropium achieved t_{max} 2 h post-administration compared with 4 h with tiotropium. At the onset of action for each compound, EC_{50} values were comparable across all four antagonists, ranging from 1.4 to 3.8 μg/mL (Table 4).

Table 2
Dissociation half-lives of [^{3}H]aclidinium, [^{3}H]glycopyrronium, [^{3}H]tiotropium and [^{3}H]ipratropium from human M_2 and M_3 receptors.

	M_2 $t_{½}$ (h)	M_3 $t_{½}$ (h)	Relative half-life at M_3 receptor[a]	$t_{½}$ M_3/M_2 ratio
Aclidinium	4.69 ± 0.29	29.24 ± 0.61	62	6.2
Glycopyrronium	1.07 ± 0.20	8.10 ± 0.45	17	7.3
Tiotropium	15.11 ± 1.57	62.19 ± 2.96	132	4.1
Ipratropium	0.08 ± 0.01	0.47 ± 0.02	1	5.9

Data are reported as mean ± standard error of the mean from three independent experiments.
$t_{½}$, dissociation half-life.
[a] Half-lives expressed relative to [^{3}H]ipratropium.

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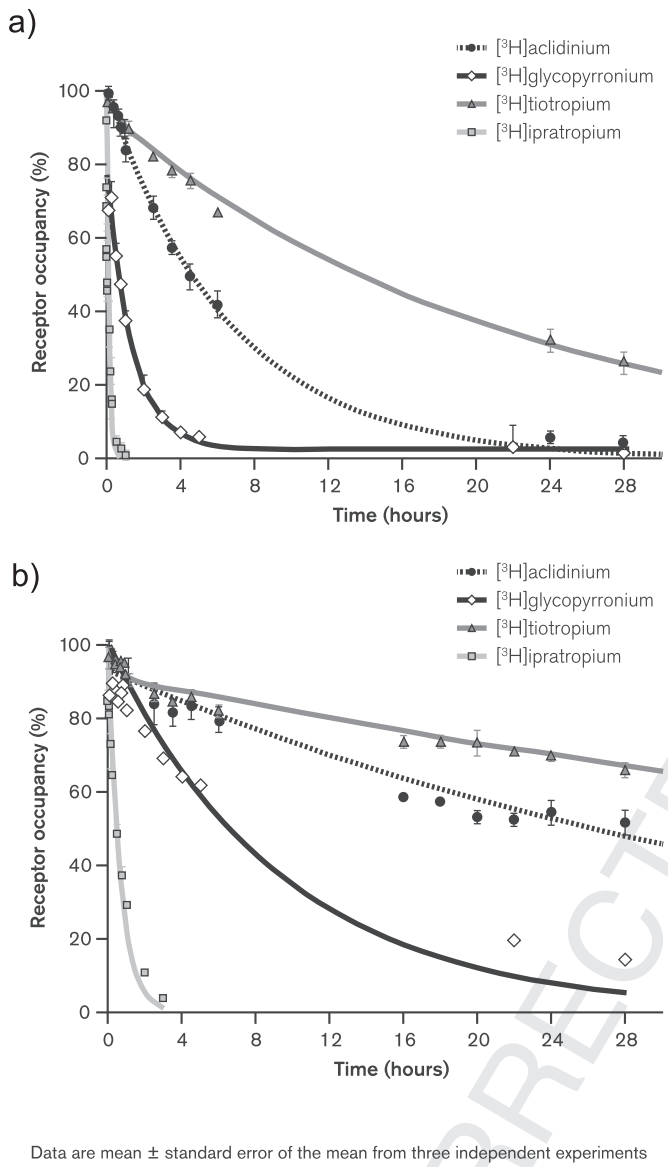

Fig. 1. Dissociation of [^{3}H]aclidinium, [^{3}H]glycopyrronium, [^{3}H]tiotropium and [^{3}H]ipratropium from (a) human M_2 receptors, and (b) human M_3 receptors.

The duration of bronchodilator action was assessed using a single concentration of each inhaled antagonist (aclidinium 100 μg/mL, glycopyrronium 100 μg/mL, tiotropium 10 μg/mL and ipratropium 30 μg/mL). At 1 h post-administration, all of the antagonists produced equi-effective (97–98%) inhibition of acetylcholine-induced bronchoconstriction at the selected doses (Fig. 2). The *in vivo* duration of bronchodilator action of aclidinium was more than 2-fold that of glycopyrronium ($t_{½}$ offset = 29 h versus 13 h, respectively; Fig. 2). Tiotropium had the longest duration of action ($t_{½}$ offset = 64 h) and ipratropium the shortest ($t_{½}$ offset = 8 h) (Fig. 2).

Table 3
Potency and duration of action of aclidinium, glycopyrronium, tiotropium and ipratropium at native M_2 receptors (isolated guinea pig left atria) and M_3 receptors (isolated guinea pig trachea).

	M_2 receptors		M_3 receptors	
	EC_{50} (nM)[a]	$t_{1/2}$ offset (min)	EC_{50} (nM)[a]	$t_{1/2}$ offset (min)
Aclidinium	17.4 ± 1.1	102	5.3 ± 1.6	>480
Glycopyrronium	17.3 ± 1.2	30	4.2 ± 0.3	>480
Tiotropium	11.8 ± 1.1	184	3.0 ± 0.6	>480
Ipratropium	19.9 ± 1.1	4	3.0 ± 0.4	42

EC_{50}, concentration required to produce 50% inhibition of the maximum carbachol-induced relaxation (M_2) or 50% inhibition of electrically stimulated contractions (M_3); $t_{1/2}$ offset, time to 50% recovery of the maximum carbachol-induced relaxation (M_2) or to 50% recovery of electrically-stimulated contractions (M_3).
[a] Data reported as mean ± standard error of the mean; n = 3–13.

3.5. Salivation in conscious rats

The ability of aclidinium, glycopyrronium and tiotropium to inhibit salivation was assessed in the rat pilocarpine-induced sialorrhea model. All three compounds inhibited sialorrhea in a dose-dependent manner (Fig. 3). However, the dose–response curve for aclidinium demonstrated a rightward shift compared with that of tiotropium. Consistent with this, the dose of aclidinium required to produce a 50% inhibition of pilocarpine-induced salivation (ED_{50}) was 43–51-fold lower than that for tiotropium and glycopyrronium (ED_{50} [μg/kg] = 38, 0.88 and 0.74 for aclidinium, tiotropium and glycopyrronium respectively; Fig. 3).

3.6. In vitro guinea pig and rat plasma stability

Fig. 4 shows the stability of all four antagonists in rat (Fig. 4a), guinea pig (Fig. 4b) and human (Fig. 4c) plasma. Plasma stability data for aclidinium, tiotropium and ipratropium in rat and guinea pig have been reported previously [22]. In rat plasma, aclidinium was rapidly hydrolysed with $t_{½}$ = 0.19 h (Table 5) whereas glycopyrronium was hydrolysed more slowly ($t_{½}$ = 12 h; Table 5). Tiotropium was hydrolysed more slowly than aclidinium, but more quickly than either glycopyrronium or ipratropium. Aclidinium was more stable in guinea pig plasma compared with rat (Table 5), whereas glycopyrronium was less stable. Aclidinium was least stable in human plasma ($t_{½}$ = 0.04 h), whereas the stability of the other three antagonists in human plasma was intermediate to that observed in rat and guinea pig plasma. The rank order of plasma stability was the same in all three species, with aclidinium < tiotropium < glycopyrronium < ipratropium (Table 5, Fig. 4a–c).

4. Discussion

The use of a LAMA in the maintenance treatment of stable COPD is well established [1]. However, until recently, tiotropium was the only LAMA approved for the treatment of COPD. The recent approval of aclidinium and glycopyrronium for use as maintenance bronchodilator treatment in patients with COPD expands the therapeutic options for these patients. Here, we compared the *in vitro* and *in vivo* profiles of aclidinium and glycopyrronium with those of tiotropium and the SAMA, ipratropium. Our results demonstrate that while all four muscarinic receptor antagonists have high affinity for M_1 to M_5 receptors and demonstrate similar kinetic selectivity for M_3 versus M_2 receptors, they have unique profiles with respect to dissociation from the therapeutic target (M_3 receptors), and *in vitro* and *in vivo* onset and duration of action. Furthermore, their propensity to inhibit salivation in a rodent model varies, which may be related, in part, to differences in the plasma stability of each compound.

Aclidinium exhibited sub-nanomolar affinity for all five receptor subtypes with no selectivity in terms of binding affinity at any of the receptors. Consistent with previous reports [21,25], glycopyrronium also had high affinity for each of the five receptor subtypes; however, its affinity was 4- to 13-fold lower than that of aclidinium across the receptors. Whilst glycopyrronium exhibited some degree of selectivity for M_3 versus M_2 receptors

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Table 4
Onset of action and potency of aclidinium, glycopyrronium, tiotropium and ipratropium in reversing acetylcholine-induced bronchoconstriction in guinea pigs ($n = 4–9$ by dose and time point).

	Onset time (h)	EC_{50}, μg/mL (95% CI)				
		1 h	2 h	4 h	18 h	24 h
Aclidinium	2	5.9 (3.7, 9.4)	2.5 (1.7, 3.5)	2.9 (1.8, 4.7)	12.4 (4.1, 37.6)	23.1 (9.3, 57.3)
Glycopyrronium	2	7.2 (4.1, 12.8)	3.8 (2.5, 5.7)	8.8 (5.2, 14.8)	68.7 (39.6, 119.2)	242.3 (162.0, 362.2)
Tiotropium	4	2.4 (1.4, 3.8)	3.9 (2.0, 7.6)	1.4 (0.7, 2.5)	1.4 (0.7, 2.9)	3.3 (2.0, 5.2)
Ipratropium	2	6.9 (4.0, 11.7)	3.4 (1.9, 5.9)	7.3 (4.0, 13.4)	689.7 (337.1, 1411.0)	NA

CI, confidence interval; Onset time, time to maximal inhibition of bronchoconstriction (h); EC_{50}, concentration required to produce 50% inhibition of bronchoconstriction induced by acetylcholine (30 μg/kg i.v.); h, hour; i.v., intravenous; NA, not available.

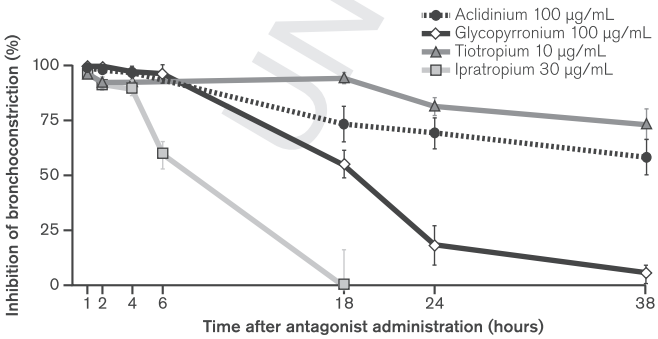
(approximately 3-fold), binding affinities at each receptor were still in the low nanomolar range. As previously reported, the affinity of aclidinium for M_1 to M_5 receptors was comparable to that of tiotropium; ipratropium was the least potent of the four compounds overall [20,21].

The difference in duration of action of LAMAs and SAMAs is thought to be primarily due to their longer residence times at human M_3 receptors [21,26]. Aclidinium, glycopyrronium and tiotropium have been reported to have a residence half-life at recombinant human M_3 receptors of between 6.1 h and 62.2 h (compared with 13.2–28.2 min for ipratropium) [20,21,26], making them suitable for once- or twice-daily dosing in the clinical setting compared with four times a day for ipratropium. In this study, the residence half-life of aclidinium at human M_3 receptors was approximately four times longer than that of glycopyrronium. Tiotropium had the longest residency time, consistent with its use as a once-daily treatment [27,28]. By contrast, ipratropium had a dissociation half-life at M_3 receptors of <1 h. The longer residency time of aclidinium versus glycopyrronium at M_3 receptors suggests a longer duration of action *in vivo* for aclidinium than glycopyrronium. Interestingly, in the clinical setting, aclidinium is administered twice daily [29,30] versus once daily for glycopyrronium [31–33]. A recent study by Sykes et al., comparing glycopyrronium and tiotropium, demonstrated that receptor-binding properties, including affinity and dissociation rates, can be overestimated under non-physiological assay conditions suggesting that other factors, in particular drug rebinding, may play an important role in determining duration of action *in vivo* [34].

Kinetic selectivity for M_3 versus M_2 receptors is considered desirable because: (i) inhibition of presynaptic M_2 receptors may facilitate cholinergic signalling in the airway by blocking negative feedback mechanisms regulating acetylcholine release from parasympathetic nerves [7,9]; and (ii) the characteristic tachycardia seen with anticholinergics is a consequence of inhibition of cardiac M_2 receptors which mediate the negative chronotropic and inotropic effects of acetylcholine in the heart [9,35]. All four muscarinic receptor antagonists have been shown previously to dissociate faster from M_2 receptors compared with M_3, conferring some degree of kinetic selectivity [20,21,26]. In this study, aclidinium and glycopyrronium had comparable kinetic selectivity for M_3 versus M_2 receptors, whereas tiotropium exhibited the lowest kinetic selectivity.

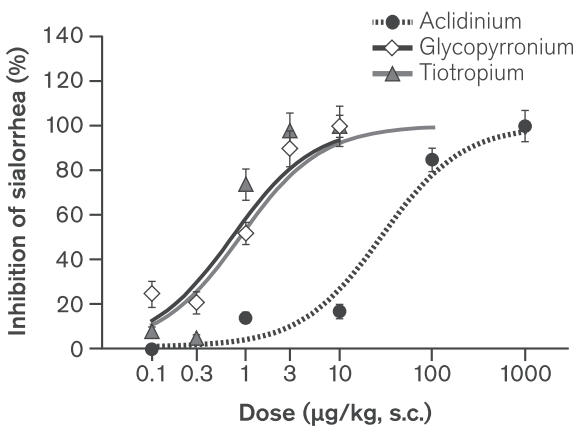
Aclidinium and glycopyrronium had similar relative potencies at native M_3 and M_2 receptors, suggesting that the higher affinity of aclidinium versus glycopyrronium for M_3 and M_2 receptors in binding experiments does not necessarily translate into improved potency at native M_3 and M_2 receptors. Both antagonists were 3- to 4-fold more potent at native M_3 receptors compared with native M_2 receptors, in contrast to the slight preference of glycopyrronium versus aclidinium for human recombinant M_3 compared with M_2 receptors. The differences in results between the binding studies and the *in vitro* potency studies may be due, in part, to the difference in receptor affinities between species. Consistent with their long residency time at recombinant human M_3 receptors, aclidinium, glycopyrronium and tiotropium had a long duration of action (>8 h) in isolated guinea pig trachea.

The faster onset of action of aclidinium and glycopyrronium compared with tiotropium in this study is consistent with the clinical profile of these compounds [36,37]. At the time of maximal effect, aclidinium, glycopyrronium, tiotropium and ipratropium were equipotent inhibitors of bronchoconstriction *in vivo*. The duration of bronchodilator action of each antagonist *in vivo* mirrored that for M_3 receptors' residency times seen in the binding



Data are mean ± standard error of the mean; n=4–9

Fig. 2. Duration of action of aclidinium, glycopyrronium, tiotropium and ipratropium in reversing acetylcholine-induced bronchoconstriction in guinea pigs.



Data are percentage of animals showing salivation normalised to vehicle-treated animals; n=6–24. Bars represent standard error of the mean. s.c., subcutaneous

Fig. 3. Effects of aclidinium, glycopyrronium and tiotropium on pilocarpine-induced salivation in conscious male Wistar rats.

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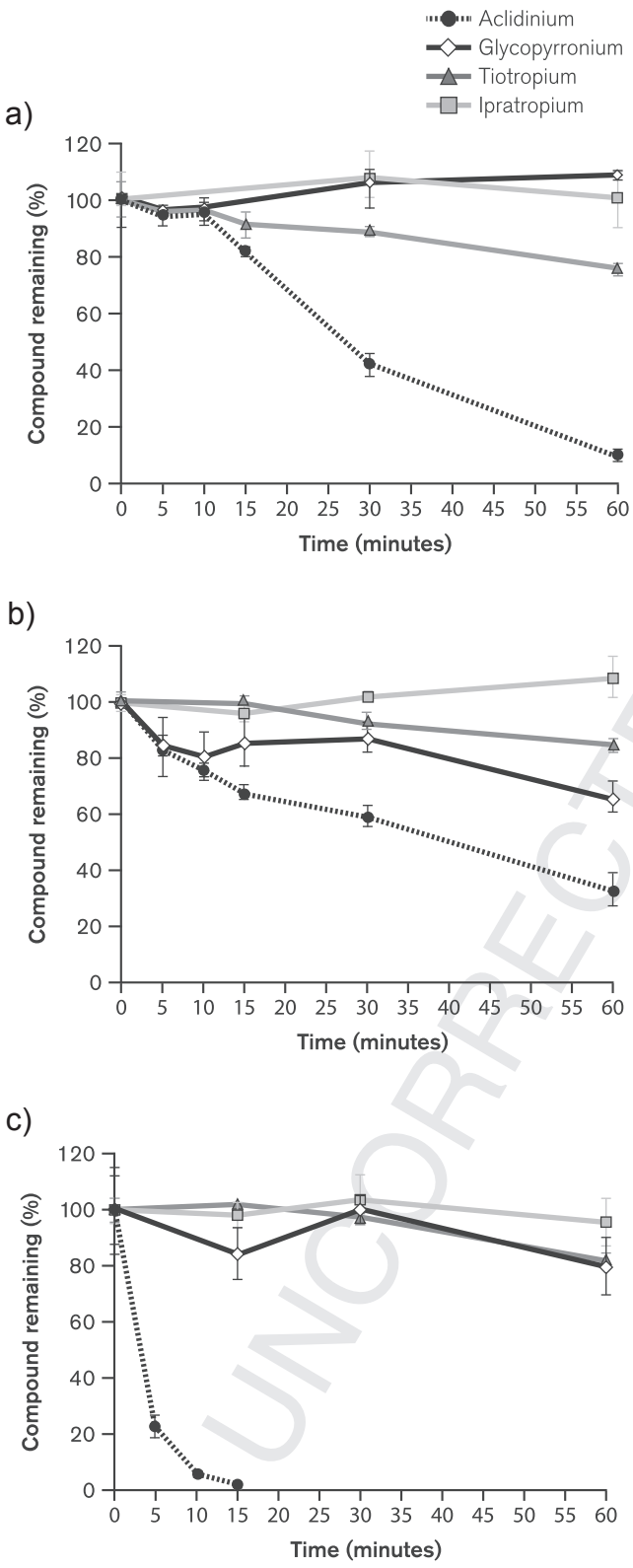

Data are mean ± standard deviation from three independent experiments

Fig. 4. Stability of aclidinium, glycopyrronium, tiotropium and ipratropium over time in (a) rat plasma, (b) guinea pig plasma and (c) human plasma.

Table 5
Estimated stability of aclidinium, glycopyrronium, tiotropium and ipratropium in rat and guinea pig plasma.

	Rat plasma $t_{½}$ (h)	Guinea pig plasma $t_{½}$ (h)	Human plasma $t_{½}$ (h)
Aclidinium	0.19	0.64	0.04
Glycopyrronium	11.6	5.5	6
Tiotropium	1.2	1.9	1.6
Ipratropium	23.6	73.4	33

h, hour; $t_{½}$, hydrolysis half-life.

studies, with aclidinium having a longer duration of effect in anaesthetised guinea pigs than glycopyrronium and ipratropium, and a shorter duration of effect than tiotropium.

Anticholinergic compounds, including tiotropium and ipratropium, are typically associated with systemic side effects such as dry mouth and tachycardia [12,13,15,16]. In a previous study, aclidinium was shown to produce a transient increase in heart rate in conscious dogs that was resolved 2.5 h post-administration, whereas tiotropium caused a significant increase that persisted for at least 6 h post-administration [20]. In this study, aclidinium was a much less potent inhibitor of salivation than either glycopyrronium or tiotropium, suggesting a lower propensity for aclidinium to cause dry mouth in the clinical setting. These preclinical observations are supported by results from Phase III clinical trials which have demonstrated that the incidence of dry mouth and cardiovascular side effects with twice-daily aclidinium was low and comparable to that with placebo [29,30]. Aclidinium was rapidly hydrolysed in rat and guinea pig plasma with a $t_{½}$ in both species 9- to 61-fold shorter than glycopyrronium and 3- to 6-fold shorter than tiotropium. Aclidinium was least stable in human plasma, with a $t_{½}$ 150-fold shorter than glycopyrronium and 40-fold shorter than tiotropium. Furthermore, in healthy volunteers aclidinium has been shown to be rapidly eliminated from plasma [38,39]. The rapid plasma hydrolysis of aclidinium results in very low systemic exposure which, coupled to its kinetic selectivity for M_3 receptors over M_2, may confer a reduced propensity for systemic side effects compared with other anticholinergic compounds.

In summary, aclidinium has high affinity for muscarinic receptors that is comparable to tiotropium but higher than glycopyrronium. While all four muscarinic antagonists have comparable kinetic selectivity, aclidinium dissociates from M_3 receptors more slowly than glycopyrronium and has a longer bronchodilatory action *in vivo*. In addition, aclidinium is more rapidly hydrolysed in plasma compared with both glycopyrronium and tiotropium, which may translate into a reduced propensity for systemic anticholinergic side effects. The availability of different LAMAs with unique pharmacological and physical properties may be important in providing additional therapeutic options for these patients.

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Disclosures

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