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Second M-3 muscarinic receptor binding site contributes to bronchoprotection by tiotropium

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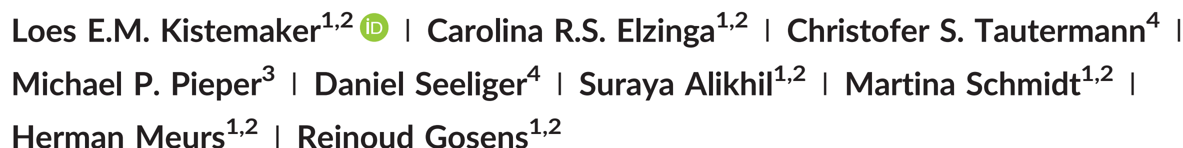

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Background and Purpose: The bronchodilator tiotropium binds not only to its main binding site on the M_3 muscarinic receptor but also to an allosteric site. Here, we have investigated the functional relevance of this allosteric binding and the potential contribution of this behaviour to interactions with long-acting β-adrenoceptor agonists, as combination therapy with anticholinergic agents and β-adrenoceptor agonists improves lung function in chronic obstructive pulmonary disease.

Experimental Approach: ACh, tiotropium, and atropine binding to M_3 receptors were modelled using molecular dynamics simulations. Contractions of bovine and human tracheal smooth muscle strips were studied.

Key Results: Molecular dynamics simulation revealed extracellular vestibule binding of tiotropium, and not atropine, to M_3 receptors as a secondary low affinity binding site, preventing ACh entry into the orthosteric binding pocket. This resulted in a low (allosteric binding) and high (orthosteric binding) functional affinity of tiotropium in protecting against methacholine-induced contractions of airway smooth muscle, which was not observed for atropine and glycopyrrolate. Moreover, antagonism by tiotropium was insurmountable in nature. This behaviour facilitated functional interactions of tiotropium with the β-agonist olodaterol, which synergistically enhanced bronchoprotective effects of tiotropium. This was not seen for glycopyrrolate and olodaterol or indacaterol but was mimicked by the interaction of tiotropium and forskolin, indicating no direct β-adrenoceptor–M_3 receptor crosstalk in this effect.

Conclusions and Implications: We propose that tiotropium has two binding sites at the M_3 receptor that prevent ACh action, which, together with slow dissociation kinetics, may contribute to insurmountable antagonism and enhanced functional interactions with β-adrenoceptor agonists.

1 | INTRODUCTION

The neurotransmitter **ACh**, which induces contraction of airway smooth muscle in the large and small conducting airways, exerts this contractile function through action at a subtype of the muscarinic receptor, the **M_3 receptor** (Belmonte, 2005; Fisher, Vincent, Gomeza, Yamada, & Wess, 2004; Kistemaker & Gosens, 2015; Roffel, Elzinga, Van Amsterdam, De Zeeuw, & Zaagsma, 1988). The M_3 receptor has two binding sites for its ligands: a functional orthosteric site within the binding pocket and a more easily accessible extracellular allosteric

Abbreviations: COPD, chronic obstructive pulmonary disease; E_{max}, maximal effect; pEC_{50}, the negative logarithm of the half maximal effective concentration; MD, molecular dynamics

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binding site (Kruse et al., 2012). These binding sites are highly conserved over various species with 100% sequence identity for the human, bovine, and rat M_3 receptor. Molecular dynamics (MD) simulations demonstrated that the bronchodilator drug **tiotropium** binds to both sites on the M_3 receptor (Kruse et al., 2012). Thus, before binding to the orthosteric site and after dissociation from the orthosteric site, tiotropium binds to the allosteric site in the extracellular vestibule (Kruse et al., 2012). It is unclear whether this behaviour has any functional relevance.

Tiotropium is a long-acting anticholinergic agent used to improve lung function in patients with obstructive lung diseases including **chronic obstructive pulmonary disease** (COPD), where it is a first line treatment, and **asthma** (Kerstjens et al., 2012; Kerstjens et al., 2015; Kistemaker & Gosens, 2015; Tashkin et al., 2008). Tiotropium is kinetically selective for the M_3 receptor, and dissociation from this receptor subtype is slower compared to that from M_1 **receptors** and in particular M_2 **receptors**, with a dissociation half-life at the M_3 receptor of over 24 hr (Casarosa et al., 2009; Tautermann et al., 2013). In addition, several other anticholinergic agents are available, including **glycopyrrolate**, which is shorter acting compared to tiotropium, with a dissociation half-life of around 6 hr from the M_3 receptor (Casarosa et al., 2009). According to COPD guidelines, treatment with anticholinergic agents can be combined with a long-acting β-adrenoceptor agonist to further improve lung function. Indeed, combination therapy with anticholinergic agents and β-adrenoceptor agonists has been shown to improve lung function in COPD, to a greater extent, compared with treatment with either of those drug classes alone (Buhl et al., 2015; Patel, 2016). Although there is consensus on the positive interactions between anticholinergic agents and β-adrenoceptor agonists on airway smooth muscle contraction, the underlying mechanism has been a matter of debate for decades (Calzetta, Matera, & Cazzola, 2015; Meurs et al., 2013; Panettieri, 2015; Pera & Penn, 2014).

Here, using MD simulation and pharmacological studies, the functional relevance of tiotropium binding to the allosteric site of the M_3 receptor was investigated. We demonstrate that tiotropium binding to the allosteric site prevents ACh entry into the orthosteric binding pocket. Together with the slow dissociation kinetics of tiotropium, this contributes to an insurmountable antagonism and is the basis for the functional interactions with β-adrenoceptor agonists.

What is already known

- Muscarinic M_3 receptors are the primary receptors mediating airway smooth muscle contraction.
- The anticholinergic agent tiotropium binds to a second binding site on the muscarinic M_3 receptor.

What this study adds

- Tiotropium binding to this secondary binding is stable and contributes to its functional effects.
- This secondary site contributes to insurmountable antagonism and enhanced functional interactions with β_2-adrenoceptor agonists, by tiotropium.

What is the clinical significance

- This is of importance for our understanding of how long-acting anticholinergic agents function as bronchodilators.
- Furthermore, it contributes to our understanding of how anticholinergics and β_2-adrenoceptor agonists functionally interact.

2 | METHODS

2.1 | MD simulations

MD simulations of binding to the M_3 receptor were set-up and performed as described previously. The initial ACh locations were chosen randomly outside the membrane and the receptor, and the starting poses of tiotropium and **atropine** have been adapted from the four predominant tiotropium binding modes to the allosteric site as published by Kruse et al. (2012). Antagonist binding to other (yet unidentified) cryptic pockets was not investigated in this study. In the set-up without antagonist, the simulation box contained 15 ACh molecules. Mixed ligand simulations contained 14 ACh molecules in solution and one tiotropium or atropine molecule bound to the extracellular vestibule. Simulation times were always 200 ns per simulation in the case of mixed antagonist/ACh systems, and for each of the four binding poses of tiotropium or atropine, five independent MD runs were performed, accumulating to 4 μs of simulation time. Simulation times for the 15 independent ACh alone MD simulations were at least 200 ns also summing up to ~4 μs. As a measure of orthosteric binding, the distance of the closest ACh entity (N atom) to Asp148 on helix 3 (Cγ atom) was measured. Asp at this position (3.32 in Weinstein–Ballesteros numbering) is a highly conserved residue in the amine receptor family, being crucially involved in endogenous ligand binding. Therefore, the distance to Asp148 is a good surrogate for the proximity to the orthosteric binding site. Distances of ~15 Å correspond to ligand locations in the loop regions, that is, the allosteric binding site.

2.2 | Contraction studies

Bovine tracheae were obtained from the local slaughterhouse, and human tracheae or main stem bronchi from anonymized lung transplantation donors were obtained from the Department of Cardiothoracic Surgery, University Medical Center Groningen, The Netherlands. Both bovine and human material are residual tissues. The study protocol was consistent with the Research Code of the University Medical Center Groningen (http://www.rug.nl/umcg/onderzoek/researchcode/index) and national ethical and professional guidelines ("Code of Conduct; Dutch Federation of Biomedical Scientific Societies";htttp://www.federa.org).

Tissue was transported to the laboratory in Krebs–Henseleit buffer (117.5 NaCl, 5.60 KCl, 1.18 $MgSO_4$, 2.50 $CaCl_2$, 1.28 NaH_2PO_4, 25.00 $NaHCO_3$, and 5.50 glucose, pre-gassed with 5% CO_2 and 95% O_2; pH 7.4), and smooth muscle strips of macroscopically identical length (1 cm) and width (2 mm) were prepared, as described previously (Gosens et al., 2004). Strips were washed and transferred to suspension culture flasks containing DMEM, supplemented with sodium pyruvate (1 mM), non-essential amino acid mixture (1:100), gentamicin (45 $\mu g \cdot ml^{-1}$), penicillin (100 $U \cdot ml^{-1}$), streptomycin (100 $\mu g \cdot ml^{-1}$), and amphotericin B (1.5 $\mu g \cdot ml^{-1}$; all from Life Technologies) and maintained at 37°C on an Innova 4000 incubator shaker (55 rpm). Strips were incubated with the muscarinic antagonists tiotropium (1–30 nM), glycopyrrolate (1–30 nM), and atropine (10–300 nM); the β-adrenoceptor agonists **olodaterol** (10 nM) and **indacaterol** (100 nM); the PKC inhibitor **GF109203X** (3 μM); the M_2 muscarinic antagonist **gallamine** (10 μM; Gosens et al., 2003, Roffel et al., 1988); and the adenylate cyclase activator **forskolin** (3 μM), for 1 and/or 24 and/or 72 hr. Compounds were provided by Boehringer Ingelheim or from Sigma-Aldrich. Isometric tension measurements were performed as described previously in 20-ml water-jacketed organ baths containing Krebs–Henseleit buffer at 37°C and continuously gassed with 5% CO_2 and 95% O_2 at pH 7.4 (Gosens et al., 2004). Strips were mounted for isometric recording in organ baths, and the resting tension was gradually increased to 3× *g*. Thereafter, strips were pre-contracted with 20- and 40-mM KCl solutions. Strips were washed three times, and the tension was readjusted to 3× *g*. After an equilibration period of 1 hr, cumulative dose–response curves with the muscarinic agonist **methacholine** (1 nM–10 mM, Sigma-Aldrich) were conducted. The next dose of methacholine was added when maximal contraction was reached, and when maximal tension was reached, strips were washed and maximally relaxed by addition of isoprenaline (0.1 mM, Sigma-Aldrich). In selected experiments, analysis of competitive inhibition of the antagonist was performed by classical Schild plot analysis using EC_{50} values calculated by GraphPad Prism 5.0 software (RRID:SCR_002798; Arunlakshana & Schild, 1959). Strips were randomly assigned to different pretreatments. The experimenter was not blinded as the combination treatments were needed to be assigned to the pretreated strip preparations.

2.3 | Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Data for contraction studies are presented as mean ± SEM. Data analysis was done in a blinded manner. Data subjected to statistical analysis were derived from a minimum of $n = 5$ separate experiments, from separate animals. The human studies have an $n = 3$ as human material suitable for contraction studies is very difficult to obtain. These data are therefore not subjected to statistical analysis, in line with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical differences between means on bovine data were calculated with Prism 5.0 software using one-way or two-way ANOVA, followed by a Bonferroni post hoc analysis where appropriate. Post hoc tests were run only if F achieved $P < .05$, and there was no significant variance inhomogeneity. An unpaired two-sided t test was used to calculate synergetic interactions between tiotropium and olodaterol or forskolin. Differences were considered significant at $P < .05$. Curve fitting was done using Prism software. When comparing a monophasic fit to a biphasic fit, Akaike's information criteria was used to evaluate the best fit

2.4 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18: GPCRs (Alexander et al., 2017).

3 | RESULTS

3.1 | MD simulation of ACh binding to the M_3 receptor

To investigate the relevance of tiotropium binding to the allosteric site or extracellular vestibule of the human M_3 receptor, we initiated MD simulation studies, based on previously published findings (Kruse et al., 2012). In the first series of simulations, spontaneous binding of ACh to the M_3 receptor was investigated. Altogether, 15 independent runs for at least 200 ns were performed, and the distance of the closest ACh to Asp148 on helix 3 was quantified as a measure of ACh binding to the two binding sites (Figure 1a and Kruse et al., 2012). In most cases, ACh bound transiently to the extracellular vestibule (Figure 1 b), followed by very rapid entry into the orthosteric binding pocket in 14 out of 15 simulations (Figure 1c and Movies S1 and S2). This is quantified in Figure 1d, demonstrating ACh binding to the extracellular vestibule at around 15 Å away from Asp148 and ACh binding to the orthosteric binding pocket at around 5 Å away from Asp148.

In the second series of simulations, we investigated ACh approaching the M_3 receptor in the presence of tiotropium bound to the extracellular vestibule. Four different predominant binding geometries of tiotropium in the allosteric site have been identified by Kruse et al. (2012). For each of these geometries, five independent runs of 200 ns each were performed. Interestingly, in the presence of tiotropium at this extracellular binding site, ACh failed to enter the orthosteric binding pocket in 18 out of 20 simulations (Figure 1f,g and Movies S1 and S2). In the two remaining simulations, ACh entry did occur, and this was associated with expulsion of tiotropium away from the extracellular vestibule binding site (Figure 1g). Thus, these data reveal that tiotropium binding to the extracellular vestibule binding site is relatively stable and is competitive with ACh in nature, suggesting that this site represents a second competitive, low affinity

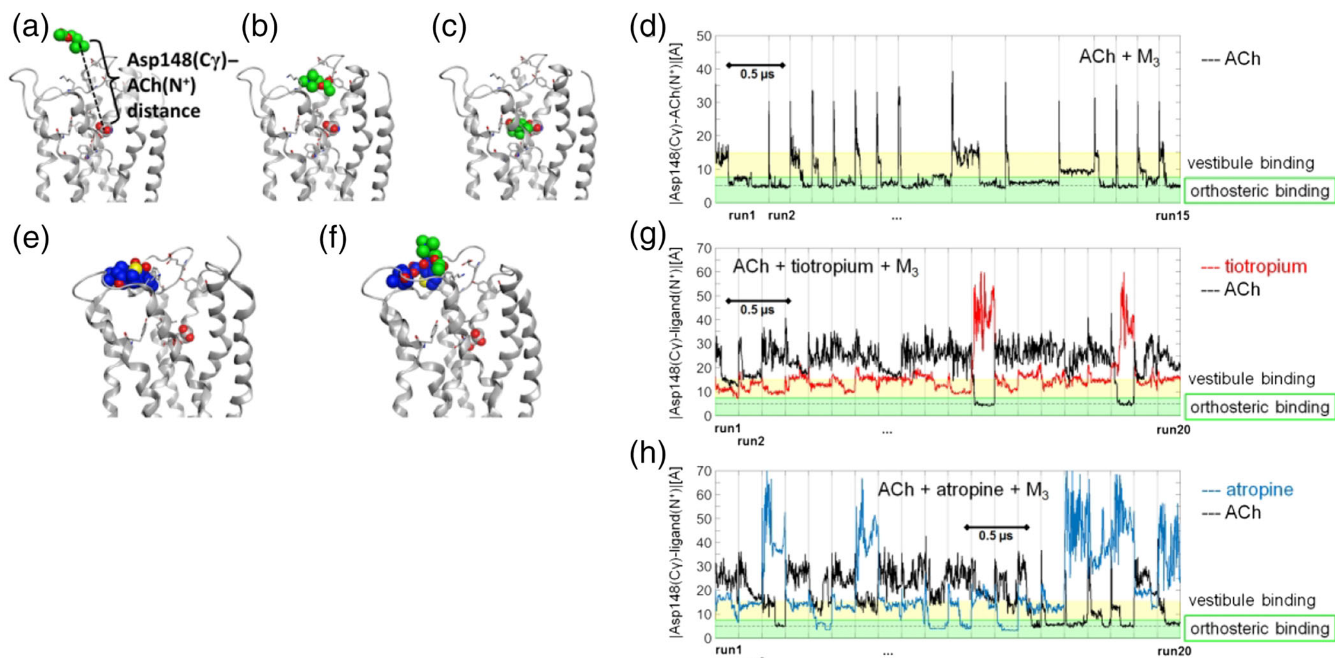

FIGURE 1 Tiotropium allosteric binding prevents ACh orthosteric binding. MD simulations of ACh binding to the M_3 muscarinic receptor. (a) Snapshot of ACh approaching the M_3 receptor, green spheres represents ACh, and the dashed line corresponds to the distance to Asp148 (grey spheres) which was used as a measure to quantify binding to the orthosteric site. (b) Transient ACh (green spheres) binding to the allosteric site. (c) ACh binding (green spheres) to the orthosteric site. (d) Quantification of ACh binding to the M_3 receptor. Binding around 15 Å represents vestibule binding, and binding around 5 Å represents orthosteric binding. Data show 15 individual runs. Note that ACh binding to the orthosteric site is quickly achieved, with a small pause in some runs at the vestibule area. (e) Snapshot of tiotropium (blue spheres) binding to the allosteric site of the M_3 receptor. (f) MD snapshot: tiotropium (blue spheres) bound to the allosteric site prevents ACh (green spheres) entry. (g) Quantification of ACh binding to the M_3 receptor in the presence of tiotropium, with tiotropium bound to the allosteric site as starting position. Data show 20 individual simulation runs. Note that when positioned at the vestibule area, tiotropium blocks entry of ACh in 18 out of 20 runs. In two runs, ACh was able to enter the orthosteric site, which was associated with dissociation of tiotropium from the vestibule area. (h) Quantification of ACh binding to the M_3 receptor in the presence of atropine, with atropine bound to the allosteric site as starting position. Data show 20 individual simulation runs. Note that binding of atropine at the vestibule area was much less stable compared to tiotropium binding. (b), (c), (e), and (f) are snapshots from simulations, see Movies S1 and S2

binding site in addition to the well-established high affinity orthosteric binding site.

To investigate whether this behaviour differentiates tiotropium from other M_3 receptor antagonists, the same MD simulations were performed using atropine as an antagonist. Interestingly, atropine binding at the vestibule was much less stable compared to tiotropium binding, with atropine moving out from the vestibule into solution in 6/20 cases (Figure 1h). ACh binding to the orthosteric site occurred in 7/20 cases, compared to only 2/20 cases for tiotropium. Furthermore, in 4/20 cases, atropine moved into the orthosteric site, which has not been observed for tiotropium simulations, further supporting the fact that tiotropium binding at the vestibule is much more stable than atropine binding (Figure 1).

3.2 | Functional studies on smooth muscle contraction in the presence of tiotropium

Given the observation that tiotropium has two binding modes at the M_3 receptor, which are both competitive with ACh, we postulated that this would be reflected by biphasic behaviour of tiotropium in pharmacological studies. To test this concept, we studied methacholine concentration–response relationships in smooth muscle strips as a model system. After 1 hr of pre-incubation with tiotropium, responses to methacholine were potently and concentration-dependently inhibited, as expected (Figure 2a). Intriguingly, the dose–response relationship to methacholine in the presence of 10-nM tiotropium showed biphasic behaviour with a methacholine pEC_{50} of 6.37 at the lower end of the dose–response relationship and a pEC_{50} of 3.80 at the higher end of the dose–response relationship (Figure 2a). Curve fitting software (Prism 5.0) confirmed this contention and demonstrated that assuming two binding sites produced the best fit for this dose–response curve (using comparison of monophasic and biphasic fit with Akaike's information criteria). In addition, maximal contraction in response to methacholine was inhibited by 33% in the presence of 10-nM tiotropium (Figure 2a). Subsequently, Schild analysis was used to assess the competitive behaviour of the antagonist (Arunlakshana & Schild, 1959). In case of competitive antagonism, a linear relationship between the size of the rightward shift (log[DR-1]) and the concentration of antagonist (−log[B]) should develop with a slope of 1.0 if agonist and antagonist use the same binding site. For the Schild analysis, we used non-linear

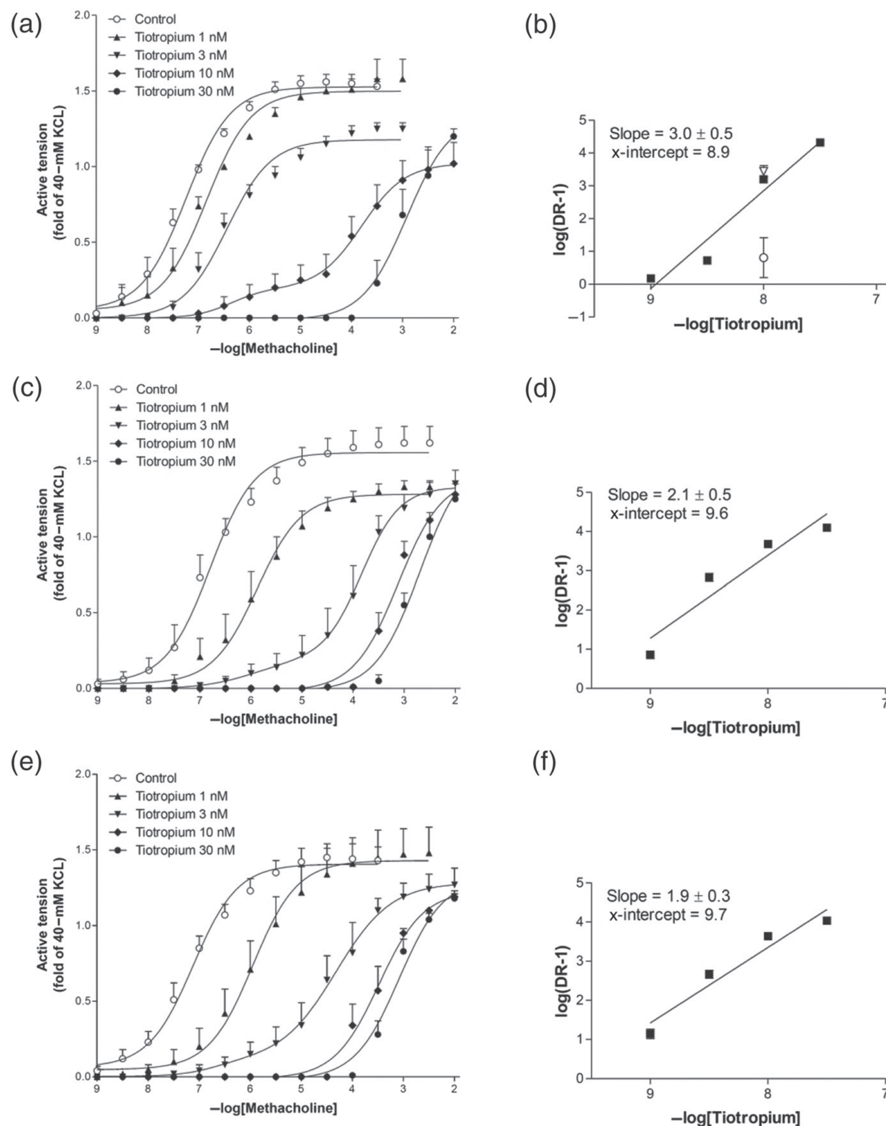

FIGURE 2 Pharmacological profile of tiotropium demonstrating biphasic behaviour. Smooth muscle strips were pre-incubated with increasing concentrations of tiotropium and methacholine dose–response curves and Schild plots were constructed. Data represent means ± SEM. (a) Methacholine dose–response curve after 1 hr of pre-incubation with tiotropium (1–30 nM) in bovine smooth muscle strips, n = 5. (b) Corresponding Schild plot after 1 hr of pre-incubation. Open symbols represent log (DR-1) values for both phases of the curve in the presence of 10-nM tiotropium. (c) Methacholine dose–response curve after 24 hr of pre-incubation with tiotropium (1–30 nM) in bovine smooth muscle strips, n = 5. (d) Corresponding Schild plot after 24 hr of pre-incubation. (e) Methacholine dose–response curve after 72 hr of pre-incubation with tiotropium (1–30 nM) in bovine smooth muscle strips, n = 5. (f) Corresponding Schild plot after 72 hr of pre-incubation

regression in Prism allowing the E_{max} to float in view of the different E_{max} values. For the 10-nM tiotropium concentration, we plotted the log (DR-1) value based on the EC_{50} assuming a monophasic fit in black symbols and the log (DR-1) values based on the EC_{50_1} and EC_{50_2} assuming a biphasic fit in white symbols. The x-intercept reflects the functional affinity and is the pA_2 value of the antagonist in case of competitive antagonism. The slope of the Schild plot of tiotropium after incubation for 1 hr was different from unity, with a slope of 3.0 ± 0.5 and an x-intercept of 8.9 (Figure 2b), as demonstrated previously (Disse et al., 1993). Intriguingly, it appeared as if the Schild analysis showed two binding sites for tiotropium with low affinity, Schild-like antagonism in a concentration range of 1–10 nM and high affinity, Schild-like antagonism in a concentration range of 10–30 nM.

Tiotropium is a long-acting anticholinergic agent with slow onset and offset of binding to the M_3 receptor, requiring prolonged time binding to establish equilibrium, which may also explain this non-Schild-like behaviour. Therefore, in subsequent experiments, the incubation period was extended to 24 and 72 hr. The functional affinity of tiotropium was higher after 24-hr incubation compared to 1-hr incubation, possibly explained by incomplete equilibrium binding at 1 hr (Figure 2c). This was not further changed using 72 hr of incubation (Figure 2e). Maximal contraction was inhibited to a similar extent as observed after 1-hr incubation, with E_{max} values being reduced up to 23% and 18% at 24 hr (Figure 2c) and 72 hr (Figure 2e), respectively. Intriguingly, the biphasic behaviour was maintained after longer pre-incubation, albeit at lower concentrations of tiotropium. This was expected, as increasing incubation time should shift the functional concentration profile of tiotropium to lower concentrations because of extending the opportunity to achieve binding equilibrium prior to the experiment. Together, these data support the hypothesis that tiotropium has a low affinity binding site and a high affinity binding site, resulting in biphasic functional behaviour.

This biphasic behaviour was examined in more detail by measuring the kinetics of methacholine-induced contraction. In all cases where this biphasic behaviour was observed, the initial low affinity binding state of tiotropium was more rapidly competed out by methacholine, whereas the high affinity binding state of tiotropium required prolonged incubation time with methacholine to develop stable

plateaus of contraction. Time to stable contraction after 24-hr incubation with 3-nM tiotropium was 2.80 ± 0.65 min for the first phase and 12.3 ± 0.62 min for the second phase in bovine strips (Figure 3a). Representative tracings support this contention and show rapid contractile responses to methacholine in control strips and in the lower end of the dose–response relationship of tiotropium pretreated strips. At higher concentrations (>$10^{-4.5}$ M methacholine), the development of contractile responses is much slower (Figure 3b). Similar findings were observed after 1 hr of pre-incubation of 10-nM tiotropium (data not shown). Thus, this aligns well with the concept of a low affinity binding site of tiotropium at the M_3 receptor, easier to compete out by a muscarinic agonist, and a high affinity binding site, from which it is more difficult to displace tiotropium. The MD and organ bath experiments indicate this is true for both ACh and methacholine.

3.3 | Functional studies using shorter acting anticholinergics

To determine whether this behaviour is related to slow dissociation kinetics of the long-acting anticholinergic agent tiotropium, we investigated the behaviour of the clinically used, medium long-acting anticholinergic agent glycopyrrolate, and of the classical short-acting antagonist atropine. Glycopyrrolate, after 1 hr of pre-incubation, concentration-dependently inhibited methacholine-induced contraction with rightward shifts in the dose–response relationship, without effects on the maximal response (Figure 4a). The characteristics of glycopyrrolate were not Schild-like at this time point, as the calculated slope was 2.6 ± 0.1 and the calculated x-intercept was 8.4 (Figure 4b). As this behaviour may reflect incomplete equilibrium binding of glycopyrrolate, the duration of the pre-incubation was extended to 24 hr. As observed previously for tiotropium, functional affinity of glycopyrrolate was increased after 24-hr incubation (Figure 4c). However, methacholine reached maximal contraction in all treatment conditions, irrespective of the concentration of glycopyrrolate applied (Figure 4c). Moreover, in contrast to tiotropium, glycopyrrolate exhibited Schild-like characteristics after 24-hr incubation, with a slope of 1.4 ± 0.1 and an x-intercept of 9.2 (Figure 4d). Thus, the nature of its competitive antagonism was dependent on the duration of the pre-incubation, but there is no evidence for biphasic behaviour.

As expected, incubation with atropine for 1 hr prior to the administration of methacholine responded with typical Schild-like characteristics. Atropine induced rightward shifts in the dose–response relationship to methacholine in a concentration-dependent fashion, and maximal response to methacholine was unaffected by atropine (Figure 4e). Schild analysis showed a slope of 1.4 ± 0.1 and an x-intercept of 8.4, similar to that reported previously (Roffel et al., 1988).

Taken together, these results on shorter acting anticholinergic agents suggest that this biphasic behaviour with insurmountable antagonism is related to slow dissociation kinetics and therefore unique to long-acting anticholinergic agents such as tiotropium. The MD data support this and indicate that tiotropium but not atropine shows stable binding at the vestibule area in the presence of ACh.

3.4 | Interactions with long-acting β-adrenoceptor agonists

Long-acting anticholinergic agents are often provided to COPD patients in combination with a long-acting β-adrenoceptor agonist,

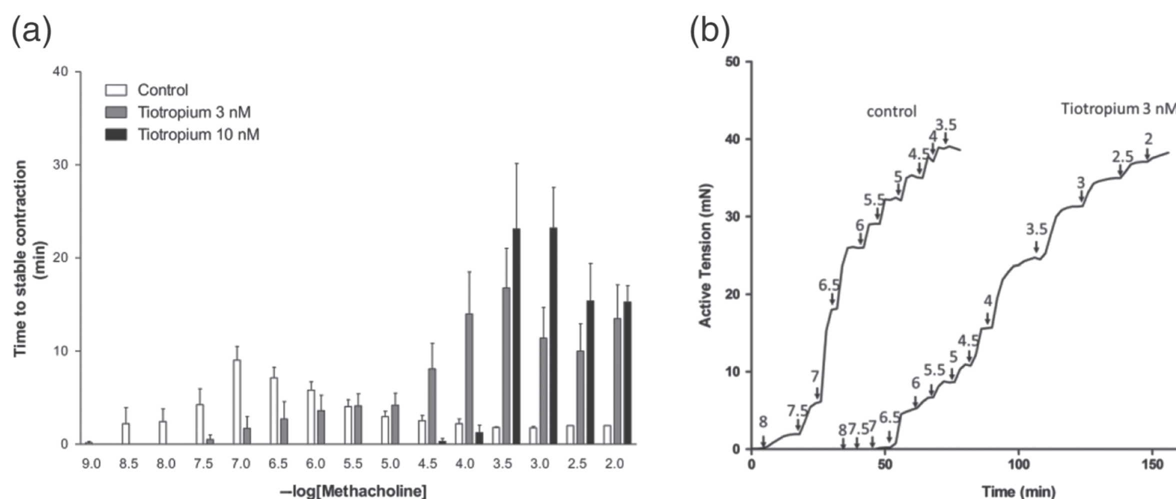

FIGURE 3 Kinetics of methacholine-induced contraction in relation to the low affinity and high affinity binding site of tiotropium. Time to stable plateau of contraction with methacholine was quantified for the individual methacholine concentrations. Data represent mean ± SEM. (a) Time to stable contraction in bovine smooth muscle strips in the absence of tiotropium and in the presence of 3-and 10-nM tiotropium after incubation for 24 hr, $n = 5$. The groups differ significantly as assessed by two-way ANOVA ($P < .05$ for tiotropium concentration, for methacholine concentration and for the interaction). (b) Representative tracings of a control and 3-nM tiotropium (24 hr) pretreated smooth muscle strip. Arrows indicate additions of methacholine (in −log[M]) to the organ bath. The tracings show rapid contractile responses to methacholine in control strips and in the lower end of the dose–response relationship of the tiotropium pretreated strip. At higher concentrations (>$10^{-4.5}$ M methacholine), the development of contractile responses is much slower

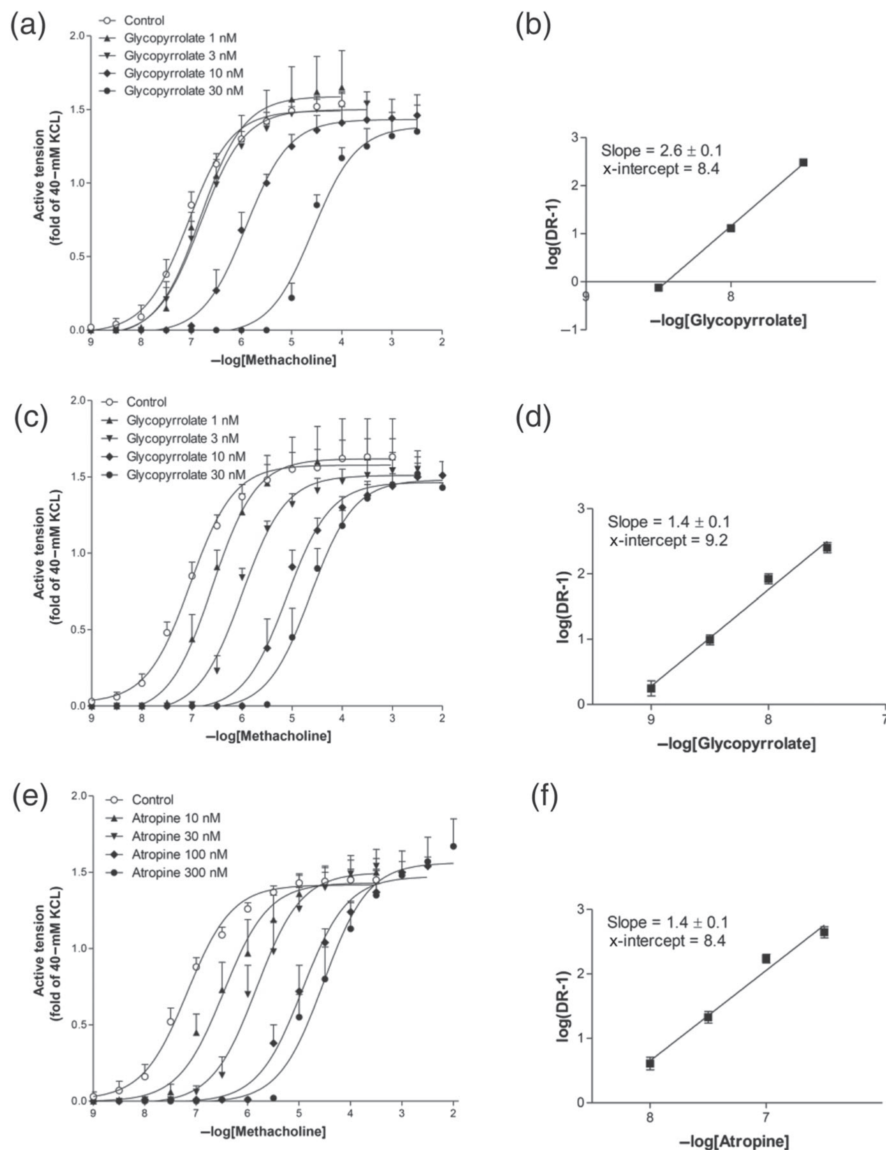

FIGURE 4 Shorter acting anticholinergic agents (glycopyrrolate and atropine) do not exhibit biphasic behaviour. Bovine smooth muscle strips were pre-incubated with increasing concentrations of glycopyrrolate or atropine, and methacholine dose–response curves and Schild plots were constructed. Data represent mean ± SEM. (a) Methacholine dose–response curve after 1 hr of pre-incubation with glycopyrrolate (1–30 nM), *n* = 5. (b) Corresponding Schild plot after 1 hr of pre-incubation. (c) Methacholine dose–response curve after 24 hr of pre-incubation with glycopyrrolate (1–30 nM), *n* = 5. (d) Corresponding Schild plot after 24 hr of pre-incubation. (e) Methacholine dose–response curve after 1 hr of pre-incubation with atropine (10–300 nM), *n* = 5. (f) Corresponding Schild plot after 1 hr of pre-incubation

which are believed to work synergistically to induce optimal bronchoprotection (Calzetta et al., 2015). We wondered whether the differential functional behaviour of long-acting anticholinergic agents would also affect the interaction with long-acting β-adrenoceptor agonists. Therefore, in our next experiments, we investigated the interactions between the clinically relevant combinations of tiotropium and olodaterol and the combination of glycopyrrolate and indacaterol. Olodaterol and indacaterol have a relatively fast onset of action showing relevant/full bronchoprotection after only 1 hr of pre-incubation. After 1-hr incubation, olodaterol significantly enhanced the bronchoprotective effect of tiotropium (Figure 5a). Thus, maximal airway contraction to methacholine in the presence of tiotropium was lowered from 1.31 (18% inhibition) to 1.02 (36% inhibition) in the presence of olodaterol, whereas the rightward shift in the dose–response relationship was enhanced from a pEC_{50} of 6.51 ± 0.09 to 6.22 ± 0.10 (Figure 5a and Table 1). For the interaction between indacaterol and glycopyrrolate, similar improvements in pEC_{50} were observed (pEC_{50} of 6.17 ± 0.07 for the combination), but no effects on maximal contraction could be seen (Figure 5b and Table 1). This suggests that because tiotropium behaves as an insurmountable antagonist, lowering methacholine efficacy, it has increased the functional effects of olodaterol on maximal contraction, which is not observed for the combination of glycopyrrolate and indacaterol.

As we have shown that these effects of tiotropium are more pronounced after 24 hr of incubation, we hypothesized that 24-hr incubation would result in increased functional interactions with olodaterol as well. Indeed, after 24 hr of pre-incubation, these effects were markedly enhanced (Figure 5c). Thus, tiotropium by itself lowered E_{max} by 31% (from 1.44 to 1.00) and induced a rightward shift from 7.08 ± 0.20 to 4.74 ± 0.15 in the absence of olodaterol. The effect on E_{max} was enhanced to 35% inhibition (0.93) in the presence of olodaterol. In addition, the combination synergistically induced a rightward shift with a pEC_{50} of 3.30 ± 0.08, which represents a ΔpEC_{50} of 3.78 ± 0.34 for the combination compared to 2.82 ± 0.38 for the

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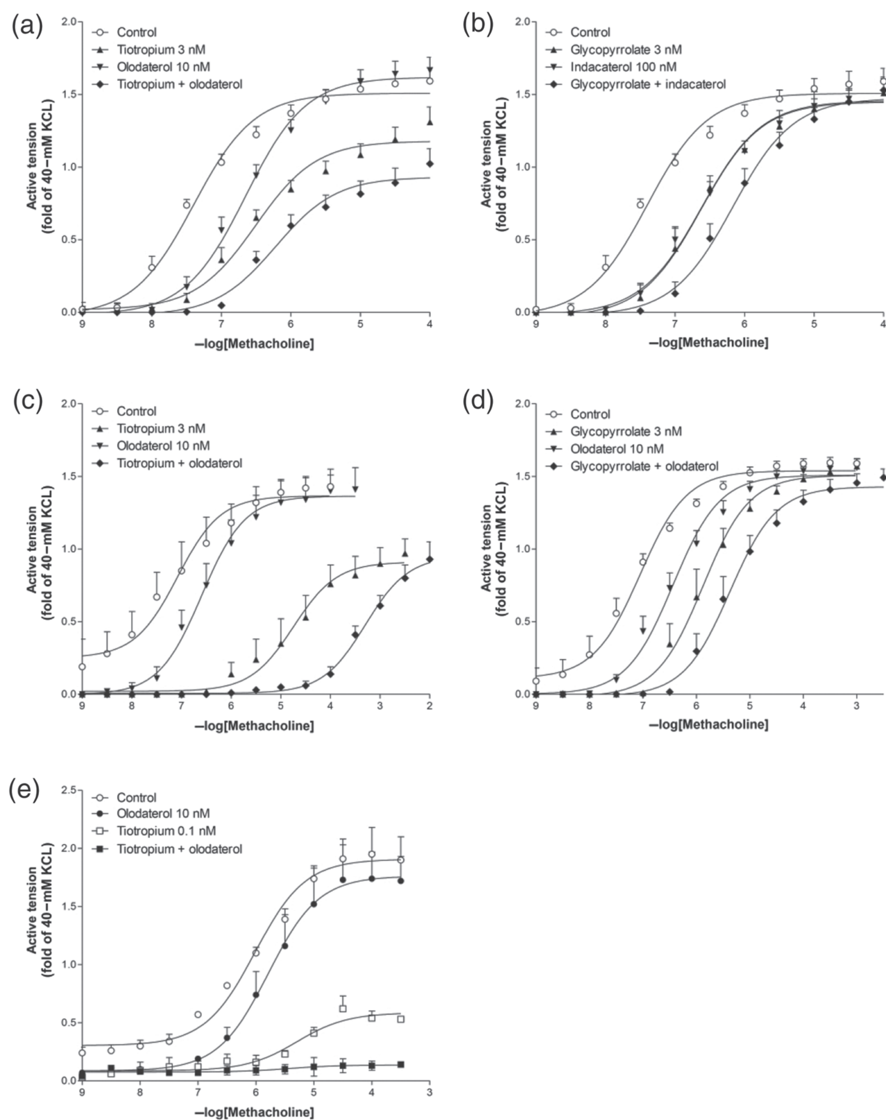

FIGURE 5 Olodaterol potentiates the bronchoprotective effects of tiotropium. (a) Methacholine dose–response curve after 1 hr of pre-incubation with tiotropium (3 nM) and/or olodaterol (10 nM) in bovine smooth muscle strips, *n* = 5. (b) Methacholine dose–response curve after 1 hr of pre-incubation with glycopyrrolate (3 nM) and/or indacaterol (100 nM) in bovine smooth muscle strips, *n* = 5. (c) Methacholine dose–response curve after 24 hr of pre-incubation with tiotropium (3 nM) and/or olodaterol (10 nM) in bovine smooth muscle strips, *n* = 5. (d) Methacholine dose–response curve after 24 hr of pre-incubation with glycopyrrolate (3 nM) and/or olodaterol (10 nM) in bovine smooth muscle strips, *n* = 5. (e) Methacholine dose–response curve after 24 hr of pre-incubation with tiotropium (0.1 nM) and/or olodaterol (10 nM) in human smooth muscle strips, *n* = 3. Data represent mean ± SEM

calculated sum of tiotropium and olodaterol alone (Figure 5c and Table 1, *P* < .01). To investigate whether these functional interactions are specific for tiotropium and not dependent on olodaterol, strips were incubated with glycopyrrolate and olodaterol. As expected, an interaction similar to that for tiotropium, was not observed for glycopyrrolate. Thus, pEC_{50} was increased from 7.06 ± 0.07 to 5.37 ± 0.07 for the combination of glycopyrrolate and olodaterol, representing a ΔpEC_{50} of 1.69 ± 0.10 compared to an calculated sum of 1.80 ± 0.09 (Figure 5d and Table 1). Furthermore, no effects on E_{max} were observed (Figure 5d). Findings for tiotropium and olodaterol were confirmed in human strips, where olodaterol increased the effect on E_{max} from 44% to 8% (Figure 5e and Table 1). Cumulatively, these results imply that functional interactions underlie the synergy observed between long-acting anticholinergic agents and long-acting β-adrenoceptor agonists, based on lowered methacholine efficacy in the presence of tiotropium, which in turn is dependent on the slow dissociation kinetics of tiotropium and its binding to the allosteric site of the M_3 receptor.

3.5 | Mechanism of interaction between anticholinergic agents and β-adrenoceptor agonists

We and many others have investigated receptor crosstalk between muscarinic receptors and β-adrenoceptors, and several mechanisms have been identified that may contribute to synergy between anticholinergic agents and β-adrenoceptor agonists (Pera & Penn, 2014). However, our current data suggest that other functional interactions, including lower intrinsic efficacy of ACh because of tiotropium vestibule binding, may be more relevant to the synergy between both drug classes. Therefore, in our final experiments, we aimed to investigate the nature of this interaction in more detail. Potential mechanisms of receptor crosstalk that are reported previously include M_3 receptor, PKC-dependent desensitization of β_2-adrenoceptors (Boterman et al., 2005), which is prevented by treatment with anticholinergic agents . Thus, in subsequent experiments, we investigated the effects of 1-hr pre-incubation with the PKC inhibitor GF109203X and/or olodaterol on methacholine-induced contraction. Whereas, as

TABLE 1 pEC_{50} and E_{max} values after incubation for 1 or 24 hr with tiotropium (3 nM), glycopyrrolate (3 nM), olodaterol (10 nM), or indacaterol (100 nM), and combinations thereof, in bovine and human smooth muscle strips

Treatment	pEC_{50}	E_{max} (%)
1 hr		
Control (bovine)	7.40 ± 0.07	100 ± 7.1
Tiotropium	6.51 ± 0.09*	82.2 ± 6.5*
Olodaterol	6.65 ± 0.06*	104.6 ± 5.6
Tiotropium + olodaterol	6.22 ± 0.10*, $	64.2 ± 6.4*, $
Control (bovine)	7.39 ± 0.07	100 ± 7.1
Glycopyrrolate	6.63 ± 0.08*	94.6 ± 6.0
Indacaterol	6.62 ± 0.08*	93.7 ± 6.5
Glycopyrrolate + indacaterol	6.17 ± 0.07*, #, $	95.8 ± 5.1
24 hr		
Control (bovine)	7.08 ± 0.20	100 ± 5.1
Tiotropium	4.74 ± 0.15*	69.4 ± 7.1
Olodaterol	6.59 ± 0.12#	98.6 ± 10.2
Tiotropium + olodaterol	3.30 ± 0.08*, #, $	64.8 ± 8.0*, $
Control (bovine)	7.06 ± 0.07	100 ± 2.3
Glycopyrrolate	5.88 ± 0.07*	100.5 ± 4.5
Olodaterol	6.44 ± 0.06*, #	98.0 ± 2.0
Glycopyrrolate + olodaterol	5.37 ± 0.07*, #, $	94.2 ± 3.9
Control (human)	6.02 ± 0.10	100 ± 11.4
Tiotropium	5.30 ± 0.19	43 ± 1.6
Olodaterol	5.79 ± 0.15	96 ± 20.0
Tiotropium + olodaterol	N/A	8.0 ± 1.0

*$P < .05$, significantly different from control;

#$P < .05$, significantly different from tiotropium/glycopyrrolate;

$$P < .05$, significantly different from olodaterol/indacaterol; one-way ANOVA, Bonferroni post hoc analysis where appropriate. $n = 5$ for bovine strips; $n = 3$ for human strips.

expected, olodaterol and GF109203X induced rightward shifts in the methacholine dose–response curve, no additional effect of the combination was observed (Figure 6a and Table 2), suggesting no major role of PKC-dependent desensitization of β_2-adrenoceptors in this model. Another hypothesis involves M_2 receptor G_i-dependent repression of adenylyl cyclase activation by β_2-adrenoceptors (Brown et al., 2013; Ehlert, 2003). Therefore, effects of the M_2 selective antagonist gallamine were investigated using the same approach. Intriguingly, the size of the rightward shift was unaffected by gallamine in combination with olodaterol, and no synergy was observed between the two compounds (Figure 6b and Table 2). These results indicate that there is no major role for M_2 and M_3 receptor-mediated crosstalk with β_2-adrenoceptors in this model.

If the interactions between long-acting anticholinergic agents and long-acting β-adrenoceptor agonists are truly independent of crosstalk at the receptor level, one would expect similar effects of direct adenylyl cyclase activation, compared to β-adrenoceptor activation. Therefore, in subsequent experiments, we investigated the bronchoprotective effects of the adenylyl cyclase activator forskolin, alone and in combination with tiotropium. As expected, forskolin pre-incubation for 1 hr induced a rightward shift in the methacholine dose–response relationship comparable to that of olodaterol, without a large effect on E_{max} (Figure 6c). Indeed, in the presence of tiotropium pre-incubated for 24 hr, strong synergistic interactions could be observed with a pEC_{50} value of 3.29 ± 0.10 compared to 4.74 ± 0.15 for tiotropium alone, representing a ΔpEC_{50} of 2.86 ± 0.66, compared to a calculated sum of 3.79 ± 0.55 (Figure 6c and Table 2,). E_{max} was inhibited by 37% (from 1.44 to 0.90) compared to 31% (1.00) for tiotropium alone (Figure 6c and Table 2). Interestingly, these values are virtually identical to those observed after combined incubation with tiotropium and olodaterol, supporting the hypothesis that the interactions are not at the receptor level but are mediated via other functional interactions between tiotropium and olodaterol.

4 | DISCUSSION

The data presented in this study reveal a specific pharmacological profile of tiotropium with implications for the subsequent functional interactions with long-acting β-adrenoceptor agonists. Using MD simulations, we demonstrate that, in contrast to atropine, tiotropium binding to the extracellular vestibule of the M_3 receptor prevents ACh binding. Furthermore, using organ bath experiments, we observed biphasic inhibition of methacholine-induced contractions, the second phase of which is insurmountable in nature. Together with slow dissociation kinetics of tiotropium, this results in lower intrinsic efficacy of muscarinic receptor agonists and primes the airway smooth muscle for subsequent bronchoprotection by β-adrenoceptor agonists. This interaction is not due to specific interactions between M_2 or M_3 receptors and β-receptors but rather the consequence of other functional interactions, which is enhanced by weaker activation of the M_3 receptor population.

Kruse et al. had identified this second allosteric binding site for tiotropium (Kruse et al., 2012), and we now demonstrate that tiotropium binding to this site inhibits ACh binding using MD simulations. We show that ACh also binds transiently to this allosteric site, before entering the binding pocket to bind to the orthosteric, functional site. ACh associates very quickly to the M_3 receptor, but this is significantly hindered when tiotropium is present at the allosteric site. This suggests that tiotropium has two, stable, and competitive antagonistic binding sites at the M_3 receptor, which may contribute to the long duration of action of tiotropium and to its biphasic behaviour.

Indeed, we observed biphasic behaviour of tiotropium in functional experiments using bovine and human airway smooth muscle strips, with a low affinity and rapidly competed out binding site at low methacholine concentrations and a high affinity slowly competed out binding site at high methacholine concentrations. Interestingly, although tiotropium did not show Schild-like behaviour over the full concentration range, both the low and high affinity states individually showed Schild-like behaviour, supporting the existence of two

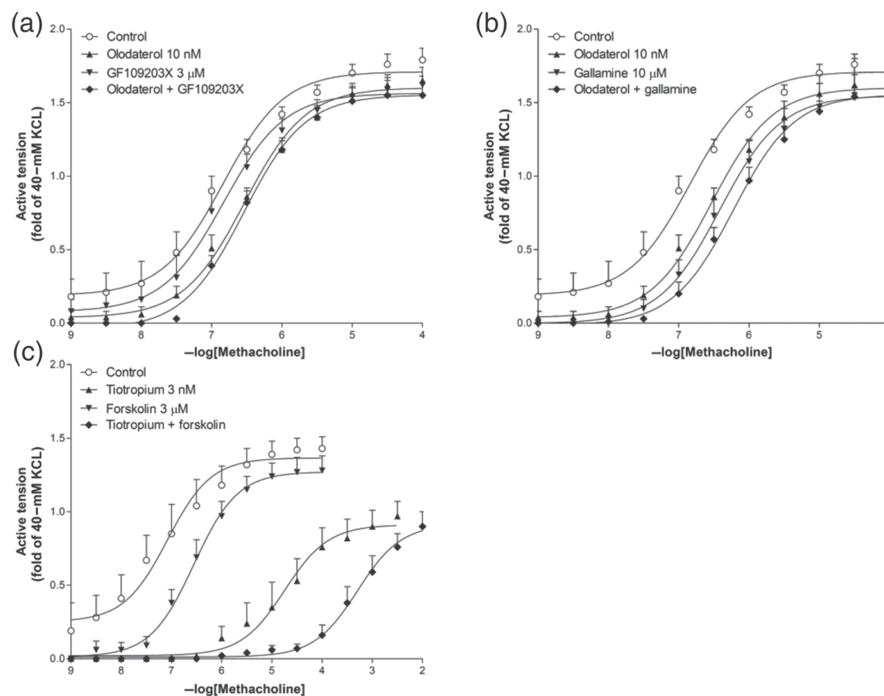

FIGURE 6 Functional interactions independent of receptor crosstalk are the basis for interactions between tiotropium and olodaterol. (a) Methacholine dose–response curve after 1 hr of pre-incubation with olodaterol (10 nM) and/or the PKC inhibitor GF109203X (3 μM) in bovine smooth muscle strips, *n* = 5. (b) Methacholine dose–response curve after 1 hr of pre-incubation with olodaterol (10 nM) and/or the M_2 muscarinic antagonist gallamine (10 μM) in bovine smooth muscle strips, *n* = 5. (c) Methacholine dose–response curve after 24 hr of pre-incubation with tiotropium (3 nM) and/or the adenylate cyclase activator forskolin (3 μM) in bovine smooth muscle strips, *n* = 5. Data represent mean ± SEM

TABLE 2 pEC_{50} and E_{max} values after incubation for 1 hr with olodaterol (10 nM) and/or GF109203X (3 μM) and/or gallamine (10 μM), and pEC_{50} and E_{max} values after incubation for 24 hr with tiotropium (3 nM) and/or incubation with forskolin (3 μM) for 1 hr in bovine smooth muscle strips

Treatment	pEC_{50}	E_{max} (%)
1 hr		
Control (bovine)	6.84 ± 0.10	100 ± 4.1
Olodaterol	6.53 ± 0.06	92.1 ± 4.9
GF	6.83 ± .010	90.3 ± 3.5
Olodaterol + GF	6.54 ± 0.07	87.4 ± 6.8
Control (bovine)	6.84 ± 0.10	100 ± 4.1
Olodaterol	6.53 ± 0.06	92.1 ± 4.9
Gallamine	6.42 ± 0.15	88.2 ± 14.5
Olodaterol + gallamine	6.22 ± 0.06*	88.5 ± 3.5
24 hr		
Control (bovine)	7.08 ± 0.20	100 ± 5.1
Tiotropium	4.74 ± 0.15*	69.4 ± 7.1*
Forskolin	6.55 ± 0.18*	90.8 ± 6.7
Tiotropium + forskolin	3.29 ± 0.10*, #, $	62.7 ± 7.2*

*P < .05, compared to control;

#P < .05, significantly different from tiotropium;

$$P$ < .05, significantly different from forskolin; one-way ANOVA, Bonferroni post hoc analysis where appropriate; *n* = 5.

separate binding sites. In further support, the time needed for re-equilibration in the assay when tiotropium and methacholine were both present was much longer for the second high affinity binding state of tiotropium, compared to the initial low affinity binding state.

Interestingly, this biphasic behaviour was specific for tiotropium and was not observed for the shorter acting antagonists, atropine and glycopyrrolate, in the present in vitro experiments. This is supported by MD simulations demonstrating that atropine binding to the vestibule is much less stable compared to tiotropium binding when using the same starting positions. Atropine either moves out of the M_3 receptor or into the orthosteric site, as is also evident from the on-rate of atropine which is about 10 times faster than that of tiotropium (Dowling & Charlton, 2006). Previously, dual binding at both binding sites was described for the M_2 receptor, were one ligand occupied both sites (Bock et al., 2016). M_2 receptors are not involved in airway smooth muscle contraction, which is solely mediated via M_3 receptors (Roffel et al., 1988). Dual binding at the two binding sites of the M_3 receptor was not observed for tiotropium in the current study, and we assume that binding of two tiotropium entities to both sites of M3 is not feasible, because of the electrostatic repulsion caused by the permanent charge of the molecule. Taken together, the slow dissociation kinetics and more stable binding of tiotropium to the vestibule hindering ACh entrance are essential to the functional effects and biphasic response of tiotropium.

In support, the bronchoprotective effects of tiotropium, alone and in combination with olodaterol, were more resistant to wash out compared to glycopyrrolate and/or indacaterol using functional experiments (unpublished observations), and the interaction between tiotropium and olodaterol was more profound than the combination of glycopyrrolate and olodaterol. Furthermore, we have shown that tiotropium inhibits the maximal methacholine effect, thus behaving as an insurmountable antagonist, which was not observed for glycopyrrolate or atropine, and is in line with previous studies (Casarosa et al., 2009; Disse et al., 1993; Salmon et al., 2013). Intriguingly, in the latter study, the depression of maximal effects was shown

to be much stronger for tiotropium compared to **umeclidinium**, despite the long duration of action of both compounds (Salmon et al., 2013). In addition, in contrast to tiotropium, umeclidinium did behave with Schild-like characteristics (Salmon et al., 2013), suggesting that this profile might be specific for tiotropium.

Long-acting anticholinergic agents and long-acting β-adrenoceptor agonists are commonly given as a combination therapy in COPD and further improve lung function when applied together, compared with monotherapy. We demonstrate,here, that the basis of this synergy is functional interactions between both drug classes, without evidence for crosstalk at the receptor level, which is confirmed by our studies using forskolin. Previously, we have shown a role for M_3 receptor, PKC-dependent desensitization of β_2-adrenoceptors (Boterman et al., 2005; Boterman, Smits, Meurs, & Zaagsma, 2006), and no role for M_2 receptors in the potential crosstalk with β-adrenoceptors (Roffel, Meurs, Elzinga, & Zaagsma, 1993; Roffel, Meurs, Elzinga, & Zaagsma, 1995). Although confirming the latter finding in our current work, it is in contrast to what we and other have published previously with respect to M_3–β_2-adrenoceptor crosstalk. We believe that the current finding is independent of the model system, as we have observed similar findings using guinea pig lung slices (unpublished observations). Most likely, this relates to the nature of the β-adrenoceptor agonists used. Whereas **isoprenaline** is a classical β-adrenoceptor agonist, activating both adenylyl cyclase and β-arrestin dependent pathways, novel long-acting β-adrenoceptor agonists including olodaterol may use β-arrestin-independent signalling, rendering them less sensitive to desensitization via this mechanism (Drake et al., 2008; Liu, Horst, Katritch, Stevens, & Wuthrich, 2012). Therefore, for combinations of anticholinergic agents with the novel class of long-acting bronchodilators, interactions may be independent of receptor crosstalk but rather dependent on other functional interactions. Furthermore, the experiments using gallamine confirm that this is an M_3 receptor-mediated effect without a role for M_2 receptors. Gallamine, which efficiently blocks M_2 receptors, has only little effect on methacholine-induced contraction, in line with previous data demonstrating that the M_2 receptor is not involved in contraction induced by methacholine in bovine airway smooth muscle (Roffel et al., 1988).

Theoretically, biphasic behaviour might be different for different ligands. We have used ACh for the MD simulations and methacholine for the organ bath experiments. The choice is based on endogenous and clinically used agonists, and there is no reason to doubt probe dependency as long as orthosteric agonists are used. We cannot exclude the possibility that agonists with more stable allosteric binding would not be differently affected. However, this is beyond the scope of the current study.

Several anticholinergic agents are available on the market, including the medium long-acting glycopyrrolate and **aclidinium** and the long-acting tiotropium and umeclidinium. There are no major differences in selectivity or onset of action between these four compounds. We reveal in our current study that the interactions with β-adrenoceptor agonists are more profound for tiotropium compared to glycopyrrolate and do not depend on the chosen β-adrenoceptor agonist in vitro. Clinically, there are no studies directly comparing different combinations of anticholinergic agents and β-adrenoceptor agonists. Fixed-dose combinations of tiotropium and olodaterol (Beeh et al., 2015; Buhl et al., 2015), glycopyrrolate and indacaterol (Bateman et al., 2013; Frampton, 2014), aclidinium and **formoterol** (D'Urzo et al., 2014; Singh et al., 2014), and umeclidinium and **vilanterol** (Decramer et al., 2014; Gras, 2014) have all been shown to be more effective than monotherapy in patients with COPD.

In conclusion, we have shown that tiotropium has two stable and competitive antagonistic binding sites at the M_3 receptor, and we propose that this may explain, at least in part, the long duration of action of tiotropium, its biphasic behaviour, its insurmountable antagonism, and the enhanced functional antagonism with long-acting β-adrenoceptor agonists.

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CONFLICT OF INTEREST

C.T., M.P., and D.S. are employees of Boehringer Ingelheim (BI). L.K. is an employee of Aquilo.

AUTHOR CONTRIBUTIONS

L.K., C.T., M.P., M.S., H.M., and R.G. designed the studies. C.T. and D.S. performed the modelling experiments. L.K., C.E., S.A., and R.G. performed the pharmacological experiments. L.K. and R.G. drafted the manuscript. All authors read and approved the final version of the manuscript.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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REFERENCES

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Marrion, N. V., Peters, J. A., … CGTP Collaborators. (2017). The Concise Guide to PHARMACOLOGY 2017/18: G protein-coupled receptors. *British Journal of Pharmacology*, *174*(Suppl 1), S17–S129. https://doi.org/10.1111/bph.13878

Arunlakshana, O., & Schild, H. O. (1959). Some quantitative uses of drug antagonists. *British Journal of Pharmacology and Chemotherapy*, *14*, 48–58. https://doi.org/10.1111/j.1476-5381.1959.tb00928.x

Bateman, E. D., Ferguson, G. T., Barnes, N., Gallagher, N., Green, Y., Henley, M., & Banerji, D. (2013). Dual bronchodilation with QVA149 versus single bronchodilator therapy: The SHINE study. *The European Respiratory Journal*, *42*, 1484–1494. https://doi.org/10.1183/09031936.00200212

Beeh, K. M., Westerman, J., Kirsten, A. M., Hebert, J., Gronke, L., Hamilton, A., … Derom, E. (2015). The 24-h lung-function profile of once-daily tiotropium and olodaterol fixed-dose combination in chronic obstructive pulmonary disease. *Pulmonary Pharmacology & Therapeutics*, *32*, 53–59. https://doi.org/10.1016/j.pupt.2015.04.002

Belmonte, K. E. (2005). Cholinergic pathways in the lungs and anticholinergic therapy for chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society*, *2*, 297–304. https://doi.org/10.1513/pats.200504-043SR

Bock, A., Bermudez, M., Krebs, F., Matera, C., Chirinda, B., Sydow, D., … Mohr, K. (2016). Ligand binding ensembles determine graded agonist efficacies at a G protein-coupled receptor. *The Journal of Biological Chemistry*, *291*, 16375–16389. https://doi.org/10.1074/jbc.M116.735431

Boterman, M., Elzinga, C. R., Wagemakers, D., Eppens, P. B., Zaagsma, J., & Meurs, H. (2005). Potentiation of β-adrenoceptor function in bovine tracheal smooth muscle by inhibition of protein kinase C. *European Journal of Pharmacology*, *516*, 85–92. S0014–2999(05)00444–9 [pii]

Boterman, M., Smits, S. R., Meurs, H., & Zaagsma, J. (2006). Protein kinase C potentiates homologous desensitization of the β_2-adrenoceptor in bovine tracheal smooth muscle. *European Journal of Pharmacology*, *529*, 151–156. S0014–2999(05)01130–1 [pii]

Brown, S. M., Koarai, A., Sturton, R. G., Nicholson, A. G., Barnes, P. J., & Donnelly, L. E. (2013). A role for M_2 and M_3 muscarinic receptors in the contraction of rat and human small airways. *European Journal of Pharmacology*, *702*, 109–115. https://doi.org/10.1016/j.ejphar.2013.01.054

Buhl, R., Maltais, F., Abrahams, R., Bjermer, L., Derom, E., Ferguson, G., … Bateman, E. (2015). Tiotropium and olodaterol fixed-dose combination versus mono-components in COPD (GOLD 2–4). *The European Respiratory Journal*, *45*, 969–979. https://doi.org/10.1183/09031936.00136014

Calzetta, L., Matera, M. G., & Cazzola, M. (2015). Pharmacological interaction between LABAs and LAMAs in the airways: Optimizing synergy. *European Journal of Pharmacology*, *761*, 168–173. https://doi.org/10.1016/j.ejphar.2015.05.020

Casarosa, P., Bouyssou, T., Germeyer, S., Schnapp, A., Gantner, F., & Pieper, M. (2009). Preclinical evaluation of long-acting muscarinic antagonists: Comparison of tiotropium and investigational drugs. *The Journal of Pharmacology and Experimental Therapeutics*, *330*, 660–668. https://doi.org/10.1124/jpet.109.152470

Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., … Ahluwalia, A. (2018). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. *British Journal of Pharmacology*, *175*, 987–993. https://doi.org/10.1111/bph.14153

Decramer, M., Anzueto, A., Kerwin, E., Kaelin, T., Richard, N., Crater, G., … Church, A. (2014). Efficacy and safety of umeclidinium plus vilanterol versus tiotropium, vilanterol, or umeclidinium monotherapies over 24 weeks in patients with chronic obstructive pulmonary disease: Results from two multicentre, blinded, randomised controlled trials. *The Lancet Respiratory Medicine*, *2*, 472–486. https://doi.org/10.1016/S2213-2600(14)70065-7

Disse, B., Reichl, R., Speck, G., Traunecker, W., Ludwig Rominger, K. L., & Hammer, R. (1993). Ba 679 BR, a novel long-acting anticholinergic bronchodilator. *Life Sciences*, *52*, 537–544. https://doi.org/10.1016/0024-3205(93)90312-Q

Dowling, M. R., & Charlton, S. J. (2006). Quantifying the association and dissociation rates of unlabelled antagonists at the muscarinic M_3 receptor. *British Journal of Pharmacology*, *148*, 927, 0706819 [pii–937. https://doi.org/10.1038/sj.bjp.0706819

Drake, M. T., Violin, J. D., Whalen, E. J., Wisler, J. W., Shenoy, S. K., & Lefkowitz, R. J. (2008). β-Arrestin-biased agonism at the β_2-adrenergic receptor. *The Journal of Biological Chemistry*, *283*, 5669–5676, M708118200 [pii]. https://doi.org/10.1074/jbc.M708118200

D'Urzo, A. D., Rennard, S. I., Kerwin, E. M., Mergel, V., Leselbaum, A. R., Caracta, C. F., & AUGMENT COPD study investigators (2014). Efficacy and safety of fixed-dose combinations of aclidinium bromide/formoterol fumarate: The 24-week, randomized, placebo-controlled AUGMENT COPD study. *Respiratory Research*, *15*, 123. https://doi.org/10.1186/s12931-014-0123-0

Ehlert, F. J. (2003). Pharmacological analysis of the contractile role of M_2 and M_3 muscarinic receptors in smooth muscle. *Receptors & Channels*, *9*, 261–277, WXGN8U2UVYPQCHN5 [pii]. https://doi.org/10.1080/10606820308265

Fisher, J. T., Vincent, S. G., Gomeza, J., Yamada, M., & Wess, J. (2004). Loss of vagally mediated bradycardia and bronchoconstriction in mice lacking M_2 or M_3 muscarinic acetylcholine receptors. *The FASEB Journal*, *18*, 711–713. https://doi.org/10.1096/fj.03-0648fje

Frampton, J. E. (2014). QVA149 (indacaterol/glycopyrronium fixed-dose combination): A review of its use in patients with chronic obstructive pulmonary disease. *Drugs*, *74*, 465–488. https://doi.org/10.1007/s40265-014-0194-8

Gosens, R., Bromhaar, M. M., Tonkes, A., Schaafsma, D., Zaagsma, J., Nelemans, S. A., & Meurs, H. (2004). Muscarinic M_3 receptor-dependent regulation of airway smooth muscle contractile phenotype. *British Journal of Pharmacology*, *141*, 943–950. https://doi.org/10.1038/sj.bjp.0705709

Gosens, R., Nelemans, S. A., Grootte Bromhaar, M. M., McKay, S., Zaagsma, J., & Meurs, H. (2003). Muscarinic M_3-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle. *American Journal of Respiratory Cell and Molecular Biology*, *28*, 257–262. https://doi.org/10.1165/rcmb.2002-0128OC

Gras, J. (2014). Umeclidinium/vilanterol fixed-dose combination for COPD. *Drugs Today (Barc)*, *50*, 231–238. https://doi.org/10.1358/dot.2014.50.3.2122119

Harding, S. D., Sharman, J. L., Faccenda, E., Southan, C., Pawson, A. J., Ireland, S., … NC-IUPHAR (2018). The IUPHAR/BPS guide to pharmacology in 2018: Updates and expansion to encompass the new guide to immunopharmacology. *Nucleic Acids Research*, *46*, D1091–D1106. https://doi.org/10.1093/nar/gkx1121

Kerstjens, H. A., Casale, T. B., Bleecker, E. R., Meltzer, E. O., Pizzichini, E., Schmidt, O., … Bateman, E. D. (2015). Tiotropium or salmeterol as add-on therapy to inhaled corticosteroids for patients with moderate symptomatic asthma: Two replicate, double-blind, placebo-controlled, parallel-group, active-comparator, randomised trials. *The Lancet Respiratory Medicine*, *3*, 367–376. https://doi.org/10.1016/S2213-2600(15)00031-4

Kerstjens, H. A., Engel, M., Dahl, R., Paggiaro, P., Beck, E., Vandewalker, M., … Bateman, E. D. (2012). Tiotropium in asthma poorly controlled with standard combination therapy. *The New England Journal of Medicine*, *367*, 1198–1207. https://doi.org/10.1056/NEJMoa1208606

Kistemaker, L. E., & Gosens, R. (2015). Acetylcholine beyond bronchoconstriction: Roles in inflammation and remodeling. *Trends in Pharmacological Sciences*, *36*, 164–171. S0165-6147(14)00211-9 [pii]

Kruse, A. C., Hu, J., Pan, A. C., Arlow, D. H., Rosenbaum, D. M., Rosemond, E., … Kobilka, B. K. (2012). Structure and dynamics of the M_3 muscarinic acetylcholine receptor. *Nature*, *482*, 552–556. https://doi.org/10.1038/nature10867

Liu, J. J., Horst, R., Katritch, V., Stevens, R. C., & Wuthrich, K. (2012). Biased signaling pathways in β_2-adrenergic receptor characterized by 19F-NMR. *Science*, *335*, 1106–1110. https://doi.org/10.1126/science.1215802

Meurs, H., Dekkers, B. G., Maarsingh, H., Halayko, A. J., Zaagsma, J., & Gosens, R. (2013). Muscarinic receptors on airway mesenchymal cells: Novel findings for an ancient target. *Pulmonary Pharmacology & Therapeutics*, *26*, 145–155. https://doi.org/10.1016/j.pupt.2012.07.003

Panettieri, R. A. Jr. (2015). Bronchodilators, receptors and cross-talk: Together is better? *Postgraduate Medicine*, *127*, 771–780. https://doi.org/10.1080/00325481.2015.1080589

Patel, H. J. (2016). An update on pharmacologic management of chronic obstructive pulmonary disease. *Current Opinion in Pulmonary Medicine*, *22*, 119–124. https://doi.org/10.1097/MCP.0000000000000240

Pera, T., & Penn, R. B. (2014). Crosstalk between β-2-adrenoceptor and muscarinic acetylcholine receptors in the airway. *Current Opinion in Pharmacology*, *16C*, 72–81. S1471-4892(14)00033-2 [pii]

Roffel, A. F., Elzinga, C. R., Van Amsterdam, R. G., De Zeeuw, R. A., & Zaagsma, J. (1988). Muscarinic M_2 receptors in bovine tracheal smooth muscle: Discrepancies between binding and function. *European Journal of Pharmacology*, *153*, 73–82. https://doi.org/10.1016/0014-2999(88)90589-4

Roffel, A. F., Meurs, H., Elzinga, C. R., & Zaagsma, J. (1993). Muscarinic M_2 receptors do not participate in the functional antagonism between methacholine and isoprenaline in guinea pig tracheal smooth muscle. *European Journal of Pharmacology*, *249*, 235–238. 0014-2999(93)90438-N [pii]

Roffel, A. F., Meurs, H., Elzinga, C. R., & Zaagsma, J. (1995). No evidence for a role of muscarinic M_2 receptors in functional antagonism in bovine trachea. *British Journal of Pharmacology*, *115*, 665–671. https://doi.org/10.1111/j.1476-5381.1995.tb14984.x

Salmon, M., Luttmann, M. A., Foley, J. J., Buckley, P. T., Schmidt, D. B., Burman, M., … Rumsey, W. L. (2013). Pharmacological characterization of GSK573719 (umeclidinium): A novel, long-acting, inhaled antagonist of the muscarinic cholinergic receptors for treatment of pulmonary diseases. *The Journal of Pharmacology and Experimental Therapeutics*, *345*, 260–270. https://doi.org/10.1124/jpet.112.202051

Singh, D., Jones, P. W., Bateman, E. D., Korn, S., Serra, C., Molins, E., … Leselbaum, A. (2014). Efficacy and safety of aclidinium bromide/formoterol fumarate fixed-dose combinations compared with individual components and placebo in patients with COPD (ACLIFORM-COPD): A multicentre, randomised study. *BMC Pulmonary Medicine*, *14*(178), 2466–14-178. https://doi.org/10.1186/1471-2466-14-178

Tashkin, D. P., Celli, B., Senn, S., Burkhart, D., Kesten, S., Menjoge, S., … UPLIFT Study Investigators. (2008). A 4-year trial of tiotropium in chronic obstructive pulmonary disease. *The New England Journal of Medicine*, *359*, 1543–1554. https://doi.org/10.1056/NEJMoa0805800

Tautermann, C. S., Kiechle, T., Seeliger, D., Diehl, S., Wex, E., Banholzer, R., … Casarosa, P. (2013). Molecular basis for the long duration of action and kinetic selectivity of tiotropium for the muscarinic M_3 receptor. *Journal of Medicinal Chemistry*, *56*, 8746–8756, 10.1021/jm401219y. https://doi.org/10.1021/jm401219y

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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