The Influence of Receptor Kinetics on the Onset and Duration of Action and the Therapeutic Index of NVA237 and Tiotropium

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ABSTRACT

Studies under nonphysiological conditions suggest that long receptor residency time is responsible for the 24-h duration of action of the long-acting muscarinic antagonist (LAMA) tiotropium. Our aim was to determine how clinically relevant dissociation rates under more physiological conditions influence the differences in onset of action between tiotropium and 3-[(cyclopentylhydroxyphenylacetyl oxy]-1,1-dimethyl-pyrrolidinium bromide (NVA237), a once-daily dry-powder formulation of the LAMA glycopyrronium bromide in development for chronic obstructive pulmonary disease. In addition, we have investigated kinetic selectivity at each of the muscarinic receptor subtypes to determine whether the improved cardiovascular therapeutic index obtained with NVA237 in animal models is attributable to differences in kinetic rate constants. The binding of radioligand \(^{3}H\)N-methyl-scopolamine was measured in the presence/absence of several concentrations of unlabeled competitors, and data were analyzed using a competition kinetic model to provide on/off rates for the competitor. We found shorter dissociation half-lives for NVA237 and tiotropium under physiological (11.4 and 46.2 min, respectively) versus nonphysiological conditions (173 and 462 min, respectively). NVA237 had a more rapid onset of action (3–4.8 times) versus tiotropium, determined in an vitro calcium and rat tracheal strip assay. Simulations suggested that the more rapid onset of NVA237 action could be explained by differences in kinetic parameters. NVA237 had greater equilibrium binding and kinetic selectivity for muscarinic type 3 (M₃) versus muscarinic type 2 (M₂) receptors, with a faster off rate from M₂ versus M₃ receptors than tiotropium, potentially affording it a more favorable therapeutic index. This study suggests that the 24-h duration of action of NVA237 and tiotropium is not solely the result of their slow dissociation from the M₁ receptor and highlights the importance of conducting in vitro experiments in conditions reflecting those in vivo.

Introduction

Bronchodilators are the mainstay of treatment for the management of chronic obstructive pulmonary disease (COPD). Inhaled long-acting muscarinic antagonists (LAMAs), considered first-line bronchodilators, are widely used for the treatment of COPD. Important features of an ideal LAMA for use in COPD include strong selectivity for muscarinic type 3 (M₃) receptors, long duration of action (preferably 24 h), fast onset of action, and the ability to deliver clinically meaningful bronchodilation with a reassuring safety profile (Donohue, 2005; Vogelmeier and Banerji, 2011; http://www.goldcopd.org/guidelines-global-strategy-for-diagnosis-management.html). A long duration of action implies both prolonged efficacy (Tashkin, 2005) and a strategy-for-diagnosis-management.html). A long duration of action (preferably 24 h), fast onset of action, and the ability to deliver clinically meaningful bronchodilation with a reassuring safety profile (Donohue, 2005; Vogelmeier and Banerji, 2011; http://www.goldcopd.org/guidelines-global-strategy-for-diagnosis-management.html). A long duration of action implies both prolonged efficacy (Tashkin, 2005) and a strategy for diagnosis and management (Tashkin, 2005). A long duration of action implies both prolonged efficacy (Tashkin, 2005) and a strategy for diagnosis and management (Tashkin, 2005).

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ABBREVIATIONS: COPD, chronic obstructive pulmonary disease; \(^{3}H\)NMS, \(^{3}H\)N-methyl-scopolamine; CHO, Chinese hamster ovary; LAMA, long-acting muscarinic antagonist; HBSS, Hanks’ balanced salt solution; NVA237, 3-[(cyclopentylhydroxyphenylacetyl oxy]-1,1-dimethyl-pyrrolidinium bromide; M₂, muscarinic type 2; M₃, muscarinic type 3.
of bronchodilators; rapid relief from symptoms provides reassurance of effect and may help improve compliance with the medication (Breekveldt-Postma et al., 2007; Bourbeau and Bartlett, 2008).

Slow receptor kinetics have been implicated in the once-daily action of tiotropium (Disse et al., 1993). Previous studies have shown that tiotropium has a slow dissociation half-life \( t_{1/2} \) from the \( M_4 \) receptor, with \( t_{1/2} \) values of 34.7 h (Disse et al., 1993) and 27 h (Casarosa et al., 2009). In contrast, NVA237 has been shown to have a dissociation \( t_{1/2} \) of 6.1 h (Casarosa et al., 2009). It has been suggested that long receptor residency time is responsible for the once-daily duration of action of tiotropium observed in the clinic (Disse et al., 1999). This observation is supported by results from in vitro studies in guinea pig trachea and human bronchi (Takahashi et al., 1994; Barnes et al., 1995), which used a washout procedure. However, such washout studies are complicated by the phenomenon of “tissue rebinding” and do not represent a true measure of receptor dissociation rate (Vauquelin and Charlton, 2010). In addition, radioligand binding studies describing the very slow dissociation of tiotropium cannot be considered representative of the physiological setting, having been performed at nonphysiological temperature in the absence of sodium ions (Disse et al., 1993; Haddad et al., 1994; Casarosa et al., 2009). It has been reported that increasing the ionic strength of the assay buffer (increases in \( \text{Na}^+ \), \( \text{Ca}^{2+} \), \( \text{K}^+ \), \( \text{Mg}^{2+} \), and \( \text{Cl}^- \) concentrations were tested) and temperature decreased the affinity of muscarinic antagonists for their receptors (Birdsall et al., 1979; Sykes et al., 2010). Evaluation of the kinetic parameters of NVA237 and tiotropium at \( M_4 \) receptors under physiological \( \text{Na}^+ \) concentration and temperature should therefore be more relevant.

Tiotropium has a relatively slow onset of action, taking up to 3 h to achieve maximal bronchodilation (Casaburi et al., 2000). Studies with NVA237 have shown the potential for a faster onset of action (Kuna et al., 2007; Overend et al., 2008; Verkindre et al., 2010). As mentioned before, tiotropium has been associated with dry mouth at therapeutic doses (Casaburi et al., 2002) and other anticholinergic adverse events at higher doses (Kesten et al., 2009). Muscarinic antagonists are also reported to have a potential for cardiac adverse effects, particularly in patients with underlying cardiac disease (Singh et al., 2008; Singh and Furberg, 2011).

The aim of this study was to determine more clinically relevant dissociation rates by characterizing the binding of long-acting \( M_4 \) receptor antagonists at the \( M_{1–5} \) receptors at 37°C in a physiologically relevant buffer containing 138 mM NaCl. We also investigated whether differences in the kinetic rate constants of NVA237 and tiotropium could explain the potential differences in onset of action, the observed improved cardiovascular therapeutic index with NVA237 in preclinical animal models (Trifilieff et al., 2007), and a potentially improved therapeutic index in humans.

## Materials and Methods

### Cell Culture and Membrane Preparation

Chinese hamster ovary (CHO) cells with stable expression of the \( M_{1–3} \) acetylcholine receptors were adherently grown in alpha minimum essential medium supplemented with 10% newborn calf serum and L-glutamine. Cells were maintained at 37°C in 5% CO\(_2\)/humidified air. Cells were routinely split 1:10, using trypsin-EDTA to lift cells, and were not used in assays beyond passage 40. Membrane preparations were carried out as described previously (Sykes et al., 2009). Membrane aliquots were maintained at \(-80 \text{°C} \) until required. CHO cell membranes expressing the \( M_4 \) and \( M_5 \) acetylcholine receptors were obtained from PerkinElmer Life and Analytical Sciences (Beaconsfield, UK) were stored as described above.

### Common Procedures Applicable to All Radioligand Binding Experiments

All radioligand experiments were conducted in 96-deep-well plates. In all cases, nonspecific binding was determined in the presence of 1 \( \mu \text{M} \) atropine. After the indicated incubation period, bound and free \([\text{H}]\text{N-methyl-scopolamine}\) \(([\text{H}]\text{NMS})\) were separated by rapid vacuum filtration using a FilterMate Cell Harvester (PerkinElmer Life and Analytical Sciences) onto 96-well GF/C filter plates previously coated with polyethyleneimine (0.5%) and rapidly washed three times with ice-cold assay buffer. After drying (4 h), 40 \( \mu \text{L} \) of Microscint 20 (PerkinElmer Life and Analytical Sciences) was added to each well, and radioactivity was quantified by using single-photon counting on a TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences). Aliquots of \([\text{H}]\text{NMS}\) were also quantified accurately to determine how much radioactivity was added to each well by using liquid scintillation spectrometry on an LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). In all experiments, total binding never exceeded more than 10% of that added, limiting complications associated with depletion of the free radioligand concentration (Carter et al., 2007).

### Kinetics of \([\text{H}]\text{NMS Binding to Membranes}\)

To accurately determine \( k_{\text{off}} \) and \( k_{\text{on}} \) values, \( k_{\text{off}} \) was calculated for at least three concentrations of \([\text{H}]\text{NMS}\), incubated with \( M_{1–5} \) CHO cell membranes (10, 30, 10, and 5 \( \mu \text{g/well}^{-1} \), respectively) in assay binding buffer, either 10 mM HEPES containing 1 mM MgCl\(_2\) at room temperature (nonphysiological temperature and sodium concentration) or Hanks’ balanced salt solution (HBSS): 1.26 mM CaCl\(_2\), 0.49 mM MgCl\(_2\), 0.41 mM MgSO\(_4\), 5.33 mM KCl, 0.44 mM KH\(_2\)PO\(_4\), 4.2 mM NaHCO\(_3\), 138 mM NaCl, 0.34 mM Na\(_2\)HPO\(_4\), and 5.56 mM d-glucose at 37°C (physiological temperature and sodium concentration), final assay volume 1 mL. An identical procedure was followed to determine the kinetic parameters of \([\text{H}]\text{NMS}\) at the rat \( M_4 \) receptor by using rat CHO cell membranes (20 \( \mu \text{g/well}^{-1} \)) in HBSS at 37°C. After incubation, radioligand bound to the membranes was separated from free radioligand by rapid filtration through GF/C filter plates (Millepore Corporation, Billerica, MA) previously coated with polyethyleneimine (0.5%). Data were globally fitted to a four-parameter logistic model to derive a single best-fit estimate for \( k_{\text{off}} \) and \( k_{\text{on}} \).

### Determination of Antagonist Affinity Constants (\( K_i \)) and Kinetic Parameters

\([\text{H}]\text{NMS} (2–20 \text{ nM}, \text{final assay volume of } 1.5 \text{ mL}) \) was incubated in the presence of the indicated concentrations of unlabeled antagonists and \( M_{1–5} \) CHO cell membranes (10, 30, 10, and 5 \( \mu \text{g/well}^{-1} \), respectively) in assay binding buffer, either 10 mM HEPES containing 1 mM MgCl\(_2\) at room temperature or HBSS at 37°C, with gentle agitation for up to 500 min. IC\(_{50}\) values were converted to equilibrium binding constants (p\( K_i \)) by using previously described methods (Dowling and Charlton, 2006). The kinetic parameters of unlabeled antagonists were assessed by using a competition kinetic binding assay (Dowling and Charlton, 2006; Sykes et al., 2009). \([\text{H}]\text{NMS} (2–5 \text{ nM, final assay volume } 0.5 \text{ mL}) \) was incubated with unlabeled competitor, and experiments were initiated by the addition of CHO membranes. All compounds were tested at various multiples of their respective p\( K_i \) values. An identical procedure was followed to determine antagonist affinity constants (p\( K_i \)) and kinetic parameters at the rat \( M_4 \) receptor by using rat CHO cell membranes (20 \( \mu \text{g/well}^{-1} \)) in HBSS at 37°C.

### Calcium Assay

CHO-M\(_4\) cells were seeded into 96-well black plates at 50,000 cells/well in minimum essential medium supplemented with 10% newborn calf serum for approximately 24 h. Cells were loaded in HBSS without phenol red containing 0.1% (w/v) bovine serum albumin, 20 mM HEPES, 2 \( \mu \text{M} \) Fluo-4 acetoxyethyl ester, 0.25 mM probenecid, and 100 \( \mu \text{M} \) brilliant black before the addition of antagonists at various time intervals. Methacholine was...
added at an EC₅₀ concentration, and changes in calcium concentration were monitored by using a fluorometric image plate reader (Molecular Devices, Sunnyvale, CA).

**Rat Tracheal Strip.** Brown Norway rat tracheal strips containing four cartilaginous rings were cut. Each segment was opened longitudinally, opposite to the smooth muscle band, and set up for recording isometric tension in 10-mL organ baths containing modified Krebs’ solution (118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose) at 37°C bubbled with 95% O₂/5% CO₂. Resting tension was maintained at 1g. After a stabilization period of 1 h, during which time the tissues were repeatedly washed, 30 μMbethanechol was added to the bath. Once the contraction stabilized, a single concentration of either NVA237 or tiotropium was added to the bath, and the tension was monitored for up to 120 min.

**Data Analysis.** Because the amount of radioactivity varied slightly for each experiment (<5%), data are shown graphically as the mean ± S.D. for individual representative experiments; all values reported are mean ± S.E.M. for the indicated number of experiments. All experiments were analyzed by either linear or nonlinear regression using Prism 4.0 (GraphPad Software Inc., San Diego, CA). Competition displacement binding data were fitted to sigmoidal (variable slope) curves by using a four-parameter logistic equation:

\[
Y = \frac{Y_{\text{max}}}{k_{\text{off}}} \left(1 + \frac{X}{k_{\text{on}} \cdot Y_{\text{max}}} \right)
\]

where \( Y_{\text{max}} \) values obtained from the inhibition curves were converted to \( K_i \) values by using the method of Cheng and Prusoff (1973). Calcium and tracheal strip data were fitted to a one-phase exponential decay function, from which \( t_{1/2} \) values were obtained.

**Determinations of the Kinetic Parameters of NVA237 and Tiotropium at M₃ Receptors under Physiological and Nonphysiological Conditions of Sodium Ion Concentration and Temperature**

To calculate the kinetic parameters \( (k_{\text{on}} \) and \( k_{\text{off}} \)) of tiotropium and NVA237, we have used the equations of Motulsky and Mahan (1984) that describe the binding between two ligands, one labeled (here using \([3H]NMS\)) and one unlabeled, competing for the same receptor site. To successfully perform this type of analysis, the kinetic parameters of \([3H]NMS\) itself had to be determined, which was achieved through construction of a family of association kinetic curves by using a range of \([3H]NMS\) concentrations. Each association curve was monitored over time until equilibrium was achieved, and the data were subsequently globally fitted to eq. 2 to derive a single best-fit estimate for the \( k_{\text{on}} \) and \( k_{\text{off}} \) of \([3H]NMS\) (data not shown).

Figure 1 shows the kinetic competition curves for NVA237 and tiotropium under nonphysiological (A) and physiological (B) conditions, respectively. These antagonists were assayed at three different concentrations to make sure that each ligand displayed competitive and reversible binding. The binding of \([3H]NMS\) influences the effect the competing ligand is having on the free receptor population (Hulme and Birdsall, 1992). When the radioligand dissociates more quickly than the competing ligand, the immediate \([3H]NMS\) binding remains unaffected by the presence of competitor. Figure 1A shows that at early time points \([3H]NMS\) binding to muscarinic receptors exceeds equilibrium; its subsequent fall to equilibrium is related to the dissociation rate of the unlabeled competitor.

The comparison of kinetic parameters at physiological versus nonphysiological conditions demonstrated that longer dissociation \( t_{1/2} \) could be obtained in the absence of sodium (Table 1). Under physiological conditions, \( t_{1/2} \) for tiotropium and NVA237 from the M₃ receptor was found to be considerably shorter than the \( t_{1/2} \) seen under nonphysiological conditions (Table 1). Dissociation of NVA237 from the M₃ receptor was four times faster than that of tiotropium under physiological conditions (Table 1), but their kinetic on rates were almost identical (Table 2). The higher \( k_{\text{off}} \) value of NVA237, therefore, seems to be the critical factor in determining its lower affinity at the M₃ receptor relative to tiotropium. This is true not only for these clinically relevant LAMAs, but also for the antagonist radioligand NMS, which has been used to quantify these kinetic parameters.

**Affinity.** \((pK_A)\) estimates for tiotropium and NVA237 at the rat M₃ receptor determined in competition kinetic binding experiments were similar to those observed at human M₃
receptors. $pK_d$ values of 9.18 ± 0.01 and 10.30 ± 0.09 were determined for NVA237 and tiotropium, respectively. The kinetic off rate ($k_{off}$) determined for NVA237 at the rat M3 receptor was almost identical to the value determined in the human ($k_{off} 1.07 \times 10^{-6} M^{-1} \cdot min^{-1}$; $k_{off} 0.071 min^{-1}$). In contrast, the kinetic $k_{off}$ rate value for tiotropium was 2-fold lower than that observed in the human (0.007 versus 0.015 min$^{-1}$). This suggests that tiotropium would have a longer residency time at the rat M3 receptor compared with the human M3 receptor, whereas the residency time of NVA237 would be similar in both species.

Figure 2 simulates the dissociation of tiotropium and NVA237 over time under nonphysiological and physiological conditions. The kinetic off rate ($k_{off}$) of each ligand was fixed to those determined in the competition binding experiments described above. The simulations in Fig. 2 represent receptor...
Onset of Action

Kinetic parameters were used to simulate the onset of action of NVA237 and tiotropium at a concentration 30-fold more than their $K_i$ (Table 1; Fig. 3A). Simulations using the kinetic rate constants predicted that tiotropium would take four to five times longer than NVA237 to equilibrate with the $M_3$ receptor. This was confirmed in the in vitro calcium assay where NVA237 demonstrated a more rapid onset of action (5-fold faster) based on its ability to inhibit a submaximal concentration of methacholine relative to tiotropium (Fig. 3B). The $t_{1/2}$ of inhibition by NVA237 was $6.1 \pm 2.1$ min ($n = 3$), whereas the $t_{1/2}$ of inhibition by tiotropium was $29.4 \pm 4.2$ min ($n = 3$). A similar profile was observed in the rat tracheal strip assay using a concentration of NVA237 3-fold higher than that of tiotropium chosen based on its approximate 3-fold higher dose in the clinic (Fig. 3C). The $t_{1/2}$ of inhibition of the bethanecol response by NVA237 was $9.8 \pm 0.8$ min, whereas the $t_{1/2}$ of inhibition by tiotropium was $24.6 \pm 2.3$ min. Simulations using the kinetic rate constants measured at the rat $M_3$ receptor predict that tiotropium will take two to three times longer than NVA237 to equilibrate with the rat $M_3$ receptor at the concentrations used in the rat tracheal study (Fig. 3D).

$M_3$ Selectivity Ratio

Determination of the Equilibrium Affinity Constants for NVA237 and Tiotropium. $IC_{50}$ values determined from competition binding experiments were converted to equilibrium binding constants ($pK_i$) by using the equation of Cheng and Prusoff (1973). The equilibrium $pK_i$ values for NVA237 and tiotropium at the $M_{1-5}$ receptors are summarized in Table 3. The $pK_i$ of tiotropium was $10.05 \pm 0.03$ for $M_2$ receptors and $10.37 \pm 0.04$ for $M_3$ receptors (selectivity ratio 2), and that of NVA237 was $8.70 \pm 0.04$ for $M_2$ receptors and $9.59 \pm 0.05$ for $M_3$ receptors (selectivity ratio 7.8). Thus, NVA237 displayed a greater $M_4$ versus $M_2$ selectivity than tiotropium (7.8- versus 2.1-fold).

Determination of the Kinetic Parameters of NVA237 and Tiotropium at $M_{1-5}$ Receptors. Using the equations described by Motulsky and Mahan (1984) and the kinetic values obtained for $[^3]H$NMS, the association and dissociation rates for NVA237 and tiotropium from the $M_{1-5}$ receptors were calculated. As mentioned previously, NVA237 has a faster off rate from the $M_3$ receptor and higher selectivity for $M_4$ versus $M_2$ receptors than tiotropium. The $t_{1/2}$ for tiotropium at $M_4$ and $M_5$ receptors was 10.8 and 46.2 min, respectively (kinetic selectivity ratio 4.3), whereas the $t_{1/2}$ for NVA237 was 1.07 and 11.4 min, respectively (kinetic selectivity ratio 10.7). Compared with tiotropium, NVA237 had a shorter $t_{1/2}$ at the $M_4$ and $M_5$ receptors (30.1 and 77.0 versus 3.14 and 12.6 min, respectively). The above paradigm assumes full receptor occupancy at both $M_2$ and $M_3$ receptors; however, depending on the compounds affinity for the various receptor subtypes, only a defined proportion of muscarinic receptors are occupied after administration of a single dose. Figure 4, A and B represents a simulation of this situation using a concentration of NVA237 and tiotropium 30-fold more than the $K_i$ for the $M_3$ receptor. In this simulation, various levels and speeds of receptor occupancy at the five muscarinic receptor subtypes can be observed; NVA237 is fully dissociated from $M_{1,5}$ receptors in less than 4 h, whereas some proportion of tiotropium is still bound to $M_4$ and $M_5$ receptors. In addition, NVA237 shows a much greater kinetic selectivity for the $M_3$ receptor over $M_2$, $M_4$, and $M_5$ receptors, compared with tiotropium in this situation (Fig. 4C). The kinetic selectivity ratios obtained from these simulations are summarized in Table 4.

$pK_i$ values from kinetic binding studies correlated well with $pK_i$ values from equilibrium binding studies ($p < 0.02$ and 0.01 for tiotropium and NVA237, respectively), suggesting that the on and off rates estimated in the competition kinetic studies are indeed accurate.

Discussion

The rationale for using a buffer with a physiological sodium chloride concentration (138 mM HBSS) is based on the
The observation that sodium ions can significantly reduce muscarinic antagonist affinity (Birdsall et al., 1979). Results from the current study confirm this important finding. Furthermore, whole-cell binding studies performed in HBSS demonstrate an almost identical affinity and kinetic off rate for [3H]NMS versus membranes studies performed under identical conditions (Charlton et al., 2011).

Studies on unlabeled compounds performed at room temperature and in the absence of sodium have predicted much longer $t_{1/2}$ values for tiotropium and NVA237 (Disse et al., 1999; Casarosa et al., 2009). The reality is that these longer $t_{1/2}$ values are the result of studying receptor kinetics under nonphysiological conditions, as shown by the results from the current study. The $t_{1/2}$ for NVA237 and tiotropium under physiological conditions (11.4 and 46.2 min, respectively) was much shorter than the $t_{1/2}$ observed under nonphysiological conditions (173 and 462 min, respectively). This contradicts the hypothesis that the clinical duration of action of LAMAs is directly governed by residency time at the receptor and suggests that there may be other factors contributing to the 24-h duration of action of these compounds. Recent studies have demonstrated that NVA237 remains bound to lung tissue over a 24-h period after intratracheal administration, despite having a shorter residency time than tiotropium (Ogoda et al., 2011).

Lung pharmacology is complex and reflects interactions related to formulation and device properties, compound dissolution within the bronchial lumen, and the kinetics of tissue uptake, storage, and clearance. Therefore, in vitro receptor binding kinetics are only one factor in the process and cannot reliably predict the duration of effect of an administered compound (Fogarty et al., 2011). This is indeed the case for clinically relevant long-acting $\beta_2$-adrenoceptor agonists used in the treatment of COPD; $t_{1/2}$ values determined for indacaterol, a once-daily $\beta_2$-adrenoceptor agonist (0.2 min), and salmeterol, a twice-daily $\beta_2$-adrenoceptor agonist (0.91 min), confirm that kinetic off rates from the $\beta_2$-adrenoceptor have no role to play in determining the duration of action of this class of molecule (Sykes and Charlton, 2012). Pharmacokinetic studies suggest that the systemic exposure achieved after inhalation of tiotropium and NVA237 is unlikely to produce functionally relevant occupancy.

### Table 3

<table>
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<tr>
<th>Receptor</th>
<th>$pK_i$</th>
<th>M$_5$ Selectivity Ratio</th>
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<tbody>
<tr>
<td>NVA237</td>
<td>10.34 ± 0.04</td>
<td>9.60 ± 0.03</td>
</tr>
<tr>
<td>Tiotropium</td>
<td>10.05 ± 0.03</td>
<td>8.70 ± 0.04</td>
</tr>
<tr>
<td>M$_3$</td>
<td>10.37 ± 0.04</td>
<td>9.59 ± 0.05</td>
</tr>
<tr>
<td>M$_4$</td>
<td>10.18 ± 0.07</td>
<td>9.06 ± 0.01</td>
</tr>
<tr>
<td>M$_5$</td>
<td>9.76 ± 0.07</td>
<td>8.91 ± 0.04</td>
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of muscarinic receptors over a full 24-h period (Disse et al., 1999; Sechaud et al., 2012). As a consequence, systemic pharmacokinetic profiles for these particular compounds do not help rationalize their 24-h duration of action in patients with COPD.

Perhaps a more compelling argument to explain the long duration of these agents comes from studies examining drug rebinding (Vauquelin and Charlton, 2010). Those studies predict that the rate of free drug elimination from the effect compartment is a key factor influencing the duration of action of a drug. The complex geometry of micro-anatomic features, such as the neuromuscular junction, may restrict the free diffusion of drug molecules away from the local environment where the receptors are concentrated, meaning that freshly dissociated drug is more likely to “rebind” to the same receptor and/or receptors nearby. The process of rebinding has been suggested to occur at a local tissue level even when drug concentrations in the bulk phase have already dropped to insignificant levels (Vauquelin and Charlton, 2010) and may explain how NVA237 and tiotropium main-

![Fig. 4. A and B, simulated association and dissociation curves for NVA237 (A) and tiotropium (B) at a concentration 30-fold more than $K_i$. The kinetic on and off rates of each ligand were fixed to those determined in the competition binding experiments (Table 2). The simulations represent the predicted muscarinic receptor occupancy over time of tiotropium and NVA237 based on the kinetic parameters measured in competition kinetic experiments. C, $M_3$ receptor kinetic selectivity ratios of NVA237 and tiotropium based on area under the curve measurements from the simulations described in A and B, using NVA237 and tiotropium at a concentration 30-fold greater than $K_i$.]

### TABLE 4

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$M_3$ Kinetic Selectivity Ratio</th>
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<tbody>
<tr>
<td>Tiotropium</td>
<td>NVA237</td>
</tr>
<tr>
<td>$M_1$</td>
<td>12.8</td>
</tr>
<tr>
<td>$M_2$</td>
<td>4.30</td>
</tr>
<tr>
<td>$M_3$</td>
<td>1</td>
</tr>
<tr>
<td>$M_4$</td>
<td>1.54</td>
</tr>
<tr>
<td>$M_5$</td>
<td>0.62</td>
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tain their 24-h duration of action in the lung, despite their relatively rapid kinetic off rates.

Simulations using kinetic rate constants predict that tiotropium will take four to five times longer to equilibrate with the M₃ receptor than NVA237. This was confirmed in the in vitro calcium assay, where the onset of action of NVA237 was 5-fold faster than tiotropium, and in the rat tracheal strip, where the onset of action for NVA237 was 3-fold faster than that of tiotropium. These data suggest that NVA237 may exhibit a more rapid clinical onset of action than tiotropium as described previously (Verkindre et al., 2010).

This study further demonstrates that NVA237 is kinetically more selective (10.7-fold) for M₃ receptors over M₂ receptors with t₁/₂ values of 11.4 and 1.07 min, respectively. In contrast, tiotropium is only 4.3-fold selective for M₃ over M₂ receptors under conditions of physiological sodium ion and temperature; this difference comes from previous reports that claimed a 10-fold kinetic selectivity for tiotropium, albeit under conditions of low sodium and at room temperature (Disse et al., 1999). It is noteworthy that, although both compounds show a reduced affinity for the M₄ and M₅ receptors compared with M₁–₃ receptors, tiotropium has a relatively long receptor residency at both M₄ and M₅ receptors versus NVA237. In addition, NVA237 has a shorter t₁/₂ on the M₃ receptor compared with tiotropium. These differences between the two compounds could have important consequences in terms of adverse-event profile. Receptor complexes involved in adverse effects should ideally be readily reversible such that occupancy of the target by its endogenous ligand is dictated by its ability to compete with the free drug molecules; NVA237 clearly has considerable advantage over tiotropium in this regard.

Evidence from preclinical studies suggests an improved in vivo therapeutic index with NVA237 compared with tiotropium. In a rabbit model, NVA237 (20 μg) and tiotropium (3 μg) both inhibited methacholine-induced bronchoconstriction, but NVA237 had no effect on the cardiovascular response to methacholine, in contrast to tiotropium (Cooper et al., 2006a,b). In Brown Norway rat pretreated intratracheally with NVA237 or tiotropium, NVA237 showed an improved therapeutic index over tiotropium, particularly for cardiovascular side effects (Trifillieff et al., 2007). This may be explained by the higher selectivity of NVA237 for M₃ over M₂ receptors, as demonstrated in the current study. Blockade of M₂ receptors attenuates the negative feedback inhibition of acetylcholine production, potentially reducing the bronchodilation produced by muscarinic agents (Belmonte, 2005). In addition to the cholinergic nerve endings, M₂ receptors are situated in the heart where they are thought to modulate pacemaker activity (Abrams et al., 2006). Thus, there is concern about the potential cardiac adverse effects that muscarinic antagonists may have in patients (Singh et al., 2008; Singh and Furberg, 2011). M₁ and M₃ receptors enhance bronchoconstriction; M₁ receptors are common in the exocrine glands and central nervous system and are associated with the anticholinergic adverse events related to muscarinic antagonists, such as dry mouth, constipation, and gastrointestinal disturbances. The competition binding data obtained in the current study suggest that NVA237 is marginally more selective for M₃ and M₁ receptors over M₂ receptors, whereas tiotropium does not seem to distinguish between these three receptor subtypes. This contrasts with previous studies performed under nonphysiological conditions, which suggest that both compounds were nonselective for all three muscarinic subtypes (Haddad et al., 1994, 1999; Casarosa et al., 2009). Furthermore, despite NVA237 having a 6-fold lower equilibrium affinity than tiotropium for the M₃ receptor, in clinical studies it is dosed only 3-fold higher (Verkindre et al., 2010), contradicting suggestions (Casarosa et al., 2009) that it is overdosed relative to tiotropium to achieve its once-daily duration of action.

In conclusion, the results of this study cast doubt over the hypothesis that the clinical duration of action of LAMAs is directly governed by receptor residency time and suggest that there are other factors contributing to the 24-h duration of action of these compounds. Indeed, studies have demonstrated that NVA237 has a 24-h duration of action, despite displaying a shorter receptor residency time than tiotropium (Verkindre et al., 2010; Fogarty et al., 2011; Vogelmeier and Banerji, 2011). In addition, with its higher kinetic selectivity for M₃ receptors over M₂ receptors and shorter residency time at M₁ and M₃ receptors, NVA237 may potentially have a more favorable therapeutic index compared with tiotropium. The results from kinetic studies have also highlighted the potential for NVA237 to show a faster rate of clinical onset compared with tiotropium. Finally, we have highlighted the importance of conducting in vitro experiments in conditions that, to the best possible extent, mimic those in vivo.

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Wrote or contributed to the writing of the manuscript: Sykes, Dowling, Kent, Trifillieff, and Charlton.

References


Casaburi R, Briggs DD Jr, Donohue JP, Serby CW, Menjoge SS, and Wittek TJ Jr...
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Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.


Cooper N, Walker I, and Knowles I (2006b) NVA237 has similar efficacy as tiotropium bromide against methacholine-induced bronchoconstriction and less systemic effect on cardiovascular variables in an anesthetized rabbit model. Eur Respir J 28 (Suppl 50):436s.


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