JPET #194456

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

David A. Sykes, Mark R. Dowling, Juliet Leighton-Davies, Toby C. Kent, Lindsay Fawcett

Emilie Renard, Alexandre Trifilieff, and Steven J. Charlton

Novartis Institutes for Biomedical Research, Wimblehurst Road, Horsham, West Sussex RH12 5AB

1

JPET #194456

Running title: The influence of receptor kinetics on NVA237 and tiotropium

*Corresponding author

Steven Charlton, Novartis Institutes for Biomedical Research, Wimblehurst Road, Horsham,

West Sussex RH12 5AB

Phone: +44 1403 324203

Fax: +44 1403 323054

Email: steven.charlton@novartis.com

Text pages: 20

Tables: 4

Figures: 4

References: 43/60

Abstract: 250/250 words

Introduction: 737/750 words

Discussion: 1,311/1,500 words

Non-standard abbreviations: [^{3}H]NMS, [^{3}H]N-methyl-scopolamine; CHO, Chinese

hamster ovary; COPD, chronic obstructive pulmonary disease; LABA, long-acting β_2

adrenergic agonist; LAMA, long-acting muscarinic antagonist; HBSS, Hank's Balanced Salt

Solution

Proposed journal for submission: Journal of Pharmacology and Experimental

Therapeutics

Section assignment: Cellular and Molecular

ABSTRACT

Studies under non-physiological conditions suggest that long receptor residency time is responsible for the 24-h duration of action of the long-acting muscarinic antagonist (LAMA) tiotropium. We aimed to determine clinically relevant dissociation rates in physiological conditions and evaluate if differences in onset of action between tiotropium and NVA237, a once-daily dry-powder formulation of the LAMA glycopyrronium bromide in development for chronic obstructive pulmonary disease, and the improved cardiovascular therapeutic index with NVA237 in animal models is attributable to differences in kinetic rate constants. The binding of radioligand [^{3}H]N-methyl-scopolamine ([^{3}H]NMS) was measured in the presence/absence of several concentrations of unlabeled competitors and data analyzed in 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 non-physiological conditions (173 and 462 min, respectively). NVA237 had more rapid onset of action (3–4.8x) versus tiotropium, determined in in 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 higher equilibrium binding and kinetic selectivity for M_3 versus M_2 receptors, and faster off rate from M_2 versus M_3 receptors versus tiotropium, potentially affording it 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 M_3 receptor and highlights the importance of conducting in vitro experiments in conditions reflecting those in vivo.

Keywords: NVA237, glycopyrronium bromide, association rate, dissociation rate, duration of action, onset of action, therapeutic index, muscarinic receptor, COPD, LAMA

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 (GOLD, 2011). Important features of an ideal LAMA for use in COPD include strong selectivity for muscarinic type 3 (M_3) 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 (Vogelmeier and Banerji, 2011; Donohue, 2005; GOLD, 2011). A long duration of action implies both prolonged efficacy (Tashkin, 2005) and a once-daily dosage regimen that has the potential to boost patient compliance (Breekveldt-Postma et al., 2007; Tamura and Ohta, 2007; Smith et al., 1996). It has also been proposed that bronchodilators with a long duration of action offer a relatively consistent improvement in airway caliber over time, in contrast to the peaks and troughs that might arise with bronchodilators that are dosed twice daily (Beeh and Beier, 2010). Tiotropium, the most widely used LAMA worldwide, has a 24-h duration of action, but has several limitations, including a slow onset of action and a relatively high incidence of anticholinergic adverse events such as dry mouth (Barr et al., 2006). NVA237 is a novel once-daily dry powder formulation of the LAMA glycopyrronium bromide, in development for the treatment of COPD. It has a rapid onset and long duration of action (> 24 h), with an acceptable safety and tolerability profile (Verkindre et al., 2010; Vogelmeier et al., 2010; Fogarty et al., 2011, D'Urzo et al., 2011). A rapid onset of action is a desirable feature 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_3 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 employed a wash-out procedure. However, such wash-out 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 non-physiological temperature in the absence of sodium ions (Disse et al., 1993; Casarosa et al., 2009 and Haddad et al., 1994). It has been reported that increasing the ionic strength of the assay buffer (increases in Na^+, Ca^{2+}, K^+, Mg^{2+} and Cl^- concentrations were tested) decreased the affinity of muscarinic antagonists for their receptors (Birdsall et al., 1979). Evaluation of the kinetic parameters of NVA237 and tiotropium at M_3 receptors under physiological 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). Recent studies with NVA237 have shown the potential for a faster onset of action (Overend et al., 2008; Verkindre et al., 2010; Kuna et al., 2007). 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).

This study aimed to determine more clinically relevant dissociation rates by characterizing the binding of long-acting M_3 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.

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 minimum essential medium supplemented with 10% newborn calf serum. 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°C until required. CHO cell membranes expressing the M_4 and M_5 acetylcholine receptors obtained from PerkinElmer 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 µM atropine. After the indicated incubation period, bound and free [³H]NMS were separated by rapid vacuum filtration using a FilterMate Cell Harvester (PerkinElmer Life and Analytical Sciences, Beaconsfield, UK) onto 96-well GF/C filter plates previously coated with polyethylenimine (0.5%) and rapidly washed three times with ice-cold assay buffer. After drying (4 h), 40 µl of Microscint 20 (PerkinElmer Life and Analytical Sciences) was added to each well, and radioactivity was quantified using single-photon counting on a TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences). Aliquots of [³H]NMS were also quantified accurately to determine how much radioactivity was added to each well using liquid scintillation spectrometry on an LS6500 scintillation counter (Beckman Coulter). 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 [³H]NMS Binding to Membranes. To accurately determine K_{on} and K_{off} values, K_{obs} was calculated for at least three concentrations of [³H]N-methyl-scopolamine ([³H]NMS), incubated with M_{1-5} CHO-cell membranes (10, 30, 10, 10 and 5 µg well⁻¹,

respectively) in assay binding buffer, either 10 mM HEPES containing 1 mM $MgCl_2$ at room

temperature (non-physiological temperature and sodium concentration) or Hank's Balanced

Salt Solution (HBSS, mM: $CaCl_2$, 1.26; $MgCl_2$, 0.49; $MgS0_4$, 0.41; KCl, 5.33; KH_2PO_4, 0.44;

$NaHCO_3$, 4.2; NaCl, 138; Na_2HPO_4, 0.34; D-Glucose, 5.56) at 37°C (physiological

temperature and sodium concentration), final assay volume 1 mL. An identical procedure

was followed to determine the kinetic parameters of [^{3}H]NMS at the rat M_3 receptor using rat

CHO-cell membranes (20 µg well^{-1}) in HBSS at 37^{0}C. Following incubation, radioligand

bound to the membranes was separated from free radioligand by rapid filtration through

Millipore GF/C filter plates previously coated with PEI (0.5%). Data were globally fitted to an

association kinetic model to derive a single best-fit estimate for K_{on} and K_{off}.

Determination of Antagonist Affinity Constants (K_i) and Kinetic Parameters.

[^{3}H]NMS (~2 nM, final assay volume of 1.5 mL) was incubated in the presence of the

indicated concentration of unlabeled antagonist and M_{1-5} CHO-cell membranes (10, 30, 10,

10 and 5 µg 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 (pK_i) using previously described

methods (Dowling and Charlton, 2006). The kinetic parameters of unlabeled antagonists

were assessed using a competition kinetic binding assay (Dowling and Charlton, 2006;

Sykes et al., 2009). [^{3}H]NMS (2–5 nM, final assay volume 0.5 mL) was incubated with

unlabeled competitor and experiments were initiated by addition of CHO membranes. All

compounds were tested at various multiples of their respective pK_i. An identical procedure

was followed to determine antagonist affinity constants (K_i) and kinetic parameters at the rat

M_3 receptor using rat CHO-cell membranes (20 µg well^{-1}) in HBSS at 37°C.

Calcium Assay. CHO-M_3 cells were seeded into 96-well black plates at 50,000

cells/well in minimum essential medium supplemented with 10% NCS for approximately 24

h. Cells were loaded in HBSS without phenol red containing 0.1% (w/v) bovine serum

albumin, HEPES (20 mM), Fluo-4AM (2 µM), and brilliant black (100 µM) before addition of antagonists at various time intervals. Methacholine was added at an EC_{80} concentration and changes in calcium concentration monitored using fluorometric image plate reader.

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 isotonic tension in 10 mL organ baths containing modified Krebs' solution (mM: NaCl, 118; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KH_2PO4, 1.2; $NaHCO_3$, 25; glucose, 11) at 37°C bubbled with 95% O_2/5% CO_2. Resting tension was maintained at 1 g. After a stabilization period of 1 h, during which time the tissues were repeatedly washed, 30 µM of bethanechol 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. As the amount of radioactivity varied slightly for each experiment (< 5%), data are shown graphically as the mean ± standard deviation (S.D.) for individual representative experiments; all values reported in the text and tables are mean ± standard error mean (S.E.M.) for the indicated number of experiments. All experiments were analyzed by either linear or non-linear regression using Prism 4.0 (GraphPad Software, San Diego, USA). Competition displacement binding data were fitted to sigmoidal (variable slope) curves using a 'four parameter logistic equation':

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / \left(1 + 10^{(\text{logEC}_{50} - X).\text{Hill coefficient}}\right) \qquad (1)$$

IC_{50} values obtained from the inhibition curves were converted to K_i values using the method of Cheng and Prusoff (1973). Dissociation data were fitted to a one-phase exponential decay function and the $t_{1/2}$ value obtained was transformed into a K_{off} rate using:

$$K_{off} = \frac{0.693}{t_{1/2}}$$

[³H]NMS association data were simultaneously fitted to Equation 2 using GraphPad Prism.
K_{on} and K_{off} were shared across the data set so that a single value for each was derived from
the whole family of curves.

Specific binding = maximal specific binding*(1-exp(-1*K_{obs}*time)) (2)

Where: K_{obs} = [Radioligand]*K_{on} + K_{off}

Association and dissociation rates for unlabeled antagonists were calculated using the
equations described by Motulsky and Mahan (1984):

$$K_A = k_1[L] + k_2$$

$$K_B = k_3[I] + k_4$$

$$S = \sqrt{\left((K_A - K_B) \wedge 2 + 4 * k_1 * k_3 * L * I * 1e^{-18}\right)}$$

$$K_F = 0.5 * (K_A + K_B + S)$$

$$K_S = 0.5 * (K_A + K_B - S)$$

$$DIFF = K_F - K_S$$

$$Q = \frac{B_{max} * K_1 * L * 1e^{-9}}{DIFF}$$

$$Y = Q * \left(\frac{k_4 * DIFF}{K_F * KS} + \frac{k_4 - K_F}{K_F} e^{(-K_F * X)} - \frac{k_4 - K_S}{K_S} e^{(-K_S * X)} \right)$$

(3)

10

Where:

X = Time (min)

Y = Specific binding [counts per minute (cpm)]

$k_1 = K_{on}$ [^{3}H]NMS

$k_2 = K_{off}$ [^{3}H]NMS

L = Concentration of [^{3}H]NMS used (nM)

I = Concentration of unlabeled antagonist (nM)

Fixing the above parameters allowed the following to be calculated:

B_{max} = Total binding (cpm)

k_3 = Association rate of unlabeled ligand (M^{-1} min^{-1})

k_4 = Dissociation rate of unlabeled ligand (min^{-1})

Simulations. The rate of antagonist binding was simulated at equi-effective concentrations using the following equation:

$$Y = Y_{max}*(1-e^{-Kobs*X})$$

Where:

K_{obs} = [radioligand]$*K_{on} + K_{off}$ and X = Time

Dissociation simulations were performed in Prism 5 using the following equation:

$$Y = (Y_0-NS)*exp(-K*X) + NS$$

Where:

X = time, Y = Binding, usually total (constrain NS to 0.0 if specific), Y_0 = Y at time 0, in units

of Y, NS = Binding at very long times, in units of Y, K = Rate constant in inverse units of X.

The half-life is 0.69/K.

Results

Determination of the Kinetic Parameters of NVA237 and Tiotropium at M_3 Receptors under Physiological and Non-Physiological Conditions of Sodium Ion Concentration and Temperature.

In order to calculate the kinetic parameters (K_{on} and K_{off}) of tiotropium and NVA237, we have employed the equations of Motulsky and Mahan (1984) which describe the binding between two ligands, one labelled (here using [3H]NMS) and one unlabelled, competing for the same receptor site. In order 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 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 Equation 2 to derive a single best-fit estimate for the K_{on} and K_{off} of [3H]NMS (data not shown).

Figure 1 shows the kinetic competition curves for NVA237 and tiotropium under non-physiological (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 ($k_2 > k_4$), the immediate [3H]NMS binding remains unaffected by the presence of competitor. Figure 1 (A) 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 non-physiological 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 M_3

receptor was found to be considerably shorter than the $t_{1/2}$ seen under non-physiological conditions (Table 1). Dissociation of NVA237 from the M_3 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_{off} value of NVA237, therefore, appears to be the critical factor in determining its lower affinity at the M_3 receptor relative to tiotropium. This is true not only for these clinically relevant LAMAs, but also the antagonist radioligand NMS which has been used to quantify these kinetic parameters.

Affinity (pK_d) estimates for tiotropium and NVA237 at the rat M_3 receptor determined in competition kinetic binding experiments were similar to those observed at human M_3 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 M_3 receptor was almost identical to the value determined in the human (K_{on} 1.07x10^8 M^{-1}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 min^{-1} versus 0.015 min^{-1}). This suggests that tiotropium would have a longer residency time at the rat M_3 receptor compared with the human M_3 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 non-physiological and physiological conditions. The kinetic off rate (K_{off}, k_2) of each ligand was fixed to those determined in the competition binding experiments described above. The simulations in Figure 2 represent receptor previously equilibrated with a saturating concentration of ligand. At t = 0, the concentration of free ligand is effectively reduced to zero and the ligand dissociates at a constant rate. This simulation demonstrates that under non-physiological conditions the majority of tiotropium has dissociated from the M_3 receptor at 24 h, whilst NVA237 has fully dissociated. However, under physiological conditions both ligands were fully dissociated from the M_3 receptors in less than 8 h. These results further

14

suggest that the receptor residency time cannot solely account for the clinical duration of action of LAMAs.

Onset of Action. Kinetic parameters were used to simulate the onset of action of NVA237 and tiotropium at a concentration 30-fold greater than their K_i (Table 1 and Fig. 3, A). Simulations using the kinetic rate constants predicted that tiotropium would take 4–5 times longer than NVA237 to equilibrate with the M_3 receptor (at equi-effective concentrations) (Fig. 3, A). 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 sub-maximal concentration of methacholine relative to tiotropium (Fig. 3, B). The $t_{1/2}$ of inhibition by NVA237 was 6.1 ± 2.1 min ($n = 3$), while the $t_{1/2}$ of inhibition by tiotropium was 29.4 ± 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. 3, C). The $t_{1/2}$ of inhibition of the bethanecol response by NVA237 was 9.8 ± 0.8 min, while the $t_{1/2}$ of inhibition by tiotropium was 24.6 ± 2.3 min.

M3 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) and the pK_d (K_{off}/K_{on}) values of [^{3}H]NMS as described in the methods. 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 ± 0.03 for M_2 receptors and 10.37 ± 0.04 for M_3 receptors (selectivity ratio: 2), and that of NVA237 was 8.70 ± 0.04 for M_2 receptors and 9.59 ± 0.05 for M_3 receptors (selectivity ratio: 7.8). Thus, NVA237 displayed a greater M_3 versus M_2 selectivity than tiotropium (7.8 versus 2-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

15

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_3 versus M_2 receptors than

tiotropium. The $t_{1/2}$ for tiotropium at M_2 and M_3 receptors was 10.8 min and 46.2 min,

respectively (kinetic selectivity ratio: 4.3), while the $t_{1/2}$ for NVA237 was 1.1 min 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 min 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 upon the compounds affinity for the various receptor

subtypes, only a defined proportion of muscarinic receptors are occupied following

administration of a single dose. Fig. 4, A and B, represents a simulation of this situation

using a concentration of NVA237 and tiotropium 30-fold greater than K_i for the muscarinic 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, while 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. 4, C). The

kinetic selectivity ratios obtained from these simulations are summarized in Table 4.

 pK_d 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 observation that sodium ions can significantly reduce muscarinic antagonist affinity (Birdsall et al., 1979). Results from the current study confirm this important finding. Further, whole-cell binding studies performed in HBSS demonstrate an almost identical affinity and kinetic off rate for [^{3}H]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 non-physiological 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 non-physiological 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 following 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 β_2-adrenoceptor agonists used in the treatment of COPD – $t_{1/2}$ values determined for indacaterol, a once-daily β_2-adrenoceptor agonist (0.2 min), and salmeterol, a twice-daily β_2-

17

adrenoceptor agonist (0.91 min), confirm that kinetic off rates from the β_2-adrenoceptor have no role to play in determining the duration of action of this class of molecule (Sykes and Charlton, 2012). Pharmacokinetic (PK) studies suggest that the systemic exposure achieved following inhalation of tiotropium and NVA237 is unlikely to produce functionally relevant occupancy of muscarinic receptors over a full 24-hour period (Disse et al., 1999; Sechaud et al., 2012). As a consequence, systemic PK profiles for these particular compounds do not help rationalize their 24-h duration of action in COPD patients.

Perhaps a more compelling argument to explain the long duration of these agents comes from studies examining drug rebinding (Vauquelin and Charlton, 2010). These 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 maintain 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 4–5 times longer to equilibrate with the M_3 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 previously described (Verkindre et al., 2010).

This study further demonstrates that NVA237 is kinetically more selective (9-fold) for M_3 receptors over M_2 receptors with $t_{1/2}$ values of 11.4 and 1.1 min, respectively. In contrast,

18

tiotropium is only 4-fold selective for M_3 over M_2 receptors under conditions of physiological sodium ion and temperature; this differs from previous reports which claimed a 10-fold kinetic selectivity for tiotropium, albeit under conditions of low sodium and at room temperature (Disse et al., 1999). Interestingly, although both compounds show a reduced affinity for the M_4 and M_5 receptors compared with M_{1-3} receptors, tiotropium has a relatively long receptor residency at both M_4 and M_5 receptors versus NVA237. In addition, NVA237 has a shorter $t_{1/2}$ on the M_1 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; Cooper et al., 2006b). In Brown Norway rat pre-treated intratracheally with NVA237 or tiotropium, NVA237 showed an improved therapeutic index over tiotropium, particularly for cardiovascular side effects (Trifilieff et al., 2007). This may be explained by the higher selectivity of NVA237 for M_3 over M_2 receptors, as demonstrated in the current study. Blockade of M_2 receptors attenuates the negative feedback inhibition of acetylcholine production, potentially reducing the bronchodilation produced by muscarinic agents (Belmonte, 2005). Besides the cholinergic nerve endings, M_2 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). M_1 and M_3 receptors enhance bronchoconstriction; M_1 receptors are common in the exocrine glands and in the CNS, 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 M_3 and M_1 receptors over M_2 receptors, while tiotropium does not appear to distinguish between these three receptor subtypes. This contrasts with previous studies performed under non-physiological conditions, which suggest that both compounds were non-selective for all three muscarinic subtypes (Haddad et al., 1999; Haddad et al., 1994; Casarosa et al., 2009). Furthermore, despite NVA237 having a 6-fold lower equilibrium affinity than tiotropium for the M_3 receptor, in clinical studies it is only dosed 3-fold higher (Verkindre et al., 2010) contradicting suggestions (Casarosa et al., 2009) that it is over-dosed relative to tiotropium to achieve its once-daily duration of action.

In conclusion, the results of this study cast doubt over the hypothesis that 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, recent 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; Vogelmeier and Banerji, 2011; Fogarty et al., 2011). In addition, with its higher kinetic selectivity for M_3 receptors over M_2 receptors and shorter residency time at M_4 and M_5 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 when 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.

Acknowledgements

Authorship Contributions

Participated in research design: DAS, MRD, JLD, TK, AT, SJC

Conducted experiments: DAS, MRD, JLD, LF, ER, AT, SJC

Performed data analysis: DAS, MRD, JLD, ER, AT, SJC

Wrote or contributed to the writing of the manuscript: DAS, MRD, TCK, ER, AT, SJC, LF

All authors read and approved the final draft of the manuscript.

Competing interests

All authors are employees of Novartis and declare no competing interests.

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FIGURE LEGENDS

Fig. 1. Example [^{3}H]-NMS competition kinetic curves for NVA237 and tiotropium under **A**, non-physiological and **B,** physiological conditions. CHO-M$_3$ membranes were incubated with ~2 nM [^{3}H]NMS and varying concentrations of competitor. Plates were incubated with constant shaking for the indicated time points, and nonspecific binding levels were determined in the presence of atropine (1 μM). Data were globally fitted to the equations described under *Materials and Methods* to calculate K_{on} and K_{off} values for the unlabeled antagonists. The whole data set is summarized in Table 1. Because the total binding varied from experiment to experiment, data are presented as the mean ± range from a representative of three or more experiments and plotted as specific bound.

Fig. 2. Simulation of M$_3$ receptor duration of action based on kinetic off rates from the M$_3$ receptor under A, non-physiological and B, physiological conditions. The kinetic off rate (K_{off}, k_2) of each ligand was fixed to those determined in the competition binding experiments, see Table 1. The simulations in Fig. 2 represent receptor previously equilibrated with a saturating concentration of ligand. At t = 0, the concentration of free ligand is effectively reduced to zero and the ligand dissociates at a constant rate.

Fig. 3. Onset of action studies. **A**, Predicted receptor occupancy rates of tiotropium and NVA237 at 30-fold greater than K_i. Data were simulated using the kinetic rate parameters shown in Table 1 and equations detailed in the simulation section of the methods. **B**, Time course of inhibition of methacholine (MCh) stimulated calcium release by tiotropium and NVA237. Tiotropium and NVA237 were preincubated at 37°C with CHO-M$_3$ cells at a concentration 30-fold greater than K_i for the indicated time period before addition of the muscarinic agonist MCh (1 nM). Data are representative of three independent experiments. **C**, Time course of inhibition of bethanechol stimulated contraction in the rat tracheal strip. The rat tracheal strip was prestimulated with bethanachol (30 μM), following stabilization of contraction tiotropium (3 nM) and NVA237 (10 nM) were added to the tissue preparation and

relaxation of contraction was monitored. Data are representative of three or more

independent experiments. **D**, Predicted receptor occupancy rates of tiotropium and NVA237

at concentrations of 3 and 10 nM respectively, as used in the rat tracheal study. Data were

simulated using the kinetic rate parameters stated in the text of the results section and

equations detailed in the simulation section of the methods.

Fig. 4. Simulated association and dissociation curves for **A**, NVA237 and **B**, tiotropium at a

concentration 30-fold greater than K_i. The kinetic on and off rate of each ligand was fixed to

those determined in the competition binding experiments, see 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**, Muscarinic

M$_3$ receptor selectivity ratios of NVA237 and tiotropium based on area under the curve

measurements from the simulations described in Fig. 4, **A** and **B**, using a concentration of

NVA237 and tiotropium at a concentration 30-fold greater than K_i.

TABLE 1

Comparison of the kinetic parameters of M_3 antagonists under non-physiological and

physiological conditions. Data are presented as the mean ± standard error from a

representative of three or more experiments.

Ligand	Non-physiological			Physiological		
	pK_d	$K_{off}(min^{-1})$	$t_{1/2}$ (min)	pK_d	$K_{off}(min^{-1})$	$t_{1/2}$ (min)
Tiotropium	11.10 ± 0.02	0.0015 ± 0.0002	462	10.37 ± 0.04	0.015 ± 0.002	46.2
NVA237	10.30 ± 0.04	0.004 ± 0.0002	173	9.64 ± 0.03	0.061 ± 0.003	11.4
[^{3}H]NMS	10.45 ± 0.02	0.017 ± 0.001	41	9.51 ± 0.05	0.31 ± 0.03	2.24

S.E., Standard Error; $t_{1/2,}$ half life.

TABLE 2

Kinetics of the interaction of [^{3}H]NMS with CHO membranes expressing the M$_{1-5}$ receptor under physiological conditions. Data are presented as the mean ± standard error from a representative of three or more experiments.

Receptor	Tiotropium				NVA237			
	K_{off} (min^{-1})	K_{on} (M^{-1}min^{-1})	Kinetic pK_d	t$_{1/2}$ (min)	K_{off} (min^{-1})	K_{on} (M^{-1}min^{-1})	Kinetic pK_d	t$_{1/2}$ (min)
M$_1$	0.0193 ± 0.002	3.37 ± 0.20 x10^8	10.23 ± 0.02	35.9	0.05 ± 0.002	1.16 ± 0.14 x10^8	9.46 ± 0.09	13.9
M$_2$	0.064 ± 0.006	8.03 ± 1 x10^8	10.13 ± 0.08	10.8	0.646 ± 0.04	6.03 ± 1 x10^8	8.72 ± 0.09	1.07
M$_3$	0.015 ± 0.002	2.29 ± 0.05 x10^8	10.20 ± 0.03	46.2	0.061 ± 0.003	1.94 ± 0.21 x10^8	9.50 ± 0.04	11.4
M$_4$	0.023 ± 0.006	3.06 ± 1.15 x10^8	10.06 ± 0.09	30.1	0.221 ± 0.034	2.55 ± 0.26 x10^8	9.07 ± 0.09	3.14
M$_5$	0.009 ± 0.001	6.60 ± 1.02 x10^7	9.83 ± 013	77	0.055 ± 0.012	7.62 ± 1.54 x10^7	9.15 ± 0.05	12.6

S.E., Standard Error; t$_{1/2,}$ half life.

TABLE 3

Affinity estimates and the M_3 selectivity ratio for tiotropium and NVA237 at muscarinic M_{1-5} receptor under physiological conditions. The M_3 selectivity ratio defines muscarinic receptor subtype selectivity as a ratio of the K_i of the individual subtypes over the K_i at muscarinic M_3 receptor. Data are presented as the mean ± standard error from a representative of three or more experiments.

Receptor	Tiotropium pK_i	NVA237 pK_i	M_3 selectivity ratio for tiotropium	M_3 selectivity ratio for NVA237
M_1	10.34 ± 0.04	9.60 ± 0.03	1.07	0.98
M_2	10.05 ± 0.03	8.70 ± 0.04	2.09	7.76
M_3	10.37 ± 0.04	9.59 ± 0.05	1.00	1.00
M_4	10.18 ± 0.07	9.06 ± 0.01	1.55	3.39
M_5	9.76 ± 0.07	8.91 ± 0.04	4.07	4.79

S.E., Standard Error.

TABLE 4

Kinetic selectivity ratios for tiotropium and NVA237 at muscarinic M_{1-5} receptors based on a concentration 30-fold greater than K_i at the muscarinic M_3 receptor under physiological conditions of sodium ion concentration and temperature based on the simulations shown in Figure 4, A and B

Receptor	Tiotropium kinetic selectivity ratio	NVA237 kinetic selectivity ratio
M_1	1.28	0.82
M_2	4.30	11.41
M_3	1	1
M_4	1.54	3.83
M_5	0.62	0.94

Fig. 1

Fig. 2

A

B

Fig. 3

A

Fig. 3

B

Change in fluorescence (% control)

Time (min)

Tiotropium (1.3 nM)

NVA237 (7.5 nM)

Fig. 3

C

Fig. 3

D

Fig. 4