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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 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 [³H]N-methyl-scopolamine ([³H]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 M₃ versus M₂ receptors, and faster off rate from M₂ versus M₃ 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 M₃ 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

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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

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(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

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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.

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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% CO₂/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,

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respectively) in assay binding buffer, either 10 mM HEPES containing 1 mM MgCl₂ at room temperature (non-physiological temperature and sodium concentration) or Hank's Balanced Salt Solution (HBSS, mM: CaCl₂, 1.26; MgCl₂, 0.49; MgSO₄, 0.41; KCl, 5.33; KH₂PO₄, 0.44; NaHCO₃, 4.2; NaCl, 138; Na₂HPO₄, 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 [³H]NMS at the rat M₃ receptor using rat CHO-cell membranes (20 µg well⁻¹) in HBSS at 37°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.

[³H]NMS (~2 nM, final assay volume of 1.5 mL) was incubated in the presence of the indicated concentration of unlabeled antagonist and M₁₋₅ CHO-cell membranes (10, 30, 10, 10 and 5 µg well⁻¹ respectively) in assay binding buffer, either 10 mM HEPES containing 1 mM MgCl₂ at room temperature or HBSS at 37°C, with gentle agitation for up to 500 min. IC₅₀ 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). [³H]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 M₃ receptor using rat CHO-cell membranes (20 µg well⁻¹) in HBSS at 37°C.

Calcium Assay. CHO-M₃ 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

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albumin, HEPES (20 mM), Fluo-4AM (2 μ M), and brilliant black (100 μ M) before addition of antagonists at various time intervals. Methacholine was added at an EC₈₀ 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; MgSO₄, 1.2; CaCl₂, 2.5; KH₂PO₄, 1.2; NaHCO₃, 25; glucose, 11) at 37°C bubbled with 95% O₂/5% CO₂. Resting tension was maintained at 1 g. After a stabilization period of 1 h, during which time the tissues were repeatedly washed, 30 μ M 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 \pm standard deviation (S.D.) for individual representative experiments; all values reported in the text and tables are mean \pm 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':

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / \left(1 + 10^{(\log \text{EC}_{50} - X) \cdot \text{Hill coefficient}} \right) \quad (1)$$

IC₅₀ 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:

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

[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.

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

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

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

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

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

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

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

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

$$\text{DIFF} = K_F - K_S$$

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

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

(3)

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Where:

X = Time (min)

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

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

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

L = Concentration of [³H]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 ($\text{M}^{-1} \text{min}^{-1}$)

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:

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

Where:

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

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

$$Y = (Y_0 - \text{NS}) * \exp(-K * X) + \text{NS}$$

Where:

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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 $0.69/K$.

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Results

Determination of the Kinetic Parameters of NVA237 and Tiotropium at M₃ 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 M₃

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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} 1.07×10^8 $M^{-1} \text{min}^{-1}$, K_{off} 0.071min^{-1}). In contrast, the kinetic K_{off} rate value for tiotropium was 2-fold lower than that observed in the human (0.007min^{-1} versus 0.015min^{-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

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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 [3H]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

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values obtained for [³H]NMS, the association and dissociation rates for NVA237 and tiotropium from the M₁₋₅ receptors were calculated. As mentioned previously, NVA237 has a faster off rate from the M₃ receptor and higher selectivity for M₃ versus M₂ receptors than tiotropium. The t_{1/2} for tiotropium at M₂ and M₃ 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 M₄ and M₅ receptors (30.1 and 77.0 min versus 3.14 and 12.6 min, respectively). The above paradigm assumes full receptor occupancy at both M₂ and M₃ 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 M₃ 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 M₁₋₅ receptors in less than 4 h, while some proportion of tiotropium is still bound to M₄ and M₅ receptors. In addition, NVA237 shows a much greater kinetic selectivity for the M₃ receptor over M₂, M₄ and M₅ 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.

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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 [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 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 -

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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,

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tiotropium is only 4-fold selective for M₃ over M₂ 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 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 t_{1/2} on the M₁ 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 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). Besides 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 in the CNS, and

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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, 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 M₃ 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 M₃ receptors over M₂ receptors and shorter residency time at M₄ and M₅ 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.

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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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References

- Abrams P, Andersson KE, Buccafusco JJ, Chapple C, de Groat WC, Fryer AD, Kay G, Laties A, Nathanson NM, Pasricha PJ, , and Wein AJ (2006) Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. *Br J Pharmacol* **148**: 565-578.
- Barnes PJ, Belvisi MG, Mark JC, Haddad EB, and O'Connor B (1995) Tiotropium bromide (Ba 679 BR), a novel long-acting muscarinic antagonist for the treatment of obstructive airways disease. *Life Sci* **56**: 853-859.
- Barr RG, Bourbeau J, Camargo CA, and Ram FS (2006) Tiotropium for stable chronic obstructive pulmonary disease: A meta-analysis. *Thorax* **61**: 854-862.
- Beeh KM and Beier J (2010) The short, the long and the "ultra-long": why duration of bronchodilator action matters in chronic obstructive pulmonary disease. *Adv Ther* **27**: 150-159.
- Belmonte KE (2005) Cholinergic pathways in the lungs and anticholinergic therapy for chronic obstructive pulmonary disease. *Proc Am Thorac Soc* **2**: 297-304; discussion 311-312.
- Birdsall NJM, Burgen ASV, and Hulme EC (1979) The effects of ions on the binding of agonists and antagonists to muscarinic receptors. *Br J Pharmacol* **66**: 337-342.
- Bourbeau J and Bartlett SJ (2008) Patient adherence in COPD. *Thorax* **63**: 831-838.
- Breekveldt-Postma NS, Koerselman J, Erkens JA, Lammers JW, and Herings RM (2007) Enhanced persistence with tiotropium compared with other respiratory drugs in COPD. *Respir Med* **101**: 1398-1405.

JPET #194456

Casaburi R, Briggs DD Jr, Donohue JF, Serby CW, Menjoge SS, and Witek TJ Jr (2000) The spirometric efficacy of once-daily inhaled tiotropium in stable COPD: a 13-week multicenter trial. The US tiotropium Study group. *Chest* **118**: 1294-1302.

Casaburi R, Mahler DA, Jones PW, Wanner A, San PG, ZuWallack RL, Menjoge SS, Serby CW, and Witek T Jr (2002) A long-term evaluation of once-daily inhaled tiotropium in chronic obstructive pulmonary disease. *Eur Respir J* **19**: 217-224.

Casarosa P, Bouyssou T, Germeyer S, Schnapp A, Gantner F, and Pieper M (2009) Preclinical evaluation of long-acting muscarinic antagonists: comparison of tiotropium and investigational drugs. *J Pharmacol Exp Ther* **330**: 660-668.

Charlton S, Sykes D, Dowling M, and Leighton-Davies J (2011) Slow off-rate from the muscarinic M₃ receptor is not sufficient to explain the once-daily duration of action of tiotropium and NVA237 *Am J Respir Crit Care Med* **183**: A1582.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) 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 (2006a) NVA237 and Tiotropium Bromide Demonstrate Similar Efficacy in an Anesthetized Rabbit Model of Methacholine-Induced Bronchoconstriction. NVA237 Demonstrates a Reduced Systemic Pharmacological Effect on Cardiovascular Parameters. *Proc Am Thoracic Soc* **3**: A117.

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 anaesthetized rabbit model. *Eur Respir J* **28(Suppl. 50)**: 436s.

JPET #194456

Disse B, Reichl R, Speck G, Traunecker W, Ludwig Rominger KL, and Hammer R (1993) Ba 679 BR, a novel long-acting anticholinergic bronchodilator. *Life Sci* **52**: 537-544.

Disse B, Speck GA, Rominger, Witek TJ Jr, and Hammer R (1999) Tiotropium (Spiriva): mechanistical considerations and clinical profile in obstructive lung disease. *Life Sci* **64**: 457-464.

Donohue J (2005) Minimal clinically important differences in COPD lung function. *COPD* **2**: 111-124.

Dowling MR and Charlton SJ (2006) Quantifying the association and dissociation rates of unlabelled antagonists at the muscarinic M3 receptor. *Br J Pharmacol* **148**: 927-937.

D'Urzo A, Ferguson GT, Kato M, Atis S, Martin C, Alagappan VKT, Banerji D, Lu Y, and Overend T (2011) NVA237 once daily provides rapid, clinically meaningful and sustained 24-h bronchodilation in patients with COPD: The GLOW1 trial [abstract]. *Eur Respir J* **38**(Suppl. 55): 3427.

Fogarty C, Hattersley H, Scala LD, and Drollmann A (2011) Bronchodilatory effects of NVA237, a once daily long-acting muscarinic antagonist, in COPD patients. *Respir Med* **105**: 337-342.

GOLD (2011) Global strategy for diagnosis, management and prevention of COPD.

Haddad EB, Mark JC, and Barnes PJ (1994) Characterization [³H] Ba 679 BR, a slowly dissociating muscarinic antagonist, in human lung: radioligand binding and autoradiographic mapping. *Mol Pharmacol* **45**: 899-907.

Haddad EB, Patel H, Keeling JE, Yacoub MH, Barnes PJ, and Belvisi MG (1999) Pharmacological characterization of the muscarinic receptor antagonist, glycopyrrolate, in human and guinea-pig airways. *Br J Pharmacol* **127**: 413-420.

JPET #194456

Hulme EC and Birdsall NJM (1992) *Strategy and tactics in receptor binding studies. In: Receptor-Ligand interactions. A practical approach.* Oxford University Press, New York.

Kesten S, Celli B, Decramer M, Leimer I, and Tashkin D (2009) Tiotropium Handihaler[®] in the treatment of COPD: a safety review. *Int J Chron Obstruct Pulmon Dis* **4**: 397-409.

Kuna P, Vinkler I, Overend T, Snape S, and Tansleyet R (2007) Efficacy and tolerability of NVA237, a once daily long-acting muscarinic antagonist, in COPD patients. *Eur Respir J* **30**(Suppl. 51): 2135.

Motulsky HJ and Mahan LC (1984) The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol Pharmacol* **25**: 1-9.

Ogoda M, Niiya R, Koshika T, and Yamada S (2011) Comparative characterization of lung muscarinic receptor binding after intratracheal administration of tiotropium, ipratropium and glycopyrrolate. *J Pharmacol Sci* **115**: 374-382.

Overend T, Fukuchi Y, Flemale A, Verkindre C, Takeda A, Johnson J, Prasad N, and Geba G (2008) Dose-ranging study to assess the efficacy and tolerability of NVA237, a once daily long-acting muscarinic antagonist, in patients with COPD. *Eur Respir J* **32**(Suppl. 52): 6306.

Smith DA, Jones BC, and Walker DK (1996) Design of drugs involving the concepts and theories of drug metabolism and pharmacokinetics. *Med Res Rev* **16**: 243-266.

Singh S, Loke YK, and Furberg CD (2008) Inhaled anticholinergics and risk of major adverse cardiovascular events in patients with chronic obstructive pulmonary disease. *JAMA* **300**: 1439-1450.

Singh S and Furberg CD (2011) Inhaled anticholinergics for chronic obstructive pulmonary disease: comment on "Inhaled anticholinergic drug therapy and the risk of

JPET #194456

acute urinary retention in chronic obstructive pulmonary disease". *Arch Intern Med* **171**: 920-922.

Sykes DA, Dowling MR, and Charlton SJ (2009) Exploring the mechanism of agonist efficacy: a relationship between efficacy and agonist dissociation rate at the muscarinic M₃ receptor. *Mol Pharmacol* **76**: 543-551.

Sykes DA and Charlton SJ (2012) Slow receptor dissociation is not a key factor in the duration of action of inhaled long acting $\beta(2)$ -adrenoceptor agonists. *Br J Pharmacol* **165**: 2672-2683.

Takahashi T, Belvishi MG, Patel H, Ward JK, Tadjkarimi S, Yacoub MH, and Barnes PJ (1994) Effect of Ba 679 BR, a novel anticholinergic agent, on cholinergic neurotransmission in guinea pig and human airways. *Am J Respir Crit Care Med* **150**: 1640-1645.

Tamura G and Ohta K (2007) Adherence to treatment by patients with asthma or COPD: comparison between inhaled drugs and transdermal patch. *Respir Med* **101**: 1895-1902.

Tashkin DP (2005) Is a long-acting inhaled bronchodilator the first agent to use in stable chronic obstructive pulmonary disease? *Curr Opin Pulm Med* **11**: 21-128.

Trifilieff A, Cope N, Bohacek B, Mazzoni L, and Collingwood S (2007) The inhaled muscarinic receptor antagonist, glycopyrrolate, has a favourable side-effect profile in a brown Norway rat lung function model when compared with tiotropium. *Chest* **132**: 530S.

Vauquelin G and Charlton SJ (2010) Long-lasting target binding and rebinding as mechanisms to prolong in vivo drug action. *Br J Pharmacol* **161**: 488-508.

Verkindre C, Fukuchi Y, Flemale A, Takeda A, Overend T, Prasad N, and Dolker M (2010) Sustained 24-h efficacy of NVA237, a once-daily long-acting muscarinic antagonist, in COPD patients. *Respir Med* **104**: 1482-1489.

JPET #194456

Vogelmeier C, Verkindre C, Cheung D, Galdiz JB, Güçlü SZ, Spangenthal S, Overend T, Henley M, Mizutani G, and Zeldin RK (2010) Safety and tolerability of NVA237, a once-daily long-acting muscarinic antagonist, in COPD patients. *Pulm Pharmacol Ther* **23**: 438-444.

Vogelmeier C and Banerji D (2011) NVA237, a long-acting muscarinic antagonist, as an emerging therapy for chronic obstructive pulmonary disease. *Ther Adv Respir Dis* **5**: 163-173.

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FIGURE LEGENDS

Fig. 1. Example [^3H]-NMS competition kinetic curves for NVA237 and tiotropium under **A**, non-physiological and **B**, physiological conditions. CHO-M₃ membranes were incubated with ~2 nM [^3H]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 \pm range from a representative of three or more experiments and plotted as specific bound.

Fig. 2. Simulation of M₃ receptor duration of action based on kinetic off rates from the M₃ 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-M₃ 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 bethanechol (30 μM), following stabilization of contraction tiotropium (3 nM) and NVA237 (10 nM) were added to the tissue preparation and

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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 .

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TABLE 1

Comparison of the kinetic parameters of M₃ 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		
	<i>pK_d</i>	<i>K_{off} (min⁻¹)</i>	<i>t</i> _{1/2} (min)	<i>pK_d</i>	<i>K_{off} (min⁻¹)</i>	<i>t</i> _{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
[³H]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 [³H]NMS with CHO membranes expressing the M₁₋₅ 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 × 10 ⁸	10.23 ± 0.02	35.9	0.05 ± 0.002	1.16 ± 0.14 × 10 ⁸	9.46 ± 0.09	13.9
M ₂	0.064 ± 0.006	8.03 ± 1 × 10 ⁸	10.13 ± 0.08	10.8	0.646 ± 0.04	6.03 ± 1 × 10 ⁸	8.72 ± 0.09	1.07
M ₃	0.015 ± 0.002	2.29 ± 0.05 × 10 ⁸	10.20 ± 0.03	46.2	0.061 ± 0.003	1.94 ± 0.21 × 10 ⁸	9.50 ± 0.04	11.4
M ₄	0.023 ± 0.006	3.06 ± 1.15 × 10 ⁸	10.06 ± 0.09	30.1	0.221 ± 0.034	2.55 ± 0.26 × 10 ⁸	9.07 ± 0.09	3.14
M ₅	0.009 ± 0.001	6.60 ± 1.02 × 10 ⁷	9.83 ± 0.13	77	0.055 ± 0.012	7.62 ± 1.54 × 10 ⁷	9.15 ± 0.05	12.6

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

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TABLE 3

Affinity estimates and the M₃ selectivity ratio for tiotropium and NVA237 at muscarinic M₁₋₅ receptor under physiological conditions. The M₃ selectivity ratio defines muscarinic receptor subtype selectivity as a ratio of the K_i of the individual subtypes over the K_i at muscarinic M₃ receptor. Data are presented as the mean ± standard error from a representative of three or more experiments.

Receptor	Tiotropium pK_i	NVA237 pK_i	M₃ selectivity ratio for tiotropium	M₃ 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.

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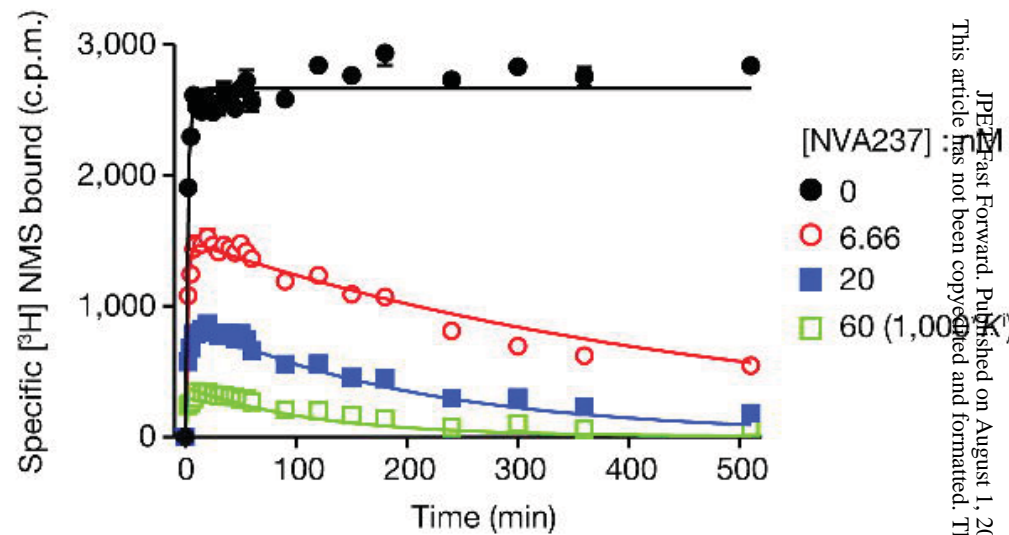
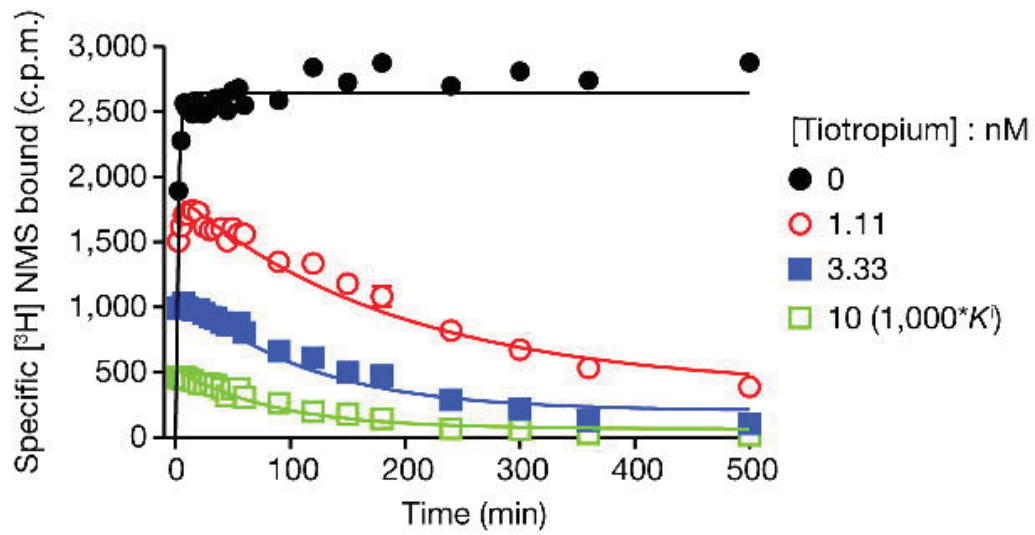
TABLE 4

Kinetic selectivity ratios for tiotropium and NVA237 at muscarinic M₁₋₅ receptors based on a concentration 30-fold greater than K_i at the muscarinic M₃ 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

A



B

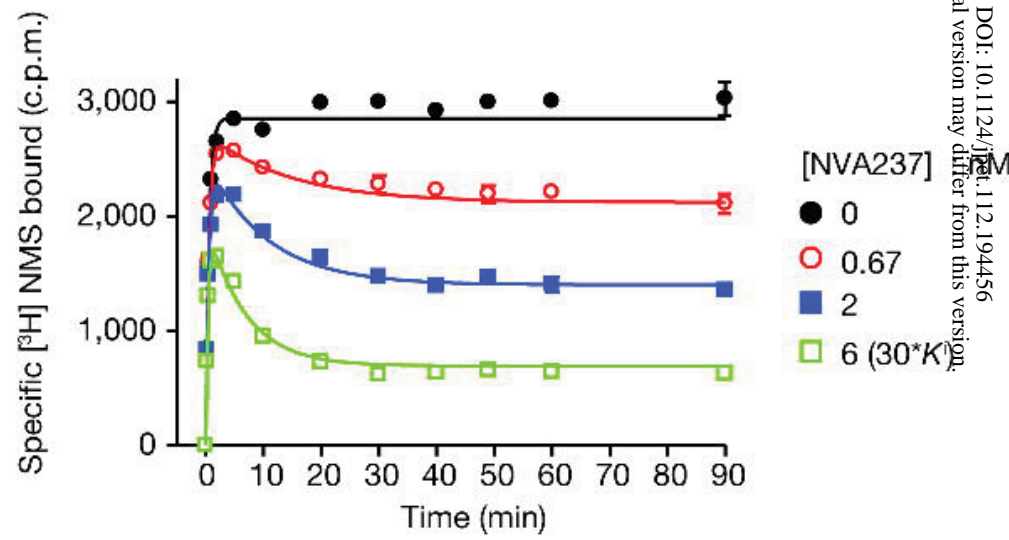
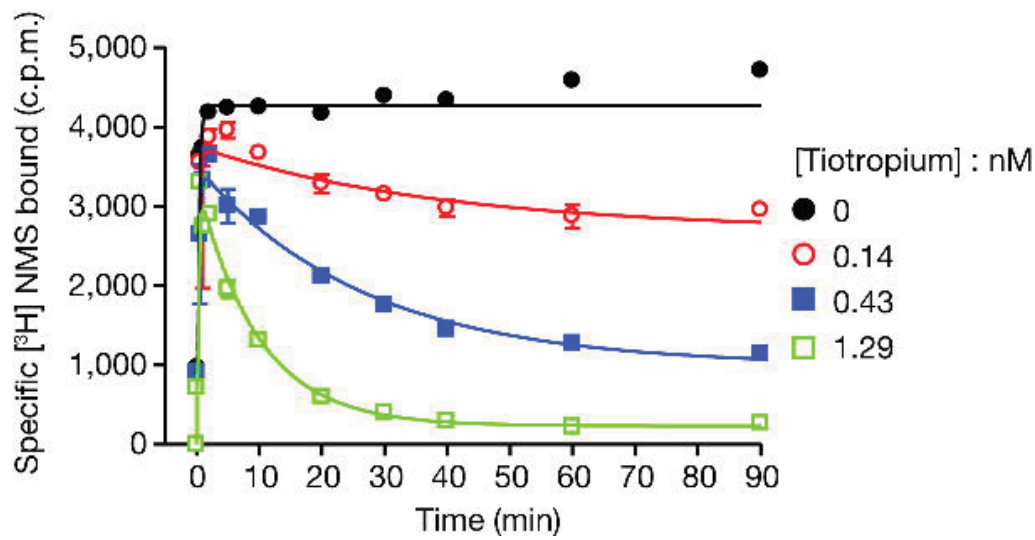
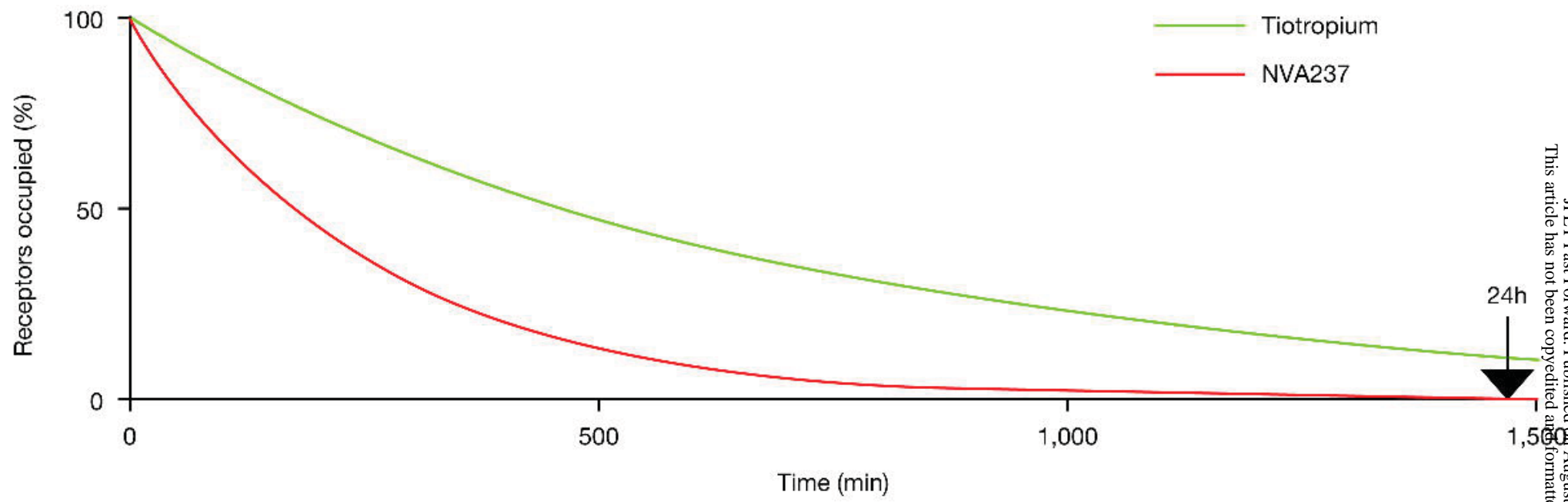
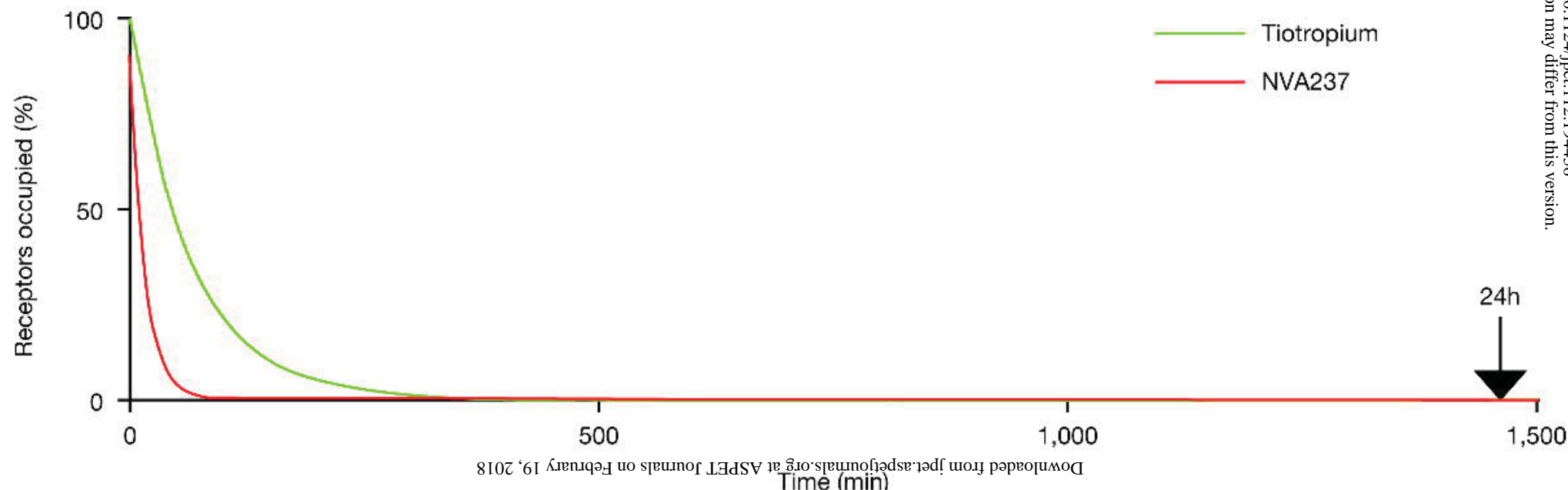


Fig. 2

A



B



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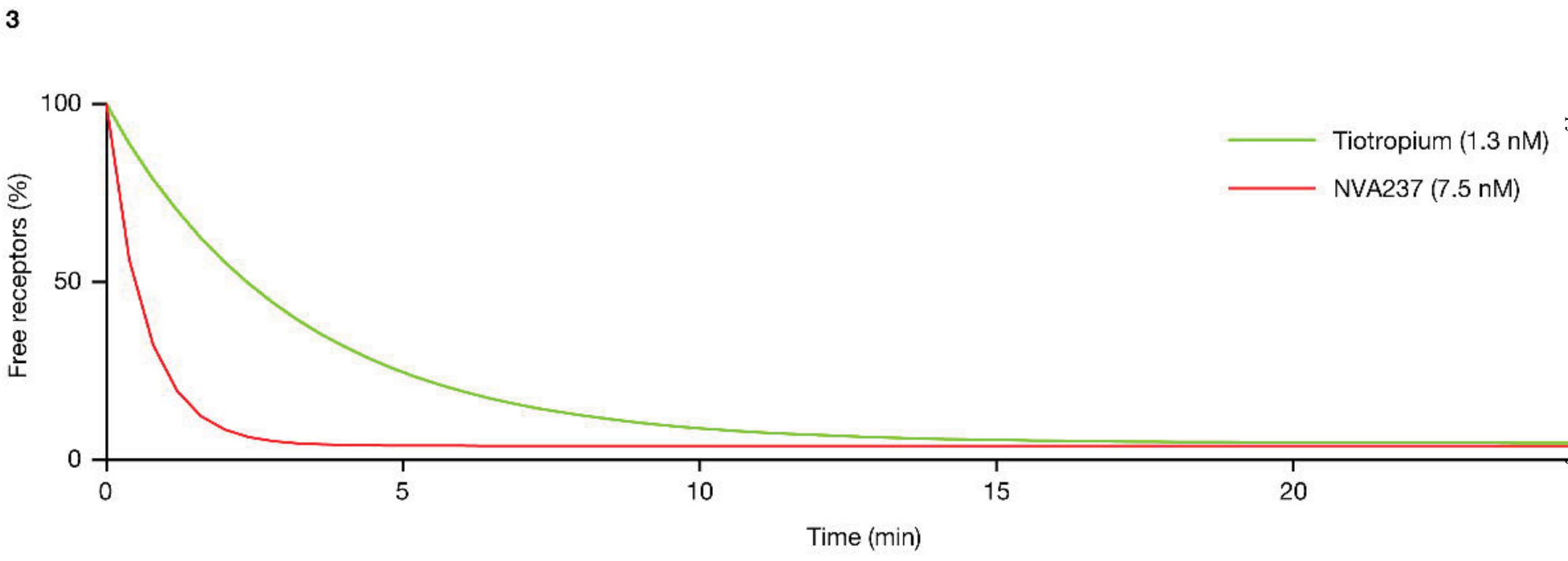


Fig. 3

B

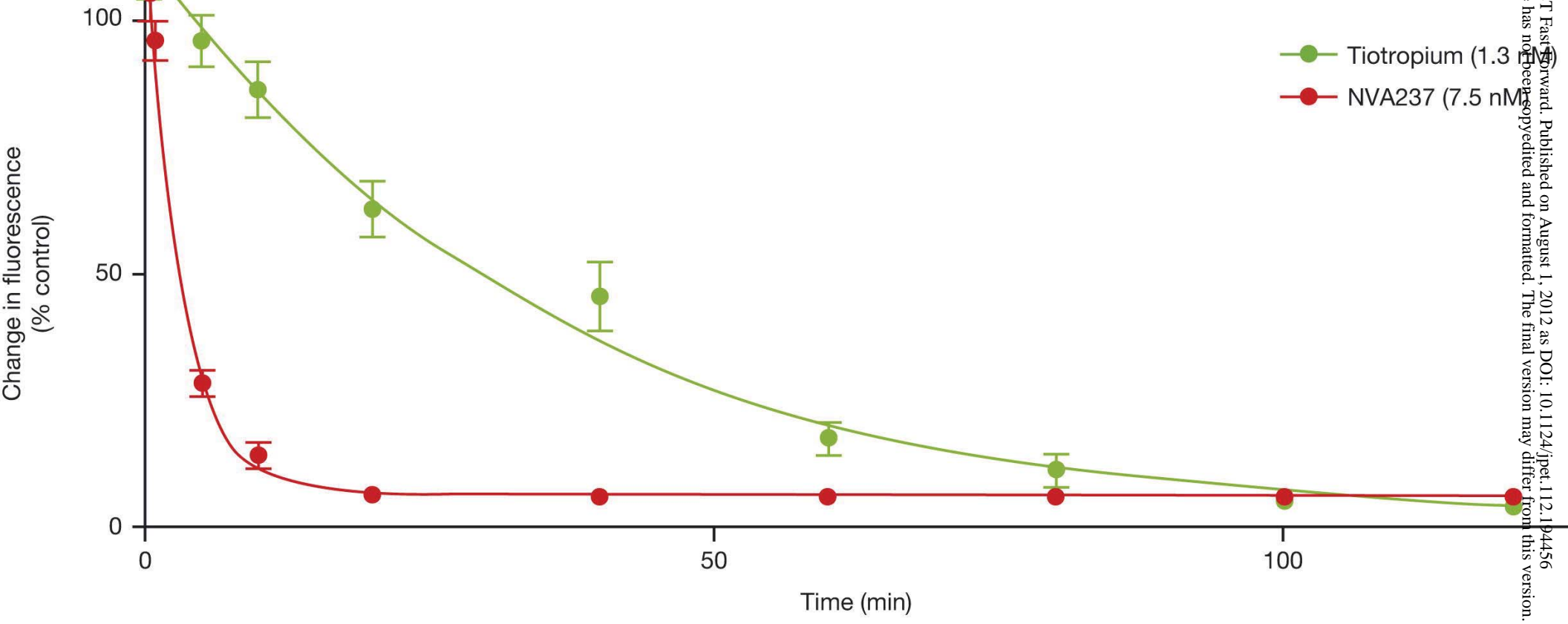


Fig. 3

C

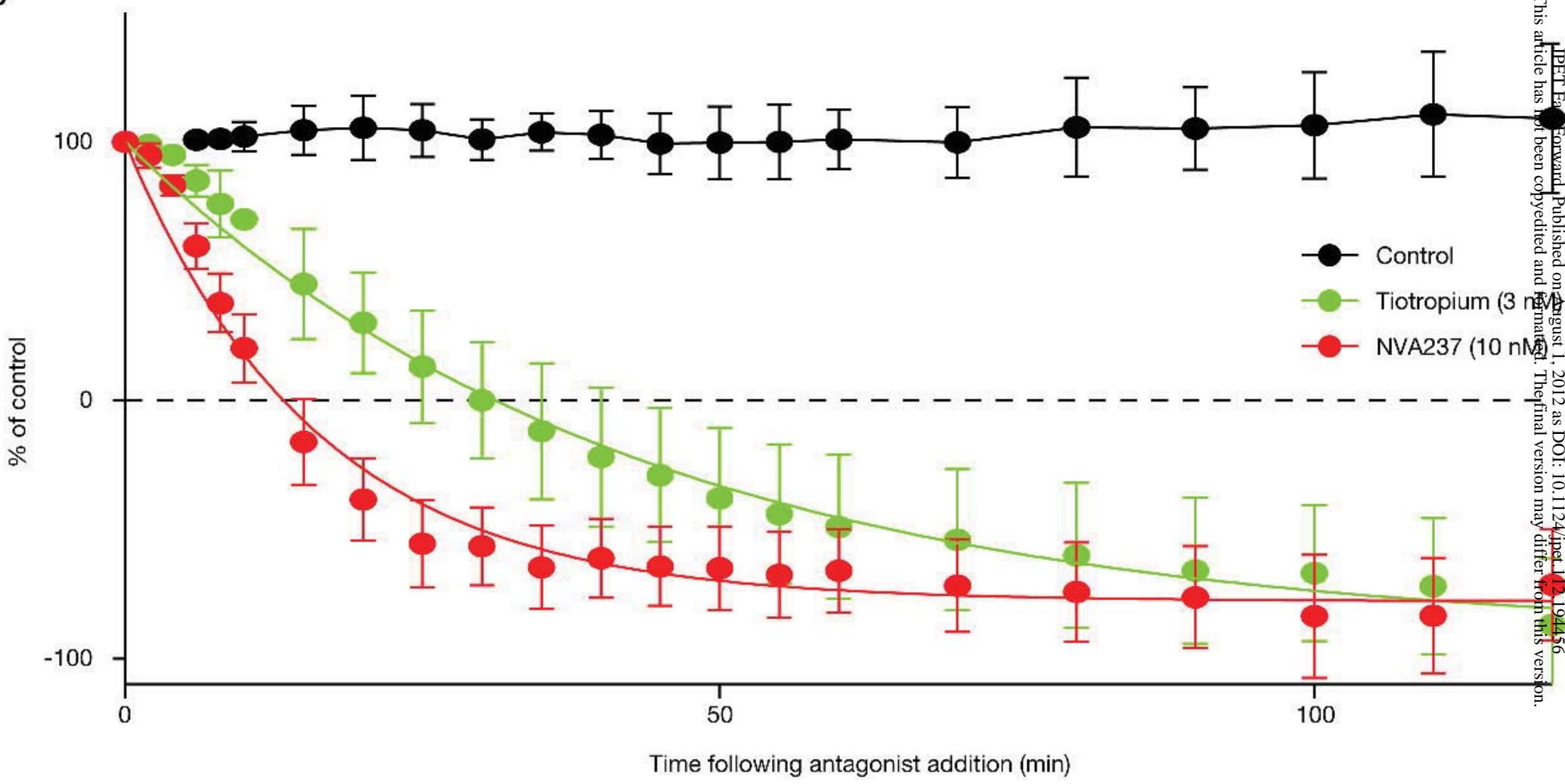


Fig. 3

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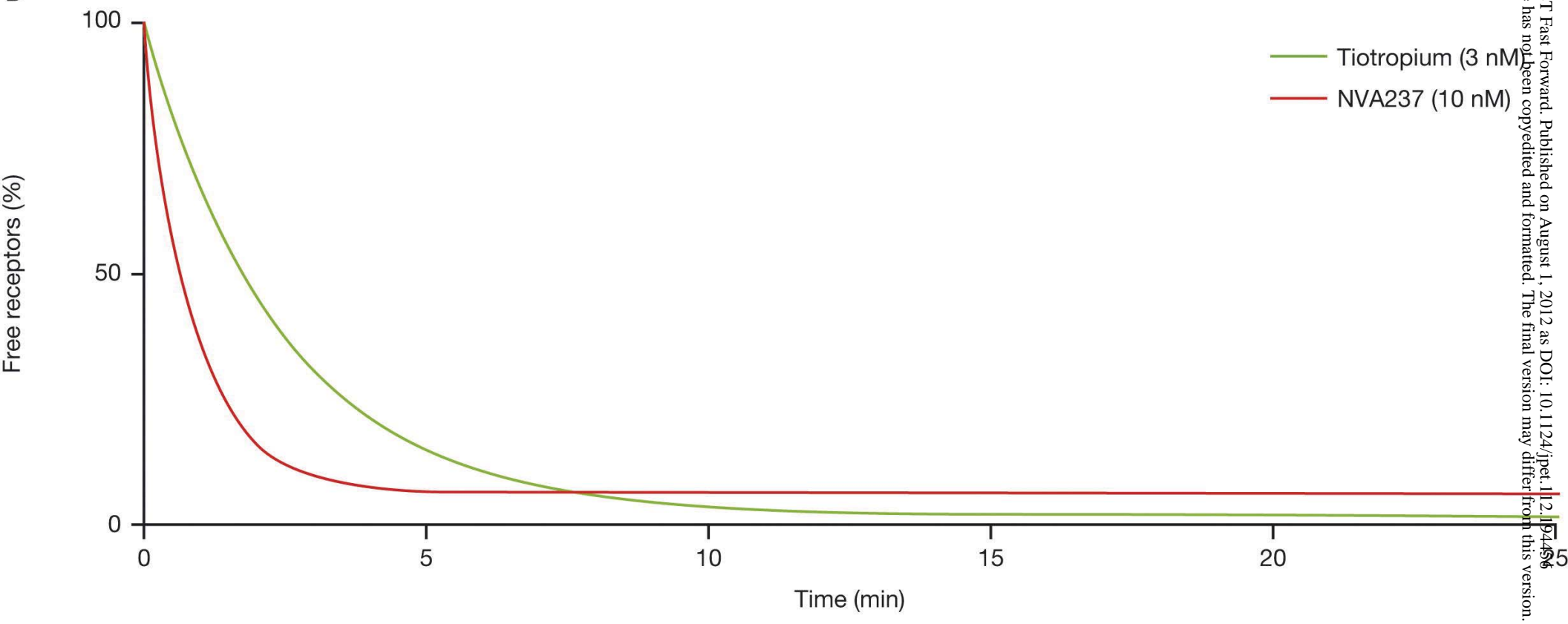
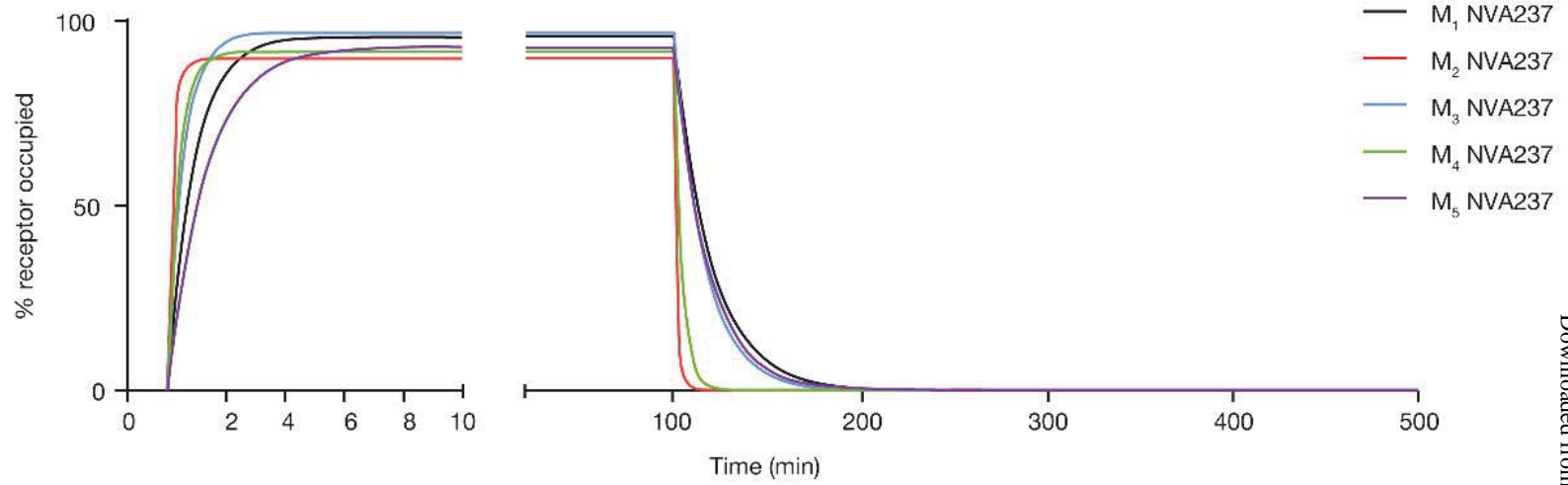
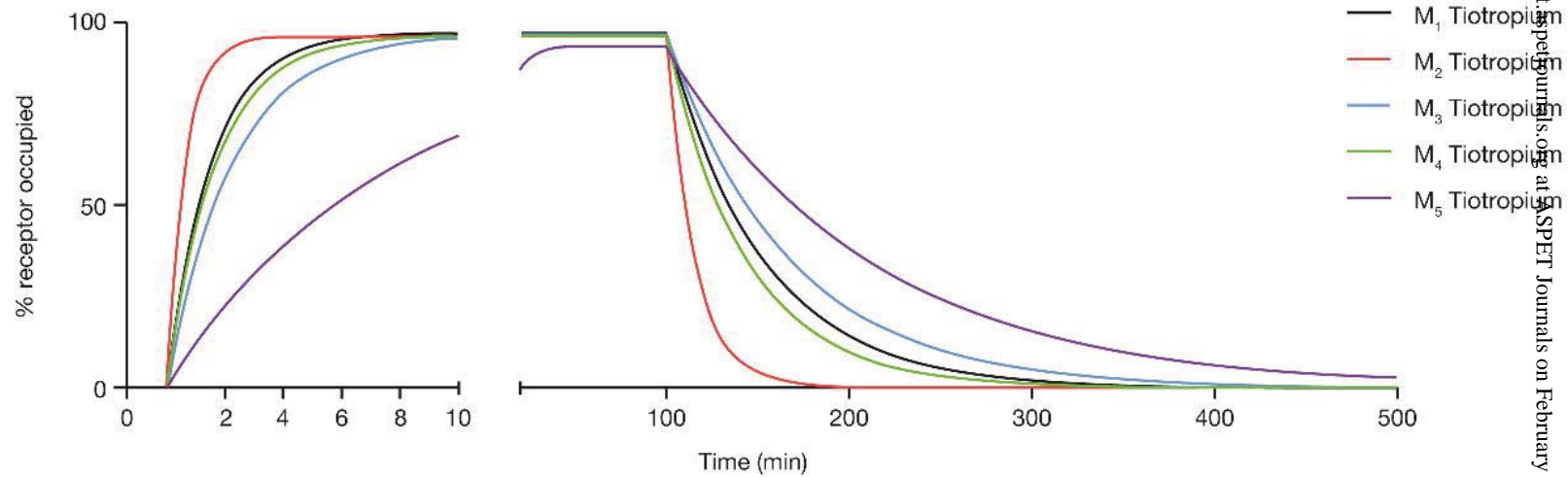


Fig. 4

A



B



C

