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Label-free kinetics: exploiting functional hemi-equilibrium to derive rate constants for muscarinic receptor antagonists

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Kinetics of mAChR ligands in binding and functional assays

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Abbreviations

4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide, ACh, acetylcholine, MCh, methacholine, NMS, N-methylscopolamine, NSB, non-specific binding, pERK, phosphorylated extracellular signal-regulated kinase, QNB, quinuclidinyl benzilate
Abstract

Drug receptor kinetics is as a key component in drug discovery, development and efficacy. However, determining kinetic parameters has historically required direct radiolabelling or competition with a labelled tracer. Here we present a simple approach to determine the kinetics of competitive antagonists of GPCRs exploiting the phenomenon of hemi-equilibrium, the state of partial re-equilibration of agonist, antagonist and receptor in some functional assays. Using functional [Ca^{2+}]_i-flux and ERK1/2 phosphorylation assays that have short incubation times and therefore prone to hemi-equilibrium ‘behaviors’, we have investigated a wide range of structurally and physico-chemically distinct muscarinic acetylcholine receptor (mAChR) antagonists. Using a combined operational/hemi-equilibrium model of antagonism to both simulate and analyse data, we derived estimates of association and dissociation rates for the test set of antagonists, identifying both rapidly dissociating (4-DAMP, himbacine) and slowly dissociating (tiotropium, glycopyrrolate) ligands. The results demonstrate the importance of assay incubation time and degree of receptor reserve in applying the analytical model. There was an excellent correlation between estimates of antagonist pK_B, k_on and k_off from functional assays and those determined by competition kinetics using whole-cell [^3H]-NMS binding, validating this approach as a rapid and simple method to functionally profile receptor kinetics of competitive antagonists in the absence of a labelled tracer.
Introduction

In recent years the importance of the kinetics of a drug binding to its receptor has become readily accepted as a key parameter in the drug discovery process. Recent literature on kinetic analysis of preclinical candidates has aided in understanding the mechanism of action of these compounds and their contribution to therapeutic efficacy. For example, GSK1004723 was identified as a potent histamine H1R and H3R antagonist for the treatment of allergic rhinitis, with a kinetically driven long duration of action (Slack et al., 2011). Tiotropium and glycopyrronium bromide (NVA237), which have both yielded positive Phase III results in the treatment of chronic obstructive pulmonary disease (Jones, 2015), have greater kinetic selectivity for the M3 muscarinic acetylcholine receptor (mAChR) in comparison to other mAChR family members, in line with the documented large therapeutic index of these drugs (Sykes et al., 2012). More recently, antagonists of the orexin-2 receptor for the treatment of insomnia could be differentiated based on their kinetic parameters, with almorexant being identified as a pseudo-irreversible antagonist (Mould et al., 2014).

Typically, kinetic parameters of compounds are rarely optimised during early stage drug discovery, as this often requires labelling the compounds with a suitable radioisotope, or addition of a fluorescent moiety. Whilst these approaches can provide direct and precise data, both can prove extremely costly, technically challenging and are normally reserved for a select subset of compounds. One alternative approach, amenable to early stage drug discovery, is the method of Motulsky & Mahan (1984). In this method, the binding of a kinetically well-defined radioligand is monitored in the presence or absence of a competitor ligand. Changes to the kinetic rates of the radioligand can be analysed and the rates of the competitor ligand determined (Dowling & Charlton, 2006). Obviously, the major limiting factor to this method is the availability of a robust, high affinity radioligand.

Hemi-equilibrium is a phenomenon in functional assays whereby a reversible, competitive antagonist produces a depression in the maximal agonist response due to insufficient agonist-
antagonist-receptor re-equilibration time between addition of agonist (to initiate a response) and the measurement of the response. Such effects are typically seen in transient assay readouts, such as calcium assays (Charlton & Vauquelin, 2010). A competitive antagonist can display a range of behaviours, from fully surmountable to fully insurmountable antagonism, subject to both the kinetics of ligand binding and assay re-equilibration time (Gaddum, 1957).

Analysis of these ‘equilibrium artefacts’ has previously revealed that the depression of the maximal agonist response is directly related to the dissociation rate of the competing antagonist (Christopoulos et al., 1999; Mathiesen et al., 2006). Accordingly, we hypothesized that this effect could be exploited to profile the binding kinetics of a range of mAChR antagonists in functional assays with different incubation times. However, functional assays are also subject to the added phenomenon of “receptor reserve”, which can mask the effects of insurmountable antagonists (Kenakin et al., 2006). For example, SR 48968, a non-peptide tachykinin NK₂ receptor antagonist has been labeled as either a surmountable or insurmountable antagonist in functional experiments depending on the different tissues and different species used (Patacchini et al., 2000).

To determine the utility of functional assays to provide quantitative estimates of ligand binding kinetics, a system is required that is amenable to profiling both in functional assays, but also using a more traditional approach by competitive radioligand binding kinetics (Dowling & Charlton, 2006). To validate our method, we used Flp-In-CHO cells stably expressing the M₁ mAChR, a physiologically relevant buffer, whole cell competition kinetics and [Ca²⁺]-flux and ERK1/2 phosphorylation functional assays. The M₁ mAChR was chosen as it is a prototypical family A G protein-coupled receptor (GPCR) that has been widely characterized in multiple binding and functional assays, and has a wide range of comprehensively studied, structurally distinct, competitive antagonists available as pharmacological tools (Christopoulos et al., 1999; Jakubik et al., 2006). Radioligand binding kinetics were analysed according to the method of Motulsky & Mahan (1984), whereas
functional data were analysed using a combined operational/hemi-equilibrium model of competitive antagonism (Kenakin, 2009). Herein we demonstrate the feasibility of using functional assays to estimate kinetic rates of unlabeled antagonists without the necessity of a suitable radioligand. This method promises significant impact on early stage drug discovery, enabling receptor kinetics to be incorporated into the traditional structure activity relationship (SAR) process.
Materials and Methods

Cell line generation and culture conditions of Flp-In-CHO-hM₁ mAChR

CHO-M₁ mAChR cell lines were generated as previously described by Avlani et al., (2007). For binding studies 7,000 Flp-In-CHO-hM₁ mAChR cells per well (100 μL final volume per well) were seeded in F-12 GlutaMax containing 10% FBS into 96-well ISOPLATE TC plates the day prior to experimentation and allowed to grow overnight at 37°C and 5% CO₂. For all functional experiments 30,000 cells per well (100 μL final volume per well) were seeded in F-12 GlutaMax containing 10% FBS into clear 96-well plates the day prior to experimentation and incubated for 8 h at 37°C and 5% CO₂ prior serum starving the cells with 100 μL of F-12 GlutaMax, overnight.

Common procedures applicable to all radioligand binding experiments

Radioligand binding experiments were performed on Flp-In-CHO cells stably expressing the hM₁ mAChR. The following day after plating, cells were washed with phosphate-buffered saline (100 μL) and re-suspended in binding buffer (110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgSO₄, 25 mM glucose, 20 mM HEPES, and 58 mM sucrose; pH 7.4 with NaOH). Due to the rapid association of [³H]-NMS at 37°C all experiments were performed at 22°C. Assay mixtures, in a total volume of 200 μL with a 1/10 dilution of drug, were incubated at 22°C. Assays were terminated by buffer removal followed by rapid washing, twice, with ice-cold 0.9% NaCl (100 μL). Plates were allowed to dry inverted for 30 min; OptiPhase Supermix scintillation cocktail (100 μL) was added, plates were sealed (TopSealTM) and radioactivity was measured in a MicroBeta2 LumiJET microplate counter.

Saturation binding experiments were performed in the absence or presence of atropine (100 μM) with 0.003–3 nM [³H]-NMS (1mCi, 37MBq). Inhibition binding experiments were performed with 0.35 nM [³H]-NMS (the approximate Kᵯ) in the presence of various concentrations of ligands. In all experiments, total binding never exceeded more than 10% of that added, limiting complications associated with depletion of the free radioligand
concentration.

**Kinetics of [3H]-NMS binding to whole cells**

To accurately determine $k_{on}$ and $k_{off}$ values, at least three concentrations of [3H]-NMS, were incubated with 7,000 Flp-In-CHO-hM$_1$ mAChR cells well per well in binding buffer at 22°C until equilibrium was reached. Assays were terminated as described above.

**Determination of antagonist affinity constants (pK$_I$) and kinetic parameters ($k_{on}$ and $k_{off}$) on whole cells**

To determine the kinetic parameters of unlabeled antagonists a competition kinetic binding assay was used (Dowling & Charlton, 2006). [3H]-NMS (0.4 nM) was incubated with 7,000 Flp-In-CHO-hM$_1$ mAChR cells well per well in binding buffer at 22°C, with gentle agitation for 22 h. IC$_{50}$ values were converted to equilibrium binding constants (pK$_I$) by using the method of Cheng & Prusoff (1973). Experiments were initiated by the co-addition of [3H]-NMS (0.4 nM) and unlabeled competitor to plated cells. 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), atropine, himbacine, ipratropium, N-methyl scopolamine (NMS) and pirenzepine were tested at 1, 3 and 10x K$_I$, glycopyrrolate and quinuclidinyl benzylate (QNB) at 10, 30 and 100x K$_I$ and tiotropium was tested at 30, 100 and 300x K$_I$, as determined in the equilibrium binding assay. Assays were terminated as described above.

**[Ca$^{2+}$]-flux assay**

On the day of the experiment, the cells were washed with 100 μL of phosphate buffered saline. The cells were then incubated with Ca$^{2+}$ assay buffer (110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgSO$_4$, 25 mM glucose, 20 mM HEPES, and 58 mM sucrose, 0.5% (w/v) bovine serum albumin (BSA), and 4 mM Probenecid, pH 7.4) containing 2 μM Fluo-4-AM for 1 h under humidified conditions at 37°C. Each assay plate was then loaded into a FLEXstation (Molecular Devices Inc., Sunnyvale, CA) with its stock compound plate. The
FLEXstation measured fluorescence over a 75 s time period using 485nm excitation and 538nm emission wavelengths and performed the addition of drugs (1:10 dilution) at the 15 s time point. For all interaction studies, mAChR antagonists were added 30 min prior agonist stimulation. For each interaction, the peak of maximum fluorescence induced by either methacholine (MCh) or acetylcholine (ACh) addition, between 15 and 75 s was chosen and corrected to the baseline (fluorescence from 0 to 14 s), then normalized to 1 mM ACh-mediated response.

**ERK1/2 phosphorylation assay**

Initial ERK1/2 phosphorylation time course experiments were performed to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by MCh or ACh. For subsequent agonist-stimulated concentration response experiments, cells were incubated at 37°C with each agonist for the 5 min required to achieve peak response. For interaction experiments, mAChR antagonists were incubated for 30 min prior agonist stimulation. In all experiments, 10% FBS was used as a positive control. The reaction was terminated by removal of drugs and lysis of cells with 80 μL SureFire™ lysis buffer (TGR Biosciences). The lysates were agitated for 1 – 2 min and 5 μL was transferred into 384-Optiplate (PerkinElmer Life and Analytical Sciences) prior addition of a mixture of Surefire™ Activation buffer, Reaction buffer and Acceptor/Donor beads (10:60:0.3:0.3) in a total volume of 13.5 μL. Plates were incubated in the dark at 37°C for 60 min, then 22°C for 10 min, before the fluorescence signal was measured using an Envision plate reader (PerkinElmer Life and Analytical Sciences) using standard AlphaScreen settings.

**Determination of cLogD<sub>pH7.4</sub> values**

These values were obtained using ChemAxon MarvinSketch version 14.11.10.0 (Budapest, Hungary), using a weighted LogP method, with this method being the arithmetic mean of methods of Viswanadhan et al., (1989), Klopman et al., (1994) and PHYSPROP© database (Syracuse Research Corporation, US).
Compounds and reagents

mAChR antagonists were purchased from Sigma Aldrich, Castle Hill, Australia. Radioligand was purchased from Perkin Elmer, Melbourne, Australia. Cell culture and molecular biology reagents were supplied by Life Technologies, Melbourne, Australia.

Data analysis

Due to the amount of radioactivity varying slightly for each experiment (<5%), data are shown graphically as the mean ± SD from a single fit to grouped data of individual experiments, and values reported in the text and tables are mean ± SD for the indicated number of experiments. All experiments were analyzed by either linear or nonlinear regression using Prism 6.0f (GraphPad Software Inc., San Diego, CA) as described below.

Saturation binding

Saturation binding isotherms were globally fitted to nonspecific and total binding data, and individual estimates for total receptor number (Bmax) and radioligand dissociation constant (Kd) were calculated:

\[ Y = \frac{B_{\text{max}} \cdot [A]}{[A] + K_A} + \text{NS} \cdot [A] \]  

where \( Y \) is the radioligand binding, \( B_{\text{max}} \) is the total receptor density, \( [A] \) is the concentration of radioligand, \( K_A \) is the equilibrium dissociation constant of the radioligand, and \( \text{NS} \) is the fraction of nonspecific radioligand binding:

Competition binding

Competition displacement binding data were fitted to sigmoidal three-parameter logistic equation:
\[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{\frac{\log IC_{50}}{\text{Top} - \text{Bottom}}}} \]  

where \( Y \) is the \% bound of radioligand. Top denotes maximal asymptotic binding and bottom denotes the minimal asymptotic binding. IC\(_{50}\) values obtained from the inhibition curves were converted to pK\(_1\) values using the method of Cheng & Prusoff (1973).

**Association binding**

[^3H]-NMS association data were globally fitted. \( k_{on} \) and \( k_{off} \) were shared across the data sets so that a single value for each was derived from the whole family of curves.

\[ Y = Y_{\text{max}} \cdot \left(1 - e^{-k_{\text{obs}} \cdot X}\right) \]  

where \( k_{\text{obs}} = [\text{radioligand}] \times k_{on} + k_{off} \); \( Y = \text{specific binding (cpm)} \); \( Y_{\text{max}} = \text{maximal specific binding} \) and \( X = \text{time} \).

**Competition binding kinetics**

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

\[ K_A = k_1 [L] + k_2 \]  
\[ K_B = k_3 [I] + k_4 \]  
\[ S = \sqrt{\left((K_A - K_B)^2 + 4 \cdot k_3 \cdot k_4 \cdot L \cdot I \cdot 1e^{-10}\right)} \]  
\[ K_F = 0.5 \cdot (K_A + K_B + S) \]  
\[ K_S = 0.5 \cdot (K_A + K_B - S) \]  
\[ DIFF = K_F - K_S \]
where $X = \text{time (min)}$; $Y = \text{specific binding (cpm)}$; $k_1 = k_{\text{on}} [^3\text{H}]$-NMS; $k_2 = k_{\text{off}} [^3\text{H}]$-NMS; $L = \text{concentration of [^3\text{H}]$-NMS used (nM)$; and $I = \text{concentration of unlabeled antagonist (nM)$.

Fixing the above parameters allowed the following to be calculated: $B_{\text{max}} = \text{total binding (cpm)}$; $k_3 = \text{association rate of unlabeled ligand (M}^{-1} \cdot \text{min}^{-1})$; and $k_4 = \text{dissociation rate of unlabeled ligand (min}^{-1}$).

**Operational model of agonism**

To estimate agonist efficacy ($\tau$), agonist concentration-response curves in both $[\text{Ca}^{2+}]_i$-flux and ERK1/2 phosphorylation assays were fitted to the following form of an operational model of agonism (Black & Leff, 1983):

$$Y = Basal + \frac{E_m - Basal}{1 + 10^{\log K_A + 10^{\log [A]} / 10^{\log \tau} \times 10^{\log ([A])}}}$$

where $E_m$ is the maximal possible response of the system (not the agonist), Basal is the basal level of response in the absence of agonist, $K_A$ denotes the functional equilibrium dissociation constant of the agonist (A), $\tau$ is an index of the coupling efficiency (efficacy) of the agonist and is defined as $R_T/K_E$ where $R_T$ is the total concentration ($B_{\text{max}}$) of receptors and $K_E$ is the concentration of agonist-receptor complex that yields half the maximum system response ($E_m$). To define the $E_m$ and $\tau$ of ACh and MCh in each assay, the $K_A$ for each agonist was constrained to equal the $K_I$ value derived from radioligand binding assays (see Results) in the nonlinear regression procedure.
Schild analysis

To estimate antagonist affinity values, agonist concentration-response data in the presence of increasing concentrations of antagonist were fitted to the following form of Schild regression analysis (Arunlakshana & Schild, 1959; Motulsky & Christopoulos, 2004):

\[ E = Basal + \frac{(E_{\text{max}} - Basal)}{1 + \left(10^{-pEC_{50}} \frac{1 + ([B]/10^{pA_2})^s}{10^{p_{\text{log}[A]}}} \right)} \]  

where \( E \) is response, \( E_{\text{max}} \) and basal are the top and bottom asymptotes of the curve, respectively, \( \log[A] \) is the logarithm of the agonist concentration, \( pEC_{50} \) is the negative logarithm of the agonist concentration that gives a response halfway between \( E_{\text{max}} \) and basal, \( s \) represents the Schild slope for the antagonist, and \( pA_2 \) represents the negative logarithm of the molar concentration of antagonist necessary to double the concentration of agonist needed to elicit the original submaximal response obtained in the absence of antagonist.

Operational/hemi-equilibrium model for competitive antagonism

If compounds showed a depression of the agonist \( E_{\text{max}} \) then data were fitted according to a combined operational/hemi-equilibrium model for competitive antagonism under non-equilibrium conditions (Kenakin, 2009; Mould et al., 2014), and estimates of antagonist affinity values, \( pK_B \), and their dissociation constant from the receptor, \( k_{\text{off}} \), were determined:

\[ \gamma = \frac{[A]/K_A \left(1 - \left(\alpha \cdot \left(1 - e^{-k_{\text{off}} \cdot \gamma \cdot \tau}\right) + \beta \cdot e^{-k_{\text{off}} \cdot \gamma \cdot \tau}\right) \cdot \tau \cdot E_m\right)}{[A]/K_A \left(1 - \left(\alpha \cdot \left(1 - e^{-k_{\text{off}} \cdot \gamma \cdot \tau}\right) + \beta \cdot e^{-k_{\text{off}} \cdot \gamma \cdot \tau}\right) \cdot \tau + 1\right) + 1} \]  

where:

\[ \alpha = \frac{[B]/K_B}{([B]/K_B + [A]/K_A + 1)} \]

\[ \beta = \frac{[B]/K_B}{([B]/K_B + 1)} \]
\[ \gamma = \frac{([B]/K_B + [A]/K_A + 1)}{([A]/K_A + 1)} \]

where \([A]\) and \([B]\) represent the concentrations of agonist, and antagonist, respectively, \(K_A\) and \(K_B\) represent the respective equilibrium dissociation constants, \(k_{\text{off}}\) is the dissociation rate constant for the antagonist (min\(^{-1}\)), \(t\) is the assay incubation time (min), \(\tau\) is the operational efficacy of the selected agonist (comprising cell- and agonist-dependent properties) and \(E_m\) is the maximal system response. All parameters were shared across all data sets except \(t\), which was fixed to the assay incubation time, 0.2 min in \([Ca^{2+}]_i\)-flux assay, and 5 min in ERK1/2 phosphorylation assay, and \(K_A\), which was constrained to equal the \(K_i\) value derived from radioligand binding assays in the nonlinear regression procedure.

**Linear correlations and statistical analysis**

Correlations between datasets were determined by calculating the Pearson correlation coefficient \((r^2)\) and associated two-tailed \(P\) value, with a \(P < 0.05\) deemed statistically significant.

**Simulations of the effects of hemi-equilibrium**

Data simulations to determine the effects of antagonist dissociation rate on agonist curve translocation were generated using the combined operational/hemi-equilibrium model of antagonism (equation 7) with the following values / ranges: \(K_A = 0.1 \mu M, K_B = 0.1 \mu M, \log \tau = 1 - 100, k_{\text{off}} = 0.001 - 0.1, E_m = 1, \) time = 1 - 100 min and \([B] = 0.1, 0.3, 1, 3 \) or \(10 \mu M\).
Results

[^3H]-NMS saturation binding and kinetic parameters using whole cells

Specific[^3H]-NMS binding to the M₁ mAChR was best described as saturable, monophasic and that a one-site model was the best fit to the data. The expression level of the M₁ mAChR recombinantly expressed in Flp-In-CHO cells was assessed by[^3H]-NMS saturation binding as 1.86 ± 0.51 x10⁶ sites cell⁻¹ per well and the equilibrium dissociation constant (pKᵯ) was determined to be 9.46 ± 0.02 (Table 1).

To enable a robust method to be used to evaluate the kinetic parameters of unlabelled mAChR antagonists (Figure 1), the kinetic parameters of[^3H]-NMS were first established. The observed association rate of a ligand is dependent on the concentration used and therefore a family of association curves of[^3H]-NMS were constructed using a range of concentrations (0.18 - 1.5 nM). Each curve was monitored until equilibrium was reached (Figure 2). Data were globally fitted to give a single best fit of \(k_{on}\) and \(k_{off}\) values. Kinetic parameters for[^3H]-NMS are shown in Table 1. The kinetically derived pKᵯ value was in excellent agreement with the pKᵯ estimated from the saturation binding analysis (9.24 ± 0.24 vs. 9.46 ± 0.02).

[^3H]-NMS equilibrium competition binding using whole cells

All mAChR antagonists produced concentration-dependent inhibition of specific binding of[^3H]-NMS. Competition displacement binding data were fitted to sigmoidal three-parameter logistic equation to obtain IC₅₀ and Hill slope parameter estimates. IC₅₀ values obtained from the inhibition curves were converted to pKᵮ values using the method of Cheng & Prusoff (1973). Data for the mAChR antagonists are summarized in Table 2.

Competition binding kinetics using whole cells

To calculate the kinetic rates of unlabelled mAChR antagonist association and dissociation rates of[^3H]-NMS in the presence and absence of competitor were determined and these values used to calculate \(k_{on}\) \((k₃)\) and \(k_{off}\) \((k₄)\) by globally fitting the individual experimental
data sets to equation 4, as described in the Methods. Grouped data curves to atropine and tiotropium are shown in Figure 3; graphs for the remaining ligands are shown in Supplemental Figure 1. Data for all ligands are summarized in Table 2. Each ligand was tested over a range of concentrations to demonstrate competitive and reversible binding (Sykes et al., 2012). Higher concentrations and increased sampling time of the slow dissociating ligands were employed to allow equilibrium to be reached. The difference in profiles is consistent with that observed for slowly dissociating ligands, where an initial overshoot occurs before equilibrium is reached (Dowling & Charlton, 2006; Mould et al., 2014).

There was an excellent correlation between kinetically derived pK_D and the equilibrium competition binding pK_I values (Figure 4a; \( r^2 = 0.98, P < 0.0001 \)). Estimates of the \( k_{on} \) and \( k_{off} \) rates of [3H]-NMS measured directly \((k_{on} = 24.84 \pm 11.79 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}, k_{off} = 0.04 \pm 0.01 \text{ min}^{-1})\) and those of unlabelled NMS measured by competition kinetics \((k_{on} = 20.88 \pm 6.26 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}, k_{off} = 0.05 \pm 0.02 \text{ min}^{-1})\) were in excellent agreement.

There was a significant correlation between equilibrium dissociation constants and both \( k_{on} \) and \( k_{off} \) values, as shown in Figure 4b \((r^2 = 0.65, P = 0.0088)\) and 4c \((r^2 = 0.50, P = 0.03)\), respectively. These data suggest that both parameters drive affinity, an observation previously made for \( \beta_2 \)-adrenoceptor ligands (Sykes et al., 2012). However, there are examples in the literature where it has been shown that affinity can be driven by \( k_{off} \) alone, for example the D_2 receptor (Kapur & Seeman, 2001), or \( k_{on} \) alone, for example the OX_2 receptor (Mould et al., 2014). Compounds in the current test set appear to fall into two clear clusters where compounds with weak affinity, for example himbacine and pirenzepine, display slow-on and fast-off kinetics, whilst compounds with high affinity, for example atropine and tiotropium display rapid \( k_{on} \) and slow \( k_{off} \) kinetics (Figure 4).

**Effect of cLogD_{pH7.4} on the rate constants**
In an attempt to understand why these ligands fell into two groups based on their kinetic rates, the effect of lipophilicity (cLogD<sub>pH7.4</sub> values, Table 2) was investigated. There was a significant correlation ($r^2 = 0.72$, $P = 0.007$) between Log $k_{on}$ and cLogD<sub>pH7.4</sub> (Supplemental Figure 2a). However, no correlation was observed between the Log $k_{off}$ and cLogD<sub>pH7.4</sub> (Supplemental Figure 2b).

**Simulations of hemi-equilibrium and the effects of stimulus-response coupling**

Our hypothesis is that the dissociation rate of unlabelled ligands can also be quantitatively estimated using the hemi-equilibrium model for competitive antagonism under non-equilibrium conditions (Kenakin, 2009). However, this method relies heavily on a significant and saturable depression of the maximal response of the control agonist in the presence of increasing concentrations of antagonist. We postulated that the effects of agonist-antagonist-receptor equilibration time, and the combined efficacy of the agonist and the total receptor density, $\tau$, as measured by the operational model of agonism (Black & Leff, 1983), could play a large part in determining the optimal conditions to observe hemi-equilibrium.

To guide our experimental design, simulations of agonist concentration-response curves in the presence of increasing concentrations of antagonist were constructed using a combined operational/hemi-equilibrium model of competitive antagonism (Kenakin, 2009; Mould et al., 2014). In an assay of modest receptor reserve ($\tau = 10$; typical of many functional screening assays) and with a fixed incubation time of 10 min, an antagonist with a receptor half-life ($t_{1/2}$) $\approx 7$ min ($k_{off} = 0.1 \text{ min}^{-1}$) is predicted to display a surmountable profile (Figure 5a). An antagonist with a receptor $t_{1/2} \approx 70$ min ($k_{off} = 0.01 \text{ min}^{-1}$) is predicted to yield a significant but saturable depression in the maximal response, characteristic of hemi-equilibrium. A compound with an extended residence time ($t_{1/2} \approx 700$ min; $k_{off} = 0.001 \text{ min}^{-1}$) would be predicted to display an essentially insurmountable antagonist profile (Figure 5c). Simulations of antagonist profiles under different assay incubation times reveal a similar pattern; an antagonist with a receptor $t_{1/2} \approx 70$ min can appear surmountable, insurmountable or display a
hemi-equilibrium profile, depending on the length of agonist-antagonist-receptor re-equilibration time (Figures 5d-f). The degree of receptor reserve present in the functional assay is also predicted to have a marked effect on the observed antagonist profile; varying the value of $\tau$ for the agonist from 1 to 100 yields a similar variety of curve-shift profiles (Figure 5g-i). Thus a number of assay parameters contribute to the observed antagonist profile, making it important to identify the correct conditions to yield, and hence analyze, hemi-equilibrium effects. This is reinforced by the observation that it is not possible to recover the parameters used to simulate data by analysis with the model for antagonists that fully collapse the agonist concentration-response curve (Figure 5j). Therefore, finding optimal experimental conditions is a pre-requisite to using the operational/hemi-equilibrium model of antagonism for kinetic parameter determinations.

To experimentally demonstrate these effects, concentration-response curves to ACh and MCh were constructed and Log $\tau$ values determined in assays with both short (15 s) and moderate (5 min) equilibration times. In the $[Ca^{2+}]_i$-flux assay Log $\tau$ values of 2.84 ± 0.06 and 1.94 ± 0.06 were determined for ACh and MCh, respectively (Figure 6a). Lower efficacies were seen in ERK1/2 phosphorylation assays, with Log $\tau$ values of 2.19 ± 0.11 and 1.35 ± 0.10 (Figure 6b) for ACh and MCh, respectively. In both the $[Ca^{2+}]_i$-flux and ERK1/2 phosphorylation assays, ACh had high efficacy and was therefore deemed a full agonist. The potencies of ACh were left shifted compared to its affinity ($pK_I = 4.55$, Table 2), suggesting a high receptor reserve within these systems for this agonist. MCh, however, had significantly lower efficacy than ACh, even though it has the same maximal response as ACh and slightly higher affinity ($pK_I = 4.80$, Table 2). As a result, MCh may be a more suitable agonist to detect the kinetic effects in assays with higher receptor reserve.

**Experimental data of hemi-equilibrium and the effects of stimulus-response coupling**
To experimentally demonstrate the effects of equilibration time and receptor reserve, concentration-response curves to ACh and MCh were constructed in the presence and absence of atropine and tiotropium as prototypical fast and slow dissociating antagonists, respectively. Atropine produced a concentration-dependent rightward shift in the ACh concentration-response curve in both the [Ca^{2+}]-flux and ERK1/2 phosphorylation assays with only a small depression in the $E_{\text{max}}$ (Figure 7a and b). Conversely, when MCh was used, atropine gave a concentration-dependent rightward shift in the MCh concentration-response curve in both assays with a markedly greater depression in the $E_{\text{max}}$ (Figure 7c and d), in accordance with the lower efficacy of this ligand. These data were analysed using the combined operational/hemi-equilibrium model of antagonism, as described in Methods, to estimate pK$_B$ and $k_{\text{off}}$ values.

Analysis of the datasets using ACh did not converge sufficiently to provide reproducible estimates of receptor $t_{1/2}$ values, whereas the data for MCh provided robust parameter estimates (14 ± 2 and 27 ± 1 min for [Ca^{2+}]-flux and ERK1/2 phosphorylation, respectively). The slowly dissociating antagonist, tiotropium, abolished the MCh response in the [Ca^{2+}]-flux assay, precluding meaningful analysis using the model (Figure 7e). However, in the ERK1/2 phosphorylation assay it produced a saturable depression of the maximal MCh response, yielding an estimated $t_{1/2} = 223 \pm 85$ min (Figure 7f).

Encouragingly, an excellent agreement was obtained for the $k_{\text{off}}$ values derived from radioligand binding and functional assays for both atropine (0.05 ± 0.01 min$^{-1}$ in [Ca^{2+}]-flux and 0.03 ± 0.01 in ERK1/2 phosphorylation vs. 0.07 ± 0.05 in binding) and tiotropium (0.003 ± 0.002 min$^{-1}$ in ERK1/2 phosphorylation vs. 0.006 ± 0.001 in binding). The pK$_B$ values for atropine from both assays were also in excellent agreement with those obtained in the competition binding kinetics (9.44 ± 0.08 in [Ca^{2+}]-flux and 9.23 ± 0.21 in ERK1/2 phosphorylation vs. 9.18 ± 0.06 in binding). A similarly excellent agreement was obtained for tiotropium (10.25 ± 0.26 in ERK1/2 phosphorylation vs. 10.05 ± 0.16 in binding).
Given the agreement across datasets for these exemplar compounds and the improved data resolution using MCh as the agonist probe, we generated kinetic parameter estimates for our set of competitive antagonists. These data are summarised in Table 3 and represented graphically in Supplemental Figure 3. Some compounds clearly demonstrate surmountable antagonism, for example 4-DAMP and himbacine, indicative of rapid dissociation. Due to their apparent rapid re-equilibration in the time frame of the assay, the t½ for these compounds could not be determined. However, a reasonable estimate would be that the half-lives for such compounds would be equal to or less than the assay incubation time.

Unsurprisingly, there was a very robust correlation between the binding kinetics derived pK_D and the functional hemi-equilibrium derived pK_B ($r^2 = 0.97$, $P < 0.0001$), which is shown in Figure 8a. More interestingly, an excellent correlation was also obtained between the radioligand binding $k_{off}$ and the functional hemi-equilibrium $k_{off}$ ($r^2 = 0.94$, $P = 0.0004$), shown in Figure 8c. Furthermore, a similarly excellent correlation was also obtained between the radioligand binding $k_{on}$ and the functional hemi-equilibrium $k_{on}$ (calculated post-hoc by $k_{off}/K_B$ values; $r^2 = 0.90$, $P = 0.0012$), shown in Figure 8b.
Discussion

Herein we provide a framework for assessing kinetic rate constants for competitive antagonists for a GPCR using functional assays. Previously, it has been shown that the dissociation rate of a competitive antagonist is proportional to its propensity to depress the maximal response to an agonist in a functional assay as a result of incomplete agonist-antagonist-receptor re-equilibration (Christopoulos et al., 1999). This phenomenon was originally termed ‘hemi-equilibrium’ (Paton & Rang, 1965; Vauquelin et al., 2002). Taking advantage of this effect, we show that application of an operational/hemi-equilibrium model of antagonism to functional data yields kinetic rate constants that are in excellent agreement with those derived from radioligand binding assays. This approach does not require a fluorescent or radiolabelled probe, rather a functional assay in which the agonist-antagonist-receptor incubation time is in the range of 1-100 times the receptor half-life for the antagonist.

In practice, most commonly used GPCR functional assays fall into this category, highlighting its generic utility.

Using the muscarinic M₁ receptor as a test system, we generated kinetic rate constant estimates for a range of antagonists by [³H]-NMS binding. Analysis revealed a wide range of affinity and rate constant estimates. Both $k_{on}$ and $k_{off}$ values significantly correlate with antagonists’ equilibrium dissociation constants (Figure 4), indicating that for this set of structurally diverse compounds, both association and dissociation rates drive equilibrium affinity. Interestingly, compounds of weak affinity, e.g. himbacine and pirenzepine, tended to display slow association, but rapid dissociation kinetics. Conversely, compounds that had higher affinity, e.g. NMS and tiotropium, displayed faster association, but slow dissociation kinetics.
In order to design an optimal functional assay under hemi-equilibrium conditions, we performed simulations of functional data using the operational/hemi-equilibrium model. These predicted the importance of antagonist $k_{\text{off}}$ values, assay incubation time and degree of stimulus-response coupling in achieving hemi-equilibrium. It is obvious that shorter assay incubation times and/or more slowly dissociating antagonists would give rise to non-equilibrium effects; simulations indicate that there is a range of these parameters that yield a saturable depression in the $E_{\text{max}}$ amenable to analysis (Figure 5). Outside of this range, there is either sufficient agonist-antagonist-receptor re-equilibration (yielding a surmountable profile) or no re-equilibration (resulting in an insurmountable profile; Figure 5). Therefore, these simulations reveal a potential “sweet spot” of $k_{\text{off}}$ values that can be identified for a given assay incubation time, where the product of incubation time, and $k_{\text{off}}$ is in the approximate range 0.01 to 1.

In functional assays, “receptor reserve” is dependent on both the level of receptor expression ([R$_T$]) and the efficiency of the agonist-receptor complex to elicit a response ($K_E$; Ariens et al., 1960; Adham et al., 1993; Umland et al., 2001). The operational parameter ‘τ’ encompasses both terms (R$_T$/K$_E$). Data simulations revealed that the degree of receptor reserve has as much influence as incubation time and antagonist $k_{\text{off}}$; very slow-off compounds could appear surmountable when τ values are high (Figure 5). In keeping with this observation, the analytical model performance improved when we used the partial agonist, MCh, in place of the full agonist, ACh, in our Flp-In-CHO-hM$_1$ functional assays. Subsequent profiling of the antagonists in the functional assays yielded estimates of dissociation rate constants that were in excellent agreement with those derived from [$^3$H]-NMS binding studies ($r^2 = 0.94$, P = 0.0004; Figure 8). It was noteworthy that compounds displaying very rapid dissociation in the radioligand binding (e.g. 4-DAMP and himbacine) appeared fully surmountable, even in the [Ca$^{2+}$]-flux assay, precluding analysis using the hemi-equilibrium model. Accordingly it would be predicted that their $t_{1/2}$ values would be equal to, or less than, the assay incubation time ($\leq 15$s). Antagonists with long $t_{1/2}$ values in...
[^3]H]-NMS binding (e.g. tiotropium and glycopyrrolate) yielded fully insurmountable profiles in [Ca^{2+}]_i-flux assays and required profiling in the ERK1/2 phosphorylation assay in order to generate estimates of dissociation rates. Finally, post-hoc calculated functional $k_{on}$ estimates were also significantly correlated with $k_{on}$ values determined by[^3]H]-NMS binding ($r^2 = 0.90$, $P = 0.0012$), demonstrating the utility of functional assays to estimate antagonist rate constants.

The revelation that common functional assays can be used to determine antagonist kinetics gives rise to several insights and opportunities. For example, it has long been apparent that agonist pharmacology is influenced by receptor reserve. Herein we demonstrate that differences in receptor reserve markedly affect antagonist pharmacology and the ability to resolve rate constants. Only when the partial agonist, MCh (rather than ACh) was used, were robust parameter estimates obtained for antagonist binding kinetics. Nonetheless, with appropriate assay design (which may include optimisation of incubation time, the level of receptor expression or efficacy of probe agonist), simple functional assays can be adapted to routinely determine antagonist kinetics for any GPCR with the same level of confidence as would be provided by more traditional radioligand binding approaches. This potentially adds value to drug discovery programs that may seek particular kinetic properties (Sykes et al., 2012; Christopher et al., 2015). Kinetic properties have also been shown to underlie discrepancies between in vitro and in vivo data for compounds (Ramsey et al., 2011; Mould et al., 2014; Sykes et al., 2014a).

Similarly, this approach may also be used to differentiate tool antagonists on the basis of their kinetics. Many of the recent advances in X-ray structure determination of GPCRs have arisen by generating receptor co-complexes with high affinity and slowly dissociating antagonists (Haga et al., 2012; Rasmussen et al., 2010; Manglik et al, 2012); our approach offers a generic method for simply assessing candidate tool compounds based on their kinetic parameters. A final consideration is that receptor kinetics may contribute to pathway-specific
affinity states and biased agonism at GPCRs. A number of ‘antagonists’ have been shown to display hitherto unappreciated agonistic effects – for example propranolol and carvedilol at the β2-adrenoceptor (Baker et al., 2003a; Wisler et al., 2007) and atropine at the muscarinic M1 receptor (Stewart et al., 2010). Furthermore, there is no a priori reason why antagonists may not display differential affinities dependent on the pathway/effector system being monitored (Kenakin, 2014); propranolol has a five-fold higher affinity to inhibit adrenaline-stimulated cAMP accumulation compared to CRE-SPAP transcription at the β2-adrenoceptor (Baker et al., 2003b) and even larger differences have been observed for α1B-adrenoceptor antagonists (Yoshiki et al., 2014). The aetiology of multiple antagonist affinity states remains largely unknown, but it is possible that the residence time of ligands may contribute to differential signal pathway activation/inhibition.

Both the radioligand binding and functional assays reveal a range of association and dissociation rates. The slower association of some ligands to the M1 receptor may be due to their physico-chemical properties; Sykes et al., (2014b) have shown that lipophilicity and membrane compartmentalization of a ligand can have profound effects on binding kinetics. For this test set of compounds, the association rate was significantly correlated with the compounds’ distribution-coefficient (cLogD7.4; \( r^2 = 0.72, P = 0.007 \)). Interestingly, there was no correlation of cLogD7.4 with dissociation rate (\( r^2 = 0.18 \)), suggesting that once bound, egress of a molecule from the M1 mACh receptor is not significantly influenced by its lipophilicity. The apparent inverse relationship between association rate and antagonist lipophilicity of the ligand is in contrast to the profile observed for the β2-adrenoceptor (Sykes et al., 2014b), and indicates that the initial interaction with the M1 mAChR may not favour lipophilic compounds. Compounds with high lipophilicity may first partition in to the cell membrane before accessing the receptor-binding site, consequently slowing their association. Conversely, compounds with low lipophilicity, and weak membrane affinity, may interact directly with the extracellular vestibule of the receptor, similar to the radioligand, [3H]-NMS. From molecular dynamics (MD) simulations, Dror et al., (2011) described, for both the β2-
and β2-adrenergic receptors, that the beta-blocker, alprenolol, bound initially to a region in the extracellular vestibule, 15Å away from the orthosteric site, before traversing to the binding pocket. Currently, our ability to measure ligand rate constants are based on competition with a ligand bound in the orthosteric-binding pocket. Consequently, we measure a combination of the complete binding process and perhaps the physico-chemical properties of a ligand play a greater part in overcoming the energy barriers, as described by Dror et al., (2011).

Investigation into these additional binding sites by use of fluorescent ligands, mutagenesis, inhibition of the orthosteric pocket by irreversible, or slowly dissociating ligands, may help elucidate the importance of these interactions and identify a ligands complete residency time at the receptor.

In conclusion, we have provided a framework for the rapid and simple assessment of competitive antagonist binding kinetics at GPCRs using widely available functional assays. With judicious experimental design, application of an analytical model of hemi-equilibrium provided estimates of muscarinic M1 receptor antagonist rate constants that were in excellent agreement with those determined by classical radioligand binding approaches. This generic method offers an approach to profile receptor kinetics without the need of labeled probes, opening up the possibility to determine kinetic parameters for GPCR antagonists as an SAR parameter in drug discovery, to discriminate tool compounds or to probe the pharmacology underlying observed biased agonism or antagonism.
Authorship Contributions

Participated in research design: Riddy, Valant, Sexton, Christopoulos, Langmead

Conducted experiments: Riddy, Valant, Rueda

Contributed new reagents or analytic tools: n/a

Performed data analysis: Riddy, Valant, Langmead

Wrote or contributed to the writing of the manuscript: Riddy, Charman, Sexton, Summers, Christopoulos, Langmead
References


Cheng Y, and Prusoff WH (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099-3108.


Legends for Figures

Figure 1
Structures of mAChR antagonists used for the study

Figure 2
Kinetics of $[^3\text{H}]$-NMS with CHO cells expressing the M$_1$ mAChR. The $k_{\text{on}}$ and $k_{\text{off}}$ values for were determined by incubating Flp-In-CHO-hM$_1$ mAChR cells with the indicated concentrations of $[^3\text{H}]$-NMS for various time periods. Association data were fitted to a global fitting model using Prism 6.0f to simultaneously to calculate $k_{\text{on}}$ and $k_{\text{off}}$, and are summarized in Table 1. Grouped data are shown ± SD (n=3).

Figure 3
$[^3\text{H}]$-NMS competition binding kinetic curves in the presence of (a) atropine and (b) tiotropium. Flp-In-CHO-hM$_1$ mAChR cells were incubated with ~0.4 nM $[^3\text{H}]$-NMS with 1, 3 and 10x $K_I$ for atropine, or 30, 100 and 300x $K_I$ for tiotropium. Plates were incubated for the indicated time points at RT. NSB levels were determined in the presence of 10 μM atropine. Data were globally fitted to the equations as described in the Methods to calculate $k_{\text{on}}$, $k_{\text{off}}$ and kinetically-derived $K_D$ values for the unlabeled agonists, and are summarized in Table 2. Grouped data are shown ± SD (n=3).

Figure 4
Correlation of (a) kinetically-derived $pK_D$ and equilibrium binding $pK_I$ (b) Log $k_{\text{on}}$ and equilibrium binding $pK_I$ and (c) Log $k_{\text{off}}$ and equilibrium binding $pK_I$ values from $[^3\text{H}]$-NMS competition binding kinetics. Data were fitted to Deming linear regression with statistical significance determined as $P < 0.05$. The values used are shown in Table 2. Grouped data are shown ± SD (n=3). Dotted line shows $y = x$. 

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Figure 5
Simulation data describing hemi-equilibrium with (a-c) varying $k_{off}$ values (d-f) a fixed $k_{off}$ value of 0.1 and varying incubation times (t), (f-i) a fixed $k_{off}$ value of 0.1 and incubation time (t) of 1 but with varying Log $\tau$ values, and (j) the effects of insurmountable antagonism on the model. All simulations were generated with the following parameters unless otherwise indicated on the figures, $K_A = 0.1$ μM, $K_B = 0.1$ μM, Log $\tau = 1$, $k_{off} = 0.1$, $Em = 1$, time = 10 min and $[B] = 0.1, 0.3, 1, 3$ or 10 μM.

Figure 6
Experimental data demonstrating the effects of $\tau$ estimates of mAChR agonists in the (a) $[Ca^{2+}]_i$-flux and (b) ERK1/2 phosphorylation assays. Experimental data was globally fitted to the equation as described in the Methods to calculate Log $\tau$ values. Grouped data are shown ± SD (n=3). Statistical significance was deemed by unpaired t-test versus the MCh response, with P < 0.05 deemed significant.

Figure 7
Concentration-response curves of ACh in the presence of increasing concentrations of atropine in (a) $[Ca^{2+}]_i$-flux and (b) ERK1/2 phosphorylation assays, or MCh in the presence of increasing concentrations of atropine or tiotropium in (c and e) $[Ca^{2+}]_i$-flux and (d and f) ERK1/2 phosphorylation assays. Flp-In-CHO-hM1 mAChR cells were incubated at 37°C with antagonist for 30 min prior to the addition of agonist and assayed for either 15 s in the $[Ca^{2+}]_i$-flux assay or 5 min for the ERK1/2 phosphorylation assay, with fluorescence being measured over a 75 s time period using 485nm excitation and 538nm emission wavelengths, or termination of the assay using the SureFire™ lysis buffer. Cell lysates were analysed using an AlphaScreen ERK1/2 phosphorylation assay kit. Data were globally fitted to the equations as described in the Methods to calculate $pK_B$ and $k_{off}$ values for the unlabeled agonists, and are summarized in Table 3. Grouped data are shown ± SD (n=3-4).
**Figure 8**

Correlation of (a) kinetically-derived pK$_D$ and functionally-derived pK$_B$, (b) binding Log $k_{on}$ and post-hoc functional Log $k_{on}$ values and (c) binding Log $k_{off}$ and functional Log $k_{off}$ values from [$^3$H]-NMS competition binding kinetics and [Ca$^{2+}$]$_i$-flux and ERK1/2 phosphorylation assays ran under hemi-equilibrium conditions. Data were fitted to Deming linear regression with statistical significance determined as P < 0.05. The values used are shown in Tables 2 and 3. Grouped data are shown ± SD (n=3-4). Dotted line shows y = x.
Tables

Table 1. Binding parameters for [$^3$H]-NMS determined by equilibrium saturation and kinetic binding experiments. Data are expressed as mean ± SD from a single fit to grouped data of three individual experiments.

<table>
<thead>
<tr>
<th>pK_D</th>
<th>B_max (x10^6 sites cell^-1)</th>
<th>k_on (k_1) (x10^7 M^-1 min^-1)</th>
<th>k_off (k_2) (min^-1)</th>
<th>t_1/2 (min)</th>
<th>Kinetic pK_D</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.46 ± 0.02</td>
<td>1.86 ± 0.51</td>
<td>24.8 ± 11.8</td>
<td>0.04 ± 0.01</td>
<td>18.9 ± 3.30</td>
<td>9.24 ± 0.24</td>
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</tbody>
</table>
Table 2. Competition binding affinities, equilibrium association and dissociation kinetics using the Motulsky & Mahan (1984) method determined using [3H]-NMS, and cLogD7.4 values of the mAChR antagonists used within this study. Data are expressed as mean ± SD from a single fit to grouped data of three to six individual experiments. cLogD7.4 values were obtained using ChemAxon MarvinSketch software as described in the Methods section.

<table>
<thead>
<tr>
<th></th>
<th>pK_{i}</th>
<th>k_{on} (k_{3}) (x10^{7} \text{M}^{-1} \text{min}^{-1})</th>
<th>k_{off} (k_{4}) (\text{min}^{-1})</th>
<th>t_{1/2} (\text{min})</th>
<th>Kinetic pK_{D}</th>
<th>cLogD_{pH7.4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>4.55 ± 0.13</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Methacholine</td>
<td>4.80 ± 0.29</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>8.55 ± 0.07</td>
<td>9.36 ± 3.88</td>
<td>0.29 ± 0.05</td>
<td>2.89 ± 0.38</td>
<td>8.51 ± 0.03</td>
<td>-0.70</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.18 ± 0.06</td>
<td>21.2 ± 4.77</td>
<td>0.07 ± 0.05</td>
<td>12.7 ± 7.36</td>
<td>9.46 ± 0.21</td>
<td>-0.41</td>
</tr>
<tr>
<td>Glycopyrrolate</td>
<td>9.43 ± 0.23</td>
<td>4.97 ± 0.40</td>
<td>0.01 ± 0.00</td>
<td>72.9 ± 4.43</td>
<td>9.70 ± 0.05</td>
<td>-1.41</td>
</tr>
<tr>
<td>Himbacine</td>
<td>6.92 ± 0.11</td>
<td>0.21 ± 0.20</td>
<td>0.47 ± 0.54</td>
<td>1.52 ± 0.83</td>
<td>6.65 ± 0.06</td>
<td>2.90</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>9.13 ± 0.06</td>
<td>17.9 ± 6.38</td>
<td>0.06 ± 0.05</td>
<td>50.7 ± 37.8</td>
<td>9.46 ± 0.41</td>
<td>-1.82</td>
</tr>
<tr>
<td>NMS</td>
<td>9.21 ± 0.07</td>
<td>20.9 ± 6.26</td>
<td>0.05 ± 0.02</td>
<td>35.0 ± 35.0</td>
<td>9.63 ± 0.31</td>
<td>-3.27</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>6.94 ± 0.09</td>
<td>0.03 ± 0.00</td>
<td>0.04 ± 0.02</td>
<td>19.0 ± 9.52</td>
<td>6.88 ± 0.37</td>
<td>0.56</td>
</tr>
<tr>
<td>QNB</td>
<td>9.41 ± 0.12</td>
<td>2.46 ± 0.69</td>
<td>0.008 ± 0.000</td>
<td>97.9 ± 2.21</td>
<td>9.49 ± 0.13</td>
<td>1.70</td>
</tr>
<tr>
<td>Tiotropium</td>
<td>10.05 ± 0.16</td>
<td>8.37 ± 1.71</td>
<td>0.006 ± 0.001</td>
<td>129 ± 29.7</td>
<td>10.13 ± 0.10</td>
<td>-1.75</td>
</tr>
</tbody>
</table>

nd = not determined
Table 3. Potency, dissociation and post-hoc association values of mAChR antagonists using a [Ca\(^{2+}\)]\(_i\)-flux or ERK1/2 phosphorylation assay under hemi-equilibrium conditions. Data are expressed as mean ± SD from a single fit to grouped data of three to four individual experiments.

<table>
<thead>
<tr>
<th></th>
<th>pK(_B) from hemi-equilibrium model</th>
<th>pK(_B) from Schild analysis</th>
<th>(k_{off}) (min(^{-1}))</th>
<th>Post-hoc (k_{on}) (x10(^7) M(^{-1}) min(^{-1}))</th>
<th>(t_{1/2}) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-DAMP</td>
<td>8.06 ± 0.14</td>
<td>7.89 ± 0.18</td>
<td>nd</td>
<td>nd</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.44 ± 0.08</td>
<td>9.10 ± 0.30</td>
<td>0.05 ± 0.01</td>
<td>14.3 ± 4.96</td>
<td>14.2 ± 2.4</td>
</tr>
<tr>
<td>Himbacine</td>
<td>6.51 ± 0.16</td>
<td>6.62 ± 0.20</td>
<td>nd</td>
<td>nd</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>9.73 ± 0.10</td>
<td>9.40 ± 0.12</td>
<td>0.05 ± 0.01</td>
<td>26.7 ± 12.7</td>
<td>14.9 ± 15.7</td>
</tr>
<tr>
<td>NMS</td>
<td>9.85 ± 0.11</td>
<td>9.24 ± 0.16</td>
<td>0.02 ± 0.01</td>
<td>16.0 ± 10.2</td>
<td>33.9 ± 4.4</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>7.80 ± 0.11</td>
<td>7.21 ± 0.15</td>
<td>0.03 ± 0.01</td>
<td>0.21 ± 0.09</td>
<td>23.3 ± 7.7</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.23 ± 0.21</td>
<td>8.88 ± 0.15</td>
<td>0.03 ± 0.01</td>
<td>20.0 ± 28.4</td>
<td>27.1 ± 1.0</td>
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<tr>
<td>Glycopyrrolate</td>
<td>10.09 ± 0.12</td>
<td>9.83 ± 0.18</td>
<td>0.007 ± 0.002</td>
<td>9.66 ± 7.93</td>
<td>98.8 ± 32.4</td>
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<tr>
<td>QNB</td>
<td>9.89 ± 0.08</td>
<td>10.19 ± 0.22</td>
<td>0.003 ± 0.001</td>
<td>1.90 ± 0.96</td>
<td>272 ± 103</td>
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<tr>
<td>Tiotropium</td>
<td>10.25 ± 0.26</td>
<td>9.74 ± 0.51</td>
<td>0.003 ± 0.002</td>
<td>3.53 ± 3.42</td>
<td>223 ± 84.9</td>
</tr>
</tbody>
</table>

\(nd\) = not determined
1,1-dimethyl-4-diphenylacetoxy piperidinium iodide (4-DAMP)

Atropine

Glycopyrrolate

Himbacine

Ipratropium

N-methylscopolamine (NMS)

Pirenzepine

Quinuclidinyl benzylate (QNB)

Tiotropium
Figure 2

The graph shows the percentage specific binding over time for different concentrations of [3H]-NMS (nM). The y-axis represents the percentage specific binding, and the x-axis represents time in minutes. The concentrations of [3H]-NMS are indicated on the right side of the graph:

- ▽ 1.48 nM
- △ 0.67 nM
- □ 0.34 nM
- ○ 0.18 nM

The graph illustrates the time course of specific binding for each concentration, with the highest concentration showing the least specific binding and the lowest concentration showing the highest specific binding.
Figure 3

(a) Atropine
- 0
- 1x Ki
- 3x Ki
- 10x Ki

(b) Tiotropium
- 0
- 30x Ki
- 100x Ki
- 300x Ki

% specific binding vs. Time (min)
Figure 4

Equilibrium binding pK\textsubscript{I} vs. Kinetically derived pK\textsubscript{D} (a)

Log k\textsubscript{on} vs. Equilibrium binding pK\textsubscript{I} (b)

Log k\textsubscript{off} vs. Equilibrium binding pK\textsubscript{I} (c)

Correlation coefficients and p-values: p\textsuperscript{2} = 0.98, P = <0.0001 (a); p\textsuperscript{2} = 0.65, P = 0.0088 (b); p\textsuperscript{2} = 0.5, P = 0.03 (c).
Figure 5

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**a** $k_{\text{off}} = 0.1$

**b** $k_{\text{off}} = 0.01$

**c** $k_{\text{off}} = 0.001$

**d** $k_{\text{off}} = 0.1$, $t = 10$

**e** $k_{\text{off}} = 0.1$, $t = 1$

**f** $k_{\text{off}} = 0.1$, $t = 0.1$

**g** $k_{\text{off}} = 0.1$, $t = 1$, $\log \tau = 0$

**h** $k_{\text{off}} = 0.1$, $t = 1$, $\log \tau = 1$

**i** $k_{\text{off}} = 0.1$, $t = 1$, $\log \tau = 2$

**j** $k_{\text{off}} = 1 \times 10^{-5}$, $t = 0.1$, $\log \tau = 0$

Calculated $\log \tau = -1.63 \times 10^{-8}$
Figure 6

(a) [Ca^{2+}]_i-flux (% ACh response)

Log τ_{ACh} = 2.8
Log τ_{MCh} = 1.9

ACh
MCh

(b) pERK1/2 (% ACh response)

Log τ_{ACh} = 2.2
Log τ_{MCh} = 1.3

ACh
MCh
Incubation time = 15 s

- [Atropine] (nM)
  - 0
  - 3
  - 10
  - 30
  - 100
  - 300

Log [ACh] (M)

- [Ca$^{2+}$]$_i$-flux (% ACh response)

Incubation time = 5 min

- [Atropine] (nM)
  - 0
  - 3
  - 10
  - 30
  - 100
  - 300

Log [ACh] (M)

- pERK1/2 (% ACh response)

Incubation time = 15 s

- [Tiotropium] (nM)
  - 0
  - 0.1
  - 0.3
  - 1
  - 3
  - 10

Log [MCh] (M)

- [Ca$^{2+}$]$_i$-flux (% MCh response)

Incubation time = 5 min

- [Tiotropium] (nM)
  - 0
  - 0.1
  - 0.3
  - 1
  - 3
  - 10

Log [MCh] (M)

- pERK1/2 (% MCh response)
Figure 8

(a) Kinetic pK$_D$ vs. Functional pK$_B$ for various AChR agonists.

(b) Log $k_{on}$ vs. Log $k_{on}$ for the same agonists.

(c) Log $k_{off}$ vs. Log $k_{off}$ for the same agonists.

For each plot, the correlation coefficient ($r^2$) and significance level ($P$) are provided.

- **A**: $r^2 = 0.97$, $P < 0.0001$
- **B**: $r^2 = 0.90$, $P = 0.0012$
- **C**: $r^2 = 0.94$, $P = 0.0004$

Agonists include Atropine, Ipratropium, Tiotropium, Glycopyrrolate, Pirenzepine, NMS, QNB, and 4-DAMP.