Label-Free Kinetics: Exploiting Functional Hemi-Equilibrium to Derive Rate Constants for Muscarinic Receptor Antagonists

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ABSTRACT

Drug receptor kinetics is as a key component in drug discovery, development, and efficacy; however, determining kinetic parameters has historically required direct radiolabeling or competition with a labeled tracer. Here we present a simple approach to determining the kinetics of competitive antagonists of G protein–coupled receptors by exploiting the phenomenon of hemi-equilibrium, the state of partial re-equilibration of agonist, antagonist, and receptor in some functional assays. Using functional [Ca²⁺]–flux and extracellular kinases 1 and 2 phosphorylation assays that have short incubation times and therefore are prone to hemi-equilibrium procedures, we investigated a wide range of structurally and physicochemically distinct muscarinic acetylcholine receptor antagonists. Using a combined operational and hemi-equilibrium model of antagonism to both simulate and analyze 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 the degree of receptor reserve in applying the analytical model. There was an excellent correlation between estimates of antagonist pKᵢ, kᵢ, and kᵦ from functional assays and those determined by competition kinetics using whole-cell [³H]N-methylscopolamine binding, validating this approach as a rapid and simple method to functionally profile receptor kinetics of competitive antagonists in the absence of a labeled 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, 4-[4-(chlorophenyl)methyl]-2-[(2R)-1-[4-(4-[3-(hexahydro-1H-azepin-1-yl)propyl]oxy)phenyl]butyl]-2-pyrrolidinyl]methyl]-1(2H)-phthalazinone (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 glycopyrrolonium bromide (NVA237), which have both yielded positive phase 3 results in the treatment of chronic obstructive pulmonary disease (Jones, 2015), have greater kinetic selectivity for the M₃ muscarinic acetylcholine receptor (mAChR) compared with 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 pseudoirreversible antagonist (Mould et al., 2014).

Typically, kinetic parameters of compounds are rarely optimized during early stage drug discovery, as this often requires labeling the compounds with a suitable radioisotope or the addition of a fluorescent moiety. Although these approaches can provide direct and precise data, both are extremely costly, technically challenging, and normally reserved for a select subset of compounds. One alternative approach, amenable to early stage drug discovery, is the method of Motulsky and 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 analyzed and the rates of the competitor ligand determined (Dowling and 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 owing to insufficient agonist-antagonist-receptor re-equilibration time between the 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 and Vauquelin, 2010). A competitive antagonist can display a range of behaviors, with their kinetic parameters, with almorexant being identified as a pseudoirreversible antagonist (Mould et al., 2014).

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ABBREVIATIONS: 4-DAMP, 1,1-dimethyl-4-diphenylacetoxyperipinidino iodide; ACh, acetylcholine; FBS, fetal bovine serum; GPCR, G protein–coupled receptor; GSK1004723, 4-[4-chlorophenyl]methyl]-2-[(2R)-1-[4-(4-[3-(hexahydro-1H-azepin-1-yl)propyl]oxy)phenyl]butyl]-2-pyrrolidinyl]methyl]-1(2H)-phthalazinone; mAChR, muscarinic acetylcholine receptor; MCh, methacholine; NMS, N-methylscopolamine; SR 48968, (S)-N-methyl-N(4-[4-acetylamino-4-phenylpiperidino]-2-[3,4-dichlorophenyl] butyl)benzamide.

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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 artifacts” 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, (S)-N-methyl-N-(4-[4-acetylamino-4-phenylpiperidino]-2-(3,4-dichlorophenyl)butyl)benzamide (SR 48968), a nonpeptide 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 while also using a more traditional approach by competitive radioligand binding kinetics (Dowling and Charlton, 2006). To validate our method, we used Flp-In-CHO cells stably expressing the hM1 mAChR, a physiologically relevant buffer, whole-cell competition kinetics, and [Ca\(^{2+}\)]-flux and ERK1/2 phosphorylation functional assays. The hM1 mAChR was chosen because 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 pharmacologic tools (Christopoulos et al., 1999; Jakubik et al., 2006). Radioligand binding kinetics were analyzed according to the method of Motulsky and Mahan (1984), whereas functional data were analyzed 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 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 7000 Flp-In-CHO-hM1 mAChR cells per well (100 \(\mu\)l final volume per well) were seeded in F-12 GlutaMax containing 10% fetal bovine serum (FBS) into 96-well ISOLATE TC plates the day before experimentation and allowed to grow overnight at 37°C and 5% CO\(_2\). For all functional experiments 30,000 cells per well (100 \(\mu\)l final volume per well) were seeded in F-12 GlutaMax containing 10% FBS into 96-well plates the day before experimentation and incubated for 8 hours at 37°C and 5% CO\(_2\) prior serum starvation the cells with 100 \(\mu\)l 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 \(\mu\)l) and resuspended in binding 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; pH 7.4 with NaOH). As a result of the rapid association of \(^{3}H\)-N-methylscopolamine (NMS) at 37°C, all experiments were performed at 22°C. Assay mixtures, in a total volume of 200 \(\mu\)l with a 1:10 dilution of drug, were incubated at 22°C. Assays were terminated by buffer removal after by rapid washing, twice, with ice-cold 0.9% NaCl (100 \(\mu\)l). Plates were allowed to dry inverted for 30 minutes; OptiPhase Supermix scintillation cocktail (100 \(\mu\)l) was added, plates were sealed (TopSeal), and radioactivity was measured in a MicroBeta2 LumiJET microplate counter. Saturation binding experiments were performed in the absence or presence of atropine (100 \(\mu\)M with 0.003–5 nM [\(^{3}H\)]NMS (1 nCi, 37 MBq)). Inhibition binding experiments were performed with 0.35 nM [\(^{3}H\)]NMS (the approximate \(K\)\(_{D}\) 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 [\(^{3}H\)]NMS Binding to Whole Cells.** To accurately determine \(k_{on}\) and \(k_{off}\) values, at least three concentrations of [\(^{3}H\)]NMS were incubated with 7000 Flp-In-CHO-hM1 mAChR cells well per well in binding buffer at 22°C until equilibrium was reached. Assays were terminated as described here.

**Determination of Antagonist Affinity Constants (\(pK_{I}\)) and Kinetic Parameters (\(k_{on}\) and \(k_{off}\) on) on Whole Cells.** To determine the kinetic parameters of unlabeled antagonists a competition kinetic binding assay was used (Dowling and Charlton, 2006). [\(^{3}H\)]NMS (0.4 nM) was incubated with 7000 Flp-In-CHO-hM1 mAChR cells well per well in binding buffer at 22°C, with gentle agitation for 22 hours. IC\(_{50}\) values were converted to equilibrium binding constants (\(pK_{I}\)) by using the method of Cheng and Prusoff (1973). Experiments were initiated by the coaddition of [\(^{3}H\)]NMS (0.4 nM) and unlabeled competitor to plated cells. Then 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), atropine, himbacine, ipratropium, N-methyl scopolamine (NMS), andpirenzepine were tested at 1, 3, and 10 \(\times\) \(K_{I}\), glycopyrrolate, and quinclidinyl benzylate at 10, 30; 100 \(\times\) \(K_{I}\) and tiotropium were tested at 30, 100, and 300 \(\times\) \(K_{I}\) as determined in the equilibrium binding assay. Assays were terminated as described here.

**[Ca\(^{2+}\)]-Flux Assay.** On the day of the experiment, the cells were washed with 100 \(\mu\)l 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 \(\mu\)M Fluo-4-AM for 1 hour 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-second time period using a 485-nm excitation and 538-nm emission wavelengths and performed the addition of drugs (1:10 dilution) at the 15-second time point. For all interaction studies, mAChR antagonists were added 30 minutes before agonist stimulation. For each interaction, the peak of maximum fluorescence induced by the addition of either methacholine (MCh) or acetylcholine (ACh), between 15 and 75 seconds was chosen, and corrected to the baseline (fluorescence from 0 to 14 seconds) and 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 minutes required to achieve peak response. For interaction experiments, mAChR antagonists were incubated for 30 minutes before agonist stimulation. For each interaction, the peak of maximum fluorescence induced by the addition of either methacholine (MCh) or acetylcholine (ACh), between 15 and 75 seconds was chosen, and corrected to the baseline (fluorescence from 0 to 14 seconds) and then normalized to 1 mM ACh-mediated response.
Kinetics of mACHR Ligands in Binding and Functional Assays

(PerkinElmer Life and Analytical Sciences, Melbourne, VIC, Australia) using standard AlphaScreen settings.

**Determination of cLogD<sub>pH7.4</sub> Values.** These values were obtained using ChemAxon MarvinSketch version 14.11.10 (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 (SRI, Inc., Syracuse, NY).

**Compounds and Reagents.** The mACHR antagonists were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Radioligand was purchased from PerkinElmer. Cell culture and molecular biology reagents were supplied by Life Technologies (Melbourne, VIC, Australia).

**Data Analysis.** Because the amount of radioactivity varied slightly for each experiment (<5%), data are shown graphically as the mean ± S.D. from a single fit to grouped data of individual experiments, and values reported in the text and tables are mean ± S.D. 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 in the following sections.

**Saturation Binding.** Saturation binding isotherms were globally fitted to nonspecific and total binding data, and individual estimates for total receptor number (B<sub>max</sub>) and radioligand dissociation constant (K<sub>D</sub>) were calculated as in eq. 1:

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

where Y is the radioligand binding, B<sub>max</sub> is the total receptor density, [A] is the concentration of radioligand, K<sub>D</sub> is the equilibrium dissociation constant of the radioligand, and NS is the fraction of nonspecific radioligand binding:

**Competition Binding.** Competition displacement binding data were fitted to sigmoidal three-parameter logistic eq. 2:

\[
Y = \frac{\text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + (10^{\log(\text{IC}_{50})} - \text{X})}}{1 + (10^{\log(\text{IC}_{50})} - \text{X})}
\]

where Y is the percent of bound radioligand. Top denotes maximal asymptotic binding, and bottom denotes the minimal asymptotic binding. IC<sub>50</sub> values obtained from the inhibition curves were converted to pK<sub>A</sub> values using the method of Cheng and Prusoff (1973).

**Association Binding.** [H]<sup>3</sup>NMS association data were globally fitted (eq. 3). k<sub>_on</sub> and k<sub>_off</sub> were shared across the data sets so that a single value for each was derived from the whole family of curves:

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

where k<sub>_obs</sub> = [radioligand] × k<sub>_on</sub> + k<sub>_off</sub>, Y = specific binding (counts per minute), Y<sub>max</sub> = maximal specific binding and X = time.

**Competition Binding Kinetics.** Association and dissociation rates for unlabeled antagonists were calculated by using the equations (eq. 4) described previously by Motulsky and Mahan (1984):

\[
K_A = k_1[I] + k_2
K_B = k_3[I] + k_4
S = \sqrt{\left((K_A - K_B)^2 + 4k_1k_3L - 1 \times 10^{-18}\right)}
K_F = 0.5(K_A + K_B + S)
K_S = 0.5(K_A + K_B - S)
DIFF = K_F - K_S
Q = \frac{B_{\text{max}}k_3L - 1 \times 10^{-9}}{\text{DIFF}}
Y = Q \left(\frac{k_1 + \text{DIFF}}{K_F} - \frac{k_1 - K_F}{K_F} + e^{-k_F(X)} - \frac{k_3 - K_S}{K_S} + e^{-k_S(X)}\right)
\]

where X = time (minutes); Y = specific binding (counts per minute); k<sub>_on</sub> = k<sub>_on</sub> [H]<sup>3</sup>NMS; k<sub>_off</sub> = k<sub>_off</sub> [H]<sup>3</sup>NMS; L = concentration of [H]<sup>3</sup>NMS used (nanomolar); and I = concentration of unlabeled antagonist (nanomolar). Fixing these parameters allowed the following to be calculated: B<sub>max</sub> = total binding (counts per minute), k<sub>_3</sub> = association rate of unlabeled ligand (M<sup>-1</sup> · min<sup>-1</sup>), and k<sub>_1</sub> = dissociation rate of unlabeled ligand (min<sup>-1</sup>).

**Operational Model of Agonism.** To estimate agonist efficacy (r), agonist concentration-response curves in both [Ca<sup>2+</sup>]<sup>+</sup>-flux and ERK1/2 phosphorylation assays were fitted to the following form of an operational model of agonism (eq. 5) (Black and Leff, 1983):

\[
Y = \frac{E_m - \text{Basal}}{1 + \left(10^{\frac{pEC_{50} - 1}{10}}\right)^{g/s}}
\]

where E<sub>m</sub> is the maximal possible response of the system (not the agonist), Basal is the basal level of response in the absence of agonist, K<sub>A</sub> denotes the functional equilibrium dissociation constant of the agonist (A), r is an index of the coupling efficiency (efficacy) of the agonist and is defined as r<sub>T</sub>/K<sub>E</sub>, where r<sub>T</sub> is the total concentration (B<sub>max</sub>) of receptors and K<sub>E</sub> is the concentration of agonist-receptor complex that yields half the maximum system response (E<sub>m</sub>). To define the E<sub>m</sub> and r of ACh and MCh in each assay, the K<sub>E</sub> for each agonist was constrained to equal the K<sub>E</sub> 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 (eq. 6) (Arunulakshana and Schild, 1959; Motulsky and Christopoulos, 2004):

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

where E is response, E<sub>max</sub> and basal are the top and bottom asymptotes of the curve, respectively, log[|A|] is the logarithm of the agonist concentration, pEC<sub>50</sub> is the negative logarithm of the agonist concentration that gives a response halfway between E<sub>max</sub> and basal, s represents the Schild slope for the antagonist, and pA<sub>2</sub> 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<sub>max</sub> data were fitted according to a combined operational/hemi-equilibrium model for competitive antagonism under nonequilibrium conditions (Kenakin, 2009; Mould et al., 2014), and estimates of antagonist affinity values, pK<sub>A</sub>, and their dissociation constant from the receptor, k<sub>_off</sub>, were determined as in eq. 7:

\[
Y = \frac{[A]/K_A(1 - \alpha(1 - e^{-k_A(t - 1)} + \beta e^{-k_A(t - 1)})) \cdot \tau E_m}{[A]/K_A((1 - \alpha(1 - e^{-k_A(t - 1)} + \beta e^{-k_A(t - 1)})) \cdot \tau + 1) + 1}
\]

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<sub>A</sub> and K<sub>B</sub> represent the respective equilibrium dissociation constants, k<sub>_off</sub> is the dissociation rate constant for the antagonist (min<sup>-1</sup>), t is the assay incubation time (minutes), τ 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 minute in $[\mathrm{Ca}^{2+}]_{i}$-flux assay, and 5 minutes 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 (eq. 7) with the following values or ranges: $K_A = 0.1 \mu M$, $K_B = 0.1 \mu M$, $\log t = 1–100$, $k_{\text{off}} = 0.001–0.1$, $E_m = 1$, time $= 1–100$ minutes, and $[B] = 0.1, 0.3, 1.0, 3.0, \text{ or } 10.0 \mu M$.

### Results

#### [3H]NMS Saturation Binding and Kinetic Parameters Using Whole Cells

Specific [3H]NMS binding to the M$_1$ mAChR was best described as saturable and monophasic and that a one-site model was the best fit to the data. The expression level of the M$_1$ mAChR recombinantly expressed in Flp-In-CHO cells was assessed by [3H]NMS saturation binding as $1.86 \pm 0.51 \times 10^6$ sites cell per well, and the equilibrium dissociation constant ($pK_D$) was determined to be $9.46 \pm 0.02$ (Table 1).

To enable a robust method to be used to evaluate the kinetic parameters of unlabeled mAChR antagonists (Fig. 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 was constructed using a range of concentrations (0.18–1.5 nM). Each curve was monitored until equilibrium was reached (Fig. 2). Data were globally fitted to give a single best fit of $k_{\text{on}}$ and $k_{\text{off}}$ values. Kinetic parameters for [3H]NMS are shown in Table 1. The kinetically derived $pK_D$ value was in excellent agreement with the $pK_D$ estimated from the saturation binding analysis ($9.24 \pm 0.24$ versus $9.46 \pm 0.02$).

#### [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 $IC_{50}$ and Hill slope parameter estimates. $IC_{50}$ values obtained from the inhibition

![Fig. 1. Structures of mAChR antagonists used for the study.](image-url)
The pK\textsubscript{a} and pK\textsubscript{b} values were obtained using ChemAxon MarvinSketch software as described in Materials and Methods. Data are expressed as mean \pm S.D. from a single fit to grouped data of three to six individual experiments. cLogD\textsubscript{pH7.4} values of the mAChR antagonists used within this study.

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TABLE 2

<table>
<thead>
<tr>
<th>Compounds</th>
<th>pK\textsubscript{a}</th>
<th>pK\textsubscript{b}</th>
<th>k\textsubscript{on}(k\textsubscript{b})</th>
<th>k\textsubscript{off}(k\textsubscript{d})</th>
<th>t\textsubscript{1/2}</th>
<th>Kinetic pK\textsubscript{T}</th>
<th>cLogD\textsubscript{pH7.4}</th>
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<tr>
<td>Acetylcholine</td>
<td>4.55 \pm 0.13</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Methacholine</td>
<td>4.80 \pm 0.29</td>
<td>nd</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 \pm 0.07</td>
<td>9.36 \pm 3.88</td>
<td>0.29 \pm 0.05</td>
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<td>8.91 \pm 0.33</td>
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<td>-0.70</td>
</tr>
<tr>
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<td>21.2 \pm 4.77</td>
<td>0.07 \pm 0.05</td>
<td>12.7 \pm 7.36</td>
<td>0.46 \pm 0.21</td>
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<td>0.01 \pm 0.00</td>
<td>72.9 \pm 4.43</td>
<td>0.70 \pm 0.05</td>
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<tr>
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<td>0.21 \pm 0.20</td>
<td>0.47 \pm 0.54</td>
<td>1.3 \pm 0.83</td>
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<td>50.7 \pm 37.8</td>
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<td>Pirenzepine</td>
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<td>Tiotropium</td>
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<td>8.37 \pm 1.71</td>
<td>0.006 \pm 0.001</td>
<td>129 \pm 29.7</td>
<td>0.13 \pm 0.10</td>
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nd, not determined; QNB, quinuclidinyl benzylate.

### Competition Binding Kinetics Using Whole Cells. To calculate the kinetic rates of unlabeled mAChR antagonist association and dissociation rates of [H]NMS in the presence and absence of competitor were determined, and these values were used to calculate k\textsubscript{on} and k\textsubscript{off} by globally fitting the individual experimental data sets to eq. 4, as described in Materials and Methods. Grouped data curves to atropine and tiotropium are shown in Fig. 3; graphs for the remaining ligands are shown in Supplemental Fig. 1. Data for all ligands are summarized in Table 2.

### Effect of cLogD\textsubscript{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\textsubscript{pH7.4}, Table 2) was investigated. Significant correlation (r\textsuperscript{2} = 0.72, P = 0.007) was found between Log k\textsubscript{on} and cLogD\textsubscript{pH7.4} (Supplemental Fig. 2A); however, no correlation was observed between the Log k\textsubscript{off} and cLogD\textsubscript{pH7.4} (Supplemental Fig. 2B).

### Simulations of Hemiequilibrium and the Effects of Stimulus-Response Coupling. Our hypothesis is that the dissociation rate of unlabeled ligands can also be quantitatively estimated using the hemi-equilibrium model for competitive antagonism under nonequilibrium 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 and Leff, 1983) could play a large part in determining the optimal conditions to observe hemiequilibrium.

To guide our experimental design, simulations of agonist concentration-response curves in the presence of increasing concentrations of antagonist were constructed using a combined...

### Kinetics of mAChR Ligands in Binding and Functional Assays.
operational/hemi-equilibrium model of competitive antagonism (Kenakin, 2009; Mould et al., 2014). In an assay of modest receptor reserve (τ = 10; typical of many functional screening assays) and with a fixed incubation time of 10 minutes, an antagonist with a receptor half-life of τ₁/₂ ≈ 7 minutes (k₂₄ = 0.1 minute⁻¹) is predicted to display a surmountable profile (Fig. 5A). An antagonist with a receptor τ₁/₂ ≈ 70 minutes (k₂₄ = 0.01 minute⁻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 (τ₁/₂ ≈ 700 minutes; k₂₄ = 0.001 minute⁻¹) would be predicted to display an essentially insurmountable antagonist profile (Fig. 5C). Simulations of antagonist profiles under different assay incubation times reveal a similar pattern; an antagonist with a receptor τ₁/₂ ≈ 70 minutes can appear surmountable or insurmountable or display a hemi-equilibrium profile, depending on the length of agonist-antagonist-receptor re-equilibration time (Fig. 5, D–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 τ for the agonist from 1 to 100 yields a similar variety of curve-shift profiles (Fig. 5, G–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 operational/hemi-equilibrium model for antagonists that fully collapse the agonist concentration-response curve (Fig. 5J). Therefore, finding optimal experimental conditions is a prerequisite 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 τ
values determined in assays with both short (15 seconds) and moderate (5 minutes) equilibration times. In the $[\text{Ca}^{2+}]_i$-flux assay, Log $\tau$ values of 2.84 ± 0.06 and 1.94 ± 0.06 were determined for ACh and MCh, respectively (Fig. 6A). Lower efficacies were seen in ERK1/2 phosphorylation assays, with Log $\tau$ values of 2.19 ± 0.11 and 1.35 ± 0.10 (Fig. 6B) for ACh and MCh, respectively. In both the $[\text{Ca}^{2+}]_i$-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 with 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 demonstrate experimentally 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\(^{2+}\)]\(_i\)-flux and ERK1/2 phosphorylation assays with only a small depression in the \(E_{\text{max}}\) (Fig. 7, A 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 \(E_{\text{max}}\) (Fig. 7, C and D), in accordance with the lower efficacy of this ligand. These data were analyzed using the combined operational/hemi-equilibrium model of antagonism, as described in Materials and Methods, to estimate \(pK_B\) and \(k_{\text{off}}\) values.

Analysis of the data sets 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 minutes for [Ca\(^{2+}\)]\(_i\)-flux and ERK1/2 phosphorylation, respectively). The slowly dissociating antagonist, tiotropium, abolished the MCh response in the [Ca\(^{2+}\)]\(_i\)-flux assay (Fig. 7E). In the ERK1/2 phosphorylation assay, however, it produced a saturable depression of the maximal MCh response, yielding an estimated \(t_{1/2} = 223 \pm 85\) minutes (Fig. 7F).

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\(^{2+}\)]\(_i\)-flux and 0.03 ± 0.01 in ERK1/2 phosphorylation versus 0.07 ± 0.05 in binding) and tiotropium (0.003 ± 0.002 min\(^{-1}\) in ERK1/2 phosphorylation versus 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\(^{2+}\)]\(_i\)-flux and 9.23 ± 0.21 in ERK1/2 phosphorylation versus 9.18 ± 0.06 in binding). A similarly excellent agreement was obtained for tiotropium (10.25 ± 0.26 in ERK1/2 phosphorylation versus 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 summarized in Table 3 and represented graphically in Supplemental Fig. 3. Some compounds clearly demonstrate surmountable antagonism, for example, 4-DAMP and himbacine, indicative of rapid dissociation. Because of their apparent rapid re-equilibration in the time frame of the assay, the \(t_{1/2}\) 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 \(pK_B\) and the functional hemi-equilibrium derived \(pK_{\text{B}}\) (\(r^2 = 0.97, P < 0.0001\)), which is shown in Fig. 8A. More interestingly, an excellent correlation was also obtained between the radioligand binding \(k_{\text{off}}\) and the functional hemi-equilibrium \(k_{\text{off}}\) (\(r^2 = 0.94, P = 0.0004\)), shown in Fig. 8C. Furthermore, a similarly excellent correlation was also obtained between the radioligand binding \(k_{\text{on}}\) and the functional hemi-equilibrium \(k_{\text{on}}\) (calculated post hoc by \(k_{\text{off}}/k_{\text{B}}\) values; \(r^2 = 0.90, P = 0.0012\)), shown in Fig. 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 and 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 radiolabeled probe but 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 M\(_1\) receptor as a test system, we generated kinetic rate constant estimates for a range of antagonists by [\(^{3}H\)]NMS binding. Analysis revealed a wide range of affinity and rate constant estimates. Both \(k_{\text{on}}\) and \(k_{\text{off}}\) values significantly correlate with antagonists’ equilibrium dissociation constants (Fig. 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.
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 $k_{off}$ 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 $E_{max}$ amenable to analysis (Fig. 5). Outside this range, there is either sufficient agonist-antagonist-receptor re-equilibration (yielding a surmountable profile) or no re-equilibration (resulting in an insurmountable profile; Fig. 5). Therefore, these simulations reveal a potential “sweet spot” of $k_{off}$ values that can be identified for a given assay incubation time, where the product of incubation time, and $k_{off}$ 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.,...
Flp-In-CHO-hM1 functional assays. Subsequent profiling of partial agonist, MCh, in place of the full agonist, ACh, in our analytical model performance improved when we used the values are high (Fig. 5). In keeping with this observation, the very slow-off compounds could appear surmountable when hoc calculated functional assay to generate estimates of dissociation rates. Finally, post assays and required profiling in the ERK1/2 phosphorylation r

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<td>Tiotropium</td>
<td>9.15 ± 0.26</td>
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<td>0.003 ± 0.002</td>
<td>3.53 ± 3.42</td>
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nd, not determined; QNB, quinuclidinyl benzylate.

Potency, dissociation, and post hoc association values of mAChR antagonists using a [Ca$^{2+}$]-flux or ERK1/2 phosphorylation assay under hemi-equilibrium conditions

Data are expressed as mean ± S.D. from a single fit to grouped data of three to four individual experiments.

1960; Adham et al., 1993; Umland et al., 2001). The operational parameter τ encompasses both terms (R$T/K_B$). Data simulations revealed that the degree of receptor reserve has as much influence as the incubation time and antagonist $k_{off}$, very slow-off compounds could appear surmountable when τ values are high (Fig. 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-hM1 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 [3H]NMS binding studies ($r^2 = 0.94$, $P = 0.0004$; Fig. 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 seconds). Antagonists with long $t_{1/2}$ values in [3H]NMS binding (e.g., tiotropium and glycopyrrolate) yielded fully insurmountable profiles in [Ca$^{2+}$]-flux assays and required profiling in the ERK1/2 phosphorylation assay to generate estimates of dissociation rates. Finally, post hoc calculated functional $k_{on}$ estimates were also significantly correlated with $k_{on}$ values determined by [3H]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 optimization 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 cocomplexes with high affinity and slowly dissociating antagonists (Rasmussen et al., 2011; Haga et al., 2012; 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 $\beta_2$-adrenoceptor (Baker et al., 2003a; Wisler et al., 2007) and atropine at the muscarinic M3 receptor (Stewart et al., 2010). Furthermore, there is no a priori reason why antagonists may not display differential affinities dependent on the pathway/effecter system being monitored (Kenakin, 2014); propranolol has a 5-fold higher affinity to inhibit adrenaline-stimulated cAMP accumulation compared with CRE-SPAP transcription at the $\beta_2$-adrenoceptor (Baker et al., 2003b), and even larger differences have been observed for $\alpha_1$-adrenoceptor antagonists (Yoshiki et al., 2014). The reason for 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
For this test set of compounds, the association rate was shown that lipophilicity and membrane compartmentalization of a ligand can have profound effects on binding kinetics.

Fig. 8. Correlation of (A) kinetically derived pK_B and functionally derived pK_B (B) binding Log k_{on} and post hoc functional Log k_{on} values and (C) binding Log k_{on} and functional Log k_{on} values from [3H]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 ± S.D. (n = 3 or 4). Dotted line shows y = x.

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 classic radioligand, [3H]NMS. From molecular dynamics simulations, Dror et al. (2011) described, for both the β2- and β2-adrenergic receptors, that the β-blocker alprenolol bound initially to a region in the extracellular vestibule, 15 Å away from the orthosteric site, before traversing to the binding pocket. Currently, our ability to measure ligand rate constants is 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 physicochemical 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, or 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.

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Authorship Contributions

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

Conducted experiments: Riddy, Valant, Rueda.

Performed data analysis: Riddy, Valant, Langmead.

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

References


association of some ligands to the M1 receptor may be due to their physicochemical properties; Sykes et al. (2014b) have shown that lipophilicy 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 (cLogD_{7.4}; r^2 = 0.72, P = 0.007). Interestingly, there was no correlation of cLogD_{7.4} 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 favor 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 lipophilicy and weak membrane affinity may interact directly with the extracellular vestibule of the receptor, similar to the radioligand, [3H]NMS. From molecular dynamics simulations, Dror et al. (2011) described, for both the β2- and β2-adrenergic receptors, that the β-blocker alprenolol bound initially to a region in the extracellular vestibule, 15 Å away from the orthosteric site, before traversing to the binding pocket. Currently, our ability to measure ligand rate constants is 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 physicochemical 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, or 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.

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

References


