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

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ABSTRACT

Drug receptor kinetics is 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 [Ca$^{2+}$]_i-flux and extracellular kinases 1 and 2 phosphorylation assays that have some functional assays. Using functional [Ca$^{2+}$]_i-flux and extracellular kinases 1 and 2 phosphorylation assays that have short incubation times and therefore are prone to hemi-equilibrium “behaviors,” 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 $pK_i$, $k_{on}$, and $k_{off}$ from functional assays and those determined by competition kinetics using whole-cell [H]$^3$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 reality 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-(3-hexahydro-1H-azepin-1-yl)propyl)oxy)phenyl)butyl-2-pyrrolidinyl(methyl)-1(2H)-phthalazinone (GSK1004723) was identified as a potent histamine H$_3$R and H$_2$R 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 3 results in the treatment of chronic obstructive pulmonary disease (Jones, 2015), have greater kinetic selectivity for the M$_3$ 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.
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 (Patachini 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 M1 mAChR, a physiologically relevant buffer, whole-cell competition kinetics, and [Ca\(^{2+}\)]-flux and ERK1/2 phosphorylation functional assays. The M1 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 384-Optiplate (PerkinElmer Life and Analytical Sciences) at 37°C for 60 minutes, then 22°C for 10 minutes, before the fluorescence signal was measured using an Envision plate reader.
(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 14.11.10.0 (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 (SRI, Inc., Syracause, 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 analyses were performed 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:

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

where Y is the radioligand binding, B<sub>max</sub> is the total receptor density, [A] is the concentration of radioligand, K<sub>a</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 sigmodial three-parameter logistic eq. 2:

\[ Y = \frac{\text{Top} - \text{Bottom}}{1 + 10^{\log I_{C_{50}} - X}} \]

where Y is the percent of bound radioligand. Top denotes maximal asymptotic binding, and Bottom denotes the minimal asymptotic binding. I<sub>C_{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):

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

\[ K_d = k_3 [L] + k_4 \]

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

\[ K_F = 0.5 (K_A + K_B + S) \]

\[ K_S = 0.5 (K_A - K_B) \]

\[ DIF = K_F - K_S \]

\[ Q = \frac{B_{\text{max}} (K_F - L) I - 1 e^{-9}}{DIF} \]

\[ Y = Q \left( k_1 + \frac{DIF}{K_F - K_S} \right) - \frac{k_2 - K_F}{K_F} \left( e^{-K_F X} - \frac{K_F - K_S}{K_S} \right) \]

where X = time (minutes); Y = specific binding (counts per minute); k<sub>1</sub> = k<sub>on</sub> [H]<sup>3</sup>NMS; k<sub>2</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 (\( \tau \)), agonist concentration-response curves in both [3H]<sup>1</sup>Binding, and ERK1/2 phosphorylation assays were fitted to the following form of an operational model of agonism (eq. 5) (Black and Lefk, 1983):

\[ Y = \frac{E_{\text{m}} - \text{Basal}}{1 + \frac{[\text{radioligand}] / C_{2}}{10^{\log K_{F}}}} \]

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), \( \tau \) is an index of the coupling efficiency (efficacy) of the agonist and is defined as R<sub>T</sub>/K<sub>t</sub>, where R<sub>T</sub> is the total concentration (B<sub>max</sub>) of receptors and K<sub>t</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 \( \tau \) of ACh and MCh in each assay, the K<sub>t</sub> for each agonist was constrained to equal the K<sub>i</sub> value derived from radioligand binding assays (see Results) in the nonlinear regression procedure.

**Schild Analysis.** To estimate agonist 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) (Arunlakshana and Schild, 1959; Motulsky and Christopoulos, 2004):

\[ E = \frac{(E_{\text{max}} - \text{Basal})}{1 + \frac{E_{\text{max}} - \text{Basal}}{10^{\log K_{F}}} \times (\frac{[\text{radioligand}] / C_{2}}{10^{\log K_{F}}})} \]

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/hemiequilibrium model for competitive antagonism under nonequilibrium conditions (Kenakin, 2009; Mould et al., 2014), and estimates of antagonist affinity values, pK<sub>50</sub>, and their dissociation constant from the receptor, k<sub>obs</sub>, were determined as in eq. 7:

\[ Y = \frac{[A]/K_A (1 - (\alpha (1 - e^{-k_d \cdot \tau}) + \beta e^{-k_d \cdot \tau}) + \gamma E_{\text{m}})}{[A]/K_A ((1 - (\alpha (1 - e^{-k_d \cdot \tau}) + \beta e^{-k_d \cdot \tau}) + \gamma + 1) + 1} \]

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>obs</sub> is the dissociation rate constant for the antagonist (min<sup>-1</sup>), t is the assay incubation time (minutes), \( \tau \) 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 minute in $[Ca^{2+}]_i$-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, $k_{cat} = 0.001$–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

$[^3]{H}$NMS Saturation Binding and Kinetic Parameters Using Whole Cells. Specific $[^3]{H}$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 $[^3]{H}$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 $[^3]{H}$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 $[^3]{H}$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 $k_{on}$ and $k_{off}$ values. Kinetic parameters for $[^3]{H}$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 $9.46 \pm 0.02$).

$[^3]{H}$NMS Equilibrium Competition Binding Using Whole Cells. All mAChR antagonists produced concentration-dependent inhibition of specific binding of $[^3]{H}$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

<table>
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<tr>
<th>1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP)</th>
<th>Atropine</th>
<th>Glycopyrrolate</th>
<th>Himbacine</th>
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<td><img src="image" alt="4-DAMP" /></td>
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<tr>
<th><img src="image" alt="pratropium" /></th>
<th><img src="image" alt="N-methylscopolamine (NMS)" /></th>
<th><img src="image" alt="Pirenzepine" /></th>
<th><img src="image" alt="Quinuclidinyl benzilate (QNB)" /></th>
<th>Tiotropium</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="pratropium" /></td>
<td><img src="image" alt="N-methylscopolamine (NMS)" /></td>
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**Fig. 1.** Structures of mAChR antagonists used for the study.

**Table 1**

<p>| Binding parameters for $[^3]{H}$NMS determined by equilibrium saturation and kinetic binding experiments |
| Data are expressed as mean ± S.D. from a single fit to grouped data of three individual experiments. |</p>
<table>
<thead>
<tr>
<th>$pK_D$</th>
<th>$K_{max}$</th>
<th>$k_{on}$ ($k_1$)</th>
<th>$k_{cat}$ ($k_2$)</th>
<th>$t_{1/2}$</th>
<th>Kinetic $pK_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$9.46 \pm 0.02$</td>
<td>$1.86 \pm 0.51$</td>
<td>$24.8 \pm 11.8$</td>
<td>$0.04 \pm 0.01$</td>
<td>$18.9 \pm 3.30$</td>
<td>$9.24 \pm 0.24$</td>
</tr>
</tbody>
</table>

| $[^3]{H}$NMS Saturation Binding and Kinetic Parameters Using Whole Cells. | Specific $[^3]{H}$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 $[^3]{H}$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 $[^3]{H}$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 $[^3]{H}$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 $k_{on}$ and $k_{off}$ values. Kinetic parameters for $[^3]{H}$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 $9.46 \pm 0.02$). | $[^3]{H}$NMS Equilibrium Competition Binding Using Whole Cells. All mAChR antagonists produced concentration-dependent inhibition of specific binding of $[^3]{H}$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... |
were used to calculate the equilibrium competition binding profiles. Association data were fitted to a global fitting model using Prism 6.0f to simultaneously calculate $k_{on}$ and $k_{off}$ and these values were derived from $C_2$ and absence of competitor were determined, and these values were used to allow equilibrium to be reached. The difference in association and dissociation rates of $[^3H]$NMS in the presence and absence of competitor were studied, and these values were used to calculate $k_{on}$ and $k_{off}$ 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 Supplemental Fig. 1. Data for all ligands are summarized in Table 2. Each ligand was tested over a range of concentrations to demonstrate competitive and increased sampling time of the slow dissociating ligands are shown in Supplemental Fig. 1. Data for all ligands were used to calculate the kinetic rates of unlabeled mAChR, antagonist association and dissociation rates of $[^3H]$NMS in the presence and absence of competitor were determined, and these values were used to calculate $k_{on}$ and $k_{off}$ 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. 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 used 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 and Charlton, 2006; Mould et al., 2014).

There was an excellent correlation between kinetically derived $pK_I$ and the equilibrium competition binding $pK_I$ values (Fig. 4A; $r^2 = 0.98, P < 0.0001$). Estimates of the $k_{on}$ and $k_{off}$ rates of $[^3H]$NMS measured directly ($k_{off} = 24.84 \pm 11.79 \times 10^7 \text{M}^{-1} \text{min}^{-1}$, $k_{off} = 0.04 \pm 0.01 \text{ min}^{-1}$) and those of unlabeled 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.

Significant correlation was found between equilibrium dissociation constants and both $k_{on}$ and $k_{off}$ values, as shown in Fig. 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 and Seeman, 2001), or $k_{off}$ alone, for example, the OX$_3$ 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, bimacine and pirenzepine, display slow-on and fast-off kinetics; compounds with high affinity, for example, atropine and tiotropium, display rapid $k_{on}$ and slow $k_{off}$ kinetics (Fig. 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$_{pH7.4}$ values, Table 2) was investigated. Significant correlation ($r^2 = 0.72, P = 0.007$) was found between $\log k_{on}$ and cLogD$_{pH7.4}$ (Supplemental Fig. 2A); however, no correlation was observed between the $\log k_{off}$ and cLogD$_{pH7.4}$ (Supplemental Fig. 2B).

**Simulations of Hem-Equilibrium 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 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

![Fig. 2. Kinetics of $[^3H]$NMS with CHO cells expressing the M$_1$ mAChR. The $k_{on}$ and $k_{off}$ values were determined by incubating Flp-In-CHO-hM$_1$ mAChR cells with the indicated concentrations of $[^3H]$NMS for various time periods. Association data were fitted to a global fitting model using Prism 6.0f to simultaneously calculate $k_{on}$ and $k_{off}$ and these values were used to allow equilibrium to be reached (Dowling and Charlton, 2006; Mould et al., 2014).](https://example.com/figure2.png)

### Table 2

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$pK_I$</th>
<th>$k_{on}$ ($k_3$)</th>
<th>$k_{off}$ ($k_4$)</th>
<th>$t_{1/2}$</th>
<th>Kinetic $pK_{T1}$</th>
<th>cLogD$_{pH7.4}$</th>
</tr>
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<tbody>
<tr>
<td>Acetylcholine</td>
<td>4.55 ± 0.13</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>Methacholine</td>
<td>4.80 ± 0.29</td>
<td>nd</td>
<td>nd</td>
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<td>nd</td>
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<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>
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$nd$, not determined; QNB, quinuclidinyl benzylate.

*Downloaded from molpharm.aspetjournals.org on December 29, 2017.*
operational/hemi-equilibrium model of competitive antagonism (Kenakin, 2009; Mould et al., 2014). In an assay of modest receptor reserve ($t_{1/2} \approx 70$ minutes) and with a fixed incubation time of 10 minutes, an antagonist with a receptor half-life of $t_{1/2} \approx 70$ minutes ($k_{off} = 0.01$ minute$^{-1}$) is predicted to display a surmountable profile (Fig. 5A). An antagonist with a receptor $t_{1/2} \approx 70$ minutes ($k_{off} = 0.01$ 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 ($t_{1/2} \approx 700$ minutes; $k_{off} = 0.001$ 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 $t_{1/2} \approx 70$ 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 $\tau$ 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 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 $\tau$
values determined in assays with both short (15 seconds) and moderate (5 minutes) equilibration times. In the $[Ca^{2+}]_i$-flux assay, Log $t$ 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 $t$ values of 2.19 ± 0.11 and 1.35 ± 0.10 (Fig. 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 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\textsuperscript{2+}]-flux and ERK1/2 phosphorylation assays with only a small depression in the $E_{\text{max}}$ (Fig. 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 $E_{\text{max}}$ (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 ($14 \pm 2$ and $27 \pm 1$ minutes for [Ca\textsuperscript{2+}]-flux and ERK1/2 phosphorylation, respectively). The slowly dissociating antagonist, tiotropium, abolished the MCh response in the [Ca\textsuperscript{2+}]-flux assay, precluding meaningful analysis using the model (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 ($0.05 \pm 0.01$ min\textsuperscript{-1} in [Ca\textsuperscript{2+}]-flux and $0.03 \pm 0.01$ in ERK1/2 phosphorylation versus $0.07 \pm 0.05$ in binding) and tiotropium ($0.003 \pm 0.002$ min\textsuperscript{-1} in ERK1/2 phosphorylation versus $0.006 \pm 0.001$ in binding). The $pK_B$ values for atropine from both assays were also in excellent agreement with those obtained in the competition binding kinetics ($9.44 \pm 0.08$ in [Ca\textsuperscript{2+}]-flux and $9.23 \pm 0.21$ in ERK1/2 phosphorylation versus $9.18 \pm 0.06$ in binding). A similarly excellent agreement was obtained for tiotropium ($10.25 \pm 0.26$ in ERK1/2 phosphorylation versus $10.05 \pm 0.16$ 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 $pK_B$ ($r^2 = 0.97$, $P < 0.0001$), 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 $k_{\alpha}$ and the functional hemi-equilibrium $k_{\alpha}$ (calculated post hoc by $k_{\text{off}}/K_B$ 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\textsubscript{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 $k_{\alpha}$ 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 $k_{\text{off}}$ 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 (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 $k_{\text{off}}$ 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., 787).
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 (even in the [Ca2+]i-flux assay, precluding analysis using the [Ca2+]i-flux model, precluding analysis using the hemi-equilibrium model. The revelation that common functional assays can be used 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 cocrystals with high affinity and slowly dissociating antagonists (Rasmussen et al., 2007; 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 β2-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 β2-adrenoceptor (Baker et al., 2003b), and even larger differences have been observed for α1-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

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>pKᵦ from hemi-equilibrium model</th>
<th>pKᵦ from Schild analysis</th>
<th>kᵦ (min⁻¹)</th>
<th>Post hoc kᵦ</th>
<th>t₁/₂ (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>14.3 ± 4.96</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>14.2 ± 2.4</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Himbacine</td>
<td>6.51 ± 0.16</td>
<td>6.62 ± 0.20</td>
<td>nd</td>
<td>33.9 ± 4.4</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>14.9 ± 15.7</td>
<td>&lt;2</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>20.0 ± 28.4</td>
<td>7.7</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>23.3 ± 7.7</td>
<td>23.7 ± 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>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>
</tr>
<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; QNB, quinuclidinyl benzylate.
Fig. 8. Correlation of (A) kinetically derived $pK_B$ and functionally derived $pK_B$ (B) binding $k_{on}$ and post hoc functional $k_{on}$ values and (C) binding $k_{on}$ and functional $k_{on}$ 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 $y = x$.

A significant correlation of some ligands to the M$_1$ receptor may be due to their physicochemical 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 ($c$Log$D_{7.4}$; $r^2 = 0.72$, $P = 0.007$). Interestingly, there was no correlation of cLog$D_{7.4}$ with dissociation rate ($r^2 = 0.18$), suggesting that once bound, egress of a molecule from the M$_1$ 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 $\beta_2$-adrenoceptor (Sykes et al., 2014b) and indicates that the initial interaction with the M$_1$ 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 lipophilicity 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 $\beta_2$- and $\beta_2$-adrenergic receptors, that the $\beta$-blocker alpranolol 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.

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 M$_1$ receptor antagonist rate constants that were in excellent agreement with those determined by classic radioligand binding approaches. This generic method offers an approach to profile receptor kinetics without the need for labeled probes, opening up the possibility to determine kinetic parameters for GPCR antagonists as a structure activity relationship 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.

**Performed data analysis:** Riddy, Valant, Langmead.

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

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Avlani VA, Gregory KJ, Martin CJ, Parker MW, Sexton PM, and Christopoulos A (2007) Critical role for the second extracellular loop in the binding of both


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.


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Supplementary Figure 1

[^3]H-NMS competition binding kinetic curves in the presence of (a) 4-DAMP, (b) glycopyrrolate, (c) himbacine, (d) ipratropium, (e) NMS, (f) pirenzepine and (g) QNB. Flp-In-CHO-hM1 mAChR cells were incubated with ~0.4 nM[^3]H-NMS with 1, 3 and 10x K_{i} for 4-DAMP, himbacine, ipratropium, NMS, and pirenzepine or 10, 30 and 100x K_{i} for glycopyrrolate and QNB. 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_{on}, k_{off} and kinetically-derived K_{D} values for the unlabeled agonists, and are summarized in Table 2. Grouped data are shown ± SD (n=3).
Supplementary Figure 2

Correlation of (a) binding Log $k_{on}$ and cLogD$_{pH7.4}$ values and (b) binding Log $k_{off}$ and cLogD$_{pH7.4}$ values from [³H]-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=3-4). *Pirenzepine was excluded from the correlation analysis due to its fast association rate.
Label-free kinetics: exploiting functional hemi-equilibrium to derive rate constants for muscarinic receptor antagonists

Supplementary Figure 3

Concentration-response curves of MCh in the presence of increasing concentrations of (a) 4-DAMP, (b) himbacine, (c) ipratropium, (d) NMS and (e) pirenzepine in the [Ca\textsuperscript{2+}]-release assay or (f) glycopyrrolate and (g) QNB in the ERK1/2 phosphorylation assay. Flp-In-CHO-hM\textsubscript{1} mAChR cells were incubated at 37°C with antagonist for 30 min prior to the addition of agonist and assayed for 5 min, 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\textsuperscript{TM} 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\textsubscript{B} and k\textsubscript{off} values for the unlabeled agonists, and are summarized in Table 3. Grouped data are shown ± SD (n=3-4).
Supplementary Figure 4

Correlation of pK_B values obtained from [Ca^{2+}]_i-release or ERK1/2 phosphorylation assays analysed by either the combined operational / hemi-equilibrium model for competitive antagonism under non-equilibrium conditions (Kenakin, 2009) or the Schild regression analysis (Arunlakshana & Schild, 1959). Data were fitted to Deming linear regression with statistical significance determined as P < 0.05. The values used are shown in Table 3. Grouped data are shown ± SD (n=3-4). Dotted line shows ŷ = x.