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Homobivalent conjugation increases the allosteric effect of 9-aminoacridine at the $\alpha_1$ adrenergic receptors

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AR, adrenergic receptor; CNS, central nervous system; GPCR, G protein-coupled receptor; NE, norepinephrine; TM, transmembrane
Abstract

The $\alpha_1$ adrenergic receptors are targets for a number of cardiovascular and CNS conditions, however current drugs for these receptors lack specificity to be of optimal clinical value. Allosteric modulators offer an alternative mechanism of action to traditional $\alpha_1$ adrenergic ligands, yet there is little information describing this drug class at the $\alpha_1$ adrenergic receptors. We have identified a series of 9-aminoacridine compounds that demonstrate allosteric modulation of the $\alpha_{1A}$ and $\alpha_{1B}$ adrenergic receptors. The 9-aminoacridines increase the rate of [$^3$H]prazosin dissociation from the $\alpha_{1A}$ and $\alpha_{1B}$ adrenergic receptors and non-competitively inhibit receptor activation by the endogenous agonist, norepinephrine. The structurally similar compound, tacrine, which is a known allosteric modulator of the muscarinic receptors, is also shown to be a modulator of the $\alpha_1$ adrenergic receptors, which suggests a general lack of selectivity for allosteric binding sites across aminergic GPCRs. Conjugation of two 9-aminoacridine pharmacophores, using linkers of varying length, increases the potency and efficacy of the allosteric effects of this ligand, likely through optimisation of bitopic engagement of the allosteric and orthosteric binding sites of the receptor. Such a bivalent approach may provide a mechanism for fine tuning the efficacy of allosteric compounds in future drug design efforts.
Introduction

Allosteric modulators offer a number of potential advantages over traditional, orthostERICALLY acting drugs including maintenance of spatio-temporal patterns of physiological signalling (Christopoulos, 2002). These advantages are of particular value in the central nervous system (CNS) where perturbation of G protein-coupled receptor (GPCR) signalling, or direct and sustained stimulation/inhibition of receptors frequently leads to limiting side-effects (Conn et al., 2014). Therefore, allosteric modulators of GPCRs are of growing interest as drugs to target validated, but otherwise intractable receptors, particularly in the CNS.

One of the main hurdles to the use of allosteric modulators remains their identification from drug screens. This, coupled with the general lack of structural information about allosteric pockets on GPCRs, means allosteric modulators remain relatively underrepresented as drugs for GPCRs. One of the few exceptions to this is the muscarinic acetylcholine receptor family, where crystal structures are publically available for 4 of the 5 subtypes (Kruse et al., 2012; Thal et al., 2016; Thorsen et al., 2014), one of which has an allosteric modulator bound (Kruse et al., 2013), and radiolabelled allosteric ligands are available (Schober et al., 2014; Tränkle et al., 2003). These serve as excellent models for fundamental properties of allosteric modulators, but many other GPCR families still lack defined allosteric sites, or many examples of allosteric ligands.

One family of GPCRs with potential CNS, as well as peripheral applications are the $\alpha_1$ adrenergic receptors. The $\alpha_1$ adrenergic receptors are three of the nine GPCRs that bind and respond to the neurohormones epinephrine and norepinephrine. The $\alpha_{1A}$ adrenergic receptor subtype has been validated as a potential target to treat seizure disorders arising in the CNS (Hillman et al., 2009; Pizzanelli et al., 2009; Zuscik et al., 2001), as well as for the treatment of heart failure (Du et al., 2006; Lin et al., 2001). However a common hurdle for the
successful targeting of this receptor family is subtype selectivity (Chen and Minneman, 2005). Even clinically relevant drugs such as prazosin, silodosin and naftopidil possess less than 100-fold selectivity for their target receptor over other \( \alpha_1 \) adrenergic receptor subtypes, as well as other biogenic amine receptors such as the 5-HT\(_{1A} \) serotonin receptor (GlaxoSmithKline, 2011; Shibata et al., 1995; Takei et al., 1999). Subtype selectivity is desirable as the \( \alpha_{1A} \) subtype which has the most therapeutic potential frequently exhibits opposing actions to the \( \alpha_{1B} \) subtype (Hillman et al., 2009; Milano et al., 1994).

Allosteric modulators have the potential to overcome many of these barriers, yet there are few examples of allosteric modulators for the \( \alpha_1 \) adrenergic receptor family (Leppik et al., 2000; Pfaffendorf et al., 2000; Sharpe et al., 2003; Waugh et al., 1999), and even less structural information regarding any allosteric site/s (Ragnarsson et al., 2015; Ragnarsson et al., 2013). Here we have employed an innovative approach to maximise the allosteric effect observable for a potential allosteric ligand. We have used a series of homobivalent derivatives of 9-aminoacridine with increasing linker lengths (Figure 1), which were previously identified as having unexpectedly high affinity for the \( \alpha_1 \) adrenergic receptors with some tissue specific differences in binding affinity (Adams et al., 1986; Adams et al., 1985). The three subtypes of the \( \alpha_1 \) adrenergic receptors had not been described at that time, but the observed differences in affinity were attributed to different binding modes of the bis(9-aminoacridine)s depending on the length of the linker, and the potential existence of a second pocket, or cleft, on tissue specific receptors. We hypothesise that the observed differences in binding affinity are attributable to subtype selective binding of the bis-(9-aminoacridine)s, and that the postulated “cleft” constitutes an allosteric site on the \( \alpha_1 \) adrenergic receptors. Additionally, we predicted that an optimally sized bivalent ligand will maximise occupancy of an allosteric site, increasing its observable effects.
Materials and Methods

Materials

COS-1 cells were purchased from ATCC (Virginia, USA). [³H]prazosin, [³H]myo-inositol, and Ultima Gold and Ultima Flo scintillation cocktails were purchased from Perkin Elmer (Waltham, MA, USA). [³H]8-OH-DPAT was purchased from GE Healthcare (Uppsala, Sweden). GF/C glass fibre filters were purchased from Whatman (Maidstone, UK). DMEM without inositol was purchased from MP Biomedicals (Santa Ana, CA, USA). FBS was purchased from Invitrogen (Carlsbad, CA, USA). (R)-epinephrine hydrochloride, (R)-norepinephrine hydrochloride, phenolamine hydrochloride, 5-methylurapidil, lithium chloride, (S)-propranolol hydrochloride, tacrine hydrochloride, DEAE dextran, ammonium formate, chloroquine diphosphate, Tris and HEPES were purchased from Sigma Aldrich (St. Louis, MO, USA). Bis(9-aminoacridine)s were synthesised as previously described (Deshpande and Singh, 1972), and are referred to throughout by the designation Cₙ bis(9-aminoacridine), where n is the number of methylenes in the linker chain. EDTA, EGTA, MgCl₂, NaCl, KCl, Na₂HPO₄, KH₂PO₄ and glycerol were purchased from Ajax Finechem (Taren Point, NSW, Australia). jetPEI was purchased from PolyPlus-transfection SA (Illkirch, France). AG 1-X8 formate resin was from Bio-Rad (Hercules, CA, USA). Human α₁A adrenergic receptor, α₁B adrenergic receptor and 5-HT₁A receptor in pcDNA were purchased from Missouri S&T cDNA Resource Centre (www.cdna.org). Human α₁D adrenergic receptor in pMV6-XL5 vector was obtained from Origene (Rockville, MO, USA).

Cell culture

COS-1 cells were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% (v/v) heat-treated FBS and 100 μg.mL⁻¹ glutamine. Cultures were kept at 37°C and 5% CO₂ and passaged as they approached confluence by standard tissue
Cells were seeded at a density of 4.5 x 10^6 cells per 175 cm² flask and transfected 24 h later using 20 µg DNA per 175 cm² flask as per the DEAE dextran method of Lopata et al. (1984). 48-72 h after transfection, cells were harvested by scraping into cold PBS and pelleted by centrifugation at 500 x g for 5 min at 4°C. The pellet was resuspended in cold HE buffer (20 mM HEPES, 10 mM EDTA, pH 7.4) using 10 mL of buffer for each 175 cm² plate scraped. Suspensions were homogenised on ice using an Ultra-Turax T125 homogeniser at 20,000 r.p.m. in three 10 s bursts. Lysate was centrifuged at 600 x g for 10 min at 4°C. Supernatant was then centrifuged at 40 000 x g for 1 h at 4°C. The final pellet was resuspended in cold 20 mM HEPES (pH 7.4) + 10% glycerol (v/v) using 200 µL per 175 cm² flask then homogenised on ice using an insulin syringe, aliquoted and stored at -80°C. Protein concentration was measured using Bradford reagent.

**Transfection and IP Accumulation**

Resuspended COS-1 cells were diluted to a concentration of 1 x 10^5 cells.mL^-1 DMEM and transfected with 1 µg construct and 2 µL jetPEI transfection reagent per 1 x 10^5 cells as per the manufacturer’s instructions. Cells were plated into 96 well plates (220 µL per well) and left at room temperature for 1 h following transfection to minimise uneven plating in the outmost wells (Lundholt et al., 2003), then transferred to a 37°C, 5% CO₂ incubator. 16-24 h post-transfection, receptor activation was determined using total soluble inositol phosphate (IP) production as previously described (Campbell et al., 2014) in the presence of 10 µM propranolol to inhibit any potential signalling from β adrenergic receptors.

**Binding assays**

All radioligand binding assays were performed at room temperature in duplicate. α₁
adrenergic receptor-expressing membranes were incubated in HEM buffer (20 mM HEPES, 1.4 mM EGTA, 12.5 mM MgCl₂, pH 7.4), unless otherwise stated, using 1-2 μg protein per reaction for α₁A and α₁B adrenergic receptors and 10-15 μg protein per reaction for the α₁D adrenergic receptor. 5-HT₁A receptor-expressing membranes were incubated in HC buffer (20 mM HEPES, 4 mM CaCl₂, pH 7.4) using 10 μg protein per reaction. Reactions were terminated by the addition of 4°C PBS and vacuum filtration through Whatman GF/C glass fibre filters.

**Saturation binding**

Saturation binding assays were performed in a final volume of 200 μL and allowed to incubate for 1 h. α₁ adrenergic receptor-expressing membranes were incubated in HEM buffer with 0.03 – 8 nM [³H]prazosin. Non-specific binding was determined using 100 μM phentolamine. 5-HT₁A receptor-expressing membranes were incubated in HC buffer with 0.1 – 12 nM [³H]8-OH-DPAT. Non-specific binding was determined using 10 μM serotonin.

**Competition binding**

Competition binding assays were performed in a final volume of 200 μL and allowed to incubate for 1 h in the presence of an approximate Kᵢ concentration of radioligand. α₁ adrenergic receptor affinity was determined in HEM buffer in competition with 250 pM [³H]prazosin. Ligand binding affinity for 5-HT₁A receptors was determined in HC buffer in competition with 1 nM [³H]8-OH-DPAT.

**Dissociation binding kinetics**

Dissociation kinetics assays were performed in HEM buffer over 80 min for the α₁A adrenergic receptor, and TE buffer (50 mM Tris, 0.5 mM EDTA, pH 7.4) over 160 min for the α₁B adrenergic receptor. Receptors were pre-equilibrated with 250 pM [³H]prazosin for 1 h at room temperature in a volume of 400 μL. [³H]prazosin reassociation was then inhibited.
by the addition of 100 μL phentolamine for a final concentration of 100 μM in the absence or presence of test compounds.

Data analysis

All binding data was analysed in GraphPad Prism 6 by non-linear regression using standard one-site curves for saturation and dissociation kinetics assays. Competition binding assays were fit by a variable-slope competition binding model (Cheng and Prusoff, 1973; Hill, 1910). All values were compared by one-way ANOVA with Tukey post-test. IP accumulation data was analysed in GraphPad Prism 6 by non-linear regression using a variable slope, four-parameter fit. Inhibitor affinity was estimated by generating a double reciprocal plot of equi-effective concentrations of agonist in the absence and presence of inhibitor at a concentration that produces an approximate 50% decrease in the maximum signal (Ehlert, 1988; Kenakin, 1997).
Results

Equilibrium binding of bis(9-aminoacridine)s

To address whether the tissue-specific differences in binding affinity originally observed for the bis(9-aminoacridine)s is attributable to subtype selectivity, their apparent affinities were determined via coincubation with radioligands for membrane-expressed human $\alpha_1$ adrenergic receptors, as well as the 5-HT$_{1A}$ receptor to assess for “off target” binding. The $\alpha_{1A}$ and $\alpha_{1B}$ adrenergic receptors expressed at similar levels ($B_{max}$: $\alpha_{1A}$ 2.3 ± 0.2, $\alpha_{1B}$ 2.4 ± 0.2 pmol.mg$^{-1}$) while the $\alpha_{1D}$ adrenergic receptors and 5-HT$_{1A}$ receptor expressed at lower levels ($B_{max}$: $\alpha_{1D}$ 0.3 ± 0.1, 5-HT$_{1A}$ 0.2 ± 0.1 pmol.mg$^{-1}$). Radioligands bound with normal affinity ([${}^3$H]prazosin $K_D$: $\alpha_{1A}$ 1.1 ± 0.1, $\alpha_{1B}$ 0.7 ± 0.1 and $\alpha_{1D}$ 0.9 ± 0.2 nM; [${}^3$H]8-OH-DPAT $K_D$ 2.2 ± 0.1 nM) and all assays were titrated so that specific binding of radioligands constituted less than 10% of free ligand. Known competitive ligands of the $\alpha_1$ adrenergic receptors and 5-HT$_{1A}$ receptor were found to display affinities comparable to previously reported values (Table 1) (Newman-Tancredi et al., 1998; Zhao et al., 1996). 9-aminoacridine, the parent monomer of the bis(9-aminoacridine)s, has an affinity of 247 nM for the $\alpha_{1A}$ adrenergic receptor, is 10-fold selective over the $\alpha_{1B}$ adrenergic receptor ($K_i$: 2.6 µM, $P<0.01$), 8-fold selective over the $\alpha_{1D}$ adrenergic receptor ($K_i$: 1.9 µM, $P<0.001$), and greater than 400-fold selective over the 5-HT$_{1A}$ receptor ($K_i$: >100 µM) (Table 1, Figure 2). To investigate whether the affinity of 9-aminoacridine is dependent upon it being fully aromatic, the affinity of its tetra-hydro derivative, tacrine, was also measured (Figure 1). Tacrine displays affinities of 1.8, 19.4, and 6.4 µM at the $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$ adrenergic receptors, respectively (Table 1, Figure 2). Thus tacrine binding is consistently weaker than 9-aminoacridine at the $\alpha_{1A}$ and $\alpha_{1B}$ adrenergic receptors ($P < 0.01$), but it still maintains selectivity for $\alpha_{1A}$ over $\alpha_{1B}$ ($P < 0.05$).

In general, the bis(9-aminoacridine)s studied show low- to sub-micromolar affinity for
all three \( \alpha_1 \) adrenergic receptor subtypes (Table 1, Figure 2). At the \( \alpha_{1B} \) and \( \alpha_{1D} \) adrenergic receptors the bis(9-aminoacridine)s have similar or greater affinity than 9-aminoacridine for all linker lengths tested, their affinity-chain length relationship producing a shallow, concave profile with maximum affinity occurring around the C5, C6, and C7 homologues (Table 1, Figure 2). In contrast, at the \( \alpha_{1A} \) adrenergic receptor this profile is more pronounced, particularly for bis(9-aminoacridine)s with linkers between 2 and 7 carbons (Table 1, Figure 2). Whereas the bis(9-aminoacridine)s display only 5- and 7-fold differences between the highest and lowest affinity compounds at the \( \alpha_{1B} \) and \( \alpha_{1D} \) adrenergic receptors, at the \( \alpha_{1A} \) adrenergic receptor there is a 91-fold difference between the highest and lowest affinity compounds, C4 and C2 bis(9-aminoacridine), respectively. At the \( \alpha_{1A} \) adrenergic receptor C2 has an affinity of 1.9 \( \mu \)M, which is significantly lower than that of 9-aminoacridine (\( P < 0.01 \)), while the affinity of C4, at 21 nM, is significantly higher than that of 9-aminoacridine as well as that of C2, C3 and all other bis(9-aminoacridine)s with linkers longer than 6 carbons (\( P < 0.05 \)). The affinity of C4 bis(9-aminoacridine) at the \( \alpha_{1A} \) adrenergic receptor is the highest observed for any of the bis(9-aminoacridine)s at all of the tested receptors (Figure 2), the ligand being 10-fold selective over the \( \alpha_{1B} \) subtype (\( P < 0.001 \)), 30-fold selective over the \( \alpha_{1D} \) adrenergic receptor (\( P < 0.001 \)), and 145-fold selective over the 5-HT\(_{1A}\) receptor (\( P < 0.001 \)). All bis(9-aminoacridine)s with a linker length of five carbons or fewer have significantly higher affinity for the three \( \alpha_1 \) adrenergic receptor subtypes than the 5-HT\(_{1A}\) receptor. Affinities of the shorter bis(9-aminoacridine)s, C2 and C3, as well as the monovalent 9-aminoacridines for the 5-HT\(_{1A}\) receptor are some of the lowest observed in the test set, resulting in substantial selectivity for the \( \alpha_1 \) adrenergic receptor subtypes.

Close observation reveals that the slopes of the competitive binding curves for some of the bis(9-aminoacridine)s at the \( \alpha_1 \) adrenergic receptors appear steeper than that generated by the competitive antagonist phentolamine (Table 2). The Hill coefficients of phentolamine
binding do not differ significantly from unity for any $\alpha_1$ adrenergic receptor, nor for serotonin binding at the 5-HT$_{1A}$ receptor (Table 2). There is a similar finding for the monomers 9-aminoacridine and tacrine (Table 2). In contrast, the Hill slope for C10 bis(9-aminoacridine) is significantly greater than 1 at the $\alpha_{1A}$ and $\alpha_{1B}$ adrenergic receptors ($P<0.01$, $P<0.05$, respectively). Likewise, C3 and C4 have Hill slopes significantly greater than 1 at the $\alpha_{1B}$ adrenergic receptor ($P<0.05$), as do C7 and C9 at both the $\alpha_{1A}$ and $\alpha_{1B}$ adrenergic receptors ($P=0.05-0.07$). The competition binding curves, in general, appear to increase in steepness as the linker increases in length, until it reaches 9 carbons, with significant correlation between linker length and Hill slope ($P<0.05$) seen at the $\alpha_{1A}$ ($r^2 = 0.8$) and $\alpha_{1B}$ ($r^2 = 0.7$) adrenergic receptors. The magnitude of the Hill coefficient tends to a value of 2 for homologues from C6 to C12 for the $\alpha_1$ adrenergic receptors, a feature not noted for binding to the 5-HT$_{1A}$ receptor (Table 2). Hill coefficients greater than 1 imply the existence of multiple ligand binding sites on the receptor, and may implicate the involvement of cooperative modes of binding (Prinz, 2010). One possibility here, is that cooperativity arises from interaction with an allosteric site via bitopic binding of the bis(9-aminoacridine)s at the $\alpha_1$ adrenergic receptors. Accordingly, we sought experimental evidence for such a mechanism in the form of bis(9-aminoacridine) modulation of the rate of dissociation of [$^3$H]prazosin from the $\alpha_1$ adrenergic receptors.

**Dissociation kinetics for [$^3$H]prazosin binding to the $\alpha_1$ adrenergic receptors**

Given that the $\alpha_{1A}$ subtype is the most validated therapeutic $\alpha_1$ adrenergic receptor target (Perez and Doze, 2011), and that the $\alpha_{1B}$ subtype is a common source of off-target activity (Carruthers, 1994; Cavalli et al., 1997), the modulatory effects of the bis(9-aminoacridine)s on prazosin dissociation rates from these two receptors were characterised. In preliminary experiments to identify appropriate experimental conditions, we found that measurements in HEM buffer yielded a half-life of 14 min for the dissociation of [$^3$H]prazosin from the $\alpha_{1A}$ adrenergic receptor: a value suitable for detecting both positive and
negative allosteric modulation. However, dissociation from the α₁B adrenergic receptor in this buffer was slow with less than 50% dissociation observed after 2 hours (data not shown). This difficulty was overcome by the use of TE buffer for this receptor, as described by Sato et al. (2012), in which the half-life was 33 min. Accordingly, HEM buffer was used for kinetic experiments with the α₁A adrenergic receptor, and TE buffer for experiments with the α₁B adrenergic receptor.

**Bis(9-aminoacridine)s increase the dissociation rate of [³H]prazosin**

The dissociation rate of [³H]prazosin was measured in the absence and presence of the bis(9-aminoacridine)s, 9-aminoacridine, and tacrine at 100 µM, this being the highest concentration possible, limited by bis(9-aminoacridine) solubility. At 100 µM, 9-aminoacridine causes a 3.2-fold increase in dissociation rate at the α₁A adrenergic receptor and a 5.5-fold increase in dissociation rate at the α₁B adrenergic receptor (Table 3). Tacrine shows a similar, albeit less efficacious, profile resulting in a 2.1-fold increase in dissociation rate from the α₁A adrenergic receptor and a 4.2-fold increase from the α₁B adrenergic receptor (Table 3). With the exception of C2 and C3, the bis(9-aminoacridine)s significantly increase the dissociation rate of [³H]prazosin from the α₁A adrenergic receptor (P < 0.05) in a linker-length dependent manner (r²=0.86, P<0.001) (Table 3). There is a similar finding for the α₁B adrenergic receptor where all the bis(9-aminoacridine)s increase the dissociation rate of [³H]prazosin (P < 0.001) in a manner dependent on chain length (r²=0.74, P<0.001) (Table 3). The C2-to-C9 bis(9-aminoacridine)s all produce a greater proportional increase in dissociation rate at the α₁B adrenergic receptor (P < 0.01), while C12 gives the greatest observed effect at the α₁A adrenergic receptor (Table 3). However, the use of different buffers in the dissociation kinetics assays for each of the subtypes makes it difficult to compare potency between the receptor subtypes as affinity and efficacy of allosteric ligands are known to change in different buffers (Ellis and Seidenberg, 2000; Schroter et al., 2000).
To investigate whether the potency of the bis(9-aminoacridine)s to modulate the dissociation rate of [3H]prazosin from the adrenergic receptors is correlated with their observed binding affinity, the $\alpha_{1A}$-selective C4 bis(9-aminoacridine), the non-selective C9 bis(9-aminoacridine), as well as the monomer 9-aminoacridine were chosen for a more thorough characterisation. Tacrine was again included to investigate whether planarity of the molecule is a necessary feature for modulation. The increase in [3H]prazosin dissociation rate caused by the monovalent ligands does not seem to be saturating at 100 µM (Figure 3), and their allosteric effects appear at concentrations higher than their apparent affinities derived from competition binding data. 9-aminoacridine has an affinity of 247 nM at the $\alpha_{1A}$ adrenergic receptor, yet allosteric properties only emerge at a minimum concentration of 30 µM. C4 bis(9-aminoacridine) causes a maximum 3.5-fold increase in dissociation rate at the $\alpha_{1A}$ adrenergic receptor but does not cause an observable increase in rate at 10 µM, well above its apparent affinity of 21 nM for this receptor. In contrast, it causes a maximum 9.0-fold increase in dissociation at the $\alpha_{1B}$ adrenergic receptor that appears to be close to saturating, producing an estimated $EC_{50(\text{diss})}$ of 22 µM (Figure 3), a concentration ~100-fold higher than its apparent affinity. C9 bis(9-aminoacridine) causes a 17.6-fold increase in dissociation rate from the $\alpha_{1A}$ adrenergic receptor at 100 µM (P<0.001) and a 62.3-fold increase in dissociation rate from the $\alpha_{1B}$ adrenergic receptor (P<0.001). This produces $EC_{50(\text{diss})}$ estimates of 32 and 6 µM for the $\alpha_{1A}$ and $\alpha_{1B}$ adrenergic receptors, respectively (Figure 3). Despite C4 bis(9-aminoacridine) having higher affinity than C9 at the $\alpha_{1A}$ adrenergic receptor, this was not translated into increased modulation potency. C9 bis(9-aminoacridine) consistently causes greater increases in [3H]prazosin dissociation, at lower concentrations, than C4 bis(9-aminoacridine) (Figure 3), implying that these two properties are dictated by ligand binding to distinct sites on the receptor, which have differing affinity for the acridine dimers.

C9 bis(9-aminoacridine) is a non-competitive antagonist of $\alpha_{1A}$ adrenergic receptor
To test whether the bis(9-aminoacridine)s are also able to perturb activation and signalling of the receptor, activation of the $\alpha_{1A}$ adrenergic receptor by norepinephrine was tested in the absence and presence of C9 bis(9-aminoacridine). C9 was chosen as it is one of the most potent and efficacious modulators of $[^3H]$prazosin dissociation identified in this study. We found it causes a significant reduction in the potency of norepinephrine at the $\alpha_{1A}$ adrenergic receptor at concentrations of 10 $\mu$M and above ($P<0.05$), as well as a decrease in the maximum observed response at concentrations of 30 $\mu$M and above ($P<0.001$) (Figure 4, Table 4). 100 $\mu$M C9 also causes a small but significant increase in receptor signalling via the IP$_3$ pathway in the absence of norepinephrine (Table 4). The IP accumulation data do not fit well to an operational model of allosterism (Leach et al., 2007), generating unrealistic or poor fit values e.g. $R^2 < 0.5$, $\beta < 0$, or $\alpha > 10^{12}$. This is possibly due to a bitopic, rather than purely allosteric binding mode of C9 bis(9-aminoacridine). Instead, the method of Ehlert (1988) was applied to the IP accumulation data to estimate affinity of C9. 30 $\mu$M C9 produces an approximate 50% decrease in the maximum signal (Figure 4), making it the most ideal concentration for use with this method (Kenakin, 1997). Generating a double reciprocal plot of equi-effective concentrations of agonist in the absence and presence of C9 bis(9-aminoacridine), yields an affinity estimate of 19.0 ± 4.9 $\mu$M, which is 71-fold higher than the apparent affinity observed for C9 at the $\alpha_{1A}$ adrenergic receptor in equilibrium binding experiments. Substituting this value for $K_B$ in the operational model does not further improve the fit. Taking all the available data together, the monovalent and homobivalent 9-aminoacridines appear to display a combination of competitive and non-competitive properties consistent with ligands capable of binding to the functionally distinct orthosteric and allosteric binding pockets.
Discussion

The bis(9-aminoacridine)s were chosen for use in this study for their potential to engage a second site of the $\alpha_1$ adrenergic receptors. It was hypothesised that the tissue-specific differences in the binding affinity of the bis(9-aminoacridine)s observed between rat brain and kidney (Adams et al., 1986) could be explained by subtype-selective binding of the ligands that reflects the tissue-specific distribution of the subtypes. Protein levels of $\alpha_1$ adrenergic receptors, like GPCRs in general, are difficult to quantify by Western blot (Jensen et al., 2009), however, mRNA transcript levels in the rat suggest that the predominant $\alpha_1$ adrenergic receptors found in the kidney are the $\alpha_{1A}$ and $\alpha_{1B}$ subtypes (Rokosh et al., 1994). 9-aminocridine and all bis(9-aminoacridine)s with linker lengths up to 5 carbons display the highest affinity at either the $\alpha_{1A}$ or $\alpha_{1B}$ subtypes, corresponding to the higher affinity displayed by these compounds for kidney tissue compared to cerebral cortex (Adams et al., 1986), suggesting that their length is suitable to make critical interactions for selective binding. In the rat brain, mRNA suggests that the predominant subtype is the $\alpha_{1D}$ adrenergic receptor (Rokosh et al., 1994). Similar to the binding profile seen for the $\alpha_{1D}$ adrenergic receptor, binding affinities of the bis(9-aminoacridine)s in the rat cortex are consistently lower than kidney for all linker lengths less than 6 carbons. Affinities in the two tissues converge for linkers of 9-10 carbons, as do the affinities at the three human $\alpha_1$ adrenergic receptors showing general consensus with the original observations made in mixed receptor tissue preparations.

In addition to competitive binding, the acridines also demonstrate allosteric effects at the $\alpha_1$ adrenergic receptors, supporting the prediction that the 9-aminoacridines are able to interact with a second site. These allosteric effects occur at free ligand concentrations well above the apparent affinity of the 9-aminoacridines indicating a relatively low affinity interaction with the allosteric site. The lack of any observable binding cooperativity for the
monovalent ligands is also consistent with a low-affinity, allosteric interaction; the orthosteric site would be saturated before any significant allosteric effect can be observed. Cooperative binding may only emerge for the bivalent ligands as a result of one acridine moiety being tethered to the higher-affinity orthosteric site, creating a high local concentration of ligand around the allosteric pocket and increasing the likelihood of an allosteric binding event. The Hill slopes most different from 1 at the $\alpha_1$ adrenergic receptors occur around the 9-carbon linker, suggesting that this length is optimal for promoting concomitant engagement of both the orthosteric and allosteric sites.

An interesting observation is that the potency and magnitude of the allosteric effect of the bis(9-aminoacridine)s correlates better with linker length than apparent affinity, viz, C4 bis(9-aminoacridine) is not the most potent modulator despite displaying the highest affinity. This implies that the allosteric interactions are associated with a binding site that is distinct from the site that is imparting high-affinity binding interactions. For the $\alpha_{1B}$ subtype, the allosteric effect appears to peak at the 9-carbon length, further reinforcing this as the optimal linker length for bitopic binding at the $\alpha_1$ adrenergic receptors. This supports our prediction that an optimally sized bivalent ligand will promote engagement of an allosteric site to exaggerate the allosteric effects of a ligand with otherwise poor efficacy or potency. Interestingly, 9 carbons was also found to be the optimum length to promote bitopic engagement of the reciprocally bivalent compound, THRX-198321 a dual $\beta_2$ adrenergic receptor agonist and muscarinic receptor antagonist (Steinfeld et al., 2011) perhaps suggesting a conserved distance between orthosteric and allosteric pockets in the biogenic amine receptor family.

One caveat to our model of bitopic binding for the bis(9-aminoacridine)s is that it may require the existence of dimers, or higher order oligomers, of the $\alpha_1$ adrenergic receptors. A bitopic model best satisfies the observed pharmacology of the bis(9-aminoacridine)s,
however, in the experimental kinetics paradigm, the orthosteric site of the receptors would already be occupied by the probe ligand, $[^3H]$prazosin, precluding any potential for tethering. Using a $[^3H]$prazosin concentration close to its $K_D$, i.e. 50% receptor occupancy, may leave dimer partners unoccupied and available for bitopic interaction with the bis(9-aminoacridine)s as would an asymmetrical binding profile. It has been demonstrated that the bitopic $D_2$ dopamine receptor ligand, SB269652 binds to one receptor and negatively modulates a dimer partner (Lane et al., 2014). $D_2$ receptors have been demonstrated to form oligomers in native tissue (Zawarynski et al., 1998) while $\alpha_1$ adrenergic receptors have been demonstrated to form dimers in transfected cells (Vicentic et al., 2002) and, so this concept is not unprecedented and we would suggest that the bis(9-aminoacridine)s are acting in a similar fashion.

We have demonstrated that a single pharmacophore can have actions at two distinct receptor binding sites. One of the most significant implications of this observed pharmacology for the 9-aminoacridines is that the orthosteric and allosteric sites of the $\alpha_1$ adrenergic receptors, and potentially many other GPCRs, share some degree of similarity. In addition, monovalent tacrine, tacrine dimers and methoctramine also show similar effects at muscarinic receptors, with mixed orthosteric-allosteric actions and the homobivalent ligands are also suggested to bind in a bitopic manner (Jakubik et al., 2014; Pearce and Potter, 1988; Tränkle et al., 2005). This observation would be in line with current understanding of ligand binding mechanisms. Recent structural biology efforts to crystallise GPCRs have yielded a structure of the $M_2$ muscarinic receptor with a bound positive allosteric modulator, LY2119620 (Kruse et al., 2013). This structure showed the allosteric modulator bound to an allosteric pocket comprised of residues from transmembrane (TM) helices TMV, TMVI, TMVII, and the second extracellular loop, which have previously been shown to constitute the primary allosteric site for the muscarinic receptors (Prilla et al., 2006; Valant et al., 2012).
This site on the M_2 muscarinic receptor also corresponds to the extracellular vestibule of the β_2 adrenergic receptor, which is a low affinity pocket identified in molecular dynamics simulations where orthosteric ligands are postulated to bind transiently before progressing to their final pose in the orthosteric pocket (Dror et al., 2011). The molecular dynamics analysis of the β_2 adrenergic receptor predicted the affinity of alprenolol to be 5 µM at the binding vestibule compared to 0.9 nM affinity observed in competitive binding assays (Baker, 2005; Dror et al., 2011). In a similar pattern, the affinity of 9-aminoacridine determined from equilibrium binding was 247 nM compared to the predicted allosteric affinity from the signalling assay of 19 µM, and the effective concentration required to increase [³H]prazosin dissociation rate also appears to be in the mid to high micromolar range.

“Pure” orthosteric and allosteric ligands may then simply be ligands with significant selectivity for one site over the other and the differences in 9-aminoacridine affinity between the two sites (~100-fold) is not substantial enough to give a single binding profile. It is possible that some effects of allosteric modulators may be attributable to interaction with the orthosteric site e.g. “allosteric agonists” may be allosteric ligands with additional, hitherto unappreciated weak orthosteric agonist activity. Such a multi-pocket mechanism of action has recently been described for some ligands of the FFA2 free fatty acid receptor (Grundmann et al.). The two identified pockets are considerably more superficial on the receptor surface, are in closer proximity and have more similar affinities than is expected for the aminergic receptors, but nonetheless demonstrate that a single ligand can have distinct actions at topographically distinct sites on a single receptor.

The observed effects of tacrine, as well as the other 9-aminoacridines, at the α_1 adrenergic receptors present an interesting conundrum. One of the purported advantages of allosteric ligands is the ability to bind to non-conserved sites of receptors, imparting increased binding selectivity (Christopoulos, 2002). However we have demonstrated that
tacrine, an allosteric ligand of the muscarinic receptors (Pearce and Potter, 1988; Potter et al., 1989; Tränkle et al., 2005), is also an allosteric ligand of the α₁ adrenergic receptors, in addition to its orthosteric activity at the two receptor families. There may therefore be conservation of allosteric sites in related receptor families, which would also explain why there appears to be a conserved distance between the orthosteric and allosteric sites in α₁ and β₂ adrenergic receptors as well as muscarinic receptors. One notable difference between the α₁ adrenergic and muscarinic receptors, however, is that tacrine acts as a positive modulator of ligand binding at the muscarinic receptors (Croy et al., 2014), while it is a negative modulator of ligand binding at the α₁ adrenergic receptors. The structural basis of positive modulation of the M₂ muscarinic receptor appears to be a “capping” effect where LY2119620 binds to the M₂ receptor in a location that directly hinders the egress of the ligand from the orthosteric binding pocket (Kruse et al., 2013). Tacrine’s actions as a negative modulator of the α₁ adrenergic receptors would imply a different mechanism whereby binding of tacrine induces a structural change in the orthosteric site that is less favourable for ligand binding.

We believe the 9-aminoacridine compounds are more likely to mimic the binding mode of the D₂ dopamine receptor negative allosteric modulator, SB269652, where the allosteric binding pocket is located closer to TMII than TMVII (Lane et al., 2014).

While the allosteric site of the α₁ adrenergic receptors remains to be fully characterised, it is clear from the observed pharmacology that the 9-aminoacridines are interacting with more than one site on the α₁ adrenergic receptors. Furthermore, by increasing or decreasing the distance between orthosteric and allosteric moieties using an appropriate linker, the magnitude of an allosteric effect can be controlled, imposing a level of control over the size of the allosteric component. The bivalent nature of the bis(9-aminoacridine)s and the linker-length dependent changes in allosteric modulation will aide in identifying the location of this allosteric pocket and such an approach may facilitate a new approach to drug design and
discovery for the $\alpha_1$ adrenergic receptors or GPCRs in general.
Acknowledgements

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Authorship contributions

Participated in research design: Campbell, Wakelin, Denny and Finch.

Conducted experiments: Campbell.

Performed data analysis: Campbell and Finch.

Wrote or contributed to the writing of the manuscript: Campbell, Wakelin, Denny and Finch.
References


Cheng Y-C and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_50) of an enzymatic reaction. *Biochem Pharmacol* **22**(23): 3099-3108.


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**Figure legends**

**Figure 1. Structures of 9-aminoacridine and derivatives**
Structure of 9-aminoacridine, tacrine (tetrahydro-9-aminoacridine), and the general structure for bis(9-aminoacridine) in their protonated state.

**Figure 2. Subtype selective binding of the bis(9-aminoacridine)s**
Binding affinities of the bis(9-aminoacridine)s at all three $\alpha_1$ adrenergic receptors and 5-HT$_{1A}$ serotonin receptor. All binding curves were fit by a variable-slope competitive binding model. Affinities were compared by one-way ANOVA with Tukey post-test. a, b, d, s: $P < 0.05$ compared to $\alpha_1A$, $\alpha_1B$, $\alpha_1D$ adrenergic receptors (AR), and 5-HT$_{1A}$ serotonin receptor, respectively. Affinity is not shown where log$K_i$ is greater than -4.

**Figure 3. Kinetics of dissociation of [$^3$H]prazosin from $\alpha_1$ adrenergic receptors**
[$^3$H]prazosin dissociation from $\alpha_{1A}$ adrenergic receptor membranes (A-H) and $\alpha_{1B}$ adrenergic receptor membranes (I-P) in the absence and presence of 9-aminoacridine (A, M), C4 bis(9-aminoacridine) (B, N), C9 bis(9-aminoacridine) (C, O), and tacrine (D, P). Membranes were preincubated with 250 pM [$^3$H]prazosin for 1 h at room temperature in 400 µL HEM buffer. [$^3$H]prazosin reassociation was inhibited by the addition of 100 µL phentolamine with or without test compound at concentrations shown. A-D and I-L show time-dependent dissociation curves. E-H and M-P show observed dissociation rates (K$_{obs}$) normalized to the control dissociation rate (K$_{off}$) measured in the absence of modulator and plotted against the log concentration of modulator. Points and error bars are the mean ± SEM of 3 individual experiments performed in duplicate.

**Figure 4. C9 bis(9-aminoacridine) modulation of norepinephrine-induced total soluble inositol phosphate accumulation**
COS-1 cells were transiently transfected with the $\alpha_{1A}$ adrenergic receptor then incubated
overnight in the presence of \[^3\text{H}\]myo-inositol. After 24 h of incubation, the medium was exchanged for serum-free DMEM in the presence or absence of C9 bis(9-aminoacridine) (C9) for 20 min followed by stimulation with norepinephrine for 30 min. Unstimulated points are shown in the left segment of the X-axis. All values were normalised between unstimulated response and maximum norepinephrine-induced response and fit by non-linear regression to a variable-slope dose-response curve using GraphPad Prism 6. Points and bars represent the mean ± SEM of n=3-5 experiments performed in triplicate.
### Table 1. Equilibrium binding affinities

<table>
<thead>
<tr>
<th></th>
<th>α₁A adrenergic receptor</th>
<th>α₁B adrenergic receptor</th>
<th>α₁D adrenergic receptor</th>
<th>5-HT₁A receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKᵢ</td>
<td>n</td>
<td>Kᵢ (nM)</td>
<td>pKᵢ</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>8.4 ± 0.06***</td>
<td>6</td>
<td>4</td>
<td>7.8 ± 0.2***</td>
</tr>
<tr>
<td>Serotonin</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>9-aminoacridine</td>
<td>6.6 ± 0.03</td>
<td>3</td>
<td>250</td>
<td>5.6 ± 0.05</td>
</tr>
<tr>
<td>C2 bis(9-aminoacridine)</td>
<td>5.7 ± 0.1***</td>
<td>3</td>
<td>1900</td>
<td>6.6 ± 0.2***</td>
</tr>
<tr>
<td>C3 bis(9-aminoacridine)</td>
<td>6.2 ± 0.09</td>
<td>3</td>
<td>700</td>
<td>6.4 ± 0.2**</td>
</tr>
<tr>
<td>C4 bis(9-aminoacridine)</td>
<td>7.7 ± 0.2***</td>
<td>3</td>
<td>21</td>
<td>6.7 ± 0.1***</td>
</tr>
<tr>
<td>C5 bis(9-aminoacridine)</td>
<td>7.5 ± 0.1***</td>
<td>3</td>
<td>33</td>
<td>7.0 ± 0.2***</td>
</tr>
<tr>
<td>C6 bis(9-aminoacridine)</td>
<td>7.2 ± 0.08</td>
<td>3</td>
<td>58</td>
<td>7.0 ± 0.1***</td>
</tr>
<tr>
<td>C7 bis(9-aminoacridine)</td>
<td>6.9 ± 0.06</td>
<td>3</td>
<td>120</td>
<td>7.0 ± 0.2***</td>
</tr>
<tr>
<td>C9 bis(9-aminoacridine)</td>
<td>6.6 ± 0.08</td>
<td>3</td>
<td>270</td>
<td>6.9 ± 0.2***</td>
</tr>
<tr>
<td>C10 bis(9-aminoacridine)</td>
<td>6.5 ± 0.07</td>
<td>3</td>
<td>290</td>
<td>6.8 ± 0.1***</td>
</tr>
<tr>
<td>C12 bis(9-aminoacridine)</td>
<td>6.4 ± 0.2</td>
<td>3</td>
<td>400</td>
<td>6.9 ± 0.09***</td>
</tr>
<tr>
<td>Tacrine</td>
<td>5.7 ± 0.06***</td>
<td>3</td>
<td>1900</td>
<td>4.7 ± 0.2***</td>
</tr>
</tbody>
</table>

pKᵢ: Negative log of apparent affinity calculated according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973).
Kᵢ: Calculated concentration of ligand required to occupy 50% of unoccupied receptors, derived from pKᵢ.
All data are presented as mean ± SEM for ‘n’ repeats, performed in duplicate. *, **, *** P < 0.05, 0.01, 0.001 compared to 9-aminoacridine, not determined for 5-HT₁A receptor. nd: not determined.
Table 2. Equilibrium binding assay slopes

<table>
<thead>
<tr>
<th></th>
<th>α&lt;sub&gt;1A&lt;/sub&gt; adrenergic receptor</th>
<th>α&lt;sub&gt;1B&lt;/sub&gt; adrenergic receptor</th>
<th>α&lt;sub&gt;1D&lt;/sub&gt; adrenergic receptor</th>
<th>5-HT&lt;sub&gt;1A&lt;/sub&gt; receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>n</td>
<td>Slope</td>
<td>n</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>1.03 ± 0.4</td>
<td>6</td>
<td>1.1 ± 0.06</td>
<td>5</td>
</tr>
<tr>
<td>Serotonin</td>
<td>nd</td>
<td></td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>9-aminoacridine</td>
<td>1.0 ± 0.08</td>
<td>3</td>
<td>1.0 ± 0.07</td>
<td>3</td>
</tr>
<tr>
<td>C2 bis(9-aminoacridine)</td>
<td>1.2 ± 0.2</td>
<td>3</td>
<td>1.2 ± 0.06&lt;sup&gt;0.089&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>C3 bis(9-aminoacridine)</td>
<td>1.1 ± 0.10</td>
<td>3</td>
<td>1.3 ± 0.04*</td>
<td>3</td>
</tr>
<tr>
<td>C4 bis(9-aminoacridine)</td>
<td>1.4 ± 0.3</td>
<td>3</td>
<td>1.3 ± 0.03*</td>
<td>3</td>
</tr>
<tr>
<td>C5 bis(9-aminoacridine)</td>
<td>1.6 ± 0.3</td>
<td>3</td>
<td>1.3 ± 0.1&lt;sup&gt;0.09&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>C6 bis(9-aminoacridine)</td>
<td>1.9 ± 0.6</td>
<td>3</td>
<td>2.0 ± 0.3&lt;sup&gt;0.06&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>C7 bis(9-aminoacridine)</td>
<td>1.5 ± 0.1&lt;sup&gt;0.05&lt;/sup&gt;</td>
<td>3</td>
<td>1.8 ± 0.2&lt;sup&gt;0.06&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>C9 bis(9-aminoacridine)</td>
<td>2.5 ± 0.4&lt;sup&gt;0.06&lt;/sup&gt;</td>
<td>3</td>
<td>2.3 ± 0.4&lt;sup&gt;0.07&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>C10 bis(9-aminoacridine)</td>
<td>2.0 ± 0.06**</td>
<td>3</td>
<td>2.0 ± 0.1*</td>
<td>3</td>
</tr>
<tr>
<td>C12 bis(9-aminoacridine)</td>
<td>2.0 ± 1</td>
<td>3</td>
<td>1.0 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>Tacrine</td>
<td>1.1 ± 0.2</td>
<td>3</td>
<td>0.80 ± 0.08</td>
<td>3</td>
</tr>
</tbody>
</table>

Hill Slope coefficient of competitive binding data fit by a variable-slope competitive binding model as per section

*, ** P < 0.05, 0.01 respectively compared to 1 by one sample t-test. P values between 0.05 and 0.1 are reported in superscript.

All data are expressed as mean ± SEM for ‘n’ repeats.
Table 3. Modulation of dissociation rates of [³H]prazosin from the α₁A and α₁B adrenergic receptors

<table>
<thead>
<tr>
<th></th>
<th>α₁A adrenergic receptor</th>
<th>α₁B adrenergic receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_{off}</td>
<td>t_{1/2}</td>
</tr>
<tr>
<td>Control</td>
<td>0.05 ± 0.01</td>
<td>13</td>
</tr>
<tr>
<td>9-aminoacridine</td>
<td>0.17 ± 0.02</td>
<td>4.1</td>
</tr>
<tr>
<td>C2 bis(9-aminoacridine)</td>
<td>0.08 ± 0.02</td>
<td>8.7</td>
</tr>
<tr>
<td>C3 bis(9-aminoacridine)</td>
<td>0.11 ± 0.03</td>
<td>6.2</td>
</tr>
<tr>
<td>C4 bis(9-aminoacridine)</td>
<td>0.18 ± 0.05</td>
<td>3.8</td>
</tr>
<tr>
<td>C5 bis(9-aminoacridine)</td>
<td>0.17 ± 0.03</td>
<td>4.0</td>
</tr>
<tr>
<td>C6 bis(9-aminoacridine)</td>
<td>0.40 ± 0.01</td>
<td>1.7</td>
</tr>
<tr>
<td>C7 bis(9-aminoacridine)</td>
<td>0.37 ± 0.07</td>
<td>1.9</td>
</tr>
<tr>
<td>C9 bis(9-aminoacridine)</td>
<td>0.96 ± 0.09</td>
<td>0.7</td>
</tr>
<tr>
<td>C10 bis(9-aminoacridine)</td>
<td>1.2 ± 0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>C12 bis(9-aminoacridine)</td>
<td>4.4 ± 1.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Tacrine</td>
<td>0.10 ± 0.01</td>
<td>6.6</td>
</tr>
</tbody>
</table>

K_{off}: Dissociation rate of [³H]prazosin (min⁻¹).
K_{obs}/K_{off}: Ratio of the observed dissociation rates in the presence and absence of 100 µM modulator.
t_{1/2}: Half-life of [³H]prazosin dissociation (minutes).
*, **, *** P < 0.05, 0.01, 0.001 compared to control.
Values are given as mean ± SEM n=3-6
### Table 4. C9 dependent changes in norepinephrine (NE) dependent and independent activation of the α1A adrenergic receptor

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>C9 10 μM</th>
<th>C9 30 μM</th>
<th>C9 100 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NE pEC&lt;sub&gt;50&lt;/sub&gt;</strong></td>
<td>6.2 ± 0.1</td>
<td>5.6 ± 0.2*</td>
<td>5.4 ± 0.1**</td>
<td>5.0 ± 0.2***</td>
</tr>
<tr>
<td><strong>NE Slope</strong></td>
<td>1.6 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>1.8 ± 0.3</td>
<td>1.8 ± 1</td>
</tr>
<tr>
<td><strong>NE E&lt;sub&gt;max&lt;/sub&gt; (%)</strong></td>
<td>100.8 ± 2</td>
<td>75 ± 10</td>
<td>46 ± 10***</td>
<td>39 ± 5***</td>
</tr>
<tr>
<td><strong>Basal (%)</strong></td>
<td>0.3 ± 0.3</td>
<td>5.4 ± 3</td>
<td>2.1 ± 1</td>
<td>17 ± 5**</td>
</tr>
</tbody>
</table>

*pEC<sub>50</sub>: negative log of the concentration required to cause 50% maximal norepinephrine mediated activation.*

Slope: slope factor of norepinephrine mediated receptor activation

E<sub>max</sub>: % maximum norepinephrine response measured in the absence of C9 bis(9-aminoacridine).

Basal: agonist independent receptor activity, expressed as % norepinephrine E<sub>max</sub>.

*, **, *** P < 0.05, 0.01, 0.001 compared to norepinephrine alone by one-way ANOVA with Tukey post-test.
Figure 1

9-aminoacridine

\[ \text{NH}_2 \]

Tacrine

\[ \text{NH}_2 \]

Cn bis(9-aminoacridine)

\[ \text{NH} \]

\[ (\text{CH}_2)_n \]

\[ \text{NH} \]
Figure 3

A. 9-aminoacridine

B. C4 bis(9-aminoacridine)

C. C9 bis(9-aminoacridine)

D. Tacrine

E. K_{on}/K_{off} vs. 9-aminoacridine log(M)

F. K_{on}/K_{off} vs. C4 bis(9-aminoacridine) log(M)

G. K_{on}/K_{off} vs. C9 bis(9-aminoacridine) log(M)

H. K_{on}/K_{off} vs. Tacrine log(M)

I. % Maximum Specific [3H]prazosin Binding vs. Time (mins)

J. % Maximum Specific [3H]prazosin Binding vs. Time (mins)

K. % Maximum Specific [3H]prazosin Binding vs. Time (mins)

L. % Maximum Specific [3H]prazosin Binding vs. Time (mins)

M. K_{on}/K_{off} vs. 9-aminoacridine log(M)

N. K_{on}/K_{off} vs. C4 bis(9-aminoacridine) log(M)

O. K_{on}/K_{off} vs. C9 bis(9-aminoacridine) log(M)

P. K_{on}/K_{off} vs. Tacrine log(M)
Figure 4

% Maximum Specific Norepinephrine Response

Norepinephrine (NE)
NE + C9 10 μM
NE + C9 30 μM
NE + C9 100 μM

Norepinephrine log(M)