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Effects of Anticholinesterases on Catalysis and Induced Conformational Change of the Peripheral Anionic Site of Murine Acetylcholinesterase

Fan Tong¹, Rafique M. Islam¹, Paul R. Carlier², Ming Ma², Fredrik Ekström³, and Jeffrey R. Bloomquist¹

¹Department of Entomology and Nematology, Emerging Pathogens Institute, University of Florida, Gainesville, FL 32611

²Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

³Swedish Defense Research Agency, CBRN Defense and Analysis, S-901 82, Umeå, Sweden

Abstract

Conventional insecticides targeting acetylcholinesterase (AChE) typically show high mammalian toxicities and because there is resistance to these compounds in many insect species, alternatives to established AChE inhibitors used for pest control are needed. Here we used a fluorescence method to monitor interactions between various AChE inhibitors and the AChE peripheral anionic site, which is a novel target for new insecticides acting on this enzyme. The assay uses thioflavin-T as a probe, which binds to the peripheral anionic site of AChE and yields an increase in fluorescent signal. Three types of AChE inhibitors were studied: catalytic site inhibitors (carbamate insecticides, edrophonium, and benzylpiperidine), peripheral site inhibitors (tubocurarine, ethidium bromide, and propidium iodide), and bivalent inhibitors (donepezil, BW284C51, and a series of *bis*(n)-tacrine). All were screened on murine AChE to compare and contrast changes of peripheral site conformation in the TFT assay with catalytic inhibition. All the inhibitors reduced thioflavin-T fluorescence in a concentration-dependent manner with potencies (IC₅₀) ranging from 8 nM for *bis*(6)-tacrine to 159 μM for benzylpiperidine. Potencies in the fluorescence assay were correlated well with their potencies for enzyme inhibition ($R^2 = 0.884$). Efficacies for reducing thioflavin-T fluorescence ranged from 23–36% for catalytic site inhibitors and tubocurarine to near 100% for ethidium bromide and propidium iodide. Maximal efficacies could be reconciled with known mechanisms of interaction of the inhibitors with AChE. When extended to pest species, we anticipate these findings will assist in the discovery and development of novel, selective bivalent insecticides acting on AChE.

Keywords

catalytic site; carbamate; donepezil; edrophonium; ethidium; fluorescence; propidium; tacrine; tacrine dimer; thioflavin-T

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Corresponding author: Jeffrey R. Bloomquist, Department of Entomology and Nematology, Emerging Pathogens Institute, 2055 Mowry Road, PO Box 100009, University of Florida, Gainesville, FL 32610-0009, jrbloomquist@epi.ufl.edu, Office No.: (352) 273-9417, Fax No.: (352) 273-9420.

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1. Introduction

Acetylcholinesterase (EC3.1.1.7, AChE) is a fast acting enzyme located at the synaptic cleft to hydrolyze the neurotransmitter, ACh, and is present in many organisms, including mammals and insects [1]. The inhibition of AChE results in the accumulation of ACh, and leads to hyperexcitation, convulsions, and death. Due to its critical function in the nervous system, AChE is the target for many insecticides, such as organophosphates and carbamates [2]. However, mammalian toxicities (both acute and chronic) and the development of resistance are major disadvantages of the use of these chemicals in agriculture and vector borne disease control [3, 4, 5, 6, 7]. Accordingly, there is a need to discover and develop alternatives to conventional AChE inhibitors.

Previous x-ray crystallographic studies found that AChE has a catalytic pocket, connected with a 20-Å, deep and narrow gorge [8, 9, 10]. In most species, there are two important ligand binding sites at each end of this gorge: the catalytic site, containing a catalytic triad (Ser²⁰³-Glu³³⁴-His⁴⁴⁷ as numbered for *Mus musculus* AChE [9]), is located at the bottom of the gorge, and the peripheral anionic site, which consists principally of one negatively charged residue (Asp⁷⁴) and multiple aromatic amino acid residues (Tyr⁷², Tyr¹²⁴, Trp²⁸⁶, and Tyr³⁴¹ [9]), is at the entrance of the gorge [8, 11, 12]. It has been reported that the binding of ligands to either site modulates the conformation or the activity of the other site [13, 14, 15, 16]. The interaction between these two ligand binding sites can be determined by using a fluorescent probe, TFT (Fig. 1), which binds to the peripheral anionic site of AChE, and increases its fluorescence over that of free TFT in solution [17]. According to Stsiapura et al. [18], TFT has a nonplanar conformation in the ground state with a torsion angle between the benzothiazole and the dimethylaminobenzene rings of around 37°. When excited by light, the TFT molecule is twisted, the torsion angle increases to about 90° if the dye is in a low viscosity microenvironment, and yields no fluorescent signal. If the TFT molecule is located in a viscous microenvironment, such as bound to AChE, the transition of the TFT molecule from the excited state to the unexcited state will be suppressed, which increases fluorescence [18]. When ligands bind to AChE, a drop in TFT fluorescence may occur either by inhibiting binding of TFT in the peripheral site, or indirectly by reducing rotational rigidity of bound TFT [17, 19].

We used this fluorescent assay to study the interaction between the peripheral anionic site and the catalytic site of murine AChE (mAChE) in the presence of various AChE inhibitors, including catalytic site inhibitors, peripheral site inhibitors, and bivalent inhibitors binding to both sites. The effects of different AChE inhibitors on mAChE were screened by using the fluorescent assay as well as enzyme activity assay to compare and contrast changes of peripheral site conformation and catalytic ability in response to AChE inhibitors.

2. Materials Methods

2.1 Enzyme and Chemicals

The mouse AChE (mAChE) was expressed in cell lines and purified by affinity and size exclusion chromatography, as described by Ekstrom *et al.* [20]. The mAChE was aliquoted into 1 mL samples, and stored at -80 °C. Immediately prior to assay, a vial with frozen mAChE was thawed and kept on ice before use.

Inhibitors of AChE in this study were selected from various categories, including peripheral site inhibitors, catalytic site inhibitors, and bivalent inhibitors that occupy both sites. The peripheral site inhibitors used here were propidium iodide, ethidium bromide, and *d*-tubocurarine. The catalytic site inhibitors were edrophonium, tacrine, benzylpiperidine,

propoxur, bendiocarb, pirimicarb, aldicarb, two experimental carbamates [21], 3-*tert*-butylphenyl methylcarbamate (**1**), and 2-(2-methylbutylthio)phenyl methylcarbamate (**2**) (Fig. 1). The bivalent inhibitors were donepezil (E2020) and BW284c51. Another group of AChE ligands is the tacrine dimers, which are two tacrine monomers separated by 2–12 methylene units, and labeled as *bis*(*n*)-tacrine, where *n* equals the number of carbon atoms in the alkyl chain (Fig. 1). The tacrine dimers are bivalent AChE inhibitors [22, 23].

Propidium iodide, ethidium bromide, *d*-tubocurarine, edrophonium, tacrine, propoxur, bendiocarb, pirimicarb, aldicarb, donepezil, BW284c51, and TFT were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Compounds **1** and **2** were prepared with methods identical or equivalent to those described previously [21], as were the *bis*(*n*)-tacrine [22].

All the candidate inhibitors except ethidium bromide and tubocurarine, which were dissolved in assay buffer, were dissolved in DMSO to make original stocks. In both fluorescence assay and enzyme activity assay with these AChE inhibitors, the final concentration of DMSO in assays was maintained as 0.1%. TFT was dissolved in methanol, and diluted with assay buffer to a methanol concentration of 0.1%.

2.2 Enzyme Fluorescence Assay

The fluorescence assay used was modified from established procedures [16, 17, 24], and were performed in black Costar 96-well plates (Corning, Tewksbury, MA, USA). The fluorescence was monitored by a SyntaxMax plate reader (BioTek, Winooski, VT, USA) in 20 mM sodium phosphate with 0.02% Triton X-100, pH 7.4, at 28 °C. Fluorescence was monitored using 450 and 490 nm wavelengths for excitation and emission, respectively, with excitation and emission slits of 10 nm (excitation) and 20 nm (emission). In order to determine the fluorescent signal resulting from the binding of TFT to the peripheral anionic site of mAChE, TFT autofluorescence, enzyme auto-fluorescence, buffer auto-fluorescence, AChE inhibitors' autofluorescence, and AChE inhibitors fluorescence with TFT as well as mAChE were all subtracted from TFT fluorescence with enzyme. Non-specific binding of TFT was also subtracted from the total binding fluorescence to obtain specific binding of TFT, by co-incubating 10 μM donepezil with mAChE and TFT for 1 hour [19, 25]. Unless otherwise indicated, 30 μL of cell lysate derived mAChE and 20 μM of TFT were used per reaction.

Equilibrium binding studies of TFT to mAChE was performed by incubating serial concentrations of TFT from 0–10 μM with mAChE. In other experiments, inhibitory dose-response curves for candidate inhibitors were determined by incubating mAChE with at least six concentrations of inhibitor for 1 hour at room temperature prior to adding 20 μM of TFT.

2.3 Enzyme inhibition assay

Inhibition of mAChE by candidate inhibitors was determined by using the Ellman assay in a 96-well plate format [26]. The mAChE samples were thawed and diluted 100-fold with 0.1 M sodium phosphate buffer, pH 7.4 before use. The dilution of mAChE (30 μL) was then preincubated with at least six concentrations of inhibitors for 1 hour at room temperature prior to adding 300 μM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 400 μM acetylthiocholine (ATCh), which were both dissolved in 0.1 M sodium phosphate buffer, pH 7.4. The kinetic reading of absorbance at 405 nm was started immediately after adding DTNB and ATCh with a Dynex Triad multimode plate reader (Dynex Technologies, Chantilly, VA, USA). The experiment was repeated in triplicate with different enzyme dilutions to obtain means and SEMs of IC₅₀ values for selected inhibitors.

2.4 Data analysis

Inhibitor concentration-response curves were performed by nonlinear regression method using GraphPad Prism 4.0c software (GraphPad Software, San Diego, CA, USA). The IC_{50} value is defined as the concentration of inhibitor for half maximal inhibition of TFT fluorescence or enzyme activity determined from nonlinear regression to a four parameter logistic equation. Saturation binding isotherms were used for measuring dissociation constant (K_d), maximum TFT fluorescence (B_{max}) using Prism. At least three separate experiments with different enzyme solutions were repeated to determine the means and standard errors of mean (SEMs) for dissociation constant (K_d), maximum TFT fluorescence (B_{max}), IC_{50} , and maximum percentage inhibition of TFT fluorescence. Results are shown as mean $\pm$ SEM ($n = 3$, separate experiments by using different enzyme aliquots and different chemical dilutions).

3. Results

3.1 Equilibrium binding of TFT to mAChE

The presence of mAChE in the incubation medium enhanced the fluorescence of TFT over that observed in free solution. Total fluorescence in the presence of enzyme and 10 μ M TFT was 1633 ± 28 units (mean $\pm$ SEM, $n = 7$), compared to 834 ± 34 units (mean $\pm$ SEM, $n = 7$) for 10 μ M TFT in free solution, an increase of 1.96-fold. Analysis of equilibrium TFT binding to mAChE (Fig. 2) found that the binding was concentration-dependent and saturable, with a K_d value of $1.4 \pm 0.2 \mu$ M, mean $\pm$ SEM ($n = 7$). The maximum specific TFT fluorescence in the presence of mAChE, B_{max} , was found to be 562 ± 23 fluorescent units (mean $\pm$ SEM, $n = 7$), under the conditions of our study. The non-specific TFT fluorescence was about 40% – 50% of the total TFT fluorescence. In control experiments, autofluorescence of mAChE was increased in the presence of 10 μ M donepezil by 170 fluorescence units, on average, and the non-specific TFT fluorescence was corrected for this effect.

3.2 AChE inhibitors reduce both enzyme activity and TFT fluorescence

As shown in Tables 1 and 2, all tested compounds showed concentration-dependent inhibitory effects on both total TFT fluorescence and mAChE activity. Overall, compounds active at the catalytic site showed varying potencies in both TFT and Ellman assays. Bendiocarb, and one of our experimental carbamates designed specifically for *Anopheles gambiae* AChE, **1**, were found to have the lowest IC_{50} values in both assays, and another experimental carbamate, **2**, showed the highest IC_{50} values in both assays. The other three commercial carbamates, propoxur, aldicarb, and pirimicarb all inhibited mAChE activity with moderate (mid nanomolar to low micromolar) affinities (Table 1). One catalytic site compound, edrophonium, was a low micromolar inhibitor in both assays.

For both TFT fluorescence assay and Ellman assay, bivalent inhibitors, such as BW284c51 and donepezil (Table 1), as well as *bis*(5)- to *bis*(8)-tacrine dimers (Table 2), showed the highest potency to quench TFT fluorescence or inhibit mAChE catalytic activity, with IC_{50} values at high picomolar to low nanomolar levels. IC_{50} values from both assays of monomer and *bis*(2)- and *bis*(3)-tacrine were similar, and then decreased with increasing tether length compared to the monomer (Table 2). The lowest IC_{50} value was found when the tether length reached 6 methylenes (TFT $IC_{50} = 7.7$ nM, Ellman $IC_{50} = 0.6$ nM), and then IC_{50} values increased again as tether length approached 12 methylenes, the tacrine dimer with the longest central linker tested (Table 2). Other bivalent inhibitors had low potency against mAChE in both assays (Table 1). The same was found for benzylpiperidine, a structural fragment of donepezil expected to bind to the catalytic site, and the peripheral site inhibitor, tubocurarine, which had the lowest affinity in both assays with IC_{50} values in the 50–70

micromolar range. For the other two peripheral site ligands, similar low potency was found with ethidium bromide, but propidium iodide was a more potent, 700 nanomolar inhibitor (Table 1).

Correlation analysis showed that the IC_{50} values of TFT assay and Ellman assay were strongly correlated with a high correlation coefficient (R^2) = 0.884. However, as shown in Tables 1 and 2, the ratios of IC_{50} values from both assays in different categories of inhibitors were different. All tacrine dimers had higher IC_{50} values in the TFT assay, which were typically 2- to 4-fold greater than in the Ellman assay, except for *bis*(5)- to *bis*(8)-tacrine, for which the TFT/Ellman ratio was 7- to 12-fold (Table 2). For other mAChE inhibitors, most of them had similar IC_{50} values of both assays that differed by less than 2.5-fold. The exceptions include propoxur, edrophonium, ethidium, and donepezil. Propoxur and edrophonium showed lower IC_{50} values (7.7-fold and 3-fold, respectively) in TFT assay than in Ellman assay, while IC_{50} values in the TFT assay for ethidium bromide and donepezil were around four times higher than in the Ellman assay for both chemicals.

3.3 Different types of AChE inhibitors varied in maximal reduction of TFT fluorescence

Different AChE inhibitors showed different maximum percentage reduction of total TFT fluorescence. As demonstrated in Fig. 3, ethidium bromide and propidium iodide, both of which are peripheral site inhibitors, were the only two inhibitors inhibiting more than 95% reduction of total TFT fluorescence. In contrast, another peripheral site inhibitor, tubocurarine, only blocked about 40% of the total TFT fluorescence. Most of the catalytic site inhibitors, including all the carbamates, benzylpiperidine, and edrophonium showed lowest efficiency to reduce TFT fluorescence (around 30%). Most bivalent inhibitors and tacrine dimers decreased the total TFT fluorescence to a moderate level with inhibition of 40% to 70% of the total fluorescence. As an indicator of specific binding of TFT, donepezil showed 46% of inhibition of the total TFT fluorescence, suggesting that the TFT specific binding should be about half of the total binding (Fig. 3). So compounds with significantly greater efficacy for inhibition of TFT fluorescence than donepezil might be inhibiting non-specific binding of TFT or quenching fluorescence unrelated to mAChE. To test this hypothesis, another experiment was performed (Fig. 4). TFT was incubated with mAChE for two minutes in assay buffer, donepezil was then added (final concentration = 10 μ M) into the reaction, which was incubated another two minutes, and then propidium iodide was added (final concentration = 30 μ M). The fluorescent signal was read at 40 seconds intervals. As shown in Fig. 4, after adding donepezil into the assay, the total TFT fluorescence was quenched to around 50%, and 30 μ M of propidium iodide continued to reduce the TFT fluorescence to 0% baseline.

4. Discussion

The equilibrium analysis of TFT binding to mAChE described the binding interaction between the fluorescent probe, TFT, and the peripheral anionic site. In the current study, we found a slightly higher equilibrium K_d value (1.4 ± 0.2 μ M, mean $\pm$ SEM, $n = 7$) compared with K_d values reported by Sultatos and Kaushik [13] and De Ferrari et al. [17] on human recombinant AChE, which were 0.36 and 0.89 μ M, respectively.

Correlation analysis showed that the IC_{50} values of TFT assay and Ellman assay were strongly correlated across multiple structural classes of inhibitors, demonstrating that modification of enzyme function was linked to reduction of TFT fluorescence. In particular, both TFT fluorescence and Ellman assay IC_{50} values for tacrine dimers were correlated, and the activity of these compounds was dependent upon tether length. Tacrine dimers have been shown to exhibit tether length-dependent inhibition of rat AChE as a consequence of simultaneous binding to the catalytic and peripheral sites of AChE [22, 23]. The tether

length dependence seen for *bis*(n)-tacrine in the Ellman assay here (Table 2) is similar to what was previously observed in rat [22].

We found that AChE inhibitors from different categories reduced the fluorescence of TFT bound to mAChE (Fig. 3). Given what is known about their actions on AChE, the reduction of TFT fluorescence induced by these anticholinesterases suggested that inhibitors bound to either of the peripheral or catalytic sites of mAChE can impact binding of TFT to the peripheral anionic site of mAChE. The peripheral site inhibitors and bivalent inhibitors likely inhibit TFT binding at mAChE, whereas the catalytic site inhibitors presumably alter the structure of the binding site of TFT allosterically, which also resulted in a reduced fluorescent signal. Sultatos and Kaushik [16] reported a competitive inhibition of TFT bound to human recombinant AChE by tubocurarine, which is a peripheral site AChE inhibitor. In addition, chlorpyrifos oxon and dichlorvos, which phosphorylate the Ser²⁰³ residue of the human catalytic triad, reduced fluorescent quantum yield by conformational interactions between the catalytic site and the peripheral site [16]. This latter mechanism explains our findings of quenched TFT fluorescence in the presence of catalytic site inhibitors that covalently modify Ser²⁰³, such as carbamates. These compounds carbamylate AChE in a reaction analogous to phosphorylation, but with a shorter half life [2]. Although the underlying principle of the Ellman assay for measuring catalysis, and the activity independent fluorescence assay is fundamentally different, the obtained IC₅₀ values were strongly correlated. This correlation suggests a close relationship between the modulation of peripheral site conformation and the inhibition of catalytic activity of mAChE.

Our TFT assay data also demonstrated that AChE inhibitors from different categories had different efficacies to maximally inhibit TFT fluorescence. Except for tubocurarine, peripheral site inhibitors and bivalent inhibitors showed higher maximal inhibition of TFT fluorescence than catalytic site inhibitors (Fig. 3), which was consistent with findings from previous studies. De Ferrari et al. [17] and Harel et al. [19] found that edrophonium, a catalytic site inhibitor, only partially quenched TFT fluorescence, similar to what we observed here. In our experiments, propidium iodide and ethidium bromide showed almost 100% inhibition of total TFT fluorescence, which suggested that these two inhibitors also inhibited non-specific binding of TFT to other protein in the enzyme preparation. As shown in Figure 4, the complete TFT fluorescence inhibition by propidium iodide indicated that propidium at a high concentration did not affect donepezil binding to mAChE, and the additional fluorescence quenching with propidium reflects competition with TFT for non-specific binding sites. Binding of ethidium and propidium-like molecules to both protein [27] and nucleic acids [28] is well established. Another peripheral site inhibitor, tubocurarine, did not show high inhibitory effect on the total TFT fluorescence (less than 40%; Fig. 3). The low efficacy of tubocurarine is possibly due to greater specificity of binding interaction, so there is less effect on non-specific sites, although high concentrations (over 1 mM) of tubocurarine had to be used in the TFT assay, and solubility limited testing of higher concentrations.

Unlike carbamates, the catalytic site inhibitors edrophonium, benzylpiperidine, and tacrine, all have a basic or quaternary nitrogen atom, and they do not covalently modify the catalytic triad of mAChE. X-ray crystallography studies demonstrate that edrophonium [19, 29] and tacrine [29] interact with the choline binding site of *Tc*AChE (Trp⁸⁴ and Phe³³⁰ residues). Based on the structure of the donepezil/*Tc*AChE binary complex [25], it seems likely that benzylpiperidine binds here as well. Accordingly, they would have different mechanisms of action for quenching TFT fluorescence than carbamates and organophosphate oxons. As noted by Harel et al. [19] in *Tc*AChE, the two aromatic rings of TFT were coplanar and packed tightly parallel to the aromatic residues along the gorge surface, including Trp²⁷⁹, Tyr³³⁴, and Phe³³⁰. The benzothiazole ring of TFT was lodged against Trp²⁷⁹, while the

dimethylaminophenyl group of TFT was close to Phe³³⁰. In the presence of edrophonium, the aromatic ring of Phe³³⁰ had to rotate in order to accommodate TFT and edrophonium in the ternary complex. This rotation was hypothesized to relax the strict coplanarity of the bound TFT, and induce partial quenching of TFT fluorescence [19]. Harel et al. [29] also found that the quaternary nitrogen of edrophonium was located between Trp⁸⁴ and Phe³³⁰, while the hydroxyl group of edrophonium formed hydrogen bonds with the nitrogen of His⁴⁴⁰ and oxygen of Ser²⁰⁰; two residues of catalytic triad. Tacrine, in contrast, was stabilized in the tacrine-AChE complex by stacking against the indole group of Trp⁸⁴, which is a choline binding site residue in *TcAChE* [29]. The other aromatic amino acid of the choline binding site, Phe³³⁰, rotated to sandwich tacrine with the Trp⁸⁴ residue [29]. These models of *TcAChE* suggest that edrophonium interacts significantly with the catalytic triad, and explain our finding that the maximum inhibition of edrophonium on TFT fluorescence is at the same level as carbamates, which interfere specifically with the catalytic triad. Tacrine does not and instead showed a closer association with the choline binding site, which would influence TFT interaction with Phe³³⁰ more efficiently than edrophonium, and therefore show greater reduction of TFT fluorescence.

Current studies are characterizing TFT interactions with a variety of ligands on AChE of *Anopheles gambiae*. If differences are observed between mammal and mosquito, we anticipate that the data will contribute to refinement of molecular models that will assist in the design of selective and high potency bivalent inhibitors of AChE.

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Abbreviations

AChE	acetylcholinesterase
IC₅₀	inhibitory concentration needed to inhibit 50% of the enzyme activity
mAChE	murine acetylcholinesterase
<i>TcAChE</i>	<i>Torpedo californica</i> acetylcholinesterase
TFT	thioflavin-T

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Highlights

- All inhibitors reduced thioflavin-T fluorescence of murine acetylcholinesterase.
- Potencies in the fluorescence assay were correlated well with potencies for enzyme inhibition.
- Maximal efficacies for reducing thioflavin-T fluorescence ranged from 23% to near 100%, depending on the compound.
- Maximal efficacies could be reconciled with known interaction of inhibitors with acetylcholinesterase.

Figure 1.

Structures of compounds referred to in the text. Thioflavin T is shown, along with the axis of rotation related to fluorescence. Other structures are experimental carbamate insecticides (**1-2**) and *bis*-tacrine dimers.

Figure 2.

Saturation equilibrium binding curve of TFT fluorescence assay with mAChE. Symbols are means, and error bars represent SEM of $n = 6$ replicates. Curve represents calculated fit to a binding hyperbola, with $R^2 = 0.93$ and d.f. = 46 (GraphPad Prism). Inset: Scatchard plot transformation of equilibrium binding data.

Figure 3.

Maximal percentage reduction of total TFT fluorescence by AChE ligands. Ligand classes are grouped by differentially shaded bars, as indicated. Bars are labeled by treatment on the abscissa, and represent means with SEM error bars of $n \geq 3$ replicates. For abscissa labels, numbers 1 and 2 refer to the corresponding structures (Fig. 1), and “bis(2)” refers to bis(2)-tacrine, etc. Inset: Representative concentration-response curves for selected inhibitors of different classes. Inhibition curves were fit to a four parameter logistic equation (GraphPad Prism): $Y = \text{bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - x) * \text{Hillslope}))}$, where x = the logarithm of the concentration and Y = the response [].

Figure 4.

Kinetic reading of TFT fluorescence inhibited by donepezil and propidium iodide, compared to control. Measurements were taken at 40 second intervals. Symbols are means and error bars represent SEM of $n = 3$ replicates. Applications of donepezil and propidium iodide used in this experiment were at times indicated by arrows and were continuously present thereafter. Controls received equal volumes of vehicle at the same treatment time.

Table 1

TFT assay and Ellman assay of AChE ligands

Chemicals	IC ₅₀ values (Mean ± SEM), nM		
	Reduction of TFT Fluorescence	Reduction of of AChE activity ^a	Ratio ^b
1	25 ± 5 (n=4)	62 ± 2.6 (n=3)	0.4
2	8,900 ± 3,025 (n=3)	10,301 ± 2,199 (n=3)	0.9
Propoxur	60 ± 15 (n=4)	466 ± 45 (n=3)	0.13
Bendiocarb	20 ± 6 (n=6)	46 ± 3.4 (n=3)	0.4
Pirimicarb	454 ± 63 (n=6)	738 ± 47 (n=3)	0.6
Aldicarb	565 ± 133 (n=5)	645 ± 0.3 (n=3)	0.9
Edrophonium	1,121 ± 198 (n=3)	3,431 ± 135 (n=3)	0.33
Tubocurarine	50,000 ± 9,800 (n=5)	69,000 ± 19,000 (n=3)	0.7
Ethidium Bromide	16,000 ± 3,400 (n=4)	4,000 ± 400 (n=3)	4.1
Propidium Iodide	670 ± 82 (n=4)	662 ± 43 (n=3)	1.0
BW284C51	8.3 ± 1.2 (n=3)	4.4 ± 0.5 (n=3)	1.9
Benzylpiperidine	159,000 ± 26,000 (n=3)	167,000 ± 6,000 (n=3)	0.95
Donepezil	78 ± 12.3 (n=3)	20 ± 2.9 (n=3)	3.9

^aInhibitors were incubated with mAChE for 1 hour at room temperature before addition of substrate.^bRatio = IC₅₀ TFT assay/IC₅₀ Ellamn assay

Table 2

TFT assay and Ellman assay of tacrine dimers

Chemicals	IC ₅₀ values (Mean ± SEM), nM		
	TFT assay	Ellman assay	Ratio ^a
Tacrine	165 ± 26 (n=4)	64 ± 7.4 (n=3)	2.6
<i>bis</i> (2)-tacrine	162 ± 24 (n=3)	77 ± 7.6 (n=3)	1.3
<i>bis</i> (3)-tacrine	163 ± 25 (n=3)	72 ± 6.4 (n=3)	2.3
<i>bis</i> (4)-tacrine	54 ± 3.2 (n=3)	13 ± 0.3 (n=3)	4.1
<i>bis</i> (5)-tacrine	22 ± 1.7 (n=3)	3 ± 0.8 (n=3)	7.3
<i>bis</i> (6)-tacrine	7.7 ± 1.8 (n=4)	0.6 ± 0.1 (n=3)	11.6
<i>bis</i> (7)-tacrine	9.8 ± 1.4 (n=4)	1.1 ± 0.1 (n=3)	8.9
<i>bis</i> (8)-tacrine	13 ± 0.9 (n=3)	1.1 ± 0.2 (n=4)	11.8
<i>bis</i> (9)-tacrine	98 ± 21 (n=3)	27 ± 2.6 (n=3)	3.6
<i>bis</i> (10)-tacrine	97 ± 19 (n=4)	35 ± 0.3 (n=3)	2.8
<i>bis</i> (12)-tacrine	831 ± 175 (n=4)	183 ± 30 (n=3)	4.5

^aRatio = IC₅₀ TFT assay/IC₅₀ Ellamn assay