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Drug-like Leads for Steric Discrimination between Substrate and Inhibitors of Human Acetylcholinesterase

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Protection of the enzyme acetylcholinesterase (AChE) from the toxic effects of organophosphate insecticides and chemical warfare agents (OPs) may be provided by inhibitors that bind at the peripheral binding site (P-site) near the mouth of the active-site gorge. Compounds that bind to this site may selectively block access to the acylation site (A-site) catalytic serine for OPs, but not acetylcholine itself. To identify such compounds, we employed a virtual screening approach using AUTODOCK 4.2 and AutoDock Vina, confirmed using compounds experimentally known to bind specifically to either the A-site or P-site. Both programs demonstrated the ability to correctly predict the binding site. Virtual screening of the NCI Diversity Set II was conducted using the apo form of the enzyme, and with acetylcholine bound at the crystallographic locations in the A-site only and in both and A- and P-sites. The docking identified 32 compounds that were obtained for testing, and one was demonstrated to bind specifically to the P-site in an inhibitor competition assay.

Key words: acetylcholine, acetylcholinesterase, AChE, docking, virtual screening

Abbreviations: AChE, acetylcholine esterase; OP, organophosphate; DMSO, dimethyl sulfoxide; NCI, National Cancer Institute.

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Acetylcholinesterase (AChE) terminates synaptic neuromuscular transmission by hydrolyzing the neurotransmitter acetylcholine (1). One focus of AChE research lies in the development of new drugs that could prevent and/or treat poisoning from organophosphates (OPs), toxic agents commonly used in insecticides as well as in chemical warfare agents (2). OP poisoning causes the inactivation of AChE that prevents synaptic transmission, leading to muscle paralysis, hypertension, and malfunction of various organ systems, ultimately leading to death.

Two ligand-binding sites in AChE have been identified, the acylation site (A-site) at the base of a deep active-site gorge and the peripheral site (P-site) near the gorge entrance through which ligands must pass on their way to the A-site (3–5). Figure 1 shows the binding gorge of human AChE with acetylcholine modeled into both the A-site (green) and P-site (pink) as described in Methods. OPs are toxic because they covalently react with S203 in the A-site. Wilson and Ginsburg (6,7) conceived complementary oxime compounds that could re-activate OP-poisoned AChE. These drugs serve as defenses against OP toxicity, but they work only on OP-inactivated AChE. It would be beneficial to develop prophylactic drugs that prevent AChE from interacting with OPs in the first place, and our objective is to design drug-like compounds to serve this function. One strategy is to obtain small molecules that bind at or near the AChE P-site. Such molecules could be modified, in principle, to sterically discriminate access to the A-site in a way that still allows entry of acetylcholine but limits the entrance of the larger OPs. This is a challenging task. While a competitive inhibitor that binds to the A-site would reduce the irreversible OP inactivation rate, it would equally reduce the rate of acetylcholine hydrolysis and provide no benefit. The binding of a ligand X to the AChE P-site is known to decrease the association and dissociation rate constants for the binding of a ligand Y to the A-site by factors of up to 400 (5,8), and it is this feature that we propose to exploit in designing a P-site ligand that would selectively block OP association with the A-site. Such a ligand would need very high affinity (pM) for the P-site to effectively block OP inactivation, and a strategy designed to evolve a series of lead compounds with progressively higher affinity is necessary. Here we report on our first steps in such an evolution.

The development of structure–activity relationships for ligands that bind specifically to the AChE P-site is in its infancy. Because of a possible AChE P-site role in promoting amyloid-β aggregation in Alzheimer's disease (9), *in silico* screening of a limited 270-member library of compounds was initiated to identify new ligands with P-site specificity (10,11). Several identified compounds as well as tetrahydrocannabinol (9) were shown experimentally to bind to the P-site.

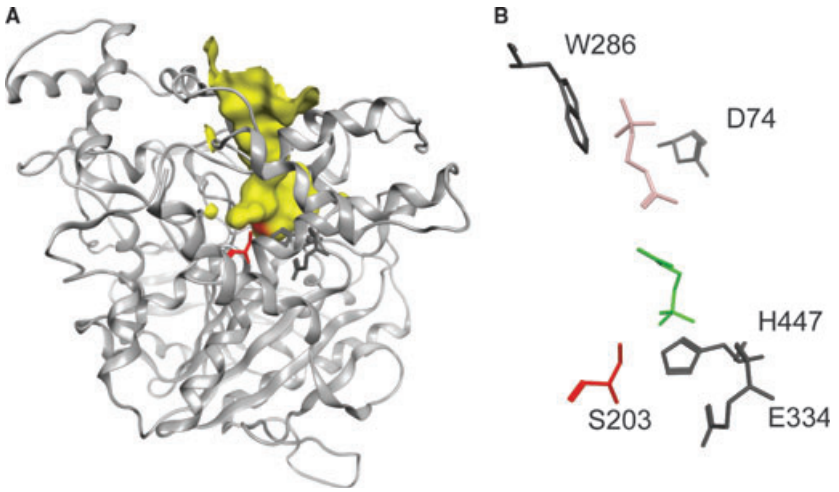


Figure 1: The AChE catalytic site is a narrow active-site gorge some 20 Å deep (yellow) that penetrates nearly to the center of the ~65 kDa catalytic subunits. Near the base of the gorge is the A-site where H447, E334, and S203 (red) participate in a triad that catalyzes the transient acylation and deacylation of S203 during each substrate turnover. The P-site is spanned by residues W286 near the mouth of the gorge and D74 near a constriction at the boundary between the P-site and the A-site. (B) Acetylcholine modeled into the A-site (green) and P-site (pink) as described in Methods.

We used a virtual screening approach to identify P-site ligands from a much larger library of compounds. To establish the ligand docking procedures used to screen the library, we selected several compounds known to bind to either the A-site or the P-site by X-ray crystallography. These were first redocked back into their original crystal structures with two docking programs, AutoDock Vina (12) and AutoDock version 4.2 (13). These compounds were then docked into human AChE to confirm that the docking programs could identify a preference for the correct site. Finally, virtual screening was used to identify compounds that bind preferentially to the P-site, and still allow acetylcholine to bind to the A-site of AChE. The NCI Diversity Set II (14) was first docked using Vina in combination with a consensus scoring approach, and after an initial round of testing, was docked using AutoDock 4.2. Candidate compounds predicted to show selective P-site binding were obtained from the NCI and tested experimentally with an AChE inhibitor-competition assay that can distinguish between inhibitor binding to the A-site or P-site.

Methods

Preparation of crystal structures

The structure of human acetylcholinesterase (AChE) was derived from the crystal structure of the AChE complexed with fasciculin-II deposited in the RCSB Protein Databank (15) (PDB id:1B41) by Kryger and co-workers (16); only the AChE was retained for our modeling studies. The locations of the A- and P-sites were defined from the crystal structure of the S203A mutant of mouse AChE complexed with acetylcholine (17) (PDB id: 2HA4), which shows one molecule of acetylcholine in each site. The 1B41 and 2HA4 structures were superimposed, and the acetylcholine molecules from 2HA4 were merged with AChE from 1B41. This formed our model of human AChE complexed with acetylcholine used in subsequent studies.

Docking controls

Crystal structures of several additional AChE-ligand complexes have been deposited in the PDB, including complexes at the A-site with edrophonium (18) (2ACK) or tacrine (19) (1ACJ) and complexes at the P-site with thioflavin T (20) (2J3Q), decidium (21) (1J07), propidium (21) (1N5R), methylene blue (22) (2W9I), or aflatoxin B1(23) (2XI4). Crystal structures of AChE complexes involving ligands that span both sites also have been obtained, but these ligands are not of interest in this study and were not examined. These structures all were obtained as complexes with *Torpedo californica* AChE (*Tc*AChE) or *Mus musculus* (*Mm*AChE). The complexes were superimposed with our human model of AChE, and therefore, all coordinates are in relation to 1B41, with the A-site and P-site defined by the acetylcholines originally from 2HA4. These ligands, shown in Figure 2, were used to test our docking procedures. In each case, the ligand was removed from the original structure and redocked into that structure with a docking box centered on the original ligand position and measuring 25.2 Å on each side, sufficiently large to encompass the ligands in this study. For the redocking controls, all crystal waters were retained.

Ability to reproduce crystal structure binding mode was assessed by molecular volume overlap, presented as a percentage of the volume of the X-ray ligand. For these calculations, we used ROCS (OpenEye Scientific Software, Sante Fe, NM, USA) to determine the absolute volume and divided the volume overlap of a ligand with itself to determine percentages. Overlap close to 100% can be interpreted as correct prediction of binding mode, while mid-range overlaps typically bind in the correct site with an incorrect conformation, and low values indicate binding in a different site.

Additionally, each of these ligands was docked into our model of human AChE under three different conditions: apo-AChE, with both the A- and P-sites free; AChE with the acetylcholine bound in the

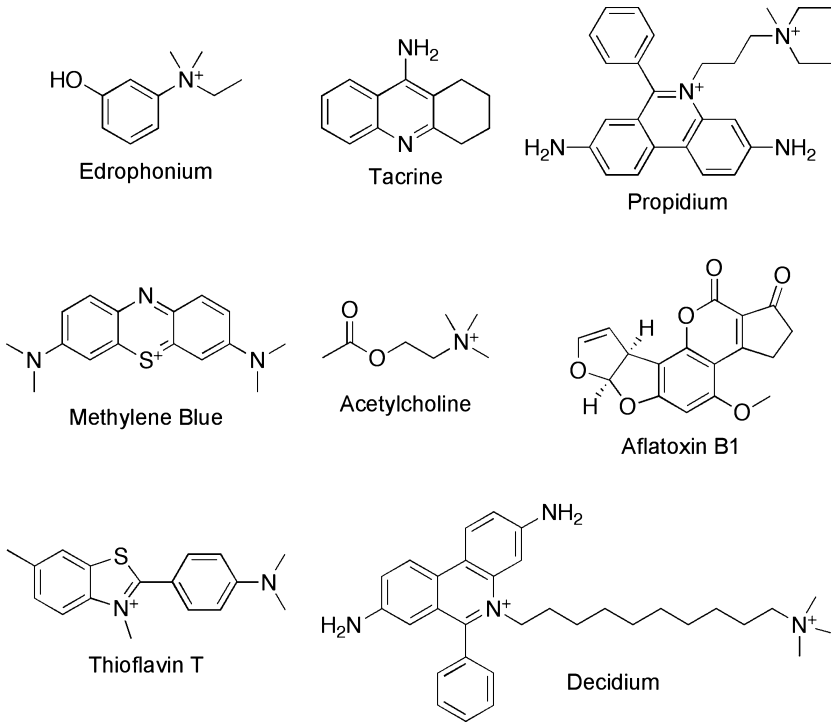


Figure 2: Ligands with crystal structures of the AChE-ligand complexes.

A-site; and AChE with acetylcholine bound at both the A-site and P-site. This docking was intended to demonstrate the ability of the docking procedure to correctly produce the known binding site of each ligand. The docking was performed with both Vina and AutoDock 4.2 using a docking box located at the center of mass of the two modeled acetylcholines (119.1, 109.1, −133.1), measuring 51.2 Å on each side, more than large enough to encompass both sites. In this case, the overlap volume was calculated as overlap with the A- and P-site modeled acetylcholines.

Virtual screening

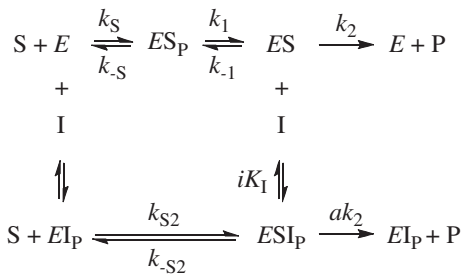
Virtual screening of the NCI Diversity Set II (14), which consists of 1364 diverse compounds selected from the full library of 140 000 compounds, was docked to identify candidate drug-like compounds that might complementarily bind at or near the P-site of human AChE. Three sets of dockings were performed: apo-AChE, with both the A- and P-site free; AChE with the acetylcholine bound in the A-site; and AChE, with acetylcholine bound at both the A- and P-sites. These conditions were selected to distinguish compounds that have a preference for each specific site, as well as those that bind outside either site. Again, the docking box was centered at the center of mass of the two acetylcholine molecules and measured 51.2 Å on each side to allow docking to any site. For each compound in each of the different docking protocols, the 20 top-scoring poses of the complexes were retained. The docking simulations were performed on the IBM cluster at the Washington University High-Performance Computing Center in Saint Louis.

A total of 27 280 poses (20 top-scoring poses for 1364 compounds) obtained from each of the docking protocols were re-ranked using a consensus scoring approach (24–26) that has been successful in virtual screening of other targets (27,28). This technique should eliminate any particular bias and increase confidence in the ranking of binding affinities and/or docking scores used as criteria to select compounds for experimental testing. In this case, we used the Vina score, X-Score (29), and CSCORE (30). Compounds were selected that had poses ranked in the top 1500 with all three scoring routines. These compounds were limited to ≤10 rotatable bonds, and filtered by Lipinski's 'Rule of 5': MW < 500, log*P* < 5, h-bond acceptors < 10, h-bond donors < 5; to help insure bioavailability (31), and selected compounds were examined in enzymatic assays with AChE.

The dockings of all 1364 NCI compounds were also conducted with AutoDock version 4.2, with or without acetylcholine bound to the A-site alone or to both the A- and P-sites. Parameters used for the dockings were defaults except for the following: npts of 256 in each dimension, grid spacing of 0.200, ls_search_freq of 0.2, ga_run of 20, ga_num_evals of 10 000 000. The center of the two acetylcholine molecules was set as the grid center for docking, with a docking box 51.2 Å on each side.

Inhibition of AChE hydrolysis of acetylthiocholine at low substrate concentrations

Recombinant human AChE was expressed as a secreted, disulfide-linked dimer in Drosophila S2 cells and purified as outlined previously (32). Recrystallized methylene blue was a gift of Dr. Lev Weiner and Dr Esther Roth at the Weizmann Institute in Rehovath, Israel, and concentrations were assigned by absorbance at 663 nm with ε663 nm = 74 000 M^{-1} cm^{-1}. Thioflavin T and edrophonium were



Scheme 1: Enzyme species considered in kinetic model.

prepared as described previously (33). Substrate hydrolysis rates v were measured with acetylthiocholine and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.33 mM) in a coupled Ellman reaction in assay buffer (20 mM sodium phosphate buffer (pH 7.0) and 0.02% Triton X-100 unless otherwise noted) at 25°C (34).

The assay used here to assess inhibition of AChE activity measures the second-order hydrolysis rate constant k_E obtained when the substrate concentration [S] is much less than the Michaelis constant K_{app} (34). Under these conditions, Scheme 1 includes all relevant enzyme species. This scheme assumes that substrate binds initially to the P-site (ES_P) before progressing to the A-site (ES) where it is hydrolyzed to products P, and it can be applied both to inhibitors that bind at the A-site ($L_P = I$, and $1/i = 0$) and to inhibitors that bind at the P-site ($L_P = I_P$) with dissociation constants K_I. Under these second-order conditions, substrate hydrolysis rates $v = k_E[E]_{tot}[S]$, where $[E]_{tot}$ is the total enzyme concentration, and k_E is measured from eqn 1 (35).

$$[S] = [S]_0 e^{-zt} \quad (1)$$

In eqn 1, $z = k_E[E]_{tot}$ and t is the time after mixing E and S. Because acetylthiocholine is hydrolyzed by AChE at nearly diffusion-controlled rates, inhibitor binding to either the A- or P-sites results in an expression for the ratio of k_E in the absence of inhibitor ($k_{E[I]=0}$) to k_E in the presence of inhibitor (k_{E+I}) given by eqn 2 (34).

$$\frac{k_{E[I]=0}}{k_{E+I}} = \left(1 + \frac{[I]}{K_I}\right) \quad (2)$$

If a series of assays are conducted with a fixed concentration of one inhibitor I1 together with varying concentrations of a second inhibitor I2, k_E in the presence of both inhibitors (here denoted k_{E+I2}) relative to k_E when only I1 is present (here denoted $k_{E[I2]=0}$) is given by eqn 3 (33).

$$\frac{k_{E+I2}}{k_{E[I2]=0}} = \frac{K_2\left(1 + \frac{[I1]}{K_1}\right)}{K_2\left(1 + \frac{[I1]}{K_1}\right) + [I2]\left(1 + \frac{[I1]}{K_{12}}\right)} \quad (3)$$

In this equation, K_1, K_2, and K_{12} are the equilibrium dissociation constants for I1 with E, I2 with E, and I1 with the E·I2 complex, respectively. The value of $[I1]/K_{12}$ will be significantly >0 only if the ternary complex E·I1·I2 can form, and this analysis, thus, can be used to detect ternary complex formation.

Assay of NCI diversity set compounds

Compounds were dissolved in dimethyl sulfoxide (DMSO) at 10–50 mM and diluted (1:100) into assay buffer. If the compound was insoluble after dilution, the DMSO stock was diluted (1:10) into methanol and then diluted further (1:10) into assay buffer. The assay solvent for the control without a compound was matched to that with compound. AChE activity was essentially unaffected by 10% methanol but was inhibited about 2-fold in the presence of 1% DMSO. However, inclusion of 10% methanol decreased the reproducibility of k_E.

Results and Discussion

Docking controls

The AChE binding sites of the compounds presented in Figure 2 are known from X-ray crystallography, and for each, the compound was removed from the structure and redocked with Vina or AutoDock 4.2. The ability to reproduce the X-ray binding mode is presented as the percent overlap of the docked ligand with the X-ray binding mode in Table 1. Docking with Vina was more successful at locating the ligand within the original binding site than AutoDock 4.2, as each docked ligand had a higher overlap with the X-ray structure. However, lack of complete overlap for most ligands describes at least a shift in position of the docked compounds, and some molecules, such as tacrine, are flipped within their correct site. The ligand ranking by docking energy agrees for the two programs except for propidium and decidium, likely due to the difficulty in docking these flexible molecules. For AutoDock 4.2, docking acetylcholine to 2HA4 with both copies of the crystallographic acetylcholine ligand removed results in consistent docking to only the A-site, even though both the A- and P-sites were available. Docking to AChE with the A-site occupied by the crystallographic acetylcholine results in the second acetylcholine docking reliably to the P-site.

Table 1: Redocking of compounds with known crystal structures

		AutoDock Vina		AutoDock 4.2	
	PDB ID	X-ray overlap (%)	Docking energy	X-ray Overlap (%)	Docking energy
A-site ligands					
Tacrine	1ACJ	98.9	−9.5	61.3	−9.0
Edrophonium	2ACK	94.6	−6.2	96.5	−6.6
P-site ligands					
Thioflavin T	2J3Q	81.8	−7.9	27.8	−7.2
Propidium	1N5R	91.4	−6.8	13.1	−8.6
Decidium	1JO7	55.0	−6.2	42.9	−10.7
Methylene Blue	2W9I	55.9	−8.5	42.5	−7.7
Aflatoxin B1	2XI4	98.6	−9.6	97.0	−9.1
Acetylcholine A-site	2HA4	83.7	−4.8	48.2	−5.5
Acetylcholine P-site	2HA4	65.3	−4.3	68.9	−3.8

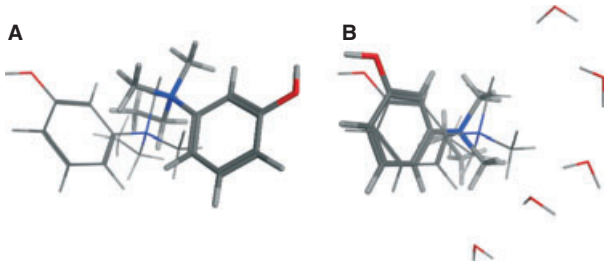


Figure 3: Docking of edrophonium into *Torpedo* apo-AChE using AutoDock 4.2 (A) with crystal waters removed; (B) with crystal waters retained. Crystal structure ligand and water in thin lines and docked results in thick lines.

Using Vina, acetylcholine is docked into both the A-site and P-site if they are unoccupied.

Crystal water molecules play an important role in accurately reproducing crystal structures in docking. This was noted specifically for AChE by Dickerson and co-workers (10), who observed that crystal waters greatly improved docking results for propidium and decidium. We observe this same behavior with several ligands, including acetylcholine and edrophonium shown in Figure 3. Without crystal waters, (Figure 3A) edrophonium docks to the A-site, but only partially overlaps with the crystal structure binding mode (28.8% volume overlap). The inclusion of only five of the crystal structure waters (Figure 3B) in the A- and P-sites results in a docked structure that much more closely matches the crystal structure ligand (86.7% overlap).

As A-site waters in AChE are well-known, inclusion of explicit waters in docking would be preferred. However, given the diverse chemotypes in our compound database, it is not possible to know prior to docking which compounds may interact with or displace the water molecules. Therefore, as an additional control calculation, we docked these same known ligands to human AChE with all waters removed to assess the ability of Vina and AutoDock 4.2 to correctly predict the preferred binding site for each ligand under virtual screening conditions.

Docking with human AChE

The three-dimensional structures of *Tc*AChE, *Mm*AChE, and human AChE are very similar, and most features of the active-site gorge are conserved between the species. Nevertheless, to confirm that the results obtained with *Tc*AChE and *Mm*AChE also apply to human AChE, docking with Vina and AutoDock 4.2 to human AChE was repeated for all the ligands in Figure 2. Docking trials were performed with three different conditions: apo-AChE, with both the A- and P-site free; AChE with the acetylcholine bound in the A-site; and AChE with acetylcholine bound at both the A- and P-site.

Docking energies for both Vina and AutoDock 4.2 are presented in Table 2 for the apo-AChE docking (no bound acetylcholine). Binding in the correct site is described by the percent overlap with acetylcholines in the A-site and P-site modeled into 1B41 through superposition of 2HA4. A measurable percent overlap indicates it is unlikely that the modeled acetylcholine and the docked ligand could bind simultaneously in the given binding mode. When only a partial overlap is observed, typically the ligand is docked immediately adjacent to the modeled acetylcholine.

All ligands are predicted to bind to the known preferred site except for tacrine docking by Vina, which was docked to the P-site. Decidium docked by Vina spans both sites, but is predominantly located in the P-site. Acetylcholine was docked by both programs into both the A- and P-sites, with a slight preference in predicted energy for the A-site, although none exactly reproduces the crystal structure binding mode. As with the redocking control, the ranking by docking energy agrees between Vina and AutoDock 4.2, with the exception of propidium and decidium.

The docking to human AChE with acetylcholine modeled into either the A-site or both the A- and P-sites produces similar results (data not shown). As each site is occupied, the docking algorithms will identify another binding location, but with less favorable energy. It

Table 2: Docking control ligands into human apo-AChE

	AutoDock Vina			AutoDock 4.2		
	A-site overlap (%)	P-site overlap (%)	Docking energy	A-site overlap (%)	P-site overlap (%)	Docking energy
A-site ligands						
Tacrine	0	84.6	−8.5	65.8	0	−8.7
Edrophonium	65.8	0	−6.3	62.2	0	−6.6
P-site ligands						
Thioflavin T	0	73.3	−8.0	4.6	25.2	−7.6
Propidium	0	15.9	−7.2	0	65.8	−10.2
Decidium	46.2	72.7	−9.1	0	82.9	−10.4
Methylene blue	0	85.7	−7.9	4.0	44.9	−7.2
Aflatoxin B1	0	84.0	−9.0	0	100	−8.9
Acetylcholine A-site	59.7	0	−4.4	43.5	0	−4.9
Acetylcholine P-site	0	29.2	−3.9	0	33.7	−4.8

should be noted that the percent overlaps in Table 1 are with the crystallographic ligands themselves but in Table 2 are with modeled acetylcholine originally from the 2HA4 crystal structure. Therefore, these values are not directly comparable except for acetylcholine itself. We do observe a decrease in percent overlap for acetylcholine between the redocking (Table 1) and human docking (Table 2), but this is expected because of the removal of crystal structure water from human AChE.

Overall, these docking controls suggest that both the Vina and AutoDock 4.2 programs may be useful for virtual screening for P-site binders of AChE. Vina more accurately predicted crystal structure binding modes, but AutoDock 4.2 was better at predicting the correct binding site. The exact orientations of some docked poses were not always reproduced, but this is not surprising considering the limitations in the scoring functions and search algorithms used in docking. To minimize some of these limitations, our Vina virtual screening protocol employed an established consensus scoring approach (27,28) that incorporates X-Score and CSCORE with the predicted binding energy from docking.

Identifying the binding site

The inhibition of second-order, substrate-hydrolysis rates k_E as measured with eqns 2 and 3 provides a convenient framework for experimentally examining the interaction of an inhibitor with free AChE. The assays are designed to assign the binding location of an unknown inhibitor by examining its competition with inhibitors whose binding sites are known from X-ray crystallography. The approach is illustrated in Figure 4, where the A-site inhibitor edrophonium and the P-site inhibitor thioflavin T are employed as the known inhibitors and methylene blue is analyzed as the unknown inhibitor. The data in Figure 4A show that $k_{E[I]=0}/k_{E+I}$ with all three inhibitors has a linear dependence on the inhibitor concentration [I], in accord with eqn 2. In this case, the simplified inhibitor-competition analysis in eqn 3 can be applied (33,34,36) to determine whether the unknown inhibitor binds to the A- and/or P-sites. The analysis is designed to detect any ternary complex formed when the unknown inhibitor and the inhibitor with a known binding site are added to AChE simultaneously. The data with methylene blue alone are plotted in the reciprocal form $k_{E+I}/k_{E[I]=0}$ in Figure 4B,C to emphasize K_I as the IC_{50}, or inhibitor concentration at which $k_{E+I}/k_{E[I]=0}$ was decreased by 50%. When a fixed concentration of edrophonium was included in the assays, the IC_{50} for methylene blue was shifted slightly to the right, indicating some competition between the two inhibitors. The affinity of methylene blue or edrophonium in the ternary complex decreased by a factor of 3.3 relative to their affinities in their respective binary complexes with the free enzyme, a small change that indicated slight steric overlap between the two binding sites. For comparison, the relative affinities of edrophonium and thioflavin T in the ternary complex with AChE were virtually identical to those in their respective binary complexes (34), and thus, these two known standards show no overlap between their binding sites. In contrast, the IC_{50} for methylene blue in Figure 4C was shifted well to the right by the addition of thioflavin T. One can calculate the extent of the shift that would result from complete competition and no ternary complex formation from the K_I values of the inhibitors individually, and these calculated shifts are shown as the dotted lines in Figure 4B,C. Methylene blue and thioflavin T essentially showed complete competition in Figure 4C. These data indicate that methylene blue binds to the AChE P-site in a fashion that is competitive with thioflavin T, but slightly overlaps edrophonium bound at the A-site. This interpretation is confirmed by the P-site binding of methylene blue complexed with AChE in the crystal structure 2W9I (22).

Virtual screening with Vina

Virtual screening of 1364 compounds that comprise the NCI Diversity Set II was initially performed with AutoDock Vina in the presence of

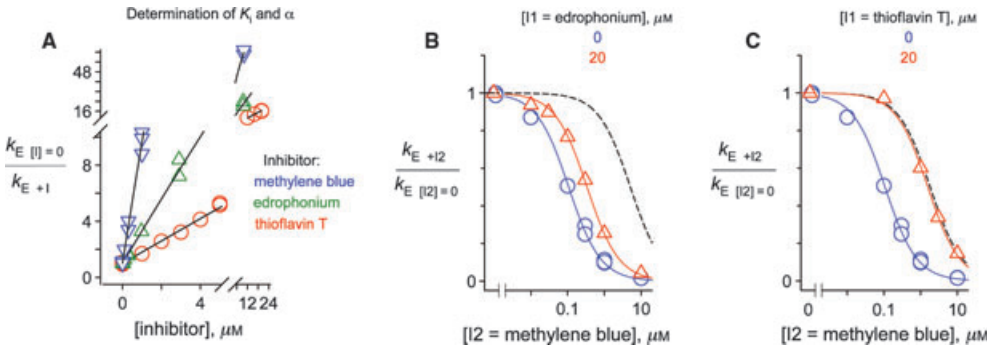


Figure 4: (A) Inhibition of substrate hydrolysis by the A-site inhibitor edrophonium and the P-site inhibitors thioflavin T and methylene blue. The substrate S was acetylthiocholine ($[S]_0$ = 10 μM), and $[E]_{tot}$ was 0.31 nM (for [I] ≤ 5 μM) or 1.6 nM (for [I] ≥ 5 μM, not shown), and the inhibitor I is indicated. Second-order rate constants k_E were obtained with eqn 1 and the ratios $k_{E[I]=0}/k_{E+I}$ were fitted to eqn 2. The K_I values obtained were 110 ± 4 nM for methylene blue (∇), 0.43 ± 0.02 μM for edrophonium (Δ), and 1.24 ± 0.04 μM for thioflavin T (O). B, C) Inhibitor competition assay for assignment of AChE binding-site specificity. Values of k_E, with inhibitor I2 in the presence (Δ) or absence (O) of inhibitor I1 as indicated, were obtained as described in Panel A, and the ratios $k_{E+I2}/k_{E[I2]=0}$ were fitted to eqn 3. K_1 and K_2 values were set as the K_Is from Panel A, and K_{12} was fitted. For edrophonium and methylene blue (panel B), the affinity in the binary complexes relative to that in the ternary complex was given by K_{12}/K_1 = 3.3 ± 0.3. For thioflavin T and methylene blue (panel C), K_{12}/K_1 = 100 ± 35. Dotted lines were calculated with the same values of K_1 and K_2 but with $[I1]/K_{12}$ fixed at 0.

acetylcholine molecules at both the A- and P-sites, in an effort to identify compounds that bound adjacent to the P-site. From consensus scoring using Vina binding energies, X-Score and CSCORE followed by Lipinski's-rule filtering, sixteen compounds with the highest scores were identified. These compounds were then docked to the human apo-AChE (with no acetylcholine bound to either the A- or P-sites) to determine if P-site docking was maintained. Most of the compounds shifted to bind predominantly at the P-site, while some shifted sufficiently to span both the A- and P- sites.

All 16 compounds were obtained from the NCI for testing. Three compounds were insoluble in the assay conditions, and eleven others were inactive (<10% inhibition at 10 μM). Two compounds shown in Figure 5 showed inhibition and were tested in the edrophonium competition assay to assess the extent to which they block the A-site. The affinity of both the compounds in the ternary complex with edrophonium and AChE decreased by a factor of >10 relative to their affinity in their respective binary complexes (Figure 5). This result indicated that the compounds overlapped strongly with edrophonium bound at the A-site, in contrast to the binding poses predicted by Vina. As the goal of this screening strategy was to identify inhibitors that did not overlap with the A-site, these compounds were not considered further.

A second round of virtual screening with Vina was also performed with acetylcholine bound only at the A-site, in an attempt to identify compounds that bound specifically at the P-site. Six compounds were predicted to bind at the P-site and were obtained for experimental assay. All six were poorly soluble and were not considered further.

Virtual screening with AutoDock 4.2

The docking-based virtual screening of NCI Diversity Set II was repeated using AutoDock 4.2, again using apo-AChE and AChE with acetylcholine in either the A-site alone or in both the A- and P-sites. This protocol was developed to indicate which compound bound preferentially at the A-site, P-site, or outside either site. Twenty docking poses were generated and clustered, and compounds with predicted binding affinities better than −7.0 kcal/mol in each of the three docking runs were selected for further analysis and visual inspection. Thirty-eight compounds met these criteria, and sixteen were obtained from the NCI for experimental assay. Of these, four were insoluble and the inhibition from ten others was too low to be of interest (<20% inhibition at 50 μM compound). Two compounds gave greater inhibition of AChE, and one (NSC_358311) had sufficient solubility for analysis with the inhibitor competition assay (Figure 6). Although the affinity of NSC_358311 for AChE was low (K_2 = ~100 μM), this assay indicated little overlap with the A-site, as NSC_358311 affinity in the ternary complex with edrophonium and AChE decreased by a factor of about 2 relative to its affinity in the binary complex with AChE (Figure 6A).

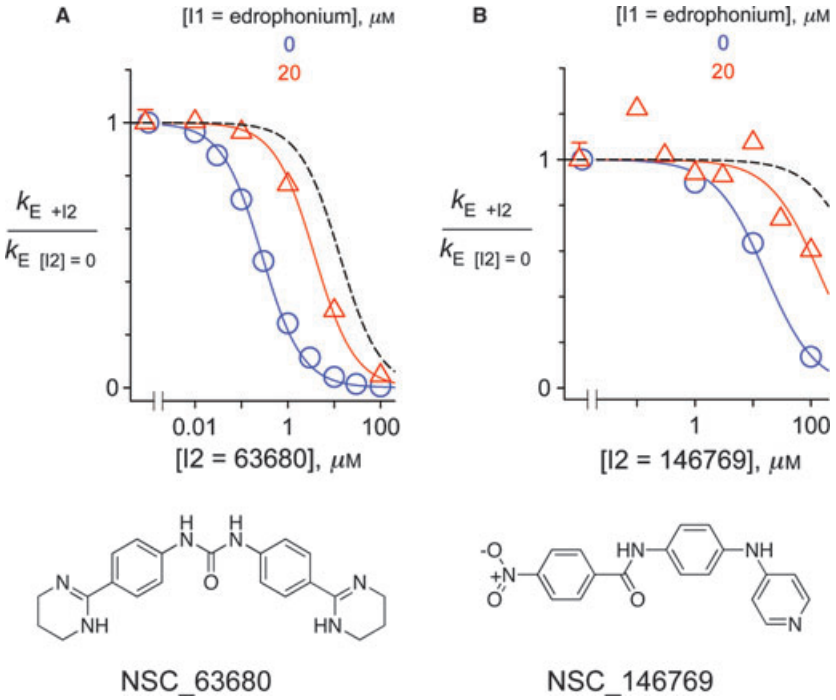


Figure 5: Inhibitor competition assay for assignment of AChE binding-site specificity for compounds selected from NCI Diversity Set II. Values of k_E, with inhibitors I1 and I2 as indicated, were obtained as described in the Figure. To maintain I2 solubility, assay buffer contained 1% dimethyl sulfoxide (DMSO) with 63 680 and 1% DMSO, 10% methanol with 146769. The ratios $k_{E\ +\ I2}/k_{E[I2]\ =\ 0}$ were fitted to eqn 3, K_1 was set from the Figure, and K_2 values in the absence of edrophonium (O) were determined from eqn 3 for 63680 (280 ± 13 nM) and 146769 (16 ± 2 μM). K_{12} was then fitted in the presence of edrophonium (Δ). The affinity in the binary complexes relative to that in the ternary complex with edrophonium (K_{12}/K_1) was 19 ± 2 for 63680 and 10 ± 5 for 146769. Dotted lines were calculated with the same values of K_1 and K_2 but with $[I1]/K_{12}$ fixed at 0.

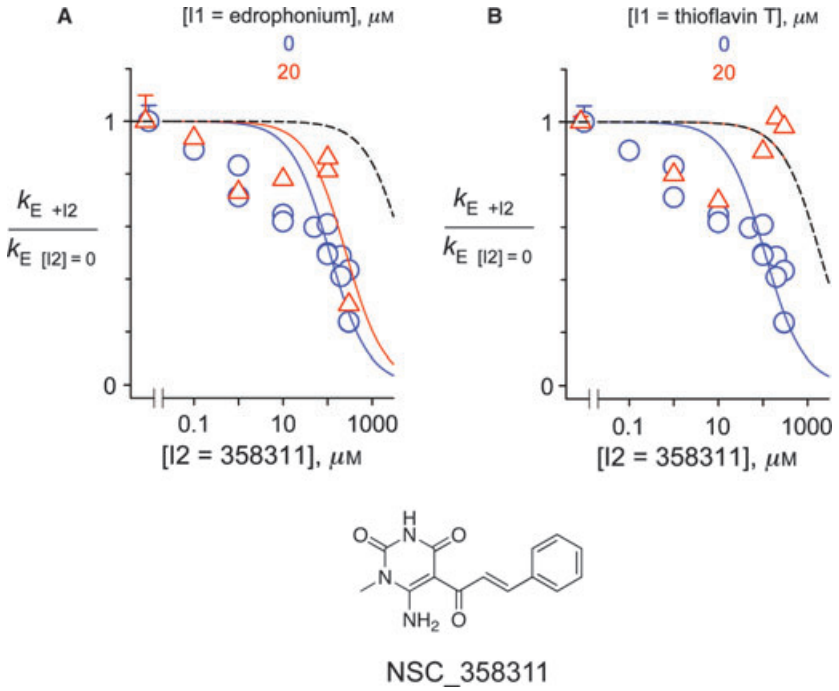


Figure 6: Inhibitor competition assay for assignment of AChE binding-site specificity for NSC_358311. Values of k_E, with inhibitors I1 and I2 as indicated, were obtained as described in Figure 5. To maintain I2 solubility, assay buffer contained 1% dimethyl sulfoxide and 10% methanol. The ratios $k_{E\ +\ I2}/k_{E[I2]\ =\ 0}$ were fitted to eqn 3, K_1 values were set from Figure. 5, and a K_2 value in the absence of I1 (O) was determined from eqn 3 as 111 ± 25 μM. K_{12} was then fitted in the presence of I1 (Δ). The affinity in the binary complexes relative to that in the ternary complex (K_{12}/K_1) was 2.4 ± 1.1 with edrophonium, but was infinite with thioflavin T (i.e., the fitted line superimposed on the dotted line calculated with the same values of K_1 and K_2 but with $[I1]/K_{12}$ fixed at 0).

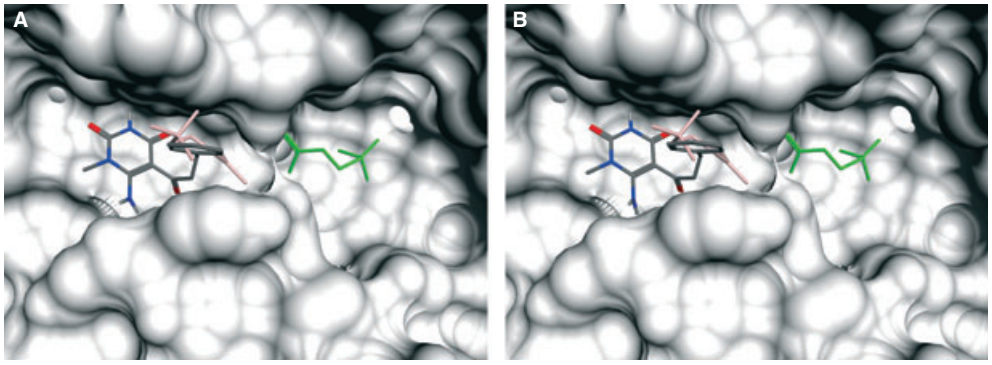


Figure 7: NSC_358311 (colored by element) docked to A) apo-AChE, B) AChE with acetylcholine modeled at the A-site. Acetylcholine molecules from crystal structure 2HA4 are superimposed at the P-site (pink) and A-site (green).

However, no ternary complex involving NSC_358311, thioflavin T, and AChE could be detected ($1/K_{12} = 0$), which shows that NSC_358311 binds only at the P-site. This is consistent with the docking result in Figure 7, which shows complete overlap with the P-site acetylcholine. This predicted binding mode would also allow a ternary complex with NSC_358311 bound at the P-site and acetylcholine at the A-site. Figure 7 also shows that NSC_358311 docks with the same binding mode both to apo-AChE (panel A) and AChE with modeled acetylcholine occupying the A-site (panel B). In this docked conformation, NSC_358311 has 65% volume overlap with the P-site (as defined previously) and zero overlap with the A-site.

Conclusion

Virtual screening of compounds with specificity for the AChE P-site with AutoDock Vina and AutoDock 4.2 was tested with compounds whose specificity for the P-site was known from X-ray crystallogra-

phy. The Vina and AutoDock 4.2 docking programs were then used for virtual screening of compounds from the NCI Diversity database, and they identified two different sets of candidates with potential P-site specificity. However, testing of two candidates predicted by Vina with an experimental inhibitor competition assay did not support this specificity. Virtual screening with AutoDock 4.2 identified NSC_358311, which the experimental binding competition assay showed to bind specifically to the P-site of AChE, while allowing a ligand to bind simultaneously at the A-site. This molecule may provide a lead for additional virtual screening, and as a good starting point for synthetic modifications that limit access to the A-site in a way that still allows entry of acetylcholine but limits the entrance of the larger OPs.

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References

1. Rosenberry T.L. (1975) Acetylcholinesterase. In: Meister A., editor. Advances in Enzymology, Vol. 43. New York, USA: John Wiley & Sons, p. 103–218.
2. Millard C.B., Broomfield C.A. (1995) Anticholinesterases: medical applications of neurochemical principles. J Neurochem;64:1909–1918.
3. Taylor P., Lappi S. (1975) Interaction of fluorescence probes with acetylcholinesterase. The site and specificity of propidium binding. Biochemistry;14:1989–1997.
4. Sussman J.L., Harel M., Frolow F., Oefner C., Goldman A., Toker L., Silman I. (1991) Atomic structure of acetylcholinesterase from *Torpedo* californica: a prototypic acetylcholine-binding protein. Science;253:872–879.
5. Szegletes T., Mallender W.D., Rosenberry T.L. (1998) Nonequilibrium analysis alters the mechanistic interpretation of inhibition of acetylcholinesterase by peripheral site ligands. Biochemistry;37:4206–4216.
6. Wilson I.B., Ginsburg S. (1955) A powerful reactivator of alkylphosphate-inhibited acetylcholinesterase. Biochim Biophys Acta;18:168–170.
7. Kitz R.J., Ginsburg S., Wilson I.B. (1965) Activity-structure relationships in the reactivation of diethylphosphoryl acetylcholinesterase by phenyl-1-methyl pyridinium ketoximes. Biochem Pharmacol;14:1471–1477.
8. Szegletes T., Mallender W.D., Thomas P.J., Rosenberry T.L. (1999) Substrate binding to the peripheral site of acetylcholinesterase initiates enzymatic catalysis. Substrate inhibition arises as a secondary effect. Biochemistry;38:122–133.
9. Inestrosa N.C., Alvarez A., Pérez C.A., Moreno R.D., Vicente M., Linker C., Casanueva O.I., Soto C., Garrido J. (1996) Acetylcholinesterase accelerates assembly of amyloid-b-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. Neuron;16:881–891.
10. Dickerson T.J., Beuscher A.E.I.V., Rogers C.J., Hixon M.S., Yamamoto N., Xu Y., Olson A.J., Janda K.D. (2005) Discovery of acetylcholinesterase peripheral anionic site ligands through computational refinement of a directed library. Biochemistry;44:14845–14853.
11. Eubanks L.M., Rogers C.J., Beuscher A.E.I.V., Koob G.F., Olson A.J., Dickerson T.J., Janda K.D. (2006) A molecular link between the active component of marijuana and Alzheimer's disease pathology. Mol Pharmacol;3:773–777.
12. Trott O., Olson A.J. (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem;31:455–461.
13. Morris G.M., Huey R., Lindstrom W., Sanner M.F., Belew R.K., Goodsell D.S., Olson A.J. (2009) AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J Comput Chem;30:2785–2791.
14. Holbeck S.L. (2004) Update on NCI in vitro drug screen utilities. Eur J Cancer;40:785–793.
15. Berman H.M., Westbrook J., Feng Z., Gilliland G., Bhat T.N., Weissig H., Shindyalov I.N., Bourne P.E. (2000) The protein data bank. Nucleic Acids Res;28:235–242.
16. Kryger G., Harel M., Giles K., Toker L., Velan B., Lazar A., Kronman C., Barak D., Ariel N., Shafferman A., Silman I., Sussman J.L. (2000) Structure of recombinant native and E202Q mutant human acetylcholinesterase complexed with the snake-venom toxin fasciculin-II. Acta Crystallogr;56:1385–1394.
17. Bourne Y., Radic Z., Sulzenbacher G., Kim E., Taylor P., Marchot P. (2006) Substrate and product trafficking through the active center gorge of acetylcholinesterase analyzed by crystallography and equilibrium binding. J Biol Chem;281:29256–29267.
18. Ravelli R.B., Raves M.L., Ren Z., Bourgeois D., Roth M., Kroon J., Silman I., Sussman J.L. (1998) Static Laue diffraction studies on acetylcholinesterase. Acta Crystallogr D Biol Crystallogr;54:1359–1366.
19. Harel M., Schalk I., Ehret-Sabatier L., Bouet F., Goeldner M., Hirth C., Axelsen P.H., Silman I., Sussman J.L. (1993) Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. Proc Natl Acad Sci U S A;90:9031–9035.
20. Harel M., Sonoda L.K., Silman I., Sussman J.L., Rosenberry T.L. (2008) Crystal structure of thioflavin T bound to the peripheral site of Torpedo californica acetylcholinesterase reveals how thioflavin T acts as a sensitive fluorescent reporter of ligand binding to the acylation site. J Am Chem Soc;130:7856–7861.
21. Bourne Y., Taylor P., Radić Z., Marchot P. (2003) Structural insights into ligand interactions at the acetylcholinesterase peripheral anionic site. EMBO J;22:1–12.
22. Weiner L., Roth E., Silman I. (2011) Targeted oxidation of *Torpedo californica* acetylcholinesterase by singlet oxygen. Photochem Photobiol;87:308–316.
23. Sanson B., Colletier J.P., Xu Y., Lang P.T., Jiang H., Silman I., Sussman J.L., Weik M. (2011) Backdoor opening mechanism in acetylcholinesterase based on X-ray crystallography and MD simulations. Protein Sci;20:1114–1118.
24. Wang R., Lu Y., Fang X., Wang S. (2004) An extensive test of 14 scoring functions using the PDBbind refined set of 800 protein-ligand complexes. J Chem Inf Comput Sci;44:2114–2125.

25. Wang R., Lu Y., Wang S. (2003) Comparative evaluation of 11 scoring functions for molecular docking. J Med Chem;46:2287–2303.
26. Oda A., Tsuchida K., Takakura T., Yamaotsu N., Hirono S. (2006) Comparison of consensus scoring strategies for evaluating computational models of protein-ligand complexes. J Chem Inf Model;46:380–391.
27. Taylor C.M., Barda Y., Kisselev O.G., Marshall G.R. (2008) Modulating G-protein coupled receptor/G-protein signal transduction by small molecules suggested by virtual screening. J Med Chem;51:5297–5303.
28. Yang R.Y.C., Yang K.S., Pike L.J., Marshall G.R. (2010) Targeting the dimerization of epidermal growth factors with small-molecule inhibitors. Chem Biol Drug Des;76:1–9.
29. Wang R., Lai L., Wang S. (2002) Further development and validation of empirical scoring functions for structure-based binding affinity prediction. J Comput Aided Mol Des;16:11–26.
30. Clark R.D., Strizhev A., Leonard J.M., Blake J.F., Matthew J.B. (2002) Consensus scoring for ligand/protein interactions. J Mol Graph Model;20:281–295.
31. Lipinski C.A. (2000) Drug-like properties and the causes of poor solubility and poor permeability. J Pharmacol Toxicol Methods;44:235–249.
32. Mallender W.D., Szegletes T., Rosenberry T.L. (1999) Organophosphorylation of acetylcholinesterase in the presence of peripheral site ligands: distinct effects of propidium and fasciculin. J Biol Chem;274:8491–8499.
33. De Ferrari G.V., Mallender W.D., Inestrosa N.C., Rosenberry T.L. (2001) Thioflavin T is a fluorescent probe of the acetylcholinesterase peripheral site that reveals conformational interactions between the peripheral and acylation sites. J Biol Chem;276:23282–23287.
34. Auletta J.T., Johnson J.L., Rosenberry T.L. (2010) Molecular basis of inhibition of substrate hydrolysis by a ligand bound to the peripheral site of acetylcholinesterase. Chem Biol Interact;187:135–141.
35. Eastman J., Wilson E.J., Cervenansky C., Rosenberry T.L. (1995) Fasciculin 2 binds to a peripheral site on acetylcholinesterase and inhibits substrate hydrolysis by slowing a step involving proton transfer during enzyme acylation. J Biol Chem;270:19694–19701.
36. Camps P., Cusack B., Mallender W.D., El Achab R., Morral J., Muñoz-Torrero D., Rosenberry T.L. (2000) Huprine X is a novel high affinity inhibitor of acetylcholinesterase that is of interest for the treatment of Alzheimer's disease. Mol Pharmacol;57:409–417.