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Hopeahainol A binds reversibly at the acetylcholinesterase (AChE) peripheral site and inhibits enzyme activity with a novel higher order concentration dependence

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Abstract

Natural product inhibitors of AChE are of interest both because they offer promise as inexpensive drugs for symptomatic relief in Alzheimer's disease and because they may provide insights into the structural features of the AChE catalytic site. Hopeahainol A is an uncharged polyphenol AChE inhibitor from the stem bark of *H. hainanensis* with a constrained, partially dearomatized bicyclic core. Molecular modeling indicates that hopeahainol A binds at the entrance of the long but narrow AChE active site gorge because it is too bulky to be accommodated within the gorge without severe distortion of the gorge as depicted in AChE crystal structures. We conducted inhibitor competition experiments in which AChE inhibition was measured with hopeahainol A together with either edrophonium (which binds at the base of the gorge) or thioflavin T (which binds to the peripheral or P-site near the gorge mouth). The results agreed with the molecular modeling and indicated that hopeahainol A at lower concentrations (< 200 μ M) bound only to the P-site, as hopeahainol A and thioflavin T were unable to form a ternary complex with AChE while hopeahainol A and edrophonium did form a ternary complex with essentially no competition between them. Inhibition increased to a striking extent at higher concentrations of hopeahainol A, with plots analogous to classic Dixon plots showing a dependence on hopeahainol A concentrations to the third- or fourth order. The inhibition at higher hopeahainol A concentrations was completely reversed on dilution and blocked by bound edrophonium. We hypothesize that bound hopeahainol A induces conformational changes in the AChE active site that allow binding

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of additional hopeahainol A molecules, a phenomenon that would be unprecedented for a reversible inhibitor that apparently forms no covalent bonds with AChE.

Graphical Abstract

Keywords

Acetylcholinesterase; hopeahainol A; peripheral site; natural product inhibitors; enzyme mechanism

1. Introduction

Acetylcholinesterase (AChE) is localized at cholinergic synapses and regulates neurotransmission through rapid hydrolysis of the neurotransmitter acetylcholine into choline and acetate [3, 4]. In Alzheimer's disease, cholinergic neurons are lost and endogenous levels of acetylcholine are decreased. Drugs that penetrate the blood brain barrier and partially inhibit AChE can increase the levels of acetylcholine, potentiate its physiological effects, and provide symptomatic relief [5]. However, more potent irreversible organophosphate (OP) inhibitors completely block AChE activity through covalent modification of the catalytic S203, and they have been used as chemical warfare nerve agents that cause death through paralysis from excessive accumulation of acetylcholine in cholinergic synapses [6]. Better insight into inhibitor interaction with AChE may provide new strategies to protect against OP inactivation of AChE.

Kinetic and thermodynamic studies have revealed that substrates and inhibitors can interact with either or both of two binding sites in AChE [7–10], and X-ray crystallography has provided information about the location of these sites [11–14]. A narrow active site gorge some 20 Å deep penetrates nearly to the center of the ~65 kDa catalytic subunits (Figure 1A). Near the base of the gorge is the acylation or A-site where H447, E334, and S203² participate in a triad that catalyzes the transient acylation and deacylation of S203 during each substrate turnover (Figure 1B). The peripheral or P-site, 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 (Figure 1B), specifically binds certain ligands like the fluorescent probes propidium [8] and thioflavin T [9, 13], and it is included in the extensive AChE surface area

²Throughout this paper we number amino acid residues according to the human AChE sequence unless otherwise noted.

bound by the neurotoxin fasciculin [15, 16]. The P-site thus far has been shown to contribute to catalytic efficiency by insuring that most substrate molecules that collide with and transiently bind to the P-site proceed on to the A-site [17–19] and, with certain bound cationic substrates, by providing a modest allosteric activation of the acylation step [20].

To assist in identifying the full range of inhibitor interactions that can occur at the P-site, we have previously examined natural products reported to inhibit AChE activity and possibly bind selectively to the P-site [21]. These include aflatoxin B1, dihydrotanshinone I, and territrem B. Using an inhibitor competition assay that can distinguish whether an inhibitor is bound to the A-site or the P-site and X-ray crystallography, we showed that aflatoxin B1 and dihydrotanshinone I bound specifically to the P-site while territrem B spanned the P- and A-sites [1, 22].

Here we investigate the natural product hopeahainol A, a polyphenol from the stem bark of *Hopea hainanensis*. Hopeahainol A was isolated and structurally characterized in 2008, and it was reported to inhibit the activity of electric eel AChE with a K_I value of about 20 μM [23]. We observe a novel inhibition pattern for human AChE with hopeahainol A which differs from that reported for any other AChE inhibitor. This inhibition pattern may reveal conformational changes in the enzyme active site relevant to future drug design.

2. Experimental methods

2.1. Hopeahainol A

Hopeahainol A was investigated both as the isolated natural product [23] and the pure synthetic compound [24]. In one early experiment it was dissolved at 25 mM in methanol; but in all subsequent experiments it was dissolved at 100 mM in fresh DMSO, and aliquots were snap frozen and stored at -20°C until use. Thawed aliquots were diluted to 2 mM stock solutions in water on the day of enzyme assays. Hopeahainol A concentrations were determined by UV spectra ($\epsilon_{307\text{ nm}} = 8130\text{ cm}^{-1}$, [23]).

2.2. Assays of substrate hydrolysis

Recombinant human AChE used in all kinetic experiments was expressed as a secreted, disulfide-linked dimer in *Drosophila* S2 cells and purified as outlined previously [25]. Crystallization attempts and size exclusion chromatography were conducted with a dimeric recombinant human AChE that was truncated at the C-terminus and contained no intersubunit disulfides [26]. Edrophonium chloride, 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, and bovine serum albumin (BSA), Fraction V, (approx. 99%) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Thioflavin T (Sigma-Aldrich) was recrystallized from water, and concentrations were assigned by absorbance at 412 nm with $\epsilon_{412\text{ nm}} = 36,000\text{ M}^{-1}\text{ cm}^{-1}$. Enzyme hydrolysis rates were measured in a coupled Ellman reaction in which thiocholine generated from acetylthiocholine in the presence of 0.33 mM DTNB was determined by formation of the thiolate dianion of DTNB at 412 nm ($\epsilon_{412\text{ nm}} = 14,150\text{ M}^{-1}\text{ cm}^{-1}$) [20]. Total AChE concentrations ($[E]$) were calculated assuming 450 units/nmol [9]. Assays were conducted at 25°C in 20 mM sodium phosphate buffer (pH 7.0) and 0.01% bovine serum albumin (assay

buffer) supplemented as described. In some experiments extending over several hr with the same diluted AChE stock, assay values of k_E or v were corrected (by up to 40%) for an observed inactivation rate of control values in the absence of inhibitor. Data fitting by nonlinear regression was conducted with SigmaPlot (vers.11.0), and analyses with either Eq. (2) or Eq. (3) were weighted assuming that the dependent variable had constant percent error.

2.3. Kinetic analysis of substrate hydrolysis rates

When the concentration of substrate (S) is low ($[S] \ll K_D$, where K_D is defined in Eq. (2) below), the most accurate way to measure the AChE-catalyzed second-order hydrolysis rate constant k_E is from Eq. (1) [27].

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

In Eq. (1), $z = k_E[E]$, where t is the time after initial mixing of enzyme (E) and S at $t = 0$. To fit hydrolysis data, the following substitutions were made in Eq. (1) and the equation was solved for A_{412} at time t : $[S] = (A_{412} - A_{412(\text{final})}) / \epsilon_{412 \text{ nm}}$, where $A_{412(\text{final})}$ is A_{412} at the conclusion of the exponential time course; and $[S]_0 = (A_{412(\text{final})} - A_{412(t=0)}) / \epsilon_{412 \text{ nm}}$.

To conduct *inhibitor competition assays* with acetylthiocholine as the substrate, a series of k_E measurements were made with a fixed concentration of one inhibitor I1 together with varying concentrations of the second inhibitor I2. The ratio of 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 then given by Eq. (2) [9, 27].

$$\frac{k_{E+I2}}{k_{E[I2]=0}} = \frac{K_2 \left(1 + \frac{[I1]}{K_1}\right)}{K_2 \left(1 + \frac{[I1]}{K_1}\right) + [I2] \left(1 + \frac{[I1]}{K_{12}}\right)} \quad (2)$$

In Eq. (2), K_1 , K_2 , and K_{12} are the equilibrium dissociation constants for I1 with E , I2 with E , and I1 with the $EI2$ complex, respectively. The value of $[I1]/K_{12}$ will be significantly greater than zero only if the ternary complex $EI1I2$ can form, and this analysis thus can be used to detect ternary complex formation.

2.4. A model for inhibition at high concentrations of hopeahainol A

To quantify pronounced increases in inhibition at higher hopeahainol A concentrations, we applied the empirical formula in Eq. (3). This equation contains terms reflecting up to a fourth-order dependence of $k_{E[I]=0}/k_{E+I}$ on the concentration of inhibitor $[I]$, and it is best applied under the second-order substrate hydrolysis conditions outlined with Eq. (1). It can also be applied if it is observed or assumed that higher concentrations of substrate have no effect on the measured extents of inhibition.

$$\frac{\nu_{[I]=0}}{\nu_{+1}} = 1 + \frac{[I]}{K_{i1}} + \frac{[I]^2}{K_{i2}} + \frac{[I]^3}{K_{i3}} + \frac{[I]^4}{K_{i4}} \quad (3)$$

The parameters K_{i1} through K_{i4} in Eq. (3) have no mechanistic significance until they are interpreted in the context of a molecular model. One model that can account for our data here is shown in Scheme 1.

In this scheme, initial binding of I to the P-site (with dissociation constant K_{i1}) is followed by a conformational change in the active site gorge ($K_{C1} = [E_1I_P]/[E_2I]$) that allows binding of additional I molecules. Here the additional I is proposed to be a dimer (I_2 , with dissociation constant $K_D = [I][I]/[I_2]$) that binds to AChE with dissociation constant K_{i2} . All species from E_1 to E_2I_2 are assumed to be in equilibrium in the initial steady state measured here by ν_0 . A second enzyme conformational change K_{C2} represents an additional step that may lead to the final steady state obtained with hopeahainol A, but this step is not included in the quantitative analysis here. Applying Scheme 1 to Eq. (3), one can show that $K_{i1} = K_{i1}K_{C1}/(1 + K_{C1})$ and $K_{i3} = K_{i1}K_{C1}K_{i2}K_{C2}K_D/(1 + K_{C2})$.

2.5. Molecular modeling

Hopeahainol A was docked into the human AChE crystal structure 1B41 [28] using GOLD (distributed by the Cambridge Crystallographic Data Centre). The docking generated 500 binding modes (poses), many of them very similar, and the best scoring and most abundant result was selected. This pose was then energy minimized, with complete protein and ligand flexibility, using the AMBER 99SB forcefield with ligand parameters from the related program GAFF.

3. Results

3.1. Molecular modeling of the interaction of hopeahainol A with AChE

Hopeahainol A is an uncharged polyphenol with a constrained, partially dearomatized bicyclic core (Fig. 2A). The absolute (*S*)- or (+)-configuration of naturally occurring hopeahainol A has been inferred from molecular modeling and the crystal structure of permethylated hopeahainol A (where four phenolic hydroxyl groups are methylated) [23, 29]. Our preliminary molecular modeling indicated that hopeahainol A is too bulky to be accommodated within the long but narrow AChE active site gorge without severe distortion of the gorge as depicted in AChE crystal structures. In a more rigorous modeling effort, hopeahainol A was docked into human AChE structure 1B41 with the docking program GOLD and minimized in the Amber 99 forcefield, a combination which has been particularly good at reproducing and predicting crystal structures. We selected the fasciculin-bound crystal structure 1B41 (with fasciculin removed) in an effort to keep the entry to the gorge as open as possible. Energy minimization then gave the structure of the binary complex shown in Fig. 2B. Hopeahainol A sits at the gorge entry with two of its phenol rings stacked and inserting into the gorge, its size preventing it from penetrating further into the gorge. Key interactions are made with W286, S293 and the backbone carbonyl of Y341.

The two inserting phenyl rings slightly overlap with the P-site location of thioflavin T in its crystal structure complex with AChE (Figure 2B).

Attempts to obtain a crystal structure of the hopeahainol A-AChE complex, either by diffusing hopeahainol A into human AChE crystals (e.g., [22]) or by crystallizing the complex directly, were unsuccessful. Crystals that were soaked in 1.3 mM hopeahainol A for four days showed no evidence of ligand binding and seemed to diffract just as well as those that were never soaked.

3.2. Inhibitor competition indicates that hopeahainol A at lower concentrations binds to the AChE P-site

In most experiments, hopeahainol A was dissolved in DMSO to minimize its degradation and diluted with water before adding it to assay solutions. Basic methanol is known to convert hopeahainol A to hopeanol through methanolysis of the χ -lactone moiety [24, 29], and incubation of hopeahainol A here in 3 mM NaPi (pH 7.0) gave UV spectral changes consistent with conversion to hopeanol [23] over a 2-day period. Hopeanol is reported to have no inhibitory activity with AChE [23, 29].

To confirm the prediction from molecular modeling that hopeahainol A binds to the AChE P-site, we employed an inhibitor competition assay. This assay is designed to detect any ternary complex formed when a new inhibitor and an inhibitor whose binding site is known from X-ray crystallography are added to AChE simultaneously [1, 9, 27, 30, 31]. The assay is conducted with a substrate like acetylthiocholine whose second-order hydrolysis at low substrate concentrations is diffusion controlled and thus is inhibited by ligands that bind to either the A-site or the P-site [27]. In Fig. 3, the A-site inhibitor edrophonium [32] and the P-site inhibitor thioflavin T [13] are employed as the known inhibitors and hopeahainol A is analyzed as the unknown inhibitor. The data with hopeahainol A alone are plotted in the form $k_{E+I}/k_{E[I]=0}$ in Figure 3A and 3B to emphasize the hopeahainol A inhibition constant K_2 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 hopeahainol A was essentially unchanged, indicating ternary complex formation with no competition between the two inhibitors.

In contrast to the results with edrophonium, the IC_{50} for hopeahainol A in Figure 3D 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 inhibition constants of the inhibitors individually, and these calculated shifts are shown as the dotted lines in Figs. 3A and 3B. Hopeahainol A and thioflavin T showed complete competition in Fig. 3B. These data are consistent with the docking locations in Fig. 2B, where bound hopeahainol A and thioflavin T overlap, while bound edrophonium at the bottom of the gorge [32] is well separated from hopeahainol A at the mouth of the gorge.

3.3. At higher concentrations, hopeahainol A shows an unprecedented increase in the extent of its AChE inhibition

Examination of the data in Fig. 3 indicates that points at high concentrations of hopeahainol A alone in the absence of a second inhibitor I1 fall considerably below the fitted line. To

show this more clearly, reciprocals of the ratios in Fig. 3 ($k_E/[I]=0/k_E+1$) from a different data set with synthetic hopeahainol A were plotted against the hopeahainol A concentration (Fig. 4A). Such plots are equivalent to classic Dixon plots of enzyme inhibition; and, with all inhibitors of acetylthiocholine hydrolysis by AChE that have been reported to date, they are linear (e.g., [1, 27, 31]). The plot in Fig. 4A, however, clearly is not linear but shows steeply increasing inhibition at higher hopeahainol A concentrations. We first addressed whether this nonlinearity might arise from an irreversible inactivation of AChE by hopeahainol A. Prolonged incubation of AChE with hopeahainol A did result in some inactivation (data not shown). The inactivation rates were strongly dependent on the container surface, with uncoated polypropylene tubes giving the slowest inactivation, but even the highest observed inactivation rate (half-time of ~100 min) was much too low to account for the nonlinearity seen in Fig. 4A after 25-min incubation. To directly assess irreversible inactivation in the incubation mixtures assayed in Fig. 4A, aliquots were diluted and hydrolysis rates v were measured and normalized for the AChE concentration (Fig. 4B). No inactivation was observed, as values of v at all hopeahainol A incubations were not significantly lower than the control value without hopeahainol A (Fig. 4B).

We next asked whether more than one step involving hopeahainol A inhibition of AChE could be detected. Hydrolysis rates v following mixing of hopeahainol A and AChE were measured in Fig. 5. An acetylthiocholine concentration of 300 μM was included so that v for controls in the absence of hopeahainol A remained essentially constant. Slow decreases in v were observed at all hopeahainol A concentrations over the 20-min assays, but at concentrations of 150 – 860 μM a faster initial decrease became apparent (Fig. 5B). Two-exponential fits to the data traces for v allowed extrapolation to the initial rate v_0 , which reflected the initial steady-state inhibition, and determination of the faster exponential decay rate constants of 0.3 – 1.5 min^{-1} (k_1 in Fig. 5B legend). We interpret this exponential decay as reflecting an additional inhibition step beyond the initial steady-state inhibition.

Analysis of the hopeahainol A concentration dependence of v_0 showed that the nonlinear inhibition seen in Fig. 4A is also apparent in the initial steady state. Assays like those in Fig. 5B were conducted with either 100 μM or 300 μM acetylthiocholine, and in both cases a striking increase in inhibition was observed at higher hopeahainol A concentrations (Fig. 6). If one assumes that this inhibition reflects binding to the free enzyme (or is unaffected by substrate binding at these acetylthiocholine concentrations), the inhibition data in Fig. 6 can be analyzed with Eq. (3). This is an empirical equation with terms that are first through fourth order in the concentration of inhibitor I. We first recognized that, as found with the pattern in Fig. 3, $v_0/[E]_{[I]=0}/v_0/[E]_{+1}$ followed a first-order dependence on $[I]$ at lower concentrations of hopeahainol A. Fitting points obtained at hopeahainol A concentrations below 200 μM gave K_{i1} values of 115 and 123 μM (Table 1), in reasonable agreement with the K_2 value of 48 μM for hopeahainol A obtained in Fig. 3 under slightly different solvent conditions. These values of K_{i1} were then fixed in Eq. (3), and the complete data sets in Fig. 6 were fitted by including individually the second-, third-, or fourth-order terms. The overall best fit was obtained by including only the third-order term, and the corresponding values of K_{i3} are shown in Table 1. Notably, inclusion of 20 μM edrophonium with 300 μM acetylthiocholine in the reaction mixtures completely eliminated the striking increase in inhibition at higher hopeahainol A concentrations and resulted in the linear Dixon plot

shown in Fig. 6. This observation also was consistent with the data in Fig. 3. The inhibition curve with edrophonium in Fig. 6 was fit to the linear version of Eq. (3) to give a K_{i1} of 109 μM (Table 1), in agreement with the K_{i1} values obtained in the absence of edrophonium. In addition, these edrophonium concentrations largely blocked the inhibition step reflected in the faster exponential decay in Fig. 5B. Only at the highest hopeahainol A concentration in Fig. 6 was a small initial segment of the v vs. t trace with a faster exponential decay observed.

4. Discussion

Interest in hopeahainol A as an inhibitor of AChE would be minimal if only the apparent inhibitor affinity were considered. Many compounds inhibit AChE in the low- to mid- μM range. However, no inhibitor has been reported previously to show steeply increasing inhibition at higher inhibitor concentrations, as characterized by Dixon plots analogous to Eq. 3 that show inhibitor concentration terms that are greater than second order. This inhibition pattern was observed with both the isolated hopeahainol A natural product [23] as well as the pure synthetic compound [24]. The observations of a third-order term in fitting the v_0 data in Fig. 6, and even a fourth-order term in fitting final steady-state v data³, indicate that hopeahainol A is interacting with AChE in a novel and highly interesting manner⁴. Yet inhibitor competition data provide no evidence that this interaction occurs outside the AChE active site gorge. Lower concentrations of hopeahainol A compete with thioflavin T binding, as expected from their binding site locations in Fig. 2, but not with edrophonium binding, as expected from its binding site observed by x-ray crystallography at the base of the gorge. Bound edrophonium nonetheless blocks hopeahainol A inhibition associated with the higher order concentration terms (Fig. 6), suggesting that it prevents further interactions of hopeahainol A with the initial hopeahainol A-AChE complex.

It is unfortunate that we have been unable to obtain crystal structure data on any complex of hopeahainol A and AChE. Failure to diffuse hopeahainol A into AChE crystals suggests that the crystal structure lattice cannot accommodate a putative AChE conformational change that accompanies hopeahainol A binding. Inclusion of hopeahainol A resulted in an AChE hydrodynamic volume decrease by size exclusion chromatography, and this decrease may involve dissociation of an AChE dimer to monomer triggered by an active site conformational change (data not shown). However, we also observed complete reversibility of the inhibition seen at all concentrations of hopeahainol A (Fig. 4B) over the course of a few minutes, indicating that any enzyme conformational change that accompanies additional hopeahainol A binding must be relatively rapidly reversible.

In Scheme 1 we offer a possible kinetic model to account for the inhibition associated with our v_0 data in Fig 6. We propose an initial P-site complex E_1I_P whose formation would be inhibited by bound thioflavin T, followed by an unfavorable conformational change in this

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⁴A reviewer suggested that hopeahainol A could be affecting AChE activity as a co-solvent, but we find this suggestion unlikely. When co-solvent effects on enzyme activity are studied, the solute concentration in aqueous solution is typically greater than 100 mM [2] and effects on activity are often irreversible. Concentrations of hopeahainol A exposed to AChE here were always less than 1 mM, and hopeahainol A inhibition was completely reversible.

complex ($K_{C1} \gg 1$) to a species E_2I in which additional hopeahainol A binding can occur. This additional hopeahainol A binding is inhibited by edrophonium bound to the A-site, so one possibility is that the bound hopeahainol A in E_2I slides into the A-site at the base of the gorge to allow additional hopeahainol A binding near the P-site in E_2II_2 . Three bound hopeahainol A molecules are necessary to account for the observed third-order dependence in Fig. 6 and Table 1, and Scheme 1 proposes the binding of one dimer in E_2II_2 to achieve this. We incorporate this proposal for simplicity, noting that hopeahainol A is predicted to undergo self-aggregation by the QSAR model of Seidler [33]. However, we have no experimental evidence that dimerization of hopeahainol A actually occurs at concentrations approaching 1 mM. For example, we saw no deviations from Beer's law at any wavelength between 275 and 412 nm for hopeahainol A up to 800 μ M. Finally, the additional inhibition step observed in Fig. 5B is not fully incorporated into Scheme 1, although another conformational change to E_3II_2 in Scheme 1 could initiate it.

Additional kinetic studies are underway to better define hopeahainol A binding by examining the ways in which hopeahainol A binding competes with other AChE ligands like fasciculin 2 and slowly equilibrating substrates like organophosphates. However, structural techniques like X-ray crystallography ultimately will be necessary to fully describe this binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

A	absorbance
AChE	acetylcholinesterase
BSA	bovine serum albumin
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)

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HIGHLIGHTS

- Hopeahainol A, an uncharged polyphenol natural product, inhibits acetylcholinesterase
- Inhibitor competition showed hopeahainol A below 200 μ M bound only to the P-site
- Inhibition at 800 μ M hopeahainol A was 100-fold higher than at 200 μ M
- All inhibition by hopeahainol A was reversible and was blocked by bound edrophonium.
- Bound hopeahainol A may alter AChE conformation to allow additional inhibitor binding

Fig. 1.

A) Cross section of AChE with the active site gorge highlighted in yellow and S203 in red. B) Additional key residues in the active site gorge are shown in gray with their residue number.² The orientation is identical to panel A. Acetylcholine molecules observed in the crystal structure of the S203A mutant [34] are inserted in the P-site (pink) and the A-site (green). This mutation does not alter the structure of native AChE but abolishes all catalytic activity, allowing acetylcholine ligands and their binding sites in AChE to be preserved.

Fig. 2.

A) Molecular structure of (*S*)-hopeahainol A. B) A complex of hopeahainol A and AChE obtained by docking with the program GOLD. Hopeahainol A is shown in stick format with carbon atoms in yellow and oxygens, in red. The AChE active site surface is colored by element with oxygens in red and nitrogens in blue. A thioflavin T molecule has been inserted based on its location in crystal structure 2J3Q of the thioflavin T-AChE complex [13], also colored by element with the addition of sulfur in yellow.

Fig. 3.

Inhibitor competition assay measuring ternary complex formation by AChE, hopeahainol A, and either the A-site inhibitor edrophonium (A) or the P-site inhibitor thioflavin T (B). Stock natural product hopeahainol A was maintained in 7.5% methanol for no more than 6 hr before assay to minimize degradation (see text). Assays were conducted in assay buffer containing 0.5–0.75% methanol, acetylthiocholine substrate S ($[S]_0 = 10 \mu\text{M}$), AChE ($[E]$ was 0.2–0.6 nM for $[I1] = 0$ or 10–20 nM for $[I1] = 20$ –22 μM), and the indicated concentration of hopeahainol A (I2) in 1.0 mL total volume. Second-order rate constants k_E were obtained with Eq. (1) as outlined in the Experimental methods, and the ratios $k_{E+I}/k_{E[I]=0}$ for hopeahainol A in the absence of a second inhibitor are shown in pink. These ratios for concentrations of hopeahainol A less than 55 μM were fitted to Eq. (2) to obtain $K_2 = 48 \pm 2 \mu\text{M}$ (solid black line). A) In the presence of 20 μM edrophonium, values of the ratios $k_{E+I2}/k_{E[I2]=0}$ (blue) were fitted to Eq. (2) with fixed $K_1 = 0.14 \mu\text{M}$ [1] and $K_2 = 48 \mu\text{M}$ to obtain a ratio of K_{I2} to K_1 of 0.7 ± 0.1 (solid blue line). B) In the presence of 22 μM thioflavin T, values of $k_{E+I2}/k_{E[I2]=0}$ (green) were fitted to Eq. (2) with fixed $K_1 = 0.92 \mu\text{M}$ [1] and K_2 again 48 μM to determine that $1/K_{I2} = 0$ (solid green line). Dotted lines were calculated with the same values of K_1 and K_2 but with $[I1]/K_{I2}$ fixed at 0.

Fig. 4.

Inhibition of AChE shows a pronounced dependence on hopeahainol A concentration and is fully reversible. A) Mixtures of AChE (0.2 – 20 nM) and synthetic hopeahainol A (60 – 600 μM) in 170 μL assay buffer containing 2% DMSO were incubated for 25 min at room temperature. A mixture of acetylthiocholine substrate S (to $[S]_0 = 20 \mu\text{M}$) and DTNB in 5 μL total was then added to 95 μL of the incubation mixture to initiate the second-order hydrolysis reaction. Values of k_E were obtained with Eq. (1), and ratios of $k_{E[I]=0}$ in the absence of hopeahainol A to k_{E+I} at the indicated concentrations of hopeahainol A are shown. B) At various times t , aliquots (13 μL) of the remaining 75- μL incubations in panel A were diluted 10- to 600-fold with assay buffer to reduce the hopeahainol A concentration to less than 10 μM and incubated for 20 min to allow hopeahainol A dissociation to approach equilibrium. These mixtures were then further diluted 10-fold with assay buffer, acetylthiocholine (to $[S] = 500 \mu\text{M}$) and DTNB, and hydrolysis rates v were measured. Plots of $\log(v)$ vs t for three time points spanning 6 h for each hopeahainol A concentration were constructed to allow linear extrapolation to v at $t = 0$. These extrapolated values of v were divided by the final AChE concentration to give $v/[E]$, and ratios of $v/[E]_{[I]=0}$ in the absence of hopeahainol A to $v/[E]_{+I}$ at the indicated concentrations of hopeahainol A are shown. The large errors observed with two points arose primarily from the nonlinearity of the $\log(v)$ vs t plots.

Fig. 5.

Initial rapid inhibition of AChE by hopeahainol A is followed a further slow inhibition step. Reactions were initiated at time 0 by mixing AChE (to 0.006 – 1.3 nM) and assay buffer containing DMSO (to 0.6%), acetylthiocholine (to 300 μM), DTNB, and the indicated concentration of synthetic hopeahainol A (HopA). A) Continuous recording of $A_{412 \text{ nm}}$ after mixing. B) The differences of $A_{412 \text{ nm}}$ at time $t - 10 \text{ s}$ from $A_{412 \text{ nm}}$ at time $t + 10 \text{ s}$ in panel A were divided by 0.33 min to give the hydrolysis rates v and plotted against t . Data traces of v were analyzed with the equation

$$v = (v_0 - v_1) * \exp(-k_1 * t) + v_1 * \exp(-k_2 * t)$$

with four fitted variables (v_0 , v_1 , k_1 , and k_2) to allow accurate extrapolation to the initial rate v_0 . Fitted values of k_1 for the faster exponential at hopeahainol A concentrations of 150 and 640 μM were 0.55 and 0.66 min^{-1} , respectively. Acetylthiocholine concentrations of at least 100 μM were necessary in this assay to maintain v essentially constant over the assay time interval and prevent substrate depletion from distorting the fitted parameters. Offsets indicate additions to all v for a given trace to avoid trace overlap.

v_0 without hopeahainol A and AChE preincubation

Fig. 6.

A pronounced dependence of AChE inhibition on hopeahainol A concentration is retained in the initial steady state but blocked by edrophonium. Values of v_0 from data traces with 300 μM acetylthiocholine like those in Fig. 5B (olive triangle) as well as from similar experiments that also included 20 μM edrophonium (blue diamond) or that utilized 100 μM acetylthiocholine (pink inverted triangle) were analyzed with Eq. (3) as outlined in Table 1. Solid black and gold lines indicate the fits for 100 and 300 μM acetylthiocholine, respectively, while the solid blue line indicates the fit for assays with edrophonium.

Scheme 1.

Table 1

Analysis of initial hopeahainol A inhibition of acetylthiocholine hydrolysis from Eq. (3).

[Acetylthiocholine] μM	[Edrophonium] μM	K_{i1} μM	K_{i3} μM^3
100	0	115 ± 10	$(9 \pm 1) \times 10^6$
300	0	123 ± 15	$(13 \pm 2) \times 10^6$
300	20	109 ± 7	-

Data from Fig. 6 were analyzed with Eq. (3). First, values of K_{i1} for the data sets without edrophonium were obtained by fitting measurements of $v_0/[E][I]=0/v_0/[E]+I$ at concentrations of hopeahainol A below 200 μM to the linear form of Eq. (3) with $1/K_{i2}$, $1/K_{i3}$, and $1/K_{i4}$ fixed at zero. These K_{i1} were then fixed, and fitting of data for all hopeahainol A concentrations to Eq. (3) was expanded to include one additional term in either $[I]^2/K_{i2}$, $[I]^3/K_{i3}$, or $[I]^4/K_{i4}$. The overall best fit as measured by the coefficient of determination Rsqr was obtained by addition of the $[I]^3/K_{i3}$ term, and corresponding values of K_{i3} are shown. The data set with edrophonium was fitted to the linear form of Eq. (3) with $1/K_{i2}$, $1/K_{i3}$, and $1/K_{i4}$ fixed at zero to give the value of K_{i1} shown. This fit was not improved with additional terms in Eq. (3).