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The role of TRP84 in catalytic power and the specificity of AChE

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Abstract

The structure–function relationship between the alkaloids physostigmine, physovenine and the three structurally related compounds were investigated by employing kinetic studies and molecular modeling. Crystallographic data from the X-ray conformation of the *Torpedo californica* acetylcholinesterase complex together with the transition state analog inhibitor *m*-(*N*,*N*,*N*,-Trimethylammonio) trifluoroacetophenone (TMTFA) was used as template onto which inhibitors were superimposed. Among the structural elements of the active site, TRP84 residue shows a versatile role. In fact, its aromatic electrons not only can be employed in π–cation interactions, as is the case for ACh, but they can also provide a polarizable surface for van der Waals and London interactions.

Keywords: Acetylcholinesterase catalysis; Acetylcholinesterase mechanism; Physostigmine analogues; Acetylcholinesterase inhibitors

1. Introduction

Very recent crystal structure of *Torpedo californica*–acetylcholinesterase (*Tc*AChE) complex provide template for speculation as to the role of the active site amino acids in the molecular recognition of the transition state. In particular the role of the catalytic triad (SER200, HIS440 and GLU327), that is located in the narrow gorge of the enzyme, has been satisfactorily discussed [1–3]. This gorge contains also other residues such as TRP84, TYR130, GLU199 and PHE330 which contribute to the anionic site and which interact with the ammonium function of ACh. Since 1991 Sussman et al. [3] have suggested that quaternary ammonium ion of acetylcholine is bound not to a negatively charged anionic site but rather, by van der Waals interactions, to some of the 14 aromatic residues that line the hydrophobic gorge. In 1993 Ordentlich et al. [4] reported that site-directed mutagenesis of human AChE showed, in addition, that TRP86 (TRP84 in *T. californica*) constitutes the classical anionic subsite.

The present studies endorse these earlier reports by analyzing the action mechanism of strong, structurally related, irreversible or slowly reversible AChE inhibitors; all these inhibitors are carbamates, a class of compounds (Fig. 1) among which belong some drugs used for the treatment of Alzheimer's disease.

No modeling study of the mechanism of AChE inhibition by these alkaloids is known. Such a study could prove interesting as these compounds possess

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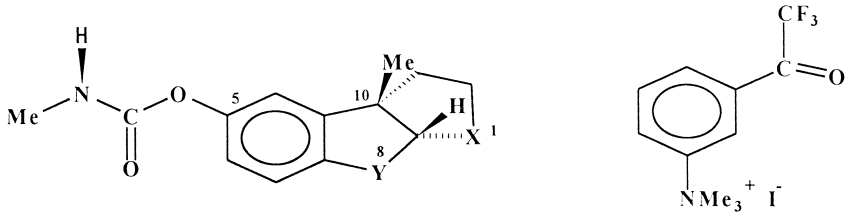

A B

Fig. 1. A: Molecular formulas of physostigmine (**1**; *X*: N–CH_3; *Y*: N–CH_3), physovenine (**2**; *X*: O; *Y*: N–CH_3), thiaphysovenine (**3**; *X*: S; *Y*: N–CH_3) and 8-carbaphysostigmine (**4**; *X*: N–CH_2–CH_3; *Y*: CH_2), nor-physostigmine (**5**; *X*: N–H; *Y*: N–CH_3). B: *m*-(*N*, *N*, *N*-trimethylammonio)–trifluoroacetophenone.

the same configuration, being very similar in structure, but different hydrophobic and electrostatic characteristics. Consequently, the study would contribute to reveal first, the role of the residues of the anionic site (TRP84, GLU199 and PHE330) and second, the transition state stabilization arising from cation–π interaction.

2. Materials and methods

2.1. Reagents

All reagents were bought from Sigma, St. Louis, MO. The authors are deeply indebted to Prof. Arnold Brossi and Prof. Nigel H. Greig (NIH, Bethesda, MD) for providing **2**, **3** and unnatural physostigmine; to Dr. Y.L. Chen (Pfizer, Groton, CT) for providing **4** The absolute configuration of natural physostigmine, **1**, is 3aS, 8aS (Fig. 1).

2.2. Molecular graphics and structure analysis

Construction of the three-dimensional molecular models was carried out at the Silicon Graphics workstation Indigo 2; analysis was performed utilizing SYBYL modeling software (Tripos). After **1** had been sketched, **2**, **3** and **5** were obtained by replacing N^1–CH_3 in O, S and N^1–H respectively; in **4** N^8–CH_3 was replaced by CH_2.

The X-ray coordinates of the TMTFA–*Tc*AChE complex were obtained from Bookhaven P.D.B., ID Code 1AMN, at 2.8 Å resolution [3]; the model was used as a basis for the modeling procedures. The inhibitors' models were fitted onto the TMTFA; then they were merged. Optimization was carried out with the special method used by ANNEAL (hot region 10 Å; interesting region 12 Å). The POWELL minimizer, who belongs to the conjugate gradient family of minimization methods, was selected. The TRYPOS force field consisted of only non-bonded energies: hydrogen-bonding, van der Waals, torsional and bending energy terms. The cut-off criterion was 8 Å radius for all interactions. The energy convergence criterion was ± 0.05 kcal mol^{-1}.

The inhibitor–AChE complexes were then subjected to energy minimization and the reported X-ray data (distance from the oxyanion of the residues GLY118, GLY119 and ALA201: 2.9, 2.9 and 3.2 Å respectively; distance $N^{\varepsilon 2}$ (H440) – O^{γ} (S200): 2.7 Å) was used as constraints. No constraint was used for the quaternary nitrogen, N^1, of **1**.

The **4**-AChE Michaelis complex proved to be very similar to the analogue **1** complex. The RMS of each model did not exceed 0.25.

2.3. Enzyme inhibition in vitro

AChE activity was determined by the colorimetric method [5] using acetylthiocholine as substrate. The assay medium contained 0.05 mM acetylthiocholine iodide; 0.25 mM dithio-bis-dinitrobenzoic acid; 0.1 M sodium phosphate, pH 8.0. The reaction was monitored at 410 nm by means of graphics software (Varian Cary 3E) at 25° using thermostated (Haake) cells. The Michaelis constant ($K_m = 0.14$ mM,

S.D. ± 0.07) at 25°C had been determined previously (average ± S.D.,). The inhibition rate depended on the inhibitor concentration used. Values represented a mean of 3–5 assays, with standard deviations not exceeding 20%. The appropriate thermodynamic and rate constants were determined according to Refs. [6–8]. Inhibition was conducted at different concentrations of inhibitors. To estimate the values of k_2 and K_d, non-linear-regression methods were used. This involved applying rates acquired from the progress curves in order to register variations in enzyme activity. The appropriate constants were obtained by fitting the experimental data to a rapid equilibrium equation. All the compounds resulted competitive inhibitors of AChE from *Electrophorous electricus*. The intrinsic dissociation constant, K_i, for inhibition of *Tc*AChE by TMTFA was 15 fM [1].

IC_{50} from literature: for **1**, 5.7×10^{-8} M [9]; for **2**, 2.8×10^{-8} M, [10,11]; for **3**, 9.9×10^{-9} M, [12]; for **4**, 3.8×10^{-8} M, [13].

3. Results

The 4 structurally related carbamates, **1**–**4**, when assayed in vitro inhibit AChE by a fairly well-proved mechanism [6–8] that, in analogy with substrate hydrolysis, is designed as follows:

$$EH + YOCON(Me)H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH - YOCON(Me)H$$

$$\xrightarrow[\text{YOH}]{k2} ECON(Me)H \xrightarrow[H_2O]{k3} EH + CO_2 + NH_2Me$$

Where EH is the enzyme, YOCON(Me)H is the inhibitor and EHYOCON(Me)H is the Michaelis complex.

The inhibition reaction is described by the dissociation constant, K_d (k_{-1}/k_{+1}), the unimolecular carbamylation rate constant k_2 (min^{-1}) and the reactivation rate constant k_3 (min^{-1}). The unimolecular constant k_2 and the equilibrium dissociation constant K_d are related to each other and to the bimolecular inhibition constant, k_i (M^{-1} min^{-1}), by its relationship to $k_i = k_2/K_d$. Even if the general nature of the catalytic mechanism were known its structural origins are largely approximated.

Our results show that the substitution of the N^1CH_3 group in **1** shows a modest decrease in the rate of inhibition reaction. This is evident by the turnover numbers for the complexes **1**, **4** (8.9 and 4.5 min^{-1}, respectively) and **2** and **3** (2.8 and 2.1 min^{-1}, respectively). These small differences indicate an almost constant binding energy. On the whole, the above kinetic results have proved significant and demonstrate that the variations in perturbations are very small. They, additionally, suggest that the amine group present in **1** may not be essential for its anti-AChE activity.

One reason for this could be that the aromatic electrons of the TRP84 indole ring modulate their charge density and their delocalization depending on the electronic environment of the ligand. Hence, the advancing ligand would induce electronic perturbations to the aromatic system of the residue TRP84 thus increasing the London dispersion interactions.

In order to verify the presence of these London interactions, the tetrahedral intermediates generated during the carbamylation step of compounds **1**–**5**, were structurally aligned on the crystallographic coordinate of the TMTFA–*Tc*AChE complex, and then subjected to field-fit optimization. From our study can be pointed out that (Fig. 2) there is a remarkable overall similarity between the orientation of the TMTFA molecule and the physostigmine analogues as the principal interactions appear to be shared.

However, the most consistent characteristic here (Fig. 2) is the peculiar effect of TRP84 on O^1 of physovenine. The *hard* lone pairs of O^1 of **2** generate a greater negative charge density than the *soft* lone pairs of the S^1 in **3**. This greater negative charge interacts with the partial negative charge of π system which characterizes the indole ring. This result, which cannot be attributed to a steric effect, modifies the conformation of the tetrahydrofuranic ring of the physovenine in order to minimize the repulsive effect. This electronic consequence justifies the previous suggestion [14] that this dispersed negative charge might be better suited to interact with a tetraalkyl ammonium cation.

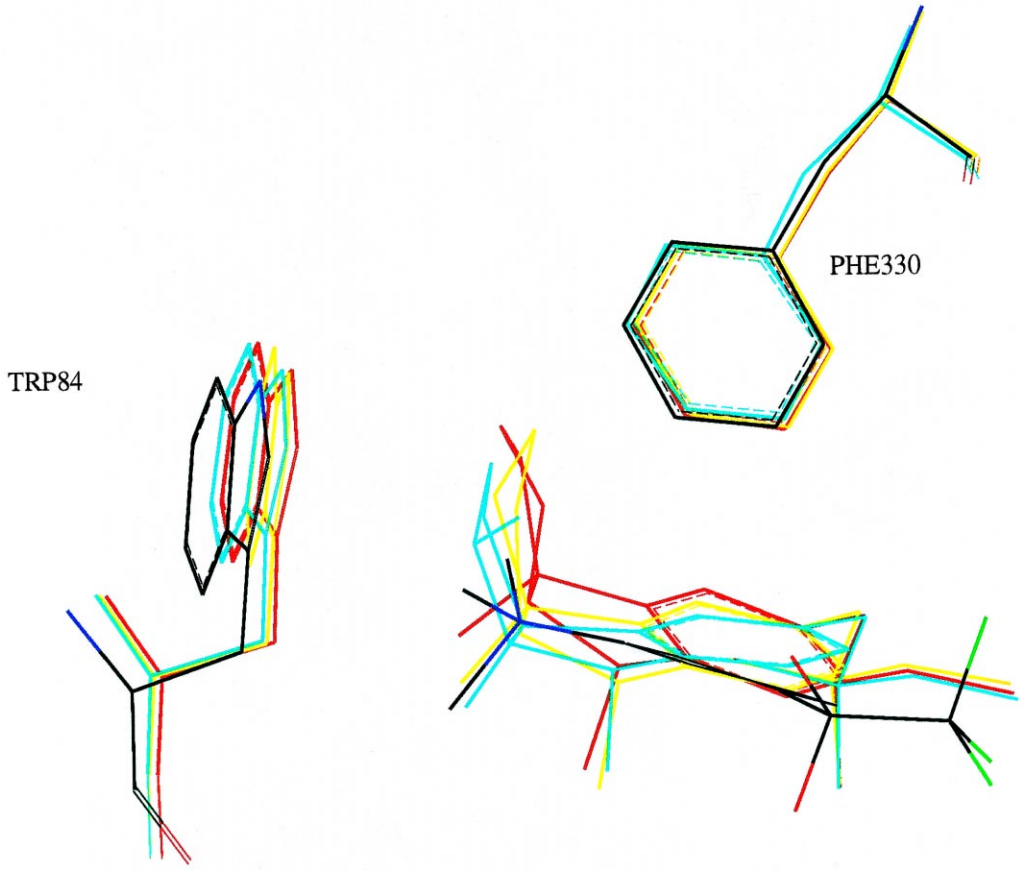

Fig. 2. Superimposition of TMTFA (black), **1** (blue), **2** (red) and **3** (yellow) minimized structure showing tetrahedral bindings only. The O^1 of alkaloid **2** results conformationally asymmetric concerning **1** and **3**.

Quantitative measures of the varying strengths of these interactions can be obtained by inhibitory catalytic carbamylation constant (k_2) values or from the k_2/K_d ratios. Our results indicate that for **1** (3aS, 8aS) the change in configuration to 3aR, 8aR show in a clear decrease in the inhibition rate of reaction (k_2 8.9 vs. 0.07 min^{-1}). This $\approx 10^2$-fold catalytic decrement of AChE carbamylation corresponds to 12.1 kJ mol^{-1}, at 298.2 K, of free energy ($\Delta\Delta G^{\ddagger} = -RT\ln(f)$, where f is the numerical ratio between constants) of stabilization of the transition state.

The relationship between the k_2/K_d (or K_i for TMTFA) and the relative shift of TRP84 from the X-ray crystallographic minimized model is reported in Fig. 3. These results suggest a direct relationship between the relative shift of TRP84 shift and the value of the inhibition constants.

From a thermodynamic point of view, natural physostigmine, **1**, and compound **4** are the most favorite inhibitors ($\Delta G_1^{\ddagger} - \Delta G_4^{\ddagger} = \Delta\Delta G^{\ddagger} = +1.1$ kJ mol^{-1}); **2** ($\Delta G_1^{\ddagger} - \Delta G_2^{\ddagger} = \Delta\Delta G^{\ddagger} = -3.8$ kJ mol^{-1}) and *3* ($\Delta G_1^{\ddagger} - \Delta G_4^{\ddagger} = \Delta\Delta G^{\ddagger} = -6.3$ kJ mol^{-1}) present, approximately, the same stability. On the other hand, from a kinetic point of view, **1** is the most efficacious ($k_2 = 8.9$ min^{-1}) AChE inhibitor. This data also shows that in the case of unnatural physostigmine, (3aR, 8aR) the stability ($\Delta G^{\circ} = 37.0$ kJ mol^{-1}) of the resulting Michaelis complex correlates directly to a reduction in the carbamylation rate. From this it can be argued that its biological affinity, reasonably high ($K_d = 3.2\ (\pm 0.4) * 10^{-7}$ M) for this class of compounds, must arise from additional geometrically confluent interactions between the enzyme and the inhibitor in the complex. However, this

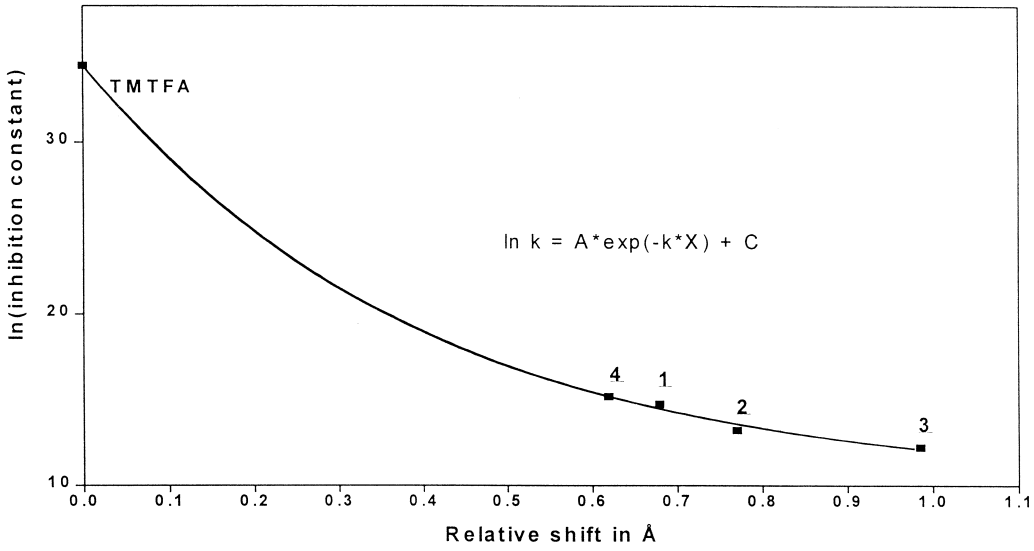

Fig. 3. Relationship between the inhibition constants (k_2/K_d for compounds **1**, **2**, **3** and **4**; the association constant, $1/K_i$, for TMTFA) and the relative shift of carbon atom CZ3 of residue TRP84. Values of parameters: $A = 24.35$; $k = 2.543$; $C = 10$. Absolute Sum of Squares: 0.2137. Along with this computer fitting the authors obtained the same graphic result using equation: $\ln k = A/(2 + X) + 12 - B/(2 + X) + 6 + C$ that recalls the van der Waals energy term used by the Tripos Force Field (Sybyl); values of parameters: $A = -19930$; $B = -1913$; $C = 9.473$. Absolute Sum of Squares: 0.2198.

increased stability has not brought about a rise in the carbamylation rate, k_2, and its mechanism of action could even not be related to enzyme carbamylation. The relatively weaker binding for physovenine ($\Delta G^\circ = 24.4$ kJ mol^{-1}) probably arises because it is not conformationally well oriented (Fig. 2). Hence the inhibitor must select a different conformation and the resulting entropic cost is higher. In fact, if we temporarily ignore the interactions with the solvent then the crucial point, which the formalism of the modeling emphasizes, is that the binding energy between the enzyme and the inhibitor is not simply a property of the interface between them. It also depends on the modifications of the internal structures of the two entities.

4. Discussion

Modeling studies of ACh hydrolysis have indicated the role of the residues TRP84, GLU199 and PHE330 in the molecular recognition of the quaternary ammonium function [1] [3]. The energetic values for the transition state stabilization have been determined by site-direct mutagenesis, following the interactions between the quaternary ammonium group and these residues. According to these studies interactions of TRP84 with the ammonium group contribute for about 20 kJ mol^{-1} [4], GLU199 for about 10 kJ mol^{-1}, and PHE330 for 3 kJ mol^{-1} [15]. In addition to these results the ionic pairing up between an anionic amino acid site and a cationic ligand has, for long time, believed essential for the catalytic activity of the enzyme [16]. However, quite recently, Ordentlich et al. [4] have evidenced the fundamental role of the aromatic TRP84 electrons in establishing interactions with quaternary ammonium function; in particular, these authors have hypothesized that the indole ring of this amino acid is the anionic site of the enzyme. They also reported that charged and uncharged substrates occupy different loci on AChE. Their hypothesis has coincided, at least partially, with analogous studies pursued by Nair et al. [17] who concluded that, during AChE activity, TRP84 residue functions neither as an anionic site [4] nor as a hydrophobic site [3]. Notwithstanding these facts, all these authors agree on the importance of the quaternary ammonium group presence in the molecule of the ligand.

The alkaloid physostigmine, **1**, as physiological pH, exists as cation and consequently its inhibition mechanism agrees with the presence of a cation–π interaction [2]. Moreover, in view of the existence of quaternary nitrogen, the **1**–AChE complex should be more stable in the transition state than the corresponding **2** or **3**–AChE complexes which are, in fact, lacking in N^1 ammonium function. Particularly, if the contributions were to be additive, **1** would have had about 30 kJ mol^{-1} of increased stability when compared with inhibitors **2** and **3**. Notwithstanding this fact **2** and **3** are still strong inhibitors of AChE; the effect on the substitution of NCH_3 for oxygen (or sulfur) is quite modest on both K_d and k_2/K_d. This factor could be the consequence of the molecular rigidity of these alkaloids, which, when reacting with the enzyme, remain blocked in the same conformational structure. This structure has led us to pre-

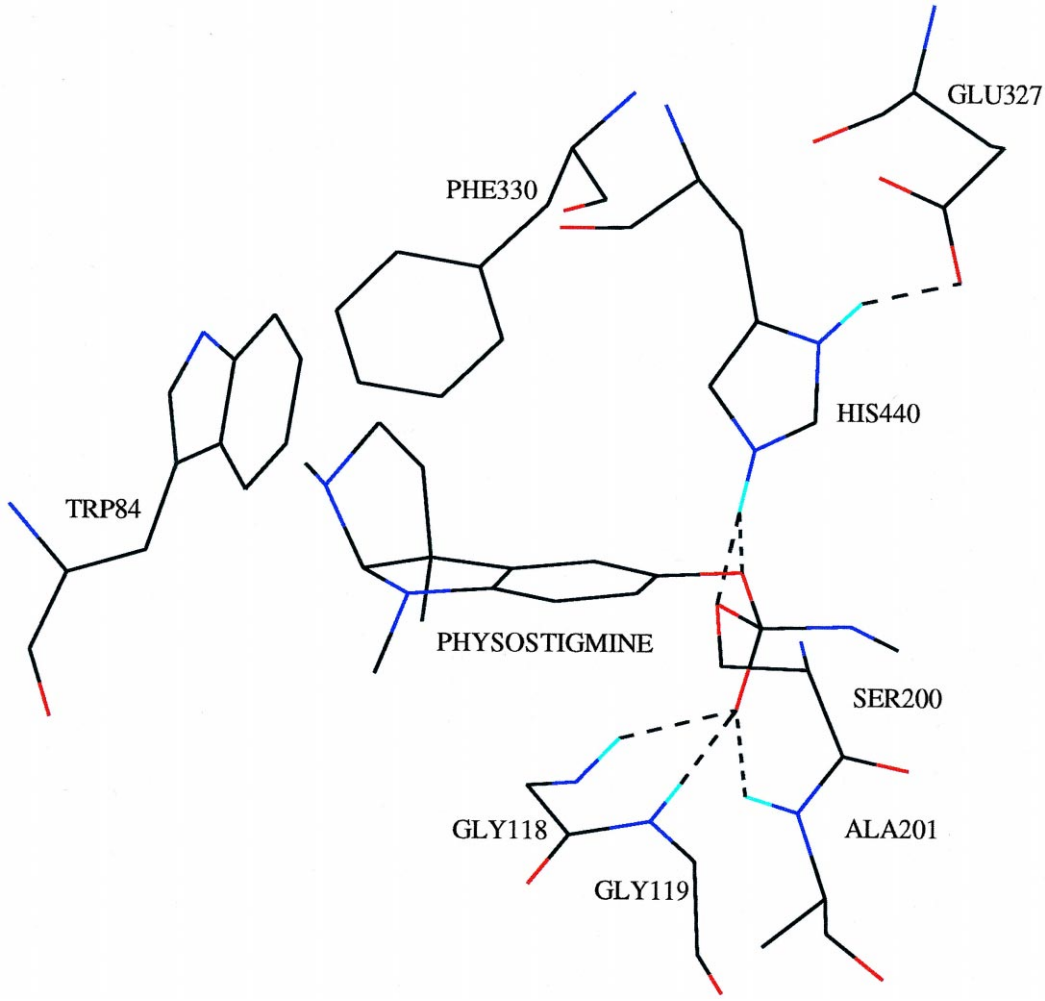

Fig. 4. Closeup of the physostigmine–*Tc*AChE complex showing its covalent bonding with SER200 in the active site. The distance SER200 Oγ-HIS440 $N^{\varepsilon 2}$ is 2.7 Å; HIS440 $N^{\varepsilon 2}$-O ester of inhibitor is 2.5 Å; $N^{\delta 1}$ HIS440-$O^{\varepsilon 1}$ GLU327 is 2.5Å. The carbamoyl group was positioned to make a tetrahedral bond with the O^{γ} of SER200. RMS 0.25.

fer, as ligand alignment for the template, the recently reported X-ray crystallographic data of the complex TMTFA–AChE as, principally, this inhibitor has a binding effect on the enzyme in a manner that resembles the tetrahedral intermediate in the carbamylation (Fig. 4) transition stage of catalysis [18]. In fact, the addition of an oxygen nucleophile, the O^{γ} of SER200 of the catalytic triad of AChE, forms a covalent bond with the carbonyl carbon of the inhibitors; hence the complex resembles a tetrahedral geometry, mimicking the intermediate in the acylating step of catalysis. Secondly, TMTFA is a transition state analog and therefore a good model for visualizing the binding interactions within the active site.

In the absence of ammonium function the strong anti-AChE activity of these inhibitors can be explained by the formation, in the transition state, of an intermediate characterized by tetrahedral geometry, and, inter alia, by weak interactions with the residues TRP84. In particular the last interaction is related to the inhibitory activity of TMTFA, **1**, **2**, **3** and **4**; as opposed to the present setting of the TMTFA–AChE complex, in its original position the TRP84 shift correlates to the decrement of the bimolecular rate constant k_i (Fig. 3 Fig. 4). Ideally, it should be possible to obtain the value of log k_i for the norphysostigmine, **5**, which resulted in being ≈ 5.9 for a relative distance (Fig. 4) of 0.68 Å.

The equilibrium dissociation constant, K_d, are influenced by the configuration of carbon atoms 3 and 8. In fact, the inhibitors **1**, **2** and **3**—which are configurationally 3S, 8S—show very similar values of their K_d (3.3, 5.2 and 10.4 μM, respectively); on the other hand, the unnatural physostigmine (configurationally 3R, 8R) shows a lower value of K_d (0.32 μM); from a thermodynamic point of view this last K_d value shows that the configuration 3R, 8R is more suitable to establish a stable Michaelis complex.

From a kinetic point of view inhibitors **1**, **2**, **3** and **4**, present similar values in the rate constants, k_2. Therefore the replacement of the N^1-amine function does not contribute to the specificity of carbamylation.

In conclusion, the present study focuses the attention on the electronic effects resulting during the catalytic mechanism of AChE. Due to the molecular rigidity charged, as **1** or **4**, and the uncharged inhibitors, **2** and **3**, appear almost isosteric. For this reason rotation of the tetrahydrofuranic ring, in the transition state complex of physovenine (Fig. 2), should be determined by the repulsion effects of the hard, high density, lone pairs of the O^1 and the TRP84 π electrons. This effect is not present in the case of **3**, probably because the two lone pairs of the sulfur atom are softer and more adaptable. Also other aromatic residues of the enzymatic gorge [3] could provide a polarizable surface for short-range dispersion interactions.

With reference to the modeling transition state it is interesting to note that the complex between enzyme and physovenine, **2**, presents a conformational change. No such substantial change is present in the case of complexes **1**, **3** and **4**.

Since what happens in the modeling study cannot be caused by steric effects, all the inhibitors present very similar configuration, the conformational change for **2** ($\alpha \approx 50°$) being justified by the electronic effects alone.

At the same time cross-talk between PHE330 and these inhibitors can result in a reorientation of this amino acid (Fig. 2) the effect of which does not seem predictable on the basis of the above kinetic results. At any rate, as we have seen above, the conformational flexibility of the aromatic residues in the ACHE gorge contributes to the activity of the enzyme.

In addition, owing to the polarization of these aromatics, as reported for the case of TRP84 π electrons, it can also be hypothesized that these amino acids interact with the same strength with charged (as in the case of ammonium function) and uncharged isosteric ligands.

Finally, the tetrahedral adduct, that forms between the carbonyl of the inhibitors and SER200 of the catalytic triad, could play a decisive role in determining the previously observed conformational changes. However, in addition to this effect on the tetrahedral adduct there is a specific attraction between the hydrophobic gorge of AChE and these alkaloids. The effect of the tetrahedral adduct reduces motion in the binding site with an ensuing increase in binding affinities; this consequence could be regarded as equivalent to lowering the temperature in the binding site.

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