

Evaluation of acetylcholinesterase and butyrylcholinesterase inhibitory activity of Huperzine-A; *in silico* and *in vitro* studies

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The present study is focused on exploring the Acetylcholinesterase and Butyrylcholinesterase inhibitory activity of Huperzine-A *in silico* and *in vitro*. In this study, Huperzine-A-A was docked with Acetylcholinesterase and Butyrylcholinesterase. Docking studies revealed the excellent interaction of Huperzine-A-A with these targets. The result of present study provides insight for the *in vitro* studies. The *in vitro* studies the enzyme kinetics of Huperzine-A-A via Lineweaver brooks plot revealed the kinetics and non-competitive inhibitory nature of the later. Further studies on Huperzine-A-A are necessary to develop and establish its role on brain cholinergic system and cognitive deficits which may serve a stepping stone in CNS medication.

Keywords: Acetylcholinesterase, Butyrylcholinesterase, Cognition, Huperzine-A

Acetylcholinesterase and Butyrylcholinesterase have been studied for decades. Early work showed that cholinesterase has a critical role in nerve impulse transmission¹. They are the chemical mediator and have an important role in entorhinal cortex and hippocampus function. It has been also claimed that Butyrylcholinesterase (BuChE) has the ability to substitute acetylcholinesterase (AChE) in the hydrolysis of ACh². The Involvement of AChE and BuChE is reported in cognitive functions and brain cholinergic³. Acetylcholine and Butyrylcholine is known as the learning neurotransmitter and is involved in muscle contraction as well. Cholinesterase inhibitors increase the amount of ACh and Buch at the neuronal synaptic cleft by inhibiting the enzyme responsible for the hydrolysis of ACh and Buch consequently improve neuronal transmission. Its potency of cholinesterase inhibition whether acetyl or butyl is similar or superior to that of physostigmine, galanthamine, donepezil and tacrine⁴. Huperzine regarded as a Nootropic agent is a plant-based alkaloid. It is a compound extracted from the herbs of the *Huperziceae* family. Huperzine-A has been used for centuries to treat fever, inflammation, blood disorders, and schizophrenia⁵. Huperzine-A acts as

a potent, highly specific and reversible inhibitor of acetylcholinesterase that crosses the blood–brain barrier, which means that it stops an enzyme from breaking down acetylcholine which results in increases in acetylcholine. Huperzine-A-A appears to be a relatively safe compound from animal studies of toxicity and studies in humans showing no side-effects at dosages routinely supplemented with. Huperzine-A is in preliminary trials for usage in fighting Alzheimer's disease as well^{6,7}. The Present study aims to study the Acetylcholinesterase and Butyrylcholinesterase inhibitory activity of Huperzine-A-A *via in silico* and *in vitro* studies.

Materials and Methods

Chemicals

Acetylcholinesterase, Butyrylcholinesterase, Galant amine, DTNB, and Huperzine-A were procured from Sigma Aldrich Company (India) and other analytical grade laboratory reagents for procured from SRL Chemicals (India).

In vitro enzyme inhibition studies

The activity of cholinergic markers AChE and BuChE were estimated spectrophotometrically using the Elisa reader according to the method of Ellman⁸. The assay is a spectrophotometric method that involves two linked reactions to produce a coloured compound. Briefly, 10 mM DTNB in 0.1 M Tris–HCl buffer (pH 8.0), 100 μ L of the supernatant, and 30 mM

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acetylthiocholine iodide as substrate were added for AChE estimation. For BuChE estimation, 10 mM DTNB in 0.1 M Tris-HCl buffer (pH 8.0), 300 μ L of supernatant, and 7.5 mM butyrylthiocholine iodide as substrate were added. Absorbance was measured at 412 nm for 3 min at 30 sec intervals using a spectrophotometer. AChE activity is expressed as micromoles of acetylthiocholine iodide (ATCh) hydrolyzed per milligram of protein per min, and BuChE activity is expressed as micromoles of butyrylthiocholine iodide hydrolyzed per milligram of protein per min. One unit is defined as 1 mole of acetylthiocholine hydrolyzed per min per milligram of protein⁹.

Enzyme inhibition kinetics

The kinetic parameters were calculated by using Lineweaver-Burk plots $1/V_0 = (K_m/V_{max}) \cdot (1/[S]) + 1/V_{max}$, where V_0 and V_{max} are the initial and maximal reaction velocities, $[S]$ is the concentration of substrate, and K_m is the Michaelis-Menten constant¹⁰. The K_m for the immobilized AChE and BuChE was determined by injecting 1 L substrate ATCh in aqueous solution at the concentration range of 0.005-5 mM. In order to determine the inhibitory potency (IC₅₀), 0.1 mM of ATCh with increasing concentration of inhibitor (0.2-500 M) was injected into both the AChE and BuChE, respectively. All samples were analyzed five times. Origin Pro 8.5 software was used to calculate K_m according to Lineweaver-Burk plots and fit the inhibition curve¹¹.

In silico docking studies

Software

Python 2.7 language was downloaded from www.python.com¹², Molecular graphics laboratory (MGL) tools and Autodock 4.2¹³ was downloaded from www.scripps.edu, Discovery Studio visualizer 4.1¹⁴ was downloaded from www.accelerys.com. Calculations were performed on Windows 8.0 Operating System.

Protein preparation

The three-dimensional crystalline structures of 4 targeted proteins (Table 1) were retrieved from the Protein Data Bank (<http://www.rcsb.org/>). The retrieved protein was PDB ID: 4EY7 for Acetylcholinesterase and PDB ID: 1ITB for Butyrylcholinesterase. The coordinates

of the structures were complexed with water molecules, and other atoms which are responsible for increased resolution and therefore the water molecules and het-atoms were removed using discovery studios and saved in .pdb format.

Docking analysis

Docking studies were performed to analyze interactions of Huperzine with Acetylcholinesterase and Butyrylcholinesterase targets¹⁵. The three-dimensional crystalline structures of 4 proteins were obtained from the Protein Data Bank (<http://www.rcsb.org/>). These protein were PDB ID: 4EY7 for Acetylcholinesterase and PDB ID: 1ITB for Butyrylcholinesterase. The structurally refined protein .pdb files were converted to .pdbqt files using grid module of autodock tools 1.5.6. Charges were assigned to the ions to the proteins manually wherever necessary. The 2D and 3D chemical structures of Huperzine (Molecular formula: C₁₅H₁₈N₂O; Molecular weight 242.32 g/M) was retrieved (<http://pubchem.ncbi.nlm.nih.gov/>). These .sdf and .mol files obtained from PubChem were converted into .pdb files using Marvin Sketch (<http://www.chemaxon.com/marvin/ketch/index.jsp>). These .pdb files were converted to .pdbqt using ligand preparation module of autodock tools 1.5.6. The docking analysis of Huperzine was carried out using the Autodock tools (ADT) v 1.5.4 and autodock v 4.2 programs. Huperzine was docked to all the target protein complexes with the molecule considered as a rigid body. The search was carried out with the Lamarckian Genetic Algorithm; populations of 100 individuals with a mutation rate of 0.02 have been evolved for ten generations. The remaining parameters were set as default. The Docked structure was then visualized using Discovery Studio 2016 for obtaining the binding interactions¹⁶.

Statistical analysis

The results are expressed as mean \pm standard error of the mean. Experiments were always performed in triplicates. Statistical comparison was performed using analysis of variance (ANOVA) followed by Bonferroni's test (* $P < 0.001$)

Results

The attachment of Covalent bond to the enzyme surface may affect its ultimate activity¹⁷. In order to verify the catalytic activity of the AChE and BuChE kinetic *i.e.* K_m , V_{max} and IC₅₀ were determined. The K_m and V_{max} values of AChE and BuChE were determined by varying the substrate ATCh

Table 1 — Molecular docking studies of Huperzine-A with acetylcholinesterase and Butyrylcholinesterase

Target	Resolution	PDB ID	Binding energy	Inhibitory constant (μ M)
Acetylcholinesterase	2.35Å	4EY7	-5.01	181.21
Butyrylcholinesterase	2.50Å	1ITB	-8.02	1.33

concentration under the described experimental conditions. The Lineweaver-Burk plots of the double reciprocal plots of $1/[\text{velocity}]$ and $1/[\text{substrate}]$ and V_{max} were found. The K_m value, which reflects the enzymatic affinities, was determined (Figs. 1 & 2). This K_m value found was promising and was in the defined range^{18,19}, showing the functional activity of the optimized AChE and BuChE. Galantamine was selected as test AChE and BuChE inhibitor for assessing the performance of the Huperzine-A for AChE and BuChE for determination of ligand affinity. Inhibition of enzymatic activity by galantamine was measured in the concentration range 0.2-500 M. As shown in the resulting dose-response curve (Fig. 1), the enzymatic activity decreased with increasing concentrations of the inhibitor galantamine, demonstrating that AChE and BuChE activity was indeed efficiently inhibited by Huperzine-A when compared to galantamine. The IC_{50} value was determined were found to be in relevance with reported

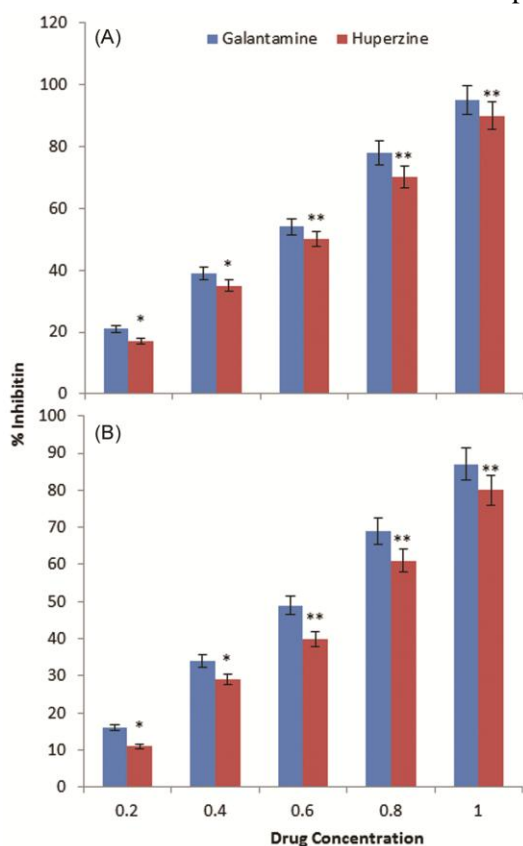


Fig. 1 — (A) Inhibitory activity of Galantamine and Huperzine-A against Ache; (B) Inhibitory activity of Galantamine and Huperzine-A against BuChE. The results are expressed as mean \pm standard error of the mean. Statistical comparison was performed using analysis of variance (ANOVA) followed by Bonferroni's test (* $P < 0.001$)

data²⁰, indicating that Huperzine-A has similar correlated enzymatic properties for inhibition of AChE and BuChE in comparison to Galantamine.

Enzyme kinetics is the systematic study of chemical processes which are catalyzed by enzymes in or out the biological system. The rate of reaction is determined and the effect of concentration on enzyme kinetics is measured. Lineweaver-Burk plot is a graphical representation of enzyme kinetics. This method helps to reveal the competitive or non competitive inhibition of enzymes by the inhibitors. The Lineweaver-Burk plot obtained for kinetic studies showed that Huperzine-A is a competitive inhibitor of both the enzymes *i.e.* ACHE and BUCHE on the comparative study with Galantamine, an established inhibitor of AChE and not BuChE²¹. The exact mechanism of the inhibition and the detailed comparison of AChE and BuChE, however, were not done. Though inhibition of BuChE can be found but the inhibition constant is too high to be reached in the body when compared to Galantamine. AChE activity can be affected more easily. It is noteworthy that Huperzine-A is not a potent inhibitor of AChE. Huperzine-A, however, a reversible inhibitor of AChE which shows binding with aromatic residues in the active site of AChE. The formation of the AChE-Huperzine-A complex is rapid while the dissociation is slow. The (Figs. 1 & 2) obtained showed that ACHE showed lesser inhibition as compared to BUCHE both in comparison to Galantamine. Initially the enzyme kinetic showed the slow enzyme and

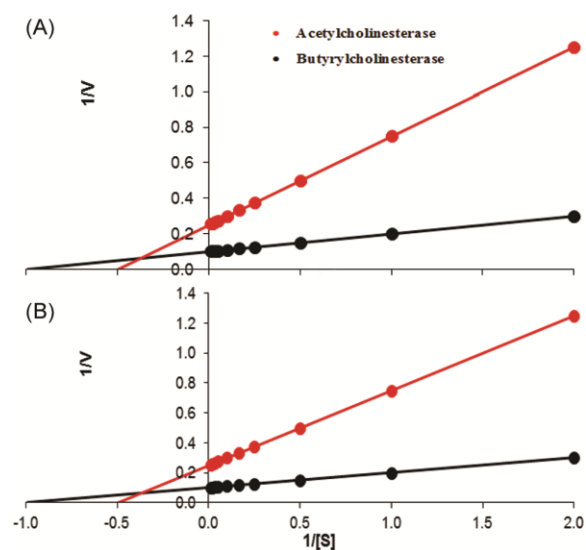


Fig. 2 — Lineweaver-Burk reciprocal plot for Acetylcholinesterase and Butyrylcholinesterase inhibition of (A) Galantamine; (B) Huperzine-A

substrate reaction and dissociation in ACHE while rapid enzyme and substrate reaction while the slow rate of dissociation. The results obtained in enzymatic studies showed that there is a higher perspective for *in vivo* studies and positive result. The rate of enzyme kinetics gave a concrete base for further studies which needs to be explored further. The enzymatic study of Huperzine-A over these enzymes established one fact that Huperzine-A is a competitive inhibitor of both ACHE as well as BUCHE in comparison to Galantamine although some differences in the enzyme-substrate reaction and dissociation.

Automated docking technique was utilized to determine the ‘orientation’ of Huperzine-A with Acetylcholinesterase and Butyrylcholinesterase. (Fig. 2) indicate the interaction of Huperzine-A with the active pocket of enzymes. Huperzine-A demonstrated minimum binding energy with enzyme *via* non-covalent interaction (Table 1). These *in silico* observation corroborate the *in vitro* non competitive and competitive enzyme inhibitory activity of Huperzine-A. The binding of Huperzine-A (Fig. 3) involves direct hydrogen bonds between OH and N2 of the ligand with 1ITB and 4EY7 respectively,

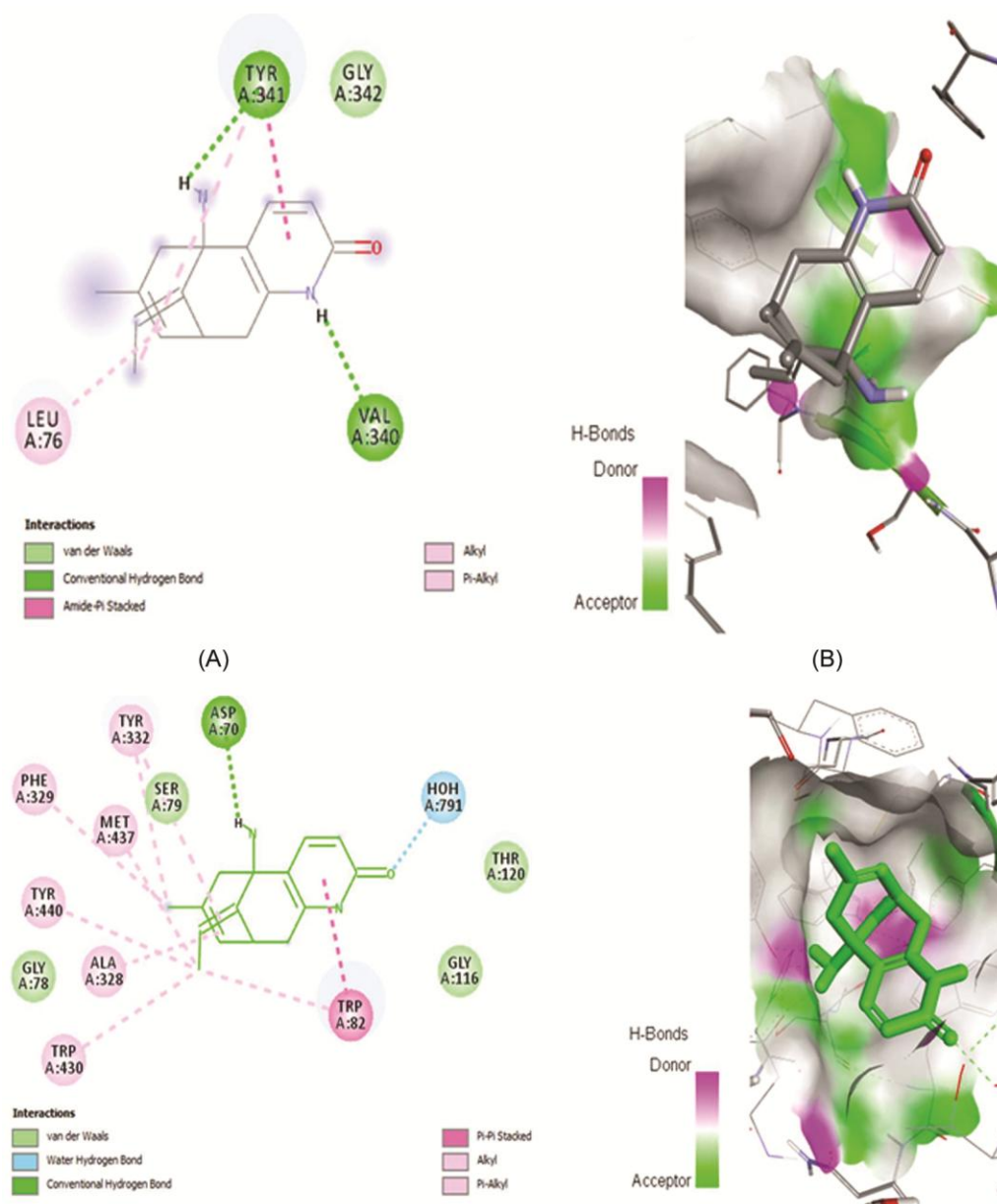


Fig. 3 — Molecular docking studies of Huperzine-A against (A) Acetylcholinesterase; (B) Butyrylcholinesterase [(A) 2D-interactions & (B) 3D-interactions]

(Fig. 2). The interaction with IITB (Fig. 3) is critical for the inhibition of the human enzyme. The crystal structure of the complex of AChE and BUCHE with optically pure Huperzine-A at 2.35 Å and 2.50 Å resolutions respectively, shows an unexpected orientation for the inhibitor with surprisingly few strong direct interactions with protein residues to explain its high affinity. This structure is compared to the native structure of AChE devoid of any inhibitor as determined to the same resolution. An analysis of the affinities of structural analogues of Huperzine-A, correlated with their interactions with the protein, shows the importance of individual hydrophobic interactions between Huperzine-A and aromatic residues in the active-site of AChE and BUCHE. The structural analysis revealed that Huperzine-A bears no resemblance to ACHE & BUCHE and that the Huperzine-A–AChE complex and Huperzine-A–BuChE complex binds to the active-site gorge of AChE with few direct contacts with the protein. Only one strong hydrogen bond is seen, as well as some hydrophobic interactions within the crystalline complex²¹. The 3D computer image of AChE–Huperzine-A, Huperzine-A–BuChE binding generated in the Raves study revealed how the Huperzine-A blocks the enzyme by sliding smoothly into the active site of AChE and BuChE where acetylcholine (ACh) is broken down, and latches onto this site *via* a large number of subtle chemical links. Further, this complex has been studied utilizing kinetic studies which revealed its competitive enzymatic inhibitory effect. The result obtained in kinetic studies *via* Ellman methods were very promising. It can be concluded that Huperzine-A shows competitive inhibition over both enzymes. The enzymatic study performed needs to be further explored with more parameters.

Discussion

With due course of increasing and developing drugs in brain cholinergic system and cognition impairment, cholinesterase inhibitor drugs have followed the finding that cholinergic pathways in the cerebral cortex and basal forebrain are played important role in cognitive deficit²². Further alteration in the cholinergic system may contribute to the enhancement of brain activity²³. Galantamine tends to be established nootropic agent which is given in many cerebral related disorders like Alzheimer's disease, Parkinson's *etc.* Drugs with single target functional components of cognition and memory always have limited success²⁴.

However, recent results for multifunctional drugs in treating neurodegenerative disorders have been encouraging and might provide greater symptomatic efficacy than a strategy of administering multiple single drugs with potentially different degrees of bioavailability, pharmacokinetics and metabolism^{25,26}. Huperzine-A is isolated from a Chinese herb, which has neuroprotective effects that go beyond the inhibition of AChE and BUCHE. Here, we have outlined the *in silico* and *in vitro* study of Huperzine-A, and demonstrated that it can target several molecular sites. The multiple effects of Huperzine-A on various sites of ACHE and BUCHE might stimulate endogenous protective processes or promote repair of damaged structures. Because most neurodegenerative diseases are characterized by progressive neuronal loss associated with the development of symptoms, the neuroprotective effects of Huperzine-A might benefit conditions such as Parkinson's disease, amyotrophic lateral sclerosis or Huntington's disease, by slowing down the neurodegenerative processes. In the *in silico* studies, it was apparent that Huperzine-A–AChE complex and Huperzine-A–BuChE complex impressively binds to the active-site showing possible structural alteration. The results obtained in docking studies showed that Huperzine-A could be non-competitive and competitive enzyme inhibitor which confirms the effect reported for the other biological molecules in numerous studies²⁷⁻³¹. Owing to the results docking the enzyme inhibitory activity was further validated by *in vitro* studies. The inhibition of enzymes further validated the result. The Lineweaver-Burk plot showed that Huperzine-A is a competitive inhibitor of ACHE and BUCHE as compared to Galantamine though there were some differences seen in the enzyme-substrate reaction and dissociation. This study concluded that the structural affinity of huperzine makes it competitive inhibitor of ACHE and BUCHE. However, further clinical study is needed before we can make this conclusion.

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