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In-vitro Evaluation and Molecular Docking Studies of Some Schiff Bases as Cholinesterase Inhibitor

Saurabh Kumar Sinha*

Department of Pharmaceutical Sciences, Mohanlal Sukhadia University, Udaipur-313003, Rajasthan, India

ABSTRACT

Some new Schiff bases of 4-aminopyridine were synthesized and evaluated for antiamnesic and cognition enhancing activity. In the current study to further understand the mechanism of action of these derivatives we have evaluated *in-vitro* acetycholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity. Enzyme kinetics and docking studies were performed for all compounds to observe their nature of inhibition. The IC_{50} value of synthesized compounds showed maximum activity of compound 4APg compared to standard drug donepezil and rivastigmine whereas its kinetic analysis of enzyme inhibition demonstrated non-competitive inhibition for both enzymes AChE and BChE. The docking study confirmed their consensual interaction with AChE and BChE active sites justifying the experimental outcome.

Keywords: 4-Aminopyridine, Antiacetycholinesterase**,** Antibutyrylcholinesterase, Enzyme kinetics.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, characterised by progressive loss of cholinergic neurons and accumulation of β-amyloid protein in the brain areas. [1] Onset starts with cognitive and short term memory destruction that slowly progresses to complete loss of cognitive function, impaired performance of activities of daily life. [2] The drug discovery concerning to AD is based either to develop an AChE inhibitor that inhibits the breakdown of acetylcholine (ACh) or improve the memory and cognition impairment [3] or to develop a β-secretase or γ-secretase inhibitor that blocks the formation of β-amyloid plaques. [4-5] It is evident that design and development of secretase inhibitors have not been achieved their true goal to treat the AD and needs to be explored as per the structure of protein. The U.S. Food and Drug Administration (FDA) have approved five medicines for the treatment of AD till date, out of which four are AChE inhibitors. It is reported that AChE could also play a vital role in accelerating senile β-amyloid peptide (Aβ) plaque deposition which is toxic to neurons. [6] BChE has also been reported to degrade ACh in healthy and AD affected brains and the role of BChE rises in the late phase of AD [7] thereby qualifying it as an additional target for the treatment. [8] From various molecular docking and dynamic studies on AChE inhibitors suggest that modulation of AChE catalytic activity is possible through binding of ligands at the peripheral anionic site (PAS) constituted by amino acid residues Tyr-72, Tyr-124, Trp-286, and Tyr-341. [9-10] Several carbamate derivatives of 4AP and Schiff bases of styrylpyridine have been synthesized and evaluated for their anticholinesterase activity. [11-12] Some 4-aminobutyric acid (GABA) and 2-indolinone derivatives of 4AP have also been reported to possess antiamnesic activity. [13] The hydrazone derivatives of

***Corresponding author: Dr. Saurabh Kumar Sinha,** Department of Pharmaceutical Sciences, Mohanlal Sukhadia University, Udaipur-313003, Rajasthan, India; **Tel.:** +91-8769338593; **E-mail:** sinsaur@gmail.com
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dihydropyridine and indolinones have been reported as potent AChE, BChE and β-amyloid aggregation inhibitory activities. [14-15] 3-methylpyridinium and 2-thionaphthol derivatives of berberine have evaluated for AChE and BChE inhibitory activity respectively. [16]

In our previous study (Sinha and Shrivastava, 2012), we had reported some new Schiff bases of 4AP for their cognition enhancing and antiamnesic activities amongst which, the pharmacological outcome of benzophenone derivative was found to be highly significant. In the present study, all the synthesized Schiff bases of the series were evaluated *in-vitro* for antiacetycholinesterase and antibutyrylcholinesterase activities followed by enzyme kinetics and docking studies to corroborate their mechanism of inhibition.

MATERIALS AND METHODS

General

All reagents and solvents used in the study were of analytical grade purity and were procured from Sigma-Aldrich (India). Donepezil standard drug was obtained as a gift sample from Cipla Ltd (Maharashtra, India).

Estimation of cholinesterase activity (*in-vitro*)

The ability of all tested compounds to inhibit acetylcholinesterase from electric eel (E.C. 3.1.1.7) and butyrylcholinesterase from human serum (E.C. 3.1.1.8) was tested and their effectiveness in inhibition could be conclusive through their IC_{50} values. The IC_{50} values were determined by the Ellman's spectrophotometric method [17] which is performed by recording the rate of increase in the absorbance at 412 nm for 5 min. The stock solution of AChE was prepared by dissolving AChE in 0.1 M phosphate buffer (pH 8.0). In case of BChE stock solution was prepared by dissolving the lyophilized powder in an aqueous solution of gelatine 0.1%. The composition of final solution for assay consisted of 0.1 M phosphate buffer pH 8.0, with the addition of 340 mM 5, 5'-dithio-bis (2-nitrobenzoic acid), 0.02 unit/mL of AChE, or BChE and 550 mM of substrate (acetylthiocholine iodide, ATCh or butyrylthiocholine iodide, BTCh, respectively). Five different concentrations of inhibitors between 20% and 80% (test compounds) were selected in order to obtain inhibition of the enzymatic activity. From the aliquots (50µL), increasing concentration of the inhibitors were added to the assay solution and were preincubated for 20 min at 37°C with the enzyme followed by the addition of substrate. Blank used in the assays consisted of all the components except AChE or BChE in order to account for the non-enzymatic reaction. Reaction rates of the assay were then compared and percent inhibition due to the presence of increasing concentrations of inhibitor was calculated. The concentration of each test compound was analyzed in triplicate, and IC_{50} values were determined graphically from log concentration percent inhibition curves. [18-19]

Enzyme kinetics study

Ellman's method of spectrophotometric analysis was used to determine the type of inhibition. Acetylcholine iodide and butyrylthiocholine iodide was used as a substrate at various concentrations both below and above but near Km in a phosphate buffer at pH 8 keeping a fixed amount of cholinesterase in the absence or presence of different inhibitors. The concentration of the inhibitor was kept close to one which corresponds to 50% inhibition of the enzyme activity (IC_{50}) and their inhibitory kinetics was evaluated by the Lineweaver and Burk method. [20]

Molecular docking studies

Preparation of the small molecules

All the molecules with their inhibition activities were taken and 3D structures were sketched using Maestro 9.3 and geometrically minimized with Macromodel 9.9 based on OPLS-2005 force field.

Preparation of the protein

The crystal structure of AChE and BChE with high resolution was retrieved from the protein data bank (pdb code: 1B41 and 1POI respectively). [18] The structure was prepared in the following procedures by protein preparation wizard in Maestro 9.3, including adding hydrogens, assigning partial charges using the OPLS-2005 force field and assigning protonation states, restrained, partial energy minimization and the resulting structure was used as the receptor model.

Docking

For the receptor structure, crystallographic and trajectory water molecules, ions and ligand compounds were removed. Proteins were prepared using Schrodinger software, Maestro 9.3 and Glide 5.8. The Glide XP algorithm was employed using a grid box volume of 10_10_10 Å. All the structures were fitted in binding pocket and the lowest energy pose for each docking run was retained. [18-19]

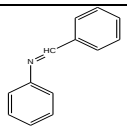
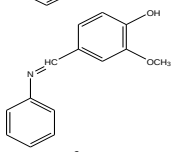
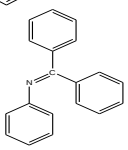
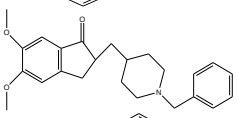
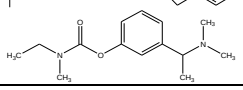
RESULTS AND DISCUSSION

The IC_{50} values of all the derivatives on AChE and BChE were determined through Ellman method and observed that compound 4APg (0.54 ± 0.88µM) was more active than standard rivastigmine (2.25µM) and less active than donepezil (0.04 ± 0.012µM) with respect to their AChE inhibitory activity. In the evaluation of BChE inhibition, compound 4APg (2.16 ± 0.64) illustrated comparable activity with rivastigmine (1.66µM) and superior activity than donepezil (15.24 ± 0.88) (Table 1).

It is worth to mention that a healthy brain contains less concentration of BChE than AChE but in AD affected brain, AChE activity decreases and BChE activity increases gradually. [21] Therefore, as AD progresses ACh regulation may become more dependent on BChE thereby paving the way for dual inhibitors to provide more persistent efficacy in early to late stage of AD than AChE selective agents. [22] The results of IC_{50} values and structure activity relationship (SAR) inferred that the activity increases in order to the number of hydroxyl and methoxy group increases on phenyl ring but more selectively the hydroxyl substituted compounds elicited better activity than

methoxy substituted compounds. The substitution of the second hydrogen from imine carbon with methyl group increases in AChE inhibitory activity but the substitution by phenyl ring results a drastic increase in both AChE as well as BChE inhibitory activity. Further, enzyme kinetics study was also performed for all derivatives to gain an insight on their nature of inhibition (Table 2).

Table 1: Anticholinesterase activity of Schiff bases of 4AP, donepezil and rivastigmine

Compound Code	Compound	AChE IC_{50} (µM)	BChE IC_{50} (µM)	Selectivity for AChE[a]
4APa		362.3 ± 4.47	>1000	-
4APf		58.56 ± 1.48	>1000	-
4APg		0.54 ± 0.88	2.16 ± 0.64	4
Donepezil		0.04 ± 0.012	15.24 ± 0.88	381
Rivastigmine*		2.25	1.66	0.74

*Data taken from Sheng et al., 2009

Table 2: Enzyme kinetics study of Schiff bases of 4AP and Donepezil.

Compound	Inhibition	AChE Ki (µM) ± SEM	Inhibition	BChE Ki (µM) ± SEM
4APa	nc	348.52 ± 4.47	nc	nt
4APf	c	52.48 ± 1.48	nc	nt
4APg	nc	0.42 ± 0.68	nc	1.85 ± 0.64
Donepezil	nc	0.056 ± 0.016	nc	12.54 ± 0.76

c= competitive, nc= non-competitive, nt= not tested

The most active compound 4APg demonstrated a non-competitive inhibition for both AChE (Ki = 0.42 ± 0.68) and BChE (Ki = 1.85 ± 0.64) enzymes. The non-competitive inhibition is attributed to a possible interaction of compound with the PAS of AChE and was also confirmed by docking studies. Since all the hydroxyl substituted compounds showed better activity than others, hence it can concluded that hydrophobicity is a necessary parameter to cross the blood brain barrier but hydrophilicity is also important for good activity.

Docking studies were carried out to provide a better interpretation of the biological profile of 4APg, donepezil and rivastigmine toward AChE and BChE and revealed that 4APg and donepezil were properly positioned into the enzyme gorge and showed interaction with the internal amino acid residue Tyr-341 and Trp-286 by means of a π-π interaction (Fig. 1). The biphenyl ring of 4APg was well placed in the hydrophobic pockets formed by Tyr-341, Trp-286, Val-294, Phe-297, Leu-289, Tyr-124, Tyr-337 and Phe-338 and exhibited hydrophobic interactions. The study clearly demonstrated that both compounds were able to bind with the key PAS residue Trp-286, Tyr-124 and Tyr-341. The pyridine nitrogen of 4APg was involved in forming a hydrogen bond with Asp-74. The methoxy group of donepezil was observed in establishing the H-bond with Phe-295 backbone. In case of other compounds 4APa-f and 4APh a reduced biological activity was observed which may be attributed to the lack of biphenyl ring.

The compound 4APg also exhibited good activity against BChE and hence to understand the differences in interactions, the compound 4APg and rivastigmine were also docked to the active site of BChE. In AChE, the acyl-binding pocket is formed by two phenylalanine molecules (Phe-295 and Phe-297), while in BChE, these two aromatic amino acid residues are replaced by two smaller amino acid residues, Leu-286 and Val-288. The key peripheral anionic site residues i.e. Trp-286, Tyr-124 and Tyr-341 of AChE were replaced by Tyr-332 and Ala-277. This structural difference causes a conformational change and thus provides a larger space in the deepest area of the BChE cleft. It was observed that, the compound 4APg and rivastigmine were properly positioned into the enzyme gorge Ser-287, Ser-72, Ala-277, Lue-274, Pro-285, Pro-84, Lue-286, Gln-119, Tyr-332, Phe-278 and Glu-276 (Fig. 2).

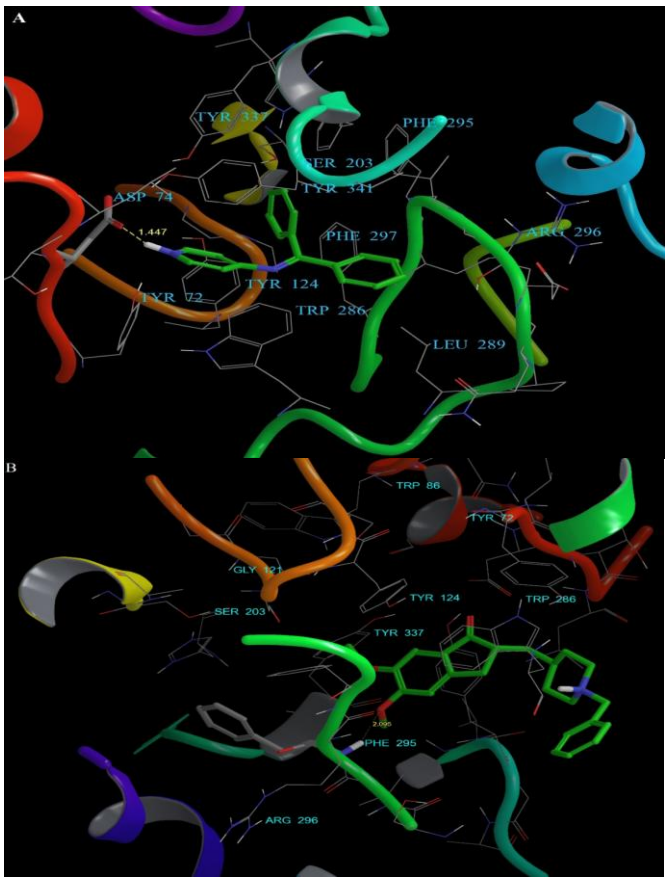


Fig. 1: The binding mode of [A] 4APg and [B] Donepezil in the AChE binding pocket. The hydrogen bonds are displayed as dashed yellow

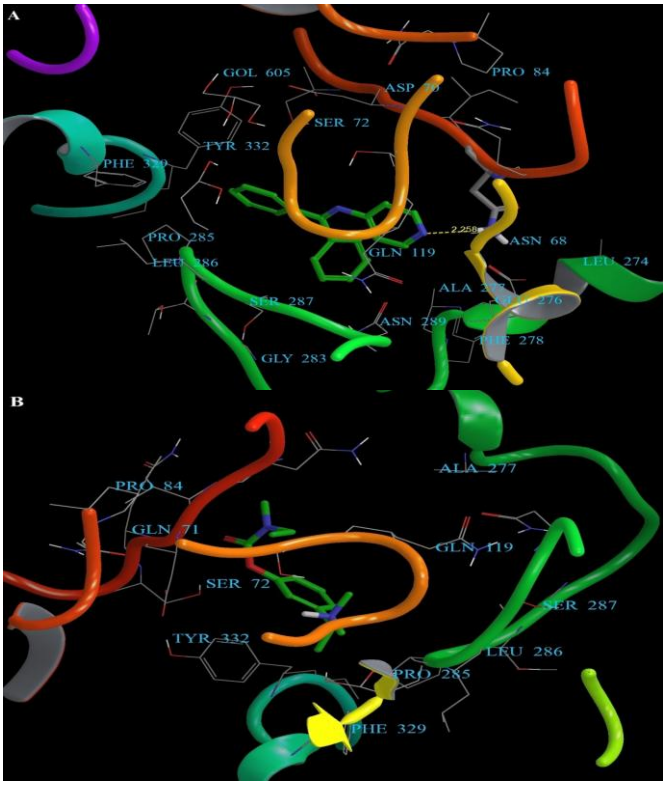


Fig. 2: The binding mode of [A] 4APg and [B] Rivastigmine in the BChE binding pocket. The hydrogen bonds are displayed as dashed yellow

4APg showed interaction with the internal amino acid residue Tyr-332 (analogous to Tyr-341 in AChE) and Ala-277 (analogous to Trp-286 in AChE) by means of a π-π staking. In addition, the pyridine nitrogen of 4APg was also involved in forming a hydrogen bond with carboxamide group of Asn-68. These results suggest that Schiff bases due to its anticholinesterase activity significantly enhance cholinergic neurotransmission and thus enhance learning and memory functions in agreement with the earliar *in-vivo* studies.

In conclusion we have identified a new class of potent anticholinesterase inhibitors among which benzophenone derivative 4APg deserves further study. Considering the role of BChE in the late phase of AD the dual inhibitor might also be beneficial for the treatment of AD and it can be a useful new lead to develop dual inhibitors with cognition enhancing and anti amnesic properties.

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