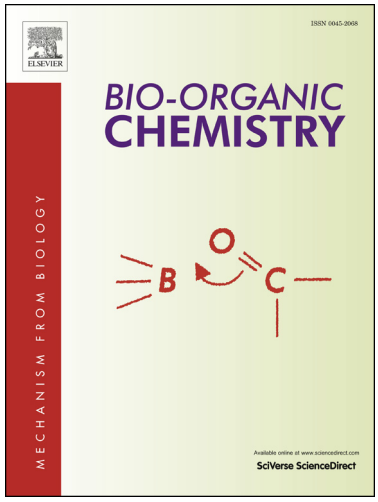

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Discovery of isoalloxazine derivatives as a new class of potential anti-Alzheimer agents and their synthesis

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ABSTRACT

This article describes discovery of a novel and new class of cholinesterase inhibitors as potential therapeutics for Alzheimer's disease. A series of novel isoalloxazine derivatives were synthesized and biologically evaluated for their potential inhibitory outcome for both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). These compounds exhibited high activity against both the enzymes AChE as well as BuChE. Of the synthesized compounds, the most potent isoalloxazine derivatives (**7m** and **7q**) showed IC_{50} values of 4.72 μM and 5.22 μM respectively against AChE; and, 6.98 μM and 5.29 μM respectively against BuChE. These two compounds were further evaluated for their anti-aggregatory activity for β-amyloid (Aβ) in presence and absence of AChE by performing Thioflavin-T (ThT) assay and Congo red (CR) binding assay. In order to evaluate cytotoxic profile of these two potential compounds, cell viability assay of SH-SY5Y human neuroblastoma cells was performed. Further, to understand the binding behavior of these two compounds with AChE and BuChE enzymes, docking studies have been reported.

Keywords: Alzheimer's disease; Isoalloxazine; Cholinesterase's inhibition; β-amyloid; Cyto-toxicity.

INTRODUCTION

Alzheimer's disease (AD) is an overwhelming neurodegenerative disorder characterized by a progressive and irreversible decline in cognitive functions. It typically develops leisurely, and gradually worsens as brain cells shrink and die. Eventually, Alzheimer's is fatal, and at present, there is no cure for it.[1] AD affects the cholinergic regions of the central nervous system (CNS) associated with cognitive functions and awareness.[2-4] Presently, majority of the therapeutic treatments for AD are aimed to inhibit acetylcholinesterase (AChE) to enhance acetylcholine (ACh) levels in brain.[5] AChE inhibitors like tacrine, donepezil, rivastigmine and NMDA receptor antagonists like memantine are currently available for AD treatment.[6] Literature supports the evidence of gradual fall in the levels of AChE in the brain of AD patients, while there occurs slight increase in the activity of butyrylcholinesterase[7,8] (BuChE). Further, post mortem tissue analysis of AD patients showed a high level of BuChE in the hallmark lesions of AD. In rats, selective BuChE inhibitor cymserine was found to elevate ACh levels and it enhanced long-term CNS potentiation and learning.[8] Therefore both these enzymes emerge as appropriate targets for the development of cholinesterase inhibitors in the treatment of AD.[9]

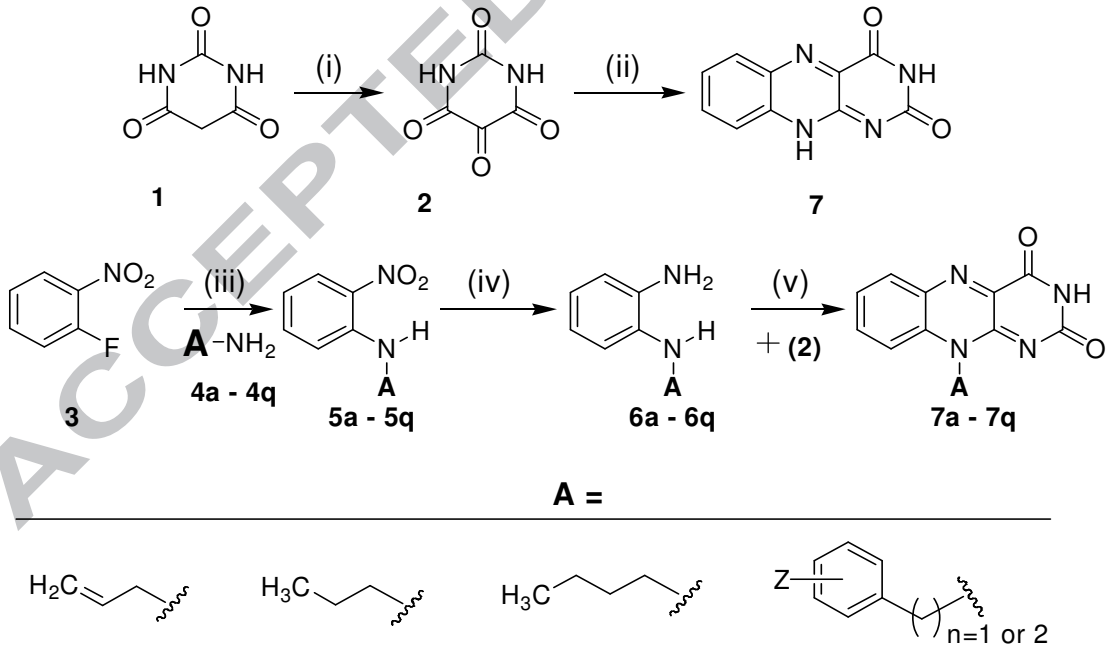
Although AChE and BuChE are produced by different genes they are highly homologous with more than 65% similarity. AChE has two major binding sub-sites, a peripheral anionic site (PAS) and the other a catalyatic anionic site (CAS) which is located in the deep gorge of the enzyme structure and is assigned to Ser-His-Glu catalytic triad. The gorge is lined by around 14 aromatic amino acids making the active site more hydrophobic, leading to better interaction with substrates. The gorge goes through half way in the enzyme and is roughly 20 Å long. Common to AChE, BuChE also has a catalytic triad consisting of Ser-His-Glu. Majority of the important features of the active site of BuChE like a triad of Ser-His-Glu, a p-cation-binding site, an oxyanion hole, and an acyl-binding pocket are similar to AChE. The acyl binding pocket of BuChE is obviously larger than that of AChE. The active sites of both the enzymes acting as nucleophiles are situated at the base of a cavity to attack the carbonyl group of the substrates.[10]

Along with tacrine, its different derivatives,[4] homo and heterodimers, and hybrids involving many heterocycles are reported to be beneficial in the AD.[11] Further, small molecules like dihydroindenones (donepezil),[12] guanidines,[13] coumarins[14-16] and their structural derivatives have also been reported for the treatment of AD. Considering the structural features of tacrine

and donepezil, along with the knowledge of the active site of the enzymes and considering parameters like lipophilicity and molecular weight as restrainers, isoalloxazine ring was derivatized and developed as anti-alzheimer's agents. Isoalloxazine is basically a pteridine based tricyclic heteronuclear moiety found mainly in flavins. It is the basic nucleus present in the structure of vitamin B2[17,18] and some of its derivatives are reported to be effective against protozoal infections particularly against human and animal trypanosomiasis and malaria.[19] Isoalloxazine derivatives are also reported for the treatment of coccidiosis in animals.[19] A piperazine spacered bis-isoalloxazine compound has been reported to suspend the association of prion proteins and Alzheimer-specific Aβ peptides.[20] Isoalloxazine {benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione} (**7**) was synthesized as the lead molecule. Compound (**7**) was observed to inhibit both AChE and BuChE with IC_{50} values of 64.45 μM and 55.57 μM respectively. To improve its activity against ChEs, orderly substitution on compound (**7**) at position 10 with different alkyl and substituted phenylalkyl groups were carried out. This was presumed to improve hydrophobic interactions with the active sites of both the enzymes.

RESULTS AND DISCUSSION

Chemistry

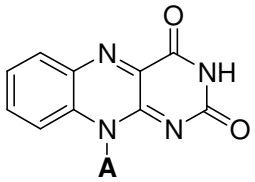


Scheme 1: Synthesis of isoalloxazine (7) and its derivatives (7a-7q).

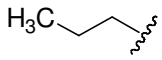
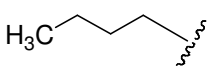
(i) CrO_3, AcOH; (ii) *o*-phenylenediamine, H_3BO_3, AcOH, RT, 8 hrs; (iii) K_2CO_3, DMF, 60°C, 4-6 hrs; (iv) Zn, AcOH, MeOH, RT, 6-8 hrs; (v) H_3BO_3, AcOH, RT, 8-10 hrs.

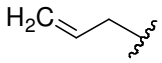
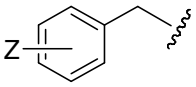
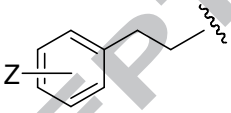
For the syntheses of isoalloxazine (**7**) and its derivatives (**7a-7q**), the followed synthetic route is outlined in **scheme 1**. Alloxan monohydrate (**2**) was prepared by oxidation of barbituric acid (**1**) according to the reported procedure.[21] The lead molecule (**7**) was prepared by the reported procedure,[22] by cyclizing compound (**2**) with *o*-phenylenediamine in presence of boric acid and acetic acid at room temperature. In order to obtain different *N*-alkylated products (**7a-7q**) at 10 position, 1-fluoro-2-nitrobenzene (**3**) was reacted with the required alkyl/arylalkyl amine (**4a – 4q**) in presence of K_2CO_3 as the base in DMF to obtain the desired *N*-alkyl/arylalkyl 2-nitrophenylamines (**5a – 5q**). Subsequently, the 2-nitro group of compounds (**5a – 5q**) was reduced in presence of Zn and AcOH in methanol as solvent at room temperature to obtain N1-substituted 1,2-diamine intermediates (**6a – 6q**). The obtained intermediates were used as such for the next step. To obtain the desired isoalloxazine derivatives (**7a – 7q**), the N1-substituted 1,2-diamine intermediates (**6a – 6q**) were reacted with **2** in presence of H_3BO_3 and AcOH at room temperature as per the procedure adopted for compound (**7**) to get the cyclized compounds (**7a – 7q**). Structures of all of the compounds were confirmed on the basis of spectral and elemental analyses. The yields of the synthesized derivatives (**7,7a-7q**) were in the range of 80-90% after recrystallization.

Table 1: ***In vitro*** **AChE and BuChE inhibitory activity of the synthesized compounds (7, 7a – 7q).**



(7 - 7q)

Compd	A	Z	IC_{50} (μM) values	
			AChE	BuChE
7	H	---	64.45	55.57
7a		---	9.32	40.38
7b		---	12.2	43.72

7c		**---**	11.22	27.24
7d		H	11.53	31.72
7e	-do-	3-CH_3	11.63	42.86
7f	-do-	4-CH_3	29.39	7.89
7g	-do-	2-F	7.28	21.64
7h	-do-	3-F	11.46	8.26
7i	-do-	3-Cl	47.26	10.49
7j	-do-	4-Cl	29.95	7.39
7k	-do-	2- OCH_3	18.82	8.39
7l	-do-	4- OCH_3	9.34	47.22
7m	-do-	**2,3-$(OCH_3)_2$**	**4.72**	**6.98**
7n	-do-	3,4-$(OCH_3)_2$	32.55	8.55
7o		H	52.75	23.41
7p	-do-	4-Cl	29.5	7.13
7q	-do-	**3,4-$(OCH_3)_2$**	**5.22**	**5.29**
Tacrine			0.056	0.0086
Donepezil			0.023	1.87

Biological Activity

Ellman's method[23] was employed to test the ability of the synthesized compounds to inhibit human AChE and equine serum BuChE using tacrine hydrochloride hydrate and donepezil hydrochloride as reference standards. The extent of inhibition was expressed as IC_{50} (μM) and was summarized in **table 1**. Taking **7** as the lead molecule, various *N*-alkyl or *N*-phenylalkyl substitutions were made in the tricyclic ring, which improved the activity against both AChE and BuChE enzymes. All the compounds in the series showed a good range of enzyme inhibition (AChE IC_{50} = 64.95 μM to 4.72 μM; BuChE IC_{50} = 55.57 μM to 5.29 μM). Simple allyl, propyl and butyl substituents were found to improve hydrophobic interactions with the enzymes and were comparatively more active on AChE than on BuChE. As compared to the lead, the benzyl and phenylethyl substituents offered improved bio-activity on both the enzymes. All the derivatives with benzyl and phenylethyl moieties showed moderate activity on both the enzymes while compounds (**7c**, **7e** and **7l**) (with allyl, 3-methylbenzyl and 4-methoxybenzyl substituents respectively) were observed to be more active on AChE than on BuChE. Whereas compounds, (**7f**, **7j**, **7n** and **7p**) (having 4-methylbenzyl, 4-chlorobenzyl, 3,4-dimethoxybenzyl and 4-chlorophenylethyl groups respectively) were found to be more active against BuChE as compared to AChE. Compounds (**7m** and **7q**) (with 2,3-dimethoxybenzyl and 3,4-dimethoxyphenylethyl groups respectively) showed the highest activity on both the enzymes [AChE (4.72 μM and 5.22 μM) and BuChE (6.98 μM and 5.29 μM) respectively]. These two compounds were choosen for further evaluation for their ability to prevent β-amyloid (Aβ) aggregation in presence and absence of *h*AChE by Thioflavin-T (ThT) and Congo red (CR) binding assays.

The two compounds (**7m** and **7q**) selected on the basis of screening results of cholinesterase inhibition assay were further assessed for their ability to prevent *h*AChE-induced $A\beta_{1\text{-}42}$ aggregation using thioflavin-T (ThT) fluorescence assay[24] in comparison to tacrine and donepezil as reference drugs. The results revealed that both the compounds (**7m** and **7q**) at a concentration of 10 μM showed significant inhibition of $A\beta_{1\text{-}42}$ aggregation (35 % and 20 % inhibition, respectively) as compared to the positive control (**Figure 1A**). This study demonstrated that these compounds (**7m** and **7q**) which were found earlier to be potent

cholinesterase inhibitors possessed moderate potential to inhibit *h*AChE induced $A\beta_{1-42}$ aggregation also. The standard drugs tacrine and donepezil caused 26.26% and 38.18% inhibition at the same concentrations under these conditions.

$A\beta_{1-42}$ aggregation was measured by Congo red binding assay. Congo red is a dye which has characteristics to bind with β-sheets of Aβ aggregates.[25] The selected compounds (**7m** and **7q**) were further evaluated using this assay along with tacrine and donepezil as reference drugs. The results demonstrated that the compounds (**7m** and **7q**) at 10 µM concentrations caused significant inhibition of $A\beta_{1-42}$ aggregation (38% and 24 % inhibition, respectively) as compared to the positive control (**Figure 1B**). This strengthened our assumption that these compounds (**7m** and **7q**) which caused significant inhibition of *h*AChE-induced $A\beta_{1-42}$ aggregation have the ability to inhibit spontaneous $A\beta_{1-42}$ aggregation in absence of *h*AChE as well. Inhibition in aggregation of $A\beta_{1-42}$ caused by the two standard drugs tacrine and donepezil was 31.82% and 42.45% under similar experimental conditions.

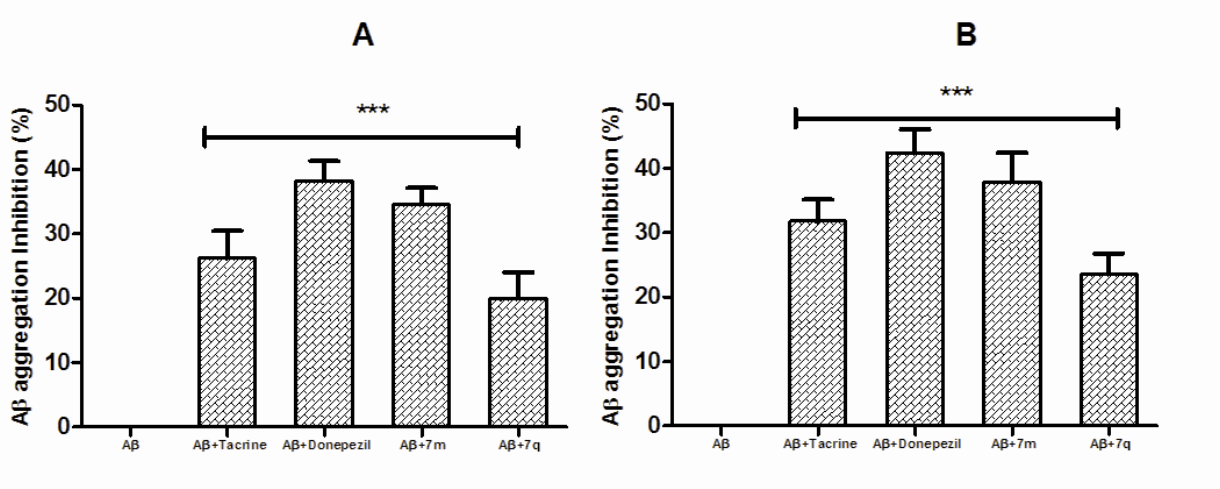


Figure-1: Percentage inhibition of $A\beta_{1-42}$ aggregation by the test and reference compounds at 10 µM concentrations with *h*AChE in (**A**) ThT assay and without *h*AChE in (**B**) CR binding assay. Data is analysed using GraphPad Prism version 5. Comparison among the groups was made by one way ANOVA followed with Bonferroni test. Data is expressed as mean±SEM. *** indicates P<0.001 vs control (Aβ).

After obtaining encouraging results from the above three studies it was planned to assess the biosafety of the two promising compounds (**7m** and **7q**), by performing the cytotoxicity studies of these compounds using MTT assay.[26] **Figure-2** represents the percentage viability of the cells treated with the test compounds compared to the control cells. In this assay, tacrine and donepezil were also used as reference compounds. At 40 µM concentration, none of the

compounds under study showed any significant toxicity. The results indicate that the compounds are relatively non-toxic and can be further evaluated using different *in vivo* animal models for Alzheimer's disease (AD) to prove their neuroprotective property.

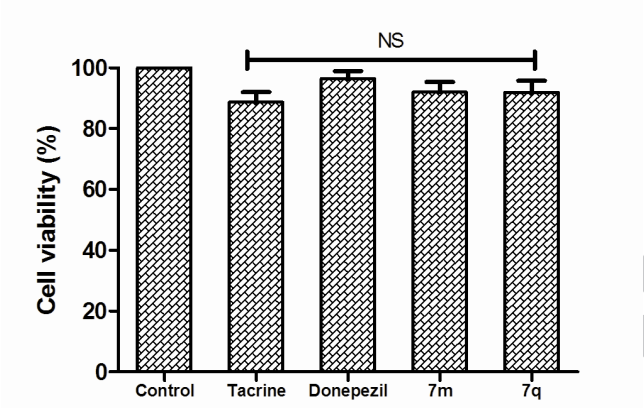


Figure-2: Percentage cell viability of the test and reference compounds assessed by MTT assay at 40 µM concentration in SH-SY5Y human neuroblastoma cell line. Data is expressed as mean ± SEM. NS indicates non-significant vs control.

Docking Study

To understand the intermolecular interactions of the promising molecules with the target enzymes leading to the inhibitory activities, molecular docking studies were performed using Glide module[27] (Schrodinger). Receptor-ligand interactions were analyzed by using LIGPLOT program.[28] As discussed earlier, both AChE and BuChE have a catalytic triad, Ser-His-Glu and certain other important features of the active sites in common. The synthesized molecules (**7m** and **7q**) along with tacrine and donepazil as standard drugs were docked into the active sites of AChE and BuChE. To understand the molecular interactions of the ligands with AChE, the said compounds were docked into the active site of the enzyme of Torpedo Californica (*Tc*AChE) (PDB Code: **1ACJ**) that was then humanized to recognize the sequence of interactions. Tacrine forms a firm complex with the enzyme by making hydrogen bond with the protonated nitrogen of acridine ring of tacrine and C=O of His 440 (*h*AChE His447). The aromatic ring of tacrine was observed to be sandwiched between Trp84 and Phe330 (*h*AChE Trp86 and Tyr337). In donepezil, the 1-benzyl moiety of donepezil was observed to be stabilized in the active site by Trp84 and Phe330 residues (*h*AChE Trp86 and Tyr337). The dihydroinden-1-one group of donepezil was stabilized by Trp279 (*h*AChE Trp286) residue through hydrophobic interactions.

The 6-methoxy group exhibited H-bonding interaction with Trp279 (*h*AChE Trp286). Compounds (**7m** and **7q**) also showed good interaction with the CAS of the enzyme. In compound (**7m**) (**figure 3A**) the tricyclic ring was observed to be stabilized within the hydrophobic pocket of CAS comprising of Trp84, Glu199, Ser200, Phe330 and His440 (*h*AChE Trp86, Glu202, Ser203, Tyr337 and His447) whereas the aromatic ring of the benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione scaffold was stabilized by π-π stacking with Trp84 and Phe330 (*h*AChE Trp86 and Tyr337). The 2,3-dimethoxybenzyl group heading towards the PAS was found to be stabilized by hydrophobic interactions with Tyr70, Tyr121 and Ser122 (*h*AChE Tyr72, Tyr124 and Ser125). Additionally the hydrogen bond between -NH of the ligand and Glu199 (*h*AChE Glu202) residue was observed to stabilize the ligand-receptor complex. In compound (**7q**) (**figure 3B**) the tricyclic ring was found to be stabilized in a similar fashion to that of **7m** within the hydrophobic pocket comprising of Trp84, Glu199, Ser200, Phe330 and His440 (*h*AChE Trp86, Glu202, Ser203, Tyr337 and His447). The aromatic ring of benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione skeleton stabilized the receptor-ligand complex by forming π-π stacking with Trp84 (*h*AChE Trp86). Strong hydrophobic interactions of 3,4-dimethoxyphenethyl group, which heads towards the PAS of the receptor, were observed with Tyr70, Tyr121, Trp279 and Phe330 (*h*AChE Tyr72, Tyr124, Trp286 and Tyr337). Additional stability to the ligand-receptor complex was bestowed by the hydrogen bond between -NH of the ligand and the Glu199 (*h*AChE Glu202) residue of the receptor's active site.

In a similar fashion, to understand the molecular interactions of the promising compounds (**7m** and **7q**) with the active site of *h*BuChE, docking studies were conducted within the active site of *h*BuChE (PDB Code: **4BDS**). Tacrine and donepazil, used as standards in biological testing, were also considered for this study. The aromatic ring of tacrine stabilized the ligand-receptor complex by π-π interactions with Trp82. H-bonding between amino N-H of tacrine and C=O of His438 imparted further stability to the complex. While in case of donepazil, the benzyl group was found to be stabilized by Trp82. The dihydroinden-1-one group by means of hydrophobic interactions with Ser198, Trp231 and Phe398 stabilized the ligand-receptor complex. In case of **7m**, the tricyclic ring was observed to be stabilized into the hydrophobic pocket of Trp82, Tyr332 and His438. The -NH of the tricyclic ring of the ligand further imparted stability to the complex by forming hydrogen bonding with Ala328. The 2,3-dimethoxybenzyl group was stabilized by the hydrophobic interactions with the Gly117, Ser198, Pro285 and

Phe329 (**Figure 4A**). Docking study of compound **7q** (**Figure 4B**) showed more prominent interactions within the hydrophobic pocket comprising of Trp82, Thr120 and Gly121. Additional stability to the ligand-receptor complex was imparted by the hydrogen bonding between –NH of the pteridine moiety and Tyr128 residue present in the enzyme. 3,4-Dimethoxyphenethyl group further added stability to the complex by hydrophobic interactions with Gly116, Pro285, Leu286 and Phe329.

Overall, it could be concluding from the docking studies that the synthesized compounds (**7m** and **7q**) showed reasonably good interactions with the active sites of AChE and BuChE. **7m** and **7q** showed interactions with the receptor active sites comparable to the standard drugs tacrine and donepezil (**Figure S1** and **Figure S2**) selected in this study for comparison purpose. Along with the biological evaluation, this docking approach also supports the potential of the test compounds (**7m** and **7q**).

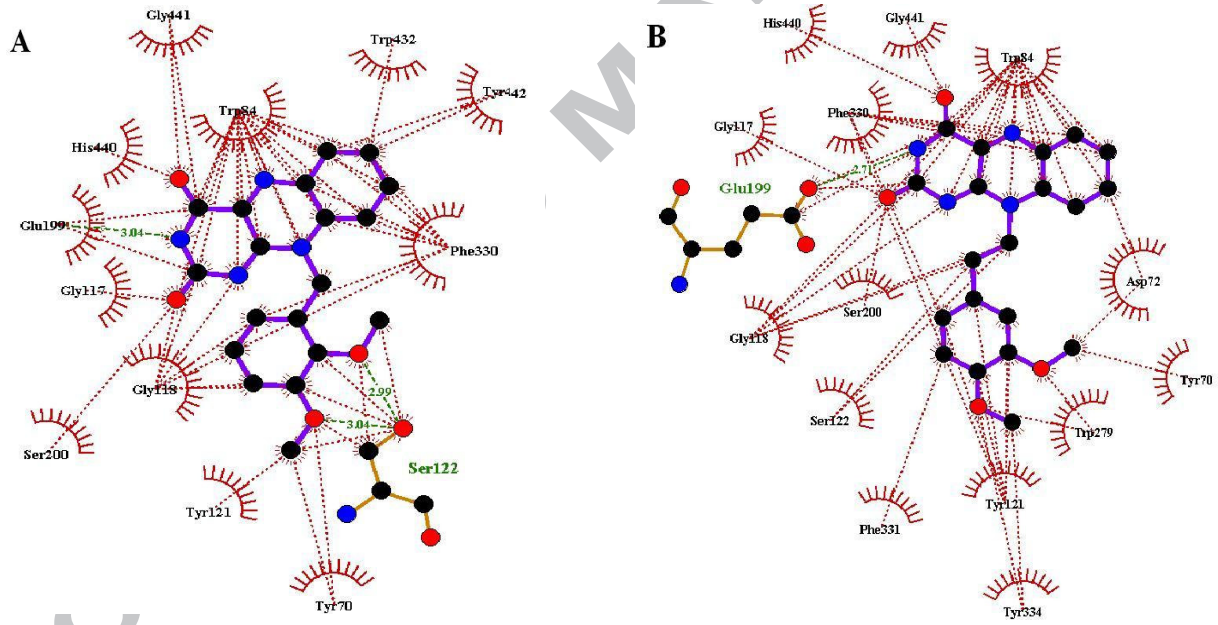


Figure 3: Docking interaction of the two compounds with the active site of AChE Eenzyme. (A) Compound (7m); (B) Compound (7q).

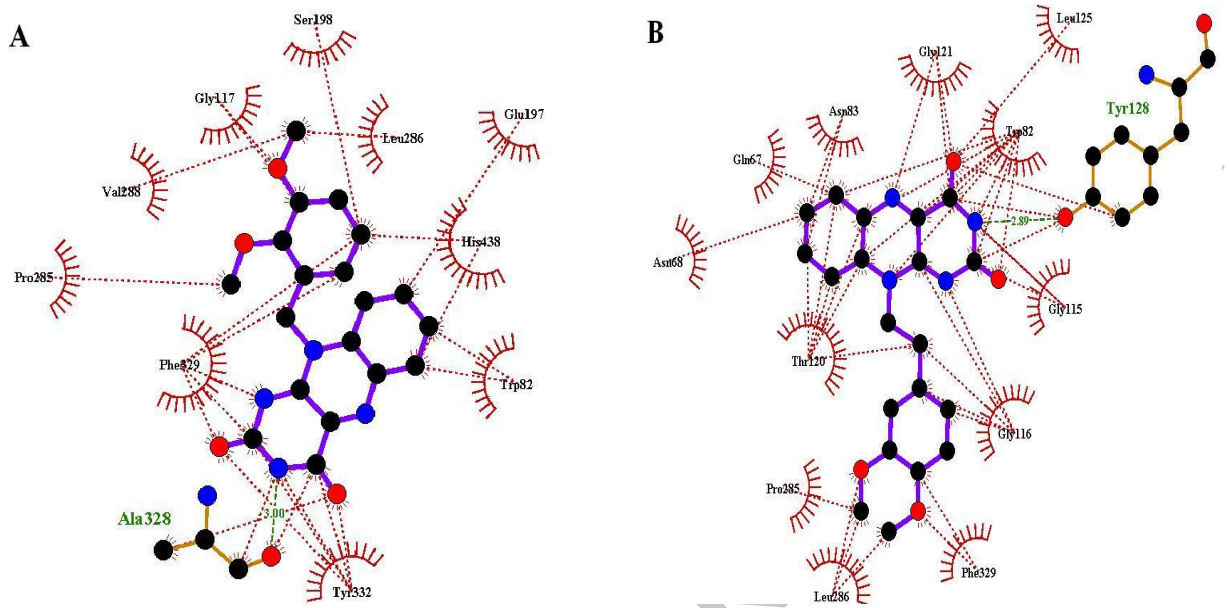


Figure 4: Docking interaction of the two compounds with the active site of BuChE enzyme. (A) Compound (7m); (B) Compound (7q).

CONCLUSION

In conclusion, this study brings to light the discovery of isoalloxazines as novel leads for the treatment of Alzheimer's disease. Along with the parent molecule isoalloxazine, different derivatives of the lead (**7**) were synthesized using various alkyl and substituted phenylalkyl groups and evaluated for their ability to inhibit AChE and BuChE enzymes by using Ellman's method. All the compounds showed moderate to good inhibitory activity in μM range against either or both of the enzymes. Amongst all, compounds (**7m** and **7q**) showed the highest inhibitory activity against AChE (4.72 μM and 5.22 μM respectively) as well as BuChE (6.98 μM and 5.29 μM respectively) enzymes. The promising compounds (**7m** and **7q**) were evaluated additionally for their ability to prevent β-amyloid (Aβ) aggregation with and without AChE by Thioflavin-T (ThT) assay and Congo red (CR) binding assay respectively. Further, cytotoxic effects of the two compounds using MTT assay were analyzed and it was found that both the compounds were non-toxic at 40 μM dose level. Molecular docking studies also confirmed that these compounds targeted both the enzymes and supported the binding potential of the synthesized compounds to the targets. On the basis of these results, further structural

modifications in isoalloxazine scaffold are in progress to design and synthesize more potential compounds in this laboratory.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found in the online version

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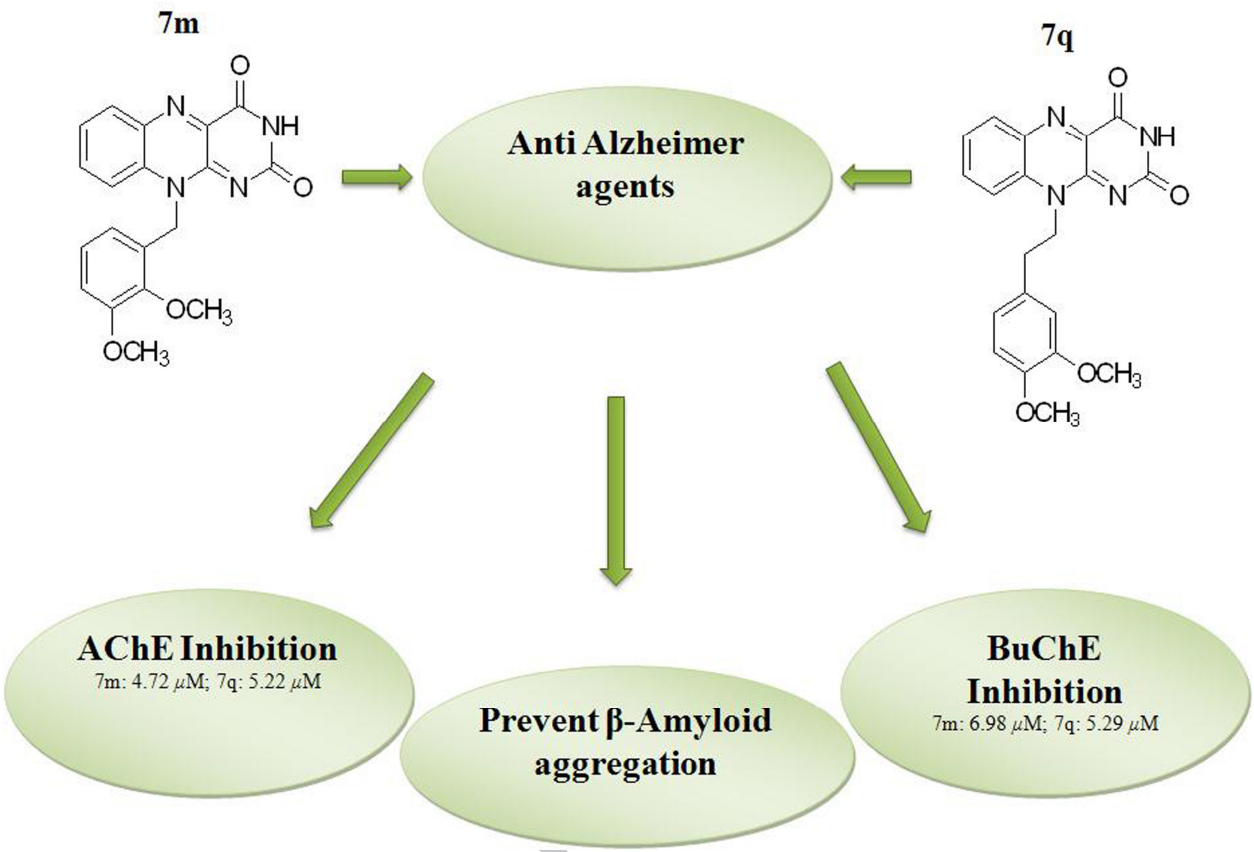

Highlights

- Isoalloxazine derivatives as novel class of cholinesterase inhibitors are reported.
- Compounds reported showed good activity against both AChE and BuChE.
- Potential therapeutic activity for Alzheimer's disease is explained.
- For most potent compounds anti-aggregatory activity for β-amyloid was performed.
- Cyto-toxicity on SH-SY5Y neuroblastoma cells, and docking study were performed.