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Novel *N*-benzylpyridinium moiety linked to arylisoxazole derivatives as selective butyrylcholinesterase inhibitors: Synthesis, biological evaluation, and docking study

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This paper is dedicated to our unique teacher in chemistry and medicinal chemistry Professor Abbas Shafiee (1937–2016).

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ABSTRACT

A novel series of *N*-benzylpyridinium moiety linked to arylisoxazole ring were designed, synthesized, and evaluated for their *in vitro* acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities. Synthesized compounds were classified into two series of **5a-i** and **5j-q** considering the position of positively charged nitrogen of pyridinium moiety (3- or 4- position, respectively) connected to isoxazole carboxamide group. Among the synthesized compounds, compound **5n** from the second series of compounds possessing 2,4-dichloroaryl group connected to isoxazole ring was found to be the most potent AChE inhibitor ($IC_{50} = 5.96\ \mu M$) and compound **5j** also from the same series of compounds containing phenyl group connected to isoxazole ring demonstrated the most promising inhibitory activity against BChE ($IC_{50} = 0.32\ \mu M$). Also, kinetic study demonstrated competitive inhibition mode for both AChE and BChE inhibitory activity. Docking study was also performed for those compounds and desired interactions with those active site amino acid residues were confirmed through hydrogen bonding as well as π-π and π-anion interactions. In addition, the most potent compounds were tested against BACE1 and their neuroprotectivity on Aβ-treated neurotoxicity in PC12 cells which depicted negligible activity. It should be noted that most of the synthesized compounds from both categories **5a-i** and **5j-q** showed a significant selectivity toward BChE. However, series **5j-q** were more active toward AChE than series **5a-i**.

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia among elderly people. The disease is characterized by the brain dysfunction leading to gradually degrade of the mental abilities such as memory loss as well as language and cognitive impairments [1]. Multiple factors have been reported to contribute to the etiology of AD. They may include cholinergic transmission [2], beta amyloid aggregation [3], microtubule associated tau protein hyper-phosphorylation [4], oxidative stress and ROS production [5], biometals dyshomeostasis [6], inflammatory [7], and pro-apoptotic pathways [8].

According to the literature, reduction of cholinergic activity in the brain was the first theory describing the feature of AD [9] in which two enzymes acetyl- and butyrylcholinesterase (AChE and BChE) played an important role in the hydrolysis of acetylcholine (ACh). AChE is an exclusive esterase which is responsible for the hydrolysis of acetylcholine and regulating cholinergic neurotransmitter in the cholinergic synapse of central nervous system [10]. On the other hand, BChE also known as "pseudo" cholinesterase is a non-specific cholinesterase or simply cholinesterase which hydrolyzes different types of choline esters, preferentially acts on the butyrylcholine [11]. In this regard, the level of AChE and BChE is crucial factor playing a fundamental role in the pathogenesis of the disease in such a manner that when the level of AChE goes down that of BChE goes up [12]. Several studies have demonstrated that the level of BChE increases in several specific regions of the brain probably to offset the role of AChE [13]. It has been reported that both AChE-knockout and wild-type mice displayed BChE activity in all parts of the brain that receive cholinergic innervations and BChE

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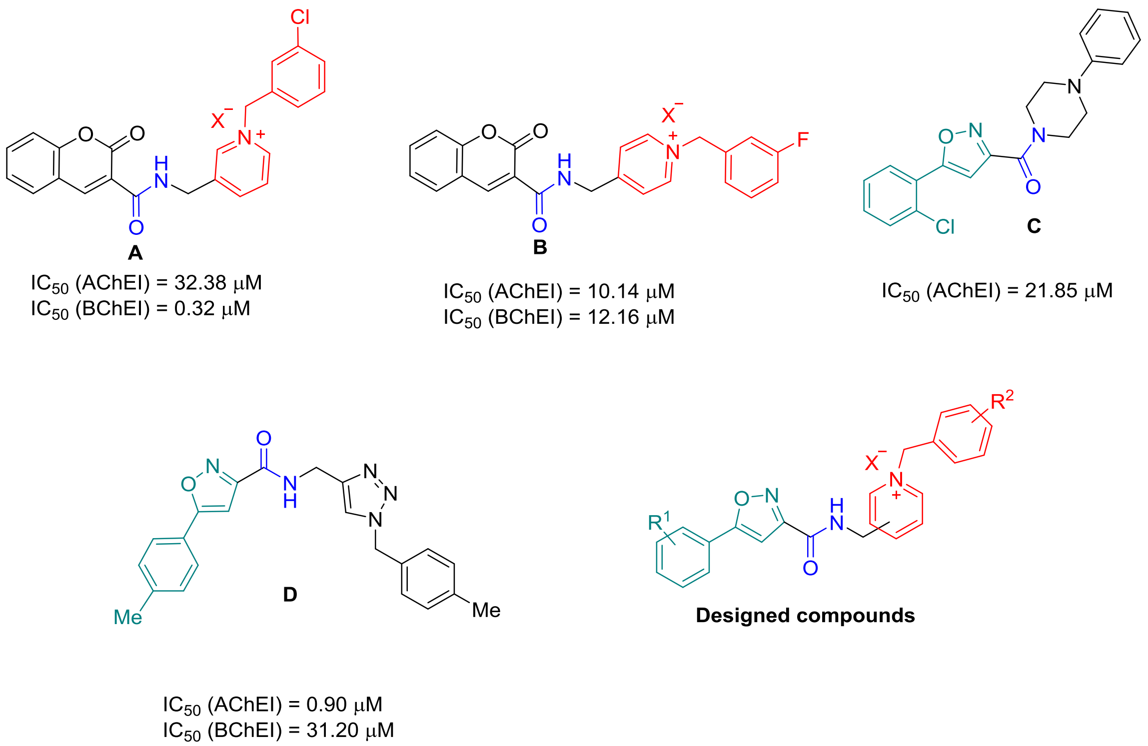


Fig. 1. The structure of compounds containing *N*-benzylpyridinium moiety as ChEIs.

could hydrolyze acetylthiocholine as the counterpart of acetylcholine [14].

Decomposition of ACh by cholinesterases (ChEs) has been appointed as one of the important implications in the onset and progression of AD [15]. Consequently, ChE inhibitors (ChEIs) have been found as the effective remedies at the early stage of AD for cognition and global change assessments. Up to now, tacrine, donepezil, galantamine, and rivastigmine have been introduced as the FDA approved ChEIs, however, tacrine was removed from the market due to toxicity. It should be noted that the efficacy of ChEIs for neuropsychiatric symptoms is not definitely accepted in patients with mild-to-moderate AD [16]. According to the literature, selective BChE inhibitors were found to be important for the treatment of moderate to severe AD because the level of AChE in the hippocampus and temporal cortex decreases while the level of BChE meaningfully increases. To overcome the adverse effects due to suppression of AChE, development of effective and selective BChEIs seems to be crucial [11,17,18].

The search for novel ChEIs as anti-AD agent led us to *N*-benzylpyridinium moiety as a highly privileged scaffold [19] specially on cholinesterase inhibitory activity [20–25] (Fig. 1). Recently, we have prepared novel coumarin-pyridinium derivatives containing amide moiety and most of them showed good to excellent ChEI activity in which predominent BChEI activity was observed (Fig. 1**A** and **B**) [26]. Also, the anti-ChE activity of isoxazole moeity has been frequently investigated by our research group (Fig. 1**C** and **D**) [27–29].

Herein, focusing on the efficacy of *N*-benzylpyridinium and arylisoxazole moieties, in continuation of our previous studies for developing new ChEIs [26–32], we reported design and synthesis of novel *N*-benzylpyridinium-isoxazole hybrids containing an amide moiety as generally selective BChE inhibitors (Scheme 1).

2. Results and discussion

2.1. Chemistry

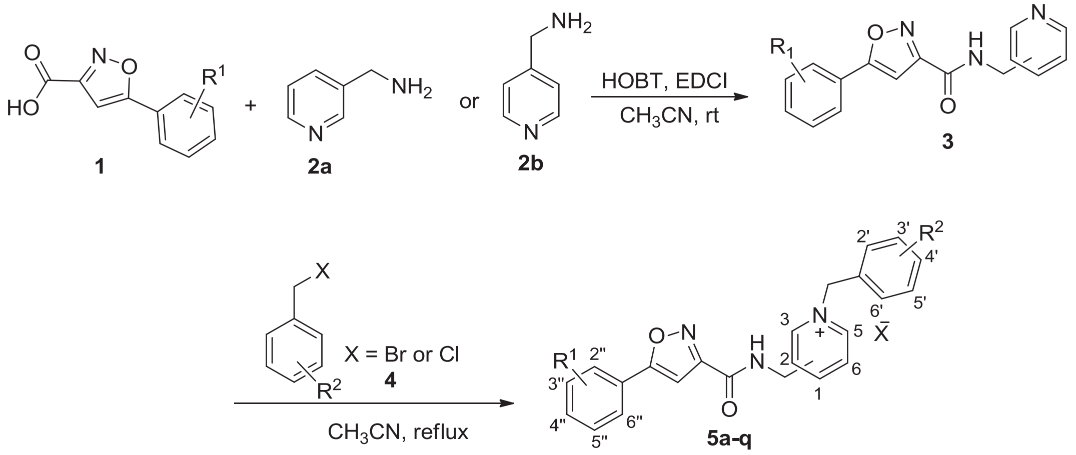
All required 5-arylisoxazole carboxylic acid derivatives **1** were prepared according to our previous report [27]. Reaction of desired carboxylic acid derivatives **1** and 3-picolylamine **2a** or 4-picolylamine **2b** in dry acetonitrile at room temperature in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and hydroxybenzotriazole (HOBt) led to the formation of arylisoxazole-pyridine carboxamide derivatives **3**. Finally, the reaction of compound **3** and appropriate benzyl halides **4** in refluxing acetonitrile afforded product **5** in good yield.

2.2. Biological activity

The *in vitro* inhibitory activity of compounds **5a-q** against ChEs was performed using modified Ellman's method [27,33] and compared with donepezil and rivastigmine as reference drugs (Table 1). As can be seen in Table 1, compounds **5a-q** can be classified into two categories depending on the position of positively charged nitrogen of pyridinium moiety connected to isoxazole carboxamide moiety: *I)* from the 3- position (**5a-i**) and *II)* 4-position (**5j-q**).

It is worth mentioning that all synthesized compounds demonstrated more significant BChEI activity than AChEI activity. To get better insight into SAR activity and effect of various substituted arylisoxazoles on the ChE inhibitory activity, most of synthesized compounds were prepared as 2-methylbenzylpyridinium salts.

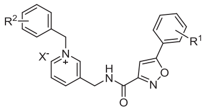
In the case of AChEI activity, the most potent compounds **5n** and **5o** were found to be in the second category of compounds with IC_{50} values of 5.96 and 6.03 μM, respectively. The most active AChE inhibitor **5n** contained 2-methylbenzylpyridinium and 2,4-dichloroaryl moieties connected to isoxazole ring. Elimination of chlorine at the 2- position of aryl ring decreased inhibitory activity and compound **5m** showed IC_{50} = 15.48 μM. Also, replacing chlorine by fluorine at 2- and 4- position of aryl ring deteriorated AChEI activity in such a manner that IC_{50} values for compounds **5k** and **5l** were calculated as 75.19 and 22.42 μM, respectively. However, the presence of fluorine at 4- position induced higher anti-AChE activity than 2-substituted derivative. It should be noted that the lack of substituents on the aryl ring led to moderate inhibitory activity in compound **5j** (IC_{50} = 16.82 μM). As



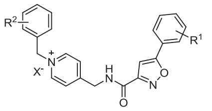
Scheme 1. Synthesis of *N*-benzylpyridinium moiety linked to arylisoxazoles **5**.

Table 1

Synthesis and cholinesterase inhibitory activity of compounds **5a-q**.[a]



5a-i



5j-q

Entry	Compound **5**	R^1	R^2	X	AChEI activity IC_{50} (μM)	BChEI activity IC_{50} (μM)
1	**5a**	4-F	2-Me	Br	75.72 ± 0.14	1.17 ± 0.73
2	**5b**	4-Cl	4-Br	Br	49.78 ± 0.03	4.51 ± 0.39
3	**5c**	4-Cl	2-Me	Br	36.57 ± 0.58	4.21 ± 0.66
4	**5d**	4-Br	2-Me	Br	99.76 ± 0.12	5.18 ± 0.68
5	**5e**	3-Me	2-Me	Br	32.76 ± 0.59	0.65 ± 0.50
6	**5f**	4-Me	2-Me	Br	80.01 ± 0.36	7.99 ± 0.80
7	**5g**	3-OMe	2-Me	Br	97.93 ± 0.61	0.90 ± 0.61
8	**5h**	4-OMe	2-Me	Br	70.42 ± 0.51	17.82 ± 0.38
9	**5i**	$3,4\text{-}(OMe)_2$	2-Me	Br	82.37 ± 0.47	2.61 ± 0.05
10	**5j**	H	2-Me	Br	16.82 ± 0.35	0.32 ± 0.06
11	**5k**	2-F	2-Me	Br	75.19 ± 0.35	5.94 ± 0.67
12	**5l**	4-F	2-Me	Br	22.42 ± 0.76	10.66 ± 0.77
13	**5m**	4-Cl	2-Me	Br	15.48 ± 0.70	12.04 ± 0.24
14	**5n**	$2,4\text{-}Cl_2$	2-Me	Br	5.96 ± 0.41	1.39 ± 0.53
15	**5o**	4-Br	2-F	Cl	6.03 ± 0.02	2.77 ± 0.01
16	**5p**	4-Br	4-Br	Br	20.22 ± 0.63	10.70 ± 0.30
17	**5q**	$3\text{-}NO_2$	2-Me	Br	9.90 ± 0.52	4.40 ± 0.403
18	Rivastigmine				11.07 ± 0.01	7.72 ± 0.02
19	Donepezil				0.03 ± 0.00	8.06 ± 0.38

[a] Data are expressed as Mean ± SD (three independent experiments).

mentioned above, compound **5o** possessing 2-fluorobenzylpyridinium and 4-bromoaryl moieties was also potent AChEI among the synthesized compounds **5**. However, it seems that 2-fluorobenzylpyridinium played an important role in the inhibitory activity since when it was replaced by 4-bromobenzylpyridinium moiety in compound **5p**, a significant decrease was observed.

Another point comes back to compound **5q** containing 3-nitroaryl moiety connected to isoxazole ring. It showed more potent inhibitory activity (IC_{50} = 9.90 μM) than its analogues **5j-m** lacking substituents or having 2-fluoro, 4-fluoro, and 4-chloro substituents. However, it demonstrated lower activity than compound **5n** possessing two Cl groups at 2- and 4- positions of aryl group connected to isoxazole ring.

According to our results reported in Table 1, compounds belonged to the first category generally showed lower inhibitory activity than the second one. In this category, compound **5e** having 2-methylbenzylpyridinium and 3-methylaryl moieties was the most potent AChE inhibitor (IC_{50} = 32.76 μM). Changing the position of methyl from 3- to 4- in compound **5f** led to much lower activity (IC_{50} = 80.01 μM). Although replacement of 4-methyl by more electron donating group (4-OMe) led to a better activity in compound **5h** (IC_{50} = 70.42 μM), changing the position of methoxy group (compound **5g**, IC_{50} = 97.93 μM) and increasing the number of that group (compound **5i**, IC_{50} = 82.37 μM) afforded lower inhibitory activity. It seems that electronic property of substituent on the aryl ring imposed a remarkable effect on the AChEI activity, as it is clear in the calculated IC_{50} values. Also, the presence of halogens on the aryl ring could not promote AChEI activity, however, they depicted different activity. Compound **5c** possessing 2-methylbenzylpyridinium and 4-chloroaryl moieties showed moderate activity (IC_{50} = 36.57 μM). Changing the halogen to F and Br in compounds **5a** and **5d** gave lower activity with IC_{50}s = 75.72 and 99.76 μM, respectively. Also, counterpart of compound **5c**, compound **5b** containing 4-bromobenzylpyridinium and 4-chloroaryl derivative induced lower activity (IC_{50} = 49.78 μM) than **5c** (IC_{50} = 36.57 μM) indicating that 2-methylbenzylpyridinium played more important role than 4-bromobenzylpyridinium moiety to induce AChEI activity.

Among synthesized pyridinium-isoxazole hybrids **5**, those derivatives contained *N*-benzylpyridinium connected from 4- position of pyridine generally showed better AChEI activity. This result was in good agreement with our report on ChEI activity of coumarin-pyridinium hybrids in which the most potent anti-AChE compound belonged to the similar category (Fig. 1, compound **B**) [26].

Comparing AChEI activity of compounds **5a-i** with compounds **5j-q** possessing the same group connected to isoxazole moiety (R^1) revealed that the second category of compounds were more potent than the first category. In this respect, **5l** > **5a**, **5m** > **5b** and **5c**, and **5o** and **5p** > **5d**.

Also, comparing our results with previously evaluated isoxazoles for their AChEI activity (Fig. 1, compound **C**) [28] confirmed that the presence of chlorinated aryl group connected to isoxazole moiety was significant for the inhibition of AChE. However, comparing compound **5n** with compound **D** (Fig. 1. [29]) showed that hybridization of isoxazole-1,2,3-triazole resulted in much more better anti-AChE activity than hybridization of isoxazole-pyridinium moiety.

In the case of BChEI activity which was appeared more significant than AChEI activity of synthesized compounds, compound **5j** from the second category of compounds having 2-methylbenzylpyridinium moiety and phenyl connected to isoxazole ring exhibited the most effective inhibitory activity (IC_{50} = 0.32 μM). In this category, lacking substituents on the aryl moiety led to lower activity. Chlorinated aryl analogues, compound **5n** possessing 2,4-dichloroaryl moiety showed good activity (IC_{50} = 1.39 μM), however, less than compound **5j**.

Elimination of Cl at the 2- position (compound **5m**) decreased BChEI activity by 8 times (IC_{50} = 12.04 μM). Introduction of fluorine into the 2- or 4- position of aryl group (compounds **5k** and **5l**) increased inhibitory activity comparing with compound **5m** and 2-fluorinated derivative (**5k**, IC_{50} = 5.94 μM) was found to be two times more potent than 4-fluorinated derivative (**5l**, IC_{50} = 10.66 μM). It should be noted that the presence of electron-withdrawing group (NO_2) afforded good BChEI activity (IC_{50} = 4.40 μM). As can be seen in Table 1, compounds lacking 2-methylbenzylpyridinium moiety, compounds **5o** and **5p** possessing 2-fluorobenzylpyridinium and 4-bromobenzylpyridinium moieties respectively did not show higher inhibitory activity than compound **5j** (the most potent compound in both categories). However, compound **5o** possessing 2-fluorobenzylpyridinium medium was approximately 4 times more potent than compound **5p**.

In the first category of compound, the best activity was observed for compound **5e** (IC_{50} = 0.65 μM) having 3-methylaryl connected to isoxazole moiety which was induced the best AChEI activity in the same group. Changing the position of methyl to 4-position of aryl group reduced activity by 13 times in compound **5f** (IC_{50} = 7.99 μM). It is interesting to mention that the presence of 3-methoxy also led to good activity (**5g**, IC_{50} = 0.90 μM) although it was lower active than compound **5e**. Insertion of methoxy group into the 4- position completely deteriorated inhibitory activity (compound **5h**, IC_{50} = 17.82 μM), however, the presence of two methoxy groups at the 3- and 4- positions led to good activity (compound **5i**, IC_{50} = 2.61 μM). To discuss efficacy of halogens at the 4- position of aryl group, the order of F > Cl > Br was observed according to the calculated IC_{50} values for compounds **5a**, **5c**, and **5d** (IC_{50}s = 1.17, 4.21, and 5.18 μM, respectively), however, higher efficiency of fluorine than chlorine and bromine is completely clear. It may be associated with the different sizes of F, Cl, and Br and steric hindrance in the active site of enzyme caused by para-substituted compound. Finally, compound **5b** should be considered as 4-bromobenzylpyridinium derivative which was found approximately as potent as (IC_{50} = 4.51 μM) its counterpart **5c** (IC_{50} = 4.21 μM).

Comparison of compounds **5a** and **5l** revealed that anti-BChE activity could be improved by derivatives belonging to the first class of compounds. It was also confirmed by comparing the BChEI activity of two analogues **5c** and **5m**.

Comparing anti-BChE of compounds **5** with those shown in Fig. 1 revealed that all compounds **5** were more active than isoxazole derivatives **C** and **D**. Also, they were relatively similar to coumarin-pyridinium hybrids such as **A** and **B** from selectivity and inhibitory activity points of view. It seems that the presence of pyridinium moiety is important for inducing better BChEI activity since the other isoxazole containing compounds did not show desired inhibitory activity. However, selectivity of compounds **5** towasrd BChE was more significant than series of **A** and **B** [26].

Comparing AChEI and BChEI activity of each series of compounds demonstrated that similar substituents did not induce the same order of inhibitory activity. For example, in the first category of compounds **5a-i**, among compounds containing 4-halogenated aryl group (**5a**, **5c**, and **5d**), compound **5a** was found to be the most potent BChE inhibitor, while, compound **5c** was the best AChE inhibitor. Similarly, their counterparts **5l** and **5m** showed the same order of activity. Compound **5l** depicted more potent BChEI activity than compound **5m** whereas compound **5m** induced better AChEI activity than compound **5l**.

2.3. Kinetic studies

To obtain more information about the mechanism of AChE inhibition, the most active AChE inhibitor **5n** was subjected to kinetic studies based on the modified Ellman's method [26].

For this purpose, the rate of enzyme activity was measured at four concentrations of inhibitor (0, 0.214, 1.284, and 2.568 μM) and different concentrations of acetylthiocholine as a substrate (ATCh, [S]). For each concentration of inhibitor, the initial velocity was measured at different substrate concentrations and the reciprocal of the initial velocity (1/v) was plotted vs the reciprocal of the substrate concentration (1/[S]). As shown in Fig. 2, the obtained double reciprocal (Lineweaver-Burk) plot represented a competitive inhibition pattern for compound **5n**. The *Ki* value was also calculated using the secondary plot (*Ki* = 0.78 μM). Similar kinetic study was performed for the most potent anti-BChE compound **5j** as described above (Fig. 3). Also, competitive inhibition pattern was observed for BChEI activity and *Ki* = 0.31 μM was calculated.

2.4. BACE1 enzyme inhibitory activity of compounds 5n and 5j

According to the significant role of β-secretase (BACE1) in AD pathogenesis [34], BACE-1 inhibitory activity of compounds **5n** and **5j** were evaluated *via* a fluorescence resonance energy transfer (FRET) based BACE including BACE-1 enzyme and specific APP based peptide substrate (Rh-EVNLDAEFK-quencher) in comparison to OM99-2 (IC_{50} = 0.014 μM) [26]. Percent inhibition values were calculated as 22.0 ± 10.4% and 21.0 ± 2.2% for compounds **5n** and **5j** at concentration of 50 μM, respectively.

2.5. Neuroprotection effect against Aβ-induced damage

Neuroprotectivity of the most potent BChE inhibitor **5j** was studied on $A\beta_{25\text{-}35}$ induced damage in PC12 cells by MTT assay [28]. It demonstrated 4.7 ± 3.2% protection at the concentration of 25 μM comparing with rutin with 13.4 ± 6.1% protection at the same concentration.

2.6. Docking study

To clarify the mode of interaction of the most potent compound **5n** in the active site of AChE, docking study was conducted using Auto dock Vina 1.1.2. For this purpose, the ligand-bonded crystallographic structure of AChE with PDB ID: 1EVE was retrieved from RCSB protein data bank.

The best docking pose of the ligand in terms of free energy was extracted and subjected to further studies of docking mode. As shown in the Fig. 4, benzyl group connected to pyridinium moiety oriented toward Phe330 residue (in the anionic subsite) through a π-π interaction. The nitrogen of the amide aligned toward Tyr130 (in the anionic subsite) *via* a hydrogen bonding. Pyridinium moiety was placed at the bottom of active site where phenyl ring interacted with Trp84 (in the CAS) through a π-π stacking interaction.

Similarly, to know the binding mode of the most potent compound **5j** in the active site of BChE, docking study was performed using Autodock Tools. In this respect, the ligand-bonded crystallographic structure of BChE with PDB ID: 4BDS was retrieved from the protein data bank. The best docking pose of the ligand in terms of free energy was extracted and subjected to further studies of docking mode.

As shown in Fig. 5, benzyl group and pyridinium moiety bound weakly through a π- lone pair and π-anion interactions with Trp82 (anionic site) and Asp70 (in the PAS), respectively. The important interaction between the ligand and receptor is between the oxygen of isoxazole moiety and Ser198 (in the catalytic triad of esteratic site) through a hydrogen bonding.

3. Conclusion

In conclusion, novel series of *N*-benzylpyridinium moiety linked to arylisoxazole derivatives were synthesized and evaluated for their anti-AChE and anti-BChE activity. Our results confirmed good to moderate ChEI activity, however, much better BChE inhibitory activity was obtained. Among the synthesized compounds, 4-((5-(2,4-dichlorophenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5n**) showed the best AChE inhibitory activity (IC_{50} = 5.96 μM) and 1-(2-methylbenzyl)-4-((5-phenylisoxazole-3-carboxamido)methyl)pyridin-1-ium bromide (**5j**) demonstrated the best BChE inhibitory activity (IC_{50} = 0.32 μM). Compounds **5n** and **5j**

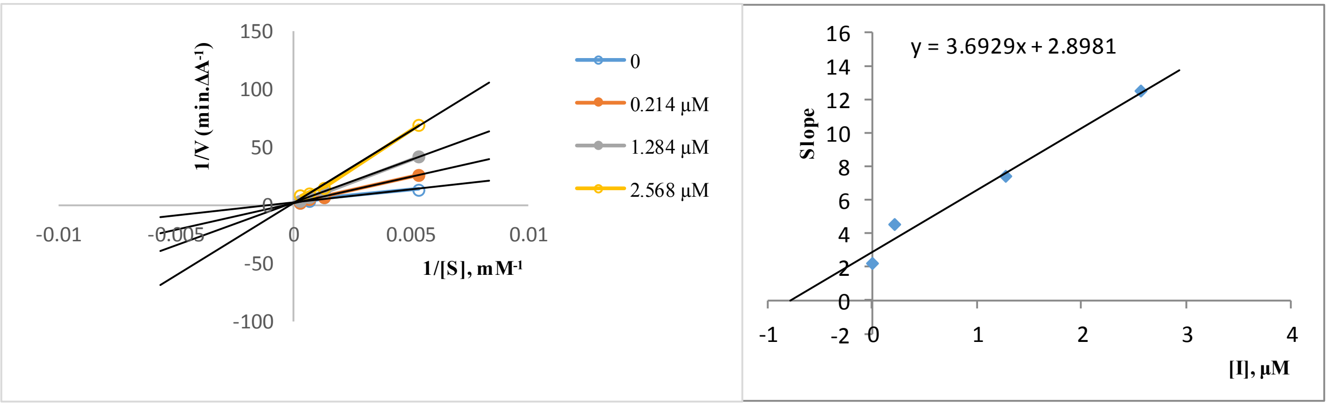


Fig. 2. Left: Lineweaver-Burk plot for the inhibition of AChE by compound **5n** at different concentrations of acetylthiocholine (ATCh). Right: Steady-state inhibition constant (Ki) of compound **5n**.

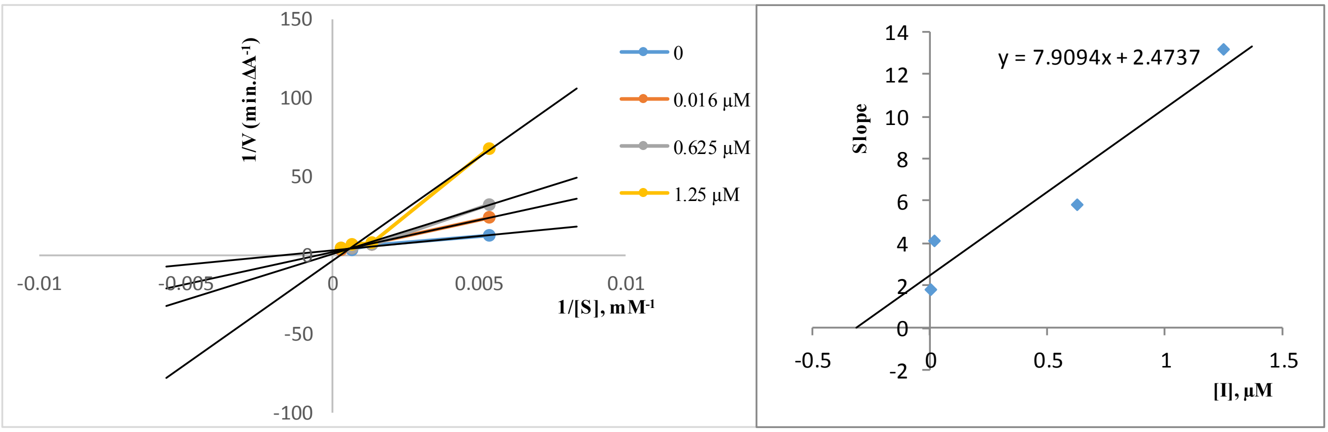


Fig. 3. Left: Lineweaver-Burk plot for the inhibition of BChE by compound **5j** at different concentrations of butyrylthiocholine (BTCh). Right: Steady-state inhibition constant (Ki) of compound **5j**.

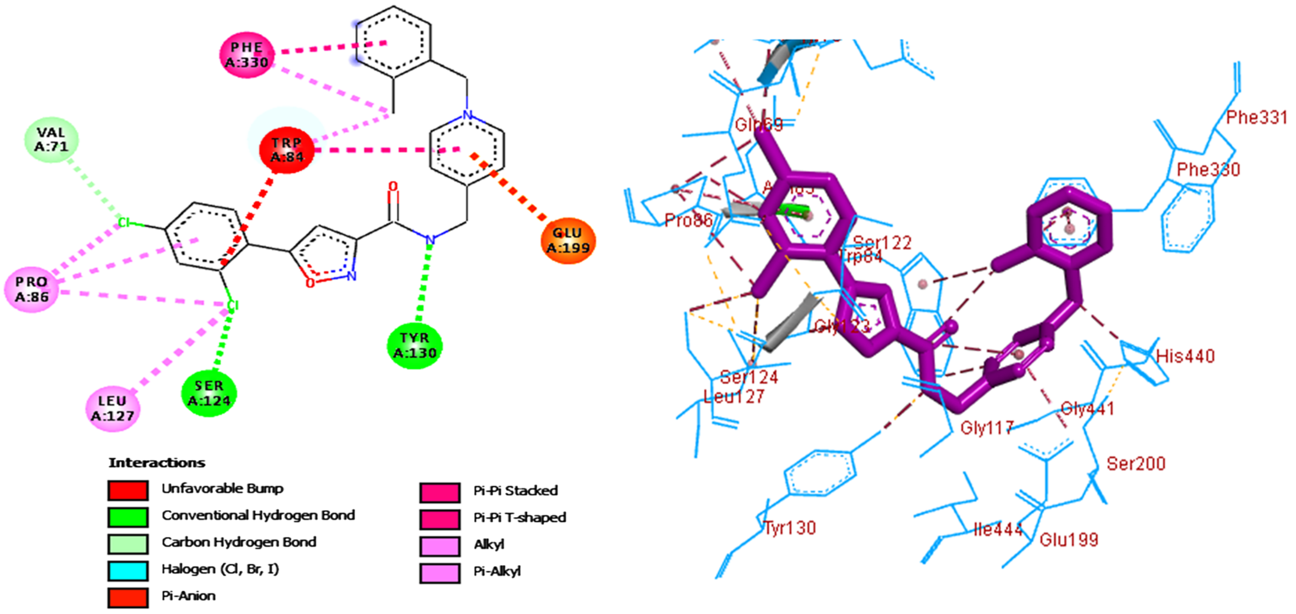


Fig. 4. The binding mode of compound **5n** in the active site of AChE.

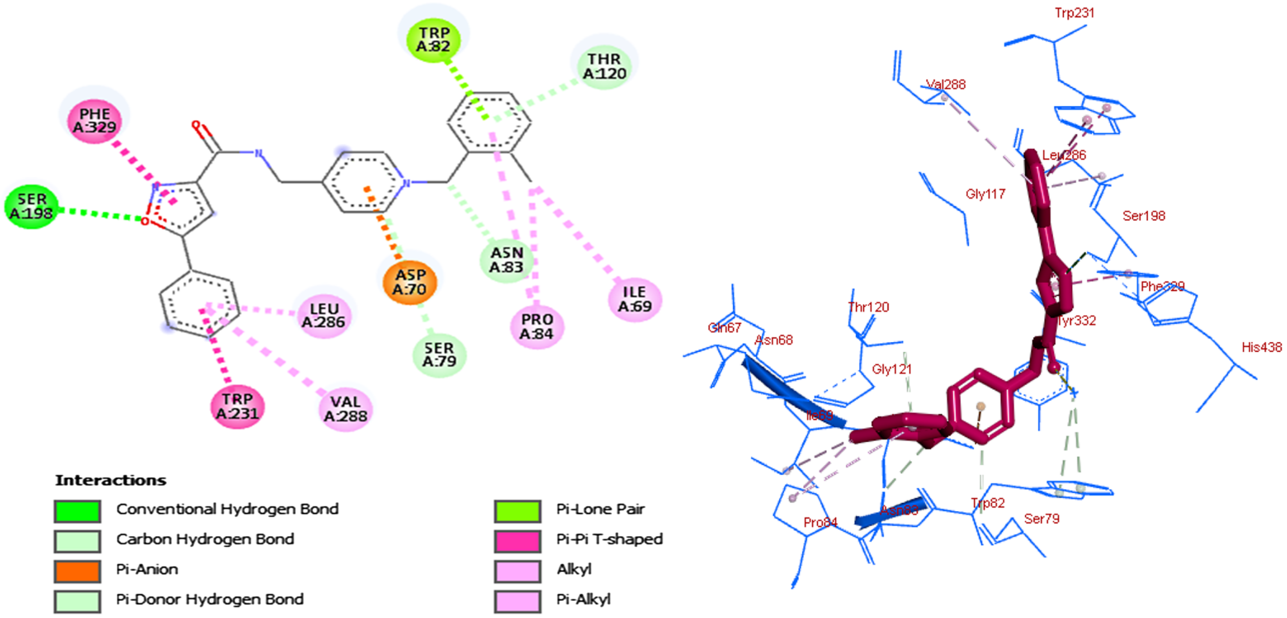


Fig. 5. The binding mode of compound **5j** in the active site of BChE.

demonstrated low BACE1 inhibitory activity and negligible neuroprotectivity on Aβ-treated neurotoxicity in PC12 cells.

4. Experimental

Melting points were measured on a Kofler hot stage apparatus and are uncorrected. ^{1}H- and ^{13}C NMR spectra were recorded on a Bruker FT-500 (Germany) using TMS as an internal standard. The IR spectra were taken on a Nicolet Magna FTIR 550 spectrometer (KBr disks). The elemental analysis was performed on an Elemental Analyzer system GmbH VarioEL CHNS mode (Germany).

*4.1. General procedure for the synthesis of pyridinium-isoxazole hybrids **5a-q***

A solution of isoxazole acid derivative **1** (1 mmol), EDCI (1.1 mmol), and HOBt (1 mmol) in dry acetonitrile (10 mL) was stirred at room temperature for 30 min. Then, 3-picolylamine **2a** or 4-picolylamine **2b** (1 mmol) was added drop wise to the mixture and the reaction was continued at room temperature for 24 h. After completion of the reaction, the solvent was reduced under vacuum and the residue was dissolved in dichloromethane and washed with sodium carbonate (10%, 3 × 20). The organic phase was dried over Na_2SO_4 and the solvent was evaporated under vacuum to give compound **3** which was completely pure. Finally, the mixture of compound **3** (1 mmol) and benzyl halide derivative **4** (1.2 mmol) in dry acetonitrile (10 mL) was heated at reflux for 10–15 h. After completion of the reaction which was monitored by TLC, the mixture was allowed to be cool and the precipitates were filtered off to afford products **5a-q** in good yields.

*4.1.1. 3-((5-(4-Fluorophenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5a**)*

Yield: 85%; mp: 218–220 °C; IR (KBr): 3297, 3021, 2985, 2959, 1679, 1613, 1543, 1504, 1450 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.58 (t, J = 7.0 Hz, 1H, NH), 9.05 (s, 1H, H_2), 8.95 (d, J = 7.0 Hz, 1H, H_6), 8.61 (d, J = 7.0 Hz, 1H, H_4), 8.18 (t, J = 7.0 Hz, 1H, H_5), 8.03 (dd, J = 7.5, 6.0 Hz, 2H, $H_{2''}$, $H_{6''}$), 7.47–7.43 (m, 3H, $H_{3''}$, $H_{5''}$, isoxazole), 7.37–7.25 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{6'}$), 7.11 (d, J = 7.5 Hz, 1H, $H_{3'}$), 5.93 (s, 2H, CH_2), 4.70 (d, J = 7.0 Hz, 2H, CH_2), 2.30 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 169.9, 163.6 (J_{C-F} = 248.7 Hz), 159.1, 158.5, 144.9, 143.7, 140.1, 136.9, 132.1, 130.9, 129.4, 128.9, 128.5, 128.3 (J_{C-F} = 8.7 Hz), 127.6, 126.7, 123.2, 116.5 (J_{C-F} = 21.2 Hz), 99.9, 61.6, 41.6, 18.8; Anal. calcd. for $C_{24}H_{21}BrFN_3O_2$: C, 59.76; H, 4.39; N, 8.71. Found: C, 59.95; H, 4.48; N, 8.38.

*4.1.2. 1-(4-Bromobenzyl)-3-((5-(4-chlorophenyl)isoxazole-3-carboxamido)methyl)pyridin-1-ium bromide (**5b**)*

Yield: 80%; mp: 205–207 °C; IR (KBr): 3241, 3047, 2996, 2934, 1662, 1610, 1542, 1486 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.55 (t, J = 6.0 Hz, 1H, NH), 9.20 (s, 1H, H_2), 9.12 (d, J = 7.0 Hz, 1H, H_6), 8.57 (d, J = 7.0 Hz, 1H, H_4), 8.16 (t, J = 7.0 Hz, 1H, H_5), 8.97 (d, J = 8.0 Hz, 2H, $H_{2''}$, $H_{6''}$), 7.66 (d, J = 8.0 Hz, 2H, $H_{3'}$, $H_{5'}$), 7.64 (d, J = 8.0 Hz, 2H, $H_{3''}$, $H_{5''}$), 7.50 (d, J = 8.0 Hz, 2H, $H_{2'}$, $H_{6'}$), 7.46 (s, 1H, isoxazole), 5.86 (s, 2H, CH_2), 4.68 (d, J = 6.0 Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 169.5, 159.1, 158.9, 144.8, 143.6, 140.1, 135.6, 133.4, 132.1, 131.2, 130.3, 129.5, 128.2, 127.6, 124.9, 122.8, 100.7, 62.5, 40.1; Anal. calcd. for $C_{23}H_{18}Br_2ClN_3O_2$: C, 49.01; H, 3.22; N, 7.45. Found: C, 48.93; H, 3.18; N, 7.38.

*4.1.3. 3-((5-(4-Chlorophenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5c**)*

Yield: 70%; mp: 240–242 °C; IR (KBr): 3297, 3021, 2985, 2959, 1679, 1613, 1543, 1504, 1450 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.62 (t, J = 6.0 Hz, 1H, NH), 9.09 (s, 1H, H_2), 8.99 (d, J = 7.0 Hz, 1H, H_6), 8.63 (d, J = 7.0 Hz, 1H, H_4), 8.19 (td, J = 7.0, 1.5 Hz), 7.99 (d, J = 8.5 Hz, 2H, $H_{3''}$, $H_{5''}$), 7.65 (d, J = 8.5 Hz, 2H, $H_{2''}$, $H_{6''}$), 7.49 (s, 1H, isoxazole), 7.35–7.25 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{6'}$), 7.13 (d, J = 7.5 Hz, 1H, $H_{3'}$), 5.97 (s, 2H, CH_2), 4.71 (d, J = 6.0 Hz, 2H, CH_2), 2.31 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 169.5, 159.2, 158.9, 144.8, 143.7, 143.5, 140.1, 136.9, 135.6, 132.3, 130.9, 129.5, 129.3, 128.9, 128.2, 127.6, 126.7, 125.0, 100.6, 61.6, 40.5, 18.8; Anal. calcd. for $C_{24}H_{21}BrClN_3O_2$: C, 57.79; H, 4.24; N, 8.42. Found: C, 57.68; H, 4.16; N, 8.33.

*4.1.4. 3-((5-(4-Bromophenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5d**)*

Yield: 65%; mp: 212–214 °C; IR (KBr): 3148, 3053, 2962, 2927, 1670, 1533, 1503, 1443 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.54 (t, J = 6.0 Hz, 1H, NH), 9.07 (s, 1H, H_2), 8.99 (d, J = 7.0 Hz, 1H, H$_6$), 8.62 (d, J = 7.0 Hz, 1H, H$_4$), 8.18 (t, J = 7.0 Hz, 1H, H$_5$), 7.83 (d, J = 8.0 Hz, 2H, H$_{3''}$, H$_{5''}$), 7.37 (d, J = 8.0 Hz, 2H, H$_{2''}$, H$_{6''}$), 7.34–7.25 (m, 4H, H$_{4'}$, H$_{5'}$, H$_{6'}$, isoxazole), 7.13 (d, J = 7.5 Hz, 1H, H$_{3'}$), 5.96 (s, 2H, CH$_2$), 4.70 (d, J = 6.0 Hz, 2H, CH$_2$), 2.38 (s, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 170.8, 159.0, 158.9, 144.8, 143.7, 143.5, 140.9, 140.1, 136.9, 132.2, 130.9, 129.8, 129.3, 128.9, 128.1, 126.6, 125.7, 123.5, 99.3, 61.5, 40.3, 18.8; Anal. calcd. for $C_{24}H_{21}Br_2N_3O_2$: C, 53.06; H, 3.90; N, 7.73. Found: C, 53.28; H, 4.22; N, 7.51.

*4.1.5. 1-(2-Methylbenzyl)-3-((5-(m-tolyl)isoxazole-3-carboxamido)methyl)pyridin-1-ium bromide (**5e**)*

Yield: 80%; mp: 215–217 °C; IR (KBr): 3217, 3095, 3017, 2940, 1664, 1609, 1536, 1450 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.59 (t, J = 5.5 Hz, 1H, NH), 9.08 (s, 1H, H$_2$), 8.98 (d, J = 7.0 Hz, 1H, H$_6$), 8.62 (d, J = 7.0 Hz, 1H, H$_4$), 8.19 (t, J = 7.0 Hz, 1H, H$_5$), 7.78 (s, 1H, H$_{2''}$), 7.74 (d, J = 7.5 Hz, 1H, H$_{6''}$), 7.45 (t, J = 7.5 Hz, 1H, H$_{5''}$), 7.38–7.25 (m, 5H, H$_{4'}$, H$_{5'}$, H$_{6'}$, H$_{4''}$, isoxazole), 7.12 (d, J = 7.5 Hz, 1H, H$_{3'}$), 5.95 (s, 2H, CH$_2$), 4.70 (d, J = 5.5 Hz, 2H, CH$_2$), 2.40 (s, 3H, CH$_3$), 2.31 (s, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 170.8, 159.0, 144.8, 143.7, 143.5, 140.1, 138.8, 137.2, 136.9, 132.2, 131.6, 130.9, 129.8, 129.4, 129.3, 128.9, 128.2, 126.7, 126.2, 123.0, 99.9, 61.6, 40.3, 20.9, 18.9; Anal. calcd. for $C_{25}H_{24}BrN_3O_2$: C, 62.77; H, 5.06; N, 8.78. Found: C, 62.89; H, 5.37; N, 8.90.

*4.1.6. 1-(2-Methylbenzyl)-3-((5-(p-tolyl)isoxazole-3-carboxamido)methyl)pyridin-1-ium bromide (**5f**)*

Yield: 80%; mp: 202–204 °C; IR (KBr): 3145, 3050, 2962, 2930, 1729, 1671, 1534, 1504 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.55 (t, J = 6.0 Hz, 1H, NH), 9.05 (s, 1H, H$_2$), 8.96 (d, J = 7.0 Hz, 1H, H$_6$), 8.61 (d, J = 7.0 Hz, 1H, H$_4$), 8.19 (t, J = 7.0 Hz, 1H, H$_5$), 7.89 (d, J = 8.5 Hz, 1H, H$_{6'}$), 7.83 (d, J = 8.0 Hz, 1H, H$_{3'}$), 7.48–7.25 (m, 5H, H$_{4'}$, H$_{5'}$, H$_{2''}$, H$_{6''}$, isoxazole), 7.13–7.10 (m, 2H, H$_{3''}$, H$_{5''}$), 5.94 (s, 2H, CH$_2$), 4.69 (d, J = 6.0 Hz, 2H, CH$_2$), 2.38 (s, 3H, CH$_3$), 2.30 (s, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 169.9, 159.9, 158.1, 144.3, 143.0, 141.0, 140.5, 139.9, 136.6, 132.3, 131.8, 130.9, 129.5, 129.7, 128.5, 126.3, 125.6, 123.5, 116.7, 57.6, 40.4, 21.3, 18.8; Anal. calcd. for $C_{25}H_{24}BrN_3O_2$: C, 62.77; H, 5.06; N, 8.78. Found: C, 62.54; H, 4.96; N, 8.63.

*4.1.7. 3-((5-(3-Methoxyphenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5g**)*

Yield: 80%; mp: 196–198 °C; IR (KBr): 3223, 3092, 3016, 2939, 1664, 1600, 1573, 1534, 1463 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.57 (t, J = 6.0 Hz, 1H, NH), 9.07 (s, 1H, H$_2$), 8.97 (d, J = 6.0 Hz, 1H, H$_6$), 8.62 (d, J = 6.0 Hz, 1H, H$_4$), 8.18 (t, J = 6.0 Hz, 1H, H$_5$), 7.53–7.48 (m, 3H, H$_{3'}$, H$_{5''}$, H$_{6''}$), 7.46 (s, 1H, isoxazole), 7.35–7.25 (m, 3H, H$_{4'}$, H$_{5'}$, H$_{6'}$), 7.14–7.11 (m, 2H, H$_{2''}$, H$_{4''}$), 5.95 (s, 2H, CH$_2$), 4.70 (d, J = 6.0 Hz, 2H, CH$_2$), 3.85 (s, 3H, OCH$_3$), 2.31(s, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 170.3, 159.6, 158.9, 144.7, 144.2, 143.5, 142.8, 139.9, 136.7, 132.1, 132.0, 130.4, 129.2, 128.8, 128.0, 127.2, 126.5, 117.8, 116.7, 110.7, 100.1, 61.4, 55.3, 42.2, 18.6; Anal. calcd. for $C_{25}H_{24}BrN_3O_3$: C, 60.74; H, 4.89; N, 8.50. Found: C, 60.89; H, 4.62; N, 8.33.

*4.1.8. 3-((5-(4-Methoxyphenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5h**)*

Yield: 80%; mp: 210–212 °C; IR (KBr): 3252, 3073, 3019, 2932, 2838, 1678, 1613, 1539, 1505, 1445 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.54 (bs, 1H, NH), 9.06 (s, 1H, H$_2$), 8.97 (d, J = 7.0 Hz, 1H, H$_6$), 8.61 (d, J = 7.0 Hz, 1H, H$_4$), 8.18 (t, J = 7.0 Hz, 1H, H$_5$), 7.88 (d, J = 7.0 Hz, 2H, H$_{2''}$, H$_{6''}$), 7.36–7.26 (m, 4H, H$_{4'}$, H$_{5'}$, H$_{6'}$, isoxazole), 7.13–7.10 (m, 3H, H$_{3''}$, H$_{5''}$, H$_{3'}$), 5.94 (s, 2H, CH$_2$), 4.69 (d, J = 5.0 Hz, 2H, CH$_2$), 3.84 (s, 3H, OCH$_3$), 2.30 (s, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 170.7, 161.2, 159.1, 159.0, 144.8, 143.7, 143.5, 140.2, 136.9, 132.3, 130.9, 129.4, 128.9, 128.2, 127.6, 126.7, 118.9, 114.8, 98.5, 61.6, 55.5, 40.2, 18.8; Anal. calcd. for $C_{25}H_{24}BrN_3O_3$: C, 60.74; H, 4.89; N, 8.50. Found: C, 60.32; H, 4.61; N, 8.62.

*4.1.9. 3-((5-(3,4-Dimethoxyphenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5i**)*

Yield: 80%; mp: 195–197 °C; IR (KBr): 3329, 3019, 2926, 2840, 1672, 1613, 1546, 1508, 1443 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.78–9.69 (m, 1H, NH), 9.38 (s, 1H, H$_2$), 9.30 (d, J = 6.0 Hz, 1H, H$_6$), 8.62 (d, J = 6.0 Hz, 1H, H$_4$), 8.20 (t, J = 6.0 Hz, 1H, H$_5$), 7.43–7.39 (m, 4H, isoxazole, H$_{4'}$, H$_{5'}$, H$_{6'}$), 7.37–7.35 (m, 2H, H$_{2''}$, H$_{6''}$), 7.139–7.10 (m, 2H, H$_{3'}$, H$_{5''}$), 5.97 (s, 2H, CH$_2$), 4.71 (d, J = 5.0 Hz, 2H, CH$_2$), 3.84 (s, 6H, OCH$_3$), 2.38 (s, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 170.6, 161.2, 159.2, 144.7, 143.6, 140.9, 140.3, 136.8, 133.2, 131.0, 130.7, 129.9, 129.2, 128.7, 128.2, 127.6, 124.1, 118.9, 114.8, 109.1, 99.4, 62.2, 55.8, 55.6, 45.2, 21.0; Anal. calcd. for $C_{26}H_{26}BrN_3O_4$: C, 59.55; H, 5.00; N, 8.01. Found: C, 59.80; H, 4.86; N, 7.83.

*4.1.10. 1-(2-Methylbenzyl)-4-((5-phenylisoxazole-3-carboxamido)methyl)pyridin-1-ium bromide (**5j**)*

Yield: 80%; mp: 195–198 °C; IR (KBr): 3388, 3128, 3049, 3008, 2860, 1677, 1641, 1573, 1542, 1450 cm^{-1}; ^{1}H NMR (DMSO- d_6, 500 MHz) δ (ppm): 9.63 (bs, 1H, NH), 9.00 (d, J = 6.0 Hz, 2H, H$_2$, H$_6$), 8.10 (d, J = 6.0 Hz, 2H, H$_3$, H$_5$), 7.94 (d, J = 7.0 Hz, 2H, H$_{2''}$, H$_{6''}$), 7.57–7.56 (m, 3H, H$_{3''}$, H$_{4''}$, H$_{5''}$), 7.42 (s, 1H, isoxazole), 7.37–7.26 (m, 3H, H$_{4'}$, H$_{5'}$, H$_{6'}$), 7.17 (d, J = 7.5 Hz, 1H, H$_{3'}$), 5.93 (s, 2H, CH$_2$), 4.79 (d, J = 6.0 Hz, 2H, CH$_2$), 2.30 (s, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 170.7, 159.2, 159.1, 159.0, 145.0, 144.6, 136.9, 132.3, 130.9, 129.9, 129.3, 129.1, 126.7, 126.2, 126.1, 125.8, 99.9, 60.9, 41.9, 18.7; Anal. calcd. for $C_{24}H_{22}BrN_3O_2$: C, 62.08; H, 4.78; N, 9.05. Found: C, 61.87; H, 4.50; N, 8.89.

*4.1.11. 4-((5-(2-Fluorophenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5k**)*

Yield: 80%; mp: 211–213 °C; IR (KBr): 3198, 3115, 3050, 2945, 1680, 1635, 1590, 1538, 1493 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.76 (t, J = 6.0 Hz, 1H, NH), 9.01 (d, J = 6.5 Hz, 2H, H$_2$, H$_6$), 8.12 (d, J = 6.5 Hz, 2H, H$_3$, H$_5$), 8.02 (td, J = 7.0, 1.5 Hz, 1H, H$_{4''}$), 7.67–7.63 (m, 1H, H$_{6''}$), 7.50 (t, J = 7.0 Hz, 1H, H$_{5''}$), 7.44 (t, J = 7.0 Hz, 1H, H$_{4'}$), 7.37–7.25 (m, 4H, H$_{5'}$, H$_{6'}$, H$_{3''}$, isoxazole), 7.15 (d, J = 7.0 Hz, 1H, H$_{3'}$), 5.93 (s, 2H, CH$_2$), 4.81 (d, J = 6.0 Hz, 2H, CH$_2$), 2.30 (s, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 165.1, 159.2, 158.5 ($J_{C\text{-}F}$ = 250.2 Hz), 157.5, 144.6, 136.9, 133.2 ($J_{C\text{-}F}$ = 7.5 Hz), 132.4, 131.0, 129.4, 129.1, 127.9, 126.7, 126.1, 125.5, 116.7 ($J_{C\text{-}F}$ = 21.2 Hz), 114.2 ($J_{C\text{-}F}$ = 12.5 Hz), 103.0, 102.9, 60.9, 42.0, 18.8; Anal. calcd. for $C_{24}H_{21}BrFN_3O_2$: C, 59.76; H, 4.39; N, 8.71. Found: C, 60.01; H, 4.61; N, 8.96.

*4.1.12. 4-((5-(4-Fluorophenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5l**)*

Yield: 80%; mp: 190–192 °C; IR (KBr): 3433, 3236, 3125, 2964, 2922, 1668, 1640, 1609, 1543, 1507, 1451 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.68 (t, J = 5.5 Hz, 1H, NH), 8.99 (d, J = 6.5 Hz, 2H, H$_2$, H$_6$), 8.10 (d, J = 6.5 Hz, 2H, H$_3$, H$_5$), 8.03 (dd, J = 8.5, 2.5 Hz, 2H, H$_{2''}$,H$_{6''}$), 7.45–7.41 (m, 3H, H$_{3''}$, H$_{5''}$, isoxazole), 7.37–7.26 (m, 3H, H$_{4'}$, H$_{5'}$, H$_{6'}$), 7.14 (d, J = 7.5 Hz, 1H, H$_{3'}$), 5.91 (s, 2H, CH$_2$), 4.79 (d, J = 5.5 Hz, 2H, CH$_2$), 2.30 (s, 3H, CH$_3$); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 169.8, 163.4 ($J_{C\text{-}F}$ = 247.5 Hz), 159.3, 159.1, 144.7, 136.9, 132.4, 131.0, 129.4, 129.1, 128.4 ($J_{C\text{-}F}$ = 8.7 Hz), 126.7, 126.1, 124.5, 122.9, 116.5 ($J_{C\text{-}F}$ = 22.5 Hz), 100.0, 60.9, 41.9, 18.8; Anal. calcd. for $C_{24}H_{21}BrFN_3O_2$: C, 59.76; H, 4.39; N, 8.71. Found: C, 59.95; H, 4.63; N, 8.63.

*4.1.13. 4-((5-(4-Chlorophenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5m**)*

Yield: 80%; mp: 187–189 °C; IR (KBr): 3218, 3119, 2965, 2913, 1667, 1640, 1610, 1541, 1490 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.70 (t, J = 6.0 Hz, 1H, NH), 9.00 (d, J = 6.5 Hz, 2H, H_2, H_6), 8.10 (d, J = 6.5 Hz, 2H, H_3, H_5), 7.99 (d, J = 7.5 Hz, 2H, $H_{2''}$, $H_{6''}$), 7.65 (d, J = 7.5 Hz, 2H, $H_{3''}$, $H_{5''}$), 7.51 (s, 1H, isoxazole), 7.37–7.26 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{6'}$), 7.15 (d, J = 7.5 Hz, 1H, $H_{3'}$), 5.92 (s, 2H, CH_2), 4.79 (d, J = 6.0 Hz, 2H, CH_2), 2.30(s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 169.6, 159.2, 159.1, 159.0, 144.6, 136.9, 135.7, 132.4, 130.9, 129.5, 129.4, 129.1, 127.6, 126.7, 126.1, 125.1, 100.6, 60.9, 41.9, 18.8, Anal. calcd. for $C_{24}H_{21}BrClN_3O_2$: C, 57.79; H, 4.24; N, 8.42. Found: C, 57.93; H, 4.45; N, 8.63.

*4.1.14. 4-((5-(2,4-Dichlorophenyl)isoxazole-3-carboxamido)methyl)-1-(2-methylbenzyl)pyridin-1-ium bromide (**5n**)*

Yield: 80%; mp: 218–220 °C; IR (KBr): 3238, 3126, 2963, 2916, 1672, 1639, 1598, 1534, 1442 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.77 (t, J = 6.0 Hz, 1H, NH), 8.98 (d, J = 6.0 Hz, 2H, H_2, H_6), 8.11 (d, J = 6.0 Hz, 2H, H_3, H_5), 7.99 (dd, J = 8.5, 2.0 Hz, $H_{6''}$), 7.93 (d, J = 2.0 Hz, $H_{3''}$), 7.67 (dd, J = 8.5, 2.0 Hz, 1H, $H_{5''}$), 7.44 (s, 1H, isoxazole), 7.37–7.26 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{6'}$), 7.14 (d, J = 8.5 Hz, 1H, $H_{3'}$), 5.91 (s, 2H, CH_2), 4.80 (d, J = 6.0 Hz, 2H, CH_2), 2.30 (s, 3H, CH_3); ^{13}C NMR (DMSO- d_6, 125 MHz) δ (ppm): 166.7, 159.2, 158.8, 144.6, 137.0, 136.9, 136.3, 132.4, 132.0, 131.1, 130.9, 130.5, 129.4, 129.1, 128.4, 126.7, 126.1, 123.9, 104.2, 60.9, 42.0, 18.8; Anal. calcd. for $C_{24}H_{20}BrCl_2N_3O_2$: C, 54.06; H, 3.78; N, 7.88. Found: C, 53.89; H, 3.62; N, 7.59.

*4.1.15. 4-((5-(4-Bromophenyl)isoxazole-3-carboxamido)methyl)-1-(2-fluorobenzyl)pyridin-1-ium chloride (**5o**)*

Yield: 80%; mp: 200–202 °C; IR (KBr): 3166, 3116, 3047, 2973, 2937, 1675, 1641, 1608, 1547, 1488 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.72 (t, J = 6.0 Hz, 1H, NH), 9.07 (d, J = 6.5 Hz, 2H, H_2, H_6), 8.09 (d, J = 6.5 Hz, 2H, H_3, H_5), 7.89 (d, J = 8.0 Hz, 2H, $H_{2''}$, $H_{6''}$), 7.78 (d, J = 8.0 Hz, 2H, $H_{3''}$, $H_{5''}$), 7.61 (t, J = 8.0 Hz, 1H, $H_{5'}$), 7.53–7.51 (m, 1H, $H_{3'}$), 7.50 (s, 1H, isoxazole), 7.31 (t, J = 8.0 Hz, 2H, $H_{4'}$, $H_{6'}$), 5.94 (s, 2H, CH_2), 4.76 (d, J = 6.0 Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 169.6, 159.5, 159.1, 158.3 ($J_{C\text{-}F}$ = 250.0 Hz), 144.8, 144.5, 139.2, 137.1, 136.5, 135.7, 132.4, 127.7, 126.1, 125.3, 124.4, 116.1 ($J_{C\text{-}F}$ = 21.5 Hz), 100.7, 57.4, 41.9, Anal; calcd. For $C_{23}H_{18}BrClFN_3O_2$: C, 54.95; H, 3.61; N, 8.36. Found: C, 55.18; H, 3.80; N, 8.19.

*4.1.16. 1-(4-Bromobenzyl)-4-((5-(4-bromophenyl)isoxazole-3-carboxamido)methyl)pyridin-1-ium bromide (**5p**)*

Yield: 80%; mp: 203–205 °C; IR (KBr): 3162, 3068, 3026, 2981, 1675, 1636, 1540, 1487 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.64 (t, J = 6.0 Hz, 1H, NH), 9.13 (d, J = 6.0 Hz, 2H, H_2, H_6), 8.08 (d, J = 6.0 Hz, 2H, H_3, H_5), 7.89 (d, J = 8.0 Hz, 2H, $H_{2''}$, $H_{6''}$), 7.77 (d, J = 8.0 Hz, 2H, $H_{3''}$, $H_{5''}$), 7.65 (d, J = 8.0 Hz, 2H, $H_{3'}$, $H_{5'}$), 7.50 (d, J = 8.0 Hz, 2H, $H_{2'}$, $H_{6'}$), 7.48 (s, 1H, isoxazole), 5.83 (s, 2H, CH_2), 4.76 (d, J = 6.0 Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 169.6, 159.2, 159.0, 144.6, 144.2, 133.5, 132.3, 131.2, 130.9, 127.8, 126.2, 125.3, 124.4, 122.8, 100.4, 61.8, 41.9; Anal. calcd. for $C_{23}H_{18}Br_3N_3O_2$: C, 45.43; H, 2.98; N, 6.91. Found: C, 45.58; H, 3.15; N, 7.17.

*4.1.17. 1-(2-Methylbenzyl)-4-((5-(3-nitrophenyl)isoxazole-3-carboxamido)methyl)pyridin-1-ium bromide (**5q**)*

Yield: 80%; mp: 200–202 °C; IR (KBr): 3442, 3164, 3042, 2995, 1674, 1641, 1527, 1443, 1349, 1314 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 9.76 (t, J = 6.0 Hz, 1H, NH), 9.03 (d, J = 6.0 Hz, 2H, H_2, H_6), 8.71 (s, 1H, $H_{2''}$), 8.43–8.38 (m, 2H, $H_{4''}$, $H_{6''}$), 8.12 (d, J = 6.0 Hz, 2H, H_3, H_5), 7.89 (t, J = 6.0 Hz, 1H, $H_{5''}$), 7.76 (s, 1H, isoxazole), 7.37–7.27 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{6'}$), 7.16 (d, J = 7.5 Hz, 1H, $H_{3'}$), 5.94 (s, 2H, CH_2), 4.81 (d, J = 6.0 Hz, 2H, CH_2), 2.31 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz) δ (ppm): 168.4, 159.3, 159.2, 158.9, 148.4, 144.6, 136.9, 132.4, 132.0, 131.2, 130.9, 129.4, 129.1, 127.5, 126.7, 126.1, 125.3, 120.4, 102.0, 60.9, 41.9, 18.8; Anal. calcd. for $C_{24}H_{21}BrN_4O_4$: C, 56.59; H, 4.16; N, 11.00. Found: C, 56.34; H, 4.37; N, 10.81.

4.2. AChE and BChE inhibition assay

All reagents, acetylcholinesterase (AChE, E.C. 3.1.1.7, Type V-S, lyophilized powder, from electric eel, 1000 unit), butyrylcholinesterase (BChE, E.C. 3.1.1.8, from equine serum), acetylthiocholine iodide (ATCI), and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich. *In vitro* anti-ChE activity was exactly performed according to the modified Ellman's method reported in our previous work [26].

4.3. Kinetic studies of AChE inhibition

The reciprocal plots of the 1/V versus 1/[S] were obtained based on the modified Ellman's method [26] using various concentrations of acetylthiocholine or butyrylthiocholine (S) and test compounds (**5n** or **5j**). Each experiment was performed by conducting the reaction within 2 min and recording the progress curve at 405 nm. Finally, double reciprocal plots (1/v vs. 1/[S]) were provided using the slopes of progress curves to obtain the type of inhibition. Also, K*i* was determined by plotting slopes of these reciprocal plots against the concentration of desired compounds and calculated as the intercept on the negative x-axis. All rate measurements were performed in triplicate and data analysis was performed with Microsoft Excel 2013.

4.4. BACE1 enzymatic assay

A FRET-based BACE1 enzyme assay kit was purchased from Invitrogen (former Pan Vera Corporation, Madison, WI) and the assay was carried out according to the manufacturer's instructions (Invitrogen. http://tools.invitrogen.com/content/sfs/manuals/L0724.pdf) [26].

4.5. Neuroprotection effect against Aβ-induced damage

The ability of compound **5n** and **5j** in protecting neuronal PC12 cells against damage induced by $A\beta_{25\text{-}35}$ was evaluated by the MTT assay as reported in our previous study [28].

4.6. Docking study

The pdb file of the AChE (1EVE) and BChE (4BDS) was retrieved from the Brookhaven protein database http://www.rcsb.org/pdb/home/home.do. Subsequently, the co-crystallized ligand and water molecules were removed and polar hydrogen were added using Discovery Studio 4.1. The structure of compounds **5n** and **5j** were sketched and saved in pdb format using Marvin Sketch 15.6.8 www.chemaxon.com. The ligand molecule **5n** and **5j** was saved in pdbqt format after adding Kollman charges. The pdbqt file of ligands was prepared in Auto Dock tools 1.5.6rc. The active site of receptor pdb file for docking was created with dimensions 60, 60, 60 with co-ordinates x, y, z of grid box was set as default. The results were visualized in Discovery Studio 4.1 as 2D and 3D diagrams.

3D structures of AChE and BChE in complex with donepezil and tacrine respectively were retrieved from Brook-haven Protein data bank (www.rcsb.org). To prepare protein for docking simulation, all co-crystallized and water molecules were removed and the protein was converted to the required pdbqt format using Auto dock Tools package (1.5.6rc). The 2D structure of the ligand was prepared using Marvin Sketch 5.8.3, 2012, Chem Axon (www.chemaxon.com); then, 2D structure was converted to 3D format by Marvin sketch and finally, pdbqt format of ligand was prepared using Auto dock Tools python script, *prepare_ligand4.py*.

Docking simulations were performed with Autodock tools (1.5.6rc) within a docking box defined by following parameters: size $x = 15$, size $y = 15$, size $z = 15$ Å, center $x = 2.023$, center $y = 63.295$, center $z = 67.062$. The exhaustiveness was set to 80. The generic algorithm parameters, number of generations runs are 20, population size 150, maximum number of evaluations 2,500,000, maximum number of generations 27,000. At the end of docking simulations, the best docking solutions were selected for further analysis of enzyme-inhibitor interactions. The graphics are depicted using Bio *via* discovery studio visualize 4.1.

Calculated docking score for compounds **5n** and **5j** was −11.05 and 13.15 kcal/mol, respectively in comparison to donepezil (−13.08 kcal/mol).

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