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Design and synthesis of selective acetylcholinesterase inhibitors: arylisoxazole-phenylpiperazine derivatives

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This paper is dedicated to the memory of our unique teacher in Chemistry and Medicinal Chemistry, Professor Abbas Shafiee (1937-2016) .

In this work, a novel series of arylisoxazole-phenylpiperazines **5a-k** were designed, synthesized, and evaluated toward acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Our results revealed that (5-(2-chlorophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5c**)

was the most potent AChE inhibitor with IC_{50} of 21.85 μM. It should be noted that most of synthesized compounds showed no BChE inhibitory activity and (5-phenylisoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5a**) was the most active anti-BChE derivative (IC_{50} = 51.66 μM). Also, kinetic studies for the AChE and BChE inhibitory activity of compounds **5c** and **5a** confirmed that they have simultaneously bound to the catalytic site (CS) and peripheral anionic site (PAS) of both AChE and BChE. Furthermore, docking study of compound **5c** showed desired interactions of that compound with amino acid residues located in the active and peripheral anionic sites. Compound **5c** was also evaluated for its BACE1 inhibitory activity and demonstrated IC_{50} = 76.78 μM. Finally, neuroprotectivity of compound **5c** on Aβ-treated neurotoxicity in PC12 cells depicted low activity.

Keyword: Arylisoxazole, Beta-secretase (BACE1), Cholinesterase, Docking, Kinetic study, Neuroprotection, Phenylpiperazine

Introduction

Alzheimer's disease (AD) is known as a controversial neurodegenerative disorder. It is the most common type of dementia among elderly people in such a manner that their quality of life is directly affected by the various drawbacks.[1] As AD has depicted enormous effects on the patients' mental ability due to gradual death of neuronal cells, a wide range of difficulties such as failure of memory and thinking skills have been emerged. In this respect, it has imposed a huge burden on the societies and health care systems.

The exact origin of AD is not recognized and several factors are involved in the pathogenesis of the diseases.[2] Among them, tau protein aggregation[3] and extracellular plaque deposits of the β-amyloid peptide (Aβ)[4] are two factors have been recently discussed and

investigated. However, treatment strategies and drug discovery have been accomplished *via* the cholinergic hypothesis[5] which is associated with the decreased level of acetylcholine (ACh) in the brain.

ACh is an essential neurotransmitter for the cognitive activities such as attention, learning, memory, and motivation. There are two enzymes known as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) which catalyze the hydrolysis of ACh leading to the reduction of synaptic availability of that substance in the brain. However, BChE plays a minor role in regulating brain ACh as two enzymes differ in rate specificity, kinetics and activity in the different parts of the brain.[6] Inhibition of AChE and BChE have been an important strategy for the management of AD and AChE inhibitors have been found as the main treatment plan. Also, non-cholinergic role of AChE and consequently AChE inhibitors (AChEIs) has attracted lots of attention due to its participation in pro-aggregating activity of Aβ.[7] Amyloid fibril formation can be achieved through various amino acid residues located in the PAS of AChE.[8]

Although AD has been characterized as a multifactorial disease, cholinesterase inhibitors (ChEIs) are still the main target for the treatment of early and moderate stages of the disease since FDA approved drugs including rivastigmine, galantamine, and donepezil are ChEIs[9] (Fig 1).

Heterocyclic compounds have been considered as the effective targets and versatile tools in the enzymatic treatment of AD. In in this respect, design, synthesis and biological evaluation of multi-target heterocycles have been the center of attention to develop potent and novel anti-AD drugs[10-12] as well as diagnostic applications.[13,14] Focusing on the cholinesterase inhibitory activity of heterocycles, design and synthesis of a wide range of compounds such as coumarins,[15] 1,2,3-triazoles,[16-19] isoxazoles,[19,20] imidazoles,[21] pyrazoles,[22] and

quinazolines,[23] etc. have been developed. In this work, following anti-ChE activity of our previously reported isoxazole derivatives **A** and **B**,[19,20] synthesis and biological evaluation of novel isoxazoles connected to phenylpiperazine moiety was reported (Fig. 2).

Design of the target compounds is based on the potent inhibitory activity of isoxazoles which can interact with the CAS and PAS of the AChE [19,20]. Also, the nitrogen atom from phenylpiperazine group plays a key role in the inhibitory activity. It is the positive charge center which is necessary in AChE inhibitory action to interact with the catalytic center of the AChE[24] (Fig. 2).

Results and discussion

Chemistry

Synthesis of desired compounds **5a-k** was carried out as depicted in Scheme 1. The synthetic procedure was started from ethyl 2,4-dioxo-4-arylbutanoate **1** which was prepared according to the literature.[19,20] The reaction of **1** and hydroxylamine hydrochloride in refluxing ethanol afforded compounds **2**. Hydrolysis of the ester group in the presence of potassium hydroxide (KOH) in refluxing MeOH gave the corresponding carboxylic acid derivatives **3**. Finally, reaction of compound **3** and 1-phenylpiperazine **4** in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and hydroxybenzotriazole (HOBt) in dry CH_3CN at room temperature yielded target arylisoxazole-phenylpiperazine derivatives **5**.

AChE and BChE assay

In vitro AChE and BChE inhibitory activity of the synthesized compounds **5a-k** was evaluated using Ellman's method comparing with donepezil and rivastigmine as the reference

drugs (Table 1).[25] The best anti-AChE activity was obtained by compound **5c** possessing 2-chlorophenylisoxazole group (IC_{50} = 21.85 μM). Changing the position of chlorine from *ortho* to *para* position of aryl group (compound **5d**) led to the reduction of AChEI activity (IC_{50} = 70.06 μM). However, compound **5e** possessing two chlorine substituents showed lower activity (IC_{50} = 34.08 μM) than compound **5c** but it was found to be stronger than compound **5d**. Substitution of different halogens such as fluorine and bromine showed versatile anti-AChE activity. Introduction of fluorine into *ortho* position of aryl group connected to isoxazole moiety (compound **5a**) deleted anti-AChE activity (IC_{50} > 100 μM) and compound **5b** possessing 4-fluorophenyl group showed lower activity than compound **5c** almost similar AChEI activity to its counterpart, compound **5d** having 4-chlorophenyl group. Increasing the size of halogen atom in compound **5f** possessing 4-bromophenyl led to lower activity comparing with other 4-halosubstituted compounds (**5b** and **5d**).

As can be seen in Table 1, introduction of electron-donating groups including methyl and methoxy groups (compounds **5g** and **5h**) as well as removal of substituents from aryl group (compound **5k**) eliminated inhibitory activity (IC_{50} > 100 μM). In the case of derivatives possessing electron-withdrawing groups (NO_2, compounds **5i** and **5j**) the position of nitro group was significant in inducing anti-AChE activity in such a manner that compound **5i** having 3-nitrophenyl group connected to isoxazole moiety depicted no activity (IC_{50} > 100 μM) whereas compound **5j** containing 4-nitrophenyl showed moderate inhibitory activity (IC_{50} = 44.14 μM).
It can be concluded that replacement of halogen substituents especially chlorine would be suitable for inducing better AChEI activity in the series of arylisoxazole-phenylpiperazines **5a-k**.

Finally, comparing our results with those reported in our previous studies on anti-AChE activity of isoxazoles **A** and **B**[19,20] revealed that the synthesized compounds **5** were found to be

lower active than 1,2,3-triazole-isoxazole hybrids **A** confirming that replacement of 1,2,3-triazole by phenylpiperazine did not afford higher activity. However, the presence of phenylpiperazine led to higher anti-AChE activity in comparison to compound **B** possessing tryptamine moiety. Also, compound **5c** similar to compound **B** possessed 2-chlorophenyl group connected to isoxazole moiety.

In the case of BChEI activity, most of synthesized compounds were inactive toward BChE. Among them, compounds **5a** and **5k** possessing 2-fluorophenyl and phenyl groups were found to be moderate BChE inhibitors with IC_{50}s of 51.66 and 72.27 μM, respectively. It should be noted these compounds were not AChEIs. Also, BChEI activity of series of compounds **5a-k** was lower than compounds **A** and **B**.

Kinetic study

The most active anti-AChE and anti-BChE compounds **5c** and **5a**, respectively were candidate for kinetic studies of enzymes inhibition using Lineweaver-Burk plots in the presence (three concentrations) and absence of those inhibitors. As indicated in Fig. 3 and 4, graphical analysis of the reciprocal Lineweaver-Burk plots depicted both increasing slopes and intercepts at increasing concentration of both compounds **5c** and **5a** (Fig. 3 and 4, respectively). The reciprocal plots confirmed mixed type of inhibition for both AChE and BChE suggesting that compounds **5c** and **5a** were able to bind both CAS and PAS of AChE and BChE. As shown in Fig. 3 and 4, the inhibition constants Ki were calculated for **5c** and **5a** from the secondary plots of the slope versus the concentration of inhibitors (38.13 μM and 18.19 μM, respectively).

BACE1 enzymatic assay

Beta-secretase 1 (BACE1) is an enzyme which catalyzes beta-site amyloid precursor protein (APP) cleaving enzyme1.[31] In this respect, development of BACE1 inhibitors has been emerged as the versatile therapeutic strategy. Compound **5c**, the most potent anti-AChE derivative was evaluated for its BACE1 inhibitory activity. The calculated IC_{50} indicated 76.78 μM ± 2.65 comparing with OM-99-2 (IC_{50} = 14.70 nM ±2.83) as the reference inhibitor.

Neuroprotective effect against Aβ-induced damage measured in PC12 cells

Neuroprotective effect of compound **5c** against damage induced by $A\beta_{25-35}$ was investigated in PC12 cells by MTT assay.[31] This compound showed negligible activity up to the concentration of 25 μM.

Docking study

1EVE was selected among 100 crystal structures of acetylcholinesterase complexed with different ligands in PDB. The most promising anti-AChE inhibitor **5c** was subjected to dock with 1EVE by using smina in Linux platform. The range of minimized affinity values of the poses of ligand is −11.42 to −9.59 kcal/mol. The Interactions of the best-docked conformation of ligand with the active site residues of acetylcholinesterase (1EVE) is depicted in Fig. 5. Target compound strongly positioned in AChE peripheral anionic site (PAS) to form hydrogen bond with Tyr121 and pi-anion interaction with Asp72 through isoxazole moiety. 2-Chlorophenyl ring surrounded by Phe330, Trp84, and Phe331 near the wall of the active gorge is responsible for the extra activities. Regarding to docking results and inhibitory activity of synthesized compounds,

aromatic ring of the molecule having lipophilic withdrawing group at *ortho* position possessed an optimum fit into the CAS and PAS.[27,28]

As demonstrated in docking study there was no interaction between an electron-rich moiety of compound **5c** and Trp279 of the PAS which is important for the inhibition of AChE-induced Aβ aggregation.[7,8,29]

Molecular docking study of compound **5c** on BACE1 (2qp8) also demonstrated -8.55 to -7.71 kcal/mol as the range of minimized affinity values of that derivative. The ligand formed hydrogen bond with Thr292 through oxygen atom of carbonyl group. Also, Pi-alkyl interaction with Pro131 and Pi-Pi interaction with Tyr132 residues were observed (Fig. 6).

Conclusion

In conclusion, a novel series of arylisoxazole-phenylpiperazines were designed, synthesized, and evaluated as anti-AChE agents. Among them, (5-(2-chlorophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5c**) showed the most potent AChEI activity (IC_{50} = 21.85 μM). However, they generally depicted no significant BChEI activity. Only, compounds **5a** and **5k** showed moderate to low activity (IC_{50}s = 51.66 and 72.27 μM, respectively) which were not active toward AChE. Kinetic as well as docking studies of compounds **5a** and **5c** confirmed their dual inhibitory activity since they could simultaneously bind to amino acid residues located at the CAS and PAS of both the AChE and BChE.

Experimental

Melting points were determined on a Kofler hot stage apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker FT-500, using TMS as an internal standard. IR

spectra were obtained on a Nicolet Magna FTIR 550 spectrophotometer (KBr disks). MS were recorded on an Agilent Technology (HP) mass spectrometer operating at an ionization potential of 70 eV. Elemental analysis was performed on an Elementar Analysensystem GmbH VarioEL CHNS mode. All chemicals were obtained from Merck and compounds **1**, **2**, and **3** were synthesized according to our previous reports.[30]

*General procedure for the synthesis of arylisoxazole-phenylpiperazines **5a-k***

A mixture of compound **3** (1 mmol), EDCI (1.1 mmol), and HOBT (1 mmol) in dry CH_3CN (10 mL) was stirred for 1 h at room temperature. Then, 1-phenylpiperazine **4** (1 mmol) was added to the reaction mixture and stirred for 24-48 h. After completion of reaction as indicated by TLC, the solvent was evaporated under vacuum, dichloromethane (50 mL) was added and the organic layer was washed with saturated $NaHCO_3$ solution (2×50 mL) following with brine (2×50 mL). The organic layer was separated, dried over Na_2SO_4, and concentrated under vacuum to yield the crude product which was purified by recrystallization from petroleum ether/ethyl acetate.

*(5-(2-Fluorophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5a**)*

Yield = 75%, mp 76-78 ºC. IR (KBr, cm^{-1}): 2971, 2914, 284, 1625, 1597. ^1H NMR (500 MHz, $CDCl_3$): 7.97 (t, J = 8.0 Hz, 1H, H4′), 7.47 (dd, J = 13.7, 8.0 Hz, 1H, H3′), 7.30 (t, J = 7.5 Hz, 2H, Ph), 7.26 (s, 1H, H4), 7.22 (t , J = 8.0 Hz, 1H, H5′), 7.03-7.04 (m, 1H, H6′), 6.95 (d, J = 7.5 Hz, 2H, Ph), 6.92 (t, J = 7.5 Hz, 1H, Ph), 4.08 (t, J = 5.0 Hz, 2H, piperazine), 3.99 (t, J = 5.0 Hz, 2H, piperazine), 3.30 (t, J = 5.0 Hz, 2H, piperazine), 3.26 (t, J = 5.0 Hz, 2H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 166.5, 162.5 (d, J_{C-F} = 250.0 Hz), 159.4, 159.3, 150.9, 132.1, 129.3, 127.5, 124.8, 122.8, 120.7, 116.8, 116.4 (d, J_{C-F} = 21.2 Hz), 104.7 (d, J_{C-F} = 11.2 Hz), 50.1, 49.5, 46.9, 42.6. MS (m/z, %): 351 (M^+, 88), 334 (20), 228 (64), 200 (55), 119 (52), 159 (100), 132

(100), 104 (100), 91 (53), 56 (100), 42 (53).Anal. Calcd for $C_{20}H_{18}FN_3O_2$: C, 68.36; H, 5.16; N, 11.96. Found: C, 68.51; H, 5.30; N, 11.76.

*(5-(4-Fluorophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5b**)*

Yield = 85%, mp 155-157 ºC. IR (KBr, cm^{-1}): 3138, 3018, 2917, 2837, 1621, 1503. ^1H NMR (500 MHz, $CDCl_3$): 7.80 (dd, *J* = 8.5, 5.2 Hz, 2H, H2′, H6′) 7.30 (t, *J* = 7.5 Hz, 2H, Ph), 7.19 (t, *J* = 8.5 Hz, 2H, H3′, H5′), 6.97-6.92 (m, 3H, Ph), 6.82 (s, 1H, H4), 3.99-4.12 (m, 4H, piperazine), 3.26-3.31 (m, 4H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 169.4, 164.0 (d, J_{C-F} = 250.6 Hz), 159.2, 159.1, 150.2, 129.2, 128.0, 123.0, 121.6, 116.8, 116.3 (d, J_{C-F} = 21.9 Hz), 100.6, 50.1, 49.5, 46.8, 42.5. MS (m/z, %): 351 (M^+, 11), 228 (15), 190 (23), 159 (50), 132 (88), 104 (100), 77 (96), 56 (80). Anal. Calcd for $C_{20}H_{18}FN_3O_2$: C, 68.36; H, 5.16; N, 11.96. Found: C, 68.10; H, 4.89; N, 12.18.

*(5-(2-Chlorophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5c**)*

Yield = 84%, mp 103-105 ºC. IR (KBr, cm^{-1}): 2913, 2847, 1636, 1598. ^1H NMR (500 MHz, $CDCl_3$): 7.96 (dd, *J* = 7.5, 3.5 Hz, 1H, H6′), 7.53 (dd, *J* = 7.5, 3.5 Hz, 1H, H3′), 7.43-7.41 (m, 2H, H4′, H5′), 7.31-7.28 (m, 3H, Ph, H4), 6.95 (d, *J* = 7.5 Hz, 2H, Ph), 6.92 (t, *J* = 7.5 Hz, 1H, Ph), 4.10 (t, *J* = 5.0 Hz, 2H, piperazine), 3.99 (t, *J* = 5.0 Hz, 2H, piperazine), 3.30 (t, *J* = 5.0 Hz, 2H, piperazine), 3.27 (t, *J* = 5.0 Hz, 2H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 166.8, 159.3, 159.0, 150.9, 132.1, 131.3, 131.0, 129.4, 129.3, 127.2, 125.6, 120.7, 116.8, 105.6, 50.1, 49.5, 46.9, 42.6. MS (m/z, %): 369 ($[M+2]^{+\cdot}$, 20), 367 (M^+, 60), 228 (73), 200 (61), 159 (90), 132 (100), 104 (97), 77 (82), 56 (100), 42 (47).

Anal. Calcd for $C_{20}H_{18}ClN_3O_2$: C, 65.31; H, 4.93; N, 11.42. Found: C, 65.51; H, 5.24; N, 11.58.

*(5-(4-Chlorophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5d**)*

Yield = 62 %, mp 168-170 ºC. IR (KBr, cm^{-1}): 3025, 2912, 2846, 1632, 1598. ^{1}H NMR (500 MHz, $CDCl_3$): 7.74 (d, *J* = 8.5 Hz, 2H, H3′, H5′), 7.47 (d, *J* = 8.5 Hz, 2H, H2′, H6′), 7.29 (t, *J* = 8.5 Hz, 2H, Ph), 6.95 (d, *J*= 8.0 Hz, 2H, Ph), 6.91 (t, *J*= 8.0 Hz, 1H, Ph), 6.85 (s, 1H, H4), 4.11 (t, *J* = 5.0 Hz, 2H, piperazine), 3.98 (t, *J* = 5.0 Hz, 2H, piperazine), 3.30 (t, *J* = 5.0 Hz, 2H, piperazine), 3.26 (t, *J* = 5.0 Hz, 2H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 169.0, 159.2, 158.8, 150.8, 136.8, 129.5, 129.3, 127.2, 125.2, 120.7, 116.8, 101.2, 50.1, 49.5, 46.9, 42.7. MS (m/z, %): 369 ($[M+2]^{+\cdot}$, 9), 367 (M^+, 27), 228 (29), 200 (25), 159 (58), 132 (97), 119 (35), 104 (74), 77 (70), 56 (100), 42 (53). Anal. Calcd for $C_{20}H_{18}ClN_3O_2$: C, 65.31; H, 4.93; N, 11.42. Found: C, 65.11; H, 5.14; N, 11.24.

*(5-(2,4-Dichlorophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5e**)*

Yield = 85%, mp 110-112 ºC. IR (KBr, cm^{-1}): 3127, 2907, 2811, 1632, 1603. ^{1}H NMR (500 MHz, $CDCl_3$): 7.90 (d, *J* = 8.5 Hz, 1H, H6′), 7.56 (d, *J* = 2.0 Hz, 1H, H3′), 7.40 (dd, *J* = 8.5, 2.0 Hz, 1H, H5′), 7.29 (t, *J* = 8.0 Hz, 2H, Ph), 7.28 (s, 1H, H4), 6.95 (dd, *J* = 8.0, 1.0, 2H, Ph), 6.92 (td, *J* = 8.0, 1.0 Hz, 1H, Ph), 4.10 (t, *J* = 5.2 Hz, 2H, piperazine), 3.98 (t, *J* = 5.2 Hz, 2H, piperazine), 3.30 (t, *J* = 5.2 Hz, 2H, piperazine), 3.26 (t, *J* = 5.2 Hz, 2H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 165.8, 159.3, 159.1, 150.8, 136.9, 132.7, 130.9, 130.1, 129.3, 127.8, 124.1, 120.7, 116.8, 105.8, 50.1, 49.4, 46.9, 42.7. MS (m/z, %): 405 ($[M+4]^{+\cdot}$, 1.5), 403 ($[M+2]^{+\cdot}$, 10), 401 (M^+, 15), 228 (21), 200 (20), 173 (14), 159 (44), 132 (85), 104 (62), 77(62), 55 (100), 42 (41). Anal. Calcd for $C_{20}H_{17}Cl_2N_3O_2$: C, 59.71; H, 4.26; N, 10.45. Found: C, 59.57; H, 4.41; N, 10.61.

*(5-(4-Bromophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5f**)*

Yield = 90%, mp 170-172 ºC. IR (KBr, cm^{-1}): 3126, 2973, 2903, 2807, 1631, 1602. ^{1}H NMR (500 MHz, $CDCl_3$): 7.67 (d, *J* = 8.0 Hz, 2H, H3′, H5′), 7.63 (d, *J* = 8.0 Hz, 2H, H2′, H6′), 7.29 (t,

J = 7.5 Hz, 2H, Ph), 6.95 (d, *J* = 7.5 Hz, 2H, Ph), 6.90 (t, *J* = 7.5 Hz, 1H, Ph), 6.87 (s, 1H, H4), 4.11 (t, *J* = 5.0 Hz, 2H, piperazine), 3.98 (t, *J* = 5.0 Hz, 2H, piperazine), 3.30 (t, *J* = 5.0 Hz, 2H, piperazine), 3.26 (t, *J* = 5.0 Hz, 2H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 165.1, 159.2, 159.1, 150.8, 132.4, 129.3, 127.3, 125.6, 125.1, 120.7, 116.7, 101.3, 50.1, 49.4, 46.9, 42.6. MS (m/z, %): 413 ($[M+2]^{+\cdot}$, 14), 411 (M^+, 14), 228 (29), 200 (22), 159 (55), 132 (92), 104 (72), 91 (40), 77 (69), 56 (100), 42 (45). Anal. Calcd for $C_{20}H_{18}BrN_3O_2$: C, 58.26; H, 4.40; N, 10.19. Found: C, 58.42; H, 4.21; N, 10.33.

*(4-Phenylpiperazin-1-yl)(5-(p-tolyl)isoxazol-3-yl)methanone (**5g**)*

Yield = 65%, mp 110-112 ºC. IR (KBr, cm^{-1}): 3030, 2921, 2833, 1649, 1602. ^{1}H NMR (500 MHz, $CDCl_3$): 7.83 (d, *J* = 7.5 Hz, 2H, H2′, H6′), 7.26-7.29 (m, 4H, H3′, H5′, Ph), 6.95-6.92 (m, 3H, Ph), 6.60 (s, 1H, H4), 3.88-3.85 (m, 4H, piperazine), 3.26-3.24 (m, 4H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 164.5, 159.5, 158.4, 150.5, 132.1, 129.6, 129.3, 127.4, 123.9, 120.8, 116.8, 95.9, 50.1, 49.4, 46.1, 42.0, 21.7. MS (m/z, %): 347 (M^+, 8), 322 (21), 161 (20), 132 (42), 119 (55), 104 (32), 91 (100), 77 (44), 56 (38). Anal. Calcd for $C_{21}H_{21}N_3O_2$: C, 72.60; H, 6.09; N, 12.10. Found: C, 72.84; H, 5.82; N, 12.38.

*(5-(3-Methoxyphenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5h**)*

Yield = 70%, mp 110-112 ºC. IR (KBr, cm^{-1}): 3035, 2966, 2906, 2809, 1630, 1574. ^{1}H NMR (500 MHz, $CDCl_3$): 7.41-7.39 (m, 2H, H5′, H6′), 7.33 (s, 1H, H2′), 7.30 (t, *J* = 7.5 Hz, 2H, Ph), 7.01 (d, *J* = 7.5 Hz ,1H, H4′), 6.95 (d, *J* = 7.5 Hz, 2H, Ph), 6.92 (t, *J* = 7.5 Hz, 1H, Ph), 6.85 (s, 1H, H4), 4.11 (t, *J* = 5.0 Hz, 2H, piperazine), 3.98 (t, *J* = 5.0 Hz, 2H, piperazine), 3.30 (t, *J* = 5.0 Hz, 2H, piperazine), 3.26 (t, *J* = 5.0Hz, 2H, piperazine). ^{13}C NMR (125MHz, $CDCl_3$): 165.0, 159.4, 159.1, 154.9, 150.9, 130.3, 129.3, 128.2, 120.7, 118.5, 116.8, 116.7, 111.1, 101.1, 55.4, 50.1, 49.5, 46.9, 42.7. MS (m/z, %): 363 (M^+, 12), 229 (17), 200 (16), 159 (38), 132 (78), 104

(81), 91 (46), 77 (100), 56 (92), 42 (18). Anal. Calcd for $C_{21}H_{21}N_3O_3$: C, 69.41; H, 5.82; N, 11.56. Found: C, 69.61; H, 5.56 N, 11.31.

*5-(3-Nitrophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5i**)*

Yield = 82%, mp 170-172 ºC. IR (KBr, cm^{-1}): 3133, 2913, 2829, 1639, 1597, 1528, 1348. ^1H NMR (500 MHz, $CDCl_3$): 8.66 (s, 1H, H2′), 8.33 (d, *J* = 8.0 Hz, 1H, H4′), 8.13 (d, *J* = 8.0 Hz,1H, H6′), 7.72 (t, *J* = 8.0 Hz,1H, H5′), 7.30 (t, *J* = 7.5 Hz, 2H, Ph), 7.04 (s, 1H, H4), 6.96 (d, *J* = 7.5 Hz, 2H, Ph), 6.93(t, *J* = 7.5 Hz, 1H, Ph), 4.13 (t, *J* = 5.0 Hz, 2H, piperazine), 3.99 (t, *J* = 5.0 Hz, 2H, piperazine), 3.31 (t, *J* = 5.0 Hz, 2H, piperazine), 3.28 (t, *J* = 5.0 Hz, 2H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 167.8, 159.5, 158.8, 150.8, 148.8, 131.4, 130.4, 129.3, 128.3, 125.1, 121.0, 120.8, 116.8, 102.8, 50.1, 49.5, 47.0, 42.8. MS (m/z, %): 378 (M^+, 6), 217 (18), 200 (12), 161 (33), 132 (56), 119 (24), 104 (100), 91 (38), 77 (69), 56 (69), 42 (35). Anal. Calcd for $C_{20}H_{18}N_4O_4$: C, 63.48; H, 4.79; N, 14.81. Found: C, 63.22; H, 4.52; N, 15.12.

*(5-(4-Nitrophenyl)isoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5j**)*

Yield = 78%, mp 201-203 ºC. IR (KBr, cm^{-1}): 3123, 3087, 2899, 2821, 1630, 1600, 1516, 1340. ^1H NMR (500 MHz, $CDCl_3$): 8.40 (d, *J* = 8.3 Hz, 2H, H3′, H5′), 8.22 (d, *J* = 8.3 Hz, 2H, H2′, H6′), 7.61 (s, 1H, H4), 7.24 (t, *J* = 7.5 Hz, 2H, Ph), 6.98 (d, *J* = 7.5, 2H, Ph), 6.83 (t, *J* = 7.5 Hz, 1H, Ph), 3.83 (t, *J* = 5.0 Hz, 4H, piperazine), 3.79 (t, *J* = 5.0 Hz, 4H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 167.8, 159.5, 158.7, 150.8, 148.8, 132.0, 129.3, 126.8, 124.5, 120.8, 116.8, 103.7, 50.1, 49.5, 47.0, 42.8. MS (m/z, %): 378 (M^+, 31), 228 (14), 200 (12), 159 (35), 132 (88), 104 (71), 91 (32), 77 (60), 56 (100), 42 (29). Anal. Calcd for $C_{20}H_{18}N_4O_4$: C, 63.48; H, 4.79; N, 14.81. Found: C, 63.60; H, 4.92; N, 14.92.

*(5-Phenylisoxazol-3-yl)(4-phenylpiperazin-1-yl)methanone (**5k**)*

Yield = 65 %, mp 118-120 ºC. IR (KBr, cm^{-1}): 3060, 3016, 2916, 2840, 1623, 1597. ^{1}H NMR (500 MHz, $CDCl_3$): 7.81 (d, *J* = 7.0 Hz, 2H, H2′, H6′), 7.48 -7.49 (m, 3H, H3′, H4′, H5′), 7.30 (t, *J* = 7.5 Hz, Hz, 2H, Ph), 6.96 (d, *J* = 7.5 Hz, 2H, Ph), 6.92 (t, *J* = 7.5 Hz, 1H, Ph), 6.86 (s, 1H, H4), 4.12 (t, *J* = 5.0 Hz, 2H, piperazine), 3.99 (t, *J* = 5.0 Hz, 2H, piperazine), 3.30 (t, *J* = 5.0 Hz, 2H, piperazine), 3.26 (t, *J* = 5.0 Hz, 2H, piperazine). ^{13}C NMR (125 MHz, $CDCl_3$): 168.1, 159.4, 158.6, 150.5, 130.7, 129.3, 129.1, 126.8, 125.9, 120.7, 116.8, 100.9, 50.1, 49.5, 46.9, 42.7. MS (m/z, %): 333 (M^+, 14), 228 (18), 200 (16), 172 (18), 159 (38), 105 (95), 91 (36), 77 (100), 56 (61), 42 (19). Anal. Calcd for $C_{20}H_{19}N_3O_2$: C, 72.05; H, 5.74; N, 12.60. Found: C, 72.22; H, 5.40; N, 12.41.

AChE and BChE inhibition assay

All chemicals required for the ChE assay were obtained from Sigma-Aldrich. The assay was performed according to our previous reports[19,20] based on the Ellman's method.[25]

Kinetic studies of AChE and BChE inhibition

For estimates of the inhibition model and inhibition constant Ki, reciprocal plots of 1/V versus 1/[S] were obtained using different concentrations of the substrate. For this purpose, all experiments were performed similar to enzyme inhibition assay.[18] The rate of enzymatic reaction was obtained with different concentrations of inhibitor and in the absence of inhibitor. For each experiment, reaction was initiated by adding substrate and progress curves were recorded at 405 nm within 2 min. Next, double reciprocal plots (1/v vs. 1/[s]) were made using the slopes of progress curves to obtain the type of inhibition. Slopes of these reciprocal

plots were then plotted against the concentration of compound, and Ki was determined as the intercept on the negative x-axis.

BACE1 enzymatic assay

The BACE1 enzyme inhibition assay was achieved using a FRET (Forster resonance energy transfer) kit, from Invitrogen (former Pan Vera corporation, Madison, WI) comparing with OM99-2 as the reference inhibitor based on the literature [31].

Neuroprotection effect against Aβ-induced damage

The ability of compound **5c** in protecting neuronal PC12 cells against damage induced by $A\beta_{25-35}$ was examined by the MTT assay as previously described [31].

Molecular modeling and docking internal validation

The 3D X-ray structure was taken from the protein data bank encoded 1EVE. For the preparation of receptor, the hydrogen atoms were added, all water molecules were removed and pH of the protein was adjusted to almost neutral (7.4). Molecular docking was performed using smina which is a fork of AutoDock Vina in Linux platform [32].The autobox ligand option is used for defining bounding box for docking. It generates a box with an 8 Å around the reference ligand in the active site. In order to confirm the validity of the docking protocol, RMSD value was measured between the crystal ligand pose and the generated nine poses of redocked crystal ligand pose. The minimized affinity values for generated poses of redocked crystal ligand pose were −11.62 to −9.81 and -8.55 to -7.71 kcal/mol for AChE (1EVE) and BACE1 (2qp8) inhibitory, respectively.

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Author Contribution Statement

Mina Saeedi contributed to the design of compounds and preparation of manuscript. Dorrin Mohtadi-Haghighi and Mohammad Mahdavi contributed to the synthesis and characterization of compounds. Seyedeh Sara Mirfazli performed the docking study. Roshanak Hariri, Hania lotfian, and Aida Iraji, performed biological tests. Najmeh Edraki and Omidreza Firuzi supervised biological tests and contributed to the preparation of the manuscript. Tahmineh Akbarzadeh supervised all phases of the study.

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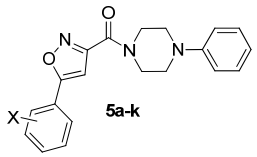
10.1002/cbdv.201800433

Table 1. Anti-cholinesterase activity of arylisoxazole-phenylpiperazines **5a-k**.[a]



Entry	Product 5	X	AChEI (IC_{50}, µM)	BChEI (IC_{50}, µM)
1	**5a**	2-F	>100	51.66±0.15
2	**5b**	4-F	69.12 ± 0.39	>100
3	**5c**	2-Cl	21.85 ± 0.15	>100
4	**5d**	4-Cl	70.06 ± 0.76	>100
5	**5e**	2,4-diCl	34.08 ±0.50	>100
6	**5f**	4-Br	84.55±1.60	>100
7	**5g**	4-Me	>100	>100
8	**5h**	3-MeO	>100	>100
9	**5i**	3-NO_2	>100	>100
10	**5j**	4-NO_2	44.14±0.75	>100
11	**5k**	H	>100	72.27±2.79
12	Donepezil		0.079 ±0.002	5.19±0.38
13	Rivastigmine		11.07±0.01	7.72±0.02

[a] Inhibitor concentration (mean ± SD of three experiments) required for 50% inactivation of AChE and BChE.

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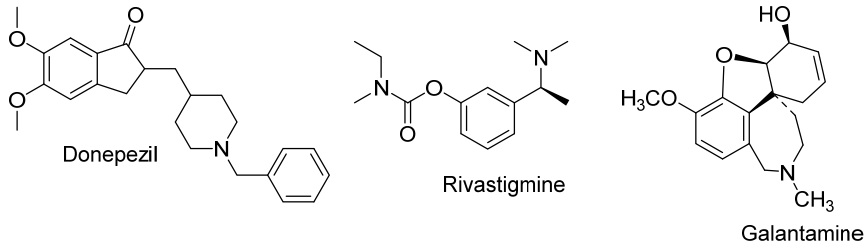

Fig. 1. Marketed cholinesterase inhibitor drugs used for management of Alzheimer's disease.

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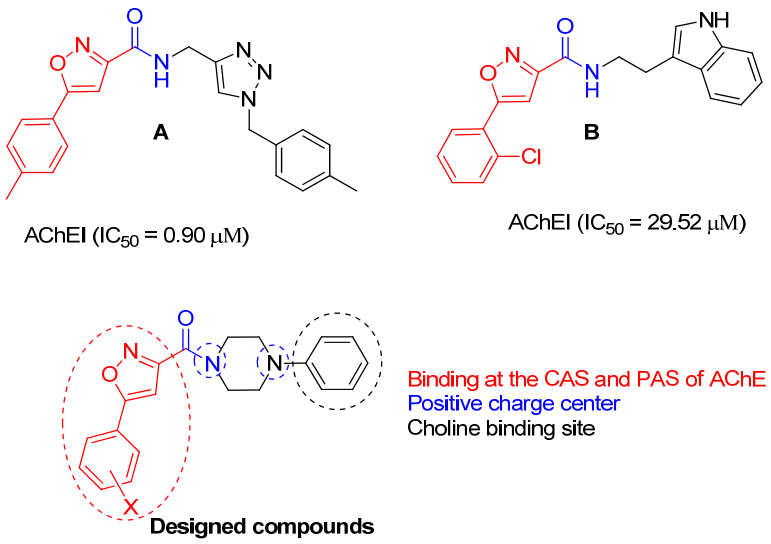

Fig. 2. Design of new isoxazole derivatives based on the anti-AChE activity of compounds **A** and **B**.

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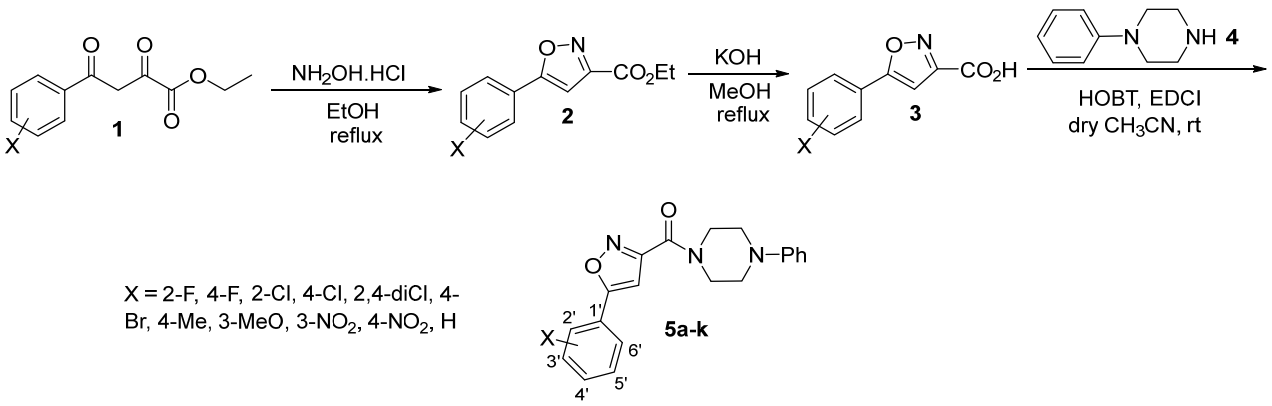

Scheme 1. Synthesis of arylisoxazole-phenylpiperazine derivatives **5a-k**.

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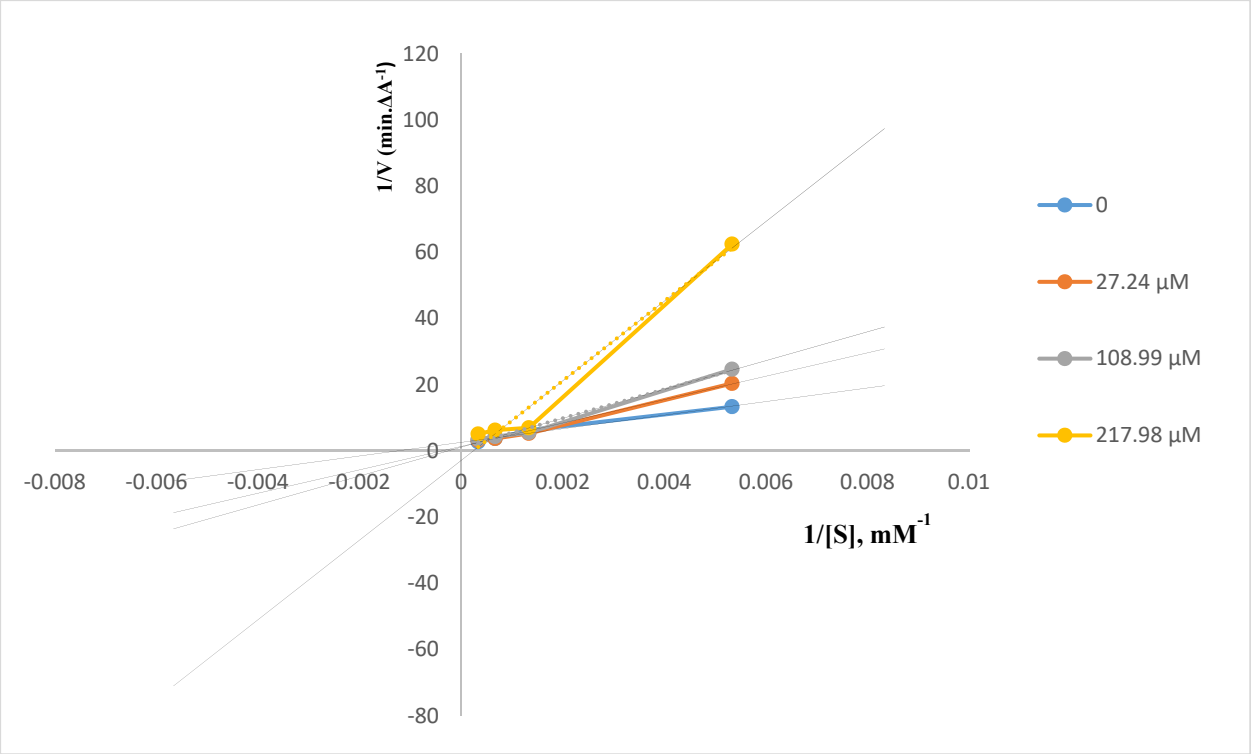
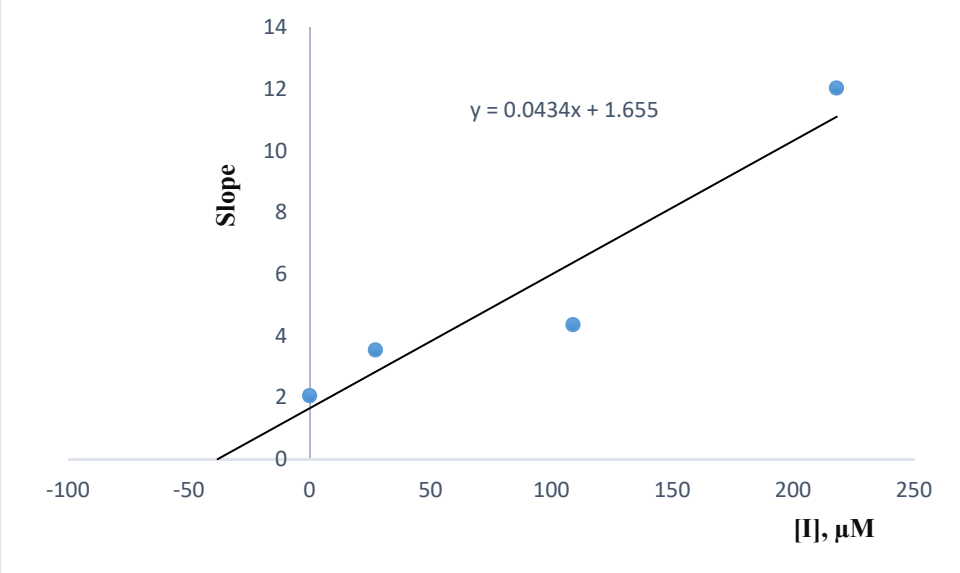


(A)



(B)

Fig. 3. (A): Lineweavere-Burk plot for the inhibition of AChE by compound **5c** at different concentrations of acetylthiocholine (ATCh). **(B):** Steady-state inhibition constant (Ki) of compound **5c**.

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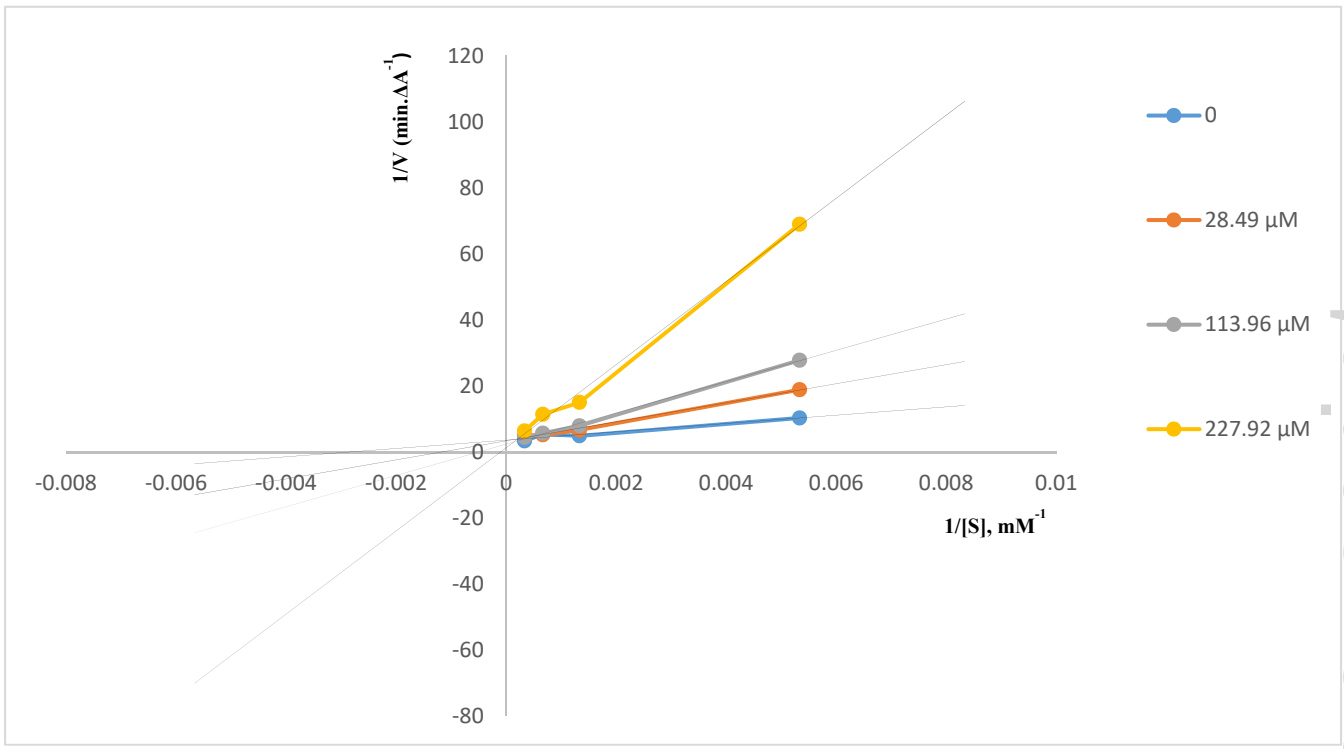
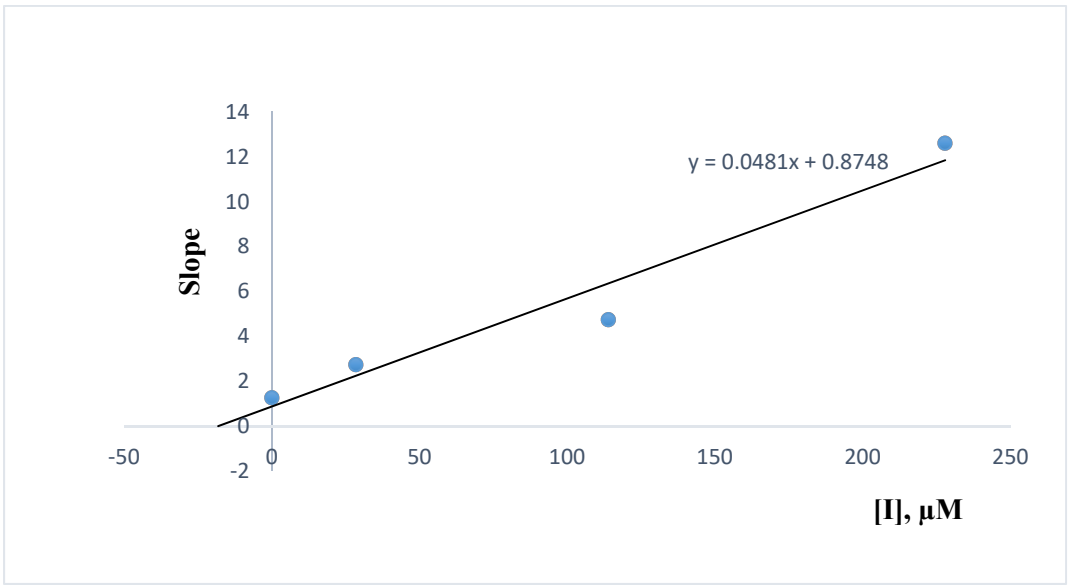


(A)



(B)

Fig. 4. (A): Lineweavere-Burk plot for the inhibition of BChE by compound **5a** at different concentrations of acetylthiocholine (ATCh). **(B):** Steady-state inhibition constant (Ki) of compound **5a**.

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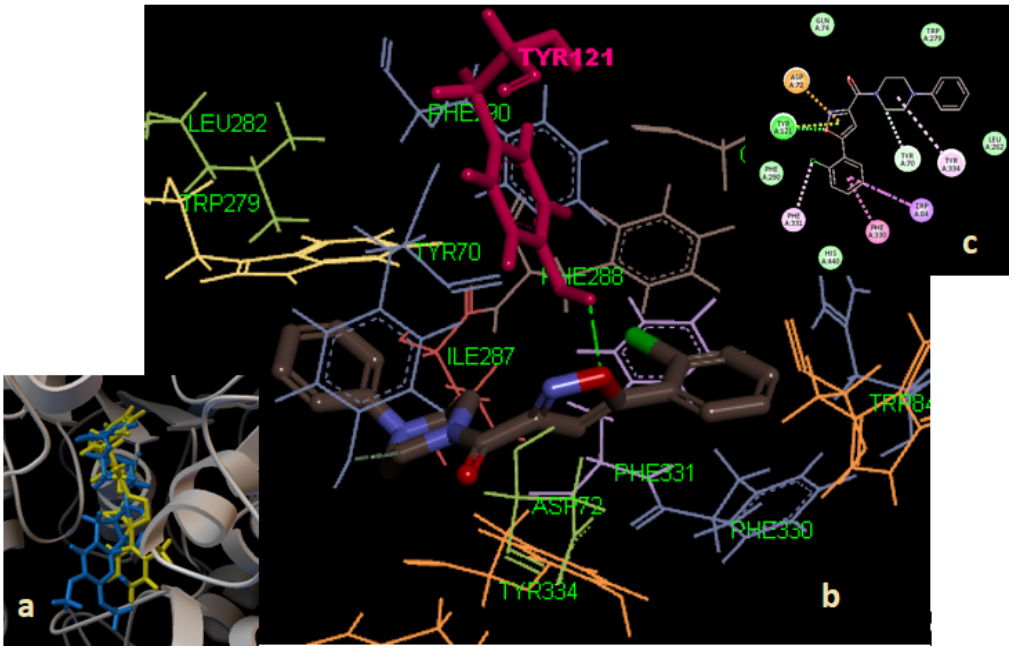

Fig. 5. a) The RMSD value between crystal ligand pose and the best pose of redocked crystal ligand pose is 0.901 b) 3D analysis of the interactions between AChE (1EVE) and compound **5c** b) 2D presentation of different interactions of compound **5c** and 1EVE

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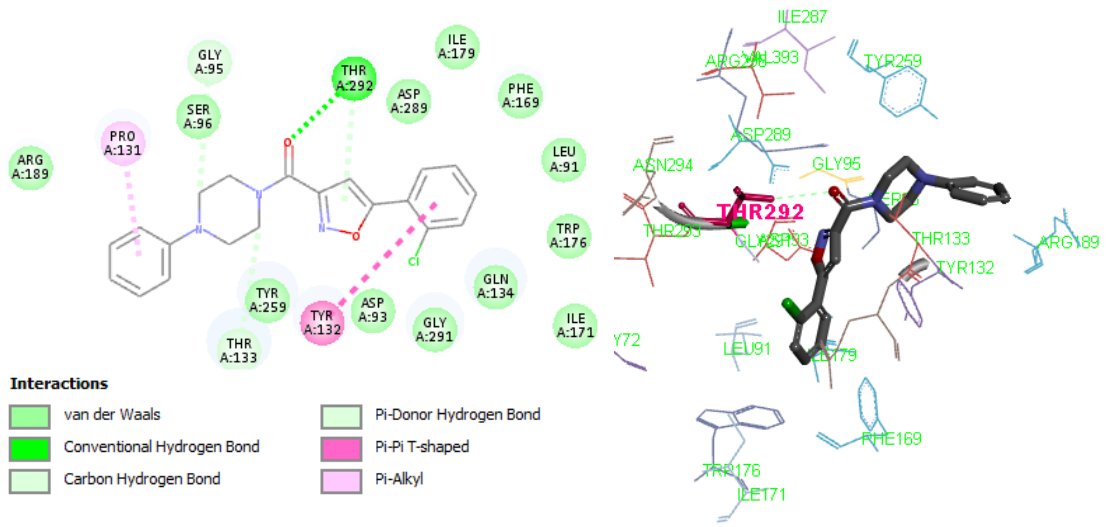

Fig. 6. The proposed orientation and binding mode of compound **5c** in the active site of BACE-1 (2qp8).