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ORIGINAL ARTICLE

Design, synthesis, in vivo and in vitro studies of 1,2,3,4-tetrahydro-9H-carbazole derivatives, highly selective and potent butyrylcholinesterase inhibitors

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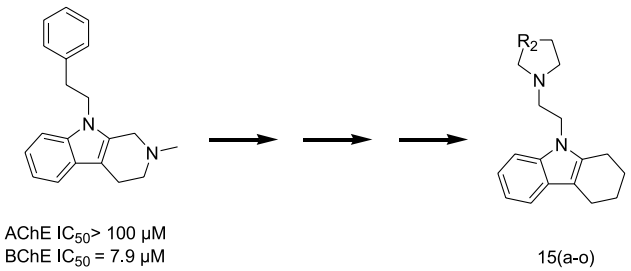
Abstract

Inhibition of butyrylcholinesterase (BChE) might be a useful therapeutic target for Alzheimer's disease (AD). A new series of 1,2,3,4-tetrahydro-9H-carbazole derivatives were designed synthesized and evaluated as BChE inhibitors. While all of the derivatives have shown for AChE IC_{50} values below the detectable limit (> 100 μM), they were selective potent BChE inhibitors. 1-(2-(6-fluoro-1,2,3,4-tetrahydro-9H-carbazole-9-yl)ethyl)piperidin-1-ium chloride (15 g) had the most potent anti-BChE activity (IC_{50} value = 0.11 μM), the highest BChE selectivity and mixed-type inhibition. Pharmacokinetic properties were accordant to Lipinski rule and compound 15g demonstrated neuroprotective and inhibition of β-secretase (BACE1) activities. Furthermore, in vivo study of compound 15g in Morris water maze task has confirmed memory improvement in scopolamine-induced impairment. All results suggest that new sets of potent selective inhibitors of BChE have a therapeutic potential for the treatment of AD.

Graphical abstract

A new series of 1,2,3,4-tetrahydro-9H-carbazole derivatives were designed synthesized and evaluated as BChE inhibitors. While all of the derivatives have shown for AChE IC50 values below the detectable limit, they were selective potent BChE inhibitors. Compound 15g had the most potent anti-BChE activity. All results suggest that new sets of potent selective inhibitors of BChE have a therapeutic potential for the treatment of AD.



Keywords Synthesis · Carbazole · Alzheimer's disease · Butyrylcholinesterase · Neuroprotective activity

Introduction

During the last decades, the number of patients suffering from AD has increased [1]. AD is characterized by neurofibrillary tangle formation, neuronal loss, the presence of plaques of amyloid β-peptide (Aβ) and decreasing

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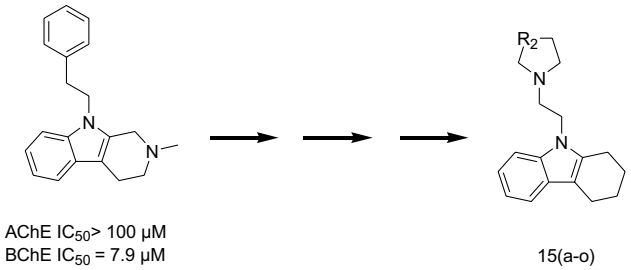
acetylcholine in some regions of the brain such as cortex and hippocampus [2, 3]. According to the cholinergic hypothesis, the dysfunction of cholinergic neurons in the brain leads to the cognitive decline observed in those with AD [4–9].

Acetylcholinesterase (AChE) is an important enzyme regulating amount of acetylcholine (Ach) in the brain. Therefore, recently there was an interest for development of AChE inhibitors [10–14]. Though butyrylcholinesterase (BChE) works as a co-regulator of cholinergic neurotransmission, recent studies show that there is an obvious increase in BChE activity in the most affected areas of the brain. Also, in progressive form of AD, the loss of AChE-activity is compensated by BChE [15]. It is reported that AChE knockout mouse model shows that mice did not suffer from increasing ACh in the absence of AChE, cause of controlling the hydrolysis of ACh by BChE [1]. On the other hand, it is claimed that selective BChE inhibitors may circumvent classical cholinergic toxicity [3]. Therefore, development of new selective BChE inhibitors can provide additional benefits in the treatment of AD. In recent years, there is an interest in development of selective inhibitors of BChE [1, 2, 16–23].

However, selective targeting of BChE over AChE is a challenging task, as both AChE and BChE share about 70% of homology with the main differences in the acyl pocket and the peripheral site. Two aromatic residues (Phe295, Phe297) in the acyl pocket of AChE are replaced with two aliphatic residues (Leu286, Val288) in BChE. This led to access of bulkier inhibitors in the catalytic site of BChE [17].

N_1-phenetylnorcymserine, phenothiazine derivatives and some quinazolinimine-based compounds are selective BChE inhibitors with tri- or polycyclic structures that have been reported previously [16, 24–26]. Also recently, selective indole-containing tricycle BChE inhibitors has been reported as a potential AD treatment [27]. But multistep synthesis of indolo-*N*-substituted carboline derivatives does not seem to be interesting attempt. Therefore, 2-methyl-9-phenethyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole [27] was taken as template, and for simpler synthesis instead of 2-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole, similar tricyclic ring named 1,2,3,4-tetrahydro-9H-carbazole were used. Furthermore, as shown in Scheme 1, our derivatives have different nitrogen-containing heterocycles at the end site of hydrocarbon chain to gain chemical modifications of the heterocyclic core. Also, substitutions on aromatic part of tricyclic ring (R1) were investigated. We have synthesis carbazole-based BChE inhibitors previously [28, 29]. In all, considering simultaneously increase selectivity and anti-BChE activity of indole-containing tricycle derivatives, we have designed a new series of tricycle derivatives.



Scheme 1 Design of compounds 15a–o based on 2-methyl-9-phenethyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole structure

Results and discussion

Cholinesterase inhibition assay

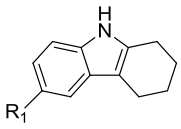
The synthesized compounds (15a–o) were tested for their inhibitory activity toward Electric eel (*Torpedo californica*) AChE (type VI-S) and BChE (E.C.3.1.1.8, from equine serum), using the Elman assay [25–27]. Also, galantamine hydrobromide was used as the reference drug. The IC_{50} values are reported in Table 1.

According to Table 1, BChE IC_{50} values were ranging from 0.11 to 27.84 μM. However, all of the derivatives have shown for AChE IC_{50} values below the detectable limit (> 100 μM). Thus, all designed compounds were selective BChE inhibitor (SI_{BChE}, IC_{50} of AChE/IC_{50} of BChE). This might be a therapeutic advantage for the treatment of AD, because in progressive form of AD, the loss of AChE-activity could be compensated by unaffected BChE [10]. The three most effective BChE inhibitors were compounds 15g, 15d and 15m (IC_{50}: 0.11 ± 0.01, 0.15 ± 0.02 and 0.17 ± 0.03 μM, respectively) with great BChE selectivity ($SI_{BChE,}$ respectively: 892.86, 645.16 and 581.39). According to Table 1, anti-BChE activities of all derivatives except 15b, 15k and 15l were more potent and selective than galantine (BChE IC_{50} of galantamine hydrobromide: 9.4 ± 2.5 μM).

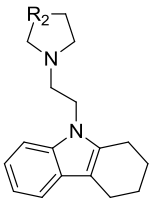
It is realized that compound 7a (2,3,4,9-tetrahydro-1H-carbazole BChE IC_{50}: 89.13 ± 0.01) did not show potent anti-BChE activity in comparison with 15a–o derivatives. So insertion of 2-(heterocycle-1-yl) ethyl moiety seems to be necessary for anti-BChE activity. It seems that anti-BChE activities have increased by replacement of hydrogen in six position of 2, 3, 4, 4a, 9, 9a-hexahydro-1H-carbazole moiety with fluorine, chlorine and methyl. However, introduction of 6-methoxy led to decreasing of anti-BChE activity.

Furthermore, introduction of 2-(piperidin-1-yl)ethyl moiety led to more potent anti-BChE activity than 2-(pyrolidine-1-yl)ethyl and 2-(morpholine-1-yl)ethyl (BChE

Table 1 The IC_{50} values of the compounds 15a–o against AChE and BChE



7a



15(a-o)

Entry	Compounds	R1	R2	AChE inhibition [IC_{50} (μM)]	BChE inhibition [IC_{50} (μM)]	SI BChE (IC_{50} AChE/ IC_{50}BuChE)
1	15a	H	CH_2–CH_2	> 100	0.41 ± 0.04	242.72
2	15b	H	CH_2–O	> 100	21.80 ± 0.01	4.59
3	15c	H	CH_2	> 100	1.88 ± 01	53.19
4	15d	Me	CH_2–CH_2	> 100	0.15 ± 0.02	645.16
5	15e	Me	CH_2–O	> 100	8.20 ± 03	12.19
6	15f	Me	CH_2	> 100	1.70 ± 0.01	58.82
7	15g	F	CH_2–CH_2	> 100	0.11 ± 0.01	892.86
8	15h	F	CH_2	> 100	1.18 ± 0.02	84.74
9	15i	F	CH_2–O	> 100	7.68 ± 0.01	13.02
10	15j	OMe	CH_2–CH_2	> 100	0.85 ± 0.02	117.65
11	15k	OMe	CH_2–O	> 100	27.84 ± 0.01	3.592
12	15l	OMe	CH_2	> 100	22.97 ± 0.02	4.35
13	15m	Cl	CH_2–CH_2	> 100	0.17 ± 0.03	581.39
14	15n	Cl	CH_2–O	> 100	2.64 ± 0.01	37.88
15	15o	Cl	CH_2	> 100	0.73 ± 0.01	136.24
16	7a	H	–	> 100	89.130 ± 0.01	1.12
17	Galantamine	–	–	1.7 ± 0.9	9.4 ± 2.5	0.18

Data are expressed as mean ± SE (three independent experiments)

IC_{50} 15b,e,I,k,n > 15c,f,h,l,o > 15a,d,g,j,m). This was in correlation with higher Pka value for nitrogen in piperidine cycle (PKa: 10.042, 9.945 and 8.006 for piperidine, pyrolidine and morpholine, respectively). Thus, it seems that nitrogen of 2-(heterocycle-1-yl) ethyl moiety should be protonated before interacting with active site of BChE.

Overall, derivatives having piperidine in 2-(heterocycle-1-yl)ethyl moiety were more potent BChE inhibitors in comparison with those having other heterocyclic rings and also the F substituent at six position of 2,3,4,4a,9,9a-hexahydro-1H-carbazole moiety due to its hydrogen binding was the most favorable one for BChE inhibition (15 g has the best BChE $IC_{50} = 0.11 \pm 0.01$ μM).

Kinetic studies of AChE and BChE inhibition

The mechanism of the BChE inhibition for the most potent derivative (15g) was determined by plotting Lineweaver–Burk curves (Fig. 1) [30]. Three different concentrations of 15g 0, 0.06, 0.14 and 0.25 μM) and butyrylcholine iodide 0.13, 0.32 and 0.69 mM) were used as inhibitor and substrate, respectively. It seems that a mixed-type inhibition could be attributed to the compound 15g. Binding of compound 15g to BChE changed both V_{max} and K_m values, a trend that is generally ascribed to mixed-type inhibition. Furthermore, the inhibition constant K_i for compound 15g was calculated ($K_i = 0.05$ μM) using plot of slope versus inhibitor concentration (Fig. 2). As Fig. 1, the Lineweaver–Burk plot reveals that the type of inhibition is mixed (competitive and non-competitive) (variable K_m and V_{max}). This fact implies that the inhibitor could interact with substrate–enzyme complex and intact enzyme as well. When it binds to intact enzyme, the inhibitor can occupy both catalytic triad and peripheral site. In complex, the catalytic triad is occupied by substrate and then the inhibitor just interacts with another site within the active site.

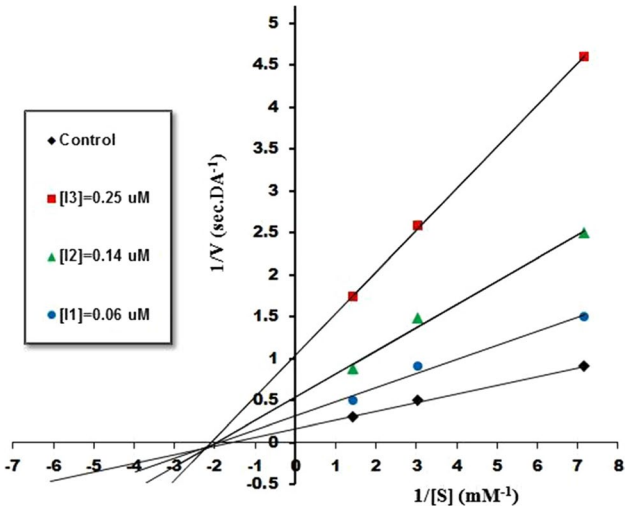


Fig. 1 Kinetic study of compound 15g on the inhibition mechanism of BChE by Lineweaver–Burk plot (1/v) sec.DA^{-1} versus (1/[s]) mM^{-1}

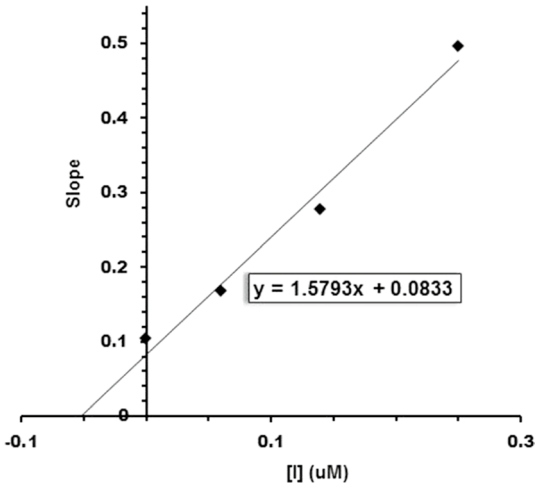


Fig. 2 The plot of the slope versus the concentration of compound 15 g (μM) for calculating K_i

Screening of pharmacokinetic properties

The polar surface area (tPSA), a number of H-bond acceptors (HBA), a number of H-bond donors (HBD), octanole/water partition coefficients (Clog P) and a number of rotatable bonds (RBC) for compound 15g as the most potent inhibitor were calculated. According to Table 2, MW < 500, HBD < 5, HBA < 10 and Clog P < 5 were reported. Thus, pharmacokinetic properties of 15 g are accordant to Lipinski's rule [31] and would have satisfactory pharmacokinetics after the oral admission as drug candidate.

Neuroprotective effect against H_2O_2-induced cell death in PC12 neurons

Additional study was performed to evaluate the neuroprotective activity of compound 15g as the most potent BChE inhibitor, using in vitro MTT assay. H_2O_2 and differentiated PC12 cells were considered as oxidative agent and in vitro model, respectively, and neuroprotective activity of the desired compound was utilized by subjecting PC12 cells to H_2O_2-induced damage. As shown in Fig. 3, data are expressed as mean ± SEM ($n = 8$) and one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparisons test was carried out to determine the level of significance. Also, the percent of cell viability was calculated in comparison with control group. According to Fig. 3, compound 15g has demonstrated good neuroprotective activity at 10 μM (cell viability = 67%, respectively, and $P < 0.01$ versus H_2O_2 treatment alone). But there was no neuroprotective activity at 1 and 100 μM.

β-secretase inhibitory activity of compound 15g

β-secretase inhibitory activity of 15g as the most potent BChE inhibitor was evaluated via a fluorescence resonance energy transfer (FRET)-based BACE-1 kit including β-secretase enzyme and specific APP-based peptide substrate (Rh-EVNL-DAEFK-quencher). Also, experiments were repeated for three times and compared with OM99-2 as the reference compound.

Compound 15g has shown inhibition against β-secretase at the concentration of 50 μM. The non-peptide structure of compound 15g is desired and acts as dual inhibitor of BChE and β-secretase (Table 3).

The in vivo study

The 10 and 20 mg/kg doses of compound 15g significantly increased the time spent in target quadrant in the probe day in compare with control and vehicle groups ($p < 0.001$ vs. scopolamine 4 mg/kg and scopolamine 4 mg/kg + DMSO 5%) but not as the donepezil positive control group (Fig. 4). The 10 mg/kg dose of compound 15g decreased significantly the

Table 2 Molecular descriptors of the compounds 15 g

Entry	Compound	HBD	HBA	ClogP	tPSA[Å2]	MW	RBC
7	15 g	0	3	5.53	6.48	300.42	3

HBD H-bond donors, *HBA* H-bond acceptors, *Clog P* calculated octanol–water partition coefficient, *tPSA* topological polar surface area, *MW* molecular weight (g mol^{-1}), *RBC* rotatable bond count

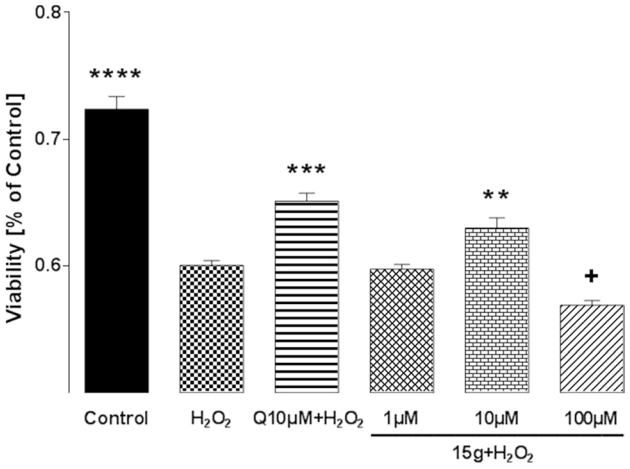


Fig. 3 Neuroprotective effect of compound 15g on the cell viability of the PC12 cells in H_2O_2-induced damage. Data are expressed as mean ± SEM ($n = 8$) and one-way analysis of variance (ANOVA) followed by the Newman–Keuls test was carried out to determine the level of significance. ****$P < 0.0001$; ***$P < 0.001$, **$P < 0.01$ all versus H_2O_2 and $^{+}P < 0.05$ versus H_2O_2 (negatively significant)

Table 3 BACE1 inhibitory activity[a] of compound 15g

Compound	Inhibition[b] at 50 μM (%)	Inhibition[b] at 10 μM (%)
15 g	18.8 ± 0.1	3.2 ± 0.1

[a]The IC_{50} of standard drug **OM99-2** was 3 nM

[b]Values represent mean ± standard error (S.E.) of three independent experiments

mean training period scape latency ($p < 0.0001$ versus scopolamine 4 mg/kg); The 20 mg/kg dose of compound 15g decreased significantly the mean training period scape latency ($p < 0.05$ versus scopolamine 4 mg/kg; Fig. 4). The 10 mg/kg dose of compound 15g decreased significantly the mean training period travelled distance ($p < 0.001$ versus scopolamine 4 mg/kg) but not as the donepezil positive control group (Fig. 4). The 20 mg/kg dose of compound 15g decreased significantly the mean training period travelled distance ($p < 0.05$ versus scopolamine 4 mg/kg). No significant difference was observed for different treatments on swimming speed (Fig. 4). Thus, in vivo study of compound 15g in Morris water maze task confirmed memory improvement in scopolamine-induced impairment [32].

Conclusion

In conclusion, a novel series of 2,3,4,9-tetrahydro-1H-carbazole derivatives were synthesized and evaluated for their anti-BChE activity. In vitro assay revealed that all of the designed compounds were selective BChE inhibitors. This might be a therapeutic advantage for the treatment of AD, because in progressive form of AD the loss of AChE-activity could be compensated by unaffected BChE [10]. Also, most of derivatives were more potent and selective BChE inhibitors than galantamine as the reference drug. Compound 15g (BChE $IC_{50} = 0.11 \pm 0.01$ μM) was the most potent BChE inhibitor. Compound 15g also has shown neuroprotective effect at 10 μM against H_2O_2-induced cell death in PC12 neurons. Also, compound 15g has shown β-secretase activity. Furthermore, in vivo study of compound 15g in Morris water maze task confirmed memory improvement in scopolamine-induced impairment. Pharmacokinetic properties of 15g are accordant to Lipinski rule and would have satisfactory pharmacokinetics after the oral admission as drug candidate. All results suggest that new selective inhibitors of BChE have a therapeutic potential for the treatment of AD.

Experimental

Chemistry

All 1H and 13C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker FT-500, using TMS as an internal standard and also IR spectra were obtained on a Nicolet Magna FTIR 550 spectrophotometer (in KBr). All reagents and solvents were obtained from Sigma-Aldrich and Merck. 2,3,4,9-tetrahydro-1H-carbazole was synthetized according to Fischer Indole Synthesis [29]. Also, all of melting points were determined by a Kofler hot-stage apparatus (Reichert, Vienna, Austria).

Synthesis of 2,3,4,9-tetrahydro-1H-carbazole derivatives (compounds 7a–e)

As shown in Scheme 2, to synthesize different 2,3,4,9-tetrahydro-1H-carbazole derivatives (compounds 7a–e), Fischer Indole Synthesis was utilized. Separately to obtain each of derivatives (7a–e), a mixture of cyclohexanone (10 mol of compound 1) and acetic acid (60 mmol) was collected, heated under reflux and stirred while one of phenyl hydrazine hydrochlorides (10 mol of compounds 2a–e) was added during 1 h. After heating each of mixtures under reflux for an additional hour, they were cooled to room temperature. The resulting solids were separately filtered with suction, washed with 50 ml of water and finally with 50 ml of 75% ethanol. Recrystallization for each solid was performed using 100 ml of methanol (yield 71–75%).

Synthesis of compounds 15a–o

To synthesize compounds 15a–o, TBAB (Tetrabutyl ammonium bromide) (2.73 mmol) was dissolved in an aqueous

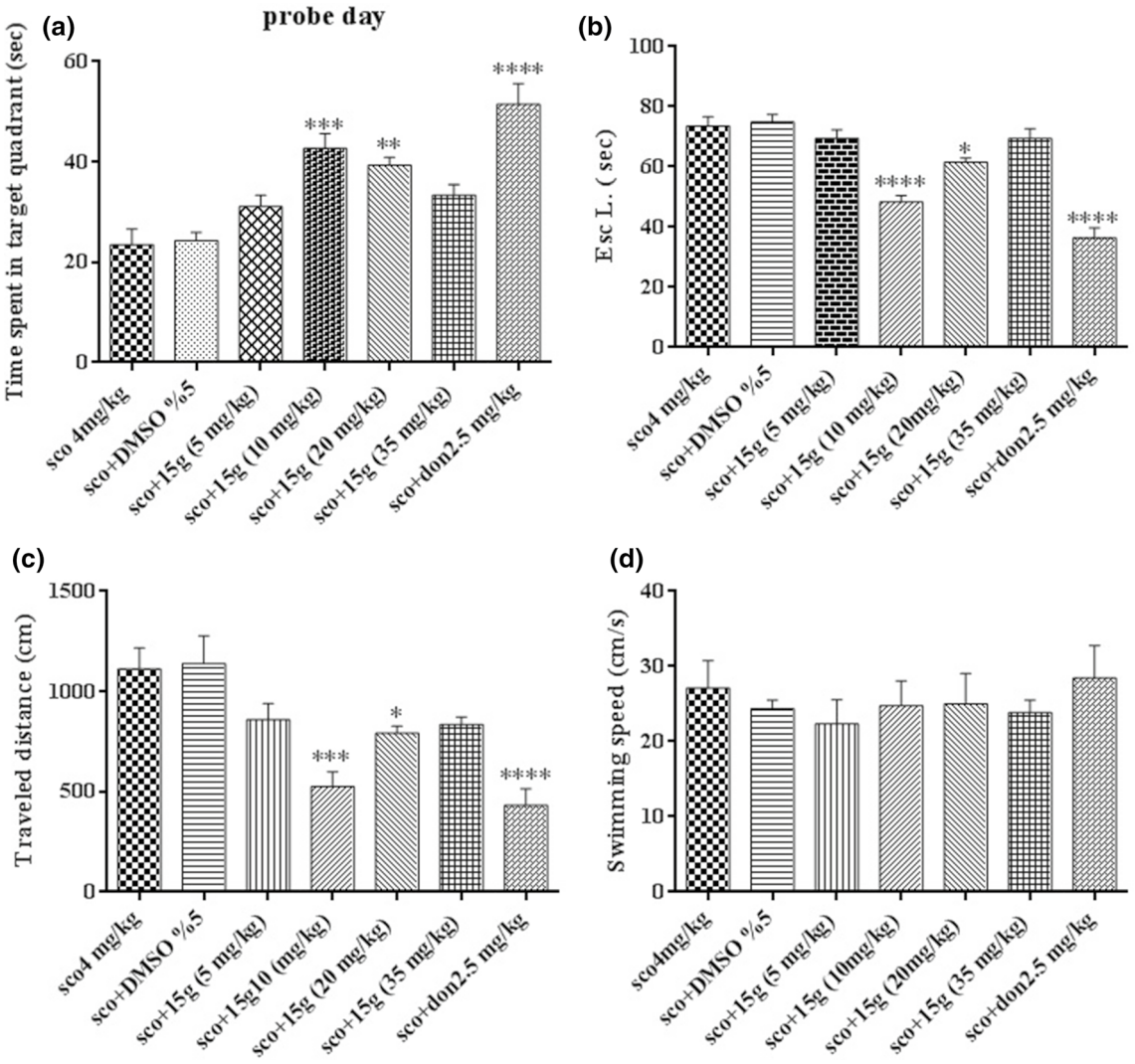


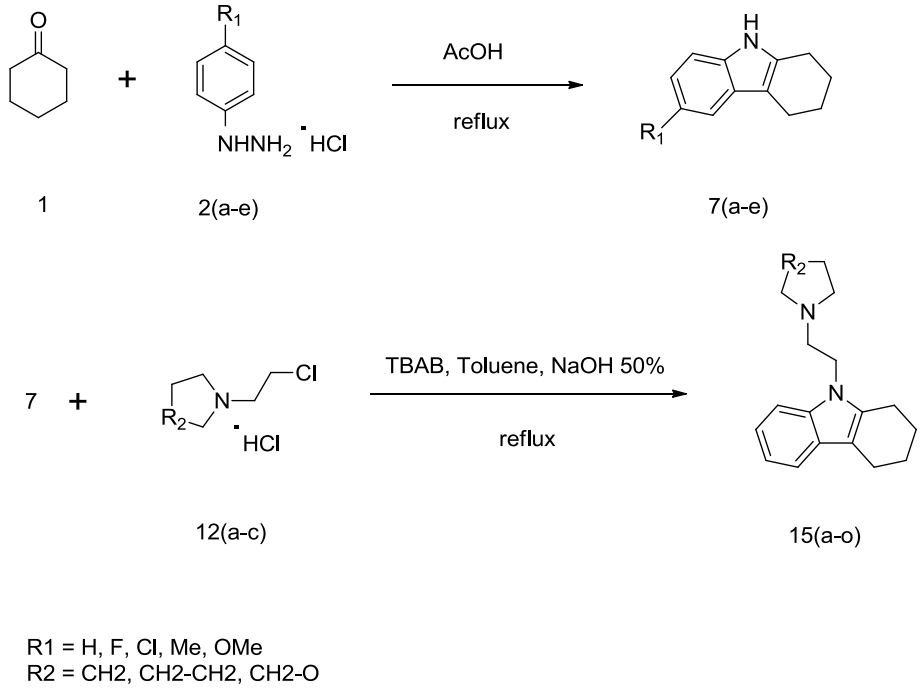
Fig. 4 The in vivo study; Morris water maze. Effects of i.p. infusion for different doses of compound 15g, vehicle (DMSO), and positive control (donepezil) on the time spent in target quadrant in the probe day (***A***), scape latency (***B***), travelled distance (***C***), and swimming speed (***D***). *$P<0.05$; **$P<0.01$; ***$P<0.001$ and ****$P<0.0001$ different from the scopolamine 4 mg/kg control group

solution of NaOH 50% (15 ml) and toluene (15 ml), then stirred for 15 min. Subsequently, a solution of compound 7a–e (5.45 mmol) in toluene (15 ml) was added to the mixture. Compound 12a–c (16.3 mmol) was added to this mixture and stirred at reflux for 6 h [33, 34]. Then, the solvent was evaporated, poured into water and extracted with chloroform (50 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent removed by evaporation. The resulting residue was purified on a silica gel plates using petroleum ether/ethyl acetate (3/1) tank to give solid (50–53% yield) (compound 15a–o).

2,3,4,9-tetrahydro-1H-carbazole (7a) White solid; yield: 70% mp = 117 °C. IR (KBr): 3455, 3048, 2931, 1612, 1580, 1468 cm^{-1}.^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 1.85–1.92$ (m, 4H, tetrahydrocarbazole-CH_2), 2.69–2.72 (m, 4H, tetrahydrocarbazole-CH_2), 7.05–7.09 (m, 2H, $H_{6,7}$), 7.11 (d, $J = 7$ Hz, 1H, H_8), 7.24 (d, $J = 8$ Hz, 1H, H_5), 7.44 (s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 21.10$, 22.20, 23.27, 23.33, 108.59, 109.42, 117.77, 118.60, 120.50, 127.47, 135.28, 136.11 ppm.

6-methyl-2,3,4,9-tetrahydro-1H-carbazole (7b) Yellow solid, yield: 69% mp = 118 °C. IR (KBr): 3408, 3022, 2934, 2849, 1680, 1617, 1472, 1442, 1373 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 1.84–1.87$ (m, 4H, tetrahydrocarbazole-CH_2), 2.43 (s, 3H, CH_3), 2.65–2.67 (m, 4H, tetrahydrocarbazole-CH_2), 6.92 (d, $J = 8$ Hz, 1H, H_7), 7.11 (d, $J = 8$ Hz, 1H, H_8), 7.23 (s, 1H, H_5), 7.45(s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 20.9$, 21.44, 23.23, 23.31, 109.65, 109.95, 117.52, 122.34, 128.06, 133.93, 134.21 ppm.

Scheme 2 Synthesis of compounds 15a–o



6-flouro-2,3,4,9-tetrahydro-1H-carbazole (7c) Yellow solid, yield: 70% mp = 120 °C. IR (KBr): 3433, 2938, 1626, 1587, 1479,1429,1371,1143 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.81–1.94$ (m, 4H, tetrahydrocarbazole-CH_2), 2.64–2.72 (m, 4H, tetrahydrocarbazole-CH_2), 6.85 (d, $J = 7$ Hz, 1H, H_7), 7.09 (d, $J = 7$ Hz, 1H, H_8), 7.25 (s, 1H, H_5), 8 (s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 20.96$, 22.24, 23.10, 23.14, 102.86 ($J_{C–F} = 22.5$), 108.405 ($J_{C–F} = 26.25$), 108.95 ($J_{C\text{-}F} = 10$), 109.62, 127.65, 132.58, 137.02, 157, 158.52 ($J_{C–F} = 231.25$) ppm.

6-methoxy-2,3,4,9-tetrahydro-1H-carbazole (7d) Red solid, yield: 69% mp = 135 °C. IR (KBr): 3416, 2851, 1617, 1584, 1479, 1222 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.86–1.90$ (m, 4H, tetrahydrocarbazole-CH_2), 2.66–2.71 (m, 4H, tetrahydrocarbazole-CH_2), 3.84 (s, 3H, OCH_3), 6.76 (d, $J = 8.5$ Hz, 1H, H_7), 6.93 (d, $J = 8.5$ Hz, 1H, H_8), 7.24 (s, 1H, H_5), 7.54 (s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 20.96$, 23.21, 23.31, 23.35, 56.01, 100.38, 110.07, 110.53, 110.90, 128.25, 130.77, 135.08, 153.91 ppm.

6-chloro-2,3,4,9-tetrahydro-1H-carbazole (7e) Yellow solid, yield: 68% mp = 128 °C. IR (KBr): 3427, 2935, 1611, 1574, 1467,785 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.85–1.92$ (m, 4H, tetrahydrocarbazole-CH_2), 2.64–2.66 (t, 2H, tetrahydrocarbazole-CH_2), 2.70–2.73 (m, 2H, tetrahydrocarbazole-CH_2), 7.04 (d, $J = 8.5$ Hz, 1H, H_7), 7.16 (d, $J = 8.5$ Hz, 1H, H_8), 7.4 (s, 1H, H_5), 7.67(s, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 20.89$, 22.18, 23.06, 23.11, 109.26, 109.50, 117.33, 120.54, 124.36, 128.46, 134.43, 136.86 ppm.

9-(2-(piperidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15a) Cream solid, yield: 60% mp = 54–55.5 °C. IR (KBr): 3459, 3048, 2931, 2850, 2775, 1612, 1580, 1468, 1446, 1375, 738 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.44$ (m, 2H, $H_{4'}$), 1.46-1.62 (m, 4H, $H_{3'}$, $H_{5'}$), 1.84–1.87 (m, 2H, tetrahydrocarbazole-CH_2), 1.91–1.95 (m, 2H, tetrahydrocarbazole-CH_2), 2.46 (t, $J = 4.5$ Hz, 4H, $H_{2'}$, $H_{6'}$), 2.57 (t, $J = 7.5$ Hz, 2H, piperidin-*N*-CH_2), 2.72–2.73 (m, 4H, tetrahydrocarbazole-CH_2), 4.14 (t, $J = 7.5$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 7.02–7.13 (m, 2H, $H_{6,7}$), 7.25 (d, $J = 8.5$ Hz, 1H, H_8), 7.43 (d, $J = 7.5$ Hz, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 21.10$, 22.20, 23.27, 23.33, 24.31, 26.03, 40.87, 55.12, 58.52, 108.59, 109.42, 117.77, 118.60, 120.50, 127.47, 135.28, 136.11 ppm, MS(EI) $m/z = 282(69, M^+)$, 198(5), 184(14), 168(16), 156(11), 128(16), 99(32), 98(100), 77(3), 55(15).

4-(2-(1,2,3,4-tetrahydro-9H-carbazol-9-yl)ethyl)morpholine (15b) Cream solid, yield: 62% mp = 101–102 °C. IR (KBr): 3446, 3044, 2923, 2855, 2830, 1609, 1577, 1468, 1374, 1113, 750 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.85–1.86$ (m, 2H, tetrahydrocarbazole-CH_2), 1.94–1.95 (m, 2H, tetrahydrocarbazole-CH_2), 2.51 (t, $J = 4$ Hz, 4H, $H_{3'}$, $H_{5'}$), 2.70 (t, $J = 6.5$ Hz, 2H, morpholine–N–CH_2), 2.72–2.73 (m, 4H, tetrahydrocarbazole-CH_2), 3.72 (t, $J = 4$ Hz, 4H, $H_{2'}$, $H_{6'}$), 4.16 (t, $J = 6.5$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 7.06–7.28 (m, 2H, $H_{6,7}$), 7.45 (d, $J = 8.5$ Hz, 1H, H_8), 7.46 (d, $J = 7.5$ Hz, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 21.02$, 22.19, 23.24, 40.51, 54.02, 58.01, 66.84, 108.47, 109.68, 117.85, 118.74, 120.60, 127.45,

135.16, 135.94 ppm, MS(EI) *m/z* = 284(66, M^+), 184(30), 168(15), 156(11), 128(9), 100(100), 77(3), 55(21).

9-(2-(pyrrolidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15c) Liquid in room temperature, yield: 61%. IR (KBr): 3607, 3388, 3051, 2924, 2847, 1612, 1587, 1464, 1372, 738 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.80$ (t, $J = 4.5$ Hz, 4H, $H_{3'}$, $H_{4'}$), 1.92 (m, 4H, tetrahydrocarbazole-CH_2), 2.57 (t, $J = 4.5$ Hz, 4H, $H_{2'}$, $H_{5'}$), 2.67 (t, $J = 7$ Hz, 2H, pyrrolidin-N-CH_2), 2.70 (m, 4H, tetrahydrocarbazole-CH_2), 4.12 (t, $J = 7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 6.79 (m, 2H, $H_{6,7}$), 7.25 (d, $J = 8$ Hz, 1H, H_8), 7.46 (d, $J = 7$ Hz, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 21.13$, 22.25, 23.29, 23.37, 24.39, 42.01, 55.40, 58.55, 109.03, 109.46, 118.60, 122.50, 127.03, 127.59, 136.01, 136.15 ppm, MS(EI) *m/z* = 268(11, M^+), 184(3), 168(3), 156(2), 128(1), 84(100), 77(1), 55(13).

6-methyl-9-(2-(piperidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15d) Cream solid, yield: 60% mp = 54.5–55.5 °C. IR (KBr): 3408, 3302, 2934, 2849, 2782, 2706, 1617, 1585, 1472, 1373 cm^{1-}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.44$ (m, 2H, $H_{4'}$), 1.46–1.62 (m, 4H, $H_{3'}$, $H_{5'}$), 1.83–1.92 (m, 4H, tetrahydrocarbazole-CH_2), 2.43 (t, $J = 4.5$ Hz, 4H, $H_{2'}$, $H_{6'}$), 2.44 (s, 3H, CH_3), 2.57 (t, $J = 7.5$ Hz, 2H, piperidin-N-CH_2), 2.68-2.69 (m, 4H, tetrahydrocarbazole-CH_2), 4.11 (t, $J = 7.5$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 6.94 (d, $J = 7$ Hz, 1H, H $_7$), 7.15 (d, $J = 7$ Hz, 1H, H_8), 7.23 (s, $J =$ s, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 21.03$, 21.37, 22.12, 23.23, 24.17, 25.84, 40.62, 54.98, 58.36, 108.19, 108.84, 117.57, 121.89, 127.58, 127.70, 34.33, 135.27 ppm, MS(EI) *m/z* = 296(71, M^+), 198(17), 182(11), 170(10), 156(8), 128(7), 98(100), 77(2), 55(9).

4-(2-(6-methyl-1,2,3,4-tetrahydro-9H-carbazol-9-yl)ethyl)morpholine (15e) Yellow solid, yield: 62% mp = 76–77 °C. IR (KBr): 3026, 2928, 2849, 2806, 2684, 1583, 1468, 1446, 1370, 1114 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.84$–1.85 (m, 2H, tetrahydrocarbazole-CH_2), 1.92–1.93 (m, 2H, tetrahydrocarbazole-CH_2), 2.44 (s, 3H, CH_3), 2.48 (t, $J = 4.5$ Hz, 4H, $H_{3'}$, $H_{5'}$), 2.61 (t, $J = 7$ Hz, 2H, morpholine-N-CH_2), 2.67–2.72 (m, 4H, tetrahydrocarbazole-CH_2), 3.71 (t, $J = 4.5$ Hz, 4H, $H_{2'}$, $H_{6'}$), 4.11 (t, $J = 7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 6.95 (d, $J = 8$ Hz, 2H, H $_7$), 7.14 (d, $J = 8$ Hz, 1H, H_8), 7.24 (d, $J = 7.5$ Hz, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 21.04$, 21.40, 22.22, 23.24, 23.29, 40.56, 54.03, 58.02, 66.85, 108.16, 109.07, 117.69, 122.00, 127.67, 127.89, 134.32, 135.26 ppm, MS(EI) *m/z* = 298(19, M^+), 198(15), 182(4), 170(4), 128(2), 100(100), 77(1), 55(7).

6-methyl-9-(2-(pyrrolidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15f) Cream solid, yield: 61% mp = 71–72 °C. IR (KBr): 3433, 2923, 2849, 2801, 2752, 2673, 1615, 1574, 1476, 1446, 1371 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.79$ (t, $J = 4.5$ Hz, 4H, $H_{3'}$, $H_{4'}$), 1.91 (m, 4H, tetrahydrocarbazole-CH_2), 2.43 (s, 3H, CH_3), 2.57 (t, $J = 4.5$ Hz, 4H, $H_{2'}$, $H_{5'}$), 2.68 (t, $J = 7$ Hz, 2H, pyrrolidin-N-CH_2), 2.69 (m, 4H, tetrahydrocarbazole-CH_2), 4.12 (t, $J = 7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 6.94 (d, $J = 7.5$ Hz, 1H, H $_7$), 7.15 (d, $J = 7.5$ Hz, 1H, H_8), 7.23 (s, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 21.03$, 21.37, 22.12, 23.23, 23.28, 23.48, 42.19, 54.45, 55.66, 108.19, 108.92, 117.59, 121.93, 127.59, 127.71, 134.38, 135.17 ppm, MS(EI) *m/z* = 282(76, M^+), 198(26), 182(14), 170(12), 128(10), 84(100), 77(4), 55(14).

6-fluoro-9-(2-(piperidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15g) Liquid in room temperature, yield: 60%. IR (KBr): 3433, 2938, 2857, 1626, 1587, 1479, 1429, 1371 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.42$ (m, 2H, $H_{4'}$), 1.60 (m, 4H, $H_{3'}$, $H_{5'}$), 1.82–1.85 (m, 4H, tetrahydrocarbazole-CH_2), 2.45 (t, $J = 4.5$ Hz, 4H, $H_{2'}$, $H_{6'}$), 2.56 (t, $J = 7$ Hz, 2H, piperidin-N-CH_2), 2.64–2.70 (m, 4H, tetrahydrocarbazole-CH_2), 4.12 (t, $J = 7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 7.05 (d, $J = 7.5$ Hz, 1H, H_7), 7.15 (d, $J = 7.5$ Hz, 1H, H_8), 7.40 (s, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 20.96$, 22.24, 23.10, 23.14, 24.13, 25.84, 29.68, 40.89, 55.10, 58.29, 102.87 ($J_{C-F} = 22.5$), 108.404 ($J_{C-F} = 26.25$), 108.93 ($J_{C-F} = 10$), 109.61, 127.62, 132.58, 137.03, 157, 158.55 ($J_{C-F} = 231.25$) ppm, MS(EI) *m/z* = 300(6, M^+), 185(4), 137(6), 98(100), 69(45), 55(11).

6-fluoro-9-(2-(pyrrolidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15h) Cream solid, yield: 61% mp = 43.2–44.3 °C. IR (KBr): 3404, 3023, 2959, 2927, 2878, 2809, 1621, 1579, 1477, 1428, 1372 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.82$ (t, $J = 4.5$ Hz, 4H, $H_{3'}$, $H_{4'}$), 1.93 (m, 4H, tetrahydrocarbazole-CH_2), 2.60 (t, $J = 4.5$ Hz, 4H, $H_{2'}$, $H_{5'}$), 2.66 (t, $J = 7$ Hz, 2H, pyrrolidin-N-CH_2), 2.72–2.75 (m, 4H, tetrahydrocarbazole-CH_2), 4.16 (t, $J = 7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 6.85 (d, $J = 7$ Hz, 1H, H_7), 7.08 (d, $J = 7$ Hz, 1H, H_8), 7.25 (s, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 20.96$, 22.24, 23.10, 23.14, 23.50, 42.32, 54.49, 55.62, 102.88 ($J_{C-F} = 22.5$), 108.403 ($J_{C-F} = 26.25$), 108.95 ($J_{C-F} = 10$), 109.62, 127.65, 132.58, 137.02, 157.00, 158.54 ($J_{C-F} = 231.25$) ppm, MS(EI) *m/z* = 286(60, M^+), 216(6), 202(12), 186(15), 146(10), 84(100), 55(18).

4-(2-(6-fluoro-1,2,3,4-tetrahydro-9H-carbazol-9-yl)ethyl)morpholine (15i) Yellow solid, yield: 62% mp = 78–79 °C. IR (KBr): 2962, 2921, 2852, 1622, 1576, 1479, 1366 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 1.81$–1.86 (m, 2H, tetrahydrocarbazole-CH_2), 1.91–1.95 (m, 2H, tetrahydrocarbazole-CH_2), 2.46 (t, $J = 4$ Hz, 4H, $H_{3'}$, $H_{5'}$), 2.57-2.60 (m, 2H, tetrahydrocarbazole-CH_2), 2.65 (t, $J = 6.5$ Hz, 2H,

morpholine-N-CH_2), 2.70-2.72 (m, 2H, tetrahydrocarbazole-CH_2), 3.69 (t, $J=4$ Hz, 4H, $H_{2'}$, $H_{6'}$), 4.09 (t, $J=6.5$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 6.85 (d, $J=7$ Hz, 1H, H_7), 7.09 (d, $J=7$ Hz, 1H, H_8), 7.15 (s, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta=20.64$, 20.93, 22.26, 23.09 ($J_{C\text{-}F}=5$), 29.64, 40.84, 53.83 ($J_{C-F}=55$), 58.02, 102.85 ($J_{C-F}=22.5$), 108.33 ($J_{C-F}=26.25$), 108.87 ($J_{C-F}=10$), 109.55 ($J_{C-F}=3.75$), 127.65 ($J_{C-F}=8.75$), 132.52, 137.06, 156.67, 158.51 ($J_{C-F}=231.25$) ppm, MS(EI) *m/z* = 302(10, M^+), 202(7), 184(3), 174(3), 133(2), 100(100), 55(6).

6-methoxy-9-(2-(piperidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15j) Liquid in room temperature, yield: 60%. IR (KBr): 3416, 2935, 2851, 1617, 1584, 1479, 1429, 1375, 1222 1151 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta=1.48$ (m, 2H, $H_{4'}$), 1.60–163 (m, 4H, $H_{3'}$, $H_{5'}$), 1.85–1.88 (m, 2H, tetrahydrocarbazole-CH_2), 1.90–1.93 (m, 2H, tetrahydrocarbazole-CH_2), 2.50 (t, $J=4.5$ Hz, 4H, $H_{2'}$, $H_{6'}$), 2.57 (t, $J=7$ Hz, 2H, piperidin-N-CH_2), 2.65-2.73 (m, 4H, tetrahydrocarbazole-CH_2), 4.15 (t, $J=7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 7.10 (d, $J=8.5$ Hz, 1H, H_7), 7.16 (d, $J=8.5$ Hz, 1H, H_8), 7.41 (s, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta=20.88$, 22.17, 23.07, 23.11, 24.14, 25.85, 40.88, 55.05, 58.28, 109.25, 109.51, 117.33, 120.54, 124.36, 128.47, 134.42, 136.85 ppm, MS(EI) *m/z* = 312(61, M^+), 214(14), 171(10), 143(6), 98(100), 77(2), 55(13).

4-(2-(6-methoxy-1,2,3,4-tetrahydro-9H-carbazol-9-yl)ethyl) morpholine (15k) Cream solid, yield: 62% mp = 94 °C. IR (KBr): 3431, 2992, 2954, 2826, 1618, 1582, 1482, 1443, 1380, 1222, 1148 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta=1.83$–1.88 (m, 2H, tetrahydrocarbazole-CH_2), 1.91-1.94 (m, 2H, tetrahydrocarbazole-CH_2), 2.48 (t, $J=5$ Hz, 4H, $H_{3'}$, $H_{5'}$), 2.60 (t, $J=7.5$ Hz, 2H, morpholine-N-CH_2), 2.67-2.72 (m, 4H, tetrahydrocarbazole-CH_2), 3.70 (t, $J=5$ Hz, 4H, $H_{2'}$, $H_{6'}$),3.85 (s, 3H, OCH_3), 4.10 (t, $J=7.5$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 6.79 (d, $J=9$ Hz, 1H, tetrahydrocarbazole-CH), 6.93 (s, 1H, H_5), 6.92 (d, $J=9$ Hz, 1H, tetrahydrocarbazole-CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta=21.08$, 22.28, 23.22, 40.72, 54.07, 56.02, 58.14, 66.90, 100.41, 109.14, 110.19, 127.68, 131.25, 135.98, 53.71 ppm, MS(EI) *m/z* = 314(81, M^+), 214(62), 171(15), 143(8), 100(100), 77(2), 55(14).

6-methoxy-9-(2-(pyrrolidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15l) Cream solid, yield: 61% mp = 66.5–66.9 °C. IR (KBr): 3429, 2991, 2948, 2924, 2835, 2775, 1618, 1579, 1484, 1455, 1221, 1146 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta=1.79$ (t, $J=4.5$ Hz, 4H, $H_{3'}$, $H_{4'}$), 1.91 (m, 4H, tetrahydrocarbazole-CH_2), 2.58 (t, $J=4.5$ Hz, 4H, $H_{2'}$, $H_{5'}$), 2.67 (t, $J=7$ Hz, 2H, pyrrolidin-N-CH_2), 2.70 (m, 4H, tetrahydrocarbazole-CH_2), 3.83 (s, 3H, OCH_3), 4.12 (t, $J=7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 6.77 (d, $J=8$ Hz, 1H, tetrahydrocarbazole-CH), 6.90 (s, 1H, H_5), 7.15 (d, $J=8$ Hz, 1H, tetrahydrocarbazole-CH) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta=22.19$, 23.21, 23.47, 42.22, 54.45, 55.70, 55.99, 100.36, 109.15, 110.14, 127.60, 131.26, 135.88, 153.65 ppm, MS(EI) *m/z* = 298(17, M^+), 214(6), 171(4), 143(2), 115(2), 84(100), 56(5).

6-chloro-9-(2-(piperidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15m) Yellow solid, yield: 60% mp = 73 °C. IR (KBr): 3427, 2935, 2847, 2807, 1611, 1574, 1467, 1364, 1304, 1271, 1231, 1123, 854, 785 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta=1.45$ (m, 2H, $H_{4'}$), 1.59–160 (m, 4H, $H_{3'}$, $H_{5'}$), 1.82–1.85 (m, 2H, tetrahydrocarbazole-CH_2), 1.90-1.93 (m, 2H, tetrahydrocarbazole-CH_2), 2.46 (t, $J=4.5$ Hz, 4H, $H_{2'}$, $H_{6'}$), 2.56 (t, $J=7$ Hz, 2H, piperidin-N-CH_2), 2.64-2.72 (m, 4H, tetrahydrocarbazole-CH_2), 4.13 (t, $J=7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 7.05 (d, $J=8.5$ Hz, 1H, H_7), 7.15 (d, $J=8.5$ Hz, 1H, H_8), 7.40 (s, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta=20.89$, 22.18, 23.06, 23.11, 24.13, 25.84, 40.88, 55.05, 58.29, 109.26, 109.50, 117.33, 120.54, 124.36, 128.46, 134.43, 136.86 ppm, MS(EI) *m/z* = 318(13, $M+2)^+$, 316(38, M^+), 218(10), 190(7), 154(10), 127(4), 98(100), 77(1), 55(14).

4-(2-(6-chloro-1,2,3,4-tetrahydro-9H-carbazol-9-yl)ethyl)morpholine (15n) Dark orange solid, yield: 62% mp = 88–89 °C. IR (KBr): 3445, 2938, 2907, 2852, 2804, 1607, 1576, 1464, 1422, 1366, 1302, 1112, cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta=1.84$ (m, 2H, tetrahydrocarbazole-CH_2), 1.92 (m, 2H, tetrahydrocarbazole-CH_2), 2.46 (t, $J=4$ Hz, 4H, $H_{3'}$, $H_{5'}$), 2.59 (t, $J=7$ Hz, 2H, morpholine-N-CH_2), 2.65–2.70 (m, 4H, tetrahydrocarbazole-CH_2), 3.69 (t, $J=4$ Hz, 4H, $H_{2'}$, $H_{6'}$), 4.09 (t, $J=7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 7.05 (d, $J=7.5$ Hz, 1H, H_7), 7.15 (d, $J=7.5$ Hz, 1H, H_8), 7.34 (s, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta=20.85$, 22.21, 23.02, 23.08, 29.66, 40.82, 54.04, 57.96, 66.86, 109.43, 117.37, 120.56, 124.41, 128.48, 134.38, 136.80 ppm, MS(EI) *m/z* = 320(10, $M+2)^+$, 318(28, M^+), 218(81), 190(5), 167(6), 127(2), 100(100), 77(1), 55(18).

6-chloro-9-(2-(pyrrolidin-1-yl)ethyl)-2,3,4,9-tetrahydro-1H-carbazole (15o) Liquid in room temperature, yield: 61μ%. IR (KBr): 3420, 2942, 2851, 1577, 1469, 1375, 1331 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta=1.85$ (t, $J=6$ Hz, 4H, $H_{3'}$, $H_{4'}$), 1.91–1.94 (m, 4H, tetrahydrocarbazole-CH_2), 2.65 (t, $J=6$ Hz, 4H, $H_{2'}$, $H_{5'}$), 2.72 (t, $J=7$ Hz, 2H, pyrrolidin-N-CH_2), 2.84 (m, 4H, tetrahydrocarbazole-CH_2), 4.20 (t, $J=7$ Hz, 2H, tetrahydrocarbazole-N-CH_2), 7.07 (d, $J=7$ Hz, 1H, H_7), 7.21 (d, $J=7$ Hz, 1H, H_8), 7.26 (s, 1H, H_5) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta=20.86$, 22.20, 23.00, 23.08, 23.47, 41.68, 54.38, 55.18, 109.51, 117.46, 120.78, 124.57, 128.56, 134.36, 136.68 ppm, MS(EI) *m/z* = 304(8,

M + 2)$^+$, 302(25, M^+), 218(6), 190(5), 168(6), 154(7), 127(4), 84(100), 77(1), 55(13).

Cholinesterase inhibition assay

AChE and BChE IC_{50} values of all derivatives were obtained using Ellman's method [35–37]. Galantamine hydrobromide was applied as reference drug, purchased from Sigma-Aldrich. Electric eel (*Torpedo californica*) AChE (type VI-S), BChE (E.C.3.1.1.8, from equine serum), acetylthiocholine iodide, butyrylthiocholine iodide and 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB) were purchased from Sigma-Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium hydroxide, and sodium hydrogen carbonate were obtained from Fluka. To obtain desired concentrations, the stock solutions were prepared by dissolving each derivative in dimethyl sulfoxide (DMSO) and then diluted to three different concentrations in absolute ethanol. This assay was performed in triplicate for each concentration to obtain 20–80% enzyme inhibitions. The assay solution consisted of 2-mL phosphate buffer (0.1 M, pH = 8), 60 µL DTNB, 30 µL test compound and 20 µL of 5 IU/mL BChE solution. Then, the above mixture was pre-incubated for 10 min at 25 °C. The reaction was then initiated by adding 20 µL of butyrylthiocholine iodide as substrate to each well (24-well plates). Blank assays were also carried out comprising all ingredients excluding substrate. The absorbance changes were scored at 412 nm for 5 min using a Synergy HTX multimode plate reader. In order to determine IC_{50} values, inhibition curves were drawn (log inhibitor concentration versus percent of inhibition) by Microsoft Excel 2010. Also, the same assay was performed to obtain anti-AChE-activity of all derivatives.

Kinetic studies of BuChE inhibition

In order to the determination of the mechanisms of BuChE inhibitory effect of the 15a–o series, 15g was studied as the most potent compound ($IC_{50} = 0.11 \pm 0.01$ µM). Four different concentrations (0, 0.06, 0.14 and 0.25 µM) were used for preparation of graph. Reciprocal plots of 1/V versus 1/[S] were drawn using for test compound and butyrylthiocholine iodide in there concentrations (0.13, 0.32 and 0.69 mM) as substrate. A secondary plot was obtained using slopes of Lineweaver–Burk plots versus different concentrations of inhibitor (15 g). The intercept of the negative *X*-axis was used for calculation of inhibition constant (K_i) value. All kinetic studies were studied in triplicate.

Computational methods

The Clog P values, tPSA, HBD, and HBA were calculated by the means of Marvine Sketch 6.2.0. Also, RBC was calculated using AutoDockTools-1.5.6.

MTT assay

Neuroprotection assay for compound 15g against H_2O_2-induced cell death in PC12 cells was performed. PC12 cell line was obtained from Pasteur Institute (Iran), and all culture media and supplements were purchased from Gibco (Europe). MTT was purchased from Sigma–Aldrich (Europe). Differentiated PC12 cells were provided as previously described method [26]. Quercetin (10 µM) was used as a positive control. Differentiated PC12 cells were incubated with 10, 50 and 100 µM of 15 g in the volume of 10 µL for 3 h. Subsequently, 375 µM of H_2O_2 was added and incubated for half day. To perform MTT assay 10 all of MTT solution (5 mg/ml) was added and left for 3 h. Then, 100 µL of the 10% SDS in 0.01 M HCl (w/v) as solubilization solution was added into each well and the plates were incubated overnight. Finally, optical density (OD) was measured at 570 nm with a 96-well ELISA plate reader. All MTT assays were repeated three times.

Study of β-secretase inhibition

β-secretase inhibition study was performed using a FRET-based BACE1 enzyme assay kit that was obtained from Invitrogen (former Pan Vera Corporation, Madison, WI). The stock solution of compound 15g was prepared in DMSO and further diluted in assay buffer to prepare different concentrations of it. To 10 µL of BACE1 substrate in separate wells of a black 96-well microplate were added 10 µL of different concentrations of 15 g solutions and mixed slowly. In order to start the reaction, 10 of µL of BACE1 was added to each well and the plates were incubated for 90 min at 25 °C. For quenching the reaction, 10 µL of sodium acetate buffer was added. A multimode microplate reader (BMG Labtech) at excitation and emission wavelength of 545 nm and 585 nm, respectively, was used for fluorescence measurements. Experiments were repeated triplicate and percentage of enzyme inhibitory activity at 10 and 50 µM concentrations of the compound 15g were calculated.

The in vivo study for compound 15g

In vivo study for compound 15g as the most potent BChE inhibitor was performed. In total, 56 adult male albino Wistar rats weighing 200–250 g were used in this experiment. Animals were housed in groups of three per cage in a 12/12 h light/dark cycle with free access to food and water.

They were kept in animal room in which the temperature was maintained at about 24 °C. The animals were randomly divided into different groups. Each animal was used only once. Rats were habituated to their new environment and handled for 3 days before the experimental procedure was started. All experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996) and were approved by the Research and Ethics Committee of Tehran University of Medical Sciences.

Drug

The i.p. 4 mg/kg dose of scopolamine hydrobromide (Sigma-Aldrich, Steinheim, Germany) was used to induce the AD. The i.p. 2.5 mg/kg dose of donepezil hydrochloride (Sigma-Aldrich) was used to treat the AD as the positive control group. The 5% dimethyl sulfoxide, DMSO was used as vehicle.

Behavioral test: Morris water maze (MWM)

Apparatus

Briefly, the Morris water maze consisted of a dark circular pool (136 cm in diameter and 55 cm high) filled with water (24 ± 1 °C) to a depth of 35 cm. A transparent Plexiglas platform (10 cm diameter) was located 1 cm below the water surface in the center of one of designed northeast (NE), southeast (SE), southwest (SW) or northwest (NW) quadrants. The platform provided the only escape from the water and was fixed in its position. Many extra-maze cues were embedded where the water maze was performed such as racks, windows, door, bookshelves, wall color and pictures on the walls surrounded the room. By these fixed cues, the rat could locate the platform to escape from the water. The movement of the animal was monitored by a camera that was mounted above the pool. Animal displacement and its time was recorded using a 3CCD camera (Panasonic Inc., Japan) placed above the MWM pool and locomotion was tracking and measured by etho-vision software (Noldus Information Technology, the Netherlands).

In these series of experiments, time spent in the target quadrant and swimming speed was recorded during 90 s.

Habituation

Twenty-four hours prior to start the training, the rats were habituated to the pool by allowing them to perform a 90-s swimming without the platform.

Procedure

The single training session consisted of four trials with different starting positions that were equally distributed around the maze.

Each rat was placed in the water facing the wall of the tank at one of the four designated starting points (north, east, south and west) and was allowed to swim and find the hidden platform located in the target quadrant of the maze. Each of four starting positions was used once in four training sessions; their order was randomized. During each trial, each rat was given 90 s to find the hidden platform. If the rats could not find the platform during this procedure, they were manually guided to the platform. After finding the platform, the animals were allowed to remain there for 20 s; then they were placed in a holding cage to pass a 30 s rest until the start of the next trial. These procedures were repeated in four consecutive days. After completion of training, the animals were returned to their home cages until the probe trial 24 h later (on the test day). In the probe trial, the hidden platform was removed and the animals were put in the counter side and allowed to swim freely for 90 s. After the probe trial, the platform was elevated above the water surface and placed in the different position and the rats were allowed to swim freely for 120 s in order to test their visual ability.

Effects of i.p. infusion of 5, 10, 20, and 35 mg/kg doses of compound 15g on spatial memory (time spent on target quadrant, scape latency, travelled distance, swimming speed) were investigated through this method [28].

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest, financial or otherwise.

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