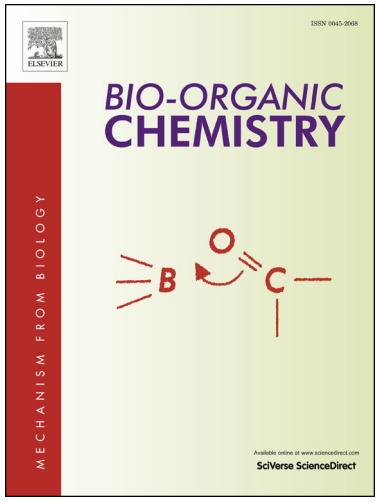

Design and synthesis of newer potential 4-(*N*-acetylamino)phenol derived piperazine derivatives as potential cognition enhancers

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Design and synthesis of newer potential 4-(*N*-acetylamino)phenol derived piperazine derivatives as potential cognition enhancers

Poonam Piplani [a*], Chhanda Charan Danta [a]

[a] *University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh-160 014, India*

[*]Corresponding author

Dr Poonam Piplani

Email: ppvohra28in@yahoo.co.in

Mobile no. +91-9357036068

Professor of Pharmaceutical Chemistry

University Institute of Pharmaceutical Sciences

Chandigarh-160 014, India

6th April, 2015

ABSTRACT

A series of novel hybrids has been designed, synthesized and evaluated for cognition enhancing activities through the inhibition of acetylcholinesterase (AChE) and by passive avoidance mouse model. All the compounds showed excellent AChE inhibition activities and potentially reversed the scopolamine induced memory deficit. Enzyme kinetic and molecular docking studies have confirmed their dual binding affinity and mixed type inhibition. Among them, compounds **1b** and **2d** displayed excellent IC_{50} values of 1.66 μM and 0.49 μM and competitive inhibitor constant *K*i 43.66 μM and 4.10 μM respectively. *Ex vivo* study confirmed their CNS penetration and brain AChE inhibition abilities. Furthermore, **1b** and **2d** showed significant antiamnesic activity at a dose of 1.0 mg/kg as compared to the reference compounds piracetam and rivastigmine. The results indicate that these two compounds emerged to be developed as cognition enhancers worthy of future pursuit.

Keywords: Acetylcholinesterase inhibitors, piperazine derivatives, cognition, passive avoidance stepdown, antiradical activity, molecular docking

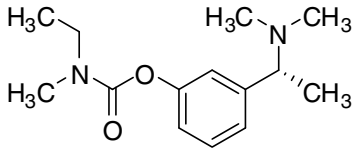
1. Introduction

Cognition is a combination of skills, including knowledge, acquisition, attention, memory, learning, language, perception, skilled motor behaviours, decision making, goal setting, planning and judgements [1]. Cognitive dysfunction is one of the most functionally infirm aspect of many neuropsychiatric disorders and neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson disease (PD), schizophrenia, depression, seizure disorders and traumatic brain injury [2]. It is a very common problem in the over 65 year age group and creates a symptomatic clinical situation that a person has complaints about memory loss and shows the evidence for cognitive impairment. It subsequently progresses towards the most devastating form of clinical dementia, i.e. Alzheimer dementia, shown by around 5% of the population and can be considered as the preclinical stage of AD and the risk factors for AD could also be risk factors for the development and progression of cognitive dysfunction [3-8].

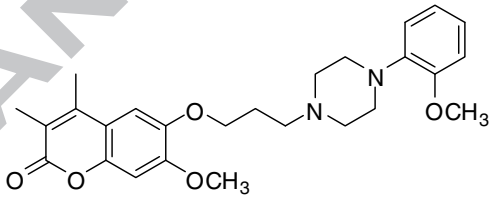
The most excited approach towards the discovery of new cognition enhancers has been based on the functions of the central cholinergic system [9]. It has been found that central cholinergic depletion is the hallmark of AD and experimentally induced cholinergic dysfunction produces cognitive deficits both preclinically and clinically [10]. Although, in the past several decades many cognition enhancers have been discovered, recently research intend has turned towards the development of new acetylcholinesterase inhibitors (AChEIs) as these are capable of suppressing the normal break down of ACh from the synaptic cleft, thereby increasing overall level of ACh available to the relevant postsynaptic receptors [11-14].

The X-ray crystallographic structure of AChE has revealed that its active site gorge lined with aromatic residues about 20 Å deep contains two ligand binding sites. *i. e.* the acylation site (AS) and the peripheral anionic site (PAS). The acylation site (Phe288, Phe290, Phe299) lies at the bottom of the gorge, consisting of a catalytic triad (His440-Glu327-Ser200), and the PAS (Trp279, Tyr70, Tyr121, Asp72, Glu197, Phe290) is located at the entrance of the gorge. The AS also has a quaternary ammonium binding locus consisting of Glu199, Phe330 and Trp84. Ligands can bind selectively to either the acylation site or the peripheral site [15-17]. However recent research has revealed that dual binding site inhibitors of AChE facilitate cholinergic transmission as well as interfere with the synthesis, deposition and aggregation of toxic beta-amyloid (Aβ). Rivastigmine, ensaculin and donepezil (**Fig. 1**) are well known clinically used

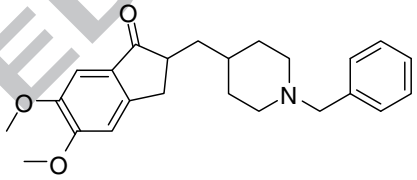
AChEIs for AD therapeutics [18]. Rivastigmine is a 'pseudo-irreversible' inhibitor of AChE and approved for the treatment of mild to moderate cognition deficit [19,20]. Similarly, ensaculin, a piperazine based molecule has shown improvement in memory and cognitive functions including slow down of progressive neurodegeneration. It is reported a dual binding site inhibitor of AChE and has been used to treat AD clinically for a long time [18]. Donepezil is a centrally acting reversible AChEI and reported as a potent anti-amnesic and neuroprotective agent [21]. On this basis, pharmacophoric hybrid (**Fig. 2**) approach has been made to design the novel molecules by hybridizing the pharmacophoric fragments of rivastigmine and ensaculin. 4-(*N*-acetylamino)phenol was selected for the aryloxy fragment with the hope of its possibility to form favourable hydrogen bond, π-π stacking interactions and simultaneously may show free radical scavenging activity. Therefore, *p*-(*N*-acetylamino)phenol derived piperazine derivatives have been synthesized and screened for cognition enhancing activities.



Rivastigmine



Ensaculin



Donepezil

Fig. 1. Structure of clinically used marketed drugs for AD therapeutics.

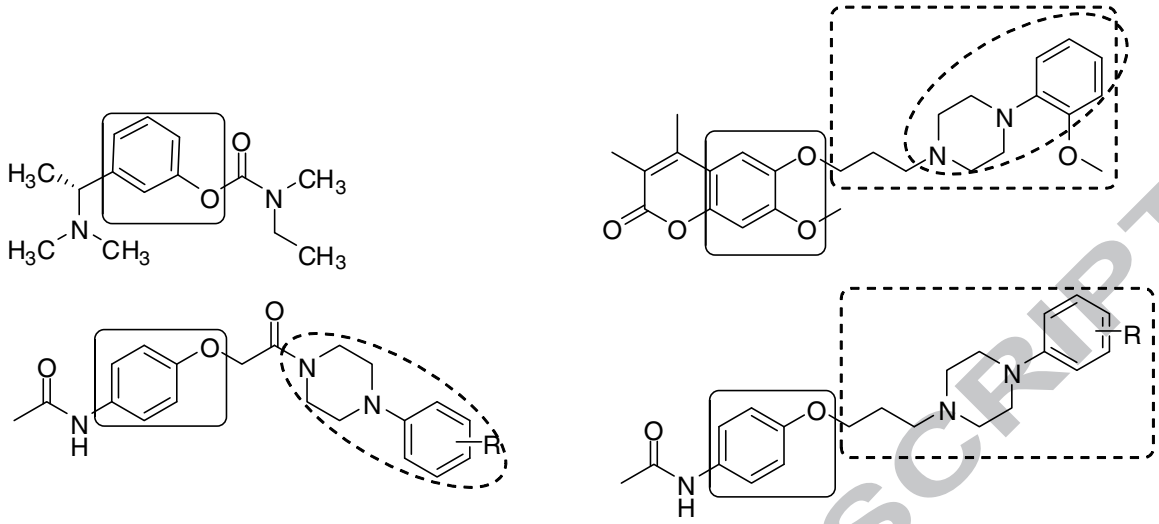
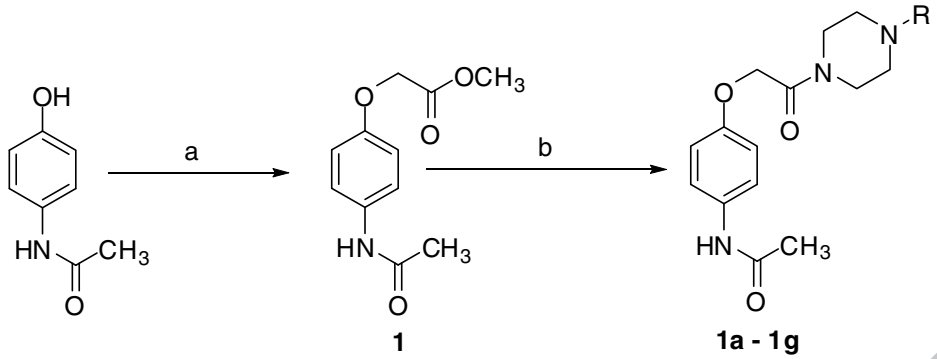


Fig. 2. Pharmacophoric hybrid design strategy of the target compounds.

2. Results and discussion

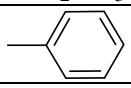
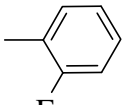
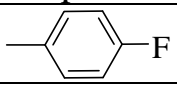
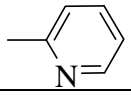
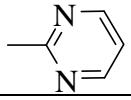
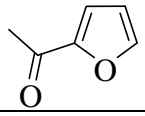
2.1. Chemistry

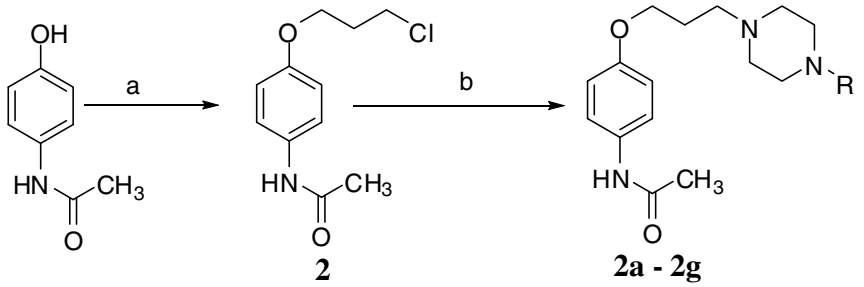
Two series of 4-(*N*-acetylamino)phenol derived piperazine derivatives were designed and synthesized as depicted in schemes **1** and **2**. In both schemes, 4-(*N*-acetylamino)phenol was used as the starting material, anhydrous K_2CO_3 as catalyst and ethyl methyl ketone as the solvent to afford the key intermediates **1** and **2**. The compounds have been synthesized by the fusion (an excellent green chemistry approach; completely avoiding use of any organic solvent) of intermediate **1** with different monosubstituted piperazines (Scheme **1**) whereas the compounds of scheme **2** were synthesized by refluxing the intermediate **2** with different monosubstituted piperazines in the presence of anhydrous K_2CO_3 in ethyl methyl ketone. All the synthesized compounds are in accordance with the IR, ^{1}H NMR, ^{13}C NMR, Mass and CHN analysis data. The synthesized ligands are enlisted in **Table 1** and **Table 2**.



Scheme 1. Reagents and reaction conditions: (a) methylchloroacetate, ethyl methyl ketone, anhyd. K_2CO_3 (b) monosubstituted piperazines, heat

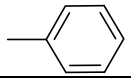
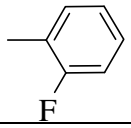
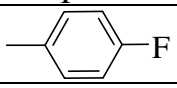
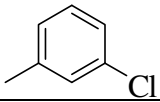
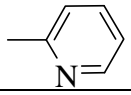
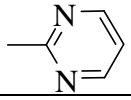
Table 1. Synthesized compounds of scheme 1.

Compounds	R
1a	$-CH_2CH_3$
1b	
1c	
1d	
1e	
1f	
1g	



Scheme 2. Reagents and reaction conditions: (a) bromochloropropane, ethyl methyl ketone, anhyd. K_2CO_3 (b) monosubstituted piperazines, ethyl methyl ketone, anhyd. K_2CO_3

Table 2. Synthesized compounds of scheme 2.

Compounds	R
1a	$-CH_2CH_3$
1b	
1c	
1d	
1e	
1f	
1g	

2.3. Docking

Docking studies of all the hybrids (**1a-1g** and **2a-2g)** on *Tc*AChE demonstrated that they emerged as good dual binding site inhibitors. The ligands were docked with *Tc*AChE through H-bond interaction and π-π stacking interaction at both AS and PAS. The carbonyl oxygen and nitrogen atoms of 4-(*N*-acetylaminophenoxy) moiety was involved in H-bond interaction with the amino acids His440, Arg289, Phe228, Tyr70, Tyr130 and Trp84, Phe228, Ser122, Ser286 respectively. Similarly, the aromatic fragments of the ligands showed π-π stacking interaction

with Trp84, Trp279, Phe330, Phe331 and Tyr334. The N_4 atom of piperazine ring showed H-bond interaction with Glu199 (**1a**, **2a**), Phe288 (**2a**) and His440 respectively. The F atom of **1c** also involved in H-bonding with Glu199. The phenoxy oxygen atom of compound **2b** showed H-bond interaction with Tyr121. Molecular docking images of most active compounds of both series **1b** and **2d** are given in (**Fig. 3** and **Fig. 4**) respectively.

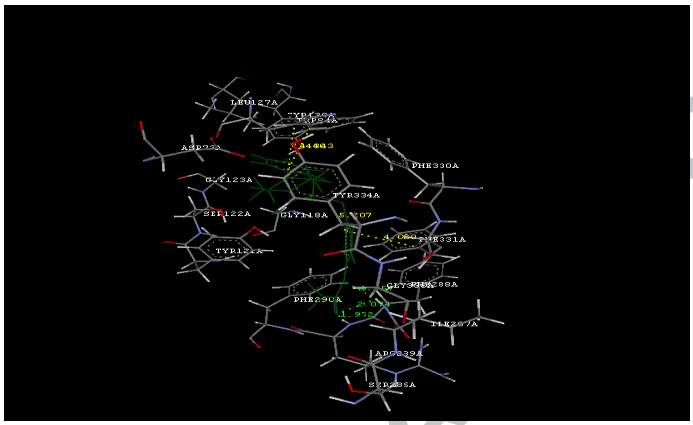


Fig. 3. Molecular docking of compound **1b** into the active sites of *Tc*AChE. Ligand is in green colour, dotted green show H-bond interaction and dotted yellow show π-π stacking interaction.

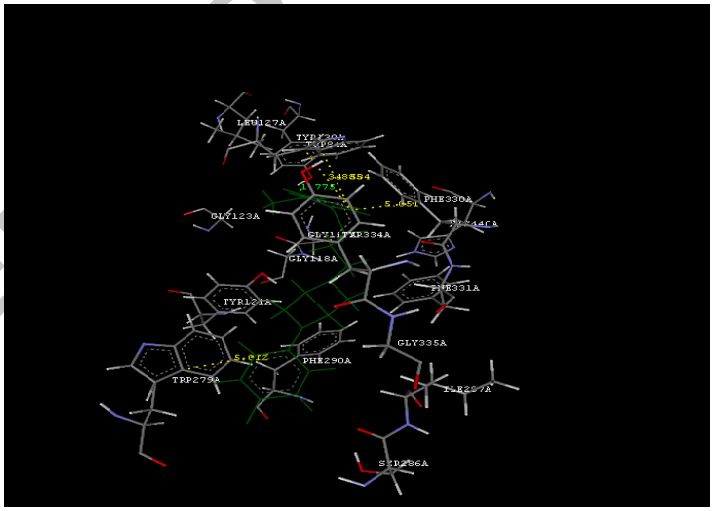


Fig. 4. Molecular docking of compound **2d** into the active sites of *Tc*AChE. Ligand is in green colour, dotted green show H-bond interaction and dotted yellow show π-π stacking interaction.

2.4. Inhibition of AChE

2.4.1. *In vitro* AChE Inhibition

To determine the AChE inhibitory activity of the synthesized compounds (**1a-1g** and **2a-2g**), *in vitro* assay was conducted using Ellman spectrophotometric method [22]. Enzyme kinetics study was also performed to elucidate the mechanism of AChE inhibition. The IC_{50} values and the kinetic parameters were calculated using GraphPad Prism 5 (**Table 3)**. All the compounds exhibited excellent IC_{50} values. Compounds **1b** and **2d** showed IC_{50} values of 1.66 µM and 0.49 µM respectively. It affirmed that the relationship between substrate concentration and reaction velocity was in good agreement with Michaelis-Menten kinetics. Overlaid reciprocal Lineweaver-Burk plots were drawn for compounds **1b** and **2d** (**Fig. 5** and **Fig. 6**) and their graphical analysis showed increasing slopes (decreased V_{max}) and increasing intercepts (higher K_m) with the increase in inhibitor concentrations which indicated of a mixed type inhibition. However, graphical analysis of rivastigmine showed unaffected V_{max} and increased K_m which confirmed competitive type inhibition. The kinetic results of the synthesized ligands strongly supported that these are dual binding site inhibitors. The competitive inhibition constant K_i of compounds **1b** and **2d** are calculated as 43.66 µM and 4.10 µM respectively.

Table 3. Enzyme Kinetics of the synthesized compounds (**1a**-**2g**) on AChE activity

Compd/Inhibitor	IC_{50} ± SEM (µM)	K_m ± SEM (µM)	V_{max} ± SEM (µmol/L/min)	K_i ± SEM (µM)	Type of inhibition
1a	57.28 ± 0.04	1.16±2.26	55±1.33	15.77±0.03	Mixed
1b	1.66 ± 0.03	13.32±3.37	51±1.65	43.66±1.25	Mixed
1c	30.24 ± 0.02	1.26±1.93	90±2.39	46.81±0.39	Mixed
1d	43.63 ± 0.05	2.16±3.25	48±2.78	5.45±0.91	Mixed
1e	51.12 ± 0.03	3.78±3.36	73±1.77	8.95±2.23	Mixed
1f	15.58 ± 0.03	1.28±2.54	46±1.67	34.83±3.42	Mixed
1g	10.66 ± 0.03	2.09±1.97	65±2.35	19.47±1.52	Mixed
2a	79.80 ± 0.03	1.56±1.92	106±3.67	117.0±3.33	Mixed
2b	1.39 ± 0.02	1.52±1.27	88±3.45	2.28±3.43	Mixed
2c	1.36 ± 0.13	1.81±2.34	140±3.95	2.54±2.51	Mixed
2d	0.49 ± 0.84	29.95±2.43	206±2.33	4.10±2.31	Mixed
2e	10.29 ± 0.07	19.30±1.13	451±1.55	9.90±1.33	Mixed

2f	16.68 ± 0.16	6.18±1.11	216±2.45	2.26±1.09	Mixed
2g	27.48 ± 0.31	1.78±3.35	88±1.75	9.04±1.03	Mixed
Rivastigmine	5.34 ± 0.37	1.82 ± 0.9	54 ± 2.7	130.9 ± 0.6	Competitive

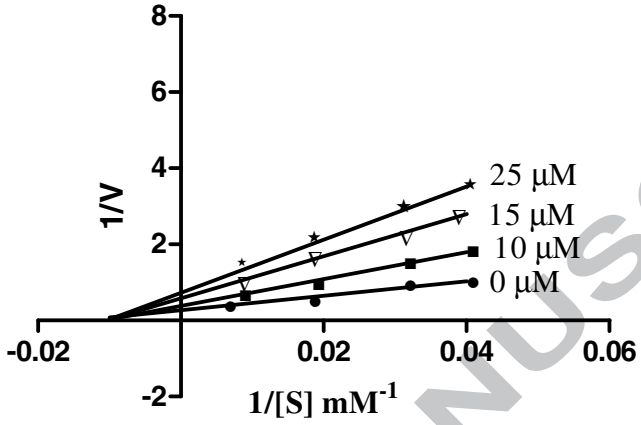


Fig. 5. Kinetic study on the mechanism of AChE inhibition by **1b**. Overlaid Lineweaver-Burk reciprocal plots of AChE initial velocity at increasing substrate concentration (ATChI, 0.2 – 0.8 mM)) in the absence of inhibitor and in the presence of 1**b** (0 - 25 µM) are shown.

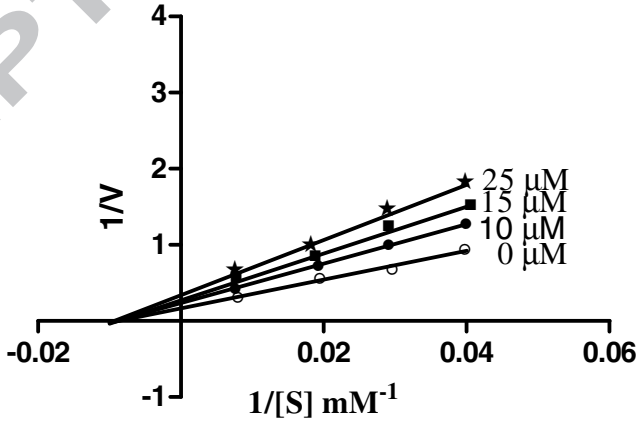


Fig. 6. Kinetic study on the mechanism of AChE inhibition by **2d**. Overlaid Lineweaver-Burk reciprocal plots of AChE initial velocity at increasing substrate concentration (ATChI, 0.2 – 0.8 mM) in the absence of inhibitor and in the presence of **2d** (0 - 25 µM) are shown.

2.4.1. *Ex vivo* AChE Inhibition

Primarily to investigate the CNS penetration ability of the synthesized compounds, **1a** and **2d** are selected for their central AChE inhibition studies by *ex vivo* assay using Ellman method. The percentage of brain AChE inhibition versus untreated controls was measured as depicted in (**Fig. 7** and **Fig. 8)** which showed both **1a** and **2d** exhibited dose-dependent brain AChE inhibition.

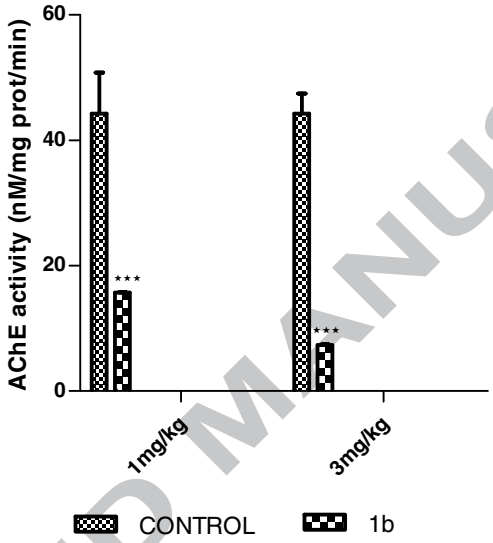


Fig. 7. *Ex vivo* determination of AChE inhibitory activity of **1b**. Data are expressed as mean ± SEM. Significance is determined by Student's t test. $^{***}p < 0.001$ as compared to control.

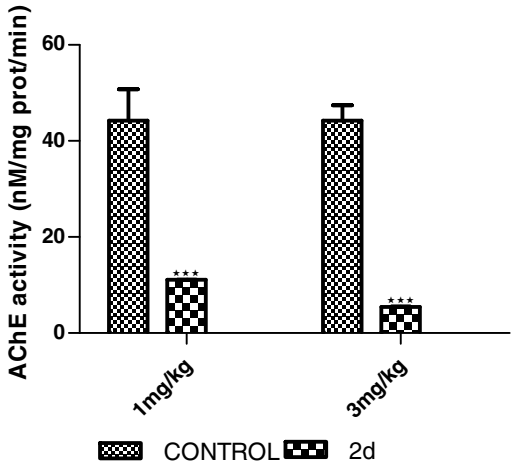


Fig. 8. *Ex vivo* determination of AChE inhibitory activity of **2d**. Data are expressed as mean ± SEM. Significance is determined by Student's t test. $^{***}p < 0.001$ as compared to control.

2.5. Passive avoidance test (The reversal of scopolamine induced memory deficit)

Over the past few decades, many efforts have been made to develop new piperazine derivatives as cognition enhancers. Some of them like DM232 and DM235 have shown outstanding potency and able to prevent amnesia at doses as low as 0.001 mg kg^{-1} subcutaneous, being several thousand times more potent than the reference compound piracetam [23,24]. Even though their pharmacological mechanism is not known, they are able to ameliorate the scopolamine induced memory deficit in animal models and can be developed as new cognition enhancer as piracetam. Likewise Ensaculin, a piperazine containing molecule acts as a cognition enhancer through the inhibition of AChE [18]. Thus, the synthesized compounds were screened using the passive avoidance step-down mice model [25] against piracetam and rivastigmine as standard drugs. The basal latency results of **1b** and **2d** are given in (**Fig**. **9** and **Fig**. **10)**. Both the compounds **1b** and **2d** showed significant improvement in cognition as compare to control and scopolamine treated groups. The results of memory parameters (latency and number of mistakes) of **1b** and **2d** are included in (**Table 4**).

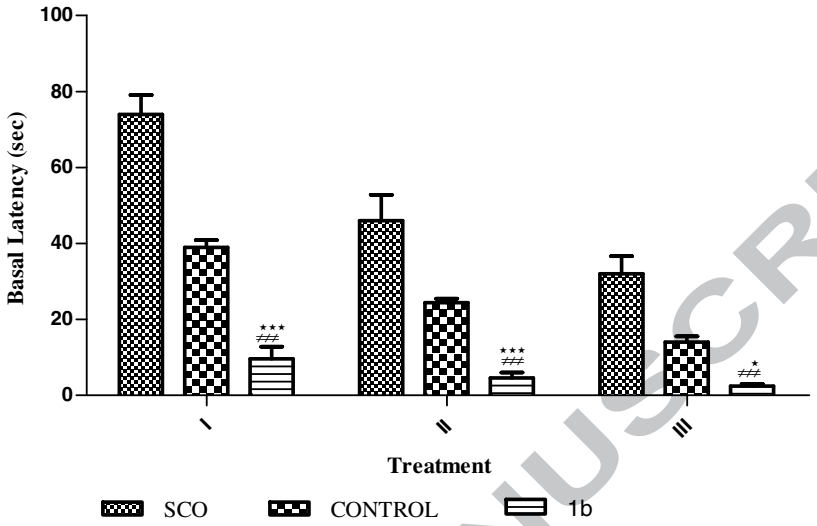


Fig. 9. Cognition enhancing activity of compound **1b** on mouse passive avoidance step down test against scopolamine induced memory loss. Data are expressed by mean ± SEM. Significance was determined by Two-way ANOVA and followed by Bonferroni posttest. It showed significant improvement in cognition: (###) $p < 0.0001$ as compare to scopolamine; (***) $p < 0.0001$ and (*) $p < 0.05$ as compare to control.

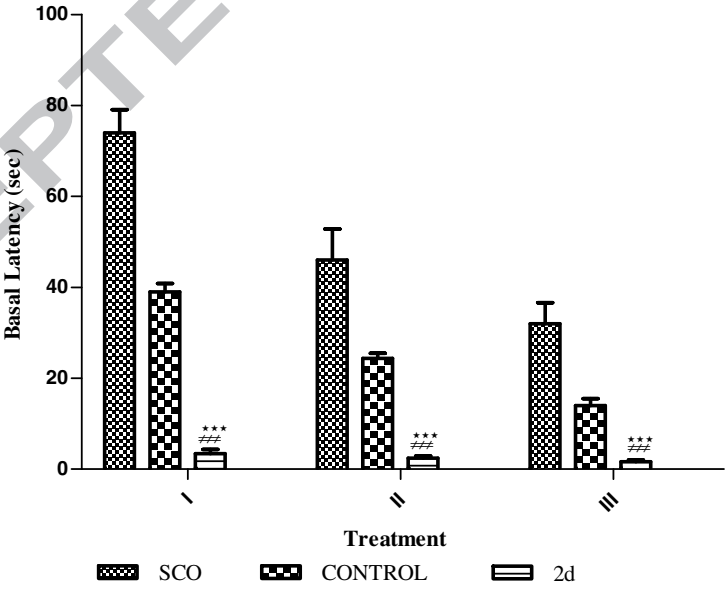


Fig. 10. Cognition enhancing activity of compounds **2d** on mouse passive avoidance step down test against scopolamine induced memory loss. Data are expressed by mean ± SEM. Significance

was determined by Two-way ANOVA and followed by Bonferroni posttest. It showed significant improvement in cognition: (###) $p < 0.0001$ as compare to scopolamine and (***) $p < 0.0001$ as compare to control.

Table 4. Cognition enhancing effect of piperazine derivatives on mouse passive avoidance test against scopolamine induced memory loss.

Experimental Group*	Treatment Groups (Dose = mg/kg, i.p.)	1st day		2nd day	
		Memory parameters (Mean ± S.E.M)		Memory parameters (Mean ± S.E.M)	
		Latency (sec)	No. of mistakes	Latency (sec)	No. of mistakes
1b	1.0	5.6 ± 3.14	2 ± 0.95	3.4 ± 1.69	1.2 ± 0.49
2d	1.0	2.2 ± 0.58	7.8 ± 2.82	1.4 ± 0.24	5.0 ± 2.84
Control	---	9.0 ± 0.63	6.6 ± 0.98	6.0 ± 1.30	6.2 ± 1.11
Scopolamine	0.5	25.8 ± 1.77	15.4 ± 0.87	21.8 ± 0.66	9.4 ± 4.20
Piracetam	2.5	2.6 ± 0.51	1.2 ± 0.97	2.2 ± 0.74	1.6 ± 2.73
Rivastigmine	1.0	1.8 ± 0.37	0.4 ± 0.40	2.6 ± 0.93	1.0 ± 2.61

*Each group contains 5 animals ($n = 5$)

2.6. Antiradical activity

Free radicals are high energetic reactive oxygen/nitrogen species (ROS/RNS). They are responsible for causing onset of life threatening disease like AD and it progresses towards the cognitive dysfunction. They also cause oxidative stress (OS) in human body which shows detrimental effects on neurons and is the basic reason of damage of nerve cells and nerve cell functions. Therefore, till date many natural and synthetic antioxidants have been developed as neuroprotective agents and they have shown beneficial results in the treatment of cognitive dysfunction [26]. Thus, to determine the antiradical capability of the synthesized compounds DPPH method was followed as reported by Shimada *et al.* [27]. The result is summarized in (**Table 5**).

Table 5. Antiradical activity of the synthesized compounds (**1a**-**2g**).

Compds	Antiradical activity (% Inhibition) (Mean ± S.E.M)					IC_{50} (µg/ml)
	1 (µg/ml)	5 (µg/ml)	25 (µg/ml)	50 (µg/ml)	100 (µg/ml)	
1a	51.07 ± 0.34	51.84 ± 0.35	53.23 ± 0.14	55.62 ± 0.04	57.05 ± 0.03	81.26 ± 0.36
1b	75.56 ± 0.21	75.84 ± 0.30	75.87 ± 0.06	77.81 ± 0.10	81.46 ± 0.04	1.76 ± 0.61
1c	50.23 ± 0.08	51.31 ± 0.10	52.11 ± 0.01	52.82±0.02	54.51 ± 0.01	123.8 ± 0.37
1d	73.61 ± 0.01	74.92 ± 0.02	75.08 ± 0.03	75.44 ± 0.03	76.58 ± 0.00	96.64 ± 0.21
1e	49.61 ± 0.01	51.98 ± 0.02	52.31 ± 0.01	53.31 ± 0.01	56.02 ± 0.02	281.4 ± 0.86
1f	48.32 ± 0.02	51.02 ± 0.02	51.51 ± 0.01	56.13 ± 0.02	57.22 ± 0.02	63.46 ± 0.56
1g	64.47 ± 0.05	77.75 ± 0.01	81.01±0.01	81.74±0.02	82.95±0.02	0.51 ± 0.44
2a	49.68 ± 0.15	50.30±0.03	51.58±0.02	51.66±0.02	51.90±0.04	8.46 ± 0.11
2b	47.56±0.24	49.08±0.02	49.82±0.08	51.56±0.01	54.24±0.04	274.4 ± 0.48
2c	47.32±0.12	50.84±0.11	52.88±0.34	51.80±0.06	52.55±0.02	0.80 ± 0.44
2d	51.82 ± 0.08	52.08 ± 0.02	52.56 ± 0.02	52.94 ± 0.01	53.86 ± 0.02	3.09 ± 0.08
2e	45.03 ± 0.02	51.32 ± 0.01	52.91 ± 0.01	53.04 ±0.02	54.96 ± 0.02	0.95 ± 0.28
2f	48.63 ± 0.18	48.98 ± 0.01	49.28 ± 0.14	49.37 ± 0.14	49.84 ± 0.02	57.78 ± 0.14
2g	50.32 ± 0.32	51.60 ± 0.01	53.03 ± 0.04	53.99 ± 0.02	55.55 ± 0.02	4.18 ± 0.13
Trolox	75.51 ± 0.04	90.41 ± 0.01	95.47 ± 0.03	96.39 ± 0.01	97.51 ±0.01	0.93 ± 0.32

3. Conclusion

Two series of novel hybrids have been designed and all the hybrids were synthesized without any difficulty. All the compounds were afforded with higher percentage yields ranging from 65.0 to 90.0%. They emerged as potential dual binding site inhibitors that have been proved by both enzyme kinetics and molecular docking studies. Based on the design approach and molecular structures, it can be strongly conclude that the presence of 4-(*N*-acetylaminophenoxy) moiety and N_4-substituted piperazine fragment seemed to enhance interactions as well as inhibitory activity, confirming the designed rationale. *Ex vivo* studies have suggested the good brain penetration and central AChE inhibition abilities of the compounds. The capability of ameliorating the scopolamine induced memory deficit has been identified from *in vivo* studies using rodent memory evaluator in mice. DPPH assay has shown good antiradical properties suggesting that they may develope as neuroprotective agents in near future. Compounds **1b** and

2d are emerged as most potent of these series which can be considered for the future drug development in search of new cognition enhancers.

4. Experimental section

4.1. Instrumentation and chemicals

All the chemicals used in the synthesis were purchased from Sigma Aldrich. The reaction progress was monitored using thin layer chromatography (TLC). TLC plates were prepared with silica gel G obtained from Merck company using ethyl acetate as solvent and activated at 110 ^{0}C for 10 min. Iodine was used for the colour visualization of the spots. Melting points were recorded on a Veego melting point apparatus and were uncorrected. Elemental analyses were done on a Perkin-Elmer-2400 model CHN analyser using acetanilide as standard. The purity of compounds was ascertained by TLC, melting point and elemental analysis methods. The molecular structure of newly synthesized compounds was analyzed by FTIR, ^{1}H NMR, ^{13}C NMR and Mass instrumental techniques. The FTIR spectra were obtained from a PerkinElmer Spectrum Version 10.03.08. The NMR spectra were obtained using a Bruker Avance II 400 MHz spectrometer in deuterated chloroform and deuterated dimethylsulfoxide as solvent and are recorded in parts per million (ppm) downfield from tetramethylsilane (Me_4Si) as internal reference. The spin multiplicities were described as singlet (s), broad singlet (br s), doublet (d), double doublet (dd), triplet (t), doublet of triplets (dt), triplet of double doublet (tdd) etc. The MASS spectrum was obtained from a TOF MS ESI mass spectrometer, and was recorded on an electrospray ionization mass spectrometer as the value m/z.

4.2. Syntheses

The syntheses of 4-(*N*-acetylamino)phenol derived piperazine derivatives **1a-1g** and **2a-2g** were carried out using the procedures as given in **Schemes 1** and **2**.

4.2.1. Methyl 2-(4-acetamidophenoxy)acetate (1)

A mixture of *N*-(4-hydroxyphenyl)acetamide (0.5 g, 3.31 mmol) and methyl chloroacetate (0.8 ml, 9.13 mmol) in the presence of anhydrous potassium carbonate (1.0 g) was refluxed in ethyl methyl ketone (15 ml) with continuous stirring at 90-100 ^{0}C for 18 h. The slurry was filtered and

the excess solvent was removed under reduced pressure and the solid obtained was crystallized from ethanol to afford **1**, 0.6 g, yield 81.1%, m.p. 133-135 ^{0}C. FTIR (KBr, cm^{-1}): 3370.2, 3100.5, 2950.8, 1750.0, 1680.6, 1605.8, 1430.2, 1540.9, 1225.6, 1080.2, 830.9, 690.8. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.60 (br s, 1H, NH). 7.49 (d, 2H, J_o = 10.52 Hz, ArH), 6.80 (d, 2H, J_o = 10.54 Hz, ArH), 4.63 (s, 2H, OCH_2), 3.76 (s, 3H, $COOCH_3$), 2.07 (s, 3H, $COCH_3$). Calcd for $C_{11}H_{13}NO_4$: C, 59.19; H, 5.83; N, 6.28. Found: C, 59.09; H, 5.73; N, 6.16%.

4.1.2. *N*-[4-{2-(4-ethylpiperazin-1-yl)-2-oxoethoxy}phenyl]acetamide (1a)

A mixture of *N*-ethylpiperazine (0.9 ml, 7.05 mmol) and compound **1** (1.0 g, 4.48 mmol) was thermally fused at 125-130 ^{0}C for 3 h with continuous stirring. Crushed ice was added to the reaction mixture and the solid obtained was crystallized from acetone to afford **1a**, 1.33 g, yield 65.0%, m.p. 141 ^{0}C. FTIR (KBr, cm^{-1}): 3256.58, 3198.61, 3076.47, 2967.27, 2804.58, 1644.25, 1549.26, 1508.06, 1373.81, 1222.05, 1083.90, 1019.16, 829.45, 772.35. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.65 (br s, 1H, NH), 7.38 (m, 2H, ArH), 6.80 (d, 2H, J_o = 9.0 Hz, ArH), 4.65 (s, 2H, OCH_2), 3.64 (t, 2H, J = 4.80 Hz, $N_1(CH_2)_2$), 3.57 (t, 2H, J = 4.96 Hz, $N_1(CH_2)_2$), 2.41 (m, 6H, -$(CH_2)_2N_4CH_2$), 2.12 (s, 3H, $COCH_3$), 1.08 (t, 3H, J = 7.18 Hz, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.91, 166.45, 154.24, 132.53, 121.82, 114.74, 67.51, 52.42, 45.20, 42.08, 24.08, 11.90. MS (ESI+), m/z: Calcd. 305.2 $[M]^+$; Obsvd. 306.3 $[M + H]^+$. Calcd for $C_{16}H_{23}N_3O_3$: C, 62.93; H, 7.59; N, 13.76. Found: C, 62.32; H, 7.43; N, 13.66%.

4.1.3. *N*-[4-{2-oxo-2-(4-phenylpiperazin-1-yl)ethoxy}phenyl]acetamide (1b)

A mixture of *N*-phenylpiperazine (0.6 ml, 3.80 mmol) and compound **1** (1.0 g, 4.48 mmol) was thermally fused at 125-130 ^{0}C for 1 h with continuous stirring. Crushed ice was added to the reaction mixture and the solid obtained was crystallized from ethanol to afford **1b**, 1.35 g, yield 85.44%, m.p. 197-198 ^{0}C. FTIR (KBr, cm^{-1}): 3259.44, 3056.23, 2841.73, 1663.22, 1599.72, 1445.27, 1512.68, 1333.70, 1155.09, 1110.52, 1027.51, 825.15, 750.50. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.58 (br s, 1H, NH), 7.44 (d, 2H, J_o = 8.96 Hz, ArH), 7.18 (m, 2H, ArH), 6.82 (m, 5H, ArH), 4.69 (s, 2H, OCH_2), 3.65 (s, 4H, $N_1(CH_2)_2$), 3.11 (d, 4H, J = 18.88 Hz, $N_4(CH_2)_2$), 2.00 (s, 3H, $COCH_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 167.92, 165.85, 153.49, 150.46, 132.84, 128.73, 120.63, 119.66, 115.9, 114.25, 66.76, 49.00, 44.38, 23.63. MS (ESI+), m/z: Calcd. 353.2 $[M]^+$; Obsvd. 354.3 $[M + H]^+$, 376.2 $[M + Na]^+$. Calcd for $C_{20}H_{23}N_3O_3$: C, 67.97; H, 6.56; N, 11.89. Found: C, 67.67; H, 6.35; N, 11.78%.

4.1.4. *N*-(4-(2-(4-(2-fluorophenyl)piperazin-1-yl)-2-oxoethoxy)phenyl)acetamide (1c)

A mixture of *N*-(2-Fluorophenyl)piperazine (0.3 g, 1.66 mmol) and compound **1** (1.0 g, 4.48 mmol) was thermally fused at 140-150 ^{0}C for 7 h with continuous stirring. Crushed ice was added to the reaction mixture and the solid obtained was crystallized from acetone to afford **1c**, 1.11 g, yield 66.86%, m.p. 128-129 ^{0}C. FTIR (KBr, cm^{-1}): 3206.24, 3140.28, 3111.51, 3009.46, 2909.58, 2856.24, 1671.13, 1626.79, 1553.24, 1513.56, 1485.01, 1428.79, 1377.56, 1328.47, 1182.92, 1111.08, 1016.60, 830.02 and743.80 cm^{-1}. ^{1}H NMR (100 MHz, $CDCl_3$): δ 7.52 (br s, 1H, NH), 7.32 (m, 2H, ArH), 6.94 (m, 2H, ArH), 6.84 (m, 2H, ArH), 6.78 (m, 2H, ArH), 4.54 (s, 2H, OCH_2), 3.68 (m, 4H, $N_1(CH_2)_2$), 2.99 (m, 4H, $N_4(CH_2)_2$), 2.05 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$,): δ 168.52, 166.58, 156.96, 154.43, 139.41, 132.14, 124.60, 123.30, 121.88, 119.25, 116.28, 116.28, 114.96, 65.61, 50.98, 45.54, 24.31. MS (ESI+), m/z: Calcd. 371.2 [M]$^+$; Obsvd. 372.3 [M + H]$^+$. Calcd for $C_{20}H_{22}FN_3O_3$: C, 64.68; H, 5.97; N, 11.31. Found: C, 64.49; H, 5.91; N, 11.11%.

4.1.5. *N*-(4-(2-(4-(4-fluorophenyl)piperazin-1-yl)-2-oxoethoxy)phenyl)acetamide (1d)

A mixture of *N*-(4-fluorophenyl) piperazine (0.6 g, 3.32 mmol) and compound **1** 1.0 g, 4.48 mmol) was thermally fused at 125-130 ^{0}C for 1 h with continuous stirring. Crushed ice was added to the reaction mixture and the solid obtained was crystallized from ethanol to afford **1d** (1.41 g, 84.94%), m.p. 210 ^{0}C. FTIR (KBr, cm^{-1}): 3257.15, 3124.62, 3048.04, 2841.98, 1661.32, 1515.66, 1445.63, 1324.98, 1156.74, 1110.26, 1029.16, 822.69, 740.91. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.78 (br s, 1H, NH), 7.48 (dd, 2H, J_o = 7.14 Hz, J_m = 1.90 Hz, ArH), 7.07 (m, 2H, ArH), 6.98 (m, 2H, ArH), 6.88 (m, 2H, ArH), 4.81 (s, 2H, OCH_2), 3.61 (t, 4H, J = 5.04 Hz, $N_1(CH_2)_2$), 3.08 (m, 4H, J = 18.88 Hz, $N_4(CH_2)_2$), 2.01 (s, 3H, $COCH_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 167.74, 165.95, 157.45, 155.10, 147.64, 132.88, 120.40, 117.72, 115.32, 114.58, 66.22, 49.42, 49.04, 44.09, 41.09, 23.75. MS (ESI+), m/z: Calcd. 371.2 [M]$^+$; Obsvd. 372.3 [M + H]$^+$. Calcd for $C_{20}H_{22}FN_3O_3$: C, 64.68; H, 5.97; N, 11.31. Found: C, 64.47; H, 5.93; N, 11.12%.

4.1.6. *N*-(4-(2-oxo-2-(4-(pyridin-2-yl)piperazin-1-yl)ethoxy)phenyl)acetamide (1e)

A mixture of 1-(2-Pyridyl)piperazine (0.3 ml, 1.96 mmol) and compound **1** (1.0 g, 4.48 mmol) was thermally fused at 110-120 ^{0}C for 1 h with continuous stirring. Crushed ice was added to the reaction mixture and the solid obtained was crystallized from ethanol to afford **1e**, 1.15 g,

72.78%, m.p. 172-173 ^{0}C. FTIR (KBr, cm^{-1}): 3298.16, 3196.31, 3134.55, 3071.81, 2918.05, 2828.77, 1655.45, 1591.80, 1539.23, 1507.71, 1474.10, 1432.31, 1373.98, 1312.69, 1215.27, 1110.14, 1016.22, 823.74, 774.30. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (dd, 1H, J_o = 4.96 Hz, J_m = 1.28 Hz, PyrH), 7.44 (m, 1H, PyrH), 7.38 (br s, 1H, NH), 7.32 (m, 2H, ArH), 6.82 (m, 2H, ArH), 6.60 (m, 2H, PyrH), 4.64 (s, 2H, OCH_2), 3.64 (m, 4H, $N_1(CH_2)_2$), 3.52 (m, 2H, $N_4(CH_2)_2$), 3.44 (t, 2H, J = 5.20 Hz, $N_4(CH_2)_2$), 2.06 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$,): δ 168.35, 166.70, 158.97, 154.44, 148.00, 137.76, 132.07, 121.89, 114.94, 114.07, 107.32, 67.99, 45.16, 41.83, 24.36. MS (ESI+), m/z: Calcd. 354.2 [M] $^+$; Obsvd. 355.3 [M + H] $^+$. Calcd for $C_{19}H_{22}N_4O_3$: C, 64.39; H, 6.26; N, 15.81. Found: C, 64.93; H, 6.21; N, 15.75%.

4.1.7. *N*-(4-(2-oxo-2-(4-(pyrimidin-2-yl)piperazin-1-yl)ethoxy)phenyl)acetamide (1f)

A mixture of 1-(2-Pyrimidyl)piperazine (0.25 ml, 1.71 mmol) and compound **1** (1.0 g, 4.48 mmol) was thermally fused at 130-135 ^{0}C for 1.30 h with continuous stirring. Crushed ice was added to the reaction mixture and the solid obtained was crystallized from ethanol to afford **1f**, 1.33 g, 83.64%, m.p. 132 ^{0}C. FTIR (KBr, cm^{-1}): 3247.55, 3134.55, 3079.20, 2915.76, 1654.74, 1585.10, 1553.16, 1509.61, 1438.85, 1363.56, 1234.70, 1076.12, 1030.33, 980.83, 835.00. ^{1}H NMR (400 MHz, $CDCl_3$,): δ 8.25 (d, 2H, J = 4.76 Hz, Pyr), 7.52 (br s, 1H, NH), 7.32 (m, 2H, ArH), 6.82 (m, 2H, ArH), 6.47 (t, 1H, J = 4.76 Hz, Pyr), 4.64 (s, 2H, OCH_2), 3.76 (m, 4H, $N_1(CH_2)_2$), 3.60 (m, 4H, $N_4(CH_2)_2$), 2.06 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.43, 166.81, 161.44, 157.81, 154.39, 132.15, 121.91, 114.92, 110.60, 68.00, 45.24, 43.90, 43.45, 42.05, 24.31. MS (ESI+), m/z: Calcd. 355.2 [M] $^+$; Obsvd. 356.3 [M + H] $^+$. Calcd for $C_{18}H_{21}N_5O_3$: C, 60.83; H, 5.96; N, 19.71. Found: C, 60.71; H, 5.88; N, 19.51%.

4.1.8. *N*-(4-(2-(4-(furan-2-carbonyl)piperazin-1-yl)-2-oxoethoxy)phenyl)acetamide (1g)

A mixture of 1-(2-Furoyl)piperazine (0.3 g, 1.66 mmol) and compound **1** (1.0 g, 4.48 mmol) was thermally fused at 100-110 ^{0}C for 2 h with continuous stirring. Crushed ice was added to the reaction mixture and the solid obtained was crystallized from acetone to afford **1g,** 1.21 g, 72.89%, m.p. 199-200 ^{0}C. FTIR (KBr, cm^{-1}): 3306.92, 3206.24, 3111.51, 2909.58, 2856.24, 1671.13, 1626.79, 1553.24, 1513.56, 1485.01, 1428.79, 1377.56, 1328.47, 1236.09, 1182.92, 1111.08, 1081.92, 1016.60, 830.02, 743.80. ^{1}H NMR (400 MHz, $CDCl_3$,): δ 7.49 (d, 1H, J = 4.76 Hz, furanoyl), 7.41 (d, 2H, J = 9.00 Hz, ArH), 7.16 (br s, 1H, NH), 7.06 (d, 1H, J = 3.6 Hz, furanoyl), 6.91 (d, 2H, J = 9.00 Hz, ArH), 6.50 (q, 1H, J = 1.74 Hz, furanoyl), 4.71 (s, 2H,

OCH_2), 3.80 (br s, 4H, $N_4(CH_2)_2$), 3.69 (br s, 4H, $N_1(CH_2)_2$), 2.16 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$,): δ 168.91, 166.53, 155.12, 153.71, 147.11, 144.12, 132.17, 122.21, 118.97, 114.45, 111.93, 68.31, 48.91, 47.53, 24.31. MS (ESI+), m/z: Calcd. 371.1 $[M]^+$; Obsvd. 372.2 $[M + H]^+$. Calcd for $C_{19}H_{21}N_3O_5$: C, 61.45; H, 5.70; N, 11.31. Found: C, 61.32; H, 5.51; N, 11.11%.

4.1.9. *N*-{4-(3-chloropropoxy)phenyl}acetamide (2)

A mixture of *N*-(4-hydroxyphenyl)acetamide (1.0 g, 4.39 mmol) and 1-Bromo-3-chloropropane (1.2 ml, 8.39 mmol) in ethyl methyl ketone (20 ml) in the presence of anhydrous potassium carbonate (2.0 g) at 90-100 ^{0}C with continuous stirring for 11 h. The slurry was filtered and the excess solvent was removed under reduced pressure and the solid obtained was crystallized from ethanol to afford **2,** 1.3 g, yield 85.5%, m.p. 120-123 ^{0}C. FTIR (KBr, cm^{-1}): 3288.23, 3134.14, 2934.49, 1660.01, 1609.61, 1407.86, 1553.69, 1509.76, 1237.70. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.48 (br s, 1H, NH), 7.38 (dd, 2H, J_o = 6.84 Hz, J_m = 2.16 Hz, ArH), 6.84 (dd, 2H, J_o = 6.84 Hz, J_m = 2.16 Hz, ArH), 4.08 (t, 2H, J = 5.84 Hz, OCH_2), 3.74 (t, 2H, J = 6.32 Hz, CH_2Cl), 2.21 (pent, 2H, $OCH_2CH_2CH_2Cl$), 2.14 (s, 3H, $COCH_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.48, 155.54, 131.22, 121.99, 114.78, 64.52, 41.56, 32.24, 24.33. Calcd for $C_{11}H_{14}ClNO_2$: C, 58.03; H, 6.20; N, 6.15. Found: C, 57.99; H, 6.17; N, 6.13%.

4.1.10. *N*-(4-(3-(4-ethylpiperazin-1-yl)propoxy)phenyl)acetamide (2a)

A mixture of compound **2** (1.0 g, 4.39 mmol) and *N*-Ethyl piperazine (0.7 ml, 5.48 mmol) was refluxed in ethyl methyl ketone (20 ml) in the presence of anhydrous potassium carbonate (2.0 g) at 90-100 ^{0}C with continuous stirring for 38 h. The slurry was filtered and the excess solvent was removed under reduced pressure and the solid obtained was crystallized from acetone to afford **2a**, 1.1 g, yield 82.08%, m.p. 190-191 ^{0}C. FTIR (KBr, cm^{-1}): 3236.37, 3182.62, 3118.65, 3055.97, 2952.66, 2832.48, 1672.71, 1600.74, 1540.92, 1511.27, 1407.55, 1308.06, 1239.76, 1173.90, 1139.37, 1039.77, 990.24, 829.33. ^{1}H NMR (400 MHz, DMSO-d_6,): δ 9.76 (br s, 1H, NH), 7.48 (d, 2H, J = 8.96 Hz, ArH), 6.79 (d, 2H, J_o = 8.96 Hz, ArH), 3.97 (t, 2H, J = 6.04 Hz, OCH_2), 3.01 (br s, 8H, $N_1(CH_2)_4N_4$), 2.6 (s, 2H, CH_2CH_3), 2.54 (t, 2H, J = 1.72 Hz, $O(CH_2)_2CH_2N_1$), 2.11 (s, 3H, $COCH_3$), 1.97 (q, 2H, J = 14.92 Hz, OCH_2CH_2), 1.27 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 167.67, 154.21, 132.47, 120.58, 114.09, 65.33, 53.49, 50.80, 50.28, 49.40, 30.52, 23.67, 9.10. MS (ESI+), m/z: Calcd. 305.2 $[M]^+$; Obsvd. 306.3 $[M + H]^+$. Calcd for $C_{17}H_{27}N_3O_2$: C, 58.03; H, 6.20; N, 6.15. Found: C, 57.99; H, 6.17; N, 6.13%.

4.1.11. *N*-[4-{3-(4-phenylpiperazin-1-yl)propoxy}phenyl]acetamide (2b)

A mixture of compound **2** (1.0 g, 4.39 mmol) and *N*-phenyl piperazine (0.7 ml, 4.44 mmol) was refluxed in ethyl methyl ketone (20 ml) in the presence of anhydrous potassium carbonate (2.0 g) at 90-100 ^{0}C with continuous stirring for 30 h. The slurry was filtered and the excess solvent was removed under reduced pressure and the solid obtained was crystallized from acetone to afford **2b**, 1.2 g, yield 77.4%, m.p. 167-169 ^{0}C. FTIR (KBr, cm^{-1}): 3296.68, 3129.44, 2828.32, 1655.00, 1602.06, 1402.24, 1508.78, 1239.40. ^{1}H NMR (400 MHz,$CDCl_3$): δ 7.34 (br s, 1H, NH), 7.30 (dd, 2H, J_o = 6.88 Hz, J_m = 6.92 Hz, ArH), 7.19 (m, 2H, ArH), 6.86 (d, 2H, J_o = 7.92 Hz, ArH), 6.78 (m, 3H, ArH), 3.93 (t, 2H, *J* = 6.32 Hz, OCH_2), 3.14 (t, 4H, *J* = 4.98 Hz, $N_4(CH_2)_2$), 2.56 (t, 4H, *J* = 4.98 Hz, $N_1(CH_2)_2$), 2.50 (t, 2H, *J* = 7.38 Hz, $O(CH_2)_2CH_2N_1$), 2.06 (s, 3H, $COCH_3$), 1.92 (pent, 2H, -$OCH_2CH_2CH_2N_1$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.41, 155.86, 151.31, 130.95, 129.13, 121.98, 119.72, 116.06, 114.77, 66.48, 55.20, 53.30, 49.14, 26.77, 24.35. MS (ESI+), m/z: Calcd. 353.2 $[M]^+$; Obsvd. 354.3 $[M + H]^+$. Calcd for $C_{21}H_{27}N_3O_2$: C, 71.36; H, 7.70; N, 11.89. Found: C, 71.23; H, 7.56; N, 11.77%.

4.1.12. *N*-(4-(3-(4-(2-fluorophenyl)piperazin-1-yl)propoxy)phenyl)acetamide (2c)

A mixture of compound **2** (1.0 g, 4.39 mmol) and *N*-(2-Fluorophenyl)piperazine (0.5 g, 2.76 mmol) was refluxed in ethyl methyl ketone (20 ml) in the presence of anhydrous potassium carbonate (2.0 g) at 90-100 ^{0}C with continuous stirring for 24 h. The slurry was filtered and the excess solvent was removed under reduced pressure and the solid obtained was crystallized from methanol to afford **2c**, 1.3 g, yield 80.1%, m.p. 109-110 ^{0}C. FTIR (KBr, cm^{-1}): 3285.21, 3133.48, 3072.37, 2935.60, 2817.16, 1656.81, 1609.96, 1510.12, 1407.93, 1372.81, 1241.26, 1172.05, 1039.06, 941.44, 831.82, 751.32. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52 (br s, 1H, NH), 7.32 (m, 2H, ArH), 6.94 (m, 2H, ArH), 6.83 (m, 2H, ArH), 6.77 (m, 2H, ArH), 4.54 (s, 2H, OCH_2), 3.71 (s, 4H, $N_4(CH_2)_2$), 3.67 (t, 4H, *J* = 4.90 Hz, $N_1(CH_2)_2$), 2.99 (m, 2H, CH_2N_1), 2.06 (s, 3H, $COCH_3$), 1.78 (pent, 2H, $OCH_2CH_2CH_2N_1$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.51, 156.96, 154.43, 139.41, 132.13, 124.60, 123.30, 121.88, 119.25, 116.29, 114.91, 65.61, 52.32, 50.98, 50.32, 45.54, 42.22, 27.77, 24.31. MS (ESI+), m/z: Calcd. 371.2 $[M]^+$; Obsvd. 372.3 $[M + H]^+$. Calcd for $C_{21}H_{26}FN_3O_2$: C, 67.90; H, 7.06; N, 11.31. Found: C, 67.86; H, 6.98; N, 11.11%.

4.1.13. *N*-(4-(3-(4-(4-fluorophenyl)piperazin-1-yl)propoxy)phenyl)acetamide (2d)

A mixture of compound **2** (1.0 g, 4.39 mmol) and *N*-(4-Fluorophenyl)piperazine (0.6 g, 3.32 mmol) was refluxed in ethyl methyl ketone (20 ml) in the presence of anhydrous potassium

carbonate (2.0 g) at 90-100 ^{0}C with continuous stirring for 32 h. The slurry was filtered and the excess solvent was removed under reduced pressure and the solid obtained was crystallized from ethanol to afford **2d**, 1.41 g, yield 86.5%, m.p. 180 ^{0}C. FTIR (KBr, cm^{-1}): 3292.11, 3056.27, 2957.16, 2829.69, 1657.50, 1607.53, 1509.74, 1374.69, 1237.56, 1152.46, 819.12, 914.33, 711.01.^{1}H NMR (400 MHz, $CDCl_3$): δ 7.13 (br s, 1H, NH), 7.30 (dd, 2H, J_o = 6.86 Hz, J_m = 2.10 Hz, ArH), 6.89 (m, 2H, ArH), 6.80 (m, 4H, ArH), 3.94 (t, 2H, J = 6.30 Hz, OCH_2), 3.06 (t, 4H, J = 4.94 Hz, $N_4(CH_2)_2$), 2.56 (t, 4H, J = 4.94 Hz, $CH_2N_1(CH_2)_2$), 2.51 (t, 2H, J = 7.36 Hz, CH_2N_1), 2.08 (s, 3H, $COCH_3$), 1.92 (pent, 2H, $OCH_2CH_2CH_2N_1$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.26, 158.34, 155.87, 147.98, 130.89, 121.93, 117.80, 115.51, 114.80, 66.45, 55.13, 53.28, 50.16, 26.77, 24.39. MS (ESI+), m/z: Calcd. 371.2 $[M]^+$; Obsvd. 372.3 $[M + H]^+$. Calcd for $C_{21}H_{26}FN_3O_2$: C, 67.90; H, 7.06; N, 11.31. Found: C, 67.85; H, 6.99; N, 11.11%.

4.1.14. *N*-(4-(3-(4-(3-chlorophenyl)piperazin-1-yl)propoxy)phenyl)acetamide (2e)

A mixture of compound **2** (1.0 g, 4.39 mmol) and *N*-(3-Chlorophenyl)piperazine hydrochloride (1.3 g, 4.53 mmol) was refluxed in ethyl methyl ketone (30 ml) in the presence of anhydrous potassium carbonate (3.0 g) at 90-100 ^{0}C with continuous stirring for 13 h. The slurry was filtered and the excess solvent was removed under reduced pressure and the solid obtained was crystallized from ethanol to afford **2e**, 1.5 g, yield 90.0%, m.p. 139-140 ^{0}C. FTIR (KBr, cm^{-1}): 3290.20, 3133.45, 3073.38, 2947.57, 2835.59, 1648.92, 1595.23, 1546.93, 1506.58, 1406.61, 1251.66, 1126.94, 1035.24, 993.47, 948.18, 829.26, 758.38. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64 (br s, 1H, NH), 7.40 (m, 2H, ArH), 7.17 (t, 1H, J_o = 8.12 Hz, ArH), 6.84 (m, 5H, ArH), 4.02 (t, 2H, J = 6.30 Hz, OCH_2), 3.21(t, 4H, J = 5.0 Hz, $N_4(CH_2)_2$), 2.60 (m, 6H, $CH_2N_1(CH_2)_2$), 2.14 (s, 3H, $COCH_3$), 1.98 (pent, 2H, $OCH_2CH_2CH_2N_1$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.56, 155.81, 152.33, 134.91, 131.03, 130.06, 122.01, 119.21, 115.67, 114.74, 113.84, 66.40, 55.12, 53.07, 48.62, 26.76, 24.31. MS (ESI+), m/z: Calcd. 387.2 $[M]^+$; Obsvd. 388.3 $[M + H]^+$. Calcd for $C_{21}H_{26}ClN_3O_2$: C, 65.02; H, 6.76; N, 10.83. Found: C, 64.97; H, 6.53; N, 10.71%.

4.1.15. *N*-(4-(3-(4-(pyridin-2-yl)piperazin-1-yl)propoxy)phenyl)acetamide (2f)

A mixture of compound **2** (1.0 g, 4.39 mmol) and 1-(2-Pyridyl)piperazine (0.3 ml, 1.96 mmol) was refluxed in ethyl methyl ketone (20 ml) in the presence of anhydrous potassium carbonate (2.0 g) at 90-100 ^{0}C with continuous stirring for 24 h. The slurry was filtered and the excess solvent was removed under reduced pressure and the solid obtained was crystallized from ethanol to afford **2f**, 1.39 g, yield 89.1%, m.p. 120-121 ^{0}C. FTIR (KBr, cm^{-1}): 3278.26, 3071.30,

2937.12, 2836.02, 1657.87, 1602.57, 1557.52, 1510.29, 1476.63, 1439.69, 1374.14, 1311.30, 1243.11, 1169.55, 1143.27, 1055.82, 936.87, 826.68, 777.36. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (dd, 1H, J_o = 4.88 Hz, J_m = 1.32 Hz, PyrH), 7.47 (m, 1H, PyrH), 7.44 (br s, 1H, NH), 7.36 (m, 2H, ArH), 6.84 (dd, 2H, J_o = 12.06 Hz, J_m = 3.14 Hz, ArH), 6.62 (m, 2H, ArH), 4.0 (t, 2H, J = 6.30 Hz, OCH_2), 3.56 (t, 4H, J = 5.06 Hz, $N_4(CH_2)_2$), 2.58 (m, 6H, $CH_2N_1(CH_2)_2$), 2.13(s, 3H, $COCH_3$), 2.01 (pent, 2H, $OCH_2CH_2CH_2N_1$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.34, 159.53, 155.84, 147.92, 137.50, 131.02, 121.93, 114.80, 113.35, 107.14, 66.45, 55.22, 53.06, 45.19, 26.69, 24.29. MS (ESI+), m/z: Calcd. 354.2 $[M]^+$; Obsvd. 355.3 $[M + H]^+$. Calcd for $C_{20}H_{26}N_4O_2$: C, 67.77; H, 7.39; N, 15.81. Found: C, 67.53; H, 7.27; N, 15.79%.

4.1.16. *N*-(4-(3-(4-(pyrimidin-2-yl)piperazin-1-yl)propoxy)phenyl)acetamide (2g)

A mixture of compound **2** (1.0 g, 4.39 mmol) and 1-(2-Pyrimidyl)piperazine (0.5 ml, 3.42 mmol) was refluxed in ethyl methyl ketone (20 ml) in the presence of anhydrous potassium carbonate (2.0 g) at 90-100 ^{0}C with continuous stirring for 23.30 h. The slurry was filtered and the excess solvent was removed under reduced pressure and the solid obtained was crystallized from ethanol to afford **2g**, 1.39 g, yield 89.1%, m.p. 180-181 ^{0}C. FTIR (KBr, cm^{-1}): 3287.64, 3150.30, 2906.96, 2808.76, 1655.22, 1592.74, 1514.35, 1440.23, 1391.32, 1360.55, 1312.94, 1253.70, 1169.51, 1140.27, 1051.26, 1012.94, 981.72, 832.63, 790.21. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (d, 2H, J_o = 4.76 Hz, PyrimH), 7.31(dd, 2H, J_o = 6.94 Hz, J_m = 2.02 Hz, ArH), 7.20 (br s, 1H, NH), 6.78 (dd, 2H, J_o = 6.90 Hz, J_m = 2.06 Hz, ArH), 6.41 (t, 1H, J_o = 4.74 Hz, PyrimH), 3.94 (t, 2H, J = 6.28 Hz, OCH_2), 3.78 (t, 4H, J_o = 10.12 Hz, $N_4(CH_2)_2$), 2.48 (m, 6H, $CH_2N_1(CH_2)_2$), 2.07 (s, 3H, $COCH_3$), 1.94 (m, 2H, $OCH_2CH_2CH_2N_1$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.25, 161.63, 157.73, 155.84, 130.95, 121.91, 114.80, 109.89, 66.40, 55.25, 53.11, 43.59, 26.65, 24.36. MS (ESI+), m/z: Calcd. 355.2 $[M]^+$; Obsvd. 356.3 $[M + H]^+$. Calcd for $C_{19}H_{25}N_5O_2$: C, 64.20; H, 7.09; N, 19.70. Found: C, 63.98; H, 7.03; N, 19.55%.

4.3. Computational study

4.3.2. Docking

Molecular docking study of the synthesized compounds (**1a-1g** and **2a-2g**) was performed against *Torpedo californica* acetylcholinesterase (*Tc*AChE, PDB ID: 1EVE) to study the binding interactions using VLife MDS 4.3 package.

4.4. Biochemical study

4.4.1. Inhibition of AChE

4.4.1.1. *In vitro* AChE Inhibition

The inhibition reactions of AChE were determined by the Ellman method. This method is based on the use of acetylthiocholine iodide (ATChI) as synthetic substrate and 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to generate a yellow chromophore (5-mercapto-2-nitrobenzoic acid) which is colorimetrically detected. Whole mouse brains of LAKA strain (25-30 g) were decapitated and rinsed with ice cold normal saline solution and homogenized in a glass Teflon homogenizer (REMI MOTORS, India) containing 50 volumes of sodium phosphate buffer (pH 8.0, 0.1 M) and centrifuged (Research centrifuge, REMI, R-24) at 10,000 rpm for 20 min at 4 ^{0}C to prepare supernatant. The obtained supernatant was used as a source of enzyme for the assay. Initially stock solutions of each test drug were prepared and diluted to various concentrations immediately before use. An aliquot of diluted solution at five different concentration (1, 5, 10, 15 and 25 µl) levels was mixed with phosphate buffer (pH 8.0, 3.0 ml), 100 µl of buffered Ellman's reagent, 100 µl of acetylthiocholine iodide solution and 50 µl of supernatant and the change in optical density was measured immediately at 412 nm for 3 minutes in a PERKIN ELMER UV/VIS spectrophotometer. The AChE activity was calculated using the formula: AChE degree = (0.05 x change in optical density) / mg protein. Additionally, the concentration of each compound required to inhibit acetylcholinesterase activity by 50% (IC_{50}) was calculated by non-linear regression of the log(concentration)-response curve using GraphPad Prism 5. Rivastigmine was used as standard drug. The protein estimation was carried out using Biuret method using Biuret reagent prepared by mixing sodium potassium tartrate (4.5 g) in 40 ml of 0.2 N NaOH, cupric sulphate pentahydrate and potassium iodide (0.5 g) and made up the volume 100 ml with 0.2 N NaOH solution. The working solution was prepared by mixing 50 µl of supernatant, 2.9 ml of normal saline and 3 ml of Biuret reagent and incubated at room temperature for 10 minutes

and measured the optical density at 540 nm in the above mentioned UV-VIS spectrophotometer. The amount of protein content (in mg) was calculated using the standard table based on optical density. Kinetic studies were performed using six concentrations of substrate ATChI (0.25 – 2.0 mM) in the presence and absence of above mentioned same concentrations of inhibitors. Kinetic parameters were calculated from the 'Enzyme kinetic' module of GraphPad Prism 5.

4.4.1.1. *Ex vivo* AChE Inhibition

To evaluate the brain permeability of the selected compounds, *ex vivo* AChE Inhibition assay was performed. Each compound was intraperitoneally (i.p.) injected to mice at doses of 1.0 mg/kg and 3 mg/kg. After 1 h animals were killed by decapitation, brain was immediately dissected, washed with ice cold saline and kept in phosphate buffer (pH 8.0). Whole brain was then homogenate and centrifuged to get the supernatant. The obtained supernatant was used for assay following Ellman method to measure the protein content and percentage of brain AChE Inhibition.

4.5. Pharmacological Evaluation (*In vivo* assay)

4.5.1. Animals

Albino mice (Laka strain, 25-30 g) of both sexes were purchased from Central Animal House, Panjab University, Chandigarh, India. All the research protocols were approved by the Institutional Animal Ethical Committee of University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India. The treatment and maintenance of animals were conducted in accordance with the Principle of Laboratory Animal Care. Animals were housed 5 per cage with food and water available *ad libitum* and maintained at constant temperature (25 ± 1 ^{0}C) and humidity (60 ± 10%) under a 12 h light / dark cycle. All behavioural task paradigms were conducted between 10.00 h and 16.00 h.

4.5.2. Passive avoidance step-down task paradigm

Passive avoidance test were carried out in a rodent memory evaluator (IMCORP, Ambala Cantt. India). All the synthesized compounds excluding the intermediates (**1** and **2**) were evaluated for antiamnesic activity using mice. Piracetam (2.5 mg/kg, obtained from Brown and Burk Pharmaceuticals) and rivastigmine (1.0 mg/kg, obtained from Sun Pharmaceutical Industries Ltd. Silvassa, India) were used as standard drugs. Scopolamine (0. 5 mg/kg i.p.) was used for memory disruption as amnesic agent. The test drugs were suspended in 0.25% carboxymethylcellulose (CMC) and administered intraperitoneally (i.p.) to animals. All of the mice were treated with scopolamine before experiments. Control group was not treated by any drug.

The rodent memory evaluator consists of a fabricated box with electric grid and a centrally located shock free zone (SFZ). It has an electric display system where the voltage current is controlled and the timer is displayed. By keeping the equipment switch off, each mouse was placed individually on the electric grid and allowed to explore the apparatus for about one minute. The equipment was turned on and switch on the latency mode. A proper voltage current (20 V) for foot shock was selected and put the animal on the electric grid and the start button was pressed. Noted down the latency (in seconds) i.e. the time taken by the mouse to reach the SFZ as displayed in the timer. The animals were rejected which took more than 2 minutes to reach SFZ. Put the animal back on the electric grid and repeated it thrice to get three basal readings to acquainted the animals with the task (trained). After one hour of the training, placed the animal on the electric grid one more time and noted the latency. Then turned on the mistake mode and count down the number of mistakes made by animal in 15 minutes as soon as touched the electric grid and felt shock. Compared the basal latency (recorded three readings **I**, **II** and **III** for each animal from each group) in reaching SFZ of each compound by control and scopolamine treated animals. Recorded basal latency data were analyzed and significance was determined by applying two-way ANOVA statistical technique followed by Bonferroni posttest for each compound with respect to control group and scopolamine treated group in a GraphPad Prism 5. Data are expressed by mean ± SEM. Memory parameters (latency and no. of mistakes) are analyzed by non-parametric method using GraphPad Prism.

4.6. Antiradical activity

Antiradical activities of the synthesized compounds were examined using DPPH (1, 1- diphenyl-2-picrylhydrazyl) method. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was used as standard. 1 ml solution of each compound at different concentration (1, 5, 25, 50 and 100 µg/ml) in methanol was mixed with 2 ml of DPPH solution (0.5 mM). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm against methanol as the blank in a spectrophotometer. Higher antiradical activity observed by the lower absorbance of the reaction mixture. Experiment was accomplished in triplicate. The antiradical activity was calculated in percentage as per the following formula: antiradical activity (%) = (absorbance of control - absorbance sample / absorbance of control) x 100.

Statistical study

Statistical analysis was performed using GraphPad Prism 5. Statistical significance was accepted for P values of < 0.05.

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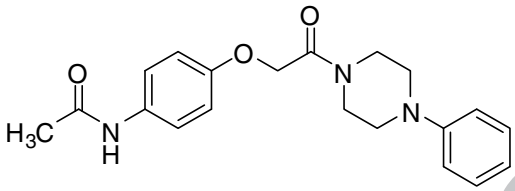
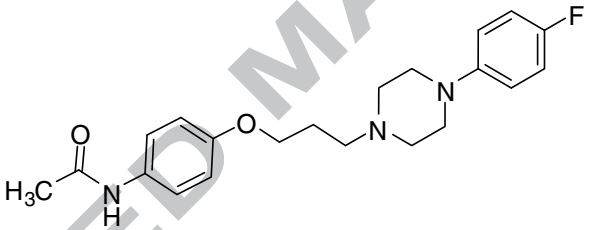
Graphical abstract



Compound 1b: Mouse AChE, IC_{50} = 1.66 μM, K_i = 43.66 μM



Compound 2d: Mouse AChE, IC_{50} = 0.49 μM, K_i = 4.10 μM

Highlights

- The synthesized piperazine derivatives were found to be potent dual binding site inhibitors of acetylcholinesterase enzyme.
- Compounds **1b** and **2d** displayed excellent IC_{50} values of 1.66 μM and 0.49 μM respectively.
- Showed significant antiamnesic activity at a dose of 1.0 mg/kg.
- Have CNS penetration and brain AChE inhibition abilities.
- May be used as leading compounds in the future drug design.