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# Design and synthesis of some acridine-piperazine hybrids for the improvement of cognitive dysfunction.

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**Design and synthesis of some acridine-piperazine hybrids for the improvement of cognitive dysfunction.**

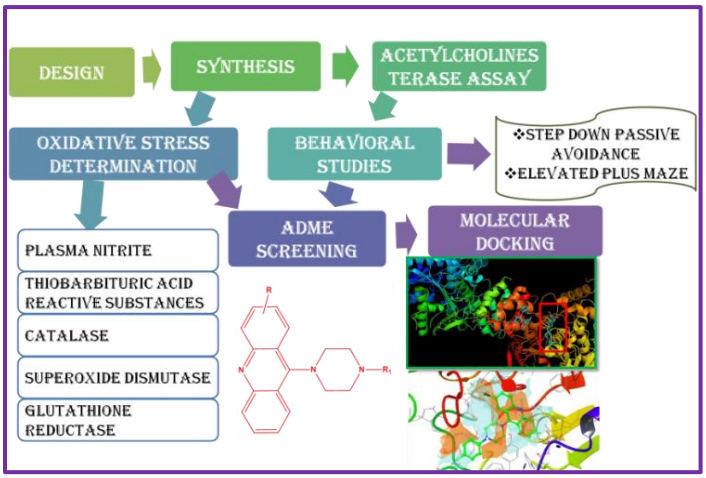
**Abstract**

A novel series of hybrid molecules (**5a-5m**) was designed, synthesized and evaluated as multifunctional cholinesterase (ChE) inhibitors against cognitive dysfunction. Heterocyclic moieties acridine and piperazine were conjugated with suitable linkers in a single scaffold and

the structures of the target compounds were confirmed by IR, $^{1}$H NMR, $^{13}$C NMR and LC-MS analysis. The pharmacological activity of synthesized compounds was evaluated using behavioral models of amnesia *viz.* step down passive avoidance and elevated plus maze at a dose 0.5mg/kg as compared to standard rivastigmine. *In vitro* acetylcholinesterase (AChE) inhibition studies using brain homogenate of mice as the enzyme source revealed that most of the compounds exhibited a significant ability to inhibit the enzyme cholinesterase with compound **5c** being the most potent ($IC_{50}$ 0.33 µM). Biochemical estimation of oxidative stress markers *viz.* plasma nitrite, thiobarbituric acid reactive substances, catalase, superoxide dismutase and glutathione has been carried out using the respective assays to see the effect of the synthesized compounds on the scopolamine induced oxidative damage. The molecular docking studies indicated the binding mode of the compounds to the catalytic site, peripheral site and mid-gorge of AChE simultaneously. The calculated ADME properties ensured the drug-likeness of the target compounds. The synthesized compounds were found to be potential cognitive enhancers, which were able to interfere with the scopolamine-induced oxidative stress also.

Keywords: Acridine; Antioxidant, Cognition Enhancer, Docking, Piperazine, Synthesis.

Graphical Abstract




HIGHLIGHTS

- A series of acridine-piperazine hybrids was designed and synthesized as potential cognition enhancers.
- The synthesized compounds were evaluated for cognitive potential using step down passive avoidance and elevated plus maze models supported by biochemical estimation for acetylcholinesterase.

- Biochemical estimation of oxidative stress markers *viz.* plasma nitrite, thiobarbituric acid reactive substances, catalase, superoxide dismutase and glutathione has been carried out.
- The $IC_{50}$ of the most potent compound **5c** against acetylcholinesterase was found to be 0.33 μM.
- Docking studies of the target compounds **5a-5m** were performed using Glide and VLife softwares to explore the binding modes within the enzyme.
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**Introduction**

Cognition (derived from the Latin word "cognitio") may be defined as the process of amassing knowledge and understanding through thought, experience, and the senses (1-3). Cognitive deterioration characterized by deficit cholinergic neurotransmission and aggregation of *β*-amyloid protein is the main testimonial evidence in various dementing disorders mainly in Alzheimer disease (AD), Parkinson's disease, stroke, schizophrenia, seizure disorders and traumatic brain injury etc (4-5). Acetylcholine (ACh) neurotransmitter plays a major role in memory and dementia. The brains of persons with severe cognition dysfunction show consistently depleted ACh levels. The deficiency of ACh at the post-synaptic receptor site is attributed to the acetate and choline due to the hydrolysis of ACh by enzyme acetylcholinesterase (AChE) (6). AChE inhibition belongs to the cholinergic replacement strategy designed for the compensation of primary selective degeneration of the cholinergic neurons. AChE inhibitors delay the inter synaptic degeneration of ACh by AChE, which leads to an increase in accessibility of ACh at the synaptic gap junction (7).

AChE has two binding sites i.e. catalytic active site (CAS) at the bottom and peripheral active site (PAS) at the entrance of gorge (8). Selectively, ligands can bind to either the CAS or the PAS and it has been hypothesized that formation of *β*-amyloid plaques and tangles is aggravated through interaction of ligands with PAS. However, literature reports have revealed that dual binding site AChE inhibitors facilitate cholinergic neurotransmission as well as interfere with the aggregation of toxic β-amyloid (9). Till now, the approval of only four AChE inhibitors *viz.* tacrine, donepezil, rivastigmine and galanthamine have been granted for the treatment of moderate to severe AD by the European and United State regulatory authorities (10). The narrowness of the current therapeutics makes treatment of AD the current biggest unmet medical need in cognitive disorders.

Literature evidences have suggested that accumulation of plaques and tangles in the brain facilitates the generation of free-radicals scavengers leading to an increase in the oxidative stress markers, which damage lipid and protein content of the neurons. This oxidative stress is responsible for neurodegeneration, nerve cell structural and functional damage and neuronal apoptosis in patients leading to cognitive dysfunction. Hence many antioxidants have been introduced both clinically and pre-clinically for the treatment of cognitive dysfunction and consideration has also been given to oxidative stress induced in cognitive deficits (11-12).

At this juncture, acridine absorbed our attention as a chemically versatile scaffold exhibiting diverse biological properties (13-14). Tacrine (acridine containing drug) was the first FDA approved non-selective AChE inhibitor for the treatment of AD (10). It remains a reference structure in developing AChE inhibitors, although the lack of selectivity and liver toxicity has limited its clinical applicability (15). In this respect, lot of efforts have been made to design and synthesize tetrahydoacridine (tacrine) based agents and its hybrids as AChE inhibitors with lesser side-effects and a better pharmacokinetic profile. The potential activity of acridine derivatives during random screening as AChE inhibitors (16-18) motivated us to explore this nucleus and pursue our research in this direction so as to minimize the side effects caused by it.

Literature further reveals the significance of piperazino moiety for its high potency, selectivity of action and excellent therapeutic profile in cognitive dysfunction, which has caught the attention of many medicinal chemists and pharmacologists to further explore this group (19-20). Easy functionalization of piperazine makes it an alluring building block for designing and synthesizing new compounds of therapeutic utility (21). This instigated the present investigator to develop acridine-piperazine hybrids devoid of unwanted side effects.

Therefore, in the present work, a series of acridine-piperazine hybrids has been synthesized and studied as potential cognition enhancers using scopolamine induced dementia, biochemical estimations of AChE and oxidative stress markers. Moreover, to further investigate the mechanism of action involving AChE, *in silico* studies have been performed.

**Experimental Section**

Melting points are uncorrected and were measured in open capillary tubes, using a Veego melting point apparatus. $^{1}H$ and $^{13}C$ NMR spectra were obtained using a Bruker Avance II 400 MHz spectrometer. Elemental analyses were done on a Thermo scientific CHNSO elemental

analyser (Model: Flash 2000) at Central Instrumental Laboratory, Panjab University, Chandigarh and results were within 0.4% of the theoretical values. Furthermore, all the compounds were checked by thin layer chromatography (TLC) and showed single spots. Taken together, both features ensure ≥ 95% of purities. The LC-MS spectra (ESI positive mode) were obtained on Waters, Q-TOF Micromass spectrometer. The FTIR spectra were obtained from a PerkinElmer Spectrum Version 10.03.08 using KBr disc method for the preparation of the sample.

*Chemistry*

*General Procedure for the synthesis of substituted anthranilic acids (**1a-1g**)*. A suspension of *o*-bromobenzoic acid (1 g, 85 mmoL), substituted anilines (25.5 mmoL) and copper powder (0.1 g) was refluxed in ethanol (20 mL) at 80 $^{o}$C for 12-18 h in the presence of anhydrous potassium carbonate (1 g) (22). The reaction was monitored by TLC. The suspension was cooled to 20-25 $^{o}$C and was poured into ice-cold water. The contents were further acidified with concentrated hydrochloric acid to yield the precipitates, which were washed with water and dissolved in 5% sodium hydroxide solution (50 mL). The resulting solution was boiled after addition of activated charcoal. The hot solution was then filtered and concentrated hydrochloric acid was added to the filtrate to obtain the crude product which was filtered, dried and recrystallized from ethanol to yield the corresponding intermediates **1a-1g**.

*General Procedure of substituted acridones (**2a-2g**)*. A mixture of various substituted anthranilic acid **1a-1g** (1 g, 4.03 mmoL) and concentrated sulphuric acid (5 mL) was heated at 100 $^{o}$C for 8-11 h. The completion of reaction was monitored by TLC. The reaction mixture was cooled to room temperature and crushed ice was further added to the obtained viscous mass to yield the crude product. It was filtered and washed with 5% aqueous solution of sodium carbonate. The precipitate obtained was filtered, dried and crystallized from ethanol. It was recrystallized with ethanol-methanol to obtain the corresponding substituted acridones **2a-2g**.

*General Procedure of substituted 9-chloroacridines **3a-3g***. Substituted acridone **2a-2g** (4.0 mmoL) was added to phosphorus oxychloride ($POCl_3$) (1 mL, 8.0 mmoL) and was refluxed at 110 $^{o}$C for 7-13 h. The resulting suspension was cooled to room temperature and crushed ice was added to the viscous mass. The solid so obtained was filtered, dried and recrystallized from ethanol to obtain **3a-3g**.

*General Procedure of acridine-piperazine hybrids **5a-5m***. A mixture of substituted piperazine (8.32 mmoL), substituted 9-chloroacridine (4.03 mmoL) and sodium iodide (0.5 g) was refluxed

at 80 $^{o}$C for 13-19 h in ethyl methyl ketone in the presence of anhydrous potassium carbonate (1 g). The completion of the reaction was monitored by TLC. The crude product obtained was filtered, washed repeatedly with water to remove unreacted amine. It was dried and crystallized from ethanol to yield **5a-5m**. The detailed spectral information of the compounds is given in the supplementary file.

**Pharmacology**

*Animals*. Albino mice (female, Laca strain) weighing 25-30 g, were purchased from Central Animal House, Panjab University, Chandigarh, India. All the research protocols were followed by the Institutional Animal Ethical Committee, Panjab University, Chandigarh, India.

*Drugs*. Rivastigmine (0.5 mg/kg, obtained from Sun Pharmaceutical Industries Ltd, Silvassa, India) was used as the standard drug. Scopolamine (0.5 mg/kg i.p.) was used as an inducer of cognition deficits, which is qualitatively similar to that occurring in senile dementic subjects (24). The test compounds were dissolved in sterile distilled water/0.1% DMSO and administered intraperitoneally (i.p.) to animals.

*Step-down passive avoidance test*. Rodent memory evaluator (IMCORP, Ambala Cantt, India) was used to carry out the behavioral test (25).

*Elevated plus maze*. The time taken by the experimental animal to enter the enclosed arm in the acquisition trial (first) was noted, so called as initial transfer latency (ITL). The retention (second) trial was performed 24 hours after the acquisition trial, so called retention transfer latency (RTL). It was expressed as the percentage of retention (26).

**Biochemical Parameters Estimation**

*Brain tissue preparation*. After behavioral assessment, animals were sacrificed by spinal dislocation and the whole brains were quickly removed, rinsed with ice-cold isotonic saline. A brain homogenate (10% w/v) was prepared using 0.03 M sodium phosphate buffer (pH 7.4) and centrifuged using an Ultra-Turrax T25 (USA) homogenizer at 10000 rpm for 15 minutes. The aliquots of supernatant were used for the biochemical estimation. Biuret method was employed to determine protein content using standard bovine serum albumin (BSA) (1 mg/mL) (27).

*Acetylcholinesterase assay*. The cholinergic dysfunction was assessed by estimation of the cholinergic marker, AChE in the whole brain according to the Ellman method (23).

*Assay for the estimation of oxidation stress parameters.* Green *et al.*, was first to develop the assay of nitrite estimation. The cytosolic fractions of different brain regions were served as an indicator of nitric oxide production (28). Malondialdehyde (MDA) content is a measure of lipid peroxidation assay and the procedure was based on the original work of Kobayashi *et al* (29). The protocol of the catalase assay was laid by Claiborne *et al* (30). Kono *et al*., developed the method to perform SOD assay and the auto-oxidation of hydroxylamine was observed by measuring the change in optical density at 560 nm for 2 min at 30-60 s intervals (31). The reduced glutathione assay has been developed by Jollow *et al*., that is based on Ellman method (32). All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

### *In Silico* Studies

ADME property prediction was carried out using QikProp module to evaluate DrugLikeness of the synthesized compounds (33).

Molecular docking studies were performed using Glide module of Schrodinger (34) and Biopredicta module of VLife MDS (version 4.6.28102016) (35).

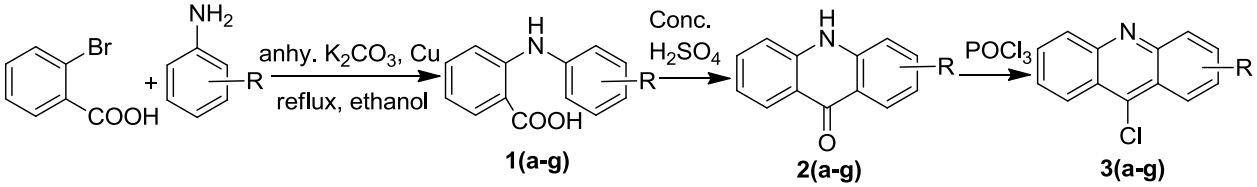
## Results and Discussion

*Molecular Design.* As reported previously in the introduction, acridine and piperazine scaffolds have emerged as good AChE inhibitors as these interact with catalytic anionic site (CAS) and peripheral active site (PAS) of AChE respectively through hydrophobic, vander wal and π-π interactions. Therefore, our research group hypothesized that amalgamating both moieties might afford more effective multifunctional molecules which could not only inhibit AChE but also exhibit neuroprotective effect. The interactions of the designed compound have been analysed using molecular docking tools by placing the compound into the binding cavity of the enzyme in a non-covalent fashion. The selection of the compounds to be synthesized has been done on the basis of high binding interactions and low binding energies. The preliminary docking studies indicated acrdine and piperazine as suitable templates to design novel cognition enhancers.

*Chemistry.* In the light of above considerations, the designed hybrids were synthesized as shown in **Scheme 1** and **2**.

Synthesis of substituted anthranilic acids **1a-1g** was carried out by copper catalysed *N*-alkylation (22) of different substituted anilines with *o*-bromobenzoic acid (85 mmoL) in the presence of anhydrous potassium carbonate and ethanol as the solvent respectively. A mixture of compounds **1a-1g** (4.03 mmoL) and concentrated sulphuric acid (5 mL) was heated at 100 $^{o}$C for 8-11 h to obtain compounds **2a-2g** respectively which were chlorinated by refluxing them with phosphorus oxychloride ($POCl_3$) (1 mL, 8.0 mmoL) at 110 $^{o}$C for 7-13 h to yield the corresponding compounds **3a-3g** (Table 1) respectively.

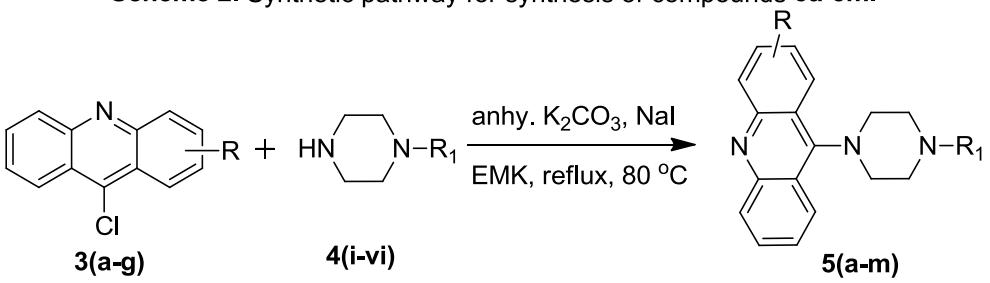
**Scheme 1.** Synthesis of compounds **3a-3g.**




R = H, 4-Cl, 4-F, 2-F, 2-Cl, 4-$CH_3$, 2,4-$CH_3$

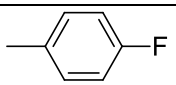
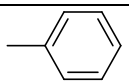
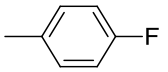
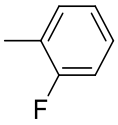
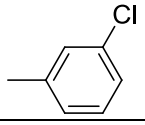
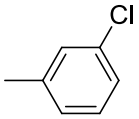
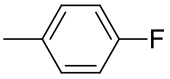
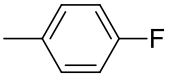
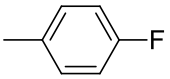
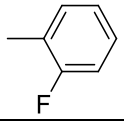
Subsequently, the target compounds **5a-5m** have been synthesized (Table 1) by refluxing the substituted chloroacridines **3a-3g** with substituted piperazines **4i-4vi** in ethyl methyl ketone in the presence of sodium iodide and anhydrous potassium carbonate respectively (**Scheme 2)**.

**Scheme 2.** Synthetic pathway for synthesis of compounds **5a-5m.**




| | $R_1$ |
|---|---|
| **4i** | 4-Fluorophenyl |
| **4ii** | 2-Fluorophenyl |
| **4iii** | -Phenyl |
| **4iv** | -Ethyl |
| **4v** | -Methyl |
| **4vi** | 3-Chlorophenyl |

**Table 1: The physicochemical data of the compounds 3a-3g and 5a-5m.**

| Code | R | $R_1$ | Molecular Formulae | Molecular weight | Percentage Yield | Melting Point ($^oC$) |
|---|---|---|---|---|---|---|
| **3a** | H | - | $C_{13}H_8ClN$ | 213.66 | 77.98 | 140-142 |
| **3b** | 4-Cl | - | $C_{13}H_7Cl_2N$ | 248.11 | 73.05 | 148-150 |
| **3c** | 4-F | - | $C_{13}H_7ClFN$ | 231.66 | 85.19 | 202-204 |
| **3d** | 2-F | - | $C_{13}H_7ClFN$ | 231.66 | 76.86 | 280-282 |
| **3e** | 2-Cl | - | $C_{13}H_7Cl_2N$ | 248.11 | 73.15 | 141-143 |
| **3f** | 4-$CH_3$ | - | $C_{14}H_9ClN$ | 227.69 | 82.72 | 123-125 |
| **3g** | 2,4-$CH_3$ | - | $C_{15}H_{14}ClN$ | 243.74 | 82.56 | 110-112 |
| **5a** | H |  | $C_{23}H_{20}FN_3$ | 357.43 | 77.84 | 180-182 |
| **5b** | 4-Cl |  | $C_{23}H_{20}ClN_3$ | 373.88 | 69.54 | 165-168 |
| **5c** | 4-Cl |  | $C_{23}H_{19}ClFN_3$ | 391.87 | 70.06 | 146-150 |
| **5d** | 4-Cl |  | $C_{23}H_{19}ClFN_3$ | 391.13 | 63.06 | 280-282 |
| **5e** | 4-Cl | -$C_2H_5$ | $C_{19}H_{20}ClN_3$ | 325.84 | 73.51 | 191-193 |
| **5f** | 4-Cl | -$CH_3$ | $C_{18}H_{18}ClN_3$ | 311.81 | 93.65 | 205-207 |
| **5g** | 4-F |  | $C_{23}H_{19}ClFN_3$ | 391.87 | 70.06 | 146-150 |
| **5h** | 2-F |  | $C_{23}H_{19}ClFN_3$ | 391.87 | 54.14 | 196-199 |
| **5i** | 2-Cl |  | $C_{23}H_{19}ClFN_3$ | 391.87 | 76.43 | 268-270 |
| **5j** | 4-$CH_3$ |  | $C_{24}H_{22}FN_3$ | 371.45 | 76.68 | 278-280 |
| **5k** | 2,4-$CH_3$ |  | $C_{25}H_{24}FN_3$ | 385.49 | 88.60 | 282-284 |
| **5l** | 2,4-$CH_3$ |  | $C_{25}H_{24}FN_3$ | 385.49 | 88.60 | 286-288 |
| **5m** | 2,4-$CH_3$ | -$C_2H_5$ | $C_{21}H_{25}N_3$ | 319.45 | 73.79 | 248-250 |

## Pharmacology

*In vitro acetylcholinesterase inhibition*. *In vitro* evaluation of the synthesised acridine-piperazine hybrids **5a-5m** was carried out using the brain supernatants of fresh or untreated mice as a source of enzyme by Ellman method (23). The concentration vs. percentage inhibition graph was plotted

by taking five different concentrations (1, 5, 10, 25, and 50 µL) of target compounds (**5a-5m**) and the standard rivastigmine according to optimal correlation coefficient to determine $IC_{50}$ (Table S1). Behavioral studies were performed on scopolamine-induced amnesic mice using step down passive avoidance and elevated plus maze test for cognitive enhancing potential of the synthesized hybrids.

*Behavioral studies on mice by step-down passive avoidance*. Three basal readings (basal latency) were recorded for the training period. After 1 h the latency was noted down and the number of mistakes made by the animals was counted for 15 minutes. Results of the basal latency and memory parameters (latency and mistakes) are shown in Table 2 and Figure 1, indicating that compounds **5a-5m** show remarkable improvement in cognitive deficit as compared to both standard rivastigmine and scopolamine treated groups.

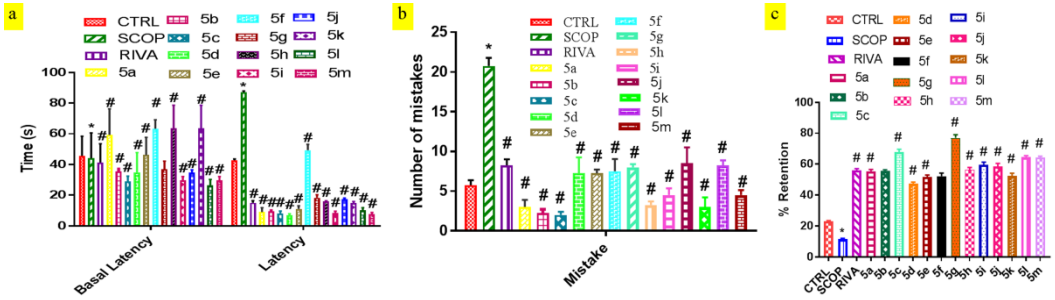



**Figure 1**. **(a)** Divergence of basal latency and latency in various groups treated by control, scopolamine, rivastigmine and target compounds **5a-5m**; **(b)** Graphical representation of the number of mistakes in the various treated groups made by the mice; **(c)** Comparison of % retention in various treated groups **5a-5m**; (*) $p < 0.0001$ as compared to control; (#) $p < 0.0001$ as compared to scopolamine (n = 5). The intergroup variation was found out using tukey's test and values were considered statistically significant (0.0001).

**Table 2: The basal latency, latency and the no. of mistakes using passive avoidance protocol and % retention in the elevated plus maze test of the compounds 5a-5m.**

| | Basal Latency | Latency | No. of Mistakes | % Retention |
|---|---|---|---|---|
| Control | 45.67±12.75 | 42.75±0.85 | 14.00±0.58 | 23.02±0.55 |
| Scopolamine | 44.08±16.50 | 87.00±0.92 | 26.00±2.31 | 11.44±0.60 |
| Rivastigmine | 41.34±6.80 | 15.00±1.47 | 8.00±0.58 | 55.94±1.21 |
| **5a** | 59.25±17.01 | 9.00±2.89 | 3.00±0.92 | 55.39±1.12 |
| **5b** | 35.50±1.71 | 9.05±0.65 | 2.25±0.48 | 55.52±0.82 |
| **5c** | 29.08±3.30 | 8.00±1.73 | 2.00±0.41 | 78.39±3.38 |
| **5d** | 46.17±11.58 | 11.00±2.16 | 7.25±0.48 | 51.72±1.26 |
| **5e** | 34.75±13.05 | 7.00±0.91 | 7.25±2.02 | 47.46±0.89 |
| **5f** | 63.25±5.91 | 49.25±3.838 | 7.50±1.55 | 52.25±1.96 |
| **5g** | 36.92±5.17 | 18.25±2.02 | 8.00±0.41 | 77.06±1.95 |
| **5h** | 63.58±15.05 | 16.00±0.41 | 3.25±0.48 | 56.10±1.75 |
| **5i** | 29.50±2.60 | 8.25±1.37 | 4.50±0.87 | 59.55±1.74 |
| **5j** | 34.67±1.89 | 17.25±0.85 | 8.50±2.02 | 58.56±1.98 |
| **5k** | 63.58±15.05 | 14.75±1.11 | 3.00±1.23 | 52.27±1.88 |
| **5l** | 26.42±3.90 | 10.25±1.65 | 8.25±0.63 | 64.49±1.05 |
| **5m** | 29.50±2.60 | 7.50±1.32 | 4.50±0.65 | 64.24±0.89 |

All results are expressed in the form of mean ± SEM (n=5). Significance for the basal latency and latency was determined by two-way ANOVA by Tukey's test and for number of mistakes and %retention was determined by one-way ANOVA followed by Tukey's test.

*Behavioral studies on mice using elevated plus maze*. Retention and memory acquisition of all the compounds was screened for antiamnesic activity. Amongst the synthesised compounds, compound **5c** was found to be an active compound and significantly improved learning and cognition with % retention of 78.39 at a dose of 0.5 mg/kg in comparison to rivastigmine (Table 2 and Figure 1).

## Biochemical estimation

*Biochemical estimation of the acetylcholinesterase enzyme.* Biochemical studies were carried out to figure out the mechanism of action of the enzyme inhibition by all the synthesized compounds using Ellman method (23). The AChE inhibitory profile of the synthesized compounds in comparison to the standard and the inducer is depicted in Figure 2 graphically. The results of biochemical studies were found to be in agreement with the behavioral studies. The active compounds (**5c** and **5d**) have shown a lower value of the μmoles of AChE (Table 3).

**Table 3: Biochemical estimation of the AChE enzyme and oxidative stress parameters.**

| S. No. | Acetylcholinesterase[a] | Plasma nitrite[b] | Lipid Peroxidation[c] | Catalase[d] | Superoxide Dismutase[e] | Glutathione Reductase[f] |
|---|---|---|---|---|---|---|
| Control | 0.00203±0.00024 | 240.17±6.57 | 0.57±0.05 | 7.53±0.33 | 10.91±0.51 | 0.0337±0.0016 |
| Scopolamine | 0.00358±0.00016 | 714.83±18.47 | 3.98±0.20 | 1.53±0.45 | 1.28±0.11 | 0.0104±0.0020 |
| Rivastigmine | 0.00182±0.00038 | 357.83±10.16 | 3.13±0.51 | 3.66±0.58 | 2.75±0.06 | 0.0159±0.0009 |
| **5a** | 0.00172±0.00028 | 477.5±5.89 | 1.93±0.28 | 4.95±0.52 | 5.61±0.71 | 0.0201±0.0029 |
| **5b** | 0.00164±0.00005 | 333.50±10.16 | 1.88±0.27 | 4.85±0.74 | 3.77±0.29 | 0.0196±0.0028 |
| **5c** | 0.00159±0.00009 | 290.83±10.54 | 1.80±0.06 | 5.14±0.38 | 5.32±0.30 | 0.0187±0.0006 |
| **5d** | 0.00154±0.00012 | 345.17±12.17 | 1.96±0.20 | 5.22±0.32 | 4.62±0.23 | 0.0204±0.0021 |
| **5e** | 0.00153±0.00035 | 336.83±8.60 | 1.81±0.26 | 5.61±0.41 | 3.32±0.29 | 0.0189±0.0027 |
| **5f** | 0.00172±0.00028 | 408.50±10.76 | 1.85±0.32 | 5.56±0.42 | 4.68±0.60 | 0.0193±0.0034 |
| **5g** | 0.00171±0.00028 | 425.83±13.64 | 1.98±0.29 | 5.32±0.12 | 4.43±0.45 | 0.0206±0.0030 |
| **5h** | 0.00175±0.00001 | 478.50±7.76 | 2.01±0.13 | 5.43±0.34 | 5.13±0.24 | 0.0209±0.0014 |
| **5i** | 0.00172±0.00003 | 408.17±11.73 | 1.94±0.20 | 5.08±0.46 | 3.48±0.40 | 0.0190±0.0022 |
| **5j** | 0.00157±0.00038 | 363.50±11.40 | 1.97±0.29 | 5.55±0.25 | 5.21±0.40 | 0.0187±0.0032 |
| **5k** | 0.00158±0.00014 | 408.50±11.26 | 1.84±0.36 | 5.33±0.37 | 3.61±0.58 | 0.0192±0.0037 |
| **5l** | 0.00159±0.00007 | 348.83±0.01 | 1.96±0.21 | 5.59±0.29 | 4.00±0.38 | 0.0184±0.0023 |
| **5m** | 0.00172±0.00022 | 471.83±10.26 | 1.87±0.21 | 5.67±0.28 | 4.47±0.33 | 0.0195±0.0022 |

All the results are expressed in the form of mean ± standard error mean (SEM). One-way ANOVA followed by Tukey's test was employed to determine significance. [a]μmoles of AchE/min/mg pr, [b]Nitrite conc. μg/mL, [c]n moles of MDA/mg protein, [d]Catalase activity/min, [e]SOD units/mg protein and [f]μmoles of GSH/mg protein

*Biochemical estimation of the parameters of oxidative stress: plasma nitrite, lipid peroxidation thiobarbituric acid reactive substances, catalase, superoxide dismutase and glutathione*. Oxidative stress is the most important factor which aggravates neurodegeneration by producing free radicals and reactive oxygen species that ultimately result in oxidative stress and damage to

the neuronal cells leading to cognitive impairments. The potential of cognition enhancers as antioxidants is an important aspect of neuroprotection which has been properly investigated by determination of biochemical markers of oxidation stress in scopolamine induced amnesia. Oxidative stress and ROS are proposed to be the major biochemical manifestations of the aging process. Scopolamine causes a significant increase in the oxidative stress markers as compared to the control group indicating a state of oxidation stress and oxidative damage.

In the present work, biochemical estimation of the parameters such as plasma nitrite, thiobarbituric acid reactive substances, catalase, superoxide dismutase and glutathione in the mice brain supernatants was carried out. A significant increase in AChE, plasma nitrite, malondialdehyde along with a remarkable decrease in activity of catalase, superoxide and glutathione reductase was observed in group of mice treated with scopolamine as shown in Figure 2 and Table 3.

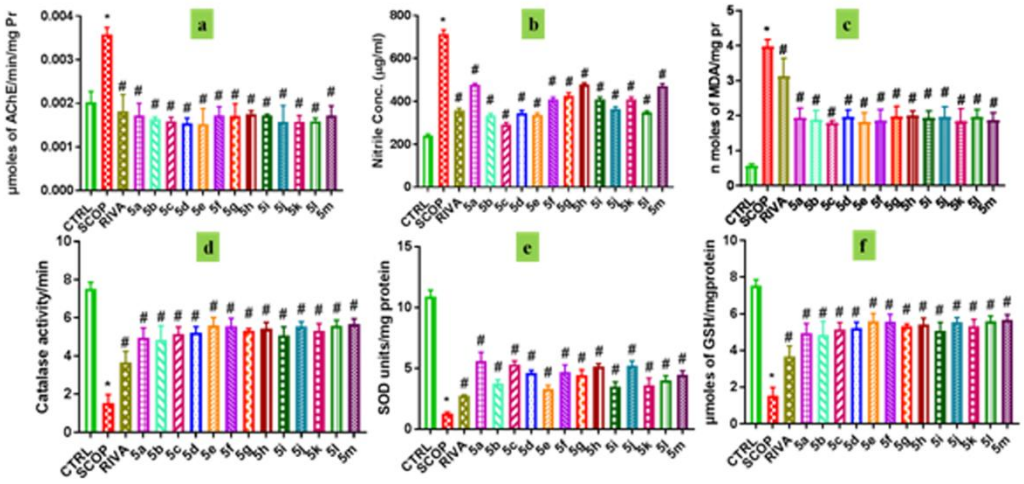



**Figure 2**. Effect of the synthesized compounds **5a-5m** treatment on **(a)** AChE, **(b)** nitrite, **(c)** malondialdehyde (MDA), **(d)** catalase, **(e)** superoxide dismutase (SOD) and **(f)** reduced glutathione (GSH) on condition of cholinergic deficit and oxidative stress induced by scopolamine. (*) $p < 0.0001$ as compared to control; (#) $p < 0.0001$ in comparison to scopolamine ($n = 5$). The intergroup variation was evaluated by employing Tukey's test and values were considered statistically significant (0.0001).

The compounds **5c** and **5e** were promising in decreasing the levels of plasma nitrite with the nitrite concentrations 290.83 µg/mL and 336.83 µg/mL as compared to 714.83 µg/mL of the scopolamine treated group. Thiobarbituric acid reactive substances (TBARS) assay is performed to estimate the lipid peroxidation products quantitatively in the mice brain homogenate sample. The absorbance was measured at 532 nm to estimate the MDA reactive products. The groups treated with compounds **5c** and **5e** exhibited lower level of MDA as compared to those treated

with scopolamine (inducer) and rivastigmine (standard). The hydrogen peroxide breakdown is measured for the biochemical estimation of catalase. As compared with the scopolamine-treated group compounds **5c**, **5e** and **5g** significantly averted the loss of catalase activity with catalase/min value of 5.16, 5.61 and 5.32 respectively. Lower value of SOD units/mg of the protein for compounds **5c** and **5e** (5.32 and 3.32) proposed the possible role of the synthesised compounds in reversal of scopolamine induced oxidative damage.

GSH is a well-known antioxidant, present in higher concentrations in the mitochondrial matrix that is synthesized in the cytoplasm. The increased oxidative stress leads to decrease the value of reduced glutathione. It is observed that the group treated with scopolamine depicted lowest value of GSH as compared to the control. GSH levels were found to be higher (0.0187 μ moles of GSH/mg protein) in the groups treated with compound **5c**, as compared to the inducer scopolamine (0.0104 μ moles of GSH/mg protein) and standard rivastigmine (0.0159 μ moles of GSH/mg protein) treated groups.

The synthesised compounds exhibited better activity as compared to the rivastigmine treated group, thus indicating that besides improving cognition, the compounds were found to be better than rivastigmine in terms of managing the scopolamine induced oxidative stress. Thus by regulating the cholinergic deficit through AChE inhibition and oxidative stress, the target compounds can be used as dual functional molecules, displaying promising role in treatment of cognitive dysfunction.

***In Silico* Studies**

*Forecasting ADME Property*. The acceptability of the compounds was estimated on the basis of the Lipinski's rule of five (36). The ADME properties of the most potent compound **5c** are shown in Table SII.

*Molecular Docking Studies*. Molecular docking studies were performed to support the data obtained from *in vitro*, *in vivo* evaluations and ADME screening for determination of the best *in silico* conformation of the most potent molecule. The PDB crystal structure of recombinant human AChE (*rh*AChE) complexed with E2020 (37) (Figure 3(a)) is taken from the protein data bank for analysis of the crucial key interactions. Figure 3(b) clearly shows the binding pocket in the form of mesh structure with the cocrystal. All the ligands were found to interact with key amino acids of the enzyme and the hypothetical modes of all the synthesised compounds showing the crucial interactions in the defined grid are depicted in Figure 3c-d. For analyzing the

interactions, the compound **5c** has been taken into consideration because all the compounds interacted in the similar manner.

The most potent compound **5c** exhibited excellent AChE inhibitory activity. Its conformation fitted suitably in the defined grid covering the CAS and PAS of the crystal structure, acting as a dual binding site inhibitor. The acridine moiety of compound **5c** interacted with the key amino acids (Trp86, Gly121, Tyr341 and Phe338) present at the bottom of the gorge and the fluoropiperazine ring showed crucial interactions at the entrance of the gorge (Trp286 and Phe297) as depicted in the 3D and 2D-ligand interaction diagram (Figure 3e and 3f). Fluorine attached to the ring formed the hydrogen bond of distance 1.486 Å with Phe299. Both the moieties showed interactions with Tyr124 amino acid present at the mid gorge site of the enzyme. It is relevant to mention here that the hybrids may follow the same mode of action as the various dual binding site inhibitors such as donepezil. In all the cases, it showed interactions with the CAS and PAS, hence it might inhibit the amyloid formation.

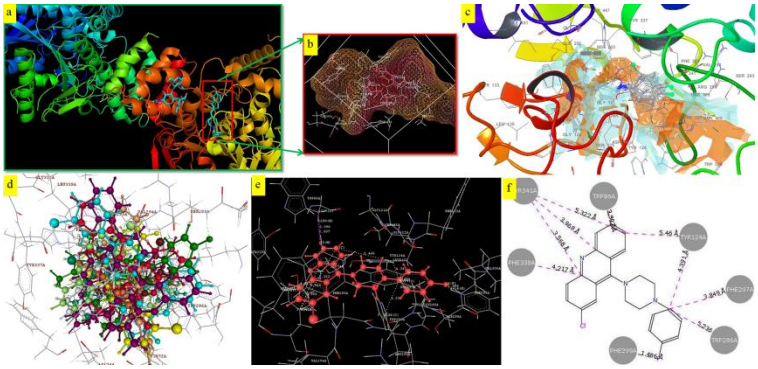


**Figure 3**. **(a)** The crystal structure of *rh*AChE (PDB ID 6EY7) with its cocrystal E2020 (marked in the box), **(b)** The key amino acids surrounding the cocrystal in the binding pocket (marked as the meshed shape) in the defined grid, **(c)** The hypothetical binding motifs of all the ligands in the enzyme pocket by Glide, **(d)** The hypothetical binding modes of all the ligands by VLife, **(e)** 3D ligand interaction diagram showing the binding orientation of the active comp **5c** with the key amino acids present in CAS and PAS of the binding pocket and **(f)** 2D-diagram showing the probable binding mode of the active comp **5c** within the enzyme pocket.

All the synthesized ligands showed Gscore ranging from -13.10 to -08.90 and dock score ranging from -4.88 to -1.17 (Table SIII) theoretically, which are comparable to the pharmacological activity. Overall, electron withdrawing substituents (like Cl and F) were found to be favorable for AChE inhibitory activity and vice-versa. The above findings suggested that the substitution of a bulky group at ninth position of tricyclic ring system might influence the electronic changes to affect the interaction with enzyme, thus influence their potency.

**Conclusion**

A **s**eries of novel acridine-piperazine hybrids has been designed, synthesized and evaluated as potential cognition enhancers. The results of *in vitro* AChE inhibition assay were also in agreement with the results obtained from the behavioral studies and *in silico* data. The role of these compounds in the management of cognitive dysfunction may be attributed to oxidative stress as illustrated by estimation of oxidative stress parameters. Based on the results of the behavioral, biochemical and *in silico* studies, compounds **5c** and **5e** have displayed appreciable AChE inhibitory activity as compared to the standard rivastigmine.

The molecular docking studies identified these active compounds as the dual binding site inhibitors displaying significant binding interactions at CAS and PAS within the binding pocket of the AChE enzyme. The present study concluded that acridine-piperazine hybrids can serve as the analogues having promising role in the management of cognitive dementia and the most potent compound **5c** can serve as promising lead in this arena of research for any further pursuit.

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