Summary and Conclusion

Chapter 6

6. Summary and Conclusions

Based on the **FA** template, we designed and developed a novel series of glycine amide derivatives and related analogs as a part of our approach to develop naturally inspired multifunctional drugs for AD management. The rationale behind the design of the novel molecules is to have improved cholinergic inhibitory activities and an increase in cLogP of **FA** analogs. The introduction of an amide linker followed by an aromatic or substituted aromatic or heterocyclic or substituted piperazine feature leads to significant improvement in the enzyme's inhibition properties. The developed molecules were tested for *in-vitro* AChE and BChE inhibitory activities. Among the tested compounds, **4d, 4f**, **4i**, **4j**, **7a** (**F24**)**,** and **7b** showed the highest inhibitory activities for AChE. All the compounds were further subjected to BChE inhibitory activity evaluation. From among all the compounds, **4f, 4i, 7a** (**F24**), and **7b** showed potent BChE inhibition properties. Enzyme inhibition studies identified compound **7a** (**F24**) as a lead molecule with preferential AChE inhibition (AChE, IC_{50} = 5.74 ± 0.13 μM; BChE, IC_{50} = 14.05 ± 0.10 μM) compared to the parent molecule **FA** (% inhibition of AChE and BChE at 20 μM, 15.19 ± 0.59% and 19.73 ± 0.91%, respectively).

The molecular docking studies revealed that **7a (F24)** could bind to peripheral and catalytic sites and expose the two different binding modes with AChE. The stabilities of the best complexes between **7a** and AChE and BChE were confirmed with the help of MD studies. The calculated physicochemical properties clearly demonstrated the druggable properties of the developed novel molecules. The data from the enzyme kinetic study proved that **7a** caused noncompetitive inhibition of AChE and mixed inhibition of BChE.

In the pH-dependent UV-based complexation study and mass spectrometric analysis, the lead molecule **7a** (**F24**) also exhibited significant antioxidant activity in the DPPH assay (IC_{50} = 57.35 ± 0.27 μM) and iron-chelation property. Based on the enzyme inhibition and antioxidant studies, **7a** (**F24**) was selected as a lead molecule for further *in-vitro* and *in-vivo* studies. The data from the AFM analysis demonstrated that **7a** (**F24**) could modulate aggregation of $A\beta_{1-42}$. The results from cell-based toxicity studies showed cytocompatibility of **7a** (**F24**) with SH-SY5Y cells at all of the tested concentrations.

Furthermore, we carried out experiments to evaluate the effect of treatment with **7a** (**F24**) on protecting the neuronal SH-SY5Y cells from toxicity induced by H_2O_2. The cell viability data from MTT experiments indicated that **7a** (**F24**) and DPZ significantly conferred the neuroprotection against H_2O_2 induced toxicity between 1 and 20 μM, both **7a (F24)** and DPZ increased cell viability significantly. **7a** (**F24**) and DPZ showed higher protection at the lower doses of 1, 2.5, and 5 μM compared to higher doses of 10 and 20 μM. Further, the incubation of SH-SY5Y cell lines with 600 μM of H_2O_2 resulted in marked morphological changes. The cells lost their shape and adherence, and neurites compared to control, which is consistent with the cytotoxicity results. Co-treatment with **7a** (**F24**) protected the cells from the cytotoxicity of H_2O_2, maintaining intact morphology of the cells at all the tested concentrations.

Elevation of intracellular ROS levels is known to induce oxidative stress and cell death. H_2O_2 causes oxidative stress, which in turn induces cell death through apoptosis [206, 207]. After 24 hours of exposure of SH-SY5Y cells with 600 μM H_2O_2 indicating mitochondrial damage before the nuclear DNA damage induced by oxidative stress. The pretreatment with the **7a** (**F24**) at the concentration of 1 and 2.5 μM protected the SH-SY5Y cells against the oxidative stress caused by H_2O_2 by significantly reducing the ROS generation compared to non-treated cells. H_2O_2 also causes

DNA lesions through the generation of oxygen free radicals [208, 209]. TUNEL assay relies on the enzymatic addition of labeled nucleotides to an end of a break TdT enzyme catalyzes the addition of dNTPs at the 3'-hydroxyl ends of double-stranded or single-stranded DNA, generating a 3' hydroxyl end, which is then assessed by TUNEL assay. There was no visible fluorescence in control untreated cells, whereas DNase and H_2O_2 treated cells showed a high fluorescence intensity due to increased apoptosis. The red fluorescence is an indication of DNA fragmentation during apoptosis. Pretreatment with different concentrations (1 to 20 μM) of **7a** (**F24**) prevented the cells from apoptosis induced by H_2O_2. **7a** (**F24**) exhibited higher protection at the lower doses of 1, 2.5, and 5 μM compared to higher doses 10 and 20 μM, which supported our earlier findings. These observations further confirmed the potent neuroprotection property of **7a** (**F24**) on the neuronal cells.

We have reported that **7a** (**F24**) can prevent $A\beta_{1-42}$ aggregation in the *in-vitro* cell-free assay. Notably, the viability of SH-SY5Y cells was reduced by 49.44%, for 55 h upon treatment with 5μM of $A\beta_{1-42}$. However, co-treatment with **7a** showed a protective effect by significantly increasing viability compared to $A\beta_{1-42}$ alone. The viabilities are demonstrated by 5 μM, and 10 μM of **7a** (**F24**) were 72.41 % and 75.3 %, respectively. The parallel artificial membrane permeability assay-blood brain barrier (PAMPA-BBB) revealed that **7a** (**F24**) could cross the BBB efficiently. We next examined the *in-vivo* neuroprotection ability of **7a** (**F24**) in reversing the retinal degeneration caused due to $A\beta_{1-42}$ toxicity. It has been shown that expression of wild type and disease-related, a variant of $A\beta_{1-42}$ in *Drosophila* neurons, leads to change in the eye phenotype [210]. To further evaluate the *in-vivo* efficacy of **7a** (**F24**) against $A\beta_{1-42}$ toxicity, we next assessed **7a** (**F24**) in $A\beta_{1-42}$ dependent toxicity in *Drosophila*. **7a** improved the eye phenotypes scored in adult *Drosophila*. Fruit flies were raised on a control food medium and food medium treated with

different concentrations of **7a** (**F24**). The improvements in rough eye phenotypes of *Drosophila* were scored in a dose-dependent manner. Among all the treated concentrations of **7a** (**F24**), the 0.8 mg/ml **7a** (**F24**) shows a substantial rescued percentage in eye tissues of AD flies. We also found concentration-dependent protection by **7a** (**F24**), with the most robust protection observed with 0.8 mg/ml concentration. Thus, our results from fly experiments corroborated with the data obtained from our previous *in-vitro* assay.

To investigate the binding interaction of **7a** with the $A\beta_{1-42}$ protofibril, computational methods like molecular docking and MD simulations were used. The molecular docking study suggests that **7a** mainly interacts with the different residues of chains C, D, and E of protofibrils through the π-π stacking, hydrogen bonds, and hydrophobic interactions. The binding free energy calculated from the MD trajectory using MM-PBSA method shows that **7a** efficiently and quiescently interacts with protofibrils; further, the per residue decomposition energy also depicts that majority of critical interacting residues contributing to final, binding energy belong to chain C, D, and E of protofibril. Further, the MD simulations of $A\beta_{1-42}$ protofibril in the presence of **7a** show that the binding of this small molecule causes considerable changes in the protofibril compared to apo-protofibril. The increase in RMSD, Rg, SASA, and RMSF of $A\beta_{1-42}$ protofibril in the presence of **7a** was observed. Similarly, the change in secondary structure *i.e.,* decrease in β-sheet of protofibrils and decrease in interchain binding free energy between the major interacting chains of protofibril to **7a**, were observed during the MD simulation study. The significant changes corresponding to structural stability and compactness, secondary structures, binding free energy profiles, and essential dynamics of $A\beta_{1-42}$ in the presence of **7a** suggest the destabilization or disaggregation of protofibrils.

In the acute toxicity studies, **7a** was found to be well-tolerated and non-toxic up to 500 mg/kg oral dose. Compound **7a** exhibited promising *in-vivo* activity upon administration through the oral route in the scopolamine-induced AD model without affecting locomotor activity in the mice. Finally, compound **7a** is able to reduce the levels of AChE and BChE significantly and had potent anti-oxidant properties in *ex-vivo* studies. To follow up on our previous study and to further evaluate the efficacy of **7a** in the AD animal model, the compound was tested in the MWM test at lower doses (1 and 2.5 mg/kg, *i.p.*). The MWM permits the accurate and reproducible study of reference memory, cognitive maps, place learning, spatial learning, and working memory. [211, 212]. Therefore, we evaluated the *in-vivo* efficacy of the **7a** through *i.p.* route at lower doses in the MWM experiment to assess the spatial working memory in mice. During the last five days of the treatment period, ELTs were recorded for the animals of different experimental groups. **7a** treated group (1 and 2.5 mg/kg) significantly shortened escape latency in the 18-22nd day of the experimental session from 59.07 ± 0.92 to 9.8 ± 1.15 s, and 59.42 ± 0.57 to 6.40 ± 1.53 s, respectively (page # 104). The escape latency produced by the 2.5 mg/kg dose of **7a** was slightly more efficacious than the 1 mg/kg. The escape latencies were significantly prolonged by the scopolamine treatment (1.4 mg/kg^{-1}, ip) in the scopolamine-treated group. DPZ (at 1 mg/kg) significantly reduced ELT ($p < 0.001$) compared to the scopolamine-treated group. Thus, compound **7a** turned out to be potent in tested doses (1 and 2.5 mg/Kg) in this animal model experiment. Therefore, we can conclude that this compound substantially improved spatial memory and cognitive abilities in the scopolamine-induced MWM test.

We have further extended the structure-activity relationship (SAR) studies of this series of molecules in a calibrated manner to improve upon the ChEs inhibition and antioxidant property to identify the novel potent multifunctional molecule. All synthesized compounds were tested for

their AChE and BChE inhibitory properties. The *in-vitro* enzyme inhibition studies suggested that the presence of tryptamine moiety could significantly improve the inhibitory activities of these molecules towards the AChE and the BChE. Several compounds from this developed latest series, for example, **18a**-**18c**, and **23a**-**23c**, can be considered dual-acting ChE inhibitors because they turned out to be effective inhibitors of both enzymes and were able to interact withthe key amino acid residues responsible for the hydrolysis of cholinesterases. Compound **23b-23c** exhibited the highest activity for the AChE/BChE in the current series. The enzyme kinetic studies demonstrated a mixed inhibitory nature of **13k** and **23b** on both the AChE and BChE. The lead molecules **13k** and **23b** also exhibited significant antioxidant activity in the DPPH assay. Interestingly, **23b** was found to be three times more potent antioxidant in a DPPH assay (IC_{50} = 20.25 ± 0.26 μM) over the earlier identified **10b** (IC_{50} = 61.98 ± 0.30), and it also was able to chelate iron. The PAMPA-BBB assay demonstrated that **13k** and **23b** could effectively cross BBB and can reach their target located in the brain. The results from cell-based cytotoxicity studies indicated that the compound had no significant effect on cell viability at all the tested concentrations. Further, in *in-vitro* assays, **23b** exhibited neuroprotection properties against H_2O_2 induced neurotoxicity in N2a cells and was able to reverse the toxicity induced through OS. Compound **23b** showed promising *in-vivo* activity in the scopolamine-induced Morris water maze AD model without affecting locomotor activity in the mice.

These findings suggest that **7a** (**F24**) and **23b** can act as lead molecules to develop naturally-inspired multifunctional molecules to manage Alzheimer's and other neurodegenerative disorders.