Section-I

Chapter-5

DISCUSSION

5. DISCUSSION

It has been previously reported that cholinesterase inhibitors are protective in AD. The chemical structures of the synthesized compounds made us to investigate their anticholinestrase property. After getting good results in the *in vitro* cholinesterase inhibition assay, we proceeded towards *in vivo* screening for the selected compounds. We studied the effect of the selected compounds in scopolamine induced amnesic mice model. The results unambiguously showed that the chosen compounds are having protective effect in Alzheimer's like condition; the *ex vivo* results advocated their anticholinestrase and antioxidant activity. Further *in vitro* ROS scavenging and anti-apoptotic property of the compounds was demonstrated against $A\beta_{1-42}$ induced neurotoxicity in rat hippocampal cells. Treatment with **TRZ-15** and **TRZ-20** showed neuroprotective ability of the compounds which was evident from the improved cognitive ability, decrease in $A\beta_{1-42}$ burden, cytochrome-c and cleaved caspase-3 levels. Data from the Wnt signalling studies suggested that GSK3b and b-catenin were dramatically improved. Further involvement of **TRZ-15** and **TRZ-20** in the Wnt signalling pathway was established by immunoflorescence and immunoblot analysis.

Further, reports of IBU-PO having protective role in $A\beta_{1-42}$ induced neurotoxicity are available [81]. These reports provided the motivation for investigating the selected compounds, with proven anticholinestrase and antioxidant property, for their protective role against $A\beta_{1-42}$ induced neurotoxicity. First, *in vitro* MTT assay was performed using rat hippocampal cells to assure that the selected compounds were not toxic to neuronal cells. The test compounds showed good cell viability which assured that the compounds were not toxic. Further *in vitro* ROS scavenging and anti-apoptotic property of the compounds was explored against $A\beta_{1-42}$ induced neurotoxicity in rat hippocampal cells followed by DAB and fluorescence staining for $A\beta_{1-42}$ and caspase using $A\beta_{1-42}$ induced toxic cells treated with the compounds. Decrease in % ROS and degenerative nuclei (Hoechst Staining) showed their protective role against $A\beta_{1-42}$ induced neurotoxicity. The immunostaining results (decrease in expression of $A\beta_{1-42}$ and caspase staining) substantiated the hypothesis that the compounds were protective against $A\beta_{1-42}$ induced neurotoxicity. Once it was known that the compounds reduced the $A\beta_{1-42}$ burden in rat hippocampal cells, the possibility that they can also be successful in *in vivo* models were also explored. Firstly the *in vivo* Alzheimer's like rat model was developed using $A\beta_{1-42}$ as inducing agent. After surgical recovery, the compounds were administered for 14 days and behaviour parameters were assessed to check the cognitive ability of the animals. The behaviour parameters substantiated the neuroprotective potential

of the compounds as the cognitive ability got increased in the treated groups. Furthermore, the immunofluorescence and DAB staining of $A\beta_{1-42}$ clearly proved the efficacy of compounds with decrease in $A\beta_{1-42}$ burden in brain sections. Additionally, fluorojade c staining was performed to count number of degenerative neurons in hippocampal region of the brain. The results clearly demonstrated that there was decrease in number of degenerating neuronal cells in the treatment groups. These findings suggested that **TRZ-15** and **TRZ-20** were effective in neuroprotection against $A\beta_{1-42}$ induced neurotoxicity.

5.1. TRZ-15 and TRZ-20 are potent ChE inhibitors and antioxidants

The major neuropathological feature of AD is cholinergic deficit which is associated with memory loss and also closely correlated with severity in cognitive dysfunction [82]. Inhibition of AChE serves as a strategy for the treatment of AD. The drugs which are approved for AD therapy, enhance acetylcholine levels in brain by counteracting acetylcholine deficit in the brain [83].

Scopolamine, a blocker of muscarinic acetylcholine (ACh) receptor, induces cognitive deficit in various animals and is widely used to evaluate the antidementia activity of newer agents on learning ability. In preliminary *in vitro* cholinesterase inhibition studies the proven potent compounds (**TRZ-15**, **TRZ-20** and **TRZ-19**) were screened *in vivo* to assess their neuroprotective potentials as cholinesterase inhibitors. In order to confirm the effects of **TRZ-15**, **TRZ-20** and **TRZ-19** on memory, Morris water maze test on spatial learning was performed. **TRZ-15**, **TRZ-20** and **TRZ-19** reduced escape latency prolonged by scopolamine in trial and probe sessions, and the number of crossings above the platform also increased. These results suggested that **TRZ-15**, **TRZ-20** and **TRZ-19** can repair the long term memory in scopolamine induced memory impairments.

To elucidate the underlying mechanisms of memory enhancing effects of **TRZ-15**, **TRZ-20** and **TRZ-19**, activities of AChE and BuChE in the brain were assessed. The results suggested the cholinesterase inhibitory property of the compounds as they significantly decreased the levels of AChE and BuChE in brain homogenates.

According to recent studies, AD is associated with inflammatory processes. Cellular constituents are damaged by ROS, a secondary messenger in inflammatory process. The use of antioxidants may be useful in the treatment of AD [84]. We further evaluated whether such impaired cognition by scopolamine is associated with altered oxidative stress indices. Scopolamine treated mice had elevated MDA levels but catalase activity was reduced. Several studies have recently shown that there are strong correlations between the patterns of oxidative damage in patients with cognitive impairment and that of scopolamine induced

amnesic mice [85, 86]. Moreover, many clinical studies suggested the involvement of oxidative stress in pathogenesis of AD. Treatment with **TRZ-15** and **TRZ-20** significantly reduced MDA levels. The results indicate that **TRZ-15** and **TRZ-20** possess potent antioxidant property by scavenging ROS and exert protective effect against oxidative damage induced by scopolamine. This shows that cognitive enhancing activities of compounds might result from regulation of ChE activity and the antioxidative defence system.

5.2. TRZ-15 and TRZ-20 showed protective role in $A\beta_{1-42}$ induced neurotoxicity

In vivo $A\beta_{1-42}$ intrahippocampal injection and *in vitro* hippocampal neuronal culture experiments have shown that the AChE–Aβ complexes induce neuronal death more dramatically than $A\beta_{1-42}$ peptide alone [87-89]. The previous results showed that the compounds (**TRZ-15** and **TRZ-20**) are potent anticholinestrase agents proven by both *in vitro* and *in vivo* experiments. In addition to this, they were also found to be potent antioxidants. This finding led to the investigation whether they were protective against Aβ induced neurotoxicity. There is plentiful evidence suggesting that Aβ contributes to the pathogenesis of AD. It is now well known that Aβ is neurotoxic to the neuronal cells via an oxidative stress-dependent apoptotic process. The neurotoxicity of Aβ has been reported to be mediated with oxygen free radicals and calmed by antioxidants and free radical scavengers [90]. The present study demonstrated that the compounds (**TRZ-15** and **TRZ-20),** which were found to be potent antioxidants and cholinesterase inhibitors in previous studies prevented hippocampal cells from Aβ induced neuronal cell injury dose dependently and that the neuroprotective effect of the compounds (**TRZ-15** and **TRZ-20**) might be through antioxidative and anti-apoptotic mechanisms. The results of MTT assay indicated that **TRZ-15** and **TRZ-20** (5-40 μM) significantly protected hippocampal neuronal cells from Aβ toxicity. The neuroprotective effects were also confirmed by analysis of morphological nuclear changes and DNA fragmentation.

Compelling evidence showed that oxidative stress in the AD brains played a key role in Aβ induced neuronal cell death [91]. It is well known that ROS induced oxidative DNA damage can cause cell apoptosis [92]. ROS, which were predominantly produced in mitochondria, led to free radical attack of membrane phospholipids and loss of mitochondrial membrane potential, which caused the release of intermembrane protein i.e. cytochrome c and this protein ultimately activated caspase-3 leading to activation of downstream cell death pathways [93, 94]. The activation of caspase-3 is also believed to be important for commitment to or execution of neuronal apoptosis. In agreement with these finding, this study demonstrated that $A\beta_{1-42}$ induce ROS generation in hippocampal neurons, and **TRZ-15**

and **TRZ-20** showed intracellular ROS scavenging property in dose dependent manner. We next examined the effect of compounds (**TRZ-15** and **TRZ-20**) on caspase-3 activity. The suppressive effect of the compounds on caspase-3 activity further suggests that the protective effect of **TRZ-15** and **TRZ-20** on neuronal cell death is related to their antioxidant & anti-apoptotic effects.

It was reported previously that, initially AChE is present in neuritic plaques in the AD brain [95, 96] where AChE can enhance $A\beta_{1-42}$ aggregation and plaque formation and may form AChE–Aβ complexes [97]. Given the various difficulties involved in evaluating disease progression vigorously in the human brain, significant work has been carried out to create animal models that might replicate at least some features of AD pathogenesis. In this regard, a significant number of transgenic mouse lines have been developed. Most of these transgenic models replicate some of the neuropathological features of the disease but non-transgenic animal models are also believed to be a useful complement to transgenic approaches to Alzheimer's pathology. In this latter respect, rodents treated with brain injection/infusion of Aβ have been frequently used as an animal model for AD-type pathology. In this regard, it has been shown that i.c.v. administration of Aβ causes memory deficits and neuronal dysfunctions [98]. Moreover, Aβ injections into the hippocampus, cortex and nucleus basalis magnocellularis or amygdala have been reported to produce neuronal loss and cholinergic degeneration [99-102].

In agreement to the previous reports we developed $A\beta_{1-42}$ induced Alzheimer's rat model. Furthermore, the therapeutic efficacy of the compounds (**TRZ-15** and **TRZ-20**) was evaluated in the $A\beta_{1-42}$ injured rats. After 14 days treatment of the compounds, spatial memory impairment got improved, as assessed by CAR using shuttle box. Additionally, number of $A\beta_{1-42}$ aggregates in hippocampus was significantly reduced in the compound (**TRZ-15** and **TRZ-20**) treated groups. Fluoro-Jade c staining for degenerating neurons in hippocampal region of brain authenticated the previous finding as the number of fluoro-jade c + cells got significantly decreased after treatment with compounds (**TRZ-15** and **TRZ-20**). These findings suggested the neuroprotective role of **TRZ-15** and **TRZ-20** against $A\beta_{1-42}$ induced neurotoxicity.

5.3. TRZ-15 and TRZ-20 are involved in regulation of the wnt signaling pathway

In general it has been accepted that $A\beta_{1-42}$ dependent neurodegeneration in the AD brain leads to loss of Wnt signaling function [103]. Previous studies established that in AD model mouse brain the expression of GSK3b and β-catenin (the two key components of the canonical Wnt signaling pathway) are altered dramatically [104], and activation of Wnt

signaling can prevent neurodegeneration induced by $A\beta_{1-42}$ fibril [105]. Supportingly, in a previous *in vivo* study the expression levels of GSK3b and β-catenin changed in AChE–Aβ induced neurotoxicity [106]. In a crucial finding, a bifunctional (AChE inhibitory and anti-inflammatory) compound, IBU-PO, prevented the loss of functioning of Wnt signaling pathway induced by $A\beta_{1-42}$ toxicity by inhibiting GSK3b and enhancing β-catenin activity [81]. These findings point out that the neuroprotection showed by AChE inhibitors is associated with modulation of the Wnt/ β-catenin signaling pathway [107]. In our study, after finding that the compounds (**TRZ-15** and **TRZ-20)** are effective AChE inhibitors, antioxidants and protectants against $A\beta_{1-42}$ induced neurotoxicity, we further explored whether **TRZ-15** and **TRZ-20** were involved in the regulation of Wnt signaling in $A\beta_{1-42}$ induced Alzheimer's rat model. In our finding we showed that **TRZ-15** and **TRZ-20** significantly inhibited GSK3b activity and stabilized the β-catenin protein levels *in vivo*. These results proved that **TRZ-15** and **TRZ-20** show neuroprotection against $A\beta_{1-42}$ induced neurotoxicity by activating the Wnt signal transduction pathway. Even though, there may be some cross linking between GSK3a and GSK3b proteins, the reports suggest that APP processing and $A\beta_{1-42}$ generation is mainly due to increased GSK3a activity [108], while tau phosphorylation and neurofibrillary tangle formation is due to activation of GSK3b [109, 110]. Our data represents that there is a significant increase in the phosphorylation levels of both GSK3a and GSK3b proteins in hippocampal regions of the **TRZ-15** and **TRZ-20** treated $A\beta_{1-42}$ induced Alzheimer's rat brain. This data advocates that **TRZ-15** and **TRZ-20** can inhibit the activity of GSK3a/b and, hence, may inhibit $A\beta_{1-42}$ generation and tau phosphorylation.

Neuroprotective targets for AD are still hard to pin down due to lack of indepth insight into the molecular events that lie beneath Aβ neurodegeneration and apoptosis [111, 112]. In our study we showed that **TRZ-15** and **TRZ-20** inactivate GSK-3b, which leads to stabilization of β-catenin, and subsequently cytosolic level of β-catenin is increased. GSK-3b and β-catenin are the two key components of Wnt signaling, and this pathway could represent a common signaling between apoptotic events and neuroprotection [113]. Compounds such as **TRZ-15** and **TRZ-20**, which mimic the activation of the Wnt signaling pathway, could eventually rescue neurons from cytotoxicity, which could be potentially beneficial for the treatment of AD patients.

In another set of this section the study deals with the pharmacological evaluation of some novel cyclic guanidine derivatives as potential cholinesterase and $A\beta_{1-42}$ inhibitors. The current study demonstrates the neuroprotective role of novel guanidine derivative (**3b)** in

AD-like animal models. Its cholinesterase inhibition property and protective role in the Aβ-induced rat model of Alzheimer's-like condition could be established. Additionally, its involvement in the Wnt signaling pathway was also evaluated.

5.4. Compound (3b) is a potential anticholinesterase and antioxidant

One of the therapeutic approaches to treat AD is AChE inhibition. Approved drugs for AD therapy enhance acetylcholine levels by inhibiting cholinesterase in the brain [83]. The *in vivo* cholinesterase inhibitory potentials of the priorly proven potent compound (**3b**) in the *in vitro* cholinesterase inhibition assay were evaluated using scopolamine induced amnesic mice model. Its effects on spatial learning and memory were evaluated using Morris water maze test. **3b** reduced escape latency, prolonged by scopolamine in trial and probe sessions, and the number of crossings above the platform also increased. These results indicated that **3b** can repair long term memory in scopolamine induced memory impaired animals.

The underlying mechanism behind memory enhancing effects of **3b** was elucidated by analysing the levels of AChE and BuChE in the brain. The results showed the cholinesterase inhibitory property of the compound as it significantly decreased the levels of AChE and BuChE in brain homogenates.

Further assessment was made to find out whether an altered oxidative stress index is associated with impaired cognition induced by scopolamine. Scopolamine treated mice had reduced catalase activity but elevated MDA levels. Several recent studies have shown that there are strong correlations between the patterns of oxidative damage in scopolamine induced amnesic mice and that of cognitive impaired patients [114]. Treatment with **3b** significantly reduced MDA levels, and increased catalase level. These results illustrated that cognitive enhancing activity of compound (**3b**) might result from regulation of ChE activity and the antioxidative effect of the test compound.

5.5. Compound (3b) shows neuroprotection in $A\beta_{1-42}$ induced neurotoxicity

Plenty of previous evidences indicate that the Aβ is a contributor in pathogenesis of AD. It is also well recognized that there is involvement of an oxidative stress-dependent apoptotic process in Aβ induced neurotoxicity identified in neuronal cells [115]. It has also been reported that oxygen free radicals are the mediators in Aβ induced neurotoxicity which can be protected by free radical scavengers and antioxidants [90]. The present study demonstrated that the compound (**3b),** proven to be a potent cholinesterase inhibitor and antioxidant in previous studies, dose dependently showed protection in Aβ injured hippocampal cells. The results of MTT assay indicated that **3b** (5-80 µM) significantly

protected hippocampal neuronal cells from Aβ toxicity. The study of morphological nuclear changes and DNA fragmentation authenticated the neuroprotective effects of the test compound. The antioxidative and anti-apoptotic mechanisms may be involved in the neuroprotection offered by the compound (**3b**).

ROS leads to loss in mitochondrial membrane potential by free radical attack on membrane phospholipids. In turn a mitochondrial intermembrane protein, cytochrome c is released and this protein ultimately activates caspase-3 leading to downstream apoptotic pathway such as neuronal apoptosis [93, 94]. In accordance with these findings, this study also confirmed that $A\beta_{1-42}$ induced ROS generation in hippocampal neurons, and **3b** showed intracellular ROS scavenging property in dose dependent manner. The suppression of cytochrome c and cleaved caspase-3 activity by compound (**3b)** further authenticated its protective role against neuronal cell death that was related to its anti-apoptotic & antioxidant effects.

Alzheimer's pathology can also be replicated by non-transgenic animal models as a substitute to transgenic approaches. In this respect, brain injection/infusion of Aβ has been used to induce AD-type pathology in rodents that is being used as the pharmacological accompaniment for transgenic approaches. Furthermore, an Aβ injection into the hippocampus, nucleus basalis magnocellularis, cortex and amygdala induces cholinergic degeneration and neuronal loss [99-102].

In agreement to the previous reports $A\beta_{1-42}$ induced Alzheimer's rat model was developed by streotaxic surgery. CAR assessment revealed an improvement from the impaired spatial memory in **3b** treated animals. Additionally, the number of $A\beta_{1-42}$ aggregates was significantly reduced in the hippocampi of **3b** treated group. Fluoro-Jade C staining for degenerating neurons in hippocampal region of brain authenticated the previous findings as the number of fluoro-jade C^+ cells got significantly decreased after treatment with compound (**3b**). These findings suggested the neuroprotective role of **3b** against $A\beta_{1-42}$ induced neurotoxicity.

5.6. Compound (3b) inhibits GSK-3 and regulates the canonical Wnt signaling pathway

Previous reports established that the expression of the two main components of canonical Wnt signaling pathway i.e. β-catenin and GSK3b have been altered in AD model mouse brain dramatically [104], and neurodegeneration induced by $A\beta_{1-42}$ fibril could be prevented by activation of Wnt signalling [105]. Additionally, in an earlier *in vivo* experiment AChE–Aβ induced neurotoxicity changed the expression levels of β-catenin and GSK3b [106]. In a critical finding, $A\beta_{1-42}$ causes neurotoxicity and leads to loss of functioning of Wnt

signaling pathway and this can be prevented by a bifunctional (AChE inhibitory and anti-inflammatory) compound IBU-PO, which enhances β-catenin activity and inhibits GSK3b [81]. These findings reveal that the neuroprotection demonstrated by AChE inhibitors is associated with activation of the Wnt/ β-catenin signaling pathway [107]. Phosphorylation of GSK-3b at Ser9 inactivates it which leads to cytosolic accumulation of β-catenin and finally activation of Wnt/β-catenin pathway. In our finding we illustrated that **3b** significantly inhibited GSK3b activity and stabilized the β-catenin protein levels *in vivo*. These results established that **3b** activated the Wnt signal transduction pathway and showed neuroprotection against $A\beta_{1-42}$ induced neurotoxicity. Although, GSK3a and GSK3b proteins have some cross linking, the reports imply that increased GSK3a activity leads to APP processing and $A\beta_{1-42}$ generation [108], while activation of GSK3b leads to tau phosphorylation and neurofibrillary tangle formation [109, 110]. Our data represents that **3b** significantly increased phosphorylation levels of pGSK3a/GSK3a ratio and pGSK3b/GSK3b ratio in hippocampal regions of Alzheimer's rat brain induced by $A\beta_{1-42}$. This data advocates that **3b** can inhibit the activity of GSK3a/b and consequently, may inhibit $A\beta_{1-42}$ generation and tau phosphorylation.

Lack of profound insight regarding events that underlie apoptosis of neuronal cells and Aβ neurodegeneration has held back the identification of neuroprotective targets for AD [111, 112]. In this study, we demonstrated that **3b** inactivated GSK-3b, leading to stabilization of β-catenin, and consequently cytosolic level of β-catenin was increased. GSK-3b and β-catenin are the key components of Wnt signaling. Further, Wnt signaling pathway commonly represents neuroprotection and anti-apoptotic events [105]. Compound (**3b)**, could ultimately rescue neurons from cytotoxicity by activation of the Wnt signaling pathway. This could offer a potential therapeutic approach for the treatment of patients suffering from AD.