Section-I

Chapter-3

MATERIALS & METHODS

3. MATERIALS AND METHODS

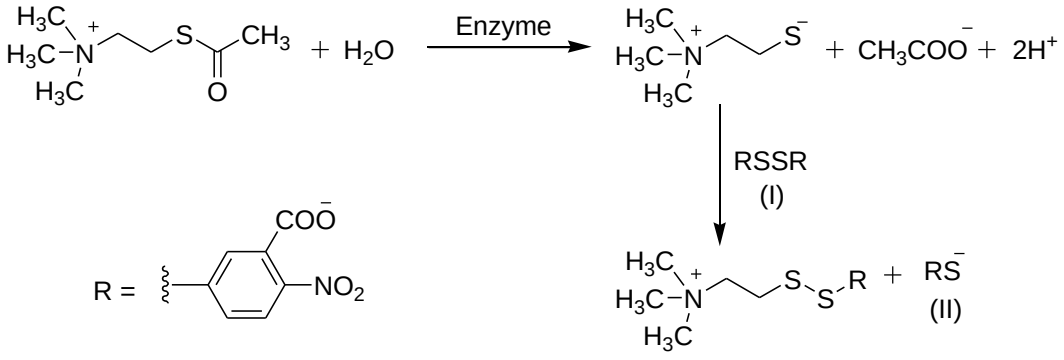
3.1. Reagents

Tacrine hydrochloride (Sigma), donepezil HCl, [5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)], [acetylthiocholine iodide (ATCl) (Sigma)], [*S*-butyrylthiocholine iodide (BTCl) (Sigma)], scopolamine hydrobromide (Sigma), mouse anti-β-actin (Sigma), rabbit anticaspase-3 (cleaved) (Sigma), rabbit anti p-GSK3, rabbit anti-Aβ (Santacruz, USA), rabbit anti-β-catenin primary antibodies, nitrocellulose membrane (0.45 µm), poly-*L*-lysine, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (MTT), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), bovine serum albumin (BSA), and normal goat serum (NGS) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12, serum free neurobasal medium, N-2 supplement, B-27 supplement, fetal bovine/calf serum, Hank's balanced salt solution (HBSS), antibiotic-antimycotic solution were obtained from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)–conjugated secondary antibody (Sigma) and Alexa Fluor 594 conjugated secondary antibodies were purchased from Molecular Probes (Invitrogen). Anti-fade mounting medium was obtained from Vector Labs. The culture wares were procured from Nunc (Roskilde, Denmark).

3.2. Cholinesterase inhibition assay

3.2.1 Principle

The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolyzed. This is accomplished by a continuous reaction of the thiol with 5*S*-dithiobis-2-nitrobenzoate ion (DTNB) (I) to produce the yellow anion of 5-thio-2-



nitrobenzoic acid (II). The rate of colour production is measured at 415 nm in a spectrophotometer. The reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme and in the concentrations used does not inhibit the enzymic hydrolysis.

3.2.2. Reagents

1. AChE enzyme (0.22U/ml): 1.2 mg of AChE procured from Sigma Aldrich was dissolved in 100 ml of 50 mM Tris buffer (*p*H 8) containing 0.1 % BSA.
2. BuChE enzyme (0.06 U/ml): 0.8 mg of BuChE procured from Sigma Aldrich was dissolved in 100 ml of 50 mM Tris buffer (*p*H 8) containing 0.1 % BSA.
3. ATCl (1.5 mM): 4.3 mg of ATCl procured from Sigma Aldrich was dissolved in 10 ml of 50 mM Tris buffer (*p*H 8).
4. BTCl (1.5 mM): 4.75 mg of BTCl procured from Sigma Aldrich was dissolved in 10 ml of 50 mM Tris buffer (*p*H 8).
5. DTNB (1.5 mM): 5.9 mg of DTNB was dissolved in 10 ml of 50 mM Tris buffer (*p*H 8).

3.2.3 Procedure

Ellman's method [69] was utilized to determine the human AChE (product number C1682, Sigma-Aldrich) and equine serum BuChE (product number C1057, Sigma-Aldrich) inhibition profile of the test compounds (IC_{50} values, µM). Appropriate reference agents like donepezil hydrochloride and tacrine hydrochloride hydrate (item number A79922 Sigma-Aldrich) were used. Buffer solution (50 mM Tris HCl, *pH* 8.0, 0.1 M NaCl, 0.02 M $MgCl_2.6H_2O$) was used to dilute the stock solutions of test compounds made in minimum volume of DMSO (1%).

Firstly, 50 µL of AChE (0.022 U/mL prepared in 50 mM Tris–HCl, *p*H 8.0, 0.1% w/v bovine serum albumin, BSA) or 50 µL of BuChE (0.06 U/mL prepared in 50 mM Tris–HCl, *p*H 8.0, 0.1% w/v BSA) with 10 µL of various concentrations of the test compounds (0.001–100 µM) were incubated in 96-well plates at room temperature for 30 min. Further, 30 µL of the substrate viz. ATCl (1.5 mM) or BTCl (1.5 mM) was added and incubated for 30 min. Lastly 160 µL DTNB (0.15 mM) was added and the absorbance was measured at a wavelength of 415 nm using Biorad microplate reader 680XR. Percent inhibition was calculated by the comparison of compound treated to various control incubations that included 1% DMSO. The concentration of the test compound causing 50% inhibition (IC_{50}, µM) was calculated from the concentration–inhibition response curve on logarithmic scale. All determinations were performed in triplicate.

3.3. *h*AChE induced Aβ aggregation inhibition studies

3.3.1. Reagents

1. Sodium phosphate buffer (0.215 M; *p*H 8.0): 3.05 gm of sodium phosphate was dissolved in 10 ml of distilled water. The *p*H was adjusted to 8.0. The final volume was made up to 100 ml with distilled water.
2. Glycine-NaOH buffer (50 mM; *p*H 8.5): 37.5 mg glycine was dissolved in 10 ml of double distilled water. The solution *p*H was adjusted to 8.5 using NaOH.

3.3.2. Procedure

Initially 2 mM lyophilized $A\beta_{1-40}$ solution was made in DMSO and diluted to 500 µM with 0.215 M sodium phosphate buffer (*p*H 8.0). 2 µL of $A\beta_{1-40}$ aliquots were then incubated with 16 µL of hAChE. 0.215 M Sodium phosphate buffer (*p*H 8.0) was used for solubilisation purpose. The incubated $A\beta_{1-40}$ and hAChE gave final concentrations of 50 µM and 230 µM respectively. For co-incubation experiments, 2 µL of the test compounds in 0.215 M sodium phosphate buffer *p*H 8.0 solution (final concentration 100 µM) were added into aliquots (2 µL) of $A\beta_{1-40}$ (final concentration 50 µM) along with 16 µL of hAChE (final concentration 230 µM). The reaction mixtures were incubated at room temperature for 24 h and 100 µL of ThT (20 µM) in 50 mM glycine-NaOH buffer (*p*H 8.5) was added. Fluorescence was monitored at 442 nm excitation wavelenth and emission at 490 nm using a Proton Technology International (PTI, Birmingham, NJ) spectrofluorometer. Each assay was run in triplicate along with tacrine as reference agent. The fluorescence intensities in the presence and absence of inhibitors (test compounds or standard) were compared using appropriate controls containing 1% DMSO and the percentage of inhibition was calculated using the following equation:

$$\% \text{ Inhibition} = 100 - (IFi/IFo \times 100)$$

Where,

IFi= Fluorescence intensity obtained for $A\beta_{1-40}$ + hAChE in presence of inhibitor.

IFo= Fluorescence intensity obtained for $A\beta_{1-40}$ + hAChE in absence of inhibitor.

3.4. Acute oral toxicity studies

See `Appendix I` and `Appendix II`

3.5. Scopolamine induced amnesic mice model

Male Swiss albino mice (20-25 g) and adult male Wistar rats (150-200 g) were used in the study. Food and water were provided *ad libitum* and the animals were maintained under standard laboratory conditions with light-dark cycles of 12 h each. All procedures were

carried out in accordance with the CPCSEA guidelines, Department of Animal Welfare, Government of India.

3.5.1. Experimental design

3.5.1.2 Preparation of scopolamine induced mice model of Alzheimer's like phenotype

Scopolamine is commonly used to induce memory impairment, so this experiment was performed to obtain Alzheimer's like phenotype animal model. Two different series of synthesized compounds were evaluated in two different sets of experimental design

3.5.1.2.1 Set 1: The animals (n=24) were divided into four different groups. Scopolamine (1.4 mg/kg) was administered to all of the groups except for the control group [0.1 % carboxy methyl cellulose (CMC)] after treatment with the standard drug and the test compound. Scopolamine (1.4 mg/kg) was given intraperitoneally. Donepezil (5 mg/kg) and **3b** (20 mg/kg) were administered orally. All the drug solutions were prepared in 0.1% CMC. Memory impairment was induced by scopolamine (1.4 mg/kg, i.p.) 30 min after administration of each drug. The same procedure was carried out for 9 days. Dose of **3b** was` decided after dose deciding pilot study.

3.5.1.2.2 Set 2: The animals (n=36) were divided into six different groups. Scopolamine (1.4 mg/kg) was administered to all of the groups except for the control group [0.1 % CMC] after treatment with the standard and the test compounds. Scopolamine was given 1.4 mg/kg intraperitoneally. Donepezil (5 mg/kg), **TRZ-15** (20 mg/kg), **TRZ-19** (20 mg/kg) and **TRZ-20** (20 mg/kg) were administered orally. All the drug solutions were prepared in 0.1% CMC. Memory impairment was induced by scopolamine (1.4 mg/kg, i.p.) 30 min after administration of each drug. The same procedure was carried out for 9 days. Doses of **TRZ-15**, **TRZ-19** and **TRZ-20** were decided after dose deciding pilot study (data not shown).

3.6. Morris water maze test

To assess learning capability, the animals were allowed to perform Morris water maze task after drug treatment. This task was adopted for mice from the concept originally described by Morris [70]. The water maze comprises of a circular pool (65 cm in diameter, 25 cm high), filled with water (26 ± 1°C) to the depth of 20 cm. The pool was made opaque by painting the inside walls black. Division of the pool was made by four quadrants. An escape platform (equidistant from middle of the pool and the side wall), was located in the middle of one quadrant. The platform was placed 1.0 cm below the water surface. During each trial same quadrant was utilized placing the platform. Reaching on the platform is the only escape condition for animals from water. In the area of the perimeter of the pool three different starting points were placed for animal. Animals were placed in all the three starting points in

a pseudorandom manner during the four days training period. Animal was placed in water facing the wall of the pool at one of the starting points to begin the trial. The animal was softly placed on the platform and allowed to stay for 15s when it failed to reach on the platform within 120 s. A time 5-10 min was given to inner trial interval. Three escape trials per day for four successive days were given to all the animals. After removing the platform, the number of crossings over the region of the platform was also observed for 120 s.

3.7. Assessment of lipid peroxidation and endogenous antioxidants

3.7.1. Assay of lipid peroxidation (MDA content)

It was estimated using the method described by Slater and Sawyer (1971) [71].

3.7.1.2 Principle

The assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA); forming a MDA-TBA_2 adduct that absorbs strongly at 532 nm.

3.7.1.3. Reagents

1. Thiobarbituric acid (0.67% v/v): 0.67 gm of thiobarbituric acid was dissolved in 50 ml of hot distilled water and the final volume was made up to 100 ml with hot distilled water.
2. Trichloroacetic acid (TCA) (10% v/v): 10 gm of trichloroacetic acid was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.
3. Standard malondialdehyde stock solution (50 mM): A standard malondialdehyde stock solution was prepared by mixing 25 µl of 1,1,3,3-tetraethoxypropane up to 100 ml with distilled water. 1.0 ml of this stock solution was diluted up to 10 ml to get solution containing 23 µg of malondialdehyde /ml. One ml of this stock solution was diluted up to 100 ml to get a working standard solution containing 23 ng of malondialdehyde/ml.

3.7.1.4 Procedure

2.0 ml of tissue homogenate (supernatant) was added to 2.0 ml of freshly prepared 10% v/v TCA and the mixture was allowed to stand in ice bath for 15 min. After 15 minutes, the precipitate was separated by centrifugation and 2.0 ml of clear supernatant solution was mixed with 2.0 ml of freshly prepared TBA. The resulting solution was heated in boiling water bath for 10 min. It was then immediately cooled on ice bath for 5 min. The colour developed was measured at 532 nm against reagent blank. Different concentrations (0-23 nM) of standard malondialdehyde were taken and processed as described above for standard graph. The values were expressed as nM of MDA/mg of protein.

3.7.2. Assay of catalase

It was estimated by the method given by Sinha (1972) [72].

3.7.2.1. Principle

Catalase is able to decompose hydrogen peroxide by two different reaction pathways. In the first, known as the "catalatic" pathway, 2 molecules of hydrogen peroxide are converted to water and oxygen (catalatic activity):

$$\text{Protein-Fe}^{3+} + H_2O_2 \longrightarrow \text{Protein-Fe}^{3+}\text{-OOH} + H_2O$$

(Primary Complex)

$$\text{Protein-Fe}^{3+}\text{-OOH} + H_2O_2 \longrightarrow \text{Protein-Fe}^{3+}\text{-OH} + H_2O + O_2$$

The overall reaction gives: $2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$

The primary complex can also decompose by another pathway (peroxidatic decomposition):

$$\text{Protein-Fe}^{3+}\text{-OOH} + AH_2 \longrightarrow \text{Protein-Fe}^{3+}\text{-OH} + H_2O + A$$

Where, AH_2 is an internal or external donor of hydrogen. Low molecular weight alcohols can serve as electron donors. The catalatic pathway is predominant when the hydrogen peroxide concentration is greater than 0.1 mM and the peroxidatic pathway is dominant when the hydrogen peroxide concentration is less than 0.1 mM or the substrate is alkyl peroxide.

3.7.2.2. Reagents

1. Phosphate buffer (50 mM/l, *p*H 7.0): (a) 6.81 gm of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in distilled water and made up to 1000 ml. (b) 8.90 gm of disodium hydrogen orthophosphate (Na_2HPO_4) was dissolved in distilled water and made up to 1000 ml. The solutions (a) and (b) were mixed in proportion of 1:15 (v/v).
2. Hydrogen peroxide (30 mM/L): 0.34 ml of 30% hydrogen peroxide was diluted with phosphate buffer up to 100 ml. This solution was freshly prepared at the time of estimation.
3. Catalase standard solution (65,000 U/mg protein; 1 mg protein/ml): Crystalline beef catalase suspension was centrifuged to isolate the crystals of the enzyme that were dissolved in 0.01 M phosphate buffer (*p*H 7.0) to give a final concentration of 1.0 mg protein/ml. Before starting the assay, it was diluted with distilled water to obtain 1000 U/ml.

3.7.2.3. Procedure

1.0 ml of hydrogen peroxide (30 mM/l) was added in 2.0 ml of diluted sample to initiate the reaction. The blank was prepared by mixing 2.0 ml of diluted sample (similar dilution) with 1.0 ml of phosphate buffer (50 mM; *p*H 7.0). The decrease in absorbance was measured at 240 nm. Catalase activity was expressed as units/mg of protein.

3.7.3. AChE and BuChE estimation in brain

Original Ellman's [69] method was used for determination of brain AChE and BuChE activity.

3.7.3.1. AChE activity in brain

3.7.3.1.2. Principle: In the presence of AChE, acetylthiocholine iodide gets converted into thiocholine**.** This thiocholine binds with DTNB (Ellman's Reagent) and makes 5-thio-2-nitrobenzoic acid which is yellow in colour and gives UV absorbance at 415nm.

Solutions: Buffer: Phosphate, 0.1 M, *p*H 8.0.

Substrate: Acetylthiocholine iodide [0.075 M (21.67 mg/ml)]: This solution was used successfully for l0-15 days if kept refrigerated

Reagent: Dithiobisnitrobenzoic acid (DTNB) 0.01 M: 39.6 mg was dissolved in 10 ml of 0.1 M phosphate buffer (*p*H 7.0) and 15 mg of sodium bicarbonate were added. The reagent was made up in buffer of *p*H 7 in which it was more stable.

Procedure: The tissue was homogenized (approximately 20 mg of tissue per ml of phosphate buffer (*p*H 8.0, 0.1 M)) in a homogenizer. For muscular tissue, considerable mincing was necessary before homogenisation.

Blank: 3 ml of phosphate buffer, 100 µl DTNB and 20 µl ATCI were taken as a blank.

1. 0.4 ml aliquot of this homogenate was added to a cuvette containing 2.6 ml of phosphate buffer (*p*H 8.0, 0.1 M).
2. DTNB reagent, 100 µl was added to the photocell. The absorbance was measured at 412 nm; when this had stopped increasing, the photometer slit was opened so that the absorbance was set to zero.
3. Of the substrate, 20 µl was added. Changes in absorbance were recorded and the change in absorbance per min. was calculated.
4. The rates were calculated as follows:

$$\text{R (moles/l. per min)} = \frac{\text{change in Absorbance per minute}}{1.36 \times 10{,}000}$$

3.7.3.2. BuChE estimation in brain

Same Ellman method was used for this assay as described above. Butyrylthiocholine iodide was used instead of ATCI. Same formula and calculation were followed as mentioned above.

3.8. Culture of hippocampal neurons

Concisely, hippocampal tissue was first dissected out from rat fetuses. The tissue was washed with cold HBSS, then pulverized and incubated in 0.1% trypsin for 30 min at 37 $^{\circ}$C. To form single cell suspension, trituration was carried out. Cells were plated at a density of 0.5 $X10^6$ viable cells/ml in serum free neurobasal medium containing B-27 supplement (2%), N-2 supplement (1%) and 1% antibiotic-antimycotic solution. The cells were allowed to grow as neurons. Cultures of neurons were placed in a humidified incubator at 37 $^{\circ}$C and 5% CO_2. The medium was changed every 3 days. The size and number of neurons were counted [73, 74].

3.8.1. Determination of cell viability

3.8.1.1. Reagents

1. MTT stock solution (5 mg/ml): 25 mg of MTT was dissolved in 5 ml of neurobasal media supplemented with 1 % N-2 supplement, 2 % B-27 supplement and 1 % antibiotic-antimycotic solution.

3.8.1.2. Procedure

In 96 well plates, the cultured hippocampal cells were pre-incubated with different concentrations (5-80 µM) of compounds (**3b**, **TRZ-15** and **TRZ-20**) for 24 h. Treatment of 20 µl of MTT stock solution (5 mg/ml) was done to the culture medium at 37 $^{\circ}$C for another 4 h. Extraction of the resulting MTT formazan was done with 200 µl DMSO and the absorbance was recorded with microtiter plate reader. In another set of experiments, cultured cells were pre-incubated with compound (5-20 µM each) for 2 h and then exposed to 16 µM of $A\beta_{1-42}$ for 24 h. After that MTT assay was performed, and apoptotic cell death and intracellular ROS generation was also distinguished [75].

3.9. Measurement of apoptotic cell death

3.9.1. Reagents

1. 2X Phosphate buffer saline (PBS): 16 gm of NaCl, 0.40 gm of KCl, 0.28 gm of KH_2PO_4 and 2.832 gm of Na_2HPO_4 were dissolved in double distilled water to make upto 1000 ml.
2. 4% Paraformaldehyde (PFA): 50 gm of paraformaldehyde was dissolved in 200 ml of double distilled water. The solution was kept in a tight closed bottle after adding

two NaOH pellets to make the solution transparent. The solution was then kept on stirrer with heating for 1 hr. After 1 hour, the solution was made up to 250 ml with double distilled water. 250 ml of 2XPBS was added to make the final volume up to 500 ml. The PFA solution was filtered.

3.9.2. Procedure

Hoechst staining was used to analyze apoptosis of hippocampal neurons. After exposure to $A\beta_{1-42}$ with or without compounds (**3b**, **TRZ-15** and **TRZ-20**) as explained above, the cells were fixed with 4% paraformaldehyde for 20 minutes and then stained with Hoechst 33258 for 15 min (Feng and Zhang 2004) and then the coverslip was applied. Fluorescence microscope was utilized to view nuclear morphology. The number of cells with apoptotic morphology *i.e.* appearing condensed or fragmented nuclei was counted [76].

3.10. Intracellular ROS generation

H2DCF-DA (2', 7'-dichlorodihydrofluorescein diacetate) assay was utilized to analyze intracellular ROS [77]. Concisely, following pre-treatment with different concentrations (5-20µM) of compounds **(3b**, **TRZ-15** and **TRZ-20**), the hippocampal cells were injured with $A\beta_{1-42}$ followed by treatment with H2DCF-DA for 15 min at 37°C. Fluorescence intensity was determined at excitation wavelength of 492 nm and emission at 495 nm using a fluorescence plate reader (PTI, Birmingham, NJ). The fluorescence intensities in the absence and presence of inhibitors were compared using appropriate controls.

3.11. Immunocytochemistry of hippocampal cells treated with $A\beta_{1-42}$

3.11.1. Principle

Immunocytochemistry (ICC) is a common laboratory technique that is used to anatomically localize presence of a specific protein or antigen in cells by use of a specific primary antibody that binds to it. The primary antibody allows visualization of the protein under a fluorescence microscope when it is bound by a secondary antibody that has a conjugated fluorophore. ICC allows researchers to evaluate whether or not cells in a particular sample express the antigen in question. In cases where an immunopositive signal is found, ICC also allows researchers to determine which sub-cellular compartments are expressing the antigen.

3.11.2. Reagents

1. 1X PBS: 8 gm of NaCl, 0.20 gm of KCl, 0.14 gm of KH_2PO_4 and 1.416 gm of Na_2HPO_4 were dissolved in double distilled water to make the volume upto 1000 ml.
2. 2X PBS: 16 gm of NaCl, 0.40 gm of KCl, 0.28 gm of KH_2PO_4 and 2.832 gm of Na_2HPO_4 were dissolved in double distilled water to make the volume upto 1000 ml.

3. 4% Paraformaldehyde (PFA): 50 gm of paraformaldehyde was dissolved in 200 ml double distilled water. The solution was kept in a tight closed bottle after adding two NaOH pellets to make the solution transparent. The solution was then kept on stirrer with heating for 1 hr. After 1 hour, the solution was made up to 250 ml with double distilled water. 250 ml of 2XPBS was added to make the final volume up to 500 ml. The PFA solution was filtered.
4. Blocking buffer: 150 mg of NGS (normal goat serum), 25 mg of BSA and 5 µl of triton X-100 was dissolved in 5 ml of 1XPBS.
5. Rabbit anti-Aβ antibody (1:500): 4 µl of rabbit anti-Aβ antibody was dissolved in 2 ml of blocking buffer.
6. Rabbit anti-cleaved caspase-3 antibody (1:500): 4 µl of rabbit anti-cleaved caspase-3 antibody was dissolved in 2 ml of blocking buffer.
7. Alexafluor secondary antibody (1:200): 10 µl alexafluor antibody was dissolved in 2 ml of 1XPBS.

3.11.3. Procedure

To assess *in vitro* neuroprotective effects of the compounds (**3b, TRZ-15 and TRZ-20**), hippocampal neuronal cultures were plated in chamber slides. The cultures were then pre-treated with donepezil and compounds **(3b**, **TRZ-15** and **TRZ-20**) at a concentration of 20 µM for 2 h followed by treatment with $A\beta_{1-42}$ at a concentration of 16 µM. The media was removed after 24 h and the cells were washed with 1X PBS. Further, the cells were fixed with 4 % paraformaldehyde for 20 minutes. Blocking buffer (PBS containing 3% NGS, 0.5% BSA and 0.1% Triton X-100) was used to block the non–specific-binding sites. Following the blocking, the sections were incubated with primary monoclonal rabbit anti-Aβ antibody (1:500) and primary rabbit anti cleaved caspase-3 antibody (1:500) for 24 h. Cultures were then incubated in peroxidase-linked secondary antibody (1:200) and alexafluor 594 (1:200, in case of cleaved caspase-3 staining) for 2 h at room temperature. The color was developed for the peroxidase-linked antibody with 3,3'-diaminobenzidine as chromogen and covered with a coverslip. The chamber slide was viewed under microscope for $A\beta_{1-42}$ and cleaved caspase-3 staining.

3.12. $A\beta_{1-42}$ induced Alzheimer's rat model

Adult male Wistar rats (150–200 g) were used in the experiment. Rats were randomly assigned into two groups for stereotaxic surgery. Isotonic saline solution was injected in the sham operated control group (n = 8). $A\beta_{1-42}$ peptide dissolved in 0.9 % normal saline solution was injected in second group of animals.

Animals were anaesthetized with ketamine (100 mg/kg body weight; i.p.) and xylazine (30 mg/kg body weight; i.p.) and mounted in a stereotaxic apparatus (Stoelting, USA). 2 µl of $A\beta_{1-42}$ (in a concentration of 4 µM/2 µl in normal saline) was administered slowly over 5 min bilaterally into the hippocampus by stereotaxic injection at the following coordinates: -4.0 mm anteroposterior, -2.5 mm mediolateral and -3.5 mm dorsoventral from bregma. The animals received $A\beta_{1-42}$ peptide and divided into two sets:

3.12.1. Set 1: Animals injected with $A\beta_{1-42}$ peptide were randomly assigned into 3 groups [vehicle, donepezil (5 mg/kg) and **3b** (20 mg/kg each); n = 8 for each group]. Rats in each group were administered with 0.9 % saline, donepezil (5 mg/kg) and **3b** (20 mg/kg) respectively. The standard drug and the test compound (**3b**) were administered perorally once every day for 14 consecutive days.

3.12.2. Set 2: Animals injected with $A\beta_{1-42}$ peptide were randomly assigned into 4 groups [vehicle, donepezil (5 mg/kg), **TRZ-15** (20 mg/kg) and **TRZ-20** (20 mg/kg each); n = 8 for each group]. Rats in each group were administered with 0.9 % saline, donepezil (5 mg/kg), **TRZ-15** (20 mg/kg) and **TRZ-20** (20 mg/kg) respectively. The standard drug and the test compounds (**TRZ-15 and TRZ-20**) were administered perorally once every day for 14 consecutive days.

3.13. Conditioned avoidance response (CAR)

The cognitive potential of the control and the drug treated animals was measured by evaluating two-way conditioned avoidance behaviour using a shuttle box apparatus (Columbus Instrument) which is a standard and sensitive psychopharmacological experiment to assess learning and memory in rats. The instrument consists of two neighbouring chambers partitioned by acrylic sheets having 1036 cm passage. At a particular time interval, a shock was delivered to one chamber only through the grid floor (intensity was adjusted according to the animal body weight); the other chamber served as the non shock chamber. Both chambers were lit by a 12 W bulb uniformly. Each trial consists of a conditioned stimulus i.e. an electric buzzer (up to 5 s) and unconditioned stimulus i.e. the foot shock (0.5 mA up to 10 s). The cognitive capability was evaluated in each group. From each group, individual rats were placed in any one of the chambers for trials. 20 Trials per day repetitively for successive days were given to the animals until the control group achieved 90% CAR. Control and the treated groups were compared by drawing 90% CAR criteria. Control rats were compared with treated rats on the day when learning or retention ability in control rats achieved 90% CAR. To interpret learning and memory, the cognitive capability was measured as percentage of CAR between control and treated groups. Animals were left for 7 days. After that memory

was assessed in all the groups as percentage CAR. Calculation of memory in treated rats was done by comparing with percent control [74].

3.14. Immunohistochemical analysis of hippocampal neurons

3.14.1. Principle

Immunohistochemistry (IHC) refers to the process of detecting antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC takes its name from the roots "immuno," in reference to antibodies used in the procedure, and "histo" meaning tissue. The procedure was conceptualized and first implemented by Dr. Albert Coons in 1941. There are two methods that are used for IHC.

The direct method is a one-step staining method and involves a labeled antibody (e.g. FITC-conjugated antiserum) reacting directly with the antigen in tissue sections. While this technique utilizes only one antibody and therefore is simple and rapid, the sensitivity is lower due to little signal amplification, in contrast to the indirect approaches. However, this strategy is used less frequently than its multi-phase counterpart.

The indirect method involves an unlabeled primary antibody (first layer) that binds to the target antigen in the tissue and a labeled secondary antibody (second layer) that reacts with the primary antibody. As mentioned above, the secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised. This method is more sensitive than direct detection strategy because of signal amplification due to the binding of several secondary antibodies to each primary antibody if the secondary antibody is conjugated to the fluorescent or enzyme reporter.

3.14.2. Reagents

1. 1X PBS: 8 gm of NaCl, 0.20 gm of KCl, 0.14 gm of KH_2PO_4 and 1.416 gm of Na_2HPO_4 were dissolved in double distilled water to make the volume upto 1000 ml.
2. 4% Paraformaldehyde (PFA): 50 gm of paraformaldehyde was dissolved in 200 ml double distilled water. The solution was kept in a tight closed bottle after adding two NaOH pellets to make the solution transparent. The solution was then kept on stirrer with heating for 1 hr. After 1 hour, the solution was made up to 250 ml with double distilled water. 250 ml of 2X PBS was added to make the final volume up to 500 ml. The PFA solution was filtered.
3. Phosphate buffer saline tween (PBST): 500 µl of tween-20 was added in 1000 ml of 1XPBS.

4. Citrate buffer (*p*H 6.0): 0.294 gm of sodium citrate was dissolved in 100 ml of double distilled water and the *p*H was adjusted to 6.0.
5. Blocking buffer: 150 mg of NGS, 25 mg of BSA and 5 µl of triton X-100 were dissolved in 5 ml of 1XPBS.
6. Rabbit anti-Aβ antibody (1:500): 4 µl of rabbit anti-Aβ antibody was dissolved in 2 ml of blocking buffer.
7. Rabbit anti β-catenin antibody (1:500): 4 µl of rabbit anti β-catenin antibody was dissolved in 2 ml of blocking buffer.
8. Alexafluor secondary antibody (1:200): 10 µl alexafluor antibody was dissolved in 2 ml of 1XPBS.

3.14.3. Procedure

Animals were sacrificed after behavioural study by transcardial perfusion under sodium pentobarbital deep anaesthesia. Animals of each group were perfused with PBS (0.1M, *p*H 7.2) followed by 4% ice-cold paraformaldehyde. After perfusion, brains were separated and stored in the same fixative overnight at 4°C. Cryopreservation of separated brains was done with 10, 20 and 30 % (w/v) sucrose solution in 1XPBS. Brain sections of 20 µm thickness were cut coronally, surrounding hippocampus region using a freezing microtome (Slee Mainz Co., Germany). Immunohistochemical analysis of the proliferating cells was done with every sixth section so that every section was 120 µm apart from each other. The sections were then taken for different staining protocols.

3.14.3.1. Diaminobenzidine (DAB) staining

The sections were washed thrice with PBST. The sections were then incubated in citrate buffer (*p*H 6.0) at 60°C for 25 min. The sections were again washed thrice with PBST. The sections were incubated with 200 µl of H_2O_2 (0.5%) for 2 hour. Freshly prepared 0.5% H_2O_2 in methanol was used to inhibit endogenous peroxidase activity. H_2O_2 was retrieved and the sections were washed thrice with PBST. The sections were incubated with blocking buffer for 2 hr to block non-specific binding sites. The blocking buffer was retrieved and the sections were incubated with primary monoclonal rabbit anti-Aβ antibody (1:500) for 24h. Primary antibody was retrieved and the sections were washed thrice with PBST. The sections were then incubated with peroxidase-linked secondary antibody (1:200) at room temperature for 2 hr. 200 µl of DAB solution was added in the sections and the sections were incubated for 10 to 12 min. Color was developed for the peroxidase-linked antibody with 3,3'-diaminobenzidine as chromogen. The sections were then washed thrice with PBST. Sections were air dried for 10-15 min. Sections were passed in C_2H_5OH (30% -100%) in a gradient

manner so as the sections were kept in each for 2 min. After passing in C_2H_5OH, the sections were kept in xylene for 1 min. Lastly, the sections were mounted with DPX mountant. The slides were air dried for 40 min and observed under microscope.

3.14.3.2. Fluorescence staining

The sections were washed thrice with PBST. The sections were then incubated in citrate buffer (*p*H 6.0) at 60^{o}C for 25 min. Again the sections were washed thrice with PBST. The sections were then incubated in 500 µl of 2N HCl at 37^{o}C for 15 min. 500 µl of borate buffer was added in the sections and the sections were incubated for 15 more min. The sections were then washed thrice with PBST. The sections were incubated in blocking buffer for 2 hr to block the non-specific binding sites. Blocking buffer was retrieved and the sections were incubated with primary monoclonal rabbit anti-Aβ antibody (1:500) and rabbit anti-β-catenin antibody (1:500) for 24 h at 4°C for immunofluoroscence analysis. Primary antibody was retrieved and the sections were washed thrice with PBST. The sections were then incubated for 2 hr. with secondary antibodies conjugated with alexafluor 594 (1:200; Molecular Probes, Invitrogen) at room temperature. The sections were washed thrice with PBST. Gelatin-coated slides were used to mount the sections, and then cover slipped with DAPI containing Hard Set anti-fade mounting medium (Vectashield, Vector Laboratories, CA). The slides were stored in dark at 4°C. Slides were analyzed for fluorescence labeling using Nikon Eclipse Ti-S inverted fluorescent microscope equipped with Nikon Digital Sight Ds-Ri1 Charged Coupled Device camera and NIS Elements Basic Research (BR) imaging software (Nikon, Japan) and confocal microscope (Carl Zheiss LSM710).

3.15. Qualitative analysis of Aβ plaques & quantification of β-catenin positive cells

Unbiased stereological method was used to quantify the stained cells. In this method, a person who is blind to the experimental groups carries out quantification of cells on coded slides. The stained sections of six animals from each group were analyzed to quantify positive stained cells. Total six sections per rat were used for analyses so that labelled cells were counted in every sixth section out of one in six sections series. The sections were kept distant by at least 120 µm to avoid duplicate counting of the same cells. The identification of the hippocampal regions was done at low magnification (X10), and an outline was drawn. Higher magnification (X60) was used to count the cells present in the cell cluster. Fluorescent images with selected paired red and blue (DAPI) were then superimposed to form color-merged overlay images using NIS Elements BR imaging software (Nikon, Japan) and Carl Zeiss LSM 710. Beta-catenin+ cells of the hippocampus region of each rat brain were

counted in sections throughout rostral caudal extent at level of bregma -3.14 to -5.20. The number of labelled cells was determined by bilaterally counting labelled cells in total six sections and averaged for each rat. The total number of β-catenin + cells in the hippocampus was estimated by multiplying the total number of + cells from six sections by the section periodicity, *i.e.*, 6, and reported as the total number of β-catenin + cells per rat.

3.16. Fluoro-jade C labeling and quantification of fluoro-jade C^+ degenerating neurons

Briefly, cryostat was used to cut 20 µm thick coronal sections across the hippocampus. The sections were mounted on slides and stained with fluoro-jade C (Millipore) as per the manufacturer's procedure. Degenerating neurons stained with fluoro-jade C were counted in six-sections per rat brain throughout rostral caudal area of the hippocampus region at levels of bregma -3.14 to -5.20. Number of fluoro-jade C labelled cells from tissue sections (averaged from total six sections per rat) were counted bilaterally to decide the number of fluoro-jade C^+ neurons where fluorescent cell bodies of fluoro-jade C^+ neurons were clearly visible. The total number of fluoro-jade C^+ neurons in the hippocampus was determined by multiplying the total number of fluoro-Jade C^+ neurons from six sections by the section periodicity, *i.e.*, 6, and described as the total number of fluoro- jade C^+ neurons per rat [74].

3.16.1. Reagents

1. Fluoro-jade c (0.01% w/v): 10 mg of fluoro-jade c was dissolved in 100 ml of double distilled water.
2. Working dye: 4 ml of the above prepared solution was added in 96 ml of 0.1 % acetic acid solution to get a final concentration of 0.0004 %)
3. $KMnO_4$ (0.06% w/v): 60 mg of $KMnO_4$ was dissolved in 100 ml of double distilled water.
4. NaOH (1% in 80% alcohol): 10 ml of 5% NaOH was added in 40 ml of absolute alcohol.

3.16.2. Procedure

Sections were mounted on gelatin coated slides, air dried for 30 min, immersed in 1% NaOH in 80% alcohol for 5 min and then incubated with 70% alcohol for 2 min. The sections were immersed in double distilled water for 2 min, incubated with $KMnO_4$ (0.06% w/v) for 10 min, rinsed with double distilled water for 2 min and incubated with fluoro-jade c for 20 min. After incubation, the sections were rinsed with double distilled water for 1 min, incubated with DAPI solution for 5 min and again rinsed with double distilled water for 2 min. The slides were dried at 50^oC in oven. Dry slides were kept in xylene for 1 min,

mounted with DPX mountant, air dried for 40 min and observed under microscope for fluoro-jade c stained degenerating neuronal cells.

3.17. Western blot analysis

3.17.1. Bicinchoninic acid (BCA) assay

3.17.1.2. Principle

The BCA assay relies primarily on two reactions. First, the peptide bonds in protein reduce Cu^{2+} ions from the cupric sulfate to Cu^{+} (a temperature dependent reaction). The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid chelate with each Cu^{+} ion, forming a purple-colored product that strongly absorbs light at a wavelength of 562 nm.

The bicinchoninic acid-Cu^{+} complex is influenced in protein samples by the presence of cysteine/cystine, tyrosine, and tryptophan side chains. At higher temperatures (37 to 60 °C), peptide bonds assist in the formation of the reaction product. Incubating the BCA assay at higher temperatures is recommended as a way to increase assay sensitivity while minimizing the variances caused by unequal amino acid composition.

The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing with protein solutions of known concentrations.

3.17.1.3 Reagents

The assay was performed using standard BCA kit.

1. Bicinchoninic acid (BCA) solution: Reagent A is a 1,000 ml solution containing bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 N NaOH (final *p*H 11.25) (as supplied by the manufacturer).
2. Copper(II) sulfate pentahydrate 4% solution
3. Reagent B is a 25 ml solution containing 4% (w/v) copper (II) sulfate pentahydrate.
4. Protein standard: BSA solution: This product is supplied in 5 flame-sealed glass ampules, each containing 1.0 ml of a solution consisting of 1.0 mg/ml bovine serum albumin in 0.15 M NaCl with 0.05% sodium azide as a preservative.

3.17.1.4. Procedure

The BCA working reagent was prepared by mixing 50 parts of reagent A with 1 part of reagent B. BCA working reagent was mixed until it was light blue in color. For the 96 well plate assay, 8 parts of the BCA working reagent was mixed with 1 part of a protein sample. The sample was a blank, a BSA protein standard, or an unknown sample. The blank consists of buffer with no protein. The BSA protein standard consists of a known concentration of bovine serum albumin, and the unknown sample is the solution to be assayed. The

absorbance was measured at 562 nm. This assay was performed to estimate total protein in the sample.

3.17.2. Western blot analysis

Tissue lysis buffer supplemented with phosphatase and protease inhibitors (Sigma) was used to homogenize the hippocampus regions of different groups of rats. Samples were homogenized, sonicated for 5s, and centrifuged at 4°C and 15000 rpm for 30 min. Equivalent amounts of proteins (100 µg) were loaded on 10% Tris-glycine gel. Tris-buffered saline/Tween-20 (TBST) (50 mM Tris-HCl, 150 mM NaCl, *p*H 7.4, 1% Tween-20) containing 5% non-fat-dried milk was used to block the membranes for 1 h at room temperature. Membranes were then incubated overnight at 4°C with anti-Aβ (1-500, Santacruz, U.S.A.), anti-cleaved caspase-3 (1:500), anti-cytochrome c (1:1000), anti-β-catenin (1:1000), anti-p-GSK-3β (1:1000), anti-GSK3 (1:1000) and anti-β-actin (1:10,000, Sigma). Further, membranes were washed thrice with TBST and incubated with HRP–conjugated secondary antibody for 1h. Immunoreactive proteins were detected using a chemiluminescent substrate (Pierce) according to the manufacturer's instructions. Protein bands were quantified using Scion Image for Windows (National Institutes of Health).

3.18. Statistical analysis

Data was statistically analysed using GraphPad InStat software (version 5.00, San Diego, CA). All data was presented as mean ± SEM. The mean significant difference in the experimental groups was determined using two way ANOVA (ELT for Morris water maze test) & one way ANOVA followed by Bonferroni test. Values of $p<0.05$ were considered statistically significant.