Section-III

Chapter-3

MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1. Animals: Wistar rats (200–250 g) of either sex were used for these studies. Animals were housed in a temperature- and humidity-controlled facility with a 12-h light/dark cycle and free access to food and water *ad libitum* throughout the study. All animal procedures were reviewed and approved by Institutional Animal Ethical Committee, Pharmacy Department, The M.S.University of Baroda. Samples of all test compounds were prepared using 2% DMSO and distilled water.

3.2. Methodology

3.2.1. Superier mesenteric artery strip preparation

Wistar rats of either sex weighing 200-300 gm were sacrificed by a dose of over-anaesthesia. Superier mesenteric artery was dissected out and immediately placed in ice-cold (4˚C) Kreb's bicarbonate solution with the following compositions (mM): NaCl (112 mM), $NaHCO_3$ (12 mM), glucose (11.1 mM), KCl (5.0 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.0 mM) and $CaCl_2$ (2.5 mM). The tissue was aerated with 95% O_2 and 5% CO_2. Before making the strip, periadditive tissue was removed with taking care not to stretch the tissue. A spinal needle was inserted in the tissue and rotated gently to denude the endothelium. Later on the tissue was cut spirally to make a helical strip (20 mm x 3 mm) using a surgical blade. The tissue was tied at both the ends using cotton thread and was suspended into the 25 ml organ tube filled with Kreb's bicarbonate solution with initial resting tension of 1 g. The *p*H of the Kreb's solution was 7.4 and maintained at 37˚C using thermostat. The tissue was equilibrated for 1 hr. The Kreb's solution in the organ tube was changed at every 10 min during this period. Isotonic contractions ware recorded using a force transducer (UGO BASILE, Italy) coupled to a Gemini 7070 recorder (UGO BASILE, Italy). The experiments were carried out as per CPCSEA guidelines, India [56, 57]

3.2.1.2. Effect of the test compounds (3a-5) on superior mesenteric artery strip

Constrictor effects of noradrenaline (1nM-1µM) were tested followed by rinsing 3 times with fresh PSS and allowed to recover to baseline for 15 min. Maximum contraction was then achieved with noradrenaline (100 nM). After reaching the plateau, endothelin denudement was tested with the relaxant effect of acetylcholine (1µM-10 µM). Prazosin (1µM) and propranolol (1 µM) were preincubated into the PSS to eliminate non specific responses of α_1 and β receptors respectively. Dopamine receptor agonism induced vasorelaxation was assessed by cumulative addition of the selective dopamine receptor agonists to the strips, which were submaximally (60 % to 80 %) preconstricted with noradrenaline.

To test the action of D_1, D_2 and D_3 receptors, cumulative concentrations of (10 nM-10 µM) of D_1-like agonist, **A-77636** [58, 59], D_2 agonist- **bromocriptine** [60, 61] , and D_3 agonist- **8-OH-PBZI** [62, 63], were added to the superior mesenteric arterial strip preconstricted by **noradrenaline**. The recepror specificities of the drug effects were determined by incubating the vessels for 30 min with a D_1 like antagonist- **R-SCH-23390** (10 µM) [64, 65], D_2 antagonist- **L-741626** (10 µM) [66] and D_3 antagonist- **SB-277011A** [67, 68]. Constrictor effects of noradrenaline (1nM to 1µM) on the superior mesenteric artery strips were assessed. During experiment, dose response curve (DRC) of noradrenaline (100 nM) was found to produce submaximal contraction (~80%). After reaching the plateau, endothelium denudement was confirmed by assessing the vasorelaxant effect of acetylcholine (1µM to 10µM). Dopamine receptor agonist induced vasorelaxant effect was assessed by cumulative addition of the selective dopamine receptor agonists to the strips, which were submaximally (80%) preconstricted with noradrenaline.

To investigate the effect of the test compounds (**3a-5**), their cumulative concentrations (10^{-9}M to 10^{-6}M) were added to the endothelium denuded rat superior mesenteric arterial strips preconstricted by noradrenaline and their pD_2 values were determined (which is the negative logarithm of base 10 of the EC_{50} of an agonist). The receptor specificity of the test compounds (**3a-5**) (10^{-6}M) was determined by pre-incubation of the strips for 30 min with a D_1 receptor antagonist- **R-SCH-23390** (10^{-6}M), D_2 receptor antagonist- **L-741626** (10^{-6}M) and D_3 receptor antagonist- **SB-277011A** (10^{-6}M) and their vasorelaxant effects were determined thereafter.

3.2.2. MTT assay

Human SH-SY5Y neuroblastoma cell lines were obtained from National Center for Cancer Research (NCCS), Pune. The neuroprotective effect of the test compound (**3d**) was assessed using human neuroblastoma SH-SY5Y cell line. The SH-SY5Y cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with (10% v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37ºC with 5% CO_2. The cells, cultured in 75cm^2 flasks, were seeded at about 5000 cells per well 100 µl of fresh medium in 96 well plates. Next day, the growth medium was replaced with fresh normal media (control cultures) or with media supplemented with 6-OHDA (100 µM) [69] for 24 h. The compound (**3d**) (5µM, 10µM and 20µM) was pre-treated for 3 hr before 6-OHDA treatment. After Incubation, MTT assay was performed to assess the cell viability [70, 71]. In brief, 20 µl of MTT (Sigma-Aldrich) solution (5 mg/ml stock solution) was added into each well and incubated for 4 h at 37 °C. Later, the culture medium was replaced by 200 µL

DMSO to dissolve the formazan. The optical density was measured at 570 nm with 630 nm reference wavelengths using a micro plate reader 680 XR (BIO-RAD, India). The absorbance of the control was considered as 100% of the cell viability [72].

3.2.3. Acute oral toxicity study

See Appendix 4

3.2.4. 6-Hydroxydopamine (6-OHDA) lesioned rat model of Parkinson's disease

3.2.4.1. Animals

Adult male Wistar rats (200–210 g) were used in the experiment. Rats were randomly allocated into two groups for stereotaxic surgery. Isotonic saline solution was injected in the sham operated control group (n=8). A second group of animals received 6-OHDA dissolved in a 0.1 % ascorbic acid-saline solution.

3.2.4.2. Lesioning

All the animals including investigational and sham-operated groups were anaesthetized with 100 mg/kg ketamine and 30 mg/kg xylazine intraperitoneally (i.p.). All the animals were mounted on a stereotaxic frame one by one and the skin overlying the skull was cut to expose it, and the coordinates for the striatum (Paxinos and Watson 1982) were précised correctly as lateral 2.5 mm, anterio-posteror 0.5 mm, dorso-ventral 4.5 mm relative to bragma and ventral from dura with the tooth bar set at 0 mm. Subsequently, all the animals in the experimental group were lesioned by injecting 10 µg 6-OHDA/2 µL in 0.1% ascorbic acid-saline into the right striatum, while the sham operated group received 2.0 µL of the vehicle. The injection rate was 1.0 µL/min maintained by injecting pump [73].

3.2.4.3. Treatment

6-OHDA injected animals were randomly assigned to 3 groups [vehicle, **A-77636** (1 mg/kg, s.c.), and the compound (**3d**) (5 mg/kg each, p.o.); n=8 for each group]. Rats in each group were administered with 0.9 % saline, **A-77636** (1 mg/kg, s.c.) and the compound (**3d**) (5 mg/kg, p.o.) respectively. After one week of surgical recovery, the drugs were administered once per day for 7 consecutive days, respectively.

3.2.5. Compound (3d) induced rotational behaviour

One week after recovery from the surgery, rats were placed in circular bowls and allowed 15 min for habituation. The total numbers of contralateral rotations were counted over 30-min period following treatment with the standard (**A77636**) and the compound (**3d**) each day for seven consecutive days respectively.

3.2.6. Neurochemical parameters

3.2.6.1. Assay of lipid peroxidation (MDA content)

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3.2.6.2. Assay of catalase

Refer Materials & Methods of section –I

3.2.6.3. Assay of superoxide dismutase (SOD)

Superoxide dismutase was estimated by method developed by Mishra and Friedovich (1972) [74].

3.2.6.3.1. Reagents

1. Carbonate buffer (0.05 M, *p*H 10.2): 16.8 gm of sodium bicarbonate and 22 gm of sodium carbonate was dissolved in 500 ml of distilled water and the final volume was adjusted to 1000 ml with distilled water
2. Ethylenediaminetetraacetic acid (EDTA) solution (0.49 M): 1.82 gm of EDTA dipotassium dihydrate salt was dissolved in 200 ml of distilled water and the final volume was made up to 1000 ml with distilled water.
3. Hydrochloric acid (0.1 N): 8.5 ml of concentrated hydrochloric acid was mixed with 500 ml of distilled water and the final volume was made up to 1000 ml with distilled water.
4. Epinephrine solution (3 mM): 0.99 gm of epinephrine bitartarate was dissolved in 100 ml of 0.1 N hydrochloric acid and the volume was adjusted to 1000 ml with 0.1 N hydrochloric acid.
5. Superoxide dismutase (SOD) standard (100 U/L): 1 mg (1000 U/mg) of SOD from bovine liver was dissolved in 100 ml of carbonate buffer.

3.2.6.3.2. Procedure

0.5 ml of tissue homogenate was diluted with 0.5 ml of distilled water, to which 0.25 ml of ice-cold ethanol and 0.15 ml of ice-cold chloroform was added. The mixture was mixed well using cyclomixer for 5 min. and centrifuged at 2500 rpm. To 0.5 ml of the supernatant, 1.5 ml of carbonate buffer and 0.5 ml of EDTA solution were added. The reaction was initiated by the addition of 0.4 ml of epinephrine and the change in optical density/minute was measured at 480 nm against reagent blank. SOD activity was expressed as U/mg protein. Change in optical density/minute at 50 % inhibition of epinephrine to adrenochrome transition by the enzyme was taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

3.2.6.4. Assay of reduced glutathione (GSH)

Reduced glutathione was determined by the method described by Moron et al. (1979) [75].

3.2.6.4.1. Reagents

1. Trichloroacetic acid (20 % w/v): 20 gm of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made up to 100 ml with distilled water.
2. Phosphate buffer (0.2 M, *p*H 8.0): 30.2 gm of sodium hydrogen phosphate was dissolved in 600 ml of distilled water. *p*H was adjusted to 8.0 by using 0.2 M sodium hydroxide solution and the final volume was made up to 1000 ml with distilled water.
3. DTNB reagent (0.6 mM): 60 mg of 5,5'-dithiobis-(2-nitrobenzoic acid) was dissolved in 50 ml of phosphate buffer and the final volume was made up to 100 ml with phosphate buffer.
4. Standard glutathione: 10 mg of reduced glutathione was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

3.2.6.4.2. Procedure

Equal volumes of tissue homogenate (supernatant) and 20 % TCA were mixed. The precipitated fraction was centrifuged and to 0.25 ml of supernatant, 2.0 ml of DTNB reagent was added. The final volume was made up to 3.0 ml with phosphate buffer. The colour developed was read at 412 nm against reagent blank. Different concentrations (10-50 µg) of standard reduced glutathione were taken and proceeded for obtaining a standard curve. The amount of reduced glutathione was expressed as nM of GSH/mg protein.

3.2.7. Immunohistochemistry

3.2.7.1. Immunohistochemical analysis of substantia nigra neurons

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 PBS. Brain sections of 20 µm thickness were cut coronally, surrounding substantia nigra 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. Non–specific-binding sites were blocked by PBS containing 3% NGS, 0.5% BSA and 0.1% Triton X-100. Tissue

sections were further incubated with rabbit anti-caspase-3(cleaved) antibody (1:500; Sigma aldrich) for 24 h at 4°C for immunofluoroscence analysis. 2H incubation with secondary antibodies conjugated with Alexa Fluor 488 (1:200; Molecular Probes, Invitrogen) was given to the sections 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 BR imaging software (Nikon, Japan).

3.2.8. Estimation of dopamine (DA) level using HPLC technique

Levels of DA in 6-OHDA-induced Parkinson's rat brains treated with compound (**3d**) and standard (**A-77636**) were estimated using HPLC with electrochemical detector (Model No.Waters 2465) (Waters Corporation, Milford, USA). Rats from all the groups were used for preparing the brain tissue samples. Homogenates were then centrifuged at 35,000 x g at 4°C for 20 min. Supernatants were filtered with 0.22 µm membrane filter and 20 µL injected via HPLC pump into a column (RP C18, 5 µm particle size, 4.6 mm i.d x 250 mm at 30°C) connected to an Electrochemical Detector at a potential of +0.80 V with glassy carbon working electrode vs. Ag/AgCl reference electrode. The mobile phase consisted of 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate, 1.4 mM sodium octylsulfonate, 0.05 mM EDTA and 16% (v/v) methanol (*p*H 4.2) at a flow rate of 1.2 ml/min. Quantification of DA levels was made by comparing peak height of the samples to a standard DA curve. The DA levels were calculated using software Breeze version 3.2 and expressed as nanograms of DA per gram of wet weight of tissue [76, 77].

3.3. Statistical Analysis

The data were expressed as mean ± SEM. Sensitivity of **A-77636**, **bromocriptine** and **8-OH-PBZI** was expressed as *p*D2 value which depicts the potency of an agonist. It is the negative logarithm to the base 10 of the EC_{50}, while the sensitivity of **R-SCH-23390**, **L-741626** and **SB-277011A** was expressed as *p*A2 value which depicts the negative logarithm to the base 10 of the molar concentration of antagonist that makes it necessary to double the concentration of agonist needed to produce the original submaximal response. Data was statistically analysed using GraphPad InStat software (version 5.00, San Diego, CA). The mean significant difference in the experimental groups was determined using two way ANOVA (Contralateral rotations) & one way ANOVA followed by Bonferroni test. Values of $p<0.05$ were considered statistically significant.