4. MATERIAL AND METHODS

4.1 Material

Novel test compounds were synthesized by the Medicinal Chemistry Laboratory, Pharmacy Department, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda. Phenylephrine hydrochloride, angiotensin II, acetylcholine chloride, deoxycorticosterone acetate were procured from Merck (Sigma Aldrich) St. Louis, MO, USA. L-Nitroarginine Methyl Ester (L-NAME) was procured from TCI chemical, Tokyo, Japan. Prazosin, Valsartan and Losartan potassium were kind gifts from Alembic Pharmaceuticals Ltd. and Zydus Cadila Healthcare Ltd. Heparin, ketamine, diazepam, tramadol, amoxicillin, doxazosin and terazosin were purchased from Adhyamaheshwar medical store, Vadodara, Gujarat. Protein kinase C, Phospho-Akt, Tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), nuclear factor kappa-β, renin, adiponectin, leptin, ELISA kits were purchased from Krishgen Biosystems, Mumbai, India. Insulin ELISA kit was purchased from Genxbio Health Services Pvt. Ltd., New Delhi, India. Cell cultures (HEK 293 and H9C2) were procured from National Cell culture (NCCS), Pune, India. Dulbecco's minimum essential medium (DMEM, High Glucose), Trypsin: EDTA solution, Fetal bovine serum, Antibiotic-antimycotic solution, tissue culture flasks (T-25 and T-75), MTT and lyophilized phosphate buffered saline (PBS, *p*H 7.2) were procured from HiMedia, Mumbai, India.

Human FXa enzyme and substrate S-2765 were procured from Molecular Innovations, USA. Thrombin assay kit was purchased from Biophen DTI, Anira Biosciences (USA). Prothrombin time and activated partial prothrombin time was measured by commercial kits purchased from Tulip diagnostics, India. Apixaban and Rivaroxaban were kind gifts from Zydus Cadila Healthcare Ltd., Dabhasa, Vadodara. Other routinely utilized chemicals were of analytical grade and purchased from companies like Himedia, Lobachemie, Sulab chemicals, etc.

4.2 Animals

Animals were procured from licensed animal breeders. Animals were housed in an air-conditioned room (25±2 °C, 50-65 % RH) in plastic cages having corncorb bedding (Shree Dutt Agro Pvt. Ltd., Vadodara, India) with 12 hr. light-12 hr. dark cycles. They had free access to pelleted diet (Pranav Agro Foods Pvt. Ltd., Pune, India) and RO water. All experimental protocols were approved by the Institutional Animal Ethics Committee

(IAEC) of Pharmacy Department, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara (Protocol No: MSU/IAEC/2016-17/1602, MSU/IAEC/2018-19/1828, MSU/IAEC/2019-20/1907). All the experimental procedures were carried out as per the CPCSEA guidelines.

4.3 Methods for screening and evaluation of designed multitargeted ligands in hypertension and cardiometabolic disorders

4.3.1 Functional antagonism assay using isolated rat thoracic aorta preparation

This assay was selected to determine the antagonistic effect of synthesized compounds against Ang II and phenylephrine mediated contractions of rat aortic strips. Male Wistar rats (14-16 weeks old; 200-250 g) were used for the study. They were sacrificed by euthanasia using sodium pentobarbital (120 mg/kg; i.p.); immediately descending thoracic aortas were removed and placed in ice-cold Kreb's bicarbonate solution of the following composition (mM): NaCl 112, $NaHCO_3$ 12, glucose 11.1, KCl 5.0, $MgSO_4$ 1.2, KH_2PO_4 1.0 and $CaCl_2$ 2.5. The pH of the Krebs's solution was 7.4 and maintained at 37±1 °C using a thermostat. The tissue was aerated with carbogen (95% O_2 and 5% CO_2) continuously. Peri-adventitious tissue was removed, taking care not to stretch the tissue. A spinal needle was inserted in the tissue and rotated gently to denude the endothelium. Following this, the tissue was cut spirally into a helical strip using surgical blade no.10. The strip was tied at both ends using a cotton thread and suspended in a 25 ml organ tube under an initial resting tension of 2 g. The Krebs's solution in the organ tube was changed at every 15 minutes during an equilibration period of about 90 minutes. Denudation of the endothelium was confirmed by observing the "absence of relaxation" on strips precontracted with phenylephrine. Isometric contractions were recorded using a force transducer (MLT002 Isometric force transducer, AD Instrument, Australia). Contractions were induced in rat aortic strips with graded, cumulative concentrations of phenylephrine or Ang II. Standard drugs or test compound was added to organ tubes 30 minutes prior to recording of the addition of phenylephrine. A similar procedure was followed to record graded cumulative response curves of Ang II. Control strips were incubated with solvent [DMSO (0.5%) or normal saline] for 30 minutes before recording the concentration response curves. pA_2 values were calculated by the method described by Arunlakshana and Schild(216),(217).

4.3.2 Docking study of compound (18) and (24) with α_1 and AT_1 receptor

To understand the molecular mechanism of dual acting anti-hypertensive agents with α_1 and AT_1 receptors antagonism, docking studies of the synthesized compounds were performed within the active sites of the respective α_1 and AT_1 receptors obtained by homology modeling.

For docking purpose, Glide offers three different levels of docking precision: HTVS (high-throughput virtual screening), SP (standard precision) and XP (extra precision). The docking studies were performed by using Glide module with XP mode. The 3D structures of ligand molecules were built within Maestro using the Build module and a single low energy conformation search was carried out for all molecules under study using OPLS2e force field at physiological pH condition using LigPrep module of Schrödinger while keeping other parameters at standard value. Docking interactions were studies for compound **(18)** and **(24)** on the active site of α_1 and AT_1 receptor.

4.3.3 *In-vivo* pressor response by invasive blood pressure measurement of compound (18) and (24)

4.3.3.1 *In-vivo* pressor response evaluation under unmasked condition

Male Wistar rats of 12 weeks weighing 250±20 g were anesthetized by ketamine 80 mg/kg, i.p.; xylazine 20 mg/kg, i.p. After confirming the anesthesia, a blunt dissection was performed on the ventral neck region and sternohyoid muscles were removed to expose the trachea. To facilitate the respiration, a partial transverse cut was put between the cartilaginous regions and a small piece of pediatric Ryle's tube was inserted in the trachea. The left jugular vein was cannulated using polyethylene (PE) catheter for administration of standard drugs and test compound dissolved in isotonic saline with 0.5% DMSO. Following this, vagotomy was performed to exclude the effect of the vagus nerve. The carotid artery was isolated and cannulated with a PE catheter attached to a pressure transducer (MLT 844 pressure transducer, AD Instrument, Australia) for measurement of intra-arterial blood pressure. The silicone membrane dome and cannula were filled with heparinized saline (100 IU/ml) to prevent clotting of blood inside cannula. The transducer was connected to Powerlab-4/35 data acquisition system (AD Instruments, Australia) for recording of the blood pressure. Baseline blood pressure was recorded for 15 minutes following which pressor responses to phenylephrine (5 µg/kg, i.v. bolus) and Ang II (5 µg/kg, i.v. bolus) were observed in absence and presence of standard drugs or test compound in different set of experiments. Inhibition of pressor response was observed at 2

dose levels; 10 µg/kg and50 µg/kg for α_1 receptor inhibition and 100 µg/kg and 200 µg/kg for AT_1 receptors inhibition. Reduction in blood pressure with antagonist treatments were normalised and statistically evaluated using one way ANOVA(218).

4.3.3.2 *In-vivo* pressor response evaluation under masked conditions

As mentioned above, surgical procedure was performed to measure invasive blood pressure. Further it was planned to evaluate the pressor-inhibition potential of selected NCE under masked conditions. *In-vivo* inhibition of selected test compounds against phenylephrine mediated arterial pressor response was measured in those animals to which 200 µg/kg losartan was pre-administered. The idea behind such a protocol was to mask the effects of test compound upon AT_1 receptor. Similarly, the other set involved measurement of inhibition of Ang II mediated arterial pressor response in those animals to which 50 µg/kgterazosin was pre-administered to mask the effects of the test compound upon α_1 receptors. Changes in blood pressure with antagonists were normalized against agonist response and statistically evaluated using one way ANOVA.

4.3.4 Cytotoxicity assay of compound (18) and (24) on HEK 293 cell culture

This assay involved treatment of cells with the test compounds at different concentrations and evaluation of cytotoxic potential on HEK293 (kidney epithelial cell line) cell culture by means of reaction with MTT. For this purpose, after reaching the required confluency, cells were trypsinized, washed with sterile PBS and counted. The final quantity of cells was adjusted with media, such that 200 µl suspension of cells contained nearly 10^4 cells/well. This final suspension was seeded onto different wells of a sterile 96-well tissue culture plate and the plate was placed in the incubator to allow adherence of cells for 24 hours. Stock solution of test compound **(18)** and **(24)** were prepared in DMSO and further dilutions (0.01 µM to 1000 µM) were prepared in serum deprived medium. The concentration of the aliquots was adjusted in such a way that 200 µl of the medium contained the required concentration of compound **(18)** and **(24)** without exceeding the total concentration limit of DMSO (3%). A concentration range of 0.01 µM to 1000 µM was utilized for the assay. After a 24-hr. incubation period, the complete media was removed from the wells by inverting the plate on tissue paper. Serum deprived medium containing the test compound was added in different wells with appropriate controls for DMSO in

triplicates. The cells were allowed to remain in contact with the test compounds for 12 hrs after which the media was removed and 200 µl of complete media containing MTT (500 µg/ml) was added to each well. The plate was then put inside the incubator and MTT was allowed to react with the cells for 4 hrs. Mitochondrial dehydrogenases from live cells metabolize MTT to insoluble formazan crystals. After 4 hrs. of incubation, the medium is removed and 200 µl DMSO (filtered through a 0.22 µm syringe filter) is added to each well to solubilize the formazan crystals. The plate is covered and shaken on a plate-shaker for 3 minutes. The lid is then removed and the purple color developed is read at 570 nm with a correction applied at 620 nm to account for unmetabolized MTT that may be present in the wells(219). Absorbance is directly proportional to the number of live cells present in each well. % Survival in each well was calculated by the following formula:

% Survival = (OD of test compound well/OD of Control well) * 100

4.3.5 Physiochemical properties and Drug-likeness prediction of compound (18) and (24)

Drug like characteristics and favorable ADME profile plays major role in the development of chemical entity for potential therapeutic alternative. The Swiss ADME software is an online tool for determining the drug-likeness and pharmacokinetic parameters (220), (221). During early stage of drug development, Lipinski's rule of 5 is considered for evaluating the drug-likeness of the compounds. The guideline was created to establish ground rules for new molecular entities in terms of drug-likeness which included molecules having H- bond donors greater than 5, H-bond acceptors greater than 10, a molecular weight larger than 500 and log P (iLog P) larger than 5. Further, parameters like topological polar surface area (TPSA) < 140 $Å^2$ and number of rotatable bonds (nRotb) were reported to have poor absorption.

4.3.6 ADME analysis and toxicity prediction of compound (18) and (24)

ADME analysis was performed by SWISS ADME and pkCSM (220). Toxicity prediction of compound **(18)** and **(24)** to check and verify the possible alarming toxic effects of chemical entities for human by pkCSM tool was also done (222). The prediction was basedon functional group similarity for the query molecules with the *in-vitro* and *in-vivo* contained in the database (223).

4.3.7 Acute toxicity study of compound (18) and (24) according to OECD guidelines

Acute toxicity study for compound **(18)** and **(24)** was carried out as per the OECD guidelines no. 423. Quinazolines scaffolds are extensively reported for their utilization with a good safety profile. Hence, a direct limit test was conducted by giving single oral dose of 2000 mg/kg. Control animals received 0.5% NaCMC at 10 ml/kg dose volume. Healthy adult female, nulliparous Wistar rats (n = 5) (220-240 g) were fasted for 8 hours with free access to water. Study animals were divided in three groups. Compound **(18)** and **(24)** were administered at the dose of 2000 mg/kg (in 0.5% NaCMC) via the oral route. Study animals were observed for 24 hrs. Parallelly, five female rats were administered with vehicle (0.5%NaCMC) to compare them with NCE treated groups.

The animals were observed for general and clinical observations such as grooming, acute allergic response, muscle activity, reflex activity, salivation, sniffing, defecations at every hour for initial 4 hours and then once daily for 14 days. Further, food and water intake were also recorded during the study period.

4.3.8 Evaluation of antihypertensive activity of compound (18) and (24) in unilateral nephrectomy (UNX) and DOCA salt induced hypertension in rats

Unilateral nephrectomy was performed as per the protocol with minor modifications. Adult Wistar rats (20-24 weeks; 200-250 g) were utilized for the study and they were anesthetized by ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.). Briefly, after confirming anesthesia through loss of righting reflex and pinching response, peritoneal laparotomy was performed around 1.2 cm dorsolateral to the diaphragm line. The left kidney was identified in peritoneum and gently brought out by holding the surrounding fat. The adhering fat and peri adventitious tissue were separated using pointed forceps. The kidney was gently pulled towards the exterior side of the peritoneal cavity to expose the ureter, renal vein and renal artery. They were clipped together using hemostatic forceps and tied towards the kidney with 2 silk sutures (no.4), one distal and the other proximal. The kidney was now severed away from the knot and the remaining portion was allowed to retract by removing the haemostatic forceps. The incision was closed by ethicon® braided-silk sutures. Since ketamine also acts as a preemptive analgesic, immediate post-surgical anesthesia was not required, however, after recovery from anesthesia a dose of tramadol (12.5 mg/kg, i.p.) was administered as post-surgical analgesic and amoxicillin (50 mg/kg; i.p.) were given as

antibiotic during recovery period. The animals were allowed to recover from surgery for one week. After the recovery period animals were divided into different groups.

Group I (normal control) and II (sham control) provided with normal RO water and food *ad-libitum* throughout the study period. Animals of group III-VIII underwent unilateral nephrectomy followed by DOCA salt treatment. Group II was subjected to surgical procedure of unilateral nephrectomy but kidney was kept intact in animals. Group III rats received 25 mg/kg DOCA salt via s.c. route twice a week for six weeks with 1% NaCl+ 0.2% KCl in drinking water. Group III served as the hypertensive control. Animals of group IV and V received 5 mg/kg and 10 mg/kg, respectively, of compound **(18)** combined with hypertensive treatment. While group VI and VII were treated with compound **(24)** at 5 mg/kg and 10 mg/kg, respectively, with hypertensive treatment. Group VIII served as standard control group and received losartan and terazosin (5 mg/kg each; p.o.; once a day) for six weeks along with DOCA salt and salt administration (224).

Table 4.1: Evaluation of compound (18) and (24) in UNX+DOCA salt induced hypertension in rats

Group	No. of animals	Group	Treatment
I	9	Normal control	Normal RO water
II	9	Sham control	Sham surgical procedure
III	9	UNX + DOCA salt [Disease Control (DC)]	25 mg/kg; s.c.; twice in a week for 6 Weeks
IV	9	DC + Compound **(18)**	5 mg/kg Oral; Once daily for 6 weeks
V	9	DC + Compound **(18)**	10 mg/kg Oral; Once daily for 6 weeks
VI	9	DC + Compound **(24)**	5 mg/kg Oral; Once daily for 6 weeks
VII	9	DC + Compound **(24)**	10 mg/kg Oral; Once daily for 6 weeks
VIII	9	DC + Losartan + Terazosin	5 mg/kg each; once daily for 6 weeks

4.3.9 Evaluation of antihypertensive activity of compound (18) and (24) in L-Nitro Arginine Methyl Ester (L-NAME) induced hypertension

Adult wistar rats (20-24 weeks; 200-250 g) were used for the study. After acclimatization period, study protocol was initiated. Hypertension was induced by administration of L-NAME hydrochloride (15 mg/kg/day) in saline via i.p. route. Treatment was carried out for 4 weeks as follows: Group I (normal control) received saline; Group II (Positive control) received L-NAME (15 mg/kg/day) for 28 days; Group III and IV received 5 mg/kg and 10 mg/kg of compound **(18)** with concomitant administration of L-NAME for 28 days. Group V and VI received 5 mg/ kg and 10 mg/kg of compound **(24)** with L-NAME. Group VII received standard drugs losartan + terazosin (5 mg/kg/day) for 4 weeks with L-NAME administration. At the end of the study invasive blood pressure measurement, serum and urine collection were carried out. Animals were euthanized; heart and kidneys were isolated for histopathological and other biochemical parameters (225).

Table 4.2: Evaluation of compound (18) and (24) in L-NAME induced hypertension in rats

Groups	No. of animals	Group	Treatment
I	9	Normal control	Equivalent volume
II	9	Disease Control (DC) (L-NAME)	15 mg/kg/day; i.p.; once daily for 4 Weeks
III	9	DC + Compound **(18)**	5 mg/kg Oral; Once daily for 4 weeks
IV	9	DC + Compound **(18)**	10 mg/kg Oral; Once daily for 4 weeks
V	9	DC + Compound **(24)**	5 mg/kg Oral; Once daily for 4 weeks
VI	9	DC + Compound **(24)**	10 mg/kg Oral; Once daily for 4 weeks
VII	9	DC + Losartan + Terazosin	5 mg/kg each; once daily for 4 weeks

4.3.10 Collection, processing and storage of samples

4.3.10.1 Collection of blood

On the penultimate day of the study, rats were fasted overnight, and the blood samples werewithdrawn from retro-orbital plexus under light anesthesia added to centrifuge tubes. Samples were divided in two parts where one tube contained 2 % EDTA solution while other tube is without EDTA solution for collection of plasma and serum samples, respectively. Blood samples were centrifuged at 2200-2500 rpm for 10 minutes. The

plasma and serum obtained was kept at -70 ºC until used. Samples were analyzed for various biochemical parameters.

Plasma samples were utilized for measurement of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and renin by rat specific sandwich ELISA immunoassay kit as described by manufacturer (Krishgen Biosystems) using BIO RAD Elisa plate reader 680XR.

Serum samples were used for the measurement of metabolic parameters such as glucose, triglycerides, total cholesterol, LDL and HDL by colorimetric kits as recommended by manufacturer (Span diagnostics Pvt. Ltd., India) using UV spectrophotometer Shimadzu 1800.

4.3.10.2 Collection of urine

On the penultimate day of the study, rats were fasted overnight and kept individually into metabolic cages with access to water. After 24 hours, the urine was collected and filtered through syringe filters and stored for further urine analysis.

Urine samples were analysed for various biochemical parameters such as uric acid, creatinine, sodium, potassium, albumin and were measured by colorimetry as recommended by manufacturer (Span diagnostics Pvt. Ltd., India) using an UV Spectrophotometer Shimadzu 1800.

4.3.11 Measurement of Hemodynamic parameters

4.3.11.1 Invasive blood pressure measurement

Invasive blood pressure was measured as explained in *in-vivo* pressor evaluation section.

4.3.12 Endothelial dysfunction

Endothelial dysfunction was measured by the relaxation of aortic strips from different groups of animals by acetylcholine. Briefly, after dissecting the aorta from the animal, periadventitious fat was removed and care was taken to preserve the endothelium in intact form while preparing the strips. The strips were mounted as mentioned in the *'functional antagonism assay'* section. Endothelial dysfunction was evaluated by means of graded, Ach-mediated relaxations on strips precontracted with phenylephrine as compared to sodium nitroprusside mediated relaxations on the same strips (226).

4.3.13 Blood and Urine parameters

4.3.13.1 Blood plasma parameters

4.3.13.1.1 Tumor necrosis factor-α (TNF-α)

TNF-α level in plasma samples were analysed by rat specific sandwich ELISA kit from Krishgen Biosystems (Cat No: KB3145).

Reagent preparation

1) Wash buffer (1X) was prepared by adding 5 ml of wash buffer (20X) to 95 ml of distilled water. This was labelled as a working solution.
2) Assay diluent (1X) was prepared by adding 1 ml of assay diluent (5X) to 4 ml of distilled water. This was labelled as a working solution.
3) Standard (Rat TNF-α, Lyophilized) Reconstitution: Lyophilized Rat TNF-α standard was reconstituted with 1.2 ml of distilled water to achieve final concentration of 2000 pg/ml. This was mixed gently and allowed to stand for 30 min before further dilution.
4) Dilutions: Reconstituted standard was used directly as a top standard (2000 pg/ml) and serial dilutions were performed in assay diluent (1X) to prepare the standard range.
5) Detection Antibody Dilution (1:200): Detection Antibody solution of 20 µl was added to 4980 µl of assay diluent (1X) to make final volume to 5 ml.
6) Streptavidin-HRP Dilution (1:200): 20 µl of Streptavidin-HRP solution was added to 4980 µl of assay diluent (1X) to make final volume to 5 ml.

Procedure

1) 100 µl/well of standards and samples were added to the plate either in duplicate or triplicate. Six 2-fold serial dilutions of the 2000 pg/ml top standard were performed, either within the plate or in separate tubes. Thus, the Rat TNF alpha standard concentrations were 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml and 62.5 pg/ml. Assay Diluent (1X) served as a blank.
2) Plates were sealed and incubated for 2 hours at room temperature (18-25 °C).
3) Plates were aspirated and washed 4 times with wash buffer (1X) and blotted for residual buffer by firmly tapping plate upside down on absorbent paper. Any liquid from the bottom outside of the microtiter wells was wiped off, as any residue can interfere in the reading step. All the washes were performed similarly.
4) 100 µl of diluted Detection Antibody solution was added to each well. The plate was sealed and incubated for 2 hours at room temperature (18-25 °C).

5) Plate was washed 4 times with wash buffer (1X) as in step 3.
6) 100 µl of diluted Streptavidin-HRP solution was added to each well. The plate was sealed and incubated for 30 minutes at room temperature (18-25 °C).
7) Plate was washed 4 times with Wash Buffer (1X) as in step 3. For this final wash, wells were soaked in wash buffer for 30 seconds to 1 minute for each wash. This helped in minimizing the background.
8) 100 µl of TMB Substrate solution was added and the plate was incubated in the dark for 15 minutes. Positive wells turned bluish. It was not necessary to seal the plate during this step.
9) Reaction was stopped by adding 100 µl of Stop Solution to each well. Positive wells turned from blue to yellow.
10) The absorbance was measured at 450 nm within 30 minutes of stopping the reaction.
11) Concentration of samples was measured by generating calibration curve using standard TNF-α.

4.3.13.1.2 Interleukin-6 (IL-6)

IL-6 level in plasma samples were analysed by rat specific sandwich ELISA kit from Krishgen Biosystems (Cat No: KB1068).

Reagent preparation

1) Wash buffer (1X) was prepared by adding 5ml of wash buffer (20X) to 95 ml of distilled water. It was labeled as working solution
2) Assay diluent (1X): Dilution: It was ready to be used.
3) Detection Antibody Dilution was prepared by adding 28 µl of detection antibody solution to 2472 µl of assay diluent to make the final volume to 2.5 ml.
4) Streptavidin-HRP dilution was prepared by adding 25 µl of Streptavidin-HRP solution to 4975 µl of Assay Diluent to make final volume to 5 ml.
5) Standard (Recombinant Rat IL-6; 440 ng/ml) upon first use, 440 ng/ml tube of recombinant standard was thawed and quick-spun; aliquots were made in polypropylene vials, and stored at -20 °C. The assay was done by thawing and diluting the recombinant protein by adding 9 µl of the standard solution in 991 µl of assay diluent to prepare the top standard solution (4000 pg/ml) to make up the final volume to 1 ml.

Procedure

1) Same procedure was followed for Interleukin-6 as per section 4.3.13.1.1.
2) Concentration of samples was measured by generating calibration curve using standard IL-6.

4.3.13.1.3 Renin

Renin level in plasma samples were analysed by rat specific sandwich ELISA kit from Krishgen Biosystems (Catalogue No: KLR0548).

Procedure

1) 50 µl of standards and 40 µl samples were pipetted into the respective wells as mentioned in the work list. Sample, biotin conjugate and streptavidin-HRP were not added to the blank well.
2) 10 µl of biotin conjugate was pipetted into each sample well. Biotin conjugate was not added into the blank and standards wells.
3) 50 µl of HRP conjugate was pipetted into each sample and standards well. HRP conjugate was not added to the blank well.
4) The plate was covered and incubated for 1 hour at 37 °C in the incubator.
5) Plate was aspirated and washed 4 times with 1X wash buffer and blotted for residual buffer by firmly tapping plate upside down on absorbent paper. Any liquid from the bottom outside of the microtiter wells were wiped off, as any residue can interfere in the reading step. All the washes were performed similarly. 50 µl substrate B was added to each well including blank well, gently mixed and incubated for 10 minutes at 37 °C in dark.
6) 50 µl of stop solution was added which resulted in a change in color from blue to yellow and was measured at 450 nm.
7) The concentration of samples was measured by generating calibration curve using standardrenin.

4.3.13.1.4 Estimation of Protein kinase C (PKC) and phospho-Akt (p-Akt) in aorta

PKC (Cat No: KLR0203) and p-Akt (Cat No: KLR1201) were analysed by rat specific sandwich ELISA kit from Krishgen Biosystems.

Procedure

1) 50 µl of standards and 40 µl samples were pipetted into the respective wells as mentioned in the work list. Sample, biotin conjugate and streptavidin-HRP were not added to the blank well.
2) 10 µl of biotin conjugate was pipetted into each sample well. Biotin conjugate was not added into the blank and standards wells.
3) 50 µl of HRP conjugate was pipetted into each sample and standards well. HRP conjugate was not added to the blank well.
4) The plate was covered and incubated for 1 hour at 37 ℃ in the incubator.
5) Plate was aspirated and washed 4 times with 1X wash buffer and blotted for residual buffer by firmly tapping plate upside down on absorbent paper. Any liquid from the bottom outside of the microtiter wells were wiped off as any residue can interfere in the reading step. All the washes were performed similarly. 50 µl substrate B was added to each well including blank well, gently mixed and incubated for 10 minutes at 37 ℃ in dark.
6) 50 µl of stop solution was added which resulted into change in color from blue to yellow and was measured at 450 nm.
7) The concentration of samples was measured by generating calibration curve using respective standard.

4.3.13.2 Blood serum parameters

4.3.13.2.1 Fasting serum glucose (GOD-POD end point assay)

Determination of glucose level in blood serum was done by using enzymatic assay kit(227) (Span or Reckon diagnostics Pvt. Ltd., India).

Procedure

1) 20 µl of either serum samples or standard were taken to labeled 2 ml microcentrifuge tubes.
2) 1.5 ml of working glucose solution was added to all the tubes.
3) All the above solutions were mixed well and incubated at 37 ℃ for 10 min.
4) Spectrophotometer was set to zero by blank solution of working glucose solution.

5) After incubation, absorbance was measured at 505 nm and glucose content was calculated by following formula.

Calculation:

Glucose (mg/dL) = (Absorbance of Test/ Absorbance of Standard) * 100

4.3.13.2.2 Cholesterol

Determination of the quantity of cholesterol in serum was done using enzymatic kit (Span or Reckon diagnostics Pvt. Ltd., India).

Procedure

1) 10 µl of serum samples and standard cholesterol was pipette into microcentrifuge tubes.
2) 0.5 ml of enzyme reagent was added into each tube and allowed to incubate for 10 minutes at 37°C.
3) After incubation, 1 ml of distilled water was added to each tube and mixed well.
4) For the preparation of blank, only enzyme reagent and distilled water was mixed and spectrophotometer was adjusted to zero with blank sample.
5) After incubation, absorbance was measured at 505 nm and total cholesterol content was calculated by following formula.

Calculation:

Total Cholesterol (mg/dl) = (OD of T / OD of S) * 200

4.3.13.2.3 HDL-cholesterol (CHOD/POD-Phosphotungstate method)

Quantitative determination of the activity of HDL-cholesterol in serum was done using enzymatic kit (Span or Reckon diagnostics Pvt. Ltd., India). Estimation of cholesterol was performed in two steps. The first steps involved the preparation of samples from serum followed by its estimation.

Procedure

Step 1

1) 0.2 ml of clear serum samples were pipetted into labeled microcentrifuge tubes and 0.3 ml of precipitating reagent was added to each tube.
2) Samples were vortexed and incubated at 25 °C for 10 minutes.

3) The samples were centrifuged at 3000 rpm for 10 minutes to get clear supernatant. If the supernatant was not clear (high TG level), the samples were diluted in 1:1 ratio with normal saline.

Step 2

1) After separating the clear supernatant, 0.5 ml of enzyme reagent was added to each microcentrifuge tube containing 20 µl of serum samples.
2) Standard was prepared by adding 1 ml of enzyme regent to 10 µl of cholesterol standard.
3) After addition, tubes were incubated at 37 ºC for 5 minutes and 1ml of distilled water was added to each tube.
4) Spectrophotometer was set to auto zero by blank solution containing 0.5 ml of enzyme regent and 1 ml of distilled water.
5) Absorbance was measured at 505 nm and HDL content was measured as per the following formula(228).

Calculation:

HDL Cholesterol (mg/dl) = (Abs. of T / Abs. of S) * 50

4.3.13.2.4 Triglycerides (GPO method)

Quantitative measurement of triglyceride concentration in serum was done by using kit (Span or Reckon diagnostics Pvt. Ltd.).

Procedure

1) 10 µl of either serum samples or standard were taken into labelled microcentrifuge tubes.
2) 0.75 ml of working reagent solution was added to microcentrifuge tubes.
3) Tubes were incubated for 15 minutes at 37 °C.
4) 0.75 ml of distilled water was added to each tube and mixed well with vortexing.
5) Spectrophotometer was set to zero by blank solution of working reagent and distilled water.
6) After incubation, absorbance was measured at 546 nm and triglyceride content was calculated as per the following formula (229).

Calculation:

Triglycerides (mg/dl) = (Abs. of Test/ Abs. of Std.) * 50

4.3.13.3 Urine sample analysis

4.3.13.3.1 Creatinine

Procedure

1) 100 µl of urine samples and standard were pipetted into microcentrifuge tubes.
2) 1 ml of creatinine working reagent was added to each tube, mixed well and aspirated immediately for measurement.
3) The analyzer was blanked with purified water and samples were measured at 520 nm.
4) Initial absorbances of the standard and test samples were measured i.e. for test samples: AT1 after 30 seconds and final absorbance (AT2) after an interval of another 120 seconds(230). (Similar procedure was followed for standard)

4.3.13.3.2 Uric Acid

Procedure

1) 20 µl of urine samples or standard was pipetted into microcentrifuge tubes.
2) 1ml of uric acid monoreagent was added to each tube and solutions were mixed well and incubated at 37 ºC for 5 minutes.
3) The analyzer was blanked with purified water.
4) Absorbance of Standard was measured followed by the test samples at 520 nm.
5) Results were calculated as per given calculation formula(231).

Calculation:

$$\text{Serum / Plasma Uric acid (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} * 6$$

Urine Uric acid (mg/day)

$$= \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} * 6 * \text{Dilution factor} * \text{24 hours urine volume in dl}$$

Conversion factor

Uric acid concentration in mmol/l = Uric acid concentration in mg/dl * 0.059

4.3.13.3.3 Sodium

Measurement of sodium was carried out in two steps as mentioned below:

Step 1: Precipitation

1) 20 µl of urine samples and standard samples were pipetted into microcentrifuge tubes.

2) 1ml of sodium precipitating reagent (L1) was added to each tube.
3) Solutions were mixed well and kept at room temperature for 5 min. with occasional shaking.
4) Samples were centrifuged at 2500 to 3000 RPM to obtain a clear supernatant.

Step 2: Color development

1) 20 µl of supernatant samples and standard samples were added into microcentrifugetubes.
2) 1 ml of acid reagent (L2) was added to each tube and mixed well.
3) 100 µl of colour reagent was added into each tube and incubated at room temperature for 5 minutes.
4) Blank sample was prepared by adding regents without sample or standard and spectrophotometer was set to auto zero with blank.
5) Absorbance was read at 546 nm and sodium content in urine samples was calculated by following formula (232).

Calculation:

$$\text{Sodium (µmol/l)} = [\text{Abs.(T)} / \text{Abs.(S)}*150] *1000$$

4.3.13.3.4 Potassium

Measurement of potassium was carried out as mentioned below:

Procedure

1) 20 µl of urine samples (T) and standard sample (S) were pipetted into microcentrifuge tubes.
2) 1 ml of potassium reagent was added to each tube.
3) Solutions were mixed well and kept at room temperature for 5 min. with occasional shaking.
4) Absorbance was measured at 630 nm within 15 minutes and concentration was determined by the given formula.

Calculation:

$$\text{Potassium (µmol/l)} = [\text{Abs.(T)} / \text{Abs.(S)} * 5] *1000$$

4.3.13.3.5 Urea

Measurement of urea was carried out as mentioned below:

Procedure

1) 100 µl of urine samples (T) and standard sample (S) were pipetted into microcentrifuge tubes.
2) 2.5 ml of solution I was added to each tube.
3) Solutions were mixed well and kept in boiling water for 10 minutes.
4) Samples were cooled down immediately with running water and brought down to room temperature with occasional shaking.
5) Absorbance was measured at 490 nm within 10 minutes.
6) Concentration was determined as per the given formula.

Calculation:

Urea (g/day) = [Abs.(T) / Abs.(S)] * 30 * dilution factor * 24 hrs. urine volume in dl

4.3.13.3.6 Albumin

Measurement of albumin was carried out as mentioned below:

Procedure

1) 100 µl of urine samples (T) and standard sample (S) (Reagent 2) were pipetted into microcentrifuge tubes.
2) 1 ml of reagent 1 was added to each tube.
3) Solutions were mixed well and kept in cold condition (15-30 ºC) for 1 minute.
4) Absorbance was measured at 630 nm and concentration was determined as per the given formula.

Calculation:

Albumin (µg/dl) = [Abs.(T) / Abs.(S)] *4*24 hrs. urine volume in dl

4.3.13.3.7 Creatinine clearance

Creatinine clearance was calculated by following formula:

Creatinine clearance

= [{(Urea creatinine*Urine output)/serum creatinine} * (1000/Body weight)/1440]

4.3.14 Estimation of oxidative stress parameters in heart and kidney homogenates

Reperfused heart and kidney homogenates were prepared in 10% w/v of ice-cold Phosphate buffer solution pH 7.4 using tissue homogenizer and centrifuged at 7000 rpm for 15 minutes at 4 ºC. Supernatants were collected and analyzed for oxidative stress parameters.

4.3.14.1 Total protein content

Total protein content was measured in heart and kidney by Lowry method (233).

Reagents

1) 0.1M NaOH: 0.4 g NaOH was dissolved in 100 ml distilled water.
2) Folin Reagent: 1 ml Folin reagent was diluted with 1 ml distilled water.
3) 1% Sodium Potassium Tartrate: 1 g sodium potassium tartrate was dissolved in 100 ml distilled water.
4) Solution A: 0.5 g Copper sulphate in 1% sodium potassium tartrate.
5) Solution B: 2 g sodium carbonate in 100 ml 0.1M NaOH.
6) Standard: 10 mg Bovine Serum Albumin was dissolved in 5 ml distilled water (2000 µg/ml). 0, 200, 400, 600, 800, 1000 and 1200 µg/ml standard solutions were prepared from stock solution.
7) Working Reagent: 2 ml of solution A was mixed with 100 ml of solution B just before use.

Procedure

To 100 µl of tissue homogenate, 0.9 ml of working reagent was added, and mixture was incubated at room temperature for 10 minutes. It was followed by the addition of 0.1 ml of Folin reagent. After incubation for 30 minutes at room temperature, the developed color wasmeasured at 660 nm against distilled water as blank.

4.3.14.2 Nitric oxide

Total NO was determined by measuring its stable metabolites, particularly, nitrite (NO^{2-}) and nitrate (NO^{3-}) based on the method of Miranda et al. (2001). (234)

Reagents

1) Ortho-phosphoric acid (OPA) (2.5% v/v): 2.5 ml of OPA was diluted up to 100 ml of distilled water.
2) Sulfanilamide (1% w/v): 1.0 g of sulfanilamide was dissolved in 50 ml of OPA.

3) Naphthyl ethylenediamine dihydrochloride (0.1% w/v): 0.1 g of NEDA was dissolved in 50 ml of OPA.
4) Griess reagent: 100 ml of Griess reagent was prepared by mixing sulfanilamide (1% w/v), NEDA (0.1% w/v) and ortho-phosphoric acid (OPA) (2.5% v/v).
5) Standard sodium nitrite: 10 mg of sodium nitrite was dissolved in 100 ml of distilled water.

Procedure

To freshly prepared 1 ml of Griess reagent, 1 ml of tissue homogenate was added and solution was incubated for 30 min at room temperature. The absorbance was measured at 540 nm and compared to those of known concentrations of sodium nitrite. The levels of tissue nitrite were expressed as µmoles/g of protein.

4.3.14.3 Malondialdehyde (MDA)

The determination of the levels of lipid peroxides, expressed as malondialdehyde (MDA), was carried out according to the thiobarbituric acid assay of Slater and Sawyer (1971). (235)

Reagents

1) Thiobarbituric acid (0.67% w/v): 0.67 g 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 (10% w/v): 10 g of trichloroacetic acid (TCA) 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: A standard MDA stock solution was prepared by mixing 25 µl of 1, 1′, 3, 3′-tetraethoxypropane with 10 ml of distilled water. 1.0 ml of this stock solution was diluted up to 100 ml to get solution containing 23 µg of malondialdehyde/ml. One ml of this stock solution was diluted up to 100 ml to prepare a working standard solution containing 23 ng of malondialdehyde/ml.

Procedure

2.0 ml of the tissue homogenate (supernatant) was added to 2.0 ml of freshly prepared TCA and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation at 3000 rpm for 15 min at 4 °C and 2.0 ml of clear supernatant solution was mixed with 2.0 ml of freshly prepared TBA. The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled

in an ice bath for 5 minutes. The color developed was measured at 532 nm against reagent blank. Different concentrations (0-23 nM) of standard MDA were taken and processed as above for standard graph. The values were expressed as nmol of MDA/mg protein.

4.3.14.4 Reduced glutathione (GSH)

Reduced glutathione level was determined as previously described by Moron and Depierre (1979). (236)

Reagents

1) Tri-chloroacetic acid (20% w/v): 20 g of TCA 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, pH 8.0): 0.2 M sodium phosphate was prepared by dissolving 31.2 g sodium phosphate ($Na_2HPO_4*2H_2O$) in 600 ml of distilled water. The pH was adjusted to 8.0 with 0.2 M sodium hydroxide solution and the final volume was adjusted up to 1000 ml with distilled water.
3) DTNB reagent (0.6 mM): 23.76 mg of 5, 5'-dithiobis (2-nitro benzoic acid) was dissolved in 50 ml of Phosphate buffer and the final volume was adjusted 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.

Procedure

Equal volumes of tissue homogenate (supernatant) and 20% TCA were mixed. The precipitated fraction was centrifuged at 4000 rpm for 10 min at 4 °C and 2 ml of DTNB reagent was added to 0.25 ml of supernatant. The final volume was made up to 3 ml with phosphate buffer. The colour developed was read at 412 nm against reagent blank. Different concentrations (10-50 µg) of standard glutathione were taken and processed as above for standard graph. The amount of reduced glutathione was expressed as µg of GSH/mg protein.

4.3.14.5 Super oxide dismutase (SOD)

Superoxide dismutase was estimated using the method described by Mishra and Fridovich (1972). (237)

Reagents

1) Potassium Phosphate buffer (0.02 M, at 25 °C pH 7.5):

2) Carbonate Buffer (0.05 M, pH 10.2): 16.8 g of sodium bicarbonate and 22 g of sodium carbonate was dissolved in 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.
3) Ethylenediaminetetraacetic acid (EDTA-Na) solution (0.49mM): 182 mg of EDTA-Na was dissolved in 200 ml of distilled water and the volume was made up to 1000 ml with distilled water.
4) Hydrochloric acid (0.1 N): 8.33 ml of conc. hydrochloric acid was mixed with 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.
5) Epinephrine solution (3 mM): 0.99 g 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.
6) Superoxide Dismutase (SOD) standard (1000 U/ml): 98µL from primary stock solution (7.3 mg/1.46 m) (14965 U/1.46 ml) of SOD from bovine liver was taken and the volume was made up to1 ml with Potassium Phosphate buffer. Stored at -20 °C. (Bottle contain 7.3 mg powder, 2050 U/mg solid, 2554 U/ mg protein).

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 vortex for 5 minutes and centrifuged at 2500 rpm for 10 min at 4 °C. To 0.5 ml of 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 units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. Calibration curve was prepared by using 5-160 units of SOD.

4.3.14.6 Catalase

It was estimated by the method of Hugo Aebi as given by Colowiek *et al.* (1984). (238)

Reagents

1) Phosphate Buffer (50 mmol/l, pH 7.0): (a) 6.81 g of potassium dihydxogen orthophosphate (KH_2PO_4) was dissolved in distilled water and made up to 1000 ml. (b) 8.90 g 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 the proportion of 1:1.5 (v/v).

2) Hydrogen Peroxide (30 mmol/1): 0.34ml of 30% hydrogen peroxide was diluted with phosphate buffer to 100 ml. This solution was prepared fresh each day.
3) Catalase standard (65,000 U/mg protein; 1mg protein/ml): Crystalline beef-liver catalase suspension was centrifuged to isolate the crystals of the enzyme that was dissolved in 0.01M phosphate buffer (pH 7.0) to give a final concentration of l.0 mg protein/ml. Before assay, it was diluted with distilled water to obtain 1000 U/ml.

Procedure

Dilute homogenate 20 times with phosphate buffer (50 mmol/l; pH 7.0). To 2 ml of diluted sample, 1ml of hydrogen peroxide (30mmol/l) was added to initiate the reaction. The blank was prepared by mixing 2 ml of diluted sample (similar dilution) with 1 ml of phosphate buffer (50 mmol/l; pH 7.0). The dilution should be such that the initial absorbance should be approximately 0.500. The decrease in absorbance was measured at 240 nm. Catalase activity was expressed as µmoles of H_2O_2 consumed/min/mg protein.

4.3.15 Histopathology study

After the treatment period, the animals were sacrificed and organs (heart, kidney and aorta) were excised, blotted free of blood and tissue fluid and preserved in 10%w/v formalin solution. The specimens were given for further processing to where routine procedure for sectioning, staining and mounting was observed by the laboratory personnel. Briefly, after a week the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70- 100%). After dehydration the tissues were cleaned in xylene and embedded in paraffin wax and examined under microscope with appropriate resolution.

4.3.16 Effect of compound (18) and (24) in 20% fructose induced Cardiometabolic disorder

4.3.16.1 Molecular docking study of compound (18) and (24) with targets of Cardiometabolic disorder

4.3.16.1.1 Protein preparation

Structure of DPP4 and PPARγ were obtained from Protein Data Bank. Selection of appropriate structure was based on the resolution and the co-crystallized ligand. We found

6B1E for DPP4, which had a vildagliptin as co-crystallized ligand. The resolution of this structure is 1.77 Å. Similarly, we obtained the structure for PPARγ- 7AWC, 7AWC had Rosiglitazone as co-crystallized ligand. vildagliptin and rosiglitazone were known to bind with DPP4 and PPARγ, respectively, so they were used to identify the binding site.

Both the structure 6B1E and 7AWC were prepared for docking using the protein preparation wizard of the maestro. During the preparation hydrogens were added, water was completely removed, selenomethionines were converted to methionines and any missing sidechain or loop were added, additionally terminals of chain were capped.

4.3.16.1.2 Grid generation

Grid was generated on the vildagliptin and rosiglitazone using the Receptor Grid Generation Wizard of maestro. All the options were kept as default.

4.3.16.1.3 Ligand preparation

Three dimensional structures of Ligands were collected from sources like pubchem and drug bank. Co-crystallized ligands were extracted from the respective prepared protein structure. All the ligands were then prepared using the ligand preparation wizard of maestro. During the ligand preparation all the options were kept default.

4.3.16.1.4 Ligand docking with maestro

Docking was performed in ligand docking wizard of maestro. Previously generated grids were used to specify the docking site. Both the standard precision and extra precision was performed. All the other options were kept default. For all the above process, OPLS4 force field was used.

4.3.16.1.5 Ligand docking with AUTODOCK VINA

Blind docking was performed in the Autodock Vina. Minimized structure from maestro was exported as pdb which was then given as input to prepare receptor script which is provided in ADFR suite. Additionally, no other argument is given except the protein structures; this script provided the pdbqt file with added gasteiger charge. Vina calculates the grid map internally using the x,y,z coordinates. These parameters were obtained manually using the AGFRgui.

Similarly, ligands were exported and prepared using the prepare ligand script. Using the vina force field, docking was performed by utilising the exhuastiveness 32, which provided higher accuracy. Docking complexes were manually analyzed using pymol, and ligplot+ to generate 2D images.

4.3.16.2 *In-vivo* activity of Compound (18) and (24) in 20% induced cardiometabolic disorder

Male Wistar rats weighing around 180-200 g were taken for study and the mean difference of weight did not differ more than 10%. At the initiation of the study, blood was withdrawn from retroorbital plexus under ether anesthesia and serum samples were separated from blood. Animals which showed abnormal metabolic profile were excluded from the study and 6 animals were allocated for each group.

Table 4.3: Experimental design for compound (18) and (24) in 20% induced cardiometabolic disorder

Groups	No. of animals	Group	Treatment
I	6	Normal control	-
II	6	20% Fructose [Disease Control (DC)]	-
III	6	DC+ Compound **(18)**	10 mg/kg Oral; Once daily for 8 weeks
IV	6	DC + Compound **(18)**	20 mg/kg Oral; Once daily for 8 weeks
V	6	DC + Compound **(24)**	10 mg/kg Oral; Once daily for 8 weeks
VI	6	DC + Compound **(24)**	20 mg/kg Oral; Once daily for 8 weeks
VII	6	DC+ standard control (Losartan + Terazosin)	10 mg/kg each respectively oral; once daily for 8 weeks

All the animals were switched to 20% fructose solution instead of RO drinking water at initiation of the study except for control animals (Group-I). While Group II animals received 20% fructose solution for 60 days. Group III and IV received dose of 10 mg/kg and 20 mg/kg respectively of compound **(18)**. Group V and VI received dose of 10 mg/kg and 20 mg/kg respectively of compound **(24)**. Group VII was administered with standard drugs for 8 weeks along with 20% fructose in drinking water.

4.3.17 Collection of blood

On the penultimate day of the study, rats were fasted overnight and the blood samples were withdrawn from retro-orbital plexus under light anesthesia added to centrifuge tubes. Samples were divided into two parts where one tube contained 2 % EDTA solution and while other tube is without EDTA solution for collection of plasma and serum samples respectively. Blood samples were centrifuged at 2200-2500 rpm for 10 minutes. The plasma and serum obtained was kept at -70 ºC until used. Samples were analyzed for various biochemical parameters.

Plasma samples were utilised for Interleukin-6 (IL-6), Tumour Necrosis Factor-α (TNF-α), renin by rat specific sandwich ELISA immunoassay kit as described by manufacturer (Krishgen Biosystems) using BIO RAD ELISA plate reader 680XR.

Serum samples were used for the measurement of metabolic parameters such as glucose, triglycerides, total cholesterol, HDL by colorimetric kits as recommended by manufacturer (Span diagnostics Pvt. Ltd., India) using UV spectrophotometer Shimadzu 1800.

4.3.17.1 Measurement of Hemodynamic Parameters

4.3.17.1.1 Invasive blood pressure measurements

Invasive blood pressure was measured as explained in *in-vivo* pressor evaluation section 4.3.11.1.

4.3.17.2 Endothelial dysfunction

Endothelial dysfunction was measured as mentioned in above section 4.3.12. (226)

4.3.17.3 Blood parameters

4.3.17.3.1 Blood serum parameters

4.3.17.3.1.1 Fasting serum Glucose (GOD-POD end point assay)

Fasting blood glucose was measured as described in section 4.3.13.2.1. (227)

4.3.17.3.1.2 Oral Glucose Tolerance Test

The oral glucose tolerance test was performed in overnight fasted rats. Glucose (1g/kg, p.o.) was administered on the 45th day. Blood was withdrawn from retro orbital plexus under light anesthesia at 0, 30, 60, 90, 120 and 240 min of glucose administration. Serum was

prepared as described above and serum glucose level was measured by enzymatic kit (Span or Reckon diagnostics Pvt. Ltd., India). (227)

4.3.17.3.1.3 HOMA-IR and QUICKI

$$HOMA\text{-}IR = (glucose \times insulin)/22.5$$

Insulin concentration was reported in μU/L and glucose in mmol/L. The constant of 22.5 is a normalizing factor; i.e, the product of normal fasting plasma insulin of 5 μU/mL, and the normal fasting plasma glucose of 4.5 mmol/L typical of a "normal" healthy individual = 22.5.

$$QUICKI = 1/[\log (Insulin\ \mu U/mL) + \log (Glucose\ mg/dL)]$$

4.3.17.3.1.4 Cholesterol

Total cholesterol level was measured as described in section 4.3.13.2.2.

4.3.17.3.1.5 Triglycerides (GPO method)

Triglycerides level in serum was measured as described in section 4.3.13.2.4. (229)

4.3.17.3.1.6 HDL-cholesterol (CHOD/POD-Phosphotungstate method)

HDL- cholesterol level was measured as described in section 4.3.13.2.3. (228)

4.3.17.3.2 Blood plasma parameters

4.3.17.3.2.1 Insulin

Insulin level was measured by rat specific sandwich ELISA kit from GeneX Bio, New Delhi, India. (239)

Reagent preparation:

1) All reagents and samples were brought to room temperature (18 – 25 ºC) before use.
2) Assay Diluent B (Item E) was diluted 5-fold with deionized or distilled water before use.
3) Sample dilution: Assay Diluent C (Item L) was used for dilution of serum samples. The suggested dilution for normal serum/plasma is 2-fold.
4) Preparation of standard: 400 µl Assay Diluent C (Item L) was added to Item C vial to prepare a 1,400 µIU/ml standard solution. The powder was dissolved thoroughly by a gentle mix. 120 µl Insulin standard (1,400 µIU/ml) was added from the vial of Item

C, into a tube with 440 µl Assay Diluent C to prepare a 300 µIU/ml standard solution. 250 µl Assay Diluent C was pipetted into each tube. The 300 µIU/ml standard solution was used to produce a dilution series of 300 µIU/ml, 150 µIU/ml, 75 µIU/ml, 37.5 µIU/ml, 18.75 µIU/ml, 9.38 µIU/ml, 4.69 µIU/ml and 0 µIU/ml. Each tube was then thoroughly mixed before the next transfer. Assay Diluent C served as the zero standards (0 µIU/ml).

5) The 20 ml of Wash Buffer Concentrate was dissolved into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
6) The 100 µl of 1X Assay Diluent B was added into the vial to prepare a detection antibody concentrate. The detection antibody concentrate was diluted 80-fold with 1X Assay Diluent B and used in step 5 of Part VI Assay Procedure.
7) HRP-Streptavidin concentrate was diluted 400-fold with 1X Assay Diluent B.

Procedure

1) All reagents and samples were brought to room temperature (18 - 25 ºC) before use.
2) The removable 8-well strips were labelled appropriately for the experiment.
3) 100 µl of each standard and sample was added into appropriate wells. The wells were covered and incubated for 2.5 hours at room temperature with gentle shaking.
4) The solution was discarded and washed 4 times with 1X Wash Solution. After the last wash, any remaining Wash Buffer was discarded by aspirating or decanting. Plate was inverted and blotted against clean paper towels.
5) Then 100 µl of 1X prepared biotinylated antibody was added to each well. It was incubated for 1 hour at room temperature with gentle shaking.
6) The solution was discarded and washed 4 times with 1X Wash Solution. Then 100 µl of prepared Streptavidin solution was added to each well. It was incubated for 45 minutes at room temperature with gentle shaking.
7) The solution was discarded and washed 4 times with 1X Wash Solution.
8) Then 100 µl of TMB One-Step Substrate Reagent was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking.
9) Then 50 µl of Stop Solution was added to each well and read at 450 nm immediately.

4.3.17.3.2.2 Leptin

Plasma leptin was measured by rat specific sandwich ELISA kit from Krishgen Biosystems. (Cat No: KLR0561)

Procedure

1) Standards Dilution: Standards Dilution: Standard dilution was prepared from 1200 pg/ml standard solution. Standard solution was diluted with buffer to 600 pg/ml and subsequently dilutions were prepared ranging from 75-1200 pg/ml. Each sample and standards were run in duplicate or triplicate.
2) The quantity of the plates depends on the quantities of samples and standards to be tested. It is suggested to remove the number of strips required for the assay.
3) 50 µl of Standards and 40 µl Samples were pipetted into the respective wells as mentioned in the work list.
4) 10 µl of biotin conjugate was added into each sample well.
5) 50 µl of HRP Conjugate was added into each sample and standards well. 7) Cover the plate and incubate for 1 hour at 37 °C in the incubator.
6) Plate was aspirated and washed 4 times with 1X Wash Buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly. Then add Substrate A 50 µl, then Substrate B 50 µl to each well including Blank well. Gently mixed, incubate for 10 min at 37 ℃ in dark.
7) 50 µl of Stop solution was added to each well and absorbance was recorded at 450 nm within 15 minutes after adding the Stop Solution blanking on the zero standards.(240, 241)

4.3.17.3.2.3 Adiponectin

Plasma Adiponectin was measured by rat specific sandwich ELISA kit from Krishgen Biosystems (Cat No: KLR 0758).

Procedure

1) Standards Dilution: Standard dilution was prepared from 1200 pg/ml standard solution. Standard solution was diluted with buffer to 600 pg/ml and subsequent dilutions were prepared ranging from 75-1200 pg/ml. Each sample and standards were run in duplicate or triplicate.
2) 50µl of Standards and 40µl Samples were pipetted into the respective wells as mentioned in the work list.
3) 10µl of Biotin Conjugate was added into each sample well.

4) 50µl of HRP Conjugate was added into each sample and standards well. 7) Cover the plate and incubate for 1 hour at 37 °C in the incubator.
5) The plate was aspirated and washed 4 times with 1X Wash Buffer and residual buffer was blotted by firmly tapping plate upside down on absorbent paper. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly. Then add Substrate A 50µl, then Substrate B 50µl to each well including Blank well. Gently mixed, incubate for 10 min at 37 °C in dark.
6) 50µl of Stop solution was added to each well and absorbance was recorded at 450 nm within 15 minutes after adding the stop solution. (240, 241)

4.3.18 Histopathology study

After the treatment period, the animals were sacrificed, and organs (Liver, pancreas, heart and aorta) were excised. Staining protocol was performed as described in section 3.3.15.

4.4 Methods for screening and evaluation of novel FXa inhibitors for anticoagulant and antithrombotic activity

4.4.1 *In-vitro* inhibition assay

4.4.1.1 *In-vitro* FXa inhibition assay

FXa inhibition study was performed using a *p*H 6.5 buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% w/v bovine serum albumin. 10µl of potential FXa inhibitor (test compounds or solvent reference) and 10µl of FXa (1µM) (FXa Human, Molecular Innovations, USA) were consecutively added. After incubating at 37 °C for 10 minutes, 20µl of FXa substrate (3 mM) (S-2765, Molecular Innovations, USA) was rapidly, added and 70µl of Tris buffer was added to make the volume upto 100µl *p*H 6.5 buffer was added to the wells, and the absorbance was measured at 405 nm using Biorad microplate reader 682XR(242).

4.4.1.2 *In-vitro* thrombin inhibition assay

In-vitro thrombin inhibition was studied by BIOPHEN DTI kit from Anira biosciences. Human thrombin enzyme and thrombin specific chromogenic substrate were prepared according to instruction manual by adding 2.5 ml distill water to enzyme and substrate vial. Solutions of standard drug and NCEs were made from 100 nM-100 µM. 50 µl of standard

drug or NCEs dilution was incubated with 50 µl of thrombin specific chromogenic substrate for 10 minutes at 37 ºC. 50µl of human thrombin enzyme was added and incubated for 2minutes with mild shaking. Reaction was stopped by adding 100 µl of 2% citric acid and immediately measured at 405 nm in Biorad microplate ELISA reader 680XR.

4.4.1.3 *In-vitro* anticoagulant activity by PT and aPTT measurement

Clotting time was determined by standard one-stage recalcification assay with a BBL Fibrosystem fibrometer (Bectone Dickinson, Sparles, MD). For PT measurement, thromboplastin was warmed and reconstituted as per the manufacturer's protocol. 2.5 µl of FXa inhibitor (1 mM), made up to 25 µl using citrated human plasma, was incubated for 30 sec at 37 ºC followed by addition of 50 µl of pre-warmed thromboplastin. For the aPTT assay, 25 µl of pre-warmed aPTT reagent (0.2% ellagic acid) was thoroughly mixed with 2.5 µl of test compound and diluted upto 50 µl with citrated human plasma and kept for incubation at 37 ºC for 4 minutes. Thereafter, immediate addition of 25 µl of pre-warmed 25 mM calcium chloride caused clotting and the clotting time was noted. Similar procedure was followed in the absence of FXa inhibitor using 2.5 µl of organic vehicle or saline(243).

4.4.2 Docking studies of novel FXa inhibitors

The novel FXa inhibitors were designed based on existing active site knowledge. Here, different structure-based approach was applied to design potential active molecules;these compounds were designed on basis of literature review and the knowledge of receptor active site and the biologically most active molecules were reanalyzed with the help of docking study.

Docking studies were executed with Glide module of Schrodinger Suite 2019.Glide is projected for screening of probable ligands based on binding mode and affinity for a given receptor molecule. It performs grid-based ligand docking and searches for promising interactions between ligand molecules and a macro molecule, typically a protein.

For docking purpose Glide offers three different levels of docking precision: HTVS (high-throughput virtual screening), SP (standard precision), and XP (extra precision). The 3D structures of ligand molecules were built within Maestro using the Build module and a single low energy conformation search was carried out for all molecules under study using OPLS2e force field at physiological *p*H condition using LigPrep module of Schrödinger, here all the parameters were kept to standard value.

The receptor crystal structure for FXa obtained from Protein data bank (PDB code: 2P16) were optimized and prepared using protein preparation wizard, using standard parameters in protein preparation wizard. The grid was generated on the active site of respective receptor structure and was validated by re-docking the pre-existing co-crystallized ligand structures. Here the docking study was performed in XP mode and interactions were visualized in visual interface.

4.4.3 Cytotoxicity assay of compound (14) and (50) on H9C2 cardiac myocytes

In order to evaluate the safety of developed compounds, MTT assay was performed for compound **(14)** and **(50)** on H9C2 cardiac myocytes according to the procedure mentioned in 4.3.4.

4.4.4 Acute toxicity study of compound (14) and (50) according to OECD guidelines

Acute toxicity was performed as per the guidelines of Organization for Economic Corporation and Development (OECD) guidelines No. 425(244). Healthy adult female, nulliparous Wistar rats (n = 5) (220-240 g) were fasted for 8 hr. with free access to water. Study animals were divided in three groups. Initially, one animal from each group received a dose of compound **(14)** and **(50)** at 2000 mg/kg (in 0.5% NaCMC) via the oral route. Study animals were observed for 24 hrs. After the interval of 24 hours, no mortality was observed amongst doses animals and subsequently four animals from each group received the same dose of compound **(14)** and **(50)**, totaling five animals per group. Parallel, five female rats were administered with vehicle (0.5% NaCMC) to compare them with NCEs treated groups.

The animals were observed for general and clinical observations such as grooming, acute allergic response, muscle activity, reflex activity, salivation, sniffing, defecations at every hour for initial 4 hours, and then after once daily for 14 days. Further, food and water intake were also recorded during the study period (244).

4.4.5 *In-vivo* evaluation of antithrombotic activity of compound (14) and (50)

4.4.5.1 $FeCl_3$ induced arterial thrombosis

Male rats (weight 200-250 g) were treated orally with selected NCEs of 15 mg/kg and 30 mg/kg dose suspended in 0.5% NaCMC and then subjected to $FeCl_3$-induced arterial thrombosis after 2 h of administration. Rats were anesthetized with ketamine and xylazine

(80 mg/kg and 20 mg/kg, respectively, intraperitoneally). A midline cervical incision was made on the ventral side of the neck, and the left carotid artery was isolated. A 2*3 mm strip of Whatman filter paper no. #1 saturated with 35% (w/v) $FeCl_3$ was kept on the carotid artery for 5 min. One hour after removal of the filter paper, the arterial thrombus was excised, blotted of excess blood and immediately weighed (243).

Table 4.4: Effect of compound (14) and (50) in $FeCl_3$ induced thrombosis in rats

Groups	No. of animals	Group	Treatment
I	6	Sham control	Equivalent volume
II	6	$FeCl_3$ (Positive Control)	35% $FeCl_3$
III	6	Positive control + Compound **(14)**	15mg/kg;p.o.
IV	6	Positive control + Compound **(14)**	30mg/kg;p.o.
V	6	Positive control + Compound **(50)**	15mg/kg;p.o.
VI	6	Positive control + Compound **(50)**	30mg/kg;p.o.
VII	6	Positive control + Apixaban	15mg/kg;p.o.
VIII	6	Positive control + Apixaban	30mg/kg;p.o.

4.4.5.2 Arteriovenous shunt (AV-SHUNT) induced thrombosis model

Most potent synthesized compounds were further evaluated by this model as per reported procedure. All rats were fasted overnight before the surgery. Rat arterial-venous shunts (silk thread model) were prepared with 2 2-cm-long polyethylene tubes (1 mm i.d.), linked by a central section (8 cm long; 2 mm i.d.) that contained a 5-cm piece of silk thread and was filled with saline solution that contained heparin 50 IU/kg. Rats were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg; i.p.) an arterial-venous shunt was placed between the right carotid artery and left jugular vein. After blood was circulated through the shunt for 15 minutes, both ends of the tube were pinched, the silk thread was removed from the shunt tube and the wet and dry weights were measured by subtracting the pre- experiment weight of the 5-cm silk thread. The rate of inhibition of thrombosis formation was calculated as inhibition (%) = (A-A1)/A*100%, where A is the thrombus weight of the thrombosis control group and A1 is the weight after treatment with the agents. The sham group did not undergo the surgery (245).

Table 4.5: Effect of compound (14) and (50) in Arteriovenous shunt induced thrombosis in rats

Groups	No. of animals	Group	Treatment
I	6	Sham control	Equivalent volume
II	6	AV shunt (Positive control)	-
III	6	Positive control + Compound **(14)**	15 mg/kg; p.o.
IV	6	Positive control + Compound **(14)**	30 mg/kg; p.o.
V	6	Positive control + Compound **(50)**	15 mg/kg; p.o.
VI	6	Positive control + Compound **(50)**	30 mg/kg; p.o.
VII	6	Positive control + apixaban	15 mg/kg; p.o.
VIII	6	Positive control + apixaban	30 mg/kg; p.o.

4.4.5.3 Tail bleeding model

Assessment of novel compounds for their bleeding risk potential was assessed by tail bleeding model in rats. Selected NCEs and apixaban were administered with therapeutic dose utilised in previous models. After 120 minutes of administration, tails of anesthetized rats were immersed vertically into saline kept at 37 °C. After 5 minutes, tails were transacted 5 mm from the tip. The time until continuous blood flow ceased for >30 s was recorded.

4.5 Statistical analysis

For all the studies, $3 \leq n \leq 9$. The results were compared by Student's *t*-test, one-way or two-way ANOVA as appropriate. Dunnet and Bonferroni's multiple comparisons were employed as the *post hoc* test wherever required. All the statistical analyses were performed using Graphpad Prism, San Diego, CA, USA (Ver. 05) or Excel Spreadsheet program, Microsoft Corp., Redmond, WA, USA (2007). Results were considered to be statistically significant when $P<0.05$. Statistical significance is denoted as:

\# = compared to normal control

* = compared to disease control

@ = compared to compound **(18)** (5 mg/kg)

$ = compared to compound **(18)** (10 mg/kg)

% = compared to compound **(24)** (5 mg/kg)

^ = compared to compound **(24)** (10 mg/kg)

& = compared to standard