EXPERIMENTAL

EXPERIMENTAL

MATERIALS

Test compounds were synthesized by the Pharmaceutical Chemistry lab of Pharmacy Dept., Faculty of Tech. & Engg., The M. S. University of Baroda. Rat aortic smooth muscle cell line, A10 clone, was procured from National Centre for Cell Sciences (NCCS), Pune, India. Phenylehprine hydrochloride, angiotensin II, prazosin, acetylcholine chloride, deoxycorticosterone acetate and bovine serum albumin were procured from Sigma Aldrich, St. Louis, MO, USA. Valsartan, olmesartan medoxomil and losartan potassium were kind gifts from Torrent Pharmaceuticals Ltd., Gujarat, India. Dulbecco's minimum essential medium (DMEM, High Glucose), Trypsin: EDTA solution, Fetal bovine serum (Australian donor herd origin), 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. 1° antibody directed against Thr-308 phosphorylated site of Akt (rabbit origin; Cat# sc-16646-R) was procured from Santacruz Biotechnology Inc., USA and 2° Alexa Fluor 594 conjugated goat anti-rabbit antibody was purchased from Life Technologies, USA. Heparin, ketamine, diazepam, tramadol, doxazosin and terazosin were procured locally. Other reagents were of analytical grade and procured from local companies.

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 (NMT 3 animals per cage) having paddy husk (Shree Dutt Agro Pvt. Ltd., Vadodara) as bedding with 12 hr light-12 hr dark cycles. They had free access to pelleted diet (Pranav Agro Foods Pvt. Ltd., Pune, India) and tap water. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Pharmacy Department, Faculty of Tech. & Engg., The M. S. University of Baroda, Vadodara. All experimental procedures were carried out as per CPCSEA guidelines.

FUNCTIONAL ANTAGONISM ASSAY USING ISOLATED RAT THORACIC AORTA PREPARATION

This assay was selected to determine the antagonistic effect of test compounds against angII 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 cervical dislocation; descending thoracic aortas were removed immediately 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 tissue was aerated with 95 % O_2 and 5 % CO_2. 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 (20 mm × 3 mm) using a surgical blade. 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 *p*H of the Kreb's solution was 7.4 and maintained at 37°C using a thermostat. The Kreb's solution in the organ tube was changed every 10 mins during an equilibration period of about 90 mins. 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 (UGO BASILE, Italy) coupled to a Gemini 7070 recorder (UGO BASILE, Italy). Contractions were induced in rat aortic strips with graded, cumulative concentrations of phenylephrine or angII. Losartan, valsartan, olmesartan or test compounds were added to organ tubes 30 mins prior to recording of the addition of phenylephrine. Similar procedure was followed to record graded cumulative response curves of angII against prazosin, doxazosin, terazosin and the test compounds. Control strips were incubated with solvent [DMSO (0.5%) or normal saline] for 30 mins before recording the concentration response curves. All the tissues were incubated with antagonists for 30 mins but incubation upto 45 mins did not affect the results. Reversal of inhibition induced by the antagonists was evaluated following repeated washings. pA_2 values were calculated by the method described by Arunlakshana and Schild (1959). Any effect mediated by the endothelium was not expected since the endothelial layer was removed while preparing the aortic strip. The primary basis for selecting this assay was cross-screening of angII blockers against phenylephrine mediated contractions and that of α1-adrenoceptor blockers

against angII mediated contractions. This assay was also utilized for screening of the test compounds for AT_1 and α_1 antagonism.

***IN VIVO* PRESSOR RESPONSE EVALUATION**

After anesthetizing the animals (ketamine 100 mg/kg, i.p; diazepam 10 mg/kg, i.p.), righting reflex and pinching response were confirmed. A blunt dissection was performed on the ventral neck region and sternohyoid muscles were removed to expose the trachea. A partial transverse cut was put between the cartilagenous regions and a PE tube was inserted in the trachea which was connected to a ventilator (70 strokes/min; stroke volume 10 ml) for allowing unobstructed respiration. Following this, vagotomy was performed to exclude the effect of vagus. The carotid artery was isolated and cannulated with a PE catheter attached to a pressure transducer for measurement of intra-arterial blood pressure. The transducer and cannula were filled with heparinized saline (100 IU/ml) to prevent accidental clotting of blood inside. The transducer was connected to Powerlab-4/35 data acquisition system (AD Instruments, Australia) for recording the blood pressure. The left femoral vein was cannulated using a scalp cannula (27 gauge; 1 inch length) for administration of test compound (MCR-1329) dissolved in saline with 0.5% DMSO. Baseline blood pressure was recorded for 30 mins following which pressor responses to phenylephrine (6 µg/kg, i.v. bolus) and angII (6 µg/kg, i.v. bolus) were observed in absence and presence of MCR-1329. Inhibition of pressor response was observed at 2 dose levels; 0.36 µmol/kg and 0.72 µmol/kg, i.v. bolus.

Masked Conditions: It was planned to evaluate the pressor-inhibition potential of MCR-1329 under masked conditions. *In vivo* inhibition of phenylephrine mediated arterial pressor response by MCR-1329 was measured in those animals in which 3.6 µM losartan was pre-administered. The idea behind such a protocol was to mask the effects of MCR-1329 on AT_1 receptor. Similarly, the other set involved measurement of inhibition of angII mediated arterial pressor response in those animals in which prazosin (0.72 µM) or terazosin (0.72 µM) were pre-administered to mask the effects of MCR-1329 upon α_1-receptor. The standard drugs and MCR-1329 were administered at equimolar concentrations. Surgical protocol remained the same as described above.

TOXICOLOGICAL EVALUATION OF MCR-1329

Single dose acute oral toxicity study-OECD 423

This study was performed using male wistar rats (10-12 weeks old, 200-230g). Although the guideline suggests that female animals may be preferred, it was decided to evaluate the test drug in male animals to avoid the protective effects of estrogens upon the cardiovascular system which may become evident when female animals are used. This study does not allow calculation of precise LD_{50} values; rather it allows determination of exposure range where lethality might be expected. The test compound (MCR-1329) was suspended freshly in Na-CMC (0.5 %) just before dosing and administered by oral gavage on day 0 in overnight-fasted animals. Urinary and biochemical parameters were recorded at day 0 and day 14. There was no reason to believe that therapeutic doses may range beyond the highest selected dose (i.e. 2000 mg/kg). During the period of study, animals were observed closely, their weight and food intake was recorded twice-a-week and any abnormal behavior, if observed, was documented. Since no preliminary information regarding toxicity of the test substance was available, it was decided to omit the limit test and directly the main test was conducted. Annex 2c of the main guideline document (OECD 423) was followed unless otherwise indicated. Accordingly, 3 animals were dosed in each group and the subsequent dosing depended on the condition of the previously dosed animals. At the end of the observation period for each group, the animals were humanely sacrificed and gross necropsy was performed by closely observing all the major organs. Histopathological and microscopic observations were performed only in case of any untoward observation. Category 5 evaluation was precluded from the evaluation since a dose beyond 2000 mg/kg was unlikely to be used for any therapeutic studies.

Repeat dose oral toxicity study – OECD 407

The purpose of this study was to evaluate the effect of the test compound when administered for chronic regimens at the selected dose. Based on preliminary acute toxicology data and literature review of related class of compounds, 10 mg/kg was chosen to be the animal therapeutic dose for pharmacodynamic studies. Accordingly, this dose was selected for this toxicity evaluation, since this dose is twice that of maximum intended therapeutic dose. The guideline mentions the use of a range finding test or a limit test with a dose of 1000 mg/kg but since such a dose level is unlikely to

be used and corresponding human dose may never be applied in practice, we preferred using a dose of 10 mg/kg. This study was performed in healthy adult wistar rats (male and female, 10-12 weeks old, 200-230 g). At the end of the dosing period, tail-cuff pressures were recorded, blood samples were collected in 3.2 % citric acid for hemoatology, and serum & urine biochemistry was performed. At the end of the study, gross necropsy was performed and major organ weights were recorded (brain, heart, lung, liver, spleen, kidney). No adverse effect was observed during the period of dosing (0-28 days) or during the post-dose observation period (29-42 days). Neither kidney nor brain (organs most likely to be affected) showed any signs of hypotension-mediated damage which was expected with this category of compounds.

PHARMACOKINETICS OF MCR-1329

Pharmacokinetic studies can be performed by non-compartmental or compartmental methods. Non-compartmental methods estimate the exposure to a drug by estimating the area under curve of a concentration-time graph whereas the compartmental methods estimate the concentration-time graph using kinetic models. Non-compartmental methods are often more versatile in that they do not assume any specific compartmental model and produce accurate results acceptable for initial drug discovery programs.

Oral dose disposition

Healthy, male wistar rats (14-16 weeks old; 200-250 g) were used for the study (n = 6). All the rats were fasted overnight and one pre-dose blood sample was withdrawn from all animals under mild ether anesthesia. Then, all the animals were administered 10 mg/kg of MCR-1329 suspended in 0.5 % solution of sodium carboxymethylcellulose in water. Following administration of the compound, blood samples were withdrawn at 1, 2, 4, 8, 12, 16 and 24 hrs from all the animals into heparinised tubes. Plasma was obtained by centrifugation at 4 °C (1200×*g* for 10 mins). Compound (MCR-1329) was extracted from plasma by protein precipitation with 3 volumes of acetonitrile, followed by centrifugation (800×*g* for 10 mins). The extract was evaporated to dryness and reconstituted with mobile phase before injection into HPLC (Purospher, reverse phase [C_{18}], Merck). The analytical column was fitted with a guard column. Detection was performed at 243 nm. For concentrated samples, required dilution was performed using blank plasma. Based on the peak areas obtained,

calibration curve was prepared by linear regression and concentration in the plasma samples was determined. AUC_{0-t}, C_{max} and t_{max} were calculated from the plasma concentration vs. time curve. The area under the plasma concentration-time curves from time zero to time infinity (AUC) was calculated by the trapezoidal rule-extrapolation method. This method employs the logarithmic trapezoidal rule for the calculation of area during the declining plasma-level phase, and the linear trapezoidal rule for the rising plasma-level phase. The area from the last data point to infinity was estimated by dividing the last concentration by the apparent terminal rate constant.

Reverse phase HPLC method for analysis of MCR-1329

The method was optimized on a binary pump Shimadzu LC-20AT instrument with Shimadzu Prominence UV detector (SPD-20A). The mobile phase chosen for analysis was 40 mM sodium phosphate buffer: acetonitrile (3:2; *p*H 3.0). Samples were injected through a 20 µl Rheodyne 7725i injection loop and eluted with the mobile phase at a flow rate of 0.8 ml/min (pressure 108-114 *kgf*). The run time was optimized to be 15 mins with an R_t of ~ 4.2 mins. Stock solution of the test compound was prepared by dissolving 10 mg of MCR-1329 in 10 ml of acetonitrile to give a final concentration of 1 mg/ml. Further aliquots from the stock were prepared by serial dilution using blank plasma as a diluent to give concentrations ranging from 0-1000 ng/ml. These aliquots were treated in the same manner as mentioned above for oral dose disposition samples and analysed on the RP-HPLC to construct a calibration curve based on the peak areas generated by the LC Solution software. All the samples were filtered through a 0.22 µm syringe filter before injection into the column. The method was validated for linearity, precision and accuracy before analysis of samples.

Human plasma protein binding/ In vitro plasma release

Binding of the test compound, MCR-1329 to plasma proteins was determined using a modified *in vitro* assay. Cellophane paper was activated by incubation in 90% ethanol for 24 hrs. It was then soaked in phosphate-buffered saline (PBS, *p*H 7.4) for another 24 hrs to remove any traces of ethanol. The cellophane paper was then folded into a cylindrical tube (available surface area 3 cm^2) and used for evaluation. To evaluate the degree of plasma protein binding, dilutions of the test compounds were prepared in PBS and spiked into blank human plasma at known concentrations and

vortexed for 10 mins and incubated at 37 °C for 4 hrs to allow proper binding of the compounds to plasma proteins. This spiked plasma was pipetted in the cellophane cylinder and the cylinder was tied at both ends in such a way that no plasma could leak from the tied ends. These cylinders were then suspended into beakers filled with PBS maintained at 37°C with stirring. Samples were withdrawn at different time points and the amount of test compounds present in it were evaluated by HPLC as mentioned in the previous paragraph. Release of the test compounds through the semi-permeable cellophane was compared over a period of 24 hrs by calculating the % cumulative drug release from the known amount spiked in each sample.

DOCA-SALT INDUCED HYPERTENSION IN RATS

Adult Wistar rats (20-24 weeks; 250-350 g) were used for the study. Left unilateral nephrectomy (UNX) was performed in all the animals and accordingly left kidney was surgically removed under anesthesia (ketamine 100 mg/kg, i.p.; diazepam 5 mg/kg, i.m.). Briefly, after confirming anesthesia through loss of righting reflex and pinching response, peritoneal laparotomy was performed 2 cm dorsolateral to the diaphragmal line (the waist region) and the left kidney was exposed. The kidney was brought out by holding the surrounding fat. The adhering fat and periadventitious tissue was separated using a pointed forcep and the adrenal gland was detached from the kidney. 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 a hemostatic forcep 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 hemostatic forcep. The incision was closed by ethicon® braided-silk sutures. Since ketamine functions as a preemptive analgesic, immediate post-surgical anaesthesia was not required, however, a dose of tramadol (12.5 mg/kg, i.p.) was administered as soon as the animal showed signs of recovery from anesthesia. The animals were allowed to recover from surgery for two weeks. Two weeks after the surgery, animals were divided into the following groups:

1) Negative control - Normal animals (no treatment or surgery)
2) Positive Control – UNX + DOCA + Salt
3) UNX Control – UNX without DOCA/Salt administration
4) Treatment group - UNX + DOCA + Salt + MCR-1329

5) Standard group - UNX + DOCA + Salt + prazosin + losartan

Animals were switched to 1 % NaCl and 0.2 % KCl in drinking water and were given injections of deoxycorticosterone acetate in olive oil (25 mg/kg, s.c., twice a week) upto 4 weeks. Uninephrectomized (UNX) rats received vehicle without DOCA or salt. To study the effect of the test compound, treatment (10 mg/kg MCR-1329 in 0.5 % sodium CMC, p.o., daily) was initiated in all the groups simultaneously, except positive (UNX+DOCA+Salt), sham and negative controls. Standard group received a combination of prazosin and losartan (5 mg/kg/day each). Non invasive tail-cuff systolic pressures were measured every week. On the penultimate day of the study, animals were placed in metabolic cages for collection of urine over a 24-hour period. At the terminal day of the study, final tail-cuff pressures were recorded, blood samples were withdrawn before sacrificing the animals, after which kidneys were removed for evaluating extracellular matrix deposition and aortae were isolated for studying endothelial dysfunction. Parameter evaluation in the DOCA-Salt hypertension mode was performed as below:

Table 17: Parameter evaluation detail in the DOCA model

Parameter	**Method/Instrument**
Systolic blood pressure-non invasive	Tail cuff apparatus, PANLAB-letica instruments, Spain
Systolic blood pressure-invasive	Arterial pressure transducer, POWERLAB-AD Instruments, Australia
Endotheilial Dysfunction-Aortic strip relaxation	Two Channel Recorder with Force Transducer, GEMINI 7070-UGO BASILE, Italy
Tubuloglomerular matrix-renal capsule	Periodic Acid – Schiff (PAS) Staining for identification of extracellular matrix
Creatinine, Uric acid, BUN-serum	Commercial kit as per manufacturer's protocol
Urine Output	Metabolic cage
Proteinuria, albuminuria, Urea Nitrogen, Na^+, K^+, creatinine, glucose-24 hr urine	Commercial kit as per manufacturer's protocol
Urine Osmolality	[2×{[Na^+ (mg/dl)]+[K^+ (mg/dl)]}]+[Urea nitrogen (mg/dl)/2.8]+[Urine Glucose (mg/dl)/18]
Creatinine Clearance	[{(Urea creatinine×Urine output)/serum creatinine}×(1000/Body weight)/1440]

Non-invasive recording of blood pressure

One week after the UNX surgery, the animals were trained for non-invasive recording of tail-cuff blood pressures. All recordings were performed using the tail-cuff pressure storage meter (Letica Instruments, Barcelona, Spain). Entire protocol for training was same as that of actual recordings, except that the readings were not recorded during the training period. For training purpose, the animals were restrained using suitable size rodent restrainers such that the tail remained freely hanging and movable from one end of the restrainer. The restrainer assembly was placed inside an external heating chamber which was maintained at 39 ± 1°C. The tail-cuff was placed towards the proximal end of the tail while the pulse transducer was placed distal to the cuff. The animal was allowed to remain in this position, until the instrument showed a ready signal. Once ready the cuff was inflated by pressing a button, whereby it deflated automatically, and recordings were stored inside the instrument. This procedure was repeated in a set of 3×3 for a period of 7 days. During actual recordings, pressure was recorded repeatedly until two consecutive readings showed the same systolic blood pressure.

Invasive recording of blood pressure

The procedure for invasive recording of blood pressure in this model is same as that mentioned in the '*in vivo pressor response evaluation'* section.

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.

CELL CULTURE AND SIGNALING STUDIES

Culture and maintenance of cells

Preparation of complete medium for cell culture: The commercially available media are incomplete and do not support the growth of cells as they lack in growth factors. Fetal bovine serum was thawed and was added to the commercially procured DMEM in a final concentration of 10 %. To this solution, antibiotic-antimycotic solution was added in a final concentration of 1 %. Since all the ingredients are available in sterile form, preparation of the final solution was performed under a laminar air-flow hood to prevent accidental contamination. Sterile Pasteur pipettes were used for effecting the transfer of solutions. If required, the final complete medium was filtered through a 0.22 µm syringe filter before use. Serum deprived medium was prepared similarly, except that the final concentration of FBS in the medium was 1 %.

Upon receipt of the cell line, the cells were observed under a light microscope for morphology and confluency, and maintained in a CO_2-incubator (95 % Air + 5 % CO_2). Healthy cells remain adherent to walls of the culture flask and exhibit fibroblastic nature with spindle shape and may be clearly observed as a monolayer. Fresh medium was replaced every week. The cells were subcultured when they reached 80-90 % confluency which was judged by visual observation under a light microscope.

Subculturing of cells: It is essential to dislodge the adherent layer of cells for subculturing. Trypsin-EDTA solution can disrupt the collagen between the cells and hence aid in dislodging cells from the adherent sites. The tissue culture flask was removed from the incubator and the medium was discarded in a waste beaker containing sodium hypochlorite. The monolayer of cells was washed twice with sterile PBS to remove traces of media as FBS present in the media can inactivate trypsin. 1 ml of Trypsin-EDTA solution was added to each flask and allowed to stand for about 5 minutes. To check whether the cells have properly dislodged or not, the flasks were tilted and checked for turbidity of solution due to presence of dislodged cells. Once it was assumed that maximum cells have dislodged, trypsin was inactivated by addition of an equal volume of the medium and the contents were gently pipetted into a microcentrifuge tube. This procedure was termed as trypsinization of cells. The cells were spinned down at 125×*g* for 5 mins to obtain a cell pellet. The pellet was washed twice with sterile PBS and then the cells were resuspended in about 900 µl of fresh

medium. The cell clumps were broken down by trituration with the pipette. This cell suspension was divided to new T-25 flasks, each already containing about 5 ml of complete medium. Normally, a subculturing ratio of 1:3 was maintained for this cell line.

Counting of cells: The cells were counted by trypan-blue exclusion method using a cytometer slide. After trypsinization of cells, the cells were centrifuged and resuspended in 1 ml of sterile PBS. An aliquote of 10 µl was taken from these cells and admixed properly with 10 µl of trypan blue solution. A coverslip was placed on the cytometer slide and small drop was allowed to aspirate by capillary action onto the slide. The cells were counted in all the four corners and their corresponding squares. The total count was multiplied by $1.1\times10^4\times2$ to give the actual number of cells/ml. If required the stock suspension of cells may be diluted to adjust the number of cells/ml.

Cytotoxicity assay

This assay involved treatment of cells with the test compound at different concentrations and evaluation of cytotoxicity produced by means of reaction with MTT. For this purpose, cells were trypsinized and counted. The final concentration of cells was so adjusted that 200 µl suspension of cells prepared in the media contained nearly 10^4 cells. This final suspension was seeded onto different wells of a sterile 96-well tissue culture plate and then the plate was placed in the incubator to allow adherence of cells. Usually, 24 hrs were required for the cells to adhere. Meanwhile a stock solution of test compound, MCR-1329, was prepared in DMSO and from this stock solution further aliquots 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 MCR-1329 and the overall concentration of DMSO did not exceed 5 %. A concentration range of 10 nM to 1 mM was utilized for the assay. After a 24-hr incubation period, the complete media was removed from the wells by inverting the plate forcibly on a paper towel. Serum deprived medium containing the test compound was added in different wells with appropriate controls for DMSO. All the concentrations were utilized in 6 replicates. 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 nearly 4

hrs. Dehydrogenases from live cells metabolize MTT to formazan crystals. These crystals are insoluble in aqueous media and may be solubilized in DMSO. 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 5 mins. 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. Higher 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

Akt signaling study

This study was undertaken to study the effect of MCR-1329 on AT_1- and α_1-receptor mediated Akt phosphorylation. The effect of phenylephrine and angII mediated stimulation of Akt phosphorylation may be studied using flow cytometry after incubation with appropriate primary and fluorochrome-conjugated secondary antibodies. The treatments and treatment protocols were followed as given below:

Table 18: Treatment indications for cell signaling studies[6]

Sr. No.	Title	Treatment
1	Control	Trypsinised cells (untreated)
2	2° Antibody	Trypsinised cells treated with vehicle and 2° antibody
3	Stim 1 (PE)	Cells treated with Phenylephrine for 30 mins
4	Stim 2 (AngII)	Cell treated with AngII for 30 mins
5	Stim1 + MCR-1329	Cell treated with phenylephrine and MCR-1329 for 30 mins
6	Stim2 + MCR-1329	Cell treated with AngII and MCR-1329 for 30 mins
7	Stim1 + MCR-1329 + LY294002	Cells treated with phenylephrine, MCR-1329 and LY294002
8	Stim2 + MCR-1329 + LY294002	Cells treated with AngII, MCR-1329 and LY294002

[6] While giving the different treatments, as and when indicated above, MCR-1329 was added first, followed by the PI_3K inhibitor LY294002 and then stimulated with respective agonists

Concentrations and dilutions utilized

Phenylephrine: 50 µM

Angiotensin II: 10 µM

MCR-1329: 100 µM

LY294002: 10 µM

1° Antibody: 1:100

2° Antibody: 1:500

Preparation of solutions for flow cytometry

Phosphate buffered saline (1X): 1.04 g of lyophilized powder of PBS was dissolved in 100 ml of double-distilled water, filtered through 0.22 µm nylon membrane and autoclaved before use. This solution may be stored at RT for 1 week.

Formaldehyde (methanol free): 2 gm paraformaldehyde was added to about 80 ml filtered 1X PBS and heated upto 60 °C. This heated mixture was intermittently sonicated to give a cloudy suspension. The cloudy suspension showed appearance of precipitate when sonication was stopped. While on the water bath, 2N NaOH was slowly added to the cloudy suspension until the solution clears completely. The solution was then allowed to cool to RT and *p*H was adjusted to 7.4 with 2N HCl. The final volume was made upto 100 ml and the solution was filtered through a 0.22 µm nylon membrane. This solution may be stored for 4-6 weeks at 2-8°C.

Incubation buffer: 500 mg of bovine serum albumin was dissolved in 100 ml of sterile 1X PBS and stored at 4°C. This solution may be filtration sterilized as BSA may precipitate upon autoclaving.

Flow cytometry protocol

Fixation of cells: All the required flasks were treated as mentioned in the above table. Treatments were given in serum-deprived media. It was necessary to fix the cells so as to freeze the protein signaling cascade. The cells were trypsinized and collected by centrifugation. The cell pellet was suspended in about 1 ml of sterile PBS and formaldehyde was added to a final concentration of 2 %. The fixation was allowed to proceed for 10 mins at RT after which the tubes were chilled on ice for 1 min.

Permeabilization of cells: To permeabilize the cells, initially the fixation was removed by centrifuging the cells and resuspending them in 90 % methanol. The cells were again

collected by centrifugation and resuspended in ice-cold methanol and permeabilization of the membranes was allowed to proceed for 30 mins on ice.

Immunostaining: The permeabilized cells were collected by centrifugation and suspended in incubation buffer. The cells were blocked in incubation buffer for about 10 mins at RT to prevent non-specific binding of 1° antibody. The unconjugated antibody against Thr-308 Akt-P was added at a dilution of 1:100 and incubated at RT for 1 hr. After the incubation period the cells were collected by centrifugation and the supernatant (which may contain unbound 1° antibodies) was saved for future studies. The cell pellet was rinsed in incubation buffer by centrifugation and then resuspended in incubation buffer containing the 2° Alexa Fluor 594 conjugated antibody (1:250). Incubation was allowed to proceed for 30 mins at RT after which the cells were collected by centrifugation and the supernatant (which may contain unbound 2° antibodies) was saved for future studies. The cell pellet was resuspended in incubation buffer and analysed on BD FACSAria III flow cytometer with an excitation laser at 590 nm and detection at 617 nm. Parameters like forward scatter, side scatter and histogram analysis were used to quantify the fluorescence emitted from cells treated with 2° antibody as compared to untreated cells.

STATISTICAL ANALYSIS

For all the studies, $3 \leq n \leq 6$. The results were compared by Student's *t*-test, one-way or two-way ANOVA as appropriate. 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 $P<0.05$.

Concentration versus area obtained from the LC Solution software after HPLC detection was treated by linear least square regression for linearity determination. Data from the validation study were presented as mean, standard deviation and % relative standard deviation [(SD*100)/mean].

EXPERIMENTAL

EXPERIMENTAL

DEVELOPMENT AND VALIDATION OF HPTLC BASED METHOD FOR QUANTIFICATION OF CHOLESTERYL ESTERS AND SCREENING OF ACAT INHIBITORS

Materials and solvents

Rat liver microsomes (500 µg protein/ml) were procured from Krishgen Biosystems, Mumbai, India. Cholesteryl oleate standard was synthesized in the Pharmaceutical chemistry laboratory of Pharmacy Department, The M. S. University of Baroda. Cholesterol and oleoyl CoA lithium salt were obtained from Sigma, St. Louis, MO. Aluminium TLC plates pre-coated with silica gel $60F_{254}$ (10×10 cm^2, 250 µm thickness) were procured from Merck, Germany. Anisaldehyde-sulphuric acid reagent was prepared in the following manner: A) 0.5 ml of anisaldehyde was dissolved in 10 ml of glacial acetic acid. B) 5 ml of conc. sulphuric acid was dissolved in 85 ml ice-cold methanol. Final working reagent was prepared by mixing solution A & B. The working reagent appears colorless and should be discarded when a pink tinge appears. Other reagents and chemicals used in the present study were of analytical grade.

Standards and working solutions

A stock solution of cholesterol oleate was prepared by dissolving 100 mg of accurately weighed cholesteryl oleate in a mixture of chloroform-methanol (2:1, v/v) to make the volume upto 100 ml. Working standards were prepared by appropriate dilutions of the stock solution with chloroform-methanol (2:1, v/v). The stock and working solutions were stored at -20 °C.

High-Performance Thin Layer Chromatography

Before sample application, chromatography plates were pre-washed using methanol as a mobile phase and dried for 10 minutes at 120 °C to activate the plates. Samples were applied to the plate as 6 mm wide bands, 10 mm apart by means of Linomat V sample applicator (Camag, Switzerland) fitted with a 100 µl Hamilton syringe. A constant rate of application of 150 nl/s was used. After sample application, the plates were dried in a current of dry air and developed in a linear ascending manner using *n*-hexane-diethyl ether-glacial acetic acid (90:10:1, v/v/v) as mobile phase. 18 ml of the mobile phase was used for development of each plate. Development was performed in a 10×10 twin-trough chamber (Camag, Switzerland) which was previously saturated with mobile phase for 30 minutes. All the steps were performed at

25 ± 2 °C and ambient relative humidity. The solvent front position was fixed at 80 mm from the point of application. After running the mobile phase, plates were dried and dipped in a solution of anisaldehyde-sulphuric acid reagent. The plates were dried and heated at 120 °C for 8 minutes. This led to the development of purple colored bands at solute fronts. Densitometric scanning was performed with Scanner III (Camag, Switzerland) in absorbance mode at 546 nm. The slit dimension was set at 6mm × 0.45mm and scanning speed was 20 mm/s. Calculations were performed with the help of WinCats software (version 1.4.4, Camag, Switzerland).

Calibration curve of cholesteryl oleate

Calibration curve was prepared over a concentration range of 100-500 ng/band. Five dilutions (100, 200, 300, 400, 500 µg/ml) were prepared from the stock solution and further aliquots of these solutions were applied to the TLC plate. This procedure was repeated 6 times. The plates were developed, scanned and the data of peak areas of the developed spots versus concentrations were treated by linear least square regression to obtain the calibration curve.

VALIDATION

Method validation parameters like linearity, range, precision, accuracy, LOD (limit of detection), LOQ (limit of quantification), specificity and robustness were checked as per ICH guidelines [Q2R1]. The methods specified in the text of the guideline were followed unless indicated otherwise.

Precision and accuracy

Repeatability of sample application was carried out by taking six replicates of the same spot (200 ng/band of cholesteryl oleate) and measurement of the peak areas. The intra- and inter-day variation for the determination of cholesteryl oleate was carried out at three different concentrations (200, 300 & 400 ng/band). Accuracy was performed by the method of standard addition. A known amount of cholesteryl oleate was added to a previously analyzed sample at three different levels and % recovery was determined. Three determinations were performed on three different occasions for each level and the results obtained were compared with the expected results.

Limit of detection and limit of quantification

LOD and LOQ were determined by the formulae 3.3σ/S and 10σ/S respectively, where σ corresponds to the average standard deviation of the y-intercepts of regression lines and S corresponds to the average slope of the regression lines.

Specificity

Specificity of the method was tested by analyzing sample and standard on the same plate. The spot for cholesteryl oleate in sample lane was confirmed by comparing the retardation factor (R_f) values. Peak purity was adjudged by comparing peak start, peak apex and peak end values of samples with that of standard. In addition, we also performed an extraction procedure on the microsomal reaction mixture in which oleoyl CoA was not added. The entire procedure was followed as stated above and the extract was then spotted on the plate and scanned in a similar manner to that of other samples. Peaks in the region surrounding the R_f of cholesteryl oleate were observed for any interference due to the biological matrix involved.

Robustness

Robustness was determined by making deliberate changes in the mobile phase composition. Mobile phases having varying compositions of *n*-hexane-diethyl ether-glacial acetic acid (90:10:0.5, 92:8:1 and 88:12:1; v/v/v) were utilized and chromatograms were obtained. Volume of mobile phase and temperature of analysis were also varied in a range of ± 5 %. The effect of these changes on peak areas and R_f value of cholesteryl oleate were observed and data presented as % RSD.

Validation of the method in plasma samples

The aforementioned method was validated for accuracy and precision using pooled human plasma. Plasma samples from 5 different voluntary male donors were provided by a local blood bank (Suraktam Blood Bank, Vadodara, Gujarat, India). Equal volumes of these samples were pooled and used for validation. Briefly, 200 µl aliquot of the collected plasma sample was subjected to extraction with 800 µl mixture of chloroform-methanol (2:1, v/v) in a centrifuge tube. Extraction was effected by vortexing the mixture for 3 mins. The lower organic phase was separated out using a pipette and a known volume of standard cholesteryl oleate was added to the organic phase and further processed as mentioned in section 2.4. The volume of standard cholesteryl oleate was such that it resulted in final concentrations of 8, 12 and 16 µg/ml

after reconstitution. This afforded concentrations of 200, 300 and 400 ng/band when the samples were spotted on the TLC plate. The inherent cholesterol esters present in the pooled plasma samples were nullified from the final results of the spiked ones. Based on these experiments, intra-day and inter-day precision, accuracy and % recovery were calculated.

Quantification of cholesteryl esters in clinical plasma samples

Plasma samples of patients were collected from local pathology laboratories. These samples were originally collected for the estimation of total cholesterol levels by different labs upon referral by physicians. This study included plasma samples for 3 patients whose total plasma cholesterol levels were reported to be above 200 mg/dl by the respective pathology laboratories. 200 µl aliquot of the collected plasma sample was subjected to extraction with 800 µl mixture of chloroform-methanol (2:1, v/v) in a centrifuge tube. Extraction was done by vortexing the mixture for 3 mins. The lower phase was separated out using a pipette and subjected to drying under a stream of N_2 gas. The remaining procedure was same as that described in the *ACAT assay* section.

ACAT assay for screening of potential ACAT inhibitors

The ACAT assay mixture consisted of potassium phosphate buffer (0.1 M), BSA (5 mg/ml), microsomal protein (200 µg) and cholesterol solubilized in 45% w/v hydroxypropyl β-cyclodextrin (2 mM). Final reaction volume was made upto 850 µl with the help of 0.1M potassium phosphate buffer. Vehicle, test compounds or the standard ACAT inhibitor (avasimibe, sigma) were added at this point (final volume NMT 10 µl) and incubated for 15 mins to allow proper binding of the inhibitor with the ACAT enzymes. The reaction was initiated by the addition of 200 µM oleoyl CoA and allowed to proceed for 10 minutes at 37°C. The reaction was terminated by the addition of 6 ml of a mixture of chloroform-methanol (2:1, v/v) to the reaction mixture. The biphasic mixture was shaken in a separating funnel and both the phases were allowed to separate. The lower organic phase was collected and evaporated to dryness under a stream of N_2 gas. The residue was then redissolved in 500 µl of chloroform-methanol (2:1, v/v) and a volume of 25 µl from this solution was spotted on the TLC plates for quantification. Each sample was applied to the TLC plates at least in triplicate. Further analysis was performed as per the method given above.

POLOXAMER-407 INDUCED LIPOPROTEIN LIPASE INHIBITION MODEL

Poloxamer-407 induced lipoprotein lipase inhibition model [Johnston and Palmer, (1993); Millar *et al*, (2005)] was used to determine change in glyceride level by the test compounds.

Materials

Poloxamer-407 was procured as a generous gift sample by Wockhardt Ltd., Aurangabad, India. Avasimibe (cat. # PZ0190) was procured from Sigma Aldrich, St. Louis, MO, USA. The test compounds synthesized in Pharmaceutical Chemistry Laboratory of Pharmacy Dept., The M. S. University of Baroda, Gujarat, India were selected on the basis of preliminary *in vitro* screening results. Triglyceride estimation was performed by using commercial kits as per manufacturer's protocol (GPO-PAP method, Coral Clinical Systems, Vapi, India).

Experimental Protocol

Experiments were performed on healthy adult wistar rats (20-24 weeks; 250-350 g). Baseline triglyceride levels were estimated in serum samples from all animals using commercial kit on Day 0. Vehicle, test compounds (20 mg/kg) and avasimibe (20 mg/kg) were suspended in 0.5 % sodium CMC and administered on Day 1 by oral gavage in fasted animals. 4 hours after the administration of agents, lipoprotein lipase inhibitor, Poloxamer-407 (1000 mg/kg), (prepared as a 10 % solution in 0.9 % NaCl) was administered intraperitoneally. On Day 2, blood samples were withdrawn to obtain serum, and triglyceride levels were estimated (method described below in the succeeding sections) in the same. Results were analyzed to determine fold-change in triglyceride levels over the 24 hour test period.

TOXICOLOGICAL EVALUATION OF TEST COMPOUND (MCR-788) *(Jonsson et al, 2013)*

Single dose acute oral toxicity study - OECD 423

This study was performed using male wistar rats (10-12 weeks old, 200-230 g). Although the guideline suggests that female animals may be preferred, it was decided to evaluate the test drug in male animals to avoid the protective effects of estrogen upon the cardiovascular system which may become evident when female animals are used.

This study does not allow calculation of precise LD_{50} values, rather it allows determination of exposure range where lethality might be expected. The test compound **(MCR-788)** was suspended freshly in 0.5% Na-CMC just before dosing and administered by oral gavage on day 0 in overnight-fasted animals. Urinary and biochemical parameters were recorded at day 0 and day 14. There was no reason to believe that therapeutic doses may range beyond the highest selected dose. During the period of study, animals were observed closely, their weight and food intake were recorded twice-a-week and any abnormal behavior, if observed, was documented. Since no preliminary information regarding toxicity of the test substance was available, it was decided to omit the limit test and directly the main test was conducted. Annex 2c of the main guideline document (OECD 423) was followed unless otherwise indicated. Accordingly, 3 animals were dosed in each group and the subsequent dosing depended on the condition of the previously dosed animals. At the end of the observation period for each group, urinary and biochemical parameters were recorded (submitted herein as appendix III to the thesis), the animals were humanely sacrificed and gross necropsy was performed by closely observing all the major organs. Histopathological and microscopic observations were performed only in case of any untoward observation. Category 5 evaluation was precluded from the evaluation since a dose beyond 2000 mg/kg was unlikely to be used for any therapeutic studies.

Repeat dose oral toxicity study – OECD 407

The purpose of this study was to evaluate the effect of the test compound when administered for chronic regimens. Based on preliminary acute toxicology data and literature review of related class of compounds, 10 mg/kg & 30 mg/kg were chosen to be the animal therapeutic doses for pharmacodynamic studies. Accordingly, a dose of 60 mg/kg was selected for this toxicity evaluation, since this dose is twice that of maximum intended therapeutic dose. The guideline mentions the use of a range finding test or a limit test with a dose of 1000 mg/kg but since such a dose level is unlikely and corresponding human dose may never be applied in practice, we preferred using a dose of 60 mg/kg. Other studies have reported appearance of cutaneous toxicity like xanthomatosis in animals dosed chronically with ACAT inhibitors due to accumulation of free cholesterol in the skin. This study was performed in healthy adult Wistar rats (male and female, 10-12 weeks old, 200-230 g). No adverse effect was observed during the period of dosing (0-28 days) or during the post-dose observation period (29-42

days). Neither liver nor skin (organs most likely to be affected) showed any signs of free cholesterol deposition-mediated damage which is expected with this category of compounds.

EFFICACY EVALUATION OF MCR-788 IN A MODEL OF DIET-INDUCED ATHEROGENESIS

Materials

The list of material used in the study and their sources are given in table 14.

Table 14: List of materials and their sources for the atherogenic-diet model

Reagent/Chemical/Ingredient	*Source*
Pelleted chow for rats	Amrut Feed, Pranav Agro, India
Cholesterol	Spectrochem, India
Coconut oil	HiMedia, Mumbai, India
Cholic acid	HiMedia, Mumbai, India
2-Thiouracil	HiMedia, Mumbai, India
Casein	SD Fine Chemicals, India
Sudan Red IV	HiMedia, Mumbai, India
Hydroxypropylcellulose (HPC)	Gift sample from Wockhardt Ltd., Aurangabad India
Test compound (**MCR-788**)	Synthesized in Pharmaceutical Chemistry Laboratory of Pharmacy Dept., The M. S. University of Baroda, Gujarat, India
Kits for Total cholesterol, triglyceride and HDL-C estimation	Coral Clinical Systems, Mumbai, India
Solvents (diethyl ether, methanol, isopropyl alcohol)	SD Fine Chemicals, India

Atherogenic Diet

The composition of the diet was as follows: Cholesterol (2%), Coconut oil (1%), Cholic acid (1%), Propylthiouracil (0.5%), Casein (16.5%), powdered chow and hydroxypropyl cellulose (HPC) binder (q.s. to 100%). After accurately weighing all the ingredients casein, cholic acid and propylthiouracil were ground in a mortar pestle until

a homogenous solid mixture was formed. This dry mixture was added to a large vessel and mixed with required amount of powdered chow. Next, cholesterol was dissolved in appropriate volume of diethyl ether and this ethereal solution was properly mixed with the powder mass. The ether was allowed to evaporate for 2-3 hours. Once the ether evaporated, required amount of coconut oil was mixed properly. For every kilogram of diet to be prepared 800 ml of 1% HPC solution was prepared by stirring for about half an hour. This solution was added at once to the dry mass and vigorously kneaded to evenly mix all the HPC. The consistency achieved was such that the lumps formed neither crumbled nor were too sticky. The lumps were hand cured to form suitably shaped lumps which looked like pelleted chow. The final pellets were dried overnight in a hot-air oven at 45-50°C. This step improves long term storage and prevents microbial growth. The pellets were stored in a perforated bag at 2-8°C. The formed pellets were not stored for more than 7 days.

Experimental protocol

The animals (adult male Wistar rats, 230-300 g) were randomized into 3 groups of 6 animals each. The groups were as follows:

1. Progression Control-received atherogenic diet only
2. Treatment Group I-received atherogenic diet containing **MCR-788** such that final daily dose was 10 mg/kg
3. Treatment Group II-received atherogenic diet containing **MCR-788** such that final daily dose was 30 mg/kg.

The protocol of the study was in the following manner:

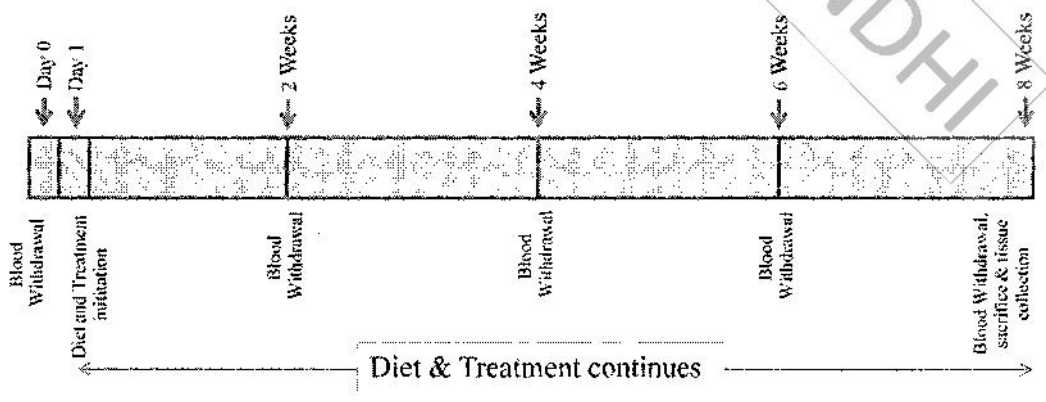

Figure 15: Protocol for diet administration, sampling and tissue collection in the atherogenic diet model.

All the blood samples were allowed to clot at room temperature for 15 mins and centrifuged at 800×*g* for 15 mins at 25°C. The supernatant serum was collected and subjected to biochemical estimation of total cholesterol, triglycerides and HDL-C using commercially available diagnostic test kits. Other parameters like VLDL, LDL-C and atherogenesis index were derived by formulae on the basis of these values. The principles and methodology of these biochemical estimations are given below. Towards the terminal stage of the study, animals were humanely sacrificed after blood sampling and thoracic aortae and aortic roots were carefully dissected.

Biochemical estimations of serum lipids and lipoproteins

Estimation of total cholesterol: (CHOD/POD-Phosphotungstate method)

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

Principle:

Cholesterol esterase (CHE) hydrolyses cholesterol esters. Free cholesterol is oxidized by the cholesterol oxidase (CHO) to choloest-4-ene-3-one and hydrogen peroxide. Hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase (POD) to produce pink colored quinoneimine dye. The intensity of color produced is proportional to cholesterol concentration.

Protocol:

Test tubes labeled Blank (B), Standard (S) and Test sample (T) were managed as shown in table 5 below:

Table 15: Protocol for total cholesterol estimation

Addition	**Blank**	**Standard**	**Test Sample**
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Cholesterol standard	-	20 µl	-
Specimen	-	-	20 µl
Incubation at 37°C for 10 minutes.			
Distilled water	2.0 ml	2.0 ml	2.0 ml

The absorbance was read against Blank at 505 nm.

Calculations:

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

Estimation of triglycerides: (GPO Method)

In vitro quantitative measurement of triglyceride (neutral fat) concentration in serum was done by using kit (Reckon diagnostics (India) Pvt. Ltd.).

Principle:

Triglycerides in the sample are hydrolyzed by microbial lipase to glycerol and free fatty acid (FFA). Glycerol is phosphorylated by adenosine 5-triphosphate (ATP) to glycerol 3-phosphate (G-3-P) in a reaction catalyzed by glycerol kinase (GK). G-3-P is oxidized to dihydroxy acetone phosphate in a reaction catalyzed by the enzyme glycerol phosphate oxidase (GPO). In this reaction hydrogen peroxide (H_2O_2) is produced in equimolar concentration to the level of triglycerides present in the sample. H_2O_2 reacts with 4-aminoantipyrine and ADPS in the reaction catalyzed by peroxidases (POD). The result of this oxidative coupling is a purple colored dye quinoneimine. The absorbance of this dye in solution is proportional to the concentration of triglycerides in sample.

Table 16: Protocol for triglyceride estimation

Addition	**Blank**	**Standard**	**Test**
Working reagent	1.0 ml	1.0 ml	1.0 ml
Standard	-	20 µl	-
Specimen	-	-	20 µl
Incubation at 37 °C for 15 minutes.			
Distilled water	1.5 ml	1.5 ml	1.5 ml

The absorbance of test and standard were read against the blank at 546 nm.

Calculations:

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

Estimation of HDL-cholesterol: (CHOD/POD-Phosphotungstate method)

In vitro quantitative determination of the activity of cholesterol in serum was done using enzymatic kit (Reckon Diagnostics Pvt. Ltd., Vadodara, India).

Principle:

The VLDL and LDL fractions of serum sample are precipitated using PTA and then HDL in the supernatant is separated by centrifugation and measured for its cholesterol content. The enzyme cholesterol ester hydrolase (CHE) hydrolyses the ester into cholesterol. Then cholesterol is oxidized by cholesterol oxidase (CHO) to cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide in presence of enzyme peroxidase (POD) reacts with 4-aminoantipyrine and phenol to produce a red colored complex, whose absorbance is proportional to HDL-cholesterol concentration.

The following samples and reagents were pipetted into microcentrifuge tubes:

Table 17: Sample preparation for HDL-cholesterol estimation

Serum	0.2 ml
Precipitating Reagent	0.3 ml

Samples were vortexed and incubated at 25°C for 10 mins. The samples were centrifuged at 300×*g* rpm for 10 minutes to get a clear supernatant. If the supernatant was not clear (high TGL level) the samples were diluted in 1:1 ratio with normal saline. Pipetted into 3 test tubes labeled Blank (B), Standard (S) and Test sample (T) as shown in table 7 below:

Table 18: Protocol for HDL-cholesterol estimation

Addition	**Blank**	**Standard**	**Test sample**
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Cholesterol standard	-	20 µl	-
Supernatant	-	-	20 µl
Incubation at 37 °C for 5 minutes			
Distilled water	2.0 ml	2.0 ml	2.0 ml

The absorbance is read against Blank at 505 nm.

Calculations:

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

Estimation of VLDL-cholesterol:

Estimation of VLDL-cholesterol was done using the Friedewald formula.

VLDL cholesterol (mg/dl) = triglycerides / 5.

Estimation of LDL-cholesterol:

Estimation of LDL-cholesterol was done using the Friedewald formula.

LDL cholesterol (mg/dl) = total cholesterol – (HDL cholesterol + VLDL cholesterol)

Estimation of Atherogenic index:

Atherogenic index = log [TG / HDL-C]

En face lipid staining

It is known that the site for atherogenesis is unpredictable along the length of the thoracic aorta, hence the entire thoracic aortae were subjected to *en face* staining with Sudan red IV solution (5 mg/ml in 70 % isopropyl alcohol, vortexed and filtered). The thoracic aortae were isolated and cleaned of periadventitious tissue as it stains heavily with Sudan red IV. Care was taken to prevent damage to intima as lipid deposits may be dislodged upon mishandling. It was split from the lumen to give a strip. The strips were pinned to a white plate and immersed in Sudan red IV solution at 37°C for 45 minutes. After this maneuver, the samples were destained for 10 mins in 40 % isopropyl alcohol. The stained tissue was photographed using a digital camera and images were analyzed using NIH (USA) image analysis software, ImageJ (ver. 1.43u, written by Wayne Rasband). Gross extent of atherosclerosis within the thoracic aorta was calculated by estimating the percent lesion area as compared to total area. Data obtained were subjected to statistical analysis.

Cryosectioning and Sudan Red IV staining

The aortic roots were immediately washed with ice-cold PBS, cleared of surrounding adventitious fat, carotid connections were severed and immersed in 10 % buffered formalin for cryosectioning. At the time of sacrifice, liver lobes were also removed to serve as support while sectioning the aortic roots. A small cavity was formed into the pith of the liver and one end of the aortic root was inserted inside the cavity. This whole lobe was now mounted on a chuck and put inside the sectioning instrument. The tissue was allowed to freeze for about 15-20 mins and then the chuck was placed near the blade-rotor assembly. The blade was moved by the rotor to give a slicing motion and the sections were collected on a slide. The sections easily stick on the slide as soon as the slides are brought to room temperature due to the proteins in the section. The sections were further stained with Sudan red IV solution (5 mg/ml in 70 % isopropyl alcohol, vortexed and filtered). The slides were put inside a coplin jar and the jar was filled with Sudan red IV solution. The jar was incubated in a water bath at 37°C for 45 mins. The slides were then destained for 10 mins in 40 % isopropyl alcohol. Slides were cleaned after removing, observed at 10X magnification and photographed.

STATISTICAL ANALYSIS

For all the studies, $3 \leq n \leq 6$. Data from the validation study were presented as mean, standard deviation and % relative standard deviation [(SD*100)/mean]. Concentration versus area obtained from the WINCATS software after HPTLC detection was treated by linear least square regression for linearity determination. Data from the ACAT screening assay and the atherogenesis study were presented in the form of mean ± SEM. Data from the lipoprotein lipase inhibition model were calculated as fold-change over a period of 24 hours and then presented as mean ± SEM.

The results were compared by Student's *t*-test, one-way or two-way ANOVA as appropriate. 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 $P<0.05$.