Pharmacological Studies on Newer Anti-Hyperlipidemic Agents

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By

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CERTIFICATE

This is to certify that the thesis entitled **"Pharmacological Studies on Newer Anti-Hyperlipidemic Agents"** and submitted by Mr. M. Anookh, ID.No. 1999PHXF014 for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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Abbreviations

Abbreviation	Description
ACAT	Acyl coenzyme A :cholesterol Acyltransferase
Ach	Acetylcholine
ADP	Adenosine diphosphate
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BBB	Blood Brain Barrier
BP	Blood Pressure
BSA	Bovine serum albumin
B.Wt	Body weight
$CaCl_2$	Calcium Chloride
CBB	Coomassie Brilliant Blue
CDP	Disodium 2-chloro-5-(4-methoxyspiro[1,2 `-(5 `-chloro)-tricyclo [3,3,1,$1^{3,7}$] decan]-4 yl)phenyl phosphate).
CE	Cholesteryl Ester
CETP	Cholesterol Ester Transfer Protein
CHD	Coronary Heart Disease
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DPPH	2,2,-diphenyl-1-picrylhydrazyl
ECG	Electro Cardiogram
ED	Effective Dose
EDRF	Endothelium Derived Relaxing factor
EDTA	Ethylene Diamine Tetra Acetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immuno Sorbent Assay
ER	Endoplasmic Reticulum
FATS	Familial Atherosclerosis Treatment Study
FBS	Fetal bovine serum
FFA	Free Fatty Acid
FH	Familial Hypercholesterolemia
FHRS	Familial Hypecholesterolemia Regression Study
HDL R	HDL-receptor

Abbreviation	Description
HDL-C	High-Density Lipoprotein-Cholesterol
HEPA	High Efficiency Particulate Air
HepG2	Human hepatoblastoma
HL	Hepatic Lipases
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
HPTLC	High Performance Thin Layer Chromatography
HR	Heart rate
IDL	Intermediat- Density Lipoprotein
INSIG-1	Insulin Induced Gene-1
IR	Infra Red
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
LCAT	Lecithin: Cholesterol Acyltransferase
LDL-C	Low-Density Lipoprotein Cholesterol
LDL-R	LDL-receptor
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein Lipase
LRP	LDL Receptor Related Protein
Mab-C7	Monoclonal antibody C7
MABP	Mean Arterial Blood Pressure
MCD	Medicinal Chemistry Department
MEM	Minimum Essential Media
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
MI	Myocardial Infarction
MTP	Microsomal Triglyceride Transfer Protein
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NaCl	Sodium Chloride
Na CMC	Sodium carboxymethyl cellulose
NADH	Nicotinamide Adenine Dinucleotide(reduced)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate(reduced)
NaH_2PO_4	Sodium Dihydrogen Phosphate
$NaHCO_3$	Sodium bicarbonate
NO	Nitric Oxide
OD	Optical Density

Abbreviation	Description
OxLDL	Oxidised LDL
PAI-1	Plasminogen activator Inhibitor 1
PBS	Phosphate Buffer Saline
PEG	Poly Ethylene Glycol
PLTP	Phospholipid Transfer Protein
PSS	Physiological Salt Solution
SCAP	SREBP-Cleavage Activating Protein
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error Mean
SMCs	Smooth Muscle Cells
SR-BI	Scavenger Receptor
SRE	Sterol Response Element
SREBP	Sterol Regulatory Element Binding Proteins
TBS	Tris Buffer Saline
TC	Total Cholesterol
TG	Triglyceride
TGRLs	Triglyceride Rich Lipoproteins
TIA	Transient Ischemic Attack
TPBS	Tween Phosphate buffer saline
TTBS	Tween Tris Buffer Saline
VCAM	Vascular Cell Adhesion Molecule
VLDL	Very Low Density Lipoprotein

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Introduction

1. Introduction

Cholesterol is a vital component of mammalian cell membrane of all tissues and also a precursor of steroid hormones. It is of critical importance that the tissues are constantly supplied with cholesterol. Its endogenous synthesis and excretion is under regulatory control and when regulatory mechanisms are not operative due to genetic or environmental causes, the level of cholesterol in the blood increases, particularly low-density lipoprotein cholesterol (LDL-C) leading to hyperlipidemia (Brown and Goldstein, 1984).

Hyperlipidemia is the most potent risk factor in the causation of atherosclerosis, ischemic heart disease, premature coronary artery disease, peripheral arterial disease and ischemic cerebrovascular diseases like transient ischemic attack (TIA) and stroke. A long established theory suggests that the higher the circulating level of low density lipoproteins (LDL), the more likely they are to gain entrance to the arterial wall and cause atherosclerosis and other related disorders (Nordestgaard et al., 1992). High-density lipoprotein (HDL) plays an anti-atherogenic role by its 'reverse cholesterol transport' activity where it removes free cholesterol from arterial wall cells and transports them to liver. It also acts as a 'sink' for some of the surface components of the hydrolysed products of the triglyceride rich lipoproteins (von Eckardstein A et al., 2001). So lowering the serum LDL and triglyceride levels and increasing the HDL levels are now the accepted means of treating patients suffering from atherosclerosis and hyperlipoproteinemias. Based on data from various clinical and epidemiological studies, the National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III guidelines state that the principal goal of risk reduction therapy is to lower LDL-C levels (Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection,

Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), 2001).

In hypercholesterolemia, the increase in blood total cholesterol level, in particular LDL cholesterol is most detrimental and is mainly responsible for the vascular pathology associated with hyperlipidemia (Nordestgaard et al., 1992). There is development of endothelial dysfunction where the endothelium of medium sized muscular arteries such as the coronary arteries lose its ability to release biologically active nitric oxide (NO), a vasodilator required for endothelium dependent relaxation of vessels in response to adenosine diphosphate (ADP), acetylcholine (Ach), hypoxia and other stimuli (Drexler, 1999). In humans, lowering the plasma concentration of LDL and other atherogenic lipoproteins that deliver harmful lipids into the vessel wall, has been reported to improve endothelium dependent vasomotor regulation (Karter Y et al., 2003). Reduction of hypercholesterolemia results in a delayed onset of atherosclerosis and a decrease in progression of atherosclerosis, thus reducing the risk of coronary heart disease in humans and other primates.

Of the lipoproteins, very low density lipoprotein (VLDL) and low-density lipoprotein (LDL) are the major carriers of triglyceride (TG) and cholesteryl ester (CE) in the plasma. About three fourth of the total cholesterol in normal human plasma is contained within LDL particles. LDL receptor is a key component of the mechanism by which animal cells maintain balanced cholesterol homeostasis. Hepatic and extra hepatic tissues have LDL receptors localised on their cell surface and the LDL that bind to these receptors is taken up by receptor-mediated endocytosis and digested by lysosomes within the cell. The CE is hydrolysed by acid lipases and free cholesterol is used by cells for membrane synthesis and in hepatic tissues for bile acid synthesis. The transcription of the LDL receptor gene is under feed back regulation by intra-cellular levels of sterol. In cells with excess sterol, transcription of this gene is repressed as well as cells own synthesis of cholesterol is

reduced, while transcription is accelerated in cells requiring cholesterol. So when hepatic LDL receptors are repressed by intracellular accumulation of dietary cholesterol, LDL is not taken up into the liver at a normal rate, and the lipoprotein builds up to high level in the blood (Brown et. al., 1986; Goldstein et al., 1990)

In humans, 70-80% of LDL is cleared from the plasma each day by the LDL receptor pathway in hepatocytes. If this uptake is blocked at the receptor level, cholesterol accumulates in the blood and can contribute to atherosclerosis (Brown and Goldstein, 1984). It has been documented that circulating LDL can get modified to small dense LDL or oxidised and picked up by scavenger cell system in phagocytic cells, these forms are much more atherogenic (Austin et al., 1988; Lamarche et al., 1997; Berliner et al., 1995).

Presently statins are undisputedly the gold standard in the treatment of dyslipidemia as more efficacious monotherapies are not available. Statins inhibit the enzyme involved in cholesterol synthesis, leading to decrease in intracellular cholesterol and this in turn cause the cells (hepatocytes) involved to over express LDL receptors. But a compensatory feedback response is elicited wherein hepatocytes synthesise increased amount of HMGCoA reductase and this thereby partly antagonise the inhibitory effect of the drug (Brown and Goldstein, 1986). Recent research suggests that around six out of ten patients with CHD receiving statin therapy were not able to achieve the LDL-C target levels as recommended by the NCEP ATP III treatment goals (Pearson et al., 2000). Dose escalation studies did not show a proportionate increase in efficacy with statins (Jones et al., 1998).

Long-term administration of these drugs causes liver dysfunction (elevated serum transaminases), rhabdomyolysis, myalgia, neuropathy and mild elevated creatine phosphokinase in some of the patients (Tobert, 1988). Studies with higher doses of statins in dog have shown development of cataract. There are reports of

development of cataract when statins are combined with erythromycin (Schlienger et al., 2001). Though long-term use of statins in the lower therapeutic range in humans does not appear to increase the risk for cataracts, safety with higher doses on long-term use has to be ascertained (Smeeth et al., 2003).

Interventions which result in selective increased expression of LDL receptors irrespective of the intracellular sterol concentration, thus allowing the liver to take up more LDL-C from blood, is a novel approach in the management of hyperlipidemia (Lin et al., 1995). *In vitro* LDL receptor up-regulation can be studied in the laboratory by cell-based assay methods where drug induced LDL receptors expression in specific cell lines, which express them, can be quantitated using monoclonal antibodies (Ashton et. al., 1996).

Hence, this study was undertaken to identify anti-hyperlipidemic molecules that directly up-regulate LDL-receptors without affecting the HMGCoA reductase activity.

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2. Review of Literature

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1. Plasma Lipoproteins – Structure, nomenclature and occurrence

1.1 Lipids

Physiologically lipids are simple or complex, the latter derived from the former by covalent association. In human plasma lipoprotein metabolism, the most important simple lipids are fatty acids, sphingosine and cholesterol whereas cholesteryl esters, triglyceride (TG), phospatidylcholine (lecithin), phosphatidylethanolamine (cephalin) and sphingomyelin are the most important complex lipids.

Lipids can be classified as polar or non-polar; this characteristic is an important determinant of where they are found within a lipoprotein. The major nonpolar lipids are TG and cholesteryl esters. Non-polar lipids form the core of plasma lipoproteins and the relative abundance of TG and cholesteryl ester in lipoprotein can affect their physical properties and metabolism. TG forms the center of lipid rich inclusion in adipocytes while cholesteryl ester is the major component of inclusions in macrophage foam cells in arterial lesions. Polar lipids are located on lipoprotein and intracellular lipid inclusion surfaces.

Some important differences in complex lipids are determined by fatty acid composition. There are three major fatty acid classes: saturated, monounsaturated and polyunsaturated. Fatty acids follow several metabolic pathways, which are determined in part by their structure. Most dietary fatty acids are delivered to cells to satisfy energy and growth requirements and some like the highly unsaturated fatty acids – eicosapentaenoate and arachidonate are precursors to, or modulators, of, bioregulators such as prostaglandins, thromboxanes and leukotrienes.

1.2 Lipoproteins

Plasma lipoproteins are complexes of lipids and proteins (apolipoproteins). With the exception of free cholesterol, the lipoprotein lipids are always complex; they include cholesteryl esters, TGs and phospholipids. Lipoproteins are classified according to their densities and electrophoretic mobilities.

In order of decreasing density they are HDL, LDL, IDL, VLDL and the chylomicron. The density of lipoproteins can shift according to changes in composition and the electrophoretic mobility can be altered by changes in the charges on the proteins. In all lipoproteins, the major fatty acids are linoleate, oleate, and palmitate in cholesteryl esters; palmitate, stearate, oleate, and linoleate in phospholipids; and oleate palmitate and linoleate in TGs. Typically, saturated fatty acids are located at the sn-1 position, where as the sn-2 position contains almost all monosaturated and polyunsaturated fatty acids. Each complex lipid is quite similar in composition among VLDL, LDL and HDL. Where differences exist in fatty acid composition, LDL and HDL are more similar to each other than to VLDL. This can be attributed to the activity of CETP, which exchanges TG and cholesteryl ester between lipoproteins (Tall, 1995). The life times of LDL and HDL (> 3 days) are sufficiently long for equilibration to occur. In contrast TG in VLDL, is rapidly removed by lipolysis, before equilibration.

Human plasma concentrations of lipids vary greatly according to nutritional status and genetically determined inter-individual differences. In the plasma of subjects who have fasted, the predominant lipids are cholesteryl esters followed by phospholipids and TG.

Much of the interest to date in plasma lipids has been related to the predictivity of plasma cholesterol concentration for CHD. Plasma cholesterol concentration is determined by complex metabolic relations among many other lipids in the plasma compartment and the regulation of cellular cholesterol synthesis by plasma factors that include both lipids and proteins. There is a growing focus on the role of TG as a cardiovascular risk factor.

1.3 Proteins

Apolipoproteins (apoproteins) are a specialized group of proteins that associate with lipids and mediate several biochemical steps associated with plasma lipid metabolism. These are very important since they provide structural stability to

the lipoproteins. They are designated by letters and roman numerals, the number refer simply to the order in which the fractions that contain them emerge from a chromatographic column in their isolations. Many of these contain determinants that regulate several activities essential for normal lipid metabolism. Some of these also stimulate enzymes that degrade plasma lipids or act as cofactors in the enzymatic process that regulate lipoprotein metabolism. Others contain the ligand that mediates the binding of plasma lipoproteins to cell surface receptors; binding is succeeded by the internalization of all or part of a lipoprotein and the regulation of intracellular lipid synthesis.

Apoprotein structure: The plasma apolipoprotein can be divided into three groups according to shared protein and gene structure. Group I comprises the soluble apolipoprotein (apo) that reversibly associate with lipid surfaces, namely apo A-I, A-II, A-IV, C-I, C-II, C-III, E. The gene structure of these proteins belongs to the same multigene family and the proteins are code by a gene that contains four exons and three introns. Additional similarities in the primary structure of all group I apolipoproteins is the numerous regions in them being helical and these regions are amphophilic (amino acid residues on one helical face are polar whereas those on the opposing helical face are nonpolar).

According to the amphophilic helical model of the soluble apoproteins, the helix associates with a lipoprotein with its axis parallel to the lipoprotein surface. The nonpolar side of the helix penetrates into the lipids beneath the lipoprotein surface; the polar residues remain in contact with the polar head groups of the phospholipids and the surrounding aqueous phase. Thus, by forming a surface film around the neutral lipids, apolipoproteins and phospholipids make the lipids soluble in an aqueous environment. X-ray crystallography of the LDL receptor-binding domain of apo E reveals a protein that forms an elongated, four-helix bundle, with the helices stabilized by a tightly packed hydrophobic core that includes leucine zipper type interactions and by salt bridges on a charged surface (Wilson et al., 1991).

Apo B-100 and apo B-48 form the group 2 plasma apolipoproteins. These two are grouped together as they are very large and are associated with the cholesteryl ester rich lipoprotein and TGRLs. Apo B-100 contains several lipid-binding domains. They have been identified in a tryptic digestion, in heparin-binding domains and in multiple sites of glycosylation. Apo B-100 contains 25 cysteines, some of which are located in a disulfide cluster. One of the cysteines form a disulfide link with apo(a). This is the means by which apo(a) is attached to LDL. Apo B-48 contains some of the glycosylation and heparin binding sites as well as the disulfide clusters of apo B-100, but it does not contain the receptor-binding domain of apo B-100 that targets LDL to cell surface receptors.

Group 3 is the reminder of the apolipoprotein for which no unifying structural or functional determinant has been identified. These are apo D and apo(a). Apo D belongs to a broad family of proteins (family includes retinal binding protein and alpha$_2$ macroglobulin) that bind to and transport various ligands. Apo(a), which is bound to a minority of LDL particles, belong to a family of proteins involved in fibrinolysis. Highly polymorphic, it bears little resemblence to any of the other apolipoproteins. Although the catalytic triad that characterizes serine proteases is present in apo(a), the potential activation site, that is cleaved by tissue plasminogen activator is modified and may not be activated.

The general hypothesis of lipoproteins structure, which is applicable to all lipoproteins, is the 'oil drop' model (Shen et al., 1977). A polar surface containing the apolipoproteins and phospholipids is thought to surround an oily core containing the neutral lipids (TGs and cholesteryl esters). Most of the free cholesterol is in a small lipoprotein such as HDL is near the surface while in lipoproteins with a larger core, a greater fraction is partitioned into the core.

There are clearly recognizable differences between lipoproteins found in hyperlipidemic and healthy subjects. In most hypertriglyceridemic subjects, there is a higher than normal ratio of proteins to neutral lipids in the LDL fraction. In FH, there is an elevation in the cholesteryl ester content of LDL.

Although all mature lipoproteins have a uniform surface monolayer of apolipoproteins and phospholipids, there is considerable heterogeneity in apolipoprotein ratio depending on the metabolic state of the subject. Larger VLDL particles contain apo B-100, apo C and apo E. Smaller VLDL particles contain less C apolipoproteins. Virtually all of the protein component of IDL is apo B-100 and apo E. LDL particle contains one molecule of apo B-100 as its sole protein. Addition of apo(a) to LDL yields Lp(a). HDL_3 is enriched in apo A-I compared to HDL_2. Postprandial HDL is less dense due to transfer of C apolipoproteins from HDL to chylomicrons.

Table 1:Characteristics of plasma lipoproteins

LIPOPROTEIN CLASS	DENSITY OF FLOTATION, g ml	MAJOR LIPID CONSTITUENT	TG/CHOL RATIO	SIGNIFICANT APOPROTEINS	SITE OF SYNTHESIS	MECHANISM(S) OF CATABOLISM
Chylomicrons and remnants	< 1.006	Dietary triglycerides and cholesterol	10:1	B-48, E, A-I, A-IV, C-I, C-II, C-III	Intestine	Triglyceride hydrolysis by lipoprotein lipase ApoE-mediated remnant uptake by liver
VLDL	< 1.006	"Endogenous" or hepatic triglycerides	5:1	B-100; E, C-I, C-II, C-III	Liver	Triglyceride hydrolysis by lipoprotein lipase
IDL	1.006-1.019	Cholesteryl esters and "endogenous" triglycerides	1:1	B-100, E, C-II, C-III	Catabolic product of VLDL	50% converted to LDL mediated by hepatic lipase 50% apoE-mediated uptake by liver
LDL	1.019-1.063	Cholesteryl esters	NS	B-100	Catabolic product of VLDL	ApoB-100-mediated uptake by LDL receptor (~75% in liver)
HDL	1.063-1.21	Phospholipids, cholesteryl esters	NS	A-I, A-II, E, C-I, C-II, C-III	Intestine, liver, plasma	Complex: Transfer of cholesteryl ester to VLDL and LDL Uptake of HDL cholesterol by hepatocytes
Lp(a)	1.05-1.09	Cholesteryl esters	NS	B-100, apo(a)	Liver	Unknown

Adapted from Mahley RW and Bersot TP: Drug therapy for hypercholesterolemia and dyslipidemia. *In* Hardman JG et al., (eds.): Goodman & Gilman's The Pharmacological Basis of Therapeutics. 10th ed. New York: McGraw-Hill, 2001a: 973.

Abbreviations: apo, apolipoprotein; CHOL, cholesterol; HDL, high-density lipoprotein; Lp(a), lipoprotein(a); LDL, low-density lipoprotein; NS, not significant (triglyceride is less than 5% of LDL and HDL); TG, triglyceride; VLDL, very-low density lipoprotein.

2. Lipid synthesis and Lipoprotein assembly

The major secreted lipoproteins are VLDL, which is assembled in the liver and the chylomicron, which is formed in the intestine. Both lipoproteins undergo substantial remodeling in the plasma compartment thus remnants or mature form of the circulating particles are found under fasting conditions. One common component of the assembly of lipoproteins is the lipids. The biosynthesis of lipids leads to one of the four compounds fatty acids, glycerol lipids, cholesterol and its esters and sphingolipids.

2.1 Lipid Synthesis

Fatty acids:- Palmitic acid is the fatty acid that is first synthesized (lipogenesis) from glucose / acetyl CoA and which through series of elongation and desaturation reaction forms long chain unsaturated fatty acids. These fatty acids are incorporated into TGs and secreted as VLDL particles, which are further remodeled through hydrolysis in the plasma compartment to FFA. They, in turn, are incorporated into adipose tissues.

Glycerol lipids:- Diacylglycerol formed from glycerol-3-phosphate is converted to phosphatidylcholine (most abundant phospholipids in plasma lipoprotein) or phosphatidylethanolamine (cephalin) when the fatty acid concentrations are low. As available fatty acids increase than diacylglycerol it is increasingly diverted to synthesis of TG (lower K_m associated with phospholipids synthesis compared to diacylglycerol acyltransferase that forms TG). The relative amounts of TG and phospholipids formed by this route appears to be important in determining whether TG is secreted as VLDL or is retained as cytoplasmic lipid droplets. Phospholipids are necessary for the formation of cell membranes and are thus critical to cell survival. Additional route for formation of TG exists in intestine. Intestinal lipases hydrolyse the fatty acids of glycerol lipids to produce water soluble

FFAs and 2-monoacyl-glycerol. The acylation of 2-monoacyl-glycerol then produces TG, which forms part of the neutral lipid core of chylomicron.

Cholesterol and its esters:- Endogenous cholesterol is derived from acetyl CoA via the rate limiting intermediate step of HMG-CoA to mevalonic acid by HMG-CoA reductase. The liver and intestine are the major source of endogenously derived lipoprotein cholesterol. A variable fraction of cholesterol can be derived from diet. Both endogenous and dietary cholesterol are involved in several important processes, including biogenesis of cell membranes, production of steroid hormones and formation of bile acids. Bile acids serve as fat emulsifiers in the digestive tract.

The rate-limiting step in the synthesis of bile salts involves the enzyme 7-α-hydroxylase and some of the steps involved in its formation occur in the liver and some that require catalysis by intestinal enzymes. Bile acids, if not sequestered, are reabsorbed from the intestinal lumen and recycled.

Free cholesterol is a substrate for plasma and intracellular enzymes that form cholesteryl esters. Acyl coenzyme A:cholesterol acyltransferase (ACAT) is a membrane bound enzyme that resides in the rough endoplasmic reticulum. Its substrates are cholesterol (membrane bound) and acyl CoA which is amphophilic. Part of acyl CoA resides in the cytoplasm as a free monomeric species or is bound to a fatty acyl binding protein and the reminder is membrane bound and this is thought to be part of the acyl CoA pool that is used by ACAT.

Dietary cholesterol is esterified by one of the two forms of the enzyme ACAT. ACAT-1 and ACAT-2. ACAT-1 is expressed in macrophages, including foam cells, skin sebaceous glands and adrenocortical cells. Although ACAT-1 esterifies cholesterol and promotes foam cell development, ACAT-1 knockout mice do not have a reduced susceptibility for developing atherosclerosis (Accad et al., 2000). ACAT-2 is found in intestine and liver, where cellular free cholesterol is esterified to be assembled in VLDL and chylomicron. In the intestine ACAT-2 regulates the absorption of dietary cholesterol (Cases et al., 1998)

Sphingolipids:- Sphingomylein, the second most abundant phospholipids component of plasma lipoproteins, is synthesized from palmitoyl CoA. Ninety percent of cellular sphingomylein resides in plasma membrane. Even LDL particles and atherosclerotic lesions are rich in sphingomylein compared to other lipoproteins and healthy arterial tissue.

2.2 Lipoprotein assembly

2.2.1 VLDL

Recent *in vitro* evidence shows a stepwise process that is dependent on adequate lipidation of a prenascent VLDL. This lipidation is dependent on a supply of fatty acids for TG synthesis and on an activity that transfers newly synthesized TG into a prenascent VLDL. This activity resides in the microsomal triglyceride transfer protein (MTP), which catalyses the initial lipidation of prenascent VLDL but is not required for the subsequent addition of most of the TG (Gordon et al., 1996). In the absence of adequate lipidation, some apo B-100 degradation occurs in the endoplasmic reticulum (Ingram and Shelness, 1996), whereas the reminder occurs in the cytoplasm through the ubiquitin-proteasome pathway, which is regulated by the molecular chaperone heat shock protein 70 (Fisher et al., 1997). Mutations in the MTP gene are responsible for the autosomal recessive disorder, abetalipoproteinemia, characterized by the inability of the liver and intestine to secrete apo B (Du et al., 1996)

2.2.2 Chylomicrons

These are formed in enterocytes from fatty acids and monoglycerides extracted from the intestinal lumen i.e. from the fatty acids of dietary TG and cholesterol absorbed from the small intestine by epithelial cells. Triglyceride synthesis is regulated by diacylglycerol transferase (Farese et al., 2000). After they are synthesized in the endoplasmic reticulum, TG are transferred by MTP

to the site where newly synthesized apoB-48 is available to form chylomicrons. There do exist some similarities between VLDL and chylomicron, both contain a TG core surrounded by a surface coat of phospholipid and protein and both requires fatty acids and MTP for formation. The major differences are; chylomicron being larger than VLDL scatters more light, so during postprandial lipemia, plasma is much cloudier though TG concentration has only doubled. They are the only lipoprotein that floats to the top of a tube of plasma that has been allowed to stand undisturbed for 12 hours. The buoyancy of chylomicron reflects their 98-99% fat content, of which 85% is dietary triglyceride.

The fatty acid composition of chylomicron is similar to that of the dietary fat load, while VLDL need not as liver can synthesize new fatty acids using its lipogenic enzymes so a fatty acid profile relatively insensitive to diet is formed. In individuals on high carbohydrate diet, increased lipogenesis leads to elevated plasma VLDL. There is an editing mechanism in the translation of the apo B-100 gene in the intestine (Chen et al., 1987). This leads to truncation of the expression, producing a protein that has only 48% of the normal apo B-100, which is found in the VLDL and LDL. Liver secretes only apo B-100 while there are unconfirmed reports of intestine secreting apo B-100. Apo B-48 is considered as a valid marker for lipoprotein of intestinal origin as neither of them transfer between lipoproteins. Other chylomicron components synthesized in the intestine include apo A-I, apo A-II and apo A-IV. These three transfer freely between chylomicrons, HDL and the aqueous compartment of plasma. The c apolipoproteins transfer between chylomicrons, HDL and VLDL. In normolipemic individuals, chylomicrons are present in plasma for 3-6 hrs after a fat containing meal has been ingested.

2.2.3 HDL

The origin of HDL is not very clear. The two probable sources of HDL – liver secretes a nascent HDL that is a bilayer disk. The other source is from the lipolysis of the triglyceride rich lipoproteins (VLDL, chylomicron) in the

plasma compartment (Patsch et al., 1978). The bilayer disk is rapidly converted to a mature lipoprotein by plasma enzymes and transfer proteins.

HDL is bimodal and appears in two distinct subclasses, HDL_2 and HDL_3 (sub classified as HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, HDL_{3c}). Another entity pre-β- HDL, which is composed mainly of apo A-I, cholesterol and phospholipids, share HDL's density but migrates with pre-β rather than α mobility. Pre--β- HDL is present in plasma only in very low concentrations but is believed to be an important mediator in the transfer of cholesterol from cells (Castro and Fielding 1988; Fielding and Fielding, 1995).

3. Remodeling of lipoproteins in plasma compartment

Transfer of lipids molecule between cells and lipoproteins occurs by spontaneous transfer or protein (enzymes) mediated transfer. The remodeling of lipoprotein in the plasma compartment by various plasma enzymes and transfer proteins is an important determinant of their metabolic fate and likely atherogenic or cardioprotective potential. Many proteins (enzymes; lipid transfer factors –CETP, PLTP; cell surface receptors and its receptor ligands – apoproteins; apoproteins as activators of some key enzymes) are required for the processing and eventual removal of lipoproteins from plasma. Lipids are also transferred between cells and lipoprotein surfaces by means of nonspecific spontaneous mechanism, which does not depend on energy, or transfer proteins. The activities that modify plasma lipoproteins, and the receptor mediated uptake of lipoproteins are both a function of the structure of the lipoprotein, especially the apoproteins component, the fluidity of the lipid surface and the covalent structure of the individual molecules cleaved by these enzymes.

3.1 Spontaneous lipid and protein transfer

Spontaneous transfer of cholesterol from cells in the vascular wall is the initial step in reverse cholesterol transport. Recent studies implicate the caveolae, which are clathrin free cell surface organelles active in transmembrane transport, as a site of preferential cholesterol efflux. Apoproteins are transferred between plasma lipoproteins by a spontaneous mechanism. Lipids that contain polar moiety (like cholesterol, phospholipids) are sparingly soluble in water, while cholesteryl esters and TGs are insoluble in water. Both cholesterol and phospholipids transfer between lipoproteins as aqueous monomers that are formed by rate limiting desorption of the lipid from the lipoprotein surface into the surrounding aqueous phase.

The time required for the transfer is inversely proportional to the aqueous solubility of the transferring species. Transfer of a phosphatidylcholine between HDL particles is on the order of 15hrs while the corresponding time for free cholesterol is about 5 min at 37°C. Solubility decreases with the increasing length and saturation of its acyl chain. Transfer time for a given lipid or protein decreases as a function of increasing diameter of the donor lipoprotein. The C apolipoproteins spontaneously transfer (rapidly) between HDL, VLDL, and chylomicrons. Transfer of apo A-I and apo A-II out of HDL is more complicated in kinetics and is slower.

3.2 Protein mediated transfer

3.2.1 Major plasma proteins of lipid transport:

Lipoprotein lipase (LPL) is the major TG hydrolyzing glycoprotein (44 residue) in human plasma. It is synthesized in tissue parenchymal cells and is secreted and transported to the capillary endothelium, where it is bound to heparin sulfate. High concentration of LPL is found in adipose tissue and striated muscle. The active form of LPL appears to be a dimer that has two bound molecules of apo C-II. Apo C-II is required for its maximum activity against TG lipoproteins. LPL hydrolyses the 1(3)-position of TGs and diglycerides. The resulting monoglyceride is sufficiently water soluble to be transferred to tissues, where cellular lipases complete the hydrolysis. In adipose tissue, LPL activity is induced by insulin.

LPL and hepatic lipases (HL) belong to a multi gene family whose members have identical active site sequence. HL a 477 residue glycoprotein, is synthesized in hepatocytes and transported to hepatic endothelial cells, where it is bound by means of heparin sulfate. Its major role appears to be the hydrolysis of TGs and phosphoglycerides of HDL and IDL, which usually lack the activator for LPL i.e. apo C-II.

Lecithin:cholesterol acyltransferase (LCAT), a 416 residue polypeptide synthesized in the liver, is the only known cholesterol esterifying activity in human plasma, where it is found in a complex with phospholipids, CETP, apo D and the apo

A-I in HDL. LCAT has two activities one is the formation of cholesteryl esters from phosphatidylcholine and cholesterol and the other is a phospholipase A_2 activity. Both the activities are activated by apoproteins. Apo A-I is the most important of the LCAT activators because of its high abundance and its high stimulatory activity.

Cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP) are factors found in the lipoprotein free fraction of human plasma that transport lipids between lipoproteins. Both transfer proteins exhibit broad specificity with respect to lipid type and fatty acid composition. Both cholesteryl esters (CE) and TGs are too hydrophobic to be transferred between lipoproteins by spontaneous mechanism. In addition, spontaneous phospholipids transfer is too slow to be important in the turnover of the TGRLs, which are relatively short-lived. CETP transfers CE, TGs, and phospholipids but phospholipids transfer appears to be less important than neutral lipid transfer. On the other hand PLTP does not transfer neutral lipids but does transfer a broad spectrum of phospholipids, sphingolipids and diglycerides. (Huuskonen et al., 1996; Rao et al., 1997). In addition PLTP transfers lipopolysaccharide and alpha-tocopherol.

Cholesterol ester transfer protein (CETP) is a 74-kDa glycoprotein with 476 amino acid residue. CETP operates by binding lipid monomers and moving between the surfaces of donors and acceptor lipoproteins. The relative amounts of CE and TG that are transported by CETP are a function of the composition of these two lipids in the surface monolayer of lipoproteins. There is rapid exchange of neutral lipids between the core and surface region and that most lipolytic and transfer activities occur in the surface. CETP transfers CE from HDL to apo B-100 containing lipoproteins and then these (B-100 lipoprotein) are removed along with the CE from plasma. CETP helps in the transfer of TG from VLDL to HDL and LDL resulting in TG rich HDL and LDL (seen in hypertriglyceridemic subjects). The transfer activity of CETP is slow (half time > 4 hrs). HDL and LDL turn over is about 5 days and 3 days respectively; as a consequence these two are virtually identical in cholesterol ester fatty acid composition. This is not observed for VLDL,

the metabolism of which is fast compared to the activity of CETP. Humans with CETP deficiency show HDL particles that are much larger than normal.

Plasma phospholipid transfer protein (PLTP) is an acidic, 476 amino acid protein belonging to a family of lipid transfer proteins that include CETP, Lipopolysaccharide-binding protein and bactericidal permeability increasing protein (Day et al., 1994; Lagrost et al., 1998). Recent studies suggest that PLTP plays an important role in lipoprotein turnover and reverse cholesterol transport and HDL is its primary target for its transfer and remodeling activity. PLTP is probably important in cholesterol transport as it facilitates the transfer of phosphatidylcholine. PLTP could be a key protein in the net transfer of phospholipid from membranes and other lipoproteins into HDL precursors. Overexpression of PLTP in mice is associated with a virtual disappearance of plasma HDL and apo A-I (Foger et al., 1997). Successive rounds of transfer and esterification by LCAT could lead to a growing particle that is a net acceptor of cholesterol and that matures into HDL_2 and HDL_3.

Serum albumin, a globular protein composed of nearly 600 amino acids crosslinked by 17 disulfide bridges, transports small hydrophobic molecules. Its major physiological role is transport of FFAs among tissue sites that require them for membrane biogenesis, energy production, synthesis of eicosanoids and thromboxanes. Albumins does not catalyse the transfer of FFAs in the same way that CETP transfer neutral lipids. Aqueous solubilities of neutral lipids are so low that virtually none are in the aqueous phase and a transport protein is required to move them between lipid surfaces. FFAs being water-soluble can move among lipid surfaces and proteins through the aqueous phase. FFAs have a plasma turn over of about 3 minutes. Another important role of albumin is to act as buffer that keeps the plasma FFAs and lysolipid concentrations low enough to minimize their cytolytic effects on tissues in contact with the plasma compartment.

3.2.2 Lipoprotein Catabolism

3.2.2.1 Processing of VLDL

The apoprotein Apo C-II, that is bound to the VLDL surface, activates LPL, which is located in the capillary endothelium. Lipolysis of TG present in VLDL liberates fatty acids and lyso-phospholipids which are transferred to albumin and this results in reduction in the size of the nonpolar core of the particle, simultaneously there is some hydrolysis of phosphoglycerolipids in the surface of the particle, during this lipid loss C apoproteins is transferred back to the HDL from where most of it was derived immediately after secretion. This leads to decrease in VLDL size and this forms IDL.

Intermediate density lipoprotein (IDL) contains one molecule of apo B-100 and variable amount of apo E. HL hydrolyses more of TGs and phosphoglycerolipids and the remaining complement of apo E is transferred to either HDL or nascent VLDL. This forms LDL.LDL is rich in sphingomyelin, which is not transferred very efficiently to other lipoproteins and for which there is no known hydrolytic activity in plasma.

3.2.2.2 Processing of chylomicron

Catabolism of chylomicron is similar to VLDL in some respects. Apo C-II stimulates LPL and releases the lipolytic products to albumin. Chylomicron hydrolysis is much faster than that of VLDL and its contribution of TGs to other lipoproteins is small. The product of chylomicron hydrolysis is the chylomicron remnant, which is removed from plasma by means of chylomicron remnant receptor in hepatocytes. Apo E in chylomicron remnant mediates its binding to remnant receptors in liver. Nascent chylomicron contains apoproteins A-I, A-II, A-IV, E and B-48. Immediately after its secretion, apo A-I and A-II are transferred to HDL spontaneously or is transferred during its initial lipolysis with LPL.

Concomitantly apo A-IV is transferred to the aqueous phase. It is not associated with any lipoprotein class. Part of apo E is lost from the chylomicron remnant to HDL. Apo B-48 remains with the chylomicron, as it is not transferred to the aqueous phase or other lipoproteins.

3.2.2.3 Processing of HDL

Since there is no non-exchangeable marker for HDL, the mechanistic picture of its processing is not very clear with respect to the initial steps. There are putative routes for the initial steps and one of these is hepatic secretion of a nascent, discoidal particle and the second is liberation of the surface components of TGRLs during lipolysis. In either case an HDL particle has pre-β mobility and is rich in phospholipid and apo A-I and it is processed to mature HDL through the activities of LCAT, HL and PLTP

As TGRLs undergo lipolysis, there surface components including cholesterol, phospholipids and apo A-I are transferred to the expanding HDL particle. LCAT converts the phosphatidylcholine and cholesterol to lysophosphatidylcholine and CE thereby increasing the volume of the core. PLTP also delivers phospholipids to HDL. In addition HDL particles obtain cholesterol and phospholipids from peripheral cells in contact with the plasma compartment thereby gradually HDL continues to increase in size ultimately forming HDL_2 and HDL_3.

3.2.2.4 Consequence of increased FFAs and CETP

Very low density lipoprotein (VLDL) generally contains a small amount of cholesterol ester, during its conversion to LDL and also it obtains some CE from HDL in exchange for TG. In hypertriglyceridemia, with most of the excess TG in VLDL, CETP mediates exchange of VLDL TG for CE of LDL and HDL. As a result LDL and HDL have very high neutral lipid core of TG. The consequence of the elevation of TG content in LDL and HDL_2 is that they become a substrate for HL, whose activity removes much of the TG core by hydrolysis resulting in small,

dense LDL and smaller HDL_3 respectively. HDL_3 remains TG rich through concomitant CETP mediated acquisition of additional TG from VLDL. Replacement of HDL CE with TG reduces the amount of cholesterol carried by HDL. This explains why two risk factors for CHD- elevated plasma TG and low plasma HDL-C are frequently associated and the virtual absence of the larger HDL_2 in moderate to severe hypertriglyceridemia.

4. Lipoprotein Catabolism

4.1 Receptor mediated uptake of lipoprotein

A major route for the removal of lipoproteins from plasma involves receptors located on the surfaces of cells in contact with blood. The receptors are recognized by a specific ligand (a protein or protein fragment) on the lipoprotein surface, and in many instances their expression is regulated by the status of the cell-that is, whether the cell needs more of the lipids that are in a given lipoprotein. Receptor mediated internalization of a lipoprotein transports many lipid molecules into a cell in a single step. In the case of LDL, each particle that enters a cell carries with it more than 1,000 molecules of cholesteryl ester and 500 molecules of free cholesterol. Particle endocytosis is an efficient route for the incorporation of lipids into cells.

Apo E and apo B-100 are the ligands that mediate the removal of plasma IDL and LDL, respectively, by means of hepatic cell surface receptors. Although VLDL contains both apo E and apo B-100, the protein conformations are not completely receptor competent. On lipolytic conversion of VLDL to IDL, the particle becomes smaller, the C apolipoproteins are transferred to HDL, it undergoes changes in its conformation and environment that make it receptor competent. Mutations in the LDL receptor are associated with type II hyperlipidemia. In type III hyperlipidemia, mutations in the ligand-binding region of apo E are associated with impaired uptake of IDL and elevations of plasma IDL (VLDL remnants). A chylomicron remnant with its complement of TG and cholesteryl ester is internalized by means of a receptor ligand in apo E; defects in apo E can lead to plasma elevations of chylomicron remnants. Expression of lipoprotein receptors differs among tissue sites and is determined by the requirements of the cells at each site for different lipids.

4.1.1 LDL receptor family

This family in addition to LDL-R also includes receptors for VLDL and the chylomicron.

4.1.1.1 LDL-Receptor

The work of Michael S. Brown and Joseph L. Goldstein (1986) has done much to elucidate the LDL receptor pathway and its role in lipid metabolism and atherogenesis as well as the molecular genetics of FH.

Each LDL contains one molecule of apo B-100 as its only protein. Most dividing cells have receptors that bind to LDL by means of a specific ligand in apo B-100. LDL binds to receptors located within coated pits on the cell surface. A coated pit closes to form a coated vesicle, which is converted to an endosome that dissociates into an LDL vesicle and an LDL receptor vesicle. The receptor vesicle recycles to the cell surface and the remaining endosome is converted into a lysosome. Within the lysosomes, acid esterases and proteases hydrolyse the lipids and proteins, respectively to free fatty acids (FFA), free cholesterol and amino acids. The free cholesterol, if in excess, is converted to cholesteryl esters. Within the endoplasmic reticulum, free cholesterol down regulates the production of LDL receptors and the rate limiting enzyme of cholesterol biosynthesis, HMG-CoA reductase. Down regulation occurs through the association of cholesterol with proteins that affect the activity of mRNA regulatory binding protein for both HMG-CoA reductase and the LDL receptor. When there are inadequate supplies of cellular cholesterol, additional receptors are synthesized. After modification in the golgi compartment, they move to the cell surface (Figure 1).

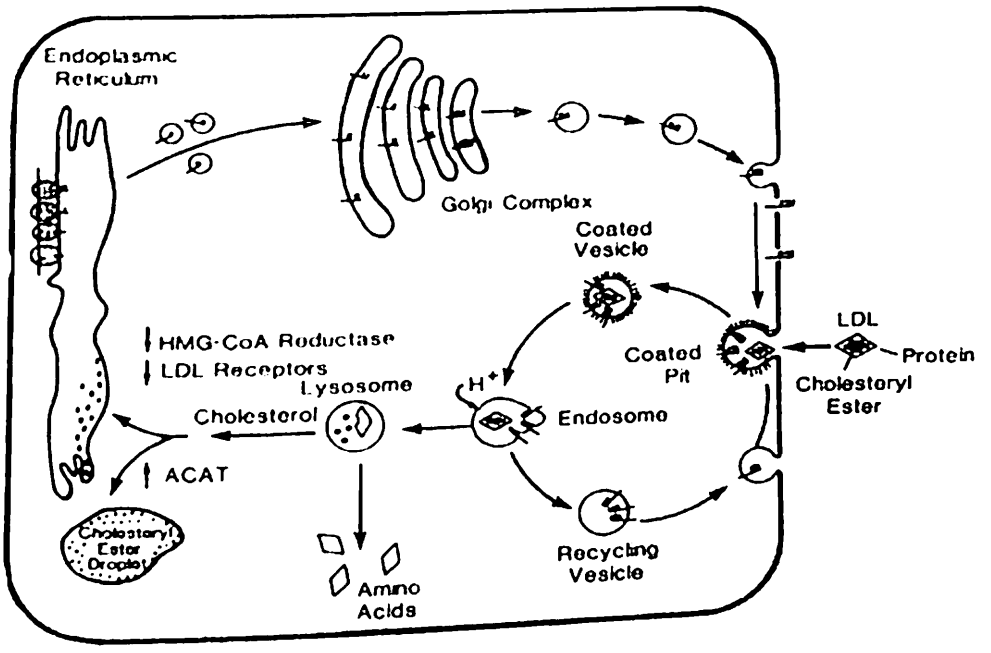

Figure 1: A schematic illustration of the route of the LDL receptor in mammalian cells. The receptor is synthesized in the endoplasmic reticulum from where it travels to the golgi complex, cell surface, coated pit, endosome, and then back to the surface. Vertical arrows indicate the direction of regulatory effects. ACAT, acyl-CoA: cholesterol acyltransferase; HMG CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase. (Adapted from Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science* 1986; **232:** 39).

In the absence of LDL receptor or its binding, there is no efficient means of removing LDL from plasma and the plasma LDL concentration rises. Also, since there is no cholesterol from LDL within the cell to down regulate cholesterol biosynthesis, the cell continues to secrete and produce cholesterol even though it is not needed.

There is a great deal of regional homology between the LDL receptor and proteins of other families. The extra cellular part of the LDL receptor is composed of an amino end that contains the ligand binding domain, which is made of seven repeats of about 40 amino acids that are arranged head to tail. Near the

carboxyl terminus of each repeat, there is a cluster of negatively charged fatty acids that bind to the positively charged amino acids of apo E and apo B. Site directed mutations of the domain have shown that the receptor determinants for the binding of LDL and β-VLDL are distinct. The second domain is highly homologous to epidermal growth factor (EGF) precursor. It is required for the acid dependent dissociation of the receptor and ligand within the endosome. It is not required for β-VLDL binding. The role of the third domain, which is small and rich in serine and threonine residues, that contains O-linked sugars, is not known. The fourth domain is the 22 residue, membrane-spanning domain that characterizes many integral membrane proteins. The carboxyl terminal domain is intracellular and targets the recycling receptor to the coated pits.

4.1.1.2 Chylomicron remnant receptor

In the plasma compartment, chylomicron TG is hydrolysed by LPL. The hydrolysis products FFAs are transferred to albumin and subsequently to adipose tissue or skeletal muscle, where they are stored or used. This process leaves a remnant particle that is enriched in cholesterol, cholesteryl esters, phospholipids, apo B-48 and apo E.

Reduction in the size of the particles during hydrolysis permits them to pass through the endothelial fenestrae and into the space of Disse, where the remnants are removed directly by receptors (de Faria et al., 1996; Choi and Cooper, 1993; Cooper, 1997). Some remnants are transiently bound to proteoglycans by means of apo E; others can be sequestered by cell surface HL by means of apo B-48. In each instance they are transferred from their sites of sequestration to the LDL receptor. Remnant uptake stimulated by HL is independent of lipolysis (de Faria et al., 1996; Krapp et al., 1996). Alternatively the sequestered remnant can acquire addition as hepatically derived apo E, which triggers particle recognition by the LDL receptor related protein (LRP), which is also the receptor for alpha$_2$ macroglobulin (Strickland et al., 1990). Apo E is required for rapid hepatic removal of remnants by

means of LRP. Binding of lipoproteins by LRP is inhibited by apo C-I, which presumably competes with apo E.

4.1.1.3 VLDL –Receptor

The VLDL receptor binds lipoproteins that contains apo E and consists of five functional domains that resemble those of the LDL receptor (Jingami and Yamamoto, 1995; Krieger and Hertz, 1994) The VLDL and the LDL receptors are almost the same in gene structure and organization. The VLDL and the LDL receptors are almost the same in gene structure and organization. Despite the presence of sterol regulatory element-1 like sequence in the VLDL receptor gene, the transcription of the gene is not down regulated by sterols. High levels of expression of its mRNA and protein in non hepatic tissue like endothelial tissue suggests that the receptor plays a role in the transport of VLDL or another constituent from the plasma compartment to adjacent tissues (Wyne et al., 1996). High expression is also seen in tissues that actively metabolise fatty acids as their source of energy. Studies have shown that it also uptake Lp(a) (Argraves et al., 1997). The VLDL receptor is also expressed in macrophages in human atherosclerotic lesions and endocytosis of Lp(a) by way of this receptor could lead to cellular accumulation of lipids within macrophages and may represent a molecular basis for the atherogenic effects of Lp(a).

4.1.2 Other receptors

4.1.2.1 HDL Receptor

Unlike in lipoproteins that contain apo B, all components of HDL are exchangeable through protein mediated or spontaneous transfer mechanisms. Therefore the lipid and proteins of HDL can be catabolised independently of one another and of the HDL particle. Studies in rats have shown kidney to be a site of selective uptake of apo A-I, whereas little or no lipid is catabolised in the kidney

(Glass et al., 1983; Ponsin et al., 1986; Pownall et al., 1991). Other studies have shown that HDL cholesteryl ester is selectively removed by the adrenal glands and kidney (Khoo et al., 1995), and a receptor for this process has been identified: the class B type I scavenger receptor (SR-BI) which binds HDL (Acton et al., 1996). SR-BI mediate selective uptake of HDL cholesteryl esters by cultured cells, and its expression is coordinately regulated by with steriodogenesis in several sites, including the adrenal glands, ovary and testis. SR-BI is the molecularly well-defined cell surface HDL receptor described. It mediates the transfer of lipid from HDL to cells by selective lipid uptake, a mechanism distinct from receptor mediated endocytosis by means of clathrin coated pits and vesicles. Free or lipid-bound apo A-I, apo A-II, and apo C-III associated, specifically with SR-BI expressing cells, with high affinity and competed for the binding of HDL (Xu et al., 1997) SR BI is fatty acylated, a property shared with other proteins that concentrate in caveolae. In Chinese hamster ovary cells stably transfected with murine SR-BI, overexpression of SR-BI promoted HDL mediated cellular cholesterol efflux (Ji et al., 1997). SR-BI colocalises with caveolae and facilitates both lipid influx and efflux.

4.1.2.2 Scavenger Receptors

Oxidatively modified LDL (OxLDL) is very different from unmodified LDL in composition, structure and metabolism. Its content is higher in fatty acids and lysophosphatidylcholine as a result of the action of an LDL associated phospholipase A_2.

Oxidatively modified LDL is characterized by reactive aldehydes, reduced cholesterol content with a reduction in the number of reactive amino groups on apo B-100, an increase in oxysterols, and fragmentation of apo B-100. The difference in the structure of OxLDL may contribute to its cytotoxicity towards fibroblasts, SMCs and endothelial cells, a cytotoxicity that may be relevant to its

effects in the arterial wall. OxLDL or its fragments have been found in animal and human atheromatous lesions (Steinberg, 1997b)

Monocytes and macrophages do not express LDL receptors. They have a receptor acetyl LDL receptor which binds and mediates removal of modified lipoproteins. This receptor is now known as macrophage scavenger receptor A because of its broad ligand specificity (Kieger and Herz, 1994). Two scavenger receptor isoforms, SRA-I and SRA-II, are generated by the alternative splicing of mRNA encoded by one gene (Emi et al., 1993). SRA –I is a homotrimeric, membrane bound protein composed of monomers of 451-454 amino acid residues. SRA-II mediates endocytosis of chemically modified LDL with high affinity and specificity similar to SRA-I. Scavenger receptor expression suppressed the diet induced rise in lipoproteins containing apo B, suggesting that hepatic overexpression of scavenger receptors may play a cardioprotective role in diet induced hyperlipidemia.

5. Regulation of Lipid synthesis

Receptor mediated uptake of lipoproteins by cells can increase cellular cholesterol content according to the number of lipoprotein endocytosed and the number of cholesterol molecule per lipoprotein particle. Transfer of cholesterol from cells to lipoproteins by spontaneous transfer will reduce the cellular cholesterol content. Through the release of sterol sensitive transcription factors, these changes in cellular free cholesterol can indirectly alter several pathways that are connected to lipid and lipoprotein metabolism. The cellular cholesterol and fatty acid homoeostasis are regulated by three transcription factors - sterol regulatory element binding protein (SREBP) isoforms, designated SREBP-1a, SREBP-1c and SREBP-2 (Brown and Goldstein, 1997; Tontonoz et al., 1993). These differ in the transcriptional activity and the transcription rate of the LDL receptor gene.

SREBP-1a and -1c are derived from a single gene located on human chromosome 17p11.2 through the use of alternative transcription start sites that produce alternative forms of exon-1 (Brown and Goldstein, 1997). SREBP-2 is derived from a second gene located on human chromosome 22q13. When cells require lipids, SREBPs transcriptionally activate a cascade of enzymes required for endogenous cholesterol, fatty acid, triacylglycerol and phospholipid synthesis.

SREBPs belong to the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors, but unlike other bHLH-Zip family members they are synthesized as approx. 1150- amino-acid precursors bound to the endoplasmic reticulum (ER). SREBP precursors are organized into the following domains:

(i) N-terminal domain containing the bHLH-Zip region,

(ii) two hydrophobic transmembrane spanning segments interrupted by a short segment that projects into the lumen of the ER, and

(iii) C-terminal segment regulatory domain. To be active, the N-terminal segment of SREBPs, designated the nuclear form (nSREBP), must be released from the membrane so that it can translocate to the nucleus of the cell.

nSREBPs are generated by a two-step cleavage process that requires at least three proteins in addition to SREBPs (Goldstein et al., 2002). The first protein, SREBP cleavage-activating protein (SCAP), serves as both an escort protein and a sterol-sensor. SCAP is anchored to the ER by eight membrane-spanning domains, five of which are important for sterol sensing (Brown and Goldstein, 1997; Goldstein et al., 2002). The C-terminal domain of SCAP contains a hydrophilic region that resembles aWD-40repeat. This region mediates the interaction with the C-terminal domain of SREBPs (Brown and Goldstein, 1997; Goldstein et al., 2002). When the cellular demand for lipids is increased, SCAP senses this need and escorts SREBPs from the ER to the Golgi. In the Golgi, the Site-1 protease (S1P), a membrane-bound subtilisin-like serine protease, cleaves SREBPs in the hydrophilic luminal loop (Goldstein et al., 2002). The second proteolytic cleavage occurs via the Site-2 protease (S2P), a zinc metalloproteinase that cleaves SREBPs in the first transmembrane segment, which releases the N-terminal segment of SREBPs from the membrane.

In most cultured cells, the predominant SREBP isoform is SREBP-1a (Shimomura et al., 1997). In most animal tissues, SREBP-1c is approx. 10-fold more abundant than SREBP-1a and approx. twice as abundant as SREBP-2 (Shimomura et al., 1997). The transcriptional activation properties of each SREBP isoform have been delineated *in vitro* and *in vivo* (Pai et al., 1998; Horton et al., 2002). All SREBP isoforms can activate an entire cascade of enzymes in the cholesterol and fatty acid biosynthetic pathways if expressed at super physiological levels. Some of the genes whose transcription is affected include HMG-CoA reductase,(Vallett et al., 1996) HMG-CoA synthase, LDL receptor, farnesyl diphosphate synthase (Spear et al., 1994; Ericsson et al., 1996), acetyl CoA carboxylase (Lopez et al., 1996), fatty acid synthase (Bennett et al.,1995; Magana and Osborne, 1996) and glycerol 3 phosphate

acyltransferase (Ericsson et al., 1997) which is the committing step in glycerol lipid synthesis.

Studies carried out in cultured cells, and in genetically manipulated mice, have demonstrated that SREBP-1c preferentially control genes involved in lipogenesis (Pai et al., 1998; Horton et al., 2002). SREBP-2 preferentially activates genes responsible for cholesterol synthesis and uptake. The physiological consequences of SREBP-2-mediated activation of this pathway were demonstrated in nSREBP-2 transgenic mice, which have a 28-fold increase in hepatic cholesterol synthesis (Horton et al., 2002). Finally, SREBP-1c and SREBP-2 activate three genes required to generate the NADPH molecules that are used for multiple reactions in both lipid biosynthetic pathways (Horton et al., 2002).

The expression of each SREBP is determined by post-transcriptional regulatory mechanisms that alter the cleavage of the precursor, and by mechanisms that regulate gene transcription. Post-transcriptional regulation involves sterol-mediated suppression of SREBP cleavage, which occurs by inhibiting the movement of the SCAP-SREBP complex from the ER to the Golgi. This form of regulation blocks all SREBP isoform activation in cells cultured in the presence of sterols and in the livers of rodents fed cholesterol enriched diets (Brown and Goldstein, 1997; Horton et al., 2002)

SREBP-1a is a potent transcriptional activator of all SREBP-responsive genes owing to the long transactivation domain encoded by exon 1a; however, SREBP-1a is constitutively expressed at very low levels in most animal tissues. This suggests that SREBP-1a may be responsible for maintaining basal levels of cholesterol and fatty acid synthesis *in vivo*. SREBP-1c has a shorter transactivation domain that reduces its overall activation strength and imparts its selectivity towards activating genes involved in lipogenesis (Horton et al., 2002). SREBP-2, like SREBP-1a, has a long transactivation domain, but its activity is more restricted to regulating genes involved in cholesterol homoeostasis (Horton et al., 2002).

6. Atherosclerosis

A principal role for LDL and its derivatives is considered central to the initiation and progression of atherosclerosis. The role for cholesterol in CHD is supported by observational epidemiological studies, findings of CHD in familial forms of hypercholesterolemia, laboratory and animal studies and clinical trials of cholesterol lowering therapy. Most people with elevated TC have elevated LDL-C and LDL is considered as the principle atherogenic lipoprotein. Strong evidence supports the initiation and promotion of atherosclerosis by LDL at every stage (Grundy, 1997).

Other major lipoprotein classes - HDL and TGRLs and the remnants and some selected factors also participate in formation of atherothrombosis.

6.1.1 Evolution of atherosclerotic lesion

The hallmark of atherosclerosis is the accumulation of cholesterol in the artery wall and oxidation or other modification of LDL particles appears to be important events in atherogenesis. Endothelial function is critical to maintaining blood flow and vascular integrity and healthy endothelium tends to favor vasodilation, antithrombosis, fibrinolysis and monocyte disadhesion (Buja, 1995; Vogel, 1997). In certain parts of the arterial tree, chronic minimal endothelial injury can result in dysfunctional endothelium characterized by increased uptake of LDL and monocyte recruitment into the vessel wall, which are both pivotal initiating events in atherosclerosis (Fernandez-ortiz and Fuster, 1996). Systemic factors that can induce such injury include hypercholesterolemia, especially minimal modified LDL and active and passive cigarette smoking, which may lead to endothelial dysfunction through an increased production of superoxide radicals by the endothelium, resulting in deactivation of EDRF/NO as well as enhancement of lipoprotein oxidation (Graham et al.,1993).

6.1.2 Monocyte migration

The initial lesion of atherosclerosis develops when leukocytes, specifically monocytes, cross the endothelial barrier to accumulate in the intima. The first readily discernible morphologic change is monocyte adhesion to a usually intact but activated endothelial surface (Ross, 1993; Faggiotto et al., 1984). After migration into the intima, monocytes are converted into macrophages, which normally are not a component of the artery wall. Macrophages imbibe large amount of lipid to become foam cells, so called because of their foamy cytoplasm (Davies, 1998; Faruqi and DiCorleto, 1993)

Monocytes are recruited to an area of dysfunctional vascular endothelium through complex changes, such as changes in vascular permeability and alteration in expression of endothelial cell adhesion receptors, coordinated by a variety of inflammatory mediators (Jones et al., 1995). The presence of minimally modified LDL is believed to be important to monocyte recruitment.

Activated endothelium expresses several proteins on its surface that increases its stickiness to blood borne cells. Among these surface adhesion molecules are selectin, integrins, and members of the immunoglobulins superfamily (such as ICAMs and VCAMs) which are important in immunology and in atherosclerosis (Schmid-Schonbein et al., 1997)

Oxidatively modified LDL (OxLDL) has both leukocyte chemoattractant and adhesion promoting roles (Lehr and Messmer, 1995)

6.1.3 Lipid insudation:

The source of cholesterol that accumulates in the artery wall is plasma lipoproteins, in particular LDL. Lipoproteins flux into and out of the artery wall as a normal function, LDL providing cholesterol and nutrients to peripheral cells. For LDL, VLDL, and IDL, flux into the arterial wall is related directly to their plasma concentrations (Nordestgaard et al.,1992). It appears, however, that selective retention of lipoproteins rather than rate of transport across the endothelium may

determine their concentration and perhaps susceptibility to modification in the artery wall (Beisiegel and St. Clair, 1996). LDL enters the atherosclerotic arteries faster than normal arteries and its accumulation in the former is greater (Nordestagaard, 1996). Proteoglycans are believed to play a major role in retaining the lipoproteins in the arterial wall (Beisiegel and St.Clair, 1996). Ultimately lipoproteins are taken up by the macrophages and the lipid stuffed macrophages contribute to formation of foam cells. LDL modification is required for the uptake by Macrophages (Goldstein et al., 1979) Acetylation and malondialdehyde conjugation can also enable *in vitro* receptor mediated macrophage uptake of LDL. Other process that can contribute by receptor or non-receptor mechanisms to macrophage uptake of LDL include phagocytosis and LDL self-aggregation, complex formation with proteoglycans, immune complex formation and degradation by hydrolytic enzymes (Steinberg, 1997a; Beisiegel and St. Clair, 1996).

The oxidative modification of trapped LDL is believed to occur in two stages, the first taking place before (and promoting) monocyte recruitment and second occurring after monocyte / macrophage contribute their great oxidative capacity (Berliner et al., 1995). Oxidised LDL in fact comprises a spectrum of oxidatively modified particles that can differ not only structurally but also functionally (Steinberg, 1997a)

6.1.3.1 Minimally Modified LDL

After LDL accumulates in the subendothelial space, its lipids can be mildly oxidized through the action of resident vascular cells, with little change in its apo B (Berliner et al., 1990, 1995; Steinberg and Witztum, 1990; Diaz et al., 1997). This minimally modified LDL, which unlike unmodified LDL is proinflammatory, induces local vascular cells to produce factors (VCAM, monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor) that stimulate recruitment of monocytes and differentiation of monocytes into macrophages in the intima (Diaz et al.,1997; Parhami et al., 1993; O'Brien et al., 1993; Nelken et al.,

1991; Rosenfeld et al.,1992). OxLDL may also exert direct effects on the recruitment of monocytes from the circulation (Steinberg,1997a; Quinn et al., 1988; Frostegard et al., 1991). LDL from subjects with risk factors such as CHD, diabetes mellitus or smoking has increased susceptibility of lipid peroxidation as does small dense LDL (Nigon et al., 1991) and LDL obtained from subjects in the post prandial state. Some circulating LDL particles are already minimally modified and could be degraded in the intima in preference to unmodified LDL, because the artery wall has a variety of mechanisms to prevent oxidation like suppression of lipoprotein oxidation by Nitric oxide, a loss of balance between pro-oxidant and anti-oxidant forces is likely important in the formation of atherosclerosis.

6.1.3.2 Fully Oxidised LDL

The second stage of LDL oxidation begins when the monocyte enters the intima and is converted into macrophages (Berliner et al., 1995). The monocyte/macrophage stimulate further peroxidation of LDL including modification of the protein portion (apo B-100) so that it is more negatively charged. The protein modification leads to a loss of recognition by the LDL receptor and a shift to recognition by the macrophage scavenger receptors, the oxLDL receptors, or both (Berliner et al., 1995; Brown and Goldstein, 1990; Sparrow et al., 1989) so that LDL is internalized by the macrophages. This uptake, unlike the uptake by LDL receptors is not subject to down regulation so that the macrophage can become heavily laden with lipids. Oxidised LDL has been demonstrated within macrophages in both humans and rabbit atherosclerotic lesions (Yla-Herttuala et al.,1989)

Oxidised LDL in addition to formation of foam cells has many other pro-atherogenic properties (Steinberg, 1997a). The contribution of biologically active molecules generated during the process of OxLDL, in addition to being a chemoattractant for circulating monocytes, it also inhibit the motility of macrophages, which might exert a trapping effect (Steinberg, 1997a). It is also a chemoattractant for T cells and is cytotoxic to a number of cell types, thus promoting

the release of lipids and lysosomal enzymes into the intimal extracellular space. OxLDL can rapidly impair endothelium dependent dilation, probably through multiple mechanisms, including direct inactivation of nitric oxide (Selwyn et al., 1997). In addition it may promote formation of thrombi (Hirose et al., 1996). A further property of oxLDL not shared by the native LDL is immunogenicity (Steinberg, 1997a). Autoantibodies to oxLDL are higher in proteins with carotid atherosclerosis than in age matched healthy subjects and the plasma concentration of immunoreactive oxLDL is higher in patients who have suffered acute MI compared with that in control subjects (Holvoet et al., 1995)

6.2 HDL

These particles are considered as anti-atherogenic. A strong inverse relationship between HDL-C concentration and risk for CHD is well established by observational epidemiology. It is likely that several mechanisms contribute to a defense by HDL particles against atherosclerosis and that there is variation depending on the stage of the disease (Anderson, 1997). Much of the effect appears to occur early in the process of the formation of lesions.

Like other lipoproteins classes, HDL particles form a heterogeneous population and cardioprotectivity could vary by subpopulation. Gradient gel electrophoresis has been used to define five subclasses: HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, HDL_{3c} in decreasing order of particle size. The HDL_2:HDL_3 ratio appears to be a reliable indicator of the efficiency of postprandial lipolysis, which evidence suggests, is related to CHD risk. Regarding protein defined subpopulation, most studies have shown CHD to be associated with decreased concentration of Lp A-I (i.e HDL particles containing apo A-I but not apo A-II, where as both of the major proteins of HDL are present in Lp A-I:A-II)

Another view holds that the inverse relation between HDL and risk for CHD may in fact reflect a positive relation between the TGRLs and CHD. Studies of subjects in

the postprandial state suggest that metabolism of the TGRLs is a major determinant of HDL-C concentration.

6.2.1 Reverse Cholesterol Transport

The cardioprotective role of HDL has been attributed to its role in reverse cholesterol transport, in which cholesterol is mopped up from the periphery back to the liver where it is metabolized. Cholesterol cannot be degraded in peripheral tissue, including the artery wall. *In vitro* HDL particles promote the efflux of cholesterol from cholesterol-laden cells and reduce the cholesterol content in foam cells. The preferred acceptor of cell cholesterol is the minor sub-population of small, pre-β-migrating particles, which contain apo A-I.

Another mechanism leading to cholesterol efflux is suggested by the binding of HDL to specific surface sites referred to as HDL receptors, the binding leads to translocation of intracellular cholesterol to the plasma membrane. From the peripheral cells, the transferred free cholesterol is transported through the lymph systems to the thoracic duct and then to the systemic circulation. The cholesterol after esterification by LCAT is transferred from HDL to LDL and finally to VLDL. The VLDL is taken up by the liver by means of LDL receptors.

6.3 Small, dense LDL

Small, dense LDL and its immediate precursor, IDL have been associated in cross-sectional studies with increased risk for CHD by clinical and angiographic indices (Austin et al., 1988; Lamarche et al., 1997). The association of LDL size with risk could simply reflect metabolic process of importance in atherogenesis. On the other hand, laboratory findings indicating increased susceptibility to oxidation or decreased LDL receptor binding have fueled speculation that small particles have an independent causal role in the development of atherosclerosis.

The formation of small, dense LDL is believed to be closely related to metabolism of TGRLs (Campos et al.,1992). The atherogenicity of dense LDL may

be related to the broader metabolic defect of impaired TG tolerance (Ebenbichler et al., 1995) or insulin resistance syndrome (Austin and Edwards, 1996). A preponderance of small LDL particles, called LDL phenotype B, is associated with a more atherogenic lipoprotein profile than is a preponderance of larger LDL (type A phenotype), including higher plasma concentrations of TG, VLDL, IDL, and apo B and lower concentrations of HDL-C and apo A-I. Small dense LDL or LDL phenotype B has been statistically related not only to CHD but also to conditions associated with CHD (like obesity, insulin resistance, diabetes mellitus). Hyperapobetalipoproteinemia (hyper apo b), a familial lipoprotein disorder strongly associated with CHD, is characterized by an increase in small, dense LDL.

Small lipoproteins may bind more readily to proteoglycans and enter the artery wall more easily. Dense LDL particles from normolipidemic subjects have reduced binding affinity for the LDL receptors compared to the more buoyant LDL particles (Nigon et al., 1991). *In vivo* smaller LDL particles are cleared from the circulation more slowly than larger LDL particles in both normal subjects and those with hyper apo B. With a longer residence in plasma, there may be prolonged exposure to free radical oxidation and easier particle uptake by means of mechanism not mediated by the LDL receptor (Rajman et al., 1994). Furthermore, dense LDL are more susceptible to oxidation *in vitro* than more buoyant LDL particles. (Packard,1994)

6.4 Lipoprotein (a)

Lipoprotein (a) [Lp(a)] is composed of an LDL particle that has a second apoprotein in addition to apo B –100 . A strong positive association exists between CHD risk and the plasma concentration of Lp(a) (Maher and Brown, 1995), a lipoprotein identical to LDL except for the addition of apo(a). The Physiologic function of Lp(a) is not known.

Given the extensive sequence homology between apo(a) and plasminogen, it has been suggested that much of the atherogenic potential of Lp(a) derives from interference in normal pathways of thrombolysis, to predispose patients to acute thrombotic complications. Prothrombotic effects of Lp(a) include interference with the binding of tissue plasminogen activator to fibrin; and stimulation of the synthesis of PAI-1

Other hypothesis include a role of Lp(a) in cholesterol delivery to the injured vessel wall and stimulation of vascular cell proliferation (Hajjar and Nachman, 1996). Lp(a) - binds lipoprotein containing apo B, avidly binds to arterial proteoglycans and fibronectin, accumulates in atherosclerotic lesions, stimulates SMC proliferation and promotes cholesterol accumulation in cells. With oxidation or modification by malondialdehyde, Lp(a) becomes a ligand, both *in vitro* and *in vivo*, for the scavenger receptor and macrophage foam cells may express a distinct Lp(a) clearance receptor (Keesler et al., 1994). Vascular lesions, induced by a lipid-rich diet, were increased 30 times in area in transgenic mice expressing human apo(a) compared with control mice. Thus Lp(a) might interfere with the normal degradation of cholesterol by way of the LDL receptor or itself be targeted to early atherosclerotic lesions, possibly through the macrophage scavenger receptor. (Hajjar and Nachman,1996). Lp(a) appears to enter the intima at about the same rate as LDL but may be retained there to a greater extent, particularly at sites of injury.

Possible risk dependence on LDL: Analysis of angiographic clinical trial data have suggested that a concomitantly high LDL-C concentration is required for Lp(a) to exert its most adverse effects. In clinical trials, FATS study, Lp(a) concentration was a dominant predictor of baseline angiographic CHD severity, its progression, and the clinical event rate in men with elevated LDL-C but lost its predictive value in patients in whom LDL-C was substantially reduced (Maher et al., 1995). In FHRS, lowering both Lp(a) and LDL-C achieved no greater angiographic benefits than lowering LDL-C alone (Thompson et al., 1995).

Maher and Brown (1995) have suggested several mechanisms for the findings that a concomitantly high LDL-C concentration is required for Lp(a) to exert its most adverse effects. Lp(a) and LDL forms aggregates which would increase their residence time in the intimal walls of the vessels. The propensity of both particles to bind arterial wall proteoglycans and entrapment in the artery intima of LDL by proteoglycans-bound Lp(a) particles with free apo(a) chain. The apo B components of Lp(a) binds proteoglycans more avidly than apo(a) component.

7. Current available therapies for hyperlipidemia:

Drug therapy in conjunction to the dietary management and modification in life style habits form the main basis of clinical management of hyperlipidemia. Several drugs have been used for lowering plasma lipids. These fall in the following categories (Mahley and Bersot, 2001b).

1. Drugs which interfere with intestinal absorption of bile salts / cholesterol:

Resins like cholestyramine, and cholestipol belongs to this class. Because of their large size resins are not absorbed from the intestine. They bind (sequester) bile salts and cholesterol (bile salts facilitate cholesterol absorption) in intestine and the bound bile acids are excreted in the stool, there by interrupting enterohepatic circulation of the bile acids. This in turn leads to increased hepatic metabolism of cholesterol to bile acids, which results in reduction of intra-cellular cholesterol. This results in up-regulation of HMGCoA reductase and LDL-R. The increase in hepatic LDL-R increases the LDL clearance and lowers the LDL-C levels.

2. Drugs which enhance lipoprotein lipase (LPL) activity:

Fibric acid derivatives come under this class of compounds. These compounds promote uptake/ degradation of TGs in VLDL and IDL through peroxisome proliferator-activated receptors (PPARα)-mediated stimulation of fatty acid oxidation, increased LPL synthesis and reduced expression of apoC-III. Secondary to increased uptake of VLDL and IDL, the LDL-C may slightly decrease.

3. Drugs which inhibit VLDL production and lipolysis:

One of the vitamin B complex, nicotinic acid, belongs to this class. In adipose tissue it inhibits the lipolysis of TG by hormone-sensitive lipase, which reduces the transport of FFAs to the liver. Hence the TG synthesis in liver reduces, resulting in reduced production of VLDL by liver, as a result the IDL and LDL is also reduced. This drug also increases activity of lipoprotein lipase.

4. HMG CoA reductase inhibitors (statins):

Statins are competitive inhibitors of HMG-CoA reductase enzyme which results in inhibition of cholesterol biosynthesis. This causes activation of the LDL R gene.

5. Miscellaneous:

Probucol has antioxidant activity so it prevents oxidation of LDL.

8. Rhabdomyolysis:

Rhabdomyolysis is a clinical and biochemical syndrome resulting from skeletal muscle injury with release of muscle contents, specifically myoglobin (oxygen-binding protein pigment) into the plasma. Myoglobin is filtered out of the bloodstream by the kidneys. It may occlude the structures of the kidney, causing damage. Myoglobin breaks down into potentially toxic compounds and frequently result in kidney damage such as acute tubular necrosis or kidney failure.

Statins inhibit the enzyme HMG-CoA reductase, many steps before the final formation of Cholesterol in the mevalonate pathway. This same pathway is used to synthesize the essential biochemical Coenzyme Q10 (CoQ10, Ubiquinone). Thus a major side effect predicted for statins is to reduce coenzyme Q10, with consequent danger to heart and skeletal muscle. (De Pinieux et al.,1996)

9. Unmet Need:

An elevated low-density lipoprotein cholesterol (LDL-C) level, in addition to other risk factors, such as advancing age, high blood pressure and diabetes, is a known risk factor for coronary artery disease, based on epidemiological studies (The Lipid Research Clinics Coronary Primary Prevention Trial results I: Reduction in incidence of coronary heart disease, 1984). The results of major primary and secondary prevention trials have shown that reducing LDL-C can decrease the overall risk for CVD morbidity and mortality by up to 40% (Mahley and Bersot, 2001c). Based on data from these clinical and epidemiologic studies, the National

Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III guidelines state that the principal goal of risk reduction therapy is to lower LDL-C levels.

Presently statins are undisputedly the gold standard in the treatment of dyslipidemia as more efficacious monotherapies are not available. However, an important unmet need lies in the effective control of patients with complicated lipid profiles. With statins unable to adequately control cholesterol in all patients, there remains a strong need for novel therapies. Recent research suggests that around six out of ten patients with CHD receiving statin therapy were not able to achieve the LDL-C target levels as recommended by the NCEP ATP III treatment goals (Pearson et al., 2000). The inter-patient variability in efficacy is very high with statins. Majority of the LDL-C lowering action occurs at the lowest statin doses, i.e. there is no dose proportionate increase in efficacy with increase in dose (Jones et al., 1998). Many of the patients are unable to tolerate statins primarily due to musculoskeletal symptoms (rhabdomyolysis). The problem was severe enough to warrant the recent withdrawal of cerivastatin from the market.

Most of the presently available drugs for the treatment of hyperlipidemia show severe gastrointestinal disturbances. Fibrates cause dermatitis, myositis, blurred vision and rhabdomyolysis. Statins though a potent drug, cause myopathy, rhabdomyolysis and hepatotoxicity (elevation in hepatic transaminases). Nicotinic acid cause cutaneous vasodilation, dyspepsia, gout, hepatotoxcity. Resins cause severe gastric intolerance. (Mahley and Bersot, 2001b)

Aims and Objectives

3. Aims and objectives

The aim of the study was pre-clinical development of newer anti-hyperlipidemic agents.

The objectives of the study were:

1. Screening of molecules in an *in vivo* model of hyperlipidemia to select molecules with appreciable efficacy.
2. Evaluate the ability of the selected molecules to increase expression of LDL receptors in a cell-based assay.
3. Choose the best molecule with desirable *in vitro* and *in vivo* activity and show its potential to be developed as an anti-hyperlipidemic agent.

Materials and methods

4. Materials and methods

4.1 Materials NCEs and experimental animals

4.2 Screening for anti-hyperlipidemic activity in hamsters

4.3 Anti-hyperlipidemic activity in hamsters - dose response

4.4 Determination of ED_{50} of TRC-7033 for reduction in LDL-C

4.5 Hypolipidemic activity of TRC-7033 in hamsters

4.6 Determination of hepatic lipids

4.7 Quantification of LDL-receptor expression

- 4.7.1 Culture of C7 hybridoma
- 4.7.2 Purification of immunoglobulin G (monoclonal antibody)
- 4.7.3 PAGE and Western blot analysis of the purified antibody
- 4.7.4 Culture of HepG2 cell line
- 4.7.5 LDL receptor assay on HepG2 cell line

4.8 Cytotoxicity of test the compounds by MTT assay

4.9 Anti-oxidant activity of the test compounds

4.10 Safety pharmacology studies on TRC-7033

- 4.10.1 Effect on HR, BP, ECG and respiratory system
- 4.10.2 Effect on spontaneous motor activity (SMA)
- 4.10.3 Effect on forced motor activity (FMA)
- 4.10.4 Effect of TRC-7033 on smooth muscles
  - 4.10.4.1 Studies on isolated rabbit aorta
  - 4.10.4.2 Studies on isolated rat uterus
  - 4.10.4.3 Studies on isolated guinea pig ileum
  - 4.10.4.4 Studies on isolated guinea pig tracheal rings

4.11 Probe toxicity studies

4.1 Materials:-

NEW CHEMICAL ENTITIES:

All the test compounds studied were synthesized by the Medicinal Chemistry Department (MCD) of Torrent Research Centre (see *Appendix - I* for details). This involved designing various classes of potential anti-hyperlipidemic agents using the Computer Aided Drug Design (CADD) facility of TRC. The HipHop module of the *Catalyst* software from MSI was used for this purpose. Subsequently the novelty of the molecules designed was confirmed by searching the CAS and STN databases. Then the synthetic feasibility of the designed molecules was explored and molecules were finalised for synthesis. The test compounds synthesised were numbered from TRC-7002 to TRC-7051.

EXPERIMENTAL ANIMALS:

The animals used for this work were obtained from the GLP-accredited Animal House Facility of Torrent Research Centre. The animal house environmental conditions were as follows: Temperature maintained at 22 ± 3°C, and relative humidity of 30-70%. HEPA filters (0.2 micron; 99.9% efficiency) were used in all the rooms. The air circulation was maintained at 20-24 (100% fresh) air changes per hour in all the animal rooms. The animal rooms were maintained at positive pressure as compared to the corridors. Lighting schedule was 12 hours artificial light: in each 24-hour period. The animals were given standard pelleted diet (Amrut feed, Sangli) and purified water (Aquaguard) *ad libitum*.

1. Hamster:

Breeder stocks of Syrian hamsters *(Mesocricetus auratus)* were procured from Central Drug Research Institute (CDRI), Lucknow. After the quarantine period they were housed in the Animal House Facility of TRC. They were housed in polypropylene cages containing paddy husk as bedding with two animals of same sex in a cage.

2. Rabbit:

New Zealand White rabbits used in the study were procured from National Institute of Nutrition (NIN) Hyderabad. After the quarantine period, the animals were housed in the Animal House Facility of TRC. Each rabbit was housed in a stainless steel cage with grilled floor.

3. Rat:

Wistar rats used in the study were procured from National Institute of Nutrition (NIN) Hyderabad. After the quarantine period, animals were housed in the Animal House Facility of TRC. They were housed in polypropylene cages containing paddy husk as bedding with two animals of same sex in a cage.

4. Guinea Pig:

Duncan Hartley guinea pigs used in the study were procured from National Institute of Nutrition (NIN) Hyderabad. After quarantine period, the animals were housed in the Animal House Facility of TRC. Guinea pigs were housed in stainless steel cages with grilled floor with two animals of same sex in a cage.

5. Mice:

Swiss albino mice 4-5 weeks old and weighing between 14-16 gm body weight were obtained from Charles River, UK, and bred in the Animal House facility of TRC. They were housed in cages containing paddy husk as bedding with five mice of same sex in a cage.

ANIMAL ETHICS:

All the experimental protocols were approved by the Institutional Animal Ethics Committee. Every care was taken to adhere to highest standards of animal ethics in all procedures carried out.

STATISTICS:

Results were expressed as group mean ± SEM. The test results were statistically analysed by independent 't' test using the SPSS (version 10) software. A value of $P<0.05$ was considered statistically significant.

4.2 Screening for anti-hyperlipidemic activity in hamsters:

The objective of the study was to investigate the anti-hyperlipidemic activity of the test compounds in the hamster model of hyperlipidemia.

Materials:

Cholesterol (Qualigens)

Coconut oil (Marico)

Method:

Male Syrian hamsters weighing 90–125g were used in the study. The animals were randomly chosen from the colony to form 72 groups of 5-6 animals per group, of these 50 groups were treated with the test compound, two with the reference compounds cerivastatin and CP-230821 and 20 with the vehicle sodium CMC. In all 50 test compounds were screened.

The test compounds were screened in batches where the number of compounds in a batch ranged from one to five. These were screened as and when they were synthesized and submitted by the MCD for the *in vivo* screening. Each study batch in addition to various test drug groups had one vehicle control group.

The reference compound, CP-230821 has been shown by Shimokawa et al. (1998), to have appreciable cholesterol lowering activity at a dose of 30mg/kg in the hamster model of hyperlipidemia. In our animal model, we administered the same dose to confirm similar activity. To screen and identify molecules with adequate efficacy, twice the equimole dose of CP-230821 (30mg/kg) was used as the dose for screening the test compounds. The other reference compound used was cerivastatin (1.25mg/kg).

The basal food intake (lab chow) of the animals was monitored for 2 days prior to the study. Body weight of each animal was registered at the beginning and at the end of the experiment. Sixteen hours prior to the experiment, food but not water was withdrawn and on the morning of the first day, blood samples were collected under light ether anesthesia by retro-orbital puncture and the samples analysed for TC, TG, and HDL. Serum total cholesterol (TC) and triglycerides (TG) levels were determined using enzymatic assay kits (Autopack, Bayer) on RA-1000 auto-analyzer. High-density lipoprotein (HDL) cholesterol was determined after removal of non-HDL cholesterol through magnesium phosphotungstate precipitation (Weingand and Daggy 1990). Serum was diluted with an equal volume of 0.9% sodium chloride before the removal of non-HDL cholesterol and similarly samples, which had TG values above 400mg/dl, were also diluted 1:1 before TG determination. The low-density lipoprotein (LDL) cholesterol was determined using the formula of Friedewald et. al.(1972).

All the animals were administered high fatty meal orally as a percent of the estimated basal food intake, i.e. 0.5% cholesterol and 5% coconut oil, in the morning. Seven hours later, the drug group received test/reference compound suspended in sodium CMC by oral gavage while the control group received only vehicle - sodium CMC. The fatty food and drug treatment was continued for the next five days (Shimokawa et al., 1998). After the last dose of drug on the fifth day, food was withdrawn and the animals were fasted for 16 hrs and blood samples collected by retro-orbital puncture. Immediately thereafter, the animals were sacrificed, the liver was dissected out, blotted free from blood and weighed. Samples of liver were frozen in liquid nitrogen and stored at –70°C until analysis. Liver from animals in the test groups that showed significant serum cholesterol lowering activity was analysed for hepatic total cholesterol (TC) and cholesteryl ester (CE) as described under *section 4.6.*

4.3 Anti-hyperlipidemic activity in hamsters - dose response:

Among the test compounds the best activity (based on lowering of TC, LDL-C, and TG) was shown by TRC-7033. Hence this compound was chosen for the dose response study along with cerivastatin.

Method:

Male Syrian hamsters weighing 90–125g were used in the study. The experiments were carried out on six drug groups and the hamsters in each group were treated with TRC-7033 at 40, 60 and 80 mg/kg and cerivastatin at 0.5, 2.5 and 5 mg/kg. Each test group had its own vehicle control group. The methodology followed was similar to the primary five-day screen in hamsters described under *section 4.2*. However in this study in addition to the lipid profile, the serum levels of the hepatic enzymes (ALT, AST, serum bilirubin and alkaline phosphatase) were also monitored before and at end of treatment.

At the end of the study, animals were sacrificed and the liver from the 80mg/kg dose group of TRC-7033 and 2.5mg/kg dose group of cerivastatin removed, blotted free from blood and weighed. Samples of liver were frozen in liquid nitrogen and stored at –70 °C, until analysis for hepatic total cholesterol (TC) and cholesteryl ester (CE) as described in *section 4.6*.

4.4 *Determination of ED_{50} of TRC-7033 for reduction in LDL-C:*

A dose response curve (DRC) for reduction in LDL-C for different doses was plotted. The ED_{50} of the chosen lead molecule, TRC-7033 was determined.

The LDL-C levels on high fat diet increased in test groups (40, 60, 80 mg/kg) as well as the vehicle group. Compared to the vehicle group, the test groups showed a dose dependent reduction in the rise in LDL-C level. This percentage reduction in LDL-C level was plotted against the corresponding test doses and the ED_{50} dose was derived. A best-fit line was constructed using a polynomial equation and the theoretical dose for 50% reduction was derived from this equation. This ED_{50} dose served as a point of reference for various doses used in the safety pharmacology studies as well as probe toxicology.

4.5 *Hypolipidemic activity of TRC-7033 in hamsters:*

TRC-7033 was screened for its hypolipidemic activity in normolipidemic hamsters. The protocol followed and parameters measured were very similar to the above anti-hyperlipidemic activity screening in *section 4.2*, except that the fatty meal was not administered to the animals in the morning.

4.6 Determination of hepatic lipids:

The accumulation of free cholesterol and cholesteryl esters in the liver at the end of drug treatment was determined by HPTLC method. (Folch et al., 1957; Schmitz et al., 1984)

Materials:

HPTLC Scanner II (Camag)

Linomat IV (Camag)

Camag twin trough chamber

HPTLC Glass plate, silica 60 F_{254} (Merck)

n-Hexane (HPLC grade, Spectrochem)

Diethyl ether (GR grade, E.Merck)

Formic acid (AR grade, WBA chemicals)

Derivatisation reagent: 3.2mg manganese chloride in 480 ml methanol, 32ml conc sulphuric acid and 480 ml water

Mobile phase: n-Hexane : n-Heptane : di-ethyl ether : formic acid (65:15:20:0.5) %v/v

Method:

The method of extraction adopted was that of Folch et al., 1957 (the details are described in *Appendix-II* -1) The neutral lipids were extracted from the liver with chloroform : methanol (2:1, v/v) mixture and the extract was further diluted 1:50 with the chloroform : methanol (2:1, v/v) mixture. This diluted sample was then used for spotting on the HPTLC plate.

A mixed standard consisting of cholesterol and cholesterol oleate was prepared in chloroform : methanol (2:1) mixture and used to identify the bands (the details are described in *Appendix-II* - 1). 10µl of each standard preparation (to get concentrations of 320, 160, 80, 40, 20 and 10ng per spot) and sample preparation was spotted on the plate using Linomat IV applicator and after drying it for 3 min at room temperature it was placed in a tank (Camag twin trough chamber) saturated with the mobile for 10mins with 5ml of mobile phase. After development (solvent front moves to 9.5cm from bottom) the plate was dried in air and then dipped in the derivatising reagent in the dip tank for 20sec. The plate was heated at 110°C for 30min and then scanned using a HPTLC scanner (densitometer).

4.7 Quantification of LDL-receptor expression:

The ability of the test compounds to upregulate the LDL-receptors in HepG2 cell line was estimated by an assay system (Ashton et al., 1996), the details are given below.

Principle:

The LDL receptors were quantified by a cell based assay- fluorescence method using the mouse monoclonal antibody to human LDL receptor. Here increase in LDL receptors on HepG2 cell line on exposure to the drug was detected using the monoclonal antibody Mab-C7 (primary antibody) and the secondary antibody used was goat antimouse antibody conjugated to alkaline phosphatase and the substrate for alkaline phosphatase was AttoPhos. Alkaline phosphatase liberates the phosphate group from the non-fluorescent AttoPhos molecule resulting in fluorescent emitter. The increase in fluorescence reading (460,590nm) denoted increase in Mab-C7 binding and hence LDL receptor number.

In order to perform the assay, certain basic preparations are needed. Initially one has to raise the primary antibody for the LDL receptor. For this purpose C7 hybridoma cells has to be cultured. Then the antibody secreted from the hybridoma needs to be purified by affinity chromatography. The presence of the antibody (protein) in the elute has to be confirmed by Western blotting after the protein was resolved by SDS-PAGE.

Hence the following prerequisites for quantitation of LDL-R was carried out:

1. Culture of C7 hybridoma and collection of Mab-C7
2. Purification of Immunoglobulin G (IgG)
3. Confirm presence of IgG by Western blotting.

The assay of the LDL-R was carried out in two steps as shown below.

1. Culture of HepG2 cell line

2. LDL receptor assay on HepG2 cell line

4.7.1 Culture of C7 hybridoma:

C7 hybridoma produces a monoclonal antibody (IgG2b) that binds to low density lipoprotein (LDL) receptors from bovine and human tissue. This clone was derived by fusing Sp2/0-Ag14 myeloma cells with spleen B-lymphocytes from a Balb/c mouse immunized with a partially purified LDL receptor from bovine adrenal cortex. The antibody recognizes an epitope in the region of repeat #1 of the ligand-binding region of the receptor.

Materials:

C7 hybridoma (CRL-1691) ATCC, USA.

Culture medium - Dulbecco's Modified Eagles Medium (DMEM) with high glucose (Hyclone)

(Details of all materials used is presented in *Appendix–II* –2)

Method:

Frozen C7 cells were revived (revival details are described in *Appendix-II* – 2.1) and maintained in DMEM with high glucose. Subculturing was carried out every second day as the cell proliferation was rapid. The contents of the flask were centrifuged and the supernatant containing the antibody of interest was collected and stored at -20°C. Simultaneously equal volume of fresh culture media too was taken and stored at -20°C for estimation of antibody (control). A part of the cell pellet obtained after removal of the supernatant was used for subculturing while the other part was used for cryopreservation (details in *Appendix – II* –2.3). The passage (subculturing) was continued only for ten generations, after which a new cryovial containing the C7 cell line was revived and subcultured. (the details are described in *Appendix - II* – 2.2).

4.7.2 Purification of immunoglobulin-G (monoclonal antibody):

The objective was to purify the antibody from the hybridoma supernatant by affinity chromatography.

Principle:

Protein A is a purified protein from the cell wall of the bacterium *Staphylococcus aureus*. This protein specifically binds to the Fc portion of immunoglobulins and its binding affinity depends on the type of immunoglobulin and the animal species from which it was raised. Protein A has high binding affinity to mouse IgG2b. This property of protein A is used in purifying IgG2b from the hybridoma supernatant.

Materials:

IgG purification kit (Bangalore Genei)

Amicon centriplus concentrators –100 (15ml capacity)

Method:

The IgG purification kit - a gel matrix of Protein A coupled (immobilized) to cross-linked agarose packed in inert polystyrene column) was used in purifying IgG2b from the hybridoma supernatant and from the fresh culture medium. The procedure used in purification was as per the instruction manual, (Bangalore Genei) supplied by the manufacturer (the details are described in *Appendix - II* – 3). The antibody in the elute was then concentrated by ultrafiltration using 100kD cutoff centriplus concentrators (the details are described in *Appendix - II* – 4). The protein content in the elute, before and after concentration was determined by *Bradford assay* (Bradford, 1976) the details are described in *Appendix - II* – 5)

4.7.3 PAGE and Western blot analysis of the purified antibody:

The objective of this study is to confirm the presence of IgG in the elute obtained in the above purification step.

Materials:

Bio-Rad mini gel apparatus

Semi dry transfer unit (Hoefer semiphor)

Nitrocellulose membrane (Amersham)

Detailed materials used is presented in *Appendix-II* - 6

Method:

The IgG purified and concentrated from the fresh culture medium was used as the control to be compared with the IgG purified and concentrated from the hybridoma supernatant.

The proteins in control and test samples were resolved on 7% SDS-polyacrylamide gels by gel electrophoresis. Two sets of gel were prepared one for direct staining (silver staining) and other for western blotting (the details are described in *Appendix - II* – 6). The second gel was used for immunoblotting, after confirmation of presence of protein in the band by silver staining the first gel.

4.7.4 Culture of HepG2 cell line:

Materials:

HepG2 cells (human hepatoblastoma) NCCS, Pune

Culture medium - Minimum essential media with Earle's balanced salts (MEM) (Hyclone)

Detailed materials used is presented in *Appendix-II* -7

Method:

Cells were maintained in MEM supplemented with fetal bovine serum and subculturing was done only when the confluency reached 75% or more (the details are described in *Appendix - II* -7).

4.7.5 LDL receptor assay on HepG2 cell line:

Materials:

Fluorescence microplate reader (Fmax)

Black fluronunc culture plates (NUNC)

Primary antibody (Mab –C7)

Sec. Antibody (goat anti-mouse IgG monoclonal Ab conjugated to alkaline phosphatase) (Bio-Rad)

AttoPhos (Roche)

Bovine serum albumin (BSA) (Sigma)

Superblock blocking buffer (Pierce)

Propidium Iodide (Sigma)

Method:

HepG2 cells were plated in sterile flat bottomed 96 well fluorescent black culture plates (on day 0) at 15,000 cells per well in 200µl culture medium (MEM)

supplemented with 5% FBS. The plate was incubated in CO_2 incubator maintained at 37°C and humidified atmosphere of 5% CO_2. Media (containing 2% FBS) renewal was done on day 2.

On day 4 when the cells show 80 percent confluency, media (containing 2% FBS) renewal was done with MEM containing 1% BSA (filtered sterilized) and then 1µl of various (200x) concentrations of the test and/or standard drug or combination of 25-hydroxycholesterol and test drug dissolved in 100% DMSO (details enumerated below) was added to the wells (in triplicate) so as to have various final concentration of these in 0.5% DMSO.

1. A concentration response curve (1µM to 0.3 nM) with TRC-7033 and cerivastatin.
2. Activity of various test compounds (TRC-7004, TRC-7007, TRC-7011, TRC-7051) and standard (CP-230821) at 1µM.
3. Various concentrations of TRC-7033 (0 to 3µM) in presence of 0.1µM cerivastatin.
4. Various concentrations of TRC-7033 (0 to 3µM) in presence of 2.5µM 25-hydroxycholesterol.
5. Cerivastatin (1 & 3µM) in presence of 2.5µM 25-hydroxycholesterol.

A modified method of Ashton et al. (1996) was used for the LDL receptor assay (flow chart given below). The cell culture was incubated with the drug for 24 hrs and then the media aspirated and the cell monolayer washed with 1x phosphate buffer saline (PBS) and fixed with 100µl of 6% formaldehyde in PBS at 4°C for 30min. The formaldehyde solution was removed and the cells were washed with ammonium sulphate solution followed by 1x PBS thrice. 100µl of the primary antibody (Mab-C7) solution – 0.5µg/ml in PBS containing 10% fetal bovine serum was added to the wells and incubated for 60 minutes at 4°C. The contents of the well were removed and 200µl of the non-specific site blocking buffer 'Superblock' was

added to the wells and incubated at 4°C for 30minutes. The Superblock was removed and cells washed with tween phosphate buffer saline (TPBS) twice and then with PBS once. The monolayer was then incubated with 100µl of goat anti-mouse antibody conjugated to alkaline phosphatase in PBS (1:6000) at 4°C for 60 minutes. The secondary antibody solution was removed and cells washed with tween phosphate buffer saline (TPBS) twice and then with PBS once. Then 100µl of alkaline phosphtase substrate – ATTOPHOS containing 20µg/ml of propidium iodide was added to the well and plate incubated at 4°C for 60 minutes. The reaction was terminated by addition of 6M sodium hydroxide. The fluorescence reading at 460 and 590 nm were taken using a fluorescence plate reader (Fmax). The cell number per well was determined by propidium iodide fluorescence at 538 and 612 nm.

The below equation was used for calculation of percent increase in LDL-receptor activity.

$$\% \text{ Increase in LDL-R Activity} = \frac{(A/C) - (B/D)}{(B/D)} \times 100$$

A= Fluorescence reading at 460,590nm in presence of test drug

B= Fluorescence reading at 460,590nm in presence of vehicle control (DMSO)

C= Fluorescence reading at 538,612nm in presence of test drug

D= Fluorescence reading at 538,612nm in presence of vehicle control (DMSO)

LDL RECEPTOR ASSAY - ESSENTIAL STEPS

15K HepG2 cells were seeded per well in 96 well plate (Media 5% FBS)

↓ Incubated at 37 ^{0}C in 5% CO_2 incubator for 48 hrs.

Media was changed (Media 2% FBS)

↓ Incubated at 37 ^{0}C in 5% CO_2 incubator for 48 hrs.

Media was changed (Media 2% FBS)

Drug was added

↓ Incubated at 37 ^{0}C in 5% CO_2 incubator for 24 hrs.

Media was removed and the cells fixed with 6% HCHO

↓ Incubated at 4 ^{0}C for 30 min.

Washed with $(NH_4)_2SO_4$ soln. two times

↓

Washed with PBS(1x) three times

↓

Primary Ab added (in 10% FBS)

↓ Incubated at 4 ^{0}C for 1 hr.

Superblock added

↓ Incubated at 4 ^{0}C for 30 min.

Washed with TPBS twice

↓

Washed with PBS(1x) once

↓

Secondary Ab added (1:6000)

↓ Incubated at 4 ^{0}C for 1 hr.

Washed with TPBS twice

↓

Washed with PBS(1x) once

↓

Attophos substrate + Propidium Iodide added

↓ Incubated at 4 ^{0}C for 1 hr.

6 M NaOH added and the plate read in F-max at 460,590 nm & 538,612 nm

4.8 Cytotoxicity of the test compounds by MTT assay:

The objective of this study is to determine the cytotoxicity of the test compounds on HepG2 cell line.

Principle:

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay is very commonly used for spectrophotometric quantification of cell growth, viability, cytotoxicity etc. The assay is based on the capacity of mitochondrial dehydrogenase enzyme (metabolically active cells) to convert the yellow water-soluble substrate - tetrazolium salt (MTT) to purple formazan crystals, which are insoluble in water. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH. The formazan crystals formed are solubilised and the resulting coloured solution quantified using an ELISA plate reader.

Materials:

ELISA plate reader (Spectramaxplus)

MTT (Sigma)

SDS (SRL)

Hydrochloric acid (Qualigen)

Method:

Preparation of MTT and solubilising reagent: A stock solution of 5 mg/ml of MTT in 1x PBS (culture grade) was prepared by slight warming (37°C) and vortexing. Similarly solubilising reagent (10% SDS+ 0.01 N HCl) was also prepared.

HepG2 cells (2.5×10^6) were seeded per T75 culture flask and after 24 hrs; HepG2 Cells were plated in 96-well flat-bottomed culture plate at 20,000 cells per well in 200µl culture medium. After 48 hrs of incubation in a CO_2 incubator maintained at 37°C and humidified atmosphere of 5% CO_2, 1µl of various (200x) concentrations of the test compound dissolved in 100% DMSO was added to the wells (in triplicate) so as to have a final concentration of drug ($1x10^{-5}$ M to $1x10^{-8}$ M) in 0.5% DMSO. The cell culture was incubated with the drug for 24 hrs and then the media was changed and cells rinsed with PBS and then 100µl of fresh media added and subsequent to this 15µl of stock solution of MTT per well and incubated for 4 hrs (at 37°C and 5% CO_2). Then 100µl of solubilising reagent was added to each well and after overnight incubation (at 37°C and 5% CO_2), the absorbance of the formazan product was measured at 570nm and the reference wavelength at 650nm.

The below equation was used for calculation of percent cytotoxicity.

$$\% \text{ Cytotoxicity} = \frac{(B-D) - (A-C)}{(B-D)} \times 100$$

A= Absorbance at 570nm in presence of test drug.

B= Absorbance at 570nm in presence of vehicle control (DMSO)

C= Absorbance at 650nm in presence of test drug.

D= Absorbance at 650nm in presence of vehicle control (DMSO)

4.9 *Anti-oxidant activity of the test compounds:*

The objective of this study is to determine the antioxidant activity of the test compounds, which will be beneficial in prevention of oxidative modification of LDL, which is more atherogenic than LDL.

Principle:

The radical scavenging activity (anti-oxidant property) of the test compound is evaluated by measuring its ability to scavenge the stable free radicals present in a methanolic solution of 2,2,-diphenyl-1-picrylhydrazyl (DPPH·). As a result of the reduction of DPPH by the anti-oxidant the absorbance at 515nm of this purple coloured solution of DPPH in methanol decreases (Brand-Williams W., 1995).

DPPH· + AH → DPPH-H + A·

(free radical) (antioxidant)

Materials:

2,2,-diphenyl-1-picrylhydrazyl (DPPH·) (Sigma)

Dimethylsulphoxide (Sigma)

Methanol (Merck)

UV-visible spectrophotometer (Spectramax plus)

Glass cuvette of 3 ml capacity

Method:

100µM solution of DPPH was prepared in methanol and various concentrations of drug solution were prepared in methanol or DMSO depending on its solubility. For the preparation of control solution, 200 µl of ethanol/methanol was added to 1800µl of DPPH radical. Similarly for preparation of test solution, 200µl of various concentration of drug solution (12.5µM, 25µM, 50µM, 100µM) in methanol

or DMSO was added to 1800µl of DPPH radical solution. The absorbance of control and test samples were recorded after incubation at 30°C for 30 minutes, at 515nm taking methanol (100%) or methanol : DMSO (9:1) as blank.

The percent antioxidant activity was calculated using the below formula.

$$\% \text{ Antioxidant activity} = \frac{(B-A)}{B} \times 100$$

A= Absorbance of test sample at 515nm

B= Absorbance of control sample at 515nm

From a plot of the concentration vs. percent anti oxidant activity, the EC_{50} for anti-oxidant activity was determined.

4.10 Safety pharmacology studies on TRC-7033:

The ED_{50} dose for reduction in LDL-C in the hamster model of hyperlipidemia served as a point of reference for various doses used in the safety pharmacology studies as well as probe toxicology.

4.10.1 Effect on HR, BP, ECG and respiratory system:

The objective of this experiment was to study the effect of TRC-7033 on the cardiovascular and respiratory system.

Materials:

MacLab 8/s system with Quadbridge and Bio Amp (AD Instruments, UK)

Pressure transducer (SensoNor 840, SensoNor a.s, Norway)

Method:

Wistar rats of either sex were used to study the cardiovascular effects of TRC-7033 The experiment was carried out on four test drug groups of which two groups were administered 65mg/kg (ED_{50} dose) and 325 mg/kg ($5xED_{50}$ dose) by oral gavage and the other two groups received 2mg/kg and 10 mg/kg of TRC-7033 by intravenous route. There were two corresponding vehicle control groups, one for the oral and the other for the intravenous formulation. Each group had 4-5 rats.

Rats were anesthetized with urethane (1.25 gms/kg) and the left common carotid artery was cannulated by a polythene cannula and was connected to a pressure transducer for the measurement of the arterial blood pressure. The jugular vein was cannulated for intravenous administration of the vehicle/drugs. The signals from the transducer were amplified using a bridge amplifier and the blood pressure was recorded using the Chart software of MacLab 8/s (AD Instruments, UK). Using

the Chart software the mean arterial blood pressure (MABP) was calculated by adding 1 3^{rd} of the systolic pressure and $2/3^{rd}$ of the diastolic pressure. Standard limb lead II ECG was recorded continuously in these animals using Bio Amp. The heart rate was also calculated as a derived parameter from the lead-II ECG. Since the sampling rates and speeds can be increased to the appropriate level, even minor changes in the ECG, heart rate (HR) and blood pressure (BP) were captured.

Studies on intravenous administration:

The drug was dissolved in vehicle (20% PEG-400, 20% tween 80 and remaining was saline) and administered by intravenous route. The effect of the drug on ECG, mean blood pressure, heart rate and respiration were monitored at doses described above.

Studies on oral administration:

The drug was administered by oral gavage as a suspension in sodium CMC. The effect of TRC-7033 on ECG, mean blood pressure, heart rate and respiration were monitored at doses described above.

4.10.2 Effect on spontaneous motor activity (SMA):

The objective of the study is to investigate the effects of TRC-7033 on the spontaneous motor activity in mice using a multi-varimex photo activity meter.

Method:

The studies were performed on Swiss albino mice, 6-8 weeks old and weighing between 17-25 gm body weight. The experiments were conducted in five groups of mice of which three were test drug group and the other two were control groups (saline and vehicle). Each group comprised of five males and 5 females. TRC-7033 was given at a dose of 65 mg/kg, 650 mg/kg and 1625 mg/kg representing ED_{50}, $10xED_{50}$ and $25xED_{50}$ by oral gavage.

Spontaneous motor activity was monitored by using a computerised activity meter "Multi-Varimex" which is an optical interface for the Columbus Instruments Event Counter hardware. It is an animal activity monitor, which operates on the IR beam principle interfaced to an I.B.M computer. The cages were made of transparent polyvinyl chloride with dimensions of 40x26x16 cm^3. The distance between two emitters or two detectors was 13.5 cm. The emitter-detector was placed at the height of 4 cm from the base of the cage. Three such pairs of infrared beams transected the cages, such that the movement of the animal would intercept the infrared beams. These interceptions were monitored by the computer.

The animals were fasted overnight before the experiments but were allowed to drink water *ad libitum*. All experiments were conducted at the same time of the day (between 9 a.m. to 12 p.m.) to minimize circadian influences. A suspension of TRC-7033 was prepared in the vehicle (Sodium carboxymethyl cellulose). Each group received the respective dose by oral gavage and each group of animals was

placed in separate cages. The system was programmed to count the interceptions at intervals of 10 minutes for a period of 120 minutes. The spontaneous motor activity was monitored after 5 min of adaptation period. The total count over a 120-min period was recorded for each group and tabulated. The effect of each dose of the test compound on the motor activity was expressed as a percentage change from the control.

4.10.3 Effect on forced motor activity (FMA):

The objective of the study is to investigate the effects of TRC-7033 on the forced motor activity in mice using a rotarod.

Method:

The studies were performed on Swiss albino mice, 6-8 weeks old and weighing between 17-25 gm body weight. The experiments were conducted in four groups of mice of which two were test drug groups and the other two were control groups (saline and vehicle). Each group comprised of five males and five females. TRC-7033 was given at a dose of 650 mg/kg and 1625 mg/kg representing $10xED_{50}$ and $25xED_{50}$ by oral gavage.

The degree of motor incoordination was determined using a standard mouse rotarod apparatus comprising of a plastic rod, 3 cm in diameter and 52 cm long, with non-slippery surface and 28 cm over the base. This rod is divided into 5 equal sections by 6 discs, thus enabling 5 mice to be tested at the same time. The rod was set to rotate at a speed of 12 rpm. The time the animal managed to spend on the rotating rod before falling was taken as the performance time. Mice were acclimatized to the procedure by training them for 4-5 times prior to the actual experiment. Normal motor coordination was defined as the ability to remain on the rotarod for 180 seconds consecutively and mice, which failed to do so during the pretest period, were excluded from the study.

The animals were fasted overnight before the experiments but were allowed to drink water *ad libitum*. After the training, rotarod scores were determined before oral administration of the drug, or saline or vehicle. A suspension of TRC-7033 was prepared in the vehicle (Sodium carboxymethyl cellulose) and administered to the

respective groups by oral gavage. After the drug/vehicle/saline administration the animals were tested on the rotarod every 20 min for 180min.

Data Analysis:

The effect of the drug on motor coordination was expressed as an activity ratio, which is defined as the ratio of the time the animal was able to remain on the rotarod after drug administration to pre-drug values.

$$\text{Activity ratio} = \frac{\text{Time (in minutes), the animal is able to remain on the rota-rod after drug treatment}}{\text{Time (in minutes), the animal is able to remain on the rota-rod before drug treatment}}$$

An activity ratio of 1 or close to 1 indicates lack of effect of test compound on motor coordination and a decreasing activity ratio indicates increasing effect of the compound resulting in motor incoordination. Data are expressed as the mean of the activity ratio ± SEM for each group.

4.10.4 *Effect of TRC-7033 on smooth muscles:*

Materials:

The test compounds were stored under refrigeration and stock solutions were prepared on a daily basis to give concentrations ranging from 10^{-8} to 10^{-5} M in a 10 or 20 ml organ bath. The reagents required for the preparation of the physiological solutions were procured from E Merck, Bombay.

4.10.4.1 *Studies on isolated rabbit aorta:*

The objective of the study was to evaluate the vasorelaxant or spasmogenic effects of the test compounds on rabbit aortic rings.

Methods:

The method adopted was a modified method of Nishikawa et al. (1982). Rabbit of either sex weighing between 1.5-2 Kgs were used throughout the study. At least 4-5 animals were used in the study.

The composition of the physiological salt solution (Krebs) was as follows: 120.3mM Nacl, 4.8mM KCl, 1.2mM $CaCl_2$, 1.03mM $MgSO_4$, 24.2mM $NaHCO_3$, 1.2mM KH_2PO_4, and 5.5mM glucose. The bathing solutions were maintained at 37± 0.5° C and bubbled with carbogen (a mixture of 95% O_2 and 5% CO_2).

The animals were sacrificed by stunning and exsanguination. The thoracic aorta was quickly removed and cut into 3-4 mm wide transverse rings. The endothelium was removed by a gentle rub on a rough stainless steel needle. Successful removal of the endothelium was confirmed by the inability of acetylcholine to induce relaxation.

Four aortic rings were mounted in a four-unit organ bath (model TSZ-04, Experimetria, Hungary) filled with 10 ml of Krebs solution, aerated with carbogen (95% oxygen and 5% carbon dioxide) and maintained at 37 C. The free end of the aortic strip was connected to the lever of a force displacement transducer (FSG-01; Experimetria, Hungary) by a silk thread. The responses from the transducers were amplified by a bridge amplifier, connected to a MacLab/8s (AD Instruments Limited, UK). The responses of the tissues after administration of the spasmogen and/or the test compounds were analysed by the Chart software version 4.2 of MacLab/8s. The data was stored in a G4 Power Macintosh computer for the analysis and interpretation.

The spasmogenic effect was studied at the dose range of $1x10^{-8}$ to $1x10^{-5}$ M. The spasmolytic effect was studied on 30mM KCl contracted tissues.

Before the experiments were carried out, the preparations were allowed to equilibrate for 30 minutes under a resting tension of 2gms in the organ baths. The Krebs solution (physiological salt solution -PSS) was changed every 10 minutes. For the spasmolytic study, the tissues in bath 1 and 2 were exposed to two primer doses of KCl (at 30 minutes interval) over the one hour period post stabilization i.e. at the end of 30 minutes stabilization the tissues were exposed to the first primer dose of 30mM KCl and as soon as the response stabilized, the tissues were washed with the PSS and at the end of 30 minutes the tissues were exposed to the second primer dose of 30mM KCl and as soon as the response stabilized, the tissues were washed with the the PSS and at the end of 30 minutes the third dose of 30mM KCl was added and the cumulative dose response curve with the test compound was recorded in bath 2 and its corresponding vehicle, in bath 1.

The relaxant response for each dose was allowed to reach a stable level before the addition of the next dose. Cumulative dose response curve for TRC-7033

was obtained from the tissue at a dose range from 10^{-8} to 10^{-5} M. From a plot of the dose vs. response (% relaxation), the ED_{50} was ascertained and the maximum relaxation obtained for the highest dose (10^{-5} M) was also determined.

To study the spasmogenic effect, tissues in baths 3 & 4 were used. After a stabilization period of 30 minutes, TRC-7033 was added in bath 4 and its vehicle in bath 3, the contractile response was allowed to stabilize before the addition of the next dose. A cumulative dose response curve for TRC-7033 was obtained from the tissues at a dose range from 10^{-8} to 10^{-5} M. From a plot of the dose vs. response (% contraction) the ED_{50} was ascertained and the maximum contraction obtained for the highest dose (10^{-5} M) was also determined.

4.10.4.2 Studies on isolated rat uterus:

The objective of the study was to determine the effect of TRC-7033 on the isolated rat uterus. The study was carried out both in estrous and diestrous stage.

Method:

Young female rats weighing between 150-200 grams were used in this study. A vaginal smear examination was done to determine the phase of estrous cycle. The estrous phase is marked by masses of cheesy looking whitish cornified cells. The diestrous stage was detected by the presence of a few dry epithelial cells in the vaginal smear. The effect of the test compound was studied on uteri from animals in estrous as well as diestrous. The spasmogenic or spasmolytic effect of the compound was studied in separate group of animals. At least 4-5 animals were used in each group.

The animal was sacrificed and the abdomen opened and the intestine pulled aside or removed, so as to expose the two horns of the uterus, which would be large if the animal was in estrous. The mesenteric attachments were cut away and the two horns of the uterus were dissected out and transferred to a dish containing De Jalon's solution (De Jalon et al, 1945) before being mounted in a two-unit organ bath system. The De Jalon's physiological salt solution contained the following 154 mM NaCl, 5.6 mM KCl. 0.55 mM $CaCl_2$, 6.0 mM $NaHCO_3$, 2.78 mM Glucose; prepared in double distilled water.

A thread was tied to each end of the uterine horn and suspended in an organ bath containing 20 ml of De Jalon's solution maintained at 32° C and aerated with carbogen. A resting tension of 0.5gms was applied onto the tissues. In every experiment, a pair of the uterine preparation was studied; one for the test drug and the other for the vehicle of the test drug.

The spasmogenic or spasmolytic properties of TRC-7033 were studied under these conditions. The spasmogenic effect was studied at the concentration range of $1x10^{-8}$ to $1x10^{-5}$ M. The spasmolytic effect was studied on 30 mM KCl contracted tissues.

After a stabilization period of 30 minutes, for the spasmolytic study two priming doses of KCl (30mM) (at 30 minutes interval) were added to bath 1 and 2. After addition of the second dose of the spasmogen, the tissues went into a stable contractile state. TRC-7033 was added in bath-2, its corresponding vehicle was added in bath-1. The relaxant responses for each dose were allowed to reach a stable level before the addition of the next dose. Cumulative dose response curve for TRC-7033 was obtained from the tissue at a dose range from 10^{-8} to 10^{-5} M. From a plot of the dose vs. response (% relaxation) the ED_{50} was ascertained and the maximum relaxation obtained for the highest dose (10^{-5} M) was also determined.

To study the spasmogenic effect separate group of animals was used. The tissues were mounted and after a stabilization period of 30 minutes, TRC-7033 was added in bath 2 and its vehicle in bath 1, the contractile response was allowed to stabilize before the addition of the next dose. A cumulative dose response curve for TRC-7033 was obtained from the tissue at a dose range from 10^{-8} to 10^{-5} M. From the plot of the dose vs. response (% contraction) the ED_{50} was ascertained and the maximum contraction obtained for the highest dose (10^{-5} M) was also determined.

4.10.4.3 Studies on isolated guinea pig ileum:

The objective of the study was to determine the effect of TRC-7033 on the isolated guinea pig ileum.

Method:

Guinea pigs (fasted for 8 hrs prior to the experiment) of either sex weighing 400-500 grams were used in the study. At least 4-5 animals were used in the study. Guinea pigs were stunned by a blow to the head and sacrificed by exsanguination. The abdomen was opened through a midline incision and the ileo-caecal junction was exposed. The terminal ileum was cut after discarding 10 cm nearest to the ileo-caecal junction because of the presence of the excitatory alpha adrenoceptors near the ileo-caecal junction. The mesenteric attachment was cut as close to the gut as possible without injury for a distance of about 20-25 cms.

The intestine was then cut across and the lumen of the isolated piece thoroughly cleaned by running warm Tyrode solution repeatedly through the proximal opening with the help of a 10 ml volumetric pipette. Care was taken so as to avoid undue stretching, ballooning or handling of the gut. The clean strip of the intestine was then placed in fresh warm salt solution for about 10 minutes for acclimatization, before being put up.

Two small segments of the ileum about 5 cms in length were cut and a thread was passed through the lumen and the wall near the mesenteric attachment at each end with the help of a fine sewing needle and tied securely without occluding the lumen. One end of the tissue was tied securely to the tissue holder and the other end was connected to a force displacement transducer. The tissues were suspended in 20 ml organ baths containing Tyrode solution, aerated with carbogen and maintained at 37^{o} C. The Tyrode solution consisted of 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$,

1.0 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.55mM Glucose and was prepared in double distilled water. Contractile responses were isometrically recorded with a resting tension of 1 gm.

The spasmogenic or spasmolytic properties of TRC-7033 were studied under these conditions. The spasmogenic effect was studied at the dose range of $1x10^{-8}$ to $1x10^{-5}$ M. The spasmolytic effect was studied on 30 mM KCl contracted tissues.

After a stabilization period of 30 minutes, for the spasmolytic study two priming doses of KCl (30mM) were added at 30-minute intervals to bath 1 and 2. The third dose of the spasmogen was added after half an hour and the tissue went into a stable contractile stage. TRC-7033 was added in bath-2, its corresponding vehicle was added in bath-1. The relaxant responses for each concentration were allowed to reach a stable level before the addition of the next dose. Cumulative dose response curve for TRC-7033 was obtained from the tissue at a dose range from 10^{-8} to 10^{-5} M. From a plot of the dose vs. response (% relaxation), the ED_{50} was ascertained and the maximum relaxation obtained for the highest dose (10^{-5} M) was also determined.

To study the spasmogenic effect, tissues in baths 3 & 4 were used. After a stabilization period of 30 minutes, TRC-7033 was added in bath 4 and its vehicle in bath 3, the contractile response was allowed to stabilize before the addition of the next dose. A cumulative dose response curve for TRC-7033 was obtained from the tissues at a dose range from 10^{-8} to 10^{-5} M. From the plot of the dose vs. response (% contraction), the ED_{50} and the maximum contraction obtained for the highest dose (10^{-5} M) were determined.

4.10.4.4 Studies on isolated guinea pig tracheal rings:

The objective of the study was to determine the effect of TRC-7033 on the isolated guinea pig tracheal rings.

Method:

Guinea pigs of either sex weighing 400-500 grams were used in the study. At least 4-5 animals were used in the study. Guinea pigs were stunned by a blow to the head and sacrificed by exsanguination. The trachea is removed and sectioned with a pair of scissors such that each piece had three tracheal rings. The rings were suspended with one hook on either side in such a way that the dorsal smooth muscle band was oriented vertical. One end of the hook was fixed to the aerator tube and the hook at the other end was tied to a force displacement transducer (FSG-01). The impulses from the tissues were detected by the transducer, amplified and viewed using ISOSYS software (Experimetria, Hungary). The rings were suspended under a resting tension of 1.0 gram in 10-ml organ baths containing Krebs solution. Four such ring preparations were mounted to the four-unit organ bath. The Krebs solution was prepared in double distilled water after addition of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 5.5 mM glucose.

The tissue baths were aerated continuously with carbogen and temperature maintained constantly at 37± 0.5°C. Two fine stainless steel pins were passed through the lumen of each preparation. One of the pins was connected to a force displacement transducer for recording of the isometric wall tension. The other pin was fixed to the tissue holder. The preparations were allowed to equilibrate for 45-60 minutes before the addition of the test compounds.

The spasmogenic or spasmolytic properties of TRC-7033 were studied under these conditions. The spasmogenic effect was studied at the dose range of $1x10^{-8}$ to $1x10^{-5}$ M. The spasmolytic effect was studied on 30 mM KCl contracted tissues.

After a stabilization period of 30 minutes, for the spasmolytic study two priming doses of KCl (30mM) (at an interval of 30 min) were added to bath 1 and 2. After the second dose of the spasmogen, the tissue went into a stable contractile stage. TRC-7033 was added in bath-2, its corresponding vehicle was added in bath-1. The relaxant responses for each concentration were allowed to reach a stable level before the addition of the next dose. Cumulative dose response curves for TRC-7033 were obtained from the tissues at a dose range from 10^{-8} to 10^{-5} M. From a plot of the dose vs. response (% relaxation) the ED_{50} and the maximum relaxation obtained for the highest dose (10^{-5} M) were determined.

To study the spasmogenic effect, tissues in baths 3 & 4 were used. After a stabilization period of 30 minutes, TRC-7033 was added in bath 4 and its vehicle in bath 3, the contractile response was allowed to stabilize before the addition of the next dose. A cumulative dose response curve for TRC-7033 were obtained from the tissues at a dose range from 10^{-8} to 10^{-5} M. From the plot of the dose vs. response (% contraction) the ED_{50} and the maximum contraction obtained for the highest dose (10^{-5} M) were determined.

4.11 Probe toxicity study:

The objective of the study was to evaluate the toxicity potential of TRC-7033 in mice.

Methods:

***Oral toxicity*:**

TRC-7033 was screened for its toxicity potential after oral administration on Swiss albino mice; 6-8 week old and weighing between 17-25 gm body weight. The experiments were conducted in three groups of mice of which two were test drug groups (low and high dose) and the other was vehicle control group. A minimum of four males and four females were used for each group. The low dose group received $10xED_{50}$ (650 mg/kg) of TRC-7033 and the high dose group received $25xED_{50}$ (1625 mg/kg) of the drug by oral gavage.

The animals were fasted overnight before the experiments but were allowed to drink water *ad libitum*. A suspension of TRC-7033 was prepared in the vehicle (sodium carboxymethyl cellulose) and administered to the respective groups by oral gavage. The animals were closely observed for one hour and the time of onset and time for signs to disappear were recorded. The animals were observed for the presence or absence of various pharmacotoxic symptoms at various time points for 72 hours post-treatment as shown in the proforma under *section 8* in the *Appendix-II*.

Intravenous toxicity:

Similarly TRC-7033 was screened for its toxicity potential after intravenous administration on Swiss albino mice; 6-8 week old and weighing between 17-25 gm body weight. The experiments were conducted in three groups of mice of which two were test drug groups and the other was vehicle control group. A

minimum of four males and four females were used for each group. The test drug groups received 10 and 20 mg kg of TRC-7033 by intravenous route.

TRC-7033 was dissolved in the vehicle (20% PEG 400 and 20% tween 80 and remaining saline) and administered to the respective groups via tail vein and the animals were closely observed for one hour and the time of onset, time for signs to disappear recorded. The animals were observed for the presence or absence of various pharmacotoxic symptoms at various time points for 72 hours post-treatment as shown in the proforma under *section 8* in the *Appendix-II.*

Results

5. Results

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5.10 Probe toxicity studies of TRC-7033.

5.1 Anti-hyperlipidemic activity of the test compounds in hamsters:

A total of 50 test molecules were screened for their anti-hyperlipidemic activity in the hamster model of hyperlipidemia. On feeding the hamsters for 5 days a high fat diet containing cholesterol and coconut oil, the serum total cholesterol (TC) levels increased by about 74%, the low density lipoprotein cholesterol (LDL-C) by 200%, the High-density lipoprotein cholesterol (HDL-C) by 34% while there was no significant change in serum triglycerides (TG) levels (Table 1). The group means are presented in the Table 1 in *Appendix-III*. The percent reduction in lipids after the drug treatment is shown in the Table 2a & 2b in *Appendix–III*.

The molecules that showed significant activity in reducing the serum cholesterol levels and low-density lipoprotein cholesterol at twice equimole dose to the reference (CP-230821) molecule were TRC-7004, TRC-7007, TRC-7011, TRC-7033 and TRC-7051. For comparisons, CP-230821 (30mg/kg) and cerivastatin (1.25 mg/kg) were used under similar conditions. The percent reduction in serum lipids is shown in the Table 2. The group means for CP-230821, TRC-7004, TRC-7007, TRC-7011 and TRC-7051 are presented in the Table 3 in *Appendix –III*. The group means for TRC-7033 and cerivastatin are presented in the Table 4 in *Appendix –III*.

TRC-7004 at the dose 80.9 mg/kg decreased the serum cholesterol by 19% and LDL cholesterol by about 22%. It did not have any significant effect on HDL cholesterol and triglycerides (Figure 1). TRC-7007 at the dose 87.6 mg/kg decreased the serum cholesterol by 20% and LDL cholesterol by about 26%. It did not have any significant effect on HDL cholesterol and triglycerides (Figure 2). TRC-7011 at the dose 80.5 mg/kg decreased the serum cholesterol by 27% and LDL cholesterol by about 40%. It did not have any significant effect on HDL cholesterol and triglycerides (Figure 3).

TRC-7033 at the dose 80 mg kg, decreased the serum cholesterol by 30% and LDL cholesterol by about 36%. It also reduced triglyceride by 44%. It did not have any significant effect on HDL cholesterol (Figure 4). TRC-7051 at the dose 71.1 mg kg, decreased the serum cholesterol by 22% and LDL cholesterol by about 22%. It did not have any significant effect on HDL cholesterol and triglycerides (Figure 5). The reference molecule CP-230821 at the dose 30 mg/kg, decreased the serum cholesterol by 39% and LDL cholesterol by about 55%. It did not have any significant effect on HDL cholesterol and triglycerides (Figure 6). The following charts summarize the results of the studies.

Table 1: The increase (%) in serum lipids and body weight in the vehicle control group after 5 days oral administration of fatty food in the hamster model of hyperlipidemia.

% Increase				
TC	HDL-C	TG	LDL-C	B. wt.
73.9 ± 3.7	34.2 ± 3.7	-3.4 ±3.5	199.8 ± 28.9	3.4 ± 0.9

Values represented are mean ± SEM for n=131. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low density lipoprotein cholesterol; B.wt, body weight. Negative values(-) represent % decrease.

Table 2: The reduction (%) of serum lipids by the test compounds in the hamster model of hyperlipidemia.

Compound	Dose (mg/kg)	Percent reduction				
		TC	HDL-C	TG	LDL-C	B.wt
TRC-7004	80.9	18.6	16.0	8.5	22.2	-0.8
TRC-7007	87.6	20.1	17.6	-0.1	26.3	-11.1
TRC-7011	80.5	27.4	17.9	-2.6	39.5	-3.3
TRC-7033	80.0	29.5	9.0	44.4	36.4	6.0
TRC-7051	71.1	21.5	12.0	27.1	22.2	16.7
CP-230821	30.0	39.3	20.5	-11.3	54.9	12.4
Cerivastatin	1.25	27.7	10.6	58.7	23.4	-1.1

Values are as compared to respective vehicle control group. (n=5-9). Negative values(-) represent % increase.

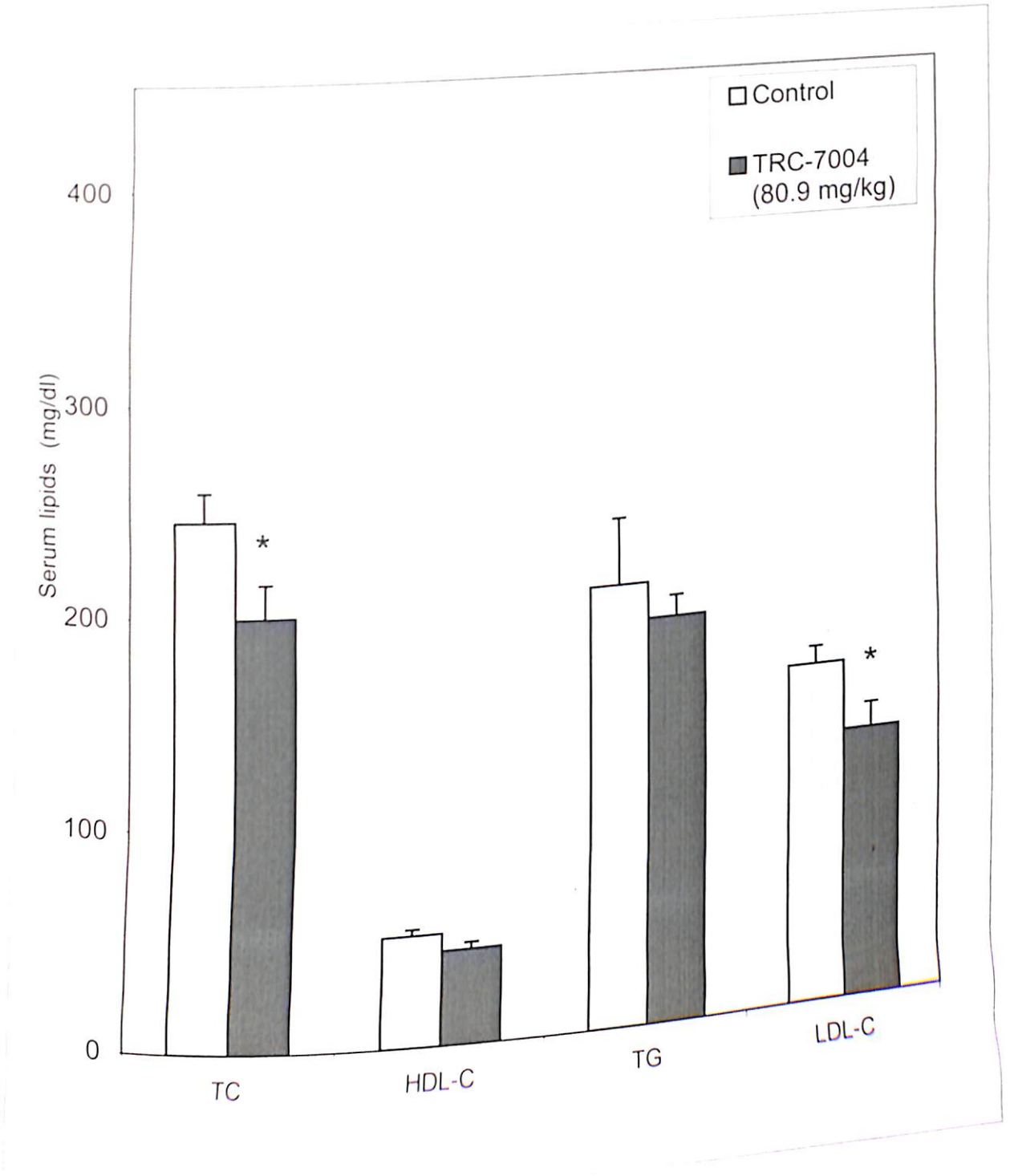


Figure 1: Effect of TRC-7004 (80.9 mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 6 observations. The error bars represent SEM. $*P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol.

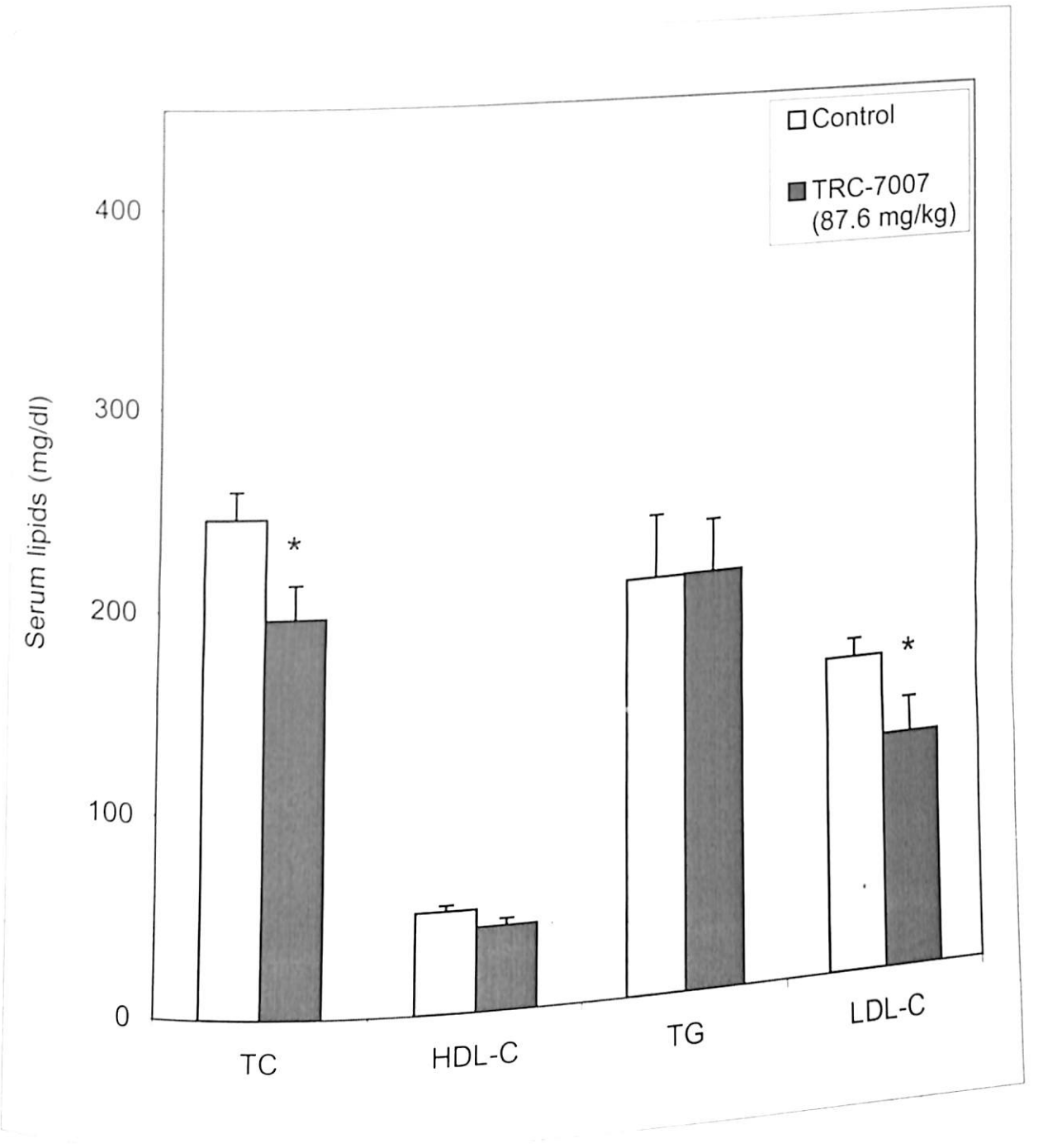


Figure 2: Effect of TRC-7007 (87.6 mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 6 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

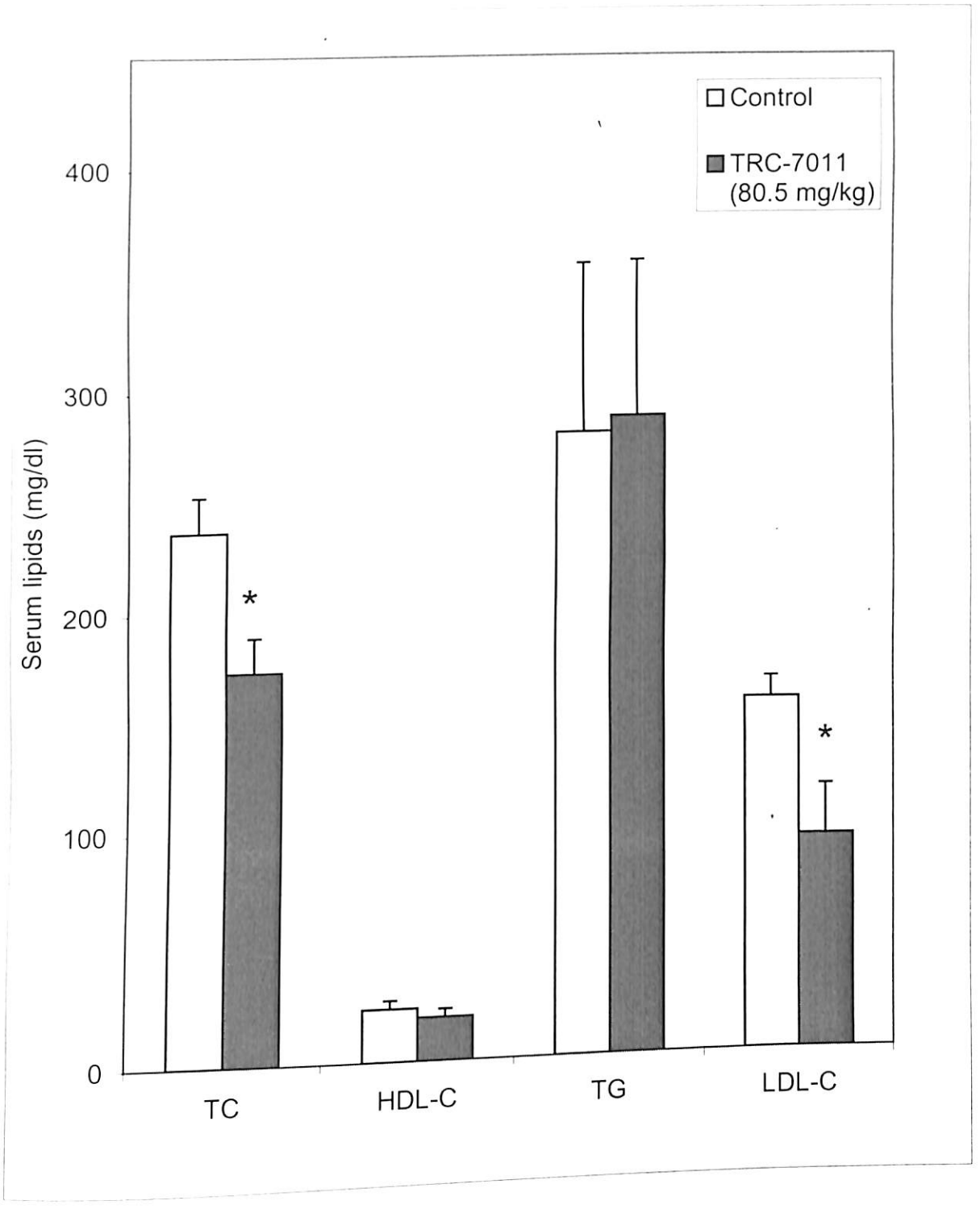


Figure 3: Effect of TRC-7011 (80.5 mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 6 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

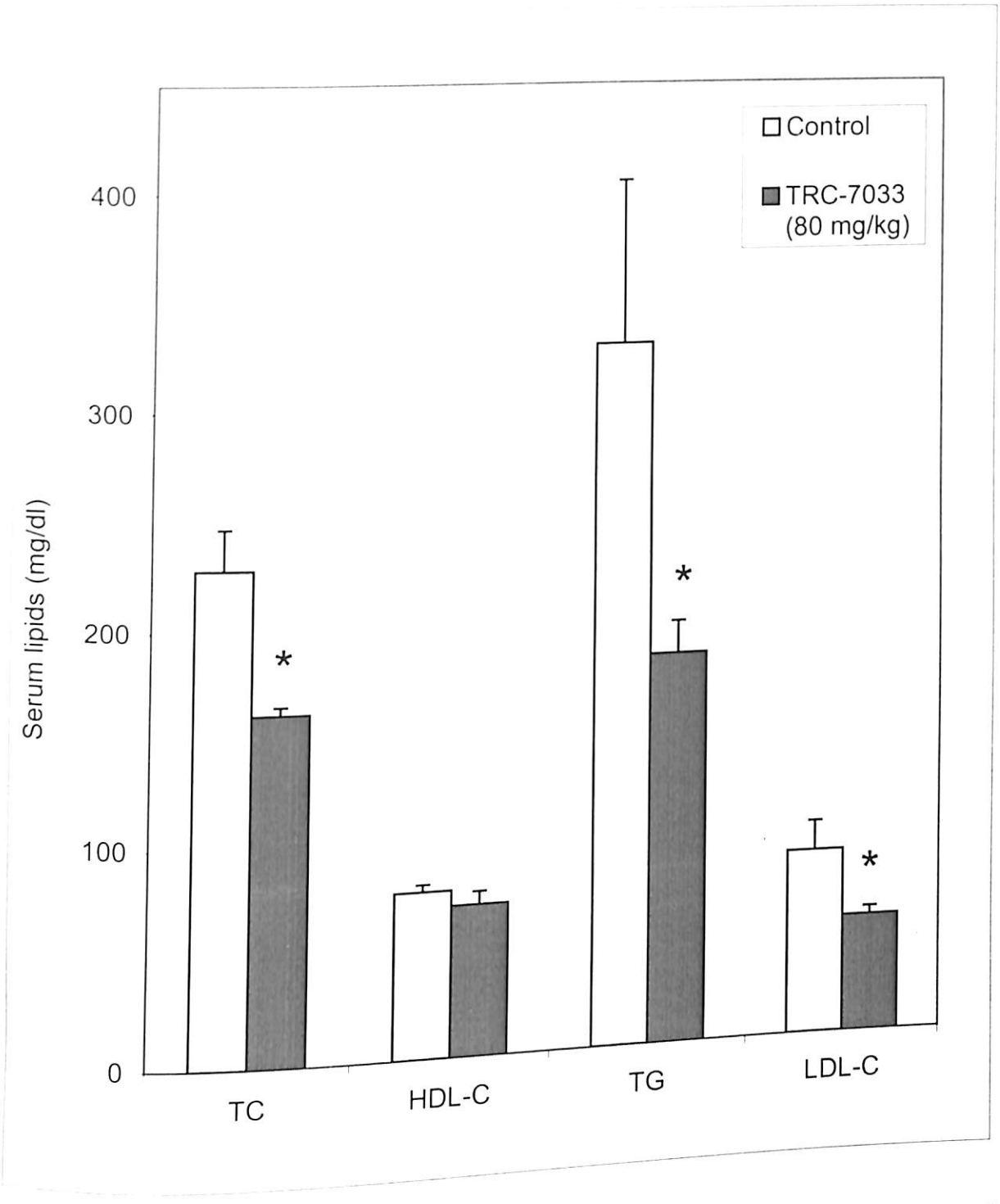


Figure 4: Effect of TRC-7033 (80mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 5 observations. The error bars represent SEM. $*P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

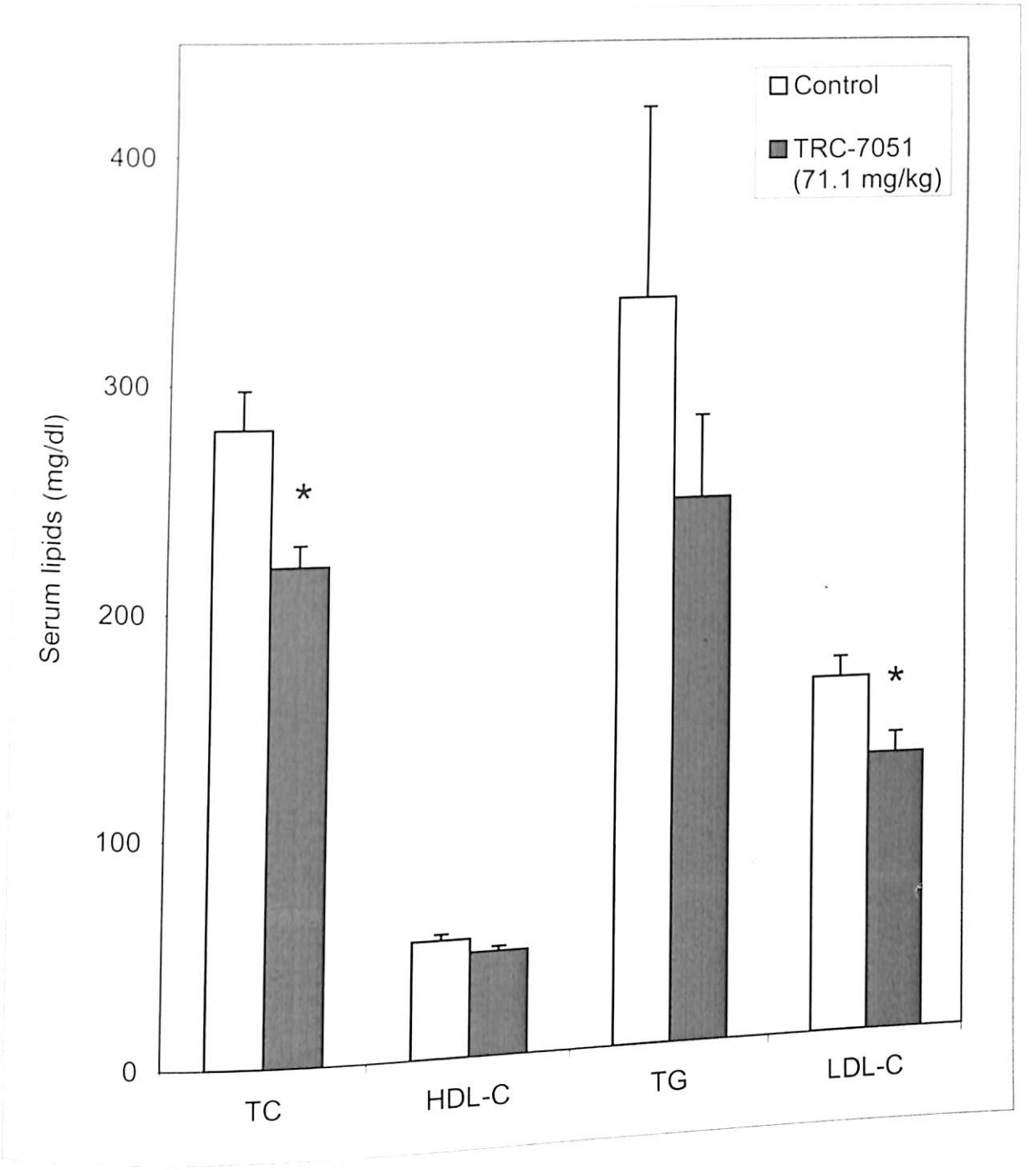


Figure 5: Effect of TRC-7051 (71.1 mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 5 observations. The error bars represent SEM. $*P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

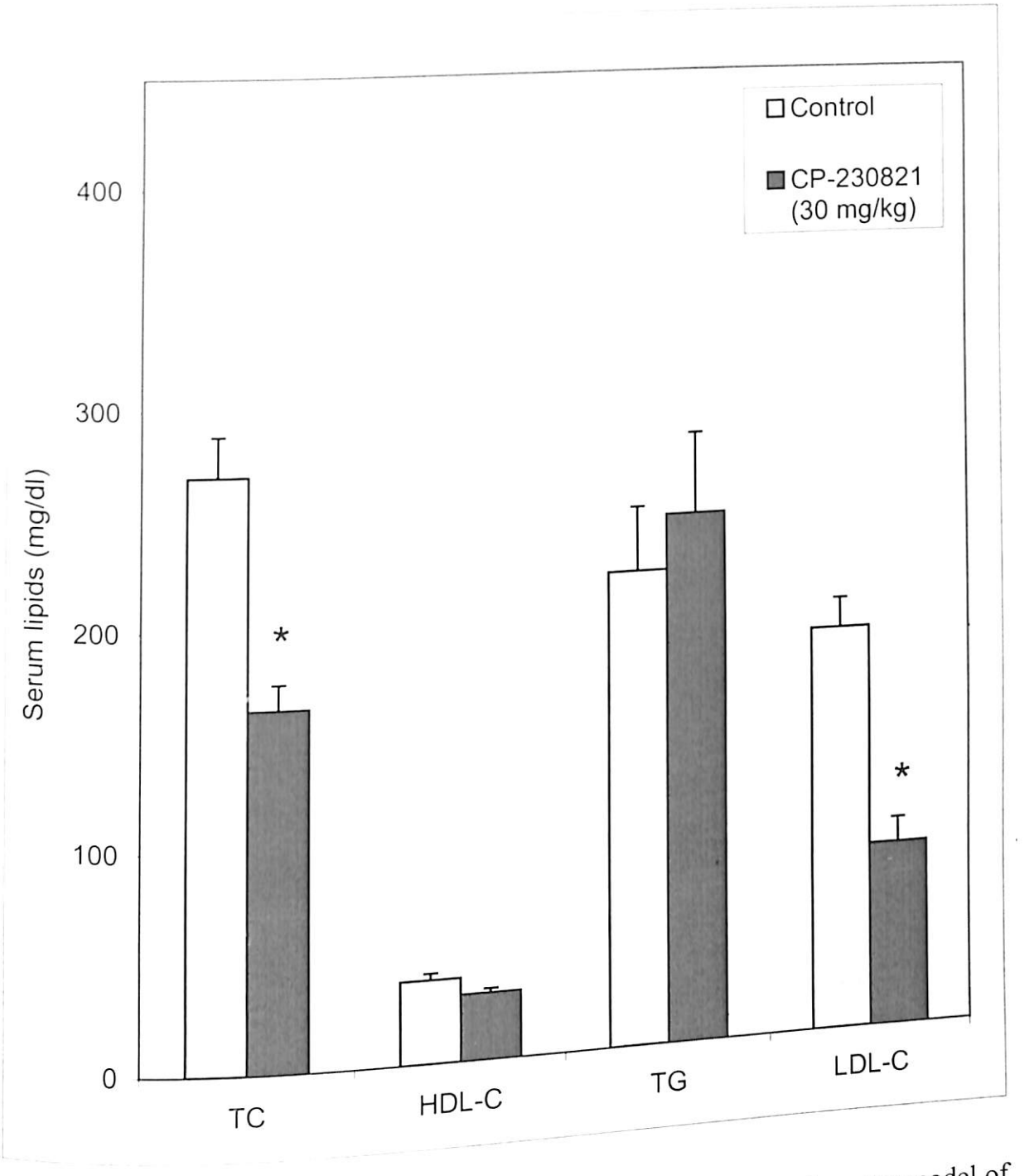


Figure 6: Effect of CP-230821 (30 mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 9 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

5.2 Anti-hyperlipidemic activity in hamsters - dose response (TRC-7033 and cerivastatin):

TRC-7033 was chosen for detailed dose response, as it had shown very good activity in reducing the serum cholesterol, LDL-cholesterol and triglyceride levels in the hamster model of hyperlipidemia compared to other molecules (Table-2). The reference molecule cerivastatin was also taken up for detailed dose response studies.

TRC-7033 at the dose 80mg/kg decreased the serum cholesterol by 30% and LDL cholesterol by about 36%. It also reduced triglyceride by 44%. It did not have any significant effect on HDL cholesterol (Figure 4). TRC-7033 at the dose 60mg/Kg decreased the serum cholesterol by 27% and LDL cholesterol by about 33%. It reduced triglyceride by 15%. It did not have any significant effect on HDL cholesterol (Figure 7). TRC-7033 at the dose 40mg/Kg decreased the serum cholesterol by 26% and LDL cholesterol by about 24%. It also reduced triglyceride by 35%. It did not have any significant effect on HDL cholesterol (Figure 8). Though dose proportionate reductions in triglycerides were not seen, TG levels were significantly reduced across all dose groups. The group means are presented in the Table 4 in *Appendix -III.*

There was no significant effect on serum total bilirubin, AST, ALT and alkaline phosphatase at any of the above doses (Table 5 in *Appendix –III).* There was no significant change in body weight or food intake (Table 4 in *Appendix –III).*

At 0.5 mg/kg, cerivastatin did not show any significant reduction in the serum cholesterol and LDL cholesterol levels while it showed significant reduction of about 39% in triglyceride levels (Figure 9). There was no significant effect on serum total bilirubin, AST, ALT and alkaline phosphatase. (Table 5 in *Appendix –III)*

Cerivastatin at the dose 1.25mg/Kg decreased the serum cholesterol by 28% and LDL cholesterol by about 23%. It reduced triglyceride by 59%. It did not have any significant effect on HDL cholesterol (Figure 10). Cerivastatin at the dose 2.5mg Kg decreased the serum cholesterol by 35% and LDL cholesterol by about 59%. The reduction seen in serum triglyceride was not significant. It did not have any significant effect on HDL cholesterol (Figure 11). ALT increased by around 70% in 2.5mg kg dose group while other parameters like serum total bilirubin, AST, alkaline phosphatase did not show any significant change (Table 5 in *Appendix –III).* There was no significant change in body weight or food intake in the 0.5, 1.25 and 2.5mg/kg doses (Table 4 in *Appendix –III).* At 5 mg/kg there was seventy percent mortality and of those that survived showed about 15-20% reduction in body weight and reduced food intake. The ALT and AST showed about 4 and 5 fold increase respectively (Table 5 in *Appendix –III).*

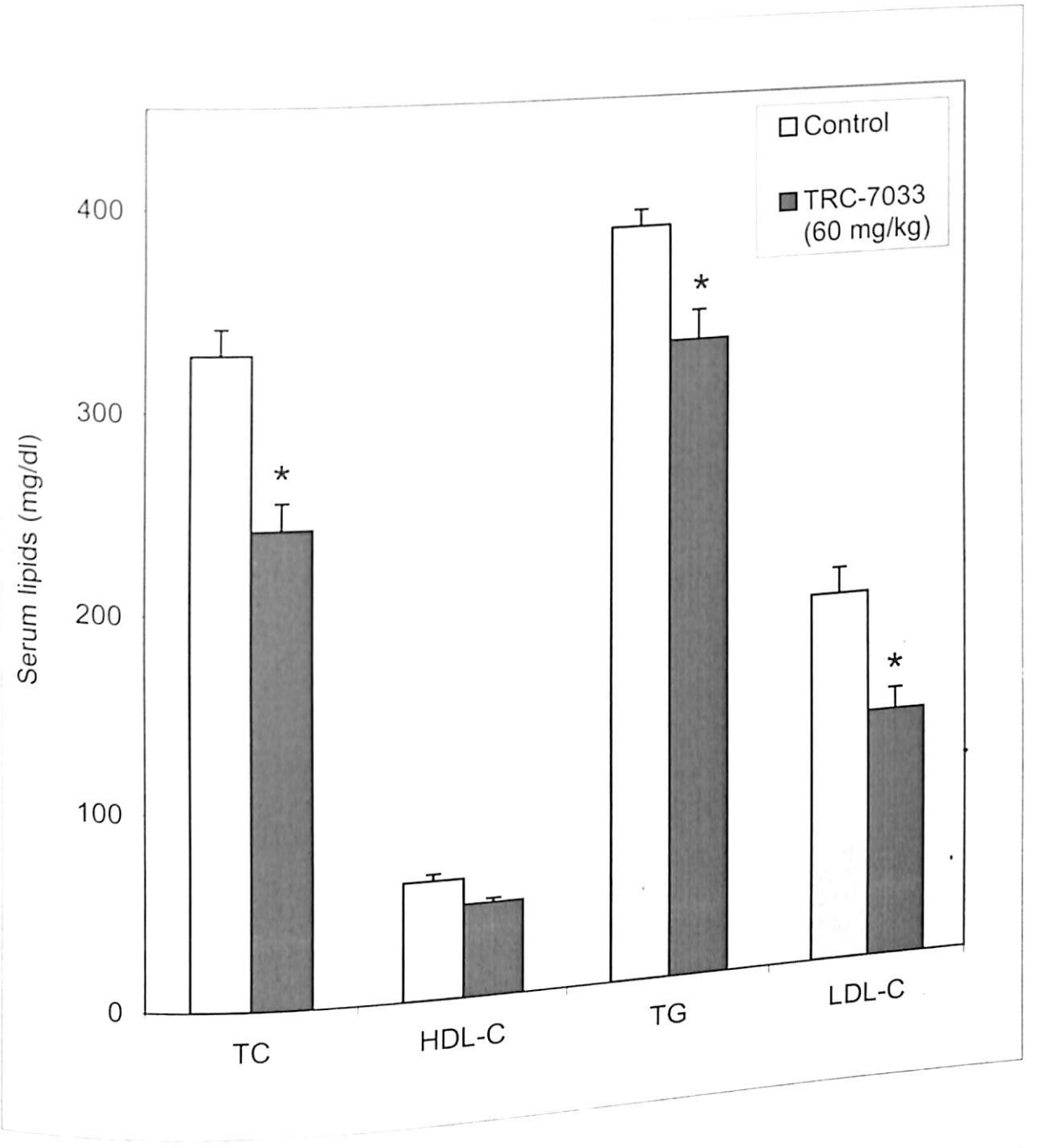


Figure 7: Effect of TRC-7033 (60mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 4-5 observations. The error bars represent SEM. $*P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

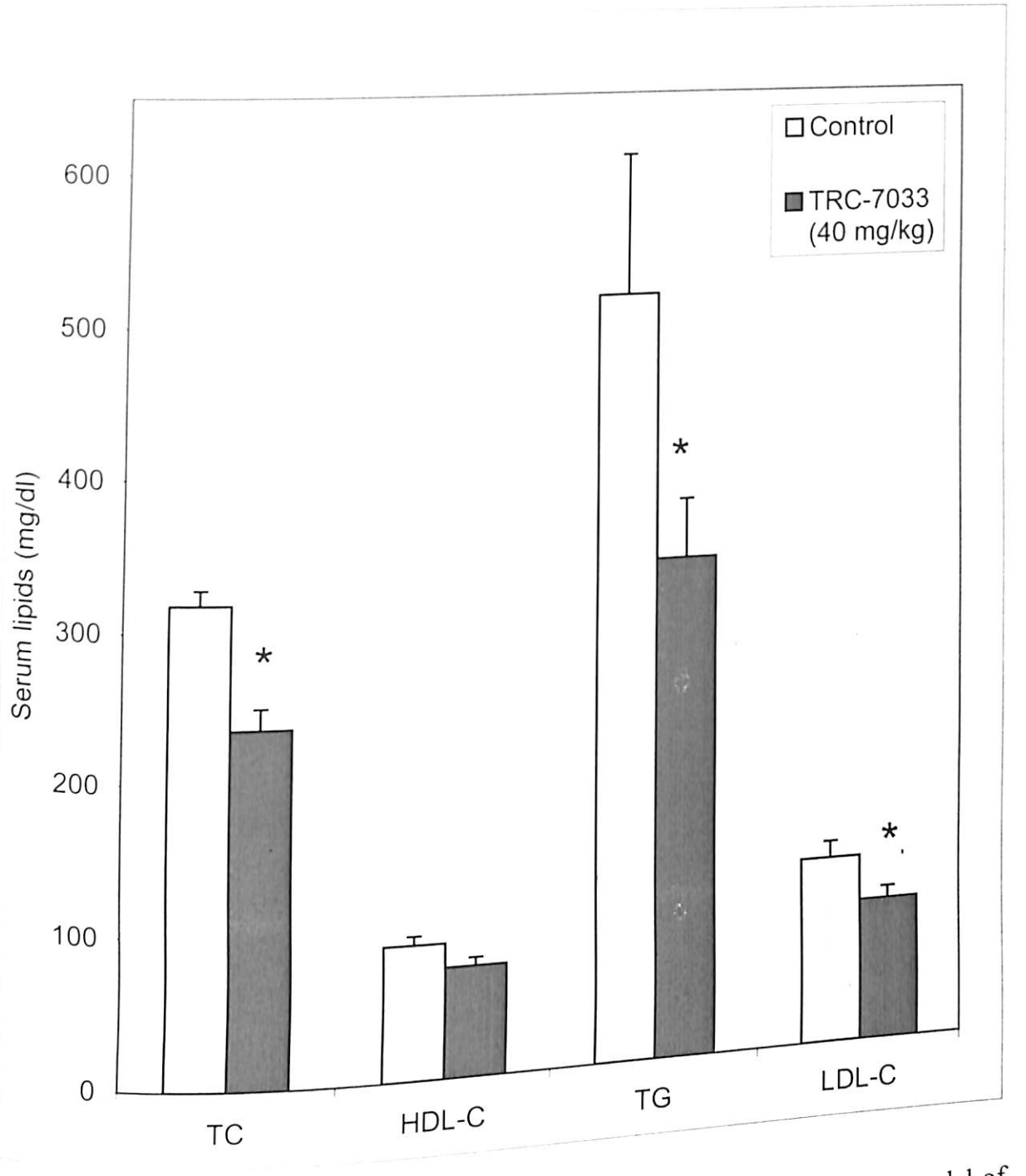


Figure 8: Effect of TRC-7033 (40mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 5-6 observations. The error bars represent SEM. $*P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

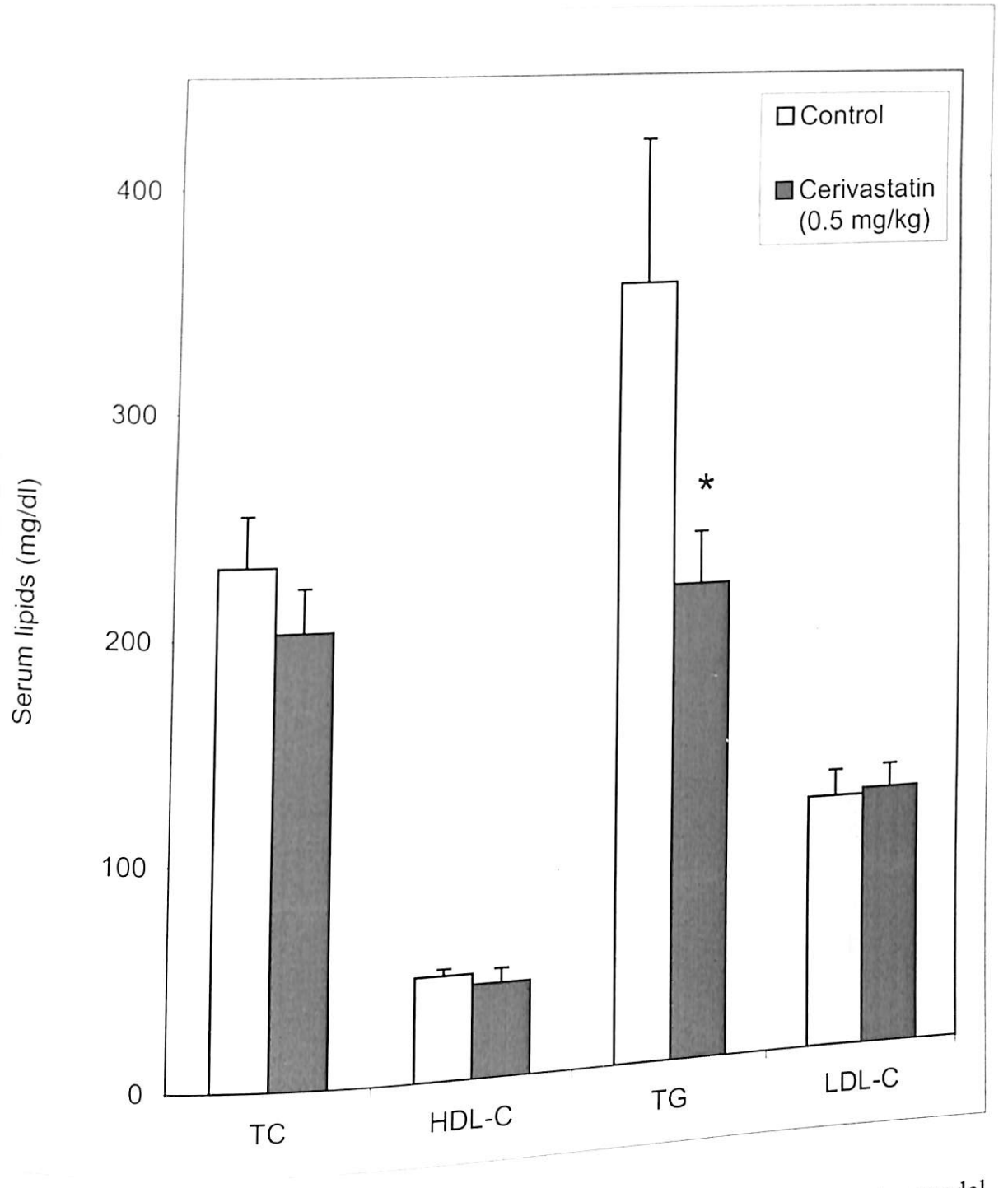


Figure 9: Effect of Cerivastatin (0.5 mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 6 observations. The error bars represent SEM. $*P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

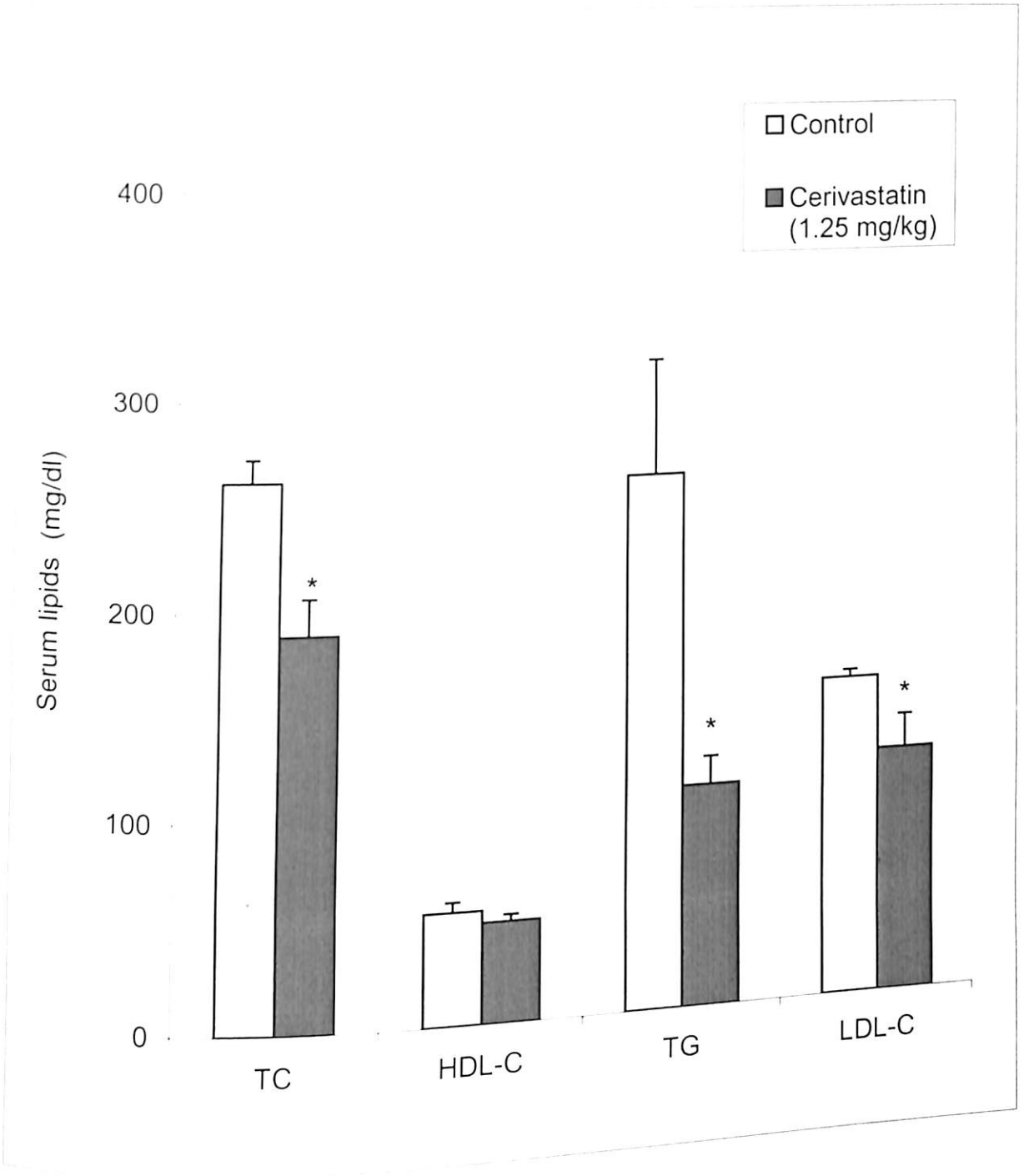


Figure 10: Effect of Cerivastatin (1.25 mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 5 observations. The error bars represent SEM. $*P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

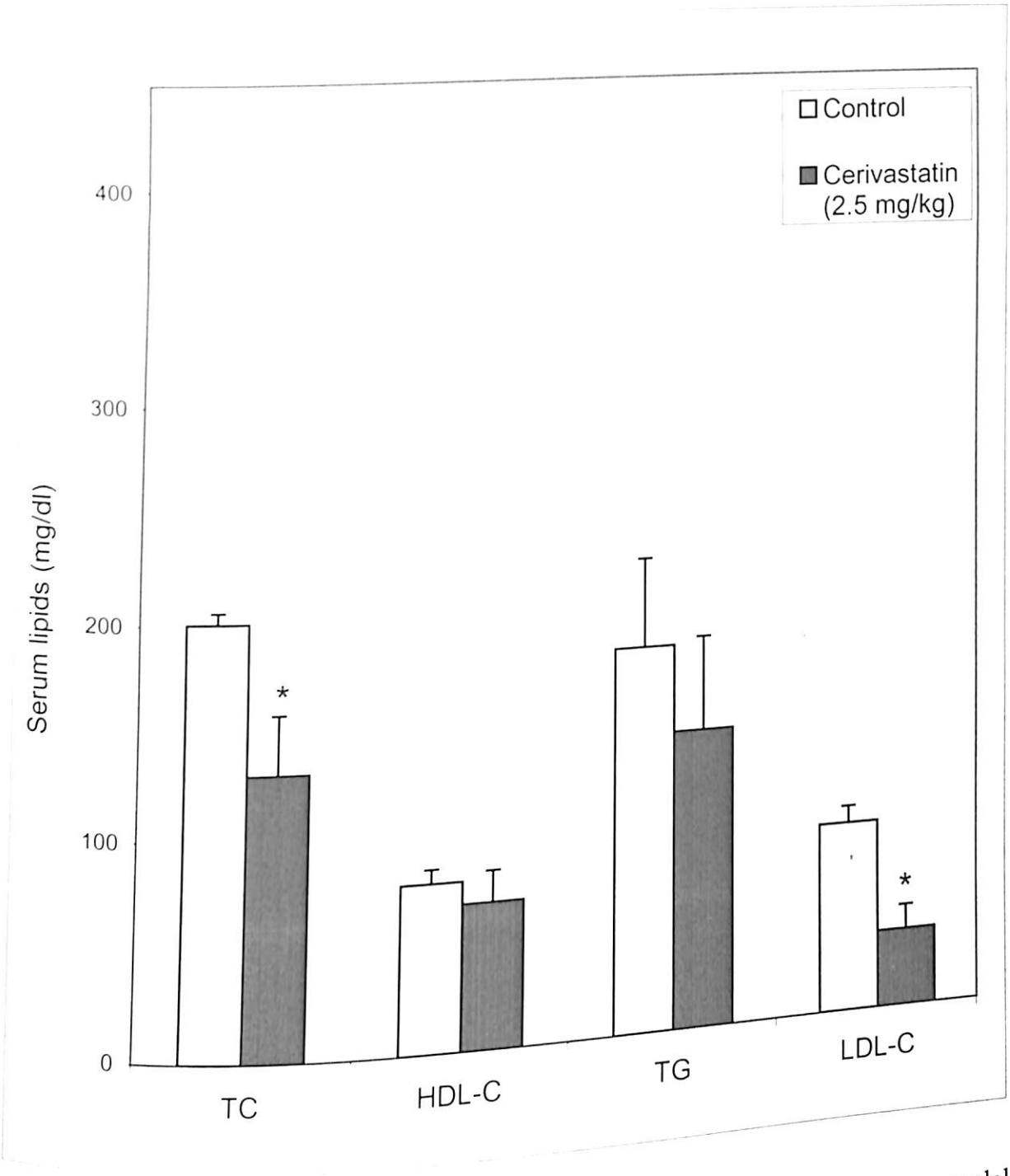


Figure 11: Effect of Cerivastatin (2.5 mg/kg) on serum lipid levels in hamster model of hyperlipidemia. Values represented are mean of 5 observations. The error bars represent SEM. * $P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

5.3 ED_{50} of TRC-7033 for reduction in LDL-C:

The ED_{50} of TRC-7033 for reduction in LDL-C was found to be 65 mg/kg (Figure-12).

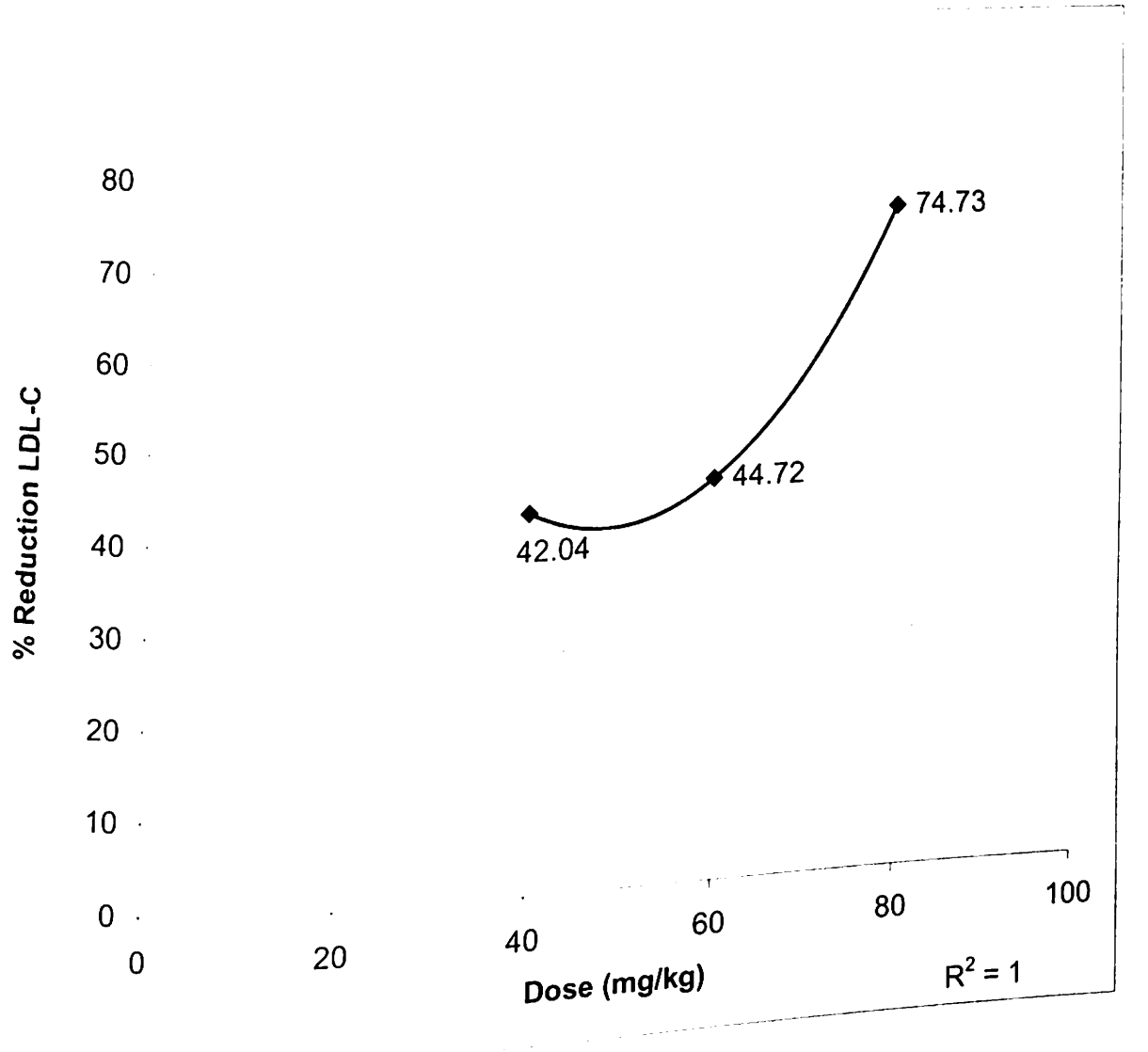


Figure 12: Dose response curve for reduction in LDL-C after oral administration of TRC-7033 in the hamster model of hyperlipidemia. The equation describing the best-fit line is $Y= 0.0342x^2-3.2823x+118.67$ and the theoretical dose for 50% reduction was derived from this equation. n=5-6 for each dose group.

5.4 Effect of TRC-7033 in normolipidemic hamsters:

Normolipidemic hamsters administered with 80 mg/kg of TRC-7033 for 5 days decreased the serum cholesterol by 18%, LDL cholesterol by about 32%. and serum triglyceride by 16%. It did not have any significant effect on HDL cholesterol. There was no significant change in body weight or food intake. The results are shown in the Figure 13. The group means are presented in Table 3.

Table 3: Serum lipid levels in normolipidemic hamster after treatment with TRC-7033 for 5 days.

Group	TC (mg/dl)	HDL-C (mg/dl)	TG (mg/dl)	LDL-C (mg/dl)	B. wt (Gms)
Control (n=5)	167±5.01	39.8±1.11	259.8±14.87	75.24±6.36	88.6±5.42
TRC-7033 (80 mg/kg) (n=6)	136.92±3.47 *	41.67±3.17	218.67±15.21*	51.52±5.09*	87±4.89

Values represented are mean ± SEM. * $P < 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; B.wt, body weight.

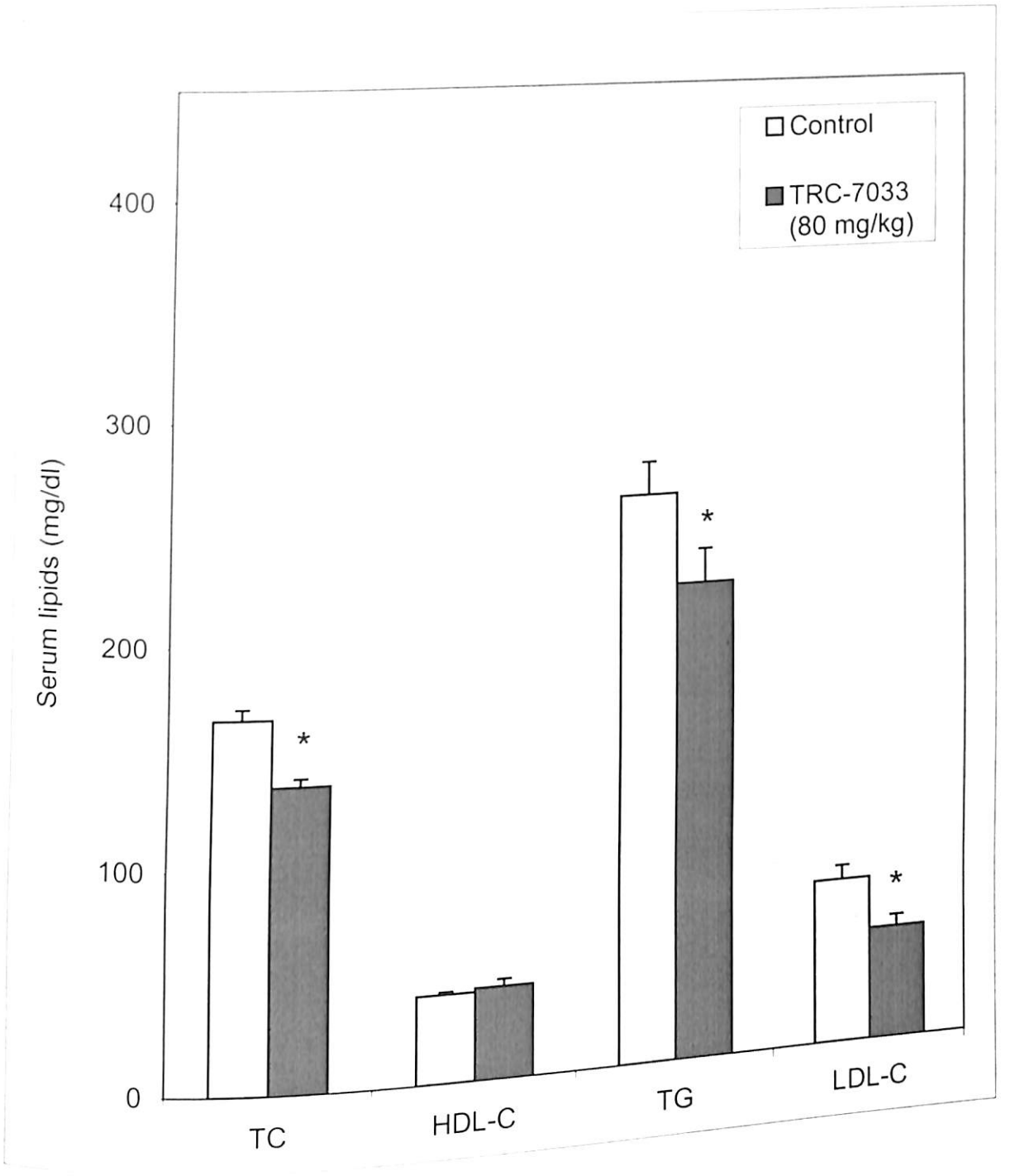


Figure 13: Effect of TRC-7033 (80mg/kg) on serum lipid levels in normolipidemic hamsters. Values represented are mean of 5-6 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol.

5.5 Hepatic lipid levels after treatment:

To examine whether the decrease in plasma lipid level results in increased hepatic lipid accumulation, the hepatic lipid contents of drug treated animals were determined and compared with the respective vehicle control group animals. The compound tested were TRC-7004, 7007, 7011, 7033, cerivastatin and CP-230821.

TRC-7004 at the dose 80.9 mg/kg decreased the hepatic free cholesterol content by 17% and the hepatic cholesterol ester (cholesterol oleate) by about 68%. The total cholesterol content in the liver reduced by about 62% (Figure 14). TRC-7007 at the dose 87.6 mg/kg had no effect on the hepatic free cholesterol content while the esterified cholesterol (cholesterol oleate) was reduced by about 88%. The total cholesterol content in the liver reduced by about 81% (Figure 15). TRC-7011 at the dose 80.5 mg/kg had no effect on the hepatic free cholesterol content while the cholesterol oleate was reduced by about 57%. The total cholesterol content in the liver reduced by about 48% (Figure 16). TRC-7033 at the dose 80 mg/kg increased the hepatic free cholesterol content by 44%. The hepatic cholesterol oleate was increased by about 69% and the total cholesterol content in the liver increased by about 65% (Figure 17).

Cerivastatin at the dose 2.5 mg/kg had no effect on hepatic free cholesterol content while hepatic cholesterol oleate was reduced by about 89% and the total cholesterol content in the liver decreased by about 81% (Figure 18). CP-230821 at the dose 30 mg/kg had no effect on hepatic free cholesterol content while hepatic cholesterol oleate was reduced by 100% and the total cholesterol content in the liver decreased by about 81% (Figure 19).

The percent reduction in hepatic lipids is shown in the Table 4. The group means are presented in the Table 6 in *Appendix-III*. Representative HPTLC chromatograms of hepatic lipid levels after vehicle/TRC-7033 treatment are shown in Figure 1 and 2 in *Appendix-III*.

Table 4: The reduction (%) of hepatic lipids by the test compounds in the hamster model of hyperlipidemia.

	Percent reduction			
Compound	**LW/BW***	**Free cholesterol**	**Cholesterol oleate**	**Total cholesterol**
TRC-7004	0.2	17.0	67.8	61.89
TRC-7011	-5.4	0.9	56.57	48.16
TRC-7007	-0.1	1.6	87.84	80.48
TRC-7033	-6.8	-43.8	-69.32	-64.52
CP-230821	20.4	7.24	88.58	89.7
Cerivastatin	-5.6	2.8	89.14	81.42

Values are as compared to respective vehicle control group. (n=5-6). Negative values (-) represent % increase. LW/BW, Liver weight/Body weight, *(LW per 100g BW)

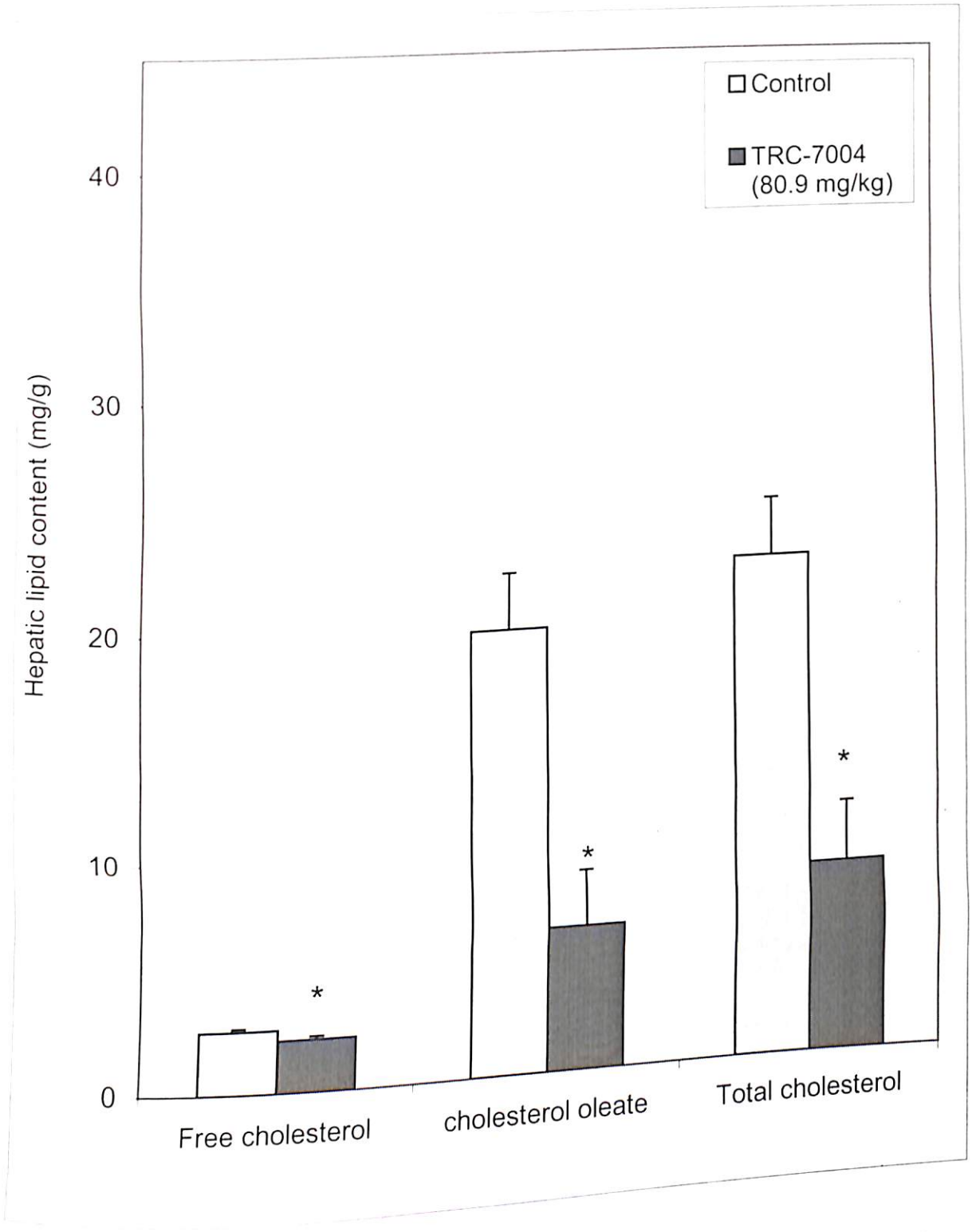


Figure 14: Effect of TRC-7004 (80.9 mg/kg) on hepatic lipid levels in hamsters. Values represented are mean of 6 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test.

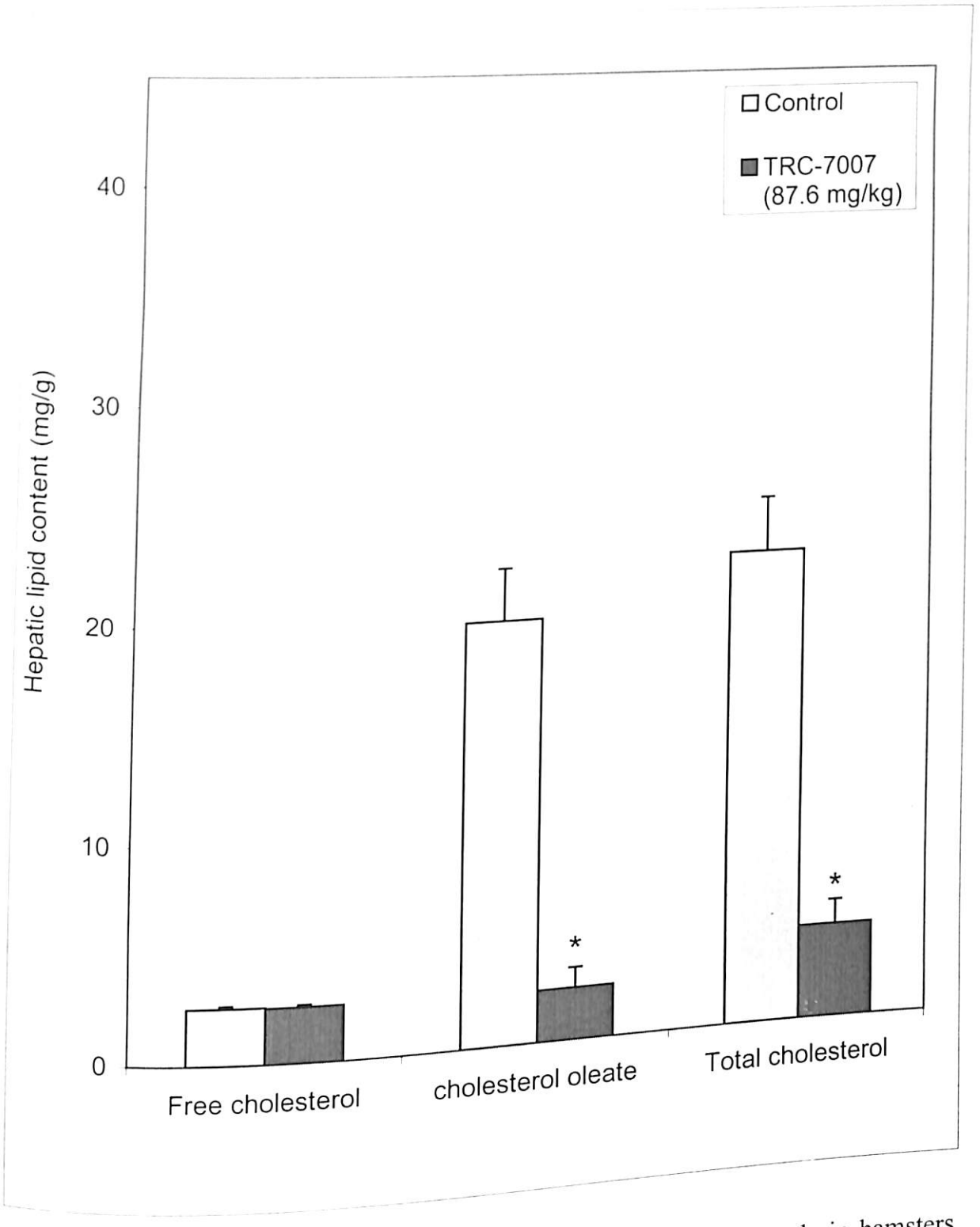


Figure 15: Effect of TRC-7007 (87.6 mg/kg) on hepatic lipid levels in hamsters. Values represented are mean of 6 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test.

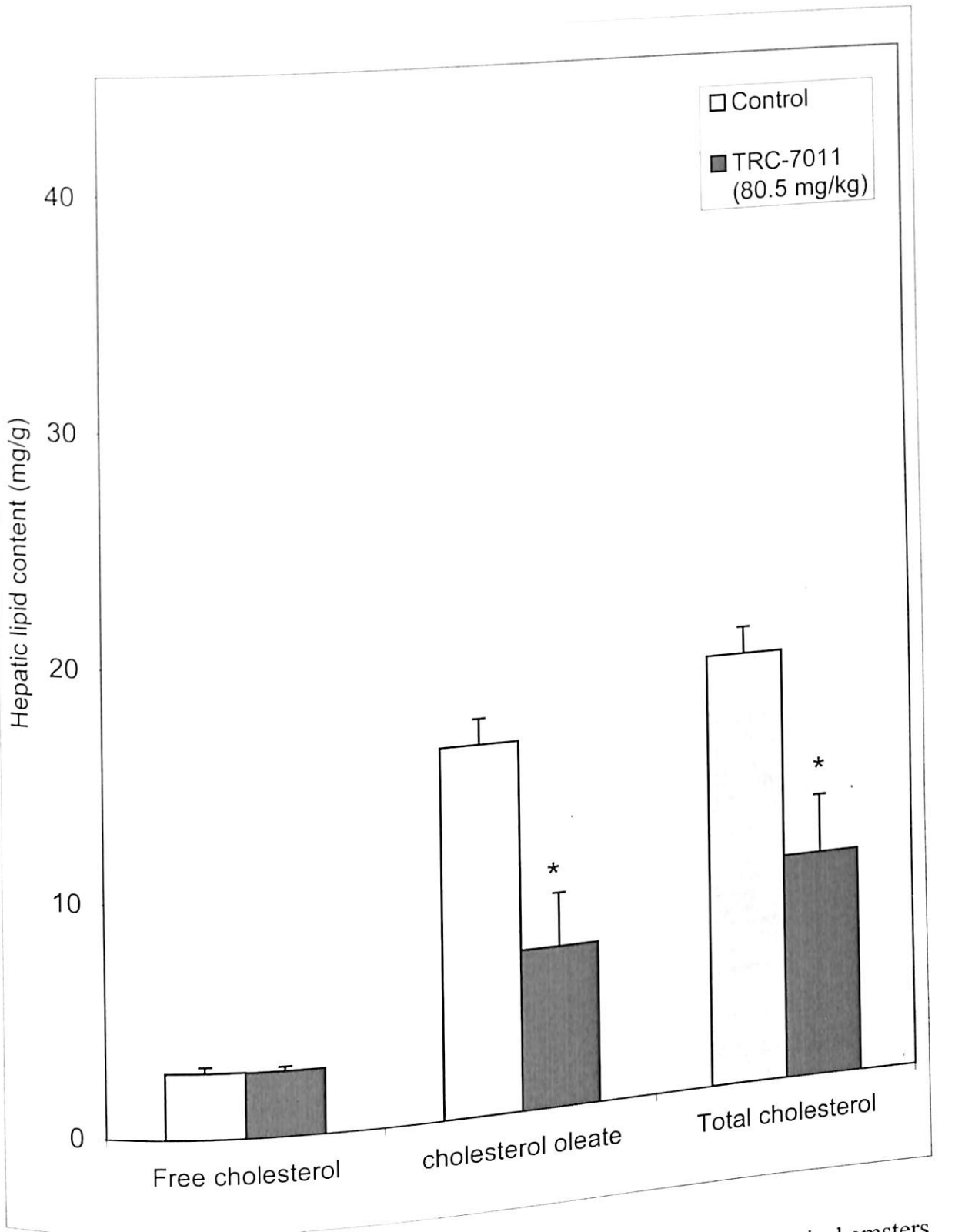


Figure 16: Effect of TRC-7011 (80.5 mg/kg) on hepatic lipid levels in hamsters. Values represented are mean of 6 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test.

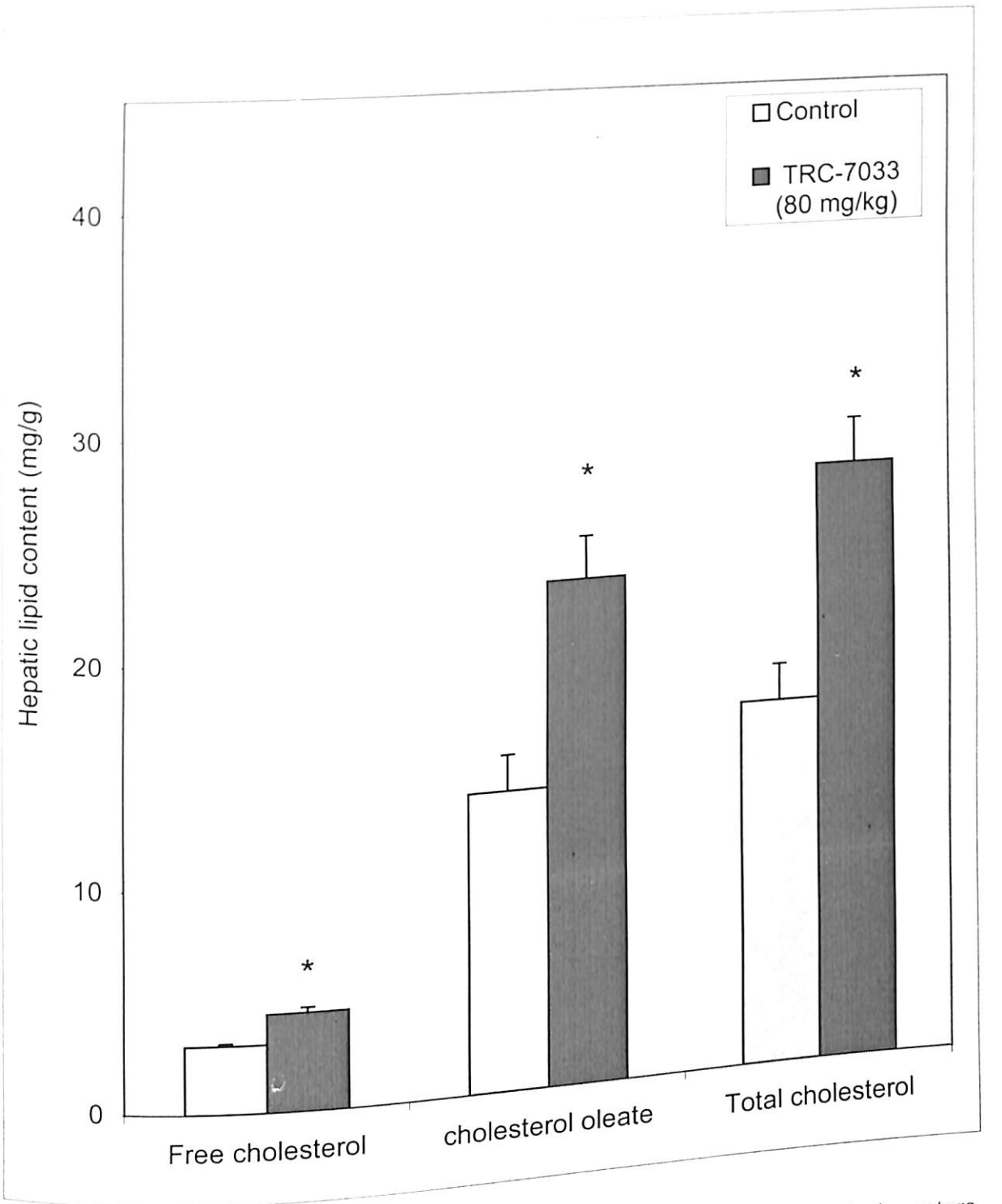


Figure 17: Effect of TRC-7033 (80 mg/kg) on hepatic lipid contents in hamsters. Values represented are mean of 5 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test.

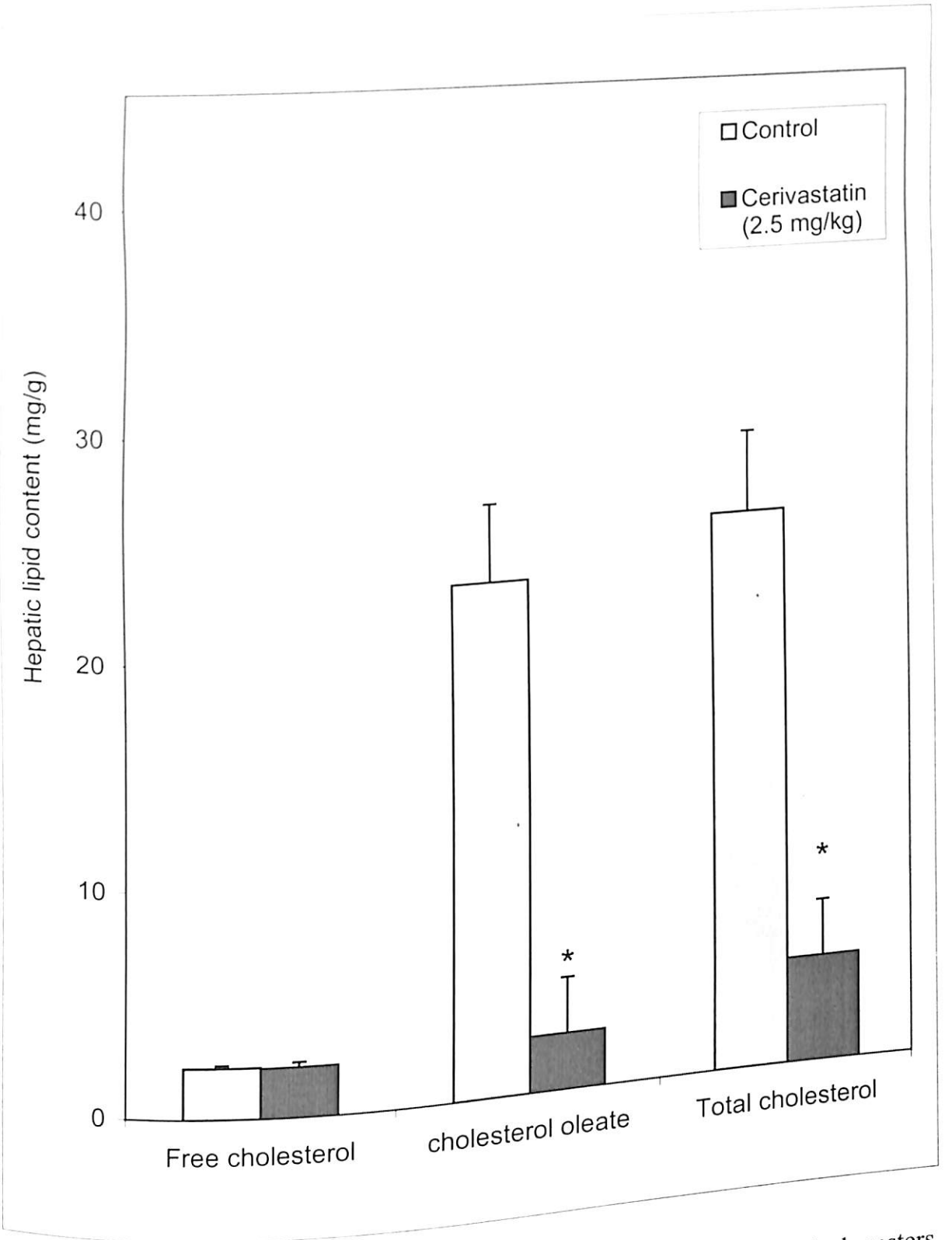


Figure 18: Effect of Cerivastatin (2.5 mg/kg) on hepatic lipid levels in hamsters. Values represented are mean of 5 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test.

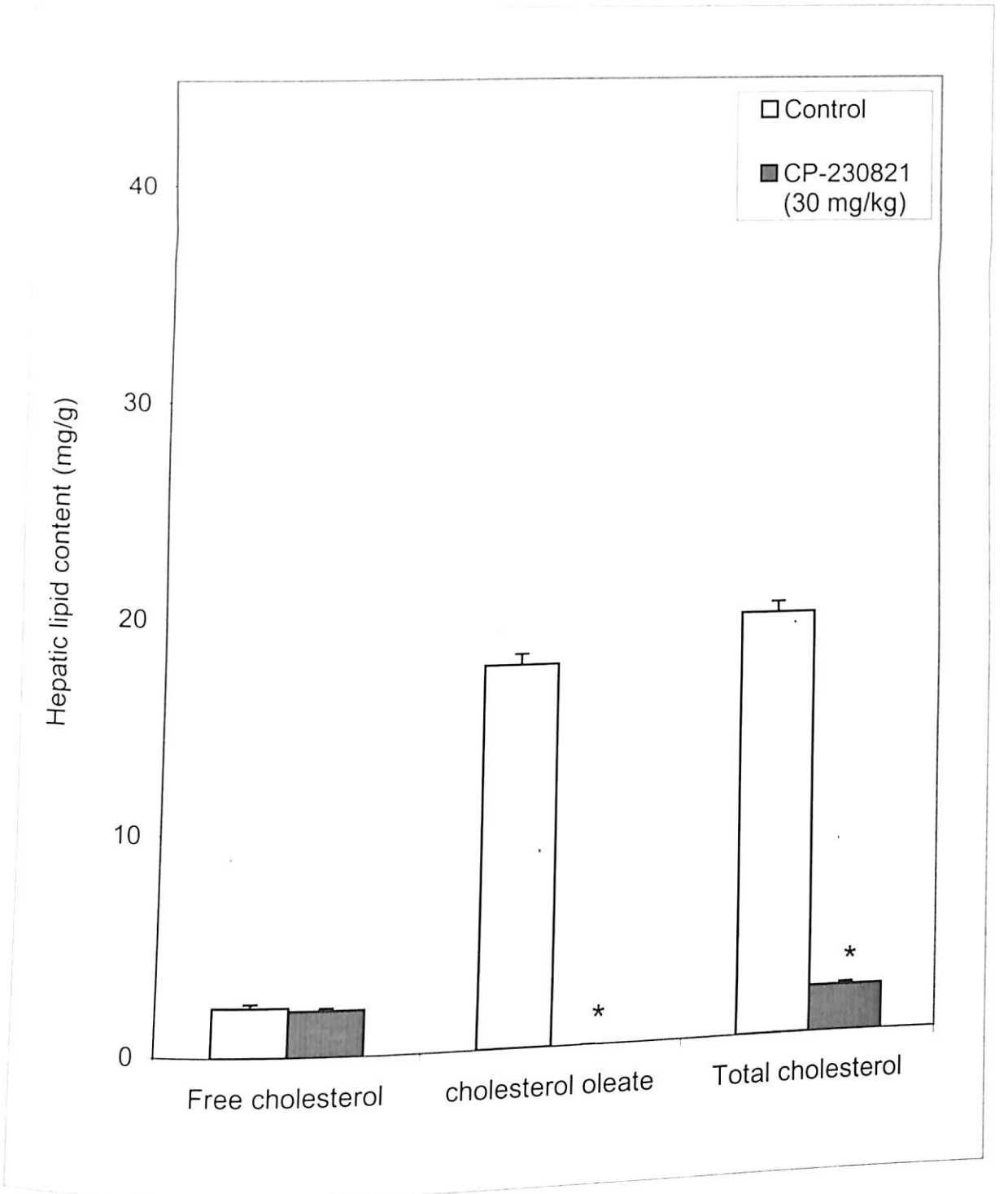


Figure 19: Effect of CP-230821 (30 mg/kg) on hepatic lipid levels in hamsters. Values represented are mean of 5 observations. The error bars represent SEM. $^{*}P< 0.05$ as compared to vehicle controls, Student's t-test.

5.6 Quantification of LDL-receptor expression:

5.6.1 Detection of monoclonal antibody:

Western blot analysis confirmed the presence of monoclonal antibody in the elute that was, obtained after the purification and concentration of the antibody from the cell supernatant.

5.6.2 Effect of TRC-7033 and cerivastatin on LDL-R expression in HepG2 cells:

A concentration response curve (1μM to 1 nM) of TRC-7033 and cerivastatin on LDL-R expression of HepG2 cell line after 24hrs of drug incubation is depicted in the Figure 20. Cerivastatin shows maximum up-regulatory activity of 70% while TRC-7033 shows 88%. Saturation in LDL-receptor up-regulatory activity was seen with cerivastatin at 0.3μM while with TRC-7033 even at 1μM it was not seen. The values are given in the Table 7 in *Appendix - III.*

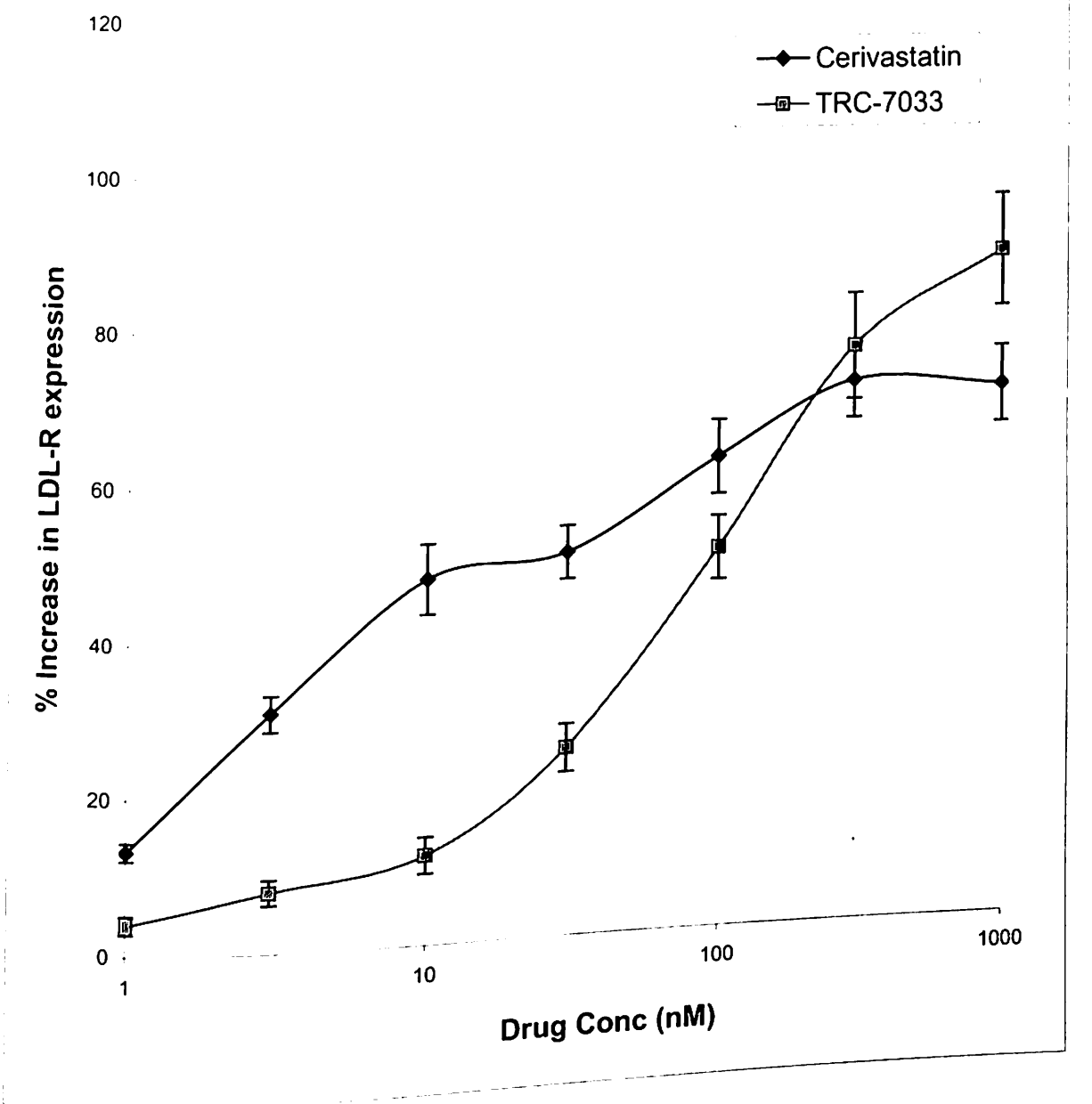


Figure 20: LDL-R expression in HepG2 cells by TRC-7033 and cerivastatin. Values represented are mean of 3 observations each done in triplicate, compared to the vehicle control (DMSO). The error bars represent SEM.

5.6.3 Effect of TRC-7033 on LDL-R expression in presence of cerivastatin:

TRC-7033 showed a concentration dependent (0 to 3μM) increase in LDL-R expression even in a maximally up-regulating concentration (0.1μM) of cerivastatin. It showed a 134% increase in up-regulatory activity at 3μM (Figure 21). The values are given in the Table 8 in *Appendix - III*.

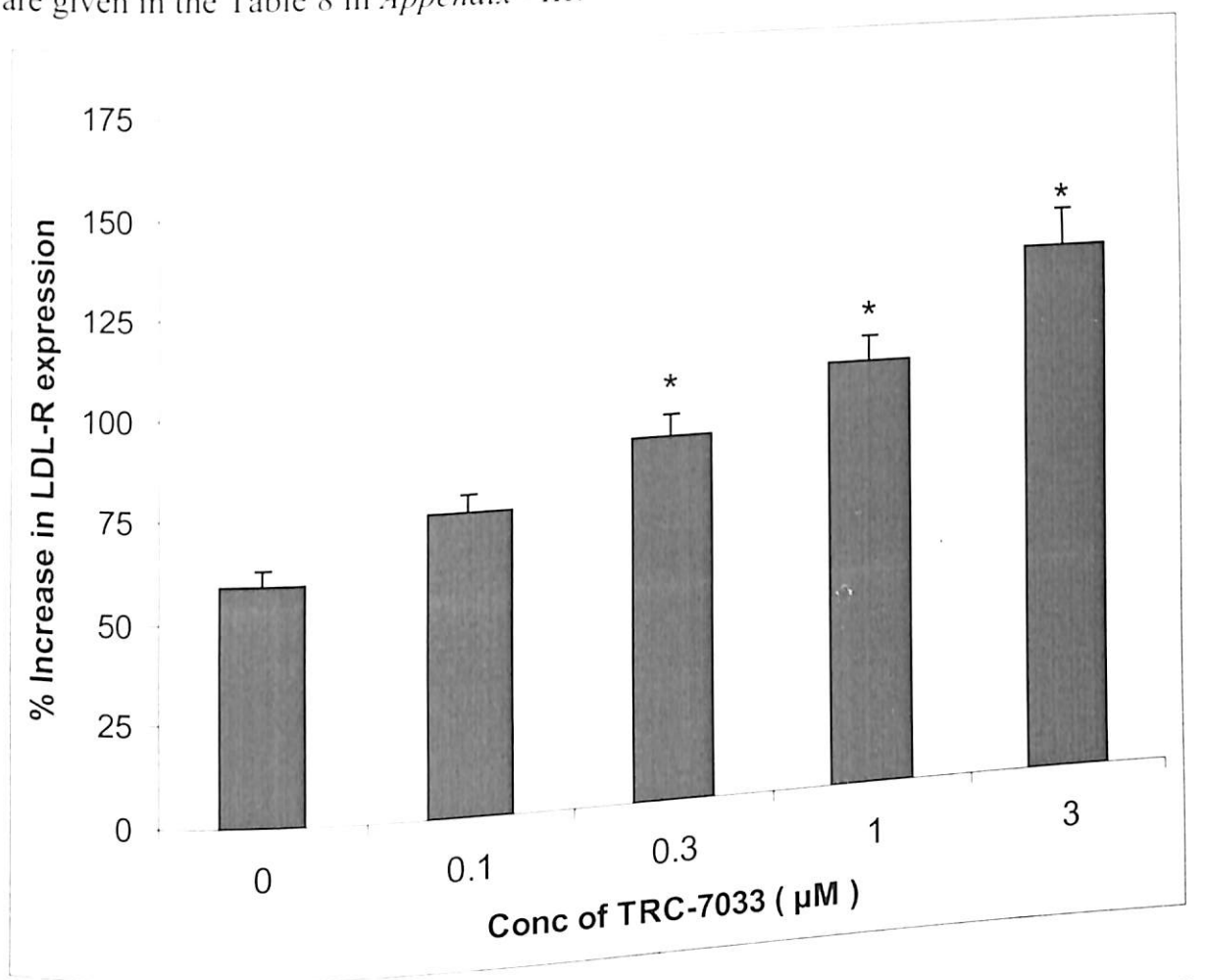


Figure 21: LDL-R Expression in HepG2 cells by TRC-7033 in presence of 0.1 μM of cerivastatin. Values represented are mean of 3 observations each done in triplicate, compared to the vehicle control (DMSO). The error bars represent SEM. *P< 0.05 as compared to 0.1μM cerivastatin alone, Student's t-test.

5.6.4 Effect of TRC-7033 and cerivastatin on LDL-R expression in presence of 25-hydroxycholesterol:

TRC-7033 showed a concentration (0 to 3μM) dependent increase in LDL-R expression even in presence of inhibitory concentration (2.5μM) of 25-hydroxycholesterol (Figure 22). It showed a 71% increase in up-regulatory activity at 3μM when compared to the vehicle (DMSO) while cerivastatin even at 3μM did not show any increase in LDL-R expression. The values are given in the Table 9 and 9a in *Appendix - III*.

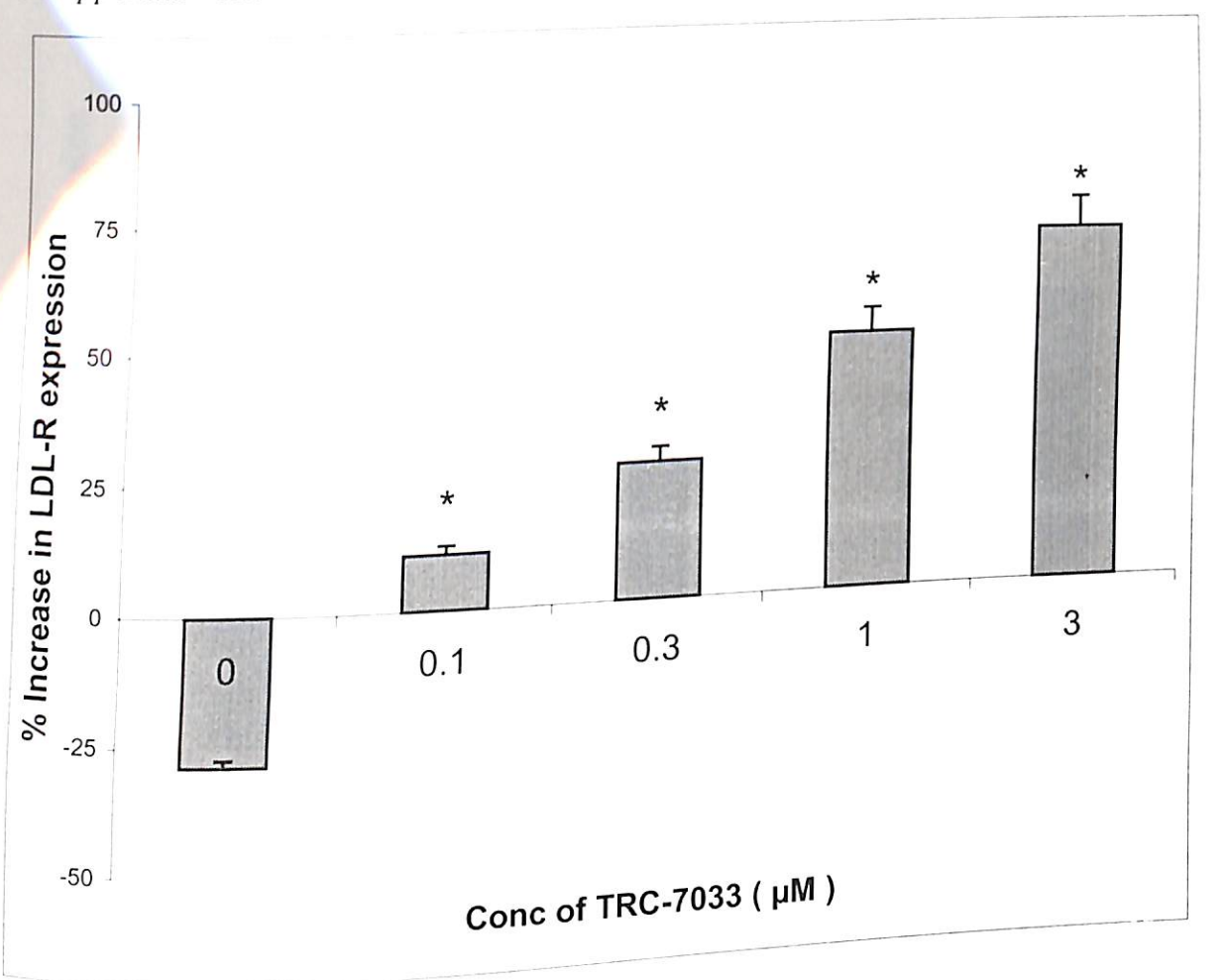


Figure 22: LDL-R Expression in HepG2 cells by TRC-7033 in presence of 2.5 μM of 25-hydroxycholesterol. Values represented are mean of 3 observations each done in triplicate, compared to the vehicle control (DMSO). The error bars represent SEM. *$P< 0.05$ as compared to 2.5μM 25-hydroxycholesterol alone, Student's t-test.

5.6.5 Effect of test compounds on LDL-R expression in HepG2 cell line:

All the compounds screened showed an LDL up-regulatory activity at 1μM. Of the test compounds screened TRC-7033 had the maximum activity of 88% while TRC-7011, 7007, 7051 and 7004 showed 69, 38, 27 and 29 percent respectively. Of the standard compounds screened CP-230821 showed about 90% while cerivastatin showed 70% (Figure 23). The values are given in the Table 10 in *Appendix - III.*

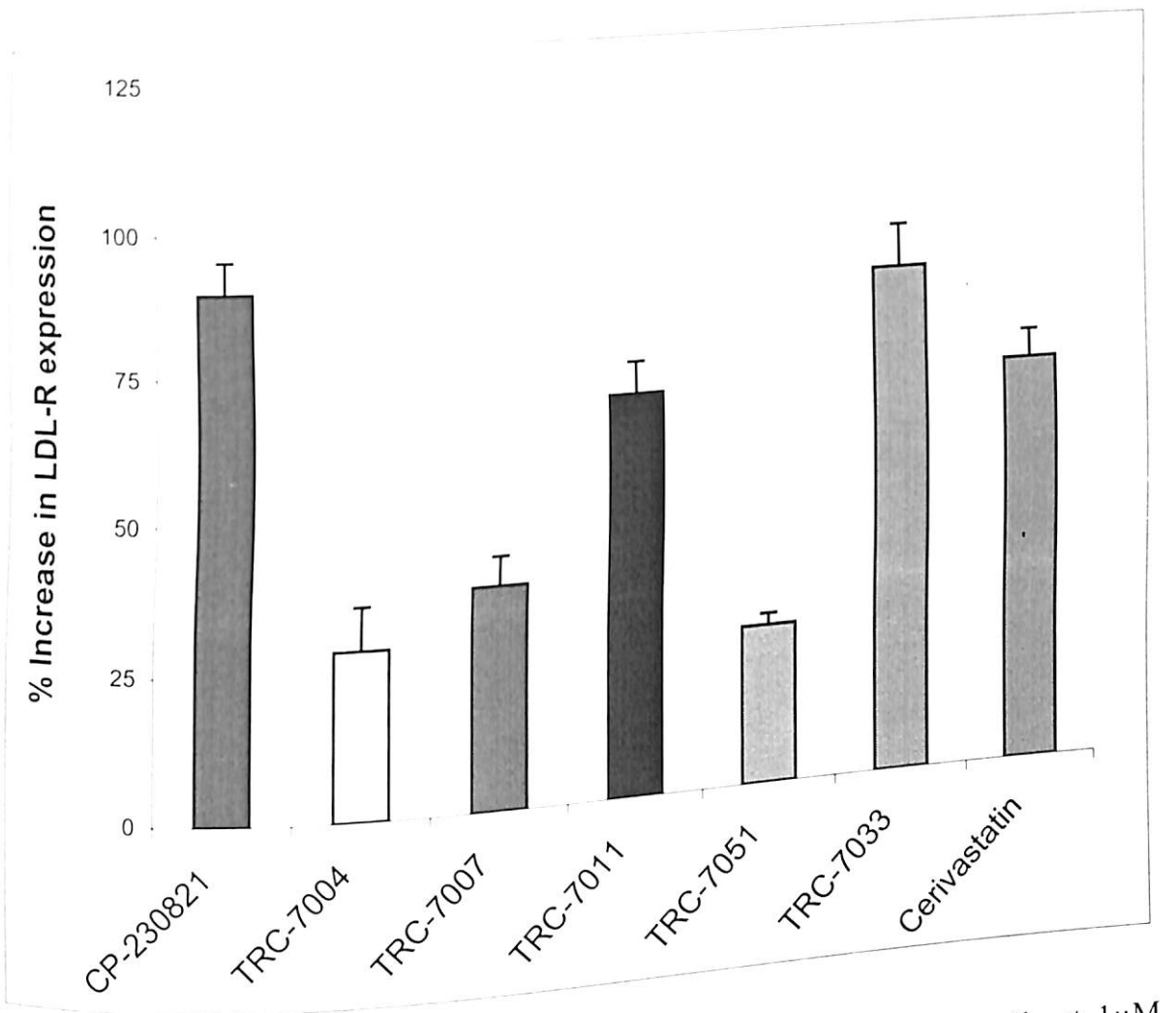


Figure 23: Effect of compounds on LDL-R expression in HepG2 cells at 1μM. Values represented are mean of 3 observations each done in triplicate, compared to the vehicle control (DMSO). The error bars represent SEM.

5.7 Cytotoxicity of test compounds in HepG2 cell line by MTT assay:

The MTT assay shows that TRC-7033 and cerivastatin did not have any cytotoxicity to HepG2 cell line after 24hrs of incubation. The results are shown in the Table 5 and Table 6.

Table 5: Cytotoxicity of cerivastatin and TRC-7033 in HepG2 cells.

Concentration (μM)	% Cytotoxicity in HepG2	
	Cerivastatin	TRC-7033
0.01	Nil	Nil
0.03	Nil	Nil
0.1	Nil	Nil
0.3	Nil	Nil
1	Nil	Nil
3	Nil	Nil
10	Nil	Nil

Note: Percent cytotoxicity below 5 was considered as nil.

Table 6: Cytotoxicity of test compounds in HepG2 cells

Test compound	Concentration (μM)	% Cytotoxicity
CP-230821	10	Nil
TRC-7004	10	Nil
TRC-7007	10	Nil
TRC-7011	10	Nil
TRC-7051	10	Nil

Note: Percent cytotoxicity below 5 was considered as nil.

5.8 Anti-oxidant activity of the test compounds:

All the test compounds were evaluated for their anti-oxidant activity and the following Figure 24 summarizes the result and the absolute values are given in the Table 11 in *Appendix - III.*

TRC-7011 had the most potent antioxidant activity; it had an EC_{50} value of 12.6μM. The other potent compounds were TRC-7029, 7007, 7034, 7038, 7025, CP-230821 in decreasing order of potency. TRC-7033 had anti-oxidant activity but was not very potent as its EC_{50} was 5.8 mM. A representative concentration response curve (TRC-7025) is shown in Figure 25.

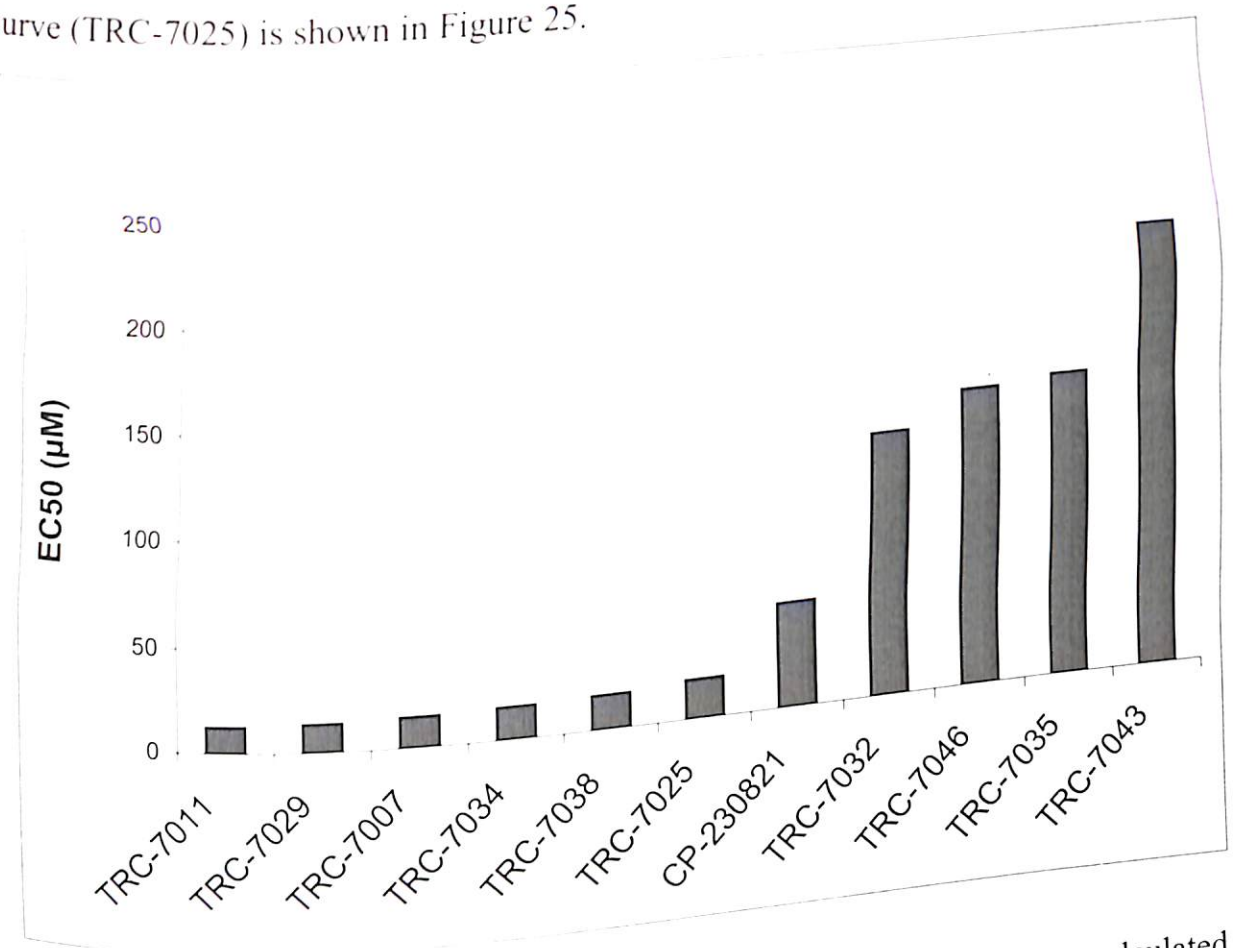


Figure 24: Anti-oxidant activity of the test compounds. The EC_{50} was calculated from a plot of concentration (12.5,25,50,100 μM) vs % anti-oxidant activity.

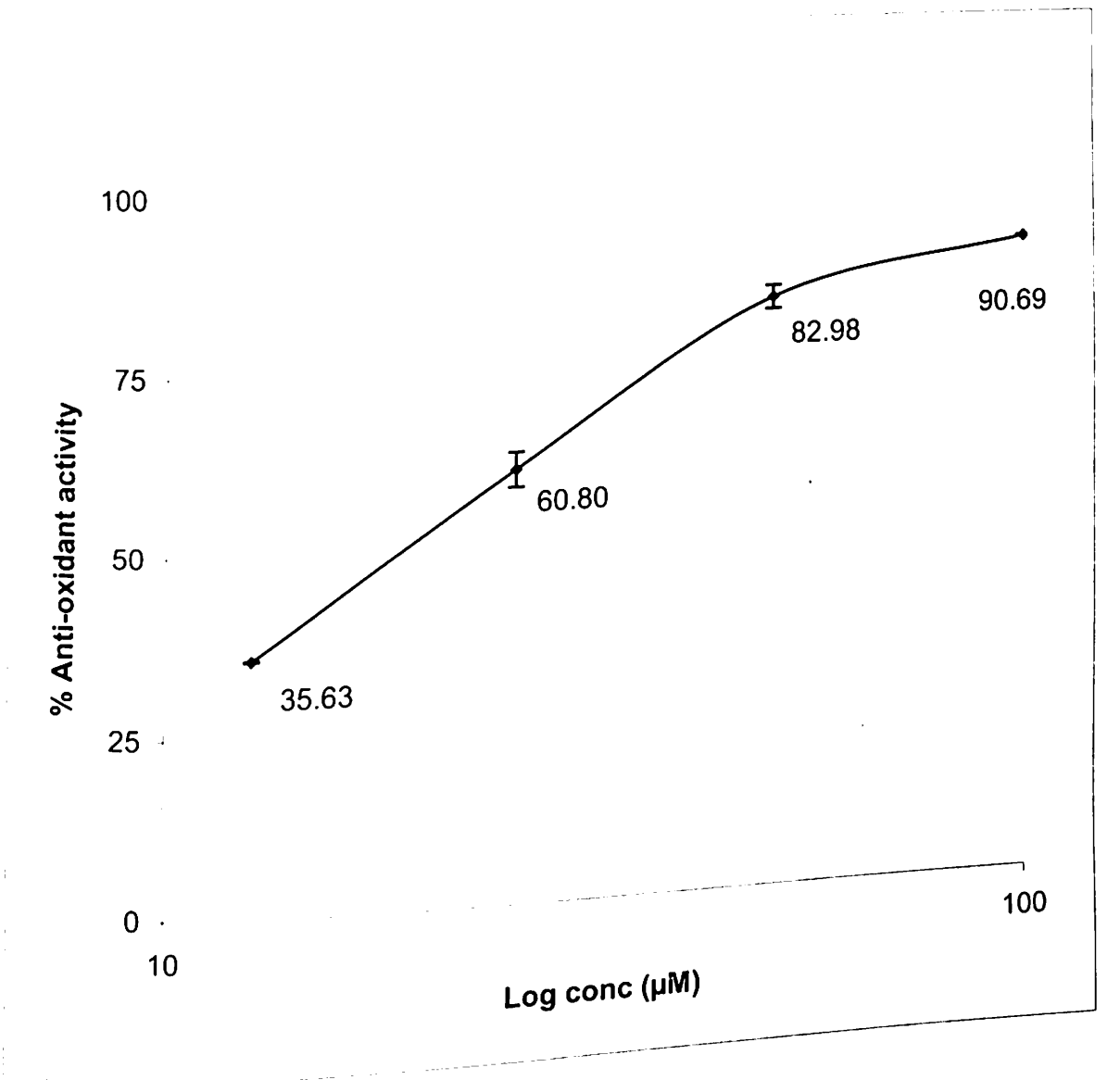


Figure 25: Anti-oxidant activity (concentration response curve) of TRC-7025. The values are mean of 2 sets of experiment done in duplicate and the error bar represent SEM.

5.9 Safety pharmacology studies on TRC-7033:

5.9.1 Effect on HR, BP, ECG and respiratory system in rats:

TRC-7033 did not have any effect on mean blood pressure, heart rate and respiration at the ED_{50} dose 65 mg/kg and 5x ED_{50} dose 325mg/kg when administered orally. TRC-7033 at a high dose of 10 mg/kg caused a significant fall in blood pressure when administered by intravenous route. The maximum fall in mean blood pressure was 54.8% while the fall in BP by the vehicle was 28.2%.

There were no significant changes in the heart rate and respiration rate after drug administration in all the three I.V. dose groups. There were no major changes in the lead II ECG pattern after the drug administration in all the oral and intravenous dose groups.

The following charts (Figures 26 and 27) summarize the result of this study. The group means are presented in the Table 12 in *Appendix-III*. A representative tracing showing various cardiovascular and respiratory parameters is shown in the Figure 3 in *Appendix-III*.

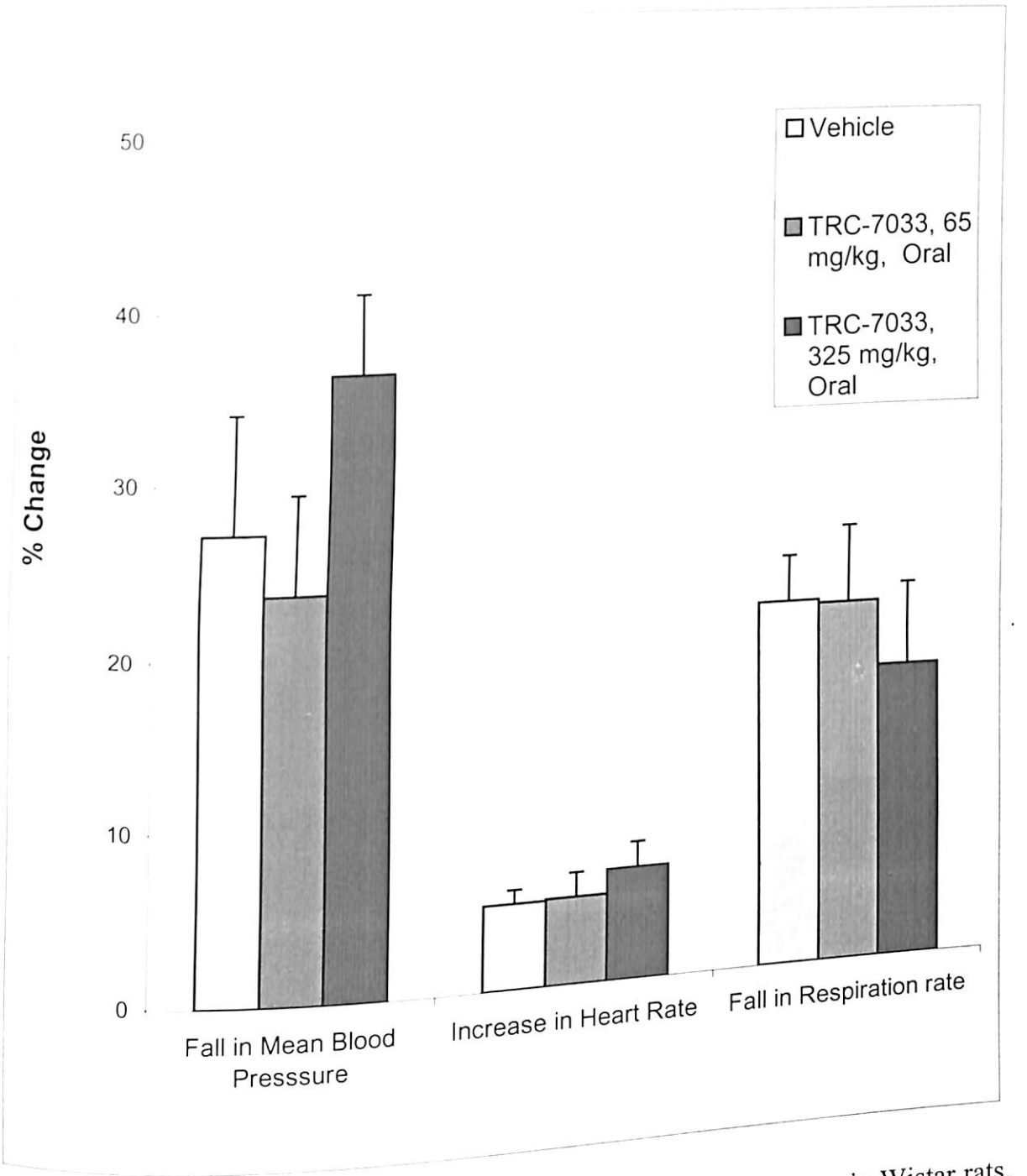


Figure 26: Effect of TRC-7033 (p.o) on CVS and respiratory system in Wistar rats. Values represented are mean of 4-5 observations. The error bars represent SEM. The responses plotted are changes (%) as compared to basal values.

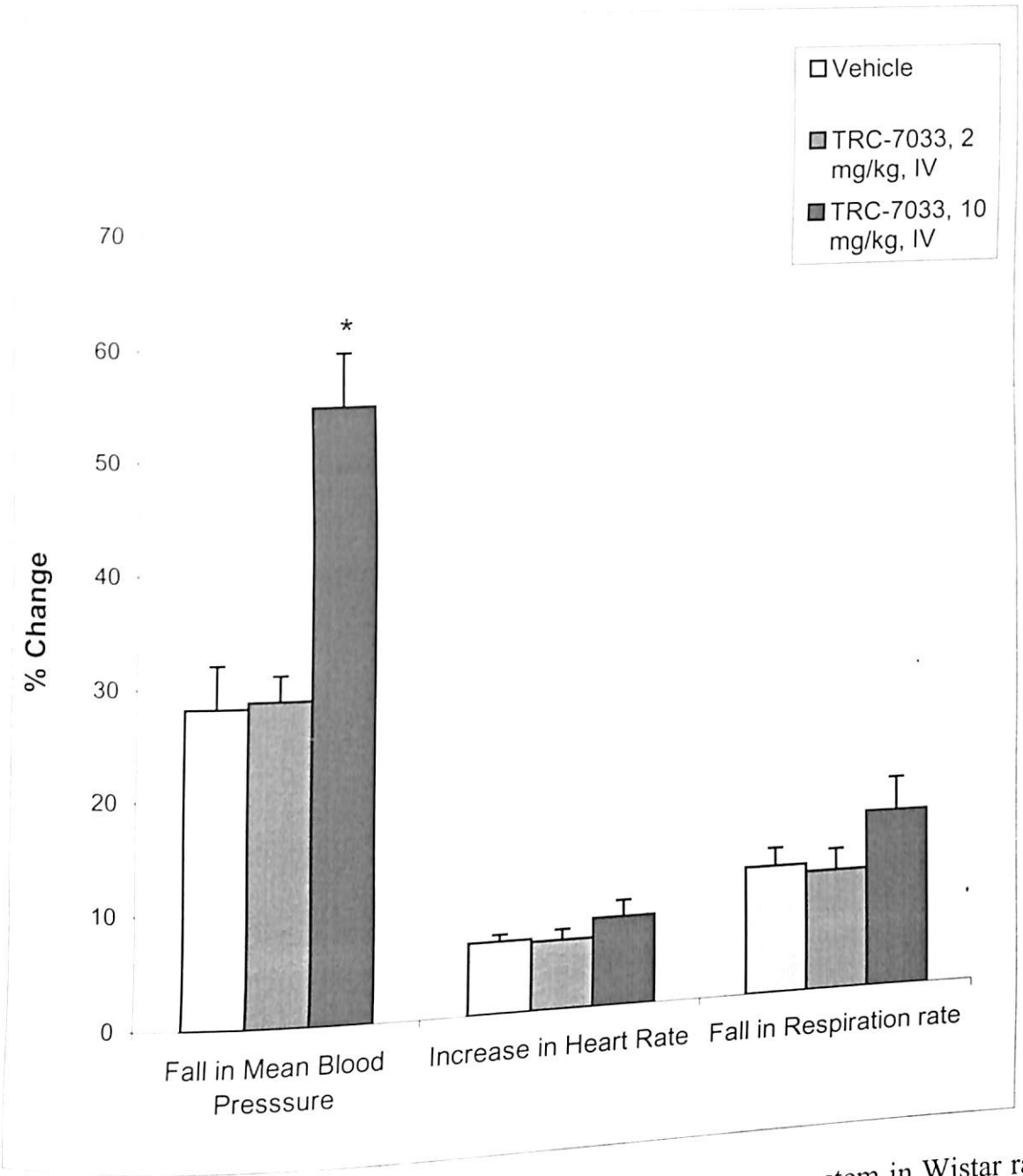


Figure 27: Effect of TRC-7033 (i.v) on CVS and respiratory system in Wistar rats. Values represented are mean of 4-5 observations. The error bars represent SEM. *$P< 0.05$ as compared to vehicle controls, Student's t-test. The responses plotted are changes (%) as compared to basal values.

5.9.2 Effect on the spontaneous motor activity in mice:

The test compound TRC-7033 did not show any significant effect on spontaneous motor activity in the low dose group – 650 mg/kg ($10xED_{50}$) and the high dose group 1625 mg/kg ($25x\ ED_{50}$) when compared to the corresponding vehicle control when given as a suspension orally. Hence it can be safely concluded that the compound does not have any major effect on the central nervous system. There was no significant difference between the activity seen for the animals treated with saline and those with the vehicle controls. The results are shown in Figure 28, the group means are presented in the Table 13, in *Appendix - III.*

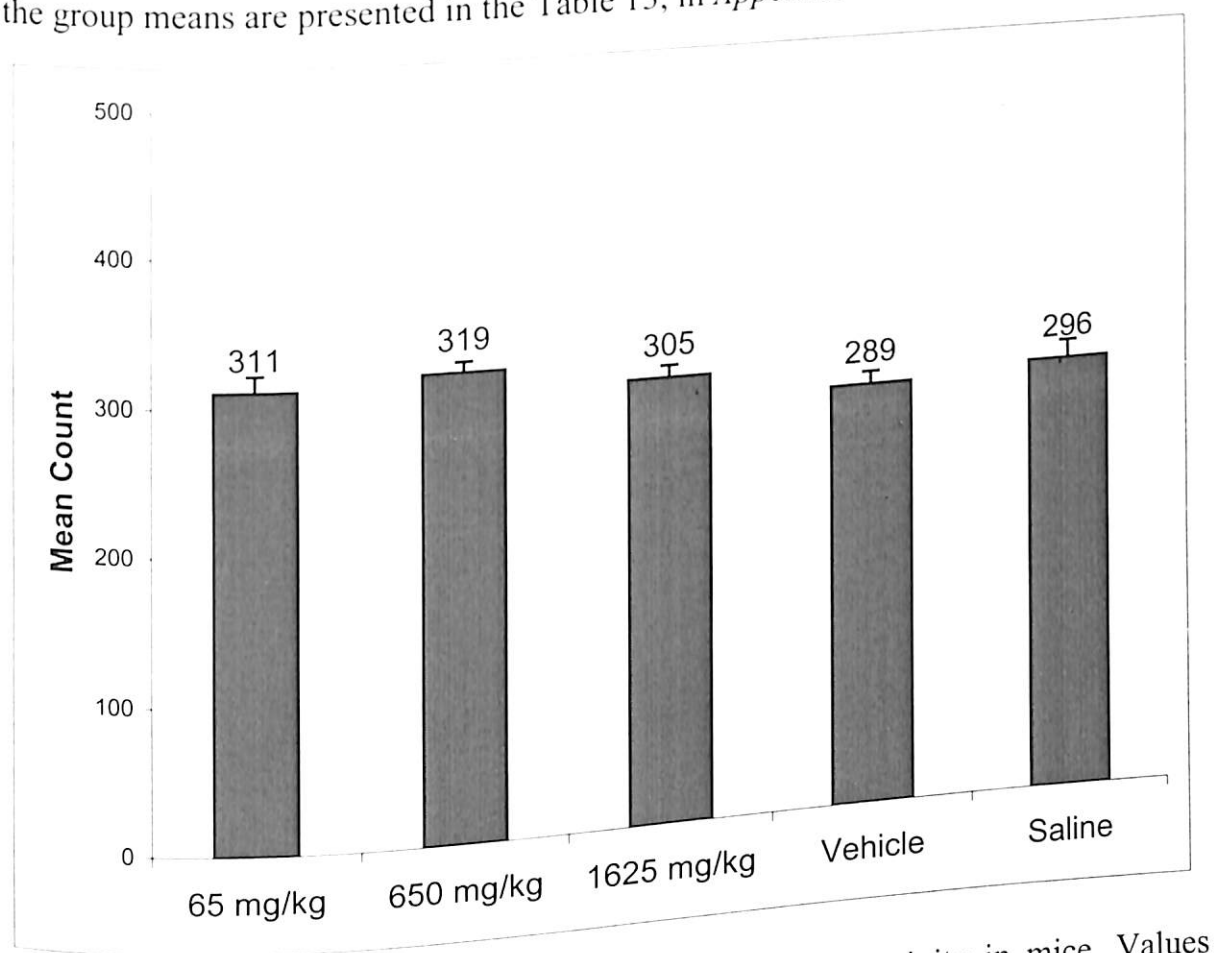


Figure 28: Effect of TRC-7033 on spontaneous motor activity in mice. Values represented are mean of 2 sets of experiment with n=10 in each dose group. The error bars represent SEM.

5.9.3 Effects on forced motor activity in mice:

TRC-7033 did not show any effect on the forced motor co-ordination of mice even at 25xED_{50} i.e.1625 mg/Kg. The results are shown in Table 7.

Table 7: Effects of TRC-7033 on forced motor activity in mice

Treatment	Activity ratio			
	0 min	60 min	120 min	180 min
Saline (n=10)	1 ± 0	1 ± 0	1 ± 0	1 ± 0
TRC-7033, 650 mg/kg (n=10)	1 ± 0	1 ± 0	1 ± 0	1 ± 0
TRC-7033, 1625 mg/kg (n=10)	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Vehicle for TRC-7033 (n=10)	1 ± 0	1 ± 0	1 ± 0	1 ± 0

Values represented are mean ± SEM for each group.

5.9.4 Effect of TRC-7033 on smooth muscles:

5.9.4.1 Studies on isolated rabbit aorta:

TRC-7033 did not relax the KCl contracted rabbit aorta at the various concentrations tested. Even the concentration as high as 10^{-5} M did not have any significant relaxant effect. It was also noted that at the concentrations tested the drug did not show any spasmogenic effect.

5.9.4.2 Studies on isolated rat uterus:

TRC-7033 showed relaxant activity on KCl precontracted tissue only at the highest concentration of 10^{-5} M. It showed a similar relaxant profile both in the estrous and diestrous states. The results are shown in Table 8. Test on spasmogenic potential of TRC-7033 on uterus (estrous and diestrous state) maintained at a resting tension of 0.5 gms showed no effect even at the highest concentration. A representative tracing showing concentration response curve of TRC-7033 is shown in the Figure 4, in *Appendix-III*

Table 8: Relaxant effects of TRC-7033 on the isolated rat uterus.

Drug	% Max relaxation at 10μM	
	Estrous state	Diestrous state
TRC-7033	39 ± 7.1*	36.3 ± 6.9*

Values represented are mean ± SEM, n=5 in each state. *P< 0.05 as compared to vehicle controls, Student's t-test.

5.9.4.3 Studies on isolated guinea pig ileum:

TRC-7033 did not relax the KCl contracted guinea pig gastrointestinal smooth muscle at the various concentrations tested. Even the concentration as high as 10^{-5} M did not have any significant relaxant effect. It was also noted that at the concentrations tested the drug did not show any spasmogenic effect. A representative tracing showing concentration response curve of TRC-7033 on isolated guinea pig ileum is shown in the Figure 5, in *Appendix-III.*

5.9.4.4 Studies on isolated guinea pig tracheal rings:

TRC-7033 did not relax the KCl contracted guniea pig tracheal rings at the various concentrations tested. Even the concentration as high as 10^{-5} M did not have any significant relaxant effect. It was also noted that at the concentrations tested the drug did not show any spasmogenic effect.

5.10 Probe toxicity study of TRC-7033:

Swiss albino mice of either sex in all the three groups (two test group and one vehicle control group) fed either an oral suspension of the test compound or vehicle did not show any abnormal symptoms. All the animals in the TRC-7033 low dose group - $10xED_{50}$ (650 mg/kg), high dose group - $25xED_{50}$ (1625 mg/kg) and vehicle control group retained their placing, righting, pinnal and other reflexes.

No abnormal behaviour was observed with animals treated with low dose of TRC-7033 (10mg/kg iv) or with its vehicle controls. Only the animals in the high dose group (20 mg/kg iv) showed a loss in the exploratory behaviour and inactivity for 10 mins after which they recovered completely. Animals showed laboured breathing, which returned to normal within fifteen minutes post drug administration. One of the animal (female) showed mild jittery behaviour immediately on drug administration but recovered within 2 minutes. On the basis of the above observations it can be concluded that the drug has a wide safety margin. A representative probe toxicity study data sheet of TRC-7033 is shown in Table 9.

Table 9: Probe toxicity study of TRC-7033 at 650 mg/kg (oral)

Probe toxicity studies

Test compound: TRC-7033
Date: 17-9-03
Time: 9.30 am
Route of admn: Oral ~~Intravenous~~
Dose: 650 mg kg
Volume : 5ml kg

Animal: Mice
Sex: F
Weight: 24g
Solvent:: Sod. CMC
Time of death: -

Strain: Swiss Albino
Age: 8 week

* √ - Present
X - Absent

GENERAL OBSERVATIONS

	0 hr	10 min	20 min	40 min	1hr	2 hr	3 hr	4 hr	6 hr	24 hr	48 hr	72hr	Onset	Disappear
Bizzare physical	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Exploratory behaviour	√	√	√	√	√	√	√	√	√	√	√	√	-	-
Aggressiveness	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Inactivity	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Convulsions (sponta:)	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Dyspnoea	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Jittery behaviour	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Blanching of	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Salivation	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Nasal discharge	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Piloerection	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Sensitivity to sound	√	√	√	√	√	√	√	√	√	√	√	√	-	-

PHYSICAL EXAMINATION

	0 hr	10 min	20 min	40 min	1hr	2 hr	3 hr	4 hr	6 hr	24 hr	48 hr	72hrs	Onset	Disappear
Altered muscle tone	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Catatonia	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Muscle tremors	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Aggressiveness	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Convulsion to touch	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Paralysis	X	X	X	X	X	X	√	√	√	√	√	√	-	-
Sensitivity to pain	√	√	√	√	√	√	√	√	X	X	X	X	-	-
Skin lesion	X	X	X	X	X	X	X	X	√	√	√	√	-	-
Reflexes Placing	√	√	√	√	√	√	√	√	√	√	√	√	-	-
Righting	√	√	√	√	√	√	√	√	√	√	√	√	-	-
Grasping	√	√	√	√	√	√	√	√	√	√	√	√	-	-
Pinnal	√	√	√	√	√	√	√	√	√	√	√	√	-	-
Coma	X	X	X	X	X	X	X	X	X	X	X	X	-	-
Death	X	X	X	X	X	X	X	X	X	X	X	X	-	-

Discussion

6. Discussion

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in large arteries. It is the primary cause of heart disease (CHD-coronary heart disease) and stroke. In the westernized societies, it is the underlying cause of about 50% of all deaths. Epidemiological studies have revealed several important environmental and genetic risk factors associated with atherosclerosis.

The early lesions of atherosclerosis consist of subendothelial accumulation of cholesterol-engorged macrophages called foam cells. The cholesterol involved here is LDL-C. In humans the fatty streak lesions are usually found in the aorta in the first decade of life, coronary arteries in the second decade and the cerebral arteries in the third or fourth decade. Because of differences in blood flow dynamics, there are preferred sites of lesion formation within the arteries. Accumulation is greater when levels of circulating LDL are raised, and both the transport and retention of LDL are increased in the preferred sites of lesion formation. This gradually leads to CHD and stroke.

A series of large-scale clinical trials using different cholesterol-lowering regimens show conclusively that correcting hypercholesterolemia profoundly reduces morbidity and mortality caused by CHD. For every 10% reduction in cholesterol level, CHD deaths are reduced by at least 15% (Gould et al., 1998)

Thus increased low-density lipoprotein cholesterol (LDL-C) is a major risk factor for atherosclerosis, the underlying cause of coronary heart disease (CHD) and most strokes (Grundy, 1998). The recognition of the lipid/lipoprotein associated risk factors for arterial disease has promoted development of a large number of lipid

lowering drugs. The most effective drugs for reducing blood levels of LDL-C are the statins, which have been shown to significantly reduce the risk of coronary events and stroke in clinical trials. These data have led to recent guidelines that expand the use of LDL-C lowering drug therapy to larger population of patients.

Despite the success with statins, there is still a need for new therapies to reduce LDL-C. Many of the patients are unable to tolerate statins (primarily due to musculoskeletal symptoms) and the recent withdrawal of cerivastatin from the market as a result of several cases of rhabdomyolysis has heightened concerns about the safety and tolerability of statins. More importantly, many patients do not achieve the LDL-C goal with statin therapy alone (Pearson et al., 2000). This is possibly because of saturation of its efficacy as detailed below.

Statins inhibit the enzyme HMGCoA reductase and inhibition of this enzyme prevents cholesterol biosynthesis in the cell, thereby leading to the reduction in the intracellular cholesterol pool. This in turn activates the transcription of the LDL receptor gene leading to appearance of increased number of LDL receptors on the hepatic cell surface (Goldstein et al., 1990; Ma et al., 1986). This leads to increased ability of liver to clear the circulating LDL-C. Thus though the statins inhibit the HMGCoA, their ability to decrease plasma levels of LDL cholesterol depends on their ability to increase the expression of LDL receptors. It is possible to visualize that due to their indirect action, statins would have a saturation or a ceiling effect on their ability to reduce the plasma LDL cholesterol as suggested above.

The over-expression of LDL receptor directly appears to be an interesting target. By directly dealing with the gene expression and related mechanisms involved in LDL receptor synthesis, one can reduce the LDL cholesterol level in the plasma. In the present study this approach has been adopted on the assumption that one can successfully develop a drug for dyslipidemia by increasing the LDL receptor

expression directly. This newer approach also is expected to reduce the side effects and possibly increase the potency. The main objective of this study was to find an ideal anti-hyperlipidemic drug, which acts by up-regulation of LDL receptor and does not inhibit HMGCoA reductase or other enzyme systems in the biosynthetic pathway of cholesterol.

Hepatic uptake of cholesterol could be increased either by increasing the number of LDL receptors or by increasing their activity. Many agents are known to directly induce LDL receptor activity. One among them is high dose estrogen, which when given to animals can dramatically stimulate LDL receptor activity in the liver, leading to marked reduction in plasma total cholesterol and LDL cholesterol levels (Ma et al., 1986). On the other hand cytokines, growth factors and hormones such as platelet derived growth factor (Mazzone et al., 1989), interleukin-1 (Moorby et al., 1992), macrophage-derived factors (Grove et al., 1991) and insulin (Mazzone et al., 1989; Wada et al., 1988) increase the expression of the LDL receptors and there by reduce the LDL cholesterol levels.

Several New Drug Discovery groups are engaged in developing NCEs to up regulate LDL receptors. Some of them are listed here below.

Company	Drug Name	Phase of development	Chemical class
1. Aventis	RPR-102359	Biological testing	Hydroxybenzamide
2. Tanabe Seiyaku	TMC-49A	Biological testing	Phenethylcarbamate
3. Taisho	MD-700	Preclinical	Isobenzofuranone
4. Pfizer	CP-230821	Preclinical	Thiosemicarbazones
5. Eli Lilly	LY295427	Preclinical	Propenylcholestan
6. Aventis	Hoe-402	Phase II	Pyrimidinecarboxamide

RPR 102359 belongs to a new series of benzamides which has shown to increase the LDL receptor expression by 80% in HepG2 cells (Ashton et al., 1996). It is chemically N-[5-[(3-cyclohexylpropionyl)amino]-2-methylphenyl]-4-hydroxybenzamide. TMC-49A was obtained from the fermentation broth of streptomyces sp. AS1345 (Koguchi et al., 1998). It is structurally N-phenethylcarbamate and exhibited low cytotoxicity. It showed LDL receptor up-regulation in HepG2 cells. MD-700 is chemically 5,7-dimethoxy-3-(13-hydroxy-10-oxotetradecyl)-1(3H)-isobenzofuranone (Murakami et al., 1999). It has shown to increase LDL receptor expression in HepG2 cells within 4hrs at 0.03μg/ml. CP-230821 belongs to a new series of thiosemicarbazones, which has shown activity in both *in vitro* and *in vivo* models (Shimokawa et al., 1997). It is chemically [2-pyridinecarbaldehyde 4-(2,3-dihydro-1,4-benzodioxin) thiosemicarbazone]. LY295427 is chemically [(3α,4α,5α)-.4-(2-propenylcholestan-3-ol)]. It was reported to derepress LDL-R expression in cell based assay models and exerts hypocholesterolemic effect in fat fed hamster models of hyperlipidemia (Bensch et al., 1999). It had no effect on normocholesterolemic hamsters. Hoe-402 is chemically 4-amino-2-[4,4-dimethyl-2-oxo-1-imidazolidinyl]-pyrimidine-5-N-[trifluoromethylphenyl] - carboxamide-monohydrochloride (Huettinger et al., 1993). It has shown hypolipidemic activity in Watanabe Heritable Hyperlipidemic (WHHL) rabbits.

Hence, new molecules were designed based on the information available from literature, synthesized and screened for desirable properties. Pharmacophore mapping was adopted as the strategy for designing the molecules. In the present study, HipHop module of Catalyst software (version 4.5) from Accelrys (MSI) Inc., USA was used for pharmacophore mapping. All the calculations were carried out on the Silicon Graphic Indigo2 Extreme workstation. On the basis of the above approach a series of substituted 3-semicarbazone and substituted 3-thiosemicarbazones were designed and synthesized by the Medicinal Chemistry Department of Torrent Research Centre.

In the conventional approach in new drug discovery, one would have initially screened very large number of molecules from various drug libraries by an *in vitro* method and then choose few molecules with appreciable *in vitro* activity for *in vivo* studies. The major drawback of this approach is that majority of the drugs fails in the *in vivo* studies due to poor pharmacokinetic profile or bioavailability. Here, in this study, the number of molecules to be screened for activity was quite modest, as a rational drug design approach was used in designing the molecules and subsequent synthesis. Hence the molecules were screened initially by an *in vivo* method to identify molecules with anti-hyperlipidemic effect, irrespective of their mechanism of action, followed by detailed studies on mechanism of action using *in vitro* techniques

HepG2 cell line is a hepatocellular carcinoma cell line. It was chosen for the studies on mechanism of action as it is of liver origin and the expression of LDL-receptors is the highest in liver cells. Hence in majority of the studies on LDL receptor expression, this cell line is used. (Huettinger et al., 1993; Ashton et al., 1996; Shimokawa et al., 1997; Koguchi et al., 1998; Bensch et al., 1999;Murakami et al., 1999)

The anti-hyperlipidemic effect of these molecules was screened in a 5-day hamster model of hyperlipidemia. The 5 day acute model of hyperlipidemia was selected as the primary screening model as it was ideal to rapidly screen the molecules and the data generated, be used in optimizing the drug design parameters instead of using the long term models of hyperlipidemia which would have delayed the drug discovery process.

The Syrian hamster (Mesocricetus auratus) is a widely used experimental animal to study the effects of drugs and diet on lipoprotein metabolism. Several

drugs approved for human use like the lipid lowering drug HMG-CoA reductase inhibitors, or cholestyramine lower plasma cholesterol in hamster. The lipoprotein and bile acid metabolism of the hamster is closer to humans than the lipoprotein and bile acid metabolism of rats and mice (Suckling et al., 1991; Bravo et al., 1994; Kris-Etheron and Dietschy, 1997). The hamster has in contrast to pig, rat and mouse, Cholesteryl Ester Transfer Protein (CETP) activity, which is similar to what is seen in humans (Ha and Barter 1982, 1986; Ahn et al., 1994).

Increase in plasma cholesterol can be easily induced by adding small, physiological amounts of cholesterol to the diet. Additional saturated fat like coconut oil has synergistic effects in the induction of hyperlipidemia (Kowala et al., 1991). A stable hyperlipidemia with a lipoprotein pattern like that of the humans can be induced in hamster within 2–3 weeks by adding 10 % coconut butter and 0.2% cholesterol in the diet. By preliminary studies involving various combinations, we arrived at an ideal composition of 0.5% cholesterol and 5% coconut oil. This diet induced the desired lipid profile within a short time in this model.

The reference molecules selected for the study were CP-230821 and cerivastatin. They were selected since they had different chemical structures and different mechanisms of action. Cerivastatin increased the expression of LDL-R indirectly by inhibiting the enzyme involved in the biosynthesis of cholesterol (Yasunobu et al., 1997). CP-230821 is a LDL receptor gene transcriptional up-regulator (Shimokawa et al., 1997). Having these potent molecules, as reference compounds would ensure selecting test molecules with higher relative activity. To screen and identify molecules with adequate efficacy, twice the equimole dose of CP-230821 (30mg/kg) was used. By this strategy it was ensured that even compounds with half as much as activity as CP-230821 would be identified and the data used in optimizing the drug design parameters

Of the fifty structurally diverse test compounds evaluated for the anti-hyperlipidemic activity, it was found that thiosemicarbazone derivatives had shown good activity. The compounds that showed significant activity in lowering the serum total cholesterol (TC) and LDL cholesterol were TRC-7004, TRC-7007, TRC-7011, TRC-7033 and TRC-7051. The maximum TC and LDL-C lowering activity was shown by TRC-7033 and TRC-7011 respectively. TRC-7033 in addition also lowered the TG levels, which were not seen with the other molecules. It was observed that none of the compounds showed significant activity in raising the HDL-C levels. CP-230821 was a very potent molecule; it showed better TC and LDL-C lowering activity compared to TRC-7033.

Though TRC-7011 had marginally better LDL-C lowering activity compared to TRC-7033, the latter was chosen as the lead as it had marginally better TC lowering activity and in addition, lowered the TG levels as well. In new drug discovery, one always looks for molecules with overall desirable properties and better profile of action rather than one isolated property. The reference molecule cerivastatin was chosen for the detailed dose response study along with TRC-7033 as it was one of the most potent anti-hyperlipidemic agents in clinical use. However, cerivastatin was withdrawn from the market recently due to more than 100 reports of fatal rhabdomyolysis, an adverse reaction involving the destruction of muscle tissue that can lead to kidney failure (Ozdemir et al., 2000).

From October 1997 to December 2000, 772 cases of rhabdomyolysis associated with the use of statins were reported to the FDA Adverse Event Reporting System (AERS). Statin-associated rhabdomyolysis occurred with each of the six statins marketed in the United States during that time. 385 occurred with statins other than cerivastatin. The toxicity of cerivastatin was increased when used in combination with gemfibrozil. Although much has been said of the increased risk of rhabdomyolysis in people using a statin and a fibrate, more than two-thirds (68%) of

the reported cases of statin-associated rhabdomyolysis cases occurred when statins were not used concurrently with fibric acid derivatives (Woodcock, 2001).

With TRC-7033, at doses of 40 mg/kg and 60 mg/kg there was no significant difference in lowering TG and LDL-C activity; however 80 mg/kg produced clearly higher effect than 40 mg/kg. So a characteristic dose response could only be shown with larger doses and increased number of animals. However in new drug discovery and development it is not uncommon to proceed ahead just with the trend of the responses established. This is due to several constraints in the drug discovery process. One of the major difficulties in new drug discovery is the availability of large quantities of the NCE for detailed studies. This is due to the fact that adequate resources are not allocated for optimizing the synthetic processes in early stages of discovery. This is due to the possibility of the molecule being abandoned latter and possibilities of replacement by a backup molecule. One does not invest heavily in synthesizing the molecules in large quantities in the early stages. But once the lead candidate is identified, adequate resources would be allocated and all earlier observations are reconfirmed/ elaborated.

Though there was significant reduction in TG levels across all dose groups with TRC-7033, the response was not dose dependent. It is possible that TG responses may be more sensitive than other responses and at the doses studied; we may be already at the top of the plateau phase of the dose response curve for TG responses. There was no change in the serum levels of hepatic enzymes at the highest dose tested, which shows that the drug doesn't induce any hepatic toxicity.

Cerivastatin at 0.5mg/kg did not show any anti-hyperlipidemic activity while at 5mg/kg it showed severe toxicity - about 70% mortality, reduction in food intake and body weight. There was several fold increase in serum transaminases, which confirms hepatic toxicity of the molecule. This shows that the therapeutic index of

cerivastatin is relatively narrow. Cerivastatin showed dose dependent reduction in TC and LDL-C at 1.25 and 2.5 mg/kg.

TRC-7033 at 80 mg/kg reduced the serum TC, LDL-C and TG levels in normolipidemic hamsters, which shows that its mechanism of action is independent of the serum or hepatic levels of the lipids. This could be due to its ability to interfere with the sterol sensing mechanism in the cell thereby impairing /circumventing the feed back controls.

TRC-7004, TRC-7007, TRC-7011 and cerivastatin did not increase the hepatic lipid accumulation post treatment; rather they caused a decrease in total hepatic cholesterol. This effect was mainly confined to the cholesterol ester (oleate). Cholesterol oleate was quantified, as it is the major ester in the liver. It is speculated that these molecules have ACAT-2 inhibitory activity preventing cholesterol taken up by the liver from being converted to cholesterol ester so the free cholesterol is available for the synthesis of bile acids and part of the free cholesterol is secreted out along with the bile salts in the bile into the intestinal lumen. It is also likely that inhibition of ACAT-2 in the intestine prevents the absorption of dietary cholesterol (Cases et al., 1998). However, these interpretations need to be confirmed by studies specifically designed to test these hypotheses.

TRC-7033 at 80mg/kg on the other hand increased the accumulation of cholesterol in liver post treatment. This result in conjunction with the result of TRC-7033 in normolipidemic hamster suggest that the reduction in serum levels of TC and LDL-C shown by this compound is through increase in uptake of cholesterol by the liver and not by inhibition of cholesterol absorption from the intestine. However a possible contribution of cholesterol absorption inhibition to the overall effect of TRC-7033 cannot be absolutely ruled out until, again, specific studies are done in this regard.

Increase in hepatic accumulation of cholesterol oleate rules out the possibility of TRC-7033 having any significant ACAT-2 inhibitory activity. If dose escalation studies show further reduction in serum cholesterol levels even in presence of this high hepatic lipid levels then it would imply that the mechanism of action of this compound is independent of the intra hepatic sterol concentration. This was seen in the cell based LDL-receptor assay, where even in the presence of an inhibitory concentration of oxysterol, TRC-7033 could increase the expression of LDL-receptors in the HepG2 cells. However, this would also mean that the drug could induce fatty liver.

The mechanism of action of TRC-7033 could be through the increased expression of LDL receptors, which hence mops up the circulating LDL cholesterol. To explore this, a receptor based assay system was selected to quantify the LDL-receptors. By this method the actual expression of the receptor can be quantified. The test compound, TRC-7033 and cerivastatin showed a concentration dependent increase in LDL-R expression. At 0.01μM cerivastatin showed 3 times more LDL receptor expression than TRC-7033 but at the maximum concentration tested (1μM), TRC-7033 showed higher expression compared to cerivastatin. Cerivastatin had reached the plateau phase of the concentration response curve at 0.3 μM, while TRC-7033 had not even at 1μM. In order to rule out the possibility of the activity of TRC-7033 being through the inhibition of cholesterol synthesis, an indirect method was adopted where a further increase in expression was measured in presence of a maximally up-regulating concentration of cerivastatin. Cerivastatin, a potent HMGCoA reductase inhibitor, at high concentrations, maximally reduces the intracellular concentration of cholesterol; this results in increased expression of LDL receptors (Mahley and Bersot, 2001a). TRC-7033 was able to further increase the expression of LDL receptors in a concentration dependent manner even when the intracellular concentration of cholesterol is minimal. In another set of studies it was

shown that TRC-7033 increases the LDL-R expression even in the presence of an inhibitory concentration of oxysterol (25-hydroxycholesterol). Oxysterols freely diffuse into the cell resulting in intracellular concentration of sterol being high, thus by negative feed back control the expression of LDL-receptor is reduced (Bowling et al., 1996; Goldstein et al., 2002). From these two studies it can be concluded that TRC-7033's mechanism of action is not through inhibition of HMG-CoA reductase or any other enzymes in the biosynthetic pathway of cholesterol.

The molecular regulation of cellular sterol metabolism has been elucidated by Brown et al., (1997). The LDLR gene promoter contains a sterol response element (SRE) that is required for regulating transcription of the gene encoding LDLR in response to cellular sterol content. Two SRE-binding proteins (SREBP-1 and -2) have been purified and cloned; they contain two transmembrane domains and are localized to the endoplasmic reticulum (ER). Another protein, termed SREBP-cleavage activating protein (SCAP), acts as a chaperone protein that transports the precursor SREBPs from the ER to the Golgi, where two proteases, site 1 and site 2 protease (SIP and S2P), sequentially cleave the SREBPs. The second cleavage liberates the mature SREBP proteins from the membrane, allowing them to enter the nucleus, bind to the SREs of target genes and, along with additional transcription factors, activate gene transcription. Responsiveness of the system to cellular sterol content is accomplished through a 'sterol-sensing domain' in SCAP. Under cholesterol replete conditions the SCAP-SREBP complex remains in an inactive form in the ER through active repression by sterols.

Recently insulin induced gene-1 (Insig-1), an intrinsic protein of the endoplasmic reticulum (ER) has been identified to regulate the proteolytic processing of SREBPs (Yang et al., 2002). When cellular levels of sterols rise, insig-1 blocks the processing of SREBP by binding to sterol-sensing domain of SCAP thereby retaining the SCAP/SREBP complex in the ER and preventing it from moving to the Golgi. More

recently Insig-2, another ER resident protein that bind the SCAP/SREBP complex and promote its ER retention when cells are treated with sterols has been identified (Yabe et al., 2002). In sterol-depleted cells, SCAP escorts SREBPs from ER to Golgi for proteolytic processing, thereby allowing SREBPs to stimulate cholesterol synthesis. Insig-1 has also been reported to play an essential role in the sterol-mediated trafficking of proteins with sterol-sensing domains, HMG CoA reductase. (Sever et al., 2003)

The test compound TRC-7033 increases the LDL-R expression even in the presence of an inhibitory concentration of oxysterol (25-hydroxycholesterol). This property was also seen with LY-295427 where the mechanism has been attributed to the upregulation of insig-1 (Janowski et al., 2002). TRC-7033 could be interfering with the binding of insig-1 to SCAP. The possible site of action of TRC-7033 is depicted in Figure 1. This property of the drug could overcome the limitations seen with statins which show ceiling effect.

The colorimetric assay using MTT was used to assess the cytotoxicity of the test compounds, as this would involve minimal use of resources while large number of compounds could be screened rapidly. The assay is based on the capacity of mitochondrial dehydrogenase enzyme (metabolically active cells) to convert the yellow water-soluble substrate - tetrazolium salt (MTT) to purple formazan crystals, which are insoluble in water. Hence, this assay basically measures the mitochondrial activity, which is an indirect indicator for cell death. Most current assays for measuring cytotoxicity are based on alterations in plasma membrane permeability and the subsequent release (leakage) of components into the supernatant, or the uptake of dyes, which are normally excluded by viable cells. A serious disadvantage of such permeability assays is that the initial damage sites of many, if not most, cytotoxic agents are intracellular. Therefore, cells may be irreversibly damaged and committed to die, while the plasma membrane is still intact. Thus, these assays tend

to underestimate cellular damage when compared to other methods. This allows the use of the colorimetric assays MTT or XTT to measure cell survival. However, apoptosis is an active mode of cell death that requires the metabolism of cells. Thus, like the permeability assays mentioned above, the colorimetric assays may underestimate cellular damage and detect cell death only at the later stages of apoptosis when the metabolic activity of the cells is reduced. Regardless of this disadvantage, the colorimetric assays are very useful for quantitating factor-induced cytotoxicity within a 24- to 96-hour period of cell culture. At 10μM, cerivastatin, a well-known drug, which was in clinical use, did not show any cytotoxicity. At similar concentrations, none of the test compounds showed cytotoxicity in HepG2 cell line. Due to limitations in solubility, cytotoxicity at higher concentrations was not explored. There are various other methods to study the cytotoxicity like the lactate dehdyrogenase (LDH) release assay, radioactive ^{51}Cr release. assay, non radioactive bromodeoxyuridine (BrdU)-labelled DNA release assay.

Lipid lowering agents in addition to their direct lipid lowering action if they have anti-oxidant activity too, then this may prevent oxidative modification of LDL, to the more atherogenic form. Oxidised LDL is cytotoxic to endothelial cells, smooth muscle cells and fibroblast and it is more potent than glycated LDL in stimulating foam cell formation after uptake by macrophage scavenger receptors (Steinberg, 1997a). Probucol, a clinically available hypolipidemic agent, prevents LDL oxidation and subsequent foam cell formation *in vitro* and in animals it prevents atherogenic lesions (Davidson, 1993; Finckh et al., 1991). Here TRC-7033 showed antioxidant activity only at very high concentrations. In this series of compounds tested, some of the molecules showed appreciable anti-oxidant activity, however the compound with the highest lipid lowering activity i.e. TRC-7033 was almost inactive as far as the antioxidant activity is concerned (EC_{50} 5.8 mM). TRC-7011 and 7007 were some of the drugs, which had potent antioxidant and anti-hyperlipidemic activity. It would be desirable to incorporate the features from the molecules, which

showed good anti-oxidant activity in TRC-7033 to improve its anti-oxidant activity.

The molecules being multifaceted could affect life support systems, so it becomes imperative that a detailed safety pharmacology study be done on these molecules before they are taken up for pre-clinical and subsequent clinical studies. The safety pharmacology study gives us a rough estimate of its toxic potential on various organs at the therapeutic dose or multiples of it. In this study, we used the ED_{50} dose (for reduction in LDL-C in the hamster model of hyperlipidemia) as the point of reference.

In the studies to explore the effect of TRC-7033 on cardiovascular system, respiratory system and in the probe toxicity studies, the compound was formulated as a suspension for oral administration due to the limitations in solubility. Though the intended route of administration in human was oral, we at this point of time did not have much information on its oral bioavailability. However TRC-7033 was bioavailable as it had shown good *in vivo* activity. So in addition to increased doses administered orally for safety evaluation, drug was also administered i.v. and safety studies were performed. Very high doses by intravenous route could not be administered due to the limitation in formulation. In the safety pharmacology studies, it was seen that TRC-7033 shows fall in blood pressure only at a high dose given by i.v route but not at high oral doses.

The test compound TRC-7033 did not affect the motor co-ordination even at 25 times its ED_{50} dose in mice. The rota-rod test, which measures the length of time that small animals are able to stay on a rotating rod, was developed to measure drug effects on the motor co-ordination. The rota-rod test is considered to be suitable for the estimation of the positive adaptability to forced motor activity (Watzman et al. 1967). When a mouse is repeatedly placed on a rod or cylinder that is rotating at a constant speed, the animal gradually learns to walk on it, adapting itself to the

rotation speed. After treatment with a central depressant, however, the animal easily falls from the rod. This procedure is called 'rota-rod test' and was first introduced by Dunham and Miya (1957) and later by Janssen (1960), for assaying the drug effects on the motor co-ordination. However, many factors, like lowered general activity, ataxia, muscle relaxation, fatigue, etc, are all known to inhibit the rotarod performance.

TRC-7033 was studied for its effects on the motor co-ordination as estimated by the rota-rod test on mice. It did not exhibit any significant changes in the muscle co-ordination even at $25xED_{50}$ dose. Similarly, TRC-7033 at $25xED_{50}$ did not show any effect in the spontaneous motor activity studies in mice. This shows that TRC-7033 has a good CNS safety profile. Since the compound is expected to be highly lypophilic as it is poorly water soluble, one would expect easy transfer of the drug across the blood brain barrier and exert actions on the CNS. Inspite of this, the drug had very little effect on rotarod performance and hence one would expect the drug to have very little potential for CNS toxicities.

TRC-7033 caused a potent relaxation of the rat uterus both at the oestrus and dioestrus states at the highest concentration tested. Whether this has any clinical implication in pre-term labour, pre-eclampsia for the inhibition of extreme uterine contractions causing foetal distress and other pregnancy related disorders cannot be concluded now as the plasma concentrations of the drug at the therapeutic dose used for its antihyperlipidemic action is to be determined. Since the drug had no effect on guinea pig tracheal rings or on rabbit aorta, neither adrenergic beta receptors nor calcium channels are involved in the uterine smooth muscle relaxant action.

The probe toxicity studies involving observations on various pharmacotoxic symptoms reveal that TRC-7033 is a safe compound. At $25xED_{50}$ dose given orally it did not show any toxic symptoms. Only at a high dose of 20mg/kg (i.v.) shows

some effects on the CNS. Clinically these high plasma concentrations may never be achieved after oral administration. The initial loss of exploratory behaviour and inactivity seen with the high intravenous dose could have been caused by hypotension as we had seen fall in blood pressure in the safety pharmacology studies at the high intravenous dose.

Since this drug is meant for long-term use as an anti-hyperlipidemic agent, the compound should be tested for sub-acute and other long-term toxicities.

Future studies on TRC-7033

A large proportion of NCE's are discarded during their discovery and development phase not because of lack of activity but. because of poor pharmacokinetic properties, toxicities etc. The pressure on drug discovery scientist is to get better lead compounds faster and at less cost. The amount of money spent on leads that fail could be quite considerable. Over 72% of the costs of drug development are on molecules that ultimately fail. One estimate is that for every 100,000 compounds screened, about 100 hits are identified. Of these 100 hits, only one makes it to the lead stage. About 40% of the lead compounds fail due to poor ADME properties, while 6% dropped due to commercial reasons, 30% due to lack of efficacy, 11% due to toxicity and 10% due to side effects. Only 10% of IND submissions get approved (Kennedy, 1997).

In the case of TRC-7033, the activity of the compound is well established in the short duration efficacy studies and the possible mechanism of action has been elucidated. The compound has desirable specificity of action and appears to be having good safety profile and related properties. The compound also seems to have the potential for ideal kinetic properties based on its *in vivo* action. To develop the molecule fully and to take it up for clinical evaluation, it would be essential to

further elucidate its safety in terms of genotoxicity and to demonstrate its explicit pharmacokinetic properties and to carry out regulatory toxicological studies. These studies would be essential before we embark on phase I clinical studies. Particularly carcinogenicity, genotoxicity, toxicokinetics, reproductive toxicology and safety pharmacology studies would be further needed to take this molecule towards the phases of clinical testing

It is concluded that the currently available therapy for treatment of hyperlipidemia, although effective in lowering lipid levels, suffers from various drawbacks, like intolerability, failure to bring the lipid levels (LDL-C) down to desired low levels. The adverse reactions like rhabdomyolysis, seen with statins are less likely to occur with TRC-7033 as its mechanism of action is different. Secondly this drug is less likely to show the ceiling effect seen with statins. This molecule could be developed as a combination therapy with drugs, which prevent intestinal reabsorption of bile acids and cholesterol thereby the dose of TRC-7033 required could be reduced to increase its acceptability.

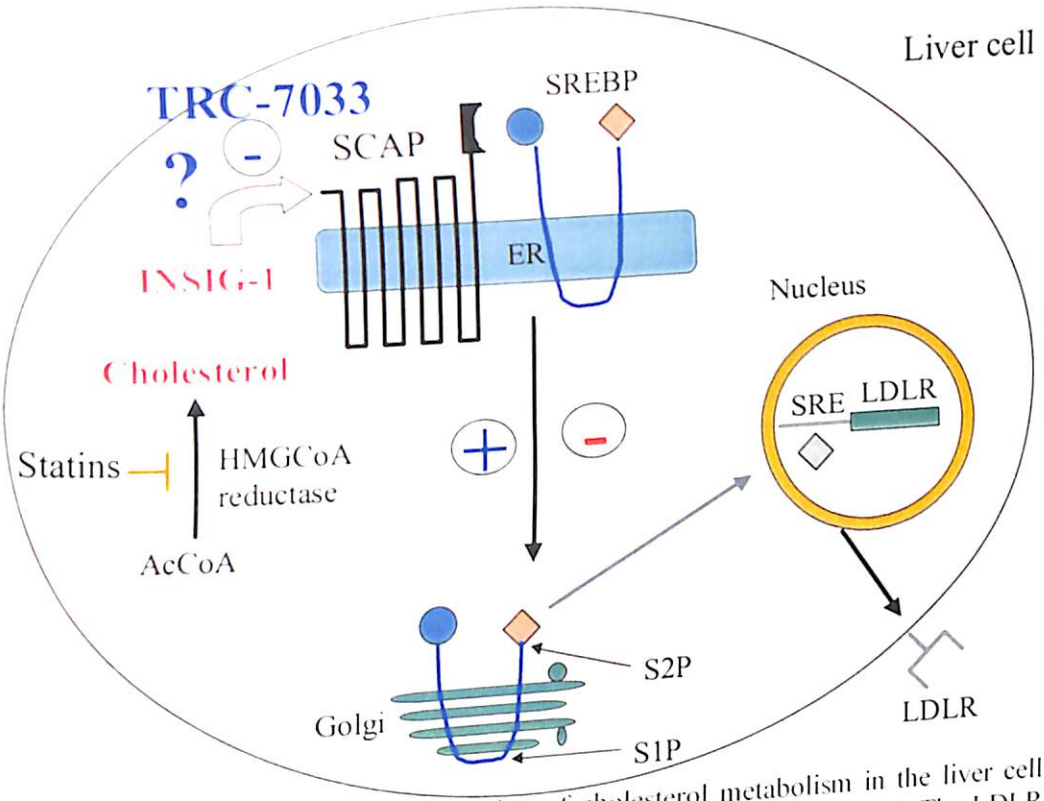


Figure 1: A schematic diagram showing the regulation of cholesterol metabolism in the liver cell The expression of LDLR on liver cells is regulated by cholesterol levels inside the cell. The LDLR gene promoter (shown inside the nucleus) contains SRE, which is required for regulating the transcription of the gene encoding LDLR in response to cellular sterol content. Two SRE-binding proteins, SREBP-1 and -2 are localized in the ER. When levels of cellular cholesterols are low, SCAP transports the precursor SREBPs from the ER to the golgi, where two proteases, S1P and S2P, sequentially cleave the SREBPs. The second cleavage liberates the mature SREBP proteins from the membrane, allowing them to enter the nucleus, binds to the SREs of the target genes such as that for LDLR. Responsiveness of the system to cellular cholesterol content is accomplished through a 'sterol-sensing domain' in SCAP. When cellular levels of sterols rise, insig-1 blocks the processing of SREBP by binding to sterol-sensing domain of SCAP thereby retaining the SCAP/SREBP complex in the ER. **TRC-7033 could be interfering with the binding of insig-1 to SCAP.** The statins inhibit HMGCoA reductase, the rate limiting enzyme in cholesterol biosynthesis, thereby reducing the levels of cellular cholesterol. Cholesterol depletion activates the translocation of SCAP-SREBP complex and results in the generation of mature SREBPs and up-regulation of LDLR.

SREBP, Sterol response element binding protein; SCAP, SREBP cleavage-activating protein; ER, endoplamic reticulum; S1P, site-1 protease; S2P, site-2 protease; AcCoA, Acetyl Coenzyme A; LDLR, Low-density lipoprotein receptor; INSIG-1, insulin induced gene-1.

Summary & Conclusions

7. Summary and conclusions

Hyperlipidemia is among the most potent risk factors in the causation of atherosclerosis, ischemic heart disease, premature coronary artery disease, peripheral arterial disease and ischemic cerebrovascular diseases like transient ischemic attack (TIA) and stroke. A long established theory suggests that the higher the circulating level of low-density lipoproteins (LDL), the more likely they are to gain entrance to the arterial wall and cause atherosclerosis and other related disorders.

The most effective drugs for reducing blood levels of low-density lipoprotein cholesterol (LDL-C) available today are the statins, which have been shown to significantly reduce the risk of coronary events and stroke in clinical trials. Statins by inhibiting the enzyme 3-Hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase prevents cholesterol biosynthesis in the cell, thereby leading to the reduction in the intracellular cholesterol pool. This results in increased expression of low-density lipoprotein receptors (LDL-R), which in turn leads to increased uptake of circulating LDL-C from blood. Despite the success with statins, there is still a need for new therapies that would address the inadequacies of the statins to reduce LDL-C. Many of the patients are unable to tolerate statins primarily due to musculoskeletal symptoms (rhabdomyolysis). The problem was severe enough to warrant the recent withdrawal of cerivastatin from the market. More importantly, many patients do not achieve the LDL-C goal with statin therapy alone. Dose escalation studies with statins did not proportionally increase the LDL-C lowering activity while the toxicity increases. This is possibly because of saturation of its efficacy.

The present study is an attempt to develop a new chemical entity, which will be useful to lower the blood levels of LDL-C by up-regulation of LDL-R and does not inhibit HMGCoA reductase or other enzyme systems in the biosynthetic pathway of cholesterol. Molecules were designed and synthesised by the Medicinal Chemistry Department of Torrent Research Centre for this purpose. Fifty molecules were screened for their anti-hyperlipidemic activity in the 5 day high fat diet induced hamster model of hyperlipidemia. The reference compounds used were cerivastatin (1.25mg/kg) and CP-230821 (30mg/kg). To screen and identify molecules with adequate efficacy after oral administration, twice the equimole dose of CP-230821 (30mg/kg) was used as the dose for screening the test compounds. The serum levels of TC, TG, HDL-C and LDL-C were determined pre and post treatment. A dose response (40, 60 and 80 mg/kg) for reduction in LDL-C was also carried out with TRC-7033, which had shown the most promising activity in the primary screen. In this study, in addition to the lipid profile, the serum levels of the hepatic enzymes (ALT, AST, serum bilirubin and alkaline phosphatase) were also monitored before and at the end of the treatment. The accumulation of lipids in the hepatic tissue was also estimated at the end of the study by High Performance Thin Layer Chromatography (HPTLC) to verify if the molecule has potential to induce fatty liver. Similarly the hypolipidemic activity of TRC-7033 in normolipidemic hamsters was also studied.

Five molecules with promising activity (TRC-7004, TRC-7007, TRC-7011, TRC-7033, and TRC-7051) were chosen for detailed studies to elucidate the mechanism of their action. The ability of the test compounds to increase the expression of LDL-R in HepG2 cell line at 1 μM concentration was estimated. The reference molecules studied were cerivastatin and CP-230821. A concentration response (1μM to 1nM) with cerivastatin and TRC-7033 was also studied. The LDL-receptors were quantified by a cell-based assay (using fluorescence method) using the mouse monoclonal antibody (Mab-C7) to human LDL receptor. The

primary antibody (Mab-C7) for the LDL-receptor assay was collected from the supernatant of C7 hybridoma cell culture and purified by affinity chromatography and its presence confirmed by western blotting.

To rule out the mechanism of LDL-receptor up-regulation of TRC-7033 being indirectly through inhibition of HMGCoA reductase enzyme, studies were carried out where further increase in LDL- receptor expression in HepG2 cells were estimated in presence of a maximally up-regulating concentration of a potent HMGCoA reductase inhibitor – cerivastatin (0.1 μM). Similarly studies were carried out where further increase in LDL-receptor expression in HepG2 cells was estimated in presence of an inhibitory concentration of oxysterol (25-hydroxycholesterol). This was done to rule out the ceiling effect with TRC-7033 as seen with statins.

The cyototoxic potential at 10μM concentration of the five promising molecules (TRC-7004, TRC-7007, TRC-7011, TRC-7033, and TRC-7051) were studied on HepG2 cell line by MTT assay. Similarly the toxicity potential in a concentration range of 10μM to 10nM with cerivastatin and TRC-7033 was also studied. The molecules were also studied for their anti-oxidant activity (concentration range of 12.5μM to 100μM) by a colorimetric assay, where its ability to scavenge the stable free radicals present in a methanolic solution of DPPH· was measured.

In addition to the above studies, safety pharmacology studies on TRC-7033 were performed to study the effect on cardiovascular, respiratory and central nervous system. The effects of TRC-7033 on BP, heart rate, ECG and respiratory system, after oral administration at ED_{50} (ED_{50} for reduction in LDL-C) and $5xED_{50}$ were studied in anaesthetised wistar rats. Similarly effects on these systems after intravenous administration at 2 and 10 mg/kg doses were also studied. Effect

of TRC-7033 on spontaneous motor activity was studied in Swiss albino mice after oral administration at ED_{50}, 10x ED_{50}, and 25x ED_{50} using a multi-varimex photo activity meter. Effect of TRC-7033 on forced motor activity was studied in Swiss albino mice after oral administration at 10x ED_{50}, and 25x ED_{50} using rota-rod apparatus.

Effects of TRC-7033 on various smooth muscles (isolated rabbit aorta, isolated rat uterus, isolated guinea pig ileum and tracheal rings) at a concentration range from 10nM to 10 μM were also studied for confirming selectivity. Finally probe toxicology was done to evaluate the toxicity potential of the molecule after oral (at 10x ED_{50} and 25x ED_{50}) and intravenous (10 and 20 mg/kg) administration.

The compounds that showed significant activity in lowering the serum total cholesterol (TC) and LDL cholesterol were TRC-7004, TRC-7007, TRC-7011, TRC-7033 and TRC-7051. The test compound TRC-7033 in addition to lowering the TC and LDL-C also lowered the TG levels. None of the compounds showed significant activity in raising the HDL-C levels. TRC-7033 at a dose of 80 mg/kg produced clearly higher effect than 40 mg/kg in reducing TG and LDL-C. There was no change in the serum levels of hepatic enzymes at the highest dose tested, which shows that the drug doesn't induce any hepatic toxicity. These studies were able to identify TRC-7033 as a molecule with good anti-hyperlipidemic activity.

TRC-7033 at 80 mg/kg reduced the serum TC, LDL-C and TG levels in normolipidemic hamsters, which shows that its mechanism of action is independent of the serum or hepatic levels of the lipids. TRC-7033 at 80mg/kg increased the accumulation of cholesterol in liver post treatment; so on long-term treatment this would mean that the drug could induce fatty liver.

Cerivastatin at 0.5mg/kg did not show any anti-hyperlipidemic activity while at 5mg/kg it showed severe hepatic toxicity. This shows that the therapeutic index of cerivastatin is relatively narrow. Cerivastatin showed dose dependent reduction in TC and LDL-C at 1.25 and 2.5 mg/kg.

The mechanism of action of TRC-7033 could be through the increased expression of LDL receptors, as a concentration dependent increase in LDL-R expression was seen after exposure to the drug. At the maximum concentration tested (1μM), TRC-7033 showed higher expression in LDL-receptors compared to cerivastatin. Cerivastatin had reached the plateau phase of the concentration response curve at 0.3 μM, while TRC-7033 did not, even at 1μM. In a maximally up-regulating concentration of cerivastatin, TRC-7033 showed further increase in LDL-R expression. TRC-7033 increases the LDL-R expression even in the presence of an inhibitory concentration of oxysterol. From these observations it is concluded that TRC-7033's mechanism of action is not through inhibition of HMG-CoA reductase or any other enzyme in the biosynthetic pathway. Hence, it is less likely to have the side effect or ceiling effect as seen with statins. TRC-7033 could be interfering with the sterol sensing mechanism in the cell. It could be interfering with the binding of insig-1 to SCAP or affecting insig-1 expression it self.

At the maximum soluble concentration tested - 10μM, neither cerivastatin nor any of the test compounds showed any cytotoxicity by MTT assay. Many of the test compounds showed anti-oxidant activity and TRC-7011 was the most potent with an EC_{50} of 12.6μM. TRC-7033 had a poor anti-oxidant activity, as its EC_{50} was 5.8mM. The compound, if it has dual activity, i.e., in addition to its direct lipid lowering action if it has anti-oxidant activity too, then this may prevent oxidative modification of LDL, to the more atherogenic form.

In the safety pharmacology studies it was seen that TRC-7033 shows fall in blood pressure only at the highest intravenous dose (10mg/kg) but not at the high oral dose ($5xED_{50}$) administered. The test compound TRC-7033 did not affect the motor co-ordination or locomotor activity even at 25 times its ED_{50} dose in mice. This shows that TRC-7033 has a good CNS safety profile. TRC-7033 caused a potent relaxation of the rat uterus only at the highest concentration tested. Since the drug had no effect on guinea pig tracheal rings or on rabbit aorta, neither adrenergic beta-receptors nor calcium channels are involved in the uterine smooth muscle relaxant action. In the probe toxicity studies at $25xED_{50}$ dose given orally, TRC-7033 did not show any toxic symptoms while at the highest intravenous dose of 20mg/kg, a momentary loss in exploratory activity was observed which could be due to hypotension, which was observed at the highest intravenous dose in the safety pharmacology studies.

TRC-7033, in addition to its efficacy, has a favourable profile of activity on other organ systems and wide safety margin. It is less likely to have the side effect or ceiling effect seen with statins. Hence the molecule is recommended to be taken up for detailed regulatory toxicity studies.

To summarise, in this study, 50 molecules were screened for anti-hyperlipidemic activity and five molecules with appreciable activity were identified. All the five molecules were shown to have the ability to increase expression of LDL receptors. One of the chosen molecule, TRC-7033, has shown good efficacy, good safety profile, specificity and other desirable properties. Hence it was concluded that the chosen lead molecule has the potential to develop as an anti-hyperlipidemic agent.

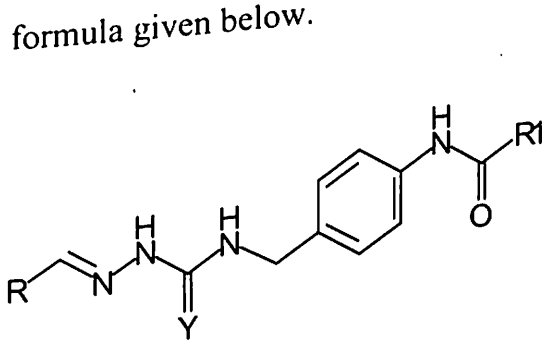
Appendix-I

Appendix - I - Chemistry of NCEs studied

All the molecules were designed and synthesised by the Medicinal Chemistry Department of Torrent Research Centre.

Pharmacophore mapping was adopted as the strategy for designing the molecules. In the present study, HipHop module of *Catalyst* software (version 4.5) from Accelrys (MSI) Inc., USA was used for pharmacophore mapping. All the calculations were carried out on the silicon graphic indigo2 extreme workstation. On the basis of the above approach a series of substituted 3-thiosemicarbazone and substituted 3-semicarbazone were designed and synthesized These molecules were evaluated for their anti-hyperlipidemic activity in the Department of Pharmacology of Torrent Research Centre.

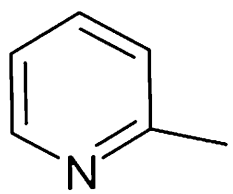
The present invention provides a new class of anti-hyperlipidemic compounds of general formula given below.



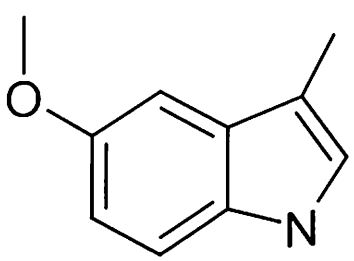
A series of substituted semicarbazone and thiosemicarbazone compounds were synthesised, where R is substituted / unsubstituted aryl and mono or fused hetroaryl. R_1 represents a group selected from alkyl, cycloalkyl, aryl and hetroaryl. "y" is independently selected from oxygen or sulphur.

The compounds show good anti-hyperlipidemic activity,

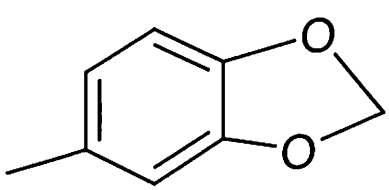
When R is a substituent like:



2-pyridyl

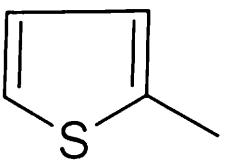


5-methoxy indolyl-3-yl



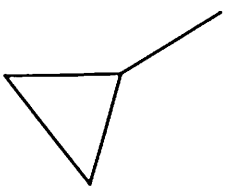
1,3 benzodioxan-5-yl

or

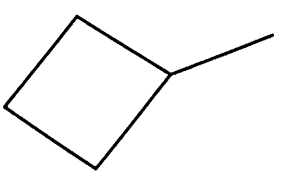


thiophene-2-yl

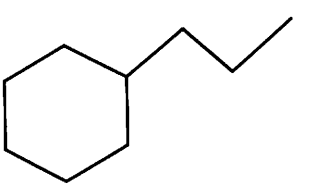
and R_1 is-



cyclopropyl

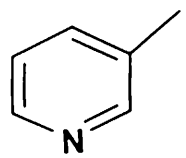


cyclobutyl



3-cyclohexyl propionyl

or



3- pyridyl

Appendix-II

1. Hepatic cholesterol and cholesteryl ester estimation :

Extraction: The method adopted was that of Folch et al., 1957. About 1 gm of the liver was homogenized in 10ml of chloroform : methanol (2:1) mixture for about 3 minutes and then the volume was made up to 25ml with chloroform : methanol (2:1) mixture. This liver homogenate was centrifuged at 3000 rpm for 20 min and 1ml of the supernatant was diluted 1:50 with chloroform : methanol (2:1) mixture. This sample was then used for spotting on the HPTLC plate. (Schmitz et al., 1984)

Preparation of standard solution of Cholesterol and cholesteryl ester:

A Stock solution of standard cholesterol 0.2mg/ml and standard cholesteryl ester (cholesterol oleate) 0.2 mg/ml was prepared in chloroform : methanol (2:1) mixture and mixed together and then further diluted with chloroform : methanol (2:1) mixture to get 32,16, 8, 4, 2 and 1 µg/ml.

Activation of the plate: The HPTLC plate was washed in methanol in the dip tank and activated for 15 min in a 110°C oven (Schmitz et al.,1984)

Spotting Parameters: Using Linomat IV

Start position	:	10mm
Band width	:	5mm
Space between bands	:	10mm
Sec/μL	:	10
Volume	:	10μL
Spot application	:	2 cm from bottom edge

Scanning Parameters:

Scanning speed	:	4mm/sec
Wavelength	:	366nm
Lamp	:	Mercury
0 Adjustment for each track	:	Yes
Span	:	25
Sensitivity	:	Automatic
Offset	:	10%
Absorbance/fluorescence	:	Absorbance
Reflection/transmission	:	Reflection
Monochromatic bandwidth	:	4
Slit width	:	4
Slit length	:	4
Optics micro/macro	:	micro

Integration parameters:

Video integration	:	Yes
Base line correction	:	Yes
Peak threshold, area	:	50
Peak threshold, slope	:	5
Data selection factor	:	1
Filter factor	:	3

A standard curve was plotted with the concentration of the standards in the x-axis and density in the y-axis and the concentration of the free cholesterol and cholesterol oleate in the test sample obtained from the standard curve.

2. Culture of C7 hybridoma:

Materials:

C7 hybridoma (CRL-1691) ATCC, USA.

Dulbecco's modified eagles medium with high glucose (Hyclone)

Sodium pyruvate (Hyclone)

Fetal bovine serum (FBS) (Hyclone)

Sodium bicarbonate (Sigma)

Dimethyl sulfoxide (DMSO) (Sigma)

Cell culture flasks 75 cm^2 (NUNC)

Plastic pipettes – 5,10, 25ml (NUNC)

Cryovials 1.8 ml (NUNC)

Cryo freezing container (Nalgene)

Media for C7 : DMEM with Glutamine (4mM) and high glucose (4.5 g/l), sodium bicarbonate (1.5 g/l), sodium pyruvate (1mM)- 90% and Fetal bovine serum -10% This is not to contain anti-fungal agent and the FBS used was not heat inactivated.

2.1 *Revival of frozen C7 cells*:

The cryovial containing the frozen C7 cells upon receipt were immediately thawed by gentle agitation in 37° C water bath. The thawing was done rapidly within 2 minutes. The surface of the vial was then decontaminated by wiping with lint free cloth dipped in 70% alcohol and the contents of vial was transferred aseptically to 75 cm^2 tissue culture flasks under laminar air flow and diluted (1:30)

with the recommended complete culture media (DMEM). The max volume of a 75 cm^2 was kept at 15 ml. Prior to addition of the vial contents to culture media, the flask with the media was placed in CO_2 incubator for 15 min to allow the medium to reach its normal pH (7.0-7.4) and thereby avoid excess alkalinity of the medium during recovery of the cells. The flask was then incubated at 37°C in 5% CO_2 incubator.

The cultures were moderately heavy after 3-4 days incubation. About 20µl was removed from the cell suspension and to it 20µl of vital stain - 0.1% trypan blue was added and this was placed on a hemocytometer (Improved Neubauer chamber) and seen under microscope for cell count and viability test.

2.2 *Subculturing procedure:*

Contents of the flask were centrifuged at 125g for 3 minutes. The supernatant containing the antibody of interest was collected and stored at -20°C. A part of the cell pellet was used for subculturing while the other part was used for cryopreservation. For subculturing the cells were re-suspended in the media (prior to use the media was kept in the CO_2 incubator at 37°C to reach its normal pH) such that the count was around $5x10^4$ viable cells/ml. The cultures were maintained at a concentration between $5x10^4$ and $5x10^5$ cells/ml and cell concentration was never allowed to exceed above $1x10^6$cells/ml. Hence split and media renewal was given every alternate day, and the supernatant collected and stored. A new 75T flask was used every eighth day for cell culture. The passage was continued only for ten generations, after which a new cryovial containing the C7 cell line was revived and subcultured.

2.3 *Cryopreservation:*

The viable cell count by trypan blue exclusion was performed from the flask containing the cells to be cryopreserved. (The pre-freeze viability should be above 70 percent as post cryopreservation viability reduces by about 20 percent).

The contents of the flask were spun at 125g for 2 minutes. The culture medium used to prepare cryopreservation media was kept at 37°C in CO_2 incubator to bring it to its normal pH before use and the DMSO was added to this media just before adding it to the cells. The cells were then re-suspended in freshly prepared cryopreserving media - 95% culture media (containing 20% fetal bovine serum) and 5% dimethylsulphoxide such that the cell concentration was $3x10^6$ cells/ml. (The recommended cell concentration for cryopreservation is 3-5 x 10^6 cells/ml). Then 1ml of cell suspension was aspirated and dispensed in 1.8 ml cryovials, these vials are then placed in controlled rate freezing chamber - cryo freezing container (cools at the rate of 1°C/min) and the chamber placed in -70°C deep freezer for 5hrs. The cryovials were immediately transferred to liquid nitrogen container and stored in the vapour phase of liquid nitrogen.

3. Purification of Immunoglobulin-G using IgG purification kit:

The frozen (-20°C) hybridoma supernatant was gradually thawed in ice over 24hrs and then mixed 1:1 with the equilibration buffer. This was loaded on to the column through which equilibration buffer had been passed and the flow rate maintained below 0.25ml per minute. The column was then rinsed with equilibration buffer prior to passage of the elution buffer through the column. The elute was collected in tubes containing neutralization buffer and stored immediately at 2-4 °C.

4. Concentration and desalting of the eluted antibody by ultrafiltration:

Concentration of protein by ultrafiltration proceeds by forcing the liquid (by using centrifugal force) in the protein solution through a membrane which retains the protein of interest. The Amicon Centriplus-100 of 15 ml capacity was used where the molecular weight cutoff was 100 kD. The buffer used to desalt was 1x PBS. The centrifugation was done at 4 °C in fixed angle rotor centrifuge (Sigma –3K15). The

sample was concentrated to 500µl and stored below 0°C after addition of 2% sodium azide to get a final concentration of 0.02%.

5. Bradford Assay (Protein estimation):

Principle:

This is a rapid and reliable dye-based assay for determining protein content in a solution (Bradford, 1976). Here the dye - Coomassie Brilliant Blue (CBB) binds to the amino group of the basic amino acids (arginine, lysine) present in the test protein and this is compared to the binding to the known concentration of the standard dye (bovine serum albumin)

Materials:-

Absolute ethanol (Herbertsons International)

Orthophosphoric acid (Qualigens)

Coomassie brilliant blue G250 (CBB) (Qualigens)

Composition of Bradford reagent:

99% Ethanol (absolute)	133.12	ml
Orthophosphoric acid	266.56	ml
CBB G250	0.1856	g
Distilled water	2265.92	ml

Method:-

A stock solution of 0.25mg/ml of bovine serum albumin (BSA) in 1x PBS was prepared and from this different volumes taken so as to get various concentrations between 2.5µg and 20µg (2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20) and the volume made up to 200µl with 1x PBS. To this 2ml of CBB was added and vortexed and the solution taken in glass or plastic cuvettes and absorbance at 595nm measured

between 5-10 minutes. A standard curve was plotted with concentration of BSA in x-axis vs optical density (OD) in y-axis and from this plot the concentration of protein in the test sample determined.

6. Gel electrophoresis and Immunoblotting (Western blotting):

Materials:-

Protein electrophoresis system (Bio-Rad)
(Mini protean II cell & Powerpac300)

TE77 Semidry transfer unit (Hoefer Semiphor – Amersham Biosciences)

Electrophoresis Power Supply – EPS-200 (Pharmacia Biotech)

Hyperfilm processor (Amersham pharmacia biotech)

Eppendorf centrifuge

Hamilton syringe

Biodancer (New Brunswick Scientific)

Hypercassette (Amersham Life Sciences)

Kodak diagnostic film T-Mat E

Nitrocellulose membarane (Amersham Pharmacia Biotech)

Tris (2-hydroxymethyl Aminomethane) –(Lancaster)

Glycine (SRL)

Methanol (Qualigen)

Acrylamide (GIBCO BRL)

Bis-acrylamide (GIBCO BRL)

APS (Ammonium Persulphate) (Sigma)

TEMED (Sigma)

Sodium dodecyl sulfate (SDS) (SRL)

Ponceau dye (Sd fine)

Bromophenol Blue (Bio-Rad)

Glycerol (Qualigens)

ß-Mercaptoethanol (Sigma)

Skimmed milk (Himedia)

High molecular weight protein markers (Bangalore Genei)

Composition of various reagents and buffers:

30% Acryl/bis

Acrylamide	58.4 g
Bis acrylamide	1.6 g
MilliQ water	200 ml

7% Seperating gel for 10 ml

30% Acryl/bis	2.334 ml
1.5 M Tris (pH:8.8)	2.4 ml
10% SDS	0.100 ml
D/W	5.17 ml
10% APS(fresh)	0.035 ml
TEMED	0.0075 ml

Stacking gel for 5ml

Acryl/bis (30%)	0.65 ml
0.5 M Tris Hcl (pH:6.8)	1.2 ml
10% SDS	0.050 ml
D/W	3.05 ml
10% APS	0.025 ml
TEMED	0.005 ml

Running gel buffer for 400 ml

Tris glycine (5x)	80 ml
10% SDS	4 ml
milli-Q	316 ml

Transfer Buffer for 200 ml

Methanol	40	ml
Tris glycine (5X)	40	ml
milli-Q (chill it at 4 °C)	120	ml

Loading dye buffer (6x)

Stacking buffer (0.5 M Tris pH 6.8)	1.4	ml
Glycerol	0.6	ml
SDS	0.2	g
ß-marcaptoethanol	0.12	ml
Bromo phenol blue	0.002	g

Detection Buffer for 50 ml

NaCl	100	mM
Tris (pH:9.5)	10	mM

Tris glycine (5x)

Tris (125mM)	15.14	g
Glycine (960mM)	72.065	g
PH – 8.3-8.6 (filter before use)		

Methods:-

Preparation of linear slab gel (prepared in duplicate): A spacer of 0.75 mm thickness was used to separate the two glass plates. 5ml of 7% separating gel solution was introduced into the gel sandwich after confirming for absence of leakage. Then butanol was poured slowly on top of the separating gel to keep the gel surface flat and prevent entry of air. After one hour at room temperature, the butanol was washed off thoroughly with repeated rinsing with milli Q water. 5ml of stacking

gel solution was pipetted on top of the separating gel and immediately the sample well comb was inserted slowly into the stacking gel and after 30 minutes the comb was removed carefully so as not to tear the well ears. The gel-casting unit with both the gels was attached to the electrode assembly and then placed in the electrophoresis chamber (tank) and the electrophoresis buffer (running gel buffer) added to the inner and outer reservoir.

Preparation of Loading samples: Standard high molecular weight (3µl) was mixed with 6x loading dye buffer and the volume made up to 20µl with 0.5 M Tris-HCl. Required concentration of the test sample protein (200ng/lane, 400ng/lane) was prepared with the loading buffer and volume made up with Tris-HCl. Similarly a volume of test sample (protein conc. between 200-400 ng) taken and volume made up to 20µl with 0.5 M Tris-HCl, similar volume of the control sample taken and volume made up to 20µl with 0.5 M Tris-HCl The eppendorf tubes containing the standard, control and test sample proteins were spun for 2 sec and then kept in boiling water for 10 min and then they were again spun for 5 sec to bring down any precipitated protein or debris if present. The volume of sample loaded into each well was 20µl. While loading using a Hamilton syringe, care was taken not to introduce any air bubbles. To avoid edge effect 10µl of 1x loading buffer was added to corner (outside) and unused wells.

Running the gel: The electrode plugs from the powerpac300 were attached to the respective electrodes (red being positive) and then power supply switched on and an initial voltage of 80V was applied till the dye front reached the margin of the separating gel, then the voltage was increased to 100V. The migration took about one and half hour. After electrophoresis the gel can be left for at most an hour before staining after which the protein may start to diffuse. After electrophoresis the power was shut off and electrode plugs disconnected. The spacer was carefully removed

and it was inserted in one corner between the plates and the glass plates pried apart and the gel was carefully detached from the glass plate.

Protein transfer: A transparency sheet with a rectangular window in the middle (10cm x 7cm) was placed in the semidry blotting apparatus (transfer unit). Above this transparency sheet, four sheets (size greater than 10cm x 7 cm) of Whatman filter paper No.3 soaked in chilled transfer buffer were placed. A nitrocellulose membrane (uncharged, pore size – 0.22μ), which was soaked for 5 minutes in chilled transfer buffer, was placed on the filter paper. The gel, which was separated out from between the glass plates, was placed slowly on the nitrocellulose membrane such that there was no air trapped between the gel and membrane. (prior to placing the gel on the membrane the loading well ear was cut off and one corner of the gel slightly sliced off to identify the marker lane).

Now another set of four Whatman filter paper No.3 soaked in chilled transfer buffer was placed one by one on the gel, then a clean test tube was rolled on the filter paper to push out air bubbles. The lid of the blotting apparatus was placed on it and the system connected to the power supply (EPS200). 40mA current was applied for 1 hr. (@ 0.8mA/cm^2 : area here being 9x6 = 54cm^2 : 0.8x54=43 mA)
The negatively charged SDS coated denatured protein migrated down towards the positive charge. At the end of 1 hr the nitrocellulose membrane was taken out from the sandwiched filter papers.

Staining for total protein: The objective was to monitor the efficiency and completeness of transfer of the protein onto the nitrocellulose membrane. An anionic dye - Ponceau S (non specific binding) was used for the staining of the nitrocellulose membrane. 0.5% Ponceau S was added on the nitrocellulose membrane for 2 minutes, and now the band was visible. The stain was washed off with tween tris buffer saline (TTBS) before the immunodetection.

Immunodetection: To prevent the non-specific binding of the antibody to the membrane a blocking agent was used. The membrane was soaked in 50ml of 5% skimmed milk in 1x Tris buffer saline (TBS) and left overnight, next morning the skimmed milk was drained off and the membrane rinsed thrice, over 5 minutes duration each with tween tris buffer saline (TTBS), placed on a biodancer. The membrane was then incubated with goat anti-mouse IgG conjugated AP (1:3000 dilution) for 1 hr on a biodancer with constant gentle agitation. The membrane was then rinsed thrice with TTBS over 5 min duration on a biodancer, subsequently it was rinsed with the detection buffer for 5 min then incubated with CDP star (1:10 dilution) for 5 minutes at room temperature. The CDP star was drained off and the membrane placed in between two thin plastic sheets and this was placed in the Hyperfilm chemiluminiscent cassette along with the x-ray film (with the film's corner chopped off) for 1 hr and the then x-ray film was fixed and developed. .

6.1 *Silver staining the gel:*

The objective here was to directly stain the gel to detect presence of small quantity of the protein in the band.

Materials:

Methanol (Qualigen)
Formaldehyde (Qualigen)
Sodium thiosulphate (Qualigen)
Silver nitrate (Qualigen)
Sodium carbonate (SRL)
Citric acid (SRL)

Composition of various Reagents:

HCHO Fixing Solution:

Methanol 40%	400 ml
37% HCHO	0.5 ml
Distilled water (D/W)	600 ml

0.02% Thiosulphate :

Na thiosulphate	0.02 g
D/W	100 ml

0.1% $AgNO_3$:

$AgNO_3$	0.1 g
D/W	100 ml

Developing Solution:

Na_2CO_3	3.0 g
0.02% thiosulphate	2.0 ml
D/W	100 ml
37% HCHO	0.05 ml (Add this just before developing the gel)

Stopping solution:

2.3 M Citric acid	4.83 g
D/W	10.0 ml

Method:

The second gel was immersed in the fixing solution with gentle agitation for 10 minutes. The fixing solution was drained off and the gel rinsed thoroughly for 10 minutes with two changes of distilled water. The gel was then soaked for 1 minute in 0.02% sodium thiosulphate with constant gentle agitation, then it was rinsed twice with distilled water over 40 sec. The gel was then soaked for 10 minutes in 0.1% silver nitrate with constant gentle agitation. The gel was then rinsed with distilled water. 37% formaldehyde was added to the other components of the developing solution and this solution was immediately used to rinse the gel, then it was drained of and the gel soaked in fresh developing solution with constant gentle agitation until the band intensifies. Now on addition of 5ml of stopping solution with constant

agitation for 5-10 minutes effervescence was observed. The gel was then washed twice with distilled water and the gel subsequently stored in distilled water till the band captured using a gel doc system.

7. Culture of HepG2 Cell line:

Materials:-

HepG2 cells (human hepatoblastoma) NCCS, Pune ,

Culture medium -Minimum essential media with Earle's balanced salts (MEM) (Hyclone)

Sodium pyruvate (Hyclone)

Fetal bovine serum (FBS) (Hyclone)

Penicillin-streptomycin solution (Hyclone)

Sodium bicarbonate (Sigma)

Dimethyl sulfoxide (DMSO) (Sigma)

Trypsin (Sigma)

Cell culture flasks 25 cm^2 (NUNC)

Plastic pipettes - 5 ml, 10 ml, 25 ml (NUNC)

Cryovials 1.8 ml (NUNC)

Culture Medium for HepG2: Minimum essential medium (Eagle) with non-essential amino acids and sodium pyruvate (1mM) with Earle's balanced salt solution, glutamine (2mM), sodium bicarbonate (1.5 g/l), 1x antibiotic (Penicillin G sodium 10,000 units/ml, streptomycin 10,000 µg/ml) - 90% and fetal bovine serum - 10%

Procedure for culturing cells in flasks: On receipt, the flask was checked for any apparent contamination and the excess media was removed and the culture checked for confluency. A split was given only if there was 75% or more confluency.

7.1 *Subculturing procedure:*

The media from the flask was removed and cells rinsed with culture grade PBS and then incubated with fresh Trypsin – EDTA solution (0.25g trypsin, 0.02g EDTA and 0.003g phenol red in 100 ml PBS, pH 7.4) for 3 min at room temperature then the trypsin solution was removed and flask was incubated at 37°C for 15 min, on confirmation of cells detachment fresh medium was added to stop trypsin action and then the contents centrifuged at 125g for 5 minutes and the supernatant discarded and the cells re-suspended in 5 ml fresh media and the subcultivation ratio of 1:4 was followed and media renewal done twice weekly.

7.2 *Cryopreservation of HepG2 cells:*

Culture flasks containing HepG2 cells in log phase of growth i.e. pre-confluent cultures below maximum cell density were selected for cryopreservation These cells were trypsinised and later the pellet resuspended in fresh culture media as per the method followed for HepG2 subculturing. A viability count by 0.1% trypan blue exclusion was performed (viability should be above 90% preferably around 95%). The culture medium used to prepare cryopreservation media was kept at 37°C in CO_2 incubator to bring it to its normal pH before use and the DMSO was added to this media just before adding it to the cells. The cells were again spun at 125g for 5 min and then the pellet was re-suspended in freshly prepared cryopreserving media - 95% culture media (containing 20% fetal bovine serum) and 5% dimethylsulphoxide such that the cell concentration was $5x10^6$ cells/ml. Then 1ml of cell suspension was aspirated and dispensed in 1.8 ml cryovials, these vials are then placed immediately in liquid nitrogen. These were later revived and sub cultured as and when required.

8. Proforma for collection of probe toxicity studies data:

Probe toxicity studies

Test compound:
Date:
Time:
Route of admn: Oral Intravenous
Dose:
Volume :

Animal:
Sex:
Weight:
Solvent::
Time of death:

Strain:
Age:

* √ - Present
X - Absent

GENERAL OBSERVATIONS

	0 hr	< 10 min	20 min	40 min	1h	2 hr	3 hr	4 hr	6 hr	24 hr	48 hr	72hr	Onset	Disappea
Bizzare physical														
Exploratory behaviour														
Aggressiveness														
Inactivity														
Convulsions (sponta:)														
Dyspnoea														
Jittery behaviour														
Blanching of														
Salivation														
Nasal discharge														
Piloerection														
Sensitivity to sound														

PHYSICAL EXAMINATION

	0 hr	<10 min	20 min	40 min	1hr	2 hr	3 hr	4 hr	6 hr	24 hr	48 hr	72hrs	Onset	Disappear
Altered muscle tone														
Catatonia														
Muscle tremors														
Aggressiveness														
Convulsion to touch														
Paralysis														
Sensitivity to pain														
Skin lesion														
Reflexes – Placing														
Righting														
Grasping														
Pinnal														
Coma														
Death														

Appendix-III

Appendix – III - Results

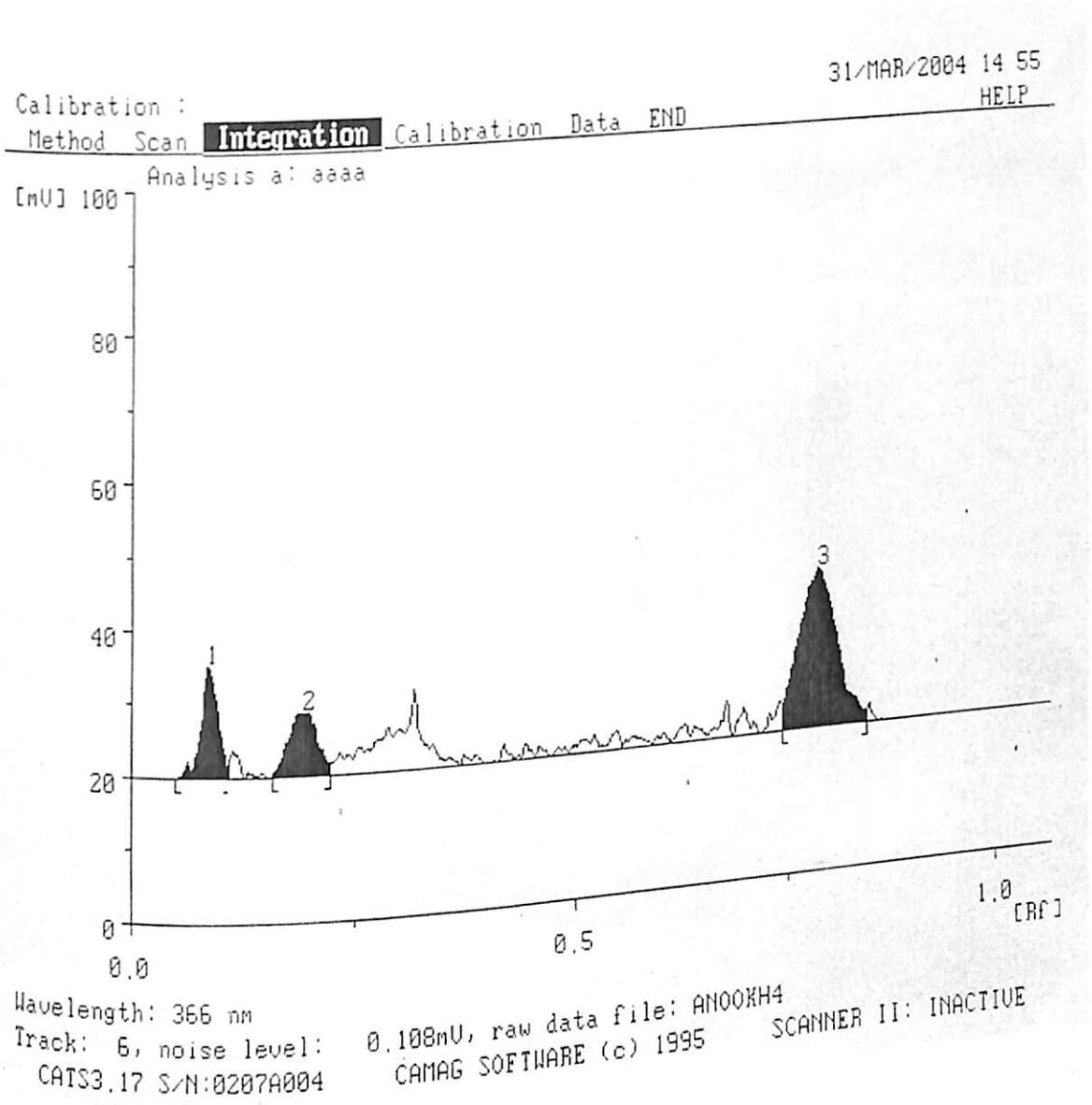


Figure 1: HPTLC chromatogram- Hepatic lipid levels after vehicle treatment in hamster. Peak (1) - Free cholesterol; (2) – Triglyceride; (3) - Cholesterol ester.

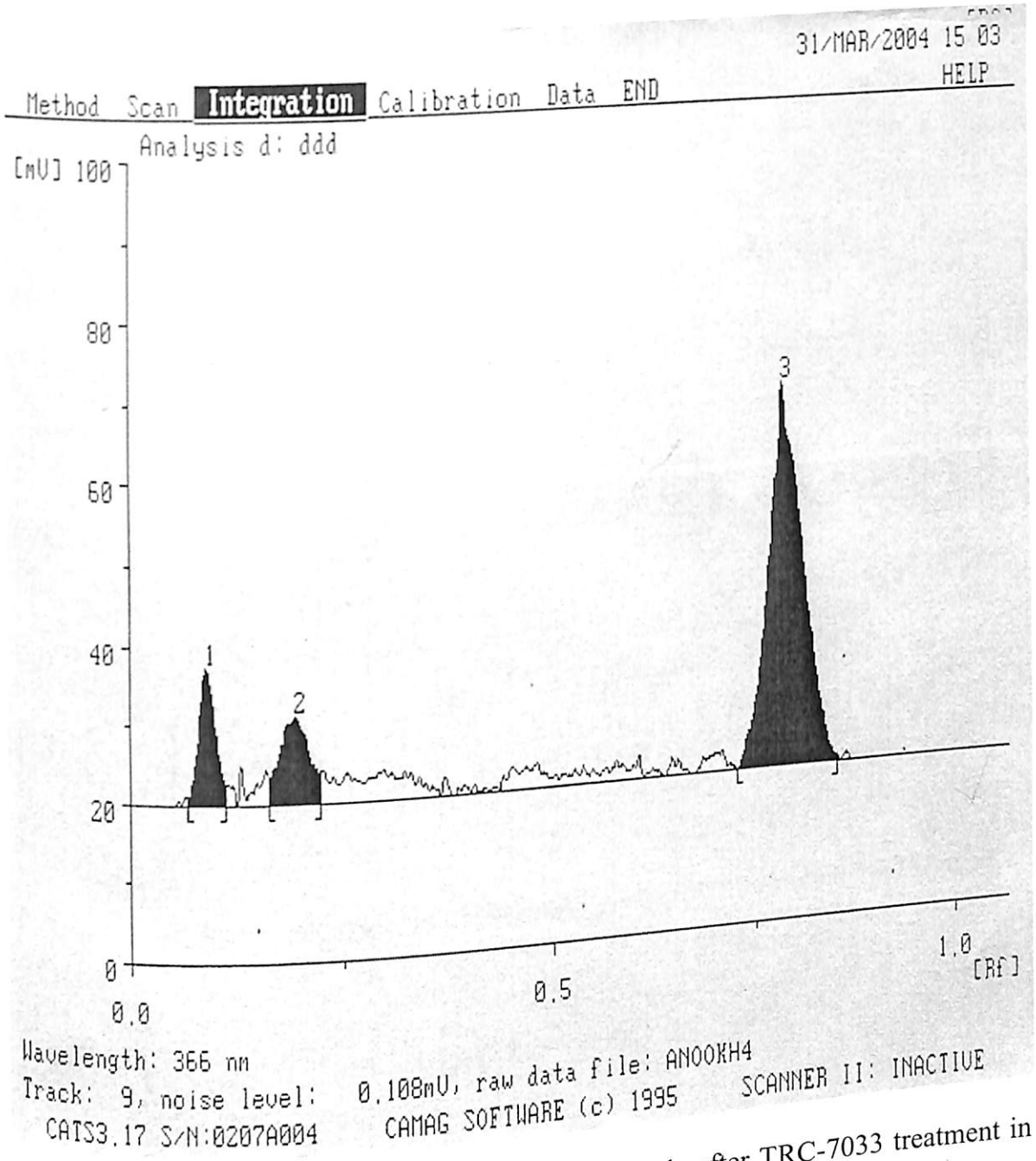


Figure 2: HPTLC chromatogram- Hepatic lipid levels after TRC-7033 treatment in hamster. Peak (1) - Free cholesterol; (2) – Triglyceride; (3) - Cholesterol ester.

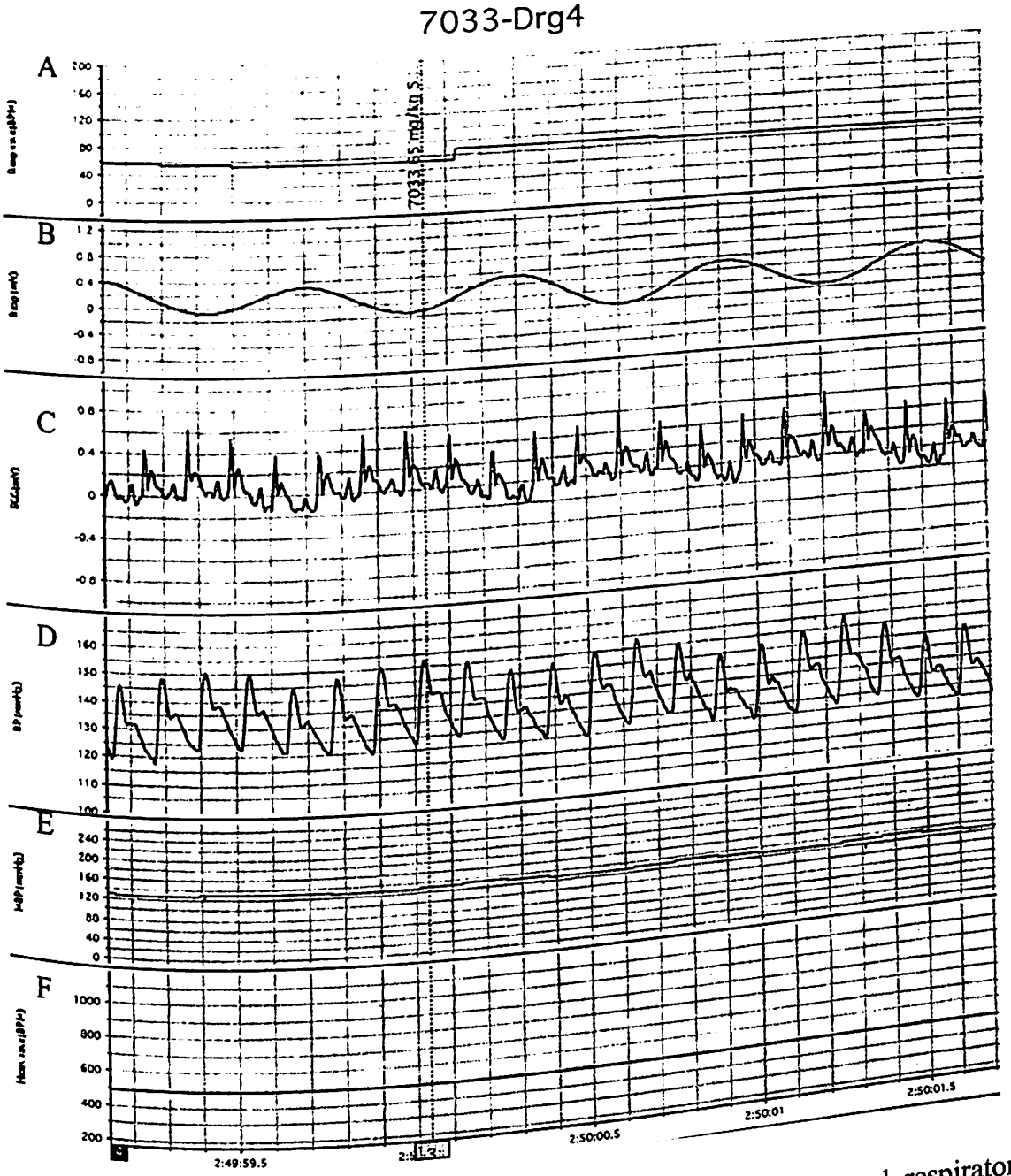


Figure 3: Representative tracing showing various cardiovascular and respiratory parameters in normal anaesthetized male wistar rat. Panel A, rate of respiration; B, respiration; C, electrocardiogram; D, blood pressure; E, mean blood pressure; F, heart rate.

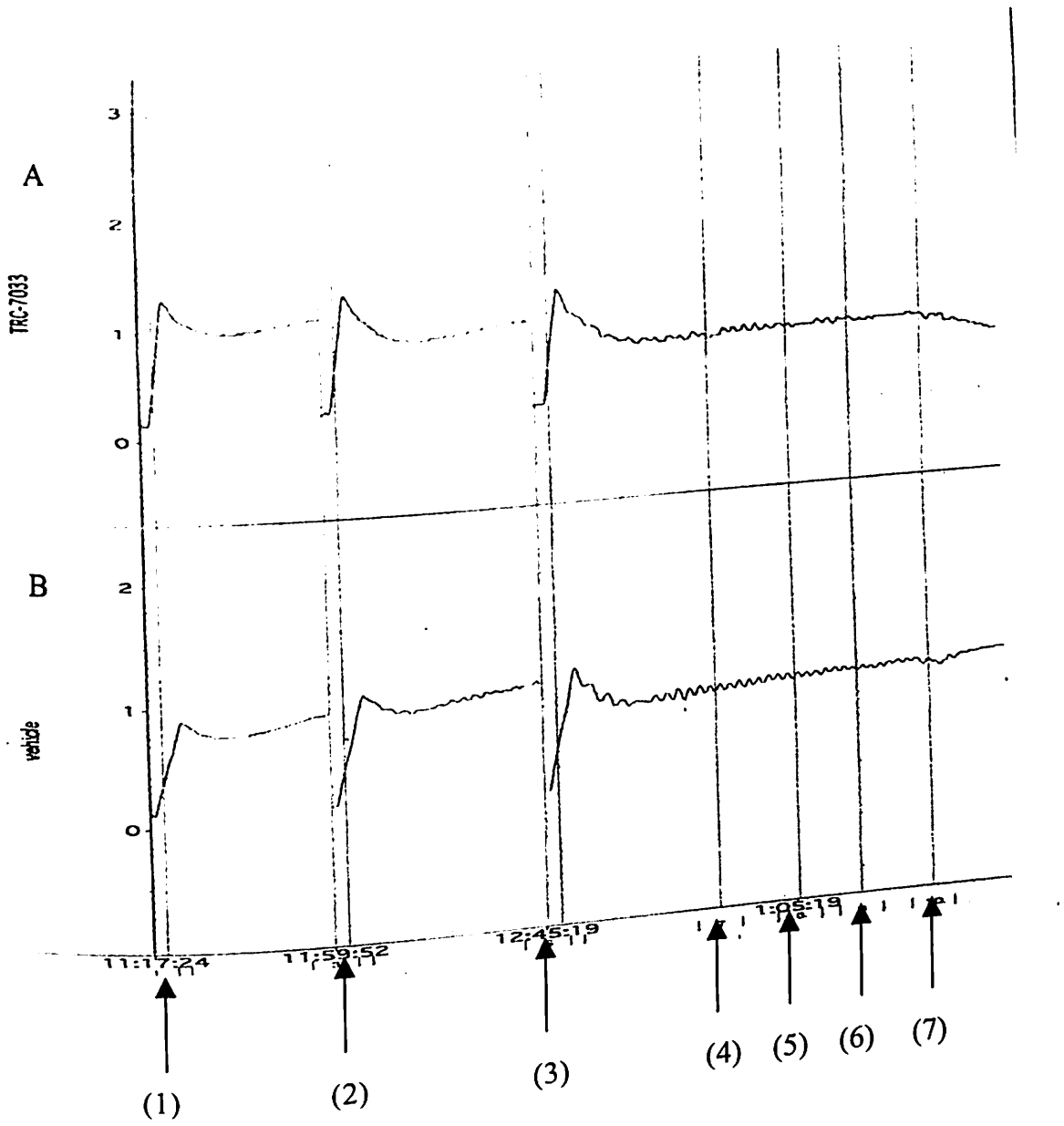


Figure 4: Spasmolytic effect of TRC–7033 on isolated estrous stage rat uterus (wistar). Response to - (1) 30 mM KCl; (2) 30 mM KCl; (3) 30 mM KCl; (4) 0.01μM of TRC-7033 / vehicle; (5) 0.1μM TRC-7033 / vehicle; (6) 1μM TRC-7033 / vehicle; (7) 10μM TRC-7033 / vehicle. Panel A, TRC-7033; Panel B, vehicle.

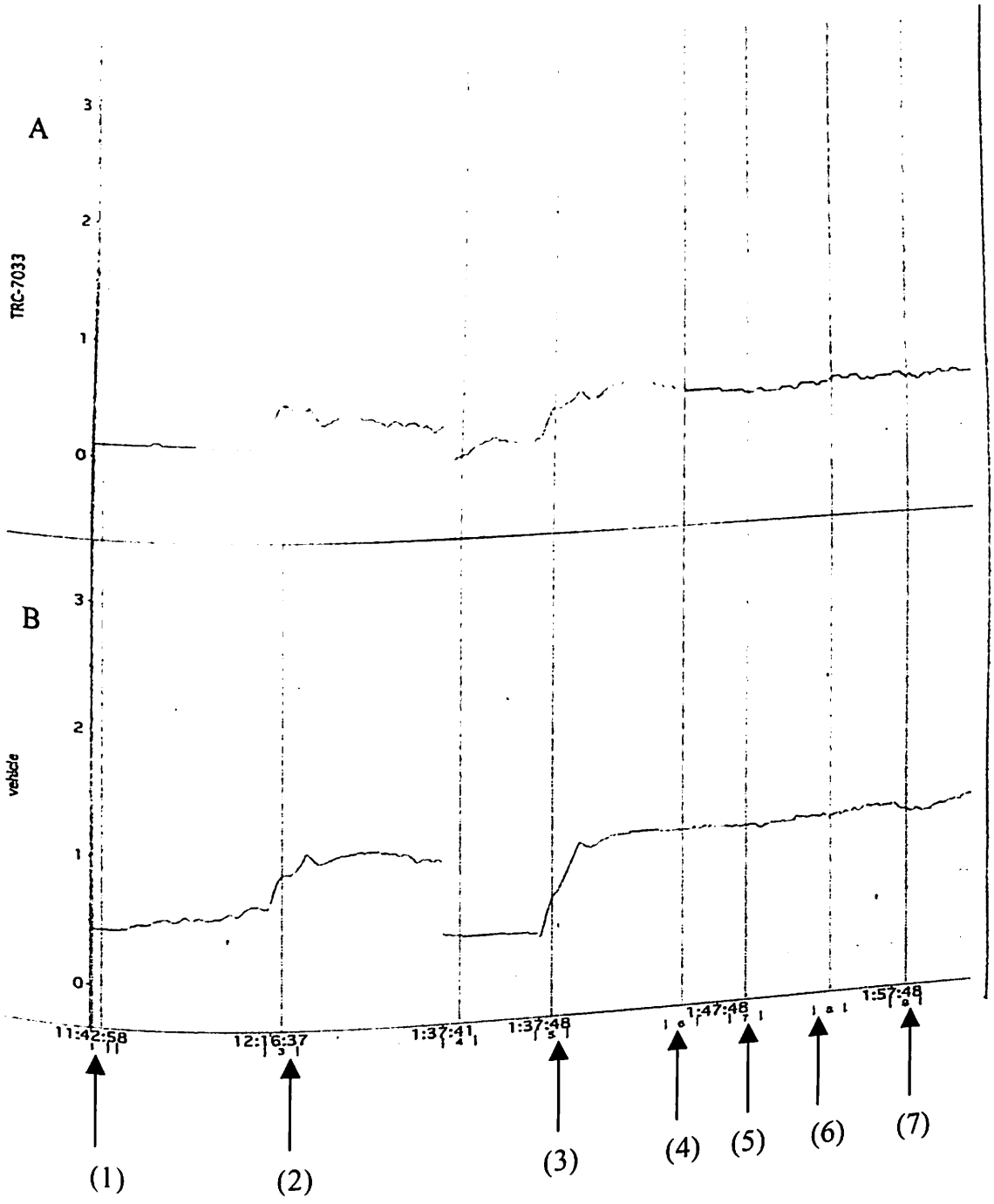


Figure 5: Effect of TRC–7033 on isolated guinea pig ileum (Duncan Hartley). Response to - (1) 30 mM KCl; (2) 30 mM KCl; (3) 30 mM KCl; (4) 0.01μM of TRC-7033 / vehicle; (5) 0.1μM TRC-7033 / vehicle; (6) 1μM TRC-7033 / vehicle; (7) 10μM TRC-7033 / vehicle. Panel A, TRC-7033; Panel B, vehicle.

Table 1: The serum lipid levels and body weight in the vehicle control group before and after 5 days oral administration of fatty food in the hamster model of hyperlipidemia

	TC (mg/dl)	HDL-C (mg/dl)	TG (mg/dl)	LDL-C (mg/dl)	B. wt. (gms)
Basal	145.2 ± 2.9	34.6 ± 0.6	285.4 ± 10.1	53.6 ± 2.2	105.5 ± 1.7
After Fat Diet	243.2 ± 3.7*	45.2 ± 1.2*	259.0 ± 10.6*	146.0 ± 3.0*	108.9 ± 1.9*

Values represented are mean ± SEM * $P < 0.05$ as compared to basal, paired Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; B.wt., Body weight.

Table 2a: The reduction (%) of serum lipids by the test compounds in the hamster model of hyperlipidemia

Test compound	Dose (mg/kg)	Percent reduction				
		B.wt	TC	HDL-C	TG	LDL-C
TRC-7002	38	6.67	14.87	3.37	30.37	7.67
TRC-7003	35.3	-12.70	2.30	3.45	1.44	2.22
TRC-7004	80.9	-0.8	18.6	16	8.5	22.2
TRC-7005	83.56	-8.80	10.19	15.01	-8.21	13.49
TRC-7006	67.49	5.08	-9.61	8.43	1.55	-18.30
TRC-7007	87.6	-11.1	20.1	17.6	-0.1	26.3
TRC-7008	70.55	6.80	-26.94	-24.16	13.72	-38.78
TRC-7009	74.57	8.22	-13.60	-16.05	-10.92	-13.63
TRC-7010	63.1	4.92	21.38	-18.29	38.04	18.43
TRC-7011	80.5	-3.3	27.4	7.9	-2.6	39.5
TRC-7012	78.2	-7.01	-5.91	18.47	-16.61	-9.32
TRC-7013	93.4	-6.11	-0.79	4.43	-2.44	-1.65
TRC-7014	67.5	-1.32	5.45	-4.81	10.44	3.59
TRC-7015	61	3.24	1.15	6.73	15.25	-4.83
TRC-7016	77.25	5.38	12.99	15.07	6.84	13.89
TRC-7017	79.06	3.90	15.56	21.39	27.50	10.74
TRC-7018	85.47	-20.37	-4.12	-2.28	0.25	-6.44
TRC-7019	57.74	5.13	-24.71	1.71	-31.37	-30.63
TRC-7020	82.981	-5.15	-25.68	-19.61	-24.55	-27.71
TRC-7021	80.1	3.63	7.47	5.50	13.33	6.61
TRC-7022	50.1	-0.81	2.68	-8.88	21.95	1.18
TRC-7023	80.11	4.17	8.28	17.48	-11.76	13.02
TRC-7024	57.17	-11.57	5.50	11.65	-31.58	17.36
TRC-7025	91.2	3.68	-2.42	13.58	0.30	-9.49
TRC-7026	59.27	1.39	-3.22	3.10	-15.65	2.11
TRC-7027	81.26	5.28	0.06	2.73	14.80	-9.71
TRC-7028	77.44	3.84	6.95	2.45	-6.03	14.38
TRC-7029	80.495	11.28	-16.67	2.04	-15.35	-23.36
TRC-7030	78.392	11.59	-25.13	13.20	-19.58	-40.27

Values are as compared to respective vehicle control group (n=5-6). TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; B.wt, body weight. When the values were more than the control values, they were shown as negative (-) values (i.e negative values represent % increase).

Table 2b: The reduction (%) of serum lipids by the test compounds in the hamster model of hyperlipidemia

Test compound	Dose (mg/kg)	Percent reduction				
		B.wt	TC	HDL-C	TG	LDL-C
TRC-7031	80.59	7.23	-54.50	31.14	-32.97	-148.27
TRC-7032	83.17	5.34	-40.56	33.72	-26.75	-118.17
TRC-7033	80	6.0	29.5	9.0	44.4	36.4
TRC-7034	90.05	7.57	-23.86	37.60	17.12	-110.70
TRC-7035	68.83	1.20	-30.42	41.99	-37.05	-90.69
TRC-7036	77.25	6.56	-1.04	20.80	-3.03	-10.40
TRC-7037	85.85	2.50	10.23	28.07	3.34	4.96
TRC-7038	85.66	-4.69	0.86	21.05	-22.26	1.79
TRC-7039	109.94	12.19	7.78	15.79	2.77	6.28
TRC-7040	87.76	6.88	4.72	46.37	10.71	-17.53
TRC-7041	75.72	6.10	2.47	0.79	-25.87	13.10
TRC-7042	72.66	0.72	-0.94	3.16	-41.55	12.24
TRC-7043	94.36	6.46	6.62	7.10	3.20	7.69
TRC-7044	75.33	1.62	1.01	-1.97	-22.04	10.17
TRC-7045	83.65	2.15	8.87	10.06	-1.86	12.31
TRC-7046	60.42	-2.84	-11.88	-34.71	-31.72	3.12
TRC-7047	78.01	6.78	7.93	12.68	-2.83	8.91
TRC-7048	93.11	-0.44	4.49	21.87	-31.96	7.29
TRC-7049	85.47	12.50	11.34	17.52	4.00	12.38
TRC-7050	82.92	14.11	22.90	18.86	20.79	25.10
TRC-7051	71.1	16.7	21.5	12.0	27.1	22.2

Values are as compared to respective vehicle control group (n=5-6). TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; B.wt, body weight. When the values were more than the control values, they were shown as negative (-) values (i.e negative values represent % increase).

Table 3: The serum lipid levels in the hamster model of hyperlipidemia on treatment with the test compounds for 5 days.

Group	TC (mg/dl)	HDL-C (mg/dl)	TG (mg/dl)	LDL-C (mg/dl)	B. wt. (gms)
Control (n=9)	269.7±18.4	37.9±4.1	219.2±29.1	188.0±13.8	105.9±11.4
CP-230821 30 mg/kg (n=9)	163.8±11.8*	30.1±3.1	244.0±37.2	84.9±11.8*	92.7±2.9
Control (n=6)	245.3±13.7	49.4±3.8	203.9±31.5	155.1±8.9	98.5±6.1
TRC-7004 80.9 mg/kg (n=6)	199.6±15.8*	41.5±4.3	186.6±9.9	120.8±12.1*	99.3±7.6
Control (n=6)	245.3±13.7	49.4±3.8	203.9±31.5	155.1±8.9	98.5±6.1
TRC-7007 87.6 mg/kg (n=6)	195.9±16.6*	40.7±4.5	204.1±26.4	114.4±17.7*	109.4±3.2
Control (n=6)	236.6±16.1	23.3±3.6	279.1±77.5	157.4±9.7	122±7.2
TRC-7011 80.5 mg/kg	171.7±16.4*	19.2±3.5	286.3±71.4	95.3±22.4*	126±4.0
Control (n=5)	280.3±17.4	52.5±3.7	334.7±85.4	160.9±9.6	99.2±7.8
TRC-7051 71.1 mg/kg (n=5)	220.1±9.2*	46.2±3.2	244.1±37.1	125.1±9.6*	82.6±7.1

Values represented are mean ± SEM * $P < 0.05$ as compared to respective vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; B.wt, body weight.

Table 4: The serum lipid levels in the hamster model of hyperlipidemia on treatment with TRC-7033 / cerivastatin for 5 days.

Group	TC (mg/dl)	HDL-C (mg/dl)	TG (mg/dl)	LDL-C (mg/dl)	B. wt. (gms)	Food Intake (gms/day)
Control (n=5)	228.8±18.8	77.4±3.3	328.2±76.1	85.8±14.1	116.2±5.9	12.8±0.7
TRC-7033 80 mg/kg (n=5)	161.4±3.9*	70.4±6.0	182.4±15.2*	54.5±3.9*	109.2±7.6	12.6±0.9
Control (n=5)	328.5±13.1	60.8±5.4	384.6±8.3	190.8±13.3	113.0±6.2	13.1±0.8
TRC-7033 60 mg/kg (n=4)	241.4±13.5*	47.6±3.9	326.3±12.0*	128.5±11.1*	107.5±1.9	12.5±0.9
Control (n=5)	318.4±10.4	89.8±6.9	514.8±93.8	125.6±11.1	115.4±4.9	11.9±0.8
TRC-7033 40 mg/kg (n=6)	235.8±14*	73.3±6.4	336.8±38.9*	95.1±8.2*	108.1±4.4	12.4±0.8
Control (n=6)	232.3±22.7	46.8±3.1	354.4±65.8	114.7±12.1	99.3±5.0	11.1±0.5
Cerivastatin 0.5 mg/kg (n=6)	202.9±19.7	41.9±6.7	217.0±23.4*	117.6±10.7	86.8±4.1	10.5±0.6
Control (n=5)	261.7±10.9	54.7±4.7	259.0±56.3	155.2±3.7	90.2±4.1	9.8±0.4
Cerivastatin 1.25 mg/kg (n=5)	189.1±17.6*	48.9±3.2	106.9±13.8*	118.8±16.3*	91.2±3.2	9.7±0.2
Control (n=5)	200.7±5.2	77.0±6.5	179.3±41.7	87.8±8.0	118.2±10.8	13.1±0.9
Cerivastatin 2.5 mg/kg (n=5)	130±27.5*	66.8±14.4	137.5±45.1	35.8±11.2*	107.3±7.3	12.3±0.7

Values represented are mean ± SEM * $P < 0.05$ as compared to respective vehicle controls, Student's t-test. TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; B.wt, body weight.

Table 5: The serum hepatic enzyme levels in the hamster model of hyperlipidemia on treatment with TRC-7033 / cerivastatin for 5 days.

Group	ALT (U/L)	T.BLB (mg/dl)	AST (U/L)	ALK. PSPT (U/L)
Control (n=5)	52.6±4.1	0.1±0.0	36.6±4.3	319.4±31.3
TRC-7033 80 mg/kg (n=5)	43.0±4.4	0.1±0.0	29.8±1.6	288.8±4.2
Control (n=5)	47.8±3.7	0.1±0.0	33.4±1.7	231.8±25.5
TRC-7033 60 mg/kg (n=4)	62.0±10.2	0.1±0.0	33.3±2	204.8±9.1
Control (n=5)	50.8±3.6	0.1±0.0	37.6±3.1	288.8±12.6
TRC-7033 40 mg/kg (n=6)	49.7±2.3	0.1±0.0	39.5±3.3	273.7±15.7
Control (n=6)	53.3±3.7	0.1±0.0	33.0±1.8	248.7±11.9
Cerivastatin 0.5 mg/kg (n=6)	60.3±3.5	0.1±0.0	37.0±3.1	260.0±14.7
Control (n=5)	54.0±2.3	0.0±0.0	43.0±4.0	213.0±27.1
Cerivastatin 2.5 mg/kg (n=5)	91.7±12.5*	0.0±0.0	50.7±10.3	226.7±25.2
Control (n=5)	54.0±2.3	0.0±0.0	43.0±4.0	213.0±27.1
Cerivastatin 5 mg/kg (n=2)	226.0±15.0*	0.1±0.0	209.0±9.0*	241.0±10.0

Values represented are mean ± SEM * $P < 0.05$ as compared to respective vehicle controls, Student's t-test. ALT, Alanine aminotransferase; T.BLB, Total bilirubin; AST, Aspartate aminotransferase; ALK.PSPT, Alkaline phosphatase.

Table 6: The hepatic lipid levels in the hamster model of hyperlipidemia on treatment with test compounds for 5 days

Group	Dose (mg/Kg)	LW/BW#	Free Cholesterol (mg/g liver)	Cholesterol Oleate (mg/g liver)	Total Cholesterol (mg/g liver)
Control (n=6)		3.97±0.1	2.60±0.1	19.76±2.4	22.36±2.5
TRC – 7004 (n=6)	80.9	3.96±0.2	2.16±0.1*	6.36±2.6*	8.52±2.7*
Control (n=6)		3.97±0.1	2.60±0.1	19.76±2.4	22.36±2.5
TRC – 7007 (n=6)	87.6	3.97±0.2	2.56±0.1	2.40±0.9*	4.36±1.2*
Control (n=6)		3.75±0.2	2.84±0.3	15.98±1.1	18.83±1.2
TRC – 7011 (n=6)	80.5	3.95±0.2	2.82±0.2	6.94±2.3*	9.76±2.5*
Control (n=5)		3.83±0.3	3.06±0.1	13.56±1.6	16.63±1.7
TRC – 7033 (n=5)	80	4.09±0.1	4.40±0.2*	22.96±1.9*	27.36±2.1
Control (n=5)		3.72±0.1	2.27±0.1	23.09±3.5	25.36±3.6
Cerivastatin (n=5)	2.5	3.93±0.3	2.20±0.2	2.51±2.5*	4.71±2.6*
Control (n=5)		4.90±0.4	2.21±0.2	17.60±1.0	19.81±1.2
CP-230821 (n=5)	30	3.9±0.2*	2.05±0.1	2.01±0.0*	2.05±0.1*

Values represented are mean ± SEM * $P < 0.05$ as compared to respective vehicle controls, Student's t-test. LW/BW, Liver weight/Body weight, #(LW per 100g BW)

Table 7: Effect of TRC-7033 and cerivastatin on LDL-R expression in HepG2 cells.

Drug concentration (in nM)	% Increase in LDL-R expression	
	Cerivastatin	TRC-7033
1000	70.4 ± 5.1	88.3 ± 7.4
300	71.3 ± 5.0	75.8 ± 7.1
100	61.7 ± 4.8	50.0 ± 4.2
30	50.2 ± 3.5	24.4 ± 3.1
10	47.6 ± 4.6	11.6 ± 2.4
3	30.8 ± 2.3	7.9 ± 1.6
1	13.4 ± 1.2	4.0 ± 1.2
0.3	1.5 ± 0.8	3.9 ± 0.7

Values represented are mean ± SEM, from 3 observations each done in triplicate. % increase as compared to vehicle control (DMSO).

Table 8: Effect of TRC-7033 on LDL-R expression in presence of cerivastatin.

Concentration of TRC-7033 (μM) in presence of 0.1 μM cerivastatin	% Increase in LDL-R expression
0	59.3 ± 3.6
0.1	75.1 ± 4.5
0.3	90.4 ± 5.3*
1	106.6 ± 6.5*
3	133.6 ± 9.8*

Values represented are mean ± SEM, from 3 observations each done in triplicate. % increase as compared to vehicle control (DMSO). *$P < 0.05$ as compared to 0.1μM cerivastatin alone (in absence of TRC-7033), Student's t-test.

Table 9a: Effect of TRC-7033 on LDL-R expression in presence of 25-hydroxycholesterol.

Concentration of TRC-7033 (μM) in presence of 2.5 μM 25- hydroxycholesterol	% Increase in LDL-R expression
0	-28.5 ± 1.4
0.1	10.9 ± 1.6*
0.3	26.5 ± 2.8*
1	50.0 ± 4.9*
3	70.7 ± 6.1*

Values represented are mean ± SEM, from 3 observations each done in triplicate. % increase as compared to vehicle control (DMSO). *P< 0.05 as compared to 2.5μM 25-hydroxycholesterol alone (in absence of TRC-7033), Student's t-test.

Table 9b: Effect of cerivastatin on LDL-R expression in presence of 25-hydroxycholesterol.

Concentration of cerivastatin (μM) in presence of 2.5 μM 25- hydroxycholesterol	% Increase in LDL-R expression
0	-28.5 ± 1.4
1	-27.6 ± 3.0
3	-25.1 ± 3.3

Values represented are mean ± SEM, from 3 observations each done in triplicate. % increase as compared to vehicle control (DMSO).

Table 10: Effect of test compounds on LDL-R expression in HepG2 cell line.

Test compounds (at 1 μM)	% Increase in LDL-R expression at 1μM
CP-230821	90.0 ± 5.6
TRC-7004	28.8 ± 7.2
TRC-7007	38.0 ± 4.9
TRC-7011	68.8 ± 5.7
TRC-7051	27.2 ± 1.9
TRC-7033	88.3 ± 7.4
Cerivastatin	70.4 ± 5.1

Values represented are mean ± SEM, from 3 observations each done in triplicate. % increase as compared to vehicle control (DMSO).

Table 11: *In vitro* anti-oxidant activity of the test compounds

Test compounds	EC_{50} for anti-oxidant activity (μM)
CP-230821	50.4
TRC-7007	14.8
TRC-7011	12.6
TRC-7029	13.6
TRC-7025	19.0
TRC-7032	127.5
TRC-7033	5797.1
TRC-7034	15.7
TRC-7035	147.3
TRC-7038	16.5
TRC-7043	217.9
TRC-7046	144.0

EC_{50} calculated from a plot of concentration (12.5,25,50,100 μM) vs % anti-oxidant activity.

Table 12: Cardiovascular and respiratory effects of TRC-7033 on oral and i.v. administration in normal Wistar rats

Treatment	% Change after drug administration		
	Fall in Mean Blood Pressure	Increase in Heart Rate	Fall in Respiration rate
Vehicle for TRC-7033, Oral (n=4)	27.2 ± 6.9	5.0 ± 0.8	21.6 ± 2.7
TRC-7033, 65 mg/kg, Oral (n=5)	23.7 ± 5.7	5.1 ± 1.4	21.4 ± 4.5
TRC-7033, 325 mg/kg, Oral (n=4)	36.3 ± 4.7	6.5 ± 1.5	17.5 ± 4.9
Vehicle for TRC-7033, IV (n=5)	28.2 ± 3.9	6.4 ± 0.6	11.4 ± 1.6
TRC-7033, 2 mg/kg, IV (n=4)	28.8 ± 2.3	6.2 ± 1.0	10.7 ± 1.9
TRC-7033, 10 mg/kg, IV (n=5)	54.8 ± 4.8*	7.8 ± 1.5	15.8 ± 3.0

Values represented are mean ± SEM. * $P < 0.05$ as compared to respective vehicle control (iv), Student's t-test. The responses plotted are changes (%) as compared to basal values.

Table 13: Effect of TRC-7033 after oral administration on spontaneous motor activity at various doses in Swiss albino mice.

Drug	Dose	No. of counts	% change in activity
TRC-7033 (ED_{50}) (n=10)	65 mg/kg	311 ± 11.4	+ 7.61
TRC-7033 (10x ED_{50}) (n=10)	650 mg/kg	319 ± 7.1	+ 10.38
TRC-7033 (25x ED_{50}) (n=10)	1625 mg/kg	305 ± 8.2	+ 5.54
Vehicle control (Na CMC) (n=10)	10 ml/kg	289 ± 8.7	- 2.36[$]
Saline control (n=10)	10 ml/kg	296 ± 12.2	-

Values represented are mean ± SEM. % change in activity compared to vehicle control.
$ Compared to saline control. Na CMC, Sodium carboxymethyl cellulose.

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