AN INVESTIGATION OF LIPOIC ACID AND IT'S DERIVATIVE AS POTENTIAL LIGANDS FOR PPARs

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PREFACE

Cardiovascular disease (CVD) remains the leading cause of mortality in developed countries. Several risk factors are associated with CVD, including type 2 diabetes, obesity, insulin resistance, dyslipidemia and hypertension. Metabolic disorders such as hyperlipidemia, atherosclerosis, diabetes and obesity rarely occur in isolation, but are usually a part of complex phenotype of metabolic abnormalities called Metabolic Syndrome or Syndrome X[1]. Different pharmacological therapies have been developed to control these risk factors.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, which belong to the nuclear receptor superfamily that controls lipid and glucose metabolism as well as inflammatory risk factors for CVD. Synthetic agonists for both PPARα and PPARγ are useful in the treatment of the diseases that are part of this syndrome. Synthetic PPARγ ligands (thiazolinediones; TZDs) are used for their potent anti-diabetic effects. These drugs decrease plasma glucose levels and improve insulin resistance in patients with diabetes. This effect depends in part on the promotion of adipocyte differentiation via PPARγ in adipocytes [1]. PPARα regulates the expression of target genes involved in lipid catabolism and plays a role in the clearance of circulating and cellular lipids as the target of fibrates, which are anti-hyperlipidemic drugs [9]. Fibrates correct dyslipidaemia, thus decreasing CVD risk. Moreover, both PPARα and PPARγ agonists exert anti-inflammatory activities in liver, adipose and vascular tissues.

Recently, it is thought that dual or cross activation of both PPARα in the liver and PPARγ in the adipocytes is important for the improvement of hyperlipidaemia and diabetes associated with obesity [83].

Alpha-Lipoic Acid has been shown to have insulin sensitizing effects in animal models of insulin resistance and appears to have an insulin-sensitizing effect in diabetic patients [81]. In my master's thesis, I propose to investigate the hypothesis that α-Lipoic Acid and its ester derivative are potential PPAR agonists by studying the effect of these compounds on activation of ligand binding domains (LBDs) of PPARs, induction of adipogenesis, gene expression of PPAR target genes and cell proliferation.

If therapeutic dosages of α-Lipoic acid and its ester derivative are able to achieve significant receptor activation of one or more PPAR isotypes, then we would be looking at potential dual PPAR agonists which can be tested in animal models and if successful could be tried in clinical trials for the treatment of global risk in patients with the metabolic syndrome or type 2 diabetes.

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INTRODUCTION

The Peroxisome Proliferator-Activated Receptors (PPARs) were first discovered less than a decade ago as key players in the response to peroxisome proliferators[1]. A variety of synthetic compounds termed as peroxisome proliferators elicit pathological changes in the livers of sensitive species [2].The effects of exposure include the proliferation of peroxisomes, hepatomegaly, and the development of carcinomas[3]. PPARs have now been identified as regulators of lipid and lipoprotein metabolism, glucose homeostasis and cellular differentiation. They have also been implicated in cancer development as well as in the control of inflammatory response and inflammation-related disorders [4].

PPARs are ligand-activated transcription factors belonging to the nuclear receptor gene family that includes the receptors for the steroid, retinoid and thyroid hormones[5,6]. The PPARs form heterodimers with another nuclear receptor, the 9-cis-retinoic acid receptor (RXR). The PPAR/RXR heterodimers recognize and bind to PPAR DNA response elements (PPRE), located in the promoter region of target genes. PPREs consist of a direct repeat of the hexameric AGGTCA recognition sequence separated by one nucleotide, known as DR-1 response elements [6,7,8]. Agonist binding to the receptor changes its conformation such that a binding cleft is created and recruitment of transcriptional co-activators occurs. This results in transcription of relevant target genes. Co-activators include members of the DRIP/TRAP complex that interact with the basal

transcription machinery and CBP and SRC that possess histone acetylase activity that can remodel the chromatin structure [9,10,11,12].

Three PPAR isotypes, which are the products of distinct genes have been identified: PPARα, PPARγ and PPARδ. PPARs possess a modular domain structure that is similar to other nuclear receptors [13]. The functional domains consist of a N-terminal region, DNA-binding domain (DBD) and Ligand-binding domain (LBD). In the C terminus of the LBD, a ligand-dependent activation domain, AF-2 is located [14]. This region is intimately involved in the generation of the receptor's coactivator binding pocket. A ligand-independent activation function, AF-1 is found in close proximity to the N-terminus of the receptor [15]. Each of the three PPAR sub-types exhibits a unique expression pattern within vertebrate tissues [6]. Sequence comparison across the sub-types of their DBDs shows that they are highly conserved, while the LBDs and the N-terminal domains have a slightly lower level of conservation [6].

PPARα

The human PPARα gene has been mapped to chromosome 22 adjacent to the region 22q12-q13.1 by somatic cell hybridization and linkage analysis [6,16]. PPARα shows elevated levels of expression in metabolically active tissues, such as liver, heart, kidney, skeletal muscle and brown fat[17]. It is also present in monocytic, vascular endothelial and vascular smooth muscle cells[9].

PPARα is activated by a structurally diverse class of compounds, such as fibrates and plasticizers[6]. The hypolipidemic fibrate drugs like Wy-14643, clofibrate, fenofibrate and bezafibrate are an important class of synthetic ligands for PPARα. Wy-14643 has been identified as a micromolar activator of murine PPARα[18]. Palmitic acid, oleic acid, linoleic acid and arachidonic acid have been identified as natural PPARα ligands[19]. PPARα also binds to a wide range of saturated fatty acids. All of the fatty acids identified to date bind to PPARα with affinities in the micromolar range. Since no single high affinity natural ligand has been identified, it has been proposed that one of the physiological roles of PPARα maybe to sense the total flux of fatty acids in metabolically active tissues [6,20].

Fibrates are clinically used hypolipidemic drugs, which efficiently decrease plasma triglyceride levels and increase HDL cholesterol concentrations [6,21]. This is achieved primarily through increased clearance and decreased synthesis of triglyceride-rich VLDL. Fibrates lower serum levels of apoCIII, a known inhibitor of VLDL clearance [22] and increase lipoprotein lipase (LPL) gene expression that is involved in lipid metabolism[23]. The effect of fibrates to increase HDL levels has been associated with an increase in apo-AI gene expression [6]. In addition to their effects on extra-cellular lipid metabolism, PPARα has been shown to play a critical role in the regulation of cellular uptake, activation and β-oxidation of fatty acids [9]. Intracellular fatty acid concentrations are determined by modulation of an import and export system that is controlled by proteins such as fatty acid transport protein (FATP) and fatty acid translocase (FAT/CD36) [24,25]. The expression of long chain fatty acid transporters is

induced in a tissue-dependent fashion in liver by PPARα. The expression of acyl-CoA synthetase (ACS), an important enzyme in fatty acid esterification, preventing their efflux from cells, is enhanced by fibrates[26]. Carnitine palmitoyltransferase type I (CPT-I), a key enzyme in mitochondrial fatty acid catabolism is up-regulated by PPARα activators [27].

A role for PPARα in atherosclerosis is suggested by clinical observations indicating that fibrate treatment lowers the progression of atherosclerotic lesion in humans, as well as in animal models [4]. Atherosclerotic lesion formation requires recruitment of monocytes into the arterial wall through expression of adhesion molecules by activated endothelial cells [28]. Expression of the adhesion molecule VCAM-1 was down-regulated by PPARα agonists in human vascular endothelial cells [29]. This process was mediated in part by inhibition of NF-κB, a ubiquitous transcription factor that transduces the effects of many proatherogenic and inflammatory stimuli. PPARα has also been shown to inhibit the actions of NF-κB in aged mice. Wy-14643 corrected the abnormal expression of genes under the control of NF-κB in these mice but was ineffective in aged PPARα^{-}/α^{-} mice [30]. PPARα agonists were shown to inhibit the IL-1 stimulated release of IL-6 and inflammatory prostaglandins in vascular smooth muscle cells [31]. Fenofibrate treatment in patients with coronery artery disease showed reduced plasma levels of IL-6, fibrinogen, and C-reactive protein possibly through negative regulation of PPARα [32].

PPARα also exerts direct anti-inflammatory activities. Activation of PPARα inhibits the inflammatory action of eicosanoids like leukotriene B4 (LTB4) by

augmenting expression of hepatic enzymes involved in their metabolism [33]. Other, non-hepatic, anti-inflammatory mechanisms have been described for PPARα ligands that may be important in maintaining vascular health such as treatment of cytokine-activated human macrophages with PPARα agonists induced apoptosis of the cells by interfering with the anti-apoptotic NF-κB signaling pathway [34]. In hyperlipidemic patients, fenofibrate treatment decreased the plasma concentrations of the inflammatory cytokine interleukin-6[35]. Additional work showed that IκBα levels were induced in vascular smooth muscle cells by fibrates, thereby offering another anti-inflammatory mechanism for PPARα agonists [36].

PPARγ

PPARγ is the most extensively studied of the three PPAR subtypes to date. The human PPAR gene has nine exons and has been mapped to human chromosome 3p25 by somatic cell hybridization and linkage analysis [37]. Three mRNA isoforms of PPARγ have been detected in humans (termed PPARγ1, PPARγ2, and PPARγ3) which arise as products of different promoter usage[38]. PPARγ1 and PPARγ3 mRNAs code for the same protein, while PPARγ2 mRNA codes for a different protein containing 28 additional amino acids at the N-terminus. PPARγ2 is expressed predominantly in adipose tissue, and PPARγ3 is found only in adipose tissue, macrophages, and colon epithelium [39]. PPARγ1 is expressed in the heart, large and small intestines, colon, kidney, pancreas, spleen, and skeletal muscle [6].

The thiazolinedione (TZD) class of antidiabetic agents, commonly referred to as 'glitazones' represent the first compounds identified as high affinity PPARγ agonists. Two glitazones , Rosiglitazone (Avandia) and Pioglitazone (Actos)bind to the receptor with high affinity with EC_{50}s of 0.043uM and 0.58uM respectively[6]. Numerous naturally occurring fatty acids, eicosanoids, prostaglandins and their metabolites are weak endogenous activators of PPARγ. PPARγ exhibits preference for essential polyunsaturated fatty acids (PUFAs) including linoleic acid, linolenic acid, arachidonic acid and eicosapentaenoic acid [40].

PPARγ is a critical transcription factor in the regulation of adipocyte differentiation [6]. Forced expression of PPARγ in fibroblasts in the presence of weak PPARγ activators results in the differentiation of the cells to adipocytes[41]. Adipogenesis appears to require coordinated expression of the PPARγ and two other groups of transcription factors, C/EBP and ADD-1/SREBP-1. Adipocyte differentiation is accompanied by the induction of several genes involved in lipid homeostasis such as aP2 [42], PEPCK [43], acyl-CoA synthetase (ACS)[44], and LPL [45]. All of these genes have been shown to contain PPAR response elements in their regulatory regions. PPARγ has also been shown to up-regulate the expression of fatty acid transporters FATP-1 and CD36 in adipocytes[46].

PPARγ has been shown to modulate a number of other genes involved in energy storage and utilization. Ligand activated PPARγ represses the expression of the *ob* gene, which codes for leptin[47]. PPARγ has been shown to up-regulate expression of the mitochondrial uncoupling proteins UCP1, UCP2, and UCP3 in cells that control cellular

energy homeostasis [48]. Activation of PPARγ in cultured adipocytes induced expression of c-Cbl associated protein, a potential signaling protein in insulin action. Thus PPARγ is key regulator of adipocyte function and lipid homeostasis [6].

TZD PPARγ agonists have been shown to enhance the sensitivity of target tissues to insulin and to reduce plasma glucose, lipid, and insulin levels in animal models of type2 diabetes as well as in humans [49]. TZDs have been shown to decrease blood pressure in a number of animal models, although the mechanism by which they exert their anti-hypertensive effects is not well understood [50, 51].

PPARγ also plays a potential role in the regulation of inflammatory processes. PPARγ agonists have been shown to reduce the expression level of TNF-α in the adipose tissue of obese rats and to block the inhibitory effects of TNF-α on insulin signaling and reduce TNF-α induced glycerol and free fatty acid release [52]. Monocytes and macrophages have a well-established involvement in inflammatory processes, including vascular wall inflammation and atherogenesis, through production of nitric oxide (NO) and inflammatory cytokines such as TNF-α, IL-1, and IL-6 [6]. Chinetti [53] found that PPARγ expression was induced upon differentiation of human monocytes into macrophages. Activation of PPARγ in macrophages by rosiglitazone resulted in induction of apoptosis due to interference with the NF-kB signaling pathway. Activation of PPARγ in macrophages has been shown to inhibit MMP-9 activity [54].Treatment of activated macrophages with PPARγ agonists resulted in a change in cell morphology and suppression of NO production [55]. PPARγ agonists block the production of inflammatory cytokines in human monocytes treated with PMA [56].

Studies based on tumor cell lines have implicated PPARγ in cell cycle withdrawal. PPARγ activation decreases the binding of the E2F/DP heterodimers to its target genes [57]. E2Fs are transcription factors which regulate the expression of genes involved in DNA synthesis [58]. The decrease in E2F/DP activity is mediated by PPARγ through the down-regulation of the PP2A protein phosphatase [59]. Inhibition of E2F/DP activity can also be achieved by activation of the RB gene. RB or the retinoblastoma protein plays a negative role in cell cycle progression by repressing the expression of the E2F target genes [57] . PPARγ ligands inhibit the phosphorylation of RB in vascular smooth muscle cells, therefore contributing to maintain RB in its active form [60]. Consequently the G1/S transition in these cells is abrogated. Another suggested mechanism for mediation of cell cycle arrest by PPARγ involves up-regulation of the cyclin-dependent kinase inhibitors p18 and p21 during adipogenesis [61]. Hence, PPARγ not only controls the expression of genes involved in the acquisition of a differentiated phenotype but also of genes involved in cell cycle control [57].

PPARγ activation reportedly inhibits the proliferation of malignant cells from different lineages such as liposarcoma, breast adenocarcinoma, prostate carcinoma, colorectal carcinoma cell lines [62, 63, 64, 65]. In adipocytes, macrophages, breast, prostate and non-small lung cancer cells, TZDs are reported to induce apoptosis [66]. PPARγ agonists also have anti-proliferative effects on human coronery artery smooth muscle cells [67].These observations suggest that activation of PPARγ may be a promising novel therapeutic approach for treating cancer [57].

PPARδ

Human PPARδ has been localized to chromosome 6p21.1-p21.2 and is expressed in liver, intestine, kidney, abdominal adipose, and skeletal muscle tissues [6]. Unlike the other subtypes, there are no known drugs that have been identified as working through PPARδ [68]. All of the PPAR agonists published to date either have low affinity for the PPARδ or lack selectivity over the other PPARs [6]. Recently, Oliver et al., reported that GW501516 was a potent, highly selective PPARδ ligand and agonist [69]. Like the other subtypes, PPARδ is a receptor for naturally occurring fatty acids. Both saturated and unsaturated fatty acids bind to the receptor. Among the polyunsaturated fatty acids, dihomo-g- linoleic acid, arachidonic acid and eicosapentanoic acid (EPA) bind with affinities in the low micromolar range. Several eicosanoids have also been shown to activate the receptor [6].

A role for PPARδ is proposed in lipid homeostasis since like the other two subtypes, fatty acids and fatty acid metabolites can activate the receptor. The potent and selective PPARδ ligand GW501516 induces a substantial increase in HDL-cholesterol levels as well as reduction in triglyceride levels in obese Rhesus monkeys. In addition , elevated levels of plasma insulin were suppressed by GW501516 treatment [69].

PPARδ may play a role in maintaining reproductive capacity in females. PPARδ is the only subtype expressed in the uterus during the implantation period in mice. Cyclooxygenase-2 (COX2) null mice show defects in implantation and decidualization. Since COX2 is involved in the synthesis of prostacyclin, it is hypothesized that COX2

null mice may be deficient in endogenous activators of PPARδ. In support of this hypothesis, PPARδ activators such as carbaprostacyclin given in combination with 9-cis-retinoic acid were shown to restore implantation in COX2 null mice [6, 70].

PPARδ has also been linked to colon cancer. PPARδ has been identified as a target of the tumor suppressor adenomatus polyposis coli (APC) gene, which is mutated in familial adenomatous polyposis, an inherited disease characterized by numerous colorectal adenomas [71]. In colorectal cancer cells which posses inactivating mutations of APC, PPARδ is highly expressed. The transcripton factors in the APC signaling pathway, β-catenin/Tcf-4, were found to interact directly with and activate the promoter of PPARδ resulting in high levels of PPARδ expression [6]. In addition, the non-steroidal anti-inflammatory drugs (NSAIDs) like sulindac, which suppresses colorectal tumorogenesis, can antagonize PPARδ. Thus PPARδ maybe a critical intermediate in the tumorogenic pathway of the APC gene and be the molecular target for the effect of NSAIDs on colorectal cancer [72].

α -Lipoic Acid and its Ester Derivative

α-Lipioc Acid (LA, 1, 2-dithiolane-3-pentanoic acid) occurs naturally in prokaryotic and eukaryotic cells [73]. LA is a potent antioxidant and free radical scavenger, and its reduced form, dihydrolipoic acid(DHLA), is an even more potent antioxidant. The LA/DHLA oxidation/reduction couple has a redox potential greater than that of α-tocopherol, vitamin C, ubiquinone(coenzyme Q), glutathione, and other well

known antioxidants [74]. Therefore, DHLA has the capacity to regenerate the reduced forms of vitamin E, vitamin C, coenzyme Q and glutathione, thereby maintaining the endogenous reduced state and opposing oxidative stress.

In chronically glucose-fed Sprague-Dawley rats, LA lowered the associated increases in heart mitochondrial superoxide anion production and advanced glycation end-product(AGE) content in the aorta, normalized superoxide anion production in aorta and prevented the fall in tissue glutathione and glutathione peroxidase activity [75]. Moreover, LA has been shown to decrease lipid peroxidation and protein glycation in high glucose-treated human erythrocytes [76]. In patients with type 2 diabetes, treatment with LA significantly decreased plasma lipid hydroperoxides, markers of oxidative stress [77].

A growing body of evidence suggests that LA may have potential therapeutic value in lowering elevated glucose levels in diabetic conditions. Oral or intravenous administration of LA was shown to modestly increase insulin sensitivity in individuals with type 2 diabetes [78]. In animal models of diabetes, chronic LA administration increased GLUT-4 protein level in muscle membranes, improved insulin-stimulated 2-deoxyglucose uptake into skeletal muscles, and reduced blood glucose level indicating that LA might enhance the capacity of insulin stimulated glucose transport and utilization in skeletal muscle [79]. Mechanistic studies conducted in insulin responsive cells in culture demonstrated that LA rapidly stimulates glucose uptake by activating elements of the insulin signaling pathway [80, 81]. Therefore, it has been proposed that the insulin-sensitizing effects of LA is a consequence of its anti-oxidative properties, its ability to

reduce oxidative stress, repair impaired antioxidant defense mechanisms and improve the imbalance between increased oxidative stress and depleted antioxidant defense in diabetes and diabetic complications. Theses beneficial effects have been attributed to alleviation of oxidative stress resulting from the pathophysiological mechanisms underlying these conditions [82].

The discovery that the thiazolinediones; rosiglitazone and pioglitazone, which are in clinical use for the treatment of type 2 (insulin resistant) diabetes, were high affinity agonists for PPARγ, led to the proposal that ligand-dependent modulation of gene transcription through PPARγ activation was an important pharmacological target in the treatment of diabetes. This concept was underscored by the fact that several insulin-dependent genes in glucose and lipid metabolism possessed PPAR gene response elements [9]. The discovery that the anti-dyslipidemic fibrates were specific PPARα agonists that modulate the expression of genes involved in fatty acid oxidation, implied an important role of PPARα in triglyceride and lipoprotein metabolism [6].

Since PPARγ ligands are proven insulin-sensitizing agents, and lipoic acid has been shown to have insulin sensitizing effects in animal models of insulin resistance and appears to have an insulin-sensitizing effect in diabetic patients, the hypothesis that LA is a PPAR ligand was investigated. Furthermore, activation of PPARs by an ester derivative of LA, diacetylethyllipoate (LA-E1) was also studied.

Structures of LA and LA-E1

The chemical structures of LA and LA-E1 are shown in Fig.1. LA-E1 was obtained by esterifying LA at three loci, the carboxyl terminus and at each sulfur of the dithiolane ring. The resulting product is an uncharged, lipid soluble pro-LA precursor. The ester bonds shown are hydrolysable under physiological conditions. LA-E1 is uncharged, and therefore more lipid soluble and cell-permeable than LA. The ester bonds of LA-E1, are presumably cleaved and the products metabolized in vivo to yield the parent compound and metabolites thereof.

Fig. 1: Structural formulae of α-lipoic acid (LA) and LA-ester(LA-E1)
The asterisk (*) indicates a chiral carbon.

MATERIALS AND METHODS

Experimental Compounds

Enantiomeric mixtures of LA and LA-E1, which was synthesized from commercially available LA(Sigma-Aldrich), were used in all experiments. Synthesis of LA-E1 was performed in the laboratory of Dr. Mitchell Avery at the University of Missippi.

Cultured Cells

CV-1 monkey epithelial cells (ATCC CCL-70), murine 3T3-L1 preadipocytes (ATCC CCL-173), human breast carcinoma cells MCF-7 (ATCC HTB-22) and human colorectal carcinoma cells HT-29(ATCC HTB-38) were obtained from American Type Culture Collection. The HepG2 human hepatoma- derived cell line stably expressing elevated levels of transfected human PPARα was kindly provided by Dr. Eric Johnson [85], human epidermal neonatal keratinocytes were obtained from Cascade Biologics and HaCaT cells were provided by Dr. Alex Markham [86].

Transient Transactivation Assay

The ability of the compounds (LA and LA-E1) to activate the ligand binding domain (LBD) of three PPAR isoforms (α, γ, δ) was studied by transient transactivation assays.The murine PPARs were tested using the PPAR-GAL4 assay system (Fig.2a). This system used receptor plasmid constructs that has the ligand binding domain of the

respective receptor fused to the DNA binding domain of the yeast GAL4 gene. The constructs were co-transfected with a luciferase reporter plasmid that contained the GAL4 binding site upstream of the luciferase gene. The human PPAR receptors were studied using the Full-Length Receptor Assay System. This system utilized full- length receptor plasmid constructs that were co-tranfected with a luciferase reporter plasmid that contained tandem repeats of PPRE upstream of the luciferase gene. In addition, in both systems a third plasmid was transfected containing the β-galactosidase gene as an internal transfection control.

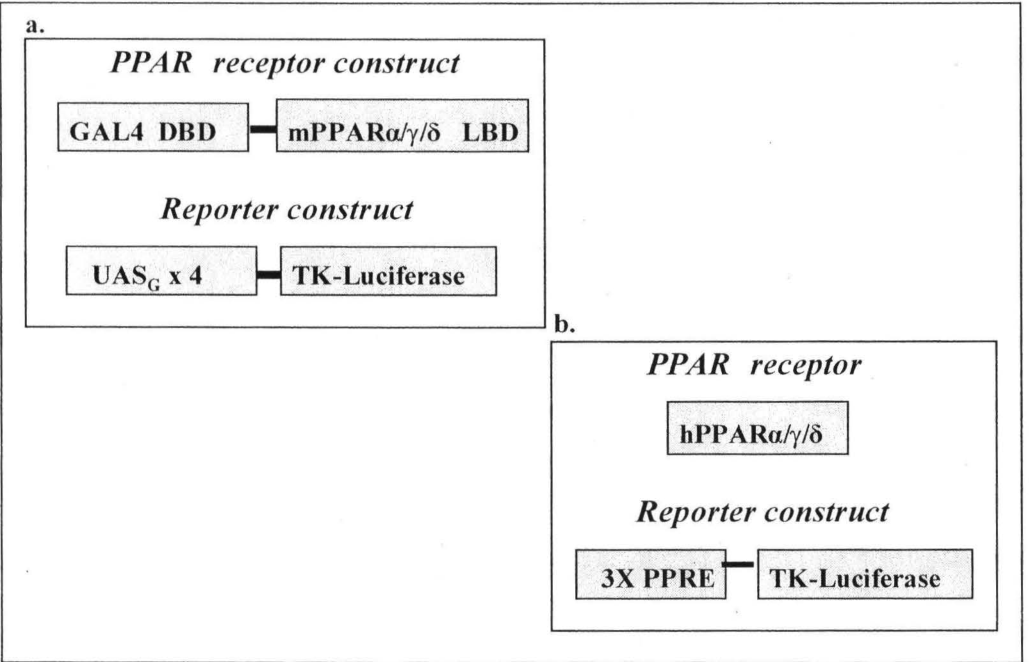


Fig.2: Transient Transactivation Assay Receptor Constructs
(a) PPAR-GAL4 Assay System (b) PPAR Full-Length Receptor Assay System

For the assays, CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% cosmic calf serum (Hyclone) and antibiotics at 37^0C and 5% CO_2. CV-1 cells were plated at a density of $2 X 10^5$ cells/well in media with 5% calf serum and antibiotics. The media in the wells was replaced the next day with DMEM containing 0.5% charcoal-dextran treated fetal bovine serum and antibiotics. After 48 hrs post-plating, the cells were transfected with a receptor expression plasmid (GAL4-mPPARα LBD [2.08μg/μl], GAL4-mPPARγ LBD [1.21μg/μl] , GAL4-mPPARδ LBD [1.18μg/μl], hPPARα[2.66μg/μl], hPPARγ [2.9μg/μl]), luciferase expression plasmid (UAS-tk-luc/3xPPRE-tk-luc) [1.37μg/μl] and a β-galactosidase expression plasmid [1.625μg/μl]. Transfections was carried out by using GenePorter I (Gene Therapy Systems) according to the manufacturer's instructions. After 24 hours post-transfection, cells were treated with the indicated compounds for 24hours and the cell extracts were prepared for luciferase and β-gal activity. Each drug concentration was represented in triplicate wells and the experiments were repeated at least twice. Luciferase activity was measured using the Promega Luciferase Assay Detection System. β-gal activity was determined by using the Promega β-galactosidase Assay Kit and was used to normalize the luciferase data.

Gene Expression Studies

The ability of LA or LA-E1 to alter transcription of genes that are regulated either by PPARα or PPARγ was determined by using quantitative real-time PCR (RT-qPCR).

Cells were treated with varying concentrations of the respective compounds and total RNA was isolated. RT-qPCR was then performed with gene-specific primers.

(A) PPARγ target gene studies: Murine 3T3-L1 cells were maintained sub-confluent in DMEM containing 10% fetal bovine serum and antibiotics. For RNA isolation cells were grown to confluence in 60mm dishes in DMEM containing 5% calf serum and antibiotics. After reaching confluence, cells were incubated for 32 hours in media containing 1μM dexamethasone (DEX), 5μg/ml insulin and 0.5mM isobutylmethylxanthine (IBMX). After 32 hours , cells were washed twice with Hanks Basal Salt Solution and incubated in media containing 5% calf serum and antibiotics and the varying concentrations of the test compounds for 48 hours. Cells were washed twice with PBS and RNA isolation was performed using the RNAqueousTM–4PCR Kit from Ambion. All steps inclusive of the DNase treatment was performed according to the manufacturer's instructions. Following isolation, each sample was quantitated spectrophotometically and checked by electrophoresis on a 1.5% denaturing agarose gel. RT-qPCR was performed on the DNA Engine Opticon™ 2 System from MJ Research Inc. using the SYBR® Green Quantitative RT-PCR Kit from Sigma. Results were analyzed by the Q-gene software[89]. PPARγ-target genes that were tested are listed in Table 1 along with the respective primer pairs and PCR conditions. All primers were designed using the Primer3 software. Product size of the amplicons was confirmed by a 2% agarose gel electrophoresis (Fig.3). The cyclophilin (peptidylprolyl isomerase A) gene was used as an internal control for normalization. Expression levels of selected genes was determined by generating an eight-point serial standard curve (each point

performed in triplicate) using vehicle-treated RNA for each gene, with the final assay concentration ranging from 3.9-500ng. This curve was used to calculate the amount of target gene mRNA in vehicle and treated sample based on RTq-PCR performed with 125ng total RNA/25µl reaction (reactions performed in triplicate). Fold changes in gene expression are displayed as the amount of normalized mRNA in LA and LA-E1 treated samples relative to that in the vehicle treated controls, which was arbitrarily defined as 1. The gene expression studies were repeated at least twice for the selected genes.

(B) PPARα target gene studies: HepG2-mPPARα cells were maintained in DMEM containing 10% fetal bovine serum, 400µg/ml G418 and antibiotics. For RNA isolation cells were grown to 70% confluence in 60mm dishes in DMEM containing 5% fetal bovine serum, 400µg/ml G418 and antibiotics. Cells were then incubated for 48 hours in media containing 5% calf serum and antibiotics and the varying concentrations of the test compounds. After 48 hours, cells were washed twice with PBS and RNA isolation and RTq-PCR was performed as described previously for PPARγ target genes. PPARα-target genes tested are listed in Table 1 along with the respective primer pairs and PCR conditions. The gene expression studies were repeated at least twice for the selected genes.

Adipocyte Differentiation Assay

The ability of LA and LA-E1 to induce differentiation, was studied in a pre-adipocyte cell line (3T3-L1).

The 3T3-L1 cells were cultured to confluence in DMEM containing 5% calf serum and antibiotics. After reaching confluence cells were treated for 32 hours in differentiation media (media containing 1μM dexamethasone (DEX), 5μg/ml insulin and 0.5mM isobutylmethylxanthine (IBMX). After 32 hours, cells were washed twice with Hanks Basal Salt Solution, followed by addition of DMEM containing 5% calf serum and antibiotics and increasing concentrations of the test compounds. Five days after treatment, cells were fixed in 10% formalin in PBS and stained with Oil Red O and qualitatively evaluated for adipogenesis [88]. Quantitative evaluation of the adipogenesis was performed using the Serum Triglyceride Determination Kit (SIGMA). The triglyceride data was normalized for cell concentration by assaying for total protein. Cells were lysed in 0.2N NaOH and protein was assayed using the Bio-Rad Protein Assay (BIO-RAD).

Cell Proliferation Assay

Cells were plated at a density of 2×10^3 cells /well in 24-well dishes in DMEM containing 10% calf serum and antibiotics. After 24 hours post-plating, the test compounds were added to each well at the appropriate concentrations. All concentrations were tested in triplicates. The cells were again shifted to fresh optimal media containing the indicated concentrations of drugs on days 3, 6 and 9 after prior exposure to the drug. When the control well (no drug compound added) was 90% confluent, the cells were evaluated for viable cell number with the Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay System.

Cytotoxicity Studies

To examine any cytotoxic effects of LA and LA-E1 cells were seeded at 2 x 10^5 cells /well in 24 well dishes in optimal media. After 24 hours post-plating, cells were treated with the various drug concentrations in optimal media for 48 hours. The cells were evaluated for cytotoxicity by the CellTiter 96® Aqueous Non-radioactive Cytotoxicity Assay System (Promega). Data was normalized by evaluating viable cell number with the Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay System.

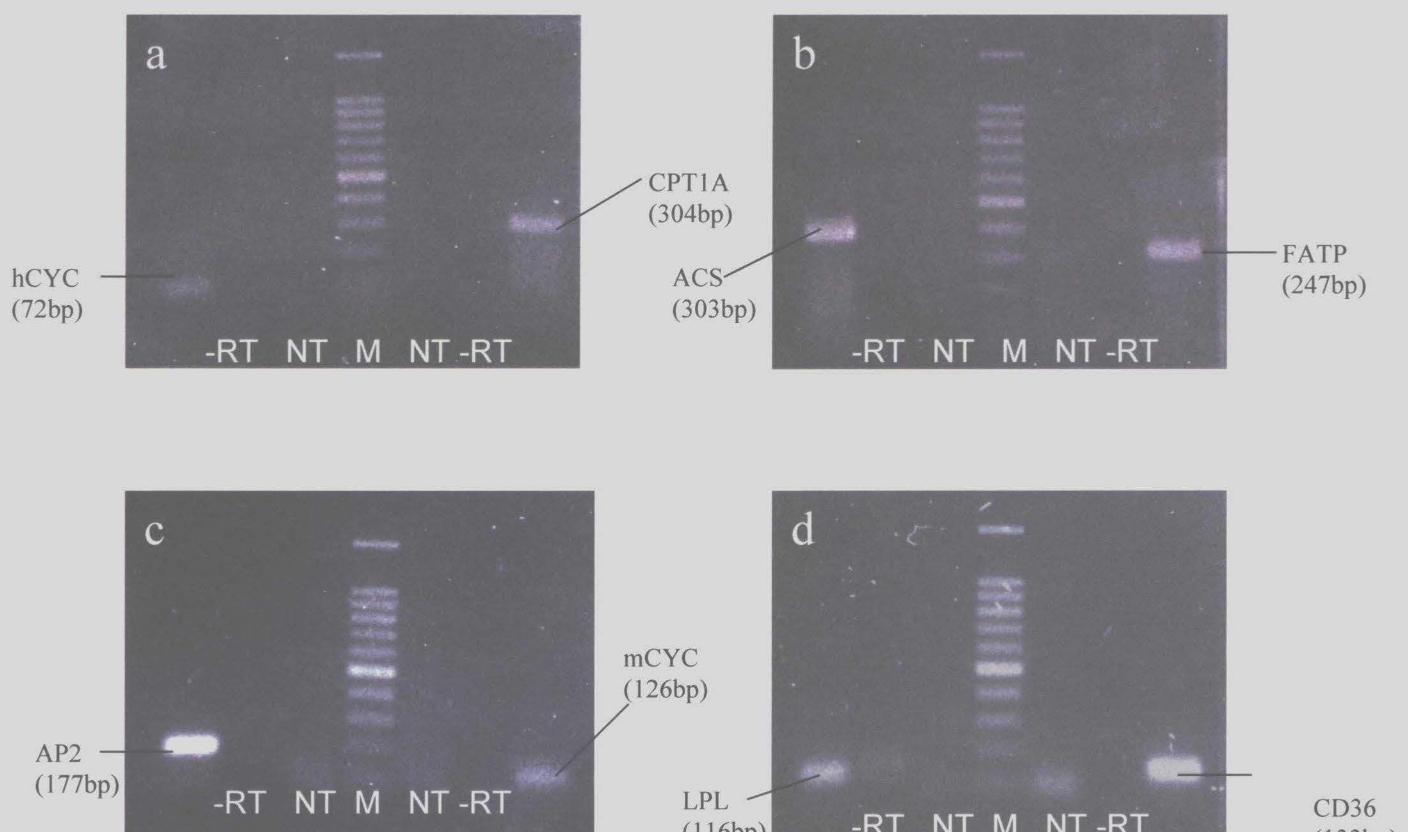


Fig.3: 2% Agarose Gel Analysis of RTq-PCR amplicons

(a) hCYC and hCPT1A genes alongwith no reverse transcriptase (–RT) and no template (NT) controls respectively (b) hACS and hFATP genes with controls (c) mCYC and AP2 genes with controls (d) mLPL and CD36 genes with controls. M= 100bp DNA Ladder.

Organism	Gene	Primer Sequence (5' → 3')		Annealing * Temp. (⁰C)
Mouse	*aP2*	Forward:	AACACCGAGATTTCCTT	55
		Reverse:	ACACATTCCACCACCAG	
	CD36	Forward:	GGAACCCATAACTGGATTCAC	55
		Reverse:	CCAGTCTCATTTAGCCACAGT	
	Cyclophilin	Forward:	AGCATACAGGTCCTGGCAT	55-60
		Reverse:	TCACCTTCCCAAAGACCAC	
	LPL	Forward:	AGGTGGACATCGGAGAAC	58
		Reverse:	TTTCACTCGGATCCTCTCG	
Human	*ACS*	Forward:	AGCAGAGCTTCGCAGCGGC	60
		Reverse:	CTGCTGTTTTCGCTGGGTCC	
	CPT1A	Forward:	AATCATCAAGAAATGTCGCACGA	60
		Reverse:	AGGCAGAAGAGGTGACGATCG	
	FATP	Forward:	GGCGCCACCCCGACAAGAC	61
		Reverse:	CGGGCTGGCATGGACCTCAC	
	Cyclophilin	Forward:	CAAATGCTGGACCCAACACA	55-62
		Reverse:	TGCCATCCAACCACTCAGTC	

Table 1 : RT-qPCR Conditions and Sequences of primers pairs
RT-qPCR Conditions for all primer sets were similar except for differences in temperature in the annealing step of the PCR reaction. The amplification conditions were as follows: one cycle of first strand synthesis at 50°C for 30 mins. and inactivation at 95°C for 15 mins.; followed by 40 cycles of denaturation at 95°C for 15 secs, *annealing for 30secs at indicated temp. and extension at 72°C for 30 secs. A final extension step was done at 72°C for 10 mins. Melt Curve Analysis was done at 68°C to 90°C.

RESULTS

LA and LA-E1 activate PPARα and PPARγ

The ability of LA and LA-E1 to activate the murine PPARs (mPPARγ, mPPARα and mPPARδ) was tested by a heterologous transactivation assay in CV-1 cells. The assay eliminates interference from endogenous receptors. Activation of mPPARγ by LA and LA-E1 was shown to be concentration dependent (Fig.4a). At 1mM, LA increased the PPARγ driven luciferase activity 5-fold greater than that seen with the vehicle-treated cells. At 2mM, LA and LA-E1 increased PPARγ activity by 8-fold and 35-fold, respectively, indicating greater efficacy of the LA-E1 compared to LA. Both compounds had narrow activation thresholds with similar EC_{50} values of 1.52mM for LA and 0.58mM for LA-E1 (Fig.4b).

Activation of mPPARα by LA and LA-E1 was also shown to be concentration dependent with both LA and LA-E1 showing similar activation over the same concentration range (Fig.5a). LA increased the activity of PPARα by 3 to 30-fold between 0.2 and 1mM, whereas LA-E1 increased mPPARα activity from 13 to 25-fold for the same concentration range. Activation thresholds for both compounds were again similar, with EC_{50}s of 0.89mM for LA and 1.06mM for LA-E1 (Fig.5b). Neither compound had any effect on mPPARδ activity (data not shown).

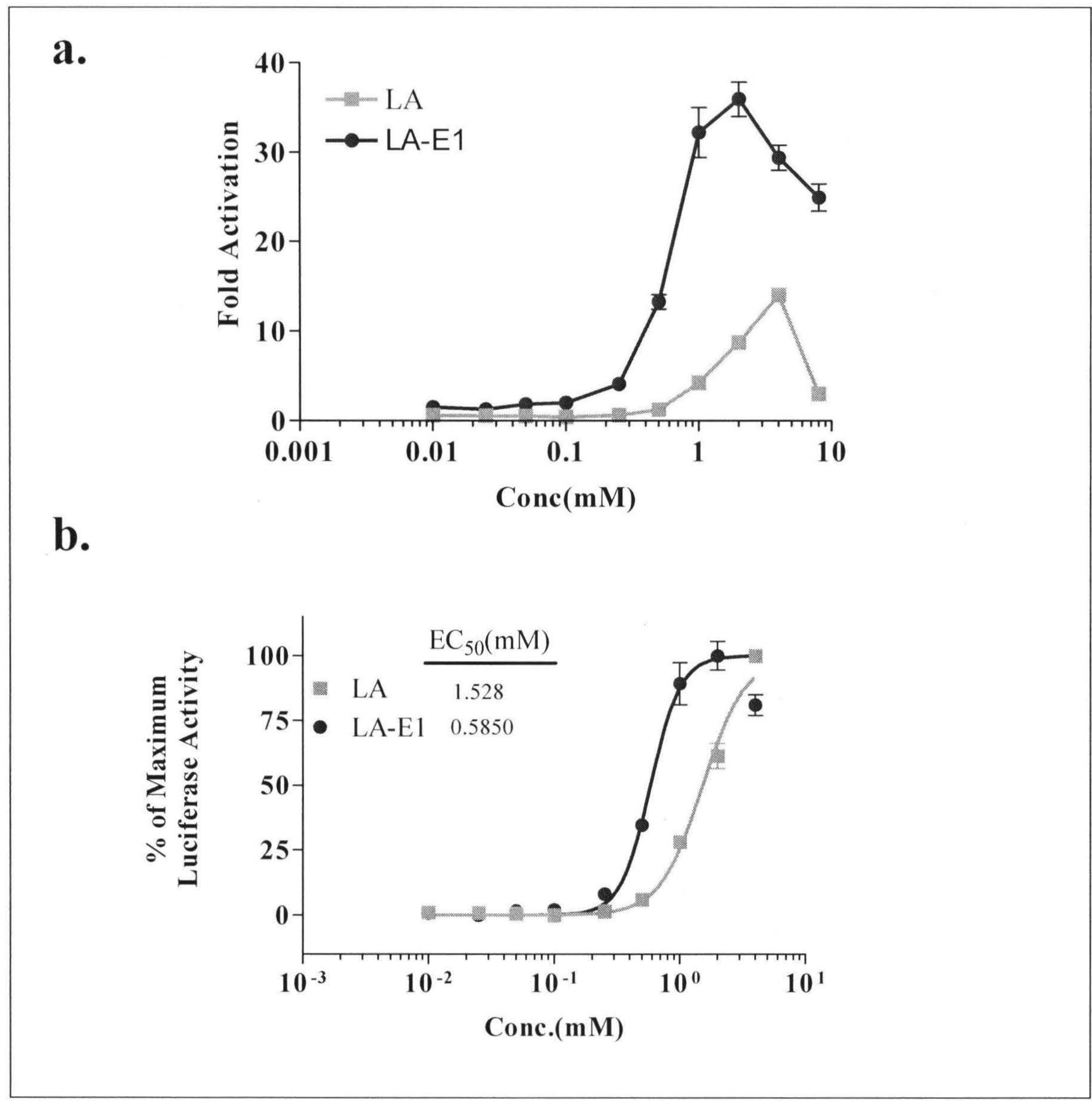


Fig.4: Activation profile of mPPARγ by LA and LA-E1
Transactivation of the GAL4-mPPARγ chimeric receptor by LA and LA-E1 in transiently transfected CV-1 cells. Transfected cells were treated with the indicated concentrations of ligand for 24 hrs and normalized total luciferase activity determined. (a) Fold activation as compared to vehicle treatment as a function of ligand concentration. (b) Data from Fig.2a presented as percent of maximal activity as a function of ligand concentration. LA (■), LA-E1 (●). Bars represent +/- S.E.M.

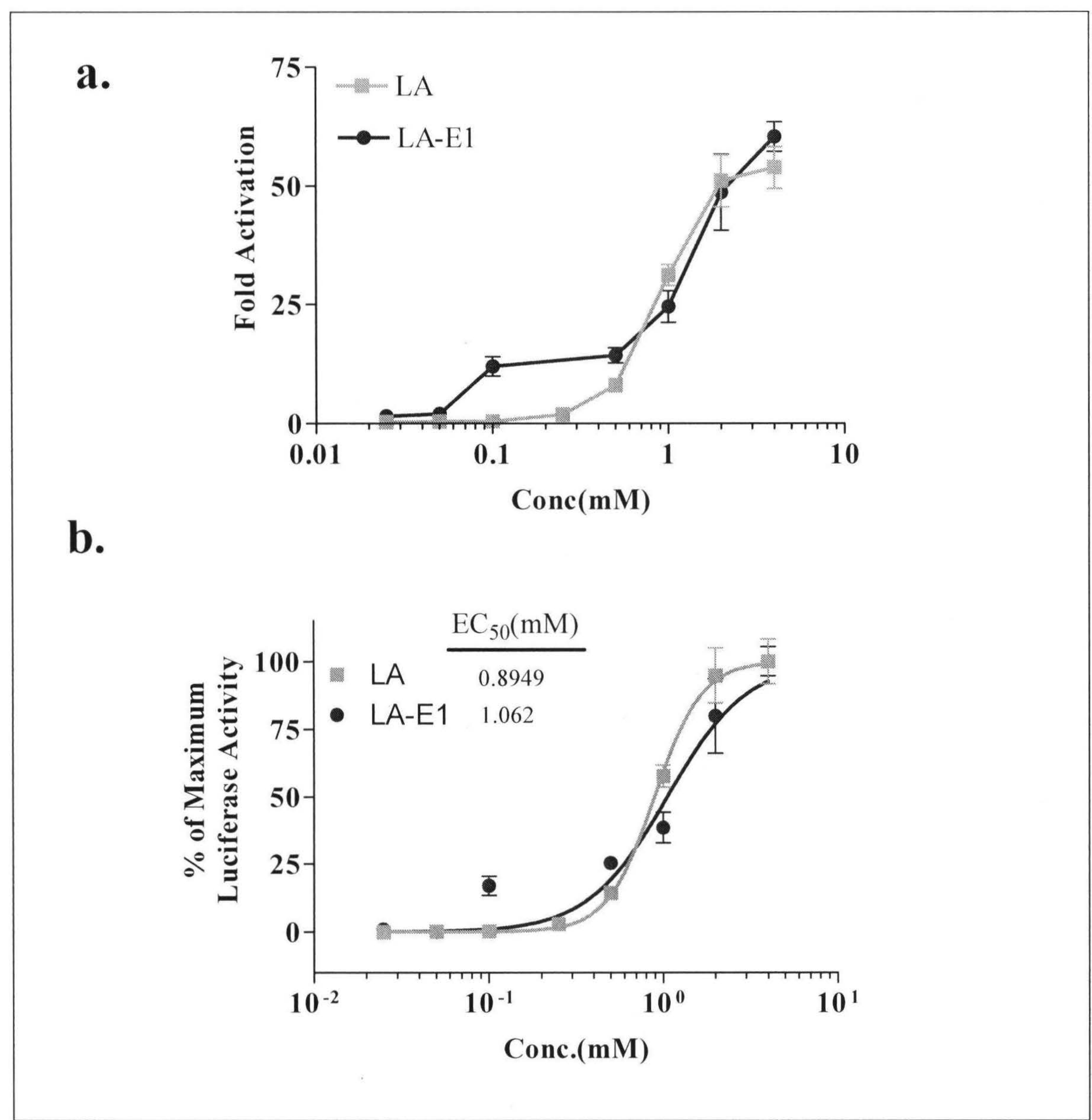


Fig.5: Activation profile of mPPARα by LA and LA-E1

Transactivation of the GAL4-mPPARα chimeric receptor by LA and LA-E1 in transiently transfected CV-1 cells. Transfected cells were treated with the indicated concentrations of ligand for 24 hrs and normalized total luciferase activity determined. (a) Fold activation as compared to vehicle treatment as a function of ligand concentration. (b) Data from Fig.3a presented as percent of maximal activity as a function of ligand concentration. LA (■), LA-E1 (●). Bars represent +/- S.E.M.

The ability of LA and LA-E1 to also activate the human PPARs (hPPARγ, hPPARα) was tested in CV-1 cells by transient co-tranfection of plasmid expressing full length receptor, PPRE 3X-tk-luciferase reporter plasmid and the β-galactosidase expression plasmid for normalization of results.

Activation of hPPARγ by LA and LA-E1 was concentration dependent (Fig.6a). At 1mM, LA increased hPPARγ activity 2-fold greater than that seen with the vehicle-treated cells. At 2mM, LA and LA-E1 increased hPPARγ activity by 2.2-fold and 3.5-fold, respectively, indicating slightly greater efficacy of the LA-E1 compared to LA. However there was a marked difference between the two compounds in terms of the activation thresholds with EC_{50}s of 0.47mM for LA and 5.16mM for LA-E1 (Fig.6b).

Activation of hPPARα by LA and LA-E1 was also shown to be concentration dependent with both LA and LA-E1 showing similar activation over the same concentration range(Fig.7a). LA increased the activity of hPPARα by 3 to 4-fold between 0.2 and 1mM, whereas LA-E1 increased hPPARa activity from 3 to 6-fold for the same concentration range. Both compounds had narrow activation thresholds for both compounds with similar EC_{50}s of 0.61mM for LA and 0.55mM for LA-E1 (Fig.7b).

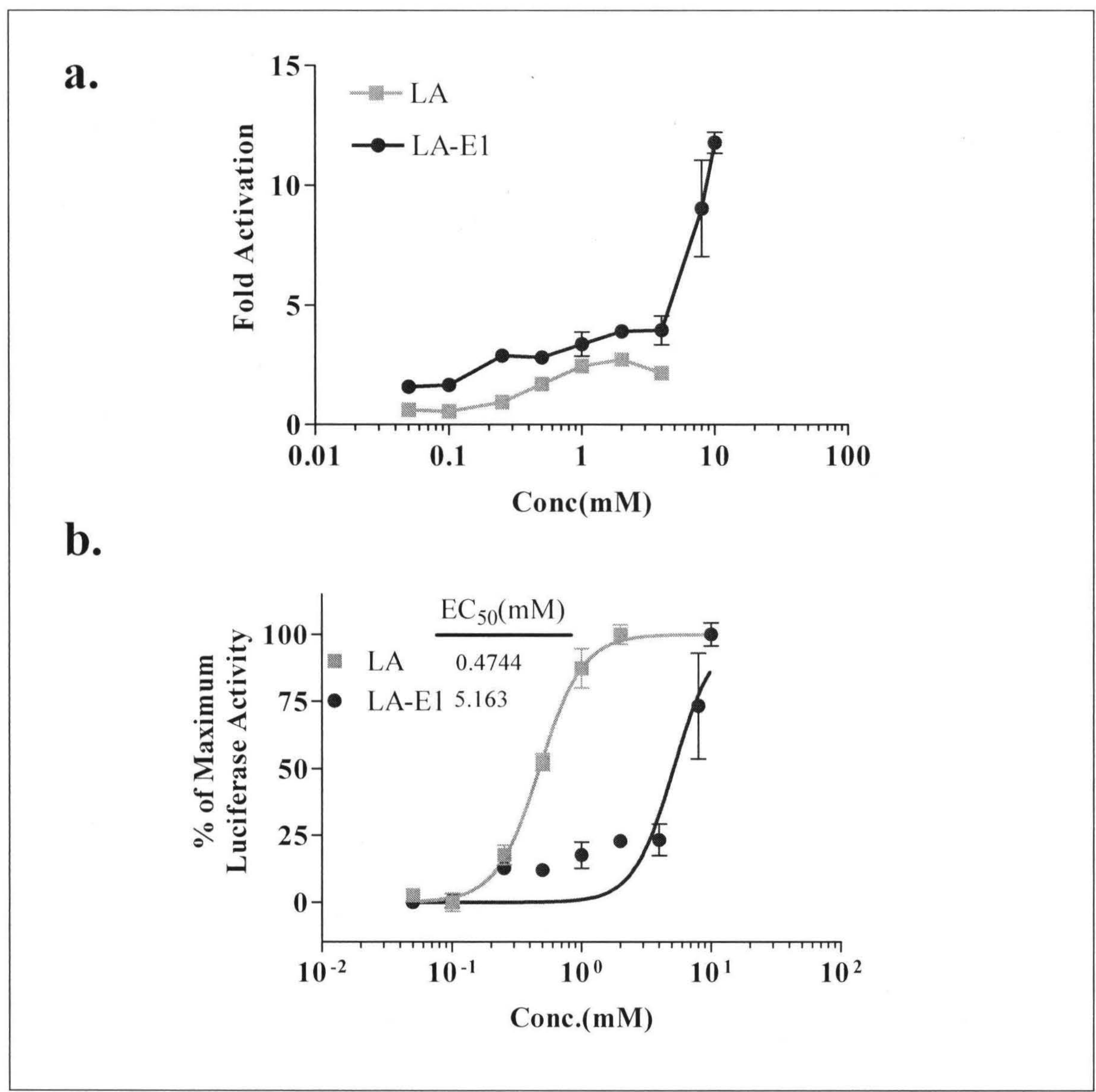


Fig.6: Activation profile of hPPARγ by LA and LA-E1

Transactivation of the full-length hPPARγ receptor by LA and LA-E1 in transiently transfected CV-1 cells. Transfected cells were treated with the indicated concentrations of ligand for 24 hrs and normalized total luciferase activity determined. (a) Fold activation as compared to vehicle treatment as a function of ligand concentration. (b) Data from Fig.4a presented as percent of maximal activity as a function of ligand concentration. LA (■), LA-E1 (●). Bars represent +/- S.E.M.

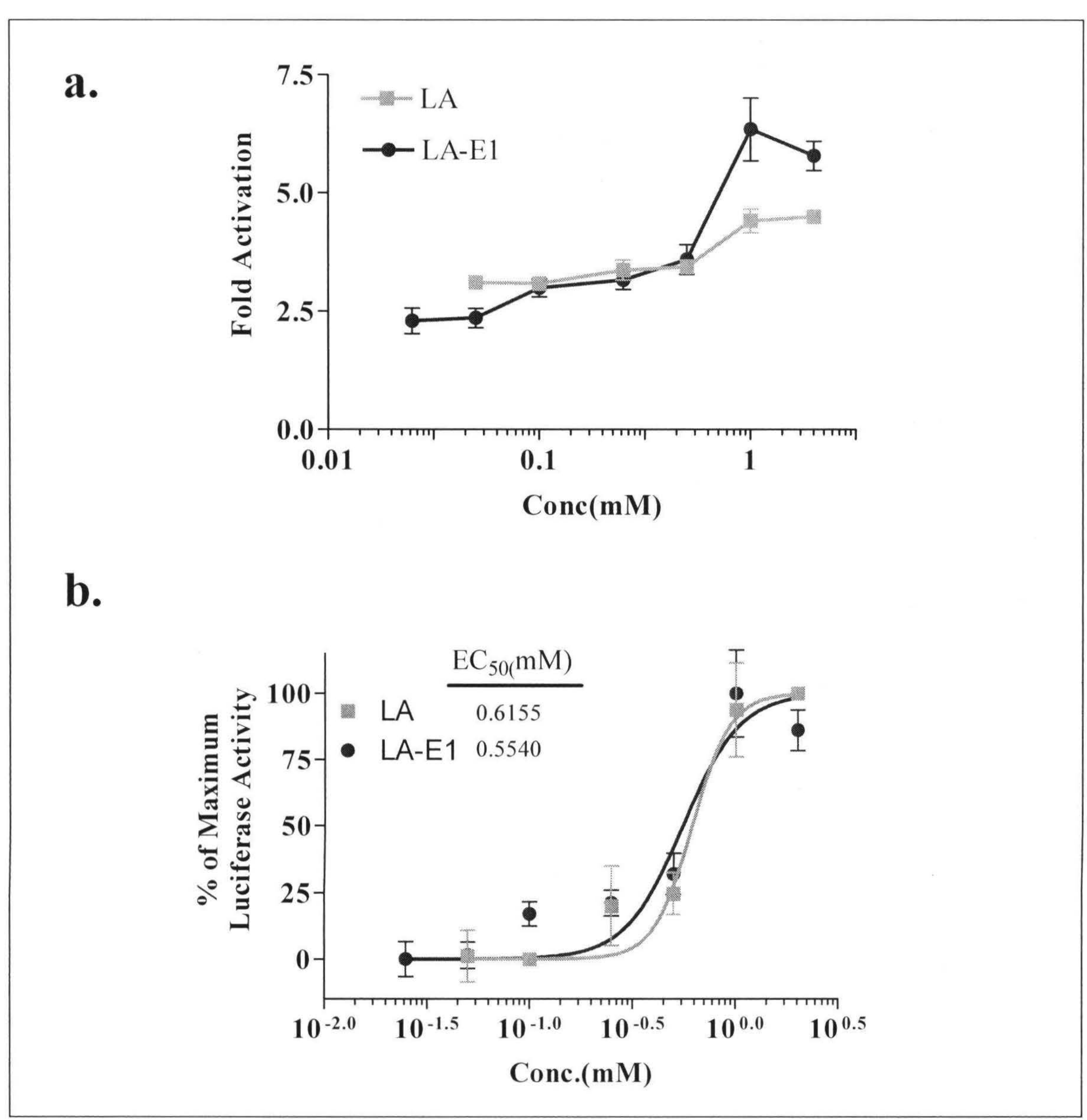
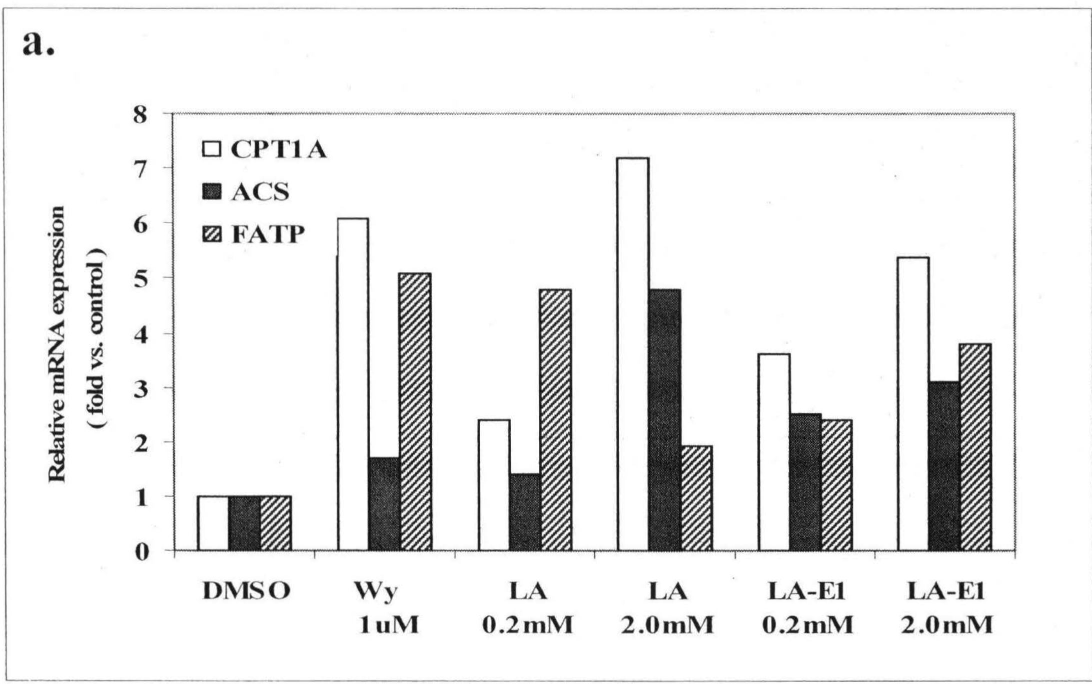


Fig.7: Activation profile of hPPARα by LA and LA-E1

Transactivation of the full-length hPPARα receptor by LA and LA-E1 in transiently transfected CV-1 cells. Transfected cells were treated with the indicated concentrations of ligand for 24 hrs and total luciferase activity determined. ***Luciferase data not normalized.***(a) Fold activation as compared to vehicle treatment as a function of ligand concentration. (b) Data from Fig.5a presented as percent of maximal activity as a function of ligand concentration. LA (■), LA-E1 (●). Bars represent +/- S.E.M.

Since LA and LA-E1 function as weak dual PPARα/γ agonists, we investigated whether these ligands would induce the expression of target genes typically activated by these receptors. HepG2 human hepatocarcinoma cells engineered to stably express PPARα at concentrations found *in vivo* were used to determine the effects of these compounds on PPARα- regulated target genes. Confluent cultures were exposed to either vehicle (DMSO), LA, LA-E1, or the known PPARα agonist, WY-14643 (positive control) for 48 hr. Compared to vehicle treatment, LA (2mM) increased the expression of CPT1A , ACS and FATP by 7.5-fold, 5-fold and 2-fold respectively (Fig.8a). Similarly, LA-E1 (2mM) increased the expression of CPT1A , ACS and FATP by 5.5-fold 3-fold and 4-fold, respectively (Fig.8a).These data indicate that LA and LA-E1 regulate the endogenous promoter region of three known PPARα responsive genes.

To investigate the effects of LA and LA-E1 on PPARγ-regulated target genes, murine 3T3-L1 preadipocytes were induced to differentiate by exposing them to differentiation medium, and then to vehicle (DMSO), LA, LA-E1, or the known PPARγ agonist, rosiglitazone. The effect of LA and LA-E1 on the expression of CD36, AP2 and LPL, is shown in Fig.8b. Rank order increase in the expression of these three genes upon exposure to 2mM LA were: LPL (26-fold) > CD36 (16-fold) > AP2 (3-fold), and upon exposure to same concentration of LA-E1 were: LPL (14-fold) > CD36 (8-fold) > AP2 (2.5-fold) (Fig.8b). Both compounds were most effective in increasing the expression of LPL and CD36, with only a modest effect on AP2 expression. These data confirm that LA and LA-E1 can enhance the transcription of at least three PPARγ-regulated genes.



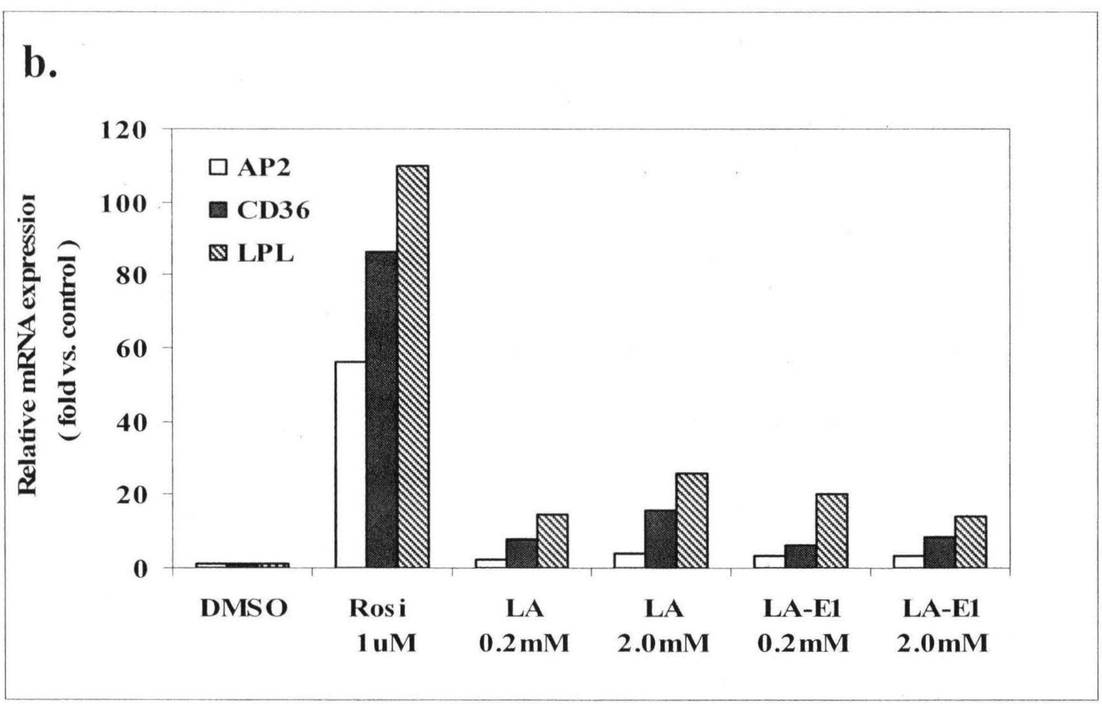


Fig.8: Induction of PPARα and PPARγ target gene expression by LA and LA-E1
(a) *PPARα target genes*: HepG2 cells were treated with varying concentrations of LA, LA-E1 or vehicle (DMSO) for 48hrs and the expression of PPARα target genes CPT1A, ACS and FATP were determined by qRT-PCR. (b) PPARγ target genes: 3T3-L1 preadipocytes were treated with varying concentrations of LA, LA-E1 and vehicle for 48 hrs and the expression of PPARγ target genes AP2, CD36 and LPL were determined by qRT-PCR. RNA levels for all target genes were normalized to levels of cyclophilin mRNA.

LA and LA-E1 can induce differentiation of 3T3-L1 preadipocytes

The hallmark property of PPARγ agonists is their ability to induce differentiation and maturation of preadipocytes. Therefore, we determined whether LA and LA-E1 could promote adipogenesis in 3T3-L1 preadipocyte fibroblasts. Both LA and LA-E1 induced adipogenesis as shown by Oil Red O staining in 3T3-L1 cells, when tested at concentrations 0.5-2mM (Fig.9).

Quantitative evaluation of the adipogenesis induced by LA and LA-E1 (Fig.10) was performed using the Serum Triglyceride Determination Kit (SIGMA). The triglyceride data was normalized for cell concentration by assaying for total protein. Both LA and LA-E1 induced adipogenesis over the concentration range 0f 0.1- 2mM at levels greater than that seen with vehicle-treated cells as indicated by the normalized serum triglyceride levels. Also LA shows elevated levels of serum triglyceride in comparison to LA-E1 over the same concentration range.

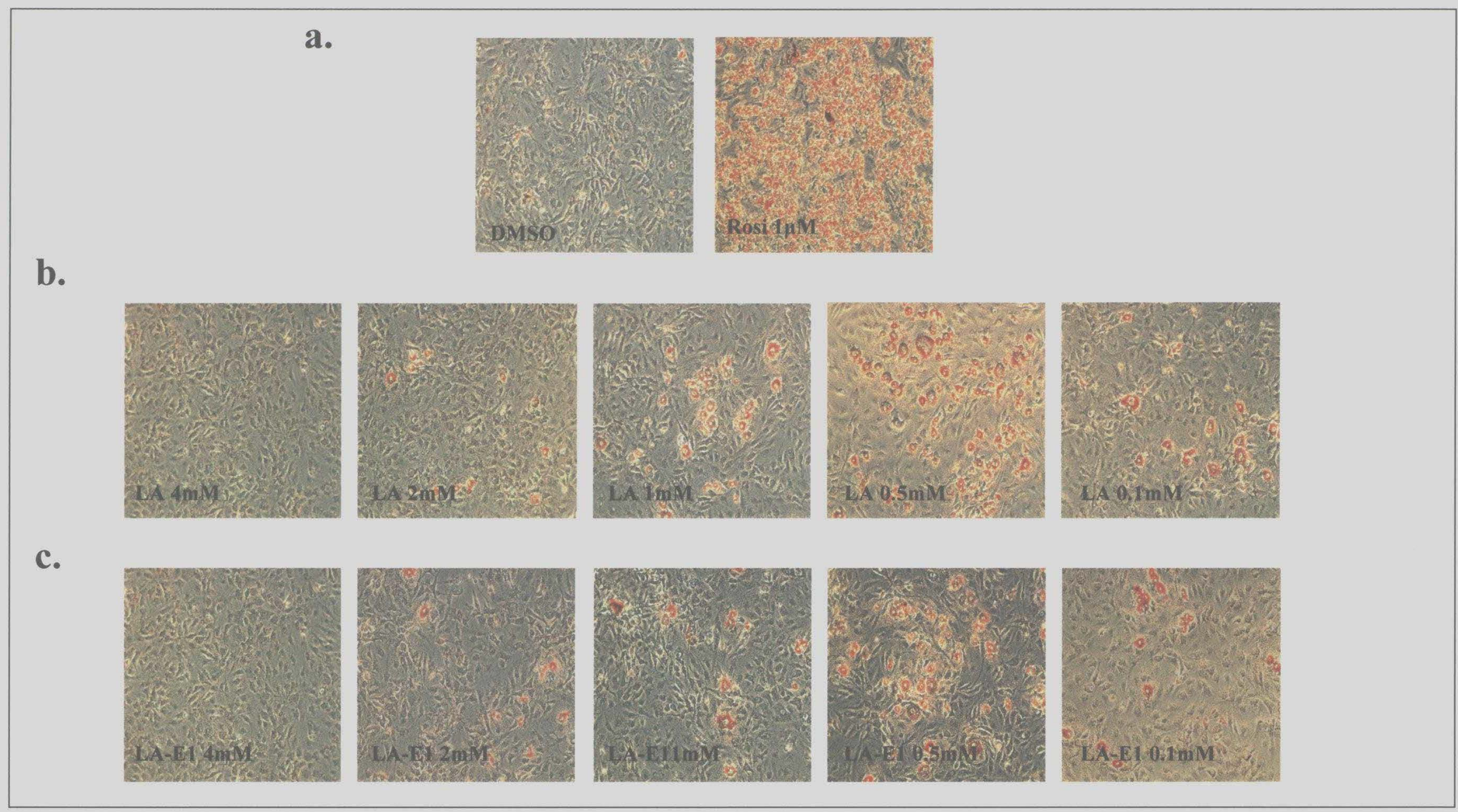


Fig.9: Induction of adipogenesis by LA and LA-E1

Confluent cultures of 3T3-L1 murine preadipocytes were treated with insulin, dexamethasone and IBMX for 32 hrs. Cells were then treated with varying concentrations of vehicle or LA or LA-E1 for 48hrs and later shifted to regular medium for 5 days. Following fixation with 10% formalin in PBS, cells were stained with Oil Red O. (a) Controls;
(b) LA; (c) LA-E1.

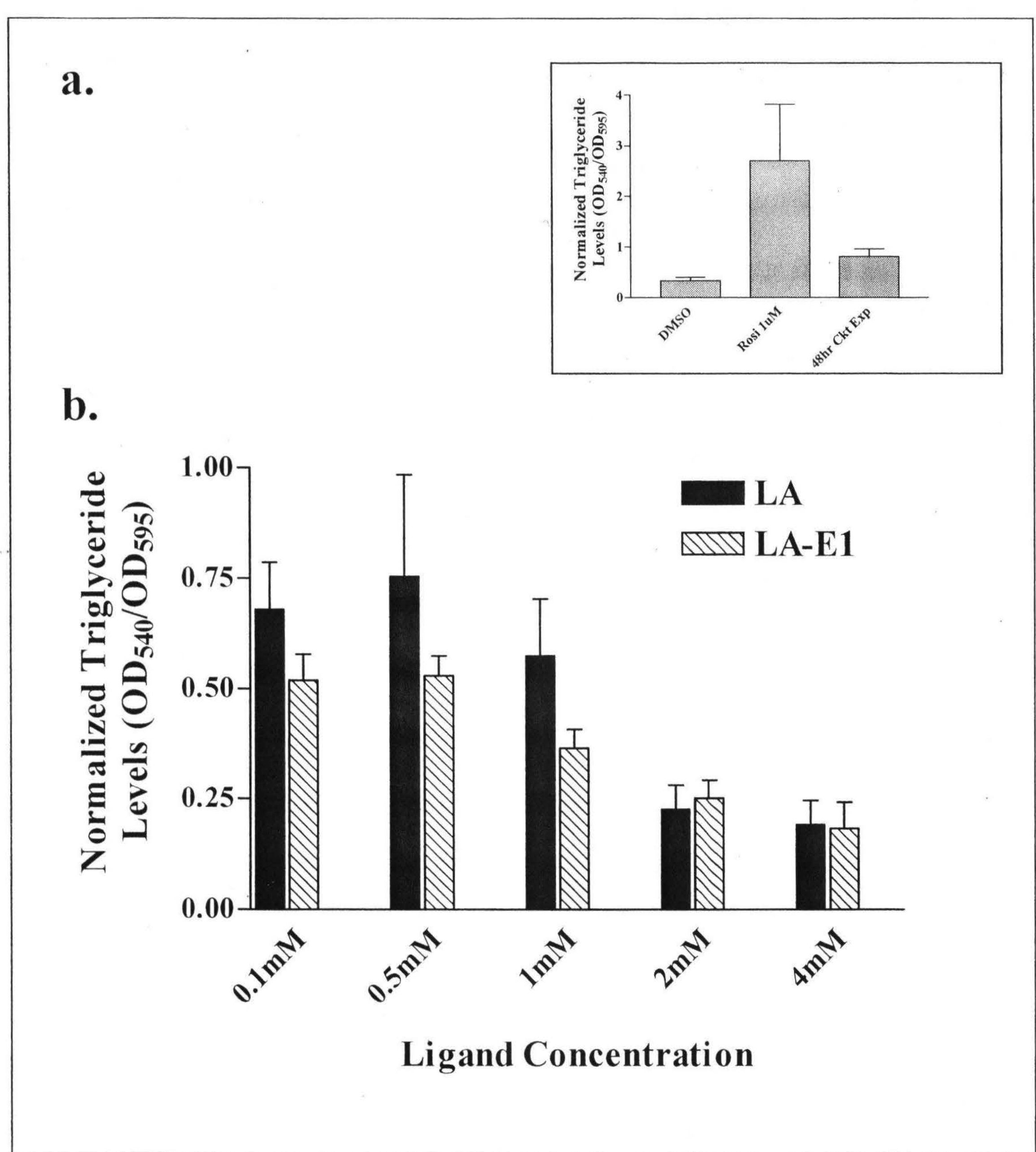


Fig.10: Quantitative evaluation of adipogenesis by LA and LA-E1
Confluent cultures of 3T3-L1 murine preadipocytes were treated with insulin, dexamethasone and IBMX for 32 hrs. Cells were then treated with varying concentrations of vehicle or LA or LA-E1 for 48hrs and later shifted to regular medium for 5 days. Total serum triglyceride levels was measured and data was normalized for cell concentration by assaying total protein. (a) Controls; (b) LA and LA-E1. Bars represent +/- S.E.M.

LA and LA-E1 Inhibit Proliferation of Several Cell Lines

PPARs are important modulators in the regulation of complex pathways of mammalian cells' metabolism, recently implicated in cancer. PPARγ ligands have been demonstrated to affect proliferation, differentiation and apoptosis of different cell types. I have therefore, studied the effect of LA and LA-E1 on several cell types [90].

The effect of LA and LA-E1 was examined on human carcinoma cell lines derived from breast (MCF-7) and colon (HT-29), continuous cell lines (CV-1 and HaCaT) and a normal cell line (HEKn). Proliferating cultures of these cell lines were treated with LA and LA-E1 as described in the materials and methods . After 2 weeks of exposure to the drug, relative cell number was measured by MTS assay.

Fig.11 shows the effect of LA and LA-E1 on cell proliferation in HT-29 cells. Both LA and LA-E1 inhibit proliferation in a dose dependent manner. At lower concentrations (0.001–0.1mM), LA-E1 was significantly more effective than LA while the two compounds showed similar efficacy at higher concentrations. At the lower concentrations 0.01mM-0.001mM LA promoted cell growth. On MCF-7 cells, both LA and LA-E1 had a dose-dependent inhibitory effect (Fig.12). Again at the lower concentrations 0.01-0.1mM LA-E1 showed higher inhibition than LA.

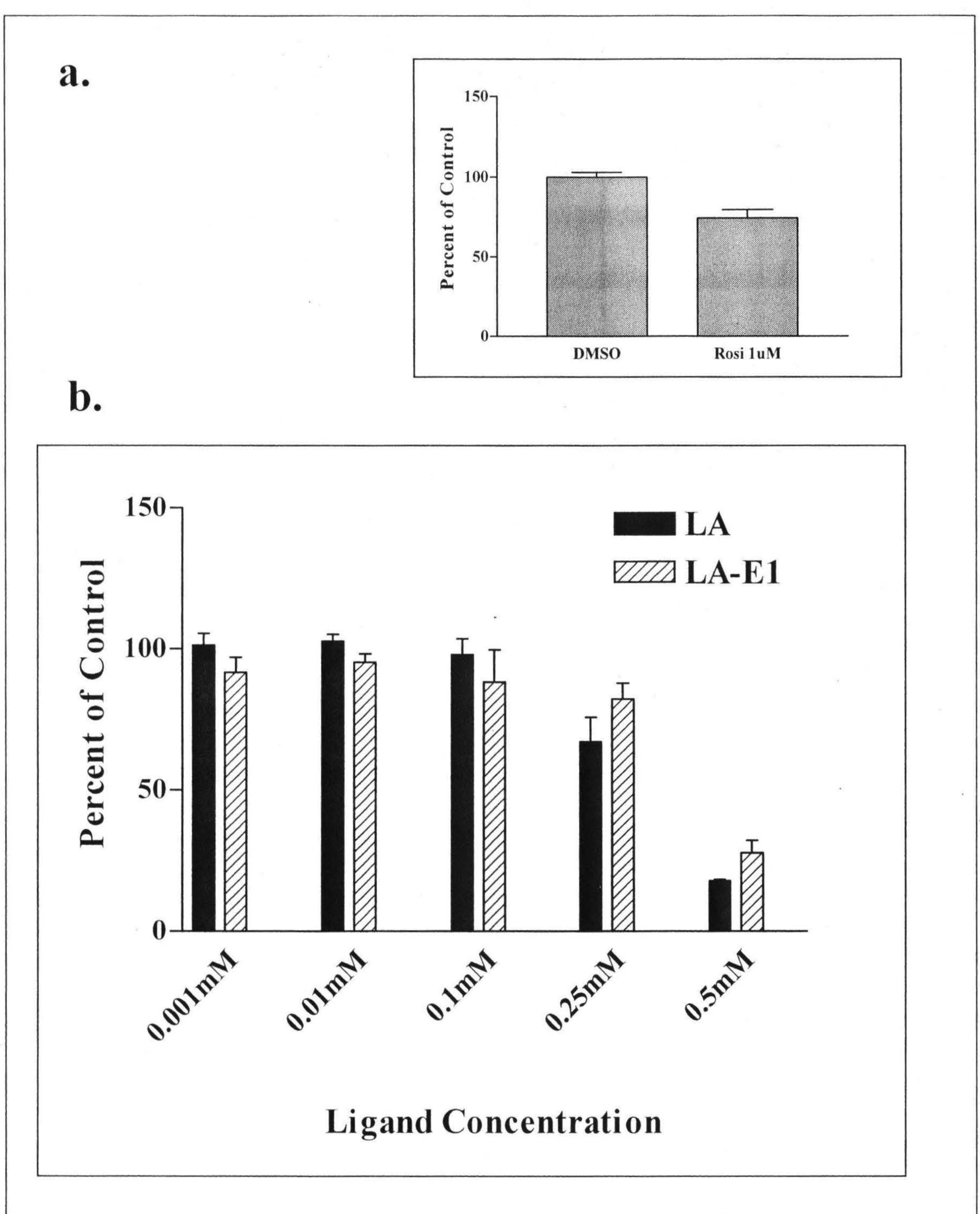


Fig.11: Effect of LA and LA-E1 on proliferation of cultured HT-29 cells
Cells were cultured for 2 weeks in the presence of varying concentrations of vehicle (DMSO) or positive control (Rosiglitazone) or LA or LA-E1. Cell number was quantitated as described in Materials and Methods and compared to vehicle treated controls. (a) Controls; (b) LA and LA-E1. Bars represent +/- S.E.M.

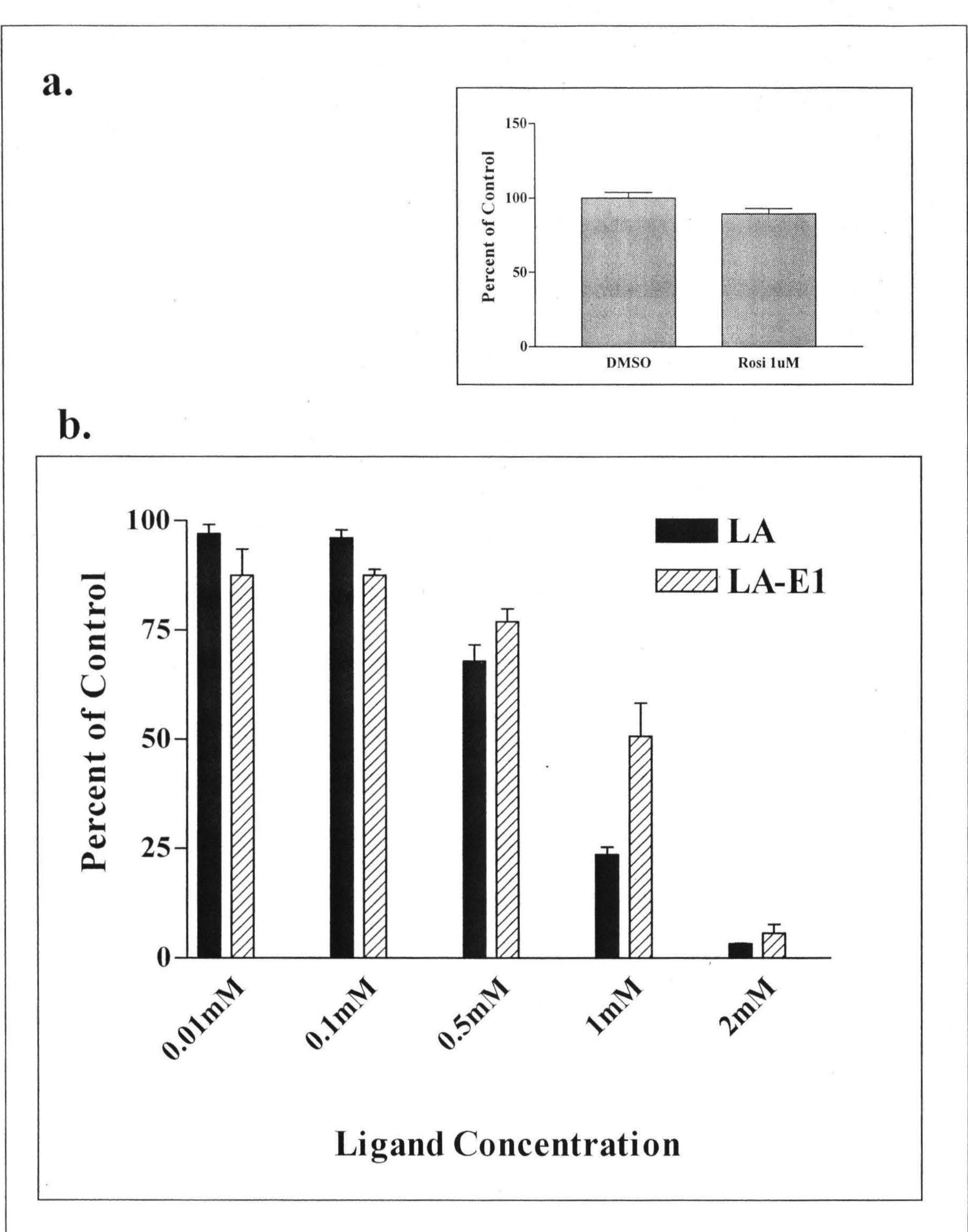


Fig.12: Effect of LA and LA-E1 on proliferation of cultured MCF-7 cells
Cells were cultured for 2 weeks in the presence of varying concentrations of vehicle (DMSO) or positive control (Rosiglitazone) or LA or LA-E1. Cell number was quantitated as described in Materials and Methods and compared to vehicle treated controls. (a) Controls; (b) LA and LA-E1. Bars represent +/- S.E.M.

The effect of LA-and LA-E1 on proliferation in the two continuous cell lines CV-1 and HaCaT and shown in Fig.13 and Fig.14 respectively. In CV-1 cells, LA and LA-E1 showed similar levels of modest inhibition at all concentrations tested. Both LA and LA-E1 showed maximum inhibition at the highest concentration of 0.5mM.

In HaCaT cells , both LA and LA-E1 along with the positive control Rosiglitazone induced proliferation. This effect was unique and limited to this cell type. There was a dose-dependent increase in proliferation at the LA and LA-E1 at all concentrations tested from 0.001mM-1.0mM.

In the one normal human keratinocyte cell line tested, HEKn, LA and LA-E1 showed a dose-dependent inhibition of proliferation (Fig.15). At lower concentrations (0.001–0.1mM), LA-E1 was significantly more effective than LA. At the lower concentrations 0.01mM-0.001mM LA promoted cell growth. The cytotoxicity assay was performed on the HEKn cells with LA and LA-E1. At all the concentrations tested from 0.001-0.1mM, none of the compounds appeared cytotoxic as determined by LDH release into the medium determined by cytotoxicity assay (Fig.16). It should be noted however that at the highest concentration of 0.5mM LA showed slightly elevated levels of LDH, indicating the potential for a cytotoxic effect at this concentration.

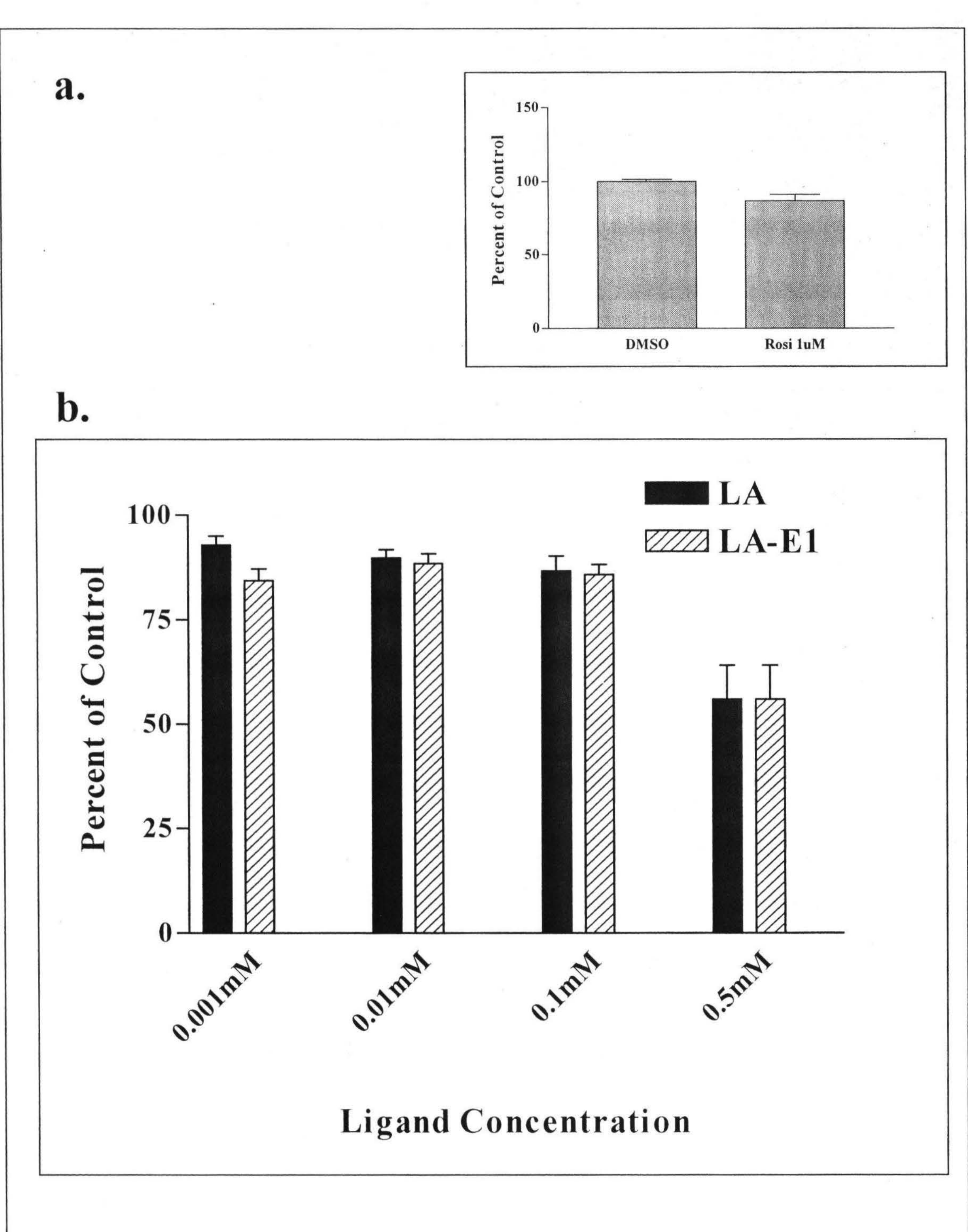


Fig.13: Effect of LA and LA-E1 on proliferation of cultured CV-1 cells
Cells were cultured for 2 weeks in the presence of varying concentrations of vehicle (DMSO) or positive control (Rosiglitazone) or LA or LA-E1. Cell number was quantitated as described in Materials and Methods and compared to vehicle treated controls. (a) Controls; (b) LA and LA-E1. Bars represent +/- S.E.M.

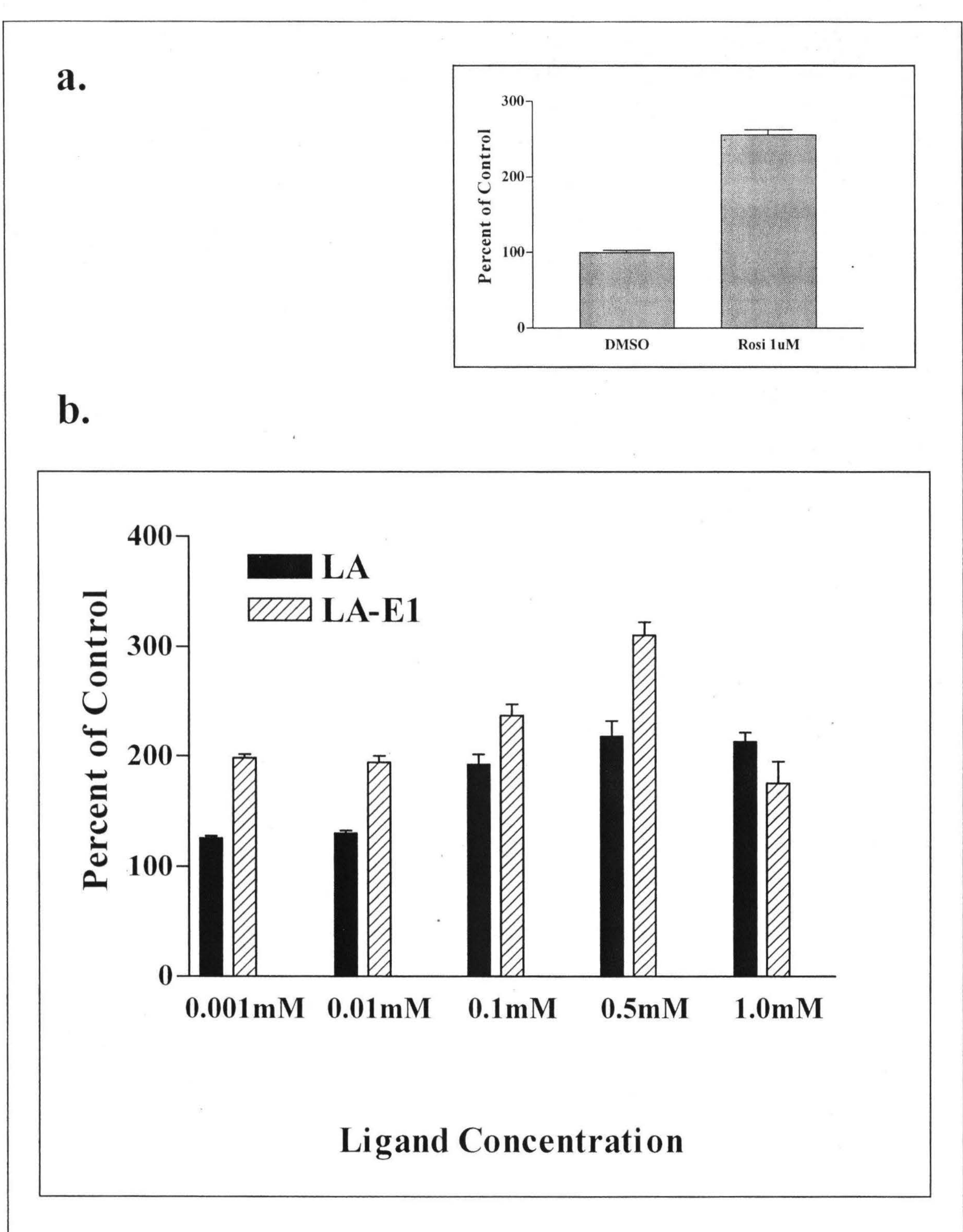


Fig.14: Effect of LA and LA-E1 on proliferation of cultured HaCaT cells

Cells were cultured for 2 weeks in the presence of varying concentrations of vehicle (DMSO) or positive control (Rosiglitazone) or LA or LA-E1. Cell number was quantitated as described in Materials and Methods and compared to vehicle treated controls. (a) Controls; (b) LA and LA-E1. Bars represent +/- S.E.M.

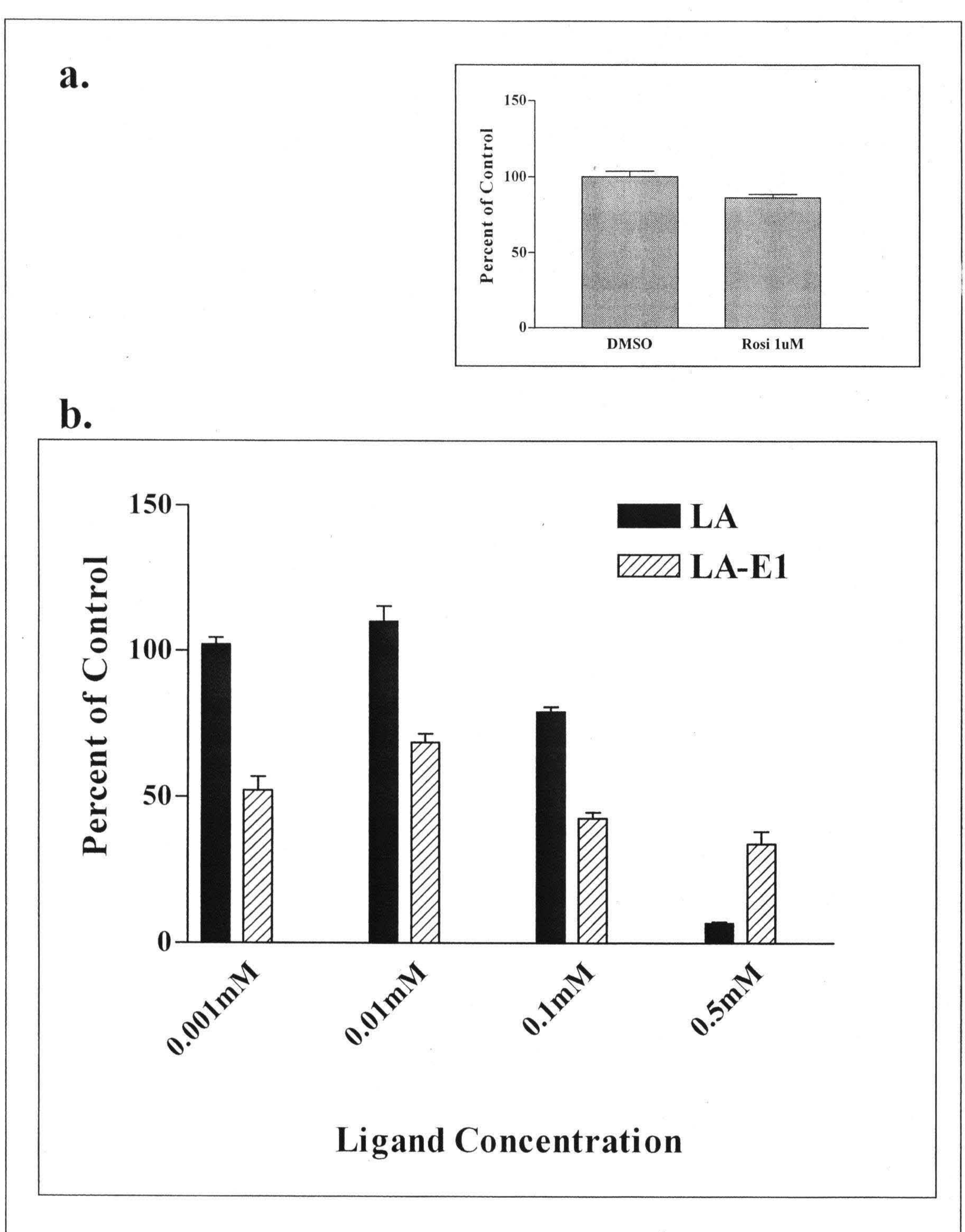


Fig.15: Effect of LA and LA-E1 on proliferation of cultured HEKn cells
Cells were cultured for 2 weeks in the presence of varying concentrations of vehicle (DMSO) or positive control (Rosiglitazone) or LA or LA-E1. Cell number was quantitated as described in

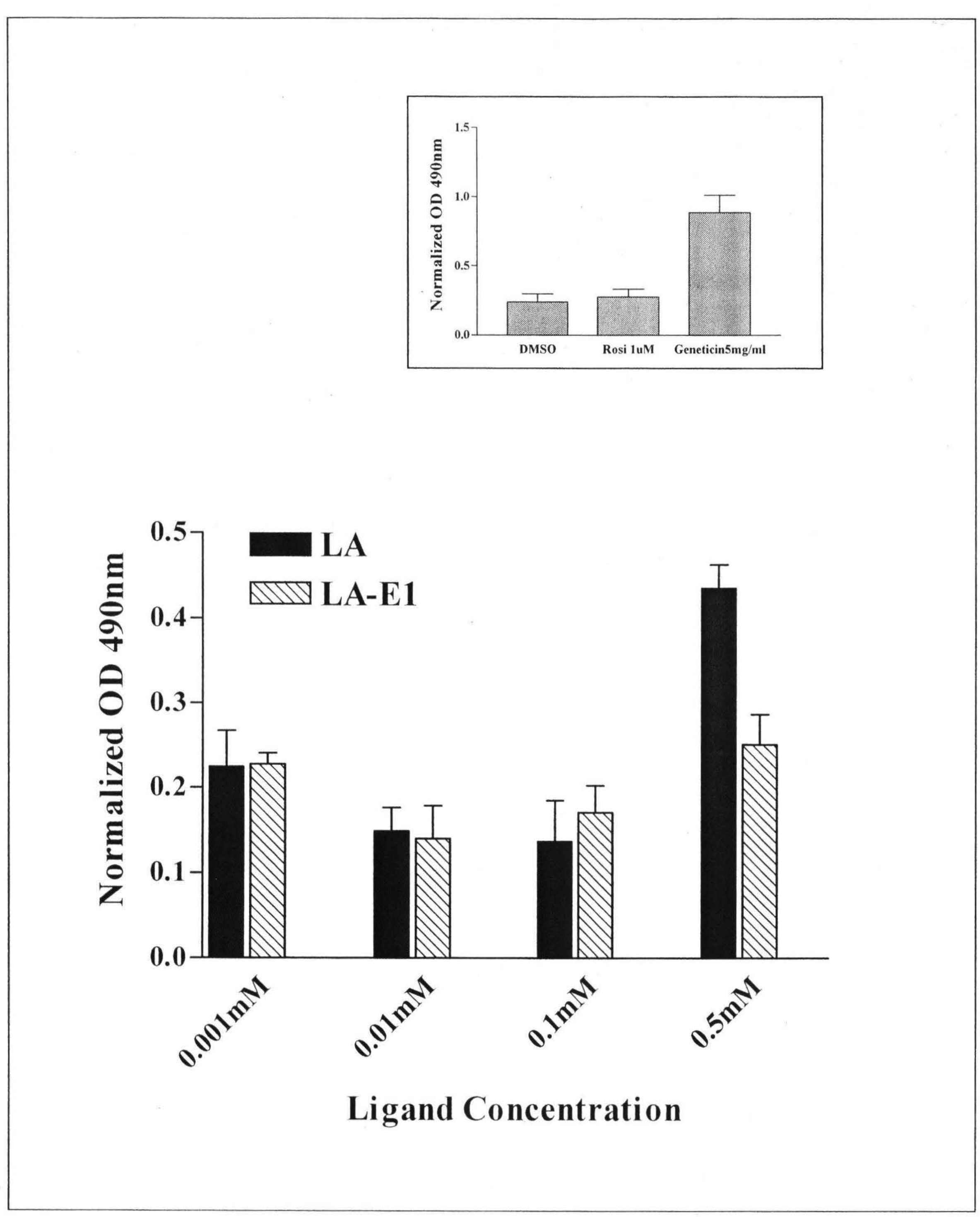


Fig.16: LA and LA-E1 cytotoxicity assay performed on HEKn cells
Cells were cultured for 48 hrs in the presence of varying concentrations of vehicle (DMSO) or positive control (Geneticin) or Rosiglitazone or LA or LA-E1. LDH released was normalized by assaying for MTS (a) Controls; (b) LA and LA-E1. Bars represent +/- S.E.M.

DISCUSSION

This study sought to determine the effects of a-Lipoic Acid (LA) and the prodrug, LA-ester (LA-E1) on a variety of PPAR mediated events including PPAR activity, adipogenesis, gene expression and cellular proliferation. The study also looks at the ability of LA-E1 to improve PPAR activity or efficacy (maximal activation) and modulate the expression of PPAR-regulated target genes.

Agonist binding to the PPAR receptor results in transcription of relevant target genes. Full agonists are drugs that produce maximal receptor activation while partial agonists induce a sub-maximal response even at full receptor occupancy. In artificial chimeric PPAR-GAL4 receptor transactivation assays, both LA and LA-E1 were found to act as weak dual PPARα/γ agonists with similar activation profiles. LA-E1 was found to have a greater efficacy than LA in activating PPARγ and similar efficacy in activating PPARα over the same concentration range (Fig. 4,5). These findings were further confirmed by the results obtained utilizing full-length human PPAR receptor constructs and the luciferase reporter gene fused to the tandemly repeated PPAR response element. Again LA-E1 showed better efficacy than LA in activating PPARγ (Fig.6) while both drugs showed comparable activation profiles for PPARα (Fig.7).

In addition to activating PPARγ and PPARα in cell-based transactivation assays, LA and LA-E1 increased the expression of known PPAR-regulated target genes. LA and LA-E1 showed a similar efficacy in increasing the expression of PPARα target genes(CPT1A, ACS and FATP) in human hepatocytes (Fig.8a). Regarding PPARγ target

genes, both LA and LA-E1 increased the expression of AP2, CD36 and LPL in adipocytes (Fig.8b). However, LA-E1 showed lower fold of increase in gene expression in comparison to LA. Neither LA nor LA-E1 activated PPARδ.

Ligand-dependent PPARγ activation triggers the genetic program for adipocyte differentiation and maturation, a signature effect of PPARγ agonists [41]. Consistent with this property of PPARγ agonists, both LA and LA-E1 induced differentiation of murine pre-adipocytes into mature adipocytes, characterized by the intracellular accumulation of lipids (Fig.9). Quantitation of adipogenesis induced by LA and LA-E1 reveals that both the compounds show maximum triglyceride accumulation in adipocytes at 0.5mM (Fig.10). This concentration is comparable to LA and LA-E1 levels at which 2-5-fold receptor activation is achieved in the mPPARγ-GAL4 transactivation assay (Fig.4).

Studies based on tumor cell lines have implicated PPARγ in cell cycle withdrawl. It is well established that PPARγ can promote cellular differentiation. The differentiation of preadipocytes into adipocytes occurs only after a prior and permanent exit from the cell cycle, an effect linked to inhibition of E2F/DP transcription factor. This decrease in in E2F/DP activity is mediated in part by PPARγ through the down-regulation of the PP2A protein phosphatase Studies have further indicated that PPARγ plays a role in cell cycle exit by up-regulating the cyclin-dependent kinase inhibitors p18 and p21 during adipogenesis [93].

PPARγ acts as an important cellular regulator by inhibiting growth and/or inducing differentiation and apoptosis of normal and tumor cells, including those of the

breast [91] and colon [92]. Treatment of breast and colon cancer cells with TZDs resulted in significant growth arrest both in culture and when implanted in nude mice [92]. Consistent with these reports, both LA and LA-E1 inhibit the proliferation of HT-29 and MCF-7 cells (Fig.11,12). They also show similar effects on CV-1 and normal human epidermal keratinocytes (Fig.13,15). However, in HaCaT cells both compounds and Rosiglitazone show proliferation (Fig.14). The reasons for same need to be researched further.

Esterification of ionized species is a widely employed method of increasing cellular permeability. Once inside the cell, the ethyl-ester linkage is enzymatically hydrolyzed to generate the corresponding carboxylic acid. This acidic moiety is apparently responsible for receptor activation, as an acidic group is essential for stabilizing PPAR ligands within the PPAR receptor ligand-binding domain [11]. The mechanism(s) whereby esterification improves efficacy of activating PPARα and PPARγ in PPAR-GAL4 receptor assays requires further study.

LA has been reported to have a marginal beneficial effect on type 2 diabetes in humans [17,18], obtained at pharmacological concentrations [19]. Intravenous infusion of LA into patients with type 2 diabetes was shown to increase insulin-stimulated glucose disposal and insulin sensitivity by 55% and 57%, respectively [20,21]. To be effective orally, LA would have to be administered at pharmacological concentrations, and formulated (e.g. as a slow release preparation) to achieve sustained therapeutic plasma concentrations required for anti-diabetic activity [22]. Thus, if the limitations of oral administration can be overcome, LA could emerge as a safe and effective adjunctive

insulin-sensitizing antidiabetic agent . These studies suggest that converting LA to various esters may be helpful in this regard. Because esters with bulky groups tend be metabolized more slowly in vivo, esterification may be a method for obtaining a more sustained plasma concentrations of LA, and possibly enhanced therapeutic efficacy. Future studies in animal models of insulin resistance and/or type 2 diabetes are needed to evaluate the pharmacokinetic profile and efficacy of various LA esters. Because PPARγ and PPARα agonists have been shown to lower triglycerides and suppress pro-inflammatory markers [23-25], it may be possible to design optimized LA-ester derivatives to improve their metabolic benefits [19,22].

The metabolic syndrome, a recently recognized high cardiovascular risk complex, consists of a cluster specific risk factors: insulin resistance, visceral obesity, elevated triglycerides, low HDL-cholesterol, and elevated blood pressure [26]. PPARα agonists such as the anti-dyslipidemic fibrates [12,25] and PPARγ agonists such as the thiazolidinediones have been shown to improve insulin resistance, lower blood pressure, improve the lipid profile and ameliorate atherosclerosis [23-25]. Consequently, it has been proposed that mixed PPARα/γ agonists are viable candidates for treating the metabolic syndrome [23,27]. Because LA-E1 is a dual PPARα/γ agonist with increased efficacy, oral administration of this LA precursor may have beneficial clinical effects in subjects with the metabolic syndrome. Moreover, it may be possible to design esters of LA to optimize their effectiveness for treating or preventing both type 2 diabetes and the metabolic syndrome.

My finding that LA can function as a dual PPARα/γ activator may, at least in part, provide a mechanistic rationale for the observed insulin-sensitizing effect of LA in type 2 diabetes. LA has previously been reported to function as a weak PPARγ activator which inhibited PPARγ transactivation induced by the TZD, troglitazone [28], consistent with the speculation that LA may function as a partial PPARγ agonist.

Despite the clinical benefits of PPARγ agonists in type 2 diabetes, the current generation of glitazone drugs is associated with side effects such as weight gain and edema, especially when used in combination with insulin [91]. This has raised concerns about the long-term usage of these drugs in diabetic patients with congestive heart failure. As a result, there is a significant interest in the design of novel drugs that modulate the activity of PPARγ to retain the insulin-sensitizing properties of the glitazones while dialing out other effects such as those on adipocyte differentiation. Support for this selective PPAR modulator (SPARMs) concept comes from the observation that synthetic ligands for nuclear receptors have been reported that show the benefits of natural steroid hormones (e.g. estrogens or androgens) but with fewer of the side effects of classical steroids. The best know examples are tamoxifen and raloxifen , two estrogen receptor modulators, that prevent the development of osteoporosis in post-menopausal women but also inhibit the estrogen-dependent proliferation of breast carcinomas [92]. In this context LA and its ester derivatives would prove to be of clinical importance in future treatment of diabetes and other major chronic human diseases such as atherogenesis and carcinogenesis.

Of interest is the recent finding that orally administered LA caused a dose-dependent reduction in the body weight of rodents by reducing food intake and enhancing energy expenditure, and that LA exerts its anti-obesity effects by suppressing hypothalamic AMPK activity [29]. Therefore, the effects of LA-E1 and other ester derivatives on body weight and hypothalamic AMPK activity should be explored. These findings provide a rationale for developing ester derivatives of LA in order to improve their efficacy for the treatment of type 2 diabetes and components of the metabolic syndrome.

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