Oleuropein and Related Compounds from Olive Plants Modulate Adipogenesis

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Abstract: Obesity is a complex multifactorial disease. Adipocytes arise from pluripotent mesenchymal stem cells (MSC), which are also capable of differentiation into bone, muscle, or cartilage. Adipogenesis involves lineage commitment, mitotic clonal expansion, and terminal differentiation. Understanding these mechanisms, as well as when and how to turn them on or off, may allow development of new therapeutic approaches to obesity, diabetes, and cardiovascular disease. The most abundant non-lipid component of olive plant is the polyphenol oleuropein (Ole). We found that Ole modulates adipocyte differentiation, fat accumulation and adipogenic gene expression in human MSCs (hMSC). Ole blocks adipogenesis in a dose-dependent manner. Using RT-PCR to monitor gene expression, we found that Ole down-regulates the expression of adipogenic genes PPARγ2, LPL (lipoprotein lipase), and aP2 (lipid binding protein), while it up-regulates PPARδ expression. In addition, in the presence of Ole, we were able to achieve transdifferentiation and de-differentiation, allowing fat cells to assume other fates. These results demonstrate the potential utility of Ole for the treatment of obesity, diabetes, and related disorders, which are associated with increased fat mass. Because it modulates adipocyte differentaion, Ole may also be useful for the treatment of cachexia and lipodystrophy.

INTRODUCTION

Obesity is a serious health problem that has become a worldwide epidemic. Using the body mass index definition of overweight and obesity, there are 1.7 billion overweight adults worldwide, of which about 400 million are obese [1]. United States is the most obese nation, with 200 million overweight and at least 100 million obese. This corresponds to two thirds (67%) of the population being overweight and one third (33%) obese [2]. It is estimated that at current trend levels, by 2030, 86.3% adults will be overweight and 51.1% will be obese; by 2048, all American adults will be overweight or obese [2].

Obesity and overweight are major contributors to a group of chronic diseases including type 2 diabetes, cardiovascular disease, hypertension, and stroke [3]. The key causes of overweight and obesity are excessive consumption of energy-rich foods high in saturated fats and sugars, and reduced physical activity. Of course, genetics and lifestyle also play important roles.

AN OVERVIEW OF ANTI-OBESITY DRUGS

The main treatment modalities for obesity and overweight are a healthy diet and physical exercise. However, for certain patients, diet and exercise alone are not sufficient or feasible, and anti-obesity drugs become necessary. Most prescription weight loss drugs are stimulants recommended only for short-term use, and thus are of limited benefit for those who need long term weight reduction over months or years. The US Food and Drug Administration (FDA)-approved weight-loss drugs can be classified according to their mechanisms [4].

Appetite Suppressants include sibutramine, phent-ermine, diethyl-propion and phendimetrazine. Phentermine and sibutramine are most prescribed in the US. However, their cardiovascular risks are significant. They may increase blood pressure and heart rate, raising the risk of heart attacks and strokes. Sibutramine was suspended from the market in January 2010. Amphetamines are appetite suppressants, but they have strong potential for abuse and dependence.

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§This work is dedicated to the memory of Professor Severo Ochoa, our respected mentor, and to Dr. An Fu Lee, our beloved mother and grandmother, for their inspiration and encouragement.

Lipase Inhibitors such as Orlistat, also known as Xenical, reduce intestinal fat absorption by inhibiting the enzyme pancreatic lipase which breaks down dietary fat. When fat is not digested, the body cannot absorb it, so it is eliminated and fewer calories are taken in.

Other drugs that are not FDA-approved for the treatment of obesity have been shown to promote short-term weight loss in clinical studies and may be prescribed off-label. These include drugs to treat depression such as Bupropion, which suppresses appetite. Drugs to treat seizures, such as topiramate and zonisamide, have also been shown to cause weight loss. Drugs to treat diabetes, such as metformin also promote weight loss in people with obesity and type 2 diabetes, though the mechanisms by which they work is clear. Thus, new safe and effective obesity drugs are urgently needed.

NATURAL PRODUCTS AS ANTI-OBESITY DRUGS

Recent Statistics

In the United States, the use of natural products and medicinal plants for health care has increased significantly recently. It accounts for about 30% of the drug market and corresponds to more than 50 billion US dollars in annual consumer spending [5]. Recent surveys show that 30-42% of obese patients with hypertension and type 2 diabetes use natural products/complementary and alternative medicine (CAM) to manage their conditions [6]. In certain ethnic populations (Navajo, Vietnamese, Hispanic), the percentages are even higher (40-66%) [7, 8].

Development of Drugs from Medicinal Plants

Over 75% of world's population uses medicinal plants for health care. In the USA, 128 FDA approved prescription drugs are derived from medicinal plants [9]. Familiar examples include aspirin from the willow tree [10], and Tamiflu, the Saras drug from the Chinese spice Star Anise [11]. There are about quarter million plant species, of which eighty thousand are medicinal plants. Of those, about ten thousand have medicinal records, and only 128 have been developed into prescription drugs. Thus, medicinal plants are a rich resource for drug development.

Medicinal Use of the Olive Plant

The olive tree lives and remains productive for thousands of years. It is the first botanical to have documented medicinal use [12]. Products of olive plant, including the olive leaf, olive fruit, and olive oil, have been used to enhance immune system, to enhance wound healing, and to reduce cardiovascular risk. They have been found to have anti-oxidant, anti-aging, anti-obesity and anti-infective properties [13-15]. What makes olive plants live so long and remain productive? What are the main ingredients for their medicinal actions? How can we apply them for human uses? How can we use modern technologies for drug discovery of this ancient plant? These are the goals for our studies.

HEALTH BENEFITS OF OLIVE COMPONENTS

The Mediterranean Diet

Epidemiological studies indicated a lower incidence of atherosclerosis, cardiovascular diseases (CVD), and diabetes in the Mediterranean region than in other parts of the world. These results have been attributed mainly to the Mediterranean diet of the local population [16-18]. An average Mediterranean diet provides up to 100 mg of Ole, hydroxytyrosol, and elenolic acid per day. This is on the order of 1.5 mg/kg body weight in man. Studies indicate that approximately 60% of ingested olive oil phenols are absorbed from the small intestine in humans.

Clinical Studies

A two year study with more than 300 participants showed that a diet with olive oil resulted in 8 lbs. more weight loss than a low carbohydrate diet, and 10 lbs. more weight loss than a low fat diet [19]. An eight year study with 13 thousand people on an olive oil diet showed an 83% reduction on type 2 diabetes [20]. Another 7-countries study showed that the olive oil diet reduced CVD by 70% [21].

Anti-Oxidant Compounds in Olive

Olive oil contains the monounsaturated fatty acid oleic acid. It lowers LDL cholesterol, triglycerides (TG) and total cholesterol, thus lowering the risk of CVD and related diseases. However, weight loss and other metabolic benefits cannot be attributed entirely to monounsaturated fatty acids. The antioxidant function of the phenolic compounds in olive plant and olive oil also play critical roles [22-24]. Olive plant products contain mono- and poly-phenolic anti-oxidants, including the simple phenolic compounds vanillic, gallic, coumaric and caffeic acids, tyrosol and hydroxytyrosol and complex phenolic compounds, the secoiridoids (oleuropein and ligstroside), and the lignans (1-acetoxypinoresinol and pinoresinol). Ole is a major anti-oxidant found in olive plants.

IDENTIFICATION AND CHARACTERIZATION OF OLIVE ANTI-OXIDANTS FROM OLIVE LEAVES

High Performance Liquid Chromatography (HPLC)

We have developed effective methods for the extraction of olive leaves, and for the purification, identification and characterization of its anti-oxidants, anti-viral and anti-obesity components [25-27]. Olive leaf extract was subjected to HPLC using a Waters two-solvent delivery system with a photodiode array detector. A Symmetry C18 column (5 μm, 3.9 x 250mm) with a Sentry Guard 3.9 x 20mm insert was used. The mobile phase was 79% distilled water and 21% acetonitrile, both acidified to pH 3 with 0.1M orthophosphoric acid. This solvent system is designed for resolution and quantitation of polyphenolic compounds. Polyphenolic compounds were monitored by absorbance at 280 nm. Fig. (**1**) shows a typical HPLC elution profile of olive leaf extract. In this figure, absorption at 280 nm was plotted against retention time in minutes.

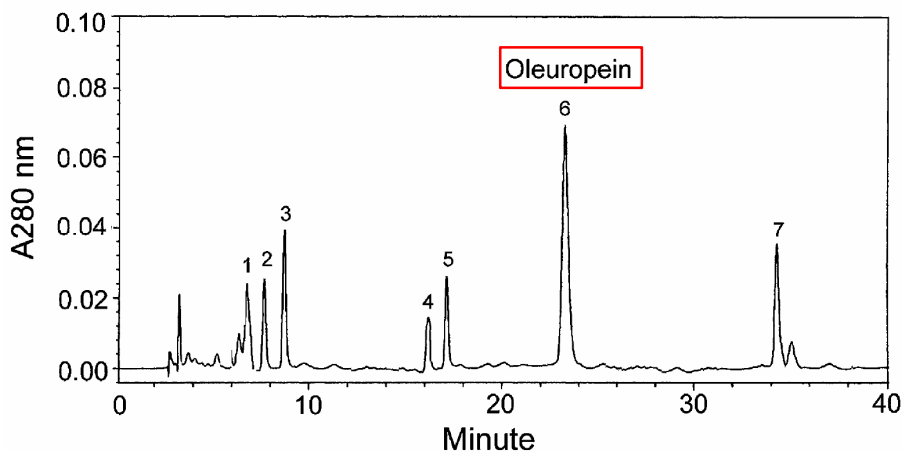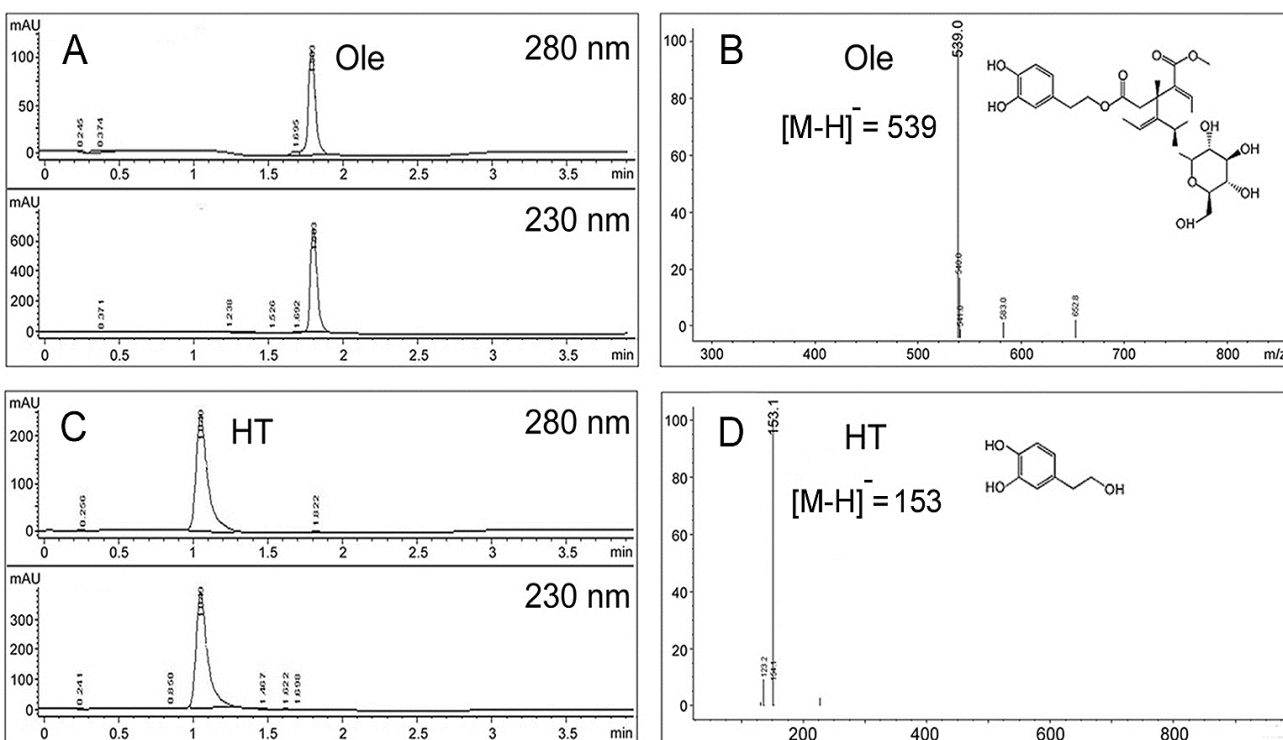**Fig. (1).** HPLC elution profile of olive leaves extract.

Fig. (2). LC-MS analysis of Oleuropein (Ole).

Seven peaks were obtained. Oleuropein (Ole) was eluted at peak 6 and it is the major component of the extract. Each peak was assayed for bioactivity, cytotoxicity, identified by Thin-layer Chromatography (TLC) and analyzed by HPLC coupled mass spectrometry (LC-MS).

**Thin-Layer Chromatography (TLC)**

To determine the identity of the phenolic compounds from HPLC, we carried out TLC using silica gel 60F254 with chloroform/methanol/acetic acid (70/30/10). Secoiridoids and flavonoids were detected by visualization under UV light at 254nm with 10% ferric chloride and 10% aminoethyl diphenylborate spray, using known standards.

Biological Activities

Biological activity of the phenolic compounds was assayed in terms of anti-viral and anti-obesity activities. Anti-viral activity was determined by inhibition of HIV core protein p24 expression in HIV infected cells and of syncytial formation during acute HIV infection [26-27]. Anti-obesity activity was measured by inhibition on fat cell formation of hMSC. Cytotoxicity was measured by cell proliferation assay [28]. Table **1** summarizes these results. It shows the retention time (Rt), identity, % (w/w), anti-HIV activity, anti-obesity activity and cytotoxicity. Ole is the major anti-oxidant, anti-HIV and anti-obesity component in olive leaf extract and it shows no cytotoxicity.

Table 1. Identity and Bioactivity of Pheonlic Compounds in Olive Leave Extract

Peak	Rt (min)	Compound	% (w/w)	Anti-HIV	Anti-obesity	Cytotoxicity
Excl	<6	Solvent front	-	-	-	-
1	6.6	Rutin	0.34	-	-	-
2	7.8	Verbascoside	0.38	-	-	-
3	8.9	Lutoelin 7-glucoside	0.68	-	-	-
4	16.0	Apigenin 7-glucoside	0.18	-	-	-
5	17.0	Flavonoid x	0.56	-	-	-
6	22.8	Oleuropein	12.80	+	+	-
7	34.0	Oleuroside	0.51	+	+	-
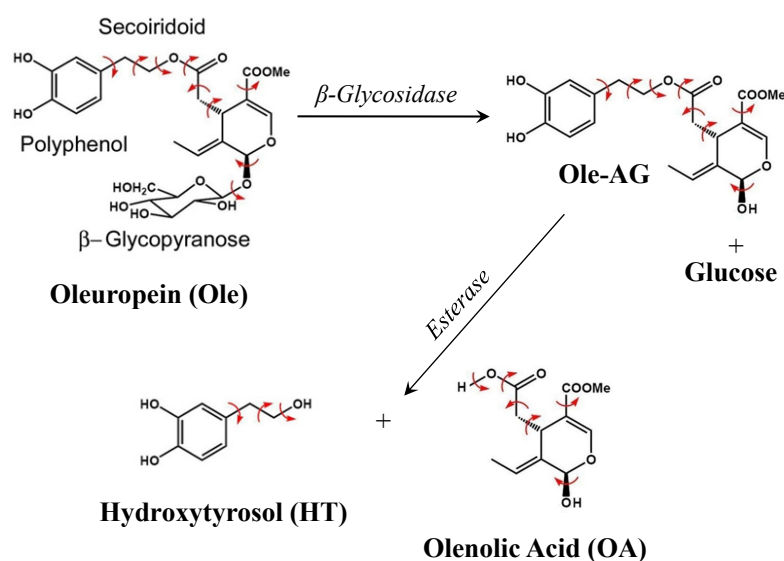
Fig. (3). Structure and metabolism of oleuropein.

Liquid Chromatography Coupled Mass Spectrometry (LC-MS)

HPLC-purified OLE was further subjected to LC-MS analysis to enhance mass resolution in the second dimension and detect multiple chemical species with the same retention time. The results are shown in Fig. (**2**).

Fig. (**2A**) shows the LC elution profile of Ole standard. Fig. (**2B**) is its MS analysis, showing one major component with a molecular mass [M-H]⁻ of 539. Fig. (**2C**) is the LC elution profile of peak 6 Ole. Fig. (**2D**) is its MS analysis. As seen in the figures, peak 6 Ole is as pure as the standard. A single major peak was observed at 1.827 min in both samples. MS analysis shows a single mass peak at [M–H]⁻ of 539. Peak 6 Ole was used in all of our experiments.

STRUCTURE, METABOLISM AND BIOAVILABILITY OF OLE

Structure and Metabolism

Fig. (**3**) shows the structure and metabolism of Ole. It consists of three functional regions, a polyphenol, a secoiridoid, and a β-glycopyranose. In metabolism, Ole is hydrolyzed by *β-Glycosidase* to give Ole-aglycone (Ole-AG) and glucose. Ole-AG is further acted on by esterase to yield olenolic acid and hydroxytyrosol.

Bioavailability and Pharmacokinetics (PK)

To define *in vivo* bioavailability and PK, data on Ole circulating concentrations and bioactive metabolites after oral administration are essential. We used LC–MS for sensitive and simultaneous detection of Ole and metabolites in rat plasma and urine following oral ingestion of 20-200 mg of olive leave extract. Groups of five animals were used for each dose levels. Blood and urine samples were collected at 0, 10, 30 minutes, 1, 2, 4, and 8 hours, extracted with 10 volumes of ethyl acetate, evaporated to dryness, resuspended in 25μl of 20% acetonitrile and 5 μl was injected onto the LC column for LC-MS analyses.

Typical results are shown in Fig. (**4**). These results demonstrate that Ole is bioavailabile, and that it is absorbed and metabolized to HT. The bioactive metabolite circulates in the blood and is secreted in the urine. The circulating concentration of HT starts to peak around one hour, whereas
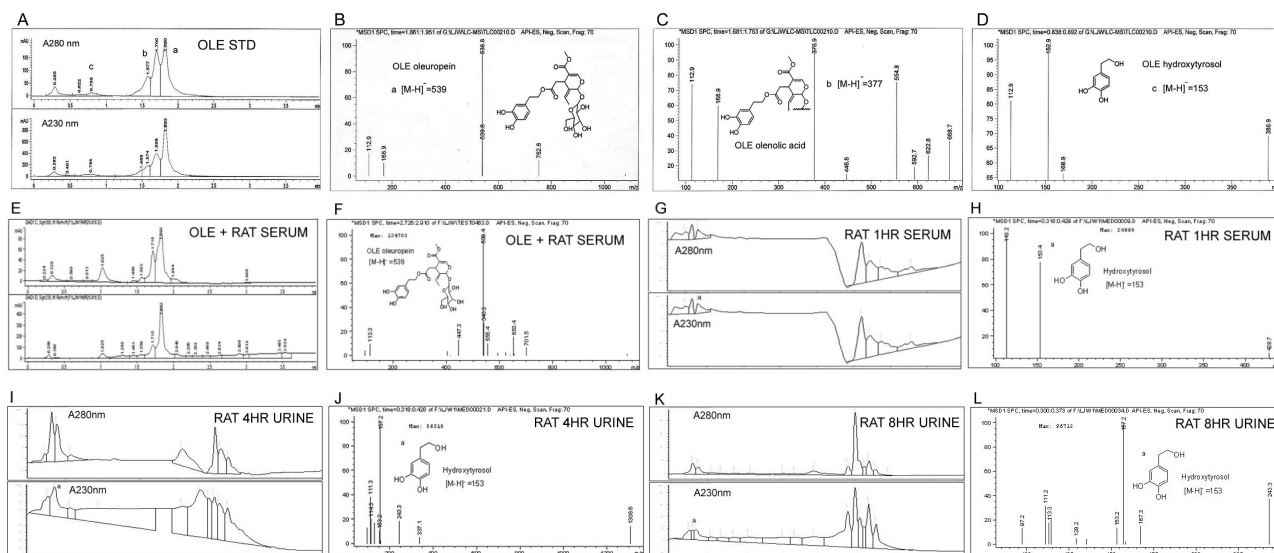
Fig. (4). LC-MS Analyses of *in vivo* bioavailability and PK of OLE in Rats.
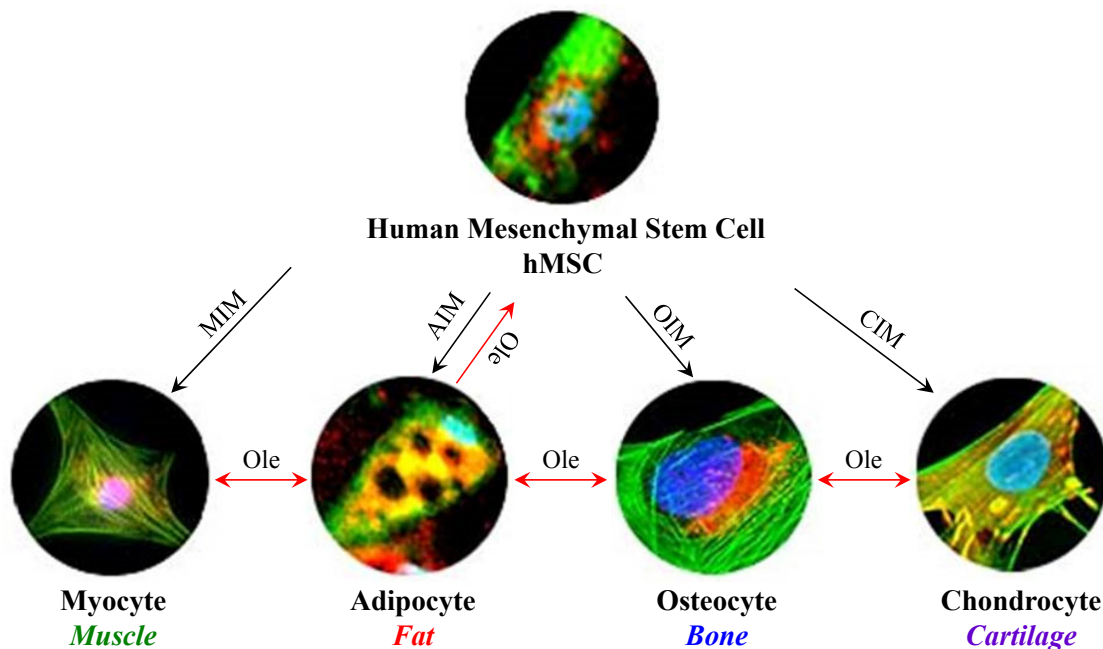

Fig. (5). *In vitro* differentiation of hMSC.

it was not detected in the 10 min and 30 min plasma samples. Secretion of the active metabolite starts at 4 hours and peaks at 8 hours.

Fig. (**4A**) shows the LC of standard olive leave extract at A280 and A230 nm. Fig. (**4B**) shows the MS analysis of peak (a) in Fig. (**4A**). It was identified as a single mass peak at [M-H]⁻ = 539 corresponding to Ole. Figs. (**4C-D**) show the MS analysis of LC peaks (b) and (c) in Fig. (**4A**). They were identified with mass peaks at [M-H]⁻ = 377 and 153 corresponding to olenolic acid and HT respectively. Fig. (**4E-F**) represent LC-MS of standard olive leave extract treated with rat plasma as internal control, showing Ole peak, indicating no degradation by the rat plasma. Fig. (**4G-H**) represent LC-MS of one hour rat serum showing the disappearance of Ole and the appearance of HT peak. Figs.

(**4 I–J**) **and** (**4K-L**) represent LC-MS of rat 4 and 8 hour urine respectively, showing the secretion of Ole metabolite HT as a function of time.

DIFFERENTIATION AND ADIPOGENESIS OF HMSCS

Adipogenesis, Obesity and Metabolic Syndromes

Fat cells (adipocytes) are responsible for the development of obesity and lipodystrophy, and contribute to the pathophysiology of metabolic syndrome. Uncontrolled expansion of fat cells leads to obesity and related diseases. The increase in adipocyte number is the result of recruitment of preadipocytes from pluripotent stem cells that reside in the
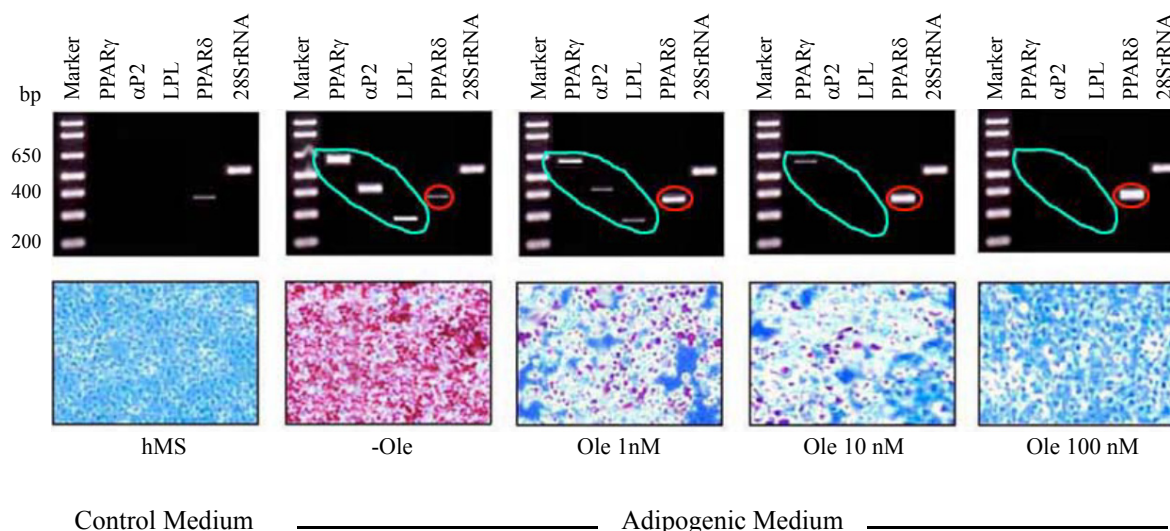
Fig. (6). Ole inhibits hMSC adipogenesis and the expression of adipogenic genes.

vascular stroma of fat (adipose) tissue. The increase in adipocyte size is the result of increased fat accumulation. These events are accompanied by increased secretion of adipocyte hormones and cytokines, including resistin and adiponectin, that affect insulin sensitivity, and angiotensin, that regulates blood pressure. These events provide direct links between obesity and CVD, type 2 diabetes, and other disorders. Thus, agents that modulate fat cell formation (adipogenesis) may hold great promise for the prevention and treatment of obesity and related diseases.

Modulation of Adipogenesis as Therapeutic Targets

Adipocytes arise from pluripotent MSC, which also give rise to precursors for bone, muscle, and cartilage cells in response to appropriate developmental cues.

We used triple fluorescent images to analyze human MSC (hMSC) differentiation. We transduced the undifferentiated hMSC with green fluorescent protein (GFP), so they could be detectable by green fluorescence. The nuclei were stained blue with Hoechst 33342 (DNA in the nucleus); mitochondria were stained red with MitoTracker Red CMXRos (seen as orange when superimposed on green). As seen in Fig. (**5**), hMSC-GFP cells cultured in myocyte inducing medium (MIM), adipocyte inducing medium (AIM), osteocyte inducing medium (OIM) or chondrocyte inducing medium (CIM), differentiate into myocyte, adipocyte, osteocyte and chondrocyte lineages, showing myosin fibers, dark fat droplets in GFP, osteofibers and chondrofibers respectively, with blue nucleus and red (or orange) mitochondria.

We hope to define the signals involved in hMSC differentiation. What is switched on or off at the commitment step? Can mature fat cells be de-differentiated? If so, this should reduce the accumulation and total numbers of fat cells. Could Ole modulate fat cell de-differentiation? If so, how? Or can fat cells be trans-differentiated into other types of cells, for example, bone cells? If so, this should not only be useful in reducing the existing pool of fat cells but also would be valuable in replenishing osteocytes, needed

due to osteoporosis from obesity, diabetes and aging. Could Ole modulate fat cell tans-differentiation? If so, how?

MODULATION OF ADIPOGENESIS BY OLE

Ole Inhibits hMSC Adipogenesis

To investigate the effect of Ole on adipogenesis, we cultured hMSC in AIM and monitored fat cell formation by Oil Red O staining for fat droplets, and by RT-PCR for the expression profile of adipogenic genes. The results are shown in Fig. (**6**).

hMSCs at 10^4 cells/cm^2 were cultured in hMSC growth medium to confluence. Adipogenesis was induced two days post confluence by culturing in AIM containing 10 μM dexamethasone, 1 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methylxanthine, in the absence or presence of Ole at 1, 10 and 100 nM. Fresh culture medium was changed every 3 days. Full differentiation of hMSC to adipocytes was detected between days 8-12 by Oil Red O staining. Oil Red O is a lipophilic dye that stains the lipid droplets containing fat cells associated adipogenesis to red color. The cells were counterstained with hematoxylin in blue.

In Fig. (**6**), hMSCs cultured in control medium do not stain with Oil Red O but are counterstained with hematoxylin in blue, while hMSCs cultured in AIM, differentiated into lipid droplet containing fat cells *via* adipogenesis. The fat cells stained red with Oil Red O. Ole demonstrated dose dependent inhibition of adipocyte differentiation.

Ole Down-regulates the Expression of Adipogenic Genes in hMSC

Differentiation of MSC to a pre-adipocytes is driven by the transcription factor peroxisome proliferator activated receptor γ (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs). In addition to assaying lipid accumulation by Oil Red O, we carried out RT-PCR to profile the effect of Ole on gene expression during adipogenesis. Table **2** shows the
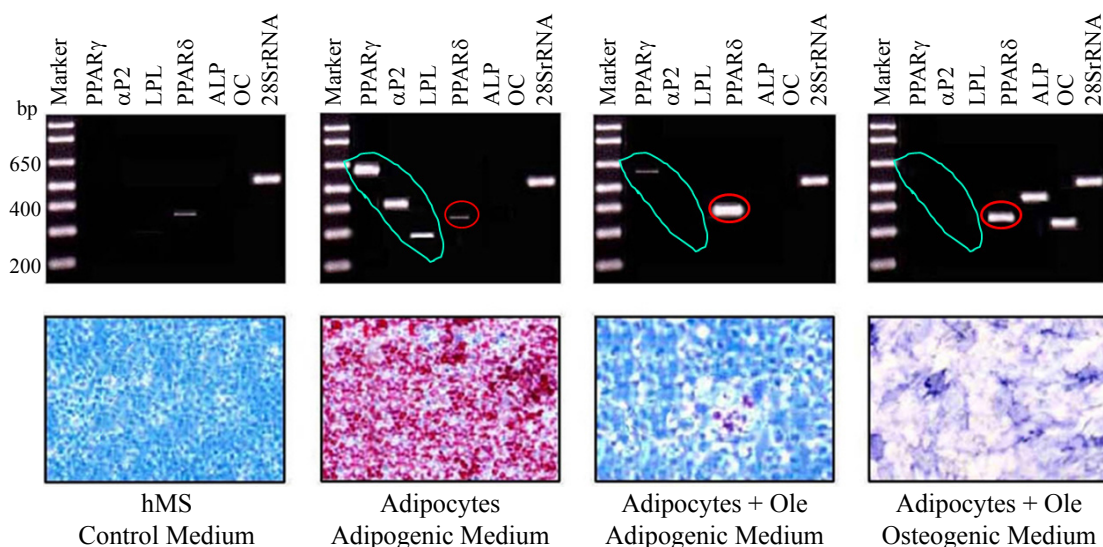
Fig. (7). Oleuropein de-differentiates adipocytes and allows trans-differentiation.

Table 2. PCR Primer for Specific Gene in hMSC Differentiation

Gene	Primer Sequence (Sense/Antisense)	Product Size (bp)	Gene ID #
Marker	1kb plus DNA ladder with 200, 300, 400, 500, 650, 850, 1000 bp	200-1000	
PPARγ Adiopogeneic	5'-GGATGTCGTGTCTGTGGAGA-3' 5'-TGAGGAGAGTTACTTGGTCG-3'	630	Bc006811
LPL Adipogenic	5'-GAGATTTCTCTGTATGGCACC-3' 5'-CTGCAAATGAGACACTTTCTC-3'	276	BC011353
αP2 Adipogenic	5'-GTACCTGGAAACTTGTCTCC-3' 5'-GTTCAATGCGAACTTCAGTC-3'	418	BC007538
PPARδ Anti-adipogenic	5'-GGTGAATGGCCTGCCTCCCTACAA-3' 5'-CACAGAATGATGGCCGCAATGAAT-3'	380	BC007578
ALP Osteogenic	5'-TGGAGCTTCAGAAGCTCAACACCA-3' 5'-ATCTCGTTGTCTGAGTACCAGTCC-3'	452	BC014139
OC Osteogenic	5'-CATGAGAGCCCTCACA-3' 5'-AGAGCGACACCTAGAC-3'	310	NM199173
28S RNA Internal control	5'-GTGCAGATCTTGGTGGTAGTAGC-3' 5'-AGAGCCAATCCTTATCCCGAAGTT-3'	589	BC000380

PCR primers of the genes we examined during hMSC adipogenesis.

The effect of Ole on adipogenic gene expression during adipogenesis is shown in the top panels of Fig. (**6**). Ole down-regulates the expression of adipogenic genes PPARγ2, LPL (lipoprotein lipase), and αP2 (lipid binding protein), up-regulates the expression PPARδ, while it does not affect expression of the internal control 28S rRNA. These results suggest that inhibition of adipogenesis by Ole is linked not only to its down-regulation of adipogenic genes but also to up-regulation of fat burning and energy uncoupling genes such as PPARδ.

Ole Promotes Adipocyte De-differentiation and Transdifferentiation

To analyze the effect of Ole on adipocyte de-differentiation and trans-differentiation, we cultured hMSCs in AIM for 12 days, allowing full differentiation into adipocytes. We then added Ole (80 nM) to the culture medium. Adipocyte de-differentiation was detected by the disappearance of lipid droplets visualized by Oil Red O staining. Ole-treated cells were collected and cultured in OIM containing 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 µg/ml L-ascorbate 2-phosphate, and 10 nM 1α, 25-dihydroxyvitamin D3 for 6-10 days.

Table 3. Tissue Distributions, Physiological Functions and Disease Targets of the PPARs

Type	Function	Distribution	Active State	Ligands	Disease Targets
PPARα	Fat Burning	Adipose, Heart, Liver, Muscle	Fasting	Fatty Acids (fibrates)	Diabetes Dyslipidemia
PPAR δ (β)	Heat Producing Energy Balacing	Ubiquitous, Brain, Brown Fat, Muscle	Movement	Fatty Acids	Dyslipidemia, Obesity
PPAR γ	Fat Storing	Adipose, Heart, Liver,	Feeding	Fatty Acids, TZD Drugs	Diabetes, Insulin resistance

N ‖ AF1 ‖ DNABD ‖ Flexible Hinge Region ‖ Ligand BD ‖ AF2 ‖ C

Y473
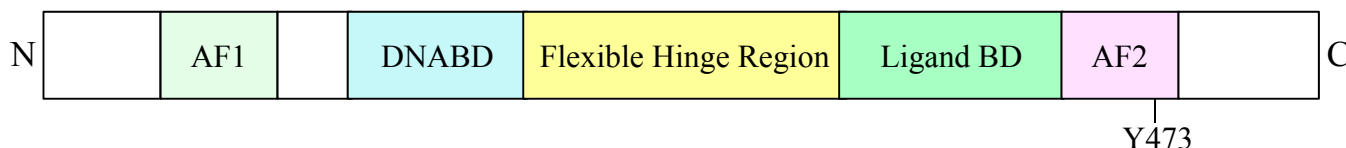
Fig. (8). Structure and Organization of PPARs.

As shown in Fig. (**7**) bottom panels, Ole promoted the de-differentiation of adipocytes. Furthermore, culturing in OIM resulted in transdifferentiation into osteoblasts. RT-PCR shown in Fig. (**7** top panels) demonstrated that 48 hours after the addition of Ole, the expression of adipogenic marker genes PPARγ2, LPL, and αP2 were down-regulated, while PPARδ was up-regulated. Upon the switch to OIM, expression of osteocalcin (OC) and alkaline phosphatase (ALP) are detected.

FAT SENSORS AND PPARS

Physiological Function and Tissue Distribution of PPARs

The PPARs are transcription factors belonging to the superfamily of nuclear receptors. They act on DNA response elements as heterodimers with the nuclear retinoic acid receptor. Dietary fat and their metabolites, such as fatty acids and lipid-derivatives, are natural activating ligands for the PPARs. Thus, the PPARs are physiological fat sensors and they play critical roles in fat cell formation and metabolism [29, 30]. Three isoforms, PPARα, PPARδ and PPARγ have been reported. Table **3** summarizes the distinct functions, tissue distributions and potential disease targets of the PPARs.

PPARα promotes fatty acid oxidation (fat burning), ketone body synthesis, and glucose sparing, in response to fasting. It is present in liver, heart, and, to a lesser extent, skeletal muscle. Fibrates, which are used to lower triglyceride levels, are ligands of PPARα. The increased fatty acid oxidation is one of the reasons why fibrates have lipid-lowering effects and why PPARα ligands could in some situations improve insulin sensitivity by reducing lipid accumulation in tissues.

PPARδ is ubiquitous. It exists everywhere, including brain, brown fat and muscle. PPARδ enhances fatty acid catabolism and energy uncoupling in adipose tissue and muscle. It also suppresses macrophage-derived inflammation. In skeletal muscle, PPARδ regulates fatty acid transport and oxidation, thermogenesis, and the formation of slow-twitch muscle fibers, resulting in enhanced endurance.

It likewise activates fatty acid transport and oxidation as well as thermogenesis in adipose tissue, retarding weight gain. PPARδ regulates the availability of BCL-6, an inflammatory suppressor protein released upon binding of PPARδ, thereby functioning as an "anti-inflammatory switch" to control macrophage-elicited inflammation and atherogenesis. In the liver, PPARδ activation suppresses glucose production by upregulating the pentose phosphate shunt. PPARδ activation also improves atherogenic dyslipidemia by raising serum HDL cholesterol levels *via* unclear mechanisms. Additionally, PPARδ activation in the heart enhances contractile function and may improve cardiomyopathy.

PPARγ promotes adipogenesis and stimulates triglyceride storage. It is expressed in adipose tissue, lower intestine, and cells involved in immunity. Activation of PPARγ induces the differentiation of preadipocytes into adipocytes and modulates adipokine expression. It increases insulin sensitivity by up-regulating the expression of adiponectin and blocking expression of TNFα and resistin. PPARγ is the molecular target for the thiazolidinedione (TZD) class of drugs clinically used to treat patients with diabetes. Although transcribed by the same gene, PPARγ is expressed in three forms through alternative splicing: γ1, γ2, and γ3. PPARγ1 is expressed in virtually all tissues. PPARγ2 is expressed mainly in adipose tissue. PPAR γ3 is expressed in macrophages, large intestine and adipose tissue.

Structural Organization of the PPARs

Like other nuclear receptors, PPARs are modular in structure and possess functional domain organization. Fig. (**8**) shows the prototypic structure and organization of the PPARs.

The N-terminal region contains a ligand-independent transactivation domain (AF-1), followed by a DNA-binding domain (DNABD, or DBD) with two zinc fingers, which bind to specific sequences of DNA known as hormone response element when the receptor is activated. At the C-terminus is situated a ligand binding domain (Ligand BD, or LBD) for dimerization and a ligand-dependent activation domain (AF-2). Tyrosine residue 473 (Y473) in AF2 plays a
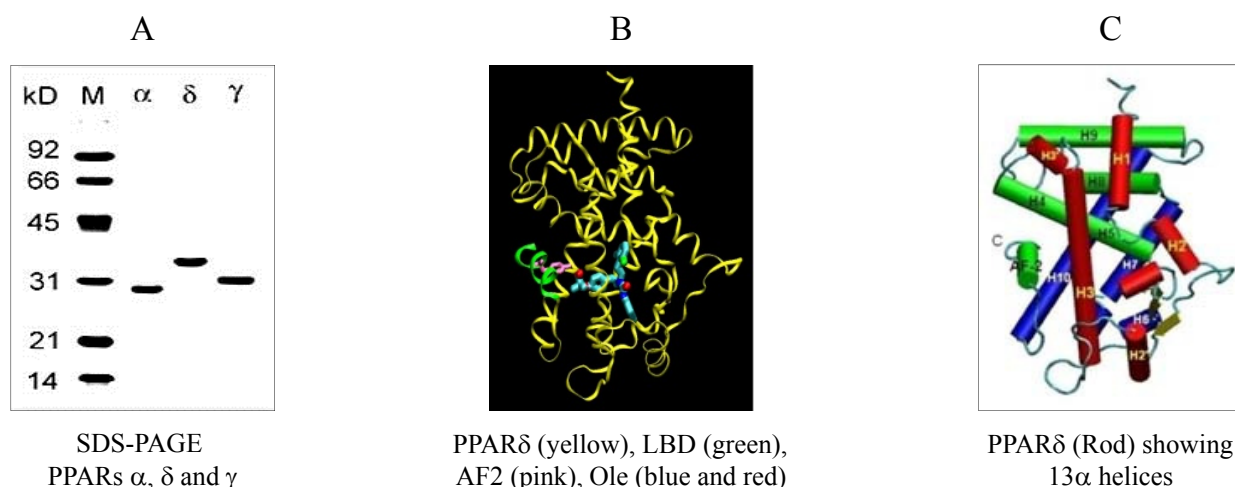
A   B   C

| SDS-PAGE | PPARδ (yellow), LBD (green), | PPARδ (Rod) showing |
| PPARs α, δ and γ | AF2 (pink), Ole (blue and red) | 13α helices |

Fig. (9). Size, Homogeneity and 3D Structure of PPARs.

critical role in ligand binding. LBD has an extensive secondary structure consisting of 13 α helices and a β sheet. Natural and synthetic ligands bind to the LBD, either activating or repressing the receptor. Between the DBD and LBD is the flexible hinge region.

Purification and 3D Structure of the PPARs

We have expressed and purified to homogeneity the LBD of PPARα (GeneBank S74349), PPARγ (GenBank NM_005037) and PPARδ (GenBank NM_006238) tagged with MKKGHHHHHHG in BL21(DE3) cells using the T7 promoter of plasmid vector pRSETA, induced with 1 mM IPTG.

Fig (**9A**) shows the SDS pattern of our purified PPARα, PPARδ and PPARγ. It demonstrates that these PPARs are homogeneous and move as single bands with molecular mass of 29, 42 and 31 kDa respectively. Fig. (**9B**) shows the 3D ribbon structure of PPARδ in yellow, bound with ligand (blue and red). The ligand binding domain is shown in green and the AF2 domain with Y473 is in pink. Fig (**9C**) shows the general 3D structure of the PPARs with 13 α helices.

MOLECULAR MODELING AND MECHANISM OF OLE ACTION

Molecular Modeling

To define the molecular mechanisms by which Ole modulates adipogenesis, we performed theoretical and computational analysis for binding of Ole to PPARα,δ,γ. We used the LBD crystal structure of the PPARs with AutoDock, Molecular Dynamics (MD) and Molecular Mechanics Poisson-Boltzmann/surface area (MM-PBSA) calculations [31-33]. The binding site is formed by several α-helices, including the C-terminal AF-2 helix. We first used molecular docking to generate several distinct binding orientations, and performed molecular dynamics simulation to further relax the complex. Then, we applied MM-PBSA to estimate the affinity for each binding mode. The binding modes with the lowest free energy are expected to be the most favorable. We then analyzed the detailed interactions

based on these binding modes. Next, we compared these binding modes with those of the respective known PPAR-ligands. The results are summarized in Fig. (**10**).

Ole Assumes Different Binding Modes than Known Ligands for PPARα

Fig. (**10A**), shows docking of Ole with PPARα in its most favorable binding mode. The PPARα backbone is represented by the gold ribbon, Ole is represented with van der Waals models (vdw) and is color coded, with carbon cyan and oxygen red. Ole adopts an extended configuration when it binds to PPARα. Ole is away from the AF2 region and it is not in contact with the AF2 Y473. Ole is also capable of assuming a Y shaped configuration when it binds to PPARα. Both of these conformations are different from the inverted U-shaped conformation of other known PPARα ligands such as GW409544. Fig. (**10D**) shows the binding of GW409544 to PPARα. The structure of GW409544 is represented with vdw modeling and is color coded carbon cyan and oxygen red. Distinct from Ole, when GW409544 binds to PPARα it assumes an inverted U conformation and it is in close contact with AF2 and forms hydrogen bonds with the Y473.

Ole Mimics the Binding Mode of High Affinity Ligands for PPARδ

Fig. (**10B**) shows docking of Ole to PPARδ in the energetically most favorable binding mode. PPARδ backbone is shown as yellow ribbon, and Ole is represented with vdw modeling. In the predicted structure, Ole occupies the Y-shaped ligand-binding pocket identical to that of known high affinity ligands such as the synthetic fibrate GW2433. Fig. (**10E**) shows the binding of GW2433 to PPARδ. The sugar group of Ole is located in a similar position and orientation as the carboxyl groups in GW2433. The two hydroxyls on the sugar ring are oriented toward the AF-2 helix and held in place through a network of hydrogen bonds with Y473, and hydrophobic interactions with L469 and T84. Both Y473 and L469 are part of the AF-2 helix.
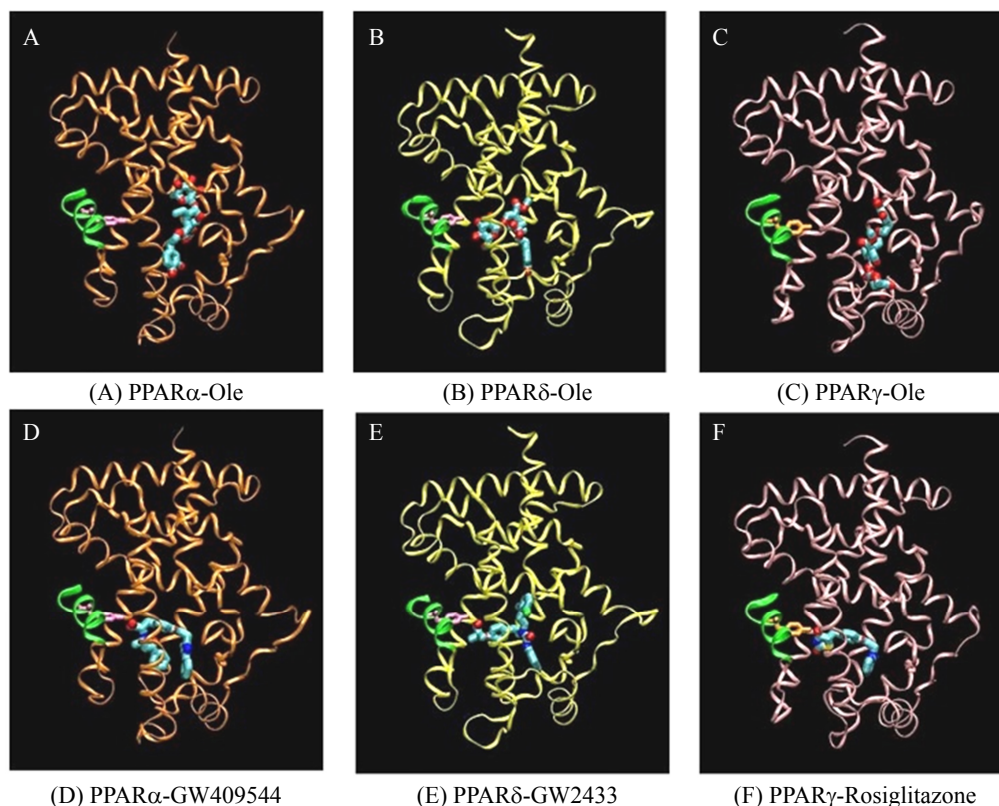
(A) PPARα-Ole   (B) PPARδ-Ole   (C) PPARγ-Ole

(D) PPARα-GW409544   (E) PPARδ-GW2433   (F) PPARγ-Rosiglitazone

Fig. (10). Molecular Modeling of Ole binding to PPARα, PPARδ, and PPARγ.

Ole Assumes Different Binding Modes than Known Ligands for PPARγ

Fig. (**10C**) shows the docking of Ole with PPARγ. The PPARγ backbone is shown as a pink ribbon, and Ole is shown with vdw modeling. Ole binds to the large PPARγ pocket in an extended (up-down) conformation rather than the inverted U-shaped conformation of known PPARγ ligands such as GI262570 and rosiglitazone. Fig. (**10F**) shows the binding of rosiglitazone to PPARγ. Unlike rosiglitazone, Ole does not interact directly with the AF-2 helix. The dihydroxyl phenol group is directed into the solvent-accessible channel between H3 and the β strands.

Molecular Modeling Results and Mechanism of Ole Action

Ole mimics precisely the binding mode of high affinity ligands for PPARδ like GW2433, with a Y-shaped configuration. As a result, the binding of the Ole molecule may shift the equilibrium toward the active configuration of PPARδ *via* direct stabilization of the AF-2 helix through hydrogen bond and hydrophobic interactions with the Ole.

In contrast, the predicted binding modes of Ole to PPARα and PPARγ involve extended "up-down" configurations, away from the AF2 helix and not in contact with Y473. This configuration is distinct from the inverted U-shaped binding modes of known high affinity ligands of both PPARα and PPARγ. As a result of such interaction, Ole may shift the equilibrium toward the less active configuration of PPARα and PPARγ by hindering the binding of the activation ligands of these PPARs.

Eicosapentanoic acid (EPA), is a natural ligand for the PPARs and it binds efficiently to all three PPARs. EPA is elongated, flexible, and can bind in either a "tail-up" or "tail-down" conformation, both of which are energetically favorable. This suggests that natural ligands such as EPA and/or other long chain fatty acids are capable of flexible binding modes. This may represent a novel mechanism for coordinated regulation of PPAR activity. Our findings that Ole binds to all of the three PPARs with distinct modes may suggest a unique mechanism for differential modulation on the activities of PPAR members. Coordinated activation and/or suppression of the PPARs by Ole binding make Ole a multifaceted therapeutic agent against obesity and related diseases. These effects suggest that the potential to control weight gain, enhance physical endurance, improve insulin sensitivity, and ameliorate metabolic syndrome, type 2 diabetes, CVD, and atherosclerosis.

CONCLUSIONS

Clinical studies indicate that a Mediterranean diet rich in olive and olive oil is linked to weight loss and reduction of type 2 diabetes and CVD. Some of these health benefits are due to the protective effect of the monounsaturated fatty acid oleic acid. However, weight loss and other metabolic beneficial effects cannot be entirely attributed to the lipid components of olive oil. The phenolic anti-oxidants, including Ole and derivatives, in olive plants and olive oil also play critical roles, especially in weight loss. How Ole promotes weight loss is not clear, and its molecular mechanism of action has not been fully understood. In this article, we report our studies on the effects of Ole on

adipogenesis and hMSC differentiation. We found that Ole inhibits adipogenesis, promotes adipocyte de-differentiation and trans-differentiation. Ole down-regulates the expression of adipogenic genes including PPARγ and up-regulates PPARδ. The PPARs are physiological fat sensors. PPARγ promotes fat cell formation and fat storage, while PPARδ enhances fat burning and energy uncoupling. In view of these results, we carried out molecular modeling to examine the interactions between Ole and the PPARs. We found that Ole binds to all three PPARs, but with distinct binding modes. Ole mimics the binding conformation of high affinity ligands for PPARδ whereas it assumes different conformations than known ligands for PPARα and PPARγ. These results provide a viable mechanism for the multifaceted effects of Ole against obesity, diabetes, CVD and related metabolic syndrome.

ABBREVIATIONS

HDL   =   High Density Lipoprotein

HPLC   =   High Performance Liquid Chromatography

HT   =   Hydroxytyrosol

LDL   =   Low Density Lipoprotein

LC   =   Liquid Chromatography

MD   =   Molecular Dynamics

MM-PBSA   =   Molecular Mechanics- Poisson-Boltzmann Surface Area

MS   =   Mass Spectrometry

Ole   =   Oleuropein

PPAR   =   Peroxisome Proliferator Activator Receptor

TG   =   Triglyceride

TLC   =   Thin Layer Chromatography

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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