A Dissertation
entitled

Characterization of the Anti-Obesity and Anti-Adipogenic Effects of the Limonoid Prieurianin

by
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DEDICATION

In loving memory of my sister and grandmother.

This work is dedicated to my parents, sisters, nephew and niece, family and friends, who have always encouraged and believed in me. Without your support I would not be here today. I love you and thank you.

ABSTRACT

Phospholipid transfer protein (PLTP) is critically important for reverse cholesterol transport (RCT), and its expression level and activity increase when mice are fed a high fat diet. RCT is the process by which accumulated cholesterol from the blood vessel walls, peripheral tissues and macrophages is transported back to the liver for excretion. Interestingly, topoisomerase I inhibitors used in chemotherapy have been shown in our laboratory to dose dependently induced PLTP expression in both *in vivo* and *in vitro* studies. Since PLTP transports phospholipid as well as cholesterol into high density lipoprotein (HDL), we asked whether elevated PLTP levels might increase the transfer of drugs into HDL via RCT, thus increasing tumor cells resistance to the drug. However, we found that camptothecin, topoisomerase I inhibitor, does not accumulate in HDL or in other lipoprotein subfractions, thus ruling out the possibility of PLTP mediating the transfer of camptothecin into HDL for liver metabolism.

The limonoid prieurianin, like topoisomerase I inhibitors, has also been shown to dose dependently increase PLTP mRNA and proteins levels, and here we show that prieurianin causes weight loss by reducing food intake in morbidly obese mice and in mice on high-calorie diet. Additionally, prieurianin is anti-adipogenic and (i) inhibits the proliferation and differentiation of preadipocytes into adipocytes, and (ii) induces either dedifferentiation or delipidation of mature adipocytes. Gene expression profiling showed that prieurianin suppresses the expression of a number of genes involved in fat metabolism, and inhibits the transcriptional activity of the adipogenesis master regulators

including the CCAAT/enhancer binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor gamma (PPARγ).

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INTRODUCTION

Obesity, Diabetes Mellitus and Insulin Resistance

Diabetes Mellitus (DM) is a heterogeneous group of disorders characterized by hyperglycemia, and is due to deficiency of insulin secretion or to resistance of the body's cells to the action of insulin, or to a combination of these. According to the NDDG/WHO classification scheme, there are four main types of DM and include type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes mellitus and other specific types of diabetes mellitus. Our primary focus is type 2 diabetes mellitus as it is closely associated with obesity.

Type 2 diabetes mellitus comprises approximately 90 to 95% of cases in the diabetes syndrome (King, Aubert, & Herman, 1998), and is caused by both genetic and nongenetic factors, such as high caloric intake, overweight and sedentary lifestyle, that culminates with insulin resistance in the liver, muscle, and adipose tissue and insulin deficiency. Patients with type 2 DM require exogenous insulin only if fasting hyperglycemia cannot be corrected with the use of diet or oral agents, and exercise. Most patients are diagnosed with the disease in adult years. There is a strong correlation between type 2 diabetes and obesity, and approximately 50 to 90% of all patients with type 2 diabetes are obese (Harris MI, 1995).

Normal fasting glucose is less than 110mg/dL, in contrast to patients with diabetes have fasting plasma glucose exceeding126mg/dL. Patients with a fasting glucose between 110 and 126mg/dL are said to have an impaired fasting glucose. According to

the NHANES III study, the prevalence of DM rose with age and peaked at 19% at age 75 years and older (Harris, et al., 1998). Further, minority populations in United States have higher rates of DM with black and Mexican Americans leading with 28% and 33% of their population (Harris, et al., 1998).

Insulin is an anabolic hormone whose main function is to maintain blood glucose within a narrow range. Following a meal, carbohydrates are digested, glucose levels rise, and insulin is secreted from pancreatic β-cells into the hepatic portal vein. Insulin regulates glucose levels through two mechanisms. First, it activates glycolytic and glycogen synthetic pathways which are responsible for the uptake, utilization, and storage of glucose in various tissues. Second, it inhibits glycogenolytic and gluconeogenic pathways involved in glucose production and output. Both of these mechanisms reduce the amount of circulating glucose in the blood. Circulating insulin is cleared by the liver through a receptor mediated endocytosis via clathrin coated vesicles. Insulin binds to the α-subunit of the insulin receptor**,** and induces a conformational change and autophosphorylation within the tyrosine kinase domain. Phosphorylation of the tyrosine residues is important for the downstream signaling proteins insulin receptor substrate (IRS) and src homology 2 domain containing protein (SHC). Insulin signals through two main pathways: (i) phosphoinositide-3-kinase (PI3K) via phosphorylation of IRS, and (ii) RAS/RAF/mitogen activated protein kinase (MAPK) pathway via phosphorylation of SHC which promotes the mitogenic action of insulin (Tanti, et al., 2004). Insulin receptor phosphorylates and activates the plasma membrane glycoprotein CEACAM1 which mediates insulin clearance in the liver (Najjar, et al., 1993).

Not surprisingly, insulin dysregulation can lead to altered fat metabolism and insulin resistance, and this is a hallmark feature of T2DM which is also associated with obesity, hyperglycemia and altered fat metabolism. In the liver, insulin decreases fatty acid oxidation by increasing malonyl-coenzyme A (malonyl Co-A) which in turn inhibit the rate limiting enzyme in control of fatty acid uptake and oxidation in the cell, carnitine palmitoyl transferase 1 (CPT-1). Further, insulin stimulates *de novo* synthesis of free fatty acids (FFA), which are then secreted into the circulation as very low density lipoprotein triglycerides (VLDL-TG) and inhibits hydrolysis of stored triglycerides in adipocytes by hormone sensitive lipase (HSL) (Kraemer & Shen, 2002; McGarry & Foster, 1980). Within the plasma membrane of the adipose tissue, lipoprotein lipase (LPL) mediates the hydrolysis and uptake of circulating trigylcerides in reponse to insulin (Eckel, 1989; Mead, Irvine, & Ramji, 2002). However, the insulin resistant state is characterized by high circulating nonesterified FFA which leads to the activation of atypical protein kinase C (PKC) then serine/threonine kinases (Griffin, et al., 1999; Itani, Ruderman, Schmieder, & Boden, 2002; Yu, et al., 2002) followed by a deactivation of the insulin signaling cascade. Thus, the net result is a loss of insulin stimuated glucose uptake and oxidation in muscle(Groop, et al., 1989; J. K. Kim, Wi, & Youn, 1996; Shulman, 2000).

Insulin resistance is associated with a state of chronic low-grade inflammation and the release of several chemical mediators such as interleukins-1, 6, 10, 18, resistin, retinol-binding protein 4 (RBP4), adipocyte-fatty acid binding protein (aP2, FABP4) and nonesterified fatty acids (NEFA), from immune cells and adipocytes. Among the pro-

inflammatory cytokines secreted is tumor necrosis factor alpha (TNFα), which like elevated nonesterified free fatty acids, stimulate the inhibitory phosphorylation of IRS-1 (Tilg & Moschen, 2008), decreases the insulin sensitizing protein and lipid senor PPARγ mRNA and DNA binding activity (Ye, 2008), as well as suppresses the transcription of adiponectin which (i) inhibits TNFα-induced NFκB activation, (ii) decreases hyperglycemia and levels of plasma FFA, and (iii) improves insulin sensitivity. TNFα-induced insulin resistance is mediated by IKKβ (Tilg & Moschen, 2008).

Obesity and Metabolic Syndrome

Metabolic syndrome is a combination of medical disorders that increase the risk of developing cardiovascular disease and diabetes, and includes abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance or glucose intolerance, prothrombotic state, and proinflammatory state (Bray & Bellanger, 2006). It is estimated that over 50 million Americans have the metabolic syndrome and are at increased risk of coronary heart disease and type 2 diabetes. The two principle risk factors for this syndrome are abdominal obesity and insulin resistance. Obesity, defined as having body-mass index (BMI) of more than 30, is an epidemic that affects an estimated 300 million people worldwide and 30% of United States adults (Haslam & James, 2005). The alarming rate of increase of obesity is due to sedentary lifestyle habits coupled with overconsumption of energy-rich foods, and is known to be associated with an increased risk for hypertension, type 2 diabetes, coronary heart disease, stroke, hyperlipidemia and certain cancers (Z. Li, et al., 2005; Spiegelman, Choy, Hotamisligil, Graves, & Tontonoz, 1993). Pharmacological intervention will undoubtedly have

numerous benefits in reducing the incidence of these comorbidities in obesity. However, today there are only two FDA-approved drugs – orlistat and sibutramine – in the market for the long-term treatment of obesity. Orlistat blocks absorption of dietary fats (Guerciolini, 1997) while sibutramine (Ryan, Kaiser, & Bray, 1995) is a serotonin and norepinephrine reuptake inhibitor that acts in the central nervous system (CNS) to reduce energy intake. These drugs have limited efficacies and side effects are commonly reported, which are further confounded by diminishing response in long-term treatment (Fernstrom & Choi, 2008; Z. Li, et al., 2005). Moreover, most of the new anti-obesity drug development continues to focus on either central or peripheral acting inhibitors of food intake (Cooke & Bloom, 2006) and would likely encounter the above problems. Thus, there is an urgent need to identify breakthrough drugs with paradigm shifting pharmacodynamics for the treatment of obesity.

The main cause of obesity is an imbalance between energy intake and energy expenditure. When energy intake exceeds expenditure, the excess is stored mainly in the form of fat in adipose tissue, either under the skin or deep in the abdomen. During adipogenesis, fat precursor cells differentiate and mature into white and brown adipose tissue. There are many in vitro cell models for the study of lipid accumulation, and a few mouse cell lines include 3T3-L1, *ob/ob* and 3T3-F442A cells. To initiate differentiation, cells are cultured to confluence and contact inhibited. Upon exposure to differentiation induction signals, the preadipocytes further undergo two to four rounds of cell division called mitotic clonal expansion, which is followed by terminal differentiation, characterized by: (i) the expression of the CCAAT/enhancer binding proteins (C/EBPs)

and peroxisome proliferator-activated receptor gamma (PPARγ) transcription factors; (ii) synthesis of lipid transport and adipocyte-specific proteins; and (iii) secretion of several cytokines including TNFα. Adipocyte differentiation culminates with a fibroblast-like to spherical phenotypic change and the accumulation of intracellular fat droplets. In the absence of differentiation inducers this process takes about two to three weeks. However, this can be shortened to five to seven days if a cocktail of three inducers are used: insulin, dexamethasone and 3-Isobutyl-1-methylxanthine (IBMX). Insulin increases cellular glucose uptake, the glucocorticoid dexamethasone downregulates preadipocyte factor 1 (Wolf, 1999), and IBMX inhibits phosphodiesterase (Beavo, et al., 1970; Chasin & Harris, 1976; Montague & Cook, 1971; H. Oka, Kaneko, Yamashita, Suzuki, & Oda, 1973; Peytremann, Nicholson, Liddle, Hardman, & Sutherland, 1973).

Signaling through nuclear factor kappa B

Nuclear factor kappa B (NFκB)/Rel proteins comprise a family of structurally-related eukaryotic transcription factors that plays a critical role in inflammation, cell migration and repair, immune response, apoptosis, and angiogenesis. These transcription factors are persistently active in a number of disease states, including chronic inflammation and heart disease. The family consists of five proteins: NFκB1 (p50 and its precursor p105), NFκB2 (p52 and its precursor p100), RelA (p65), RelB, and c-Rel.

All NFκB/Rel proteins contain Rel homology (RH) domains located near the N-terminus. RH domain is required for dimerization, DNA-binding, and interaction with IκB. NFκB/Rel proteins can be divided into two classes: NFκB (p105 and p100) and Rel

(RelA (p65), RelB, and c-Rel) proteins. Members of the NFκB class have long C-terminal domains that contain multiple copies of ankyrin repeats which inhibit NFκB activation and nuclear translocation. Members of the NFκB class become active, shorter DNA-binding proteins (p105 to p50, p100 to p52) by either limited proteolysis or arrested translation. Members of the NFκB class are not activators of transcription generally, except when they form dimers with the Rel proteins. Rel proteins contain C-terminal transcription activation domains which promotes transcription by recruiting coactivators and displacing repressor proteins.

In most cells, NFκB is present as an inactive heterodimer complexed to IκBα in the cytoplasm. When a cell receives any of a multitude of extracellular signals, NFκB rapidly enters the nucleus and activates gene expression. Within the nucleus, acetyltransferases including p300 and cyclic AMP response element-binding protein (CREB)-binding protein (CBP) acetylate and activate NFκB. The NFκB complex binds to promoter and enhancer regions containing the consensus sequence GGGRNNYYCC (N=any base, R=purine, and Y=pyrimidine) (Hayden & Ghosh, 2004), called κB sites and this results in the activation of NFκB responsive genes such as TNFα and IκBα. In the classical pathway, the newly-synthesized IκBα enters the nucleus, removes NFκB from DNA, and exports the complex back to the cytoplasm to restore the original latent state. Thus, the activation of the NFκB pathway is generally a transient process, lasting from 30-60 minutes in most cells.

One of the most potent activators of NFκB is tumor necrosis factor-alpha (TNFα), a soluble protein first isolated in 1975, that was discovered to be cytotoxic to tumor cells and promote regression of mouse tumors (Carswell, et al., 1975). TNFα is a 17kDa trimer protein and proinflammatory cytokine that is expressed in a number of tissues including the bladder, heart, lung, lymph node and adipose tissue. TNFα blocks the action of insulin by inhibiting insulin receptor tyrosine kinase activity and insulin-sensitive glucose transporter (GLUT4) expression (Hotamisligil, Shargill, & Spiegelman, 1993; Sethi, et al., 2000; Stephens & Pekala, 1991). Consequently obese individuals develop insulin resistance, have high plasma insulin levels, and are at considerable risk for developing noninsulin-dependent diabetes mellitus also called type 2 diabetes (Cseh, Winkler, Melczer, & Baranyi, 2000; Uysal, Wiesbrock, & Hotamisligil, 1998; Uysal, Wiesbrock, Marino, & Hotamisligil, 1997). Furthermore, TNFα causes cachexia (tissue wasting), and *in vitro* inhibits preadipocyte differentiation (Kawakami, et al., 1989), and stimulates lypolysis and de-differentiation of differentiated adipocytes. To date, there is no known small molecule that mimics TNFα action.

The cellular action of the trimer TNFα protein is mediated by two ubiquitously expressed and distinct receptors: TNFR1 (55kDa) and TNFR2 (75kDa). TNFRs are classed as type I transmembrane protein and lack any enzymatic activity. They possess an extracelluar amino-terminus with a cysteine-rich domain. However, their intracellular carboxy-terminus is dissimilar and lacks sequence homology. TNFR1 can activate NFκB, mediate apoptosis, and function as a regulator of inflammation. The adaptor

proteins TNFR-associated death domain (TRADD) and TNFR-associated factor 2 (TRAF2) have been shown to interact with TNFR1.

TNFR1 is one of the major receptors for ligand TNFα. When the cell receives an extracellular signal, TRADD recruits TRAF2 and receptor-interacting protein (RIP). TRAF2 in turn recruits IKK, enabling the serine-threonine kinase RIP to activate it. IKK phosphorylates IκBα, which is normally bound to NFκB. The phosphorylated IκBα is subsequently degraded, and the released NFκB translocates to the nucleus and activates NFκB responsive genes.

In contrast, the binding of TNFα to TNFR2 results in the formation of heterocomplex between TNFR2 and TNFR1 that mediates the recruitment of the anti-apoptotic protein inhibitors of apoptosis 1 (IAP1). IAP1 possesses E3 ubiquitin ligase activity, and mediates the ubiquitination and proteasomal degradation of TRAF2 (X. Li, Yang, & Ashwell, 2002).

Limonoids and Prieurianin

Limonoids occur naturally only in plants species of the Rutaceae and Meliaceae plant families. The term limonoids was derived from limonin, first isolated in 1938 by Highby from Washington navel orange, and later showed it as the bitter principle of navel orange juice in 1949. Limonoids are highly oxygenated, modified terpenoids, and have attracted much attention because compounds belonging to this group have exhibited a broad range of biological activities such as insecticidal, insect antifeedant and growth

regulating activity on insects, as well as antibacterial, antifungal, antimalarial, anticancer, antiviral and numerous other pharmacological activities on humans such as antineoplastic, anitoxidant and hypocholesterolemic activity (Koul, Singh, Singh, Daniewski, & Berlozecki, 2004; Manners, 2007)

Prieurianin is isolated from *Turraea obtusifolia* which belongs to the Meliaceae (Mohogany) family. The genus *Turraea* is named after Georgio della Turre, while the species *obtusifolia* means blunt-leaved in Latin and refer to the often blunt tip of the leaves. Although *Turraea obtusifolia* is said be very poisonous, its leaves, bark and rootbark are used in traditional medicine to treat stomach and intestinal ailments. The leaves contain limonoids which serves as an insect repellant to protect crop plants from insect damage. Prieurianin, a small molecule with molecular weight of 762, has been shown to deter insect feeding behavior by acting as antagonist to the molting hormone 20-hydroxyecdysone in the central nervous system (Sarker, Savchenko, Whiting, Sik, & Dinan, 1997).

CHAPTER I. Role of PLTP in drug-induced resistance in cancer.

ABSTRACT

Accumulated cholesterol from the blood vessel walls, peripheral tissues and macrophages is transported back to the liver for excretion via the reverse cholesterol transport (RCT) pathway. A number of enzymes and transfer proteins are involved in this process, one of which is called the phospholipid transfer protein (PLTP). The function of PLTP is to shuttle phospholipids from triglyceride-rich lipoproteins to the high density lipoprotein (HDL), thereby remodeling HDL for RCT. Interestingly, topoisomerase I inhibitors used in chemotherapy have been shown in our laboratory to dose dependently induced PLTP expression in both *in vivo* and *in vitro* studies. Since PLTP transports phospholipid as well as cholesterol into HDL, we raise the hypothesis that PLTP may shuttle topoisomerase I inhibitors such as camptothecin, into HDL for further metabolism in the liver, thus increasing tumor cells resistance to the drug. Our results may yield insights into a novel mechanism of drug resistance in cancer.

INTRODUCTION

Plasma phosopholipid transfer protein (PLTP) is a 53kDa secreted glycoprotein that has two main functions. Firstly, it mediates the conversion of HDL_3 particles into larger HDL_2 particles and smaller poorly lipidated prebeta-HDL particles, which are efficient acceptors of cholesterol from peripheral cells and thus acts as an anti-atherogenic factor preventing cellular cholesterol overload (von Eckardstein, et al., 1996). Secondly, PLTP transfers surface phospholipids from triglyceride-rich lipoproteins, chylomicrons and very low-density lipoproteins (VLDL) to high density lipoprotein (HDL). Frequently referred to as the good cholesterol, HDL (i) plays a key role in the process of reverse cholesterol transport (RCT), which promotes the efflux of excess cholesterol from peripheral tissues and returns it to the liver for biliary excretion, and (ii) is inversely related to risk of atherosclerotic cardiovascular disease. Thus, PLTP can be envisioned to play an important role in the prevention of atherosclerosis. Secreted PLTP can be found in the plasma and cerebrospinal fluid. There are two transcript variants encoding different PLTP isoforms in human and size-exclusion chromatography analysis revealed that there are two populations of PLTP – one catalytically active and the other inactive (Attia, et al., 2007; Huuskonen, Olkkonen, Jauhiainen, & Ehnholm, 2001; Jiang, 2002; Karkkainen, et al., 2002; T. Oka, et al., 2000). Active PLTP has an average molecular mass of 160kDa. In contrast, inactive PLTP has an average molecular mass of 520kDa and contains 70% of the total PLTP.

Surprisingly, PLTP also has been reported to be a pro-atherogenic factor. In humans, increased plasma PLTP activity is associated with increased risk for coronary heart disease (Schlitt, et al., 2003), and this is corroborated in transgenic mice studies showing that overexpressing human PLTP decreased plasma HDL and increased VLDL, and therefore elevates the susceptibility to atherosclerosis (Foger, et al., 1997; Jaari, et al., 2001; Lie, et al., 2002; Lie, et al., 2004; van Haperen, et al., 2002; van Haperen, et al., 2000; Yang, et al., 2003). PLTP knockout mice fed either regular chow or high fat diet have (i) decreased plasma cholesterol, (ii) decreased expression of proinflammatory genes, and (iii) increased cholesterol accumulation in macrophages (Ogier, et al., 2007; Shelly, Royer, Sand, Jensen, & Luo, 2008), thus these studies further suggest that PLTP is pro-atherogenic. On the other hand, numerous studies suggest that PLTP regulates HDL metabolism and acts as an anti-atherogenic factor preventing cellular cholesterol overload by generation of prebeta-HDL in vivo (Jiang, et al., 1999; Tu, et al., 1999; van Haperen, et al., 2000), and total serum PLTP mass protects against coronary heart disease (Yatsuya, et al., 2004)

PLTP is nearly ubiquitously expressed in all cell types and tissues, as well as a broad range of tumors such as bladder, breast, cervix, colorectal, esophageal, gastrointestinal, glioma, kidney, liver, lung, ovary, pancreas, prostate, skin and uterus. In addition to being a secreted protein, PLTP is also widely expressed in the central nervous system and the cerebrospinal fluid (Vuletic, et al., 2003). However, very little is known about potential intracellular PLTP functions. Recently, Vuletic et al showed that PLTP is present in the nucleus of neuronal, kidney and ovarian cells, and confirmed that

intracellular PLTP is active in phospholipid transfer (Vuletic, et al., 2003). This suggests that PLTP may be involved in regulation, transport and distribution of phospholipids and cholesterol in the nucleus. Besides phospholipds, PLTP also facilitates the transfer of other lipophilic molecules such as vitamin E or α-tocopherol (Jiang, et al., 2002). α-tocopherol is also present in the nuclei and its antioxidant properties aids in the preservation of DNA integrity by preventing damage to DNA which may lead to the development of cancer caused by free radicals. Since PLTP is critical for transfer of α-tocopherol to the cells, it is possible that it may be also involved in its transfer to the nucleus. Not surprisingly, recent studies have shown that PLTP mRNA transcript, protein levels and activity are decreased in the pathophysiology of various diseases including Alzheimer's disease (Vuletic, et al., 2003) and breast cancer (Ferkingstad, Frigessi, & Lyng, 2008).

Second to heart disease, cancer is the next leading cause of death in United States. Surgical tumor resection, radiotherapy and chemotherapy are the main therapeutic strategies in cancer treatment, and are very successful when patients are diagnosed in the early stages of localized cancers. However, patients after long term treatment develop drug resistant cancer cells, which unfortunately result in disease relapse and ultimately death. The molecular mechanisms underlining multidrug resistance (MDR) is a current topic of great interest for improving clinical therapies against cancers, and numerous investigators have delineated many signaling molecules that may contribute to this aberration. Previously, we observed that the chemotherapeutic topoisomerase I inhibitor and camptothecin derivative, topotecan induces the expression of PLTP in HepG2 liver

cells by DNA micorarray analyses. Additionally, we have shown that topotecan and other camptothecin derivatives induce both the expression of PLTP mRNA as well as the PLTP promoter fused to a luciferase reporter construct transfected into HepG2 liver cells suggesting that topotecan regulates PLTP expression at the transcriptional level. By contrast, inactive derivatives of camptothecin and protoberberines (topoisomerase I and II inhibitor) failed to enhance the expression of PLTP indicating that PLTP induction was specific to camptothecin derivatives. Since camptothecin is cytotoxic to normal cells, we hypothesized that the increase PLTP expression maybe to facilitate the transfer of drugs from the peripheral tissues to the liver for detoxification and in so doing contribute the drug-induced tolerance observed with camptothecin treatment.

MATERIALS AND METHODS

Construction of PLTP Adenovirus. The PLTP adenovirus was generated using the ViraPower Adenoviral Expression System (Invitrogen) according to the manufacturer's instructions. The encoding sequence of PLTP was cloned into the pENTR vector (Invitrogen). The cDNA was transferred into the pAd/CMV/V5-DEST vector (Invitrogen) using the Gateway LR Clonase II enzyme mix according to the manufacturer's directions (Invitrogen). The ligation reaction was transformed into One Shot TOP10 chemically competent *Escherichia coli*. Recombinant adenoviral DNA was recovered from the TOP10 *E. coli* cells and transfected into 293A cells (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's directions. The 293A cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO_2. Cell lysis was typically apparent in 5–7 days post-transfection. Cells and media were collected and subjected to three freeze/thaw cycles. The cell debris was pelleted at 3000 rpm for 15 min. Adenovirus was amplified and viral stock stored in 1mL aliquots at -80°C.

Western Blot Analysis. Proteins from HepG2 cells were harvested in lysis buffer consisting of 50 mM Tris-Cl, pH 8.0, 150mM NaCl, 1% Nonidet P-40 and diluted protease inhibitor mixture (Roche 11697498001). Cell debris were removed by centrifugation for 10 min at 4°C. An equal amount of protein (50ug) was loaded onto a 12% SDS-PAGE gel and transferred to a 0.45 micron pure nitrocellulose membrane (Bio-Rad). Blots were probed with anti-PLTP antibodies (BioVision, #3595-100) in phosphate-buffered saline containing 5% nonfat dry milk powder and incubated with

horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Biosciences, #NA934). Protein bands were resolved using HyGLO HRP detection kit (Denville, #34080 or 34094) and quantified by Image J software (open source Image J software available at http://rsb.info.nih.gov/ij/). Actin-peroxidase (42 KDa) (Sigma, # A3854) was used as loading control for each lane.

PLTP Activity Assay. PLTP activity was measured using a fluorescence-based assay kit (Cardiovascular Targets Inc., New York, NY, USA). The kit contains both donor and acceptor particles, and a fluorescent phospholipid that is in the quenched state when associated with the donor. Briefly, 3uL of human plasma (positive control) or 50μg HepG2 conditioned media was incubated with donor and acceptor particles. Incubation of donor and acceptor particles with plasma results in PLTP-mediated transfer of fluorescent phospholipid from donor to acceptor particle, and increased fluorescence intensity.

Camptothecin Transport in HepG2. Briefly, HepG2 cells were seeded in 24-well plate at 85% confluency (~250,000 cells/well). *Day 0*, media was refreshed and 20uL adenovirus added. After 24 hours (*Day 1*) of standard cell culture conditions an additional 1mL media was added. *Day 2*, add 1uCi/well radioisotope. *Day 3*, conditioned media and cell lysates were counted for radioactivity using a liquid scintillation counter.

Fractionation of Plasma Lipoproteins by Density Gradient Ultracentrifugation in Swing-out Rotors. Male C57/B6J mice weighing 18-20 g were injected intra-peritoneal with 50uCi of [1,2-^{3}H(N)] cholesterol (Moravek, # MT 912) or [14-^{3}H(N)]-campothecin (Moravek, # MT 1733) in 0.2ml of saline buffer per mice; the mice were kept for up to 36

hr in cages until the animals were killed by CO2 asphyxiation and exsanguinated at 0.5, 2, 6, 12, 24, and 36 hours by cardiac puncture using a 27-gauge TB syringe (BD, #305945) placed into the heart ventricular cavity and withdrawing the blood slowly to prevent the heart from collapsing. About 0.5 to 1mL of blood was collected from each donor into BD Vacutainer PST (BD, #367960) containing the anti-coagulant heparin for plasma separation and centrifuged at 3000 rpm for 10 minutes at 4°C to yield plasma. The lipoprotein fractionation was completed within 36 hours of collection using a modified method from Kleinveld et al (Kleinveld, Duif, Pekelharing, & van Rijn, 1996). After adjustment of the density of plasma samples to 1.24kg/L using KBr, solution was overlayered with a stepwise NaCl-KBr-gradient (density 1.12-1.06kg/L) in a preparative centrifuge tube (Beckman, #344060). After 9-h centrifugation (36,000 ×*g*) in a swing-out SW-40 rotor, the very low density (VLDL), low density (LDL), and high density lipoproteins (HDL) were well resolved from each other in samples. Using a 23G needle, a hole was punctured at the bottom of the tube and twenty-four 0.5mL fractions collected. The distribution of tritiated cholesterol or camptothecin among the lipoproteins was determined by counting each fraction using liquid scintillation counter and correcting for background radioactivity and quench. Fractions 8, 18, 24 correspond to peaks of HDL, LDL and VLDL fractions respectively.

RESULTS

Overexpression of human PLTP in HepG2.

To evaluate the capacity of Ad-PLTP to enhance the expression and activity of PLTP in cells, HepG2 cells were infected with Ad-PLTP or infected with Ad-LacZ or Ad-GFP (Fig. 1) as a control. Forty-eight hours after infection, cell lysates were prepared and subjected to Western blot analysis. Compared to cells without infection or infected with Ad-GFP, the expression of PLTP in the total cell extract and supernatant from cells infected with Ad-PLTP was increased 40-fold and 15-fold respectively (Fig. 2A). Similar results were obtained by measuring PLTP phospholipid transfer activity (Fig. 2B). In the conditioned media of cells infected with Ad-PLTP, transfer of fluorescent phospholipid was 67pmol/min/mg of protein compared to the uninfected control 25pmol/min/mg of protein. However, no appreciable PLTP phospholipid transfer activity was detected for total cell extracts.

Effect of human PLTP overexpression on Camptothecin Transport in HepG2.

Human hepatoma HepG2 cells are a liver-derived cell line that assembly and secrete lipoproteins, and PLTP mediate the transfer of cholesterol from cells to nacent HDL. To test the role of plasma transfer protein-mediated drug induced tolerance, the effect of PLTP on camptothecin transport was investigated. HepG2 were transduced for 24 hours with adenoviruses and incubated for an additional 24 hour with 1μCi camptothecin. There was neither an increased efflux or uptake in the cells overexpressing PLTP (Fig. 3).

Plasma lipoprotein analysis by ultracentrifugation.

Many cancers overcome chemotherapy by drug-induced tolerance mechanisms. Here we test another possible mechanism for tolerance. We hypothesize that plasma transfer proteins shuttle the drug via lipoproteins to the liver for detoxification and in so doing contribute the drug-induced tolerance observed with camptothecin treatment. To investigate if plasma transfer proteins would facilitate the transfer of camptothecin, like cholesterol, into lipoproteins we injected C57/B6J mice with radiolabeled cholesterol and camptothecin. Figure 4 illustrates the lipoproteins profile of nonfasting plasma from C57/B6J mice injected with radioactive cholesterol and camptothecin. In agreement with previously published data, cholesterol is distributed amongst all lipoprotein samples, and this increases with a function of time. No cholesterol is associated with HDL and VLDL at 30 minutes after injection (Fig 4B), although a small fraction seem to be associated with LDL and intermediate density lipoprotein (IDL). Two hours after injection, cholesterol is associated with LDL and VLDL fractions, and increases to a maximum of 15,000cpm and 25,000cpm at 24h for LDL and VLDL respectively (Fig 4B). In contract to the LDL and VLDL, cholesterol is detected only in the HDL fractions between 12 and 24 hours (Fig. 4B).

On the other hand, camptothecin has a maximum between fractions 1-4 and this quickly tappers to negligible traces of radioisotopes by as early as fraction 12 at 2 hours suggesting that camptothecin may have lightly diffused across the gradient and is not associated with any major lipoprotein fraction. The maximum camptothecin

concentration (22,000cpm) observed in fraction 2 at 2 hours may thus be a reflection of the time for the drug to enter the blood supply. Taken together with the aforementioned, this data suggests that camptothecin, unlike cholesterol, is not a substrate for plasma protein mediated transfer to lipoproteins.

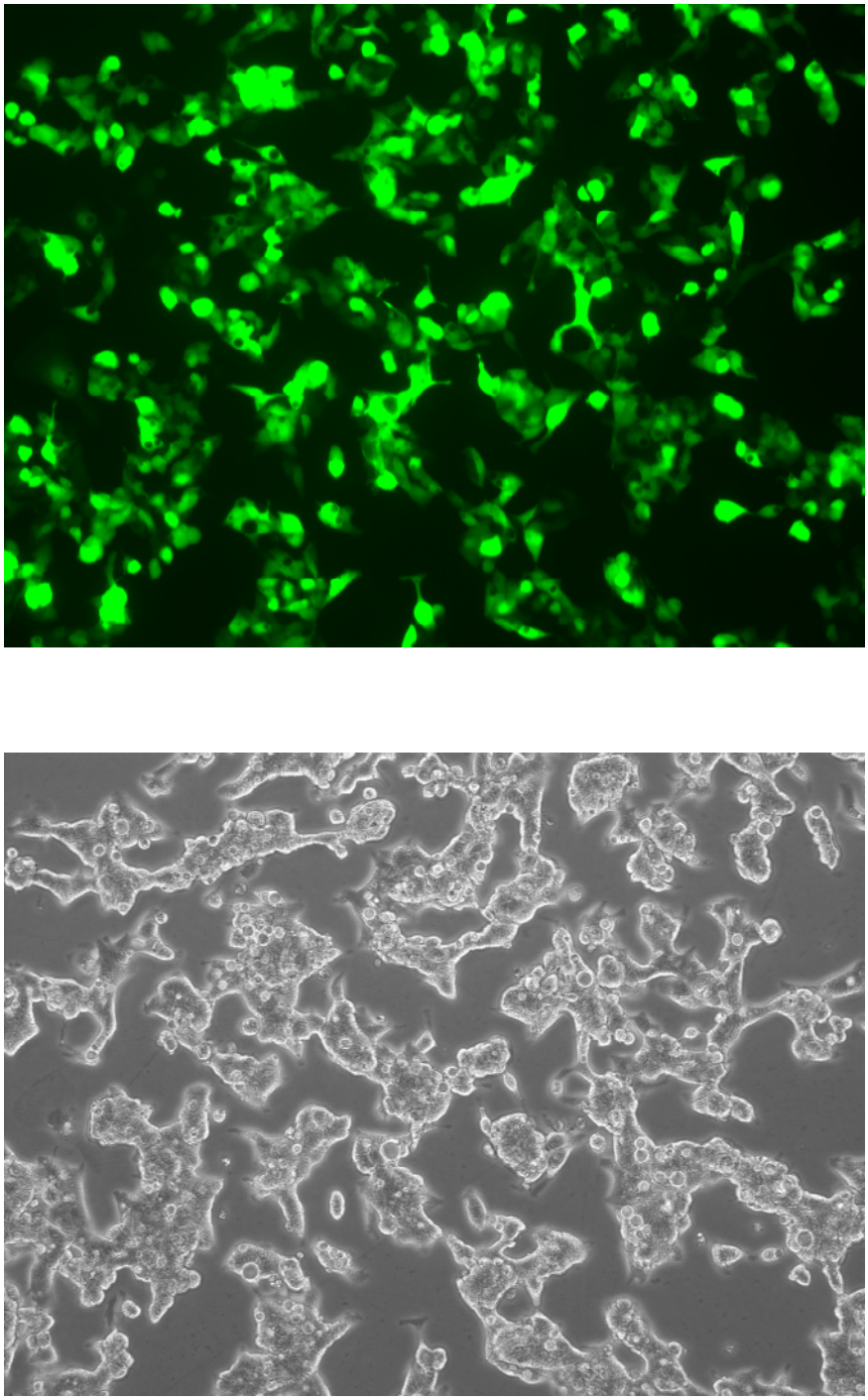


Figure 1. Infection of hepatocellular carcinoma HepG2 cells with recombinant adenovirus encoding green fluorescent protein (GFP). The expression of GFP was observed in 95-100% of the infected HepG2 cells at 20X magnification.

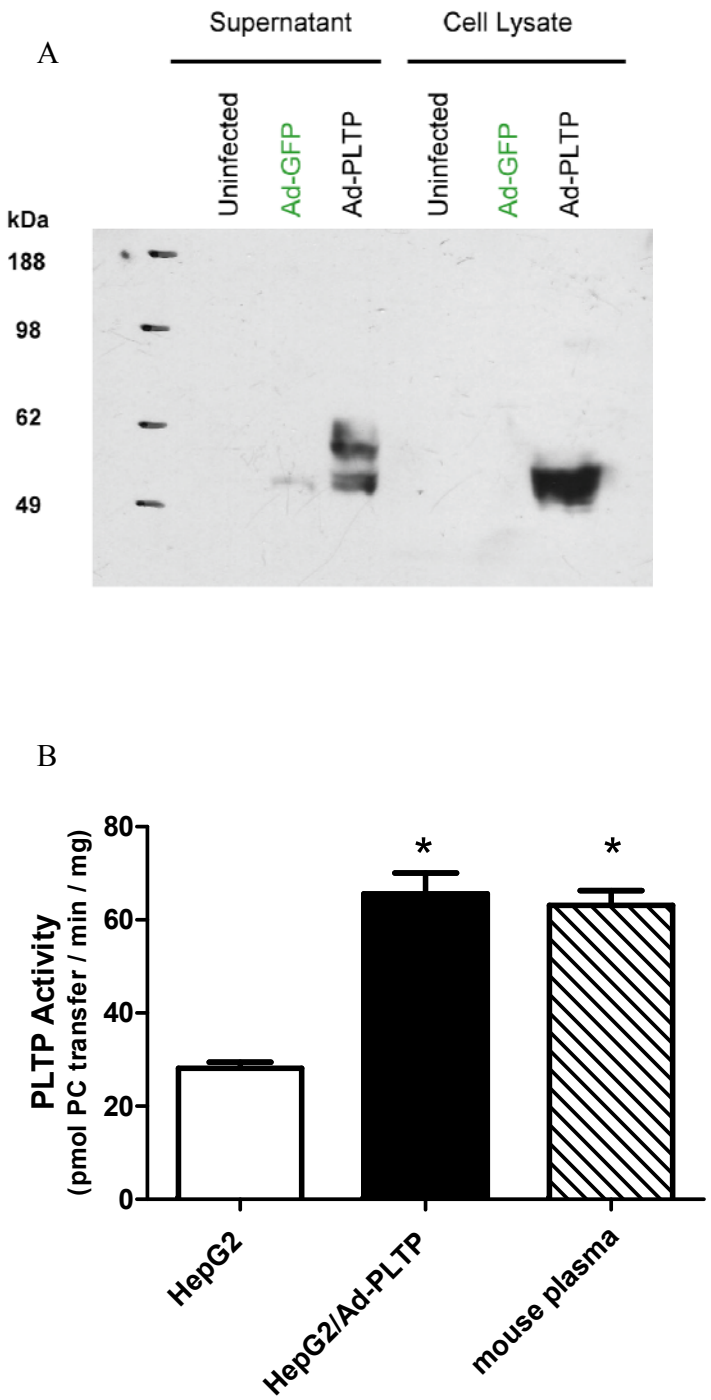


Figure 2. Infection of hepatocellular carcinoma HepG2 cells with recombinant adenovirus encoding human PLTP protein. (A) HepG2 cells were infected with Ad-

PLTP virus. Forty-eight hours later a cell extract was prepared. Cell extracts from HepG2 cells infected with Ad-GFP were used as a control. Fifty micrograms of protein was loaded onto each lane for 12% SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis with polyclonal rabbit anti-human PLTP antibody as described under "Materials and methods". (B) Conditioned media from uninfected cells or cells infected with Ad-PLTP for 48 h were used to determine the activity of secreted PLTP. To measure PLTP catalytic activity, we incubated fluorescent phospholipid that is in the quenched state when associated with the donor together with acceptor particles and cell culture supernatant. Results reflect the mean ± S.E.M. * indicates p value less than 0.05

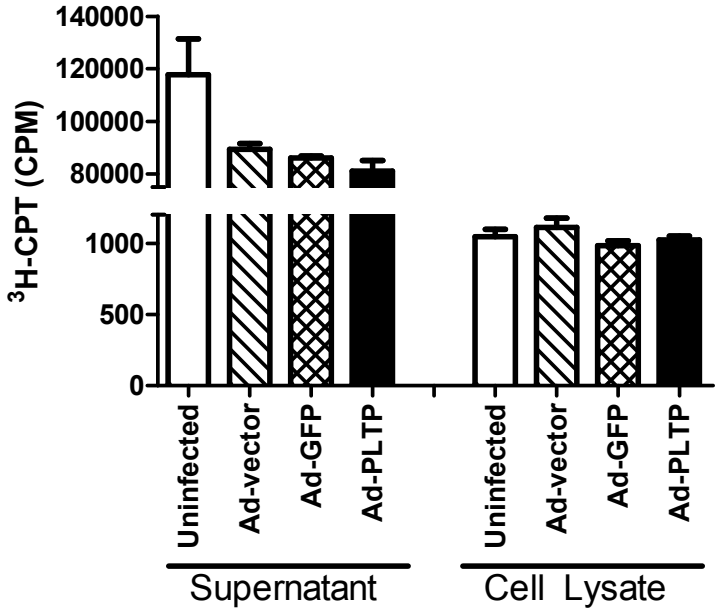


Figure 3. Camptothecin Transport in HepG2 overexpressing human PLTP. Human hepatoma HepG2 cells were transduced with Ad-empty vector, -GFP or -PLTP as described in 'Methods' and camptothecin (CPT) efflux in the cells and medium was assessed. Open bars, uninfected cells; crossed bars, cells infected with empty vector; crosshatched bars, GFP-infected; filled bars, PLTP-infected cells. Result reflects mean ± S.E.M. * indicates p value less than 0.05, while NS indicates that there is no significant difference.

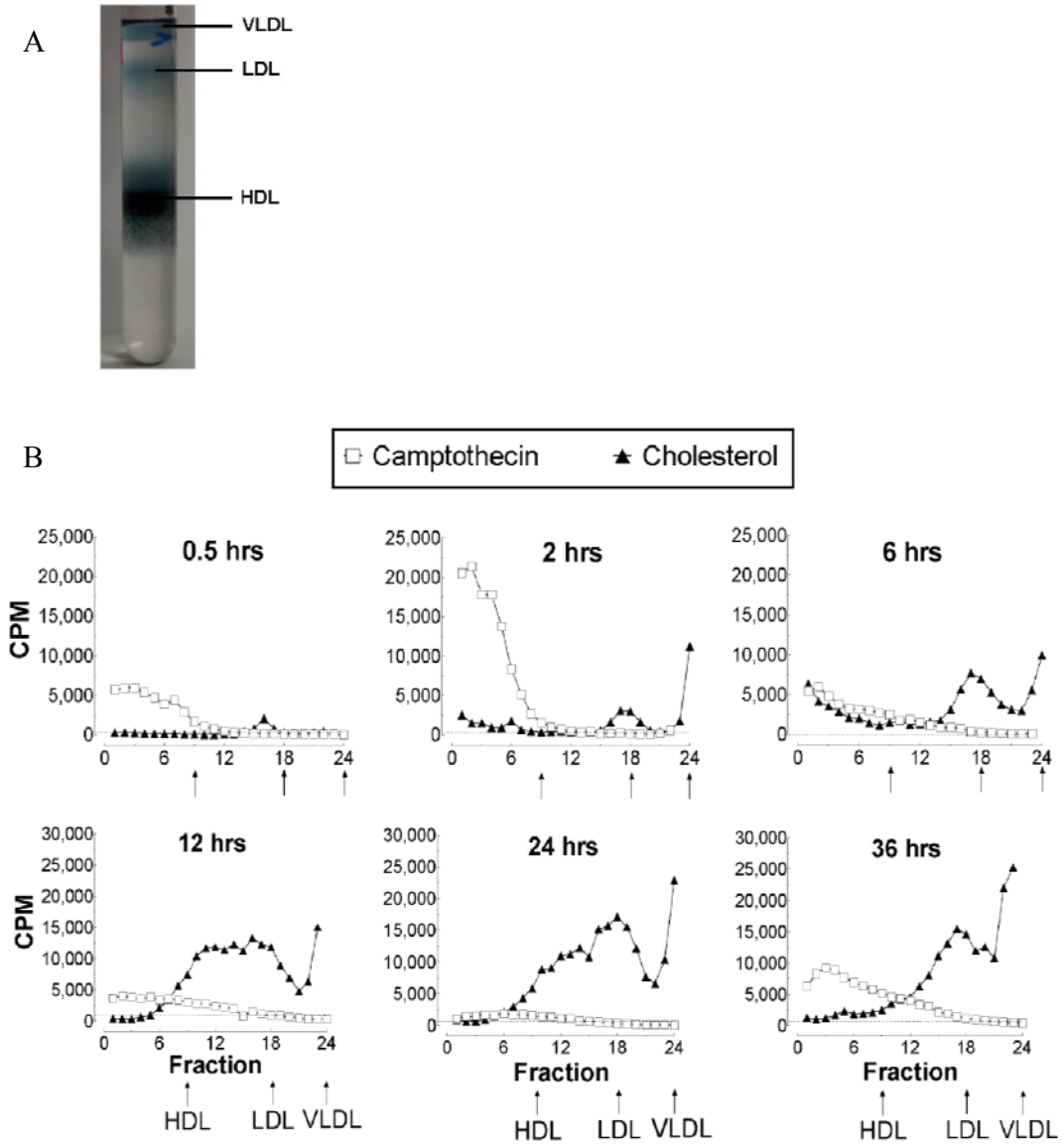


Figure 4. Cholesterol and camptothecin distribution in plasma lipoproteins from C57/B6J mice. (A) The relative positions of VLDL, LDL and HDL fractions after separated by ultracentrifugation. (B) The plasma lipoproteins distribution in mice injected with radioactive cholesterol (closed triangles) and camptothecin (open squares). The elution positions of VLDL, LDL, HDL lipoproteins are indicated.

DISCUSSION

Drug resistance in cancer is a complex process involving the genetic alteration of multiple cellular factors and aberrantly perturbed biological pathways during tumorigenesis. Therefore, understanding how drug resistance against cytotoxic chemotherapeutic agents develops in cancer is of paramount importance in improving cancer treatment and outcome. In this report, we successfully overexpressed human PLTP using an adenoviral expression system in HepG2 and demonstrated that the protein is functionally active in mediating the transfer of the fluorescent labeled phospholipid molecule from donor to acceptor (figure 2). In addition to transferring neutral lipids and phospholipid, PLTP has been recently shown to transport vitamin E (Jiang, et al., 2002). This seminal study together with our previous findings that camptothein induced PLTP mRNA and protein expression, and increases HDL-cholesterol in C57/B6J mice led us to postulate that PLTP might increase the transfer of other small molecules and drugs such as camptothecin from the peripheral tissues into HDL, which is normally returned to the liver for metabolism by reverse cholesterol transport process. However, both our *in vitro* and *in vivo* results nullified our hypothesis. Our *in vitro* studies showed that human PLTP overexpression in HepG2 cells do not increase camptothecin efflux (figure 3). While in our *in vivo* studies, mouse plasma PLTP, which has markedly high PLTP activity, did transport cholesterol (positive control) into HDL, LDL or VLDL, but failed to shuttle camptothecin into lipoprotein particles (figure 4).

These observations pose the possibility that the induction of PLTP gene expression by camptothecin is merely the result of bystander effect following drug exposure, which activates the expression of hundreds genes (Guo, et al., 2006), and some of which may have either no physiological consequences, such as PLTP, or whose functional impacts remain to be identified. However, these studies are not absolute and do not rule out that PLTP may indeed facilitate the transfer of other small molecules into lipoproteins. However, it is clear that PLTP neither shuttles camptothecin into lipoproteins nor aids in its cellular efflux. Additionally, PLTP may have failed to transport camptothecin because lack of an adequate stoichiometric ratio, incompatibility of camptothecin to dock in the PLTP binding pockets or unfeasibly high activation energy required for the transfer reaction to occur. While it may be possible that water-soluble camptothecin derivatives such as topotecan, which is also a more potent inducer of PLTP mRNA and protein expression, maybe transported by PLTP, there was, however, no commercially radiolabeled topotecan was available at the time of the study. Undoubtedly, as the role of PLTP in cancer becomes clearer, we may learn about its role in drug transport and cholesterol metabolism.

Previously we showed that the water-soluble camptothecin derivative topotecan unexpectedly raised serum cholesterol and triglyceride levels, and a severely depleted serum HDL in mice treated with high dose of topotecan. The abrupt rise in serum triglyceride is associated with the onset of acute pancreatitis (Cappell, 2008). Though under-reported and under-appreciated, drug-induced acute pancreatitis are commonly encountered in drug therapy (Eland, van Puijenbroek, Sturkenboom, Wilson, & Stricker,

1999). The etiologic cause of most drug-induced acute pancreatitis and atherosclerosis may be associated with hyperlipidemia, resulting from increased total serum triglyceride and cholesterol levels (Yadav & Pitchumoni, 2003). The mechanisms of drug-induced hyperlipidemia are unknown. Increase in PLTP activity has been linked to hypertriglyceridemia (Jonkers, et al., 2003; Kaser, et al., 2004), which may trigger the onset of acute pancreatitis (Miller, 2000; Yadav & Pitchumoni, 2003). It is noteworthy that acute pancreatitis has been reported in cancer patients treated with topoisomerase I inhibitors such as irinotecan (Govindan, Read, Faust, & Mc Leod, 2003a) and exatecan (De Jager, et al., 2000), as well as other chemotherapeutic agents including Lasparaginase (Jain, Naithani, Kapoor, & Nath, 2009; Parsons, et al., 1997), tamoxifen (Alagozlu, Cindoruk, & Unal, 2006), interferon α (Wong, Jakowatz, & Taheri, 2004), and capecitabine (Koutras, Habeos, Vagenakis, & Kalofonos, 2006). These observations, together with our results, suggest that hypertriglyceridemia induced acute pancreatitis may be associated with the increase in PLTP, thus offering the possibility that PLTP may serve as a biomarker for camptothecin and other drug-induced hypertriglyceridemia and acute pancreatitis.

In summary, our results here showed that though camtothecin induced PLTP expression may have no functional consequence in drug resistance, but may be associated with drug-induced hyperlipidemia and the onset of acute pancreatitis. Monitoring changes in serum PLTP levels or activity, therefore, may serve as an important biomarker for drug-induced hyperlipidemia and the development of acute pancreatitis. How

increased PLTP expression causes hyperlipidemia (hypercholesterolemia and hypertriglyceridemia) and lowers HDL levels needs to be further investigated.

CHAPTER II. Characterization of the anti-obesity and anti-adipogenic effects of the limonoid prieurianin.

ABSTRACT

The sharp rise in the number of overweight and obese people in the last decade has become one of the most serious public health risks worldwide. Currently approved therapies for obesity exhibit modest efficacy and limiting side effects. We show here that prieurianin, a limonoid, causes weight loss by reducing energy intake in morbidly obese mice and in mice on high-calorie diet. Prieurianin is also anti-adipogenic by inhibiting the proliferation and differentiation of preadipocytes into adipocytes, and induces either dedifferentiation or delipidation of mature adipocytes. Gene expression profiling showed that prieurianin suppresses the expression of a number of genes involved in fat metabolism, and inhibits the transcriptional activity of the adipogenesis master regulators including the CCAAT/enhancer binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor gamma (PPARγ). The effects of prieurianin are reminiscent of proinflammatory cytokines tumor necrosis factor alpha (TNFα) that regulates appetite and adipogenesis.

INTRODUCTION

The alarming rate of increase in obesity, largely due to sedentary lifestyle habits coupled with overconsumption of energy-rich foods, has significantly raised the risk and incidence of associated comorbidities such as diabetes, hypertension, cardiovascular and metabolic diseases (Bray & Bellanger, 2006). Despite the wealth of information and understanding that had been gained in the past decade about the hormonal and transcriptional mechanisms that regulate energy metabolism, there are only two drugs currently approved by the FDA for the treatment of obesity, which include orlistat that blocks the absorption of dietary fat (Guerciolini, 1997), and sibutramine, a specific reuptake inhibitor for norepinephrine and serotonin that acts in the central nervous system (CNS) to reduce energy intake (Ryan, et al., 1995). These drugs have limited efficacies and side effects are commonly reported, which are further confounded by diminishing response in long-term treatment (Fernstrom & Choi, 2007; Z. Li, et al., 2005). Moreover, most of the new anti-obesity drug development continues to focus on either central or peripheral acting inhibitors of food intake (Cooke & Bloom, 2006), which likely would encounter the above problems (Pi-Sunyer, Aronne, Heshmati, Devin, & Rosenstock, 2006). There is, therefore, an urgent need to identify breakthrough drugs with paradigm shifting pharmacodynamics for the treatment of obesity.

While studying the pharmacological response of tumor cells to anticancer drugs, we found by DNA microarray a striking induction of the phospholipid transfer protein (PLTP) gene expression by topotecan, a topoisomerase I inhibitor and camptothecin

derivative (Fig. 1). PLTP is critically involved in reverse cholesterol transport. Subsequently, we identified by high throughput screen a natural product small molecule, prieurianin, which transcriptionally activates PLTP gene expression. Prieurianin has been shown to be an insect feeding deterrent by antagonizing 20-hydroxyecdysone in *Drosophila* cells (Koul, Daniewski, Multani, Gumulka, & Singh, 2003; Sarker, et al., 1997). These results prompted us to raise the hypothesis that the anti-feedant effects of prieurianin can be exploited for the treatment of obesity.

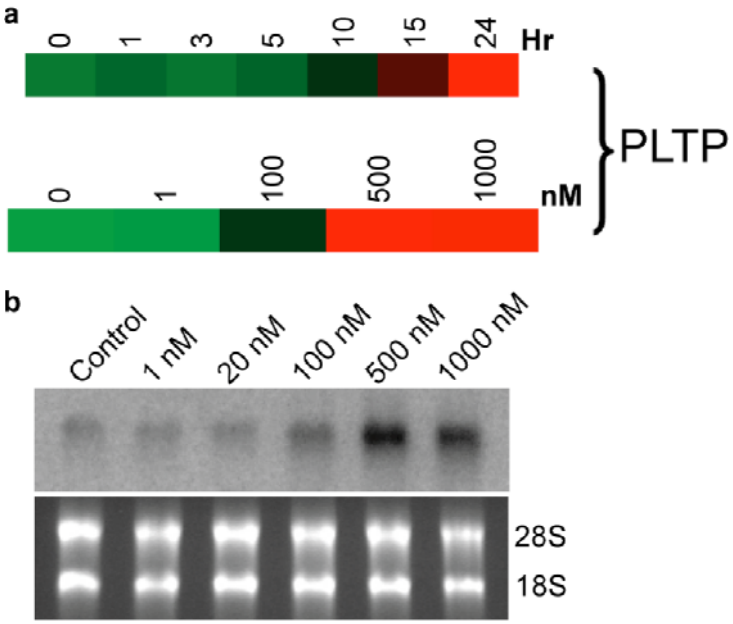


Figure 1. Induction of PLTP gene expression by topotecan. a, Dendrograms of gene expression profile of the pharmacological effects of topotecan in HepG2 cells by DNA microarray in time-course and dose-response studies showing the induction of PLTP by topotecan. **b,** Northern blot analysis confirmed the induction of PLTP gene expression by topotecan dose-dependently.

MATERIALS AND METHODS

High-throughput screen. A transgenic cell line, derived from the human hepatoblastoma HepG2 cells, harboring a luciferase reporter driven by the 1.5kb promoter of the phospholipid transfer protein (PLTP) gene fused to the neomycin selectable marker cassette and stably transfected into HepG2 cells was generated. The transgenic line was used for a high-throughput screen with an all natural product library (MicroSource Discovery Systems, Inc., Gaylordsville, CT). Identified hits were subjected to further validation with the reporter cell line by dose-response and time-course, as well as Northern and Western blots analyses for PLTP expression. Prieurianin (MicroSource Discovery Systems, Inc., Gaylordsville, CT), the most potent hit, was selected for the *in vitro* NIH-3T3/L1 (L1) cell culture adipogenesis assay and *in vivo* efficacy study in mouse models of obesity.

Animals and diets. All animal procedures were approved by the Institutional Animal Care and Use Committee. For all *in vivo* efficacy studies, prieurianin was given intraperitoneally as a mixture in Cremophor to the animals and vehicle-treated controls received equivolume injections of 0.5% of DMSO in Cremophor. Eight to nine weeks old genetically obese *ob/ob* and *db/db* mice (The Jackson Laboratory) were acclimatized for at least one week on a 12-hour light/dark cycle at 68-72^{o}F before the start of experiments. For DIO studies, C57BL/6 and *Cc1*$^{-/-}$ male mice were fed a high-fat diet until their mean body weight reached approximately 30 g, and remained on the high-fat diet for the duration of the study. Mice had access to the 45% kcal diet *ad libitum* during

treatment in all DIO studies. Blood glucose and insulin levels were measured and adipose tissues were harvested for weighing at the end of the experiments. Food intake and body weight were measured every two days for the duration of the study. Mice were euthanized by CO_2 asphyxiation according to AAALAC guidelines. The *Cc1*$^{-/-}$ mice treated with 5 mg kg^{-1} prieurianin showed dramatic decrease in food intake and body weight loss a week following treatment, and pronounce reduced physical activity. All the animals in this group were euthanized at the end of one week of treatment. However, postmortem necropsy showed no organ toxicity in the heart, kidney and liver.

Cell culture and adipocyte differentiation. NIH 3T3-L1 (L1) (American Type Culture Collection) and OP9 stromal (a gift of Dr. Perry Bickel, University of Texas Health Science Center, Houston, TX) cells were cultured at 37°C with 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Invitrogen), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 μg mL^{-1} streptomycin sulfate, and 100 U mL^{-1} penicillin. To assess preadipocytes proliferation, cells were plated in 12 well dishes and then treated with various concentrations of prieurianin. Cell growth was measured daily on a Coulter cell counter. To differentiate L1 cells into adipocytes, cells were incubated with 250 nM dexamethasone, 450 μM 3-isobutyl-1-methylxanthine, and 167 nM insulin for 2 days, followed by 167 nM insulin for an additional 3 days. For OP9 cells, differentiation was initiated with a serum replacement medium composed of MEM-a with 15% KnockOut SR (Invitrogen, Carlsbad, CA), 100 μg mL^{-1} streptomycin sulfate, and 100 U ml^{-1} penicillin, for 2 days and then replenished in the propagation medium as above. Nile Red

(Sigma) staining following differentiation was performed as described (Gonzales & Orlando, 2007) by adding a 1 mg mL^{-1} stock solution to cultured cells to a final concentration of 5 μg mL^{-1} and then visualized under fluorescence microscope. To determine the effect of prieurianin on dedifferentiation/lipolysis, preadipocytes were differentiated into adipocytes as above and further cultured for 5-6 days, and then treated with or without drug for an additional 5-6 days before Nile Red staining.

Microarray analysis. L1 cells were differentiated in the presence of prieurianin (2 μM) in a time course analysis from 0, 0.25, 0.5, 1, 2, 3, 5, 9, 12, 15, 24, 36, 48, 96, 144, 192, 240, to 288 hours, and compared to untreated and vehicle-treated controls. Total RNA was purified at the indicated time using RNeasy Mini kit (Qiagen) and then labeled and hybridized to the Mouse OneArray, a whole genome array, (Phalanx Biotech Group, Palo Alto, CA) and analyzed as previously described (Zheng, et al., 2002). Raw microarray data is available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih. gov/geo/) under series accession no. GSE15018).

Glucose, insulin and adipose tissues. Fed glucose and insulin levels were measured from blood samples taken at the end of the treatment period. Glucose was measured using CardioChek meter (PTS, Indianapolis, IL). Insulin levels were measured in plasma using an ELISA kit (Crystal Chem Inc., Downers Grove, IL). Post-mortem necropsy analysis of two fat depots, subcutaneous and visceral fat, were performed by weighing the dissected tissues.

RT-PCR. The RNA from 0, 1, and 288 hr time points from the microarray analysis was selected for amplification using Superscript III (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The reverse-transcription reaction was carried out at 50ºC for 30 minutes, followed by 25 cycles of 95ºC for 30 seconds, 58ºC for 30 seconds, 72ºC for 60 seconds, and a final extension at 72ºC for 10 minutes. Primer sequences were as follows (5' to 3', sense, antisense):

Lipe (CTCCATTGACTGTGACATCTCG, TCATGGACCCTCTTCTACCAC),

Hsd11b1 (AACCACATCACTCAGACCTC, TGATCTTCCTTCCTGGGTTC),

Cd36 (TTTGTTCTTCCAGCCAATGC, GCAACAAACATCACCACTCC),

Erg1 (AATCCCAGCTCATCAAACCCAG, GGGATGGGTAAGAAGAGAGTGAAG),

Fabp4 (ATGTGTGATGCCTTTGTGGGAAC, CTCTTGTGGAAGTCACGCCT),

Fasn (CCTGCTGGACGCCCTTTTTGA, CTCCCGAATGTGCTTGGCTTGGTA),

Pparg2 (ACTGCCTATGAGCACTTCAC, CAATCGGATGGTTCTTCGGA),

Cebpa (ACCACGACTTCCTCTCCGAC, ACAAGTTCCGCAGGGTGCTG),

Ppargc1b (GAGGAGGAGGAAGAAGAAGAAG, TTGGCTTGTATGGAGGTGTG),

β-Actin (GAGACCTTCAACACCCCAGC, CACGGAGTACTTGCGCTCAG).

Reporter assay. Human TNFα construct (gift from Dr. Sonia Najjar, University of Toledo – Health Science Campus, Toledo, OH) was PCR cloned into pGEX-6P-2 vector (Amersham Biosciences, Piscataway, NJ, # 27-4598-01) using BamHI and EcoRI linkers, expressed in BL21-CodonPlus (DE3)-RIPL *E. coli* competent cells (*S*tratagene, La Jolla, CA, # 230280), and purified by affinity chromatography using Glutathione Sepharose™ 4 Fast Flow (Amersham Biosciences, Piscataway, NJ, # 17-0618-02) and PreScission

Protease (Amersham Biosciences, Piscataway, NJ , # 27-0843-01). Oligonucleotides containing three CEBP- and PPARγ -response elements in tandem followed by TATA box were subcloned into the pGL3-Luc reporter expression plasmid using XhoI and HindIII restriction sites.

CEBP-response element (CEBPRE):

5'-ATTG**CGCAAT**ATTG**CGCAAT**ATTG**CGCAAT**AGATCTGGGTA**TATAA**TGGAAGCT-3'

PPARγ -response elements (PPRE):

5'-TGAAACT**AGGGTAAAGTTCA**TGAAACT**AGGGTAAAGTTCA**TGAAACT**AGGGTAAAGTTCA**AGATC TGGGTA**TATAA**TGGAAGCT-3'

The expression plasmids C/EBPα–pEMBL19, C/EBPβ-pCMV-Sport6, and PPARγ-pBabe were gifts from Drs. Manohar Ratnam, James Wiley and Ivana de la Serna. respectively. All constructs were sequence-verified using a forward primer from gene and reverse vector specific primer by Eurofins MWG Operon. Reporter gene assays were performed in the melanoma Mel501 cells. In a typical experiment, 250 ng of reporter plasmids were mixed with 250 ng of expression constructs for transcription factors either in the presence or absence of prieurianin, and then transfected into Mel501 cells using LTX (Invitrogen) in 24-well plate. Equal amounts of DNA were used for all transfection by adding the appropriate amount of salmon sperm DNA and the Renilla luciferase reporter, pRL-CMV vector (Promega, # E2261) as internal control. Relative luciferase activities were determined 24 h following transfection and normalized to the *Renilla* luciferase activity. All transfection experiments were performed in triplicates.

Statistical analyses. Statistical analyses were performed using commercially available software package (PRISM, GraphPad Software, Inc., La Jolla, CA) or the data analysis add-in for Microsoft Excel. Either single factor ANOVA or Student's t-tests were used for all analysis. P values of less than or equal to 0.05 were considered statistically significant.

RESULTS

Anorexigenicity

We examined the effects of prieurianin on feeding in four different mouse models of obesity by administering the drug to the morbidly obese leptin-deficient *ob/ob(Halaas, et al., 1995; Y. Zhang, et al., 1994)* and the leptin receptor-deficient *db/db* (H. Chen, et al., 1996; Chua, et al., 1996; Lee, et al., 1996) mice, as well as the diet-induced obese (DIO) insulin resistant *Cc1*$^{-/-}$, with null mutation for *Ceacam*1 gene (DeAngelis, et al., 2008; Leung, et al., 2006), and the C57BL/6 (B6) mice. Prieurianin was given intraperitoneally to 12-14 week-old normal B6 and the *ob/ob* mice (2 or 5 mg kg^{-1}) three times a week for three weeks, and body weight and food intake were measured every three days. We observed a dose-dependent weight loss in *ob/ob* mice after three weeks of treatment with either 2 mg kg^{-1} (-7.4%) or 5 mg kg^{-1} (-9.5%) prieurianin (Fig. 2b). Food intake was decreased by 14.3% and 56% in the 2 and 5 mg kg^{-1} treated group, respectively, relative to the untreated or vehicle-treated controls (Table 1). In contrast, a modest weight loss (1.5%) (Fig. 2a) and hypophagia (18%) were observed in both 2 and 5 mg kg^{-1} prieurianin-treated B6 mice compared to untreated and vehicle-treated controls (Table 1). Further, *ob/ob* mice are hyperglycemic compared to B6 mice (Fig. 2e) and prieurianin treatment normalized fed blood glucose level to that of B6 (Fig. 2e). Prieurianin also significantly lowered insulin level in *ob/ob* mice compared to controls and B6 mice (Fig. 2f). In the leptin receptor-deficient *db/db* mice, no weight loss was observed with prieurianin treatment, but weight gain was attenuated by 50% while untreated or vehicle-treated mice continued to gain weight (Fig. 2c).

Table 1. Food Intake in mice measured over 48 hours (g/mouse/48 hrs). * B6 and *ob/ob*, 2 and 5 mg kg^{-1}; and *db/db* and $Cc1^{-/-}$, 3 and 5 mg kg^{-1}. Values shown in table represent average food consumption of ten mice ± SEM

Treatment	B6	*ob/ob*	*db/db*	*Cc1*$^{-/-}$
Untreated	7.4 ± 0.9	10.0 ± 1.8	13.7 ± 2.2	6.0 ± 1.2
Vehicle	7.9 ± 1.3	10.3 ± 1.7	11.5 ± 1.8	5.7 ± 0.9
2 or 3 mg kg^{-1}*	6.3 ± 1.0	8.7 ± 0.9	11.3 ± 1.5	1.6 ± 0.3
5 mg kg^{-1}	6.2 ± 0.8	4.5 ± 0.5	10.6 ± 1.8	1.0 ± 0.3

DIO model has been widely used to investigate the underlying mechanisms of obesity in human (Collins, Martin, Surwit, & Robidoux, 2004), and we examined the effects of prieurianin in the B6 and the insulin resistant *Cc1*$^{-/-}$ mice. We fed *Cc1*$^{-/-}$ mice a high calorie diet (45% kcal) for 4 weeks for fattening. Daily treatment with prieurianin (3 or 5 mg kg^{-1}) for 3 weeks resulted in approximately 20-26% body weight loss (Fig. 2d) and >50% reduction in food intake (Table 1), compared to untreated and vehicle-treated controls. Fed glucose and insulin levels were also markedly reduced at the end of treatment period (Fig. 2g, h).

DIO B6 mice were given high fat diet for 15 weeks and then treated daily with either 1 or 3 mg kg^{-1} prieurianin for three weeks. Prieurianin caused a dose-dependent weight loss (Fig. 3a) that was accompanied by 70-80% decrease in food consumption,

which eventually returned to normal levels by the end of the treatment period (Fig. 3b). Notably, a gradual regain in body weight was observed following the first week of treatment (Fig. 3a), suggesting the onset of drug-induced tolerance.

One of the major unresolved problems for the pharmacotherapy of obesity is the diminishing efficacy of drugs with chronic treatment (Fernstrom & Choi, 2007). To assess whether drug-induced tolerance can be circumvented by "drug holidays", mice were treated with either 3 or 5 mg kg^{-1} prieurianin for 5 or 3 days, respectively, followed by 5 days of drug withdrawal, and repeating this regimen for 4 cycles (Fig. 4). DIO B6 mice from the experiments in Fig. 3a were maintained on the high calorie diet for four additional weeks without treatment and then subjected to the drug holiday treatment cycle. This treatment protocol, at either 3 or 5 mg kg^{-1} prieurianin, produced a greater response than daily treatment, with up to 20% weight loss at either dosage, and no observable weight regain at the end of the treatment cycle (Fig. 3c). Food consumption decreased precipitously initially, but gradually returned to approximately 60-70% of normal feeding and maintained at that level for the duration of the treatment cycle (Fig. 3d). Fed glucose and insulin levels also showed a trend of decrease at the end of treatment period (Fig. 3e, f).

Anti-adipogenesis

Postmortem necropsy showed >50% decrease in subcutaneous and visceral fat depots in prieurianin-treated *ob/ob* and DIO B6 mice, compared to untreated and vehicle-treated controls (Fig. 5a), while no significant effects was observed in B6 mice on normal chow

(Fig. 5b). The ability of prieurianin to suppress appetite and reduce fat mass is reminiscent of the cachexic effects of some cytokines such as tumor necrosis factor α(TNFα) (Spiegelman & Hotamisligil, 1993) and macrophage inhibitory cytokine-1 (MIC-1) (Johnen, et al., 2007). Moreover, differentiation of preadipocytes into mature adipocytes is completely inhibited by TNFα, IL-1β, IFNγ or TGFβ1 (Bortell, Owen, Ignotz, Stein, & Stein, 1994; Simons, van den Pangaart, van Roomen, Aerts, & Boon, 2005). We examined the effects of prieurianin in adipogenesis using either the cultured NIH-3T3/L1 (L1) preadipocytes (Green & Meuth, 1974) or the OP9 mouse stromal cells (Wolins, et al., 2006), that are capable of differentiating into adipocytes. Prieurianin inhibited the proliferation of 3T3-L1 cells in a dose-dependent manner (Fig. 6a) and differentiation of OP9 stromal cells into adipocytes, as evident from the reduced Oil Red O stained lipid-accumulating adipocytes relative to the untreated/undifferentiated and the differentiated controls (Fig. 3c). TNFα also inhibited OP9 cells differentiation (Fig. 6c). These effects of prieurianin were recapitulated in the L1 preadipocytes (Fig. 7a). The decrease in adipocytes was not a result of prieurianin-induced apoptosis, as indicated by the lack of annexin V binding to phosphatidylserine, in contrast to stromal cells treated with doxorubicin, a cytotoxic drug that causes apoptosis (Fig. 6b). Similarly, apoptosis was not observed in prieurianin-treated L1 preadipocytes (Fig. 7b). Prieurianin also inhibited the postconfluent mitosis and clonal expansion of the L1 preadipocytes, evident from the reduced ^{3}[H]-thymidine uptake following differentiation induction (Fig. 7c). In contrast, the appetite suppressant, sibutramine, did not inhibit preadipocytes differentiation into adipocytes (Fig. 8a).

To assess the effects of prieurianin in mature adipocytes, L1 preadipocytes were differentiated into adipocytes followed by treatment with either TNFα or prieurianin. Consistent with previous report (Ryden & Arner, 2007), TNFα induced rapid lipolysis, resulting in significant loss of Nile Red stained adipocytes (Fig. 6d). Though less potent, prieurianin also reduced Nile Red positive adipocytes compared to differentiated control and vehicle-treated cells (Fig. 6d).

Gene expression profiling of adipogenesis

These data demonstrate that prieurianin causes weight loss in obese mice by suppressing appetite, thereby reducing energy intake, and disrupting adipogenesis by inhibiting preadipocytes proliferation and differentiation, and dedifferentiation/delipidation of adipocytes. To determine that prieurianin is anti-adipogenic, we performed whole-genome gene expression profiling to determine how prieurianin disrupted the transcription program of adipogenesis in L1 preadipocytes undergoing differentiation across time. A subset of expression changes was verified by polymerase chain reaction with reverse transcription (RT-PCR) (Fig. 9).

Global gene expression changes following the onset of differentiation exhibited a temporal pattern of early, intermediate and late response clusters (Fig. 10a), suggestive of an ordered and hierarchical transcriptional program that control adipogenesis. Distinct induction and suppression of gene expression by prieurianin were also observed (Fig. 10b). The inhibition of preadipocytes differentiation by prieurianin was accompanied by the suppression of C/EBPα, β, and δ, and PPARγ expression, a core group of

transcriptional regulators of adipogenesis (Tontonoz & Spiegelman, 2008) (Fig. 10c). It is notable that Krox20, previously identified to be necessary for adipogenesis (Z. Chen, Torrens, Anand, Spiegelman, & Friedman, 2005), though induced early transcriptionally during differentiation (Fig. 10c), its expression persisted, however, despite the inhibition of differentiation by prieurianin (Fig. 10c), thus raising question about its role in adipogenesis under these conditions.

Whole-genome RNA mediated interference (RNAi) analysis of all 16,757 genes in *Caenorhabditis elegans* for genes required for normal fat storage has identified 305 genes, whose inactivation resulted in decreased body fat accumulation (Ashrafi, et al., 2003). We examined these genes in the expression profile of prieurianin-treated preadipocytes. The anti-adipogenic characteristic of prieurianin is further supported by its striking inhibition of the expression of a cluster of the mammalian homologues of these fat regulatory genes in *C. elegans* (Fig. 10d). Repression of these fat metabolism genes in preadipocytes are consistent with the inhibition of their differentiation into adipocytes, and also the reduced fat accumulation in prieurianin-treated adipocytes (Fig. 6d), indicated by the downregulation of genes that promote lipolysis including insulin receptor substrate-1 (van Harmelen, et al., 2007), hormone sensitive lipase(Arner & Langin, 2007), and cell death-inducing DFFA-like effector c (Puri, et al., 2007). In addition, prieurianin differentially regulated the expression of Tnfrsf1b, Trf5, Agt (Serazin, Dos Santos, Morot, & Giudicelli, 2004), Tgfb1i1 (Drori, et al., 2005), Ilf2 and Irak2 (J. A. Kim, et al., 2005), genes of the TNFα, TGFβ and interleukin cytokine signalling pathways that also control satiety and adipogenesis.

Induction of NFκB reporter plasmid by prieurianin

The transcription factors C/EBPα, β, and δ, and PPARγ are key regulators of adipogenesis. Since prieurianin disrupts the temporal patterns of gene expression and inhibits preadipocytes differentiation, we wondered if it might pharmacologically repress the transcriptional regulation of adipogenesis mediated by the C/EBPs and PPARγ. Using luciferase reporters transcriptionally driven by either C/EBPα. and β, or PPARγ from their response elements, C/EBPRE and PPRE, respectively, we found prieurianin directly inhibited transactivation from the promoters by these transcription factors (Fig. 11a, b). Sibutramine, in contrast, showed no effect on the promoter activity (Fig. 8b). DNA microarray global expression profile analysis shows that tumor necrosis factor receptor type 2 (TNFR2) (Fig. 12) and TNFR-associated factor 5 (TRAF5) expressions are upregulated with prieurianin treatment. These results suggest that the prieurianin-induced inhibition of adipogenesis may be mediated through the TNFR-NF-κB signaling pathway. Based on these observations, we further speculate that an early transcriptional repressor induced by prieurianin might inhibit the expression of C/EBPs and PPARγ (Fig. 4c) and their transcriptional activity (Fig. 5a, b).

To further investigate the mechanism of action of prieurianin, and we examined if prieuriain signals through the TNFR-NFκB pathway. To do this we transfected SW620 cells with the NFκB reporter plasmid. Our results show that prieurianin dose-dependently transactivated the NFκB reporter plasmid expression (Fig. 13A). Additionally, the addition of IKK® inhibitor (Calbiochem, CA) reduced the prieurianin-induced NFκB

activation (Fig. 13B). In our studies, TNFα is used as positive controls since it is known to activate the NFκB signaling. Bufalin, similar to prieurianin in structure, also activates PLTP promoter activity. However, bufalin neither inhibits 3T3-L1 adipocyte differentiation (data not shown) nor activates NFκB signaling, and serves as a negative control. To confirm our findings that prieurianin activates NFκB, we stimulated HeLa cells for 30 minutes and isolated the cytoplamic and nuclear fractions for immunoblot anaylsis (Figure 14). We showed that both TNFα and prieurianin treatment led to a substantial loss, of detectable IκBα in cytoplasmic extracts. Since IκBα associates with and inhibits NFκB-p65 activation and nuclear translocation, we next immunoblotted for cytoplasmic and nuclear NFκ- p65. As expected, the changes in IκBα levels measured in cytoplasmic cell extracts were correlated with the appearance in nuclear extracts of the NFκB-p65 subunit. Thus, while nuclear NFκB-p65 protein was almost undetectable on Western blots with extracts prepared from unstimulated cells, after prieurianin and TNFα stimulation there were a strong induction within 30 minutes.

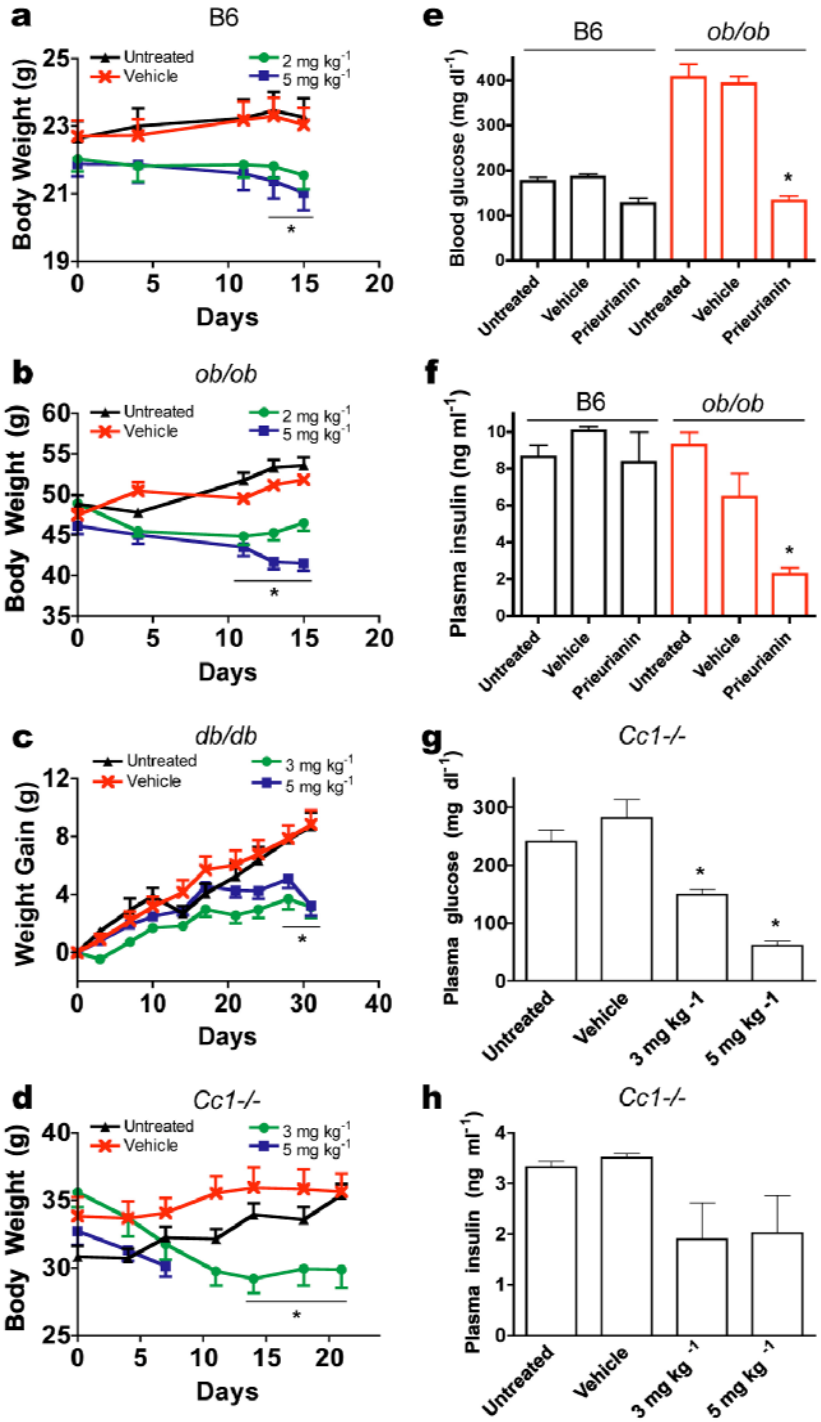


Figure 2. Prieurianin in genetically obese and DIO *Cc1-/-* mice. a-d, Prieurianin causes weight loss. Body weights of B6 (**a**), *ob/ob* (**b**), *db/db* (**c**), and *Cc1-/-* (**d**) mice given either 2, 3 or 5 mgkg-1 of prieurianin for two (**a**,**b**) or three (**c**, **d**) weeks.

Genetically insulin resistant *Cc1-/-* knockout mice were fed high fat diet for fattening for 4 weeks prior to prieurianin treatment. **e-h**, Decreased plasma glucose (**e**, **g**) and insulin (**f**, **h**) levels in B6, *ob/ob*, and *Cc1-/-* mice measured at the end of prieurianin treatment. All studies consisted of ten mice per group. Statistics were conducted as student *t*-test. Asterisk, $P<0.05$ versus untreated and vehicle-treated controls. Error bars indicate s.e.m.

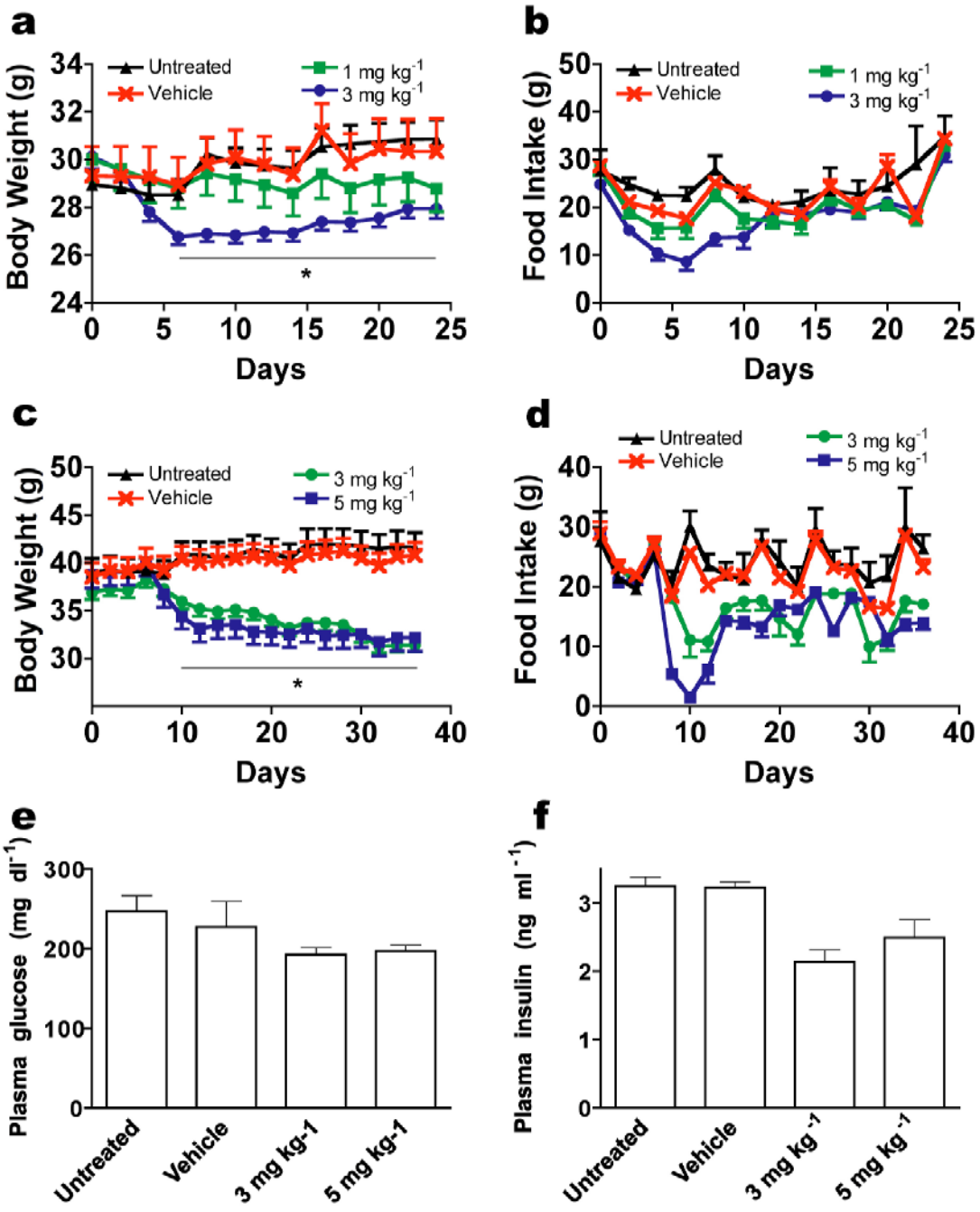


Figure 3. Prieurianin in mouse model of diet-induced obesity. a, b, Prieurianin causes weight loss and reduced food consumption in DIO B6 mice compared to untreated and vehicle-treated controls. **c, d,** A drug holiday treatment protocol with prieurianin

circumvented potential drug-induced tolerance and maintained weight loss and reduced food intake. **e**, **f**, Plasma glucose (**e**) and insulin (**f**) levels measured at the end of prieurianin treatment. All studies consisted of 10 animals per group, except for the 3 (**a, b**) and 5 (**c, d**) mgkg-1 treated group (n=20). Statistics were conducted as student *t*-test. Asterisk, $P<0.05$ versus untreated and vehicle-treated controls. Error bars indicate s.e.m.

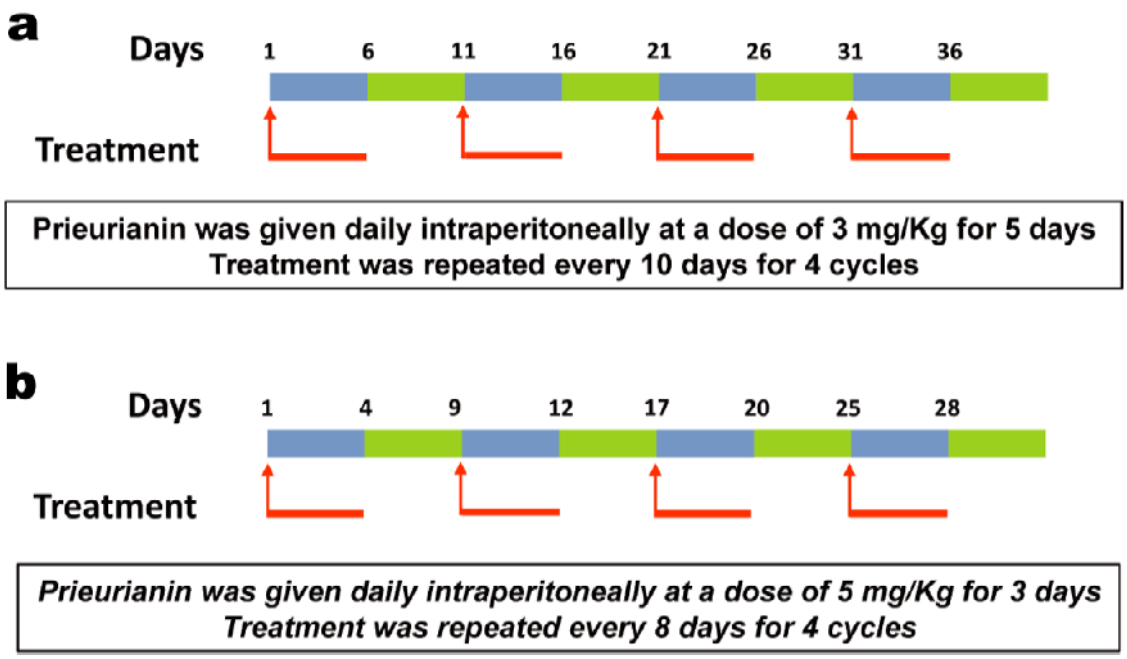


Figure 4. A "drug holiday" treatment protocol for overcoming drug-induced tolerance. a, b, This on-off or cyclical treatment strategy comprised of specified doses of treatment for a defined duration coupled with intermittent drug holiday, can overcome drug-induced tolerance, desensitization, or lack of response in the long-term treatment of obesity.

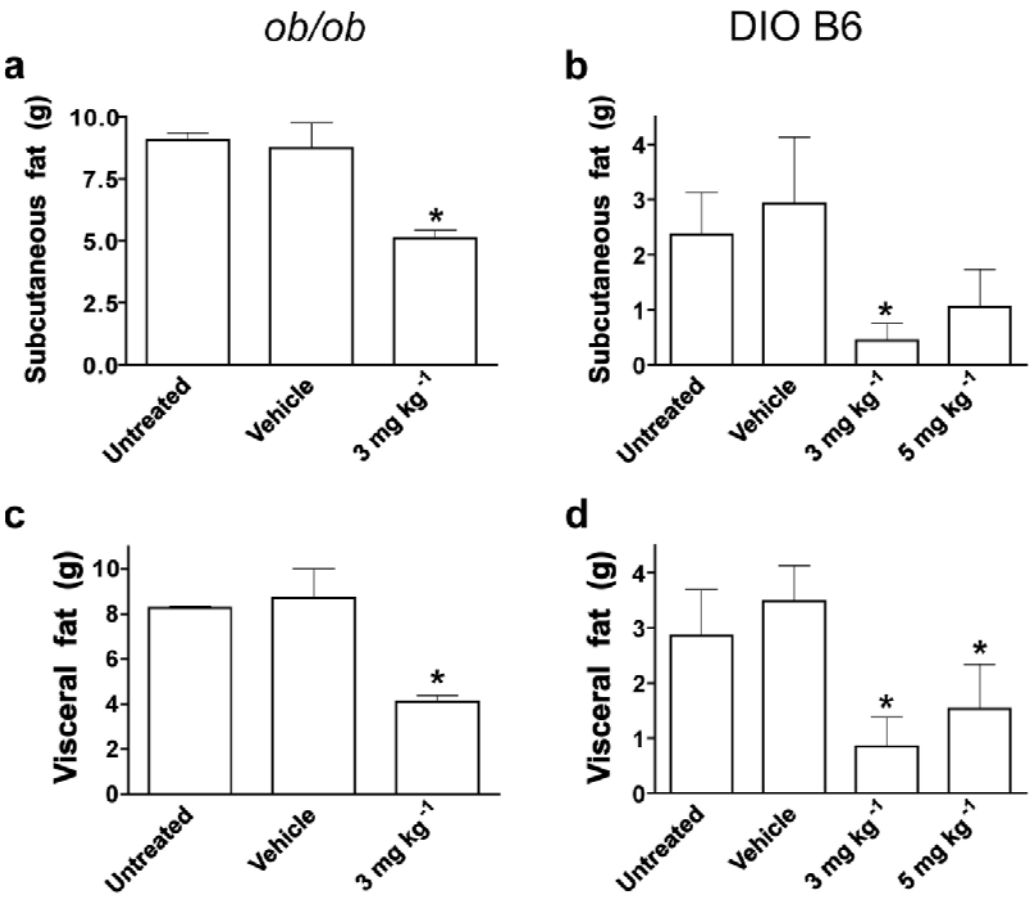


Figure 5. Prieurianin reduces adipose mass in genetically obese ob/ob and high fat diet fed mice. Significant reduction was observed in post-mortem distribution of adipose mass in subcutaneous (**a**, **b**) and visceral (**c**, **d**) compartments of prieurianin treated *ob/ob* (**a**, **c**) and DIO B6 (**b**, **d**) mice compared to untreated and vehicle (DMSO)-treated mice. Asterisk, $P<0.05$ versus untreated controls. Error bars indicate s.e.m.

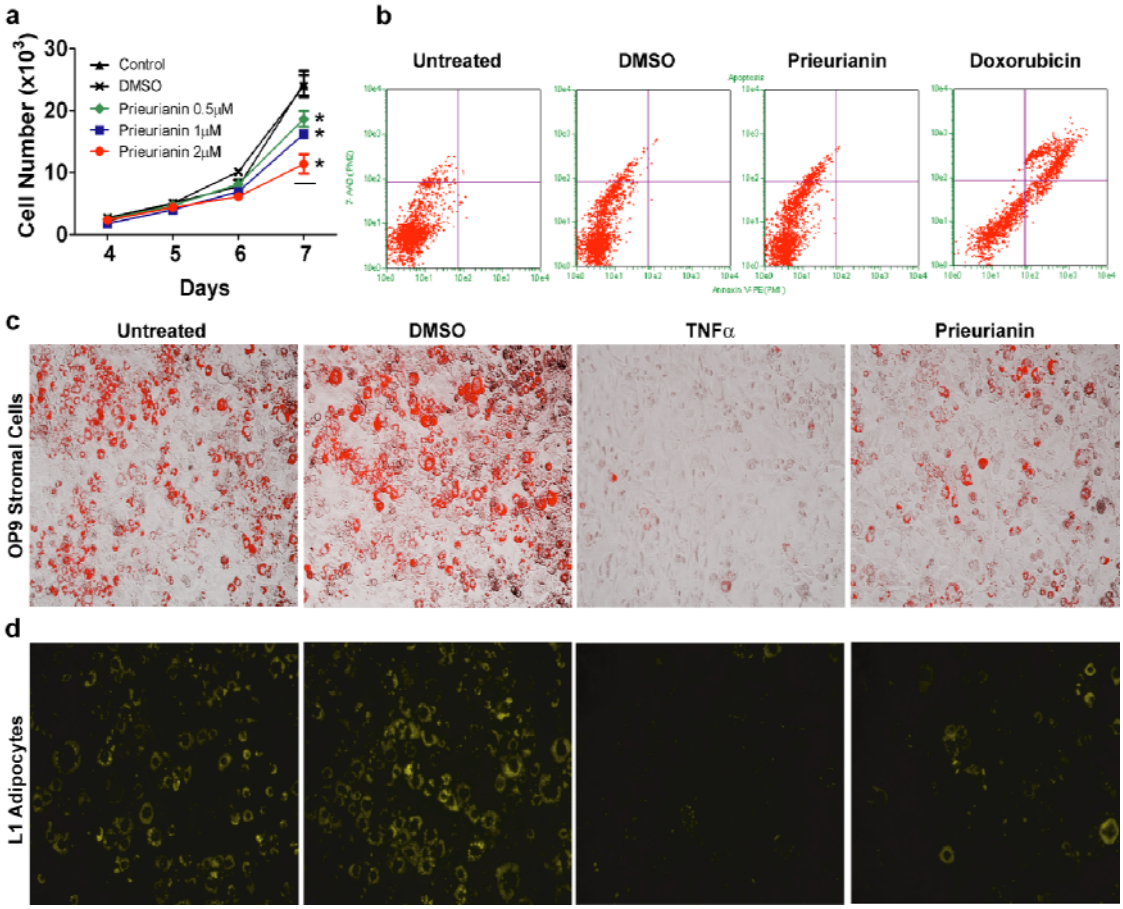


Figure 6. Prieurianin is anti-adipogenic. a, Prieurianin inhibits L1 preadipocytes proliferation. **b,** Prieurianin does not cause apoptosis in L1 cells as measured by annexin V binding to cell surface phosphatidylserine, compared to the cytotoxic drug, doxorubicin. **c,** Prieurianin inhibits the differentiation of OP9 cells into adipocytes, stained with Oil Red O. TNFα, a cytokine and potent inhibitor of differentiation, is used as control. **d,** Prieurianin causes either dedifferentiation or delipidation of differentiated adipocytes, stained with Nile Red, compared to untreated and vehicle-treated adipocytes. TNFα, which causes lipolysis, serves as control.

Statistics were conducted as student *t*-test. Asterisk, $P<0.05$ versus untreated and DMSO-treated controls. Error bars indicate s.e.m.

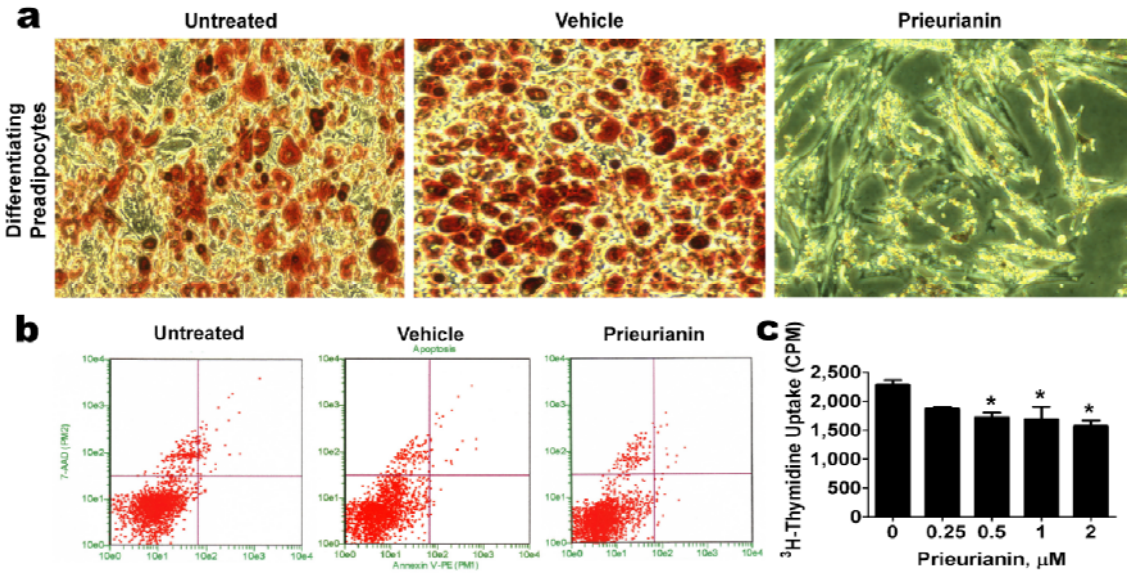


Figure 7. Prieurianin is anti-adipogenic. **a,** Prieurianin inhibits the differentiation of L1 preadipocytes into adipocytes compared to untreated and vehicle-treated controls. **b,** Treatment of preadipocytes undergoing differentiation with prieurianin does not cause apoptosis as measured by the lack of annexin V binding to cell surface phosphatidylserine. **c,** Prieurianin inhibits the postconfluent mitosis and clonal expansion of L1 preadipocytes. Asterisk, $P<0.05$ versus untreated controls. Error bars indicate s.e.m.

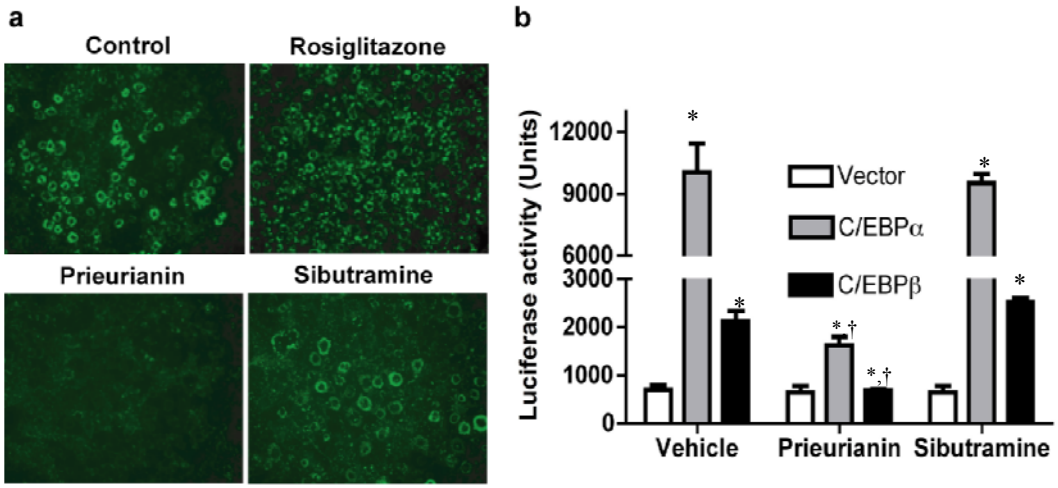


Figure 8. Sibutramine does not inhibit adipogenesis. **a,** In contrast to prieurianin, sibutramine, an appetite suppressant, does not inhibit the differentiation of L1 preadipocytes into adipocytes compared to untreated control. **b,** Sibutramine also did not inhibit the transactivation potential of CEBPα and β from a promoter reporter plasmid containing CEBP-response elements. Error bars indicate s.e.m. * indicates p value less than 0.05, and † indicates p value less than 0.05 compared to respective vehicle treated and C/EBPα- or β-transfected controls

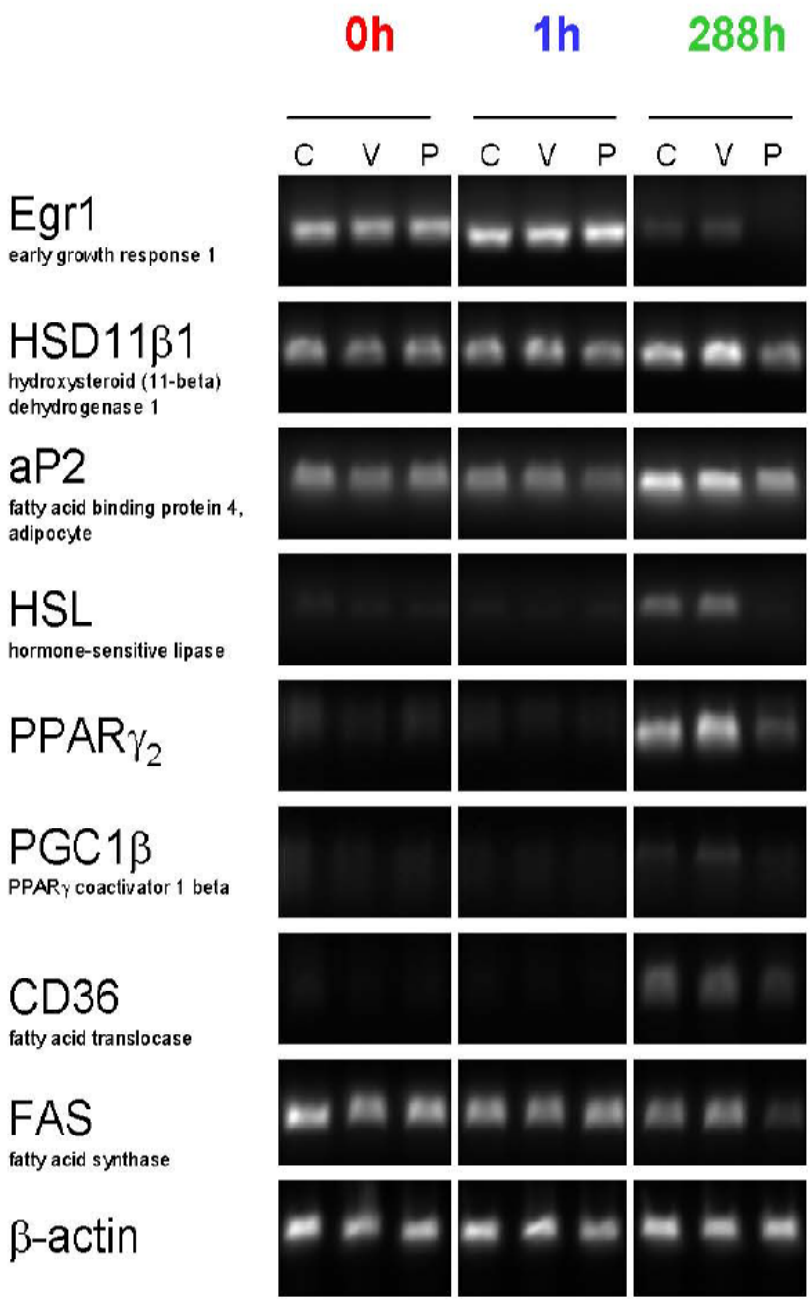


Figure 9. Validation of microarray results by RT-PCR. A subset of genes from the gene expression profiling study is subjected to validation by RT-PCR. The time points of 1 and 288 hrs following drug treatment were compared to the 0 hr control. Levels of β-actin were indistinguishable in all samples.

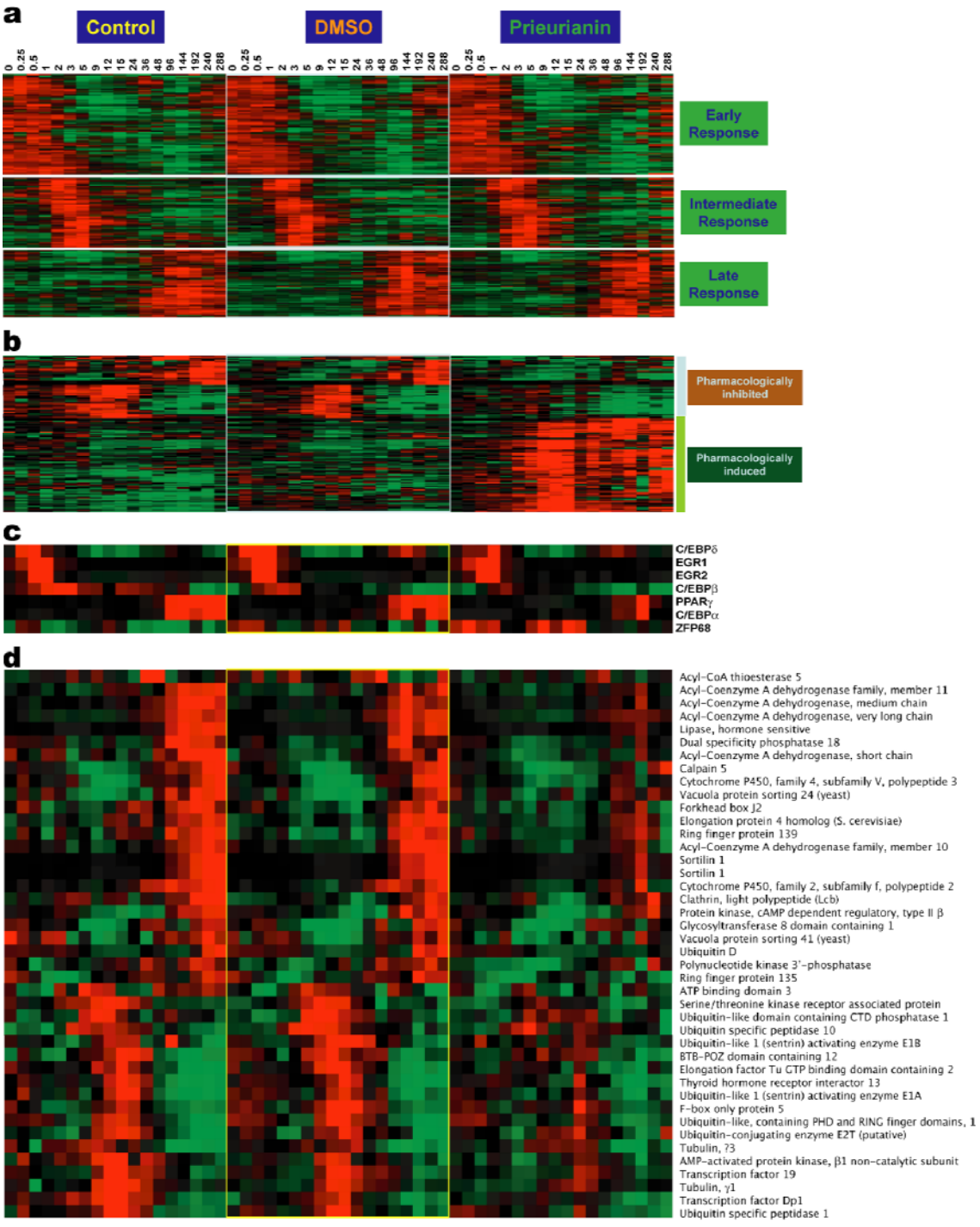


Figure 10. Genome-wide gene expression profiling shows that prieurianin disrupts the transcriptome of adipogenesis. Dendrograms of gene expression changes during

differentiation of L1 preadipocytes showing specific alterations induced by prieurianin compared to DMSO or untreated control. **a**, Distinct temporal changes in gene expression were clustered into early, intermediate and late responses during preadipocytes differentiation into adipocytes. **b**, Specific clusters of genes whose expression were either induced or inhibited by prieurianin. **c**, Attenuated expression of C/EBPα, β, and δ, and PPARγ, transcriptional regulators of adipogenesis, and the induction of Zfp68 expression by prieurianin. EGR1 and 2 expressions were not affected by prieurianin. **d**, Inhibition of fat metabolism genes by prieurianin that correspond to those inactivated by genome-wide RNAi in *C. elegans*, which causes reduced body fat accumulation. The complete data set is available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under series accession

no. GSE15018).

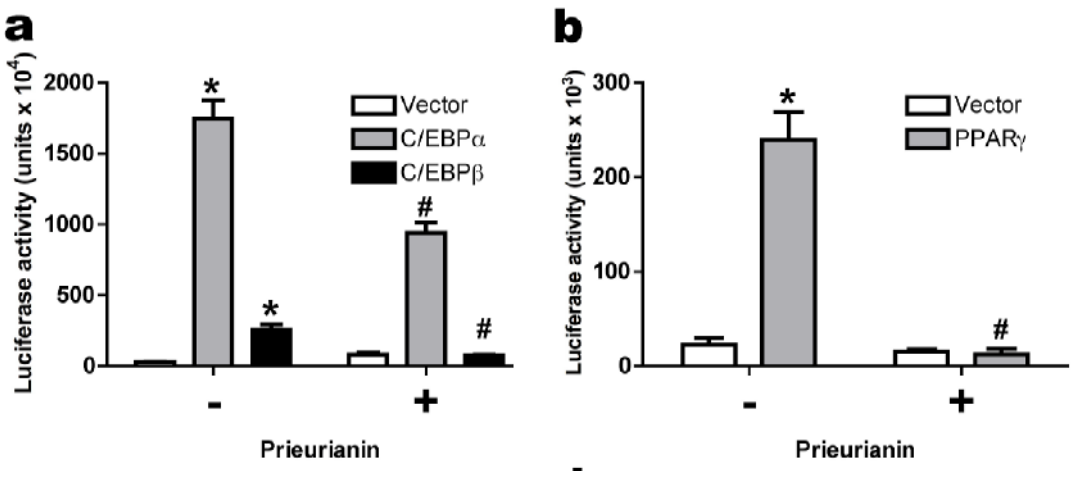


Figure 11. Prieurianin suppress transcriptional activity of C/EBPs and PPARγ. a, b, Prieurianin suppresses the transactivation potential of C/EBPα and β from the C/EBP-response element (**a**) and PPARγ-transactivated PPAR-response element (**b**) luciferase reporters. Statistics were conducted as student *t*-test. Asterisk, P<0.05 versus vector; hash, P<0.05 versus the respective C/EBPα-, β-, or PPARγ-transfected controls. Results are means ± s.e.m., normalized to *Renilla* luciferase activity.

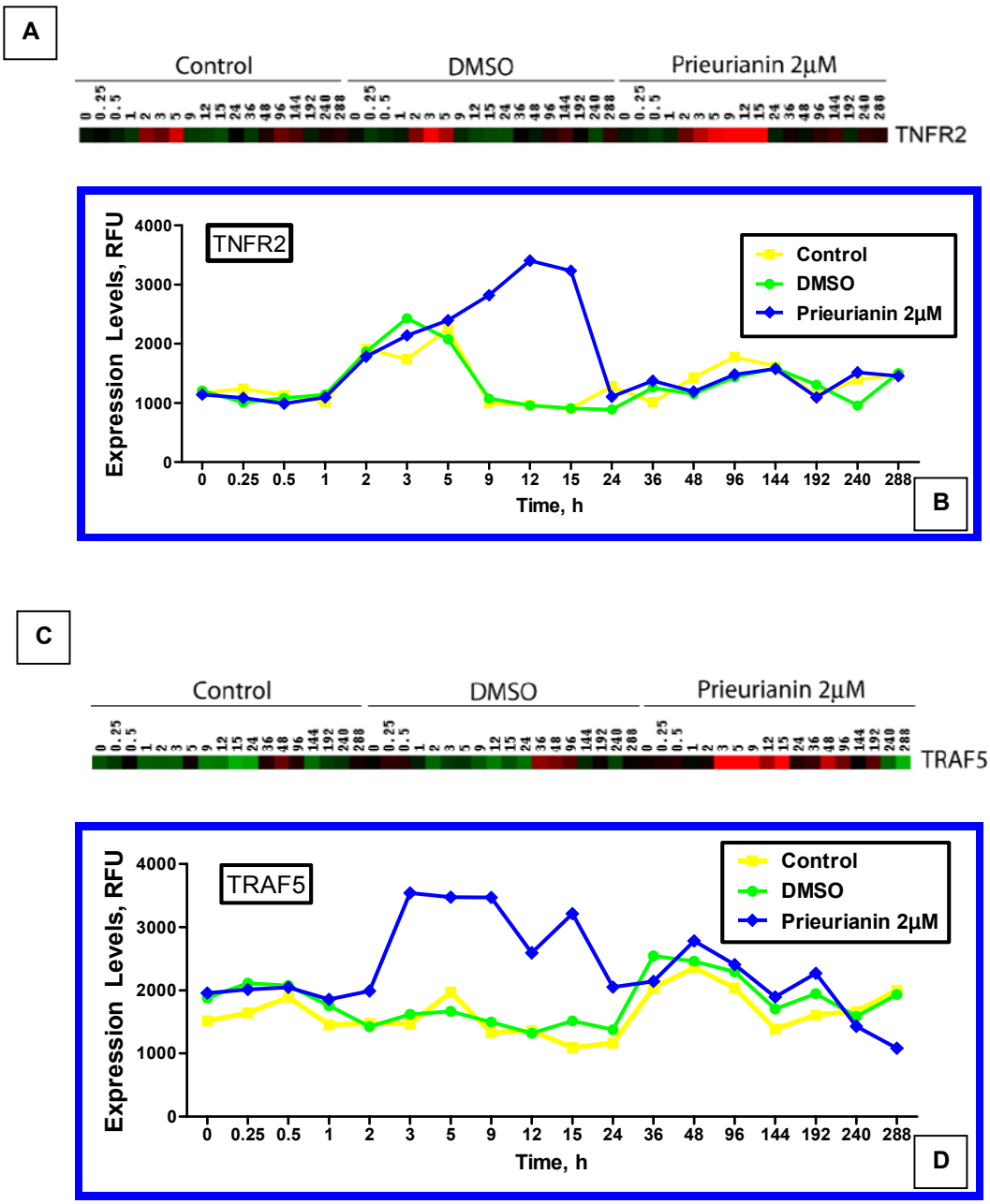
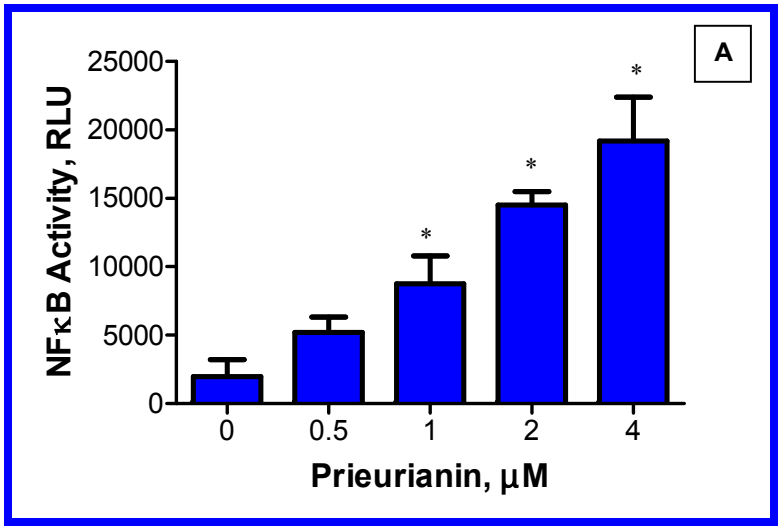


Figure 12. Expression profiling of pharmacological response of 3T3-L1 cells to prieurianin by DNA microarray. 3T3-L1 cells were induced to differentiate to adipocytes in the presence of DMSO (vehicle control) or 2µM prieurianin. RNA was isolated at the indicated times (in hours). Dendrograms of TNFR2 and TRAF5 expression changes are shown (A, C) and a plot of actual values (B, D) respectively.



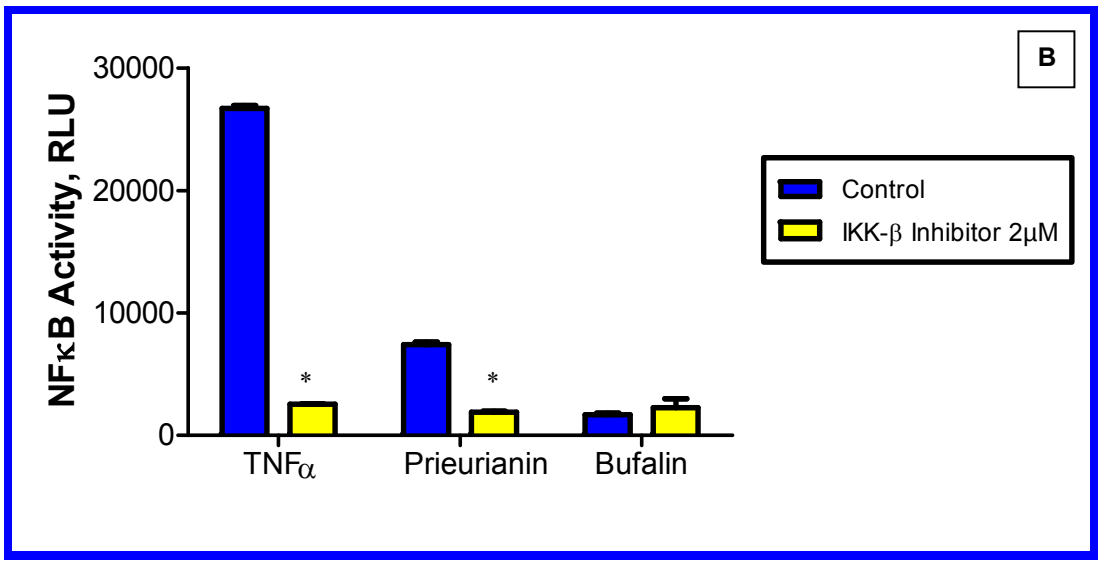


Figure 13. Transactivation of NFκB by prieurianin in SW620. (A) The NFκB consensus sequence fused to a luciferase reporter was transactivated by prieurianin dose-dependently. (B) TNF- and prieurianin-induced NFκB activation is inhibited by IKKβ inhibitor. Results are the mean ± S.E. of three experiments. * indicated p value less than 0.05.

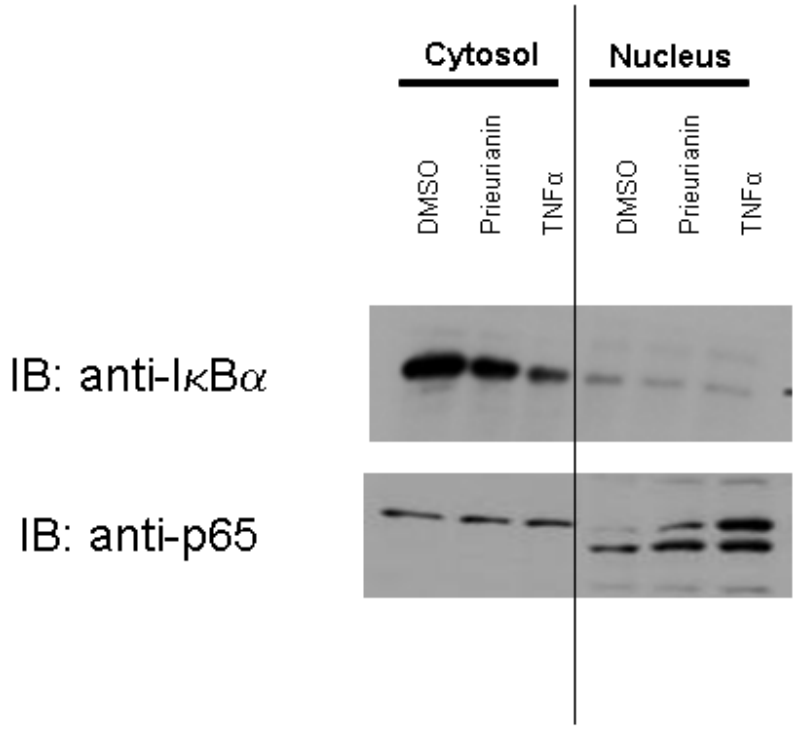


Figure 14. Prieurianin induces p65 nuclear translocation and IκBα degradation in HeLa. Prieurianin treatment, like TNFα, led to a substantial loss, of detectable IκBα in cytoplasmic extracts by Western blotting within 30 minutes. The changes in IκBα levels measured in cytoplasmic cell extracts correlate with the appearance of NFκB-p65 in nuclear extracts.

DISCUSSION

Obesity, the result of an imbalance in storage energy, is associated with chronic low grade inflammation and insulin resistance. Obese adipose tissues are characterized by the enhanced infiltration of macrophages, and a paracrine loop involving the secretion of monocyte chemoattractant protein-1 (MCP-1), TNFα, and the free fatty acid between adipocytes and macrophages. This signalling loop is accompanied by down-regulation of PPARγ, glucose transport 4 (GLUT4) and IRS-1. Needless to say, the pathogenesis of this disease is complex and there are several contributory genetic and environmental factors. It should not be surprising that over 50% of diabetic patient are obese, owing to the strong correlation between the development of insulin resistance and obesity. Thus, elucidating mechanisms for insulin resistance has been an intense area of research in the field of diabetes, and undoubtedly it would be of great benefit in the treatment of obesity in the future.

The unique pharmacological profile of prieurianin in suppressing energy intake in mice, and inhibiting the proliferation and differentiation of preadipocytes, and causing dedifferentiation/delipidation of adipocytes, reveals a first-in-class anti-obesity drug. The dual anorexigenic and anti-adipogenic action of prieurianin is also unique among currently available pharmaceutical therapies and pipeline products in development that either suppress satiety or inhibit energy homeostasis (Cooke & Bloom, 2006). With the cytokine-mimicking effects on weight loss and lipolysis (Johnen, et al., 2007; Spiegelman & Hotamisligil, 1993), as exemplified by prieurianin, the limonoids hold the promise to be a novel class of small molecules that target multiple physiological mechanisms

including modulation of the neuronal input that controls anorexigenic function, and the induction of a *de novo* transcriptional repressor that inhibits adipogenesis. The precise molecular target of prieurianin, its long-term efficacy and toxicology profile remain to be determined.

Our results further demonstrate that drug-induced tolerance resulting from chronic pharmacotherapy for obesity can be overcome by a cyclical drug holiday treatment strategy, which produces a greater and a more durable therapeutic response and the maintenance of weight loss and reduced energy intake. Hence, the on-off treatment protocol may potentially restore the efficacy of currently available anti-obesity drugs as well as other experimental pharmacotherapeutics that are prone to drug-induced tolerance in the long-term treatment of obesity. In addition, despite significant advances in the transcriptional regulation of adipogenesis, many gaps remain, specifically in the anti-adipogenic factors that control adipose tissue development. The activation of NFκB responsive genes have previously been shown to be a repressor of C/EBPs and PPARγ activity and demonstrates that prieurianin targets the regulatory factors that arrest adipocyte differentiation. Thus, prieurianin may constitute an excellent pharmacological tool for probing the transcriptional process that regulates adipogenesis. Taken together, the findings in this study show that prieurianin is a novel anti-obesity therapeutic that exhibits efficacy against obesity of various etiologic conditions. Its dual anorexigenic and anti-adipogenic properties that parallel those of inflammatory cytokines raise the possibility of a novel class of more potent and efficacious treatment for obesity with the limonoids than currently available pharmacotherapeutics.

DISCUSSION

Part I

Drug resistance in cancer is a complex phenotypic process involving the genetic alteration of multiple cellular factors and aberrantly perturbed biological pathways that evolve during tumorigenesis. Therefore, understanding how drug resistance emerges in cancer is of paramount importance in improving cancer treatment and outcome. In this report, we identified the transcriptional induction of PLTP gene expression by camptothecin (CPT) and its derivatives that are active inhibitor of the topoisomerase I enzyme (Figs. 1 and 2). There are two types of topoisomerases (type I and type II), and these enzymes function to unwind DNA and to facilitate (i) DNA replication, and (ii) mRNA transcription and subsequent translation during protein synthesis. Topotecan and irinotecan are two examples of commonly used chemotherapy drugs that work by interfering with topoisomerase I in cancer cells. Topotecan is the water-soluble derivative of camtothecin and used to treat ovarian and lung cancer, as well as other cancer types. It acts by forming a stable covalent complex with the DNA/topoisomerase I aggregate and this process leads to breaks in the DNA strand resulting in apoptosis (Tolis, Peters, Ferreira, Pinedo, & Giaccone, 1999). According to the WHO, in 2004 lung cancer was the most common cause of cancer-related death in men and women, and responsible for 1.3 million deaths worldwide annually. The vast majority of lung cancers are carcinomas—malignancies that arise from epithelial cells, and the two main types of lung cancer are small cell lung carcinoma (17%) and non-small cell lung carcinoma (80%). While primary lung cancers most commonly metastasize to the adrenal glands, liver,

brain, and bone, the lungs are a common place for metastasis of tumors from other parts of the body such as the breast. Because the lungs are a major site expressing PLTP mRNA in humans and mice, this suggests that this protein might have an important role in maintaining normal function of this organ. Using an emphysematous animal model, Jiang et al showed that the lung of human collagenase transgenic mice expressed PLTP mRNA 3-fold higher than in control mice but the mRNA in other tissues was not changed (Jiang, et al., 1998). Thus, these observations suggest that a hypoxic stimulus occurring in emphysema may be contribute to enhanced expression of PLTP. Our studies showed that induction of PLTP expression by CPTs was not only drug-specific but also restricted to the human liver cancer HepG2 cells only. We further demonstrated that topotecan activated PLTP expression is mediated through the PKC signaling pathway and requires specific PKC isozyme for its transcriptional regulation (Fig. 3). PLTP expression is also induced *in vivo* in mice following treatment with topotecan that is accompanied by a rise in serum triglyceride and cholesterol, and a marked loss of HDL (Fig. 5). In addition, CPT induced increase in PLTP level did not result in the transfer of CPT to HDL.

The profound induction of PLTP expression by CPTs is intriguing and led us to initially hypothesize that topoisomerase I inhibitors induced their own metabolism via the reverse cholesterol transport pathway. Moreover, it has been shown that cholesterol is a substrate for PLTP (Tu, Nishida, & Nishida, 1993), and high cholesterol diet increases PLTP activity and PLTP mRNA in mice (Jiang & Bruce, 1995; Tu, et al., 1999). In addition, crystal structure data of the bacterial permeability increasing (BPI) protein (Beamer, Carroll, & Eisenberg, 1997; Huuskonen, et al., 1999) and the cholesteryl ester

transfer protein (CETP) (Qiu, et al., 2007) showing that proteins in this family, including PLTP, contain intrinsic lipid binding sites that appear to act as carrier proteins or channels that shuttle and redistribute lipids between lipoproteins. These observations raised the possibility that CPTs might be substrates for PLTP-mediated transfer into HDL and subsequently transported to the liver for metabolism via the reverse cholesterol transport pathway. However, our results showed that, unlike cholesterol, CPT was not found in HDL or other lipoproteins, suggesting that CPTs are not substrates for PLTP and ruling out the possibility of PLTP-mediated transfer of CPT into HDL for liver metabolism. These observations pose the possibility that the induction of PLTP gene expression by CPTs is merely the result of bystander effect following drug exposure, which inadvertently activates the expression of many genes, and some of which including PLTP may have no physiological consequences or whose functional impacts are unknown.

Besides topotecan, it has previously been reported that PLTP expression is induced by hypoxia (Jiang, et al., 1998), fenofibrate (Bouly, et al., 2001), and the liver X receptor (LXR) agonist, T0901317 (Cao, et al., 2002). Fenofibrates are drug of the fibrate class, that is amphipathic carboxylic acids, and like other fibrates they are used to reduce low-density lipoprotein (LDL), very low density lipoproteins (VLDL) and triglycerides levels, as well as to increase high-density lipoproteins (HDL) levels, and these effects are mediated by an increase in PLTP mRNA and activity (Bouly, et al., 2001). The liver X receptors (LXR) belong to the nuclear receptor superfamily that can regulate important lipid metabolic pathways. Cao et al showed that T0901317, a specific

LXR agonist, has been shown to elevate HDL cholesterol and phospholipid in C57/BL6 mice and generated enlarged HDL particles that were enriched in cholesterol, ApoAI, ApoE, and phospholipid, and this was closely correlated with the increased plasma PLTP activity and liver PLTP mRNA levels (Cao, et al., 2002). These observations suggest that PLTP expression is responsive to stimulation by small pharmacological molecules. While PLTP mRNA is found abundantly in a variety of tissues (Albers, et al., 1995), however, the induction by topotecan is cell-type specific. The underlying reason for this conditional expression pattern is unclear, and we speculate that it may be dependent on the small pharmacological molecules as well as the presence of specific transcriptional regulators in the respective tissues or cell-types.

Another curious observation in our study is the unexpected rise in serum cholesterol and triglyceride levels, and a severe depletion of serum HDL in mice treated with a high dose of topotecan. The abrupt rise in serum triglyceride is known to be associated with the onset of acute pancreatitis (AP), a sudden inflammation of the pancreas. (Cappell, 2008). There are many etiological risk factors for AP, including a history of alcohol abuse, gallstones, endoscopic retrograde cholangiopancreatography and manometry, trauma or surgical procedures near the pancreas, certain medications, hyperlipidemia, infection, and chronic hypercalcemia (Sekimoto, et al., 2006). The pancreas exocrine function is to produce digestive enzymes for release into the gastrointestinal tract (Berardi RR, 2005). The acinar cells within the pancreas are responsible for producing the proenzymes, which then are packaged into storage vesicles called zymogens. The zymogens travel through the pancreatic duct and are secreted into the

duodenum. Within the duodenum, enterokinase converts trypsinogen to trypsin, and then active trypsin facilitates the conversion of the other pancreatic proenzymes to the active form. AP can occur if there is damage to the acinar cells and/or injury to the pancreatic duct that leads to inappropriate accumulation and activation of proenzymes within the pancreas. The activated pancreatic enzymes digest the cell membranes of the pancreas and activate an inflammatory response, which increases the vascular permeability of the pancreas. Hemorrhage, edema, ischemia, and necrosis can result (Berardi RR, 2005). In severe AP, patients progress to systemic inflammatory response syndrome, sepsis, and multiple organ failure (Neoptolemos, Raraty, Finch, & Sutton, 1998). About 3% to 13% of AP cases progress to chronic pancreatitis (Sekimoto, et al., 2006). Though under-reported and under-appreciated, drug-induced acute pancreatitis are commonly encountered in drug therapy (Eland, et al., 1999). The etiologic cause of most drug-induced acute pancreatitis and atherosclerosis may be associated with hyperlipidemia, resulting from increased total serum triglyceride and cholesterol levels (Yadav & Pitchumoni, 2003). The mechanisms of drug-induced hyperlipidemia are unknown. Increase in PLTP activity has been linked to hypertriglyceridemia (Jonkers, et al., 2003; Kaser, et al., 2004), which may trigger the onset of acute pancreatitis (Miller, 2000; Yadav & Pitchumoni, 2003). It is noteworthy that acute pancreatitis has been reported in cancer patients treated with topoisomerase I inhibitors such as irinotecan (Govindan, Read, Faust, & Mc Leod, 2003b) and exatecan (De Jager, et al., 2000), as well as other chemotherapeutic agents including L-asparaginase (Jain, et al., 2009; Parsons, et al., 1997), tamoxifen (Alagozlu, et al., 2006), interferon α (Wong, et al., 2004), and capecitabine (Koutras, et al., 2006). These observations, together with our results,

suggest that hypertriglyceridemia induced acute pancreatitis may be correlated with the increase in PLTP, thus suggesting that PLTP may serve as a biomarker for CPTs and other drug-induced hypertriglyceridemia and acute pancreatitis.

In summary, our results showed that CPTs induced PLTP expression has no functional consequence in drug resistance, but may be associated with drug-induced hyperlipidemia and the onset of acute pancreatitis. Monitoring changes in serum PLTP levels or activity, therefore, may serve as an important biomarker for drug-induced hyperlipidemia and the development of acute pancreatitis. How increased PLTP expression causes hyperlipidemia (hypercholesterolemia and hypertriglyceridemia) and lowers HDL levels needs to be further investigated.

Part II

There are currently only two FDA-approved pharmacotherapy for obesity in U.S. Recent FDA issued heart risk warning in U.S. for sibutramine and its withdrawal in Europe, as well as new concerns for potential liver toxicity with orlistat, further reduced the armamentarium for the combat of obesity. Sibutramine is an orally administered appetite suppressant used for the treatment of obesity, and is classified as a schedule IV controlled substance in the United States, despite having no potential for abuse due to its lack of appreciable dopaminergic effects. Sibutramine is well absorbed from the GI tract (77%), but undergoes considerable first-pass metabolism, reducing its bioavailability.

The drug itself reaches its peak plasma level after 1 hour and has also a half-life of 1 hour. Sibutramine is metabolized by cytochrome P450 isozyme CYP3A4 into two pharmacologically-active primary and secondary amines (called active metabolites 1 and 2) with half-lives of 14 and 16 hours, respectively. Peak plasma concentrations of active metabolites 1 and 2 are reached after three to four hours. The following metabolic pathway mainly results in two inactive conjugated and hydroxylated metabolites (called metabolites 5 and 6). Metabolites 5 and 6 are mainly excreted in the urine. Sibutramine is a neurotransmitter reuptake inhibitor that reduces the reuptake of serotonin (by 53%), norepinephrine (by 54%), and dopamine (by 16%), thereby increasing the levels of these substances in synaptic clefts and helping enhance satiety; the serotonergic action, in particular, is thought to influence appetite. Older anorectic agents such as amphetamine and fenfluramine force the release of these neurotransmitters rather than affecting their reuptake (Heal, et al., 1998). Despite having a mechanism of action similar to tricyclic antidepressants such as selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), sibutramine has failed to demonstrate antidepressant properties in animal studies. In contrast, the primary function of orlistat is to prevent the absorption of fats from the human diet, thereby reducing caloric intake. It is intended for use in conjunction with a physician-supervised reduced-calorie diet. Orlistat is the saturated derivative of lipstatin, a potent natural inhibitor of pancreatic lipases isolated from the bacterium *Streptomycen toxytricini* (Barbier & Schneider, 1987). However, due to simplicity and stability, orlistat rather than lipstatin was developed into an anti-obesity drug (Pommier, Pons, & Kocienski, 1995). Orlistat works by inhibiting gastric and pancreatic lipases, the enzymes that break down triglycerides in the intestine.

When lipase activity is blocked, triglycerides from the diet are not hydrolyzed into absorbable free fatty acids, and are excreted undigested instead. Only trace amounts of orlistat are absorbed systemically; the primary effect is local lipase inhibition within the GI tract after an oral dose. The primary route of elimination is through the feces. Orlistat prevents approximately 30% of dietary fat from being absorbed. Drug repositioning is the application of known drugs and compounds to new diseases, and this field has been growing in importance in the last few years as an increasing number of drug development and pharmaceutical companies see their drug pipelines drying up and realize that many previously promising technologies have failed to deliver 'as advertised'. A significant advantage of drug repositioning over traditional drug development is that since the repositioned drug has already passed a significant number of toxicity and other tests, its safety is known and the risk of failure for reasons of adverse toxicology are reduced. Using drug repositioning, pharmaceutical companies have achieved a number of successes, for example Pfizer's Viagra in erectile dysfunction and Celgene's thalidomide in severe erythema nodosum leprosum. The use of repositioned drugs comprising of combinatorial mixture of anticonvulsant, antidepressant, or psychoactive stimulant is also being contemplated for the treatement of obesity, however questions arise of unknown long-term safety concerns, as these drugs all have various known risks and side effects (Robinson & Niswender, 2009). These issues all point to an urgent need for the development of more efficacious and safe pharmacotherapeutics for obesity.

We described here the unexpected identification of a natural plant limonoid product, prieurianin, which has a unique pharmacological profile of suppressing energy

intake in mice, and inhibiting the proliferation and differentiation of preadipocytes, and causing dedifferentiation/delipidation of adipocytes. Further, we showed that prieurianin mechanism of action is mediated in part by NFκB signaling. These findings ushered in a novel first-in-class anti-obesity drug candidate. The dual anorexigenic and anti-adipogenic action of prieurianin is unique among currently available pharmaceutical therapies and pipeline products in development that focused on either suppressing satiety or inhibiting energy homeostasis (Cooke & Bloom, 2006). Further, with the cytokine-mimicking effects on weight loss and lipolysis (Johnen, et al., 2007; Spiegelman & Hotamisligil, 1993), prieurianin holds promise to be a novel class of small molecules that target multiple physiological mechanisms including the potential modulation of neuronal input that controls anorexigenic function, and the prevention of new adipose mass development by inhibiting adipogenesis. The precise molecular target of prieurianin, its long-term efficacy and toxicology profile remain to be determined.

Long-term efficacy and effectiveness of obesity treatments are known to have poor outcome (Mauro, Taylor, Wharton, & Sharma, 2008). The potential of drug-induced tolerance associated with anti-obesity therapeutics, though recognized, but circumvention of its development has not been fully addressed (Fernstrom & Choi, 2007, 2008). Our results demonstrate that drug-induced tolerance resulting from chronic pharmacotherapy for obesity can be overcome by a cyclical drug holiday treatment strategy, which produces a greater and a more durable therapeutic response and the maintenance of weight loss and reduced energy intake. A drug holiday is when a patient stops taking a medication(s) for a period of time; anywhere from a few days to many months or even

years if they feel it is in their best interests. Planned drug holidays are used in numerous fields of medicine. They are perhaps best known in HIV therapy, after a study showed that stopping medication may stimulate the immune system to attack the virus. Further, a drug holiday may be used because (i) it is part of a progression toward treatment cessation, (ii) to reduce drug side effects, or (iii) to permit a drug to regain effectiveness after a period of continuous use, and to reduce the tolerance effect that may require increased dosages. Hence, the on-off treatment protocol may potentially restore the efficacy of currently available anti-obesity drugs as well as other experimental pharmacotherapeutics that are prone to drug-induced tolerance in the long-term treatment of obesity. Besides, decreasing the dosage and frequency of drug exposure using the drug holiday treatment protocol may reduce the incidence and disposition to the toxicities of these anti-obesity drugs. Therefore, new trials should be initiated to test the potential benefits of this approach in the treatment of obesity.

It is noteworthy that prieurianin was recently shown to inhibit endocytosis and also block brassinosteroid-induced gene expression in germinating seedlings of *Arabidopsis thaliana* (Robert, et al., 2008). It is unclear how prieurianin-inhibited endocytosis translates into its anti-obesity effects. Nevertheless, the potential action of prieurianin to inhibit both the hormone-induced transcription in germinating seedlings and the transcriptionally regulated adipogenesis in preadipocytes, further suggests its unique pharmacodynamics in disrupting gene expression program during differentiation.

Hence, identifying the molecular target(s) of prieurianin might yield insights into the rate-limiting factor that drives the development of adipose tissue.

NF-kB is well known as a master regulator of gene transcription in white blood cells and lymphocytes involved in inflammation and innate immunity. Recently, many studies have shown that NF-κB signaling plays a role in obesity, a pathophysiological state associated with a chronic low-grade inflammation accompanied by increasing levels of proinflammatory cytokines (Hotamisligil, 2006; Shoelson, Herrero, & Naaz, 2007; Wellen & Hotamisligil, 2005). According to the National Health and Nutrition Examination Survey in the United States, it was estimated that over 65% of adults were overweight or obese, and 16% of children were overweight (CDC, 2002). Obesity, particularly that caused by visceral fat accumulation is a serious risk factor for diabetes, coronary heart diseases, hyperlipidemia, hypertension and cancer (Nakamura, et al., 1994). Further, insulin resistance is considered to be the most common underlying cause, and is influenced by both genetic and environmental factors, as well as changes in diet and exercise (Uauy & Diaz, 2005). While weight loss via diet and exercise improves insulin sensitivity, the precise mechanisms modulating this improvement are not completely understood, but there is mounting evidence that NF-κB signaling plays a crucial role (Chiang, et al., 2009; X. Zhang, et al., 2008). NF-κB may be activated by toll-like receptor-4 (TLR4) due to interactions with dietary fatty acids (F. Kim, et al., 2007; Tsukumo, et al., 2007). Adipose tissue responds to overnutrition by secreting cytokines and chemokines that recruit macrophages to adipose tissue (Lumeng, DelProposto, Westcott, & Saltiel, 2008). In turn, macrophages secrete more cytokines

and chemokines that attenuate insulin action, and increase lipolysis and free fatty acid release (Feingold, Doerrler, Dinarello, Fiers, & Grunfeld, 1992). Thus, the inflammatory response is exacerbated. Interestingly, in our studies when C57/BL6 mice were treated with topotecan, triglycerides levels decreased and HDL-cholesterols increased. This suggests that PLTP may have played a role in preventing the accumulation of excess fatty acids and cholesterol in the plasma, and mitigated the NF-κB induced inflammatory response. Because of these desirable physiological effects, we set out to screen natural compound libraries to identify compounds that can similarly increase PLTP gene expression, reduced triglycerides and LDL-cholesterols, and increase HDL-cholesterols, but are neither cytotoxic nor DNA damaging agents. From the screen of 720 natural compounds and small molecules, prieurianin emerged the clear winner.

Prieurianin is a limonoids and occur naturally only in plants species of the Rutaceae and Meliaceae plant families. It reduces food consumption in rodent models of obesity by possibly diverting carbohydrates and fatty acids that would have become fat in liver into hepatic glycogen. In turn, this metabolic change may send a signal to the brain that results in appetite suppression. Our pair fed experiments suggests that prieurianin in addition to suppressing food intake, by preventing mice from efficiently transforming the nutrients fed into their own biomass, also stimulates the breakdown of adipose stores. Paradoxically, the hydrolysis of the fat droplets and tissue was not accompanied by a subsequent efflux of fatty acids and glycerol, the hallmark clues of lipolysis and thus raise the question, so where did the fat go? One possibility is that prieurianin increased the cell's β-oxidative capacity. However, a number of the genes essential to β-oxidation

particularly PPARα was not upregulated in our studies. Another more plausible explanation is that after lipolysis the excess glycerol was converted to glycogen, which would not have been stained by either oil red-O or nile red, neutral lipids and triglycerides staining dyes, rather peroidic acid-Schiff (PAS) staining should be used. Lipolysis is a transient and rapid process, and usually can be evident in a few minutes to hours. On the other hand, adipocytes treated with prieurianin takes 5-7 days before marked differences can be observed when compared to untreated or vehicle treated controls suggesting that the kinetics and action of prieurianin is slow. Lipogenesis is a reciprocal biochemical process that goes hand in hand with lipolysis to maintain lipid homeostasis, and the process is regulated by hormones and lipid metabolites that signal chiefly to the lipogenic transcription factors PPARγ and C/EBPα, and β. Our studies showed that prieurianin, in addition to stimulating the loss of lipid droplets, blocked the transcriptional expression of these keys lipogenic transcription factors and in turn blocks the differentiation of adipocytes.

Taken together, the findings in this study show that prieurianin is a novel anti-obesity therapeutic candidate that exhibits efficacy against obesity of various etiologic origins. Its dual anorexigenic and anti-adipogenic properties that parallel those of inflammatory cytokines raise the possibility of a novel class of more potent and efficacious treatment for obesity with the limonoids than currently available pharmacotherapeutics.

CONCLUSION

1. Prieurianin, like camptothecin, increases PLTP mRNA and protein levels in HepG2. However, it does not affect blood cholesterol levels. PLTP levels are associated with increased adiposity and decreases with weight loss.

2. Prieurianin reduces adipose tissue mass in four animal models of obesity, and decreases plasma insulin and glucose levels.

3. Prieurianin inhibits the expression of key adipogenic transcription factors including C/EBP-α, -β, and –δ, and PPARγ

4. The prieurianin-induced block of the adipogenic transcription machinery is an NFκB-p65 dependent event.

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