Investigation into lipolytic compounds

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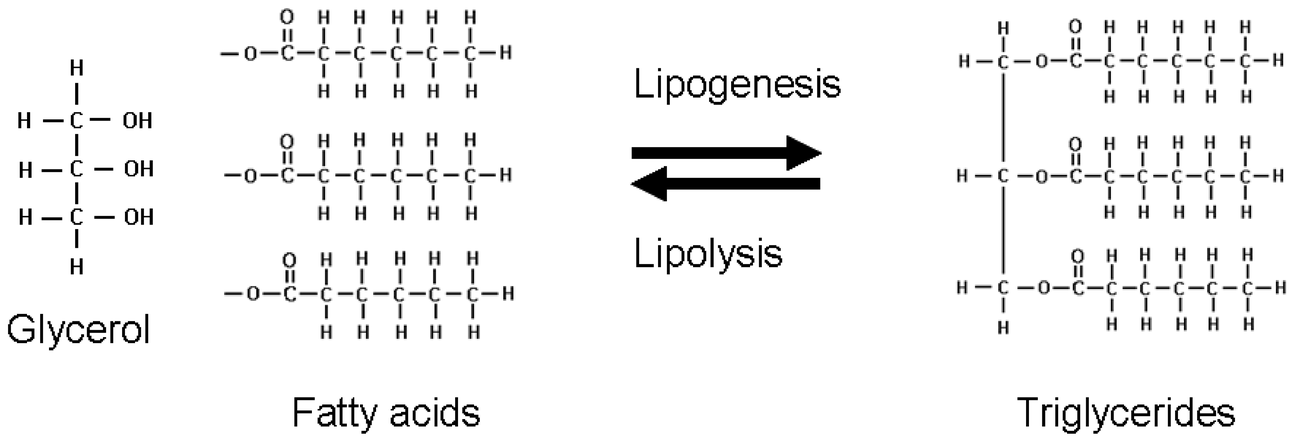
Introduction

The adipose tissue is specialized connective tissue that functions as the major storage site for fat in the form of triglycerides. In adult mammals, the major bulk of adipose tissue is a loose association of lipid-filled cells called adipocytes. Approximately 60 to 85% of the weight of white adipose tissue is lipid, with 90% being triglyceride. The size of adipose tissue mass is a function of both adipocyte number and size. The increase in number occurs primarily by mitotic activity in precursor cells whereas the increase in size occurs primarily by lipid accumulation within the cell. Cellulite is a disorder of the connective tissue and occurs mainly on the lower limbs, pelvic region, and abdomen, and is characterized by an “orange peel” or “cottage cheese” appearance. Approximately 85% of women over the age of 20 have some degree of cellulite.

Adipogenesis or lipogenesis, the differentiation process of adipocytes from precursor cells, provides constant renewal of adipocytes and contributes to the increase of adipose tissue mass. Insulin plays a predominant role in the adipogenic process. Lipolysis, on the other hand, is the chemical decomposition and release of fat from adipose tissue by hydrolysis of the ester bonds in triglycerides. This process predominates over adipogenesis when additional energy is required. The triglycerides within the adipocytes are acted upon by a multi-enzyme complex called hormone sensitive lipase (HSL), which hydrolyzes the triglyceride into free fatty acids and glycerol. Cellulite is a complex condition that can result from the accumulation of degraded fatty tissue in the skin.

Methylxanthines including caffeine, aminophylline, and theophylline are phosphodiesterase inhibitors commonly used in anti-cellulite products due to their proposed effect on adipocyte lipolysis via inhibition of phosphodiesterase, and increasing cyclic adenosine monophosphate (cAMP) levels. Isoflavones like genistein are antioxidants from plant extracts that are widely used in slimming products. Both genistein and another antioxidant resveratrol were shown to be effective in inhibiting adipogenesis in 3T3-L1 cell with genistein also being effective in induction of adipocytes lipolysis. Systemic drugs that have been developed for various heart and respiratory conditions, including isoproterenol (a beta-adrenergic agonist), aminophylline (a phosphodiesterase inhibitor) and theophylline (a phosphodiestrase inhibitor similar in structure to caffeine), have been developed for cellulite treatment as part of a mesotherapy regimen. Here we describe a novel group of small molecules, fatty amides in particular, for modulating adipocytes such as through the stimulation of triglycerides breakdown by increasing the lipolytic processes as well as slow down of lipid accumulation or reduce the size of adipocytes by promoting antiadipogenesis . The synergistic effects of these fatty amides in combination with naturals are also discussed.

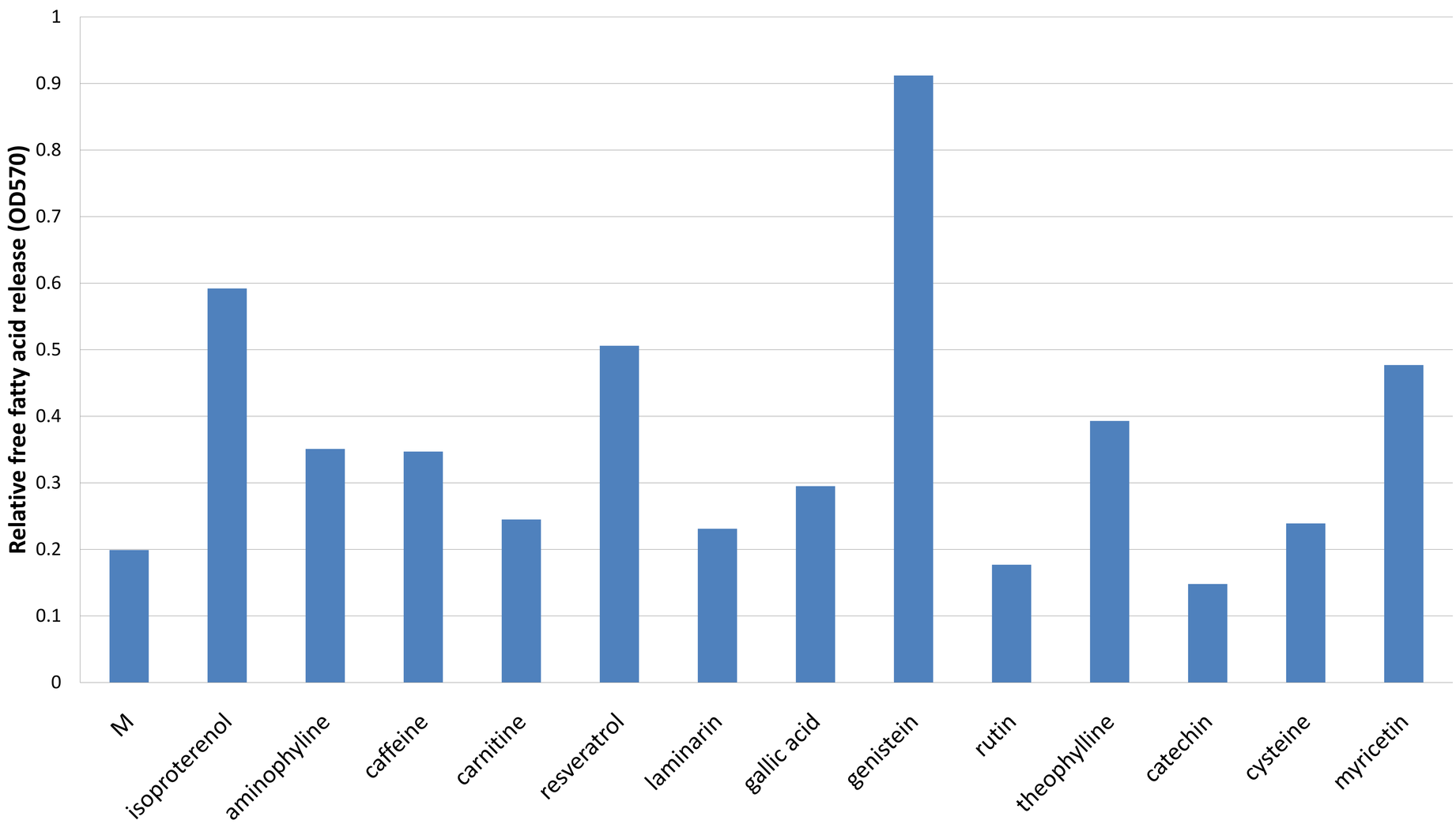
FIGURE 1. Illustration of adipocyte lipolysis and lipogenesis



STIMULATION OF LIPOLYSIS

In this study, molecules were characterized by their ability to increase lipolysis in differentiated 3T3-L1 adipocytes using the detection of generated free fatty acids as the end point. A wide range of natural and synthetic molecules have been documented to modulate lipolysis and as a result have been proposed to have beneficial effects upon adipose based conditions such as cellulite. As part of determining the lipolytic activity of LCF011 we also compared that activity to 13 compounds commonly used in dermatology and/or mesotherapy. As seen in Figure 2, compounds eliciting a 1-1.5 fold increase included carnitine, catechin, cysteine, gallic acid, laminarin and rutin. A 1.5-2.0 increase: aminophylline, theophylline and caffeine. A 2.0-2.5 fold increase: myrecetin and alpha MSH, and a greater than 2.5 fold increase: isoproterenol, resveretrol and genistein

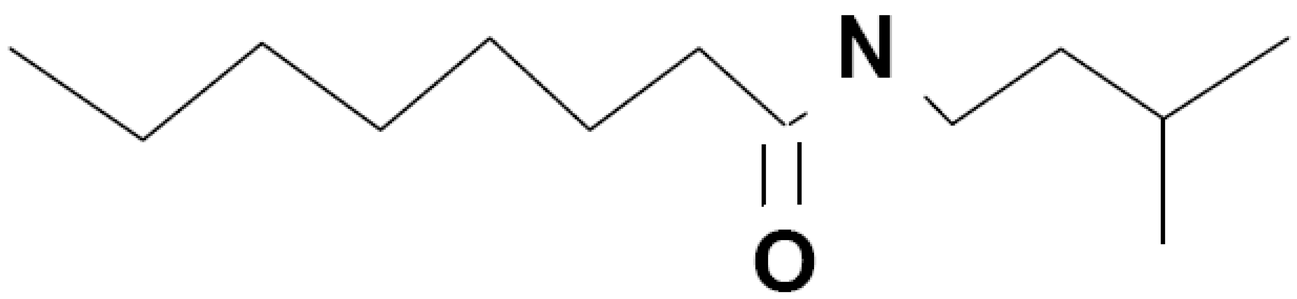
FIGURE 2. Comparative lipolytic activity of commonly used actives



STIMULATION OF LIPOLYSIS

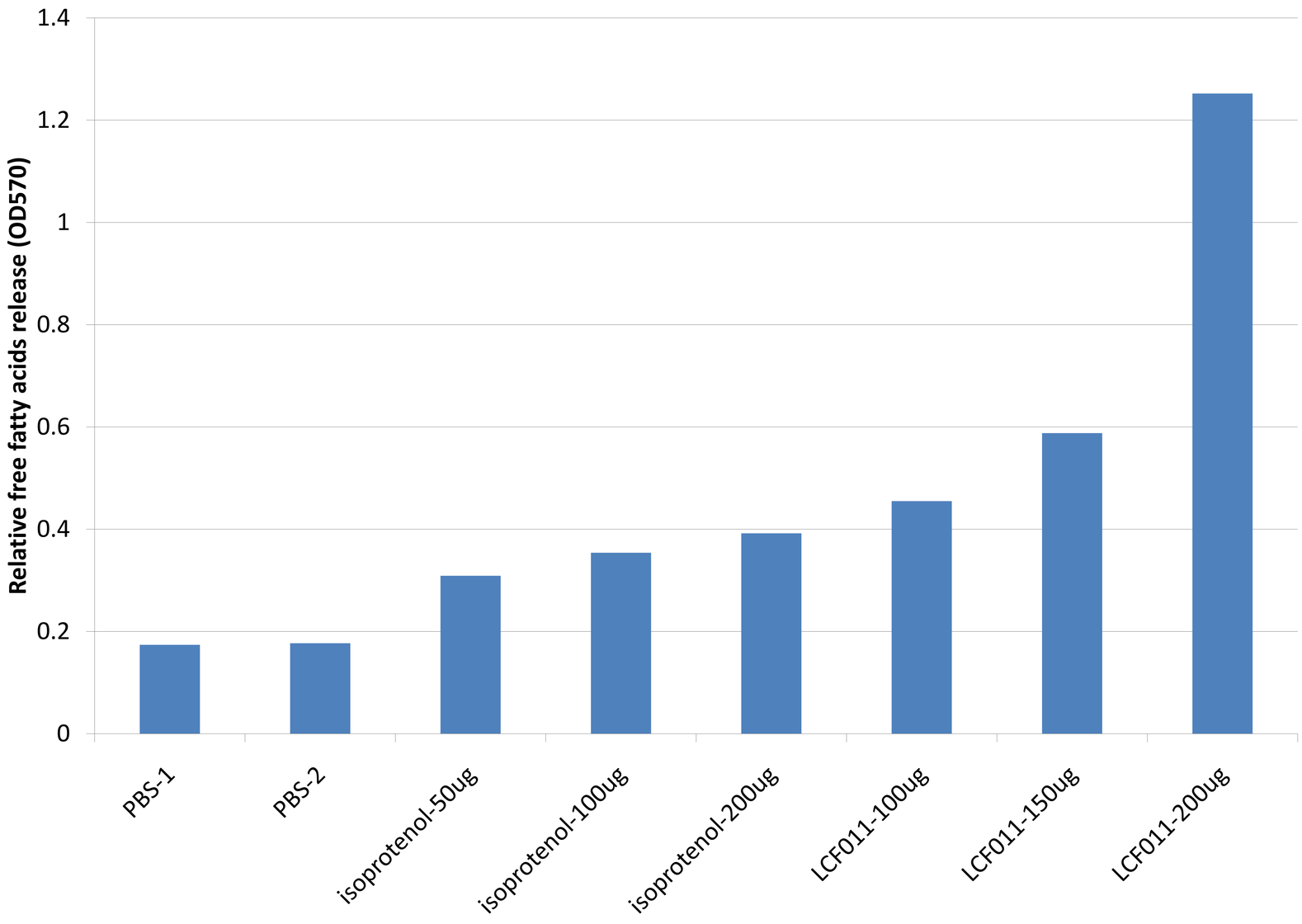
A family of novel compounds was designed by modification of a free amino acid such as leucine with mid-chain fatty acids. A series of compounds were characterized with the basic structure shown in Figure 3 below. One such molecule, LCF011, was designed and synthesized based upon the rationale that due to its structure it cannot be incorporated into triglyceride. The lipolytic activity of LCF011 was compared to the activity to the 13 compounds commonly used in dermatology and/or mesotherapy. LCF011 increased the level of lipolysis in 3T3-L1 adipocytes by a maximum of 5 fold and did so in a dose dependant manner from 0.25mM to 1mM (Figure 4). This activity was shown to be synergistic with phosphodiesterase inhibitors (Figure 5).

FIGURE 3. Basic structure of novel lipolytic compounds



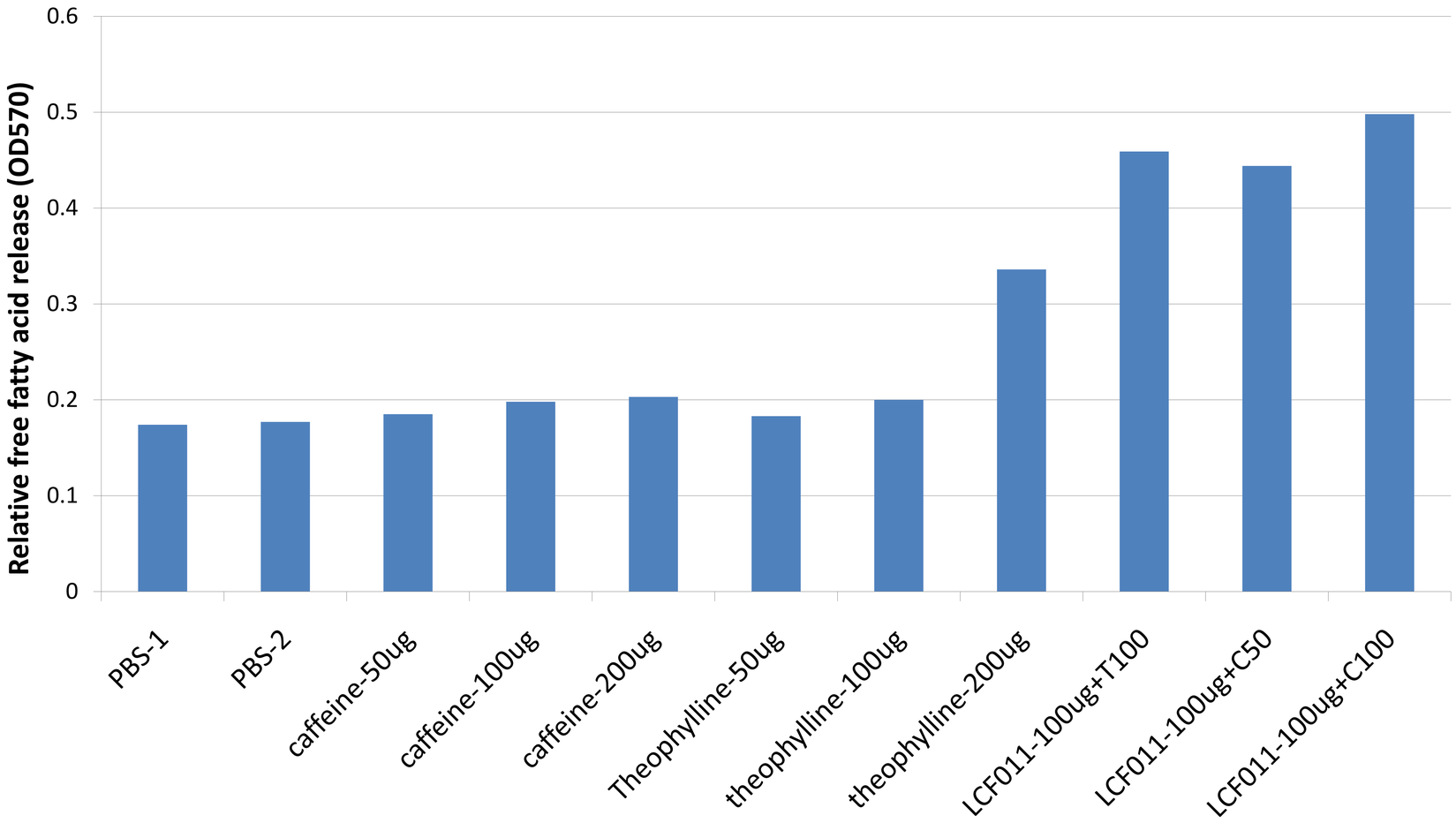
STIMULATION OF LIPOLYSIS

FIGURE 4. Lipolytic activity of LCF011 compared to isoproterenol



STIMULATION OF LIPOLYSIS

FIGURE 5. Synergy of LCF011 lipolytic activity with phosphodiesterase inhibitors



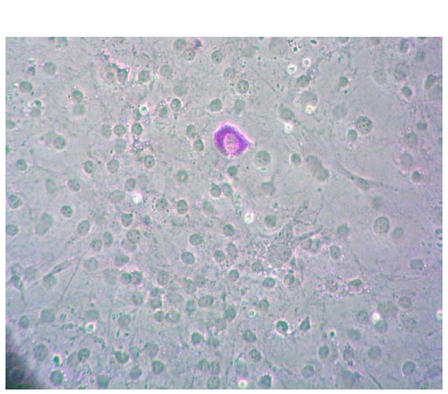
Abbreviations
T100: Theophylline 100ug
C50: Caffeine 50ug
C100: Caffeine 100ug

INHIBITION OF ADIPOGENESIS

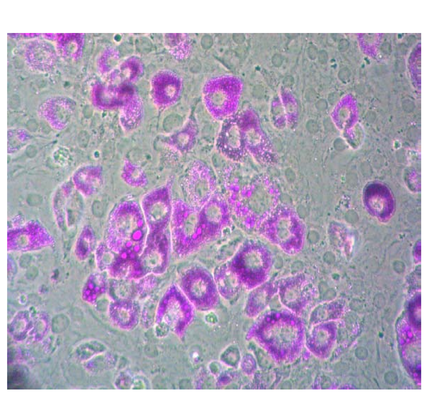
Adipogenesis is the process of cell differentiation by which preadipocytes become adipocytes. In vitro, adipocyte differentiation is routinely induced by a combination of dexamethasone, insulin, and isobutylmethylxanthine. In the presence of this induction cocktail 3T3-L1 differentiation process follows a cell morphology change. The preadipocytes are fibroblast-like cells and such morphology disappears during early stage of differentiation and is replaced by a characteristic morphology with enlarged and rounded shaped of cells of varying size. These rounded cells start to accumulate lipid-droplets. The adipose tissue mass is a function of both adipocyte number and size. An increase in adipose tissue mass can occur by hyperplastic growth, an increase in adipocyte number. This increase occurs primarily by mitotic activity in precursor cells. Adipose tissue mass can also increase by hypertrophic growth, which is an increase in adipocyte size. This increase in size occurs primarily by lipid accumulation within the cell.

LCF011 significantly inhibited or slowed down the preadipocyte differentiation process in the presence of adipogenic factors. Such inhibition activity was achieved by slowing down the formation of enlarged cells as well as the accumulation of lipid droplets inside the adipocytes. This occurred in a concentration dependent manner. Of the cells that were treated with low concentration of LCF011 a significant number of cells became differentiated however the intensity of lipid staining was significantly reduced as indicated with Oil red O stain method.

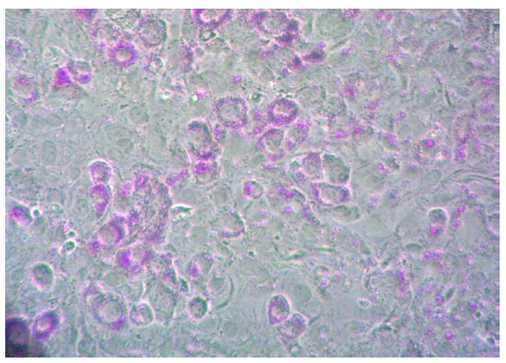
FIGURE 6. Inhibition of lipogenesis by LCF011



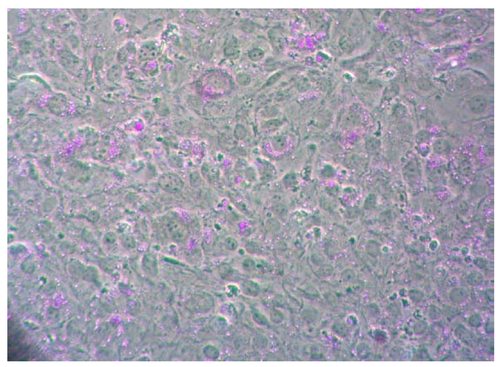
No induction



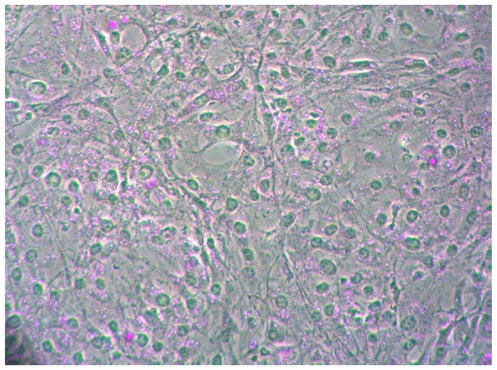
Induction control



Induction+100ug/ml LCF011



Induction +150ug/ml LCF011

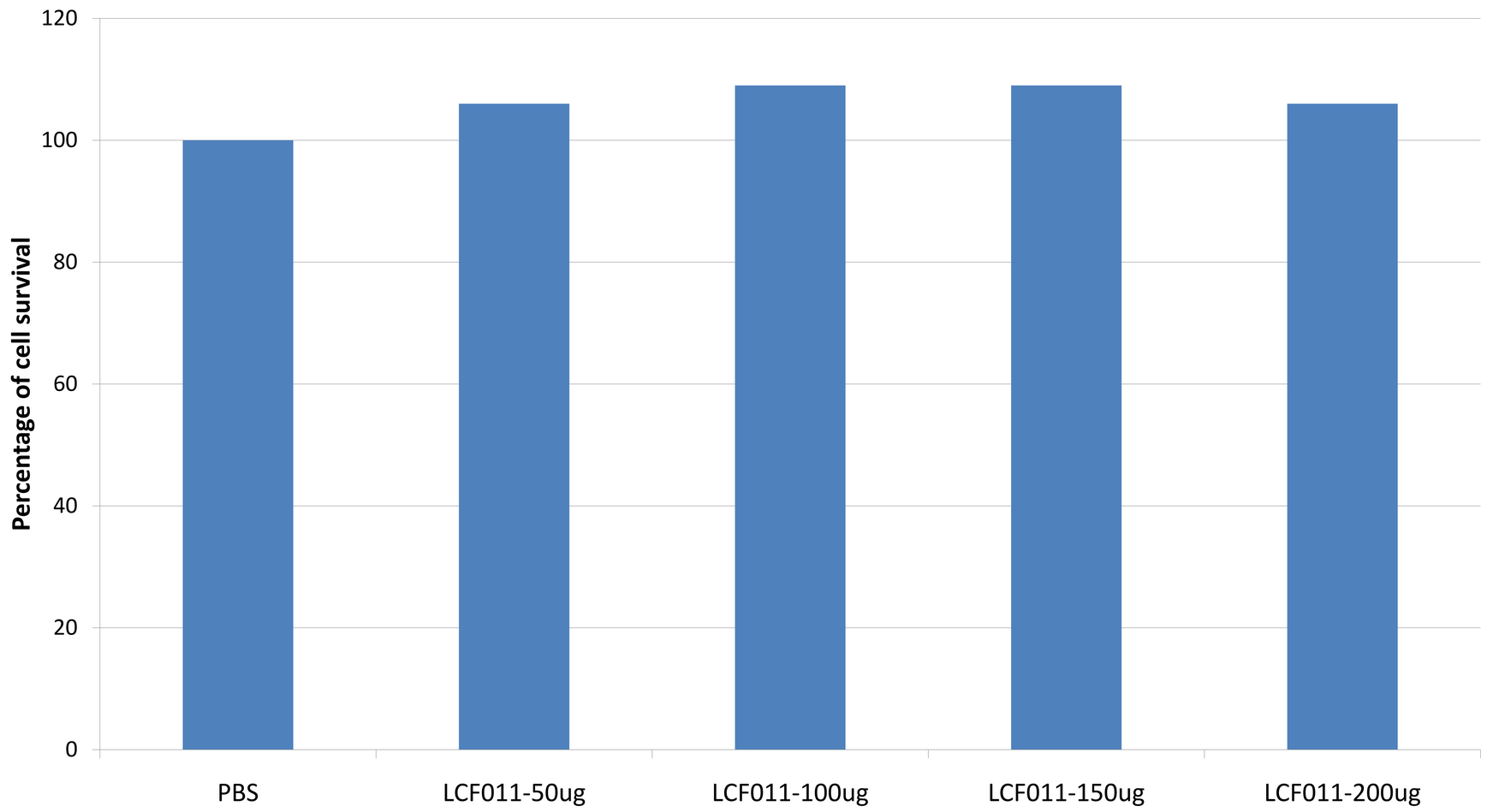


Induction+200ug/ml LCF011

CYTOTOXICITY

To ensure that the effects of LCF011 observed on lipolysis and adipogenesis were not due to toxicity or inhibition of cell function a standard cytotoxicity assay was performed on the cell line exposed to LCF011. The ATCC (Manassas, VA) MTT cell cytotoxicity assay kit was used following the manufacturer's instructions. Briefly, cells were grown to 100% confluence then exposed to peptide for 24hr. The tetrazolium salt, MTT, was added to each well, including controls, the plates re-incubated for a further 2-4hrs, during which time the cells were observed for the appearance of purple precipitate. Once developed and clearly visible by microscopy, solubilizing detergent was added prior to a further 2-4 hrs incubation at room temperature in darkness. Absorbance was determined at 570nm. All assays were performed in triplicate and PBS (phosphate buffered saline) treated controls were set at 100% with peptide treatment presented as percentage of control. As seen in Figure 7 LCF011 showed no signs of decreasing cell viability.

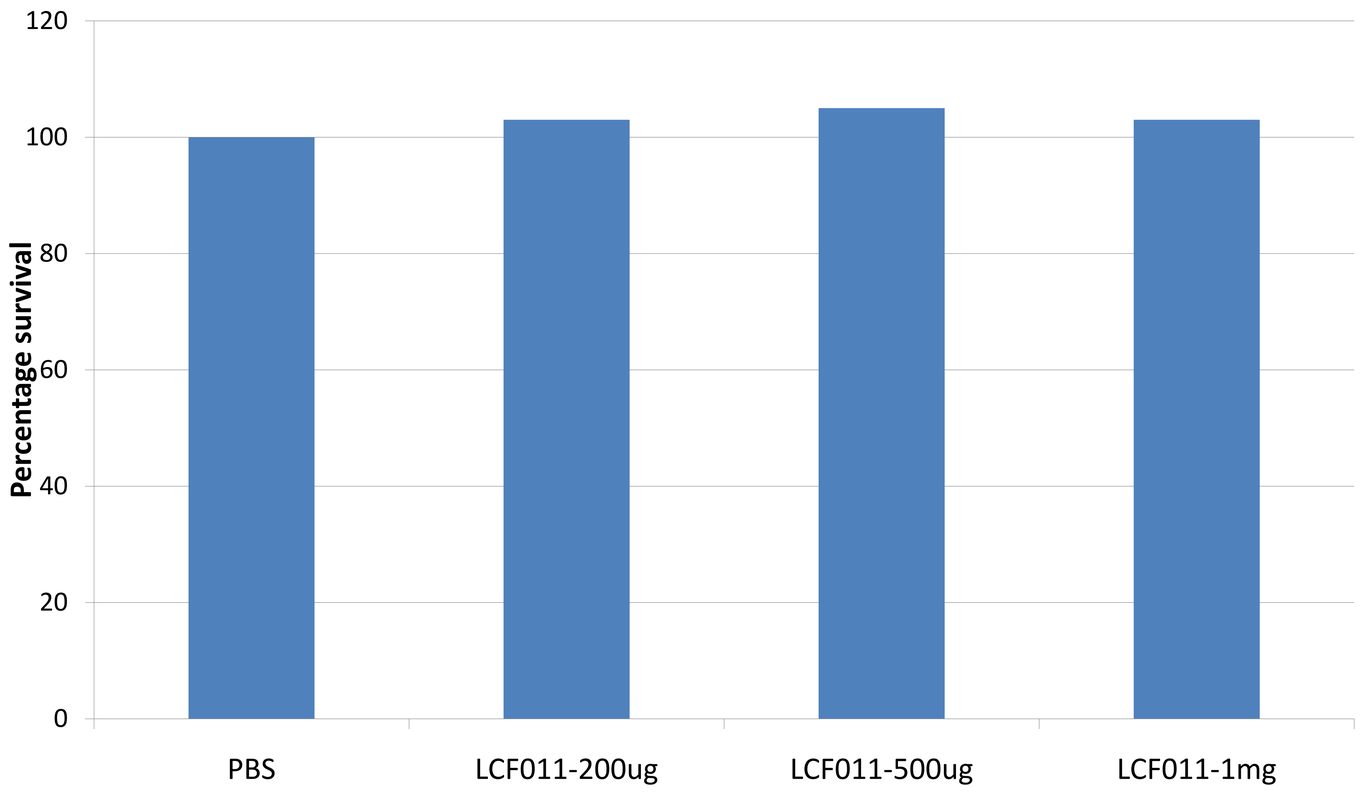
FIGURE 7. Lack of cytotoxicity of LCF011 on 3T3-L1 adipocytes



Dermal Toxicity

Potential for dermal irritancy was determined using the EpiDerm™ Skin Model (MatTek, MA) in combination with a modified MTT assay. The EpiDerm™ Skin Model exhibits *in vivo-like* morphological and growth characteristics which are uniform and highly reproducible. EpiDerm™ consists of organized basal, spinous, granular, and cornified layers analogous to those found *in vivo.* The tissues were treated with LCF011 at various concentrations for 24hr prior to MTT tissue viability assay. As seen in Figure 8, even high concentrations (1mg/ml), LCF011 exhibited no negative effects upon tissue viability. It should be noted that LCF011 was shown to be negative in the AMES test (data not shown).

FIGURE 8. Lack of toxicity of LCF011 in EpiDerm™ skin model



EpiDerm™ skin tissue

CONCLUSION

LCF011 is a small novel molecule (approx. 200mw), non-cytotoxic and non-mutagenic, that modulates adipocyte lipolysis and lipogenesis. It provides a starting point for a strategy of triglyceride reduction in subcutaneous adipocytes. It can be combined with modulatory molecules for enhanced activity. It should be noted that cellulite is not only related to adipocyte function but is a multifaceted disorder including issues associated with connective tissue matrix, the microcirculatory system and the lymphatic system. As a result, a combination of different active components is required to influence the different aspects of cellulite pathophysiology. The combination of targeting both adipogenesis and lipolysis with the activation of extracellular matrix rebuilding could provide the basis of synergistic treatment regimes.

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