Identification of a Pharmacological Inducer of Lipoic Acid Synthase that Impacts Mitochondrial Function: Metabolic Benefits and Body Weight Loss without Changes in Caloric Intake

Indira Padmalayam1*, Rebecca Hamm1, D. Srinivas Reddy2, Deb Nath Bhuniya2, Angela Vines1, Sujatha Seenu1, Ranjan Chakrabarti1, Uday Saxena1 and Sivaram Pillarisetti1

1Discovery Research, Reddy US Therapeutics, Inc. 3065 Northwoods Circle, Norcross, GA 30071, USA
2Dr. Reddy’s Laboratories Limited, Hyderabad, India

Abstract

Objective: Lipoic Acid Synthase (LASY) was recently shown to play a critical role in inflammation, mitochondrial function and insulin resistance. In this study using a pharmacological inducer of LASY as a tool, we studied the effects of LASY induction in vivo in rodent models of obesity and type 2 diabetes.

Methods: We identified a small molecule that increases LASY mRNA and lipoic acid levels in vitro. Effect of the inducer (compound 1) on body weight, food intake, energy expenditure and metabolic parameters was studied in rodent models of genetic and diet-induced obesity.

Results: Compound 1 was identified as a transcriptional inducer of LASY. In vitro, compound 1 increased LASY mRNA expression, mitochondrial lipoic acid levels and improved mitochondrial function in cultured skeletal muscle cells. LASY mRNA and lipoic acid levels were significantly increased in skeletal muscle and adipose tissue of animals treated with compound 1. In genetic and diet-induced rodent models of obesity, treatment with compound 1 significantly reduced body weight gain and fat mass without changes in food intake. Whole-animal calorimetry revealed that compound 1-treated animals had a significant increase in energy expenditure over vehicle control. Compound 1 treatment was also accompanied by improvements in plasma metabolic parameters and increase in lean mass.

Conclusion: Our data suggests that the beneficial effects of compound 1 are mediated by increase in mitochondrial lipoic acid levels, although general effects on mitochondrial function cannot be ruled out. These results establish the in vivo relevance of LASY induction and show the potential of a peripheral mediated approach for achieving many of the desired effects in treating obesity. Future studies focusing on the mechanism(s) by which compound 1 exerts its beneficial effects will help to reveal if these benefits are specific to LASY induction or are due to non-specific effects on mitochondrial function.

Keywords: Lipoic acid synthase; Diabetes; Lipoic acid

Introduction

Obesity is the result of an imbalance between energy intake and expenditure. It plays a causal role in the metabolic syndrome which encompasses various risk factors such as insulin resistance, type 2 diabetes, dyslipidemia, hypertension and cardiovascular dysfunction. Although the genetic and etiological reasons for obesity are varied and still under investigation, mitochondrial dysfunction is a key perpetrator, forming a well-established link between oxidative stress and obesity [1]. Obesity is associated with increased oxidative stress and mitochondrial dysfunction in adipocytes, which are considered as early instigators of the metabolic syndrome [1,2]. This suggests that alleviating oxidative stress and consequentially improving mitochondrial function would be a novel treatment strategy for obesity and ensuing metabolic syndrome.

Lipoic acid (LA), a short chain fatty acid, is a potent endogenous antioxidant that functions as a cofactor of major mitochondrial enzyme complexes such as the pyruvate dehydrogenase complex (PDC) and the alpha-ketoglutarate dehydrogenase complex (KDH). Both LA and its reduced form, dihydrolipoic acid (DHLA) also have powerful antioxidant properties, and can regenerate other antioxidants such as vitamin C, vitamin E and glutathione [3]. Supplementation with LA has been shown to have numerous therapeutic benefits, including amelioration of insulin resistance in rodent models [4,5] and in human studies [6]. Many of these therapeutic benefits are likely mediated by its powerful antioxidant function. Recently, dietary supplementation with LA has also been shown to have beneficial effects in lowering body weight gain in obese rodent models, by reducing energy intake [7]. Lipoic acid synthase (LASY) is the mitochondrial enzyme that is responsible for endogenous synthesis of LA by catalyzing the final step in the generation of LA from the precursor form, octanoic acid, by the insertion of two sulfur atoms [8]. Homozygous knock out of LASY in mice causes embryonic lethality, while heterozygotes appear to be normal, except for significantly reduced erythrocyte glutathione levels [9]. Thus, although a single copy of the LASY gene is sufficient for survival, deficiency of LASY contributes to aberrations in the endogenous antioxidant capacity [9]. Defects in LA biosynthesis in humans results in a fatal mitochondrial disease, as observed recently in ten infants who were found to have a point mutation in a protein, NFU1, ten infants who were found to have a point mutation in a protein, NFU1.
that is necessary for maturation of Fe-S cluster proteins such as LASY and succinate dehydrogenase (complex II of the ETC) [10]. Of interest is the fact that, these were the only Fe-S proteins whose activities were affected by this mutation. This defect resulted in a significant reduction in mitochondrial LA levels presumably due to impairment in the activity of LASY, which led to decreased activities of the LA-requiring complexes such as PDHC, α-KGDH and H-protein of the glycine cleavage system. Due to the importance of these enzyme complexes in mitochondrial function, reduction in their activities would have serious metabolic consequences which alter the intracellular fate of glucose. In another recent report, LASY deficiency due to a polymorphism identified in a human subject caused significant reduction in mitochondrial LA levels, and was associated with several metabolic abnormalities [11]. A key abnormality was impairment in mitochondrial metabolism due to decreased PDHC activity and reduced oxidation of pyruvate [11]. These observations highlight the critical role played by LASY in mitochondrial respiration and maintenance of metabolic homeostasis. Deficiencies in endogenous synthesis of LA cannot be complemented by exogenously supplied (dietary) LA [9], which further underscores the importance of the LA biosynthetic mechanism. We have reported that LASY is down regulated in animal models of obesity [12]. In vitro studies suggested that increased inflammation and oxidative stress (hall marks of obesity) contribute to down regulation of LASY. We also presented evidence that overexpression of LASY decreases inflammation in vitro [12]. Endogenous synthesis of LASY could have potential benefits over dietary LA supplementation because of the availability of LA within the mitochondria, within proximity of mitochondrial enzymes and as well as reactive intermediates which cause oxidative stress. This would help to fulfill the two critical functions of LA, namely as a cofactor and as an antioxidant. With this goal, we went about looking for a small molecule to fulfill the two critical functions of LA, namely as a cofactor and as an antioxidant. We identified a small molecule that is a transcriptional inducer of LASY which has therapeutic benefits in alleviating obesity and associated risk factors. We identified a small molecule that is a transcriptional inducer of LASY, and report the effects of this molecule in rodent models of genetic and diet-induced obesity.

Research Methods and Procedures

Chemicals

Compounds used in the study were synthesized at Dr. Reddy's laboratories, Hyderabad, India. Unless otherwise stated, reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Animals, diet and housing: All animal protocols were reviewed and approved by the Internal Animal Care and Use Committee (IACUC). Rodents were purchased from Jackson laboratories, Bar Harbor, Maine. Animals were housed in groups of 2-4 upon arrival. Mice for all studies were housed at 24°C with a 12-hour light-dark cycle (lights on at 07:00). All animals were allowed to feed ad libitum. Male ob/ob mice were fed a regular chow diet (Laboratory rodent diet, 5001, LabDiet, Inc.). For the ob/ob study, food and water intake were measured daily and body weight measurements were done once in seven days. Animals were euthanized by CO₂ inhalation. Necropsies were performed immediately (within 5 minutes) after euthanization and tissues were removed, diced and quick frozen (in liquid nitrogen) immediately (within 2 minutes of collection). Tissues collected were blood, skeletal muscle (soleus), adipose tissue (visceral fat pad) and liver sections. Blood was collected by orbital bleed. Blood for gene expression analysis was collected in tubes containing an RNA stabilization reagent. Blood for other assays was collected in tubes containing EDTA.

Real-time PCR assay

Validated real time qPCR primer sets were purchased from SABiosciences, Frederick, MD. Real-time PCR assay for LASY were performed using Mx3000P (Stratagene, La Jolla, CA) and using a one-step SYBR green kit (Stratagene, Inc.,La Jolla, CA). The PCR amplification parameters consisted of 1 cycle of 30 minutes at 50°C, 1 cycle of 10 minutes at 95°C and 40 cycles of 30 seconds at 95°C, 1 minute at 50-60°C, 30 seconds at 72°C. The dissociation program consisted of incubating the amplified product for 1 minute at 95°C, ramping down to 55°C at a rate of 0.2°C/sec. The dissociation curve was generated by completing 81 cycles of incubation where the temperature was increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C. The duration of each cycle was 30 seconds. Annealing temperatures for the primers for Adiponectin, Acyl-Coenzyme A Oxidase 1 (ACOX1), Carnitine palmitoyltransferase-1 (CPT-1), LASY and Actin were 60°C.

Cell growth and differentiation

Human skeletal muscle myoblasts (HSMM) (Cambrex Corporation, East Rutherford, NJ) were grown and differentiated according to Cambrex's protocol. Mouse C2C12 myoblasts (American Type Culture Collection (ATCC), Manassas, VA, no. CRL-1772) were cultured at 37°C in an atmosphere of 5% CO₂ in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin). Confluent cells were differentiated in differentiation medium consisting of DMEM supplemented with 2% horse serum, 10 µg/ml insulin, and 5 µg/ml transferrin.

Cell treatments

For HSMM and C2C12 myoblasts, treatments with compounds were done on differentiated cells. Cells were treated with either DMSO, or compound 1 in treatment medium (HSMM basal medium +1% fetal...
bovine serum (FBS)). Treatments were done for 18 hours at 37°C/5% CO₂.

RNA isolation

RNA was isolated from cultured cells using the Absolutely RNA™ 96 microprep kit (Stratagene, Inc., La Jolla, CA) following the manufacturer’s protocol. RNA from tissue samples was isolated using tissue-specific kits from Qiagen, Inc., Valencia, CA.

LASY promoter cloning and transfection

A 200 base pair region upstream of the LASY translational start site was identified as the minimal LASY promoter by deletion cloning. This region was cloned into pGL3-Basic (promoter-less) vector upstream of the luciferase gene. The resulting construct (pGL3-200) was transfected into HEK-293 cells to confirm luciferase expression. pGL3-200 was then transfected into HSM M by using Targeffect F1 reagent and peptide enhancer (Targeting Systems, Inc. El Cajon, CA). Transfections were allowed to proceed for 24 hours before adding treatments. Western Blot Analysis and antibodies: Western blot was done as described earlier [12]. Lipoic acid (LA) Western blot was performed with polyclonal anti-rabbit antibodies to LA bound to E2 subunits of Pyruvate Dehydrogenase Complex (PDC) and α-Keto Dehydrogenase complex (KDH) purchased from Calbiochem (EMD Biosciences, Gibbstown, NJ). Monoclonal mouse antibodies specific for PDH (E2/E3 subunits) were purchased from MoleculProbes (Invitrogen), Carlsbad, CA. Purified PDC (E2 subunit) (Bovine heart) was purchased from Globozymes, Carlsbad, CA. Secondary, HRP-conjugated IgG (H+L) antibodies were purchased from Zymed laboratories, San Francisco, CA.

Phosphorylated AMPK Western blot

Liver and adipose tissue samples for 5' AMP-activated protein kinase (AMPK) Western blot were processed as described in Supplementary method 2. Phosphorylated AMPK and AMPK antibodies were purchased from Cell Signaling Technology, Danvers, MA. Immunoblotting was done using conditions suggested by the antibody manufacturer.

Lipoic acid ELISA

We developed a sandwich-ELISA based assay to quantitate the amount of lipoic acid LA that was bound to the pyruvate dehydrogenase complex. The protocol for this assay is outlined in Supplementary method 3.

Detection of mitochondrial membrane potential

Changes in mitochondrial membrane potential were determined using a JC-1 assay kit (Biotium, Inc., Hayward, CA) as described by the manufacturer.

Beta oxidation assay

The beta oxidation assay was done according to protocols available in the literature [14,15] with the following modifications. C₃H₁₀ cell myoblasts were grown and differentiated in T25 flasks. Cells were pretreated with compounds for 22 hours in DMEM +10% FCS. Cells were then grown in media containing final concentration of 0.25 µCi/ml of [14C] Oleoyl CoA along with compounds or DMSO for 2 hours. Media containing radioactivity was discarded and cells were washed with phosphate buffered saline (PBS). 14CO₂ was captured and radioactivity measured according to the protocol described in the references.

Dual energy X-ray absorptiometry (DEXA) scan

DEXA scan was performed at the Jackson Laboratories using DEXA Scanner from Lunar/ PIXImus, Lunar PIXImus Corporation / GE Medical Systems, Madison, WI. Body composition measurements were performed on all animals at the end of the study. Mice were sedated with isoflurane during the procedure.

Comprehensive Laboratory Monitoring system (CLAMS)

Indirect calorimetry was performed on groups of three from each group for 48 hours each using the Oxymax Equal Flow

![Image](https://example.com/figure1.png)

**Figure 1: Induction of LASY expression and Lipoic acid (LA) levels in HSM M treated with compound 1**

A: Induction of LASY mRNA levels in HSM M treated with compound 1. Cells were grown and differentiated in 96 well plates and treated with either DMSO, 50, 100 or 250 µM of compound 1 in treatment medium (HSM M basal medium +1 % fetal bovine serum (FBS)). Treatments were done for 18 hours at 37°C/5% CO₂. Cells were lysed in lysis buffer and RNA extracted using Absolutely RNA 96 well RNA isolation kit (Stratagene, Inc., La Jolla, CA). Real time PCR quantification was done using the SYBR green real time PCR kit (Stratagene). Ct values obtained from LASY primers were corrected for Ct values from Actin primers. Fold changes compared to DMSO control are shown. Results shown are averages of three independent experiments and represent means ± standard deviation (SD). P values were calculated using one way ANOVA with post-hoc testing. *P=0.000003 for compound 1 250 µM versus DMSO control; P=0.0000003 for compound 1 250 µM versus DMSO control

B: Induction of LASY promoter by compound 1. HSM M were grown and differentiated in 24 well plates. A pGL3 construct containing the 200 base pair LASY promoter region (pGL3-200) was transfected into the differentiated cells for 24 hours, followed by treatment with either DMSO or 250 µM of compound 1 in treatment medium (HSM M basal medium +1 % fetal bovine serum (FBS)). A pGL3 control vector was used as transfection control. Luciferase expression was quantitated in terms of luminescence. All values were normalized for protein content. Values obtained for pGL3-200 were subtracted from values obtained for the pGL3 control vector to correct for background luminescence. Results shown are averages of three independent experiments and represent means ± SD. P values were calculated using a two-sample, paired t-test. *P=0.007 for compound 1 versus DMSO control

C: Induction of PDC-associated LA levels in HSM M treated with compound 1. Cells were grown and differentiated in 24 well plates and treated as described above for RNA. Cells were lysed in T-PE R protein lysis buffer (Pierce, Inc.) containing protease inhibitors. Protein was quantitated using a BCA kit (Pierce). LA ELISA was used to quantitate LA amounts in samples according to the protocol described in Supplementary methods. LA concentration associated with PDC (µg/ml) is shown. Results shown are averages of three independent experiments, and represent means ± SD. P values were calculated using one way ANOVA with post-hoc testing. *P<0.01 for compound 1 100 µM versus DMSO control; P=0.0001 for compound 1 250 µM versus DMSO control
Compound 1 increases expression of LASY and accumulation of lipoic acid in cultured skeletal muscle cells: We utilized promoter and real time PCR based 96 well plate assays to identify compounds that induce LASY expression. Compound 1 dose dependently induced LASY expression in primary cultures of human skeletal muscle myoblasts (HSMM) (3 fold induction at 100 μM (P=0.000002) and 6 fold induction at 250 μM (P=0.0000003) (Figure 1A). The compound also significantly induced LASY promoter activity (Figure 1B, P=0.007) suggesting that increase in LASY expression is due to transcriptional activation. Lipoic acid is an essential cofactor of major mitochondrial enzymes such as pyruvate dehydrogenase complex (PDC). Since LASY is the key enzyme in the generation of endogenous lipoic acid (LA), we also studied LA levels associated with PDC. PDC associated LA levels were determined by ELISA. As shown in (Figure 1C), there was an approximately 3-fold and 6-fold increase in PDC-associated LA with the 100 μM (P=0.01) and 250 μM (P=0.0001) doses, respectively. Thus increase in LASY expression translates to increase in endogenous LA.

Pharmacokinetic (PK) studies in mice revealed that compound 1 which is an ester converted to its acid in vivo (supplementary table).
Since the acid had better PK properties than the salt, we used the acid form of compound 1 for in vivo efficacy studies.

**Compound 1 decreases body weight in genetic models of obesity in rodents without reducing food intake:** We studied the effect of compound 1 on body weight in ob/ob (leptin deficient) mouse model of type 2 diabetes and obesity. As shown in (Figure 2A), compound 1 caused a significant stabilization of body weight by day 19 compared to vehicle (P=0.04). Reduction in body weight by compound 1 was sustained at day 26 (P=0.01). The decrease in body weight was independent of food intake since there was no change in food consumption over the period of the study (Figure 2B).

**Improvement in plasma metabolic parameters by compound 1 in the ob/ob model:** We studied the effect of compound 1 on various metabolic parameters that indicate the degree of metabolic control in type 2 diabetes and obesity. Treatment with compound 1 significantly decreased serum triglycerides, as well as low density lipoprotein cholesterol (LDL) levels and significantly increased high density lipoprotein cholesterol (HDL) levels (Table 1). Decrease in four-hour fasting glucose levels was not significant (P=0.06). However, an oral glucose tolerance test (OGTT) done at the end of the study indicated a significant decrease in plasma glucose at 30 minutes (P=0.009) and 60 minutes (P=0.02) in the group treated with compound 1 (Figure 2C). The simulated glucose area measured as area under the curve (AUC) was significantly lower for the compound 1 treated group (Figure 2D, 18%, P=0.03) suggesting a positive effect on glucose homeostasis.

**Upregulation of LASY expression and lipoic acid (LA) levels in skeletal muscle and adipose tissue by compound 1:** Induction of LASY expression and LA accumulation was studied in skeletal muscle and adipose tissue of ob/ob mice that were treated with compound 1. Skeletal muscle and adipose tissue were collected from ob/ob mice at the end of the study and analyzed for expression of LASY by real time PCR. Treatment with compound 1 resulted in a 39% and 50% induction of LASY expression in skeletal muscle and adipose tissue of treated animals, respectively, compared to vehicle group (Figure 3A and B, P=0.01 for skeletal muscle and adipose tissue). This increase was reflected as a significant increase in LA associated with pyruvate dehydrogenase (LA-PDH) and alpha ketoglutarate dehydrogenase (LA-KGD) in both tissues (Figure 3C), skeletal muscle (P=0.02 and 0.03 for LA-PDH and LA-KGD, respectively); (Figure 3D), adipose tissue, (P=0.02 for LA-PDH and LA-KGD). This increase have been established. We used real time PCR to study expression of adiponectin (Acrp30), a major insulin sensitizing hormone in adipose tissue from ob/ob mice treated with compound 1. As shown in Table 2, there was a significant increase in expression of Acrp30 with compound 1 treatment compared to vehicle-treated mice. We also studied the activity of AMPK, by measuring levels of phosphorylated protein in adipose tissue. There was no detectable increase in AMPK activity.

**Gene expression and activity studies:** To further understand the mechanism by which compound 1 exerts its beneficial effects on body weight, glucose and lipid homeostasis, we studied the expression and activity of genes for which definite roles in obesity and diabetes have been established. We used real time PCR to study expression of adiponectin (Acrp30), a major insulin sensitizing hormone in adipose tissue from ob/ob mice treated with compound 1. As shown in Table 2, there was a significant increase in expression of Acrp30 with compound 1 treatment compared to vehicle-treated mice. We also studied the activity of AMPK, by measuring levels of phosphorylated protein in adipose tissue. There was no detectable increase in AMPK activity.
**Figure 4: Effect of compound 1 on body weight, energy intake, body composition and insulin resistance in DIO mice**

**A:** Body weight change in animals treated with vehicle, rimonabant or compound 1 over the study period. Average body weight of 8 mice per group is shown in grams. Means ± SD for each time point are shown. Statistical significance for each time point was analyzed by using repeated measures ANOVA procedure with post-hoc testing. Body weight change for compound 1 versus vehicle was significant from day 14 onwards (*P=0.02 and P=0.007 for days 14 and 35, respectively). Body weight change for rimonabant versus vehicle and compound was significant from day 4 onwards (*P=0.02 and P=0.002 for day 8 and day 35, respectively).

**B:** Food consumed by animals treated with vehicle, rimonabant or compound during study period. Food intake was measured daily for animals which were housed in groups of 4 per box. Average food consumption per box was calculated. The average daily food intake of 8 mice (2 boxes) per group is shown in grams. Means ± SD for each time point are shown. Statistical analysis was performed for each time point by using repeated measures ANOVA procedure with post-hoc testing. Difference between rimonabant and vehicle-treated mice was significant for days 1-8.

**C:** Data from B represented as bar graph. Average food intake for each group is averaged for days 1-8 and 9-33. *P=0.0004 for Rimonabant versus vehicle for days 1-8. Food intake differences for Rimonabant for days 9-33 were not significant. No significant difference between compound 1 and vehicle.

**D:** Energy intake expressed in terms of body weight: Average energy intake in kilocalories during 10 day period divided by body weight X 100. *P=0.03 for compound 1 versus vehicle for days 21-33.

**E:** Lean (fat-free) mass (in grams) quantified by DEXA. Statistical analysis was performed using the two sample, paired t test. *P=0.0001 for rimonabant and P=0.002 for compound 1 compared to vehicle.

**F:** Fat mass (g) measured by DEXA. Statistical analysis was performed using the two sample, paired t test. *P=0.0000004 for day 4 and P=0.007 for days 14 and 35.

**G:** Lean mass (g) quantified by DEXA. Statistical analysis was performed using the two sample, paired t test. *P=0.001 for rimonabant and P=0.02 for compound 1 compared to vehicle.

**H:** Effect of compound 1 on insulin resistance: Fasting glucose and insulin levels were determined at the end of the study. N=8 per group. HOMA-IR was calculated from fasting glucose and insulin values using the formula, (HOMA-IR): (fasting insulin (pM) X fasting glucose (mM))/135. Statistical analysis was performed using the two sample, paired t test. *P=0.02 for compound 1 versus vehicle.
with compound 1 treatment in adipose tissue (Table 2), liver or skeletal muscle (data not shown). Similarly, there was no significant increase in AMPK activity in vitro in L6 muscle skeletal muscle cells and liver cell line (HePG2) cells treated with compound 1 (data not shown). We also observed that neither peroxisome proliferator-activated receptor (PPAR) α nor PPARα regulated genes (ACOX1 and CPT1) were significantly affected (data not shown) by compound 1 treatment.

**Compound 1 significantly decreases body weight gain in a mouse model of diet-induced obesity (DIO) without reducing caloric intake:** Based on the decrease in body weight seen in the genetic model of obesity, we next studied the effects of compound 1 on body weight in C57BL/6J mice that were fed a high fat diet as a model for diet induced obesity (DIO). In this study body weight effects and food intake were compared to that of an obesity compound, Rimonabant (SR141716). Rimonabant is a selective CB1 receptor endocannabinoid blocker which has been demonstrated to effectively reduce body weight and fat accumulation. This was further substantiated by significantly lower visceral fat pad weights of compound 1 and rimonabant treated animals compared to vehicle-treated animals (data not shown). Compound 1-treated animals also had significantly more lean mass than vehicle-treated animals (Figure 4G, P<0.02 compared to vehicle). This was in contrast to rimonabant-treated animals which showed significantly less lean mass than vehicle-treated animals (P=0.001 compared to vehicle).

**Compound 1 decreases insulin resistance in DIO mice:** We studied the effect of compound 1 on insulin resistance (IR) in the DIO mouse model by homeostasis model assessment (HOMA) method using the formula indicated in the Research Methods and Procedures section. Results are expressed as counts per minute (CPM) per milligram (mg) of protein per hour (hr). Data is the average of three independent experiments, and represent means ± SD. P values were calculated using two sample, paired t test. *P<0.00000009 compound 1-treated cells versus DMSO-treated control cells. A: Mitochondrial membrane potential in HSMM treated with compound 1. Differentiated HSMM cells were treated with 250 μM of compound 1. Mitochondrial membrane potential was determined using the JC-1 assay. Results are expressed as ratio of red to green fluorescence of JC-1. Data is the average of three independent experiments, and represent means ± SD. P values were calculated using two sample, paired t test. *P=0.00000009 compound 1-treated cells versus DMSO-treated control cells.

**Compound 1 increases energy expenditure in DIO mice:** Since compound 1 did not reduce food intake, we hypothesized that it may cause increased energy expenditure (EE) which could account for the reduction in body weight and fat accumulation. We used indirect calorimetry to measure the effect of compound 1 on oxygen consumption, carbon dioxide output, heat production and metabolic rate. The metabolic parameters such as glucose, triglycerides, low density lipoprotein (LDL), high density lipoprotein (HDL), total cholesterol (TC), high-density lipoprotein (HDL), and triglycerides were measured in 14-week-old ob/ob mice following treatment with compound 1 for 4 weeks. Mean ± SD are reported. N=8 animals per group.

**Table 1: Compound 1 improves plasma metabolic parameters in ob/ob mice**

<table>
<thead>
<tr>
<th>Plasma Metabolic Parameters</th>
<th>Vehicle</th>
<th>Compound 1</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>323.4 ± 92.8</td>
<td>205.75 ± 65.7</td>
<td>0.06</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>123.639 ± 44.8</td>
<td>63.178 ± 8.9</td>
<td>0.03</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>35.98 ± 2.71</td>
<td>23.67 ± 4.25</td>
<td>0.008</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>155.87 ± 6.52</td>
<td>176.94 ± 21.19</td>
<td>0.0001</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>17.67 ± 1.9</td>
<td>12.74 ± 1.5</td>
<td>0.004</td>
</tr>
</tbody>
</table>

**Table 2: Compound 1 increases Acrp30 expression in ob/ob mice**

<table>
<thead>
<tr>
<th>Gene expression/protein activity</th>
<th>Vehicle</th>
<th>Compound 1</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrp30 %</td>
<td>100 ± 20</td>
<td>160 ± 35</td>
<td>0.01</td>
</tr>
<tr>
<td>Phospho AMPK %</td>
<td>100 ± 15</td>
<td>93 ± 9</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Figure 5:** Effect of compound 1 on mitochondrial membrane potential and fat oxidation in skeletal muscle cells.

A: Mitochondrial membrane potential in HSMM treated with compound 1. Differentiated HSMM cells were treated with 250 μM of compound 1. Mitochondrial membrane potential was determined using the JC-1 assay. Results are expressed as ratio of red to green fluorescence of JC-1. P values were calculated using two sample, paired t test. *P=0.00000009 compound 1-treated cells versus DMSO-treated control cells.

B: Beta oxidation in C2C12 myotubules treated with compound 1. C2C12 compound 1-treated cells versus DMSO-treated control cells. P values were calculated using two sample, paired t test. *P=0.00000009 compound 1-treated cells versus DMSO-treated control cells.
Table 3: Compound 1 increases metabolic rate and energy expenditure in DIO mice

<table>
<thead>
<tr>
<th>RER</th>
<th>VO$_2$ (ml/g/h)</th>
<th>VCO$_2$ (ml/g/h)</th>
<th>Heat / LBM (cals /h/g)</th>
<th>Maximal aerobic capacity (ml O$_2$/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.77 ± 0.043</td>
<td>2308.0 ± 349.69</td>
<td>2386.75 ± 292.10</td>
<td>22.7 ± 7.8</td>
</tr>
<tr>
<td>Compound 1</td>
<td>0.782 ± 0.038</td>
<td>3861.00 ± 760.21</td>
<td>2881.72 ± 542.77</td>
<td>25.9 ± 0.45 *</td>
</tr>
</tbody>
</table>

Oxygen uptake (VO$_2$), Carbon dioxide output (VCO$_2$), respiratory exchange ratio (RER) and heat generation were measured by indirect calorimetry after 4 weeks of compound treatment in groups of 3 DIO mice from each group. Values were normalized to lean body mass (LBM), and represent means ± SD. Maximal aerobic capacity was calculated as milliliter (ml) O$_2$/minute (min)/per gram (g) LBM. Statistical significance was calculated by using the two sample, paired t test. P values shown are for compound 1 versus vehicle group. *P=0.04 for heat production and P=0.02 for maximal aerobic capacity.

In this study we report that pharmacological induction of LASY has beneficial effects on body weight and metabolic parameters in genetic and diet-induced rodent models of obesity. We have shown previously that LASY expression levels as well as levels of endogenous LA are lower in animal models of diabetes and obesity compared to lean, normoglycemic controls [12]. In the current study, we used a small molecule (compound 1) to demonstrate that induction of LASY expression in these animal models leads to significantly reduced body weight gain and better metabolic control. Our in vitro and in vivo data suggests that induction of LASY expression leads to increase in mitochondrial levels of LA, a powerful mitochondrial antioxidant with reported beneficial effects in obesity, type 2 diabetes and other diseases associated with oxidative stress [3-8]. In previous studies we demonstrated that increased endogenous LA levels restores depressed activities of antioxidant enzymes such as superoxide dismutase and catalase, which would, conceivably, lead to further alleviation of oxidative stress. Since oxidative stress is one of the earliest instigators of metabolic syndrome, reduction in oxidative stress in accumulated fat would be one of the first and most effective means of reducing body weight and improving the metabolic abnormalities associated with excess weight. Increase in endogenous LA would reduce inflammation, an effect which is also mediated by reduction of oxidative stress. Decreased oxidative stress and inflammation would interdependently contribute to better insulin sensitivity and improved glucose and fat metabolism, and eventually to body weight control. We have consistently observed an increase in expression of Acrp30 in adipose tissue from obese mice (Table 2) and rats treated with compound 1 compared to vehicle-treated mice. This could be a consequence of the relief in oxidative stress due to LASY induction, since Acrp30 expression is reportedly susceptible to reduction due to increased oxidative stress [19]. Increased expression of Acrp30, in itself, has numerous therapeutic effects in obesity and the metabolic syndrome such as weight loss, improved insulin sensitivity and cardiovascular benefits [20]. Thus increase in Acrp30 expression would augment the beneficial effects of LASY induction.

Interestingly, the decrease in body weight observed with compound 1 treatment was achieved without significant effects on food consumption in both genetic and diet-induced obesity models. We have shown that the ratio of energy intake to body weight is in fact significantly higher in compound 1 treated animals, indicating that in these animals body weight gain is not proportional to their food intake. Interestingly, these results are in contrast to the effects of exogenously administrated (dietary) LA, which lowers body weight by reducing caloric intake via inhibition of hypothalmic AMPK [21]. This study therefore highlights the mechanistic differences between the actions of dietary LA and LA which is endogenously synthesized. This is consistent with earlier findings that dietary LA is unable to compensate for deficiency of LA due to knock-down of LASY [9]. It is possible that dietary LA, for unknown reasons, cannot be incorporated into mitochondrial enzymes to fulfill the co-factor function of prosthethically-bound lipoamide. In contrast, based on our observation that compound 1 increases enzyme-bound LA, we infer that LA which is synthesized endogenously within the mitochondria is in a form that is conducive to its incorporation into mitochondrial enzymes. Future studies will reveal if the increase in enzyme bound LA due to compound 1 treatment translates to increase in the enzyme activities. It is likely that the antioxidant function of LA is distinct from its cofactor function, which is borne out by many studies including human trials showing efficacy of LA in oxidative stress mediated abnormalities [22].

The fact that compound 1 reduces body weight without decreasing energy intake suggests that the animals are metabolizing the excess calories consumed rather than converting it to fat. This turned out to be the case since we observed an increase in energy expenditure (EE) (measured as heat produced and maximal aerobic capacity) in DIO mice that were treated with compound 1. Although there was no significant changes in basal, whole body respiratory exchange rate (RER) determined by measuring VO$_2$ (oxygen consumption) and VCO$_2$ (Carbon dioxide output), there was a significant increase in average heat production and maximal aerobic capacity (Table 3), suggesting that compound 1 treatment increases peripheral energy utilization. This effect may be mediated by increased fat oxidation and increased mitochondrial uncoupling, with the oxidation energy being dissipated as heat. It also suggests an overall improvement in skeletal muscle mitochondrial function. Based on this data, we suggest that increasing energy expenditure without restricting food intake is a viable approach to managing obesity. Thus, in contrast to current research paradigms that focus mainly on central mechanisms for body weight reduction.
such as those involving CB1 receptor inverse agonists [23,24], serotonin receptor agonists [25] or melanin-concentrating hormone (MCH) receptors [26], pharmacological induction of LASY offers a novel approach whereby body weight reduction can be achieved without affecting food consumption. Our approach is not without precedent since there have been other reports in the literature demonstrating the feasibility of achieving weight reduction without energy intake reduction [27-30]. Calculation of the polar surface area of compound 1 revealed that this area is too large for it to cross the blood brain barrier (supplementary table). Therefore, it is unlikely that it activates any receptors in the brain. Moreover, in vitro radioligand binding assays revealed that compound 1 was inactive at human cannabinoid (CB) 1, melanocortin (MC) 4, Serotonin 5-hydroxytryptamine (5-HT2c) and Neuropeptide Y1 receptors which are associated with central regulation of appetite. In the DIO model, the effects of compound 1 paralleled that of rimonabant (SR141716), a known anti-obesity compound. The body weight reducing effect of rimonabant is primarily due to its blockage of CB1 receptors in the central nervous system, although peripheral receptor mediated effects have also been reported [23,24]. Although the net effect on body weight reduction mediated by compound 1 was not as dramatic as that with rimonabant, paradoxically, the effect on accumulation of adipose tissue was comparable, as revealed by DEXA analysis and comparison of fat pad weights with those of vehicle treated animals. DEXA analysis also revealed that compound 1-treated animals had significantly more (9%) lean mass compared to vehicle-treated animals. In contrast, rimonabant-treated animals had significantly less lean mass (4%) compared to vehicle-treated animals. It is conceivable that the increase in lean mass could obscure the actual loss in body weight following compound 1 treatment. This observation suggests that induction of LASY could have a beneficial effect in increasing lean mass, in conjunction with reducing body fat.

We speculate that increase in lean mass may be due to increase in muscle mass as a result of improvement in mitochondrial function that we observed as increased mitochondrial aerobic capacity in vivo. We demonstrated a significant improvement in mitochondrial function in a human skeletal muscle cell line that was treated with compound 1, providing in vitro validation for this hypothesis. The correlation between mitochondrial function and muscle mass is well established, with oxidative stress mediated deterioration in mitochondrial function being associated with decreased muscle mass [31-38]. Conversely, alleviation of oxidative stress through LASY induction would improve mitochondrial function and increase muscle mass. Although the data presented in this study strongly suggests a positive correlation between induction of LASY and improvement in mitochondrial function, it is conceivable that compound 1 may also have “broader” effects on mitochondria that are not limited to LASY induction. For example, at the present time we don’t know if compound 1 impacts other effectors of mitochondrial biogenesis. Thus LASY induction could in part account for improvement in mitochondrial function, but further studies will reveal if compound 1 has additional effects.

The increase in EE that we observed in this study could involve increased fat oxidation. This is consistent with in vitro studies done with C2C12 myotubes and liver cells (HepG2) which revealed that compound 1treatment significantly increases beta oxidation (Figure 5B). The extent of increase in beta oxidation was similar to the effects of the PPAR α agonist, fenofibrate in liver cells. However, unlike fenofibrate, compound 1 did not have a significant effect on expression of PPAR α or genes activated by PPAR α, such as ACOX or CPT1. There was, however, a significant increase in expression of CPT2 which is involved in the transfer of long-chain fatty acids into the mitochondria. Since CPT2 expression is not dependent on PPAR α activation [39], it is likely that increase in beta oxidation by compound 1 may be through a mechanism that is independent of PPAR α activation.

Body weight reduction due to compound 1 treatment was accompanied by improvement in metabolic parameters in obese mice. In genetically obese, diabetic mice (ob/ob), there were significant reductions in plasma glucose in the OGTT and insulin assay, accompanied by improvements in lipid profiles (triglyceride, low density lipoprotein and high density lipoprotein). In the DIO model, there was significant reduction in insulin resistance by HOMA-IR. Conceivably, these effects could be attributable to increased fat oxidation which would result in reduced accumulation of fat in muscle and liver. Based on the established correlation between muscle lipid content and insulin resistance [40], reduction in muscle fat would ameliorate insulin resistance. It is also known that inflammation is associated with dyslipidemia. In an earlier study we showed that over expression of LASY have anti-inflammatory effects [12]. Therefore, it is likely that compound 1 by increasing LASY expression, would reduce inflammation and contribute to improved lipid profile. Although we currently don’t have the data to support this hypothesis, it is possible that compound 1 corrects dyslipidemia in obesity/diabetes by restoring insulin-mediated malonyl CoA activity (due to increase in insulin sensitivity), and decreasing mobilization of free fatty acids (FFAs) from adipose tissue to the liver. Increased mobilization of FFAs and aberrant distribution of lipids among the major liproteins are primarily responsible for the increase in LDL and decrease in HDL which is prevalent in obesity/diabetes. Specifically, increased mobilization of FFAs results in the overproduction of very low density lipoprotein (VLDL) which promotes transfer of cholesteryl esters from high density lipoprotein (HDL) and low density lipoprotein (LDL) to VLDL in exchange for triglycerides (TG). Triglyceride enrichment of HDL causes dissociation of ApoA-I and its rapid clearance, reducing the availability of HDL for reverse cholesterol transport [41]. Increased TG-enriched LDL can undergo lipolysis to yield small, dense LDL. Low levels of HDL and the presence of small dense LDL are each independent risk factors for cardiovascular disease [41]. By preventing TG-enrichment of HDL and LDL, treatment with compound 1 would ensure the proper distribution of lipids, so that HDL is available for reverse cholesterol transport and LDL is in a form that ensures its clearance.

Based on our studies with the LASY promoter, it appears that induction of LASY by compound 1 is at the level of transcription. We have identified the minimal LASY promoter as a 200 base pair (bp) region upstream of the LASY translational start site. In addition, using site-directed mutagenesis, we have identified cis-acting regulatory/promoter elements within this region that are critical for LASY expression. One of these is the heat shock element (HSE) which is bound by the transcription factor, heat shock factor-1 (HSF-1). There are two overlapping HSEs within the 200 bp LASY promoter. Using an electrophoretic mobility shift assay (EMSA) we found that treatment with nuclear extracts from cells treated with compound 1 caused a shift in a biotin-labeled HSE probe (unpublished observation). This suggests that compound 1 activates HSF-1, which is a key step that enables its translocation into the nucleus to bind to the HSE and initiate the transcription of heat shock genes. Although the exact mechanism by which compound 1 activates HSF-1 is not currently known, this study suggests that induction of LASY transcription is,
at least in part, mediated by activation of HSF-1. Two other elements within the 200 bp region that we have identified as being necessary for LASY transcription are the stress response element (STRE) and PAX4 (unpublished observation).

In conclusion, pharmacological induction of LASY offers a novel peripheral approach for management of obesity and associated metabolic abnormalities. We present data demonstrating the beneficial effects of compound 1 treatment in genetic and diet-induced obesity models. This data supports the concept that changes in energy expenditure alone without energy intake restriction are sufficient to produce significant reduction in body weight with ensuing improvements in metabolic status.

Acknowledgement

We thank Ravi Krishna Babu for his help with the oral glucose tolerance test and ob/ob study. We acknowledge the assistance of Xiaoyan Ma and Matt Rycyk in harvesting animal tissues. We thank Dr. Sathyavani and Priyanka Tilak for their assistance with statistical analysis. Data in this manuscript was presented at the 67th Scientific Sessions of the American Diabetes Association, Chicago, IL.

References


Mitochondrial function and apoptotic susceptibility in aging skeletal muscle. Aging Cell 7: 2-12.


