Novel Small-Molecule PGC-1α Transcriptional Regulator With Beneficial Effects on Diabetic db/db Mice

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Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) has been shown to influence energy metabolism. Hence, we explored a strategy to target PGC-1α expression to treat metabolic syndromes. We developed a high-throughput screening assay that uses the human PGC-1α promoter to drive expression of luciferase. The effects of lead compound stimulation on PGC-1α expression in muscle cells and hepatocytes were investigated in vitro and in vivo. A novel small molecule, ZLN005, led to changes in PGC-1α mRNA levels, glucose uptake, and fatty acid oxidation in L6 myotubes. Activation of AMP-activated protein kinase was involved in the induction of PGC-1α expression. In diabetic db/db mice, chronic administration of ZLN005 increased PGC-1α and downstream gene transcription in skeletal muscle, whereas hepatic PGC-1α and gluconeogenesis genes were reduced. ZLN005 increased fat oxidation and improved the glucose tolerance, pyruvate tolerance, and insulin sensitivity of diabetic mice. Hyperglycemia and dyslipidemia also were ameliorated after treatment with ZLN005. Our results demonstrated that a novel small molecule selectively elevated PGC-1α in myotubes and skeletal muscle and exerted promising therapeutic effects for treating type 2 diabetes.

RESEARCH DESIGN AND METHODS

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See accompanying commentary, p. XXX.
of 24 h, luciferase substrate was added to each well and the released luciferin signal then was detected using an EnVision microplate reader.

Luciferase enzyme assay. Luciferase was diluted to 20 units and incubated with compounds in a 384-well plate. Then, luciferase substrate was added, and the released luciferin signal was detected using an EnVision microplate reader.

Measurement of fatty acid oxidation. The assay was initiated by adding [9,10-\textsuperscript{3}H]-palmitic acid to a final concentration of 250 \mu M in L.0µL and 1.5 \muCi per well in Dulbecco’s modified Eagle’s medium (DMEM). After incubation with the compound for 4 h in differentiated L6 myotubes, a sample from each well was added to charcoal slurry for centrifugation and then the radioactivity was measured.

Transfection. For plasmid transfection, L6 myoblasts were transfected with Lipofectamine 2000 containing the pGL-3 basic vector, PGC-1\textalpha promoter luciferase, delta MEF, or delta CRE promoter luciferase. Six hours after transfection, the medium was changed for differentiation. After being cultured overnight, the cells were incubated with compound for 24 h. For transfection of small interfering RNA (siRNA), 20 nmol/L siRNA (sense: GAUUCAAACUGACAGAUU; antisense: AAUCGUCUGAGUUUGAAUC) targeting PGC-1\textalpha was transfected on day 4 of L6 myotube differentiation using siLentFect lipid in serum-free DMEM. Six hours after transfection, the medium was replaced with normal medium. The next day, the cells were treated with compound for 24 h for studies.

Animal experiments. The animal experiments were approved by the Animal Ethics Committee of the Shanghai Institute of Materia Medica. Male C57BKS db/db and lean littermate C57BKS mice were housed in a temperature-controlled room (22 ± 2°C) with a 12-h light/dark cycle. For the pharmacokinetic studies, a single dose of ZLN005 was administered to db/db mice. The plasma and tissue samples were collected at different time points, and the compound concentrations were determined by liquid chromatography–mass spectrometry/mass spectrometry. For chronic treatment, six to eight 8-week-old mice were assigned randomly to the various treatment groups by body weight and glucose levels. Lean and db/db mice received oral administration of either vehicle (0.5% methylcellulose), ZLN005, or metformin for 6 weeks. Body weight and food intake were recorded daily. Fasting blood glucose levels were measured after 6 h of fasting. At the 3rd week of treatment, the effect of chronic ZLN005 administration on the respiratory exchange ratio (RER) of db/db mice was measured using an eight-chamber indirect calorimeter. The glucose tolerance test (GTT) (0.5 g. kg\textsuperscript{-1} sodium pyruvate i.p.) and insulin tolerance test (1 unit. kg\textsuperscript{-1} insul in i.p.) were performed after 6 h of starvation. The pyruvate tolerance test (PTT; 1.5 g. kg\textsuperscript{-1} sodium pyruvate i.p.) was performed in mice that had been fasted overnight. Plasma concentrations of nonesterified fatty acids (NEFAs), triglycerides, and insulin were measured as previously described (26). Plasma 

PGC-1\textalpha is a powerful transcriptional coregulator of GLUT4 and mitochondrial genes, including components of the electron transport system. As seen in Fig. 1D, the mRNA levels of GLUT4, NRF1, estrogen-related receptor \alpha (ERR\alpha), cytochrome c oxidase 5b (cox5b), and acyl-CoA oxidase (24,34) were increased by ZLN005 (10 \muM/L).

Because GLUT4 plays a crucial role in glucose uptake in skeletal muscle, we investigated this effect of ZLN005 further. As shown in Fig. 1E, ZLN005 stimulated glucose uptake dose-dependently after 24 h of treatment, with 20 \muM/L of ZLN005 resulting in a 1.8-fold improvement. Because the induction of the expression of genes involved in mitochondrial biogenesis and fatty acid oxidation (FAO) (35) was observed. As shown in Fig. 1F, ZLN005 increased oxidation of palmitic acid dose-dependently, with 20 \muM/L ZLN005 resulting in a 1.28-fold increase after 24 h compared with control. These results demonstrated that ZLN005 stimulated the expression of PGC-1\textalpha and downstream genes in skeletal muscle cells and improved glucose utilization and FAO.

RESULTS

ZLN005 increases expression of the PGC-1\textalpha gene in L6 myotubes. Studies have shown an inverse correlation between levels of PGC-1\textalpha in muscle and mitochondrial activity, insulin resistance, and T2DM (32,33). Therefore, we created an HEK293 stable cell line containing luciferase expression driven by the human PGC-1\textalpha promoter (PGC-1\textalpha-luc); then the luciferase reporter assay was optimized for application in automated HTS. After we randomly screened a library containing 45,000 pure synthetic compounds with diverse structures, the novel compound ZLN027 (Supplementary Fig. 1A) was found to increase the PGC-1\textalpha promoter reporter by 1.7-fold (Supplementary Fig. 1B). Forskolin and dexamethasone, which were reported to stimulate expression of PGC-1\textalpha, had stimulatory effects at 1.4- and 2.0-fold, respectively. By checking the cell survival rate, we found obvious cytotoxicity for ZLN027 in HEK293 and L6 myotubes. Extensive data mining identified another compound, ZLN005 (Fig. 1A), which had the same chemical backbone but did not have obvious cytotoxicity (Supplementary Fig. 1D and E). ZLN005 was observed to potently inhibit luciferases, but ZLN027 was not (Supplementary Fig. 1C). These results might explain the discrepancy between the two compounds in the PGC-1\textalpha promoter reporter assay. The transcriptional modulatory effect of ZLN005 in L6 myotubes also was investigated. As shown in Fig. 1B, ZLN005 increased PGC-1\textalpha mRNA levels in a dose-dependent manner; 20 \muM/L ZLN005 caused a threefold increase over the control after 24 h. At 10 \muM/L, the PGC-1\textalpha mRNA levels were increased to almost the same extent at 16 to 48 h (Fig. 1C).

PGC-1\textalpha is a powerful transcriptional coregulator of GLUT4 and mitochondrial genes, including components of the electron transport system. As seen in Fig. 1D, the mRNA levels of GLUT4, NRF1, estrogen-related receptor \alpha (ERR\alpha), cytochrome c oxidase 5b (cox5b), and acyl-CoA oxidase (24,34) were increased by ZLN005 (10 \muM/L).

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ZLN005 did not increase the expression of the PGC-1\textalpha gene in rat primary hepatocytes. Gluconeogenesis plays an important role in regulating glucose levels, and PGC-1\textalpha is known to stimulate this process (36). ZLN005 had no effect on levels of PGC-1\textalpha in rat primary hepatocytes after 24 h of treatment (Fig. 2A). The expression of the key gluconeogenic enzyme coregulated by PGC-1\textalpha, phosphoenolpyruvate carboxykinase (PEPCK), was not affected by ZLN005 (Fig. 2B), nor did ZLN005 increase glucose production (Fig. 2C) in primary hepatocytes. The contrary effects of ZLN005 in L6 myotubes and primary hepatocytes suggested that expression of PGC-1\textalpha was regulated in a cell type-specific manner.

AMP-activated protein kinase is involved in the mechanism inducing PGC-1\textalpha in L6 myotubes. To evaluate the dependence of PGC-1\textalpha in the effects of ZLN005, L6 myotubes were transfected with PGC-1\textalpha siRNA. ZLN005-stimulated expression of the PGC-1\textalpha gene and oxidation of palmitic acid was blocked upon PGC-1\textalpha silencing (Fig. 3A and B). This indicated that ZLN005-stimulated FAO was mediated by PGC-1\textalpha.

To clarify the mechanism of ZLN005 acting on PGC-1\textalpha, transfection of the PGC-1\textalpha promoter harboring truncated mutations in the MEF2 and CREBP binding sites were analyzed (27). Because ZLN005 inhibits luciferase activity, luciferase protein levels were investigated as the direct
readout for transcriptional activity. The PGC-1α promoter drives luciferase induction upon treatment with ZLN005 (10 μmol/L), but the promoter constructs with the truncated CREBP binding site showed little difference compared with the PGC-1α promoter. However, a truncated MEF2 binding site resulted in an ablation of the increase in luciferase protein in response to treatment with ZLN005 (Fig. 3C). This suggested that ZLN005 stimulation of PGC-1α expression was dependent on the MEF2 binding site.

Studies have reported that p38 MAPK, the energy sensor protein AMP-activated protein kinase (AMPK), and cAMP-responsive CREBP signaling pathways control expression of PGC-1α (37,38). Therefore, we determined whether treatment of L6 myotubes with ZLN005 (10 μmol/L) would lead to activation of AMPK, p38 MAPK, or CREBP. Phosphorylation levels of p38, which phosphorylates and activates MEF2 (39) and phosphorylates PGC-1α protein (40), slightly decreased, suggesting the p38 pathway was not the

FIG. 1. ZLN005 increases expression of the PGC-1α gene in L6 myotubes. L6 myotubes were differentiated for 4–6 days. A: The structure of ZLN005 (molecular weight 250.3). B: Dose-dependent effect of ZLN005 on PGC-1α mRNA levels. L6 myotubes were treated for 24 h with different doses of ZLN005 or 1 mmol/L AICAR as a positive control. C: Time course of ZLN005 on PGC-1α mRNA levels. D: Effect of ZLN005 (10 μmol/L) on relative mRNA levels. E: Dose-dependent effect of ZLN005 on glucose uptake over 24 h. Insulin (100 nmol/L) was added in the last 30 min. F: Dose-dependent effect of ZLN005 on palmitic acid oxidation over 24 h. Radioactive medium containing compounds of interest was changed at the start of the last 4 h. Two millimolar AICAR also was added in the last 4 h. *P < 0.05, **P < 0.01 compared with DMSO.
cause of the increased expression of PGC-1α. Phosphorylation of CREBP remained unchanged, which was consistent with the previously observed lack of effect on luciferase expression for the CREBP binding site. Meanwhile, the phosphorylation of AMPK and its downstream acetyl-CoA carboxylase (ACC) were increased significantly (Fig. 3D).

The dose-dependent activation of AMPK by ZLN005 was confirmed at 24 h (Fig. 3E). Thus we examined whether the effects of ZLN005 on glucose uptake were dependent...
on AMPK using the AMPK inhibitor compound C. Pre-
treatment with compound C attenuated the ability of
ZLN005 to induce phosphorylation of AMPK and expres-
sion of PGC-1α (Fig. 3F and G). These results sugges-
ted that PGC-1α expression stimulated by ZLN005 in L6
myotubes was dependent on the AMPK pathway. To in-
vestigate the mechanism of AMPK activation by ZLN005,
we measured the direct effect of ZLN005 on AMPKα2β1γ1
and AMPKα2β2γ1 on a molecular level, and no catalytic
kinase activation was observed (data not shown). We
found it could increase the ADP-to-ATP ratio in L6 myotubes
(Fig. 3H) and mildly increase oxygen consumption in iso-
mated muscle mitochondria (Fig. 3I). This suggests that
ZLN005 increases the ADP-to-ATP ratio to activate AMPK
by uncoupling mitochondria respiration in L6 myotubes.

Chronic effects of ZLN005 on the RER. Pharmacoki-
etic studies of db/db mice showed that ZLN005 was
absorbed into the plasma quickly, reaching a concentra-
tion of 3.7 μmol/L within 15 min. The concentration de-
clined to 0.44 μmol/L within 4 h (Fig. 4A) after a single oral
dose of 15 mg/kg. ZLN005 reached a fairly high concen-
tration of 60.9 μmol/L within 15 min in liver tissue, which
then declined to 10.9 μmol/L within 4 h (Fig. 4B). The
concentration of ZLN005 in muscle tissue was stable at
approximately 3–4 μmol/L over 4 h.

Because PGC-1α is a key regulator of oxidative metab-
olism, we studied the effects of ZLN005 on whole-body fat
oxidation using indirect calorimetry. The db/db mice were
dosed with ZLN005 (15 mg · kg⁻¹ · day⁻¹) or vehicle for
2 weeks. The last administration was given 4 h before the
experiment. The animals were monitored for oxygen
consumption and CO₂ production for 21 h (between 1700
and 1400 h). There were no significant changes in VO₂ and
VCO₂ (Fig. 4C and D), but a decrease in the RER was ob-
served, indicating a shift to fatty acid use (Fig. 4E). The
heat was almost unchanged during the 21-h measurement,
except for a slight increase during cycles 11 to 20 (Fig. 4F).

Chronic antidiabetic efficacy of ZLN005. To assess the
antidiabetic efficacy of ZLN005 in vivo, we investigated the
effect of chronic ZLN005 administration in db/db mice and
lean mice. ZLN005 (15 mg · kg⁻¹ · day⁻¹) was administered
orally for 6 weeks, and metformin (250 mg · kg⁻¹ · day⁻¹)
was used as a positive control. During the treatment,
ZLN005 did not affect body weight gain or food intake in
either db/db mice or lean mice (Fig. 5A and B). In lean
mice, plasma glucose generally was unchanged by ZLN005
treatment. In db/db mice, however, random blood glucose
and fasting blood glucose levels decreased significantly
over 4 weeks by ZLN005 and metformin treatment (Fig. 5C
and D). ZLN005 did not alter glucose tolerance in lean
mice, but in db/db mice it improved glucose clearance, as
evidenced by the approximately 14% decrease in the area
under the curve (AUC) (Fig. 5E). An insulin tolerance test
revealed that treatment with ZLN005 significantly de-
creased insulin resistance in db/db mice, as evidenced by
the approximately 18% decrease in the AUC (Fig. 5F).
A PTT also was performed in db/db mice, and ZLN005
improved pyruvate tolerance, as evidenced by the 16% de-
crease in the AUC (Fig. 5G).

In db/db mice, plasma NEFA and triglyceride levels were
decreased by 20% and 37%, respectively, and cholesterol
was decreased by 10% (Table 1) with ZLN005 treatment.
Plasma insulin and β-hydroxybutyrate content, liver/body-
weight index and adipose composition, and muscle and
liver triglyceride levels, however, were not ameliorated by
treatment with ZLN005 or metformin.

Together, these results indicated that ZLN005 had anti-
hyperglycemia and antihyperlipidemia effects and in-
creased insulin sensitivity in db/db mice but not lean mice.
These results suggested that ZLN005 might act selectively
in the diabetic mouse model and have no adverse effects
on normal lean mice.

Tissue-specific efficacy of ZLN005 in skeletal muscle
and liver tissue of diabetic db/db mice. To clarify the
tissue-specific efficacy, we found that chronic treatment
with ZLN005 led to significant induction of PGC-1α mRNA
expression and PGC-1α target genes in the gastrocnemius,
including GLUT4 and mitochondria OXPHOS genes such as
ERRα, cytochrome-c, cox5b, ATPase-F1α, and uncoupl-
ing protein 3. Genes related to FAO, such as medium-
chain acyl-CoA dehydrogenase (MCAD) and long-chain
acyl-CoA dehydrogenase, also were induced. However,
NRF1 and carnitine palmitoyl transferase 1 remained un-
changed (Fig. 6A).

We also investigated gene expression in liver tissue.
Unlike in skeletal muscle, PGC-1α mRNA was down-
regulated by 34% compared with the vehicle group. Its
downstream gluconeogenic genes, including glucose-6-
phosphatase (G6Pase) and PEPC, were reduced by 31% and
27%, respectively (Fig. 6B), in response to ZLN005
treatment. PGC-1α is known to coordinate the mitochon-
dria OXPHOS and FAO in liver, so the expression of mi-
tochondria OXPHOS genes, such as cytochrome-c, ERRα,
cox5b, and ATPase-F1α, also were investigated. However,
no reduction was observed in the mRNA level of these
genes. Similarly, no changes in carnitine palmitoyl trans-
ferase 1, MCAD, and acyl-CoA oxidase were observed.

PGC-1α has emerged as a master regulator of mito-
ochondria, and mitochondrial biogenesis resulting from an
increased number of mitochondrial DNA copies. Indeed,
mitochondrial DNA was increased (31%) by ZLN005 in the
gastrocnemius of db/db mice, an effect that was not ob-
served in liver (Fig. 6C). We also measured AMPK and
ACC phosphorylation in abdominal muscle and liver tissue
of db/db mice. Consistent with the results found at the
cellular level, AMPK and ACC phosphorylation were in-
creased in abdominal muscle but not in liver tissue (Fig.
6D and E). These differing effects suggested that ZLN005
increased PGC-1α expression in a tissue-specific manner
in vivo.

DISCUSSION

Transcription cofactor PGC-1α is precisely regulated in
a number of metabolic tissues and physiological contexts,
such as in response to fasting in the liver and exercise in
muscle. It is dysregulated in pathological states, such as
mitochondrial dysfunction in skeletal muscle and hyper-
gluconeogenesis. It may, therefore, be a potential target for
drug discovery in the treatment of metabolic syndromes.
Arany et al. (34) recently set up an HTS method for the
identification of small molecules that induced
PGC-1α expression and showed positive effects on
PGC-1α transcriptional regulation. During this process
they identified a luciferase assay to identify small molecules and demonstrate positive effects on
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PGC-1α transcriptional regulation.
FIG. 4. Chronic effects of ZLN005 on RER in db/db mice. A: Mean plasma concentration time profiles of ZLN005 after a single oral dose (15 mg·kg⁻¹) in db/db mice (n = 3). B: Distribution of ZLN005 in tissues harvested after a single oral dose (n = 3). C and D: For RER measurement, 8-week-old db/db mice were gavaged with vehicle (0.5% methylcellulose) or ZLN005 (15 mg·kg⁻¹·day⁻¹) for 2 weeks. After a 4-h rest, mice were placed in a metabolic chamber and observed over a 21-h period (n = 6–8). Energy expenditure was evaluated by oxygen consumption (VO₂) and carbon dioxide release (VCO₂). E and F: Changes in RER and heat throughout the monitoring period (white circle = vehicle, black circle = ZLN005). The adjacent bar graphs represent the average for each group. *P < 0.05, **P < 0.01 compared with vehicle.
FIG. 5. Antidiabetic effects of ZLN005 in db/db mice. Eight-week-old db/db mice were gavaged with vehicle (0.5% methylcellulose), ZLN005 (15 mg · kg⁻¹ · day⁻¹), or metformin (250 mg · kg⁻¹ · day⁻¹) (n = 6–8) and lean mice (wt) were gavaged with vehicle (0.5% methylcellulose) and ZLN005 (15 mg · kg⁻¹ · day⁻¹).

A: Body weight. B: Food consumption. C: Random blood glucose. D: Fasting blood glucose. E: Blood glucose levels after an intraperitoneal glucose load (1.5 g · kg⁻¹) performed after 4 weeks of treatment. The areas under the curve are indicators of glucose clearance. F: Blood glucose levels after an intraperitoneal insulin load (1 unit insulin · kg⁻¹) performed after 5 weeks of treatment in db/db mice. The areas under the curve are indicators of insulin clearance. G: Blood glucose levels after an intraperitoneal sodium pyruvate load (1.5 g · kg⁻¹) given after 5 weeks of treatment in db/db mice. The areas under the curve are indicators of pyruvate clearance (white circle = db/db vehicle, black circle = db/db ZLN005, white square = db/db metformin, white triangle = wt vehicle, black triangle = wt ZLN005). *P < 0.05, **P < 0.01 compared with vehicle.
beneficial in metabolic disorders. First, we chose L6 myotubes to validate ZLN005. We found that it increased PGC-1α and downstream gene expression. Consistent with increased gene expression, FAO and glucose uptake also were up-regulated in L6 myotubes. An siRNA study then demonstrated that ZLN005-stimulated oxidation of palmitic acid was dependent on PGC-1α.

In the chronic treatment of db/db mice, the GLUT4 and mitochondrial OXPHOS genes were up-regulated in the gastrocnemius. Some of the regulators of FAO, such as long-chain acyl-CoA dehydrogenase and MCAD, also were up-regulated by ZLN005, consistent with ZLN005 promoting palmitic acid oxidation in L6 myotubes. The number of mitochondria was increased simultaneously, demonstrating that ZLN005 might promote mitochondria biogenesis.

Liver gluconeogenesis is known to be highly regulated by PGC-1α (19). Excessive gluconeogenesis is one of the symptoms of T2DM. We observed down-regulation of hepatic expression of PGC-1α, PEPCK, and G6Pase and improvement of PTT results in db/db mice. Several studies have shown that liver-specific down-regulation of PGC-1α leads to defects in lipid metabolism and hepatic insulin resistance (41), suggesting PGC-1α regulation must be tightly connected to lipid homeostasis and glucose metabolism.

Because decreased expression of PGC-1α was observed in liver tissue, the influence of PGC-1α on FAO and mitochondrial biogenesis genes was explored next. It is noteworthy that these expression levels remained unchanged. It has been reported that mice with a germ line-disruption of PGC-1α exhibit normal mitochondrial abundance and morphology in the liver (42). Thus, compensatory pathways to maintain the function of liver mitochondria might exist. It also was reported that increased insulin sensitivity might elicit the phosphorylation and inhibition of PGC-1α through kinase Akt2/protein kinase B-β (43). In contrast to the effects observed in db/db mice, ZLN005 had no effect on the expression of PGC-1α and PEPCK in primary hepatocytes. Therefore, we speculated that alterations in PGC-1α in the liver tissue of db/db mice might be a secondary phenomenon.

The effect of ZLN005 incorporation in vivo in lean mice and db/db mice was tested. After a 2-week treatment, a reduction in the RER was observed, which reflected the preferential use of lipids over carbohydrates as a source of energy. After a 6-week treatment, ZLN005 improved glucose homeostasis and insulin sensitivity, possibly from muscle glucose uptake because an increase in GLUT4 expression was observed. This effect also was possible through diminished production of hepatic glucose, a principal constituent of whole-body glucose homeostasis. The improvement in PTT values may have resulted from down-regulation of PEPCK and G6Pase gene expression. Lipid profiles, such as triglycerides, NEFAs, and cholesterol levels, were reduced, potentially as a consequence of enhanced use of whole-body fat.

Because PGC-1α is activated in pancreatic β-cells in animal models of obesity and T2DM, poor transcriptional regulation might lead to a deficiency in insulin secretion (44). The unchanged plasma insulin levels suggested that ZLN005 might not show adverse effects on pancreatic β-cells. In addition, the antidiabetic action of ZLN005 was not a direct consequence of food intake because body weight gain and food intake differences between groups were minimal. Furthermore, ZLN005 had no major side effects in lean mice, indicating that it worked specifically in diabetic mice. For preliminary safety evaluation, Sprague-Dawley rats were administered either vehicle or ZLN005 (75 mg·kg⁻¹·day⁻¹) orally for 14 days. All the animals survived, and no obvious change in the body weight gain or metabolic parameters was observed (Supplementary Tables 2–4).

At the PGC-1α promoter, there is a MEF binding site for the transcription factor MEF2, an insulin receptor substrate binding site for forkhead box class-O and a CRE binding site for activating transcription factor 2 and CREB, all of which enhance PGC-1α transcription (32). In this case, we investigated the effect of ZLN005 on CRE and MEF binding sites and found that MEF was necessary for the up-regulation of PGC-1α in L6 myocytes.

A number of intracellular pathways are known to impinge on PGC-1α, including signaling by cAMP, AMPK, Ca²⁺, and p38 MAPK (40,45,46). To investigate the mechanism by which ZLN005 enhanced PGC-1α expression in L6 myotubes, we sought to identify signaling pathways and found that phosphorylation of Thr-180/Tyr-182 in p38 MAPK, which phosphorylates and activates MEK2 and activating transcription factor 2, was not increased after treatment with ZLN005. In addition, phosphorylation of Ser-133 of CREB, which can be stimulated by Ca²⁺ signaling through calmodulin-dependent protein kinase IV.

### Table 1: Chronic effects of ZLN005 and metformin on metabolic variables in db/db mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vehicle</th>
<th>ZLN005</th>
<th>Metformin</th>
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<tbody>
<tr>
<td>Plasma triglyceride (mmol)</td>
<td>0.52 ± 0.07</td>
<td>0.33 ± 0.02**</td>
<td>0.36 ± 0.03*</td>
</tr>
<tr>
<td>Plasma NEFPA (mEq/L)</td>
<td>1.02 ± 0.07</td>
<td>0.82 ± 0.07*</td>
<td>0.85 ± 0.04*</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol)</td>
<td>2.38 ± 0.02</td>
<td>2.14 ± 0.09*</td>
<td>2.21 ± 0.07</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>1.41 ± 0.18</td>
<td>2.26 ± 0.73</td>
<td>2.38 ± 0.59</td>
</tr>
<tr>
<td>Plasma β-hydroxybutyrate (mmol)</td>
<td>0.65 ± 0.09</td>
<td>0.64 ± 0.06</td>
<td>0.62 ± 0.12</td>
</tr>
<tr>
<td>Liver (% body weight)</td>
<td>4.83 ± 0.16</td>
<td>4.82 ± 0.14</td>
<td>5.24 ± 0.04*</td>
</tr>
<tr>
<td>Epididymal fat (% body weight)</td>
<td>4.96 ± 0.18</td>
<td>4.76 ± 0.15</td>
<td>4.93 ± 0.03</td>
</tr>
<tr>
<td>Perirenal fat (% body weight)</td>
<td>2.57 ± 0.09</td>
<td>2.62 ± 0.08</td>
<td>2.44 ± 0.07</td>
</tr>
<tr>
<td>Subcutaneous fat (% body weight)</td>
<td>6.36 ± 0.37</td>
<td>6.09 ± 0.33</td>
<td>7.44 ± 0.41*</td>
</tr>
<tr>
<td>Hepatic triglyceride (μmol/g)</td>
<td>161.3 ± 14.7</td>
<td>135.3 ± 11.2</td>
<td>206.2 ± 20.9*</td>
</tr>
<tr>
<td>Muscle triglyceride (μmol/g)</td>
<td>130.4 ± 7.5</td>
<td>126.4 ± 19.0</td>
<td>122.1 ± 13.5</td>
</tr>
</tbody>
</table>

The data represent the mean ± SE of 6–8 mice. *P < 0.05; **P < 0.01 compared with the vehicle group.
and calcineurin A, also did not change (32). However, AMPK was activated by ZLN005 in L6 myotubes and the muscle tissue of db/db mice, and PGC-1α expression and glucose uptake are dependent on AMPK activation at the cellular level. To further address this issue, we found ZLN005 activated AMPK by mildly uncoupling mitochondria to increase the cellular ADP-to-ATP ratio. The weak uncoupling of mitochondria leading to a beneficial effect on metabolism was reported previously (47). AMPK is an energy sensor that is activated upon depletion of energy (48) and phosphorylates PGC-1α on Thr-177 and Ser-538 in skeletal muscle (49). It is plausible that AMPK phosphorylated PGC-1α and that PGC-1α then acted through positive feedback, binding with MEF2C to increase its own gene expression in myotubes (27). It is interesting that the AMPK pathway did not seem to be affected in rat primary hepatocytes (Supplementary Fig. 2) or the liver tissue of db/db mice, possibly because of the different energy states of the two types of cells. Considering the discrepancy in L6 myotubes and primary hepatocytes, and because MEF2 proteins are expressed predominantly in muscle and the brain (50), we speculated that ZLN005 regulates PGC-1α through muscle cell–specific transcription factors such as MEF2. Thus, the down-regulation of PGC-1α mRNA in the liver tissue of db/db mice might occur as a result.

Overall, our results demonstrated that small molecule–mediated tissue-specific activation of PGC-1α in skeletal muscle is feasible and that the novel compound ZLN005 exerts beneficial effects in an animal model of T2DM. These results further support the potential of PGC-1α as a drug target for the treatment of T2DM and metabolic syndromes.
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L.-N.Z. contributed to research data, discussion, and preparation of the manuscript. F.W., C.-M.X., and T.-C.D. contributed to the animal experiments. H.-Y.Z. contributed to the synthesis of compounds. Y.-Y.F., Y.-Y.L., M.G., and L.-Y.W. contributed to the discussion and research data. J.-Y.L., J.-K.S., and J.L. contributed to the study design, discussion, and editing of the manuscript.

J.-Y.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis.

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