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L312, a novel PPARγ ligand with potent anti-diabetic activity by selective regulation

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ABSTRACT

Background: Selective PPARγ modulators (sPPARγM) retains insulin sensitizing activity but with minimal side effects compared to traditional TZDs agents, is thought as a promising strategy for development of safer insulin sensitizer.
Methods: We used a combination of virtual docking, SPR-based binding, luciferase reporter and adipogenesis assays to analyze the interaction mode, affinity and agonistic activity of L312 to PPARγ *in vitro*, respectively. And the anti-diabetic effects and underlying molecular mechanisms of L312 was studied in db/db mice.
Results: L312 interacted with PPARγ-LBD in a manner similar to known sPPARγM. L312 showed similar PPARγ binding affinity, but displayed partial PPARγ agonistic activity compared to PPARγ full agonist pioglitazone. In addition, L312 displayed partial recruitment of coactivator CBP yet equal disassociation of corepressor NCoR1 compared to pioglitazone. In db/db mice, L312 (30 mg/kg·day) treatment considerably improved insulin resistance with the regard to OGTT, ITT, fasted blood glucose, HOMA-IR and serum lipids, but elicited less weight gain, adipogenesis and hemodilution compared with pioglitazone. Further studies demonstrated that L312 is a potent inhibitor of CDK5-mediated PPARγ phosphorylation and displayed a selective gene expression profile in epididymal WAT.
Conclusions: L312 is a novel sPPARγM.
General significance: L312 may represent a novel lead for designing ideal sPPARγM for T2DM treatment with advantages over current TZDs.

1. Introduction

Thiazolidinediones (TZDs), including rosiglitazone and pioglitazone, are the only clinically available agent controlling hyperglycemia by improving insulin resistance, which is regarded as hallmark and early etiologic basis for T2DM. Unfortunately, the use of TZDs has been beset with adverse side effects, including increased cardiovascular risk, fluid retention, bone fracture and body weight gain [1,2]. Thus, a need for a safer insulin sensitizer is apparent.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the NR1C subgroup which includes PPARα and -β. These receptors form heterodimers with the retinoid X receptor and modulate the transcription of divergent genes [3]. PPARγ is predominantly expressed in adipose tissue, with lower expression in liver, muscle, and other tissues [4]. PPARγ is a clinically validated target for treatment of insulin resistance and TZDs have been demonstrated to behave as the PPARγ full agonists [5], which activate PPARγ leading to a wide spectrum of genes expression changes both involved in insulin sensitizing activity [3,5] and adverse side effects [2,6,7]. However, distinctive properties of PPARγ interaction molecular manner provide the chance for selective modulation of the receptor such that benefited therapeutic effects may be attained without the unwanted effects of full activation. Indeed, the LBD of PPARγ includes an activation function 2 motif (AF2) that displays great flexibility in response to diverse ligands, resulting in different conformation change, coactivators recruitment, transcriptional regulation of downstream PPARγ target genes and leading to ultimate specific physiological effects [8]. Researchers proposed the concept of selective PPARγ modulator (SPPARγM), which is the development of PPARγ ligands that could modulate the transcriptional activity of PPARγ toward insulin sensitizing activity, and not toward side effects [8]. This concept is reminiscent of the selective estrogen receptor modulator (SERM) concept that have been demonstrated that different estrogen receptor ligands can have different agonist or antagonist

Abbreviations: BAT, Brown adipose tissue; CBP, cAMP responsive element binding protein (CREBP) binding protein; CDK5, Cyclin-dependent kinase 5; EC_{50}, half-maximal effective concentration; FFA, Free fatty acids; HOMA-IR, HOMA of insulin resistance; ITT, Insulin tolerance test; LBD, Ligand-binding domain; NCoR, Nuclear receptor corepressor; OGTT, Oral glucose tolerance test; PPAR, Peroxisome proliferator-activated receptor; pSer273PPARγ, Phosphorylation at serine 273 in PPARγ; sPPARγM, Selective PPARγ modulator; SPR, Surface Plasmon resonance; T2DM, Type 2 diabetes mellitus; TG, Triglycerides; TZDs, Thiazolidinediones; WAT, White adipose tissue

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properties depending on the cell context and the specific target genes in question [9]. In brief, sPPARγMs bind in distinct manners to the ligand-binding pocket of the PPARγ receptor, leading to differential cofactor displacement and recruitment to the receptor, ultimately resulting in specific gene expression profile and physiological effects in theory.

Characterization and identification of several PPARγ ligands with partial classical agonistic activity (partial agonist) but with similar insulin sensitizing effects to TZDs, verified the concept of SPPARγM in fact [10–14], which further suggested that agonistic activity of PPARγ is not strictly associated with insulin sensitivity and sPPARγM could act as partial agonist to avoid undesirable effects. However, the mechanistic understanding of sPPARγM to selectively retain insulin sensitizing activity is still to be obscure. Until recently, cyclin-dependent kinase 5 (CDK5)-stimulated phosphorylation at serine 273 of PPARγ (pSer273PPARγ), which lead to dysregulation of a set of gene expression in adipose tissue, especially epididymal white fat tissue (eWAT), was revealed as the critical link between obesity and insulin resistance [15]. Furthermore, TZDs and several sPPARγMs were demonstrated to inhibit p273SerPPARγ equally, indicating that the mechanism underlying PPARγ ligands resulted in insulin sensitizing activity, largely depended on the inhibition of pSer273PPARγ [15], which gives rise to a novel viewpoint to understand the mechanisms underlying sPPARγM mediated insulin sensitizing activity. Moreover, changes of pSer273PPARγ had no effect on PPARγ agonistic activity. Collectively, PPARγ ligands interact with PPARγ in a unique mode, leading to less classical agonistic activity but potent inhibitive effects on p273SerPPARγ will be promising candidates for sPPARγM [16,17].

In the present study, we reported a novel PPARγ ligand L312 that retains the benefits of improving insulin resistance by inhibiting pSer273PPARγ, but that minimizes the common side effects of TZDs by alleviating PPARγ agonistic activity.

2. Methods

2.1. Docking simulations of PPARγ with target compounds

The molecular docking was performed using the program eHiTS v12 from SimBioSys Inc. eHiTS (http://www.simbiosys.com/ehits) [18,19] is an exhaustive flexible docking algorithm with a scoring function which incorporates both empirical and knowledge-based features, and it was used for the active site detection and docking. Open Babel (http://openbabel.org) was used for manipulating the ligand chemical formats and acquiring the ligand 3D structures. No special preparation of the 3D structures was applied, since eHiTS automatically evaluates all the possible protonation states for the ligands and targets. PyMol (http://www.pymol.org/) was used for visual inspection of the results and the graphical representations.

The crystal structure of PPAR-γ in complex with MRL124 (a reported typical sPPARγM) (PDB ID: 2Q5P) was selected for the docking study. The eHiTS software package was used for flexible docking. Active site detection was carried out using the "-complex" parameter. The program automatically detected the ligand in the complex and selected the part of target protein within a 7 Å margin around the ligand to be the active site. The compound was then docked into the active site using the highest accuracy mode of docking ("-accuracy" parameter set to 6). The scoring was according to the eHiTS Score that is included in the eHiTS software package. We selected the compound with the best score and speculated the detail binding patterns.

2.2. Chemical compounds

The compound L312 ((S)-2-(4-chlorobenzamido)-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl) propanoic acid) was synthesized by the new drug design center of our institute. Reference compounds (pioglitazone, GW409544, GW1516) were obtained from a same source. Purity and structure were confirmed by high performance liquid chromatography, mass spectrometry and H-nuclear magnetic resonance. The structure of L312, pioglitazone is shown in Fig. 1. And the PPARγ antagonist GW9662 was purchased from EFEBIO (Shanghai, China).

2.3. Ligand binding assay with surface plasmon resonance (SPR)

The binding affinities of L312 and pioglitazone for PPARγ-LBD (Cayman Chemical, Michigan, MI, USA) were assayed using SPR-based Biacore T100 (GE Healthcare/Biacore, Uppsala, Sweden) as described previously [20,21]. BIA evaluation software version 2.0.3 (GE Healthcare/Biacore) and steady state affinity fitting analysis were used to determine the equilibrium dissociation constant (*Kd*) of compounds.

2.4. Luciferase reporter assay

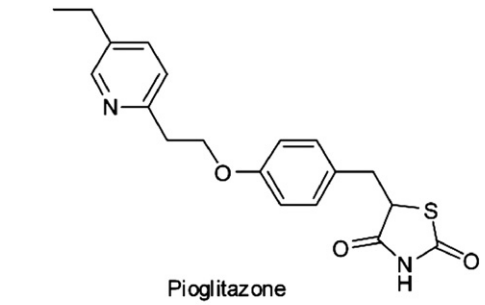
Cell based transactivation of PPARs was assessed in reporter assay as described before [22,23]. In brief, HEK293T cells were seeded in 96-well plate at the concentration of 20,000/well. The expression plasmid pCMX-Gal4-PPARγ-LBD, pM-Gal4-PPARα-LBD, pM-Gal4-PPARβ-LBD or pSV-sport-mPPARγ2 and reporter plasmid tk- UAS × 4Luc or PPRE × 3Luc were transfected, respectively. The pRL-tk was used as internal control. Luciferase activity was tested using Dual-Glo® Luciferase Assay kit (Promega, Madison, WI, USA).

2.5. Mammalian two-hybrid assays

HEK293T cells were transfected with the mixture of expression vectors pACT-hPPARγ2, pBIND-hCBP and pBIND-hNCORI,, the reporter construct pG5-luc and reference plasmid pRL-tk. 8–12 h after transfection, cells were exposed to L312 or rosiglitazone or pioglitazone and incubated for an additional 18 h. Cell extracts were assessed for luciferase activity as described above.

2.6. Adipogenesis assay

Two days post-confluent 3T3-L1 cells were subjected to differentiation as described before [16]. During the differentiation, cells were treated with or without compounds at the dosage of 10 μM, at the end



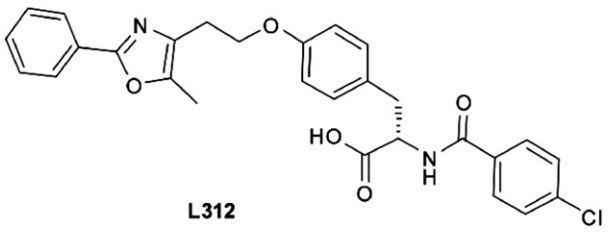


Fig. 1. Chemical structure of pioglitazone and L312 ((S)-2-(4-chlorobenzamido)-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl) propanoic acid).

of differentiation, adipocyte were stained with oil Red O as described before [23]. To investigate the compound effects on PPARγ target genes, differentiated adipocytes were incubated for 24 h with indicated amounts of compounds.

2.7. RNA extraction, cDNA synthesis and Real-time PCR

Total RNA was isolated from cells or adipose tissue using the high purity total RNA extraction kit (BioTeke Corporation, Beijing, China) according to the manufacture's instruction. RNA was reversely transcribed into cDNA applying the High Capacity cDNA Reverse Transcription Kit (Transgenes Corporation, Beijing, China). Real-time PCR was carried out on the ABI 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (Roche Molecular Biochemicals, Mannheim, Germany). The relative gene expression levels were normalized using β-actin gene and quantified by the $2^{-\Delta\Delta Ct}$ method, primers sequences used were listed as Table 1.

2.8. Preparation of cell and tissue lysates and Western blot

Differentiated adipocyte 3T3-L1 cells were treated as described before [16], in summary, cells were pretreated with PPARγ ligands for 45 min, and stimulated with TNFα (50 ng/ml) (PEPROTECH, NJ, USA) for 30 min. Cells and eWAT were homogenized in 10% SDS buffer with protease and phosphatase inhibitors. For Western blotting, a phospho-specific antibody against PPARγ Ser273 was produced by Abmart Inc. (Shanghai, China), with a synthetic phosphopeptide as previously reported [15]. Total tissue lysates were analyzed with an anti-PPARγ antibody (Santa Cruz Biotechonology, Santa Cruz, CA, USA). Bands were detected using ECL enhanced chemiluminescence detection reagents (Applygen, Beijing, China) and scanned on an Alpha Imager 5500 (Alpha Innotech, San Leandro, CA, USA) imaging densitometer.

2.9. Animal studies

Eight week-old male and female homozygous C57BLKS/J db/db mice were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). Animals were maintained under a temperature, humidity, and light controlled conditions (22 °C, 50% humidity, 12 h day/night schedule), mice had *ad libitum* access to standard mouse diet and water. Mice received once daily oral doses of pioglitazone (30 mg/kg) or L312 (10 or 30 mg/kg) for continuously 4 weeks. Body weight (BW) and food intake was recorded regularly during the treatment period. After 14 days treatment, mice were fasted for 12 h prior to oral glucose tolerance tests (OGTT). Blood glucose was monitored at 0, 30, 60, 90 and 120 min after oral administration of 1 g/kg dextrose. After 21 days treatment, mice were subjected for insulin tolerance test (ITT), and fasted for 6 h prior i.p injection of $1U \cdot kg^{-1}$ insulin, blood glucose were estimated as in OGTT. At the end of the experimental period, mice were fasted 12 h and blood samples were taken from the orbital vein for assessment of serum biochemical parameters. Hemoglobin was analyzed by an automatic blood cell counter MEK-7222 K, (Nihon Kohden, Tokyo, Japan) using whole blood collected into a capillary tube with EDTA. The interscapular brown adipose (iBAT), epididymal white adipose tissue (eWAT), subcutaneous white adipose tissue (sWAT), liver and heart were excised, weighed and rapidly frozen in liquid nitrogen for subsequent gene expression and western blot. eWAT depot were fixed in 10% formaldehyde for histological analysis and embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with hematun/eosin, the cell area were calculated by the software ImageJ.

All animal experiments were conducted strictly in accordance with the guidelines of the Animal Experimentation Ethics Committee of Beijing Institute of Pharmacology and Toxicology for animal care, handling and termination.

Table 1
Primer sequences used for qPCR.

Gene	Forward primer	Reverse primer
aP2	AAGGTGAAGAGCATCATAACCCT	TCACGCCTTTCATAACACATTCC
Adiponectin	GGAGAGAAAGGAGATGCAGGT	CTTTCCTGCCAGGGGTTC
Fgf21	AGATGGAGCTCTCTATGGATCG	GGGCTTCAGACTGGTACACAT
Adipsin	CATGCTCGGCCCTACATGG	CACAGAGTCGTCATCCGTCAC
Pdk4	CGCTTAGTGAACACTCCTTCG	CTTCTGGGCTCTTCTCATGG
Cytc	CCAAATCTCCACGGTCTGTTC	ATCAGGGTATCCTCTCCCCAG
Ucp1	GGCCTCTACGACTCAGTCCA	TAAGCCGGCTGAGATCTTGT
Ucp2	CCGGGGCCTCTGGAA AG	CCCAAGCGCAGAAAGGA
Ucp3	TACCCAACCTTGGCTAGACG	GTCCGAGGAGAGAGCTTGC
Cycp2f2	GTCGGTGTTCACGGTGTACC	AAAGTTCCGCAGGATTTGGAC
Rarres2	GCCTGGCCTGCATTAAAATGG	CTTGCTTCAGAATTGGGCAGT
Selenbp	ATGGCTACAAAATCACAAAGTG	CCTGTGTTCCGGTAAATGCAG
Car3	TGACAGGTCTATGCTGAGGGG	CAGCGTATTTTACTCCGTCCAC
Peg10	TGCTTGCACAGAGCTACAGTC	AGTTTGGGATAGGGGCTGCT
Cidec	ATGGACTACGCCATGAAGTCT	CGGTGCTAACACGACAGGG
Cd24a	GTTGCACCGTTTCCCGGTAA	CCCCTCTGGTGGTAGCGTTA
Acyl	CAGCCAAGGCAATTTCAGAGC	CTCGACGTTTGATTAACGGTCT
Nr1d2	TGAACGCAGGAGGTGTGATTG	GAGGACTGGAAGCTATTCTCAGA
Ddx17	TCTTCAGCCAACAATCCCAATC	GGCTCTATCGGTTTCACTACG
Aplp2	GTGGTGGAAGACCGTGACTAC	TCGGGGGAACTTTAACATCGT
Nr3c1	AGCTCCCCCTGGTAGAGAC	GGTGAAGACGCAGAAACCTTG
Rybp	CGACCAGGCCAAAAAGACAAG	CACATCGCAGATGCTGCATT
Txnip	TCTTTTGAGGTGTCTTCAACG	GCTTTGACTCGGGTAACTTCACA
Rvrα	ACGACCCTGGACTCCAATAA	CCATTGGAGCTGTCACTGTAGA
β-actin	TAAAGACCTCTATGCCAACACAGT	CACGATGGAGGGGCCGGACTCATC

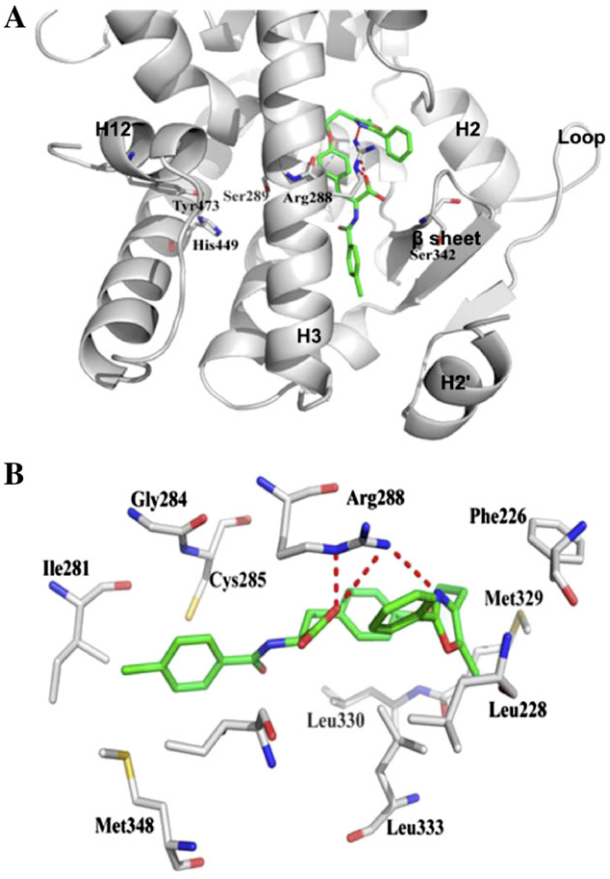


Fig. 2. L312 interacts with PPARγ-LBD in a distinct mode. (A) Structure of the PPARγ: L312 complex. L312 binds to PPARγ-LBD between helix 3 and the β-sheet. The key residues in ligand binding pocket of PPARγ are shown in gray. The C-backbone of L312 is shown in green. Hydrogen interactions with PPARγ are shown in red. (B) Atomic details of ligand recognition. Hydrogen bonds stabilizing the complex are shown as dashed lines.

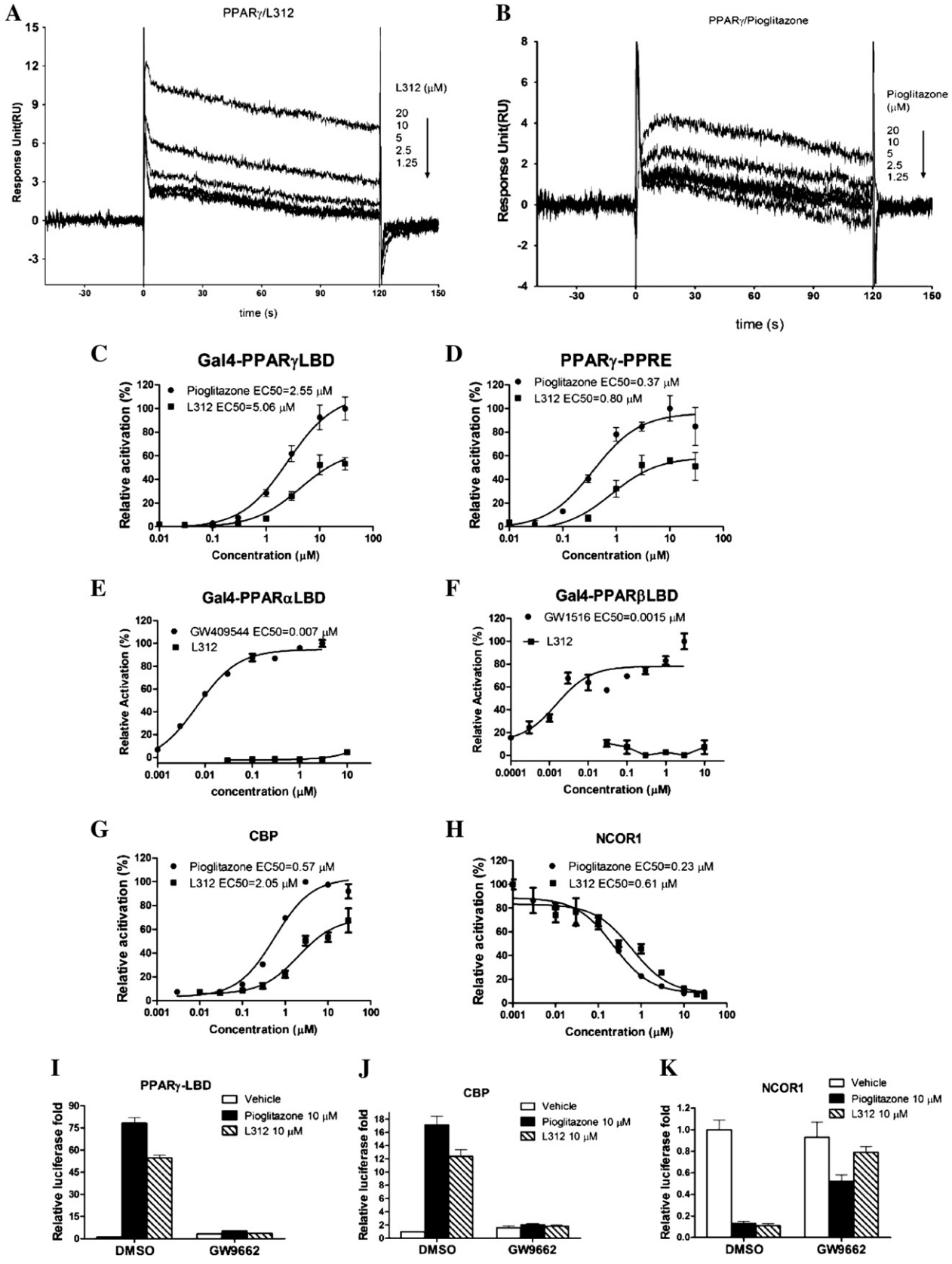


Fig. 3. Identification of L312 as a novel PPARγ ligand. (A) Ligand binding affinity of L312 at concentrations of 1.25, 2.5, 5, 10 and 20 μM, (B) and pioglitazone at concentrations of 1.25, 2.5, 5, 10 and 20 μM to PPARγ-LBD in SPR-based Biacore. (C–F) Receptor-specific transactivation by L312. HEK293T cells were cotransfected with the reporter plasmids and plasmids encoding different isotypes of PPARs-LBD or full length PPARγ. (G–H) Cofactor recruitment and dissociation by L312. HEK293T cells were transfected with plasmids encoding pACT-hPPARγ2, pBIND-hCBP or pBIND-hNCORI, and the reporter construct pG5-luc. After transfection, cells were treated with L312 or ligands specific for each receptor at various concentrations. Luciferase activity is reported normalized to renilla activity encoded by the plasmid pRL-tk as an internal control. Values are shown as means ± SE (n = 3). (I–K) The transactivation of PPARγ, recruitment of CBP and displacement of NCoR1 induced by L312 and pioglitazone (10 μM) was reversed by GW9662 (10 μM).

2.10. Metabolic parameter measurements in serum

Serum triglyceride (TG), free fatty acids (FFA), cholesterol and glucose were measured by using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. Serum insulin was quantified by radioimmunoassay using a rat RIA insulin assay kit (Linco Research Inc.). The HOMA-insulin resistance (IR) was determined according to HOMA-IR = fasting blood glucose ($mmol \cdot L^{-1}$) × fasting insulin ($pmol \cdot L^{-1}$)/156.

2.11. Statistical analyses

All results are expressed as mean ± SE. For two group comparison, statistical significance was examined by unpaired two-tailed Student's *t* test, if not otherwise stated. For multiple comparisons statistical analysis was performed by one-way ANOVA followed by Tukey multiple comparison tests with GraphPad Prism 5.0 software. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. L312 interacts with PPARγ-LBD in a specific mode

Firstly, we performed virtual docking simulations to screen a library of compounds targeting PPARγ kept in our lab to obtain candidate compounds. This approach has been used successfully in the identification of small molecule active compounds of other nuclear receptor targets [24–26]. We selected MRL24-PPARγ crystal structure (PDB ID: 2q5p) in the reported crystal structures of sPPARγM, which has been validated to potentiate inhibition of pSer273PPARγ, as a template for the virtual molecular docking, mainly based on the similarity of the structure features between our candidate compounds and MRL24, both containing an acid portion. The acid portion of MRL24 contacts the key amino acid residues Ser-342 of the β-sheet in PPARγ-LBD with several hydrogen bonds, which was demonstrated to play a key role in the determination of interaction mode of PPARγ-LBD: MRL24. Validation test runs demonstrate that eHiTS can reproduce X-ray structures and provide good alignment accuracy with low RMSD (1.13 < 2.0 Å). Interestingly, our results indicated that L312 interacted with PPARγ-LBD in a manner similar to MRL24 as shown in Fig. 2A.

The docking simulation showed that L312 did not create the classical and critical hydrogen bonds with Tyr-473 in the C-terminal of helix H12, but adopted a conformation within the receptor that allowed the nitrogen atom of oxazole and the acidic head group to form three hydrogen bonds with Arg 288 on helix 3 (Fig. 2A–B). Furthermore, L312 interacted with a number of key amino acid residues (Phe-226, Leu-228, Ile-281, Gly-284, Cys-285, Met-329, Leu-330, Leu-333, Ile-341 and Met-348) in a lipophilic pocket formed by helices 2′ and 3, and the β-sheet (Fig. 2B). These data indicated that L312 interacted with the PPARγ-LBD in a manner that was different from TZDs.

3.2. L312 is a novel PPARγ partial agonist

In order to ensure that L312 interacts directly with PPARγ-LBD, we performed SPR-based Biacore binding assays to detect the affinity of L312 to PPARγ-LBD. In this assay, L312 dose-dependently bind to PPARγ-LBD with equilibrium dissociation constant (*Kd*) values of 8.70 μM, (Fig. 3A). Under the same conditions, pioglitazone, a commonly used TZDs, was demonstrated to bind to the receptor with a *Kd* = 3.22 μM (Fig. 3B), indicating that L312 had a similar affinity to PPARγ-LBD compared to pioglitazone.

To evaluate the transcriptional activity of L312 on PPARγ, we performed a luciferase reporter assay. In Gal4-PPARγLBD and tk-UAS-Luc plasmids based assay, treatment with L312 significantly induced the transcriptional activity of PPARγ, but to a lesser extent than pioglitazone, the maximal transcriptional potency induced by L312 was approximately 60% relative to that of pioglitazone. Half maximal effective concentration (EC_{50}) in transfected HEK293T cells was 5.06 μM for L312, compared with 2.55 μM for pioglitazone (Fig. 3C). In full length PPARγ and PPRE reporter plasmid based assay, L312 also showed about 60% maximal transcriptional activity relative to pioglitazone and the value of EC_{50} is 0.80 μM and 0.37 μM for L312 and pioglitazone, respectively (Fig. 3D). In addition, L312 showed highly selective PPARγ activation, which did not show any effect on transcriptional activity of Gal4-PPARαLBD (Fig. 3E) and Gal4-PPARβLBD (Fig. 3F) at concentrations up to 10 μM in HEK293T cells.

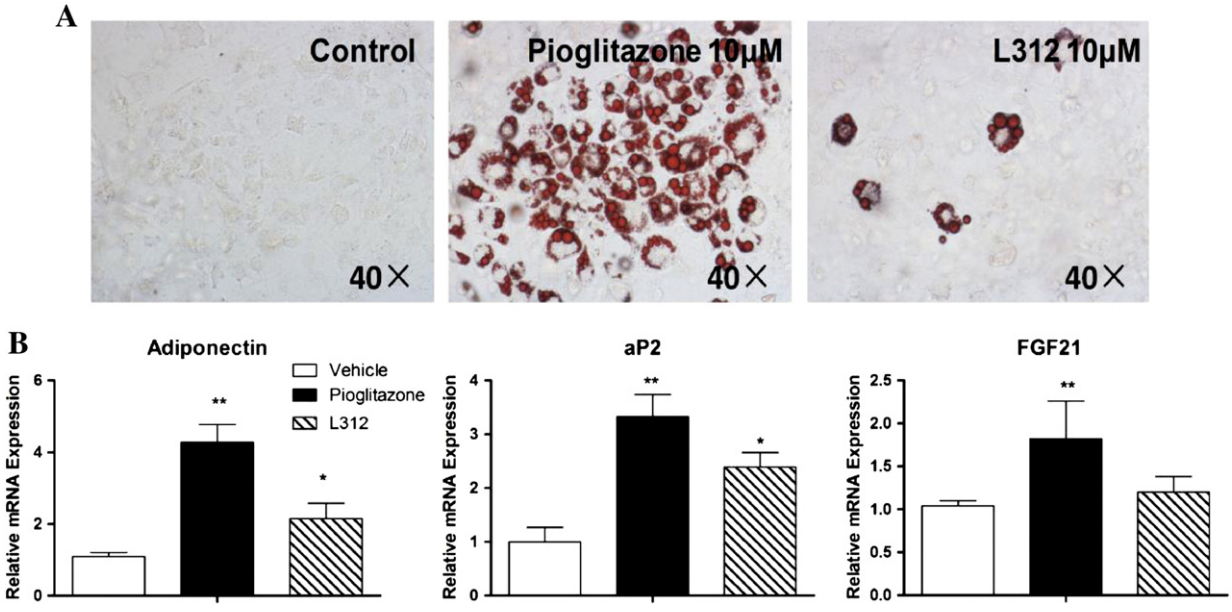


Fig. 4. L312 functions as a partial agonist with weak effect on adipogenesis and adipogenic genes expression. (A) Effect of L312 on adipogenesis. Preadipocyte 3T3-L1 cells were subjected to differentiation medium with vehicle, pioglitazone (10 μM) or L312 (10 μM) during the differentiation period. At the end, cells were stained with oil Red O. (B) Effect of L312 on adipogenic genes expression. Differentiated 3T3-L1 adipocytes were treated with pioglitazone or L312 at a concentration of 10 μM for 24 h. RNA was extracted and used to detect the expression of the adipogenic-related genes adiponectin, aP2 and fgf21. Values are shown as means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle.

Further, the effect of L312 on coactivator CBP recruitment and corepressor NcoR1 diassociation was evaluated in mammalian cell based two-hybrid assay, the result indicated that L312 mediated maximal CBP recruitment level is approximately 60% relative to that conferred by pioglitazone (Fig. 3G). EC_{50} of L312 and pioglitazone on CBP recruitment is 2.05 μM and 0.57 μM, respectively. However, L312 abolished recruitment of the corepressor NcoR1 showing maximal dissociation potency is similar to that of pioglitazone (Fig. 3H), IC_{50} of L312 and pioglitazone on NcoR1 dissociation is 0.61 μM and 0.23 μM, respectively.

GW9662 is an antagonist for PPARγ, which could covalently interact with PPARγ-LBD in the same position as agonist and irreversibly antagonizes the binding of agonist to PPARγ. In our study, L312 induced PPARγ transactivation, CBP recruitment and NCoR1 displacement in cell based reporter assay all could be abrogated by co-treatment with GW9662 (Fig. 3I–K), which confirmed that the effect of L312 on PPARγ functions through direct binding to PPARγ-LBD.

3.3. L312 weakly promotes adipogenesis and adipogenic genes expression

TZDs have been shown to promote the conversion of preadipocyte cell lines into adipocyte. This response has been widely used as a sensitive cellular test for PPARγ agonism activity. The murine preadipocyte cell line 3T3-L1 was subjected to differentiation medium with L312 or pioglitazone at concentrations of 10 μM, which showed maximal transactivation on PPARγ in luciferase reporter assay, during the differentiation period. At the end of the treatment, Oil red O staining

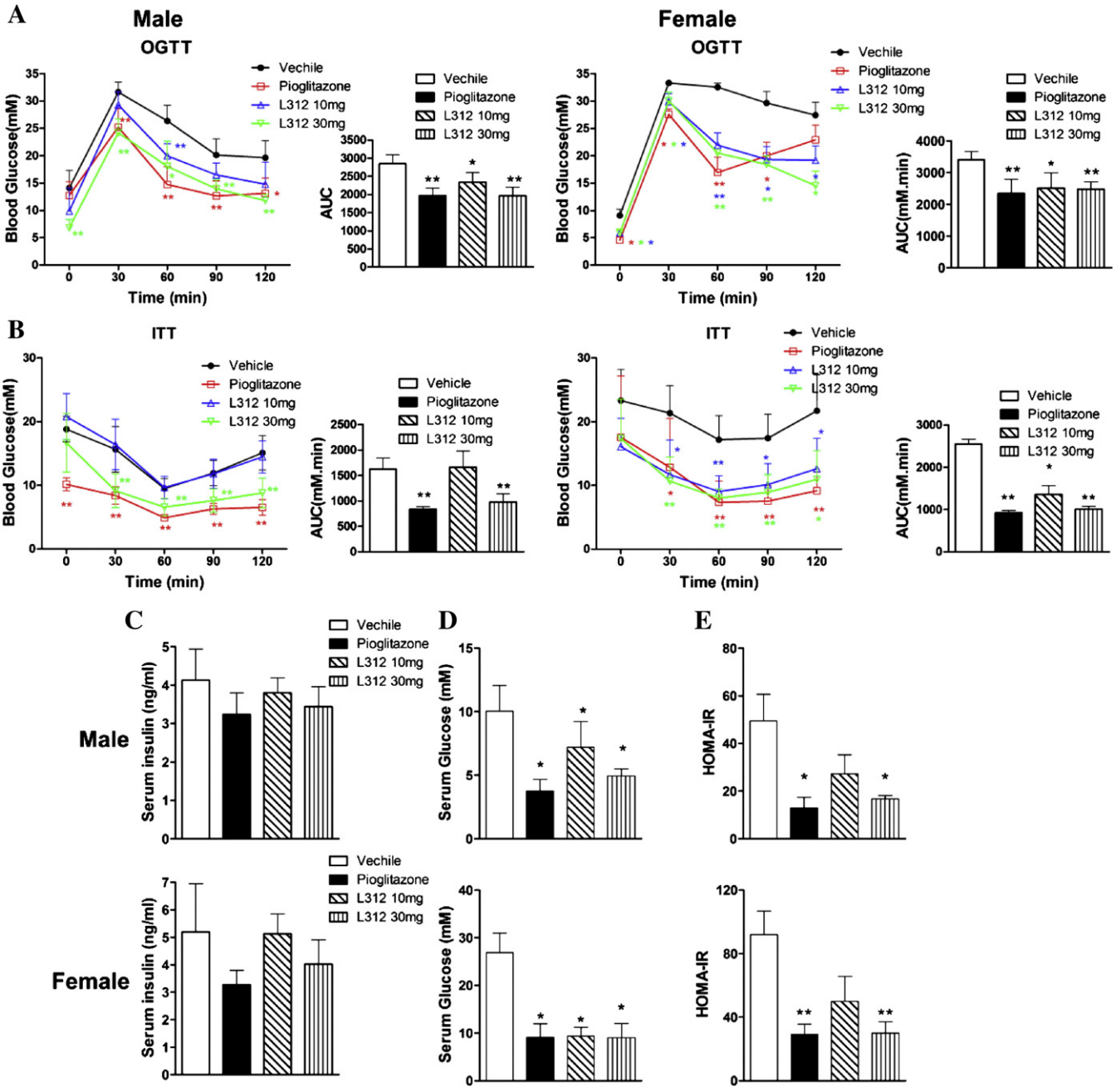


Fig. 5. L312 improves glucose tolerance and insulin resistance in db/db mice. (A) Oral glucose tolerance tests (OGTT) were performed in male and female db/db mice after 14 days treatment with vehicle, pioglitazone (30 mg/kg), or L312 (10 mg/kg or 30 mg/kg). Glucose (1 g/kg) was administrated by gavage to 12-h-fasted db/db mice (n = 5 for each group), and the area under the curve was calculated. (B) The insulin tolerance test (ITT) was performed after 21 days treatment. Insulin (1 U/kg) was administrated by i.p. injection to 6-h-fasted db/db mice. (C) Insulin levels, (D) serum glucose levels, (E) and HOMA-IR were determined at the end of the 28-day treatment. Values are shown as means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle.

indicated that pioglitazone at a concentration of 10 μM induced massive lipid accumulation. In contrast, L312 treatment induced only little lipid accumulation at 10 μM (Fig. 4A). The stimulation of the expression of adipogenic genes was also apparent using pioglitazone, as illustrated by an increased expression of adipogenic-related genes (*Adiponectin*, *aP2* and *Fgf21*). In contrast, L312 induced a much weaker effect on these genes (Fig. 4B). These results confirmed that L312 is a partial agonist on the behalf of the adipogenic activity.

3.4. L312 effectively improves insulin resistance in db/db diabetic mice model

Next, we evaluated the physiological effects of L312 *in vivo*. The severe T2DM model of leptin receptor deficient db/db mice was used to study the insulin sensitizing activity, side effects and the molecular mechanisms in detail. Males and females have many biological differences because of sex hormones such as estrogen, which affects many pathophysiological processes including obesity and type 2 diabetes and might play roles in drug responding [27]. In this study, we choose both genders to evaluate the insulin sensitizing activity to understand the effects of the L312 comprehensively *in vivo*. Eight-week-old male and female db/db mice were treated with vehicle, pioglitazone (30 mg/kg), or L312 (10 mg/kg or 30 mg/kg) for 28 days. Our data showed that L312 improved insulin resistance in a dose-dependent manner and exhibited comparable maximal efficacy with equal dose pioglitazone with regard to OGTT and ITT both in males and females (Fig. 5A–B). L312 considerably enhanced glucose tolerance (31% and 28% decreases in glucose area under the curve (AUC) *vs.* vehicle in males and females, respectively) during OGTT (Fig. 5A) and insulin sensitivity (40% and 47% decreases in glucose AUC *vs.* vehicle in males and

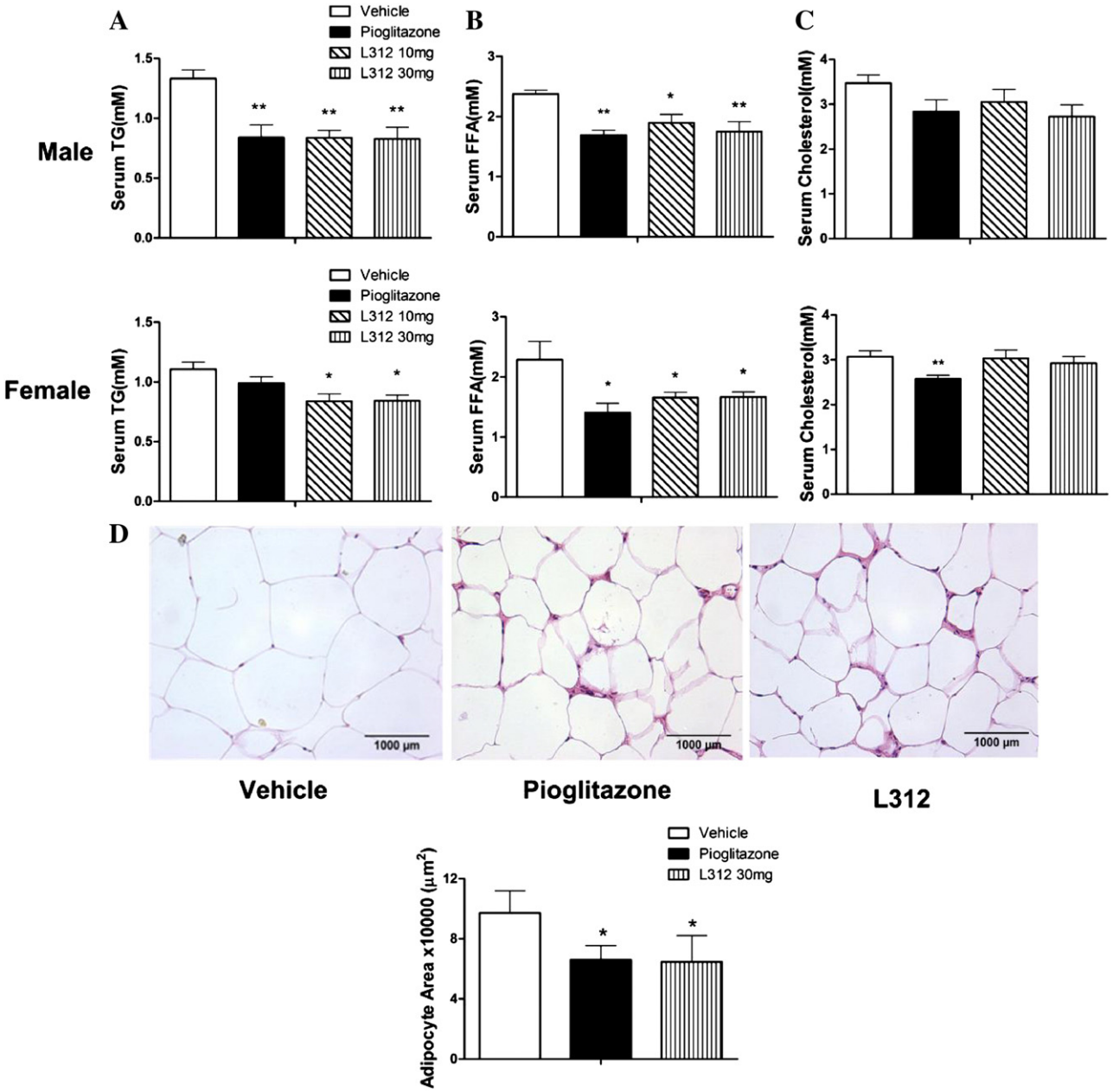


Fig. 6. Effect of L312 treatment on serum lipids levels and adipocyte morphology in db/db mice. (A) Fasting triglycerides, (B) free fatty acids and (C) cholesterol after 28 days of treatment in males and females (n = 5 each group). (D) Epididymal white adipose tissue were sectioned and stained with HE and the average adipocyte area was calculated by ImageJ. Values are shown as means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle.

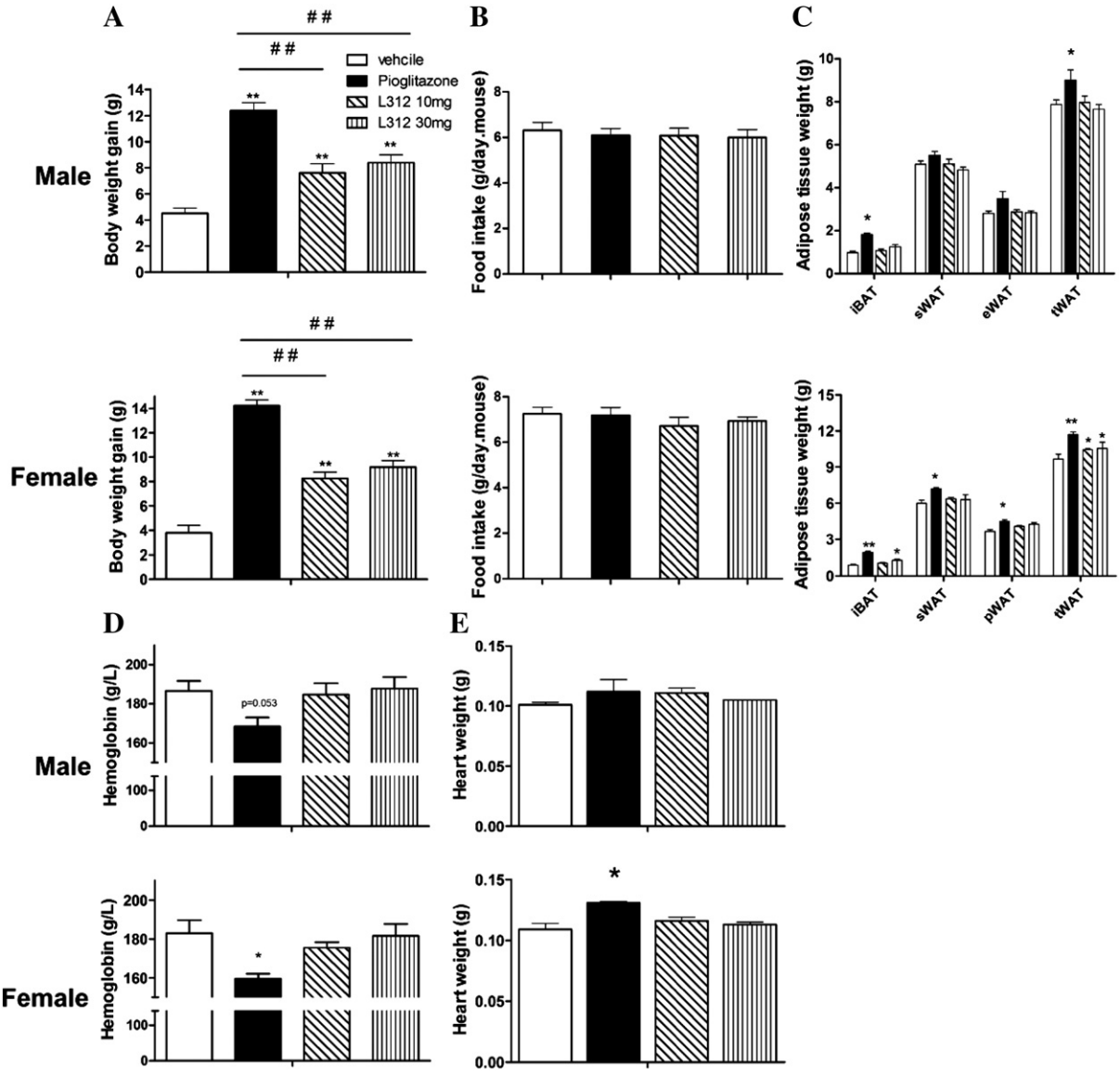


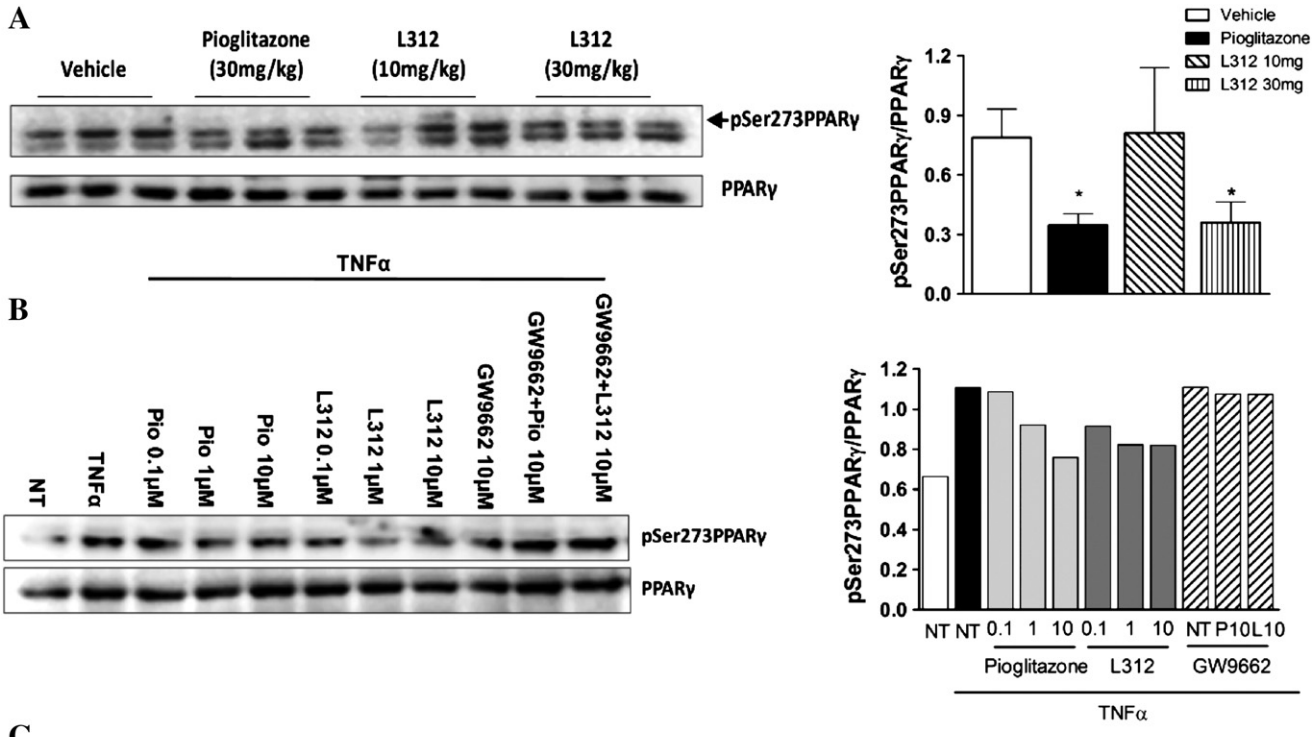
Fig. 7. L312 shows reduced side effects that are commonly associated with classical full PPARγ agonists. (A) Body weight gain, (B) food intake, (C) interscapular BAT (iBAT), epididymal/parametral WAT (eWAT/pWAT) and subcuteous inguinal WAT (sWAT) weight, (D) blood hemoglobin, (E) heart weight were determined after 28 days of treatment both in males and females with vehicle, pioglitazone (30 mg/kg), or L312 (10 mg/kg or 30 mg/kg) (n = 5 each group). Values are shown as means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle; $\#p < 0.05$, $\#\#p < 0.01$ compared with pioglitazone.

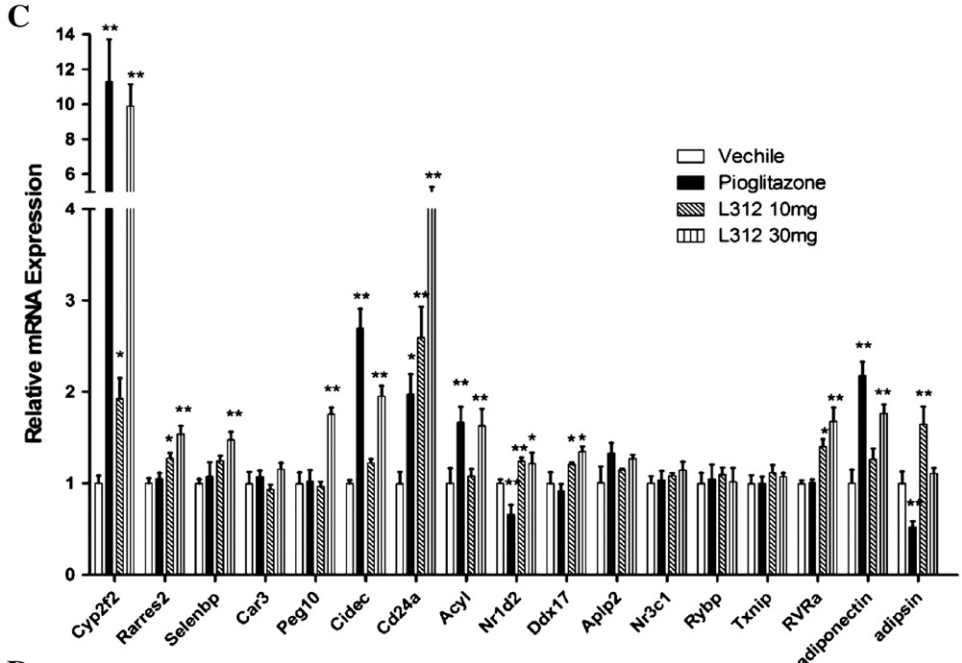
females, respectively) during ITT (Fig. 5B). Moreover, at the end of treatment, both pioglitazone and L312 treatments showed a trend toward lowered serum insulin levels (Fig. 5C) and led to a significant reduction in fasting serum glucose levels (Fig. 5D). Insulin resistance index, as estimated by HOMA-IR, showed a significant and dose-dependent improvement of insulin resistance by L312 and the maximal magnitude is similar to pioglitazone both in male and female (Fig. 5E).

On the other hand, L312 attenuated hyperlipidemia accompanied with insulin resistance. Levels of serum TG, FFA were down-regulated, and the maximal reduction efficacy was comparable to pioglitazone both in males and females (Fig. 6A–C). However, L312 and pioglitazone both has no effects on cholesterol level in males, and pioglitazone could significantly decreased cholesterol in females but L312 did not. HE staining of eWAT paraffin sections of male mice indicated that both L312 and pioglitazone reduced adipocyte size compared with vehicle treatment, which is negatively correlated with insulin sensitivity [28] (Fig. 6D).

3.5. L312 does not induce cardiomegaly or hemodilution, and induces less adipogenesis and weight gain in db/db mice

During treatment, body weight and food intake were regularly recorded. Pioglitazone induced a significant body weight gain compared with vehicle treatment, the net weight gain being 8 and 10 g more in males and females, respectively (Fig. 7A). However, L312 showed much less effect on weight gain, both L312 dosages induced similar body weight gain, which was approximately 50% or 60% less than that of pioglitazone (Fig. 7A) in males and females, respectively. On the other hand, both pioglitazone and L312 had no effect on food intake (Fig. 7B). Quantification of adipose tissue indicated that pioglitazone induced a significant increase in weight of total WAT (sum of epididymal/parametral and inguinal WAT) and brown adipose tissue (BAT) (interscapular) both in males and females (Fig. 7C). On the contrary, L312 had absolutely no effect on adipose tissue weight in males and showed a lesser weight gain in female (Fig. 7C). As for edema,


A
Vehicle
Pioglitazone (30mg/kg)
L312 (10mg/kg)
L312 (30mg/kg)
pSer273PPARγ
PPARγ
Vehicle
Pioglitazone
L312 10mg
L312 30mg
pSer273PPARγ/PPARγ
B
TNFα
NT
TNFα
Pio 0.1μM
Pio 1μM
Pio 10μM
L312 0.1μM
L312 1μM
L312 10μM
GW9662 10μM
GW9662+Pio 10μM
GW9662+L312 10μM
pSer273PPARγ
PPARγ
pSer273PPARγ/PPARγ
NT NT 0.1 1 10 0.1 1 10 NT P10L10
Pioglitazone
L312
GW9662
TNFα


C
Relative mRNA Expression
Vechile
Pioglitazone
L312 10mg
L312 30mg
Cyp2f2
Rarres2
Selenbp
Car3
Peg10
Cidec
Cd24a
Acyl
Nr1d2
Ddx17
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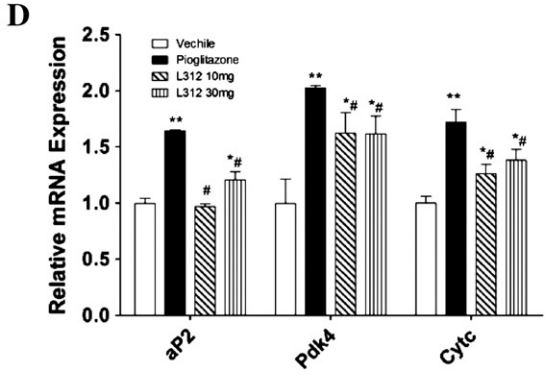
Rybp
Txnip
RVRa
adiponectin
adipsin


D
Relative mRNA Expression
Vechile
Pioglitazone
L312 10mg
L312 30mg
aP2
Pdk4
Cytc

hemoglobin (Hb) concentration in blood analysis showed that pioglitazone induced a significant decrease of Hb in females, and a trend toward a decrease in males ($p = 0.053$), but L312 had no effect on blood Hb (Fig. 7D). In our study, we observed that pioglitazone induced significant heart weight gain in females, but not in males (Fig. 7E). Treatment with L312 had no effect on heart weight in both males and females (Fig. 7E).

3.6. L312 blocks CDK5-mediated phosphorylation of PPARγ at Ser273

We detected the level of Cdk5-mediated phosphorylation of PPARγ in eWAT. As similar physiological effects were observed both in male and female mice, the male mice were subjected to explore the molecular mechanisms next. Our data showed that L312 inhibited pSer273PPARγ dose-dependently and the dose of 30 mg/kg as pioglitazone (30 mg/kg) (Fig. 8A). In addition, we also detected effects of L312 on pSer273PPARγ in 3T3-L1 adipocyte. Cells were stimulated with TNFα for 30 min and pretreated with pioglitazone or L312 for 45 min at concentrations ranging from 0.1 to 10 μM. Pioglitazone and L312 could effectively blocked pSer273PPARγ at concentrations 1, 10 μM and the effects could be neutralized by co-treatment with PPARγ antagonist GW9662 (Fig. 8B), suggesting pioglitazone and L312 induced inhibition of pSer273PPARγ dependent on direct binding to PPARγ-LBD and confirmed the result observed *in vivo*.

Furthermore, we detected the change of genes corresponding to pSer273PPARγ in epididymal WAT [15,16], and observed that treatment with L312 attenuated expression of these genes in the direction predicted to block pSer273PPARγ dose-dependently, which is in accordance with the blockade of pSer273PPARγ at protein level. L312 (30 mg/kg) caused a change of 11/17 (65%) of these genes compared to that pioglitazone caused a change of 5/17 (29%) (Fig. 8C). On the other hand, we also detected genes (*aP2*, *Pdk4* and *Cytc*) responding to agonistic activity [16] and the result indicated that L312 induced much less expression of these genes compared to pioglitazone (Fig. 8D), which suggested that L312 selectively regulate genes expression in epididymal WAT.

4. Discussion

In this study, we characterized and identified that L312 is a novel sPPARγM with partial PPARγ agonistic activity and potent inhibition of pSer273PPARγ both *in vitro* and *in vivo*, which potentiated equal insulin sensitizing activity and less side effects compared to full agonist pioglitazone.

The specific binding mode of the ligand with PPARγ-LBD determines the specific biological effects [8,29]. Previous studies delineated that stabilization of helix H12 of PPARγ-LBD was demonstrated to correlate with increased stability of AF2 surface of the receptor, which facilitates coactivators recruitments and is positively correlated to PPARγ full agonism [30,31]. On the other hand, stabilization of helix H3 and β-sheet was shown to protect Ser273 from CDK5 [15,16,32–34] and that the proximity of ligand to amide Arg288 within helix H3 or Ser342 in β-sheet correlated to increased stability of the helix2–helix2′ loop, which contains the Ser273 in PPARγ, and the increased stability of the helix2–helix2′ loop protects Ser273 from CDK5 [16], which has been demonstrated in previous studies such as non-agonistic ligand SR1664 was demonstrated to interact with Ser342 in the β-sheet [16], plant-derived sPPARγM, amorfrutin links to Ser342 and Arg288 with hydrogen bonds and salt bridge [34] and another sPPARγM GQ16 stabilizes helix H3 by π-stacking with Arg288 [32]. In this study, we used a virtual docking approach that has been successfully validated in the identification of small molecule active compounds of androgen receptor [24–26], and data from preliminary virtual docking indicated that L312 entered into the ligand binding pocket of PPARγ in a manner similar to the sPPARγM MRL24, which located between helix H3 and the β-sheet, but without interaction with Tyr473 in helix H12. Further analysis indicated that L312 stabilized the helix H3 *via* linking to Arg288 with three hydrogen bonds, which is a novel interaction mode that correlated with increased stability of the helix 2–helix 2′ loop and protect Ser273 from CDK5. In summary, these data indicated that L312 interacted with PPARγ in a different mode with TZDs, and suggested that L312 could avoid full agonism and retain potent inhibition of CDK5 mediated phosphorylation at Ser273 of PPARγ in a specific interaction mode.

A desirable sPPARγM should include high-affinity interaction with PPARγ-LBD in a manner that leads to partial agonistic activity [8]. In this study, L312 was demonstrated to bind to PPARγ-LBD with the affinity similar to full agonist pioglitazone in SPR-based biacore assay, but which potentiated partial transcriptional activity in luciferase reporter assay and displayed weaker activation to induce preadipocyte maturation. These results suggested that L312 bound to PPARγ-LBD in a distinct manner and lead to a conformation which potentiated less affinity to coactivators participated in the PPARγ mediated classical agonism, such as adipogenesis. CBP is a coactivator that is regarded to be indispensable for the full activation of PPARγ and adipocyte differentiation [35,36]. We took cell-based mammalian two-hybrid assays to test the recruitment of CBP upon L312 stimulated PPARγ and demonstrate that L312 mediated partial potency compared to that of pioglitazone, which is consistent with reported partial agonists [13,34]. Collectively, these data demonstrated that L312 is a novel potent PPARγ ligand with partial agonistic activity.

Reserved potent insulin sensitizing activity is the essential characterization of sPPARγM. Treatment with L312 in T2DM db/db mice displayed considerable effects on lowering glucose, exerting insulin sensitizing activity and improving dyslipidemia as pioglitazone to a similar degree. In contrast, treatment with L312 induced less known TZD like side effects such as body weight gain and adipogenesis compared to pioglitazone, which is in accordance with partial agonistic activity as demonstrated *in vitro*. Furthermore, L312 did not induce any hemodilution and cardiomegaly compared to pioglitazone, which has been regarded as the main risk factor for PPARγ full agonists associated congestive heart failure clinically, the very reason that rosiglitazone was restricted to use [1]. Therefore, L312 is identified to be a novel sPPARγM according to the pharmacological effects *in vivo*.

Inhibition of CDK5 mediated pSer273PPARγ is regarded as one molecular mechanism underlying PPARγ ligands mediated insulin sensitizing activity [15]. Molecular mechanism characterization demonstrated that L312 mediated a potent inhibition of pSer273PPARγ both *in vitro* and *in vivo*, and improved genes expression responding to pSer273PPARγ in a better way than pioglitazone but not genes associated to agonistic activity in eWAT, which is similar to previously reported natural product-derived sPPARγM amorfrutin that is demonstrated to be more efficient than rosiglitazone in reversing genes expression induced by pSer273PPARγ [34]. On the other hand, L312 mediated specific striking disassociation efficacy to the corepressor NCoR1 in the cell-based mammalian two-hybrid assay probably plays an important role in the inhibition of pSer273PPARγ as NCoR1 interacts with CDK5 and disassociation of NCoR1 prevents the CDK5 interacting with PPARγ [37]. Hence, L312 is a sPPARγM to attenuate insulin resistance *via* striking

Fig. 8. L312 blocks the phosphorylation of PPARγ at Ser273 and mediates a selective gene expression profile in epididymal WAT. (A) Detection of PPARγ phosphorylation in epididymal WAT and quantification of phosphorylation compared with total PPARγ in male. pSer273PPARγ, phosphorylation of PPARγ at Ser273. Total epididymal WAT lysates were obtained from compound-treated db/db mice (n = 3 for each group) and subjected to western blot. (B) Inhibition of pSer273PPARγ stimulated by TNFα in 3T3-L1 adipocyte after treatment with pioglitazone, L312 or GW9662 & pioglitazone/L312. NT, no treatment. (C) The epididymal WAT of treated db/db mice were analyzed by quantitative PCR for expression of genes responding to pSer273PPARγ. (D) Quantification of expression of genes responding to agonistic activity (n = 3 for each group). Values are shown as means ± SE. *p < 0.05, **p < 0.01 compared with vehicle. #p < 0.05, ##p < 0.01 compared with pioglitazone.

inhibition of pSer273PPARγ and selectively improving genes expression responding to pSer273PPARγ. Moreover, the potent inhibition of pSer273PPARγ confirmed the result obtained from virtual docking. Of course, the virtual modeling data is suggestive, the accurate L312: PPARγ-LBD crystal structure characterizations are seeking to validate these results. And subsequent studies will determine the roles of resides in inhibiting pSer273PPARγ.

Overall, the here identified selective PPARγ modulation pattern, in combination with suppression of hyperglycemia, hyperlipidemia and with less side effects like TZDs, makes L312 an interesting lead compound with a good potential to design novel sPPARγM for T2DM.

Acknowledgments

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