WSF-7 Inhibits Obesity-Mediated PPAR\(\gamma\) Phosphorylation and Improves Insulin Sensitivity in 3T3-L1 Adipocytes

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Peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)), the molecular target for antidiabetic thiazolidinediones (TZDs), is a master regulator of preadipocyte differentiation and lipid metabolism. The adverse side effects of TZDs, arising from their potent agonistic activity, can be minimized by PPAR\(\gamma\) partial agonists or PPAR\(\gamma\) non-agonists without loss of insulin sensitization. In this study, we reported that WSF-7, a synthetic chemical derived from natural monoterpene \(\alpha\)-pinene, is a partial PPAR\(\gamma\) agonist. We found that WSF-7 binds directly to PPAR\(\gamma\). Activation of PPAR\(\gamma\) by WSF-7 promotes adipogenesis, adiponectin oligomerization and insulin-induced glucose uptake. WSF-7 also inhibits obesity-mediated PPAR\(\gamma\) phosphorylation at serine (Ser)273 and improves insulin sensitivity of 3T3-L1 adipocytes. Our study suggested that WSF-7 activates PPAR\(\gamma\) transcription by a mechanism different from that of rosiglitazone or luteolin. Therefore, WSF-7 might be a potential therapeutic drug to treat type 2 diabetes.

Key words WSF-7; peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)); glucose uptake; insulin sensitivity; adiponectin

INTRODUCTION

Diabetes is characterized with chronic hyperglycemia arising from insufficient production of insulin in type 1 diabetes or insulin resistance in type 2 diabetes (T2DM). Thiazolidinediones (TZDs), synthetic ligands for peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)), are effective insulin sensitizers currently used as frontline treatment for T2DM. PPAR\(\gamma\) is a ligand-dependent transcription factor required for activating the transcription of genes involved in diverse cellular processes, such as preadipocyte differentiation and lipid metabolism. Conformational changes caused by the binding of a ligand to PPAR\(\gamma\) ligand-binding domain (LBD) in transactivation domain AF-2 promotes the binding of co-activators, leading to increased expression of PPAR\(\gamma\) target genes.

TZDs are highly potent in improving insulin sensitivity. However, they carry adverse side effects, including fat accumulation, bone loss, edema, fluid retention, and even tumor risks. PPAR\(\gamma\) partial agonists and non-agonists have similar insulin-sensitizing effects as full agonists, such as TZDs, but with fewer side effects. The antidiabetic effects of these PPAR\(\gamma\) ligands are primarily mediated by blocking PPAR\(\gamma\) phosphorylation at Ser273 by CDK5, suggesting that obesity-mediated Ser273 phosphorylation may be the casual to insulin resistance. Compounds that inhibit Ser273 phosphorylation might be potential anti-diabetic drugs that target PPAR\(\gamma\).

As a strategy to discover novel PPAR\(\gamma\) agonists, we screened for synthetic chemicals that inhibit Ser273 phosphorylation of PPAR\(\gamma\). WSF-7 (5,5,7-trimethyl-3-(p-tolyl)-3,3a,4,5,6,7-hexahydro-4,6-methanobenzo[c]isoxazol-7-ol), derived from natural monoterpene \(\alpha\)-pinene, was identified as one of the candidates. In this study, we demonstrated that WSF-7 is a novel PPAR\(\gamma\) agonist with weak adipogenic activity. Activation of PPAR\(\gamma\) by WSF-7 promotes preadipocyte differentiation, adiponectin oligomerization and insulin-induced glucose uptake. We provide evidence that WSF-7 can improve insulin sensitivity of 3T3-L1 adipocytes.

MATERIALS AND METHODS

Materials Antibodies specific for phospho-PPAR\(\gamma\) Ser273 were purchased from Bioss (Beijing, China). Antibodies against aP2, PPAR\(\gamma\), Akt, pAkt (Ser473) and glucose transporter type 4 (GLUT4) were from Cell Signaling (Shanghai, China). Rosiglitazone, luteolin, GW9662, insulin, wortmannin and tumor necrosis factor (TNF)-\(\alpha\) were purchased from Sigma (Shanghai, China).

Preparation of 4-(4’-Methylbenzylidene)-2-hydroxy-3-pinanone 1.68 g (10.0 mmol) 2-hydroxy-3-pinanone, 1.44 g 3-pinanone, 1.68 g p-methyl benzaldehyde, 10 mL tert-butanol, and 0.31 g potassium tert-butoxide were successively added into 50 mL three-neck flask and heated at 70°C for 3h under protection of N\(_2\). The reaction was monitored with GC until the conversion ratio of 2-hydroxy-3-pinanone reached 99.3%. Reaction was extracted with 75 mL × 3 ethyl acetate. Saturated brine was used to wash the combined organic layer until neutrality. The residue was dried with Na\(_2\)SO\(_4\) then recrystallized in acetyl acetate–petroleum ether (1 : 2 (v/v)) to get 2.12 g of white crystal, yield 78.4%, purity 99.0%, mp 125.8–126.4°C; IR (KBr, \(cm^{-1}\)): 3474, 2975, 2920, 2869, 1697, 1611, 1509, 1469, 1052, 1011, 841, 811; \(^1\)H-NMR (CDCl\(_3\), 500MHz) \(\delta\) = 0.84 (3H, t), 1.38 (3H, s), 1.39 (3H, s), 1.83 (d, \(J =10.45\) Hz, 1H), 2.10 (t, \(J =6.11\) Hz, 1H), 2.51–2.55 (m, 1H), 3.29 (t, \(J =6.11\) Hz, 1H), 5.35 (s, 1H), 7.20–7.25 (m, 4H), 7.47
(s, 1H); 13C-NMR (CDCl3, 125 MHz) δ: 20.79, 23.71, 25.10, 26.83, 28.29, 39.50, 42.73, 50.53, 74.68, 129.06, 129.25, 131.82, 132.08, 137.92, 140.16, 201.20; MS (70 eV) m/z (%): 270 (11), 252 (6), 237 (4), 227 (29), 212 (9), 201 (21), 199 (6), 185 (5), 171 (38), 165 (8), 157 (14), 143 (31), 141 (20), 129 (25), 115 (23), 105 (32), 99 (100), 91 (12), 79 (12), 65 (6).

**Synthesis of WSF-7** 0.68 g (2.5 mmol) of 4-(4-methylbenzylidene)-2-hydroxy-3-pinacoline, 0.21 g (3.0 mmol) of hydroxymethylalcohol chloride, 5 mL 20% aqueous NaOH, and 10 mL anhydrous ethanol were added into 50 mL three-neck flask and heated at 60°C for 1–2 h under N2 protection. Ethyl acetate was used for extraction. The organic layer was dried with Na2SO4 after being washed with saturated brine until neutrality. The residue was recrystallized in cyclohexane-ethyl acetate to get white needle crystal WSF-7, yield 69.8%, purity 99.3%, mp 130.8–131.4°C; IR (KBr, ν cm⁻¹): 3439, 2973, 2922, 2872, 1706, 1517, 1472, 1043, 839, 576, 733; 1H-NMR (CDCl3, 500 MHz) δ: 0.69 (s, 3H), 0.99 (s, 3H), 1.34 (s, 3H), 1.53 (t, J = 6.05 Hz, 1H), 1.88 (d, J = 10.7 Hz, 1H), 1.98 (t, J = 6.05 Hz, 1H), 2.25 (s, 3H), 2.34–2.38 (m, 2H), 3.57 (d, J = 9.7 Hz, 1H), 5.40 (s, 1H), 7.07 (d, J = 8.0 Hz, 2H), 7.13 (d, J = 7.95 Hz, 2H); 13C-NMR (CDCl3, 125 MHz) δ: 20.64, 23.10, 24.45, 26.88, 39.81, 39.66, 46.16, 50.85, 52.71, 75.05, 127.46, 128.22, 133.42, 135.95, 213.02; MS (70 eV) δ: 267 (48), 266 (22), 252 (58), 238 (8), 224 (79), 210 (5), 199 (9), 184 (44), 171 (11), 170 (57), 158 (13), 157 (18), 143 (60), 134 (22), 129 (13), 128 (23), 118 (20), 109 (25), 105 (51), 103 (100), 91 (50), 78 (99), 77 (87), 67 (35), 65 (31), 54 (25), 53 (34), 51 (24).

**Cell Differentiation and Treatment** 3T3-L1 preadipocytes (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) supplemented with 10% fetal bovine serum (FBS) (GIBCO) at 37°C in 5% CO₂ and induced to differentiate in DMEM containing 0.5 mM IBMX, 1 mM DEX, 5 mg/mL insulin and 10% FBS. To examine the effect on differentiation, 3T3-L1 preadipocytes were induced to differentiate in DMEM containing 0.5 mM IBMX, 1 mM DEX, 5 mg/mL insulin and 10% FBS. To examine the effect on differentiation, 3T3-L1 preadipocytes were induced to differentiate in the presence of WSF-7 or 0.5 µM rosiglitazone. At Day 8 post-differentiation, the cells were stained with Oil Red O. To examine the effect on adiponectin oligomerization, mature 3T3-L1 adipocytes were harvested and 3T3-L1 adipocytes were incubated with sample buffer containing 10 mM dithiothreitol (DTT) for 1h at room temperature, followed by heat denaturation at 95°C for 10 min. Adiponectin monomer was detected using antibody raised against the N-terminal peptide. WPAR was detected by antibodies for Ser273 pPPARγ or total PPARγ. Akt was detected using antibodies specific for Ser473 pAkt or total Akt. The amount of each protein was quantified by analyzing the western blots using the NIH ImageJ software. All experiments were performed at least three times and the representative results were presented.

**PPARγ Ligand Binding Assay** In vitro binding of WSF-7 to PPARγ was analyzed by a Lanthascreen™ time-resolved fluorescent energy transfer (TR-FRET) PPARγ competitive binding assay kit (Invitrogen, Shanghai, China), in accordance with the manufacturer’s instructions. WSF-7 or rosiglitazone was mixed with Fluormone Pan-PPAR Green, GST-PPARγ-LBD, and Tb-GST antibody in a black 384-well assay plate and incubated at room temperature for 2h. The fluorescent emission signal was measured by PerkinElmer, Inc. EnSpire™ Multimode Microplate Reader at 490 and 520 nm, using a 340 nm excitation filter.

**Luciferase Reporter Assay** 293T cells were transfected with PPRE-TK-Luciferase reporter along with PPARγ and retinoid X receptor α (RXRα) expression vectors. 24h after transfection, the cells were treated with WSF-7 or rosiglitazone in the presence or absence of GW9662. The cells were harvested for the luciferase assay after treatment for 24h. Luciferase activities were normalized to Renilla activities cotransfected as an internal control.

**Glucose Uptake Assay** 3T3-L1 adipocytes were treated with WSF-7 or rosiglitazone in the presence or absence of 25 µM GW9662 or 2.5 µM wortmanin for 24h, then incubated in glucose-free DMEM for 2h, followed by stimulation with 5mg/mL insulin for 10min. Glucose uptake was measured by a bioluminescent assay, using the Glucose Uptake-Glo™ Assay Kit (Promega, Beijing, China).

**Membrane Isolation** 3T3-L1 adipocytes were harvested for membrane isolation after treatment with WSF-7 or rosiglitazone for 24h. Total membranes were prepared using Membrane and Cytosol Protein Extraction Kit (Beyotime, Shanghai, China), in accordance with the manufacturer’s instructions. Membrane-bound and cytoplasmic GLUT4 were detected by anti-GLUT4 antibody.

**Site-Directed Mutagenesis** Site-directed mutants were generated in PPARγ expression vector using Fast Mutagenesis System (TransGen Biotech, Beijing, China). DNA sequencing was used to confirm the presence of the desired mutations in the LBD of mouse PPARγ, including K288A, F310A, and H489A.

**Statistical Analysis** All data were expressed as the means ± standard deviation (S.D.). Student’s t-test was used to compare two samples. Statistical comparisons among three samples of the same treatment group were performed using one-way ANOVA with Dunnett’s test as posthoc test. In all cases, a p-value less than 0.05 was considered to be statistically significant.

**RESULTS**

WSF-7 Is a Novel Partial PPARγ Agonist WSF-7, derived from natural monoterpene α-pinene (Fig. 1A), was identified as a potential PPARγ agonist in our screen for synthetic chemicals that inhibit Ser273 phosphorylation of PPARγ. To confirm that WSF-7 is a ligand for PPARγ, we examined in vitro binding of WSF-7 to PPARγ-LBD in a TR-FRET PPARγ competitive binding assay. Similar to rosiglitazone, WSF-7 binds directly to the LBD of PPARγ with an IC50 value of 99 nM (Fig. 1B).
Fig. 1. WSF-7 Binds Directly to PPARγ and Activates the Transcriptional Activity of PPARγ by a Novel Mechanism

(A) Synthesis of WSF-7. (B) In vitro binding of WSF-7 or rosiglitazone (Rosi) to PPARγ was examined by TR-FRET PPARγ competitive binding assay. The fluorescent emission signal was measured at 490 and 520 nm, using a 340 nm excitation filter. The ratio of 520/490 nm was calculated and presented. (C) PPARγ and RXRa expression plasmids and PPRE-TK-Luciferase reporter were cotransfected into 293T cells. The luciferase activity of the transfected cells was measured after treatment with WSF-7 for 24h. The results were presented as mean ± S.D. *p < 0.05, **p < 0.01. (D) PPARγ (wild-type or mutant) and RXRa expression plasmids and the reporter plasmid were transected into 293T cells, followed by treatment with WSF-7 (20µM), rosiglitazone (0.5µM) or luteolin (Lut) (20µM) for 24h. Luciferase activity was examined and presented.

Fig. 2. WSF-7 Promotes Preadipocyte Differentiation

WSF-7 was used to treat 3T3-L1 preadipocytes with or without 20µM GW9662 for eight days. (A) Oil Red O was used to stain differentiated cells, with quantification results shown in B. (C) PPARγ and aP2 were detected by Western blot with specific antibodies. *p < 0.05, **p < 0.01. (Color figure can be accessed in the online version.)
Fig. 3. WSF-7 Promotes Adiponectin Oligomerization

3T3-L1 adipocytes were treated with WSF-7 for 48h. (A) HMW, MMW and LMW adiponectin were separated under native conditions and detected by antibodies specific for the C-terminal globular domain. N-terminal peptide antibody was used to detect adiponectin monomer (Total Ad). (B) The ratio of HMW/Total Ad was presented. *p < 0.05, **p < 0.01.

Fig. 4. WSF-7 Enhances Insulin-Induced Glucose Uptake in 3T3-L1 Adipocytes

WSF-7 was used to treat 3T3-L1 adipocytes for 24h, followed by insulin stimulation for 10min. (A) Glucose uptake was examined by a bioluminescent assay and presented relative to the non-treated control (WSF-7 0μM, GW9662 0μM) or GW9662-treated control (WSF-7 0μM, GW9662 20μM). (B) Total membrane or cytoplasmic proteins were analyzed by anti-GLUT4 or anti-PPARγ antibody. (C) 3T3-L1 adipocytes were treated with WSF-7 in the absence or presence of 2.5μM wortmanin. Glucose uptake and the level of membrane-bound GLUT4 were examined. (D) 3T3-L1 adipocytes were treated with WSF-7, rosiglitazone or insulin for 1h. Phosphorylated Akt at Ser473 (pAkt) and total Akt were detected using anti-pAkt or anti-Akt antibodies, respectively. *p < 0.05, **p < 0.01.
agonists were reported to promote adipogenesis.\(^{11,18,19}\) As a mechanism, high molecular weight (HMW) is closely associated with insulin resistance, there-fore, HMW adiponectin possesses the most potent insulin-sensitizing effect.\(^{20}\) PPAR\(\gamma\) agonists were reported to elevate the level of adiponectin mRNA via a PPRE (PPAR\(\gamma\)-responsive regulatory element) present in promotor. PPAR\(\gamma\) agonists also upregulate expression of DsbA-L and Ero1-L\(\alpha\), which have been demonstrated to be involved in promoting adiponectin oligomerization.\(^{21}\)

After treatment for 48h, the level of adiponectin monomer (Total Ad) was elevated by WSF-7 (Fig. 3A), confirming that WSF-7 upregulates adiponectin expression. Adiponectin oligomerization was also promoted by WSF-7, as can be seen by increased level of LMW, MMW and HMW oligomers. Rosiglitazone was reported to enhance insulin sensitivity by increasing the HMW/totol adiponectin. WSF-7 treatment also increased this ratio (Fig. 3B). The effects of WSF-7 on adiponectin were abolished by GW9662 (Fig. 3). Therefore, WSF-7 promotes adiponectin oligomerization by PPAR\(\gamma\) activation in 3T3-L1 adipocytes.

**WSF-7 Enhances Insulin-Induced Glucose Uptake in 3T3-L1 Adipocytes** Insulin-induced glucose uptake, an important function of adipocytes, can be activated by PPAR\(\gamma\) agonists.\(^{22,23}\) To investigate the effect of WSF-7, 3T3-L1 adipocytes were treated with WSF-7 for 24h, then induced with insulin for ten minutes. We found that WSF-7 promoted insulin-stimulated glucose uptake and that WSF-7-mediated effect was abrogated by GW9662 (Fig. 4A).

GLUT4 plays a key role in facilitating insulin-induced glucose uptake in adipocytes.\(^{24}\) As a PPAR\(\gamma\) target gene, GLUT4 expression was increased by WSF-7, which was inhibited in the presence of GW9662 (Fig. 4B), consistent with previous reports that PPAR\(\gamma\) agonists increase the expression of GLUT4 by activating PPAR\(\gamma\).\(^{25,26}\) The level of membrane-bound GLUT4 was also increased by WSF-7 (Fig. 4B).

To investigate whether WSF-7 activates the phosphati-dylinositol 3-kinase (PI3K)/Akt pathway, 3T3-L1 adipocytes were treated with WSF-7 together with wortmanin, a PI3K inhibitor. Insulin-induced glucose uptake was inhibited by wortmanin, however, the level of membrane-bound GLUT4 was still increased by WSF-7 in the presence of wortmanin (Fig. 4C). No WSF-7-induced Akt phosphorylation was detected.
(Fig. 4D). Therefore, WSF-7 does not activate the PI3K/Akt pathway directly. The inhibition of insulin-induced glucose uptake by wortmannin might be caused by the indirect inhibitory effect on the insulin signaling pathway, independent of WSF-7. Our results demonstrate that WSF-7 enhances insulin-induced glucose uptake via GLUT4 upregulation in 3T3-L1 adipocytes. 28) Our results suggest that cdk5 non-agonist WSF-7 exhibits much weaker PPARγ antagonism (Fig. 2C). Therefore, WSF-7 stimulates the endogenous PPARγ activity both during and after adipogenesis. However, WSF-7 inhibits Ser273 phosphorylation of PPARγ in 3T3-L1 adipocytes.

**DISCUSSION**

In this paper, we found that WSF-7 binds directly to PPARγ and activates the transcriptional activity of PPARγ (Fig. 1). We demonstrated that WSF-7 stimulates the endogenous PPARγ activity of mature 3T3-L1 adipocytes by the following results: (1) WSF-7 increases PPARγ expression (Fig. 4B); (2) PPARγ-responsive genes, such as adiponectin and GLUT4, are also upregulated by WSF-7 (Figs. 3A, 4B); (3) GW9662 suppresses WSF-7-mediated effect on gene expression. In addition, we found that WSF-7 increases the expression of PPARγ and aP2 (another PPARγ target gene) during preadipocyte differentiation (Fig. 2C). Therefore, WSF-7 stimulates the endogenous PPARγ activity both during and after adipogenesis. However, WSF-7 exhibits much weaker PPARγ-agonistic effects than rosiglitazone (Figs. 1, 2). Therefore, WSF-7 is a partial PPARγ agonist.

PPARγ activities are selectively regulated by the binding of specific ligands at LBD. A small four-stranded β-sheet and 12 α-helices in a three-layer sandwich are present in PPARγ LBD, which provides a large binding pocket and multiple points to interact with ligands. Full agonists, like TZDs, stabilize C-terminal helix 12, which is part of a binding cleft for coactivator in AF2, leading to efficient recruitment of transcriptional coactivator. There is an alternate site for ligand binding in LBD, which is adjacent to a flexible loop with about 15 amino acid residues between helices H2’ and H3. 27) When luteolin binds to PPARγ, the amino-terminal portion of AF2 is incompletely folded into α-helical conformation. 29) Our results suggest that WSF-7 might activate PPARγ by a novel mechanism (Fig. 1D). We are currently in the process of determining how WSF-7 binds to the LBD of PPARγ.

PPARγ phosphorylation at Ser273 has been linked to insulin resistance. 30) PPARγ agonists, both full and partial, specifically inhibit this phosphorylation. 6-8,11) Moreover, non-agonist PPARγ ligands also improve insulin sensitivity by inhibiting this phosphorylation. 31) The adverse side effects of TZDs have been reported to be reduced significantly by PPARγ partial agonists or even non-agonist without loss of insulin sensitization. Therefore, blocking Ser273 phosphorylation without classical agonism is a powerful strategy for developing novel antidiabetic drugs targeting PPARγ. We have previously identified several natural products from traditional Chinese medicine as partial PPARγ agonists. 11,14) WSF-7, the first synthetic PPARγ agonist we have identified in our screen, also inhibits phosphorylation of PPARγ at Ser273 (Fig. 5). It is possible that the binding of WSF-7 at the LBD causes the conformational change of PPARγ, blocking the accessibility of Cdk5 to Ser273. The effect of WSF-7 on the insulin sensitivity of diabetic rats is currently under investigation.

Insulin-induced glucose uptake, with GLUT4 as the major glucose transporter, is very important for maintaining glucose homeostasis in adipocytes. 29) The level of GLUT4 correlates highly with insulin sensitivity. 30) When blood glucose level is low, GLUT4 is sequestered in the membranes of small internal vesicles. Insulin, secreted from pancreatic β-cells in response to high glucose, induces these vesicles to move to the plasma membrane, with which they fuse, increasing the number of membrane-bound GLUT4. As a result, glucose uptake is stimulated. Our results demonstrate that WSF-7 promotes insulin-induced glucose uptake through activating the transcriptional activity of PPARγ, leading to increased expression and translocation of GLUT4 (Fig. 4), as reported previously for other PPARγ agonists. 8,25,26) We failed to detect WSF-7-induced Akt phosphorylation (Fig. 4D). Therefore, WSF-7 does not activate the PI3K/Akt pathway directly. We also demonstrate that WSF-7 promotes adiponectin oligomerization by increasing the level of HMW adiponectin (Fig. 3), which possesses the most potent insulin-sensitizing effect. Therefore, in addition to inhibiting PPARγ Ser273 phosphorylation, WSF-7 also improves insulin sensitivity of adipocytes by increasing the HMW/total Ad ratio and promoting insulin-stimulated glucose uptake. WSF-7 can be easily synthesized from natural monoterpene α-pinene, which is found in the oils of many coniferous trees. These characteristics, combined with its weak PPARγ-agonistic but effective insulin-sensitizing activities, make WSF-7 a potential safer drug for T2DM treatment.

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**Conflict of Interest** The authors declare no conflict of interest.

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