C333H Ameliorated Insulin Resistance through Selectively Modulating Peroxisome Proliferator-Activated Receptor γ in Brown Adipose Tissue of db/db Mice

Ning Zhang, Wei Chen, Xinbo Zhou, Xiaolin Zhou, Xinni Xie, Aimin Meng, Song Li, and Lili Wang*

Department of Molecular Nuclear Medicine, Tianjin Key Laboratory of Molecular Nuclear Medicine, Tianjin 300192, P. R. China: and Beijing Institute of Pharmacology and Toxicology, Beijing 100850, P. R. China.

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Peroxisome proliferator-activated receptor γ (PPARγ) is a unique target for insulin sensitizer agents. These drugs have been used for the clinical treatment of type 2 diabetes for almost twenty years. However, serious safety issues are associated with the PPARγ agonist thiazolidinediones (TZDs). Selective PPARγ modulators (SPPARMs) which retain insulin sensitization without TZD-like side effects are emerging as a promising new generation of insulin sensitizers. C333H is a novel structure compound synthesized by our laboratory. In diabetic rodent models, C333H has insulin-sensitizing and glucose-lowering activity comparable to that of TZDs, and causes no significant increase in body weight or adipose tissue weight in db/db mice. In diabetic db/db mice, C333H elevated circulating high molecular weight adiponectin isoforms, decreased PPARγ 273 serine phosphorylation in brown adipose tissue and selectively modulated the expression of a subset of PPARγ target genes in adipose tissue. In vitro, C333H weakly recruited coactivator and weakly dissociated corepressor activity. These findings suggest that C333H has similar properties to SPPARMs and may be a potential therapeutic agent for the treatment of type 2 diabetes.

Key words  C333H; peroxisome proliferator-activated receptor γ (PPAR γ); insulin resistance; selective PPARγ modulator; type 2 diabetes

Thiazolidinediones (TZDs) are unique insulin sensitizing drugs used for the treatment of type 2 diabetes and other metabolic syndromes associated with insulin resistance. The γ subtype of peroxisome proliferator activated receptors (PPARγ) acts as target for TZDs and is key regulator of glucose homeostasis and adipocyte differentiation. As a nuclear receptor, PPARγ firstly interacts with retinoid X receptor α (RXRα) to form a heterodimer, which in turn recruits specific coactivators or corepressors depending on physiological environment. It then binds to specific response elements (PPREs) within promoter region of target genes to control gene transcription. However, the unwanted effects of TZDs including body weight gain, edema, increased risks of congestive heart failure, and increased rate of bone fracture restrict the clinical usage of these drugs.

It has been known for several years that the insulin sensitizing effects can be obtained by PPARγ ligands without the risk of TZD-like side effects. Genetic and epidemic studies have shown that the side effects of TZDs are closely related to overactivation of PPARγ, whereas physiologically appropriate PPARγ activity is beneficial in terms of reducing insulin resistance and other risks associated with type 2 diabetes. More recently, it has been reported that the inhibition of CDK5 mediated PPARγ 273 serine phosphorylation, rather than PPARγ agonism and lipogenic effects, constitutes the key target for insulin sensitization and the antidiabetic effects of PPARγ agonists. The same workers reported that a non-agonist PPARγ ligand, SR1664, inhibited PPARγ 273 serine phosphorylation, and enhanced insulin sensitivity without causing TZD-like body weight gain and increased haematocrit in vivo. Moreover, several other selective PPARγ modulators (SPPARMs) without significant TZD-like side effects, for example MBX102 and INT131, have already entered Phase II or III clinical development.

C333H (2-(3-furan-2-yl-acyrloylamino)-3-[(4-[(5-methyl-2-phenyl-oxazol-4-yl)-ethoxy]-phenyl)-propioniacid; Fig. 1) is a novel PPARγ dual agonist (EC50 for PPARα and PPARγ is 0.399±0.006 and 0.155±0.057μM respectively in transcript activation assay). C333H predominately exhibited effective glucose lowering effects in diabetic db/db mice. Here we report the outcomes of studies conducted to further explore its insulin sensitizing effect and action mechanism in db/db mice, and in insulin-resistant monosodium glutamate (MSG) rats. We further demonstrated that C333H displayed equivalent antidiabetic potency to that of rosiglitazone, but did not significantly increase body weight and adipose tissue weight. Further studies indicated that C333H showed important differences to rosiglitazone in terms of regulation of lipogenic gene transcription in white and brown adipose tissues, and in terms of selectively at inhibiting phosphorylation of PPARγ 273 serine in brown adipose tissue of db/db mice. In vitro studies demonstrated that C333H showed weak potency to PPARγ coactivator recruitment and corepressor dissociation.

MATERIALS AND METHODS

Materials  C333H and rosiglitazone were synthesized by the New Drug Design Centre at the Beijing Institute of Pharmacology and Toxicology. Purity and structure were confirmed by high-performance liquid chromatography, mass spectrometry and H-nuclear magnetic resonance.

Cell Lines and Cell Culture  Hela and A293T cells were maintained at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) and RPMI 1640 medium, respec-
Plasmid Construction Human PPARγ2 (total length) was obtained from a human lung tissue cDNA library by polymerase chain reaction (PCR) and cloned into pACT-CMV plasmid (Promega, U.S.A.) to generate a pACT-hPPARγ2 chimera. Human CBP (1–115 amino acid), human nCoR1 (2239–2300 amino acid), and human SRC2 (618–826 amino acid) were obtained from a Hela cell cDNA library by PCR and cloned into pBIND-CMV to generate pBIND-hCBP, pBIND-hnCoR1, pBIND-hSRC2 chimeras. The PPARγ2 forward primer set was 5′-TAG TCG ACT GGT GTA AACT CTT GGG-3′ and the corresponding reverse primer was 5′-GCC CTC TAG ACT AGC GG AGC GAG CGC-3′. The CBP forward primer was 5′-ATT CTC GAC TGT CTC GGG GCG CGT TTT-3′ and the corresponding reverse primer was 5′-TAT CTA GAC GCC-3′. The nCoR1 forward primer was 5′-CTT GTG TAC AAG GCT CTC AG-3′ and its reverse primer was 5′-AAC TGT CCT ATG AGG TGT GGG CAG TAC CAG-3′. The SRC2 forward primer was 5′-CCG GAT CCT TCC CCC GGC CCT GAG CAG TGG AG-3′ and its reverse primer was 5′-GGG GGA TCA CCT CTC GTG TCT GGG AAA AGC TGT G-3′.

Mammalian Two-Hybrid Assay A293T cells were transiently transfected with 0.2 μg of the expression vector pACT-hPPARγ2, and with 0.2 μg of the expression vectors pBIND-hCBP, pBIND-hSRC2 or pBIND-hnCoR1, using lipofectamine 2000 (Invitrogen, U.K.). The transfection process also included 0.2 μg of the reporter construct pGLuc and 0.2 μg of reference plasmid pRL-CMV vector. Twenty-four hours after transfection, cells were exposed to C333H or rosiglitazone (10 μg/kg) for 5 weeks prior to performing the euglycemic–hyperinsulinemic clamp experiment.

For the euglycemic–hyperinsulinemic clamp experiment, rats were fasted overnight and cannulated in the jugular vein and opposite carotid artery. Blood samples were taken from the carotid catheter to measure basal glucose. Experiments were initiated following infusion of insulin (4 μU/kg/min) into the jugular vein. The glucose (20% dextrose) infusion was initiated when blood glucose fell below basal levels. The glucose infusion rate (GIR) was adjusted empirically to achieve blood glucose level of 5.0±0.5 mmol/L within the next 1.5 to 2 h. To facilitate this process, blood samples were taken from the carotid catheter at 10-min intervals for immediate plasma glucose measurements using a glucometer. This continued until the end of the study. The clamp was defined by the five or six consecutive glucose measurements that were within the range 5.0±0.5 mmol/L.

Serum Adipokine Detection Serum leptin and insulin were measured by FlexMAP3D (Luminex, xMAP Technology, U.S.A.) using a MILLIPLEX MAP Mouse Metabolic Magnetic Bead Panel kit (Millipore, U.S.A.). Serum high molecular weight (HMW) adiponectin was measured by Spectra MAXM5 (Molecular Device, U.S.A.) using a Mouse total, HMW adiponectin ELISA Kit (ALPCO Diagnostics, U.S.A.). The insulin sensitivity index (ISI) was calculated from the values of overnight fasting blood glucose (FBG) and fasting blood insulin (FBI). ISI was calculated as: 1/(FBG×FBI)×1000, where FBG was expressed as mg/dL and FBI as mU/L.

Gene Expression Analysis Total RNA was isolated from cells or tissues using a high purity total RNA extraction kit (BioTeke Corporation, China) according to the manufacturer’s instructions, and was purified using a RQI RNA-Free DNase I system (Promega). cDNA synthesis from oligodT primers was performed using an M-MLV RTase cDNA synthesis kit.
The glucose infusion rate needed to maintain euglycemia was significantly lower in MSG treated rats than in normal litters. C333H treatment markedly increased the glucose infusion rate in a dose dependent manner (Fig. 2A), suggesting that it ameliorates insulin resistance in MSG rats.

**Effect of C333H on Blood Glucose and Insulin Sensitivity in db/db Mice** The in vivo therapeutic potential of C333H on metabolic dysfunction was evaluated in genetic T2DM db/db mice. C333H was administered to db/db mice for 15 d and blood glucose levels were monitored at indicated time points. C333H significantly decreased fasting blood glucose levels in a time- and dose-dependent manner (Fig. 2B). C333H also significantly improved glucose tolerance and reduced the area under the curve (AUC) obtained from an oral glucose test after 12 d of treatment (Figs. 2C, D). This was accompanied by a dose-dependent significant decrease in fasting blood glucose and serum insulin levels (Figs. 2E, F) and a significant increase in the insulin sensitivity index (Fig. 2G). These results indicate that C333H ameliorates glucose disorders and insulin resistance in db/db mice.

**Effect of C333H on Body Weight Gain and Adipose Tissue Weight in db/db Mice** Rosiglitazone significantly increased body weight from the second day of treatment until the end of experiment, whereas no significant change in body weight was seen during treatment with C333H (Fig. 3A). Rosiglitazone significantly increased BAT weight but no significant changes in WAT or BAT weights were seen with C333H. The weight of BAT did not change significantly after 12 d of treatment (Fig. 3B).

**Effect of C333H on Serum Adiponectin and Leptin Levels in db/db Mice** Adipokines (which are mainly secreted by adipocytes) are able to completely reverse insulin resistance in diabetic rodents. Elevated leptin levels are considered to represent leptin resistance, which enhances insulin resistance in leptin receptor deficient db/db mice. C333H significantly increased the serum HMW adiponectin in diabetic db/db mice, to concentrations slightly higher than in normal mice (Fig. 3A). Rosiglitazone significantly increased BAT weight but no significant changes in WAT or BAT weights were seen with C333H (Fig. 3B).

**Effect of C333H on Serum Adiponectin and Leptin Levels in db/db Mice** Adipokines (which are mainly secreted by adipose tissue), such as adiponectin and leptin play vital roles in regulating energy homeostasis and insulin sensitivity. Adiponectin, especially the high molecular weight (HMW) isoform, has a strong association with insulin resistance, and levels of circulating adiponectin are lowered in diabetic animal models and patients. It has been shown that physiological concentrations of reconstituted adiponectin are able to elevate levels of circulating adiponectin in diabetic rodents. Elevated leptin levels are considered to represent leptin resistance, which enhances insulin resistance in leptin receptor deficient db/db mice. In our experiments C333H significantly increased the serum HMW adiponectin in diabetic db/db mice, to concentrations slightly higher than in normal mice (Fig. 4A). Elevated serum leptin levels in db/db mice were significantly decreased by rosiglitazone and insignificantly increased by C333H (Fig. 4B).
Fig. 2. Effects of C333H Treatment on Fasting Blood Glucose and Insulin Sensitivity in Rodent Models

(A) Glucose disposal was evaluated in MSG rats using the hyperinsulinemic–euglycemic clamp method as described in Materials and Methods. Glucose infusion rates (GIR) were measured at steady state after administration of vehicle (MC; 2% DMSO), rosiglitazone (RSG; 10 mg/kg), C333H (C; 1, 3 or 10 mg/kg) to MSG rats and vehicle-administrated normal littermates (NC). Each bar represents mean±S.D. n=6–8, *p<0.05 vs. MC.

(B) Fasting blood glucose (for 4 h) following changes of vehicle (MC; 2% DMSO), rosiglitazone (RSG; 10 mg/kg) or C333H (C; 5, 10 or 20 mg/kg) in db/db mice were measured at indicated times during 15 d of treatment.

(C) An oral glucose tolerance test (OGTT) was performed after 12 d of treatment in db/db mice. (D) The area under the curve (AUC) of the OGTT was calculated. (E) Fasting blood glucose (overnight) and (F) fasting serum insulin levels (overnight) were measured at the end of treatment in db/db mice. (G) The insulin sensitivity index (ISI) was calculated. Each bar represents mean±S.D. n=8, *p<0.075, *p<0.05 vs. MC.
reduced by C333H (Fig. 4B). These results suggest that C333H improves circulating adipokine dysfunction in db/db mice.

Impact of C333H on PPARγ Target Gene Expression in db/db Mice
To identify the molecular mechanism and distinct pharmacological profile of C333H, the mRNA levels of representative PPARγ target genes involved in glucose utilization and lipid metabolism were analyzed in white and brown adipose tissues of diabetic db/db mice. Rosiglitazone and C333H both attenuated induction of the mRNA levels of fatty acid transport protein (FATP) in white adipose tissue. They also increased levels of lipid droplet associated protein cell death-inducing DFFA-like effector c (Cidec), and the energy expenditure related protein Rev-Erb alpha (NR1D1). C333H and rosiglitazone both increased the mRNA levels of thermogenic gene uncoupling protein 1 (UCP1) and glucose transport protein 4 (Glut4), and reduced the mRNA levels of glucogenic protein phosphoenol pyruvate carboxykinase (PEPCK) and lipolytic protein hormone-sensitive lipase (HSL). C333H markedly reduced the mRNA levels of fatty acid binding protein 2 (aP2) and lipoprotein lipase (LPL) (Fig. 4C).

In brown adipose tissue, C333H, unlike rosiglitazone significantly reduced mRNA levels of LPL, FATP, aP2, and PEPCK but had no impact on mRNA levels of UCP1. C333H and rosiglitazone both increased mRNA levels of NR1D1 and Cidec and both decreased Glut4 mRNA levels (Fig. 4D).

Effect of C333H on the Phosphorylation of PPARγ Serine 273 and Total PPARγ Level in Brown and White Adipose Tissues of db/db Mice
Phosphorylation of PPARγ Serine 273 by CDK5 has been shown to alter the effects of PPARγ and deregulates a subset of PPARγ target genes.6,7) The effects of C333H on phosphorylation of PPARγ 273 serine and total PPARγ levels were detected by western blot analysis in the white and brown adipose tissues of db/db mice. Rosiglitazone increased the total PPARγ level and insignificantly reduced PPARγ phosphorylation in WAT, whereas C333H had no impact on total PPARγ levels or PPARγ phosphorylation in WAT (Fig. 5A). However, both C333H and rosiglitazone increased total PPARγ levels and decreased PPARγ phosphorylation in BAT (Fig. 5B).

The Impact of C333H on PPARγ Cofactor Recruitment
The dissociation of corepressors and subsequent recruitment of coactivators are prerequisites for regulating gene expression by ligand-dependent PPARγ activation. Selective cofactor recruitment is a hallmark of SPPARM.2) To evaluate the effects of C333H on cofactor recruitment by PPARγ, ligand-induced interactions of human PPARγ with SRC2, CBP and nCoR1 were each examined using a mammalian two-hybrid system in A293T transfected cells. Rosiglitazone and C333H significantly induced the recruitment of coactivators SRC2 and CBP. C333H also induced the dissociation of corepressor nCoR1, but to a lesser extent than rosiglitazone (Figs. 5C, D).

DISCUSSION
SPPARMs provide insulin-sensitizing effects with minimal TZD-like side effects. However its molecular mechanism of action is still poorly understood even though their pharmacological properties are widely accepted. SPPARMs are generally effective at lowering blood glucose. They ameliorate adiponectin dysfunction with minimal body weight gain and lipogenic effects, and selectively induce the recruitment of PPARγ cofactors which distinguishes them from the full agonists. Importantly, SPPARMs also selectively regulate PPARγ target gene transcription in response to specific cofactor recruitment.18–20)

In present report, the insulin sensitizing properties of C333H were further confirmed in diabetic db/db mice and insulin resistant obese MSG rats. In db/db mouse, C333H was at least as effective as the full PPARγ agonist rosiglitazone in ameliorating the glucose intolerance and increasing ISI. Moreover, C333H improved insulin resistance in obese MSG rats and increased the GIR in the euglycemic–hyperinsulinemic
clamp test. These effects were accompanied by the increase in serum HMW adiponectin concentrations, to the levels of normal littermates. However, unlike rosiglitazone, C333H did not significantly increase body weight and BAT weight in db/db mice, indicating that C333H enhanced insulin sensitivity, without promoting lipogenesis.

Cofactor recruitment is essential for the regulation of PPARγ target genes. It has been shown that TIF-2 and CBP/P300 favor fat accumulation, while nCoRI and/or SMRT inhibit PPARγ action and adipogenesis. SPPARMs cause minimal recruitment of TIF-2 and CBP/P300 together with only partial release of NCoRI.21) These three coactivators were therefore selected to appraise the impact of C333H on cofactor recruitment. Using mammalian two-hybrid assays, we demonstrated that C333H weakly promotes dissociation of the PPARγ corepressor NCoRI, and weakly promoted recruitment of coactivators CBP/P300 and SRC2. These effects may partially explain its antidiabetic efficacy and relative absence of TZD-like side effects.

Promoting dephosphorylation of PPARγ 273 serine is
thought to be the key mechanism for selective PPARγ modulation, insulin sensitization and the antidiabetic effects of SPPARMs.\(^6,7\) Recent studies indicate that the newly developed SPPARMs, such as SR1664, MRL24 and MBX102 exhibit similar insulin sensitizing effects to rosiglitazone despite causing less PPARγ activation.\(^6\) In addition these agents do not appear to induce lipogenesis or body weight gain. The studies above also shown inhibition of CDK5 mediated phosphorylation of adipocyte PPARγ 273 serine selectively induces expression of specific subsets of PPARγ target genes that are responsible for the pharmacological effects of SPPARMs.\(^5,7\) This mechanism involves selective induction of the expression of adipocyte thermogenic genes and selective inhibition of adipogenic gene expression in adipose tissue.\(^22\) In present study, we analyzed the phosphorylation of PPARγ 273 serine and investigated expression of representative PPARγ target genes in white and brown adipose tissues of db/db mice. We found that C333H promoted dephosphorylation of

![Fig. 5. Effects of C333H on PPARγ Phosphorylation in White and Brown Adipose Tissues of db/db Mice and in Vitro Cofactor Recruitment to Human PPARγ](image)
PPARγ 273 serine in brown adipose tissue to the same degree as rosiglitazone, but C333H did not exert this effect in white adipose tissue. In terms of regulation of target genes, C333H, in common with SPPARMs, selectively decreased the pool of adipogenic genes, and at the same time increased thermogenic genes in white and brown adipose tissues. Meanwhile, we also further studied PPARα target genes’ expression in the liver of db/db mice after 3 and 15 d treatment of C333H respectively. It was observed that different from that of rosiglitazone, C333H also significantly increased expression of PPARα target genes (such as CPT1a, ACO and LPL) (data not shown), which promote lipolysis and fatty acid oxidation. These results demonstrated the PPARα agonistic activity of C333H in vivo also contribute to its insulin sensitization, and at the same time antagonize some side effects (such as body weight and adipose weight increase) of the pure PPARγ agonist.

Taken together the evidence presented indicates that C333H retains the beneficial insulin sensitizing effects of the TZDs while displaying minimal adipogenic capacity. The SPPARM properties of C333H may be of potential benefit in the treatment of type 2 diabetes.

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