A Nonthiazolidinedione Peroxisome Proliferator-Activated Receptor α/γ Dual Agonist CG301360 Alleviates Insulin Resistance and Lipid Dysregulation in db/db Mice


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ABSTRACT

Activation of peroxisome proliferator-activated receptors (PPARs) have been implicated in the treatment of metabolic disorders with different mechanisms; PPARα agonists promote fatty acid oxidation and reduce hyperlipidemia, whereas PPARγ agonists regulate lipid redistribution from visceral fat to subcutaneous fat and enhance insulin sensitivity. To achieve combined benefits from activated PPARα on lipid metabolism and insulin sensitivity, a number of PPARα/γ dual agonists have been developed. However, several adverse effects such as weight gain and organ failure of PPARα/γ dual agonists have been reported. By use of virtual ligand screening, we identified and characterized a novel PPARα/γ dual agonist, (R)-1-(4-(2-(5-methyl-2-tolyloxazol-4-yl)ethoxy)benzyl)piperidine-2-carboxylic acid (CG301360), exhibiting the improvement in insulin sensitivity and lipid metabolism. CG301360 selectively stimulated transcriptional activities of PPARα and PPARγ and induced expression of their target genes in a PPARα- and PPARγ-dependent manner. In cultured cells, CG301360 enhanced fatty acid oxidation and glucose uptake and it reduced pro-inflammatory gene expression. In db/db mice, CG301360 also restored insulin sensitivity and lipid homeostasis. Collectively, these data suggest that CG301360 would be a novel PPARα/γ agonist, which might be a potential lead compound to develop against insulin resistance and hyperlipidemia.

Introduction

Insulin resistance is an abnormal physiological phenomenon caused by defects in insulin action devoid of the deficiency of insulin (Kahn, 1994). Diminished insulin activity leads to elevated circulating glucose and free fatty acids (FFAs), which are crucial factors for various metabolic disorders, including type 2 diabetes, hyperlipidemia, hyperglycemia, atherosclerosis, and cardiovascular diseases (DeFronzo and Ferrannini, 1991). In the last several decades, numerous drugs or therapeutic agents targeting the above disorders

ABBREVIATIONS: FFA, free fatty acid; TZD, thiazolidinedione; PPAR, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; WY14643, prinixic acid; GW501516, 2-[2-methyl-4-[[4-(difluoromethoxy)phenyl]-1,3-thiazol-5-yl]methylsulfanyl]phenoxypyruvate; CG301360, (R)-1-(4-(2-(5-methyl-2-p-tolyloxazol-4-yl)ethoxy)benzyl)piperidine-2-carboxylic acid; TNF, tumor necrosis factor; LPS, lipopolysaccharide; GPDH, glyceroldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; DMEM, Dulbecco’s modified Eagle’s medium; PDB, Protein Data Bank; qRT-PCR, quantitative real-time polymerase chain reaction; TG, triacylglyceride; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBST, Tris-buffered saline with 0.1% Tween 20; VAT, white adipose tissue; FGF21, fibroblast growth factor 21; siRNA, small interfering RNA.
have been developed. For instance, fibrates have been prescribed to treat hyperlipidemia and lipid dysregulation (Guérin et al., 1996), whereas thiazolidinediones (TZDs) have been used to relieve insulin resistance and type 2 diabetes (Saltiel and Olefsky, 1996). Fibrates and TZDs act as ligands for peroxisome proliferator-activated receptor (PPAR) α and γ, respectively, which are key transcriptional regulators of glucose and lipid metabolism (Issemann and Green, 1990; Lehmann et al., 1995).

Although TZDs potently sensitize insulin action in peripheral tissues to ameliorate insulin resistance, several negative concerns, such as weight gain, hepatotoxicity, edema, and heart failure, have been reported (Watkins and Whitcomb, 1998; Hirsch et al., 1999; Tang et al., 2003; Shim et al., 2006). Unlike TZD, PPARα agonists mediate fatty acid oxidation to alleviate hypertriglyceridemia and also mildly reduce body adiposity (Guerre-Millo et al., 2000). Given the facts that selective activation of PPARα and PPARγ enhance lipid and glucose metabolism, respectively (Chaput et al., 2000; Carmona et al., 2005), combination of PPARα and PPARγ agonists would be expected to achieve beneficial effects on restoring metabolic disorders. Hence, a number of PPARα/γ dual agonists have been designed and developed (Murakami et al., 1998; Goldstein et al., 2006; Harrity et al., 2006). However, recently identified PPARα/γ dual agonists were ineffective because of undesirable side effects during preclinical or clinical trials. For example, muraglitazar, a synthetic PPARα/γ dual agonist, was aborted during clinical trials because of increased mortality, fluid retention, edema, and cancer (Mitra et al., 2007; Tannehill-Gregg et al., 2007), and tesaglitazar was reported to cause fibrosarcoma in subcutaneous tissues (Hellmold et al., 2007). Furthermore, it has recently been reported that several types of TZDs induce tissue toxicity (Lloyd et al., 2002; Nissen and Wolski, 2007). Therefore, we aimed to identify non–TZD-based PPARα/γ dual agonists to overcome and/or minimize previously reported side effects of PPARα/γ dual agonists. To screen and identify novel PPARα/γ dual agonists, we used virtual ligand screening and identified several candidate compounds that might fit into the ligand-binding domain (LBD) of PPARα.

In the present study, we characterized a novel PPARα/γ dual agonist, (+)-1-(4-(2-(5-methyl-2-p-tolyloxazol-4-yl)ethoxy)benzyl)piperidine-2-carboxylic acid (CG301360), and examined its effects on insulin sensitivity and lipid metabolism through in vitro and in vivo studies. As a modest PPARα/γ dual agonist, CG301360 selectively activated the transcriptional activities of both PPARα and PPARγ. In obese and diabetic db/db mice, CG301360 rescued severe insulin resistance, hyperlipidemia, and hyperglycemia through stimulation of glucose uptake and fatty acid oxidation. Furthermore, CG301360 also suppressed pro-inflammatory responses, which are closely related to insulin sensitivity. Together, our findings suggest that CG301360 would be a useful chemical for the treatment of insulin resistance and lipid dysregulation.

**Materials and Methods**

**Reagents.** CG301360 was designed in Crystal Genomics (Seoul, Korea) and synthesized by Korea Research Institute of Chemical Technology (Daejeon, Korea). Rosiglitazone and pirinixic acid (WW14643) were obtained from Cayman Chemical (Ann Arbor, MI), and 2-[2-methyl-4-[(4-methyl-2-[(trifluoromethyl)phenyl]-1,3-thiazol-5-yl)methylsulfanyl]phenoxycacetic acid (GW501516) was kindly provided by Dr. J. B. Seo (Boram Pharmaceutical Co., Seoul, Korea). Tumor necrosis factor (TNF) α and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO). Antibodies for adiponectin and GAPDH were purchased from Cell Signaling Technology (Danvers, MA) and Ab Frontier (Seoul, Korea), respectively. All media and additives for cell culture were purchased from Hyclone (Logan, UT). Adiponectin-ELISA kit was acquired from Adipogen (Incheon, Korea).

**Cell Culture and Glucose Uptake Assay.** All cells [human embryonic kidney (HEK) 293, FAO, C2C12, 3T3-L1, and RAW 264.7] used in this study were obtained from American Type Culture Collection (Manassas, VA). HEK 293 and 3T3-L1 cells were cultured in DMEM supplemented with 10% bovine calf serum and 1% penicillin/streptomycin, and the other cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. C2C12 and 3T3-L1 cells were cultured as described previously (Yoon et al., 2006). Glucose uptake assay was performed in differentiated 3T3-L1 adipocytes as described previously (Jeong et al., 2009).

**Gene Transfer and Reporter Assay.** Transfections and reporter assay in HEK293 cells were performed as described previously (Seo et al., 2004). To suppress PPAR expression, specific siRNAs (Bioneer, Daejeon, Korea; the siRNA sequences are available upon request) were transfected with a Microporter (Digital Bio, Seoul, Korea) as follows: FAO cells were pulsed once at 1400 V for 20 ms and 3T3-L1 cells were pulsed twice at 1300 V for 20 ms. After transfection, cells were maintained with growth medium in the absence of antibiotics for overnight.

**Docking Simulation.** Docking simulation was performed with Discovery Studio 1.7 computer program. Docking calculation was constructed according to LigandFit module implemented in the receptor-ligand interaction protocol. Cocrystral structures of the ligand-bound ligand binding domains of human PPARα and human PPARγ (PDB ID: 3FEI and 3FEJ, respectively) were employed, and the binding sites were defined from each receptor cavities. For estimating the binding affinity of each chemical, the potential of mean force (Muegge and Martin, 1999) was used as a docking score function. All parameters used in calculation are also provided.

**qRT-PCR Analysis.** Total RNA was prepared using Tri-Reagent (Molecular Research Center, Cincinnati, OH), and cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase kit (RevertAid; Fermentas, Glen Burnie, MD) following to manufacturer’s instruction. qRT-PCR was performed using Micro-IQ thermocycler (Bio-Rad Laboratories, Hercules, CA). The amplifying reaction was conducted as follows; 40 repetitive thermal cycles (94°C for 20 s, 60°C for 20 s, 72°C for 20 s) with SYBR green (Invitrogen, Carlsbad, CA). The primers used in this study were synthesized from Bioneer. The sequences of qRT-PCR primers are available upon request.

**Animal Experiments.** All animal experiments were approved by the Seoul National University Animal Experiment Ethics Committee. C57BLKS/J-Leprdb db/heterozygous male mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea). They were housed at 22 ± 2°C, 55 ± 5% relative humidity, with a light/dark cycle of 12 h, and they were fed ad libitum. C57BLKS/J-Leprdb/db male mice, at 10 weeks of age, were administered 50 mg/kg WW14643, rosiglitazone, or CG301360 daily, and an equivalent volume of vehicle (distilled water) by oral gavage for 30 days.

For pharmacokinetic analysis of CG301360, male ICR mice were injected with CG301360 (5 mg/kg body weight dose) or vehicle (distilled water) via oral gavage. Blood samples were collected at 0.167, 0.5, 1, 2, 4, 6, 8, and 24 h after injection. The quantification of the CG301360 compound in plasma was monitored using a liquid chromatography/tandem mass spectrometry system. Pharmacokinetic parameters (area under the time-concentration, time to reach maximum concentration, maximum plasma concentration, and mean residence time were calculated).
Measurement of Blood Lipid Profiles. Blood samples were used to determine triacylglyceride (TG), FFAs, and total cholesterol levels with Infinity reagents (Thermo Fisher Scientific, Melbourne, Australia). Plasma insulin level was measured with insulin enzyme-linked immunosorbent assay kit according to manufacturer's instruction (Merodia, Uppsala, Sweden). The level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma were measured as described previously (Kim et al., 2009).

Glucose Tolerance Test and Insulin Tolerance Test. For the glucose tolerance test, PPAR agonist-administered mice were fasted for 24 h. Glucose (Sigma) solution (5%) was administered orally, and the blood glucose level was measured with Freestyle blood glucose meter (Therasense, Indianapolis, IN). For the insulin tolerance test, mice were fasted for 6 h before injection of 1 U of human insulin (Lilly, Indianapolis, IN). The blood glucose level was measured from the mice by Freestyle blood glucose meter (Therasense, Alameda, CA).

Western Blot. Western blot analysis was performed as described previously (Jeong et al., 2009) with minor modifications. In brief, an equal amount of protein separated on SDS-polyacrylamide gel electrophoresis gels was transferred to nitrocellulose membrane. Blots were blocked with Tris-buffered saline with 0.1% Tween 20 (TBST) containing 0.5% nonfat milk at room temperature for 15 min and incubated with primary antibodies at 4°C overnight. After washing with TBST three times, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma) at room temperature for 2 h and followed by washing with TBST an additional three times. The membrane was incubated with enhanced chemiluminescence reagents and exposed to X-ray film (Fujifilm, Tokyo, Japan).

Fatty Acid Oxidation Assay. Cells were incubated for 24 h in palmitate (Sigma)-saturated α-minimal essential medium containing 0.1 mM 9,10-[3H]palmitate (5 μCi/ml; PerkinElmer Life and Analytical Sciences, Waltham, MA) and 2% bovine serum albumin. After incubation, the medium was precipitated with an equal volume of 10% trichloroacetic acid (Sigma). Then the supernatants were transferred to open 1.5-ml microcentrifuge tubes placed in a scintillation vial containing 0.5 ml of water and incubated at 55°C for 10% trichloroacetic acid (Sigma). Then the supernatants were transferred to open 1.5-ml microcentrifuge tubes placed in a scintillation vial containing 0.5 ml of water and incubated at 55°C for overnight. After removal of the open microcentrifuge tubes, the content of 3H2O content was measured in a scintillation counter.

Statistical Analysis. Results are representative data from independent experiments repeated at least three times. Error bars represent S.D. and P values < 0.05 (calculated from analysis of variance followed by Student-Newman-Keuls comparison test) were considered statistically significant.

Results

Transcriptional Activity of PPARα and PPARγ Is Up-Regulated by CG301360 through Their Ligand Binding Domains. To design novel PPARα/γ dual agonists, we analyzed the known PPAR agonists and their modes of action. For the specific interactions between ligands and nuclear receptors, the most important interaction is the hydrogen bond networks linking agonists and amino acid residues in the AF2 helix of nuclear receptors, which induce conformational change of the receptor. We adopted a phenoxy as a linker and 5-methyl-2-phenyl-oxazole as a hydrophobic tail, similar to other PPAR agonists. As a new acid head, piperidine-2-carboxylic acid was introduced to make an additional hydrophobic interaction in the binding pocket. Chemical compounds were synthesized on the basis of virtual information and evaluated through transactivation reporter assays with three PPAR isoforms. Among potential candidates for PPARα/γ dual agonists, compound CG301360 was selected after evaluating various cellular effects. As shown in Fig. 1A, CG301360 has a non–TZD-based structure. To con-
firm the specificity and sensitivity of CG301360 in the regulation of transcriptional activities of PPARs, luciferase reporter assays were performed. CG301360 effectively activated PPARα (Fig. 1B) and PPARγ (Fig. 1C), whereas CG301360 did not significantly activate PPARδ (Supplemental Fig. 1). Dose-response analyses showed that CG301360 acted as an effective agonist for PPARα and PPARγ when CG301360 was compared with the known PPARα and PPARγ agonists WY14643 and rosiglitazone, respectively (Supplemental Fig. 2, A and B). Moreover, CG301360 enhanced the transactivation of GAL4-PPARα and GAL4-PPARγ LBD constructs in a dose-dependent manner (data not shown; EC50 of CG301360 is given in Supplemental Table 1), suggesting CG301360 as a modest and selective dual agonist for PPARα and PPARγ through the interaction with their LBD domains. However, CG301360 barely stimulated the transcriptional activity of PPARδ up to 1 μM (Supplemental Fig. 2C). In addition, when pharmacokinetic properties of CG301360 were evaluated, time to reach maximum concentration was 0.167 h, implying that CG301360 is rapidly absorbed after administration (Supplemental Table 2).

To investigate whether CG301360 is able to indeed interact with the LBDs of PPARα and PPARγ, we simulated the docking study of CG301360 with the reported structures of PPARα LBD and PPARγ LBD (PDB ID: 3FEI and 3FEJ, respectively) (Grether et al., 2009). As shown in Fig. 1, D and E, overall structures of LBDs of PPARα and PPARγ containing CG301360 were similar to those of human PPARα and PPARγ with previously reported PPARα/γ dual agonist, phenylthiazole derivative (Grether et al., 2009). The electrostatic potential surface (molecular volume = 346.7 Å3) of CG301360 nicely fits into the LBDs of PPARα and PPARγ (Supplemental Fig. 3). In particular, the typical four hydrophobic bonds linking carboxylate of CG301360 and the Ser, His, and Tyr residues of PPARα/γ are within a distance of ≤3 Å. Moreover, CG301360 seems to dock into the hydrophobic pockets of PPARα/γ, which are composed of Met, Cys, and Gly residues. In addition, when we calculated the docking scores of CG301360 toward PPARα/γ by comparison with known PPAR ligands such as WY14643 and rosiglitazone (Supplemental Table 3), the data obtained clearly suggest that CG301360 would be properly accommodated into the LBD of PPARα and -γ as a potential dual ligand.

**CG301360 Stimulates Expression of PPARα and PPARγ Target Genes.** Because CG301360 effectively stimulated the transactivation of both PPARα and PPARγ, we next examined the effects of CG301360 on the expression of PPARα and PPARγ target genes. In hepatoma (FAO) and myocytes (C2C12), CG301360 augmented the expression of PPARα target genes such as acyl-CoA oxidase, carnitine-palmitoyl transferase, medium-chain acyl-CoA dehydrogenase, and PPARα itself in a dose-dependent manner (Fig. 2, A and B). Furthermore, CG301360 stimulated the expression of PPARα target genes in differentiated 3T3-L1 (Fig. 2C) and 3T3-F442A adipocytes (data not shown), implying that CG301360 might promote fatty acid oxidation even in fat cells. As expected, in cultured adipocytes, CG301360 significantly augmented the expression of PPARγ target genes such as adipocyte fatty acid binding protein, adiponectin, lipoprotein lipase, and CD36 in a dose-dependent manner (Fig. 2D). These data indicate that CG301360 could act as a dual activator of PPARα and PPARγ in cultured cells.

**CG301360 Restores Dysregulated Glucose and Lipid Metabolism in Diabetic Mice.** To assess the in vivo effect of CG301360, CG301360 was administered to obese and diabetic db/db mice every day for 1 month (from 10 to 14 weeks of age). Without significant change in food intake (data not shown), increase in body weight and white adipose tissue (WAT) weight was observed in CG301360-treated db/db mice, albeit to a lesser extent than by rosiglitazone, a well known PPARγ agonist (Fig. 3, A and B) (Chaput et al., 2000; Shim et al., 2006).

Next, plasma glucose and lipid profiles were analyzed to examine whether CG301360 was able to modulate metabolic abnormalities in obese and diabetic subjects. In db/db mice, CG301360

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**Fig. 2.** Increased expression of PPARα and PPARγ target genes by CG301360. FAO rat hepatoma cells (A), C2C12 myotubes (B), and 3T3-L1 adipocytes (C and D) were incubated with WY14643 (WY; 1 μM), rosiglitazone (Rosi; 1 μM), or CG301360 (CG360; 0.5, 1, and 5 μM) for 24 h. The relative mRNA level of each gene was analyzed by use of qRT-PCR and normalized by GAPDH. Each bar represents mean ± S.D. of duplicates. Similar results were obtained from at least three independent experiments. +, P < 0.05 versus negative control; ++, P < 0.01 versus negative control. □, vehicle; diamond-filled box, WY14643 (1 μM); □, rosiglitazone (1 μM); □, CG301360 (0.5 μM); □, CG301360 (1 μM); □, CG301360 (5 μM). ACO, acyl-CoA oxidase; CPT, carnitine-palmitoyl transferase; mCAD, medium-chain acyl-CoA dehydrogenase; FATP, fatty acid transport protein; ap2, adipocyte fatty acid binding protein; Acp30, adiponectin; LPL, lipoprotein lipase.
significantly reduced plasma glucose, insulin, TG, and FFA levels (Fig. 3, C–F). These effects of CG301360 on plasma profiles were more potent than those of rosiglitazone, a PPARγ agonist, and WY14643, a PPARα agonist. Moreover, CG301360 even slightly decreased the level of plasma ALT and AST levels (Fig. 3, G and H) (Al-Salman et al., 2000). Collectively, these data suggest that CG301360 in db/db mice would ameliorate hyperglycemia and hyperlipidemia.

CG301360 Mitigates Glucose and Insulin Intolerance in Diabetic Mice. Because CG301360 greatly reduced plasma glucose and lipid levels in db/db mice (Fig. 3, C–F), we next conducted glucose tolerance and insulin tolerance tests to examine the effect of CG301360 on systemic glucose and insulin sensitivity. As illustrated in Fig. 4, A and B, CG301360 was as effective as rosiglitazone in alleviating glucose and insulin tolerance in db/db mice. Increased glucose uptake through activation of PPARγ has been suggested as a mechanism of reducing circulating glucose level (Tamori et al., 2002). Next, we tested the effect of CG301360 on glucose uptake. In 3T3-L1 adipocytes, CG301360 significantly enhanced insulin-stimulated glucose uptake (Fig. 4C) as well as in 3T3-F442A adipocytes (data not shown). Moreover, expression of adiponectin was measured because it is known to enhance insulin sensitivity by promoting glucose uptake and fatty acid oxidation (Yamauchi et al., 2002). CG301360 elevated the level of adiponectin both in vitro and in vivo (Fig. 4, D and E). Taken together, these data suggest the role of CG301360 in improving insulin sensitivity and glucose metabolism as a PPARγ activator.

CG301360 Suppresses Proinflammatory Responses. Because pro-inflammatory response is a causal factor for insulin resistance (Shoelson et al., 2006), we examined the effect of CG301360 on the inflammatory responses of macrophages and adipocytes. In RAW 264.7 macrophages and 3T3-L1 adipocytes, enhanced expression of pro-inflammatory genes such as TNFα, interleukin-1β, interleukin-6, inducible NO synthase, monocyte chemoattractant protein-1, cyclooxygenase 2, and matrix metalloprotease 9 upon inflammatory stimuli were markedly reduced in the presence of CG301360 (Fig. 5, A and B).

Although CG301360 slightly increased adipose tissue mass in vivo (Fig. 3B), it effectively reduced expression level of macrophage marker genes including CD68, F4/80, CD11b, and CD11c and pro-inflammatory genes such as TNFα, inducible NO synthase, and monocyte chemoattractant protein-1 in adipose tissue of db/db mice (Fig. 5, C and D). These data suggest that CG301360 would effectively suppress pro-inflammatory responses, eventually leading to improve insulin resistance in obese subjects.

CG301360 Promotes Fatty Acid Oxidation. As a PPARα activator, CG301360 elevated the expression of fatty acid oxidation genes in various cell lines (Fig. 2). In db/db mice, CG301360 augmented the expression of several β-oxidation genes in peripheral tissues such as liver, skeletal muscle, and WAT (Fig. 6, A–C), indicating that CG301360 might stimulate fatty acid oxidation in peripheral tissues. Consistent with the results from gene expression profiles, CG301360 indeed promoted fatty acid oxidation in various cell types, including hepatoma, myocytes, and adipocytes (Fig. 6, D–F). The effect of CG301360 on β-oxidation seems to be mainly through PPARα activation, because rosiglitazone rarely promoted expression of fatty acid oxidation genes (Fig. 6, D–F). It is noteworthy that in CG301360-treated db/db mice, the expression of fibroblast growth factor 21 (FGF21), which induces β-oxidation and reduces the levels of glucose and TG in diabetic animals (Xu et al., 2009), was significantly up-regulated in liver (Fig. 6G), providing further supporting evidence that CG301360 is capable of improving hyperlipidemia by facilitating lipid consumption.

CG301360 Selectively Potentiates PPARα and PPARγ Activities. CG301360 exhibited several beneficial effects on both glucose and lipid metabolism in db/db mice. To further confirm whether these favorable effects of CG301360 on glucose and lipid metabolism are mediated by PPARα and/or PPARγ activation, we decided to use PPAR knockdown with siRNAs. As a control experiment, we examined whether CG301360 might influence activity of PPARγ, which shares several target genes and some features in lipid catabolism with PPARα (Evans et al., 2004). As shown in Fig. 7A, GW501516, a synthetic PPARγ ligand, failed to increase the expression of β-oxidation genes in PPARγ-suppressed FAO cells. Unlike GW501516, CG301360 still stimulated the expression of genes involved in fatty acid oxidation (Fig. 7A, and average fold induction is denoted in Supplemental Table 4). In contrast, cells transfected with PPARα siRNA evi-
dently blunted the expression of genes related to β-oxidation induced by CG301360 (Fig. 7B). These results propose that the effects of CG301360 on the expression of fatty acid oxidation genes would be primarily required for PPARα rather than PPARδ. In addition, knockdown of PPARγ also reduced the expression of both of basal and CG301360-induced PPARγ target genes in differentiated adipocytes (Fig. 7C).

To further confirm the suppressing effects of PPARα and/or PPARγ activity via each knockdown, we assessed β-oxidation (for PPARα) and adipogenesis (for PPARγ). PPARα siRNA remarkably inhibited fatty acid oxidation in FAO cells (Fig. 7D), and knockdown of PPARγ reduced CG301360-mediated adipogenesis (Fig. 7E and Supplemental Fig. 4). Together, these data strongly support the idea that the effects of CG301360 on the regulation of insulin sensitivity and lipid metabolism are mediated by activation of both PPARα and PPARγ.

**Discussion**

The importance of PPARs on metabolic diseases has been well established by genetic studies. PPARα(-/-) mice fail to meet energy demands during fasting by exhibiting hyperketonemia, hyperlipidemia, and fatty liver (Kersten et al., 1999). Tissue-specific PPARγ-null mice are severely insulin-resistant (He et al., 2003; Hevener et al., 2003), whereas whole-body PPARγ(-/-) mice are embryonic lethal (Barak et al., 1999), implying the importance of PPARγ in adipose tissue during development. On the other hand, PPARδ-overexpressing transgenic mice have enhanced lipid metabolism and are resistant to obesity and tissue steatosis, whereas PPARδ deficiency exhibits obesity and causes defect in energy uncoupling (Wang et al., 2003). Thus, it is very likely that tight regulation of PPAR activity would be crucial to treat metabolic diseases including obesity, hyperlipidemia, and insulin resistance.

Through simultaneous activation of both PPARα and PPARγ, PPARα/γ dual agonists have been expected to alleviate obesity and diabetes (Watkins and Whitcomb, 1998; Hirsch et al., 1999; Al-Salman et al., 2000; Tang et al., 2003; Shim et al., 2006). However, side effects of PPARα/γ dual agonists still make them unsuitable for treatment of obesity and insulin resistance (Hellmold et al., 2007; Mittra et al., 2007; Tannenhill-Gregg et al., 2007). In this regard, the failure of former PPARα/γ dual agonists prompted us to develop novel PPARα/γ dual agonists without (or with less) deleterious effects by screening different backbone structures of PPAR ligands.

The LBD of nuclear hormone receptors undergoes conformational change upon interaction with their ligands, leading to activate nuclear receptors (Bourguet et al., 2000). By using virtual screening, we found optimal chemicals to fit into the LBD of PPARα. After screening, we isolated the non–TZD-based CG301360, which is capable of activating both PPARα and PPARγ. In the current study, we demonstrate that this novel PPARα/γ dual agonist effectively and selectively activates PPARα and PPARγ and shows favorable effects on insulin sensitivity and lipid metabolism in db/db mice. Unlike many previous PPARα/γ dual agonists (Hellmold et al., 2007; Mittra et al., 2007), CG301360 moderately activates PPARα and PPARγ. Furthermore, given that CG301360 action may not be primarily mediated through PPARδ, the failure of CG301360 might be free from PPARδ-mediated side effects.

As a PPARα activator, CG301360 is able to alleviate hyperlipidemia by enhancing lipid catabolism. In fact, CG301360 increased the expression of PPARα target genes.
as well as FGF21, a PPARα-responsive cytokine regulating lipid metabolism (Xu et al., 2009), and CG301360 augmented fatty acid oxidation, implying that CG301360 would promote lipid oxidation to improve hyperlipidemia in obese and/or diabetic animals. Furthermore, CG301360 also improves glucose metabolism by activation of PPARγ. The possible mechanisms responsible for CG301360-dependent glucose sensitivity are probably by 1) enhancing insulin sensitivity and 2) stimulating adiponectin production and secretion, which are not mediated by PPARα activation. Collectively, it is feasible to speculate that CG301360 might increase insulin sensitivity as a PPARγ activator, whereas it relieves lipid dysregulation by primarily activating PPARα.

One of the side effects of TZDs is tissue damage, which often results from fluid retention and edema (Hirsch et al., 1999; Tang et al., 2003; Mittra et al., 2007). Although some TZDs induce liver failure (Watkins and Whitcomb, 1998; Hirsch et al., 1999; Al-Salman et al., 2000), CG301360 slightly but substantially decreased the level of ALT and AST in db/db mice. Furthermore, CG301360 is ascertained not to cause fluid retention, edema, and related tissue failures such as renal tubular damage and cardiac muscle damage in db/db mice, (Supplemental Fig. 5). Although experiments of long-term administration are needed to clearly elucidate whether CG301360 might confer fluid retention or not, this
notion reflects that CG301360 would relieve insulin resistance without acute organ failure in diabetic mice (at least within a month of administration).

One of important features of CG301360 is its anti-inflammatory property. Because pro-inflammatory responses play key roles in insulin resistance, it is important to suppress pro-inflammatory responses to restore insulin resistance. In fact, several anti-inflammatory reagents relieve insulin resistance (Jeong et al., 2009). Both in vitro and in vivo, CG301360 showed anti-inflammatory effects, probably by activating PPARα and PPARγ.

In conclusion, we identified a novel PPARα/γ dual agonist, CG301360, that provides favorable effects on insulin sensitivity and lipid homeostasis. CG301360, a modest activator of PPARα and PPARγ, ameliorates hyperglycemia and hyper-insulinemia by enhancing glucose and lipid metabolism via increased glucose uptake and fatty acid oxidation. CG301360 also suppresses pro-inflammatory responses to restore insulin resistance in vitro and in vivo. Taken together, these data lead us to suggest that CG301360 would be a potential agent targeting insulin resistance and related metabolic disorders through activation of both PPARα and PPARγ.

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References


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