Anti-Diabetic Actions of a Non-Agonist PPARγ Ligand Blocking Cdk5-Mediated Phosphorylation

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Abstract

PPARγ is the functioning receptor for the thiazolidinedione (TZD) class of anti-diabetes drugs including rosiglitazone and pioglitazone[1]. These drugs are full classical agonists for this nuclear receptor, but recent data has shown that many PPARγ-based drugs have a separate biochemical activity, blocking the obesity-linked phosphorylation of PPARγ by Cdk5[2]. Here we describe novel synthetic compounds that have a unique mode of binding to PPARγ, completely lack classical

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transcriptional agonism and block the Cdk5-mediated phosphorylation in cultured adipocytes and in insulin-resistant mice. Moreover, one such compound, SR1664, has potent anti-diabetic activity while not causing the fluid retention and weight gain that are serious side effects of many of the PPARγ drugs. Unlike TZDs, SR1664 also does not interfere with bone formation in culture. These data illustrate that new classes of anti-diabetes drugs can be developed by specifically targeting the Cdk5-mediated phosphorylation of PPARγ.

Keywords

diabetes; anti-diabetic agent; PPARγ; agonism; synthetic ligands; structure

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PPARγ is a member of the nuclear receptor family of transcription factors and is a dominant regulator of adipose cell differentiation and development[3,4]. It is also the functioning receptor for the thiazolidinedione (TZD) class of anti-diabetic drugs such as rosiglitazone and pioglitazone[1,5]. These anti-diabetes drugs were developed specifically to have high affinity and full agonism toward PPARγ before their molecular modes of action were known[6]. It has therefore been assumed that their therapeutic actions result from their functional agonism on this receptor. From a clinical perspective, rosiglitazone (Avandia) and pioglitazone (Actos) are both highly effective oral medications for type 2 diabetes and are well tolerated by the majority of patients[7]. Unfortunately, a substantial number of patients experience side effects from these drugs, including fluid retention, weight gain, congestive heart failure and loss of bone mineral density[8,9]. While some of the non-TZD full agonists have good anti-diabetic activity, they also cause many of the same side effects, including fluid retention.

The therapeutic role of classical agonism of PPARγ was made somewhat confusing by the development of several compounds that have less than full agonist properties (partial agonists) but retain substantial insulin-sensitizing and anti-diabetic actions in experimental models[10,11]. Furthermore, we have recently shown that many anti-diabetic PPARγ ligands have a second, distinct biochemical function: blocking the obesity-linked phosphorylation of PPARγ by cyclin-dependent kinase 5 (Cdk5) at serine 273[2]. This is a direct action of the ligands and requires binding to the PPARγ ligand binding domain (LBD), causing a conformational change that interferes with the ability of Cdk5 to phosphorylate serine 273. Rosiglitazone and MRL24 (a selective partial agonist toward PPARγ) both modulate serine 273 phosphorylation at therapeutic doses in mice. Furthermore, a small clinical trial of newly diagnosed type 2 diabetics showed a remarkably close association between the clinical effects of rosiglitazone and the blocking of this phosphorylation of PPARγ. Thus, the contribution made by classical agonism to the therapeutic effects of these drugs and to their side effects is not clear.

These data suggest that it might be possible to develop entirely new classes of anti-diabetes drugs optimized for the inhibition of Cdk5-mediated phosphorylation of PPARγ while lacking classical agonism. Here we describe the development of synthetic small molecules that bind tightly to PPARγ yet are completely devoid of classical agonism and effectively inhibit phosphorylation at serine 273. These compounds have a unique binding mode in the

ligand binding pocket of PPARγ. An example from this series, SR1664, exhibits potent and dose-dependent anti-diabetic effects in obese mice. Unlike TZDs and other PPARγ agonists, this compound does not cause fluid retention or weight gain *in vivo* or reduce osteoblast mineralization in culture.

In order to develop a suitable ligand, we optimized compounds for (*i*) high binding affinity for PPARγ, (*ii*) blocking the Cdk5-mediated PPARγ phosphorylation and (*iii*) lacking classical agonism. We first identified published compounds that bind tightly to PPARγ and have favorable properties as a scaffold for extensive chemical modifications. Classical agonism is defined here, as is standard in the nuclear receptor field, as an increased level of transcription through a tandem PPAR response element luciferase reporter. Of particular interest was compound **7b** described by Lamotte et al. as an extremely potent and selective PPARγ partial agonist (30% activation as compared to rosiglitazone)[12]. A modular synthesis approach was used to make a series of analogs of compound **7b**; these compounds were tested *in vitro* and in adipose cells (Supplementary Fig. 1c and 1d). Using a LanthaScreen competitive binding assay, SR1664 (Fig. 1a) had an IC_{50} of 80nM (Supplementary Figs. 1a and 1b). As shown in Fig. 1b, when compared to rosiglitazone or MRL24 (a partial agonist) in a classical transcriptional activity assay, SR1664 had essentially no transcriptional agonism at any concentration. Rosiglitazone and SR1664 both effectively blocked the Cdk5-mediated phosphorylation of PPARγ *in vitro* with half-maximal effects between 20 and 200 nM (Fig. 1c). In contrast, they had no effect on the phosphorylation of a well-characterized Cdk5 substrate, the Rb protein (Fig. 1d)[13]. This suggested that these compounds do not disrupt the basic protein kinase function of Cdk5. In addition, SR1664 was also effective at blocking Cdk5-mediated phosphorylation of PPARγ in differentiated fat cells (Fig. 1e) with no measurable difference in phosphorylation of Rb (Supplementary Fig. 1e). Additional analogs were synthesized and four compounds were identified that have similar *in vitro* profiles (Supplementary Fig. 1b). SR1824 (Fig. 1a) was further characterized for its ability to block Cdk5-dependent phosphorylation of PPARγ (Fig. 1b-e). These data demonstrate that ligands can be made that potently block Cdk5-dependent phosphorylation of PPARγ in cells while demonstrating little to no classical agonism.

Of the four compounds identified as non-agonist inhibitors of Cdk5-mediated PPARγ phosphorylation, SR1664 had adequate pharmacokinetic properties to move forward to biological and therapeutic assays. Adipogenesis was the first known biological function of PPARγ[3] and agonist ligands for PPARγ have been shown to potently stimulate the differentiation of pre-adipose cell lines; this response has been widely used as a sensitive cellular test for PPARγ agonism[1,14,15]. As shown in Fig. 2a, rosiglitazone potently stimulated fat cell differentiation, as evidenced by Oil Red O staining of the cellular lipid. In contrast, SR1664 did not stimulate increased lipid accumulation or changes in morphology characteristic of differentiating fat cells. The stimulation of fat cell gene expression was also apparent with rosiglitazone, as illustrated by an increased expression of genes linked to adipogenesis. In contrast, SR1664 induced little or no change in the expression of these genes (Fig. 2b).

Another well-known effect of both rosiglitazone and pioglitazone is that they decrease bone formation and bone mineral density leading to an increase in fracture risk[8,16]. TZDs have

also been shown to decrease bone mineralization in cultured osteoblasts[17]. As shown in Fig. 2c, rosiglitazone treatment reduced the mineralization of mouse osteoblastic cells, as measured by Alizarin red staining. Moreover, the expression of genes involved in the differentiation of these cells was impaired (see Supplementary Fig. 2). Importantly, treatment with SR1664 did not affect the extent of calcification or the expression of this osteoblast gene set in MC3T3-E1 cells.

Co-crystallography, mutagenesis and hydrogen/deuterium exchange (HDX) have all demonstrated that full agonists of PPARγ affect critical hydrogen bonds within the C-terminal helix (H12) of the receptor[18–21]. This interaction stabilized the AF2 surface (helix 3–4 loop, C-terminal end of H11 and H12) of the receptor facilitating co-activator interactions. Interestingly, high affinity partial agonists have been identified that do not make these interactions yet still possess some level of classical agonism, and several of these have been shown to bind the backbone amide of S342 (S370 in PPARγ2) within the β-sheet of the LBD[18]. More recently, we demonstrated that the proximity of ligand to the amide of S342 correlated with increased stability of the helix 2-helix 2′ loop, the region of the receptor containing S273 (S245 in PPARγ1) as determined by HDX[2]. Surprisingly, HDX analysis of SR1664 and SR1824 increased the conformational mobility of the C-terminal end of H11, a helix that abuts H12 (Fig. 2d); in contrast, the full and partial agonists stabilized the same region of H11 (Supplementary Fig. 3).

In silico docking studies were carried out to understand the structural basis of SR1664 interactions in the PPARγ1 ligand binding (Supplementary Fig. 4). In this model, the phenyl substituted nitro group of SR1664 clashes with hydrophobic side chains of H11 such as Leu452 and Leu453 (Leu480 and Leu481 in PPARγ2, respectively) as well as Leu469 and Leu465 (corresponding to Leu497 and Leu493 in PPARγ2) of the loop N- terminal to H12. This potentially explains the lack of stabilization of H12 and the destabilization of the region of H11 near His449 as seen by HDX. Despite the altered mode of binding, SR1664 and rosiglitazone both bind to the same core residues within the PPARγ LBD. This is demonstrated by the ability of SR1664 to attenuate the transcriptional activity of rosiglitazone on PPARγ in the context of a competitive ligand binding assay (Supplementary Fig. 4b).

To determine whether the altered transcriptional activity of SR1664 may be attributed to differences in DNA binding or coactivator recruitment, we compared the chromatin association of PPARγ or steroid receptor co-activator-1 (SRC1) within the aP2 promoter. As expected, rosiglitazone significantly increased SRC1 occupancy without impacting PPARγ occupancy. However, SR1664 treatment did not influence the occupancy of PPARγ or SRC1 recruitment to aP2 promoter suggesting that SR1664 has a very different activity of co-regulator recruitment (Supplementary Fig. 4c).

We next asked whether SR1664 had anti-diabetic properties *in vivo*. Wild-type mice fed a high-fat high-sugar diet become obese and insulin-resistant, with activation of Cdk5 in their adipose tissues[2]. Fig. 3a demonstrates that SR1664, injected twice daily for 5 days, caused a dose-dependent decrease in the Cdk5-mediated phosphorylation of PPARγ at serine 273 in adipose tissue. Moreover, SR1664 treatment also caused a trend toward lowered (and

normalized) glucose levels, and a significant reduction in the fasting insulin levels. Insulin resistance, as computed by HOMA-IR, showed a clear and dose-dependent improvement with SR1664 (Fig. 3b). These changes occurred without significant differences in body weight compared to vehicle treated mice (Supplementary Fig. 5).

The most accurate method for measuring changes in insulin sensitivity *in vivo* is the hyperinsulinemic-euglycemic clamp[22]. As shown in Fig. 3c and in Supplementary Fig. 6, the glucose infusion rate (GIR) needed to maintain euglycemia in the mice treated with SR1664 was significantly greater than in animals treated with the vehicle alone, indicating improved whole-body insulin sensitivity. Suppression of hepatic glucose production (HGP), an important component of insulin action, was improved by SR1664. While no difference in whole-body glucose disposal was detected from calculations of ^{3}H-glucose turnover, analysis of tissue-specific ^{14}C-2-deoxyglucose transport demonstrated improved insulin-stimulated glucose disposal in adipose tissue of SR1664 treated mice. Similarly, reductions in both basal and clamped plasma FFA levels, as well as a 20% greater suppression of lipolysis in response to insulin, suggested improved adipose tissue insulin sensitivity in SR1664 treated mice. Together, these data indicate that SR1664 improves insulin sensitivity.

Using cells expressing the S273A mutant of PPARγ, we previously defined a gene set in cultured adipose cells that was most sensitive to the phosphorylation at this site[2]. Treatment of mice with SR1664 caused changes in the expression of 11/17 (65%) of these genes, *all* in the direction predicted for the inhibition of the PPARγ S273 phosphorylation (Fig. 3d). *Adiponectin* and *Adipsin*, genes long recognized as being reduced in obesity[23,24] are both induced by SR1664. We also defined a separate set of genes reflective of a full agonist (rosiglitazone) on cultured fat cells. SR1664 caused changes in expression of 6/19 genes in this "agonist" gene set; importantly, three of these changes were in the same direction as expected for an agonist, but three were changed in the opposite direction (Fig. 3e). Taken together, these data show that SR1664 has an insulin-sensitizing effect with preferential regulation of the gene set sensitive to the phosphorylation of PPARγ by Cdk5.

A more severe model of obesity is the leptin-deficient ob/ob mouse. These animals are very obese and insulin-resistant, with substantial compensatory hyperinsulinemia. Preliminary pharmacokinetic and pharmacodynamic experiments showed comparable drug exposures at 40mg/kg for SR1664 and 8mg/kg for rosiglitazone, both injected twice daily (Supplementary Fig. 7). Functional analyses were performed at days 5 and 11 after the start of treatments. As shown in Fig. 4a, both drugs caused a similar reduction in PPARγ phosphorylation at S273. After five days of treatment, there were no overt differences in fasting body weight or glucose levels (Fig. 4b). Control mice receiving only the vehicle remained hyperinsulinemic, but both rosiglitazone and SR1664 substantially reduced these insulin levels (Fig. 4b). Glucose tolerance tests were markedly improved with both rosiglitazone and SR1664, and the areas under these glucose excursion curves were statistically indistinguishable, without changing body weight (Fig. 4c).

Weight gain and fluid retention caused by TZD drugs like rosiglitazone are suspected to be key factors in their increased cardiac risk[9,25]. After recovering from the glucose tolerance test on day 5, rosiglitazone-treated mice began to show an increase in body weight (Fig. 4d).

This increased mass is accounted for primarily by fluid retention, quantified by a decrease in hematocrit seen with hemodilution (Fig. 4f). However, an increase in body fat was also observed by MRI (Figs. 4e and f). Importantly, SR1664 treatment did not cause the weight gain seen with the rosiglitazone treatment. Furthermore, SR1664 treatment showed no decrease in the hematocrit or change in body adiposity. These results were confirmed by measurements showing a decreased concentration of hemoglobin in the mice treated with rosiglitazone but not those treated with SR1664 (Supplementary Fig. 8). Taken together, these data indicate that SR1664, a non-agonist PPARγ ligand, has anti-diabetic actions in two murine models of insulin-resistance. Furthermore, this non-agonist does not stimulate two of the best documented side-effects of the PPARγ agonist drugs *in vivo.*

The TZD class of drugs has been important for the treatment of type 2 diabetes[26]. While these drugs function as full agonists for PPARγ, the role of agonism in their therapeutic effects has been called into question recently. Rosiglitazone and partial agonists like MRL24 both block the obesity-linked phosphorylation of PPARγ at serine 273[2]. The tight correlation between inhibition of this phosphorylation and the therapeutic effects of these drugs in both mouse and man suggested that it might be possible to create new classes of non-agonist ligands for PPARγ which are effective for the treatment of diabetes and cause fewer side effects. Hence, this paper addresses three key questions: first, is it possible to create novel PPARγ ligands that block Cdk5-mediated PPARγ phosphorylation yet have no classical agonism? Second, would such compounds have robust anti-diabetic activity? Finally, would non-agonist compounds have fewer side effects than classical full agonists like rosiglitazone?

We show here that it is possible to create new ligands that have high affinity for PPARγ, block the Cdk5-mediated phosphorylation and completely lack classical agonism. SR1664 does not function as an agonist and has no adipogenic action *in vitro*. The structural requirements for the non-agonist actions of SR1664 and SR1824 are particularly interesting. Ligands that function as classical full agonists, like rosiglitazone, have been shown to alter the conformation and HDX kinetics of H12, the major agonist helix. Surprisingly, ligands that do not affect the conformational dynamics of H12 are not non-agonists, rather they seem to function as partial agonists[18,21,27]. This strongly suggests that when engaged by ligands, other structural features of the AF2 surface such as H3, H3–H4 loop, and the C-terminal end of H11 contribute to partial agonism of the receptor. As expected SR1664 and SR1824 do not interact with H12 in any detectable way, but unexpectedly both ligands cause an increase in the conformational mobility of H11, which is part of the AF2 surface and directly abuts H12. Hence, it seems likely that the destabilization of H11 distorts the AF2 surface enough to block partial agonism. Whether there are other alternative modes of ligand binding that would lead to a complete lack of classical agonism remains to be determined.

That classical agonism is not required for strong anti-diabetic actions of a PPARγ ligand is now clear. In both diet-induced and genetically obese animals, SR1664 has strong anti-diabetic actions. The ability to improve adipose tissue insulin sensitivity is similar to the effects shown for rosiglitazone[28]. SR1664 has inferior pharmacokinetic properties compared to rosiglitazone, so an absolute quantitative comparison of their efficacy is difficult. However, using our best calculations to get approximately equal exposure to the two drugs

in vivo, SR1664 has very robust anti-diabetic activity, roughly equivalent to rosiglitazone in the experiments shown here. The unfavorable pharmacokinetic properties of SR1664 strongly suggest that this compound will never be administered to patients but it proves that non-agonist compounds can have robust therapeutic effects.

Analysis of the side effects of PPARγ ligands can be difficult because some of these (like cardiovascular disorders) do not occur in mice while others (like loss of bone mineral density) take many months of treatment to manifest. However, weight gain and fluid retention occur rapidly in both humans and mice. Increased body weight, increased accretion of fat tissues and increased fluid retention all occur in mice within 11 days of treatment with rosiglitazone (Fig 4). The non-agonist SR1664 shows none of these side effects, even as it effectively improves glucose homeostasis. Unlike rosiglitazone, SR1664 does not affect bone cell mineralization in culture (Fig. 2c). Taken together, these data argue that many of the known side effects of the TZD drugs occur as a consequence of classical agonism on target genes. Whether ligands directed at the Cdk5-mediated phosphorylation have their own problems remains to be determined. Still, these studies illustrate that the development of entirely new classes of PPARγ-targeted drugs is feasible.

Methods Summary

Cell Culture

Adipocyte differentiation in 3T3-L1 or PPARγ-null mouse embryonic fibroblasts (MEFs) expressing PPARγ[2] was induced by treating cells with 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 850 nM insulin for 48 h and cells were switched to the maintenance medium containing 850 nM insulin for 6 days.

Gene expression analysis

Total RNA was isolated from cells or tissues using Trizol reagents (Invitrogen). The RNA was reverse-transcribed using ABI reverse transcription kit. Quantitative PCR (qPCR) reactions were performed with SYBR green fluorescent dye using an ABI9300 PCR machine. Relative mRNA expression was determined by the   -Ct method using tata-binding protein (TBP) levels.

Animals

All animal experiments were performed according to procedures approved by Beth Israel Deaconess Medical Center's Institutional Animal Care and Use Committee. 4 to 5 week-old male C57BL/6J and C57BL/6J-*Lep^{ob/ob}* mice were obtained from the Jackson Laboratory. C57BL/6J mice were fed a high fat, high sucrose diet (60% kcal fat, D12492, Research Diets Inc.). For glucose tolerance tests, mice were intraperitoneally (i.p.) injected with rosiglitazone or SR1664 for 5 days, and fasted overnight before i.p. injection of 1 g/kg D-glucose.

Full methods and associated references are available in the online version of the paper at www.nature.com/nature

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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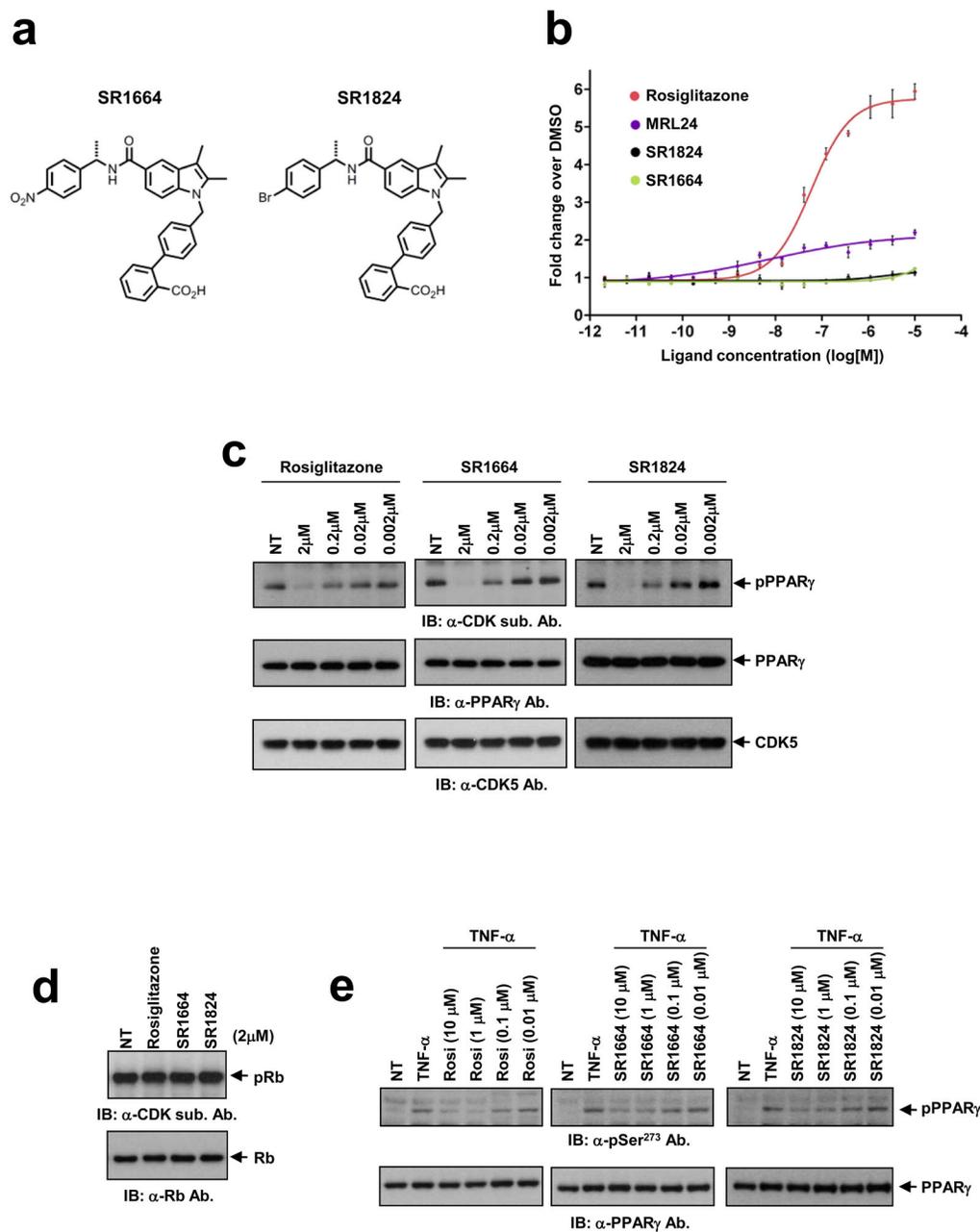
Figure 1. Novel PPARγ ligands lack classical agonism, block phosporylation at Ser273
a, Chemical structures of SR1664 and SR1824. **b**, Transcriptional activity of a PPAR-derived reporter gene in COS-1 cells following treatment with rosiglitazone, SR1664 or SR1824 (n=3). **c** and **d**, *In vitro* Cdk5 assay with rosiglitazone, SR1664 or SR1824 with PPARγ or Rb substrates. **e**, TNF-α-induced phosphorylation of PPARγ in differentiated PPARγ KO MEFs expressing PPARγ^{WT} treated with rosiglitazone, SR1664 or SR1824. Error bars are s.e.m.
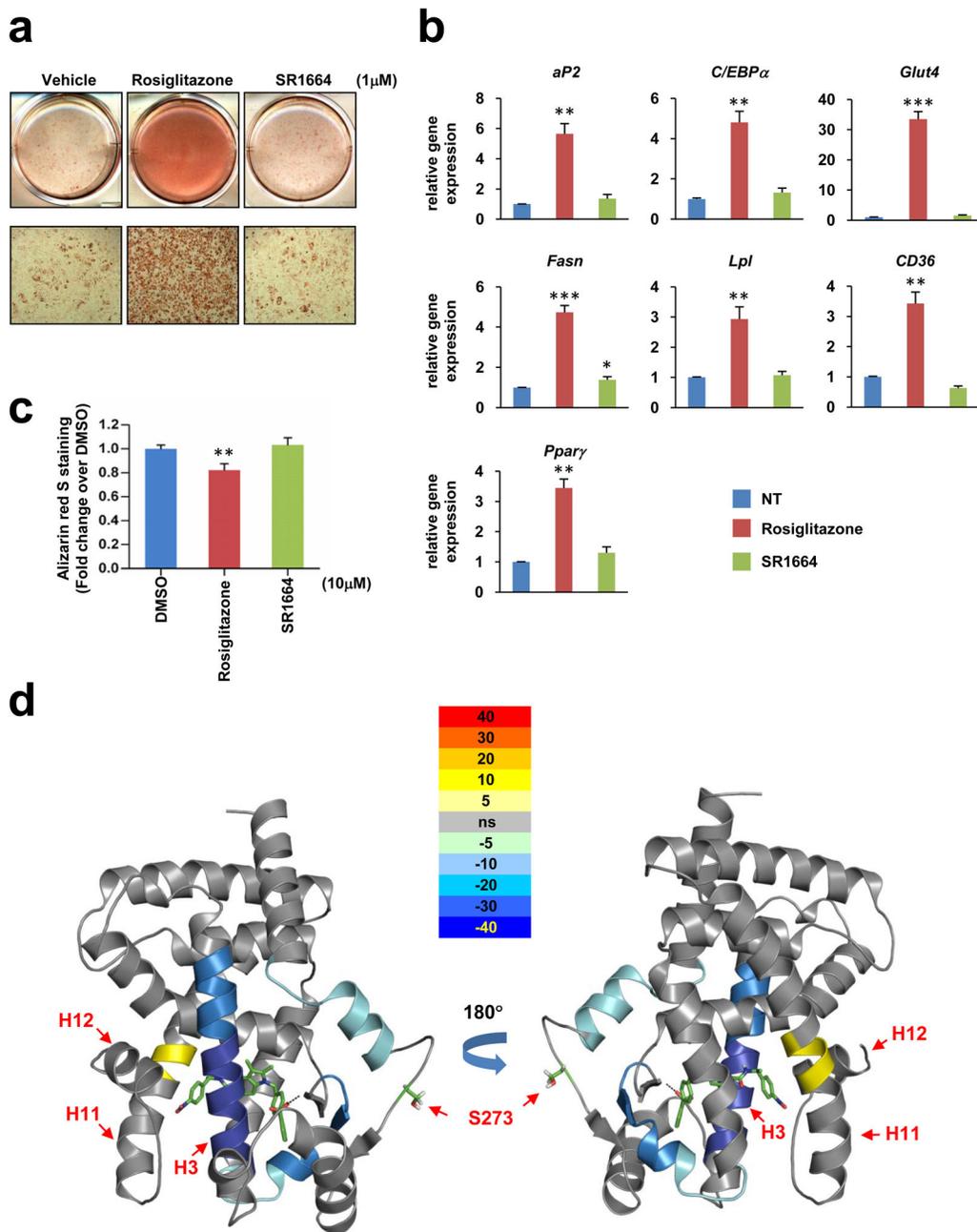
a

b

c

d

Figure 2. Structural and *in vitro* functional analysis of SR1664

a, Lipid accumulation in differentiated 3T3-L1 cells treated with rosiglitazone or SR1664 following Oil-Red-O staining. **b**, Expression of adipocyte-enriched genes in these cells was analyzed by qPCR (n=3). **c**, Mineralization of MC3T3-E1 osteoblast cells as determined by Alizarin Red-S. Error bars are s.e.m.; *$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s.; not significant. NT, no treatment. **d,** Overlay of differential HDX data onto the docking model of 2hfp bound to SR1664 (see Supplemental Fig. 3). This overlay depicts the difference in HDX between ligand-free and SR1664 bound PPARγ LBD. Perturbation data are color coded and

plotted onto the backbone of the PDB file according to the key. Observed changes in HDX were statistically significant ($p<0.05$) in a two tailed t-test (n=3).
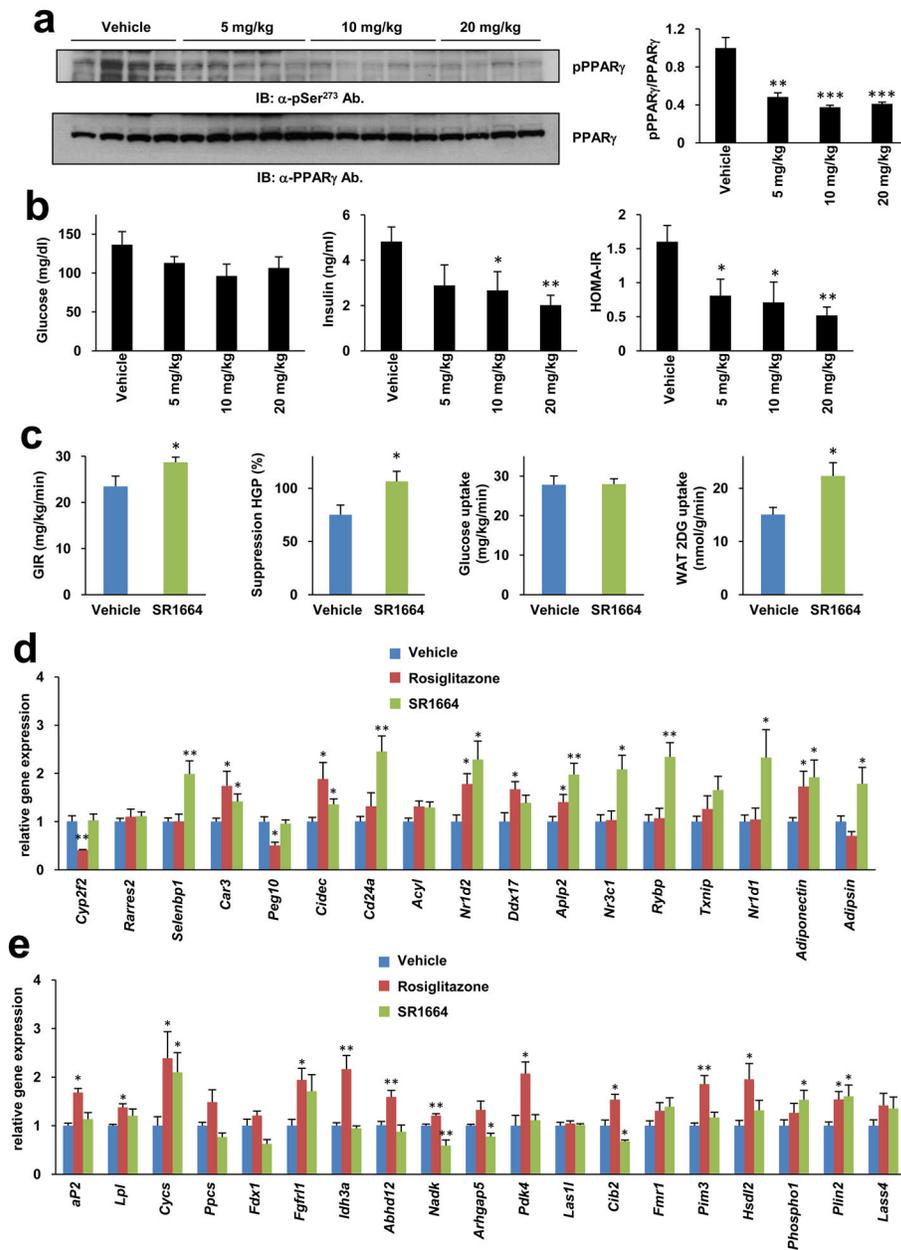
Figure 3. Anti-diabetic activity of SR1664 in high-fat diet (HFD) mice
a, Dose-dependent inhibition of phosphorylation of PPARγ by SR1664 in white adipose tissue (WAT). Quantification of PPARγ phosphorylation compared to total PPARγ (right). **b**, Ad libitum fed glucose (*p*=0.062 at 10mg/kg), insulin and HOMA-IR in HFD mice. **c**, Glucose infusion rate (GIR), suppression of hepatic glucose production (HGP), whole body glucose disposal and WAT 2-deoxyglucose tracer uptake during hyperinsulinemic-euglycemic clamps. **d**, Expression of a gene set regulated by PPARγ phosphorylation in WAT. **e**, Expression of an agonist gene set (see Methods) in WAT. Error bars are s.e.m.; *p<0.05, **p<0.01.
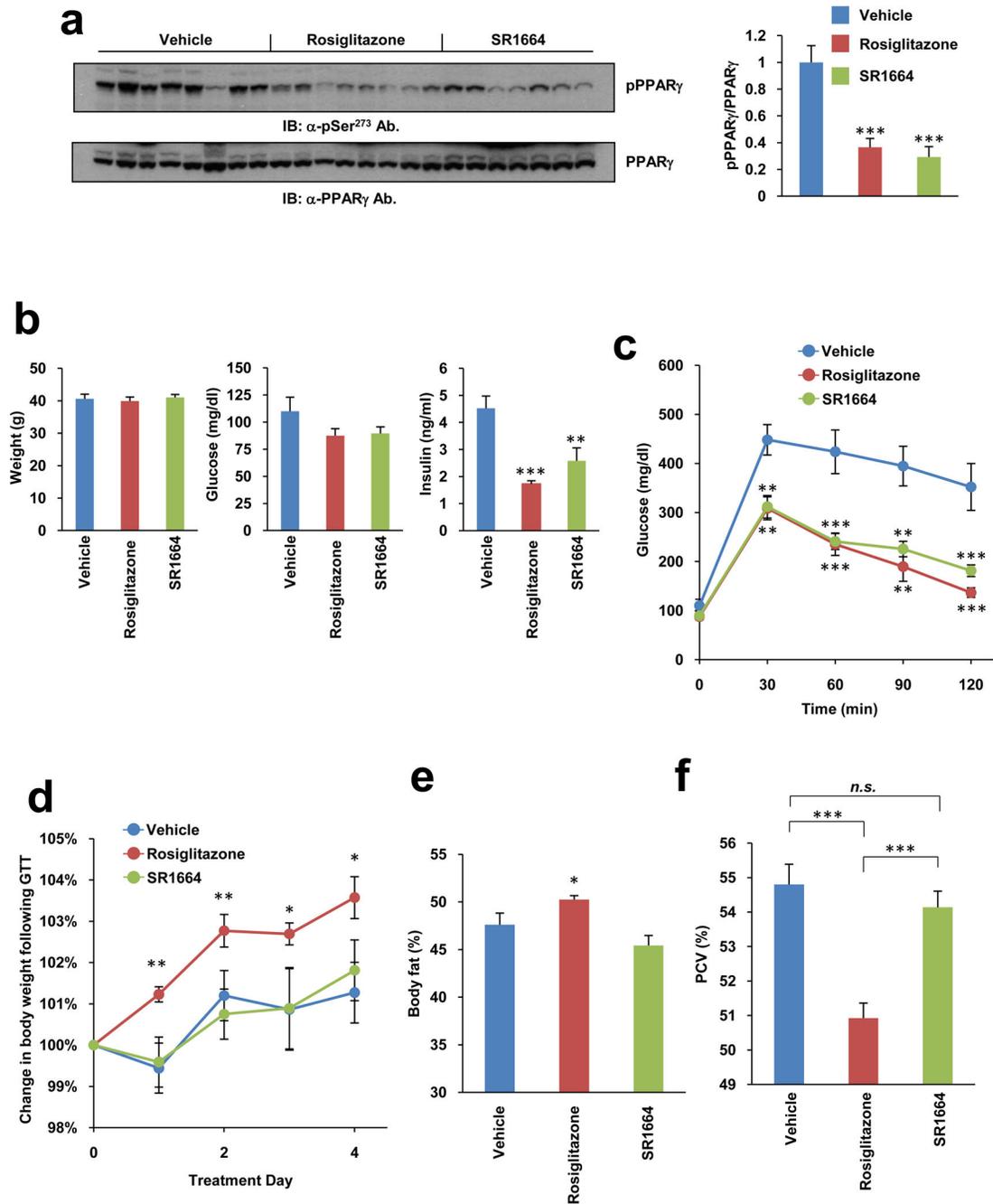
Figure 4. SR1664 has potent anti-diabetic activity and does not promote fluid retention in ob/ob mice

a, Phosphorylation of PPARγ in WAT (left). Quantification of PPARγ phosphorylation compared to total PPARγ (right). **b and c**, Fasting body weight, blood glucose and insulin levels prior to glucose-tolerance tests (GTT) in ob/ob mice treated with vehicle, rosiglitazone or SR1664 (n=8). Whole-body weight (**d**) and fat change (**e**) with continued drug administration following the GTT. **f**, Packed cell volume (PCV) in whole blood from

ob/ob mice treated with vehicle, rosiglitazone or SR1664. Error bars are s.e.m.; *p<0.05, **p<0.01, ***p<0.001. n.s.; not significant.