LETTER

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Antidiabetic actions of a non-agonist PPARγ ligand blocking Cdk5-mediated phosphorylation

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PPARγ is the functioning receptor for the thiazolidinedione (TZD) class of antidiabetes drugs including rosiglitazone and pioglitazone[1]. These drugs are full classical agonists for this nuclear receptor, but recent data have shown that many PPARγ-based drugs have a separate biochemical activity, blocking the obesity-linked phosphorylation of PPARγ by Cdk5 (ref. 2). Here we describe novel synthetic compounds that have a unique mode of binding to PPARγ, completely lack classical transcriptional agonism and block the Cdk5-mediated phosphorylation in cultured adipocytes and in insulin-resistant mice. Moreover, one such compound, SR1664, has potent antidiabetic 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 antidiabetes drugs can be developed by specifically targeting the Cdk5-mediated phosphorylation of PPARγ.

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 antidiabetic drugs such as rosiglitazone and pioglitazone[1,5]. These antidiabetes drugs were developed specifically to have high affinity and full agonism towards 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]. Whereas some of the non-TZD full agonists have good antidiabetic 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 antidiabetic actions in experimental models[10,11]. Furthermore, we have recently shown that many antidiabetic PPARγ ligands have a second, distinct biochemical function: blocking the obesity-linked phosphorylation of PPARγ by cyclin-dependent kinase 5 (Cdk5) at serine 273 (ref. 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 towards 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 indicate that it might be possible to develop entirely new classes of antidiabetes 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, shows potent and dose-dependent antidiabetic 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.

To develop a suitable ligand, we optimized compounds for (1) high binding affinity for PPARγ, (2) blocking the Cdk5-mediated PPARγ phosphorylation and (3) lacking classical agonism. We first identified published compounds that bind tightly to PPARγ and have favourable 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 previously as an extremely potent and selective PPARγ partial agonist (30% activation compared to rosiglitazone)[12]. A modular synthesis approach was used to make a series of analogues of compound **7b**; these compounds were tested *in vitro* and in adipose cells (Supplementary Fig. 1c, d). Using a LanthaScreen competitive binding assay, SR1664 (Fig. 1a) had a half-maximum inhibitory concentration (IC_{50}) of 80 nM (Supplementary Fig. 1a, b). 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 indicated 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 analogues 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

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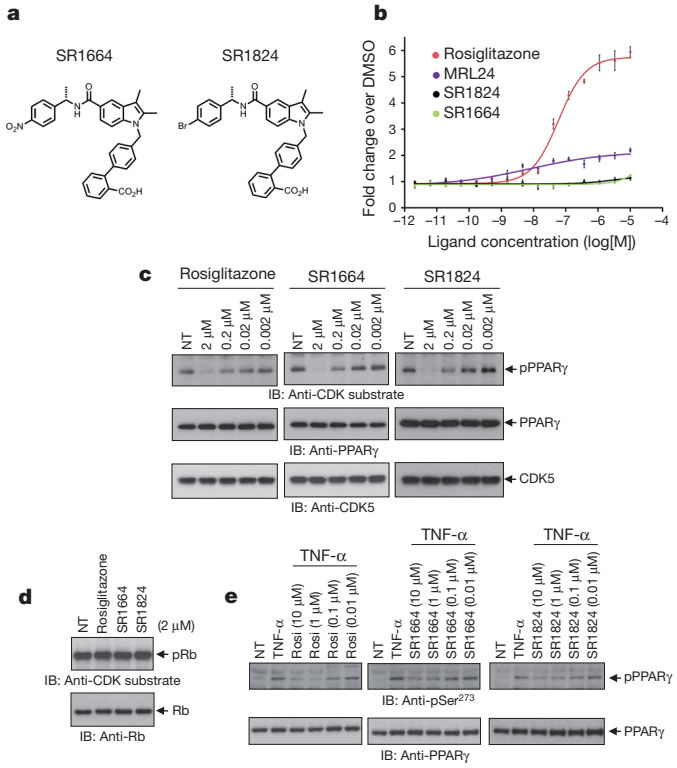

Figure 1 | Novel PPARγ ligands lack classical agonism, block phosphorylation at Ser 273. **a**, Chemical structures of SR1664 and SR1824. **b**, Transcriptional activity of a PPAR-derived reporter gene in COS-1 cells following treatment with rosiglitazone, SR1664 or SR1824 ($n = 3$). **c**, **d**, *In vitro* Cdk5 assay with rosiglitazone, SR1664 or SR1824 with PPARγ or Rb substrates. IB, immunoblot; NT, not treated; pPPARγ, phosphorylated PPARγ; pRb, phosphorylated Rb. **e**, TNF-α-induced phosphorylation of PPARγ in differentiated PPARγ knock-out MEFs expressing wild-type PPARγ treated with rosiglitazone, SR1664 or SR1824. Error bars are s.e.m.

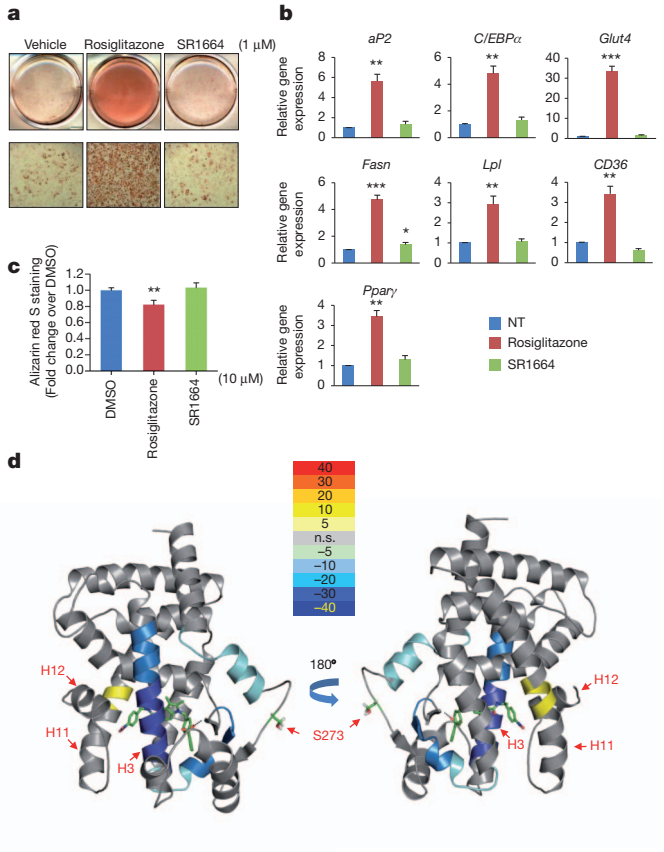

Figure 2 | Structural and *in vitro* functional analysis of SR1664. **a**, Lipid accumulation in differentiated 3T3-L1 cells treated with rosiglitazone or SR1664 following Oil Red O staining. **b**, Expression of adipocyte-enriched genes in these cells was analysed by qPCR ($n = 3$). **c**, Mineralization of MC3T3-E1 osteoblast cells as determined by Alizarin Red-S. Error bars are s.e.m.; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. 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 colour coded and plotted onto the backbone of the PDB file according to the key. n.s., not significant. Observed changes in HDX were statistically significant ($P < 0.05$) in a two-tailed *t*-test ($n = 3$).

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 stimulate potently 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 domain (Supplementary Fig. 4). In this model, the phenyl-substituted nitro group of SR1664 clashes with hydrophobic side chains of H11 such as Leu 452 and Leu 453 (Leu 480 and Leu 481 in PPARγ2, respectively) as well as Leu 469 and Leu 465 (corresponding to Leu 497 and Leu 493 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 His 449 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 affecting PPARγ occupancy. However, SR1664 treatment did not influence the occupancy of PPARγ or SRC1 recruitment to the aP2 promoter, indicating that SR1664 has a very different activity of co-regulator recruitment (Supplementary Fig. 4c).

We next asked whether SR1664 had antidiabetic 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]. Figure 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 towards 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).

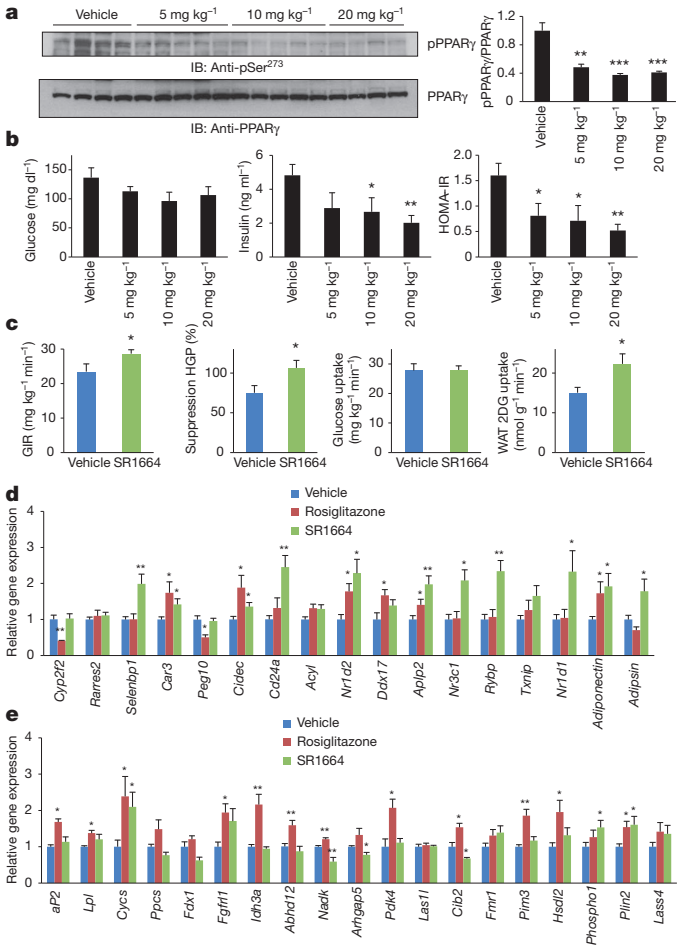

Figure 3 | Antidiabetic activity of SR1664 in high-fat diet (HFD) mice. **a**, Dose-dependent inhibition of phosphorylation of PPARγ by SR1664 in white adipose tissue (WAT). Quantification of PPARγ phosphorylation compared to total PPARγ (right). **b**, *Ad libitum*-fed glucose ($P = 0.062$ at 10 mg kg^{-1}), 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 hyperinsulinaemic-euglycaemic 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.; $*P < 0.05$, $**P < 0.01$.

The most accurate method for measuring changes in insulin sensitivity *in vivo* is the hyperinsulinaemic-euglycaemic clamp[22]. As shown in Fig. 3c and in Supplementary Fig. 6, the glucose infusion rate (GIR) needed to maintain euglycaemia 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. Whereas 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 free fatty acids levels, as well as a 20% greater suppression of lipolysis in response to insulin, indicated 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 hyperinsulinaemia. Preliminary pharmacokinetic and pharmacodynamic experiments showed comparable drug exposures at 40 mg kg^{-1} for SR1664 and 8 mg kg^{-1} 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 5 days of treatment, there were no overt differences in fasting body weight or glucose levels (Fig. 4b). Control mice receiving only the vehicle remained hyperinsulinaemic, 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 haematocrit seen with haemodilution (Fig. 4f). However, an increase in body fat was also observed by magnetic resonance imaging (Fig. 4e, f). Importantly, SR1664 treatment did not cause the weight gain seen with the rosiglitazone treatment. Furthermore, SR1664 treatment showed no decrease in the haematocrit or change in body adiposity. These results were confirmed by measurements showing a decreased concentration of haemoglobin in the mice treated with rosiglitazone, but not in those treated with SR1664 (Supplementary Fig. 8). Taken together, these data indicate that SR1664, a non-agonist PPARγ ligand, has antidiabetic actions in two murine models

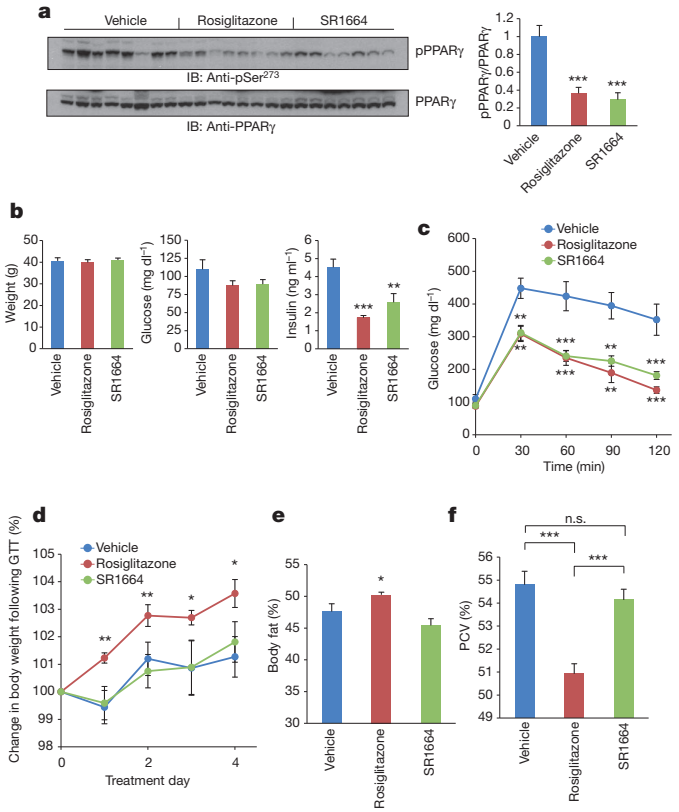

Figure 4 | SR1664 has potent antidiabetic activity and does not promote fluid retention in ob/ob mice. **a**, Phosphorylation of PPARγ in WAT (left). Quantification of PPARγ phosphorylation compared to total PPARγ (right). **b**, **c**, Fasting body weight, blood glucose and insulin levels before glucose-tolerance 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.

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]. Whereas 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 (ref. 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 antidiabetic 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 loops 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 antidiabetic actions of a PPARγ ligand is now clear. In both diet-induced and genetically obese animals, SR1664 has strong antidiabetic 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 antidiabetic activity, roughly equivalent to rosiglitazone in the experiments shown here. The unfavourable 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 whereas 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 indicate 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 reagent (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 $\Delta\Delta$-C_t 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. Male C57BL/6J and C57BL/6J-*Lep*$^{ob/ob}$ mice (4- to 5-week-old) 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 injected intraperitoneally (i.p.) with rosiglitazone or SR1664 for 5 days, and fasted overnight before i.p. injection of 1 g kg^{-1} D-glucose.

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

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

SR1664. (*S*)-4′-((5-((1-(4-nitrophenyl)ethyl)carbamoyl)-1*H*-indol-1-yl)methyl)-[1,1′-biphenyl]-2-carboxylic acid. Commercially available ethyl 2,3-dimethyl-1*H*-indole-5-carboxylate was *N*-alkylated with commercially available *tert*-butyl 4′-(bromomethyl)biphenyl-2-carboxylate using NaH in DMF. The corresponding ethyl ester was hydrolysed using aqueous NaOH in ethanol to give the acid, which was coupled to (S)-1-(4-nitrophenyl)ethanamine using 2-(3H-[1,2,3]triazolo [4,5-b]pyridin-3-yl)-1,1,3,3-tetramethylisouronium hexafluorophosphate(V) (HATU) and diisopropylethylamine in CH_2Cl_2 to give the amide. Final deprotection of the *tert*-butyl ester using 30% trifluoroacetic acid in CH_2Cl_2 and purification by flash chromatography (ethyl acetate/hexanes 10–100%) afforded SR1664. Electrospray ionisation coupled with mass spectrometry (ESI-MS; *m/z*): 576 $[M+H]^+$; ^{1}H NMR (400 MHz, dimethylsulphoxide (DMSO)-d_6): δ (p.p.m.) 8.83 (d, $J = 7.6$ Hz, 1H), 8.25 (m, 1H), 8.16 (d, $J = 1.2$ Hz, 1H), 7.74-7.68 (m, 4H), 7.57 (dt, $J = 1.6$, 7.2 Hz, 1H), 7.51 (d, $J = 8.4$ Hz, 1H), 7.46 (dt, $J = 1.2$, 7.2 Hz, 1H), 7.36 (dd, $J = 0.8$, 7.6 Hz, 1H), 7.28 (m, 2H), 7.03 (m, 2H), 5.52 (s, 2H), 5.32 (quint, $J = 7.2$ Hz, 1H), 2.36 (s, 3H), 2.34 (s, 3H), 1.57 (d, $J = 6.8$ Hz, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 170.5, 167.9, 154.5, 147.2, 141.5, 140.7, 138.7, 138.2, 135.1, 133.2, 131.8, 131.5, 130.0, 129.6, 128.6, 128.2, 128.1, 126.8, 125.8, 124.4, 121.4, 118.8, 109.7, 108.3, 49.4, 46.7, 22.9, 11.0, 9.7.

SR1824. (*S*)-4′-((5-(1-(4-bromophenyl)ethylcarbamoyl)-2,3-dimethyl-1*H*-indol-1-yl) methyl)biphenyl-2-carboxylic acid (1824) was synthesized in the same manner using (*S*)-1-(4-bromophenyl)ethanamine. ESI-MS (*m/z*): 581/583 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 1.48 (d, $J = J = 6.8$ Hz, 3H, CH_3 (4-bromophenyl)ethylcarbamoyl), 2.28 (s, 3H, CH_3 indole), 2.32 (s, 3H, CH_3 indole), 5.17 (quintuplet, $J = 7.6$ Hz, 1H, CH (4-bromophenyl)ethylcarbamoyl), 5.47 (s, 2H, CH_2-biphenyl), 6.99 (d, $J = 8$ Hz, 2H, H_7 and H_9 biphenyl), 7.24 (d, $J = J = 8$ Hz, 2H, H_6 and H_{10} biphenyl), 7.31 (d, $J = 7.6$ Hz, 1H, H_7 indole), 7.36–7.55 (m, 7H, H_2, H_3 and H_4 biphenyl, H_6 indole and H 4-bromophenyl), 8.10 (d, $J = J = 1.6$ Hz, 1H, H_4 indole), 8.65 (d, $J = 8$ Hz, 1H, NH amide). ^{13}C NMR (400 MHz, DMSO-d_6): δ (p.p.m.) 169.5, 166.7, 144.9, 140.5, 139.7, 137.6, 137.3, 134.0, 132.2, 131.0, 130.8, 130.4, 129.0, 128.6, 128.4, 127.6, 127.2, 125.9, 125.0, 120.3, 119.4, 117.7, 108.7, 107.3, 47.9, 45.7, 22.1, 10.1, 8.6.

Cell culture. COS-1, 3T3-L1 and HEK-293 cells were obtained from ATCC. 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 with 10% FBS in DMEM for 48 h and cells were switched to the maintenance medium containing 10% FBS and 850 nM insulin. Lipid accumulation in the cells was detected by Oil Red O staining. All chemicals for cell culture were obtained from Sigma unless otherwise indicated.

***In vitro* kinase assay.** Active Cdk5/p35 was purchased from Millipore. *In vitro* CDK kinase assay was performed according to the manufacturer's instructions (Cell Signaling Technology). Purified PPARγ (0.5 μg; Cayman Chemicals) were incubated with active CDK kinase in assay buffer (25 mM Tris-HCl pH 7.5, 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) containing 20 μM ATP for 15 min at 30 °C. PPARγ ligands were pre-incubated with the specified substrates for 30 min before the assay was performed. Rb (Cell Signaling Technology) was used as a positive control.

LanthaScreen. PPARγ competitive binding assay (Invitrogen) was performed according to the manufacturer's protocol. A mixture of 5 nM glutathione *S*-transferase fused with the PPARγ ligand binding domain (GST–PPARγ–LBD), 5 nM Tb-GST-antibody, 5 nM Fluormone Pan-PPAR Green, and serial dilutions of SR1664 beginning at 10 μM downwards was added to wells of black 384-well low-volume plates (Greiner) to a total volume of 18 μl. All dilutions were made in TR-FRET assay buffer C. DMSO at 2% final concentration was used as a no-ligand control. Experiments were performed in triplicate and incubated for 2 h in the dark before analysis in Perkin Elmer ViewLux ultra HTS microplate reader. The FRET signal was measured by excitation at 340 nm and emission at 520 nm for fluorescein and 490 nm for terbium. The fold change over DMSO was calculated by 520 nm/490 nm ratio. Graphs were plotted as fold change of FRET signal for each compound over DMSO-only control.

Cell-based transactivation assay. COS-1 cells were cotransfected in batch by adding 4.5 μg full-length murine PPARγ2-pSV Sport or full-length human PPARγ2-pSport6, with 4.5 μg 3× multimerized PPRE-luciferase reporter and 27 μl X-treme Gene 9 transfection reagent in serum-free Opti-mem reduced serum media (Gibco). After 18-h incubation at 37 °C in a 5% CO_2 incubator, transfected cells were plated in triplicate in white 384-well plates (Perkin Elmer) at a density of 10,000 cells per well. After replating, cells were treated with either DMSO vehicle only or the indicated compounds in increasing doses from 2 pM–10 μM for mouse receptor or 220 pM–2 μM for the human receptor. After 18-h incubation, treated cells were developed with Brite Lite Plus (Perkin Elmer) and read in 384-well Luminescence Perkin Elmer EnVision Multilabel plate reader. Graphs were plotted in triplicate as fold change of treated cells over DMSO-treated control cells.

Ensemble docking. PPARγ co-crystal structures (68 in total) with unique ligands were identified in the Protein Data Bank (PDB) (as of 3 January 2011). Four structures were selected based on the maximum similarity of the co-crystal ligands to SR1664; specifically 3kmg (ligand 538, 0.98 similarity), 2hfp (ligand NSI, similarity of 0.91), 1fm9 (ligand 570, 0.90 similarity), 2pob (ligand GW4, 0.88 similarity). SR1664 was prepared using Schrodinger LigPrep generating tautomers and ionization states (pH range 7 ± 2). Flexible ligand docking of SR1664 against the four structures was performed using Schrodinger Glide. At least one of the two constraints Arg 288 and Ser 342 (Arg 316 and Ser 370 in PPARγ^2) was required to score docking poses. The best docking score (Glide docking scores are meant to correspond to binding affinity) of −9.21 was achieved with the PPARγ structure 2hfp and SR1664 forms a hydrogen bond to Ser 342 (shown in Fig. 2). Unconstrained docking produced almost the same docking pose with the preserved hydrogen bonding to Ser 342 and a slightly less favourable docking score of −8.99 indicating Ser 342 as a critical ligand binding element.

Differentiation of MC3T3-E1. After reaching confluence, cells were grown in α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 200 μM ascorbic acid and 10 mM β-glycerophosphate. The cells were treated with either rosiglitazone (10 μM) or SR1664 (10 μM) or left in vehicle at the start of differentiation. The cells were collected 7 days post-differentiation for gene expression analysis and 21 days post-differentiation for mineralization. The mineralization of MC3T3-E1 cells was determined by Alizarin red S staining (Millipore catalogue no. ECM815) as per manufacturer's instructions.

Preparation of cell or tissue lysates and immunoblotting. Differentiated adipocytes were pre-treated with PPARγ ligands for 45 min, and incubated with TNF-α for 30 min. For tissue lysates, WAT from mice was homogenized in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS with protease and phosphatase inhibitors). For western blotting, a phospho-specific antibody against PPARγ Ser 273 was used[2]. Total tissue lysates were analysed with an anti-PPARγ antibody (Santa Cruz).

Gene expression analysis. Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen). The RNA was reverse-transcribed using the ABI reverse transcription kit. Quantitative PCR reactions were performed with SYBR green fluorescent dye using an ABI9300 PCR machine. Relative mRNA expression was determined by the $\Delta\Delta$-C_t method normalized to TATA-binding protein (TBP) levels. The sequences of primers used in this study are found in Supplementary Table 1.

ChIP. Differentiated 3T3-L1 adipocytes were treated on day 6 with 1 μM of compounds or vehicle for 24 h. The samples were prepared using manufacturer's protocol (ChampionChIP One-Day Kit, Qiagen). Briefly, cross-linked chromatin was sonicated and 5 μg of antibody was used to immunoprecipitate the pre-cleared samples. The following antibodies were used: normal rabbit IgG, PPARγ (Santa Cruz), SRC-1 (Abcam). The promoter region of aP2 for PPAR-γ binding was amplified using PCR with reverse transcription (RT–PCR). The primers used for aP2 were aP2 forward 5′-AAATTCAGAAGAAAGTAAACACATTATT-3′; aP2 reverse 5′-ATGCCCTGACCATGTGA-3′.

Gene sets from microarray. We performed a microarray with total RNA isolated from PPARγ-null fibroblasts expressing wild-type or S273A mutant of PPARγ or WT cells treated with 1 μM rosiglitazone for 24 h (ref. 2). To create refined gene sets regulated by phosphorylation of PPARγ or rosiglitazone, we first calculated *P*-values as well as fold-change of gene expression in wild-type versus S273A mutant cells or wild-type versus wild-type /Rosiglitazone cells, and we plotted −log *P*-value versus $\log_2$ fold-change. From this list of genes, we selected genes which were changed in magnitude (≥1.4 fold difference) and statistical significance ($P < 0.05$). The selected genes were validated in cells by using qPCR, the resulting gene sets (phosphorylation-dependent or agonist-dependent gene sets) were analysed in WAT of mice using qPCR.

Animals. All animal experiments were performed according to procedures approved by Beth Israel Deaconess Medical Center's Institutional Animal Care and Use Committee. Male C57BL/6J or C57BL/6J-*Lep*$^{ob/ob}$ mice (4- to 5-week-old) were obtained from the Jackson Laboratory. C57BL/6J mice were fed a regular diet (10% kcal fat, D12450B, Research Diets Inc.) or a high-fat, high-sugar diet (60% kcal fat, D12492, Research Diets Inc.) for either 8, 10 or 18 weeks. The mice were intraperitoneally (i.p.) injected twice daily with 4 mg kg^{-1} rosiglitazone or 20 mg kg^{-1} SR1664 for 6 days before gene expression analysis or hyperinsulinaemic-euglycaemic clamp experiments. Clamps were performed essentially as previously described with one exception to the standard protocol[29]. As the mice were fed a high-fat diet for 8 weeks before the clamp studies, a higher insulin infusion rate of 4 mU (kg-min)$^{-1}$ was used instead of the typical 3 mU (kg-min)$^{-1}$ for standard chow studies. For glucose tolerance tests, 6-week-old male C57BL/6J-*Lep*$^{ob/ob}$ mice were i.p. injected twice daily with 8 mg kg^{-1} rosiglitazone or 40 mg kg^{-1} SR1664 for 6 days, and fasted overnight before i.p. injection of 1 g kg^{-1} D-glucose. Glucose was measured by tail vein bleeds at the indicated intervals using a Truetrack

glucometer. Serum insulin concentrations were determined by ELISA (Crystal Chem).

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