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An alternate binding site for PPAR γ ligands

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PPAR γ is a target for insulin-sensitizing drugs such as glitazones, which improve plasma glucose maintenance in patients with diabetes. Synthetic ligands have been designed to mimic endogenous ligand binding to a canonical ligand-binding pocket to hyperactivate PPAR γ . Here we reveal that synthetic PPAR γ ligands also bind to an alternate site, leading to unique receptor conformational changes that impact coregulator binding, transactivation and target gene expression. Using structure–function studies we show that alternate site binding occurs at pharmacologically relevant ligand concentrations, and is neither blocked by covalently bound synthetic antagonists nor by endogenous ligands indicating non-overlapping binding with the canonical pocket. Alternate site binding likely contributes to PPAR γ hyperactivation *in vivo*, perhaps explaining why PPAR γ full and partial or weak agonists display similar adverse effects. These findings expand our understanding of PPAR γ activation by ligands and suggest that allosteric modulators could be designed to fine tune PPAR γ activity without competing with endogenous ligands.

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Nuclear receptors (NRs) are ligand-regulated transcription factors that function as allosteric protein scaffolds recruiting chromatin remodeling machinery to promoter regions of target genes¹. Recent studies have explored the structural features of full-length NR complexes^{2–6}, yet ligand-driven structural changes that are critical for determining the molecular basis for the pharmacological and functional response to ligands remain unclear. Endogenous and synthetic ligands are understood to compete for binding to an internal hydrophobic ligand-binding pocket (LBP) located within the NR ligand-binding domain (LBD). Ligands bind the LBP, which is a hydrophobic core within the 12 helix bundle LBD⁷, altering the hydrogen bond network that can stabilize a key switch helix, helix 12 (H12). In the absence of ligand, H12 is highly dynamic and in equilibrium among many conformations ranging from active to inactive. Ligand binding can stabilize the dynamics of helix 12 and alter the coregulator protein interaction surface of the LBD, which is referred to as the activation function-2 (AF-2) surface. The AF-2 surface is a three-dimensional (3D) surface that includes H3, H3-4 loop, H11 and H12. The active conformation favours coactivator binding, and when the equilibrium is shifted towards the inactive state corepressor binding is favoured. These specific interactions affect the transactivation activity of the receptor and modulate the expression of receptor target genes.

Ligand-induced activation of NRs was initially viewed as a straightforward ‘on-off’ switch^{8,9}. However, more complex mechanisms of ligand-induced regulation of NR function are becoming clear. For example, we have shown that PPAR γ ligands can differentially affect the structural conformation of two distinct surfaces important to interaction with various PPAR γ partners and the post-translational modification status of PPAR γ ^{10,11}. We also described a dynamic mechanism by which ligand binding partially stabilizes the PPAR γ AF-2 surface for partial agonist activity^{12,13}. In addition, serotonin and fatty acid metabolites can bind simultaneously to distinct sub-regions within the PPAR γ LBP, suggesting a mechanism to integrate signals from distinct signalling pathways¹⁴.

Here we demonstrate that synthetic ligands designed to mimic the activity of endogenous ligands via binding to the canonical LBP in PPAR γ can also bind to an alternate site. Alternate site binding occurs via three mechanisms of potential pharmacological relevance, including (1) binding two molar equivalents of ligand to PPAR γ , one to the canonical LBP and a second to the alternate site; (2) binding when the canonical LBP is ‘blocked’ by a covalently bound irreversible antagonist; and (3) binding when the canonical LBP is covalently bound to an endogenous ligand. Alternate site binding likely contributes to the pharmacological response of PPAR γ ligands, which could impact the interpretation of studies examining the function of PPAR γ using synthetic ligands and opens up the possibility of fine tuning PPAR γ activity with synthetic allosteric modulators in the presence of endogenous ligands.

Results

PPAR γ binds two molar equivalents of synthetic ligand. We used ¹⁹F NMR to monitor the binding of two synthetic PPAR γ ligands, MRL20 and MRL24 (Fig. 1a; compounds 20 and 24 (ref. 15)), to PPAR γ . Stoichiometric addition of MRL20 (Fig. 1b,c and Supplementary Fig. 1) or MRL24 (Fig. 1d,e) to PPAR γ reveals a sequential saturation of two ¹⁹F NMR resonances. Population of the second MRL20 ¹⁹F NMR resonance did not decrease the peak intensity or volume of the first ¹⁹F NMR resonance, but it caused a slight change in chemical shift and linewidth of the first ¹⁹F NMR resonance (~20 Hz). The canonical LBP-binding affinity of MRL20 is 2 nM (ref. 15). Using ¹⁹F NMR, we calculated the

binding affinity of the second MRL20 binding event to be ~4 μ M. These MRL20-binding affinities are confirmed by time-resolved Förster resonance energy transfer (TR-FRET) experiments below. The sequential population of two MRL20 ¹⁹F NMR resonances is consistent with two MRL20 ligands binding to PPAR γ with ~1,000-fold difference in binding affinity. We previously proposed that multiple MRL20 NMR resonances could correspond to a single ligand-binding event with multiple binding modes¹², and we confirmed that this indeed occurs at 1:1 ligand:PPAR γ (Supplementary Fig. 2). In addition, these new data implicate a second ligand-binding event that occurs within the context of the PPAR γ /RXR α LBD heterodimer (Fig. 1f) and full-length PPAR γ (Fig. 1g), which is specific as no binding was observed to a control protein, lysozyme (Fig. 1h).

Structural mapping of the alternate binding site in PPAR γ .

We performed a series of 2D [¹H,¹⁵N]-TROSY-HSQC NMR experiments to map the effect of the second ligand-binding event. Using additional NMR data and other assignments¹⁶, we extended PPAR γ NMR chemical shift assignments for MRL20 and MRL24 (ref. 12) in the following regions: H2'–H3, β -sheet region and loops, the Ω loop (a flexible loop region comprising ~15 residues between H2' and H3 spanning D260–K275), H11 and H12. Titration of MRL20 up to 1:1 (ligand:protein) reveals a transition in slow exchange on the NMR timescale (Fig. 2a), indicating a high-affinity binding event consistent with a 2 nM LBP affinity¹⁵. Higher stoichiometries reveal a second transition where some peaks are affected by subtle chemical shift changes and line broadening, consistent with exchange effects expected¹⁷ for a ligand with ~4 μ M binding affinity.

To determine the location of the alternate ligand-binding site, we compared NMR peak intensities for backbone NH groups in 2D [¹H,¹⁵N]-TROSY-HSQC (Fig. 2b) and 3D TROSY-HNCO (Fig. 2c), and methyl groups in 2D [¹H,¹³C]-CHD₂-HSQC (Fig. 2d,e) data for PPAR γ bound to one or two molecules of MRL20. The most significant drop in HNCO peak intensity occurs for residues in β -strand 1 (β 1), H2', the Ω loop, β 2-4 and the surrounding loops (Fig. 2f; red and purple), indicating this is likely the alternate binding site for the second MRL20 ligand. A pocket is present at this site (Fig. 2g) posited to be the ligand entry/exit site to the canonical LBP^{18,19}. Molecular docking demonstrates a second MRL20 molecule can bind to this pocket (Fig. 2h), which is bound on one side by the Ω loop—a flexible loop region between H2' and H3 in the LBD that is commonly unobserved in crystal structures due to its high mobility. This alternate ligand-binding site encompasses an area not thought of as part of the canonical LBP. Several other regions distant from this alternate binding site display a notable decrease in NMR peak intensity (Fig. 2f; orange and blue), though not as significant as the alternate site, including the AF-2 surface. MRL24 induces similar alternate site-binding effects (Fig. 3). These data implicate an allosteric structural coupling between alternate site binding and the AF-2 coregulator-binding surface.

Covalent antagonists do not block alternate site binding.

GW9662 and T0070907 (Fig. 4a) are synthetic irreversible PPAR γ antagonists that covalently attach to Cys285 (refs 20,21, which points into the PPAR γ LBP (Fig. 4b). Covalent antagonists block binding of other ligands to the LBP (Fig. 4c), and because they are thought to completely block ligand binding to PPAR γ they are used as chemical tools to determine whether or not ligand binding to PPAR γ is involved in a particular functional response. If another PPAR γ ligand shows activity when co-administered with a covalent antagonist, the ligand is said to have

Figure 1 | MRL20 and MRL24 bind to PPAR γ with a 2:1 stoichiometry. (a) Chemical structure of MRL20 and MRL24. Titration of (b,c) MRL20 or (d,e) MRL24 results in the population of two PPAR γ LBD-bound ^{19}F NMR resonances, where (c,e) are a plot of the total ^{19}F peak area in (b) and (d), normalized to the area of the saturated canonical peak; concentrations beyond two molar equivalents of ligand are corrected (free ligand signal subtracted and adjusted for differential longitudinal relaxation; see methods for details). (f,g) MRL20 populates two ^{19}F NMR resonances within the context of the (f) PPAR γ /RXR α LBD heterodimer and (g) full-length PPAR γ . (h) A single, sharp ^{19}F resonance is observed for free MRL20 in buffer or added to lysozyme, indicating that alternate site binding of MRL20 to PPAR γ occurs in a specific manner.

PPAR γ -independent, or off-target, functional effects. Analysis of the GW9662-PPAR γ co-crystal structure suggested that GW9662 or T0070907 would block MRL20 binding to the canonical LBP but not the alternate site. To test this we exposed PPAR γ to GW9662 or T0070907, added a saturating amount of MRL20 and performed ^{19}F NMR. MRL20 (Fig. 4d) or MRL24 (Fig. 4e) binding to PPAR γ covalently bound to an antagonist populates a single ^{19}F NMR resonance. This effect is dependent on Cys285, as pretreatment of a Cys285Ala mutant with covalent antagonist shows two peaks when saturated with MRL20. The MRL20 ^{19}F chemical shift in the presence of GW9662 (61.95 p.p.m.) and T0070907 (62.55 p.p.m.) is similar to the chemical shift of the second MRL20 (Fig. 1b) that binds to the alternate site (62.1 p.p.m.), indicating they likely bind within the same alternate site. The only difference between GW9662 and T0070907 is a single nitrogen substitution (Fig. 4a). Thus, the different alternate-site-bound ^{19}F chemical shifts for MRL20 bound to GW9662-PPAR γ versus T0070907-PPAR γ suggest that these antagonists are in close structural proximity to MRL20 bound to the alternate -site, which is consistent with a docking model (Fig. 4f).

We confirmed that MRL20 binds to GW9662-PPAR γ LBD using 2D [^1H , ^{15}N]-TROSY-HSQC NMR (Fig. 4g). MRL20 binding to the alternate site of GW9662-PPAR γ causes line broadening and chemical shift perturbations for residues within the vicinity of the alternate site and the AF-2 surface. Although

alternate site binding of MRL20 to GW9662-PPAR γ causes substantial line broadening for residues within the alternate site, other resonances distant from this alternate site display a transition in slow exchange on the NMR timescale (Fig. 4h). An interpretation of the mixed intermediate/slow exchange effects is that the binding affinity of MRL20 for the alternate site is higher when the LBP is blocked with GW9662 compared with when the LBP is occupied by MRL20. This is confirmed by TR-FRET measurement below. However, despite the higher alternate site affinity, the ^{19}F NMR resonance for MRL20 bound to the alternate site is broader when bound to GW9662-PPAR γ (Fig. 4d) compared with MRL20-bound PPAR γ (Fig. 1b). This indicates a large degree of conformational heterogeneity when bound to the alternate site of GW9662-PPAR γ , such as multiple binding modes²² or motion/exchange on the μs – ms timescale¹⁷, providing an explanation for the intermediate exchange observed for residues near the alternate site.

Alternate site binding stabilizes the AF-2 surface. These NMR data demonstrate that covalent antagonists block binding of MRL20 to the canonical PPAR γ LBP but not the alternate site, which we confirmed using molecular docking (Fig. 4f). Thus, treatment of PPAR γ with these covalent antagonists allows the study of alternate site binding specifically on the structure and function of PPAR γ . Approximately half of the NMR resonances are missing in GW9662-PPAR γ (Supplementary Fig. 3). We

Figure 2 | Mapping the alternate MRL20 binding site in PPAR γ . (a) Titration of MRL20 into ^{15}N -PPAR γ LBD monitored by 2D $[^1\text{H}, ^{15}\text{N}]$ -TROSY-HSQC NMR reveals two binding transitions. The first slow exchange transition corresponds to the canonical LBP binding event (apo to 1:1), and the second intermediate exchange transition to the alternate site binding event ($>1:1$ stoichiometry). (b) Comparison of 2D $[^1\text{H}, ^{15}\text{N}]$ -TROSY-HSQC spectra for ^{15}N -PPAR γ LBD bound to one or two molecules of MRL20 (black and orange, respectively). (c) NMR chemical shift footprinting reveals a decrease in peak intensity between 3D TROSY-HNCO experiments collected for $^2\text{H}, ^{13}\text{C}, ^{15}\text{N}$ -PPAR γ LBD bound to one or two molecules of MRL20 (black/pink and orange/grey, respectively, for positive/negative peak amplitudes) and reveals residues affected by the alternate site-binding event. (d) Comparison of 2D $[^1\text{H}, ^{13}\text{C}]$ -methyl CHD₂-detected HSQC data for $^2\text{H}, ^{13}\text{C}, ^{15}\text{N}$ -PPAR γ LBD bound to one or two molecules of MRL20. (e) Residues with methyl NMR resonances affected upon binding a second MRL20 ligand. (f) NMR chemical shift footprinting changes mapped onto the PPAR γ LBD structure reveals the site of interaction (red) and regions allosterically affected by alternate site binding (blue, orange); spheres represent methyl groups affected, and regions coloured black have unassigned NMR chemical shifts likely due to dynamics on the NMR intermediate exchange regime; the dynamic Ω loop, which comprises part of the alternate site, is illustrated with a dotted line. (g) The alternate site is formed by a solvent-accessible pocket on the PPAR γ LBD surface. (h) Molecular docking of a second MRL20 ligand (yellow) into the alternate site, which is also shown in (f).

therefore used hydrogen/deuterium exchange (HDX) mass spectrometry (MS) to monitor changes in solvent accessibility and hydrogen bonding caused by binding of MRL20 to the alternate site of GW9662-PPAR γ . Incubation of PPAR γ with GW9662 resulted in a mass shift of the H3 peptide containing

Cys285 by +241.062 Da. We were unable to detect unmodified H3 peptide confirming complete covalent attachment of GW9662 to PPAR γ . MRL20 induced robust protection from HDX in GW9662-PPAR γ within several structural regions (Fig. 4i and Supplementary Table 1) including the alternate site and the AF-2 surface. Qualitatively, the pattern of HDX protection on the AF-2 surface caused by alternate site binding of MRL20 to GW9662-PPAR γ is similar to what we observed previously for MRL20 binding to the canonical LBP using a 10 $\times$ molar excess of ligand^{12,13}. One exception is a peptide from H6/7, which is protected upon binding MRL20 to the LBP but not to the alternate site of GW9662-PPAR γ . This peptide is in close proximity to the canonical LBP-bound MRL20 but not the alternate site, possibly explaining why it is not affected by alternate site binding of MRL20 to GW9662-PPAR γ .

Alternate site binding affects coregulator interaction. To determine whether alternate site ligand binding can affect the interaction between coregulator proteins and PPAR γ , we performed a TR-FRET assay to monitor the ability of ligands to affect coregulator peptide binding to the PPAR γ LBD. MRL20 causes a concentration-dependent increase in association between a peptide fragment 'NR box' of the TRAP220 coactivator (13 nM EC₅₀) (Fig. 5a,b). Furthermore, there is a subtle but notable effect on TRAP220 binding around the expected alternate site K_d (~2 μ M; Fig. 5c). MRL20 binding to the alternate site of GW9662-PPAR γ and T0070907-PPAR γ causes a potent and robust increase in TRAP220 binding (361 nM and 2 μ M EC₅₀, respectively), indicating the phenyl (GW9662) to pyridyl (T0070907) group modification affects alternate site-binding affinity. Antagonist treatment of a Cys285Ala mutant does not affect MRL20 EC₅₀ values, confirming that covalent attachment to Cys285 is necessary for the right-shifted EC₅₀ values (Fig. 5d). In addition, the alternate site response occurs for full-length PPAR γ with and without its heterodimer partner, RXR α (Fig. 5e).

Profiling a diverse set of synthetic PPAR γ ligands. We next determined whether functional effects of alternate site binding occur with other synthetic PPAR γ ligands by monitoring NCoR or TRAP220 peptide recruitment to PPAR γ as a function of ligand concentration for a diverse set of PPAR γ ligands and control non-PPAR γ ligands. Antagonist treatment increases NCoR recruitment (Fig. 5f) and decreases TRAP220 recruitment (Fig. 5a) compared with apo PPAR γ . Most synthetic PPAR γ ligands profiled display some degree of alternate site binding effects to antagonist-bound PPAR γ with varying potencies (Supplementary Fig. 4). Ligands that show alternate site binding effects in a saturating and concentration-dependent manner with potencies near or below 1 μ M include SR9034 (65 nM; compound 7b²³), MRL24 (175 nM), nTZDpa (264 nM), SR1988 (1; 356 nM), SR1664 (ref. 10) (384 nM), SR2088 (2; 461 nM) and BVT.13 (2.44 μ M). The binding modes of ligands that have published co-crystal structures overlap with that of GW9662, although for

Figure 3 | Mapping the alternate MRL24-binding site in PPAR γ .

(a) Comparison of 2D [1H, 15N]-TROSY-HSQC spectra for 15N-PPAR γ LBD bound to one or two molecules of MRL24 (black and orange, respectively). (b) Comparison of 2D [1H, 13C]-methyl CHD2-detected HSQC data for 2H, 13C, 15N-PPAR γ LBD bound to one or two molecules of MRL24. (c) NMR chemical shift footprinting reveals a decrease in peak intensity between 3D TROSY-HNCO experiments collected for 2H, 13C, 15N-PPAR γ LBD bound to one or two molecules of MRL24 (black/pink and orange/grey, respectively, for positive/negative peak amplitudes) and reveals residues affected by the alternate site-binding event.

Figure 4 | Covalent antagonists do not block alternate site ligand binding to PPAR γ . (a) Chemical structures of GW9662 and T0070907. (b) GW9662 (cyan) covalently attaches to PPAR γ residue C285 (yellow). (c) When GW9662 (cyan) is covalently bound to PPAR γ , it sterically blocks MRL20 (magenta) from binding to the LBP. (d,e) ^{19}F NMR reveals (d) MRL20 and (e) MRL24 bind to PPAR γ LBD bound to a covalent antagonist and populate a single resonance. (f) Molecular docking of MRL20 ligand into the alternate site of PPAR γ LBD covalently bound to GW9662. (g) 2D ^1H , ^{15}N -TROSY-HSQC NMR confirms that MRL20 binds to ^{15}N -PPAR γ LBD covalently bound to GW9662. (h) Titration of MRL20 into GW9662-bound ^{15}N -PPAR γ LBD monitored by 2D ^1H , ^{15}N -TROSY-HSQC NMR reveals MRL20 binding effects occurring in slow exchange at residues remote from the alternate binding site. (i) HDX-MS analysis reveals that alternate site binding of MRL20 to PPAR γ LBD covalently bound to GW9662 causes protection from HDX in the alternate site region and the PPAR γ AF-2 surface.

nTZDpa and BVT.13 the overlap is less severe. This implies that although these ligands do not bind to GW9662-PPAR γ using the crystalized binding modes, the alternate site binding modes for these ligands have a potent functional effect. PPAR γ full agonists GW1929 and thiazolidinediones (TZDs) rosiglitazone, pioglitazone, troglitazone and ciglitazone displayed less potent and/or efficacious alternate site functional effects. Two naturally occurring plant compounds reported to modulate PPAR γ activity, daidzein and resveratrol, show prominent alternate site functional effects. Finally, we tested several non-PPAR γ ligands, including ligands of other NRs, most of which showed no

significant alternate site response. Thus, the functional response to alternate site binding is more potent and efficacious for synthetic PPAR γ ligands versus non-PPAR γ ligands. For some ligands, it is possible that alternate site effects in this assay could be attributed to ligand aggregation or precipitation at high concentrations. However, this does not appear to be the case at least for MRL20, MRL24 and rosiglitazone (Supplementary Figs 5 and 6).

Alternate site binding affects transactivation. Luciferase reporter assays are used to measure NR-dependent transcription,

Figure 5 | Alternate site binding affects PPAR γ -coregulator interaction. (a) TR-FRET assay showing the effect of MRL20 on TRAP220-2 peptide binding to PPAR γ protein in the absence or presence of a covalent antagonist, performed in duplicate and plotted as the average ($\pm$ s.d.). (b) Same data as (a) but normalized to illustrate the biphasic response for MRL20 to apo PPAR γ and the EC₅₀ differences between all conditions. (c) Same as in (b) but focused on the alternate site response for apo PPAR γ . (d) PPAR γ C285A mutation serves as a control to show that C285 is critical for covalent antagonist attachment and blocking MRL20 binding to the LBP in the TR-FRET assay. (e,f) TR-FRET assay shows an alternate-site response for MRL20 using (e) full-length PPAR γ protein and full-length PPAR γ protein heterodimerized to RXR α LBD covalently bound to T0070907 performed in duplicate and plotted as the average ($\pm$ s.d.). (f) Covalent attachment of T0070907 increases the basal interaction of NCoR-3 peptide right shifts the MRL20 IC₅₀ compared with apo PPAR γ , performed in duplicate and plotted as the average ($\pm$ s.d.). All data fit to a sigmoidal dose response curve.

Figure 6 | Alternate site binding affects PPAR γ transactivation and target gene expression. (a,b) Luciferase reporter assay showing the concentration-dependent effects of MRL20, MRL24 and rosiglitazone on PPAR γ transactivation (a) without GW9662 pretreatment and (b) with GW9662 pretreatment, performed by cotransfection of Gal4-PPAR γ LBD and UAS:*luc* reporter plasmid, performed in triplicate and plotted with the average ($\pm$ s.e.m.) and fit to a sigmoidal dose response curve. (c) qRT-PCR analysis of C/EBP α expression in Jurkat cells, performed in quadruplicate, plotted with the average ($\pm$ s.e.m.), and analysed using Bonferroni *post hoc* comparison; ns = not significant. (d-g) qRT-PCR analysis of PPAR γ target genes in NIH-3T3-L1 cells harvested 3 days after inducing differentiation, performed in quadruplicate, plotted with the average ($\pm$ s.e.m.), and analysed using one-way ANOVA with Tukey *post hoc* comparison; ns = not significant. T007 = T0070907.

or transactivation, in cells. In general, rosiglitazone, MRL20 and MRL24 display concentration-dependent increases in PPAR γ transactivation with potencies (EC₅₀) consistent with their

canonical LBP-binding affinities (Fig. 6a)¹². However MRL24, a poor activator of PPAR γ transcription, still shows an increase in transactivation at concentrations >200-fold higher than its 2 nM

canonical LBP binding affinity¹⁵, indicating there may be contributions from alternate site binding. To further determine whether alternate site ligand binding can affect PPAR γ transactivation, we performed a cotransfection assay by preincubating cells with GW9662 before treating cells with ligand (Fig. 6b). GW9662 did not block the action of MRL20 and MRL24 with EC₅₀ values consistent with the TR-FRET coregulator assay (317 nM and 62 nM, respectively). Also consistent with TR-FRET coregulator binding data (Supplementary Fig. 4j), GW9662 blocks the action of rosiglitazone at concentrations up to $\sim 2 \mu\text{M}$, whereas higher rosiglitazone concentrations elicit an increase in PPAR γ transactivation.

Alternate site binding affects target gene expression. We next assessed the effect of MRL20 binding to the alternate site of endogenous PPAR γ in Jurkat T-lymphocyte cells. In the absence of covalent antagonist, MRL20 causes a concentration-dependent increase in *C/EBP α* expression (Fig. 6c). Coadministration of a covalent antagonist (GW9662 or T0070907) and MRL20 did not block the action of MRL20 on *C/EBP α* expression, but rather caused a right-shift in the efficacy of MRL20. Consistent with our TR-FRET data (Fig. 5a,b), the rank order of potency for MRL20 inducing *C/EBP α* expression is no covalent antagonist > GW9662 > T0070907.

We also assessed the action of alternate site binding in the NIH-3T3-L1 preadipocyte cell line, which is a commonly used cell model to assess endogenous PPAR γ function related to adipocyte differentiation. NIH-3T3-L1 cells were differentiated in the presence of MRL20 or rosiglitazone with or without coadministered covalent antagonist and harvested 3 days after initiating differentiation. In the absence of antagonist both MRL20 and rosiglitazone increase *C/EBP α* expression (Fig. 6d). However, coadministration of T0070907 significantly reduced the efficacy of rosiglitazone, but not MRL20, on inducing *C/EBP α* expression. This occurs without significantly affecting PPAR γ expression (Fig. 6e). We also tested the effect of MRL20, with and without T0070907 coadministration, on the expression of *aP2* (Fig. 6f) and *adiponectin* (Fig. 6g), which are genes regulated by classical PPAR γ -driven transcriptional agonism and phosphorylation of PPAR γ , respectively^{10,11}. MRL20 alone and MRL20 coadministered with T0070907 both significantly increased the expression of these genes. This indicates that binding of MRL20 to the alternate site can affect the activity of endogenous PPAR γ in cells and that alternate site binding affects markers of both classical PPAR γ transcriptional agonism (*aP2*) and PPAR γ -driven anti-diabetic effects that occur through blocking phosphorylation of PPAR γ at Ser273 (*Adiponectin*).

Concurrent binding of endogenous and synthetic ligands.

Several endogenous PPAR γ ligands have been identified that, similar to GW9662 and T0070907, covalently bind to Cys285 in the PPAR γ LBP. These include the lipid 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 (15d-PGJ2) and oxidized fatty acids such as 5-oxoeicosatetraenoic acid (5-oxoETE), 15-oxoETE and 9-oxooctadecadienoic acid (9-OxoODE)^{24–26}. Synthetic antagonists covalently bind to PPAR γ through a halogen exchange reaction²⁰, whereas endogenous ligands covalently bind through a Michael addition²⁶. We performed ¹⁹F NMR to determine whether MRL20 can bind to the alternate site of PPAR γ covalently bound to an endogenous ligand (Fig. 7a). Similar to studies with covalent antagonists, ¹⁹F NMR shows that MRL20 binds to PPAR γ covalently bound to one of several endogenous ligands. Alternate site binding to PPAR γ bound to an endogenous ligand results in the appearance of a single ¹⁹F MRL20 resonance

Figure 7 | Alternate site binding of MRL20 to PPAR γ bound to an endogenous ligand. (a) ¹⁹F NMR analysis reveals that MRL20 binds to PPAR γ LBD covalently bound to endogenous ligands. (b,c) TR-FRET assay demonstrates that alternate site MRL20 binding affects the interaction between (b) TRAP220-2 and (c) NCoR-3 for PPAR γ LBD covalently bound to 15-oxoETE, but not 5-oxoETE. PPAR γ LBD protein not covalently bound to an endogenous ligand but exposed to vehicle (ethanol) was used as a control; performed in duplicate, plotted as the average ($\pm$ s.d.), and fit to a sigmoidal dose response curve.

with a chemical shift similar to the alternate site ¹⁹F resonances populated when PPAR γ binds two equivalents of MRL20 or when MRL20 binds to GW9662-PPAR γ or T0070907-PPAR γ . In the case of 15d-PGJ2, a lowly populated peak is observed corresponding to the chemical shift of MRL20 bound to the canonical LBP, which likely represents protein not completely modified by 15d-PGJ2 as previously observed²⁶.

We performed a TR-FRET coregulator-binding assay to determine whether alternate site binding of MRL20 to PPAR γ covalently bound to an oxidized fatty acid can affect coregulator interaction. Covalent attachment of 5-oxoETE, but not 15-oxoETE, significantly increases basal binding of TRAP220 peptide to PPAR γ (Fig. 7b), and both endogenous ligands

Figure 8 | Possible structural mechanism for indirect stabilization of the PPAR γ AF-2 surface via the alternate site. (a) Ligands such as rosiglitazone that bind to the PPAR γ canonical LBP form hydrogen bonds with residues in the helix 12 pocket (Y473 and H449) to directly stabilize helix 12 and the AF-2 surface. **(b)** Ligands that bind to the alternate site may indirectly stabilize the AF-2 surface by stabilizing helix 3, facilitating hydrogen bond formation between side chains of residues on helix 3 to residues in the helix 11-12 loop, particularly in the presence of a bound coregulator, which could affect helix 12/AF-2 stabilization.

decrease the basal binding of NCoR peptide (Fig. 7c). This indicates that 5-oxoETE, but not 15-oxoETE, may impart significant AF-2 stabilization upon binding to PPAR γ . This is consistent with the likely binding modes of 5-oxoETE and 15-oxoETE in the PPAR γ LBP (Supplementary Fig. 7). Addition of MRL20 to 5-oxoETE bound PPAR γ did not affect coregulator recruitment, likely because the AF-2 surface is already maximally stabilized by 5-oxoETE as indicated by robust TRAP220 recruitment to 5-oxoETE-bound PPAR γ . In contrast, MRL20 increased TRAP220 binding and decreased NCoR binding to 15-oxoETE bound PPAR γ (~ 20 – $30 \mu\text{M}$ EC₅₀). Thus, 15-oxoETE-bound PPAR γ resembles apo PPAR γ in terms of TRAP220 and NCoR binding, and therefore alternate site binding of MRL20 can impart significant additional stabilization of the PPAR γ AF-2 surface and significantly affect coregulator peptide recruitment.

Discussion

The glitazones were first discovered using phenotypic screens to look for small molecules with anti-diabetic efficacy, and it was only later discovered that they activate PPAR γ ²⁷. Subsequent studies using crystallography²⁸ or competitive ligand-binding assays revealed that these synthetic ligands and others bind to the

canonical LBP of PPAR γ , a variable-sized pocket within the interior of LBD conserved across the NR superfamily. Around the same time, the first identification of endogenous PPAR γ ligands were made^{29,30}, and more recent studies have revealed that endogenous ligands and synthetic ligands have overlapping binding sites in the PPAR γ canonical LBP^{24,26,31–33}. Thus, synthetic ligands are presumed to bind to PPAR γ by competing with endogenous ligands for the same binding site. Our studies here demonstrate that PPAR γ ligands can also bind to an alternate site and that this occurs via three pharmacologically relevant scenarios discussed below.

First, alternate site binding can occur through binding two molar equivalents of the same ligand to PPAR γ , one to the canonical LBP at high affinity and a second to the alternate site at a lower but potentially pharmacologically relevant affinity. This observation could affect the interpretation of studies examining the pharmacological function of PPAR γ ligands. Although many PPAR γ ligands bind to the canonical LBP with high affinity, often in the low nM range, in cell-based studies ligands are typically administered at high pharmacological concentrations that exceed their canonical LBP binding affinity ranging from $10 \mu\text{M}$ to $150 \mu\text{M}$ ³⁴. For *in vivo* animal model studies it is difficult to know *a priori* the tissue-specific ligand concentrations in mice dosed with ligand on the order of several hundred mg per kg several times a day over many months. In these cases, it is possible that ligand concentrations could be high enough to elicit an alternate site functional effect through binding a second ligand.

Second, alternate site binding can also occur when the PPAR γ LBP is ‘blocked’ by covalently binding synthetic antagonists. Our studies demonstrate that PPAR γ -dependent activity could be expected for some ligands well below $10 \mu\text{M}$ in the presence of a covalent antagonist. This calls into question whether the activity of a PPAR γ ligand should be classified as PPAR γ -independent if it shows activity when co-administered with a covalent antagonist. The action of daidzein on PPAR γ is thought to occur by an LBD-independent mechanism because it activated the receptor in the presence of T0070907 (ref. 35). However, our studies indicate that daidzein binds and functionally activates the PPAR γ LBD when the LBP is blocked by a covalent antagonist. For some ligands, such as BVT.13 and nTZDpa, covalent antagonist coadministration significantly enhances the ability of the ligand to increase coactivator binding to PPAR γ (Supplementary Fig. 4c,g).

Finally, we show for the first time that alternate site binding can occur when the canonical LBP is bound by an endogenous ligand. Although the physiological role of covalently binding endogenous PPAR γ ligands is not completely clear²⁶, our data suggest that the alternate site could be a target for allosteric modulators if PPAR γ is occupied by covalently binding endogenous ligands. In this realm, anti-cancer efficacies of PPAR γ ligands in cell models are reported to be 1,000–10,000-fold higher than the ligand’s canonical LBP binding affinity^{36–39}. In fact, coadministration of covalent antagonist with another PPAR γ ligand does not block the anti-cancer activity and in some cases has been shown to synergize⁴⁰. A characteristic feature of cancer cells is their ability to enhance *de novo* fatty acid and lipid biosynthesis, which can increase the concentration of oxidized fatty acids via β -oxidation, which bind covalently to the PPAR γ LBP²⁶. Thus, it is tempting to speculate that the mechanism of action affording the anti-cancer activity of PPAR γ ligands occurs through the alternate binding site. In addition, obese individuals display enhanced *de novo* fatty acid and lipid biosynthesis or increased bioavailability from dietary sources, increasing the probability of PPAR γ occupancy by oxidized fatty acids. In scenarios such as these, the alternate site may be the only means to modulate the activity of PPAR γ using synthetic ligands.

Figure 9 | Alternate site-bound ligands uniquely protrude around helix 3 to affect the conformation of the Ω loop. Ligands bound deep in the canonical LBP are coloured orange, and the second bound ligand, whether it occupies the alternate site region or not, are coloured blue; Ω loop region, if observed in the crystal structure, from M257-V277 is coloured green. Alternate site-bound ligands that affect the Ω loop conformation are also circled. **(a)** Co-crystal structure of the PPAR γ LBD bound to one molecule of MRL20 (PDB: 2Q59). **(b)** Co-crystal structure of the PPAR γ LBD bound to one molecule of T2384 (PDB: 3K8S; chain A). **(c)** Co-crystal structure of the PPAR γ LBD with MRL20 (orange) (PDB: 2Q59) docked with a second MRL20 ligand (blue). **(d)** Co-crystal structure of the PPAR γ LBD bound to two molecules of T2384 in the canonical LBP (orange) and alternate site (blue) (PDB: 3K8S; chain B). **(e)** Co-crystal structure of the PPAR γ LBD bound to two molecules of 9-(S)-HODE (PDB: 2VSR). **(f)** Co-crystal structure of the PPAR γ LBD bound to 5-methoxy-indole acetate (orange) and 15-oxoETE (blue) (PDB: 3ADW).

Our structural data show that alternate site binding affects NMR chemical shifts and amide proton exchange via HDX (that is, affects the hydrogen bond network) of residues that compose the AF-2 surface, which indicates an effect on the AF-2 surface conformation. The functional impact of this second ligand-binding event may be more notable for non-full agonists, which do not robustly affect the AF-2 surface or coactivator recruitment through binding to the canonical LBP. Mechanistically, there is evidence of an allosteric network running from the Ω loop to H3 via F287, to H12 and the AF-2 surface³², which also comprises a region called the 'H3 electrostatic cluster'⁴¹. Ligand binding to the LBP can directly stabilize the H12/AF-2 surface through formation of hydrogen bonds with residues within a 'H12 subpocket' near the AF-2 surface, including Y473 and H449 (Fig. 8). However, the stabilization induced by alternate site binding likely occurs through an indirect mechanism, perhaps via the H3 cluster. Our data also indicate that graded PPAR γ agonists have higher affinity for the alternate site than full agonists. This may be because PPAR γ full agonists form a hydrogen bond with a specific residue on H12, Tyr473, which is a major contributor to their binding energy. Graded PPAR γ agonists do not typically hydrogen bond with Tyr473, but instead use other bonding features to bind to the hydrophobic PPAR γ LBP with high affinity that may be more conducive for alternate site binding. Of note, our previous PPAR γ HDX studies used a $10 \times$ molar excess of

ligand^{10–13}, and thus observations made in these previous studies likely include a mixture of canonical LBP and alternate site binding effects.

Our work here extends several of our previous studies, including structure–function work revealing that PPAR γ partial agonists display H12-independent activation mechanisms^{12,13}. Data presented in these papers, performed at a $10 \times$ excess of ligand, clearly show the involvement of regions of the ligand-binding pocket remote of AF-2, which we now know contains the alternate binding site. We have also shown that PPAR γ ligands can affect PPAR γ transcriptional activation and PPAR γ -driven anti-diabetic efficacy through distinct mechanisms^{10,11}. These latter studies addressed the paradox that exists for drugs that are anti-diabetic and activate PPAR γ even though there are no loss-of-function mutations in the receptor of most type 2 diabetics. Phosphorylation of Ser273 (Ser273 ~ P) represses the expression of a subset of PPAR γ target genes, and full and partial agonists interfere with this post-translational modification to approximately the same degree. Blocking Ser273 ~ P by ligands correlates more closely with the ability to increase the expression of adiponectin and other anti-diabetic effects than with the magnitude of transactivation of classical pro-adipogenic genes such as aP2. These observations suggest that a compound that binds and alters the level of Ser273-P independent of the degree of agonism of adipogenic genes can be anti-diabetic.

We confirmed this in a subsequent report of SR1664, a compound that can block S273-P and is anti-diabetic even though it does not activate expression of adipogenic genes in cell culture and in obese mice¹⁰. We now show here that alternate site binding affects both classical PPAR γ transcriptional agonism (increased receptor transactivation in a reporter assay and increased expression of *aP2*) and PPAR γ driven anti-diabetic efficacy (increased expression of *Adiponectin*)^{10,11}. These observations indicate that the development of compounds that block PPAR γ phosphorylation with little transcriptional activation can be complicated by alternate site binding. If alternate site binding contributes to the hyperactivation of PPAR γ *in vivo*, where tissue/cell-specific drug concentrations are unknown, alternate site binding could explain why anti-diabetic partial agonists have not progressed to the clinic due to hints of side effects associated with full agonists and TZDs. This would make consideration of the alternate binding site critical to the development of a PPAR γ modulator that only blocks Ser273-P without activating AF-2 regulated (for example, adipogenic) genes.

Nearly all liganded PPAR γ crystal structures have been solved with a single ligand bound to the canonical LBP. A crystal structure of PPAR γ bound to a ligand at an alternate site would provide strong and direct evidence for the mechanism proposed in Fig. 8. Despite our attempts, we were unable to obtain a co-crystal structure of MRL20 or MRL24 bound in the alternate site. However, a few published examples have revealed more complex structural mechanisms by which ligands bind to PPAR γ 's large Y-shaped canonical LBP and the alternate site we identified here (Fig. 9). A serotonin and a fatty acid metabolite can bind to distinct sub-regions in the canonical LBP¹⁴, and two non-covalently binding endogenous ligands have been crystalized within the PPAR γ LBP²⁶. However, in both of these cases, the ligands occupy a space within the canonical LBP similar to that of a single molecule of MRL20 bound in the LBP¹³ and do not protrude out of the LBP near H3. Interestingly, the PPAR γ partial agonist T2384 was crystalized with two molecules bound to one PPAR γ . However, the authors did not explore the possibility that the alternate site T2384-binding event could affect function, this despite observing a bell-shaped response in a TR-FRET coregulator-binding assay with a high- and a low-affinity inflection point⁴². As mentioned above, the Ω loop has been implicated as part of an allosteric network that connects H3 to the AF-2 surface³², and our NMR studies as well as the T2384-bound PPAR γ crystal structure⁴² reveal that alternate site binding affects the conformation of the Ω loop. Our attempts to separate the independent functions of the canonical and alternate binding sites via mutagenesis were complicated because the mutant proteins behaved differently compared with wild-type PPAR γ . Thus, we chose to use covalent antagonists to separate the function of the two sites by blocking binding to the canonical LBP, as they can be used as a pharmacological tool to study alternate site-binding *in vitro* and *in vivo*.

The observation of alternate ligand-binding sites in general is relatively new in the NR field, and there is no general consensus on the importance of these sites. Though there is growing evidence to suggest that alternate binding sites are present in other NRs⁴³, including ER^{44–46}, AR^{47–49}, TR^{50–52} and VDR^{53–57}, there is a lack of comprehensive studies to determine whether alternate site binding events can affect the structure and function of NRs. One main issue is how can alternate site ligand-binding events be detected? Most NR ligand-binding assays employ a ligand displacement assay, where a ligand that is radiolabelled or tagged with a fluorophore is competed with the ligand of interest. However, these displacement assays only report on the displacement of labelled ligand bound in the canonical LBP and

will not generally detect an alternate site-binding event. As we demonstrate here, structural and biophysical assays can be employed to observe alternate site-binding events.

In conclusion, our studies show that ligand binding to an alternate site can affect the structure and function of PPAR γ . These observations reveal that ligand binding to PPAR γ is not a simple competition between individual ligands and that the pharmacological properties of currently used synthetic PPAR γ ligands are more complex than previously understood. Recognizing that this alternate binding site can affect PPAR γ function may guide more informed development of a new generation of PPAR γ drugs.

Methods

Protein preparation and ligands. PPAR γ LBD (residues 203–477, isoform 1 numbering), full-length PPAR γ (isoform 2, residues 1–505) and RXR α LBD (residues 223–462) were expressed in *Escherichia coli* BL21(DE3) cells as TEV-cleavable hexahistidine-tagged fusion proteins using protocols previously described¹² except that TCEP was used as a reducing agent. The final NMR buffer (Buffer C) consisted of 20 mM KPO₄ (pH 7.4) and 50 mM KCl. Ligands were either purchased from commercial sources or synthesized in-house based on prior reports^{10,15,23,58,59}, including compounds 1 and 2 (Supplementary Figs. 8 and 9). Herein, references to structural studies use PPAR γ 1 sequence numbering, and references to functional studies use PPAR γ 2 numbering (Ser273 in γ 2 = Ser245 in γ 1; Cys313 in γ 2 = Cys285 in γ 1).

NMR spectroscopy. NMR was performed on 400 and 700 MHz (¹H frequency) Bruker NMR instruments at 298 K, calibrated using using an established relationship between the chemical shift difference of methanol-d₄ resonances and temperature⁶⁰, equipped with a BBO or QNP probe (400 MHz) or BBO, TXI or QCI probe (700 MHz). Ligands were dissolved in DMSO-d₆ with the final sample concentrations containing < 1% DMSO except in the ¹⁹F observed titrations of MRL20 and MRL24 where direct addition of ligand to concentrated protein necessitated higher DMSO concentrations (< 3%). Protein NMR and ¹⁹F NMR (ligand-observe) experiments were performed as described previously¹² using pulse sequences provided with Bruker Topspin 3.0 and standard experimental parameters provided in Bruker Topspin 3.0 as well as methyl CHD₂-detected experiments⁶¹. For quantitative comparison of relative ¹⁹F NMR populations, a 10 s relaxation delay was used, otherwise 1 s delays were used. In addition to the raw value, corrected values for some titration points where free ligand is likely at or near maximum in Fig. 1 and Supplementary Fig. 1 were plotted. The correction was accomplished as follows. First, the signal from free ligand was subtracted, which was 0.44 (75 μ M MRL24 solubility/171 μ M PPAR γ used) and 0.19 (30 μ M MRL20 solubility/162 μ M PPAR γ used) canonical area units (signal from saturated canonical site) for MRL24 and MRL20, respectively. Second, the final titration point was re-run using a longer relaxation delay (10 s) and the relative change in area between the alternate site/free ligand signal and canonical site signal (due to different longitudinal relaxation rates) was determined and values were adjusted for these higher titration points as well (MRL20: 0.16 area units, MRL24: 0.2 area units).

¹⁹F NMR calculation of alternate site-binding affinity. The alternate site binding affinity using the relationship $K_D = ([L][R])/[LR]$, where $[L]$ and $[R]$ are the concentration of free ligand and receptor and $[LR]$ is the concentration of receptor bound to ligand. We assumed one additional binding site (two MRL20 molecules per PPAR γ). The integral (area) of the ¹⁹F NMR peaks indicates site occupancy⁶². ¹⁹F NMR titration of MRL20 into buffer alone provided an estimate of 30 μ M MRL20 solubility (Supplementary Fig. 5), which is $[L]$. We saturated 330 μ M PPAR γ LBD with MRL20 and collected a ¹⁹F NMR spectrum, which showed 89% occupancy of the alternate site compared with the canonical (after subtraction of signal from $[L]$). Occupancy of the alternate site decreases with a degree expected for the calculated K_D upon dilution and re-concentration of PPAR γ LBD. Definitions: $[LR] = (I_a/I_{ha})[R_0] - [L]$; $[R_0]$ is the overall protein concentration; I_a and I_{ha} are the integrals of the low- and high-affinity peaks, respectively; $[R] = [R_0] - [LR]$; $[L] = 30 \mu$ M.

Hydrogen/deuterium exchange mass spectrometry (HDX-MS). Solution-phase amide HDX experiments were carried out using a fully automated system⁶³. Ten micromoles of His-PPAR γ LBD protein (20 mM KPO₄ pH 7.4, 50 mM KCl) were pre-incubated with 1:2 molar excess of GW9662 at 4 °C overnight; this was the reference sample for HDX used to compare binding of MRL20. MRL20 was added at a 1:3 molar excess for 1 h. Five microlitres of protein solution were mixed with 20 μ l of D₂O-containing HDX buffer (20 mM KPO₄, pH 7.4, 50 mM KCl) and incubated at 4 °C for 10, 30, 60, 900 and 3,600 s. Following on-exchange, unwanted forward or back exchange was minimized and the protein was denatured by dilution with 25 μ l of quench solution (0.1% v/v TFA in 3 M urea). Samples

were passed through an in-house prepared immobilized pepsin column⁶⁴ at 200 $\mu\text{l min}^{-1}$ (0.1% v/v TFA, 15 °C) and the resulting peptides were trapped on a C₈ trap column (Hypersil Gold, Thermo Fisher). Bound peptides were gradient-eluted (5–50% CH₃CN w/v and 0.3% w/v formic acid) across a 2 mm $\times$ 50 mm C₁₈ HPLC column (Hypersil Gold, Thermo Fisher) for 5 min at 4 °C. The eluted peptides were then subjected to electrospray ionization directly coupled to a high resolution Orbitrap mass spectrometer (Exactive, Thermo Fisher). Each HDX experiment was carried out in triplicate and the intensity weighted average *m/z* value (centroid) of each peptide isotopic envelope was calculated with in-house HDX Workbench software⁶⁵. MRL20-induced changes in deuterium uptake were determined by subtracting average percentage deuterium uptake for 64 peptides spanning PPAR γ LBD from the GW9662-PPAR γ LBD sample and visualized with PyMOL (DeLano Scientific).

Time-resolved Förster resonance energy transfer. TR-FRET was measured using a PerkinElmer EnVision multilabel plate reader in 384-well plate format using peptides derived from the second LXXLL motif in the TRAP220 coactivator (TRAP220-2; residues 638–656; NTKNHPMLMNLKDNPAQD) or the third CoNR motif in the NCoR corepressor (NCoR-3; residues 2256–2278; DPASNLGLEDIIRKALMGSEFDDK) containing a N-terminal FITC label with a six-carbon linker (Ahx); the C terminus was amidated for stability. Experiments contained 10 μl of protein mixture A: 50 nM hexahistidine-PPAR γ LBD (or full-length PPAR γ) + 5 nM Anti-hexahistidine antibody labelled with Lumi4 Tb (Cis-bio), 300–400 nM FITC-peptide (Lifetein) in TR-FRET buffer (0.1% BSA + Buffer C). Mixture A was plated, followed by the application of 10 μl of 2 $\times$ ligand or vehicle in TR-FRET buffer. Final concentration of DMSO (vehicle) was constant in all wells and <1%. The Lumi4–terbium donor was excited at 340 nm, its emission was monitored at 486 nm, and the acceptor FITC was measured at 520 nm. Fluorescence was measured after a 1–4 h incubation for PPAR γ and then in some cases RXR α LBD + 9-*cis*-retinoic acid was added and TR-FRET was measured again after a 4–12 h incubation. There was little change in signal in control wells where nothing was added during this second incubation. Plates were incubated at 4 °C and measured directly after removal from the cold in a room temperature instrument. In some cases, a gradual temperature-dependent reduction in the assay window occurred (for example, NCoR in the presence of GW9662) as the plate warmed during the longer plate readings. Ligand concentrations were varied in the direction of read (along rows and read along rows) when possible to minimize this effect within a ligand titration. Plates were also measured after equilibration at room temperature, which gave the same results. In some experiments, TR-FRET experiments were performed using biotinylated peptides for TRAP220 coactivator (TRAP220-2; residues 625–657; Biotin-KGGTPPPVSSMAGNTKNHPMLMNLKDNPAQDF) and NCoR corepressor (NCoR-3; residues 2,250–2,283; Biotin-KGGFADPASNLGLEDIIRKALMGSEFDDKVEDHG) with a streptavidin-d2 acceptor (CisBio).

Ligand docking. Ligand docking was performed using AutoDock Vina⁶⁶ using standard parameters with the degree of exhaustiveness set to 30. MRL20 was docked into the co-crystal structure of MRL20-PPAR γ LBD (RCSB Protein Data Bank accession code 2Q59) or GW9662-PPAR γ LBD (RCSB Protein Data Bank accession code 3B0R).

Cell-based transactivation and gene expression. 293T cells were batch cotransfected with Gal4-PPAR γ and UAS::luciferase reporter, plated, and after 18 h of incubation were treated with vehicle control (DMSO) or compound for 18 h. Luciferase activity was quantified 18 h after addition of compound with Britelite Plus (Perkin Elmer). For studies involving the covalent antagonist GW9662, before incubation with PPAR γ ligands MRL20, MRL24 and rosiglitazone, cells were first pre-incubated with 5 μM GW9662 or vehicle control for 3 h. Jurkat T cells (ATCC TIB-152) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 units per ml penicillin/streptomycin and plated at 5 $\times$ 10⁵ cells (1 ml final volume). Cells were treated with ligand or vehicle control (DMSO) for 24 h and harvested for RNA isolation. NIH-3T3-L1 cells (provided by A. Chakraborty) were grown in DMEM media containing 10% fetal calf serum and 50 units per ml penicillin and streptomycin (Growth Medium). After 48 h, cells were switched to differentiation medium (Growth Medium with 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine and 877 nM insulin but with 10% fetal bovine serum (FBS) instead of fetal calf serum) containing ligands or vehicle (DMSO) control. After another 48 h, cells were switched to a maintenance medium (Growth Medium with 877 nM insulin, also with FBS) containing ligands or vehicle control, and cells were harvested 3 days after inducing differentiation. Total RNA was isolated from cells using the RNeasy kit (Qiagen). The RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR reactions were performed with Faststart Universal SYBR Green Master (Roche) using an Applied Biosystems 9300 Real-Time PCR instrument with four biological replicates per condition. Relative mRNA expression was determined by the ddC_t method normalized to GAPDH. Statistical analysis was performed using GraphPad Prism via one-way ANOVA with Tukey *post hoc* comparison of all treated conditions (NIH-3T3-L1 analysis) or Bonferroni *post hoc* comparison of selected

conditions (Jurkat dose response analysis) using log transformed data. The sequences of primers used in this study are found in Supplementary Table 2.

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Author contributions

T.S.H. expressed and purified proteins. Y.S., A.-L.B. and T.M.K. synthesized ligands; for full chemical characterization, T.S.H. performed NMR analysis and T.M.K. performed MS. T.S.H., I.M.S.d.V. and D.J.K. performed NMR spectroscopy. D.P.M. performed HDX-MS. T.S.H. and I.M.S.d.V. performed the TR-FRET assay. D.S.K. performed the cell-based transactivation assay. P.K.G. performed the gene expression analysis. D.J.K. performed the ligand docking. T.S.H., P.K.G., I.M.S.d.V., D.P.M., D.S.K., T.P.B., P.R.G. and D.J.K. contributed to data analysis and interpretation. T.S.H. and D.J.K. conceived the experiments and wrote the manuscript.

Additional information

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