Published in final edited form as:

J Med Chem. 2016 November 23; 59(22): 10335–10341. doi:10.1021/acs.jmedchem.6b01340.

Probing the Complex Binding Modes of the PPAR γ Partial Agonist 2-chloro-*N*-(3-chloro-4-((5-chlorobenzo[d]thiazol-2-yl)thio)phenyl)-4-(trifluoromethyl)benzenesulfonamide (T2384) to Orthosteric and Allosteric Sites with NMR Spectroscopy

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Abstract

In a previous study, a co-crystal structure of PPAR γ bound to 2-chloro-*N*-(3-chloro-4-((5-chlorobenzo[d]thiazol-2-yl)thio)phenyl)-4-(trifluoromethyl)benzenesulfonamide (1, T2384) revealed two orthosteric pocket binding modes attributed to a concentration-dependent biochemical activity profile. However, 1 also bound an alternate/allosteric site that could alternatively account for the profile. Here, we show ligand aggregation afflicts the activity profile of 1 in biochemical assays. However, ligand-observed fluorine (^{19}F) and protein-observed NMR confirms 1 binds PPAR γ with two orthosteric binding modes and an allosteric site.

Keywords

peroxisome proliferator-activated receptor gamma; ligand binding; alternate site; allosteric pocket; orthosteric pocket; crystallography; nuclear receptor; drug target; binding mode; Nuclear Magnetic Resonance (NMR) spectroscopy

Members of the human nuclear receptor (NR) transcription factor superfamily are targets for approximately 10% of FDA approved drugs.¹ The thiazolidinedione (TZD) class of peroxisome proliferator-activated receptor gamma (PPAR γ) ligands, which includes the

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Supporting Information

Experimental details for protein expression and purification; luciferase assay; TR-FRET; fluorescence polarization; ligand solubility; NMR spectroscopy; spectral characterization of 1; wild-type and G284I 2D NMR data; and methods references (PDF)
Molecular formula strings (CSV)

Notes

The authors declare no competing financial interests.

FDA approved drugs rosiglitazone, troglitazone, and pioglitazone, are full agonists that hyperactivate PPAR γ . Despite their desired anti-diabetic effects, TZDs and other PPAR γ agonists cause side effects in patients, which has spurred the development of new PPAR γ ligands with a wide spectrum of distinct activation profiles.²

To understand the mechanism of action of PPAR γ -binding drugs and ligands, many ligand-bound co-crystal structures have been determined to detail the molecular contacts that ligands make when they bind to the PPAR γ ligand-binding domain (LBD). The NR LBD is described as an alpha helical sandwich fold containing a canonical, orthosteric ligand-binding pocket located in the internal core of the LBD, which is the binding site for endogenous lipids and synthetic ligands, including FDA-approved drugs.³ However, using solution-based structural methods, including NMR spectroscopy, we recently demonstrated that some synthetic PPAR γ ligands not only bind to the orthosteric pocket but can also bind to an alternate binding site, which is a surface exposed region capped by the conformationally flexible Ω -loop located between helix 2' and helix 3, and affect PPAR γ function.⁴ Ligand binding to this alternate site does not compete with endogenous ligand binding to the orthosteric site indicating it is an allosteric site.

A previous study reported a crystal structure of PPAR γ bound to **1** (T2384; Figure 1A), a PPAR γ partial agonist.^{5, 6} In this structure, PPAR γ crystallized as a homodimer in the crystal lattice, and **1** adopted different binding modes within the orthosteric site of the two PPAR γ molecules. These different orthosteric binding modes were described to have different intrinsic affinities and provide distinct coregulator interaction patterns at low and high ligand concentrations in a biochemical assay. In addition, for one of the PPAR γ molecules in the crystal lattice, a second molecule of **1** also bound weakly, as inferred by weak electron density, outside of the orthosteric site within the allosteric site we identified.⁵ We posited that the binding of **1** with lower affinity to this allosteric site, rather than **1** adopting different conformations in the orthosteric site, could account for the concentration-dependent coregulator selectivity. Herein, we recapitulated and extended the biochemical coregulator interaction assay and also used solution-state NMR spectroscopy to examine the complex binding modes of **1** to PPAR γ in solution.

RESULTS AND DISCUSSION

Compound **1** (Figure 1A) is a conformationally flexible PPAR γ partial agonist capable of adopting different binding modes as observed in a co-crystal structure (Figure 1B).⁵ In this structure, **1** is bound to PPAR γ LBD in the orthosteric site (Figure 1C), which is the same site where natural ligands such as fatty acids bind to PPAR γ . In chain A, **1** adopts a U-shaped orthosteric conformation where the benzothiazole ring is tucked under the β -sheet surface of the pocket through hydrophobic contacts. However, **1** adopts an S-shaped orthosteric conformation in chain B where the benzothiazole ring tucks into a subpocket above the β -sheet surface through hydrophobic contacts (Figure 1D). Furthermore, in chain B a second molecule of **1** is bound to PPAR γ in an alternate/allosteric site (Figure 1D). Here, the central chlorophenyl ring of **1** packs between helix 3 and the β -sheet surface, and the benzothiazole ring points towards the flexible Ω -loop making hydrophobic contacts with helix 2' and 3.

The U-shaped orthosteric binding mode of **1** in chain A is in a conformation that would clash with **1** bound to the allosteric site. This suggests the U-shaped conformation may have blocked a second molecule from binding to chain A in the crystal structure, and the S-shaped conformation may allow binding of a second molecule to the allosteric site. A mutant protein containing three mutations in the PPAR γ orthosteric site (L228W, A295W, L333W) rotated the binding orientation of **1** to the U-shaped conformation and blocked the lower affinity biochemical response.⁵ Although the crystal structure of this mutant protein is shown in the previous report,⁵ the coordinates were not deposited in the Protein DataBank (PDB). A G284I mutant protein was also created to disrupt **1** binding in the U-shaped orthosteric binding mode.⁵ In a biochemical assay, the G284I mutant only showed the low affinity response while also negatively affecting the ability of rosiglitazone to displace the NCoR peptide. A crystal structure of **1** bound to the G284I mutant, also not deposited in the PDB, revealed only the S-shaped orthosteric binding mode, indicating the mutation may block the U-shaped orthosteric binding mode. Notably, in this study the bimodal response for PPAR γ was posited to originate from rotation between the U-shaped and S-shaped orthosteric binding modes without considering the second molecule bound to the allosteric site.

To determine if the origin of the bimodal functional response is due to **1** binding to the orthosteric site at high affinity and the allosteric site at lower affinity, we used a similar biochemical time-resolved fluorescence resonance energy transfer (TR-FRET assay) to that used in the previous study,⁵ as well as ligand- and protein-observed nuclear magnetic resonance (NMR) spectroscopy to structurally explore these complex binding modes in solution.

We synthesized **1** following published protocols⁶ and using a cell-based transcription assay (Figure 2) confirmed its activity as a PPAR γ partial agonist relative to the agonist (*S*)-2-(2-((1-(4-methoxybenzoyl)-2-methyl-5-(trifluoromethoxy)-1*H*-indol-3-yl)methyl)phenoxy)propanoic acid (**2**, MRL20) that we studied previously that also binds to the allosteric site.^{4, 7} 2-chloro-5-nitro-*N*-phenylbenzamide (**3**, GW9662) is a commercially available PPAR γ antagonist that binds to the orthosteric site of PPAR γ through a covalent thioether bond with Cys285,⁸ the only cysteine residue present in the PPAR γ LBD. We previously showed that **3** blocks the binding of many PPAR γ ligands, including **2**, to the orthosteric site but not to the allosteric site.^{4, 9} Structural overlay of PPAR γ LBD crystal structures bound to **1** or **3** (Figure 3) indicates **3** would likely block binding of **1** to the orthosteric site in the U-shaped or S-shaped conformation but not the allosteric site. We therefore used **3** as a chemical tool to block orthosteric binding of **1** in the biochemical TR-FRET assay and NMR experiments.

We used a TR-FRET biochemical assay similar to the previously reported HTRF assay⁵ to study how **1** affects the interaction between PPAR γ LBD and a peptide derived from the NCoR corepressor protein. We observed a biphasic functional response profile of **1** in binding to PPAR γ (Figure 4A) in the absence or presence of increasing amounts of bovine serum albumin (BSA), a carrier protein that binds small molecule ligands that is frequently used in biochemical assays, similar to the previously reported HTRF assay using 0.01% BSA.⁵ **1** causes a concentration-dependent decrease in signal at lower concentrations,

indicating decreased binding of NCoR to PPAR γ . At higher concentrations an increase in signal is observed, indicating increased binding of NCoR. Notably, however, when increasing the concentration of the non-ionic detergent Tween 20 instead of using BSA, signal variability and a muting of the lower and higher concentration responses was observed (Figure 4B). These experimental phenomena are suggestive of ligand aggregation^{10, 11} and indicate that the solubility of **1** plays a role in these response curves. Indeed, using an HPLC-based UV detection assay, the solubility limit of **1** in assay buffer was determined to be ~2-4 μ M. In the presence of Tween 20, the solubility of **1** was more variable but did not significantly improve. Furthermore, when the TR-FRET assay is performed with protein pretreated with **3** to block the orthosteric site, in the presence of BSA we observed a decrease in signal indicating that binding of **1** to the allosteric site displaces the NCoR peptide (Figure 4C), similar to what occurs for **2**.⁴ However, both PPAR γ (Figure 4A) and **3**-bound PPAR γ (Figure 4C) show an increase in TR-FRET at **1** concentrations >5-10 μ M, likely due to compound aggregation.

We also performed TR-FRET with the PPAR γ G284I mutant LBD and observed only the lower affinity response (Figure 4D) as previously reported.⁵ The initial TR-FRET ratio is lower than wild-type protein (Figure 4A), suggesting the G284I mutation decreases the affinity for the NCoR peptide. We verified this using a fluorescence polarization assay (Figure 4E), which revealed the G284I mutation weakens the NCoR binding affinity (K_d = 3.1 μ M) relative to wild-type protein (1.4 μ M). Thus, in addition to potentially blocking U-shaped binding mode of **1**, the absence of a high affinity response for the G284I mutant is due to the decreased affinity for NCoR and relatively low concentration of NCoR peptide used in the assay (200 nM in the previous study,⁵ 400 nM in Figure 4D). Indeed, using a higher NCoR peptide concentration (800 nM) we observed a concentration-dependent bimodal response for the G284I mutant similar to what was observed for wild-type protein. As discussed above, the increase in TR-FRET signal at concentrations of **1** higher than 5-10 μ M is likely due to compound aggregation as the same increase is observed for wild-type (with or without **3**) and the G284I mutant. Thus, the G284I mutant weakens the NCoR peptide binding affinity but does not prevent **1** from displacing the peptide.

The TR-FRET biochemical assay uses a protein concentration (4 nM) much lower than the affinity of **1**, and when the concentration of **1** is high enough to bind PPAR γ compound aggregation afflicts the results. The presence of BSA in the assay buffer accentuates the lower concentration response, but a similar increase in TR-FRET ratio at high concentrations of **1** is still observed. In contrast to the low nanomolar protein concentrations used in TR-FRET biochemical assays, solution NMR spectroscopy requires much higher protein concentrations (mid-to-high micromolar). Detection of **1** binding by NMR is therefore more straightforward because when the protein is at high concentration, **1** will bind when added at sub-stoichiometric amounts and is less prone to issues with compound aggregation that are problematic in the TR-FRET biochemical assay where ligand concentrations greatly exceed the protein concentration. We therefore studied binding of **1** to PPAR γ LBD and the G284I mutant using solution NMR.

Taking advantage of the trifluoromethyl group in **1** (Figure 1A), we performed a ligand-observed ¹⁹F NMR titration into PPAR γ LBD. Titration of **1** up to one equivalent resulted in

the appearance of two NMR peaks (Figure 5A, green dashed lines). Titration of a second equivalent of **1** resulted in the appearance of a third intense NMR peak (Figure 5A, blue dashed line). In the presence of **3** to block the orthosteric site, a peak corresponding to PPAR γ bound to **1** is observed (Figure 5A, orange dashed line) that is shifted upfield from the two orthosteric peaks but closer to the third “allosteric” peak that appears when two equivalents of **1** is added in the absence of **3** (Figure 5A, orange dashed line). A minor peak is also observed that could correspond to another allosteric binding mode or site; notably, this peak has a distinct chemical shift from the orthosteric-bound chemical shifts observed at one equivalent of **1**. When two equivalents of **1** is added to **3**-bound PPAR γ , a broadening of the spectrum is observed likely due to a component of the signal resulting from chemical exchange between the allosteric site bound form of **1** and free **1** that may be aggregated in solution. The broad peak could also correspond to exchange between other allosteric binding modes or different allosteric sites.

We also performed the ligand-observed ^{19}F NMR titration into PPAR γ G284I LBD. Similar to wild-type PPAR γ , two NMR peaks are observed at one equivalent of **1** (Figure 5B, green dashed lines) and a third peak is populated at two equivalents (Figure 5B, blue dashed line). In the presence of **3** to block the orthosteric site, we again observed an upfield shifted NMR peak near the third peak that appears when two equivalents of **1** are added in the absence of **3** (Figure 5A, orange dashed line). Comparison of ^{19}F chemical shift values of **1** bound to PPAR γ LBD and the G284I mutant (Figure 5A,B) reveals a large change in the allosteric site chemical shifts in the absence or presence of **3**, but not the orthosteric site chemical shifts populated at one equivalent of **1**. This is consistent with the co-crystal structure of PPAR γ LBD bound to **1** because the G284I mutation (Figure 1, black arrow) is situated closest to **1** bound to the allosteric site (Figure 1, red circles).

Taken together, these data indicate **1** can bind to both wild-type PPAR γ LBD and the G284I mutant, adopt two orthosteric binding modes (populated at one equivalent), and bind to the allosteric site (at two equivalents, or upon binding to **3**-bound PPAR γ LBD). Notably, the previously reported G284I mutant LBD crystal structure⁵ showed only the S-shaped orthosteric conformation of **1**, possibly because this was a thermodynamically stable conformation with favorable crystallization properties. However, our NMR data reveal that both orthosteric and allosteric binding modes are accessible in solution.

To structurally map the locations affected by the two binding events of **1** in solution, we performed a protein-observed NMR titration using 2D [^1H , ^{15}N]-TROSY-HSQC NMR experiments. Titration of **1** into ^{15}N -labeled PPAR γ LBD up to one equivalent resulted in chemical shift perturbations (Figure 6A) consistent with other orthosteric site-binding PPAR γ ligands.^{4, 7} Titration of **1** beyond one equivalent resulted in additional changes (Figure 6B) consistent with ligand binding to the allosteric site.⁴ For example, residues in the β -strand region, a boundary between the orthosteric and allosteric sites, show two transitions that saturate with one or two equivalents of **1** (Figure 6C,D; Gly344 and Gly346). Similar transitions are observed for other residues in this region, including Val248 and Gly258 (Figure 6C,D). Two transitions are also observed for residues near the activation function-2 (AF-2) coregulator-binding surface (Figure 6C,D; Gly399 and Val307). Furthermore, analysis of NMR line shapes indicate some residues exchange between

multiple conformations at one equivalent of **1**, which are further affected at two equivalents of **1** (Figure 7). That is, some peaks appear as doublets at one and two equivalents of **1**, and others show more complex line shapes, supporting the concept that **1** can bind in two orthosteric binding modes resulting in multiple **1** bound conformations at one equivalent of **1** in particular.

We also performed 2D [^1H , ^{15}N]-TROSY-HSQC NMR experiments in the presence of **3** to block the orthosteric site. **1** binding to ^{15}N -labeled **3**-bound PPAR γ LBD causes a number of NMR chemical shift perturbations (Figure 8A,B) that are similar to what was observed when ligands bind to the allosteric site of PPAR γ .⁴ This includes residues proximal to **1** bound to the allosteric site in the co-crystal structure, the LBD core, and distal from the allosteric site near the AF-2 coregulator-binding surface.

Finally, we performed 2D [^1H , ^{15}N]-TROSY-HSQC NMR experiments of **1** binding to ^{15}N -labeled PPAR γ G284I LBD without (Figure 9A,B) or with **3** (Figure 9C). The G284I mutation stabilizes intermediate exchange time scale dynamics for some residues relative to wild-type apo-protein and thus alters the LBD conformation relative to wild-type protein, but importantly for spectral analysis peaks on the spectral periphery are conserved (Figure S1). Consistent with the ligand-observed ^{19}F NMR data, **1** added at one and two equivalents shows two binding events to the G284I mutant, as well as binding in the presence of **3**. Similar to experiments on wild-type protein, when the G284I mutant is bound to one equivalent of **1** some of the NMR peaks show multiple populations that are distinct from the apo-protein spectra. Taken together, these data further indicate the G284I mutant used for crystallography⁵ may adopt a thermodynamically stable conformation that prefers the S-shaped **1** orthosteric binding mode, but in solution **1** binds to the G284I mutant at orthosteric (one equivalent of **1**) and allosteric (two equivalents of **1**) sites with two apparent orthosteric binding modes (i.e., two ^{19}F NMR peaks are observed).

CONCLUSIONS

Here, we used a TR-FRET biochemical assay and solution-state NMR spectroscopy to study the complex binding of **1** to PPAR γ . Our studies using BSA and Tween 20 in the biochemical assay with protein concentrations much lower than the binding affinity of **1** indicate these data, and particularly the bimodal response at high ligand concentrations, are variable and likely afflicted by compound aggregation. However, our NMR data using higher protein concentrations support the complex crystallized binding modes of **1**. Our ^{19}F NMR data show that **1** can adopt two different orthosteric conformations (two NMR peaks at one equivalent of **1**) corresponding to high affinity of **1** binding to PPAR γ , consistent with the U- and S-shaped crystallized conformations. Furthermore, a third ^{19}F NMR peak is populated at two equivalents of **1**, supporting an allosteric binding event with lower affinity than the orthosteric binding event. Using the covalent orthosteric antagonist **3** as a chemical tool to block the orthosteric site, we further confirmed that **1** binds to the allosteric site of PPAR γ . Notably, the G284I mutation did not block the two peaks populated by the orthosteric binding event at one equivalent of **1** or the third peak populated at two equivalents of **1**. This indicates that the crystallization conditions likely selected only the S-shaped conformation when bound to G284I mutant,⁵ but in solution both of the orthosteric

peaks observed in the wild-type ^{19}F NMR data at one equivalent of **1** are observed in the G284I mutant.

This work shows the utility of solution NMR structural analysis to validate complex ligand binding modes with target proteins, in particular in cases where compound aggregation afflicts biochemical results. Furthermore, NMR studies may be of interest in targeting ligands to bind alternate or allosteric sites in NRs, for which there is a growing appreciation.¹²⁻²⁰

EXPERIMENTAL SECTION

Chemical Synthesis

Compound **2** was previously synthesized following published methods⁹ and used previously by our groups.^{4, 7} Compound **1** was synthesized following published methods.⁶ Compound identity and purity was determined to be >95%) as verified by HPLC, ^1H NMR, and high resolution ESI-MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We thank Prof. Ben Shen for access to a high-resolution mass spectrometer. This work was supported by NIH/NIDDK awards R01DK101871 (DK), F32DK097890 (TH), K99DK103116 (TH), R00DK103116 (TH), and F32DK108442 (RB); and AHA awards 12POST12050025 (TH) and 16POST27780018 (RB).

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Figure 1. Compound **1** (A) bound to PPAR γ LBD (PDB ID 3K8S) adopts multiple binding modes (B) in orthosteric and allosteric sites (C,D). U- and S-shaped orthosteric conformations are orange and cyan, respectively; **1** bound to an allosteric site is magenta. A red oval denotes the allosteric site; a black arrow denotes the G284I mutation (modeled using PyMOL).

Figure 2. Transcriptional activity of **1** and **2**. Gal4-PPAR γ LBD/5xUAS-*luciferase* luciferase reporter gene assay in HEK293T cells. Data represent the mean $\pm$ s.e.m (n=4).

Figure 3. Structural overlay PPAR γ LBD crystal structures bound to the orthosteric antagonist **3** (PDB ID 3B0R) or **1** (PDB ID 3K8S). (A) Chemical structure of **3**. (B,C) PPAR γ LBD co-crystal structure bound to **1** (**1** shown as in Figure 1) superimposed with the PPAR γ LBD bound to **3** (**3** shown as blue/red spheres).

Figure 4.

TR-FRET biochemical assay data (A-D) performed with (A,C,D) increasing concentrations of BSA with (A) PPAR γ LBD, (C) 3-bound PPAR γ LBD, and (D) PPAR γ G284I LBD; or (B) PPAR γ LBD with increasing concentrations of Tween 20. (E) Fluorescence polarization peptide interaction assay to determine NCoR peptide binding affinity. (F) TR-FRET assay with PPAR γ G284I LBD at higher NCoR peptide concentration (800 nM vs. 400 nM in panels A-D). Data represent the mean $\pm$ s.e.m (n=2).

Figure 5.

^{19}F NMR titration of **1** into (A) PPAR γ LBD and (B) PPAR γ G284I LBD (green, up to 1 equivalent (eq); blue, up to 2 eq; orange, pre-bound to **3**). Peaks populated at 1 and 2 eq of **1**, in the presence of **3**, or free in buffer (marked with #) are noted with green, blue, orange, or grey vertical dotted lines, respectively; a minor peak (marked with *) in the **3** + 1 eq of **1** (A) is also observed.

Figure 6. 2D ^1H , ^{15}N -TROSY-HSQC NMR data of ^{15}N -labeled PPAR γ LBD (A) ± 1 eq of **1**; or (B) 1 or 2 eq of **1**. (C) Zoomed in regions at the indicated molar equivalents; orange and blue arrows denote changes. (D) Residues labeled in (A-C) plotted on the PPAR γ LBD co-crystal structure bound to **1** (PDB ID 3K8S); chain A and B are structurally superimposed with all ligands shown.

Figure 7. Multiple peak populations observed in the 2D ^1H , ^{15}N -TROSY-HSQC NMR data with (A) 1 eq or (B) 2 eq of **1**. Brackets denote multiple peak populations, and orange arrows denote transition between one and two equivalents.

Figure 8.

2D [^1H , ^{15}N]-TROSY-HSQC NMR data (A) of ^{15}N -labeled **3**-bound PPAR γ LBD $\pm$ 2 eq of **1**. (B) Residues (green spheres) labeled in (A) plotted on the PPAR γ LBD co-crystal structure bound to **1** (PDB ID 3K8S; chain B cartoon, allosteric site **1** as sticks) superimposed with PPAR γ LBD structure bound to **3** (PDB ID 3B0R; **3** is shown as blue/red spheres).

Figure 9.
2D [^1H , ^{15}N]-TROSY-HSQC NMR data of ^{15}N -labeled PPAR γ G284I LBD (A) $\pm$ 1 eq of **1**; (B) with 1 or 2 eq of **1**; and (C) bound to **3** $\pm$ 2 eq of **1**.