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Indazole MRL-871 interacts with PPARγ via a binding mode that induces partial agonism

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ABSTRACT

The nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) plays a central role in metabolic processes. PPARγ full agonists have side effects, arguing for the discovery of PPARγ partial agonists with novel chemotypes. We report the unique binding mode of the known allosteric retinoic acid receptor-related orphan receptor gamma t (RORγt) ligand MRL-871 to PPARγ. MRL-871 binds between PPARγ helices 3, 5, 7 and 11, where it stabilizes the beta-sheet region with a hydrogen bond between its carboxylic acid moiety and PPARγ Ser370. Its unique binding mode differs from that of the benzoyl 2-methyl indoles which are well-studied, structurally similar, PPARγ ligands. MRL-871's high affinity for PPARγ induces only limited coactivator stabilization, highlighting its attractive partial agonistic characteristics. Affinity comparison of MRL-871 and related compounds towards both RORγt and PPARγ indicates the possibility for tuning of selectivity, bringing MRL-871 forward as an interesting starting point for novel PPARγ ligands.

Introduction

The peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear receptor (NR) superfamily and plays a major role in metabolic processes including adipogenesis, lipid metabolism and insulin sensitivity.[1] This makes PPARγ an interesting drug target for example for type 2 diabetes. Synthetic PPARγ agonists of the thiazolidinedione (TZD) class[2] have been used for their insulin-sensitizing effects in the clinic since the 1990s. However, these TZDs come with side effects mainly ascribed to their full agonistic nature, which could potentially affect the transcription of hundreds of genes.[3] As such, there is a need for conceptually novel PPARγ targeting drugs, potentially with a mode of action shifted towards partial agonism or selective PPARγ modulation that can uncouple insulin sensitizing actions from adverse effects.[1,4–7]

Bruning et al. discovered, through hydrogen/deuterium exchange experiments, that while full agonists stabilize helix 12, which directly leads to stabilization of the coactivator binding surface, certain partial agonists can instead stabilize the β-sheet region of the PPARγ LBD.[4] Choi et al. revealed that increased PPARγ phosphorylation at serine 273 (Ser245 in PPARγ1), which is adjacent to the β-sheet region, leads to dysregulation of genes involved in insulin sensitivity.[8] Molecules that do not display classical PPARγ agonism and stabilize the β-sheet, which "freezes" that region in a configuration less optimal for Cdk5 phosphorylation, have antidiabetic effects in obese mice.[8–9] These observations have directed the search towards PPARγ ligands with minimal coactivator recruitment activity, but tight interactions with the β-sheet region. A highly relevant feature of the PPARγ LBD in this respect is the presence of a so-called "alternate" binding site.[10] This site, first described by Hughes et al., partially overlaps with one of the arms of the orthosteric binding site, but extends towards a solvent exposed pocket formed by the Ω-loop (Figure 1A).[10] Typically, this alternate pocket can get occupied after ligand binding to the orthosteric pocket, either by the same compound, a different (endogenous) ligand or a covalent antagonist.[10–11]

The PPARγ LBD is relatively permissive towards ligands of diverse chemical structures, albeit typically with lower affinities.[5,12–13] As such, we were intrigued by an off-target PPARγ activity in a class of indazole-compounds that were discovered as novel allosteric modulators for the retinoic acid receptor related orphan receptor (ROR)γt.[14–15] This activity was found using cell-based NR reporter assays and PPARα and PPARβ/δ were not affected.[15] Notwithstanding certain structural similarities with benzoyl 2-methyl indole PPARγ ligands such as MRL20,[16]

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the significant molecular differences with archetypical PPARγ ligands brought forward the potential of a unique PPARγ binding mode for these indazoles. Here, we elucidate the binding mode of the prototypical indazole-compound MRL-871 to PPARγ using a combination of X-ray crystallography and biochemical assays. MRL-871 addresses a unique set of interactions in the PPARγ ligand binding pocket, resulting in a PPARγ binding affinity stronger than the TZD rosiglitazone, but with a much weaker agonistic effect on coactivator recruitment.

Protein X-ray crystallography was utilized to obtain the co-crystal structure of PPARγ in complex with MRL-871. The crystal structure (Table S1, PDB: 6TDC) shows the complete LBD of PPARγ, with MRL-871 binding between helices 3, 5, 7, and 11 and the beta sheet region (Figure 1A, S1). MRL-871 is wrapped around helix 3, stabilized by multiple hydrophobic interactions with helix 3 and helix 7. Its benzoic acid moiety is directed towards the β-sheet region, while the bis-*ortho*-substituted phenyl ring is pointing in the opposite direction, mostly interacting with helix 3, but also with helices 5 and 7. In addition to these hydrophobic contacts, there are also relevant polar interactions. Most prominently, the carboxylic acid of MRL-871 partakes in a hydrogen bond with the backbone amide of Ser370 (Figure 1C). Proximity of ligands to Ser370 is known to correlate with stabilization of the beta sheet region and the helix 2-helix 2′ loop.[8] Such a binding mode, where the ligand is not extending towards and interacting with helices 11 and 12, also called branch I, is usually associated with partial agonistic behavior (Figure S2).[6] Comparison of the binding modes of MRL-871 and full agonist rosiglitazone (Figure 1D), reveals this clear difference in the degree of protrusion towards helix 12. By extending in that direction, rosiglitazone stabilizes helix 12 favoring coactivator binding.[17] In contrast, MRL-871 is not interfacing with helix 12. Helix 12 is as such not stabilized in the active agonistic position, but points away from its own LBD to interact with the cofactor binding groove of a symmetry mate PPARγ (Figure S3). In solution the overall conformation of helix 12 is likely to be flexible, resulting in partial agonism (vide infra).[18] Figure 1E shows an overlay between the binding positions of MRL-871 and MRL20 (PDB 2Q59).[4] Despite structural similarities of these compounds (Figure 1B), their binding modes are profoundly distinct. MRL-871 is generally directed more towards helix 7, while MRL20 is extended alongside helix 3 and protrudes further into PPARγ branch I. Another interesting feature of the binding mode of MRL-871 is the protrusion of its indazole towards PPARγ helix 7. Upon overlaying all 252 currently available PPARγ crystal structures, it can be seen that the protrusion at this location is further than any other compound seen

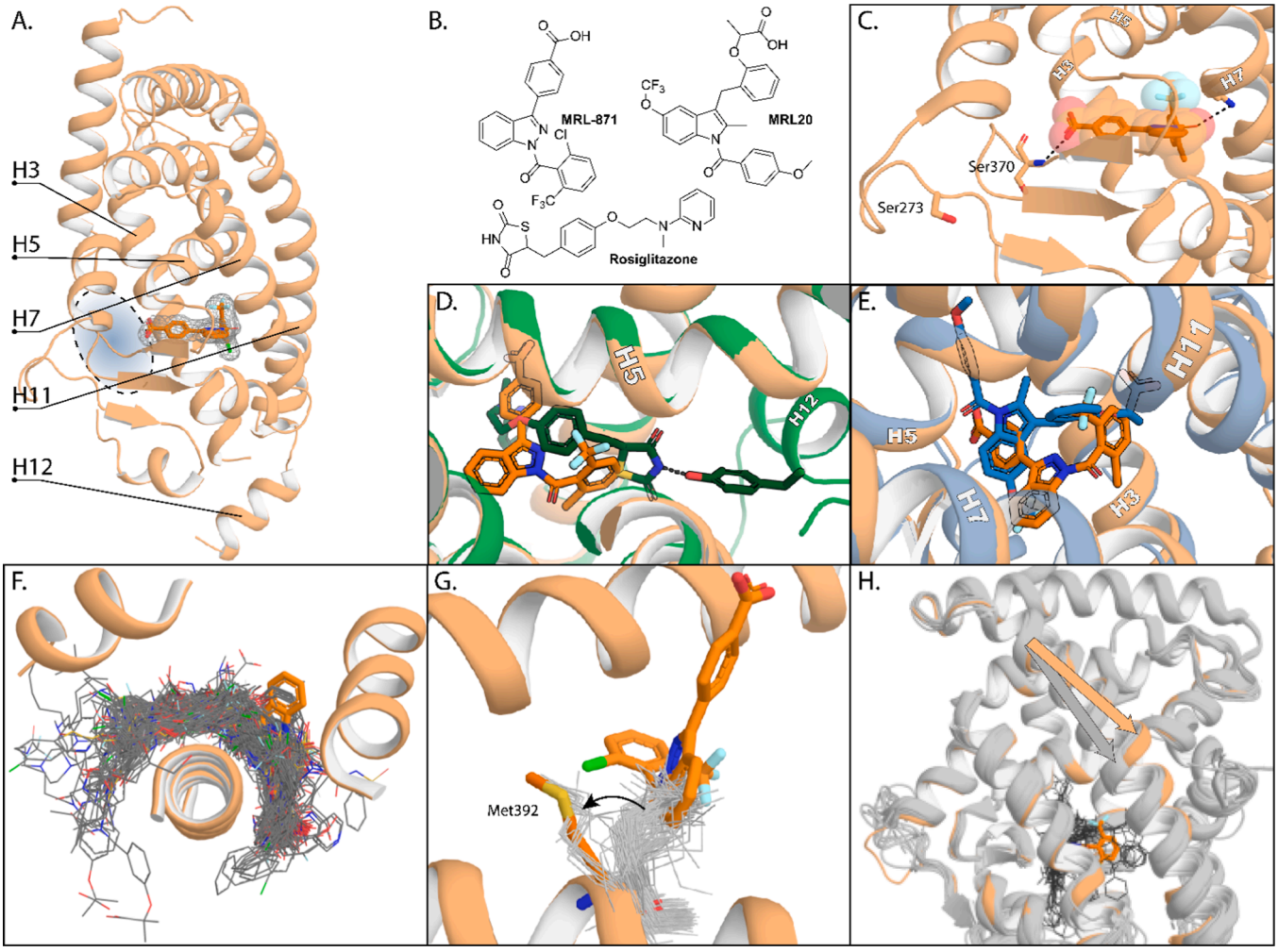


Figure 1. Co-crystal structure of PPARγ (light orange) with MRL-871 (dark orange) (PDB: 6TDC). (A) Overview of the binding location of MRL-871 in the full LBD of PPARγ. The electron density map around the compound is shown as an isomesh. The blue region with the dotted oval indicates the alternate site. (B) Molecular structures of MRL-871, Rosiglitazone and MRL20 (C) Close-up of the binding position. Hydrogen bonds are indicated as black dotted lines. (D) Overlay of the binding positions of MRL-871 (orange) and rosiglitazone (green, PDB: 2PRG). (E) Overlay of the binding positions of MRL-871 (orange) and MRL20 (blue, PDB: 2Q59). (F) Overlay of the ligand binding pocket of all PPARγ structures, MRL-871 is shown in orange. (G) Position of the Met392 residue. (H) Comparison of the direction of helix 10–11 between MRL-871 and other partial agonists (PDB: 2FVJ, 2G0H, 2I4Z, 2P4Y, 2POB, 2Q5P, 2Q6S, 3B1M, 4F9M, 3FUR, 3OSW, 3R8A, 3S9S, 3VSO, 4A4W, 4PRG).

so far (Figure 1F). The only compound class that extends somewhat in this direction, 1.0 Å less than MRL-871, are the cercosporamides.[19–20] As a result of this protrusion, Met392 on helix 7 has to flip to the other side compared to its direction in most other structures (Figure 1G). Furthermore, there seems to be a correlation between the distance of the compound to helix 7 and the course of the antiparallel helix 10–11. For both the cercosporamides and MRL-871 this helix lies at a slightly different angle compared to other compounds which might contribute to the partial agonist nature of these compounds (Figure 1H).

Biochemical studies were conducted to determine the functional implications of the interaction between MRL-871 and PPARγ. Rosiglitazone (Figure 1B) was used as a reference compound because of its well-studied agonistic binding mode. First, a thermal stabilization analysis was performed.[21–23] The ligand-induced stabilization was analyzed using differential scanning fluorimetry (DSF) for which the PPARγ LBD was incubated in the presence and absence of MRL-871 or rosiglitazone (Figure 2A).[24–25] Without compound, the PPARγ LBD had a melting temperature of 47.5 ± 0.7 °C. Four equivalents of rosiglitazone (14 μM) increased the melting temperature to 48.8 ± 1.0 °C (1.3 °C increase, P = 0.01), while the same amount of MRL-871 increased the melting temperature to 50.2 ± 0.7 °C (2.7 °C increase, P < 0.0001). The binding of MRL-871 thus has a significant stabilizing effect on PPARγ, stronger than the established drug compound. Of note; these data also reflect that MRL-871 has a strong affinity to PPARγ in the absence of coactivator.

A compound concentration dependent heat challenge assay was performed to obtain dissociation constants (K_D) for the ligand PPARγ interaction. After the heat challenge folded and denatured protein were separated by means of centrifugation and the remaining soluble protein in the supernatant was quantified using Bradford reagent (Figure 2B).[26] Using the calculations set up by Bai et al. dissociation constants were determined.[27] After a 3 min heat challenge of 50.5 °C, 71 ± 3 % of the apo PPARγ LBD was aggregated. The addition of either rosiglitazone or MRL-871 protected the protein from unfolding. Rosiglitazone featured a K_D of 3.3 ± 1.2 μM and MRL-871 had a ten-fold lower K_D value of 250 ± 110 nM. This low K_D testifies to the potential of MRL-871 as a high affinity PPARγ ligand.[28] The weaker affinity of rosiglitazone in this assay reflects the strong preference of rosiglitazone to bind PPARγ in the presence of coactivators (vide infra).

The agonistic activity of both compounds towards stabilizing the protein–protein interaction between PPARγ and coactivators was assessed using a fluorescence anisotropy (FA) assay. Here, PPARγ LBD was titrated to a fixed concentration of FITC-labeled peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) peptide (Figure 2C). In absence of compound, the peptide bound to PPARγ with a K_D of 1.6 ± 0.1 μM. In the presence of rosiglitazone (10 μM) this K_D between peptide and protein decreased to 370 ± 30 nM; a 4.4-fold increase of the PPARγ-PGC-1α affinity, indicating agonistic behavior. MRL-871 lowered the K_D to 710 ± 50 nM, representing a weaker 2.3-fold increase in affinity. Since rosiglitazone is classified as a full agonist, this indicates that MRL-871 acts as a partial agonist on this PPARγ–PGC-1α interaction.

Ligand potency towards the PPARγ-PGC-1α interaction was further assessed using a homogeneous time resolved fluorescence resonance

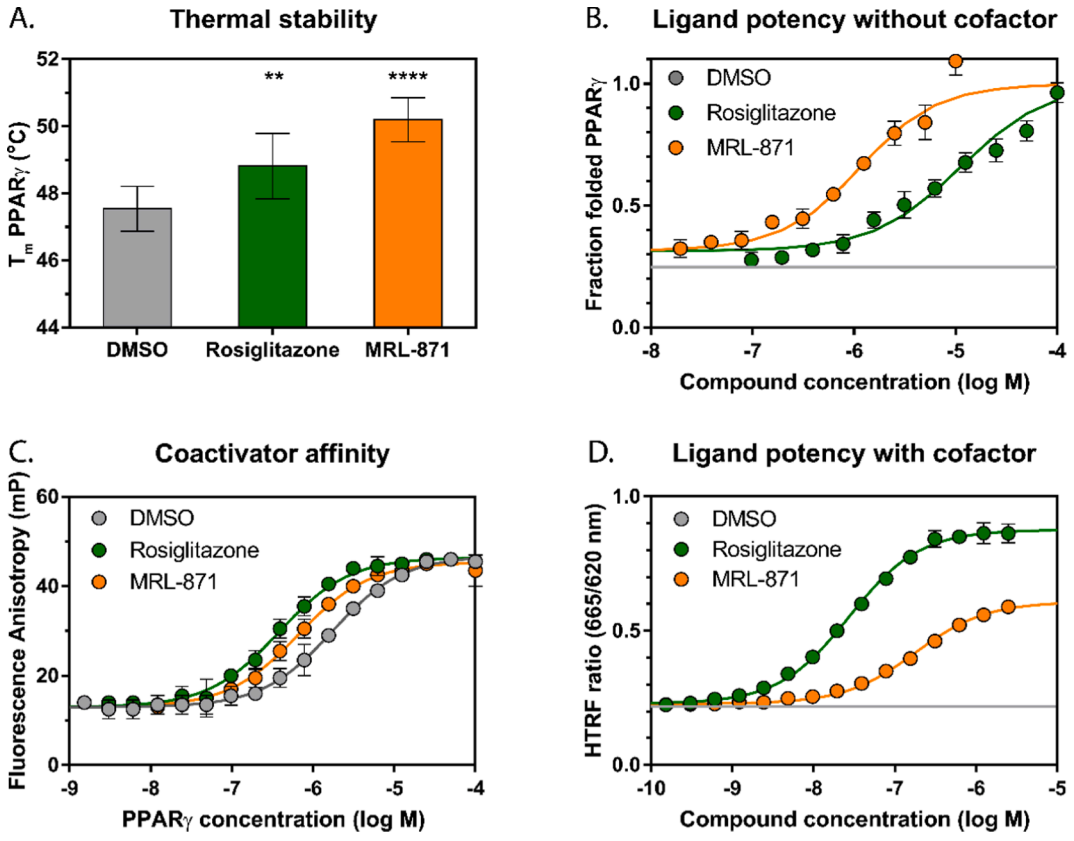


Figure 2. MRL-871 interacts with PPARγ in biochemical assays. (A) Differential scanning fluorimetry assay of PPARγ LBD (3.5 μM) melting temperatures in absence and presence of rosiglitazone or MRL-871 (14 μM) ($n \geq 3$; mean ± s.d; significances between with and without compound assessed using unpaired *t*-test). (B) PPARγ LBD (600 nM) thermal stability assay using Bradford readout after a 3-minute heat-challenge at 50.5 °C ($n = 3$, representative data shown as mean ± s.d. of one experiment in duplicate). (C) Fluorescence anisotropy coactivator interaction assay between PPARγ LBD and fluorophore labelled PGC-1α peptide (10 nM) in absence (DMSO) or presence of rosiglitazone (10 μM) or MRL-871 (10 μM) ($n = 3$; representative data shown as mean ± s.d. of one experiment in triplicate). (D) HTRF coactivator recruitment of PPARγ LBD (10 nM) and PGC-1α peptide (200 nM) with ligand titration ($n = 3$, representative data shown as mean ± s.d. of one experiment in triplicate).

energy transfer (HTRF) assay, which allows compound evaluation in a more befitting concentration window than FA (Figure 2D). Rosiglitazone featured an EC_{50} for PPARγ-PGC-1α stabilization of 26 ± 6 nM, while the EC_{50} of MRL-871 was 170 ± 10 nM. Importantly, and further testifying to a partial agonistic character of MRL-871 on the PPARγ-PGC-1α interaction, MRL-871 induced only a partial increase in the HTRF ratio. Thus while the MRL-871–PPARγ binding is strong, it does not very effectively induce a conformation of PPARγ to bind PGC-1α.

MRL-871 and rosiglitazone bind partly to similar parts of the PPARγ binding pocket (Figure 1D). The competitiveness of these binding modes was evaluated using a competition HTRF assay. MRL-871 was titrated to PPARγ in presence of fixed concentrations of rosiglitazone (Figure 3). An increased concentration of rosiglitazone shifted the EC_{50} values of MRL-871 to higher concentrations. Applying the Cheng-Prusoff equation, which corrects for the amount and affinity of rosiglitazone, showed that the calculated "inhibition constant" (Ki) of MRL-871 remained constant and in the same range as observed for the cofactor free potency assay.[29] These results thus confirm that the two ligands cannot bind simultaneously to PPARγ.

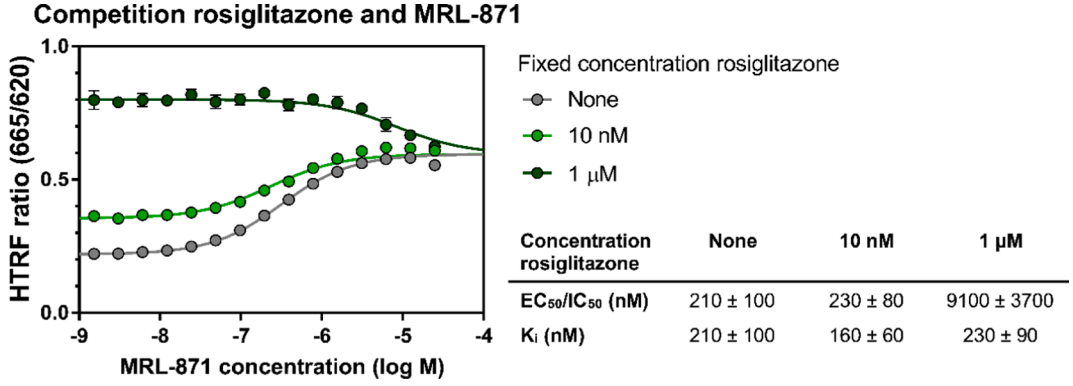
Certain ligands have been reported to be able to bind to the alternate site (Figure 1A) within the large PPARγ binding pocket even when the orthosteric binding site is occupied.[10–11,30] To further explore the behavior of MRL-871 in this matter, two tool compounds were covalently fused to the cysteine in the PPARγ ligand binding pocket (Figure S4). GW9662 is known to block the orthosteric pocket of PPARγ, but leave enough room for compounds to bind to the alternate site.[10,31] SR16832 is bigger and specifically designed to simultaneously block both sites.[32] DSF and HTRF were used to quantify binding of MRL-871 to the covalently blocked PPARγ LBDs. In DSF (Figure 4A) the covalent blockers themselves lead to a thermal stabilization of 3.9 °C ($P < 0.0001$) for GW9662 and 4.4 °C for SR16832 ($P < 0.0001$). Addition of MRL-871 to the GW9662 modified PPARγ still caused an increase in PPARγ stability of 1.1 °C ($P = 0.03$). When the LBD is covalently linked to SR16832, no significant change in thermal stability was observed (-0.6 °C, $P = 0.2$) upon addition of MRL-871. In contrast, Rosiglitazone was not able to increase the thermal stability of PPARγ linked to GW9662 (0.0 °C, $P = 0.9$) nor to SR16832 (-0.1 °C, $P = 0.6$). Combined, this confirms that rosiglitazone only binds to the orthosteric binding site while MRL-871 can function by addressing a site distinct from the orthosteric site. This is further confirmed by HTRF experiments. PPARγ covalently ligated to GW9662 is hardly responsive to rosiglitazone anymore; rosiglitazone's EC_{50} decreases to 44 ± 35 μM, a more than 1800-fold loss (Figure 4B). Any residual binding might be caused by small amounts of unlinked PPARγ (Figure S4). In contrast, MRL-871 still has an EC_{50} of 1.3 ± 0.2 μM for PPARγ covalently ligated to GW9662, only an 8.1-fold decrease compared to apo PPARγ. SR16832 again inhibits the binding of both compounds (Figure 4C). Together these data show that even when the orthosteric pocket is blocked, MRL-871 can bind to PPARγ. This distinct position is potentially, in part, generated by the GW9662 ligation, but provides entry points for chemical exploration.

In recent years, next to MRL-871, several other allosteric RORγt inverse agonists, with differing central scaffold structure, were developed. As a starting point to look into PPARγ vs RORγt selectivity of such compounds, we compared three allosteric RORγt inverse agonists (Figure 5).[33–34] In HTRF, the Glenmark compound 13 (CPD13) has a relatively similar EC_{50} as MRL-871 (300 ± 10 nM respectively 200 ± 80 nM) for PPARγ, while the EC_{50} of FM26 for PPARγ is significantly weaker (5.6 ± 0.8 μM) (Figure 5A). MRL-871 is also the most potent compound on RORγt, (4.7 ± 2.2 nM), but FM26 binds here a bit stronger than CPD13 (330 ± 100 nM respectively 620 ± 100 nM) (Figure 5B). In DSF, a similar pattern can be observed. MRL-871 and especially CPD13 efficiently stabilize PPARγ, while FM26 is not able to do so at the used concentration (Figure 5C). For RORγt, the increase in stability by MRL-871 is significantly larger than the increases caused by CPD13 and FM26. This differentiated binding profile for PPARγ and RORγt indicates that of these three compounds, CPD13 appears to be the most nonselective. FM26 in contrast is most RORγt selective, while MRL-871 is the most potent compound on both NRs. These data thus indicate that, while administering unaltered MRL-871 would probably be problematic when targeting PPARγ due to RORγt binding, and possibly vice versa, PPARγ vs. RORγt selectivity can be tuned for these classes of compounds.

In conclusion, MRL-871 binds to PPARγ in a binding mode distinct to that previously found for other PPARγ ligands, including its most close counterpart the benzoyl 2-methyl indole compounds. The MRL-871 binding site in PPARγ is located between helices 3, 5, 7 and 11 and the beta sheet region with a hydrogen bond to Ser370 and without protrusion into branch I. Additionally, MRL-871 protrudes further towards helix 7 than any other compound seen to date. This binding mode causes MRL-871 to bind with high affinity, independent of the presence of coactivators. As a result, MRL-871 features only weak coactivator stabilization for PPARγ. This partial agonism is highly desirable for novel compound development endeavors. PPARγ vs RORγt subtype selectivity is tunable in these compounds classes, which provides a route towards a differentiated PPARγ pharmacology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Concentration rosiglitazone	None	10 nM	1 μM
EC_{50}/IC_{50} (nM)	210 ± 100	230 ± 80	9100 ± 3700
K_i (nM)	210 ± 100	160 ± 60	230 ± 90

Figure 3. MRL-871 competes for the binding location of rosiglitazone in HTRF. HTRF coactivator recruitment assay showing MRL-871 dependent change in PPARγ LBD (10 nM), PGC-1α peptide (200 nM) interaction in presence of fixed concentrations of rosiglitazone ($n = 3$, representative data shown as mean ± s.d. of one experiment in triplicate).

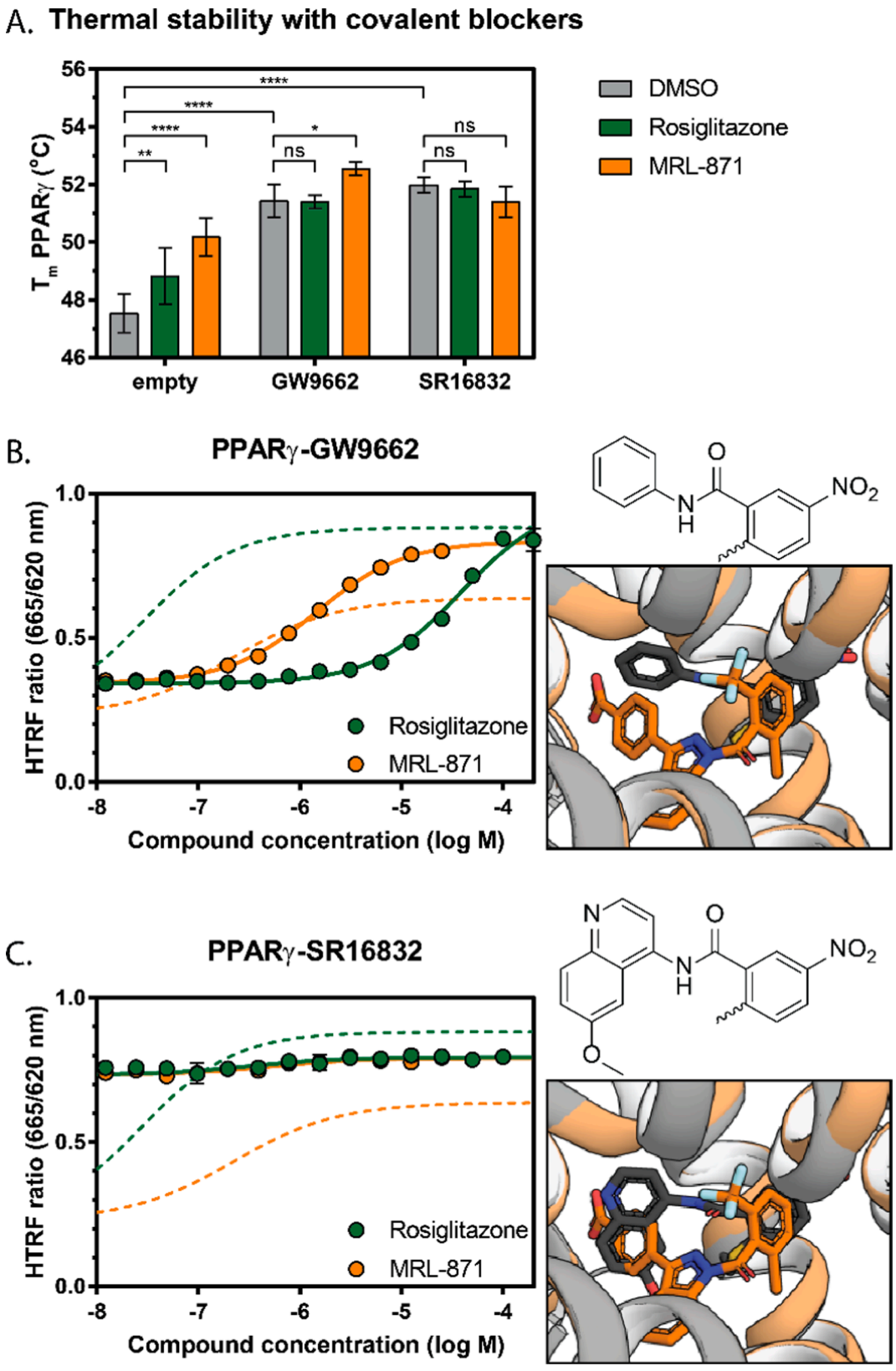


Figure 4. MRL-871 still binds PPARγ with micromolar affinity when the orthosteric pocket is blocked by GW9662. (A) DSF melting temperatures of PPARγ LBD (3.5 μM) in absence and presence of rosiglitazone or MRL-871 (14 μM) and covalently bound to GW9662 or SR16832 ($n \geq 3$; mean ± s.d; significance difference between with and without compound assessed using unpaired *t*-test). (B-C) HTRF coactivator recruitment assay showing ligand dependent change in PPARγ LBD (10 nM), PGC-1α peptide (200 nM) interaction with the PPARγ LBD covalently bound to either (B) GW9662 or (C) SR16832. Dotted lines show HTRF curves without a covalent blocker ($n = 3$, representative data shown as mean ± s.d. of one experiment in triplicate).

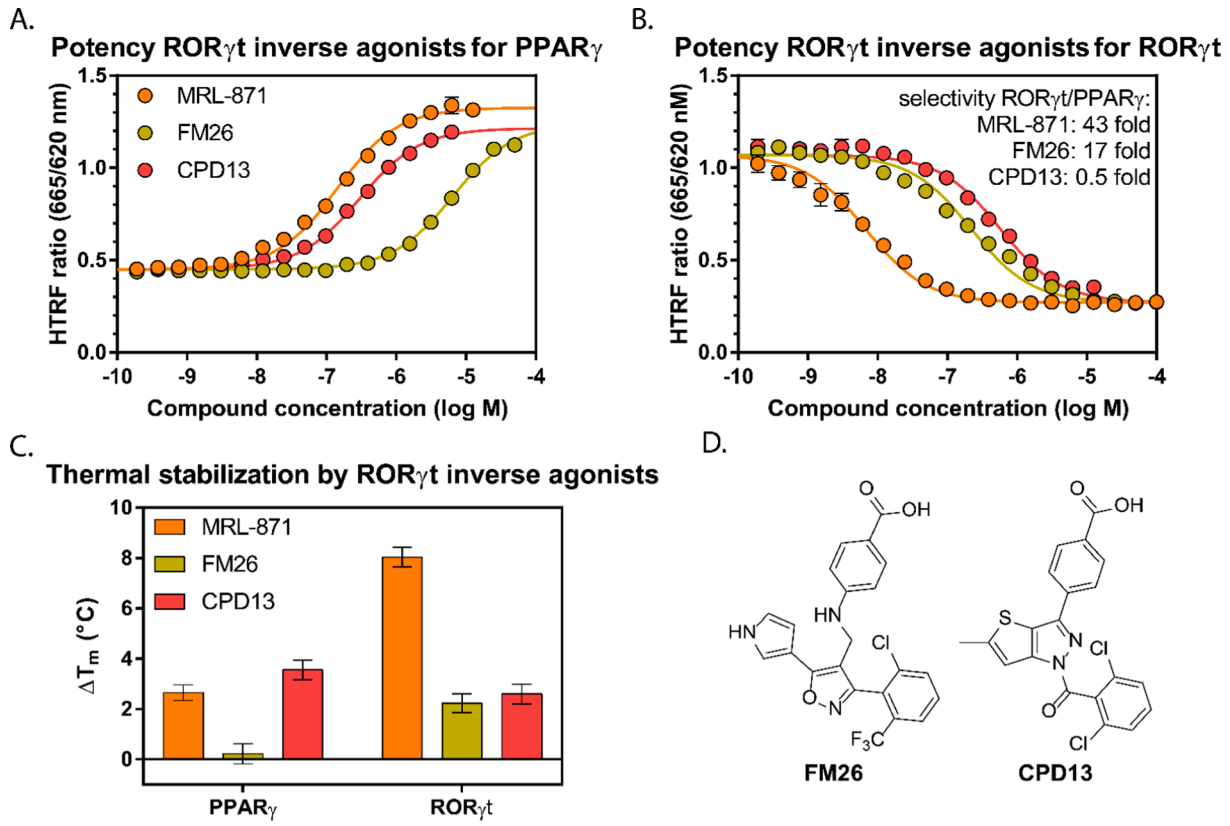


Figure 5. PPARγ and RORγt selectivity. (A) Ligand dependent HTRF PPARγ-coactivator recruitment assay with PPARγ (10 nM), PGC-1α peptide (200 nM) ($n = 3$, representative data shown as mean ± s.d. of one experiment in triplicate). (B) Ligand dependent HTRF RORγt-coactivator recruitment assay with RORγt (20 nM), SRC1B2 peptide (100 nM) ($n = 3$, representative data shown as mean ± s.d. of one experiment in triplicate).

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2022.116877.

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