Comparative Molecular Profiling of the PPARα/γ Activator Aleglitazar: PPAR Selectivity, Activity and Interaction with Cofactors

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear hormone receptors that control the expression of genes involved in a variety of physiologic processes, through heterodimerization with retinoid X receptor and complex formation with various cofactors. Drugs or treatment regimens that combine the beneficial effects of PPARα and γ agonism present an attractive therapeutic strategy to reduce cardiovascular risk factors. Aleglitazar is a dual PPARα/γ agonist currently in phase III clinical development for the treatment of patients with type 2 diabetes mellitus who recently experienced an acute coronary event. The potency and efficacy of aleglitazar was evaluated in a head-to-head comparison with other PPARα, γ and δ ligands. A comprehensive, 12-concentration dose–response analysis using a cell-based assay showed aleglitazar to be highly potent, with EC50 values of 5 nM and 9 nM for PPARα and PPARγ, respectively. Cofactor recruitment profiles confirmed that aleglitazar is a potent and balanced activator of PPARα and γ. The efficacy and potency of aleglitazar are discussed in relation to other dual PPARα/γ agonists, in context with the published X-ray crystal structures of both PPARα and γ.

Peroxisome proliferator-activated receptors (PPARs) belong to a family of ligand-regulated nuclear hormone receptors that control the expression of genes involved in a variety of physiologic processes, including lipid and glucose homeostasis, inflammation, and cell differentiation.[1,2] The fibrates, which act as PPARα agonists, are used clinically to treat dyslipidemia and associated cardiovascular risk.[3,4] Thiazolidinedione (TZD) PPARγ agonists, such as pioglitazone and rosiglitazone, improve insulin sensitivity and glucose homeostasis,[5,6] and exhibit anti-inflammatory[7] and antihypertensive effects.[8,9] However, the use of TZDs is associated with weight gain, increased incidence of edema, and risk of congestive heart failure.[10,11] Illustrating the distinct compound-specific effects of TZDs, pioglitazone has been shown to reduce atheroma in patients with type 2 diabetes mellitus[12,13] and to decrease cardiovascular events in some studies,[14] whereas rosiglitazone increases the risk of myocardial infarction.[15] Clinical trials and post-marketing surveys support the notion that rosiglitazone and pioglitazone do not share the hepatotoxic profile of the prototype TZD PPARγ agonist troglitazone,[16] further highlighting the distinct compound-specific effects of TZDs.

Drugs or treatment regimens that combine the beneficial effects of PPARα and γ agonism present an attractive therapeutic strategy.[17,18] Several dual PPARα/γ agonists, namely muraglitazar, tesaglitazar and aleglitazar,[19] have reached late-stage clinical trials. The development of muraglitazar and tesaglitazar was discontinued due to compound-specific side effects that included elevated risk of cardiovascular events for muraglitazar[20] and decreased renal function for tesaglitazar. Moreover, in clinical studies, both muraglitazar and tesaglitazar increased weight gain and edema to a similar or even greater degree than pioglitazone.[22,23] In contrast, in the phase II SYNCHRONY trial (NCT00388518) in patients with type 2 diabetes mellitus, aleglitazar caused less weight gain and demonstrated better lipid effects than pioglitazone at doses achieving similar glycemic control, although the study was not designed to assess significant differences between the two treatments.[24]

The regulation of PPAR activity is complex. PPARs are regulated through mechanisms including phosphorylation and dephosphorylation,[25,26] ligand- and cell-specific interactions with cofactors of the p160 family,[27] and heterodimerization with members of the retinoid X receptor (RXR) family.[27] The specific cofactors recruited to PPAR–RXR complexes in response to dif-

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ferent ligands are suggested to lead to major differences in transactivation of target genes.\(^{27-29}\) Many of these cofactors have been shown in their own right to be key players in metabolic regulation.\(^{30, 31}\) It has thus been hypothesized that the balance between efficacy and side-effect profiles of each specific PPAR agonist might relate, at least in part, to its potency, PPAR isoform selectivity, and/or pattern of cofactor recruitment. New molecules designed taking these factors into account have the potential to become superior therapeutics that sufficiently separate efficacy from side effects, leading to a broader therapeutic window. This concept has led to efforts to identify selective PPAR modulators, such as the partial PPAR\(\gamma\) agonists INT131,\(^{32}\) MK0533,\(^{33}\) and ATx008-001/FK614.\(^{33}\) INT131 recruits DRIP205, a co-activator involved in adipocyte differentiation, with an efficacy of about 20–25\% that of prototypical full PPAR\(\gamma\) agonists, including rosiglitazone and pioglitazone.\(^{32}\) In animal models of diabetes, INT131 caused less weight gain compared with pioglitazone or rosiglitazone, while retaining efficacy to reduce plasma glucose.\(^{32, 34}\) The aim of dual PPAR\(\alpha/\gamma\) agonist treatment is to simultaneously capture the glycemic benefits of targeting PPAR\(\gamma\) and the lipid benefits of targeting PPAR\(\alpha\). Indeed, there is evidence indicating distinct but overlapping gene signature profiles for different PPAR agonists.\(^{35-37}\) In this context, aleglitazar, a dual PPAR\(\alpha/\gamma\) agonist currently in phase III development, has been shown to induce transcriptional signatures different from those of other dual PPAR\(\alpha/\gamma\) treatments.\(^{35}\) This could underlie the favorable efficacy/side-effect profile observed in preclinical and clinical investigations.\(^{24, 38}\)

We recently solved the X-ray structures of the ternary complexes of aleglitazar with a peptide fragment of the receptor co-activator SRC1 and the ligand binding domains of both PPAR\(\alpha\) and \(\gamma\).\(^{19}\) As shown in the structural representations in Figure 1, the C-terminal activation helix 12 in both PPARs adopts a full-agonist conformation, mediated by a direct interaction of the carboxylate head group of aleglitazar with Tyr464 and Tyr473 of the \(\alpha\)- and \(\gamma\)-isoforms, respectively. This arrangement of helix 12 generates a hydrophobic interaction surface to which the LxxLL motif of the co-activator SRC1 fragment peptide binds. The carboxylate of aleglitazar engages in three additional strong hydrogen-bonding interactions with Ser280, Tyr314 and His440 residues of PPAR\(\alpha\) or Ser289, His323 and His449 residues of PPAR\(\gamma\). Moreover, the extended aleglitazar structure has excellent shape complementarity with both the PPAR\(\alpha\) and PPAR\(\gamma\) ligand binding pockets. The central benzothiophene and terminal phenyl ring of aleglitazar make additional hydrophobic interactions that contribute to greater binding efficiency in both receptors compared with other ligands.

Here, we describe the relative potency and efficacy of aleglitazar in a head-to-head comparison with key PPAR ligands, including dual PPAR\(\alpha/\gamma\) agonists previously in development, as well as marketed PPAR drugs (Table 1). Comprehensive transcriptional transactivation and cofactor recruitment studies confirm the high potency and balanced activity of aleglitazar on PPAR\(\alpha\) and \(\gamma\) and suggest, particularly for PPAR\(\alpha\), that ale-
glitazar possesses a unique profile compared with other ligands.

**Results**

**Effects on PPAR transcriptional activity**

The results indicate that aleglitazar is a highly potent agonist of both PPARα and γ transcriptional activity, with half-maximal activation (EC_{50}) values of 5 nM and 9 nM, respectively (Figure 2). The other dual PPARα/γ agonists tested, muraglitazar and tesaglitazar, are substantially less potent. EC_{50} values against PPARα and PPARγ were found to be 5680 nM and 243 nM for muraglitazar and 4780 nM and 3420 nM for tesaglitazar, respectively (Figure 2, Table 2), indicating selectivity towards PPARγ activation, particularly for muraglitazar.

Aleglitazar was also more potent towards PPARα than fenofibrate acid (EC_{50} = 22,400 nM) and reference compound RO4899100 (EC_{50} = 193 nM) but had lower maximal activity, suggesting a partial PPARα agonist profile. Comparisons between the tested dual PPARα/γ agonists showed that aleglitazar elicits a lower maximum PPARα activation of sevenfold.
versus muraglitazar and tesaglitazar at 11- and 12-fold baseline, respectively, consistent with this hypothesis. PPARγ was activated by aleglitazar to a similar degree as by the PPARγ agonists pioglitazone and rosiglitazone, achieving a maximum activity of 26–29-fold baseline. However, aleglitazar was the most potent of the three, with an EC50 value of 9 nM; values for rosiglitazone and pioglitazone were 245 nM and 1160 nM, respectively.

Aleglitazar was found to have a low potential to activate PPARδ (Table 2). Its maximum activation was low compared with the positive control GW501516 (sixfold and 100-fold maximum activity, respectively). Pioglitazone and rosiglitazone were less potent against PPARδ but had five times more activity than aleglitazar, with a maximum activation of >30-fold.

The EC50 values of aleglitazar towards both PPARα and γ observed in this study are lower than we previously reported; however, they are confirmed by time-resolved fluorescence resonance energy transfer (TR-FRET) data described in this report. The data reported here represent a more accurate analysis, since a more comprehensive 12-concentration (vs 8) dose–response analysis, including lower concentrations of ligands, was carried out.

### Cofactor recruitment

There were some differences in cofactor-peptide recruitment between PPARα and γ. Receptor-associated co-activator (RAC) 3_M1, nuclear receptor co-repressor (NCoR) 1, and silencing mediator of retinoid and thyroid receptor (SMRT) 1 were not recruited by either PPAR isoform (Table 3 and Figure 3). Eight of the 16 cofactor peptides tested were recruited to PPARα; these were steroid receptor co-activator (SRC) 1_M1, SRC1_M3, etc.

### Table 2. Summary of transcriptional activation potency and activity of different PPAR ligands.

| Compd       | PPARα EC50 [nM] | MFA[a] | PPARγ EC50 [nM] | MFA[b] | PPARδ EC50 [nM] | MFA[b] |
|-------------|-----------------|--------|-----------------|--------|-----------------|--------|
| Aleglitazar | 5               | 7      | 9               | 29     | 376             | 6      |
| Tesaglitazar| 4780            | 12     | 3420            | 40     | 51000           | 10     |
| Muraglitazar| 5680            | 11     | 243             | 25     | 16400           | 13     |
| RO4899100   | 193             | 15     | 19900           | 10     | 51000           | 12     |
| Fenofibric acid | 22400       | 12     | 1470           | 3^c    | 1060           | 2.5^c  |
| Pioglitazone| 11600           | 7      | 1160           | 26     | 9210           | 30     |
| Rosiglitazone| 15000         | 6.5    | 245             | 26     | 8630           | 40     |
| GW501516    | NA              | NA     | NA              | NA     | 36              | 100    |

[a] Maximum fold activation (MFA). [b] Value is inaccurate due to low maximal activation. [c] GW501516 activity was determined for PPARδ only. NA, not available.
transcription intermediary factor (TIF) 2_M1, TIF2_M2, TIF2_M3, RAC3_M2, NCoR2, and SMRT2 (Table 3). The following peptides were not recruited by PPARγ in the assay: SRC1_M2, SRC1_M4, RAC3_M2, SRC1_M4, TIF2_M1, SRC3_M2, SRC3_M3, CBP, TRAP220, NCoR2 and SMRT2 (Table 3). The results confirmed that aleglitazar induces partial recruitment of TIF2_M2 versus full recruitment by RO4899100. Moreover, increasing concentrations of aleglitazar were able to compete with the full recruitment induced by RO4899100 at 100 nm down to the partial recruitment profile characteristic of aleglitazar alone (figure S1a in the Supporting Information). Both aleglitazar and RO4899100 were able to completely displace NCoR2, demonstrating that both compounds fully occupy the PPARα receptors (figure S1b in the Supporting Information). These results suggest that the differential recruitment of TIF2_M2 is indeed due to different conformations of the complexes of aleglitazar and RO4899100 with PPARα.

Rosiglitazone, pioglitazone, edegalitazone and farglitazone have a clear PPARγ agonist profile, with predominant PPARγ activity and little (edegalitazone/farglitazone) or no (rosiglitazone/pioglitazone) PPARα activity (Table 4). Furthermore, in assays with PPARγ, aleglitazar was a more potent agonist than the reference compounds edegalitazone, tesaglitazone or muraglitazone, and it exhibited superior potency to pioglitazone (Figure 4b,d and Table 4). No significant differences in maximal efficacy were observed between the agonists, with the exception of farglitazone, the most efficacious ligand in the cofactor assays (Table 4).

The potency of several ligands (e.g., pioglitazone and rosiglitazo) differs when compared with earlier reports, but the activities are roughly in line with subsequent literature. Our study, employing two independent assays (cell-based transactivation and receptor recruitment assays), indicates that aleglitazar exhibited a slightly greater potency towards PPARα, whereas both tesaglitazone and muraglitazone showed greater potency towards PPARγ (Table 5). This was in general agreement with the ratios of EC50 values as determined in the cell-based transcriptional transactivation assays (Table 2).


c| Motif| Cofactor peptides sequence| Recruited by: |
|----|----------------|------------------|
| SRC1 | M1 | 623DSKYSQTSKHLQVLQITLTTAQDLQRLK | + + |
| M2* | 721DLASKSKEDEQDLRLLQDLQSKGPS | – – |
| M3 | 142TGQFTCAQGKLQLQQLLQDSV | + + |
| M4* | 2081AVQEHASTNMGLEAIIRKALMGKYD | – – |
| TIF2 | M1 | 630SRLHSDKQKTQLLLQTLTSDK | + + |
| M2* | 677STGTSLKEKHILLHRLLQDS | – – |
| M3* | 730SFQKKENALRYLRLDDPSDV | + + |
| RAC3 | M1 | 615SGSKKHLQLLLQTCSS | – – |
| M2* | 670SNKHKQDLQKHLQLKLLQDS | – – |
| M3 | 720PKKENALLRLYLRLDDPSDV | + + |
| TRAP220 | M2* | 637GNTKHNMLLLNNKLPQDF | – – |
| CBP | –* | 55SGNLVPSDAASKHLQSELSTGGGS | – – |
| NCoR | ID1 | 2289HLILNASLEQKIRLLQLLQ OGNGSL | – – |
| ID2 | 2081ADPASNLGLEDIKALMSQ | – – |
| SMRT | ID1 | 2312HQRVSQTLQHSETVIQDITTTH | – – |
| ID2 | 2081AVQEHASTNMGLEAIIRKALMGKYD | – – |

[a] Steroid receptor co-activator 1 (SRC1); transcriptional intermediary factor 2 (TIF2); receptor-associated co-activator 3 (RAC3); thyroid hormone receptor-associated protein complex 220 kDa component (TRAP220); cAMP responsive element binding protein (CREB)-binding protein (CBP); nuclear receptor co-repressor (NCoR); silencing mediator of retinoid and thyroid hormone receptors (SMRT). [b] Peptides differentially recruited by only one of the PPARs are indicated by *. All other peptides were recruited by both PPARs (or neither, for example, RAC3_M1 peptide). [c] Peptides and corresponding amino acid position within each cofactor.

whereas muraglitazone showed a fivefold greater potency towards SRC1_M1/PPARγ compared with SRC1_M1/PPARα but a similar potency towards TIF2_M1/PPARα and TIF2_M1/PPARγ.

The ratio of PPARγ to PPARα EC50 values calculated from the cofactor recruitment assays indicates that aleglitazar exhibited a slightly greater potency towards PPARα, whereas both tesaglitazone and muraglitazone showed greater potency towards PPARγ (Table 5). This was in general agreement with the ratios of EC50 values as determined in the cell-based transcriptional transactivation assays (Table 2).
vation and non-cell-based FRET), produced reasonably concordant data. For example, the EC50 value of rosiglitazone against PPARγ is 245 nm in the transactivation assay (Table 2) and 256 nm in the cofactor experiments (Table 5). The corresponding values for pioglitazone were 1160 nm and 2061 nm, respectively, while aleglitazar showed the highest potency at 9 nm and 10 nm, respectively. Notable disparities, for example, are values for muraglitazar and tesaglitazar, which showed higher potency in the cofactor recruitment assay compared with values determined from the results of transactivation experiments. These observations highlight the value of head-to-head comparative testing since comparisons across studies are complicated by alternative protocols, employing, for example, different cell backgrounds or full-length versus chimeric receptors.

Discussion

The current study demonstrates that aleglitazar shows a potent and balanced activity for both PPARα and γ, as analyzed by transcriptional transactivation and cofactor recruitment assays. In transactivation assays, aleglitazar was found to have EC50 values of 5 nm and 9 nm against PPARα and γ, respectively. Aleglitazar was the most potent agonist for PPARα and γ of a panel of seven ligands, which included compounds in clinical use, such as fenofibrate and pioglitazone, and also the prototype dual PPARα/γ agonists muraglitazar and tesaglitazar.

The high and balanced potency of aleglitazar for both PPARα and γ can be rationalized from the X-ray co-crystal structures. The negatively charged carboxylate head group of aleglitazar is involved in four strong hydrogen bonds with PPARα and PPARγ, effectively minimizing desolvation penalties. While the TZD head group, as present in pioglitazone and rosiglitazone, is able to interact efficiently with PPARγ, it shows significantly reduced affinity for PPARα due to an amino acid difference (His 323 to Tyr 314) in this polar recognition region. The bulkier Tyr 314 present in PPARα results in a smaller ligand binding pocket, which can be much better accommodated by the smaller carboxylate head group of aleglitazar compared with the TZD head group. The remaining residues lining the extended ligand pocket are mostly hydrophobic. In comparison with pioglitazone and rosiglitazone, aleglitazar contains greater buried surface area, making additional hydrophobic interactions through an annulated thiophene at the central phenyl ring and an additional terminal phenyl moiety. Together, these features appear to explain the remarkably high and, most importantly, balanced affinity of aleglitazar for both PPARα and PPARγ.
Table 4. Cofactor-dependent recruitment potency and activity towards PPARα and PPARγ (delta ratio) [a] 

| Compd             | EC50 [nM] | SRC1_M1 | SRC1_M2 | SRC1_M3 | SRC1_M4 | TIF2_M1 | TIF2_M2 | TIF2_M3 | RAC3_M2 | RAC3_M3 | CBP   | TRAP220 | NCoR2 | SMRT2 |
|-------------------|-----------|---------|---------|---------|---------|---------|---------|---------|---------|---------|-------|--------|-------|-------|
|                  |           |         |         |         |         |         |         |         |         |         |       |        |       |       |
| Aleglitazar       | 2.9 (94)  | 2.1 (53)| 1.6 (53)| 2.3 (56)| 1.6 (53)| 2.3 (56)| -       | 1.7 (53)| 14.1 (51)|       |       |       |       |       |
| Tesaglitazar      | 427 (100)| 111 (52)| 162 (54)| 377 (55)| 182 (54)| 377 (55)| -       | 646 (54)| 1780 (51)|      |       |       |       |       |
| RO4899100         | 16.1 (100)| 4.9 (100)| 8.6 (100)| 5.9 (100)| 12.7 (100)| 25.9 (100)| -       | 18.5 (100)|       |       |       |       |       |
| Fenofibric acid   | 4363 (39) | 3452 (47)| 2161 (34)| 5198 (44)| 1293 (25)| -       | 2622 (97)| 3211 (70)|       |       |       |       |       |
| Edaglitazone      | 867 (41)  | -       | 4.9 (100)| 38.1 (100)| 86.5 (100)| -       | 38.1 (100)| 9.8 (100)|       |       |       |       |       |
| Farglitazar       | 8.6 (195)| 3.8 (105)| 12.1 (160)| 3.3 (120)| 16.1 (517)| -       | 6.5 (132)| 8.7 (157)|       |       |       |       |       |
| Pioglitazone      | 2256 (92)| 196 (93)| 2116 (77)| 222 (105)| 951 (87) | -       | 1071 (86)| 1865 (77)|       |       |       |       |       |
| Rosiglitazone     | 240 (116)| 18.5 (91)| 241 (107)| 233 (102)| 79.1 (101)| 431 (94)| 9422 (44)| 346 (96)|       |       |       |       |       |

[a] Compounds were tested at a range of concentrations. The EC50 values are given as the concentration that elicits 50% of the maximum response.}
file of aleglitazar on PPARα, combined with its ability to act as a balanced activator of PPARα/γ, underlies its favorable clinical profile observed in SYNCHRONY.

The results from the cofactor recruitment assay highlight distinct pharmacologic features of aleglitazar versus other PPARα/γ agonists and might explain, at least in part, the unique gene expression profile observed with the different ligands. However, it must be acknowledged that these data are derived using the ligand binding domains of the PPARs and LxxLL-containing peptide motifs of the cofactor panel. Cofactors such as SRC1 and TIF2 contain several binding motifs available for interacting with PPAR, and in full-length proteins with a complete cofactor–receptor interaction, these motifs might produce a synergistic effect not observed with the use of short, synthetic peptides. Therefore, additional studies are required to confirm the effects using full-length cofactor proteins, including the finding that aleglitazar acts as a partial PPARα agonist.

It is well established that treatment of type 2 diabetes mellitus patients with TZD PPARγ agonists is associated with weight gain, in part due to increased adipogenesis/fat mass. Consider-
able research has focused on identifying PPARγ activators that maintain the beneficial metabolic features without increasing adipogenesis. One approach is the use of moderate activation of PPARα, for example by differential co-activator recruitment with decreased adipogenic capacity, or via partial agonism of the receptor. The latter so-called selective PPAR modulation might alleviate the weight-gain effect, and possibly other side effects, associated with TZDs.\cite{17, 42, 47}

Several selective PPAR modulators (MK0533,\cite{48} AXt008-001/FK614,\cite{33} MBX-102\cite{33} and INT131\cite{32} have progressed into clinical development. In particular, preclinical studies in animal models of diabetes showed INT131 to be efficacious in reducing plasma glucose while resulting in fewer side effects as compared with rosiglitazone.\cite{32, 34} However, a phase I multiple ascending-dose study showed that INT131 at the highest dose—10 mg per day for four weeks—caused significant weight gain as well as modest edema in 25% of patients, concurrent with significant lowering of plasma glucose.\cite{46} Thus, the concept of using selective PPAR modulators to reduce side effects is intriguing, but the incidence of side effects in clinical trials for INT131, despite having an apparently improved safety profile in animal studies, demonstrates the complexity of mechanisms involved in PPAR cofactor recruitment and regulation of gene expression.

Recently, the role of classical receptor agonism in the action of PPARα ligands has come under scrutiny as a cyclin-dependent kinase 5 (CdK5)-mediated decrease in PPARα phosphorylation was found to correlate with the antidiabetic effect of some PPARα ligands (MRL24 and rosiglitazone) independently of receptor agonism.\cite{50} As no differences were observed in the DNA binding for different phosphorylation states of PPARα, it was suggested that other factors, such as cofactor recruitment, might be regulated in a phosphorylation-dependent manner.\cite{50} It would be interesting to determine the effect of aleglitazar, and other ligands that show differential effects on adipogenesis/weight gain, on CdK5-dependent phosphorylation sites on PPARα and to analyze their correlation with cofactor recruitment. However, it must be noted that the investigations of changes in phosphorylation status have been limited to mainly preclinical studies, with assessment in only a small cohort of subjects treated with rosiglitazone. The same mechanism does not apply to either PPARα or PPARβ, suggesting transcriptional agonism will still be required for the additive, added lipid benefits of dual activation of PPARα along with PPARγ.

Conclusions

The results of this study have characterized aleglitazar as a potential dual PPARα/γ agonist with a unique profile in terms of potency and balance, as evaluated in transactivation and cofactor recruitment assays. Furthermore, the in vitro binding and cofactor recruitment profile of aleglitazar show qualitative differences compared with the profiles of other agonists that might explain the effects of aleglitazar observed in preclinical and clinical studies.

### Experimental Section

**General:** The structures of aleglitazar, tesaglitazar, muraglitazar, 2-methyl-2-(3-(methyl-[2-methyl-6-(4-trifluoromethyl-phenyl)pyridin-3-ylmethyl]carbamoyl)phenoxy)propionic acid (RO4899100, an experimental PPARα-selective compound),\cite{31} fenofibric acid, edgagitazone, farglitazone, pioglitazone, rosiglitazone and GW501516 are shown in Table 1. RO4899100 and edgagitazone were selected as reference compounds for assays with PPARα and PPARγ, respectively. GW501516 was chosen as reference for PPARβ experiments. Compound stock solutions were prepared in dimethyl sulfoxide (DMSO) at a final concentration of 10 mM, such that the final concentration of DMSO in assays did not exceed 0.1% v/v. LANCE EuW1024-labeled anti-glutathione-S-transferase (GST) antibody (3.9 μM) and SureLight allophycocyanine streptavidin were obtained from PerkinElmer (Waltham, MA, USA). Purified GST–PPARα and γ fusion proteins were produced in-house.

### Transactivation assay

**Expression plasmids:** The preparation of plasmid constructs expressing PPARα, γ and δ has been described previously.\cite{52} Briefly, the DNA binding domain of the yeast Gal4 transcription factor was fused in-frame to the N terminus of the ligand binding domains of human PPARα (amino acids 167–469), mouse PPARγ (amino acids 174–476) or human PPARδ (amino acids 139–442) receptors. Mouse and human PPARα are 97% identical in their ligand binding domains and 100% identical in their ligand binding pockets.

**Assay protocol:** Baby hamster kidney cells (BHK21) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C and 5% CO2. Cells were distributed in six-well plates at a density of 10^5 cells per well then transfected using FuGENE 6 reagent (Roche Molecular Biochemicals, Rotkreuz, Switzerland) with the pFR-Luc luciferase reporter plasmid and expression plasmids for PPARα, γ or δ ligand binding domains. Cells were harvested by trypsination 6 h post-transfection and then distributed in 96-well plates at a density of 10^4 cells per well. After incubation for 24 h to allow attachment of cells, the medium was removed and replaced with 100 μL of phenol red-free medium containing the test or reference compound. Following 24 h incubation,

### Table 5. Summary of cofactor recruitment potency of different PPAR ligands.

| Compd         | EC50a (nM) | PPARα | PPARγ | Ratioγ/α |
|---------------|------------|--------|--------|-----------|
| Aleglitazar   | 2.0        | 10.3   | 5.1    |           |
| Tesaglitazar  | 377        | 230    | 0.61   |           |
| Muraglitazar  | 118        | 38     | 0.32   |           |
| RO4899100     | 12.3       | >10000 | >810   |           |
| Fenofibric acid| 4557       | >10000 | >2.2   |           |
| Edgagitazone  | 1053       | 35.6   | 0.034  |           |
| Farglitazone  | 479        | 8.7    | 0.018  |           |
| Pioglitazone  | >10000     | 2061   | <0.21  |           |
| Rosiglitazone | >10000     | 256    | <0.026 |           |

[a] Median EC50 values were determined from the EC50 value of each ligand against PPARα or PPARγ and select cofactors. For PPARα, these were SRC1_M1, SRC1_M4, TIF2_M1, TIF2_M2, RAC3_M3 and NCoR2. For PPARγ, the cofactors were SRC1_M1, SRC1_M4, TIF2_M1, RAC3_M3, TRAP220 and NCoR2. (b) Ratio of EC50 values for PPARα/PPARγ.

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tion in the presence of ligand, 50 μL of the medium was replaced with 50 μL of luciferase constant-light reagent (Roche Molecular Biochemicals) to lyse cells and initiate the luciferase reaction. Luminescence was detected in a TopCount microplate reader (Perkin Elmer).

Data analysis: Comprehensive 12-concentration dose–response curves with quadruplicate values per concentration were produced for all compounds to ensure accurate comparative values. Transactional activation in the presence of a test compound was expressed as fold-change in luminescence compared with cells incubated at low, non-activating concentrations of the test compound. Data were calculated as the mean ± standard error of the mean (SEM) of luciferase values and were then converted to fold activation. Potencies for EC₅₀ values of receptor transcriptional activity were calculated using the XLfit program (ID Business Solutions Ltd., Guildford, UK) using a one-site dose–response model.

Time-resolved fluorescence resonance energy transfer assay

Homogeneous TR-FRET represents a highly sensitive and robust assay format for determination of peptide–peptide or peptide–protein interactions. TR-FRET assays were used here to determine the interaction signatures of the cofactor peptides (co-activators and co-repressors) with the purified ligand binding domains of PPARα and γ in response to all ligands.

Cofactor peptides: A total of 16 FRET peptides corresponding to potential PPAR interaction motifs previously identified in several transcriptional co-activators (SRC1, TIF2, RAC3, TRAP220 and CBP) and transcriptional co-repressors (NCoR and SMRT) were synthesized by Jerini (Berlin, Germany) or Biosynth (Berlin, Germany). All cofactor-derived peptides were 20–25 amino acids in length (sequences given in Table 3) and were labeled with biotin separated by an aminohexanoic acid spacer at the N-terminus. Stock solutions (200 μM in DMDSO) were stored at −20 °C.

Assay protocol: Homogeneous TR-FRET was performed with either GST–PPARα (20 nm) or GST–PPARγ (20 nm) in each well in 384-well microtiter plates (Corning, Corning, NY, USA) by incubating for 60 min at 37 °C in the presence of agonist and the individual cofactor (500 nm) and a cocktail containing Eu-W1024-la beled anti-GST antibody (0.76 nm) and allophycocyanin streptavidin (40 nm) in HEPES-buffered solution (50 mM HEPES (pH 7.4), 25 mM NaCl, 0.1 mg/mL fatty acid-free bovine serum albumin (BSA), 1 mM dithiothreitol). Fluorescence was quantified at an excitation wavelength of 337 nm and at emission intensities of 665 nm (allophycocyanine) and 620 nm (europium cryptate) using a Nan o-Scan time-resolved fluorescence plate reader (IOM, Berlin, Germany).

Ligand effects on cofactor peptide–PPAR interactions

In a pilot experiment, the binding ability of the 12 co-activator and four co-repressor peptides to PPARα and PPARγ was determined in the presence of 10 μM of each PPAR ligand (Table 3). Following identification of the subset of peptides that showed ligand-dependent recruitment to either PPARs or PPARγ, detailed concentration–response experiments were performed to calculate both the potency (EC₅₀) and the maximal signal versus baseline for all peptides shown to be recruited to each PPAR in the pilot experiments.

Data analysis: Comprehensive 12-concentration dose–response curves with quadruplicate values per concentration were produced for all compounds on all PPARα– or PPARγ–cofactor pairs to ensure accurate comparative values. Data were calculated as the mean ± standard deviation (SD) for each concentration. Binding potency was determined as the EC₅₀ value from the FRET signal for each ligand on each receptor–cofactor pair. Curves were fit to a one-site dose–response model using XLfit. Median EC₅₀ values for each ligand were calculated from the mean EC₅₀ values determined from several cofactor peptides. For PPARα, they were SRC1_M1, SRC1_M4, TIF2_M1, TIF2_M2, RAC3_M3 and NCoR2; for PPARγ, they were SRC1_M1, SRC1_M4, TIF2_M1, RAC3_M3, TRAP220 and NCoR2. For comparison of the magnitude of the TR-FRET signals induced by ligands as potentially indicative of specific ligand-induced conformations, the fold-change in signal for each PPAR–peptide combination versus baseline was calculated and normalized to the positive controls RO4899100 (PPARα) or edaglitazone (PPARγ), for which the values were set to 100%.

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