T0070907, A Selective Ligand for Peroxisome Proliferator-Activated Receptor γ, Functions as an Antagonist of Biochemical and Cellular Activities

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Running title: PPARγ antagonist

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Abstract

The nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ [NR1C3]) plays a central role in adipogenesis and is the molecular target for the thiazolidinedione (TZD) class of antidiabetic drugs. In a search for novel non-TZD ligands for PPARγ, T0070907 was identified as a potent and selective PPARγ antagonist. With an apparent binding affinity (concentration at 50% inhibition of [3H]rosiglitazone binding or IC50) of 1 nM, T0070907 covalently modifies PPARγ on cysteine 313 in helix 3 of hPPARγ2. T0070907 blocked PPARγ function in both cell-based reporter gene and adipocyte differentiation assays. Consistent with its role as an antagonist of PPARγ, T0070907 blocked agonist-induced recruitment of coactivator-derived peptides to PPARγ in an HTRF-based assay, and promoted recruitment of the transcriptional corepressor NCoR to PPARγ in both GST pull-down assays and a PPARγ/RXRα dependent gel shift assay. Studies with mutant receptors suggest that T0070907 modulates the interaction of PPARγ with these cofactor proteins by affecting the conformation of helix 12 of the PPARγ ligand-binding domain. Interestingly, while the T0070907-induced NCoR recruitment to PPARγ/RXRα heterodimer can be almost completely reversed by the simultaneous treatment with RXRα agonist LGD1069, T0070907 treatment
has only modest effects on LGD1069-induced coactivator recruitment to the PPARγ/RXRα heterodimer. These results suggest that the activity of PPARγ antagonists can be modulated by the availability and concentration of RXR agonists. T0070907 is a novel tool for the study of PPARγ/RXRα heterodimer function.
Introduction

Peroxisome proliferator-activated receptor γ (PPARγ [NR1C3]) is a member of the nuclear hormone receptor (NHR) superfamily of ligand-activated transcription factors (1, 2). At least two PPARγ isoforms exist, γ1 and γ2, result from transcription from two different promoters upstream of the PPAR gene (3, 4). PPARγ2 possesses 30 additional amino acids at its amino terminus. PPARγ1 is expressed broadly in many tissues, while PPARγ2 is expressed predominantly in adipose tissue. Both "gain of function" and "loss of function" studies strongly support a critical role for PPARγ in adipocyte gene expression and differentiation (5).

Like other members of the NHR superfamily, PPARγ binds to a DNA response element (PPAR-response-element or PPRE) upstream of the coding regions of target genes and forms a heterodimeric complex with one of the three retinoid X receptor (RXR) proteins (1). Binding of ligands to PPARγ causes conformational changes in the receptor, in particular to α-helix 12 (H12), which is located at the C-terminal end of the protein and forms part of the transcriptional activation function (AF-2). When agonists bind to PPARγ, H12 along with H3, H4, and H5 form a charge clamp and a hydrophobic pocket that allows the recruitment of coactivator protein complexes which are essential for transcriptional activation of PPARγ target
genes (6). Although PPARγ, in isolation, is capable of binding to transcriptional corepressor proteins NCoR and SMRT in the absence of ligand, PPARγ does not interact with these corepressors in the context of the RXR heterodimer, nor does the PPARγ/RXR heterodimer repress transcription of PPARγ target genes, unlike heterodimers of RXR with TR or RAR (7). Two explanations for the difference in PPARγ/corepressor interaction on and off DNA have been offered. The orientation of PPARγ and RXR on a PPRE could simply inhibit the binding of corepressor (8). Alternatively, PPARγ may be unable to stabilize a conformation of RXR that is permissive for corepressor interaction; unlike TR and RAR, PPARγ is unable to interact with H12 from RXR (9). Because other NHR antagonists stabilize the interaction of corepressors with their cognate receptors, PPARγ antagonists or inverse agonists would be useful tools to study PPARγ/corepressor interaction. One way to test these hypotheses is to study the effects of a PPARγ antagonist or inverse agonist on corepressor binding. This avenue has not yet been explored.

Both natural and synthetic ligands have been reported for PPARγ (reviewed in (10)). Naturally-occurring compounds that have been reported to bind PPARγ include a number of fatty acids and eicosanoid derivatives such as 9- or 13-hydroxyoctadienoic acid (9-HODE or 13-HODE) and prostaglandin
derivative 15-deoxy-Δ13,14-prostaglandin J2 (15d-PGJ2). The most widely used synthetic agonists of PPARγ are members of a class of antidiabetic agents known as thiazolidinediones (TZDs), including rosiglitazone, troglitazone, and pioglitazone. More recently, a series of tyrosine-based PPARγ agonists exemplified by GI262570 have been shown to be among the highest-affinity PPARγ ligands described thus far. Synthetic partial agonists identified include GW0072 (11), L-764406 (12), 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO, 13), and FMOC-L-Leucine (14). Several synthetic antagonists have also been described, these include bisphenol A diglycidyl ether (BADGE, 15), GW9662 (10), and PD068235 (16). However, relatively little is known about how these compounds affect PPARγ/RXR heterodimer function.

Here, we describe a novel, potent, and selective PPARγ ligand, T0070907. Using a variety of biochemical and cell-based assays, we demonstrate that T0070907 is a PPARγ antagonist. Our studies suggest that T0070907 modulates the interaction of PPARγ with cofactor proteins by affecting the conformation of helix 12 of the PPARγ ligand-binding domain (LBD). Finally, our studies reveal a functional asymmetry between the effects of PPARγ and RXR ligands on the activity of the permissive PPARγ/RXRα heterodimer.
EXPERIMENTAL PROCEDURES

Plasmid Construction. All PPARγ and RXRα proteins produced by in vitro translation and used in gel mobility shift assay were expressed from pET28b-based plasmids (Novagen, Madison, WI) containing inserts cloned into NcoI and NotI sites. For PPARγ, the following constructs were made: hPPARγ1, full-length human PPARγ1; hPPARγ1 ΔH12, (amino acids 1-461 of hPPARγ1); hRXRα, full-length human RXRα; hRXRα ΔH12 (amino acids 1-443 of hRXRα). The construct used to produce human NCoR (hNCoR) protein from the baculovirus expression system was made by inserting hNCoR DNA encoding amino acids 1948-2440 into the BamHI and NotI sites of the pFASTBAC HTa vector (GibcoBRL, Carlsbad, CA). GST-PPARγ LBD was constructed by inserting hPPARγ1 DNA encoding amino acids 175-471 into the BamHI site of pGEX-2TK vector (sequence at the junction is 5’-GGATCCCATATG-hPPARγ1 amino acid 175).

Mass Spectrometry. After incubation with 10 μM T0070907 for 4 h at room temperature in 50 mM Tris pH7.9, 50 mM KCl, 1 mM EDTA, GST-PPARγ (12 μg) was purified on an SDS-polyacrylamide gel. The excised gel fragment containing PPARγ was digested with trypsin at 37°C for 12 h.
without reduction and alkylation in 100 mM ammonium bicarbonate using an enzyme:substrate ratio of 1:50 (w/w). Analysis of covalent binding of T0070907 to PPAR\(\gamma\) was performed with a Voyager-DE\textsuperscript{TM} MALDI-TOF Mass Spectrometer (PerSeptive Biosystems, Framingham, MA) and an Esquire\textsuperscript{TM} Nano-electrospray Tandem Mass Spectrometer (Bruker Daltonik, Billerica, MA).

The MALDI matrix was prepared by mixing \(\alpha\)-cyano-4-hydroxy-trans-cinnamic acid (HCCA, 40 mg/ml in acetone), nitrocellulose (20 mg/ml in acetone), and 2-propanol at a ratio of 2:1:1 (v:v:v). An aliquot (0.5 µl) of the sample/matrix was spotted and mixed on a MALDI sample plate. After drying completely, samples were washed with 3 µl of 5% formic acid and then with Milli-Q water (Millipore, Bedford, MA). The MALDI-TOF was operated in reflectron mode with delay extraction. The mass spectrometer was calibrated externally using des-Arg-Bradykinin (m/z 904.4681), Angiotensin I (m/z 1296.6853) and Glu-Fibrinopeptide B (m/z 1570.6774) (17).

The in-gel tryptic digest was desalted and concentrated prior to analysis by Nano-ESI-MS/MS. The in-gel digests were extracted twice with 10 µl of a 50% acetonitrile (ACN)/5% trifluoroacetic acid (TFA) solution. All extracts were pooled and dried to 5 µl with a SpeedVac. An additional 15 µl of a
0.1% acidic acid solution was added, and a 10-µl sample was loaded into a Ziptip™ (Millipore, Bedford, MA) with C_{18} resin for desalting. After washing the column with 1% TFA (in H_{2}O), 5 µl of 50% acetonitrile/0.1% acidic acid was used to elute the sample into a nanospray needle. On the basis of the MALDI-TOF mass analysis results, the tandem mass spectrometric sequencing was only acquired on select precursor ions (18).

**Homogeneous Time-Resolved Fluorescence (HTRF) Assay.** HTRF assays were performed as previously described (19) with the following modifications. Reaction conditions were as follows: a 100-µl reaction volume contained 50 mM Tris pH 7.9, 50 mM KCl, 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin (BSA), 800 ng/ml anti-GST-(Eu)K antibody (PerkinElmer, Wellesley, MA), 1 ng/ml GST-PPARγ, 1.5 µg/ml Streptavidin conjugated with allophycocyanin (Streptavidin-APC, PerkinElmer, Wellesley, MA), 200 nM biotin-peptide, and 5 µl compound of interest in dimethylsulfoxide (DMSO) as indicated in the Figure legends. GST-PPARγ/anti-GST-(Eu)K (20 µl) and biotin-peptide/Streptavidin (20 µl) were incubated separately for 1 h at room temperature (rt) before being combined with the remaining components, and the complete mixture was incubated for an additional 1 h at rt. Reactions were carried out in 96-well
plates (black polypropylene, Whatman Inc., Clifton, NJ), and fluorescence was measured on a LJL Analyst (LJL BioSystems, Sunnyvale, CA). Data were expressed as the ratio of the emission intensities at 665 nm and 620 nm multiplied by a factor of 1000,

_Corepressor Recruitment Assay (Pull-Down Assay)._ Purified GST-PPARγ fusion protein (15 µg) was incubated with 10 µl of glutathione-Sepharose beads (50% slurry in GST binding buffer, Amersham Pharmacia Biotech, Piscataway, NJ) in GST binding buffer (20 mM HEPES pH7.7, 100 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl2, 0.01% Nonidet P-40, 2 mM dithiothreitol (DTT), 10% glycerol) for 90 min at rt. After washing 5 times (5 min each wash) with 1 ml of binding buffer, the bead-bound GST-PPARγ protein was incubated with 7.5 µl [35S]methionine-labeled in vitro-translated hNCoR protein (TNT T7 Rabbit Reticulocyte Lysate Translation System, Promega Corp., Madison, WI) and the indicated ligand concentration in a final volume of 300 µl at rt for 2 h. After washing with binding buffer as indicated above, the bound protein was eluted with 20 µl 2x SDS buffer at 95°C, separated on a 10% SDS-polyacrylamide electrophoresis (SDS-PAGE) gel, and analyzed by autoradiography.
**Ligand-Binding Assay.** To determine the binding affinity of T0070907 to the PPARs, scintillation proximity assay (SPA) was performed as described (20, 12) with the following modifications. A 90-µl reaction contained SPA buffer (10 mM K₂HPO₄, 10 mM KH₂PO₄, 2 mM EDTA, 50 mM NaCl, 1 mM DTT, 2 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] (CHAP), 10% (vol/vol) glycerol, pH 7.1), 50 ng of GST-PPARγ (or 150 ng of GST-PPARα, GST-PPARδ), 5 nM [³H] radioligands, and 5 µl of T0070907 in DMSO. After incubation for 1 h at rt, 10 µl of polylysine-coated SPA beads (at 20 mg/ml in SPA buffer) were added, and the mixture was incubated for 1 h before reading in Packard Topcount. [³H]rosiglitazone was used for PPARγ, and [³H] GW2433 (21) was used for PPARα and PPARδ.

**Gel Mobility Shift Assay (GMSA).** Our GMSAs were similar to previous studies (22, 23) with the following modifications. The sequence of the DNA probe used in the GMSAs was derived from the PPRE of the acyl-CoA oxidase gene (5’-AGCTGGACCAGGACAAAGGTCACGTTCAGCT-3’). In vitro--translated PPARγ (0.5 µl) and RXRα (0.5 µl) were incubated in 20 mM Tris, pH8.0, 1 mM EDTA, 50 mM KCl, 0.05% NP-40, 10% glycerol, 2 mM DTT, 50 µg/ml poly(dI-dC), labeled probe (typically 40,000 cpm per
reaction), various ligands, and with or without baculovirus-expressed NCoR (5 µg) (final volume, 20 µl) for 30 min at rt. Reaction mixtures were loaded on a 5% (38:2)polyacrylamide nondenaturing gel in 1x TGE (50 mM Tris, pH8.5, 40 mM glycine, 2 mM EDTA) and separated in 1x TGE by electrophoresis at 4 °C. Gels were dried prior to autoradiography.

**Transient Transfection and 3T3L1 Differentiation Assay.** Luciferase reporter assays were carried out following transient transfection of HEK293 cells using GenePORTER2 reagent (GTS Inc., San Diego, CA) according to the manufacture’s protocol. 3T3-L1 preadipocytes were cultured and induced to differentiate as described (24) with the following modifications. 3T3-L1 cells were grown to confluence in Delbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), and induced to differentiate with 0.25 µM dexamethasone (DEX), 0.5 mM isobutylmethylxanthine (IBMX), and 1 µg/ml insulin. Medium was replaced 2 days post-induction (and every 2-3 days thereafter) with DMEM/10% FBS supplemented with 1 µg/ml insulin.
RESULTS

**T0070907 Is a Novel and Selective PPARγ Ligand.** In a search for novel non-TZD ligands for PPARγ, T0070907 was identified to bind PPARγ with high affinity, capable of displacing [$^3$H]rosiglitazone with an apparent $K_i$ of 1 nM as shown in Fig. 1. Furthermore, T0070907 shows high selectivity among PPAR subtypes with a >800 fold preference for PPARγ over PPARα and PPARδ. In competition with the PPARα and PPARδ co-ligand [$^3$H]GW2433 (21), T0070907 has an apparent $K_i$ of 0.85 µM to PPARα and 1.8 µM to PPARδ (Fig. 1B).

**T0070907 Is a Specific Potent PPARγ Antagonist in a Transient Transfection Assays.** The effect of T0070907 on the transcriptional activity of PPARγ in a cell-based reporter gene assay was examined. HEK293 cells were transiently transfected with an expression construct that contained the PPARγ LBD fused to the Gal4-DNA binding domain, together with a luciferase reporter gene under the transcriptional control of the Gal4 upstream activating sequence (Gal4-UAS). As shown in Fig. 2A, rosiglitazone activated transcription up-to 20-fold, whereas, T0070907 has no effect (or perhaps even a slight inhibitory effect) on basal transcription.
In addition, T0070907 is a potent inhibitor (IC50 value in the nM range) of PPARγ transactivation in the presence of rosiglitazone (Fig. 2A). This inhibition is not due to cytotoxicity as the concentration required to kill 50% of cells is greater than 10 µM (data not shown). The specificity of T0070907 was also examined in cell-based reporter gene assays. HEK293 cells were transiently transfected with a GAL4-UAS reporter and expression constructs encoding the LBDs of PPARα, PPARδ, FXR, LXRα, LXRβ, or PXR fused to the Gal4-DNA binding domain. As shown in Fig. 2A, T0070907 at 1 µM has no effect on the transcriptional activity of any other receptor besides PPARγ. These results demonstrate that T0070907 is a PPARγ specific antagonist.

**T0070907 Blocks Hormone-Mediated Differentiation of the Adipogenic Cell Line 3T3-L1.** We next investigated whether T0070907 could block the induction of adipogenesis by various treatment of the adipogenic cell line 3T3-L1. As shown in Fig. 2B, the standard treatment of dexamethasone, 3-isobutyl-1-methylxanthine, and insulin (Dex/Mix/Ins) promoted lipid accumulation in 3T3-L1 cells. In contrast, lipid accumulation in these cells was completely inhibited when cells were treated with both 1 µM T0070907 and the differentiation cocktail. Similar inhibitory effects of T0070907 were
observed when adipogenesis was induced by treatment with the PPARγ agonist, rosiglitazone (data not shown).

*T0070907 Covalently Modifies PPARγ on Cys313.* To understand the mechanism by which T0070907 antagonizes PPARγ function, its binding properties were first examined. That rosiglitazone was unable to displace T0070907 prebound to PPARγ suggested that the binding of T0070907 was irreversible (data not shown). To verify the covalent nature of the interaction between T0070907 and PPARγ, and to identify the site of covalent attachment, we performed proteolytic mapping studies via mass spectrometry. The covalent binding of T0070907 (Mw 277.7 Da) to PPARγ would result in a mass change of the modified tryptic peptide(s) by 241.1 Da. By comparing the tryptic digests of PPARγ with and without T0070907 treatment, a candidate peptide containing the T0070907 attachment site (AA 272-280, IFQGCQFR, m/z 998.49 Da) was identified based on its mass shift to m/z 1239.56 (data not shown). The precise binding site on this peptide was determined with ESI-tandem mass spectrometry (Fig. 3A). The calculated dominant y- and b-ion fragments of this peptide are shown at the top of Fig. 3A, with the ions observed in the mass spectrum underlined. The mass difference between the y3 and y4-ions (m/z 344.1) identified Cys313 as
the site of modification by T0070907. In addition, several double-charged y-ions and small internal fragment ions obtained also confirmed this conclusion.

To confirm the importance of Cys$^{313}$ in T0070907 binding to PPARγ, a mutant PPARγ was constructed in which Cys$^{313}$ was converted to a serine residue, and the corresponding recombinant GST-PPARγ LBD (C313S) fusion protein was expressed and purified. [3H]-labeled T0070907 was first incubated with either wild (wt) type PPARγ or the C313S mutant as described, and the reaction mixtures were separated by SDS-PAGE electrophoresis. As shown in Fig. 3B, [3H]T0070907 was only able to modify the wild type protein (upper panel, autoradiograph), although equal amounts of wild type and C313S mutant PPARγ were added to each reaction (lower panel, Coomassie staining). Thus, Cys$^{313}$ is necessary for the binding of T0070907 to PPARγ.

The specificity of the covalent modification was examined in a whole-cell extract (WCE) made from HEK293 cells. Purified GST-PPARγ LBD fusion protein (Fig. 3C) was mixed with the WCE and 2 µM [3H]T0070907, and then separated on an SDS-PAGE gel. As shown in Fig. 3C, the exogenously added PPARγ was preferentially modified by [3H]-T0070907 (upper panel,
autoradiograph), despite the presence of many other proteins in the WCE (lower panel, Coomassie staining).

*TO070907 Behaves as an Inverse Agonist of PPARγLBD in vitro.* Using the homogeneous time-resolved fluorescence (HTRF) technology, we developed an assay to study the effects of PPARγ ligands on the interaction of PPARγ with fragments of coactivator or corepressor proteins. Reporter peptides of approximately 20 amino acids in length were synthesized from sequences derived from various coactivator and corepressor proteins (Table 1) (25, 26). The effects of various ligands on PPARγ binding to this collection of peptides are shown in Fig. 4A. The patterns that emerged from this peptide profiling have allowed us to distinguish between different functional classes of PPARγ ligands. First, known PPARγ agonists such as rosiglitazone, troglitazone, and the GSK compound GI262570 (27) showed very similar peptide profiles. Rosiglitazone and troglitazone, which both belong to the TZD chemical class, were more similar to each other than to tyrosine-based GI262570. GI262570 recruited additional peptides (peptides #11, #19, #23, and #27), suggesting perhaps that PPARγ adopted a slightly different conformation when bound to GI262570, compared to the conformation assumed by the receptor when bound to the TZD compounds. In contrast,
the novel PPARγ ligand T0070907 shows a unique peptide profile (Fig. 4A), exclusively promoting recruitment of peptides derived from corepressor proteins NCoR and SMART (peptides #2 and #3 respectively). Furthermore, compared to the DMSO control, T0070907 seems to suppress the basal interactions between PPARγ and coactivator-derived peptides. In order to confirm these results, more extensive titration and competition experiments were carried out with two peptides derived from a representative coactivator and a representative corepressor (peptides #1 and #2). As shown in Fig. 4, panels B and C, rosiglitazone promoted the dose-dependent recruitment of peptide derived from coactivator DRIP205 to PPARγ, while suppressing the interaction between PPARγ and a peptide derived from corepressor NCoR. In contrast, T0070907 suppressed the interaction between PPARγ and the coactivator-derived peptide in the absence of ligand, while promoting the recruitment of the NCoR-derived peptide to PPARγ. T0070907 also effectively antagonized the effects of rosiglitazone in a dose-dependent manner. To independently confirm these observations using an alternative nonfluorescence-based format, the effects of T0070907 on PPARγ/NCoR interactions were examined using a GST pull-down assay. As shown in Fig. 4D, rosiglitazone suppresses the interaction between the GST-PPARγLBD
and NCoR in a dose-dependent fashion, with an IC50 (concentration at 50% inhibition) consistent with its binding affinity to PPARγ. On the other hand, T0070907 promoted a dramatic increase in NCoR binding to GST-PPARγ consistent with the results observed in the HTRF assay.

Effects of T0070907 and LGD1069 on PPARγ and RXRα Heterodimer in GMSAs. Having shown that T0070907 strongly promotes recruitment of NCoR to the PPARγ LBD in both HTRF and pull-down assays, we next used a GMSA (gel mobility shift assay) to examine whether this could also occur in the context of the PPARγ/RXRα heterodimer. As shown in Fig. 5A, in vitro-translated PPARγ and RXRα can bind simultaneously to a PPRE-containing DNA fragment derived from the promoter of acyl-CoA oxidase gene (lane 2). This shift in fragment mobility is absolutely dependent on the presence of both PPARγ and RXRα (data not shown), indicating the proper formation of a functional PPARγ/RXRα heterodimer under these conditions. While NCoR could not bind efficiently to the PPARγ/RXRα heterodimer in the absence of ligand (compare lanes 2 and 3, and similar to previous report)(9), T0070907 was able to promote a significant increase in the recruitment of NCoR to the heterodimeric complex (compare lanes 3 and 4).
To ensure that this increased NCoR recruitment was the result of T0070907 binding to PPARγ and to understand the nature of the PPARγ conformational changes associated with T0070907 binding, we next investigated the effects of deleting H12 from both receptors on the binding of NCoR to the heterodimer. The deletion of PPARγ H12 (PPARγ ΔH12) increased the basal interaction of NCoR with the heterodimer; however, T0070907 did not provide further enhancement of binding (Fig. 5A, lanes 5-7). In contrast, the PPARγ wt/RXRα ΔH12 heterodimer responded to T0070907 and almost all PPARγ wt/RXRα heterodimer could be super-shifted to form the PPARγ/RXRα/NCoR complex in the presence of the antagonist (Fig. 5B, lanes 2-4). Complexes containing H12 deletions in both PPARγ and RXRα interacted very efficiently with NCoR in the absence of ligand, but, as in the case of the PPARγ wt/RXR ΔH12 complex, T0070907 had almost no effect on NCoR recruitment (Fig. 5B, lanes 5-7).

The allosteric effects between PPARγ and RXRα were studied next by examining the effects of simultaneous treatments of RXRα agonist and PPARγ antagonist on the recruitment of coactivator and corepressor proteins to the heterodimer. Since the RXRα ΔH12 containing heterodimers interacted much more strongly with NCoR than the wild-type containing heterodimers (Fig. 5B), we examined the effects of an RXRα agonist,
LGD1069 (28), on T0070907-induced NCoR recruitment to PPARγ wt/RXRα ΔH12 and PPARγ ΔH12/RXRα ΔH12 complexes. Strikingly, the addition of LGD1069 dramatically inhibited NCoR binding to both pairs of heterodimer complexes (Fig. 5C, lanes 5-10 and lanes 14-19). Importantly, LGD1069 was not able to completely inhibit corepressor binding to the PPARγ ΔH12/RXRα ΔH12 complex as it was in the PPARγ wt/RXRΔH12 complex. To ensure that the effect of LGD1069 on NCoR recruitment was not due to blocking T0070907 from binding to the PPARγ/RXRα heterodimer, experiments were performed during which PPARγ was treated with T0070907 for an extended period prior to the addition of other components of the GMSA reaction mixture and LGD1069 to saturate all available binding sites on PPARγ. No difference on the recruitment of NCoR to the PPARγ/RXRα heterodimer was observed with or without preincubation with T0070907 (data not shown).

The effects of T0070907 binding to PPARγ on the ability of RXRα to interact with coactivators were also studied. In the presence of the RXRα agonist LGD1069, the coactivator protein SRC-1 can be recruited to the PPARγ/RXRα heterodimer, as evidenced by the super-shifted complex observed in GMSAs (Fig. 4D, lanes 5-10). This super-shifted complex was not observed in the presence of rosiglitazone (BRL, lane 4) or in reactions
containing an RXR H12 mutant (23) suggesting that SRC-1 was specifically recruited to the RXRα subunit. When added to the GMSA reaction mixture together with LGD1069, T0070907 seems to have a modest effect on formation of the PPARγ/RXRα/SRC-1 super-shifted complex induced by LGD1069 (compare lanes 5-10 and lanes 12-17).
DISCUSSION

We have identified a specific, high-affinity PPARγ antagonist, T0070907, that blocks PPARγ activity in both biochemical and cell-based assays. T0070907 is highly selective for PPARγ over PPARα, PPARδ, other NHRs, and proteins in an HEK293 WCE. Proteolytic mapping indicated that T0070907 irreversibly modifies PPARγ on Cys313 in helix 3 of the LBD, a residue that is conserved in all three PPAR subtypes. This indicates that other residues in the binding pocket confer the specific binding of T0070907 to PPARγ. Interestingly, this is also the site of covalent modification by L-764406, a PPARγ partial agonist that was previously described (12).

T0070907 functions as a PPARγ antagonist in cell-based assays. It effectively blocked TZD-induced transactivation by the GAL4-PPARγ LBD, as well as adipogenesis in 3T3-L1 cells treated with a differentiation cocktail. Overall, these results further support the important role for PPARγ in fat cell differentiation. The antagonist properties of T0070907 were also demonstrated in a variety of in vitro biochemical assays using the PPARγ LBD. T0070907 suppressed agonist-induced interactions between the PPARγ LBD and coactivator-derived peptides, and promoted recruitment of
corepressor-derived peptide in HTRF assays (Fig. 4). The effect of T0070907 on assembly of corepressor NCoR/PPARγ complex was also observed in the pull-down assay (Fig. 4D).

Previous studies have suggested that corepressors bind to a hydrophobic groove on NHR LBDs formed by H3, H5, and H6. This binding site partially overlaps with that utilized by coactivators (29, 30, 31). NHR agonists disrupt the interaction between NHR LBDs and corepressors and it is believed that the conformation of H12 stabilized by agonists partially occludes the corepressor binding site. In the unliganded state, H12 of NHR LBDs is thought to exist in multiple conformations, including the agonist-bound conformation. Consistent with this hypothesis, H12 is inhibitory for NCoR binding to most NHRs. Mutations and deletions of H12 from either PPARγ or RXRα significantly increase the recruitment of NCoR to the PPARγ/RXRα heterodimer (32, 9) (Fig. 5). T0070907 can also promote NCoR recruitment to PPARγ/RXRα heterodimer, but T0070907 can only promote recruitment of NCoR to complexes containing wt PPARγ but not to complexes containing PPARγ ΔH12. These results suggest that the effect of T0070907 on the heterodimer is indeed mediated through PPARγ and that T0070907 induced NCoR recruitment requires H12. Indeed, T0070907
treatment or the deletion of H12 stabilize NCoR recruitment to comparable extents (Fig 5) suggesting that T0070907 most likely acts on PPARγ by preventing H12 from adopting the agonist-bound conformation. The deletion of RXRα H12 domain has a synergistic effect with either T0070907 treatment or PPARγ ΔH12 on the recruitment of NCoR to PPARγ/RXRα heterodimer (Fig. 5B). Two NHR binding motifs are present on NCoR protein (29, 30, 31), the deletion of H12 from RXRα together with either T0070907 treatment or the deletion of H12 from PPARγ perhaps allows the cooperative binding of both motifs to PPARγ/RXRα heterodimer.

In order to dissect the contributions of the PPARγ and RXRα to NCoR recruitment to the heterodimer, the effects of simultaneous treatment with T0070907 and LGD1069 were determined. Notably, LGD1069 dramatically inhibited the T0070907-mediated increase in NCoR recruitment to the PPARγ wt/RXRα ΔH12 and PPARγ ΔH12/RXRα ΔH12 heterodimers in a dose-dependent manner (Fig. 5C). Recent X-ray crystallographic studies of the apo-RXRα LBD (unliganded), the holo-RXRα LBD (agonist bound), and a PPARγ/RXRα heterodimer (each bound to agonist) suggest possible molecular mechanisms for these effects. The rosiglitazone bound PPARγ/9-cis retinoic acid (9cRA) bound RXR heterodimer interface which is largely
composed of residues from H10 and H11 of both receptors contains several important salt bridges. In particular, a salt bridge formed between the carboxylic acid of Y477 from PPARγ H12 and K431 from RXRα H10 stabilizes the positioning of H12 from PPARγ in the agonist-bound conformation (33). In addition, comparison of the apo- and holo-RXRα LBD structures reveal that ligand-binding triggers several large conformational changes. For example, H11, which partially fills the ligand binding pocket in the apo-RXRα structure, moves out of the binding pocket and rotates by ~180° around its own axis upon binding of 9cRA, allowing H10 and H11 to form an almost continuous helix (34). Although the structure of a PPARγ/apo-RXRα heterodimer has not yet been described, these structural results suggest that LGD1069 binding could lead to significant alterations in the PPARγ/RXRα heterodimer interface. Given that K431 is located near the site of the conformational changes involving H10 and H11, RXR agonists could also influence the stability of the Y477/K431 salt bridge and hence the positioning of PPARγ H12. Thus, we suggest that LGD1069 binding inhibits binding of NCoR to the wild-type heterodimer (with or without T0070907) by orienting PPARγ and RXRα such that binding of NCoR is disfavored and by stabilizing PPARγ H12 in the agonist-bound conformation. Consistent with this view, the effect of
LGD1069 on T0070907-induced recruitment of NCoR is more potent on the PPARγ wt/RXRα ΔH12 heterodimer than on PPARγ ΔH12/RXRα ΔH12 heterodimer. In addition, a residual amount of NCoR remained on PPARγ ΔH12/RXRα ΔH12 heterodimer even at the highest LGD1069 concentrations (Fig. 5C), and LGD1069 was also ineffective in preventing NCoR binding to PPARγ ΔH12/RXRα wt heterodimer complexes (data not shown).

The effect of T0070907 on LGD1069-induced recruitment of coactivator SRC-1 was also tested. While the T0070907 induced NCoR recruitment to PPARγ/RXRα heterodimer can be almost completely reversed by the simultaneous treatment with RXRα agonist LGD1069, the effects of T0070907 on LGD1069 induced coactivator recruitment to the PPARγ/RXRα heterodimer are more modest by comparison. These results suggest that RXRα agonists may have a greater influence on the conformation of the PPARγ/RXRα heterodimer than do PPARγ antagonists, and more importantly, PPARγ antagonist activity could be modulated by the availability and concentration of RXRα agonist, in vivo relevance of these effects are the focus of our current and future studies.
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**Table 1.** List of amino acid sequences and sources of peptides used in the HTRF peptide profiling assay. The LXXLL motif is in bold. Peptides are tagged on the N-termini with biotin.

**Figure legends**

Fig. 1. Chemical structure of T0070907 and analysis of its binding to PPARs. *A*, chemical structure of T0070907. *B*, the affinities of T0070907 for the various PPAR subtypes were determined with an SPA assay. $[^{3}H]$GW2433 (5 nM) was used as a ligand for PPAR$\alpha$ and PPAR$\delta$, and $[^{3}H]$rosiglitazone (5 nM) was used for PPAR$\gamma$.

Fig. 2. T0070907 is a specific potent PPAR$\gamma$ antagonist. *A*, effects of T0070907 on transcriptional activities of the LBDs of PPAR$\gamma$, PPAR$\alpha$, PPAR$\delta$, FXR, LXR$\alpha$, LXR$\beta$, or PXR$\gamma$ fused with Gal4 protein on Gal4-UAS-luciferase reporter gene expression in HEK293 cells. *B*, effects of T0070907 on Dex/Mix/Ins-induced 3T3-L1 cell differentiation. Top panel, 3T3-L1 differentiation protocol. Cells were stained with Nile Red and photographed under light microscope.
Fig. 3. T0070907 covalently binds to PPARγ at Cys^{313}. A, Nano-ESI-MS/MS spectrum of tryptic fragment modified by T0070907. The calculated y- and b-ions of this peptide are shown at the top of the figure, with ions observed in the mass spectrum underlined. Cys^{313} is indicated by *. B, wt or C313S mutant PPARγ proteins were incubated with [³H]T0070907 and subsequently separated on an SDS-polyacrylamide gel, which was then subjected to autoradiography. Upper panel, autoradiography of the SDS-polyacrylamide gel. [³H]T0070907 bound covalently only to wt PPARγ. Lower panel, coomassie blue-stained same gel showing equal loading of wt and mutant PPARγ proteins. C, purified GST-PPARγ mixed with whole-cell extract (WCE) derived from HEK293 cells was incubated with 2 µM [³H]T0070907 and subsequently separated on an SDS-polyacrylamide gel, which was then subjected to autoradiography. Upper panel, autoradiography of the gel. Only GST-PPARγ was preferentially modified by [³H]T0070907. Lower panel, coomassie blue staining of the same gel.

Fig. 4. T0070907 is an inverse agonist of the GST-PPARγ LBD in vitro. A, HTRF peptide profiling of T0070907, rosiglitazone, troglitazone, and GI262570. All compounds were tested at 1 µM, and peptides (y-axis) were in numeric order as listed in Table 1. Corepressor-derived peptides #2 and
#3 are shown in black.  

B, dose-response of rosiglitazone and T0070907 in the presence and absence of 1 μM rosiglitazone in an HTRF assay with GST-PPARγ LBD and peptide #1 (DRIP205).  

C, same as in (B) except that the HTRF peptide is #2 (NCoR).  

D, dose-responses of rosiglitazone and T0070907 in a GST pull-down assay that measures the interaction between GST-PPARγ and NCoR protein.  An arrow indicates the position of the NCoR protein.

Fig. 5.  Effects of T0070907 on PPARγ/RXRα heterodimer in a GMSA.  

A, effects of T0070907 on the recruitment of NCoR to the wt PPARγ/wt RXRα and PPARγ ΔH12/wt RXRα heterodimers.  

B, effects of T0070907 on the recruitment of NCoR to the wt PPARγ/RXRα ΔH12 and PPARγ ΔH12/RXRα ΔH12 heterodimer complexes.  

C, effects of LGD1069 on T0070907 induced recruitment of NCoR to the wt PPARγ/RXRα ΔH12 heterodimer complex.  

D, effects of T0070907 on LGD1069-induced recruitment of SRC-1 to the PPARγ/RXRα heterodimer complex.  Arrows indicate the positions of free probe and various complexes.
References

1. Rosen, E. D., and Spiegelman, B. M. (2001) *J Biol Chem* **276**, 37731-37734
2. Nuclear Receptors Nomenclature Committee. (1999) *Cell* **97**, 161-163
3. Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, K., Lefebvre, A. M., Saladin, R., Najib, J., Laville, M., Fruchart, J. C., Deeb, S., Vidal-Puig, A., Flier, J., Briggs, M. R., Staels, B., Vidal, H., and Auwerx, J. (1997) *J Biol Chem* **272**, 18779-18789
4. Fajas, L., Fruchart, J. C., and Auwerx, J. (1998) *FEBS Lett* **438**, 55-60
5. Lowell, B. B. (1999) *Cell* **99**, 239-242
6. Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998) *Nature* **395**, 137-143
7. Zamir, I., Zhang, J., and Lazar, M. A. (1997) *Genes Dev* **11**, 835-846
8. DiRenzo, J., Soderstrom, M., Kurokawa, R., Ogliastro, M. H., Ricote, M., Ingrey, S., Horlein, A., Rosenfeld, M. G., and Glass, C. K. (1997) *Mol Cell Biol* **17**, 2166-2176
9. Zhang, J., Hu, X., and Lazar, M. A. (1999) *Mol Cell Biol* **19**, 6448-6457
10. Willson, T. M., Lambert, M. H., and Kliwer, S. A. (2001) *Annu Rev Biochem* **70**, 341-367
11. Oberfield, J. L., Collins, J. L., Holmes, C. P., Goreham, D. M., Cooper, J. P., Cobb, J. E., Lenhard, J. M., Hull-Ryde, E. A., Mohr, C. P., Blanchard, S. G., Parks, D. J., Moore, L. B., Lehmann, J. M., Plunket, K., Miller, A. B., Milburn, M. V., Kliwer, S. A., and Willson, T. M. (1999) *Proc Natl Acad Sci U S A* **96**, 6102-6106
12. Elbrecht, A., Chen, Y., Adams, A., Berger, J., Griffin, P., Klatt, T., Zhang, B., Menke, J., Zhou, G., Smith, R. G., and Moller, D. E. (1999) *J Biol Chem* **274**, 7913-7922
13. Wang, Y., Porter, W. W., Suh, N., Honda, T., Gribble, G. W., Leesnitzer, L. M., Plunket, K. D., Mangelsdorf, D. J., Blanchard, S. G., Willson, T. M., and Sporn, M. B. (2000) *Mol Endocrinol* **14**, 1550-1556
14. Rocchi, S., Picard, F., Vamecq, J., Gelman, L., Potier, N., Zeyer, D., Dubuquoy, L., Bac, P., Champy, M. F., Plunket, K. D., Leesnitzer, L. M., Blanchard, S. G., Desreumaux, P., Moras, D., Renaud, J. P., and Auwerx, J. (2001) *Mol Cell* **8**, 737-747
15. Wright, H. M., Clish, C. B., Mikami, T., Hauser, S., Yanagi, K., Hiramatsu, R., Serhan, C. N., and Spiegelman, B. M. (2000) *J Biol Chem* **275**, 1873-1877
16. Camp, H. S., Chaudhry, A., and Leff, T. (2001) *Endocrinology* **142**, 3207-3213
17. Papac, D. I., Wong, A., and Jones, A. J. (1996) *Anal Chem* **68**, 3215-3223
18. Wilm, M., Neubauer, G., and Mann, M. (1996) *Anal Chem* **68**, 527-533
19. Zhou, G., Cummings, R., Li, Y., Mitra, S., Wilkinson, H. A., Elbrecht, A., Hermes, J. D., Schaeffer, J. M., Smith, R. G., and Moller, D. E. (1998) *Mol Endocrinol* **12**, 1594-1604
20. Janowski, B. A., Grogan, M. J., Jones, S. A., Wisely, G. B., Kliewer, S. A., Corey, E. J., and Mangelsdorf, D. J. (1999) *Proc Natl Acad Sci U S A* **96**, 266-271
21. Brown, P. J., Smith-Oliver, T. A., Charifson, P. S., Tomkinson, N. C., Fivush, A. M., Sternbach, D. D., Wade, L. E., Orband-Miller, L., Parks, D. J., Blanchard, S. G., Kliewer, S. A., Lehmann, J. M., and Willson, T. M. (1997) *Chem Biol* **4**, 909-918
22. Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) *Nature* **358**, 771-774
23. Yang, W., Rachez, C., and Freedman, L. P. (2000) *Mol Cell Biol* **20**, 8008-8017
24. Rubin, C. S., Hirsch, A., Fung, C., and Rosen, O. M. (1978) *J Biol Chem* **253**, 7570-7578
25. Robyr, D., Wolffe, A. P., and Wahli, W. (2000) *Mol Endocrinol* **14**, 329-347
26. Rosenfeld, M. G., and Glass, C. K. (2001) *J Biol Chem* **276**, 36865-36868
27. Willson, T. M., Brown, P. J., Sternbach, D. D., and Henke, B. R. (2000) *J Med Chem* **43**, 527-550
28. Boehm, M. F., Zhang, L., Badea, B. A., White, S. K., Mais, D. E., Berger, E., Suto, C. M., Goldman, M. E., and Heyman, R. A. (1994) *J Med Chem* **37**, 2930-2941
29. Perissi, V., Staszewski, L. M., McInerney, E. M., Kurokawa, R., Krones, A., Rose, D. W., Lambert, M. H., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1999) *Genes Dev* **13**, 3198-3208
30. Nagy, L., Kao, H. Y., Love, J. D., Li, C., Banayo, E., Gooch, J. T., Krishna, V., Chatterjee, K., Evans, R. M., and Schwabe, J. W. (1999) *Genes Dev* **13**, 3209-3216
31. Hu, X., and Lazar, M. A. (1999) *Nature* **402**, 93-96
32. Gurnell, M., Wentworth, J. M., Agostini, M., Adams, M., Collingwood, T. N., Provenzano, C., Browne, P. O., Rajanayagam, O., Burris, T. P., Schwabe, J. W., Lazar, M. A., and Chatterjee, V. K. (2000) *J Biol Chem* **275**, 5754-5759
33. Gampe, R. T., Jr., Montana, V. G., Lambert, M. H., Miller, A. B., Bledsoe, R. K., Milburn, M. V., Kliewer, S. A., Willson, T. M., and Xu, H. E. (2000) *Mol Cell* **5**, 545-555
34. Egea, P. F., Mitschler, A., Rochel, N., Ruff, M., Chambon, P., and Moras, D. (2000) *Embo J* **19**, 2592-2601
Fig. 1A

T0070907

Fig. 1B

Fig. 2A
Day 0                    Day 2                  Day 4                                                   Day 13
Plate cells           DEX/MIX/INS                INS                                                    Harvest

Fig. 2B

DMSO                   DEX/MIX/INS               DEX/MIX/INS + T0070907
Fig. 3A

Fig. 3B

\[^{3}H\] T070907 [1\mu M] + +
C313S GST-hPPAR\(\gamma\) LBD +
WT GST-hPPAR\(\gamma\) LBD +

Coomassie

1 2
Table 1

| Protein      | Sequence                  |
|--------------|---------------------------|
| 1  DHRP205   | GNTKNHPLMNLLKDNPADQFC     |
| 2  NCOR      | ADPSNLGLEDIRKALMGSCF     |
| 3  SMRT      | EAHSTMGLEAIRKALMGKYC     |
| 4  TIF1      | NANYPRSLTSLGNSSQSTC     |
| 5  NGFI-B    | GEPQASCLSLGGKLPERTC     |
| 6  Nurr-1    | GGLNRPNLKLGKPLERTC     |
| 7  SRC1-1    | KSQSTSHKLQLTTTAEQGLC   |
| 8  SRC1-2    | SLTERKHLRLLQEGSPDIC     |
| 9  SRC1-3    | KESKDHLLRKYLDKEKDLRC    |
| 10 SRC2-1   | HDSKGQTLQGLLLTTSQDMEC   |
| 11 SRC2-2   | SLKEKHLRHLLQDSSPVDIC    |
| 12 SRC2-3   | PKKKENALRLYLLDKDDTDICI  |
| 13 SRC3-1   | LSEKGHKLLQQLTCSSDRGC   |
| 14 SRC3-2   | LLQEKHRILHKLQLQGNSPAC   |
| 15 SRC3-3   | PKKVENALLRLYLLDREPDIC   |
| 16 CBP       | DAASKHQLSSELRGGSGSCI    |
| 17 p300-mutant | DAAASKHQLSSELLRGSSPSNL |
| 18 CBP-mutant | DAASKHQLSSEARGGSGSCI   |
| 19 Tip60     | GHERAMKLRLRDSKCLHC      |
| 20 Jun-a     | EVSYFKSSDLKKLLLWNYWGC   |
| 21 Jun-j     | FKSSDLKLLLELWNYWVNTC    |
| 22 NKF3      | MPVKRSKLQGLLEENSFPSC    |
| 23 ASC-2 (2) | AMLREAPTLSSLQDLNSGAPNV  |
| 24 APA70     | LQQGAQQLYSLLQGFOCNLTHC  |
| 25 RIP1 40 (1) | QDSIYTVLEQLMHQAAGGS     |
| 26 RIP1 40 (2) | KGKQDSTLLASSLQSFSSRLQC  |
| 27 RIP1 40 (3) | CVGASHLKTLKKSKVKDQC    |
| 28 RIP1 40 (4) | KPSVACSLALLSSHAEHLQQC  |
| 29 RIP1 40 (7) | NLEERTYQLLLGNTKGRVC    |
| 30 RIP1 40 (8) | FSFSNGLSSRLRLONQDSYL    |
| 31 RIP1 40 (9) | RESKSFNYKLQLLLSENSVCRDC |
| 32 PEA       | GPRGMGTAKLKLLAGAVAYGC   |
Fig. 4A

- **DMSO**
- **Rosiglitazone**
- **Troglitazone**
- **GI262570**

The graphs show the HTRF units for different peptides across the x-axis, ranging from 1 to 31, with y-axis values ranging from 0 to 160.
**Fig. 5C**

| Condition                  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|----------------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|
| PPARγ wt                   | - | + | + | + | + | + | + | + | + | +  | +  | -  | +  | +  | +  | +  | +  | +  | +  | +  |
| PPARγ ΔH12                 | - | + | + | + | + | + | + | + | + | +  | +  | -  | +  | +  | +  | +  | +  | +  | +  | +  |
| RXRα ΔH12                  | - | + | + | + | + | + | + | + | + | +  | +  | -  | +  | +  | +  | +  | +  | +  | +  | +  |
| NCoR                       | - | + | + | + | + | + | + | + | + | +  | +  | -  | +  | +  | +  | +  | +  | +  | +  | +  |
| T0070907 (0.3 μM)          | - | + | + | + | + | + | + | + | + | +  | +  | -  | +  | +  | +  | +  | +  | +  | +  | +  |
| LGD1069                    | - | + | + | + | + | + | + | + | + | +  | +  | -  | +  | +  | +  | +  | +  | +  | +  | +  |

**Free Probe**
Fig. 5D

|                  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|
| PPARγ/RXRα      | - | + | + | + | + | + | + | + | + | +  | +  | +  | +  | +  | +  | +  | +  |
| SRC-1           | - | + | + | + | + | + | + | + | + | +  | +  | +  | +  | +  | +  | +  | +  |
| BRL (3μM)       | + |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| T0070907 (1μM)  | + | + | + | + | + | + | + | + | + | +  | +  | +  | +  | +  | +  | +  | +  |
| LGD1069         | + |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

Free Probe

1-17
T0070907, A selective ligand for peroxisome proliferator-activated receptor g, functions as an antagonist of biochemical and cellular activities
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