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Synthesis of a High-Affinity Fluorescent PPARγ Ligand for High-Throughput Fluorescence Polarization Assays

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Abstract—Members of the peroxisome proliferator activated receptor (PPAR) family of transcription factors are under investigation as molecular targets for the treatment of numerous diseases including Alzheimer's, asthma, atherosclerosis, inflammation, multiple sclerosis, cancer, and diabetes. We employed the X-ray crystal structure of the PPARγ subtype complexed with the potent small molecule agonist GI262570 (farglitazar) to design and synthesize a novel fluorescent and high-affinity probe for homogeneous and high-throughput fluorescent polarization (FP) assays. Examination of this X-ray structure revealed that the phenyl carbon atom meta to the oxazole moiety of GI262570 is exposed to solvent at the bottom of a narrow protein cavity. A derivative of GI262570 was synthesized bearing a linear phenylacetylene-derived side chain comprising propargylamine coupled to fluorescein. This fluorescent analogue was designed to project the fluorophore into the adjacent protein cavity with minimal effects on receptor affinity and maximal effects on fluorescence polarization properties. The recombinant PPARγ ligand binding domain protein bound tightly and specifically to this probe with $K_d = 61 \pm 14$ nM as determined by FP measurements. Competition binding assays with known PPARγ ligands provided K_i values that were highly correlated with analogous values obtained by scintillation proximity (SP) assays. This fluorescent PPARγ probe enables high-throughput and homogenous FP assays for the identification of novel endogenous and exogenous PPARγ ligands, and this rational ligand design approach may be applied to other therapeutically important members of the nuclear hormone receptor superfamily.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-regulated transcription factors.[1–7] These proteins comprise an important class of therapeutic targets thought to be involved in Alzheimer's disease, asthma, atherosclerosis, inflammation, multiple sclerosis, cancer, and diabetes.[8–11] The PPARγ subtype is involved in glucose and lipid homeostasis, adipocyte differentiation, and insulin sensitivity.[12] The binding of endogenous ligand(s) such as Prostaglandin J_2 to PPARγ results in the formation of heterodimers with RXR proteins, interaction with co-activators, and regulation of the expression of target genes.[11,13–19] Several antidiabetic drugs including the thiazolidinediones[20–27] and the more recently described GI262570[28–30] (**1**, Fig. 1) exert their therapeutic effects by targeting the PPARγ subtype. Given the importance of PPARγ in human disease, significant effort is currently focused on the discovery of improved therapeutics that modulate activities of this receptor and the identification of natural endogenous ligands. To facilitate these studies, we report here the design, synthesis, binding properties, and utility of compound **2**, a derivative of GI262570 as a fluorescent PPARγ ligand suitable for high-throughput fluorescence polarization (FP) assays directed at the discovery of novel endogenous and exogenous PPARγ ligands.

Ligand Design

We employed the X-ray crystal structure of the heterodimeric ligand binding domain (LBD) co-complex of RXRα/9-*cis*-retinoic acid, PPARγ/GI262570, and an

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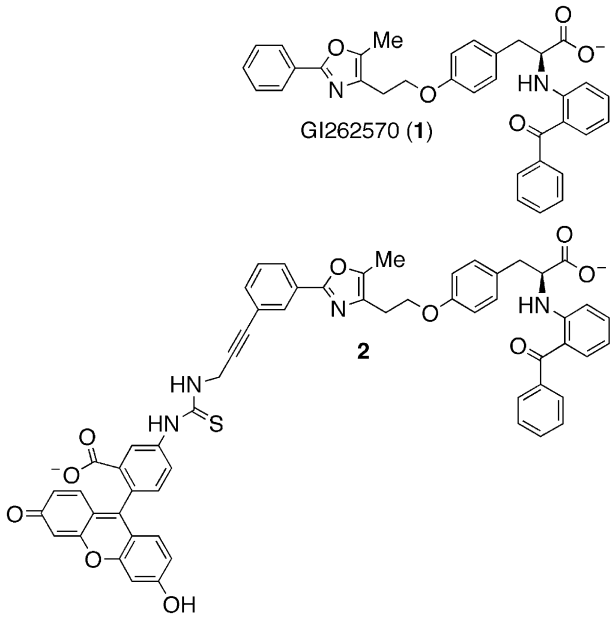


Figure 1. Structures of PPARγ ligands. The novel fluoresent probe **2** was designed from the X-ray crystal structure of PPARγ-bound **1**.

SRC-1 peptide,[10,31,32] to design the fluorescent ligand **2** as a PPARγ probe. The GI262570 agonist was chosen for modification because of its high affinity ($K_i = 1.2$ nM)[28] for PPARγ and the availability of high-resolution structural information regarding the protein-bound complex (Fig. 2). As shown in Figure 2, examination of the PPARγ–GI262570 subunit revealed that the protein-bound drug is nearly completely encapsulated by protein except on one face of the phenyl oxazole moiety. A phenyl carbon atom meta to the oxazole moiety is exposed to the aqueous environment, and this carbon lies at the bottom of a ~11-Å deep cavity with minimal dimensions of ~4 by ~9 Å (Fig. 2). This structural information indicated that a linear functional group might be installed at this meta carbon to project a fluorophore into the adjacent empty protein cavity to minimize unfavorable interactions between the fluorophore-terminated side chain and the protein. Moreover, modeling indicated that the relatively flat fluorophore derived from fluorescein isothiocyanate might be rotationally restricted upon protein binding, potentially providing a strong effect on the fluorescence polarization properties of the probe.

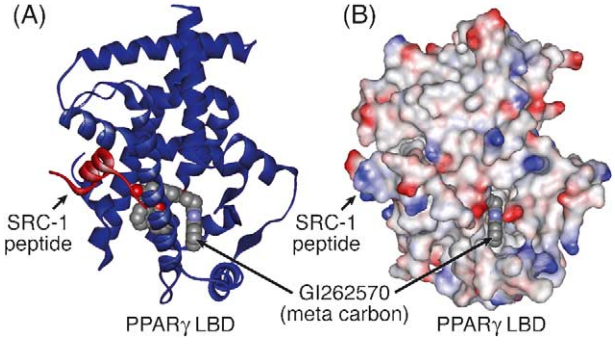


Figure 2. X-ray crystal structure of the PPARγ GI262570 complex (PDB# 1FM9). The solvent-exposed carbon atom meta to the oxazole of bound GI262570 is shown in the ribbon model (A) and electrostatic surface model (B).

Synthesis

Preparation of **2** commenced with optimization of the synthesis of 3-iodobenzamide **6**[33,34] as outlined in Scheme 1. This product was cyclized with bromoketoester **4** prepared from **3** as previously reported[35] to afford the *meta*-iodophenyloxazole **7**. Reduction of **7** with lithium borohydride provided the primary alcohol **8**, which was subjected to a Mitsunobu reaction[36–38] with phenol **9**, prepared as previously reported.[28] The linked methyl ester product **10** was hydrolyzed to acid **11**. This acid (**11**) was synthesized as an analogue of GI262570 (**1**) to provide a high-affinity iodo-modified ligand of PPARγ for subsequent competition binding experiments. Ester **10** was also modified by Sonagashira coupling[39,40] with Boc-protected propargylamine (**12**)[41] to provide the side-chain coupled product **13**. The methyl ester group of **13** was hydrolyzed under basic conditions, the Boc-carbamate removed under acidic conditions, and the resulting amine acylated with fluorescein isothiocyanate (FITC) to afford the fluorescein-modified GI262570 derivative **2** (Scheme 1) in 7.8% yield over 10 steps. This compound was isolated as the weakly fluorescent free acid form shown in Scheme 1. Dissolution of **2** in buffer at pH 7.4 generates the highly fluorescent anionic tautomer shown in Figure 1.

Fluorescence Polarization Assay Development

To examine the potential effectiveness of compound **2** as a fluorescent probe, the FP properties of this compound were initially evaluated as a function of glycerol concentration. This highly viscous solvent slows the molecular tumbling rate, and these solutions provided a method to examine the dynamic range of FP values.[42] As shown in Figure 3, the FP of **2** was highly sensitive to glycerol, indicating that a reduction in tumbling rate resulting from protein binding might be readily detected by FP measurements. This suggested that **2** would comprise a good candidate for FP studies in biochemical systems.

To optimize the conditions required for use of **2** as a fluorescent PPARγ probe, fluorescence intensity and fluorescence polarization (mP) values were quantified as a function of the concentration of **2**. As shown in Figure 4 (Panel A), fluorescence intensity increased linearly with respect to compound concentration whereas FP values were insensitive to concentrations of **2** at or below 400 nM. Furthermore, a probe concentration of 100 nM appeared to be optimal for further experiments. To examine binding of PPARγ to this probe, the murine PPARγ LBD was added to buffer containing **2** at 100 nM. The affinity (K_d) of **2** for the PPARγ LBD was determined to be 61 ± 14 nM by non-linear least squares curve fitting of the data shown in Figure 4 (Panel B). Competition with 50 μM of the non-fluorescent iodo-substituted precursor (**11**) substantially reduced FP values,

confirming the specificity of the small molecule–protein interaction. Based on these results, the optimal PPARγ concentration for subsequent competition experiments was determined to be ~400 nM (~15 ng/μL).

To validate the high-affinity probe **2** in a competition assay format, the K_i of the iodo precursor **11** for the PPARγ LBD was measured. Displacement of the bound probe **2** by specific ligands such as **11** should diminish observed mP values. As shown in Figure 5 (Panel A), titration of **11** provided $K_i = 50 \pm 10$ nM, which confirmed that specific PPARγ ligands can be identified by competition with **2**. Competition studies with a series of known PPARγ ligands such as conjugated linoleic acids (CLA) analyzed in high-throughput format on a Packard Fusion microtiter plate reader provided the displacement curves shown in Figure 5 (Panel B). The structures of these ligands are shown in Figure 6.

The K_i values determined from competition binding experiments were compared with previously reported scintillation proximity (SP) competition assays.[43] As shown in Table 1, an excellent correlation was generally observed between the K_i values determined by FP and analogous SP measurements. However, analysis of these compounds by FP assay was found to better predict the biological effects of compounds under investigation. For example, the 9Z11Z–CLA isomer is known to exhibit significant activity in a PPARγ-mediated model of adipogenesis.[44] In contrast, the 9E11E–CLA isomer

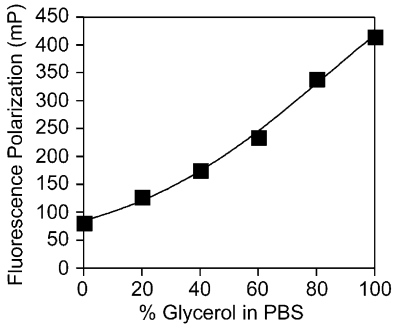


Figure 3. Fluorescence polarization calibration curve of **2** (100 nM) in glycerol/phosphate-buffered saline (pH 7.4). The high viscosity of glycerol decreases the tumbling rate of the fluorescent small molecule to enhance the measured FP value.

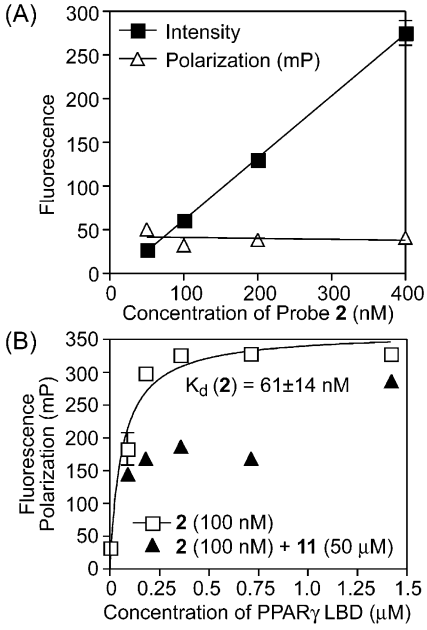
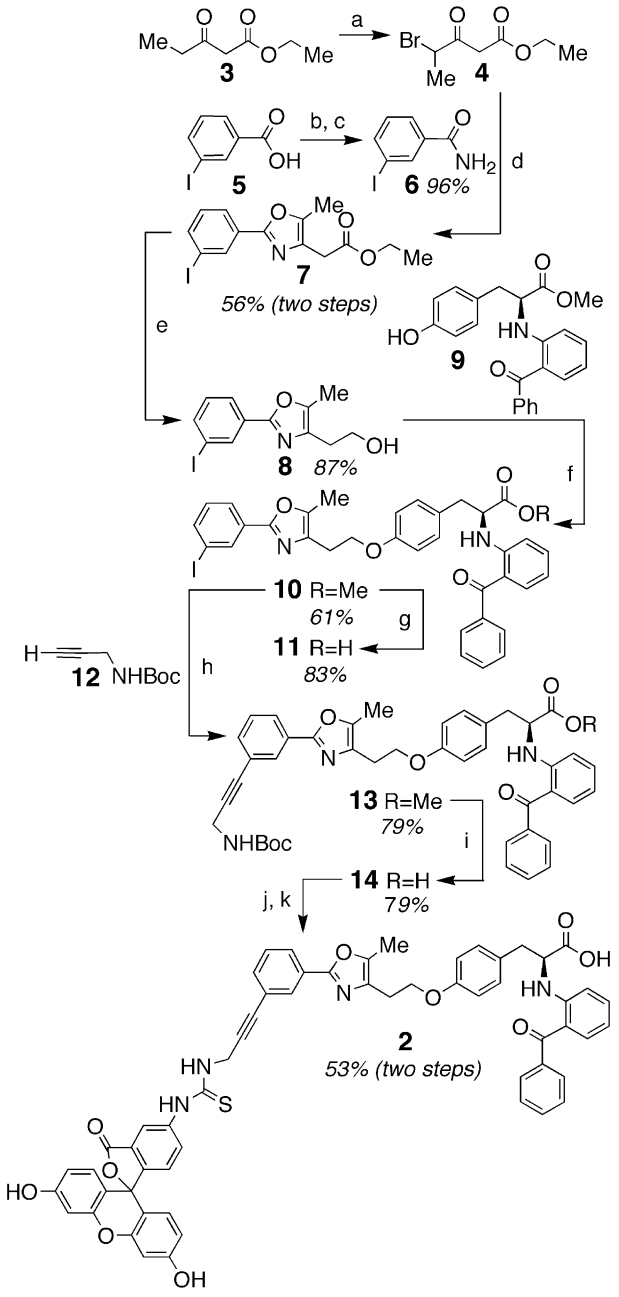


Figure 4. Optimization of conditions for fluorescence polarization assays. Panel A: Fluorescence intensity and polarization values as a function of the concentration of **2** in PBS (pH 7.4). Panel B: Fluorescence polarization values from addition of mPPARγ LBD to **2** (100 nM) in PBS. In competition experiments, the non-fluorescent compound **11** was added at a final concentration of 50 μM.



Scheme 1. Reagents: (a) $Br_2/CHCl_3$, 0–25 °C, 17 h; (b) $SOCl_2$, reflux, 3 h; (c) liquid NH_3, −78 to −55 °C; (d) 125 °C, 4 h; (e) $LiBH_4$, THF, 50 °C, 1.5 h; (f) DEAD, PPh_3, THF, 24 h; (g) LiOH, THF/MeOH (3:1), 3 h. (h) $Pd(PPh_3)_2Cl_2$, CuI, TEA, 60 °C, 0.5 h; (i) LiOH, THF/MeOH, (3:1) 3 h; (j) TFA, CH_2Cl_2, 0.5 h; (k) FITC, TEA, EtOH/THF (3:2) 3.5 h.

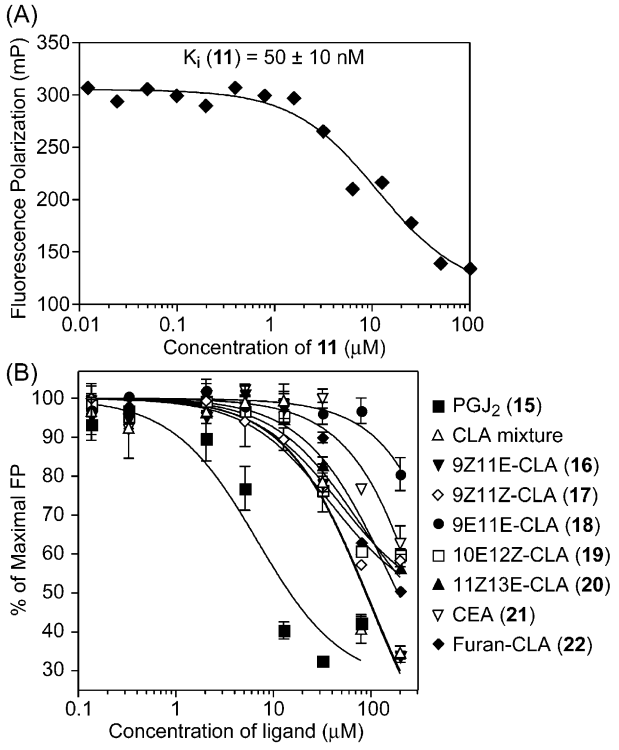


Figure 5. High-throughput competition binding experiments employing fluorescent probe **2** (100 nM). Panel A: Determination of K_i for compound **11**. Panel B: Competition assays with known PPARγ ligands. PGJ_2, prostaglandin J_2. CLA, conjugated linoleic acid. The CLA mixture includes 9Z11E–CLA (35%), 10E12Z–CLA (35%), and 9Z11Z–CLA + 9E11E–CLA (5%).

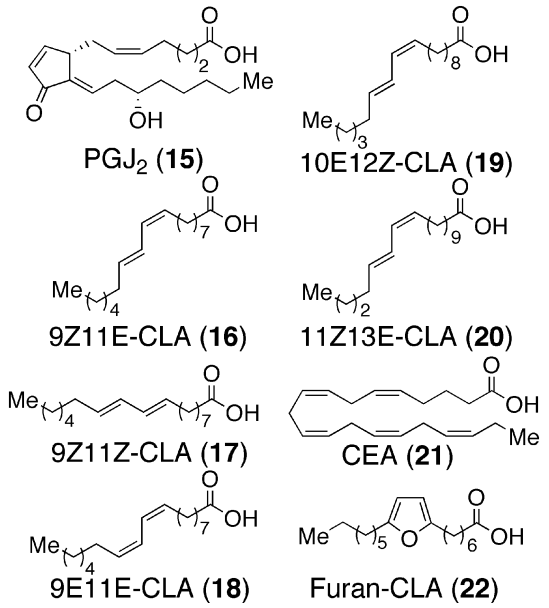


Figure 6. Structures of prostaglandin J_2 (**15**) and conjugated linoleic acid (**16**–**22**) ligands of PPARγ.

is known to exhibit much lower activity in this model. Although the SP assay does not indicate significant differences between these structurally similar isomers, the FP assay with **2** indicated a 200-fold difference in K_i (Table 1). These results illustrate the advantages of **2** as a probe for high-throughput screening by FP, and

Table 1. Comparison of fluorescence polarization (FP) and previously reported[43] scintillation proximity (SP) assays with PPARγ in a competitive binding format

PPARγ ligand	FP assay K_i (μM)	SP assay K_i (μM)
PGJ_2	0.63	0.34
CLA mixture	9.22	N/A
9Z11E-CLA	9.14	6.45
9Z11Z-CLA	3.63	N/A
9E11E-CLA	731	4.70
10E12Z-CLA	4.54	7.10
11Z13E-CLA	7.03	N/A
Furan-CLA	14.6	5.50
CEA	62.8	N/A

N/A, data not available.

demonstrate that this probe is sensitive to subtle differences in molecular interactions in solution that allow detailed analysis of equilibrium binding events.

Discussion

We report the synthesis of a fluorescent PPARγ ligand (**2**) suitable for high-throughput fluorescence polarization (FP) assays. This assay format was investigated because FP has recently emerged as one of the best methods for quantifying molecular interactions in solution.[42] The FP method enables rapid, non-radioactive determination of the affinities of small molecules for proteins in homogeneous solution-phase assays using either direct or competitive binding experiments. This technique exploits the observation that fluorescent molecules excited with plane-polarized light emit photons in the same plane provided that the molecule remains stationary throughout the excited state (~4 nanoseconds for the fluorescein fluorophore).[45] Small fluorescent molecules tumble rapidly in solution, which causes photons to be emitted in a different plane from that of the initial excitation. In contrast, large molecules such as proteins move little during the exited state interval, and emitted photons remain highly polarized with respect to the excitation plane. Hence, small fluorescent molecules are depolarized when free in solution due to rapid tumbling but become highly polarized upon binding to a slowly tumbling protein receptor. This change in FP allows highly accurate determination of the affinities for protein receptors of both fluorescent small molecules and non-fluorescent small molecules that compete for binding with a fluorescent probe to a macromolecular target. The homogeneous format of FP assays facilitates high-throughput screening of small molecule ligands against cognate protein receptors.

Compound **2** was designed based on an X-ray crystal structure of the PPARγ ligand binding domain bound to the small molecule agonist GI262570. A solvent exposed atom of this bound compound at the bottom of an open and narrow protein channel was modified with a linear alkyne linker that was coupled to the fluorescein fluorophore. This modification was designed to minimize loss of affinity upon binding of the fluorescent probe to the protein. Comparison of the K_i value of the related iodo-substituted precursor **11** (50±10 nM) with

the K_d of fluorescent probe **2** (61 ± 14 nM) revealed that modification of this compound with the linear side chain and appended fluorophore resulted in only a slight reduction in affinity for the receptor. The K_i values of known PPARγ ligands were measured in high-throughput competition FP assays and compared with previously reported scintillation proximity (SP) assays. This comparison revealed that the K_i values measured by FP more closely paralleled the biological activity of PPARγ ligands in whole-cell assays. The application of fluorescent probe **2** in high-throughput assay formats may significantly facilitate the discovery of endogenous and exogenous PPARγ ligands. Moreover, the rational ligand design approach described herein holds significant potential for the generation of other fluorescent probes of nuclear hormone receptors.

Materials and Methods

General

All reactions, except those in aqueous media, were performed under an argon atmosphere using standard techniques for the exclusion of moisture. All commercial reagents for synthesis were purchased from Aldrich (Milwaukee, WI) or Acros (Morris Plains, NJ) and used without further purification. The protein ligands Prostaglandin J_2 (BioMol, Plymouth Meeting, PA), CLA mixture (Pharmanutrients, Lake Bluff, IL), and conjugated linoleic acids (Sigma) were obtained from commercial sources. Tetrahydrofuran and diethyl ether were distilled from sodium benzophenone ketyl under nitrogen. Dichloromethane, triethylamine, and hexanes were distilled from calcium hydride under nitrogen. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (EM Science, Kansas City, MO, 0.25 mm). ICN (Costa Mesa, CA) SiliTech Silica Gel (32–33 μm) was employed for column chromatography. All ^1H and ^{13}C NMR Spectra were recorded using Bruker DPX-300, AMX-360, DRX-400, and AMX-2-500 MHz spectrometers at ambient temperature (22 °C). NMR signals were referenced to internal $CHCl_3$ (δ_H 7.27) and $CDCl_3$ (δ_C 77.23) or DMSO-d_5 (δ_H 2.50) and DMSO-d_6 (δ_C 39.5) peaks in parts per million (ppm). Mass spectral data was obtained using a Quattro II (Micromass Inc., St. Paul, MN) and Mariner (Perseptive Biosystems, Foster City, CA) spectrometers from either The University of Texas at Austin Mass Spectrometry Facility (FAB) or The Pennsylvania State University Mass Spectrometry Facility (ESI and CI). Infrared spectra were obtained with a Perkin–Elmer 1600 Series FTIR. Elemental analyses were performed by Midwest Microlab, LLC (Indianapolis, IN, USA). Melting points are uncorrected.

3-Iodobenzamide (6). 3-Iodobenzoic acid (20 g, 80.6 mmol) was refluxed in excess $SOCl_2$ (150 mL) for 3 h. The reaction was cooled to room temperature and the remaining $SOCl_2$ was evaporated in vacuo to afford the acid chloride as a yellow liquid that was used immediately without further purification. This acid chloride (21.5 g, 80.6 mmol) was dissolved in THF (300 mL) and cannulated over 45 min into freshly distilled, liquefied ammonia (500 mL) at −78 °C. This mixture was allowed to stir for 45 min and then warmed to −40 °C for 15 h. Excess ammonia was removed by evaporation at 22 °C to yield an off-white solid that was suspended in dH_2O (300 mL), filtered, and washed with dH_2O (4×50 mL). Evaporation in vacuo gave **6** as a white solid (19.2 g 97%). Mp 185–186 °C; ^1H NMR (DMSO-d_6, 400.13 MHz) δ 8.22 (s, 1H), 8.06 (s, 1H), 7.87 (d, 2H, J = 5.8 Hz), 7.47 (s, 1H), 7.26 (t, 1H, J = 5.9); ^{13}C NMR (DMSO-d_6, 75.57) δ 166.4, 139.7, 136.3, 135.9, 130.4, 126.8, 94.6; IR (KBr): ν_{max} 3337.6, 3158.0, 1665.6, 1561.1, 658.2 cm^{-1}; low-resolution MS (ESI^+) m/z 248 (MH^+). Anal. calcd for C_7H_6INO: C, 34.03; H, 2.45; N, 5.67. Found: C, 34.13; H, 2.42; N, 5.55.

Ethyl[2-(3-iodophenyl)-5-methyl-1,3-oxazol-4-yl]acetate (7). A solution of Br_2 (5.82 g, 36.4 mmol) in $CHCl_3$ (10 mL) was added dropwise over 2 h to a stirring solution of ethyl propionylacetate (**3**) (5.25 g, 36.4 mmol) in $CHCl_3$ (30 mL) at 0 °C. The reaction mixture was allowed to stir for an additional 30 min and warmed to 22 °C for 17 h. The reaction vessel was exposed to the atmosphere, and air was bubbled in for 1 h. The remaining liquid was dried (Na_2SO_4) and concentrated in vacuo to afford **4** as a crude yellow liquid, which was used immediately without further purification. Compound **6** (5.0 g, 20.2 mmol) was combined with **4** (8.13 g, 36.4 mmol) and the slurry was heated to 125 °C. After 4 h, the resulting dark-red solution was allowed to cool to 22 °C and immediately purified by column chromatography (150 g SiO_2) eluting with Et_2O/hexanes (1:9) followed by Et_2O/hexanes (3:10) to afford **7** as a tannish-white solid (4.2 g, 56%). Mp 68–69 °C; ^1H NMR ($CDCl_3$, 400.13 MHz) δ 8.34 (t, 1H, J = 1.5 Hz), 7.92 (d, 1H, J = 6.7 Hz), 7.71 (d, 1H, J = 6.8 Hz), 7.14 (t, 1H, J = 7.9 Hz), 4.18 (q, 2H, J = 7.2 Hz), 3.55 (s, 2H), 2.36 (s, 3H), 1.27 (t, 3H, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ 170.4, 157.0, 146.4, 138.8, 134.9, 130.4, 130.0, 129.5, 125.2, 94.4, 61.3, 32.3, 14.4, 10.5; IR (KBr): ν_{max} 2983.3, 1730.0, 1648.1, 1543.3, 1413.0, 1372.2, 797.7, 722.9 cm^{-1}; low-resolution MS (ESI^+) m/z 372 (MH^+). Anal. calcd for $C_{14}H_{14}INO_3$: C, 45.30; H, 3.80; N, 3.77. Found: C, 45.24; H, 3.79; N, 3.65.

2-[2-(3-Iodophenyl)-5-methyl-1,3-oxazol-4-yl]ethanol (8). A solution of $LiBH_4$ in THF (2 M, 6.7 mL, 12.0 mmol) was slowly added via a syringe to a stirring solution of **7** (2.21 g, 6.0 mmol) in THF (20 mL) at 0 °C. The reaction mixture was allowed to warm to 22 °C and then heated to 50 °C for 1.5 h. The solution was cooled, poured into ice–water (100 mL), acidified to pH ~2 with aqueous HCl (1 M), and extracted with EtOAc (3×25 mL). The organic extract was dried (Na_2SO_4) and concentrated to give a yellow oil that was purified by column chromatography (50 g SiO_2) with EtOAc/hexanes (7:3) to afford **8** (1.72 g, 87%) as a white solid. Mp 102–103 °C; ^1H NMR ($CDCl_3$, 400.13 MHz) δ 8.31 (t, 1H, J = 1.6 Hz), 7.92 (d, 1H, J = 6.4 Hz), 7.72 (d, 1H, J = 6.8 Hz), 7.15 (t, 1H, J = 7.9 Hz), 3.92 (t, 1H, J = 5.8 Hz), 3.17 (bs, 1H), 2.72 (t, 2H, J = 5.73), 2.32 (s, 3H); ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ 157.0, 144.0, 138.9, 134.8,

134.4, 130.5, 129.4, 125.2, 94.5, 61.9, 28.4, 10.3; IR (KBr): ν_{max} 3329.9, 1961.7, 1641.9, 1543.5, 1467.1, 1130.5, 1056.0, 886.1, 732.4 cm^{-1}; low-resolution MS (ESI^+) m/z 330 (MH^+). Anal. calcd for $C_{12}H_{12}INO_2$: C, 43.79; H, 3.67; N, 4.26. Found: C, 43.91; H, 3.57; N 4.15.

Methyl (2*S*)-2-[(2-benzoylphenyl)amino]-3-(4-{2-[2-(3-iodophenyl-5-methyl-1,3-oxazol-4-yl]ethoxy}phenyl) propanoate (10). A solution of diethyl azodicarboxylate (1.2 g, 7.0 mmol) in THF (70 mL) was added over 0.5 h to a solution of **9** (1.74 g, 4.6 mmol), **8** (1.68 g, 5.1 mmol), and PPh_3 (1.8 g, 7.0 mmol) in THF (170 mL) at 22 °C. The resulting yellow solution was stirred for 24 h and concentrated in vacuo to give a viscous yellow oil that was purified by column chromatography (75 g SiO_2) eluting with EtOAc/hexanes (3:17) to afford **10** as a yellowish foam (1.91 g, 61%). Mp 104–105 °C; ^1H NMR ($CDCl_3$, 400.13 MHz) δ 8.90 (d, 1H, J = 8.1 Hz), 8.34 (t, 1H, J = 1.6 Hz), 7.94 (d, 1H, J = 8.7 Hz), 7.73 (d, 1H, J = 8.8 Hz), 7.62–7.59 (m, 2H), 7.54–7.43 (m, 4H), 7.36–7.32 (m, 1H), 7.19–7.14 (m, 3H), 6.83 (d, 2H, J = 9.5 Hz), 6.64 (d, 1H, J = 9.3 Hz), 6.58 (t, 1H, J = 8.3 Hz), 4.09 (q, 1H, J = 7.9 Hz), 4.20 (t, 2H, J = 7.3 Hz), 3.70 (s, 3H), 3.24–3.09 (m, 2H), 2.95 (t, 2H, J = 7.3 Hz), 2.36 (s, 3H); ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ 199.4, 173.2, 157.0, 157.9, 150.4, 145.9, 140.5, 138.8, 135.7, 135.1, 134.8, 133.3, 131.1, 130.5, 130.4 (×2), 129.8, 129.3 (×2), 128.6, 128.2 (×2), 125.2, 118.4, 115.1, 114.8 (×2), 111.9, 94.5, 66.7, 58.2, 52.4, 38.3, 26.5, 10.5; IR (KBr): ν_{max} 3289.7, 2951.2, 2872.1, 1737.8, 1627.7, 1569.8, 936.8, 750.4 cm^{-1}; low-resolution MS ($APCI^+$) m/z 687 (MH^+). Anal. calcd for $C_{35}H_{31}IN_2O_5$: C, 61.23; H, 4.55; N, 4.08. Found: C, 61.11; H, 4.59; N, 4.00.

(2*S*)-2-[(2-Benzoylphenyl)amino]-3-(4-{2-[2-(3-iodophenyl)-5-methyl-1,3-oxazol-4-yl]ethoxy}phenyl) propanoic acid (11). Aqueous LiOH (2 M, 0.33 mL) was added to a solution of **10** (150 mg, 0.22 mmol) in THF/MeOH (3:1, 8 mL) and the reaction stirred at 22 °C for 3 h. This reaction mixture was diluted with dH_2O (25 mL), acidified to pH ~2 with aqueous HCl (1 M), and extracted with EtOAc (3×15 mL). The organic extracts were dried (Na_2SO_4) and concentrated to give a yellow oil that was purified by column chromatography (30 g SiO_2) eluting with $MeOH/CH_2Cl_2$ (1:19) to afford **11** as a crude yellow oil. Trituration of this oil with Et_2O/hexanes (1:1, 4×25 mL) yielded a yellowish-orange foam that was washed with Et_2O/hexanes (1:1, 3×15 mL) to afford **11** as a bright yellow solid (123 mg, 83%). Mp 144–145 °C; ^1H NMR ($CDCl_3$, 400.13 MHz) δ 8.86 (bs, 1H), 8.32 (s, 1H), 7.91 (d, 1H, J = 7.9 Hz), 7.72 (d, 1H, J = 7.9 Hz) 7.59 (d, 2H, J = 7.1 Hz), 7.53–7.42 (m, 4H), 7.35 (t, 1H, J = 7.7 Hz), 7.21 (d, 2H, J = 8.5 Hz), 7.13 (t, 1H, J = 7.9 Hz), 6.80 (d, 2H, J = 8.4 Hz), 6.68 (d, 1H, J = 8.5 Hz), 6.61 (t, 1H, J = 7.5 Hz), 4.38 (bs, 1H), 4.14 (t, 2H, J = 6.4 Hz), 3.29–3.13 (m, 2H), 2.93 (t, 2H, J = 6.4 Hz), 2.33 (s, 3H); ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ 199.5, 176.3, 158.03, 158.01, 150.2, 146.0, 140.3, 138.9, 135.7, 135.2, 134.9, 133.2, 131.2, 130.6 (×2), 130.5, 129.5, 129.4 (×2), 128.4, 128.3 (×2), 125.3, 118.7, 115.6, 114.9 (×2), 112.3, 94.5, 66.7, 58.1, 37.0, 26.3, 10.5; IR (KBr): ν_{max} 3323.0, 2935.3, 1733.9, 1625.0, 1572.8, 1251.7, 1179.1, 701.0 cm^{-1}; low-resolution MS ($APCI^-$) m/z 671 (MH^-). Anal. calcd for $C_{34}H_{29}INO_5$: C, 60.72; H, 4.35; N, 4.17. Found: C, 60.73; H, 4.42; N, 4.09.

Methyl (2*S*)-2-[(2-benzoylphenyl)amino]-3-(4-{2-[2-(3-{3-[(*tert* - butoxycarbonyl)amino] - 1 - propynyl}phenyl) - 5 - methyl - 1,3 - oxazol - 4 - yl]ethoxy}phenyl)propanoate (13). Compounds **10** (405 mg, 0.59 mmol) and **12** (114 mg, 0.74 mmol) were added to $Pd(PPh_3)_2Cl_2$ (20.7 mg, 5 mol%) in triethylamine (30 mL) and this solution was extensively degassed under Ar. CuI (11.2 mg, 10 mol%) was added with degassing under Ar, and this mixture was heated to 60 °C for 0.5 h. The reaction was cooled to 22 °C, diluted with dH_2O and extracted with EtOAc (3×20 mL). This organic extract was dried (Na_2SO_4) and concentrated in vacuo to afford a faint red oil that was purified by column chromatography (40 g SiO_2) eluting with EtOAc/hexanes (3:7) to afford the product as a yellow oil (332 mg, 79%). ^1H NMR (DMSO-d_6, 400.13 MHz) δ 8.65 (d, 1H, J = 7.9 Hz), 7.87 (d, 2H, J = 7.9 Hz), 7.59–7.34 (m, 10H), 7.09 (d, 2H, J = 8.4 Hz), 6.81 (d, 3H, J = 8.4 Hz), 6.62 (t, 1H, J = 7.4 Hz), 4.64 (q, 1H, J = 6.4 Hz), 4.14 (t, 2H, J = 6.4 Hz), 4.01 (d, 2H, J = 5.1 Hz), 3.64 (s, 3H), 3.15–3.03 (m, 2H), 2.88 (t, 2H, J = 6.3 Hz), 2.31 (s, 3H), 1.40 (s, 9H); ^{13}C NMR (DMSO-d_6, 100.61 MHz) δ 198.3, 172.3, 157.4, 157.2, 155.3, 149.5, 145.5, 140.0, 135.1, 134.7, 132.9, 132.4, 131.1, 130.3 (×2), 129.6, 128.7 (×2), 128.2 (×2), 128.1, 128.0, 127.5, 125.2, 123.2, 117.4, 114.9, 114.3 (x2), 112.4, 88.6, 80.6, 78.3, 66.0, 56.3, 52.0, 36.7, 30.1, 28.2 (×3), 25.5, 9.8; IR (film): ν_{max} 3307.1, 2975.9, 1710.9, 1624.8, 1249.3, 735.0, 702.4 cm^{-1}; high-resolution MS ($APCI^+$), Mass calcd for $C_{43}H_{43}N_3O_7$: 713.310. Found (MH^+) 714.315.

(2*S*)-2-[(2-Benzoylphenyl)amino]-3-(4-{2-[2-(3-{3-[*tert*-butoxycarbonyl)amino]-1-propynyl}phenyl)-5-methyl-1,3-oxazol-4-yl]ethoxy}phenyl)propanoic acid (14). Aqueous LiOH (2 M, 0.55 mL) was added to a solution of 13 (262 mg, 0.37 mmol) in THF/MeOH (3:1, 16 mL) and the reaction stirred at 25 °C for 3 h. This mixture was diluted with dH_2O (20 mL), quickly acidified with ice cold aqueous HCl (1 M), and immediately extracted with EtOAc (3×15 mL). This organic extract was dried (Na_2SO_4), the solvent removed in vacuo, and the residue purified by column chromatography (30 g SiO_2) eluting with MeOH/hexanes (1:49) to give **14** as a crude yellow oil, which was triturated with a mixture of Et_2O/hexanes (1:1, 4×15 mL) to afford a yellowish-white solid. This solid was collected and washed with Et_2O/hexanes (1:1, 3×10 mL) to provide **14** as a bright yellow solid (205 mg, 79%). ^1H NMR (DMSO-d_6, 400.13 MHz) δ 8.68 (d, 1H, J = 7.6 Hz), 7.87–7.85 (m, 2H), 7.58–7.47 (m, 7H), 7.40 (t, 2H, J = 7.3 Hz), 7.34 (d, 1H, J = 7.6 Hz), 7.11 (d, 2H, J = 8.4 Hz), 6.84–6.79 (m, 3H), 6.58 (t, 1H, J = 7.5 Hz), 4.52 (q, 1H, J = 6.3 Hz), 4.12 (t, 2H, J = 6.6 Hz), 4.02 (d, 2H, J = 5.4 Hz), 3.18–3.01 (m, 2H), 2.87 (t, 2H, J = 6.4 Hz), 2.30 (s, 3H), 1.40 (s, 9H); ^{13}C NMR (DMSO-d_6, 100.61 MHz) δ 198.2, 173.3, 157.4, 157.1, 155.3, 149.7, 145.5 (×2), 139.8, 135.0, 134.8, 132.9, 132.4, 131.0, 130.4, 129.6, 128.7 (×2), 128.6, 128.2 (×2), 128.1, 127.5, 125.2, 123.1, 117.3, 114.5, 114.2 (×2), 112.4, 88.6, 80.7, 78.3, 65.0, 56.3, 36.5, 30.2,

28.2 (×3), 25.5, 9.8; IR (film): ν_{max} 3415.4, 2257.0, 1653.6, 1248.5, 1166.4, 1025.6, 827.1, 765.3 cm^{-1}; high-resolution MS ($APCI^+$), Mass calcd for $C_{42}H_{41}N_3O_7$: 699.294. Found (MH^+) 700.302.

(2*S*)-2-[(2-Benzoylphenyl)amino]-3-{4-[2-(2-{3-[3-({[3′,6′-dihydroxy-3-oxo-3H-spiro[2-benzofuran-1,9′-xanthen-5-yl)amino]carbonothioyl}amino) - 1 - propynyl]phenyl} - 5 - methyl-1,3-oxazol-4-yl)ethoxy]phenyl} propanoic acid (2). Compound **14** (47 mg, 0.07 mmol) was treated with trifluoroacetic acid/'wet' CH_2Cl_2 (3:47, 10 mL) at 25 °C for 1 h. The solution was diluted with NaOH/dH_2O (1:9, 10 mL) and extracted with EtOAc (3×10 mL). The organic extract was dried (Na_2SO_4) and concentrated in vacuo to give the free amine as a crude oil. This amine (40.3 mg, 0.07 mmol) was added to 5-fluorescein isothiocyanate, isomer I (26 mg, 0.07 mmol) in EtOH/THF (3:2, 5 mL), cooled to 0 °C, and triethylamine (10 μL, 0.07 mmol) was added. The resulting orange reaction mixture was warmed to 22 °C and stirred in the dark for 4 h. The reaction was concentrated in vacuo in the dark to give an orange solid that was purified by column chromatography (20 g SiO_2) eluting with AcOH/MeOH/CH_2Cl_2 (2:3:95) to provide **2** as a yellowish-orange solid (37 mg, 55%). ^1H NMR (DMSO-d_6, 500.13 MHz) δ 8.65 (d, 1H, J = 7.0 Hz), 8.47 (s, 1H), 7.90 (s, 1H), 7.76 (t, 2H, J = 8.3 Hz), 7.56–7.45 (m, 6H), 7.41–7.36 (m, 2H), 7.32–7.31 (m, 1H), 7.16 (d, 1H, J = 8.4 Hz), 7.10 (d, 2H, J = 8.3 Hz), 6.83–6.80 (m, 4H), 6.67–6.66 (m, 2H), 6.60–6.53 (m, 5H), 5,17 (s, 2H), 4.47 (d, 1H, J = 5.7 Hz), 4.13 (t, 2H, J = 6.6 Hz), 3.34 (bs, 4H), 3.16–2.99 (m, 2H), 2.89 (t, 2H, J = 6.4 Hz), 2.33 (s, 3H); ^{13}C NMR (DMSO-d_6, 125.76 MHz) δ 198.1, 173.3, 172.0, 168.8, 159.5 (×2), 158.2, 157.1, 152.5, 151.9 (×2), 149.8, 145.5, 145.3, 142.6, 139.8, 139.7, 137.1, 135.0, 134.7, 132.8, 131.0, 130.4 (×2), 129.5, 129.2, 129.1, 128.8, 128.6 (×2), 128.2 (×2), 127.6, 127.2, 125.3, 124.4, 123.6, 123.4, 117.2, 116.8, 114.4, 114.2 (×2), 112.6, 112.4, 112.0, 109.8 (×2), 102.2 (×2), 83.1, 69.7, 66.1, 56.4, 36.5, 25.6, 21.1, 9.9; IR (KBr): ν_{max} 2923.2, 1611.3, 1452.8, 1249.5, 1179.3, 832.0, 752.8 cm^{-1}; high-resolution MS (FAB^+), Mass calcd for $C_{58}H_{44}N_4O_{10}S$: 988.278. Found (MH^+) 989.284.

Production of bacterially expressed PPARγ

The DNA encoding the murine PPARγ ligand binding domain (regions D–F, amino acids 203–505, NP_035276) was cloned into the *Nde*I and *Bam*HI restriction sites downstream of the hexahistidine tag of plasmid pET-15b (Novagen). This construct was confirmed by automated dideoxynucleotide sequencing and expressed in *Escherichia coli* strain BL21(DE3). Cultures were grown at 37 °C to an OD_{600} = 0.4–0.6. Protein expression was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was subsequently grown for 16 h at 23 °C to minimize the formation of inclusion bodies. Bacterial cells from a 1 L culture were harvested by centrifugation and resuspended in 10 mL of loading buffer (20 mM HEPES, pH = 7.9) containing NaCl (250 mM), and imidazole (10 mM). Lysozyme (400 μg/mL) was added and the cell slurry was incubated on ice for 30 min. The slurry was then subjected to three cycles of sonication at 4 °C (30 s, micro-tip installed, duty cycle = 50%, output = 5) with a 30-s rest period between cycles. This extract was frozen in liquid N_2 for 2 min followed by warming in a 37 °C water bath until thawed. This lysate was applied to a column of Ni-agarose (1 mL bed volume per liter of bacterial culture) that had been pre-equilibrated with HEPES loading buffer until the OD_{260} was reduced to 0.05. The loaded column was washed with buffer (20 mM HEPES, pH = 7.9) containing imidazole (25 mM) and glycerol (10%) until the OD_{260} was reduced to 0.01. The PPARγ–LBD was eluted in buffer (20 mM HEPES, pH = 7.9) containing imidazole (200 mM) and glycerol (10%). The protein concentration was determined by BCA assay (Pierce, bovine serum albumin standard) and protein purity was verified by SDS-PAGE (purity visualized by coomassie staining >90%).

Fluorescence polarization assays

Assays employed compound **2** (conc = 100 nM for K_i and K_d measurements) in binding buffer (10 mM HEPES, pH = 7.9, 159 mM NaCl, 2 mM $MgCl_2$, 5 mM DTT). The data shown in Figure 4 and the competition data with **11** in Figure 5 (Panel A) was obtained with a Panvera Beacon 2000 instrument. The competition data with PGJ_2 (**15**), CLA compounds (**16**–**22**), and the CLA mixture (Figure 5, Panel B) was obtained on 96-well plates with a Packard Fusion Universal Microplate Analyzer. Values of K_d and K_i were calculated by non-linear least squares curve fitting using binding models from GraphPad Prism 3.0 (San Diego, CA, USA).

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