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RESEARCH RESOURCE

Identification and Characterization of a Selective Peroxisome Proliferator-Activated Receptor β/δ (NR1C2) Antagonist

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The identification of small molecule ligands for the peroxisome proliferator-activated receptors (PPARs) has been instrumental in elucidating their biological roles. In particular, agonists have been the focus of much of the research in the field with relatively few antagonists being described and all of those being selective for PPARα or PPARγ. The comparison of these agonist and antagonist ligands in cellular and animal systems has often led to surprising results and new insights into the biology of the PPARs. The PPARβ/δ receptor is emerging as an important regulator of energy metabolism, inflammation, and cell growth and differentiation; however, only agonist ligands have been described for this receptor thus far. Here we describe the first report of a PPARβ/δ small molecule antagonist ligand. This antagonist ligand will be a useful tool for elucidating the biological roles of PPARβ/δ. (*Molecular Endocrinology* 22: 523–529, 2008)

THE PEROXISOME proliferator-activated receptor β/δ (PPARβ/δ NR1C2) is a fatty acid-regulated nuclear receptor implicated as a key regulator of fatty acid utilization, glucose disposal, and inflammation. Because of these biological functions, PPARβ/δ is a promising target for the discovery of medicines for metabolic diseases. The synthetic ligand GW501516 has been shown to correct the metabolic syndrome in obese primates, treat the diabetic symptoms in rodent models of type 2 diabetes, and reduce inflammation in animal models of atherosclerosis (1–4). Recently, it has been shown to regulate high-density lipoprotein levels in normal healthy volunteers (5). In addition, PPARβ/δ signaling has been implicated in regulating cell proliferation and angiogenesis (6, 7). Thus, there are multiple potential therapeutic uses for PPARβ/δ ligands.

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Abbreviations: APC, Allophycocyanin; ChIP, chromatin immunoprecipitation; NCOR, nuclear receptor corepressor; PPARs, peroxisome proliferator-activated receptors; PPRE, peroxisome proliferators response element; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors.

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The biological pathways regulated by PPARβ/δ that contribute to the pharmacological activities on metabolism are becoming better understood. It is now well appreciated that PPARβ/δ regulates the expression of genes involved in fatty acid β-oxidation, reverse cholesterol transport, and carbon substrate utilization in skeletal muscle (7, 8). In addition, there is evidence that PPARβ/δ can regulate skeletal muscle fiber type (9, 10). Conversely, PPARβ/δ has been implicated as a lipogenic transcription factor in the liver (11). Together, these activities are thought to contribute to the positive effects of PPARβ/δ agonists in animal models of metabolic disease.

The development of small molecule regulators of the PPAR family has been instrumental in deciphering the biological roles of these receptors. Most of this work has focused on the discovery of agonists of PPARα, PPARγ, and PPARβ/δ, whereas relatively few antagonists have been described. The molecule GW9662 is an antagonist of PPARγ, and GW6471 is an antagonist of PPARα (12–14). GW9662 acts by binding covalently to cysteine residues in the ligand binding pocket, whereas GW6471 directly contacts the activation function 2 (AF2) helix of PPARα and blocks it from assuming the active conformation. This increases corepressor binding and is mechanistically linked to the antagonist activity of the molecule.

Antagonists often have unexpected pharmacological activity in animal disease models, and thus they have the potential to be good drug development leads themselves (15, 16). Selective antagonist ligands have not been described for PPARβ/δ. Agonist regulation of PPARβ/δ has also been implicated in regulating cell growth and tumor formation, but considerable controversy surrounds this area of research, and both carcinogenic and tumor suppressor activities have been attributed to PPARβ/δ agonists (6, 7). A selective antagonist of PPARβ/δ would provide a valuable pharmacological tool for elucidating the role of PPARβ/δ in cellular growth and homeostasis. Here we report the identification and characterization of the first antagonist molecule for PPARβ/δ. We suggest that this antagonist will be a valuable chemical tool for the continued elucidation of the biological roles of PPARβ/δ.

RESULTS

We identified new PPARβ/δ ligands via a high-throughput ligand displacement screen. One of the identified molecules, GSK0660, was of a unique chemotype, and we focused on its characterization (Fig. 1A). We determined the pIC50 and selectivity of GSK0660 for the PPARs in an *in vitro* ligand displacement assay (Table 1). GSK0660 is a potent binder of PPARβ/δ with a pIC_{50} of 6.8 (~160 nM) and is nearly inactive on PPARα and PPARγ with IC_{50}s above approximately 10 μM. These data are the result of six to eight repeated experiments. In a standard cell-based Gal4 chimera/reporter agonist assay, GSK0660 was inactive on all three PPARs. This result was surprising considering the potent binding the molecule exhibited on the PPARβ/δ ligand binding domain (LBD). This result suggested that GSK0660 could be an antagonist of PPARβ/δ. We therefore determined the activity of GSK0660 in a standard cell-based Gal4 chimera/reporter antagonist assay in which an EC_{80} dose of a standard PPARβ/δ agonist (GW1516) is added to the cells and the antagonist test compound is dosed on top of the agonist. In this assay, GSK0660 antagonized 100% of the activity of PPARβ/δ with a pIC_{50} of 6.53 (average of $n = 4$ experiments) and was inactive on PPARα and PPARγ. Thus, GSK0660 is an antagonist of the PPARβ/δ receptor (Table 1).

The methyl ester analogs of specific carboxylic acid PPAR ligands have been demonstrated to exhibit a significant decrease in binding affinity but similar potencies in functional reporter assays (17). This type of result has been attributed to the fact that esters are often converted to carboxylic acids via cellular enzymes during the incubation time of the Gal4 chimera/reporter assay. We surmised that the corresponding acid analog of GSK0660 was likely to be an active ligand with increased binding affinity. As such, we synthesized the carboxylic acid analog GSK1491 and measured its ability to bind and activate each PPAR subtype. Surprisingly, this acid analog failed to bind or elicit *in vitro* functional PPAR activity (data not shown). These results suggest that this new class of PPARβ/δ ligand has a binding mode distinct from other known PPAR ligands.

We also determined the *in vivo* bioavailability of GSK0660 in rodents. Unfortunately, GSK0660 was rapidly cleared and did not accumulate in the blood (data not shown). Thus, we focused on cell culture systems for our analysis of GSK0660.

We next determined the effect of GSK0660 on the expression of PPARβ/δ target genes in skeletal muscle cells. We examined the expression of *CPT1a*, *ANGPTL4*, and *PDK4* at 1, 10, 100, and 1000 nM of GSK0660 or a single saturating dose of the PPARβ/δ agonist GW0742 (100 nM). GW0742 (18) robustly induced target gene expression consistent with this ligand being a very potent and efficacious agonist (Fig. 1B). In contrast, GSK0660 reduced *CPT1a* expression below the basal vehicle-treated level by approximately 50% at 100 nM. This is in contrast to GW0742, which induced *CPT1a* expression by approximately 15-fold. *ANGPTL4* expression was also reduced below the basal level by approximately 30% with maximal suppression at approximately 100 nM, whereas GW0742 induced expression by approximately 8-fold. Interestingly, *PDK4* expression was not altered by GSK0660 but it was induced by approximately 10-fold in the presence of GW0742.

We next determined whether GSK0660 can antagonize the agonist activity of GW0742 on gene expression. Skeletal muscle cells were treated with a near saturating dose (10 nM) of GW0742 to induce gene expression to approximately 80% of that of the saturating dose. Different concentrations of GSK0660 were added to the cells and the effects on gene expression determined as above. The activity of GW0742 was antagonized by GSK0660 for all three genes tested in a dose-dependent manner and reduced expression by approximately 60% for *ANGPTL4* and approximately 80% for *CPT1a* and *PDK4* (Fig. 2A). Thus, GSK0660 can antagonize the binding of GW0742 at the PPARβ/δ receptor on native target genes.

CPT1a is a critical regulator of fatty acid β oxidation, and it is well established that PPARβ/δ agonists positively regulate *CPT1a* expression and increase fatty acid β oxidation (19). Our gene expression results with *CPT1a* suggest that GSK0660 may have an impact on fatty acid β oxidation. We tested this hypothesis by performing a $^{14}C\ CO_2$ capture β oxidation assay with C2C12 myotubes treated with ^{14}C-labeled oleate and GW0742 or GSK0660. Treatment with GW0742 increased the rate of $^{14}C\ CO_2$ release by approximately 40% over vehicle treatment as expected (Fig. 2B). Treatment with GSK0660 at 100, 500, and 1000 nM resulted in a dose-dependent decrease in the rate of $^{14}C\ CO_2$ release to approximately 50% below the vehicle level (Fig. 2B). Thus, GSK0660 can inhibit the expression of PPARβ/δ target gene *CPT1a*, and this

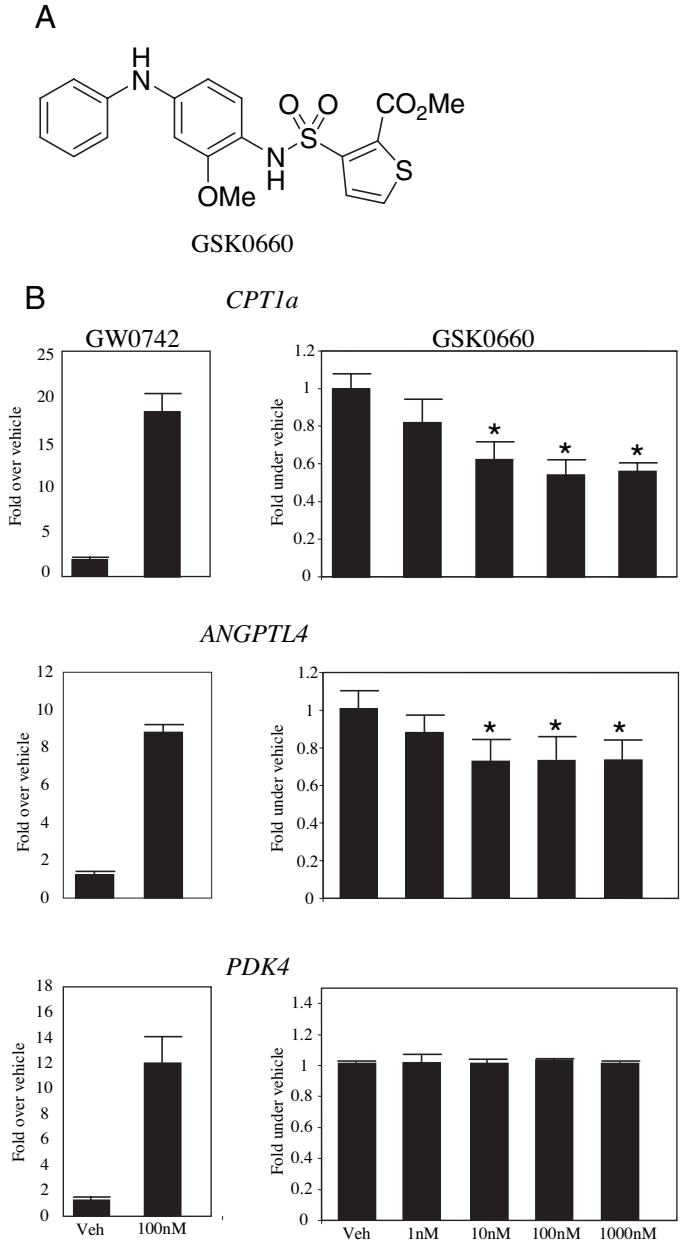

Fig. 1. Structure of GSK0660 and Its Activity in Primary Human Skeletal Muscle Cells

A, Structure of GSK0660, methyl 3-({[2-(methoxy)-4 phenyl]amino}sulfonyl)-2-thiophenecarboxylate. B, Effects of GW0742 and GSK0660 on gene expression in human skeletal muscle cells. GW0742 robustly induces the expression of known PPARβ/δ target genes, whereas GSK0660 reduces the basal level of gene expression for *ANGPTL4* and *CPT1a*, but not *PDK4* in a dose-responsive manner. Gene expression analysis was performed using Taqman. *, $P < 0.05$ by a *t* test.

action is correlated with fatty acid β oxidation activity of the cell.

We next tested the activity of GSK0660 in a variety of cell types to ascertain whether it is a broadly active antagonist of PPARβ/δ. In the human hepatoma line HuH7, GW0742 increased the expression of *CPT1a* and *ANGPTL4* and codosing GW0660 with GSK0742 blocked the action of the agonist (Supplemental Fig. 1, A and B, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Similarly, in rat L6 myotubes, GW0742 increased the expression of *CPT1b* and *PDK4*, but co-dosing GW0660 with GSK0742 antagonized the activity of GW0742 (Supplemental Fig. 1, D and E). In mouse bone marrow-derived macrophages, GW0742 increased *CPT1a* expression and GSK0660 antagonized *CPT1a* expression when dosed alone (Supplemental Fig. 1C). This is consistent with the antagonist activity of GSK0660 noted in THP-1 macrophages (20). Additionally, ^{14}C palmitate oxidation

Table 1. Activity and Selectivity of GSK0660

	PPARα	PPARβ/δ	PPARγ
Binding assay IC_{50}	>10 μM	155 nM	≥10 μM
Agonist assay EC_{50}	ia	ia	ia
Antagonist assay IC_{50}	nt	300 nM (100)	nt

Binding activity in a ligand displacement assay and activity in a GAL4 LBD chimera assay of GSK0660A. Antagonist assays are performed in the presence of an EC_{80} dose of the PPARβ/δ agonist GW1516. *Number in parentheses* is percent antagonism. ia, Inactive at 10 μM; nt, not tested.

that is induced by GW0742 is antagonized by GSK0660 (Supplemental Fig. 1F). Thus, GSK0660 acts as an antagonist of PPARβ/δ in multiple cell types from multiple species.

The conformation of helix 12 in the LBD of nuclear receptors is the primary factor determining the activation state of a nuclear receptor. Fluorescent labeling of helix 12 is an established technique used to detect conformational changes in helix 12 of nuclear receptors upon binding of ligands or other cofactors (21, 22). To monitor the conformation of the PPARβ/δ AF2 helix by fluorescence anisotropy, we attached a fluorescein-labeled cysteine to the C terminus of the PPARβ/δ LBD by intein-mediated protein ligation as has been done previously with PPARγ (21). Titration of 5 nM of the Helix 12-labeled PPARβ/δ LBD with the agonist GW0742 decreases the steady state fluorescence anisotropy of the PPARβ/δ LBD in a dose-dependent manner (Supplemental Fig. 2A). We also observed a dose-dependent decrease in anisotropy for over a dozen PPARβ/δ agonists (data not shown). In addition, a saturating concentration of an LxxLL-containing coactivator peptide from PGC-1α (PPARγ, coactivator 1α) induces a similar decrease in anisotropy as the agonist compound (Supplemental Fig. 2A). Changes in fluorescence anisotropy are due to a variety of factors including local dynamics of the fluorophore, and the radius of rotation of the macromolecule to which the fluorophore is attached. We believe that this observed reduction in anisotropy caused by both agonist ligands and coactivator peptide are a result of helix 12 orienting in its active conformation. Titration of the fluorescein-labeled PPARβ/δ LBD with GSK0660 results in a dose-dependent increase in the observed anisotropy. This clearly demonstrates that GSK0660 induces a different conformation of helix 12 than observed with the PPARβ/δ agonists.

To determine whether the functional consequences of the altered conformation of the GSK0660-bound PPARβ/δ LBD, we examined the interaction of the LBD with the LxxLL coactivator peptide and CoRNR box corepressor peptide by fluorescence resonance energy transfer. Agonists typically reduce the affinity of the receptor for corepressors and increase its affinity for coactivators, thus leading to enhanced transcription of target genes (23). As expected, GW0742 increased the interaction between the PPARβ/δ LBD and the LxxLL peptide, whereas the interaction with the CoRNR box was reduced. In contrast to GW0742, GSK0660 reduced the association between the PPARβ/δ LBD and the LxxLL peptide, whereas the

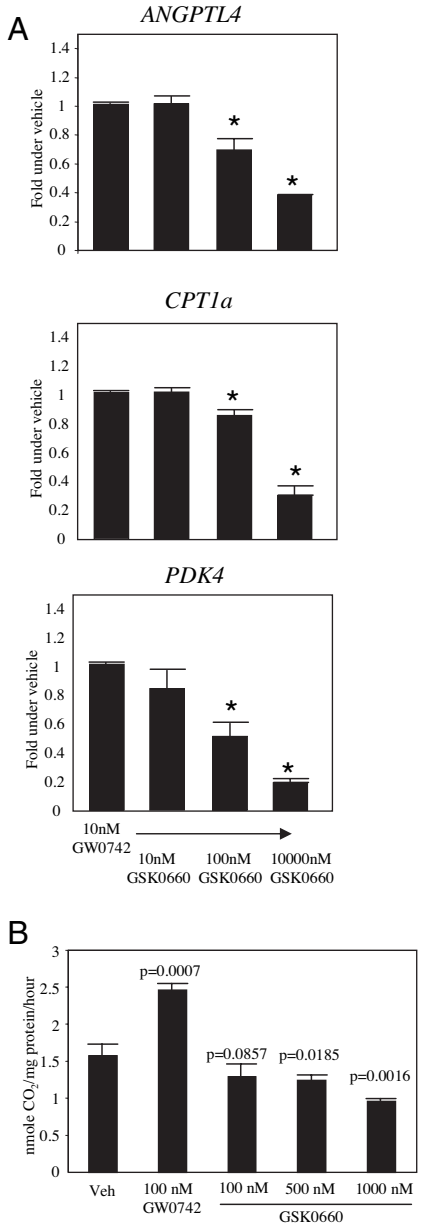

Fig. 2. Antagonism of GW0742 and Effects on Fatty Acid Oxidation by GSK0660

A, Human skeletal muscle cells were dosed with 10 nM GW0742 without or with the addition of various concentrations of GSK0660 and PPARβ/δ target gene expression was determined as in Fig. 1. The level of gene induction over vehicle for GW0742 alone is set to 1. GSK0660 can compete with GW0742 and decreases the efficacy of GW0742 agonism. B, Fatty acid oxidation was determined in C2C12 myotubes treated with GW0742 or increasing doses of GSK0660. The *P* values were calculated using a *t* test *vs.* the vehicle control; *, $P < 0.05$.

interaction with the CoRNR box peptide was increased (Supplemental Figs. 2, B and C, and 3). This activity is consistent with GSK0660 acting as an antagonist of the PPARβ/δ.

Given that *in vitro* GSK0660 increases the association between the PPARβ/δ LBD and a corepressor peptide, we next investigated the molecular mechanism by which GSK0660 inhibits the expression of *CPT1a* and *ANGPTL4*. Primary human skeletal muscle cells were treated with 100 nM GW0742 or 1000 nM GSK0660 for 16 h and then cross-linked and prepared for chromatin immunoprecipitation (ChIP) analysis. We hypothesized the GSK0660 may strengthen corepressor interactions with the peroxisome proliferators response element (PPRE) containing regulatory regions of the *CPT1a* and *ANGPTL4* genes to inhibit their expression, analogous to other nuclear receptor antagonists (14, 24, 25). In the basal state, the corepressor SMRT (silencing mediator of retinoic acid and thyroid hormone receptors) was bound to the PPRE regions of both *CPT1a* and *ANGPTL4* (Supplemental Fig. 4, A and B). We were unable to detect nuclear receptor corepressor (NCOR) bound to this region (data not shown). Upon treatment with GW0742, SMRT occupancy of both regulatory regions is decreased severalfold below that of the vehicle-treated cells as would be expected for a strong agonist (Supplemental Fig. 4, A and B). In contrast, GSK0660 did not affect occupancy of the chromatin by SMRT (Supplemental Fig. 4A). In addition, we did not detect recruitment of NCOR in the presence of GSK0660 (data not shown). Thus, the mechanism of repression by GSK0660 does not involve increased recruitment of SMRT or NCOR. We also determined whether GSK0660 alters the occupancy of the PPRE. PPARβ/δ was highly enriched on the *CPT1a* and *ANGPTL4* PPREs; however, neither GW0742 nor GSK0660 altered PPARβ/δ occupancy of the PPRE region (Supplemental Fig. 4C). To demonstrate the specificity of the PPARβ/δ ChIP, the liver X receptor response element region in the sterol regulatory element-binding protein 1 gene was not enriched by ChIP with αPPARβ/δ antibodies.

DISCUSSION

We have identified and characterized the first selective antagonist ligand for PPARβ/δ, and we suggest that it will be an important tool molecule for determining the biological roles of PPARβ/δ in cultured cells. This ligand is of a distinct chemical structure from previously reported PPARβ/δ ligands and may have a previously unknown binding mode within the receptor. We used fluorescence anisotropy measurements of the PPARβ/δ LBD-labeled at the AF2 helix to determine the effect of coactivator peptide, agonist, and the antagonist GSK0660 on receptor structure. Both the coactivator peptide and the agonist ligand reduced the anisotropy of the PPARβ/δ LBD, suggesting that this signal is characteristic of the receptor in the agonist conformation. Remarkably, GSK0660 increased the anisotropy of the LBD, suggesting that the AF2 is in a conformation that is different from that of the agonist-bound LBD and that the positive shift in anisotropy is a signature of the receptor in an antagonist conformation. The ability of GSK0660 to place the AF2 in a unique conformation is a property shared with other NR antagonists and modulators such as 4-hydroxytamoxifen in the estrogen receptors and the PPARα antagonist GW6471 (14, 22).

We observed that GSK0660 can reduce the basal expression of *CPT1a* and *ANGPTL4* but not of *PDK4*, suggesting selective activity on different gene regulatory regions. The activity of GSK0660 on *CPT1a* and *ANGPTL4* implies that it promotes a mechanism by which PPARβ/δ represses gene express. This may be via the potentiation of an intrinsic repressive activity mediated by PPARβ/δ (26). In our ChIP assays, we were able to detect basal levels of SMRT bound to the regulatory regions of *CPT1a* and *ANGPLT4*, consistent with the observed repressive activity of apo-PPARβ/δ (26). However, we did not detect increased SMRT binding to these regulatory regions when the cells were treated with GSK0660. One possible explanation for this result is that a previously uncharacterized corepressor may be recruited to the receptor by GSK0660. Given that SMRT is already occupying at least some of the PPARβ/δ bound to the regulatory regions, there may be a portion of PPARβ/δ that is not bound by SMRT and available to interact with other corepressors.

An explanation for the lack of activity on the *PDK4* gene is that *PDK4* is expressed at very low basal levels in the muscle cell cultures, and it may already be maximally repressed. *PDK4* is a target of PPARβ/δ regulation in these cells because it is readily induced upon agonist treatment, and GSK0660 was able to block the effects of the agonist on *PDK4* expression. Thus, a lack of PPARβ/δ involvement in regulating the *PDK4* gene is not an explanation for the lack of inverse agonist activity of GSK0660 in this context.

We have described the first selective PPARβ/δ antagonist molecule and demonstrated that it can compete with agonist in a cellular context and that when dosed by itself it exhibits inverse agonist activity. This ligand will be a valuable tool for the elucidation of PPARβ/δ signaling pathways *in vitro*. Unfortunately, the lack of *in vivo* bioavailability will limit the use of the ligand to cellular systems (data not shown). Future work will capitalize on the knowledge gained here to identify a selective, bioavailable PPARβ/δ antagonist.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Fluorescein-Labeled Human PPARβ/δ LBD

PPARβ/δ residues 164 to 441 with a N-terminal hexahistidine tag was cloned into the *Nde*I/*Sap*I site of the pTYB1

vector (New England BioLabs, Boston, MA). This plasmid expresses 6× His PPARβ/δ LBD with a C-terminal fusion of a self-splicing intein and a chitin binding domain. The plasmid was transformed into BL21-DE3. Overnight cultures were used to inoculate 1 liter of Superbroth. After 8 h at 23 C, the temperature was lowered to 17 C before induction with 250 μM isopropyl β-D-1-thiogalactopyranoside. Cells were harvested after 24 h at 17 C. The PPARβ/δ-intein-chitin binding domain fusion protein was purified by chromatography on a 5 ml Ni-chelating column (His-TRAP; GE Health Sciences, Little Chalfont, UK). Next 3 mg of the fusion protein were incubated with 5 mM Cys-Lys-Fluorscein (SynPep, Dublin, CA) in 50 mM Tris (pH 8.5)/50 mM NaCl/50 mM sodium 2-sulfonylethanesulfonte at room temperature overnight. The free label was removed by chromatography on a Ni-chelating column. The identity of the resulting protein: N-terminal hexahistidine-tagged PPARβ/δ LBD (164–441) with the attached Cys-Lys-fluorescein peptide was confirmed by mass spectrometry.

The fluorescence anisotropy assays were conducted in 40 μl total volume in black 384-well plates. A 5-nM concentration of the fluorescein-labeled PPARβ/δ LBD with varying concentrations of ligand or peptide in 50 mM Tris (pH 7.0)/50 mM KCl/2 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate/2.5% dimethylsulfoxide were incubated at room temperature. After 1 h the fluorescence anisotropy was measured using an Envision Multilabel Plate reader (PerkinElmer, Foster City, CA).

PPARβ/δ Fluorescence Resonance Energy Transfer Assay

Allophycocyanin (APC)-labeled PPARβ/δ LBD was generated by incubating an equimolar concentration of biotinylated PPARβ/δ LBD with APC-streptavidin (PerkinElmer) followed by blocking with free biotin. Europium-labeled LxxLL (DGTPPPQEAEEPSLLKKLLLAPANT) or CoRNR box (GHSFADPASNLGLEDIIRKALMGSF) peptides were generated by incubating equimolar concentration of biotinylated peptide and Europium-labeled steptavidin W8044 (PerkinElmer) followed by blocking with free biotin. The effect of ligands on the binding of PPARβ/δ LBD to cofactor peptides was measured by titrating the ligand with 20 nM APC-PPARβ/δ LBD and 40 nM Eu-labeled LxxLL or CoRNR box peptide in 40 μl final volume in 50 mM Tris (pH 7.0)/50 mM KCl/2 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate/2.5% dimethylsulfoxide. The plates were incubated for 4 h at room temperature and time-resolved fluorescence intensities were determined in a Victor V Multilabel Counter. Plots of fluorescence intensity ratio (intensity at 665 nm/intensity at 610 nm) *vs.* ligand concentration were constructed.

Cell Culture and RNA Purification. Human skeletal muscle cells were purchased from Cambrex and grown according to the manufacturer's directions. The day before drug dosing, cells were plated at a density of 10–15,000 cells per well of a 96-well culture plate in DMEM plus 10% FBS. The next day, the cells were dosed, and the following day they were lysed for RNA purification (SV96; Promega, Madison, WI). Established cell lines were cultured according to established protocols (ATCC, Manassas, VA). Mouse bone marrow-derived macrophages were isolated and cultured as previously described (27).

Gene Expression Analysis

Gene expression analysis was carried out using 384-well Taqman (ABI, Foster City, CA) and specific primer probe combinations for each gene as previously described (5).

Fatty Acid Oxidation

Fatty acid oxidation was performed as previously described (5), except that C2C12 myotubes were used for these experiments.

ChIP

ChIP was performed as described previously (28). Cross-linking was performed with 1% formaldehyde. Samples were analyzed by real-time PCR using SYBR green dye on an ABI Prism 7900. Unprecipitated input samples from each condition were pooled, diluted serially, and used as the standard for all PCRs. Antibodies: SMRT (catalog no. PA1-842; Affinity BioReagents, Golden, CO), TRAP220 (catalog no. sc-8998; Santa Cruz Biotechnology, Santa Cruz, CA). Primer sequences: ANGPTL4 (adjacent to the PPRE in intron 3) CCCGCCAAGTAGGAGAAAGT, TTCCTCCCATCCAGTAAGGA; CPT1a (flanks the PPRE in the intron separating exons 1b and 2) CTCCGGAAGGTCTCTGTGG, AAAGTAGGGGAAAGGTCAGCA; RPLP0 (alias 36B4, within coding sequence) ACGCTGCTGAACATGCTCAA, GATGCTGCCATTGTCGAACA.

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Conflict of Interest Statement: B.G.S., J.M.W., T.B.S., D.C.L., D.A.G., M.A.I., T.M.W., and A.N.B. are employees and share holders in GlaxoSmithKline. D.J.S. and M.A.L. received research support funds from GlaxoSmithKline.

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