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High-Affinity Peroxisome Proliferator-Activated Receptor β/δ-Specific Ligands with Pure Antagonistic or Inverse Agonistic Properties

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ABSTRACT

Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) is a ligand-regulated nuclear receptor with essential functions in metabolism and inflammation. We have synthesized a new derivative [methyl 3-(*N*-(4-(hexylamino)-2-methoxyphenyl)sulfamoyl)thiophene-2-carboxylate (ST247) structurally related to the published PPARβ/δ inhibitory ligand methyl 3-(*N*-(2-methoxy-4-(phenylamino)phenyl)sulfamoyl)thiophene-2-carboxylate (GSK0660). ST247 has a higher affinity to PPARβ/δ than GSK0660, and at equimolar concentrations, it more efficiently 1) induces the interaction with corepressors both in vitro and in vivo, 2) inhibits the agonist-induced transcriptional activity of PPARβ/δ, and 3) downregulates basal level expression of the peroxisome proliferator responsive element-driven PPARβ/δ target gene *ANGPTL4*. Methyl 3-(*N*-(4-(*tert*-butylamino)-2-methoxyphenyl)sulfamoyl)thiophene-2-carboxylate (PT-S58), another high-affinity derivative from our series, also efficiently inhibits agonist-induced transcriptional activation, but in contrast to ST247, it does not enhance the interaction of PPARβ/δ with corepressors. PT-S58 rather prevents corepressor recruitment triggered by the inverse agonist ST247. These findings classify ST247 as an inverse agonist, whereas PT-S58 is the first pure PPARβ/δ antagonist described to date. It is noteworthy that ST247 and PT-S58 are also effective on PPRE-independent functions of PPARβ/δ: in monocytic cells, both ligands modulate expression of the activation marker *CCL2* in the opposite direction as an established PPARβ/δ agonist. The possibility to differentially modulate specific functions of PPARβ/δ makes these novel compounds invaluable tools to advance our understanding of PPARβ/δ biology.

Introduction

Nuclear receptors are of particular pharmacological interest, because they are often instrumental in the etiology of major human diseases and can easily be modulated because of their intrinsic ability to interact with regulatory ligands. This explains the success of a large number of drugs targeting, for instance, the estrogen or androgen receptors for treating hormone-sensitive cancers (McDonnell and Wardell, 2010) or the peroxisome proliferator-activated receptors (PPARs). PPARα is the target of the fibrate class of hypolipidemic drugs that function by up-regulating fatty acid catabolism, leading to decreased serum lipids and improved insulin resistance (Staels et al., 1998). Likewise, PPARγ is the target of anti-type II diabetes thiazolidione drugs, which improve insulin resistance and decrease blood glucose (Stumvoll and Haring, 2002). The third member of the PPAR fam-

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ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; SMRT, silencing mediator of retinoic and thyroid hormone; Bcl-6, B-cell lymphoma 6; DMEM, Dulbecco's minimal essential medium; GSK3787, 4-chloro-*N*-(2-((5-trifluoromethyl-2-pyridyl)sulfonyl)ethyl)benzamide; GSK0660, methyl 3-(*N*-(2-methoxy-4-(phenylamino)phenyl)sulfamoyl)thiophene-2-carboxylate; GW501516, [2-methyl-4-[[[4-methyl-2-[4-(trifluoromethyl)phenyl]-5-thiazolyl]methyl]thio]phenoxy]-acetic acid; GW1929, *N*-(2-benzoylphenyl)-*O*-[2-(methyl-2-pyridinylamino)ethyl]-L-tyrosine hydrochloride; GW7647, 2-(4-(2-(1-cyclohexanebutyl)-3-cyclohexylureido)ethyl)-phenyl-thio)-2-methyl-propionic acid; M-CSF, macrophage–colony-stimulating factor; PBS, phosphate-buffered saline; TGM, thioglycollate-elicited macrophages; RT-qPCR, real-time quantitative polymerase chain reaction; ChIP, chromatin immunoprecipitation; HDAC3, α-histone deacetylase 3; TR-FRET, time-resolved fluorescence resonance energy transfer; ID2, interaction domain 2; LBD, ligand binding domain; DMSO, dimethyl sulfoxide; GST, glutathione transferase; Angptl4, angiopoietin-like 4; PT-S58, methyl 3-(*N*-(4-(*tert*-butylamino)-2-methoxyphenyl)sulfamoyl)thiophene-2-carboxylate; ST247, methyl 3-(*N*-(4-(hexylamino)-2-methoxyphenyl)sulfamoyl)thiophene-2-carboxylate.

ily, PPARβ/δ, also plays an essential role in lipid and glucose metabolism, and several selective agonist drug candidates have been developed and are currently in clinical trials for the treatment of metabolic diseases (Bedu et al., 2005; Billin, 2008).

PPARβ/δ also has essential functions in other disease-associated biological processes, including cell differentiation, proliferation, apoptosis, and immune regulation (Peters et al., 2000; Di-Poi et al., 2002; Burdick et al., 2006; Kilgore and Billin, 2008; Müller et al., 2008b). Consistent with this conclusion, genetically engineered PPARβ/δ-deficient mice show defects in placenta development (Nadra et al., 2006), wound healing (Michalik et al., 2001), and intestinal Paneth cell differentiation (Varnat et al., 2006). Furthermore, PPARβ/δ also seems to modulate intestinal tumorigenesis (Peters et al., 2000; Barak et al., 2002; Di-Poi et al., 2002; Gupta et al., 2004; Wang et al., 2004; Burdick et al., 2006) and chemically induced skin carcinogenesis in different mouse models (Kim et al., 2004; Bility et al., 2008), as well as in tumor stroma organization (Abdollahi et al., 2007; Müller-Brüsselbach et al., 2007). PPARβ/δ also plays a key role in the differentiation and/or function of specific immune cells, including macrophages (Kang et al., 2008; Odegaard et al., 2008) and T-helper cells (Kanakasabai et al., 2010). These observations indicate that PPARβ/δ may represent a drug target for the treatment of major human diseases.

Like the other PPAR subtypes, PPARβ/δ forms heterodimers with the nuclear retinoid X receptor that bind to peroxisome proliferator response elements (PPREs) in target genes. A major function of agonists in this context is to induce a conformational change in PPARβ/δ that can result in the dissociation of interacting corepressors, such as silencing mediator for retinoid and thyroid hormone receptors (SMRT), and/or the induced interaction with specific coactivators, such as histone acetyl transferases, resulting in transcriptional activation (Yu and Reddy, 2007; Zoete et al., 2007). PPARβ/δ also regulates genes by mechanisms independent of DNA binding, for instance by sequestering the transcriptional repressor Bcl-6 in macrophages in a ligand-dependent fashion (Lee et al., 2003).

Although several high-affinity, subtype-specific, and bioavailable synthetic agonists have been developed, the PPARβ/δ inhibitory ligands described to date do not fulfill the same criteria. Thus, 2-(2-methyl-4-((4-methyl-2-(naphthalen-1-yl)thiazol-5-yl)methylthio)phenoxy)acetic acid (SR13904) (Zaveri et al., 2009) and 4-chloro-*N*-(2-((5-trifluoromethyl-2-pyridyl)sulfonyl-)ethyl)benzamide (GSK3787) (Palkar et al., 2010; Shearer et al., 2010) are not specific for PPARβ/δ. Moreover, GSK3787 is an irreversible inhibitor, which is undesirable in clinical applications. In contrast, 3-(((2-methoxy-4-(phenylamino)phenyl)amino)sulfonyl)-2-thiophenecarboxylate (GSK0660) (Shearer et al., 2008) is PPARβ/δ subtype-specific but is not bioavailable and requires the use of relatively high concentrations to achieve good inhibitory effects. All these compounds have been described as antagonists but may indeed be classified as inverse agonists. This is suggested by their effect on the basal expression of PPARβ/δ target genes (Shearer et al., 2008) and an increased recruitment of transcriptional corepressors (Palkar et al., 2010). However, on the basis of the available data, other explanations cannot be ruled out.

We have synthesized and tested new derivatives of GSK0660 and show that several of these compounds possess greatly improved inhibitory properties at equimolar concentrations as the parent molecule. Although one of these compounds [methyl 3-(*N*-(4-(hexylamino)-2-methoxyphenyl)sulfamoyl)thiophene-2-carboxylate (ST247); Fig. 1] is an inverse agonist, another compound [methyl 3-(*N*-(4-(*tert*-butylamino)-2-methoxyphenyl)sulfamoyl)thiophene-2-carboxylate (PT-S58); Fig. 1] was identified as the first pure PPARβ/δ antagonist. The novel inhibitory PPARβ/δ ligands described in the present study should represent invaluable tools to advance our understanding of the PPARβ/δ-regulated transcriptional network, its biological functions and its potential as a drug target.

Materials and Methods

Ligands. [2-Methyl-4-[[[4-methyl-2-[4-(trifluoromethyl)phenyl]-5-thiazolyl]methyl]thio]phenoxy]-acetic acid (GW501516) was purchased from Axxora (Lörrach, Germany), *N*-(2-benzoylphenyl)-*O*-[2-(methyl-2-pyridinylamino)ethyl]-L-tyrosine hydrochloride (GW1929) and L165,041 from Biozol (Eching, Germany), and 2-(4-(2-(1-cyclohexanebutyl)-3-cyclohexylureido)ethyl)-phenyl-thio)-2-methyl-propionic acid (GW7647) from Sigma-Aldrich (Steinheim, Germany). Synthesis of GSK0660 derivatives (Shearer et al., 2008) are described elsewhere (Toth et al., 2011).

Cell Culture. WPMY-1 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C and 5% CO_2.

Isolation and Cultivation of Mouse Bone Marrow Cells. Bone marrow cells were isolated from C57BL6 mice as described previously (Weischenfeldt and Porse, 2008) with some modifications. Mice were sacrificed by cervical dislocation, the tibiae and femurs were removed, and the bone marrow was flushed out with ice-cold PBS using a 26-gauge needle. Fibroblasts and macrophages were removed by subtractive adherence to cell culture plates. To remove aggregated cells and bone fragments, cells were passed through a 100-μm cell strainer (Thermo Fisher Scientific, Schwerte, Germany) and subsequently centrifuged for 5 min at 450*g*. The supernatant was removed, and the cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 1 mM sodium pyruvate, and 20 ng/ml mouse recombinant macrophage colony-stimulating factor (M-CSF) from Biozol (Eching, Germany) and antibiotics as above. Cells were then plated in six-well culture plates (Primaria; BD Biosciences, Heidelberg, Germany) in a humidified incubator at 37°C and 5% CO_2.

Isolation and Culture of Human Monocytes. Heparinized blood was obtained from healthy donors in 8-ml Vacutainer tubes (BD Biosciences). Sixteen milliliters of whole blood were diluted in 16 ml of PBS in a 50-ml tube and processed according to published procedures (Davies and Gordon, 2005) with modifications. In brief, the diluted blood was centrifuged for 30 min at 400*g* through a 16-ml cushion of Ficoll-Paque PREMIUM 1.084 (GE Healthcare, Munich, Germany). The peripheral blood mononuclear cell fraction was collected, transferred into a fresh 50-ml tube, and washed twice with PBS. The cells were resuspended in RPMI 1640 medium supplemented with 4% human serum type AB (Sigma, Hamburg, Germany), and 3×10^7 cells were plated in 60 × 15-mm culture dishes (Primaria; BD Biosciences). After a 30-min incubation at 37°C, the medium was removed and cells were washed with PBS until only strongly adherent cells remained. Four milliliters of RPMI 1640 medium supplemented with 2% human serum type AB was added. The cells were cultured for 7 days in the presence of 20 ng/ml human recombinant M-CSF (Enzo Life Sciences, Inc., Lörrach, Germany) in a humidified incubator at 37°C and 5% CO_2.

Mouse Strains. *Ppard*-null and wild-type mice have been described previously (Peters et al., 2000).

Thioglycollate-Elicited Macrophages. Brewer thioglycollate medium [4% (w/v); BD Difco] was prepared in distilled water, boiled to dissolve all solids, autoclaved, and stored in the dark for at least 3 months at 4°C before use. One milliliter of 4% Brewer thioglycollate medium was injected intraperitoneally through a 23-gauge needle. TGM cells were flushed from the peritoneal cavity 2 days later and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 1 mM sodium pyruvate, and antibiotics as described under *Cell Culture*.

Plasmids. LexA-PPARβ/δ and 7L-TATAi have been described previously (Jérôme and Müller, 1998; Fauti et al., 2006). LexA-PPARα and LexA-PPARγ were constructed in an analogous way to LexA-PPARβ/δ.

Transfections and Luciferase Reporter Assays. Transfections were performed with polyethylenimine (average mol. wt., 25,000; Sigma-Aldrich). Cells were transfected on 12-well plates at 70 to 80% confluence in Dulbecco's minimum essential medium plus 2% FCS with 2.5 μg of plasmid DNA and 2.5 μl of polyethylenimine (1:1000 dilution, adjusted to pH 7.0 and preincubated for 15 min in 100 μl of phosphate-buffered saline for complex formation). Four hours after transfection, the medium was changed, and cells were incubated in normal growth medium for 48 h. Luciferase assays were performed as described previously (Gehrke et al., 2003), except that a *Renilla reniformis* luciferase plasmid (pRL-SV40; Promega, Mannheim, Germany) was cotransfected for standardization. Values from three assays were combined to calculate averages and S.D.

Real-Time Quantitative PCR. cDNA was synthesized from 0.25 to 1 μg of RNA using oligo(dT) primers and the Omniscript kit (QIAGEN, Hilden, Germany). qPCR was performed in a Mx3000P RT-qPCR system (Stratagene, La Jolla, CA) for 40 cycles at an annealing temperature of 60°C. PCRs were carried out using the Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany) and a primer concentration of 0.2 μM following the manufacturer's instructions. L27 primers were used for normalization. Expression values were statistically analyzed by Student's *t* test (two-tailed, equal variance). Primer sequences have been published previously (Rieck et al., 2008; Kaddatz et al., 2010). The mouse *Ccl2* and human *CCL2* primers were as follows: *Ccl2*: forward, 5′-ACT CAC CTG CTG CTA CTC ATT CAC; reverse, 5′-AAC TAC AGC TTC TTT GGG ACA CCT; *CCL2*: forward, 5′-TTC TGT GCC TGC TGC TCA T; reverse, 5′-GGG GCA TTG ATT GCA TCT.

Chromatin Immunoprecipitation Analysis. ChIP was performed essentially as described previously (Kaddatz et al., 2010; Stockert et al., 2011) using the following antibodies: IgG pool (IgG from rabbit serum, reagent grade; Sigma-Aldrich); anti-histone deacetylase 3, H99 (Santa Cruz Biotechnology, Heidelberg, Germany); and anti-SMRT, ChIP grade (Abcam Cambridge, UK). Primer sequences covering the *ANGPTL4* PPREs were as described previously (Kaddatz et al., 2010).

Time-Resolved Fluorescence Resonance Energy Transfer Assays In Vitro. Ligand binding was determined by TR-FRET in vitro (Stafslien et al., 2007) using the Lanthascreen PPARα, PPARβ/δ, and PPARγ competitive binding assays as described previously (Rieck et al., 2008; Naruhn et al., 2010). The interaction of the PPARβ/δ LBD with coregulator peptides was determined using the Lanthascreen TR-FRET PPARβ/δ coregulator assay with either a fluorescein-labeled coactivator peptide (C33 peptide provided by the manufacturer) or a fluorescein-labeled corepressor peptide derived from the SMRT-interaction domain 2 (SMRT-ID2; Naruhn et al., 2010). Assays were carried out in 100 mM KCl, 20 mM Tris, pH 7.9, 0.01% Triton X-100, and 1 μg/μl bovine serum albumin for 30 min. Measurements were performed on a VICTOR3 V Multilabel Counter (WALLAC 1420; PerkinElmer Life and Analytical Sciences, Waltham, MA) with instrument settings as described in the manufacturer's instructions for LanthaScreen assays.

Results

PPAR Subtype-Specific Interaction of GSK0660 Derivatives with PPAR Ligand Binding Domains In Vitro. To be able to select suitable compounds derived from GSK0660 (Fig. 1) for a detailed analysis, we first determined their binding to the LBD of PPARβ/δ in an in vitro TR-FRET-based competitive ligand binding assay. In this assay, the terbium-labeled PPARβ/δ LBD interacts with the fluorescent PPAR ligand Fluormone Pan-PPAR Green, which produces FRET from terbium (495 nm) to Pan-PPAR Green (520 nm). Displacement of the fluorescent ligand by an unlabeled test compound results in a quantifiable attenuation of FRET, as indicated by a decreased ratio of the fluorescence intensities at 520 nm and 495 nm. Two of our GSK0660-derived compounds showed a significantly enhanced competition efficiency in this assay (Fig. 2A). These are ST247 and PT-S58, with IC_{50} values of 93 nM and 98 nM, respectively, compared with 310 nM for GSK0660. In contrast, methyl 3-(*N*-(2-methoxy-4-morpholinophenyl)sulfamoyl)thiophene-2-carboxylate (PT-S77) was considerably less efficient with an IC_{50} value of 2700 nM (Fig. 2A).

We next tested GSK0660, ST247, and PT-S58 for PPAR

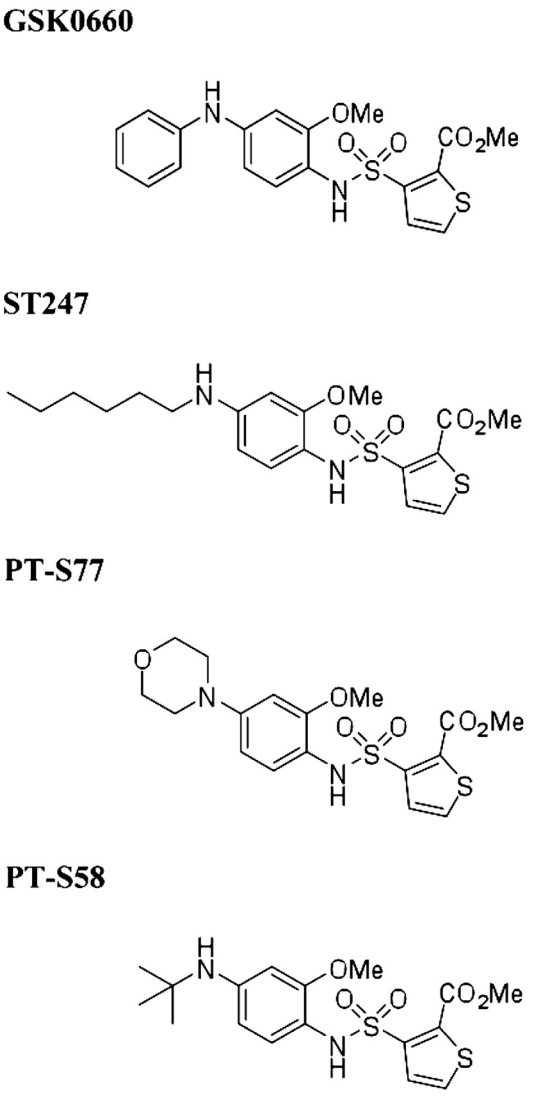

Fig. 1. Chemical structures of compounds analyzed in the present study.

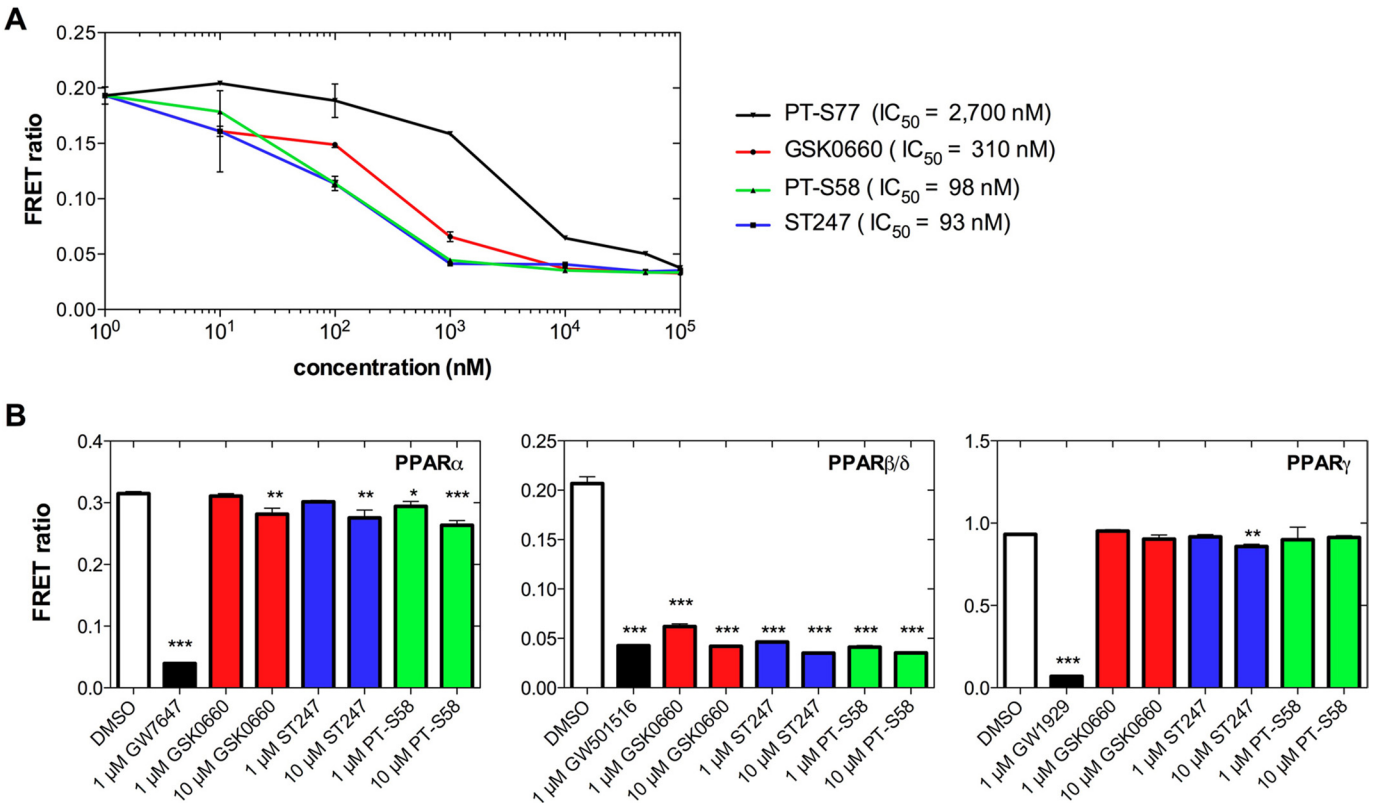

Fig. 2. Competitive in vitro PPAR ligand binding assays. A, displacement of the Fluormone Pan-PPAR Green PPAR ligand from recombinant GST-PPARβ/δ by the indicated compounds was determined by TR-FRET. B, competition for Fluormone Pan-PPAR Green by GSK0660, ST247, and PT-S58 (1 and 10 μM each) to PPARα, PPARβ/δ, and PPARγ compared with solvent (DMSO) only or 1 μM the PPARα agonist GW7647 (left), the PPARβ/δ agonist L165,041 (middle), or the PPARγ agonist GW1929 (right). Results ("FRET ratio") are expressed as the ratio of fluorescence intensity at 520 nm (fluorescein emission excited by terbium emission) and 495 nm (terbium emission). All data points represent averages of triplicates (± S.D). IC_{50} values were determined by nonlinear regression analysis using Prism software (ver. 5.0; GraphPad Software, San Diego, CA) . ***, **, and *, significant difference from DMSO-treated sample ($P < 0.001$, $P < 0.01$, and $P < 0.05$ by t test, respectively).

subtype selectivity using the same competitive binding assay. The data in Fig. 2B show that at 1 μM, all compounds showed only marginal competition, if any, in the PPARα or PPARγ binding assays, whereas strong competition was seen for PPARβ/δ, as expected. At 10 μM, a very weak, but statistically significant binding to PPARα was observed with all three compounds, and a marginal effect was seen with ST247 and PPARγ. However, because ST247 and PT-S58 reach their maximal binding efficacy for PPARβ/δ below 1 μM (Fig. 2A), both compounds can be considered highly subtype-selective. The validity of the assay was confirmed by demonstrating that the PPARα agonist GW7647, the PPARβ/δ agonist GW501516, and the PPARγ agonist GW1929 strongly and specifically interacted with the respective PPAR subtypes (Fig. 2B).

Coregulator Peptide Recruitment In Vitro. To assess how the chemical alterations would affect the inhibitory properties of GSK0660, we investigated the effect of these compounds on the interaction of PPARβ/δ with a synthetic corepressor peptide by TR-FRET. This assay measures the interaction of the PPARβ/δ LBD (indirectly labeled by terbium) with the fluorescein-labeled SMRT-ID2 peptide, derived from the interaction domain 2 of the corepressor SMRT, which was previously identified as a peptide strongly interacting with the PPARβ/δ LBD in response to binding of the antagonist GSK3787 (Palkar et al., 2010). The result of this assay is a measure of the intensity of the fluorescence emission (520 nm) by fluorescein (SMRT-ID2 peptide) excited by terbium-emitted fluorescence (495 nm). An increased ratio of the intensities at 520 and 495 nm therefore indicates an increased interaction. The data obtained by this assay (Fig. 3) closely mirror the results obtained by the competitive binding assay (Fig. 2) for ST247, which induced a clearly enhanced interaction of the PPARβ/δ LBD with the SMRT-ID2 peptide (EC_{50} = 10 nM) compared with the parent compound GSK0660 (EC_{50} = 65 nM). In contrast, PT-S58 was unable to trigger corepressor peptide recruitment (Fig. 3), even though it strongly competed in the ligand binding assay (Fig. 2).

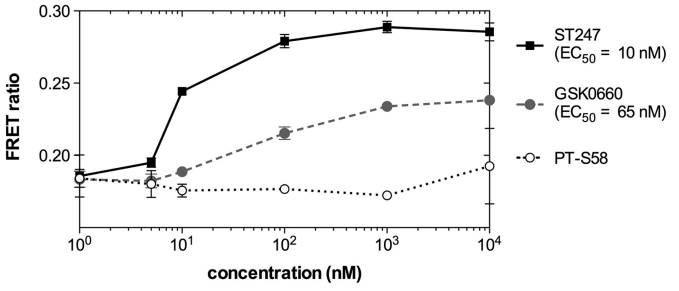

Fig. 3. Ligand-induced binding of a corepressor derived peptide to the PPARβ/δ LBD in vitro. Interaction of fluorescein labeled SMRT-ID2 peptide and recombinant GST-PPARβ/δ bound by a terbium-labeled anti-GST antibody was determined by TR-FRET. Results are expressed as the "FRET ratio" determined as in Fig. 2. All data points represent averages of triplicates (± S.D). EC_{50} values were determined by nonlinear regression analysis using Prism software.

The latter finding suggests that PT-S58 antagonizes ligand binding without direct effects on the receptor that would enable coregulator interactions. These features are characteristic of pure competitive antagonists. In this case, PT-S58 should prevent not only the agonist-mediated interaction with coactivators, but also the recruitment of corepressors induced by inverse agonists. We therefore tested the effect of PT-S58 on coregulator recruitment triggered by a synthetic agonist (L165,041) or an inverse agonist (ST247). The data in Fig. 4 indeed confirm the prediction, because PT-S58 efficiently inhibited both the L165,041-induced recruitment of the coactivator peptide C33 and the ST247-triggered interaction with the SMRT-ID2 corepressor peptide. We therefore conclude from these in vitro studies that 1) PT-S58 classifies as a pure antagonist, 2) ST247 is an inverse agonist, and 3) PT-S77 is largely inactive. We therefore focused all subsequent studies on ST247 and PT-S58.

Specific Inhibition of the Transcriptional Activity of PPARβ/δ. We next analyzed the effect of ST247 and PT-S58 on the agonist-induced transcriptional activity of the three PPAR subtypes in human WPMY-1 myofibroblasts. In this assay, a luciferase reporter construct with multiple LexA binding sites upstream of a minimal basal promoter is cotransfected with a plasmid expressing a transcriptional activator consisting of the PPARα, PPARβ/δ, or PPARγ LBD fused to a LexA DNA binding domain. As shown in Fig. 5, treatment with subtype-specific agonists resulted in a 2- to 2.5-fold induction of transcriptional activity for all three fusion proteins (leftmost lanes). Although neither of the two GSK0660 derivatives had any significant effect on PPARα or PPARγ driven transcription, they both efficiently antagonized the L165,041-mediated transcriptional activation of PPARβ/δ. These data are consistent with the results of the in vitro ligand binding assay described above (Fig. 2) and con-

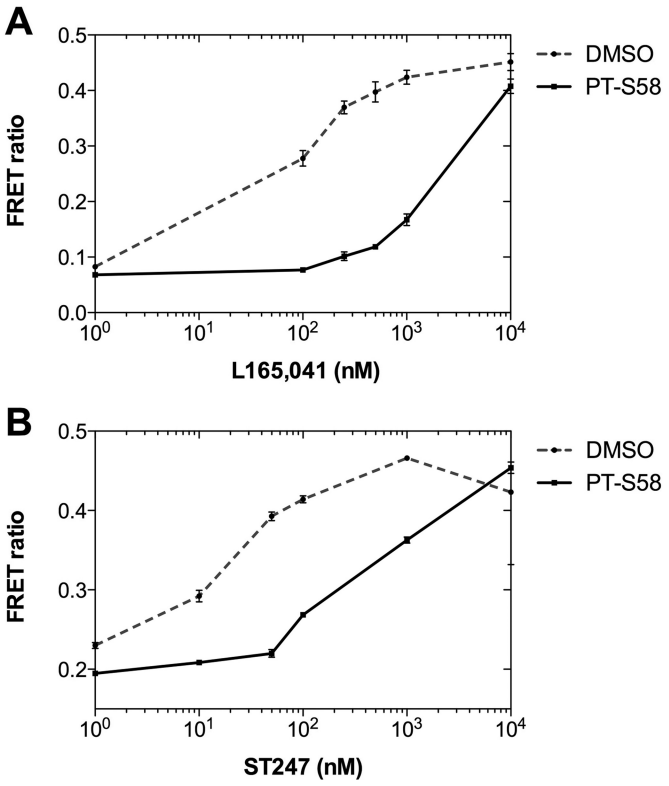

Fig. 4. Inhibition by PT-S58 of both agonist-induced coactivator recruitment (A) and inverse agonist-triggered corepressor binding (B). Coregulator peptide recruitment was determined by TR-FRET in vitro as in Fig. 3, except that in A, the C33 coactivator peptide was used. The assay was performed in the presence of a constant concentration of PT-S58 (1 μM) and increasing levels of the agonist L165,041 (A) or the inverse agonist ST247 (B).

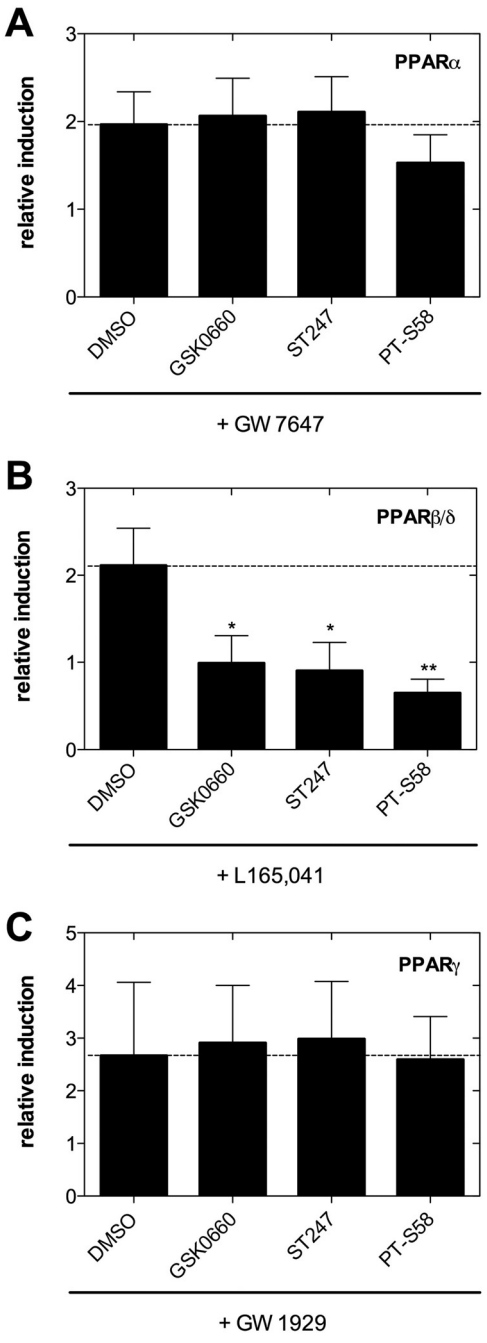

Fig. 5. Effects of GSK0660, ST247 and PT-S58 on the agonist-induced transcriptional activity of LexA-PPARα (A), LexA-PPARβ/δ (B), and LexA-PPARγ (C). WPMY-1 cells were transiently transfected with a luciferase reporter plasmid containing multiple LexA binding sites. Four hours after transfection, the cells were treated with the indicated compounds (500 nM) for 48 h, followed by 300 nM GW7647 (PPARα agonist), 500 nM L165,041 (PPARβ/δ agonist), or 300 nM GW1929 (PPARγ agonist) or agonist solvent. Induction values represent luciferase activities of agonist-treated cells relative to cells treated with agonist solvent. ** and *, significant difference from untreated sample ($P < 0.01$ and $P < 0.05$ by t test, respectively).

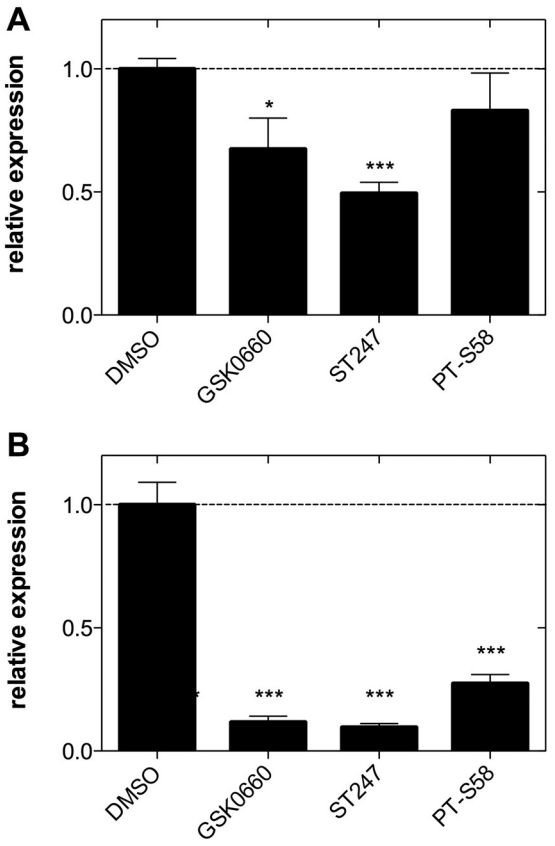

Fig. 6. Impact of GSK0660, ST247, and PT-S58 on the expression of the endogenous PPARβ/δ target gene *ANGPTL4*. Cells were treated for 24 h with the indicated compounds (1 μM), RNA was isolated and analyzed by qPCR using *L27* for normalization. A, human myofibroblasts (WPMY-1); B, TGMs. Cells were treated for 6 h, and RNA was analyzed as in A. Expression values were calculated relative to DMSO-treated cells and represent averages of triplicates (± S.D.). *** and *, values significantly different ($P < 0.001$ and $P < 0.05$ by t test, respectively) between DMSO- and ligand-treated cells.

firm that both derivatives have retained the specificity for PPARβ/δ.

Inhibition of Endogenous PPARβ/δ Target Genes. *ANGPTL4* is one of the best established PPARβ/δ target genes (Mandard et al., 2004; Kaddatz et al., 2010) and was therefore used for testing the regulatory properties of GSK0660 derivatives in an endogenous context. *ANGPTL4* is repressed by PPARβ/δ under basal conditions because of the formation of a repressive PPARβ/δ complex at its PPREs, which is abolished either by siRNA-mediated PPARβ/δ depletion or by treatment with a synthetic agonist (Kaddatz et al., 2010).

ST247 reduced *ANGPTL4* expression in WPMY-1 myofibroblasts by approximately 50% (Fig. 6A) and thus showed significantly stronger effects than GSK0660 (30%) at equimolar concentrations (1 μM). In contrast, the pure antagonist PT-S58 was largely ineffective. The data are consistent with the ability of inverse agonists GSK0660 and ST247 to trigger corepressor recruitment in vitro, as determined by TR-FRET (Fig. 3). In view of PPARβ/δ's pivotal function in macrophages and inflammation we also tested the same compounds in TGMs, where we observed a considerably stronger effect compared with WPMY-1 cells. Both GSK0660 and ST247 reduced *ANGPTL4* expression in TGMs by approximately 90% (Fig. 6B). Moreover, a clear effect (63% inhibition) was also seen with PT-S58, which is conceivable in view of the high concentrations of PPARβ/δ ligands in macrophages, notably (±)15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (Chapkin et al., 1988; Laviolette et al., 1988; Huang et al., 1999; Naruhn et al., 2010).

We also performed titration experiments to determine the IC_{50} values for GSK0660 and ST247 in WPMY-1 cells (Fig. 7A) and TGMs (Fig. 7B). This analysis clearly revealed the superior effect of ST247 (right) compared with the parent compound (left), with IC_{50} values of 210 nM for GSK0660 and

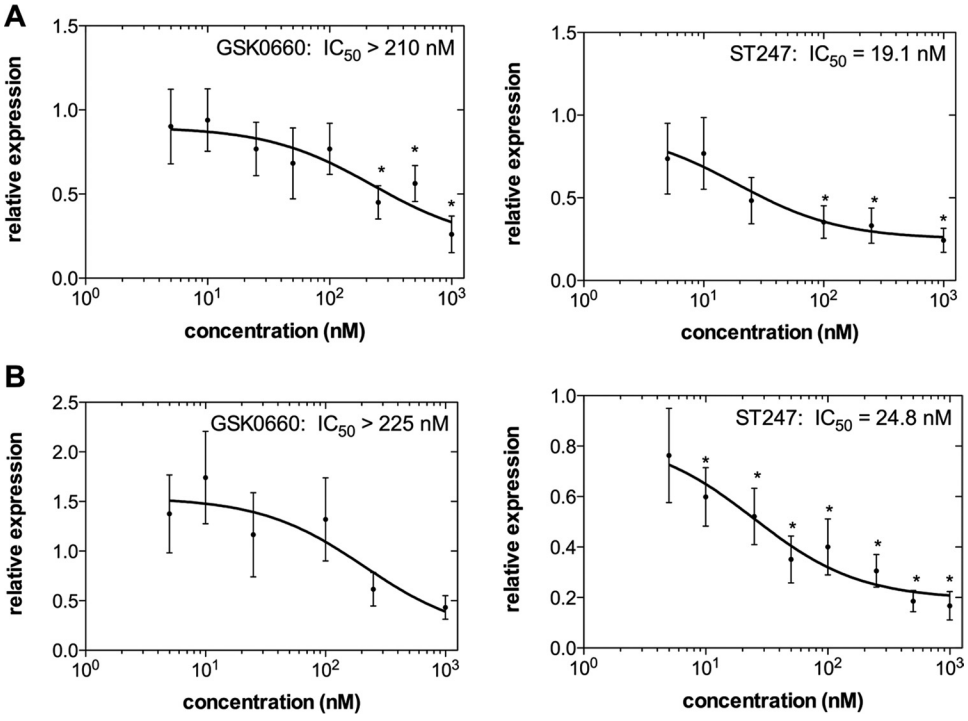

Fig. 7. Titration of GSK0660 and ST247 effects on endogenous *ANGPTL4* expression. A, WPMY-1 cells. B, TGMs. The experimental setup was as in Fig. 6. Expression values were calculated relative to DMSO-treated cells and represent averages of triplicates (± S.D.). *, values significantly different ($P < 0.05$ by t test) between DMSO- and ligand-treated cells. IC_{50} values were determined as in Fig. 2.

19 nM for ST247 in WPMY-1 cells, and IC_{50} values in TGMs of >225 and 25 nM, respectively.

Because none of the GSK0660-derived compounds has any detectable effect on PPARα and PPARγ (Fig. 5), it is very likely that the observed inhibition of *Angptl4* expression is mediated though PPARβ/δ. This is further supported by our observation that the inhibitory effect of ST247 on *Angptl4* expression was abolished in macrophages lacking PPARβ/δ (Fig. 8A). Furthermore, the effect of ST247 is not gene-specific, because similar results were obtained for a second PPARβ/δ target gene, *Adrp* (Fig. 8B). Finally, the rapid action of ST247 on *Angptl4* expression with a reduction of >60% within 1 h of treatment (Fig. 8C) argues in favor of a direct effect on PPARβ/δ, which leads to an instant reorganization of chromatin-associated transcription complexes upon ST247 binding.

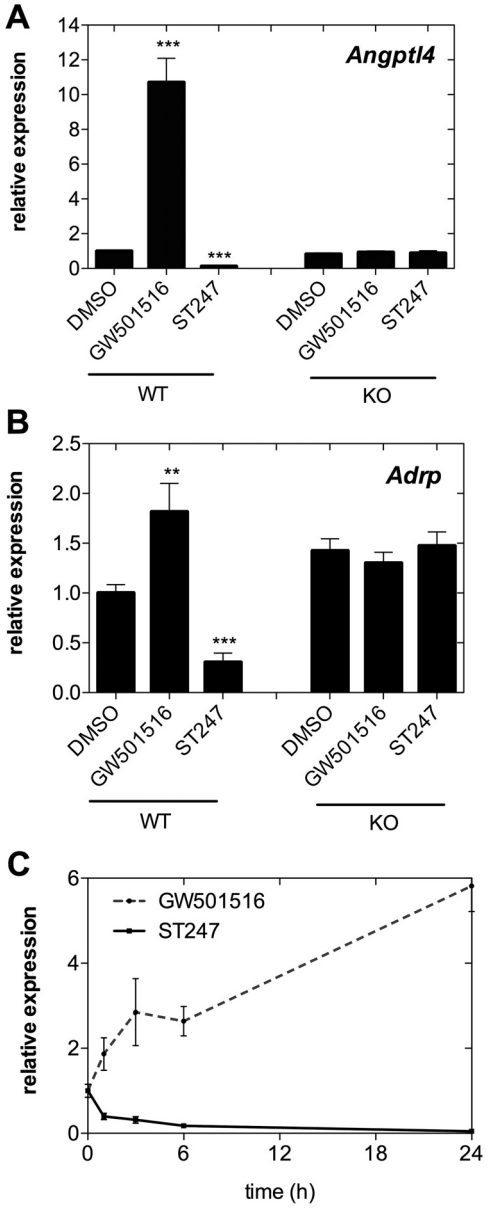

Fig. 8. Dependence on PPARβ/δ and kinetics of target gene repression by ST247. TGM from *Ppard* wild-type (WT) and *Ppard*-null (KO) mice were treated with GW501516 (300 nM), ST247 (500 nM), or solvent (DMSO) for 6 h, and the expression of *Angptl4* (A) and *Adrp* (B) was determined by RT-qPCR. C, TGMs were treated with GW501516 (300 nM) or ST247 (500 nM) for different times, and the expression of *Angptl4* relative to DMSO-treated cells was plotted against the time of treatment. *** and **, significant difference from untreated sample ($P < 0.001$ and $P < 0.01$ by t test, respectively).

Inverse Agonist-Triggered Corepressor Recruitment to Chromatin-Associated PPARβ/δ. To investigate the effect of GSK0660 and its derivatives on the assembly of chromatin-associated corepressor complexes, we analyzed the in vivo recruitment of HDAC3, a central component of SMRT/nuclear receptor corepressor complexes (Guenther et al., 2000; Li et al., 2000; Wen et al., 2000; Shi et al., 2002; Codina et al., 2005). Consistent with the transcriptional repression induced by these compounds, ChIP analyses of human WPMY-1 cells (Fig. 9A) demonstrated an enhanced recruitment of HDAC3 to the *ANGPTL4* PPREs compared with

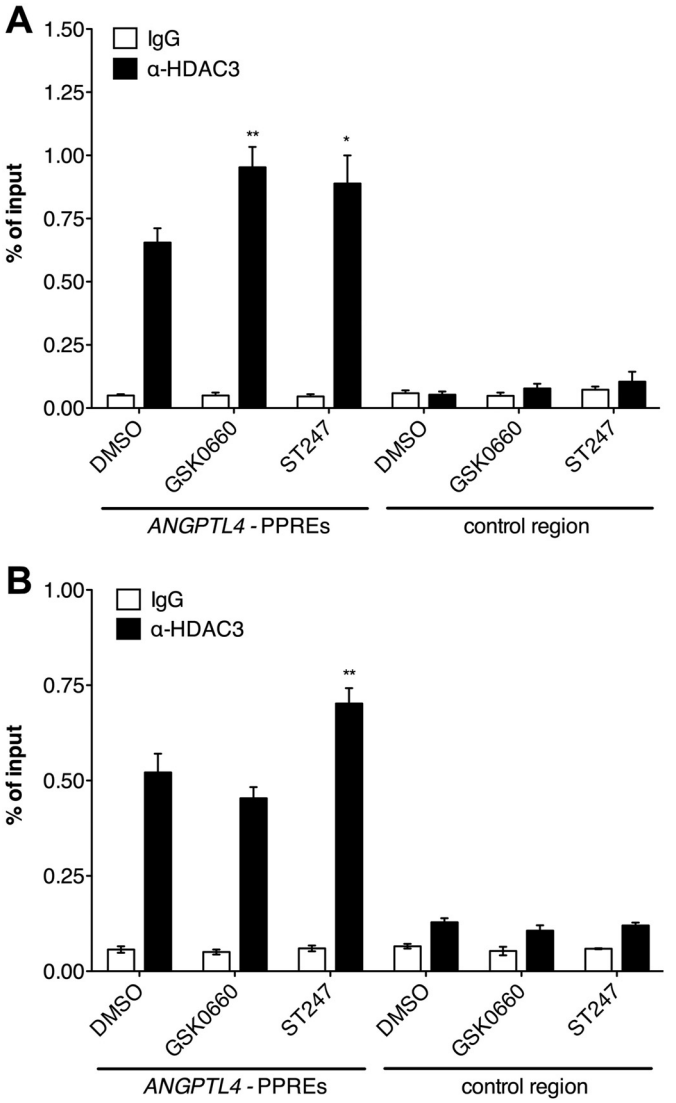

Fig. 9. Impact of GSK0660 and ST247 on corepressor recruitment to the *ANGPTL4* promoter in WPMY-1 myofibroblasts. Cells were treated with the indicated compounds (A, 1 μM; B, 100 nM) for 45 min. ChIP was carried out using antibodies against HDAC3 or with a nonspecific rabbit IgG pool (negative control). DNA was amplified with primers encompassing the *ANGPTL4* PPREs or a control region. Relative amounts of amplified DNA in immunoprecipitates were calculated by comparison with 1% of input DNA. Results are expressed as percentage input and represent averages of triplicates (± S.D). ** and *, significant difference from untreated sample ($P < 0.01$ and $P < 0.05$ by t test, respectively).

cells treated with solvent (DMSO). The specificity of the ChIP assay was shown by the lack of antibody binding to an irrelevant region of the *PDK4* gene (Fig. 9A, control region). Furthermore, PT-S58 failed to induce HDAC3 recruitment in vivo (Fig. 10), which is consistent with the in vitro corepressor peptide interaction data in Fig. 3. The data in Fig. 9A also indicate that GSK0660 and ST247 have similar effects at 1 μM. However, lowering the concentration to 100 nM abrogated the effect by GSK0660 but did not affect ST247-triggered HDAC3 recruitment (Fig. 9B), indicating that the latter compound is more efficient at inducing corepressor complex assembly. This concentration-dependent effect is in agreement with the in vitro titration experiment in Fig. 3.

Finally, we analyzed the effect of all three inhibitory compounds with differential inverse agonistic properties on the recruitment of the corepressors SMRT and HDAC3 (Fig. 10). As expected, enrichment of both corepressor proteins was found at the *ANGPTL4* PPREs in WPMY-1 cells in response to ST247, but not after treatment with PT-S58, confirming the classification of the latter compound as a pure PPARβ/δ antagonist lacking inverse agonistic properties.

Effect of PPARβ/δ Ligands on Monocytic Activation. The experiments reported above all address the function of PPARβ/δ in the context of PPRE-regulated transcription. However, PPARβ/δ also has PPRE-independent functions, as exemplified by the sequestration of the transcriptional repressor BCL6 in macrophages (Lee et al., 2003). The ligand-regulated interaction of these two proteins reportedly plays an essential role in regulating the inflammatory status of the cells by modulating the expression of BCL6 target genes (Lee et al., 2003). We were therefore interested to investigate the effect of the inhibitory PPARβ/δ ligands ST247 and PT-S58 on the BCL6 target and monocytic activation marker *CCL2* (also termed *MCP1*) in two experimental systems (i.e., mouse bone marrow cells and human blood monocytes stimulated with M-CSF).

As expected, *CCL2* expression was strongly repressed by a PPARβ/δ agonist (L165,041, 1 μM; GW501516, 0.3 μM) in both the murine (67% inhibition by L165,041) and human system (73% down-regulation by GW501516). In the experiment setting, 1 μM ST247 produced the opposite effect and increased *CCL2* expression by 79 and 103%, respectively. In the murine system, we also tested the effect of the agonist PT-S58 and found a clear stimulatory effect (123%), which is presumably attributable to the presence of high levels of endogenous PPARβ/δ ligands in monocytic cells, as discussed above for the data in Fig. 6B. In agreement with these data, we also observed a partial reversal of the agonist-mediated CCL2 repression by both ST247 and PT-S58 (Fig. 11A). Taken together, these data clearly demonstrate that the inhibitory ligands described in the present study modulate a

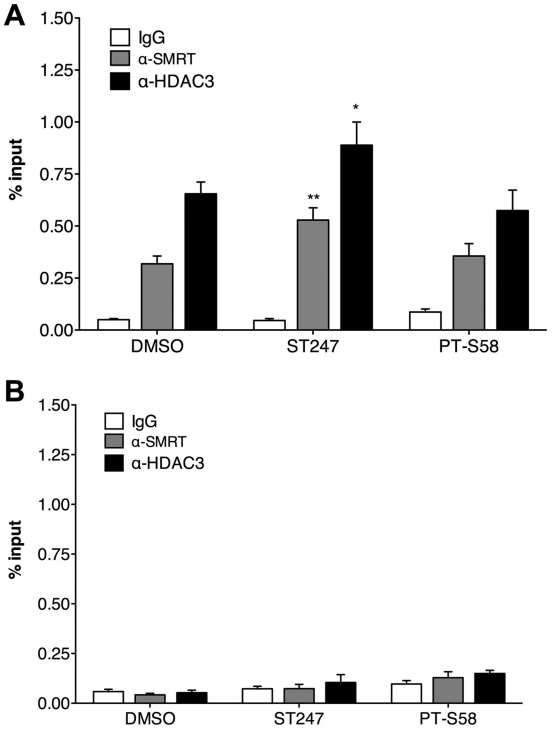

Fig. 10. Enhancement of corepressor recruitment to the *ANGPTL4* promoter by the inverse agonist ST247, but not by the antagonist PT-S58, in WPMY-1 myofibroblasts. All compounds were used at a concentration of 1 μM. DNA was amplified with primers encompassing the *ANGPTL4* PPREs (A) or a control region (B). The experimental conditions were as in Fig. 9, except that an antibody against SMRT was included. ** and *, significant difference from untreated sample ($P < 0.01$ and $P < 0.05$ by t test, respectively).

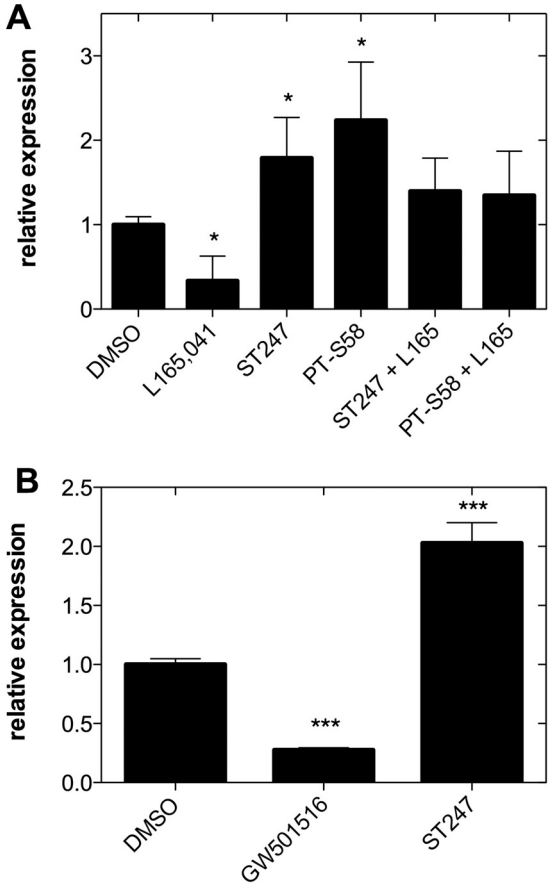

Fig. 11. Effect of PPARβ/δ ligands on expression of the monocytic activation marker gene *CCL2*. A, mouse bone marrow cells were induced to monocytic differentiation by M-CSF and treated with the PPARβ/δ agonist L165,041, the inverse agonist ST247, the antagonist PT-S58 (1 μM each), or solvent (DMSO) for 24 h. B, human blood monocytes were induced to macrophage differentiation by M-CSF for 7 days in the presence of the PPARβ/δ agonist GW501516 (300 nM), the inverse agonist ST247 (1 μM), or solvent (DMSO). Cells were harvested and *CCL2* mRNA expression was determined by RT-qPCR. ***, **, and *, significant difference from DMSO-treated sample ($P < 0.001$, $P < 0.01$, and $P < 0.05$ by t test, respectively).

monocytic activation marker in the opposite direction than a PPARβ/δ agonist, providing further evidence for the specificity of these ligands.

Discussion

Due its functions in disease-related biological processes, including metabolism and inflammation, PPARβ/δ is a potentially interesting pharmacological target. GSK0660 is a subtype-specific inhibitory ligand for PPARβ/δ, but its mechanism of action is not fully understood. We have synthesized a series of GSK0660 derivatives (Fig. 1) and show that several of these compounds at equimolar concentrations display improved inhibitory properties relative to the parent compound but retain PPAR subtype selectivity. It is noteworthy that one of these compounds turned out as a bona fide antagonist, whereas several other high-affinity derivatives show strong inverse agonistic properties.

In vitro ligand binding and coregulator interaction studies. Key to the development of the compounds described in the present study was our observation that replacement of the 4-aminophenyl group in GSK0660 by a different 4-aminoalkyl substituent resulted in a clear gain in affinity for PPARβ/δ in vitro, as determined by a competitive TR-FRET assay (Fig. 2A). Thus, the introduction of a 4-*n*-hexylamino side chain in ST247 or a 4-(*tert*-butylamino) group in PT-S58 greatly increased the affinity for PPARβ/δ, as indicated by a >3-fold lower IC_{50} value in vitro. It is noteworthy that the increased affinity of ST247 correlated with an enhanced interaction of PPARβ/δ with a corepressor-derived peptide (Fig. 3), which provided the first indication that these compounds retained their inhibitory properties. On the other hand, incorporation of the 4-amino group into a ring system in PT-S77 resulted in a clearly decreased affinity to PPARβ/δ (Fig. 2A).

There is no strict correlation, however, between the affinity of a given compound for PPARβ/δ and the induction of corepressor recruitment, as suggested by PT-S58, which performed well in the competitive TR-FRET assay (Fig. 2), but failed to trigger the interaction with the SMRT-ID2 peptide (Fig. 3). This suggests that PT-S58 possesses competitive antagonistic properties, but in contrast to the other compounds, lacks inverse agonistic properties. PT-S58 differs from ST247 by a shorter aminoalkyl substituent with a branching point close to the 4-amino group, suggesting that the more extended side chain of ST247 allows for the establishment of additional hydrophobic drug-protein interactions that stabilize a PPARβ/δ conformation favoring corepressor interactions, thus enabling inverse agonistic functions.

PPAR Subtype-Specific Inhibitory Effects in Cell Culture. It is noteworthy that both ST247 and PT-S58 retained their ability to enter cells and to inhibit the agonist-induced transcriptional activity of PPARβ/δ but did not affect ligand-activated PPARα and PPARγ (Fig. 5), which is consistent with the marginal effects on PPARα and PPARγ seen in vitro (Fig. 2B). We also assessed the efficacy of ST247 to inhibit the endogenous PPARβ/δ target gene *ANGPTL4* in human myofibroblasts and murine macrophages relative to GSK0660. The titration experiments in Fig. 6B clearly demonstrated the superiority of ST247 over the parent compound, as reflected by a ~10-fold higher IC_{50} value for GSK0660. The effect of ST247 on both *ANGPTL4* and *ADRP* transcription was completely abolished in macrophages from *Ppard* knockout mice (Fig. 8A), confirming the high selectivity of the compound. Nevertheless, it cannot be ruled out that ST247 has other unidentified off-target effects due to unknown interactions with other proteins.

ChIP analyses showed that the GSK0660 and ST247-mediated down-regulation of *ANGPTL4* correlated with an increased recruitment of the transcriptional corepressors HDAC3 and SMRT (Figs. 9 and 10), indicating that these compounds enhance the repressor function of PPARβ/δ. This effect was seen at a concentration of 1 μM with both compounds (Fig. 9A). However, decreasing the concentration to 100 nM canceled the effect of GSK0660 but did not affect ST247 (Fig. 9B). This concentration-dependent effect is consistent with the considerably better performance of ST247 in the TR-FRET-based corepressor peptide recruitment assay (Fig. 3) and may also explain published observations (Shearer et al., 2008), suggesting that GSK0660 does not induce corepressor recruitment.

Antagonistic versus Inverse Agonistic Properties. By definition, an inverse agonist binds to the same receptor binding site as an agonist and reverses constitutive activity of the receptor, thereby exerting the opposite effect as a receptor agonist. In contrast, a receptor antagonist does not induce a response itself upon receptor binding but merely blocks agonist-mediated effects. Consequently, antagonist binding will disrupt the interaction with, and inhibit the function of, both agonists and inverse agonists. Based on these criteria, ST247 was classified as an inverse agonist and can be clearly distinguished from PT-S58, classified as a full antagonist. Thus, ST247 strongly enhanced corepressor recruitment in vivo and in vitro, whereas PT-S58 did not (Figs. 3 and 10). Likewise, basal level *ANGPTL4* transcription was efficiently blocked by ST247 in WPMY-1 cells but was not significantly altered by PT-S58 (Fig. 6A). On the other hand, inhibited basal level *ANGPTL4* expression in macrophages (Fig. 6B), which, unlike WPMY-1 cells, produce high amounts of endogenous PPARβ/δ ligands, such as (±)15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (Chapkin et al., 1988; Laviolette et al., 1988; Huang et al., 1999; Naruhn et al., 2010). PT-S58 also bound to the PPARβ/δ LBD with high affinity (Fig. 2A), blocked the agonistic effect of L165,041 in WPMY-1 cells (Fig. 5), and prevented corepressor recruitment induced by ST247 (Fig. 4), clearly demonstrating the antagonistic properties of PT-S58.

It has previously been proposed that PPARs have high basal activity because of a ligand-independent stabilization of helix 12 in the LBD, resulting in ligand-independent coactivator association (Molnar et al., 2005). Although this could in principle explain the inverse agonistic properties of ST247, this is unlikely, because we were unable to detect changes in CBP recruitment to the *ANGPTL4* gene upon ST247 treatment (data not shown). We therefore favor instead a different scenario explaining the inverse agonistic effects. In WPMY-1 cells, the *ANGPTL4* gene is repressed under basal conditions by PPARβ/δ because of the absence of substantial levels of endogenous PPARβ/δ agonists (Kaddatz et al., 2010). It is likely that ST247 potentiates PPARβ/δ-mediated repression through a conformation-mediated enhancement of corepressor association, which is consistent with our TR-FRET (Fig. 3) and ChIP analyses (Figs. 9 and 10). Of course, the two models (i.e., coactivator displacement

and corepressor recruitment) are not mutually exclusive, and different scenarios may exist in different cell types, depending on their precise molecular composition.

Perspectives. Two main conclusions can be drawn from the present study. First, replacement of the 4-aminophenyl group in GSK0660 by appropriate substituents results in a dramatic gain in affinity for PPARβ/δ, concomitant with a greatly improved inhibitory potential in cultured cells. Second, depending on the substituent, either inverse agonists (such as ST247) or bona fide antagonists (like PT-S58) can be generated. These new compounds now provide highly efficient and specific probes to investigate the regulation and functions of PPARβ/δ, which includes the possibility to discriminate between effects on transcriptional activation by endogenous agonists and ligand-independent regulation.

Furthermore, in view of their high affinity and specificity, these compounds may represent interesting new tools to investigate the potential of PPARβ/δ as a drug target. The antagonist PT-S58 may be of particular interest in this context, in that it may be used to modulate PPARβ/δ selectively in cells exposed to high levels of endogenous agonists, which is a frequent feature of pathological processes. GSK0660 is poorly bioavailable in mice, making its use in animal models difficult (Shearer et al., 2008). Pharmacokinetic studies have shown that the bioavailability of ST247 is as poor as that of the parent compound (study performed at Cerep, Redmond, WA; data not shown). However, the analysis of further derivatives has provided preliminary evidence that certain positions in the molecule can be modified without a loss of affinity or specificity (Toth et al., 2011). The linkage of suitable side groups to these positions is a potential strategy to improve the bioavailability of ST247 and is currently pursued in our laboratories.

ST247 and PT-S58 may also represent interesting new lead structures for drug development. However, adverse side effects of inhibiting PPARβ/δ are likely in view of its positive role in insulin sensitization (Lee et al., 2006) and its frequently inhibitory function in both inflammation (Kilgore and Billin, 2008) and tumor cell proliferation (Peters and Gonzalez, 2009). It should be noted, however, that the pathophysiological role of PPARβ/δ is not entirely clear, and its contribution to tumorigenesis is partly controversial (Müller et al., 2008a). Furthermore, PPARβ/δ regulates its target genes by different mechanisms, including target gene-specific effects of ligands (Adhikary et al., 2011). Owing to these complexities, the biological response to PPARβ/δ inhibitors is difficult to predict but is likely to be less severe than the global genetic inactivation of the *Ppard* gene. Moreover, PPARβ/δ antagonists or inverse agonists do not necessarily have opposite effects as PPARβ/δ agonists, because the outcome is determined by the availability of transcriptional coregulators and endogenous ligands.

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Authorship Contributions

Participated in research design: Naruhn, Klebe, Müller-Brüsselbach, Diederich, and Müller.

Conducted experiments: Naruhn, Toth, Adhikary, Kaddatz, Pape, and Dörr.

Performed data analysis: Naruhn, Adhikary, and Kaddatz.

Wrote or contributed to the writing of the manuscript: Naruhn and Müller.

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