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A Widely Used Retinoic Acid Receptor Antagonist Induces PPAR γ Activity

Michael Schupp, Joshua C. Curtin, Roy J. Kim, Andrew N. Billin, Mitchell

A. Lazar

Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine and
Department of Genetics, and The Institute for Diabetes, Obesity, and Metabolism, University
of Pennsylvania School of Medicine, Philadelphia, PA USA (M.S., J.C.C., R.J.K., A.N.B.,

M.A.L)

Division of Endocrinology, Endocrinology, Children's Hospital of Philadelphia, Philadelphia,
PA USA (R.J.K.)

Department of High-throughput Biology, GlaxoSmithKline, Research Triangle Park, NC
USA (A.N.B.)

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Running title: An RAR α antagonist activates PPAR γ

Address Correspondence to: Mitchell A. Lazar, M.D., Ph.D., University of Pennsylvania

School of Medicine, 611 CRB, 415 Curie Blvd. Philadelphia, PA 19104-6149. Phone: (215)

898-0198; Fax: (215) 898-5408; e-mail: lazar@mail.med.upenn.edu

25 text pages,

2 tables,

6 figures,

40 references

Words: abstract 229, introduction 306 and discussion 496

Abbreviations: Nuclear receptors (NRs), Peroxisome Proliferator-Activated Receptor (PPAR), Retinoic Acid Receptor (RAR), thiazolidinediones (TZDs), adipose protein 2 (aP2), 3-isobutyl-1-methylxanthine (IBMX), Scintillation Proximity Assay (SPA), PPAR-Ligand Binding Domains (LBDs), Simpson Golabi Behmel Syndrome (SGBS), all-trans retinoic acid (atRA), PPAR γ response element (PPRE)

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ABSTRACT

Nuclear receptors (NRs) are transcription factors whose activity is regulated by the binding of small lipophilic ligands, including hormones, vitamins, and metabolites. Pharmacological NR ligands serve as important therapeutic agents; for example, all-trans retinoic acid (atRA), an activating ligand for Retinoic Acid Receptor α (RAR α), is used to treat leukemia. Another RAR α ligand, RO 41-5253, is a potent antagonist that has been a useful and purportedly specific probe of RAR α function. Here we report that RO 41-5253 also activates the Peroxisome Proliferator-Activated Receptor γ (PPAR γ), a master regulator of adipocyte differentiation and target of widely prescribed antidiabetic thiazolidinediones (TZDs). RO 41-5253 enhanced differentiation of mouse and human preadipocytes, and activated PPAR γ target genes in mature adipocytes. Like the TZDs, RO 41-5253 also downregulated PPAR γ protein expression in adipocytes. In addition, RO 41-5253 activated the PPAR γ -ligand binding domain (LBD) in transiently transfected HEK293T cells. These effects were not prevented by a potent RAR α agonist nor by depleting cells of RAR α , indicating that PPAR γ activation was not related to RAR α antagonism. Indeed, RO 41-5253 was able to compete with TZD ligands for binding to PPAR γ , suggesting that RO 41-5253 directly affects PPAR activity. These results vividly demonstrate that pharmacological NR ligands may have "off-target" effects on other NRs. RO 41-5253 is a PPAR γ agonist as well as an RAR α antagonist, whose pleiotropic effects on NRs may signify a unique spectrum of biological responses.

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INTRODUCTION

The peroxisome proliferator-activated receptors (PPARs) and the retinoic acid receptor (RARs) are members of the NR superfamily of ligand-activated transcription factors (Germain et al., 2006; Michalik et al., 2006). PPAR γ is expressed at its highest levels in white adipose tissue and is required for adipocyte differentiation (Chawla et al., 1994; Tontonoz et al., 1994). Ligands for this receptor, the antidiabetic drugs thiazolidinediones (TZDs), were found to be high-affinity ligands for PPAR γ promoting adipogenesis (Lehmann et al., 1995). PPAR γ heterodimerizes with retinoid X receptor (RXR), and RXR ligands can both enhance or attenuate the activity of PPAR γ responsive genes (Hondares et al., 2006; Yamauchi et al., 2001). In contrast, RAR α activation by all-trans retinoic acid (atRA), or by synthetic ligands, prevents differentiation of murine preadipocytes (Kamei et al., 1994; Schwarz et al., 1997). Unlike RXR ligands, which directly bind and activate the PPAR γ /RXR heterodimer, the mechanism by which RAR ligands block this activity of PPAR γ is less clear, and is likely to be indirect (Schwarz et al., 1997).

In order to better understand the effects of RA on adipocyte differentiation, we utilized RO 41-5253 which, originally synthesized by Hoffman LaRoche, is a specific antagonist for RAR α with little affinity for RAR β and RAR γ (Apfel et al., 1992). This compound has been widely used to dissect the role of RAR α in RA-dependent biological processes (Emionite et al., 2003; Engedal et al., 2004; Higuchi et al., 2003; Lu et al., 2005; Shang et al., 1999). Surprisingly, we found that RO 41-5253 stimulated the adipogenic differentiation of mouse 3T3-L1 preadipocytes, as well as a human preadipocyte cell line. Remarkably, this function of RO 41-5253 is RAR α -independent. In exploring potential RAR α -independent mechanisms, we discovered that RO 41-5253 is, unexpectedly, an agonist ligand for PPAR γ . Among other

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things, this finding suggests that biological studies that employed this eccentrically pleiotropic ligand may require re-interpretation.

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MATERIALS AND METHODS

Cell Culture and Differentiation. Reagents were obtained from Invitrogen (Carlsbad, CA) unless otherwise noted. 293T and murine 3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (U.S. Bio-Technologies Inc, Parkerford, PA), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown to confluence and induced to differentiate 2 days after confluence with media containing 0.4 µM dexamethasone, 3 µg/ml bovine insulin, and 0.25 mM 3-isobutyl-1-methylxanthine (IBMX, all Sigma, St. Louis, MO) for 2 days and for additional 2 days in insulin only. In differentiation studies, IBMX was replaced by either pioglitazone or RO 41-5253 for the first 4 days. Culturing and differentiation of human preadipocytes from the Simpson Golabi Behmel Syndrome (SGBS) were described elsewhere (Kim et al., 2006) and ligands were present for the first 7 days of differentiation. Oil Red-O staining was performed as previously described (Li and Lazar, 2002).

Transfections and Luciferase Assay. 3T3-L1 adipocytes and 293T cells were transfected by electroporation (Nucleofector II, AMAXA). Adipocytes were detached from culture dishes with 0.25% trypsin and 0.5 mg collagenase/ml in PBS, washed twice, resuspended in electroporation buffer (solution V, AMAXA), mixed with 2 µg pGL3- 3xAOxPPRE plasmid, electroporated and seeded into 12-well plates and incubated for 24h with compounds as indicated. 293T cells were electroporated with 2nmol non-targeting or human smart-pool RAR α oligonucleotides (both Dharmacon, Lafayette, CO) and seeded into 24-well plates and used for transactivation assays 24h later. pGal4-hPPAR γ -LBD, pGal4-hPPAR β/δ -LBD and pGal5-TK-pGL3 were transfected in 293T cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and incubated for 24h with compounds as indicated.

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All transfection were normalized to cotransfected pRL-CMV and measured using the Dual-luciferase reporter assay (Promega, Madison, WI).

Immunoblot analysis and antibodies. Protein were isolated and separated in 4-20% SDS polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membrane (Invitrogen). After incubation with the primary antibodies for PPAR γ (sc7273, Santa Cruz, CA), RAR α (sc-551, Santa Cruz) or the ubiquitously expressed GTPase RAN (BD Biosciences, San Jose, CA), a secondary horseradish-conjugated antibody (Invitrogen) was added, and an enhanced chemiluminescent substrate kit (Amersham, UK) was used for detection.

Quantitative Polymerase Chain Reaction. RNA was purified with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). cDNA was generated using the Sprint Powerscript System (Clontech, Mountain View, CA). Primers and probes for adipose protein 2 (aP2), PPAR γ_2 and 36B4 for normalization were described elsewhere (Chui et al., 2005; Schupp et al., 2005). All PCR reactions were carried out using Taqman Universal Polymerase Master Mix (Applied Biosystems, Foster City, CA) and the PRISM 7900 instrument (Applied Biosystems) and evaluated according to the standard curve method.

Scintillation Proximity Assay (SPA) for the PPAR-Ligand Binding Domains (LBDs). The measurement of ligand displacement was performed as previously described (Nichols et al., 1998). The radio ligands were [3 H]-rosiglitazone for PPAR γ and [3 H]-GW2433 for PPAR α and PPAR β/δ (Xu et al., 1999).

Statistical Analysis. Representative results of at least three independent experiments are shown. All results are expressed as mean \pm SD of triplicates. Statistical significance was

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determined using either the 2-tailed Student's *t* test or ANOVA, as appropriate, and $P < 0.05$ was deemed significant (* $p < 0.05$, ** $p < 0.01$).

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RESULTS

RO 41-5253 is an inducer of adipocyte differentiation. RO 41-5253 was used previously in 3T3-L1 cells to block the inhibitory effect of atRA on differentiation (Kamei et al., 1994). We tested the possibility that this compound has enhancing effects on differentiation itself. 3T3-L1 and human SGBS preadipocytes were therefore induced to differentiate into fat cells by exposing them to the hormonal inducers. Using a differentiation mix devoid of IBMX diminished the grade of adipocyte differentiation (Hamm et al., 2001) (Fig.1A). The presence of pioglitazone or RO 41-5253 could rescue the ability of differentiation as shown in increased Oil Red O staining, PPAR γ_2 mRNA expression in 3T3-L1 cells (Fig.1A), and PPAR γ protein expression in human SGBS cells (Fig.1B). However, pioglitazone was more efficient than RO 41-5253 in promoting the adipocyte phenotype (Fig. 1A and 1B).

RO 41-5253 induces aP2 expression and downregulates PPAR γ protein levels in adipocytes. To examine whether the enhancing effects on differentiation were mediated by PPAR γ we measured the expression of the PPAR γ target gene aP2 and PPAR γ protein expression after incubation with pioglitazone and RO 41-5253 in 3T3-L1 adipocytes. PPAR γ levels were shown to decrease upon activation in an autoregulatory manner (Hauser et al., 2000). RO 41-5253, like pioglitazone, significantly upregulated aP2 mRNA and downregulated PPAR γ protein expression in adipocytes. Pioglitazone had more pronounced effects than RO 41-5253 (Fig. 2A and 2B).

RO 41-5253 activates endogenous PPAR activity. Consistent with the effects of RO 41-5253 on adipocyte differentiation and aP2 expression, RO 41-5253 increased the activity of endogenous PPAR γ on a transfected AOx-PPAR response element (PPRE) in 3T3-L1 adipocytes (Fig.3).

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RO 41-5253 activates the PPAR γ - but not the PPAR β/δ -LBD. 3T3-L1 adipocytes express both PPAR γ and PPAR β/δ (Yan et al., 2007) . We therefore investigated whether RO 41-5253 is able to directly activate the PPAR γ or β/δ -LBDs. We transfected the Gal4-PPAR γ and β/δ -LBDs and the corresponding reporter in 293T cells and incubated with increasing concentrations of pioglitazone or the PPAR β/δ agonist GW 610742 (Sznaidman et al., 2003; van der Veen et al., 2005) and RO 41-5253.

The RAR α antagonist potently activated the PPAR γ -LBD but with much less efficiency than pioglitazone (Fig. 4A). Although the concentrations necessary for half maximal activation (EC₅₀) for both ligands are in the same range, the maximal activation of RO 41-5253 over vehicle treated cells was less than 30% of the activation of pioglitazone (Table 1). Consistently, the full activation of the PPAR γ -LBD induced by 1 μ M pioglitazone was attenuated by co-treatment with increasing concentrations of RO 41-5253 (Fig. 4C). This strongly suggests that pioglitazone and RO 41-5253 may both act via the same PPAR γ activating mechanism. On the contrary, RO 41-5253 could not activate the PPAR β/δ -LBD (Fig. 4B). We noticed a slight decrease of the basal PPAR β/δ -LBD activity with high concentrations of RO 41-5253. Accordingly, there was a reduction in the full PPAR β/δ -LBD activation induced by 20nM GW 610742 by co-treatment with 10 μ M RO 41-5253 to 53% (data not shown).

RO 41-5253 activates the PPAR γ -LBD independent of RAR α . We next addressed the question whether RAR α was involved in the specific PPAR γ activating property of RO 41-5253 using a pharmacological approach. 293T cells were transfected with the Gal4-RAR α -LBD and the corresponding reporter and titrated for activating/repressing concentrations of RO 41-5253 and the RAR α agonist AM-580. The strong repression elicited by 500nM RO

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41-5253 was completely abolished by co-incubation with 5 μ M AM-580 (Fig. 5A, compare the RO 41-5253 and the RO 41-5253 + AM-580 repression). The same concentration of the RAR α agonist AM-580 had little effect of the pioglitazone- as well as RO 41-5253- induced activation of the PPAR γ -LBD (Fig. 5B) showing that RAR α antagonism is dispensable for the activation of the PPAR α -LBD by RO 41-5253.

To provide further evidence for the RAR α -independent mechanism, we depleted RAR α in 293T cells (Fig. 6A, compare protein levels of cells electroporated with either siControl or siRAR α). This depletion did not prevent the activation of the PPAR γ -LBD by RO 41-5253, proving that RAR α is not involved in the PPAR γ activation (Fig. 5B). In contrary, it slightly increased the efficiency by which RO 41-5253 activated the PPAR γ -LBD.

RO 41-5253 competes with specific ligands for direct binding to PPAR γ . Since RAR α was nonrelevant for the PPAR γ activation we questioned whether Ro 41-5253 directly interacts with the PPAR γ protein. We therefore measured the competition with specific radiolabelled ligands to human PPAR-LBDs by a scintillation proximity assay and calculated the concentrations for half maximal displacement. RO 41-5253 was able to bind to PPAR γ and PPAR β/δ with IC₅₀ in the low micromolar range (Table 2).

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DISCUSSION

In this study, we have shown that RO 41-5253 is a strong inducer of adipogenesis in mouse and human preadipocytes. We provide evidence that RO 41-5253 can bind and activate PPAR γ , the master regulator of adipogenesis. On the other hand, RO 41-5253 could bind PPAR β/δ but was not able to activate the PPAR β/δ -LBD Gal4 fusion protein. Using pharmacological and biochemical interventions, we can exclude the involvement of RAR α in the PPAR γ activating property of RO 41-5253. Thus, RO 41-5253 is not only an antagonist for RAR α , but also an agonist for PPAR γ .

Although the potency of RO 41-5253 was comparable to pioglitazone (Table 1), its efficiency of promoting lipid accumulation, target gene expression and activation of the PPAR γ -LBD was consistently lower than by using pioglitazone, a full agonist (Fig. 2A and 4A). Although RO 41-5253 seems to bind the PPAR γ -LBD in a manner that is similar to TZDs, TZD-induced activation could be diminished with increasing concentrations of RO 41-5253, which is the classical definition of partial agonist behavior. The exact mechanism for this partial agonism of PPAR γ by RO 41-5253 may involve selective or reduced interaction with NR coactivators as has been shown for other partial agonists such as F-Moc leucine (Rocchi et al., 2001), MCC-555 (Reginato et al., 1998) and certain angiotensin receptor blockers (Schupp et al., 2005).

There is no endogenous ligand for NRs known which has both RAR α antagonizing and PPAR γ activating properties. However, it is intriguing to think of the existence of endogenous pleiotropic ligands, considering the functional antagonism of RAR and PPAR for instance during adipogenesis (Chawla and Lazar, 1994; Xue et al., 1996). Furthermore, RO 41-5253,

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chemically derived from atRA, has no obvious similarity with any synthetic PPAR γ activator. On the other hand, it shares structural elements with arachidonic metabolites like PGJ₂, which have been shown to activate PPAR γ (Forman et al., 1995; Kliewer et al., 1995; Yu et al., 1995).

Finally, the unexpectedly pleiotropic effects of RO 41-5253 that we have uncovered indicate that the caution must be applied to the interpretation of effects elicited by RO 41-5253 and previously attributed to specific RAR α antagonism. This caution pertains not only to studies of adipogenesis (Kamei et al., 1994), but to many other cell types. For example, several studies have used RO 41-5253 as an ostensibly specific probe of RAR α function in breast cancer (Emionite et al., 2004; Lu et al., 2005; Schneider et al., 2000; Shang et al., 1999; Toma et al., 2005), where PPAR γ is expressed at significant levels, not only in cell lines such as MCF-7, MDA-MB-231 or ZR-75.1 (James et al., 2003; Kilgore et al., 1997; Nwankwo and Robbins, 2001) but in primary and metastatic breast adenocarcinomas (Kilgore et al., 1997; Mueller et al., 1998). The fact that RO 41-5253 was able to bind β/δ in the low micromolar range adds to the concerns about overinterpretation of its effects as RAR α -specific in biological systems. Unfortunately, to our knowledge there is no other RAR antagonist commercially available.

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ACKNOWLEDGEMENTS

We thank Dr. M. Wabitsch (Pediatric Endocrinology, University of Ulm, Germany) for the SGBS preadipocyte cell line.

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FOOTNOTES

Address correspondence to Mitchell A. Lazar, Division of Endocrinology, Diabetes, and Metabolism, University of Pennsylvania School of Medicine, 615 Curie Boulevard, Philadelphia, PA 19104, Ph. 215-898-0198, Fax 215-898-5408, Lazar@mail.med.upenn.edu.

This work was supported by NIH R01 DK49780 (to M.A.L). M.S. was supported by Mentored Fellowship award from the American Diabetes Association, and J.C.C was supported by NIDDK training grants (5-F32-DK-070405-03, 5-RO1-DK-045780-12).

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FIGURE LEGENDS

Figure 1. RO 41-5253 induces differentiation of mouse and human preadipocytes. 3T3-L1 preadipocytes were treated with the full differentiation mix (Mix) or Mix without IBMX and supplemented with either 500nM pioglitazone or RO 41-5253. Differentiation was evaluated by phase contrast microscopy, Oil Red O staining and PPAR γ_2 expression at day 8 after initiation of differentiation (A). Human SGBS preadipocytes were treated for 14 days with Mix without IBMX and supplemented with 500nM ligands for the first 7 days. Protein levels of PPAR γ were determined and cells assessed by phase contrast microscopy.

Figure 2. RO 41-5253 increases PPAR γ target gene expression in 3T3-L1 adipocytes and downregulates PPAR γ protein. Day 8 adipocytes were incubated for 24h with vehicle, 500nM pioglitazone or RO 41-5253. mRNA expression of aP2 (as fold induction over vehicle treatment) and PPAR γ protein expression were measured (A and B).

Figure 3. RO 41-5253 increases endogenous PPAR activity in adipocytes. 3T3-L1 adipocytes were electroporated with either empty vector (pGL3) or a PPAR- response element containing reporter (pGL3- 3x AOX-PPRE) and incubated for 24h with vehicle and 500nM pioglitazone or RO 41-5253 and assayed for luciferase activity. Data represent the fold induction of luciferase activity over vehicle treatment of the PPRE-containing plasmid.

Figure 4. RO 41-5253 partially activates the PPAR γ -LBD but not the PPAR β/δ -LBD and competes with TZD for activation. 293T cells were transiently transfected with pGal4-hPPAR γ _DEF or pGAL-hPPAR β/δ _DEF and the pGal5-Tk-pGL3 reporter followed by stimulation with pioglitazone or GW 610742 (black squares in A and B) or RO 41-5253 (red

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circles) as indicated for 24h. Data represent the fold induction of luciferase activity over vehicle treatment for activation of PPAR γ (A) or PPAR β/δ (B). Transfected cells were incubated with 1 μ M pioglitazone and increasing concentrations of RO 41-5253 for 24h and assayed for luciferase activity (C).

Figure 5. RO 41-5253 activates the PPAR γ -LBD in presence of the RAR α agonist AM-580. 293T cells were transiently transfected with the pGal4-hRAR α _DEF (A) or pGal4-hPPAR γ _DEF (B) and pGal5-Tk-pGL3 reporter. Cells were treated with 500nM RO 41-5253, 5 μ M of the RAR α agonist AM-580 or both for 24h. Data represent the fold repression over vehicle treated cells (A). Activation the PPAR γ -LBD was measured after 24h incubation with 0.03, 0.3 and 3 μ M of either pioglitazone or RO 41-5253 in the presence of vehicle or 5 μ M AM-580. Data represent the fold induction over vehicle treated cells (B).

Figure 6. RO 41-5253 activates the PPAR γ -LBD in cells depleted of RAR α . Reduction of RAR α protein levels by electroporating siRNA control or siRNA RAR α in 293T cells (A). After 24h, cells were transfected with pGal4-hPPAR_DEF and pGal5-Tk-pGL3 and incubated for another 24h with 3 μ M of RO 41-5253. Data are shown as fold induction over vehicle treated cells (B).

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Table 1. EC₅₀ and maximal activation of the hsPPAR γ -LBD in transient transfections

Dose response curves from the transiently transfected 293T cells with the pGal4-hPPAR_DEF and pGal5-Tk-pGL3 reporter (fig.4) were used to calculate values for the EC₅₀. The maximal activation represents the maximal relative activation of RO 41-5253 in comparison to pioglitazone (=100%).

compound	EC₅₀	maximal activation
pioglitazone	0.58μM	100%
RO 41-5253	0.81μM	28%

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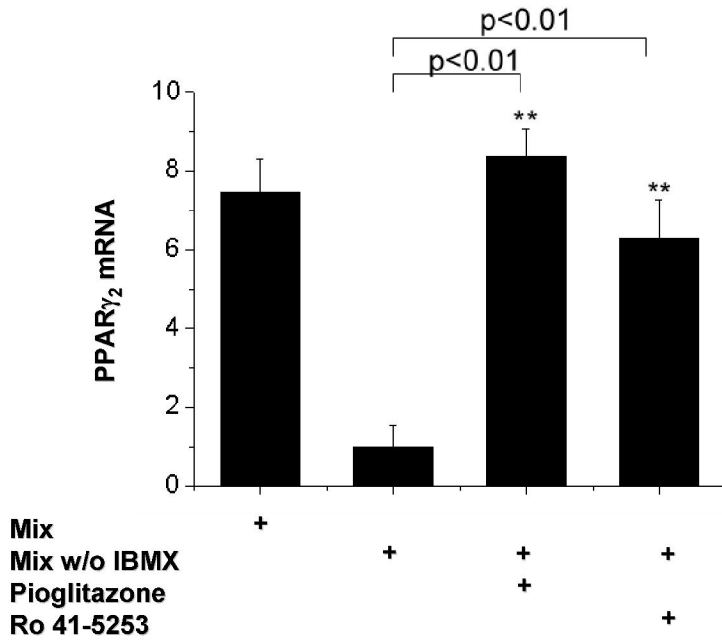
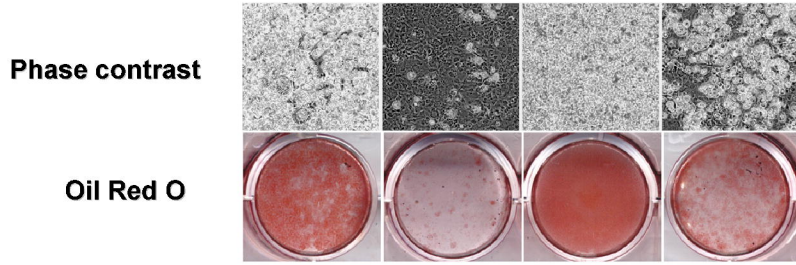
Table 2. IC₅₀ of RO 41-5253 in displacing specific [³H]-ligands from hsPPAR isoforms

RO 41-5253 displaces specific ligands from hsPPAR-LBDs as measured by a SPA assay.

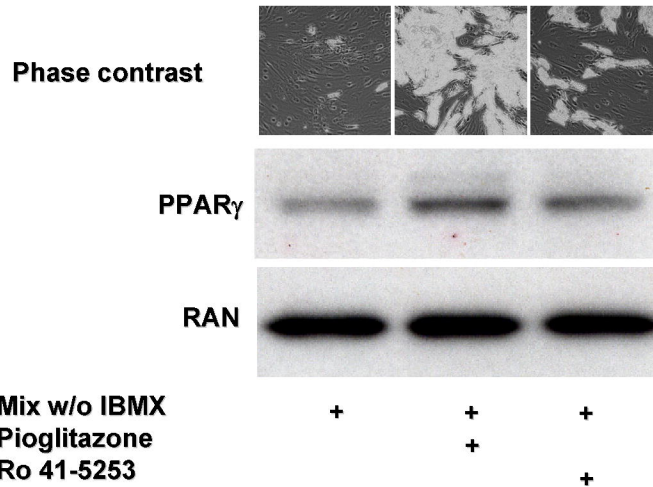
Data represent mean IC₅₀ values of 3 independent experiments.

isoform	IC₅₀
PPARα	>25μM
PPARβ/δ	7.73μM
PPARγ	5.07μM

A



B



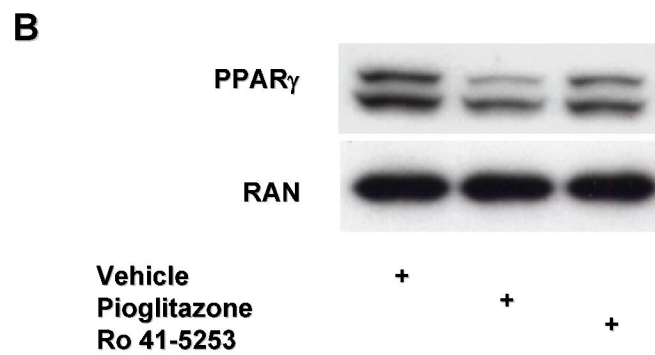
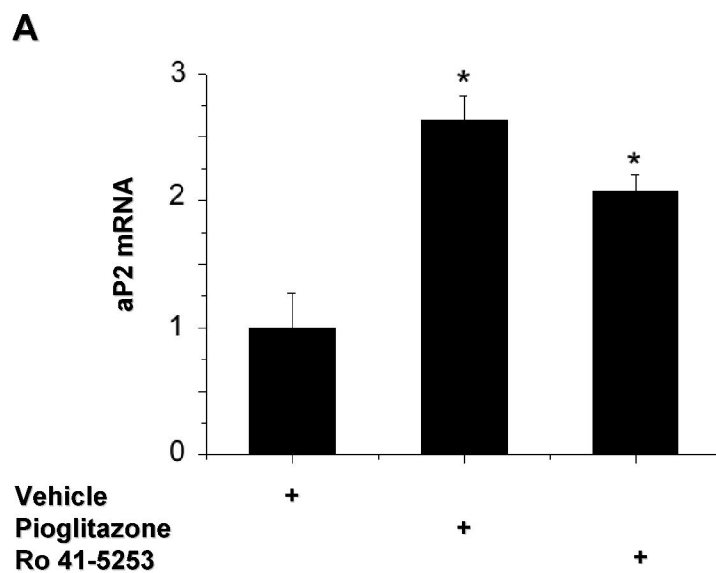
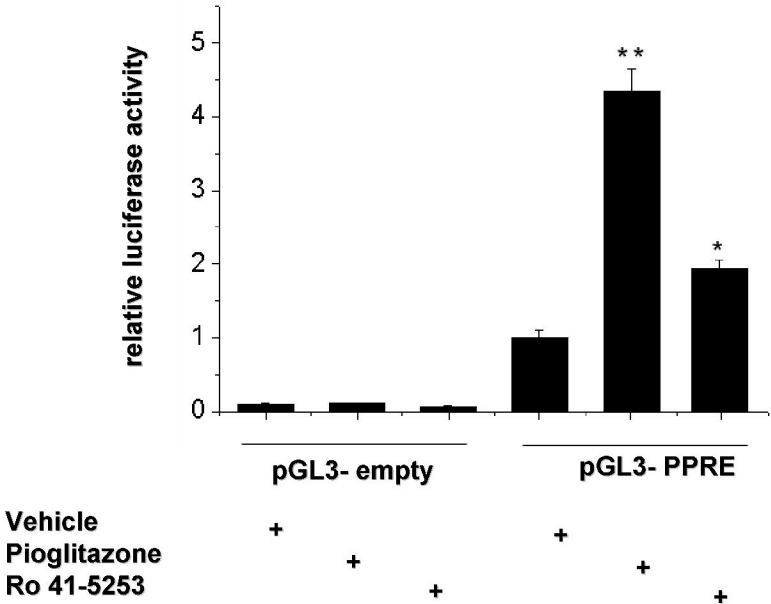
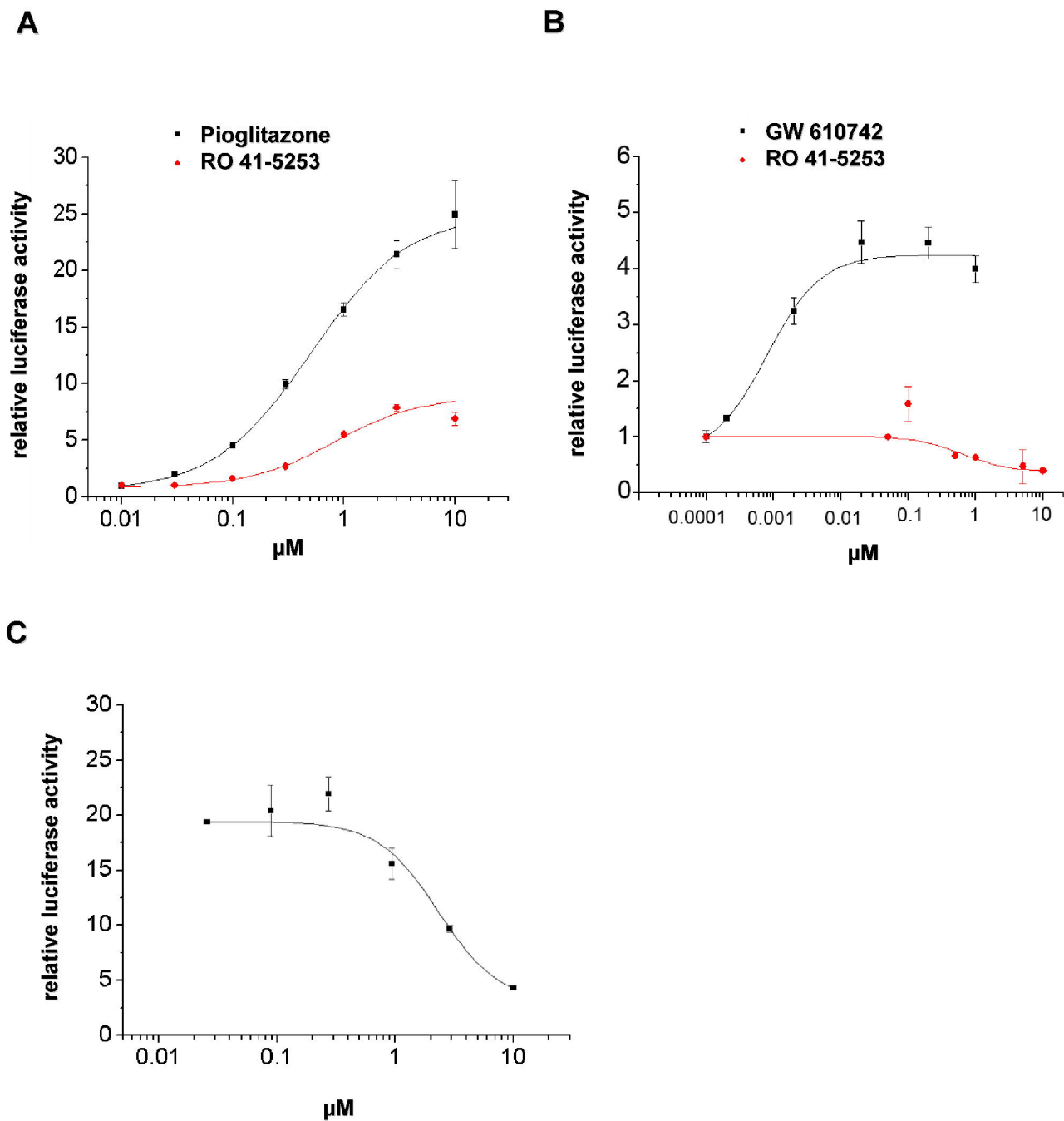


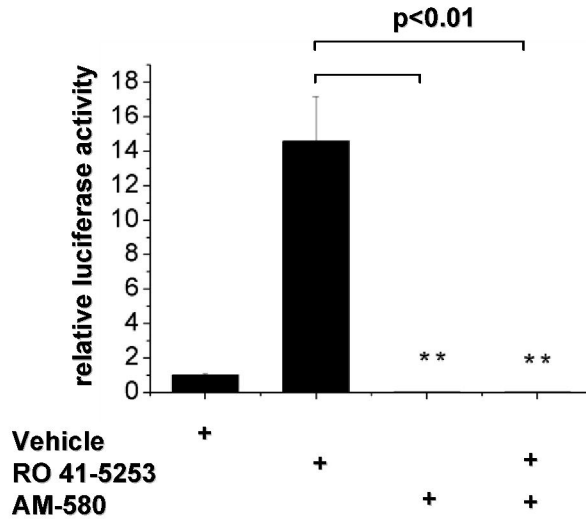
Figure 3

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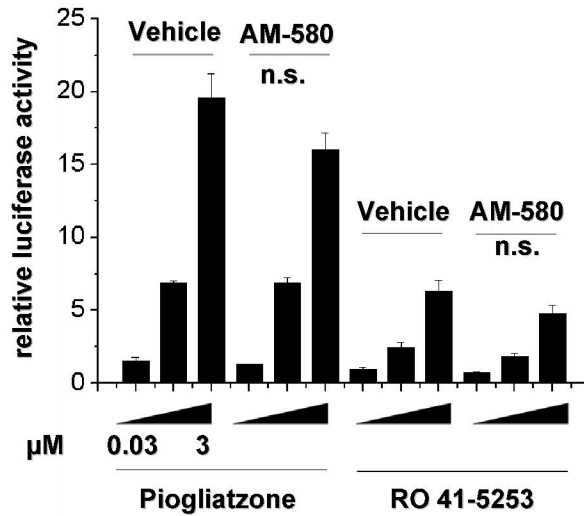




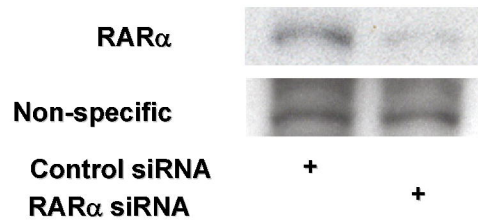
A RAR α -LBD transrepression



B PPAR γ -LBD transactivation



A



B

