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A Widely Used Retinoic Acid Receptor Antagonist Induces PPARγ Activity

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Running title: An RARα antagonist activates PPARγ

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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), PPARγ response element (PPRE)

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 (RARα), is used to treat leukemia. Another RARα ligand, RO 41-5253, is a potent antagonist that has been a useful and purportedly specific probe of RARα function. Here we report that RO 41-5253 also activates the Peroxisome Proliferator-Activated Receptor γ (PPARγ), 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 PPARγ target genes in mature adipocytes. Like the TZDs, RO 41-5253 also downregulated PPARγ protein expression in adipocytes. In addition, RO 41-5253 activated the PPARγ-ligand binding domain (LBD) in transiently transfected HEK293T cells. These effects were not prevented by a potent RARα agonist nor by depleting cells of RARα, indicating that PPARγ activation was not related to RARα antagonism. Indeed, RO 41-5253 was able to compete with TZD ligands for binding to PPARγ, 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 PPARγ agonist as well as an RARα 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). PPARγ 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 PPARγ promoting adipogenesis (Lehmann et al., 1995). PPARγ heterodimerizes with retinoid X receptor (RXR), and RXR ligands can both enhance or attenuate the activity of PPARγ responsive genes (Hondares et al., 2006; Yamauchi et al., 2001). In contrast, RARα activation by all-trans retinoid 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 PPARγ/RXR heterodimer, the mechanism by which RAR ligands block this activity of PPARγ 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 RARα with little affinity for RARβ and RARγ (Apfel et al., 1992). This compound has been widely used to dissect the role of RARα 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). Surpisingly, 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 RARα-independent. In exploring potential RARα-independent mechanisms, we discovered that RO 41-5253 is, unexpectedly, an agonist ligand for PPARγ. 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 RARα oligonucleotides (both Dharmacon, Lafayette, CO) and seeded into 24-well plates and used for transactivation assays 24h later. pGal4-hPPARγ-LBD, pGal4-hPPARβ/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 PPARγ (sc7273, Santa Cruz, CA), RARα (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), PPARγ$_2$ 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 PPARγ and [^{3}H]-GW2433 for PPARα and PPARβ/δ (Xu et al., 1999).

Statistical Analysis. Representative results of at least three independent experiments are shown. All results are expressed as mean ± 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, PPARγ_2 mRNA expression in 3T3-L1 cells (Fig.1A), and PPARγ 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 PPARγ protein levels in adipocytes. To examine whether the enhancing effects on differentiation were mediated by PPARγ we measured the expression of the PPARγ target gene aP2 and PPARγ protein expression after incubation with pioglitazone and RO 41-5253 in 3T3-L1 adipocytes. PPARγ 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 PPARγ 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 PPARγ on a transfected AOx-PPAR response element (PPRE) in 3T3-L1 adipocytes (Fig.3).

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

The RARα antagonist potently activated the PPARγ-LBD but with much less efficiency than pioglitazone (Fig. 4A). Although the concentrations necessary for half maximal activation (EC_{50}) 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 PPARγ-LBD induced by 1μM 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 PPARγ activating mechanism. On the contrary, RO 41-5253 could not activate the PPARβ/δ-LBD (Fig. 4B). We noticed a slight decrease of the basal PPARβ/δ-LBD activity with high concentrations of RO 41-5253. Accordingly, there was a reduction in the full PPARβ/δ-LBD activation induced by 20nM GW 610742 by co-treatment with 10μM RO 41-5253 to 53% (data not shown).

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

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

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

RO 41-5253 competes with specific ligands for direct binding to PPARγ. Since RARα was nonrelevant for the PPARγ activation we questioned whether Ro 41-5253 directly interacts with the PPARγ protein. We therefore measured the competition with specific radiolabelled ligands to human PPAR-LBDs by a scintiallation proximity assay and calculated the concentrations for half maximal displacement. RO 41-5253 was able to bind to PPARγ and PPAR β/δ with IC_{50} 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 PPARγ, the master regulator of adipogenesis. On the other hand, RO 41-5253 could bind PPARβ/δ but was not able to activate the PPARβ/δ-LBD Gal4 fusion protein. Using pharmacological and biochemical interventions, we can exclude the involvement of RARα in the PPARγ activating property of RO 41-5253. Thus, RO 41-5253 is not only an antagonist for RARα, but also an agonist for PPARγ.

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 PPARγ-LBD was consistently lower than by using pioglitazone, a full agonist (Fig. 2A and 4A). Although RO 41-5253 seems to bind the PPARγ-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 PPARγ 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 RARα antagonizing and PPARγ 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 PPARγ activator. On the other hand, it shares structural elements with arachidonic metabolites like PGJ_2, which have been shown to activate PPARγ (Forman et al., 1995; Kliewer et al., 1995; Yu et al., 1995).

Finally, the unexpectedly pleotropic 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 RARα 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 RARα 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 PPARγ 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 RARα-specific in biological systems. Unfortunately, to our knowledge there is no other RAR antagonist commercially available.

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FOOTNOTES

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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 PPARγ_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 PPARγ were determined and cells assessed by phase contrast microscopy.

Figure 2. RO 41-5253 increases PPARγ target gene expression in 3T3-L1 adipocytes and downregulates PPARγ 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 PPARγ 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 PPARγ-LBD but not the PPARβ/δ-LBD and competes with TZD for activation. 293T cells were transiently transfected with pGal4-hPPARγ_DEF or pGAL-hPPARβ/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

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

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

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

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Table 1. EC_{50} and maximal activation of the hsPPARγ-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 EC_{50}.The maximal activation represents the maximal relative activation of RO 41-5253 in comparison to pioglitazone (=100%).

compound	EC_{50}	maximal activation
pioglitazone	**0.58µM**	**100%**
RO 41-5253	**0.81µM**	**28%**

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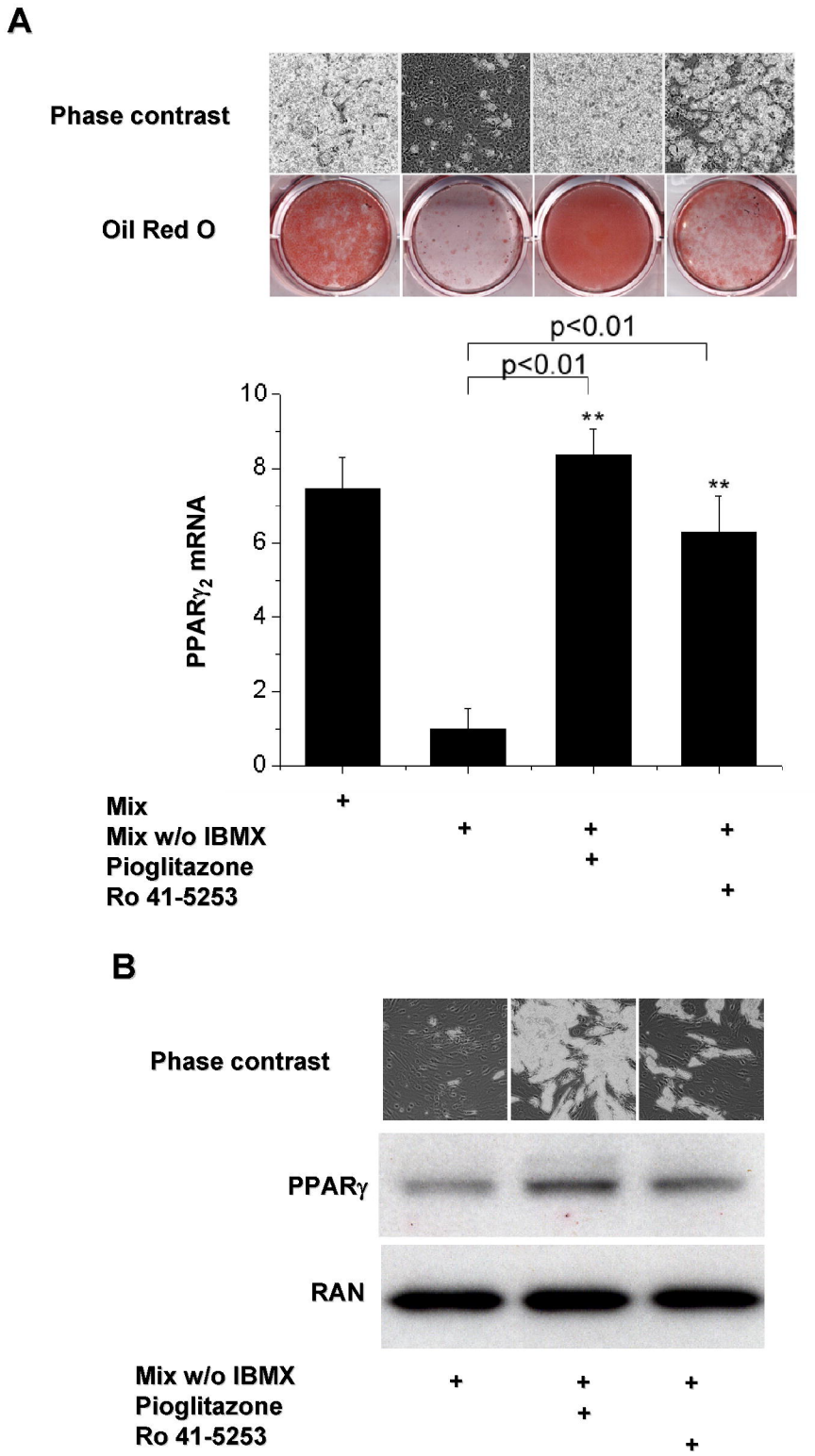
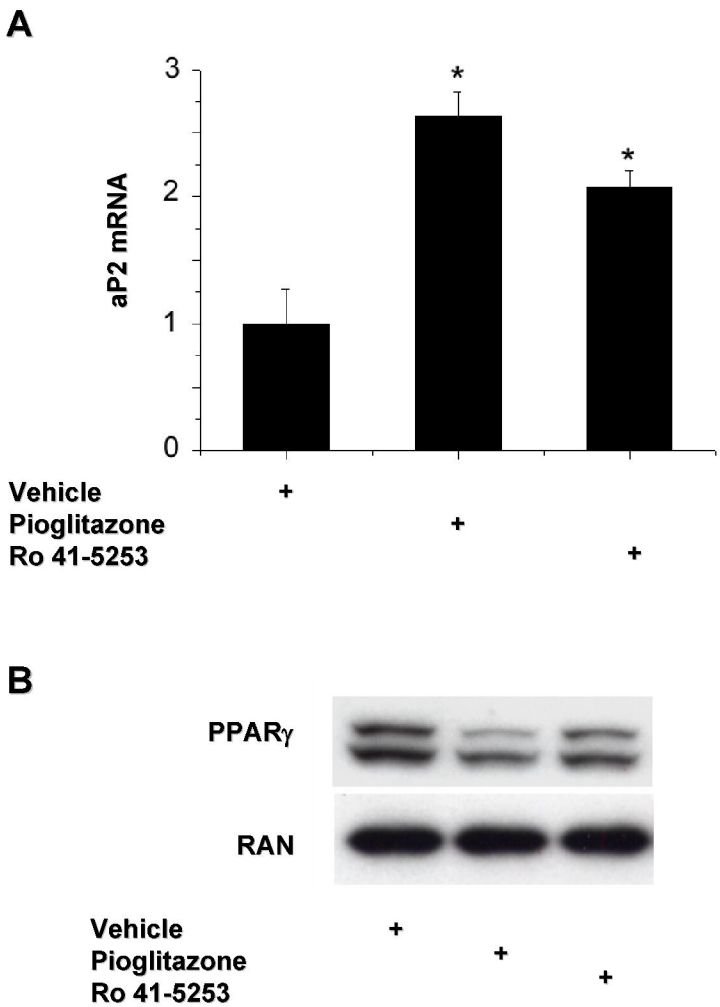
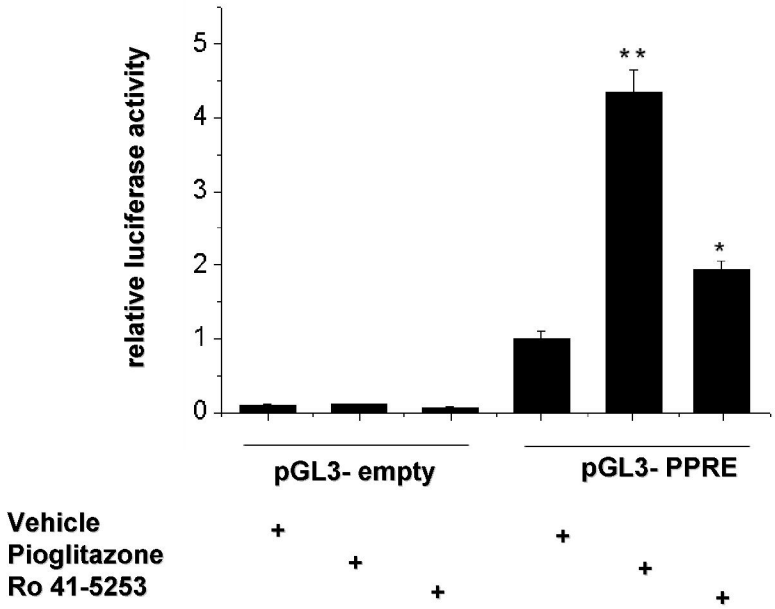
Table 2. IC_{50} of RO 41-5253 in displacing specific [^{3}H]-ligands from hsPPAR isoforms

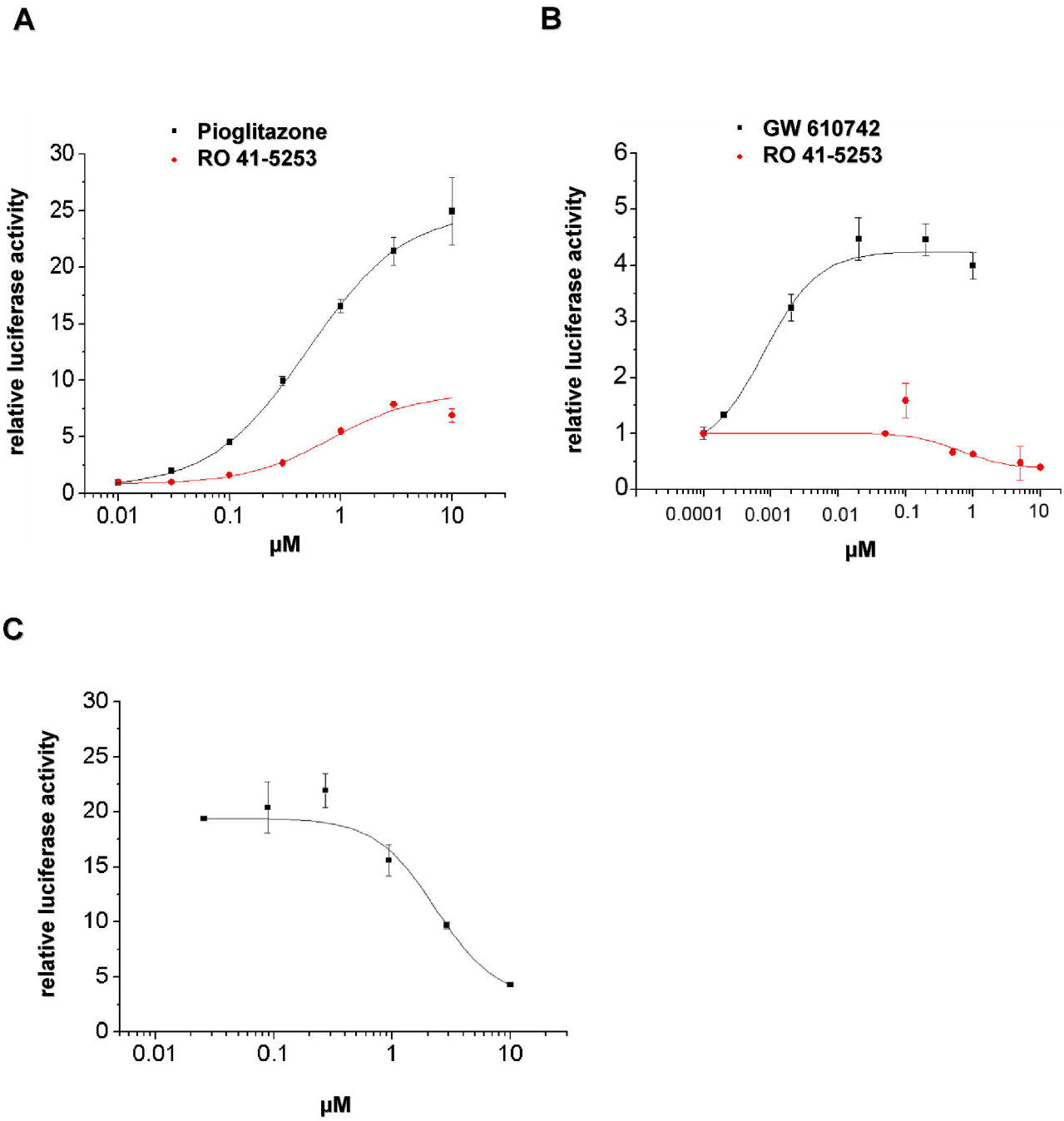
RO 41-5253 displaces specific ligands from hsPPAR-LBDs as measured by a SPA assay. Data represent mean IC_{50} values of 3 independent experiments.

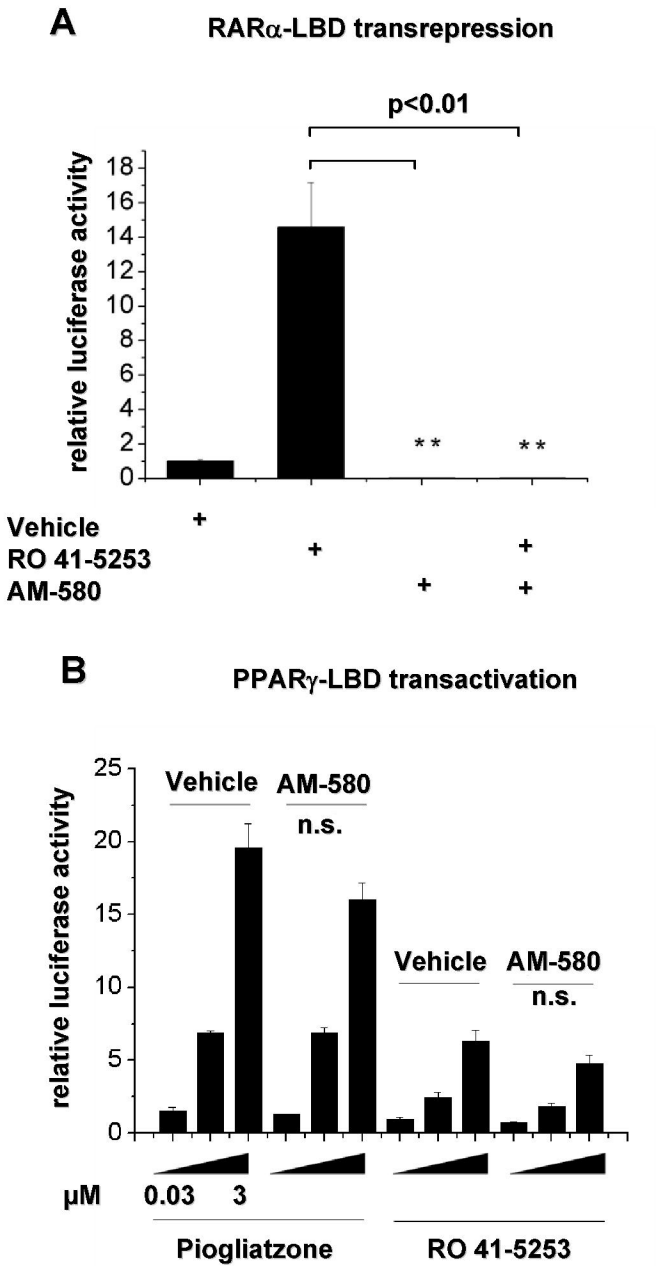
isoform	IC_{50}
PPARα	>25μM
PPARβ/δ	7.73μM
PPARγ	5.07μM

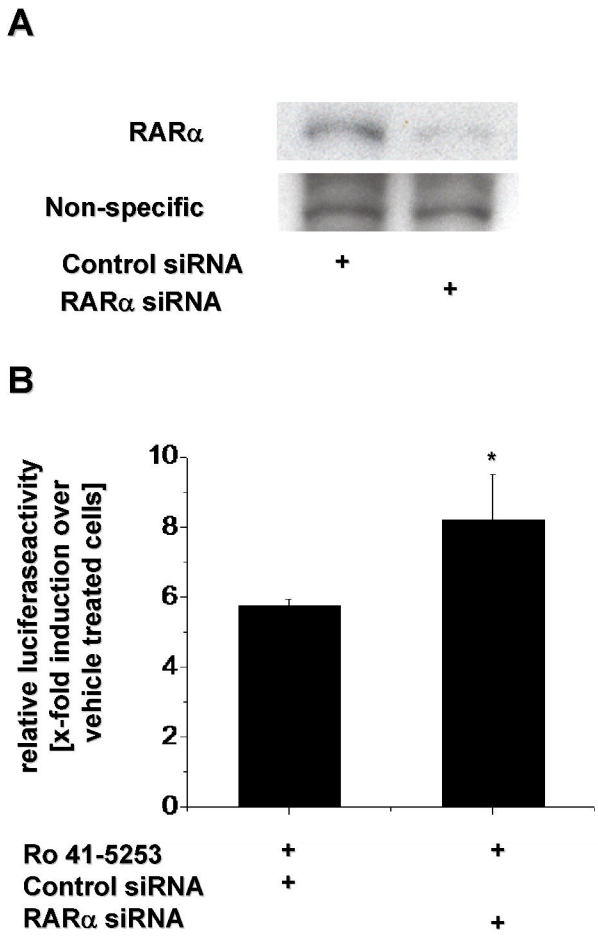

A
Phase contrast
Oil Red O
p<0.01
p<0.01
PPARγ2 mRNA
10
8
6
4
2
0
**
**
Mix
Mix w/o IBMX
Pioglitazone
Ro 41-5253
B
Phase contrast
PPARγ
RAN
Mix w/o IBMX
Pioglitazone
Ro 41-5253


A
aP2 mRNA
3
2
1
0
*
*
Vehicle
Pioglitazone
Ro 41-5253
+
+
+
B
PPARγ
RAN
Vehicle
Pioglitazone
Ro 41-5253
+
+
+


relative luciferase activity
0
1
2
3
4
5
**
*
pGL3- empty
pGL3- PPRE
Vehicle
Pioglitazone
Ro 41-5253
+
+
+
+
+
+


A
Pioglitazone
RO 41-5253
relative luciferase activity
0
5
10
15
20
25
30
0.01
0.1
1
10
μM
B
GW 610742
RO 41-5253
relative luciferase activity
0
1
2
3
4
5
6
0.0001
0.001
0.01
0.1
1
10
μM
C
relative luciferase activity
0
5
10
15
20
25
30
0.01
0.1
1
10
μM


A
RARα-LBD transrepression
p<0.01
relative luciferase activity
18
16
14
12
10
8
6
4
2
0
**
**
Vehicle
RO 41-5253
AM-580
+
+
+
+
+
B
PPARγ-LBD transactivation
relative luciferase activity
25
20
15
10
5
0
Vehicle
AM-580
n.s.
Vehicle
AM-580
n.s.
μM
0.03
3
Piogliatzone
RO 41-5253


A
RARα
Non-specific
Control siRNA
RARα siRNA
+
+
B
relative luciferaseactivity
[x-fold induction over
vehicle treated cells]
10
8
6
4
2
0
*
Ro 41-5253
Control siRNA
RARα siRNA
+
+
+
+
+