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A Widely Used Retinoic Acid Receptor Antagonist Induces Peroxisome Proliferator-Activated Receptor-γ Activity

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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, an activating ligand for retinoic acid receptor α (RARα), is used to treat leukemia. Another RARα ligand, (*E*)-*S*,*S*-dioxide-4-(2-(7-(heptyloxy)-3,4-dihydro-4,4-dimethyl-2*H*-1-benzothiopyran-6-yl)-1-propenyl)-benzoic acid (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 down-regulated PPARγ protein expression in adipocytes. In addition, Ro 41-5253 activated the PPARγ-ligand binding domain in transiently transfected HEK293T cells. These effects were not prevented by a potent RARα agonist or 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.

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 (Yamauchi et al., 2001; Hondares et al., 2006). 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).

To better understand the effects of atRA on adipocyte differentiation, we used Ro 41-5253; originally synthesized by Hoffman LaRoche, it 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 atRA-dependent biological processes (Shang et al., 1999; Emionite et al., 2003; Higuchi et al., 2003; Engedal et al., 2004; Lu et al., 2005). We were surprised to find that Ro

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ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; TZD, thiazolidinedione; RXR, retinoid X receptor; NR, nuclear receptors; atRA, all-*trans* retinoid acid; Ro 41-5253, (*E*)-*S*,*S*-dioxide-4-(2-(7-(heptyloxy)-3,4-dihydro-4,4-dimethyl-2*H*-1-benzothiopyran-6-yl)-1-propenyl)-benzoic acid; IBMX, 3-isobutyl-1-methylxanthine; SGBS, Simpson Golabi Behmel Syndrome; LBD, ligand binding domain; aP2, adipose protein 2; GW2433, 2-(4-{3-[1-[2-(2-chloro-6-fluoro-phenyl)-ethyl]-3-(2,3-dichloro-phenyl)-ureido]-propyl}-phenoxy)-2-methyl-propionic acid; PPRE, peroxisome proliferator-activated receptor response element; AM-580, 4-[(5,5,8,8-tetramethyl6,7-dihydronaphthalene-2-carbonyl)amino]benzoic acid.

41-5253 stimulated the adipogenic differentiation of mouse 3T3-L1 preadipocytes as well as a human preadipocyte cell line. 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. This finding suggests, among other things, that biological studies that employed this eccentrically pleiotropic ligand may require re-interpretation.

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 from 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 described previously (Li and Lazar, 2002).

Transfections and Luciferase Assay. 3T3-L1 adipocytes and 293T cells were transfected by electroporation (Nucleofector II; Amaxa Biosystems, Gaithersburg, MD). Adipocytes were detached from culture dishes with 0.25% trypsin and 0.5 mg of collagenase/ml in phosphate-buffered saline, washed twice, resuspended in electroporation buffer (solution V; Amaxa Biosystems), mixed with 2 μg of pGl3–3xAOxPPRE plasmid, electroporated, seeded into 12-well plates, and incubated for 24 h with compounds as indicated. 293T cells were electroporated with 2 nmol of nontargeting or human smart-pool RARα oligonucleotides (Dharmacon, Lafayette, CO) and seeded into 24-well plates and used for transactivation assays 24 h 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. 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 to 20% SDS polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membrane (Invitrogen). After incubation with the primary antibodies for PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA), RARα (Santa Cruz Biotechnology) 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, Chalfont St. Giles, 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 have been 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 were evaluated according to the standard curve method.

Scintillation Proximity Assay for the PPAR-Ligand Binding Domains. The measurement of ligand displacement was performed as described previously (Nichols et al., 1998). The radioligands 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 ± S.D. of triplicates. Statistical significance was determined using either the 2-tailed Student's t test or ANOVA, as appropriate, and $P < 0.05$ was deemed significant.

Results

Ro 41-5253 Was 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. 1, A and B).

Ro 41-5253 Induced aP2 Expression and Down-Regulated 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 up-regulated aP2 mRNA and down-regulated PPARγ protein expression in adipocytes. Pioglitazone had more pronounced effects than Ro 41-5253 (Fig. 2, A and B).

Ro 41-5253 Activated 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).

Ro 41-5253 Activated the PPARγ-LBD 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 was 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). The full activation of the PPARγ-LBD induced by 1 μM pioglitazone was consistently attenuated by cotreatment with increasing concentrations of Ro 41-5253 (Fig. 4C). This strongly suggests that pioglitazone and Ro 41-5253 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 20 nM GW 610742 by cotreatment with 10 μM Ro 41-5253 to 53% (data not shown).

Ro 41-5253 Activated the PPARγ-LBD Independent of RARα. We next addressed the question of 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 500 nM Ro 41-5253 was com-

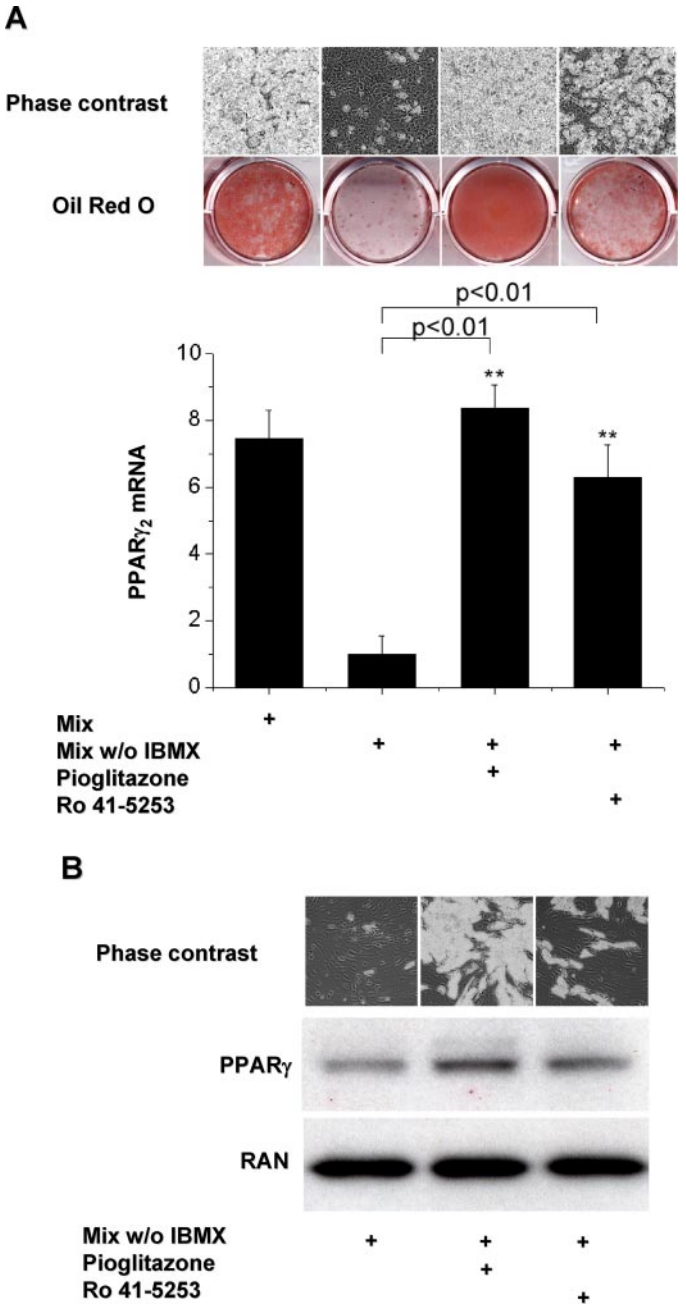

Fig. 1. Ro 41-5253 induces differentiation of mouse and human preadipocytes. 3T3-L1 preadipocytes were treated with the full differentiation mix (Mix) or Mix without IBMX and supplemented with either 500 nM pioglitazone, or 500 nM Ro 41-5253. Differentiation was evaluated by phase contrast microscopy, 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 500 nM ligands for the first 7 days. Protein levels of PPARγ were determined and cells assessed by phase contrast microscopy. *, $p < 0.05$; **, $p < 0.01$.

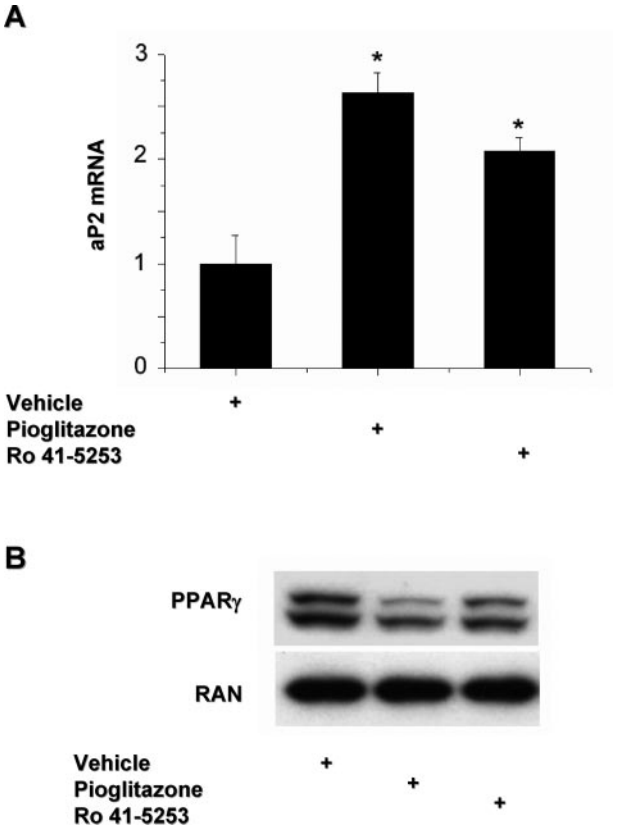

Fig. 2. Ro 41-5253 increases PPARγ target gene expression in 3T3-L1 adipocytes and down-regulates PPARγ protein. Day 8 adipocytes were incubated for 24 h with vehicle, 500 nM pioglitazone, or 500 nM Ro 41-5253. mRNA expression of aP2 (as -fold induction over vehicle treatment) and PPARγ protein expression were measured (A and B). *, $p < 0.05$.

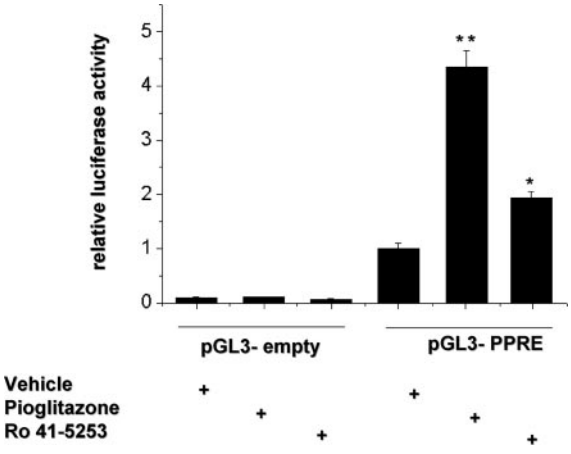

Fig. 3. Ro 41-5253 increases endogenous PPAR activity in adipocytes. 3T3-L1 adipocytes were electroporated with either empty vector (pGL3) or a PPAR- response element containing reporter (pGL3–3xAOx-PPRE) and incubated for 24h with vehicle and 500 nM pioglitazone or 500 nM Ro 41-5253 and assayed for luciferase activity. Data represent the -fold induction of luciferase activity over vehicle treatment of the PPRE-containing plasmid. *, $p < 0.05$; **, $p < 0.01$.

pletely abolished by coincubation with 5 μM 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 not necessary 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). On the 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. Because 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 radiolabeled ligands to human PPAR-LBDs by a scintillation proximity assay and calculated the concentrations for half-maximal displacement.

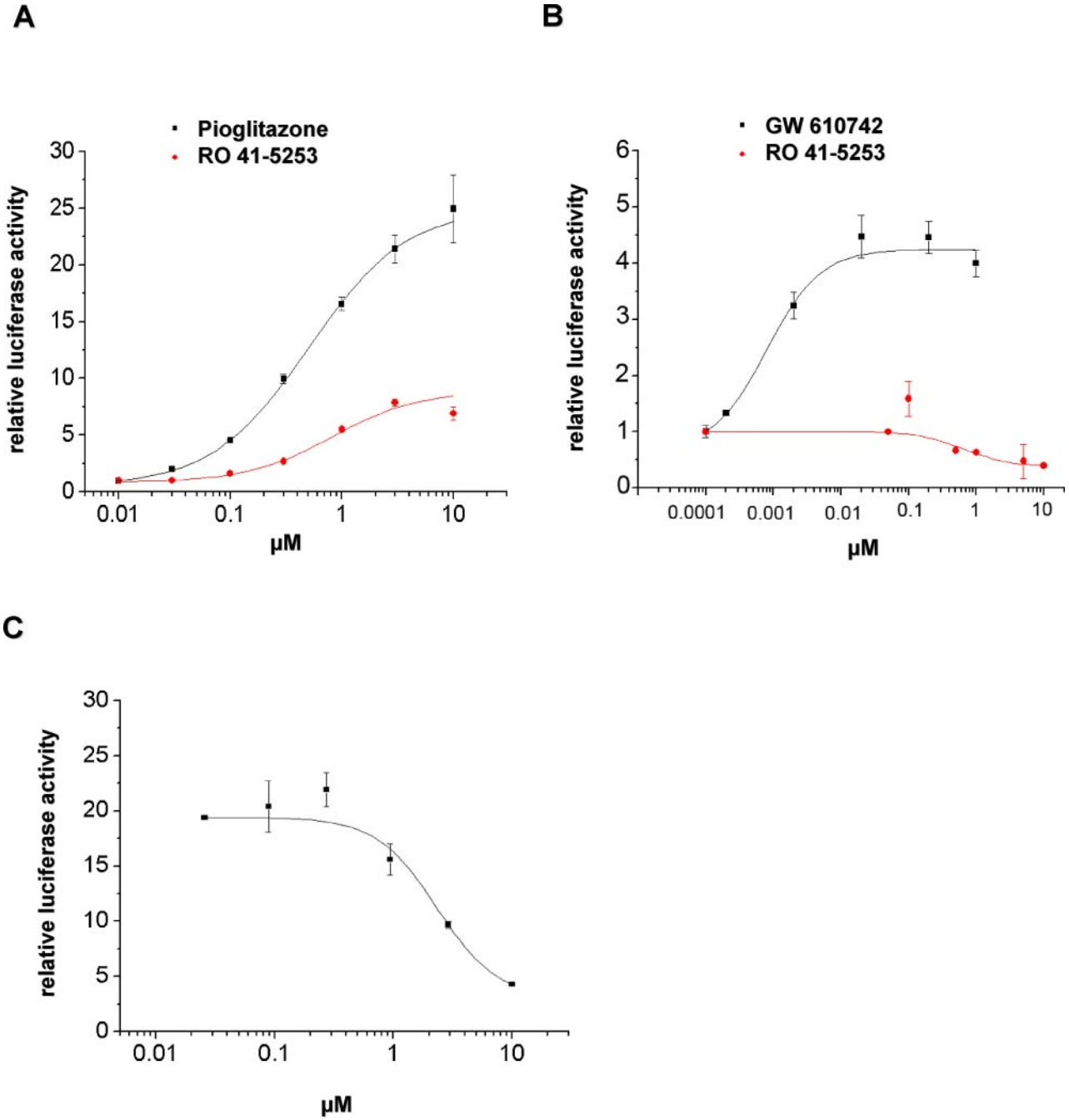

Fig. 4. Ro 41-5253 partially activates the PPARγ-LBD but not the PPARβ/δ-LBD and competes with TZD for activation. 293T cells were transiently transfected with pGal4-hPPARγ_DEF or pGAL-hPPARβ/δ_DEF and the pGal5-Tk-pGL3 reporter followed by stimulation with pioglitazone or GW 610742 (black squares in A and B) or Ro 41-5253 (red circles) as indicated for 24 h. 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 1 μM pioglitazone and increasing concentrations of Ro 41-5253 for 24 h and assayed for luciferase activity (C).

TABLE 1

EC_{50} and maximal activation of the human PPARγ-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
	μM	%
Pioglitazone	0.58	100
Ro 41-5253	0.81	28

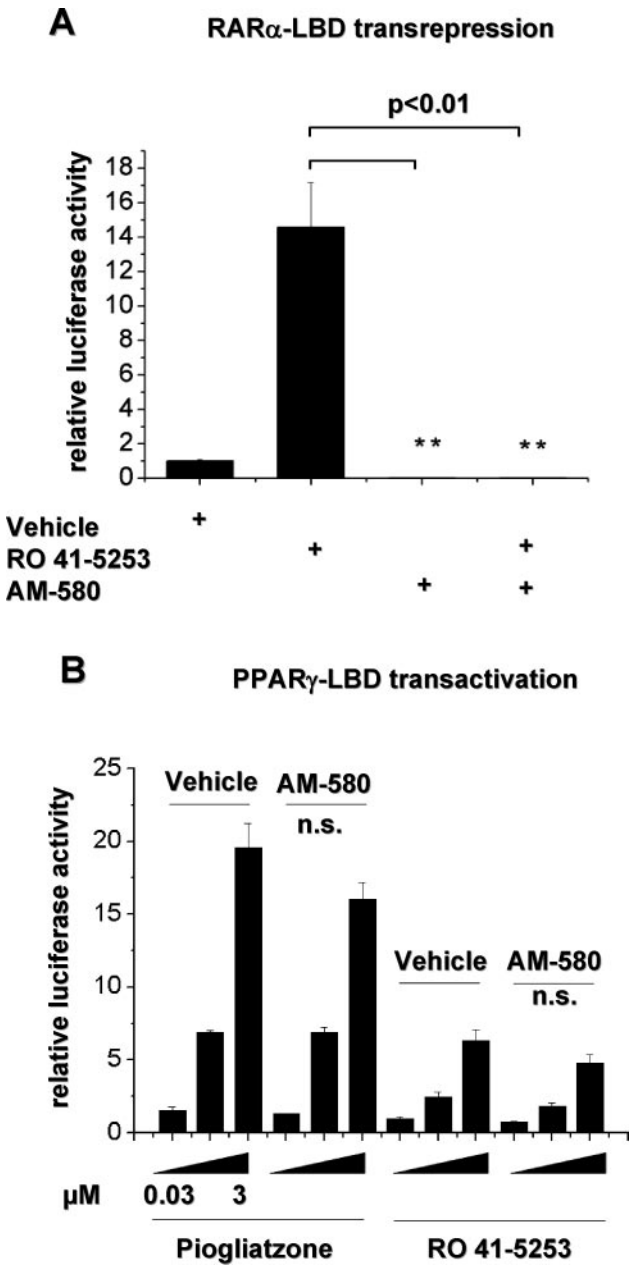

Fig. 5. Ro 41-5253 activates the PPARγ-LBD in presence of the RARα agonist AM-580. 293T cells were transiently transfected with the pGal4-hRARα_DEF (A) or pGal4-hPPARγ_DEF (B) and pGal5-Tk-pGL3 reporter. Cells were treated with 500 nM Ro 41-5253, 5 μM AM-580 (a RARα agonist), or both for 24 h. Data represent the -fold repression over vehicle-treated cells (A). Activation the PPARγ-LBD was measured after 24-h incubation with 0.03, 0.3, and 3 μM concentrations of either pioglitazone or Ro 41-5253 in the presence of vehicle or 5 μM AM-580. Data represent the -fold induction over vehicle treated cells (B). **, $p < 0.01$.

Ro 41-5253 was able to bind to PPARγ and PPARβ/δ with IC_{50} values in the low micromolar range (Table 2).

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 with 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 (Figs. 2A and 4A). Although Ro 41-5253 seems to bind the PPARγ-LBD in a manner similar to that of

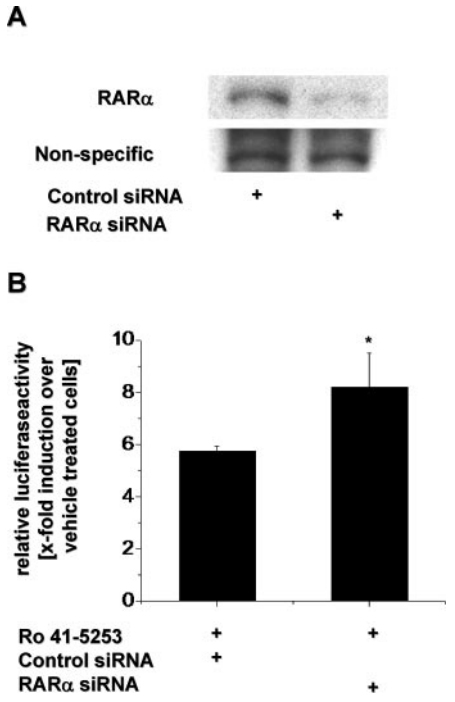

Fig. 6. Ro 41-5253 activates the PPARγ-LBD in cells depleted of RARα. Reduction of RARα protein levels by electroporating siRNA control or siRNA RARα in 293T cells (A). After 24 h, cells were transfected with pGal4-hPPAR_DEF and pGal5-Tk-pGL3 and incubated for another 24 h with 3 μM Ro 41-5253. Data are shown as -fold induction over vehicle-treated cells (B). *, $p < 0.05$.

TABLE 2

IC_{50} of Ro 41-5253 in displacing specific ^{3}H-labeled ligands from hsPPAR isoforms

Ro 41-5253 displaces specific ligands from hsPPAR-LBDs as measured by a scintillation proximity assay. Data represent mean IC_{50} values of three independent experiments.

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

TZDs, TZD-induced activation could be diminished with increasing concentrations of Ro 41-5253, which is the traditional 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 *N*-(9-Fluorenylmethoxycarbonyl)-L-leucine (Rocchi et al., 2001), MCC-555 (Reginato et al., 1998) and certain angiotensin receptor blockers (Schupp et al., 2005).

No known endogenous ligand for NRs 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, chemically derived from atRA, has no obvious similarity with any synthetic PPARγ activator. On the other hand, it shares structural elements with arachidonic metabolites, such as prostaglandin J_2, that have been shown to activate PPARγ (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 RARα antagonism. This caution pertains not only to studies of adipogenesis (Kamei et al., 1994) but also 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 (Shang et al., 1999; Schneider et al., 2000; Emionite et al., 2004; Lu et al., 2005; 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 (Kilgore et al., 1997; Nwankwo and Robbins, 2001; James et al., 2003) but also in primary and metastatic breast adenocarcinomas (Kilgore et al., 1997; Mueller et al., 1998). Ro 41-5253 was able to bind β/δ in the low micromolar range, which adds to the concerns about overinterpretation of its effects as RARα-specific in biological systems. Unfortunately, to our knowledge, no other RAR antagonist is commercially available.

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