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The Benzenesulfoamide T0901317 [*N*-(2,2,2-Trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide] Is a Novel Retinoic Acid Receptor-Related Orphan Receptor-α/γ Inverse Agonist

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ABSTRACT

Retinoic acid receptor-related orphan receptors (RORs) regulate a variety of physiological processes including hepatic gluconeogenesis, lipid metabolism, circadian rhythm, and immune function. Here we present the first high-affinity synthetic ligand for both RORα and RORγ. In a screen against all 48 human nuclear receptors, the benzenesulfonamide liver X receptor (LXR) agonist *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide (T0901317) inhibited transactivation activity of RORα and RORγ but not RORβ. T0901317 was found to directly bind to RORα and RORγ with high affinity (K_i = 132 and 51 nM, respectively), resulting in the modulation of the receptor's ability to interact with transcriptional cofactor proteins. T0901317 repressed RORα/γ-dependent transactivation of ROR-responsive reporter genes and in HepG2 cells reduced recruitment of steroid receptor coactivator-2 by RORα at an endogenous ROR target gene (*G6Pase*). Using small interference RNA, we demonstrate that repression of the gluconeogenic enzyme glucose-6-phosphatase in HepG2 cells by T0901317 is ROR-dependent and is not due to the compound's LXR activity. In summary, T0901317 represents a novel chemical probe to examine RORα/γ function and an excellent starting point for the development of ROR selective modulators. More importantly, our results demonstrate that small molecules can be used to target the RORs for therapeutic intervention in metabolic and immune disorders.

Several members of the nuclear receptor (NR) superfamily regulate the expression of key genes involved in carbohydrate and lipid metabolism in response to ligands such as fatty acids, bile acids, cholesterol metabolites, and steroid hormones. For example, the nuclear receptors liver X receptors (LXRs) α and β (NR1H3 and NR1H2) bind oxidized cholesterol and function as sensors for excess intracellular oxysterols (Janowski et al., 1996; Kalaany et al., 2005). Many studies have demonstrated that the LXRs are involved in the regulation of a wide variety of physiological processes, including cholesterol metabolism and transport, lipogenesis, gluconeogenesis, and inflammation, making these receptors attractive targets for the development of synthetic ligands for the treatment of disorders such as dyslipidemia, atherosclerosis, and diabetes (Mohan and Heyman, 2003). An early result of drug discovery efforts on LXRs was the discovery of two potent synthetic agonists, the benzenesulfonamide T0901317 and the tertiary amine GW3965, which have been used extensively to help expand our understanding of the physiological roles of the LXRs (Schultz et al., 2000; Collins et al., 2002). However, recently, it has been shown that

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ABBREVIATIONS: NR, nuclear receptor; ROR, retinoic acid receptor-related orphan receptor; ChIP, chromatin immunoprecipitation; SRC2, steroid receptor coactivator 2; LXR, liver X receptor; G6Pase, glucose 6-phosphatase; T0901317, (2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide; GW3965, 3-[3-[[[2-chloro-3-(trifluoromethyl)phenyl]methyl](2,2 -diphenylethyl) amino]propoxy]benzeneacetic acid hydrochloride; siRNA, small interference RNA; PCR, polymerase chain reaction; DBD, DNA binding domain; LBD, ligand binding domain; HEK, human embryonic kidney; DMSO, dimethyl sulfoxide; RORE, retinoic acid receptor-related orphan receptor response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin.

in addition to potent modulation of the LXRs, T0901317 but not GW3965 is a potent agonist of both the farnesoid X receptor and the xenobiotic receptor pregnane X receptor (Houck et al., 2004; Mitro et al., 2007), bringing into question conclusions drawn from pharmacological studies using this compound.

The promiscuity of T0901317 and other nuclear receptor ligands indicates that there are privileged structures (chemotypes) that bind to a range of these receptors. Because it is possible to use these promiscuous ligands as points to initiate the development of receptor-selective ligands, we set out to profile the activity of a collection of well characterized NR ligands against all human nuclear receptors. Our laboratory has developed a GAL4 nuclear receptor library containing all 48 human receptors to facilitate the selectivity profiling of putative NR modulators that emerge from HTS campaigns at the Scripps Research Molecular Screening Center. In an effort to demonstrate the usefulness of the NR library, a collection of 65 well characterized NR modulators including the LXR agonist T0901317 was assembled. It is interesting that when this chemical set was tested against the GAL4 NR library, it was discovered that in addition to its expected activity, T0901317 was a potent inhibitor of the nuclear receptors retinoid-related orphan receptors α and γ (RORα and RORγ; NR1F1 and NR1F3) yet afforded little or no activity on RORβ (NR1F2).

The RORs are orphan nuclear receptors for which the endogenous ligand has yet to be described. Because the RORs are constitutive activators of transcription in the absence of ligands, it has been suggested that the coactivator binding surface, or activation function 2, is locked in the holo-conformation (Harris et al., 2002), circumventing the need for ligand interaction to transactivate target genes. However, the cocrystal structures of RORα LBD bound to cholesterol and cholesterol sulfate have been solved, suggesting that like the LXRs, the RORs can bind and may respond to metabolites of cholesterol (Kallen et al., 2002, 2004).

The RORs have emerged as attractive drug targets for the treatment of metabolic disorders and inflammatory disease. Here we demonstrate, for the first time, that a synthetic ligand can bind directly to and modulate the transcriptional activity of RORα and RORγ. T0901317 was found to directly bind to RORα and RORγ with high affinity (K_i = 132 and 51 nM, respectively), resulting in the modulation of the receptor's ability to interact with transcriptional cofactor proteins. T0901317 repressed RORα/γ-dependent transactivation of ROR-responsive reporter genes and in HepG2 cells reduced the recruitment of steroid receptor coactivator-2 (SRC2) by RORα at an endogenous ROR target gene. Using small interference RNA (siRNA), we demonstrate that repression of the gluconeogenic enzyme glucose-6-phosphatase in HepG2 cells by T0901317 is ROR-dependent and is not due to the compound's LXR activity.

In summary, T0901317 represents a novel chemical probe to examine RORα/γ function. In addition, this compound, with a chemically tractable scaffold, represents an excellent starting point for medicinal chemistry toward the development of ROR-selective modulators. More importantly, our results demonstrate for the first time that small molecules can be used to target the RORs for potential therapeutic intervention in metabolic and immune disorders.

Materials and Methods

Reagents

T0901317 was purchased from Sigma-Aldrich (St. Louis, MO). 25-Hydroxycholesterol was purchased from MP Biomedicals (Irvine, CA). Radioligand 25-[26,27-^{3}H]hydroxycholesterol was from PerkinElmer Life and Analytical Sciences (Waltham, MA). Fifty-five endogenous and synthetic ligands from the Sigma Nuclear Receptor Signaling Ligand set (Sigma-Aldrich) were used to build the 65-compound chemical set run against the Gal4NR library. For ligand binding studies, RORα ligand binding domain (amino acids 304–556) was PCR-amplified and cloned into a pGEX-2T (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) encoding an N-terminal GST-Tag according to the manufacturer's instructions. The protein was induced with 1 mM isopropyl β-D-thiogalactoside in BL21 gold (DE3) cells (Invitrogen, Carlsbad, CA) and purified by affinity chromatography with Protino GST/4B column (Macherey-Nagel, Bethlehem, PA) followed by size-exclusion chromatography with HiLoad 26/60 Superdex 200 column (GE Healthcare). The protein was eluted, concentrated, and stored in 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM dithiothreitol, and 10% glycerol. For AlphaScreen studies (PerkinElmer Life and Analytical Sciences), recombinant His-RORα-LBD protein and Biotin-RIP140 box B peptide containing the LYYML motif were gifts from Eli Lilly and Company (Indianapolis, IN). The histidine (Nickel) detection kit was purchased from PerkinElmer Life and Analytical Sciences.

GAL4 NR Library Compound Profiling

The GAL4 NR library was built by replacing the endogenous N terminus and DNA binding domain (DBD) of all 48 receptors with a GAL4 DBD. The fusion constructs consist of the GAL4 DBD, the hinge domain, and LBD (and F domain if applicable) of the human receptors. The library was plated in triplicate on 384-well plates, and HEK293T cells were reverse-transfected with the well-specific construct and the UAS luciferase reporter pGL4.31 (Promega, Madison, WI) using Fugene6 transfection reagent (Roche Applied Sciences, Indianapolis, IN) in a final volume of 40 μl. Control wells contained constructs encoding for the GAL4 DBD alone (pBind) or GAL4 fused to VP16 were also analyzed. After 24 h, optimized compounds (2 μM final concentration) or DMSO was added to the plates and allowed to incubate for 20 h before the addition of 40 μl of BriteLite (PerkinElmer Life and Analytical Sciences) to measure luciferase activity. Compounds that attenuate the GAL4-VP16-dependent luciferase activity in the positive control were considered promiscuous or cytotoxic. Each compound was evaluated using two plates of the GAL4 NR library providing six replicates and was averaged for luciferase and compared with DMSO-only controls. Compounds with mean signals 3 S.D. from the DMSO controls were considered hits in this assay.

Radioligand Receptor Binding Assay

Forty-five or 90 ng of purified GST-RORα or GST-RORγ was incubated with various concentrations of [^{3}H]25-hydroxycholesterol in assay buffer (50 mM HEPES, pH 7.4, 0.01% bovine serum albumin, 150 mM NaCl, and 5 mM $MgCl_2$) to determine the K_d value. Nonspecific binding was defined in the absence of protein and excess of nonradioactive 25-hydroxycholesterol and was shown to be identical. The assays were terminated by rapid filtration through presoaked Whatman GF/B filters (0.5% polyethylenimine in phosphate-buffered saline) in Multiscreen plates (Millipore, Billerica, MA) and were washed (3 × 0.1 ml) with ice-cold assay buffer. The radioligand binding results were analyzed using Prism software (GraphPad Software, Inc., San Diego, CA). For the competition assay, various concentrations of T0901317 were incubated with receptor in the presence of 3 nM [^{3}H]25-hydroxycholesterol.

AlphaScreen

The assays were performed in triplicate in white opaque 384-well plates (PerkinElmer Life and Analytical Sciences). The final volume was 20 μl for the generation of compound dose-response curves (0.5–7.5 μM). All dilutions were made in assay buffer (100 mM NaCl, 25 mM HEPES, and 0.1% bovine serum albumin, pH 7.4). The final DMSO concentration was 0.25%. A mix of 12 μl of His-RORα-LBD (75 nM), beads (30 μg/ml of each), and 4 μl of increasing concentrations of compound (0.02–8 μM) was added to the wells, and the plates were sealed and incubated for 1 h at room temperature in the dark. After this preincubation step, 4 μl of Biotin-RIP140B (25 nM) was added, the plates were sealed and further incubated for 2 h at room temperature in the dark. The plates were read on a PerkinElmer Envision 2104, and data were analyzed using Prism software.

Cell Culture and Transcriptional Assays

RIP140 Modulation of RORα Activity. Luciferase reporter assays were conducted using a pBind Gal4-tagged RORα LBD construct, UAS luciferase reporter, and pSport6 full-length RIP-140 cotransfected into HEK293T cells. Reverse transfections were performed in bulk using 10^6 cells in 6-cm plates; 3 μg of total DNA in a 1:1:1 ratio of receptor, reporter, and corepressor, respectively; and FuGene6 (Roche) in a 1:3 DNA/lipid ratio. As controls, separate transfections containing either reporter only or receptor/reporter were performed using pBind or pSport6 empty vectors in place of receptor and corepressor, respectively. After 24-h bulk transfection, cells from different transfection conditions were counted and plated in 384-well plates at a density of 10^4 cells/well. After additional 24-h incubation, luciferase levels were assayed by one-step addition of 20 μl of BriteLite (PerkinElmer Life and Analytical Sciences) and read using an Envision multilabel plate reader (PerkinElmer Life and Analytical Sciences). Data were normalized to luciferase signal from UAS luciferase reporter/pBind control empty vector and displayed as the -fold change over UAS luciferase reporter. Unpaired *t* tests were performed on all data sets, and significance of differences between Gal4RORα and Gal4RORα/RIP140 was determined at $p < 0.001$.

RORα Modulation of Glucose 6-Phosphatase Wild-Type and Mutant Reporters. For the *glucose 6-phosphatase* promoter, wild-type promoter or ROR-response element (RORE) mutant was used to transfect HEK293T cells with SRC2 as coactivator in the presence or absence of full-length RORα and were treated as defined in the figure legends (Chopra et al., 2008). Likewise, for *Cyp7B1*, the wild-type or RORE mutant promoter was transfected in HEK293T cells in the presence or absence of full-length RORα. The *Cyp7B1* promoter constructs were a gift from Dr. Wen Xie (University of Pittsburgh, Pittsburgh, PA) and have been described previously (Wada et al., 2008).

RORα Modulation of IL-17 Reporter. HEK293 cells were grown in 96-well plates (1×10^6/well) and were transiently transfected using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Cells were transfected with a total of 200 ng of DNA per well consisting of the pGL4 mIL-17 firefly luciferase reporter construct, the pGL4 mIL-17 + CNS-5 firefly luciferase reporter construct, or the pGL4 mIL-17 2kB RORE mutant (100 ng/well) (Addgene, Cambridge, MA), an actin promoter *Renilla reniformis* luciferase reporter (50 ng/well), and either control vector alone or the test DNA (full-length RORα or full-length RORγ at 50 ng/well). The IL-17 reporters have been described previously (Zhang et al., 2008) and were obtained from Addgene. Cells were treated with T0901317 for 24 h and then lysed and read using the Dual-Glo Luciferase assays system (Promega) 48 h after transfection. These results were normalized (firefly/*R. reniformis* ratio).

Reduction of Endogenous Gene Expression by Small Interference RNAs

To reduce endogenous ROR expression, HepG2 cells were seeded onto a 12-well plate (2.5×10^5/well) and transfected the next day with siRNAs against human RORα and RORγ (Dharmacon RNA Technologies, Lafayette, CO) at 50 nM according to the instructions for Dharma-FECT 1 transfection reagent. Forty-two hours after transfection, cells were treated with vehicle (DMSO) or T0901317 (10 μM) for 6 h. Cells were harvested, and total RNA was isolated. Quantitative reverse transcriptase PCR was performed to analyze mRNA levels of human RORα, RORγ, GAPDH, and *glucose 6-phosphatase* (*G6Pase*) using SYBR Green technology. The primers used for quantitative PCR analysis are as follows: human RORα, GTAGAAACCGCTGCCAACA (forward) and ATCACCTCCCGCTGCTT (reverse); human RORγ, CCCCTGACCGATGTGGACT (forward) and CAGGATGCTTTGGCGATGA (reverse); human *G6Pase,* TCATCTTGGTGTCCGTGATCG (forward) and TTTATCAGGGGCACGGAAGTG (reverse); and GAPDH, TGCACCACCAACTGCTTAGC (forward) and GGCATGGACTGTGGTCATGAG (reverse).

ChIP/re-ChIP

HepG2 cells were infected with Flag-RORα adenovirus for 24 h and then treated with vehicle (DMSO) or T0901317 (10 μM) for another 24 h. Re-ChIP assays were performed by using the kit from Active Motif Inc. (Carlsbad, CA). Anti-FLAG (Sigma-Aldrich) antibody was used to do the first immunoprecipitation for all of the samples. The second immunoprecipitation was performed by using anti-mouse IgG (Millipore), anti-RNA Pol II (Millipore), or anti-SRC2 (Bethyl Laboratories, Montgomery, TX). The *G6Pase* primers used in PCR were CCCTGAACATGTTTGCATCA (forward) and CATTCCTTCCTCCATCCTCA (reverse).

Results

Using a cell-based GAL4-NR LBD cotransfection assay, we found that T0901317 (2 μM) was a potent repressor of both GAL4-RORα and GAL4-RORγ (Fig. 1). It is interesting that T0901317 inhibited the constitutive transactivation activity of both GAL4-RORα and GAL4-RORγ with little or no activity on GAL4-RORβ (Fig. 1). We observed that 1 μM T0901317 repressed RORα by almost 70% and by approximately 90% at 10 μM (see Fig. 1, inset). In control cells transfected with GAL4-VP16 and the UAS reporter, no repression of GAL4-VP16 transactivation of the luciferase gene was observed, suggesting that the repression induced by T0901317 is not a result of nonspecific luciferase effects or cellular toxicity (data not shown). As illustrated in Fig. 2, A and B, treatment of cells expressing GAL4-LXRα, GAL4-RORα, or GAL4-RORγ with increasing concentrations of T0901317 demonstrated an excellent dose response, with an estimated EC_{50} of 0.25 μM (LXRα) and estimated IC_{50} values of 2.0 (RORα) and 1.7 μM (RORγ), respectively.

To determine whether the repression of RORα by T0901317 is due to direct binding to the receptor, we carried out a competitive radioligand binding assay. Previous studies using mass spectroscopy indicated that 25-hydroxycholesterol binds to RORα (Bitsch et al., 2003), and we developed a radioligand binding assay using [^{3}H]25-hydroxycholesterol. We demonstrate that 25-hydroxycholesterol binds to RORα and RORγ with a K_d value of 3.3 $\pm$ 0.89 and 5.1 $\pm$ 0.71 nM, respectively, as determined from a saturation binding curve (Fig. 3, A and C). More importantly, T0901317 dose-dependently competed with [^{3}H]25-hydroxycholesterol for RORα

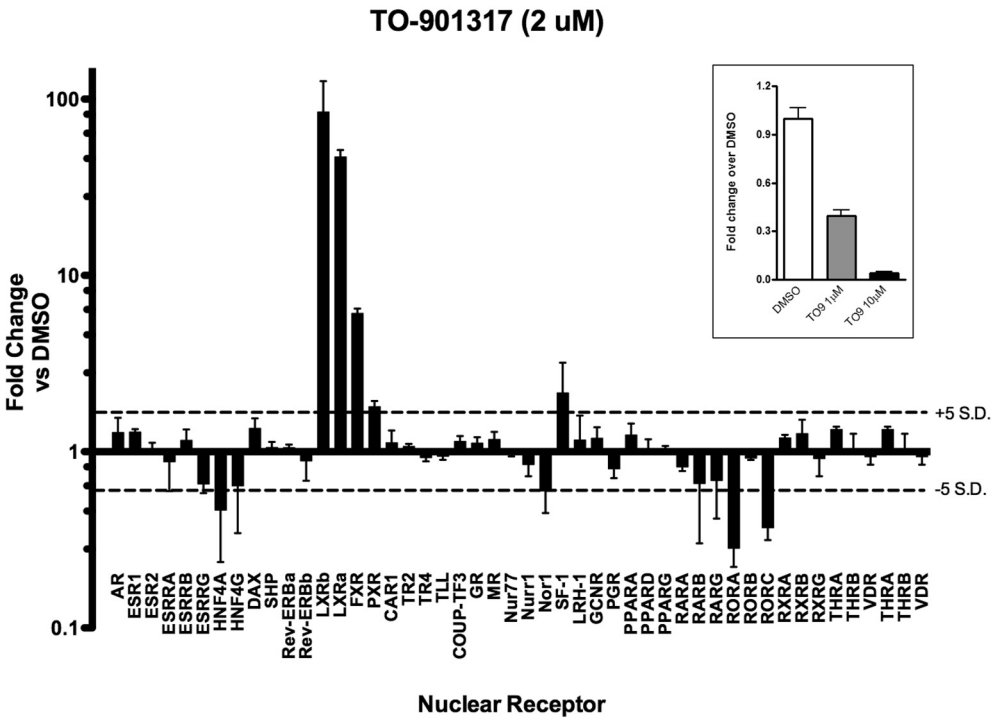

Fig. 1. Gal4 nuclear receptor profiling of T0901317. RORα and RORγ transactivation is repressed by T0901317 in a GAL4 NR library selectivity panel. Gal4 NR clones were reverse-transfected with a UAS reporter construct into HEK293T cells. After 24 h, the LXRα agonist T0901317 (2 μM final concentration) or DMSO was added and incubated for 20 h. The luciferase activity of each construct was measured and normalized to the mock (vector alone), then the -fold change in signal compared with DMSO was calculated (n = 6). Inset, HEK293T-transfected cells were separately treated with either 1 or 10 μM T0901317 or vehicle for 20 h followed by luciferase activity measurement (data shown is mean ± S.E.M., n = 6). Horizontal dashed lines represent ±5 S.D. of the control (mock transfected).

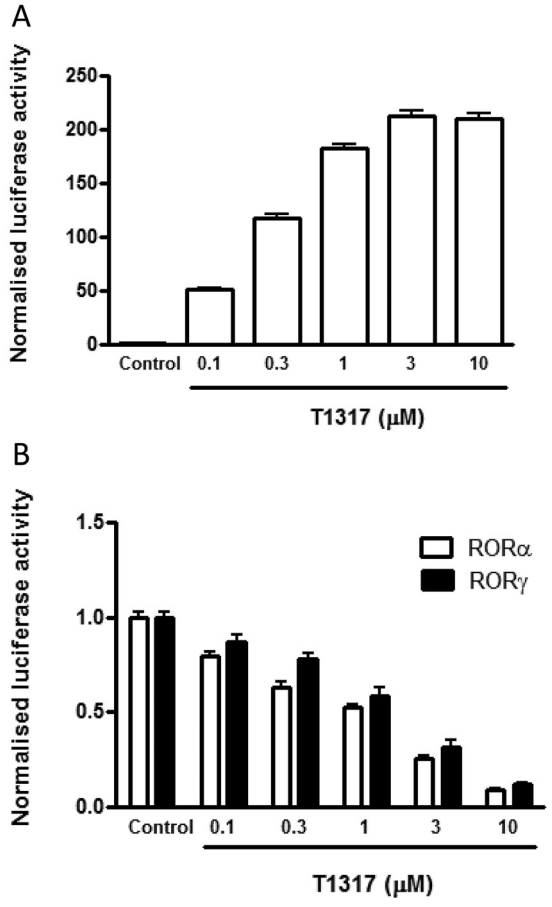

Fig. 2. Dose-response curves for transactivation of LXRα and suppression of RORα and RORγ by T0901317. 293T cells were cotransfected with UAS-luciferase and Gal4-LXRα (A) or Gal4-RORα/γ (B) and were treated with various concentration of T0901317 for 20 h followed by luciferase activity measurement. Relative change was determined by normalizing to control vector treated with vehicle. Each data point was performed in eight replicates and is represented as the mean ± S.E.M., n = 8.

and RORγ binding with an approximate IC_{50} of 254 and 81 nM (K_i = 132 and 51 nM, respectively) (Fig. 3, B and D).

The activity of nuclear receptors can be modulated by ligand-induced cofactor protein interaction where, for example, agonist recruits coactivator protein, and antagonist either blocks coactivator interaction or facilitates the recruitment of corepressor. Corepressors such as NCoR1, NCoR2, and RIP140 have been shown to interact with the RORs (Jetten, 2009). RIP140, also known as nuclear receptor-interacting protein 1, is a nuclear protein that has been shown to specifically interact with the activation function 2 domain of nuclear receptors and repress their activity. A screen of peptides derived from the NR boxes of coactivators and corepressors using Luminex technology revealed that two NR box peptides representative of the ligand-dependent nuclear receptor binding domain of RIP140 (RIP140-B and RIP140-9) interacted strongly with the RORs (data not shown). Using AlphaScreen technology, we sought to determine the ability of T0901317 to modulate receptor-corepressor interaction. The interaction of the NR box peptide, RIP140-B, with the histidine-tagged LBD of RORα was monitored in response to increasing concentrations of T0901317. As shown in Fig. 4A, T0901317 modulated the interaction of RIP140-B with RORα in a dose-dependent fashion. In the absence of receptor, the AlphaScreen counts are at baseline. The AlphaScreen data along with the radioligand binding results demonstrate that T0901317 binds directly to RORα and can induce a conformational change in the LBD that modulates interaction with the NR box peptide derived from the repressor RIP140. We then sought to confirm the ability of RIP140 to repress RORα activity in cells. In cotransfection studies, full-length RIP140 effectively represses the transactivation activity of Gal4RORα on the UAS luciferase reporter (Fig. 4B).

It has been shown that *G6Pase* gene expression is regulated by RORα along with the coactivator SRC2 (Chopra et al., 2008). Therefore, we examined the ability of T0901317

to modulate a *G6Pase* reporter in an RORα-dependent fashion in the presence of the coactivator SRC2. As expected, cotransfection of RORα strongly stimulated the *G6Pase* reporter gene and, as shown in Fig. 5A, T0901317 dose-dependently repressed the *G6Pase* promoter activity with approximately 31% repression at 10 μM. In the absence of RORα or in the presence of a *G6Pase* promoter-reporter containing a mutation of RORE binding site, the repressive effect of T0901317 was eliminated (data not shown), suggesting strongly that this effect was mediated via RORα. Likewise, we analyzed yet another RORα-responsive gene, the cytochrome P450 7B1 (*Cyp7B1*) (Wada et al., 2008). Again, cotransfection of RORα stimulated *Cyp7B1* reporter gene activity by more than 8-fold, and T0901317 dose-dependently repressed the *Cyp7B1* promoter activity with approximately 35% repression at 10 μM (Fig. 5B). The repressive effect of T0901317 was nearly abolished in the absence of RORα or when the RORE binding site of the *Cyp7B1* promoter was mutated (data not shown).

To examine the RORα/γ component of T0901317 pharmacology, we identified a target gene that is regulated by RORα

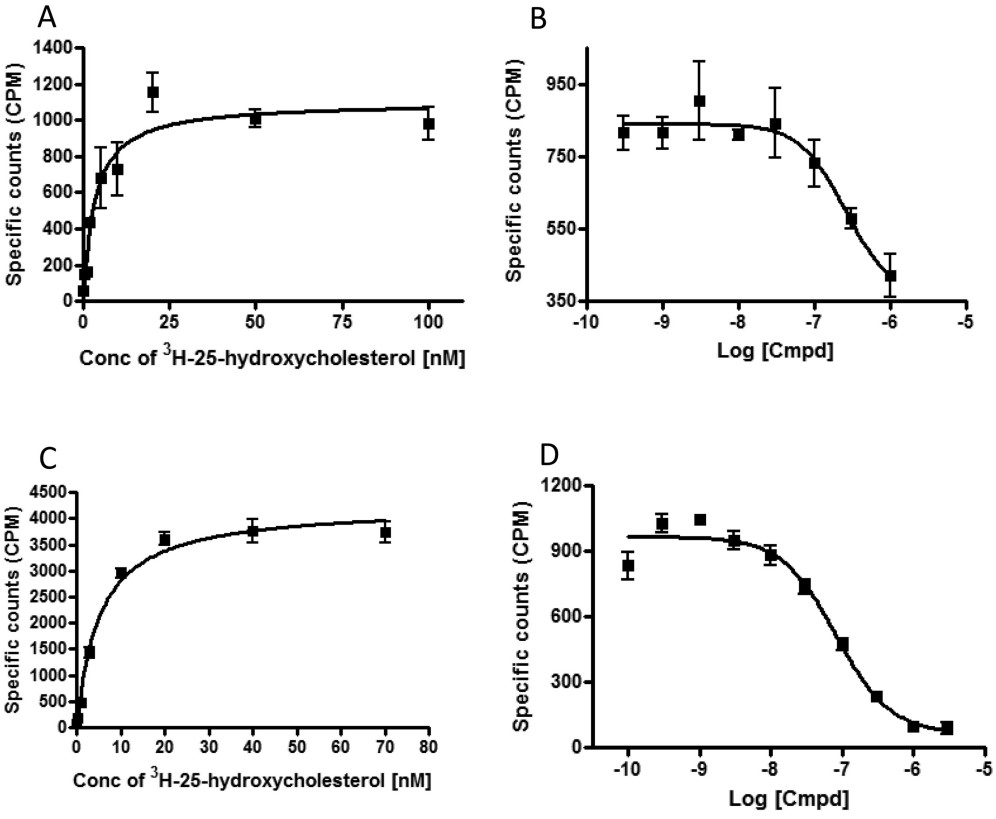

Fig. 3. Radioligand binding assay with GST-RORα and GST-RORγ. Saturation curves for [^{3}H]25-hydroxycholesterol was generated with 45 ng of GST-RORα (A) or 90 ng of GST-RORγ (C) in the assay buffer as mentioned in *Materials and Methods*. K_d value was 3.3 and 5.1 nM for GST-RORα and GST-RORγ, respectively. Competition assay was performed to determine the K_i value of T0901317 for RORα and GST-RORγ. Increasing concentrations of T0901317 were incubated with 3 nM [^{3}H]25-hydroxycholesterol and 45 ng of GST-RORα (B) or 90 ng of GST-RORγ (D). Estimated IC_{50} values for T0901317 were 254 nM and 81 nM for GST-RORα and GST-RORγ, respectively. Data shown are representative results from two independent experiments performed in duplicate. The results were analyzed using Prism software.

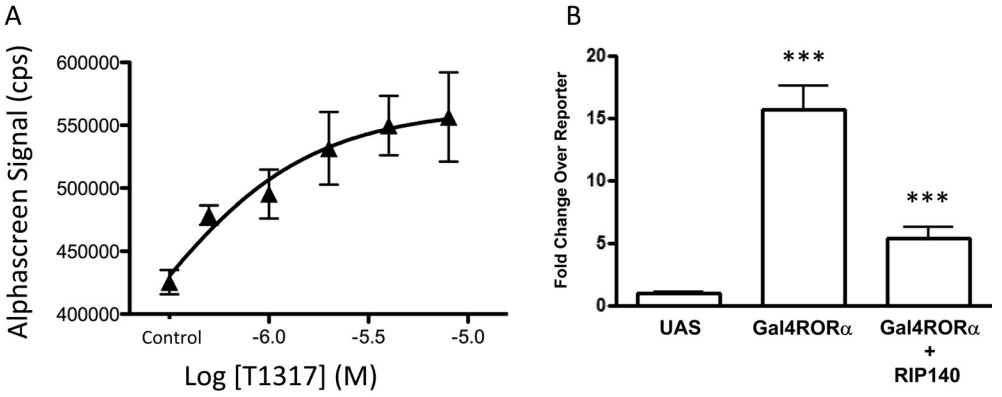

Fig. 4. RORα is repressed by RIP140, and T0901317 modulates RORα interaction with RIP140. A, increasing concentrations of T0901317 were incubated with RORα (75 nM) and 30 μg/ml AlphaScreen beads for 1 h at room temperature in the dark as described under *Materials and Methods*. The Biotin-RIP140B peptide (25 nM) was added to each well, and the plates were incubated in the dark for an additional 2 h at room temperature. Representative data are shown as mean ± S.D. of three independent experiments. B, cell-based luciferase reporter assays measuring the effects of RIP140 on RORα-dependent transactivation. HEK293T cells were reverse-transfected with 3 μg of total DNA composed of either UAS luciferase reporter only, Gal4RORα and UAS luciferase reporter, or Gal4RORα, UAS luciferase reporter, and full-length RIP140. After bulk transfections, cells were replated, and assays were conducted in 384-well format with $n = 6$. Data were normalized to luciferase signal from UAS/pBind vector control. Differences in transactivation between Gal4RORα and Gal4RORα/RIP140 were determined to be significant with $p < 0.001$ (***), as determined by unpaired t test.

and RORγ but not LXR. IL-17 is a well characterized target gene for RORα and RORγ, harbors an essential RORE in the promoter (Yang et al., 2008; Zhang et al., 2008), and displays no responsiveness to LXR (data not shown). HEK293 cells were transfected with an IL-17 promoter-driven luciferase reporter containing the RORE, and these cells were treated with compound or DMSO for 24 h. As shown in Fig. 6, T0901317 was able to repress activation of the IL-17 promoter induced by either RORα or RORγ in a dose-dependent fashion. Unfortunately, there are no good T-cell-derived cell lines that are readily able to be transfected, and there are no cell lines derived from Th17 cells that express significant levels of RORα/RORγ. However, as reported previously, HEK293 cells have been shown to be a good model to study ROR-dependent regulation of the IL-17 promoter (Ichiyama et al., 2008).

With the recent finding that the gluconeogenic enzyme *G6Pase* gene expression is regulated by RORα (Chopra et al., 2008), we examined the ability of T091317 to suppress endogenous *G6Pase* expression in HepG2 cells and to determine whether the effects of T091317 on *G6Pase* are ROR-dependent. Therefore, we monitored mRNA levels of *G6Pase* before and after knockdown of endogenous RORα and RORγ by siRNA. As shown in Fig. 7A, expression of endogenous *G6Pase* was reduced in HepG2 cells by treatment with T091317 (10 μM). Transfection of these cells with a nontargeting siRNA did not interfere with the ability of T091317 to reduce the expression of *G6Pase*. Transfection with the nontargeting siRNA did not have an effect on the expression levels of either RORα or RORγ (data not shown). Treatment of HepG2 cells with siRNAs targeting RORα and RORγ reduced the expression of both receptors by more than 60% but had no effect on GAPDH expression levels (data not shown). In agreement with previous studies (Chopra et al., 2008), reduction of expression levels of RORα and RORγ reduced the expression of G6Pase (Fig. 7A). More importantly, the ability of T0901317 to repress G6Pase expression in HepG2 cells was lost when the RORs were knocked down (Fig. 7A). These results demonstrate that T091317 modulation of G6Pase is ROR-dependent and is not related to the compound's LXR activity, as has been suggested previously.

Finally, the ability of T0901317 to modulate RORα recruitment of the p160 coactivator SRC2 to the *G6Pase* promoter was assessed using a sequential ChIP assay (ChIP/reChIP). Treatment with T0901317 did not affect the level of RORα occupancy of the *G6Pase* promoter (Fig. 7B); however, in the reChIP using the SRC2 antibody, substantial decrease in the amount of SRC2 occupancy was noted in the presence of the T0901317. These results demonstrate that T0901317 decreases the ability of RORα to recruit the SRC2 coactivator to the *G6Pase* promoter and thus decreases the expression of the gene.

Discussion

The first ROR (RORα) was discovered in the early 1990s based on sequence similarities to the retinoic acid receptor and the retinoid X receptor, hence the name "retinoic acid receptor-related orphan receptor" (Beckerandre et al., 1993; Giguere et al., 1994), soon followed by the identification of RORβ and RORγ (Carlberg et al., 1994; Hirose et al., 1994). Each ROR gene generates multiple isoforms as a result of alternative promoter usage and splicing. In humans, four forms of RORα have been detected (α1–α4), yet only α1 and α4 are found in the mouse (Jetten et al., 2001). Two forms of RORβ are found in the mouse (β1 and β2), but only β1 is present in humans (Jetten et al., 2001). Two forms of RORγ are found in human and mouse (γ1 and γ2), with the γ2 form often referred to as RORγt because it is primarily expressed in the immune system. This isoform has garnered much attention lately because of its role in Th17 cells (Jetten et al., 2001; Miller and Weinmann, 2009). All three isoforms display a high degree of sequence similarity, yet surprisingly, as we demonstrate here, T0901317 can modulate the activity of both RORα and RORγ but not that of RORβ, suggesting the possibility for the development of synthetic molecules that would be ROR isoform-selective modulators.

The three RORs display distinct patterns of expression, suggesting nonredundant functions. RORα is expressed in the liver, skeletal muscle, skin, lungs, adipose tissue, kidney, thymus, and brain (Hamilton et al., 1996; Steinmayr et al., 1998), whereas RORβ expression is restricted to the central nervous system (Andre et al., 1998a,b). RORγ is highly expressed in the thymus; however, significant expression is also found in the liver, skeletal muscle, adipose tissue, and

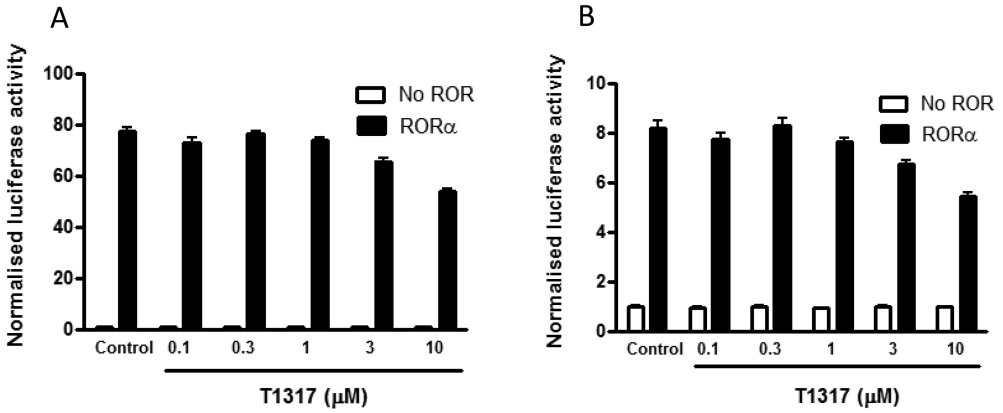

Fig. 5. Modulation of RORα-mediated *G6Pase* promoter activity and *CYP7B1* promoter activity by T0901317 (T1317). 293T cells were cotransfected with pS6 control plasmid or pS6 containing full-length RORα along with *G6Pase* promoter (A) or *CYP7B1* promoter (B) as detailed under *Materials and Methods*. SRC2 as a coactivator was also cotransfected with *G6Pase* promoter. Dose-response curve was determined by treating the transfected cells with varying concentrations of T0901317 for 20 h. Luciferase activity was measured, and relative change was determined by normalizing to cells treated with vehicle only. Each data point was performed in eight replicates and is represented as mean ± S.E.M., $n = 8$.

kidney (Medvedev et al., 1996). All RORs recognize and bind to specific sequences of DNA, termed ROREs as monomers, and these ROREs typically consist of an AGGTCA "half-site" with a 5′ AT-rich extension (Carlberg et al., 1994; Giguere et al., 1994; Hirose et al., 1994). When bound to their element within the promoter of a target gene, all three RORs constitutively recruit coactivators such as the p160 coactivator SRC2, resulting in constitutive activation of transcription of their target genes (Atkins et al., 1999).

A role for RORα in the regulation of metabolic pathways was revealed by studies in the *staggerer* (ROR$\alpha^{sg/sg}$) mouse. This natural mutant mouse strain carries an intragenic insertion within the RORα gene that results in a frameshift and a premature stop codon, rendering RORα inactive (Hamilton et al., 1996). Detailed examination of the *staggerer* mouse revealed alterations in lipid metabolism evidenced by low levels of total plasma cholesterol, triglycerides, apoCIII (an apolipoprotein involved in triglyceride-rich metabolism), high-density lipoprotein, and apoA1 (the major apolipoprotein in high-density lipoprotein) (Mamontova et al., 1998). *Staggerer* mice are less susceptible to hepatic steatosis and have a reduced body fat index relative to wild-type mice, despite higher food consumption (Lau et al., 2008). The size of both brown and white adipose cells are smaller in these animals, and hepatic triglyceride content is lower (Lau et al., 2008). Consistent with this phenotype, the animals are less susceptible to high-fat diet-induced obesity and hepatic steatosis (Lau et al., 2008).

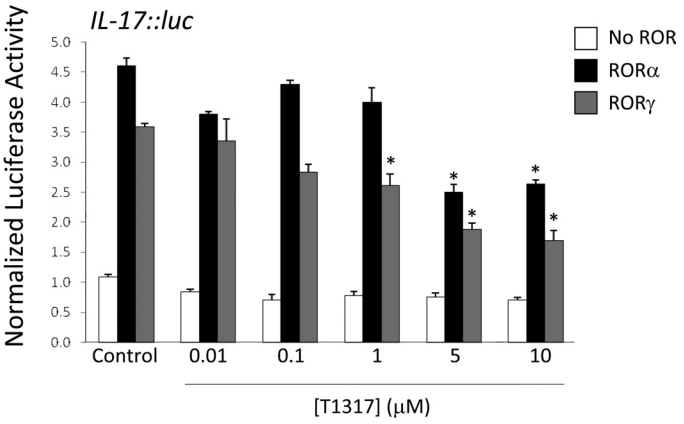

Fig. 6. T0901317 (T1317) suppresses RORα- and RORγ-mediated IL-17 transcription. HEK293 cells were transiently transfected with the IL-17-dependent reporter construct, *R. reniformis* luciferase, and vectors containing full-length RORα (RORα), full-length RORγ (RORγ), or empty vector alone (endogenous). Twenty-four hours later, cells were treated with DMSO or increasing concentrations of T0901317. Twenty-four hours after treatment, IL-17 activity was determined by dual luciferase assay. The data are normalized to the vehicle (DMSO)-treated cells. Differences in transactivation were determined to be significant with $p < 0.05$ (*), as determined by unpaired t test.

RORγ-null mice exhibit normal levels of plasma cholesterol and triglycerides (Kang et al., 2007). An interesting metabolic phenotype was revealed when *staggerer* mice were crossed with RORγ-null mice, effectively creating an RORα/γ double knockout. Although neither individual strain showed significant alterations in plasma glucose levels, the double knockout was hypoglycemic, illustrating a role for these receptors in maintaining glucose homeostasis (Kang et al., 2007). This study also demonstrated that RORα and RORγ display significant redundancy in function, which is consistent with plasma glucose levels remaining unaffected unless both receptors are lost. More recently, a role for RORα in the regulation of glucose metabolism was characterized when Chopra et al. (2008) found that loss of the p160 family coactivator SRC2 in mice led to a phenotype similar to von Gierke's disease, which is associated with severe hypoglycemia and abnormal accumulation of glycogen in the liver. Loss of expression of the enzyme *G6Pase* is responsible for 80% of the diagnosed von Gierke's disease cases. It is noteworthy that SRC2 was required for RORα to regulate this gene in a normal manner (Chopra et al., 2008).

Both RORα and RORγ regulate key physiological pathways and are also involved in pathogenic processes. RORα regulates lipid and glucose metabolism and is believed to play a role in protection against the development of atherosclerosis (Jetten, 2009). This receptor also is critical for normal function of the mammalian clock and is involved in the

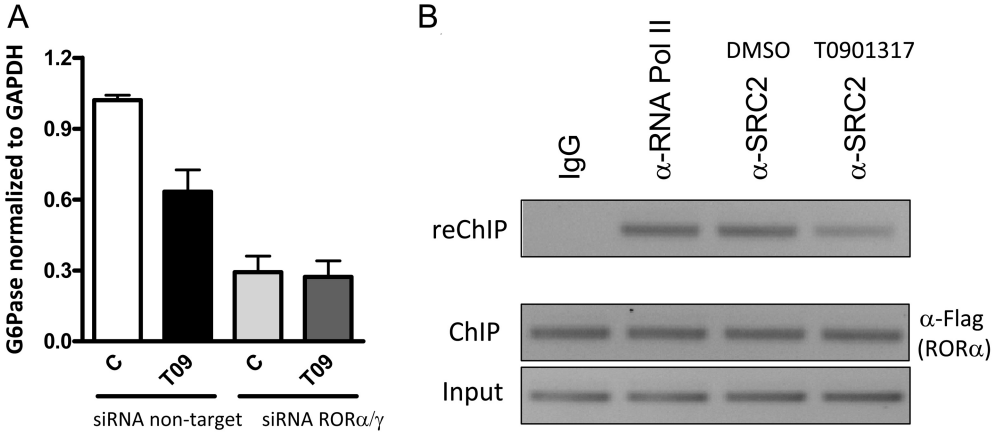

Fig. 7. Modulation of endogenous G6Pase mRNA expression and endogenous *G6Pase* promoter activity by T0901317 in HepG2 cells. A, expression of endogenous *G6Pase* in HepG2 cells was reduced by treatment with siRNA against both human RORα and RORγ. siRNA oligonucleotides against RORs were transfected into HepG2 cells at 50 nM. Forty-two hours after transfection, cells were treated for 6 h with either vehicle (DMSO) or T091317 (10 μM). Total RNA was prepared from these cells and was subjected to reverse transcriptase PCR to measure the mRNA levels. B, sequential chromatin immunoprecipitation (ChIP/reChIP) assay illustrating that T0901317 (10 μM) treatment reduces the ability of RORα to recruit SRC2 to a *G6Pase* gene promoter. HepG2 cells overexpressing Flag-tagged RORα were treated with vehicle (DMSO) or 10 μM T0901317 for 24 h, followed by sequential ChIP. The first immunoprecipitation was performed using α-Flag antibody, and the second immunoprecipitation was performed using α-SRC2 antibody. Mouse IgG was used as a negative control, and anti-RNA pol II antibody was used as a positive control.

modulation of immune function (Yang et al., 2008; Jetten, 2009). The most prominent role for RORγ is the regulation of immune function, especially in the development of the Th17 cells that are believed to play an important role in autoimmunity (Ivanov et al., 2007). RORγ also helps to coordinate lipid and glucose metabolism in concert with RORα (Kang et al., 2007; Jetten, 2009). RORα and RORγ have also been implicated in bone development and cancer (Jetten, 2009). Thus, the development of small-molecule ligands that modulate the activity of these two orphan receptors has held significant interest for those pursuing the role of RORα/γ in the areas of metabolic diseases, autoimmunity, osteoporosis, and cancer. Our finding that T0901317 binds directly to both RORα and RORγ and modulates their transcriptional activity provides the first step toward the development of chemical tools to determine the ability to pharmacological target these receptors for these diseases.

The pharmacology of T0901317 has been characterized in detail in the literature with much of its activity attributed to the activation of LXRα and LXRβ (Michael et al., 2005). However, we described previously that this compound also activates farnesoid X receptor (Houck et al., 2004), and it was later reported that it also activates pregnane X receptor (Mitro et al., 2007). This degree of receptor promiscuity that provided us a critical advantage for the identification of a synthetic ligand for an orphan nuclear receptor creates difficulties for the interpretation of results obtained with this compound, especially in animal models. For example, T0901317 has been used to show that activation of LXR may lead to decreased severity of experimental autoimmune encephalomyelitis (Hindinger et al., 2006) by decreasing Th17 function (Xu et al., 2009). Based on our results, these effects may be due to the ability of T0901317 to suppress the activity of RORα and RORγ that is required for Th17 cell proliferation and IL-17 production.

It is unclear what the relative contribution of inhibition of RORα/γ activity is to the pharmacology to the array of animal studies examining the role of T0901317 on lipid and glucose metabolism. The results presented here demonstrate that the T0901317 effects on repression of *G6Pase* are in fact ROR-dependent and are not a result of the compound's LXR activity.

Conclusion

RORs regulate a variety of physiological processes, including hepatic gluconeogenesis, lipid metabolism, circadian rhythm, and immune function. Here we demonstrate that T0901317 represents the first synthetic ligand for RORα and RORγ, and this compound is a potent inverse agonist of these two orphan nuclear receptors. This was demonstrated by competitive radioligand binding assay and cell-based assays in which T0901317 repressed RORα/γ-dependent transactivation of reporter genes driven by the ROR-responsive promoters from the *G6Pase* and *Cyp7b1* genes. Moreover, repression of *G6Pase* by T0901317 was relieved after knockdown of both RORs, concluding that this compound's effects on this gluconeogenic enzyme are ROR-dependent. Finally, we show that T0901317 reduces recruitment of the p160 coactivator SRC2 by RORα at the *G6Pase* promoter, thus providing a mechanism for control of this important enzyme by the RORs.

The pharmacology of T0901317 has been extensively studied in animal models, with the compound exhibiting acceptable pharmacokinetic properties. More importantly, the benzenesulfonamide scaffold is amenable to a modular synthetic chemistry optimization (Michael et al., 2005). Therefore, T0901317 represents a novel chemical tool to examine RORα/γ function, and our findings offer an excellent starting point for the design of potent and selective ROR ligands with potential application in the treatment of metabolic and immune disorders.

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