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# The benzenesulfoamide T0901317 is a novel RORα/γ Inverse Agonist

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**Running Title:** Characterization of a Synthetic RORα/γ Inverse Agonist

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**Abbreviations:** Retinoic acid receptor-related orphan receptors (ROR), chromatin immunoprecipitation (ChIP), steroid receptor coactivator 2 (SRC2), liver X receptor (LXR), nuclear receptor (NR), glucose 6-phosphatase (*G6Pase***),** cytochrome p450 7B1 (Cyp7B1).

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## ABSTRACT

Retinoic acid receptor-related orphan receptors (RORs) regulate a variety of physiological processing 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 forty-eight human nuclear receptors, the benzenesulfoamide LXR agonist 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 nM and 51 nM, respectively) resulting in 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 the coactivator SRC2 by RORα at an endogenous ROR target gene. Using siRNA, we demonstrate that repression of the gluconeogenic enzyme glucose-6-phosphatase in HepG2 cells by T0901317 is ROR-dependent and 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 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.

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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 LXRα and LXRβ (liver X receptors; 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 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 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 [N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-Benzenesulfonamide] and the tertiary amine GW3965 [3-[3-[[[2-Chloro-3-(trifluoromethyl)phenyl]methyl](2,2 -diphenylethyl)amino]propoxy]benzeneacetic acid hydrochloride], which have been used extensively to help expand our understanding of the physiologic roles of the LXRs (Collins et al., 2002; Schultz et al., 2000). However, recently it has been shown that in addition to potent modulation of the LXRs, T0901317 but not GW3965 is a potent agonist of both the farnesoid X receptor (FXR) and the xenobiotic receptor PXR (Houck et al., 2004; Mitro et al., 2007) bringing into question conclusions drawn from pharmacological studies using this compound.

The promiscuity of T0901317 as well as other nuclear receptor ligands indicates that there are privileged structures (chemotypes) that bind to a range of these receptors. Since it is possible to utilize these promiscuous ligands as points to initiate 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. Recently, our laboratory developed a GAL4 nuclear receptor library containing all 48 human receptors to facilitate selectivity profiling of putative NR modulators that emerge from HTS campaigns at the Scripps Research Molecular Screening Center. In an effort to demonstrate the utility of the NR library, a collection of 65 well characterized NR modulators including the LXR agonist T0901317 was assembled. Interestingly, when this chemical set was tested

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against the GAL4 NR library, it was discovered that in addition to its expected activity, T0901317 was a potent inhibitor of the nuclear receptors RORα and RORγ (retinoid-related orphan receptor-alpha and -gamma; [NR1F1] and [NR1F3]) yet afforded little or no activity on RORβ (retinoid-related orphan receptor-beta; [NR1F2]).

The RORs are orphan nuclear receptors for which the endogenous ligand has yet to be described. As 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 (AF2), is locked in the holo-conformation (Harris et al., 2002) circumventing the need for ligand interaction to transactivate target genes. However, the co-crystal 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., 2004; Kallen et al., 2002).

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 nM and 51 nM, respectively) resulting in 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 the coactivator SRC2 by RORα at an endogenous ROR target gene. Using siRNA, we demonstrate that repression of the gluconeogenic enzyme glucose-6-phosphatase in HepG2 cells by T0901317 is ROR-dependent and not due to the compounds LXR activity.

In summary, T0901317 represents a novel chemical probe to examine RORα/γ function. Also, this compound, with a chemically tractable scaffold, represents an excellent starting point for medicinal chemistry towards 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.

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## Materials and Methods

**Reagents.** T0901317 [N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-Benzenesulfonamide] was purchased from Sigma. 25-hydroxycholesterol was purchased from MP Biomedicals. 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 (L02218, 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) encoding an N-terminal GST-Tag following the manufacturer instructions. The protein was induced with 1 mM IPTG in BL21 gold (DE3) cells (Invitrogen), and purified by affinity chromatography with Protino GST/4B column (Macherey-Nagel) 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, 2mM DTT, and 10% glycerol. For AlphaScreen studies, recombinant His-RORα-LBD protein and Biotin-RIP140 box B peptide containing the LYYML motif were gifts from Eli Lilly and Company. The histidine (Nickel) detection kit was purchased from Perkin Elmer.

**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) 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 hours, optimized compounds (2 µM final concentration) or DMSO were added to the plates and allowed to incubate for 20 hours prior to addition of 40 µl BriteLite (Perkin Elmer, Waltham, MA) to measure luciferase activity. Compounds that attenuate the GAL4-VP16-dependent luciferase

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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 to DMSO only controls. Compounds with mean signals three standard deviations from the DMSO controls were considered hits in this assay.

**Radioligand Receptor Binding Assay.** Forty-five or ninety nanograms 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. Non-specific binding was defined in the absence of protein as well as excess of cold 25-hydroxycholesterol and were shown to be identical. The assays were terminated by rapid filtration through pre-soaked Whatman GF/B filters (0.5% PEI in PBS) in Multiscreen plates (Millipore) and were washed (3 x 0.1 ml) with ice-cold assay buffer. The radioligand binding results were analyzed using GraphPad Prism software. For the competition assay, various concentration of T0901317 were incubated with receptor in the presence of 3 nM of [$^3$H]-25-hydroxycholesterol.

**AlphaScreen.** The assays were performed in triplicate in white opaque 384-well plates (Perkin Elmer). The final volume was 20 µL for generation of compound dose-response curves (0.02 uM to 8 uM). All dilutions were made in assay buffer (100 mM NaCl, 25 mM Hepes, 0.1% BSA, pH 7.4). The final DMSO concentration was 0.25%. A mix of 12 uL of His-RORα-LBD (75 nM), beads (30 µg/mL of each), and 4 µL of increasing concentrations of compound (0.02 µM to 8 µM) were added to the wells, the plates were sealed and incubated for 1h at room temperature in the dark. After this pre-incubation step, 4 µL of Biotin-RIP140B (25 nM) was added, the plates were sealed and further incubated for 2h at room temperature in the dark. The plates were read on PerkinElmer Envision 2104 and data analyzed using GraphPad Prism software (La Jolla, CA).

**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 $1x10^6$ cells in 6 cm

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plates, 3μg 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. Following 24 hour bulk transfection, cells from different transfection conditions were counted and plated in 384 well plates at a density of 10,000 cells/well. Following additional 24 hour incubation, luciferase levels were assayed by one-step addition of 20μL BriteLite (Perkin Elmer) and read using an Envision multilabel plate reader (Perkin Elmer). Data was normalized to luciferase signal from UAS luciferase reporter/pBind control empty vector and displayed as 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 RORE mutant were used to transfect HEK293T cells with SRC-2 as co-activator in the presence or absence of full length RORα and were treated as defined in figure legends (Chopra et al., 2008). Similarly, for *Cyp7B1*, the wild type or RORE mutant promoters were 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) and have been previously described (Wada et al., 2008).

*RORα modulation of IL-17 reporter in* - HEK293 cells were grown in 96 well plates ($1 \times 10^6$/well) and were transiently transfected using Lipofectamine (Invitrogen) following the manufacturer's protocol. Cells were transfected with a total of 200*n*g of DNA/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*n*g/well) (Addgene, Cambridge, MA), an -actin promoter *Renilla* luciferase reporter (50*n*g/well), and either control vector alone or the test DNA (full-length RORα or full length RORγ at 50*n*g/well). The IL-17 reporters have been previously described (Zhang et al., 2008) and were obtained from Addgene (Cambridge, MA). Cells were treated with T0901319 for 24 hours and then lysed and read using the Dual-Glo

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Luciferase assays system (Promega) 48 hours after transfection. These results were normalized (FireFly:Renilla ratio).

***Reduction of Endogenous Gene Expression by Small Interference RNAs.*** To reduce endogenous ROR expression, HepG2 cells were seeded into a 12-well plate (2.5 x $10^5$/well) and transfected the next day with small interference RNAs (siRNAs) against human RORα (#L-003440-00-0005) and RORγ (#L-003442-00-0005; Dharmacon RNA Technologies, Lafayette, CO) at 50 nM following the instructions for Dharma-FECT 1 transfection reagent. Forty-two hours post transfection, cells were treated with vehicle (DMSO) or T0901319 (10 μM) for 6 hours. Cells were harvested and total RNA was isolated. Quantitative RT-PCR was performed to analyze mRNA levels of human RORα, RORγ, GAPDH, and *G6Pase* using SYBR Green technology. The primers used for quantitative PCR analysis are: human RORα, GTAGAAACCGCTGCCAACA (Forward) and ATCACCTCCCGCTGCTT (Reverse); human RORγ CCCCTGACCGATGTGGACT (Forward) and CAGGATGCTTTGGCGATGA (Reverse); human *G6Pase* TCATCTTGGTGTCCGTGATCG (Forward) and TTTATCAGGGGCACGGAAGTG (Reverse); GAPDH TGCACCACCAACTGCTTAGC (Forward) and GGCATGGACTGTGGTCATGAG (Reverse).

**ChIP/re-ChIP**. HepG2 cells were infected with Flag-RORα adenovirus for 24 hours and then treated with vehicle (DMSO) or T1317 (10 μM) for another 24 hours. Re-ChIP assays were performed by using the kit from Active Motif®. Anti-FLAG (Sigma) antibody was used to do the first immunoprecipitation for all the samples. The second immunoprecipitaion was performed by using anti-mouse IgG (Millipore), anti- RNA Pol II (Millipore) or anti-SRC2 (Bethyl Lab). The *G6Pase* primers used in PCR were CCCTGAACATGTTTGCATCA (forward) and CATTCCTTCCTCCATCCTCA (Reverse).

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## Results

Using a cell-based GAL4-NR LBD co-transfection assay we found that T0901317 (2 μM) was a potent repressor of both GAL4-RORα and GAL4-RORγ (**Figure 1**). Interestingly, T0901317 inhibited the constitutive transactivation activity of both GAL4-RORα and GAL4-RORγ with little or no activity on GAL4-RORβ (**Figure 1**). We observed that 1 μM T0901317 repressed RORα by almost 70% and approximately 90% at 10 μM (see **Figure 1** insert). 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 non-specific luciferase effects or cellular toxicity (data not shown). As illustrated in **Figure 2A 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) as well as estimated $IC_{50}$s of 2.0 μM (RORα) and 1.7 μM (RORγ), respectively.

In order to determine if 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±0.89 nM and 5.1±0.71 respectively as determined from a saturation binding curve (**Figure 3A and 3C**). More importantly, T0901317 dose-dependently competed with [$^3$H]-25-hydroxycholesterol for RORα and RORγ binding with an approximate $IC_{50}$ of 254 nM and 81 nM ($K_i$ 132 nM and 51 nM respectively) (**Figure 3B and 3D**).

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 NRIP1 (Nuclear receptor interacting

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protein 1), is a nuclear protein that has been shown to specifically interact with the AF2 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 His tagged LBD of RORα was monitored in response to increasing concentrations of T0901317. As shown in **Figure 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 co-transfection studies, full length RIP140 effectively represses the transactivation activity of Gal4RORα on the UAS luciferase reporter (**Figure 4B**).

Recently, it has been shown that *G6Pase* gene expression is regulated by RORα along with the co-activator SRC2 (Chopra et al., 2008). Therefore, we examined the ability of T0901317 to modulate a *G6Pase* reporter in a RORα-dependent fashion in the presence of the co-activator SRC2. As expected, co-transfection of RORα strongly stimulated the *G6Pase* reporter gene and as shown in **Figure 5A**, T0901317 dose-dependently repressed the *G6Pase* promoter activity with approximately 31% repression at 10μM. In the absence of RORα or 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. Similarly, we analyzed yet another RORα responsive gene, the cytochrome p450 7B1 *(Cyp7B1)* (Wada et al., 2008). Again co-transfection 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 10μM (**Figure 5B**). The repressive effect

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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).

In order to examine the RORα/γ component of T0901317 pharmacology, we identified a target gene that is regulated by RORα 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 **Figure 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 previously reported 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 if the effects of T091317 on *G6Pase* are ROR-dependent. Therefore we monitored mRNA levels of *G6Pase* before and after knockdown of endogenous RORα and RORg by siRNA. As shown in **Figure 7A**, expression of endogenous *G6Pase* was reduced in HepG2 cells by treatment with T091317 (10 μM). Transfection of these cells with a non-targeting siRNA did not interfere with the ability of T091317 to reduce expression of *G6Pase*. Transfection with the non-targeting siRNA did not have an effect on expression levels of either RORα or RORγ (data not shown). Treatment of HepG2 cells with siRNAs targeting RORα and RORγ reduced expression of both receptors by more than 60% while having 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 (**Figure 7A**). More importantly, the ability of T0901317 to repress G6Pase expression in HepG2 cells was lost when the RORs were

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knocked down (**Figure 7A**). These results demonstrate that T091317 modulation of G6Pase is ROR-dependent and not related to the compounds LXR activity as previously suggested.

Finally, the ability of T0901317 to modulate RORα recruitment of the p160 coactivator SRC-2 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 (**Figure 7B**); however, in the reChIP using the SRC-2 antibody substantial decrease in the amount of SRC-2 occupancy was noted in the presence of the T0901317. These results demonstrate that T0901317 decreases the ability of RORα to recruit SRC-2 coactivator to the *G6Pase* promoter and thus decreases the expression of the gene.

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**Discussion**

The first ROR (RORα) was discovered in the early 1990s based on sequence similarities to the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), 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 as it is primarily expressed in the immune system. This isoform has garnered much attention lately due to 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 development of synthetic molecules that would be ROR isoform selective modulators.

The three RORs display distinct patterns of expression suggesting non-redundant 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), while RORβ expression is restricted to the central nervous system (Andre et al., 1998a; Andre et al., 1998b). RORγ is highly expressed in the thymus, however, significant expression is also found in the liver, skeletal muscle, adipose tissue and kidney (Medvedev et al., 1996). All RORs recognize and bind to specific sequences of DNA termed ROR response elements (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 regulation of metabolic pathways was revealed by studies in the *staggerer* ($ROR\alpha^{sg/sg}$) mouse. This natural mutant mouse strain carries an intragenic

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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), HDL, and apoA1 (the major apolipoprotein in HDL) (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).

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 a RORα/γ double knock-out. Although neither individual strain showed significant alterations in plasma glucose levels, the double knock-out 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 regulation of glucose metabolism was characterized when Chopra et al. found that loss of the p160 family coactivator SRC-2 (steroid receptor coactivator-2) 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 (Chopra et al., 2008). Loss of expression of the enzyme glucose-6-phosphatase (*G6Pase*) is responsible for 80% of the diagnosed von Gierke's disease cases. Importantly, SRC-2 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 development of atherosclerosis (Jetten, 2009). This receptor also is critical for normal function of the mammalian clock and is involved in modulation of immune function (Jetten, 2009; Yang et al., 2008). The most prominent

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role for RORγ is regulation of immune function, especially in development of the Th17 cells that are believed to play an important role in autoimmunity (Ivanov et al., 2007). RORγ also helps coordinate lipid and glucose metabolism in concert with RORα (Jetten, 2009; Kang et al., 2007). 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 towards 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 activation of LXRα and LXRβ (Michael et al., 2005). However, we previously described that this compound also activates FXR (Houck et al., 2004) and it was later reported that it also activated PXR (Mitro et al., 2007). This degree of receptor promiscuity that provided us a critical advantage for identification of a synthetic ligand for an orphan nuclear receptor creates difficulties for 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 are 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. Our results presented here demonstrate that the T0901317 effects on repression of G6Pase are in fact ROR-dependent and not a result of the compounds LXR activity.

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## Conclusion

Retinoic acid receptor-related orphan receptors (RORs) regulate a variety of physiological processing 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 where T0901317 repressed RORα/γ-dependent transactivation of reporter genes driven by the ROR responsive promoters from the *glucose-6-phosphatase* (*G6Pase*) and *cytochrome p450 7b1* (*Cyp7b1*) genes. Moreover, repression of G6Pase by T0901317 was relieved following knockdown of both RORs concluding that this compounds effects on this gluconeogenic enzyme are ROR-dependent. Finally, we show that T0901317 reduces recruitment of the p160 coactivator SRC-2 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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**FOOTNOTES**

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## FIGURE LEGENDS

**Figure 1.** *Gal4 Nuclear Receptor Profiling of T0901317*. RORα and RORγ transactivation are 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 hours, the LXRα agonist T0901317 (2 μM final concentration) or DMSO was added and incubated for 20 hours. The luciferase activity of each construct was measured and normalized to the mock (vector alone), then the fold change in signal compared to DMSO is calculated (n=6). Inset shows HEK293T transfected cells were separately treated with either 1 or 10 μM T0901317 or vehicle for 20 hr followed by luciferase activity measurement (data shown is mean ±SEM, n = 6). Horizontal dashed lines represent +/- 5 standard deviations of the control (mock transfected).

**Figure 2.** *Dose response curves for trans-activation 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 and were treated with various concentration of T0901317 for 20 hr followed by luciferase activity measurement. Relative change was determined by normalizing to control vector treated with vehicle. Each data point was performed in 8 replicates and represented as mean ±SEM, n = 8.

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

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**Figure 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 of AlphaScreen beads for 1h at room temperature in the dark as described in 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 2h at room temperature. Representative data are shown as mean ± S.D. of 3 independent experiments. B) Cell-based luciferase reporter assays measuring effects of RIP140 on RORα dependent transactivation. HEK293T cells were reverse transfected with 3μg of total DNA comprised of either UAS luciferase reporter only, Gal4RORα and UAS luciferase reporter or Gal4RORα, UAS luciferase reporter and full-length RIP140. Following bulk transfections, cells were re-plated and assays conducted in 384 well format with n=6. Data was 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.

**Figure 5**. *Modulation of RORα mediated Glucose-6-Phosphatase (G6Pase) promoter activity and CYP7B1 promoter activity by T0901317.* 293T cells were co-transfected with pS6 control plasmid or pS6 containing full length RORα along with *G6Pase* promoter (A) or *CYP7B1* promoter (B) as detailed in Material and Methods. SRC-2 as a coactivator was also co-transfected 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 represented as mean ±SEM, n=8.

**Figure 6**. *T0901317 Suppresses RORα and RORγ mediated IL-17 transcription.* HEK293 cells were transiently transfected with the IL-17-dependent reporter construct, *renilla* 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 post-

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treatment, IL-17 activity was determined by dual luciferase assay. The data are normalized to the vehicle (DMSO) treated cells.

**Figure 7.** *Modulation of endogenous G6Pase mRNA expression and endogenous Glucose-6-Phosphatase (G6Pase) promoter activity by T0901317 in HepG2 cells.* (A) Expression of endogenous *G6Pase* in HepG2 cells was reduced by treatement with siRNA against both human RORα and RORγ. siRNA oligos against RORs were transfected into HepG2 cells at 50nM. Forty-two hours post transfection cells were treated for 6 h with either vehicle (DMSO) or T091317 (10 μM). Total RNA was prepared from these cells and subjected to RT-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 SRC-2 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 immuneprecipitation was performed using α-SRC-2 antibody. Mouse IgG was used as a negative control and anti-RNA pol II antibody was used as a positive control.

Figure 1

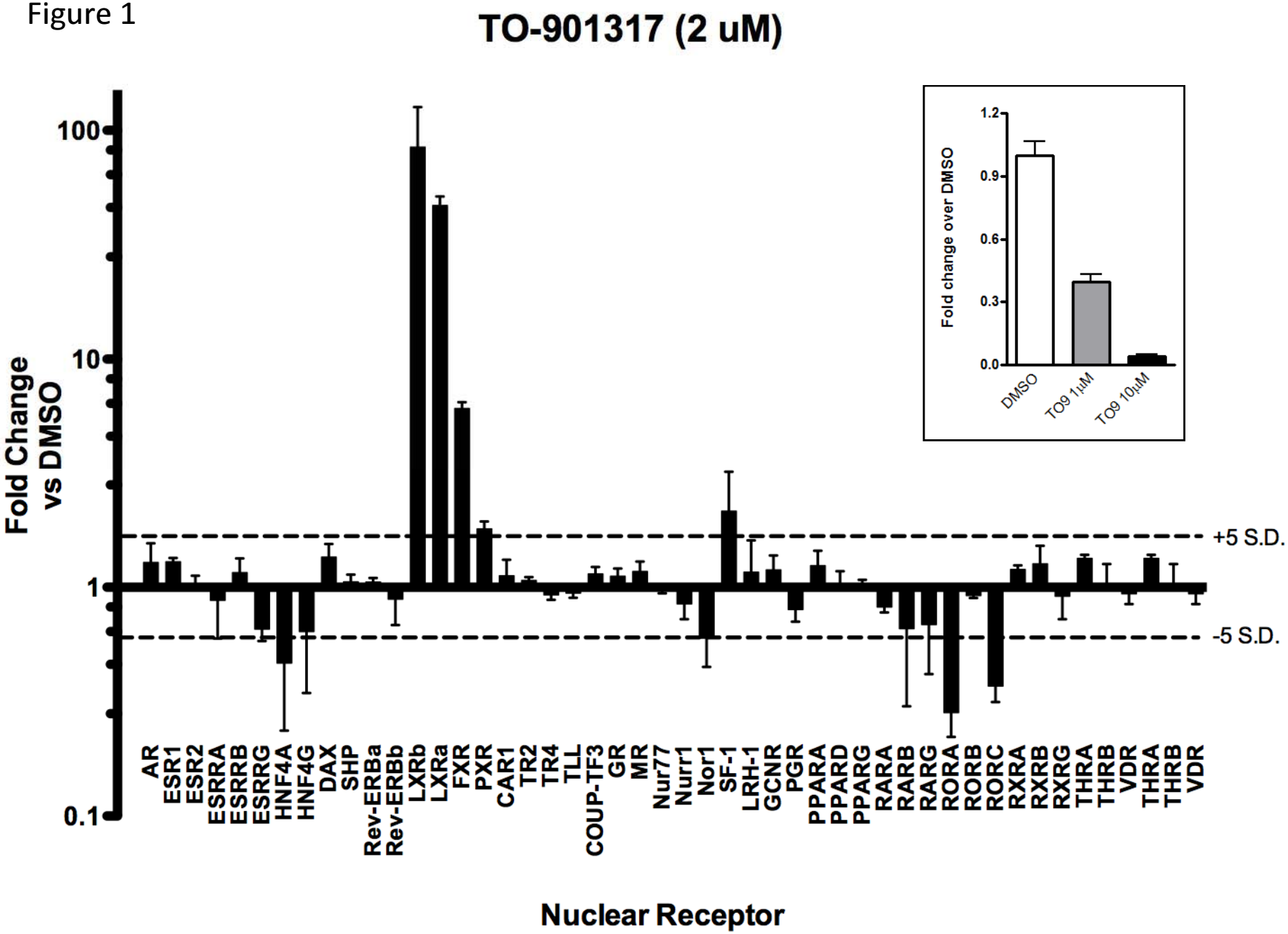
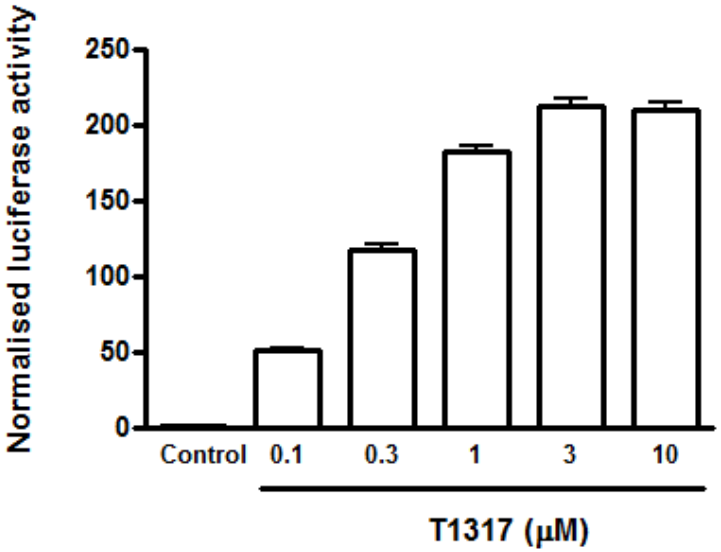


Figure 2

A)

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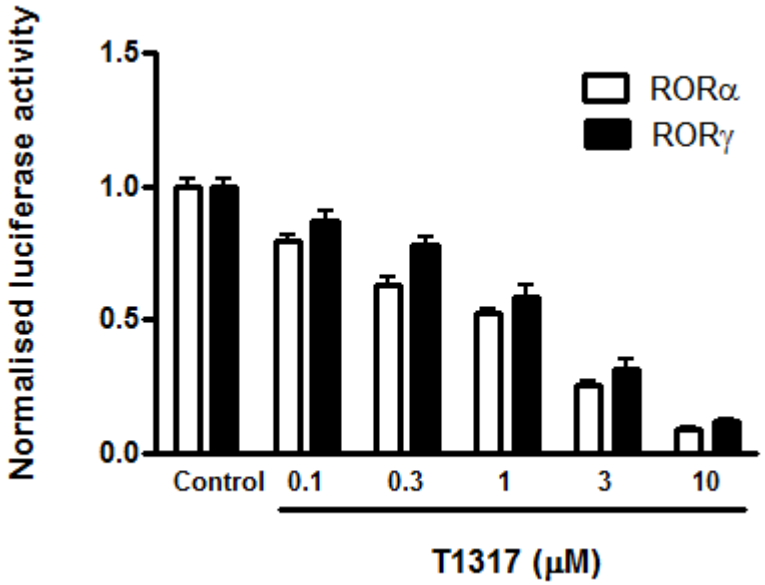


Figure 3

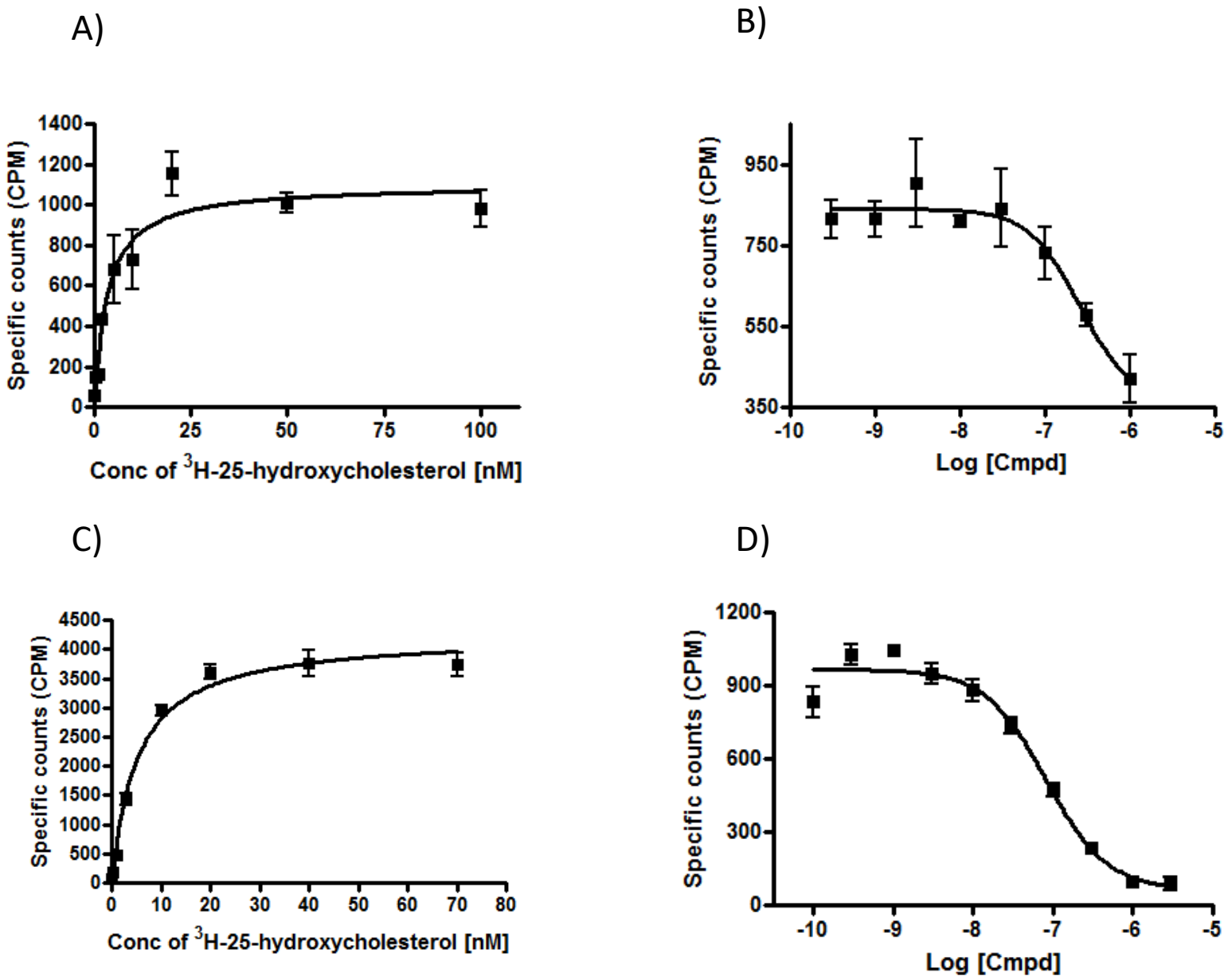
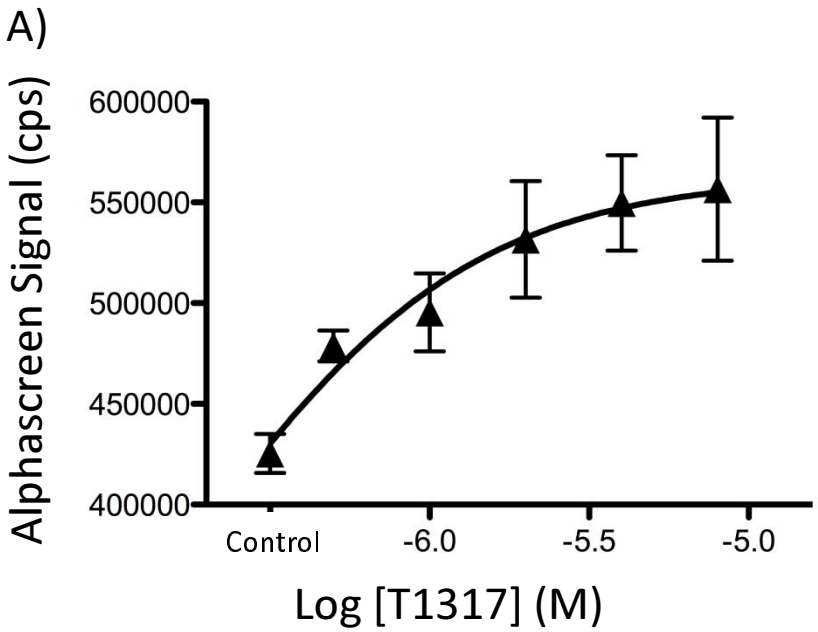


Figure 4



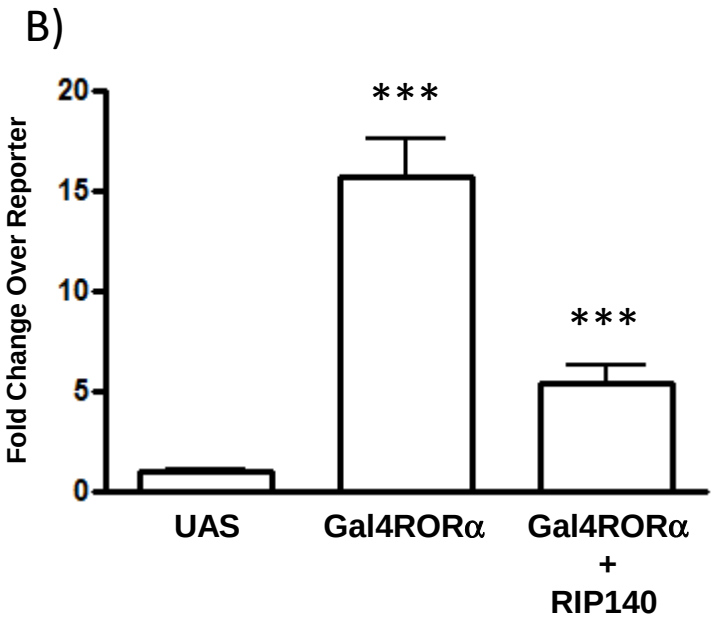



Figure 5

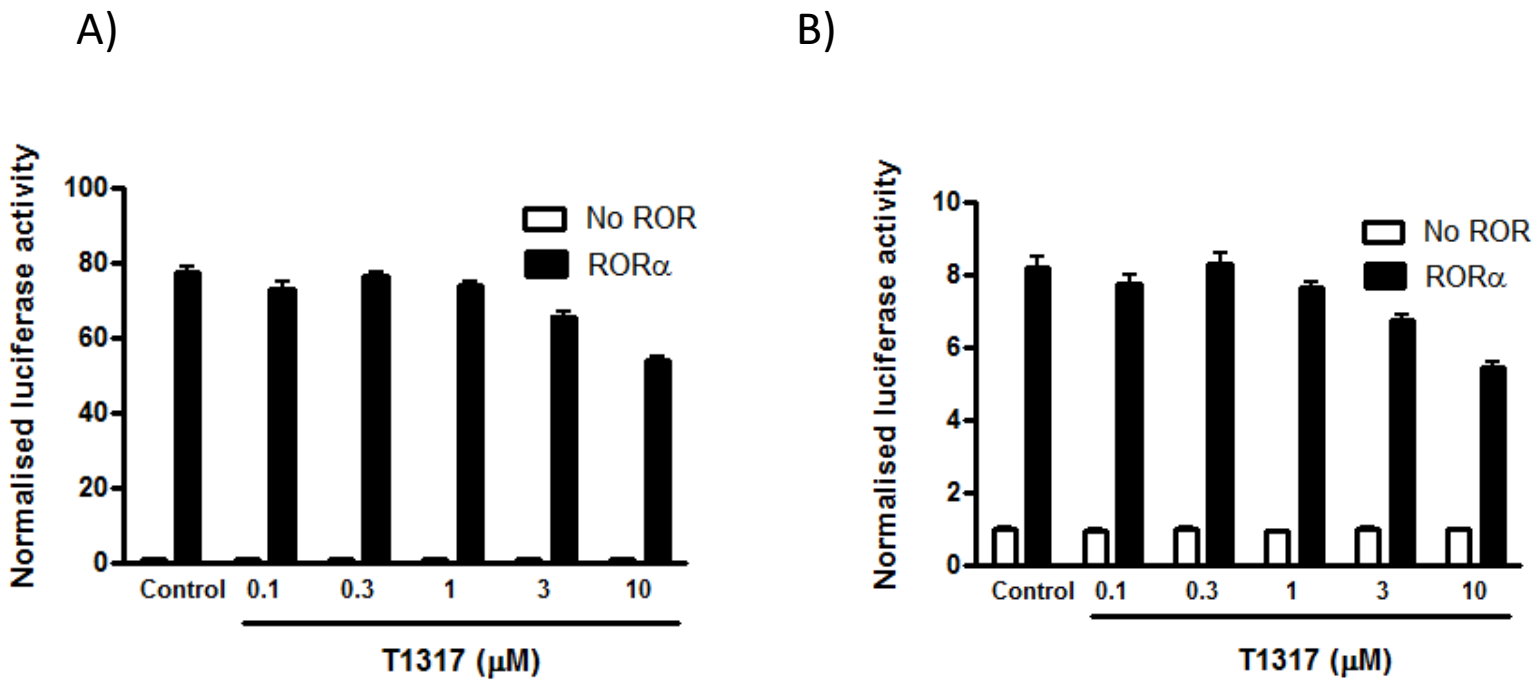


Figure 6

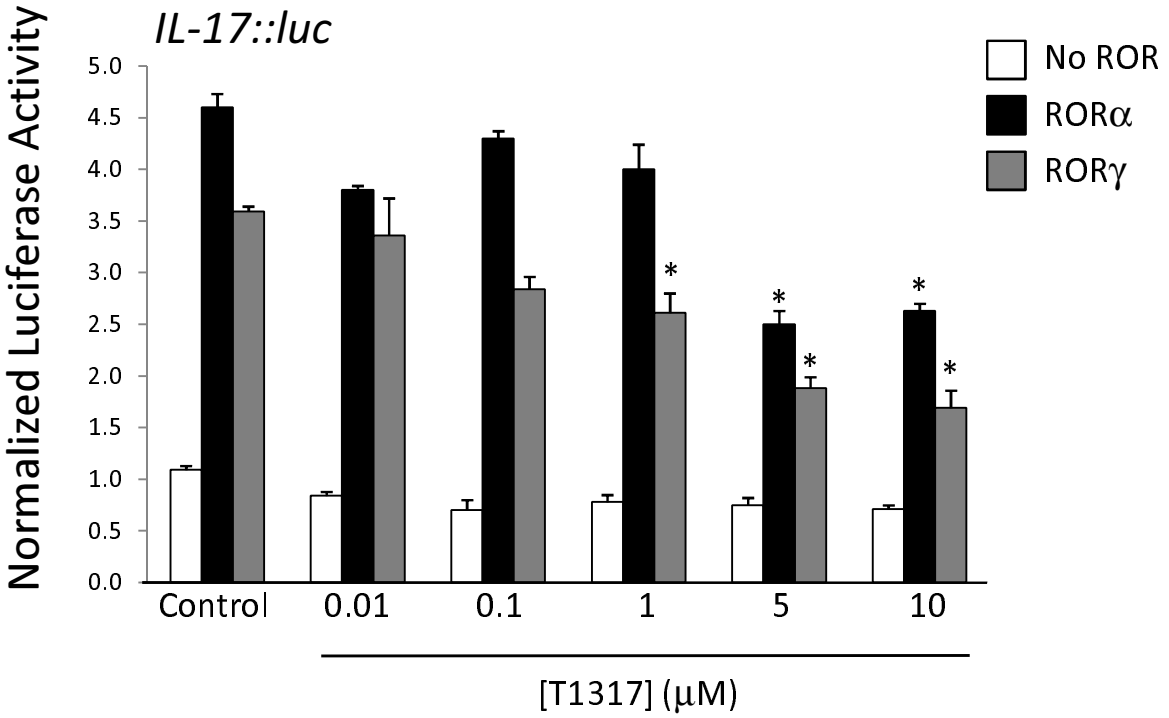


Figure 7