Published in final edited form as:
ACS Chem Biol. 2010 November 19; 5(11): 1029–1034. doi:10.1021/cb100223d.

Identification of a Synthetic Agonist for the Orphan Nuclear Receptors RORα and RORγ, SR1078

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Abstract

The retinoic acid receptor-related receptors (RORs) are members of the nuclear receptor (NR) superfamily of transcription factors. Several NRs are still characterized as orphan receptors since ligands have not yet been identified for these proteins. Here, we describe the identification of a synthetic RORα/RORγ ligand, SR1078. SR1078 modulates the conformation of RORγ in a biochemical assay and activates RORα and RORγ driven transcription. Furthermore, SR1078 stimulates expression of endogenous ROR target genes in HepG2 cells that express both RORα and RORγ. Pharmacokinetic studies indicate that SR1078 displays reasonable exposure following injection into mice and consistent with SR1078 functioning as a RORα/RORγ agonist, expression of two ROR target genes, *glucose-6-phosphatase* and *fibroblast growth factor 21*, were stimulated in the liver. Thus, we have identified the first synthetic RORα/γ agonist and this compound can be utilized as a chemical tool to probe the function of these receptors both *in vitro* and *in vivo*.

Members of the nuclear receptor (NR) superfamily display a conserved domain structure with highly conserved DNA-binding and ligand-binding domains. Members of this family include the receptors for the steroid hormone, thyroid hormone as well as for bile acids and oxysterols. Although many of the 48 NRs found in the human are characterized as ligand activated transcription factors, a significant number of these proteins still have uncharacterized ligands. The retinoic acid receptor-related orphan receptors α and γ (RORα and RORγ) are two of these orphan receptors that have been demonstrated to play important roles in regulation of metabolism and immune function (1,2).

Cholesterol and cholesterol sulfate have been suggested to be natural ligands for RORα (3,4) and our recent work identified various oxysterols that bind to both RORα and RORγ with high affinity and regulate their activity (5,6). There is some controversy as to the nature of the constitutive activity of the RORs observed in cell-based assays. Our data indicates that RORs display the constitutive activity in biochemical assays under conditions where the receptor would be expected to have no endogenous ligand present (denatured and refolded receptor) (5), but others have suggested that endogenous oxysterol ligands may copurifiy leading to the observed constitutive activity (7). Although the physiological significance of these natural ligands for the RORs is unclear, the potential utility of synthetic ligands that modulate the activity of these receptors is apparent. For example, loss of RORα in the staggerer mice results in mice resistant to weight gain and hepatic steatosis when placed on a high fat diet (8). RORγ has been shown to be involved in development of Th17 cells that are implicated in autoimmune diseases and loss of RORγ yields animals that are resistant to development of these diseases (9,10). RORα has been shown to be required for normal bone

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development and staggerer mice lacking functional RORα are osteopenic (11) suggesting that RORα agonists may have utility in the treatment of osteoporosis.

We recently identified the first synthetic ligand that binds to and modulates the activity of RORα and RORγ, T0901317 (T1317) (Fig. 1A) (12). T1317 was originally identified as a liver X receptor agonist (LXR), an NR that serves as a physiological receptor for oxysterols (13). We later showed that T1317 showed a degree of promiscuity and also activated another NR that serves as a receptor for bile acids, FXR (14). In an NR specificity screen examining the activity of T1317 at all 48 NRs, we noted that T1317 displayed inverse agonist activity on RORα and RORγ (12). Since both RORs and LXRs bind to oxysterols with high affinity it is not unexpected that they may also display cross-reactivity with regard to synthetic ligands; however, our data indicate that T1317 serves as a very efficacious agonist of LXR while performing as an inverse agonist on RORs. Clearly, T1317 does not display the appropriate pharmacological profile to be used as a chemical tool to probe ROR function since it displays significant activity on LXR and FXR. However, we believed that the benzenesulfonamide scaffold to be a suitable initiation point for development of the first selective ROR ligands.

Using the T1317 as an initial scaffold, we synthesized an array of compounds and assessed their activity at RORα, RORγ, FXR, LXRα, and LXRβ. One compound of particular interest was the amide SR1078 (Fig. 1A) that displayed a unique pharmacological profile indicating a high potential to be used as a chemical probe for assessment of ROR function. The synthetic scheme for SR1078 is shown in Fig. 1B (15). This compounds was initially identified based on its ability to inhibit the constitutive activity of RORα/γ. In a biochemical coactivator interaction assay using Alpha screen technology, increasing doses of SR1078 resulted in a concentration dependent reduction in the ability of RORγ to recruit the TRAP220 coactivator LXXLL NR box (Fig. 1B). In a cell-based chimeric receptor Gal4 DNA-binding domain – NR ligand binding domain cotransfection assay, SR1078 significantly inhibited the constitutive transactivation activity of RORα and RORγ, but had no effect on the activity of FXR, LXRα and LXRβ (Fig. 1C). These data clearly demonstrate that we developed a compound that selectively targeted RORα and RORγ and no longer functioned as a LXR/FXR agonist.

In order to examine the activity of SR1078 in more detail, we performed additional cotransfection assays where we transfected cells with full-length RORα or RORγ and luciferase reporter genes driven by promoters derived from known ROR target genes. Two distinct reporter constructs were utilized: one driven by the *glucose-6-phosphatase* (*G6Pase*) promoter and one driven by the *fibroblast growth factor-21* (*FGF21*) promoter. Both of these genes have been shown to be direct target genes of ROR and have characterized ROR response elements within their promoters (5,16,17). Unexpectedly, we noted that SR1078 functioned as a ROR agonist, not inverse agonist, when used in the context of the full-length receptors. As shown in Fig. 2A, in a RORα cotransfection assay, treatment of cells with SR1078 resulted in a significant increase in transcription. Similarly, in the RORγ cotransfection assay, SR1078 treatment resulted in a stimulation of RORγ-dependent transcription activity (Fig. 2B). In both cases, these effects were clearly mediated by ROR since the effect was lost when the RORE was mutated in the *G6Pase* promoter. Consistent with the *G6Pase* data, when the *FGF21* promoter was used in the cotransfection assay, SR1078 behaved as a RORα/γ agonist stimulating ROR activity (Fig. 2C). With both RORα and RORγ using either promoter, we noted that the effects of SR1078 were dose-dependent as shown in Figs 3A-3D. Transcription was stimulated at concentrations of 2 to 5 μM and above. The lack of correlation between recruitment of a cofactor peptide and agonist vs. antagonist activity in cell-based assays has been observed previously with REV-ERBα

where the natural ligand heme causes displacement of the cofactor peptide but the ligand acts like an agonist in cells (18,19).

In order to confirm that SR1078 is indeed an agonist in a more "physiological" context, we tested its activity in an assay that detects its effect on the expression of actual target genes in a target cell line expressing endogenous levels of RORα and RORγ. HepG2 cells were treated with SR1078 for 24h followed by assessment of *G6Pase* and *FGF21* gene expression. As shown in Fig. 4A, SR1078 treatment resulted in a significant 3-fold increase in *FGF21* mRNA expression. *G6Pase* mRNA expression was also significantly stimulated ~2-fold by SR1078 treatment (Fig. 4B). These data support the results from the cotransfection data indicating that SR1078 functions as a ROR agonist unlike its precursor T1317 which functions as an inverse agonist is these same assays.

We examined the pharmacokinetic properties of SR1078 in mice and noted significant *in vivo* exposure. Plasma concentrations reached 3.6 μM 1h after a 10 mg/kg i.p. injection of SR1078 and sustained levels of above 800 nM even 8h after the single injection (Fig. 4C). These levels were sufficient to perform a proof-of-principle experiment to determine if SR1078 treatment would stimulate ROR target gene expression in an animal model. Mice were treated with SR1078 (10 mg/kg i.p.) and 2h after the injection the livers were harvested and mRNA purified for assessment of *G6Pase* and *FGF21* gene expression. As shown in Fig. 4D & 4F the expression of both *FGF21* and *G6Pase* was significantly stimulated by SR1078 treatment vs. vehicle control.

In summary, we report the identification of a synthetic ligand for RORα and RORγ that functions as an agonist in the context of the full-length receptors. Thus, SR1078 represents the first synthetic ligand that is able to function as an ROR agonist. In cotransfection assays, SR1078 activates the transcription driven by ROR target gene promoters in a RORE-dependent manner. Furthermore, treatment of cells that express RORα and RORγ endogenously with SR1078 results in stimulation of expression of ROR target genes. It is worth noting that this compound activates the receptor beyond the level of its constitutive activity that is normally observed. The fact that the initial lead compound, T1317, functions as a RORα/γ inverse agonist and SR1078 functions as a RORα/γ agonist indicates that it is possible to develop synthetic ligands that will either suppress the constitutive activity of the receptors or further activate the receptors. Our work leading to the identification of SR1078 also demonstrates that it is possible to develop ROR selective synthetic ligands that lack activity at related receptors such as LXR and FXR. This degree of promiscuity that is displayed by T1317 limits the ability to utilize this particular compound as a chemical tool to probe the function of the RORs. Additionally, we show that SR1078 displays pharmacokinetic properties that allow it to be used in vivo and as would be expected for a RORα/γ agonist, administration of this compound to mice results in an increase in expression of ROR target genes in the liver. This proof-of-principle study demonstrates that additional experiments are warranted to examine the pharmacological profile of this compound *in vivo*.

Methods

Synthesis and Physical Characterization of SR1078

Under argon, to a solution of 4-(1-Hydroxy-1-trifluoromethyl-2,2,2-trifluoroethyl)aniline (1.5M in THF, 128 μL, 0.193 mmol) in CH2Cl2 (275 μL) were successively added at room temperature N,N-Diisopropylethylamine (37 uL, 0.212 mmol) and 4-(Trifluoromethyl)benzoyl chloride (30 μL, 0.193 mmol). The mixture was stirred for 8 hours and concentrated under reduce pressure. The crude residue was directly purified by column

chromatography on silica gel without any work-up by Hexane/AcOEt (8/2) to obtain 59 mg (71%) of SR1078 as a white powder.

FTIR—3404, 3214, 1671, 1602, 1529, 1417, 1322, 1272, 1206, 1190, 1176, 1138, 1117, 1065, 1016, 973, 964, 948, 902, 857, 830, 765, 752, 737, 704, 692 cm^{-1}.

1H NMR (400 MHz, $(CD_3)_2SO$)—7.68 (d, *J* = 8.2 Hz, 2H), 7.89-7.96 (m, 4H), 8.16 (d, *J* = 8.2 Hz, 2H), 8.66 (s, 1H), 10.67 (s, 1H).

^{13}C NMR (100 MHz, $(CD_3)_2SO$)—120.12 (2C), 125.4 (q, *J* = 4.0 Hz, 2C), 125.8, 127.3 (2C), 128.7 (2C), 131.5 (q, *J* = 31.9 Hz, 1C), 138.4, 140.4, 164.7. There are four carbons missing for the description of SR1078. They correspond to the three carbons of the (1-Hydroxy-1-trifluoromethyl-2,2,2-trifluoroethyl) moiety and the (trifluoromethyl)benzene. The fluorine coupling with these carbons give multiplets that are really difficult to see on the ^{13}C spectrum even with a prolonged number of scans.

MS (ES-) m/z = 430 (found for $C_{17}H_{10}F_9NO_2$-H^+).

Mp = 169°C.

Cell Culture and Cotransfections

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37 °C under 5% CO2. HepG2 cells were maintained and routinely propagated in minimum essential medium supplemented with 10% (v/v) fetal bovine serum at 37 °C under 5% CO2. In experiments where lipids and sterols were depleted, cells were maintained on charcoal treated serum (10% (v/v) fetal bovine serum) and treated with 7.5 μM lovastatin and 100 μM mevalonic acid. 24 h prior to transfection, HepG2 or HEK293 cells were plated in 96-well plates at a density of 15×10^3 cells/well. Transfections were performed using LipofectamineTM 2000 (Invitrogen). 16 h post-transfection, the cells were treated with vehicle or compound. 24 h post-treatment, the luciferase activity was measured using the Dual-GloTM luciferase assay system (Promega). The values indicated represent the means ± S.E. from four independently transfected wells. The experiments were repeated at least three times. The ROR and reporter constructs have been previously described (5,12).

cDNA Synthesis and Quantitative PCR

Total RNA extraction and cDNA synthesis as well as the QPCR were performed as previously described (18,20).

Coactivator Interaction Assay

The ALPHA screen assays were performed as previously described(12,21-23). Assays were performed in triplicate in white opaque 384-well plates (Greiner Bio-One) under green light conditions (<100 lux) at room temperature. The final assay volume was 20 μL . All dilutions were made in assay buffer (100 mM NaCl, 25 mM Hepes, 0.1% (w/v) BSA, pH 7.4). The final DMSO concentration was 0.25% (v/v). A mix of 12 μL of GST-RORγ-LBD (10 nM), beads (12.5 μg/ml of each donor and acceptor), and 4 μL of increasing concentrations (210 nM – 50 μM) of compound SR1078 were added to the wells, the plates were sealed and incubated for 1h. After this preincubation step, 4 μL of Biotin-TRAP220-2 peptide (50 nM) was added, the plates were sealed and further incubated for 2h. The plates were read on PerkinElmer Envision 2104 and data analyzed using GraphPad Prism software (GraphPad software).

Animal Studies

Plasma levels of SR1078 were evaluated in C57BL6 mice (n = 3 per time point) administered by i.p. injection. After 1, 2, 4, and 8h blood was taken. In the 2h time point liver was taken for target gene analysis. Plasma was generated using standard centrifugation techniques, and the plasma and tissues were frozen at −80°C. Plasma and tissues were mixed with acetonitrile (1:5 (v/v) or 1:5 (w/v), respectively), sonicated with a probe tip sonicator, and analyzed for drug levels by liquid chromatography/tandem mass spectrometry. All the procedures were conducted in the Scripps vivarium, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and were approved by the Scripps Florida Institutional Animal Care and Use Committee.

Acknowledgments

The efforts of P.R.G. and W.R.R. were supported by the National Institutes of Health (NIH) Molecular Library Screening Center Network (MLSCN) grant U54MH074404 (Hugh Rosen, Principal Investigator). This work was also supported by NIH grants DK080201 (T.P.B.) and GM084041 (P.R.G).

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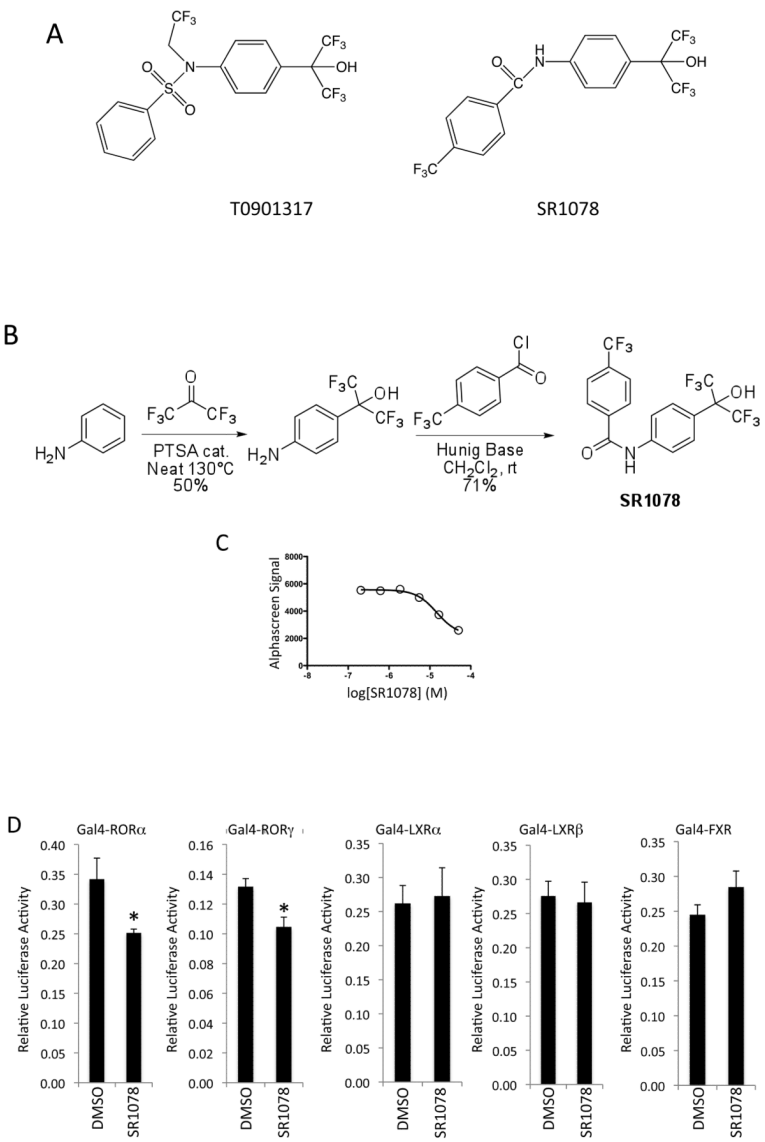

Figure 1.

Identification of a selective RORα/γ synthetic ligand, SR1078. A) Comparison of the chemical structure of T0901317 (T1317) to SR1078. B) Biochemical coactivator interaction assay examining the ability of the RORγ LBD to interact with the LXXLL domain peptide derived from the TRAP220 coactivator protein. ALPHA Screen technology was used for this assay. Increasing levels of SR1078 result in a conformational change that results in a dose-dependent decrease in recruitment of the peptide. C) Cotransfection assays in HEK293 cells demonstrate RORα/RORγ selectivity. Gal4 DBD-NR LBD chimeric receptors were transfected into cells along with a luciferase reporter responsive to Gal4. RORα, RORγ, LXRα, LXRβ and FXR chimeric receptors were examined. SR1078 (10 μM) resulted in reduced activity of RORα and RORγ, but had no effect on LXRα, LXRβ or FXR activity. *, indicates $p<0.05$.

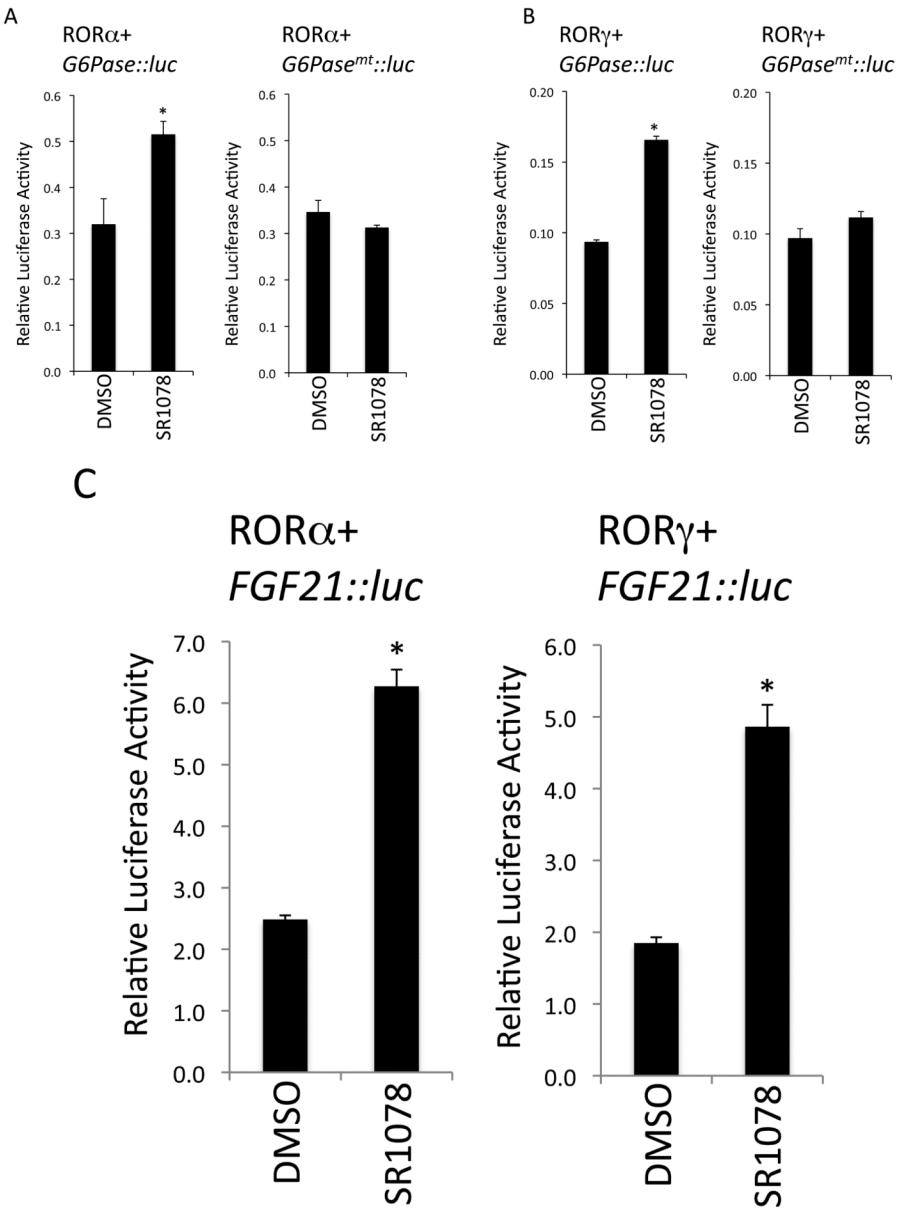

Figure 2.

SR1078 is a RORα/γ Agonist. A) Cotransfection of HEK293 cells with RORα and a reporter consisting of the *G6Pase* promoter upstream of a luciferase reporter gene. Addition of 10 μM SR1078 results in stimulation of transcription. This effect is dependent on the RORE since no activity was noted in a reporter that is identical except for the deletion of the RORE (*G6Pase*^{mt}*::luc*). B) Cotransfection of HEK293 cells with RORγ and a reporter consisting of the *G6Pase* promoter upstream of a luciferase reporter gene. Addition of 10 μM SR1078 results in stimulation of transcription. This effect is dependent on the RORE since no activity was noted in a reporter that is identical except for the deletion of the RORE (*G6Pase*^{mt}*::luc*). C) Cotransfection of HEK293 cells with RORα or RORγ and a reporter consisting of the *FGF21* promoter upstream of a luciferase reporter gene. Addition of 10 μM SR1078 results in stimulation of transcription. *, indicates $p<0.05$.

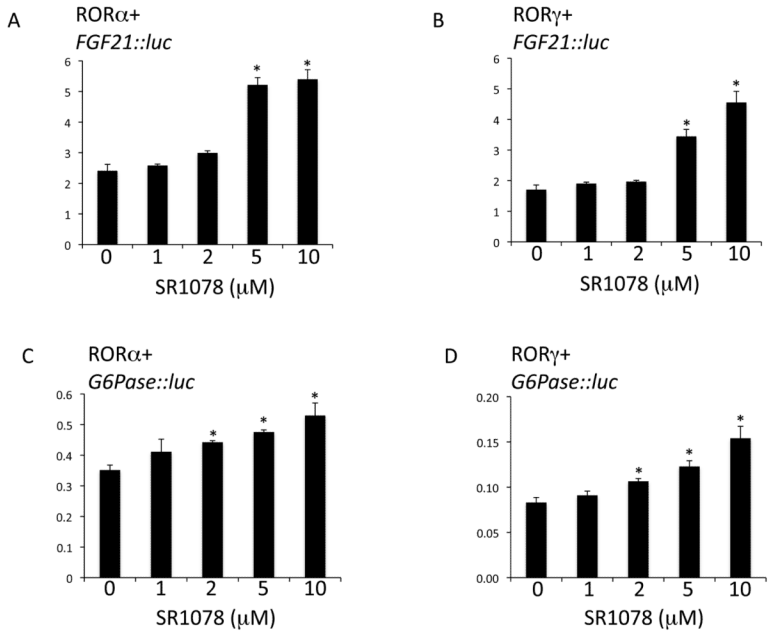

Figure 3.

SR1078 dose-dependently activates RORα and RORγ directed transcription. HEK293 cells were cotransfected with full length RORα (A and C) or RORγ (B and D) along with the FGF21 (A and B) and G6Pase (C and D) reporter as described in Figure 2. In all cases, SR1078 induced expression of the target gene reporter dose-dependently with significant activation occurring in the range of 2–5 μM. *, indicates $p<0.05$.

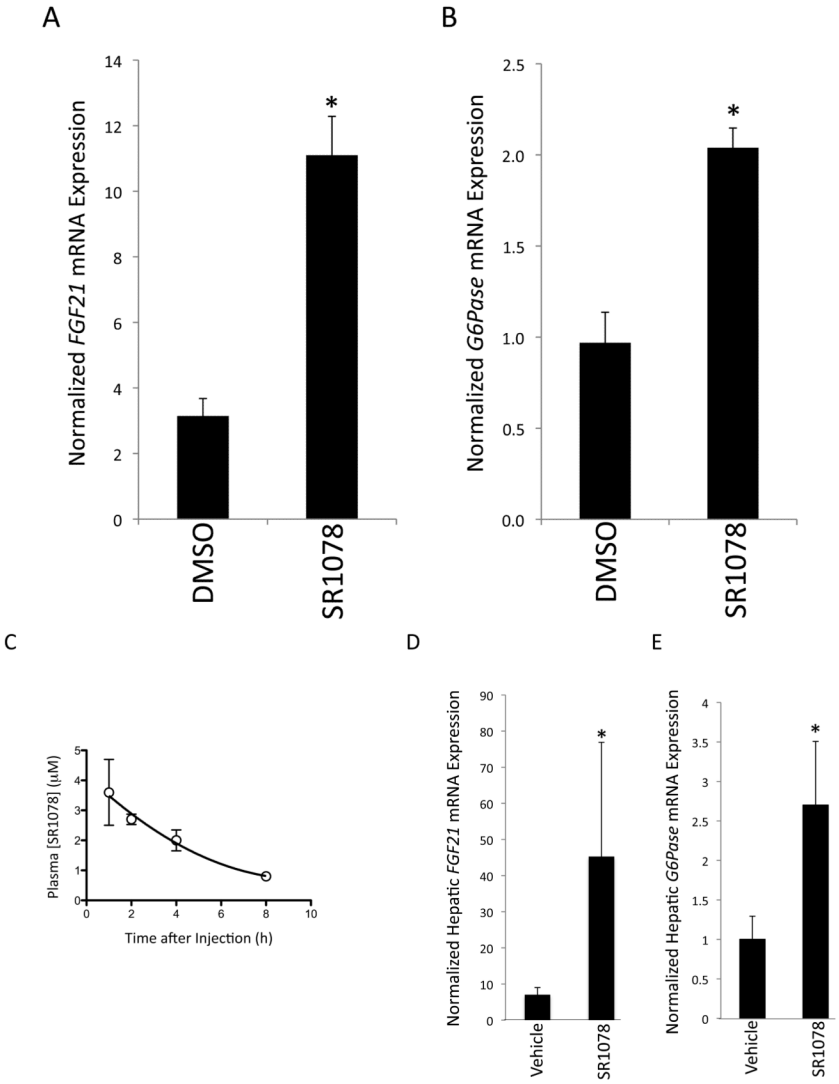

Figure 4.

SR1078 activates ROR target gene transcription both *in vitro* and *in vivo*. HepG2 cells expressing natural levels of RORα and RORγ were treated with 10 μM SR1078 for 24h followed by assessment of either *FGF21* (A) or *G6Pase* (B) gene expression. The expression of both of these ROR target genes was stimulated by the ROR agonist. C) Analysis of plasma levels of SR1078 following i.p. injection of the compound at a dose of 10 mg/kg in mice. D and E) Levels of expression of *FGF21* (D) and *G6Pase* (E) mRNA 2h following injection (i.p. 10 mg/kg) of SR1078.