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Analysis of the Transcriptional Activity of Retinoic Acid-related Orphan Receptors (RORs) and Inhibition by Inverse Agonists

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Abstract

Here, we describe several assays to analyze the transcriptional activity of retinoic acid-related orphan receptors (RORs) and the effect of inverse agonists on their activity. One assay measures the effect of an inverse agonist on the transcriptional activation of a luciferase reporter by RORs in a Tet-On cell system. A mammalian two-hybrid assay analyzes the interaction of the ROR ligand binding domain with a co-activator peptide. Two additional assays examine the effect of an inverse agonist on the activation of a luciferase reporter under control of the promoter of the ROR target gene, *IL17*, and on ROR-mediated activation using a mammalian monohybrid assay.

Keywords

Reporter assays; (inverse) agonist; retinoic acid-related orphan receptor; ROR α ; ROR γ ; luciferase reporter; transcriptional activation; promoter; mammalian two-hybrid and monohybrid assays

1 Introduction

The retinoic acid-related orphan receptors alpha, beta, and gamma (ROR α - γ encoded by *RORA-C* or *NR1F1-3*) constitute a subfamily of nuclear receptors [1]. By alternative promoter usage and alternative splicing the *ROR* genes generate several isoforms that exhibit different patterns of expression and regulate distinct biological functions. RORs regulate gene transcription by binding to ROR-response elements (ROREs), consisting of an RGGTCA consensus sequence preceded by an A/T-rich region, in the promoter regulatory region of target genes. The interaction of RORs with ROREs is mediated by two (C2-C2)-type zinc finger motifs that constitute the DNA-binding domain (DBD). The RORs contain a transcription activation domain at their C-terminus that interacts with co-activators or co-repressors. RORs are involved in the regulation of a wide range of biological functions, including embryonic development, glucose and lipid metabolism, circadian rhythm, and immunity [1]. For example, several lipid and glucose metabolic genes, including glucose-6-phosphatase (*G6PC*) and ELOVL fatty acid elongase 3 (*ELOVL3*), are direct targets for ROR γ transcriptional regulation [2, 3]. Both ROR α and ROR γ are implicated in the control of circadian clock gene expression. ROR α and ROR γ directly regulate the expression of neuronal PAS domain protein 2 (*NPAS2*), aryl hydrocarbon receptor nuclear translocator like (*ARNTL*, also named *BMAL1*), and cryptochrome 1 (*CRY1*) [4]. ROR γ is required for the differentiation of naïve T cells into T helper 17 (Th17) cells and has been demonstrated to regulate interleukin 17 (*IL17*) transcription by interacting with an RORE in the *IL17* promoter [5, 6].

Recently, ROR α and ROR γ were shown to function as ligand-dependent transcription factors [7–13]. Several cholesterol intermediates and metabolites have been shown to interact with the ligand binding domain (LBD) at the C-terminus of RORs and to act either as agonists or inverse agonists of ROR α and/or ROR γ . For example, 7 α -hydroxycholesterol and 24,25-epoxycholesterol function as inverse agonists for ROR α and/or ROR γ , whereas cholesterol and 25-hydroxycholesterol act as agonists of ROR α and ROR γ , respectively. Several vitamin D3 metabolites have also been reported to function as weak ROR α / γ inverse agonists [14]. Based on studies showing that Th17 cells and IL17 play a critical role in autoimmune disease, that ROR γ is required for Th17 cell differentiation and IL-17 production, and that ROR γ knockout mice are protected against autoimmunity, raised the prospect that ROR γ inverse agonists might potentially be an attractive therapeutic strategy in the management of autoimmune disease. This resulted in an extensive search for synthetic ROR γ inverse agonists and led to the discovery of a number of high affinity inverse agonists [9, 12, 15–20]. Treatment with ROR γ inverse agonists was shown to greatly alleviate Th17-dependent inflammation in several experimental autoimmune disease models in rodents, including Th17-mediated experimental autoimmune encephalomyelitis (EAE), skin inflammation in imiquimod-induced psoriatic models, and inflammation in collagen- and antigen-induced arthritis models [9, 17, 18, 20–26]. Recently, phase 2 clinical trials with ROR γ inverse agonists showed statistically significant efficacy in psoriatic patients supporting the concept that ROR γ inverse agonists might provide a new therapeutic strategy in the management of inflammatory autoimmune disease [27].

In addition to methods that analyze direct ROR-ligand interactions, several reporter assays have been developed to study ROR transcriptional activity. These assays are very useful for examining the effect of certain ROR mutations on ROR activity as well as the effect of ROR (inverse) agonists on ROR-mediated transcriptional activation. In this chapter, we describe several of such assays, including a Tet-On ROR-inducible cell reporter system. This test system is using CHO cells stably expressing a (RORE)₅-LUC reporter and ROR α or ROR γ under the control of the tetracycline (Tet)-regulated transactivator (rTA), which allows doxycycline-inducible expression of ROR α / γ . Upon their induction, ROR α / γ subsequently bind to the ROREs leading to activation of the luciferase reporter. This cell system provides a very valuable assay to study the effects of ROR (inverse) agonists on ROR-mediated transcriptional activation. In addition, we describe a mammalian two-hybrid reporter assay that examines the interaction of RORs with a LXXLL-coactivator peptide, a monohybrid assay, and an assay in which the reporter is under the control of the promoter of the ROR target gene, interleukin 17 (*IL17*).

2 Materials

2.1 Cell lines

1. CHO Tet-On 3G cells (Clontech/Takara).
2. Chinese hamster ovary (CHO-K1) cells (ATCC, CCL-61).
3. Acute T cell leukemia cells, Jurkat, clone E6.1 (ATCC, TIB-152).

2.2 Media/cell culture

1. RPMI-1640 and Ham's F-12 (F-12) media (Gibco).
2. Fetal bovine serum (FBS) (Hyclone).
3. Tet System Approved FBS (Clontech/Takara).
4. Penicillin/streptomycin (10,000 U/ml/10,000 µg/ml) (Gibco).
5. GlutaMAX (200 mM) (Gibco).
6. 0.25% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA) disodium salt (Gibco).
7. Doxycycline (20 mM)(Sigma-Aldrich).

2.3 Antibiotics

1. Geneticin (G418) (50 mg/ml) (Gibco).
2. Hygromycin B (50 mg/ml stock solution) (Gibco).
3. Puromycin (10 mg/ml stock solution) (Sigma-Aldrich).

2.4 Plasmids

1. pGL4.27-(UAS)₅ reporter plasmid containing five tandem repeats of the GAL4 upstream activating sequence (UAS) upstream of a minimal promoter.
2. pM-RORγ(LBD) and pM-LXXLL containing the GAL4-DNA(DBD) fused to, respectively, the RORγ(LBD) or the LXXLL co-activator peptide VESEFPYLLSLLGEVSPQP [28].
3. pVP16-RORα(LBD), pVP16-RORγ(LBD), containing the VP16 activation domain fused to the LBD of RORα or RORγ [28].
4. pCMV-β-Gal.
5. pTRE2pur-RORα and pTRE2pur-RORγ expression vectors containing the full-length mouse RORα4 and RORγ1, respectively, under the control of a tetracycline-response element (TRE). pTRE2pur (Clontech)[26].
6. The reporter plasmid pGL4.27(RORE)₅-Luc (pRORE-Luc) containing five copies of the ROR response element (RORE). pGL4.27 and pGL4.14 (Promega) [26].
7. p3X-FLAG-CMV10-RORα and p3X-FLAG-CMV10-RORγ encoding the full-length mouse RORα4 and RORγ1, respectively, tagged with 3 adjacent FLAG epitopes.
8. pGL4.14(*IL17-CNS*) reporter plasmid containing human *IL17*-3kb-CNS promoter [26].

2.4 Reporter assays

1. 96-well, 24-well, and 6-well cell culture plates (Nalgene, Denmark).

2. Luciferase Assay System (Promega).
3. Luminescent β -galactosidase Detection Kit II (Clontech/Takara).
4. Passive lysis buffer (5X) (Promega).
5. Modulus microplate reader (Promega) or equivalent luminometer/plate reader.
6. Transfection agents: Lipofectamine 2000 (Thermo Fisher Scientific).

2.4 Inverse agonist

1. The ROR γ selective inverse agonist GSK2981278 (Cayman Chemical).

3 Methods

3.1 Generation of ROR-Tet-On Cell Lines

1. Plate 4×10^4 CHO Tet-On 3G cells in 6-well plates in F12 medium supplemented with 10% FBS.
2. Next day, transfect cells with 4 μ g pTRE2pur-ROR α or pTRE2pur-ROR γ expression plasmid DNA using 10 μ l Lipofectamine 2000 following the manufacturer's protocol.
3. Two days after transfection, select pTRE2pur-ROR expressing cells in medium containing puromycin (1–2 μ g/ml) till resistant cell colonies have developed and all cells in nontransfected cells are killed.
4. Trypsinize resistant cell colonies, replate each of them to expand the cell number in the medium supplemented with puromycin.
5. Transfect cells obtained under (4) with 4 μ g of pGL4.27-(ROR) $_5$ reporter plasmid DNA using 10 μ l Lipofectamine 2000.
6. Two days after transfection, select pGL4.27-(ROR) $_5$ reporter integrated cells in the medium containing hygromycin B (600–900 μ g/ml) till resistant cell colonies have developed and all nontransfected cells are killed.
7. Trypsinize individual hygromycin-resistant colonies, replate in 6 well dishes, and expand in F12/10% FBS medium.
8. After expansion of each colony, trypsinize cells, and plate them at a density of 1×10^4 cells per well in 500 μ l F12/10% FBS medium in a 24-well plate. Triplicate wells are treated with vehicle or doxycycline. Replate the remaining cells in a T25 culture flask.
9. Next day, add doxycycline (1 μ M final) or vehicle (water) to the triplicate wells (see Note 1).
10. Twenty hr later determine the luciferase activity with the Luciferase Assay System (Fig. 1).

11. Select flasks with cells of the colonies with the highest fold induction in relative luciferase activity (*see* Note 2) for further expansion, aliquot, and store in liquid nitrogen.
12. Cells integrated with both the pGL4.27-(RORE)₅ reporter and pTRE2pur-ROR α or pTRE2pur-ROR γ are referred to as CHO ROR α -Tet-On and CHO ROR γ -Tet-On, respectively.

3.2 Reporter Assay 1

1. Plate 2×10^3 of the CHO ROR α -Tet-On and CHO ROR γ -Tet-On cells (*see* Note 3) in 96 well microplate in 100 μ l F12 medium supplemented with 10% FBS approved for the use in the Tet-On system (*see* Note 4).
2. The next day, replace medium with 100 μ l fresh medium containing 1 μ l of doxycycline (1 μ M final concentration) and 1 μ l of vehicle (DMSO) or a dilution series of the ROR γ inverse agonist GSK2981278 (1.0, 3.1, 10, 31, 100 nM, 0.3, 1.0, 3.1 and 10 μ M) or other test compounds in triplicate wells.
3. After twenty hr incubation, gently aspirate medium, wash cells with phosphate-buffered saline and add 25 μ l 1X passive lysis buffer.
4. Transfer 20 μ l aliquots of the cell lysate to a new 96-well microplate to measure the relative luciferase activity in a plate reader using the Luciferase Assay System following the manufacturer's instructions (Fig. 1).
5. Calculate the percent repression in the induction of relative luciferase activity by the inverse agonist (*see* Note 5).
6. Plot the percent repression as a function of the inverse agonist concentration (Fig. 2A)

3.3 Reporter Assay 2 (Mammalian Two-hybrid Assay)

1. Plate CHO-K1 cells in 24-well cell culture plates at a density of $1-2 \times 10^5$ cells per well in 500 μ l F-12 medium containing 10% FBS.
2. Next day, co-transfect cells with a pGL4.27-(UAS)₅ reporter plasmid (0.16 μ g), pCMV- β -Gal (0.16 μ g), pM-LXXLL (0.24 μ g), and pVP16-ROR γ (LBD) (0.24 μ g) with 2 μ l lipofectamine 2000 for each well. In controls, co-transfect cells with pGL4.27-(UAS)₅ and pCMV- β -Gal with empty vectors instead of pM-LXXLL and/or pVP16-ROR γ (LBD).
3. Three hr after transfection, add to triplicate wells 1 μ l of vehicle (DMSO) or a dilution series of the ROR γ inverse agonist GSK2981278 (1.0, 10, 100 nM and 1.0 μ M) or other test compounds.
4. After twenty four hr incubation, measure luciferase and β -galactosidase activities using the Luciferase Assay System and Luminescent β -galactosidase Detection Kit II, respectively (*see* Note 6).

5. Calculate the relative luciferase activity (*see* Note 7) and normalize to empty control.
6. Plot relative luciferase activity against the GSK2981278 concentration (Fig. 2B).

3.4 Reporter Assay 3

1. Plate $4-8 \times 10^5$ Jurkat cells in 24-well cell culture plate in 500 μ l of RPMI1640 medium supplemented with 10% FBS.
2. Next day, co-transfect cells with pGL4.14(*IL17-CNS*) reporter plasmid DNA (0.16 μ g), pCMV- β -Gal (0.16 μ g), and p3X-Flag-CMV10-ROR γ expression vector (0.48 μ g) or p3X-Flag-CMV10 empty vector (control) using 2 μ l lipofectamine 2000.
3. Three hr after transfection, add to triplicate wells 1 μ l vehicle (DMSO) or 1 μ l of a dilution series of ROR γ inverse agonist GSK2981278 (1.0, 10, 100 nM and 1.0 μ M final concentration) or other test compounds.
4. After twenty four hr incubation, measure luciferase and β -galactosidase activities with the Luciferase Assay System and Luminescent β -galactosidase Detection Kit II, respectively, as described under 3.3 (*see* Note 6).
5. Calculate the relative luciferase activity (*see* Note 7) and normalize to empty control.
6. Plot relative luciferase activity against the GSK2981278 concentration (Fig. 2C).

3.5 Reporter Assay 4 (Mono-hybrid assay)

1. Plate CHO-K1 cells in 24-well cell culture plates at a density of $1-2 \times 10^5$ cells per well in 500 μ l F-12 medium containing 10% FBS.
2. Next day, co-transfect cells with a pGL4.27-(UAS)₅ reporter plasmid (0.16 μ g), pCMV- β -Gal (0.16 μ g), and pM-ROR γ (LBD) (0.48 μ g) with 2 μ l lipofectamine 2000 for each well. In controls, co-transfect cells with pGL4.27-(UAS)₅ and pCMV- β -Gal with pM empty vector instead of pM-ROR γ (LBD).
3. Three hr after transfection, add to triplicate wells 1 μ l of vehicle (DMSO) or a dilution series of the ROR γ inverse agonist GSK2981278 (1.0, 10, 100 nM and 1.0 μ M) or other test compounds.
4. After twenty four hr incubation, measure luciferase and β -galactosidase activities using the Luciferase Assay System and Luminescent β -galactosidase Detection Kit II, respectively, as described under 3.3 (*see* Note 6).
5. Calculate the relative luciferase activity (*see* Note 7) and normalize to empty control.
6. Plot relative luciferase activity against the GSK2981278 concentration (Fig. 2D).

4 Notes

1. Doxycycline is unstable in water, so solution should be prepared fresh every time the assay is performed to maintain reproducibility.
2. The highest fold induction is determined by the ratio [luciferase activity in the presence of doxycycline / luciferase activity in the presence of vehicle]. Colonies with the highest ratio are selected for expansion and storage.
3. CHO Tet-On 3G cells expand rapidly during the assay and are very sensitive to nutrient shortage in the medium. Therefore, plating the cells at low density increases reproducibility of the luciferase assays.
4. CHO Tet-On 3G cells are cultured in F12 medium supplemented with low tetracycline 10% FBS approved for the use in the Tet-On system to reduce basal activation of RORs expression in the control samples not treated with doxycycline.
5. The percent repression is defined as $100 \times [\text{RLU of (CHO ROR Tet-On cells treated with doxycycline and DMSO/vehicle)} - \text{RLU of (CHO ROR Tet-On cells treated with doxycycline and test compound)}] / \text{RLU of (CHO ROR Tet-On cells treated with doxycycline and DMSO/vehicle)}$. RLU indicates relative luciferase units. The RLU of CHO
6. β -galactosidase activity is determined to adjust for variables, such as cell number and transfection efficiency between the wells.
7. The relative luciferase activity is calculated by the ratio [relative luciferase units (RLU) / β -galactosidase activity].

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Fig. 1.

Schematic of the Tet-On ROR-inducible cell reporter assay. This assay uses CHO cells stably expressing a (RORE)₅-LUC reporter and ROR α or ROR γ under the control of the tetracycline (Tet)-regulated transactivator (rTA), which allows doxycycline (Dox)-inducible expression of ROR γ . Upon their induction, ROR γ subsequently binds to the ROREs leading to activation of the luciferase reporter. Treatment with doxycycline in the presence of an ROR γ (inverse) agonist inhibits ROR γ -mediated transcriptional activation.

Fig. 2.

Inhibition of ROR γ -mediated transcriptional activation by the ROR γ inverse agonist GSK2981278. (a) Reporter assay 1. The effect of GSK2981278 on the transcriptional activity of ROR α and ROR γ was examined in CHO ROR α -Tet-On and CHO ROR γ -Tet-On cells. GSK2981278 selectively inhibits the transcriptional activity of ROR γ , but that of ROR α . (b) Reporter assay 2. The effect of GSK2981278 on the transcriptional activity of ROR γ was determined by mammalian two-hybrid analysis using the pGL4.27-(UAS)₅ reporter, pM-LXXLL, and VP16-ROR γ (LBD) in CHO-K1 cells. (c) Reporter assay 3. The effect of GSK2981278 on ROR γ -mediated transcriptional activation of the *IL17-CNS* promoter was examined in Jurkat cells. (d) Reporter assay 4. The effect of GSK2981278 on the transcriptional activity of ROR γ was determined by mammalian mono-hybrid analysis using the pGL4.27-(UAS)₅ reporter and pM-ROR γ (LBD). Data represent mean $\pm$ SEM (standard error of the mean). Significant inhibition was determined by Student's *t* test, *

$P < 0.01$. The data in the four panels shown are from Fig. 2 of a previously published paper (Reference 26; <https://www.ncbi.nlm.nih.gov/pubmed/26870941>).

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