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(Inverse) Agonists of Retinoic Acid–Related Orphan Receptor γ : Regulation of Immune Responses, Inflammation, and Autoimmune Disease

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Abstract

Retinoic acid–related orphan receptor γ t (ROR γ t) functions as a liganddependent transcription factor that regulates multiple proinflammatory genes and plays a critical role in several inflammatory and autoimmune diseases. Various endogenous and synthetic ROR γ (inverse) agonists have been identified that regulate ROR γ transcriptional activity, including many cholesterol intermediates and oxysterols. Changes in cholesterol biosynthesis and metabolism can therefore have a significant impact on the generation of oxysterol ROR γ ligands and, consequently, can control ROR γ t activity and inflammation. These observations contribute to a growing literature that connects cholesterol metabolism to the regulation of immune responses and autoimmune disease. Loss of ROR γ function in knockout mice and in mice treated with ROR γ inverse agonists results in reduced production of proinflammatory cytokines, such as IL-17A/F, and increased resistance to autoimmune disease in several experimental rodent models. Thus, ROR γ t inverse agonists might provide an attractive therapeutic approach to treat a variety of autoimmune diseases.

Keywords

retinoic acid–related orphan receptor; ROR γ ; Th17 cells; autoimmune disease; (inverse) agonists; cholesterol biosynthesis; inflammation

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INTRODUCTION

The retinoic acid–related orphan receptor (ROR) subfamily consists of three members: ROR α , ROR β , and ROR γ , which are encoded by the *RORA–C* (or *NR1F1–3*) genes, respectively (1–7). Each of these receptors functions as a ligand-dependent transcription factor (8, 9). RORs regulate gene transcription by binding as monomers to retinoic acid–related orphan receptor response elements (ROREs) containing an AGGTCA consensus preceded by an A/T-rich sequence in the regulatory regions of target genes. This binding is mediated by two C₂C₂-type zinc finger motifs within the N-terminal DNA-binding domain (DBD) that are highly conserved among nuclear receptors (3, 8, 9). In the last ten years, significant breakthroughs have been made in identifying several (oxy)sterols and synthetic ligands that act as either ROR γ agonists or inverse agonists (8, 9).

The *RORC* gene is located on chromosome 1q21.3 and encodes two isoforms in both humans and mice that arise through the use of alternative promoters (2,7–10). ROR γ 1 contains 518 amino acids, whereas ROR γ t (ROR γ 2) lacks the first 21 amino acids of ROR γ 1 and also differs from ROR γ 1 at the first three amino acids (MetArgThr instead of MetAspArg). Both isoforms regulate the transcription of target genes by binding a RORE consensus and recruit many of the same coactivators and corepressors (Figure 1). The existence of alternative promoters likely explains the different patterns of cell type–specific expression and the regulation of distinct physiological processes and target genes exhibited by the two isoforms. ROR γ 1 is expressed, often together with ROR α , in many peripheral tissues, including adipose, liver, kidney, and muscle tissue, where they regulate the transcription of a number of circadian and metabolic genes (3, 6, 11–16). In contrast, the expression of ROR γ t is confined to several different immune cell populations, including T helper 17 (Th17) cells, lymphoid tissue inducer (LTi) cells, innate lymphoid 3 cells (ILC3s), and $\gamma\delta$ T cells (3, 6, 17–24) (Figure 2).

Mice with impaired ROR γ t function are protected against the development of autoimmune diseases (23, 25). This, together with the discovery that treatment with small-molecule ROR γ t inverse agonists mimics the effects observed in ROR γ t-deficient mice, launched a search to identify additional high-affinity ROR γ t inverse agonists in the hope that this might lead to new therapeutic strategies in the management of several autoimmune diseases. In this review, we provide a short overview of ROR γ t and its (inverse) agonists and discuss their roles in regulating immune cell functions, particularly Th17 cells, and their relationship to autoimmune disease and inflammation.

ROLES OF ROR γ t IN IMMUNE CELLS

Th17 Differentiation

Naïve CD4⁺ T cells that exit the thymus can differentiate into several discrete T helper (Th) lineages defined primarily by their master transcription factors and the cytokines they produce (Figure 2). Th17 cells contain ROR γ t; produce the interleukins (ILs) IL-17A, IL-17F, and IL-22; and protect hosts against infections by extracellular bacteria and fungi. Th17-derived IL-21 also activates T follicular helper cells, which promote humoral immunity in secondary lymphoid tissue. Treatment of naïve T cells with transforming

growth factor (TGF)- β together with either IL-6 or IL-1 drives Th17 differentiation by inducing *Rorc* expression (25–29). IL-6 induces the phosphorylation of STAT3 (pSTAT3), which then moves to the nucleus to activate the transcription of *Rorc* and other Th17-associated genes (30, 31). IL-1 represses the suppressor of cytokine signaling (SOCS)3, an inhibitor of pSTAT3, thereby increasing *Rorc* expression (32). IL-23 stabilizes Th17 cells and contributes to their pathogenicity, in part by inducing expression of the transcription factor Runx1, which in turn stimulates *Rorc* expression and, together with ROR γ t, promotes Th17 differentiation (33). TGF- β promotes Th17 differentiation in part by acting through SKI, a transcriptional repressor that forms a complex with SMAD and inhibits H3K9 acetylation and transcription at the *Rorc* locus (34). TGF- β signaling leads to the degradation of SKI and the consequent derepression of the *Rorc* locus. Upon its synthesis, ROR γ t is recruited to ROREs at several Th17-associated loci, including *Il17a*, *Il17f*, *Irf4*, and *Il23r*, thereby directly regulating their transcription (35). In ROR γ t-deficient mice, the differentiation of Th17 cells is greatly impaired as is the transcription of Th17-type inflammatory cytokines (23, 25). Many transcription factors, including ROR γ t, are unable to bind chromatin until it has been opened through the actions of pioneer transcription factors. BATF (36) and IRF4 (37) are pioneer transcription factors that bind cooperatively to overlapping sites in chromatin near several Th17-related genes, including *Rorc* and *Il17*. This increases chromatin accessibility at these sites and allows pSTAT3 to bind the *Rorc* promoter and induce its transcription (38, 39).

It is well established that differentiation into the regulatory T cell (Treg) or Th17 lineages is controlled by the level of FOXP3 and ROR γ t, respectively (26–28, 40, 41). *Foxp3* expression and Treg development are favored in cultures containing high levels of TGF- β , IL-2, and retinoic acid (RA), whereas *Rorc* expression and Th17 development are promoted by low amounts of TGF- β together with proinflammatory cytokines, IL-6 and IL-1. However, recent studies have shown that these lineages are not necessarily fixed and that T cells can display substantial plasticity, with some Th17 cells expressing FOXP3 as well as ROR γ t. In addition to promoting the expression of genes associated with Tregs, FOXP3 can also interact with ROR γ t through a nuclear receptor interaction-like motif and repress *Il17* promoter activation by ROR γ t (42). The factors that control the relative abundance of FOXP3 and ROR γ t, and therefore the balance of Tregs and Th17 cells, are currently an area of intense investigation.

Innate Lymphoid Cells

Another active area of investigation is the development of innate lymphoid cells (ILCs), a class of non-B, non-T lymphocytes that are derived from the common lymphoid progenitor and possess typical lymphoid morphology but lack antigen-specific receptors and their associated molecules. There are some differences between the development of mouse ILCs and human ILCs, although the former is better understood (17) (Figure 2). An important function of ILCs is to provide a source of cytokines during the time required by antigen-specific effector T cells to develop from naïve T cells and undergo clonal expansion. The three major classes of human ILCs, as well natural killer (NK) cells, are derived from a common innate lymphoid cell progenitor (17, 24, 43) (Figure 2). There are several subsets of ILC3s and ROR γ t⁺ ILC3s. ROR γ t is selectively expressed in ILC3s that produce

IL-17A, IL-17F, IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor and in IL-22-producing NKp46⁺ cells (24,44,45). GATA3 is required for the development of ROR γ t⁺ ILC3s as well as for the other ILC subtypes (46). The transcription factor RUNX3 is also required for ILC3 development and regulates ROR γ t expression by inducing its transcription (47). ROR γ t in turn directly regulates the transcription of the aryl hydrocarbon receptor (*Ahr*) gene, which is required for ILC3 as well as other ILCs. ILCs are strategically located at sites of pathogen invasion, including the skin and the mucosa of the gut and lung. Although the involvement of ILCs in disease is still poorly defined, roles in infectious and inflammatory lung diseases and in the maintenance of the mucosal barrier homeostasis have emerged (44, 48). ILCs are heterogeneous and display significant plasticity. For example, ILC3s from both humans and mice can convert into ILC1-like cells (49–51). This transition follows a decrease in ROR γ t and a corresponding increase in T-bet and Notch signaling. Conversely, Notch ligand treatment of ILC2s induces ROR γ t and the consequent production of IL-17.

Lymphoid Tissue Inducer Cells

The first discovered member of the ILC3 group is the LT α i cell, a type of CD4⁺CD3[−] cell that expresses lymphotoxin α 1 β 2. LT α i cells are required for the development of secondary lymphoid organs, including Peyer's patches and intestinal lymphoid follicles (19, 43, 52, 53). ROR γ t is required for LT α i development, and ROR γ t-deficient mice consequently fail to develop secondary lymphoid organs (3, 21, 22). *Rorc* expression is induced by RA, which signals through RA receptors that bind to and activate the *Rorc* promoter (54).

Thymopoiesis

During thymopoiesis, T cells undergo a series of maturation steps in which immature CD4[−]CD8[−] double-negative thymocytes differentiate into maturing double-positive (DP) CD4⁺CD8⁺ cells and then into mature single-positive CD4⁺ or CD8⁺ T cells (Figure 2). ROR γ t is selectively expressed in DP cells and is required for their expression of the antiapoptotic gene *Bcl-X_L* (21, 22). DP thymocytes lacking ROR γ undergo accelerated apoptosis both in vivo and in vitro due to the downregulation of *Bcl-X_L*. Consequently, ROR γ -null mice have reduced numbers of DP cells and their descendants, including CD4⁺ and CD8⁺ Th cells.

$\gamma\delta$ T Cells

In addition to Th17 cells, some other immune cells can also express IL-17 in a ROR γ t-dependent manner. This includes $\gamma\delta$ T cells (Figure 2), which exhibit features of both adaptive and innate immune cells. In contrast to $\alpha\beta$ T cells, which do not produce IL-17 until they differentiate into Th17 cells, $\gamma\delta$ T cells emerge from the thymus fully capable of producing IL-17 in response to stimulation by IL-23 and IL-1 β without engagement of the T cell receptor (55). This induction of IL-17 is mediated by increased ROR γ t expression by IL-23. The ability to rapidly produce IL-17 in response to innate cytokines allows these cells to contribute to pathogen clearance before conventional Th17 cells have had time to develop. $\gamma\delta$ T cells have also been implicated in autoimmune disease (56). RNA for *Il17a* and *Il17f* as well as their encoded proteins, IL-17A and IL-17F, are elevated in psoriatic skin (57), and ILC3s and Th17 and $\gamma\delta$ T cells are important sources of IL-17 (58).

REGULATION OF ROR γ ACTIVITY AND FUNCTION

Several studies have demonstrated that posttranslational modifications play a critical role in modulating ROR γ protein activity. ROR γ is phosphorylated at multiple sites and can be ubiquitinated, sumoylated, and acetylated (9, 59–63) (Figure 1). Although the impact of the multiple ROR γ phosphorylation sites on ROR activity and function has yet to be established, several studies have recently provided insights into the role of ROR γ t ubiquitination in ROR γ activity and its regulation of Th17 cells. The HECT E3 ubiquitin ligase, ITCH, interacts with ROR γ and catalyzes its K48-polyubiquitination, thereby promoting its proteasomal degradation (62). Conversely, deubiquitination of the K48-linked polyubiquitination of ROR γ t by the deubiquitinase ubiquitinspecific protease USP4 stabilizes ROR γ t and promotes its activation of Th17-associated genes, whereas inhibition of USP4 activity diminishes Th17 differentiation (64). The E3 ubiquitin ligase tumor necrosis factor receptor–associated factor 5 (TRAF5) stabilizes ROR γ t through K63-linked polyubiquitination (65). Accumulation of the deubiquitinating enzyme DUBA (OTUD5) negatively regulates ROR γ t stability and IL-17 expression in T cells by stabilizing the ubiquitin ligase UBR5, which ubiquitinates ROR γ t in response to TGF- β , leading to ROR γ t degradation and decreased IL-17 expression (66).

The ubiquitin-specific protease USP17 reduces polyubiquitination of ROR γ t at Lys³⁶⁰ and prevents its degradation by proteasomes, whereas downregulation of USP17 decreases ROR γ t protein and expression of IL-17 (67). Ubiquitination of ROR γ t at Lys⁴⁴⁶ inhibits its interaction with NCOA1 and negatively regulates Th17 differentiation (68). The mutation of Lys⁴⁴⁶ to Arg or the deubiquitination of Lys⁴⁴⁶ by USP15 promotes NCOA1 recruitment and Th17 differentiation.

(De)acetylation also plays a critical role in the control of ROR γ t expression, its transcriptional activity, and its regulation of Th17 differentiation and inflammation. ROR γ t transcription is dependent on STAT3; STAT3 acetylation at Lys⁶⁸⁵ by p300 is required for its nuclear translocation, transcriptional activity, induction of ROR γ t, and Th17 differentiation. Activation of the NAD-dependent deacetylase Sirtuin 1 (SIRT1) promotes its interaction with STAT3 and induces deacetylation at Lys⁶⁸⁵, thereby inhibiting STAT3 translocation to the nucleus and consequently reducing the transcriptional activation of *RORC* (69). These studies indicate that (de)acetylation plays a critical role in regulating *RORC* transcription and the balance between Th17 and Treg differentiation.

Another report identified a different role for SIRT1 in regulating ROR γ t activity (61). This study showed that the histone acetylase p300 (KAT3B) interacts with and acetylates ROR γ t at several positions within the DBD and impairs its interaction with RORE and thus its ability to induce Th17 differentiation and IL-17A expression. SIRT1 physically interacts with the ROR γ ligand-binding domain (LBD) and promotes deacetylation of the ROR γ t DBD, which restores ROR γ t transcriptional activity and consequently promotes the generation of Th17 cells, thereby exacerbating autoimmunity (61). Recently, ROR γ t activity was found to be also modulated by sumoylation (70). Sumoylation of ROR γ t by SUMO3, but not SUMO1, at Lys³¹ catalyzed by the E3 Sumo ligase PIAS4 promotes recruitment of the lysine acetyl transferase KAT2A (GCN5) and NCOA1, which enhance ROR γ t

transcriptional activity and stimulate Th17 differentiation. These ROR γ t-mediated activities were greatly diminished when Lys³¹ was mutated to Arg. Loss of SUMO3 expression was shown to significantly reduce Th17 differentiation. A recent report identified a different mechanism by which sumoylation influences ROR γ t activity. This study showed that SUMO-conjugating enzyme, Ubc9 (UBE2I), binds ROR γ t via a conserved GKAE motif and targets it for sumoylation. This subsequently leads to the recruitment of HDAC2 by ROR γ t and suppression of ROR γ t transcriptional activity and *IL17* expression (71). Consistent with these observations are findings showing that sumoylation-defective Th17 cells are colitogenic upon transfer to Rag1^{-/-} mice.

A recent study identified an indirect way by which phosphorylation affects NCOA1–ROR γ interaction and the balance between Th17 and Treg lineage determination (72). Phosphorylation of NCOA1 by protein kinase C theta (PKC- θ or PRKQC) stimulates its interaction with ROR γ t, causing the stabilization of ROR γ t–RORE binding and the dissociation of bound FOXP3 and its subsequent proteasomal degradation. This induces a shift from Treg toward Th17 differentiation and increased IL-17A expression.

OXYSTEROLS AS ENDOGENOUS (INVERSE) AGONISTS OF ROR γ

X-ray crystallography studies demonstrating that cholesterol and cholesterol sulfate are associated with the ligand-binding pocket of ROR α and function as ROR α agonists (73, 74) provided the first evidence for a connection between RORs and cholesterol and its derivatives. These observations suggested that cholesterol-related intermediates or metabolites might also function as ligands of ROR γ and regulate its transcriptional activity and functions. Subsequent studies revealed that 20 α -hydroxycholesterol (-OHC), 22R-OHC, and 25-OHC all effectively enhance the interaction between ROR γ LBD and the NCOA1 coactivator peptide containing an LXXLL nuclear receptor–interacting motif (75). The naturally occurring oxysterols 7 α ,27-OHC, 7 β ,27-OHC, and 27-OHC were identified as potent and selective ROR γ agonists (76) based on their ability to reverse the repression of ROR γ transcriptional activity by the inverse agonist ursolic acid. In contrast, the oxysterol 7 α ,25-OHC was inactive, while 7 α -OHC, 7 β -OHC, 25-OHC, and 24S-OHC showed either weak-to-moderate agonist or inverse agonist activity (76–78).

Additional studies showed that several intermediates of the cholesterol biosynthesis pathway function as endogenous ligands for ROR γ (79–81) (Figure 3). Zymosterone, zymosterol, 7dehydrocholesterol, and desmosterol were among the sterols activating ROR γ , while lanosterol and T-MAS showed little activity. 4 α -carboxy, 4 β -methyl zymosterol (4ACD8), and 25-OHC were identified as some of the most effective ROR γ agonists (80). Sulfated derivatives of these sterols were about twice as effective in enhancing ROR γ activity. Interestingly, the expression of the sterol sulfotransferase Sult2B1 was increased, while expression of the sterol sulfatase STS was decreased during Th17 differentiation, bringing about a shift toward more active, sulfated sterol formation that would lead to enhanced ROR γ t activation.

In the skin, 7-dehydrocholesterol can be converted by UVB to previtamin D3 and subsequently by CYP2R1, CYP27A1, and CYP27B1 to the active form 1,25-(OH)₂D3. Both

can then be converted by CYP11A1 and CYP24A1 to various additional hydroxylated metabolites such as 20-OHD3 and 20,22-(OH)₂D3 (82). Alternatively, UVB can convert D3 to lumisterol (L3), which is subsequently metabolized by CYP11A1 to various hydroxylumisterols such as 20(OH)L3 (83). 1,25-(OH)₂D3, in addition to acting as an agonist of the vitamin D receptor, also functions as a weak inverse agonist of ROR γ and ROR α (80, 82). Several hydroxylated vitamin D3 and L3 metabolites also exhibit ROR inverse agonist activity (8, 82, 83).

MODULATION OF CHOLESTEROL METABOLISM AFFECTS ROR γ t ACTIVITY AND INFLAMMATION

Observations showing that various intermediates of the cholesterol biosynthetic pathway function as ROR γ t (inverse) agonists suggested that changes in this pathway would affect the generation of ROR γ (oxy)sterol (inverse) agonists and, consequently, ROR γ transcriptional activity and functions, including Th17 differentiation. Consistent with this hypothesis were findings showing that when naïve T cells differentiate into Th17 cells, the expression of many enzymes of the cholesterol biosynthetic pathway is increased, while the expression of those involved in cholesterol export is decreased (79, 84). Furthermore, inhibition of the mevalonate–cholesterol biosynthetic pathway negatively affects the differentiation and function of T cells. Statins, such as simvastatin and lovastatin, which inhibit the synthesis of cholesterol isoprenoid precursors, reduce ROR γ t expression and IL-17 production (79, 85) (Figure 3). Moreover, deficiency in certain cholesterol biosynthetic enzymes can have either a positive or negative effect on ROR γ t activity by regulating the availability of ROR γ t agonists. Deficiency in CYP51 or treatment with variousazole fungicides (e.g., clotrimazole and hexaconazole), inhibitors of CYP51, reduces ROR γ transcriptional activity and decreases Th17 differentiation and IL-17 production by inhibiting cholesterol biosynthesis and the formation of ROR γ t agonists (79, 80, 86) (Figure 3). This inhibition of Th17 cells could be reversed by the addition of ROR γ agonists such as zymosterol, which also induces ROR γ t-dependent IL-17 expression in $\gamma\delta$ T cells. The observed reduction in LT α cells and lymph node anlagen, which are also dependent on ROR γ t, in CYP51-deficient mice is consistent with the reduced ROR γ t activity in these mice (80). In addition, CYP51 ablation reduces both ROR α and ROR γ activities in mouse liver (87), while inhibition of CYP51 by ketoconazole reduces IL-17 production by $\gamma\delta$ T cells and improves inflammation in imiquimod-induced psoriasis in mice (80). Likewise, squalene synthetase (FDF1) deficiency, as well as the knockdown of SC4MOL or NSDHL, reduces ROR γ t transcriptional activity (Figure 3). Deletion of SC4MOL in all ROR γ t-expressing cells, or only in CD4⁺ T cells, showed that CD4⁺ T cells lacking SC4MOL have a greatly reduced ability to differentiate along the Th17 lineage and that this could be reversed by the addition of 4ACD8.

Deficiency in the scavenger receptor cysteine-rich family member CD5L, which regulates lipid homeostasis, enhances the expression of several cholesterol biosynthetic enzymes, including CYP51A1 and SC4MOL, and subsequently increases availability of ROR γ t agonists and IL-17 expression in pathogenic Th17 cells (8, 88). The transcription factor SREBF2, which increases cholesterol biosynthesis by enhancing the transcription of several

cholesterol biosynthetic genes, including *HMGCR* and *HMGCS*, contributes to a proinflammatory response (89) that might in part be due to increased synthesis of ROR γ t agonists. These observations further suggest that stimulation of cholesterol biosynthesis by a high-fat or hypercholesterolemic diet might result in higher levels of ROR γ t agonists, Th17 differentiation, and the production of proinflammatory cytokines. This is consistent with a report showing that apolipoprotein E-deficient mice, which have elevated levels of cholesterol, exhibit an increased susceptibility to type II collagen-induced arthritis that is in part due to the activation of Th17 inflammatory responses (90). Together, these studies indicate that ROR γ transcriptional activity and ROR γ -regulated physiological functions can be modulated by changes in the cholesterol biosynthetic pathway. Thus, ROR γ t functions as a mediator that plays a central role in linking the control of cholesterol metabolism with regulation of the immune system; immune responses; and, consequently, autoimmune disease (8, 81) (Figure 1).

INVERSE AGONISTS

The discovery that ROR γ functions as a ligand-dependent transcription factor, together with findings showing that the loss of ROR γ function protects against autoimmune disease, initiated a search for small-molecule inverse agonists that would inhibit ROR γ t transcriptional activity as well as proinflammatory responses regulated by ROR γ t, protect against autoimmune diseases, and provide a new therapeutic approach in managing these diseases. High-throughput screens led to the discovery of several series of ROR γ (inverse) agonists. The cardiac drug digoxin and its derivatives β -acetyldigoxin 20,22-dihydrodigoxin-21,23-diol [Dig(dhd)] and digoxin-21-salicylidene [Dig(sal)] were among some of the first synthetic ROR γ -selective inverse agonists identified (91) (Figure 4). In contrast to its inverse agonist effect, a recent study reported that, at low concentrations, digoxin exhibited agonist activity in HepG2 cells (92).

In another study, the LXR agonist T0901317 was identified as an inverse agonist of both ROR α and ROR γ but not of ROR β (93). As a result of this discovery, a series of chemical derivatives were synthesized and their effect on ROR activity analyzed. This led to the identification of several additional (inverse) agonists, including the ROR α and ROR γ inverse agonist SR1001 and agonist SR1078 and the selective ROR γ inverse agonist SR2211 (94). Several natural products have been reported to act as ROR γ ligands. Ursolic acid, a pentacyclic triterpenoid found in many plants, acts as a ROR γ -selective inverse agonist, whereas ganoderone A, an antiviral triterpenoid produced by the fungus *Ganoderma pfeifferi*, acts as a very potent ROR γ agonist (95, 96) (Figure 4).

Subsequent studies by many different laboratories led to the discovery of a number of additional small-molecule ROR γ (inverse) agonists (35, 97–113) (Figure 4). For a more comprehensive review of studies that identified and characterized ROR γ t inverse agonists, the reader is referred to several sources (8, 9, 94, 114–117).

The configuration of H12 plays a critical role in determining the activator or repressor state of nuclear receptors. Structure analysis of the ROR γ LBD in complex with several oxysterols and chemical ligands showed that agonists stabilize the active position of H12,

which becomes tightly packed against the LBD, thereby allowing it to interact with the LXXLL motif of coactivators, such as NCOA1/2 (8, 9, 75, 80, 91, 96, 97, 118, 119) (Figure 1). Agonists can bind to and regulate ROR γ activity through different mechanisms. Many agonists act via a canonical mechanism in which they bind to the ligand-binding pocket of ROR γ , allowing for the recruitment of coactivators and consequent transcriptional activation of ROR γ target genes (75, 76, 96) (Figure 1). However, in certain instances, agonist-bound ROR γ can also bind corepressors such as nuclear receptor interacting protein 1 (NRIP1, also referred to as RIP140), thereby repressing transcription (96).

Binding inverse agonists induces a conformational change in the ROR γ LBD that leads to the displacement of coactivators and recruitment of corepressors such as NCOR1/2 and the histone deacetylases HDAC1/2 (8, 95, 96). In the canonical mechanism, inverse agonists, including some (oxy)sterols, TMP778, TMP920, and GSK805, interact with the ROR γ ligand-binding pocket but suppress ROR γ activity in two different ways. TMP778- and GSK805-bound ROR γ t are still able to bind ROREs in the promoter regions of target genes, whereas TMP920-bound ROR γ t is no longer capable of interacting with ROREs (35) (Figure 4). However, certain inverse agonists suppress ROR γ activity via a noncanonical mechanism. For example, MRL-871 does not interact with the canonical ligand-binding pocket but with a distal, allosteric binding site, causing a change in the ROR γ LBD conformation that prevents recruitment of coactivators (120). Small changes in the chemical structure of oxysterols or synthetic ROR γ ligands can determine whether they act as inverse agonists or agonists, as reported for several tertiary sulfonamide ROR γ modulators (121) (e.g., see Figure 4).

Administration of ROR γ inverse agonists mimics many of the same effects observed in ROR γ -deficient mice, including inhibition of Th17 differentiation and repression of the transcription of Th17 signature genes such as *IL17A*, *IL17F*, and *IL23R* (6, 35, 75, 76, 91, 95, 122–126) (Figure 1). In contrast to the suppression of Th17 signature genes, inverse agonists enhance the expression of signature genes from other CD4⁺ lineages, including Treg cells (35). The latter indicates that inverse agonists cause a shift toward Treg differentiation, which is consistent with the concept that the Th17 and Treg lineages are reciprocally controlled by the levels of ROR γ t and FOXP3. The repression of *IL17A* and *IL23R* transcription correlates with several epigenetic changes at their promoter regions, including a reduction in H3AcK9/14 and H3K4m3, transcription-permissive acetylation, and methylation signatures (25, 127). In addition to the effects on Th17 cells, treatment with inverse agonists also mimics other phenotypes seen in ROR γ -null mice. For example, inverse agonists inhibit the stimulation of ROR γ t-dependent IL-17 in $\gamma\delta$ T cells by IL-23 and IL-1 β (125, 128, 129). In addition, treatment of isolated CD4⁺CD8⁺ DP thymocytes with the inverse agonist Cpd 1 induces a significant increase in apoptosis, as has been observed in ROR γ -null mice (21, 22, 123).

ROR γ t, INVERSE AGONISTS, AND INFLAMMATORY DISEASE

Mice deficient in ROR γ t function have been shown to be protected against multiple autoimmune diseases (23, 25). Several ROR γ t-dependent IL-17⁺ immune cells, including Th17, ILC3, and $\gamma\delta$ T cells, contribute to different degrees to the development and

progression of autoimmune diseases (56, 57, 130, 131). Multiple sclerosis (MS) is a disease of the central nervous system, involving demyelination of and consequent damage to nerve cells. Experimental autoimmune encephalomyelitis (EAE) has been the most widely used animal model of MS to study the role of ROR γ t, and it has proven to be extremely informative for uncovering the pathogenic mechanisms that drive MS, including the role of ROR γ t. Th17 cells are thought to be of central importance in this disease because the Th17-produced proinflammatory cytokines IL-17A/F, IL-23, and IL-22 play a critical role in the pathogenicity of EAE as well as in other autoimmune diseases (132, 133). This is supported by studies demonstrating that antibodies against IL-17A and IL-23 or their receptors are effective in the treatment of several autoimmune diseases. Mice lacking ROR γ are significantly protected from EAE (23, 25), type II collagen-induced arthritis, and IL-23-induced psoriasis-like lesions (126, 128). ROR γ -null mice also displayed reduced numbers of eosinophils and CD4⁺ lymphocytes in an ovalbumin-induced model of allergic airway inflammation (134). Together, these studies raised the prospect that ROR γ (inverse) agonists might be useful in the management of allergic and autoimmune diseases (35,76,80,135,136). This was confirmed by several studies demonstrating that small-molecule ROR γ t inverse agonists ameliorate disease in several rodent models of autoimmunity. Thus, treatment with the ROR α /ROR γ inverse agonist SR1001 and ROR γ -selective inverse agonists, including ursolic acid, digoxin, MRL-248, TMP778, TMP920, Cpd 1, and dehydrodiconiferyl alcohol, suppresses *IL17* transcription and IL-17 production and significantly reduces the severity of EAE (35, 91, 95, 137, 138) (Figure 1).

The inverse agonist GSK2981278, a very effective, selective inhibitor of ROR γ activity, attenuates inflammation in an imiquimod-induced, psoriasis-like mouse model when treated topically (124). GSK2981278 significantly reduced the levels of several proinflammatory cytokines, including IL-17A/F, IL-22, and IL-1 β , in treated skin and reduced epidermal hyperplasia and skin thickness. Topical treatment with GSK2981278 also inhibited Th17-type cytokine production and IL-17-driven inflammation in human tissue-based assays and skin explants from psoriatic patients (124). However, in a human randomized, double-blind trial, topical treatment with GSK2981278 did not change psoriatic infiltrate thickness or the level of several Th17-type genes such as *IL17A/F* (139), possibly because longer and more frequent treatment or systemic treatment is needed to be effective. Another ROR γ inverse agonist, BIO-0554019, inhibited imiquimod-induced skin inflammation when applied as a cream (129). Subcutaneous treatment with TMP776 also attenuated imiquimod-induced skin inflammation (125). In both cases, this correlated with the inhibition of *IL17A/F* expression in Th17 cells as well as $\gamma\delta$ T cells, which are critical in psoriasis (140). Two different studies showed that oral administration of the inverse agonists VTP-43742 and A213 attenuated psoriatic lesions in mouse models (126, 141). A213 mitigated phorbol ester-induced psoriasis and IL-23-induced dermatitis in K5.Stat3C transgenic mice that constitutively expressed active Stat3 in epidermal cells, and VTP-43742 statistically significant efficacy in psoriatic patients in a phase 2a trial carried out by Vitae Pharmaceuticals (142). A different ROR γ t inverse agonist, JNJ-54271074, has also been reported to decrease IL-23-induced psoriasis-like skin inflammation (128), and using a translational model of human epidermal and skin-homing T cells from psoriatic patients, JNJ-54271074 inhibited streptococcus extract-induced IL-17A/F.

Using a model of antigen (methylated BSA)-induced arthritis in rats, oral gavage with the ROR γ t inverse agonist Cpd 1 reduced paw swelling and numbers of IL-17-producing cells in a dose-dependent manner, consistent with Cpd 1-mediated suppression of pathogenic T cell development (127). A different ROR γ t inverse agonist, JNJ-54271074, suppressed IL-17A production in peripheral blood mononuclear cells (PBMCs) from human rheumatoid arthritis patients and suppressed pathologic scores in collagen-induced arthritis in mice (128). Similarly, the inverse agonist SR2211 has also been reported to diminish inflammation in a collagen-induced arthritis mouse model (143), while MRL-248 and MRL-367 were effective in reducing Th17 function in PBMCs isolated from patients with rheumatoid arthritis (144).

Crohn's disease (CD) is a chronic and relapsing disease of the intestine that affects more than 4 million people worldwide. While management of this disease has improved in recent years, there is still a need for more effective therapies. However, blocking IL-17 has generally proven to be ineffective, and in some CD patients it has led to worse pathology, possibly because of an increased incidence of intestinal infections (145). The orally available ROR γ inhibitor GSK805 diminished inflammation and tissue damage in the colon in response to infection with *Citrobacter rodentium* (146). Although ROR γ t promotes the development of both Th17 cells and ILC3s, GSK805 reduced the number of IL-17A-producing cells in the colon lamina propria and mesenteric lymph node without affecting the number of IL-17-producing ILC3s. GSK805 also attenuated spontaneous colonic inflammation in *Il10*^{-/-} mice as well as intestinal inflammation resulting from CBir1 T cell transfer. In addition, GSK805 inhibited the number Th17 cells and IL-17 expression in tissues resected from the colon of CD patients (146). Similarly, oral administration of the ROR γ t inhibitor BI119 improved clinical signs in a colitis model of CD4⁺CD45RB^{high} T cell transfer into immune-deficient, X-linked, severe combined immunodeficiency (SCID) mice (147). Together, these studies suggest that ROR γ t inverse agonists might provide an attractive therapeutic approach to treat inflammatory bowel disease and other chronic inflammatory diseases.

While the inhibition of ROR γ -dependent transcription has tremendous potential for treating a wide variety of autoimmune diseases, ROR γ -deficient mice have a high incidence of developing thymic lymphomas that rapidly metastasize to the liver and spleen (148). Lymphoblastic lymphomas also develop in a conditional knockout mouse model in which the immune system develops normally, with ROR γ being deleted when animals reach adulthood (149). In addition, long-term treatment of rats with an inverse agonist (Cpd 1) caused thymic preneoplastic hyperplasia similar to that observed in ROR γ -null mice (123,148). It remains to be seen whether long-term treatment with ROR γ t inverse agonists also causes lymphoma in humans, but if so, this deleterious effect of such compounds might preclude their long-term use. Additional studies will be needed to assess these risks and to weigh them against the great promise short-term inhibition of ROR γ has already shown in multiple autoimmune diseases.

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Figure 1.

ROR γ t functions play a central intermediary role in linking the control of cholesterol biosynthesis and metabolism to the regulation of immune responses and autoimmune disease. Many endogenous oxysterols generated by the cholesterol biosynthetic pathway and cholesterol metabolism act as effective ROR γ t agonists, leading to the recruitment of coactivators such as NCOA1 and CBP and the transcriptional activation of ROR γ t target genes, including *IL17*, *IL21*, *IL23R*, and *AHR*. The increase in proinflammatory cytokines promotes inflammation and autoimmune disease. Many synthetic ROR γ ligands and some oxysterols, such as 24*S*-OHC and several hydroxylated metabolites of vitamin D3, function as inverse agonists. Inverse agonists prevent coactivator binding, promote the recruitment of corepressors such as NCOR1 and HDAC1/2, and repress the transcription and production of proinflammatory cytokines, thereby reducing inflammation and protecting against autoimmune disease. Changes in cholesterol biosynthesis and metabolism, for example, by treatment with statins or a high-cholesterol/fat diet, affect the generation of ROR γ t agonists and thereby ROR γ t activity and the development of inflammation and autoimmune disease. Posttranslational modifications can also affect ROR γ t activity by changing ROR γ t protein stability or its ability to recruit coactivators. Abbreviations: OHC, hydroxycholesterol; ROR, retinoic acid–related orphan receptor; RORE, ROR response element.

Figure 2.

RORγt-dependent development of T cells and ILCs from the CLP. Cells expressing RORγt are indicated. (*top*) Development of the indicated T cells, including CD4⁺CD8⁺ DP αβTCR⁺ T cells and γδTCR⁺ T cells in the thymus as well as αβTCR⁺ CD4⁺ Th17 cells. (*bottom*) Innate immune cells that also arise from the CLP, including NKp46⁺ NK cells derived from NKP cells, the three major ILC subsets derived from the ILCP, and LTi cells arising from the CHILP. Abbreviations: CHILP, common helper ILC progenitor; CLP, common lymphoid progenitor; DN, double-negative; EILP, early innate lymphoid progenitor; HSC, hematopoietic stem cell; ILC, innate lymphoid cell; ILCP, ILC progenitor; LTi, lymphoid tissue inducer; NK, natural killer; NKP, NK cell progenitor; ROR, retinoic acid–related orphan receptor; TCR, T cell receptor; Th, T helper.

Figure 3.

Intermediates of the cholesterol biosynthetic pathway function as ROR γ agonists. Acetyl CoA is converted to mevalonate by HMGCR and then into farnesyl pyrophosphate and squalene via several steps, which are subsequently converted into cholesterol via multiple steps. Statins reduce cholesterol biosynthesis by inhibiting the formation of mevalonate. Zymosterol, desmosterol, zymosterone, 4ACD8, 25OHC, and 7-dehydrocholesterol are among the most effective endogenous ROR γ agonists. Deficiency in CYP51A1, or its inhibition by azoles, and deficiency in FDFT1 inhibit cholesterol biosynthesis and limit the availability of ROR γ agonists. Abbreviations: ACAT1, acetyl-CoA acetyltransferase 1; CH25H, cholesterol 25-hydroxylase; CYP51A1, cytochrome P450 family 51 subfamily A1; DHCR24, 24-dehydrocholesterol reductase; DHCR7, 7-dehydrocholesterol reductase; EBP, emopamil binding protein; FDFT1, farnesyl-diphosphate farnesyl-transferase 1; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; HSD17B7, hydroxysteroid 17- β dehydrogenase 7; LSS, lanosterol synthase; NSDHL, NAD(P)-dependent steroid dehydrogenase-like; ROR, retinoic acid—related orphan receptor; SC4MOL, fatty acid hydroxylase domain containing 2; SC5D, sterol-C5-desaturase; SQLE, squalene epoxidase; TM7SF2, transmembrane 7 superfamily member 2.

Figure 4.

Chemical structures of several retinoic acid-related orphan receptor (ROR) inverse agonists: digoxin (91), SR1001 (a ROR α and ROR γ inverse agonist) and SR2211 (135), ursolic acid (95), VTP-43742 (141), GSK2981278 (124), BIO-0554019 (129), TMP778 and TMP920 (35), JNJ-54271074, Cpd-1 (123), and A213 (126). The structures of a sulfonamide inverse agonist antagonist (121) and the agonist ganoderone A (96) are also shown.