Review

Action of RORs and their ligands in (patho)physiology

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The retinoic-acid-receptor-related orphan receptors (RORs) are members of the nuclear receptor (NR) superfamily whose activity has been implicated in several physiological and pathological processes. The RORs, specifically RORα and RORγ, are considered to be master regulators of T_H17 cells, a recently described subset of $CD4^+$ T helper cells that have been demonstrated to have a pathological role in autoimmune disease. As with most members of the NR superfamily, RORs are ligand-regulated, suggesting that their activity can be modulated by synthetic ligands. Recent advances in the field have established that selective inhibition of the RORs is a viable therapeutic approach for not only the treatment of autoimmune disorders but also ROR-mediated metabolic disorders.

Introduction

The human NR superfamily (see Glossary) is a highly conserved family of transcription factors composed of 48 members. NRs function as ligand-dependent transcription factors and share considerable amino acid sequence homology [1]. General structural characteristics of NRs are a variable N-terminal A/B domain, a central, highly conserved DNA-binding domain (DBD, also termed a C region), a hinge region (D), and a C-terminal ligand-binding domain (LBD, or E region). The LBD is responsible for recognition and binding of the receptor ligand as well as for ligand-dependent transcriptional activity. Some receptors contain an additional C-terminal region, or F domain, of which the function is poorly understood.

Approximately half of the NR superfamily have well-characterized natural ligands, whereas the remaining receptors are considered to be 'orphan' receptors and remain the focus of intense research [2]. The majority of NRs with identified natural ligands are also validated targets for clinical purposes and are a rich source of therapeutics aimed at the treatment of a great number of diseases, including inflammation, cancer, and metabolic disorders. Orphan NRs are an active area of research due to the potential for identification of ligands that may be used to modulate these receptors with the goal of developing targeted therapeutics for various diseases [3]. Over the past few years there have been significant breakthroughs in the identification of novel ligands, both natural and synthetic, for several orphan NRs. This review examines the progress made in the identification of ligands for the RORs and their roles in immune and metabolic processes.

RORs: the basics

The ROR family comprises three members – RORα (NR1F1), RORβ (NR1F2), and RORγ (NR1F3). These are considered to be 'orphan' receptors because their endogenous ligands have yet to be agreed upon definitively. Owing to their known roles in metabolic and immune processes, there is significant interest in the identification of ligands that regulate the RORs due to their potential for clinical utilization. Unlike most family members, the RORs recognize and bind as monomers to specific sequences of DNA, termed ROR response elements (ROREs), typically consisting of an AGGTCA 'half site' with a 5′ AT-rich extension in the regulatory region of the target gene [4–6]. When bound to this element within the promoters of their target genes, RORs constitutively recruit coactivators, leading to

Glossary

Coactivator: a nuclear receptor coactivator is a transcriptional coregulatory protein that contains nuclear receptor-interacting domains. The coactivator is unable to bind to DNA by itself but assists nuclear receptors to bind to HREs on target gene promoter sites and increase transcription.
Corepressor: a nuclear receptor corepressor, similar to a coactivator, contains nuclear receptor-interacting domains. The corepressor assists nuclear receptors in the downregulation of target gene expression.
Diet-induced obesity (DIO): a mouse model of prediabetic type 2 diabetes and obesity with elevated blood glucose and impaired glucose tolerance.
Experimental autoimmune encephalomyelitis (EAE): a mouse model of autoimmunity. Symptoms and disease progression in EAE are similar to those experienced by multiple sclerosis patients.
Hormone response element (HRE): a short DNA sequence in the promoter of a gene that binds a specific NR complex and regulates transcription. An HRE is most commonly composed of two inverted repeats separated by three nucleotides, which allows the receptor to bind as a dimer.
Ligand-binding domain (LBD): a domain found in NRs that is highly conserved between the various NR where ligands bind and modulate gene transcription. The LBD contributes to the dimerization interface of the receptor and in addition binds coactivator and corepressor proteins.
Nuclear receptors (NRs): highly conserved transcription factors that generally regulate gene transcription in a ligand-dependent manner. Steroid hormones are perhaps the most recognized members of the NR superfamily.
Orphan receptors: a NR is considered to be an orphan receptor when it has no known, or generally agreed upon, endogenous ligand(s).
Retinoic acid receptor-related orphan receptor (ROR): a member of the NR superfamily. There are three isoforms of ROR – ROR-α, -β, and γ – encoded by different genes. RORs bind as monomers to hormone response elements as opposed to the majority of other nuclear receptors which bind as dimers.
T helper 17 cells (T_H17): a subset of T helper cells that are developmentally distinct from T_H1 and T_H2 cells and that produce interleukin 17 (IL-17). T_H17 cells are thought to play a key role in autoimmune disease such as multiple sclerosis, psoriasis, juvenile diabetes, rheumatoid arthritis, and Crohn's disease.
Type II collagen-induced arthritis (CIA): an animal model of polyarthritis that is induced by immunization of susceptible mice and rats with type II collagen.

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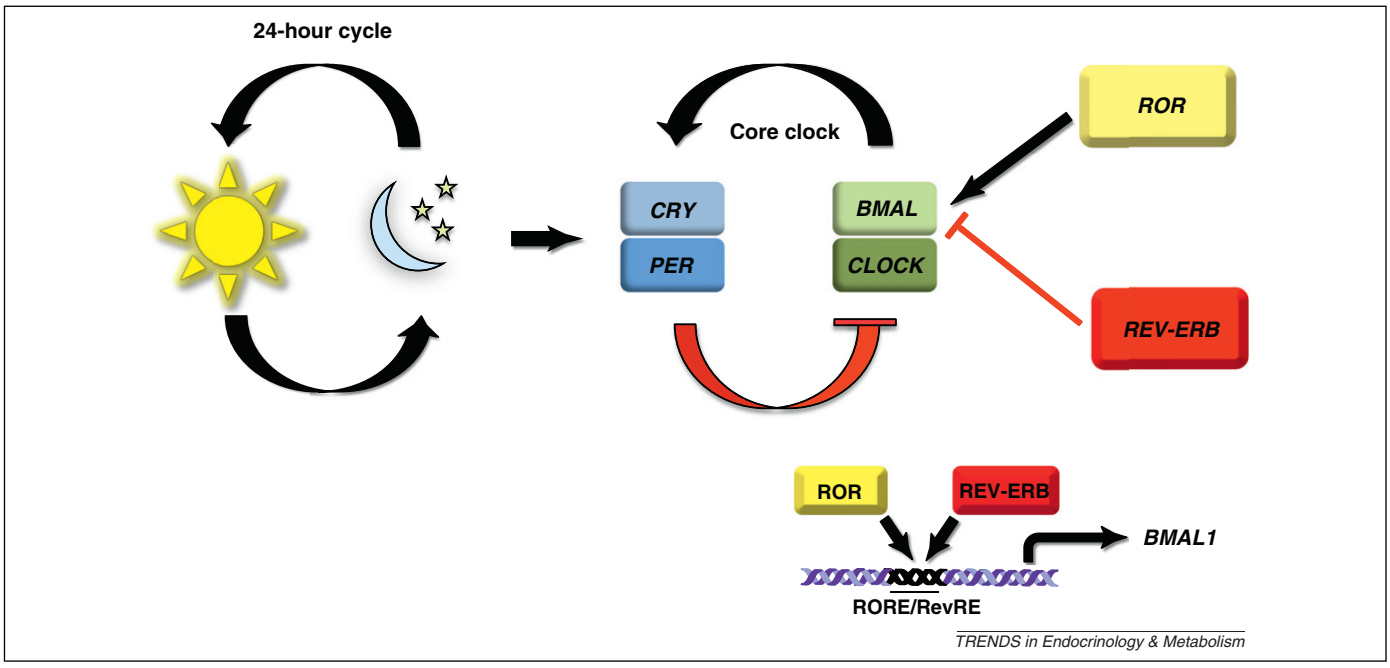


Figure 1. Retinoic acid receptor-related orphan receptor (ROR) regulation of the circadian rhythm. Circadian rhythms are biological processes that display endogenous oscillations of approximately 24 h and are regulated by a core circadian clock. The master circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. There are several interconnected transcriptional autoregulatory feedback loops controlling the circadian cycle. Heterodimers of BMAL1 and CLOCK activate the expression of *CRY* and *PER* genes. Once the level of CRY/PER heterodimers reaches a critical threshold they enter the nucleus and repress BMAL/CLOCK transactivation. RORα and REV-ERBα have been demonstrated to regulate positively and negatively the expression of BMAL1, respectively. RORα competes with REV-ERBα for binding of their shared DNA response element in the *BMAL1* promoter. The oscillating pattern of RORα and REV-ERBα in the SCN dictates the circadian pattern of BMAL1 expression. This RORα/REV-ERBα feedback loop interconnects the positive and negative arms of the core circadian clock.

continual activation of transcription of their target genes [7,8]. Another group of NRs, the REV-ERBs, recognize the same response elements as the RORs and are coexpressed in many tissues [9–11]. The REV-ERBs are ligand-dependent transcriptional repressors and, in many cases, functionally antagonize the action of the RORs [12–14].

The three RORs display significant sequence similarity and conservation between species. Each ROR generates multiple isoforms based on alternative promoter usage and exon splicing, with all of the isoforms varying only in the N-terminal region of the receptor [7]. The RORs display distinct patterns of tissue expression and are involved in the regulation of various physiological processes. RORα is widely expressed and is found in liver, skeletal muscle, skin, lungs, adipose tissue, kidney, thymus, and brain [15,16]. The expression of RORβ is extremely restricted and is limited to the central nervous system [17,18]. RORγt has been the focus of considerable attention due to its role in T helper 17 cell (T_H17) development and autoimmune disease pathology. RORγ, specifically RORγ2 (also termed RORγt), is highly expressed in immune tissues, including the thymus, but there is significant expression of RORγ in the liver, skeletal muscle, adipose tissue, and kidney [7]. Due to significant sequence and functional similarities, ROR subtypes coexpressed in cells may exhibit functional overlap [7]. However, the physiological relevance and responsiveness of all of the different isoforms of each ROR have yet to be clarified.

ROR regulation in circadian rhythms

Circadian rhythms are daily cycles of biochemical, behavioral, and physiological processes controlled by endogenous 'clocks' that play essential roles in the regulation of the physiology of an organism, including metabolism (Figure 1) [19]. In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Aberrant circadian rhythms are associated with numerous disorders in humans, including sleep and mood disorders. The circadian rhythm is generated by a feedback loop where heterodimers of BMAL1 and CLOCK (the positive arm) activate the expression of the cryptochrome (*Cry*) and period (*Per*) genes (the negative arm). RORα is a core part of the clock machinery that positively regulates the expression of BMAL1 [20,21]. RORα competes with REV-ERBα for binding to their shared DNA response element in the *BMAL1* promoter, resulting in REV-ERBα-mediated repression or RORα-mediated activation of BMAL1 expression [21–23]. This oscillating expression of RORα and REV-ERBα in the SCN leads to the circadian pattern of *BMAL1* expression, thus interconnecting the positive and negative arms of the core circadian clock (Figure 1). Therefore, RORα influences the period length and stability of the clock [20].

Genetic models in which the RORs are either modified or have been deleted have been instrumental in identifying their roles in the circadian rhythm. The *staggerer* mouse ($RORα^{sg/sg}$) is a natural mouse mutant that carries an intragenic insertion within the RORα gene, and this results in a frameshift and premature stop codon, rendering RORα inactive [15]. *Staggerer* mice exhibit severe cerebellar ataxia as well as a shortened period length when placed under constant dark conditions [20]. $RORβ^{-/-}$ mice also exhibit aberrant circadian rhythm, such that under constant dark conditions $RORβ^{-/-}$ mice have a longer

period length than wild-type (wt) mice [17]. Although no overt circadian abnormalities were apparent in RORγ$^{-/-}$ mice, recent work has demonstrated that RORγ directly regulates neuronal PAS domain protein 2 (Npas2) *in vivo* suggesting a regulatory role for this receptor in Npas2-dependent physiological processes [7,24]. Likewise, several lines of evidence suggest a link between disrupted circadian rhythms and cardiovascular disease, metabolic disturbances, and mood disorders [25]. Given its extensive role in the regulation of the circadian rhythm, targeted modulation of RORα appears a feasible means by which to regulate these disorders.

RORs in metabolism and metabolic disease

The aforementioned genetic models have also been invaluable in identifying the roles of the RORs in physiological processes. On a normal diet, *staggerer* mice display hypo-α-lipoproteinemia, have lower total plasma cholesterol levels, lower high-density lipoprotein (HDL), apolipoprotein AI (Apoa1, the major constituent of HDL), lower apolipoprotein CIII levels (Apoc3), Apoa2, and triglycerides, compared to wt mice [26–28]. *Staggerer* mice have decreased expression of the reverse cholesterol transporters Abca1 and Abca8/g1 in their liver and intestine, and are much less susceptible to hepatic steatosis and weight gain, compared to wt mice [29]. Sterol regulatory element-binding protein 1, isoform c (Srebp-1c) is reduced in the liver and muscle of *staggerer* mice as is the enzyme fatty acid synthase (Fas) [29,30]. Expression of the coactivators peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α and β, proteins involved in the regulation of oxidative metabolism and gluconeogenesis, are increased in *staggerer* mice [31]. Furthermore, expression of the P450 enzyme Cyp7b1 is reduced in *staggerer* mice. RORα directly regulates Cyp7b1 expression by binding to a functional RORE in the promoter regulatory region of the *Cyp7b1* gene [30,32]. These observations suggest that RORα functions as a positive regulator of *Cyp7b1* function [30,32]. *Staggerer* mice also have smaller brown and white adipose cells than wt mice and, when fed a high-fat diet, *staggerer* mice are resistant to weight gain and hepatic steatosis [29].

Evidence supporting a role for RORα in glucose metabolism derives from studies in steroid receptor coactivator-2 (SRC-2) knockout mice. These mice display symptoms similar to von Gierke's disease, which is associated with severe hypoglycemia and abnormal accumulation of glucose in the liver. SRC-2 controls the expression of hepatic glucose-6-phosphatase (G6Pase), an enzyme that is crucial for maintaining fasting blood sugar levels by increasing hepatic glucose production and coactivates RORα bound to the RORE on the G6Pase (*G6PC*) gene promoter [33]. Finally, it was recently demonstrated that RORα controls the expression and secretion of fibroblast growth factor 21 (FGF21), a hepatic hormone that regulates peripheral glucose tolerance and hepatic lipid metabolism [34]. Because RORα is crucial in regulating the expression of key enzymes in the gluconeogenic pathway, suppression of RORα activity may lead to a decrease in the elevated hepatic glucose-output levels observed in type 2 diabetes (T2D).

Initial characterization of RORγ$^{-/-}$ mice revealed that they display normal cholesterol and triglyceride levels, but have slightly lower blood glucose levels than their wt counterparts [30]. However, recent evidence suggests that RORγ may indeed have a role in metabolism through the regulation of adipogenesis and insulin sensitivity. Meissburger *et al.* demonstrate that RORγ is a negative regulator of adipocyte differentiation *in vitro*. When overexpressed during adipocyte differentiation, RORγ decreases the amount of differentiated adipocytes. However, *in vivo* differentiation of adipocyte precursors in RORγ$^{-/-}$ mice was enhanced but showed decreased size. The smaller adipocytes were insulin sensitive and protected the mice from obesity-induced hyperglycemia and insulin resistance [35]. Moreover, analysis of adipose stromal–vascular fractions from obese human subjects demonstrated a positive correlation between RORγ expression and adipocyte size that was negatively correlated with adipogenesis and insulin sensitivity. These findings suggest that RORγ may be a novel target for the treatment of obesity-associated insulin resistance [35].

Deletion of both RORα and RORγ leads to similar changes in cholesterol, triglyceride, and blood glucose levels as in single-knockout mice. Gene expression analysis from livers of double-knockout (DKO) mice suggests a degree of functional redundancy between RORα and RORγ which is most probably due to the similarities in RORE binding affinities [30]. However, the recent evidence regarding obesity and insulin resistance in the RORγ$^{-/-}$ mice highlights the differences between the two NRs in metabolic processes.

RORs and (auto)immunity

Host defense against invading pathogens is largely dependent upon distinct adaptive immune responses facilitated by the differentiation of CD4$^+$ T cells into specific lineages of effector T helper cells (T_H1, T_H2, and T_H17 cells) [36]. Both RORα and RORγ, specifically RORγt, have generated significant attention over the past few years due to their essential role in the development of T_H17 cells. Until recently, it was generally thought that there were only two T helper subsets within the CD4$^+$ T cell repertoire, T_H1 and T_H2. T_H1 cells mediated cellular immunity against intracellular bacteria and viruses whereas T_H2 cells were thought to be involved in the humoral response to parasitic pathogens [36]. However, T_H1 cells that respond to self-antigen can lead to autoimmune diseases whereas dysregulation of T_H2 responses to allergens and parasites can cause specific allergic and parasitic pathology. T_H1 cells had long been thought to be the mediators of tissue damage in autoimmune disease [36]. Key experiments using two established mouse models of autoimmunity, experimental autoimmune encephalomyelitis (EAE) and type II collagen-induced arthritis (CIA), largely led to the discovery of another T helper subset known as T_H17 cells (Figure 2). In this setting, T_H17 cells were crucial mediators of much of the pathology associated with EAE and CIA. T_H17 cells are defined by a specific cytokine profile and secrete IL-17, IL-9, IL-21, IL-22, IL-26, and CCL20 [37]. These mediators are responsible for several different effector functions in host defense as well as in autoimmune diseases.

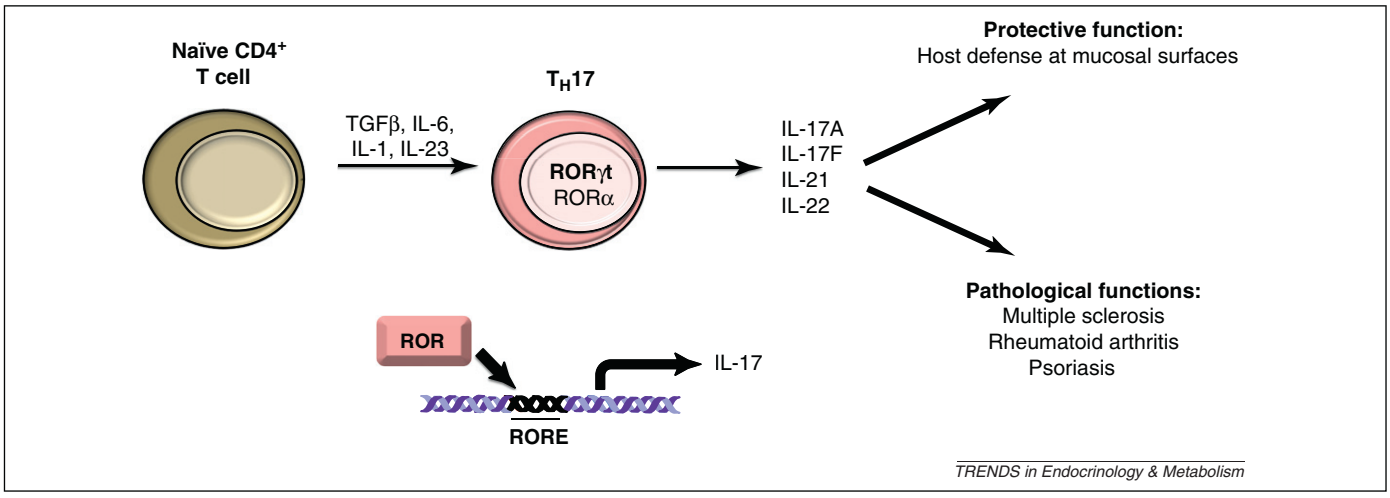


Figure 2. Retinoic acid receptor-related orphan receptor (ROR)α and RORγ in T_H17 cell differentiation. In the presence of several exogenous factors, including TGFβ, IL-6, and IL-1, naïve $CD4^+$ T cells differentiate into T_H17 cells. Exogenous IL-23 is necessary for the propagation of pathogenic T_H17 cells. The expression of RORα and particularly RORγt is necessary for T_H17 cell differentiation and for the expression of IL-17A and IL-17F, among other cytokines. T_H17 cells play a significant role in host defense against extracellular pathogens at mucosal surfaces. However, aberrant T_H17 cell activity has been associated with the pathology of several autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and psoriasis.

Despite the negative implications for T_H17 cells, this cell type plays a significant role in host defense against extracellular pathogens, specifically Gram-negative bacteria at mucosal surfaces, as well as against obligate intracellular pathogens, including intracellular bacteria and fungi [38]. In addition, T_H17 cells have been shown to exhibit general tissue-protective functions [37].

Key factors in the development of T_H17 cells involve the RORs, specifically RORα and RORγt, one isoform of RORγ that is exclusively detected in a few distinct types of cells in the immune system [39]. Overexpression of RORγt in naïve $CD4^+$ T cells was demonstrated to drive the induction and development of T_H17 cells [40]. Furthermore, RORγt$^{-/-}$ mice display impaired T_H17 cell development [40]. Mice deficient in both RORα and RORγ completely lack T_H17 cells and are resistant to the development of several autoimmune diseases, including EAE [41,42]. Collectively, these data suggest that targeted inhibition of RORα and RORγ with specific synthetic ligands could potentially provide a means for reducing autoimmune pathology.

Regulation of RORs by endogenous ligands

The ligand-binding domains of NRs are multifunctional. Typically, ligand binding induces a conformational change in the receptor resulting in dissociation of corepressors and recruitment of coactivators [1]. However, RORs are constitutively active – meaning that they are in an active conformation in the absence of ligand, and that ligand binding might actually repress receptor activity (inverse agonist; Box 1). Although identification of the endogenous ligands for RORs has been controversial, recent evidence suggests that, similarly to the liver X receptors (LXRs), oxygenated sterols may function as high-affinity ligands. Indeed, 7-oxygenated sterols [7α-OHC (7α-hydroxycholesterol), 7β-OHC, and 7-ketocholesterol] function as inverse agonists for both RORs. The 7-oxygenated sterols bind to both RORα and RORγ isoforms with a significantly greater affinity than do cholesterol and cholesterol sulfate, and suppress their transactivation properties. It was also shown that both RORα and RORγ are constitutively active in the absence of ligand, and are able to bind coactivator peptides and activate transcription. Furthermore, the 7-oxygenated sterols modulated the expression of RORα/γ-dependent target genes in a receptor-dependent manner [8] and were able to induce the conformational change necessary to alter cofactor binding and transcriptional activity, a core prerequisite for a *bona fide* ligand (Figure 3).

Box 1. Definition of an NR ligand

NRs are generally characterized as ligand-dependent transcription factors. Typically, NR ligands are small hydrophobic molecules including steroid hormones, fatty acids, and lipophilic vitamin derivatives. True ligands bind in the LBD of NRs inducing a conformational change within the receptor, thereby providing an interface for cofactor binding. Cofactors can be either coactivators or corepressors. Ligands are classified according to their ability to regulate the transcriptional activity of specific NRs.

Agonist: an agonist binds to the LBD and induces a conformational change resulting in increased recruitment of coactivator proteins. This results in maximal alterations in target gene transcription.

Antagonist: an antagonist does not provoke a response from the receptor. Instead, an antagonist binds to the LBD and blocks the ability of an agonist to bind and activate the receptor.

Inverse agonist: an inverse agonist binds within the LBD of a given receptor, but inhibits the basal constitutive activity of the receptor. This generally describes a ligand for a particular NR that is not bound by any ligand in its basal conformation but is able to interact with a cofactor protein (either coactivator or corepressor), leading to constitutive transcriptional activity. An inverse agonist induces a conformational change within the receptor that decreases the affinity of the receptor for a cofactor protein and thereby represses transcription.

Partial agonists: partial agonists bind to and activate a receptor, but only with partial efficacy relative to a ligand that elicits a maximal response.

The RORs have intrinsic transcriptional activity, meaning that they are constitutively active, because it has been demonstrated that they bind coactivator proteins in the absence of ligand. Ligand binding represses the transcriptional activity of the receptor.

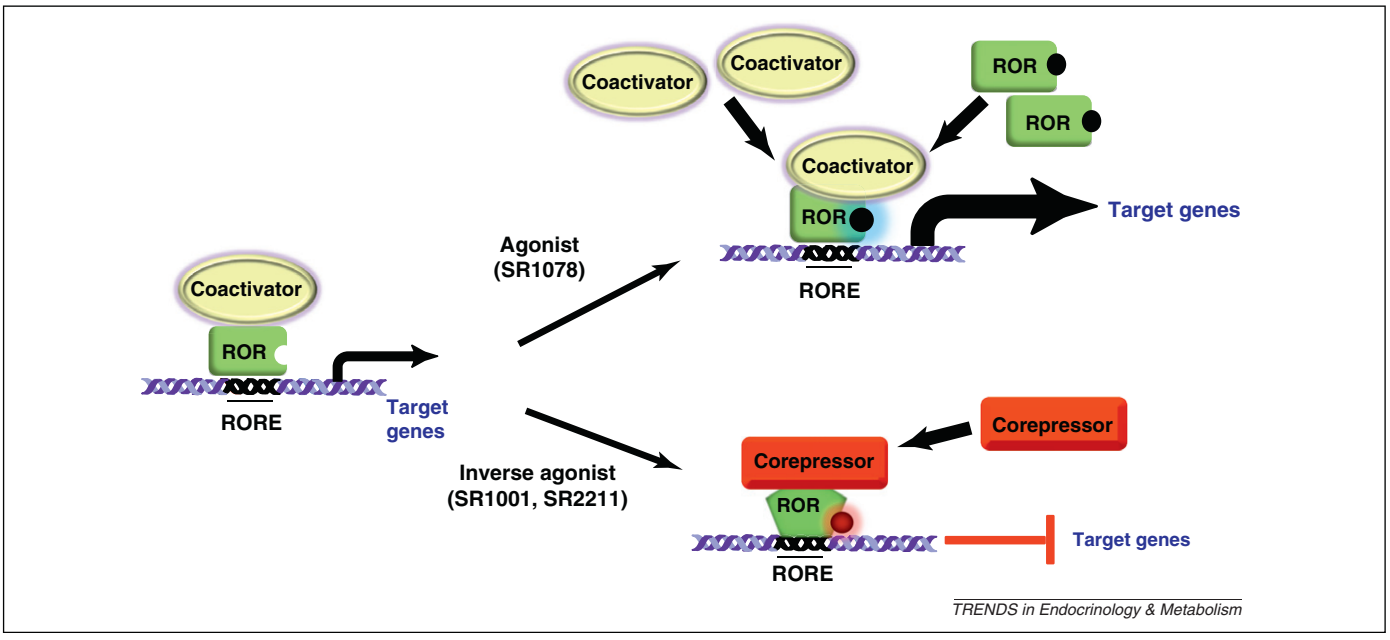


Figure 3. Regulation of retinoic acid receptor-related orphan receptor (ROR) activity with synthetic ligands. The RORs are considered to have intrinsic transcriptional activity, meaning that they are constitutively active and bind coactivators in the absence of ligand. However, owing to the ubiquitous expression of putative ROR ligands, it remains to be determined whether the RORs are ever in an unbound state or require ligand for receptor stability. Treatment with an agonist (SR1078) would result in the recruitment of more coactivator proteins, thereby enhancing transcriptional activity. Inverse agonists, when bound to the ROR LBD, induce a conformational change in the receptor resulting in dissociation of coactivator proteins and recruitment of corepressor proteins. Inverse agonists repress the activity of the receptors.

Several other endogenous RORα and RORγ ligands have been described recently. 24*S*-hydroxycholesterol (24*S*-OHC) is a high-affinity ligand for RORα and RORγ, and, similarly to the 7-oxygentated sterols, 24*S*-OHC acts as an inverse agonist and dose-dependently reduces RORα and RORγ constitutive activity [43]. As a consequence, expression of *BMAL1* and *REV-ERBα* mRNAs are also reduced. In a similar manner, 24*S*,25-epoxycholesterol (24,25-epoC) and 24*R*-cholesterol (24*R*-OHC) also selectively bind to and regulate the activity of RORγ [43].

20α-OHC, 22*R*-OHC, and 25-OHC were also shown to be putative endogenous ligands for RORγ [44] because all three ligands dose-dependently increased the recruitment of coactivator peptides to RORγ *in vitro* [44]. In addition, elucidation of the RORγ crystal structure revealed that these three ligands bind to RORγ in a similar manner. The RORγ crystal structures also demonstrated that the AF-2 domain at the C terminus of the receptor, together with helices H3, H4, and H5, form a charge clamp pocket, the area that facilitates binding of coactivator proteins to NRs. Mutational studies of this region revealed that an intact charge clamp pocket was required for these hydroxycholesterols to affect RORγ activity [44] (Table 1).

Despite the identification of these putative ligands for the RORs, their physiological significance and whether they are regulatory or structural is not clear. For instance, several studies using RORα LBD purified from insect cells identified and characterized cholesterol, cholesterol sulfate, and several other cholesterol derivatives as endogenous putative ligands [45,46]. Although subsequent studies have since established that several of these ligands are fortuitous, they suggest a requirement for ligand-bound LBD for receptor stability [47]. Mutations in several key amino acids known to be involved in ligand binding abolishes the constitutive activity of the receptor, and this could be attributed to the instability of the receptor in the absence of ligand [48]. Furthermore, much of the work describing ROR ligands has been performed using artificial systems *in vitro* with luciferase reporter systems. To date, only the 7-oxygenated sterols and 24*S*-OHC, 24,25-epoC, and 24*R*-OHC were shown to affect ROR target gene expression *in vitro* [8,43]. It is also difficult to envisage how many of these putative ligands could function as regulatory ligands given their abundance within tissues. Whether these ligands associate with the RORs *in vivo* has yet to be determined. These questions need to be answered to determine whether the described ROR ligands are deemed 'endogenous' and regulatory. With this in mind, the use of these ligands as 'tools' to understand the biology of the RORs should be met with caution. Despite some caveats, these data suggest that the RORs may function as lipid sensors and thus play a major role in the regulation of lipid metabolism.

Modulation of ROR activity with synthetic ligands

The identification of endogenous ligands to RORα and RORγ intensified the search for synthetic ligands that could modulate ROR activity (Table 1). The synthetic LXR agonist T0901317 was the first synthetic inverse agonist identified for both RORα and RORγ. Despite its potency at activating RORα and RORγ, T0901317 displays promiscuity and binds to several NRs including LXR, farnesoid X receptor (FXR), and pregnane X receptor (PXR), thus limiting its use as a chemical tool to explore the activity of the RORs in physiological settings [49,50].

A focused medicinal chemistry approach to develop analogs of T0901317 that activated RORs but not other NRs led to the development of several ROR-selective modulators. The first RORα/γ-specific synthetic ligand characterized was the amide SR1078 (Figure 3). SR1078 was

Table 1. Structure of ROR ligands – both natural and synthetic ligands are presented

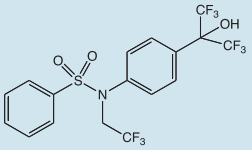
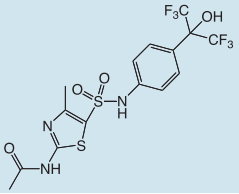
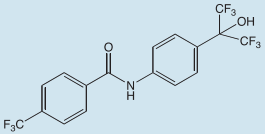
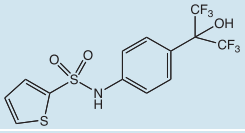
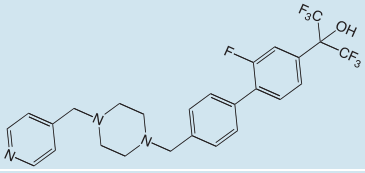
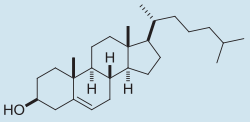
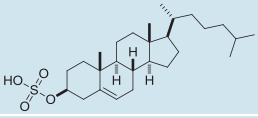
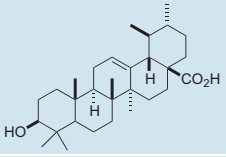
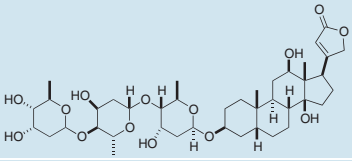
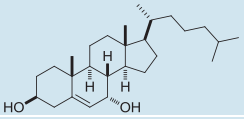
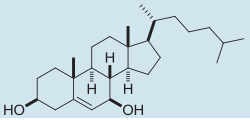
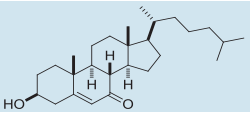
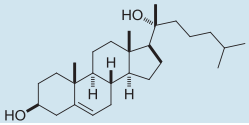
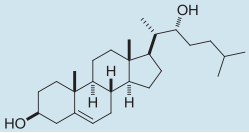
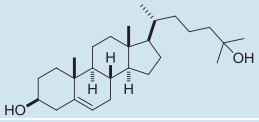
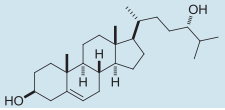
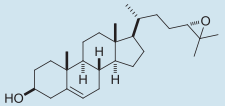
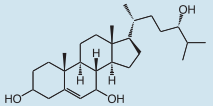
Name	Structure	Origin	Receptor preference	Ligand type	Affinity (Ki)	Refs
T0901317		Human NR specificity screen	RORα RORγ LXRα LXRβ PXR FXR other	RORs: inverse agonist LXRs, PXR, FXR: agonist	RORα: 132 nM RORγ: 51 nM	[49,50,66,67]
SR1001		Synthetic small-molecule analog of T0901317	RORα RORγ	Inverse agonist	RORα: 172 nM RORγ: 111 nM	[55]
SR1078		Synthetic small-molecule analog of T0901317	RORα RORγ	Agonist	IC_{50} 1–3 μM	[51]
SR3335		Synthetic small-molecule analog of T0901317	RORα	Inverse agonist	220 nM	[54]
SR2211		Synthetic small-molecule analog of T0901317	RORγ	Inverse agonist	105 nM	[61]
Cholesterol		Sf-9 insect cells Ubiquitously expressed in all mammalian cells	RORα	Agonist	EC_{50} 200 nM	[44,45]
Cholesterol sulfate		Sf-9 insect cells Ubiquitously expressed in all mammalian cells	RORα	Agonist		[46]
Ursolic acid		Small chemical library screen, carboxylic acid expressed in plants	RORγ	Inverse agonist	IC_{50} 680 nM	[58]
Digoxin		Chemical screen, isolated from the foxglove plant	RORγ	Inverse agonist	K_d 109 nM	[56]
7α-Hydroxycholesterol		Screen of oxysterols for ROR activity	RORα RORγ	Inverse agonist	RORα: 12–18 nM RORγ: 17–31 nM	[8]
7β-Hydroxycholesterol		Screen of oxysterols for ROR activity	RORα RORγ	Inverse agonist	RORα: 12–18 nM RORγ: 17–31 nM	[8]
7-Ketocholesterol		Screen of oxysterols for ROR activity	RORα RORγ	Inverse agonist	RORα: 12–18 nM RORγ: 17–31 nM	[8]

Table 1 (*Continued*)

Name	Structure	Origin	Receptor preference	Ligand type	Affinity (Ki)	Refs
20α-Hydroxycholesterol		Alpha screen of cholesterol and hydroxycholesterols	RORγ	Agonist	EC_{50} 20–40 nM	[44]
22R-Hydroxycholesterol		Alpha screen of cholesterol and hydroxycholesterols	RORγ	Agonist	EC_{50} 20–40 nM	[44]
25-Hydroxycholesterol		Alpha screen of cholesterol and hydroxycholesterols	RORγ	Agonist	EC_{50} 20–40 nM	[44]
24S-Hydroxycholesterol		Screen of oxysterols for ROR activity	RORα RORγ	Inverse agonist	25 nM	[43]
24,25-Epoxy-cholesterol		Screen of oxysterols for ROR activity	RORγ	Inverse agonist	20 nM	[43]
24R-Hydroxycholesterol		Screen of oxysterols for ROR activity	RORγ	Inverse agonist	102 nM	[43]

initially identified as an inverse agonist because it repressed the constitutive activity of RORα and RORγ and inhibited the recruitment of coactivators to RORγ in a dose-dependent manner [51]. However, further examination revealed that SR1078 acts as an agonist and stimulated expression of two ROR target genes, *G6Pase* and *FGF21*, in the liver. Pharmacokinetic studies revealed that SR1078 displays reasonable plasma exposure, thus enabling its use as a chemical tool to probe the function of RORα and RORγ both *in vitro* and *in vivo* [51].

RORα expression is induced in response to some types of cellular stress and is downregulated in several breast, prostate, and ovarian cancer cell lines [52]. Interestingly, activation of RORα by SR1078 in this setting results in an increase in p53 levels and apoptosis, suggesting that RORα represents a novel target for the development of cancer therapeutics [53].

The inverse agonist, SR3335 (Table 1), was initially identified based on its ability to inhibit the constitutive activity of RORα. Furthermore, SR3335 bound directly to the LBD of RORα, with little effect at RORγ, and suppressed expression of RORα target genes involved in hepatic gluconeogenesis – including G6Pase (*GCPC*) and phosphoenolpyruvate carboxykinase (*PCK2*). Pharmacokinetic studies revealed that SR3335 had reasonable plasma exposure and administration of this ligand to diet-induced obese (DIO) mice led to reduced plasma glucose levels following a pyruvate tolerance test (PTT), an indicator of gluconeogenesis [54]. Given that elevated glucose output is observed in T2D, suppression of RORα activity with novel ligands such as SR3335 may hold utility in the treatment of metabolic disorders, including T2D.

With the accumulating evidence surrounding the roles of RORα and RORγt role in T_H17 cell development and autoimmune pathology, identification of a dual and highly selective RORα/γ inverse agonist that inhibits T_H17-mediated pathology is extremely enticing and such efforts led to the identification and characterization of SR1001 (Table 1), a first-in-class RORα/γ-specific inverse agonist (Figure 3). SR1001 binds directly to the LBD of both RORα and RORγ, resulting in a conformational change that decreases affinity for coactivators and increased affinity for corepressors [55]. When screened against all 48 human nuclear receptors, SR1001 displayed activity only at RORα and RORγ. *In vitro*, SR1001 inhibited IL-17 expression and T_H17 cell development without affecting the differentiation and function of any of the other T helper cell lineages [55]. More importantly, *in vivo* administration of SR1001 delayed the onset and severity of EAE through inhibition of T_H17 cell development and function. These data demonstrate that small-molecule inhibitors of ROR activity are effective at suppressing T_H17-mediated autoimmune diseases [55].

Huh *et al*. identified the well-known cardiac glycoside digoxin (Table 1), a small-molecule inhibitor of RORγ activity. Currently, digoxin is used clinically in the treatment for various heart conditions. Digoxin normally competes with K^+ ions for the same binding site on the Na^+/K^+ ATPase pump, thereby altering electrical conduction in the heart. Digoxin suppressed RORγ-mediated activity only, and displayed no activity for RORα, *Drosophila* hormone receptor 3 (DHR3), the *Caenorhabditis elegans* nuclear hormone receptor Dauer formation-12 (DAF-12), or the androgen receptor [56]. Digoxin inhibited T_H17 cell differentiation and function and delayed the onset and severity of EAE [56]. Despite its efficacy in this model, major drawbacks with digoxin are its toxicity, the occurrence of adverse drug reactions associated with use of this drug, and a

narrow therapeutic window. Given these issues, less-toxic digoxin analogs have been derived that are able to inhibit RORγ activity and T_H17 cell differentiation and function, *in vitro* [56]. The crystal structure of the RORγ LBD bound to digoxin was recently resolved demonstrating the mechanism by which digoxin inhibited RORγ activity. Similarly to SR1001, when bound to RORγ, digoxin inhibited coactivator binding [57]. These findings demonstrate the feasibility of targeting RORγ or both RORα and RORγ with small molecules for the treatment of T_H17-mediated autoimmune disorders.

Ursolic acid has also been demonstrated to target RORγ and thus inhibit T_H17 cell differentiation. When administered *in vivo*, mice treated with ursolic acid exhibited a delay of onset with decreased severity of symptoms of EAE. Biochemical assays indicate that ursolic acid effectively binds to the LBD of RORγ, leading to displacement of coactivator binding, whereas it had little effect at RORα [58]. Ursolic acid, which is present in many plants, including apples, was originally described as a potential anticancer therapeutic able to inhibit various types of cancer cells by inhibiting STAT3 activation [59]. Further examination suggested that ursolic acid reduced the expression of matrix metalloproteinase-9 (MMP-9) potentially by acting through the glucocorticoid receptor (GR) [60]. Given the steroidal-like structures of both ursolic acid and digoxin, the possibility that both compounds exhibit activity at GR complicates the *in vivo* interpretations. Glucocorticoids are very effective at inhibiting symptoms of EAE and are in fact routinely prescribed by neurologists to reduce the severity and duration of relapses in MS patients.

Although SR1001 was effective at delaying the onset and reducing the severity of EAE, there was some concern that this compound, which modified the activity of RORα, would induce a phenotype similar to that of the *staggerer* mouse, including ataxia and a disrupted circadian rhythm [15,55]. Furthermore, although several RORγ selective modulators had already been described, their utility as candidates for further drug development was limited. Therefore, further development of RORγ modulator was warranted. SR2211 is a selective RORγ modulator that binds to the LBD of RORγ and functions as an inverse agonist to suppress receptor activity [61]. Therefore, SR2211 is a potent and efficacious RORγ modulator with potential utility in the treatment of T_H17-mediated autoimmune disorders.

Despite their high-profile roles in T_H17-mediated autoimmunity, RORγt and RORα expression is not restricted to this cell type, nor are all T_H17 cells pathogenic. Recent evidence links RORα to the maintenance of IgA^+ memory B cells [62]. Particular types of innate lymphoid cells, including lymphoid tissue inducer cells (LTi), γδ T cells, and intestinal epithelial cells (IEPs), express RORγt [63,64]. Innate lymphoid cells play important roles in tissue surveillance and can be the first line of defense against several invading pathogens [64]. Similarly, T_H17 cells have proved to be essential for host defense against some Gram-negative bacteria and fungal infections at mucosal surfaces [65]. Given the increasing number of immune cells expressing the RORs, inhibiting their activity during particular immune-system assaults may be detrimental. Therefore, careful assessment of the infection and invading pathogen(s) may be warranted before administration. Alternatively, the control of some infections due to specific Gram-negative bacteria or fungi could exploit ROR agonists to amplify the immune response from these ROR-restricted cell types.

Concluding remarks

To date, several groups have developed or described numerous small-molecule ligands for RORα and RORγ. Collectively, these data demonstrate that these orphan NRs are not only valid drug targets, but have efficacy at suppressing T_H17 cell development and function both *in vitro* and *in vivo*. Although further optimization of the small molecules is still needed, it is obvious that targeting the RORs for the treatment of T_H17-mediated autoimmune disorders represents a promising endeavor. Current treatments for known T_H17-mediated autoimmune diseases, including multiple sclerosis, use agents that are general immunosuppressants, and thus the side-effect profile is significant. Targeting of the RORs presents a significant advantage over the current therapies because they specifically target the one arm of the immune system that mediates disease instead of the immune system as a whole. Finally, extensive analysis of the use of these ligands *in vivo* has yet to be carried out. Although genetic studies are valuable tools for elucidating the roles these receptors play in physiology, the roles of the RORs during metabolic and autoimmune disease progression can be extensively studied through *in vivo* use of specific synthetic ligands.

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