REVIEW

The therapeutic potential of RORγ modulators in the treatment of human disease

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Abstract: Nuclear receptors (NR) are ligand-regulated transcription factors that bind DNA in proximity to their target genes and exert their effects as a result of binding by small molecule ligands such as sterols, lipids, fatty acids, retinoids, and steroid hormones. The retinoic acid receptor-related orphan receptors or RORs (NR1F1–NR1F3) are nuclear receptors that regulate multiple cellular processes, including metabolism, cellular differentiation, and apoptosis, in a range of tissues and organs. These receptors bind as monomers to ROR response elements commonly called ROREs present in promoter regions of target genes and tether chromatin remodeling enzymes, facilitating recruitment of transcription machinery. Several recent reports have highlighted the potential role for RORs in human disease, and more importantly, studies have demonstrated that these receptors can be modulated by exogenous synthetic ligands, paving the way for development of novel therapeutics. Here we review the current status of synthetic ligand development as well as the structural aspects governing modulation of ROR signaling pathways as they relate to metabolic diseases and autoimmune disorders.

Keywords: retinoic acid receptor-related orphan receptors (ROR), nuclear receptor (NR) modulator, synthetic ligand, inverse agonists, metabolic disorder, autoimmune disorder, ligand-binding domain (LBD)

Introduction

Members of the nuclear receptor (NR) superfamily regulate the expression of target genes involved in a range of physiological processes such as development, metabolism, and immunity. All members of the superfamily are multidomain proteins, with many consisting of a highly variable N-terminal "A/B domain" that contains a ligand-independent activation function domain (AF-1) that can engage transcriptional coregulators regardless of receptor-ligand status, a two zinc finger DNA-binding "C domain," a highly variable hinge "D domain," and an "E domain" ligand-binding domain (LBD) that contains the ligand-dependent activation function-2 cofactor-binding surface (Figure 1).[1,2] Several NRs, such as the estrogen receptor, contain an additional C-terminal "F domain" whose functional role is poorly defined.

The LBDs of the nuclear receptors are multifunctional (eg, bind to ligands and interact with coregulators and corepressors) and have the secondary domain structure (predominately α-helical) that is characteristic of all NRs. While there are some NRs that likely do not bind ligands, most NRs activate or repress transcription of their target genes in response to binding small molecules or upon specific post-translational modifications. Endogenous NR ligands include small molecules such as fatty acids, bile acids, cholesterol metabolites, and steroid hormones. Synthetic ligands that function as

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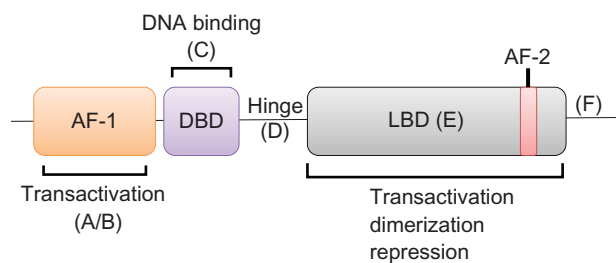Fax +1 561 228 3081
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Figure 1 Canonical domain structure of nuclear receptors (NRs).
Notes: Most members of the nuclear receptor superfamily contain the following functional domains: an N-terminal domain containing activation function 1 (AF1, also known as the A/B domain), a highly conserved zinger finger containing DNA-binding domain or C domain, a highly variable hinge or D domain, and a C-terminal ligand-binding domain (LBD) or E domain that contains activation function 2 (AF2). Several NRs contain a highly variable C-terminal F domain.

activators (agonists that increase activity over basal levels), neutralizers (antagonists, that keep receptor activity at basal levels), and inhibitors or repressors (inverse agonists that lower activity below basal levels) have been developed for many of the NR family members. Several examples of such modulators are used clinically in the treatment of diabetes and dyslipidemia (NR1C1;PPARα and NR1C3;PPARγ), inflammation (NR3C1;GR), cancer (NR3C3;PR), and osteoporosis (NR3A1;ERα).

Of the 48 genes in the human NR superfamily, several are still considered "orphan receptors" because their endogenous ligands have not yet been identified. For instance, the NR1F subfamily containing the retinoic acid receptor-related orphan receptors (RORs), named for their sequence homology to the retinoic acid receptor, referred to as RAR, are considered orphan receptors, although strong evidence has shown that these receptors potently bind to and are modulated by oxysterols.[3] The ROR subfamily contains three major isoforms: RORα (NR1F1), RORβ (NR1F2), and RORγ (NR1F3).[4–7] The RORs display significant sequence similarity, and each ROR gene generates several isoforms, differing only in their amino termini, as a consequence of alternative promoter usage and exon splicing. Four different RORα isoforms have been shown to be expressed in lung, muscle, brain, heart, peripheral blood leukocytes, spleen, liver, and ovary.[5,8] In contrast, RORβ exhibits a more restricted neuronal-specific expression pattern in brain, retina, and pineal gland.[5,9] RORγ has been shown to be highly expressed in thymus (the thymus-specific isoform is referred to as RORγt), muscle, testis, pancreas, prostate, heart, and liver.

The RORs have been shown to bind to DNA as monomers on half-site elements with a 5′-A/T-rich extension, which is identical to the response elements of the orphan NRs known as the Rev-erbs.[6,10] Like other NRs, the RORs have been shown

to interact with transcriptional coactivators such as SRC1 (NCOA1),[11] SRC2 (NCOA2 or TIF2),[12] PGC1α,[13] and p300/CBP, and with transcriptional corepressors such as NCOR1, SMRT (NCOR2),[14] RIP140,[15] and neuronal interacting factor X (NIX1) (Figure 2).[16] RORα is considered to be constitutively active, with the ability to interact with coregulatory proteins without the need to bind ligand. Structural studies have suggested that the coactivator-binding surface (AF2) of RORα is locked in a holo or active conformation,[17] circumventing the need for ligand binding to facilitate coactivator interaction. Interestingly, however, the co-crystal structures of RORα LBD bound to cholesterol and cholesterol sulfate suggest that this receptor can bind to metabolites of cholesterol.[18,19] It is not clear that these metabolites of cholesterol modulate the function of the receptor. Recent studies suggest that RORγ is not constitutively active but that this receptor requires binding to ligands such as 25-hydroxycholesterol (25-OHC) for activation and interaction with coregulators.[20] While intriguing, the nature of the endogenous ligand for RORγ remains controversial. Regardless, we and others have shown that a range of oxysterols can potently bind to and modulate the function of RORγ.[20] The crystal structure of RORβ bound with stearate in complex with a SRC1 LXXLL motif NR box peptide has been solved.[21] The presence of stearate in the receptor's ligand-binding pocket (LBP) was described as an artifact of the protein expression system, as stearate has low affinity for RORβ and this ligand does not modulate the activity of the receptor.

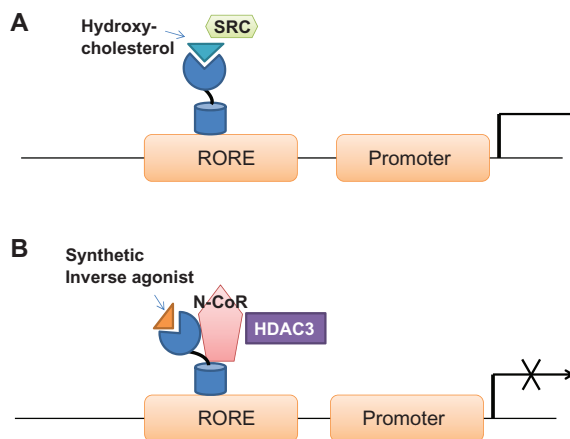In 2010, it was shown that the LXR agonist T0901317 was a potent repressor (inverse agonist) of both RORα

Figure 2 Mechanism of repression of RORs by synthetic ligands. (**A**) ROR agonists drive recruitment of transcriptional coactivators such as SRC2. (**B**) Inverse agonists of ROR displace coactivator and drive recruitment of transcriptional repressors.
Abbreviations: SRC, steroid receptor coregulator; ROR, receptor-related orphan receptors; RORE, ROR respon element; NCoR, nuclear receptin co-repression; HDAC3, histone deacetylase.

and RORγ.[22] Interestingly, T0901317 does bind potently to the LBD of RORβ; however, in cotransfection assays, T0901317 did not modulate the activity of RORβ (unpublished data). A caveat is that RORβ has little to no constitutive activity when transfected into cells, so in these assays, it may not be possible to detect repression. More recently, selective synthetic modulators of RORα and RORγ have been described, such as the RORα-selective inverse agonist SR3335,[23] the dual RORα/RORγ inverse agonist SR1001,[24] and the RORγ-selective inverse agonists SR1555[25] and SR2211.[26] The natural products digoxin and ursolic acid have been described as RORγ-selective inverse agonists.[27,28] However, their utility as candidates for further development is limited, as digoxin displays significant adverse drug reactions with a very narrow therapeutic index, and ursolic acid activates other nuclear receptors.[29,30] Surprisingly, unlike the advances made for RORα and RORγ, few advances have been made, to date, in the development of potent and selective modulators of RORβ.

RORs in metabolic disease

A role for RORα in the regulation of metabolic pathways was revealed by studies in the staggerer (RORα[sg/sg]) mouse.[31,32] This natural mutant mouse strain carries an intragenic insertion within the RORα gene that results in a frame shift and a premature stop codon causing the RORα protein to be inactive. Staggerer mice are less susceptible to hepatic steatosis and have a lower body fat index relative to wild-type mice, despite their having higher food consumption. A role for RORα in the regulation of glucose metabolism is suggested by studies showing that loss of the steroid receptor coregulator SRC2 in mice leads to a phenotype similar to von Gierke's disease (glycogen storage disease-1a), a human disorder caused by mutations in the RORα target gene *glucose-6-phosphatase (G6Pase)* and associated with severe hypoglycemia and abnormal accumulation of glycogen in the liver.[33] Interestingly, treatment of hepatocytes with T0901317 repressed the expression of G6Pase and chromatin immunoprecipitation (ChIP re-ChIP), which demonstrated that this synthetic ligand diminishes the presence of SRC2 at the G6Pase promoter in an ROR-dependent fashion.[22] Consistent with these studies, treatment of HepG2 cells with oxysterols – putative endogenous modulators of RORs – resulted in repression of the expression of G6Pase and the displacement of ROR-dependent SRC2 at the G6Pase promoter.[34] To further support these findings, treatment of murine primary hepatocytes with oxysterols resulted in repression

of both PEPCK and G6Pase and resulted in a reduction of glucose output from these cells by 24%.[34]

All three ROR isoforms bind to the identical response elements, so it is predicted that each receptor can compensate for the other in modulating target gene levels when coexpressed. RORα and RORγ are both expressed in skeletal muscle, a tissue that accounts for approximately 40% of total body mass and 50% of energy expenditure, and in the liver, an organ that is a major site of fatty acid and glucose oxidation.[35] It has been shown that RORγ controls expression of genes that regulate muscle activity, fat mass, and lipid homeostasis (fatty acid-binding protein 4 [FABP4], CD36, and LPL) and plays a role in the regulation of reactive oxygen species (ROS).[36] Microarray analyses of liver tissue from RORα[sg/sg], RORγ^{−/−}, and RORα[sg/sg]/RORγ^{−/−} double knockout mice[37] revealed that RORα and RORγ are critical regulators of hepatic genes encoding several phase I and phase II metabolic enzymes, including 3β-hydroxysteroid dehydrogenases, cytochrome P450 enzymes, and sulfotransferases. Mice deficient in RORγ also exhibit reduced blood glucose levels.[37] These findings suggest that RORγ may have a role in glucose metabolism, through regulation of adipogenesis and insulin sensitivity. In mice, double knockout of RORα and RORγ showed a similar reduction in cholesterol, triglyceride, and blood glucose levels, compared to single gene knockout. Additionally, RORs were shown to affect the expression of several genes involved in steroid, bile acid, and xenobiotic metabolism, suggesting that RORs are promising targets for the treatment of obesity-associated insulin resistance and metabolic disease.[37,38] As muscle and liver are critical mediators of insulin sensitivity, lipid metabolism, and energy balance,[39,40] targeting RORα and RORγ for the treatment of metabolic disease holds great promise.

RORs in autoimmunity

The T-cell-specific RORγ isoform, RORγt, is the key lineage-defining transcription factor for the differentiation program of Th17 cells.[41] The Th17 cell, which produces interleukin-17 (IL-17) and IL-22, has been implicated in inflammatory conditions and autoimmune disorders, including arthritis, multiple sclerosis, asthma, and inflammatory bowel disease.[42,43] Mice lacking IL-17 are resistant to developing experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis and collagen-induced arthritis (CIA) a model of rheumatoid arthritis. Furthermore, neutralizing IL-17 with a targeted antibody suppressed autoimmune inflammation, joint damage, and bone destruction.[44–46] Recently, a study using an adoptive transfer model of colitis revealed the

importance of RORγ in immunity. In this study, IL-17A-null T cells were transferred to RAG1-null mice, leading to a severe colitis phenotype, similar to mice transferred with wild-type T cells. However, transfer of RORγ-null T cells into these mice failed to increase mucosal IL-17 cytokine levels, and induction of colitis was not detected. Subsequent treatment of these animals with IL-17 resulted in induction of colitis.[47] Consequently, RAG1^{-/-} mice reconstituted with bone marrow cells from RORα or RORγ-deficient mice were shown to be less susceptible to EAE than mice reconstituted with wild-type bone marrow.[48] These data are consistent with earlier work showing that RORα and RORγ (but not RORβ) are expressed in bone marrow-derived mesenchymal stem cells.[49] Combined, these observations suggest that controlling Th17 differentiation and IL-17 expression is important to the prevention of autoimmune disorders. Consequently, inhibition of RORγ function offers a potential therapeutic target for the treatment of immune disorders such as rheumatoid arthritis, multiple sclerosis, and chronic colitis (Figure 3).

Macrophages are specialized differentiated mononuclear phagocytic cells that perform key roles in antimicrobial defense, autoimmunity, and inflammatory disease.[50] It has been shown that macrophages can produce IL-17,[51] and studies have been published exploring the role of RORs in regulating macrophage activation.[52] In particular, a role for RORα in macrophages was suggested by studies of RORα[sg/sg] mice. Following lipopolysaccharide (LPS) stimulation, macrophages from these animals produce more IL-1β and TNFα

Figure 3 Proposed model for RORγ inhibition in rheumatoid arthritis therapy.
Abbreviations: IL, interleukin; IFNγ, interferon gamma; TNFd, tumor necrosis factor alpha; LPS, lipopolysaccharide; TLR4, toll-like receptor 4.

than macrophages from control animals.[53–55] Moreover, overexpression of RORα in human primary smooth muscle cells inhibits TNF-α-induced expression of IL-6, IL-8, and cyclooxygenase-2 (COX-2), and up-regulates IκB.[56] Induction of IκB following LPS exposure has been suggested to be involved in the downregulation of cytokine/chemokine expression.[57,58] These studies suggest that RORα may be a negative regulator of proinflammatory cytokine production in endotoxin-primed macrophages. Comprehensive gene-expression profiling using quantitative PCR revealed that RORα and RORγ but not RORβ are expressed in resting macrophages. Eight hours post-LPS treatment, the expression of all three ROR isoforms was increased significantly.[59] Similar to LPS, interferon gamma (IFNγ) is a classic macrophage activator. IFNγ binding to its cognate receptor results in increased expression of major histocompatibility complex class II (MHC), the chemokine receptor CCR2, and the toll-like receptors TLR2 and TLR4. LPS stimulation of IFNγ-primed macrophages results in increased secretion of the antimicrobial cytokines IL6 and TNFα as well as nitric oxide. Interestingly, the expression of RORα peaks at just one hour post-stimulation with the IFN-γ, which is significantly earlier than the expression peak of >20 hours observed post-LPS exposure. This stimulus-dependent expression profile demonstrates that monitoring ROR expression can serve as a dynamic marker of macrophage activation. LPS stimulation of macrophages enhances autoimmune response through activation of toll-like receptor (TLR) signaling, and mice deficient in either TLR2 or TLR4 are resistant to CIA and streptococcal-cell-wall-induced arthritis.[60] Studies also suggest that RORs regulate the stimulation of macrophages by regulating NF-κB signaling, a pathway triggered by LPS exposure.[55,56] Consequently, these findings suggest that targeted inhibition of RORα or RORγ with synthetic ligands could provide a means for reducing autoimmune pathology (Figure 3).
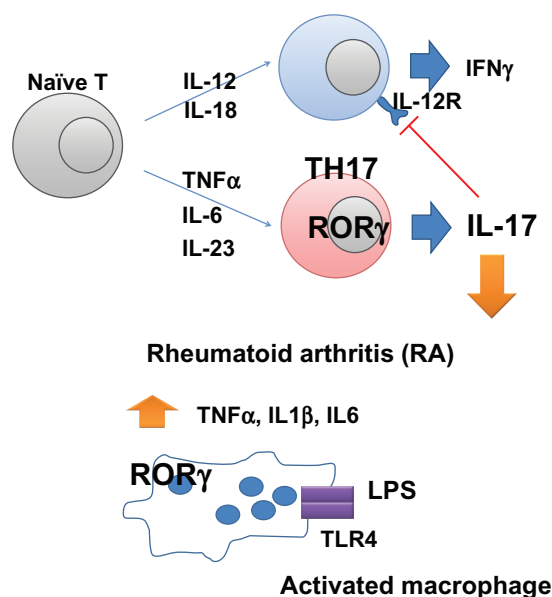
Ligand modulation of RORs

As shown in Table 1, several putative endogenous and synthetic ligands have been described for the RORs. The synthetic LXR, PXR, FXR agonist T0901317 was shown to repress both RORα and RORγ,[22,61] and this compound has demonstrated therapeutic potential in the CIA model.[62] However, in this study, it was not clear whether the compound's efficacy was due to repression of RORα and RORγ or if the activation of LXR played a role. Medicinal chemistry efforts focused on the T0901317 scaffold led to the development of several nonsterol, nonnatural-product

Table 1 Natural and synthetic ligands of RORα and RORγ

Name	Pharmacology	Origin	RORα IC$_{50}$ (µM)	RORγ IC$_{50}$ (µM)	PMID
7-Hydroxy cholesterols	Endogenous RORα/ RORγ ligand	Screen of Oxysterols for ROR activity	0.162	EC50: 0.02–0.04	20203100 21297105
Ursolic acid	RORγ inverse agonist	Plants	NR (not reported)	0.68	21566134
Digoxin	RORγ inverse agonist	Foxglove plant	No inhibitory effect	1.98	21441909
T0901317	RORs: inverse agonist LXR, PXR, FXR: agonist	Human NR specific screen	0.132	0.051	19887649 21735608
SR1078	RORα/RORγ agonist	Synthetic Small-molecule Analog of T090131	1–3	1–3	20735016 22509368
SR1001	RORα/RORγ inverse agonist	Synthetic Small-molecule Analog of T090131	0.172	0.111	21499262
SR3335	RORα inverse agonist	Synthetic Small-molecule Analog of T090131	0.480	No effect	21090593
SR2211	RORγ inverse agonist	Synthetic Small-molecule Analog of T090131	>10 µM (no effect on RORa transcriptional activity at 10 µM)	approx. 0.320	22292739
SR1555	RORγ inverse agonist	Synthetic Small-molecule Analog of T090131	No effect	approx. 1.5	22769242

ROR-selective modulators. All of the Scripps Research (SR) analogs of T0901317 described below were shown to be devoid of LXR binding and agonism. SR3335 was identified as an inverse agonist of RORα with little effect on RORγ.[23] It is reported to suppress the expression of RORα target genes involved in hepatic gluconeogenesis, such as *G6Pase* (*G6P*) and *phosphoenolpyruvate carboxykinase 2* (*PCK2*).[23] The dual RORα/RORγ inverse agonist SR1001 and the RORγ-selective inverse agonist SR2211 demonstrated inhibition of inflammatory cytokine expression, particularly IL17. SR1001 was shown to be efficacious in the EAE model in rodents.[23] More recently, the RORγ-selective inverse agonist SR1555 was shown to positively modulate Tregs.[25] These studies strongly suggest that inhibition of the RORs has potential therapeutic value for the treatment of Th17-derived autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

Structural insights into modulation of RORs

The first atomic structure of an ROR LBD to be solved was that of RORβ bound with stearic acid.[21] Subsequently, all-trans retinoic acid (ATRA) and a synthetic analog (ALRT 1550) were identified as putative functional ligands, and their binding mechanisms were shown using crystal structures.[63]

Interestingly, there have been no follow-up publications on these putative RORβ ligands. The characterization of the RORα LBD structure identified cholesterol and cholesterol sulfate as potential ligands and implicated RORα in lipid metabolism.[18,19] In spite of the high sequence similarity (63%) and similarly sized ligand-binding pockets (722Å^3 and 766Å^3, respectively; Table 2) of the LBDs of RORα LBD and RORβ, cholesterol has no effect on RORβ activity.[63]

Within the past few years, the crystal structure of RORγ with oxysterols (20α OHC, 22R-OHC, and 25-OHC) as agonists and of digoxin as antagonist or inverse agonist[64] were solved by two separate groups. Comparison of these two structures provided important insights into the agonist and antagonist modes of ligand binding. Agonist (25-OHC)-bound RORγ positions its AF2 helix (helix 12) in a conformation that facilitates coactivator binding by a conserved-charge clamp groove involving helices 3, 4, 5, and 12.[20] In contrast, antagonism (digoxin) results from disruption of the active conformation of the highly dynamic AF2 helix. Occupying the same cavity as the agonist, digoxin interferes with the key cation-π interaction between residues of helix 11 and 12. In addition, the large extension of the digoxin molecule sterically hinders formation of a proper LBD-coactivator interface necessary for functional activation. This critical positioning of the AF2 helix in either agonist or antagonist

Table 2 List of ROR X-ray crystal structures in the PDB database

Protein isoform	Resolution/PDB ID	Peptide	LBD pocket size	Ligand identified	Reference
RORβ	1.9/1K4W	SRC-1	766 Å³	Stearic acid	21
RORα	1.63/1N83	–	722 Å³	Cholesterol	19
RORβ	2.1/1N4H	SRC-1	753 Å³	All-trans retinoic acid	63
RORβ	1.5/1N4Q7	SRC-1	820 Å³	7-(3,5-ditert-butylphenyl)-3-methylocta-2,4,6-trienoic acid	63
RORα	2.2/1SOX	–	ND	Cholest-5-en-3-yl hydrogen sulfate	18
RORγ	2.35/3KYT	SCR2-2	ND	20-hydroxycholesterol	20
RORγ	2.4/3LOJ	SCR2-2	ND	(3alpha,8alpha,22R)-cholest-5-ene-3,22-dio	20
RORγ	1.74/3LOL	SCR2-2	ND	25-hydroxycholesterol	20
RORγ	2.2/3BOW	–	ND	Digoxin	64

Abbreviation: ND, not determined; LBD, ligan binding domain; PDB ID, protein data bank identifier.

mode, resulting in coactivator or corepressor binding, has been reported for other nuclear receptors.[65,66]

It will be interesting to see if a structure-based design will lead to the improved potency and selectivity of agonists and antagonists of the RORs with the predicted biological outcomes. Lusher et al showed that the extent of antagonism (partial or full) for a set of chemically related progesterone-receptor modulators depended on the degree of disruption of interaction with a critical methionine (AA 909) residue on helix 12.[66] Thus, it is likely that the structure-guided optimization of synthetic ligands can facilitate the development of potent and isoform-selective ROR modulators with targeted tissue activity that avoids unwanted side effects.

Concluding remarks

A number of small molecule ligands for RORα and RORγ have been reported in recent years, highlighting the potential for this NR subfamily as a therapeutic drug target in metabolic disease. Recent animal studies have indicated that compounds that repress the RORs also suppress Th17-cell development and offer efficacy in models of autoimmunity. Several studies suggest that such compounds may also inhibit proinflammatory genes in activated macrophages. Although further optimization of these small molecule ligands is required, it is clear that targeting RORs for the treatment of Th17-mediated immune disorders may serve as appropriate therapy and avoid the side-effect profile of current clinically used immunosuppressants. It has been shown that the ROR isoform, RORγt, plays a critical function in thymic T-cell (helper and cytotoxic T cells) survival, which is critical for Th17-cell differentiation. Ideally one would like to selectively target RORγt for use in treatment of autoimmune disease; however, these receptors share identical LBD domains and differ only slightly on their N-termini, making it unlikely that

selective modulators of RORγt can be developed. Regardless, chemical probes that are pure isoform-selective activators or repressors will provide a means to fully dissect the biology of the ROR isoforms.

Disclosure

The authors report no conflicts of interest in this work.

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