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The REV-ERBs and RORs: molecular links between circadian rhythms and lipid homeostasis

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Abstract

Research efforts spanning the past two decades have established a clear link between nuclear receptor function, regulation of the circadian clock and lipid homeostasis. As such, this family of receptors represents an important area of research. Recent advances in the field have identified two nuclear receptor subfamilies, the REV-ERBs and the 'retinoic acid receptor-related orphan receptors' (RORs), as critical regulators of the circadian clock with significant roles in lipid homeostasis. In this review, the latest information garnered from cutting-edge research on these two nuclear receptor subfamilies will be discussed. Through direct targeting of the REV-ERBs and RORs with synthetic ligands, generation of novel tools aimed at characterizing their function *in vivo* have been developed, which may lead to novel therapeutics for the treatment of metabolic disorders.

Nuclear receptors: ligand-activated transcription factors

Nuclear receptors (NRs) are a family of highly conserved transcription factors that regulate gene transcription in response to various environmental stimuli. There are 48 NRs that exist in humans, but some organisms such as worms contain nearly 300 members of the family [1]. NRs share considerable amino acid sequence homology with highly conserved domain structures, including a variable amino-terminal A/B domain, a central, highly conserved DNA-binding domain containing two zinc fingers, a hinge region and a carboxy-terminal ligand-binding domain (LBD, or E region) [2]. Several NRs contain an additional C-terminal F domain, the function of which is poorly understood. Examples of classical and well-characterized NRs include the estrogen receptor (ER), progesterone receptor, glucocorticoid receptor and thyroid receptor.

Nuclear receptors work in concert with other proteins to regulate the expression of genes important for an organism's development, homeostasis and metabolism. NRs bind directly to specific DNA response elements in the regulatory region of their target genes and regulate the rate of transcription in a ligand-dependent manner. Most NRs bind to DNA as dimers: either homodimers, as is the case for steroid hormones, or heterodimers with another member of the NR superfamily (the retinoid X receptors [RXRs]) [3,4]. A small number of NRs have the ability to bind to DNA as monomers. Ligand binding induces a

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conformational change within the LBD, exposing a surface for specific cofactor interactions, which results in the recruitment of additional proteins that subsequently modify the chromatin and contact the basal transcriptional machinary to alter gene transcription. NR ligands are small, hydrophobic molecules, such as steroid hormones, fatty acids, lipophilic vitamin derivatives, dietary metabolites, as well as antibiotics and synthetic drugs. Ligands are classified according to their ability to regulate a NR's transcriptional activity. Agonists bind to the LBD and induce a conformational change that results in increased recruitment of cofactor proteins that result in alterations in target gene transcription eliciting a 'maximal' response. An antagonist does not provoke a response of the receptor, rather it blocks the ability of an agonist to bind and generate a response. If a particular receptor has ligand-independent 'basal' activity, inverse agonists may also be identified. An inverse agonist binds to the receptor but inhibits the constitutive activity of the receptor. This class of NR ligands can be observed when a particular receptor has a basal conformation that permits interaction with a cofactor protein (coactivator or corepressor) leading to basal activity. An inverse agonist induces a conformational change within the receptor that decreases the affinity of the receptor for the cofactor protein. Partial agonists bind and activate a receptor, but have only partial efficacy at the receptor relative to a maximally efficacious 'full' agonist. In addition, analogous to partial agonists, partial inverse agonists display only limited ability to block the basal activity of a receptor. Thus, there are many different types of ligands that can modulate the transcriptional activity of a NR, the commonality being their ability to bind a NR. Approximately half of the NRs found in humans have identified endogenous ligands [1]. The remaining NRs are considered 'orphan' receptors, as they have no known, or generally agreed upon, ligands.

Orphan NRs represent an active area of research due to the potential for identification of ligands that may be used to modulate these receptors and may lead to treatments for various diseases. Recently, heme was identified as the ligand for the orphan receptors **REV-ERB**α/β, the well-known regulators of the circadian clock and lipid metabolism [5,6]. Another orphan subclass, the 'retinoic acid receptor-related orphan receptors' (RORs), recognize the same DNA binding sites as the REV-ERBs and are often coexpressed in the same tissues as the REVERBs [2,7]. The RORs are also well known regulators of the circadian clock and recent evidence has associated their activity with metabolic processes, including lipid homeostasis. De-orphanizing these receptors will further serve to advance our knowledge of the two NRs, their dynamic interplay and ultimately their roles in an organism's physiology. Furthermore, the information garnered from these studies will help increase the opportunities for therapeutic intervention of disorders caused by aberrant ROR and REV-ERB activity.

The RORs & REV-ERBs

The first member of the ROR subfamily, RORα, was identified in the early 1990s based on sequence similarities to the retinoic acid receptors (RARs) and the RXRs [7,8]. The highly similar receptors, RORβ and RORγ, were identified soon thereafter [9,10].

The three RORs display significant sequence similarities. Each gene generates multiple isoforms based on alternative promoter usage and exon splicing, with all of the isoforms varying only in the amino-terminal region of the receptor [11]. In humans, four isoforms of RORα exist (α1–α4), while only α1 and α4 are present in mice. Tissue expression of RORα includes the liver, skeletal muscle, skin, lungs, adipose tissue, kidney, thymus and brain. Two isoforms of RORβ are found in mice (β1 and β2) whereas only β1 is present in humans. The expression of RORβ is limited to the CNS. Finally, two isoforms of RORγ are found in both humans and mice (γ1 and γ2). RORγ2 is often regarded as RORγt and found primarily in the immune system. RORγ is most highly expressed in the thymus (mainly RORγt) but is

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also expressed in the liver, skeletal muscle, adipose tissue and kidney [11]. RORγt has been the focus of considerable attention due to its role in T_H17 cell development and its exclusive expression in key cells in the immune system. The three RORs and their various isoforms display distinct patterns of tissue expression and are involved in the regulation of physiological processes utilizing specific target genes. However, owing to significant sequence similarities, in cells where RORs are coexpressed, the different RORs may exhibit functional overlap [11]. RORs exhibit a typical NR domain structure (Figure 1A), as discussed in the introduction. All 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 [8–10]. When bound to this element, the RORs recruit coactivators resulting in constitutive activation of transcription of their target genes [11,12].

Another NR subclass, the REV-ERBs (REVERBα and REV-ERBβ), display significant crosstalk with the RORs. The REV-ERBs were originally identified as orphan receptors based on homology to the canonical NR domain structure (Figure 1B). REV-ERBα and REV-ERBβ have overlapping expression patterns and are found in adipose tissue, skeletal muscle, the brain and liver. However, the receptors display different relative tissue-specific expression levels; for example, REV-ERBα is broadly expressed at the same level in many different tissues, whereas REV-ERBβ is very highly expressed in certain tissues, showing high expression in parts of the brain (pineal and prefrontal cortex), thyroid, uterus and pituitary.

REV-ERBs regulate the expression of many target genes in a circadian manner. However, in contrast to the RORs, the REV-ERBs are transcriptional repressors (Figure 2). This is in part because REV-ERBs lack the activation-function 2 (AF-2) region, which is involved in coactivator binding [13]. Thus, the REV-ERBs bind corepressor proteins such as the nuclear receptor corepressor (NCoR) [14,15]. REV-ERBs bind to the same RORE DNA response elements as the RORs [16]. REV-ERBs can bind as a monomer to an AGGTCA 'half site' with a 5′AT-rich extension. Although this form has been shown to be transcriptionally inactive, REVERB monomers function as transcriptional silencers [17]. REV-ERBs can also bind as a homodimer to a RORE direct repeat 2. They are highly stable homodimers that function as transcriptional repressors [17].

The roles of RORs & REV-ERBs in lipid homeostasis, metabolism & circadian rhythms

Circadian rhythms are daily cycles of biochemical, behavioral and physiological processes in living organisms regulated by endogenous 'clocks'. Regulation of the circadian clock is important for an organism's physiological function and behavior since it relies on it to anticipate and adapt to predictable daily changes such as day/night and activity/rest cycles, metabolism, hormone secretion, insulin sensitivity, blood pressure and feeding time. Abnormalities in circadian rhythm have been implicated in a number of diseases including sleep and mood disorders, diabetes, obesity and cancer [2,18].

Numerous studies have established links between expression of a certain number of NRs driven by the circadian clock, NR function and participation in the circadian control of various metabolic processes. In particular, RORα and REV-ERBα are part of the core clock machinery. In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Semi-autonomous clocks in the periphery can be entrained to signals from the SCN as well as other cues including nutrient status [18]. There are several interlocked transcriptional and post-translational auto-regulatory feedback loops controlling the circadian cycle. The Per, Arnt and Single-minded (PAS) domain basic helix-

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loop-helix transcription factor circadian locomotor output cycles kaput (CLOCK), brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein 1 (BMAL1), and the CLOCK paralog neuronal PAS domain protein 2 (NPAS2), form a heterodimer and control the positive limb of the circadian cycle by activating transcription of the *Period (PER) 1*, *PER2*, and *Cryptochrome (CRY) 1* and *CRY2* genes through their E-box DNA elements in their promoters [19,20]. Once the PER/CRY heterodimer concentration reaches a critical level, the PER/CRY heterodimers enter the nucleus and repress BMAL1/CLOCK transactivation as well as their own transcription. This results in relief of BMAL1/CLOCK inhibition and allows a new cycle to start. This feedback loop results in the oscillations in expression of BMAL1/CLOCK and CRY/PER that follow a circadian pattern [21].

RORα and REV-ERBα are major regulators of the cyclic expression of *BMAL1*, the secondary feedback loop in the circadian cycle [22,23]. REV-ERBα transcription is activated by the BMAL1/CLOCK heterodimer and transrepressed by CRY/PER, resulting in circadian oscillations of REV-ERBα. In turn, REV-ERBα represses *BMAL1* and *CLOCK* transcription. REV-ERBβ expression also oscillates in a circadian fashion and can repress *BMAL1* transcription [22]. RORα competes with REV-ERBα for binding of their shared DNA binding elements, the RORE, in the *BMAL1* promoter leading to *BMAL1* expression being repressed by REVERBα and activated by RORα. The oscillating expression of RORα and REV-ERBα in the SCN leads to the circadian pattern of BMAL1 expression [23,24]. This REV-ERBα/RORα feedback loop interconnects the positive and negative limbs of the core circadian clock.

NPAS2, like CLOCK, forms heterodimers with BMAL1 and effectively functions in the regulation of mammalian circadian rhythms. As BMAL1 forms obligate heterodimers with NPAS2 or CLOCK, it was unclear as to how the expression of these two proteins was coordinated with BMAL1 expression. We recently demonstrated that NPAS2, like BMAL1, is a RORα and REV-ERBα target gene. We discovered two functional ROREs within the *NPAS2* promoter and demonstrated that both RORα and REVERBα regulate the expression of *NPAS2,* thus suggesting a mechanism by which RORα and REV-ERBα coordinately regulate the positive limb of the circadian clock [25]. The importance of these proteins in circadian rhythms has been established through genetic knock-out experiments. *Rev-erb*α-deficient mice display altered circadian rhythms characterized by an increase in *Bmal1* and *Clock* expression and shorter period lengths when compared with wild-type mice [26]. *Ror*α– and *Ror*β-deficient mice display aberrant circadian behavior, while no abnormalities are apparent in *Ror*γ-deficient mice [23,24,27–29].

An organism's energy homeostasis depends upon a coordinated control of key metabolic processes. Organs with high metabolic activity, including liver and adipose tissue, display circadian rhythm in the expression of genes involved in key metabolic pathways [30,31]. Growing evidence suggests that RORs play a critical role in the regulation of several metabolic pathways. *Staggerer* mice (RORα$^{sg/sg}$), a natural mouse mutant in which RORα is rendered inactive due to a deletion within the *ROR*α gene, has shed some light on the role of RORα in metabolism [32]. When fed a normal diet, *staggerer* mice display hypo-α-lipoproteinemia and have lower levels of total plasma cholesterol, the high-density lipoprotein, Apoa1, the major constituent of high-density lipoprotein, ApoCIII, *Apoa2* and triglycerides, compared with wild-type mice [33–35]. In addition, expression of the reverse cholesterol transporters *Abca1* and *Abca8/G1* are decreased in the liver and intestine of *staggerer* mice [36]. These mice are less susceptible to hepatic steatosis and despite their higher food consumption, have reduced body fat [36]. Fat cells in brown and white adipose tissue are smaller and liver triglyceride levels lower in these mice than their wild-type counterparts. When fed a high fat diet (HFD), *staggerer* mice are resistant to HFD-induced obesity and hepatic steatosis [36]. However, despite their resistance to weight gain, these

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mice develop severe atherosclerosis when fed a HFD, suggesting that RORα may play an atheroprotective role [36]. *Ror*γ$^{-/-}$ mice display normal cholesterol and triglyceride levels, however, their blood glucose levels are slightly lower than wild-type mice [37]. Mice deficient in both *Ror*α and *Ror*γ (double knock-out) exhibit similar changes in cholesterol, triglyceride and blood glucose levels as the single knock-out mice. Gene expression analysis from livers of double knock-out mice suggests a degree of functional redundancy between the two RORs, most likely due to their similarities in RORE binding affinities [37].

The effect on cholesterol and triglyceride homeostasis in the *Ror* knock-out mice is most likely due to RORα's regulation on a number of genes involved in the control of lipogenesis and fatty acid oxidation. For instance, *sterol regulatory element-binding protein 1, isoform c* (*Srebp-1c)* is reduced in the livers and muscle of *staggerer* mice as is *fatty acid synthase* (*Fas*) [36,38]. Expression of the coactivators PGC1α and β, proteins involved in the regulation of oxidative metabolism and gluconeogenesis, is increased in *staggerer* mice [29]. Finally, RORs have been demonstrated to regulate the oxysterol 7α-hydroxylase gene (*Cyp7b1*), the gene product is an enzyme that plays a crucial role in the alternative pathway of cholesterol metabolism. Expression of *Cyp7b1* is reduced in *staggerer* mice and to a lesser degree in *Ror*γ$^{-/-}$ mice [37–39]. One intriguing notion is that the regulated conversion of cholesterol into bile acids, yielding hydroxycholesterols as intermediates, may regulate the circadian rhythm via modulation of ROR activity. Circadian control of cholesterol biosynthesis has been well documented and it is believable that the levels of hydroxycholesterols may also follow a circadian pattern [40,41]. Therefore, expression of *Cyb7a1* and *Cyp7b1* may regulate themselves through a dynamic feedback loop involving the RORs and REV-ERBs and ultimately be another mechanism of control of the circadian rhythm.

Recent evidence has established a role for RORα in glucose metabolism. Chopra *et al.* found that loss of the NR coactivator steroid receptor coactivator 2 (SRC-2) resulted in a murine phenotype similar to von Gierke's disease. This disease is associated with severe hypoglycemia and abnormal accumulation of glucose in the liver [42]. Furthermore, our group demonstrated that RORα regulates both the expression and secretion of FGF21, a hepatic hormone that regulates peripheral glucose tolerance, torpor and hepatic lipid metabolism [39,43,44].

REV-ERBs play a central role in regulating clock genes, as discussed earlier, which in turn affects many processes coordinated by the circadian clock. Although currently there have been no specific reports detailing the precise role of REV-ERBs in clock-associated diseases, dysfunction of the circadian lock (perhaps via REVERB dysfunction) can have profound effects on energy homeostasis and metabolic disorders. In this sense, *Rev-erb*α$^{-/-}$ mice display a 0.5 h shorter period and are more sensitive to phase shifts induced by light pulses [26,45].

Several studies have shown that REV-ERBα plays an important role in lipid metabolism. *Rev-erb*α$^{-/-}$ mice display elevated very low-density lipoprotein triglyceride levels, which correlates with elevated levels of *Apoc3* mRNA in the serum and liver [46,47]. *Apoc3* is a key player in serum triglyceride metabolism, and in combination with data from RORα$^{sg/sg}$ *staggerer* mice, suggests that REV-ERBs and RORs are both important in the regulation of *Apoc3* expression and lipid metabolism [46,48]. REV-ERBα is also a regulator of other genes involved in metabolism, including *Apoa1* (in certain species; mentioned above for ROR), as well as *Elov3* (a very long-chain fatty acid elongase) and *Pai-1* (regulator of the fibrinolytic system and modulator of inflammation and atherosclerosis) [49–51].

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REV-ERBα has also been shown to be a direct regulator of *Fgf21* expression. Overexpression of the receptor results in decreased *Fgf21* mRNA levels in primary mouse hepatocytes in a heme-dependent manner, and is dependent on *Alas1* expression levels [52]. *Alas1*, a PGC-1α target gene, produces a product that functions as a rate-limiting enzyme in the synthesis of heme, the natural ligand for REV-ERBs. PGC-1α regulates the expression of *Alas1*, thus linking the roles for both REV-ERB and PGC-1α in *Fgf21* expression [53]. *Rev-erb*β knock-out mice have not yet been reported, but additional studies have provided insights into its role in metabolism. Expression of a dominant negative form of REV-ERBβ lacking the LBD results in decreased expression of several genes involved in lipid metabolism. These loss-of-function studies identified *Fatty acid translocase* (*Fat/CD36*), *Fabp3*, *Fabp4 Ucp3*, *Srebp-1c* and *Scd-1* as REV-ERBβ target genes [54].

Discovery of endogenous ligands

The LBDs of NRs are multifunctional. They play a role not only in ligand binding, but also nuclear localization, receptor dimerization and they provide an interface for coactivator and corepressor binding. Ligand binding induces a conformational change in the receptor resulting in helix 12 in the LBD (also referred to as the AF-2 region) making contact with the ligand. For agonist ligands, this results in the dissociation of corepressor molecules to be replaced by coactivator molecules [55,56]. In the case of most NRs, coactivator/corepressor interaction is ligand-dependent. In addition to the typical 12 canonical α-helices, the RORs contain two extra helices, H2′ and H11′ [11]. However, the RORs have intrinsic transcriptional activity, where ligand binding actually represses the activity of the receptor, meaning that the RORs are constitutively active and that coactivators bind to the AF-2 surface in the absence of ligand.

Identification of endogenous ligands for the RORs has been controversial (Figure 3A). In the mid 1990s, two putative ligands for RORα, melatonin and thiazolidinedione, were suggested [57]. However, these data have yet to be confirmed. Later, x-ray crystallographic studies provided insights into the structure, size and potential ligands for RORα's LBD (Figure 4). When the RORα LBD protein was expressed in Sf-9 insect cells, crystallographic analysis revealed that RORα co-purified with cholesterol, which was bound in the ligand-binding pocket, thus suggesting a potential endogenous ligand for RORs [58]. In addition to cholesterol, 7-dehydrocholesterol, cholesterol sulfate and 25-hydroxycholesterol were also identified as RORα agonists [58,59]. All ligands were found to bind RORα in a reversible manner and enhanced RORE-dependent transcriptional activation by RORα. These findings suggested that RORα may function as a lipid sensor, thus implicating RORα in the regulation of lipid metabolism. In fact, recent reports indicate that RORα regulates the expression of several genes involved in lipid metabolism [35–37,58]. Regardless, these data suggest that RORα may bind to a number of sterols leaving open the question as to which ligands are physiologically relevant.

Putative ligands for RORβ have been revealed through studies involving x-ray crystallography (Figure 5). Similar to cholesterol for RORα, stearic acid co-purified with recombinantly expressed RORβ LBD protein [60]. Crystallographic analysis revealed that the stearic acid molecule bound to the ligand-binding pocket in RORβ. However, functional experiments suggest that stearic acid likely acts more as a 'filler' than a functional ligand [60]. Several retinoids, including all-*trans* retinoic acid, and the synthetic retinoid ALRT 1550 (Allergan Ligand Retinoid Therapeutics, Inc.), were identified as functional inverse agonists as they reversibly bound RORβ in the ligand-binding pocket with high affinity and altered RORβ-mediated transcriptional activation. These retinoids were also able to bind to RORγ not RORα, and inhibit RORγ-mediated transactivation [61]. Further analysis of these

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ligands is needed, since it is still unclear whether they act as genuine physiological ligands for RORβ and RORγ and can regulate ROR target genes in a ligand-dependent manner.

Despite the reports describing cholesterol and cholesterol sulfate as ligands for RORα, it was still unclear as to whether these two sterols were physiologically relevant ligands. Recently, we demonstrated that the 7-oxygenated sterols (Figure 3A) function as high-affinity ligands for both RORα and RORγ, demonstrating K_i values in the range of 10–20 nM as determined by radioligand-binding assays. Our data indicate that the 7-oxygenated sterols (7α-OHC, 7β-OHC and 7-ketocholesterol) bind to RORα and RORγ with higher affinity than cholesterol sulfate, with cholesterol binding barely detectable. We found that RORα and RORγ, when produced in *Escherichia coli*, were devoid of any endogenous sterols yet displayed constitutive activity as evidenced by their ability to bind to coactivator peptides and were transcriptionally active in the absence of a ligand. The 7-oxygenated sterols functioned as inverse agonists to both receptors while cholesterol and cholesterol sulfate failed to modulate the activity of RORα and RORγ in cell based assays. Finally, the 7-oxygenated sterols modulated the expression of RORα/γ-dependent target genes in a receptor-dependent manner [12]. Our data suggest that while cholesterol and cholesterol sulfate bind RORα and RORγ, these sterols lack the ability to induce the conformational change necessary to alter cofactor binding and transcriptional activity.

We also discovered that 24*S*-hydroxycholestrol (24*S*-OHC; Figure 3A) is a high-affinity ligand for RORα and RORγ, binding to these receptors with K_i values of approximately 25 nM [62]. 24*S*-OHC acts as an inverse agonist for both RORα and RORγ, as it dose-dependently reduces the constitutive activity of these receptors in Gal4 cotransfection assays, as well as *Bmal::luc* and *Glucose-6-Phosphatase* (*G6Pase)::luc* reporter assays. In addition, 24*S*-OHC reduces the expression of *BMAL1* and *REV-ERB*α mRNA levels in a RORα-dependent manner, as shown by siRNA knock-down of RORα, via decreased SRC-2 recruitment to promoter regions, as shown in a *BMAL1* ChIP-reChIP assay. In this report, we also showed that 24*S*,25-epoxycholesterol (24,25-epoC) and 24*R*-hydroxycholesterol (24*R*-OHC) (Figure 3A) selectively bind and regulate the activity of RORγ, with similar Ki values (20 and 102 nM, respectively) and IC_{50} values in a Gal4 dose titration assay (280 and 90 nM, respectively) [62].

Alongside these discoveries, another group discovered that 20α-hydroxycholesterol (20α-OHC), 22*R*-hydroxycholestrol (22*R*-OHC) and 25-hydroxycholestrol (25-OHC) (Figure 3A) are ligands for RORγ [63]. These ligands dose-dependently increased the recruitment of coactivator peptides in an AlphaScreen biochemical assay. Crystal structures of RORγ bound to these ligands were determined (Figure 6), which revealed that all three ligands bound to RORγ in a similar manner. Mutagenesis studies validated the role of several key residues involved in the interaction with the hydroxycholestrol ligands, including one mutation (Ile397Asn) that created a new hydrogen bond with 22R-OHC; for this mutation, all ligands except 22R-OHC lost activity [64].

For many years, REV-ERBs were thought to function in a ligand-independent manner, much like the RORs. Their function as constitutive transcriptional repressors was attributed to their lack of anAF-2 region, or helix 12 in the LBD. However, in 2007, our laboratory, as well as the Lazar laboratory, discovered that REV-ERBs are indeed ligand-regulated and identified the porphyrin heme as a physiological ligand (Figure 7) [5,6]. We showed that heme binds to REV-ERBs with a K_d of 2–3 μM and thereby induced a conformational change in the receptor, increasing recruitment of the corepressor NCoR and leading to suppression of the expression of REV-ERB target genes. Additional data from the Lazar laboratory showed that heme regulates the expression of additional REV-ERB target genes,

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including the gluconeogenic genes *G6Pase* and *PEPCK*, as well as interaction with the NCoR–HDAC3 corepressor complex.

Crystal structures of REV-ERBα and REVERBβ provided a structural validation of heme as a ligand for REV-ERBs (Figure 8), including some molecular details concerning the repression-only function of these receptors. A structural study of REV-ERBβ bound to heme demonstrated one heme molecule hexa-coordinated within the ligand-binding pocket, making contacts to the side chains of Cys-384 and His-568 (Figure 8A) [65]. In the absence of heme (Figure 8B), side chains of aromatic residues line the REV-ERBβ ligand-binding pocket (Phe-405, Phe-409, Phe-443 and Phe-450), which stabilizes the ligand-free form of the receptor [66], supported by circular dichroism thermal melt plots showing only a slight increase in the thermal stability of REV-ERBs upon binding heme [5].

More recently, the structure of REV-ERBα bound to the NCoR interaction domain 1 (ID1) peptide was reported in the absence of heme (Figure 8C) [67]. The NCoR ID1 domain forms an antiparallel β-sheet interface with the C-terminal 'Y domain' of REV-ERBα. This type of antiparallel β-sheet interface has only been observed in one other structural study (RAR bound to the NCoR ID1 peptide) and may be a general structural mechanism for corepressor binding. However, an interesting point to note is that this interaction is facilitated in the absence of REV-ERB's physiological ligand, heme. The authors suggest that the NCoR ID1 region may enable REV-ERB to perpetuate a basal level of repression in the absence of heme. In agreement, we previously noted that heme displaced the NCoR ID1 peptide in a FRET-based biochemical assay [5] – suggesting that the ID1 region may prefer to bind to heme-free receptor. The authors also suggest that heme binding may induce a conformational change resulting in the recruitment of the NCoR ID2 region, perhaps by displacing the ID1 region.

Discovery of synthetic ligands

The wide range of physiological processes regulated by NRs and pathological consequences resulting from aberrant NR activity have made this family of proteins a popular area of research as potential targets for drug development. The NRs contain naturally occurring hydrophobic, ligand-binding pockets that when bound by small hydrophobic molecules can be turned 'on' or 'off'. The most effective drugs are small hydrophobic compounds that can cross the plasma membrane. The steroid receptors, including the mineralcorticoid, estrogen, androgen, glucocorticoid and progesterone receptors have been successful targets for a range of diseases including cancer, inflammation and cardiovascular disease. Thyroid receptor, peroxisome proliferator-activated receptors, RARs and RXRs have been successful targets for the development of drugs for the treatment of metabolic and skin disorders. Accordingly, a tremendous amount of effort has been generated in order to elucidate and determine the function of human NR ligands.

Recently, we identified the first high-affinity synthetic ligand for both RORα and RORγ. Using a NR-specificity screen containing all 48 human NRs, we identified the benzene-sulfonamide liver X receptor (LXR) agonist, T0901317 (Figure 3B), as a RORα/γ inverse agonist. We determined that T0901317 bound with high affinity to both RORα and RORγ (K_i = 132 and 52 nM, respectively) resulting in the modulation of the transcriptional activity of each receptor and their ability to interact with cofactor peptides. Furthermore, T0901317 repressed the expression of ROR-dependent target genes, including the gluconeogenic enzyme *G6Pase* [68]. While we were the first to discover that T0901317 modulates ROR activity, it had already been well characterized as a LXRα and LXRβ agonist. Furthermore, T0901317 acts as a potent agonist to the farnesoid X receptor and pregnane X receptor

[69,70]. Although the promiscuity of T0901317 makes it difficult to use this compound as a selective probe, it lays the groundwork for the development of ROR-selective modulators.

Using the benzenesulfonamide as a scaffold, we synthesized an array of compounds and assessed their activity on RORα, RORγ, farnesoid X receptor, LXRα and LXRβ. Using this approach, we discovered one compound, the amide SR1078 (Figure 3B), to be a selective RORα/γ modulator as it displayed no activity on the other NRs tested. Further examination of SR1078 revealed that it is a RORα/γ agonist as it stimulated the expression of two ROR target genes in the liver, *G6Pase* and *FGF21* [71]. Additional work from our group has elucidated a selective RORα synthetic ligand, SR3335 (Figure 3B). Similar to T091317, treatment with SR3335 suppressed the expression of *G6Pase* and *PhosPhoenolpyruvate Carboxykinase (PEPCK)*, RORα target genes involved in hepatic gluconeogenesis. Furthermore, in a diet-induced obesity mouse model, SR3335 treatment led to reduced plasma glucose levels following a pyruvate tolerance test, and indicator of gluconeogenesis [72]. These studies prove the feasibility of developing ROR-selective modulators as tools to probe the function of these receptors *in vitro* and *in vivo*. Currently, the design and synthesis of compounds with improved physicochemical properties for the individual ROR family members is underway and would further increase their utility as therapeutics in the treatment of ROR-mediated metabolic disorders, including Type II diabetes.

The first synthetic REV-ERB compound, GSK4112 (Figure 7B), was identified in 2008 in a FRET-based biochemical assay as a ligand that dose-dependently increased the interaction of a peptide derived from the NCoR corepressor with REV-ERBα [73]. This study also showed that treatment of Rat-1 cells with GSK4112 reduces the expression of the REV-ERB target gene *Pai-1*. GSK4112 treatment also resets the circadian expression of a *Per2:Luc* reporter in Rat-1 cells and primary lung fibroblasts.

Both ROR and REV-ERB have been shown to play an important role in regulation of adipogenesis [74–78]. We tested the pharmacology of GSK4112 within the context of a REV-ERB responsive gene, *Bmal1*, in a recent paper characterizing the ability of GSK4112 to regulate adipogenesis in 3T3-L1 cells in a REV-ERB-dependent manner [79]. First, we biochemically verified the ability of GSK4112 to dose-dependently increase the interaction of the receptor with a NCoR corepressor peptide revealing that GSK4112 is a dual REV-ERBα/β agonist. GSK4112 decreases the activity of a luciferase reporter under the control of the *Bmal1* reporter in a cotransfection assay with a REVERBα expression vector in both HepG2 and HEK293 cells. Furthermore, GSK4112 treatment of HepG2 cells decreases the expression of *Bmal1* in a dose-dependent manner [80,81]. In addition, ChIP analysis reveals that GSK4112 treatment of HepG2 cells increases the occupancy of NCoR on the *Bmal1* promoter. These data reveal that GSK4112 acts as a REV-ERB agonist, regulating the expression of REVERB responsive target genes and increases the recruitment of the NCoR corepressor complex in a manner similar to REV-ERB's physiologically ligand, heme [5]. In this study, we also showed that GSK4112 induces adipogenesis in 3T3-L1 cells in a synergistic manner with the peroxisome proliferator-activated receptor γ agonist rosiglitazone.

Later, GSK4112 was further characterized and suggested that it could be used as a chemical probe to define the function of REVERB *in vitro* [80]. In this study, data were presented showing that heme and GSK4112 compete for binding in FRET-based biochemical assay with a NCoR peptide. In addition, the authors present some information regarding the structure–activity relationship in the discovery of GSK4112. Data are also presented, validating our prior study, showing that GSK4112 decreases the expression of the REV-ERB target gene *Bmal1* in a REV-ERBα-dependent manner, and further show it decreases the expression of other REV-ERB target genes, including *G6Pase*, *PEPCK* and *PGC1*α.

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GSK4112 also increases the occupancy of HDAC3 to the G6Pase promoter. Furthermore, GSK4112 treatment represses glucose output in primary mouse hepatocytes, as well as decreases the expression of *Bmal1* and *G6Pase*. In addition, GSK4112 represses the circadian expression of *Bmal1*, *Cry1* and *Pgc1*α in primary mouse hepatocytes.

Our unpublished studies and the findings of other groups reveal that GSK4112 displays poor pharmacokinetic properties [80]. In particular, high clearance and rapid metabolism lead to low plasma drug levels. Thus, the poor pharmaco-kinetic properties of this compound preclude it from use as a probe of REV-ERB function *in vivo*. However, these studies reveal that small-molecule compounds can indeed be used to define the function of REV-ERB, providing a basis for future REV-ERB-based medicinal chemistry efforts with significantly better *in vivo* pharmacokinetic/pharmacodynamic properties.

We recently identified a compound, SR8278, which functions as a REV-ERB antagonist (Figure 7B) [81]. In a Gal4 cotransfection assay, where the LBDs of REV-ERBα and REV-ERBβ are fused to the Gal4 DNA-binding domain, the GSK4112 agonist causes repression, whereas the SR8278 antagonist stimulates transcription. We further verified the antagonist activity of SR8278 using luciferase reporters containing the promoter sequences for the REV-ERB target genes *BMAL1*, *G6Pase* and *PEPCK*. Similar to the observation in the Gal4 assay, SR8278 stimulates transcription for the REV-ERB responsive promoters and does so in a dose-dependent manner that competes with GSK4112 and heme, the endogenous agonist. We further characterized the antagonist activity of SR8278 by treating HepG2 cells with the compounds. Whereas GSK4112 decreased the expression of *PEPCK* and *G6Pase*, SR8278 stimulated the expression of these genes. Unfortunately, SR8278 also displays poor pharmacokinetic properties that will limit its use to *in vitro* and biochemical studies. However, this discovery of the first antagonist of REV-ERB activity, and future structure–activity relationship studies to find compounds with favorable pharmacokinetic properties, will allow the field to better define the function of REV-ERBs *in vivo*.

Metabolic disorders associated with RORs and REV-ERBs

The circadian rhythm plays an essential role in the regulation of metabolism. Several lines of evidence suggest that the incidence of cardiovascular disease and metabolic disturbances is higher in individuals who alter their normal sleep–wake pattern for shift work [82–84]. Additional studies have uncovered the prevalence of metabolic syndrome, increased body-mass index and cardiovascular events in shift workers, raising the possibility that disrupted cycles of rest and activity and fasting and feeding may contribute to the initiation and progression of metabolic syndrome [85,86]. Circadian control of glucose metabolism is a well-recognized aspect of clinical diabetes management. Individuals diagnosed with Type I diabetes mellitus are acutely aware of this pattern as patients must adjust their daily insulin requirements to accommodate the fluctuations of insulin in accordance with the light–dark cycle and timing of meals [87]. One hallmark of Type II diabetes is alterations in the normal patterns of glucose regulation/tolerance [88]. Further understanding of the physiological and molecular mechanisms regulating circadian control of glucose tolerance may lead to better strategies for diabetes management.

Insight into RORs regulation of glucose metabolism comes from studies of patients with Von Gierke’s disease, a genetic disorder resulting from a deficiency in the enzyme glucose-6-phosphatase. Deficiency of this enzyme leads to the inability of the liver to produce free glucose from glycogen and from gluconeogenesis. Therefore, reduced glycogen breakdown results in increased glycogen storage in both the liver and kidneys, causing enlargement of both organs and severe hypoglycemia. Both organs function normally in childhood but are susceptible to a variety of problems in adulthood. Mice

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deficient in the NR coactivator, SRC-2, develop symptoms similar to those with von Gierke's disease. Chopra *et al.* demonstrated that RORα, along with SRC-2, was required to regulate *G6Pase* in a normal manner [42].

The 7-oxygenated sterols (7α-hydroxycholesterol [7α-OHC], 7β-hydroxycholesterol [7β-OHC], and 7-ketocholeseterol [7-KC]) function as high-affinity ligands for both RORα and RORγ, behaving as inverse agonists, thus suppressing the constitutive activity of the receptors [89]. The 7-oxygenated sterols play a very important role in both bile acid biosynthesis and in the pathology of atherosclerosis. 7β-OHC and 7-KC are the two most enriched oxysterols found in atherosclerotic plaques following 27-OHC. 7-KC is the most enriched oxysterol in oxidized low density lipoprotein and in the macrophage foam cells [90,91], and has been shown to exert an atherogenic activity by inhibiting sterol efflux from these cells [92]. Plasma levels of 7β-OHC are elevated in patients with cardiovascular disease [93]. Clearly modulators of RORs would prove beneficial for elucidation of the function of RORs *in vivo* and provide the basis for therapeutic intervention of ROR-mediated disorders.

To date, no human diseases or disorders have been exclusively attributed to aberrant REVERB activity. However, empirical evidence links REV-ERB function with the regulation of lipid homeostasis and metabolism. *Rev-erb*α$^{-/-}$ mice display a dyslipidemic phenotype with elevated very low-density lipoprotein triglyceride levels along with increased liver and serum ApoCIII expression [34,47]. In addition, *Rev-erb*α$^{-/-}$ mice exhibit decreased levels of expression of cholesterol 7α-hydroxylase (*Cyp7a1*), the enzyme that catalyzes the rate-limiting step of bile acid biosynthesis [94,95]. Furthermore, REV-ERB has been shown to suppress the expression of *G6Pase* and *PEPCK* and hepatocyte glucose output. Clearly, modulators of this NR could also serve as useful treatments for metabolic disorders.

Future perspective: targeting RORs & REV-ERBs

As more information surrounding the biology of the RORs and REV-ERBs comes to light, it becomes more apparent that these two NR subfamilies play important roles in numerous physiological processes, specifically the regulation of circadian rhythms, lipid homeostasis and glucose homeostasis. Several studies have revealed that the activity of these NRs can be regulated by both endogenous and synthetic ligands. Recent breakthroughs in the development of synthetic ligands selective to the RORs and REV-ERBs promises to provide us with both chemical tools to probe the function of the receptors as well as develop therapeutics towards the treatments of disorders in which the RORs and REV-ERBs play significant roles.

One major challenge in the development of selective synthetic ligands for the treatment of ROR-mediated disorders is our limited knowledge of the function of each subfamily member. Genetic evidence suggests that RORα and RORγ, may have overlapping roles in the regulation of metabolism. However, exactly how much these two NRs overlap in terms of function has yet to be established. While the generation of dual RORα/γ agonists and inverse agonists will significantly aid in elucidating the function of the RORs in a biological context, development and optimization of synthetic ligands, both agonists and inverse agonists, for RORα as well as RORγ will help define the specific roles that each member plays in metabolic processes. Application of the synthetic ligands as chemical tools will prove useful. For example, *in vitro* use will enhance our knowledge of the function of the receptors at the molecular and cellular level. Furthermore, the physiochemical properties of the ligands must be optimized, including their pharmacokinetics, drug solubility and plasma

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protein binding. Once optimized, administration of the compounds *in vivo* will be more telling of the function of the RORs in an organism's physiology.

While there has been successful development of synthetic ligands for RORα and RORγ, little is still known about RORβ. Genetic experiments have provided some insight into the function of this third ROR family member, however, ligands specific to RORβ will aid in our understanding of this NR. Considering the rapid development of synthetic compounds for RORα and RORγ, it is conceivable that the next few years will see the development of compounds specific to RORβ.

Similarly, our recent advances in developing synthetic REV-ERB ligands will allow us to probe the physiological function of these receptors as well as to evaluate the clinical utility of these compounds as potential drugs. Currently, there are only two synthetic ligands available (the agonist GSK4112 and the antagonist SR8278) and both of these ligands have poor pharmacokinetic properties limiting their use *in vivo*. In addition, both demonstrate activity at REV-ERBα and REV-ERBβ. Current efforts in our laboratory are focused on developing REVERB ligands with greater potency/efficacy and improved pharmacokinetic properties as well as receptor subtype selectivity.

In conclusion, the data generated from the *in vitro* characterization of the ROR and REVERB synthetic ligands are extremely promising. However, how these ligands affect the physiology of the organism *in vivo* has yet to be extensively studied. While studies in knockout animals are extremely telling regarding the roles these receptors play in the development and an organism's overall physiology, the ultimate roles of these two opposing NRs in disease progression and resolution can only be determined through *in vivo* use of specific synthetic analogs. Efforts in our laboratory to characterize ROR and REV-ERB compounds *in vivo* are currently underway and are yielding promising, exciting and unexpected results, making the study of the RORs and REV-ERBs even more stimulating.

Key Term

REV-ERBs
Initially, those in the field of the nuclear receptor research thought that REV-ERBs had no endogenous ligands and were ligand-independent constitutive transcriptional repressors. However, recent studies revealed that REV-ERBs respond to an endogenous ligand, heme, by repressing the transcription of their target genes. Without heme binding, the REV-ERBs are transcriptionally inactive, thus there is no basal transcription activity. All cells have some intracellular heme present, thus leading to the appearance that REV-ERBs have constitutive repressor activity

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Executive summary

- Nuclear receptors (NRs) are ligand-dependent transcription factors that regulate the expression of genes important in development, homeostasis and metabolism.
- Ligands to NRs are classified according to their ability to regulate a NRs transcriptional activity. For example, they can be agonists, antagonists, inverse agonists and partial agonists.
- Two subfamilies of NRs, the retinoic acid receptor-related orphan receptors (RORs) and REV-ERBs, have been demonstrated to play critical roles in the regulation of circadian rhythms and lipid homeostasis.
- Identification of endogenous ligands for the RORs has proven controversial and includes various sterols, including cholesterol and its derivatives, as well as several retinoids.
- Recently, heme was identified as an endogenous ligand for the REV-ERBs, thus de-orphanizing this NR.
- Discovery of synthetic compounds to the RORs and REV-ERBs (e.g., T0901317 and GSK4112, respectively) has led to an expansion of structure–activity relationship studies aimed at the identification of novel compounds for both the RORs and REV-ERBs.
- An array of new selective synthetic ligands for ROR and REV-ERBs have recently been discovered creating an opportunity to evaluate these receptors as drug targets for metabolic diseases.

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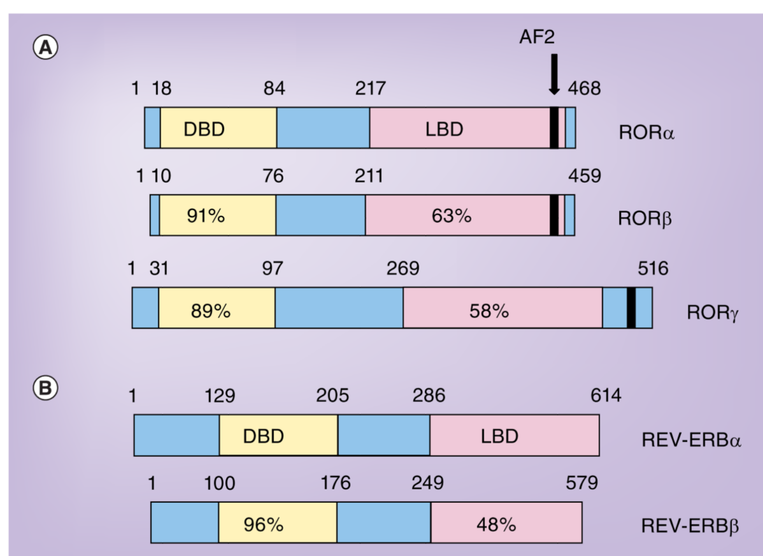

Figure 1. Comparison of human retinoic acid receptor-related orphan receptors and REV-ERB nuclear hormone receptors

(A) The numbers indicate amino acid identity relative to RORα. RORα4, RORβ1 and RORγ1 are shown. **(B)** The numbers indicate amino acid identity relative to REV-ERBα. AF: Activation-function; DBD: DNA-binding domain; LBD: Ligand-binding domain; ROR: Retinoic acid receptor-related orphan receptor.

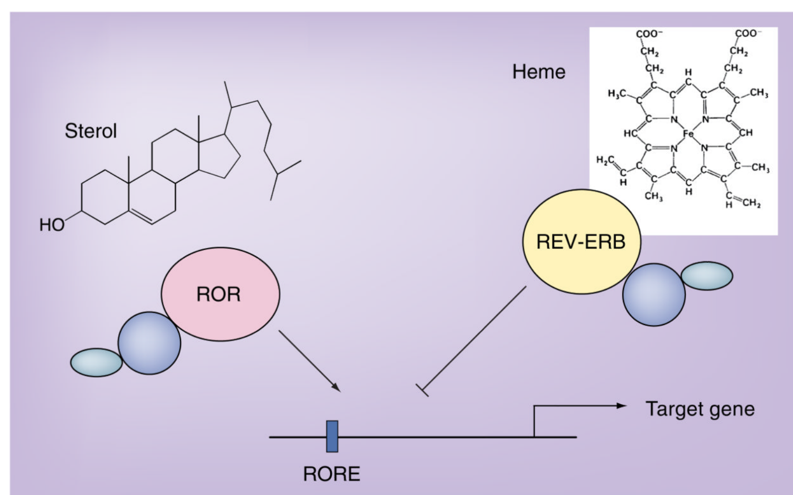

Figure 2. Model illustrating the regulation of a target gene by both retinoic acid receptor-related orphan receptors and REV-ERB

REV-ERB functions as a receptor for heme, which is required for its activity as a repressor. Several sterols have been suggested as ligands for the RORs. In this cartoon, sterols regulate the constitutive activity of the RORs.

ROR: Retinoic acid receptor-related orphan receptor; RORE: ROR response element.

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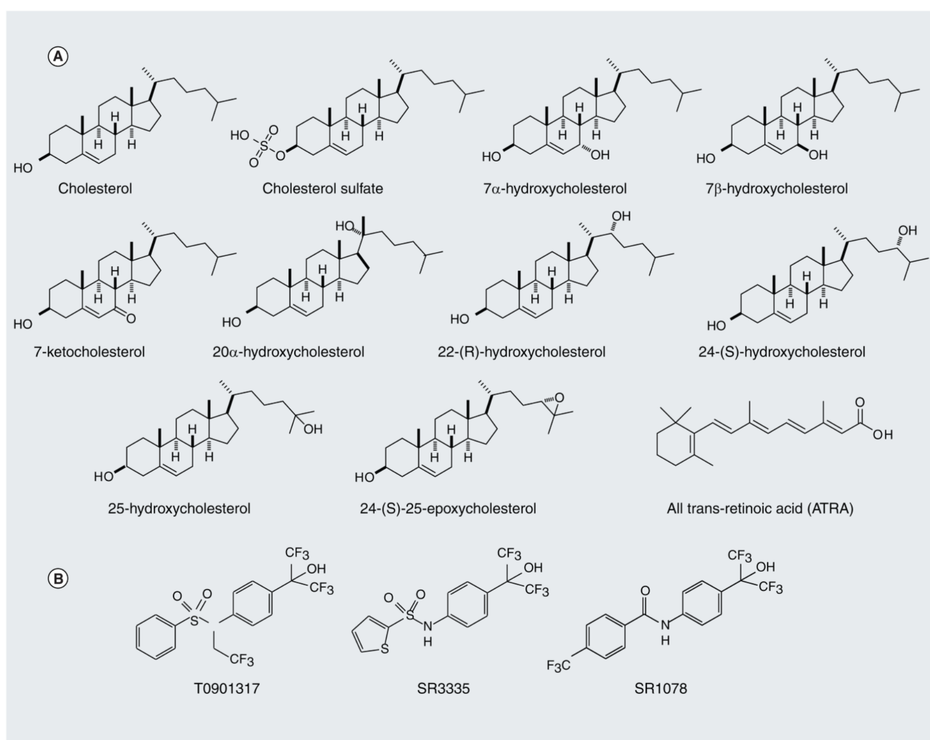

Figure 3. Examples of retinoic acid receptor-related orphan receptor ligands

(A) Endogenous ROR ligands that have been proposed. **(B)** Synthetic ROR ligands that have been identified.

ROR: Retinoic acid receptor-related orphan receptor.

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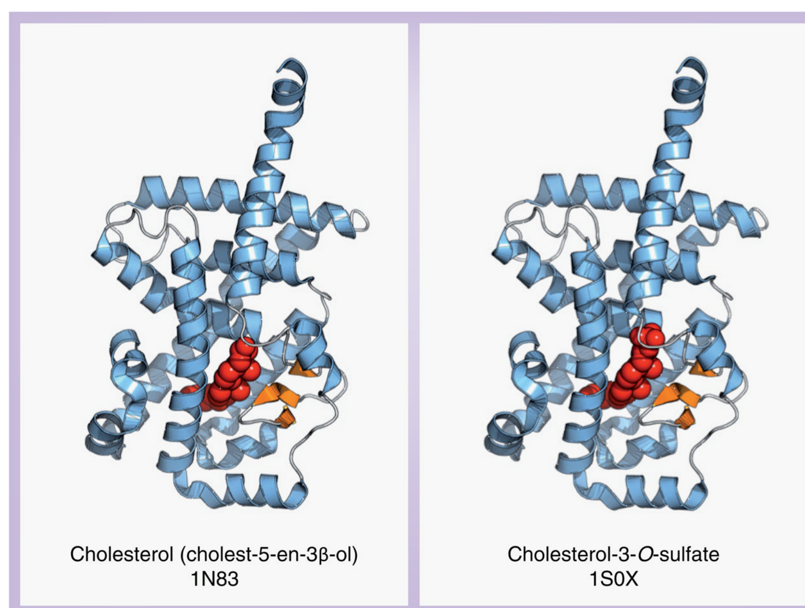

Figure 4. Crystal structures of retinoic acid receptor-related orphan receptor α with two proposed endogenous ligands within the ligand-binding pocket

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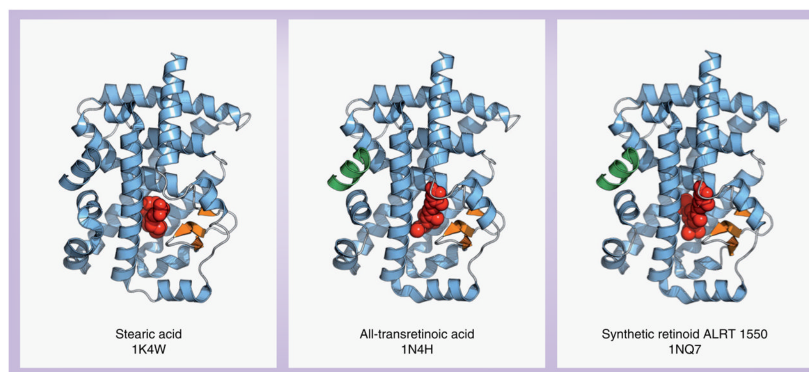

Figure 5. Crystal structures of retinoic acid receptor-related orphan receptor β with three proposed ligands

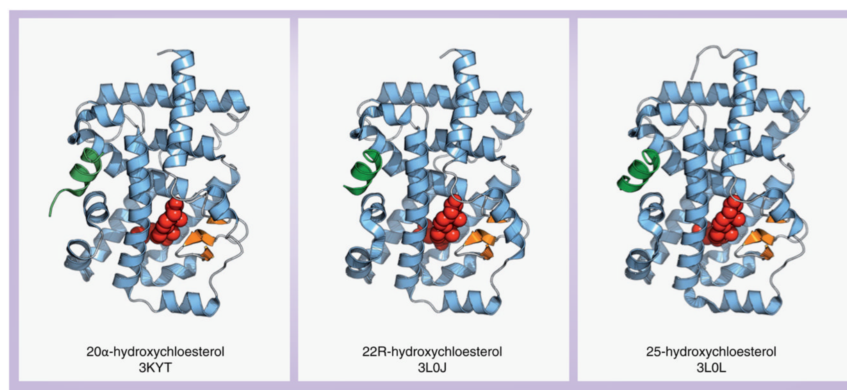

Figure 6. Crystal structures of retinoic acid receptor-related orphan receptor γ with three proposed endogenous ligands within the ligand-binding pocket

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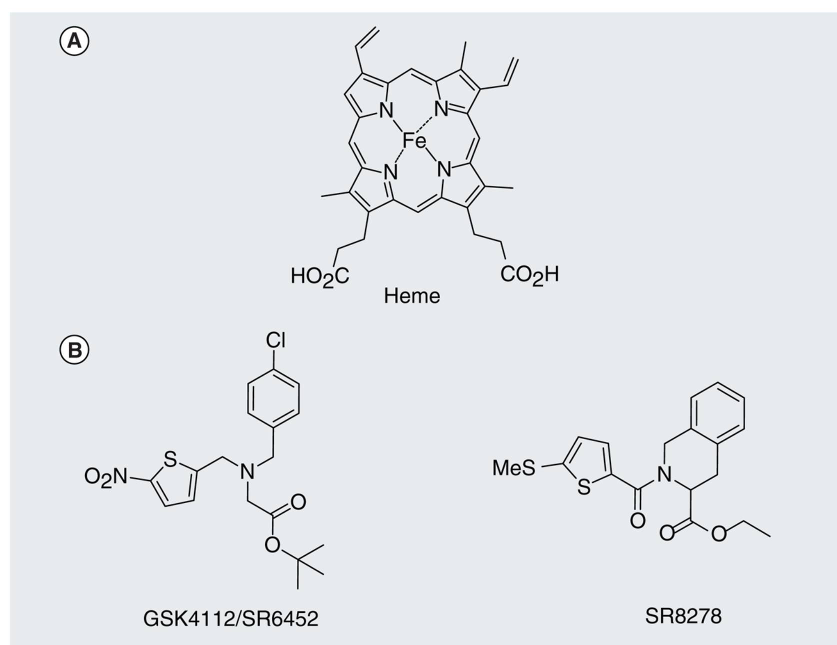

Figure 7. Examples of REV-ERB ligands

(**A**) Heme, the identified endogenous ligand for REV-ERB. (**B**) Two recently characterized REV-ERB-specific synthetic ligands.

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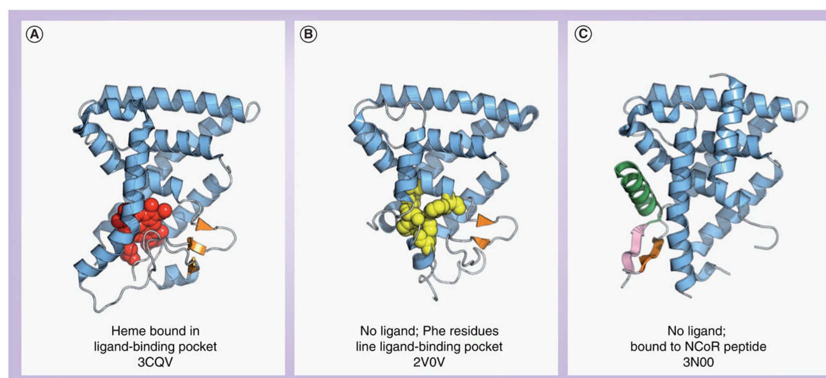

Figure 8. Crystal structures of REV-ERB ligand-binding domain

(**A**) Heme bound in the ligand-binding pocket of REV-ERBβ, (**B**) REV-ERBβ in the absence of a ligand. (**C**) NCoR bound to REV-ERBα in the absence of heme. NCoR: Nuclear receptor corepressor.