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REV-ERB and ROR nuclear receptors as drug targets

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Abstract

The nuclear receptors REV-ERB (consisting of REV-ERBα and REV-ERBβ) and retinoic acid receptor-related orphan receptors (RORs; consisting of RORα, RORβ and RORγ) are involved in many physiological processes, including regulation of metabolism, development and immunity as well as the circadian rhythm. The recent characterization of endogenous ligands for these former orphan nuclear receptors has stimulated the development of synthetic ligands and opened up the possibility of targeting these receptors to treat several diseases, including diabetes, atherosclerosis, autoimmunity and cancer. This Review focuses on the latest developments in ROR and REV-ERB pharmacology indicating that these nuclear receptors are druggable targets and that ligands targeting these receptors may be useful in the treatment of several disorders.

Nuclear receptors are generally classified as ligand-regulated transcription factors, as many members of the nuclear receptor superfamily serve as receptors for physiological ligands, including steroid hormones, lipids and fatty acids. The nuclear receptor superfamily is one of the primary classes of therapeutic drug targets for human disease. Among the drugs that target nuclear receptors are the anti-inflammatory glucocorticoids, steroidal contraceptives and hormone replacement therapies, as well as the fibrate class of lipid-lowering agents. Members of the nuclear receptor family have a conserved modular domain structure (FIG. 1a). The binding of ligands to a region called the ligand-binding domain (LBD) causes a conformational change in this domain, which results in a cascade of downstream events. For some nuclear receptors, such as the glucocorticoid receptor and other steroid receptors, these events include dissociation from heat shock proteins and translocation of the receptor from the cytoplasm to the nucleus. Subsequent to ligand binding, the conformational change in the receptor facilitates the recruitment of transcriptional co-regulatory proteins to receptor-specific gene promoter complexes to activate or repress transcription. However, many other nuclear receptors, such as thyroid hormone receptors and peroxisome proliferator-activated receptors (PPARs), are localized in the nucleus regardless of whether or not they are bound to a ligand and constitutively interact with DNA response elements[1-3].

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When various hormones such as thyroid hormones and the steroid hormones (oestrogens, progestins, glucocorticoids, androgens and mineralocorticoids) were identified, it was not known that they targeted members of the nuclear receptor superfamily; indeed, they were identified before the existence of the superfamily was even known. Even today, the physiological ligands are known for only half of the nuclear receptor superfamily (of which there are 48 members in humans). The development of drugs that target ligand-regulated nuclear receptors led to the design of many therapeutic compounds, which prompted substantial interest in the identification of either natural or synthetic ligands for the orphan members of the superfamily that could be used as chemical tools to probe receptor function and to understand the potential therapeutic value of these receptors. In many cases, these efforts have led to the development of synthetic ligands with pharmacokinetic and pharmacodynamic profiles that are appropriate for their testing as therapeutic modulators in several animal models of disease. This chemical biology strategy has been successful in characterizing several orphan receptors as potential drug targets, including PPARδ, liver X receptor (LXR), retinoid X receptor and the farnesoid X receptor (FXR; also known as bile acid receptor)[4-6].

The chemical biology strategy has recently been applied to additional orphan receptors, including REV-ERBs and retinoic acid receptor-related orphan receptors (RORs). These two classes of nuclear receptors share many of the same target genes and thus have substantial overlap in functions that are known to include regulation of the circadian rhythm, metabolism and immune function[7-10].

In this Review, we discuss the physiological and pathological roles of these two classes of nuclear receptors and the discovery of their natural ligands, as well as the development of synthetic ligands targeting these receptors and their use in cellular and *in vivo* models of disease. Finally, we highlight potential future directions for therapeutics targeting REV-ERB and ROR for the treatment of autoimmune diseases, central nervous system (CNS) disorders, diabetes and obesity.

Roles of REV-ERB and ROR

REV-ERBs

The REV-ERBs acquired their unusual name owing to the unique genomic organization of *NR1D1*, which encodes REV-ERBα. REV-ERBα is encoded by the opposite DNA strand of the *ERBA* (also known as *THRA*) oncogene[11-13], and hence its name is derived from ‘reverse strand of *ERBA*’. *ERBA* encodes the thyroid hormone receptor-α and thus REV-ERBα is encoded by sequences of DNA on the opposite strand of the gene that encodes thyroid hormone receptor-α. Both REV-ERBα and the closely related REV-ERBβ (encoded by *NR1D2*), which was identified a few years after REV-ERBα, have an atypical LBD that lacks the carboxy-terminal activation function 2 (AF2) region[14-16] (FIG. 1b). Because the AF2 region recognizes co-activators that are necessary for transcriptional activation, REV-ERBα and REV-ERBβ are generally characterized as being unable to activate transcription. Indeed, the REV-ERBs are constitutive repressors of transcription owing to their constant binding of co-repressors such as the nuclear receptor co-repressor 1 (NOR1)[17].

The recruitment of co-repressors to the target gene by a nuclear receptor (via the DNA response element) leads to repression of the target gene owing to histone deacetylation and condensation of chromatin[17,18]. Unlike many other nuclear receptors that function as obligate heterodimers (either as homodimers or as heterodimers with retinoid X receptor) and recognize two copies of a core sequence of nucleotides that are organized in either a palindromic or a repeated manner (termed the half site), REV-ERBs typically function as monomers and recognize a half site that consists of a single 5′ extended AGGTCA sequence[7]. However, REV-ERB homodimers have been reported to occur under some conditions[18,19]. REV-ERBs have overlapping patterns of temporal and spatial expression, which is consistent with our current understanding of their substantial overlapping functions. Both REV-ERBα and REV-ERBβ are widely expressed throughout the body and, interestingly, both receptors have a circadian pattern of expression that is essential for their role in the circadian regulation of transcription[20-23].

RORs

The three members of the ROR subfamily — RORα, RORβ and RORγ — have sequence similarities to the retinoic acid receptor[24-27] and each receptor can constitutively activate transcription through the ligand-independent recruitment of transcriptional co-activators (FIG. 1b). RORα is widely expressed in many tissues, including cerebellar Purkinje cells, the liver, thymus, skeletal muscle, skin, lung, adipose tissue and kidney[28,29]. RORγ has a similar broad pattern of expression but is observed at very high levels within the thymus. RORβ has a more restricted pattern of expression relative to the other RORs, and is found in regions of the CNS that are involved in the processing of sensory information, the retina and the pineal gland[30].

There is considerable overlap in the DNA response elements that are recognized by REV-ERBs and RORs, and both receptors are often co-expressed in the same tissues[31]. Because RORs constitutively activate transcription, whereas REV-ERBs repress transcription, the balance of ROR and REV-ERB activity is crucial for the dynamic regulation of target genes containing the DNA response elements that are responsive to both classes of receptors (FIG. 2). Owing to the substantial overlap in expression patterns as well as the target genes that are regulated by these receptors, REV-ERBs and RORs are often involved in the regulation of similar physiological processes, as outlined below.

Regulation of the circadian rhythm

Circadian rhythms have an essential role in the sleep–wake cycle, feeding behaviour and metabolism, as well as in the control of body temperature, blood pressure and renal function[32]. The circadian rhythm is generated by feedback loops in the expression patterns of genes encoding proteins that make up the so-called molecular clock (FIG. 3). Heterodimers of two transcription factors, brain and muscle ARNT-like 1 (BMAL1; also known as ARNTL) and circadian locomotor output cycles protein kaput (CLOCK), induce the expression of the cryptochrome genes (*CRY1* and *CRY2*) and the period circadian clock genes (*PER1, PER2* and *PER3*) genes. As CRY and PER proteins reach crucial levels, they repress the stimulatory effect of the CLOCK–BMAL1 dimer on the expression of their

respective genes. The dynamic interplay between the opposing circadian patterns of expression and the opposing transcriptional activity of RORs and REV-ERBs, resulting in the positive and negative regulation of gene transcription, is readily apparent in this feedback loop as both classes of receptors have been shown to regulate *BMAL1* expression[7].

As well as RORs and REV-ERBs, various other nuclear receptors have been implicated in the modulation and/or regulation of the circadian rhythm. Over half of the nuclear receptor superfamily members are expressed in a circadian manner[33], and given their role as transcription factors this probably leads to rhythmic expression of their target genes. Other direct links between nuclear receptor activity and circadian clock function have been identified. These include the direct interaction of the glucocorticoid receptor with CRY1 and CRY2, which mediates the rhythmic repression of glucocorticoid receptor transcriptional activity; this effect is essential for normal glucocorticoid signalling, which follows a clear circadian pattern[34]. Additionally, PER2 has been shown to interact with PPARα and REV-ERBα at promoter sites to regulate their transcriptional activity[35].

REV-ERBα represses the transcription of *BMAL1* (REFS 22,36) through its actions on two DNA response elements that are located in the *BMAL1* promoter. The circadian feedback loop shows additional complexity given that REV-ERBα expression is itself regulated by BMAL1–CLOCK heterodimers via E box DNA response elements found within the *Nr1d1* promoter[37,38]. *Nr1d1*$^{-/-}$ mice have aberrant expression of *Bmal1* and alterations in the period and phase of their circadian locomotor behaviour[36]. *Nr1d2*$^{-/-}$ mice have a much more subtle circadian phenotype, but the *Nr1d1*$^{-/-}$*Nr1d2*$^{-/-}$ double knockout mice are arrhythmic[39] and have a similar phenotype to *Bmal1*$^{-/-}$ mice[40], *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice[41] and *Per1*$^{-/-}$*Per2*$^{-/-}$ mice[42]. Indeed, the expression of genes encoding the REV-ERBs is driven by E-box DNA response elements in their promoter elements, which are similar to those that drive the circadian expression of the *CRY* and *PER* genes. These data suggest that the genes that encode REV-ERBs should be considered as core clock genes per se rather than components of an accessory loop that merely modulates the pattern of expression of the core clock genes.

In contrast to the REV-ERBs, RORs stimulate *BMAL1* expression[43]. Mice with a loss-of-function mutation in RORα (*Rora*$^{sg/sg}$ mice; also known as staggerer mice) have alterations in the circadian oscillator, indicating an essential role for this receptor in normal circadian function[43]. REV-ERBα (the repressor) and RORα (the activator) are expressed in an oscillatory fashion 12 hours out of phase with each other, leading to alternating activation and repression of *BMAL1* expression[36,43]. RORβ-null mice also have a circadian deficit with a longer period (τ) than wild-type mice[44,45], and RORγ has also been implicated in the regulation of the circadian rhythm[46]. Given that RORs and REV-ERBs are regulated by ligands, synthetic ligands that act at these nuclear receptors could be used to modulate the circadian rhythm as well as to treat diseases that are associated with disrupted circadian rhythms, such as sleep disorders, metabolic disease and behavioural disorders. The initial studies that have aimed to test this hypothesis are addressed below.

Regulation of metabolism

Circadian rhythms are intricately linked to the regulation of metabolism, and genetic perturbations of core clock genes lead to a range of abnormal metabolic phenotypes in mice, including obesity, dyslipidaemia and glucose intolerance[47-51]. In humans, circadian disruption caused by shift work[52-54] or manipulated under controlled conditions causes metabolic disturbances[55,56]. The role of RORs and REV-ERBs in the regulation of metabolic pathways is well characterized. Both receptors are crucial components of the clock that link the core circadian oscillator to the regulation of clock-controlled genes, which in turn regulate metabolic pathways.

Loss-of-function studies both *in vitro* and *in vivo* clearly demonstrate that REV-ERBs have a crucial role in lipid metabolism. REV-ERBα-null mice have dyslipidaemia with elevated levels of very-low-density lipoprotein (VLDL) triglyceride and increased serum levels of apolipoprotein C3 (APOC3)[57,58]. *Rora*$^{sg/sg}$ mice have the opposite phenotype with reduced APOC3 expression and lowered triglyceride levels[58]. APOA1, a component of high-density lipoprotein (HDL), is also regulated by both REV-ERBα and RORα[59]. The expression of several genes involved in lipid metabolism was suppressed in a myocyte cell line expressing a dominant negative form of REV-ERBβ[60]; these genes included fatty acid translocase (*FAT*; also known as *CD36*), fatty acid binding protein 3 (*FABP3*; also known as *MDGL*), *FABP4* (also known as *ALBP*), mitochondrial uncoupling protein 3 (*UCP3*; also known as *SLC25A9*), sterol regulatory element-binding transcription factor 1 (*SREBF1*) and stearoyl-CoA desaturase (*SCD*). Although it is unclear whether these are direct target genes of REV-ERBβ, this study clearly demonstrates that REV-ERBβ is involved in the regulation of genes that are involved in fatty acid and lipid absorption, energy expenditure and lipogenesis in muscle.

Hepatic glucose metabolism is regulated by REV-ERBα, which directly regulates the expression of the genes encoding the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (*PCK*) and glucose-6-phosphatase (*G6PC*)[61]. Mice that are deficient in either REV-ERBα or both REV-ERBα and REV-ERBβ have increased plasma glucose levels[39,62]. This phenotype has been examined in more detail in REV-ERBα-null mice, yet no alteration in insulin sensitivity was noted[62]. In contrast to the REV-ERBα- and REV-ERBβ-deficient mice, both the *Rora*$^{-/-}$ mice and *Rora*$^{-/-}$*Rorg*$^{-/-}$ mice have reduced blood glucose levels[63]. *Rora*$^{sg/sg}$ mice also have improved insulin sensitivity with increased glucose uptake in skeletal muscle[64].

REV-ERB is a key regulator of the oxidative capacity of skeletal muscle and mitochondrial biogenesis[65]. REV-ERBα-null mice had reduced mitochondrial content and oxidative function, which resulted in reduced exercise capacity[65]. RORα and RORγ are both expressed in skeletal muscle. The expression of a dominant negative form of RORα in muscle resulted in altered expression of genes involved in lipid metabolism[66]. *Rora*$^{sg/sg}$ mice develop muscular atrophy but the exact mechanism underlying this is unclear.

REV-ERBs are also involved in adipogenesis. REV-ERBα expression is highly induced during adipogenesis[67], and overexpression of REV-ERBα in 3T3-L1 cells results in

increased expression of markers of adipogenesis, such as FABP4, PPARγ and CCAAT/enhancer binding protein-α (C/EBPα), as well as an increase in lipid accumulation[68]. Furthermore, overexpression of REV-ERBα in these cells synergized with the effects of a PPARγ ligand to increase markers of adipogenesis[68]. Although REV-ERBα expression is required for adipogenesis in cell-based models, REV-ERBα deficiency *in vivo* is associated with increased adiposity and increased weight gain owing to a high-fat diet[62]. This apparent discrepancy may be due to a dual role for REV-ERBα in adipogenesis, where REV-ERBα expression is increased in the initial stages of adipogenesis but the protein is degraded in the late stages of the process to allow for efficient development of the fat cells[69]. Interestingly, the degradation of REV-ERBα in late-stage adipogenesis seems to be dependent on gradually increasing levels of the natural ligand for REV-ERBα[70,71].

The phenotypes of the knockout mice described above are consistent with the often opposing roles of REV-ERB and ROR. *Rora*$^{sg/sg}$ mice have reduced adipose mass and are less susceptible to weight gain on a high-fat diet[72]. RORγ also seems to be a negative regulator of adipocyte differentiation, and mice deficient in RORγ are resistant to weight gain induced by a high-fat diet[73] and have increased sensitivity to insulin. REV-ERBα-deficient mice have substantial hepatic steatosis[74], whereas *Rora*$^{sg/sg}$ mice seem to be less susceptible to hepatic steatosis[72]. Given the diverse roles of these nuclear receptors in the regulation of metabolism, it is clear that ligands that modulate REV-ERB and ROR activity may hold utility in treatment of several metabolic disorders, including obesity, type 2 diabetes and atherosclerosis.

Regulation of immune function

RORα and RORγt (an isoform of RORγ, encoded by *RORC*) are crucial for the development of T helper 17 cells (T_H17 cells), which have an essential role in the development of many autoimmune disorders, including multiple sclerosis, psoriasis and rheumatoid arthritis[75,76]. Overexpression of RORγt in naive $CD4^+$ T cells is sufficient to drive the induction and development of T_H17 cells[77]. Furthermore, the development of T_H17 cells is impaired in *Rorc*$^{-/-}$ mice[77]. Mice that are deficient in RORα and RORγ lack T_H17 cells altogether and are resistant to the development of autoimmune diseases[78]. These data suggest that the development of ROR-targeted inhibitors with the potential to suppress T_H17 cell development might hold utility in the treatment of autoimmune diseases.

The knowledge of the role that REV-ERBs have in the regulation of the immune system is not as well developed as for RORγ. However, REV-ERBα has been demonstrated to regulate the production and release of the pro-inflammatory cytokine interleukin-6 (IL-6) in macrophages[79]. Additionally, genome-wide analysis of REV-ERBα- and REV-ERBβ-binding sites in macrophages revealed that these receptors were involved in the complex regulation of target genes, which suggests that REV-ERBα and REV-ERBβ have an important role in this cell type[80]. Given the opposing roles of the RORs and REV-ERBs, it is likely that REV-ERBs may directly repress T_H17 cell development. In fact, T_H17 cell differentiation is altered in REV-ERBα-null mice[81], and further work will be required to determine the exact role of these receptors in the regulation of T_H17 cell function and autoimmunity. Knockdown of *Nr1d1* in haematopoietic cells followed by bone marrow

transplantation into LDL receptor (LDLR)-null mice revealed that REV-ERB has a crucial role in the development of atherosclerosis[82]. Atherosclerotic plaque development was increased in these mice, but lipid levels were unaffected. This effect was attributed to altered macrophage function, as overexpression of REV-ERBα led to increased levels of anti-inflammatory M2 macrophages[82]. These data suggest that increasing the repressive activity of REV-ERB may be useful for the treatment or prevention of atherosclerosis.

Endogenous ligands for REV-ERB and ROR

The RORs and REV-ERBs were both initially identified as orphan receptors, and at the time it was not clear that these nuclear receptors were regulated by small-molecule ligands. The subsequent identification of endogenous ligands for these proteins provided clear evidence that a chemical biology approach could be taken to design synthetic ligands with the ability to regulate the activity of these receptors.

Identification of haem as a physiologically relevant REV-ERB ligand

Studies using the *Drosophila melanogaster* orthologue of REV-ERB suggested that the human receptor might bind to haem[83]. Indeed, direct binding of haem to REV-ERBs was demonstrated using several biochemical and biophysical methods, including mutation studies that identified a key amino acid residue in REV-ERBα that was necessary for haem binding[61,84], transcriptional repressor function and repression of target gene transcription[61,84]. Moreover, reduction of intracellular haem levels decreased REV-ERB-mediated repression of REV-ERB target genes (that is, delta-aminolevulinate synthase 1 (*ALAS1), BMAL1*, elongation of very-long-chain fatty acid elongase 3 (*ELOVL3*), *G6PC* and *PCK*), decreased the interaction between REV-ERB and the NCOR–HDAC3 (histone deacetylase 3) co-repressor complex in cells, and impaired the recruitment of NCOR to REV-ERB target gene promoters[61,84]. These studies, together with additional biophysical studies examining the affinity of haem for REV-ERB, suggested that haem acts as a bone fide ligand for the REV-ERBs.

Adipogenesis is regulated by haem in a REV-ERB-dependent manner[71]. Intracellular haem levels are increased during adipogenesis, and inhibition of haem biosynthesis has been shown to inhibit adipogenesis. Increased REV-ERB levels reduce intracellular haem levels through direct modulation of PPARγ co-activator 1α (PGC1α; also known as PPARGC1A) levels[85,86], suggesting a mechanism by which REV-ERBs regulate the biosynthesis of their endogenous ligand, haem. Haem may also have an important role in the regulation of the circadian function of REV-ERB. Haem levels and REV-ERB expression are regulated in a circadian manner, which suggests that oscillations in the availability of haem may regulate the transcriptional activity of REV-ERB[87].

The crystal structures of REV-ERBs in the apo structure form (FIG. 4a) and bound to haem (FIGS 4b,c) have provided some insight into the molecular details of haem coordination by REV-ERBs, and indicated how REV-ERB might be targeted by synthetic ligands. The structure of the haem-bound LBD of REV-ERBβ (FIG. 4b,c) revealed that the REV-ERB ligand-binding pocket is located in the same structural region as other nuclear receptors[88]. The structure revealed that haem is coordinated by two key residues, a histidine residue on

helix 11 and a cysteine residue on helix 3. Mutations in these residues prevent haem from binding to REV-ERB and result in loss of REV-ERB activity[61,84,88,89]. The ligand-binding pocket undergoes a large expansion to accommodate the haem ligand, and the bulky hydrophobic residues that make up the inaccessible pocket in the apo structure were found to form key hydrophobic interactions with the porphyrin ring of the haem molecule. However, porphyrin-like synthetic ligands have not been pursued because many proteins use haem as a cofactor, which could lead to potential specificity issues.

The crystal structure of the LBD of the apo form of REV-ERBα complexed to an NCOR fragment (co-repressor nuclear receptor (CoRNR) box motif) (FIG. 4d) was reported, which was unexpected because cell-based studies indicate that NCOR recruitment is dependent on haem. This structure suggests that apo REV-ERBs can indeed bind to co-repressors. Notably, the authors were unable to obtain crystals for the REV-ERBα LBD complexed with an NCOR fragment in the presence of haem[90]. Although cell-based studies have revealed that the binding of haem to REV-ERB increases the recruitment of co-repressor proteins such as NCOR, leading to transcriptional repression[61,84], these structural studies (as well as other biochemical studies) suggest that haem binding results in a loss of co-repressor binding. However, it should be noted that structural and biochemical studies use isolated LBDs and peptide fragments of the co-repressor proteins NCOR and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor; also known as NCOR2), which are clearly distinct from the full-length co-repressor proteins that are present under physiological circumstances[61,84,88]. Thus, the precise mechanism of action of haem-dependent REV-ERB-mediated transcriptional repression remains unclear, and the discrepancies noted above may be due to differences in the conformation of the receptor when it is bound to relatively short NCOR peptide fragments versus the full-length NCORs.

REV-ERB as a gas sensor

The functions of many haem-binding proteins are regulated by small-molecule diatomic gases, such as carbon monoxide and nitric oxide. Indeed, REV-ERBs are responsive to diatomic gases, which repress REV-ERB-mediated transcription[88], and are sensitive to the redox state of the iron-haem centre[88,89,91]. Cell lines treated with the chemical nitric oxide donor diethylenetriamine have increased expression of *BMAL1, NR1D1* and *NR1D2*, and REV-ERB-dependent transcriptional repressor activity was suppressed, which suggests that nitric oxide acts as an antagonist of REV-ERB activity[88]. Similar to the results obtained in haem-dependent biochemical and cell-based studies, nitric oxide — which acts as an antagonist of REV-ERB activity in cells — increased the interaction between REV-ERB and co-repressor peptides in biochemical studies, which indicates that it acts as an agonist rather than an antagonist of REV-ERB activity in this setting[88]. Thus, the molecular mechanism by which a small-molecule diatomic gas regulates REV-ERB activity, as well as its physiological significance, remains unclear.

Before REV-ERB was identified as a haem-binding protein, its *D. melanogaster* orthologue E75 was shown to bind to haem in an obligate manner[83]. Nitric oxide regulated E75 activity by altering its ability to interact with its heterodimer partner, the *D. melanogaster* orphan nuclear receptor HR3 (DHR3), thus altering the transcriptional activity of the dimers[83].

Unlike REV-ERB, haem seems to function as an obligate cofactor in the LBD of E75. Given the role of nitric oxide in the regulation of the circadian rhythm[92], it is possible that nitric oxide may be a physiological ligand for REV-ERB. This would lead to a unique model of nuclear receptor regulation where REV-ERB is regulated by haem levels as well as nitric oxide levels. Thus, REV-ERB may only be responsive to nitric oxide when haem levels are sufficiently elevated to occupy the LBD of the receptor.

Sterol ligands for RORs

Cholesterol has been demonstrated to act as a ligand for RORα by binding to the LBD[93] of the receptor (FIG. 4e,f). Other cholesterol metabolites, including cholesterol-3-*O*-sulphate, also bind to the LBD of RORα[94].

More recent studies, including mechanistic analyses, have revealed that several oxysterols are high-affinity endogenous modulators of ROR activity[95-97] (TABLE 1). Oxysterol ligands bind directly to the LBD of RORα and RORγ and modulate the interaction of co-regulators. For example, 7-oxygenated sterols inhibit *G6PC* expression by reducing the recruitment of nuclear receptor co-activator 2 (NCOA2; also known as SRC2 or TIF2) by RORα and RORγ on the *G6PC* promoter[96]. 24*S*-hydroxycholestrol reduces the expression of *BMAL1* by reducing SRC2 recruitment by RORα[95]. These actions of sterols reduce the transcriptional activity of RORs and the expression of ROR target genes in cells; through these actions, oxysterol ligands function as ROR inverse agonists. Structure-based studies have provided further evidence that cholesterol-based ligands induce a conformational change in RORα that is consistent with a reduction in the affinity of co-activators for this receptor. However, it seems that cholesterol or other sterols do not act as ligands for RORβ[98].

Interestingly, a recent study that screened a commercially available plant extract library for ROR ligands identified a natural product plant sterol called neoruscogenin as a RORα agonist[99]. In a biochemical assay measuring the recruitment of the cofactor SRC2 to RORα, neoruscogenin had an EC_{50} (effector concentration for half-maximum response) value of 0.11 μM. In cell-based assays, neoruscogenin induced transcription driven by a chimeric receptor, Gal4–RORα, and also increased the transcription of RORα target genes in HepG2 cells[99]. Although neoruscogenin had activity against the pregnane X receptor, it was selective versus other nuclear receptors and it also induced the expression of hepatic RORα target genes when it was orally administered to mice[99].

Endogenous ligands for RORβ

The first ligand that was demonstrated to bind to any ROR was stearic acid, which was fortuitously discovered when the LBD of the RORβ protein that was expressed in *Escherichia coli* crystallized with a stearate ligand that bound to the putative ligand-binding pocket of the receptor[100]. However, it was unclear at the time — and is still unconfirmed at present —whether stearate is similar to a putative physiological ligand found in humans, such as a fatty acid. Because RORs are evolutionarily related to the retinoic acid receptors, which serve as receptors for retinoic acid and other metabolites, a mass spectrometry approach was used as a screening tool to determine whether retinoids may also serve as endogenous ligands for RORβ[98]. This approach revealed that all-*trans* retinoic acid (ATRA)

(TABLE 1) and the synthetic retinoid ALRT 1550 (TABLE 1) bind to the RORβ LBD, which was verified by co-crystal structures of RORβ bound to these ligands. In addition to these two ligands, all-*trans*-4-oxoretinoic acid binds to RORβ, and all three ligands have been shown to function as inverse agonists of RORβ in a cell-based cotransfection reporter assay. Retinoids do not, however, bind to RORα or RORγ, which suggests that RORβ has a subtype-specific ligand preference compared to RORα and RORγ; this may allow for the development of subtype-specific synthetic ligands targeting these receptors.

Synthetic ligands for REV-ERB and ROR

The discovery that REV-ERBS and RORs are liganded receptors has led us and others to develop screens to find ligand scaffolds that could be used to identify potent ligands. Such potent ligands could be used as chemical tools to probe the role of REV-ERBs and RORs in physiological and disease models.

Synthetic REV-ERB ligands

REV-ERB agonist: GSK4112

The first synthetic REV-ERB-targeting ligand to be identified was 1,1-dimethylethyl *N*-[(4-chlorophenyl)methyl]-*N*-[(5-nitro-2-thienyl) methyl]glycinate[101]. It was first published with no other moniker and was subsequently synthesized and characterized by our group and given the name SR6452. The compound was renamed GSK4112 in a follow-up paper from the group[92] that initially identified it, and we suggest that this name should now be used. GSK4112 was identified from a fluorescence resonance energy transfer (FRET) biochemical screen in which it increased the binding of an NCOR peptide to REV-ERBα in a concentration-dependent manner[101]. Further mechanistic studies investigated the pharmacological effects of this compound in cell-based assays[71,102] (TABLE 2).

GSK4112 increases the recruitment of NCOR to the *BMAL1* promoter[71] and increases the recruitment of HDAC3 to the *G6PC* promoter[102], providing a mechanism for the repressive effect of the compound on REV-ERB target genes. Primary mouse hepatocytes treated with GSK4112 have decreased expression of several gluconeogenic REV-ERB target genes and a decreased hepatic glucose output, which suggests that GSK4112 has the potential to be used as an antidiabetic compound[102]. Additionally, the compound modulated the expression levels of several key genes involved in the circadian rhythm, providing a potential pharmacological approach for the modulation of the circadian rhythm[102]. As REV-ERB has been shown to be involved in the differentiation of 3T3-L1 cells to adipoctyes, the effect of the compound on adipogenesis was examined[71]. Both rosiglitazone (a PPARγ agonist) and GSK4112 induced the expression of several key gene markers of adipogenesis, including *FABP4, PPARG, PCK*, adiponectin C1Q and collagen domain containing protein (*ADIPOQ*), *FAS* and resistin (*RETN*), and they also increased the lipid content of the cells. Additional studies have demonstrated that rosiglitazone and GSK4112 synergistically induce adipogenesis[71].

Unfortunately, GSK4112 does not have a favourable pharmacokinetic profile following intraperitoneal administration (it has a low systemic exposure) and it displayed weak

efficacy in terms of REV-ERB agonism, which limits its use as a tool to probe the function of REV-ERBs *in vivo*. Therefore, we — and other groups — undertook a comprehensive structure–activity relationship (SAR) analysis to identify potent and efficacious REV-ERB modulators based on the GSK4112 scaffold that had pharmacokinetic and pharmacodymamic properties that would make them suitable for *in vivo* studies[103,104]. Below, we describe several key tool compounds that were identified in SAR campaigns and that led to subsequent studies providing insight into REV-ERB function *in vitro* and *in vivo*.

REV-ERB agonists: SR9009 and SR9011

Only two REV-ERB ligands have been characterized *in vivo*[103-105], although other compounds have been shown to be bio-available, as discussed below (TABLE 1). Both of these compounds used GSK4112 as an initial scaffold, with several modifications to improve potency, efficacy and pharmacokinetic properties (increased systemic exposure). SR9009 and SR9011 are dual synthetic agonists of REV-ERBα and REV-ERBβ, and they have been used to demonstrate that pharmacological targeting of REV-ERBs may be useful in the treatment of circadian disorders, including metabolic diseases and sleep disorders[105].

SR9009 and SR9011 are three- to fourfold more potent than GSK4112, and are threefold more efficacious in driving REV-ERB repression in a reporter gene luciferase assay. In addition, they had no significant cross-activity in a specificity assay against the 46 other mem bers of the human nuclear receptor superfamily[105,106]. Studies to determine the effects of the compounds on circadian behaviour in mice revealed that a single intraperitoneally administered dose of either compound, given when REV-ERBα expression levels peak (that is, in the middle of the sleep period), results in loss of wheel running activity during the subsequent wakeful period. In addition, both compounds were shown to affect the circadian expression of several core clock genes in the hypothalamus of mice, including the suppression of *Cry2*, enhancement of *Per2*, a phase shift in the expression of *Bmal1* and complete elimination of the circadian expression pattern of neuronal PAS domain-containing protein 2 (*Npas2*)[105]. Together, these data indicate that SR9009 and SR9011 substantially affect the circadian oscillator through the modulation of REV-ERB activity.

Consistent with the range of metabolic effects noted in REV-ERBα-null mice, pharmacological activation of REV-ERB with SR9009 and SR9011 had additional metabolic effects in mice. Most notable was the weight loss in diet-induced obese mice, which was associated with an increase in energy expenditure without alterations in locomotor behaviour or food intake[105]. A decrease in plasma triglycerides, total cholesterol and non-esterified fatty acids was also noted in the obese mice after treatment. These metabolic changes correlated with changes in the expression of key factors in these metabolic pathways, including decreased expression of lipogenic enzymes (fatty acid synthase (*Fasn*) and *Scd*) and cholesterologenic regulator proteins (3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*) and *Srebf2*), as well as increased expression of genes encoding enzymes involved in fatty acid and glucose oxidation (carnitine palmitoyltransferase 1b (*Cpt1b*), *Ucp3*, PPAR-gamma co-activator 1-beta (*Ppargc1b*), M2 isoform of pyruvate kinase muscle (*Pkm2*) and hexokinase 1 (*Hk1*))[105].

During the development of SR9009 and SR9011, we identified almost 50 additional REV-ERB ligands, several of which had substantial *in vivo* exposure (including in the CNS) when administered intraperitoneally[104]. A more recent study has shown that the activation of REV-ERB with SR9009 *in vivo* leads to increased oxidative metabolism and mitochondrial biogenesis in skeletal muscle, resulting in increased exercise endurance[65], which is consistent with the metabolic alterations and weight loss observed[105].

Additional REV-ERB agonists

GlaxoSmithKline has also pursued the GSK4112 scaffold and recently described four additional compounds that can be used to probe REV-ERB function (TABLE 1). Four of these have sufficient pharmacokinetic properties to be used *in vivo* and they are orally bioavailable[107]. Three of these compounds have similar pharmacokinetic properties to SR9009 (compounds 10, 16 and 23), whereas one compound (compound 4) has an increased half-life, area under the curve (AUC) and maximum peak plasma concentration (C_{max}). In addition, it has an approximately tenfold higher bioavailablity than the other compounds. Other than SR9009, these compounds have not yet been tested in animal models of disease.

GSK4112, SR9009 and SR9011 — and almost all of the analogues reported in REFS 104,107 — contain a nitrothiophene group, which carries a potential toxicological liability[104,107]. We sought to mitigate this potential toxicological liability and recently described a distinct chemical series of tetrahydroisoquinoline-based REV-ERB agonists lacking the nitrothiophene group, with potencies (that is, half-maximal inhibitory concentration (IC_{50}) values) of ~70 nM in a REV-ERB co-transfection assay[103] (TABLE 1). Although these tetrahydroisoquinoline-based REV-ERB agonists are not orally bioavailable, many of them have reasonable plasma exposure and half-lives of ~2 hours when administered intraperitoneally[103]. As of yet, no dedicated toxicological studies have been performed for any of the REV-ERB ligands.

Specificity of SR9009 and SR9011

Although the original report of SR9011 and SR9009 examined the specificity of these compounds in a Gal4-chimeric nuclear receptor assay panel and observed no activity other than at the REV-ERBs[105], a recent report indicates that these two ligands may have some agonist activity at the LXR[107]. In the original paper that reported the identification of GSK4112, it was indicated that the compound did not modulate LXRα or LXRβ in reporter assays[101], but it was unclear whether these data were taken from a chimeric Gal4 LBD or a full-length REV-ERB co-transfection assay. In a follow-up study, GSK4112 had an IC_{50} value of 5 μM in an LXRα radioligand binding assay and increased ATP-binding cassette subfamily A member 1 (*ABCA1*) mRNA expression in THP-1 cells, which was attributed to LXR activity[107]. SR9009 and SR9011 had similar activity as GSK4112 in these assays[107]. However, we have not observed LXR activation by either SR9011 or SR9009 in assays that use chimeric Gal4 LBD co-transfections[105] or with studies that use full-length LXR co-transfections (T.B., unpublished observations). We therefore believe, as suggested by several additional studies, that these compounds do not have substantial LXR agonist activity. Treatment of HepG2 cells with SR9009 or SR9011 suppressed (rather than activated) the well-characterized LXR target gene *Srebf1* (REF. 105). Additionally, when SR9009 was

administered to mice, hepatic expression of *Srebf1* and several other direct LXR target genes, including cytochrome P450 family 7 subfamily A polypeptide 1 (*Cyp7a1*), *Fasn* and *Scd*, was suppressed; this finding is inconsistent with SR9009 acting as an LXR agonist[105]. Although one study indicated that SR9009 and SR9011 were active in an LXR radioligand binding assay (with IC_{50} values of 6–13 μM), concentration–response data were not presented[107].

It should also be considered that binding does not necessarily equate to modulation of receptor function and that there is often a substantial reduction in potency (ten-fold or greater) when one compares the binding potency with cell-based potency for nuclear receptor ligands. In the paper reporting that SR9009 and SR9011 may have some agonist activity at the LXR, the calculation of REV-ERB specificity was based on data from a biochemical REV-ERB NCOR recruitment assay in which SR9009 and SR9011 were inactive[107], even though the compounds SR9009 and SR9011 directly interact with REV-ERB with submicromolar K_d (dissociation constant) values, based on other biochemical data (circular dichroism thermal shift assay). In our view, the specificity of SR9009, SR9011 and GSK4112 should have been calculated using a functional transcriptional response.

REV-ERB antagonist: SR8278

Only one REV-ERB antagonist has been described to date. SR8278 is a synthetic antagonist of REV-ERB activity[108] (TABLE 2). SR8278 increased the activity of REV-ERBα and REV-ERBβ in a Gal4-based co-transfection reporter assay, and in an assay that used full-length REV-ERBα and luciferase reporters driven by the promoters of three REV-ERB target genes: *BMAL1, PCK* and *G6PC*. Treatment of HepG2 cells with SR8278, which express REV-ERBs endogenously, increases the expression of *G6PC* and *PCK*. A further study showed that activation of REV-ERBα by haem and GSK4112 stimulated glucose-induced insulin secretion in MIN-6 mouse insulinoma cells, which was inhibited by SR8278 (REF. 109). SR8278 was used, together with haem and GSK4112, to show that activation of the connexin 43 promoter via interaction with the transcription factor SP1 occurs in a ligand-independent manner and does not involve the LBD of REV-ERBα[110].

Synthetic ROR ligands

The first report describing ligands for RORs demonstrated that melatonin and a synthetic thiazolindinedione (CGP 52608) bound to these receptors[111-113] (TABLE 3). However, beyond these initial reports, ligands for these receptors have not been well validated, with the exception of a recent study suggesting that CGP 52608 activates RORα at a potency >100,000-fold weaker than originally reported (>100 μM versus 1–3 nM)[114]. Nevertheless, several studies have identified several synthetic ligands that target RORs and have *in vitro* and *in vivo* activity on ROR function.

RORα and RORγ inverse agonist: T0901317

T0901317 was the first validated synthetic ligand that was shown to bind to and regulate the function of an ROR[115] (TABLE 3). T0901317 was originally identified as an agonist for LXRs[116], and subsequent studies demonstrated that this ligand also functions as an agonist

for FXR[117] and pregnane X receptor[118]. Only later was it determined that T0901317 was also an inverse agonist of RORα and RORγ[115]. As T0901317 is a nonspecific ligand for several nuclear receptors, it was used as a starting point for developing ROR-selective compounds, as described below.

RORα and RORγ agonist: SR1078

SR1078 (TABLE 3) is a direct agonist of RORα and RORγ — it increased the expression of the ROR target genes *G6PC* and fibroblast growth factor 21 (*FGF21*) in HepG2 cells — and has no activity for FXR, LXRα and LXRβ[119] in functional transcription assays. SR1078 displays acceptable pharmacokinetic properties (plasma concentrations of 3.6 μM 1 hour after an intraperitoneal injection of 10 mg per kg, and sustained levels above 800 nM 8 hours after a single injection) that allowed for a proof-of-concept analysis of the compound in animals. SR1078 was also used to characterize the *FGF21* gene as a direct target gene of RORα[120]. For example, *in vivo* administration of FGF21 in rodent models of diabetes improved glucose and triglyceride levels as well as insulin sensitivity[121]; administration of FGF21 to diabetic non-human primates leads to a similar improvement in the metabolic profile[122]. Thus, small-molecule RORα agonists could be one approach for modulating the expression of this hormone, which has therapeutic potential in the treatment of obesity and diabetes.

Expression levels of RORα are decreased or down-regulated in several types of cancers and cancer cell lines, including breast, ovarian and prostate cancers[123], and this has been linked to RORα-mediated regulation of the transcription factor SOX4 (which regulates tumour suppressor p53 activity). Interestingly, treating cancer cells with SR1078 leads to p53 stabilization and induces apoptosis[124], which suggests that further studies are warranted to investigate the potential of this agonist in models of cancer.

RORα inverse agonist: SR3335

Additional modifications of the T0901317 and SR1078 scaffolds led to the discovery of SR3335 (TABLE 3), the first potent RORα-specific inverse agonist[125]. SR3335 had acceptable pharmacokinetic properties (plasma concentrations of 9 μM 30 minutes after an intraperitoneal injection of 10 mg per kg, and sustained levels above 360 nM 4 hours after a single injection), and so it was tested in a mouse model of diet-induced obesity. Mice treated with SR3335 (15 mg per kg, intraperitoneally administered twice daily) for 6 days had significantly reduced plasma glucose levels compared to vehicle control-treated animals, including a decrease in *Pck*, the rate-limiting enzyme in gluconeogenesis.

RORα and RORγ inverse agonist: SR1001

Previous studies with synthetic ROR ligands were limited to basic proof-of-concept *in vivo* studies described above. The discovery and report of SR1001 (REF. 126) (FIG. 5; TABLE 1) represented the first robust analysis demonstrating that a ROR ligand can be used to probe the role of RORs in mouse models of disease. This compound directly binds to RORα and RORγ and acts as an inverse agonist that suppresses RORα and RORγ reporter activity, decreasing the interaction between the receptor and co-activators.

Consistent with the role of RORα and RORγt in T_H17 cell development[78], SR1001 inhibited the differentiation of splenocytes that were cultured under conditions to produce T_H17 cells, and this inhibition was associated with inhibited expression of several cytokines, including *IL17A, IL17F, IL21* and *IL22* (FIG. 6). SR1001 inhibited IL-17 protein production and secretion in splenocytes as well as in human peripheral blood mononuclear cells. Notably, SR1001 did not affect the differentiation of splenocytes into inducible regulatory T cells (T_{Reg} cells) or other T_H cell lineages, including T_H1 cells and T_H2 cells, and so it specifically targets T_H17 cells. In a mouse model of T_H17-mediated multiple sclerosis[127,128], SR1001 treatment (25 mg per kg administered intraperitoneally) delayed the onset and clinical severity of disease and was associated with reduced expression of the cytokines *Il17a, Il21* and *Il22*.

SR1001 targets both RORα and RORγ, and both of these receptors are required for the development of T_H17 cell-mediated autoimmune diseases[77,78]. However, it is clear that RORα has additional activities, such as a more robust regulation of circadian rhythm compared to RORγ, as well as a role in cerebellar development. We therefore pursued the development of RORγ-specific inverse agonists to determine whether these compounds may hold utility in suppressing T_H17 cell development. This led to the identification of two RORγ-specific ligands, SR2221 (REF. 129) and SR1555 (REF. 130), which are discussed below.

RORγ inverse agonist: digoxin

Digoxin (TABLE 3) was identified as an inhibitor of the transcriptional activity of RORγ in a screen of over 4,000 compounds using a *D. melanogaster* cell-based reporter system[131], and was subsequently shown to occupy the ligand-binding pocket of RORγ[132]. In a mouse model of multiple sclerosis, digoxin delayed the onset and reduced the severity of disease progression. However, digoxin is toxic to humans at the concentrations required for influencing RORγ activity and it is known to raise intracellular calcium levels by interacting with the $(Na^+ + K^+)$ATPase[133,134]. Although the toxic effects limit its use as a tool and potential therapeutic compound, this proof-of-concept study provided evidence that RORγ-targeted ligands can be used to probe the activity of RORγ *in vivo*.

RORγ inverse agonist: ursolic acid

Ursolic acid (TABLE 3) was identified in a cell-based screen as a compound that inhibited T_H17 cell development and the expression of *Il17* in T_H17 cells[135]. Structural similarity between ursolic acid and hydroxycholesterols led the authors to posit that ursolic acid functions through the RORs. RORγ was confirmed as the target of ursolic acid by carrying out biochemical assays and studies showing that ursolic acid inhibited *Il17* and *Il17f* expression only when T cells were differentiated via overexpression of RORγt. Although ursolic acid delays the onset and decreases the severity of disease symptoms in a model of multiple sclerosis, it has many other cellular targets, including the liver kinase B1 (LKB1; also known as STK11)–AMP-activated protein kinase (AMPK) pathway[136], the NFE2-related factor 2 (NRF2) pathway[137], nuclear factor-κB (NF-κB)[138,139], signal transducer and activator of transcription 3 (STAT3)[140,141] and glucocorticoid receptor[142], which suggests that it would not be well suited as a RORγ-selective probe *in vivo*.

RORγ inverse agonist: SR2211

Additional work on the SR1001 scaffold, directed at designing RORγ-selective inverse agonists, led to the discovery of SR2211 (TABLE 3), a compound that displays exquisite selectivity for RORγ over RORα in both biochemical and cell-based assays, with a K_i (inhibition constant) value of 105 nM at RORγ and no detectable binding to RORα[129]. In EL-4 cells, SR2211 treatment repressed the expression of *Il17a* and *Il23R*, as well as intracellular IL-17 protein levels, and suppressed T_H17 cell differentiation[130]. This compound was recently shown to be active in a mouse model of collagen-induced rheumatoid arthritis; SR2211 administered twice daily for 15 days by intraperitoneal injection significantly reduced joint inflammation in these mice[143].

RORγ inverse agonist: SR1555

SR1555 (TABLE 3) binds to and represses the activity of RORγ. Although it is considerably less potent than SR2211, it has no RORα[130] activity. Interestingly, although SR1001 (REF. 126) and SR2211 (REF. 130) specifically target and suppress T_H17 cell differentiation only, SR1555 affects both T_H17 cells (suppressing their differentiation) and T_{Reg} cells (increasing the frequency of inducible T_{Reg} cells)[130]. SR1555 is the first RORγ ligand to have an effect on T_{Reg} cells, which suggests that RORγ-specific ligands can be further optimized to have specific effects on different classes of T cells.

RORγ inverse agonist: ML209

A quantitative high-throughput screen of 310,000 compounds identified a series of diphenylpropanamides as selective inhibitors of RORγ activity using a cell-based RORγ reporter assay[144]. The initial hit displayed an IC_{50} value of 3.3 μM in the RORγ assay with no activity in control assays, including the RORα assay. The initial hit also inhibited T_H17 cell differentiation, which indicated that this was a viable scaffold for developing SAR studies. These efforts led to the discovery of ML209 (TABLE 3), an RORγ inhibitor with an IC_{50} value of 0.5 μM. Notably, in a panel of 20 nuclear receptors, only weak activity was reported for oestrogen-related receptor-α (ERRα), LXRα, thyroid hormone receptor-α and thyroid hormone receptor-β. ML209 displaces 25-hydroxycholesterol from RORγ, which indicates that it binds directly to the LBD of RORγ. In RORγ-dependent T_H17 cell differentiation assays, ML209 inhibited T_H17 cell differentiation without affecting T_H1 cells and T_{Reg} cells. The compound also inhibited the expression of the RORγ target gene *Il17a* and protein levels of IL-17A, but it did not affect the expression of RORα target genes, which further indicates that ML209 is specific for RORγ. The pharmacokinetic properties of ML209 were not provided in this initial report, so it is currently unknown whether this compound could be an effective tool to probe RORγ activity *in vivo*.

Aryl amide RORγ agonists

Several small-molecule activators of transcription driven by the *IL17* promoter have been identified and shown to act as direct ligands of RORγ via a circular dichroism thermal shift assay[145]. Three compounds (1a, 1b and 1c) increased *IL17* promoter-driven transcription in a co-transfection assay with a potency of ~100 nM (TABLE 3). Compound 1b also increases T_H17 cell differentiation[145]. A novel inhibitor of *IL17* promoter-driven transcription was

identified in the same assay, but little specific information was provided with regard to its mechanism of action. Inhibitor Y (TABLE 3) was used to compete with the aryl amide agonists in the study, but it is not clear whether this compound is a direct RORγ ligand. Further studies are needed to determine the specificity of the compounds described in this study for RORγ.

Conclusions

Substantial advances have been made over the past several years in our understanding of how ligands can regulate two classes of nuclear receptors, RORs and REV-ERBs, which were once considered orphan receptors. Not only have we been able to determine the identity of endogenous, high-affinity ligands for these receptors, we have also been able to use classic methods of drug discovery to generate synthetic ligands that have been used to probe the potential of these receptors as bona fide drug targets for the treatment of several human diseases (FIG. 7). Even though synthetic ligands have only been available for use in animal models of disease for the past few years, it is clear that improved and optimized ligands may have utility for the treatment of autoimmune diseases and metabolic disorders. Given the role of these receptors in the regulation of the circadian rhythm, additional studies are under-way to investigate the potential of these compounds in targeting the central mammalian clock, with the possibility of treating behavioural and sleep disorders. Progress is being made to improve the drug-like properties of these compounds, and some of these improved compounds or their analogues have a substantially high potential to enter clinical trials in the not too distant future.

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Glossary

DNA response element	A short sequence of DNA that is specifically recognized and bound by a particular transcription factor. DNA response elements are often found in the promoter regions of genes, and confer the responsiveness of a gene to regulation by a particular transcription factor.
E box	A particular DNA response element that is recognized by transcription factors belonging to the basic helix–loop–helix domain-containing family, such as circadian locomotor output cycles protein kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1).
Period (τ)	The time that elapses for one complete oscillation or cycle of a particular activity (for example, locomotor activity). Typically, the period for a circadian rhythm is almost 24 hours. In the absence of any extrinsic stimuli that act to ‘entrain’ the circadian rhythm (such as light), the period may differ; for example, mice typically have a period of slightly less than 24 hours in the absence of entrainment.

ROR response element	A particular DNA response element that is recognized by retinoic acid receptor-related orphan receptors (RORs) and REV-ERBs.
T helper 17 cells	(T_H17 cells). A subset of T_H cells that produce interleukin-17 (IL-17) and provide microbial immunity at mucosa and epithelial barriers. They have been implicated in the development of autoimmune disease.
T cell	A type of lymphocyte that has a crucial role in cellular immunity. T cells can be distinguished from other lymphocytes based on the expression of the T cell receptor on their plasma membrane.
Apo structure	A receptor structure that is free from a bound ligand.
Inverse agonists	Ligands that suppress the basal activity of a receptor.
Wheel running activity	A measure of locomotor activity as defined by rodents running on a wheel within a cage.
Phase shift	A discrete alteration in an oscillation in locomotor activity or other measurable physiological activity along the time axis within a circadian rhythm.
Area under the curve	(AUC). The area under the curve that is generated by plotting the concentration of a drug in plasma against time.
Melatonin	A hormone that is produced by the pineal gland in a circadian manner and is associated with entrainment of the circadian rhythm.
Fibroblast growth factor 21	(FGF21). A hormone that has several metabolic activities. FGF21 protects animals from diet-induced obesity and lowers blood glucose and lipid levels when administered to diabetic rodents.
T_{Reg} cells	A subset of T cells that produce interleukin-10 (IL-10) and transforming growth factor-β (TGFβ) and have an important role in immune tolerance.
T_H1 cells	A subset of T helper (T_H) cells that produces interferon-γ (IFNγ) and has an important role in cellular immunity.
T_H2 cells	A subset of T helper (T_H) cells that produces interleukin-4 (IL-4), IL-5 and IL-13, and has an important role in humoral immunity.

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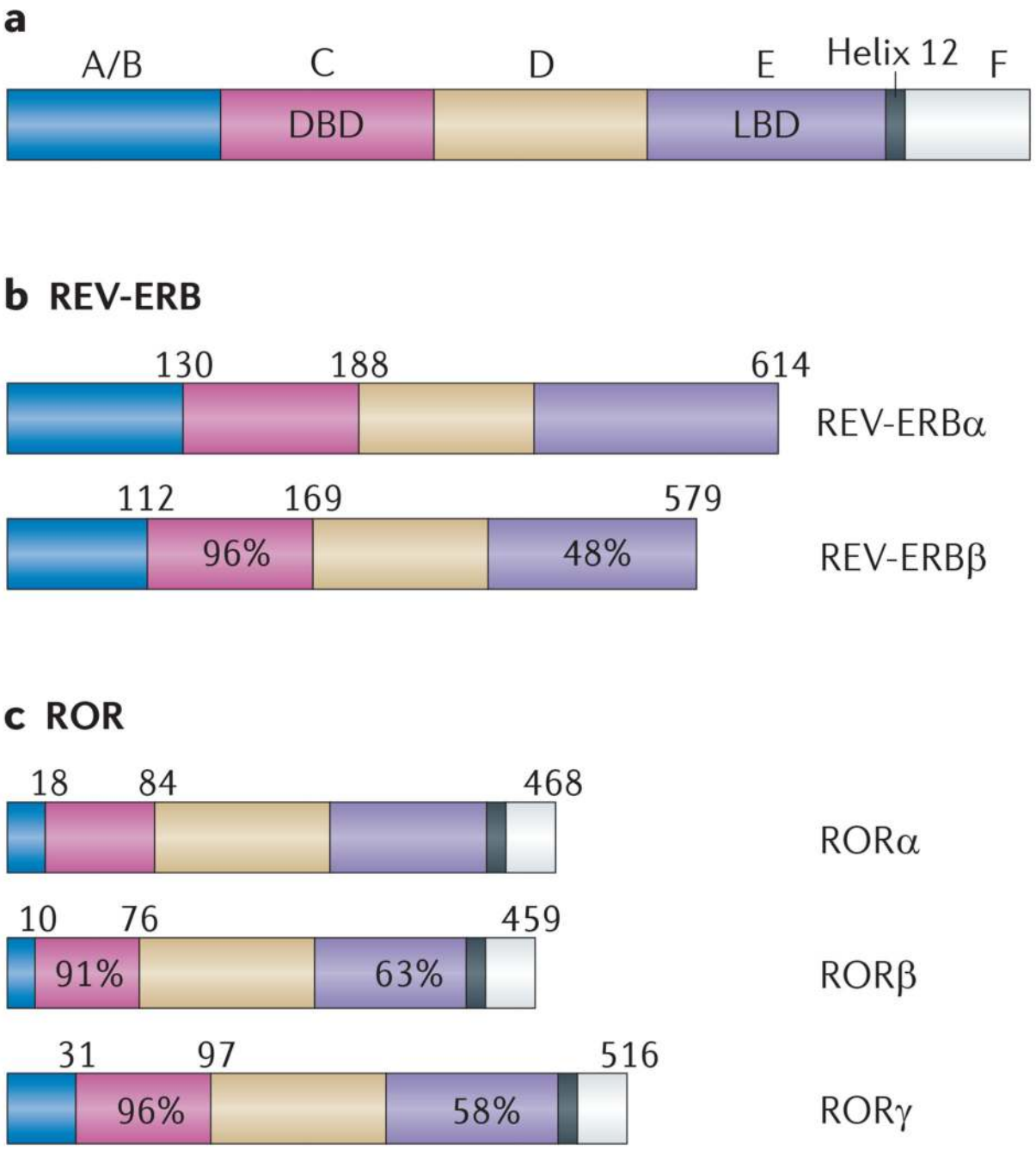


Figure 1. Structure of the RORs and REV-ERBs

a | The general organizational structure of members of the nuclear receptor superfamily. **b** | Structure of the REV-ERBs. **c** | Structure of the retinoic acid receptor-related orphan receptors (RORs). Numbers above each receptor represent the amino acid position. Percentages indicate amino acid identity within a particular domain relative to either REV-ERBα or RORα. A/B, C, D, E and F refer to classically defined regions in the nuclear receptor domain structure. DBD, DNA-binding domain; LBD, ligand-binding domain.

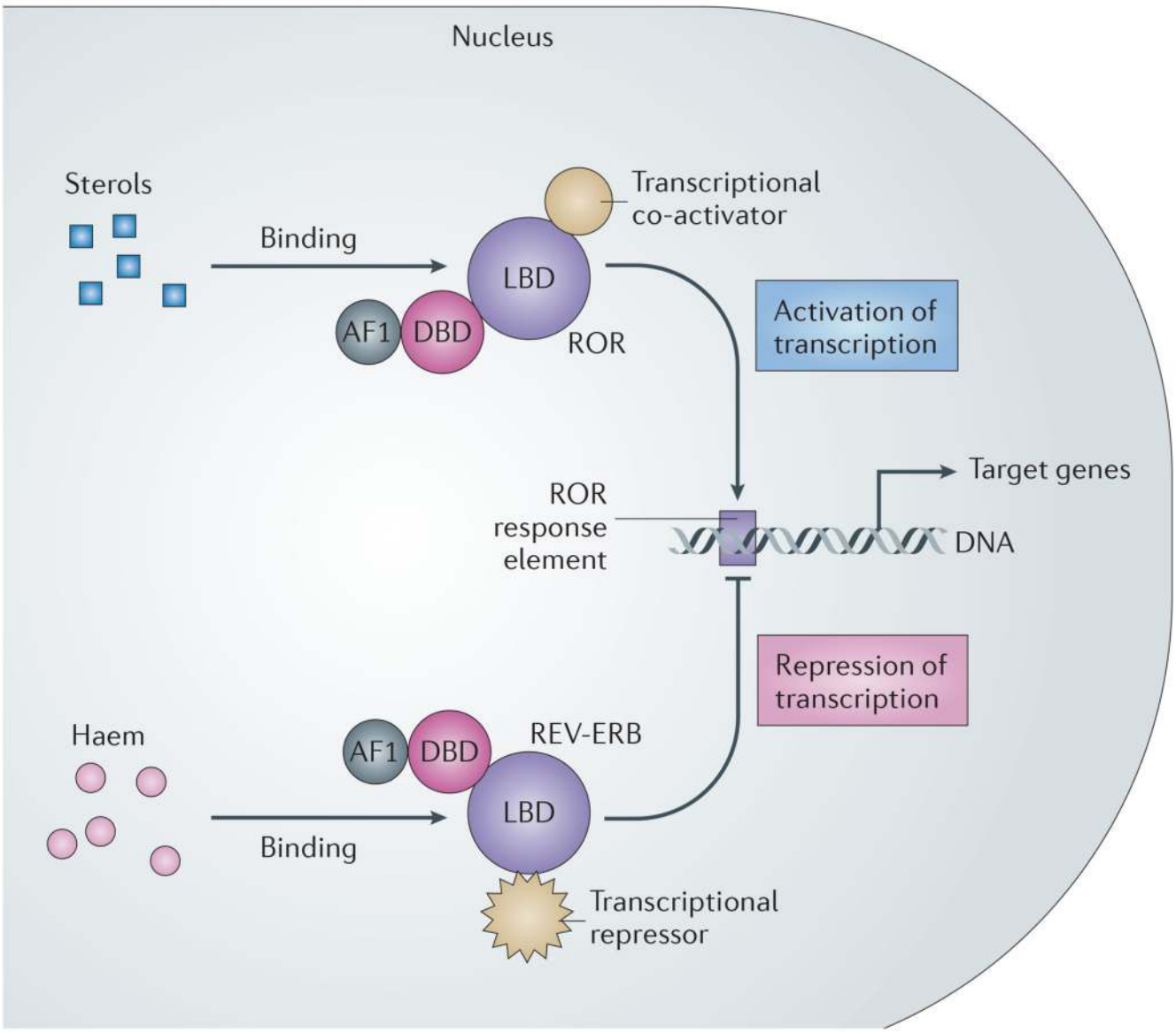


Figure 2. Molecular mechanism of action of the RORs and REV-ERBs

Retinoic acid receptor-related orphan receptors (RORs) and REV-ERBs are involved in transcriptional regulation and are regulated by ligands. Haem functions as a ligand for REV-ERBs, whereas sterols (cholesterol, cholesterol sulphate and various oxysterols) function as ligands for RORs. Both classes of receptors recognize a similar DNA response element, typically denoted as a ROR response element. ROR activates transcription (via recruitment of transcriptional co-activators), whereas REV-ERB silences transcription (via recruitment of transcriptional co-repressors). REV-ERB functions as a ligand-dependent transcriptional repressor (haem binding is required for the recruitment of the co-repressor and transcriptional repression), whereas ROR typically functions as a constitutive activator of transcription, and the binding of oxysterol ligands results in decreased activity. AF1, activation function 1; DBD, DNA-binding domain; LBD, ligand-binding domain.

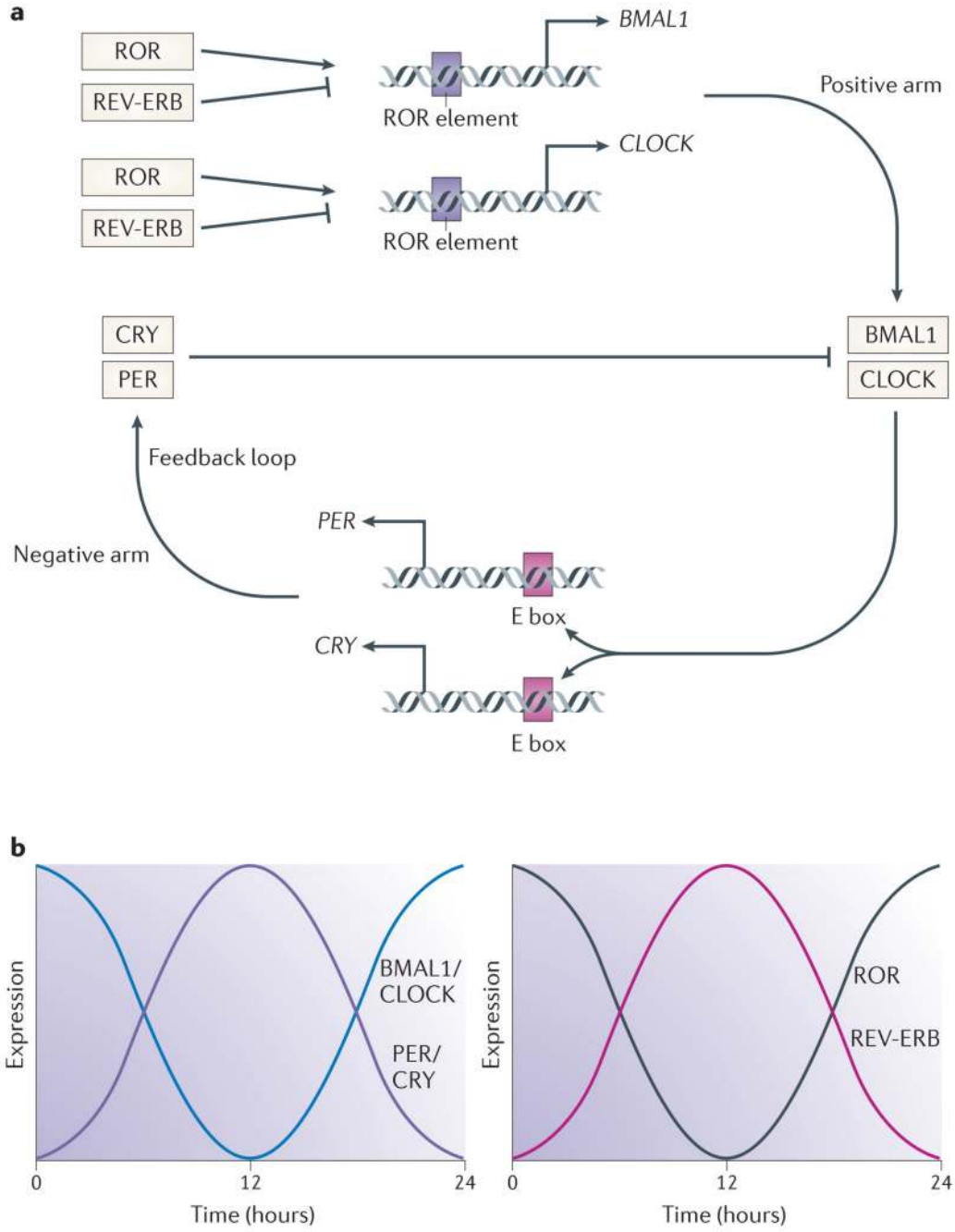


Figure 3. Role of RORs and REV-ERBs in regulation of the mammalian clock

a | The core mammalian clock is composed of a heterodimer of the transcription factors circadian locomotor output cycles protein kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1) (known as the positive arm), which activate the transcription of period circadian clock (*PER*) and cryptochrome (*CRY*) genes via E box sequences within their promoters. PER and CRY proteins (known as the negative arm) form dimers and directly interact with the CLOCK–BMAL1 heterodimers, thus suppressing their activity. This feedback loop follows a 24-hour rhythm where peak expression of the CLOCK–BMAL1 complex is 12

hours out of phase with peak PER and CRY expression. A retinoic acid receptor-related orphan receptor (ROR) response element within the *BMAL1* promoter is responsive to both ROR and REV-ERB (encoded by the genes *NR1D1* and *NR1D2*); ROR activates the transcription of *BMAL1*, whereas REV-ERB suppresses its transcription. The expression of ROR and REV-ERB also oscillates in a circadian manner (12 hours out of phase with one another), reinforcing the core circadian oscillator. The REV-ERB promoter also contains an E box, allowing direct regulation of *NR1D1* and *NR1D2* transcription by BMAL1–CLOCK. PER2 has also been demonstrated to directly interact with REV-ERB at REV-ERB-responsive promoters and to regulate its activity. **b** | The expression of PER and CRY as well as BMAL1 and CLOCK oscillates over the course of 24 hours. REV-ERB and ROR expression also undergoes circadian oscillations.

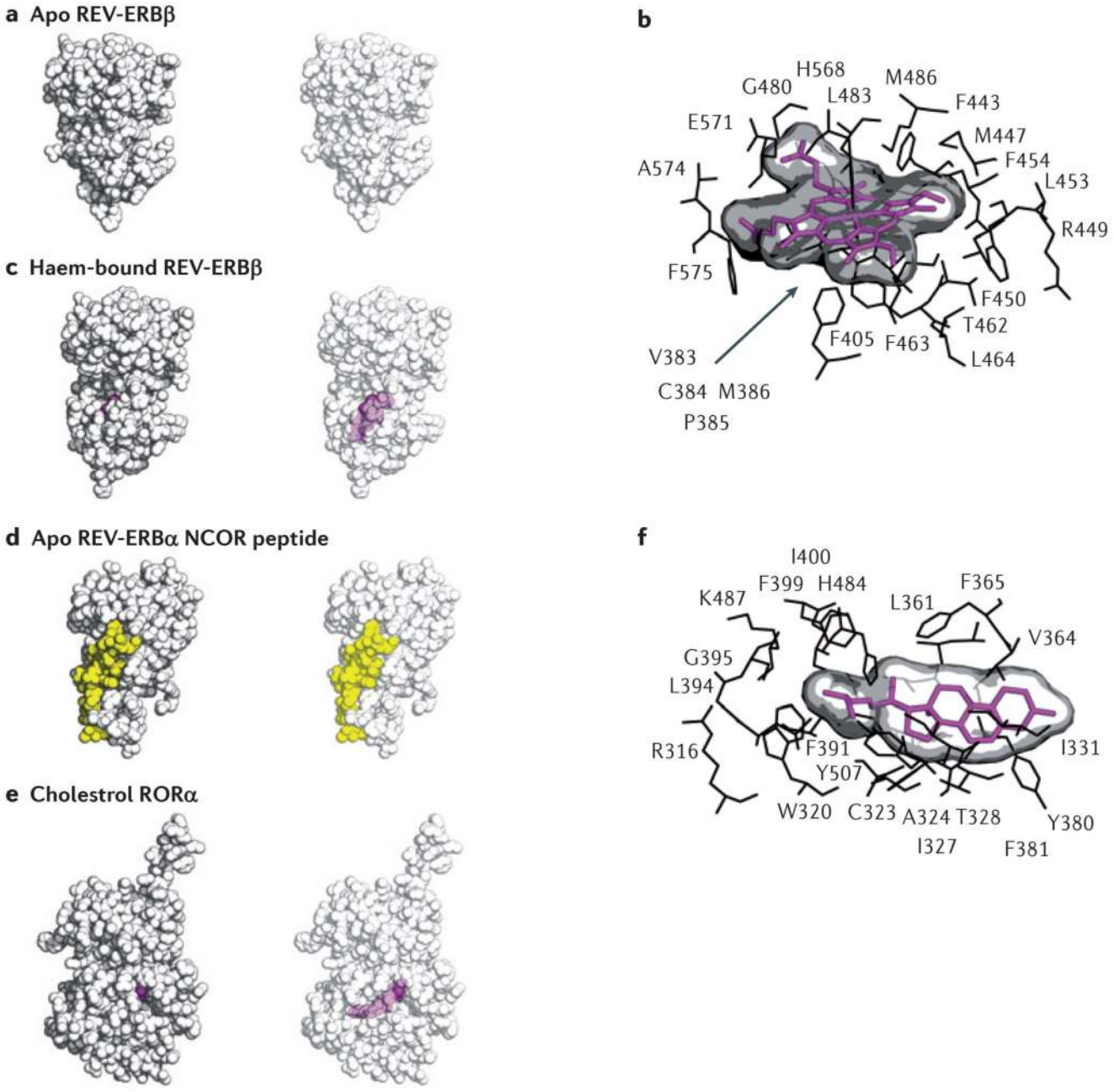


Figure 4. Structures of REV-ERB and ROR demonstrate their capacity to bind to natural ligands

The apo structure of REV-ERBβ (part **a**) indicated that the putative ligand-binding pocket was filled with large hydrophobic residues and thus devoid of the space that would be necessary for a ligand to bind. However, the haem-bound REV-ERBβ structure (parts **b** and **c**) shows that its ligand-binding pocket can profoundly change its shape to accommodate haem, a large porphyrin natural ligand. Intriguingly, although studies indicate that the binding of haem to REV-ERB increases its interaction with the nuclear receptor co-repressor (NCOR)[11,61], the structure of apo REV-ERB bound to an NCOR peptide (part **d**) indicates

that the binding of haem may not be an absolute requirement for mediating the REV-ERB–NCOR interaction. The co-crystal structure of the ligand-binding domain of retinoic acid receptor-related orphan receptor-α (RORα) bound to cholesterol (parts **e** and **f**) sets the stage for other studies indicating that various cholesterol derivatives, such as 7-oxygenated sterols, may act as physiological ligands to influence ROR activity. Structures are shown as space-filling models (parts **a**, **c**, **d** and **e**), with and without transparency to allow visualization of ligands bound to the internal ligand-binding pocket (haem-bound REV-ERBβ and cholesterol-bound RORα). A snapshot of the residues mediating the interaction of haem with REV-ERB (part **b**) illustrates that the repositioned hydrophobic residues that were originally thought to block the ligand-binding pocket in fact cooperate in binding to the large hydrophobic porphyrin haem scaffold. Protein Data Bank (PDB) codes: apo REV-ERB, 2V0V; haem-bound REV-ERB, 3CQV; apo REV-ERB with an NCOR fragment (co-repressor nuclear receptor (CoRNR) box motif peptide, 3N00; cholesterol-bound ROR, 1N83.

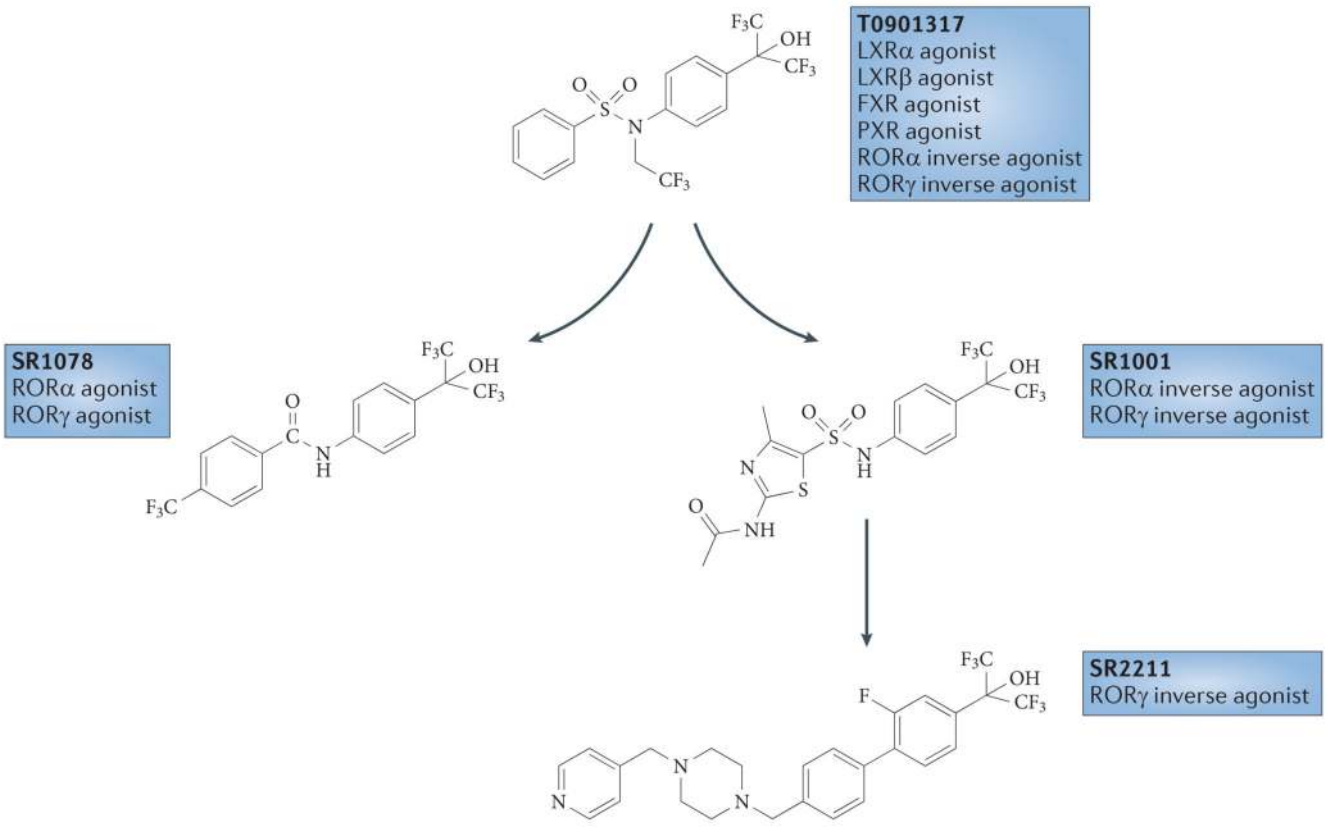


Figure 5. Development of selective ROR ligands

Following a screen of known nuclear receptor ligands against the entire nuclear receptor superfamily, the liver X receptor (LXR) agonist T0901317 was identified as a retinoic acid receptor-related orphan receptor (ROR) ligand. T0901317 has substantial promiscuity against other nuclear receptors. Various alterations in the structure led to the discovery of an agonist of RORα and RORγ (SR1078), an inverse agonist of RORα and RORγ (SR1001), and a RORγ-selective inverse agonist (SR2211). FXR, farnesoid X receptor; PXR, pregnane X receptor.

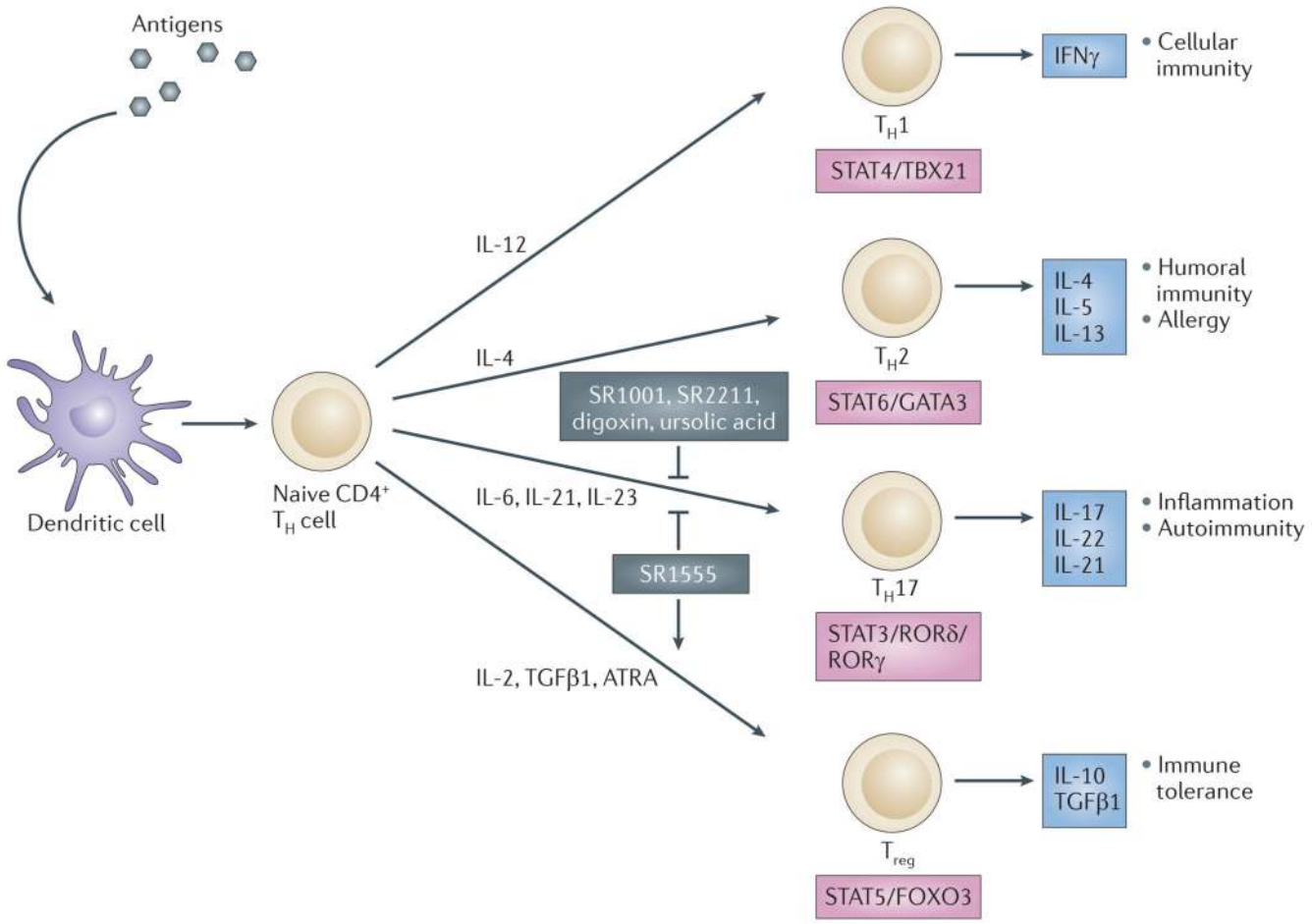


Figure 6. ROR inverse agonists alter T_H cell development

T helper 1 (T_H1), T_H2, T_H17 and regulatory T (T_{Reg}) cells develop from naive $CD4^+$ T_H cells. The differentiation of naive $CD4^+$ T_H cells into these effector $CD4^+$ T cells is initiated via an interaction of dendritic cells with naive $CD4^+$ T_H cells. Effector cell types are defined by their production of specific cytokines, function, modulation of distinct signalling pathways and the expression of distinct transcription factors. Retinoic acid receptor-related orphan receptor (ROR) inverse agonists suppress T_H17 cell differentiation and function. The RORγ inverse agonist SR1555 promotes T_{Reg} cell differentiation as well. ATRA, all-*trans* retinoic acid; FOXO3, forkhead box protein O3; GATA3, GATA-binding protein 3; IFNγ, interferon-γ; IL, interleukin; STAT, signal transducer and activator of transcription; TBX21, T-box protein 21 (T-bet); TGFβ1, transforming growth factor-β1.

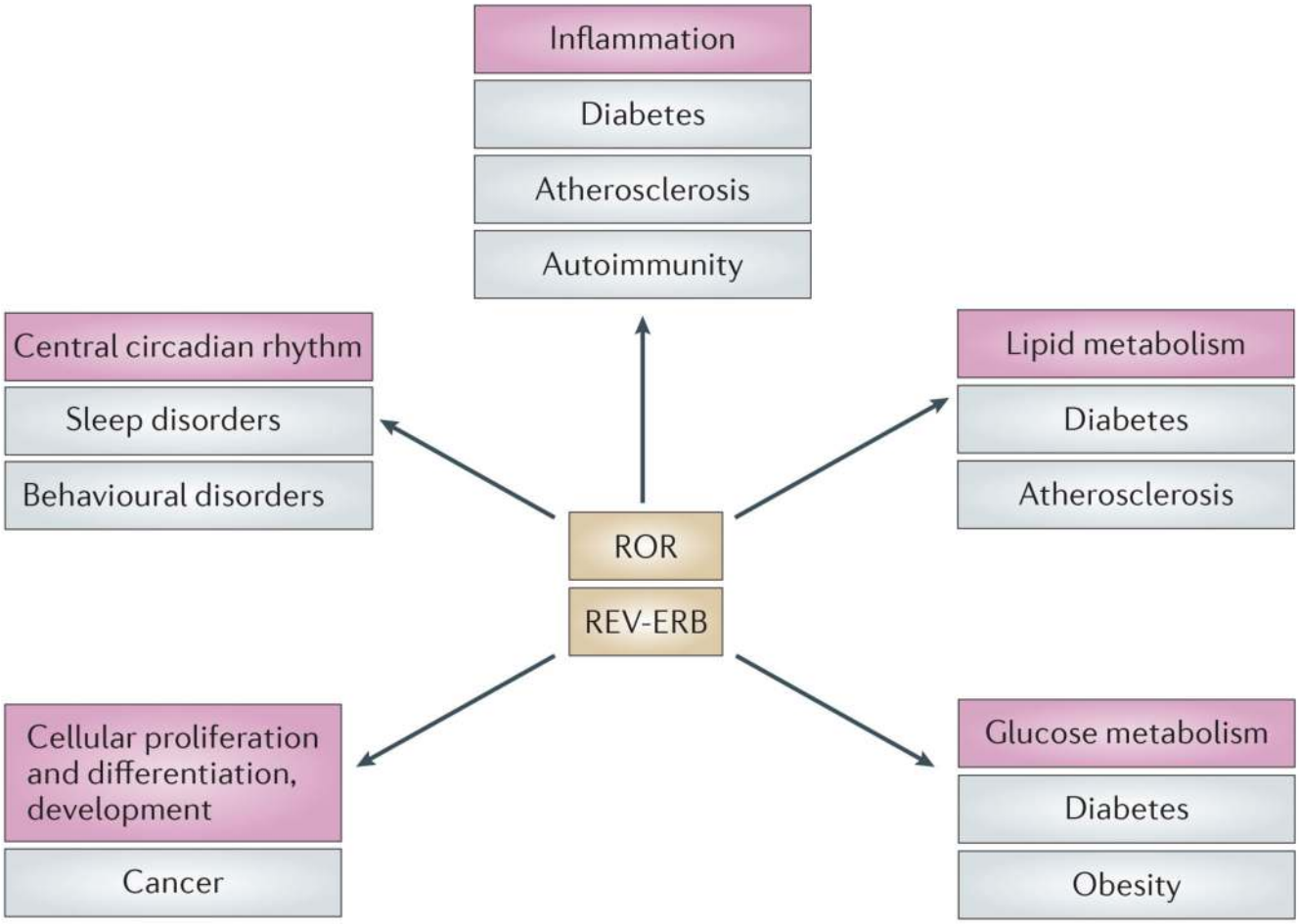
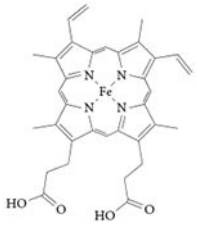
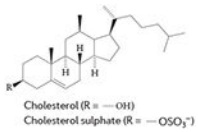
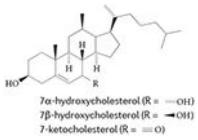
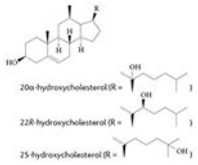
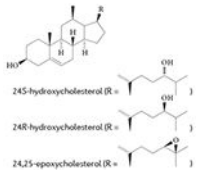
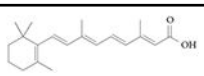
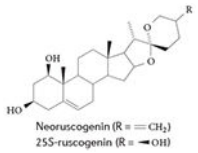


Figure 7. ROR and REV-ERB in the regulation of physiological processes

Physiological processes are shown in pink boxes, and the potential therapeutic indications of synthetic ligands that target retinoic acid receptor-related orphan receptor (ROR) and the nuclear receptor REV-ERB are indicated in grey boxes.

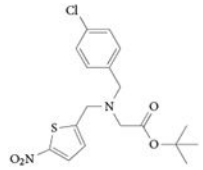
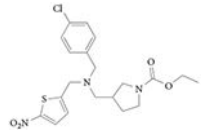
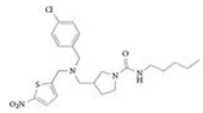
Table 1
Natural ligands of the nuclear receptors REV-ERB and ROR

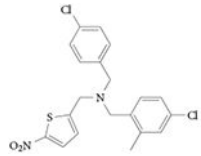
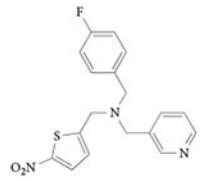
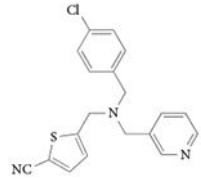
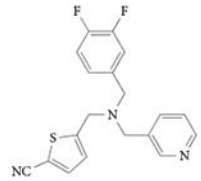
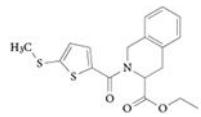
Ligands	Structure or scaffold	Receptor (or receptors)	Refs
• Haem		REV-ERBα, REV-ERBβ	17,84
• Cholesterol • Cholesterol sulphate		RORα	93
• 7α-hydroxycholesterol • 7β-hydroxycholesterol • 7-ketocholesterol		RORα, RORγ	96
• 20α-hydroxycholesterol • 22*R*-hydroxycholesterol • 25-hydroxycholesterol		RORα	97
• 24*S*-hydroxycholesterol • 24*R*-hydroxycholesterol • 24,25-epoxycholesterol		RORα, RORγ	96
• Stearic acid		RORβ	99
• All-*trans* retinoic acid		RORβ	98
• Neoruscogenin • (25*S*)-ruscogenin		RORα	99

ROR, retinoic acid receptor-related orphan receptor.

Table 2

Synthetic REV-ERB ligands

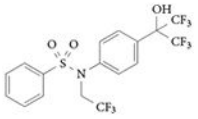
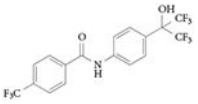
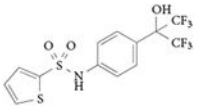
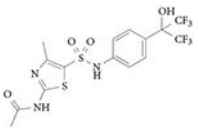
Compound	Structure	Comments (activity, affinity, EC_{50} value, IC_{50} value and actions)	Refs
GSK4112 (also known as SR6452)		• Agonist • Targets REV-ERBα and REV-ERBβ • REV-ERBα EC_{50} = 0.4 μM (measured by FRET assay) • REV-ERBα EC_{50} = 2.3 μM (measured by *Bmal1* luciferase reporter assay) • Suppresses expression of REV-ERB target genes in cells • Induces adipogenesis in 3T3-L1 cells • Lowers glucose output in primary mouse hepatocytes • Resets circadian oscillation of REV-ERB target genes in cells • Has a limited *in vivo* exposure, which limits its use as a chemical tool *in vivo*	71,101, 102
SR9009		• Agonist • REV-ERBα EC_{50} = 0.67 μM (measured by Gal4 reporter assay) • REV-ERBα EC_{50} = 0.71 μM (measured by full-length *Bmal1* reporter assay) • REV-ERBα K_d = 0.8 μM (measured by circular dichroism binding assay) • REV-ERBβ EC_{50} = 0.8 μM (measured by Gal4 reporter assay) • Increases recruitment of CoRNR peptide fragment of NCOR • Suppresses expression of REV-ERB target genes *in vitro* and *in vivo* • Does not exhibit significant activity at other nuclear receptors (Gal4–UAS luciferase specificity panel) • Causes loss of locomotor activity in the current circadian cycle after a single injection • Causes weight loss in mice (due to decreased fat mass) without affecting food intake; increases oxygen consumption and decreases plasma lipids	105
SR9011		• Agonist • REV-ERBα EC_{50} = 0.79 μM (measured by Gal4 reporter assay) • REV-ERBα EC_{50} = 0.62 μM (measured by full-length *Bmal1* assay) • REV-ERBβ EC_{50} = 0.56 μM (measured by Gal4 reporter assay) • Suppresses expression of REV-ERB target genes *in vitro* and *in vivo* • Increases recruitment of CoRNR peptide fragment of NCOR • Does not exhibit significant activity at other nuclear receptors (measured by Gal4–UAS luciferase specificity panel) • Suppresses the amplitude of circadian oscillations in SCN explants from a *Per2*-luciferase reporter mouse and in *Per2*-luciferase fibroblasts • Causes loss of locomotor activity in the current circadian cycle after a single injection • Causes weight loss in mice (due to decreased fat mass) without affecting food intake; increases oxygen consumption and decreases plasma lipids	105

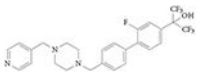
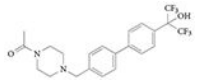
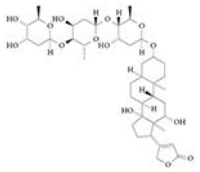
Compound	Structure	Comments (activity, affinity, EC_{50} value, IC_{50} value and actions)	Refs
GSK2945		• REV-ERBα EC_{50} = 50 nM (measured by NCOR peptide recruitment) • Reduces oscillation of BMAL-luciferase • Inhibits IL-6 secretion • Pharmacokinetics suitable for 20–30 mg per kg dosing daily	107
GSK0999		• REV-ERBα EC_{50} = 160 nM (measured by NCOR peptide recruitment) • Reduces oscillation of BMAL-luciferase • Inhibits IL-6 secretion • Pharmacokinetics suitable for acute dosing	107
GSK5072		• REV-ERBα EC_{50} = 200 nM (measured by NCOR peptide recruitment) • Reduces oscillation of BMAL-luciferase • Inhibits IL-6 secretion • Pharmacokinetics suitable for acute dosing	107
GS2667		• REV-ERBα EC_{50} = 200 nM (measured by NCOR peptide recruitment) • Reduces oscillation of BMAL-luciferase • Inhibits IL-6 secretion • Pharmacokinetics suitable for acute dosing	107
SR8278		• Antagonist • REV-ERBα IC_{50} = 2.3 μM (measured using full-length *Bmal1* reporter assay) • Increases expression of REV-ERB target genes in cells • Limited *in vivo* exposure, which limits its use as a chemical tool *in vivo*	108

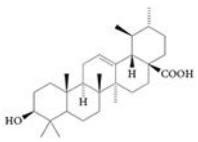
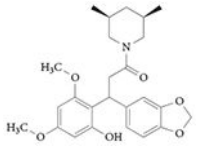
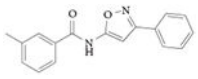
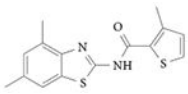
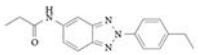
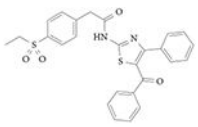
BMAL1, brain and muscle ARNT-like 1; CoRNR, co-repressor nuclear receptor; EC_{50}, effector concentration for half-maximum response; FRET, fluorescence resonance energy transfer; IC_{50}, half-maximal inhibitory concentration; IL-6, interleukin-6; NCOR, nuclear receptor co-repressor; PER2, period circadian clock; ROR, retinoic acid receptor-related orphan receptor; SCN, suprachiasmatic nucleus; UAS, upstream activating sequence.

Table 3

Synthetic ROR ligands

Compound	Structure	Comments (activity, affinity, EC_{50} value, IC_{50} value and actions)	Refs
T0901317		• RORα and RORγ inverse agonist • RORα IC_{50} = 2.0 μM (measured by Gal4–UAS luciferase assay) • RORγ IC_{50} = 1.7 μM (measured by Gal4–UAS luciferase assay) • RORα K_d = 132 nM (measured by radioligand displacement) • RORα K_d = 51 nM (measured by radioligand displacement) • Does not bind to RORβ • Also acts as LXRα and LXRβ agonist, FXR agonist and PXR agonist • Increases the interaction between RORα and peptide fragment of the RIP140 co-repressor • Suppresses *G6PC* and *IL17* promoter activity • Suppresses *G6PC* gene expression and decreases recruitment of SRC2 co-activator to the *G6PC* promoter	115
SR1078		• RORα and RORγ agonist • Decreases interaction between RORγ and the peptide fragment of TRAP220 co-activator • Suppresses transcriptional activity of RORα and RORγ in assays that use Gal4-chimeric receptors, but increases transcriptional activity of RORα and RORγ in assays that use full-length receptors • Increases the expression of RORα and RORγ target genes *in vitro* and *in vivo* • Has sufficient *in vivo* exposure for use as a chemical tool • No activity at other nuclear receptors as defined in a Gal4-chimeric receptor assay	119
SR3335 (also known as SR3335/ML176)		• RORα inverse agonist • K_i = 220 nM (measured by radioligand binding assay) • IC_{50} = 480 nM (measured by Gal4–UAS luciferase assay) • Does not exhibit significant activity at other nuclear receptors (measured by Gal4–UAS luciferase specificity panel) • Suppresses *G6PC* and *PCK* promoter activity • Suitable *in vivo* exposure for proof-of-principle experiments • Lowers plasma glucose levels in mouse model of diet-induced obesity • Suppresses *PCK* expression *in vivo* • No activity at other nuclear receptors as defined in a Gal4-chimeric receptor assay	125
SR1001		• RORα and RORγ inverse agonist • RORα K_i = 172 nM (measured by radioligand binding assay) • RORγ K_i = 111 nM (measured by radioligand binding assay) • Inhibits RORγ activity on the *IL17* promoter in a concentration-dependent manner • RORγ IC_{50} = 117 nM (recruitment of TRAP220 peptide to RORγ LBD) • Suppresses expression of *IL17A* and *G6PC* in cell culture	125

Compound	Structure	Comments (activity, affinity, EC_{50} value, IC_{50} value and actions)	Refs
		• Inhibits NCOR recruitment while promoting the recruitment of RORα and RORγ to the *IL17A* promoter in cells • Affects the structural conformation of RORγ LBD and decreases interaction of SRC2 with RORγ • Inhibits expression of *IL17A, IL17F, IL21* and *IL22* in cells • Inhibits T_H17 cell differentiation without affecting other T_H cell lineages • Inhibits secretion of IL-17 from $CD4^+$ T cells • Suppresses experimental autoimmune encephalomyelitis • No activity at other nuclear receptors in a Gal4-chimeric receptor assay	
SR2211		• RORγ inverse agonist • K_i = 105 nM (measured by radioligand binding assay) • IC_{50} = 320 nM (measured by Gal4–UAS luciferase assay) • Weak activity on LXRα; no activity on FXR • Affects the structural conformation of RORγ LBD • Suppresses five copies of ROR response element and *IL17* promoter in a luciferase assay in a concentration-dependent manner • Suppresses *IL17* expression and IL-17 production • Suppresses T_H17 cell differentiation • No activity at other nuclear receptors in a Gal4-chimeric receptor assay	129, 130, 143
SR1555		• RORγ inverse agonist • No activity at LXR, FXR and RORα • IC_{50} = 1.5 μM • K_i = 1 μM (measured in a radioligand binding assay) • Suppresses *IL17* promoter driven luciferase activity • Suppresses *IL17A, IL21* and *IL22* expression in cells • Increases *FOXP3* expression in cells • Inhibits IL-17 protein expression • Inhibits T_H17 cell differentiation • Increases the frequency of T_{Reg} cells • No activity at other nuclear receptor in a Gal4-chimeric receptor assay	130
Digoxin		• RORγ inverse agonist • IC_{50} = 1.98 μM (measured in a Gal4–UAS luciferase assay) • IC_{50} = 4.1 μM (fluorescence polarization displacement assay) • Suppresses IL-17A protein expression • Suppresses *IL23R, IL17A, IL17F* and *IL22* • Inhibits T_H17 cell differentiation without affecting the differentiation of other T cell lineages • Suppresses experimental autoimmune encephalomyelitis • Binds to the RORγt LBD	131, 132

Compound	Structure	Comments (activity, affinity, EC_{50} value, IC_{50} value and actions)	Refs
Ursolic acid		• RORγ inverse agonist • IC_{50} = 680 nM (binding of SRC1 peptide to RORγt LBD) • IC_{50} = 560 nM (T_H17 cell differentiation assay) • Suppresses RORγt activity on an *IL17* promoter-driven luciferase reporter • Suppresses *IL17* expression • Inhibits IL-17 protein production • Inhibits T_H17 cell differentiation • Suppresses experimental autoimmune encephalomyelitis • Has reported activity at the glucocorticoid receptor and non-nuclear receptor targets	135
ML209		• RORγ inverse agonist • IC_{50} = 500 nM (measured in a Gal4–UAS luciferase assay) • IC_{50} = 110 nM (measured in a fluorescence polarization displacement assay) • Minimal activity on oestrogen-related receptor-α (IC_{50} = 4.5 μM), LXRα (IC_{50} = 10 μM), thyroid hormone receptor-α (IC_{50} = 4.5 μM), and thyroid hormone receptor-β (IC_{50} = 13 μM, in a Gal4–UAS luciferase assay) • Suppresses *IL17A* expression • Inhibits T_H17 cell differentiation • Specificity not published	144
Compound 1a		• RORγ agonist • EC_{50} = ~100 nM (*IL17* reporter assay) • Shift of 0.49 °C in a circular dichroism thermal shift assay	145
Compound 1b: *N*-(4,6-dimethyl-benzo[d]thia-zol-2-yl)-3-methyl-thiophene-2-carboxamide		• RORγ agonist • EC_{50} = ~100 nM (measured in an *IL17* reporter assay) • Shift of 2.26 °C circular dichroism thermal shift assay • Augments IL-17 production in a dose-dependent manner (maximum effect was 220% at 3 μM)	145
Compound 1c: *N*-(2-(4-ethyl-phenyl)-2*H*-benzo-[d][1,2,3]triazol-5-yl) propionamide		• RORγ agonist • EC_{50} = ~100 nM (measured in a *IL17* reporter assay) • Shift of 2.9 °C in a circular dichroism thermal shift assay	145
Inhibitor Y: *N*-(5-benzoyl-4-p henylthiazol-2-yl)-2-(4-(ethylsulfonyl) phenyl)acetamide		• Possibly a RORγ inverse agonist • Inhibits the actions of compounds 1a, 1b and 1c in a concentration-dependent manner	145

EC_{50}, effector concentration for half-maximum response; FOXP3, forkhead box protein P3; FXR, farnesoid X receptor; G6PC, glucose-6-phosphatase; IL-17, interleukin-17; IL-23R, interleukin-23 receptor; IC_{50}, half-maximal inhibitory concentration; K_d, dissociation constant; K_i, inhibition constant; LXR, liver X receptor; NCOR, nuclear receptor co-repressor; PCK, phosphoenolpyruvate carboxykinase; PXR, pregnane X receptor; RIP140, receptor-interacting protein 140; ROR, retinoic acid receptor-related orphan receptor; T_H, T helper; TRAP220, thyroid hormone receptor-associated protein complex 220 kDa component; UAS, upstream activating sequence.