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Digest

The transcriptional repressor REV-ERB as a novel target for disease

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ABSTRACT

REV-ERB is a member of the nuclear receptor superfamily of transcription factors involved in the regulation of many physiological processes, from circadian rhythm, to immune function and metabolism. Accordingly, REV-ERB has been considered as a promising, but difficult drug target for the treatment of numerous diseases. Here, we concisely review current understanding of the function of REV-ERB, modulation by endogenous factors and synthetic ligands, and the involvement of REV-ERB in select human diseases. Particular focus is placed on the medicinal chemistry of synthetic REV-ERB ligands, which demonstrates the need for higher quality ligands to aid in robust validation of this exciting target.

The misregulation of transcription plays a key role in the development and maintenance of many human diseases. Direct regulators of transcriptional events are therefore often considered as interesting drug targets. Despite this, many such transcription factors are labelled as 'undruggable'[1] for a range of reasons grounded in the logistics of small molecule drug discovery. These factors range from intrinsic disorder at the protein level, to lack of suitable binding pockets for small molecule ligands. While such an undruggable designation is driving further innovation in medicinal chemistry,[2] it is clearly false to label all transcription factors as such. Nuclear receptors are transcription factors that are responsible for sensing molecules (such as hormones) and, in response, directly regulating the expression of specific subsets of genes[3]. By virtue of their control by small molecules, they are inherently druggable and have long been recognised as drug targets for new medicines.[1,4] There are a large number of approved drugs across many disease indications that target nuclear receptors; from ligands of the estrogen receptor (for example, Tamoxifen) for the treatment of breast cancer, to ligands of the glucocorticoid receptor (for example, Dexamethasone) for anti-inflammatory usage. Nonetheless, there are many more opportunities for the therapeutic exploitation of the nuclear receptor superfamily.

REV-ERB is a member of the nuclear receptor family consisting of two similar proteins: REV-ERBα[5] and REV-ERBβ.[6] The name REV-ERB is derived from "reverse-ERB", since the *NR1D1* gene that codes for REV-ERBα was mapped on the antisense DNA strand of the *ERBA* proto-oncogene (THRA, thyroid hormone receptor-α).[5] The closely related *NR1D2* gene encodes for the other isoform, REV-ERBβ. REV-ERBα and REV-ERBβ share almost complete identity in their DNA binding domains (DBD), while only 71% amino acid sequence identity in their ligand binding domains (LBD) (Fig. 1). Moreover, they show similar expression patterns within tissues.[7] Potential partial redundancy has been shown in knock out mice, where the presence of REV-ERBβ can compensate the loss of REV-ERBα, at least in certain tissues.[8]

As is the case for all nuclear receptors, DNA binding by REV-ERBα/β is mediated by two zinc fingers and is responsible for recognising and binding to specific genomic regions of target genes.[9] Unlike most other nuclear receptors however, REV-ERBs lack the carboxy terminal activation function 2 (AF2) at the C-terminal end of the LBD (Fig. 1 left).[10] Because the AF2 region recognises co-activator protein partners responsible for transcriptional activation, the REV-ERBs are thought to be unable to activate transcription. Instead, they act as constitutive repressors of transcription through binding via the DBD to the ROR response element DNA sequence (RORE). This binding site creates an interesting dynamic interplay with the retinoic acid receptor-related orphan receptor (ROR), which binds to many of the same target genes, but results in transcriptional activation.[11] Two REV-ERB molecules, usually present as a dimer, compete with ROR for the binding to the RORE region.[8] Their successful binding to the DBD results in the recruitment of a nuclear receptor corepressor (NCOR1). The NCOR1 complex then recruits histone deacetylase 3 (HDAC3), which leads to chromatin condensation and transcription repression through histone deacetylation (Fig. 1).[12,13]

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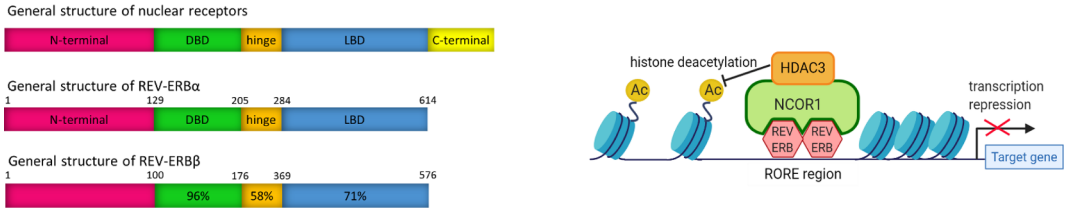

Fig. 1. On the left, general structure of nuclear receptors and the structure of REV-ERB, lacking activatory function 2 (AF2) in the C-terminal domain. The values reported represent % in amino acid identity for each domain comparing REV-ERBα and β. The *N*-terminal domains were too variable for comparison. Sequences were obtained from Uniprot. Query covers and amino acid identity were calculated via BLASTP algorithm using NCBI BLAST webtool. On the right, the mechanism of transcriptional regulation by REV-ERB. REV-ERB binds as a dimer to the RORE region of target genes, driving recruitment of NCOR1, followed by recruitment of HDAC3, which deacetylates histone tails and leads to chromatin condensation and transcription repression of target genes.

REV-ERB has been implicated as a potential target in a range of diseases. Whether activation or inhibition of the repressive action of REV-ERB is required depends on the disease in question, as outlined below. Furthermore, as has previously been identified for other nuclear receptors, some ligands show opposing effects in different cell types, which further complicates analysis and target validation.[14,15] Therefore, high quality small molecule activators and inhibitors of REV-ERB function (*vide infra*) are both useful tools and would potentially be applicable in different disease contexts. A brief discussion of select examples that showcase the potential of REV-ERB as a drug target are given below. For more detailed discussion, readers are directed to several excellent reviews on the topic.[8,11,16]

Shortly after its discovery in 1989,[5] REV-ERB was seen to fluctuate in a circadian manner, suggesting a role in circadian rhythm; a 24 h oscillatory cycle that regulates multiple biological processes. The direct role of REV-ERB in the circadian cycle is now well established, with other essential regulators being rhythmically regulated by REV-ERB in tightly controlled feedback loops (Fig. 2).[17] The dimer formed by circadian regulators CLOCK or NPAS2, together with BMAL1 (also known as ARNTL) activates transcription of *CRY, PER, REV-ERB* and *ROR*. Once CRY and PER reach threshold levels, the CRY/PER dimer inhibits the action of BMAL1/CLOCK and BMAL1/NPAS2, leading as well to reduced levels of CRY and PER themselves. On the other hand, REV-ERB acts as a repressor of *BMAL1, CLOCK* and *NPAS2* expression, while ROR binds competitively to the RORE region to activate their transcription.[18,19]

Given the role of REV-ERB in this tightly regulated system, the targeting of REV-ERB holds promise to modulate many of the biological

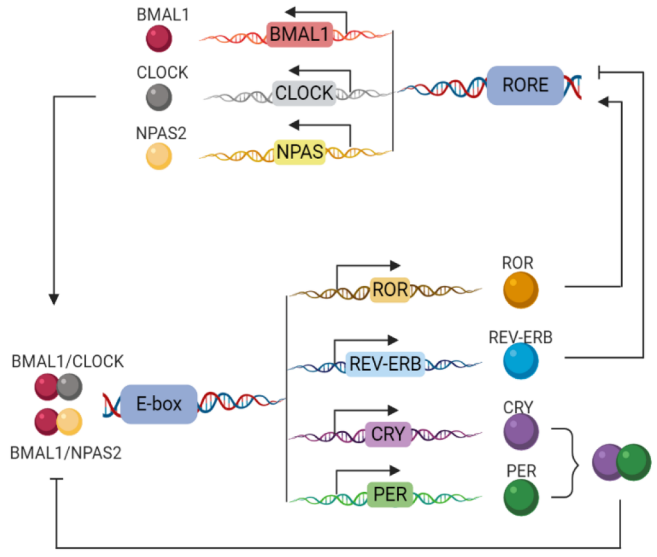

Fig. 2. Mechanism of the mammalian circadian clock illustrating the feedback loops that control expression of the key genes *BMAL1*, *CLOCK*, *NPAS2*, *ROR*, *REVERB*, *CRY* and *PER*.

processes dependent on the circadian cycle. Metabolism is well known to be under circadian control,[20,21] where REV-ERB regulates lipid[22] and glucose metabolism,[23,24] as well as adipocyte differentiation,[25–27] making it a target for the treatment of metabolic diseases. In this context, the use of REV-ERB activating agonists was reported to lead to downregulation of expression of the lipogenic gene *Srebf1* and the fatty acid synthase gene *Fasn*, as well as elevated transcriptional levels of the fatty acid transport gene *Fatp1*.[21] The apolipoprotein A1 (*ApoA1*) and apolipoprotein C3 (*ApoC3*) genes also play a major role in cholesterol metabolism and their repression by REV-ERB is yet another mechanism of regulation of lipid metabolism mediated by this transcription factor.[22] Similarly, conversion of cholesterol to bile acids, which facilitate digestion of dietary fats and oils, is achieved via downregulation of gene expression of the transcription factor *E4BP4* in the liver and the signalling molecule *SHP* by REV-ERB.[28] Overall, the enhancement of REV-ERB activity by synthetic agonist compounds has been reported to lead to weight loss, reduced cholesterol and improved metabolic functions.[21] Furthermore, high levels of REV-ERB have been correlated with increased insulin secretion and reduced glucose concentration, highlighting the potential use of REV-ERB agonists for the treatment of type 2 diabetes.[24]

Many immune-related diseases also follow a circadian pattern and REV-ERB is now well established as a regulator of inflammation.[29] Repression of *Tlr4* in macrophages by REV-ERB decreases IL-6 production, blocking the inflammatory response. REV-ERB degradation under inflammatory conditions results in *Tlr4* gene expression and successful inflammatory response, but, in turn, drives downregulation of many other circadian processes.[30,31]

Also in the field of immune regulation, T_H17 cells have been established as REV-ERB regulated effectors.[32–34] T_H17 are proinflammatory immune cells that produce Interleukin 17 (IL17) cytokines and play an important role in host defence against infection. However, the long half-life and strong cytokine secretion of T_H17 cells are often related to autoimmune complications.[33] REV-ERB has been reported to negatively regulate T_H17 development by competing with RORγt for the RORE region of key T_H17 genes, reducing IL17 and other associated proinflammatory cytokines such as IL6 or IFNγ.[33,34] In this scenario, the use of REV-ERB activatory agonist ligands could be used to supress T_H17-mediated autoimmune diseases, such as multiple sclerosis or psoriasis.

REV-ERB also appears to play a complex role in muscle function. REV-ERB overexpression is known to increase mitochondrial content and activity, by blocking autophagy.[35] REV-ERB deficiency leads to a compromised exercise capacity and low cellular energy levels.[35] Given this, it could be hypothesized that activation of REV-ERB with a synthetic agonist would enhance mitochondrial biogenesis, which could be beneficial to muscle function. Despite this, in the context of dystrophic muscle, it was observed that inhibition of REV-ERB had a beneficial effect, stimulating muscle regeneration and function. Curiously, such REV-ERB inhibition was seen to promote mitochondrial biogenesis, previously ascribed to REV-ERB overexpression.[36,37] This apparently contradictory effect was related to the different role of REV-ERB in

proliferating vs. differentiated muscle cells, as well as normal vs. dystrophic environments.

Beyond these examples, we note that control of REV-ERB activity was recently reported to be beneficial in breast cancer cell models,[38] for the prevention of bone loss[39] and for improved cognitive function in Alzheimer's disease.[40,41]

Perhaps unsurprisingly for a key component of the molecular clock, multiple signalling pathways and environmental stimuli have been reported to have direct regulatory roles on REV-ERB. Initially assigned as an orphan receptor, in 2007 it was reported that heme is the endogenous ligand of REV-ERB,[42] which activates it towards transcriptional repression through NCOR1 recruitment. The precise regulatory role of heme remains to be fully elucidated however, with apparently conflicting results in cellular and purified systems.[43] To reconcile these results, it has been suggested that there might be an unidentified cellular component that is required for heme-dependent NCOR1 recruitment.[44]

Lazar and co-workers have used crystallography to analyse the relation between heme and NCOR1 binding to REV-ERB.[43] They successfully co-crystalized REV-ERBα with an NCOR1-derived peptide, in particular the region ID1 that is responsible for binding to the REV-ERB LBD. The resultant structure indicates the formation of a β-sheet interaction between REV-ERB and NCOR1. Comparison of this structure with heme-bound and apo-structures (Fig. 3) suggests that in the presence of heme, α-helix 3 shifts, potentially disrupting the β-sheet interaction and destabilizing NCOR1-ID1 binding. This result is apparently contradictory, given the assignment of heme as a ligand that activates REV-ERB through NCOR1 recruitment. To reconcile this result, Lazar and co-workers suggest that NCOR1 is able to bind REV-ERB in the absence of ligand, allowing for a basal repression state. In the presence of heme, NCOR1 binding could be stabilized through interaction with regions other than ID1. Unfortunately, there are currently no further crystal structures of the full-length protein to corroborate this theory. As a final element of complexity, heme has also been reported to increase the rate of REV-ERB degradation via a ubiquitin-dependent pathway; heme therefore can also have an indirect regulatory role.[45,46]

Redox fluctuations have been reported to have a direct impact on heme affinity and REV-ERB function. Two sites have been characterised as susceptible to redox events: the iron atom within heme and key cysteine residues in the REV-ERB pocket (Fig. 4). Under normal conditions, Fe^{3+}-heme binds to REV-ERB, in a binding mode that involves a

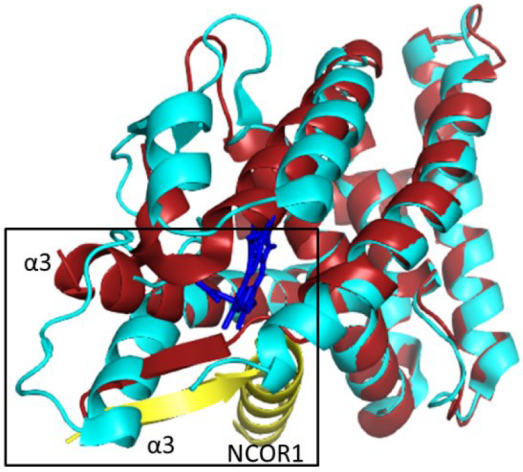

Fig. 3. Comparison of heme-bound REV-ERBβ (3CQV, cyan) and NCOR1-ID1 bound REV-ERBα (3 N00, red for REV-ERB, yellow for NCOR1-ID1). In region defined by the black box, a β-sheet interaction occurs between REV-ERBα and NCOR1 for 3N00, while in 3CQV (REV-ERB + heme) α3 undergoes a significant shift, potentially disrupting this interface.

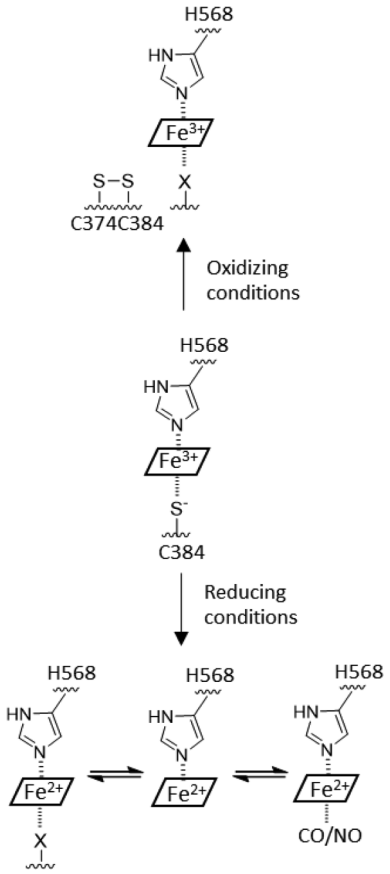

Fig. 4. Redox effects on heme binding to REV-ERB LBD, as shown in (47). Thiol-reduced form of REV-ERB binds Fe^{3+}-heme in a hexacoordinate complex with H568 and C384, with a K_d constant of ~100 pM. Under oxidizing conditions C384 forms a disulfide bridge and Fe^{3+}-heme remains bound to REV-ERB in a hexacoordinated complex with a K_d constant of ~14 nM. Under reducing conditions, Fe^{2+}-heme is thought to exist either as part of a pentacoordinate complex, or in a hexacoordinate complex through binding to another ligand, such as a diatomic gas molecule, with a K_d between 24 and 500 nM.

hexacoordinate iron complex with residues H568 and C384 as axial ligands.[47] Heme bound in this complex has been measured to have a dissociation constant from REV-ERB in the range of 100 pM.[48] Under oxidizing conditions however, C384 dissociates from the iron centre and forms a disulphide bridge with the neighbouring C374. Fe^{3+}-heme remains part of a hexacoordinate complex, with C384 being replaced by an, as yet, unidentified ligand.[47] The dissociation constant of heme in this context is reduced by around 70-fold to ~14 nM.[48] An equivalent effect is observed in a reducing environment, where Fe^{3+} is reduced to Fe^{2+}. The resultant Fe^{2+}-heme is thought to participate in a pentacoordinate complex, or a hexacoordinate complex, but now bound to an unknown ligand, or to diatomic gases such as CO or NO.[47] This complex has a dissociation constant ranging from 24 to 500 nM.[48] Given that the K_d values under both reducing and oxidizing conditions are much higher than the predicted levels of heme available in the nucleus (under 2.5 nM),[49] it has been suggested that under those conditions, REV-ERB would be found as an apoprotein. However, an alternative hypothesis is that REV-ERB binds Fe^{2+}-heme in the cytosol, where heme levels are higher, and this complex is then oxidized to Fe^{3+}-heme.[44]

The fact that heme is the ligand for REV-ERB leads to the potential for sensing of other chemical signals through iron coordination, as introduced above. As for many other heme-binding proteins, REV-ERB function is sensitive to diatomic gases, particularly CO and NO.[50–52] Structural and spectroscopic studies have suggested this control to occur via coordination of the CO or NO ligand to the Fe^{2+}-heme centre

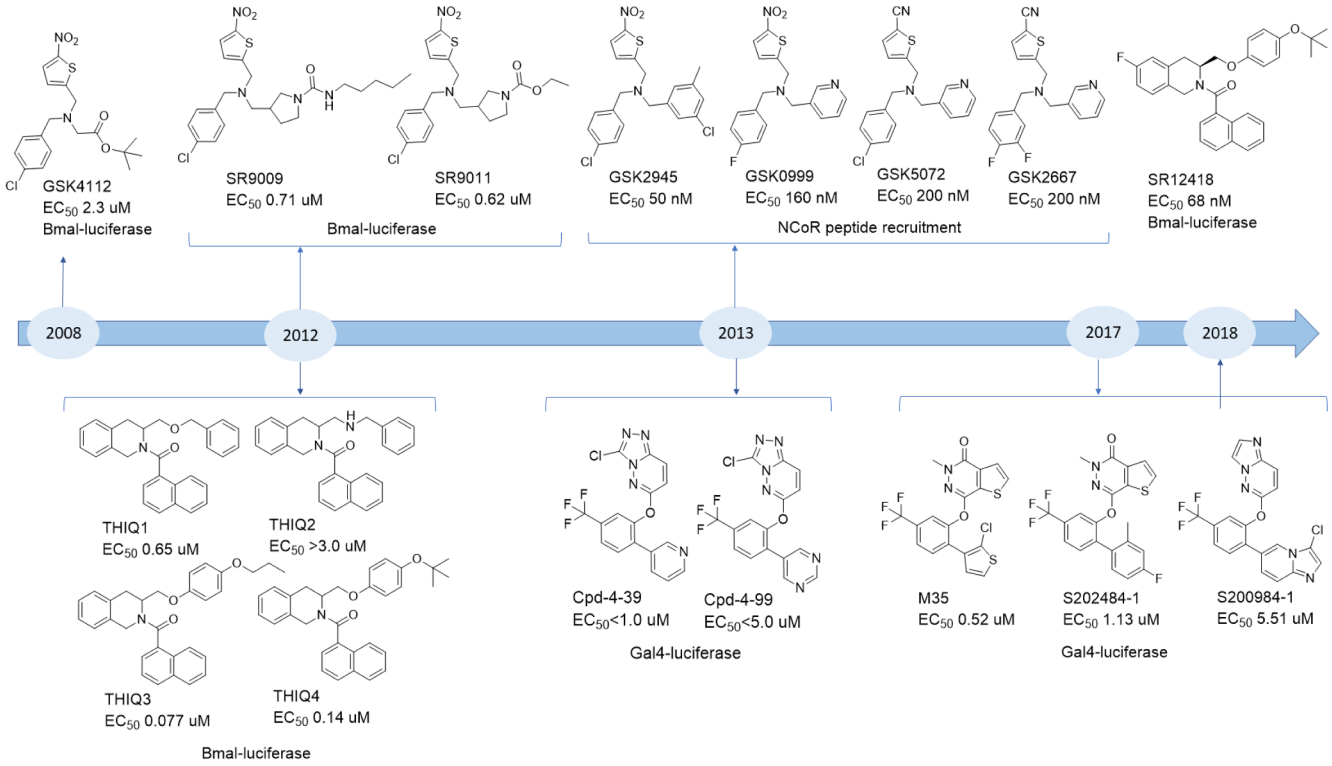

Fig. 5. Timeline of REV-ERB agonist ligands discovered to date, with corresponding structures, EC_{50} values and biological assay employed to obtain such EC_{50} values.

in REV-ERB under reducing conditions.[47] In cell-based assays, addition of NO or CO reduces the repressive activity of REV-ERB. However, in extracellular biochemical assays, it increases interactions between NCOR1 and REV-ERB.[52] While the precise functional role of this apparent dichotomy remains to be further defined, it is likely such binding events provide an additional layer of regulatory control for REV-ERB. Thus, it has been suggested that NO/CO binding, in addition to heme binding and redox sensitivity, act as cues to dynamically regulate the metabolic and circadian functions of REV-ERB.[52]

Further investigations of the heme ligand have examined cobalt (CoPP) and zinc porphyrins (ZnPP) as alternative REV-ERB binders. While the sole difference between these ligands and heme is the metal centre, such small changes result in a reduced affinity for NCOR1 and a loss of repressor activity, suggesting these Co/Zn porphyrins act as REV-ERB antagonists.[53] The binding of CoPP and ZnPP to REV-ERB causes only subtle conformational changes however, when compared to heme; for example a planar porphyrin ring in the Co/Zn porphyrin bound structure, compared to the puckered ring conformation in heme-REV-ERB. We note that a similar planar conformation is reported to occur after the binding of diatomic gases (such as NO) to heme-protein complexes.[54] CoPP and ZnPP, on the other hand, have been reported to have a reduced ability to bind diatomic gases.[53] These results further link the coordination geometry, environment and redox status as a regulatory feature in REV-ERB repressor activity.

Signalling pathways also play a direct regulatory role on REV-ERB via post-translational modifications. Various protein kinases have been reported to phosphorylate REV-ERB with a variety of functional outcomes.[55] For example, phosphorylation of T275 by CDK1 leads to proteasome-dependent degradation,[56] whereas CDK9 suppresses binding of REV-ERB to its target genes, resulting overall in an amplification of circadian oscillation.[57] By contrast, phosphorylation of serine 55 and 59 by GSK3β stabilizes REV-ERB, and inhibition of GSK3β targets REV-ERB for proteasomal degradation.[58] Beyond phosphorylation, the inflammatory process has been reported to trigger SUMOylation of REV-ERBα, resulting in enhanced degradation of the protein through ubiquitin-dependent proteasomal degradation.[31]

Ultimately, these multiple levels of regulatory control lead to a significant degree of complexity when it comes to understanding REV-ERB dependent biology. Nonetheless, such events also open up a number of opportunities for manipulating the function of REV-ERB via the generation of synthetic ligands.

Synthetic ligands to control REV-ERB function

As highlighted above, activation or inhibition of REV-ERB function may be useful depending on the particular disease context and a summary of key developments in small molecule REV-ERB ligand development is given below. Despite significant ligand development, to the best of our knowledge, detailed mechanistic work is largely missing from the studies concerning many of the ligands reported. In general terms, agonists are compounds that bind to a nuclear receptor and stimulate its transcriptional activity by inducing a conformation that favours binding of co-regulatory proteins. Antagonists, on the other hand, block the effect of agonists through competitive binding to the agonist binding site, preventing the binding of co-regulatory proteins. Finally, ligands which reduce the basal level of nuclear receptor activity (i.e. activity in the absence of agonists) are known as inverse agonists. In the discussion below we use the terms “agonist”, “antagonist” and “inverse agonist” as they were reported in the corresponding literature. However, particularly for the inhibitors of REV-ERB function, it is incompletely understood whether the reported ligands function via antagonism, inverse agonism, or another mechanism. Given that REV-ERB is a transcriptional repressor, it should be emphasised that agonists that increase activity of REV-ERB will decrease the transcription of target genes whereas inverse agonists/antagonists will result in increased transcription of target genes.

Above this, there are many further uncertainties with respect to the activity of the ligands described below. For example, medicinal chemistry studies do not routinely assay against both REV-ERBα and REV-ERBβ. The fact that there is only a 71% amino acid sequence identity between REV-ERBα and REV-ERBβ in the LBD means that there will most likely be differences in ligand selectivity for the two isoforms; at

least for ligands that bind in the LBD. Unfortunately, in many cases the precise binding site of the ligands is unknown, as is knowledge of whether ligand binding occurs in the presence or absence of heme.

Activators of REV-ERB function

The majority of ligand discovery and development work for REV-ERB has focused on compounds that act as agonists (Fig. 5). GSK4112 was the first synthetic REV-ERB agonist, described in 2008 by Loudon, Ray and co-workers.[59] A fluorescence resonance energy transfer (FRET) assay was used for screening efforts and GSK4112 was identified as enhancing NCOR1 recruitment in a dose dependent manner, with an EC_{50} of 0.4 μM.[60] In a cellular reporter gene assay, measuring the expression of BMAL1-luciferase, GSK4112 was found to have an EC_{50} of 2.3 μM.[61] It is worth noting that such reporter gene assays are perhaps the most commonly used assays in nuclear receptor ligand discovery. GSK4112 was found to lower hepatic glucose levels, induce adipogenesis and reset the circadian cycle in accordance with the role for REV-ERB in glucose and lipid metabolism as well as being a core component of the circadian clock.[27,59,60] An unfavourable pharmacokinetic (PK) profile, including high plasma clearance (353 mL/Kg min), and poor oral bioavailability in rats,[62] however, meant GSK4112 was non-optimal for *in vivo* usage.

Following GSK4112, Burris and co-workers published an expanded structure–activity relationship (SAR) study of this tertiary amine REV-ERB agonist series.[63] Amongst the compounds studied, SR9009 and SR9011 were identified as the best agonist compounds, with improved potency and efficacy compared to the parent compound GSK4112.[20] EC_{50} values of 0.71 and 0.62 μM respectively, were reported using a BMAL1-luciferase reporter gene assay.[20] Plasma exposure levels suggested an improved PK profile compared to GSK4112, allowing for further *in vivo* work. These studies showed a loss in locomotor activity of mice after administration, resulting in a 1–3 h delay in initiation of diurnal activity, consistent with the role of REV-ERB in circadian behaviour. Furthermore, use of these compounds reduced fat mass in obese mice, consistent with the role of REV-ERB in metabolism regulation.[20]

Despite this early promise, SR9009, SR9011 and GSK4112 have all been reported to exhibit off-target binding to the nuclear receptor LXRα at ~10 μM,[64] a nuclear receptor involved in the inflammatory response. Furthermore, studies by Lazar and co-workers have reported that SR9009 has strong REV-ERB-independent effects on embryonic stem cells and hepatocyte viability and proliferation, affecting metabolism, gene expression and mitochondrial respiration.[65]

Given these limitations, further efforts were made to optimise this series of REV-ERB agonists by Trump and co-workers, focused on improving compound PK and nuclear receptor selectivity.[64] SAR studies focused on optimizing the three amine substituents, with the main structural optimizations summarized in Fig. 5.

From this study, four lead agonists were identified, GSK2945, GSK0999, GSK5072 and GSK2667, which had improved activity over GSK4112 in a NCOR1 peptide recruitment assay and showed reduced activity against LXRα, with over 100-fold selectivity for REV-ERB. EC_{50} values were obtained using an NCOR1 peptide recruitment assay, with the new compounds showing improved values of 50, 160, 200 and 200 nM respectively, compared to 500 nM for GSK4112. Suppression of BMAL1 expression was also analysed via the standard luciferase reporter gene assay and found to be improved over GSK4112.[64] Despite some improvement in LXR selectivity, GSK0999, 5072 and 2667 display similar PK profiles to SR9009, with short half-lives (~0.7 h), low oral bioavailability (~3%) and high clearance (~45 mL/Kg min). However, GSK2945 demonstrated a longer half-life (2 h) and an improved oral bioavailability of 23%, suitable for chronic *in vivo* dosing both intravenously and orally.[64]

Cell activity data reported for GSK2945 is, however, representative of the challenges encountered when studying a target involved in circadian biology and that exhibits cell-type specific effects.[15,31] For example, Trump and co-workers used a U2OS cell line stably expressing a BMAL1-luciferase reporter gene assay. In this assay, an increase in potency for recruitment of the NCOR1 peptide correlated with a 21% suppression of BMAL1 expression after 40 h of treatment using 20 μM GSK2945. However, this assay also allows for observation of real-time oscillations of synchronized cells. Inspection of this data shows that quantification of BMAL1 expression is clearly time dependent and the degree of suppression varies following the oscillation pattern.[64] More recently, Wu and co-workers reported that GSK2945 has an opposite "antagonistic" activity, based on a dose-dependent increase in BMAL1-luciferase expression.[66] The difference between these outcomes – agonist versus antagonist – has not yet been reconciled; but we note that Wu and co-workers used a transient (rather than a stable) reporter gene assay for their data and different cell lines (HEK293 and HepG2 vs. U2O2 previously used).

Burris and co-workers reported an alternative study aimed at increasing the activity of this agonist series through restricted rotation of the scaffold; specifically connecting two of the amine sidechains to provide a tetrahydroisoquinoline (THIQ) core[62] (Fig. 5). The SAR

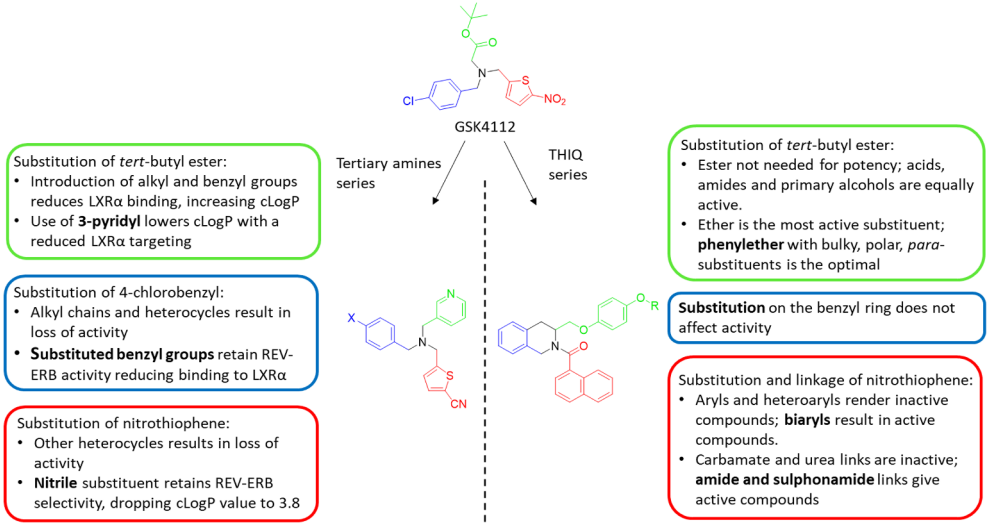

Fig. 6. SAR developments of two main REV-ERB agonist series, tertiary amines on the left, and tetrahydroisoquinolines on the right, starting from parent compound GSK4112, on top. SAR efforts focused on three areas, depicted in green, blue and red.

studies conducted on this series are also summarized in Fig. 6. Four molecules, here referred to as THIQ1, THIQ2, THIQ3 and THIQ4, gave EC_{50} values of 0.65, 3.0, 0.077 and 0.14 μM in a BMAL1-luciferase reporter assay, and were selected for further *in vivo* PK studies in rats. In general, THIQ1 showed the most promising results compared to GSK4112 (see above), with a lower clearance rate (39 mL/Kg min), reduced volume of distribution (3.8 L/Kg), and a longer half-life (1.9 h); although oral bioavailability was still poor.[62] In an effort to further optimise these compounds, Solt and co-workers recently reported SR12418, which had an EC_{50} of 68 nM in a BMAL1-luciferase reporter gene assay. Plasma exposure levels in mice showed a modest improvement compared to SR9011 and successful suppression of T_H17-driven autoimmunity *in vivo*.[33]

Since these studies on the major REV-ERB agonist chemotype were published, several additional scaffolds have been reported. Bourotte et al. reported a series of 6-substituted [1,2,4]triazolo[4,3-*b*]pyridazines as REV-ERB agonists (Fig. 5), developed as a potential treatment of type 2 diabetes.[24] Cpd-4–39 and Cpd-4–99 were reported to activate REV-ERB in a Gal4-REV-ERB luciferase assay: EC_{50} between 0.1 and 1.0 μM for Cpd4-39 and between 1.0 and 5.0 μM for Cpd-4–99. It should be noted that unlike the BMAL1 reporter assay, which uses full length REV-ERB to regulate the luciferase reporter gene, the GAL4 reporter relies on fusion of the yeast GAL4 transcription factor DBD to the REV-ERB LBD. Hence the results may not be directly comparable. Metabolic stability studies show more than 40% remaining of parent compound after 1 h incubation in mouse microsomes. The lead molecules, Cpd-4–39 and Cpd-4–99, were shown to be able to reduce glucose concentrations *in vivo* in diabetic mice.[24]

Ducrot and co-workers used a computational approach, where they applied molecular dynamics (MD) to study the free energy and binding modes of 35 new compounds to the REV-ERB/NCOR1 complex.[67] Four structurally diverse compounds were selected for synthesis and biological evaluation. Three of them were successfully reported as new REV-ERB agonists, referred to as M35, S200984-1 and S202484-1 (Fig. 5). These compounds were reported to activate the GAL4-REV-ERB LBD fusion, with EC_{50} of 0.52, 1.13 and 5.51 μM respectively. Furthermore, FRET was used to confirm interaction between REV-ERB and NCOR1. Mechanistically, Ducrot et al. suggest that the ligands bind to REV-ERB at the interface with the NCOR1 binding site, in turn promoting the recruitment of the co-repressor and stabilizing the complex. While mostly justified by computational modelling, the authors state that crystal structures of these ligand-bound complexes have been obtained, although these structures have not yet been released into the public domain. Such structures would represent the first ligand-bound REV-ERB crystal structures that exclude heme, and, as such, may help develop further understanding of REV-ERB ligand binding and regulatory biology.

Despite the similarity between the compounds reported by Bourotte and co-workers and Ducrot and co-workers, there is no reported relationship between the two series. Nonetheless, it would seem that such bicyclic heterocycles could represent a promising alternative approach for the design of REV-ERB ligands, especially given the limitations of the tertiary amines/THIQ series.

Inhibitors of REV-ERB function

One particularly interesting aspect of the THIQ series reported by Burris and co-workers, was the identification of SR8278[61] (Fig. 7). In contrast to the agonist series, SR8278 treatment resulted in *increased* expression of REV-ERB target genes in cells, with an EC_{50} of 0.47 μM in the BMAL1-luciferase reporter gene assay. This suggests that SR8278 acts as an inhibitor of REV-ERB function. It was assigned as an "antagonist" on the basis that SR8278 eliminated the activity of agonist GSK4112 in cellular competition assays. A further study showed that agonist-based activation of REV-ERBα stimulated glucose-induced insulin secretion in MIN-6 mouse insulinoma cells, which was inhibited by SR8278.[68] However, the precise binding site of SR8278 and competition with heme remain to be determined. We also note that peptide recruitment assays in the presence of SR8278, which may be helpful in determining precise mechanism of action (*vide infra*), have not been reported. While SR8278 certainly represents an important and exciting development in REV-ERB ligand discovery – the possibility to inhibit rather than activate REV-ERB function – the compound's unfavourable PK profile has limited its usage *in vivo*. For example, a PK study of SR8278 reported a short half-life (0.17 h), large volume of distribution (44.4 L/Kg) and high clearance rate (55 mL/Kg min).[69]

SR8278 was reported as a singleton, with no associated SAR. However, we note that two other related compounds with "antagonist" activity were reported in a patent by Burris and co-workers. These compounds, B17 and B166 (according to their numbering in the original patent) were able to repress REV-ERB activity in a BMAL1-luciferase reporter gene assay, with an EC_{50} of 1.3 μM for B166 (value not reported for B17).[70]

While investigating the role of REV-ERB in cancer proliferation, Grimaldi and co-workers performed *in silico* screening aimed at the identification of new inhibitors of REV-ERB function. These studies led to the identification of a new chemical series with a spirocyclic cyclopentane, including ARN5187 (Fig. 7), which shows modest inhibition of REV-ERBβ activity, with an EC_{50} of 30 μM in a RORE-driven luciferase assay.[71] Further SAR studies led to additional derivatives with improved activity over ARN5187, reducing the EC_{50} to 2.10 μM.[72] In general, these compounds were reported to have dual activity in the breast cancer cell line BT-474, where they inhibited both REV-ERBβ activity and autophagy, leading to an increased *in vitro* cytotoxicity.

In 2018, another REV-ERB inhibitor, GSK1362, was described by Loudon, Ray and co-workers.[31] GSK1362 was shown to inhibit the interaction of NCOR1 with the LBD of REV-ERB in a FRET assay, with an approximate EC_{50} of 150 nM. This was stated to be representative of the action of an inverse agonist and therefore GSK1362 was assigned as such. GSK1362 was also found to upregulate the transcription of *BMAL1* with an EC_{50} of 2.5 μM in the BMAL1-luciferase reporter gene assay. Additional target engagement for GSK1362 was demonstrated using a cellular thermal shift assay (CETSA) assay, revealing a change in REV-ERBα protein stability resulting from GSK1362 exposure. Unlike some of the agonists described above, GSK1362 was reported to have good selectivity for REV-ERB over 20 nuclear receptors including LXR. Complex outcomes were observed in isolated macrophages and bronchial epithelial cells however, that were used to suggest that there may be undetermined off-target effects of GSK1362. Nonetheless, GSK1362 was able to assist in the regulation of pulmonary inflammation by inhibiting cytokine production such as IL6 from alveolar macrophages, as well as reversing enhanced proteasomal degradation of REV-ERBα by blocking SUMOylation. Unfortunately, GSK1362 was reported to have a poor predicted pharmacokinetic profile, therefore *in vivo* experiments using this compound may be limited.

Conclusions and future perspectives

Nuclear receptors are ligand-gated transcription factors with a rich history as targets for the development of successful medicines. REV-ERBα and REV-ERBβ stand out as fascinating members of this superfamily, with structural divergence from common nuclear receptors and an interesting regulatory ligand – heme (as opposed to hormones) – which appears important for the sensitivity of REV-ERB to redox status. Due to its central position in circadian biology, REV-ERB holds much potential as a target against diverse diseases, from cancer to diabetes to

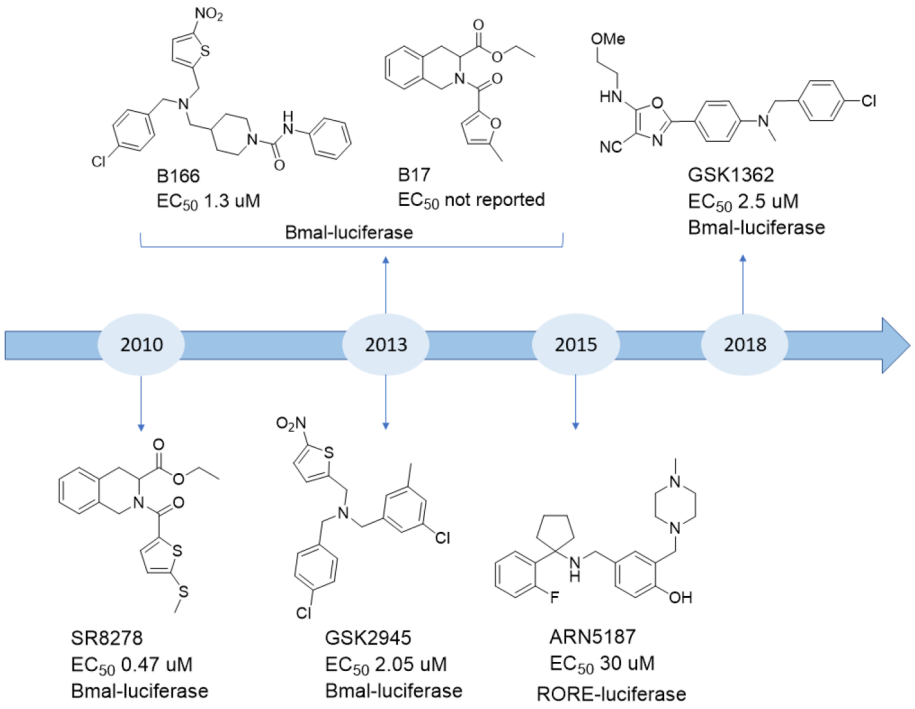

Fig. 7. Timeline of REV-ERB antagonist ligands discovered to date, with corresponding structures, EC_{50} values and biological assay employed to obtain such EC_{50} values.

inflammation. However, it seems that the regulatory roles of REV-ERB are complex and cell-type specific. Furthermore, the α and β isoforms might have particular roles; all of which makes robust target validation both challenging and essential. For example, while knock out of the *REV-ERBα* gene can be potentially compensated by the presence of REV-ERBβ, gene deletion is a different perturbation to small molecule protein inhibition and so target validation using knock out studies alone may be misleading – as is the case for many drug targets. Ultimately, as is often observed for emerging targets, high-quality chemical tools will be invaluable for such target validation work, while also laying the groundwork for subsequent drug development.

Despite this need, there is much to be done in the medicinal chemistry of synthetic REV-ERB ligands. The early-stage discovery work summarised in this review presents numerous scaffolds that both activate and inhibit the function of REV-ERB. Some series have been more heavily studied and developed than others. While these developments provide important insight with respect to the druggability of REV-ERB, many of the compounds fall short of the criteria for a high-quality chemical tool[73]. Issues include poor selectivity over related and unrelated off-targets, undesirable toxophore functionality (i.e. nitro aromatics), (off-target) cell killing, etc. Additionally, while not necessarily a criterion for a useful chemical probe, many of the compounds reported also display a poor PK profile. This, ultimately, limits target validation and proof of concept data in animal models. Therefore, there is still significant need for further medicinal chemistry work against REV-ERB moving forward. While further SAR work on established scaffolds may prove useful, we anticipate that significant value would come from further detailed mechanistic work. Key questions, such as precise ligand binding site(s), selectivity in targeting of the α and β isoforms, potential competition with heme, and how the redox status of REV-ERB influences activity, remain ill-defined for many, if not all, of the ligands to date. Answers to these questions will no doubt fuel further compound discovery and development strategies against this exciting transcriptional regulator in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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