Recent advances in modulators of circadian rhythms: an update and perspective
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ABSTRACT
Circadian rhythm is a universal life phenomenon that plays an important role in maintaining the multiple physiological functions and regulating the adaptability to internal and external environments of flora and fauna. Circadian alignment in humans has the greatest effect on human health, and circadian misalignment is closely associated with increased risk for metabolic syndrome, cardiovascular diseases, neurological diseases, immune diseases, cancer, sleep disorders, and ophthalmic diseases. The recent description of clock proteins and related post-modification targets was involved in several diseases, and numerous lines of evidence are emerging that small molecule modulators of circadian rhythms can be used to rectify circadian disorder. Herein, we attempt to update the disclosures about the modulators targeting core clock proteins and related post-modification targets, as well as the relationship between circadian rhythm disorders and human health as well as the therapeutic role and prospect of these small molecule modulators in circadian rhythm related disease.

1. Introduction
Circadian rhythm is the result of natural selection during the long-term evolution of organisms, enabling organisms to better adapt to changes in the external environment. Various behaviours and physiological functions of the body show obvious circadian rhythms, such as the sleep-wake cycle, food intake and other autonomous activities, as well as physiological activities including blood pressure, blood lipids, coagulation-fibrinolysis balance, heart rate, body temperature, locomotor activity, hormone levels, cell metabolism, and cell proliferation. The generation, maintenance, and regulation of circadian rhythms depend on the synergy of the circadian clock system, circadian input system, and circadian output system at the overall level (Figure 1) and at the cellular level, relying on the precise regulation of the endogenous circadian clock gene network (Figure 2). Any abnormalities in these intrinsic rhythms can cause disturbances in the circadian rhythm.

The physiological basis for the generation and maintenance of circadian rhythms comprises the central and peripheral circadian clock systems, rhythm input systems, and rhythm output systems. The rhythm input system senses and transmits environmental synchronisation signals represented by light signals to the central circadian clock system. The central biological clock system acts as the circadian rhythm pacemaker through the output system to transmit the generated rhythm signals to the periphery, and cooperates with the endogenous biological clock system of the peripheral organs to maintain the physiological activity of the body (Figure 1). The circadian clock system is composed of the central circadian clock and the peripheral circadian clock. In mammals, the apex of this system is the suprachiasmatic nuclei (SCN) master pacemaker, which is considered the central or master clock. The SCN integrates the environmental time information (primarily light) via the retina to revamp or entrain its phase, and then mastermind other oscillators in extra-SCN brain regions and peripheral organs. The rhythm output system is regulated by SCN, which can regulate gene expression, cellular function, metabolism, physiology, activity, behaviour, and sleep-wake cycles. Additionally, the rhythm output systems in turn can affect the SCN master pacemaker. For example, the arrhythmic food intake, excessive exercises, and sleep/circadian disorders affect SCN by remodel clock-controlled circuit.

Circadian rhythm production and maintenance are regulated by circadian clock genes. The molecular mechanism of the mammalian circadian clock is produced by a cell-autonomous feedback loop. The periodic oscillation of circadian rhythm depends on the precise regulation of the circadian clock gene and the clock-controlled gene regulatory network, including transcriptional-translational feedback loops and the non-transcription mechanism of post-translational modification. As shown in Figure 2, the transcriptional-translational feedback loops include a core loop and a secondary stabilisation loop.

In mammals, the transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1) form a heterodimer, which binds to E-box enhancers to activate the target gene transcription of circadian clock gene Period (including Per1 and Per2) and Cryptochrome (including CRY1 and CRY2). When PER and CRY proteins accumulate to a certain extent, they could be further transferred from the cytoplasm to the nucleus, and the PER/CRY heterodimer as a negative regulator directly interacts with CLOCK/BMAL1 to inhibit its transcriptional activity. In the stabilisation loop, the CLOCK/BMAL1 heterodimer can also induce the expression of nuclear receptors REV-ERBα and RORα. As a negative regulator, REV-ERBα can bind to the retinoic acid receptor-related orphan receptor binding element (RRE)
Figure 1. The physiological basis for the generation and maintenance of mammalian circadian rhythm. Reproduced from Chen et al.19

Figure 2. Molecular clock loops and their potential targets with representative small molecule modulators. CLOCK: circadian locomotor output cycles kaput; BMAL1: brain and muscle ARNT-like 1; CRY: cryptochrome; PER: period; ROR: RAR-related orphan receptor; RRE: retinoic acid receptor-related orphan receptor binding element; CCGs: clock-controlled genes; CK1: casein kinase 1; CDKs: cyclin-dependent kinases; GSK3β: glycogen synthase kinase 3β; DNA TOPs: DNA topoisomerases. Reproduced from He and Chen49. Copyright 2016 American Chemical Society.
(sequence AGGTCA) in the BMAL1 promoter region and block the transcription of BMAL1. Conversely, RORs can be used as a positive regulator to bind to the RRE of the BMAL1 promoter region to promote the transcription of BMAL1, thereby forming an auxiliary loop for the transcription and translation oscillations of the circadian clock gene. However, beyond that, post-translational modifications (phosphorylation/dephosphorylation, acetylation/deacetylation, etc.) and degradation (ubiquitination/proteasome pathway) of various circadian proteins enable fine-tuning of the transcriptional-translational feedback loops (such as adjusting the expression phase and the period of oscillation), so it can also play an important role in the cyclical circumadian rhythms. For example, PER and CRY proteins can be phosphorylated by casein kinase 1ε (CK1ε)/casein kinase 1ε (CK1i), which affects the increase in the continuous length of the cycle. Silent information regulator 1 (SIRT1) regulates the expression of the clock gene BMAL1, Cry1, and Per2 by interacting with the CLOCK/BMAL1 complex and catalysing the deacetylation and degradation of the PER protein.

Circadian alignment in humans has great effect on human health, and circadian misalignment has been involved in metabolic syndrome, cardiovascular disease, acute lung injury and inflammation, cancer, neurological diseases, and immune diseases. While accumulating evidence indicates that circadian misalignment has been involved in metabolic syndrome, cardiovascular disease, acute lung injury and inflammation, cancer, neurological diseases, and immune diseases, the highly active compound 2-(9H-carbazol-9-yl)-N-(2-fluorophenyl)ethan-1-amine (Compound 1) can lengthen the circadian period, repress Per2 activity, and stabilise CRY better than compound 1. More interestingly, another group discovered a series of small molecule modulators of circadian rhythms.

| Table 1. Modulators targeting CRYs. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Name            | Activity        | Actions         | Physiological effects                  | Reference       |
| KL001 (Compound 1) | IC_{50} = 14U/M/0.82U/M (measured by BMAL1-dLuc and Per2-dLuc reporter U2OS cells, Agonist) | Stabilise CRY, lengthen period, reduce amplitude | Inhibit glucagon-induced gluconeogenesis in primary hepatocytes | Hirota et al.52, Nangle et al.53 |
| KL002 (Compound 2) | IC_{50} = 5.9U/M/1.2U/M (measured by BMAL1-dLuc and Per2-dLuc reporter U2OS cells, Agonist) | Stabilise CRY, lengthen period, reduce amplitude | Inhibit glucagon-induced gluconeogenesis in primary hepatocytes | Hirota et al.52 |
| KL003 (Compound 3) | IC_{50} = 4.4U/M/0.66U/M (measured by BMAL1-dLuc and Per2-dLuc reporter U2OS cells, Agonist) | Stabilise CRY, lengthen period, reduce amplitude | Inhibit glucagon-induced gluconeogenesis in primary hepatocytes | Hirota et al.52 |
| FAD (Compound 4) | / (Inhibitor) | Stabilise CRY proteins by competing with FBXL3 | Lengthen the circadian period, repress Per2 activity, and stabilise CRY | Hirano et al.54 |
| KL004 (Compound 5) | log[IC_{50}(M)] = -7.32 (Agonist) | / (Inhibitor) | Light-independent mechanisms of FAD regulate CRY | Lee et al.55 |
| GO044 (Compound 6) | / (Inhibitor) | Shorten period | / | Oshima et al.56 |
| GO200 (Compound 7) | / (Inhibitor) | Stabilise CRY | / | Oshima et al.56 |
| GO211 (Compound 8) | / (Inhibitor) | Stabilise CRY | / | Oshima et al.56 |
| KS15 (Compound 9) | EC_{50}=0.49U/M (Inhibitor) | Attenuate circadian oscillation, inhibit the repressive function of CRY1/2 | Enhance E-box-mediated transcription | Chun et al.57 |
| 50 (Compound 10) | EC_{50} = 0.363U/M (measured by Per2-dLuc reporter U2OS cells, Agonist) | Lengthen the circadian period, repress Per2 activity, and stabilise CRY | Inhibit glucagon-induced gluconeogenesis | Humphries et al.58 |
| KL101 (Compound 11) | log[IC_{50}]= -5.79 (measured by BMAL1-dLuc cells, Agonist) | Stabilise CRY1 and lengthen period | Enhance brown adipocyte differentiation | Miller et al.59 |
| TH0301 (Compound 12) | log[IC_{50}]= -6.03 (measured by BMAL1-dLuc cells, Agonist) | Stabilise CRY1/2 and lengthen period | Enhance brown adipocyte differentiation | Miller et al.59 |
compound 1 derivatives, compounds 6–8, which can shorten the period by targeting cryptochrome in the mammalian circadian clock\(^\text{36}\). Unfortunately, no physiological effects were reported by subsequent studies. The novel derivative of 2-ethoxypropanoic acid, compound 9, can inhibit the target CRY1 and CRY2\(^\text{57}\). Compound 9 can enhance E-box-mediated transcription and attenuate the rhythm without affecting the period. Recently, the potent compound 1–(3-(3,6-difluoro-9H-carbazol-9-yl)-2-hydroxypropylimidazolidin-2-one (compound 10) significantly enhanced glucose clearance at 100 mg/kg in an oral glucose tolerance test\(^\text{58}\). Furthermore, the compound N-(2-(2,4-dimethylphenyl)-2,6-dihydro-4H-thieno[3,4-c]pyrazol-3-yl)cyclopentane-1-carboxamide (compound 12) as moderately selective agonist for CRY2 than CRY1 were reported by using human U2OS cells with a Bmal1 promoter-luciferase (Bmal1-dLuc) reporter\(^\text{59}\). The X-ray crystal structures of CRY1 in complex with compound 11 and compound 12 show that these molecules were located in the FAD-binding pocket. As a useful tool for high selectivity against CRY isoform, the compound 11 and compound 12 were proved to facilitate brown adipocyte differentiation. Altogether, the modulators including the agonist or inhibitor of CRYs may be useful tools to treat circadian clock-related diseases through its action on CRY (see Figure 3 and Table 1).

Figure 3. The structure of modulators targeting CRYs.
2.1.2. Modulators for REV-ERBs

Endogenous ligands for REV-ERBs. In 2007, compound 13 was confirmed as a physiological ligand of nuclear receptors REV-ERβ (encoded by nuclear receptor subfamily 1, group D, member 1 (NR1D1)) and REV-ERβ (Nuclear receptor subfamily 1, group D, member 2 (NR1D2)) by two research groups, Rastinejad et al. and Lazar et al. Multiple biochemical and biophysical methods were used to demonstrate the association of compound 13 with ligand-binding domains of REV-ER receptors, including mutation studies, transcriptional repressor function and repression of target gene transcription, ultraviolet-visible spectroscopy, mass spectrometry, isothermal titration calorimetry (ITC), and circular dichroism. Soon afterward, the crystal structure of REV-ERβ in complex with compound 13 was also reported. All the results disclosed suggest that compound 13 can bind the REV-ERRs and is indeed a physiological ligand of nuclear receptors REV-ERRs. In mammalian cells, compound 13 can cause the recruitment of the co-repressor nuclear receptor corepressor-1 (NCoR) by targeting REV-ERβ, giving rise to repression of target genes including BMAL1 (also known as ARNTL). Moreover, by targeting the REV-ERRs, compound 13 can suppress the expression of hepatic gluconeogenic gene and the output of glucose. These findings would facilitate the development of small molecule modulators against REV-ERRs to treat diseases related to the dysfunctional disorder of metabolism and the mammalian clock.

Synthetic ligands for REV-ERRs. In 2008, the compound 1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate was reported by using REV-ERβ–NCoR fluorescence resonance energy transfer (FRET) assay, which showed an EC50 value of 250 nM. This compound was the first agonist of REV-ERβ and was competitive with compound 13. In subsequent studies, this compound was successively named SR6452 or GSK4112 (compound 14) (Table 2 and Figure 4). Compound 14 can induce adipocyte differentiation in 3T3-L1 cells, enhance the recruitment of nuclear receptor co-repressor (NCoR) to REV-ERβ, and inhibit expression of the circadian target gene Bmal1. In addition, similar to compound 13, compound 14 also repressed the expression of gluconeogenic genes in liver cells and reduced glucose output in primary hepatocytes. These studies suggest that compound 14 may be used to treat diabetes or to modulate the circadian rhythm.

Although compound 14 was used as a probe to investigate the pharmacological effects in vitro, it has a poor pharmacokinetic profile with rapid clearance (Cint > 1.0 ml min⁻¹ mg⁻¹ protein) in rat liver microsomes and lower oral bioavailability (F ≤ 1% in mice). Therefore, a series of analogues of compound 14 were synthesised by medicinal chemists to explore the applicable pharmacokinetics and pharmacodynamics used in in vivo studies.

The analogues of compound 14, the potent compounds 15–16, were disclosed by Burris et al., which were the first REV-ER agonists with in vivo activity. Compounds 15–16 can generate loss of locomotor activity during the subject dark phase and 1–3 h delay in the onset of nocturnal locomotor activity. The two compounds can alter the expression of the core clock genes, including Per2, Bmal1, Clock, Cry2, and Npas2. The ability of REV-ERβ agonists in modulating the circadian behaviour of C57BL/6 mice may be used as a drug to treat sleep disorders and jet lag. Indeed, compound 15 was found to be able to induce wakefulness and reduce paradoxical sleep–rapid eye movement (REM) and slow-wave sleep.

As previously reported, the double-knockout REV-ERβ and REV-ERβ mice can also markedly alter metabolic effects. The administration of the agonist of REV-ERβ and REV-ERβ, compound 16, gives rise to increase in energy expenditure and weight loss. In obese mice, including diet-induced obese mice and genetic model of obesity (OB/OB mice), REV-ERβ agonist treatment results in a decrease in fat mass and plasma lipids. Recently, a study investigated further the metabolic profile of the nuclear receptor REV-ERβ agonist. The results of the experiment show that the enzymatic isoforms mainly involved in the compound 15 phase I biotransformation pathways are cytochrome P450 3A4 (CYP3A4), cytochrome P450 3A5 (CYP3A5), cytochrome P450 2C19 (CYP2C19), and cytochrome P450 2D6 (CYP2D6).

With the further study of REV-ERβ agonist, compound 15 was associated with heart failure, cancer, atherosclerosis, chikungunya and O’nyong-nyong virus and autoimmune disease. However, Lazar et al. discovered that compound 15 can decrease cell viability, rewire cellular metabolism, and alter gene transcription in hepatocytes and embryonic stem cells lacking both REV-ERβ and REV-ERβ, which means that the effects of compound 15 cannot be used solely as surrogate for REV-ERβ activity. Therefore, more efforts are needed to explore its mechanism of action. Highly selective compounds also need to be developed urgently.

According to published papers in the same period as compound 15, Kamenecka et al. also conducted structure-activity relationship analysis on compound 14. Compounds 18–19 show slightly better plasma and brain exposure as compound 14, but they displayed the best CNS exposure with brain penetration of 100% or 67%, respectively. The analogue of compound 14, compound 17, was reported by Tomkinson et al., which shows > 1000-fold selectivity over liver X receptor (LXRα) and is a potent agonist with REV-ERβ activity (EC50 = 0.05 μM), which may be the best compound with high selectivity and may serve as a pharmacological toolbox to investigate the biology of REV-ERβ. Recently, the novel small molecular compound 20 was disclosed, which can reinforce REV-ERβ activity by acting in a RORE-dependent manner, though not by the same mechanism as known REV-ERβ agonists. It may also provide a new way of exploring the REV-ERβ modulator.

The first antagonist of REV-ERβ is compound 21. Compound 21 was derived from compound 14 based on the tertiary amine scaffold. In HepG2 cells, compound 21 could increase the expression of either glucose 6-phosphatase (G6Pase) or phosphoenolpyruvate carboxykinase (PEPCK) mRNAs by blocking the action of the endogenous agonist. Compound 21 also caused significant increases in the expression levels of growth/differentiation factor 10 (GDF10) and Growth and differentiation factor 15 (GDF15) in uterine endometrial stromal cells (UESCs). These results show that cellular oscillators may serve an important role of regulating the expression of downstream genes during the differentiation of UESCs.

Although the pharmacokinetic properties of small molecular compound 21 is poor, which has also been confirmed by our group, compound 21 serves as a useful probe to explore the REV-ERβ function by others. In vesicular stomatitis virus (VSV)-induced encephalitis model, administration of compound 21 increased C-C motif chemokine ligand 2 (CCL2) mRNA expression and decreased mice survival, which is associated with neuroprotective effects and lifetime. The molecular connection between the circadian timing system and mood regulation was identified by Kim et al. The circadian nuclear receptor REV-ERβ is associated with bipolar disorder, as it influences midbrain dopamine production and mood-related behaviour in mice. Treatment with compound 21 induced mania-like behaviour in association with a central hyperdopaminergic state. The evidence suggests that
| Name                | Activity | Actions                                                                 | Physiological effects                                                                 | Reference       |
|---------------------|----------|--------------------------------------------------------------------------|----------------------------------------------------------------------------------------|-----------------|
| Heme (Compound 13) | IC_{50} = 0.05μM (measured by FRET assay, agonist)                       | Represses activity of REV-ERB\textsubscript{a} LBD                                  | Regulates interaction between REV-ERB\textsubscript{a} and NCoR-HDAC3                    | Raghuram et al.60, Yin et al.61 |
| GSK4112/SR6452      | EC_{50} = 0.25μM (measured by FRET assay, agonist)                       | Resets the circadian oscillation of REV-ERB target genes, suppresses expression of REV-ERB target genes in cells | Inhibits expression of the circadian target gene \textit{bmal1}                       | Meng et al.64, Kumar et al.65, Grant et al.66 |
| SR9009 (Compound 15)| IC_{50} = 0.67/0.89μM (measured by GaIA reporter assay for REV-ERB\textsubscript{a} and REV-ERB\textsubscript{b}, agonist) | Amplitude reduction, suppresses RRE-mediated transcription | Improves glucose homeostasis in obese mice, promotes wakefulness, reduces anxiety | Solt et al.67   |
| SR9011 (Compound 16)| IC_{50} = 0.79/0.56μM (measured by GaIA reporter assay for REV-ERB\textsubscript{a} and REV-ERB\textsubscript{b}, agonist) | Amplitude reduction, suppresses RRE-mediated transcription | Improves glucose homeostasis in obese mice, promotes wakefulness, reduces anxiety | Solt et al.67   |
| GSK2945 (Compound 17)| EC_{50} = 0.05μM (measured by NCOR peptide recruitment for REV-ERB\textsubscript{a}, agonist) | Suppression and shift of the BMAL oscillation curve | Inhibits IL-6 production from human THP-1 cells                                      | Trump et al.68   |
| 12e (Compound 18)   | EC_{50} = 0.7μM (measured by full-length \textit{bmal1} reporter assay for REV-ERB\textsubscript{a}, agonist) | Suppresses expression of REV-ERB target genes in cells | Inhibits expression of the circadian target gene \textit{bmal1}                       | Shin et al.69    |
| 6j (Compound 19)    | EC_{50} = 0.077μM (measured by full-length \textit{bmal1} reporter assay for REV-ERB\textsubscript{a}, agonist) | Suppresses expression of REV-ERB target genes in cells | Inhibits expression of the circadian target gene \textit{bmal1}                       | Noel et al.70    |
| KK-S6 (Compound 20) | IC_{50} = 3.95μM (measured by cell-based assay using the \textit{wtBmal1: Luc}-transfected NIH3T3 cells, agonist) | Alters the amplitude of circadian oscillations of \textit{Bmal1} and \textit{Per2} | Represses RORE-dependent transcriptional activity of m\textit{Bmal1} promoter and reduces endogenous BMAL1 protein expression | Lee et al.71     |
| SR8278 (Compound 21)| IC_{50} = 0.47μM (measured using full-length \textit{Bmal1} reporter assay for REV-ERB\textsubscript{a}, Antagonist) | Increases expression of REV-ERB target genes in cells | Reduces glucagon secretion from mouse alpha cells                                      | Kojetin et al.72  |
| ARN5187 (Compound 22)| IC_{50} = 17.5μM (measured using luciferase-based reporter assay, dual autophagy/REV-ERB inhibitor) | Direct interaction with the LBD of REV-ERB\textsubscript{a} | Enhances the expression of BMAL1, \textit{Per1}, and \textit{Pepck}, and blocks autophagy by disrupting the lysosomal function and preventing autophagolysosome final maturation | De Mei et al.73  |
| 30 (Compound 23)    | IC_{50} = 1.34μM (measured using luciferase-based reporter assay, dual autophagy/REV-ERB inhibitor) | Direct interaction with the LBD of REV-ERB\textsubscript{a} | Enhances the expression of BMAL1, \textit{Per1}, and \textit{Pepck}, and blocks autophagy by disrupting the lysosomal function and preventing autophagolysosome final maturation | Torrente et al.74 |
| GSK1362 (Compound 24)| inverse agonist | Protects REV-ERB\textsubscript{a} protein from degradation | Increases transcription of \textit{Bmal1}                                               | Pariollaud et al.75 |
| Chelidamic acid     | EC_{50} = 0.36μM (measured using mammalian cell-based two-hybrid system, agonist) | Binds specifically to the LBD site of REV-ERB\textsubscript{a} receptor               |                                                        | Hering et al.76   |
targeting REV-ERBα may be beneficial to the treatment of circadian rhythm-related affective disorders. Compound 21 could slow the progression of muscular dystrophy by increasing lean mass and muscle function and decreasing muscle fibrosis and muscle protein degradation in C57BL/10ScSn-Dmdmdx/J (mdx) mice. This research suggests that the antagonist compound 21 of REV-ERB may be a profound agent for the treatment of Duchenne muscular dystrophy (DMD). In conclusion, these results suggest that compound 21 is a unique chemical tool. However, it must be clearly recognised that poor pharmacokinetic properties of...
compound 21 also limit the further development of the compound. It is urgent to discover novel and potent compounds against REV-ERBs.

The novel dual autophagy/REV-ERB inhibitor compound 22 was revealed in 201493. Compound 22 can relieve the clock transcriptional repression mediated by REV-ERB and enhance the expression of REV-ERB target genes, Bmal1, Per1, and phosphoenolpyruvate carboxykinase (PEPCK), in BT-474 cells. It can also block autophagy by disrupting the lysosomal function and preventing autophagosome some final maturation. Although the potency of compound 22 is under micromolar range, this compound provides an uncoiling the new measures to treat cancers. Therefore, Grimaldi et al.74 carried out structure–activity relationship (SAR) studies of compound 22 and finally obtained the potent compound 23 (1-(4-Fluorophenyl)-N-[1-[3-[(1-methyl-4-piperidyl)methyl]phenyl]methyl]-cyclopentanamine) with 15-fold greater REV-ERB/β-inhibitory and cytotoxic activities compared to compound 22.

Recently, a novel oxazole inverse agonist of REV-ERB, compound 24, was discovered by Ray et al.75 based on fluorescence resonance energy transfer (FRET) assay. Compound 24 showed a high selectivity over 20 nuclear receptors, which can reverse the degradation of REV-ERBβ protein mediated by inflammatory stimuli. Subsequently, Gul et al.76 established a mammalian cell-based two-hybrid assay system and found compound 25 as a novel agonist of REV-ERB. In addition, three other compounds against REV-ERB, compounds 26–28 (Figure 4), were found using this method. Compound 28 was confirmed as an antagonist, and compounds 26–27 were confirmed as agonists. Although the three compounds showed a poor selectivity against other targets, these compounds present a new kind of scaffold and can be used as a profound hit to reveal a drug-like compound.

2.1.3. Modulators for RORs

Natural ligands for RORs. In 2002, the first ligand of RORα, compound 29, was proved by X-ray structure (PDB entry 1N83). It is present in the ligand-binding pocket (LBP) and is important in designing the ligand targeting RORs94. The analogue of compound 29, compound 30, can also bind to RORα as confirmed by the crystal structure (PDB entry 150X)95. Other sterols including oxysterols as ROR inverse agonists and neoruscogenin as RORα agonist were found and reviewed in other papers96,97. The representative structure of sterols (compounds 31–37) is presented in Figure 5 to analyse the structure for researchers. In 2001, the first ligand of RORβ, compound 38, was proved by X-ray structure (PDB entry 1K4W)98. This crystal structure of compound 38 and the ligand-binding domain (LBD) of the rat RORβ shed new light on the development of ligands against RORs. Subsequently, the crystal structure of the complex between compound 38 and RORβ (PDB entry 1N4H) was solved by Schule group99. They also solved the crystal structure of the complex between synthetic analog compound 40 and RORβ (PDB entry 1NQT). All these two-crystal structures present similar results, namely, the compound 39 and analogs were binding to the RORβ ligand-binding domain (LBD). Hydroxysterol compounds (compounds 41–43) were binding to the RORβ LBD using the same method in 2010, with access code 3KYT (RORβ/Compound 41), 3LOJ (RORβ/Compound 42), and 3LOL (RORβ/Compound 43), respectively100. Recently, the natural compound 44 as an agonist for the ROR was reported by using ClockΔ19/+ cells with PER2::Luc reporter101,102. The potent natural compound and all these crystal structures of the complex between natural ligand and ROR have inspired researchers to search for potent and selective small molecule modulators targeting RORs (Figure 5).

Synthetic ligands for RORs. In 2010, using cell-based GAL4-NR LBD cotransfection assay, Griffin et al.103 found the first RORα/β inverse agonist compound 45, which was also the agonist of the liver X receptor (LXR)104. Compound 45 was binding to RORα/β but not to RORβ. This compound provided the scaffold to further exploit the potent and selective ligands targeting ROR. A compound with multiple targets is not an ideal tool to disclose the function of protein. Therefore, the core scaffold of compound 45 was optimised, and a round of agonists or inverse agonists against RORα/β, RORβ, and RORγ were reported. These compounds have been reviewed elsewhere96,97. The representative compounds can be found in Figure 6 and Table 3 to systematically review the research studies.

The first synthetic RORα-selective partial inverse agonist compound 48 based on the core scaffold of compound 45103 and compound 46105 was reported in 2010107. Compound 48 can inhibit the constitutive transactivation activity of RORα with an IC50 of 480 nM, but it cannot inhibit the activity of LXRα, RORβ, and RORγ. Compound 48 can suppress hepatic gluconeogenesis and improve glucose homeostasis in vivo, suggesting that compound 48 may be a potential tool to treat type 2 diabetes.

After structure–activity relationship (SAR) studies of compound 47, the potent and selective inverse agonist compound 49 targeting RORγ was obtained, which can reduce the conformational mobility of RORγ LBD. The other potent and selective agonists, inverse agonists, or inhibitors of RORγ were reviewed elsewhere50,112. Recently, 4-(isoaxazol-3-yl) butanoic acid derivatives as high selective inhibitors of RORγ were reported. The potent compound 50 showed commendable anti-inflammatory effects in a mouse dermatitis model. A novel compound 52, 2-(4-(ethylsulfonyl)phenyl)-N-(2′-fluoro-4′-(1,1,1,3,3,3-hexafluoro-2-(trifluoromethyl)propan-2-yl)-1,1′-biphenyl)-4-yl)acetamide, in complex with the RORγ ligand binding domain (LBD), was reported111. Compound 52 possesses good metabolic stability and pharmacokinetic profile, and shows a significant tumour growth inhibition in vivo.

2.2. Small molecule modulators with other or unknown targets

Compounds targeting other proteins including kinase, epigenetic proteins, and others can also alter circadian characteristics. All these compounds are summarised as follows.

2.2.1. Modulators for kinases

Casein kinase 1 (CK1). The casein kinase family comprises seven proteins, and others can also alter circadian characteristics. All these proteins have been reviewed elsewhere119. The representative compounds can be found in Table 1 and Figure 7 to systematically review the research studies. Compound 60 (Table 4 and Figure 7) was also proven to lengthen the period in cultured cells and were reviewed in other papers59,116. Recently, compound 61 was identified as a regulator to increase period length in mammalian cells and larval zebrafish assay117. Compound 62 lengthens the period through CK1 inhibition118. All these studies reveal that the role of CK1 is important in the regulation of circadian rhythm119.

Cyclin-dependent kinases (CDKs). The cyclin-dependent kinase family comprises 11 distinct genes encoding CDK isoforms (1–11)113. CDKs has been reported to directly phosphorylate
CLOCK\textsuperscript{126}, inhibitor compound \textbf{63} targets CDK1, CDK2, and CDK5, and compound \textbf{64} targets CDK2, CDK4, and CDK5 can lengthen the circadian period\textsuperscript{123}. However, the multi-target inhibitors, compounds \textbf{65}–\textbf{66} targeting both CDK and GSK3, were proved to shorten the circadian period. Recently, compound \textbf{67}, an inhibitor of CDK7 and CDK9, has been reported to increase period length in mammalian cells\textsuperscript{124}.

Other kinases. As other studies in the literature have reported, glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)) can also regulate the circadian clock, which can phosphorylate CLOCK, PER, REV-ERB, and CRY proteins\textsuperscript{114}. The selective GSK3\(\beta\) inhibitors, compounds \textbf{68}–\textbf{70}, have been reported to shorten the circadian period\textsuperscript{125}. Other kinase inhibitors including compounds \textbf{71}–\textbf{72} targeting p38 and compound \textbf{73} targeting CLK1 have been reported to increase period length\textsuperscript{125}. Recently, compounds \textbf{74}–\textbf{76} selective BCR-ABL tyrosine kinase inhibitor were found to shorten the circadian period\textsuperscript{51} (Table 4 and Figure 7).
2.2.2. Modulators for epigenetic proteins and others

Silent information regulator 1 (SIRT1) has been found to contribute to circadian control, which regulates circadian clock gene expression through PER2 deacetylation. SIRT1 activator compound 77 is involved in physiological rhythms and clock gene expression. The potent SIRT1 activators, compounds 78–81, also show that they can reduce circadian expression, lengthen period, and reduce amplitude. Recently, SIRT6 was also found to regulate circadian rhythms via Per2. However, the small molecules of SIRT6 have not been tested by researchers.

In a recent study, peroxisome proliferator-activated receptor γ (PPARγ) was involved in regulating the expression of Bmal1 and REV-ERBα, and its agonist compound 82 can induce expression of Bmal1. Compounds 83–84, DNA topoisomerase (TOP) inhibitors, were also found to enhance the circadian expression and lengthen the circadian period. Recently, the androgen antagonist and oestrogen activator compound 85 was found to shorten the circadian period (Table 5 and Figure 8).

3. Implications in circadian rhythm-related diseases

Circadian rhythm plays a very important role in the normal maintenance of organisms, but physical and psychological influences including jet lag, shift work, and diseases can cause a misalignment of the intrinsic oscillators. Jet lag occurs in individuals...
travelling across multiple time zones, who may suffer from some symptoms including disruption of sleep, gastrointestinal disturbances, decreased vigilance and attention span, a general feeling of malaise, and an increased risk of cancer and heart disease. Shift work is apparent among people employed in factories or social event firms and work from 7 pm to 9 am. Shift work has become a common phenomenon in society, and was found to be involved in cancer, cardiovascular disease, depression, and infertility. Jet lag and shift work induce rhythm disorder, which can cause a mass of psychological, nervous system, mental health, and physical health problems. Beyond all that, diseases are closely related to circadian rhythms. Diseases can cause disturbances in circadian rhythms, and disorder in circadian rhythms, in turn, further aggravate the severity of the disease. This section will focus on the relationship between disease and circadian rhythm disorders (Figure 9).

### 3.1. Metabolic diseases

Circadian rhythm has been associated with homeostasis and physiology, which is closely related to physical health. Numerous lines of evidence are emerging that circadian dysfunctions are closely associated with increased risk for metabolic disease such as obesity and diabetes. Evidence that the circadian rhythm is associated with energy homeostasis, glucose homeostasis, and lipid homeostasis has been found. Homozygous Clock mutant mice can lead to type 2 diabetes mellitus, with metabolic syndromes of hyperleptinemia, hyperlipidaemia, hepatic steatosis, and hyperglycaemia, with insufficient compensatory insulin production. Clock mutant animals can induce obesity, hyperphagia, reduced energy expenditure, adiposity, as well as dysregulation of glucose and lipid metabolism. The core clock genes Clockmut or Bmal1+/- depress and abolish

| Table 4 Representative modulators targeting kinases. |
|---|---|---|---|
| Name | Activity | Physiological Effects | Reference |
| IC261 (Compound 53) | Inhibiting CK1ζ | Period lengthening | Eide et al. |
| Cki-7 (Compound 54) | Inhibiting CK1ζ | Period lengthening | Vanselow et al. |
| D4476 (Compound 55) | Inhibiting CK1ζ | Period lengthening | Reischel et al. |
| PF-4800567 (Compound 56) | Inhibiting CK1ζ | Period lengthening | Meng et al. |
| LH846 (Compound 57) | Inhibiting CK1ζ | Period lengthening | Lee et al. |
| 1-3 (Compound 58-60) | Inhibiting CK1ζ | Period lengthening | Chen et al. |
| A002195858 (Compound 61) | Inhibiting CK1 | Period lengthening | Morsos et al. |
| B-AZ (Compound 62) | Inhibiting CK1 | Period lengthening | Ono et al. |
| Roscovitine (Compound 63) | Inhibiting CKD1, CKD2 and CKD5 | Period lengthening | Hirota et al. |
| Puralanol A (Compound 64) | Inhibiting CKD2, CKD4 and CKD5 | Period lengthening | Hirota et al. |
| Indirubin-3'-oxime (Compound 65) | Inhibiting CKD and GSK3 | Period shortening | Hirota et al. |
| Kenpaullone (Compound 66) | Inhibiting CKD and GSK3 | Period shortening | Hirota et al. |
| PHA676491 (Compound 67) | Inhibiting CKD7/CDK9 | Period shortening | Uehara et al. |
| Chir99021 (Compound 68) | Inhibiting GSK3β | Period shortening | Hirota et al. |
| indirubin (Compound 69) | Inhibiting GSK3β | Period shortening | Hirota et al. |
| SB203580 (Compound 70) | Inhibiting p38 | Period shortening | Isoijima et al. |
| PD169316 (Compound 71) | Inhibiting p38 | Period shortening | Isoijima et al. |
| TG003 (Compound 72) | Inhibiting CLK1 | Period shortening | Isoijima et al. |
| Nilotinib (Compound 73) | Inhibiting BCR-ABL | Period shortening | Tamai et al. |
| Imatinib (Compound 74) | Inhibiting BCR-ABL | Period shortening | Tamai et al. |
| Bafetinib (Compound 75) | Inhibiting BCR-ABL | Period shortening | Tamai et al. |
Figure 7. Development and structure of synthetic modulators targeting kinases.
Gluconeogenesis also induced hypertriglyceridaemia in animal models. REV-ERBα knockout mice also displayed altered lipid and bile metabolism. Subsequent studies have shown that double knockout mice (REV-ERBα and REV-ERBβ) have disorganised lipid homeostatic gene networks. The other core circadian rhythm gene ROR also turns out to be related to the regulation of energy homeostasis and several lipid and glucose metabolic genes. Mutant RORα mice (also known as staggerer mice) display hypo-α-lipoproteinemia. Recent studies have shown that RORα accommodates peripheral glucose tolerance, torpor, and hepatic lipid metabolism by regulating the expression of fibroblast growth factor 21 (FGF21). All of these pieces of evidence suggest that the circadian rhythm is associated with metabolism and that clock proteins can be as drug targets to treat metabolic diseases.

Many small molecule modulators of circadian proteins have been found to be useful in metabolic diseases. The CRY’s activator compound 1 has been shown to inhibit glucagon-induced gluconeogenesis, which may provide a foundation for the treatment of diabetes. Aside from the ligands of CRYs, the ligands of circadian nuclear receptors REV-ERB and RORs also demonstrated that they can be conducive to regulate metabolism in vivo. Compounds 15–16 as agonists of REV-ERBα and REV-ERBβ proved highly effective in the improvement of the metabolic profile in obese mice. Recently, Chen et al. identified that compound 44 as an agonist for ROR can potently protect against metabolic syndrome and remodel the circadian and metabolic gene expression in diet-induced obese mice. Subsequently, they demonstrate that compound 44 can serve as a potential drug to treat the metabolic disorders and age-related diseases.
decline by regulating cholesterol and bile acid metabolism \(^{148}\) and overcome the metabolic challenge by enhancing mitochondrial respiration in skeletal muscle \(^{149}\). Therefore, with an in-depth study of the mechanism for clock proteins and the discovery of selective and potent small molecule modulators, it is believed that in the near future, the ligands of CRYs, REV-ERBs, or RORs will provide first-class treatment for metabolic diseases such as obesity and diabetes.

| Name                  | Activity     | Physiological effects                                      | Reference     |
|-----------------------|--------------|------------------------------------------------------------|---------------|
| Resveratrol (Compound 77) | SIRT1 activator | Modulate physiological rhythms and clock gene expression     | Chang et al.\(^ {128}\) |
| SRT2183 (Compound 78)  | SIRT1 activator | Reduce circadian expression                                 | Bellet et al.\(^ {130}\) |
| SRT1720 (Compound 79)  | SIRT1 activator | Reduce circadian expression                                 | Bellet et al.\(^ {130}\) |
| SRTCD1023 (Compound 80) | SIRT1 activator | Reduce circadian expression                                 | Bellet et al.\(^ {130}\) |
| SRTCL1015 (Compound 81) | SIRT1 activator | Reduce circadian expression                                 | Bellet et al.\(^ {130}\) |
| Rosiglitazone (Compound 82) | PPAR\(\gamma\) agonist | Reduce circadian expression Lengthen period                  | Wang et al.\(^ {131}\) |
| Camptothecin (Compound 83) | TOPI inhibitor | Enhance the circadian expression and lengthen the circadian period | Onishi et al.\(^ {132}\) |
| Harmine (Compound 84)   | TOPI inhibitor | Enhance the circadian expression                            | Onishi et al.\(^ {132}\) |
| DHEA (Compound 85)      | Androgen antagonist and oestrogen activator | Shorten the circadian period                                | Tamai et al.\(^ {51}\) |

Figure 8. Development and structure of synthetic modulators targeting epigenetic proteins and others.
3.2. Sleep disorders

Sleep plays a very important role in the biological process of all creatures; it is regulated by circadian rhythm and homeostatic mechanisms. Normal circadian rhythms play an irreplaceable role in sleep. Circadian misalignments such as jet lag, shift work, and sleep deprivation have resulted in sleep disorders. Kessling’s group and Yamaguchi’s group identified that the different organs of mice showed heterogeneity entrainment kinetics in an experimental paradigm for jet lag. The rhythm gene has been linked to sleep disorders. Mutations in both PER2 (PER2 S662G) and CSNK1D (CK1δ T44A) have been involved in familial advanced sleep phase syndrome (FASPS). Recent studies indicate that the core clock gene expression has a close association with sleep apnoea (SA). Canales et al. identified that the Per3 expression of SA was lower than that in the normal group. Pharmacological treatment targeting the mammalian clock has been shown to have beneficial effects on sleep architecture. Compound 16 as an agonist of REV-ERBα and REV-ERBβ displays increase in wakefulness and reduction of paradoxical sleep-rapid eye movement (REM) sleep and slow-wave sleep in vivo. Therefore, the REV-ERB ligands may be beneficial in treating sleep disorders.

3.3. Ophthalmic diseases

As widely appreciated, light has profoundly influenced the mammalian circadian rhythm. Light is mainly received by intrinsically photosensitive retinal ganglion cells (ipRGCs). A large number of studies show that the knockout of the rhythm gene affects retinal processing of light information. The circadian rhythm is involved in ophthalmic diseases including glaucoma, macular degeneration, cataract, retinitis pigmentosa, diabetic retinopathy, and optic nerve atrophy. Evidence is accumulating that glaucoma directly damages the light input into the circadian system and causes optic nerve dysfunction. Recently, a mass of transcripts of nocturnal rodents and diurnal primates with daily and circadian oscillations were presented by RNA Sequencing (RNA-Seq) technology. In previous studies, we found that compound 21 as an antagonist of REV-ERBα can enhance corneal wound healing. Therefore, the small molecule modulators of circadian proteins provide a potential solution for the treatment of ophthalmic diseases.

3.4. Other diseases

The impact of the circadian system on immune diseases, mood disorders, neurovascular diseases, aging, renal diseases (such as hypertension, chronic kidney disease, renal fibrosis, and kidney stones), and cancer has been reviewed by others. As described in section 2, small molecule modulators of circadian proteins supply pharmacological tools to treat these diseases. For example, the REV-ERB ligand compound 14 can regulate innate immune responses by repressing interleukin 6 (Ile6). Interestingly, Kim et al. identified that the pharmacological inhibition of REV-ERBα activity produces mania-like behaviour. The mice showed more hyperactive behaviour after the administration of REV-ERBα antagonist compound 21. The REV-ERBα agonist may be useful for mood regulation.

4. Perspectives and concluding remarks

In this review, we detailed all aspects of the physiological basis, molecular clock loops, biological function, potential targets, and small molecule modulators of circadian rhythm. The generation, maintenance, and regulation of circadian rhythms depend on the synergy of the circadian clock system, circadian input system, and circadian output system at the overall level. In particular, the circadian clock system is composed of the central circadian clock and the peripheral circadian clock. The apex of this system is the SCN master pacemaker in mammals. The periodic oscillation of circadian rhythm depends on the precise regulation of the circadian clock gene and the clock-controlled gene regulatory network, including transcriptional-translational feedback loops and the non-transcription mechanism of post-translational modification.

Extensive research has been performed on the relationship between circadian clock disorder and disease. Circadian clock genes knockout has confirmed that circadian misalignment is involved in metabolic syndrome, cardiovascular diseases, acute lung injury and inflammation, neurological diseases, immune diseases, cancer, mood disorders, sleep disorders, and ophthalmic diseases. As summarised in this article, circadian rhythms are important for human health, which suggests that the development of small molecules is imminent and could be used to treat circadian rhythm related diseases.

More importantly, a large number of small molecule modulators of circadian rhythm have been discovered, and most modulators have potential therapeutic effects on disease. In order to identify hits of the circadian clock, hundreds of thousands of compounds have been filtered by cell-based high-throughput circadian assays. The effectiveness of chemical biology approaches contributed to the discovery of the small molecule modulators of circadian rhythm. In recent years, with the emergence and popularisation of some new technologies, biophysical methods (such as differential scanning fluorimetry, differential scanning calorimetry, isothermal titration calorimetry, and surface plasmon resonance) and computer-aided drug design will help in the discovery of more modulators targeting clock proteins. It is believed that in the near future, small molecule modulators will
be a useful tool in the treatment of circadian rhythm related diseases.

Author contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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