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Development and Therapeutic Potential of Small-Molecule Modulators of Circadian Systems

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Abstract

Circadian timekeeping systems drive oscillatory gene expression to regulate essential cellular and physiological processes. When the systems are perturbed, pathological consequences ensue and disease risks rise. A growing number of small-molecule modulators have been reported to target circadian systems. Such small molecules, identified via high-throughput screening or derivatized from known scaffolds, have shown promise as drug candidates to improve biological timing and physiological outputs in disease models. In this review, we first briefly describe the circadian system, including the core oscillator and the cellular networks. Research progress on clock-modulating small molecules is presented, focusing on development strategies and biological efficacies. We highlight the therapeutic potential of small molecules in clock-related pathologies, including jet lag and shiftwork; various chronic diseases, particularly metabolic disease; and aging. Emerging opportunities to identify and exploit clock modulators as novel therapeutic agents are discussed.

Keywords

circadian clock; high-throughput screen; chemical derivatization; chronotherapy; clock-related diseases; aging

INTRODUCTION

In biology, as in society, timing is crucial for optimal performance. In response to the daily environmental changes of our planet, a biological timer called the circadian clock has evolved in virtually all organisms (1, 2). These clocks are characterized by both stable periodicity and robust entrainment. The periodicity is primarily synchronized by the physical rhythm of Earth's rotation. To align with the 24-h external cycle, the circadian system is endowed with exceedingly complex mechanisms to ensure period constancy. These include

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feedback gene regulation at all levels (ranging from transcriptional initiation to protein degradation), genetic redundancy (gene duplication), and coupling of intracellular and intercellular oscillators (3). By contrast, the clock can be readily entrained by cellular and external cues to adjust to environmental and (patho)physiological signals. Light is a dominant *zeitgeber* (time giver) for photosynthetic organisms and light-sensing organs in animals. Since the seminal study establishing peripheral oscillators in cell lines (4), a wide spectrum of external factors and endogenous metabolites capable of resetting the intrinsic clocks have been described (5–7). Analogous to a well-manufactured watch, the circadian system is both precise and adjustable.

In the natural environment, intact clock functions are required for optimal growth, predator avoidance, and protection against environmental challenges such as redox and irradiation (8–12). Studies conducted with laboratory animals and human subjects have shown acute and chronic adverse effects on health and fitness when circadian systems are misaligned or attenuated by genetic or environmental perturbation (13, 14). Although it is clearly important for species to survive and thrive, circadian timekeeping in the realm of drug development is mainly limited to chronotherapeutic applications, namely optimizing the schedule for established therapies to attain maximum therapeutic index (15). As our knowledge of the organization, regulation, and function of circadian systems grows, researchers are increasingly interested in developing small molecules to target the circadian system directly for therapeutic gains. In addition to jet lag, circadian dysregulation is implicated in various chronic diseases as well as age-related decline (16–19). The purpose of this review is to synthesize key research advances in small-molecule modulators of the mammalian clock and illustrate opportunities for further small-molecule identification and therapeutic developments.

MAMMALIAN CIRCADIAN SYSTEM

Hierarchical Organization and the Cell-Autonomous Oscillator

At the apex of the hierarchical mammalian circadian system (**Figure 1**) is the suprachiasmatic nucleus (SCN) master pacemaker, a pair of neuron clusters bilaterally located in the anterior of the hypothalamus (20). Blue light activates melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina, transmitting signals via the retinohypothalamic tract to the SCN (21). In postsynaptic SCN neurons, the *N*-methyl-D-aspartic acid (NMDA) glutamatergic and pituitary adenylate cyclase-activating polypeptide (PACAP) signaling pathways involving Ca^{2+} and cAMP (22) play predominant roles to induce immediate early genes, including the core clock gene *Period1* (*Per1*), and subsequently reset SCN cellular oscillators. Compared with peripheral tissues, SCN oscillators are tightly coupled (23). Several neurotransmitters have been implicated in intercellular SCN coupling, with the vasoactive intestinal peptide (VIP) being the most prominent (24). The SCN functions to synchronize tissue and cellular clocks throughout the body via neural and hormonal signals (25). For example, the SCN directly or indirectly projects to several cell bodies in the hypothalamus, regulating the rhythmic release of glucocorticoid hormone (26).

The operational unit of the mammalian circadian system is the cell-autonomous molecular oscillator (**Figure 2**), found in not only SCN pacemaker neurons but also virtually all other peripheral cells (4, 27). The molecular oscillator is characterized by interconnected negative feedback loops (1). In the core loop, each of the paralogous basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) transcription factors circadian locomotor output cycles kaput (CLOCK) and neuronal pas protein 2 (NPAS2) heterodimerizes with brain and muscle ARNT-like 1 (BMAL1) through their bHLH and PAS domains to activate expression of *Period (Per)* and *Cryptochrome (Cry)* genes via E-box promoter elements. The PER and CRY proteins in turn heterodimerize and translocate into the nucleus to inhibit transcriptional activities of CLOCK/BMAL1 and hence their own transcription. In the stabilization loop, two families of nuclear receptors, REV-ERB α/β and RAR-related orphan receptor $\alpha/\beta/\gamma$ (ROR $\alpha/\beta/\gamma$), compete for binding to shared consensus elements (RORE and RevDR2) on the promoter of *Bmal1* and other target genes throughout the genome (28). The negative feedback is mediated via robust circadian expression of the genes encoding REV-ERBs driven by CLOCK/BMAL1 (26). Other feedback loops, including a third transcriptional loop involving the proline and acidic amino acid-rich basic leucine zipper (PARzip) protein, D-box binding protein (DBP), and E4 promoter-binding protein 4 (E4BP4)/NFIL3 (29), as well as post-translational (NAD⁺-dependent SIRT1) mechanisms (30–32), also intersect with these two loops to confer further regulation (**Figure 2**).

In specific tissues, the master pacemaker and local oscillators are normally coordinated to drive systemic and tissue-autonomous gene expression programs (33). The clock-controlled genes (CCGs) vary greatly from tissue to tissue, with the overlap of CCGs between tissues at approximately 10% (34). However, when 12 different tissues were examined, 43% of all genes were found to oscillate in at least 1 tissue in mice (35). Such rhythmic gene expression programs dictated by the circadian clock in turn govern the daily oscillation of metabolism, physiology, and behavior.

Physiological Inputs and Molecular Mechanisms to Reset the Clock

As mentioned above, the circadian clock is receptive to a diverse array of input signals (5), providing the initial hint that novel small-molecule modulators can be identified to manipulate the clock. The underlying regulatory network is emerging, including transcription cofactors, epigenetic regulators, and enzymatic activities involved in various steps of gene expression (33). Diverse regulators with histone modification (e.g., acetylation, methylation, and monoubiquitination and their respective reverse modifications) and nucleosome remodeling activities are involved in circadian regulation (36, 37), particularly on the E-box during the transcriptional cycle (1, 38). On the RORE elements, several cofactor complexes have also been identified, including HDAC3 and NCOR1 (36). Recent studies have also shown that lineage-specific enhancers and transcription factors cooperate with the core circadian factors to govern circadian transcription in individual tissues (28, 39). For example, in mouse liver, DNA binding-deficient REV-ERB α remained tethered to promoter regions enriched for the consensus sites for the liver transcription factor HTF6 (28), suggesting an indirect strategy to recruit core circadian factors to cell type-specific regulatory regions. Posttranscriptional and posttranslational mechanisms also play an important regulatory role (40–42). In particular, degradation of PERs and CRYs is a key

mechanism governing circadian period length. PERs are phosphorylated by casein kinase 1 (CK1) prior to their proteasomal degradation by the F-box proteins β -TRCP1/2 (41). Likewise, CRY proteins are substrates of AMPK (43), and their subsequent degradation is coordinately mediated by paralogous F-box proteins FBXL3 and FBXL21 in an antagonistic manner (44–48). As exemplified by the CK1 inhibitors detailed below, these key regulators of circadian clocks are excellent targets for small-molecule development.

Circadian robustness, or amplitude, serves an important physiological function, as dampened amplitude is strongly correlated with chronic diseases and aging (16, 49, 50). Genetic redundancy, intercellular coupling, and stoichiometric balance between positive and negative factors are important to sustain robust cycling. Paralogous circadian components play overlapping roles to safeguard rhythmicity. For example, CLOCK and NPAS2 are paralogous transcription factors with redundant functions in both SCN and peripheral cells (51–53). For both SCN and peripheral tissues (3, 23), coupling of oscillators [e.g., by VIP in the SCN (54)] renders greater resistance to genetic perturbation and noise compared with single-cell oscillators (27, 55, 56). Within the oscillator, stoichiometric ratios between positive and negative limbs are important for high-amplitude gene oscillation (57). In particular, REV-ERBs and RORs of the stabilization loop play important roles in amplitude regulation. REV-ERBs show strong oscillation in both mRNA expression and protein degradation and compete with RORs to regulate occupancy and activity at target gene promoters (28, 54). REV-ERBs and RORs also engage a facilitated recruitment mechanism in which a rate-limiting step mediated by ROR/BMAL1 and transcription cofactors SRC-2/PBAF permits REV-ERB binding to open chromatin (58). Recruitment of REV-ERB by the activators ROR/BMAL1 likely facilitates transcriptional repression at the end of the activation phase, ensuring robust gene oscillation.

SMALL-MOLECULE MODULATORS OF CIRCADIAN SYSTEMS

Identification of Small-Molecule Clock Modulators

The circadian system is highly amenable to resetting signals, including environmental changes, intracellular mechanisms, and endogenous metabolites [e.g., flavin adenine dinucleotide (FAD), heme, and cholesterol act as natural ligands for CRYs, REV-ERBs, and RORs] (59, 60). Novel small molecules can modulate the circadian clock directly on the oscillator, via input pathways or through feedback mechanisms from output functions (**Figure 1**). If the entry point is too far removed from the core oscillator, the small molecules would likely exhibit pleiotropic effects separate from its circadian function. Therefore, we focus our discussion on chemical compounds targeting core clock components (**Table 1**), key regulators, and the oscillator (**Table 2**).

Small-molecule clock modulators (**Figure 3**) have been identified via chemical screening of libraries of varying sizes using different primary screening assays (17, 61). In phenotypic functional assays, stable cell lines express either luciferase from an exogenous clock gene promoter (62–65) or PER2::luciferase fusion from the endogenous *Per2* promoter (66–68), reporting circadian transcription and protein oscillation, respectively. Following cell synchronization, circadian bioluminescence rhythms are monitored continuously over several days (a so-called kinetic assay), and cardinal circadian parameters, including period,

phase, and amplitude, are quantified by data analysis tools. Such screens using cell-based assays have several advantages. They are not restrictive with regard to compound targets and mechanisms (**Figure 2**), and circadian effects are well defined for the reporter assay. The most significant challenge, however, is the elucidation of the direct targets for novel small molecules. The second cell-based screening strategy entails endpoint assays measuring specific transcription activities. In these assays, reporter cell lines are constructed to express luciferase under the control of the circadian gene promoter or promoter element (69, 70), and compounds are screened for endpoint transcriptional readout (up- or downregulated). Secondary assays (such as the kinetic assays mentioned above) can be carried out to validate their circadian effects. Finally, in vitro biochemical assays can identify compounds that specifically target certain clock components. For example, GSK4112 (71), the first synthetic agonist of REV-ERB, was identified via a fluorescence assay measuring the biochemical interaction between REV-ERB and an interacting peptide of its cofactor NCOR. Compared with cell-based assays, biochemical assays directly identify compounds for the known protein targets.

Primary hits are prioritized through secondary and counterscreen assays. Validated modulators suggest privileged scaffolds that can be subjected to chemical modification, aided by available knowledge of ligand structure, ligand-protein interaction, or both (59, 72). Improved pharmacokinetics and pharmacodynamics are typically required before in vivo applications, particularly those of therapeutic potential. In some instances, the scaffolds are available without the initial screening. For example, whereas IC261 and CKI-7 are CK1 inhibitors known to lengthen the clock period with moderate potency and minimal selectivity (**Table 2**), a focused medicinal chemistry effort led to a selective inhibitor of CK1 ϵ , PF-4800567, which confers >20-fold greater inhibition over that of CK1 δ (73–75).

Circadian Efficacies and Targets of Small-Molecule Modulators

Phenotypic screens have identified a diverse array of compounds showing effects on circadian period, phase, and/or amplitude (63, 65, 76–79). A major group of clock-modulating small molecules are the ones that alter, and especially lengthen, circadian period length. Consistent with genetic evidence showing a key role of PERs and CRYs in period regulation, compounds that affect PER/CRY levels were found in several chemical screens to alter circadian periods. For example, screens of known drugs have revealed numerous kinase inhibitors that potently lengthened circadian period (62, 63, 67). Although many drugs are known to target other kinases, they showed robust inhibition of CK1 kinase activity (63). It is possible that CK1 inhibition plays a predominant role in their period-lengthening activity. A central role of CK1 in determining circadian period is further illustrated by two studies that used pull-down or activity assays to identify new period-lengthening compounds that serve as CK1 inhibitors (64, 66). Moreover, KL001, a carbazole derivative, was identified in a cell-based screen to lengthen period and reduce amplitude, and pull-down experiments revealed CRY proteins as its target (65) (**Table 1**). Structural studies demonstrated that KL001 binding to the FAD-binding pocket of CRY interferes with its recognition by FBXL3 (80), thereby repressing CRY ubiquitination and proteasomal degradation. Compounds that target other cellular proteins or unknown targets with period-lengthening or -shortening effects have also been reported (61, 62).

Whereas period changes lead to long-term phase alteration, acute circadian phase resetting is primarily mediated via cAMP signaling, which leads to induction of the immediate early genes, including *Per1* (26, 66). Compounds that acutely advance or delay circadian phase act at different points of the pathway, including upstream kinases [e.g., extracellular signal-regulated kinase (ERK) and Ca²⁺/calmodulin-dependent protein kinase II (CamKII)], adenylyl cyclase, and phosphodiesterase (61). Furthermore, researchers used high-throughput screening to discover a group of sulfonamide compounds, opsinamides, that antagonize retinal binding to melanopsin and inhibit its photoreceptor functions in vitro and in vivo (81). *Per*-independent phase resetting pathways have also been described (82, 83), and modulatory compounds (e.g., SB432542) may function to reset circadian phase (**Table 2**).

Cell-based phenotypic screens have identified several clock amplitude-enhancing small molecules (CEMs) (61). The first group of synthetic CEMs potentiated cellular and tissue reporter rhythms (66), and the benzimidazole compound CEM3 in particular displays a unique capability to potentiate SCN reporter rhythms. A more recent screen identified the natural flavonoid Nobiletin (NOB) and its analog Tangeretin as CEMs (68). NOB increased mRNA and protein oscillatory amplitude in both fibroblast cells and liver, and filter-binding and functional assays established ROR receptors as the direct targets of NOB (68), reaffirming an important function of RORs in circadian amplitude regulation. Despite a strong NOB-ROR interaction, NOB-mediated activation of ROR target genes, including both core clock (e.g., *Bmal1*) and clock-controlled output genes, was generally moderate, likely because of the autoregulatory feedback loop within the core circadian clock (68).

Chemical modification of privileged scaffolds has led to various ligands targeting REV-ERBs and RORs with improved specificity and potency (**Table 1**) (59). For example, a tertiary amine series was reported to function as agonists of REV-ERB α (71, 76, 84, 85). However, for many of these modified REV-ERB and ROR ligands, their effects on the circadian clock are not well characterized. An inverse agonist of REV-ERBs, SR9011, was found to diminish circadian amplitude (76) despite its similar function with NOB in tipping the balance between REV-ERBs and RORs toward the latter. Together with the moderate induction of NOB in association with amplitude enhancement, this finding is in agreement with the limit-cycle nature of the clock, in which positive and negative limbs must balance and synergize to achieve greater amplitude over a sustained period.

THERAPEUTIC POTENTIAL OF SMALL-MOLECULE CLOCK MODULATORS

Circadian Clock as Drug Target

A majority of top-selling drugs in the United States target proteins that are encoded by oscillatory genes, and almost half of these drugs have short half-lives (<6 h) (35). Molecular studies have also provided strong evidence that xenobiotic metabolism is subjected to circadian regulation (86). These observations are part of our growing knowledge on circadian pharmacokinetics and pharmacodynamics, indicating an important role of circadian dosing time for drug efficacy (15, 77). However, clock-modulating small molecules can directly manipulate the circadian system to improve clock-regulated output

processes, alleviating disease symptoms and physiological decline (16–18, 61). Below, we begin our discussion of therapeutic potential with metabolic disease to highlight key lessons.

Distinct Clock-Modulating Small Molecules Against Metabolic Disorders

Circadian clocks and metabolism are interdependent (87, 88). In human subjects and laboratory mice, dysregulation of circadian timing by phase misalignment or genetic manipulation compromised energy homeostasis and increased the risk of metabolic disorders. Circadian clocks, both central and local, drive cyclic expression of metabolic genes in important metabolic tissues (32, 89). Mechanistic studies have unveiled molecular interaction between the clock and metabolic pathways. For example, it was recently found that the clock-NAD⁺ loop regulates the NAD⁺-dependent SIRT3 to modulate the acetylation state of key proteins involved in mitochondrial respiration (90). Together with previous studies on clock regulation by SIRT1 (30, 31), these observations underscore an important role of sirtuins in circadian metabolic control.

Several studies have reported distinct clock-modulating small molecules as potential therapeutics for metabolic disease. The first strategy targets circadian components with an established role in metabolic regulation, including REV-ERBs and RORs (36, 76, 91). Two synthetic agonists of REV-ERBs, SR9011 and SR9009, markedly improved energy homeostasis and acutely altered circadian behavior in diet-induced obese (DIO) mice (76). Consistent with the effect of these REV-ERB agonists, an inverse agonist of RORs, SR1555, was found to reduce weight and increase activity in DIO mice (92). Several other ROR α / γ ligands can also modulate hepatic metabolism (93, 94). Another clock modulator, the CRY stabilizer KL001, lengthened circadian period and repressed amplitude (65), and a bioavailable derivative was recently shown to improve glucose tolerance in DIO mice (72). In addition to functioning as the major repressor in the circadian core loop, CRYs are also known to modulate gluconeogenesis in the liver by directly interacting with the G α subunit of G protein-coupled receptors to modulate cAMP signaling (95).

A different strategy focused on enhancing the clock amplitude. Several lines of evidence suggest a relationship between dampened amplitude and metabolic disorder, including human studies showing that attenuated oscillation in insulin secretion rhythm correlates with exaggerated diabetes risk (96). *Clock*^{-19/19} mutant mice suffering a broad array of metabolic dysfunctions experienced a profound attenuation of circadian gene expression and feeding rhythm (97). Whereas ad libitum high-fat diet feeding repressed circadian gene oscillation in rodents, time-restricted feeding (TRF) during nighttime enhanced circadian amplitude and improved metabolic health (6, 98, 99). Consistent with previous studies (100), the clock-enhancing compound NOB showed considerable efficacy in mitigating weight gain and improving energy homeostasis in both DIO mice and *db/db* diabetic mice, corresponding to enhanced circadian functions (68, 101, 102). Together, these findings support the notion that clock enhancement by NOB contributes to metabolic improvement (103).

Several lessons have emerged from these studies. First, distinct strategies targeting either specific clock components or the overall circadian systemic feature (e.g., amplitude) can lead to metabolic improvements. The fact that these metabolic-promoting compounds

showed distinct effects on circadian phenotypes (e.g., amplitude) suggests a circadian metabolic network that is both extensive and flexible. Second, ligand activity and physiological output may not follow a simple correlation, and each compound should be evaluated experimentally. Several ROR inverse agonists and REV-ERB agonists (59, 78) improved energy metabolism in metabolic disease models (92, 104), whereas NOB showed similar efficacies as an ROR agonist. Underscoring the need for detailed understanding of compound-specific mechanisms, researchers recently found that three antagonists of ROR γ t employ varying mechanisms to modulate ROR γ t promoter binding and target gene expression (105). Finally, genetic manipulation may not be extrapolated to pharmacological effects, and vice versa. The three ROR γ t antagonists mentioned above displayed different mimicry of genetic ablation (105). In a classical example (106), both peroxisome proliferator-activated receptor γ (PPAR γ) haplodeficiency and the PPAR γ agonists thiazolidine-diones (TZDs) led to insulin sensitizing, which was later found to involve distinct regulatory mechanisms of hepatic and adipose tissue functions, respectively. ROR activation by NOB seemingly presents a paradox because ROR deficiency appears to retard body weight gain (107). Of note, global ROR deficiency, although shown to improve certain metabolic parameters, is deleterious to overall fitness, including immunity, motor function, development, and cardiovascular health (91).

Jet Lag and Shift Work

Transmeridian long-distance flights lead to jet lag, characterized by misalignment of internal rhythms, which remain locked to the origin, and the destination solar and social cycles. When the circadian misalignment becomes chronic, as in shift work, prevalent health problems can arise, including serious sleep and gastrointestinal disturbances, increased risk of several cancers (e.g., breast cancer), metabolic syndrome (being prone to weight gain), cardiovascular disease, and mood disorders (14, 88). The pineal hormone melatonin can facilitate sleep adjustment after jet lag, likely involving its phase-resetting activity in the SCN, where its receptors are expressed (108). However, the effects of melatonin are typically minor, and several side effects, such as headache and daytime drowsiness, are common. Because the primary circadian phenotype in jet lag and shift work is phase misalignment, small molecules with appropriate phase-resetting activities are candidates for drug development. In an in vivo screen to identify signaling pathways for jet lag, arginine vasopressin and its receptors (V1a and V1b receptors) were found to play an important role in the intercellular cross talk in mouse SCN (109), and the receptor double knockout mice displayed immediate phase shift under experimental jet lag conditions. Treatment of mice with an antagonist mix (OPC-21268 and SSR 149415 for V1a and V1b, respectively) shortened the phase shift time by half, suggesting a therapeutic strategy for jet lag and shift work.

Sleep Disorders

Sleep is an essential function governed by both circadian and homeostatic mechanisms (110). Human and mouse genetic studies have shed light on clock-associated sleep disorders, particularly familial advanced sleep phase syndrome (FASPS) and delayed sleep phase syndrome (111). FASPS is characterized by circadian period shortening, and mutations in both *PER2* (PER2 S662G) and *CSNK1D* (CK1 δ T44A) have been identified as an

underlying genetic cause (111). The CK1 δ -specific inhibitor PF-670462 induced behavioral rhythms in arrhythmic mice either subjected to constant light exposure or harboring the *Vipr2*^{-/-} mutation (75), suggesting a pharmacological agent could target CK1 δ to prolong the period in FASPS patients or animal models. In a recent forward genetic screen (112), two mouse mutants expressing mutant forms of the SIK3 kinase and the sodium channel NALCN displayed long sleep and shortened REM sleep phenotypes. Interestingly, both genes exhibit circadian oscillatory expression under normal conditions (113), consistent with an integrative control of sleep by both the circadian clock and the homeostatic pathway. Circadian modulators that influence their expression can be evaluated for sleep improvement.

Immune Disorders

Both innate and adaptive immune functions are regulated by the circadian clock (114). For example, the clock regulates the timing and duration of the expression of various proinflammatory cytokines such as IL-6, IL-17, tumor necrosis factor, and CXCL1. REV-ERB α plays a regulatory role in macrophage transcription (59), and mice deficient in REV-ERB α expression lost the gating of IL-6 induction following lipopolysaccharide (LPS) challenge, as evidenced by a highly elevated trough level at CT0 (85). ROR γ t is a master regulator for the development of the T helper cell 17 (Th17), an important immune cell type for autoimmune disorders, and ligands of REV-ERBs and RORs have shown beneficial effects on immune functions. The REV-ERB ligand GSK4112 repressed LPS induction of IL-6, CXCL11, CXCL6, and CCL2 in primary human macrophages (85). Digoxin and ursolic acid, as ROR γ inverse agonists, ameliorated autoimmune disorders, including arthritis and encephalomyelitis, via suppression of Th17 differentiation (115, 116). Several optimized ROR α / γ ligands attenuated expression of downstream cytokines and strongly alleviated autoimmune disease symptoms (117, 118), suggesting that targeting these clock components constitutes a valid strategy against inflammation and autoimmune diseases.

Mood Disorders

Circadian occurrence of psychiatric episodes is well documented, often most pronounced in the morning or around sunset (119, 120). During winter months, depressed seasonal affective disorder patients (121) show dampened rhythms in feeding, sleep, body temperature, and hormone release. Consistent with disrupted circadian rhythms often found in human mania (122), similar behaviors (123) were observed in clock-disrupted *Clock*^{-19/19} mice. Release of glucocorticoid hormones regulating stress response and mood balance is subject to circadian control. In a recent study, enhanced circadian amplitude of glucocorticoid rhythm, without increase of total glucocorticoid levels (124), showed anxiolytic effects, potentially due to the marked glucocorticoid reduction during the descending phase of its high-amplitude oscillation. In addition to environmental and behavioral therapies such as bright light therapy and sleep deprivation, small-molecule modulators of the clocks have also been used in mood disorders. Lithium, a mood stabilizer to treat bipolar disorder, showed complex effects on the circadian system, including enhancement of circadian reporter amplitude in both the SCN and the periphery (125). Another demonstrated molecular target of lithium is GSK-3 β , a broad-acting kinase previously shown to phosphorylate and stabilize REV-ERB α (126). Agonists of REV-ERBs

also displayed anxiolytic effects (127), consistent with genetic evidence showing enhanced anxiety in REV-ERB β knockout mice. Interestingly, acute administration of a REV-ERB antagonist, SR8278, to mouse ventral midbrain was also able to reduce anxiety and promote maniac-like behavior (128, 129). These findings are reminiscent of the aforementioned observations that both NOB, an agonist of ROR, and certain inverse agonists of RORs can improve energy metabolism, likely through distinct mechanisms. Given the strong correlation between circadian amplitude dampening and mood imbalance, the therapeutic potential of CEMs would be an interesting area for further investigation.

Aging

Aging, marked by progressive decline in metabolic, physiological, and behavioral functions, leads to widespread circadian changes such as sleep phase advance in humans. In both humans and rodents, the response to entraining cues is markedly weaker and slower with age (130), which correlates with age-related impediment in circadian synchronization and amplitude attenuation (19, 49). Clock-regulated physiological and behavioral processes, including SCN firing rate, hormone secretion (e.g., cortisol and melatonin), sleep architecture, and body temperature, display reduced amplitude with age (49, 131). At the molecular level, there is also broad dysregulation of clock gene expression (131), particularly in peripheral clocks (132, 133). In genetic studies, *Bmal1* knockout mice and α MUPA transgenic mice provide contrasting examples of premature aging and longevity, with strong correlations between aging effects and respective circadian functions (134, 135).

Dietary interventions, including caloric restriction (CR) and TRF, have shown antiaging effects (19). CR universally promotes longevity; similar to TRF, timed CR is accompanied by highly consolidated food intake within a few hours and enhances the amplitude of circadian metabolic rhythms (136, 137) and core clock gene oscillation (138). CR involves several pivotal metabolic regulators, including SIRT1, AMPK, AKT, and mTORC1, all of which functionally interact with the clock (32, 137, 139). For example, SIRT1 was found to interact with PGC-1 α to control *Clock* and *Bmal1* gene expression in the SCN, regulating CLOCK/BMAL1 target genes (140). Various SIRT1-activating small molecules such as resveratrol show longevity effects (141, 142). Pharmacological agents shown to promote or mimic clock-enhancing manipulations, such as CR, TRF, and exercise, are a rich venue for discovery of additional clock-targeting agents (136, 137, 143).

FUTURE DIRECTIONS AND CONCLUDING REMARKS

Developing New Modulators

Opportunities to identify new clock-modulating small molecules include chemical screens to target regulatory steps (e.g., intracellular translocation via high-content screening), new circadian regulators or targets (e.g., epigenetic factors and cofactors, enzymes regulating mRNA and protein levels, and SIK3), and new cell types (e.g., disease-associated or neuronal cells). Focused ligand modification can be applied to existing ligands or factors such as REV-ERBs and RORs with natural ligand-binding domains. For example, multiple structural studies have revealed molecular details of ROR isoforms (α , β , and γ) binding to their natural ligands, including cholesterol and stearic acid. Both ROR α and ROR γ are

widely expressed, and their ligand-binding domains are highly similar, with the former slightly smaller in volume (144). It is possible to develop isoform-specific ROR ligands and differentiate functional roles.

Structural studies have provided rich insight into the core oscillator and, importantly, novel regulatory surfaces involved in ligand binding or protein-protein interactions (PPIs) (**Figure 3**). Mammalian CRY proteins retain low-affinity FAD binding in the photolyase homology region (145), and KL001 competes with the FBXL3 C terminus for binding at the FAD pocket (80), exemplifying a structural motif for compound development. In the CLOCK-BMAL1 crystal structure, the PAS-B domains of these molecules engage an asymmetrical interaction between the BMAL1 β -sheet and CLOCK α -helix (146). This interaction creates a fairly large internal hydrophobic surface (approximately 700 Å²). More recently, a detailed crystal structure study revealed ubiquitous internal pockets in the PAS-A and PAS-B domains of bHLH-PAS proteins, including CLOCK and BMAL1 (147). These pockets, varying in volume, can be targeted by functional ligands. For example, a buried pocket in the PAS-B domain of hypoxia-inducible factor 2 α (HIF2 α) is accessible by synthetic ligands to modulate HIF2 α /ARNT interaction (148). PAS domains first evolved in bacteria and plant species for ligand binding. The structural studies described above revealed a remnant ligand-binding ability in mammalian PAS domains as a promising target for drug discovery (149).

Although the presence of cardinal or covert ligand-binding cavities or clefts on core clock proteins allows in silico screening or chemical optimization of known ligands, the discovery of GSK4112 exemplifies another drug discovery strategy targeting PPIs (71, 150). Compared with biologics, small-molecule modulators of PPIs pose greater challenges, especially in terms of tertiary sequences and allosteric sites. Finding the interaction hot spots that confer disproportionally high binding energy is important for small-molecule perturbation (151). In the PER-CRY crystal structure, several regulatory interaction surfaces can be distinguished from the extensive interaction throughout the CRY-binding domain of PER (152), including interaction near the CRY secondary pocket that binds to CLOCK/BMAL1 and a competitive binding with FBXL3 for access to the extreme C-terminal surface on CRY. As PPIs are important for both clock protein activities and core complex formation on chromatin, advanced structural understanding will offer a rich source of drug discovery targets.

Exploring Therapeutic Potential

The list of clock-associated diseases continues to grow. Cardiovascular functions, including blood pressure, heart rate, cardiac contractility, and metabolism, exhibit robust circadian oscillation (153), and several key regulators, including Hsd3b6 and KLF15, are subjected to direct clock control (154, 155). There is also growing evidence for a relationship between clock dysregulation and tumorigenesis (156, 157). For example, a chronic jet-lag paradigm induced several cancer types, including hepatocellular carcinoma, and metabolic dysregulation partly mediated by the constitutive androstane receptor promoted progression of tumorigenesis from the initial fatty liver phenotype (158). Neurodegenerative diseases (16, 159) have been shown to correlate with dampened circadian amplitude and may represent new venues for CEMs. Finally, for the existing chronotherapies, small-molecule

modulators can be coadministered to manipulate circadian timing to better align with target expression or reduce drug metabolism.

As discussed above, detailed mechanistic studies are important for the development and eventual application of therapeutically active small molecules (17, 18). For compounds isolated via phenotypic screening, it is invaluable to identify direct molecular targets. For compounds identified for specific targets, their circadian effects and cellular effectors should be characterized. Such mechanistic knowledge will have predictive values toward specific applications of the small molecules in certain disease areas. Chemical optimization of lead compounds is typically required to minimize polypharmacology and improve bioavailability (63, 79, 117) for in vivo animal and human studies. With the growing number of new clock modulators showing favorable pharmacokinetics and efficacies in mouse disease models, human trials will be the exciting goal in the near future to evaluate the therapeutic potential of circadian manipulation.

In conclusion, small-molecule modulators target clock components or clock-associated cellular pathways to modulate circadian characteristics and output functions. Target identification and mechanistic studies of new small-molecule modulators will unveil a key regulatory nexus in the circadian network and facilitate their therapeutic applications, alone or in combination, for clock-related diseases.

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Figure 1.

Mammalian circadian system. The circadian system consists of input pathways, the clock, and output functions. Light is a major input signal for the circadian system and activates the master pacemaker SCN. The SCN coordinates the cellular oscillator network throughout the body via neuronal and hormonal signals. The clock drives expression of clock-controlled genes in a tissue-specific manner, thereby regulating tissue physiologies and systemic functions. Many output functions such as activity, metabolism, and temperature also reciprocally regulate the clock. Small-molecule modulators can alter the input pathway, oscillator, and output functions with feedback activities. Abbreviation: SCN, suprachiasmatic nucleus.

Figure 2.

Core and regulatory components of the circadian oscillator and representative small-molecule modulators. The circadian clock oscillator is composed of a network of transcriptional-translational feedback loops, including the core loop (BMAL1/CLOCK/ NPAS2 and PERs/CRYs), the stabilization loop (BMAL1/CLOCK, REV-ERBs, and RORs), and the auxiliary loop (DBP, E4BP4, REV-ERBs, and RORs). Various protein regulators and ligands have been determined to regulate core clock components and circadian functions. Modified from Reference 50 under Creative Commons Attribution license. Abbreviations: BMAL1, brain and muscle ARNT-like 1; CLOCK, circadian locomotor output cycles kaput; CRY, cryptochrome; DBP, D-box binding protein; E4BP4, E4 promoter-binding protein 4; NPAS2, neuronal PAS protein 2; PER, period; ROR, RAR-related orphan receptor.

Figure 3.

Development of clock-modulating small molecules. Two general strategies can be deployed to identify clock-modulating small molecules. The modulators can be identified via kinetic phenotypic screens measuring circadian parameters or endpoint activity screens measuring transactivation. Elucidation of the protein target is important for mechanistic and functional studies. Small molecules can also be developed via biochemical assays measuring protein-protein interaction, known ligands or scaffolds, or structure-based design. Circadian activities and mechanistic pathways can be characterized for prioritized hits. Depending on pharmacokinetic profile, target, and circadian function, small molecules can be applied to in vivo models of clock-associated diseases and aging.

Table 1Representative small-molecule modulators of core clock proteins^a

Clock proteins	Modulators	Circadian or target activities	Physiological effects	References
CRY1/2	KL001 and CRY-stabilizing derivative	Stabilize CRY Lengthen period Reduce amplitude	Improve glucose tolerance in obese mice	65, 72
	GO044 GO200 GO211	Variable effects on CRY Shorten period	NA	160
	2-ethoxypropanoic acid KS15	Inhibit CRY Activate E-box transcription Shorten period Reduce amplitude	Inhibit breast cancer cell growth	70, 161
REV-ERBs	GSK4112	Enhance REV-ERB and NCOR peptide interaction	Inhibit gluconeogenesis and inflammatory response in primary cells	59,71,85
	SR9009 SR9011	Derived from GSK4112 Selective agonists for REV-ERB Alter circadian behavior and gene expression	Improve glucose homeostasis in obese mice Promote wakefulness Reduce anxiety	76,127
	GSK2945 GSK0999 GSK5072 GSK2667	Derived from GSK4112 Selective agonists for REV-ERB Reduce Bmal1 expression	Inhibit inflammatory response	79
	SR8278	GSK4112-derived antagonist	Reduce glucagon secretion from mouse alpha cells Reduce anxiety and promote maniac-like behavior	59, 128, 162
	ARN5187	REV-ERB β agonist	Cytotoxic against cancer cells	163
RORs	SR1078	ROR agonist	Induce apoptosis Inhibit hepatoma cell growth Improve autistic behavior in mice	164, 165
	Nobiletin	Agonist Enhance amplitude and lengthen period	Improve metabolic homeostasis in obese/diabetic mice Show broad efficacies against tumors, inflammation, and cardiovascular disease	68,100–102
	Neoruscogenin	Agonist promoting ROR interaction with NCOA2/TIF2 Activate <i>Bmal1</i> expression	Activate hepatic expression of ROR metabolic target genes such as <i>Cyp7b1</i> , <i>G6Pase</i> , <i>Lpin2</i> , and <i>Angptl4</i>	166
	Compound 1a	ROR γ agonist	Promote Th17 cell differentiation	167
	T0901317	Inverse agonists of RORs and agonist of LXR	Diverse biological activities regulated by LXR	118
	SR1001	Derived from T0901317 with high inverse agonist activity and selectivity for ROR α and ROR γ	Inhibit Th17 cell differentiation and autoimmunity	117
	SR2211 SR1555 Digoxin Ursolic acid ML209	ROR γ inverse agonist	Inhibit Th17 cell differentiation	59
	SR3335	ROR α inverse agonist	Reduce blood glucose in obese mice	93

Abbreviations: BMAL1, brain and muscle ARNT-like 1; CRY, cryptochrome; LXR, liver X receptor; NA, not applicable; ROR, RAR-related orphan receptor.

^aFor additional related compounds and references, please refer to a recent review (59).

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Table 2Representative small-molecule modulators with other or unknown targets^a

Small molecules	Target activity	Circadian phenotype	Physiological effects	References
Opsinamides	Melanopsin inhibitors	Suppress light input to the SCN	Generally suppress nonvisual photoreponse	81
SB432542	ALK5 inhibitor	Attenuate phase delay	Inhibit TGF- β signaling	82
AICAR, metformin	AMPK activator	Lengthen period Reduce amplitude	AICAR: exercise mimetic Metformin: antidiabetic	43, 168
CKI-7, IC261, D4476, PF-670462, longdaysin, LH846, Compound 1-3, and others	CK1 inhibitor	Lengthen period	CK1 is broadly involved in various pathophysiologies, including familial sleep and mood disorders	75, 78
PF-4800567	Selective CK1 α inhibitor	Blocks PER2 degradation but shows minimal effects on circadian period	Increases sensitivity to psychostimulants and opioids	73
DRB, DMAT	CK2 inhibitor	Lengthen period	Antineoplasia	78, 169
Lithium	GSK3 β inhibitor	Lengthen period Enhance amplitude	Mood disorders	125, 170
Chir99021, 1-azakenpaullone, indirubin	GSK3 β inhibitor	Shorten period	Improve glucose metabolism, anti-inflammatory and antiangiogenesis	62
L-Methyl selenocysteine	Enhance <i>Bmal1</i> transcription	Induction of BMAL1 protein level and activity	Improved survival of cyclophosphamide-sensitive <i>Clock</i> mutant mice	69
Resveratrol	Sirt1 activator	Modulate physiological rhythms and clock gene expression	Broad physiological and antiaging efficacies	140
SRT2183, SRT1720, SRTCD1023, SRTCL1015	Sirt1 activator	Reduce circadian expression Lengthen period Reduce amplitude	Improve circadian clock gene expression Reduce inflammation in chronic obstructive pulmonary disease	143, 171
OPC-21268, SSR 149415	Antagonists for arginine vasopressin receptors V1a and V1b, respectively	Accelerate entrainment to jet lag	Accelerate reentrainment after jet lag and shift work	109
Compound 5, a benzodiazepine derivative	GABA receptor agonist Acts on an unknown target	Lengthens period in both SCN and peripheral clocks	NA	66
Compound 10/CEM3	Unknown	Enhance amplitude and shorten period in both SCN and peripheral clocks	NA	61,66

Abbreviations: AMPK, AMP-activated protein kinase; CEM, clock-enhancing small molecule; CK1, casein kinase 1; CK2, casein kinase 2; GABA, γ -aminobutyric acid; GSK3 β , glycogen synthase kinase 3 β ; NA, not applicable; PER, period; SCN, suprachiasmatic nucleus; TGF- β , transforming growth factor- β .

^aFor additional related compounds and references, please refer to a recent review (78).