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Molecular targets for small-molecule modulators of circadian clocks

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Abstract

Background—Circadian clocks are endogenous timing systems that regulate various aspects of mammalian metabolism, physiology and behavior. Traditional chronotherapy refers to the administration of drugs in a defined circadian time window to achieve optimal pharmacokinetic and therapeutic efficacies. In recent years, substantial efforts have been dedicated to developing novel small-molecule modulators of circadian clocks.

Methods—Here, we review the recent progress in the identification of molecular targets of small-molecule clock modulators and their efficacies in clock-related disorders. Specifically, we examine the clock components and regulatory factors as possible molecular targets of small molecules, and we review several key clock-related disorders as promising venues for testing the preventive/therapeutic efficacies of these small molecules. Finally, we also discuss circadian regulation of drug metabolism.

Results—Small molecules can modulate the period, phase and/or amplitude of the circadian cycle. Core clock proteins, nuclear hormone receptors, and clock-related kinases and other epigenetic regulators are promising molecular targets for small molecules. Through these targets small molecules exert protective effects against clock-related disorders including the metabolic syndrome, immune disorders, sleep disorders and cancer. Small molecules can also modulate circadian drug metabolism and response to existing therapeutics.

Conclusion—Small-molecule clock modulators target clock components or diverse cellular pathways that functionally impinge upon the clock. Target identification of new small-molecule modulators will deepen our understanding of key regulatory nodes in the circadian network. Studies of clock modulators will facilitate their therapeutic applications, alone or in combination, for clock-related diseases.

Keywords

Circadian clock; small-molecule modulator; molecular target; nuclear receptor; metabolic syndrome; drug metabolism

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

Introduction

Circadian clocks are endogenous timing systems that drive daily rhythms in a variety of metabolic, physiological and behavioral processes [1–3]. In mammals, the cell-autonomous molecular oscillator is the basic unit of the clock system, comprised of transcriptional-translational feedback loops including a core loop and a secondary stabilization loop (Fig. 1) [2, 4]. Briefly, heterodimers of transcription factors CLOCK/BMAL1 and NPAS2/BMAL1 bind to E-box DNA elements on the promoters of *Period1/2* (*Per1/2*) and *Cryptochrome1/2* (*Cry1/2*) genes and activate their expression. PER1/2 and CRY1/2 proteins in turn form complexes and inhibit CLOCK/BMAL1 and NPAS2/BMAL1 activities and thus their own expression, completing one cycle and priming for the next. In the stabilization loop, CLOCK/BMAL1 and NPAS2/BMAL1 drive the expression of genes encoding the nuclear receptor REV-ERBs and RORs. REV-ERBs and RORs respectively repress and activate transcription of *Bmal1* and several other clock genes, through direct binding to the RORE elements on their promoters. In addition, the transcriptional-translational feedback loops are subjected to regulation at various levels including post-translational modifications such as phosphorylation, ubiquitination and acetylation [5, 6]. Post-translational modifications of clock proteins provide the necessary plasticity to fine-tune the clockwork and respond to environmental changes. These cell-autonomous circadian oscillators regulate oscillatory expression of clock-controlled genes (CCGs) in a tissue-specific manner, a process coordinated by the SCN (suprachiasmatic nuclei) master pacemaker in the hypothalamus.

Importantly, circadian misalignment in humans has been shown to contribute to sleep disorders, metabolic syndrome, cancer and other pathologies [7–9]. For example, human subjects who lived on an enforced 28-hr rhythm for 10 days were found to exhibit impaired glucose tolerance and hyperinsulinemia [8]. These experimental data are well aligned with epidemiological evidence showing increased disease risk in shift workers [10]. Furthermore, circadian mutant mice with genetic disruption in clock genes displayed a variety of physiological deficits [11–13]. For example, $Bmal1^{-/-}$, $Cry1/2^{-/-}$ and $Clock^{\Delta 19/\Delta 19}$ mutant mice harboring disruptive mutations in the core loop have been reported to exhibit a spectrum of metabolic disorders including obesity, hyperlipidemia, hepatic steatosis and hyperglycemia [14–18]. These findingstogether indicate thata robust circadian clock is necessary for health .

The therapeutic relevance of circadian clocks has traditionally been limited to the domain of chronotherapy, which entails selecting the best time during the daily cycle for drug administration to achieve the optimal therapeutic index and pharmacokinetic profile [19]. For example, standard weekly treatments of a broad-spectrum anti-cancer drug gemcitabine often lead to hematologic toxicity, which could be significantly diminished by treating patients at 9AM as opposed to 3PM [20]. More recently, increasing attention has turned to the discovery and exploitation of small molecule modulators which can manipulate the circadian timing to prevent or alleviate clock-related pathological deficits [21]. Several endogenous molecules such as fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin, heme, melatonin and cAMP are known to modulate the circadian clock [22], providing proof-of-principle for the identification of novel natural or synthetic small-molecule modulators. Indeed, a number of small molecule modulators have been

successfully discovered through circadian phenotype-based screening or target-based design [21, 23, 24]. In this review, we will enumerate modulators of key components or regulatory factors of the mammalian circadian clock and review their efficacies in manipulating circadian rhythms and ameliorating clock-related diseases.

Targets of small-molecule clock modulators

Below we describe small molecule modulators which directly interact with core clock proteins or key regulatory factors of the circadian clock.

1. Core clock proteins

The core clock proteins include components of the transcriptional-translational feedback loops, such as BMAL1, NPAS2, CLOCK, PER1/2, CRY1/2, REV-ERBs and RORs. Synthetic or natural ligands for CRY1/2, REV-ERBs and RORs have been reported (Table 1 and Fig. 1).

1.1 CRY1/2 proteins—CRY1/2 proteins function as thenegative regulator sof the core circadian feedback loop. In an unbiased cell-based circadian phenotypic screen, a carbazole compound KL001 was shown to specifically interact with CRY proteins. KL001 stabilized CRY1/2 by preventing ubiquitin-dependent CRY degradation, and consequently lengthened the circadian period and reduced the circadian amplitude [25]. Among KL001 derivatives directly targeting CRY, KL044 and GO214 also lengthened the circadian period, whereas GO044, GO200 and GO211 showed the opposite effects [26, 27]. In another study, compound 15, a derivative of 2-ethoxypropanoic acid, was reported to bind to the C-terminal tail of CRY1/2 and inhibit their repressive function, resulting in slight shortening of the circadian period accompanied by significant reduction of the circadian amplitude [28]. These studies describe CRY-interacting small molecules showing distinct biochemical mechanisms and functional outcome, yet interestingly dampening the rhythms in both cases. Further studies are required to fully understand the mechanisms of these compounds withinthe molecular oscillator .

1.2 REV-ERB receptors—The REV-ERB nuclear receptors (consisting of REV-ERBα and REV-ERBβ) repress *Bmal1* transcription through binding to the promoter RORE elements [13, 29]. Synthetic REV-ERB agonists SR9009 and SR9011, optimized from the lead compound GSK4112, reduced the circadian amplitude in SCN slices from *Per2::Luciferase* reporter mice (PER2::LUC) without affecting the circadian period [30, 31]. SR9011 also disrupted wheel-running activity immediately after single administration [30]. Moreover, several other derivative compounds, including GSK2945, GSK0999, GSK5072 and GSK267, activated REV-ERBα and diminished the oscillation of the Bmal1-Luciferease reporter in U2OS cells. Paradoxically however, a more recent study showed that SR9009 and SR9011 displayed greater selectivity for LXRα than REV-ERBα [31, 32]. Further experiments are required to resolve this discrepancy. In addition to agonists, structure-activity analysis focusing on the GSK4112 tertiary amine scaffold also identified an REV-ERB antagonist SR8278 displaying inhibitory activities in a luciferase-based cellular assay [33]. These findings reveal that tertiary amine derivatives are rich sources for the identification of REV-ERB agonists or antagonists.

1.3 ROR receptors—The retinoic acid receptor-related orphan receptors (RORs; consisting of RORα, RORβ and RORγ) enhance *Bmal1* transcription through direct competition with REV-ERBs for binding to RORE elements [34, 35]. A number of RORs ligands have been identified via circadian phenotype-based screening or target-based design [31, 36–38]. For example, T0901317 and SR1001 act as inverse agonists for RORα and RORγ [36]. Among isoform-specific ligands, SR2211, SR1555, digoxin, ursolic acid and ML209 function as RORγ inverse agonists [31], whereas SR3335 is a RORα inverse agonist [38]. Ligands activating RORs are also emerging in recent literature; for example, SR1078 was shown to activate both RORα and RORγ [37]. How these RORs agonists or inverse agonists modulate circadian clocks remains to be characterized.

2. Kinases

Phosphorylation of core clock proteins is a prevalent regulatory mechanism for the circadian clock [39]. Therefore, ligands targeting cognate kinases can profoundly alter circadian characteristics(Table 2 and Fig. 1).

2.1 Casein kinasel (CKI)—In mammals, several genes encode CKI isoforms (α, β, γ1, γ2, γ3, δ and ε), among which α, δ and ε have been implicated in the regulation of BMAL1, PERs, and CRYs [39]. CK1δ/ε inhibitors IC261, CKI-7 and D4476 previously showed period lengthening effects in cultured cells [40–42]. Moreover, PF-670642 and LH846, as potent CKIδ inhibitors, also significantly lengthened molecular oscillations in U2OS cells and mouse tissue explants *in vitro* and circadian locomotor activity rhythms *in vivo* [43, 44]. Interestingly, PF-4800567 strongly inhibited CKIε yet displayed only a minimal effect on the circadian clock [45]. In our previous study, compounds 1–3 showed robust CKIε inhibitory activities and markedly lengthened the period in cultured cells and mouse tissue explants; it is possible that these compounds also inhibit CK1δ [46]. These studies together indicate that among the several CKI isoforms implicated in clock regulation, CKIδ, and perhaps CKIε to a lesser degree, may bepromising targetsfor pharmacological modulation of circadian clocks.

2.2 Casein kinase 2 (CK2)—Previous biochemical studies revealed that CK2 regulates circadian clocks by phosphorylating BMAL1 and PER proteins [39]. Downregulating CK2 expression by RNAi has been shown to lengthen the circadian period [47]. Pharmacological inhibition of CK2 by DRB and DMAT also resulted in period lengthening [40, 47], indicating a strong correlation between clock lengthening and CK2 inhibition .

2.3 Glycogen synthase kinase3 (GSK3)—Among the two mammalian GSK3 isoforms (GSK3α and GSK3β), GSK3β has been reported to regulate the circadian clock by phosphorylating CLOCK, PER, REV-ERBα and CRY proteins [39]. Lithium, a weak GSK3β inhibitor, lengthened the circadian period in cultured cells and *in vivo*. However, GSK3β-selective inhibitors including Chir99021, 1-azakenpaullone and indirubin shortened the circadian period [40]. Because lithium broadly inhibits inositol monophosphatase, GSK3 and other phosphomonoesterases [48], the period-lengthening effect of lithium might be mediated by target protein(s) other than GSK3β .

2.4 5' AMP-activated protein kinase (AMPK)—AMPK, a heterotrimeric protein kinase consisting of a catalytic (α) subunit and two regulatory (β , γ) subunits, has been shown to phosphorylate and destabilize PER and CRY proteins [39, 49]. AICAR, an AMPK activating metabolite, reduced the oscillatory amplitude and lengthened the period [49]. Furthermore, metformin, a broadly used diabetes drug and an AMPK activator, potentiated the degradation of PER2 and altered the expression of clock genes in mouse muscle, liver and heart [50, 51]. In the diabetic *db/db* mice, metformin also restored the expression pattern of clock genes in white fat via the AMPK-Nampt-Sirt1 pathway [52]. The precise role of AMPK in the clock, via PER or CRY phosphorylation or another target pathway, requiresfurther study .

2.5 Cyclin-dependent kinases (CDKs)—Recent studies revealed that CDK5, one of 11 CDK isoforms, regulates E-box dependent transcriptional activation by directly phosphorylating CLOCK [53]. Indirubin-3'-oxime and kenpaullone, inhibitors of both CDK and GSK3, were found to shorten the circadian period [40]. In contrast, CDK inhibitors with varying target specificity such as roscovitine (for CDK1, CDK2 and CDK5) and puralanol A (for CDK2, CDK4 and CDK5) significantly lengthened the circadian period [40]. NU6102, a selective inhibitor of CDK1 and CDK2, was incapable of lengthening the period [40]. Therefore, additional studies are necessary to clarify the complex roles of CDK inhibitors and determine relative contributions of CDK isoforms tocircadian regulation .

2.6 other kinases—A number of other kinase inhibitors, including the p38 inhibitors SB203580 and PD169316, the JNK inhibitor SP600125, and the CLK inhibitor TG003, were identified as period-lengthening small molecules in NIH-3T3 and U2OS cells [42]. However, these compounds also seemed to inhibit CK1δ/ε other than their primary targets [42] and the exact role of p38, JNK and CLK, if any, in period lengthening is unclear. SB432542, an inhibitor of activinreceptor -like kinase (ALK), was found to attenuate phase delays through acute induction of the circadian transcriptional regulator DEC1 [54]. Furthermore, a kinase inhibitor screen established inhibitors against CK1δ/ε (IC261), CK2 (DMAT and DRB) and p38 (SB202190) as period-lengthening compounds, and also revealed similar effects by those of PI3-kinase (LY294002) and Akt (BML-297) [55]. In general, however, the prevalent promiscuity of kinase inhibitors presents a significant challenge to pinpoint the causal relationship regarding circadian clockeffects .

3. Nuclear receptorsother than REV -ERBs and RORs

A growing number ofnuclear receptors have been reportedto play important regulatory roles in circadian clocks. In addition to REV-ERBs and RORs acting in the stabilization loop of the core oscillator, glucocorticoid receptor (GR), estrogen receptors (ERs), peroxisome proliferator activated receptors (PPARs), retinoic acid receptors (RARs) and retinoid X receptors (RXRs) have also been shown to regulate the clock [56], therefore implicating their ligands as clock modulators (Table 2 and Fig. 1). An in-depth understanding of the roles of nuclear receptor ligands in the clock system will enhance their therapeutic potential for clock-related disorders.

3.1 Estrogen receptors(ER s)—In tissue explant cultures from PER2::LUC reporter mice, 17β-estradiol, an ERα/β agonist, shortened the period of rhythmic PER2::LUC expression in the uterus but not in the SCN, possibly through regulation of the *Per2* promoter [57]. However, PPT, a specific ERα agonist, lengthened the circadian period [42]. Since PPT also acts on CK1δ/ε [42], determining the exact function of ERα/β on circadian period requires improved pharmacological and genetic tools.

3.2 Peroxisome proliferator activated receptors (PPARs)—PPARα regulates the expression of *Bmal1* and *Rev-erbα* via binding to the PPRE promoter elements [58]. Fenofibrate, a synthetic PPARα agonist, was shown to induce circadian clock gene expression *in vitro* and to up-regulate hepatic *Bmal1 in vivo* [58]. In addition, PPARγ also directly regulates the expression of *Bmal1* in the vascular system and thus participates in the circadian regulation of blood pressure and heart rate [59]. In accordance, administration of the PPARγ agonist rosiglitazone induced aortic expression of *Bmal1* [59]. However, the natural PPARγ ligand 15-deoxy-Delta12, 14-prostaglandin J2 entrained cellular circadian clocks in a PPARγ-independent manner, suggesting an off-target effect[60].

3.3 Retinoic acid receptors (RARs) and retinoid X receptors (RXRs)—In the vasculature, RARα and RXRα were shown to negatively regulate the BMAL1/CLOCK-mediated transcriptional activation of clock gene expression through direct interaction with CLOCK [61]. All-trans retinoic acid (ATRA), a ligand for both RAR and RXR, can phase-shift *Per2* mRNA rhythm in serum-induced smooth muscle cells and *in vivo* [61]. In addition, 9-cis retinoic acid and 13-cis retinoic acid, ligands for both RAR and RXR, are also able to entrain circadian rhythms *in vitro* and *in vivo* [61]. These studies collectively suggest that ligands for both RAR and RXR modulate circadian clocks by altering the interaction between RAR/RXR and CLOCK. This mode of action contrasts with that of other nuclear receptor ligands which function primarily by altering binding of nuclear receptors to cognate promoterelements of clock genes.

4. Silent Information Regulator 1 (SIRT1)

SIRT1 is a sirtuin family deacetylase, directly recruited to the CLOCK/BMAL1 chromatin complex to suppress circadian transcription [62, 63]. Apart from histones, BMAL1 has also been identified as a SIRT1 substrate. Previously, several SIRT1 activators were characterized. For example, SRT2183 and SRT1720 decrease d circadian gene expression, and SRTCD1023 and SRTCL1015 also reduced the amplitude and lengthened the period of circadian rhythms. In contrast, SRTCE1022 did not activate SIRT1 in biochemical or cell-based assays, and showed no effects on circadian amplitude and period [64]. These results demonstrate a negative role of SIRT1 incircadian gene expression.

5. DNA topoisomerases(TOPs)

Topoisomerases, including type I (TOPI) and type II (TOPII), are key enzymes for DNA replication, transcription, recombination and chromatin remodeling [65]. In a recent study, it was shown that TOPI suppressed *Bmal1* transcription via binding to an intermediate region between the two RORE elements in the promoter. Camptothecin, a TOPI inhibitor, enhanced *Bmal1* transcription and lengthened the circadian period [65]. In addition, the TOPI inhibitor

harmine also lengthened the circadian period by enhancing the trans-activating function of RORα *in vitro* and *in vivo* [66, 67]. In comparison, three TOPII inhibitors including etoposide, mitoxantrone and amsacrine have been shown to shorten the circadian period [40], suggesting differential functions of TOPI and TOPII in the circadian clock.

Therapeutic potentials of small-molecule clock modulators

In this section, we firstdescribe *in vitro* and mouse studies demonstrating the therapeutic potential of clock-modulating small molecules in several clock-related disorders. We will then consider their possible application in traditional chronotherapy where drug metabolism is a predominant factordetermining efficacy and toxicity .

1. Clock-related disorders

1.1 Metabolic syndrome—To combat the current global epidemic of metabolic disorders, circadian research has demonstrated novel interventions via directly manipulating circadian rhythms [30, 68]. For example, nighttime-only intake of high-fat diet (HFD) was shown to protect mice against metabolic disease and improve their motor coordination compared with unrestricted feeding [68]. It has been hypothesized that restricting caloric intake during the rest phase when energy expenditure is suppressed prevents storage of excess nutrients and thus reduces body weight gain. Interestingly, the oscillatory amplitude of clock and metabolic gene expression was significantly enhanced in mice fed with time-restricted intake of HFD, raising the possibility that small molecules inducing amplitude enhancement may also protect against metabolic diseases. On the other hand, the REV-ERB agonist SR9009 has been shown to elicit beneficial metabolic effects in diet-induced obese mice, yet acutely repressed circadian behavior and oscillatory amplitude [30]. These data on the one hand suggest that targeting the clock machinery may be efficacious against metabolic disorders, on the other also reveal mechanistic complexity, particularly regarding the functional relationship between clock amplitude and metabolic output.

1.2 Immune disorders—The clock is known to regulate the expression of various pro-inflammatory cytokines such as IL-6, TNF, IL-17 and CXCL1 [69]. In a recent study, the REV-ERB ligand GSK4112 was found to inhibit expression of IL6, CXCL11, CXCL6 and CCL2 in primary human macrophages with LPS stimulation [70]. In addition, digoxin and ursolic acid, as RORγ inverse agonists, ameliorated autoimmune disorders including arthritis and encephalomyelitis via suppression of Th17 differentiation [71–74]. These studies indicate that targeting clock componentsmay constitute a valid strategy against inflammation and autoimmune diseases.

1.3 Sleepdisorders—In addition to acute sleep disturbance such as jetlag, familial advanced sleep phase syndrome (FASPS) or delayed sleep phase syndrome (DSPS) are among the circadian sleep disorders with a genetic basis [75]. FASPS is characterized by circadian period shortening due to a T44A mutation in the CK1δ protein [75]. Interestingly, the CK1δ inhibitor PF-670462 has been shown to induce behavioral rhythms in mice rendered arrhythmic by constant light exposure or the *Vipr2*$^{-/-}$ mutation [43], indicating pharmacological targeting of CK1δ constitutes a therapeutic modulation of perturbed circadian behavior. It will be interesting to investigate whether PF-670462 can prolong the

period in FASPS patients or model animals and alleviate the sleep symptom. Likewise, other small molecule modulators capable of altering circadian period and/or phase may be efficacious for the treatment of circadian sleep disorders.

1.4 Cancer—Circadian clocks have been postulated to serve tumor suppressor functions. Epidemiological and genetic studies have provided evidence that circadan disruption increases cancer risk and clock gene expression is altered in various tumors [9, 76]. For example, several studies revealed that the incidence of breast cancer is higher among women who work night shifts [77]. It has also been reported that REV-ERBβ is over-expressed in various breast cancer cell lines, as well as in tumor cell lines derived from skin, liver and prostate [78]. Interestingly, treatment of breast cancer cell lines with the REV-ERB agonist SR9011 resulted in a significant decrease in Cyclin A2 levels and caused a dose-dependent reduction in cell viability [79]. Although not directly targeting core clock proteins, L-methyl selenocysteine (MSC) is a clock-modulating compound that upregulates *Bmal1* transcription and importantly protects against toxicity induced by the chemotherapeutic agent cyclophosphamide in mice [80]. Further efforts should be dedicated to exploiting pharmacological modulation of circadian clocksas an anti -cancer strategy.

2. Drug metabolism

Circadian pharmacokinetics and pharmacodynamics studies have revealed administration time-dependent efficacy and toxicity for a growing number of drugs, facilitating chronotherapeutic applications for various diseases such as rheumatoid arthritis, asthma, ulcer, cardiovascular disease, metabolic disease and cancer [19]. In accordance, transcriptome analysis of mouse liver indicated that hepatic drug-processing genes involving in absorption, biotransformation, and excretion of exogenous and endogenous compounds are rhythmically expressed in liver [81]. Genetic studies further demonstrate a regulatory role of the circadian clock in the expression of drug metabolism genes. For example, RORα and RORγ deficient mice displayed perturbed expression of genes encoding several phase I and phase II metabolic enzymes, including 3β-hydroxysteroid dehydrogenases, cytochrome P450 enzymes, and sulfotransferases [82]. Likewise, mice deficient for the PAR-bZip family circadian transcription factors (DBP, TEF and HLF) showed reduced basal expression of genes involved in drug metabolism and detoxification, including cytochrome P450 enzymes, carboxylesterases, and constitutive androstane receptor (CAR); furthermore, the mice are hypersensitive to xenobiotic challenges [83]. These studies together indicate that circadian expression of cytochrome P450s (CYPs) and other xenobiotic metabolism enzymes regulates drug absorption, distribution, metabolism and elimination (ADME), contributing to chronotherapeutic effects[84].

In a mouse genetic study, deficiency in *Clock* or *Bmal1* rendered mice highly sensitive to the toxic effect of the anticancer agent cyclophosphamide, whereas mice with *Cry1/2* double knockout were more resistant to the same treatment [85]. These results suggested that the functional interplay between the positive and negative circadian components impactsdrug toxicity by regulating expression of genes encoding either enzymes important for drug metabolism or targets of the drug. In that regard, it is interesting to note that 56 of the top 100 best-selling drugs are known to target proteins encoded by rhythmically expressed genes

[86]. Clock-modulating small molecules can be exploited to fine-tune the functional state of the clock, thereby optimizing both drug metabolism and response, i.e., pharmacokinetics and pharmacodynamics. In essence, these clock modulators can serve in combination therapies to enhance the therapeutic index of existing drugs(see also below) .

Future directions and conclusions

Small molecule modulators are useful probes targeting a diverse array of clock components including core clock proteins and other regulatory factors [21, 87]. Several important points should be reiterated. First, compounds with known primary targets may act on other targets to exert circadian effects. Whereas small-molecule modulators targeting core clock proteins such as CRYs may be expected to be more specific, cross-reactivity among kinases or nuclear receptors is not uncommon. For example, the effects of the p38 inhibitors SB203580 and PD169316, JNK inhibitor SP600125 and CLK inhibitor TG003 on circadian clocks may be mainly attributable to inhibition of CK1δ/ε rather than their primary targets [42]. For REV-ERB and ROR receptors with ligand binding domains sharing sequence homology with other receptors, selectivity of derived ligands toward related receptors should be experimentally evaluated [32]. Second, regulatory proteins of the circadian loops may also possess non-clock functions, suggesting clock-independent effects of their pharmacological agents. The aforementioned kinases again exemplify this point as they are known to regulate various cellular pathways in addition to the clock. Finally, given the multilayer feedback regulation embedded in the oscillator, significant challenge remains in discerning the precise biochemical mechanism and oscillator-wide function of these compounds. For example, the clock gene expression and overall amplitude depends on the stoichiometric ratio of positive and negative factors [88]. Conceivably, excessive activation of the positive factors or suppression of the negative factors will likely disrupt the balance within the oscillator, causing the cyclic pattern to collapse or dampen.

Despite these challenges, clock-targeting pharmaceuticals show preventive and therapeutic potentials against clock-related disorders. Continuing efforts can be dedicated to development of modulators for other clock proteins and their functional complexes, such as PERs, BMAL1, CLOCK and NPAS2 proteins, and PER/CRY, BMAL1/NPAS2 and BMAL1/CLOCK complexes. Two areas of studies should be conducted to fully exploit the potential of small-molecule modulators for therapeutic application. First, *in vivo* studies are required to characterize the effects of small-molecule modulators on the circadian clock and clock-related pathologies, e.g., using animal models for sleep disorders, cancer, obesity and diabetes. Synthetic REV-ERB agonists were previously found to alter circadian and metabolic gene expression and improve metabolic homeostasis in high-fat diet induced obese mice but not in *ob/ob* mice [30]; it will be interesting to investigate why REV-ERB agonists display distinct effects on these two models and whether other REV-ERB agonists also improve the metabolic syndrome. Second, as mentioned above, small molecule modulators may improve efficacy and reduce toxicity of existing therapeutic agents. For example, the expression of MCT1, a lactate acid transporter, is required for tumor cell survival and known to be regulated by the clock [89, 90]. It is possible that small molecules modulating MCT1 circadian expression could enhancethe th erapeutic index of the MCT1 inhibitor AZD3965. In addition, given the demonstrated circadian control of CYP expression

[81–84], small-molecule modulators may reduce the toxicity of drugs when co-administeredwith other drugs.

In conclusion, small-molecule clock modulators target clock components or diverse cellular pathways functionally impinging upon the clock. Target identification of new small-molecule modulators will deepen our understanding of key regulatory nodes in the circadian network. Importantly, studies of clock modulators will also facilitate their therapeutic applications, alone or in combination, for clock-related diseases.

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ABBREVIATIONS

ALK	Activin receptor-like kinase
AMPK	5' AMP-activated protein kinase
ATRA	All-trans retinoic acid
CCGs	Clock controlled genes
CDKs	Cyclin-dependent kinases
CK1	Casein kinase 1
CK2	Casein kinase 2
Cry	Cryptochrome
CYPs	cytochrome P450s
DSPS	Delayed sleep phase syndrome
EGF	Epidermal growth factor
ERs	Estrogen receptors
FASPS	Familial advanced sleep phase syndrome
FGF	Fibroblast growth factor
GR	Glucocorticoid receptor
GSK3	Glycogen synthase kinase 3
Per	Period
PPARs	Peroxisome proliferator activated receptors
RARs	Retinoic acid receptors
RORs	Retinoic acid receptor-related orphan receptors
RXRs	Retinoid X receptors
SIRT1	Silent information regulator 1

TOP DNA topoisomerase

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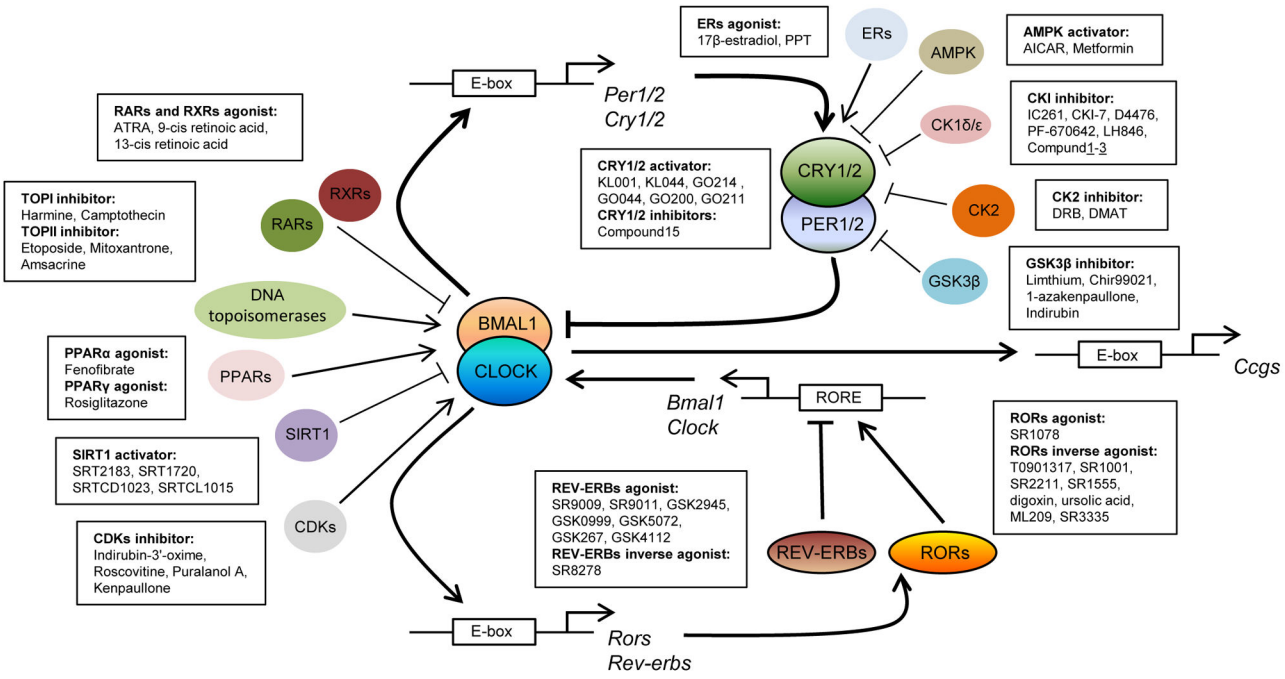

Fig. 1. Circadian clock loops and possible molecular targets of small-molecule modulators

The circadian clock oscillator is composed of transcriptional–translational feedback loops including a core loop (BMAL1/CLOCK and PERs/CRYs) and a stabilization loop (REV-ERBs and RORs). In addition to core clock components, various cellular pathways that modulate the molecular feedback loops can be targeted by small molecules leading to changes in the amplitude, phase, and period of circadian rhythms. Results from circadian phenotype-based screening or target-based rational design will continue to expand potential druggable pathways, which could lead to novel therapeutic strategies for clock-related disorders.

Table 1

Small-molecule modulators targeting core clock proteins

Molecular target	Target activity	Compound	Circadian phenotype	Reference
CRY1/2	activator	KL001	Period lengthening Amplitude reducing	[25]
		KL044	Period lengthening	[26, 27]
		GO214	Period lengthening	[26, 27]
		GO044	Period shortening	[26, 27]
		GO200	Period shortening	[26, 27]
		GO211	Period shortening	[26, 27]
	Inhibitor	2-ethoxypropanoic acid	Period shortening Amplitude reducing	[28]
REV-ERBs	Agonist	SR9009	Amplitude reducing	[30]
		SR9011	Amplitude reducing	[30]
		GSK2945	Amplitude reducing	[31, 32]
		GSK0999	Amplitude reducing	[31, 32]
		GSK5072	Amplitude reducing	[31, 32]
		GSK267	Amplitude reducing	[31, 32]
		GSK4112	N/A	[31]
	Inverse agonist	SR8278	N/A	[33]
RORs	Agonist	SR1078	N/A	[37]
	Inverse agonist	T0901317	N/A	[31]
		SR1001	N/A	[36]
		SR2211	N/A	[31]
		SR1555	N/A	[31]
		Digoxin	N/A	[31]
		Ursolic acid	N/A	[31]
		ML209	N/A	[31]
		SR3335	N/A	[38]

Table 2

Small-molecule modulators targeting regulators of circadian clocks

Molecular target	Target activity	Compound	Circadian phenotype	Reference
ALK	Inhibitor	SB432542	Attenuated phase delay	[54]
AMPK	Activator	AICAR	Period lengthening Amplitude reducing	[49]
		Metformin	Induced circadian expression	[50, 51]
CDKs	Inhibitor	Indirubin-3'-oxime	Period shortening	[40]
		Kenpaullone	Period shortening	[40]
		Roscovitine	Period lengthening	[40]
		Puralanol A	Period lengthening	[40]
CKI	Inhibitor	IC261	Period lengthening	[40, 41]
		CKI-7	Period lengthening	[40–42]
		D4476	Period lengthening	[40–42]
		PF-670642	Period lengthening	[43, 44]
		LH846	Period lengthening	[43, 44]
		Compund1–3	Period lengthening	[46]
CK2	Inhibitor	DRB	Period lengthening	[40, 47]
		DMAT	Period lengthening	[40, 47]
ERs	Agonist	17β-estradiol	Period shortening	[57]
		PPT	Period lengthening	[42]
GSK3β	Inhibitor	Lithium	Period lengthening	[40]
		Chir99021	Period shortening	[40]
		1-azakenpaullone	Period shortening	[40]
		Indirubin	Period shortening	[40]
PPARs	Agonist	Fenofibrate	Induced circadian rhythm	[58]
		Rosiglitazone	Induced circadian expression	[59]
RARs and RXRs	Agonist	ATRA	Phase shift	[61]
		9-cis retinoic acid	Induced circadian rhythm	[61]
		13-cis retinoic acid	Induced circadian rhythm	[61]
SIRT1	Activator	SRT2183	Reduced circadian expression	[64]
		SRT1720	Reduced circadian expression	[64]
		SRTCD1023	Period lengthening Amplitude reducing	[64]
		SRTCL1015	Period lengthening Amplitude reducing	[64]
TOPI	Inhibitor	Harmine	Period lengthening	[66, 67]
		Camptothecin	Period lengthening	[65]
TOPII	Inhibitor	Etoposide	Period shortening	[40]
		Mitoxantrone	Period shortening	[40]
		Amsacrine	Period lengthening	[40]