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REVIEW

Small molecule modifiers of circadian clocks

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Abstract Circadian clocks orchestrate 24-h oscillations of essential physiological and behavioral processes in response to daily environmental changes. These clocks are remarkably precise under constant conditions yet highly responsive to resetting signals. With the molecular composition of the core oscillator largely established, recent research has increasingly focused on clock-modifying mechanisms/molecules. In particular, small molecule modifiers, intrinsic or extrinsic, are emerging as powerful tools for understanding basic clock biology as well as developing putative therapeutic agents for clock-associated diseases. In this review, we will focus on synthetic compounds capable of modifying the period, phase, or amplitude of circadian clocks, with particular emphasis on the mammalian clock. We will discuss the potential of exploiting these small molecule modifiers in both basic and translational research.

Keywords Metabolites · Synthetic compounds · Period · Phase · Amplitude · Clock-associated diseases · Chronotherapy

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Abbreviations

ARNT	Aryl hydrocarbon receptor nuclear translocator
bHLH PAS	Basic helix–loop–helix PER-ARNT-SIM
BMAL1	Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like
CEM	Clock-enhancing molecule
CLOCK	Circadian locomotor output cycles kaput
CREB	cAMP response element-binding protein
CRY	Cryptochrome
FASPS	Familial advanced sleep phase syndrome
HIF	Hypoxia-inducible factor
NPAS2	Neuronal PAS domain protein 2
PER	Period
ROR	Retinoid acid receptor-related orphan receptor
SCN	Suprachiasmatic nuclei

Introduction

To cope with daily environmental changes due to the earth's rotation, virtually all living organisms have evolved an intrinsic time-keeping mechanism called the circadian clock [1–8]. The fundamental unit of animal clocks is a cell-autonomous oscillator consisting of transcriptional-translational feedback loops [9, 10]. In the primary feedback loop of the mammalian oscillator, heterodimeric transcription factors CLOCK/BMAL1 and NPAS2/BMAL1 activate expression of the *Period1/2* and *Cryptochrome1/2* genes. The resulting protein products, PER1/2 and CRY1/2, translocate to the nucleus where they inhibit CLOCK/BMAL1 and NPAS2/BMAL1 and repress their own expression. Various transcriptional and post-transcriptional mechanisms impinge on this primary loop to generate the ~24-h rhythms [11–16]. In particular, nuclear hormone

receptors REV-ERBs and RORs act antagonistically to regulate transcription of several core clock genes, including *Bmal1*, through the shared RORE promoter element [17]. PER and CRY protein turnover is also tightly regulated via coupled phosphorylation/ubiquitination pathways. For example, CRYs have been shown to be phosphorylated by AMP-activated kinase AMPK [18] prior to ubiquitination by the F-box E3 ligase FBXL3 [19–21]. Likewise, sequential phosphorylation by NEMO/NEMO-like kinase and Casein kinase I primes PER proteins for ubiquitination by SLIMB/β-TRCP E3 ligase and proteosomal degradation [13, 22–25]. At the organismal level, the molecular oscillators throughout the body that perform tissue-specific physiological functions are coordinated by the central pacemaker in the hypothalamic SCN [26–29].

Given their key roles in coordinating cellular and physiological processes in anticipation of environmental rhythms, clocks are critical for the well-being and even survival of organisms. Desynchrony between intrinsic and environmental rhythms has been found to render growth disadvantage for cyanobacteria and plants and shortened lifespan in mice [30–33]. Ablation of the SCN central clock in chipmunks adversely affected their survival in the wild, likely attributable to impairments in foraging and predator avoidance [34]. Whereas genetic disruption of clock genes does not lead to acute lethality in laboratory settings, circadian mutant mice show a wide spectrum of physiological deficits [6], including metabolic syndromes in *Clock*Δ*19* mutant mice and premature ageing in *Bmal1* knockout mice [35–39]. In humans, epidemiological and laboratory studies have also demonstrated increased risks of metabolic and cardiovascular diseases and cancer as a result of circadian disruption [40–43]. For example, within 10 days of living on an enforced 28-h rhythm, human subjects were found to suffer impaired glucose tolerance and hyperinsulinemia [41], similar to that seen with dysregulated pyruvate tolerance in a 2-week mouse model of shift work [44].

It is now well accepted that clocks play a fundamental role in metabolic regulation [45]. For photosynthetic organisms, nitrogen fixation is highly sensitive to oxygen and thus temporally sequestered from daytime photosynthesis. In mammals, hepatic gluconeogenesis takes place in the resting phase to maintain blood glucose homeostasis [46]. Concordantly, genomic and metabolomic studies have found tissue-specific oscillation of mRNA and metabolite accumulation in metabolically active tissues [47–53]. On the other hand, the clock is also highly amenable to reciprocal regulation by metabolites [45, 54, 55]. A number of metabolites can activate upstream signaling pathways that feed into the core oscillator, thereby altering cellular and physiological rhythms [55]. For example, cAMP levels were found to oscillate in the SCN, and the cAMP signaling pathway reciprocally resets the clock by inducing immediate early genes such as *Per1* [56–58]. Importantly, certain metabolites can directly modulate clock protein functions by serving as endogenous ligands, including adenosine dinucleotides (NAD and FAD), heme and diatomic gases (NO and CO), and cholesterols [59–69]. For example, NAD levels oscillate in cells, as *Nampt*, the gene encoding nicotinamide phosphoribosyltransferase that catalyzes the rate-limiting step of NAD biosynthesis, is subject to direct transcriptional control by CLOCK/BMAL1 via its E-box promoter element [70, 71]. Oscillatory NAD levels in turn modulate the activities of NAD-dependent protein modifying enzymes SIRT1 and PARP1 that respectively deacetylate and poly(ADP-ribosyl)ate clock proteins [59, 60, 66], closing the NAD-centric feedback loop imposed on the transcriptional loop.

The revelation that circadian clocks are susceptible to manipulation by small molecule metabolites ushers in an exciting era to develop synthetic small molecule clock modifiers [72, 73]. A number of promising chemical modifiers have been uncovered in recent years, through either phenotypic functional screens or targeted ligand development. In this review, we discuss these small molecule modifiers of the circadian clock and their potential therapeutic application in clock-associated diseases.

Overview of synthetic compounds as clock modifiers

Whereas classical genetics produces inherited changes in the sequence and/or abundance of the target protein, most synthetic small molecule modifiers allosterically alter the protein in a reversible, time-controlled and dose-dependent manner. Small molecules may also bind to a particular domain and consequently modulate the cognate function of a multi-domain protein, leaving the other parts of the protein and associated functions intact. If the binding surface is conserved among multiple paralogous proteins, small molecules can concurrently regulate their activities to circumvent functional redundancy commonly observed in classical genetic studies. Thus, the small molecule-based chemical genetic approach is a powerful tool to perturb the system of interest [72, 74, 75].

Two complementary methods have been utilized to identify small molecule modifiers of the clock. The first approach, based on phenotypic functional assays, interrogates broad chemical space via screening of diverse chemical libraries. In published studies, the reporter assays involved stable cell lines expressing either luciferase alone from an exogenous *Bmal1* promoter [76–80] or PER2::luciferase fusion proteins from the endogenous *Per2* promoter [81], corresponding to mRNA or protein rhythm, respectively. Bioluminescence is monitored over several

days in the so-called kinetic, as opposed to end-point, assay to visualize circadian reporter rhythms. Changes in key clock parameters, including period, phase, and amplitude, can then be measured to identify small molecule modifiers. In these screens, small molecule modifiers may act on an intracellular target in the upstream input pathway, the core oscillator, or any output pathways with feedback regulatory functions, such as metabolism (Fig. 1). Furthermore, novel screening assays targeting additional clock regulatory pathways will likely lead to an enriched repertoire of clock modifiers.

Small molecule modifiers can also be identified based on direct interaction with particular clock proteins or regulatory factors. For example, IC261 and CKI-7 have been shown to lengthen the clock period as expected from their known CKI inhibitory activities [22, 82] (see also Tables 1, 2). On the other hand, to generate novel and/or improved ligands for a particular target, it is often necessary to conduct deliberate chemical derivatization of small molecule analogs based on prior knowledge of known ligands and/or binding cavity structures [83]. An interesting example is the development of a selective inhibitor of casein kinase Iε, PF-4800567 which confers >20-fold selective inhibition over CKIδ [84–86]. More recently, this approach has been successfully applied to the nuclear hormone receptors REV-ERBs and RORs, which constitute the stabilization loop of the core oscillator [17]. Whereas the endogenous ligands are known for these proteins (heme and cholesterols respectively) [63–65], small molecule ligands are highly desirable to circumvent intracellular complications that altering metabolites commonly incurs, including nonspecific actions, cytotoxicity and redox imbalance [87]. Starting with privileged scaffolds known to target ligand binding domains of nuclear hormone receptors, investigators were able to identify tertiary amines with three lipophilic substituents as agonists of REV-ERBα [87–90].

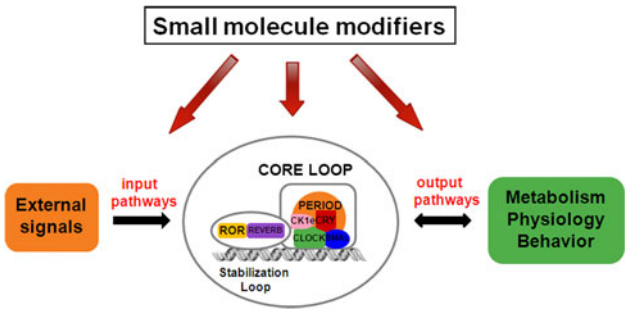

Fig. 1 Small molecule modifiers of circadian clocks. In the mammalian circadian clock system, external signals are transmitted via input pathways to the molecular oscillator consisted of interlocked feedback loops. The molecular oscillator in turn orchestrates output functions which may reciprocally regulate the clock via feedback regulation. Small molecule modifiers of the clocks may target the input pathways, the core clock, or output pathways with feedback regulatory functions

Novel RORα/γ ligands, most of them sulfonamide derivatives, have also been shown to modulate hepatic metabolism [91, 92] or to attenuate expression of downstream cytokines and alleviate autoimmune disease symptoms [93, 94]; however, the role of the clock in these settings is currently unknown. In an attempt to correlate bona fide clock effects of small molecules with physiological consequences, we describe below known small molecule clock modifiers based on their activities in modifying the three major clock characteristics, namely period, phase, and amplitude. The classification is based on their primary, most pronounced phenotype since many small molecules are able to co-regulate more than one clock parameter.

Period-altering modifiers

Circadian period has been a reliable assay parameter traditionally in rodent genetic studies in rodents and more recently in high-throughput chemical screens [6, 81]. In several independent chemical screens, small molecules showing the most significant period-lengthening activities were found to be predominantly CKI inhibitors (Table 1) [76–82, 95]. These compounds show diverse scaffold structures and are able to prolong the period of luciferase reporter rhythms to 48 h at 25 μM [77]. Inhibition of CKI slows down PER protein turnover, thus decelerating clock progression and lengthening the circadian period [6, 13]. The mechanistic convergence of these potent period-lengthening molecules highlights the central role of PER degradation cycles in setting the clock speed.

Kinase inhibitors are known to be promiscuous in target selectivity, and most CKI inhibitors appear to target paralogous CKI enzymes [58, 77, 78, 84]. In contrast, the selective CKIε inhibitor PF-4800567 caused insignificant period lengthening in cells and mice [84, 86], consistent with genetic evidence supportive of a predominant role of CKIδ in determining circadian speed [96]. Recently, three period-lengthening compounds (Cmpd-1, -2, -3) were shown to inhibit CKIε in vitro [81]; given their robust period-lengthening effects, it is possible that they also target CKIδ. Unlike Cmpd-1 and Cmpd-2, Cmpd-3 significantly increased the levels of *Per2* mRNAs, suggesting a divergent mechanism for this CKI inhibitor in addition to PER protein stabilization. In addition to CKIδ and CKIε, casein kinase 2 (CK2) has also been shown to directly phosphorylate PER and regulate its nuclear localization and turnover in *Drosophila* and mammalian cells; in agreement, inhibitors of CK2 were also found to lengthen the circadian period [25, 97, 98]. Furthermore, a number of period-lengthening small molecules are known to inhibit CKIα, ERK, CDKs, p38, or c-JNK [25, 77, 78, 97] (Table 1). Since most of these kinase inhibitors also acts on

Table 1 Summary of small molecules capable of altering the circadian period

Name	CAS #	Molecular targets	Period effects	References
IC261	186611-52-9	CKIδ/ε	Lengthening	[22]
CKI-7	1177141-67-1	CKIδ/ε	Lengthening	[82]
CK01	N/A	CKIδ/ε	Lengthening	[152]
D4476	301836-43-1	CKIδ/ε	Lengthening	[95]
DMAT	749234-11-5	CK2, CKI?	Lengthening	[25, 97]
PF-4800567	1188296-52-7	CKIε	Lengthening	[84]
PF-670462	950912-80-8	CKIδ/ε	Lengthening	[96]
Roscovitine	186692-46-6	CDK, CKIδ/ε	Lengthening	[77]
TG003	300801-52-9	CLK, CKIδ/ε	Lengthening	[77]
SB202190	152121-30-7	P38, CKIδ/ε	Lengthening	[77]
PD169316	152121-53-4	P38, CKIδ/ε	Lengthening	[77]
SU5416	204005-46-9	VEGFR PTK, CKIδ/ε	Lengthening	[77]
DRB	53-85-0	CK2, CKIδ/ε	Lengthening	[77]
SP600125	129-56-6	JNK, CKIδ/ε	Lengthening	[77]
CGS-15943	104615-18-1	AR agonist, CKIδ/ε	Lengthening	[77]
PPT	263717-53-9	ERα agonist, CKIδ/ε	Lengthening	[77]
Calyculin A	101932-71-2	PP2A	Lengthening	[22]
17-OHP	68-96-2	Progesterone receptor	Lengthening	[77]
Vincristine	57-22-7	Tubulin/microtubule	Shortening	[76]
Etoposide	33419-42-0	DNA topoisomerase II	Shortening	[76]
Mitoxantrone	65271-80-9	DNA topoisomerase II	Shortening	[76]
Amsacrine	51264-14-3	DNA topoisomerase II	Shortening	[77, 103]
PMA	16561-29-8	PKC agonist	Shortening	[76]
SKF-96365	130495-35-1	Ca channel	Shortening	[76]
Indirubin-3'-oxime	160807-49-8	CDK, GSK-3β	Shortening	[76, 83]
Kenpaulone	142273-20-9	CDK, GSK-3β	Shortening	[76]
SB216763	280744-09-4	GSK-3β	Shortening	[76, 77]
Longdaysin	1353867-91-0	CKIδ/α, ERK2, CDK7	Lengthening	[76]
LH846	639052-78-1	CKIδ	Lengthening	[79]
KL-001	309928-48-1	CRY	Lengthening	[80]
Cmpd-1	683807-31-0	CKIε, CKIδ?	Lengthening	[81]
Cmpd-2	892293-00-4	CKIε, CKIδ?	Lengthening	[81]
Cmpd-3	422279-51-4	CKIε, CKIδ?	Lengthening	[81]
Cmpd-4	533873-00-6	$GABA_AR$ agonist and ?	Lengthening	[81]
Cmpd-7	416879-98-6	Unknown	Shortening	[81]

Except as otherwise indicated, the small molecules herein negatively regulate their respective targets. For analog series, representative compounds are listed. The clock modifying activities of IC261, CKI-7, D4476, DMAT, and Calyculin A were specifically tested based on their known enzymatic targets. These small molecules were not identified via circadian-based screening or chemical derivatization approaches

CKIδ, the exact contribution of inhibiting these other kinases to period lengthening of these compounds requires further study.

Apart from the above kinase inhibitors, several carbazole derivatives also lengthened the circadian period, but appeared to function via potentiating the transcriptional repression by CRY proteins [80]. In this study, purified CRY proteins were found to directly bind to affinity resins conjugated with an active derivative KL001; furthermore, point mutation in the FAD binding pocket of CRY1 strongly attenuated its binding to the conjugated resin. Previously, hypomorphic mutations in *Fbxl3*, encoding the F-box E3 ligase FBXL3 required for CRY degradation, were found to lengthen the circadian period [19–21]. These studies thus indicate that activation of the primary repressor CRYs in the mammalian clock, by either binding small molecule agonists or blocking its turnover, lengthens the circadian period. Together with the above studies on CKI inhibitors, identification of CRY agonist molecules highlights the importance of the clock proteins in the negative arm of the feedback loop (PERs and CRYs) in setting the speed of the clock.

The target and mechanism of a period-lengthening benzodiazepine derivative, Cmpd-4, is currently unclear [81]. In central neurons, it appears to act as a canonical agonist for $GABA_A$ receptors, contributing moderately to

period lengthening. Its predominant activity, however, is likely mediated by a novel target, leading to significant period lengthening in peripheral, non-neuronal cells where $GABA_A$ receptors are not abundantly expressed. This dual action by Cmpd-4 highlights the complexity of circadian regulation, and also reveals unexpected versatility of small molecules.

In comparison, period-shortening small molecules are less common. In contrast to early studies showing inhibition of GSK-3β activity by lithium or via genetic manipulation caused period lengthening [99, 100], several selective inhibitors of GSK-3β, including Indirubin-3′-oxime, Chir99021, Kenpaullone, and SB216763, were recently found to shorten the circadian period in reporter cell assays [72, 77, 83]. GSK-3β has been shown to phosphorylate both PER2 and CRY2 proteins [101, 102], modulating their nuclear localization and proteosomal degradation, respectively. Given the important roles of PER and CRY proteins in period regulation as mentioned above, it will be of interest to determine the specific mechanism by which GSK-3β inhibitors modulate circadian progression.

Three DNA topoisomerase II inhibitors and chemotherapeutic agents, namely etoposide, mitoxantrone, and Amsacrine, have also been shown to cause period shortening and phase advance [76, 77, 103]. It has been proposed that DNA damage constitutes a circadian resetting cue, or zeitgeber, capable of altering circadian progression [104]. For example, γ-irradiation and the radiomimetic agent methylmethane sulphonate (MMS) were shown to cause phase advance in mouse and *Neurospora*, respectively, when administered during the subjective day [105, 106]. On the other hand, circadian clock genes have been implicated in mediating the DNA damage response and cell cycle gating [107–112]. For example, the clock has been shown to transcriptionally regulate a key nucleotide excision repair factor XPA, conferring robust defense against cisplatin-induced DNA damage in the late afternoon [110]. Likewise, a yeast metabolic clock also gates cell cycle progression as a means of minimizing oxidative DNA damage [8, 113, 114]. Further investigation of the circadian function of the above DNA damage and chemotherapy agents may reveal important insight into the detailed mechanism underlying the reciprocal relationship between the clock and the DNA damage response/cell cycle progression.

Phase-altering modifiers

Phase-resetting mechanisms allow the clocks to respond to environmental changes, conferring crucial adaptability in physiology and behavior. Whereas period changes can cause chronic phase delay or advance, acute phase shifts independent of sustained period changes play a predominant role in entrainment of the clock in response to the environment. Compounds that perturb the input pathways or downstream processes with feedback functions may transiently alter the circadian phase of the core oscillator. In mammals, acute phase resetting, or entrainment, of SCN clocks involves immediate early induction of *Per1*, and more weakly *Per2*, by the cAMP/CREB signaling pathway [10, 26]. Following the initial discovery of serum-induced synchronization of circadian gene oscillation in Rat-1 cells [57], many chemicals, including a number of kinase inhibitors (Table 2), have been shown to synchronize peripheral clocks and induce phase shifts in vitro [58, 72, 115–118]. Many such compounds also converge on the cAMP/CREB pathway and induce *Per* expression, such as U0126 (ERK inhibitor) and KN-62 (CamKII inhibitor) [119–123]. In fibroblast cells, two cAMP-inducing compounds, Cmpd-5 and Cmpd-6, were found to cause acute induction of *Per1* mRNA levels and PER2::Luc reporter bioluminescence, followed by significant phase delay and amplitude damping of reporter rhythms [81]. These observations are reminiscent of the effects seen previously in SCN slices treated with the adenylyl cyclase activator Forskolin [56]. Furthermore, an inhibitor of the cAMP-catabolizing enzyme phosphodiesterase 4 (PDE4), Rolipram, also caused acute bioluminescence induction and subsequent phase delay [81]. In the SCN, the guanine exchange factors Epac1/2, but not the hyperpolarizing cyclic nucleotide-gated ion channels (HCN), were previously found to be involved in mediating cAMP-induced clock resetting [56]. Elucidation of the direct targets and downstream effectors for Cmpd-5 and Cmpd-6 requires further investigation.

Phase resetting independent of acute *Per* induction has also been reported. SB431542, an inhibitor of activin receptor-like kinase (ALK), was found to attenuate alkaline shock-induced phase delays via SMAD3-dependent acute induction (within 20 min to 1 h) of the circadian transcriptional regulator *Dec1*, but not *Per1* [124]. DEC1 and its paralog DEC2 were initially found to play a role in suppressing *Per1* transcription [125]. On the other hand, double knockout of *Dec1* and *Dec2* severely attenuated photic induction of *Per1*, also suggesting a potential positive role of DECs in *Per1* transcription [126]. In a photic phase resetting experiment, a 30-min light pulse administered at night was able to acutely induce *Dec1* in the SCN [125], mimicking the well-established light induction of *Per1*. In contrast, a light pulse showed no effects on Dec2. These observations together suggest that different cues may differentially cause acute induction of *Per1* and/or *Dec1* to reset the circadian phase. In the case of SB431542, whether *Per1* contributes to its overall phase resetting effects requires further studies.

Table 2 Small molecules capable of altering the circadian phase and/or amplitude

Name	CAS #	Molecular targets	Circadian effects	References
U0126	109511-58-2	ERK	Attenuated phase shift	[119–121]
KN-62	127191-97-3	CaMKII	Attenuated phase shift	[122]
KT5823	126643-37-6	PKG	Attenuated phase advance	[118]
SB431542	301836-41-9	ALK	Attenuated phase delay	[124]
Cmpd-5	361469-09-2	cAMP inducer	Phase delay	[81]
Cmpd-6	443097-13-0	cAMP inducer	Phase delay	[81]
Rolipram	61413-54-5	PDE	Phase delay	[81]
GSK4112	1216744-19-2	REV-ERBα	Amplitude reduction	[87, 89]
SR9011/SR9009	1379686-29-9/1379686-30-2	REV-ERBs	Amplitude reduction	[90]
T0901317	293754-55-9	RORα/γ	N/A	[93]
SR1001	1335106-03-0	RORα/γ	N/A	[94]
SR1078	1246525-60-9	RORα/γ agonist	N/A	[91]
SR3335	293753-05-6	RORα	N/A	[92]
CEM1/Cmpd-8	329903-11-9	Unknown	Amplitude enhancement, period shortening	[81]
CEM2/Cmpd-9	687581-48-2	Unknown	Amplitude enhancement, period shortening	[81]
CEM3/Cmpd-10	305334-67-2	Unknown	Amplitude enhancement, period shortening	[81]
CEM4/Cmpd-11	892267-62-8	Unknown	Amplitude enhancement, period shortening	[81]

Except as otherwise indicated, the small molecules herein negatively regulate their respective targets. For analog series, representative compounds are listed. The clock modifying activities of U0126, KN-62, KT5823, and SB431542 were specifically tested based on their known enzymatic targets. These small molecules were not identified via circadian-based screening or chemical derivatization approaches

Amplitude-altering compounds

Amplitude represents the robustness of oscillation, clearly an important characteristic of any rhythmic process. The amplitude of mouse free-running activity rhythms can be measured as the relative power of the circadian component via fast-Fourier transformation (FTT) algorithms [127]. More recently, circadian reporter assays based upon cycling core clock elements allow convenient measurement of rhythm amplitude as the difference between the peak and the trough [10, 128]. In our previous chemical screen, 4 compounds (Cmpd-8, -9, -10, -11; Table 2) were identified to dose-dependently enhance the amplitude of PER2:luciferase reporter rhythm in fibroblast cells and pituitary explants [81], hereafter renamed as clock-enhancing small molecules (CEMs). These CEMs showed only modest stimulatory effects on *Per2* transcript levels, suggesting post-transcriptional mechanisms required for the induction of PER2::Luc reporter bioluminescence. In addition, CEMs showed distinct effects on Bmal1-luc reporter rhythms in U2OS cells as well as transcript oscillation of *Bmal1* target genes *Dbp* and *Rev-erbα*. For example, whereas CEM1 appeared to strongly induce Bmal1-luc oscillatory amplitude, CEM4 appeared to increase the magnitude (absolute value) of both trough and peak reporter expression, leading to only minor enhancement in amplitude (the difference between peak and trough). These observations underscore the complexity of the clock feedback regulatory circuit, particularly with regard to clock amplitude.

Apart from amplitude effects, CEMs also caused period shortening [81]; for example, at the concentration of 5 μM, CEMs were able to shorten the circadian period by 1–3 h in fibroblast cells. Whereas reciprocal regulation between amplitude and phase shifts has been demonstrated in rodents and humans [129–131], the relationship between amplitude and period is not well understood. Previously, classical mouse genetic studies have shown that overexpression of a bacterial artificial chromosome (BAC) transgene of *Clock* shortened the circadian period by approximately 1 h [132]. In flies, attaching a strong transcriptional activator VP-16 to CYCLE, the equivalent of mammalian BMAL1, or increasing the copy number of *dClock*, has been shown to enhance circadian transcription and reporter oscillatory amplitude [133]. Interestingly, enhanced transcription under these conditions correlated with shorter periods, likely attributable to accelerated dPER accumulation and subsequent transcriptional repression. These studies suggest that potentiating the activities or levels of the positive factors can both enhance the amplitude and shorten the period, primarily by accelerating the circadian phase when these factors are active. On the other hand, in a detailed biochemical study of mouse embryonic fibroblast (MEF) cells [134], CLOCK and BMAL1 were found to be enriched relative to PERs

and CRYs. Ectopic expression of CLOCK and BMAL1 by adenoviral expression specifically increased the basal levels and thus dampened the overall rhythm of the PER2::Luc reporter. In contrast, overexpression of the less abundant PERs in MEFs enhanced the reporter rhythms. Therefore, maximum circadian amplitude appears to depend on stoichiometic levels of the positive and negative factors in the core clock feedback loop [134]. Observations from these studies can be unified if CLOCK or CYCLE are limiting in flies. Regardless, future mechanistic studies using CEMs will reveal key insights into the regulatory mechanisms of clock amplitude and period.

Elucidation of the direct signaling pathways or proteins targeted by CEMs is of significant interest. Previously, in an siRNA functional genomic screen using a U2OS cell line containing a Bmal1-driven destabilized luciferase reporter, over 50 genes were identified whose knockdown increased the circadian amplitude [128]. Examination of the gene list reveals highly divergent intracellular processes, suggesting that clock amplitude regulation is subjected to broad network control. Furthermore, the central SCN clock in the mammalian clock system has been shown to be particularly robust due to intercellular coupling [135] and resistant to genetic perturbation [136]. Among the identified CEMs showing general efficacy in peripheral clocks, only CEM3 appeared to enhance the reporter rhythm in SCN explants. At the cellular level, the failure of other CEMs to activate SCN clocks could result from the lack of expression of the protein target in the SCN (assuming they are not core clock proteins), or the inability to overcome the strong coupling among the SCN neurons. Future studies will investigate the effects of these CEMs on single-cell bioluminescence [137] to distinguish between these possibilities. Such studies will also shed new light onto the effects of CEMs on rhythm damping in cultured cells, generally considered to be a consequence of loss of synchrony.

As opposed to CEMs, an inverse agonist of REV-ERBs, SR9011, was recently found to significantly repress oscillation amplitude without altering the clock period [90], consistent with the notion that the secondary feedback loop, consisting of REV-ERBs and RORs, functions to confer robustness and stability of the clock [138]. SR9011 also disrupted wheel-running activity immediately after administration. Notably, SR9011 appeared to promote energy expenditure and reduce weight gain in a diet-induced obesity model, providing an interesting example of beneficial effects of repressing the clock amplitude on energy metabolism. Whether this represents a general strategy or a specific case involving a derivatized nuclear receptor ligand remains to be seen. In the above chemical screen [81], a significant number of small molecules, estimated to be 1–3 % of the total compounds screened, strongly reduced the amplitude of reporter rhythms (data not shown). Visual examination at the end of the experiments revealed widespread cytotoxicity in these samples. Therefore, identification and utilization of amplitude-repressing compounds will require careful selection of secondary screens to eliminate cytotoxic and other complicating factors.

Therapeutic potentials in clock-related diseases

Circadian disruption is well known to contribute to pathologies with a strong temporal basis such as jetlag, sleep disorders, and seasonal affective disorders [6]. In recent years, a host of exciting studies have provided key mechanistic insights into circadian control of other physiological processes, and thus greatly expanded the spectrum of clock-related diseases. For example, the dominant negative *Clock*Δ*19* mutation or *Bmal1* knockout led to impaired pancreatic insulin secretion and caused diabetic glucose intolerance in mice [37, 139, 140]. The *Clock*Δ*19* mutant mice are also prone to diet-induced obesity [141], perhaps in part due to the increased intestinal absorption of monosaccharides and lipids in these mice [142]. In a recent study, the Krüppel-like transcription factor 15 (KLF15) was found to be directly activated by CLOCK/BMAL1 via the E-box promoter element, and KLF15 in turn regulated the transcription of the gene encoding KvCHIP2, an important component of the cardiac ion channel required for myocardial repolarization [143]. Disruption of this transcriptional cascade was shown to render increased susceptibility to ventricular arrhythmias, thus providing a mechanistic explanation for the high incidence rates of myocardial infarction in the early morning. Recent studies have also revealed circadian controls of key regulators of immune responses in both mice and plants [144, 145]. These advances in circadian biology lay the foundation for applying clock-based therapies to a wide variety of diseases.

There are two general strategies in exploiting circadian rhythms to combat clock-related diseases. Traditional chronotherapy entails optimizing the circadian timing for existing therapies, such as cancer chemotherapy, to improve efficacy and/or reduce toxicity [73, 146]. On the other hand, small molecule modifiers with desirable pharmacokinetic and pharmacodynamic characteristics afford a novel strategy involving direct manipulation of the clock to improve output pathophysiology intrinsic to disease etiology. The small molecule modifiers may be administered by themselves or in conjunction with complementary therapies. The general rationale is to match the phenotypic or molecular function of small molecules with corresponding diseases with known clock dysfunctions. Jetlag is fundamentally a phase misalignment and therefore can be

targeted by phase-resetting molecules. Given the reciprocal relationship between phase shift and amplitude, an amplitude repressor could be co-administered to augment or accelerate a phase shift. Another clock-related disorder is the familial advanced sleep phase syndrome (FASPS), characterized by short circadian periods, and in one family linked to a T44A missense mutation in human CK1δ located within the N-terminal ATP-binding motif [147, 148]. Paradoxically, this FASPS mutation repressed CK1δ kinase activity, suggesting distinct effects on PER proteins and circadian period compared with the aforementioned CKI inhibitors. It would be interesting to investigate whether known CKI inhibitors can act on this mutant CK1δ to prolong the period and alleviate the sleep syndrome. One good candidate is the selective CK1δ inhibitor PF-670462. Previously, daily dosing of PF-670462 has been shown to induce behavioral rhythms in mice that were arrhythmic due to either constant light exposure or disruption in the *Vipr2* gene encoding a G protein-coupled receptor required for SCN pacemaker functions [86], indicating in vivo activity in a circadian mouse mutant.

Several studies have revealed a strong correlation between clock dampening (reduced amplitude) and various pathological conditions [37, 129]. In particular, the *ClockΔ19* mutant mice are known to exhibit damped amplitude and lengthened period of circadian rhythms, accompanied by various physiological and behavioral deficiencies [129, 141, 142, 149–151]. Using this mouse line as a disease model, a recent study showed that a CKIδ/ε inhibitor CK01, similar to PF-670462, was able to alleviate the manic-like behaviors in these mice [152]. In cell culture, *ClockΔ19/+* heterozygous cells displayed approximately threefold reduction in reporter rhythm amplitude relative to wild-type cells [81], and CEM treatment largely restored the normal amplitude in *ClockΔ19/+* cells. Moreover, CEM3 also enhanced reporter amplitude in *ClockΔ19/+* SCN explants. Certain CEMs were able to acutely induce reporter expression followed by a descending phase in *ClockΔ19/ClockΔ19* or even *Bmal1*-deficient cells using a daily dosing protocol (Fig. 2). Detailed circadian gene analysis will help elucidate whether and how such CEM-induced reporter oscillations resemble canonical circadian cycles. It is possible that even slight amplitude enhancement of individual cellular oscillators can combine to elicit significant physiological improvement in patients with partially impaired clocks.

Future directions

It is useful to expand the ensemble of small molecules capable of manipulating the clock by chemical screening or targeted ligand development. For example, new screens can utilize neuronal (or SCN derived) stable reporter cells [153], additional clock promoters (e.g., *Dec2*) [154], nuclear localization via high content screening [155], or simply exploring new chemical space. For ligand development, one particularly interesting target is the PER-ARNT-SIM (PAS) domains present in PER proteins and the bHLH-PAS family of transcription factors including CLOCK/NPAS2, BMAL1, HIF, and ARNT. In microorganisms and plants, PAS domain proteins are required for photic and two-component signaling pathways [156, 157]. In mammals, PAS domains mainly function in protein–protein interaction and recruitment [158, 159]. However, recent crystal structures of HIF2α-ARNT PAS domains revealed a buried internal pocket in the PAS-B domain of HIF2α, and artificial bicyclic ligands were capable of allosterically modulating heterodimer formation [160, 161]. More recently, the crystal structure of the full-length CLOCK/BMAL1 heterodimer also showed highly conserved structural features in the asymmetrically positioned CLOCK/BMAL1 PAS-B domains [158]. For example, Trp427 on the BMAL1 PAS-B domain inserts into a binding pocket on the CLOCK PAS-B domain that resembles cofactor-binding motifs in other PAS proteins. Interestingly, the corresponding Tryptophan residue on the CLOCK PAS-B domain protrudes away from the CLOCK:BMAL1 dimer and may interact with the PAS domains of CRY [158, 162]. These findings suggest a central role of the binding pockets on PAS-B domains during dynamic circadian complex formation. It will be of strong interest to derive ligands capable of binding PAS domains of clock proteins.

Small molecule modifiers are useful probes to understand basic circadian biology. As mentioned above, detailed characterization of how the modifiers regulate the core oscillator will reveal important insight into the regulatory mechanism of clock amplitude and its relationship with circadian period. Ultimately, the holy grail of small molecule studies is to identify the cellular pathways or proteins that are directly targeted by small molecules. Both functional genomic screens (siRNA, shRNA libraries) [74] as well as chemical proteomics [80] have been successfully utilized to identify small molecule targets. On the other hand, we can now envision using circadian mouse mutants as disease models to investigate whether restoring normal clock functions by small molecules will improve clock output physiology. The next step will be to directly applying small molecule modifiers including CEMs to canonical disease models, e.g., *ob/ob* mice in obesity and diabetes. A rational approach is to characterize the circadian features, at both molecular and physiological levels, of these disease models [163] in order to select small molecules with the best chance of therapeutic efficacy, either alone or in combination. Humans display a wide range of

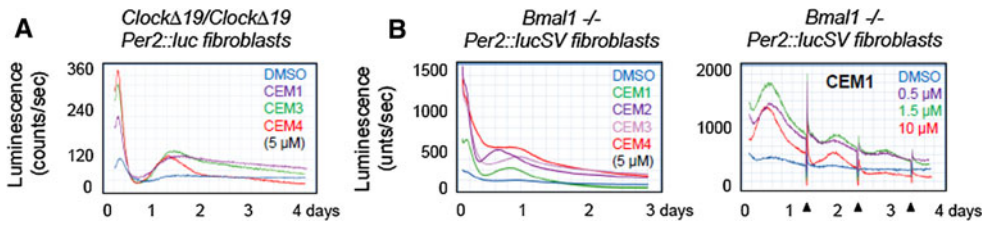

Fig. 2 Stimulatory effects of clock-enhancing small molecules (CEMs) on reporter rhythms in homozygous *Clock∆19/Clock∆19* (**a**) and *Bmal1* −/− (**b**) fibroblast cells. Luminescence recording was carried out as previously described [81]. Compared with the *Per2::luc* reporter, *Per2::lucSV* contains an exogenous SV40 polyA element which increases reporter luminescence [81]. The *X*- and *Y*-axes indicates time and luminescence, respectively. In (**b**) *right panel*, daily dosing times of CEM1 are indicated by the *arrowheads* corresponding to the spikes in bioluminescence recordings

circadian phenotypes [164, 165]; of note, the period lengths of fibroblast cells from human subjects have been shown to correlate well with behavioral chronotypes. Therefore, application of small molecules in human fibroblast cells constitutes an in vitro experimental system toward the ultimate goal of pharmacologically manipulating human circadian rhythms.

In conclusion, small molecule modifiers have taught us much about how clocks are intricately constructed and broadly regulated. Identification of their underlying mechanisms will continue to unravel key regulatory nodes in the clock network. As we increasingly appreciate the importance of timing in biology and disease, the timing is also opportune to fully exploit small molecule modifiers for exciting advances in both basic research and therapeutic development.

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