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Review

Chemical chronobiology: Toward drugs manipulating time

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ABSTRACT

Circadian clocks are endogenous timing systems orchestrating the daily regulation of a huge variety of physiological, metabolic and behavioral processes. These clocks are important for health – in mammals, their disruption leads to a diverse number of pathologies. While genetic and biochemical approaches largely uncovered the molecular bases of circadian rhythm generation, chemical biology strategies targeting the circadian oscillator by small chemical compounds are increasingly developed. Here, we review the recent progress in the identification of small molecules modulating circadian rhythms. We focus on high-throughput screening approaches using circadian bioluminescence reporter cell lines as well as describe alternative mechanistic screens. Furthermore, we discuss the potential for chemical optimization of small molecule ligands with regard to the recent progress in structural chronobiology.

1. Introduction

Circadian clocks are endogenous oscillators that drive daily rhythms in physiology, metabolic functions and behavior in a variety of species. In mammals, the master clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus orchestrating subsidiary clocks in peripheral organs. At the molecular level circadian oscillators are composed of interconnected transcriptional translational negative and positive feedback loops generating an about 24 h (circadian) periodicity. Within this gene-regulatory network, a precise timing of gene expression, protein–protein interactions as well as post-transcriptional and post-translational events is essential for sustaining normal circadian dynamics [1–3].

In the last decade systems biology approaches have been useful in creating a more comprehensive view of processes necessary for generating circadian rhythms. Transcriptome and proteome analyses helped to characterize circadian parameters of a huge number of genes in different organisms and tissues [4–10]. Furthermore, large scale forward and reverse genetic screens have identified new components and modulators relevant for the robustness and fine tuning of circadian timing as well as linking the circadian clock to a variety of other biological processes [11–13].

The importance of a robust circadian timing for health is increasingly recognized; therefore the identification of small molecules capable of modulating circadian clocks became an emerging topic. Misalignment of the circadian oscillator with the natural light dark cycle as a result of modern lifestyle (shift-work, (social) jetlag, as well as irregular food intake) is associated with various pathologies such as cancer, metabolic syndrome or depression [14–16]. In addition, animal models such as *Bmal1* knockout mice (premature ageing), *Clock* (obesity and metabolic syndrome) and *Per2* (cancer) mutants strongly support these findings [17–19]. In humans, Familial Advanced Sleep Phase Syndrome (FASPS) and Delayed Sleep Phase Syndrome (DSPS) are directly linked to mutations in *Per* (*Per2* or *Per3*) and *CKI* (ε and δ) genes, respectively [19–23].

Small molecule chemical compounds represent tools of great potential to not only better understand the molecular clockwork but also to serve as lead structures for the development of drugs targeting these clock-associated diseases. While genetic approaches mostly involve irreversible changes of the DNA sequence, small molecules have been successfully used in a reversible, dose- and time-dependent manner [24–26]. Complementary to "manipulating time" by targeting the molecular oscillator *per se* with small molecules, more classical chronotherapy "exploits time" by considering time-of-day dependent variations in pharmacokinetics and pharmacodynamics of many drugs [27].

In this review we will provide a comprehensive view on the strategies that led to the identification of small molecules modulating the circadian clock (Fig. 1). We put the focus on high-throughput screening approaches for compounds that alter circadian parameters such as period or amplitude in mammalian cell culture models. Furthermore, we discuss the potential for chemical optimization of small molecule ligands with regard to the recent progress in structural chronobiology.

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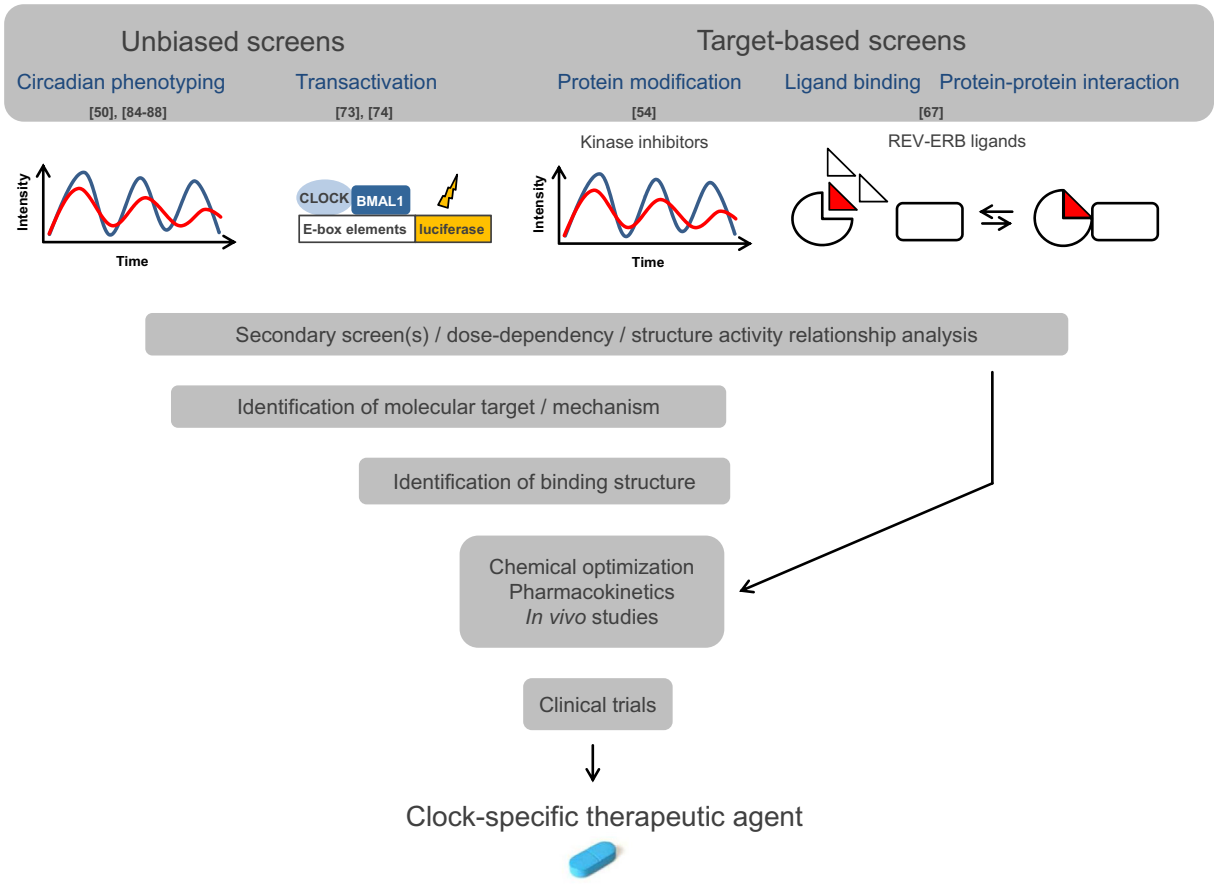

Fig. 1. Steps toward circadian drug development highlighting different identification strategies.

2. The mammalian molecular clockwork

Circadian rhythms in mammals are cell-autonomously generated with a delayed negative feedback loop in gene regulation as a central mechanism. The transcription factors CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (or ARNTL) (Brain and Muscle Aryl hydrocarbon Receptor Nuclear Translocator (ARNT)-Like 1) as a heterodimer activate the transcription of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes *via* E-box enhancer elements in their promoters. PER and CRY proteins form large complexes that inhibit their own synthesis by binding directly to the CLOCK/BMAL1 complex at the end of a circadian cycle. In addition to the negative feedback mechanism an interconnected loop composed of the nuclear receptors RORs (RAR-related Orphan Receptors) and REV-ERBs (NR1D1/2) regulates *Bmal1* and *Clock* expression anti-phasic to *Per* and *Cry* genes [1–3]. The delay in negative feedback is necessary to generate self-sustained ~24 h oscillations and is achieved by various post-transcriptional and post-translational events [28]. This core clock machinery also drives rhythmic output of so called "clock controlled genes" in almost every tissue. About 5–10% of all expressed transcripts and up to 20% of the proteins display a circadian expression pattern in a given tissue [4,7–10].

Phosphorylation of clock proteins has been demonstrated to have a huge impact on circadian dynamics probably because it regulates many essential events such as complex formation, subcellular localization, stability or transcriptional activity [28]. Kinases such as casein kinase I (CKI) isoforms (α/ε/δ), casein kinase II (CKII) as well as glycogen synthase kinase 3-beta (GSK3-β) were shown to modify PER, CRY and REV-ERB proteins as well as the CLOCK/BMAL1 heterodimer [29], whereas AMP-activated kinase (AMPK) seems to target specifically CRYs for proteasomal degradation [30]. Upon phosphorylation of PER proteins, the F-box proteins β-TRCP1 and β-TRCP2 initiate their time-of-day dependent degradation *via* the ubiquitin–proteasome pathway [31] while the F-box proteins FBXL3 and FBXL21 predominately regulate the stability of CRY proteins [32–35]. Interestingly, it was long believed that phosphorylation-supported degradation of negative elements at the end of the circadian cycle is a prerequisite for the next cycle to start, but recent findings in *Neurospora crassa* challenge this view [36,37].

It is widely accepted that kinase activities control clock speed, however much less is known about the role of de-phosphorylating enzymes [29]. Protein phosphatase 1 (PPP1) was shown to regulate the abundance of mammalian BMAL1 as well as nuclear localization and stability of PER2 [38–40], while protein phosphatase 5 (PPP5) acts indirectly on PER phosphorylation *via* the activation of CKIε [41]. Despite the fact that many kinase inhibitors can affect circadian period in oscillating cells, selective inhibition of phosphatases *via* pharmacology still remains a promising yet challenging strategy to regulate circadian dynamics. In addition to the kinase-phosphatase balance also the chromatin state, i.e. the accessibility of promoter regions, is regulated in a circadian fashion [42–44]. For example, CLOCK has a histone acetyltransferase (HAT) property that is enhanced by BMAL1 [45]. Also, clock proteins such as PER2 and BMAL1 can be acetylated – counterbalanced by the nutrition sensing NAD^+-dependent de-acetylase Sirtuin1 (SIRT1) [46,47]. Thus, the regulation of acetyl- or methyl-transferases or their counterparts using chemical compounds might also be a possible strategy to manipulate the

molecular clockwork. Moreover, the adenosine 3′,5′-monophosphate (cAMP) signaling pathway is regulated in a time-of-day dependent manner within the SCN and peripheral tissues feeding back to the molecular clockwork. *Via* this mechanism clock controlled fluctuations in cellular cAMP levels contribute to the robustness of circadian rhythm generation of circadian output at the transcript level [48].

The more we learn about the molecular and structural mechanisms within the circadian oscillator, the more potential targets for pharmacological intervention may be considered. The crucial questions for successful chemical chronobiology are therefore: (i) which of these targets are accessible to small molecule compounds; (ii) which targets allow a specific manipulation of the circadian oscillator without affecting other cellular mechanisms (or cellular health); and (iii) which molecular events are so important for the clock that chemical manipulations elicit a sufficiently large response.

3. Identification of chemical circadian modulators

3.1. Target-based rational design

3.1.1. Protein kinases

Protein kinases are global regulatory enzymes in cells that affect many physiological processes including circadian rhythms. In mammals, *Ck1* mutant alleles were among the first to be described with altered circadian rhythmicity [49]. In addition, the Familial Advanced Sleep Phase Syndrome (FASPS) in humans, where affected individuals show a 4–6 h advance in e.g. sleep behavior, is associated with single point mutations in the *Per2* or *Ck1δ* genes [19,20,23]. Both mutations affect the phosphorylation status of PER proteins leading to altered circadian periods and phases of entrainment. In addition, the importance of GSK3-β and CKII for circadian period underlines the essential role of post-translational modifications for the circadian oscillator [12,50]. Thus, it is not a surprise that pharmacological attempts to manipulate circadian rhythms have been focusing on kinases as targets.

Inhibiting CKI with small molecules (such as CKI-7 or others) led to long circadian periods in reporter cells [51]. However, these inhibitors were not specific for a particular casein kinase isoform. To discriminate between the contributions of CKIε or CKIδ within the circadian oscillator, a specific CKIε inhibitor (PF-4800567) was developed that surprisingly affected circadian period only moderately. In contrast, a specific CKIδ inhibitor (PF-670462) drastically slowed down circadian rhythms in oscillating cells supporting genetic data for this kinase isoform [52]. In addition, specific inhibitors of CKII (such as DMAT) helped to characterize the role of this kinase for circadian dynamics and PER2 stability [12,53]. Based on results with lithium treatment, it was long believed that GSK3-β inhibition causes long circadian periods. More recently, however, the application of selective GSK3-β small molecule inhibitors was reported to cause a period shortening in line with genetic data [50].

Analyzing the impact of kinases on circadian rhythm generation more systematically, Yagita et al. performed a mini-screen applying 84 known or assumed kinase inhibitors to oscillating rat cells stably expressing an *mPer2* promoter luciferase reporter [54] (Fig. 1). They identified inhibitors of CKIε/δ (IC261), CKII (DMAT), PI3K (LY294002) and c-Jun N-terminal kinase (JNK) (SP600125), which all lengthen the circadian period suggesting a role for these kinases within the molecular oscillator. Mechanistic studies by Yoshitane and colleagues underpinned the role of JNK for circadian rhythm generation by identifying BMAL1 as its molecular target [55].

Such examples vindicate the use of specific pharmacological inhibitors of known or assumed circadian regulatory enzymes to elucidate the underlying molecular mechanisms of circadian clock function.

3.1.2. Nuclear receptors

RORs (retinoic acid receptor-related receptors) and REV-ERBs are nuclear receptors involved in the transcriptional regulation of many cellular processes, such as inflammation, cell proliferation, metabolism and circadian rhythmicity. Therefore, their pharmacological modulation with e.g. derivatives of their natural ligands is of high interest for a variety of diseases such as diabetes, cancer and circadian dysfunction [56,57].

The roles of these receptors for circadian rhythm generation has been extensively studied with genetic approaches in cell culture and in vivo [58–61]. Within the molecular oscillator both types of receptors are part of the gene-regulatory oscillator network and primarily regulate *Bmal1* and *Clock* expression. Transcription of *Bmal1* and *Clock* is either activated by RORs or repressed by REV-ERBs *via* competition at ROR response elements (RREs) in their promoters [59,62]. The activity of RORs and REV-ERBs is regulated by specific ligands that lead to conformational changes modulating e.g. the binding to co-activators or co-repressors such as the nuclear receptor co-repressor 1 (NCOR1) [63]. In the last decade, heme and various sterols have been identified as *bona fide* ligands of REV-ERBs and RORs, respectively [56]. The determination of the three-dimensional structures of REV-ERBβ and RORα in their ligand-free and heme- or cholesterol-bound states, respectively, provided insights how synthetic molecules might occupy the ligand binding domains and consequently modulate their transcriptional function [64–66].

The first synthetic REV-ERB agonist (GSK4112) was identified from a medium-throughput compound screen that was focused on a library of pre-selected potential nuclear receptor ligands. The principle of this screen was based on the fact that REV-ERBα binding to its co-repressor NCOR1 is enhanced by its ligand heme. Thus, small molecule compounds were tested for their ability to modulate the binding affinity of REV-ERBα to an NCOR1-derived peptide using a FRET (fluorescence resonance energy transfer) reporter assay [67]. Identified compounds (GSK4112 and – after further optimization – also SR9009 and SR9011) indeed specifically supported the association of REV-ERBs to the NCOR1 fragment and enhanced REV-ERB's repressor function toward *Bmal1* transcription [68] (Fig. 1). The effect of these REV-ERB agonists on circadian dynamics and output was then demonstrated by a battery of experiments: (i) SR9011 reversibly reduced the circadian amplitude in SCN slices from *Per2::Luciferase* mice without affecting circadian period; (ii) SR9009 and SR9011 altered clock gene expression and disrupted circadian behavior when injected into mice at the circadian phase when REV-ERB expression is peaking; (iii) SR9011 increased energy expenditure and weight loss in vivo and reduced fat mass in diet-induced obese mice – accompanied by altered circadian gene expression in metabolic tissues such as liver and skeletal muscle [69].

Although for ROR agonists and antagonists there have been successful screening efforts [56], little is known about the ability of the identified compounds to modulate circadian rhythmicity. However, it is conceivable that such compounds also have effects on circadian dynamics given their effects on e.g. transcriptional regulation of ROR target genes such as G6PC (glucose 6-phosphatase, alpha) and FGF21 (fibroblast growth factor 21), [70]. Future studies will tell.

4. High-throughput chemical library screens

4.1. Mechanism-based screens

Circadian oscillations are generated by daytime-dependent CLOCK/BMAL1 transactivation activity at E-box elements in the promoter regions of *Per* and *Cry* genes, whose protein products subsequently form complexes to inhibit their own transcription

[71,72]. This mechanism guided the development of reporter-based functional assays for chemical interference. CLOCK/BMAL1 transactivation in cell culture was monitored using E-box-luciferase reporters and tested for modulation by chemical compounds.

Up to now, two screening studies reported the identification of chemical compounds affecting CLOCK/BMAL1 transactivation [73,74] (Fig. 1). In the first study Hu et al. aimed to identify enhancers of CLOCK/BMAL1 transactivation activity since high CLOCK/BMAL1 activity has been associated with reduced side-effects during chemotherapy [75]. CLOCK/BMAL1 transactivation of a *Per1*-luciferase reporter was analyzed in murine fibrosacroma cells in the presence of LOPAC library compounds (Library of Pharmacologically Active Compounds; 1280 compounds). Fourteen inhibitors and 17 activators of *Per1* promoter activity were identified including known circadian clock modifiers such as forskolin, glucocorticoids *etc*. Interestingly, an organic form of selenium, L-methyl-selenocysteine, was among the activators, in line with previously known chemopreventive effects of various forms of selenium. Mechanistically, L-methyl-selenocysteine interferes with TIEG1-mediated repression of *Bmal1* transcription thus upregulating BMAL1 protein levels. In wild-type but not in *Bmal1*$^{-/-}$ mice, L-methyl-selenocysteine treatment protects from cyclophosphamide-induced mortality indicating that the well-known chemopreventive function of selenium forms [76] are at least in part mediated by their effects on CLOCK/BMAL1 transcriptional activity. It would be interesting to test whether this compound also has an impact on circadian dynamics.

A second study by Chun et al. reported the identification of a chemical substance that also acts positively on CLOCK/BMAL1 mediated transcription yet *via* a different mechanism [74]. Here, the authors screened about 1000 drug-like compounds in NIH3T3 fibroblasts stably expressing CLOCK/BMAL1 and an artificial E-box luciferase reporter. About 70 compounds that either enhanced or reduced the bioluminescence signals were identified. The specificity of the compound effects toward CLOCK/BMAL1 transactivation was tested in a secondary screen using wild-type or *Bmal1*-deficient mouse embryonic fibroblasts harboring the same reporter. A biotinylated version of the most potent substance (derivate of 2-ethoxypropanoic acid) allowed the identification of CRYs as predominant targets in pull-down assays suggesting that the normal repressor function of CRYs toward CLOCK/BMAL1 is attenuated by this substance. As a result, this compound reduced the circadian amplitude dose-dependently in oscillating murine fibroblasts. As CRYs have been reported to regulate the expression of tumor suppressor genes, the authors speculated that chemical modulators of CRY function could also have positive implications for cancer treatment [77–79].

Such mechanistic screening approaches increase the likelihood that active compounds indeed target the circadian core oscillator directly rather than acting on cellular pathways that only indirectly affect circadian rhythms. Therefore, further screens addressing other important steps such as protein–protein interactions or subcellular localization of circadian clock proteins should be considered.

4.2. Circadian phenotype-based screens

Circadian rhythms can be monitored in cell-based assays by the use of reporters that are driven by the endogenous clockwork. Especially, *Bmal1* and *Per1/2* promoters fused to luciferase were successfully used as a readout for circadian function in a variety of cell lines with robust circadian rhythmicity [12,13,80]. In addition, tissues and cells derived from *Per2::Luciferase* knock-in mice in which a PER2-LUCIFERASE fusion protein is expressed from the endogenous *Per2* promoter allow the tracing of the circadian clock function at the protein level [81,82]. Tissue explants from these mice permit the analysis of compound effects on the more rigid oscillator network in the SCN and clocks located in the periphery [83]. In general, reporter cells or tissues are synchronized with dexamethasone, forskolin or serum shock and bioluminescence rhythms are monitored over several days. With appropriate time series analysis tools circadian parameters such as period, amplitude, phase and damping are extracted.

To date, six chemical biology studies with circadian dynamics as readout have been described [50,84–88] (Fig. 1 and Table 1). All studies screened chemical compound libraries with human osteosarcoma U-2 OS or murine fibroblasts (NIH3T3 or primary cells) as reporter cells.

Hirota and colleagues [50] have pioneered chemical biology approaches to analyze circadian dynamics in U-2 OS cells carrying the *Bmal1*-promoter luciferase construct. They initially screened the LOPAC (1280) library and were primarily interested in circadian period phenotypes. Eleven of the 13 primary compound hits showed a dose-dependent shortening or lengthening of the circadian period. Most of the identified active compounds have been described to affect the activity of kinases such as cyclin dependent kinase (CDK) and glycogen synthase kinase 3 (GSK3), the latter being a putative clock regulating enzyme. Other identified compounds are known to induce DNA damage or inhibit cellular Ca^{2+} entry or microtubule assembly. To investigate whether the period shortening phenotype of the GSK3 inhibiting compounds was specifically due to its effect on GSK3 (and not on CDK) the authors (i) tested further GSK3 inhibitors and (ii) silenced GSK3-β using RNA interference. Both, GSK3 (but not CDK) pharmacological inhibition as well as siRNA targeting GSK3-β mRNA resulted in short circadian periods indicating that indeed GSK3 is essential for normal circadian rhythms in mammalian cells potentially by regulating CRY2 and REV-ERBα abundance [50].

In 2009, Isojima et al. have been using the same library with both murine NIH3T3 fibroblasts and U-2 OS *Per2*-promoter luciferase reporter cells. The ten most potent compounds with effects in both cell lines lengthened the circadian period (also in primary cells and SCN slice cultures) and again were associated with the inhibition of kinase activity. TG003 and SP600125 reduced CKIε/δ activity dramatically and SP600125 (likely therefore) also increased PER2 protein stability [84]. These results are in line with previous reports describing the critical role of CKIε/δ-mediated PER phosphorylation in setting clock speed [89]. Although Isojima et al. [84] and Hirota et al. [50] both screened the identical LOPAC library, the overlap of their identified compounds was small – only the kinase inhibitors roscovitine (inhibits CDK), SB 202190 (inhibits p38 MAPK) and DRB (inhibits CKII) caused circadian phenotypes in both screens. This is typical for screening endeavors [90] and probably has multiple causes including different cell lines, different reporters and different cutoffs in hit definition.

The first truly large-scale screen for chemical circadian modulators was reported again by Hirota et al. [85]. They screened about 120 000 uncharacterized chemicals in human U-2 OS cells with a *Bmal1*-promoter luciferase reporter. Among several identified compounds affecting circadian period, the authors focused on a purine derivate that drastically lengthened the circadian period in a dose-dependent manner. A structure–activity relationship (SAR) analysis revealed a three times more potent compound, which they named *longdaysin*. To identify the molecular target of *longdaysin*, the compound was conjugated to agarose beads and binding proteins from cell lysates were analyzed using affinity purification followed by mass spectrometry. This resulted in the identification of several kinases as *longdaysin* binding partners, of which CKIδ, CKIα and ERK2 can be inhibited by *longdaysin*. The effect of *longdaysin* on circadian dynamics is likely due to a combined influence on these kinases, since knocking them down with

Table 1
Circadian phenotype-based high-throughput chemical biology screens.

Library	Cell line(s)	Circadian phenotype(s)	Targets	Most active compound(s)	Mechanism	References
LOPAC (1280 compounds)	U-2 OS *Bmal1*-luc	Short period (6 hits) Long period (5 hits)	Kinases	Indirubin-3′-oxime Kenpaulone SB216763 Chir99021	Inhibition of GSK3-β	[50]
LOPAC (1260 compounds)	*Primary screen:* NIH3T3 *Per2*-luc U-2 OS *Per2*-luc *Follow-ups:* $PER2^{Luc}$ SCN and MEFs	Long period (10 hits)	Kinases	SP600125 TG003	Inhibition of CKIε/δ Stabilization of PER2	[84]
120000 uncharacterized compounds	*Primary screen:* U-2 OS *Bmal1*-luc *Follow-ups:* $PER2^{Luc}$ cells and tissues	Long period (1 hit selected)	Kinases	Longdaysin	Inhibition of CKIδ, CKIα, ERK2 Stabilization of PER1 *via* inhibition of CKIδ and CKIα	[85]
~500000 drug-like compounds	U-2 OS *Bmal1*-luc	Long period (1 hit selected)	Kinases	LH846	Inhibition of CKIδ Stabilization of PER1	[86]
~200000 compounds	*Primary screen:* $PER2^{lucSV}$ fibroblasts *Follow-ups:* U-2 OS *Bmal1*-luc $PER2^{Luc}$ SCN/peripheral tissues $Clock^{\Delta 19/+}$ reporter cells	Long period (4 hits) Reporter induction (2 hits) Short period (5 hits), four of them also enhanced amplitude	Kinases Unknown Unknown	Compounds 1–3 Compounds 5 and 6 Compounds 7–11	Inhibition of CKIε Induction of cAMP Unknown	[87]
~60,000 compounds	*Primary screen:* U-2 OS *Bmal1*-luc *Follow-ups:* U-2 OS *Per2*-luc NIH3T3 *Bmal1*-luc/*Per2*-luc $PER2^{Luc}$ SCN/lung	Long period/reduced amplitude (1 hit selected)	CRY1/2	KL001	Inhibition of CRY1-FBXL3 binding Stabilization of CRYs	[88]

siRNA individually had only subtle effects on circadian period, while a combined knockdown – similar to *longdaysin* – drastically lengthened the circadian period. To search for targets of CKIα and ERK2 within the core circadian oscillator (for CKIδ, PER proteins as targets were already known) the authors performed co-immunoprecipitation experiments and also found PER proteins as predominant binding partners. Similar to the effect of inhibiting CKIδ, also CKIα (but not ERK2) inhibition by *longdaysin* prevented PER1 from proteasomal degradation – a likely molecular cause for the period lengthening effect. Remarkably, the effects of *longdaysin* on circadian dynamics have also been tested in vivo: reporter zebrafish larvae show dose-dependent period lengthening when treated with *longdaysin* [85].

In 2011, the same group (Lee et al.) reported yet another chemical biology screen applying over 500000 drug-like compounds again in U-2 OS expressing the *Bmal1*-promoter luciferase reporter. Here, the benzothiazole derivate LH846 was shown to lengthen the circadian period by over ten hours without affecting the amplitude. Affinity purification of cellular targets of LH846 as described above identified CKIδ as the primary target. LH846 inhibited kinase activity and had a stabilizing effect on PER1 protein. As in the case of *longdaysin* the modulation of PER stability by LH846 might explain the substantially longer period [91].

In 2012, Chen and colleagues published a library screen with about 200000 synthetic compounds using murine fibroblasts from *Per2::lucSV* reporter mice, where a PER2-luciferase fusion protein is expressed from the endogenous *Per2* locus. Out of the 530 primary hits that affected period and/or increased amplitude, the authors focused on those eleven compounds, whose effects were (i) dose-dependent and (ii) similar in U-2 OS *Bmal1*-luciferase reporter cells. Three of the four compounds found to lengthen the circadian period (in SCN and peripheral tissues) also inhibited CKIε activity. Two compounds that initially strongly induced reporter signals were shown to enhance intracellular cAMP levels dose-dependently. Five compounds shortening the period were more effective in peripheral tissues than in SCN slices (none of them are structurally related to GSK3 β inhibitors). The authors called four of these compounds "clock enhancing molecules" since they also increased the circadian amplitude to different degrees. In fact, one even "rescued" the low amplitude of SCN explants derived from ClockΔ19/+ reporter mice. Thus, the authors reasoned that such compounds might be valuable for the treatment of clock-associated (e.g. metabolic) diseases [87].

Finally, in 2012 Hirota et al. screened about 60000 compounds in *Bmal1*-luciferase U-2 OS cells and found one that specifically targets a core clock protein [88]. The carbazole derivate KL001 lengthened the circadian period dose-dependently and reduced amplitude (also in different cell lines) but was less effective on the SCN clock. An SAR analysis allowed the generation of an agarose conjugate of KL001 that was successfully used to identify CRY proteins as binding partners for KL001. Interestingly, the co-factor FAD (flavin adenine dinucleotide) pocket of CRY1 seems to be the binding site for KL001: application of FAD as a competitor as well as mutations in the FAD binding site of CRY1 resulted in reduced affinity of KL001 to CRY1. In addition, the application of KL001 led to the stabilization of CRY proteins probably *via* interfering with their FBXL3-mediated proteasomal degradation, which is likely the cause of the long circadian periods. Indeed, recent X-ray structural analyses of CRY2 in complex with FBXL3 or KL001 could explain the mechanistic basis of KL001 activity (for details see below) [92,93]. To explore a potential therapeutic function of KL001 beyond its effect on the circadian core oscillator, Hirota et al. studied the effect of KL001 on gluconeogenesis in hepatocytes. CRYs normally inhibit the glucagon-induced upregulation of key gluconeogenetic enzymes *Pck1* and *G6pc*, an effect that is augmented when applying KL001. As a consequence, glucagon-induced glucose production is attenuated in KL001-treated hepatocytes. These results demonstrate the ability of small molecules to regulate clock associated cellular physiology setting the stage for the development of therapeutic agents.

In conclusion, circadian phenotype-based chemical screens have been successfully used to identify various compounds

affecting circadian rhythmicity. Why did these screens mostly reveal compounds that inhibit kinases rather than e.g. inhibitors of CLOCK/BMAL1 transactivation activity? It is conceivable (and to some extent even likely) that phosphorylations of clock proteins are key events for regulating the clock speed [29] – more important than any other step in the circadian circuit. However, it is also possible that kinase inhibition has pleiotropic effects on many different cellular pathways that directly or indirectly affect circadian rhythms. In addition, compounds affecting cellular health might alter e.g. the metabolic state of the cells, which also could feed back to the circadian oscillator. It is a difficult endeavor to vigorously tease apart specific effects of identified compounds on clock protein kinases from indirect effects toward other cellular processes. Therefore, it is important to identify the molecular targets of bioactive compounds to analyze the mechanistic basis of their action. A third, probably equally important aspect is that circadian phenotype-based screens likely suffer from a selection bias toward target proteins with binding pockets for small molecules, i.e. proteins with catalytic clefts such as kinases, phosphatases or other enzymes. Nevertheless, while most of these limitations are absent in mechanistic screens with defined targets, circadian phenotype screens have the potential to uncover novel clock mechanisms in an unbiased manner.

5. Structural basis of target binding and chemical optimization

In recent years the analysis of the circadian oscillator function at a structural level became an emerging topic. X-ray crystallography experiments revealed high-resolution structures of circadian clock proteins either alone or with binding partners [92,94–97] – a prerequisite for rational design as well as structure-based chemical optimization of small molecule compounds targeting clock proteins. The first set of studies reported the structure determination of PER-ARNT-SIM (PAS, A and B) domains required for protein–protein interactions among PER proteins (*Drosophila* PER and mPER2). While PAS domains of PER proteins are conserved between mouse and *Drosophila*, they show differences in homodimeric PAS domain interactions [98,99]. In addition, structural studies of mPER PAS-domains have also revealed diverse modes of homodimerisation of the three PER homologes indicating specific circadian functions [100]. In 2012, Huang and colleagues reported the crystal structure of CLOCK/BMAL1-derived fragments each containing one bHLH (basic helix-loop-helix) and two tandem PAS-A and PAS-B domains showing three interaction interfaces. Interestingly, the PAS-B domain of CLOCK contains a specific binding pocket, which is occupied by tryptophan (Trp427) of the BMAL1 PAS-B domain – a putative structural target region for small molecules aiming to disrupt CLOCK/BMAL1 interaction [94]. In general, PAS domains seem to be ideal for challenging circadian clocks with small molecule compounds, since (i) they are integral constituents of many crucial protein–protein interactions within the circadian oscillator and (ii) their evolutionary old role as sensors of light, redox and other stimuli led to a specific PAS fold with the capability of binding various natural small-molecule metabolites.

The structural analysis of the mouse FBXL3 CRY2 complex [92] and the mouse CRY1 structure indicate that the FAD co-factor binding pocket of CRY proteins is involved in FBXL3 binding [95]. The carbazol compound (KL001) identified by Hirota et al. was thought to prevent the binding of FBXL3 to CRY1 resulting in a more stable CRY [88]. Indeed, a subsequent crystal structure of CRY2 in complex with KL001 showed that one part of KL001 binds to the FAD pocket of CRYs mimicking FAD while the other part of KL001 is imitating the tail of FBXL3. These structural insights set the stage for further chemical optimization of KL001. The authors propose the introduction of additional functional groups to the carbazole ring of KL001 to even better imitate the pyrimidine structure of the FAD isoalloxazine, which would likely improve its potency [93].

More recently, the crystal structure of mouse CRY1 in complex with a C-terminal mouse PER2 fragment revealed four interaction interfaces and may guide the rational design of small molecules interfering with this interaction. Interestingly, the PER2 CRY1 complex formation is regulated by the interplay of zinc binding and disulfide bond formation within CRY1, which may allow the circadian oscillator to sense the redox state of the cell [96]. mPER2 encloses the helical mCRY1 domain covering the binding sites of FBXL3 and the CLOCK/BMAL1 heterodimer while the FAD binding pocket is not engaged in PER2 binding. The PER2 CRY1 complex structure provides further insights how alternative binding of CRY1 to PER2, FBXL3, CLOCK/BMAL1 and/or FAD might regulate the clock, which could be exploited for the design and chemical optimization of small molecules specifically targeting those interactions.

6. Future perspectives

Which are the right targets for identifying chemical compounds with clock-modulating properties? First, the target should be druggable, i.e. (i) it should be accessible to binding small-molecules that are ideally orally bioavailable and (ii) small molecule binding should alter the activity of the target. Second, the target should modulate clock dynamics or influence input or output pathways. Third, for a compound being specifically targeting the clock other cellular pathways that might cause side effects upon inhibition should not or only minimally be affected. While the druggability of e.g. kinases has long been known and kinase inhibitors are frequently used as drugs especially for cancer treatment, it remains to be seen whether such inhibitors will be successful clock drugs given their multiple roles in many cellular pathways. On the other hand, many key players of the circadian core oscillator, whose modulation potentially would lead to more specific clock effects, need yet to be shown to be druggable. So far, small molecule binding to clock proteins – apart of kinases – has only been shown for CRYs and nuclear receptors. It remains to be investigated, whether other clock proteins show structural features (e.g. clefts or binding grooves) necessary for binding small-molecule ligands with a reasonable degree of potency.

The chemical circadian screens reported so far led to the identification of small molecules that predominantly affect circadian period in oscillating cells. The characterization of novel inhibitors of period-modifying regulatory enzymes such as CKI or GSK3-β has elucidated mechanistic features of the molecular clockwork. For example, the identification of CKIα as period-modulating and PER stabilizing enzyme was a direct result of a high-throughput chemical screen. Interestingly, "clock enhancing molecules" have restored rhythmicity in a clock mutant mouse, whereas the CRY stabilizing effect upon KL001 treatment also affected glucagon-induced glucose synthesis in hepatocytes. These data highlight the potential of chemical circadian modulators to be developed as effective remedies for a broad range of clock associates diseases.

The application of compounds on both SCN neurons and peripheral cells became state of the art in investigating the effects of chemical substances on clock dynamics. In principle, a small molecule drug affecting circadian dynamics could act in several ways: (i) phase shifting agents might be useful, e.g. to treat jetlag or alleviate shift-work associated problems; (ii) molecules that reduce circadian amplitude specifically in the SCN might be beneficial for re-entrainment in jetlag-type situations since *Zeitgebers* have

a larger effect on low amplitude oscillators; (iii) agents that enhance circadian amplitude might have positive effects in situations where the circadian amplitude is reduced (elderly individuals or certain disease states such as cancer or metabolic syndrome); (iv) compounds that alter circadian period might be used for treatment of genetic syndromes such as FASPS or DSPS that are associated with altered circadian periods.

Unbiased screens with circadian dynamics as readout have identified a variety of compounds, however follow-up experiments charcterizing the molecular targets are laborious and time-consuming. Instead, mechanistic screens for small molecules that perturb a specific molecular event might be the experiment of choice in the future, as more and more crystal structures of clock proteins become available allowing for virtual screening and hence more focused compound libraries. To date, the best characterized compound-clock target interaction is KL001 binding to CRYs, where structural information is available. Other essential clock proteins and their interactions might be equally suitable targets for future chemical screens including PER-CRY, CLOCK-BMAL1, CRY-CLOCK/BMAL1 with classical protein–protein interaction readouts. In addition, enzymes performing essential posttranslational events such as acetylation, deacetylation or de-phosphorylation might be putative targets, however, here specificity toward the clock is likely difficult to achieve.

Depending on the application (see above) the development of a tissue-specific drug targeting the clock might be desirable. For example, for treating jetlag after travel across time zones, a drug should phase shift the clocks while preserving the phase relationship between SCN and peripheral clocks. This is probably very difficult since SCN and peripheral clocks have been shown to have different sensitivities toward *Zeitgebers*; thus, it might be necessary to develop SCN-specific clock targeting compounds. These might include compounds affecting the coupling between SCN neurons (e.g. VIP and its receptor), targeting SCN-specific proteins (e.g. other G-protein coupled receptors) or second messenger pathways (e.g. cAMP or Ca^{2+} signaling) that modulate circadian rhythmicity. Novel high-throughput compatible SCN cell lines would be a very helpful tool to achieve these goals.

A next step after identifying small molecules manipulating circadian oscillator dynamics is to test whether they would have beneficial effects on diseases associated with circadian clock perturbation. Animal models for cancer, metabolic dysfunction (diabetes, obesity) as well as mental disorders can be used to study such effects. A first step in this direction was the application of "clock enhancing molecules" on SCN slices of *Clock* mutant mice: Here, indeed, low circadian amplitude was "rescued"; yet it remains to be seen whether this would also alleviate the metabolic dysfunctions observed in this mouse model. Also, synthetic REV-ERB agonists that altered the circadian pattern of metabolic genes improved the metabolic status of high-fat diet induced obese mice, although it needs to be investigated what is cause and what is effect.

In summary, small molecules targeting the circadian oscillator *per se* will not only be invaluable basic research tools – in addition to genetic approaches – to further characterize the molecular basis of circadian rhythm generation; they will also allow us to develop novel treatment strategies for those types of diseases and syndromes that are associated with altered or disrupted clocks.

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References

[1] Reppert, S.M. and Weaver, D.R. (2002) Coordination of circadian timing in mammals. Nature 418, 935–941.
[2] Zhang, E.E. and Kay, S.A. (2010) Clocks not winding down: unravelling circadian networks. Nat. Rev. Mol. Cell Biol. 11, 764–776.
[3] Partch, C.L., Green, C.B. and Takahashi, J.S. (2014) Molecular architecture of the mammalian circadian clock. Trends Cell Biol. 24, 90–99.
[4] Hughes, M.E., DiTacchio, L., Hayes, K.R., Vollmers, C., Pulivarthy, S., Baggs, J.E., Panda, S. and Hogenesch, J.B. (2009) Harmonics of circadian gene transcription in mammals. PLoS Genet. 5, e1000442.
[5] Zhang, R., Lahens, N.F., Ballance, H.I., Hughes, M.E. and Hogenesch, J.B. (2014) A circadian gene expression atlas in mammals: implications for biology and medicine. Proc. Natl. Acad. Sci. USA 111, 16219–16224.
[6] Keller, M., Mazuch, J., Abraham, U., Eom, G.D., Herzog, E.D., Volk, H.D., Kramer, A. and Maier, B. (2009) A circadian clock in macrophages controls inflammatory immune responses. Proc. Natl. Acad. Sci. USA 106, 21407–21412.
[7] Robles, M.S., Cox, J. and Mann, M. (2014) In-vivo quantitative proteomics reveals a key contribution of post-transcriptional mechanisms to the circadian regulation of liver metabolism. PLoS Genet. 10, e1004047.
[8] Reddy, A.B. et al. (2006) Circadian orchestration of the hepatic proteome. Curr. Biol. 16, 1107–1115.
[9] Mauvoisin, D. et al. (2014) Circadian clock-dependent and -independent rhythmic proteomes implement distinct diurnal functions in mouse liver. Proc. Natl. Acad. Sci. USA 111, 167–172.
[10] Chiang, C.K. et al. (2014) The proteomic landscape of the suprachiasmatic nucleus clock reveals large-scale coordination of key biological processes. PLoS Genet. 10, e1004695.
[11] King, D.P. et al. (1997) Positional cloning of the mouse circadian clock gene. Cell 89, 641–653.
[12] Maier, B., Wendt, S., Vanselow, J.T., Wallach, T., Reischl, S., Oehmke, S., Schlosser, A. and Kramer, A. (2009) A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. Genes Dev. 23, 708–718.
[13] Zhang, E.E. et al. (2009) A genome-wide RNAi screen for modifiers of the circadian clock in human cells. Cell 139, 199–210.
[14] Bechtold, D.A., Gibbs, J.E. and Loudon, A.S. (2010) Circadian dysfunction in disease. Trends Pharmacol. Sci. 31, 191–198.
[15] Takahashi, J.S., Hong, H.K., Ko, C.H. and McDearmon, E.L. (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nat. Rev. Genet. 9, 764–775.
[16] Maywood, E.S., O'Neill, J., Wong, G.K., Reddy, A.B. and Hastings, M.H. (2006) Circadian timing in health and disease. Prog. Brain Res. 153, 253–269.
[17] Kondratov, R.V., Kondratova, A.A., Gorbacheva, V.Y., Vykhovanets, O.V. and Antoch, M.P. (2006) Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. Genes Dev. 20, 1868–1873.
[18] Turek, F.W. et al. (2005) Obesity and metabolic syndrome in circadian Clock mutant mice. Science 308, 1043–1045.
[19] Fu, L., Pelicano, H., Liu, J., Huang, P. and Lee, C. (2002) The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell 111, 41–50.
[20] Xu, Y. et al. (2005) Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. Nature 434, 640–644.
[21] Ebisawa, T. et al. (2001) Association of structural polymorphisms in the human period3 gene with delayed sleep phase syndrome. EMBO Rep. 2, 342–346.
[22] Takano, A. et al. (2004) A missense variation in human casein kinase I epsilon gene that induces functional alteration and shows an inverse association with circadian rhythm sleep disorders. Neuropsychopharmacology 29, 1901–1909.
[23] Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptacek, L.J. and Fu, Y.H. (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science 291, 1040–1043.
[24] Antoch, M.P. and Chernov, M.V. (2009) Pharmacological modulators of the circadian clock as potential therapeutic drugs. Mutat. Res. 680, 109–115.
[25] Chen, Z., Yoo, S.H. and Takahashi, J.S. (2013) Small molecule modifiers of circadian clocks. Cell. Mol. Life Sci. 70, 2985–2998.
[26] Antoch, M.P. and Kondratov, R.V. (2013) Pharmacological modulators of the circadian clock as potential therapeutic drugs: focus on genotoxic/anticancer therapy. Handb. Exp. Pharmacol., 289–309.
[27] Ortiz-Tudela, E., Mteyrek, A., Ballesta, A., Innominato, P.F. and Levi, F. (2013) Cancer chronotherapeutics: experimental, theoretical, and clinical aspects. Handb. Exp. Pharmacol., 261–288.
[28] Gallego, M. and Virshup, D.M. (2007) Post-translational modifications regulate the ticking of the circadian clock. Nat. Rev. Mol. Cell Biol. 8, 139–148.
[29] Reischl, S. and Kramer, A. (2011) Kinases and phosphatases in the mammalian circadian clock. FEBS Lett. 585, 1393–1399.
[30] Lamia, K.A. et al. (2009) AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. Science 326, 437–440.
[31] Reischl, S., Vanselow, K., Westermark, P.O., Thierfelder, N., Maier, B., Herzel, H. and Kramer, A. (2007) Beta-TrCP1-mediated degradation of PERIOD2 is essential for circadian dynamics. J. Biol. Rhythms 22, 375–386.
[32] Hirano, A. et al. (2013) FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. Cell 152, 1106–1118.

[33] Busino, L., Bassermann, F., Maiolica, A., Lee, C., Nolan, P.M., Godinho, S.I., Draetta, G.F. and Pagano, M. (2007) SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. Science 316, 900–904.

[34] Siepka, S.M., Yoo, S.H., Park, J., Song, W., Kumar, V., Hu, Y., Lee, C. and Takahashi, J.S. (2007) Circadian mutant overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. Cell 129, 1011–1023.

[35] Yoo, S.H. et al. (2013) Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm. Cell 152, 1091–1105.

[36] Larrondo, L.F., Olivares-Yanez, C., Baker, C.L., Loros, J.J. and Dunlap, J.C. (2015) Circadian rhythms. Decoupling circadian clock protein turnover from circadian period determination. Science 347, 1257277.

[37] Kramer, A. (2015) Circadian rhythms. When the circadian clock becomes blind. Science 347, 476–477.

[38] Wallach, T., Schellenberg, K., Maier, B., Kalathur, R.K., Porras, P., Wanker, E.E., Futschik, M.E. and Kramer, A. (2013) Dynamic circadian protein-protein interaction networks predict temporal organization of cellular functions. PLoS Genet. 9, e1003398.

[39] Schmutz, I., Wendt, S., Schnell, A., Kramer, A., Mansuy, I.M. and Albrecht, U. (2011) Protein phosphatase 1 (PP1) is a post-translational regulator of the mammalian circadian clock. PLoS One 6, e21325.

[40] Gallego, M., Kang, H. and Virshup, D.M. (2006) Protein phosphatase 1 regulates the stability of the circadian protein PER2. Biochem. J. 399, 169–175.

[41] Partch, C.L., Shields, K.F., Thompson, C.L., Selby, C.P. and Sancar, A. (2006) Posttranslational regulation of the mammalian circadian clock by cryptochrome and protein phosphatase 5. Proc. Natl. Acad. Sci. USA 103, 10467–10472.

[42] Aguilar-Arnal, L. and Sassone-Corsi, P. (2014) Chromatin landscape and circadian dynamics: spatial and temporal organization of clock transcription. Proc. Natl. Acad. Sci. USA.

[43] Eckel-Mahan, K. and Sassone-Corsi, P. (2013) Epigenetic regulation of the molecular clockwork. Prog. Mol. Biol. Transl. Sci. 119, 29–50.

[44] Sahar, S. and Sassone-Corsi, P. (2013) The epigenetic language of circadian clocks. Handb. Exp. Pharmacol., 29–44.

[45] Doi, M., Hirayama, J. and Sassone-Corsi, P. (2006) Circadian regulator CLOCK is a histone acetyltransferase. Cell 125, 497–508.

[46] Asher, G. et al. (2008) SIRT1 regulates circadian clock gene expression through PER2 deacetylation. Cell 134, 317–328.

[47] Nakahata, Y., Kaluzova, M., Grimaldi, B., Sahar, S., Hirayama, J., Chen, D., Guarente, L.P. and Sassone-Corsi, P. (2008) The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. Cell 134, 329–340.

[48] O'Neill, J.S., Maywood, E.S., Chesham, J.E., Takahashi, J.S. and Hastings, M.H. (2008) CAMP-dependent signaling as a core component of the mammalian circadian pacemaker. Science 320, 949–953.

[49] Ralph, M.R. and Menaker, M. (1988) A mutation of the circadian system in golden hamsters. Science 241, 1225–1227.

[50] Hirota, T., Lewis, W.G., Liu, A.C., Lee, J.W., Schultz, P.G. and Kay, S.A. (2008) A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3beta. Proc. Natl. Acad. Sci. USA 105, 20746–20751.

[51] Vanselow, K. et al. (2006) Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS). Genes Dev. 20, 2660–2672.

[52] Walton, K.M. et al. (2009) Selective inhibition of casein kinase 1 epsilon minimally alters circadian clock period. J. Pharmacol. Exp. Ther. 330, 430–439.

[53] Tsuchiya, Y., Akashi, M., Matsuda, M., Goto, K., Miyata, Y., Node, K. and Nishida, E. (2009) Involvement of the protein kinase CK2 in the regulation of mammalian circadian rhythms. Sci. Signal. 2, ra26.

[54] Yagita, K., Yamanaka, I., Koinuma, S., Shigeyoshi, Y. and Uchiyama, Y. (2009) Mini screening of kinase inhibitors affecting period-length of mammalian cellular circadian clock. Acta Histochem. Cytochem. 42, 89–93.

[55] Yoshitane, H. et al. (2012) JNK regulates the photic response of the mammalian circadian clock. EMBO Rep. 13, 455–461.

[56] Kojetin, D.J. and Burris, T.P. (2014) REV-ERB and ROR nuclear receptors as drug targets. Nat. Rev. Drug Discov. 13, 197–216.

[57] Marciano, D.P., Chang, M.R., Corzo, C.A., Goswami, D., Lam, V.Q., Pascal, B.D. and Griffin, P.R. (2014) The therapeutic potential of nuclear receptor modulators for treatment of metabolic disorders: PPARgamma, RORs, and Rev-erbs. Cell Metab. 19, 193–208.

[58] Schmutz, I., Ripperger, J.A., Baeriswyl-Aebischer, S. and Albrecht, U. (2010) The mammalian clock component PERIOD2 coordinates circadian output by interaction with nuclear receptors. Genes Dev. 24, 345–357.

[59] Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U. and Schibler, U. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110, 251–260.

[60] Cho, H. et al. (2012) Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. Nature 485, 123–127.

[61] Kornmann, B., Schaad, O., Bujard, H., Takahashi, J.S. and Schibler, U. (2007) System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. PLoS Biol. 5, e34.

[62] Guillaumond, F., Dardente, H., Giguere, V. and Cermakian, N. (2005) Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J. Biol. Rhythms 20, 391–403.

[63] Privalsky, M.L. (2004) The role of corepressors in transcriptional regulation by nuclear hormone receptors. Annu. Rev. Physiol. 66, 315–360.

[64] Pardee, K.I. et al. (2009) The structural basis of gas-responsive transcription by the human nuclear hormone receptor REV-ERBbeta. PLoS Biol. 7, e43.

[65] Kallen, J.A., Schlaeppi, J.M., Bitsch, F., Geisse, S., Geiser, M., Delhon, I. and Fournier, B. (2002) X-ray structure of the hRORalpha LBD at 1.63 A: structural and functional data that cholesterol or a cholesterol derivative is the natural ligand of RORalpha. Structure 10, 1697–1707.

[66] Kallen, J., Schlaeppi, J.M., Bitsch, F., Delhon, I. and Fournier, B. (2004) Crystal structure of the human RORalpha Ligand binding domain in complex with cholesterol sulfate at 2.2 A. J. Biol. Chem. 279, 14033–14038.

[67] Grant, D. et al. (2010) GSK4112, a small molecule chemical probe for the cell biology of the nuclear heme receptor Rev-erbalpha. ACS Chem. Biol. 5, 925–932.

[68] Noel, R. et al. (2012) Synthesis and SAR of tetrahydroisoquinolines as Rev-erbalpha agonists. Bioorg. Med. Chem. Lett. 22, 3739–3742.

[69] Solt, L.A. et al. (2012) Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists. Nature 485, 62–68.

[70] Wang, Y., Kumar, N., Nuhant, P., Cameron, M.D., Istrate, M.A., Roush, W.R., Griffin, P.R. and Burris, T.P. (2010) Identification of SR1078, a synthetic agonist for the orphan nuclear receptors RORalpha and RORgamma. ACS Chem. Biol. 5, 1029–1034.

[71] Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S. and Weitz, C.J. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. Science 280, 1564–1569.

[72] Griffin Jr., E.A., Staknis, D. and Weitz, C.J. (1999) Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. Science 286, 768–771.

[73] Hu, Y. et al. (2011) Selenium is a modulator of circadian clock that protects mice from the toxicity of a chemotherapeutic drug via upregulation of the core clock protein, BMAL1. Oncotarget 2, 1279–1290.

[74] Chun, S.K. et al. (2014) Identification and validation of cryptochrome inhibitors that modulate the molecular circadian clock. ACS Chem. Biol. 9, 703–710.

[75] Gorbacheva, V.Y., Kondratov, R.V., Zhang, R., Cherukuri, S., Gudkov, A.V., Takahashi, J.S. and Antoch, M.P. (2005) Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends on the functional status of the CLOCK/BMAL1 transactivation complex. Proc. Natl. Acad. Sci. USA 102, 3407–3412.

[76] Hatfield, D.L., Tsuji, P.A., Carlson, B.A. and Gladyshev, V.N. (2014) Selenium and selenocysteine: roles in cancer, health, and development. Trends Biochem. Sci. 39, 112–120.

[77] Lee, J.H., Gaddameedhi, S., Ozturk, N., Ye, R. and Sancar, A. (2013) DNA damage-specific control of cell death by cryptochrome in p53-mutant ras-transformed cells. Cancer Res. 73, 785–791.

[78] Ozturk, N., Lee, J.H., Gaddameedhi, S. and Sancar, A. (2009) Loss of cryptochrome reduces cancer risk in p53 mutant mice. Proc. Natl. Acad. Sci. USA 106, 2841–2846.

[79] Aad, G. et al. (2011) Search for dilepton resonances in pp collisions at radicals = 7 TeV with the ATLAS detector. Phys. Rev. Lett. 107, 272002.

[80] Relogio, A. et al. (2014) Ras-mediated deregulation of the circadian clock in cancer. PLoS Genet. 10, e1004338.

[81] Mohawk, J.A., Green, C.B. and Takahashi, J.S. (2012) Central and peripheral circadian clocks in mammals. Annu. Rev. Neurosci. 35, 445–462.

[82] Yoo, S.H. et al. (2005) A non-canonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. Proc. Natl. Acad. Sci. USA 102, 2608–2613.

[83] Abraham, U., Granada, A.E., Westermark, P.O., Heine, M., Kramer, A. and Herzel, H. (2010) Coupling governs entrainment range of circadian clocks. Mol. Syst. Biol. 6, 438.

[84] Isojima, Y. et al. (2009) CKIepsilon/delta-dependent phosphorylation is a temperature-insensitive, period-determining process in the mammalian circadian clock. Proc. Natl. Acad. Sci. USA 106, 15744–15749.

[85] Hirota, T. et al. (2010) High-throughput chemical screen identifies a novel potent modulator of cellular circadian rhythms and reveals CKIalpha as a clock regulatory kinase. PLoS Biol. 8, e1000559.

[86] Lee, J.W. et al. (2011) A small molecule modulates circadian rhythms through phosphorylation of the period protein. Angew. Chem. Int. Ed. Engl. 50, 10608–10611.

[87] Chen, Z. et al. (2012) Identification of diverse modulators of central and peripheral circadian clocks by high-throughput chemical screening. Proc. Natl. Acad. Sci. USA 109, 101–106.

[88] Hirota, T. et al. (2012) Identification of small molecule activators of cryptochrome. Science 337, 1094–1097.

[89] Etchegaray, J.P. et al. (2009) Casein kinase 1 delta regulates the pace of the mammalian circadian clock. Mol. Cell. Biol. 29, 3853–3866.

[90] Bushman, F.D. et al. (2009) Host cell factors in HIV replication: meta-analysis of genome-wide studies. PLoS Pathog. 5, e1000437.

[91] Lee, S., Jang, Y., Lee, Y.M., Lee, J., Lee, H., Kim, G.H. and Kim, J.J. (2011) Rice straw-decomposing fungi and their cellulolytic and xylanolytic enzymes. J. Microbiol. Biotechnol. 21, 1322–1329.

[92] Xing, W., Busino, L., Hinds, T.R., Marionni, S.T., Saifee, N.H., Bush, M.F., Pagano, M. and Zheng, N. (2013) SCF(FBXL3) ubiquitin ligase targets cryptochromes at their cofactor pocket. Nature 496, 64–68.

[93] Nangle, S., Xing, W. and Zheng, N. (2013) Crystal structure of mammalian cryptochrome in complex with a small molecule competitor of its ubiquitin ligase. Cell Res. 23, 1417–1419.
[94] Huang, N. et al. (2012) Crystal structure of the heterodimeric CLOCK:BMAL1 transcriptional activator complex. Science 337, 189–194.
[95] Czarna, A., Berndt, A., Singh, H.R., Grudziecki, A., Ladurner, A.G., Timinszky, G., Kramer, A. and Wolf, E. (2013) Structures of Drosophila cryptochrome and mouse cryptochrome1 provide insight into circadian function. Cell 153, 1394–1405.
[96] Schmalen, I. et al. (2014) Interaction of circadian clock proteins CRY1 and PER2 is modulated by zinc binding and disulfide bond formation. Cell 157, 1203–1215.
[97] Nangle, S.N., Rosensweig, C., Koike, N., Tei, H., Takahashi, J.S., Green, C.B. and Zheng, N. (2014) Molecular assembly of the period-cryptochrome circadian transcriptional repressor complex. Elife 3, e03674.
[98] Yildiz, O. et al. (2005) Crystal structure and interactions of the PAS repeat region of the Drosophila clock protein PERIOD. Mol. Cell 17, 69–82.
[99] Hennig, S., Strauss, H.M., Vanselow, K., Yildiz, O., Schulze, S., Arens, J., Kramer, A. and Wolf, E. (2009) Structural and functional analyses of PAS domain interactions of the clock proteins Drosophila PERIOD and mouse PERIOD2. PLoS Biol. 7, e94.
[100] Kucera, N. et al. (2012) Unwinding the differences of the mammalian PERIOD clock proteins from crystal structure to cellular function. Proc. Natl. Acad. Sci. USA 109, 3311–3316.