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Pharmacological Modulators of Molecular Clock and their Therapeutic Potentials in Circadian Rhythm-Related Diseases

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Abstract

Circadian rhythms regulate a wide range of biological processes and play a fundamental role in mammalian behavior, physiology, and metabolism. The hierarchically organized circadian timing system in mammals, with a master pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus and subsidiary oscillators in extra-SCN brain regions and peripheral tissues, mediates periodicities in physiological processes. It has been well recognized that disruption or misalignment of the intrinsic rhythms leads to diverse pathological states. Since mammalian circadian clock genes were first identified in the 1990's, genetic and biochemical approaches have uncovered the molecular bases of the cell-autonomous and self-sustainable rhythms that are generated by two interlocking feedback loops of clock proteins. With our understanding of key features underlying the overt circadian rhythm and physiological outputs, it has emerged that pharmacological control of the circadian clock may provide a novel therapeutic strategy to treat a variety of circadian rhythm-related human diseases such as neuropsychiatric, metabolic, cardiovascular, and immune/inflammatory diseases, and even cancer. Pharmaceutical approaches to circadian clock may involve either development of drugs to treat such circadian-related disorders or combinational uses with existing therapeutic strategies to improve their therapeutic efficacy via the intrinsic clock-dependent mechanisms. In this review, we will focus on recent progress in discovery of small molecule chemical compounds that can pharmacologically modulate molecular circadian clock and their potential to be developed into therapeutic drugs.

Keywords: Circadian rhythm; Circadian clock; Circadian rhythm-related diseases; Cryptochrome (CRY); REV-ERB; Small molecule chemical compounds

Circadian Timing System in Mammals

Circadian rhythms are comprised of ubiquitous biological oscillations with an approximately 24-h period that are evolutionarily conserved from cyanobacteria to humans. Such daily rhythm is not a simple response to alternating changes of day and night. Rather, it arises from an intrinsic and genetically operated timekeeping system referred to as a "circadian clock". This internal timekeeping system allows organisms to anticipate environmental changes, thereby optimizing their physiology and behavior at the right time of day. The biological clock also greatly contributes to ensuring that certain biological processes take place in coordination with others. The circadian clock is cell-autonomous and self-sustainable by an elaborate cooperation of genetic components, but it can be also entrained by external time cues (called *zeitgebers* meaning 'time givers'). Most cells in multi-cellular organisms harbor their own cell-autonomous oscillators, which are hierarchically organized into a circadian timing system. At the apex of the mammalian circadian system, the suprachiasmatic nucleus (SCN) in the hypothalamus composed of densely packed neurons generates self-sustaining rhythms by both genetic and neural mechanisms and thus is considered as the central or master clock [1]. The SCN central clock receives the environmental time information (primarily light) to adjust or entrain its phase and then orchestrates other oscillators in extra-SCN brain regions and peripheral tissues (referred to as local or peripheral clocks) to exhibit overt circadian rhythms such as the rest-activity cycle, periodic daily variations in metabolism and body temperature, and the rhythmic secretion of hormones [2,3].

It has been well established that a robust circadian timing is prerequisite for human health and disruption of the intrinsic rhythms leads to diverse pathological states. For instance, misalignment of the intrinsic oscillators by shift-work, jetlag (either physical or social) or irregular food intake is strongly associated with various human diseases such as sleep disorders, metabolic syndrome, affective disorders and even tumorigenesis [4,5]. Phenotypic analyses on mutant mice models with defective clock genes along with human genetic studies also supported the above notion and revealed mechanistic links between disrupted circadian clock and the onsets of these circadian rhythm-related diseases. As a result of extensive studies on circadian clock and its functional roles in the last decades, the identification of small molecule chemical compounds capable of modulating circadian clocks either directly or indirectly has become an emerging issue. In this review, we will therefore focus on recent progress in discovery of small chemical compounds that can pharmacologically modulate circadian timing system and their therapeutic potential with special emphases on core components of the molecular circadian clock.

Molecular Basis of Cell-Autonomous Circadian Rhythms

Key components of the molecular circadian clock in mammals

The intrinsic and self-sustainable nature of the circadian timing system is primarily attributed to the presence of the molecular circadian

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oscillator based on genetic components. The molecular circadian clock is composed of a group of clock proteins, which are required for the generation and maintenance of the cell-autonomous rhythm [2]. The molecular clock mechanism in mammals is currently understood as a molecular feedback loop formed by at least ten genes. The clock proteins form two interlocked positive and negative transcription/translation feedback loops, thereby promoting periodic gene expression (Figure 1). Among these clock proteins, CLOCK (Circadian Locomotor Output Cycle Kaput) and BMAL1 (Brain Muscle Aryl hydrocarbon receptor nuclear translocator-Like 1 or ARNTL1), which belong to bHLH–PAS (basic helix-loop-helix–PER-ARNT-SIM) family of transcription factor, are the uppermost regulators of the molecular oscillator. CLOCK and BMAL1 form heterodimers, bind to E-boxes (5'-CACGTG-3') and E'-boxes (5'-CACGTT-3') enhancer elements and then activate transcription of the target genes through epigenetic activation of their promoters. These downstream genes include the negative limb of the feedback loop including *Periods* (PERs: PER1-3) and *Cryptochromes* (CRYs: CRY1-2). Accumulated PER and CRY proteins form large complexes that repress the E-box-mediated transcription by binding directly to CLOCK/BMAL1 complex. Degradation of PER and CRY proteins terminate the repressive phase and reinitiate a new cycle of transcription [6-9] (Figure 1).

In addition to the core loop, the amount of BMAL1 protein is also adjusted by an auxiliary or stabilizing feedback loop consisting of sets of the circadian nuclear receptors such as retinoic acid receptor-related orphan nuclear receptors (RORs; RORα, β and γ) and REV-ERBs (REV-ERBα and β encoded by *NR1D1* and *NR1D2* genes, respectively), which are also transcriptionally controlled by the CLOCK/BMAL1 heterodimer. REV-ERBs and RORs compete with each other to bind the ROR/REV-ERBs-responsive elements (RREs) present in the 5′ flanking region of *Bmal1* gene; RORs transcriptionally activates Bmal1 mRNA expression, whereas REV-ERBs strongly suppresses it [10-12]. This second loop was originally considered as an accessory loop due to moderate phenotypes observed in mutant mice bearing null alleles of any one of these genes. However, more recent studies with inducible double knockout strategies for both *Rev-erbα* and *β* genes revealed that the subtle phenotypes are primary due to their compensatory functions and REV-ERBs are required for normal period regulation of circadian behavioral rhythmicity [13]. REV-ERBs also have a key role in controlling various circadian outputs by cooperation with a variety of cell type-specific transcriptional regulators [14,15]. Taken together, these two interlocked feedback loops provide a molecular basis for the self-sustaining circadian oscillations with a period of approximately 24 hours.

Post-translational regulatory mechanisms influencing the circadian clock

Along with the canonical transcription/translation feedback loops, a wide range of auxiliary proteins such as protein kinases, chromatin-modifying proteins and RNA-binding proteins are related to the control of protein stability, subcellular trafficking, and transcriptional activity of clock proteins, thereby contributing to fine and precise control of the cellular circadian rhythms [16-18]. Among these post-translational regulatory mechanisms, phosphorylation state of the negative limb proteins, PERs and CRYs is key to setting the period because phosphorylation-dependent degradation of PER and CRY proteins is required to terminate the repression phase leading to initiation of a new cycle of transcription (Figure 1). For example, extensive studies have shown that phosphorylation by casein kinase 1δ and 1ε (CK1δ/ε) and subsequent ubiquitin-dependent degradation of the PER proteins are the determinants of circadian period length

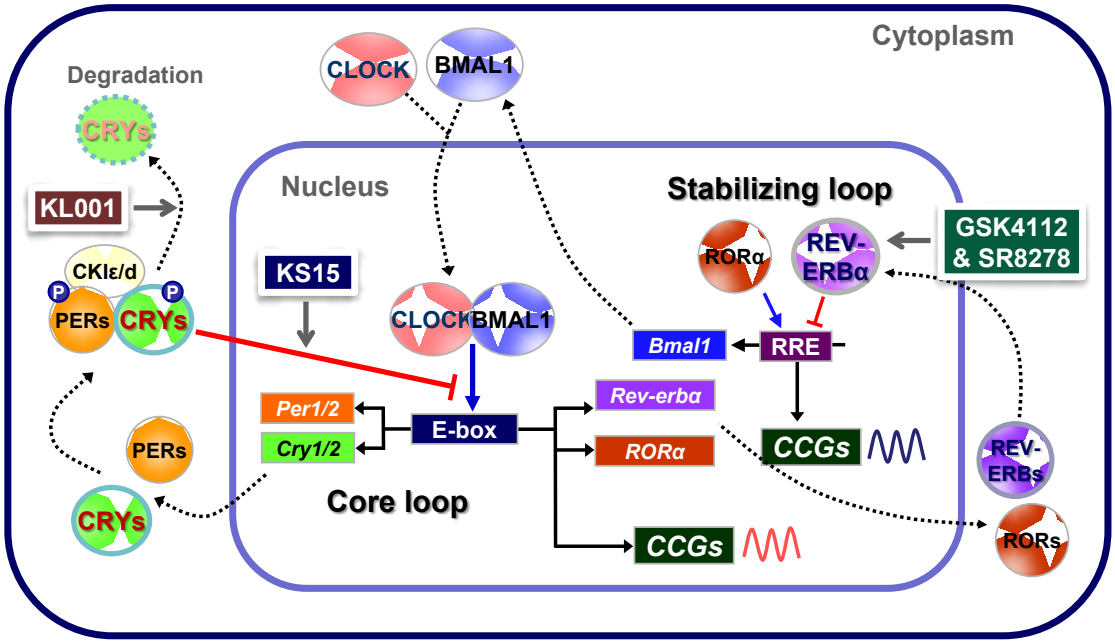


Figure 1: Molecular circadian clock in mammals. Mammalian circadian clock is composed of two interlocking transcription/translation feedback loops: i.e., core and stabilizing/auxiliary loops. CLOCK and BMAL1, the integral components of the core loop form a heterodimer, then induce E-box-mediated transcription of the negative regulators *PERIODs* (PERs) and *CRYPTOCHROMEs* (CRYs). Accumulated PER and CRY proteins repress E-box-mediated transcription until they are sufficiently cleared by proteasome-mediated degradation. In addition, CLOCK and BMAL1 also control the expression of the nuclear receptors RORs and REV-ERBs, which modulate the *BMAL1* mRNA levels by competitive actions on the RRE element residing in the *Bmal1* promoter. Collectively, the cycling of clock components determines the levels of various Clock-Controlled Genes (CCGs) by transcription via the E-box and/or RRE, thus generating rhythmic physiological outputs. Among these core clock proteins, we will mainly focus on CRYs and REV-ERBs that have been recently identified as targets of small molecule modifiers of the circadian clock. CCG: clock-controlled genes; KL001: a CRYs stabilizer; KS15: a CRY inhibitor; GSK4112 and SR8278: synthetic ligands for REV-ERBs.

[19-21]. The importance of PERs phosphorylation by CKIδ/ε for circadian rhythms has been manifested in humans; it was discovered that Familial Advanced Sleep-Phase Syndrome (FASPS) is caused by a mutation in the CKIδ/ε binding site of PER2 [20]. Similarly, with PERs, phosphorylation-dependent degradation of CRY proteins is also important for clock period. Mutation approaches identified the F-box protein-encoding gene *Fbxl3*, of which abrogation leads to long-period phenotypes in mice [22,23]. FBXL3 causes ubiquitination of CRY proteins, thereby targeting them for proteasome-mediated degradation [24]. Delayed expression of CRY proteins can be attributed to the combinatorial effect of the active degradation as well as delayed transcription activation.

CLOCK and BMAL1 proteins are also subjected to post-translational regulations. For example, rhythmic phosphorylation of the BMAL1 protein by casein kinase 2α (CK2α) was shown to be required for acetylation of BMAL1 and cyclic gene transcription by the CLOCK/BMAL1 complex in both central and peripheral clocks [25,26]. Sumoylation of BMAL1 promotes its interaction with CLOCK, exclusive nuclear accumulation in promyelocytic leukemia (PML) nuclear bodies, transactivation, and ubiquitin-dependent degradation [27,28]. On the other hand, Ca^{2+}-dependent protein kinases C (PKC)-mediated phosphorylation of CLOCK and subsequent recruitment of transcription co-factors such as p300 and CBP appear to be important for the phase resetting of periodic clock mRNA expression [29-31]. Another level of post-translational regulation of CLOCK and BMAL1 involves protein acetylation, which is also associated with epigenetic controls of gene expression [32,33]. Taken together, post-translational regulation of core clock proteins are important for precise control of circadian rhythms, and several small molecules targeting these regulators has been identified to modulate the period of the molecular rhythm [34].

Small Chemical Compounds Modulating Molecular Circadian Clock

Small molecule modifiers of the circadian clock

Since accumulating evidence based on human genetic and animal studies points to the pivotal roles of the circadian clock in affecting a variety of biological functions and dysfunctions, it is not surprising that accompanying studies have attempted to pharmacologically fix a broken clock for therapeutic purposes. Earlier studies have utilized the screening strategies based on phenotypic assays to identify small molecule modifiers inducing changes in key parameters of biological rhythmicity such as period, phase, and amplitude. For instance, the circadian reporter assays monitoring bioluminescence driven by rhythmic clock genes such as *Bmal1* and *Per2* enabled high-throughput screenings of large scale chemical libraries. However, the functional screenings more prominently resulted in hit compounds that indirectly influence the upstream input pathways or any outputs with feedback functions to the central oscillators such as cellular metabolism, rather than target the core component of the molecular clock. Indeed, several compounds were identified to alter circadian period at the cellular levels through such chemical biology approaches, but many of the compounds modifying the period were a series of CKI inhibitors [35-37]. By contrast, small molecules shortening the period acted on a wide range of targets including microtubule, DNA topoisomerase II, PKC, GSK-3β, and calcium channel, indicating the involvement of indirect or feedback mechanisms to the core oscillators [35,38]. Some of small molecules also influenced the circadian phase [37]. Identification of such targets, which influence the core circadian oscillators and can be also pharmacologically targeted, greatly contributed to our understanding of the post-translational mechanisms underlying the circadian clock and expanding a repertoire of clock modifiers by revealing novel clock regulatory pathways. However, there have been attempts to develop small molecule modifiers directly targeting the core components of the mammalian circadian clock with more benefits for therapeutic applications.

CRYs: Key targets of chemical compound acting on the core loop

Various small molecules influencing molecular circadian properties of living cells have been identified from the 2000s as noted above. Nevertheless, all of the compounds acted via signaling molecules that can post-translationally modify a certain component(s) of the mammalian circadian clock. Since structural features and critical domains of core clock proteins has been successfully identified, it has been also suggested that some of them may be targeted by small chemical probes. In this regard, identification of a key chemical scaffold capable of specifically binding to CRY proteins is remarkable.

KL001 and its derivatives: Small molecule activators/stabilizers of CRY proteins: KL001, a carbazole derivative was identified to bind to both isoforms of CRYs. It was the first small molecule targeting the core components of the mammalian molecular clock [39]. To identify small molecule modulators of the circadian clock, Kay and colleagues had examined the effects of ~60,000 structurally diverse compounds on molecular circadian rhythms using a *Bmal1* promoter-driven luciferase reporter, and then found three related carbazole derivatives (denoted as KL001 to KL003) to lengthen the period. Subsequent studies demonstrated a direct binding of these compound with CRY proteins and continuous treatment with them led to significant period lengthening and amplitude reduction of both *Bmal1* and *Per2* promoter activities in cultured SCN explants and fibroblast cells, implying activation of endogenous CRY proteins. KL001, the representing compound among three (Table 1) could bind to CRY protein through the FAD-binding pocket, which is known to be recognized by FBXL3 and mediate proteasomal degradation [22,24,40]. The co-crystal structure of the KL001-CRY2 complex revealed that KL001 compete with FAD to occupy the FAD binding site and then interferes with the binding of FBXL3 C-terminal to CRY, thereby stabilizing CRY proteins [39,41].

As KL001 was the first-in-class molecule targeting mammalian CRY proteins, it served as an initial scaffold for understanding the Structure-Activity Relationship (SAR) analyses and identification of more potent and efficacious derivatives. Indeed, accompanying SAR studies have developed several KL001 derivatives with better pharmacokinetic properties. Kay and colleagues carried out extensive SAR analyses of KL001 derivatives leading to the development of a highly active derivative denoted as KL044 (Table 1) [42]. KL044 provided a potent chemical probe applicable for studies on CRY protein functions, and moreover the 3D-QSAR models with KL044 in combination with the CRY-KL001 co-crystal structure greatly expanded our understanding of the mechanistic features of CRY regulation. Interestingly, another SAR study discovered a series of period-shortening compounds based on their own panel of KL001 derivatives [43]. In the study, they employed cutting-edge C-H activation chemistry to construct a focused library of KL001 derivatives, and identified several period-shortening KL001 derivatives such as GO044, GO200 and GO211. Considering that CRY1 or CRY2 knock-out showed opposite effect on the period length, it is plausible that period-lengthening and shortening derivatives of KL001 may have differential affinity to either of CRY isoforms.

KS15, the first CRYs inhibitor: Independently of KL001-based SAR studies to develop CRY activators/stabilizers, we have recently

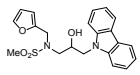
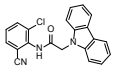
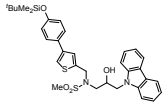
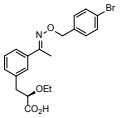
Name	KL001	KL044	GO200	KS15
Structure				
Actions	• Stabilizer • Period lengthening	• Stabilizer • Period lengthening	• Stabilizer • Period shortening	• Inhibitor • Amplitude dampening
EC_{50}/IC_{50}	• 0.69 μM (Period effect)	• 0.048 μM (Period effect)	• N.D.	• 0.49 μM (E-box-mediated transcription)
Potential applications	• Metabolic disorders • Obesity (weight and fat loss)		• N.D.	• Cancers
References	[39,41]	[42]	[43]	[44,90]

Table 1: Synthetic CRYs modulators.

identified a chemical scaffold inhibiting the feedback actions of CRYs to CLOCK/BMAL1-mediated transcriptional activation [44]. We tried to identify novel small molecule modulators influencing the CLOCK/BMAL1 heterodimer-mediated transcription by use of the artificial E-box-driven luciferase reporter described in our previous study [45] to screen more than approximately 1000 drug-like scaffolds. A derivative of 2-ethoxypropanoic acid (initially designated as compound 15 and renamed later as KS15) was identified (Table 1). KS15 directly binds to CRY1 and CRY2 proteins as revealed by substantial pull-down assay, enhanced E-box-mediated transcription in a CRYs-dependent manner, and most importantly attenuated the circadian oscillations of *Bmal1* and *Per2* promoter activities. In contrast to KL001 and its derivatives strengthening the feedback actions of CRYs, KS15 increased basal promoter activities, indicating that it reduces the amplitudes of the molecular rhythms by inhibiting repressive actions of CRYs on CLOCK/BMAL1-mediated cycles of transcription. Although our previous work demonstrated its binding to the C-terminal domains of CRY proteins, it is still unclear how KS15 inhibits CRYs actions. The CRYs are composed of highly conserved N-terminal photolyase homology region (PHR) and variable C-terminal extension domain [46]. Putative coiled-coil (CC) domain is located at the beginning of C-terminal tail and is highly conserved even between CRY1 and CRY2. Previous studies suggested that the C-terminal tails of CRYs including the CC domain are important for nuclear localization and interaction with other core clock proteins such as PERs and BMAL1 [46,47]. The C-terminal tail can be also recognized and phosphorylated by protein kinases such as GSK3β and MAPK to regulate stability of CRY proteins [48,49]. It is, therefore apparent that an exact and detailed inhibitory mechanism(s) of KS15 should be clarified for further SAR studies to develop derivatives with improved pharmacokinetic properties.

REV-ERBs, circadian nuclear receptors as targets of small molecule probes

The circadian nuclear receptors REV-ERBs and RORs are involved in many physiological processes including metabolism, development, immunity, and cognition as well as the circadian rhythm. Members of nuclear receptor superfamily can be considered as ligand-activated transcription factors that serve as intracellular receptors for cell-permeable ligands. The nuclear receptors have been thus considered as one of the primary molecular classes suitable for drug targets. Interestingly, both REV-ERBα and β have atypical ligand-binding domains (LBDs) and lack C-terminal activation function 2 (AF2) domain, through which transcriptional co-activators can be recruited. As a result, REV-ERBs dominantly interact with co-repressors and thus function as constitutive repressors of transcription upon binding to their target gene promoters, while RORs predominantly activate transcription of the targets. Recent studies have identified endogenous ligands for both REV-ERBs and RORs, thereby stimulating the development of synthetic ligands and their therapeutic applications to a variety of circadian rhythm-related diseases. Although both families of these nuclear receptors are required for normal functioning of the circadian timing system and believed to be therapeutic targets for circadian rhythm-related diseases as recently reviewed elsewhere [50], we will mainly focus on REV-ERBs for the following reasons. First, REV-ERBs serve as determining factors for RRE-mediated cyclic gene transcription. In spite of periodic accumulation of both REV-ERBs and RORs in many tissues, REV-ERBs usually elicit more robust rhythms in terms of amplitude and robustness of their cyclic expression [12,51]. An atypical mode of action found in REV-ERBs-mediated transcriptional repression is also considered for development of small molecule synthetic ligands. Unlike other canonical nuclear receptors that usually recognize a palindromic or a repeated copy of a core sequence (or the half site) as dimeric forms (either as homodimers or as heterodimers with retinoid X receptors), REV-ERBs mainly act as monomers and recognize only a half site containing AGGTCA sequence [50]. This features may reduce the complexity of actions and unexpected consequences by an indirect mechanism. Finally, potential developmental defects should be considered for therapeutic applications; for example, biological roles of RORα were first identified by examination of the well-known *staggerer* mutation with cerebellar lesions, which have an ataxic gait, hypotonia, and sometimes smaller size compared to littermates [52], while such developmental defects have not been, thus far reported in REV-ERBα and/or β-defective mutant mice.

Identification of an endogenous ligand for REV-ERBs: REV-ERBs were initially identified as orphan nuclear receptors, but heme is suggested as an endogenous ligand from the study with the *Drosophila* orthologue of these orphan receptors [53]. Accompanying studies have then revealed that heme can also bind to the LBD of mammalian REV-ERBs and identified key amino acid residues in REV-ERBα [54,55]. However, there is still some controversy on the precise mechanism of action of heme-dependent transcriptional repression of REV-ERBs. Nevertheless, decrease in intracellular heme levels impaired REV-ERBs-mediated transcriptional repression by reducing the recruitment of the co-repressor complex to their target gene promoters. In accordance, heme has a role in regulating the circadian functions of REV-ERBs; both intracellular heme concentration and REV-ERBs expression are regulated in a circadian manner, which suggests that periodicity found in heme availability may constitute an additional mode of the circadian activation of REV-ERBs [56]. REV-ERBs and heme are likely to form a negative feedback mechanism underlying tight control of intracellular heme levels, which is critical for proper mitochondrial functions [57]. Increased REV-ERBs expression or enhanced their activity by heme led to repression of PPARγ co-activator 1α (PGC1α)

expression, thereby reducing expression of the rate limiting enzyme for heme biosynthesis, aminolevulinic acid synthase 1 (ALAS1), while low intracellular heme levels caused impairment of REV-ERBs activity and then enhanced PGC1α-mediated biosynthesis of their endogenous ligand. The feedback mechanism underlying heme homeostasis by REV-ERBs/PGC1 α also provide a molecular basis for coordination of metabolic and circadian pathways. For example, adipogenesis was shown to be regulated by heme in a REV-ERBs-dependent manner [58]. Intracellular heme levels were increased during adipogenesis, and inhibition of heme biosynthesis inhibited adipogenesis. Another example is FGF21, a hormone produced in liver and fat that dramatically improves peripheral insulin sensitivity and lipid metabolism. PGC1α-mediated transcriptional repression of hepatic FGF21 expression was dependent on the expression of ALAS1 and REV-ERBα [59].

Development of synthetic REV-ERBs ligands: The discovery of endogenous ligand for REV-ERBs led to identification of chemical scaffolds that could be used as potent synthetic ligands. The first identified synthetic REV-ERBs ligand was GSK4112 (Table 2) [60]. It was identified by a fluorescence resonance energy transfer (FRET)-based *in vitro* assay, in which it enhanced the binding of REV-ERBα with NCOR peptide. Accompanying cell-based assays revealed that GSK4112 acted as an agonist for REB-ERBs and could increase the recruitment of NCOR and HDAC3 to the REV-ERBs target gene promoters and then repressed transcriptional activities and/or mRNA levels of the targets such as *BMAL1*, *PCK* and *PGC1α* [61]. Although GSK4112 open the chance for the development of synthetic REV-ERBs ligands, it did not exhibit a favorable pharmacokinetic profile sufficient for applications *in vivo*. In attempt to improve potency, efficacy and pharmacokinetic properties, Burris and colleagues have identified two REV-ERBs agonists (denoted as SR9009 and SR9011) that are suitable for *in vivo* applications (Table 2). Both compounds were developed from GSK4112 and successfully used as therapeutic applications of small molecule REV-ERBs modulators in the treatment of circadian-related disorders such as metabolic diseases and sleep disorders [62]. Indeed, SR9009 and SR9011 appears to be 3-fold more potent and efficacious compared with GSK4112 in *in vitro* assays. More importantly, even a single intraperitoneally administration of either compound in a rest period impaired locomotor activities during the subsequent active period and, in good agreement with the behavioral alterations, significantly affected the circadian expression of core clock genes in the murine hypothalamus, indicating that SR9009 and SR9011 sufficiently and selectively enhance REV-ERBs-mediated transcriptional repression *in vivo*.

In addition to the profound effects on the circadian timing system, it was remarkable that pharmacological activation of REV-ERBs had significant metabolic impacts on mice implying that pharmacological and systemic activation of these nuclear receptors can be a novel therapeutic strategy to treat metabolic disorders. Treatment with the REV-ERBs agonists resulted in weight loss in diet-induced obese mice, presumably due to an increase in energy expenditure without alterations in motor activity or food intake [62]. A significant decrease in circulating levels of triglycerides, total cholesterol and non-esterified fatty acids was also observed. In consistence with the metabolic alterations and substantial weight loss, a more recent study also showed that the activation of REV-ERBs with SR9009 increased oxidative metabolism and mitochondrial biogenesis in skeletal muscle *in vivo*, leading to improved exercise endurance [63]. Although chemical biology approaches have further identified a series of GSK4112 analogues to improve pharmacokinetic properties and bioavailability, it should be noted that almost all of the analogues contain a nitrothiophene group implying a potential toxicity. It is therefore obvious that toxicological properties should be extensively examined for these REV-ERBs ligands to make a next step toward clinical applications.

In contrast to the rapid expansion of REV-ERBs agonists, only one antagonist, SR8278 has been thus far identified (Table 2). SR8278 inhibited the transcriptional repression activity of REV-ERBα and REV-ERBβ in a cell-based assay using chimeric receptors composed of the DNA-binding domain of GAL4 (GAL4-DBD) fused to the ligand binding domain (LBD) of either of REV-ERBs and also in an assay that used full-length REV-ERBα and luciferase reporters driven by the target promoters of REV-ERBs [64]. Since SR8278 has limitations for its applications *in vivo*, its potential for clinical applications cannot be discussed yet. However, it provided a convenient tool to temporally inhibit REV-ERBs activity in target tissues or cells of interest. Treatment of HepG2 cells, an immortalized hepatic cell line, with SR8278 induced the expression of REV-ERBs target genes such *BMAL1*, *G6PC* and *PCK*. A subsequent study revealed that activation of REV-ERBα by heme and/or synthetic agonists stimulated glucose-induced insulin secretion in MIN-6 mouse insulinoma cells, and the effects could be antagonized by SR8278 [65]. More recently, we revealed that REV-ERBα expressed in midbrain dopaminergic (DAergic) neurons have a key role in controlling DA-dependent emotional phenotype. Local inhibition of REV-ERBα activity by infusion of SR8278 into the ventral midbrain was sufficient to produce mania-like behaviors as well as hyper DAergic state as REV-ERBα-defective mutant mice did [14].

Therapeutic Implications in Circadian Rhythm-Related Diseases

Because the circadian system underlies a wide range of biological phenomena, and these are hierarchically organized, it may not be

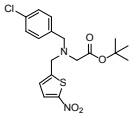
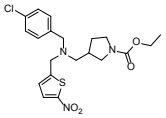
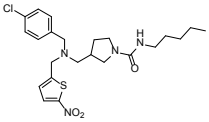
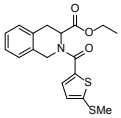
Name	GSK4112	SR9009	SR9011	SR8278
Structure				
Actions	• Agonist • Suppressing RRE-mediated transcription	• Agonist • Suppressing RRE-mediated transcription	• Agonist • Suppressing RRE-mediated transcription	• Antagonist • Increased RRE-mediated transcription
EC_{50}/IC_{50}	• 2.3 μM (Bmal1-Luc)	• 0.71 μM (Bmal1-Luc)	• 0.62 μM (Bmal1-Luc)	• 2.3 μM (Bmal1-Luc)
Potential applications	• Limited *in vivo* exposure	• Metabolic disorders including. obesity • Sleep and anxiety disorders		• Cancers
References	[60,61]	[62]	[62]	[64]

Table 2: Synthetic ligands for REV-ERBs.

surprising that a robust circadian timing is prerequisite for human health and disruption of the intrinsic rhythms leads to a diverse pathological states. Indeed, sleep and circadian disruptions are widely observed in association with various human diseases [4,5]. Increasing evidence obtained from studies on mutant mice as well as human genetic studies points to the causal role of circadian dysfunctions on the onset and symptoms of such circadian rhythm-related human diseases. Even before the discovery of molecular and genetic basis of the circadian rhythms, environmental strategies to fix or strengthen the intrinsic rhythmicity such as bright light therapy, sleep deprivation and scheduled meals has been often employed to treat such diseases [66]. In this regard, small molecule modifiers of the molecular circadian clock can be used to rescue de-synchrony of the intrinsic rhythms causing the circadian-related diseases and/or to optimize the internal time for a certain pharmacological treatment. In this section, we are summarizing some examples for the current efforts to utilize clock modulators for therapeutic applications.

Metabolic and cardiovascular diseases

It is well established that circadian dysfunctions are tightly linked with a wide spectrum of metabolic and cardiovascular diseases such as obesity, type 2 diabetes, and atherosclerosis. In accordance with numerous epidemiological studies, mutant mice bearing defective clock genes have revealed integral roles of molecular circadian clock in metabolic regulation. The first direct link between circadian clock and metabolism was discovered in mice with *Clock*$^{\Delta 19/\Delta 19}$ mutation. Along with attenuated diurnal feeding rhythm as well as arrhythmic locomotor behaviors under constant darkness, the *Clock*-defective mutant mice displayed abnormal metabolic phenotypes including hyperphagia, hyperlipidemia, hyperleptinemia, hepatic steatosis and hyperglycemic hypoinsulinemia [67,68]. Tissue-specific manipulation of core clock proteins has provided strong evidence supporting that peripheral oscillators have key roles in maintaining the metabolism and energy balance, while their dysfunctions lead to the onset of metabolic diseases through the extensive disruption of orchestrated gene expression in metabolic organs. For example, hepatocytes-specific abrogation of *Bmal1* gene resulted in loss of periodic expression of key metabolic genes and more importantly, hypoglycemia during the rest periods due to impaired gluconeogenesis and exaggerated glucose clearance [68,69]. Overexpression of the *ClockΔ19* allele in cardiomyocytes altered heart rate variability and contractility, indicating a role of the peripheral circadian gene network in the control of myocardial functions [70]. Mice lacking *Rev-erbα* also exhibited altered lipid and bile metabolism [71], whereas liver-specific overexpression of REV-ERBα caused alterations in expression profiles of genes mediating xenobiotic detoxification, carbohydrate and energy metabolism, and lipid homeostasis [72].

Considering the reciprocal links between circadian clock and metabolism, small molecule modifiers of circadian clock identified thus far, have targeted metabolic disorders as well as circadian misalignment for their therapeutic applications. Kay and colleagues demonstrated impacts of KL001, a CRYs activator/stabilizer on hepatocyte carbohydrate metabolism and its therapeutic potential for diabetes [39]. Indeed, CRY proteins are known to regulate fasting hormone–induced transcription of the *Pck1* and *G6pc* genes encoding rate-limiting enzymes of gluconeogenesis [73,74], and *CRY2* gene locus is significantly associated with fasting blood glucose concentrations and presentation of type 2 diabetes in the human genome-wide association studies [75]. KL001 suppressed glucagon-dependent induction of *Pck1* and *G6pc* genes without affecting their basal expression in cultured mouse primary hepatocytes and repressed glucagon-mediated activation of glucose production, suggesting the potential of KL001 to control fasting hormone-induced gluconeogenesis [39].

In addition to the CRYs activator, pharmacological ligands for the circadian nuclear receptors were also shown to be beneficial to modulate body metabolism *in vivo*. Burris and colleague demonstrated the metabolic effects of their REV-ERB agonists (SR9009 and SR9011) in mice models. Chronic treatment with these agonists resulted in weight loss and reduced fat mass with increased energy expenditure. In accordance, modulation of REV-ERBs activity by the synthetic agonist significantly altered daily expression of an array of genes related to glucose and lipid metabolism, collectively implying decreased lipogenesis and cholesterol/bile acid synthesis in the liver, increased lipid and glucose oxidation in the skeletal muscle, and decreased triglyceride synthesis and storage in the adipose tissues. Notably, REV-ERB agonists were also effective in a high fat diet-induced obesity model. Repeated treatment with the compounds significantly decreased plasma glucose, triglycerides, total cholesterol, non-esterified fatty acids and leptin, leading to a severe reduction in body weight and adiposity in the rodent model of obesity [62]. Therefore, it is plausible that pharmacological activation of CRYs or REV-ERBs may provide a first-in-class therapeutic strategy to treat obesity and related metabolic and cardiovascular diseases in near future.

Sleep disorders

Dysregulations of circadian rhythmicity are associated with neuropsychiatric disorders including depressive disorders, anxiety, schizophrenia, and sleep disorders [76]. It is still unclear whether the impacts of the circadian timing system on various brain functions related to these disorders are manifested as an output of the dysregulated circadian rhythm or represent additional functions independent of timekeeping. It has been, however, suggested that effects of the mammalian clock genes on brain functions are attributable to local oscillators located in discrete brain regions as well as the SCN central clock.

Given that circadian timing system is one of two major mechanistic facets of sleep, small molecule modulators of molecular clock may greatly contribute to better understanding of sleep. Indeed, abnormal sleep phenotypes have been reported for defective alleles of core clock genes and also of various genes involved in post-translational controls of the clock proteins in both human and rodents [77]. For example, it is well known that FASPS can be caused by phosphorylation-defective mutations in human PER2 gene and also by mutant alleles for protein kinases such as CK1δ acting on the sites as mentioned above [20,21]. A variation in human *PER3* gene was also reported to be associated with differences in sleep homeostasis particularly after sleep deprivation [78]. Therefore, one may expect that pharmacological manipulation of circadian clockworks can be used for treatment of circadian rhythm-related sleep disorders including sleep fragmentation, abnormal sleep phase syndromes and non-24-hour sleep-wake rhythm disorders. In this regard, it should be noted that manipulation of REV-ERBs expression/activity resulted in significant alterations in sleep architecture. *Rev-erbα* knock-out mice displayed advanced sleep/wake distributions relative to the light-dark cycle and reductions in both EEG delta power and sleep consolidation after sleep onset in baseline and also after sleep deprivation, implying a slower increase of homeostatic sleep need during wakefulness [79]. In association with the notion, pharmacological activation of REV-ERBs during daytime significantly induced wakefulness and suppressed both slow-wave sleep (SWS) and rapid eye movement (REM) sleep [80].

Mood and anxiety-related disorders

Affective disorders such as Major Depressive Disorder (MDD), Bipolar Disorder (BPD) and Seasonal Affective Disorder (SAD) would be one of the most attractive targets for small molecule modulators of circadian clock to be used for therapeutic purpose. Patients with affective disorders such as BPD and MDD commonly suffer from disrupted sleep-wake cycles and dysregulated diurnal mood variations [81,82]. A variety of human genetics studies has reported close associations of clock genes with the onset rates and the symptoms of the mood-related disorders. It has been well established that midbrain DAergic circuits are key mediators of circadian modulation of mood as well as therapeutic targets for the mood disorders. For instance, *Clock*$^{\Delta 19/\Delta 19}$ mice showed mania-like behaviors, characterized by hyperactivity, decreased depression- and anxiety-related behaviors and higher cocaine sensitization with enhanced DAergic transmission [83]. More recently, we have revealed that REV-ERBα plays a key role in linking the molecular circadian clock with the mood-controlling DAergic system by demonstrating mania-like phenotypes of *Rev-erbα*-deficient mice [14]. Considering *Rev-erbα* gene expression is primarily controlled by CLOCK/BMAL1 heterodimer in a periodic manner and highly attenuated in the *ClockΔ19* mice [84], it is most plausible that REV-ERBα expressed in the DAergic neurons may have some determining roles. REV-ERBα evoked daily variations in DA biosynthesis through circadian control of tyrosine hydroxylase (TH) expression, which is regarded as a rate-limiting enzyme for catecholamine biosynthesis. It is also important that, in this process REV-ERBα compete with NURR1, a well-known nuclear receptor prerequisite for DAergic neuronal development and functions, to produce circadian transcription of sets of DAergic genes, indicating a more versatile regulator of nuclear receptor signaling and functional circadian outputs than previously thought. In addition to DA production, monoamie oxidase (MAO) mediating DA clearance in the postsynaptic sites were found to be under the circadian control of NPAS2 and BMAL1 [85]. Taken together, these findings indicate that biosynthesis, transmission and even turnover of DA are tightly controlled by the circadian clock.

In addition to the evidence obtained from the mutant mice, even acute administration of REV-ERB antagonist (SR8278) to the ventral midbrain was sufficient to produce mania-like behaviors with increased DA production. Furthermore, the pharmacological inhibition of REV-ERBs activity increased neural firings of midbrain DA neurons, thereby leading to enhanced DA release. This finding strongly suggest that REV-ERBα expressed in the DAergic neurons appears to control DA production and release in synchrony [14]. However, it is not a simple situation because both REV-ERBs agonist and antagonist reduced anxiety-like behaviors in wild-type mice, while REV-ERBs agonist did not produce decreased despair-based behaviors [14,80]. The discrepancy may be originated from the presence of two REV-ERB isoforms and the synthetic ligands thus far act on both isoforms. In contrast to our previous study with REV-ERBs antagonist micro-infused selectively into midbrain regions, Banerjee et al. [80] systemically treated mice with REV-ERBs agonist. In spite of co-expression of both isoforms in many cell types, REV-ERBα appears to be dominantly expressed in the ventral midbrain, where DA neurons are located. Therefore, it is plausible that systemically injected REV-ERB agonists may differentially act on other targets affecting anxiety such as hypothalamic nuclei, some cortical regions, amygdala, habenula and even peripheral tissues including the adrenal gland producing stress hormones. These findings collectively suggest a therapeutic potential of the circadian clock modulators for the emotion-related disorders, but are also warning a potential risk in their clinical applications to circadian rhythm-related sleep and metabolic diseases.

Cancer

A close relation between circadian dysfunction and the risk of tumorigenesis is now widely accepted and strongly supported by extensive epidemiological studies. Indeed, chronic shift work or repeated exposures to jet-lag has been reported to be associated with increased incidence of breast cancer in women, and similarly also significantly to be correlated with the risk of developing prostate cancer among middle-aged men. In accordance with the findings, genetic mice models and human clinical studies provided more direct evidence supporting that the dysregulation of circadian clock by either genetic or epigenetic mechanism is virtually linked with various types of cancer models as reviewed elsewhere [86]. The relationship appears to be reasonable because both molecular circadian rhythm and cell cycle constitute major components of cellular cycling and, more importantly they are coupled with each other. Major pathways commonly involved in various types of oncogenesis usually altered the molecular clock, whereas many proto-oncogenes and/or tumor suppressors including RAS, p53 and MYC are normally under the tight control of the cellular clock machinery [87]. Considering the close relationship and circadian features found in pharmacokinetics and pharmacodynamics of anticancer agents, chronotherapeutic strategies based on an individual property of the circadian timing system have been claimed for efficacious treatment of cancers [88].

Identification of small molecule clock modulators added an additional possibility to the chronotherapeutic approaches to cancer by providing pharmacological tools to actively adjust cellular states for the efficacious actions of a given anti-cancer agent. In this context, it is noteworthy that dual inhibition of REV-ERBβ and autophagy was proposed as a novel pharmacological approach to induce cytotoxicity in cancer cells [89]. Grimaldi and colleagues in their study found that REV-ERBβ expression was increased and predominant in a number of tumor types including several breast cancer cell lines compared with non-cancer control cells. Overexpressed REV-ERBβ appeared to have a protective function, particularly conferring resistance to chloroquine, a clinically relevant lysosomotropic agent suppressing autophagy. Based on these findings, they identified an ARN5187 with dual inhibitory activity toward RRE-mediated transcriptional repression and autophagy as well. Subsequently, they clearly demonstrated potency and efficacy of this novel lysosomotropic REV-ERBβ ligand in eliciting cytotoxicity in cancer cells. More recently, we also demonstrated that pharmacological inhibition of CRY proteins displayed anti-proliferative and pro-apoptotic actions on certain types of human breast cancer cells [90]. Treatment with KS15 altered expression profiles for various cell cycle regulators, proto-oncogenes and tumor suppressor genes in well accordance with reduced cellular growth and viability in MCF-7 cells, but not in MCF-10A cells. More importantly, KS15 increased chemo sensitivity of MCF-7 cells to anti-tumor drugs such as doxorubicin and tamoxifen. Even though finding cancer-specific alterations in cellular clockworks is not sufficient to have anti-cancer effects by itself, combinational uses of circadian clock modulators with well-established anti-cancer drugs can be more beneficial by enhancing potency and efficacy, and reducing toxicity of the drugs. It is therefore plausible that characterization of a mode of circadian disruption in a given tumor type and an accompanying application of proper clock modulators combined with anti-cancer agents may provide practical approaches to a broad array of drug-resistant cancers.

Concluding Remarks

Circadian clock is evolutionarily conserved internal timekeeping machinery that adapts internal systems to daily environmental

cycles. The clock network is present in almost all tissues and governs a remarkable variety of biochemical, physiological, and behavioral processes. A growing body of evidence indicates that proper function of central and peripheral clocks is crucial for the well-being of the organism. Disruption of circadian rhythmicity or misalignment of internal oscillators has been implicated in the pathogenesis of human diseases. Therefore, a better understanding of the role of the molecular clock in regulation of daily physiological processes will enable development of novel treatment schemes, more efficacious therapeutic delivery, and better preventative strategies for management of the circadian rhythm-related diseases. In this regard, identification of chemical clock modulators has provided valuable tools to better understand the functional relevance of the molecular clockwork by reversibly controlling circadian clock in certain tissues of interest, and more importantly to serve as lead structures for developing novel therapeutics applicable for the numerous circadian rhythm-associated diseases. It can be therefore postulated that the concept of "classical chronotherapy" considering time-of-day effects in terms of pharmacokinetics and pharmacodynamics can be expanded to the "active chronotherapy", in which the internal time can be pharmacologically manipulated to be optimized for a certain pharmacological treatment.

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Conflict of Interest

There is no conflict of interest.

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