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Identification of a novel circadian clock modulator controlling BMAL1 expression through a ROR/REV-ERB-response element-dependent mechanism

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ABSTRACT

Circadian rhythms, biological oscillations with a period of about 24 h, are maintained by an innate genetically determined time-keeping system called the molecular circadian clockwork. Despite the physiological and clinical importance of the circadian clock, development of small molecule modulators targeting the core clock machinery has only recently been initiated. BMAL1, a core clock gene, is controlled by a ROR/REV-ERB-response element (RORE)-dependent mechanism, which plays an important role in stabilizing the period of the molecular circadian clock. Therefore, we aimed to identify a novel small molecule modulator that regulates *Bmal1* gene expression in RORE-dependency, thereby influencing the molecular feedback loop of the circadian clock. For this purpose, we carried out a cell-based screen of more than 1000 drug-like compounds, using a luciferase reporter driven by the proximal region of the mouse *Bmal1* promoter. One compound, designated KK-S6, repressed the RORE-dependent transcriptional activity of the m*Bmal1* promoter and reduced endogenous BMAL1 protein expression. More importantly, KK-S6 significantly altered the amplitude of circadian oscillations of *Bmal1* and *Per2* promoter activities in a dose-dependent manner, but barely affected the period length. KK-S6 effectively decreased mRNA expression of metabolic genes acting downstream of REV-ERBα, Pai-1 and Citrate synthase, that contain RORE *cis*-element in their promoter. KK-S6 likely acts in a RORE-dependent manner by reinforcing the REV-ERBα activity, though not by the same mechanism as known REV-ERB agonists. In conclusion, the present study demonstrates that KK-S6 functions as a novel modulator of the amplitude of molecular circadian rhythms by influencing RORE-mediated BMAL1 expression.

1. Introduction

Most biological processes in mammals have circadian rhythms with periods of about 24 h. The circadian timing system is organized hierarchically into multiple oscillator networks. Coordination of the circadian system across the body involves regulation of signals emanating from the suprachiasmatic nuclei (SCN), a central, master pacemaker in the anterior hypothalamus, and subsidiary clocks in the extra-SCN brain regions and peripheral organs, which regulate physiological and behavioral outputs [1,2].

Abbreviations: BMAL1, brain-muscle-ARNT-like protein 1; CLOCK, circadian locomotor output cycles kaput; CRY, cryptochrome; DEX, dexamethasone; DMSO, dimethylsulfoxide; MEFs, mouse embryonic fibroblast cells; PER, period; ROR, retinoic acid receptor-related orphan receptor; RORE, ROR/REV-ERB-response element; SCN, suprachiasmatic nuclei.

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The circadian molecular clockwork in conserved among most cells, and consists of two interlocked positive/negative feedback loops. In the core clock loop, CLOCK and BMAL1 form heterodimers that positively regulate the transcription of *Periods* (*Per1*, *Per2*, and *Per3*) and *Cryptochromes* (*Cry1* and *Cry2*) genes by activating a *cis*-element, the E-box, in the promoters of these genes. PERs and CRYs are phosphorylated by casein kinases and can dimerize [3]. This complex translocates into the nucleus where it negatively regulates the transcriptional activity of the CLOCK:BMAL1 heterodimer. In addition, the circadian nuclear receptors, RORs and REV-ERBs, compete for binding of the RORE in the *Bmal1* promoter region; REV-ERBs suppress *Bmal1* transcription, whereas ROR induces it, forming a stabilizing loop [4]. In addition to the transcriptional regulation underlying the molecular makeup of the clock machinery, the post-translational modification processes, including the phosphorylation [5], ubiquitination, sumoylation [6], signal transduction [7], and subcellular trafficking [8] of the clock proteins have been extensively studied.

Extensive studies on the phenotypes of mutant mice with defective clock genes, as well as pathophysiological consequences of circadian disruption, have highlighted the therapeutic potential of circadian molecular clockwork for treating a wide range of human diseases such as metabolic disorders, cardiovascular diseases, immune system dysfunction, neuropsychiatric disorders, and even cancers. Both endogenous and synthetic small molecules have recently been identified as circadian clock modulators. For example, heme [9], cAMP [10], and NAD [11] have been shown to affect the periodicity of the molecular circadian clock. Casein kinase I inhibitors such as longdaysin [12] and LH846 [13] were also shown to change the circadian period. More recently, small molecules directly targeting core clock components have been developed; synthetic ligands of REV-ERBs, including GSK4112, SR8278, SR9009, and SR9011 [14–16] were developed as promising molecular clock modulators. Small molecules stabilizing CRYs, such as KL001 and its derivatives, suppress the transcriptional activity of the CLOCK:BMAL1 heterodimer by strengthening the negative feedback inhibition of CRYs, and are currently in preclinical or clinical trials [17]. By screening our library of drug-like chemical compounds, we recently identified KS-15, which can directly bind to CRYs and impair their feedback of the CLOCK:-BMAL1 heterodimer [18].

In the present study, we develop a novel modulator, KK-S6 that selectively affects RORE-mediated transcriptional activity by cell-based screening of our in-house chemical library. We also validated its functional significance and REV-ERBα-dependency, providing a novel mechanism in the control of circadian rhythms through modulating the RORE-mediated activity by KK-S6.

2. Materials and methods

2.1. Plasmid construction

The minimal mouse *Bmal1* promoter was fused to the luciferase reporter (wtBmal1::Luc) by inserting a 171-bp proximal fragment of the mouse *Bmal1* promoter containing two copies of *RORE* (−106 to +65 from the transcriptional start site) into the pGL3-promoter vector (Promega) by use of the *Xho*I and *Nhe*I restriction sites. Primer sequences are shown in Table 1. The RORE-mutated reporter constructs (m1Bmal1::Luc, m2Bmal1::Luc, and m1/2Bmal1::Luc) were generated by site-directed mutagenesis with the primers (Table 1).

2.2. Cell culture

NIH3T3 and HepG2 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen) at 37 °C under 5% CO_2. NIH3T3 cells were transfected with the wild-type or mutated reporter along with the pcDNA3.1 plasmid, and stably transfected cells were established by selection in the presence of G418 (400 μg/ml) for 3 wks. Stably transfected cells were then maintained in medium (DMEM supplemented with 10% FBS and 2% penicillin/streptomycin) containing G-418 (100 μg/ml).

2.3. Measurement of luciferase reporter activity

For the conventional luciferase reporter assay, each indicated luciferase reporter and expression construct was co-transfected together with 400 ng of a *Renilla* luciferase reporter construct (pRL-basic), as an internal control for transfection efficiency, into NIH3T3 cells using Lipofectamine Plus reagent (Invitrogen). After 48 h, cell extracts were prepared by incubation in 0.1 mL of 1 × lysis buffer (Promega) for 20 min at room temperature. Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay Kit (Promega). Reporter-driven firefly luciferase activities were normalized relative to *Renilla* luciferase levels.

For real-time bioluminescence monitoring, stably transfected cells were seeded in 35 mm dishes. Cell cultures were synchronized using 1 μM DEX for 2 h before and their medium was replaced with fresh medium containing 0.1 mM D-luciferin (Promega) and either vehicle (0.05% DMSO) or chemical compounds at the indicated concentrations [18].

2.4. Cell-based screening of in-house chemical library

A small, druggable, scaffold-based library containing 1128

Table 1
Sequences of PCR primers used in the generation of mutated RORE reporter constructs and amplification of genes acting downstream of REV-ERBα.

Primer name		Sequence
1 **Primers for site-directed mutagenesis**		
RORE1 mutant	F	5′-CGG ATT GGT CGG AAA GTA **CA**T TAG TGG TGC GAC ATT TAG-3′
	R	5′-CTA AAT GTC GCA CCA CTA A**TG** TAC TTT CCG ACC AAT CCG-3′
RORE2 mutant	F	5′-GGA AGG CAG AAA GTA **CA**T CAG GGA CGG AGG C-3′
	R	5′-GCC TCC GTC CCT GA**T G**TA CTT TCT GCC TTC C-3′.
2 Primers for RT-PCR		
Bmal1	F	5′-TTA AGA GGT GCC ACC AAT CC-3′
	R	5′-TTC CCT CGG TCA CAT CCT AC-3′
Pai-1	F	5′-AAG ACT CCC TTC CCC GAC TC-3′
	R	5′-GGG CGT GGT GAA CTC AGT ATA G-3′
Citrate synthase	F	5′-TAG TGC TTC CTC CAC GAA TTT G′-3′
	R	5′-CCA CCA TAC ATC ATG TCC ACA G-′3′
Tbp	F	5′-CGG CTG TTT AAC TTC GCT TC-3′
	R	5′-TTC TTG GCA AAC CAG AAA CC-3′

compounds was described in our previous study [18]. Each compound was dissolved in DMSO and used at the indicated dosage. Compounds were screened by a cell-based assay using the wtBmal1::Luc-transfected NIH3T3 cells in 24-well plates. Transfected cells were treated with vehicle (0.05% DMSO) or chemical compounds for 24 h.

2.5. RNA isolation and real-time quantitative PCR

RNA expression was analyzed according to our previous study [19]. Total RNA was isolated using the single-step acid guanidinium thiocyanate-phenol-chloroform method. Real-time PCR was carried out in the presence of SYBR Green I (Sigma). Gene expression levels were normalized against expression of TATA box-binding protein (*Tbp*). Primer sequences used for real-time RT-PCR are shown in Table 1. The reactions were first incubated at 94 °C for 10 min, followed by 50 cycles at 95 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s.

2.6. Western blot analysis

Whole-cell extracts were separated using 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) in a Bio-Rad Trans-Blot electrophoresis apparatus using Towbin's buffer. The blots were blocked in 1 × Tris-buffered saline containing 0.3% Tween-20 with 10% skimmed milk, then incubated with primary antibody at room temperature for 90 min. They were then washed four times with 1 × TBS/0.3% Tween-20. Antibody binding was subsequently detected by incubation with secondary antibodies linked to horseradish peroxidase (Jackson Immuno Research Laboratories). Expression levels were visualized using an ECL detection kit (Pierce). Anti-BMAL1 and anti-α-actin (s.c.-56459; Santa Cruz) were obtained commercially. Anti-BMAL1 antibody was raised by immunization of rabbits and affinity-purified, as described previously [20].

2.7. Statistical analysis

Data were statistically evaluated by Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey post-hoc tests using GraphPad Prism software. P-values <0.05 were considered to be significant. All data shown are means ± SEM.

3. Results

3.1. Characterization of the wild-type and mutated luciferase reporters

To identify novel circadian clock modulators affecting BMAL1 expression in an RORE-dependent manner, we prepared wild-type and RORE-mutated luciferase reporter constructs (wtBmal1::Luc, m1Bmal1::Luc, m2Bmal1::Luc, and m1/2Bmal1::Luc) based on the 171 bp proximal promoter region of the mouse *Bmal1* gene (Fig. 1A). The short *Bmal1* promoter region, containing minimal promoter elements and the essential RORE *cis*-element required for rhythmic

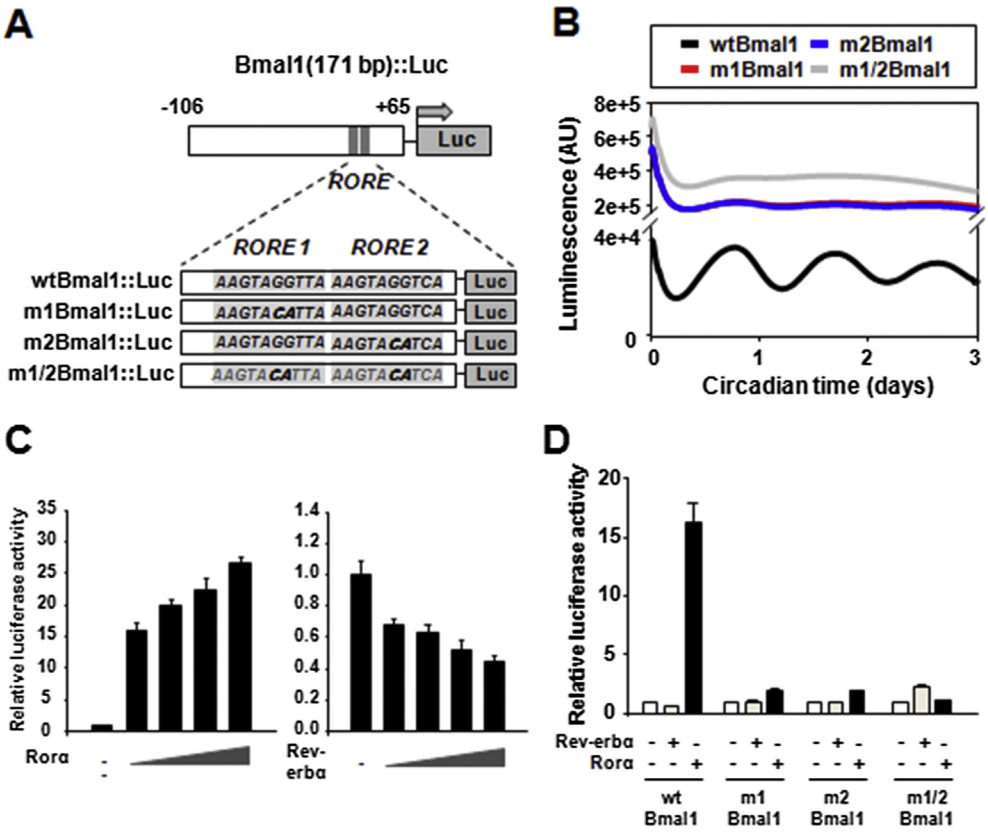

Fig. 1. Validation of the luciferase reporter harboring the minimal *Bmal1* promoter region. (A) A schematic representation of the Bmal1::Luc reporter construct with wild-type (wtBmal1::Luc) or abrogated (m1Bmal1::Luc, m2Bmal1::Luc, and m1/2Bmal1::Luc) RORE. (B) Circadian oscillations on wild-type RORE or mutated RORE luciferase reporter in fibroblast cells measured using a real-time bioluminescence monitoring system. (C) Dose-dependent changes in the transcriptional activity of the wtBmal1::Luc reporter when co-expressed with ROR or REV-ERBα in NIH3T3 cells. (D) Comparison of changes in wild-type RORE and mutated RORE luciferase activity by ectopic expression of REV-ERBα or RORα. (n = 6).

transcription, was employed to minimize the effects mediated by other enhancer elements. Cultured NIH3T3 cells transfected with the wild-type reporter displayed a circadian oscillation when induced by DEX, which requires intact RORE elements (Fig. 1B). Transient expression of RORE-acting transcriptional regulators also influenced the wtBmal1::Luc reporter activities in a RORE-dependent manner: RORα induced reporter activity, whereas REV-ERBα repressed it (Fig. 1C). On the contrary, a GG/CA mutation in RORE1 and/or RORE2 completely abrogated the effects of REV-ERBα or RORα on reporter activity and circadian oscillation profiles (Fig. 1D).

3.2. Identification of a synthetic compound modulating the RORE-dependent Bmal1 transcription

In an initial attempt to identify circadian modulator(s), we conducted a screen of 1128 previously described compounds [18] at 20 μM using wtBmal1::Luc-transfected cells (data not shown). Intriguingly, a series of structurally-similar compounds was shown to significantly alter the reporter activity, and was designated the KK-S series of compounds (Fig. S1). We then investigated the dose–response relationships of selected compounds in the KK-S subfamily (designated as KK-S1–S10) at concentrations ranging from 0.02 to 20 μM. All compounds except KK-S5 produced a dose-dependent decrease in luciferase activity driven by the RORE (Fig. 2A). When we compared the effects of the selected KK-S compounds on the wild-type versus mutated *Bmal1* promoter activities, only KK-S6 resulted in a RORE-specific repression of luciferase activity (Fig. 2B). KK-S6 consists of two aromatic systems linked by an ethylene bridge. The R-group of KK-S6 contains a unique alkyne (triple bond) unit, which can display a π-π interaction as well as a hydrophobic interaction (Fig. 2C and S2).

3.3. Impact of KK-S6 on endogenous circadian clockwork

Next, we examined whether KK-S6 can influence the endogenous circadian molecular clockwork. The effect of KK-S6 on endogenous *Bmal1* expression was assessed in comparison with the effects exerted by known REV-ERB modulators. Treatment with KK-S6 significantly reduced *Bmal1* mRNA expression, to a similar extent as heme, the endogenous ligand, or GSK4112, a synthetic REV-ERB agonist (Fig. 3A). In accordance, KK-S6 and heme also decreased BMAL1 protein levels, whereas treatment with lithium increased BMAL1 protein expression (Fig. 3B). Lithium is known to be an inhibitor of glycogen synthase kinase 3β (GSK3β), and phosphorylation by GSK3β stabilizes REV-ERBs from degradation

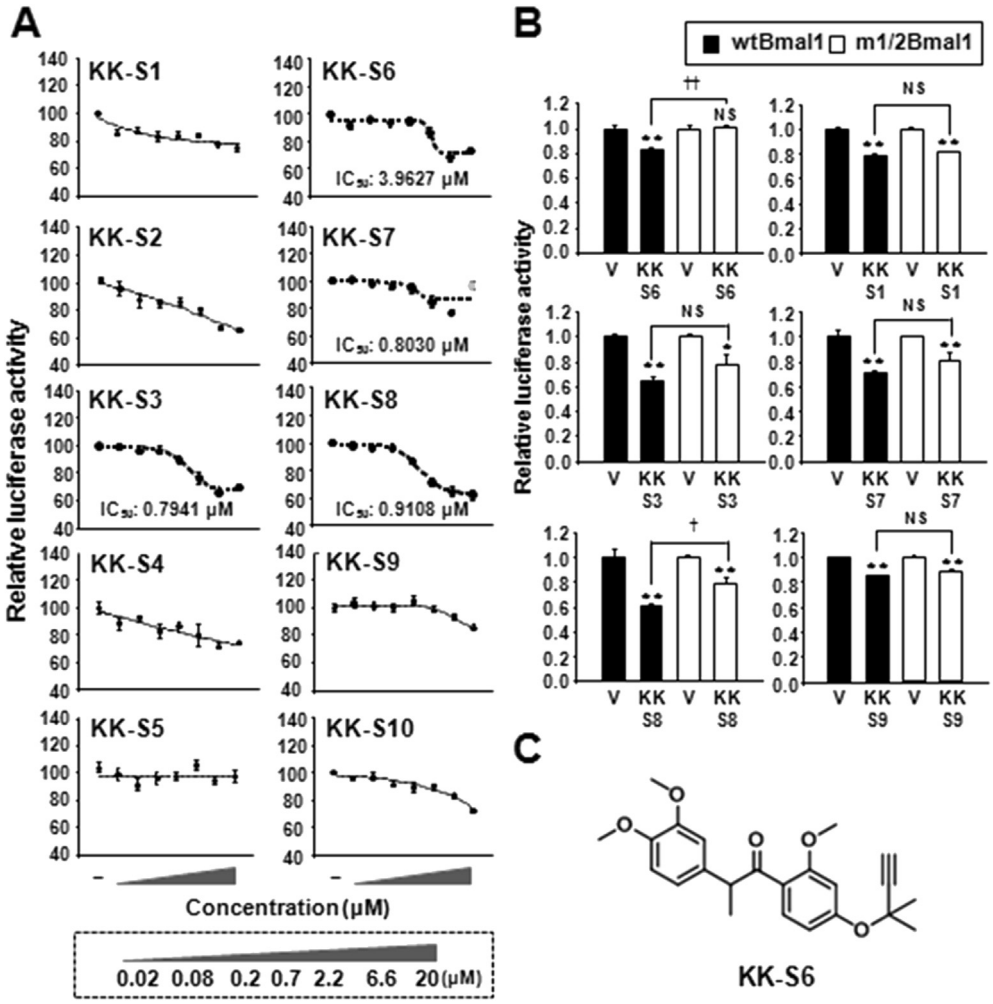

Fig. 2. Identification of KK-S6. (A) Dose-response curves showing the effects of the ten structurally-related compounds, designated the KK-S series, on RORE-mediated transcription. Luciferase levels of wtBmal1::Luc reporter were examined in the presence of indicated concentrations of a compound (7 concentrations of a 3-fold dilution series in DMSO). The grey dot indicates cellular damage by treatment with a high concentration of the compound. (B) Effects of selected KK-S compounds (5 μM) on wtBmal1:Luc and m1/2Bmal1::Luc activities. $^{**}p < 0.01$ compared to vehicle-treated group, ††$p < 0.01$ and †$p < 0.05$ vs wtBmal1::Luc receiving the same treatment. (C) Structure of KK-S6.

[21]. We then examined the temporal oscillations of reporter activity driven by the mouse *Bmal1* or *Per2* promoter, to explore the functional relevance of KK-S6 in the regulation of circadian molecular rhythms. Stable cell cultures harboring either wtBmal1::Luc or Per2::Luc reporter were synchronized using 1 μM DEX for 2 h, then bioluminescence signals were continuously monitored in the absence or presence of KK-S6 at various concentrations. KK-S6 caused an apparent dose-dependent attenuation of the amplitude of rhythmic activity levels of both reporter constructs, while it barely affected the period length of the rhythm (Fig. 3C and D).

3.4. KK-S6 strengthens the transcriptional repression by REV-ERBα

We next assessed whether KK-S6 affected RORE-mediated transcription by modulating the activity of REV-ERBs, which belong to the nuclear receptor superfamily and are well known as key transcriptional repressors of RORE-mediated downstream gene expression [22]. Overexpression of Rev-erbα to NIH3T3 cells significantly reduced wtBmal1::Luc reporter activity, and treatment with KK-S6 further impaired the reporter activity (Fig. 4A). Moreover, KK-S6 suppressed Bmal1::ELuc (emerald luciferase) activity in WT mouse embryonic fibroblast cells (MEFs), but its repressive effect on RORE-mediated transcription was lost in MEFs derived from Rev-erbα knock-out mice (Fig. 4B). These data indicate that KK-S6 may reinforce the transcriptional repression activity of REV-ERBs. We further attempted to determine whether KK-S6 could modulate mRNA expression levels of two genes that act on downstream of REV-ERBα and contain the RORE *cis*-element in their promoter in HepG2 cells. Sequence analysis shows that like a *Bmal1* promoter, *Plasminogen activator inhibitor-1* (Pai-1) [23] promoter has two putative ROREs, whereas that of *Citrate synthase* [24] has one. All three genes can be directly regulated by REV-ERBα [25]. Treatment of KK-S6 repressed the transcription levels of these genes as strongly as heme, the endogenous ligand, and GSK4112, a REV-ERB agonist (Fig. 4C).

GSK4112, a synthetic agonist, inhibited the activity of a luciferase reporter containing a GAL4 binding promoter (GAL4-UAS-Luc) in the presence of a chimeric protein, in which the DNA-binding domain (DBD) of GAL4 was fused to the ligand-binding and transcriptional repression domains of REV-ERBα (GAL4-REV). In contrast to GSK4112, KK-S6 did not affect the activity of GAL4-UAS-Luc reporter (Fig. 4D). Therefore, KK-S6 does not seem to be a classical REV-ERB agonist, suggesting a non-canonical or indirect mechanism.

4. Discussion

The present study identifies a novel compound that suppresses *Bmal1* gene expression, thereby attenuating the circadian rhythmicity of molecular clockworks. KK-S6 reduced both BMAL1 mRNA and protein expression by a RORE-dependent mechanism and, more importantly, impaired the amplitude of rhythmic E-box and RORE-mediated transcription. The inhibitory effect of KK-S6 on BMAL1 expression appears to involve enhanced transcriptional repression by REV-ERBs, which are key negative regulators of *Bmal1* gene transcription. KK-S6 is, however, distinct from recently identified endogenous and synthetic agonists of REV-ERBs in terms of its mode of action as well as its structural features [14,16].

REV-ERBs play an important role in stabilizing the period of circadian rhythms by controlling cyclic expression of BMAL1, an indispensable transcription factor of circadian molecular clockwork. In addition to circadian rhythmicities, recent studies demonstrated that REV-ERBs are heavily involved in the regulation of a wide range of physiological processes such as metabolic and energy homeostasis, inflammation, cardiovascular function, and neural function [16]. It was proposed that synthetic REV-ERB

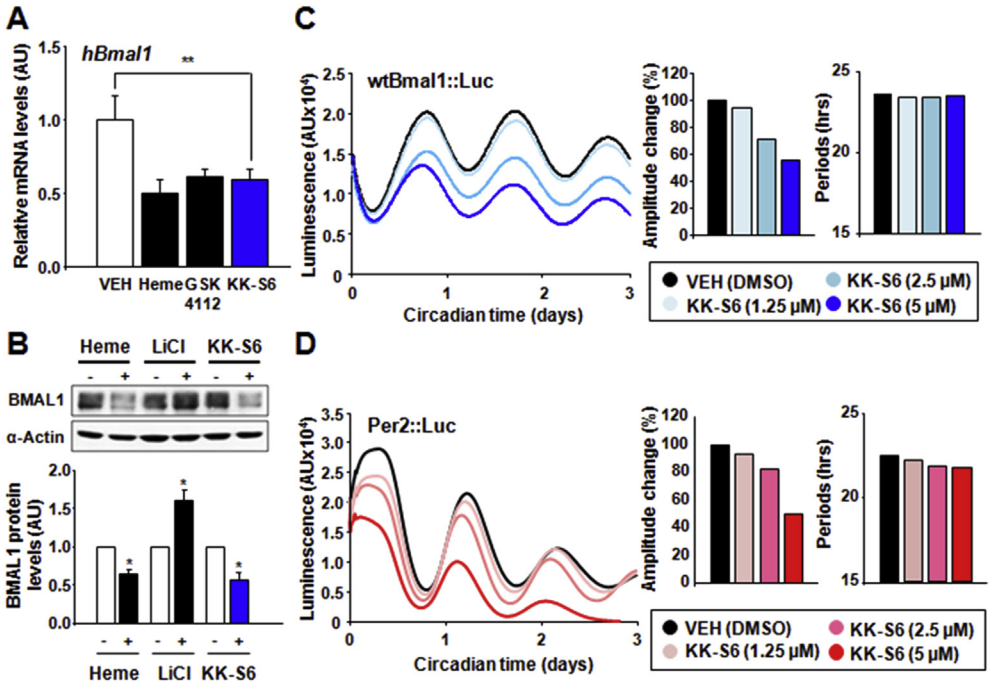

Fig. 3. Influence of KK-S6 on endogenous circadian clockworks. (A) Changes in *Bmal1* mRNA expression in HepG2 cells by treatment with vehicle (0.05% DMSO) or KK-S6 (5 μM) for 24 h. Heme (30 μM) and GSK4112 (15 μM) were used for comparison. (n = 4–9). **p < 0.01 compared to vehicle -treated group. (B) Alteration of BMAL1 protein levels by treatment with heme (30 μM), lithium (10 mM), or KK-S6 (5 μM) for 24 h *p < 0.05 compared to vehicle-treated group. (C and D) Dose-dependent effects of KK-S6 on cycling expression of wtBmal1::Luc (C) and Per2::Luc (D) reporters. NIH3T3 cells harboring the indicated luciferase reporter were synchronized with 1 μM DEX for 2 h then treated with vehicle (0.05% DMSO) or KK-S6 (1.25, 2.5, or 5 μM).

ligands may be beneficial in treatment of metabolic diseases as well as sleep disorders. Two metabolic genes acting downstream of REV-ERBα were significantly decreased by KK-S6 at the transcriptional level in Fig. 4. In fact, *Pai-1* plays a critical role in inflammation and the fibrinolytic system, whereas *Citrate synthase*, a citrate producer, is heavily involved in lipogenesis and cholesterologenesis [23,24]. It is thus worth noting that KK-S6 could control metabolic homeostasis, as REV-ERBα is a link between the circadian timing system and metabolism.

Furthermore, our previous study showed that modulating REV-ERBα activity, particularly in the midbrain, had an impact on the regulation of mood states [19]. It is reasonable that RORE-mediated transcriptional repression of downstream genes, including *Bmal1*, may underlie the effect of REV-ERBs on such a wide variety of biological processes. In addition to REV-ERB agonists, KL001, a recently developed small molecule CRY stabilizer, could reinforce feedback inhibition of CRYs on the E-box-mediated transcriptional activity of the CLOCK:BMAL1 heterodimer [17].

It should be noted that the mode of action and chemical structure of KK-S6 are distinct from previously reported synthetic REV-ERB agonists, which act on the heme-binding site in the ligand-binding domain [14,16]. Although KK-S6 enhanced REV-ERB function, and as a result repressed RORE-mediated transcriptional activation, our mammalian one-hybrid assay based on a chimeric REV-ERBα-LBD fused with the GAL4 DBD suggested that it may not act on REV-ERBα LBD as shown in Fig. 4. Rather, KK-S6 may affect the post-translational modification of REV-ERBs, and thus modulate their activity and/or stability. One possibility is that REV-ERBs can undergo ubiquitin-dependent proteasomal degradation by E3 ligases such as ARF-BP1 and PAM, which is phosphorylation state-dependent by KK-S6. For example, GSK3β stabilizes REV-ERBα by phosphorylation, thereby suppressing *Bmal1* transcription; whereas lithium, an inhibitor of GSK3β, can accelerate degradation of the protein [26]. Notably, treatment with lithium increased the period length of both Per2::Luc and Bmal1::Luc rhythms, but it barely affected the amplitude of the Bmal1::Luc rhythm [27]. Since KK-S6 mainly influenced the amplitude of both Per2::Luc and Bmal1::Luc rhythms rather than the length, it is unclear whether the mechanism of action of KK-S6 is related to GSK3β-mediated phosphorylation of REV-ERBs. Another possibility involves a direct allosteric effect of KK-S6 on REV-ERB function, which is a phenomenon seen in various other members of the nuclear receptor superfamilies [28]. REV-ERBs form complexes with co-repressors such as NCoR and HDAC3, which suppress the transcription of target genes upon activation by a ligand [9]. Therefore, it is also plausible that the effect of KK-S6 is produced by an allosteric stabilization of the binding of co-repressors and/or endogenous ligands to REV-ERBs.

In conclusion, we discovered a novel modulator of the mammalian clock by conducting a cell-based screening of our in-house chemical library and characterized a mechanism of action that exhibited capacity to modulate RORE-mediated activity.

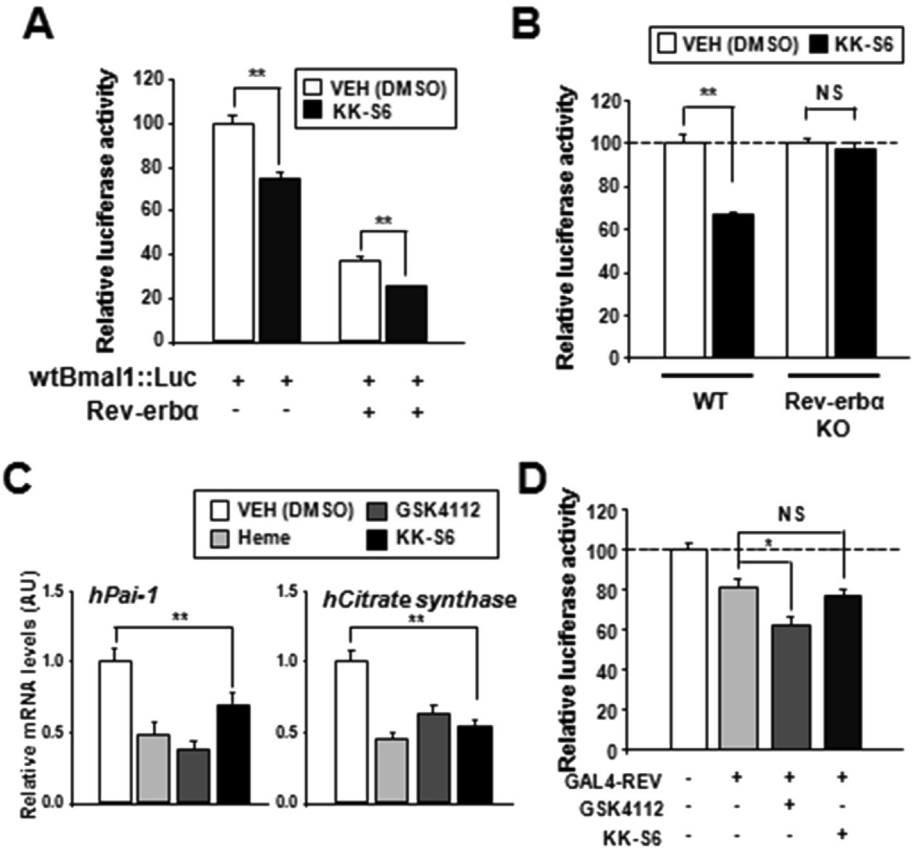

Fig. 4. Effects of KK-S6 on REV-ERBα. (A) Enhanced REV-ERBα activity by treatment with KK-S6. NIH3T3 cells were transfected with wtBmal1::Luc and/or Rev-erbα and treated with vehicle (0.05% DMSO) or KK-S6 (10 μM). (n = 6). **p < 0.01 compared to vehicle-treated group. (B) Effect of KK-S6 on REV-ERBα. Wild-type or *Rev-erbα* knock-out mouse embryonic fibroblast cells (MEFs) were obtained from crosses between Bmal1::ELuc/*Rev-erbα*$^{+/-}$ and *Rev-erbα*$^{+/-}$ mice and treated with KK-S6 (5 μM). (n = 3). **p < 0.01 compared to vehicle-treated group. (C) Effect of KK-S6 on mRNA expression of Pai-1 and Citrate synthase genes affected by REV-ERBα. HepG2 cells were treated with vehicle (0.05% DMSO) or KK-S6 (5 μM) for 24 h. Heme (30 μM) and GSK4112 (15 μM) were used for comparison. (n = 4–9). **p < 0.01 compared to vehicle-treated group. (D) Effect of KK-S6 on chimeric REV-ERBα-LBD harboring the GAL4-DBD (GAL4-REV). NIH3T3 cells were co-transfected with GAL4-responsive luciferase reporter with GAL4-REV then treated with GSK4112 (15 μM) or KK-S6 (10 μM). (n = 6). *p < 0.05 compared to control group.

Evidence obtained in the present study strongly suggests KK-S6 has a distinct mode of action, presumably involving post-translational and/or allosteric mechanisms. As reinforcing REV-ERB activity and impairing the transactivation by the CLOCK:BMAL1 heterodimer have been proposed as new strategies to treat circadian rhythm-related diseases, including metabolic disorders, KK-S6 may provide a tool to study the regulation of REV-ERB-dependent physiology and aid development of molecular clockwork-based therapeutics.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.12.030.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.12.030.

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