

A Small Molecule Modulates Circadian Rhythms through Phosphorylation of the Period Protein**

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Many living organisms including bacteria, fungi, plants, and animals have observable, genetically determined biological rhythms. The rhythm affects many aspects of mammalian behavior and physiology including sleep, physical activity, alertness, hormone levels, body temperature, immune function, and digestive activity.^[1] These circadian rhythms are controlled by cell-autonomous circadian clocks.^[2] In mammals, the central circadian pacemaker controlling behavioral rhythms is located within the suprachiasmatic nucleus (SCN) of the anterior hypothalamus.^[3] Peripheral clocks are located in most major organs and control key physiological processes such as blood pressure, hepatic glucose levels, and heart rate.^[4] Clock genes and their gene products are involved in negative and positive regulatory feedback loops.^[5] For example CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like protein 1) heterodimerize and activate the expression of the clock genes period (*Per*) 1, 2, and 3, and cryptochrome (*Cry*) 1 and 2. PER and CRY proteins enter the nucleus and repress CLOCK-BMAL1-driven transcription.^[6]

Perturbation of clock function is implicated in numerous pathologies including circadian sleep disorders, cardiovascular disease, cancer, and metabolic disease.^[7] Thus, the identification of small molecules that modulate clock function may not only provide additional insights into the role of circadian rhythms in human physiology, but also lead to new

treatments for diseases that have an underlying circadian disorder.^[8] To date, a number of small molecules have been demonstrated to be modulators of circadian rhythms including the CDK inhibitor roscovitine, a JNK inhibitor SP600125, and longdaysin, which lengthens circadian period by inhibiting CKI α , CKI δ , and ERK2.^[9–11] Furthermore, several intracellular small molecules such as heme,^[12a,b] cAMP,^[12c] and NAD^[12d,e] are known to be circadian modulators.

To identify novel small molecules that induce circadian period changes, we exploited a previously developed circadian cell-based assay with a luminescent readout^[10] (see the Supporting Information). Human U2OS cells stably expressing a *Bmal1-dLuc* reporter were treated with approximately 500 000 druglike compounds,^[13] and luminescence intensity was measured every two hours for three days. Primary hit compounds were further tested in assays in an eight point serial dilution format to determine compound potency and dose response.

We identified a number of different scaffolds that lengthened the circadian period in a dose-dependent manner in U2OS cells. The benzothiazole derivative LH846 (8 μ M) lengthened the period (10 h) with minimal effect on the amplitude of both *Per2-dLuc* and *Bmal1-dLuc* rhythms in U2OS cells (Figure 1). A preliminary structure activity relationship (SAR) study was performed to identify sites which could be derivatized without significant loss in activity to generate affinity probes for target identification (see

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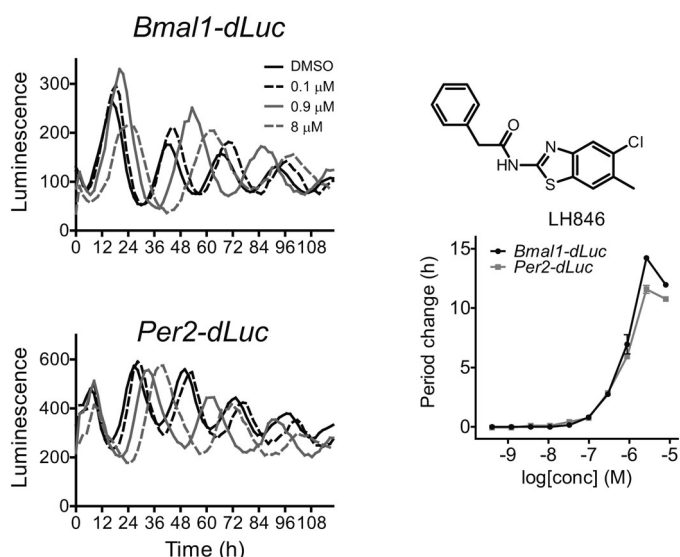


Figure 1. Period effect and dose response of LH846 on *Per2-dLuc* and *Bmal1-dLuc* U2OS cells. LH846 lengthened circadian period of *Per2-dLuc* and *Bmal1-dLuc* rhythms in a dose-dependent manner.

Table 1S in the Supporting Information). Twenty six benzothiazole analogues were synthesized and tested in a dose response format in the U2OS cell-based assay. We defined the potency of the compound by calculating the concentration which causes 1 h period lengthening. Methyl and methoxy substitution on the benzothiazole led to a decrease in activity. In contrast, chloro and bromo substitution at the 5- or 6-positions of the benzothiazole ring appear to be tolerated or slightly increase activity, whereas, chloro substitution at the 4-position decreased activity. Substitution of the phenylacetamide group with benzamide or phenylsulfonamide moieties resulted in a complete loss in activity. Also, substitution with benzothiazole and benzamide moieties (LH25 and LH26) led to a complete loss in activity. Some derivatives of the phenylacetamide group with methoxy at the *meta* or *para* position had only slightly decreased activity (see Table 1S in the Supporting Information). Based on the SAR, the *para* position of the phenylacetamide moiety was used for attachment of LH846 to solid support through a diethylene glycol linker. Although the diethylene glycol derivative (LH846-linker) was around 10-fold less potent than LH846, it retained a significant period lengthening effect and was therefore used in pull-down assays.^[14]

An agarose conjugate of LH846-linker (Figure 2a) was prepared and incubated for 3 h with U2OS cell lysate in the absence or the presence of 300 μ M LH846 as competitor. Proteins that bound to the affinity resin were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining. One band at about 45 kDa was observed, and binding of the protein(s) to the LH846-linker-agarose resin was blocked by free LH846 (Figure 2b), indicating specific binding to LH846. Analysis of the band by linear ion trap mass spectrometry (LTQ)^[15] suggested casein kinase 1 delta (CKI δ) as the target. An independent affinity chromatography pull-down experiment followed by Western blotting with anti-CKI δ antibody confirmed binding of CKI δ to the affinity resin which was blocked in the presence of LH846 (Figure 2c). In vitro kinase profiling against a panel of around 50 kinases revealed that LH846 strongly inhibited CKI δ and, with less potency CKI α and ROCK2 (see Table 2S in the Supporting Information). Determination of the half maximal inhibitory concentration (IC₅₀) based on an in vitro assay revealed that LH846 inhibited CKI δ with an IC₅₀ of 290 nM, CKI α with an IC₅₀ of 2.5 μ M, CKI ϵ with an IC₅₀ of 1.3 μ M and had no effect on CK2 (see Figure 1S in the Supporting Information). Compounds with similar structures to LH846 have been reported as inhibitors of ubiquitination.^[16] However, we found that an active ubiquitination inhibitor (PC4)^[16] had no effect on circadian period and did not inhibit CKI δ activity (see Figure 2S in the Supporting Information). In contrast, an inactive ubiquitination inhibitor (PC43) that is also similar to LH846 inhibited CKI δ activity and lengthened circadian period (see Figure 2S in the Supporting Information). Furthermore, a commercially available cell-permeable ubiquitin E1 inhibitor PYR-41 showed no effect on period length (see Figure 2S in the Supporting Information). Taken together, these results suggest that CKI δ is the biological target of LH846.

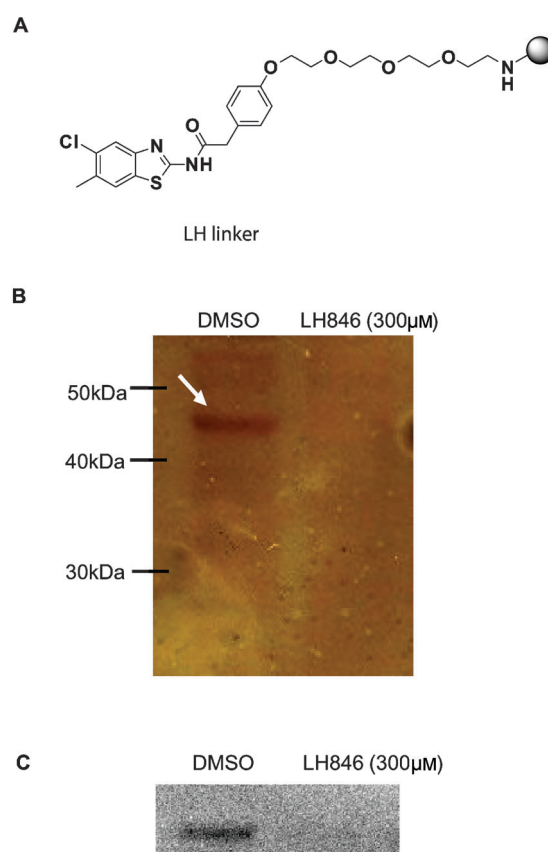


Figure 2. Identification of a cellular protein that interacts with LH846. A) Structure of the LH846-linker. B) The LH846-linker matrix was incubated with cell lysate of U2OS cells in the presence or absence of LH846 (300 μ M). Bound proteins were separated by SDS-PAGE followed by silver staining. C) The LH846 matrix was incubated with cell lysate of U2OS cells in the absence or presence of LH846 (300 μ M). Proteins bound to the LH846 matrix were immunostained with anti-CKI δ antibody.

CKI δ is a well-characterized kinase in period regulation of the circadian clock; modulation of its activity by genetic mutation or CKI inhibitor is known to cause period change.^[17,18] We therefore knocked down expression of the CKI δ gene (*CSNK1D*) by RNA interference and determined the effect on period length in the *Per2-dLuc* U2OS cell-based assay. siRNA-mediated knockdown of CKI δ showed a significant period lengthening effect while reduction of CKI ϵ did not alter period length (Figure 3a,b). We confirmed specific knockdown of CKI δ (60%) and CKI ϵ (80%) by measuring cellular mRNA levels (Figure 4c). The observed difference in period change caused by LH846 (around 10 h) and CKI δ knockdown (< 1 h) may be due to partial inhibition of CKI δ by siRNA-mediated knockdown (Figure 3c) or a difference because of inhibition of kinase activity (LH846) versus reduction of mRNA level (siRNA).^[19]

Because CKI δ is known to cause PER1 proteasomal degradation upon phosphorylation of PER1 protein,^[18,20] we next investigated the effect of LH846 on CKI δ -mediated PER1 phosphorylation using a phosphorylation-dependent mobility shift assay. Coexpression of PER1 with CKI δ

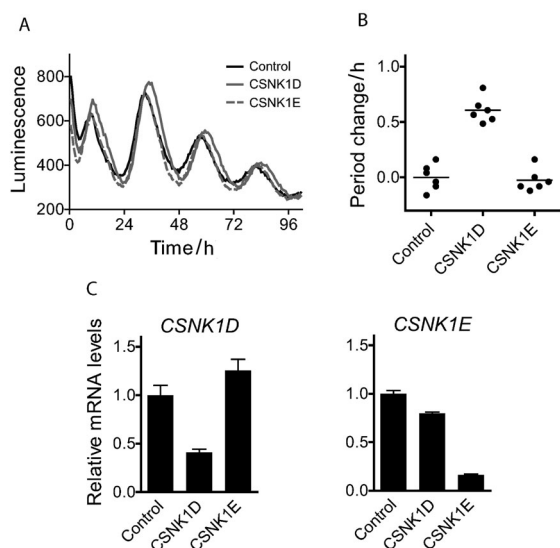


Figure 3. Effect of siRNA-mediated knockdown of CKI δ on circadian period of *Per2-dLuc* U2OS cells. A and B) Knockdown of *CSNK1D* showed a period lengthening effect, and *CSNK1E* showed no period effect. C) The knockdown efficiency was measured by quantitative polymerase chain reaction (qPCR) analysis.

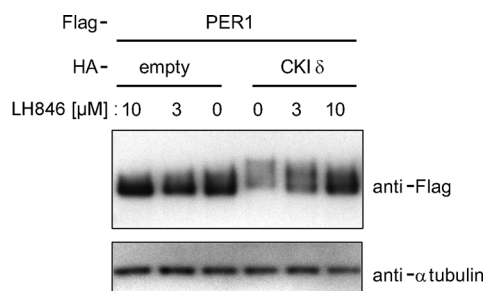


Figure 4. Effect of LH846 on CKI δ -dependent phosphorylation of PER1. HEK293T cells were reversely transfected on 12-well plates by Lipofectamine 2000 with expression vectors for C-terminally 3XFlag-tagged PER1 protein and N-terminally HA-tagged CKI δ . The cells were treated with 3 or 10 μ M LH846 or 0.1% DMSO for 7 h, and a part of the cell extract was analyzed by Western blot to detect the PER1-Flag protein.

resulted in a mobility shift and lower abundance of PER1 protein (Figure 4). Treatment with LH846 reduced the PER1 mobility shift and PER1 clearance in a dose-dependent manner, indicating that CKI δ -dependent phosphorylation and subsequent degradation of PER1 protein is inhibited by LH846 treatment. Previous genetic studies demonstrated an important role of CKI-dependent phosphorylation of PER protein in period regulation,^[17,18] supporting the period lengthening effect of LH846 through PER1 protein phosphorylation.

The CKI family of serine and threonine kinases is ubiquitously expressed and genetically conserved in eukaryotes.^[21] CKI α , CKI δ , and CKI ϵ are all known to play roles in modulating circadian rhythms. CKI δ and CKI ϵ phosphorylate PER and trigger its degradation;^[22] mutations in each affect the clock in vivo.^[17b,23] The relatively nonselective casein

kinase inhibitors IC261 (IC₅₀ of 6.0, 4.7, and 31 μ M for CKI α , CKI δ , and CKI ϵ , respectively) and D4476 (IC₅₀ of 2.5, 1.1, and 11 μ M for CKI α , CKI δ , and CKI ϵ , respectively) cause period lengthening in cell culture, but to a significantly lesser degree than LH846. The CKI ϵ selective inhibitor PF-4800567 showed that CKI ϵ does not contribute significantly to period regulation,^[24] while longdaysin^[11] which inhibits CKI α , CKI δ , and ERK2 showed a combinational effect of these kinases on period length comparable to that of LH846.

LH846 is therefore a relatively potent and selective inhibitor of CKI δ that has a significant effect on period length (10 h) in cell culture. Because behavioral studies mediated by CKI δ activity are restricted because of the lethality of known CKI δ -null mutations,^[18] LH846 and its analogues may prove useful tools for studies of clock function in more complex organismal systems and may ultimately lead to chronotherapeutic agents.

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