Structure–Activity Relationships

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C–H Activation Generates Period-Shortening Molecules That Target Cryptochrome in the Mammalian Circadian Clock**

Tsuyoshi Oshima, Iori Yamanaka, Anupriya Kumar, Junichiro Yamaguchi, Taeko Nishiwaki-Ohkawa, Kei Muto, Rika Kawamura, Tsuyoshi Hirota, Kazuhiro Yagita, Stephan Irle, Steve A. Kay, Takashi Yoshimura,* and Kenichiro Itami**

Dedicated to Professor Takao Kondo

Abstract: *The synthesis and functional analysis of KL001 derivatives, which are modulators of the mammalian circadian clock, are described. By using cutting-edge C–H activation chemistry, a focused library of KL001 derivatives was rapidly constructed, which enabled the identification of the critical sites on KL001 derivatives that induce a rhythm-changing activity along with the components that trigger opposite modes of action. The first period-shortening molecules that target the cryptochrome (CRY) were thus discovered. Detailed studies on the effects of these compounds on CRY stability implicate the existence of an as yet undiscovered regulatory mechanism.*

The circadian rhythm is an approximately 24-hour cell-autonomous biological oscillation observed in almost all living organisms.[1] This rhythm regulates various physiological behaviors, such as sleep–wake cycles, hormone secretion, metabolism, and seasonal reproduction.[2,3] The circadian rhythm is driven by a circadian oscillator, which consists of multiple transcriptional–translational feedback loops.[3,4] The main feedback loop of the mammalian circadian clock is shown in Figure 1. The CLOCK–BMAL1 heterodimer activates the transcription of the *period* (*Per*) and *cryptochrome* (*Cry*) genes. PER and CRY form a complex and suppress their own gene expression by inhibiting CLOCK–BMAL1-mediated transcription. FBXL3, an F-box-type ubiquitin ligase subunit, recognizes CRY at the flavin adenine dinucle-

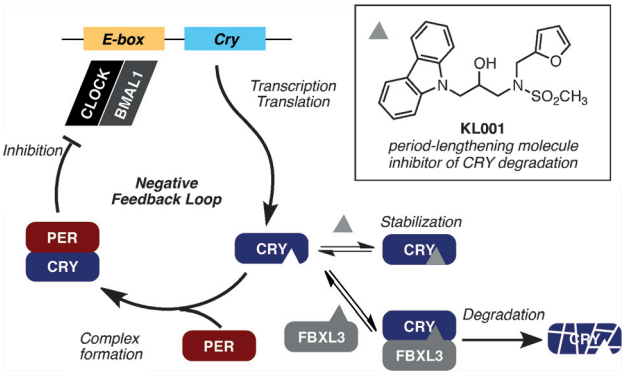

Figure 1. The core feedback loop of the mammalian circadian clock and the effect of KL001 on the cryptochrome (CRY).

[*] T. Oshima,[+] Dr. I. Yamanaka,[+] Dr. A. Kumar, Dr. J. Yamaguchi, Dr. T. Nishiwaki-Ohkawa, K. Muto, R. Kawamura, Dr. T. Hirota, Prof. Dr. S. Irle, Prof. Dr. S. A. Kay, Prof. Dr. T. Yoshimura, Prof. Dr. K. Itami
Institute of Transformative Bio-Molecules (WPI-ITbM)
Nagoya University
Chikusa, Nagoya, 464-8601 (Japan)
E-mail: sirle@chem.nagoya-u.ac.jp
takashiy@agr.nagoya-u.ac.jp
itami@chem.nagoya-u.ac.jp

T. Oshima,[+] Dr. J. Yamaguchi, K. Muto, Prof. Dr. S. Irle, Prof. Dr. K. Itami
Department of Chemistry, Graduate School of Science
Nagoya University
Chikusa, Nagoya, 464-8602 (Japan)

Dr. T. Nishiwaki-Ohkawa, R. Kawamura, Prof. Dr. T. Yoshimura
Graduate School of Bioagricultural Sciences
Nagoya University
Chikusa, Nagoya, 464-8601 (Japan)

Dr. T. Hirota
JST, PRESTO
Chikusa, Nagoya, 464-8602 (Japan)

Prof. Dr. K. Yagita
Department of Physiology and Systems Bioscience
Kyoto Prefectural University of Medicine
Kyoto 602-8566 (Japan)

Prof. Dr. S. A. Kay
Dornsife College of Letters, Arts and Sciences
University of Southern California
3430 S. Vermont Avenue, Los Angeles, CA 90089-3301 (USA)

Prof. Dr. T. Yoshimura
National Institute for Basic Biology
Myodaiji-cho, Okazaki 444-8585 (Japan)

Prof. Dr. K. Itami
JST, ERATO, Itami Molecular Nanocarbon Project
Chikusa, Nagoya, 464-8602 (Japan)

[+] These authors contributed equally to this work.

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otide (FAD) binding pocket,[5] promoting proteasomal degradation of CRY.[6–8] Recently, a period-lengthening small molecule, KL001, that directly targets CRY was discovered.[9] KL001 stabilizes CRY by binding to the FAD-binding pocket in competition with FBXL3,[10] thereby extending the circadian period in mammals.[9]

Provided that KL001 is the first-in-class small-molecule modulator targeting CRY with a unique molecular structure, there are huge opportunities for KL001 and its derivatives not only as tools to understand the mechanism of the mammalian circadian clock, but also in a number of other applications. However, very limited information regarding the structure–activity relationships (SARs) of KL001 has been available up to now. Herein, we report the first functional analysis of a range of KL001 derivatives enabled by cutting-edge C–H activation chemistry.[11–13] This campaign led us to uncover the sites on KL001 derivatives that are critical for its rhythm-changing activity and the rhythm-lengthening/shortening selectivity, along with the discovery of the first period-shortening molecules targeting CRY.

The established general synthetic scheme for KL001 derivatives is shown in Figure 2a. Arylmethylamines **1** were first mesylated with CH_3SO_2Cl and then subjected to N alkylation with epibromohydrin and a subsequent epoxide ring-opening reaction with amines to afford KL001-like core structures **2**. The furan and carbazole moieties can be easily modified in this reaction sequence by changing **1** and the amines in the epoxide ring-opening step. SAR studies on the hydroxy group of **2** can be carried out by subsequent oxidation or substitution reactions to provide **3** and **3′**, respectively. Although not present in the parent KL001 molecule, the introduction of substituents at the furan or thiophene moieties might enable the tuning of the period-changing properties of the molecule. An ideal method to obtain various substituted derivatives would be a late-stage C–H activation[11] reaction on the heterocyclic groups. We successfully introduced a range of aryl groups at the C5 position of the heterocycles to afford arylated compounds **4** by treating **2** with aryl iodides in the presence of $[PdCl_2(PPh_3)_2]$, $AgNO_3$, and KF in DMSO (Mori's conditions).[14] The introduction of the aryl group at the least reactive C4 position of the five-membered heterocycle is known to be extremely challenging. Nevertheless, we achieved this by employing our recently developed catalytic system.[15] Thus, the treatment of **2** with aryl boronic acids in the presence of $[Pd(OAc)_2]$, 2,2′-bipyridyl, and 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) in $C_6H_5CF_3$ afforded the target C4 arylation products **5** (see the Supporting Information for details).

By following the above-mentioned synthetic scheme, we synthesized over 50 KL001 derivatives. The effects of these new compounds on the circadian-clock activity were investigated by a cell-based luminescence assay using *Bmal1–dLuc* reporter U2OS cells.[9,16–18] The results of the SAR study and the period-changing activities of representative molecules at a substrate concentration of 10 μM are summarized in Figure 2b and 2c, respectively (see the Supporting Information for the results of other compounds). The carbazole moiety of KL001 was found to be critical for the period-changing activity: Replacing the carbazole group with diphenylamine (GO203) or 2-phenylindole led to a complete loss of activity.

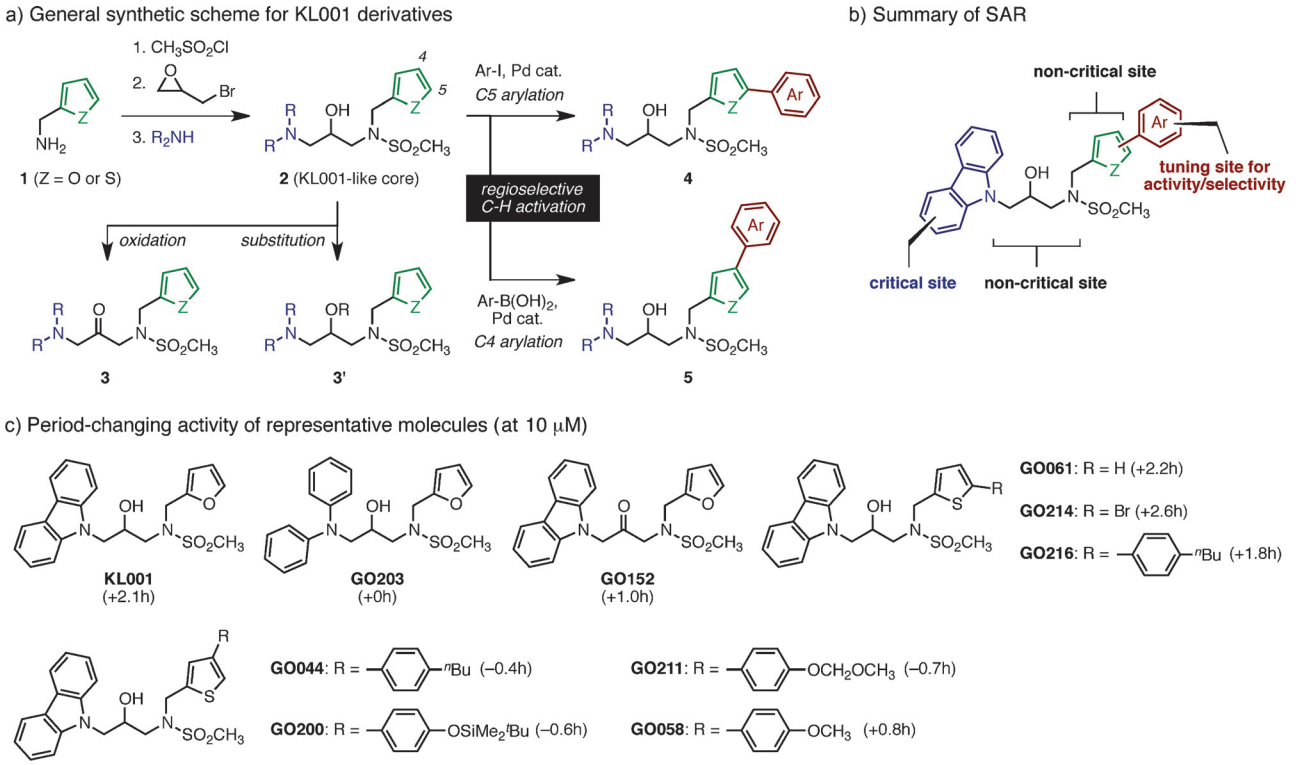

Figure 2. a) General synthetic scheme for KL001 derivatives. b) Summary of the SAR study. c) Period-changing activity of representative molecules in the initial screening.

The hydroxy substructure turned out to be a significant, but non-critical site as ketone (GO152) and alkoxy analogues sustained the period-lengthening activity, albeit with less potency. The furan part was found not to be a critical site as thiophene analogue GO061 and a phenyl-substituted analogue also exhibited period-changing effects. Fortunately, as expected, it was found that additional substituents on the C5 position led to an increase in period-changing activities. For example, the C5-bromo-substituted derivative GO214 induced a higher period-lengthening effect at the same concentration. C5-aryl-substituted derivative GO216 had an activity similar to that of KL001. Interestingly, we discovered that some aryl substituents at the C4 position could induce the opposite effect (period shortening), albeit with low efficiency. For example, when a 4-*n*-butylphenyl group was attached at the C4 position (GO044), the period was shortened by 0.4 hours. Given the opposite activity of C5 isomer GO216, the rotation around the thiophene ring may be rather restricted at the binding site. We also found that the substituent effect at the C4 position was rather elusive. Whereas the introduction of some *para*-substituted aryl groups (GO200 and GO211) also induced a period-shortening effect, a small *para* substituent, such as the methoxy group (GO058), led to a period-lengthening effect.

With these exciting preliminary results in hand, we examined the effects of the following representative compounds in further detail: one period-lengthening compound, GO214, and three period-shortening compounds, GO044, GO200, and GO211. We first studied the effects of these four compounds at three different concentrations (Figure 3a, b). At 10 μM, the period-lengthening effect of GO214 was significantly greater than that of KL001 (Supporting Information, Figure S1). On the other hand, GO044, GO200, and GO211 shortened the period by 0.3 to 1 hour at 10 μM (Figure 3a, b). These effects were also confirmed in the *mPer2* promoter-driven luciferase assay (Figure S2).

We next added period-lengthening and -shortening compounds simultaneously to a cell culture to examine whether these compounds compete with each other. When we added a 1 μM solution of KL001 together with a 1 μM solution of either GO044, GO200, or GO211, the period-lengthening effect of KL001 was attenuated, and the resulting period was comparable to that observed in vehicle-treated cells (Figure 3c and Figure S3a). The period-lengthening effect of GO214 was also cancelled out by the addition of GO200 or GO211 (Figure S3b). These results suggest that the period-shortening compounds may compete with KL001 to bind to the same FAD-binding pocket.

Therefore, to elucidate the binding mode of the derivatives to CRY in more detail, we performed docking simulations for the representative compounds (period-lengthening GO214 and period-shortening GO044, GO200, and G0211) with the CRY protein structure (PDB X-ray structure 4MLP).[10] Technical details of these simulations are described in the Supporting Information. The optimal docking pose of the ligands was obtained by performing a conformational search allowing for torsional flexibility. Using this method, the binding pose of the crystal structure for KL001 was successfully reproduced. The best docking pose and the most

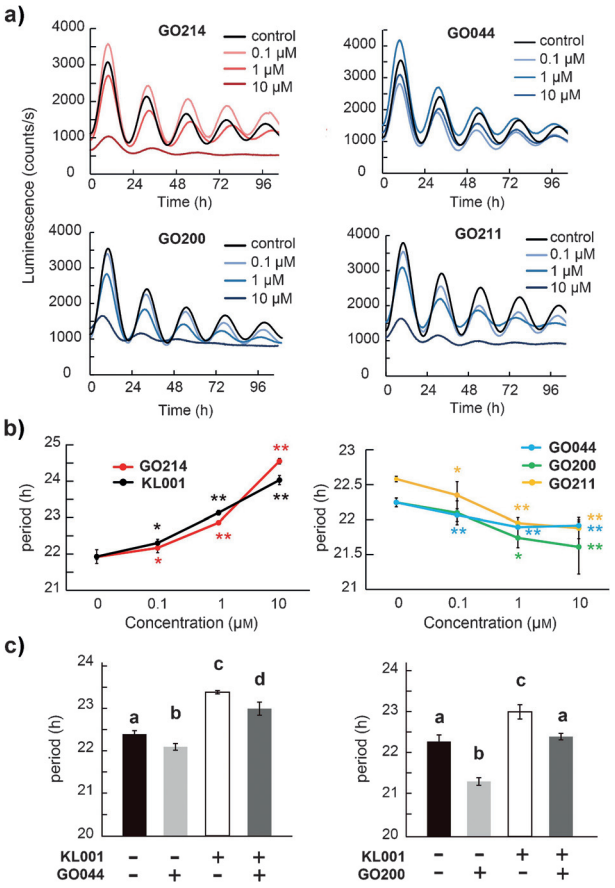

Figure 3. Discovery of period-lengthening and -shortening molecules. a) Representative luminescence traces and b) the changes in the circadian period in *Bmal1-dLuc* U2OS cell lines in the presence of each compound. Measurements were done in triplicate or quadruplicate. Data are presented as mean ± SD (* $P<0.05$, ** $P<0.01$; one-way ANOVA vs. vehicle). c) Competition assay between KL001 and period-shortening compounds. *Bmal1-dLuc* U2OS cells were simultaneously treated with KL001 (1 μM) and either GO044 or GO200 (1 μM). Measurements were done in triplicate or quadruplicate. Data are presented as mean ± SD. Different characters (a–d) indicate significant differences ($P<0.05$; one-way ANOVA). For each compound, we performed three independent experiments, and representative results are shown.

important amino acid residue interactions of GO214, GO044, GO200, and G0211 are shown as snapshots of the binding pocket in Figures 4a, 4b, S4a, and S4b, respectively.

The binding free energies of KL001, GO214, GO044, GO200, and GO211 were determined to be −10.41, −11.39, −13.58, −8.44, and −12.27 kcal mol^{-1}, respectively, indicating that all four compounds bind to the FAD-binding pocket of CRY. In a similar manner to KL001, the hydroxy moiety and the sulfonyl moiety in GO214 and GO044 form a hydrogen bond with S414 and H377, respectively. Furthermore, the mesyl moiety forms a CH–π interaction[19] with W417 and W310 (within < 4 Å), which is similar to the interaction of P426 in the FBXL3–CRY complex (PDB: 4I6J).[20] In the case of GO200, two hydrogen bonds were observed, one between the mesyl group and H377 and the second one between the hydroxy moiety and H373 instead of S414, owing to a torsional change to fit into the active site. The superimposition of the

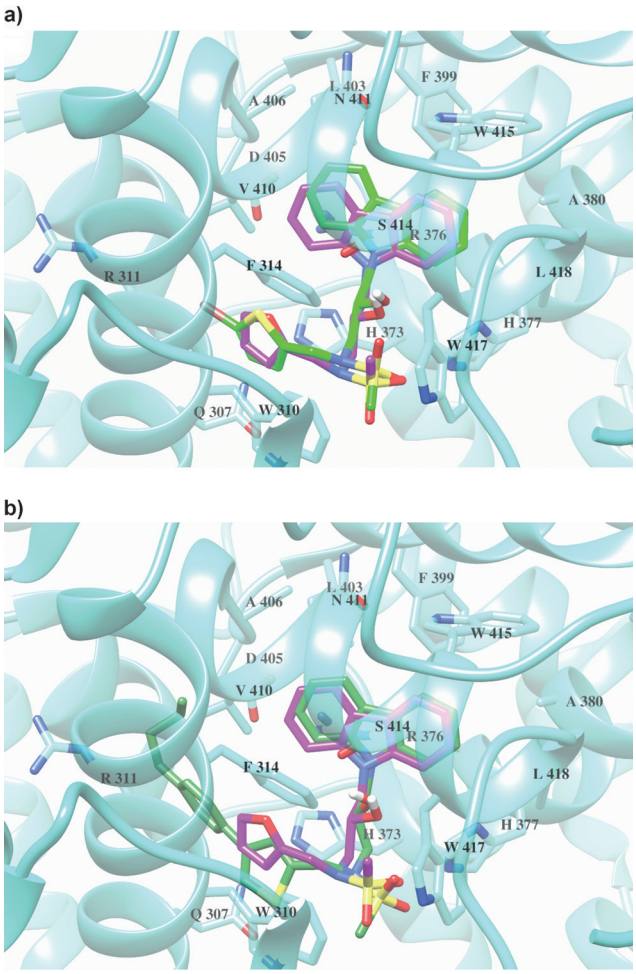

Figure 4. The overlaid docking poses of a) GO214 (light green) and b) GO044 (dark green) with KL001 (magenta) bound to the CRY protein (PDB: 4MLP).

best binding pose of GO200 to CRY (PDB: 4MLP) with that of FAD bound to CRY (PDB: 4I6G)[20] shows that the *tert*-butyl moiety of GO200 occupies the position of the sugar moiety of FAD in the FAD-binding site (Figure S5 a). Hence, more hydrogen-bond acceptors at this position could enhance the binding of GO200 and mimic the interactions of FAD. In the case of GO211, which showed similar behavior to KL001, two hydrogen bonds are formed, with one observed between the mesyl group and H377 and the second one between the hydroxy moiety and S414. The superposition of the best binding mode of GO211 to CRY (PDB: 4MLP) with that of FAD bound to CRY (PDB: 4I6G), shows that the methoxymethoxy moiety of GO211 bends towards the flavin ring in FAD (Figure S5 b). The silyloxyphenyl moiety in GO200 and the methoxymethoxyphenyl moiety in GO211 occupy the position of a water molecule and form a hydrogen bond with another water molecule. Surprisingly, the longer side chains of the period-shortening compounds, that is, GO044, GO200, and GO211, fit reasonably well into the binding pocket of FAD. To fit into the binding pocket, the conformation of the thiophene moiety in GO044 and GO211 was flipped without a reduction in the binding strength. On the other hand, in GO200, there was no flip of the thiophene moiety, but a hydrogen bond with S414 was lost, which results in a lower binding free energy. Therefore, novel compounds that probe new conformational space for additional interactions in the active site are potential CRY modulators.

Although we cannot disregard other possibilities, both the competition assay (Figures 3 c and S3) and the docking study (Figure 4) suggest that our representative compounds (period-lengthening GO214 and period-shortening GO044, GO200, and G0211) target the FAD-binding pocket of CRY. KL001 inhibits the FBXL3- and ubiquitin-dependent degradation of the CRY protein.[9,10] Therefore, we further examined whether KL001 derivatives also affect the stability of CRY. When we examined the effect of period-lengthening compound GO214 on the CRY1 degradation rate using a HEK293 cell line stably expressing the CRY1–luciferase fusion protein (CRY1–LUC),[9] the half-life of CRY1–LUC increased in a dose-dependent manner (Figures 5 and S6)

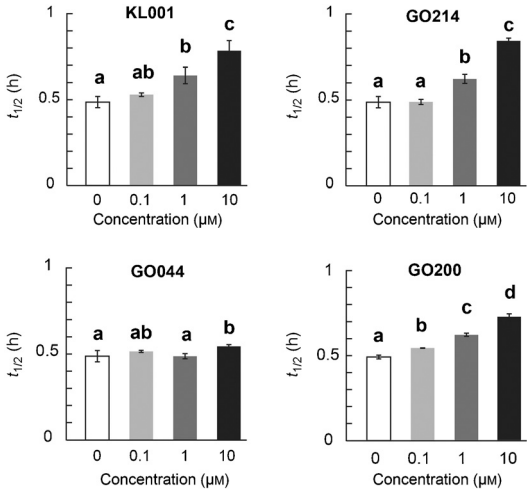

Figure 5. Effects of KL001 derivatives on the half-live of CRY1 ($t_{1/2}$ (h)) in the HEK293 stable cell line expressing the CRY1–LUC fusion protein. Measurements were done in triplicate or quadruplicate, and data are presented as mean ± SD. Different characters (a–d) indicate significant differences ($P < 0.05$; one-way ANOVA vs. vehicle). Data are representative of four to eight independent experiments.

without affecting LUC stability (Figure S7). This suggests that GO214 lengthens the circadian period in a similar manner to KL001. Unexpectedly, however, period-shortening molecules did not exhibit CRY1-destabilizing activity; GO044 had little effect on the stability of CRY1, and both GO200 and GO211 increased the half-life of CRY1 in a dose-dependent manner (Figures 5 and S6). It is possible that these molecules regulate the circadian period not only by the degradation of CRY but also through some other unknown processes mediated by CRY. Although further extensive studies are necessary to elucidate the as yet unknown CRY-mediated period-shortening mechanism, it is clear that C–H activation chemistry unveiled an untapped chemical space of biological importance.

In summary, we have succeeded in uncovering the sites of KL001 derivatives that are critical for their rhythm-changing

activities and period-lengthening/shortening selectivities towards the CRY-mediated circadian clock regulation. We envisage that these compounds provide a tool to study the regulatory mechanism of CRY in the circadian timekeeping mechanism. Furthermore, the modulation of the circadian period is expected to improve animal production and to form the basis of therapeutic applications. Last but not least, we wish to emphasize that the present study represents the fruitful merging of synthetic chemistry, circadian-clock science, and theoretical chemistry, and also showcases the power of new C–H activation processes in discovering new biofunctional molecules (the first period-shortening molecules targeting the CRY were discovered in this study). With the recent advent of a number of game-changing C–H activation reactions,[12,13] there are significant opportunities to use these methods to accelerate circadian-clock science.

Keywords: C–H activation · circadian clock · cryptochrome · small-molecule modulators · structure–activity relationships

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