Lycorine, a Candidate for the Control of Period Length in Mammalian Cells

Yoshiaki Onishi, Yasuhiro Kawano and Yoshimitsu Yamazaki

Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Higashi, Tsukuba

Key Words

Circadian rhythm • Alkaloid • Transcription

Abstract

Plants of the Amaryllidaceae family have been used as therapeutic agents against CNS related maladies such as Alzheimer's disease. The known primary alkaloid constituents have significant biological activity. We identified the *Lycoris* alkaloids lycorine and lycoricidinol from Amaryllidaceae using a real-time reporter gene assay system based on NIH3T3 cells. These alkaloids have a wide spectrum of pharmacological actions and dose-dependently lengthen the circadian period. When cells that had been incubated with lycorine or lycoricidinol were washed and then incubated without these alkaloids, period length reverted to that of control cells, suggesting that elongation of the circadian period induced by lycorine and lycoricidinol is reversible. Although one of its major activities is the inhibition of protein synthesis, lycorine induced dose-dependent period elongation regardless of the presence of cycloheximide and moreover, cycloheximide, itself did not affect period length, suggesting that lycorine

dose-dependently extends the circadian period by a mechanism other than translational inhibition. Real-time RT-PCR showed that lycorine enhanced *ROR α* and *Bmal1* transcription, and exogenous expression and knockdown of *Bmal1* also caused long and short periods, respectively, thus confirming the phenotype indicated by lycorine. These data indicate that lycorine and lycoricidinol modulate *Bmal1* transcription and the circadian period, and also suggest that *Lycoris* alkaloids are novel contributors to the control of period length in mammalian cells.

Copyright © 2012 S. Karger AG, Basel

Introduction

Circadian clocks align behavioral and biochemical process with the day/night cycle. The molecular mechanism of the circadian oscillator consists of autoregulatory transcriptional and translational feedback loops that have both positive and negative elements. Among the core clock genes, *Bmal1* is apparently

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2012 S. Karger AG, Basel
1015-8987/12/0294-0407\$38.00/0

Accessible online at:
www.karger.com/cpb

Yoshiaki Onishi
Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Higashi 1-1-1, Tsukuba, Ibaraki 305-8566 (Japan)
Tel. +81-29-861-6527, Fax +81-29-861-6053
E-Mail y-onishi@aist.go.jp

essential and non-redundant in the mammalian clock and its expression level robustly oscillates in central and peripheral clock cells [1]. Therefore, *Bmal1* transcription should be closely associated with circadian rhythms. The *Bmal1* promoter contains two recognition motifs for ROR and REV-ERB orphan nuclear receptors (ROREs) as the critical elements for *Bmal1* oscillatory transcription [2]. In addition, the ROREs are embedded in a unique chromatin structure consisting of GC-rich open chromatin, with which the nuclear matrix-like structure at the 3'-flanking region cooperates to regulate *Bmal1* transcription [3]. The master clock that generates circadian rhythms in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus where it controls all aspects of physiology such as sleep-wake cycles, body temperature, hormone secretion, blood pressure and metabolism. Coordination among such aspects of physiology by the circadian clock is essential to optimize metabolic responses and strengthen inherent homeostatic regulatory mechanisms [4]. The circadian clock generates robust rhythms coupled with changes in the cellular environment. Thus, circadian dysfunction is considered to contribute to the incidence and severity of a wide range of clinical and pathological conditions including sleep disorders, cancer, depression, metabolic syndrome and inflammation [5]. Symptoms of disturbed circadian rhythms such as day-time agitation, night-time insomnia and restlessness often develop at some stage of Alzheimer's disease [6].

Various herbaceous plant families have been used as therapeutic agents against CNS-related maladies such as Alzheimer's disease for thousands of years [7]. Members of the Amaryllidaceae family are among the top 20 most widely applied medical plants, from which many pharmacologically active compounds including alkaloids, phenols, lectines and peptides have been isolated. Almost 500 structurally diverse alkaloids with significant biological activity have been isolated from the Amaryllidaceae family [8]. Galantamine, an alkaloid isolated from the bulbs and flowers of *Galanthus woronowii* (Amaryllidaceae), has been an effective treatment for mild to moderate Alzheimer's disease [9].

Natural products, especially alkaloids from herbal plants exhibit a range of biological activities. Recently, we found that the herbal alkaloid, harmine has potential as anti-inflammatory agents that can suppress TNF- α and NO production [10]. We also found using a custom-designed assay for circadian oscillation *in vitro* that harmine extends the circadian period [11]. The

Amaryllidaceae alkaloid, lycorine (3, 12-didehydro-9, 10-[methylenebis(oxy)]-galanthan-1 α , 2 β -diol) also suppressed TNF- α and NO production, indicating powerful anti-inflammatory activity [10]. Lycorine also has antiviral and antimalarial properties as well as the ability to inhibit protein synthesis and cell growth [12]. In addition, lycorine inhibits acetylcholinesterase activity, which might restore the acetylcholine levels and cholinergic functions of the brains of patients with Alzheimer's disease [13]. Lycorine might affect circadian rhythms, but this has yet to be proven.

Here, we determined the effects of the *Lycoris* alkaloids, lycorine and lycoricidinol on the circadian oscillation of *Bmal1* transcription *in vitro*.

Materials and Methods

Chemicals

Restriction and modifying enzymes were purchased from TOYOBO (Osaka, Japan). Cell culture materials were obtained from Invitrogen. Lycoricidinol was prepared as described [10]. Briefly, lycoricidinol was extracted from bulbs of *Lycoris radiata* with ethanol and purified by silica gel column chromatography (methanol/ethyl acetate) followed by reverse phase column chromatography (Wakogel 100C18) with 0 - 50% methanol in water as the solvent. Other chemicals used were of the highest quality available from Sigma.

Cell culture

Stable cells containing the luciferase reporter gene driven by the *Bmal1* promoter region (-197 to +27) were established from NIH3T3 cells as described [11]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and a mixture of penicillin and streptomycin in a humidified incubator at 37°C under a 5% CO₂ atmosphere.

Real-time reporter gene assays

Real-time reporter gene assays proceeded as described [3]. Stable reporter cells were stimulated with 100 nM dexamethasone for 2 h and then incubated with DMEM containing 0.1 mM luciferin (Promega), 25 mM HEPES (pH 7.2) and 10% FBS. Bioluminescence was measured and integrated for 1 min at 10-min intervals using a Kronos AB-2500 (ATTO). Data were detrended by subtraction of a best fit line and subsequent fitting to a sine wave to determine circadian period length as described [14].

Cell growth assay

Stable cells (2 x 10⁴) were incubated with lycorine in 96-well plates and then cell growth was assayed using the Cell Counting Kit-8 (DOJINDO). The water-soluble tetrazolium salt, WST-8 (10 μ l) was added to each well and incubated at 37°C under 5% CO₂ in a humidified incubator for 1 h. Absorbance

was measured using a microplate reader (Bio-Rad) at 450/655 nm.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR proceeded as described [3]. Total RNA was prepared using acid guanidinium thiocyanate-phenol-chloroform. First strand cDNA synthesized from RNA (1 µg) using PrimeScript RT-PCR kit (TAKARA, Osaka, Japan) was amplified using a LightCycler (Roche) and the Light Cycler-FastStart DNA Master SYBR Green I kit (Roche) with the following primer sequences: *Actin*, 5'-TAC GCCAACACAGTGCTGTCTG-3' and 5'-TTTCTCTGCGCAAGTTAGGTTTGTGTC-3'; *Bmal1*, 5'-GGA CTT CGC CTC TAC CTG TTC A-3' and 5'-AAC CAT GTG CGA GTG CAG GCG C-3'; *RORα*, 5'-CCA ACC GTG TCC ATG GCA GAA C-3' and 5'-GCA CAC AGC TGC CAC ATC ACC T-3'. The PCR products cloned into the pGEM-T Easy vector (Promega) served as an authentic template. Relative expression levels were evaluated using Light Cycler software version 3.5.

Western blotting

SDS-PAGE and Western blotting proceeded as described [11]. Briefly, proteins were resolved by 9% SDS-PAGE and transferred to PVDF membranes (Amersham). Non-specific binding was blocked with 3% dry milk in PBS. Proteins were probed with anti-BMAL1 antibody (Santa Cruz Biotechnology), anti-RORα antibody (Santa Cruz Biotechnology), anti-ACTIN antibody (Millipore) or anti-LAMIN A/C antibody (Santa Cruz Biotechnology) and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-goat IgG (Upstate). Immunoreactive proteins were visualized using ECL (Amersham) according to the manufacturer's instructions.

Excess and knockdown of *Bmal1* expression

The *Bmal1* plasmid that were used for overexpression has been described [15]. The Stealth RNAi siRNA duplex for *Bmal1* knockdown and BLOCK-iT Fluorescent Oligo as a negative control were purchased from Invitrogen. The stealth RNAi was the mixture of 3 siRNAs and these sequences were as follows: 5'-AUAACAUGAUGUACC UAG AAG UUC C-3' and 5'-GGA ACU UCU AGG UAC AUC AUG UUA U-3'; 5'-UUGUCU GGC UCA UUG UCU UCG UCC A-3' and 5'-UGG ACGAAGACAAUGAGC CAGACAA-3'; 5'-UUU GGA UGC AGG UAG UCA AAC AAG C-3' and 5'-GCU UGU UUG ACU ACC UGC AUC CAA A-3'. The plasmid and siRNAs were introduced into cells using HilyMax (DOJINDO).

Results

Lycorine and lycoricidinol extend the circadian period

To determine whether or not the *Lycoris* alkaloids, lycorine and lycoricidinol affect circadian rhythms, lycorine and lycoricidinol were analyzed using real-time reporter gene assays and an established stable clone [11]. Figure

Fig. 1. Dose-dependent period elongation. Stable clones were stimulated with 100 nM dexamethasone for 2 h, and then bioluminescence was measured without (gray) or with 5 µM lycorine (black in A) or with 0.1 µM lycoricidinol (black in C). Fit curve data of detrended results are representative of triplicate experiments. Stable clones were analyzed by real-time reporter gene assays with various concentrations of alkaloids (B, lycorine; D, lycoricidinol) and period parameters obtained by curve fitting were plotted against final alkaloid concentrations. Values are means $\pm$ SEM of triplicate assays.

1A shows representative results of real-reporter gene assays using stable cells with lycorine, indicating that the period length of the cells incubated without (gray) and with 5 µM lycorine (black) were 25.1 ± 0.98 and 30.6 ± 0.73 h, respectively. Moreover, lycorine dose-dependently extended the circadian period for up to 8 h (Fig. 1B) and lycoricidinol, another derivative of *Lycoris* alkaloid also dose-dependently extended the circadian period (Fig. 1C and D). We next investigated whether or not the period extended by lycorine and lycoricidinol is transient. Stable reporter cells that have been incubated once with these alkaloids and then washed out were used in real-time reporter assays. The period lengths after washout were 28.5 ± 1.17 and 24.5 ± 0.59 h, respectively whereas those with lycorine and

Fig. 2. Reversible period elongation by lycorine and lycoricidinol. Stable clones were stimulated with 100 nM dexamethasone for 2 h, and then bioluminescence was measured with 5 μ M lycorine or 0.1 μ M lycoricidinol. Cells were washed and the culture medium was replaced with recording medium without lycorine or lycoricidinol 96 h later (indicated as arrows). Black and gray lines indicate results with and without cell wash and medium change, respectively. Fit curve data of detrended results are representative of triplicate experiments.

lycoricidinol before washout were 33.8 ± 1.64 and 30.0 ± 1.50 h, respectively (Fig. 2). On the other hand, the period length of the cells incubated with vehicle did not differ between before and after washout (Fig. 2). These results

Fig. 3. Effects of cycloheximide on circadian period. Stable clones were stimulated with 100 nM dexamethasone for 2 h, and then bioluminescence was measured with various concentrations of cycloheximide. Black, blue, cyan, green, orange and red lines indicate results with 0, 25, 50, 100, 500, 1000 ng/ml of cycloheximide, respectively. Fit curve data of detrended results are the representative of triplicate experiments (Top). Period parameters obtained by curve fitting were plotted against final alkaloid concentrations and values are means $\pm$ SEM of triplicate assays (Bottom).

Addition	Period (h)		
	Control	Lycorine (2.5 μ M)	Lycorine (10 μ M)
None	25.5 ± 1.41	29.3 ± 1.13	34.1 ± 1.76
Cycloheximide	25.7 ± 0.73	29.4 ± 1.27	34.3 ± 1.94

Table 1. Effect of cycloheximide on lycorine-induced period elongation.

indicate that the period length elongated by lycorine or lycoricidinol was shortened almost to that of the control cells, suggesting that elongation of the period induced by lycorine and lycoricidinol is reversible.

Fig. 4. Effect of lycorine on cell growth. Stable cells were incubated with various concentrations of lycorine in 96-well plates, and cell growth was assayed using Cell Counting Kit-8. Values are means $\pm$ SEM of triplicate assays. Open and closed circles, closed and open squares and closed and open triangles indicate 0, 0.6, 1.3, 2.5, 5.0 and 10 μ M lycorine, respectively.

Period elongation is independent of protein synthesis inhibition

A major actions of lycorine is the inhibition of protein synthesis [16] and therefore, the amplitude of circadian oscillation increased after washing the alkaloids (Fig. 2). To evaluate the effect of protein synthesis on the circadian period, we studied the effect of cycloheximide (a protein synthesis inhibitor) on circadian rhythms using real-time reporter gene assays. Inhibition of protein synthesis of 5 μ M lycorine [16] is comparable to that of 25 ng/ml cycloheximide [17]. According to the concentration of cycloheximide, the phase of circadian oscillation was delayed and its amplitude was reduced (Fig. 3). On the other hand, circadian period did not significantly change, suggesting that the length of the circadian period was not dependent on the effect of cycloheximide. Moreover, lycorine induced dose-dependent period elongation regardless of the presence of cycloheximide, which did not additively affect period elongation induced by lycorine (Table 1). Although inhibition of translation blocks cell proliferation [17] and lycorine induces apoptosis of tumor cells [18], cell growth assays showed that the cells proliferated in the presence of 1 μ M lycorine and that the cell viability incubated with 10 μ M of lycorine at 4 days was 77.2% (Fig. 4). Taken together, these results suggest that lycorine dose-dependently extends the circadian period via a mechanism other than translational inhibition.

Fig. 5. Lycorine and lycoricidinol enhance *Bmal1* transcription. NIH3T3 cells were incubated with 5 μ M lycorine or 0.1 μ M lycoricidinol for 12 or 24 h and expression of *Bmal1* (A) and *RORα* (B) was analyzed by real-time quantitative RT-PCR. Levels of RNA were normalized to *Actin* expression. Expression levels are indicated relative to that without these compounds. Values are means $\pm$ SEM of triplicate assays. *P < 0.05 (Student's *t* test).

*Lycorine and lycoricidinol enhance *Bmal1* transcription*

Elongation of the circadian period of *Bmal1* transcription indicates a change in *Bmal1* transcription. Both lycorine and lycoricidinol activated *Bmal1* expression up to 3.5-fold after 24 h (Fig. 5A). To determine how *Bmal1* transcription increased, we analyzed *RORα*, a major positive transcription factor for *Bmal1*, recognition sequences for which are found in the reporter gene [11]. Real-time RT-PCR experiments showed that lycorine and lycoricidinol increased *RORα* transcription, particularly after their 24 h, implying that both of them activate *Bmal1*

Fig. 6. Western blot analysis of BMAL1 and ROR α . NIH3T3 cells were cultured with 5 μ M lycorine or 0.1 μ M lycoricidinol for indicated periods, and then whole cell lysate (A) or nuclear (Nuc.) and cytosolic (Cyto.) fractions were prepared as described [11] (B) and analyzed by Western blotting. Triangles and asterisks indicate phosphorylated and unphosphorylated forms, respectively. BMAL1 with anti-BMAL1 antibody; ROR α , with anti-ROR α antibody; Actin, anti-actin antibody; Lamin, with anti-lamin A/C antibody.

Fig. 7. Level of *Bmal1* expression affects period length. *Bmal1* expression plasmid (A) or siRNA (B) was transfected into stable clones and at 24 h after transfection circadian periods were started to analyze using real-time reporter gene assays. Fit curve data of detrended results are representative of triplicate experiments (control transfection, gray; *Bmal1* plasmid or *Bmal1* siRNA, black).

transcription via the induction of ROR α transcription (Fig. 5B). To evaluate whether or not BMAL1 and ROR α protein levels are enhanced by lycorine, Western blot analysis using anti-BMAL1 and anti-ROR α antibodies proceeded. As reported previously [19], several phosphorylated forms of BMAL1 were detected and an increase of BMAL1 protein (especially phosphorylated forms) was observed by lycorine and lycoricidinol (Fig. 6A), suggesting that lycorine and lycoricidinol enhance BMAL1 protein level. Nuclear phosphorylated ROR α slightly increased at 12 h after incubation with lycorine and lycoricidinol whereas neither phosphorylated nor unphosphorylated form of cytosolic ROR α showed the change of protein level (Fig. 6B). These results also support the above hypothesis that lycorine and

lycoricidinol activate *Bmal1* transcription.

We then evaluated the relationship between the level of *Bmal1* gene expression and period length. A *Bmal1* expression plasmid was transfected into stable reporter cells to increase *Bmal1* expression, and then the circadian period was analyzed using real-time reporter assays. Figure 7A shows that the circadian periods of cells transfected with the *Bmal1* expression vector (black) or with control vector (gray) were 25.9 ± 0.59 and 24.5 ± 0.50 h, respectively ($p = 0.0019$). Knockdown experiments using siRNA to reduce *Bmal1* expression and real-time reporter gene assays with siRNA showed that the circadian periods of the cells harboring *Bmal1* siRNA (black) and control siRNA (gray) were 23.2 ± 0.51 and 25.2 ± 0.53 h, respectively (Fig. 7B). These data indicate that the phenotype of the erratic *Bmal1* gene regulation was associated with significantly longer and shorter circadian periods, respectively and that the amount of *Bmal1* expression is related to circadian rhythms.

Discussion

Amaryllidaceae alkaloids

Various plant families have been used as therapeutic agents against various diseases for thousands years and extracts of the Amaryllidaceae family have been used as herbal remedies. The diverse alkaloids of this family have a wide range of biological activities [8]. Galanthamine was the first commercially available selective, reversible and competitive acetylcholine esterase inhibitor generated from Amaryllidaceae alkaloids that was used to treat Alzheimer's disease. Lycorine was the first antiviral alkaloid discovered in the Amaryllidaceae family and it is also a powerful inhibitor of cell division and growth in higher plants [20]. Lycoricidinol, also known as narciclasine was isolated in 1967 from *Narcissus* (Amaryllidaceae species) bulbs based on its ability to inhibit the growth of wheat grain radicles [21]. These *Lycoris* alkaloids arise from the common intermediate, norbelladine, which undergoes different cyclization, possibly followed by the rearrangement and/or elimination of two carbons, ring opening, and/or recyclization to provide a variety of skeletons [12]. *Lycoris* alkaloids have various biological properties such as protein biosynthesis inhibition [16], interference in vitamin C biosynthesis [22], pro-apoptotic effects [18], anti-apoptotic activity [23] and DNA interaction [24]. Thus, *Lycoris* alkaloids should have potential as novel anti-cancer [12], antiviral,

anti-inflammatory and antimalarial agents [20]. In addition, lycorine inhibits acetylcholine esterase activity, which might be expected to restore the acetylcholine levels and cholinergic functions of the brain among patients with Alzheimer's disease [13].

Here, we showed that lycorine and its derivative, lycoricidinol dose-dependently extended the circadian period (Fig. 1), and this is the first report to describe a circadian modulating function of *Lycoris* alkaloids. Analyses of structure-activity relationships confirmed the importance of the chemical structure, such as the presence of a positively charged nitrogen atom, molecular planarity and appropriately positioned alkoxy functions for the biological activities of *Lycoris* alkaloids [25]. Both lycorine and lycoricidinol extended the circadian period, suggesting that they have structural similarity for this activity.

Circadian rhythm and protein synthesis

The clock system consists of autoregulatory transcriptional and translational feedback loops and the protein modification of clock components such as phosphorylation which affects the protein stability or the protein amount and thus is important for circadian rhythms [26]. Figure 3 shows that cycloheximide, a protein synthesis inhibitor, little affected the circadian period whereas the phase of circadian oscillation was delayed and the amplitude was reduced. Cycloheximide at $0.02 \mu\text{g/ml}$ reduces protein synthesis by 50% in NIH3T3 cells [17], which are the parental cells of the stable cells used in this study. Recently proposed clock system in *Gonyaulax* controls protein synthesis at the translational level [27]. Even in this system, pulses of protein synthesis inhibitors cause phase shifts and not period changes, findings that are supported by our results (Fig. 3), suggesting a common feature of the circadian system. Inhibition of de novo protein synthesis stabilizes mRNAs with a short half-life [28] and we also observed an increase of *Bmal1* mRNA by lycorine and lycoricidinol (Fig. 5A). The BMAL1 protein level increased after treatment with lycorine and lycoricidinol (Fig. 6A), whereas protein synthesis inhibitors result in the rapid loss of proteins [29]. Although the inhibition of protein synthesis has been considered the major pharmaceutical activity of lycorine [16], cycloheximide did not affect lycorine-induced elongation of the circadian period (Table 1). Taken together, these results suggest that lycorine dose-dependently extends the circadian period by a novel mechanism other than translational inhibition.

Circadian rhythms and cell growth

Lycoris alkaloids have promising anti-tumor properties through arresting the cell cycle and inducing apoptosis [18]. Lycoricidinol has more powerful anti-tumor activity than lycorine [10] and elongates the period elongation at a lower concentration (Fig. 1), implying a relationship between the effect of period elongation and anti-tumor activities. Unsal-Kacmaz et al. have proposed two models for coupling of circadian and cell cycles [30]. One is the serial coupling model. In this mode of synchronization of the two cycles, proteins or reactions belonging primarily to the circadian cycle regulate the expression of genes controlling the cell cycles. The other is the direct coupling model. In this mode a protein such as Tim directly participates in the molecular machineries of both cycles, and both cycles might collapse as a consequence of eliminating this protein.

The growth of stable cells incubated with 10 μ M of lycorine was arrested; the cell viability at 4 days was 77.2% (Fig. 4) and the period elongation was 8.6 h. In contrast, the cells proliferated in the presence of 1 μ M lycorine (Fig. 4), and the period elongation was 1.3 h. Increasing the concentration of cycloheximide causes total depletion of cells at the G1 and S phases [17] but does not affect the circadian period (Fig. 3). These results do not indicate the direct relationship between period elongation and anti-tumor activities, suggesting that the period elongation is a novel pharmaceutical activity of lycorine.

*Circadian regulation of *Bmal1* transcription*

Bmal1 transcription shows circadian oscillation, whose critical *cis*-element is the RORE [2], and the *Bmal1* promoter region of the reporter gene contains ROREs, where ROR and REV-ERB families can bind [11]. Lycorine and lycoricidinol enhanced *Bmal1* transcription after a 24-h incubation (Fig. 5A) and gradually increased the transcription of ROR α , the major *Bmal1* activator (Fig. 5B), suggesting that *Lycoris* alkaloids enhance *Bmal1* transcription via ROR α activation. This was also supported by the result that *Lycoris* alkaloids enhanced nuclear ROR α level (Fig. 6B).

Enhanced *Bmal1* expression in the stable reporter cells represent the long period phenotype (Fig. 7A), which is consistent with the effects of lycorine and lycoricidinol. These and our previous findings that enhanced ROR α expression in stable reporter cells represent the long period phenotype are consistent [11]. Recently, Lee et al. have reported that the stoichiometric relationship between clock proteins is critical for the robustness of circadian rhythms

and demonstrated that overexpression of BMAL1 significantly lengthens the circadian period [31]. Furthermore, knockdown of *Bmal1* expression by siRNA resulted in the short period phenotype (Fig. 7B). Taken together, these results suggest that the modulation of *Bmal1* expression affects the circadian period, which plays a role in period elongation induced by *Lycoris* alkaloids. The huge difference between 8 h (10 μ M lycorine effect) and 1.4 h (*Bmal1* overexpression) existed (Fig. 1B and 7A), whereas lycorine treatment did not cause higher BMAL1 and nuclear ROR α levels than that resulted from *Bmal1* overexpression (Fig. 6). *Lycoris* alkaloids enhanced BMAL1 and nuclear ROR α levels and both were phosphorylated forms (Fig. 6). Functions of BMAL1 [19] and ROR α [32] are modulated by phosphorylation. The importance of PER phosphorylation kinetics for the circadian period determination has recently been described [33]. These data imply the possibility of phosphorylation of clock proteins by *Lycoris* alkaloids. Effects of *Lycoris* alkaloids on the protein phosphorylation has not been fully elucidated yet and it has been reported that lycorine shows the inhibition of p38 and STATs, or dephosphorylation of these proteins [34], implying that the circadian period elongation by *Lycoris* alkaloids may be brought by the synergic effect, enhancement of BMAL1 and other biochemical activities such as protein phosphorylation. A more precise mechanism of period elongation should be elucidated and *Lycoris* alkaloids will be useful tools with which to analyze the circadian mechanism.

Chronotherapeutic insight into phyto-alkaloids

Plant extracts have been used for millennia as herbal remedies and alkaloids are their major phyto-constituents. *Lycoris* alkaloids are acetylcholine esterase inhibitors with anti-inflammatory, antimalarial and antitumor activities. Both biological events and drug metabolism are under the control of the circadian clock and therefore not only circadian rhythms, but also the pharmacokinetics, effects and safety of chronopharmaceuticals (pharmaceuticals that focus on biological rhythms) should be considered in terms of risk for disease [35].

Galanthamine, an Amaryllidaceae alkaloid is a long acting, selective, reversible and competitive acetylcholinesterase inhibitor that has been marketed as a hydrobromide salt under the name Reminyl for the treatment of Alzheimer's disease. Circadian rhythms are often disturbed in patients with Alzheimer's disease [6]. In addition, modulating the natural circadian fluctuations of central cholinergic transmission is an important factor for improvement when using galanthamine to stimulate

central cholinergic transmission in Alzheimer's disease [36]. Here, we showed that *Lycoris* alkaloids also have an acetylcholinesterase inhibition activity [13] and elongate the circadian period (Fig. 1) and our findings provide new insight into achieving optimal benefits from chronopharmaceutical therapy using Amaryllidaceae alkaloids by avoiding interference with circadian rhythms.

Acknowledgements

We wish to thank S. Maruyama and K. Oishi (AIST) for helpful discussions. This study was supported by a grant from KAKENHI (23592756) and by operational subsidies from AIST.

References

- Bunger MK, Wilbacher LD, Moran SM, Clendenen C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA: Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 2000;103:1009-1017.
- Ueda HR, Chen W, Adachi A, Wakamatsu H, Hayashi S, Takasugi T, Nagano M, Nakahama K, Suzuki Y, Sugano S, Iino M, Shigeyoshi Y, Hashimoto S: A transcription factor response element for gene expression during circadian night. *Nature* 2002;418:534-539.
- Onishi Y, Hanai S, Ohno T, Hara Y, Ishida N: Rhythmic SAF-A binding underlies circadian transcription of the Bmal1 gene. *Mol Cell Biol* 2008;28:3477-3488.
- Reppert SM, Weaver DR: Coordination of circadian timing in mammals. *Nature* 2002;418:935-941.
- Bechtold DA, Gibbs JE, Loudon AS: Circadian dysfunction in disease. *Trends Pharmacol Sci* 2010;31:191-198.
- Weldemichael DA, Grossberg GT: Circadian rhythm disturbances in patients with Alzheimer's disease: a review. *Int J Alzheimers Dis* 2010;2010:pii:716453.
- Adams M, Gmunder F, Hamburger M: Plants traditionally used in age related brain disorders-a survey of ethnobotanical literature. *J Ethnopharmacol* 2007;113:363-381.
- Jin Z: Amaryllidaceae and Scleptium alkaloids. *Nat Prod Rep* 2007;24:886-905.
- Heinrich M, Lee Teoh H: Galanthamine from snowdrop-the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *J Ethnopharmacol* 2004;92:147-162.
- Yamazaki Y, Kawano Y: Inhibitory Effects of Herbal Alkaloids on the TNF- α and Nitric Oxide Production in Lipopolysaccharide-stimulated RAW264 Macrophages. *Chem Pharm Bull (Tokyo)* 2011;59:388-391.
- Onishi Y, Oishi K, Kawano Y, Yamazaki Y: The harmala alkaloid, harmine is a modulator of circadian Bmal1 transcription. *Biosci Rep* 2011;32:45-52.
- Lamoral-Theys D, Decaestecker C, Mathieu V, Dubois J, Kornienko A, Kiss R, Evidente A, Pottier L: Lycorine and its derivatives for anticancer drug design. *Mini Rev Med Chem* 2010;10:41-50.
- Lopez S, Bastida J, Viladomat F, Codina C: Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and Narcissus extracts. *Life Sci* 2002;71:2521-2529.
- Welsh DK, Imaizumi T, Kay SA: Real-time reporting of circadian-regulated gene expression by luciferase imaging in plants and mammalian cells. *Methods Enzymol* 2005;393:269-288.
- Ohno T, Onishi Y, Ishida N: The negative transcription factor E4BP4 is associated with circadian clock protein PERIOD2. *Biochem Biophys Res Commun* 2007;354:1010-1015.
- Vrijnsen R, Vanden Berghe DA, Vlietinck AJ, Boeye A: Lycorine: a eukaryotic termination inhibitor? *J Biol Chem* 1986;261:505-507.
- Adolph S, Brusselbach S, Muller R: Inhibition of transcription blocks cell cycle progression of NIH3T3 fibroblasts specifically in G1. *J Cell Sci* 1993;105:113-122.
- Liu J, Hu WX, He LF, Ye M, Li Y: Effects of lycorine on HL-60 cells via arresting cell cycle and inducing apoptosis. *FEBS Lett* 2004;578:245-250.
- Tamaru T, Isojima Y, van der Horst GT, Takei K, Nagai K, Takamatsu K: Nucleocytoplasmic shuttling and phosphorylation of BMAL1 are regulated by circadian clock in cultured fibroblasts. *Genes Cells* 2003;8:973-983.
- Toriizuka Y, Kinoshita E, Kogure N, Kitajima M, Ishiyama A, Otoguro K, Yamada H, Omura S, Takayama H: New lycorine-type alkaloid from *Lycoris traubii* and evaluation of antitrypanosomal and antimalarial activities of lycorine derivatives. *Bioorg Med Chem* 2008;16:10182-10189.
- Ceriotti G: Narciclasine: an antimitotic substance from *Narcissus bulbs*. *Nature* 1967;213:595-596.
- Arrigoni O: Ascorbate system in plant development. *J Bioenerg Biomembr* 1994;26:407-419.
- Yui S, Mikami M, Kitahara M, Yamazaki M: The inhibitory effect of lycorine on tumor cell apoptosis induced by polymorphonuclear leukocyte-derived calprotectin. *Immunopharmacology* 1998;40:151-162.
- Karadeniz H, Gulmez B, Sahinci F, Erdem A, Kaya GI, Unver N, Kivcak B, Ozsoz M: Disposable electrochemical biosensor for the detection of the interaction between DNA and lycorine based on guanine and adenine signals. *J Pharm Biomed Anal* 2003;33:295-302.
- Zee-Cheng RK, Yan SJ, Cheng CC: Antileukemic activity of ungeremine and related compounds. Preparation of analogues of ungeremine by a practical photochemical reaction. *J Med Chem* 1978;21:199-203.
- Kojima S, Shingle DL, Green CB: Post-transcriptional control of circadian rhythms. *J Cell Sci* 2011;124:311-320.
- Hastings JW: The Gonyaulax clock at 50: translational control of circadian expression. *Cold Spring Harb Symp Quant Biol* 2007;72:141-144.

- 28 Xavier S, Gilbert V, Rastaldi MP, Krick S, Kollins D, Reddy A, Bottinger E, Cohen CD, Schlondorff D: BAMB1 is expressed in endothelial cells and is regulated by lysosomal/autolysosomal degradation. *PLoS ONE* 2010;5:e12995.
- 29 Nguyen T, Sherratt PJ, Huang HC, Yang CS, Pickett CB: Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J Biol Chem* 2003;278:4536-4541.
- 30 Unsal-Kacmaz K, Mullen TE, Kaufmann WK, Sancar A: Coupling of human circadian and cell cycles by the timeless protein. *Mol Cell Biol* 2005;25:3109-3116.
- 31 Lee Y, Chen R, Lee HM, Lee C: Stoichiometric relationship among clock proteins determines robustness of circadian rhythms. *J Biol Chem* 2011;286:7033-7042.
- 32 Duplus E, Gras C, Soubeyre V, Vojdani G, Lemaigre-Dubreuil Y, Brugg B: Phosphorylation and transcriptional activity regulation of retinoid-related orphan receptor alpha 1 by protein kinases C. *J Neurochem* 2008;104:1321-1332.
- 33 Lee HM, Chen R, Kim H, Etchegaray JP, Weaver DR, Lee C: The period of the circadian oscillator is primarily determined by the balance between casein kinase 1 and protein phosphatase 1. *Proc Natl Acad Sci USA* 2011;108:16451-16456.
- 34 Kang J, Zhang Y, Cao X, Fan J, Li G, Wang Q, Diao Y, Zhao Z, Luo L, Yin Z: Lycorine inhibits lipopolysaccharide-induced iNOS and COX-2 up-regulation in RAW264.7 cells through suppressing P38 and STATs activation and increases the survival rate of mice after LPS challenge. *Int Immunopharmacol* 2012;12:249-256.
- 35 Ohdo S: Chronopharmaceutics: pharmaceuticals focused on biological rhythm. *Biol Pharm Bull* 2010;33:159-167.
- 36 Nieoullon A, Bentue-Ferrer D, Bordet R, Tsolaki M, Forstl H: Importance of circadian rhythmicity in the cholinergic treatment of Alzheimer's disease: focus on galantamine*. *Curr Med Res Opin* 2008;24:3357-3367.