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Shikonin shortens the circadian period: Possible involvement of Top2 inhibition

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ABSTRACT

The naphthoquinone pigment, shikonin, is a natural product derived from *Lithospermum erythrorhizon* and an active component of a Chinese traditional herbal therapeutic. We identified shikonin as a candidate for shortening the circadian period using real-time reporter gene assays based on NIH3T3-derived stable reporter cells. Period length that became shortened in cells incubated with shikonin or etoposide reverted to that of control cells after continued incubation without these compounds. These findings indicated that shikonin and etoposide shorten the circadian period reversibly and through similar mechanisms. Topoisomerase II (Top2)-specific decatenation assays confirmed that shikonin, liker etoposide, is a Top2 inhibitor. Shikonin was incorporated into the nucleus and Top2 was located in the *Bmal1* promoter, suggesting the relationship between *Bmal1* transcription and Top2 inhibition. *Top2a* siRNA also shortened period length, suggesting that *Top2* is involved in this process. Promoter assays showed that *Top2a* siRNA, etoposide and shikonin reduce *Bmal1* promoter activity. These findings indicated that Top2 is involved in *Bmal1* transcription and influences the circadian period, and that shikonin is a novel contributor to the control of period length in mammalian cells.

1. Introduction

Circadian clocks align behavioral and biochemical process with the circadian cycle and control 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 [1]. Thus, perturbation of the circadian clock has been implicated in numerous pathologies including cardiovascular and metabolic diseases, circadian sleep disorders and cancer [2]. The master clock that generates circadian rhythms in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The circadian clock resides at the cellular level, and single cells exhibit circadian rhythms in a cell-autonomous manner [3]. The molecular mechanism of the circadian oscillator is based on interlocking transcriptional and translational feedback loops that have both positive and negative elements. The mammalian core clock proteins BMAL1 and CLOCK heterodimerize, bind to E-boxes and activate the transcription of *Pers* and *Crys*. The core clock proteins PERs and CRYs heterodimerize, associate with other partners in the nucleus and repress BMAL:CLOCK-driven activation, thus generating a negative autoregulatory feedback loop [1]. The BMAL:CLOCK heterodimer also binds to E-boxes in many clock-controlled genes and dictates their expression. Thus, circadian dysfunction is considered to contribute to the incidence and severity of a wide range of clinical and pathological conditions [4].

Among the core clock genes, the expression of *Bmal1* that oscillates in the SCN and in peripheral clock cells is closely associated with circadian rhythms [5]. The *Bmal1* promoter contains two recognition motifs for ROR and REV-ERB orphan nuclear receptors (ROREs) that are critical elements for *Bmal1* oscillatory transcription [6]. We previously found that the ROREs are embedded in a unique GC-rich open chromatin structure, with which a nuclear matrix-like structure at the 3′-flanking region cooperates to regulate *Bmal1* transcription [7], and that the chromatin architecture of clock genes including DNA topology is a determinant of these rhythms [8]. Several chemicals with topoisomerase inhibition show a wide range of pharmaceutical activity [9]. Changing the DNA topology of clock genes, and of *Bmal1* in particular, implies modifications of circadian rhythms, and we previously evaluated relationships between topoisomerase inhibition and these rhythms. For instance, harmala alkaloids inhibit topoisomerase activity [10] and we reported that the harmala alkaloid harmine modulates circadian *Bmal1* transcription [11]. Hirota et al. found that the Top2-inhibitor etoposide shortens the circadian period [12]. We recently found that Top1 modulates the chromatin structure of the *Bmal1* promoter, regulates *Bmal1* transcription and

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influences the circadian period, whereas the Top1 inhibitor camptothecin lengthens the circadian period [8].

The naphthoquinone pigment, shikonin is a natural product derived from *Lithospermum erythrorhizon* and an active component of the Chinese herbal therapeutic, Zicao which has historically been used to treat measles, sore-throat, carbuncles and burns. Shikonin has a broad spectrum of pharmacological activities including DNA topoisomerase inhibition [13]. Here, we studied the effects of shikonin on the transcriptional oscillation of *Bmal1*. We then evaluated the relationship between Top2 inhibition and *Bmal1* transcription using etoposide, a standard Top2 inhibitor or *Top2a* siRNA.

2. Materials and methods

2.1. Reagents

Shikonin (HPLC > 99.0%) was purchased from Wako Pure Chemical Industries. Other chemicals used were of the highest quality available commercially.

2.2. Cell culture

Stable cells containing the luciferase reporter gene driven by the *Bmal1* promoter region (−197 to +27) were derived from NIH3T3 cells [7]. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and a mixture of penicillin and streptomycin in a humidified incubator at 37 °C under a 5% CO_2 atmosphere.

2.3. Real-time reporter gene assays

Real-time reporter gene assays proceeded as described [7]. 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 as described by subtracting a best fit line followed by subsequent fitting to a sine wave to determine circadian period length [11].

2.4. Cell growth assays

The growth of stable cells (1×10^5) incubated with camptothecin in 96-well plates and was assayed using Cell Counting Kit-8 (DOJINDO). The water-soluble tetrazolium salt, WST-8 (10 μl) was added to the wells and incubated at 37 °C under a 5% CO_2 atmosphere in a humidified incubator for 1 h. Absorbance was measured using a microplate reader (Bio-Rad) at 450/655 nm.

2.5. Top2-mediated decatenation assays

These assays proceeded as described [14]. Briefly, 5 units of Top2 protein (TopoGEN) and various concentrations of shikonin or etoposide were incubated with 0.2 μg of kinetoplast DNA (TopoGEN) in 20 μl reaction mixtures (50 mM Tris–HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol and 30 μg/ml bovine serum albumin) at 37 °C for 10 min. The reactions were terminated by adding 5 μl of 2.5% sarkosyl and 10 mM EDTA. We resolved a 2.5 kb decatenated DNA by electrophoresis on 1% agarose gels, and transferred the DNA onto a membrane for hybridization and detection using ^{32}P-labelled kinetoplast DNA as a probe. Top2 activity was determined by measuring radioactivity in the 2.5 kb decatenated DNA.

2.6. Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation assays proceeded as described [15]. Briefly, NIH3T3-derived stable cells were incubated with 1% formaldehyde for 10 min at room temperature to cross-link proteins and DNA. The cells were lysed and chromatin was fragmented and immunoprecipitated using the SimpleChIP enzymatic chromatin IP kit (Cell Signaling Technology) and anti-Top2α or anti-Top2β antibodies (Santa Cruz Biotechnology). Purified DNA was analyzed by PCR using the following primers: *Bmal1*, 5′-GAACGCGAATTGGTTTGGGTTGTCCG-3′ and 5′-ACACTCACCGTGGCTCGCTGCGAGC-3′; −5 kb of the transcription unit of *Kcnd2*, 5′-CGTTGTAGACCAGTAGTGAGTGTAGG-3′ and 5′-ATTGGACTGGGATCCAGTTAGTGC-3′. The −5 kb region of *Kcnd2* was used as the Top2-nonbound region [16].

2.7. Fluorescence microscopy

Briefly, NIH3T3 cells (1×10^5) seeded in 35 mm glass-bottom dishes were washed with FBS-free DMEM twice and incubated with 25 μM shikonin for 5 min. The cells were then washed and fixed with 100% methanol at −30 °C for 1 min. Cells were visualized by differential interference contrast (DIC) and fluorescence imaging using an IX70 microscope (Olympus). Low and high magnification images were acquired using a standard UPlanSApo objective (20× magnification, 0.75 N.A.) and a UPlanApo water immersion objective (60× magnification, 1.20 N.A.), respectively. Shikonin was detected using a U-MWG2 cube comprising a 510–550 nm band pass excitation filter and a 570-nm long pass emission filter (Olympus). Digital images were captured using an Orca ER CCD camera system (Hamamatsu Photonics) and processed using Adobe Photoshop.

2.8. Knockdown of Top2a expression

The Stealth RNAi siRNA duplex for *Top2a* knockdown and BLOCK-iT Fluorescent Oligo as a negative control were purchased from Invitrogen. Stealth RNAi comprises a mixture of siRNAs with the following sequences: 5′-CCAGCCUGACUUAUCUAAGUUUAAA-3′ and 5′-UUUAAACUUAGAUAAGUCAGGCUGG-3′; 5′-GCGAGAAGUGAAGGUUGCCCAGUUA-3′ and 5′-UAACUGGGCAACCUUCACUUCUCGC-3′; 5′-GGGAGUGAAGAAGACAGCAACCAAA-3′ and 5′-UUUGGUUGCUGUCUUCUUCACUCCC-3′. The siRNAs were introduced into cells using HilyMax (DOJINDO).

2.9. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR proceeded using a LightCycler (Roche) and the Light Cycler-FastStart DNA Master SYBR Green I kit (Roche) as described [7] with the following primer sequences: *Actin*, 5′-TACGCCAACACAGTGCTGTCTG-3′ and 5′-TTTTCTGCGCAAGTTAGGTTTTGTC-3′; *Top2a*, 5′-CACAATTGGCCATCTCTTCTGCGAC-3′ and 5′-TTCCTTAGCTTCCTTTGATGTGC-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.

2.10. Transient reporter gene assay

The luciferase reporter gene plasmid driven by the *Bmal1* promoter region (−197 to +27) and the internal control plasmid, pRL-CMV (Promega) were transfected into NIH3T3 cells. Luciferase was measured using the Dual Luciferase Reporter Assay System (Promega) as described [17]. Transcriptional activities were normalized relative to *Renilla* luciferase activities.

3. Results

3.1. Shikonin shortens period length of Bmal1 transcriptional oscillation

We analyzed the effects of shikonin on period length using real-time reporter gene assays. The period lengths of Bmal1 transcriptional oscillation in cells incubated with and without (control) 0.5 μM shikonin were 24.3 ± 0.29 and 24.9 ± 0.24 h, respectively, indicating that shikonin shortened period length by about 30 min (Fig. 1A). Shikonin (0.5 μM) was not cytotoxic to stable cells (Fig. 1B; filled triangles).

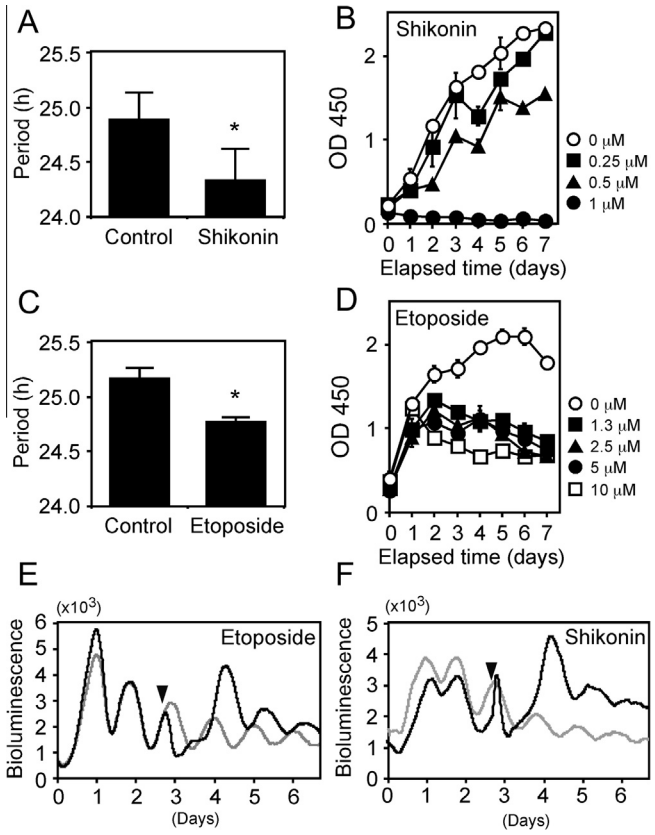

Fig. 1. Shikonin and etoposide reversibly shorten period length. (A) Effect of shikonin on transcriptional oscillation of *Bmal1*. NIH3T3-derived stable clones were stimulated with 100 nM dexamethasone for 2 h and then 0.5 μM shikonin was added and bioluminescence was measured. Circadian period was measured from fit curve data of detrended results. Values are means ± SEM of triplicate assays. $^{*}P < 0.05$ (Student's *t* test). (B) Effect of shikonin on cell growth. Stable cells were incubated with various concentrations of shikonin in 96-well plates, and then growth was assayed using Cell Counting Kit-8. Values are shown as means ± SEM of triplicate assays. Unfilled circles, filled squares, triangles and circles indicate 0, 0.25, 0.5 and 1 μM shikonin, respectively. (C) Effect of etoposide on transcriptional oscillation of *Bmal1*. NIH3T3-derived stable clones were stimulated with 100 nM dexamethasone for 2 h, 5 μM etoposide was added and then bioluminescence was measured. Circadian period was measured from fit curve data of detrended results. Values are shown as means ± SEM of triplicate assays. $^{*}P < 0.05$ (Student's *t* test). (D) Effect of etoposide on cell growth. Stable cells were incubated with various concentrations of etoposide in 96-well plates, and cell growth was assayed using Cell Counting Kit-8. Values are shown as means ± SEM of triplicate assays. Unfilled circles, filled squares, triangles, circles and unfilled squares indicate 0, 1.3, 2.5, 5 and 10 μM etoposide, respectively. (E and F) Reversible period shortening. Stable clones were stimulated with 100 nM dexamethasone for 2 h, and then bioluminescence was measured after incubation with 5 μM etoposide (E) or 0.5 μM shikonin (F). Cells were washed and culture medium was replaced with DMEM without these compounds 3 days later (arrows). Black and gray lines, with and without cell washout and medium change, respectively. Results are representative of triplicate experiments.

Etoposide also shortens period length [12]. Our real-time reporter assays found that the period lengths of *Bmal1* transcriptional oscillation in cells incubated with or without (control) 5 μM etoposide were 24.7 ± 0.05 and 25.2 ± 0.1 h, respectively, indicating that 5 μM etoposide also shortened period length by 30 min, which was consistent with previous findings (Fig. 1C). Cells incubated with etoposide grew at any concentration for 1 day and then growth was arrested at ⩽5 μM (Fig. 1D; filled circles). Etoposide became cytotoxic at 10 μM (Fig. 1D; unfilled squares). The effects of etoposide and shikonin on cell growth (Fig. 1B and D) suggested that the cytotoxic mechanisms differed between the two compounds. Stable reporter cells were incubated once with 5 μM etoposide or 0.5 μM shikonin, and then both were washed out to evaluate period length in real-time reporter assays. The period lengths of *Bmal1* transcriptional oscillation in the cells incubated with 5 μM etoposide or 0.5 μM shikonin were 24.5 ± 0.01 and 24.1 ± 0.08 h, respectively, whereas those after continued incubation without these compounds were 25.2 ± 0.09 h and 25.3 ± 0.09 h, respectively, indicating that removing these compounds elongated period length (Fig. 1E and F). These findings suggest that etoposide and shikonin induce shorten the circadian period *via* similar mechanisms. We did not find dose-dependent effects of either etoposide or shikonin on period shortening because the changes were very subtle.

3.2. Shikonin inhibits topoisomerase II activity

Etoposide is a major (Top2) inhibitor [18]. Chen et al. found using DNA relaxation assays that shikonin is a minor groove binder and that it inhibits topoisomerase activity [19]. Harmine has DNA binding properties and it significantly inhibits Top1 but does not affect Top2 [10]. We assayed decatenation in which only Top2 activity can be measured, to assess whether shikonin inhibits Top2 activity. Kinetoplast DNA was treated with Top2 in the presence of increasing concentrations of shikonin or etoposide. Fig. 2A shows that the concentrations of etoposide and shikonin required to achieve 50% inhibition were about 100 and 125 μM, respectively, indicating that shikonin inhibits Top2 activity similar to etoposide, the standard Top2 inhibitor. We next confirmed Top2 binding around the ROREs *in vivo* using ChIP assays with anti-Top2α and β antibodies in NIH3T3-derived stable cells. Fig. 2B shows that the *Bmal1* promoter region was amplified in ChIP assays using anti-Top2 antibodies, indicating Top2 binding within the *Bmal1* promoter region *in vivo*. All cells were stained with shikonin, suggesting that shikonin easily permeates cells (Fig. 3A). The intensity of intracellular shikonin staining was highest in the nucleus although entire cells were stained, suggesting that shikonin can act in the nucleus (Fig. 3B and C). Taken **together**, these lines of evidence suggest that Top2 inhibition is responsible for circadian period shortening by shikonin.

3.3. Effects of Top2 on circadian period

We evaluated the effects of Top2 on the circadian period using *Top2* siRNA. Fig. 4A shows that levels of the *Top2* transcript were reduced about 40% in stable cells incubated with *Top2* siRNA. Real-time reporter assays showed that the period lengths of cells incubated without and with *Top2a* siRNAs were 24.6 ± 0.33 and 24.2 ± 0.24 h, respectively, indicating that the reduction of *Top2* by siRNA causes shortening of the circadian period (Fig. 4B). These results are consistent with the findings obtained using Top2 inhibitors that shortened the period by reducing Top2 activity and suggest that *Top2* is involved in the control of circadian rhythms. We previously reported that *Bmal1* promoter activity is related to the period length of *Bmal1* transcriptional oscillation [8]. Here we assessed the effects of *Top2a* siRNA (Fig. 4C) and Top2 inhibitors such as etoposide and shikonin (Fig. 4D) on *Bmal1* promoter activity. We

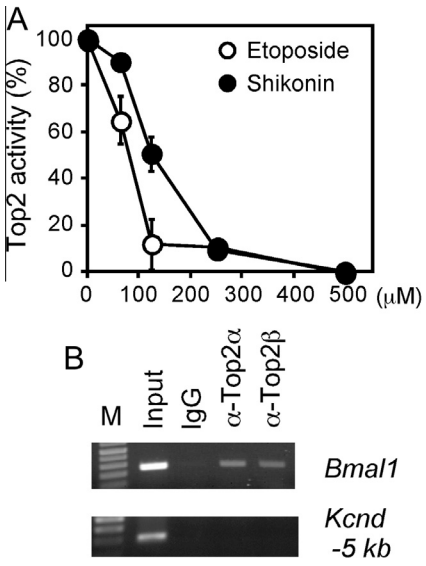

Fig. 2. Shikonin inhibits Top2 activity. (A) Decatenation by Top2 assayed using various concentrations (500, 250, 125, 62.5 μM) of etoposide or shikonin. Reaction products were resolved in agarose gels and visualized by Southern blotting. Levels of Top2 activity are shown, and value for Top2 activity without etoposide or shikonin was set at 100%. Unfilled and filled circles indicate Top2 activities in cells incubated with etoposide and shikonin, respectively. Values are shown as means ± SEM of triplicate assays. (B) Top2 localizes in *Bmal1* promoter region. Top2-binding on *Bmal1* promoter in NIH3T3-derived stable cells was analyzed using ChIP assays and PCR products were resolved on 2% agarose gels. M, 100-bp ladder marker; IgG, mouse immunoglobulin; α-Top2α, anti-Top2α antibody; α-Top2β, anti-Top2β antibody.

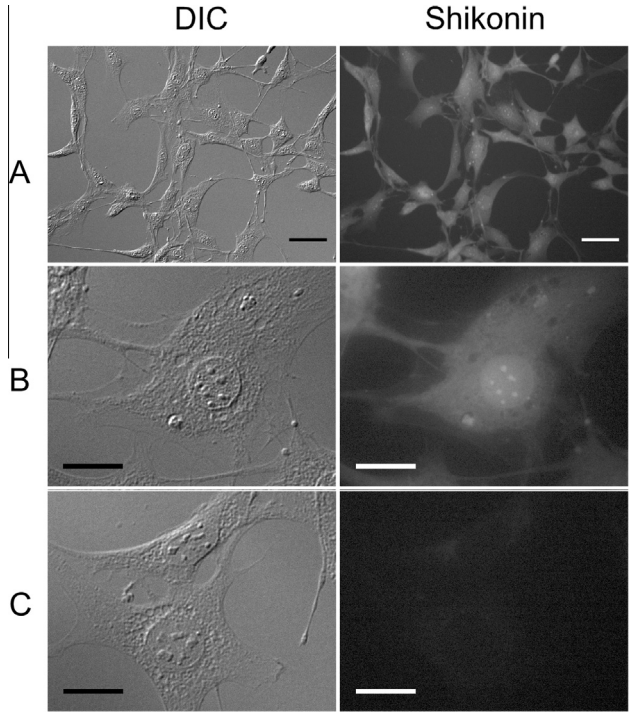

Fig. 3. Fluorescent and DIC images of NIH3T3 cells stained with shikonin. NIH3T3 cells were stained with shikonin and fixed with methanol. (A) low magnification; (B and C) high magnification. Panel C shows auto fluorescence from unstained cells under identical conditions to other panels. Bar in panel A, 50 μm; bars in panel B and C, 20 μm.

found that *Bmal1* promoter activity tended to be reduced in cells incubated with *Top2a* siRNA, etoposide or shikonin, supporting

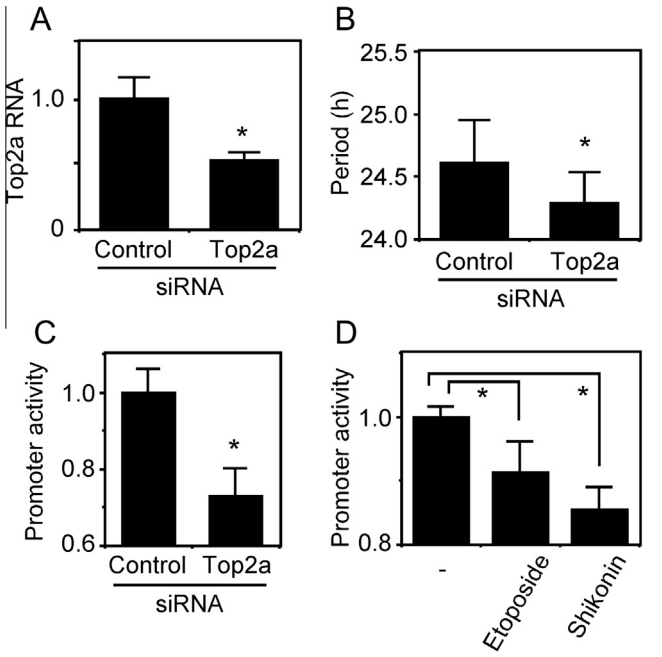

Fig. 4. *Top2* expression affects period length. Stealth RNAi siRNA duplex for *Top2a* knockdown (Top2a) and BLOCK-iT Fluorescent Oligo as a negative control (Control) were transfected into stable clones. *Top2a* expression was analyzed 24 h after later using real-time quantitative RT-PCR. Levels of RNA were normalized to those of *Actin* expression and value for cells transfected with BLOCK-iT Fluorescent Oligo was set at 1. Values are means ± SEM of triplicate assays. $^{*}P < 0.05$ (Student's *t* test) (A). At 24 h after siRNA transfection, stable clones were stimulated with 100 nM dexamethasone for 2 h and then bioluminescence was measured. Circadian period was measured from fit curve data of detrended results. Values are shown as means ± SEM of triplicate assays. $^{*}P < 0.05$ (Student's *t* test) (B). Reporter plasmids and siRNA described above were introduced into NIH3T3 cells and luciferase activities were measured 24 h later. Normalized expression levels were calculated relative to luciferase activities of control siRNA transfectants. Values are shown as means ± SEM of triplicate assays. $^{*}P < 0.05$ (Student's *t* test) (C). NIH3T3 cells transfected with reporter plasmids were incubated with 5 μM etoposide or 0.5 μM shikonin for 24 h and then reporter assays proceeded. Normalized expression levels were calculated relative to luciferase activities of transfectants incubated without compounds. Values are means ± SEM of triplicate assays. $^{*}P < 0.05$ (Student's *t* test) (D).

the notion that *Bmal1* promoter activity is related to the period length of *Bmal1* transcriptional oscillation.

4. Discussion

4.1. Shikonin shortens period length

Cell-based assay systems to identify compounds that potentially affect circadian clock function have recently been developed [12,20] and some circadian modulators have been described. Hirota et al. reported that small molecular inhibitors of glycogen synthase kinase 3 (GSK-3) consistently cause a powerful, short-period phenotype [12].

Chen et al. identified five period-shortening compounds that do not inhibit GSK-3β kinase [21]. These data indicate that more than one mechanism is involved in circadian period shortening. We found here that shikonin also shortens the circadian period and it is not structurally related to known GSK-3β kinase inhibitors [12]. The abilities of shikonin and etoposide to inhibit Top2 were comparable (Fig. 2A). We and others [12] found that the major Top2-inhibitor, etoposide is also a period shortener (Fig. 1). Thus, we speculated that Top2 inhibition is related to circadian period shortening. The period-shortening compounds identified by Chen et al. contain phenolic groups that seem to interact with DNA, and thus, whether they can inhibit Top2 is of interest.

4.2. Topoisomerases in the Bmal1 promoter

We previously found that the ROREs are embedded in a unique GC-rich open chromatin structure, with which a nuclear matrix-like structure at the 3′-flanking region cooperates to regulate *Bmal1* transcription [7] and that the chromatin architecture of clock genes including DNA topology is a determinant of these rhythms [8]. Here, we showed that Top2 inhibition shortens circadian period length (Fig. 4B) whereas Top1 inhibition leads to circadian period elongation [8]. In addition, *Top2a* knockdown suppressed (Fig. 4C), whereas Top1 knockdown enhanced [8] *Bmal1* promoter activity. These results indicate that Top1 and Top2 have independent functions, and rather contrary effects on *Bmal1* transcription and the period length of its oscillation, supporting our previous findings that modulating *Bmal1* expression affects the circadian period [22]. We previously localized the Top1 binding site among ROREs that are critical elements for circadian transcription of the *Bmal1* gene [8] and here we showed that Top2 protein also localizes in the *Bmal1* promoter region (Fig. 2B). Sperling et al. reported that Top2 binds nucleosome-free DNA [23], supporting the notion that Top2 binds to the unique GC-rich open chromatin structure of the *Bmal1* promoter region [7]. On the other hand, Kawano et al. showed a Top2β RNA-dependent association with heterogeneous nuclear ribonucleoprotein U (hnRNP U) [24]. This also supported the notion that Top2 binds to the *Bmal1* promoter region because hnRNP U/SAF-A binds to the 3′-flanking region of the *Bmal1* promoter with circadian timing [7]. Taken together, these results suggest that Top2 on the *Bmal1* promoter affects *Bmal1* transcription (Fig. 4C and D). However, how Top2 modulates *Bmal1* transcription remains unclear and thus requires further investigation.

4.3. Shikonin

Zicao (Purple gromwell) is a popular herbal medicine, and shikonin is one of its main components with various medical properties. Here, we evaluated the shortening effect of shikonin on the circadian period. Shikonin inhibits proteasomes [25], acts as a radical scavenger [26] and inhibits protein tyrosine kinase [27] in addition to topoisomerase (Fig. 2A). Weinch et al. found that shikonin targets mitochondria and causes mitochondrial dysfunction in cancer cells [28]. Shikonin mainly localized to the nucleus of our cell line derived from NIH3T3 cells and was distributed to some extent throughout the cytosol (Fig. 3), suggesting that these actions of shikonin on circadian rhythms are concentration-dependant. These activities might provide new insights into pharmaceutical applications of shikonin.

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