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Oncogenic and Circadian Effects of Small Molecules Directly and Indirectly Targeting the Core Circadian Clock

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Abstract

Circadian rhythms are essential for controlling the cell cycle, cellular proliferation, and apoptosis, and hence are tightly linked to cell fate. Several recent studies have used small molecules to affect circadian oscillations; however, their concomitant cellular effects were not assessed, and they have not been compared under similar experimental conditions. In this work, we use five molecules, grouped into direct versus indirect effectors of the circadian clock, to modulate periods in a human osteosarcoma cell line (U2OS) and determine their influences on cellular behaviors, including motility and colony formation. Luciferase reporters, whose expression was driven via *Bmal1*- or *Per2*-promoters, were used to facilitate the visualization and quantitative analysis of circadian oscillations. We show that all molecules increase or decrease the circadian periods of *Bmal1* and *Per2* in a dose-dependent manner, but period length does not correlate with the extent of cell migration or proliferation. Nonetheless, molecules that affected circadian oscillations to a greater degree resulted in substantial influence on cellular behaviors (ie, motility and colony formation), which may also be attributable to noncircadian targets. Furthermore, we find that the ability and extent to which the molecules are able to affect oscillations is independent of whether they are direct or indirect modulators. Because of the numerous connections and feedback between the circadian clock and other pathways, it is important to consider the effects of both in assessing these and other compounds.

Keywords

circadian rhythm, small molecule modulators, clock period, luminescent reporters, U2OS cells, cell proliferation, cell migration, tumor growth

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Introduction

Circadian clocks are endogenous time-keeping systems that cycle with an approximate period of 24 hours.[1] These autonomous clocks help organisms adapt to environmental changes, such as day-night cycles[2] or the changing of seasons.[3] Light and temperature inputs regulate circadian rhythms through the hypothalamic suprachiasmatic nucleus (SCN), which is the master clock regulator for the entire organism.[4] Circadian clocks coordinate daily changes to control many physiological, behavioral, and metabolic functions, including the sleep-wake cycle,[5] body temperature,[6] blood pressure,[7] food intake,[8] and humoral secretion.[9] Disruption of circadian rhythms via night-shift work,[10] exposure to light at night,[11] or chronic jet lag[12] has been shown to elicit a number of pathological developments, including cancer,[13] metabolic disorders,[14,15] and cardiovascular diseases.[16] While accumulating evidence indicates that circadian disruptions lead to malignant transformations in cells and organs, the molecular relationship between the two is not well understood.

The circadian system in mammals is hierarchically organized into two major components: a central pacemaker SCN and peripheral oscillators within cells.[17] When the retina receives photons from the environment, it signals the SCN to synchronize peripheral clocks via neural and humoral pathways.[18] At the cellular level, molecular clocks

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are regulated by transcriptional/translational feedback loops (TTFLs) integrated in clock genes. Core clock proteins aryl hydrocarbon receptor nuclear translocator–like protein 1 (ARNTL/BMAL1) and circadian locomotor output cycles kaput (CLOCK) heterodimerize and bind to the E-box promoter, which initiates the expression of *Period* (*Per*) and *Cryptochrome* (*Cry*). PER and CRY accumulate over time to reach a high concentration and form heterodimers, which translocate back into the nucleus and inhibit BMAL1 and CLOCK activity.[19] As a result, the core circadian machinery oscillates with a period of approximately 24 hours. In cell culture, intrinsic and self-sustained circadian clocks are persistent even in the absence of external time cues.[20] To synchronize clocks in cultured cells, treatments of high concentration serum[21] or chemical reagents (eg, dexamethasone[22] or forskolin[23]) are frequently used.

Circadian rhythmicity is tightly associated with posttranslational modifications of clock proteins.[24,25] Phosphorylation of most clock proteins occurs in a rhythmic manner; thus, alteration of clock protein phosphorylation can also result in changes to circadian periods.[26] Genetic manipulations of the posttranslational regulators of clock proteins have been shown to affect circadian functions and periodicity.[26,27] However, conventional genetic approaches (eg, mutation, knockout, and overexpression) may also result in fatality (cell death), pleiotropy,[28] functional redundancy,[29] and compensation effects.[30] Pleiotropy is the ability of individual genes to influence multiple functions or characteristics of an organism, and it is often considered a by-product of genetic manipulation processes.[28] At the same time, in some cases, genetic modifications may yield no detectable phenotypes or false-negative results due to gene redundancy.[29] Additionally, a compensatory mechanism may be activated when a paralog exists, maintaining function although the target gene was knocked out or down.[30] Alternatively, chemical modulation of these regulators via small molecules can reversibly manipulate clock functions in time- and dose-dependent manners without incurring these effects.

Recently, the identification of small molecule modulators of circadian rhythms has garnered much interest.[31,32] A few directly interact with clock proteins, including CRYs,[32,33] REV-ERBs,[34] and RORs,[35] while the others target kinases (eg, CK2, GSK3β, and AMPK)[36-38] that regulate clocks via posttranslational modifications.[39,40] These molecules have promoted the study of posttranslational mechanisms underlying the circadian system and have been used to discover new clock-regulatory pathways.[24,41] Some circadian-modulating molecules have shown therapeutic potential as well. It has been recently reported by Sulli et al[42] that SR9009 and SR9011, two agonists of REV-ERBs (secondary clock components), have potent anticancer effects in various cancer cell lines, and no toxicity in normal cells or tissues. Oshima et al[36] discovered GO289, which regulates circadian rhythms via inhibition of CK2, finding that it suppressed cancer cell proliferation in a cell type–dependent manner. But, among currently published studies, the biological and circadian effects of most small molecules, including those studied here, have not been addressed concurrently; nor have they been studied in similar systems using the same modes of analyses. Hence, we present a systematic assessment, whereby we have thoroughly evaluated a small panel of these molecules, also addressing whether concentration ranges resulting in circadian effects yield cellular changes and vice versa.

In this study, we used five small molecules to affect circadian period (the time interval for completing one oscillation cycle), and assessed concomitant changes to the oncogenic traits of cancer cells. Among the selected compounds, two, KL001[33] and PF-670462,[43] directly interact with circadian proteins and the other three, SP600125,[44] Chir99021, and etoposide,[45] target entities that regulate core circadian proteins in a posttranslational manner (Figure 1). A human bone osteosarcoma epithelial cell line, U2OS, was separately transfected with *Bmal1:luc* and *Per2:luc* reporters to facilitate high-resolution tracking of circadian rhythms, and used throughout this work. We confirmed that the five small molecules all either increase or decrease the periods of *Bmal1* and *Per2* in these cells, to varying degrees. However, we found that conditions resulting in circadian effects did not necessarily yield changes to oncogenic features. This leads us to posit that circadian periods may not be correlated with cell motility or growth. To determine whether this is the case, and in further studies using small molecules to affect and study circadian rhythms, molecules directly targeting additional core clock components should be identified and employed. These may be combined with genetic approaches to uncover the molecular details connecting circadian disruptions with cancer development.

Materials and Methods

Cell Culture

The U2OS cell line was obtained from Prof Patricia Wadsworth (Biology, University of Massachusetts Amherst). Cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco), with 10% fetal bovine serum (FBS; Corning), 10% L-glutamine (Gibco), 1% penicillin-streptomycin (Gibco), 1% nonessential amino acids (HyClone), and 1% sodium pyruvate (Gibco). The HEK293T cell line was obtained from Prof D Joseph Jerry (Veterinary and Animal Sciences, University of Massachusetts Amherst). Cells were maintained in DMEM/F12 (Gibco), with 10% FBS, 1% penicillin-streptomycin, and 0.015 mg/mL gentamicin (Gibco). All cells were incubated at 37 °C under 5% CO_2, except where otherwise noted.

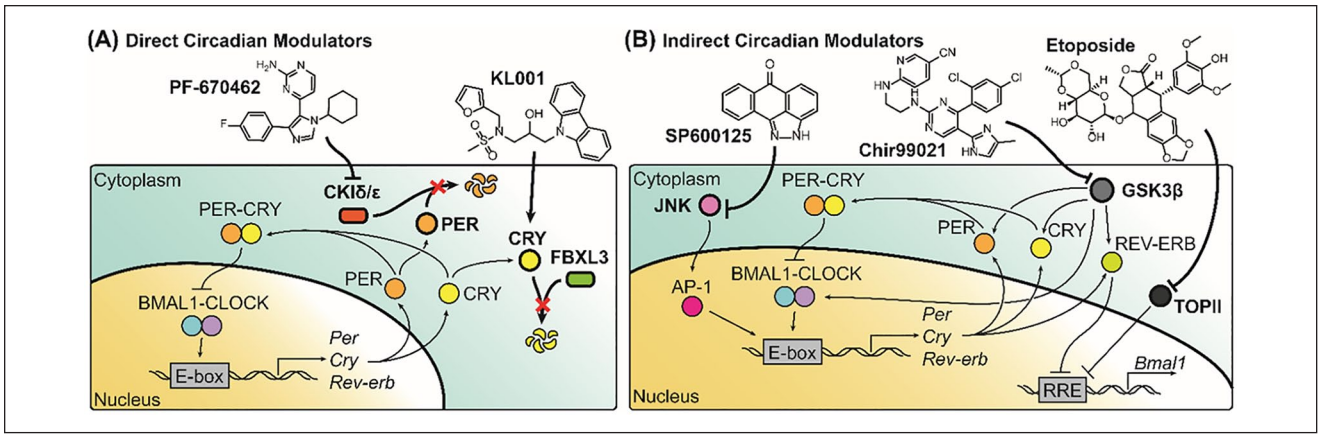

Figure 1. Structures and targets of direct circadian modulators (A) and indirect circadian modulators (B) used in these studies. KL001 directly binds to CRY, preventing FBXL3-mediated degradation, and PF-670462 binds CKIδ/ε resulting in PER stabilization. SP600125, Chir99021, and etoposide indirectly influence circadian rhythms by binding to kinases that are involved in circadian pathways.

Plasmid and Recombinant DNA

The *Bmal1* promoter–driven luciferase reporter construct (pABpuro-BluF, *Bmal1:luc*) was obtained from Addgene (plasmid #46824, deposited by Dr Steven Brown).[46] Our generation of the *Per2* promoter–driven luciferase (*Per2:luc*) reporter construct was described previously.[47]

Lentiviral Transductions

3×10^6 HEK293T cells were seeded in 60-mm culture dishes and transiently transfected with 3 µg psPAX2 packaging plasmid, 2 µg pMD2.G envelope plasmid (both from Prof D Joseph Jerry, Veterinary and Animal Sciences, University of Massachusetts Amherst), and 3 µg *Bmal1:luc* or *Per2:luc* reporter constructs using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions. Lentiviral particles were harvested from supernatant 48 hours after DNA-lipid complexes were added to cells; this virus-containing supernatant was passed through a 0.45-µm filter. 9 mL of lentivirus-containing supernatant were mixed with 9-mL DMEM culture medium containing 4 µg/mL polybrene (Sigma-Aldrich). U2OS cells were seeded in T25 culture flasks at 2×10^5 cells/mL and incubated under conditions above until 70% to 80% confluence was reached. Culture medium was removed, and 6 mL of lentivirus-containing media was added to each flask. After 2 days of infection, the medium was replaced with selection medium (DMEM with all growth supplements plus 4 µg/mL puromycin [Gibco]), in which cells were incubated for 3 to 6 weeks for selection.

Bioluminescence Recording

Cells were seeded in 35-mm culture dishes with 2 mL of 2×10^5 cells/mL and incubated to reach 100% confluence. After 24 hours, culture media was replaced with bioluminescence recording media, (powdered DMEM [Sigma-Aldrich] with 4 mM sodium bicarbonate [Gibco], 5% FBS, 1% HEPES [HyClone], 0.25% penicillin-streptomycin, 53.5 mM D-luciferin [Pierce]). 100 nM dexamethasone (Sigma-Aldrich) was added to recording media for synchronization. Dishes were sealed with 40-mm sterile cover glass using silicone vacuum grease and subjected to monitoring using a LumiCycle 32 System (Actimetrics) at 36.5 °C for 5 to 7 days. Raw traces were detrended using the 24-hour moving average method via LumiCycle Analysis v.2.56 software. All period analyses were performed via simultaneous Levenberg-Marquardt least squares parameter optimization with damped sinusoidal waveform using the same package.

Small Molecule Cell Treatments

SP600125 (Sigma-Aldrich), KL001 (Tocris), PF-670462 (Sigma-Aldrich), Chir99021 (Tocris), and etoposide (Santa Cruz Biotech), were prepared in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a concentration of 50 mM and stored at −20 °C in single-use aliquots. When dosing cells, each was diluted in culture media (or recording media for bioluminescence recording) to a final DMSO concentration of 0.2%. The solution was mixed well and added to the seeded cells.

Wound Healing Assay

Cells were seeded in 24-well plates at 2×10^5 cells/mL and incubated for 24 to 48 hours. After cells reached 100% confluence, wounds were generated using a 1 mL micropipette tip. Culture media was removed, cells were washed with 500 µL phosphate-buffered saline (Gibco), and 500 µL of new culture media containing indicated molecules was

added into each well. Images were acquired immediately following for the first time point ($T = 0$) and then every 6 hours for 24 hours via Biotek Cytation 3 cell imaging multimode reader. Wound closure rates were determined by normalizing wound area at each time point to $T = 0$ via ImageJ.

Colony Formation Assay

Cells were suspended in agar and incubated until colonies formed, as previously described.[48] Briefly, 3% 2-Hydroxyethyl Agarose (Sigma-Aldrich) was prepared and stored in a water bath at 45 °C. Cell culture media at 37 °C was used to dilute agarose to 0.6%, and 2 mL was added to each well of a 6-well plate, which was incubated at 4 °C until agar solidified (first, bottom layer). DMSO solutions of molecules were added to 1 mL of 4×10^4 cells/mL suspended U2OS cells in warm culture media, followed by addition of 0.6% agarose solution in a 1:1 ratio. 1 mL drug-cell-agarose solution was dispensed per well and incubated at 4 °C for 30 minutes until the agar layer solidified (second, drug cell–containing layer). Then plates were incubated at 37 °C under 5% CO_2. A 1 mL feeder layer of 0.3% agarose in culture media containing small molecules at designated concentrations was added to each well once every 7 days for 4 weeks. When imaging colonies, 1 mL of 0.005% crystal violet dye (Fisher Scientific) in 20% methanol (Fisher Scientific) was added to wells and incubated on a shaker at room temperature for 1 hour. Wells were then washed 4 to 6 times with 2 mL ddH_2O. Eight images were acquired per well using a Biotek Citation 3 multimode cell imaging reader. Each condition was performed with 3 biological replicates. Images were stitched using Adobe Illustrator CS6, and colony numbers/sizes were analyzed using ImageJ.

Results

Both Direct and Indirect Circadian Modulators Alter Periods in a Dose-Dependent Manner

To evaluate changes to circadian periods caused by the small molecules used, real-time luminometry assays were performed using human U2OS osteosarcoma cells harboring *Bmal1:luc* or *Per2:luc* reporters (Supplementary Figure S1, available online). Among the molecules, KL001[33] and PF-670462[43] are categorized as direct circadian modulators, because their targets, CRY and CKIδ/ε, are essential elements for completing endogenous circadian TTFLs. SP600125,[44,49] Chir99021, and etoposide[45] are classified as indirect circadian modulators, because their targets, JNK, GSK3β, and topoisomerase type II (TOPII), respectively, exert downstream effects on endogenous circadian TTFLs.

Treatment with KL001 was observed to both increase period and dampen *Bmal1:luc* amplitude in a dose-dependent manner (Figure 2A and B; Supplementary Figure S2 and Supplementary Table S1, available online). Similar period lengthening effects were also observed following treatment with PF-670462 (Figure 2C and D; Supplementary Figure S3 and Supplementary Table S1). Compared with KL001, however, PF-670462 did not affect amplitudes and resulted in more consistent oscillations, even at longer periods. The longest periods observed for KL001 were 29.2 ± 3.3 hours, while for PF-670462, they were 34.4 ± 0.8 hours. We also ascertained the molecules' abilities to lengthen the periods of *Per2*, via U2OS-*Per2:luc* reporter cells (Supplementary Figure S4 and Supplementary Table S2). As anticipated, both compounds significantly increased the periods of *Per2;* period enhancements and amplitude effects were similar for both reporters (Supplementary Figure S5).

The JNK inhibitor SP600125 also increased *Bmal1* periods in a dose-dependent manner (Figure 3A and B; Supplementary Figure S6 and Supplementary Table S3). Like PF-670462, SP600125 did not dramatically affect amplitudes and yielded consistent outputs and periods, even at high concentrations and longer periods. At the highest concentration tested (10 μM), the period increased to 33.9 ± 0.5 hours. Whereas the other period-lengthening molecules resulted in similar effects on *Bmal1* and *Per2* oscillations, SP600125 affected *Bmal1* periods to a greater extent (~2 hours longer) at the same concentrations (Supplementary Figures S7 and S8, and Supplementary Table S4).

While KL001, PF-670462, and SP600125 all produced period-lengthening effects, Chir99021 and etoposide shortened periods. Chir99021 dramatically decreased *Bmal1* periods in a dose-dependent manner (Figure 3C and D; Supplementary Figure S9 and Supplementary Table S3). It resulted in decreased amplitudes at higher concentrations, but still resulted in consistent, substantially reduced periods (18.5 ± 0.2 hours at the highest concentration evaluated, 3 μM). In contrast, etoposide reduced periods but not in a statistically significant manner, with a wider distribution of data. (Figure 3E and F; Supplementary Figure S10 and Supplementary Table S3). It yielded variable periods at higher concentrations of up to 7 μM but appeared to have an overall decreasing trend. Both molecules resulted in similar effects on *Per2* transcription (Supplementary Figures S7 and S8, and Supplementary Table S4). Taken together, all five small molecules demonstrated an ability to modulate circadian periods of *Bmal1* and *Per2* to varying extents. Evaluation of viability showed no toxicity effects following treatment (Supplementary Figure S11).

Effects of Small Molecules on Oncogenic Characteristics of Cells

In parallel with the circadian studies described above, we evaluated the effects of compound treatments at the same concentrations on cellular characteristics. These included

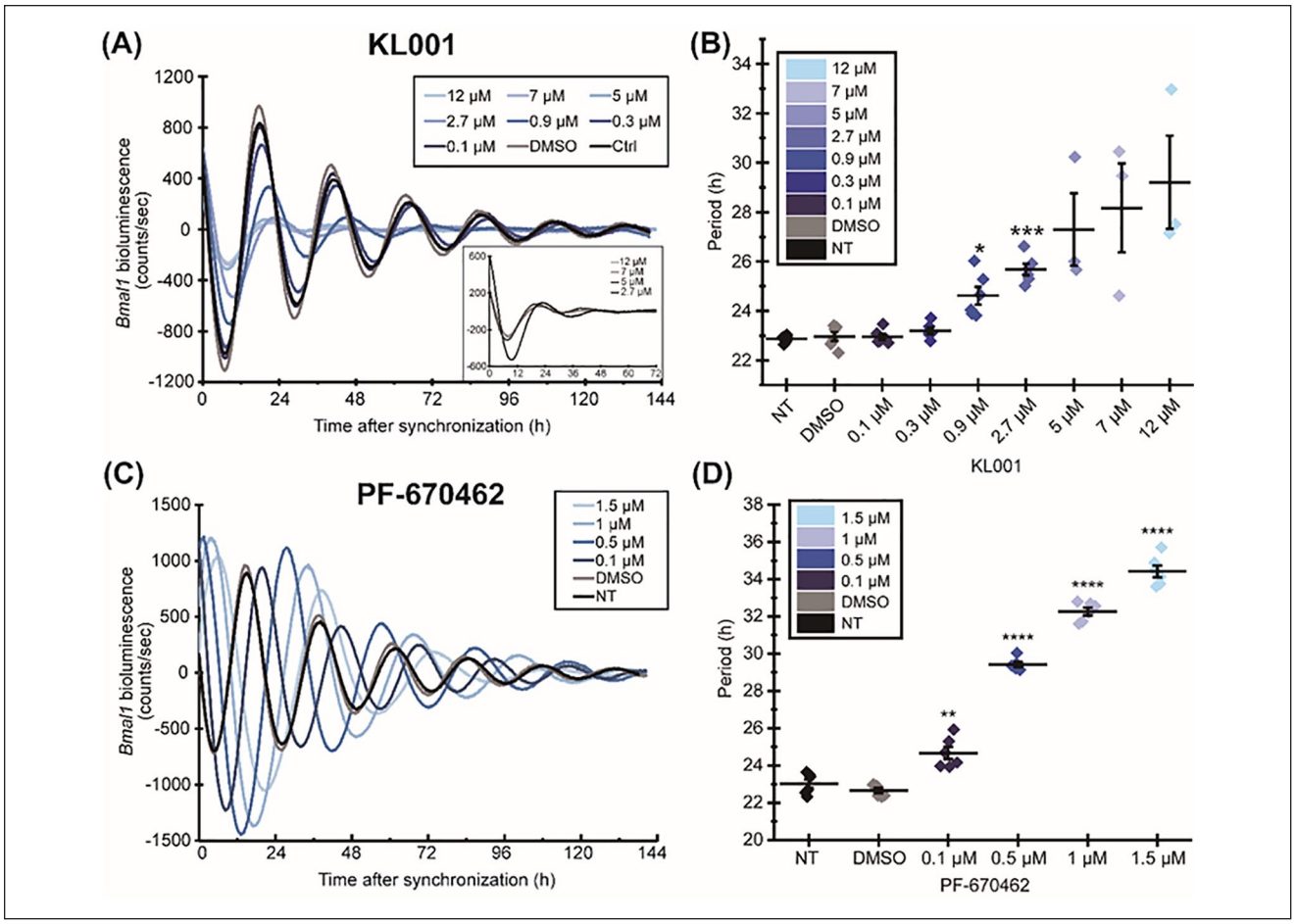

Figure 2. Direct circadian modulators, KL001 (A, B) and PF-670462 (C, D), increase the circadian period of *Bmal1* in a dose-dependent manner. The inset in (A) shows an enlarged version of the first three cycles for treatments at the three highest concentrations. Representative traces are shown; replicate traces (raw and detrended) for each condition are in Supplementary Figures S2 and S3. The lines (B, D) represent mean of the data ± 1 standard error. Statistical significance was evaluated via Welch's *T* test followed by Bonferroni Correction, **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. NT, non-treated; DMSO, dimethyl sulfoxide–only control (0.2%). Period and goodness of fit (GOF) for each condition are shown in Supplementary Table S1.

motility, two-dimensional proliferation, and anchorage-independent proliferation. One of the primary features of more aggressive/malignant cancer cell types is their migratory ability; metastasis is the migration of primary cancer cells to other, secondary sites. To assess the effects of small molecules on this feature, wound healing/scratch assays were performed. These are well-established methods to determine collective cell migration, which is composed of complex cellular interactions that regulate wound repair, cancer invasion, metastasis, immune responses, and angiogenesis.[50-52] For the motility assay, we assessed multiple concentrations of each compound, as it was unknown whether or which concentrations would result in effects, and if they would exist here, even in the absence of circadian changes. Our results showed that higher concentrations of KL001 yielded slightly increased cancer cell migration relative to controls at early time points following treatment ($T = 6$ and 18 hours) but resulted in no significant overall (24 hours) change (Figure 4A). We also observed that 2.7 μM KL001 treatment did not affect cell proliferation (Supplementary Figure S12A).

Concurrently, we found that 1.5 μM PF-670462 treatment showed decreased cell motility but not in a statistically significant manner when compared with the non-treated condition (Figure 4B). While DMSO/vehicle treatments did not result in significant changes from non-treated, there were differences observed in comparing compound treatments versus each of the two controls. Here, 1 and 1.5 μM PF-670462 treatments exhibited changes compared with the vehicle-treated conditions. A concentration of 1 μM was $P < .01$ at $T = 12$ and $P < .05$ at $T = 18$; 1.5 μM was $P < .01$ at $T = 12$, 18, and 24 hours (not shown in the figure). While it inhibited U2OS cell migration (Figure 4B), similar treatments resulted in promotion of cell proliferation

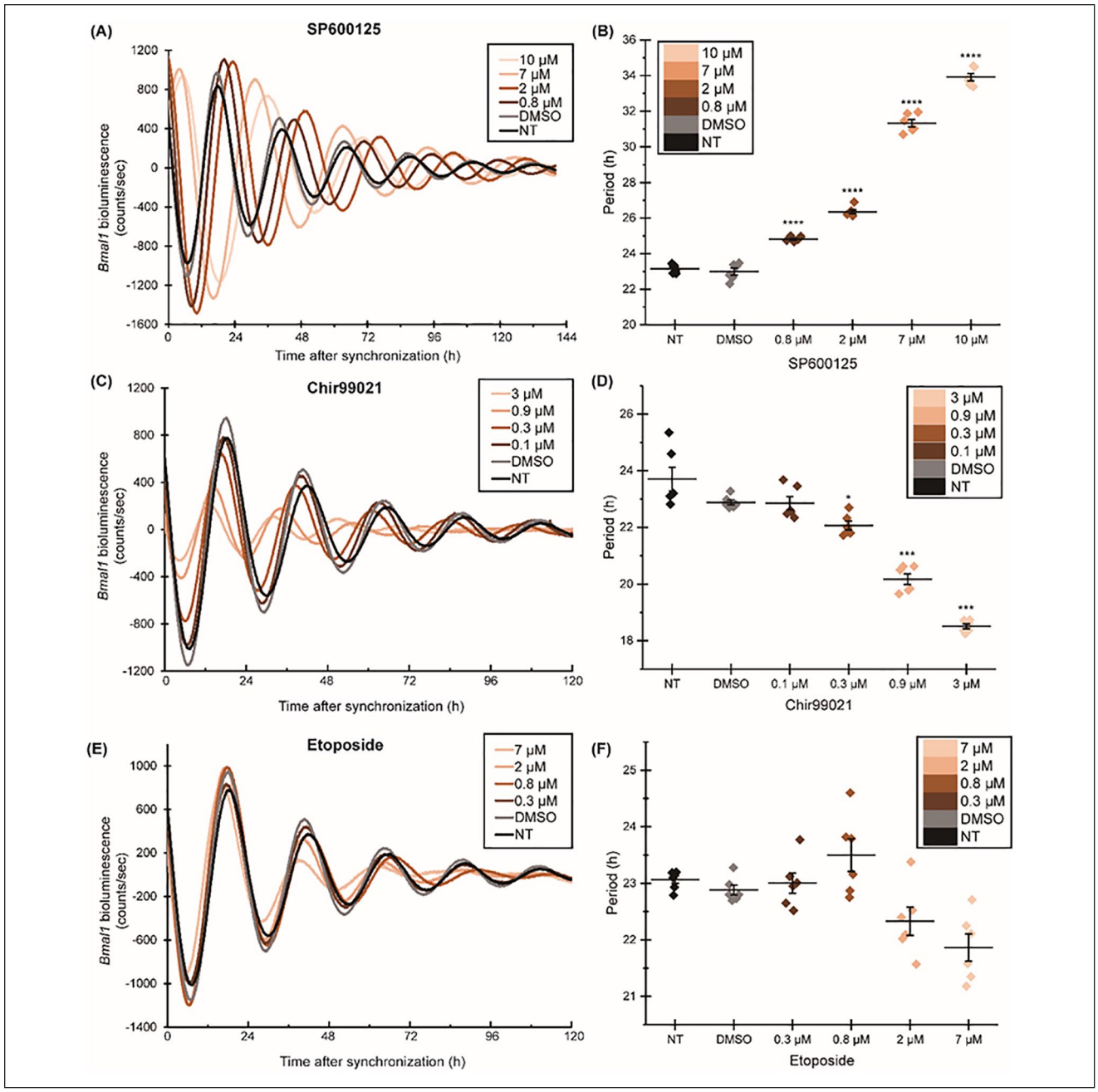

Figure 3. Three indirect circadian modulators, SP600125 (A, B), Chir99021 (C, D), and etoposide (E, F), increase or decrease the period of *Bmal1* in U2OS cells. Representative traces are shown; replicate traces (raw and detrended) for each condition are shown in Supplementary Figures S6, S9, and S10. The lines (B, D, F) represent mean of the data ±1 standard error. Statistical significance was evaluated via Welch's *T* test followed by Bonferroni Correction, **P* < .05, ***P* < .01, and *****P* < .0001. NT, non-treated; DMSO, dimethyl sulfoxide–only control (0.2%). Individual period and goodness of fit (GOF) for each drug treatment are shown in Supplementary Table S3.

(Supplementary Figure S12B), also supported by our viability assay (Supplementary Figure S11).

Of the indirect modulators, Chir99021 and SP600125 affected motility, while etoposide had negligible effects. In contrast, etoposide affected cell proliferation, while the other two did not (Supplementary Figure S13). Chir99021 significantly suppressed cell migration at the 3 μM dosage; the 0.9 μM treatment showed inhibition but not in a statistically significant manner when compared with the non-treated condition (Figure 4C). However, 0.9 μM Chir99021 treatment exhibited statistical significance ($P < .05$) at $T =$ 18 and 24 hours when compared with vehicle-treated

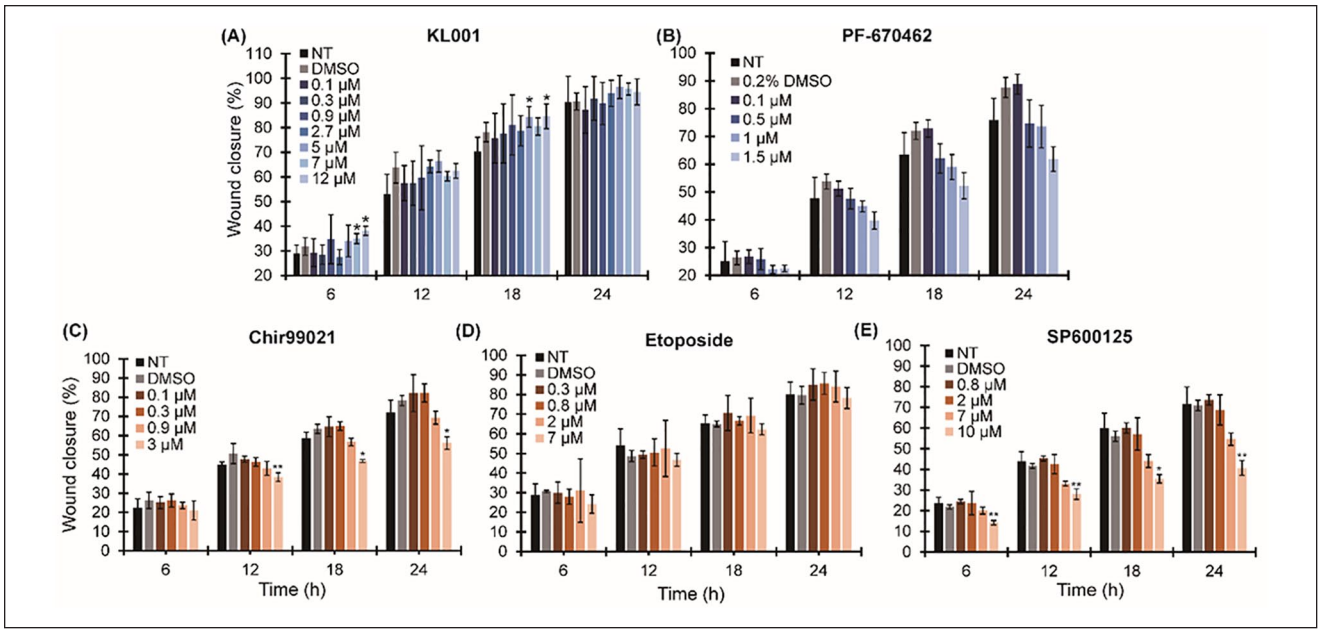

Figure 4. Two direct circadian modulators, KL001 (A) and PF-670462 (B), and three indirect circadian modulators, Chir99021 (C), etoposide (D), and SP600125 (E), influence cell motility to various extents. KL001 at concentrations 5, 7, and 12 μM promotes cell motility at $T = 6$, 12, and 18 hours (A). PF-670462 at 1.5 μM concentration reduces cell motility (B). SP600125 and Chir99021 treatments significantly inhibit cell migration (C, E), while etoposide shows no effect (D) in U2OS cells. Data are representative of three biological replicates; error bars represent standard deviation. Statistical significance was evaluated via Welch's *T* test followed by Bonferroni Correction, $^{*}P < .05$ and $^{**}P < .01$. NT, non-treated; DMSO, dimethyl sulfoxide–only control (0.2%).

samples (not shown in the figure). Etoposide showed no significant change at any of the concentrations tested (Figure 4D) but significantly suppressed cell proliferation in an assay evaluating that property (Supplementary Figure S13A). SP600125 significantly inhibited cell migration at the highest concentration, 10 μM (Figure 4E) when compared with the non-treated condition. SP600125 treatment at 7 μM concentration showed statistical significance ($P <$ .01) at $T = 12$, 18, and 24 hours when compared with the vehicle-treated condition (not shown in the figure). Among the molecules that showed effects on cell migration, SP600125 inhibited it to the greatest extent (30.9% at 10 μM when compared with non-treated condition), while Chir99021 and PF-670462 resulted in 15.8% and 13.9% reductions, respectively.

Small Molecules Affect Formation of Cancerous Colonies

Anchorage-independent proliferation is another important characteristic of aggressive cancers. While normal cells are unable to survive in the absence of adhesion to the extracellular matrix, malignant cells can grow into colonies due to loss of this dependence. While several of the molecules studied here affected cancer cell migration, we wanted to assess cell proliferation in a three-dimensional environment. By using a soft agar or colony formation assay, we can evaluate cancer cell proliferation in semisolid matrices, mimicking what may occur in in vivo microenvironments. After four weeks of incubation, U2OS cells generally form "tumor-like" colonies in the agar gels. For this assay, we utilized only the highest concentration of each drug (except for KL001) used in other assays performed here, since those concentrations led to the greatest period changes. For KL001, concentrations greater than 2.7 μM showed variable results, including in circadian assessments, wide distributions of periods, and significantly damped amplitudes (Figure 2B), resulting in our decision to use the lower concentration.

We observed that neither 2.7 μM KL001 nor 1.5 μM PF-670462 altered colony formation (Figure 5). 10 μM SP600125 and 3 μM Chir99021 decreased colony sizes by 59% and 32%, respectively, but had no effects on numbers of colonies. These data indicate that Chir99021 and SP600125 may inhibit cell proliferation in three dimensions (Figure 5) but not in monolayer cultures (Supplementary Figure S13B and C). This is not surprising given that it is well established that cells in two versus three dimensions may respond differently to drugs,[53] underscoring the importance of this type of assessment. Additionally, treatment of 7 μM etoposide significantly decreased colony size by 75%, from 206 ± 70 μm to 51 ± 5 μm (Figure 5B). We suspect this was related to inhibition of cancer cell proliferation, not observed with other molecules (Supplementary Figures S12 and S13). Etoposide likely resulted in greater (but not

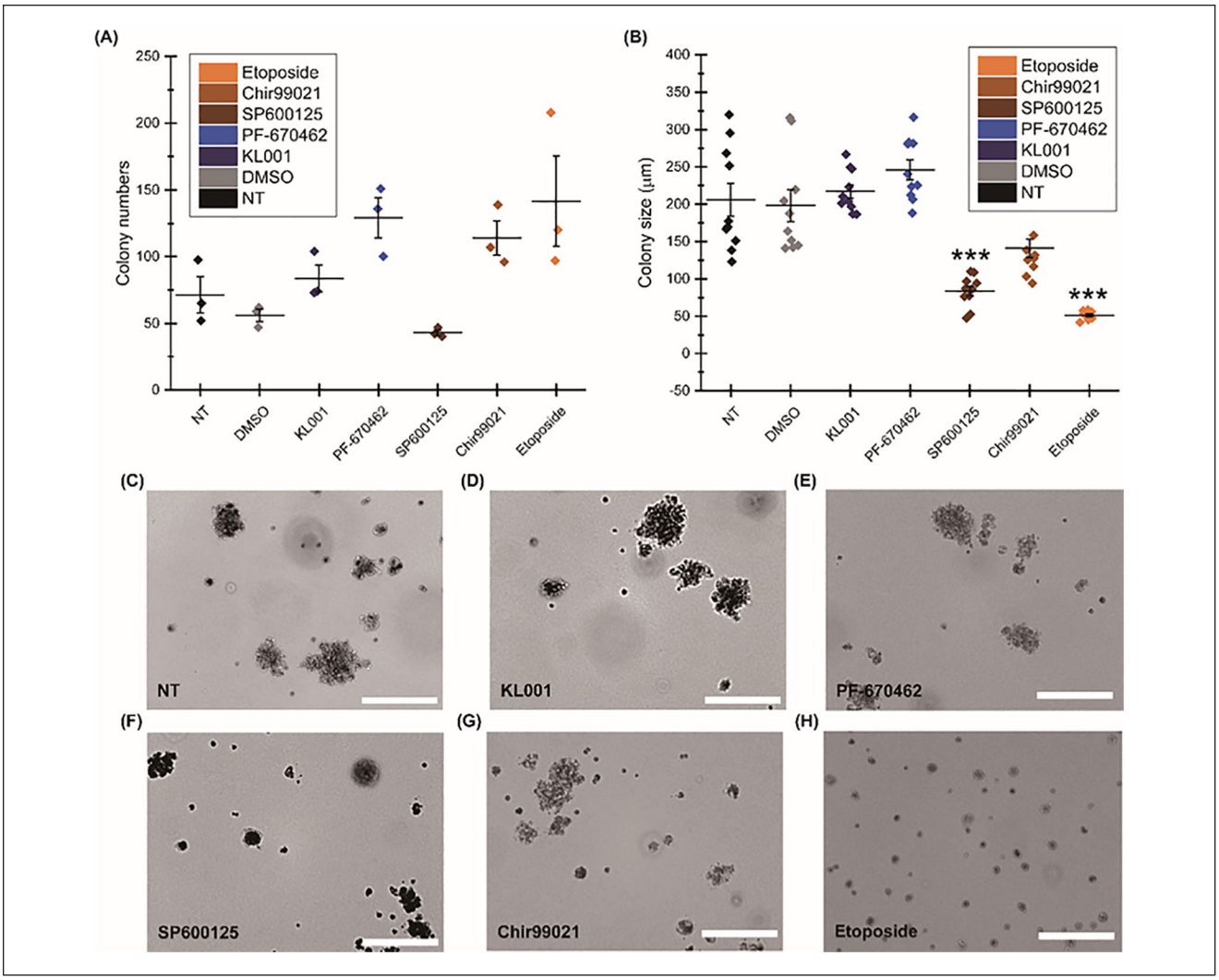

Figure 5. Small molecule modulators of circadian rhythms affect tumor formation in a soft agar assay. After four weeks of incubation, colony numbers (A) and sizes (B) were evaluated. PF-670462 and etoposide significantly increased colony numbers, while SP600125, Chir99021, and etoposide reduced colony sizes. Statistical significance was evaluated via Welch's *T* test followed by Bonferroni Correction (***$P < .001$). NT, non-treated; DMSO, dimethyl sulfoxide–only control (0.2%). Representative images for various treatments are shown (C-H). Scale bars are 100 μm.

statistically significant) colony numbers, (and also had a broader distribution) on account of cancer cell and colony growth suppression, which resulted in a lack of aggregation-forming clusters (Figure 5A, B, and H).

Discussion

While many small molecules have been developed to modulate or shown to affect circadian rhythms, to date, a systematic evaluation using the same concentrations in a parallel comparison of their effects on cellular and oncogenic characteristics is lacking. In this study, five small molecules were used to affect circadian rhythms, with a focus on period effects (ie, increase or decrease circadian cycle periods) in a commonly used circadian model, the human osteosarcoma U2OS cell line. We found that they influence periods and cancer cell migration, proliferation, and anchorage-independent growth to varying extents.

KL001 directly binds to CRY, inhibiting F-box and leucine-rich repeat protein 3 (FBXL3)-mediated CRY degradation.[33] Prior mathematical and experimental data both showed that stabilization of CRY via KL001 could increase circadian periods.[33] The model predicted that the period would increase by ~10% (ie, 2-3 hours) and was consistent with experimental data in *PER2::Luc* fibroblasts, which showed a ~2 hour enhancement with continuous KL001 treatment, but not in *Bmal1:luc* U2OS cells, where it resulted in ~6 hours lengthening.[24,33] Our data were

similar to that of the fibroblasts and another study, discussed further below.

While perturbation of the FBXL3-CRY pathway is known to affect circadian period,[54] the other negative clock regulatory pathway, casein kinase I (CKI)-PER, has been linked to period length as well.[26] We showed that PF-670462 treatment also resulted in a dose-dependent period lengthening effect. It has been reported that PF-670462 has an IC_{50} of 0.55 μM for CKIε and an IC_{50} of 0.14 μM for CKIδ in whole cells.[55] This molecule is proposed to increase circadian periods by specifically inhibiting CKIδ-mediated phosphorylation of PER, not through CKIε. Meng et al[43] showed that selective inhibition of CKIδ via PF-670462 stabilized the nuclear localization of endogenous PER2 in both SCN and peripheral cells, indicating that the molecule can act as a PER stabilizer and lead to period enhancement. This has been evaluated mathematically[56] and experimentally.[43,56,57] The model predicted dose-dependent period lengthening, with the following increases resulting from indicated treatments: 1 hour for 0.1 μM; 3 hours for 0.5 μM; 7 hours for 1 μM. These results were similar to both experimental validation in the same study,[56] and other experiments,[43,57] including our own.

Since circadian effects of the molecules have been reported in the literature, it was initially surprising to us that in some instances, our results differ from published data. KL001 has been reported to increase the period of *Bmal1* in U2OS cells, whereupon 2.7 μM treatment enhanced it by ~6 hours.[24,33] Our data showed a ~2.5-hour period enhancement in the same model (Figure 2B), similar to the ~2 hour change observed in *PER2::Luc* fibroblasts.[33] In this instance, variability likely results from use of different analysis algorithms, and possibly the model used. Currently, there is no standard analysis method for calculating circadian periods, leading to differing results even for the same cell model and reporter combination.

Despite employment of different models, our PF-670462 results were similar to period changes in previously published data. Meng et al showed that 1 μM PF-670462 lengthened the period of *PER2::Luc* by ~10 hours in primary lung fibroblasts,[43] while both Kim et al[56] and Patton et al[57] showed that the same treatments exhibited similar period enhancements of ~7 to 8 hours in *PER2::Luc* SCN slices. Our data displayed a similar change (~9.5 hours) in the period of *Bmal1:luc* in U2OS cells (Figure 2D).

While both direct modulators significantly increased the periods of *Bmal1* and *Per2* in U2OS cells, they exhibited opposing effects on cell migration. At $T = 6$ and 18 hours, KL001 promoted cancer cell migration but had no effects on cell proliferation or colony formation. One recent study assessed the effects of KL001 on cancer: Dong et al[58] showed that KL001 effectively inhibited glioblastoma stem cell proliferation, but normal brain cells were affected to a lesser extent. Similarly, Xue et al[59] used KL001 to impair mitosis and epithelium repair in a model of corneal wound healing. Collectively, it is likely that these changes occur in a cell-dependent manner. The growth suppression effects of KL001 may be due to its stabilization of CRY proteins, which are generally characterized as tumor suppressors. Huber et al showed that CRY2 and FBXL3 cooperatively destabilize c-MYC (a critical oncoprotein for cancer cell proliferation), and that disruption of CRY2 may contribute to elevated c-MYC and higher cancer susceptibility in shift workers and animals.[60] Separately, Chun et al[61] found that KS15, which inhibits interactions between CRY and the CLOCK:BMAL1 heterodimer, suppressed breast cancer cell proliferation and increased drug responses to chemotherapies; the mechanism by which these effects occurred has not yet been investigated.

Conversely, PF-670462 reduced cell migration but enhanced cell proliferation and colony numbers. As Rodriguez et al[62] showed that PF-670462 significantly suppressed ovarian cancer cell proliferation and migration, and Cheong et al[63] found that it weakly inhibited fibrosarcoma cell proliferation, we were surprised by our results. PF-670462's targets, CKIε and δ, are key regulators for many biological processes, including circadian rhythms, cell cycle, cell adhesion, cytoskeleton construction, and receptor-coupled signal transduction.[64] However, CKI has been implicated in numerous oncogenic signaling networks,[64] and contradictory evidence has implied that the CKI family may play dual roles as tumor drivers and suppressors.[65,66] Hence, the seemingly contradictory effects of PF-670462 in different models may stem from the diverging functions of its targets.

SP600125, Chir99021, and etoposide, the three indirect circadian modulators, all altered the periods of *Bmal1* and *Per2* in U2OS cells. SP600125 inhibits JNK-1, JNK-2, and JNK-3 (cell-free IC_{50} of 40 nM for JNK1 and 2, and 90 nM for JNK3).[67] It has been shown that activated JNK isomers directly phosphorylate the BMAL1-CLOCK complex to regulate the speed and phase of circadian oscillations in cells;[68] thus, treatment with SP600125 increased periods.

On the other hand, Chir99021 specifically inhibits GSK3α/β (cell-free IC_{50} of 10 nM for GSK3α and 6.7 nM for GSK3β), which prevents phosphorylation of PER2,[69] CRY2,[70] CLOCK,[71] BMAL1,[41] and REV-ERBα[72] leading to a period-shortening effect. Interestingly, a mathematical model previously developed showed that inhibition of GSK3β did not alter the phosphorylation of PER2,[73] which was contrary to experimental data that indicated its prevention.[69]

Etoposide has been previously reported to result in decreased periods,[45] and it may affect the clock via its interaction with TOPII (cell type–dependent IC_{50} ranges from 0.1 to > 100 μM), which has been identified as a regulator of *Bmal1* transcriptional activity.[74]

Variability was also observed here when comparing our results with previously published data. 7 μM SP600125 exhibited a ~3 hour period enhancement in U2OS-*Bmal1:luc* cells,[45] while we found the same treatment increased it by ~8 hours (Figure 3B). We were surprised to find that in contrast, *Per2* was only lengthened by ~6 hours; however, no other studies addressed changes in oscillations for both *Per2* and *Bmal1*. 3 μM Chir99021 showed a ~3 hour decrease in U2OS-*Bmal1:luc* cells,[45] while the same treatment resulted in a ~4.5-hour reduction in our work (Figure 3D). In the same evaluation, 7 μM etoposide showed a ~3.5-hour decrease in the U2OS-*Bmal1:luc* cells,[45] but we only found the change to be ~1 hour via similar treatment (Figure 3F). Again, use of different analysis algorithms may contribute to these differences.

In evaluating their effects on cancer cell behaviors, we found that both SP600125 and Chir99021 treatments inhibited cell migration and resulted in diminished colony sizes, which were consistent with published observations.[75,76] On the other hand, etoposide showed no effect on cell migration but significantly reduced cell proliferation and colony size. Inhibition of cell proliferation via etoposide treatment has been observed previously[77,78] and may support our results. To the best of our knowledge, no previous work has investigated the effects of etoposide on cell migration.

Our data appear to indicate that circadian period length may not be correlated with cell growth and migration, and that pharmacological targets may play a dominant role in controlling cell motility. It is notable that PF-670462, SP600125, and Chir99021 all affected circadian periods and motility to greater extents (1.5 μM PF-670462 increased *Bmal1* period to 34.4 ± 0.8 hours, 10 μM SP600125 increased *Bmal1* period to 33.9 ± 0.5 hours, and 3 μM Chir99021 decreased *Bmal1* period to 18.5 ± 0.2 hours), while KL001 and etoposide only resulted in minor changes for both (12 μM KL001 increased the period of *Bmal1* to 29.2 ± 3.3 hours, and 7 μM etoposide decreased it to 21.9 ± 0.6 hours). Based on our results, it may be possible that some cellular effects are only observed upon significant deviation from the 24-hour cycle. To be certain, further studies, including perturbations directly targeting core clock proteins, are required.

It is also possible that directly targeting circadian proteins or inducing circadian period effects may not be sufficient to affect cancerous phenotypes, and that noncircadian pathways have a greater influence on cellular behavior. There is no shortage of work to be done in this area, including assessment of other circadian alterations, such as amplitude and phase, use of additional small molecules directly targeting circadian proteins that have greater effects on period, utilization of next-generation sequencing methods to further assess connections between small molecule–affected cancer and circadian pathways, and employment of combined genetic and chemical biology approaches via knockdown of noncircadian targets and chemical modulator treatments to further distinguish circadian-based effects from alternative pathway-based effects. Understanding the contributions of circadian rhythms and their effects on cellular phenotypes will be critical to understanding roles of the clock in homeostasis and disease. However, we note that in further studies of small molecules targeting the circadian machinery or otherwise, it is highly merited to evaluate both cellular and circadian effects.

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Supplemental Material

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