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Summary: Chemical Modulation of Circadian Rhythms and Assessment of Cellular Behavior via Indirubin and Derivatives

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Abstract

We present a summary of our protocol for employment of small-molecule circadian rhythm modulators. The abilities of compounds to affect oscillations have received significant attention. We outline assessments of circadian changes using indirubin-3'-oxime (**IO**), 5-iodo-indirubin-3'-oxime (**5I-IO**), and 5-sulfonic acid-indirubin-3'-oxime (**5SA-IO**), analyzing effects on *Bmal1* and *Per2* oscillations and oncogenic features.

Background

Circadian clocks control various functions with 24-h rhythmicity.[1-3] The mammalian clock is comprised of a transcription-translation feedback loop, with proteins BMAL1 and PER cycling in anti-phase. Indirubins have high affinities for many kinases involved in tumorigenesis,[5] and possess anti-cancer effects.[6,7] Two previous studies measured their period shortening abilities: one used 6-bromo-indirubin-3'-oxime analogues ;[8] the other included indirubin-3'-oxime and 5-iodo-indirubin-3'-oxime.[9]

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Results and Discussion

We studied indirubin compounds in U2OS cells bearing luciferase reporters (*Bmal1:luc* and *Per2:luc*), tracking oscillations via real time luminometry.[10] Raw luminescence data were pre-processed by removing initial transiently high bioluminescence counts (first 24-h), baseline trend (by removing a 24-h moving average), and noise (with a 3-h moving average smooth). At each stage, we visualized the data and effects of processing to confirm the approach used. For trend removal, we compared multiple methods (including discrete wavelet transform[11,12] and low-order polynomials) and chose the method leading to the most sinusoidal time-series. At low doses of **IO** and all of **5SA-IO,** individual recordings were similar, while high doses of **IO** and all of **5I-IO** led to later and more varied peak and trough times (**Figure 1A, C, E**). Average recordings for each condition (**Figure 1B, D, F**) clarified trends. Amplitude, phase, and period effects of **IO** and **5I-IO** are apparent, while effects of **5SA-IO** are negligible.

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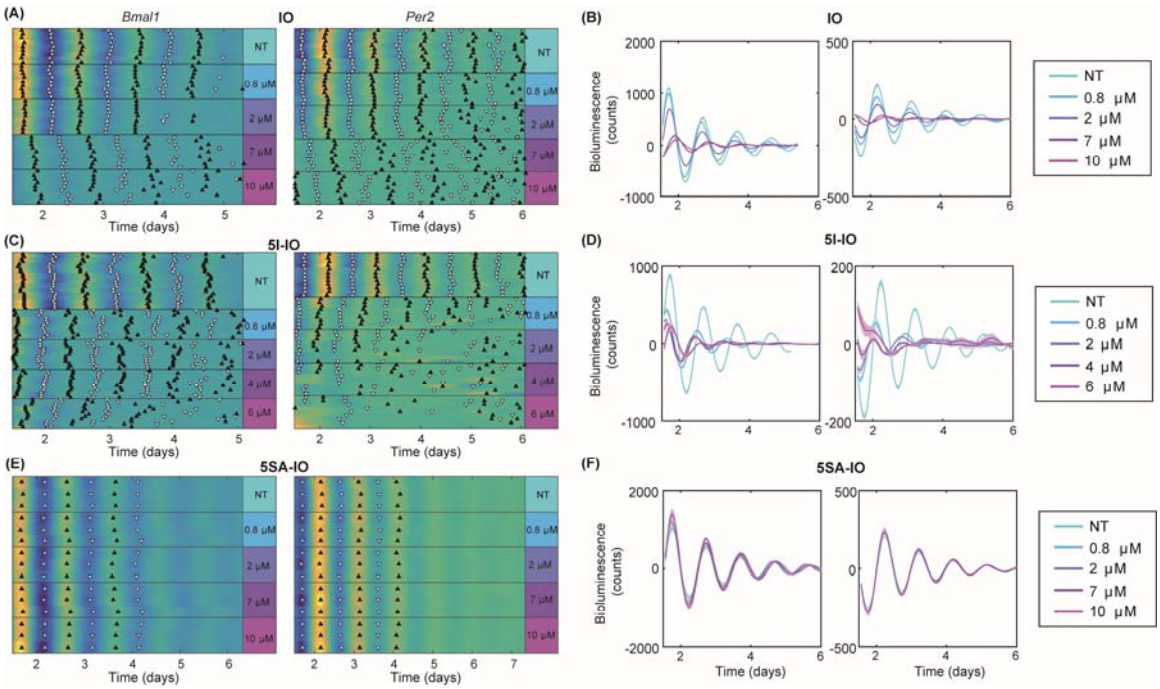

Figure 1. De-trended, smoothed time-series from cells treated with (top) **IO**, (middle) **5I-IO**, and (bottom) **5SA-IO**. Heatmaps (left) display recordings individually, by treatment condition. Each is scaled separately: blue indicates minimum and yellow maximum per reporter/treatment. Peaks are black and troughs inverted white triangles. Time-series plots (right) show average recordings per reporter and condition. SEM measurements form envelopes around the line and are nearly invisible, indicating that within a condition, behavior is similar. NT=non-treated.

To quantify treatment effects, it is important to determine reliable estimates of circadian parameters. We focused on period, using results of three methods that use different approaches. 1. Determining time between peaks -- this is straightforward and easy to examine visually; it can be misleading if the waveform has small changes from cycle to cycle. Fitting a curve to the data can alleviate such problems and is resistant to noise remaining in the signal, so 2. fit to a

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damped sine curve. 3. Wavelet analysis, which represents a widely used class of methods (spectral), and can handle oscillations whose period changes over the course of recording. For all methods, we excluded time-series with periods outside the circadian range of 16-32 h. Period estimates were different, depending on the method, but all three revealed similar trends (**Figure 2**).

To quantify treatment effects on average periods, we used a randomization test for difference in means, comparing each treatment to non-treated. Regardless of method, 0.8 μM **IO** leads to shorter periods (**Figure 2A**). In most cases, 2 μM **IO** also leads to shorter periods. Distributions of periods are wider for both 7 μM and 10 μM treatments, and *Per2:luc* differences are mostly not statistically significant. Likewise, **5I-IO** treatment shortens periods (**Figure 2B)**. Trends are consistent across measures for *Bmal1:luc*, with the exception of the highest dose (6 μM), which shows a wide range. For *Per2:luc*, 0.8 μM **5I-IO** leads to significantly shorter periods, but at higher doses, there are fewer than 5 measurements each. As a statistical test would lack power to find significance at 5%, none is used. In contrast, **5SA-IO** treatment produces no period effects (**Figure 2C**).

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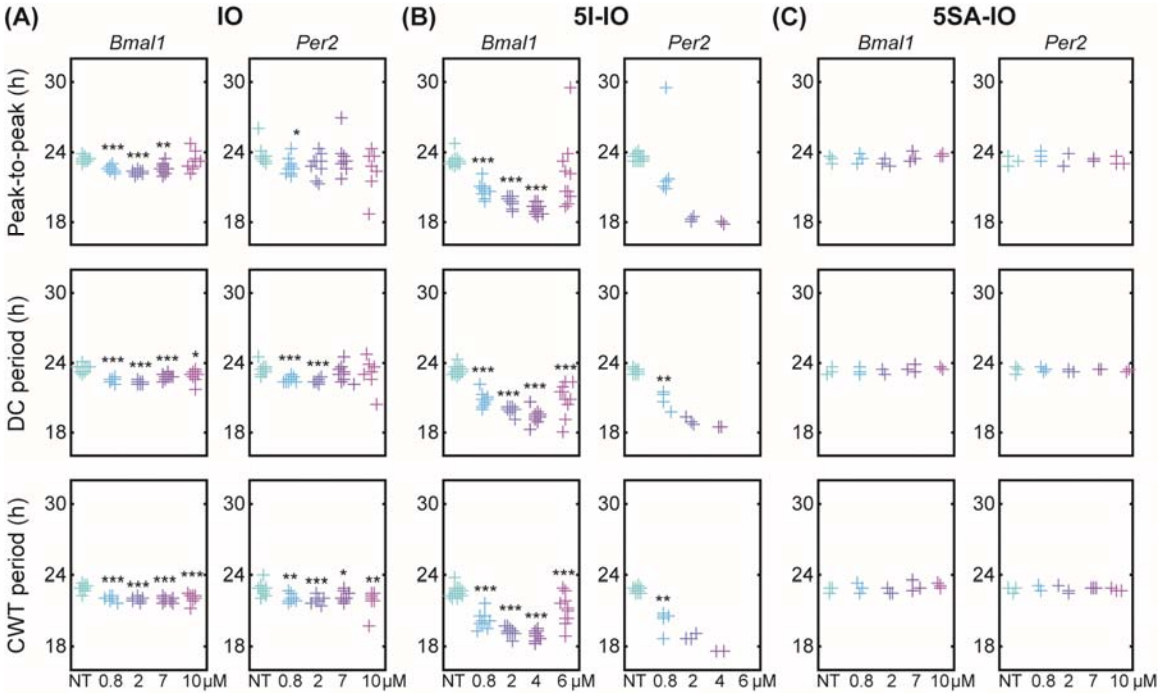

Figure 2. Jitter plots measuring period changes across treatments. Periods are estimated using average time from peak to peak (top), damped cosine-fitting (middle), and continuous wavelet transformation (bottom). Asterisks indicate statistically significant differences between each treated condition versus non-treated. P-values were computed using a randomization test for difference in means and Bonferroni corrected (*p<0.05; ** <0.01, *** < 0.001).

We used wound healing and colony formation assays to assess accompanying cellular effects (data not shown). **IO** and **5I-IO** significantly reduced cell migration (confirmed by 2-tailed Student's T-Test), while **5SA-IO** did not. In a soft agar colony formation assay, **IO** and **5I-IO** showed potent effects; **5SA-IO** was excluded from the assay. With increasing concentrations, compound-treated cells formed fewer and smaller colonies. Welch's (2-tailed) T-test with Bonferroni correction was used to assess differences between non-treated and each

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treatment. Significant area reductions occurred for 7 and 10 μM **IO** and all **5I-IO**. Low doses led to variable colony numbers, while significant reductions occurred at high doses.

Conclusion

We have summarized a workflow and case study assessing small molecule circadian modulators in cell culture. We highlighted important steps in circadian time-series analysis and assessments of cellular effects.

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