

ORIGINAL ARTICLE

Modeling and Validating Chronic Pharmacological Manipulation of Circadian Rhythms

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Circadian rhythms can be entrained by a light-dark (LD) cycle and can also be reset pharmacologically, for example, by the CK1 δ/ϵ inhibitor PF-670462. Here, we determine how these two independent signals affect circadian timekeeping from the molecular to the behavioral level. By developing a systems pharmacology model, we predict and experimentally validate that chronic CK1 δ/ϵ inhibition during the earlier hours of a LD cycle can produce a constant stable delay of rhythm. However, chronic dosing later during the day, or in the presence of longer light intervals, is not predicted to yield an entrained rhythm. We also propose a simple method based on phase response curves (PRCs) that predicts the effects of a LD cycle and chronic dosing of a circadian drug. This work indicates that dosing timing and environmental signals must be carefully considered for accurate pharmacological manipulation of circadian phase.

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The timing of activities such as waking, sleeping, body temperature, blood pressure, hormone expression, and feeding show circadian (daily) rhythms.^{1,2} These circadian rhythms are regulated by the master circadian clock in the suprachiasmatic nuclei where transcriptional activators CLOCK and BMAL1 drive the expression of repressors period (Per) and cryptochrome (Cry).³ This feedback system includes the PER1/2 proteins, which are phosphorylated by the CK1 δ/ϵ , dimerize with the CRYs, then translocate to the nucleus to inhibit BMAL1/CLOCK and repress the transcription of Per and Cry.⁴ Further phosphorylation by CK1 δ/ϵ signals PER degradation that releases BMAL1/CLOCK transcriptional inhibition and resumes transcription of Per and Cry.²

This endogenous timekeeping system can be synchronized to the earth's 24-h periodic environment through external cues, known as zeitgebers (e.g., light-dark (LD) cycle and temperature cycle).^{5,6} To maintain clock-environment synchrony, zeitgebers induce changes in the concentrations of the molecular components of the clock to levels consistent with the appropriate stage in the 24-h cycle. Misalignments of circadian timing with the external environment can cause significant physiological problems, such as jet lag, depression, insomnia, coronary heart disease, neurodegenerative disorders, and cancer.⁷ In particular, mood disorders and bipolar disorders appear to be tightly related to disrupted circadian rhythms.^{8–11} To treat the misalignment of circadian clocks with the external environment, pharmacological manipulation of circadian clocks has received much attention.^{12–15} We previously showed that acute dosing of PF-670462 (CK1 δ/ϵ inhibitor) can delay circadian behavior, as well as re-establish a circadian rhythm in *Vipr2*^{-/-} mice that are arrhythmic under dark-dark (DD) cycle or light-light

(LL) cycle.^{16–18} To extend this work to real-life situations that proceed under LD cycles with seasonal variation, we need to study the effect of CK1 δ/ϵ inhibition on circadian rhythms under different LD cycles.

Light and inhibition of CK1 δ/ϵ simultaneously affect multiple components in the molecular feedback loops in circadian clocks.^{4,17–21} Light induces transcription of Per1 and Per2, whereas inhibition of CK1 δ/ϵ decreases the degradation rate and the nuclear translocation rate of PER as well as the binding rate between PER and CRY.² To understand these interactions systematically, mathematical modeling has played important roles. For instance, the correct function of the *tau* mutation in CK1 ϵ was identified by the Forger–Peskin model, which was later confirmed experimentally.^{22,23}

Here, we study how light stimuli and CK1 δ/ϵ inhibition affect mammalian circadian timekeeping with a combination of experiments and simulations using a mathematical model of intracellular mammalian circadian clocks.²⁴ We find that acute pharmacologic inhibition of CK1 δ/ϵ via PF-670462 immediately delays all clock gene expression in suprachiasmatic nuclei and locomotor activity under LD cycles. The opposing actions of pharmacological delay and light can yield a constant stable delay of circadian behavior when CK1 δ/ϵ is inhibited chronically under LD cycles. The occurrence and magnitude of a stable phase delay depend on dosing amount, dosing timing, and day lengths. We also find that complex behaviors induced by the LD chronic dosing can be predicted with phase response curves (PRCs) to light stimuli and CK1 δ/ϵ inhibition. This work provides a way to determine a dosing strategy of chronic CK1 δ/ϵ inhibition to treat the misalignment of circadian clocks by modulating the phase of circadian rhythms.

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RESULTS

The counteracting effects of light on multi-day dosing of PF-670462

We previously showed that the CK1 δ/ϵ inhibitor, PF-670462 delays circadian phase under a DD cycle regardless of dosing timing.^{16–18} Here, we examine the effects of multi-day dosing of PF-670462 at CT11 (ZT11), the dosing timing that yielded the largest phase delay¹⁶ in both the DD and LD cycles (Figure 1a). CT and ZT refer to timing with respect to a circadian signal or external signal, respectively (e.g., for a nocturnal animal, time of the onset of activity and lights off define CT12 and ZT12, respectively).⁵ In a LD cycle, 10 and 32 mg/kg dosing induce phase delays of 1.3 and 2.9 h, respectively (Figure 1b and Supplementary Table S1 online). Treatment in DD, however, yields phase delays of 1.5 and 3.9 h at 10 and 32 mg/kg, respectively, which are greater in comparison to LD dosing (Figure 1b and Supplementary Table S1 online). These multi-day-dosing experiments indicated that a LD cycle partially counterbalances the effect of PF-670462 on circadian rhythms.

Chronic dosing of PF-670462 induces a constant stable delay under LD cycles

The 3-day dosing experiments indicates that the phase delays from multiple days of dosing are additive. Given the

significant effect of light to partially counteract the delay in activity, we sought to determine whether daily dosing in LD would be continuously additive, as has been previously shown for extended DD dosing.¹⁸ Daily dosing of PF-670462 was performed in both the LD and DD cycles for up to 3 weeks (Figure 2a). The LD experiment was terminated after 2 weeks upon the observation of a constant daily phase delay, whereas the dosing in DD was continued for a third week. In DD, large accumulated delays of 7.6 and 13 h were detected at 10 and 32 mg/kg, respectively (Figure 2b,d and Supplementary Table S2 online). In a LD cycle, the reduced accumulated delays of 1.8 and 6 h were observed with 10 and 32 mg/kg, respectively (Figure 2c,d and Supplementary Table S2 online). This stable delay of LD chronic dosing, which was also observed in a previous study with rats,²⁵ indicates that equilibrium has been reached by balancing light-induced phase advances and pharmacological-induced phase delays.

Effect of acute CK1 δ/ϵ inhibition on gene expression and animal behavior under LD cycles

To extend our experimental results to different LD cycles or different dosing timings, we used an accurate and detailed mathematical model of the intracellular mammalian circadian clock,²⁴ which accurately predicts many phenotypes of

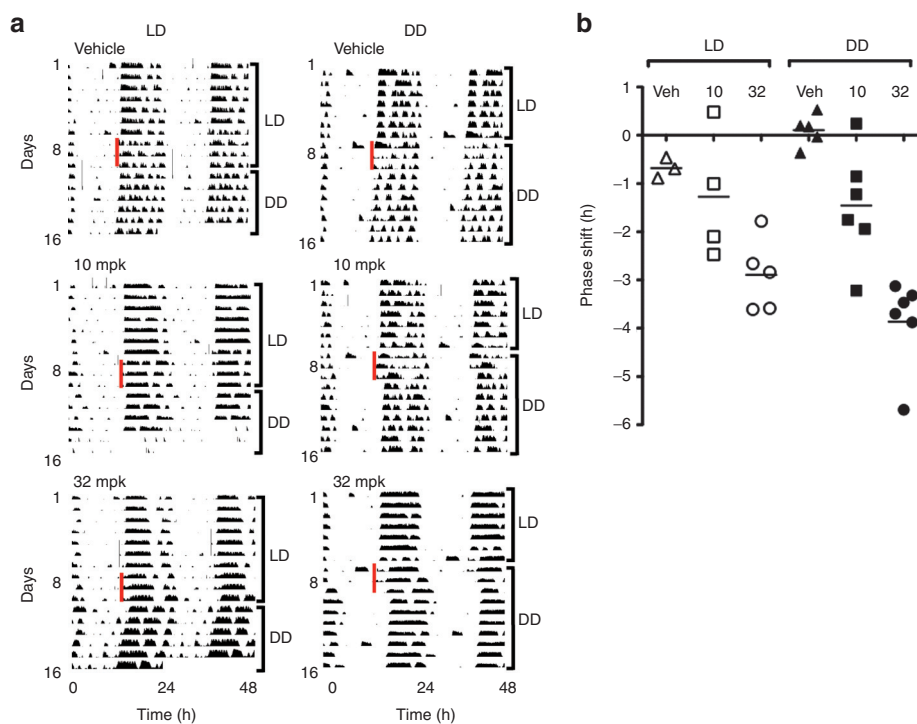


Figure 1 Counteracting effects of light on multi-day dosing of PF-670462. (a) Double-plotted actograms of mice activity for 16 days, where black vertical bars represent the activity of mice. The actogram is double plotted so that there are 2 days per line, which facilitates the identification of circadian rhythms. Further details of actograms can be found in ref. 5. Mice were maintained in 12:12 LD and circadian measures followed for 7 days (see Supplementary Methods online for details of measurement method). In each experiment, animals were either shifted into DD or maintained in LD and treated with vehicle or PF-670462 (10, 32 mg/kg) at CT11 (ZT11) for 3 days (days of dosing indicated by red dash). After the last day of dosing, mice in LD were shifted into DD and all groups followed an additional 7 days to measure phase shifts. Note CT and ZT are used to describe the dosing timing under DD and LD, respectively⁵ and dosing amount is selected based on our previous study.¹⁷ (b) Phase shifts were calculated as the time difference in behavior onset between the predosing week and after dosing as analyzed by the Clocklab software. The PF-670462-induced phase delay is attenuated by light (Supplementary Table S1 online). DD, dark-dark cycle; LD, light-dark cycle.

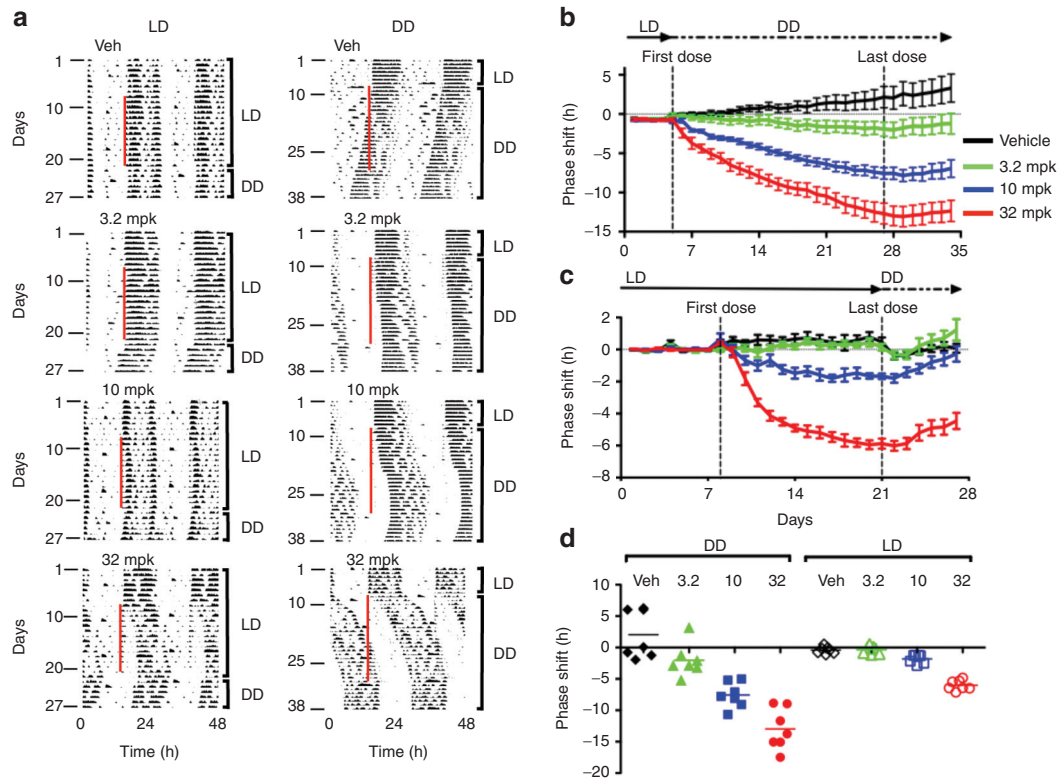


Figure 2 Chronic dosing of PF-670462 induces a constant stable delay under LD, but not under DD. (a). Mice were maintained in 12:12 LD and circadian measures followed for 7 days before dosing. In each experiment, mice were either shifted into DD or kept in LD, and daily treated with vehicle or PF-670462 (3.2, 10, 32 mg/kg) at CT11 (ZT11), red dash indicates days of dosing. After the last dose, mice in LD were shifted into DD and all groups followed for an additional 7 days. Daily phase angle was measured as the start of the circadian signal relative to that defined by the 7 days before dosing in the same way used in Figure 1. (b–d) DD chronic dosing induces continually accumulating delays regardless of dosing amount (in b), but LD chronic dosing induced the constant stable phase delays (in c). DD, dark-dark cycle; LD, light-dark cycle.

circadian mutations including the *tau* mutation in CK1 ϵ . To use the molecular model, we first tested whether the effects of CK1 δ/ϵ inhibition in animals entrained to a LD cycle were reflected both in gene expression and in whole animal locomotion since intracellular timekeeping is often not fully reflected in whole animal behavior.²⁶

Mice housed in a 12:12 LD cycle were treated with PF-670462 at ZT11. Starting at 4h following dosing, the hypothalamus, a region of the brain which includes the master circadian clock of mice, was harvested at 3-h intervals and the expression of eight clock genes was measured. Animals continued in the LD cycle until hypothalamus harvesting to test the effect of both the PF-670462 and LD cycle together. All the genes examined showed a dose-responsive delay in expression under LD cycles (Supplementary Figure S1 online and Supplementary Table S3 online). In particular, *Rev-erb α* and *DBP* showed the clearest shifts in the peak times of expression. While *DBP* showed no shift of peak time for 32 mg/kg dosing, other timepoints were shifted, indicating the overall phase shift might be <3h, the time interval used to measure gene expression (Figure 3a).

We next examined how the delayed circadian gene expression is reflected in circadian behavior. Under treatment conditions identical to the gene expression studies, activity was measured by telemetry. Following treatment and one

additional 12:12 LD cycle, reflecting the day of hypothalamus harvesting in the gene expression studies, the mice were transferred into DD to assess phase shifts (Figure 3b). Circadian behavior also showed a dose-response delay under a LD cycle (Supplementary Table S4 online). Delays of 1.3 and 6.7h, for dosing of 32 and 100 mg/kg were found, respectively. Although the shift induced by 32 mg/kg is not significant (Supplementary Table S4 online), the behavioral shifts are remarkably consistent with the gene expression shifts of *Rev-erb α* and *DBP* (Figure 3c).

Taken together, these behavioral and gene expression results are consistent with a previous study showing that *Rev-erb α* and *DBP* expression best reflect altered locomotor activity in a natural LD cycle.²⁷ The data also indicate that circadian behavior is strongly linked to the timing of clock genes when gene expression is altered by CK1 δ/ϵ inhibition in an acute manner even under LD cycles.

A systems pharmacology model of the mammalian circadian clock including PF-670462

Since our experiments showed a tight relationship between molecular and behavioral rhythms in response to CK1 δ/ϵ inhibition under a LD cycle (Figure 3), we hypothesized that our molecular model would give accurate predictions of both molecular timekeeping and whole animal behavior.²⁴ Since

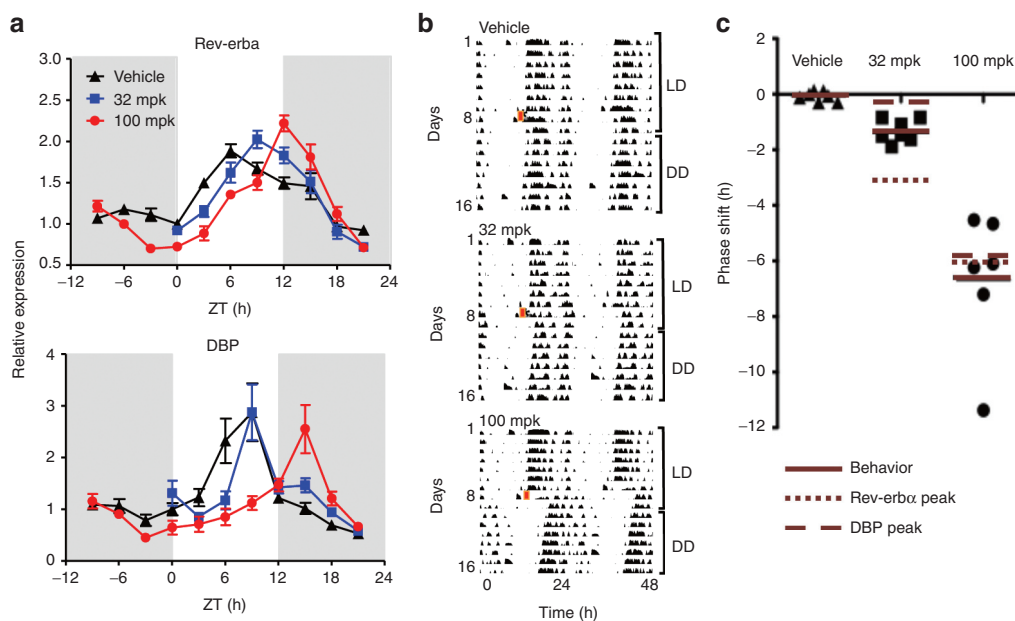


Figure 3 Direct relationship between the delay of circadian gene expression and circadian behavior for pharmacological inhibition of CK1 δ/ϵ under a LD cycle. (a) Mice were treated with either vehicle or PF-670462 (32, 100 mg/kg) at ZT11, maintained in a LD cycle and the hypothalamus was taken for gene expression analysis over the subsequent 36h. While the expression of each circadian gene examined shows a dose-responsive delay in expression (Supplementary Figure S1 online and Supplementary Table S3 online), those of Rev-erba and DBP showed the clearest shifts. (b) Similar to the gene expression study, mice were maintained in 12:12 LD and circadian measures followed for 7 days, then treated with vehicle or PF-670462 (32, 100 mg/kg) at ZT11. One day after dosing, mice were shifted into DD and followed for an additional 7 days to measure phase shift. (c) PF-670462 induced the similar phase shift of gene expression and behavior under LD cycle. Here, lines indicate average phase shifts of behavior, peak timing of Rev-erba and DBP. DD, dark-dark cycle; LD, light-dark cycle.

this model does not include PF-670462, a multi-compartment pharmacokinetic/pharmacodynamic model was included into our model to describe disposition of PF-670462 and its interaction with CK1 δ/ϵ ²⁸ (Supplementary Figure S2 online and Supplementary Methods online). This adds 11 parameters that describe the pharmacokinetics and pharmacodynamics of PF-670462 in the model (Supplementary Table S5 online). The values of these new parameters were estimated by fitting to experimental data via a simulated annealing (SA) method.²⁹ See Methods and Supplementary Table S5 online for details of description and estimation of new parameters.

The estimated parameters reflect the pharmacokinetics and pharmacodynamics of PF-670462 accurately. Our parameters are fitted to the disposition profiles of PF-670462 in plasma and brain tissue following a single 32 mg/kg s.c. (Figure 4a). Furthermore, interestingly, the model correctly predicts CK1 δ/ϵ occupancy, the fraction of bound CK1 δ/ϵ by PF-670462 (Figure 4b), suggesting accurate prediction of the binding affinity between PF-670462 and CK1 δ/ϵ .

Next, we compared simulations of our model with previous *in vitro* experimental studies that measured the effect of PF-670462 on clock gene expression.¹⁸ Our model successfully predicts dose-dependent period changes of clock gene expression in suprachiasmatic nuclei (Figure 4c). Matching previously published data,¹⁸ the model also predicts that period prolongation by CK1 δ/ϵ inhibition is mainly due to the prolongation of the interval between the peaks of Per2 and Bmal1 (Figure 4d).

Finally, we compared the phase shifts of behavior produced by light and CK1 δ/ϵ inhibition in the model and in the experiments. For this, we explored PRCs, which are measured by giving a stimulus (e.g., light) to circadian rhythms at different times and measuring the effect on the phase of rhythms.^{30,31} Previous studies have shown that PRCs of circadian rhythms to light pulses have both the advance and delay regions,^{32–34} but the PRC to dosing of PF-670462 shows only delays.¹⁶ That is, light stimuli can advance or delay circadian rhythms depending on the timing of stimuli, but a dose of PF-670462 always delays the phase, regardless of dosing timing. Our mathematical model successfully reproduced these two PRCs (Figure 4e) and was also able to reproduce both a constant stable delay induced by LD chronic dosing and a cumulative increasing delay induced by DD chronic dosing at ZT11 (Figures 2 and 4f). To test the reliability of estimated parameters, we also simulated PRCs to dosing of PF-670462 in the presence of parameter perturbations. Even with the significant perturbations of parameters, the model successfully produced PRCs that show only delays (Supplementary Figure S3 online). These simulations indicate that the model can accurately and robustly reproduce behavioral data in response to CK1 δ/ϵ inhibition in a LD cycle.

Predictions about chronic dosing of a PF-670462

Our mathematical model was successfully able to reproduce experimental data on the effects of a light pulse or CK1 δ/ϵ inhibition on circadian rhythms (Figure 4). With our mathematical model, we simulated chronic dosing of PF-670462 under various conditions. First, we investigated whether

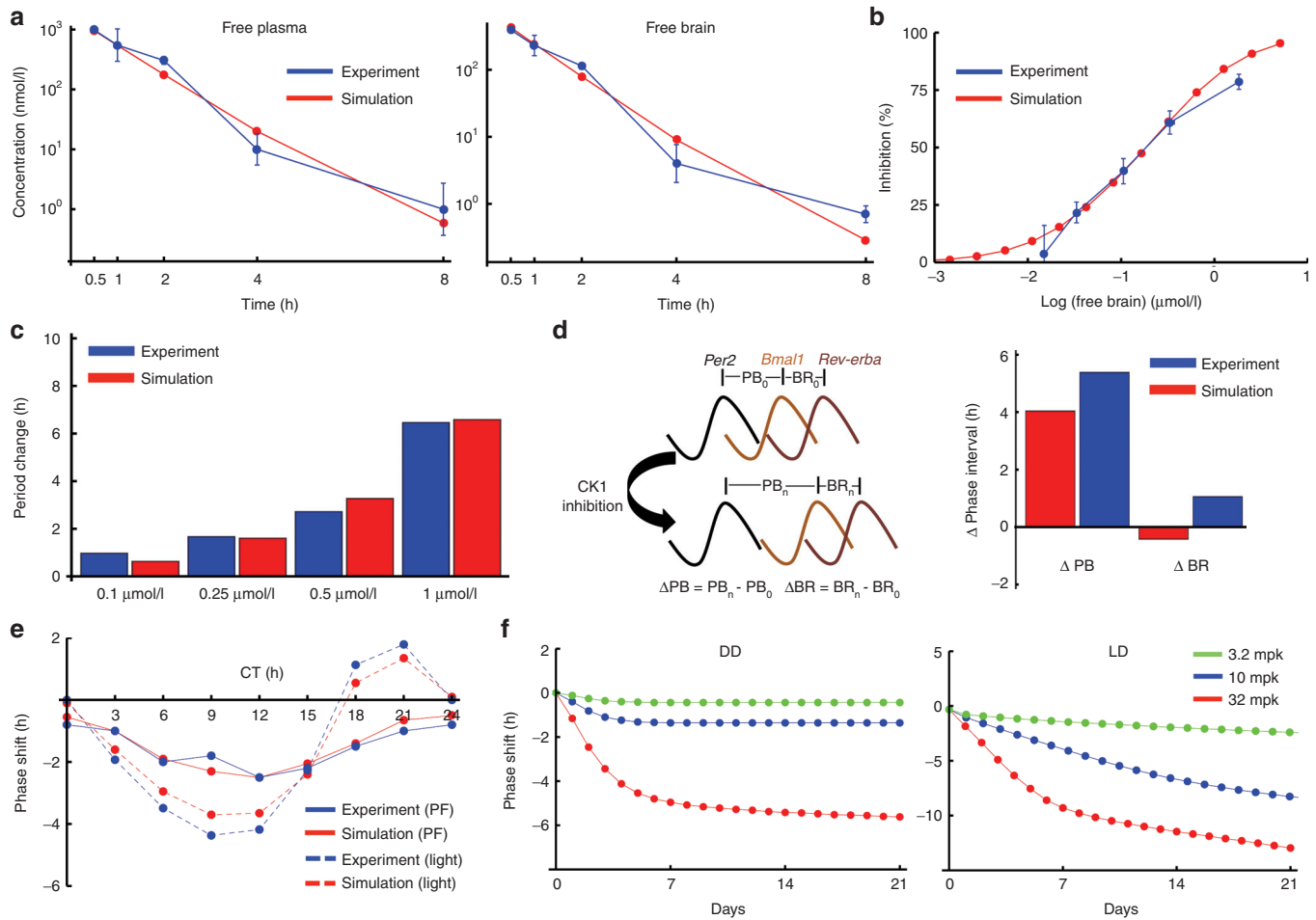


Figure 4 Comparison between simulations and experimental data: **(a,b)** PK/PD of PF-670462, **(c,d)** clock gene expression phase change, and **(e,f)** behavioral phase changes. **(a)** Experimentally measured disposition profiles of free PF-670462 in plasma and brain tissue after a single 32 mg/kg s.c. dose were compared with simulations. The units of concentrations of simulations were chosen that match experimental data (see **Supplementary Methods** online for details). **(b)** The model correctly predicts CK1 occupancies corresponding to concentrations of free brain PF-670462. **(c)** The model predicts dose-dependent prolongation of circadian period, matching a previous experimental study of SCN slice.¹⁸ **(d)** The model predicts that PF-670462-induced prolongation of period in **(c)** is mainly due to the lengthened internal phase relationship between the *Per2* peak and the *Bmal1* peak (ΔPB), matching the experimental data of the previous study.¹⁸ PB_0 and PB_n represent the difference of peak timing of *Per2* and *Bmal1* mRNA without and with 1 $\mu\text{mol/l}$ dosing of PF-670462, respectively. ΔPB is the difference between PB_n and PB_0 . **(e)** The model successfully reproduces PRCs to a 12-h, 100 lux light pulse³⁴ and a 50 mg/kg dose of PF-670462.¹⁶ **(f)** Simulations of 32 mg/kg chronic dosing of PF-670462 at ZT11 induces a constant stable delay under 12:12 LD, but not under DD, matching our experimental data (**Figure 2**). The experimental data of **a,e**, and **f** were used in estimation of parameters. See Methods for details of parameter estimation. DD, dark-dark cycle; LD, light-dark cycle; PK/PD, pharmacokinetics/pharmacodynamics; PRC, phase response curve; SCN, suprachiasmatic nuclei.

LD chronic dosing with 32 mg/kg of PF-670462 can induce a constant stable phase delay regardless of dosing timing. Based on our experiments showing that LD chronic dosing at ZT11 caused a constant stable phase delay (**Figure 2c**),¹⁶ one might assume that LD chronic dosing at other times might also lead to constant stable phase delays. However, the model surprisingly predicted that this was not the case. In fact, our model predicts that stable entrainment does not occur, i.e., no stable relationship between the LD cycle and circadian phase is achieved, when the inhibitor is applied during the early night (**Figure 5a**). This contrasts with late night dosing which is predicted to produce minimal phase shifts. The model also predicts that stable entrainment can occur during daytime dosing, however, the magnitude of the phase delay is predicted to vary greatly depending on the dosing

timing (**Figure 5a**). Thus, we predict that dosing timing must be very carefully controlled to achieve a desired phase delay.

Since the timing of exact daily dosing is predicted to have a significant effect on the ability of the circadian clock to entrain to a LD cycle, we next wondered whether the accuracy of the dosing timing was also an important factor. To explore this, we simulated 32 mg/kg chronic dosing that varies somewhat but centers around either ZT2, ZT5, ZT8 or ZT11 under a 12:12 LD cycle (**Figure 5b**). While entrainment could still be seen in the presence of variations in dosing timing, the phase of the circadian clock was, as expected, less controlled particularly near ZT11 (**Figure 5b**). In fact, the variability in dosing timing often prevented entrainment from occurring at ZT11 (**Figure 5b**). These results indicate that LD chronic dosing in the morning or early afternoon is

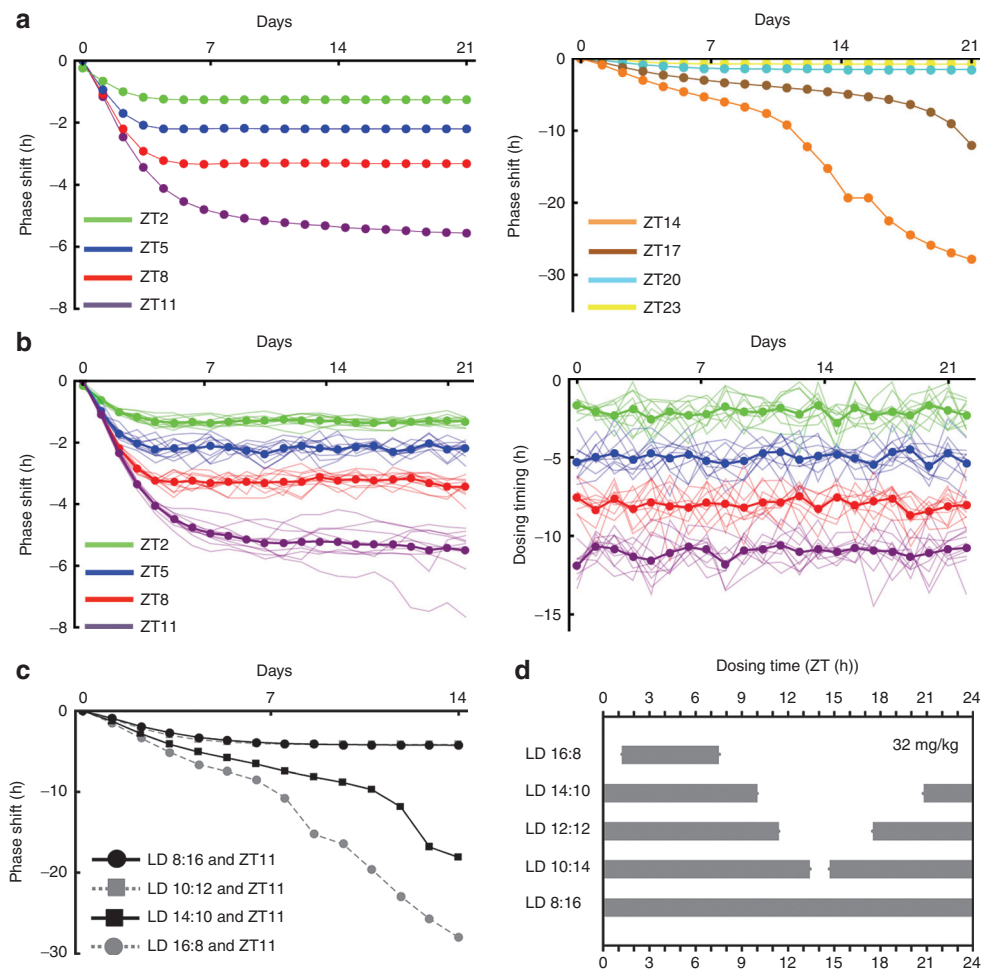


Figure 5 Simulations of chronic dosing under various conditions: (a) different dosing timings, (b) less-controlled dosing timings, and (c) different day lengths. (a) A total of 32 mg/kg chronic dosing under 12:12 LD with different dosing timings were simulated. The model predicts that the chronic dosing in the morning, but not at early night induces a constant stable phase delay. (b) The same simulations of Figure 5a for day dosing were performed in the presence of perturbations in dosing timing (left panel). Here, the dotted lines represent the average of 10 individual simulations (thin lines). Dosing timings were perturbed daily by choosing dosing timing from normal distribution with mean of a original dosing timing and SD of 1 h. The perturbations of dosing timings are illustrated in the right panel: thin lines are perturbed dosing timings and dotted lines are the average dosing timing of 10 individual simulations. (c) A total of 32 mg/kg chronic dosing at ZT11 under various day lengths were simulated. The model predicts that the chronic dosing under short days, but not long days induces a constant stable delay. (d) The simulated ranges of dosing timings that induce a stable entrainment for 3 weeks dosing (32 mg/kg) are represented as gray bars under various day length conditions. DD, dark-dark cycle; LD, light-dark cycle.

more likely to induce a stable and robust phase delay in the presence of a less-controlled dosing schedule.

Finally, the effect of the seasonal change of day length on chronic dosing was explored. Since light opposes the phase delays induced by chronic dosing and produces the stable entrainment (Figures 2c and 5a), we initially expected that increasing the amount of available light (e.g., to a 16:8 LD cycle) would allow for entrainment over a wider range of dosing times. However, our simulations showed the opposite results: as the light duration lengthens, entrainment is less likely to occur, and was lost for ZT11 dosing (Figure 5c). The ranges of dosing timings that induce the stable entrainment become narrower as day length increases (Figure 5d). These results indicate that dosing schedules should be adjusted according to the short and long day lengths, corresponding to winter and summer, to ensure stable entrainment.

Understanding complex behavior of LD chronic dosing via PRC analysis

We have shown that the interaction of two zeitgebers, light and dose of PF-670462 can lead to complex behavioral patterns, which depend on dosing amount, dosing timing, and day length (Figure 5). With a fixed relationship between the lighting schedule and the dosing timing, we can consider the light and dosing as part of one combined signal to shift the circadian clock. The effect of this combined signal can be understood through PRC analysis. As an example, we can consider dosing at ZT5 under a 12:12 LD cycle as a combined signal by a 12-h 300 lux light stimulus and subsequent CK1δ/ε inhibition occurring 5 h after light onset. To measure phase shifts induced by the combined signal at different phases, we simulated a PRC to the combined signal (Figure 6a). If the 12-h light

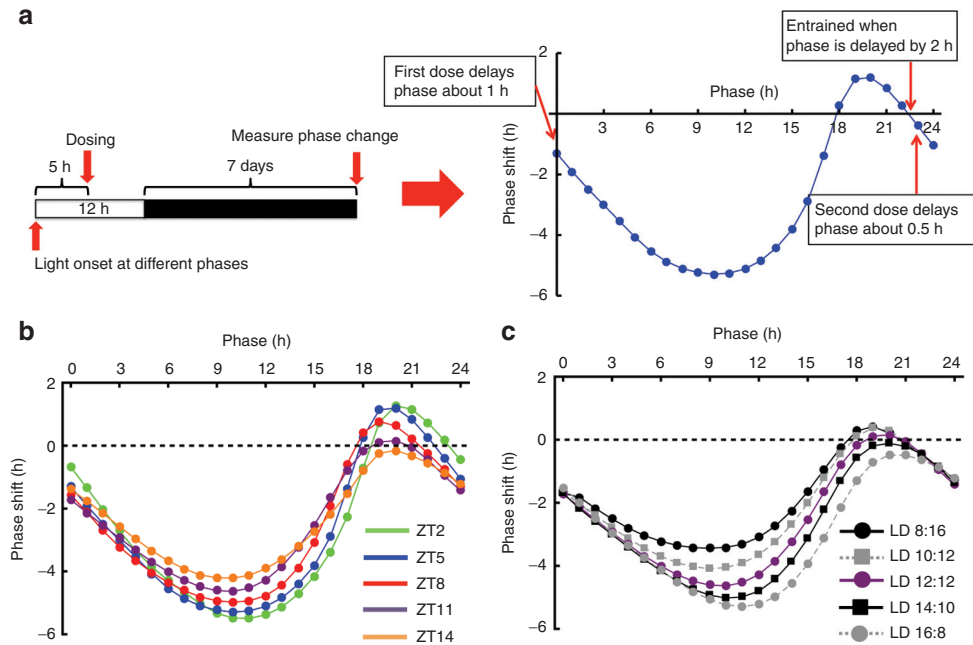


Figure 6 The PRCs to the combined signal of light and PF-670462 explain complex behaviors of LD chronic dosing. (a) The PRC to the combined signal of light and 32 mg/kg dosing of PF-670462 at ZT5 is simulated. The location of zeros of the PRC predicts that LD chronic dosing at ZT5 will induce about 2-h delay. (b) The PRC to the combined signal of 12-h light and 32 mg/kg dose of PF-670462 at different timings were simulated. The zeros of each PRC predict the magnitudes of delay induced by the LD chronic dosing. In particular, the PRC with ZT14 dosing does not take the value of zero, indicating that LD chronic dosing at ZT14 cannot yield a constant stable delay. (c) The PRCs to light with different durations (8, 10, 14, and 16 h) and 32 mg/kg dosing at ZT11 were simulated. As light durations increase, PRCs move down. Finally, the PRCs to light with 14- or 16-h duration do not have zeros, which explains why LD chronic dosing did not yield a constant stable delay under long days. DD, dark-dark cycle; LD, light-dark cycle; PRC, phase response curve.

pulse begins at circadian phase 0 (CT0), with subsequent dosing 5 h later, the PRC predicts a 1-h delay in phase due to the combined signal (**Figure 6a**). Thus, on the second day, the light onset begins at CT23. The PRC at CT23 predicts a phase delay of about 0.5 h after the second day of dosing. After several days, light onset begins near CT22, where the PRC is zero, no more phase shifting occurs, and entrainment is reached. This matches our ZT5 chronic-dosing simulation (**Figure 5a**).

Similar arguments can be used to predict the combined signal at different phase relationships between the dosing time (ZT2, ZT5, ZT8, ZT11, and ZT14) and LD cycle (**Figure 6b**). All of these PRCs correctly predicted whether the constant stable delays of the circadian rhythms occur and if a constant stable delay occurs, to what extent the phase is delayed. That is, the zero crossing of the PRCs correctly predicts the extent of phase delay with LD chronic dosing (**Figures 5a** and **6b**). Moreover, the fact that the PRC with ZT14 dosing never takes the value of 0 explains why stable entrainment would not occur with LD chronic dosing at ZT14 (**Figures 5a** and **6b**).

Next, to understand the effect of day length on chronic dosing, we simulated PRCs to a 300 lux light pulse with different durations and the subsequent 32 mg/kg PF-670462 dosing 11 h after light onset (ZT11). Interestingly, as light duration lengthens, the PRCs move downward and the PRCs to long light duration did not have zero crossings (**Figure 6c**). This explains why the ZT11 chronic dosing was not able to yield a stable entrainment during long days (**Figure 5c**).

The analysis of combined PRCs can also be used to predict the effect of free-running period from 24 h. If free-running period is longer than 24 h, the phase would be delayed every day, and this difference in period from 24 h must be made up by phase shifts from light and the inhibitor to achieve entrainment. We can incorporate this into our combined PRC analysis by subtracting the difference in free-running period from 24 h at all points. Due to the subtraction, the PRCs have less of a chance to have zeros, so stable entrainment is less likely to occur as the free-running period becomes longer, which matches simulations of the model (**Supplementary Figure S4** online).

Estimation of the combined PRCs from two separate PRCs

We have shown that PRCs to a combined stimulus of light and PF-670462 dosing can be used to understand and predict phase changes caused by LD chronic dosing (**Figure 6**). However, experimentally measuring these combined PRCs for all combinations of different dosing timing and light duration require a tremendous amount of experimental work. Thus, we were curious whether the combined PRCs could be estimated from the already measured individual PRCs to light and PF-670462, which were successfully reproduced by our model (**Figure 4e**).^{16,34}

To estimate the combined PRCs to a 12-h light pulse and ZT5 dosing, we added the PRC to 12-h light and a 5-h translated PRC to 32 mg/kg PF-670462 (**Figure 7a**). For example, the phase response to light at ZT0 and the phase response to the PF-670462 at CT5 are added. The summation of these

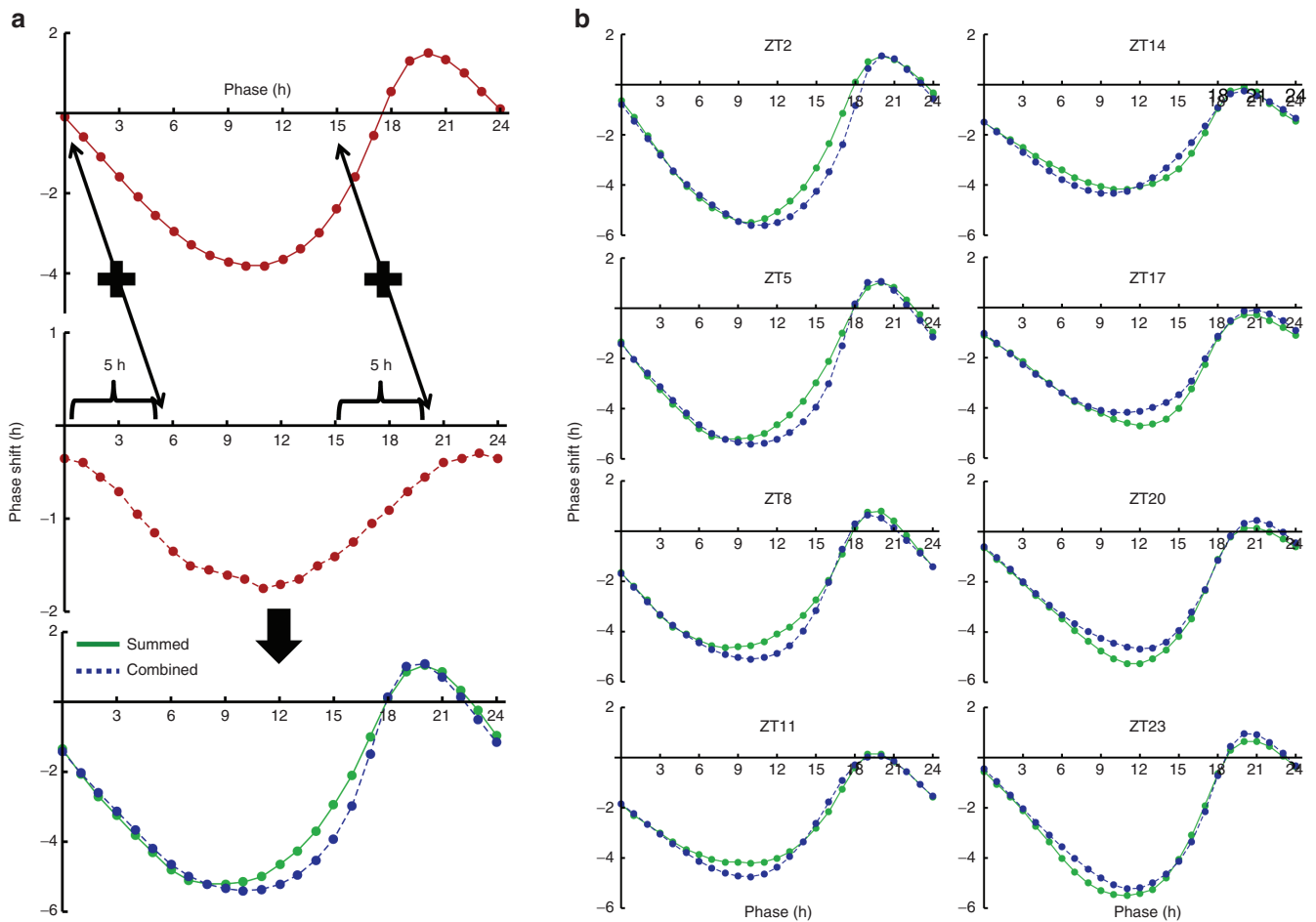


Figure 7 The PRC to the combined signal of light and PF-670462 can be predicted from the two individual PRCs to light and PF-670462. (a) To estimate the PRC to the combined signal of light and ZT5 dosing, we need to sum the effect of light and the effect of dosing 5 h after light onset. This can be estimated by summing the PRC to light with the shifted PRC to PF-670462 by 5 h. (b) The summed PRC and PRC to the combined signal give similar predictions for all dosing timings. In particular, zeros of PRCs to the combined signal are correctly predicted in the summed PRCs. DD, dark-dark cycle; LD, light-dark cycle; PRC, phase response curve.

two PRCs well matches the combined PRC with ZT5 dosing (Figure 7a). In a similar way, we added the light PRC and the PF-670462 PRC with time translations of 2, 8, 11, 14, 17, 20, and 23h to estimate the combined PRCs to ZT2, ZT8, ZT11, ZT14, ZT17, ZT20, and ZT23 dosing, respectively (Figure 7b). The summed PRCs also nicely match with the combined PRCs and, in particular, correctly predict the zero crossings of combined PRCs. Thus, we can understand and predict LD chronic dosing-induced phase change by measuring the separate light PRC and PF-670462 PRC. For instance, as light duration lengthens, the advance region of light PRC of mice becomes smaller,³⁴ which indicates that the summation of light PRCs and PF-670462 PRCs is more likely to be all delay or zeros of PRC is less likely to exist. This explains why stable entrainment was less likely to occur during chronic dosing as light duration lengthens (Figure 5c).

DISCUSSION

We have employed gene expression quantitation, behavioral measures, and mathematical modeling together to study how the phases of circadian rhythms are modulated

by two zeitgebers, the LD cycle, and pharmacological CK1 δ/ϵ inhibition. By using acute inhibition of CK1 δ/ϵ under a LD cycle, we have shown a direct and immediate relationship between the delay of circadian gene expression and circadian behavior (Figure 3). Our mathematical intracellular model also successfully reproduced the altered behaviors associated with CK1 δ/ϵ inhibition in LD cycles (Figure 4).

The inhibition of CK1 δ/ϵ over several days increased the phase shift in behavior. This behavioral shift is attenuated when the animal is maintained in LD (Figure 1). The counterbalancing of the delay by a LD environment is particularly dramatic when it occurs over multiple weeks. While chronic dosing of PF-670462 at ZT11 under DD cycle yielded a continuously accumulating delay, those under LD cycle induced a constant stable behavior shift in a dose-dependent manner, consistent with previous studies (Figure 2).^{18,25} The effect suggests that LD chronic dosing can be used to treat circadian rhythm sleep disorders, in particular advanced sleep phase disorder which is characterized by a several hour earlier sleep schedule than what is desired. Currently, treatment of advanced sleep phase disorder involves timed exposure to bright light in the evening

(chronotherapy) or dosing of melatonin in the morning, each of which have their own limitations due to the lengthy process and sedating effect, respectively.^{13,35–37} These issues may be addressed by the LD chronic dosing of PF-670462 (**Figure 2**). Although there are side-effect concerns since CK1 δ/ϵ are key regulators of diverse cellular growth and survival processes,³⁸ a recent study showed that PF-670462 has only a modest effect on cell proliferation.³⁹

To reliably use the LD chronic dosing of PF-670462 to modulate the phase of circadian rhythms, we explored the effect of the chronic dosing under various conditions. With an accurate and detailed mathematical model of mammalian circadian clock,²⁴ we studied how dosing amount, dosing timing, and day length affect the chronic dosing-induced phase delay (**Figure 5**). Since chronic dosing at ZT11 under a 12:12 LD cycle showed a constant stable delay (**Figure 2**), dosing at other times was also expected to yield a constant stable delay since acute dosing at ZT11 induces the largest delay (**Figure 4e**).¹⁶ Moreover, considering the counterbalancing effects of PF-670462 and light on phase, a constant stable delay is expected as the day length becomes longer (**Figure 1**). However, simulations predict that the stable entrainment is less likely to occur when PF-670462 is dosed at early night and when day length becomes longer (**Figure 5a,c**). Furthermore, simulations predict that stable entrainment is less likely to occur as free-running period becomes longer (**Supplementary Figure S4** online).

We found that these complex patterns can be understood by analyzing PRCs to a combination of light pulse and dosing of PF-670462. That is, the occurrence of a constant stable delay during LD chronic dosing depends on the existence of zeros of the combined PRC and to what extent phase delay can be estimated from the position of zeros (**Figure 6**). Moreover, we showed that these combined PRCs are successfully approximated by simple summation of a light PRC and a translated PF-670462 PRC (**Figure 7**). This result indicates that if we can measure a light PRC and a PF-670462 PRC for other organisms including humans, we can determine the optimized dosing timing and amount of LD chronic dosing to modulate the phase of circadian rhythms. Since the PRCs to light or PF-670462 may vary from species to species,³² the results of LD chronic dosing of PF-670462 may also vary from species to species. Testing whether the results of chronic dosing can be predicted from a PRC to light and a PRC to PF-670462 for other species will be an interesting future work. Similarly PRCs to other circadian drugs could be tested and incorporated into a similar model.

We demonstrate in our model that entrainment or the lack of entrainment is predicted to occur in response to LD chronic dosing. Periodically forced clocks can also show more complex behaviors including chaos.⁴⁰ It would be an interesting future work to find whether these complex behaviors can be seen in the model or in experimental data.

Many studies have shown that bipolar disorders and mood disorders are associated with disrupted circadian rhythms.^{8–10,41} Specifically, phase misalignment is frequently present in mood disorders and the amplitude of circadian rhythms is often reduced.^{11,41,42} In particular, a recent study observed attenuated amplitudes in *Rev-erba* and *DBP* gene expression in fibroblasts of bipolar disorder patients.⁴³

Interestingly, our study showed that dosing of PF-670462 increases the amplitude of these genes' expression under a LD cycle (**Figure 3a**). This suggests that the chronic dosing of PF-670462 under a LD cycle could be a potential way to treat mood disorders on the basis of modulating the phase stably and increasing the amplitude of circadian rhythms. Indeed, recent studies showed that the chronic inhibition of CK1 δ/ϵ rescues pathological behaviors in animal models of mania and alcoholism.^{44,45} Future work should test whether the chronic dosing of PF-670462 can treat these and other models of mood disorders.^{46–50}

METHODS

Modeling studies

Estimation of parameters. The values of newly added parameters are estimated by using SA method in two steps.²⁹ In the first round, SA found 10's of parameter sets that simulate time course of PF-670462 in plasma and brain, matching experimental data (**Figure 4a**). Among these parameter sets, about 10 parameter sets also generated all delayed PRC to 50 mg/kg PF-670462 seen in the experimental data (**Figure 4e**).¹⁶ We used these parameter sets as initial parameter sets for the second round of SA. In the second round, SA found a final parameter set (**Supplementary Table S5** online) that matches the chronic-dosing experimental data (**Figures 2** and **4f**) as well as generates all delayed PRC to 50 mg/kg PF-670462 (**Figure 4e**) and pharmacokinetic data of PF-670462 (**Figure 4a**). During SA, we did not fit simulated PRC to the experimentally measured PRC of rats since the original model is based on experimental data of mice. Nevertheless, the simulated PRC with the final parameter set is a good fit with the measured PRC of rats (**Figure 4e**). This indicates that mice and rats may have similar PRCs to PF-670462.

Simulation. All the simulation and parameter search were done with 150×8 Ghz CPU using MATHEMATICA 8.0 (Wolfram Research, Champaign, IL). The code of the model is available in MATHEMATICA format from the ModelDB (Access code: 148320).

Model description. Details can be found in **Supplementary Methods** online, **Supplementary Tables S5–7** online, and **Supplementary Figure S2** online.

Experimental studies. Details can be found in the **Supplementary Data** online and **Supplementary Table s8** online.

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C.C. performed the research; J.K.K., D.B.F., M.M., D.W., A.D., T.W., C.C., and K.M.W. analyzed the data; J.K.K., D.B.F., C.C., and K.M.W. wrote the manuscript; J.K.K., D.B.F., M.M., D.W., A.D., T.W., C.C., and K.M.W. contributed new reagents/analytical tools.

Conflict of interest. M.M., A.D., T.W., and C.C. are employees of Pfizer, Inc. The other authors declared no conflict of interest.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON TOPIC?

- ✓ Both PF-670462 and light can change the phase of circadian rhythms in their own way; however, the combined effect of these two signals on circadian rhythms is not clearly understood.

WHAT QUESTION THIS STUDY ADDRESSED?

- ✓ Here, we study how to pharmacologically manipulate circadian phase in the presence of the real world signals that also set circadian phase.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

- ✓ This study proposes a systems pharmacology model that accurately predicts the effects of PF-670462 and light.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS

- ✓ We find that the effects of pharmacological manipulation of circadian phase are highly dependent on the environmental lighting conditions. These effects could be predicted by using phase response curves and a systems pharmacology model, which can guide clinical dosing regimens of PF-670462.

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