Selective Inhibition of Casein Kinase 1ε Minimally Alters Circadian Clock Period

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ABSTRACT

The circadian clock links our daily cycles of sleep and activity to the external environment. Deregelation of the clock is implicated in a number of human disorders, including depression, seasonal affective disorder, and metabolic diseases. Casein kinase 1 epsilon (CK1ε) and casein kinase 1 delta (CK1δ) are closely related Ser-Thr protein kinases that serve as key clock regulators as demonstrated by mammalian mutations in each that dramatically alter the circadian period. Therefore, inhibitors of CK1ε may have utility in treating circadian disorders. Although we previously demonstrated that a pan-CK1 inhibitor, 3-(3-chloro-phenoxymethyl)-1-(tetrahydro-pyran-4-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (PF-4800567), a novel and potent inhibitor of CK1ε (IC50 = 32 nM) with greater than 20-fold selectivity over CK1δ. PF-4800567 completely blocks CK1ε-mediated PER3 nuclear localization and PER2 degradation. In cycling Rat1 fibroblasts and a mouse model of circadian rhythm, however, PF-4800567 has only a minimal effect on the cycling outputs of the suprachiasmatic nucleus, not fully identified, directly by light via the retinohypothalamic tract. The cycling outputs of the suprachiasmatic nucleus, not fully identified, regulate multiple downstream rhythms, such as those in sleep and awakening, body temperature, and hormone secretion (Schibler et al., 2003; Ko and Takahashi, 2006). As anyone who has experienced jet lag knows, misalignment of the internal clock with the external environment profoundly affects well being. Furthermore, diseases, such as depression, seasonal affective disorder, and meta...
bolic disorders, may have a circadian origin (Barnard and Nolan, 2008; Green et al., 2008).

The Drosophila kinase double time (Dbt) was among the earliest proteins identified as having a molecular role in the clock, with point mutants producing either short or long daily cycles (Kloss et al., 1998; Price et al., 1998). Soon after the identification of Dbt, its mammalian homolog, CK1ε, was identified as mediating the phenotype of the hamster with the τ mutation (Ralph and Menaker, 1988; Lowrey et al., 2000). Although a normal hamster has a 24-h period, i.e., the time from the beginning of one active cycle to the next, the τ hamster has a markedly short period of 20 h. This phenotype caused by a point mutation in the gene for CK1ε indicates that the role of this kinase in the circadian clock is conserved across evolution. Subsequently, a mutation in CK1δ, a kinase highly related to CK1ε, was found to mediate a human genetic trait surprisingly similar to the hamster τ phenotype (Xu et al., 2005). This trait, familial advanced sleep phase disorder, was found to be inherited as a dominant trait with incomplete penetrance (Green et al., 2008).

suggests that CK1ε/δ phosphorylates several proteins, including PER1–3 (Vielhaber et al., 2005). This results in decreased expression of the circa- oscillator. Thus, in the presence of a fully functioning CK1/o, the circadian cycle begins anew. Until recently, unique roles for CK1ε and CK1δ in the circadian clock were unclear. However, recent investigations with a CK1ε knockout mouse indicate the clock runs well without the kinase (Meng et al., 2008a). Although this finding suggests that the kinases may be redundant, the potential exists for compensation by CK1δ in an animal that never expressed CK1ε. An alternative to address this issue is acute, pharmacological inhibition. Several previously identified CK1δ/ε inhibitors, however, have only modest potency and cell penetration, and none exhibits sufficient selectivity to be useful in identifying identifying functions (Badura et al., 2007). This is not surprising because the kinases are 85% similar overall, with 98% similarity in the kinase domain (Knippschild et al., 2005). However, we now report the development of a selective CK1ε inhibitor, PF-4800567. Our studies demonstrate that acute CK1ε inhibition has little effect on circadian period, whereas a pan-CK1δ/ε inhibitor dramatically delays the clock. Thus, in the presence of a fully functioning CK1δ, CK1ε activity is superfluous for establishing the period of the oscillator.

Materials and Methods

CK1δ/ε Cloning and Purification. Full-length human CK1δ isoform 1 gene (accession number NP_001884A), mammalian codon optimized, was synthesized by DNA2.0 (Menlo Park, CA) and cloned in-frame into pcDNA/HisA (Invitrogen, Carlsbad, CA) at the PstI and ApaI sites. This expressed residues 2 to 415 of the full-length CK1δ protein.

A COOH-terminally truncated kinase domain construct of human CK1ε isoform, including residues 1 to 317, was subcloned into the PET15 vector (Novagen, Madison, WI) containing an NH2-terminal Hisa tag and thrombin cleavage site and was recombinantly expressed in Escherichia coli at 20°C. The resulting cell pellet was solubilized at 4 mL/g cells in a lysis buffer consisting of 100 mM Tris, pH 7.5, 0.5 M NaCl, 2 mM TCEP, EDTA-free protease inhibitors (Roche Applied Science, Indianapolis, IN), 1 mM phenylmethylsulfonyl fluoride, and 10 mM imidazole and lysed with a single pass through a prechilled microfluidizer at 18,000 p.s.i. The lysate was clarified by centrifugation and loaded at 2 mL/min onto a 5-mL Hi Trap Ni2+-Sepharose Fast-Flow column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) that was pre-equilibrated with a wash buffer consisting of 50 mM Tris-HCl, 0.5 M NaCl, 1 mM TCEP, and 100 mM imidazole, pH 7.8. The column was washed with ~50 column volumes of wash buffer and then eluted with a 15-column-volume gradient of wash buffer containing 500 mM imidazole. The fractions containing CK1δ based on SDS-polyacrylamide gel electrophoresis were pooled and then dialyzed into a size exclusion chromatography buffer consisting of 30 mM Tris-HCl, 0.3 M NaCl, 5 mM β-mercaptoethanol, 1 mM TCEP, and 1 mM EDTA, pH 7.8. The dialyzed fractions were then treated with 1 μl of bovine pancreatic high-activity thrombin (Haematologic Technologies, Inc., Essex Junction, VT) for 2 h at room temperature or overnight at 4°C. The cleavage reaction was halted by slow addition of a 200 mM stock solution of phenylmethylsulfonyl fluoride to a final concentration of 1 mM. The sample was concentrated, loaded onto a S200 2660 column equilibrated in size exclusion chromatography buffer and purified at a flow rate of 1 mL/min. Finally, the size exclusion chromatography fractions containing CK1δ based on SDS-polyacrylamide gel electrophoresis analysis were pooled, concentrated to ~1 mg/mL, aliquoted, and flash-cooled in liquid nitrogen for storage at −80°C. All purification steps were conducted at 4°C unless otherwise noted. CK1δ cloning and purification has been described previously (Badura et al., 2007).

Kinase Assays. The CK1ε and CK1δ kinase assays were performed in a 20-μl volume in buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM DTT, 10 μM ATP, and 42 μM peptide substrate, PLSRETTLASLPLG (Flotow et al., 1990). The final enzyme concentrations were 2.5 nM for CK1ε and 2 nM for CK1δ. Assays were run in a panel format in the presence 1 μl of CK1 inhibitor or 5% DMSO. The reactions were incubated for 2 h at room temperature, followed by detection with use of 20 μl of the Kinase Glo Plus Assay reagent (Promega, Madison, WI) according to the manufacturer’s instructions. Luminescence was measured by using Enhanced Lum detection on an Envision plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Nuclear Translocation. CK1ε- and CK1δ-dependent PER3 nuclear translocation was run as described previously for CK1ε (Badura et al., 2007), with alterations as indicated below. CK1δ-dependent PER3 nuclear translocation was performed with human full-length CK1δ-pcDNA4/HisA cotransfected into COS-7 cells with the GFP-tagged murine PER3. The sensitivity of the assay was increased by incorporating an anti-GFP antibody-staining step. After the plates were fixed with 4% paraformaldehyde and washed in PBS, cells were blocked and permeabilized in PBS with 4% goat serum and 0.1% Triton X-100 for 1 h at room temperature. Blocking buffer was then replaced with a polyclonal anti-GFP antibody (Alexis), diluted 1:1000 in blocking buffer, and incubated for 2 h. After three washes in PBS, goat anti-rabbit Alexa Fluor 488 secondary antibody was added and incubated for 1 h at room temperature. Cells were washed again and fluorescent images were acquired on a Bio-Rad Radiance 2000 laser scanning confocal microscope.
Protein Degradation. Real-time monitoring of PER2::YFP degradation in COS-7 cells was performed essentially as described previously (Meng et al., 2008a). Cells cotransfected with PER2::YFP and CK1ε expression plasmids were treated first with compound and subsequently (1 h later) with 20 μg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO). Fluorescence recording was initiated 30 min later, at 10-min intervals, for 8 h by time-lapse microscopy using Openlab software (Improvision, Warwick, UK). Single-cell fluorescence data were analyzed by IPLab software (BD Biosciences Bioreactor, Rockville, MD), and background was corrected. Overall, 25 to 30 separate cells in three independent experiments were measured for each experimental condition. Normalization, one-phase exponential decay curve fitting and statistics were performed in Prism (GraphPad Software, San Diego, CA). Data were plotted as best-fit decay curves or as calculated degradation rate constants K ± S.E.M.

In Vitro Circadian Bioluminescence Real-Time Recording. To report in real time the transcriptional rhythms of the mouse Per2 gene. Rat1 cells were stably transfected with a mPer2::luc expression plasmid as described previously (Meng et al., 2008b). Confluent Rat1 stable cells in 35-mm dishes were synchronized by treatment with 200 nM dexamethasone (Sigma-Aldrich, St. Louis, MO). Fluorescence recording was initiated 30 min later, at 10-min intervals, for 8 h by time-lapse microscopy using Openlab software (Improvision, Warwick, UK). Single-cell fluorescence data were analyzed by IPLab software (BD Biosciences Bioreactor, Rockville, MD), and background was corrected. Overall, 25 to 30 separate cells in three independent experiments were measured for each experimental condition. Normalization, one-phase exponential decay curve fitting and statistics were performed in Prism (GraphPad Software, San Diego, CA). Data were plotted as best-fit decay curves or as calculated degradation rate constants K ± S.E.M.

Pharmacokinetic Characterization. The concentration-time profiles of PF-4800567 and PF-670462 in plasma and whole brain were determined in male C57BL/6J mice after administration of a single subcutaneous dose. PF-4800567 was administered at a dose of 100 mg/kg with use of 20% hydroxypropyl β-cyclodextrin as dosing vehicle. PF-670462 was administered at a dose of 32 mg/kg with use of 5:5:90 DMSO/cremophor/20% hydroxypropyl β-cyclodextrin as dosing vehicle. The dosing volume for both compounds was 10 ml/kg. Plasma and whole brains were collected from mice while they were receiving i.s. treatment at 0.5, 1, 2, 4, 8, and 24 h after dosing (n = 4 per time point). Plasma was obtained after centrifugation of whole blood. Whole brains were diluted in a 4× volume (w/v) 60% isopropyl alcohol and homogenized by use of a Mini-Beadbeater-96 (Biospec Products, Bartlesville, OK). Both plasma and brain homogenate samples were quantified with use of a liquid chromatography/mass spectrometry method following protein precipitation. Concentration and pharmacokinetic results are presented as mean ± S.E.M. AUC0-Tlast values were calculated with use of the linear trapezoidal rule.

Protein binding experiments were conducted with mouse plasma and brain homogenate with use of high-throughput 96-well equilibrium dialysis (Banker et al., 2003). Aliquots (n = 6 per matrix) of plasma and brain homogenate spiked with 1 μM compound were dialyzed against an equal volume of buffer for 6 h at 37°C. The dialysis membranes had a molecular cutoff of 12 to 14 kDa (Spectrum Laboratories Inc., Rancho Domínguez, CA). After incubation, donor and receiver samples were transferred to a 96-well block containing an equal volume of the opposite matrix and internal standard. The diluted samples were then analyzed by liquid chromatography/mass spectrometry after protein precipitation. Free fraction in plasma was calculated as the ratio of instrument response between donor and receiver samples. Determination of undiluted brain-free fraction was calculated as described previously (Kalvass and Maurer, 2002).

Synthesis of PF-4800567. The synthesis of PF-4800567 begins with commercially available starting materials, as illustrated in Supplemental Fig. 1. Condensation of 2-benzoyl-β-acetyl chloride with malononitrile using NaN₃ as the base gave 2 in a 75% isolated yield (1.8 kg). Methylation of 2 with trimethylorthofomate in the presence of p-toluene sulfonic acid gave 3 in a 44% isolated yield (500 g). Reaction of 3 with known hydrazine, (tetrahydro-pyran-4-yl)-
from each other (Meng et al., 2008a). This prompted the midine (6) temperature to effect a second ring closure to give the pyrazolopyrimidine (5) in a 80% crude yield (190 g). Reaction of 5 with formamidate at elevated temperature effected a second ring closure to give the pyrazolo[3,4-d]pyrimidin-3-ylmethanol (7) in a 73% crude yield (73 g). Completion of the synthesis was accomplished by reaction of 7 with 3-chlorophenol under Mitsunobu conditions yielding the title compound, 3-(3-chlorophenoxy)methyl)-1-[(tetrahydro-pyran-4-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-y]lamine (PF-4800567), in a 77% isolated yield. See supplemental data for the detailed synthetic scheme.

PF-670462 was synthesized by the Department of Medicinal Chemistry (Pfizer Global Research and Development). Through Pfizer’s Gift program, both PF-670462 and PF-4800567 may be available for those interested in using them as tools to better understand the role of CK1ε and CK1δ in vitro and in vivo systems. If interested, please contact the authors for details on the program.

Results

Selectivity of PF-4800567. Recently published data suggest that the kinases CK1ε and CK1δ may have roles distinct from each other (Meng et al., 2008a). This prompted the initiation of efforts to identify compounds that preferentially inhibited one of these kinases. Through directed chemistry efforts, PF-4800567 (Fig. 1) was identified. When tested in an in vitro assay against purified CK1ε and CK1δ, this compound had an IC50 of 32 nM for CK1ε, whereas its CK1δ IC50 was 711 nM (Fig. 2; Table 1). This revealed a 22-fold greater potency toward CK1ε for both compounds determined to be ATP competitive (data not shown). PF-4800567 was tested against 50 additional kinases at 1 and/or 10 μM (Fig. 3; Table 2). These kinases represent a highly diverse subset of the major branches of the kinome tree. At the lower concentration, PF-4800567 showed significant activity, although less potent, toward only one additional kinase in this panel, epidermal growth factor receptor. The broad nature of this kinase panel suggests that a similar degree of selectivity for CK1ε exists in the rest of the kinome. PF-4800567 has greater selectivity than PF-670462 does among several kinases, including protein kinase A, protein kinase C, p38, and GSK3β.

Inhibition of PER3 Nuclear Translocation. CK1ε and CK1δ have multiple roles in the circadian clock. One of these is to phosphorylate the PER proteins in the cytoplasm, resulting in their translocation into the nucleus. We wanted to test whether the in vitro preference of PF-4800567 for CK1ε would translate to selective inhibition of this kinase in intact cells. Each kinase effectively promotes the nuclear localization of PER3 (Fig. 4, A and B, DMSO control). At the highest concentration of each compound (10 μM), the nuclear localization of the GFP-tagged PER3 by each kinase is effectively blocked, resulting in the absence of nuclear fluorescence (Fig. 4, A and B). For PF-670462, the nuclei are clearly visible down to 0.1 μM; even down to 0.01 μM, the nuclei are more visible than in the DMSO control (Fig. 4B). Likewise, PF-4800567 effectively blocks nuclear translocation mediated by CK1ε down to 0.01 μM (Fig. 4A, top). However, it is less effective against CK1δ at this concentration and, even at 0.1 μM, does not block CK1δ-mediated translocation of PER3 (Fig. 4A, bottom). Quantitation of the relative PER3 nuclear intensity shows that PF-670462 has comparable IC50 values for both kinases, whereas PF-4800567 is over 20-fold more potent for CK1ε (Fig. 4, C and D; Table 1). For both inhibitors, the ratio of the kinase IC50 values against the purified enzymes was essentially the same as the ratio of the kinase IC50 values determined in the whole-cell assay. These data support the translatability of the selectivity data measured with the enzymes to the more complex whole-cell environment.

Inhibition of PER2 Degradation. Another key role for CK1ε in the circadian clock is the phosphorylation-dependent degradation of the PER proteins. Subsequent to the phosphorylation event that occurs in the cytoplasm to induce PER translocation to the nucleus and repression of CLOCK/BMAL1, this second phosphorylation takes place in the nucleus to promote ubiquitin-mediated degradation of PER and relief of the CLOCK/BMAL1 transcriptional repression (Eide et al., 2005). Coexpression of CK1ε with PER2-YFP significantly increases the rate of PER2 degradation by approximately 4-fold (Fig. 5). Both PF-4800567 and PF-670462 can completely block the enhanced PER2 degradation. These
data, along with the nuclear localization studies, show that each of the modulatory roles that CK1ε has on the PER proteins can be potently inhibited by these compounds.

**CK1ε inhibition by PF-4800567 Does Not Alter Circadian Period in Vitro.** With confirmation of the selective inhibition of CK1ε by PF-4800567 and its ability to block the roles of CK1ε in the clock, we then wanted to investigate the effect of CK1ε inhibition on the cycling clock itself. Extensive studies of the circadian clock have been done in Rat1 cells (Ripperger and Schibler, 2006; Meng et al., 2008b). After the quiescence of these cells by serum starvation, addition of dexamethasone initiates a synchronized circadian rhythm. In the cells used here, a stable line expressing PER2-luciferase shows the rhythmic cycling of this signal over several days (Fig. 6, A and B). Addition of the pan-CK1ε inhibitor PF-670462 causes a robust lengthening of period, as shown by the shifting of the peak of activity over several days (Fig. 6B). Under the same conditions, PF-4800567 causes a minimal shift in the cycling of PER2-luciferase, even at the highest doses (Fig. 6A). The period of the cycle in the cells can be calculated be measuring the time peak to peak. The dose-responsive effect on the period shows a dramatic increase by PF-670462 at concentrations as low as 1 μM, but PF-4800567 shows only a minor effect at concentrations up to 30 μM (Fig. 6C). When these data are analyzed as a function of the compound concentration relative to the whole-cell IC50 for each kinase, the ability of the compound to dissect the role of each kinase on the cycling of the circadian clock is highlighted (Fig. 6, D and E). PF-670462 begins to increase the period of the clock at approximately 3- to 10-fold over the whole-cell CK1ε IC50 and approximately 3-fold lower for CK1ε. PF-4800567 parallels this effect on period up to approximately 3-fold the CK1ε IC50, although it falls off at the highest concentration. However, at the doses where PF-4800567 shows a small increase in period, its concentration is nearly 100-fold over its whole-cell CK1ε IC50 (Fig. 6D). These

### TABLE 2

Selectivity comparison between PF-4800567 and PF-670462

Data shown are the percentage of inhibition at the indicated concentration of each inhibitor.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>PF-4800567 (% inhibition at 1 μM)</th>
<th>PF-670462 (% inhibition at 1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK1γ2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>GSK3β</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>p38 cascade</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>MLCK</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>PRKC B2</td>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>PKAα</td>
<td>1</td>
<td>78</td>
</tr>
<tr>
<td>EphA2</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>SRC</td>
<td>9.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>11</td>
<td>55</td>
</tr>
<tr>
<td>LGK</td>
<td>32</td>
<td>57</td>
</tr>
<tr>
<td>HGK</td>
<td>20</td>
<td>75</td>
</tr>
<tr>
<td>EGFR</td>
<td>69</td>
<td>76</td>
</tr>
</tbody>
</table>

**CK1γ2, casein kinase 1γ2; GSK3β, glycogen synthase kinase 3β; p38 cascade, p38α coupled to MAPKAPK2; MLCK, myosin light-chain kinase; PRKC B2, protein kinase C B2; PKAα, protein kinase A catalytic subunit α; VEGFR2, vascular endothelial growth factor receptor 2 tyrosine kinase; EGFR, epidermal growth factor receptor tyrosine kinase; N.D., not determined; LGK, lymphocyte-specific protein tyrosine kinase; HGK, HPR/GCK-like kinase.**
data indicate that, in this circadian model, CK1ε inhibition alone does not drive changes in the period of the circadian clock.

Characterization of PF-4800567 as an in Vivo Tool.

To use PF-4800567 to test the role of CK1ε in the clock, its in vivo exposure must be sufficient to inhibit the kinase in the brain. To assess this, we determined in C57BL/6J mice the pharmacokinetics of this compound after a single 100 mg/kg subcutaneous dose and compared it with the pan-CK1ε/H9254ε inhibitor PF-670462 dosed at 32 mg/kg (Fig. 7; Table 3). The $T_{\text{max}}$ for both compounds in plasma and brain were observed at the first time point (0.5 h), indicating rapid absorption and distribution. The brain-to-plasma ratios, in general, were constant throughout the 24-h time course, with an average value of 2.1 and 1.3 for PF-4800567 and PF-670462, respectively. Conversion of the time course data to reflect free-tissue concentrations showed that free brain $C_{\text{max}}$ of PF-4800567 was nearly 3-fold over the whole-cell CK1ε $IC_{50}$ (Fig. 7; Table 4). For CK1ε, however, the free $C_{\text{max}}$ in the brain is below its whole-cell CK1ε $IC_{50}$, reaching only 0.2-fold. In contrast, PF-670462 was 3-fold above its CK1ε whole-cell $IC_{50}$, the same level as PF-4800567 was for CK1ε, along with a 0.7-fold CK1ε whole-cell $IC_{50}$. This indicates that in vivo, at the doses tested, PF-670462 should inhibit most of the CK1ε and CK1ε activity, whereas PF-4800567 should only reach levels to selectively inhibit CK1ε. Similar studies at both lower and higher doses indicate that the central and peripheral exposure is dose-related for both compounds and that for PF-4800567, the higher dose would significantly inhibit CK1ε and thereby lose the selective nature of the compound (data not shown). At the 24-h time point, PF-4800567 is undetectable in the brain, and free compound in the plasma is less than 1 nM. Therefore, no significant accumulation of the compound would be expected with daily dosing of 100 mg/kg. Likewise, no significant accumulation of PF-670462 dosed at 32 mg/kg is expected with daily dosing (Badura et al., 2007; data not shown).

Selective Inhibition of CK1ε Has Little Effect on Circadian Timing in Vivo.

Confirmation that PF-4800567 and PF-670462 can be dosed in mice to similar multiples of their whole-cell CK1ε and CK1ε $IC_{50}$s, respectively, allowed us to test the effects of this inhibition on in vivo circadian rhythm. Mice were kept in a controlled 12:12 LD (light and dark cycles of 12 h each) environment for several weeks to ensure that their circadian rhythms were synchronized. They were subsequently shifted into DD (24 h, dark) to isolate them from the entraining effects light has on circadian rhythm, thereby providing the greatest sensitivity for the effects of the compounds on the endogenous circadian rhythm. After
the first 24-h shift into DD, the mice were dosed daily for 3 days with either 100 mg/kg PF-4800567 or 32 mg/kg PF-670462 and their respective vehicles at CT11, i.e., 1 h before the time that the lights had previously been turned off, which is also the beginning of their active period. After the 3rd day of dosing, the mice were maintained in DD to determine the effects of the compounds on their endogenous circadian clock, as indicated by the shift in the start of their active period after dosing versus before dosing. PF-4800567 delayed the active period by only 0.5 h when dosed at 3- and 0.2-fold its whole-cell CK1ε and CK1δ IC_{50}s, respectively (Fig. 8; Table 5). In contrast, PF-670462 delayed the active period by 6.5 h when dosed to 0.7- and 3-fold its whole-cell CK1ε and CK1δ IC_{50}s, respectively. The compound-induced delay of the circadian clock did not have any after-dose effects on the animals’ (period), i.e., the time from the start of one active period to the start of the next active period (Table 5). This suggests that the changes are confined to an acute effect on the clock only when the compounds are present.

Discussion

Phosphorylation of circadian clock proteins is an essential element in controlling the cyclical rhythm of the clock.
Netic analyses of the hamster and the human familial advanced sleep-phase syndrome mutations confirmed CK1ε and CK1δ, respectively, as being able to drive the mammalian clock (Lowrey et al., 2000; Xu et al., 2005). Consistent with the central role of these kinases in circadian rhythm, chronic administration of a potent, dual inhibitor of CK1ε and CK1δ, PF-670462, dramatically slows down the clock in cycling fibroblasts in vitro (Fig. 6). More significantly, acute treatment in vivo induces phase delays in a rodent model of circadian rhythm (Fig. 8) (Badura et al., 2007). These data, however, do not clarify whether CK1ε and CK1δ are redundant or whether one is the primary driver of the mammalian circadian clock. The study presented here demonstrates that selective inhibition of CK1ε by PF-4800567 does not affect the circadian clock in vitro and yields only a small phase shift in vivo. At 3-fold its whole-cell CK1ε IC50, PF-4800567 caused less than one-tenth of the phase shift that was induced by PF-670462 at 3- and 0.7-fold its whole-cell CK1δ and CK1ε IC50s, respectively. It is interesting to note that the large in vivo effect by PF-670462 is at a concentration that has only a very small effect on Rat1 cells in vitro. This may indicate that the in vivo clock is much more sensitive to changes in CK1ε/δ inhibition. If so, the in vivo shift by PF-4800567 may be caused by a small but direct effect on CK1δ added on to its CK1ε inhibition. At the dose tested, PF-4800567 is at 0.2-fold its whole-cell CK1δ IC50. A 0.5-h phase shift via this low-level CK1δ inhibition is consistent with our observations that phase shift responds in a linear fashion to compound concentration (data not shown). Additional support for the minor in vivo activity being the result of CK1δ inhibition is that the kinase panel used to characterize the selectivity of PF-4800567 indicates that it has little activity against most other kinases tested, including other kinases implicated in circadian function, such as GSK3β and ERK2 (Gallego and Virshup, 2007).

Although both CK1ε and CK1δ may need to be inhibited to delay the clock, the effects of PF-670462 and its preferential inhibition of CK1δ over CK1ε suggest that CK1δ is the predominant mediator of clock period. The limited separation between its potency for these kinases, however, makes this claim uncertain. Evaluating the role of CK1δ in the clock has been difficult, because the knockout of this kinase is embryonic.

**TABLE 3**

| Pharmacokinetic parameters of PF-4800567 and PF-670462 after subcutaneous administration to mice |
|--------------------------------------------------|----------------|----------------|----------------|----------------|
| Dose (mg/kg) | PF-4800567 | PF-670462 | PF-4800567 | PF-670462 |
| AUC (ng*h/ml) | 100 | 100 | 11,400 | 11,400 |
| Total Cmax (ng/ml) | 5250 | 5250 | 9250 | 9250 |
| Tmax (h) | 0.5 | 0.5 | 0.5 | 0.5 |
| free Cmax (nM) | 1007 | 1007 | 411 | 411 |

**TABLE 4**

| Comparison of the observed free Cmax concentrations in mice relative to whole-cell CK1δ IC50 values |
|--------------------------------------------------|----------------|----------------|----------------|----------------|
| PF-4800567 and PF-670462 were dosed at 100 and 32 mg/kg, respectively. Values presented are -fold greater than whole-cell IC50. |
|--------------------------------------------------|----------------|----------------|----------------|----------------|
| Dose (mg/kg) | PF-4800567 | PF-670462 | PF-4800567 | PF-670462 |
| Free Plasma | Free | Free | Plasma | Brain |
| CK1δ | 8 | 0.4 | 2 | 0.7 |
| CK1δ | 3 | 0.2 | 7 | 3 |

**Fig. 8.** Selective CK1ε inhibition by PF-4800567 has only minor effects on shifting in vivo phase, whereas preferential CK1δ inhibition by PF-670462 induces a large phase shift. Mice (n = 5) were maintained in 12:12 LD for several weeks to get a baseline of activity. The lights were subsequently turned off, and the mice kept in DD conditions for testing. They were given 100 mg/kg PF-4800567 or 32 mg/kg PF-670462 or the appropriate vehicle for 3 days, once a day. They were maintained another week in DD, and shifts in the start of their active periods after the dose were determined relative to the start predose; this is defined as the phase shift. A, quantitation of the phase shifts. B, representative temperature analyses shown as double-plotted actograms from each of the treatment groups. Asterisks indicate time of dosing. By convention, an advance in the active period is positive, and a delay is negative.
with the CRYs inhibit transcription of CLOCK/BMAL1-in
2) In the nucleus, the phosphorylated PERs in combination
phosphorylation is required for PER translocation to the nucleus.
most extensively and has several specific effects. 1) Phos-
CK1 also phosphorylates BMAL1, although
ment of additional pharmacologic or genetic tools.
controller? Answering this question requires the develop-
in the absence of CK1 ε/H9254 itself, the question remains whether it is also true for CK1 ε/H9254
selective inhibition of CK1 ε/H9254

Both CK1ε and CK1δ phosphorylate the PER and CRY proteins, and CK1ε also phosphorylates BMAL1, although CK1δ has yet to be tested against BMAL1. Among these clock pathway substrates, PER phosphorylation is studied the most extensively and has several specific effects. 1) Phosphorylation is required for PER translocation to the nucleus. 2) In the nucleus, the phosphorylated PERs in combination with the CRYs inhibit transcription of CLOCK/BMAL1-induced genes. 3) Further phosphorylation leads to PER degradation and the restart of transcriptional induction by the CLOCK/BMAL1 complex (Vielhaber et al., 2000; Akashi et al., 2002; Eide et al., 2005). In vitro, the lengthening of period by inhibiting both CK1ε and CK1δ with PF-670462 (Fig. 6) is consistent with the slowing of the cycle via chronic inhibition of PER phosphorylation. This would slow down the rate of movement through each step but still allow the cycle to continue. In vivo, the half-lives of the compounds indicate that they would only slow down the cycle for the short time the compound is present in sufficient concentration. This would yield an acute delay in the clock each day, with a cumulative shift over the treatment days. This aligns with the observed effect of PF-670462 (Fig. 8).

The effect of these compounds on the clock is consistent with the characterization of the hamster τ mutation as having a gain-of-function CK1ε phenotype (Gallego et al., 2006; Meng et al., 2008a). If the τ mutation induced shorter periods via a kinase with decreased activity, it would be expected that a CK1ε kinase inhibitor would have the same effect. On the contrary, CK1ε inhibition has little or no effect on period. The lengthening of period by CK1δ/ε inhibition, via decreased PER phosphorylation, aligns with the shortened period of the τ mutation being mediated by increased PER phosphorylation. With regard to the lack of an effect with CK1δ inhibition alone, loss of CK1δ phosphorylation of PER could be compensated simply by the increased presentation of unphosphorylated PER to CK1δ. In the τ mutation, however, compensation of increased PER phosphorylation by CK1δ would require the concomitant decrease of CK1δ activity, and the kinase may not be able to respond in this manner.

Despite the observation that selective inhibition of CK1ε has little effect on setting period, it should not be construed to mean that CK1ε is dispensable in circadian biology. With the central role that phosphorylation plays in the clock (Gallego and Virshup, 2007), there are other aspects of circadian function not tested here in which CK1ε may yet reveal a primary role. A recently reported genetic association between CK1ε and stimulant sensitivity suggests that the kinase may have a significant role in the ability of stimulants to entrain behavior (Falcón and McClung, 2008; Bryant et al., 2009).

The identification of a selective CK1ε inhibitor opens up other key regulatory pathways for exploration into the function of this kinase. For example, CK1ε has a key role in Wnt signaling. The Wnt pathway stimulates changes in gene expression through the induction of β-catenin translocation to the nucleus, leading to effects on proliferation, survival, and cell fate (Price, 2006). Addition of the peptide Wnt to cells activates CK1ε (Swiattek et al., 2004). This results in phosphorylation of Dishevelled and translocation of β-catenin to the nucleus (McKay et al., 2001; Price, 2006). It is unclear whether both CK1δ and CK1ε have a role in the Wnt pathway. It was claimed that the CK1ε τ mutation has a loss of function in this pathway, but no related effects were reported in the τ hamster or τ transgenic mice. It is possible, however, that the loss of activity is compensated by CK1δ. Although the CK1δ inhibitor D4476 can block Wnt signaling, it does not have selectivity between CK1ε and CK1δ (Bryja et al., 2007). PF-4800567 provides a tool to probe for separate roles of these kinases in this pathway.

In general, CK1ε and CK1δ have been treated as interchangeable because of their high degree of sequence identity and the lack of tools to tease apart their unique functions. The development of PF-4800567 as a pharmacological tool that can distinguish between these two kinases now creates an opportunity to explore the differences between these kinases in greater detail.

References


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