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Brief Article

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Structural Basis for the Potent and Selective Inhibition of Casein Kinase 1 Epsilon

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Supporting Information

ABSTRACT: Casein kinase 1 epsilon (CK1ε) and its closest homologue CK1δ are key regulators of diverse cellular processes. We report two crystal structures of PF4800567, a potent and selective inhibitor of CK1ε, bound to the kinase domains of human CK1ε and CK1δ as well as one apo CK1ε crystal structure. These structures provide a molecular basis for the strong and specific inhibitor interactions with CK1ε and suggest clues for further development of CK1δ inhibitors.

INTRODUCTION

The casein kinase 1 (CK1) family of highly conserved serine/threonine protein kinases has six human isoforms α, γ1, γ2, γ3, δ, and ε that are involved in the regulation of diverse cellular processes including Wnt signaling, circadian rhythms, cellular signaling, membrane trafficking, cytoskeleton maintenance, DNA replication, DNA damage, and RNA metabolism.[1] CK1δ and CK1ε (CK1δ/ε), two closely related CK1 isoforms, have recently gained increased attention as potential drug targets. CK1δ/ε phosphorylate certain clock-related proteins as part of a complex arrangement of transcriptional/translational feedback loops that compromise the circadian oscillator in mammals.[2] CK1δ/ε are also two key mediators of the Wnt signaling pathway, and several studies have highlighted their roles in cell survival and cancer progression.[3] Moreover, both CK1δ/ε were suggested to be involved in psychostimulant-induced behaviors by acting on the Darpp-32-PP1 signaling pathway to regulate AMPA receptor phosphorylation in the nucleus accumbens.[4] Thus, inhibition of the kinase activity of CK1δ/ε is a promising strategy for multiple therapies in the treatment of abnormal circadian behavior, cancer, and drug use disorders.

PF670462 (**1**, Figure 1A), a novel and potent small molecule pan-CK1δ/ε inhibitor (IC_{50} = 13−80 nM) with good selectivity over other kinases,[5] was shown to alter CK1δ/ε dependent processes in circadian rhythms in both cultured cells and in animals.[6] Intriguingly, **1** significantly reduces the growth rate of ovarian cancer cells independent of the wnt/β-catenin pathway[7] but only modestly inhibits the proliferation of some other cancer cells despite being a potent inhibitor of Wnt signaling.[8] **1** also blocks methamphetamine/amphetamine induced locomotion and decreases Darpp-32 phosphorylation in mice.[4] These results suggest that CK1δ/ε are important in the regulation of circadian clock, ovarian cancer growth/survival, and sensitivity to drug abuse. However, it remains unclear whether one of the CK1δ/ε kinases has a predominant role because PF670462 is very potent for both CK1δ (IC_{50} = 13 nM) and CK1ε (IC_{50} = 80 nM). A potent and selective small-molecule inhibitor of CK1ε has been recently reported and has proved useful in probing unique roles between CK1δ and CK1ε. PF4800567 (**2**, Figure 1A) had an IC_{50} of 32 nM for CK1ε and displayed greater than 22-fold selectivity over CK1δ (IC_{50} = 711 nM) and even greater selectivity against other kinases.[5b] While **1** causes a significant phase delay in animal models of circadian rhythm, **2** has only a minimal effect on the circadian clock at concentrations substantially over its CK1ε IC_{50}, indicating that CK1ε is not the predominant mediator of circadian timing compared to CK1δ.[5b,9] **2** has a similar growth inhibition effect on ovarian cancer cells as **1**, demonstrating the likely importance of CK1ε in regulating their proliferation, which is also confirmed by shRNA knockdown of CK1ε.[7] Both **2** and CK1ε knockout increase the locomotor stimulant response to methamphetamine whereas **1** has the opposite effect, suggesting that CK1ε and CK1δ are negative and positive regulators respectively of sensitivity to psychostimulants.[10]

Crystal structures of CK1δ in the apo form and in complex with **1** revealed that the side chain of the gatekeeper residue Met82 is rotated toward Pro66 in the complex structure to avoid a steric clash with **1** and engages in strong hydrophobic interactions with **1** instead.[11] The side chain rotation of Met82 is only possible due to the small side chain of Pro66, and only a few kinases (such as CK1ε) have residues with small side chain at the position equivalent to Pro66 in CK1δ, resulting in the high selectivity of **1** over other kinases. However, no crystal structure of CK1ε has been reported, and it is not obvious from the CK1δ crystal structures why **2** is more than 22-fold more selective for CK1ε over CK1δ despite identical ATP binding site and >98% sequence identity between these two kinases (Figure 1B). Herein, we report the crystal structures of human CK1δ and CK1ε in complex with **2** as well as apo CK1ε and describe the structural basis for the inhibitor's potency and selectivity for CK1ε over CK1δ and other kinases, which are further validated by mutagenesis analysis.

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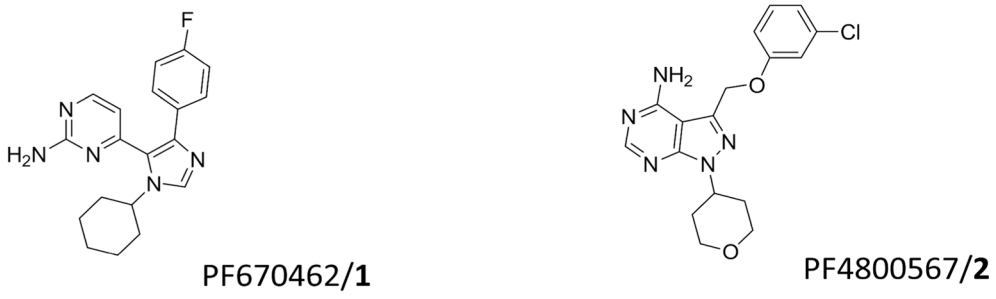

B

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                 10        20        30        40        50        60
                  |         |         |         |         |    *    |
CK1δ    MELRVGNRYRLGRKIGSGSFGDIYLGTDIAAGEEVAIKLECVKTKHPQLHIESKIYKMMQ
CK1ε    MELRVGNKYRLGRKIGSGSFGDIYLGANIASGEEVAIKLECVKTKHPQLHIESKFYKMMQ

                 70        80        90       100       110       120
                  |         |         |         |         |         |
CK1δ    GGVGIPTIRWCGAEGDYNVMVMELLGPSLEDLFNFCSRKFSLKTVLLLADQMISRIEYIH
CK1ε    GGVGIPSIKWCGAEGDYNVMVMELLGPSLEDLFNFCSRKFSLKTVLLLADQMISRIEYIH

                130       140       150       160       170       180
                  |         |         |         |         |         |
CK1δ    SKNFIHRDVKPDNFLMGLGKKGNLVYIIDFGLAKKYRDARTHQHIPYRENKNLTGTARYA
CK1ε    SKNFIHRDVKPDNFLMGLGKKGNLVYIIDFGLAKKYRDARTHQHIPYRENKNLTGTARYA

                190       200       210       220       230       240
                  |         |         |         |         |         |
CK1δ    SINTHLGIEQSRRDDLESLGYVLMYFNLGSLPWQGLKAATKRQKYERISEKKMSTPIEVL
CK1ε    SINTHLGIEQSRRDDLESLGYVLMYFNLGSLPWQGLKAATKRQKYERISEKKMSTPIEVL

                250       260       270       280       290
                  |         |         |         |         |
CK1δ    CKGYPSEFATYLNFCRSLRFDDKPDYSYLRQLFRNLFHRQGFSYDYVFDWNMLK
CK1ε    CKGYPSEFSTYLNFCRSLRFDDKPDYSYLRQLFRNLFHRQGFSYDYVFDWNMLK
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Figure 1. (A) Molecular structure of **1** and **2**. (B) Sequence alignment of CK1δ and CK1ε and kinase domains, with nonidentical residues highlighted.

■ RESULTS AND DISCUSSION

Co-crystallization of dephosphorylated CK1δ kinase domain with **2** yielded crystals that diffracted to 2.07 Å resolution. The two CK1δ/**2** complexes observed in this crystal structure are highly similar (with a backbone rmsd of 0.8 Å). The CK1δ/**2** complex structure (Figure 2A) reveals that **2** is anchored in the ATP binding site of CK1δ by interactions similar to those observed in CK1δ/**1** complexes. The amino pyrimidine group of **2** forms two hydrogen bonds with the carbonyl oxygen of Glu83 and the main chain NH of Leu85 from the interlobe linker (hinge) polypeptide of CK1δ. The *meta*-chlorophenyl moiety of **2** is buried deeply in CK1δ, forming strong hydrophobic interactions with the side chains of Met80 and Met82. The side chain of Met82 is rotated toward Pro66. The interactions of the meta chlorophenyl group of **2** with residues Met82 and Pro66 of CK1δ, similar to those of the para fluorophenyl of **1**, likely account for the high selectivity of **2** over other kinases. However, the structure also shows that the eight residues (amino acid 8, 27, 28, 31, 55, 67, 69, and 249) that are different between CK1δ and CK1ε in the kinase domain are more than 9 Å from **2**, making it difficult to explain the >22-fold selectivity of **2** for CK1ε over CK1δ.

To better understand this selectivity of **2**, we determined the crystal structure of CK1ε kinase domain in complex with **2** at 2.74 Å resolution (Figure 2B). Dephosphorylated CK1ε (1−294) complexed with **2** was cocrystallized in a crystal form that is different from that of CK1δ (1−294) complexed with **2**. The two CK1ε/**2** complexes observed in this crystal structure are highly similar. Reminiscent of the CK1δ/**2** structure, the amino pyrimidine group of **2** forms two hydrogen bonds with the interlobe linker of CK1ε and the *meta*-chlorophenyl moiety of **2** is buried deeply in CK1ε, forming strong hydrophobic interactions with the side chains of Met80 and Met82. The side chain of Met82 is also rotated toward Pro66 in the CK1ε/**2** structure. Strikingly, structural comparison of the CK1ε/**2** and the CK1δ/**2** complexes reveals one obvious difference: the DFG motif of CK1ε in the CK1ε/**2** complex is flipped by ∼180° compared to the DFG motif of CK1δ in the CK1δ/**2** complex and is involved in additional strong hydrophobic interaction with the *meta*-chlorophenyl group. This provides an explanation for why **2** is a much more potent inhibitor for CK1ε than CK1δ. On the other hand, structural superposition of the CK1ε/**2** and the CK1δ/**1** complexes suggests that the side chain of the Phe150 of the "flipped" DFG motif in CK1ε would clash with the imidazole of **1**, and it is likely that the DFG motif of CK1δ adopts the same conformation as that of CK1ε when **1** is bound. This is consistent with **1** being an equally potent inhibitor of CK1ε and CK1δ.

This unique conformation of the DFG motif of CK1ε adopted in the CK1ε/**2** structure was also not observed in the unliganded CK1ε structure determined at 2.77 Å resolution (Figure 2C). The 12 CK1ε molecules observed in this apo crystal structure are very similar to each other, and all the DFG

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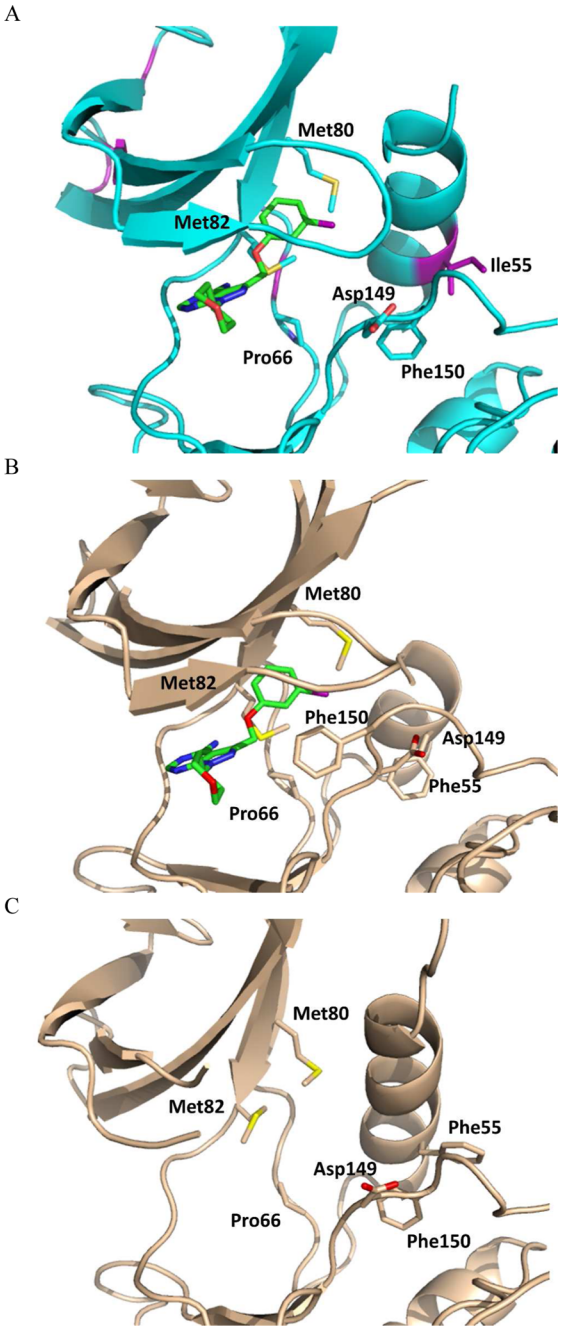

Figure 2. (A) The ATP binding site, showing interactions between **2** and CK1δ residues. For **2**, carbon, nitrogen, and chlorine atoms are shown in green, blue, and purple, respectively. Residues that are different from CK1ε are colored in purple. The DFG motif adopts a "DFG-in" conformation. (B) The ATP binding site, showing interactions between **2** and CK1ε residues. The DFG motif adopts a "DFG-out" conformation. (C) The ATP binding site of apo CK1ε. The DFG motif adopts a "DFG-in" conformation.

motifs adopt a "DFG-in" conformation where the side chain of Phe150 of the DFG motif is buried in a deep hydrophobic pocket formed by the side chains of Tyr56, Met59, Ile115, Ile119, His126, and Ile147. The same "DFG-in" conformation has been adopted by the DFG motif of CK1δ in both apo and complex structures. In comparison, the DFG motif in the CK1ε/**2** complex adopts a "DFG-out" conformation where the Phe150 is flipped out of this hydrophobic pocket to be involved in hydrophobic interactions with the inhibitor and the side chain of Phe55 from the αC helix is rotated into the hydrophobic pocket to maintain similar hydrophobic interactions. The DFG motif and the hydrophobic pocket are completely conserved between CK1ε and CK1δ, but Phe55 of CK1ε is replaced by Ile55 in CK1δ, which could not engage in the same strong interactions with the hydrophobic pocket to stabilize the "DFG-out" conformation of the DFG motif, resulting in much weaker inhibition of CK1δ by **2**.

To evaluate the importance of residue 55 in the inhibition of **2** between CK1ε and CK1δ, we mutated Phe55 to Ile55 in CK1ε and Ile55 to Phe55 in CK1δ and then carried out a differential scanning fluorimetry (DSF) binding assay to monitor whether these mutations altered the interactions with **2** (Table 1). DSF measures the shifts of melting

Table 1. Differential Scanning Fluorimetry (DSF) Binding Studies of 1 and 2 to Wild-Type and Mutant CK1δ/ε

ΔT_m (°C)		**1**	**2**
CK1δ	wild-type	9.6 ± 0.1	4.8 ± 0.1
	I55F	9.6 ± 0.1	13.2 ± 0.1
	T67S	9.6 ± 0.1	4.8 ± 0.1
	R69K	9.6 ± 0.1	4.8 ± 0.1
CK1ε	wild-type	8.6 ± 0.1	11.5 ± 0.1
	F55I	10.4 ± 0.1	6.4 ± 0.1
	S67T	8.6 ± 0.1	11.5 ± 0.1
	K69R	8.6 ± 0.1	11.5 ± 0.1

temperature (T_m) of proteins in the presence of compounds, and it has been previously used for rapid chemical profiling of protein kinases.[12] Wild-type CK1δ was stabilized significantly by **1** (ΔT_m = 9.6 °C) but less so by **2** (ΔT_m = 4.8 °C), in good agreement with the enzymatic data that **1** (IC_{50} = 13 nM) inhibits CK1δ much more strongly than **2** (IC_{50} = 711 nM).[5b] Wild-type CK1ε was stabilized significantly by **1** (ΔT_m = 8.6 °C) and even more by **2** (ΔT_m = 11.5 °C), consistent with the enzymatic data that **2** (IC_{50} = 32 nM) is an even more potent inhibitor than **1** (IC_{50} = 80 nM) for CK1ε.[5b] In sharp contrast, the T_m of CK1ε Ile55Phe was shifted by 9.6 °C in the presence of **1** and by 13.2 °C in the presence of **2**, similar to the wild-type CK1ε, while the T_m of CK1δ Phe55Ile was shifted by 10.4 °C in the presence of **1** and by only 6.4 °C in the presence of **2**, reminiscent of the wild-type CK1δ. In comparison, mutations of other nonconserved residues such as 67 and 69 of CK1ε and CK1δ, which are slightly closer to the inhibitors than residue 55, did not change the stabilization profile at all. These results further support that the difference of residue 55 between CK1ε and CK1δ accounts for the selectivity of **2** for CK1ε over CK1δ. Phe55 is also not conserved in other kinases, and it is not surprising that **2** displayed much greater selectivity for CK1ε over other kinases than **1**.

Our CK1ε/**2** complex structure also suggests routes to further optimize CK1ε inhibitors. As mentioned above, the *meta*-chlorophenyl is involved in hydrophobic interaction with the side chain of Phe150 of the "flipped" DFG motif of CK1ε. It appears that ortho substitutions such as fluoro or methyl at position 2 of the *meta*-chlorophenyl could form an additional hydrophobic interaction with the side chain of Phe150, resulting in a potential improvement in potency and selectivity.

In summary, the present structural and mutagenesis studies provide insights into the molecular basis for the interactions of **2** with CK1ε, which will be important for further optimization of CK1ε inhibitors.

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EXPERIMENTAL SECTION

Human CK1δ (1−294) was expressed and purified as previously described.[11] Human CK1ε (1−294) was expressed and purified similarly to human CK1δ (1−294), except that the expression was carried out in Rosetta(DE3). **2** (>99% purity by HPLC) was purchased from Tocris Bioscience, and crystals of CK1δ complexed with **2** were obtained in hanging drops with 100 mM citrate pH 5.0, 1.0 M LiCl, 20−30% PEG6000. These crystals belong to the space group *C*2 with unit cell parameters of a = 171.22, b = 48.10, c = 80.47 Å, and β = 110.67°. Crystals of CK1ε/**2** were obtained in hanging drops with 100 mM Hepes pH 7.5, 3% ethanol, and 1.1−1.6 M $(NH_4)_2SO_4$. These crystals belong to the space group $P4_32_12$, with unit cell parameters of a = b = 111.11 and c = 155.43 Å. Crystals of CK1ε were obtained in hanging drops with 100 mM Hepes pH 7.5, and 1.0−1.5 M $(NH4)_2SO_4$. These crystals belong to the space group *C*2 with unit cell parameters of a = 180.36, b = 142.50, c = 232.46 Å, and β = 73.86°. Paratone-N mineral oil was used as cryoprotectant. Diffraction data for all crystals in this work were collected on beamline 21-ID-F at the Advanced Photon Source (APS) and processed and scaled with HKL 2000. All three structures were solved by molecular replacement with PHASER using our CK1δ/**1** (PDB code 3UZP) structure as the template. Model building was carried out with QUANTA, and refinement was done using CNS. Details on data processing and refinement statistics are given in the Supporting Information, Table S1. $2f_o - f_c$ electron density maps of the ATP binding sites before any ligand building are shown in Supporting Information, Figure S1.

CK1δ mutants such as Ile55Phe and CK1ε mutants such as Phe55Ile were expressed and purified as wild-type CK1δ and CK1ε, respectively. Proteins were diluted to 0.1 mg/mL in buffer containing 100 mM Hepes, pH 7.5, and 150 mM NaCl containing 1:1000 SyproOrange (Invitrogen). Then 25 μL of protein solution were added to all wells in 96-well clear plates (Roche Diagnostics), each well containing 0.25 μL 10 mM compound solution (thus 100 uM final compound concentration) or 0.25 μL pure DMSO as the references. The plates were spun at 1000 rpm and then sealed by tape (Roche Diagnostics). Thermal stability was measured by monitoring SyproOrange fluorescence ($\lambda_{excitation}$ = 465 nm and $\lambda_{emission}$ = 580 nm) while heating the samples from 20 to 90 °C in increments of 1 °C/min in a real-time PCR system LightCycler480 (Roche Diagnostics). Each experiment was carried out in triplicate.

ASSOCIATED CONTENT

Supporting Information

Details on data processing and refinement statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Coordinates for the CK1δ and **2**, CK1ε and **2**, and CK1ε structures have been deposited in the Protein Data Bank with access codes 4HNF, 4HNI, and 4HOK.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CK1, casein kinase 1; T_m, melting temperature; ΔT_m, shift in melting temperature

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