Specific CLK Inhibitors from a Novel Chemotype for Regulation of Alternative Splicing

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SUMMARY

There is a growing recognition of the importance of protein kinases in the control of alternative splicing. To define the underlying regulatory mechanisms, highly selective inhibitors are needed. Here, we report the discovery and characterization of the dichloroindolyl enaminonitrile KH-CB19, a potent and highly specific inhibitor of the CDC2-like kinase isoforms 1 and 4 (CLK1/CLK4). Cocrystal structures of KH-CB19 with CLK1 and CLK3 revealed a non-ATP mimetic binding mode, conformational changes in helix αC and the phosphate binding loop and halogen bonding to the kinase hinge region. KH-CB19 effectively suppressed phosphorylation of SR (serine/arginine) proteins in cells, consistent with its expected mechanism of action. Chemical inhibition of CLK1/CLK4 generated a unique pattern of splicing factor dephosphorylation and had at low nM concentration a profound effect on splicing of the two tissue factor isoforms flTF (full-length TF) and asHTF (alternatively spliced human TF).

INTRODUCTION

There are about 23,000 protein-coding genes in the human genome. However, the human proteome consists of a far larger number of unique protein sequences. In fact, some 90% of all transcribed genes may undergo alternative splicing and more than 80% may have at least 15% abundance of minor splicing forms (Shi et al., 2008). In many cases, alternative splicing leads to the expression of several protein isoforms with different and sometimes antagonistic functions (Pajares et al., 2007). Notable examples include pro- and antiapoptotic isoforms of Bcl-2 family members (Akgul et al., 2004) and pro- and antiangiogenic forms of VEGFA (Harper and Bates, 2008). This plasticity plays a fundamental role in tissue development and the cellular response to external stimuli, for example in the control of blood clotting (Eisenreich et al., 2009) and insulin action (Jiang et al., 2009). Not surprisingly, the deregulation of alternative splicing has also been linked to numerous human pathologies (Ward and Cooper, 2010).

The regulation of alternative splicing is highly complex. In addition to the essential enzymatic step of RNA breakage and ligation, the spliceosome must recognize the exon and intron boundaries precisely and in a controlled fashion. Not surprisingly, the splicing machinery involves hundreds of auxiliary factors that control splice site selection, spliceosome assembly and the splice reaction (Wahl et al., 2009; Bourgeois et al., 2004). Indeed, the spliceosome alone rivals the ribosome and chromatin remodeling complexes in its complexity (Ritchie et al., 2009). But what distinguishes the spliceosome is its very dynamic nature. During the different stages of the splicing process, dozens of proteins get recruited or dissociated from the spliceosomal complex (Wahl et al., 2009). The availability and posttranslational modification status of these regulatory proteins define the outcome of the splicing reaction and link it to extracellular signaling (Blaustein et al., 2007). One group of proteins regulating the selection of alternatively spliced exonic or intronic premessenger (mRNA) sequences in response to environmental changes are serine/arginine-rich (SR) proteins (Bourgeois et al., 2004). The group name relates to the serine/arginine-rich sequences present in these proteins (Long and Caceres, 2009). The serine residues in these sequence patches are phosphorylated by several protein kinase families, most notably the serine/arginine-rich protein kinases (SRPKs) and the CDC2-like kinase family (CLKs) (Colwill et al., 1996; Gui et al., 1994). The phosphorylation status of SR proteins regulates in turn their cellular localization and activity (Stamm, 2008).

The phosphorylation-dependent signal transduction is a recurrent theme in cell signaling and the control of alternative splicing appears to be no exception. Given the recent success in designing selective kinase inhibitors, several efforts have...
been made to target CLKs. Muraki et al. (2004) reported a cell permeable benzothiazole compound (TG003) with 20 nM and 15 nM potency for CLK1 and CLK4, respectively. However, more comprehensive profiling of this compound revealed strong inhibition of TG003 for all CLK family members except for CLK3 but also cross reactivity with casein kinase (CK1\(\alpha\) and CK1\(\beta\)), dual-specificity tyrosine phosphorylation-regulated kinase (DYRK1B), Yeast Sps1/Ste20-related kinase (YSK4) and proviral insertion site in Moloney Murine Leukemia Virus (PIM) kinase isoforms (Mott et al., 2009). The latter paper reported also a series of substituted 6-arylquinazolines with low nM potencies inhibiting all CLKs as well as DYRK1A and DYRK1B and the tyrosine kinase EGFR. In addition, a number of nonselective inhibitors have been reported together with the crystal structures of CLK1 and CLK3 (Bullock et al., 2009). However, to date there are still no potent and highly selective CLK inhibitors with the submicromolar cellular activity available that would be required for use in vivo experiments. Chemical probes with such characteristics may help to decipher the role of CLKs not only in splicing regulation, but also in the control of viral infections (Karlas et al., 2010) as well as cellular metabolism (Rodgers et al., 2010).

Here, we describe a novel class of CLK inhibitors (dichlorindolyl enaminonitriles), with high specificity for CLK1/CLK4 isoforms and a unique binding mode to the kinase hinge region. The lead compound shows single-digit nanomolar activity in modulating alternative splicing in human endothelial cells.

**RESULTS**

Natural compounds provide a rich source for novel chemical scaffolds which offer an excellent foundation for rational structure-based design. Recently, we reported a novel class of potent and selective class III histone deacetylase (sirtuin) inhibitors, which are structural hybrids between a common kinase inhibitor scaffold and the \(\beta\)-carboline alkaloid bauerine C (Figure 1A), having a unique 7,8-dichloro substitution pattern (Huber et al., 2010a). Bauerine C was originally isolated from the blue-green alga *Dichothrix baueriana* and has been reported to have antiproliferative as well as antiviral properties (Larsen et al., 1994). In this study, we envisaged to prepare a library of novel bioactive compounds using 4-cyano-bauerine C (3), an easy-to-functionalize derivative of the alkaloid bauerine C, as a basis for structural diversification (Figure 1B).

For the preparation of 4-cyano-bauerine C (3) we started from ethyl 3-cyanomethyl-6,7-dichloro-1-methyl-1H-indole-2-carboxylate (1) (Huber et al., 2010b), which was reacted with Bredereck’s reagent (tert-butoxy-bis(dimethylamino)methane) to give the tertiary enaminonitrile 2 as a mixture of E/Z isomers. This intermediate was then heated with ammonium acetate and glacial acetic acid in a microwave reactor to give 4-cyano-bauerine C (3). Both 3 and the intermediate tertiary enaminonitrile 2 were screened against a panel of 106 kinases using a thermal shift assay and showed only interaction with CLK family members (see Table S1 available online). Primary enaminonitrile KH-CB20 (as an E/Z mixture) was originally isolated as a side product in the synthesis of 3 and also screened against the kinase panel. Serendipitously, the kinase assay revealed KH-CB20 to be a potent and selective inhibitor of CLK1 and the closely related isoform CLK4, with significantly reduced affinity to CLK2 and CLK3 (Figure 2; Table S1). Thus the procedure for the synthesis of KH-CB20 was optimized. Addition of sulfuric acid to the reaction mixture and shorter reaction time largely prevented cyclisation to 4-cyano-bauerine C (3). Both 3 and the intermediate tertiary enaminonitrile 2 were screened against a panel of 106 kinases using a thermal shift assay and showed only interaction with CLK family members (see Table S1 available online). Primary enaminonitrile KH-CB20 (as an E/Z mixture) was originally isolated as a side product in the synthesis of 3 and also screened against the kinase panel. Serendipitously, the kinase assay revealed KH-CB20 to be a potent and selective inhibitor of CLK1 and the closely related isoform CLK4, with significantly reduced affinity to CLK2 and CLK3 (Figure 2; Table S1). Thus the procedure for the synthesis of KH-CB20 was optimized. Addition of sulfuric acid to the reaction mixture and shorter reaction time largely prevented cyclisation to 4-cyano-bauerine C (3) and led to predominant formation of the primary enaminonitrile KH-CB20 as a 71:29 mixture of E- and Z-isomers. Separation of the E-isomer KH-CB19 could be achieved by selective recrystallization from toluene. The pure E-isomer KH-CB19 and the E/Z-mixture KH-CB20 had similar kinase binding activity (Table S1).

Direct measurements of kinase inhibition in enzymatic assays revealed low nM potencies. Both KH-CB20 and KH-CB19 showed potent inhibition of CLK1 with an IC\(_{50}\) of 20 nM, and for the pure isomer KH-CB19, almost 100-fold selectivity...
against the CLK3 isoform (Figure 2 and Table 1). Using temperature shift assays, cross-screening against 129 kinases revealed only strong interaction with CLK family members, in particular CLK1 and CLK4. We were interested to assess how temperature shift data across such a wide and diverse panel of kinases correlate with binding affinities. To do this, we used the large panel of binding data that has been made available by AMBIT (Fabian et al., 2005; Karaman et al., 2008) and temperature shift data that have been published previously by our laboratory (Fedorov et al., 2007). As shown in Figure 2C, the thermal shift data showed good overall correlation (R = 0.95) with published AMBIT binding constants. However, weaker hits identified in temperature shift assays sometimes still correspond to potent inhibitors in enzyme kinetic assays. Unfortunately, this was also the case for DYRK1A which showed a temperature shift of 5.4°C that corresponded to an IC50 of 55 ± 6 nM in enzyme kinetic assays (Table 1). To further confirm specificity, KH-CB19 was profiled against a panel of 71 protein kinases (see Manley et al. [2010] for panel members) using an enzymatic activity assay. No additional kinases from the panel were inhibited confirming the inhibitor selectivity for CLKs. The exceptionally specific activity and unique chemical structure, which does not resemble any known kinase inhibitor, prompted us to determine the crystal structure of KH-CB19 complexes with both CLK1 and CLK3. In addition, we determined the cocrystal structure of CLK3 with a typical ATP mimetic triazole diamine inhibitor, K00546 (5-amino-3-{[4-aminosulfonyl]phenylamino}-N-2,6-difluorophenyl)-1H-1,2,4-triazole-1-carbothiamide), which has been published as a potent CDK1 and CDK2 inhibitor (Lin et al., 2005) (for refinement and data collection statistics, see Table 2). The cocrystal structure with KH-CB19 revealed that the inhibitor bound to the ATP binding site in CLK1 and CLK3 (Figure 3A). However, due to the lack of hydrogen bond donors or acceptors at the carbocyclic ring, KH-CB19 did not interact with the hinge region with a canonical ATP mimetic binding mode (Figure 3B). Instead, KH-CB19 formed a halogen bond with the main chain carbonyl of Glu242. The Cl-O distance was 2.9 Å, below the sum of van der Waals radii (3.3 Å) of carbon-bound chlorine and sp2-hybridized oxygen. The linear C-Cl/O geometry also fulfilled the geometrical criteria for a chlorine halogen bond to the kinase backbone (Voth and Ho, 2007). Interestingly, superimposition with the triazole diamine cocrystal structure revealed one Cl atom in the same position as the primary amine nitrogen that forms a hydrogen bond with the hinge backbone. Similarly, the role and geometry of this halogen bond for KH-CB19 kinase interaction was evident from superimposition with the CLK1/hymenialdisine (K0010) cocrystal structure (Bullock et al., 2009) (Figure 3C). In both cases, the chlorine atom occupied the position of the hydrogen bond donor of common kinase inhibitors, mimicking the NH2 group of ATP. The contribution of halogen bonds to ligand affinity and specificity has not been fully determined and may vary (Bissantz et al., 2010). The second chlorine atom of KH-CB19 was positioned outside of halogen bond range and formed more common lipophilic interactions. Overall, the inhibitor was well defined by electron density (Figure 3D).
Instead of the canonical polar interactions of ATP-mimetic inhibitors with the kinase hinge, the hydrophilic groups of KH-CB19 were oriented toward the back of ATP pocket (Figure 3). In particular, the cyano moiety formed a hydrogen bond with the catalytic residue Lys191 (CLK1 numbering), while the amino group made bidentate bonds to the backbone of Glu292 and side chain of Asn293. Cyano moieties that interact with the catalytic lysine are also present in the non-ATP competitive MEK inhibitor U0126, but since this compound does not occupy the ATP site it coordinates the lysine $\omega$-NH$_2$ group from the allosteric binding pocket adjacent to the MEK ATP site (Fischmann et al., 2009). The CLK binding geometry packed the N- and C-terminal kinase lobes tightly, making a critical contribution to the overall binding affinity. Another interesting feature of the complex was the wedge-like contact between Phe172 (CLK1) and the inhibitor. Conserved aromatic residues in the Phe172 position on the tip of the phosphate binding loop (P loop) have been proposed to contribute to kinase inhibitor binding (Pogacic et al., 2007; Yamaguchi et al., 2006). For example, the loop dynamics have been postulated to determine kinase isozyme selectivity (Doudou et al., 2010). Comparison of the CLK1 and CLK3 structures further supported this hypothesis. The conformation of the P loop was identical in the two CLK1 structures, the complex with KH-CB19 (Figure 4) and the structurally different and nonselective kinase inhibitor hynenialdisine. The CLK3-KH-CB19 complex also superimposed well, suggesting that the CLK1 P loop conformation was optimal for KH-CB19 binding. In contrast, superimposition of the two CLK3 complexes, with KH-CB19 (Figure 4) and CDK1/2 inhibitor, revealed the preference for CLK3 to adopt a more open conformation with the P loop moving away from the ATP binding site. Therefore, the markedly decreased affinity of KH-CB19 for CLK3 may reflect the energetic penalty associated with its induced fit.

**Effects of CLK Inhibition on SR Protein Phosphorylation**

To assess the phosphorylation state of SR proteins, western blotting was performed 2 min poststimulation of human microvascular endothelial cells (HMEC-1) with TNF-$\alpha$ (Figure 5A). SRp75, SRp55, SRp40, SC35, SF2/ASF, and SRp20 were detected in HMEC-1 using antibodies that selectively recognize phosphorylated variants of these proteins (Figure 5A, lane 1). Treatment of nonstimulated cells with 10 $\mu$m KH-CB19 led to a reduced phosphorylation of SRp75, SRp55, and SRp20.

### Table 1. Effect of the Studied Inhibitors on Enzymatic Activity

| Inhibitor | CLK1 [nM] | CLK3 [nM] | DYRK1A [nM] |
|-----------|-----------|-----------|-------------|
| KH-CB19   | 19.7 ± 6  | 530 ± 140 | 55.2 ± 6    |
| KH-CB20   | 16.5 ± 3  | 488 ± 120 | 57.8 ± 2    |
| TG003     | 48.6 ± 16 | >4000     | 156.1 ± 23.0|
| K00546    | 8.9 ± 3   | 29.2 ± 8  | ND          |

$I_{50}$ values are shown in nM and values were average from three independent experiments. Literature values for TG003 according to Muraki et al. (2004): CLK1, 20 nM, CLK4 15 nM, CLK3 >10 $\mu$m. ND, not determined.

### Table 2. Data Collection and Refinement Statistics

#### Data Collection

| PDB ID | Target | Inhibitor | Space group | Cell dimensions: a, b, c (Å) | Resolution (Å) | Unique observations | Completeness (%) | Redundancy (%) | Rmerge (%) | I/σI
|--------|--------|-----------|-------------|----------------------------|----------------|---------------------|-----------------|--------------|------------|--------|
| 2VAG   | CLK1   | KH-CB19   | C2          | 90.95, 64.11, 78.89         | 1.80 (1.92–1.80) | 36,979 (5380)       | 99.9 (99.9)     | 3.8 (3.0)    | 0.090 (0.613) | 11.6 (1.8) |
| 2WU6   | CLK3   | KH-CB19   | C2          | 89.15, 62.33, 74.15         | 1.92 (2.02–1.92) | 30,158 (4343)       | 97.1 (96.2)     | 4.3 (4.4)    | 0.132 (0.761) | 7.3 (2.0)  |
| 2WU7   | CLK3   | K00546    | C2          | 90.00, 118.17, 90.00        | 2.23 (2.28–2.23) | 19,441 (861)        | 99.9 (97.8)     | 6.59 (4.45)  | 0.058 (0.581) | 7.26 (2.1) |

#### Refinement

| Resolution (Å) | Rwork / Rfree (%) | Number of atoms(protein/other/water) | B factors (Å$^2$)(protein/other/water) | Rmsd bonds (Å) | Rmsd angles (°) | Ramachandran favored (%) | Allowed (%) | Disallowed (%) |
|----------------|-------------------|---------------------------------------|---------------------------------------|----------------|-----------------|--------------------------|-------------|---------------|
| 1.80           | 1.92 / 2.23       | 18.2/22.5                             | 2645/22/230                           | 0.013          | 1.404           | 96.04                    | 2.74        | 1.22          |

*Values in parentheses correspond to the highest resolution shell.*
compared with nonstimulated controls, whereas the phosphorylation of SRp40, SC35, and SF2/ASF was unaffected under basal conditions (lane 2). Pretreatment of HMEC-1 with 10 μM TG003, a previously identified CLK inhibitor, only reduced the phosphorylation of SRp20, but had no effect on the phosphorylation state of other SR proteins under normal conditions (lane 3). Stimulation of HMEC-1 with TNF-α led to an increase in the phosphorylation of all detected SR proteins only 2 min post induction (lane 4). Pretreatment of cells with KH-CB19 or TG003 led to a reduction of the TNF-α-induced increase in phosphorylation of all analyzed SR proteins (lanes 5 and 6) compared with TNF-α-stimulated controls (lane 3). However, the effect of 10 μM KH-CB19 was far greater under both normal and proinflammatory conditions as compared to cells treated with 10 μM TG003. Dose response of KH-CB19 was tested using SRp75 and SRp55. As shown in Figure 5C phosphorylation levels of these two proteins in TNF-α-stimulated cells were significantly reduced at increasing concentration of the inhibitor. In contrast, TG003 had little effect on SRp75 and SRp55 phosphorylation at the tested concentrations.

**DISCUSSION**

Despite the substantial effort in developing targeted kinase inhibitors, the task of selective inhibitor design remains highly challenging (Morphy, 2010). As a result, only a handful of reported kinase inhibitors can be classified as truly specific agents (Smyth and Collins, 2009; Karaman et al., 2008). One, albeit not insurmountable challenge, is the overreliance on ATP-mimetic hydrogen bonding to the kinase hinge region. Few kinases have been successfully targeted by other binding mechanisms. A prominent example is PIM1, which has a unique proline residue in the +3 hinge donor position which breaks the classical hydrogen bonding pattern leading to reorientation of inhibitors and formation of polar contacts with the opposing face of the ATP binding pocket (Bullock et al., 2005; Jacobs et al., 2005).

**Effect of KH-CB19 and TG003 on Alternative Tissue Factor pre-mRNA Splicing in Human Endothelial Cells**

Unstimulated HMEC-1 constitutively express both tissue factor (TF) isoforms, the soluble asHTF as well as the membrane bound full-length TF (fTF) at the mRNA level (Figure 5B, lane 2). Stimulation of HMEC-1 with 10 ng/ml TNF-α led to an increased mRNA expression of both TF isoforms compared with nontreated controls (lane 3). Treatment of resting cells with 10 μM KH-CB19 significantly reduced the basal expression of flTF as well as asHTF (lane 4). Pharmacologic inhibition of CLKs using KH-CB19 also lowered TNF-α-induced expression of both TF mRNA splice variants to baseline (lane 5). Treatment of HMEC-1 cells with 10 μM TG003 also reduced the basal expression of fTF and asHTF mRNA (lane 6), and showed only slightly reduced mRNA expression in TNF-α-induced cells 1 hr post stimulation (lane 7).
These unusual binding modes have been associated with the unique PIM hinge region which does not allow formation of a second hydrogen bond with ATP or ATP mimic ligands. Here, we report that CDC2-like kinases, which have seemingly nondistinguished and standard sequence around the ATP binding site can be successfully targeted by inhibitors that do not mimic the canonical hydrogen bond pattern of ATP mimic inhibitors. Crystal structures suggest that this binding mode is optimally satisfied by an inward conformation of the P loop which provides additional interaction through CLK1 Phe172. This work highlights the opportunity to develop very potent and specific inhibitors with new chemical profiles. Comparisons of inhibitor cross reactivity revealed a very favorable selectivity profile for KH-CB19 when compared with typical ATP mimetic ligands.

An interesting feature of the KH-CB19 binding mode is the presence of a halogen bond formed with one CLK hinge region backbone carbonyl. Halogen bonds are short-range molecular interactions involving polarized halogens. Such contacts occur frequently in inhibitor target complexes but have only recently been recognized as intermolecular interactions that may favorably contribute to ligand affinity (Hernandes et al., 2010). Well-known examples are halogen bonds that have been described in tetrabromobenzimidazole casein kinase 2 (CK2) complexes (Battistutta et al., 2005). However, more theoretical and experimental studies are needed to understand the biophysical nature of halogen bonding and how these interactions can be exploited in structure based drug design.

Alternative splicing is an essential regulatory process influencing the functional diversity and plasticity of the proteome in response to environmental changes (Black, 2003). CLKs regulate alternative splicing by phosphorylating SR proteins, thereby modulating their cellular localization and splicing activity (Prasad et al., 1999; Tardos et al., 2008; Bourgeois et al., 2004; Eisenreich et al., 2008). The pharmacological inhibition of CLKs is feasible (Muraki et al., 2004) and has been shown to influence alternative splicing of important vascular proteins, such as TF and VEGF (Eisenreich et al., 2009; Nowak et al., 2008). Here, we show that KH-CB19 suppresses SR protein phosphorylation by CLKs under proinflammatory conditions and that this inhibition is sufficient to modulate the differential expression of the TF isoforms, asHTF and iTF in human endothelial cells. The pharmacologic inhibition of CLKs by TG003 was also shown previously to reduce the expression of both TF isoforms and to reduce phosphorylation of SRp75, SRp55 and SF2/ASF (Eisenreich et al., 2009). The selectivity of this pharmacologic inhibition to the CLK family was verified by specific siRNA-mediated inhibition of CLK1 as well as CLK4. Here, we demonstrate that the inhibitory effect of KH-CB19 is more selective and efficacious in cellular assays than inhibitors that have been reported previously (Eisenreich et al., 2009). Thus, KH-CB19 represents an excellent tool compound for examining the role of CLKs, especially in their regulation of alternative splicing and for further development as a lead compound in drug discovery.

**SIGNIFICANCE**

Kinases have been in the focus of drug discovery for more than two decades. Despite the large effort in this target area, only a few highly selective inhibitors have been described. In this study, we identified the dichloroindolyl enaminonitrile KH-CB19 as highly selective inhibitor for CLK kinases. Methylation of the indole nitrogen precluded the canonical ATP mimetic binding mode. Cocrystal structures revealed that hinge interaction of KH-CB19 is mediated by halogen bonding. CLK kinases are key regulators of protein splicing. Consistent with its expected mechanism of action, KH-CB19 effectively suppressed phosphorylation of SR (serine/arginine) splicing factors in cells and significantly altered splicing of the two tissue factor isoforms iTF (full-length TF) and asHTF (alternatively spliced human TF). The discovered inhibitor class is therefore a useful model and an excellent probe compound for the development of inhibitors that target protein splicing. Furthermore, the described binding mode of the discovered dichloroindolyl enaminonitrile inhibitors may serve as a template for the development of selective inhibitors for other kinase targets that explore alternative splicing.
non-ATP mimetic interactions with the kinase active site and halogen bonding with the hinge backbone.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification
CLK1 and CLK3 were prepared as described (Bullock et al., 2009). In brief, the kinase domains of human CLK1 (residues 148–484 (C terminus) and CLK3 (residues 275–632) were subcloned by ligation independent cloning into a pET-derived expression vector, pLIC, and expression performed in BL21 (DE3) with 1 mM IPTG induction for 4 hr at 18°C. Cells were lysed using a high-pressure homogenizer and cleared by centrifugation and the lysates were purified by Ni-NTA chromatography. The eluted proteins were treated with lambda phosphatase together with TEV protease overnight to remove phosphorylation and the hexahistidine tag, respectively. The proteins were further purified by size exclusion chromatography using a S75 16/60 HiLoad column.

Thermal Stability Shift Assay
Thermal denaturation experiments were carried out in an Mx3005p real-time PCR machine (Agilent) using a protein concentration of 2 μM and an inhibitor concentration of 10 μM. Samples were buffered in 10 mM HEPES (pH 7.5), 500 mM NaCl and a 1:1000 dilution of SyproOrange (Invitrogen). The assay and data evaluation were carried out as described (Bullock et al., 2005)

Kinase Inhibition Assay
Phosphorylation reactions were monitored using a coupled-enzyme assay in which ADP production is coupled to NADH oxidation by pyruvate kinase (PK) and lactate dehydrogenase (LDH) as described (Bullock et al., 2009). The reaction was started by addition of 0.1 mM ATP after a 10 min preincubation of the reaction mixture at 25°C. The consensus peptide for CLK1 (AFREWESPGEAK) and the DYRK1A substrate peptide (YRSAEPESPRPPA-amide) were used as substrates at a concentration of 100 μM. Inhibitors, dissolved in DMSO, were added at the beginning of the preincubation period resulting in a DMSO concentration of 2% in the assay. Kinetic analysis was performed by nonlinear regression fitting using GraphPad Prism 5 and least-squares fits to sigmoidal dose response curves with variable slope equation:

\[ Y = \min + \frac{\max - \min}{1 + 10^{(\log EC_{50}/x) \cdot \text{Hillslope}}} \]

where max and min corresponds to maximal and minimal absorbance value. K00546 is CDK1/2 inhibitor III purchased from Merck Biosciences (cat. # 217714). TG003 was purchased from Merck Biosciences (cat. #219479).

Enzymatic kinase selectivity screening was carried out using the Caliper mobility shift assay which is based on the difference in capillary electrophoresis.
electrophoresis mobility of a fluorescent tagged peptide as a result of the addition of a phosphate moiety by the studied kinase. The kinase reactions were started by addition of 4.5 μl substrate mix consisting of ATP and peptide substrate in assay buffer (50 mM HEPES [pH 7.5], 0.02% bovine serum albumin, 1 mM DTT, 0.02% Tween 20, 0.01 mM Na2VO3, 10 mM beta-glycerophosphate) and 4.5 μl enzyme solution in assay buffer. The peptide concentration was 2 μM. Concentrations for the enzyme, as well as for MgCl2 and MnCl2, were adjusted specifically to the requirements of the individual enzyme.

ATP concentrations were adjusted to the Km values of the specific enzyme. After incubation for 60 min at 30°C the kinase reactions were stopped by addition of 16 μl stop solution (100 mM HEPES [pH 7.5], 5% DMSO, 0.1% coating reagent [Caliper Lifescience]) 10 mM EDTA [pH 8.0], 0.015% Brij35). Initial models were built by ARP/wARP (Perrakis et al., 1999) and MOSFLM (Leslie and Powell, 2007) [CLK1/KH-CB19, CLK3/KH-CB19] or in-house on a Bruker system equipped with a Microstar generator (Caliper Lifescience) 10H NMR spectra, chemical shifts are given in General Information.

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Data Collection and Structure Solution
Crystals were cryoprotected using the well solution supplemented with additional ethylene glycol and were flash frozen in liquid nitrogen in all cases. Data were collected at the Swiss Light Source on beamline X1OSA using a MAR225 detector at 1.00629 Å [CLK1/KH-CB19], at the Diamond Light Source on beamline I02 using a ADSC Q315 detector at 0.9050 Å [CLK3/K00546], or in-house on a Bruker system equipped with a Microstar generator and a Pt135 detector at 1.54 Å. Indexing and integration was carried out using MOSFLM (Leslie and Powell, 2007) [CLK1/KH-CB19, CLK3/K00546] or XPREP (Sheidrick, 2008) (CLK3/K01762) and scaling was performed with SCALA (Evans, 2007). Initial phases were calculated by molecular replacement with PHASER (McCoy et al., 2005) using the known model of CLK1 (PDB ID 1257). Initial models were built by ARP/WARP (Perakis et al., 1999) and building was completed manually with COOT (Emsley and Cowtan, 2004). Refinement was carried out in REFMACS (Murshudov et al., 1997) or BUSTER (Bricogne, 1993). In all cases, thermal movements were analyzed using TLSMD (Painter and Merritt, 2006) and hydrogen atoms were included in late refinement cycles. Data collection and refinement statistics can be found in Table 2.

Chemical Synthesis

General Information
NMR spectra were recorded using a Jeol JNMN-GX400 and Jeol JNMN-GX500 (Jeol, Peabody, MA). E/Z ratios were determined by integration of the corresponding peaks in the 1H NMR spectra, chemical shifts are given in Hertz. Mass spectra (electronic ionization, El, 70 eV) were recorded using a Hewlett Packard 5989 A Mass Spectrometer with a 59980 B Particle Beam LC/MS-interface (Agilent Technologies, Palo Alto, CA). High-resolution mass spectra were obtained using a Jeol Mass Spec 700. Melting points were determined with a Büchi 5-640 apparatus (Büchi, Flawil, Switzerland) and are uncorrected. Microwave reactor: CEM Discover (CEM, Matthews, NC). Purification by flash column chromatography (FCC) was done using Silica gel 60 (Merck, Darmstadt, Germany). All solvents and chemicals were purchased from Sigma-Aldrich, Fluka, and Acros.

(E/Z)-Ethyl 6,7-Dichloro-3-[1-Cyano-2-(Dimethylamino)Vinyl]-1-Methyl-1H-Indole-2-Carboxylate (2)

Under nitrogen, 2.00 g (11.57 mmol) Bredereck’s reagent (tert-butoxy-bis(dimethylamino)methane) were added to a solution of 2.00 g (6.43 mmol) ethyl 3-(cyanomethyl)-6,7-dichloro-1-methyl-1H-indole-2-carboxylate (1) (Huber et al., 2010b) in 10 ml anhydrid DMF and the mixture was stirred at 80°C for 12 hr. The solvent was removed by rotary evaporation and the crude product recrystallized from toluene to give 1.60 g (69%) of 2 as yellow crystals. MP 154°C; 1H NMR (400 MHz, CDCl3, TMS) δ 7.60 (J = 8.6 Hz, 0.82 x 1 H, 4-H, Z), 7.47 (J = 8.6 Hz, 0.18 x 1 H, 4-H, E), 7.27 (J = 8.6 Hz, 0.18 x 1 H, 5-H, E), 7.24 (J = 8.6 Hz, 0.82 x 1 H, 5-H, Z), 6.93 (0.18 x 1 H, 2′-H, Z), 4.39 (br s, 1 H, 2′-H, Z), 3.45 (0.18 x 3 H, 1-CH3), 3.28 (0.18 x 3 H, 1-CH3), 2.33 (0.18 x 3 H, 1-CH3), 1.42 (t, J = 7.1 Hz, 3 H, CH2-N(CH3)2); 13C NMR (100 MHz, CDCl3, TMS) δ 162.0 (C=O, Z), 161.7 (C=O, E), 153.6 (C-2, E), 153.2 (C-2a, Z), 134.9 (C-7a, E), 131.1 (C-6, E), 130.8 (C-6, Z), 130.5 (C-2, E), 129.6 (C-2, Z), 129.4 (C-3a, E), 128.4 (C-3a, Z), 123.7 (CN, E), 123.4 (C-5, E), 122.9 (C-5, Z), 121.5 (C-7, Z), 120.9 (C-4, E), 120.6 (C-4, Z), 119.2 (C-3, Z), 116.6 (C-7, E), 115.6 (C-7, Z), 115.7 (C-3, E), 68.2 (1′-C′, E), 65.5 (1′-C, Z), 61.9 (CH2, Z), 61.8 (CH2, E), 42.5 (2′-N(CH3)), 35.6 (1-CH3). E/Z ratio (%) 18.82%: MS El m/z (relative intensity, %) 369 [M+H] (10), 367 [M+H] (68), 365 [M+H] (100), 292 (27).

7.8-Dichloro-9-Methyl-1-Oxo-2,9-Dihydro-1-H-Pyrido[3,4-b]Indole-4-Carboxamide (3)

890 mg (2.43 mmol) (E/Z)-ethyl 6,7-dichloro-3-[1-Cyano-2-(dimethylamino)vinyl]-1-methyl-1H-indole-2-carboxylate (2), 8.0 g ammonium acetate and 2 ml glacial acetic acid were irradiated in a microwave reactor at 112°C and 150 W power for 45 min. The mixture was poured into ice-water and the precipitate filtered off. The crude product was resuspended in toluene and the solvent removed to give 200 mg (28%) as a beige solid. MP 410°C (decomp); 1H NMR (500 MHz, DMSO-d6, TMS, 70°C) δ 11.97 (br s, 1 H, C-16), 8.16 (d, J = 8.5 Hz, 1 H, 5-H, E), 8.01 (s, 1 H, 3-H, Z), 7.52 (d, J = 8.5 Hz, 1 H, 6-H, Z), 4.61 (s, 3 H, N-CH3); 13C NMR (500 MHz, DMSO-d6, TMS, 70°C) δ 153.6 (C-2, Z), 136.3 (C-3, Z), 131.7 (C-7, Z), 127.4 (C-6a, E), 127.4 (C-6a, Z), 121.2 (C-2b, Z), 120.1 (C-4a, E), 116.7 (C-6, Z), 115.9 (C-6, Z), 84.0 (C-4), 34.3 (N-CH3); MS El m/z (relative intensity, %) 295 [M+H] (10), 293 [M+H] (68), 291 (100).
Cell Culture
Human microvascular cells (HMEC-1) were cultured in endothelial cell (EC) growth medium containing 5% fetal calf serum at 37°C in a humidified incubator (5% CO₂, 95% air). Cells from passages 2 to 6 were used. For inhibition experiments, HMEC-1 endothelial cells were switched to EC basal medium (without fetal calf serum) for 1 hr. After that, cells were pretreated with the CLK inhibitor KH-CB19 (1 nM to 100 μM) or TG003 (10 μM; Calbiochem, Darmstadt, Germany), respectively, for 1 hr and then stimulated with 10 ng/ml TNF-α (Sigma Aldrich, St Louis, MO). Analysis of the TF isoform mRNA expression was done 1 hr post stimulation with TNF-α and assessment of the phosphorylation state of SR proteins by western blotting was performed 2 min post induction of the cells. Positive controls were stimulated only with TNF-α, and negative controls were untreated.

TF Isoform-Specific Real-Time RT-PCR
Real-time PCR employing RTF, asHTF, and GAPDH-specific primers and probes was performed as described previously (Szotowski et al., 2009).

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