Chemistry & Biology
Review

New Protein Kinase CK2 Inhibitors: Jumping out of the Catalytic Box

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Protein kinases are central components of signal transduction cascades often dysregulated in cancer, and they represent some of the most promising drug targets. However, the target selectivity is a major concern because most described kinase inhibitors target the highly conserved ATP-binding pocket. Recently, new classes of inhibitors that do not compete with ATP and exhibit different mechanisms of action have been described. Overexpression of protein kinase CK2 is an unfavorable prognostic marker in several cancers. Consequently, CK2 has emerged as a relevant therapeutic target. Several classes of ATP-competitive inhibitors have been identified, showing variable effectiveness. The molecular architecture of this multisubunit enzyme could offer alternative strategies to inhibit CK2 functions, and this review illustrates these emerging possibilities.

Introduction

Protein phosphorylation is a recurrent theme in biology and is recognized as a major regulatory device that controls nearly all aspects of cellular growth, division, and differentiation. This reversible protein modification is catalyzed by protein kinases. These enzymes use ATP, as a phosphate group donor, and protein alcohol groups (on serine and threonine) and phenolic groups (on tyrosine), as phosphate group acceptors, to generate phosphate monoesters. In eukaryotic cells, protein kinases are central components of signaling pathways regulating cellular homeostasis. Unfortunatly, perturbation of protein kinase-mediated signaling networks can lead to various disease states. Therefore, protein kinases have emerged as some of the most promissing drug targets. Indeed, diverse strategies to inhibit protein kinase functions have been developed by using genetic techniques such as RNAi and knockout animals. Although they are powerful means of perturbing protein kinase-based signaling pathways, these strategies have limitations, as the knockout very often exhibits the opposite phenotype from the one observed when only the catalytic activity of a kinase is inhibited by using small molecules (Knight and Shokat, 2005) (Table 1). A new era of molecularly targeted agents begun when several chemical kinase inhibitors demonstrated powerful clinical activity in tumors in which the target kinase was deregulated (Cohen, 1999; Bogoyevitch and Fairlie, 2007), thus ensuring a prominent position for chemical inhibition of protein kinases in pharmacology. Several kinase inhibitor drugs have been approved for treatments in oncology, and the success of marketed small-molecule kinase inhibitors, such as Gleevec, Irressa, and Tarceva, has recently attracted burgeoning interest (Klein et al., 2005; Krause and Van Etten, 2005). However, the target selectivity is a major concern because most small-molecule kinase inhibitors target the highly conserved ATP-binding pocket of this enzyme family, which is similar to ATP-binding sites of nonkinases, resulting in unforeseen crossreactivities. Less specific inhibitors would be expected to exhibit undesirable toxicities due to an off-target impact that would limit their potential utility as therapeutic agents. For example, Gleevec, a great therapeutic success, has a relatively broad specificity profile that was suggested to lead to side effects such as cardiotoxicity (Kerkela et al., 2006). Thus, there are compelling reasons to identify new classes of protein kinase inhibitors that do not target the ATP-binding site. Consequently, recent years have witnessed considerable efforts to identify kinase inhibitors with substantially different mechanisms of action (Table 2). Some of them are allosteric inhibitors that alter kinase conformation and prevent protein substrate binding. As an illustration, the crystal structure of a PD184352 (2-[2-chloro-4-iodo-phenylamino]-*N*-cyclopropylmethoxy-3,4-difluoro-benzamide) analog in complex with MEK1 and ATP shows that this compound binds to a site adjacent to the ATP-binding pocket, and the low degree of sequence conservation of this MEK1 region explains the high selectivity of this kind of compound (Ohren et al., 2004). Several classes of nanomolar, noncompetitive inhibitors of p38α kinase have been described along with detailed structural characterization of their binding sites. Remarkably, one of these compounds, CMPD1 (2′-fluoro-N-[4-hydroxyphenyl]-(1,1′-biphenyl)-4-butanamide), is substrate selective, blocking the activity of p38α against only a subset of its targets (Davidson et al., 2004). Other inhibitors directly compete with protein substrate binding or target the phosphorylation domain on the protein substrate (Bogoyevitch and Fairlie, 2007).

Extensive studies have established that protein kinase CK2 (formerly casein kinase 2) participates in the regulation of fundamental cellular processes in eukaryotic cells. Indeed, a significant proportion of the cellular phosphoproteome can be attributed to the catalytic activity of CK2, and this ubiquitously expressed protein kinase could be described as a master-mind enzyme, regulating crosstalk among multiple signaling pathways critical for cell differentiation, proliferation, and survival (Litchfield, 2003). CK2 is a tetramer composed of two catalytic subunits, CK2α and CK2α′, and two regulatory, CK2β, subunits. Genetic studies in yeast have shown that knockout of CK2α and CK2α′ results in lethality, providing evidence for an essential role of CK2 catalytic activity for survival (Glover, 1998; Lou et al., 2008). In mice, knockout of CK2α′ resulted in

Table 1. Different Strategies to Inhibit Protein Kinase Functions

Method	Advantages	Limitations
Antisense oligonucleotides	Specifity	Variable efficiency
	In vivo uses	
siRNA/shRNA	Highly specific (isoform-specific targeting)	Inhibit protein expression: all protein functions are blocked
	In vivo uses	No successful clinical uses up to now
Knockout	Highly specific (organ- and isoform-specific targeting)	Irreversible
	Physiologically relevant	Redundancy may lead to absence of phenotype
Microinjection of blocking antibodies	Easy to set up	Time consuming
		Restricted to cell culture
Transfection of mutants/negative dominant	Relatively fast	Restricted to cell culture and simple model organisms (yeast, zebrafish)
		Physiological expression hardly achievable
Small molecules	In vivo uses	
	Wide spectrum	Development is costly and time consuming
	Clinical use achievable	Absolute specificity is rarely obtained
	Reversibility: study of dynamic phenomenons possible	
	Functions can be independently blocked (i.e., kinase activity versus regulatory subunit interaction)	

siRNA, small interfering RNA; shRNA, small hairpin RNA.

viable animals with defects in spermatogenesis (Escalier et al., 2003), and knockout of CK2β resulted in embryonic lethality, revealing the functional importance of this regulatory subunit (Buchou et al., 2003).

A variety of experimental evidence supports the view that disregulated CK2 is functionally linked to cancer. Elevated CK2 activity has been uniformly documented in different human cancer types and has been correlated with aggressive tumor

Table 2. Selected Kinase Inhibitors with Different Mechanisms of Action

Protein-Kinase	Compound Name	Targeted Domain	Reference
Akt	AFQ5	Allosteric site	Lindsley et al. (2005)
	Compound 36	Allosteric site	Siu et al. (2008)
BCR-Abl	GNF-2	Myristate-binding site	Adrian et al. (2006)
	ON012380	Substrate-binding site	Gumireddy et al. (2005a)
B-Raf	Sorafenib	Allosteric site	Wilhelm et al. (2006)
ERK MAPK	G8935	Allosteric site	Han et al. (2005)
	G0328	Allosteric site	Han et al. (2005)
GSK3b	TDZD	Allosteric site	Castro et al. (2008)
	CMTK17	Allosteric site	Conde et al. (2003)
IGFR-1	SBL02	Substrate-binding site	Steiner et al. (2007)
iκB Kinase	BMS-345541	Allosteric site	Burke et al. (2003)
MEK1	U0126	Allosteric site	Favata et al. (1998)
	PD184352	Allosteric site	Spicer et al. (2007)
	PD098059	Allosteric site	Favata et al. (1998)
	PD318088	Allosteric site	Ohren et al. (2004)
p38MAPK	BIRB796	Allosteric site	Pargellis et al. (2002)
	CMPD1	Allosteric site	Davidson et al. (2004)
Plk1	ON01910	Substrate-binding site	Gumireddy et al. (2005b)
VEGFR	AAL-993	Inactive conformation of ATP-binding site	Manley et al. (2004)

TDZD, ThiaDiaZoliDinones; CMTK, ChloroMethylThienylKetones; AFQ: Amino-Functionnalized Quinoxaline.

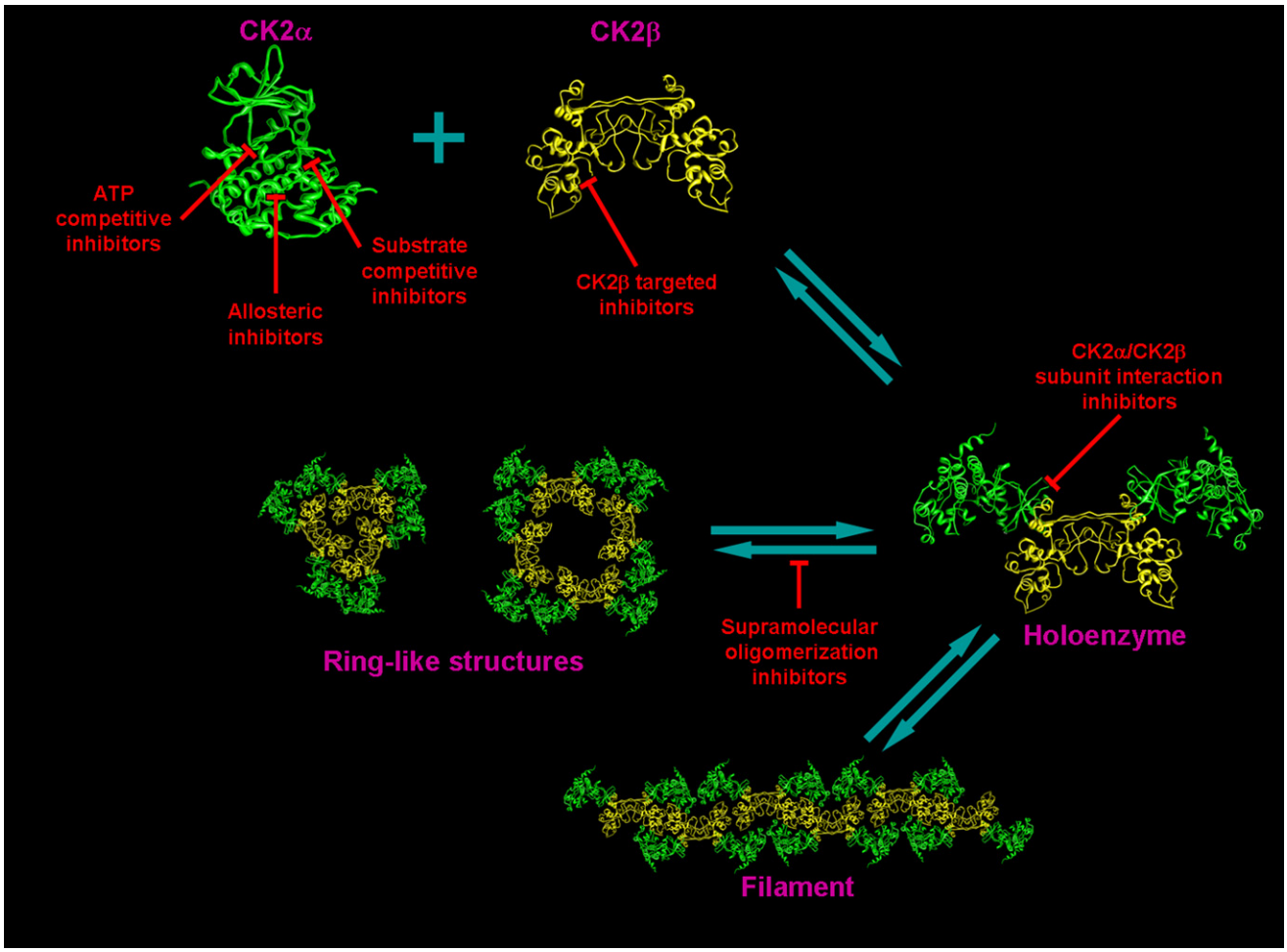


Figure 1. Different Modes of CK2 Inhibition by Small-Molecule Inhibitors
The catalytic (CK2α) and regulatory (CK2β) subunits are subjected to various interaction forces that lead to the reversible formation of different molecular forms like the tetrameric holoenzyme and high-ordered oligomers (ring-like structures and filaments). This dynamic molecular organization offers several opportunities to target the surface area of each subunit with small-molecule inhibitors.

behavior (Tawfic et al., 2001; Faust and Montenarh, 2000). Cancer cells bearing activated CK2 signaling pathways show distinctive features such as growth advantage, enhanced survival, and dynamic adaptation to stress. CK2 exerts a key influence in the progression of oncogenesis by promoting cell growth via the regulation of various oncogenes, tumor suppressor proteins, and protection of antiapoptotic proteins from caspase-mediated cleavage (Duncan and Litchfield, 2008). Of particular interest are reports demonstrating that CK2 overexpression is an unfavorable prognostic marker in prostate cancer (Laramas et al., 2007), in lung cancer (O-charoenrat et al., 2004), and in acute myeloid leukemia (Kim et al., 2007). Additionally, several viral proteins are CK2 targets, indicating a role for this kinase in viral infections (Wadd et al., 1999; Souquere-Besse et al., 2002; Nuesch and Rommelaere, 2007; Medina-Palazon et al., 2007). Overexpression studies of kinase-inactive CK2α and CK2α′, as well as antisense or siRNA knockdown studies of the different CK2 subunits (CK2α, CK2α′, or CK2β) showed effects on the cell cycle progression and induction of an epithelial-to-mesenchymal transition in epithelial cells (Yde et al., 2007; Deshiere et al., 2008). The viability of targeting CK2 for cancer therapy was demonstrated when CK2 antisense-mediated diruption of CK2 activity in vitro led to the induction of apoptosis (Faust et al., 2000; Wang et al., 2001) In addition, it was also found that the same CK2α antisense oligodeoxynucleotide in xenograft models of prostate cancer induced dose- and time-dependent tumor cell death (Slaton et al., 2004). However, these studies also suggested the limitations of CK2 knockdown strategies by showing variable effectiveness to reduce CK2 expression levels (Duncan et al., 2008). In contrast, small molecules can inhibit CK2 activity without affecting other protein domains that might be affected by a knockdown, and diverse strategies to inhibit CK2 functions by small molecules have been explored in preclinical studies (for an example, see Duncan et al., 2008) (Figure 1). Therefore, the pharmacological inhibition of CK2 appears as a promissing alternative strategy in deciphering its various cellular functions. This review highlights recent advances that take advantage of the peculiar molecular architecture of CK2 to discover small molecules targeting different surface areas of CK2 subunits.

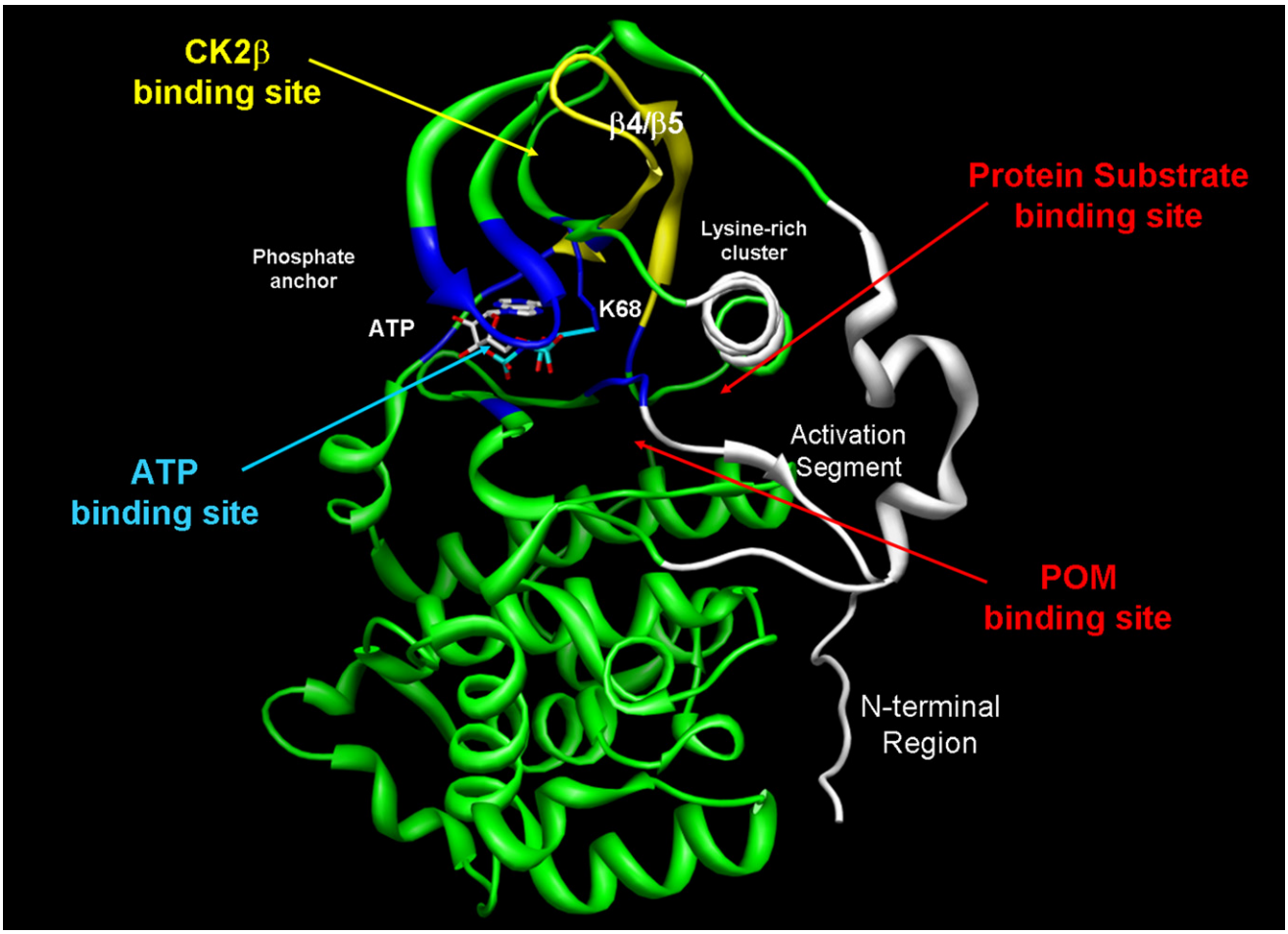


Figure 2. Representation of the CK2α Structure Illustrating Its Different Surface Areas Targeted by New Potent Inhibitors
The phosphate anchor domain is in blue, and key structural elements, like the lysine-rich cluster, the activation segment, and the N-terminal region, are in gray. The CK2β-binding site formed by the β4/β5 loop is in yellow.

ATP-Competitive Inhibitors

The degree of homology across the entire family of human protein kinases is relatively high, especially within their catalytic cores. Nonetheless, the majority of small-molecule protein kinase inhibitors interact with the conserved ATP-binding site of their target. These compounds mainly act as adenine mimics, targeting the ATP-binding pocket by virtue of a complex network of hydrophobic interactions and hydrogen bonding. However, even though major specificity determinants, such as a small hydrophobic pocket located at the back of the ATP-binding site commonly blocked by a side chain of a so-called "gatekeeper" residue, are known in some cases, a complete specificity of these compounds is hard to achieve, and in vivo assessment of compound selectivity remains a challenge (Cohen and Goedert, 1998; Knight and Shokat, 2005; Fabian et al., 2005).

In the case of CK2, a number of different classes of chemical compounds that target its active site have been characterized as ATP-competitive inhibitors, such as halogenated compounds, 4,5,6,7-tetrabromobenzimidazole (TBB) derivatives (Pagano et al., 2004), condensed polyphenolic derivatives (Meggio et al., 2004), and indoloquinazoline-based compounds (Sarno et al., 2003; Vangrevelinghe et al., 2003). They are efficient in the low micromolar range and display high specificity for CK2. Some of them are cell permeable but differ in their physiological effects in cells, probably reflecting their off-target activity toward other ATP-binding proteins with similar ATP-binding sites (Sarno et al., 2002; Duncan et al., 2008).

Alternative Strategies to Inhibit CK2

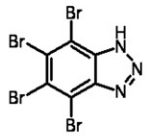
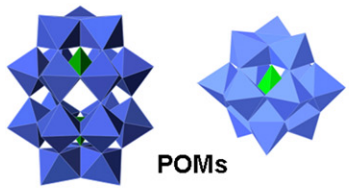
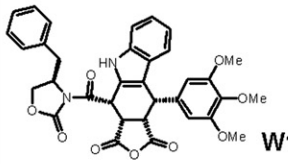
There is now an increasing interest in identifying more specific next-generation CK2 inhibitors that do not directly compete with ATP and exhibit different mechanisms of action (Figure 2).

Non-ATP-Competitive CK2 Inhibitors

Small-molecule inhibitors that block CK2-dependent phosphorylations without directly targeting ATP binding have been recently identified (Figure 2 and Table 3).

Targeting the CK2 Substrate. A distinctive feature of CK2, which could be exploited to inhibit its activity, is its acidophilic substrate specificity. Peptides that inhibit CK2-catalyzed phosphorylation by binding to the acidic phosphoacceptor site on CK2 substrates have been identified by screening of a random cyclic peptide phage library by using the HPV-16 E7 oncoprotein as a target (Perea et al., 2004). One active peptide, P15, abrogates the phosphorylation of the E7 protein by CK2 (Table

Table 3. Examples of Small-Molecule Inhibitors Differentially Targeting the Two CK2 Subunits

Mode of Inhibition	Class of molecule	Examples	References
ATP-competitive inhibitor	Organic molecules	 TBB	Pagano et al., 2004
Allosteric inhibitors	Inorganic molecules	 POMs	Prudent et al., 2008
CK2β-targeted inhibitor	Peptide	GKMNGVLPLAWPSLYLRL P1 Peptide	Martel et al., 2006
CK2αCK2β subunit interaction inhibitors	Cyclic peptide & Organic molecules	GCRLYGFKIHGCG Pc Peptide  W16	Laudet et al., 2007 Laudet et al., 2008
Substrate-targeted inhibitor	Cyclic peptide	CWMSPRHLGTC P15 Peptide	Perera et al., 2008

TBB, 4,5,6,7-tetrabromo-1*H*-benzotriazole; POMs, polyoxometalates; W16, podophyllotoxine indolo-analog.

3). In addition, when fused to the cell-penetrating peptide Tat, P15 behaved as a proapoptotic peptide in tumor cells and exhibited antitumor activity after systemic administration both in cancer animal models and in a clinical trial on patients with cervical malignacies (Perera et al., 2008; Perea et al., 2008). However, it is not clear whether P15 has a specific or global effect on CK2-mediated phosphorylations in living cells. Furthermore, a direct link between the proapoptotic and antitumor properties of this peptide and the targeting of the phosphoacceptor domain in relevant physiological CK2 substrates remains to be established.

Targeting an Exosite on CK2α. Using high-throughput screening of highly diverse chemical libraries, polyoxometalates (POMs) belonging to a unique class of inorganic compounds have been identified as potent CK2 inhibitors (Prudent et al., 2008). POMs are complexes of early-transition metal ions (mainly M = V^{v}, Mo^{vi}, W^{vi}) and oxo ligands (Pope, 2003), which are generated in solution by condensation reactions of the oxo anion MO_4^{n-} (Table 3). Several polyoxometalate derivatives inhibit CK2 in the nanomolar range and exhibit high specificity for CK2. Steady-state kinetic analysis showed a mixed inhibition of CK2 by POMs with respect to ATP, indicating that these compounds are not ATP-competitive inhibitors. Also, POMs are not peptide substrate-binding site-directed inhibitors. Different biochemical analysis suggested that POMs bind outside the ATP/peptide-binding pocket and the CK2α/CK2β interface. Further analysis with proteolytic degradation of the CK2α-POM complex and site-directed mutagenesis revealed that inhibitor-interacting regions contain key structural elements, like an activation segment (Figure 2). In CK2, this segment is stabilized by contacts to the N-terminal region that maintain CK2 in an active state (Niefind et al., 2001; Duncan and Litchfield, 2008). Thus, docking of POMs to the activation segment may disrupt these contacts, locking CK2 in an inactive conformation, and although the intramolecular interactions between the activation loop and the N-terminal segment are very strong they are easily overcome by low concentrations of POMs. Therefore, POMs are effective CK2 inhibitors in terms of both efficiency and selectivity, interacting with CK2 in a unique way. The binding

mode of these nonclassical kinase inhibitors may provide an exploitable mechanism for developing potent drugs with desirable properties, such as enhanced selectivity relative to ATP-mimetic inhibitors.

Targeting the CK2β Subunit

CK2 has a molecular architecture of multisubunit protein kinases with a quaternary structure consisting of two catalytic subunits (CK2α and CK2α′) and two regulatory subunits (CK2β) (Pinna, 2002). Consequently, different strategies based on this molecular architecture can be exploited to inhibit CK2 functions (Figure 2 and Table 3). For example, although CK2 catalytic subunits possess a significant basal activity, CK2β is a central component responsible for docking and/or recrutment of CK2 substrates or protein partners, modulating substrate selectivity and catalytic activity (Bibby and Litchfield, 2005). This raises the possibility that CK2α could be locally and transiently recruited into multimolecular complexes in which the CK2β dimer serves as a scaffold for high-affinity interactions with substrate or nonsubstrate protein partners (Litchfield, 2003; Filhol et al., 2004). In mice as well as in *Caenorhabditis elegans*, a functional loss of CK2β is lethal, illustrating the dramatic phenotypic consequences of disrupting the interactions in which this protein is involved. Therefore, reagents that could block interactions between CK2β and specific partners in vivo would help to elucidate the functional significance of CK2 in cell signaling pathways. Using a two-hybrid screening of a combinatorial peptide aptamer library, we could isolate a peptide (P1 peptide) that binds with high affinity (K_D = 0.4 μM) to the N-terminal domain of CK2β without disrupting the formation of the CK2 holoenzyme (Table 3) (Martel et al., 2006). This is comparable with K_D values for other biologically important interactions, including those between various SH2 domains and receptor-mimicking phosphopeptides ($K_D \approx$ 0.3–130 μM) (Ladbury et al., 1995; Zhou et al., 1995). Expression of P1 peptide in different mammalian cell lines activates p53 phosphorylation on Ser15, induces an upregulation of p21, and triggers apoptosis through the recruitment of a p53-dependent apoptosis pathway (Martel et al., 2006). The crystal structure of CK2β revealed its zinc finger-mediated dimerization and the presence of solvent-exposed and conserved residues that may represent binding surfaces for multiple protein ligands previously identified as CK2β partners (Chantalat et al., 1999; Grein et al., 1999; Filhol et al., 2004). The significant sequence homology between P1 and the murine cytomegalovirus IE2 protein raises the possibility that these two ligands might interact with a common domain on CK2β. Indeed, the IE2 protein is known to interact with the N-terminal region of CK2β and to trigger cell apoptosis (Shimada et al., 2004; Chiou et al., 2001). Therefore, binding of P1 peptide or viral proteins to the N-terminal domain of CK2β may compete for a binding site on the protein for endogenous ligands. It is noteworthy that most drugs and leads achieve their activity by competing for a structurally defined binding site on a protein with endogenous ligands (Hopkins and Groom, 2002). Thus, specifically targeting the regulatory CK2β subunit could represent a strategy to block specific interaction between CK2 and some of its partners in vivo. In addition, this study provides the first peptide lead that can be exploited as a guide for the design of small chemical molecules for CK2β-targeted inducers of apoptosis.

Disrupting the Subunit Interaction

Protein-protein interactions play a key role in cellular signal transduction pathways. Albeit challenging, the ability to interfere with specific protein-protein interactions has already provided a powerful means of influencing the functions of selected proteins within the cells. The CK2β subunit enhances CK2 signaling by bringing CK2α into proximity with its partners and substrates. In addition, the crystal structure of the CK2 holoenzyme complex as well as live cell imaging studies (Niefind et al., 2001; Filhol et al., 2003) suggested that CK2 subunits can coexist in the cell without forming the holoenzyme complex, even though they readily bind with high affinity in vitro (Guerra et al., 1999; Filhol et al., 2004). Thus, this intersubunit flexibility and the existence of a subtle equilibrium between different forms of CK2 subunits is a likely point of regulation that should be amenable to drug-discovery efforts. Structural properties of the holoenzyme showed that the CK2 subunit interface is relatively small, thus offering attractive opportunities for the identification of small molecules that modulate this interaction. In the CK2 holoenzyme structure, a segment located in the CK2β tail points away from the protein core and forms a β hairpin loop that inserts deep into a shallow hydrophobic groove present in the β4/β5 sheets of CK2α (Niefind et al., 2001). Site-directed mutagenesis and functional assays have revealed that only a small set of primary hydrophobic residues present in this segment of CK2β dominates affinity. Further characterization of these hotspots led to the structure-based design of CK2β-derived cyclic peptides that are potent inhibitors of the CK2 subunit interaction (Table 3). One of these conformationally constrained cyclic peptides, Pc peptide, was able to efficiently antagonize the assembly of the CK2 holoenzyme complex and to strongly affect its substrate preference (Laudet et al., 2007). The Laudet et al. study was the first step to set the framework for the discovery and development of chemical inhibitors of this interaction. The existence of a druggable pocket within the interface area of CK2 was corrrobored by a recent structural study showing that one of the oldest, albeit nonspecific, CK2 inhibitors, D-ribofuranosylbenzimidazole (DRB), binds, in addition to the canonical ATP cleft, to an allosteric site that was identified in the CK2α/CK2β interface. Inhibition kinetic studies demonstrated the dual binding mode of this inhibitor. However, the affinity of DRB for this binding site is not high enough to inhibit the subunit interaction (Raaf et al., 2008). Screening of a library of podophyllotoxine indolo-analogs led to the identification of the first chemical inhibitors of this interaction (Laudet et al., 2008). Three lead compounds were shown to block the interaction between the two CK2 subunits through direct binding to CK2α (the structure of the most potent compound [W16] is shown in Table 3). Interestingly, kinetic analysis revealed that these compounds trigger a noncompetitive inhibition of CK2α without interfering with the ATP cleft. This inhibition was alleviated by CK2β or by the CK2β-derived cyclo peptide (Pc peptide), suggesting that this class of inhibitors binds to the CK2α/CK2β interface in/near the CK2β-binding pocket on CK2α. As this binding site is located close to the ATP-binding pocket, docking of the compounds into this binding site may induce subtle allosteric conformational changes in CK2α that affect its activity. More recently, using a structure-based virtual screening approach, we could identify a cluster of chemical

inhibitors of the CK2α/CK2β interaction that exhibit cell-permeant properties (manuscript in preparation). Structural insights from CK2α-inhibitor complexes would be essential to reveal the mode of CK2 inhibition by these compounds and may provide a platform for the structure-based design of a next generation of inhibitors.

The existence of higher-ordered quaternary structures of CK2 holoenzymes (ring-like structures and filaments) has been observed both in solution (Glover, 1986; Valero et al., 1995) and in CK2 holoenzyme crystals (Niefind and Issinger, 2005). Importantly, the catalytic activity was shown to be significanty enhanced in the ring-like structures or downregulated in the filaments (Valero et al., 1995). Formation of this supramolecular oligomers depends on electrostatic interactions that are modulated by spermine or $MgCl_2$ concentrations (Leroy et al., 1994), suggesting that CK2 may exist in vivo in the form of inactive or active oligomers that could be targeted by small-molecule inhibitors (Figure 1).

Summary and Outlook

CK2 is endowed with specific structural features that have motivated the current efforts in this exciting field of early drug discovery. New ways to screen for, or design, inhibitors of CK2 on the basis of a detailed understanding of its catalytic and regulatory properties is likely to become more and more important. The design, synthesis, and testing of compounds that interact differentially with the surface areas of CK2α outside its catalytic gorge region already led to the identification of new potent inhibitors (Figure 2 and Table 3). They promise exciting opportunities by exploiting new mechanisms of action that may allow for greater specificity. Obviously, structural and biophysical characterization of their binding sites is urgently needed. Interfering with specific interactions between CK2β and some of its partners with peptide ligands or chemical compounds could also provide an efficient strategy for disrupting CK2 functions. The dynamic CK2 subunit interaction is also a likely point of regulation. Therefore, structure-based design of CK2β-derived peptides that antagonize the CK2 subunit interaction has been utilized to produce pharmacophore and scaffold structures, allowing for the identification of chemical inhibitors of this interaction by in silico screening. It is expected that these compounds will be substrate selective, inhibiting the activity of the kinase against a subset of its substrates.

Overall, the emergence of a new generation of CK2 inhibitors provides a fondation for a new paradigm for CK2 inhibition and a unique opportunity to predict how their different modes of inhibition will translate into phenotypes in a physiological setting.

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