Structural Exploration of Different Binding Pockets Suitable to Affect Protein Kinases CK2α and CK2α' With Peptides and Small Molecules

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“*Per aspera ad astra*”

Lucius Annaeus Seneca

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Abbreviations

Table 1. List of Abbreviations used in this thesis.

Abbreviation	Explanation
°C	Degree Celsius
Å	Angstrom 1 Å = 0.1 nm
ACN	Acetonitrile
ADME-Tox	Absorption, distribution, metabolism, excretion, and toxicity
ADP	Adenosine diphosphate
AKAP	A-kinase-anchoring protein
AMP	Adenosine monophosphate
AMP-PNP	Adenosin-5′-(β,γ-imido)triphosphate, a non-hydrolyzable ATP analogon
APC	Adenomatous polyposis coli, a tumor suppressor
ATP	Adenosine triphosphate
BOC	*tert*-Butyloxycarbonyl
B-RAF	Rapidly accelerated fibrosarcoma B, a serine/threonine protein kinase
BRMS1	Breast cancer metastasis suppressor 1, a transcriptional repressor
BTK	Bruton's tyrosine kinase
Caco-2	A human colorectal adenocarcinoma cell line
cAMP	Cyclic adenosine monophosphate
CD5	Cluster of differentiation 5, a T-cell surface glycoprotein
CDC7	Cell division cycle 7-related protein kinase
CDK	Cyclin dependent kinase
CF	5(6)-Carboxyfluorescein
cGMP	Cyclic guanosine monophosphate
CIGB-325	A peptide inhibitor that impairs CK2-mediated phosphorylation of certain substrates
CK2α	Casein kinase 2 alpha (catalytic subunit)
CK2α'	Casein kinase 2 alpha' (catalytic subunit), a paralog of CK2α
CK2β	Casein kinase 2 beta (regulatory subunit)
CKIP-1	Casein kinase 2 interacting protein 1
CMGC	Kinase family named after: CDK, MAPK, GSK3 and CLK
CPP	Cell penetrating peptide
C-RAF	A serine/threonine protein kinase and member of the tyrosine-kinase-like (TKL) group of EPKs
DAG	Diacylglycerol
DAPK	Death-associated protein kinase
dbPTM	Database for post-translational modifications of proteins
DCM	Dichloromethane
DIC	*N*,*N*′-Diisopropylcarbodiimide
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid, a carrier of genetic instructions
DPA	2-(3,4-dichlorophenyl)ethan-1-amine, an αD pocket binding ligand for CK2
DRB	5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole
e.g.	Latin "*exempli gratia*" means "for example"
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF2β	Eukaryotic initiation factor 2 subunit beta
EPK	Eukaryotic protein kinase
et al.	*Et alii*, and other
EtOH	Ethanol
FACT	Histone chaperone facilitates chromatin transcription protein
FBLD	Fragment-based lead discovery screenings
FCS	Fetal calf serum
FDA	U.S. Food and Drug Administration
Fmoc	Fluorenylmethoxycarbonyl
g	Gram
GDP	Guanosine diphosphate
GMP-PNP	Guanosine 5′-[β,γ-imido]triphosphate, a non-hydrolyzable GTP analogon
GSK3	Glycogen synthase kinase 3
GTEx	A database for tissue-specific gene expression and regulation
h	Hour
HATU	(1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate)
HEK	An immortalized human embryonic kidney cell line
HeLa	A human cervical cancer cell line
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV1	Herpes simplex virus 1
HUGO	Human genome organization
i.e.	Latin "*id est*" means "that is"
IC_{50}	Half maximal inhibitory concentration of a compound
IGFR-1R	Insulin-like growth factor 1 receptor
IP_3	Inositol 1,4,5-trisphosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside, a synthetic sugar that binds to the lac operon
ITC	Isothermal titration calorimetry
JAK1	Janus kinase 1, a tyrosine kinase
JAK2	Janus kinase 2, a tyrosine kinase
K_d	Dissociation constant
Ki	Inhibition constant between an inhibitor and an enzyme
KN2	Bivalent CK2 inhibitor, addressing the co-substrate and the αD pocket
KSR1	Kinase suppressor of Ras 1
KSR2	Kinase suppressor of Ras 2
l	Liter
LiCl	Lithiumchlorid
m	Meter
M	Molar
MAPK	Mitogen-activated protein kinase
MB002	3-(4,5,6,7-tetrabromo-1H-benzotriazol-1-yl)propan-1-ol, an ATP-competitive inhibitor, also used as a crystallization chaperone for CK2α'
MEK	Mitogen-activated protein kinase kinase
MeOH	Methanol
MES	2-(*N*-morpholino)ethanesulfonic acid
min	Minute
mol	(1 mole = 6.023 * 10^{23} particles)
N.D.	Not defined

NaCl	Sodium chloride
NF-κB	Nuclear factor 'κ-light-chain-enhancer' of activated B-cells
nm	Nanometer
no.	Number
NSP3	The nonstructural protein 3
nTPM	Average normalized transcripts per million
OCNDS	Okur-Chung Neurodevelopmental Syndrome
OD_{600}	Optical density at 600 nm
O-GlcNAc	O-linked β-N-acetylglucosamine, a post translational modification
p21	Cyclin-dependent kinase inhibitor 1
P53	Transformation-related protein 53, a tumor suppressor
Pc	Cyclic CK2β-mimicking peptide (Laudet *et al*. 2007)
PDB	Protein data bank
PEG	Polyethylene glycol, a precipitant used in crystallization experiments
Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 regulates the conformation of its substrates after previous phosphorylation
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
POBINDS	Poirier-Bienvenu neurodevelopmental syndrome
PTEN	Tumor suppressor phosphatase and tensin homolog
rpm	Revolutions per minute
RSV	Human respiratory syncytial virus
s	Second
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
sC18	Cathelicidin-derived cell-penetrating peptide
SD	Standard deviation
SEM	Standard error of the mean
siRNA	Small interfering RNA
S_N2	A bimolecular type of nucleophilic substitution reactions
ssRNA	Single-stranded ribonucleic acid
SUMO	Small ubiquitin-related modifier protein
Tat	Trans-activating transcriptional activator-derived cell-penetrating peptide
TBB	4,5,6,7-tetrabromo-1*H*-benzotriazole
TBI	4,5,6,7-tetrabromo-1*H*-benzimidazole
tBu	*tert*-butanol
TFA	2,2,2-Trifluoroethanoic acid
TK	Tyrosine kinases
TKL	Tyrosine kinases like
Tris	Tris(hydroxymethyl)aminomethane
VSV	Vesicular stomatitis virus
WHO	World Health Organization
Wnt	Signaling pathway (the name is composed of the words Wingless and Int-1)
λ	Wavelength (in nm or Å)

Abstract

The eukaryotic protein kinase CK2, previously known as casein kinase 2, is a ubiquitously expressed, acidophilic serine/threonine kinase belonging to a branch of the group of CMGC kinases. The enzyme features several peculiarities, one of which is its extraordinary pleiotropic character. A plethora of biological substrates have been described for CK2 to date and these are, among others, involved in cell proliferation, angiogenesis, apoptotic processes, viral infections, and DNA-damage repair. Several of these substrates play key roles in the development and progression of a diverse spectrum of diseases. The ubiquitous presence of CK2, combined with its unusual constitutive activity, presents a highly interesting pharmacological profile as a promising drug target. In particular, neoplastic diseases are significantly driven by high levels of CK2 and the importance of the search for suitable molecules to alter the enzymatic properties of CK2 is therefore evident and a subject of current research. This thesis also contributes to this field, focusing primarily on the investigation of the structural aspects of various protein-ligand interactions at different binding sites of the enzyme.

An important role in these structural studies is accounted by CK2α', a paralog of the catalytic subunit CK2α. Although the two paralogs are highly similar in many respects, CK2α' has been neglected in CK2 research over the past decades due to its poor biochemical handleability and its insufficient crystallization properties. Therefore, in this work, for the first time, a crystallization protocol was developed that reliably yields CK2α structures with an atomic resolution of 1.0 Å and thereby outperforming all previously existing CK2α structures to date. This protocol has proven to be an extremely valuable crystallographic tool to study the precise binding mode of a wide variety of CK2 inhibitors from different substance classes, including high and low-affinity compounds. As an example, the exact binding site and binding mode of different 2-aminothiazole derivatives could be elucidated. These compounds belong to a class of CK2 inhibitors that were erroneously assumed to bind outside the cosubstrate pocket.

In addition to crystallographic studies, organic syntheses were also conducted as part of the research for this thesis. This includes the synthesis of halogenated cyclic peptides which address the α/β interface area of the catalytic subunits, interfering with CK2β binding and thus with the tetrameric holoenzyme assembly. Furthermore, by conjugating with the cell-penetrating peptide sC18, it was possible to investigate the impact of some of these compounds on different cell lines.

Moreover, different 4,5,6,7-tetrabromobenzimidazole derivatives were synthesized and studied. In particular, the bivalent inhibitor KN2, which simultaneously occupies the cosubstrate binding pocket as well as the recently discovered αD binding pocket, proved to be exceptionally high in affinity and outstandingly selective. The aspect of selectivity has always been a particular challenge for kinase inhibitors due to the high degree of conservation of the cosubstrate binding region among eukaryotic protein kinases. The inclusion of the αD binding pocket is currently one of the most promising approaches to overcome this challenge since the high plasticity in this region has exclusively been described for CK2. In this thesis, it was shown for the first time that this is not only true for CK2α, but rather for both paralogs.

Finally, the crystallization successes with CK2α' and an eight-month desalting procedure led to the discovery of a novel binding site, located close to the N-terminus. However, the suitability of this cryptic site for the design of future generations of CK2 inhibitors requires further studies.

Chapter 1 – Introduction

1.1 Eukaryotic Protein Kinases

1.1.1 Physiological Relevance of Protein Kinases to Eukaryotic Cells

The countless biochemical processes that take place in all kinds of cells form a highly complex and dynamic network that is subject to constantly changing environmental conditions. The vast number of reactions constantly needs to be regulated depending on the type and abundance of available resources, the prevailing abiotic parameters, the influence of cellular signaling, and numerous other external and internal stimuli. As a result, transcription and expression levels, as well as protein activities, must be adequately regulated and fine-tuned with respect to the current conditions, both inside and outside of the cell. For this purpose, cells can draw on an extensive repertoire of more than 400 post-translational modifications that alter the chemical properties of the respective proteins and can thus modulate their activities, lifespan, folding, localization, or binding properties [1] [2]. These modifications contribute significantly to the structural and functional diversity of the human proteome, which is much more complex than the number of underlying genes that make up the human genome. Hence, more than 19 000 protein-coding genes have been approved and cataloged by the Human Genome Organization (HUGO) to date [3], whereas the human proteome is often estimated to comprise millions of protein species [4] [5], which are also known as proteoforms [6]. The numerous post-translational modifications are – to a large extent – enzymatically catalyzed and include, among others, the transfer of organic as well as inorganic groups, covalent binding of macromolecules, cyclization reactions, the cleavage of functional groups or peptide chains, and even the conjugation of entire proteins such as the small ubiquitin-related modifier protein (SUMO) or ubiquitin [7]. By far the most frequent modification in the human proteome is the phosphorylation of the hydroxyl group bearing amino acids serine, threonine, and tyrosine, which is catalyzed by protein kinases. However, the rarer, and to some extent, chemically and thermally more labile phosphorylations of histidine, lysine, arginine, cysteine, aspartate, and glutamate have also been detected in human cells [8]. Based on more than 2.2 million experimentally determined posttranslational protein modifications deposited in the database for post-translational modifications of proteins (dbPTM), phosphorylation accounts for

approximately 72.2% of all entries[a] [9]. A commonly recurring statement in the literature in the field of phosphoproteomics indicates that approximately one third of all human proteins are phosphorylated at any time and although this view is often supported by references, these do not support this claim with any primary data [10] [11] [12] [13]. In recent mass spectrometry studies by Sharma *et al*. [14], it has become apparent that the number of phosphorylated human proteins could be significantly underestimated since at least 75% of all proteins expressed in HeLa cells were found to be phosphorylated. Moreover, some proteins can even be subject to heavy multisite phosphorylation, such as the transcription factor and tumor suppressor P53, for which more than 30 potential phosphorylation sites have been described [15] [16]. Vlastaridis *et al*. [17] recently published a survey paper in which a thorough evaluation of more than 1 000 publications, including 187 high-throughput phosphoproteomic studies, is described. The authors conclude that there are approximately 13 000 phosphoproteins and 230 000 non-redundant phosphorylation sites in human cells, resulting in a statistical average of approximately 17.7 possible phosphorylation sites per phosphoprotein.

The vast dimensions of the phosphoproteome are also reflected by the large number of more than 500 protein kinase encoding genes, which represent approximately 2% of human genome [18]. In addition, according to Chen *et al*. [19], there are 264 protein phosphatases, including several pseudophosphatases, which also account for more than 1% of the human genome. Protein phosphatases are enzymatic opponents of kinases and are responsible for the removal of phosphate groups from proteins via hydrolysis, thereby ensuring that the effects of kinases on cellular processes can be reversed under certain conditions. Protein kinases were classified according to Manning *et al*. [18], based on their sequence similarities, structural aspects of kinase domains, and functional properties. All studied kinases were systematically divided into groups: the AGC, CAMK, CMGC, TK, TKL, STE, and CK1, which form the superfamily of eukaryotic protein kinases (EPKs), and the atypical kinases (Figure 1) [18]. The phylogenetic tree developed by Manning *et al*. [18] was, however, based on the seminal work of Hanks and Hunter [20], who had already classified EPKs into four major groups in 1995. Another approach is to categorize kinases according to their substrate specificity. Thus, a distinction can be made between serine/threonine kinases, tyrosine kinases, and the more rarely occurring kinases of dual substrate specificity, for instance, mitogen-activated protein kinase kinases (MEK), which are capable of phosphorylating serine, threonine, and tyrosine [21] [22]. Although the designation “EPK” implies that these proteins can exclusively be found in eukaryotes, many exceptions have been

[a] https://awi.cuhk.edu.cn/dbPTM/statistics.php; accessed: 06.12.22

discovered in other kingdoms [23]. Vice versa, members of the superfamily of histidine kinases are more common in prokaryotes but are also not limited to them [24]. In fact, although the term “EPK” has become established, it is strictly speaking not scientifically accurate and should therefore not be understood too literally.

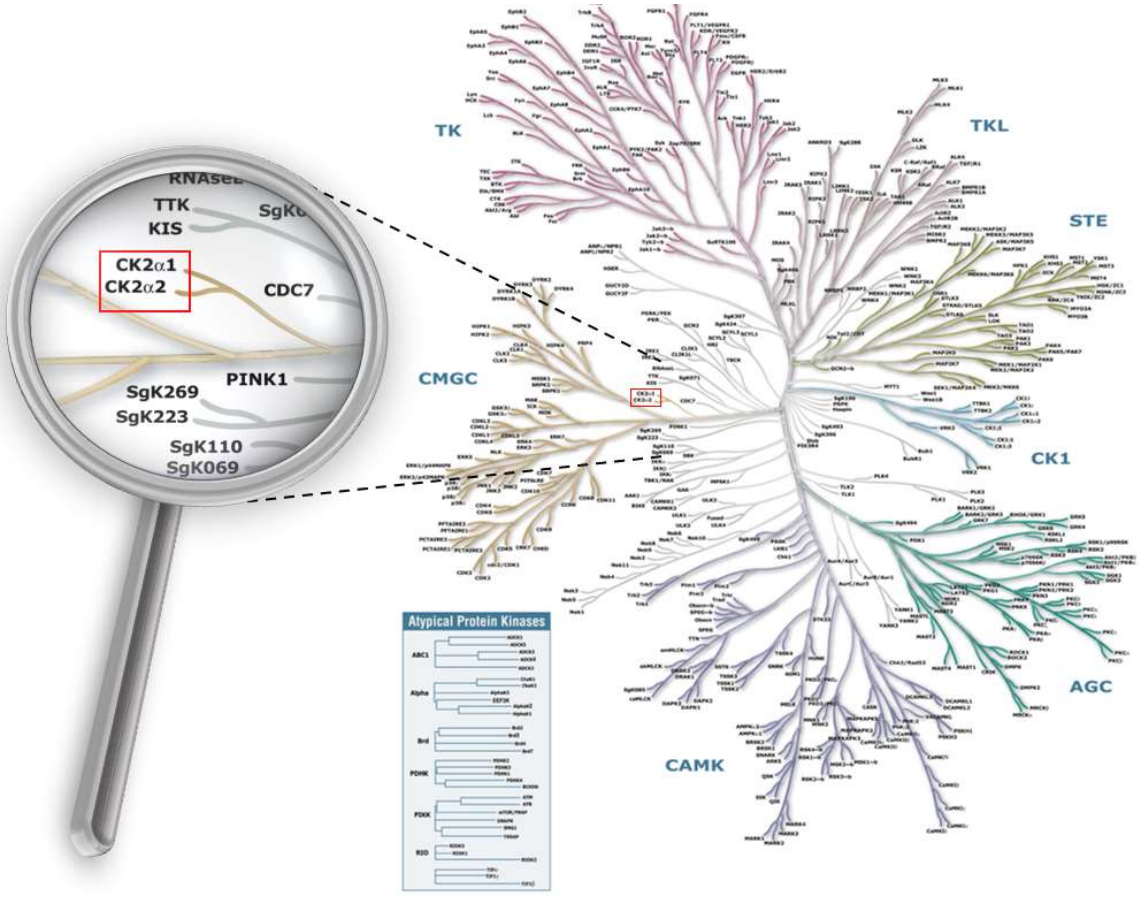


Figure 1. The phylogenetic tree of the human kinome according to Manning *et al*. [18] (Illustration reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com)). The superfamily contains over 500 members, which can be classified into the following groups: the AGC (PKA, PKG, and PKC families), CAMK (calcium/calmodulin-dependent kinases), CMGC (CDK, MAPK, GSK3, and CLK families), TK (tyrosine kinases), TKL (tyrosine kinase-like), STE (homologs of Sterile 7/11/20 kinases from yeast), CK1 (casein kinase 1 family), and the atypical kinases (represented in a separate box). The two CK2α paralogs (CK2α1 and CK2α2 in this thesis referred to as CK2α and CK2α’), which are the focus of this thesis, are magnified and highlighted by a red frame.

The sheer number of human kinases and phosphosites that have been discovered highlights the inestimable importance of this post-translational modification, which has been evolutionarily

proven as a successful biochemical strategy for regulating and maintaining cellular homeostasis. However, a considerable degree of biochemical context is still unknown, e.g., only 3% of the human phosphosites found so far have been linked to a specific kinase [25]. Therefore, a great deal of research effort is still required to fully understand this complex network of phosphorylation and dephosphorylation.

1.1.1 Structure and Function of EPKs

In this subsection, the amino acid numbering of CK2α will be used to describe and illustrate the structural elements that are relevant for the catalytic activity or the scaffolding of EPKs. CK2α was chosen as a representative since it features the typical structural elements of EPKs and it is the focus of interest of this thesis along with CK2α'.

The core of EPKs is highly conserved and composed of two clearly distinguishable folding domains (Figure 2) [26]. The C-terminal domain, usually referred to as the C-lobe, is rich in α-helices and represents the larger of the two domains. The N-lobe, on the other hand, is characterized by a central, five-stranded beta sheet with an antiparallel orientation and a structurally and functionally crucial helix, which is known as helix αC. Both lobes form a deep cleft in between, which serves as the binding site for substrates and thus acts as the enzyme's catalytic center. The two parts are connected by a hinged linker, which is a loop that connects to beta strand β5 on one side and to helix αD on the other. This region serves as an architectural pivot point for EPKs, allowing the two subdomains to perform considerable rotational motions relative to each other, known as kinase breathing, as a function of substrate and cosubstrate loading [27] [28].

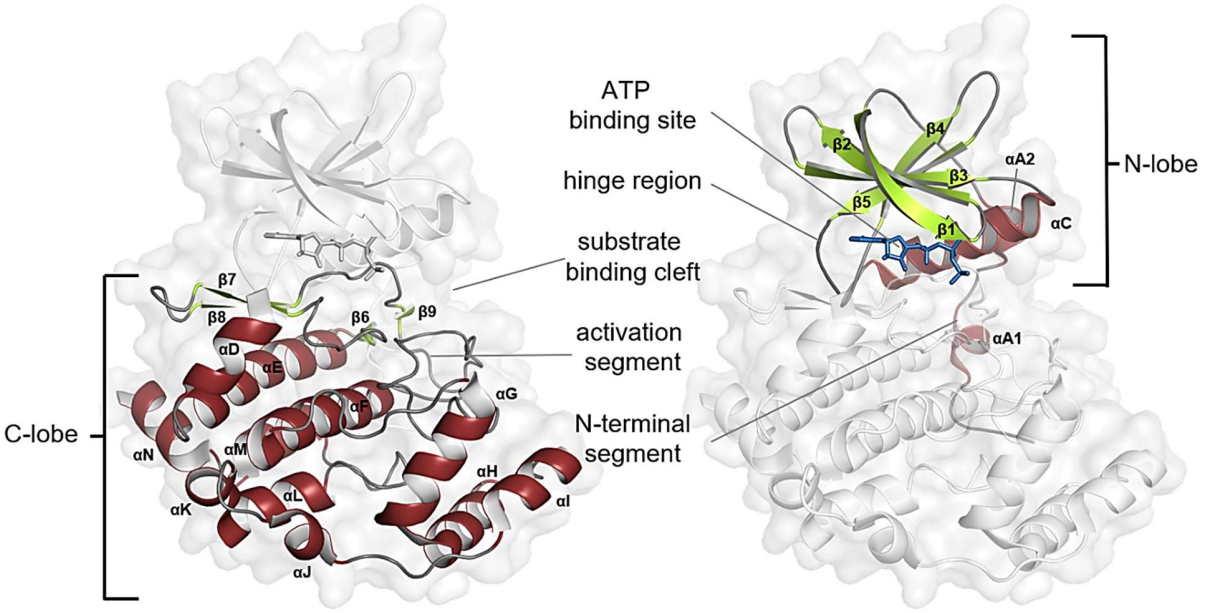


Figure 2. The highly conserved core of EPKs is exemplified by a complex structure of protein kinase CK2α with the hydrolysis-stable ATP analog AMP-PNP (PDB 1LP4). The core can be divided into two structurally distinct parts, the C-lobe (left) which mainly consists of alpha helices (red), and the N-lobe (right) which harbors a central beta-sheet (yellow-green).

The EPK's catalytic cycle includes the γ-phosphoryl group transfer from a nucleoside triphosphate, usually adenosine triphosphate (ATP), to a hydroxyl group of a suitable substrate. This requires a well-defined orientation of the cosubstrate and an appropriate coordination of the triphosphate moiety to allow an appropriate charge balancing of the phosphate chain (Figure 3a). For this reason, the ATP binding pocket of EPKs is subject to particularly strong chemical constraints and is structurally highly conserved [29], a circumstance that will be illuminated in more detail in Subsection 1.3.2. The adenine ring is held in position by backbone interactions with the adjacent hinge region and hydrophobic interactions, whereas the triphosphate moiety is bound in an active orientation by polar interactions and hydrogen bonds with conserved residues such as Lys68 or Lys158. In addition, the high density of negative charges on the triphosphate moiety is stabilized by the presence of two divalent cations, usually Mg^{2+}, which bind with different affinities [30] [31]. Extensive studies on protein kinase A (PKA) by Knape *et al*. [32] have shown that other divalent cations e.g., Ca^{2+}, Cd^{2+}, Fe^{2+}, Ni^{2+}, Mn^{2+}, or Zn^{2+} are also capable of supporting phosphoryl transfer to bound substrates. However, the resulting enzyme-product complex appears to dissociate very poorly in the presence of ions other than Mg^{2+} [32].

On the N-lobe side, the phosphate chain of the nucleotide is in contact with the glycine-rich loop extending from residue Gly46 to Ser51 and connecting beta strands β1 and β2 (Figure 3b). The outstanding flexibility of this loop, due to the low steric hindrance of the glycine residues, provides high adaptability in the binding process of the nucleotide [33]. Moreover, the glycine-rich loop facilitates the γ-phosphoryl group transfer though interactions with the substrate chain, which brings the chain closer to the cosubstrate and the catalytic aspartate. It is a recurring and unique structural element for kinases that facilitates nucleotide binding via the sequence GxGxxG [34] [35]. A study by Huang *et al*. [29] encompassing 469 kinases showed that the first two glycines in the sequence are found with 93% and 96% probability, respectively while the third glycine was still present in 74% of the analyzed kinases. In many CMGC kinases, a related sequence (GxGx**Y**G) can be found with the defined tyrosine serving as a potential regulatory phosphorylation site. Cyclin-dependent kinases, for example, CDK1[b], first need to be dephosphorylated at this particular residue to reach their peak activity [36].

[b] CDK1 is referred to as CDC2 in the older literature, as in Mueller *et al*. 1995.

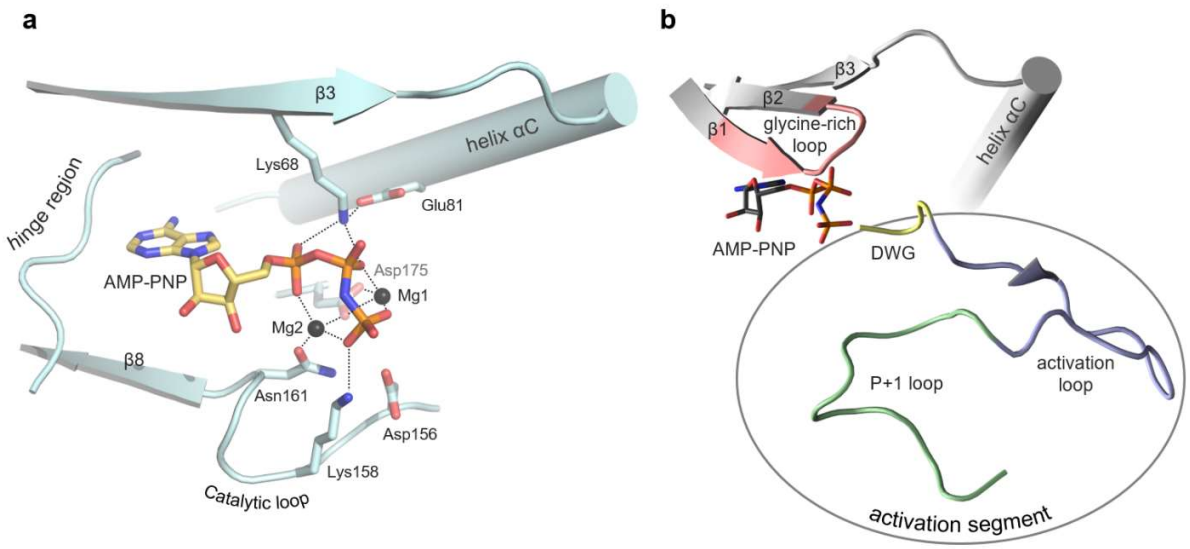


Figure 3. (**a**) The nucleotide-binding site of CK2α (PDB 1LP4) from *Zea mays* as a representative of EPKs. Both direct and magnesium ion-mediated interactions of the ATP analog AMP-PNP with the protein are indicated by dashed lines. In addition, a salt bridge is shown between helix αC and the beta-strand ß3, which is a key switching element for establishing an active conformation in EPKs. (**b**) The activation segment extends from the DFG motif, in the case of CK2α from the DWG motif (yellow), through the activation loop (blue) to the end of the P+1 loop (green). The segment plays a central role in the activation status of many kinases as well as in the recognition of suitable substrates. The exceptionally flexible glycine-rich loop (pink) participates in the cosubstrate binding as well as γ-phosphoryl group transfer.

A key function in the coordination of both the α and β-phosphate groups of the cosubstrate is occupied by Lys68, which is localized on strand ß3. Moreover, Lys68 forms a crucial salt bridge with the nearby Glu81, thereby enabling EPKs to adopt an active conformation. This glutamate is part of the helix αC, which is located between the strands β3 and β4. It is a structural switching element in nearly all EPKs that can flip to an outward position (αC-out) upon the transition to an inactive state which disrupts the salt bridge described above [37]. Together with Asp175, which coordinates the β and γ-phosphate groups, and Asp156, which serves as a catalytic base to deprotonate the substrate's acceptor hydroxyl group, the aforementioned amino acids are so indispensable for the activity of the enzyme that their absence can be used as a reliable indicator for predicting catalytically inactive pseudokinases [38].

In accordance with its function, the region in which the catalytic aspartate is located is also referred to as the "catalytic loop", which is in close proximity to the so-called activation segment that extends from the DFG motif, or in the case of CK2 from the DWG motif, to the activation loop and

ends after the P+1 loop (Figure 3b) [39]. The most important positions of the substrate's consensus sequence can be located on the C-terminal or N-terminal of the phosphorylation site. For most CMGC kinases, these key substrate positions are usually in the N-terminal direction, referred to as the P+(1, 2,...) positions, and are recognized and bound in particular by the P+1 loop via amino acids with the same hydrophobicity or opposite electrostatic properties [35]. The activation loop plays a crucial role in the regulation of most EPKs [40]. Apart from the orientation of the helix αC, the activation loop is a second central switching element for the activity of many kinases. Typically, one or more residues in the activation loop must be phosphorylated before a catalytic event can occur. This results in conformational changes, and the substrate binding site becomes accessible. The phosphorylated residue forms electrostatic interactions with positive amino acids such as the arginine of the HRD motif, which is located one position upstream of the catalytic aspartate [41]. As a result of dephosphorylation, the activation segment can collapse into the active site of the kinases, leading to a blocked or partially blocked substrate binding cleft [42].

In addition to the previously mentioned structural elements of EPKs, this subsection will lastly deal with two well-conserved cross-domain features of EPKs. According to the spine theory, the C-lobe is connected to the N-lobe by two stacks of hydrophobic amino acids (Figure 4) [43]. The so-called catalytic spine or short C-spine is anchored to the hydrophobic helix αF, which is located in the center of the C-lobe. While the C-spine is complemented by the adenine ring of ATP, the second spine is only complete if helix αC is locked in an active position with an established salt bridge between Glu81 and Lys68 as described earlier. Since this salt bridge directly correlates with the activity state of the enzyme, it is called the regulatory spine or R-spine.

The two paralogs CK2α and CK2α' are characterized by structural peculiarities with respect to spine theory. The proteins' C-spines can either be completed by Phe121 or by the less hydrophobic Tyr125 in CK2α respectively Phe122 and Tyr126 in CK2α'. This represents a plasticity that has not yet been found in any other kinase. A third possible state is the complete opening of this region. In this case, neither of the two amino acids complement the C-spine, which in turn opens a narrow binding pocket, as resented in more detail in Subsection 1.2.1 and Chapter 7.

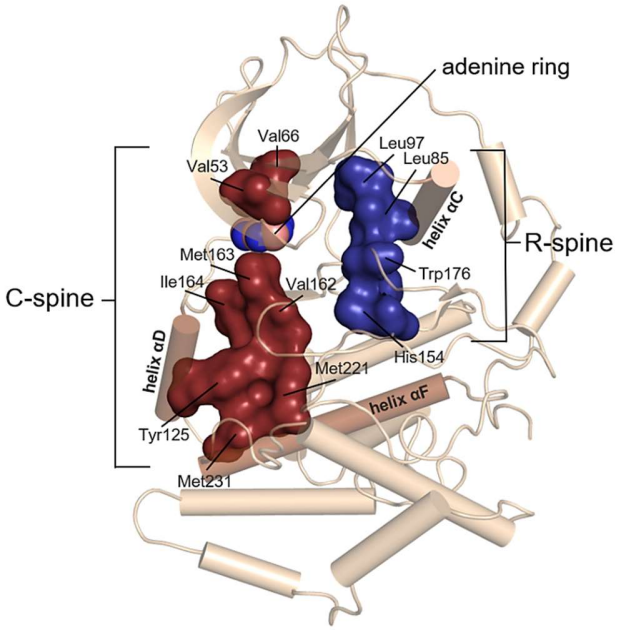


Figure 4. The catalytic spine (red) and regulatory spine (blue) of the eukaryotic protein kinase CK2α (*Zea mays*) are depicted as a surface representation (PDB 1LP4). The co-crystallized ATP analog, AMP-PNP, was trimmed in the illustration to the adenine ring, which completes the C-spine. The helices αC, αD, and αF serve as anchors for the structural integrity of the two spines. In the case of CK2α and CK2α', however, there is an unusually large plasticity in the region of the C-spine, insofar as it can be completed by either Phe121 or Tyr125 in CK2α and Phe122 or Tyr126 in CK2α' respectively.

1.1.2 Regulatory Mechanisms

As described in Subsection 1.1.1, EPKs play key roles in the regulation of almost all cellular processes and must therefore themselves be subject to strict control and regulation. Dysregulated EPKs can lead to various diseases with a wide range of symptoms including, among others, cardiovascular [44] or neurodegenerative diseases [45] as well as immunodeficiencies [46]. Particularly prevalent, however, are neoplastic diseases, which are the result of – or favored by – an acquired constitutive or otherwise abnormal activity of one or more EPKs [47] [48] [49]. In healthy cells, kinases are therefore regulated and kept in check by different mechanisms. In the following, this will primarily be exemplified by PKA, an extensively studied serine/threonine kinase of the AGC kinase group.

Cofactors and second messengers – Cofactors or second messengers including Ca^{2+}, cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), diacylglycerols

(DAG), or phospholipid derivatives such as IP_3 and PIP_3 are among the most important effectors for many EPKs [50]. Ca^{2+} is a particularly prominent example among EPKs, which even lent its name to the group of Ca^{2+}/calmodulin-dependent kinases (CAMK). However, not all kinases of this group are sensitive to the presence of high Ca^{2+} concentrations [51]. The death-associated protein kinase 3 (DAPK3), for example, is one of several kinase members for which the group name is misleading since DAPK3 does not even feature a calmodulin-binding site in contrast to its closest relatives DAPK1 and DAPK2 [52].

One of the earliest discovered modulations of protein kinases with second messenger molecules was the activating effect of cAMP on PKA, published in 1968 by Krebs *et al*. [53]. In its inactive state, PKA forms heterotetramers consisting of two catalytic subunits (C-subunits) and two regulatory subunits (R-subunits). The latter contains the substrate (consensus sequence: RRx**S**) or pseudosubstrate (RRxA/G) sequences to block the active site of PKA [54]. Furthermore, each of the two R-subunits features two high-affinity cAMP-binding domains. Even at nanomolar concentrations, up to four molecules of cAMP can bind to the R-subunits, reducing the affinity for the catalytic subunits significantly, eventually causing the complex to disassemble and allowing the PKA monomers to become active. To prevent an uncontrolled progression of phosphorylation, one of the substrates recognized by the enzyme is cAMP-phosphodiesterase which, upon activation by PKA, begins to convert cAMP to non-cyclic AMP, thus starting a negative regulatory feedback mechanism.

Protein-protein interactions – The influence of regulatory proteins represents a second commonly encountered mechanism. In contrast to PKA and its R-subunits, interaction partners do not necessarily have to be influenced by small molecules such as cAMP, nor does the interaction have to be inhibitory. For example, protein kinase CK2 only reaches its full activity via association with the regulatory CK2β dimer [55]. A similar picture emerges for most of the 20 human cyclin-dependent serine/threonine kinases, which can interact with a specific subset of cyclins, often depending on the prevailing state of the cell cycle [56]. However, the main difference between the previous two examples is that CK2 undergoes an increase in activity through the heterotetrameric structure, whereas the catalytic subunits are even active as monomers. For most CDKs, however, the cyclin-mediated change in activity is of a binary nature, i.e., the enzymatic activity can be entirely suppressed in the absence of the respective cyclins.

Oligomerization – Oligomeric kinases represent a special form of protein-protein interactions, which can be found among several kinase families. In particular, dimerization often occurs in receptor tyrosine kinases, followed by trans-autophosphorylation leading to a transition into an active state. Dimerization is usually initiated by the binding of a signal molecule to an extracellular

dimerization domain. A well-studied example of this mechanism is the binding of the growth hormone EGF, to the epidermal growth factor receptor (EGFR) [57] [58]. Subsequently, dimerization takes place, whereby the phosphoracceptor, typically a residue in the activation segment of the corresponding kinase, is inevitably located in close proximity to the active site of the adjacent kinase. An extremely high substrate concentration apparently arises due to the local proximity between the active site of the first kinase and the directly adjacent substrate. As a result, the trans-autophosphorylation reaction is usually very rapid. Surprisingly, this effect is so substantial that the surrounding amino acids of the phosphorylated position do not even necessarily have to match the inherent consensus sequence of the executing kinase, but can deviate significantly from it [59]. An example of this phenomenon is given, e.g., by CHK2 [60]. In addition to the effect of the locally increased substrate concentration, the kinase counterpart can also have a structurally stabilizing effect and can, for example, lead to a reorganization of the helix αC, whereby the R-spine becomes complemented as described in Subsection 1.1.1. Thus, certain kinases can enter an active state after binding, even if the binding partner is a pseudokinase with no enzymatic activity of its own. An example of this is represented by members of the rapidly accelerated fibrosarcoma (RAF) kinases, such as B-RAF or C-RAF, which convert into an enzymatically productive state after homo- or heterodimerization or side-to-side binding with pseudokinases such as KSR1 and KSR2 [61]. The different mechanisms behind oligomerization are also reflected in the diversity of the interaction domains of the kinases, and various interface regions between the monomers have been described in the literature, including side to side, back--to-back, and head-to-tail binding modes [62]. Moreover, the number of kinase molecules involved in the oligomer is also not limited to two. For example, the kinase CaMK2 forms dodecamers, which trans-phosphorylate very rapidly at appropriate Ca^{2+} concentrations [63].

Post-translational modifications – Another regulatory mechanism, which can again be demonstrated using the example of PKA, was already mentioned in the previous passage about kinase oligomerization. It concerns the phosphorylation in important catalytic and regulatory regions of the kinases. Like most other proteins, kinases are also subject to post-translational modifications, and as previously described, some CMGC kinases may have a phosphotyrosine in the glycine-rich loop that effectively inactivates the kinase (see Subsection 1.1.1). Similarly, phosphorylation sites in the activation loop often determine whether the entire activation segment is in an active or inactive conformation. Many kinases require one or more phosphorylations in this region to be catalytically active, including PKA, for which an important phosphothreonine (p-

Thr198)[c] has been described [64] [65]. Based on crystal structures, it was shown that p-Thr198 is essential for a stable active conformation, as it establishes important polar contacts to His88 of helix αC as well as further interactions with Lys190 of beta-strand β9, and Arg166 of the catalytic loop (Figure 5). Helix αC and the DFG motive are hereby stabilized in an αC-in/DFG-in position that ensures the complementation of the R-spine. In addition, the corresponding Asp185 is pulled into a position that is favorable for the binding of ATP and both magnesium ions. In accordance with the structural data, site-directed phosphomimetic mutagenesis of Thr198 to Asp198 in PKA by Adams *et al*. [66] resulted in an activity increase of two magnitudes and there are, in fact, numerous examples in which even multiple phosphorylations are required to increase kinase activity [67] [68]. In contrast, other kinases such as CK2 lack serines or threonines as potential phosphoacceptor sites in the described region whereby these are not mandatory for an active conformation.

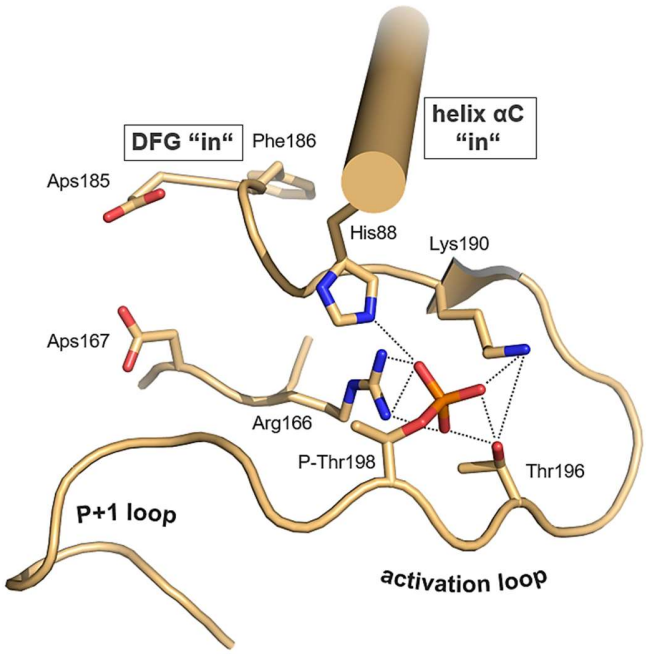


Figure 5. The human prototype kinase PKA exhibits a crucial phosphorylation of Thr198 in the activation loop, which is required for the activation segment to adopt an active conformation (PDB 3OOG). Polar contacts are indicated as dashed lines. Equivalents to p-Thr198 have been identified in a large number of different EPKs.

[c] The amino acid Thr198 is often referred to as Thr197 in the older literature, which depends on whether or not the initial starting methionine is included in the numbering.

Localization within the cell – Finally, the possibility to regulate kinase activity via subcellular localization should be mentioned. There are at least 50 different A-kinase anchoring proteins (AKAPs) that direct PKA, together with its regulatory subunits, to discrete cellular compartments and thereby limit the availability of potential substrates within the kinase's sphere of influence. AKAPs are a diverse group of structurally highly diverse proteins that primarily function as binding platforms for the formation of multi-protein complexes [69].

1.2 Protein Kinase CK2

1.2.1 CK2 Represents an Unusual Member of the EPKs

The serine/threonine kinase CK2, formerly known as casein kinase 2, is a member of the CMGC group of EPKs. The enzyme is closely related to the mitogen-activated kinases (MAPK), the glycogen synthase kinase 3 (GSK3), and the CDKs that are of utmost physiological relevance, especially in terms of cell cycle control and transcriptional regulation [70]. Comparable biochemical importance is also attributed to CK2. A clear indication of its key role is already provided by the high degree of conservation and the ubiquitous expression of the enzyme among eukaryotes [71]. This is supported by knockout experiments with CK2α in yeast [72], slime molds [73], and mice [74] [75], which demonstrated that the enzyme's activity appears to be vital for eukaryotes. Compared to other EPKs, CK2 is characterized by some peculiarities of a structural and functional nature that are highlighted in this subsection.

Tetrameric holoenzyme structure – The heterotetrameric holoenzyme structure of CK2 (Figure 6a), is one of the most striking and, from an evolutionary point of view, very interesting feature, which makes this protein kinase unique. It is composed of two catalytic subunits, CK2α or its paralog CK2α', and two regulatory CK2β subunits. CK2β does not exhibit any significant sequence homologies with other known proteins except for the stellate protein of *Drosophila melanogaster*, which when expressed in male specimens results in an *in vivo* crystal formation influencing spermatogenesis [76]. CK2β forms a central dissociation-stable dimer, which is complexed at opposite sides through a relatively small contact area of 832 $Å^2$ by the N-lobes of the two catalytic

subunits [77]. Binding of CK2β results in both an increase in catalytic activity and a certain level of protection against thermal and chemical denaturation [78] [79] [80].

While most EPKs are monomers in their active state, there are some exceptions such as the receptor tyrosine kinases described in Subsection 1.1.2, which dimerize upon binding of a suitable ligand and become active after autophosphorylation. The insulin receptor IGF-1R can be mentioned as an example of a kinase with a heterotetrameric structure [81]. It consists of two extracellular α-subunits, which act as binding sites for insulin, and two transmembrane β-subunits, which exert intracellular tyrosine kinase activities. However, unlike CK2, both subunits are covalently linked via disulfide bridges. Moreover, as indicated in Subsection 1.1.2, PKA also features a heterotetrameric structure but, in contrast to the CK2 holoenzyme, these complexes are entirely inactive, whereas CK2 is enzymatically active as a monomer and as a heterotetramer. Early experiments in which isolated CK2α was detected in large amounts in nuclei of animal cells, already indicated that both forms could coexist in cells [82]. This assumption is consistent with the empirical K_d value of 5.4 nM for the α/β-interaction, first measured by Martel *et al*. [83] via plasmon resonance spectroscopy and later confirmed by Raaf *et al*. [80] via isothermal titration calorimetry (ITC), which revealed the transient nature of the complex. A dynamic equilibrium between the monomeric and heterotetrameric forms was therefore suspected early on and was later supported by live-cell imaging [84] studies as well as an observed imbalance in subunit expression [85], whereby the tetrameric form appears to be predominant *in vivo* [86].

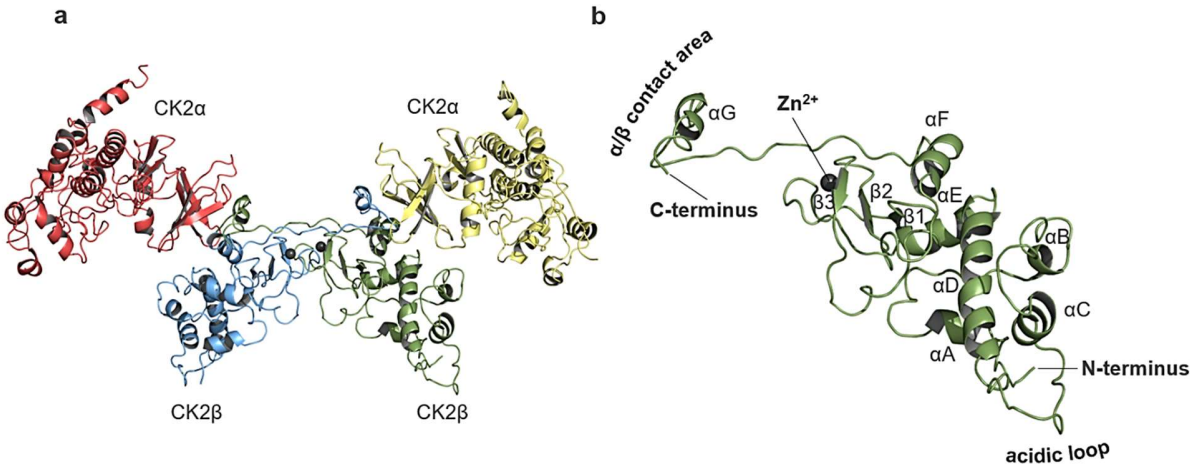


Figure 6. The CK2 holoenzyme structure (**a**) consists of two catalytically active CK2α or CK2α' subunits (red and yellow) and two regulatory CK2β subunits (blue and green), which form a dissociation-stable dimeric core of the heterotetramer (PDB 1JWH). (**b**) CK2β interacts to a significant extent with the CK2α subunits via its C-terminus and with helix αF. It also possesses a zinc-binding motif, which is important for the dimerization of CK2β, and a highly acidic loop in the N-terminal region.

Substrate recognition and pleiotropy – Interestingly, the holoenzyme assembly of CK2 leads to a modulation of the substrate preferences compared to isolated CK2α or CK2α'. Accordingly, the known substrates of CK2 were grouped into three substrate classes defined by Pinna [87]. Class I substrates can be recognized and phosphorylated by the monomer or the holoenzyme. On the other hand, class II substrates can only be phosphorylated in the absence of CK2β. A well-studied example from this class is calmodulin, for which a strong inhibitory effect of CK2β has been verified several times [88] [89]. In contrast, only the heterotetramer is capable of phosphorylating class III substrates, such as the guanine nucleotide-exchange factor eIF2β that regenerates the eukaryotic initiation factor eIF2 by an exchange of GDP against GTP [90]. Due to a lack of ternary complex structures with suitable substrates, the exact mechanisms behind these shifts in substrate specificity depending on the presence of CK2β are yet to be elucidated. However, there is some evidence that the acidic loop of CK2β (Figure 6b) interacts with basic regions of potential substrates, thus bringing the phosphorylation site into spatial proximity to the active center of CK2α [91]. Moreover, steric reasons could serve to explain the rejection of class II substrates by the holoenzyme, since the CK2β dimer evidently has a certain space requirement that might not be compatible with some substrates.

In general, CK2 can be described as an extraordinarily pleiotropic EPK with a constantly growing list of several hundred substrates that have been identified, at least *in vitro* [92] [93] [94]. All these substrates have an acidic sequence in common, which is required for the recognition by CK2. The corresponding consensus sequence can be defined as (**S**/**T**-D/E-X-D/E) with "**S**/**T**" being the phosphorylated serine or threonine [95]. The positions P+1 and P+3, which demand acidic amino acids, are particularly important and characteristic of CK2 activity [96]. The outstanding pleiotropic character results from the rather short consensus sequence containing certain degrees of freedom whereby "X" can represent any amino acid in this context. However, as shown by Meggio *et al*. [94], acidic amino acids are also significantly favored in the P+2, P+4, P+5, P+6, and even P+7 positions. Due to its distinct pleiotropic character, CK2 is involved in a remarkable number of cellular processes including, in particular, aspects of cell proliferation [97], apoptosis [98] [99], angiogenesis [100] [101], DNA damage response [102] [103], viral infections [104], and many more. In addition to the phosphorylation of alcoholic side chains, some reports of phenolic substrates have been published [105] [106]. While most observations were made *in vitro*, there is also evidence of tyrosine phosphorylation in mammalian cells, although the physiological significance of this finding remains unclear [107].

Constitutive activity – Another hallmark of CK2 is its constitutive activity, a phenomenon that contradicts the principles of strict EPK regulation highlighted in Subsection 1.1.2. The question arises concerning which structural characteristics of CK2 lead to such constitutive activity. A distinctive difference from other EPKs is the DWG motif, which is located in the activation loop of CK2 instead of an EPK-typical DFG-sequence. The frequently observed conformational plasticity of the DFG-sequence region in EPKs, which correlates with the activity state of kinases, has never been observed in CK2 in the numerous structures derived to date [108]. Based on crystal structures, it was demonstrated that the nitrogen in the indole ring of Trp176 forms an additional hydrogen bond with the backbone oxygen of Leu173, which locks the activation loop in an active "DWG-in" conformation [109]. A second critical reason for the constitutive activity is the conformation-stabilizing contact between the activation segment and the N-terminus [110]. The corresponding interactions include hydrophobic attractions as well as strong hydrogen bonds, e.g. between Tyr182 of the activation loop and the backbone nitrogen of Ala9 or the side chain of Asn16. These interactions contribute significantly to the observed rigidity in the canonical key structural elements of CK2, which exhibit regulatory plasticity in other EPKs.

Regulation – Unlike other EPKs, in the case of CK2 no classical secondary messengers, such as cAMP or Ca2+, that cause an enzymatic deactivation have yet been found. Phosphorylation in the

activation loop is also not required, and the catalytic subunits are constitutively active both with and without the contribution of CK2β. This raises the question of how the concept of a permanently active kinase is compatible with the extensive regulation of so many target proteins and biochemical pathways. As an interim conclusion, all observations and structural findings to date point to the central assumption that CK2 is indeed always active in eukaryotic cells. However, the constitutive activity must not lead to the misconception that CK2 is not subject to any fine regulation. In addition to the activating effect of CK2β, the holoenzyme is also capable of undergoing supramolecular oligomerization to form $(CK2\alpha_2\beta_2)_n$ ring-like structures and filaments (Figure 7) [111]. A distinction can thus be made between a primary interaction between the CK2 subunits and a secondary interaction between the holoenzyme units based on electrostatic forces. While the intrinsic activity of CK2 decreases with increasing salt concentration, higher ionic strengths disrupt the secondary interactions between the holoenzyme units. The enzyme's active centers are thus more accessible as isolated heterotetramers. These two opposing trends result in a concentration optimum of approximately 300 mM of NaCl. Depending on the salt concentration, the superstructures can be entirely converted into each other. Especially compounds with primary amines such as spermine or polylysines were found to stimulate CK2 activity [112] [113]. However, the physiological relevance as well as the question of a general regulatory mechanism behind these high-ordered structures is not fully clarified.

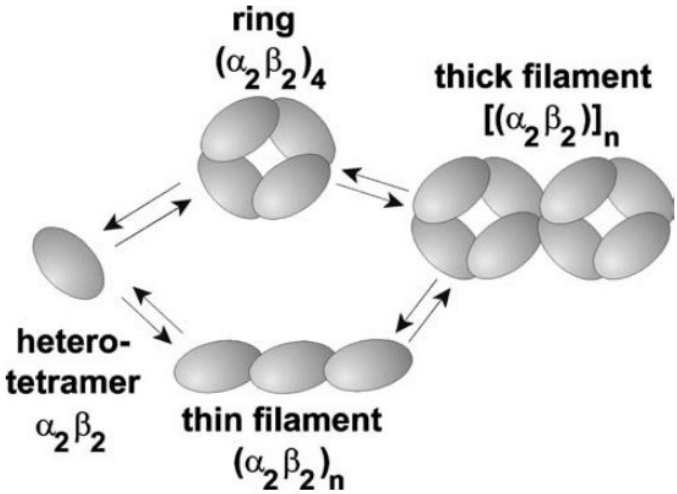


Figure 7. CK2 occurs in different higher-order aggregates depending on the ionic strength of the solvent. Low salt concentrations up to 0.1 M stabilize the formation of filamentous structures. Between 0.1 M and 0.2 M, ring-shaped aggregates are observed, which reversibly dissolve again to isolated heterotetramers at higher salt concentrations. Adapted from Niefind *et al*. [111] based on data from Valero *et al*. [113].

Moreover, it is known that CK2 can to some extent be regulated by a variety of intrinsic and extrinsic posttranslational modifications at different residues [114] including, for instance, phosphorylations at Ser2, Ser3, or Ser4 on CK2β [115]. In addition to activating covalent modifications such as phosphorylation and acetylation, inactivating SUMOylation as well as an O-GlcNAc glycosylation at position Ser347 that suppress a potentially activating phosphorylation by CDK1 have been described [116]. Numerous extrinsic modifications as well as protein-protein interactions further expand the regulatory "fine-tuning-spectrum" of CK2. Among the best-known interaction partners, to name but a few, are the positive regulatory proteins MEK [117], cyclin-dependent kinase inhibitor 1 (p21) [118], histone chaperone facilitates chromatin transcription protein (FACT) [119], cluster of differentiation 5 (CD5) [120] or the casein kinase interacting protein 1 (CKIP-1) [121], which, interestingly, only binds to CK2α but not to CK2α'. As counterexamples, the beta-catenin controlling protein adenomatous polyposis coli (APC) or the isomerase peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) can be highlighted as restraining interaction partners [122]. Overall, a very complex and multifactorial picture emerges, showing that CK2 can be fine-tuned on many levels, including that of supramolecular organization, post-translational modifications, and diverse protein-protein interactions. In addition, there is also evidence of regulation at the transcriptional and translational levels [123].

Dual cosubstrate specificity – For the actual process of phosphorylation, CK2 can recruit not only ATP but also GTP as an energy-rich phosphate source. In 2013, a study encompassing more than 200 kinases was conducted by Becher *et al*. [124] with the purpose of determining kinase affinities for the cofactors ATP, ADP, and GTP. The investigated kinases were derived from cell culture extracts of two human cell lines and were mixed with the cosubstrates of interest. Subsequently, an immobilized, promiscuous kinase inhibitor mixture (kinobeads^{TM}) was used to compete with the free nucleotides to enrich those kinases with unsaturated cosubstrate binding sites [125]. Apart from CK2, for which a dual cosubstrate specificity had been described much earlier [126], the kinases MEK1 and B-RAF attracted attention in the screening. However, in follow-up experiments, B-RAF was found to be significantly less able to utilize GTP as a phosphate source compared with CK2, and MEK1 emerged as entirely incapable of doing so [124]. Although there are indeed very few counterexamples in the literature, the ability to effectively utilize both ATP and GTP thus appears to be an exception among kinases [127] [128]. The structural aspect that allows CK2 to utilize GTP lies in the hinge region of the protein and was elucidated by co-crystallization with GMP-PNP (Figure 8a) by Niefind *et al*. [129] in 1999. Sequential differences compared to other kinases in this region up to the end of the helix αD ensure that the purine ring of the GTP can rotate outward by a few degrees and form a hydrogen bond with the main chain

of Val116. This provides the necessary space for a water molecule in the binding pocket to move into the position normally occupied by the exocyclic nitrogen of the purine base. Consequently, the water molecule creates an additional hydrogen bond between the GTP and the binding pocket, thus mimicking the binding situation that occurs in the case of ATP binding.

Unique plasticity of helix αD – The hinge region is not only of interest because it allows dual cosubstrate specificity, but also because of its uniquely high adaptability that includes the adjacent helix αD [130]. In previous studies, it has repeatedly been observed that the C-spine of CK2α can be complemented either by Phe121 (closed conformation) or partially by Tyr125 (open conformation) of helix αD (Figure 8b) [131], depending on the concentration of kosmotropic salts in the respective crystallization approach (see Chapter 3) [132]. Upon conformational opening, the N-terminus of the helix αD is tilted away from the cosubstrate binding site, thus increasing the access to a binding pocket, which, however, is still substantially occupied by Tyr125. In the search for selective CK2 inhibitors, the open conformation has recently attracted increasing attention (see Subchapter 1.3.2) [133]. Indeed, it was shown that suitable organic molecules can induce further conformations that, in extreme cases, can even lead to a melting of the entire helix αD (see Chapter 7). This plasticity has been observed for CK2α and more recently for CK2α', and is a feature that distinguishes the two proteins from all other EPKs according to the current state of knowledge.

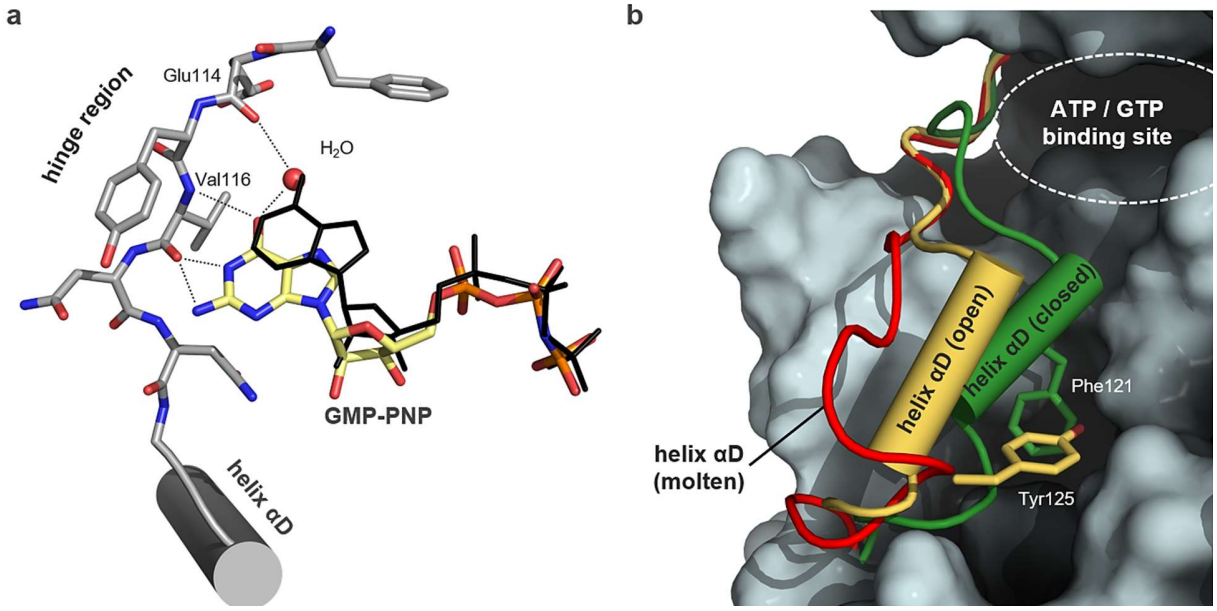


Figure 8. Hinge region with the adjacent helix αD. (**a**) Co-crystallization of the GTP analog GMP-PNP with CK2α from *Zea mays* (PDB 1DAY). For comparison, the orientation of the ATP analog AMP-PNP from a reference structure was inserted as black sticks (PDB 1LP4). Associated with a well-coordinated water molecule, GTP is able to mimic the binding mode of ATP (**b**) Helix αD and parts of the hinge loop of CK2 can adopt an open (yellow) (PDB 1PJK) or closed (green) conformation (PDB 3BQC) depending on the salt concentration. Binding of αD pocket-affine compounds (molecule not shown) can even melt the helix entirely (red) (PDB 7ATV).

1.2.2 CK2α and CK2α': Two Paralogous Catalytic Subunits With Distinct Differences

CK2α and CK2α' are paralogous catalytic subunits of CK2 which are encoded by the genes CSNK2A1[d] and CSNK2A2, located on chromosomes 20 (p-arm) and 16 (q-arm), respectively [134]. Both proteins are characterized by a remarkably high similarity on the primary structure level in the conserved catalytic EPK core. However, a major difference can be found in the C-terminal region of CK2α', which is, compared to CK2α, truncated by 41 amino acids. An overall sequence identity of 75% can thus be calculated if the C-terminus is taken into account and 86% if only the canonical core from sequence positions 1–330 is compared (see Figure 2 in Chapter 4). Interestingly, the C-terminal extension of CK2α contains four alcoholic residues (Thr344,

[d] The designations used in 1994 by Yang-Feng *et al*. for the CK2α and CK2α'-coding genes differ from those used today, as CK2α was referred to as CSNK2A and CK2α' as CSNK2A1.

Thr360, Ser362, and Ser370), which are temporarily phosphorylated by CDK1 in a cell cycle-dependent manner, especially during the prophase and metaphase [135] [136]. St-Denis *et al*. [137] further demonstrated the physiological relevance of this by showing that phosphomimetic point mutants of the corresponding amino acids resulted in mitotic catastrophes, thereby leading to cell death. Consistent with the high sequence identity, except for the C-terminus, the two kinases are structurally also almost identical to an EPK-typical architecture. The most striking differences can be found in the loop region between beta strands β4 and β5, where CK2α' prefers an open conformation, which is usually observed in holoenzyme structures, whereas CK2α tends to adopt a closed conformation [138]. Accordingly, CK2α must first undergo a conformational change within this region prior to CK2β dimer binding.

The cosubstrate binding pocket region differs only in two amino acids, His115 and Val116 in CK2α versus Tyr116 and Ile117 in CK2α',[e] both located in the hinge region. Although this observation is interesting at first, especially with regard to the coordination of nucleotides and possible pharmacologically applicable ATP-competitive compounds, the difference is difficult to exploit, since the corresponding interactions with the hinge region are usually mediated by the main chain backbone and not by the side chains [139]. In fact, there are only a few reports of small molecules in which a slight discriminatory interaction with the paralogs has been observed [140] [141] [142] (see Chapter 4).

Moreover, the characteristics mentioned in Subsection 1.2.1 fully apply to both proteins, i.e., both kinases form heterotetrameric structures with CK2β, both are extremely pleiotropic and acidophilic, both are constitutively active and can use GTP as a cosubstrate, and both show the described hypermobility of the helix αD. From a medical point of view, both kinases are known to be unfavorable prognostic markers for certain cancers (see Subchapter 1.2.3). Considering all these similarities, it seems very surprising that, since the discovery of protein kinase CK2, the literature has been flooded with findings concerning CK2α, whereas its paralog, CK2α', is largely overlooked. Hence, CK2α' was not included in a large number of experiments performed with CK2α, which raises the question of how such an uneven distribution of attention can be explained.

To address this question, further differences between the paralogs need to be discussed:
(I) A fundamental difference between the two subunits, which has significantly slowed down research efforts for a long time, was the poor solubility of CK2α' compared to CK2α and the resulting challenges in recombinant expression and purification. A preliminary approach was

[e] The numbering of the amino acids of CK2α and CK2α' is shifted by one position due to an insertion of a single amino acid at the N-terminus of CK2α'.

provided by Nakaniwa *et al*. [143], who generated a soluble version of CK2α' by truncating the C-terminus upstream of Gln334. To retain the C-terminal part, Bischoff *et al*. [138] later introduced the point mutant Cys336 to Ser336, which prevented the full length protein from dimerizing by a disulfide bridge but still allowed its full catalytic functionality.

(II) A publication in 1999 by Xu *et al*. [74] shifted the focus significantly toward CK2α. The authors showed that knockout of CK2α has lethal consequences for mice embryos during midgestation because of heart and neural tube defects, whereas knockout of CK2α' only leads to infertility in males due to severe globozoospermia. This created the impression that the physiological importance of CK2α' is minor and could at least partially be replaced by CK2α activity. However, the sheer conservation of both proteins in many species, especially in vertebrates, and the fact that paralog-specific interaction partners have been found provides strong evidence that both proteins have their own essential roles in the organism and are therefore worth studying [144].

(III) Also in 1999, another article by Guerra *et al*. [145] was published in which transcriptional and translational levels of CK2α and CK2α' in different tissues were qualitatively investigated using Northern and Western blots. The respective publication has repeatedly been cited up to the present date, giving the impression that CK2α' is only present in testis and brain tissue, whereas CK2α is omnipresent. However, according to more recent findings, this impression is no longer tenable as countless data sets are available underlining the fact that both paralogs are present in all tissues, both as mRNA (Figure 9) [146] and protein (Figure 10) [147], although the levels of CK2α' are usually slightly lower in most tissues.

(IV) Finally, the aspects of structural elucidation and crystallization behavior should be addressed. For a long time, structural insights concerning CK2α' were either not available or very limited because CK2α' was a crystallographically very problematic candidate, which always crystallized in the form of unusable fine and fragile needles. A large number of different crystallization conditions for CK2α' were found but the morphological results as well as the diffractive power were consistently poor - a fundamental problem which is an essential subject of the research efforts summarized in this thesis. Further information on this challenging issue and the solutions that have been developed for a reliable method of CK2α' crystallization with atomic resolution can be found in Chapter 4.

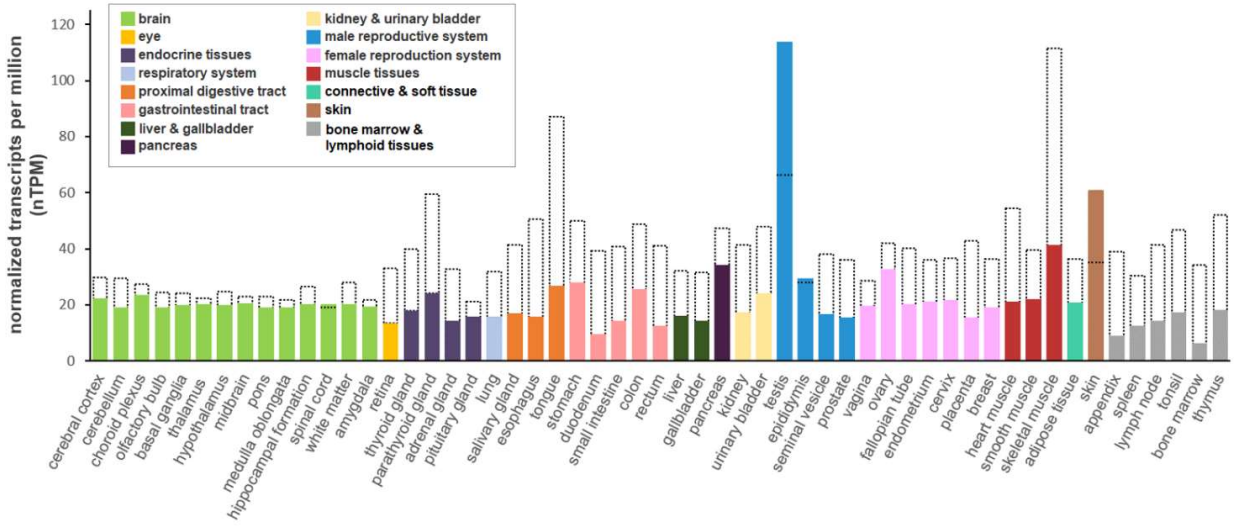


Figure 9. Overview of the average normalized transcription levels of human CK2α' (colored bars) compared with human CK2α (black dashed bars) in different tissues. The tissues shown are color-coded according to their function and respective organs in the human body. Data were taken from the Human Protein Atlas without modification and are based on entries from the Human Protein Atlas database (HPA) and the Genotype Tissue Expression Project (GTEx) [293]. Prior to publication in the protein atlas, noncoding RNAs were subtracted. Subsequently, the data were scaled to one million to ensure the comparability of datasets from different sources. The values shown are average normalized transcripts per million (nTPM) and standard deviations were not available in the database.

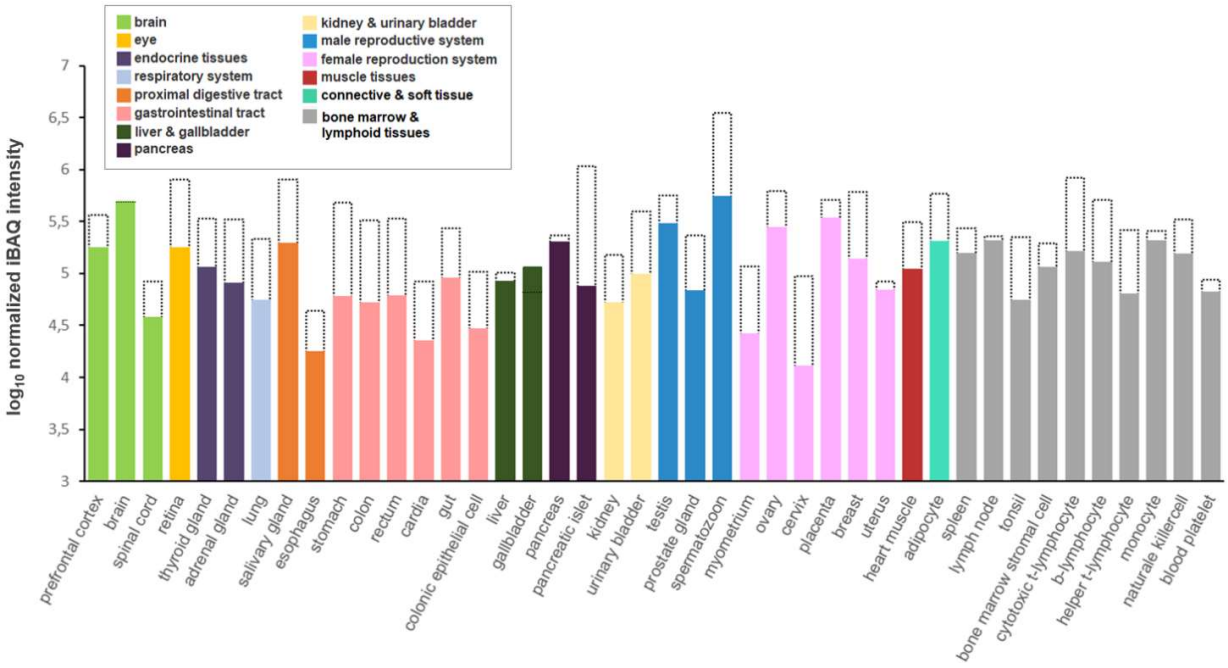


Figure 10. Overview of normalized median CK2α' expression levels (colored bars) in different tissues compared to CK2α (black dashed bars). The tissues shown are color-coded according to their function and respective organs in the human body. MS1-based values, processed by the iBAQ method, were obtained without further modification from the Proteomics DB database[f]. Standard deviations are only incompletely annotated in the database and can be looked up there [294].

1.2.3 Role of CK2 in Cancer

Due to its distinct pleiotropy, CK2 is involved in a variety of biochemical processes, which in turn leads to its role in a plethora of diseases, among others neurodegenerative diseases such as Alzheimer's disease [148], Parkinson's disease [149], amyotrophic lateral sclerosis [150], and Huntington's disease [151]. In line with this, two neurodevelopmental disorders, termed Okur-Chung neurodevelopmental syndrome (OCNDS) and Poirier-Bienvenu neurodevelopmental syndrome (POBINDS), which are predominantly caused by de novo point mutations in CK2α and CK2β, should also be mentioned [152]. In addition, a link has been found between CK2 activity and inflammatory processes [153] [154], diabetes mellitus [155], obesity [156], cardiovascular diseases [157], and infections with a wide spectrum of bacterial [158] and viral pathogens [159] [160] [161], including the proliferation of the severe acute respiratory syndrome coronavirus 2

[f] https://www.proteomicsdb.org; version 3.0; accessed 04/13/22 - 11:15

(SARS CoV 2) virus, which has unleashed the most severe global pandemic of the 21st century to date (Subsection 1.2.4). However, apart from the listed diseases several others [162], the impact of CK2 on the progression of various types of cancer has been studied by far most extensively since malignant neoplastic diseases are considered the most promising pharmacological indication for CK2-inhibitor-based-therapies.

Cancer is one of the leading diseases with the highest mortality in the developed world, with approximately 19.3 million new cases and 10 million deaths in 2020, and the number of diagnosed cancer cases is expected to increase to 28.4 million in 2040 [163]. The main difficulty in developing new chemotherapeutics for effective therapy is that cancer is not a single disease, but rather a complex and diverse group of hundreds of diseases with different manifestations and causes. What all these neoplasms have in common is their uncontrolled proliferation and invasiveness, the ability to thrive under low oxygen levels, to procure nutrients via blood vessel formation, and their escape from the immune system [164]. To ensure the optimal utilization of a new drug for chemotherapy, a common molecular target is needed that applies to as many different cancer types as possible and allows a certain selectivity over benign cells.

The search for a “magic bullet” against cancer was fueled by CK2 research since highly elevated CK2 levels were detected in several tumors originating from different tissues [165] [166] [167] [168] [169]. This raised the hope that CK2 might be the desired common denominator for many types of cancer, which could subsequently be pharmacologically exploited. CK2 has long been known for its ability to suppress apoptosis [170] and its massive stimulation of proliferation and angiogenesis [101], whereby the levels of CK2 activity correlate significantly with the degree of malignancy [171], the resistance to chemotherapeutic agents [172] [173] [174], the rate of progression, and the metastatic spread in advanced stages of cancer [175]. In this context, it has been demonstrated multiple times that CK2 overexpression has not only diagnostic but also prognostic qualities [176] [177] [178] [179] [180]. It was further suggested that during tumor evolution, a spiral-like process sets in, in which higher CK2 levels dictate a more aggressive phenotype, while at the same time these cells fall into a certain dependency on such excessive CK2 activity [181]. This dependency is sometimes referred to as CK2 “addiction,”, a term originally coined by Bernard Weinstein in the context of oncogenes in general [182]. At this point, however, it must be clearly emphasized that CK2 is not a proto-oncogene by a classical definition, since CK2 is characterized by its constitutive activity even without any sequence alterations. So far, no CK2 gain-of-function mutant has been reported that is responsible for a neoplastic transformation of healthy cells. Nevertheless, the available data suggest that CK2 activity generally creates a

tumor-driving environment, as several key players of tumor-promoting signal transduction pathways are recognized as substrates by CK2. Major signaling pathways affected by CK2 include the nuclear factor “κ-light-chain-enhancer” of activated B-cells (NF-κB) [183], the JAK2/STAT3 [184], the PI3K/Akt [185], the Wnt/β-catenine [183], the Hedgehog pathway [186], and various DNA damage response mechanisms [187]. Moreover, caspase substrates are phosphorylated near the recognition site by CK2 and thus lose their suitability and can no longer be cleaved to initiate apoptotic events [188]. CK2 activity can therefore be considered as a direct antagonist of caspases. Moreover CK2 was found to activate the chaperone CDC7 which in turn stabilizes many true oncokinases in their active states [189]. In other words, even if one or more true oncogenes were originally the underlying trigger for the cell’s malformation, it may nevertheless become dependent on CK2, a phenomenon also known as “non-oncogene addiction” [190].

In summary, although CK2 is neither the cause nor the consequence of cellular malignancy, an overexpression of CK2 promotes tumorigenesis by the stimulation of multiple pro-survival and anti-apoptotic signals. However, to be more precise, overexpression typically refers to CK2α and CK2β, whereas CK2α’ can either be upregulated or downregulated depending on the type of cancer, which again is indicative of different physiological roles of the two paralogs, even in cancer cells [180]. These different functions were also pointed out by Vilk *et al*. [191], who overexpressed death mutants of CK2α and CK2α’ in malignant osteosarcoma cells, with a reported loss of viability by overexpressed CK2α’, whereas this was not observed for CK2α. In addition, Brown *et al*. [192] also demonstrated that the downregulation of CK2α in squamous cell carcinoma of the head and neck resulted in marked sensitization to suboptimal doses of cisplatin. A lower proliferation rate, apoptosis susceptibility, and impaired cell migration were also observed by the authors in a wounding assay, whereby none of these observations applied for CK2α’. In contrast, there are some reports of certain cancer types that rely heavily on CK2α’ activity. For example, in 2016, Liu *et al*. [175] reported that CK2α’ drives metastasis in non-small cell lung cancer, thereby adopting a role of which CK2α is incapable. This is due to a paralog-specific interaction between CK2α’ and breast cancer metastasis suppressor 1 (BRMS1), whereupon the latter is phosphorylated at Ser30, subsequently exported from the nucleus, and degraded in a ubiquitin-mediated manner.

Currently, different CK2 inhibitors are undergoing clinical trials with encouraging preliminary results such as the orphan drug silmitasertib, formerly CX-4945 (Figure 11a/c), which has completed phase II clinical trials [g] for hard-to-treat bile duct cancer, also known as cholangiocarcinomas [193]. However, the fundamentals of these promising CK2 inhibitors – along with the whole concept of CK2 as a therapeutic target – have recently been challenged by an ATP-competitive inhibitor of extraordinary selectivity. The inhibitor SGC-CK2-1 (Figure 11b/c) recently introduced by Wells *et al*. [194] is based on a pyrazolopyrimidine scaffold and showed an IC_{50} value of 16 nM and 36 nM for CK2α and CK2α', respectively, determined via NanoBRET™ assay [195]. The compound is thus comparably potent to silmitasertib, but the number of detected off-targets is unsurpassed with only five hits out of a tested panel of 403 kinases. The compound is thereby even more selective than the current gold standard of CK2 inhibition, silmitasertib, which already stands out from many other inhibitors due to its excellent selectivity with 28 significant off-targets. Unexpectedly, in an extensive assay with 140 cell lines from 18 different tissues, SGC-CK2-1 showed very poor cytotoxicity for almost all cancer cell lines, thereby calling the general suitability of CK2 inhibitors and the indispensability of the physiological role of CK2 for cellular processes in cancer cells into question [194] [196]. This led to the idea that previous therapeutic successes with CK2 inhibitors may be due to the unintended off-target-inhibition of other oncologically relevant kinases. However, on the basis of all currently available data regarding the involvement of CK2 in tumor biology, Salvi *et al*. [197] strongly warned against hasty conclusions based on the findings of Wells and coworkers [194].

[g] https://www.ClinicalTrials.gov Identifier: NCT02128282

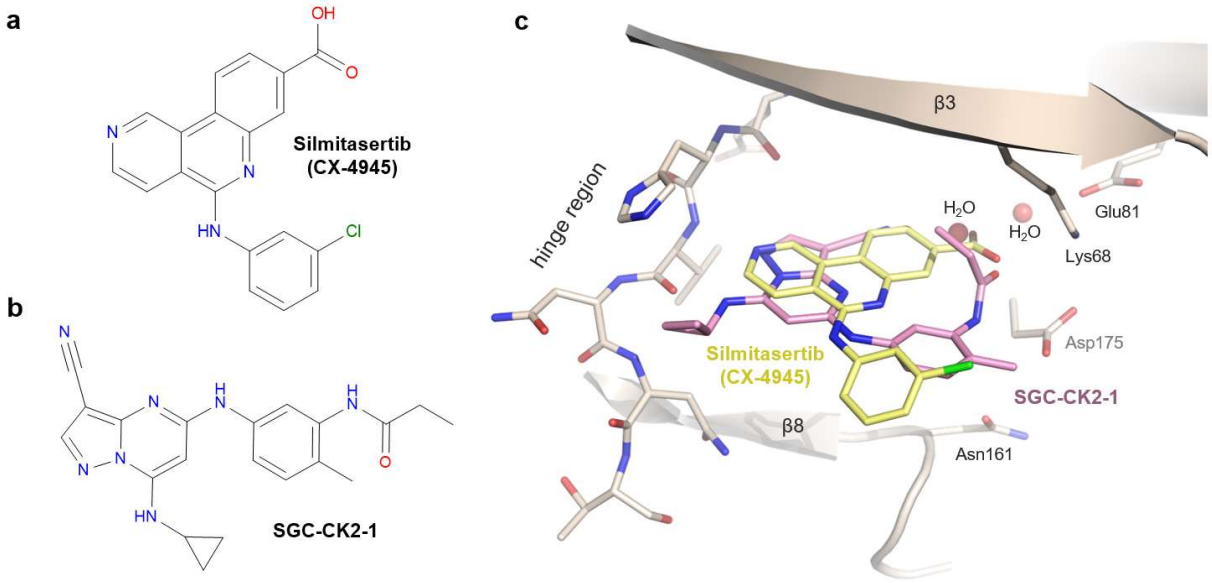


Figure 11. The structures of the two CK2 inhibitors (a) silmitasertib and (b) SGC-CK2-1 are shown. Silmitasertib has held an orphan drug status for the treatment of cholangiocarcinoma since 2017 and is currently being tested in further clinical trials for its efficacy against SARS-CoV-2, among other indications. SGC-CK2-1 has even greater selectivity compared to silmitasertib but has not been shown to be very effective in inhibiting the proliferation of various cancer lines in cell studies to date. (c) Both inhibitors bind to the cosubstrate binding site of CK2 (superimposition of the structures PDB 6z83 with SGC CK2 1 [pink] and PDB 3PE1 with silmitasertib [yellow] is shown).

In summary, three major points were criticized regarding the publication from Wells *et al*. [194]. (I) Non-pharmacological downregulation of CK2, e.g., by using siRNA or overexpression of dead mutants, has repeatedly been found to decrease survival and proliferation rates and to increase susceptibility to chemotherapeutic agents [198]. Moreover, attempts to generate tumor cells entirely without CK2 subunits have consistently failed.

(II) Furthermore, the fact that CK2 is massively overexpressed in all cancers was not sufficiently considered by Wells *et al*., and a random occurrence of this hallmark without physiological significance seems very implausible.

(III) Finally, insufficient data and misinterpretation of previous data were criticized. Thus, a discrepancy between the *in vitro* inhibition of recombinant CK2 and the required concentration for cellular inhibition of proliferation is also present for silmitasertib (K_i of 223 pM [199] but antiproliferative effect only at several magnitudes higher concentration). Therefore, the expected cytotoxicity may have been inconsistent with respect to the tested concentrations. Furthermore,

as a counterexample, it was pointed out that a silmitasertib derivative has recently been described, which exhibits increased selectivity along with an improved inhibition of tumor cell proliferation [200].

While the search for novel potent and selective CK2 inhibitors was also a central component of this thesis, whether or not the inhibition of CK2 via small molecules will ultimately prove to be a successful strategy for therapeutic purposes cannot be fully clarified at this point. Nevertheless, increasingly selective inhibitors, possibly including paralog-specific inhibitors, provide valuable probes for fundamental research regarding the biological functions of CK2.

1.2.4 A Possible Role of CK2 in SARS-CoV-2 Infections and Covid-19

Since the end of 2019, the world has been gripped by the COVID-19 pandemic. It is the most devastating pandemic of the 21th century to date, with severe economic consequences and over six million confirmed deaths so far (as of December 2022). The rapid spread of infections prompted the World Health Organization (WHO) to declare a “Public Health Emergency of International Concern” as early as January 30, 2020, one month after the discovery of the novel virus that causes COVID-19 [201]. This respiratory syndrome is caused by the SARS-CoV-2 virus, an enveloped (+) single-stranded ribonucleic acid (ssRNA) virus (Baltimore class IV) from the genus of Betacoronavirus. With more than half a billion people infected by April 2022, there is a constant risk of overburdening the healthcare systems around the world, and frequently occurring mutations undermine the safety gains of vaccines, which usually require time-consuming development and testing phases. Thus, in addition to a prophylactic immunological approach, a spectrum of potent drugs suitable for the treatment of critically ill patients is needed. Various antibodies and small molecules that target different steps in the viral replication cycle have already been tested for this purpose and have yielded some promising results [202]. As of April 2022, more than a dozen such drugs have already received an emergency use authorization for the treatment of COVID-19 infections from the U.S. Food and Drug Administration (FDA)[h]. Interestingly, one of these candidates is baricitinib, a kinase inhibitor affecting the Janus kinases JAK1 and JAK2 [203].

[h] https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization#coviddrug

The efficacy of baricitinib indicates that kinase inhibitors can also make an important contribution to the arsenal of drugs in the fight against COVID-19, whereby the therapeutic potential of kinases in the context of viral diseases has been recognized long before the outbreak of the current pandemic. For example, in a review article, Keating *et al*. [204] describe a variety of viral diseases in which the respective viruses rely on phosphorylation by host cell kinases or viral kinases. Interestingly, CK2 is mentioned several times by the authors in the context of the dengue virus, hepatitis C, the vesicular stomatitis virus (VSV), the human respiratory syncytial virus (RSV), measles, influenza A, rotavirus A, the human immunodeficiency virus (HIV), the human papillomavirus (HPV), the human adenovirus, the varicella zoster virus (VZV), herpes simplex (HSV1), the human cytomegalovirus, the Epstein-Barr virus (EBV), and the Kaposi's sarcoma-associated herpes virus (KSHV).

Consistent with the role of hijacked host cell CK2 in many infections, a large-scale study of the phosphorylation patterns in SARS-CoV-2 infected Vero E6 cells by Bouhaddou *et al*. [205] revealed a substantial increase in CK2 activity, while many mitotic kinases, such as CDKs, were strongly downregulated or completely silenced to ensure cell cycle arrest. The authors also noticed that infected Caco-2 cells, a lineage of immortalized colorectal adenocarcinoma cells, showed overly pronounced and highly branched protrusions, called filopodia, which are associated with viral budding [206]. Interestingly, CK2 was colocalized with the viral N protein in these protrusions, leading the authors to speculate about an allosteric interaction to enhance CK2 activity. It is hypothesized that CK2 activity drives actin polymerization in the cell, as both α-catenin and the motor protein myosin IIA are substrates for CK2 as shown in the literature [207] [208]. Such reorganization of the actin skeleton is typical of the viral replication cycle in general and is required for viral particle egress [209] [210]. In addition, multiple phosphoproteomic studies indicated that the viral nucleocapsid protein (N), the membrane protein (M), the nonstructural protein 3 (NSP3), and the spike protein (S) are phosphorylated by CK2, although the underlying functions are not yet fully understood [211] [212] [205].

As the evidence for the essential role of CK2 in SARS-CoV-2 infections grew stronger with each phosphoproteomics dataset obtained, it was a logical next step to investigate the effect of selective CK2 inhibitors that are already in clinical trials. The approach of repurposing drugs that are already approved (or in clinical phases) greatly accelerates the potential for use as many studies, for example on the toxicological profile of the compounds, do not have to be repeated [202]. Two intensively studied inhibitors are silmitasertib and CIGB-325. Surprisingly, silmitasertib showed antiviral activity (IC_{50} = 2.34 µM) [205], which qualified the compound for further investigation in

different clinical trials on patients with severe and moderate COVID-19 symptoms[i]. CIGB-325 is actually not a CK2 inhibitor in the classical sense, as this partially-cyclic peptide binds to CK2 substrate sequences [213]. This peptide was tested by intravenous administration with a group of 10 COVID-19 patients. Comparison with a control group also showed that even a low dose of 2.5 mg/kg body weight had a significant antiviral effect [161]. Most strikingly, on average, lesions in the lung tissue decreased by 42% after seven days of treatment with CIGB-325, whereas patients from the control group who received a standard therapy showed only a 33% decrease. However, much more data are needed, including clinical trials with a larger group of patients, to provide a final estimation of the efficacy of CIGB-325.

[i] https://ClinicalTrials.gov; Identifier: NCT04668209

1.3 CK2 Inhibitors and General Druggability

1.3.1 Inhibitor Types and Current Trends

Kinase inhibitors were originally classified into three groups according to their binding abilities (inhibitor types I, II, and III) [214]. This definition was extended by the work of Zuccotto *et al*. [215] by including type I½ inhibitors. Type I to II inhibitors are characterized by their affinity for the cosubstrate binding pocket, where they usually complement the C-spine and compete with ATP or GTP. Type I and I½ inhibitors are both specific for kinases with a DFG-in conformation but, depending on the overall activity state of the kinase, either one or the other type is able to bind (see Table 2). Typically, such inhibitors form hydrogen bonds or halogen bonds with the hinge region and additionally interact with hydrophobic amino acids in the binding pocket. In addition, many compounds, including those developed for CK2, possess one or more proton acceptors, such as carboxylic acid functions or keto groups, which form hydrogen bonds with a conserved lysine in the cosubstrate site, i.e., Lys68 for CK2α or Lys69 for CK2α'.

Table 2. Different types of inhibitors and their characteristics according to Roskoski [216]. The constitutively active character of CK2 leads to a permanent DFG-in/αC-in state, precluding types I½, II, and III from being considered. Type IV inhibitors are also incompatible with the currently available warheads of covalently binding inhibitors, as they require a nearby accessible cysteine.

	Type I	**Type I½**	**Type II**	**Type III**	**Type IV**	**Type V**	**Type VI**
Type of Inhibitor	ATP competitive	ATP competitive	ATP competitive	Near the ATP/GTP site (allosteric)	Apart from the ATP/GTP site ("truly" allosteric)	Bivalent inhibitor connecting two sites	Covalent inhibitor
Kinase Activity	Active	Inactive	Inactive	N.D.	N.D.	N.D.	N.D.
DFG Motive	In	In	Out	N.D.	N.D.	N.D.	N.D.
Helix αC	In	N.D.	N.D.	Out	N.D.	N.D.	N.D.
Spines	Completed	Distorted	Usually distorted	Distorted	N.D.	N.D.	N.D.
Reversible	Yes	Yes	Yes	Yes	Yes	Yes	Usually not
Applicable for CK2	Yes	No	No	No	Yes	Yes	Probably not

In contrast, type II inhibitors take advantage of the structural features of a DFG-out conformation, which opens an additional hydrophobic pocket that was previously occupied by the phenylalanine of the DFG motif [216]. A particularly well-known type II inhibitor is Imatinib, formerly also known as Gleevec[j] (Figure 12a), which became famous in the 1990s for its novel binding mode and high efficacy for the treatment of Bcr-Abl1-positive chronic myeloid leukemia and acute lymphoblastic leukemia [217]. Imatinib was developed by rational drug design and was derived from an initial hit in a fragment-based high-throughput screening. Unfortunately, most inhibitors that address the cosubstrate binding site suffer from an inherent low selectivity, which will be discussed in more detail in the next subsection. For a long time, it was assumed that type II inhibitors might have particularly favorable prerequisites for high selectivity, since the conformations of active kinases are naturally very similar, whereas the inactive states can be very heterogeneous. However, a study in 2014 by Zhao *et al*. [218] conducted with more than 200 different kinases could not confirm this theory. Instead, the authors showed that type I and type II kinase inhibitors strongly overlap regarding their selectivity profiles. Moreover, a large fraction of the human kinome has the ability to adopt a DFG-out conformation [219]. However, in the case of the constitutively active CK2, no naturally occurring inactive conformation has been described, thus excluding the development of type I½ and II inhibitors per se in this case.

Allosteric inhibitors were initially classified as a single group (type III inhibitors). However, this definition was subsequently further refined by Simard *et al*. [220] and, henceforth, type III inhibitors were defined as compounds that bind in close proximity to the cosubstrate binding pocket but still within the cleft between the two lobes.

Type IV inhibitors, on the other hand, address regions further away from the cosubstrate binding pocket. Such “true” allosteric inhibitors have been investigated several times for CK2, for example in the form of cyclic peptides that bind to the α/β-binding site of the N-lobe and thus prevent the heterotetrameric assembly of CK2. These peptides, originally developed by the Cochet group [221] [222], were optimized in the course of this thesis (see Chapter 2). Inhibitors of these types have the potential to achieve high selectivity since regions of the protein can be targeted that show a significantly lower degree of conservation [223].

[j] Referred to as “Glivec” in the older literature, but the spelling was changed at the request of the FDA to avoid confusion with the diabetes agent Glyset.

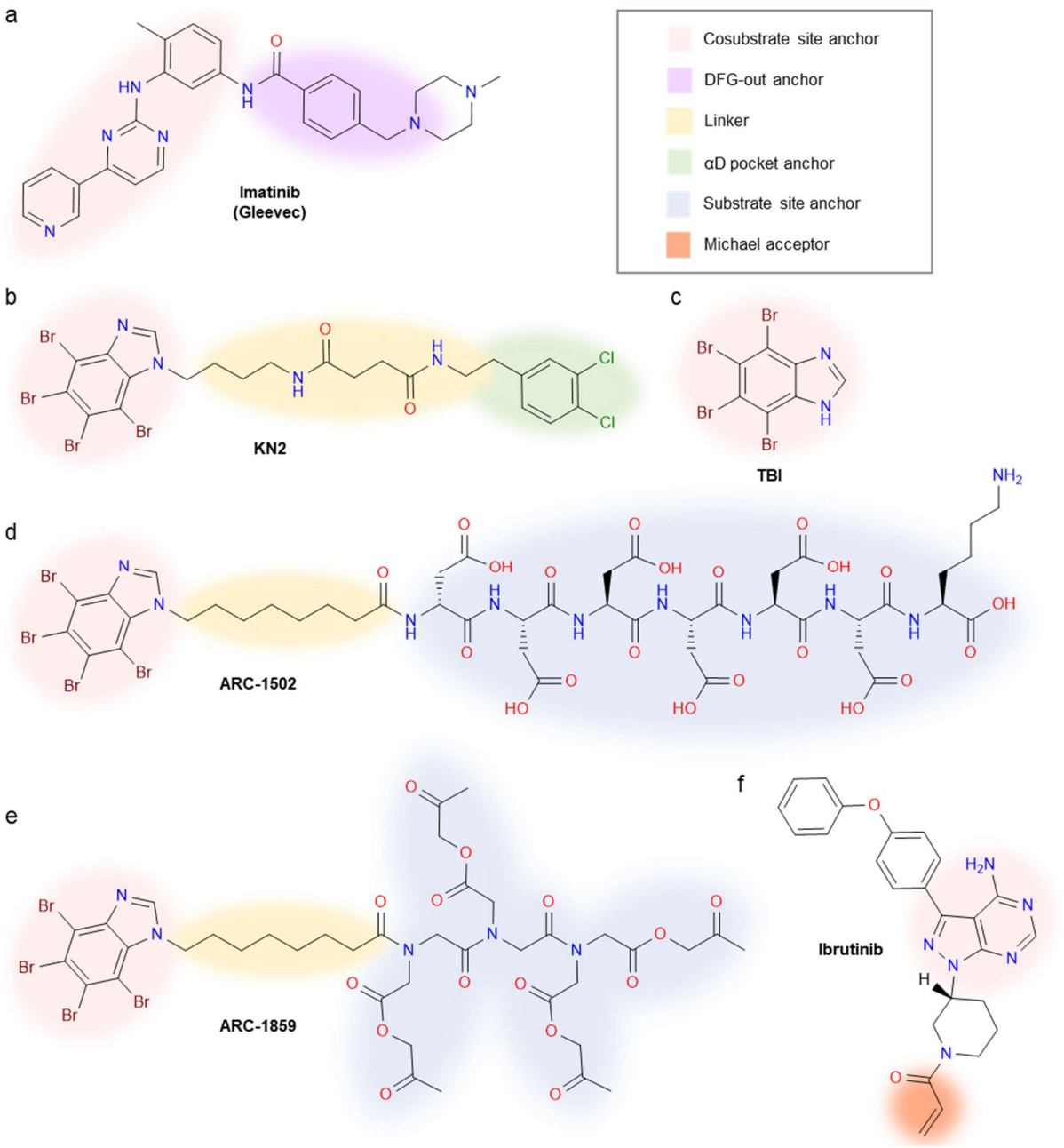


Figure 12. Different types of kinase inhibitors. (**a**) Imatinib has gained great popularity as the first type II kinase inhibitor approved by the FDA. (**b**) KN2 is a recently developed bisubstrate inhibitor that binds to both CK2α and CK2α' with low nanomolar affinity. Its ATP-competitive anchor is derived from TBI (c). The compounds ARC-1502 (d) and ARC-1859 (e) are shown exemplarily as bisubstrate inhibitors, a subcategory of bivalent type V inhibitors, with the latter having improved stability as well as cell permeability due to a modification of the peptide structure. (f) The FDA-approved agent Ibrutinib is a covalently binding type VI kinase inhibitor that binds to Bruton's tyrosine kinase (BTK) and occupies the cosubstrate binding pocket. The α,β-unsaturated carbonyl allows the formation of a covalent bond with a nearby cysteine in the course of a Michael addition.

A reliable strategy to combine the selectivity advantages of allosteric inhibitors with additional affinity is provided by type V inhibitors. Two binding fragments, connected by a linker, allow a variety of possible combinations for a kinase with multiple known binding sites, such as CK2 [224]. The potency of bivalent inhibitors is usually several magnitudes greater compared to the single non-conjugated fragments [225], which is largely due to the fact that the binding energies of the two conjugated fragments add up for a bivalent ligand [226]. In addition, a bivalent inhibitor is able to displace multiple molecules from the binding pockets, which provides a favorable entropic contribution [227] and allows for the design of inhibitors with both high affinity and excellent selectivity. KN2 is a novel type V inhibitor, presented in this thesis (Figure 12b), that combines an ATP/GTP-competitive 4,5,6,7-tetrabromobenzimidazole or TBI moiety (Figure 12c) with an anchor of a nearby exosite, namely the αD pocket which was already described in Subsection 1.2.1 (also see Chapter 7). However, the design of a suitable linker can be a significant challenge for this inhibitor type as a variety of parameters, such as chemical and enzymatic stability, the flexibility and the solubility, among other properties, have to be taken into account, especially when bridging larger distances between the two binding sites [228].

In general, type V inhibitors are further subdivided into generic bivalent inhibitors, including KN2, which link two binding sites in any combination, and bisubstrate inhibitors, which occupy the cosubstrate and the substrate binding site per definition and often show structural similarity with the enzyme's substrates [223]. The first bisubstrate inhibitor developed for CK2 is called ARC-1502 (Figure 12d) and was introduced in 2012 by Enkvist *et al*. [229]. It is composed of an ATP/GTP-competitive TBI moiety and an extremely acidic heptapeptide. Despite the outstanding affinity (K_i = 0.5 nM compared to 462 nM of isolated TBI [141]) and an acceptable selectivity with nine kinases being inhibited by more than 50% from a panel of 140 kinases tested at 1 µM, the compound has been criticized for two major reasons that prevent its pharmacological use. First, the peptide is easily proteolytically degraded, and second, membrane permeability is not given. As a result, three years later, the improved compound ARC-1859 (Figure 12e) was described in the literature [230], which is based on a protease-stable peptoid scaffold and also hides the negative charges of the carboxylic acid functions by acetoxymethyl esters, to improve cellular uptake. This approach involves an intracellular conversion into the active form by means of esterase activity. Nowadays, the spectrum of bivalent and bisubstrate CK2 inhibitors has become very broad and is one of the most promising inhibitor groups.

The sixth group of kinase inhibitors comprises covalently binding componds, which usually have a leaving group that allows them to react with cysteines in the course of an S_N2 reaction, or via a Michael system to form a sulfur ether. The advantage of this inhibition strategy is the irreversible nature of the kinase inactivation. Especially for tyrosine kinases such as EGFR, several type VI agents, such as Ibrutinib (Figure 12f), have been approved [231]. However, these inhibitors require an accessible sulfhydryl group of a cysteine in the area of binding, which is unfortunately not present in the case of CK2. Nevertheless, research is progressing rapidly to expand the repertoire of available warheads for covalent drugs in the future so that residues other than cysteines could be targeted by this type of inhibitor and kinases such as CK2 could be addressed. [232].
Very recently, a seventh group was defined by Lu *et al*. [233]. Type VII inhibitors also have an allosteric character and differ from type IV inhibitors insofar as they bind to extracellular domains of tyrosine kinases and thus represent a special case of allostery. However, it remains to be seen whether this definition will prevail in light of future research.

1.3.2 The Disadvantages of ATP-competitive Compounds

Most of the CK2 inhibitors developed so far address the cosubstrate binding pocket (see Figure 13). This is not surprising considering that it is the most distinct binding pocket of the enzyme, which, due to its sheer depth and the hydrophobic regions [234], is particularly suited for the development of high-affinity compounds. In addition, the cosubstrate pocket is permanently accessible and pre-formed, i.e., no conformational changes are necessary that would present an energy barrier during the binding process. Moreover, since it is an essential region for kinase activity, replacing the nucleotide with adequate inhibitors leads to an enzymatic inactivation. The first lead compounds that were discovered in many respects resembled the structure of ATP and were easily optimized in terms of affinity by rational drug design and the use of structural data.

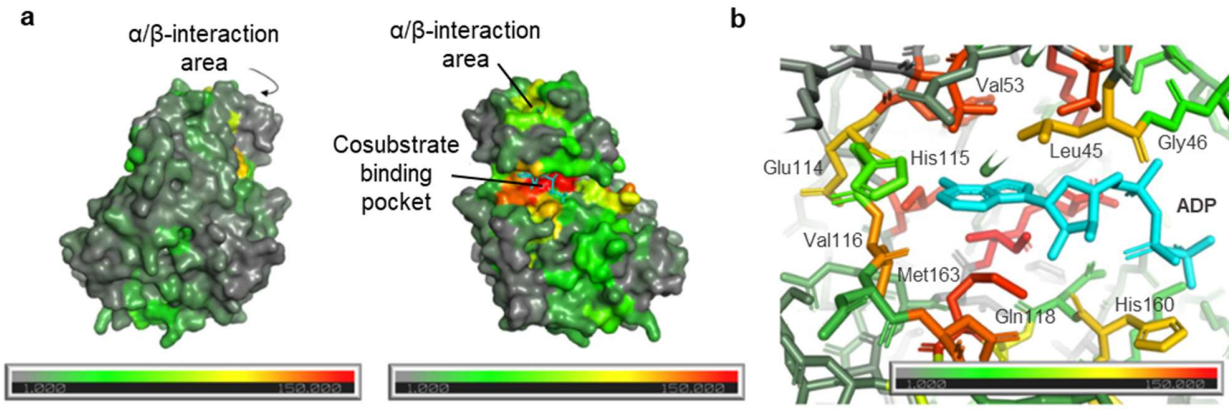


Figure 13. Color-coded visualization of the binding sites of 180 different ligands of CK2α deposited in the PDB, excluding any nucleotides. Contact between an amino acid and the respective ligand was defined in such a way that atoms of the ligand must be 4 Å or less away from the interacting amino acid. The results of the study were presented as a surface representation (**a**) or as a stick model focusing on the cosubstrate binding pocket (**b**). The PDB entry 5CU6 was selected here as a structure for the heat-map projection. Gray areas indicate the absence of ligand interactions whereas, in contrast, red areas are particular hotspots of ligand binding. For better orientation, a stick model of ADP is included (cyan). The figure is adapted in a modified form from Atkinson *et al*. [235] (MDPI open access article).

The nucleoside analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was the first known synthetic CK2 inhibitor derived in the mid-1980s (see Figure 14) [236], although its binding properties needed to be improved (K_i = 4.5 µM determined *in vitro*). A few years later, TBI and the analogous triazole, i.e., 4,5,6,7-tetrabromobenzotriazole (TBB), were introduced, which already deviated significantly from the original nucleoside structure [237] [238]. Similar to DRB, a benzimidazole or a very similar benzotriazole backbone was used. The halogens of DRB also proved to be remarkably useful since they form strong halogen bonds with main chain oxygens of the hinge region and were therefore extended by two additional halogens. In contrast to DRB, however, ribose was omitted as it only pointed into the solvent and had little electron density in crystal structures [239]. These simple changes already minimized the K_i value by about one magnitude compared to DRB. Over the years, this optimization has been successfully advanced so that today's inhibitors, such as silmitasertib, bind in the picomolar range (K_i = 360 pM).

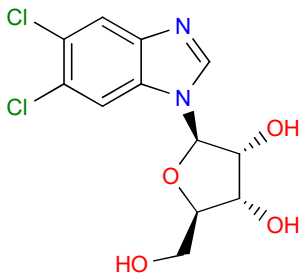
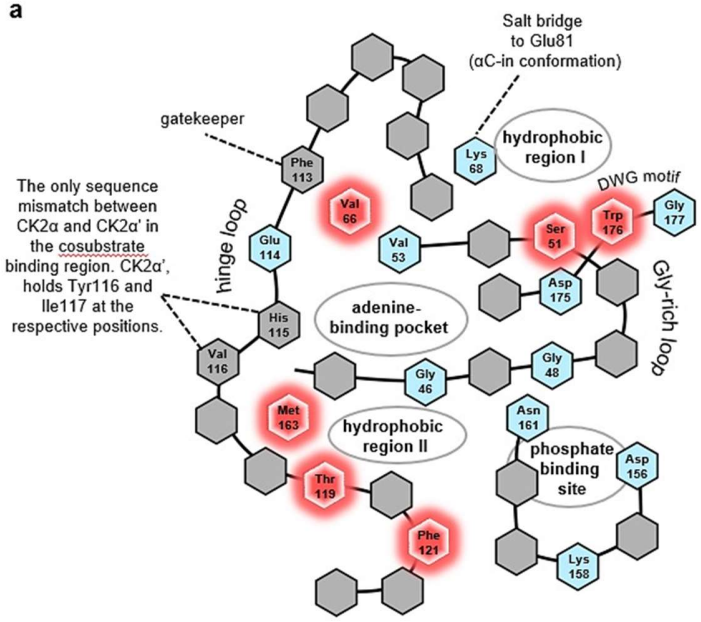


Figure 14. Structure of the first synthetic CK2 inhibitor, DRB, which is derived from ATP.

However, a selectivity optimization of ATP-competitive compounds is much more challenging, and it is not only a problem in the case of CK2 but for any other ATP-binding protein, including EPKs in general. This is also the reason for some well-documented side effects of FDA-approved kinase inhibitors, whereby particularly cardiotoxicity, hepatotoxicity, and hematotoxicity are repeatedly reported in patients undergoing treatment with kinase inhibitors [240], which, in many cases, leads to otherwise promising drug candidates failing to obtain FDA approval. In the most severe cases, cardiotoxicity can even result in heart failure [241]. Because of the common side effects, ATP-competitive kinase inhibitors are sometimes used as combination therapy or as second-line agents in the absence of a response to initial treatment or in the case of an acquired resistance.

The main issue in terms of selectivity is the highly conserved structure of the cosubstrate binding pocket (see Figure 15), which in turn is caused by evolutionary and chemical constraints. A certain selectivity can be achieved, for example, by using a deeply buried cavity of the binding pocket (known as hydrophobic region I) [234], if this part is accessible by means of small preceding amino acids. A key determinant in EPKs is the gatekeeper residue, which unfortunately is very bulky in the case of CK2 (Phe113 for CK2α and Phe114 for CK2α'). Together with Val66, which is usually an alanine in other kinases, these amino acids block the hydrophobic region I so that this area cannot be exploited for selectivity gains.

a



b

Amino acid in *homo sapiens* CK2α	Corresponding amino acid in other EPKs	Degree of conservation [%]
Gly46	Gly	93
Gly48	Gly	96
Ser51	**Gly**	**74**
Val53	Val	89
Val66	**Ala**	**92**
Lys68	Lys	97
Glu114	Gln	77
Thr119	**Gly**	**71**
Phe121	**Leu**	**80**
Asp156	Asp	96
Lys158	Lys	75
Asn161	Asn	98
Met163	**Leu**	**80**
Asp175	Asp	97
Trp176	**Phe**	**85**
Gly177	Gly	95

Figure 15. Schematic representation of the cosubstrate binding pocket of CK2α (**a**). Nonconserved amino acids are shown in gray, and conserved amino acids are colored. For the blue amino acids, the conserved residues match those of CK2; in the case of red positions, there is a deviation from other EPKs. In the adjacent table (b), the amino acids of CK2 are compared with the corresponding amino acids of other EPKs and their degree of conservation. The data have been adopted and the figure was prepared according to Huang *et al*. [29].

In summary, CK2 does not have the best prerequisites for the development of highly selective ATP-competitive inhibitors. It has a highly conserved cosubstrate binding pocket, no cysteines in this region that would allow covalent binding, several bulky amino acids that prevent deeper penetration of the binding pocket. Nevertheless, recently developed inhibitors such as SGC-CK2-1 show that high selectivity is possible in principle. In this context, it should also be mentioned that the dual cosubstrate specificity of CK2, which is barely found in any other kinase, may already indicate that the few sequence peculiarities of CK2 may be sufficient for the synthesis of selective inhibitors. However, the synthesis of new selective compounds remains a general challenge, and the current trend is increasingly to target other binding sites, so-called exosites, and make them pharmacologically accessible.

1.3.3 Exosites in the Focus of Research

By definition, exosites are binding sites located apart from the cosubstrate binding region [242]. In an effort to achieve selectivity, various exosites have been studied over the years and evaluated for their suitability for inhibitor development. The following subsection provides a systematic overview of these sites and their inherent advantages and disadvantages.

The α/β-interface area of CK2α – The CK2β binding site is an architecturally striking exosite of CK2α which, due to its rareness, is a promising target for selective inhibitors. Significantly, in crystallographic studies, the first synthetic CK2 inhibitor, DRB, was found to target both the cosubstrate binding site and the α/β-interface region of CK2α (see Figure 16). Although this was only an incidental finding, Raaf *et al*. [239] showed that the dual binding mode can be utilized to disrupt the tetrameric holoenzyme structure and thus, the idea arose to search for further compounds to investigate the effects of CK2β-competitive molecules on CK2 activity *in vitro* and *in vivo*. In this context, the bisubstrate inhibitor ARC-3140 with a DRB-related tetraiodobenzimidazole moiety, also unexpectedly bound with low micromolar affinity to the α/β-interface region of CK2α as recently described by Pietsch *et al*. [243]. Considering that CK2β boosts the activity of CK2α and also increases the overall stability of the protein, it can be assumed that the disruption of the holoenzyme assembly leads to a general decrease in cellular CK2 activity, to which “addicted” malignant cells should respond with particular sensitivity [181]. However, it must be assumed that not all substrates would be equally affected, and instead phosphorylation of type III substrates would be most impaired, whereas type II substrates would mostly be unaffected. A particular difficulty for the development of CK2β-competitive inhibitors arises from the fact that only a very shallow hydrophobic cavity is present, which, in the case of the canonic ligand CK2β, is occupied by only one phenylalanine residue and one peripheral tyrosine (Phe190 and Tyr 188). As a result, small molecule design has been extremely challenging due to the minimal contact area and the large solvent exposition. Only a few compounds have been identified to date, including W16 and various podophyllotoxin derivatives [244] which have never structurally been confirmed as CK2β-competitive and show binding in the low micromolar range, as well as compound 6 from Kufareva *et al*. (K_d = 30 µM) [245].

Apart from these small molecule compounds, several peptides have been developed that bind significantly tighter to CK2α. These peptides are derived from Pc, a 13-meric cyclic peptide that mimics the c-terminal binding region of the CK2β [221]. Over time, extensive optimizations have been introduced to Pc, which are in parts also presented in this thesis (see Chapter 2). Different groups tried to improve cellular uptake by adding cell-penetrating sequences such as Tat or sC18,

as in the case of TAT-Pc [246]. Moreover, the chemical stability was enhanced by replacing the disulfide cyclization, as demonstrated by CAM7117 or TAT-Pc, and the affinity was increased by making the peptide more rigid or though the introduction of halogen bonds to enable additional interactions with the protein backbone [247]. Although the peptide derivatives never reached very low nanomolar to picomolar affinity, they nevertheless bound in a superior way compared to small molecule inhibitors. This is not surprising since peptides are well known to be more suitable for interacting with targets with flat or extended epitopes due to their large surface area [248]. However, since peptides are only suitable as therapeutic agents to a limited extent and the affinity would still have to be significantly increased, there is a considerable need for further optimization of these compounds towards peptidomimetics. However, these efforts seem worthwhile as cytotoxicity in different cancer cell lines has been reported for different cell penetrating Pc derivatives [246] [247].

Substrate binding site – As previously presented in the context of antiviral drugs against SARS CoV 2, peptides and peptidomimetic compounds such as CIGB-325 can also serve as substrate-competitives although, strictly speaking, CIGB-325 does not bind directly to the substrate binding cleft of CK2 but rather binds directly to the corresponding recognition sequences of the target proteins and thereby prevents phosphorylation by blocking the phosphoacceptor serine or threonine. Nevertheless, CIGB-325 is one of the best-studied substrate-competitive compounds and the only candidate of this type showing significant antitumor activity that has made it into clinical trials to date [249] [250]. In general, selectivity for substrate-competitive compounds that actually interact with the binding cleft of CK2 can be generated via the distinct acidophilic character of CK2. The highly clustered occurrence of basic amino acids around the substrate cleft, as exemplified by the basic strand of CK2 that extends from amino acid Lys74 to Arg80 and contains a remarkable cluster of lysins, is the reason why compounds with a particularly negative net charge, such as polyglutamyl peptides or heparin [251] [95], are repeatedly described as CK2-binding molecules in the literature.

However, these negative charges are also problematic in terms of cellular uptake. Another obstacle to the design of substrate-competitive compounds is the lack of depth of the binding pocket. The affinities described, for example, for modern ATP-competitive compounds have so far not been achieved for the compounds addressing the substrate binding cleft. A way out of this dilemma might be given by bisubstrate inhibitors such as ARC compounds, as described previously using the examples of ARC-1502 and ARC-1859. Further improved ARC compounds also contain ATP-competitive 4,5,6,7-tetraiodo moieties, e.g. ARC-3140 or feature a silmitasertib conjugation [252]. However, urgently needed structural data are still lacking, since the

substrate-competitive parts of these bisubstrate compounds have never been covered with electron density in crystallographic studies (for more information, see Subsection 8.5).

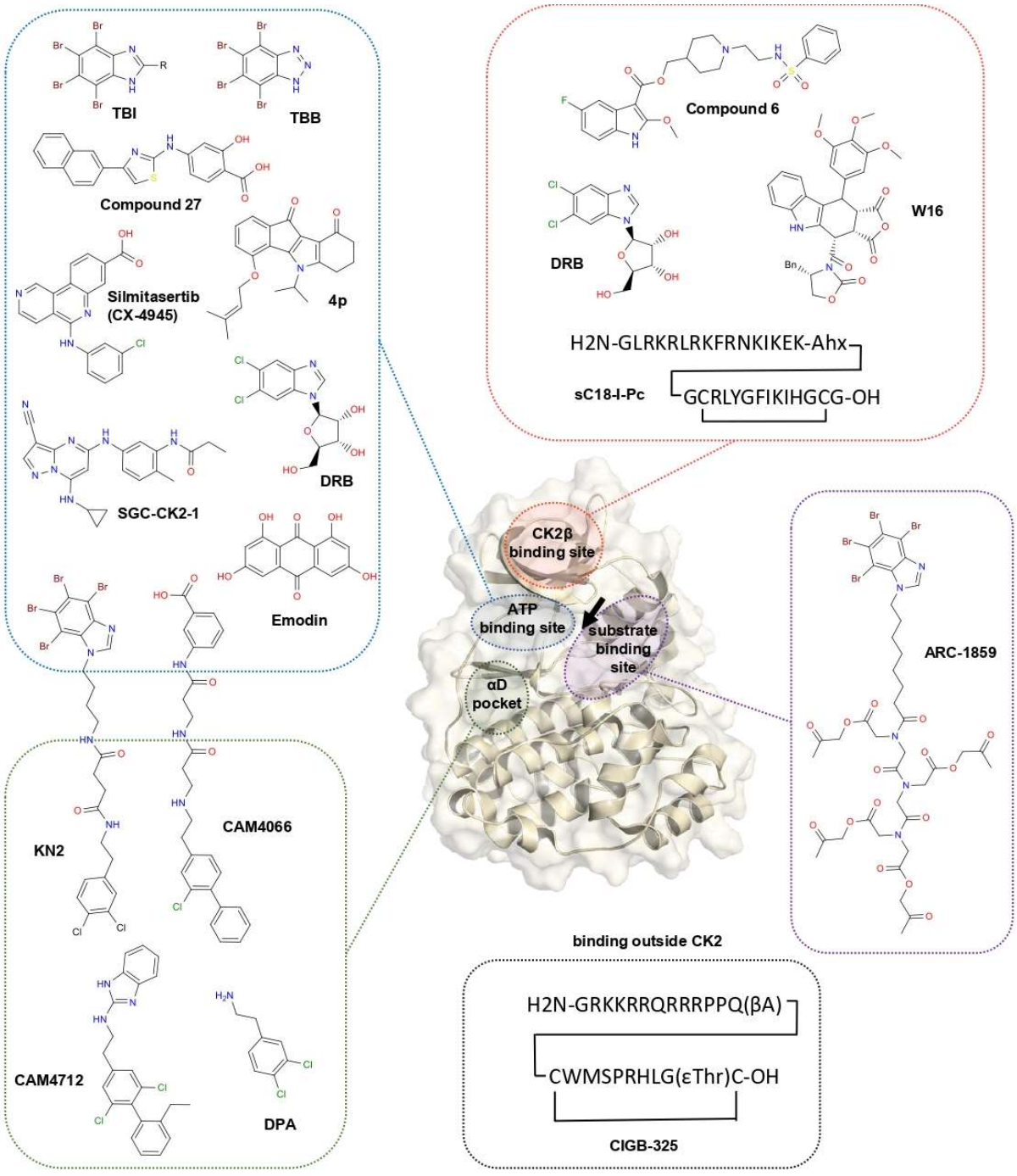


Figure 16. The various binding sites of CK2 are highlighted in different colors and exemplary inhibitors are shown for each of the binding sites. The peptides sC18-I-Pc and CIGB-325 are shown in the one-letter code, and black lines symbolize covalent bonds. The black arrow next to the ATP binding pocket of CK2 indicates the area that was assumed to be the binding site for the 2-aminothiazole compounds.

αD pocket – Another promising exosite is the recently discovered αD binding pocket, which has already been presented in Subsection 1.2.1. According to current knowledge, helix αD melting is only found in CK2α and CK2α' and therefore provides an ideal opportunity to generate selectivity. Furthermore, it is a very deep and rather narrow binding pocket, which provides ideal conditions for optimizing the currently known lead compounds towards excellent affinity. Initial work in this area has already been published by Iegre *et al.* [253]. However, addressing the αD binding pocket comes with two (not insignificant) drawbacks. First, it is a cryptic binding pocket that is not preformed and thus presents a certain energy barrier for the inhibitor's entry process. Additionally, fragments binding to the αD pocket do not exert any inhibitory effect per se. However, Iegre *et al*. [253] demonstrated that an extended αD-compound, designated CAM4712 that reaches into the ATP-binding pocket, has an inhibitory effect on the enzyme (K_d = 4 µM, IC_{50} = 7 µM at 25 µM of ATP; experiments were performed with isolated CK2α), although the protruding head moiety does not directly interact with the ATP-binding pocket. Due to its close proximity to the cosubstrate binding site, this binding pocket is particularly interesting and promising for the development of bivalent inhibitors that also bind to the cosubstrate binding site, as already demonstrated with KN2 (K_i = 6.1 nM, IC_{50} = 19.3 nM at 100 µM of ATP; experiments were performed with isolated $CK2\alpha_2\beta_2$) or CAM4066 (K_d = 320 nM, IC_{50} = 370 nM at 25 µM of ATP; experiments were performed with isolated CK2α).

Further exosite – In 2019, Bestgen *et al*. attracted a lot of attention with two associated publications [254] [255] in which the authors claimed to have found a group of 2-aminothiazole-based compounds, which are hypothesized to act as allosteric modulators of CK2. The authors performed various experiments to localize the putative binding site and came up with a concrete suggestion of where it *might* be located (see Figure 16), but all attempts to obtain a corresponding co-crystal structure with any of the several dozens derivatives failed. It was, however, possible to obtain these lacking data in the course of this thesis (see Chapter 6) and thereby disprove the hypothesis concerning a novel allosteric binding site.

In summary, the repertoire of available CK2 inhibitors now comprises a very large number of substance classes and a wide variety of inhibitor types and binding sites. Both selectivity and affinity have been further optimized over the past 40 years since the commencement of inhibitor development and it remains to be seen whether this will be reflected in further clinical studies beyond silmitasertib and CIGB-325.

1.4 Objectives

The serine/threonine kinase CK2, which is ubiquitous in eukaryotes, is a special kinase in many respects; for example, it is probably one of the most pleiotropic EPKs of the human kinome. Among its substrates are several key proteins of signaling pathways that stimulate cell proliferation or control the initiation of apoptosis. For this reason, CK2 activity has been linked to a variety of different diseases, especially the rapid progression and malignancy of different types of cancer. Current research is therefore primarily driven by the pharmacological interest in the development of potent and selective inhibitors, to which this thesis also contributes, as described in the next six chapters. Various inhibitors from different substance classes, targeting different binding sites of CK2, were synthesized and characterized.
Methodologically, the main focus is on gaining structural insights that are the foundation for the optimization of future inhibitor generations. In this context, special attention was paid to the structural exploitation of CK2α', a paralog of CK2α, which so far has been considered extremely problematic from a crystallographic point of view and for which only a very limited number of PDB entries with moderate resolutions are available.

Design of CK2β-Mimicking Peptides as Tools To Study the CK2α/CK2β Interaction in Cancer Cells

Chapter 2 describes the optimization of a cyclic peptide that targets the α/β interface region of CK2α and thereby interferes with the holoenzyme assembly. The aim was to investigate the effects on binding affinity by the introduction of different halogens at a key position with respect to CK2α binding and to achieve membrane permeability by conjugation of the cell-penetrating peptide sC18. Besides radiometric and fluorescence anisotropy-based *in vitro* measurements with isolated CK2, a study of the cellular effects, especially uptake and cytotoxic properties in a direct comparison of two cell lines, was undertaken.

Unexpected Binding Mode of a Potent Indeno[1,2-b]indole-Type Inhibitor of Protein Kinase CK2 Revealed by Complex Structures with the Catalytic Subunit CK2α and Its Paralog CK2α′

The work in Chapter 3 aims to characterize the CK2 inhibitor 4p, a member of the indeno[1,2-*b*]indole class, in terms of its cellular permeability and binding mode. Structural data were collected under high salt conditions with CK2α and under low salt conditions with both paralogs. Although a certain binding mode had been predicted based on *in silico* docking, no co-crystallization experiments with indeno[1,2-*b*]indoles and CK2 had been published until point.

Diacritic Binding of an Indeno[1,2-*b*]indole Inhibitor by CK2α Paralogs Explored by a Reliable Path to Atomic Resolution CK2α' Structures

This chapter aims to structurally characterize an isoform-specific difference in the inhibitory potency of THN27, an ATP-competitive indeno[1,2-*b*]indole-type inhibitor, based on crystal structures with CK2α and CK2α', respectively. As CK2α' has always been an extremely challenging protein in terms of crystallization, a novel crystallization protocol was presented, which yields easily reproducible CK2α' crystals to generate structures with atomic resolution. These crystals were subsequently soaked with inhibitors of different affinities to test the potential of this novel crystallographic tool.

Synthesis, biological properties and structural study of new halogenated azolo[4,5-*b*]pyridines as inhibitors of CK2 kinase

The fifth chapter deals with the synthesis and testing of novel halogenated CK2 inhibitors based on a pyridine scaffold. Compared to the structurally closely related compounds TBI and TBB, improved solubility in the aqueous medium was expected due to the pyridine nitrogen at the N4 position. Therefore, it was aimed to investigate how this change in the backbone and the associated loss of one of the halogens would affect the aspects of affinity as well as the cytotoxicity in different cancer cell lines. The crystallization protocol presented in Chapter 4 was used for a detailed structural investigation of the binding modes of the most promising compounds.

Structural and Mechanistic Basis of the Inhibitory Potency of Selected 2-Aminothiazole Compounds on Protein Kinase CK2

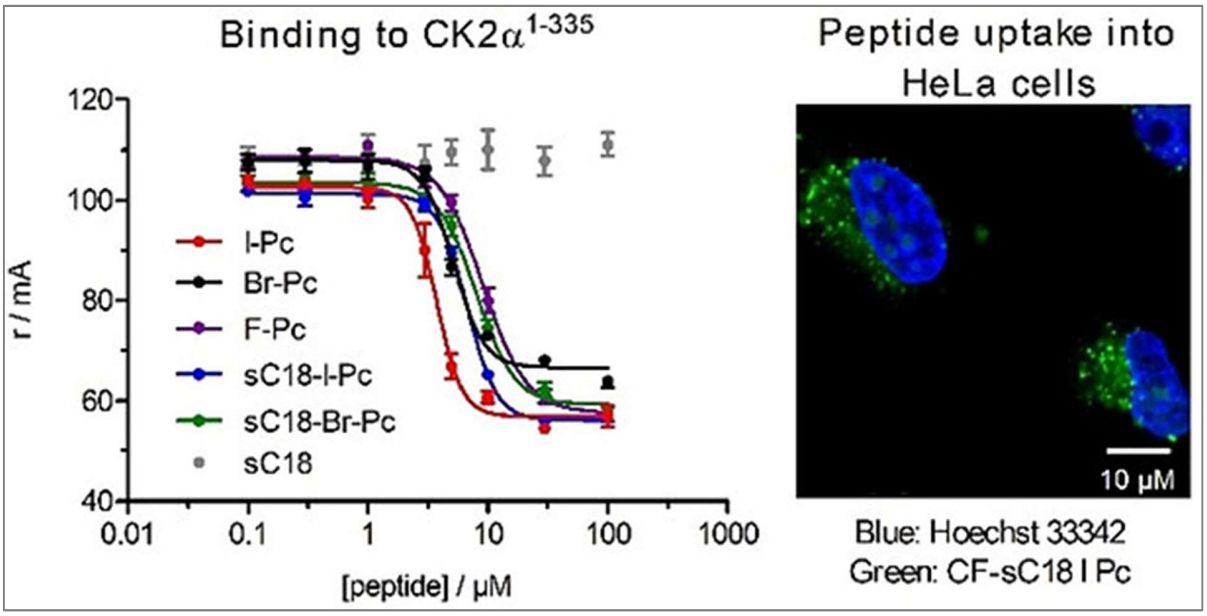
In 2019, Bestgen *et al*. claimed to have identified 2-aminothiazoles as non-ATP-competitive inhibitors of CK2 through extensive studies. However, the authors did not achieve structural elucidation. The research presented in this chapter aimed to provide follow-up crystallographic data for CK2α and CK2α' and to localize the precise binding site of these compounds. In addition, the presented inhibition kinetics should be validated once again.

Molecular Plasticity of Crystalline CK2α' Leads to KN2, a Bivalent Inhibitor of Protein Kinase CK2 with Extraordinary Selectivity

The novel crystallization protocol for CK2α' was used to address the question of whether ligand-induced melting of the αD-helix is exclusive to CK2α or whether this structural phenomenon occurs in both paralogs. The resulting CK2α' complex structure containing the crystallization chaperone MB002 and the αD ligand 2-(3,4-dichlorophenyl)ethan-1-amine (DPA) was used for the development of KN2, a bivalent inhibitor connecting the cosubstrate binding pocket with the αD pocket. Subsequently, KN2 was tested *in vitro* and *in cellulo* for its suitability as a CK2 inhibitor with regard to its affinity, cytotoxicity, and cellular uptake. Furthermore, the selectivity was determined by a kinome-wide profiling against a panel of 83 different kinases, including known off-targets for the inhibitors Silmitasertib and TBI.

Chapter 2 – Design of CK2β-Mimicking Peptides as Tools To Study the CK2α/CK2β Interaction in Cancer Cells

Graphical abstract



This chapter corresponds to the following publication:

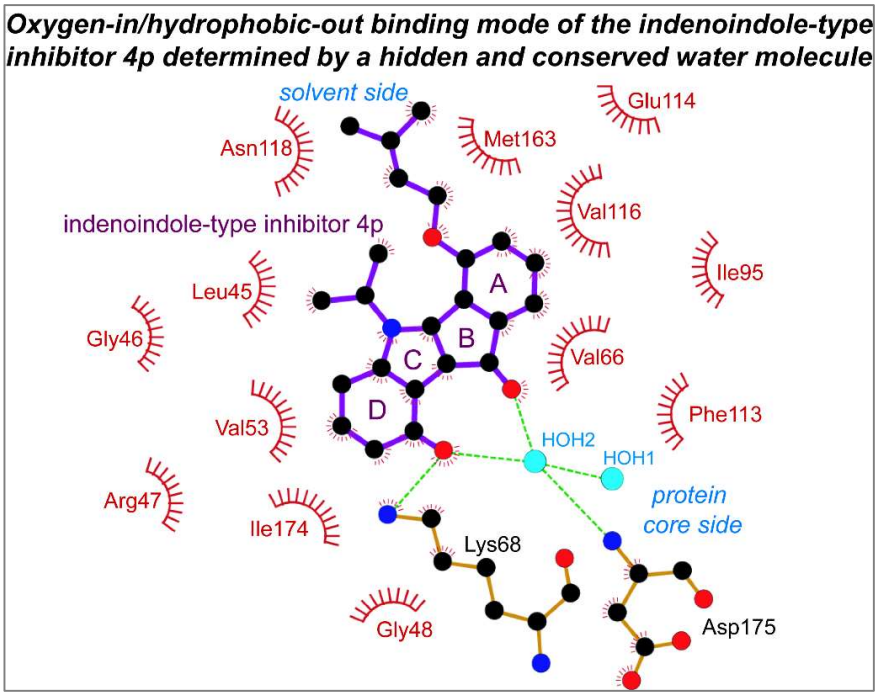
D. Lindenblatt, M. Horn, C. Götz, K. Niefind, I. Neundorf, and M. Pietsch, “Design of CK2β-Mimicking Peptides as Tools To Study the CK2α/CK2β Interaction in Cancer Cells”, ChemMedChem, vol. 14, no. 8, pp. 833–841, Apr 17 2019, doi: 10.1002/cmdc.201800786.

Quick access to the article

2.1 Supporting Information for Chapter 2

Chapter 3 – Unexpected Binding Mode of a Potent Indeno[1,2-*b*]indole-Type Inhibitor of Protein Kinase CK2 Revealed by Complex Structures with the Catalytic Subunit CK2α and Its Paralog CK2α′

Graphical abstract



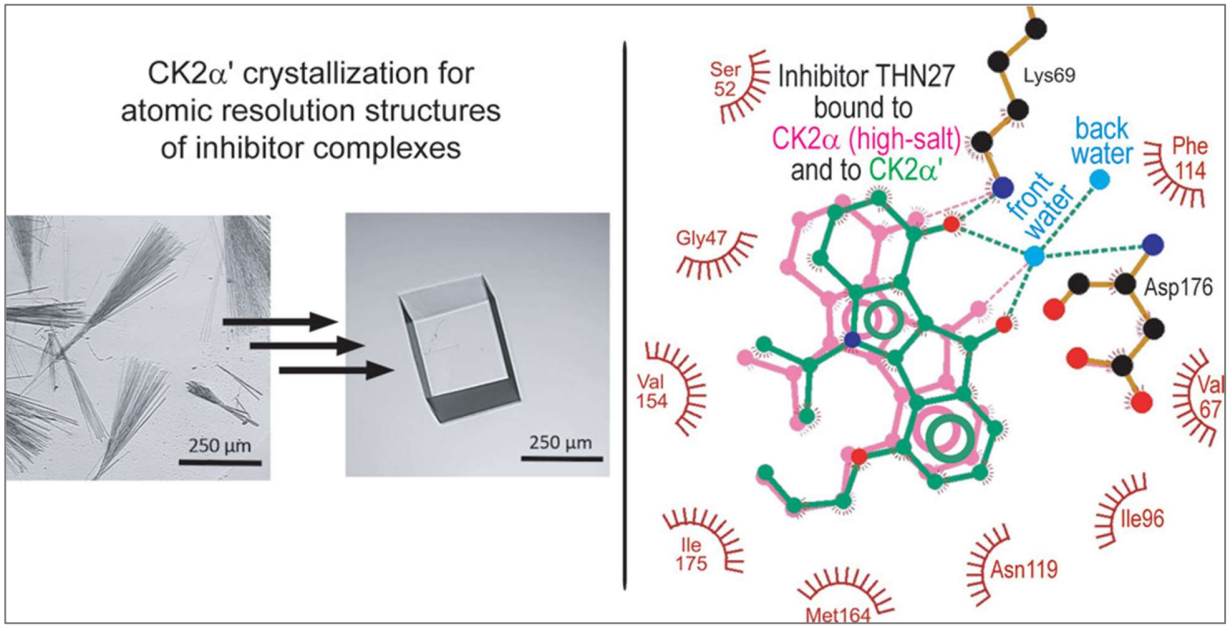
This chapter corresponds to the following publication:

J. Hochscherf, D. Lindenblatt, B. Witulski, R. Birus, D. Aichele, C. Marminon, Z. Bouaziz, M. Le Borgne, J. Jose and K. Niefind, "Unexpected Binding Mode of a Potent Indeno[1,2-b]indole-Type Inhibitor of Protein Kinase CK2 Revealed by Complex Structures with the Catalytic Subunit CK2α and Its Paralog CK2α'", Pharmaceuticals (Basel), vol. 10, no. 4, 98, Dec 13 2017, doi: 10.3390/ph10040098.

Quick access to the article

Chapter 4 – Diacritic Binding of an Indenoindole Inhibitor by CK2α Paralogs Explored by a Reliable Path to Atomic Resolution CK2α' Structures

Graphical abstract



This chapter corresponds to the following publication:

D. Lindenblatt, A. Nickelsen, V. M. Applegate, J. Hochscherf, B. Witulski, Z. Bouaziz, C. Marminon, M. Bretner, M. Le Borgne, J. Jose and K. Niefind, "Diacritic Binding of an Indenoindole Inhibitor by CK2α Paralogs Explored by a Reliable Path to Atomic Resolution CK2α' Structures", *ACS Omega,* vol. 4, no. 3, pp. 5471-5478, Mar 31 2019, doi: 10.1021/acsomega.8b03415.

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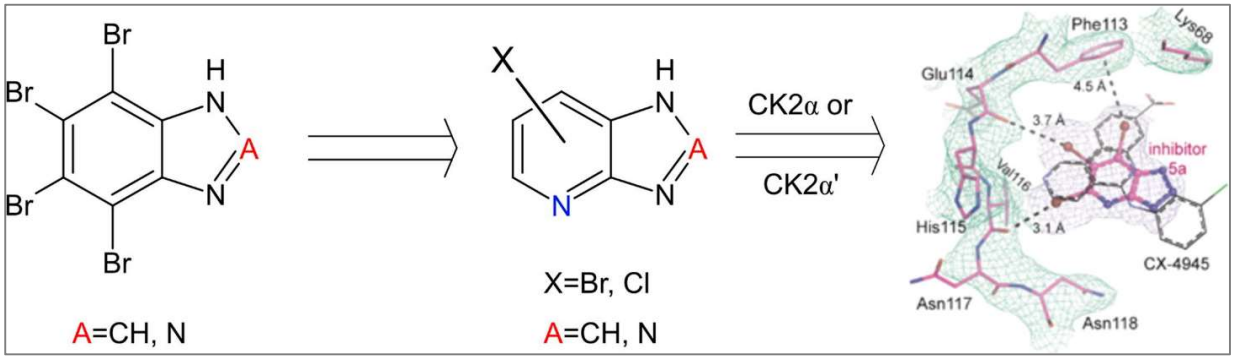
4.1 Supporting Information for Chapter 4

Chapter 5 - Synthesis, Biological Properties and Structural Study of New Halogenated Azolo[4,5-*b*]pyridines As Inhibitors of CK2 Kinase

Graphical abstract



This chapter corresponds to the following publication:

K. Chojnacki, D. Lindenblatt, P. Wińska, M. Wielechowska, C. Toelzer, K. Niefind and M. Bretner, "Synthesis, biological properties and structural study of new halogenated azolo[4,5-b]pyridines as inhibitors of CK2 kinase", Bioorg Chem, vol. 106, p. 104502, Jan 2021, doi: 10.1016/j.bioorg.2020.104502.

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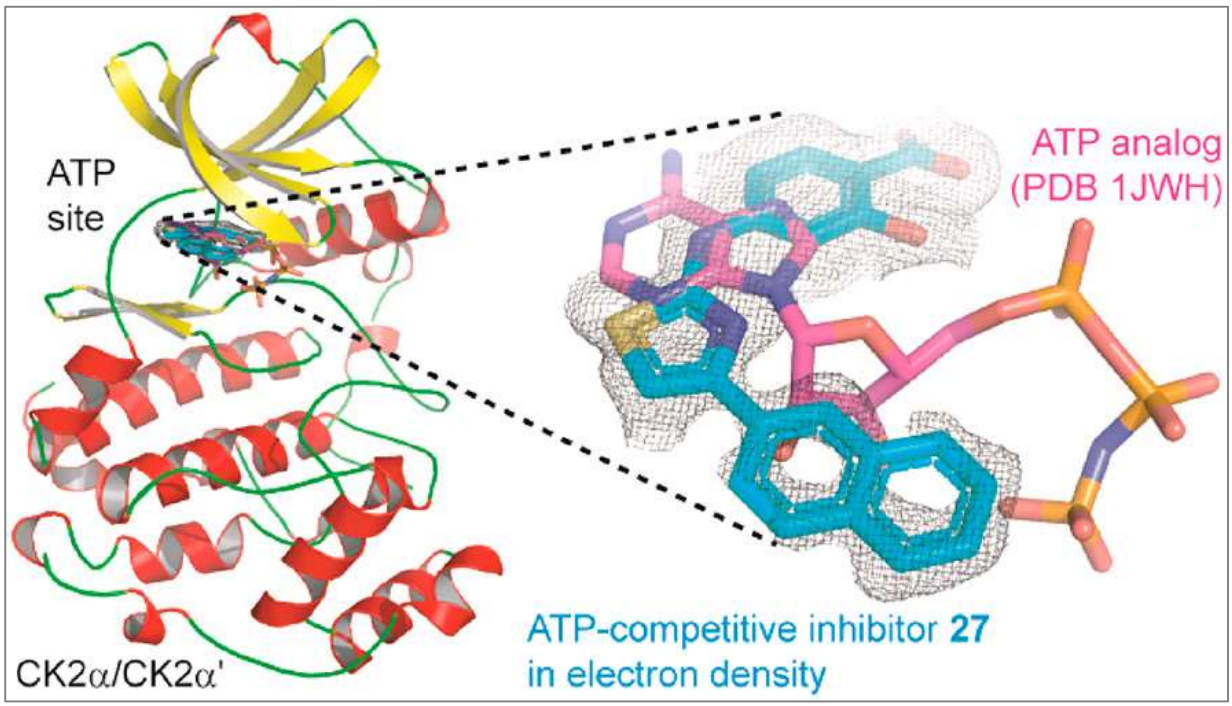
5.1 Supporting Information for Chapter 5

Chapter 6 – Structural and Mechanistic Basis of the Inhibitory Potency of Selected 2-Aminothiazole Compounds on Protein Kinase CK2

Graphical abstract



This chapter corresponds to the following publication:

D. Lindenblatt, A. Nickelsen, V. M. Applegate, J. Jose, and K. Niefind, “Structural and Mechanistic Basis of the Inhibitory Potency of Selected 2-Aminothiazole Compounds on Protein Kinase CK2”, J Med Chem, vol. 63, no. 14, pp. 7766-7772, Jul 23 2020, doi: 10.1021/acs.jmedchem.0c00587.

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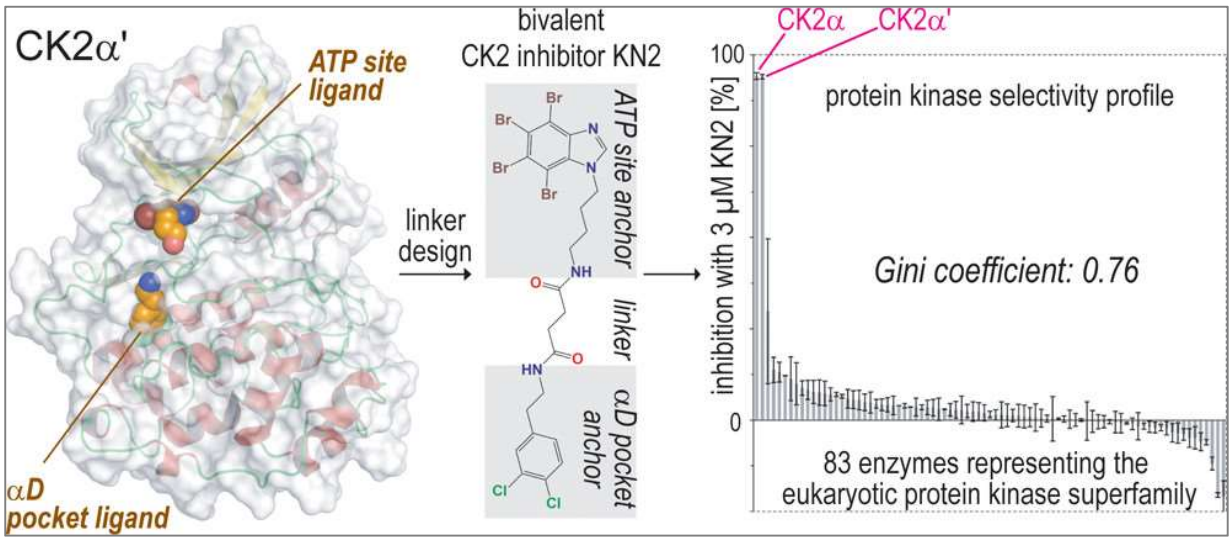
6.1 Supporting Information for Chapter 6

Chapter 7 – Molecular Plasticity of Crystalline CK2α' Leads to KN2, a Bivalent Inhibitor of Protein Kinase CK2 with Extraordinary Selectivity

Graphical abstract



This chapter corresponds to the following publication:

D. Lindenblatt, V, Applegate, A, Nickelsen, M, Klußmann, I, Neundorf, C. Götz, J. Jose and K. Niefind, "Molecular Plasticity of Crystalline CK2α' Leads to KN2, a Bivalent Inhibitor of Protein Kinase CK2 with Extraordinary Selectivity", J Med Chem, vol. 65, no. 2, pp. 1302-1312, Jan 27 2022, doi: 10.1021/acs.jmedchem.1c00063.

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7.1 Supporting Information for Chapter 7

Chapter 8 – Discussion and Conclusion

8.1 Advances and Limitations in Addressing the α/β Interaction Area With Peptides and Small Molecules

As described in Chapter 2, several derivatives of the Pc peptide were synthesized and tested for their binding affinity for CK2α using a fluorescence anisotropy-based assay [256]. The most suitable compound was then conjugated to sC18, a cell-penetrating peptide (CPP), and characterized *in vitro* and *in cellulo*. It could be demonstrated that the presence of the resulting chimeric peptide sC18-I-Pc limited the CK2 holoenzyme's ability to catalyze the phosphorylation of class III substrates, which are defined by the fact that their phosphorylation requires catalysis by the intact heterotetrameric form of CK2. These results indicate that CK2β can indeed be outcompeted by compounds such as this chimeric peptide. In addition, using a 5(6)-Carboxyfluorescein-labeled version of sC18-I-Pc, endosomal internalization was detected within 30 min in HeLa and HEK-293 cells. Interestingly, HeLa cells, which were selected as a model for malignant cells, showed significantly higher sensitivity towards sC18-I-Pc compared to HEK-293 cells. An IC_{50} value of 37 µM was extrapolated for HeLa cells, whereas HEK cells did not show any decrease in viability at the highest tested concentration of 50 µM. This is consistent with the fact that HeLa cells are known in the literature to be "addicted" to high CK2 levels in contrast to non-cancerous cells such as HEK-293 [257].

A comparison of the measured binding affinities of Pc derivatives with those of previously developed small molecules, such as compound 6 from Kufareva *et al*. [245] confirms the conclusion that peptides are much better suited to address large and predominantly flat protein-protein interfaces because their size allows for the utilization and coverage of a broad surface area and, in addition, their high flexibility facilitates their adaptation to the site [258]. This may suggest that peptides are ideal drug candidates, offering tremendous pharmacological opportunities in terms of targeting proteins, including CK2. However, the FDA statistics of the past decades convey a completely different picture. Between 1960 and 2020, only 90 peptide drugs were approved by the FDA, although the rate of approvals has risen steadily over time [259] [260] [261]. Between 2000 and 2020, 56 peptides were approved and in 2021 a historic record of 10 approved peptide drugs was reported, representing a remarkable share of 20% with a total of 50 FDA-approved compounds for that year. This historic trend raises the question of why the

emergence of peptide drugs has been so slow and what this implies for CK2β-competitive peptide compounds such as sC18-I-Pc.

Peptides are associated with several inherent drawbacks, which is why they have long been considered very poorly suited for pharmacological applications. Whereas the search for promising CK2 inhibitors described above mainly focuses on the two aspects of affinity and selectivity, pharmaceutical compounds need to have several additional properties. A detailed characterization requires data regarding the compound's administration, distribution in the various tissues, metabolization, excretion, and toxicological profile, which is also known as the compound's ADME-Tox properties [262].

The first point already addresses a critical challenge that the development of peptide drugs faces. The oral bioavailability of peptides is usually very poor and the digestive system can easily degrade peptides because of their enzymatic instability towards proteases. However, even when peptides enter the body via intravenous administration and have been chemically modified to protect them from protease-mediated degradation, these compounds usually suffer from rapid plasma clearance. The short biological half-life is due to the fact that the glomeruli of the kidneys have a molecular cutoff of <2-25 kDa, reslting from their pore size of 8 nm, and peptide drugs thus face a rapid renal excretion [263]. Furthermore, the great flexibility of peptides may impair the binding process to certain targets compared to more rigid small molecules. Finally, it should be mentioned that peptides cannot cross certain boundaries in the body or can only do so very poorly. For example, their mostly hydrophilic character prevents them from crossing the blood-brain barrier and, with the exception of CPPs such as sC18, they normally cannot cross cell membranes. Especially with regard to the latter point, it must be emphasized that no membrane-permeable peptide compound has received FDA approval to date.

While the list of drawbacks is relatively long, organic chemical synthesis has found solutions to each of these challenges. These include, to mention only a few, the incorporation of non-natural amino acids or termini modifications to hide the peptide from protease recognition, or the conjugation of long polymers or hydrophobic serum-albumin-binding anchors to avoid renal excretion [264]. Similarly, small tags, e.g. polyfluorinated alkanes [265], are now known facilitate cellular permeability without large, and thereby cost-intensive, CPP moieties, although some CPP-conjugated peptides are also currently being investigated in clinical trials. Interestingly, such chimeric peptides mostly are constructs with the oldest known CPPs such as TAT [266], for example in CIGB-325, penetratin, or artificial polyarginine repeats. Matijass and Neundorf [267] speculated that the reason for using the older CPPs might be due to the fact that these peptides

are well studied and thus have been subjected to extensive pharmacological fine-tuning, especially in light of the fact that FDA-approved peptide compounds require, on average, a 12 year preliminary lead time [268]. However, Matijass and Neundorf also noted that a larger repertoire of CPPs is used for clinically tested antitumor compounds than for other applications because some CPPs have inherent antitumor properties. In line with this point, some CPPs are repeatedly described in the literature as having a certain selectivity towards malignant cells [269].

In conclusion, the study presented in Chapter 2 showed that CK2β-competitive peptide inhibitors do have the potential to affect CK2 activity in cells and thus to affect addicted malignant cells. However, the findings indicate that there is still a long way to go to develop a CK2β mimicking peptidomimetic compound with a "true" drug character, which is why sC18-I-Pc was only presented as a tool for cellular studies. Numerous modifications would still need to be made to improve the ADME-Tox properties and the redox stability, as well as to increase the affinity of sC18-I-Pc and similar CK2β-competitive peptide compounds towards CK2. However, the growing number of peptide drugs and the ongoing clinical trials on CPP conjugates are grounds for optimism that increasing numbers of intracellular targets will soon be addressed with peptides.

8.2 A Reliable Approach to Atomically Resolve CK2α' Complex Structures With Diverse Inhibitors Offering Further Untapped Opportunities

Despite the high sequence similarity of ~86% in the canonical EPK core, the two paralogs CK2α and CK2α' show completely different crystallization behaviors. CK2α has a very pronounced crystallization tendency over a wide range of conditions, yielding a variety of useful crystal forms with a rather high diffraction power. Crystallization of CK2α', on the other hand, has always been a challenging task [138]. Although crystals form under a variety of conditions, they usually occur as crystalline showers or extremely fragile needles, which do not withstand any external influences such as the slightest mechanical forces and are therefore essentially impossible to handle. Rarely occurring, thicker needles are hardly reproducible, as in the case of the ATP-competitive compound 4p in complex with CK2α' (PDB 5OOI). When the data on 4p were published in 2017, only four structures of CK2α' in the resolution range of 3.2 Å to 2.0 Å had been deposited in the PDB (see Figure 17). In comparison, information concerning more than 90 CK2α and CK2α based holoenzyme structures had been published at the same time. The development of a novel

crystallization protocol for CK2α' was thus highly desirable to remedy this imbalance in the available structural information. More detailed insights provide the opportunity for a better understanding of the molecular behavior and functions of CK2α' and may help to explain long-known differences between paralogs in the future.

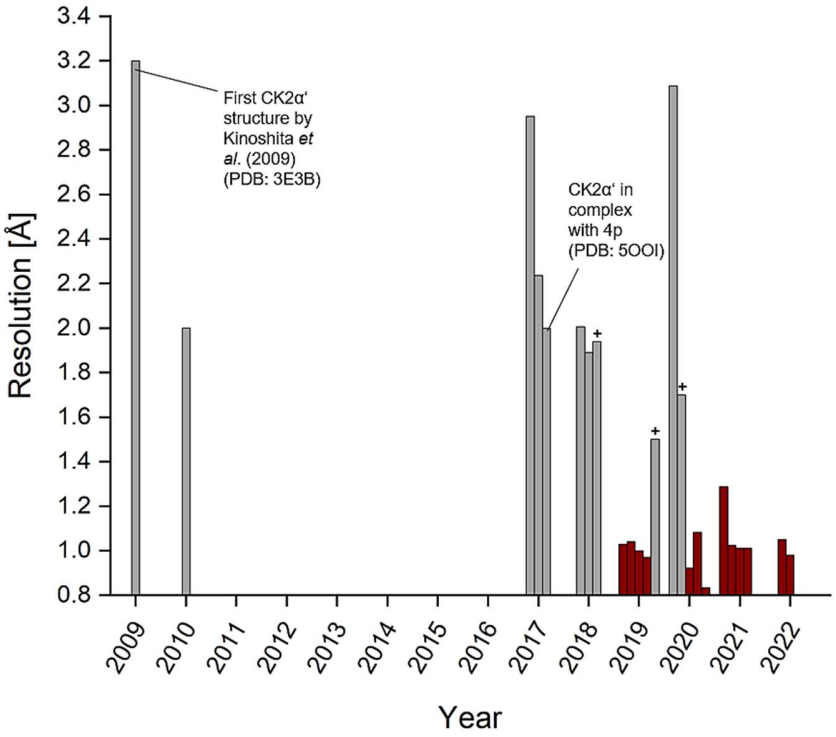

Figure 17. Chronological overview of all CK2α' structures deposited in the PDB by December 2022. The red bars represent all structures that were obtained using the novel crystallization protocol presented in Chapter 4. These structures are characterized by excellent resolutions of approximately 1.0 Å and thus provide significantly more details compared to previous structures. All entries marked in red as well as the structure "5OOI" were produced in the course of this thesis. All bars marked with (+) represent structures that have been uploaded to the PDB but have not yet been published elsewhere. From left to right, the entry codes are: (2009) – 3E3B; (2010) – 3OFM ; (2017) – 5M4U, 5M56, 5OOI; (2018) – 5Y9M, 5YF9, 5YWM; (2019) – 6HMB, 6HMC, 6HMD, 6HMQ, 6QY9; (2020) – 6L20, 6QY8, 6TE2, 6TEW, 6TGU; (2021) – 7A1B, 7A1Z, 7A22; and (2022) – 7AT9, 7ATV.

Recently, the inhibitor THN27, a derivative of 4p, was surprisingly found to have a certain paralog selectivity, with an approximately twofold inhibition of CK2α' compared to CK2α (IC_{50} = 273 nM vs. 607 nM). This was unexpected insofar as the previously conducted crystallization studies with the cell-permeable inhibitor 4p in complex with CK2α under both high and low salt conditions and

in complex with CK2α' showed a fully conserved binding mode (see Chapter 3). In addition, THN27 and 4p both possess an indeno[1,2-*b*]indole scaffold as well as the two keto functions, which were found to be crucial for the orientation in the cosubstrate pocket by interacting with Lys68 of CK2α and Lys69 of CK2α', respectively. Another aspect that contradicted the observed selectivity was the fact that the paralogs showed only two amino acid exchanges in the vicinity of the cosubstrate binding site, both located in the hinge region. This loop, however, is not involved in the binding process of 4p and there is no evidence from the molecular structure that this should be different for THN27. For a more detailed structural study of this phenomenon, the crystallization protocol described in Chapter 4 was developed in a time-consuming effort. The novel protocol has not only been immensely helpful for studying the slightly different binding mode of THN27 in both paralogs, but it has also triggered a wave of new CK2α' PDB entries with an atomic resolution of approximately 1.0 Å since 2019. In addition, the novel crystal form offers some major advantages, which have not yet been fully exploited experimentally:

(I) First, the CK2α' crystals can be prepared in crystal libraries before the inhibitors of interest are added to the wells. This saves time and resources in contrast to classical co-crystallization approaches including the screening for suitable conditions.

(II) Moreover, the crystals reliably provide atomically resolved structures, which are suitable for *ab initio* phasing.

(III) The crystal size can easily be scaled up to edge lengths of approximately 1 mm, which facilitates the measurement of neutron diffraction data sets [270]. This may be important for resolving some specific questions involving the position of hydrogens. Just recently, the data concerning such a structure were published for CK2α by Shibazaki *et al*. and could thus serve as a comparator for CK2α' neutron diffraction structures [271]. Shibazaki *et al*. postulate a proton network traversing the C-terminus of CK2α, which is believed to serve the removal of protons released during the phosphorylation reaction. This putative proton channel includes two water molecules that have not previously been seen in X-ray structures. However, the fact that these water molecules are also absent in all atomically resolved CK2α' structures so far casts some doubt on the authors' hypothesis.

(IV) Another advantage of the new CK2α' structures is that all binding sites are accessible to small molecules by inhibitor soaking. MB002, which is required for initial crystallization according to the novel protocol, can even be replaced by the presence of low-affinity inhibitors. This has already been demonstrated by the example of AR18, a weakly binding indeno[1,2-*b*]indole [272].

Unfortunately, the CK2β binding site is blocked by a crystal contact. However, it was later found that a slightly different crystallization condition with lower LiCl concentrations, around 700 mM, allowed CK2β-competitive binding of small molecules as well, caused by a different crystal packing (unpublished data) (see Figure 18).

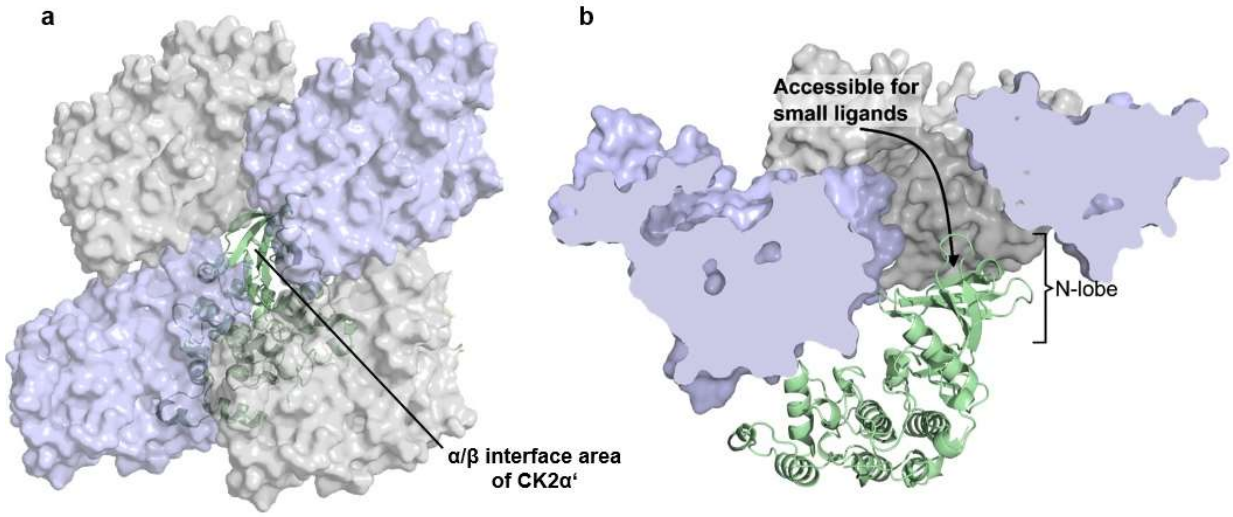

Figure 18. (**a**) CK2α' crystallizes at slightly lower LiCl concentrations of 700 mM in the space group P1 21 1 instead of P1 at higher LiCl concentrations. In these crystals, CK2α' is surrounded in the N-terminal region by four symmetry equivalents (shown as surface representations and in different colors for better differentiation). A solvent exposed channel between the proteins allows accessibility of the of the N-terminal α/β interface region of CK2α' for small molecules. (**b**) Cross section though the structure shown under (a) for a better visualization of the channel leading to the α/β interface region.

(V) In addition, ligand exchange or other adjustments in the mother droplet, such as desalting or an increase in the organic content of the solvent, can be carried out without a loss of the diffraction performance, since the crystals often tolerate even harsh changes in conditions without suffering any damage, even an opening of the αD-pocket in the crystalline state, as recently shown by the example of the bivalent inhibitor KN2 [273], could not damage the structural integrity of the crystals (see Chapter 7).

As a further possible application, it should also be considered to introduce point mutations into the CK2α' sequence to mimic the structural setting in the corresponding binding pockets of CK2α. The advantages of this valuable crystallographic tool could thus be applied to CK2α in future

experiments. Fortunately, there are only very few sequence differences in the ATP binding pocket, namely His115 and Val116 in CK2α versus Tyr116 and Ile117 in CK2α'. In addition, there is an important sequence difference in the αD pocket, namely Ile140 in CK2α and Leu141 in CK2α', which is discussed in more detail in Subchapter 8.4. However, none of these differences are localized in the crystal contact regions. Therefore, it seems unlikely that the crystal packing will change as a consequence of the mutation. The expression of high-resolution CK2α-mimicking crystal structures should thus be tested in the near future as a promising approach to extend the advantages of the novel CK2α' crystallization protocol to both paralogs.

Another opportunity presented by the reliable crystallization combined with the potentially record-breaking resolution is the performance of fragment-based lead discovery screenings (FBLD), an X-ray-based search for currently unknown ligands or binding sites. In recent years, this strategy has evolved into a powerful method for detecting small-molecule ligands due to rapid technological developments, such as faster detectors, more reliable processing pipelines, and more brilliant synchrotron beams [274] [275]. It should be emphasized that small lead compounds which are able to bind to certain exosites are rarely detected by classical binding assays. Moreover, such small, non-optimized compounds usually do not have a very high affinity. These fragments are therefore easily overlooked with their potential not being recognized. For this reason, FBLD screenings usually have a much higher hit rate, around 1-4%, which is superior by a factor of 10 to 1 000 compared to conventional binding assays [276]. Such an approach is therefore auspicious for the discovery of completely new classes of compounds, exosites, or even unknown conformations of CK2α' as already successfully demonstrated in the case of CK2α by Brear *et al*. with the discovery of the αD binding pocket [277]. Similarly, it would be possible to detect new hidden binding sites, so-called cryptic sites (see Subsection 8.5).

Recently, the enormous potential of FBLD was repeatedly demonstrated in the search for new antiviral compounds targeting the SARS CoV 2 virus. For example, in their search for ligands for the viral NSP13 helicase, Newman *et al*. [278] found 65 novel fragments in 52 datasets from a total of 648 screened crystals. Douangamath *et al*. [279] even surpassed this number by setting up 1 742 soakings and 1 139 co-crystallizations in their search for ligands for the 3C main protease. As a result, the authors were able to publish 96 complex structures in the PDB, some of which provide promising starting points for the development of new antiviral drugs. A similar plentiful output might also be possible for CK2α' by using the novel crystallization protocol discussed within the scope of this thesis.

8.3 The Potential of Bivalent Inhibitors to Overcome Selectivity Problems

In principle, all combinations of CK2 binding pockets are possible for the development of bivalent inhibitors, whereby the respective pair should block at least one functionally important binding site. However, to date, only bisubstrate inhibitors and inhibitors such as KN2 that cover the ATP-site and αD-binding pocket have been realized [280] [252]. While a combination including the α/β-binding area is also conceivable, in this case, the development of an adequate linker could be a particular challenge. The linker would have to be very long, for example >30 Å to reach the cosubstrate binding site, which could negatively influence the binding energy of the compound. In the case of linking the α/β-binding area and the substrate binding site, the linker would have to be even longer. The advantage in this case would be that the degree of conservation of the ATP binding pocket would not negatively affect the selectivity of the bivalent inhibitor. Nevertheless, the bivalent inhibitor KN2 presented in Chapter 7 showed that very high levels of selectivity can be achieved even by simultaneously addressing the ATP-binding pocket as proved by the high Gini coefficient of KN2.

The Gini coefficient has become widely accepted as a measure for quantifying and comparing the selectivity of different kinase inhibitors [281]. It is a statistical expression to describe the selectivity in a single number, by plotting the cumulative fraction of total inhibition against the cumulative fraction of tested EPKs (Figure 19a). This number can range from 0 to 1, whereby 0 corresponds to a perfect equidistributional line, i.e., a maximum of non-selectivity, and 1 corresponds to ideal selectivity, i.e., no kinase other than the target kinase is inhibited. This numeric range, which is especially easy to grasp, facilitates a certain comparability between the selectivity profiles of different compounds. However, it should also be noted that the Gini coefficient depends on multiple arbitrary parameters which again limits the level of comparability between different studies. The freely selectable parameters include the spectrum of kinases examined, and the ATP or inhibitor concentration. KN2 was tested against a panel of 83 kinases at an inhibitor concentration of 3 µM, resulting in a notably high Gini coefficient of 0.76 (Figure 19b). Remarkably, in previous studies, the corresponding isolated ATP-competitive binding moiety TBI only yielded a Gini coefficient of 0.31 with 70 EPKs being tested at an inhibitor concentration of 10 µM. Thus,

a significant increase in selectivity could be achieved by incorporating the αD-binding moiety. In a direct comparison, CAM4066 only demonstrates a marginally higher Gini coefficient of 0.82, although CAM4066 was tested against a considerably smaller panel of only 52 EPKs and a lower inhibitor concentration of 2 µM. According to Brear *et al*. [277], the affinity of the bivalent inhibitor should not be dominated by the ATP-competitive part, because otherwise, the selectivity problems of the ATP-binding pocket would again move into the foreground. However, the high selectivity of KN2 despite the rather affine ATP-competitive moiety reveals that the assumption formulated by Brear *et al*. is not universally valid. Finally, it should be noted that both bivalent inhibitors have a significantly better Gini coefficient compared to silmitasertib, which was long considered the gold standard in terms of selectivity. However, to achieve better comparability, ideally the entire kinome would need to be included in the study of each kinase inhibitor.

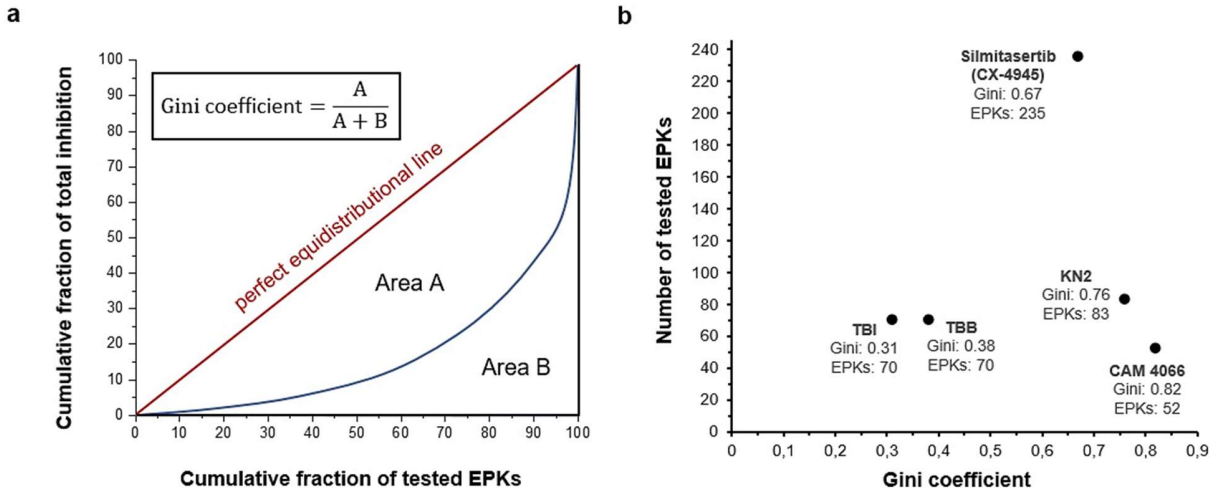

Figure 19. (**a**) The Gini coefficient describes the selectivity of a kinase inhibitor based on experimentally determined inhibition data, based on a panel of several different kinases. The cumulative fraction of total inhibition is plotted against the cumulate fraction of the tested kinases. The resulting curve defines two areas under the perfect equidistributional line, from which the Gini coefficient can be calculated according to the given formula. (**b**) The Gini coefficients of different CK2 inhibitors are plotted against the size of the tested kinase panels. The figure indicates the different levels of selectivity of the inhibitors but also the differences in the experimental design of the studies. Inhibitors compared with each other are: KN2 [273], silmitasertib [199], CAM 4066 [277], TBB [199] [282] and TBI [199] [282].

In conclusion, KN2 can be considered as a provider of further evidence that bivalent inhibitors have the potential to finally overcome the selectivity problems of CK2 inhibitors. As a result, testing additional combinations of binding sites may be rewarding and should be pursued further. In this

regard, the compound class of 2-aminothiazoles that was described in 2019 by Bestgen *et al*. [254] [255] as being non ATP-competitive inhibitors initially looked promising since they seemed to broaden the spectrum of allosteric ligands available for the development of new bivalent inhibitors. However, crystallization efforts with both paralogs and the help of the new CK2α' crystallization protocol disproved the binding site originally suspected by the authors. It appears as if their obtained data were misinterpreted by the authors, which was indicated by the crystal structures presented along with the supporting kinetic measurements in the course of this thesis (Chapter 6). Without exception, the co-crystal structures of all tested 2-aminothiazoles, including the best binding inhibitor, presented by Bestgen *et al*. [255], revealed by excellently defined electron density maps that these compounds are located in the cosubstrate binding pocket rather than an allosteric site. This result is unfortunate because the originally suspected binding site for 2-aminothiazole would have been very close to the substrate and cosubstrate binding site. Otherwise, the design of bivalent or even trivalent inhibitors would have been apparent due to the proximity of the sites. Nevertheless, in this thesis, a promising novel finding concerning an additional binding site is presented. This putative new exosite is located in the N-terminal segment and could be combined with the substrate binding site to derive a novel bivalent inhibitor. Further details concerning these findings are presented in Subsection 8.5.

8.4 Structural Basis for the Development of Paralog-Specific CK2 Inhibitors

Inhibitors able to discriminate between CK2α and CK2α' would provide valuable tools to study the complex biological functions of the two paralogs independently and are thus highly desirable. On the one hand, such compounds could help to answer the question of the evolutionary conservation of the two paralogs in vertebrates as, for example, other species such as insects can thrive with CK2α only. Furthermore, it would be very interesting to study the pharmacological impact on different human cell lines, especially on malignant cells, which show a severe, acquired "addiction" to one or both paralogs. A potent and selective inhibition of the respective paralog might have a strong impact on the viability of these cells, whereas the unaffected paralog could nevertheless fulfill its functions in the cells and thus possibly exert a certain protective effect for benign cells. However, such an assumption is highly speculative at present and would need to be verified

experimentally with suitable compounds. Unfortunately, the cosubstrate pocket is not very favorable for the design of discriminatory inhibitors. First, there are only two sequence mismatches in this region, more specifically in the hinge loop; second, the corresponding amino acids normally only interact with ATP-competitive inhibitors via their main chain atoms. Moreover, the observed differences in inhibitory activity, as in the case of THN27, are usually rather small and arise from subtle changes in the binding mode, which, however, might be difficult to optimize (see Chapter 4). Moreover, the example of THN27 shows how the presence of CK2β is able to nullify such discriminating properties.

The α/β-interaction region does not appear to be more suitable as a starting point for the development of discriminatory inhibitors. So far, only minor differences of approximately one magnitude in the affinity of β-competitive compounds have been reported, such as derivatives of the CK2β mimicking peptide Pc [283]. This is consistent with the observed difference in binding affinity of the natural ligand, CK2β, which binds also 10-fold stronger to CK2α compared to CK2α' [138]. Most importantly, the general problems associated with the development of CK2β-competitive inhibitors must be considered, such as the fact that mainly class III substrates would be affected by such compounds (see Subsection 1.3.3). Furthermore, in contrast to the ATP binding pocket, the K_d value of the α/β-interaction is in the low nanomolar range, which means that only extremely high-affinity compounds would qualify as CK2β-competitive moieties.

Hopes for the synthesis of paralog-specific inhibitors may be pinned on the αD pocket, which exhibits a subtle yet consequential sequence difference, namely Ile140 in CK2α to Leu141 in CK2α' located at helix αE. The side chain of Ile140 protrudes deeper into the αD pocket by the length of about one methyl group compared to CK2α' (see Figure 20). The local constriction of the binding pocket forces the *meta*-chlorine of the αD binding ring of KN2 to perform a rotation of about 160° and thus to point away from Ile140. The same can be observed for similar compounds such as the lead molecule DPA, discovered by Brear *et al*. [277]. Interestingly, this alternative binding mode is only possible because Leu128 on the opposite side of the binding pocket moves aside and thereby exposes a small bulge, which roughly equals the volume loss caused by Leu140 and is enough for the *meta*-chlorine to fit in.

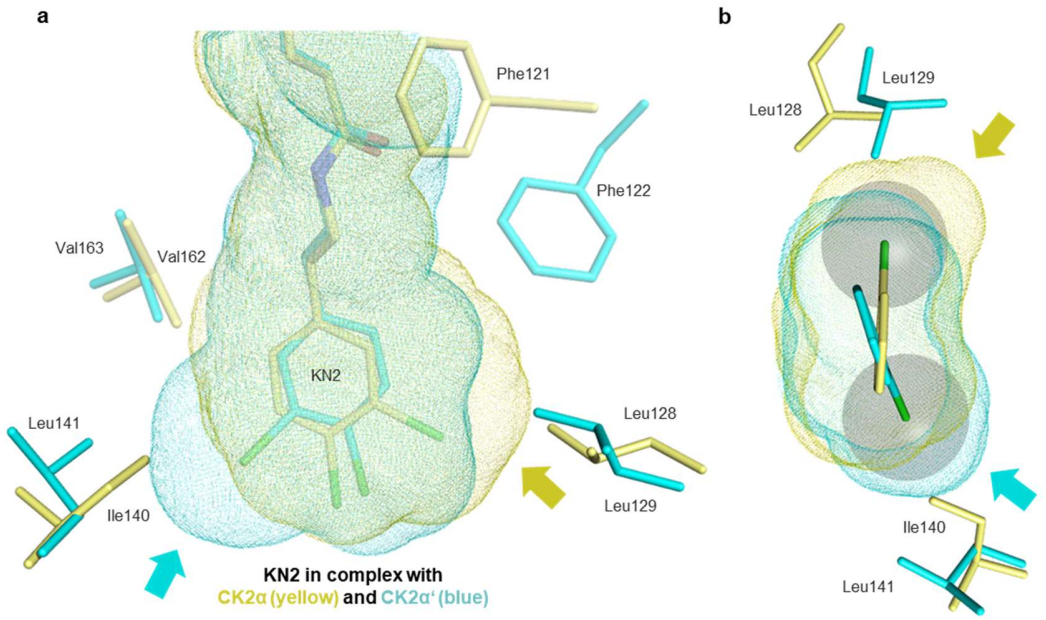

Figure 20. The αD pocket is shown in a side view (**a**) and a cross-section (**b**). The structures shown are complexes of KN2 with CK2α (PDB 7AT5, shown in yellow) and with CK2α' (PDB 7ATV, shown in blue). The inner outline of the αD-pocket, i.e., the freely accessible space for ligands is depicted as a fine mesh, with the encoded colors corresponding to the color scheme of the models, i.e., yellow regions are only accessible in the CK2α structure and blue regions only to CK2α' [k]. The enlarged binding region in CK2α' caused by the sequence difference, Ile140 to Leu141, is marked with a blue arrow. The bulge opening on the opposite side in CK2α, which serves as an alternative binding region for the *meta*-chlorine atom of KN2, is marked with a yellow arrow. To illustrate the positions of the chlorine atoms in the pockets, the Van der Waals radii of the *meta*-halogens are illustrated as gray spheres in (**b**).

Thus, a unilateral expansion of the αD pocket binding moiety by replacing the *meta*-chlorine substituents with bigger groups in order to increase the paralog specificity would probably not be effective in terms of paralog selectivity. It would rather support the different orientations of the aromatic ring in the two paralogs but it might not lead to an increased paralog selectivity. In fact, both the *meta* and *meta'*-positions would have to carry larger substituents to preclude CK2α binding for steric reasons. However, as a factor of uncertainty, it must be considered that even more conformational states of the flexible helix αD might exist and an even more extensive "volume compensation" in the region around Leu128 could be induced. The current pocket volume of approximately 300 Å^3 might thereby not be the end of the line. Another problem could result from the fact that a significantly enlarged αD moiety could generally lead to a hampered binding

[k] The representation of the inner contours of the binding pockets was calculated using the program MoloVol [284].

process to the narrow pocket, which would need a considerably wider initial opening for the inhibitor to move in.

In conclusion, the αD pocket currently offers the most promising possibility to generate paralog specific inhibitors by systematic derivatization in the meta and meta' positions. Strictly speaking, however, high selectivity can only be expected for CK2α' due to the larger αD pocket volume, which in turn would be of particular pharmacological interest for CK2α'-addicted cancer subtypes such as some lung, colorectal, and prostate cancers [175] [180].

8.5 A Novel Exosite Revealed by a CK2α'/ARC780 Co-crystal Structure

To date, several bisubstrate inhibitors including many ARC compounds have been crystallized with CK2α and CK2α' [252]. In the course of these experiments, various attempts have been made to modify the crystallization conditions to obtain a complex structure with the inhibitor fully covered with electron density. Unfortunately, the substrate-competitive parts of such types of inhibitors have so far not been sufficiently visualized [229] [243]. Similarly, other attempts to obtain protein/substrate or even ternary complexes with different substrates have also never been successful. Nevertheless, there is a growing list of examples of such complexes for other kinases in the PDB [285] [286] [287]. Due to the similar architecture of EPKs, these structures provide indications concerning the substrate binding mode of CK2, thereby allowing the putative location of substrate peptides at least to be modeled *in silico*. In addition, anions such as sulfates have repeatedly been observed in well-defined positions, indicating which amino acid residues interact with the acid functions of the substrate's P+1 and P+3 positions [288]. This prediction is also consistent with the recently published structure of a CK2α$^{1-335}$/heparin complex, in which the putative P+1 position was occupied by a very well-defined acid function of heparin [95].

A recurring observation in crystallization experiments with bisubstrate inhibitors was that between the ATP-competitive part and the substrate-competitive moiety, small parts of the linkers were often visible and, in most cases, pointed toward the solvent channels, i.e., away from the enzyme. Thus, the interaction with the corresponding regions of CK2α or CK2α' is probably not sufficiently strong under the prevailing conditions in the crystallization droplet. The failure to observe electron

density for the peptidic parts of bisubstrate inhibitors is not too surprising, assuming that the interactions between the basic amino acids of CK2α in the substrate binding region and the acidic residues of the substrate sequence are mostly of an ionic nature. The mother droplet is a highly saline mixture, and in the case of CK2α, it usually has a salt concentration of several hundred millimolar. The storage buffer of CK2α alone already contains 500 mM sodium chloride as well as 25 mM Tris (pH 8.5) and to this, the salts of the precipitation solution must be added. It is likely that these high salt concentrations significantly disrupt the anticipated intermolecular interactions between the substrate and the enzyme, thereby resulting in the observed absence of electron density to date. It is also consistent that the published complex structure with heparin has required a reduction in the concentration of salts and their replacement with malonate [95].

However, a unique opportunity to obtain the desired complex structures in which the substrate binding site might be occupied emerged from the novel crystallization protocol for the CK2α'. Not only do these crystals scatter extremely well but they can also withstand external influences such as large amounts of organic solvents or osmotic pressures to a high degree, as mentioned in Chapter 4 and Subsection 8.2. For this reason, various ARC substances, including ARC780 (Figure 21a), were soaked in CK2α' crystals and then subjected to a desalting procedure over a period of eight months (Figure 21b). Here, the lithium chloride content of the reservoir solution was reduced in a stepwise manner from 900 mM to 50 mM and the original sodium chloride content of 500 mM, which was added via the storage buffer of the protein, was completely removed.

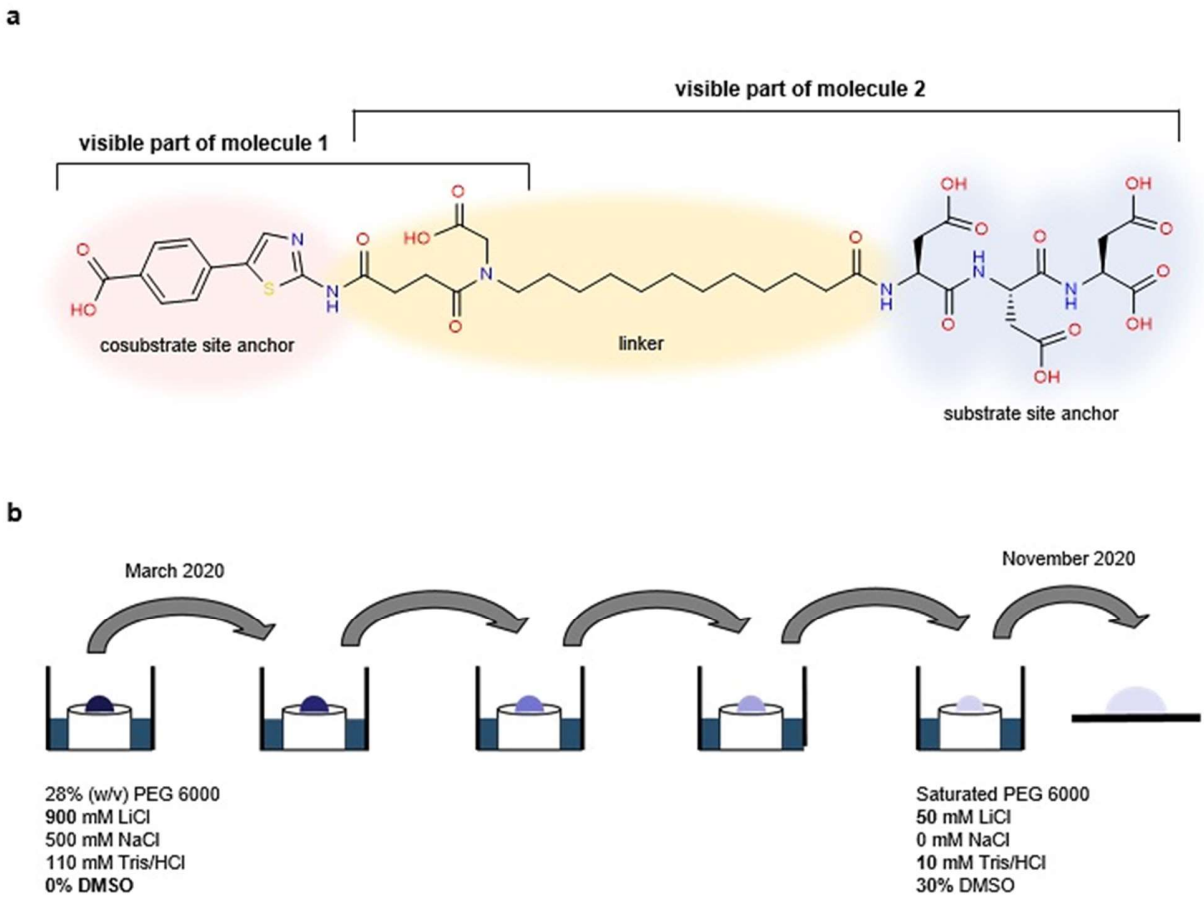

Figure 21. (**a**) ARC780 is a bisubstrate inhibitor that is composed of three parts. On one side, the inhibitor has an ATP-competitive head group (red) consisting of two aromatic rings and an acid function. On the other side, the inhibitor ends in a tripeptide (blue), which is composed of three aspartic acid units according to the substrate preference of CK2. Both parts are connected by a hydrophobic and rather flexible linker (yellow). Although two ARC780 ligands were found in the derived co-crystal structure, both of them were only partially coated with electron density. The visible regions are indicated in the figure. (**b**) The obtained structure is the result of an eight month desalting procedure. In the course of this process, the salt concentration was stepwise reduced whereas the concentration of PEG 6000 and DMSO was significantly increased.

This extensive desalting procedure finally allowed the localization of the peptide part of ARC780 (statistical data are presented in Table 3, Subsection 10.1). However, the structure obtained is surprising in two respects. First, not one, but two inhibitor molecules are bound to the enzyme (Figure 22a). As in previous crystallization experiments, one ligand binds to the cosubstrate binding pocket, where it is well-defined by electron density (Figure 22b). As expected from previous crystallization experiments, the planar rings of ARC780 fit very well into the narrow

cosubstrate binding pocket of CK2α', in which they interact with the amino acids of the binding pocket via hydrophobic interactions. The orientation of the inhibitor is determined by a typical ionic interaction between the acid function of the benzoate and the sidechain of Lys69, comparable to silmitasertib or the 2-aminothiazoles described in Chapter 6 and many other examples. However, as is usual in the case of CK2α structures in complex with bisubstrate inhibitors, only the first few atoms of the linker close to the ATP-competitive moiety are covered with electron density, and this part is oriented toward the solvent rather than toward the substrate binding site.

For the second bound ligand, exactly the opposite applies: the complete linker including the peptide part is visible, whereas the ATP-competitive part is not covered with electron density. An even more surprising finding was that the peptide moiety and the linker of the second inhibitor molecule are not located in the substrate binding area as expected, but rather they are in fact located in the region of the N-terminal segment in a groove that has not previously been described for CK2 in the literature (Figure 22c). To permit access to this newly discovered cryptic binding site, residues Gln187 and His184 had to tilt to the side, thereby extending the substrate binding cleft up to the N-terminal segment. The peptide part of ARC780 is excellently defined, even though it only forms a single hydrogen bond with Ser18. This can be explained by the presence of a crystal contact in the N-terminal region that allows the peptide part of the ARC780 molecule to interact with a symmetry equivalent of CK2α'. On one side, the acid function occupying the P+3 site of the enzyme provides a strongly coordinated anchor; on the other side, a peptide nitrogen forms a hydrogen bond with Ser18. The connecting hydrocarbon chain is stabilized by hydrophobic interactions, e.g., by being sandwiched in between the aromatic residues Trp25 and His184.

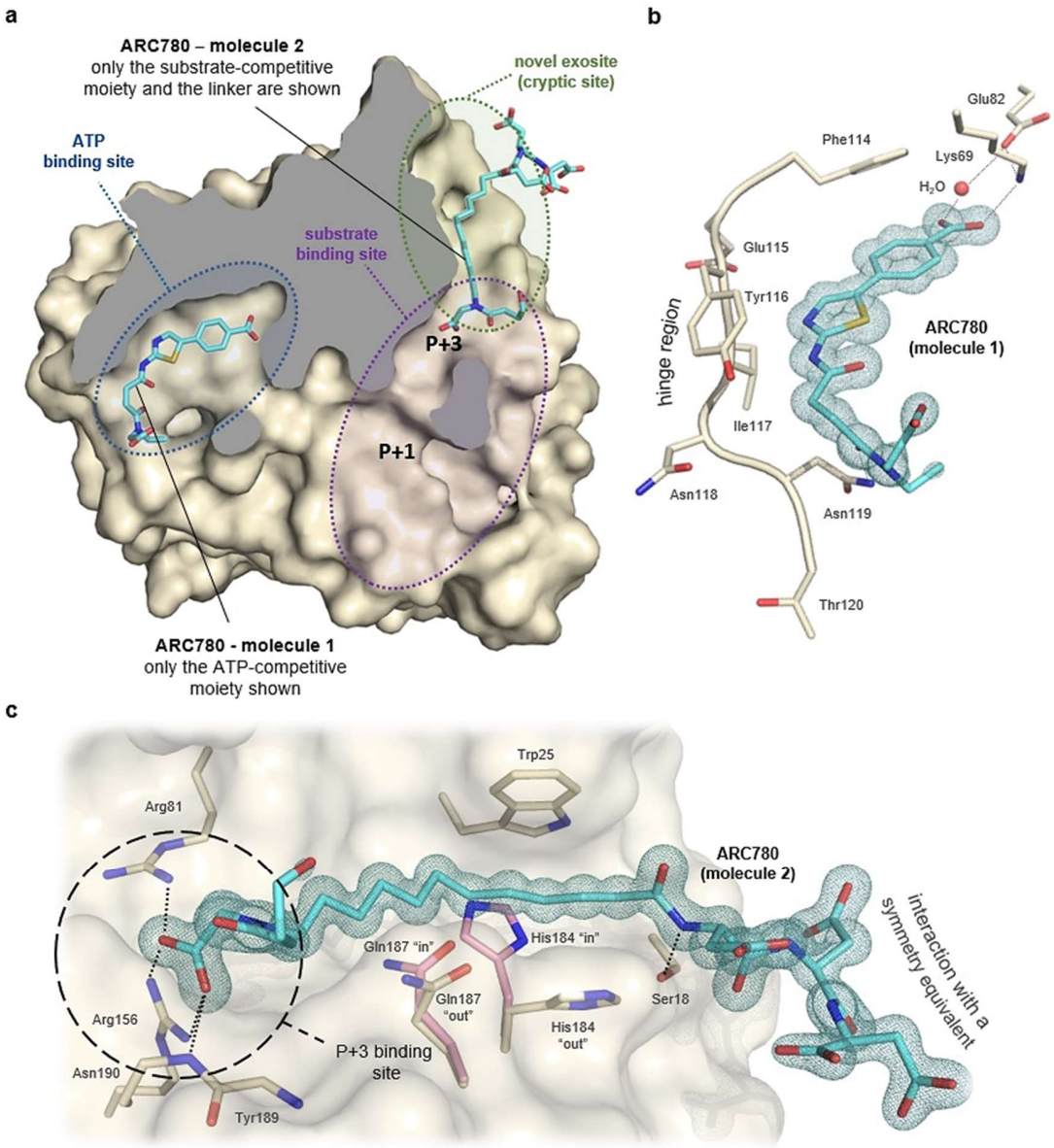

Figure 22. CK2α'/ARC780 complex structure obtained after an eight-month desalting procedure. (**a**) Cross-section through the enzyme with the N-terminus cleaved off. Two ARC780 ligands are bound to the enzyme, although both inhibitors are reduced to the defined parts that were covered with electron density. One molecule is bound in the cosubstrate binding pocket (circled in blue), and one molecule occupies a previously unknown exosite (circled in green). (**b**) Detailed view of the cosubstrate binding pocket. The ligand (molecule 1) binds as observed in previous experiments and forms a hydrogen network with Lys69, a coordinated water, and Glu82 (cutoff level 1.5 σ). Another hydrogen bond is found between the side chain of Asn119 and a peptide oxygen of the ligand. (**c**) Detailed view of the novel exosite with the second ARC780 ligand bound (cutoff level 1.5 σ). Amino acids Gln187 and His184 are involved in the opening and closing of the binding pocket. The corresponding conformations are superimposed and labeled as "in" for the closed conformation (pink) and "out" for the opened conformation. The closed conformation was retrieved from the PDB code 6HMQ.

The novel exosite shown here was not previously described in the literature for CK2, and rarely for other kinases. However, in the case of Aurora A kinase, two ligands were recently found at a comparable location. In 2016, Rennie *et al*. [289] noticed a MES buffer molecule (2-(N-morpholino)ethanesulfonic acid) from the crystallization solution at a similar site. Just one year later, McIntyre *et al*. [290] found a single hit out of a total of 59 hits from an X-ray-based fragment screening, although this ligand was approximately 8 Å apart from the ARC780 binding region. A direct comparison of the Aurora A and the CK2α'/ARC780 complex structure does not lead to any further conclusions, since the Aurora A kinase is structurally significantly different compared to CK2α' in the region of the novel binding site and the N-terminal segment.

The question arises why ARC780 fails to bind as expected, covering the substrate and cosubstrate binding sites. The reason might be the linear design of the ATP-competitive moiety of ARC780, which already forces the linker to point outward into the solvent. Therefore, it would make sense to rather bind the thiazole ring in the ortho or meta-position to the benzoic acid ring, so that the ATP-competitive part already features a fixed angle that guides the linker in the desired direction, towards the substrate binding site. It should be noted, however, that the inhibitor design was based on many assumptions and simplifications in the absence of a ternary structure, leading to uncertainties regarding the linker length or the structure and sequence of the substrate--competitive part.

The low-salt complex structure of CK2α' with ARC780 that was obtained can nevertheless be considered a serendipitous finding and the novel binding site may serve as a starting point for a new generation of monovalent or bivalent inhibitors covering the adjacent substrate binding site. In this context, ARC780 can serve as a lead structure, and it is advisable to extend the linker in the direction of the substrate binding site with further peptide units or similar artificial acidic monomer units, so that the P+1 position can also be exploited as an anchor site. The peptide part of ARC780 and the cosubstrate binding part can, however, be removed, as these will probably not contribute to binding in solution. The aligned residues Trp25 and His184, on the other hand, offer a unique opportunity to create an additional interaction by forming a so-called face-to-face or parallel-displaced multiple ring π-π stacking, which can be energetically favorable [291]. The incorporation of an aromatic ring into the hydrocarbon chain could significantly increase the affinity of a second-generation inhibitor. As an alternative, the attachment of a positively charged group would also be a conceivable interaction with the aromatic rings [292].

Despite the fact that the original experimental objectives concerning ARC780 as a bisubstrate inhibitor were not achieved, valuable information was derived from the time-consuming

crystallographic experiment. It was demonstrated that a drastic lowering of the salt concentration led to a change in the binding behavior of the ligands. The novel binding site that was found could represent the starting point for the development of a completely new group of potent and potentially selective inhibitors. Furthermore, it is yet another example of the high value of the novel CK2α' crystallization protocol for the structural study of CK2 in general. For decades, CK2α' has largely remained overlooked, and had been crystallographically neglected. However, the efforts to crystallize CK2α' in the context of this work have ultimately proven to be highly rewarding.

Chapter 9 – References

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Chapter 10 – Appendix

10.1 Crystallographic Supplementary Tables

Table 3. Statistics of the CK2α'/ARC780 complex structure after performing an eight-month desalting procedure. The corresponding values for the highest-resolution shells are written in parentheses. Since the structure is still unpublished, no PDB code is available yet.

Crystallization	
Sitting drop composition before equilibration	10 µl reservoir + 20 µl CK2α^{C336S}/MB002 mixture (180 µl 6 mg/ml CK2α^{C336S}, 500 mM NaCl, 25 mM Tris/HCl, pH 8.5, mixed and pre-equilibrated with 20 µl 10 mM inhibitor in dimethyl sulfoxide)
Vapor diffusion reservoir composition	28 % (w/v) PEG 6000, 900 mM LiCl, 100 mM Tris/HCl, pH 8.5
Complex formation	Extensive soaking with ARC780
Desalting target	Stepwise exchange of the reservoir solution to a final composition of saturated PEG 6000, 90 mM LiCl, 10 mM Tris/HCl, pH 8.5; subsequent washing of the crystals with reservoir solution mixed with ARC780
X-ray Diffraction Data Collection	
Wavelength [Å]	0.9677
Synchrotron (beamline)	ESRF (ID30A-3)
Space group	P1
Unit cell a, b, c [Å] | α, β, γ [°]	46.232 47.511 50.6 | 66.598 89.596 88.127
Resolution [Å]	32.65 -1.231 (1.275 - 1.231)
R-merge	0.04334 (1.358)
R-meas	0.05043 (1.587)
R-pim	0.02568 (0.815)
$CC^{1}/_{2}$	0.999 (0.435)
Signal-to-noise ratio (I/σI)	13.94 (0.66)
No. of unique reflections	86431 (625)
Completeness [%]	71.60 (5.47)
Multiplicity	3.8 (3.6)
Wilson B-factor [Å^2]	11.61
Structure Refinement	
Reflections used in refinement	82006 (626)
Reflections used for R-free	1542 (17)
R-work	0.1256 (0.3942)
R-free	0.1504 (0.3580)
Number of non-hydrogen atoms	3192
macromolecules	2806
ligands	109
solvent	277
Protein residues	328
RMS (bonds)	0.011
RMS (angles)	1.09
Ramachandran favored [%]	97.85
Ramachandran allowed [%]	2.15
Ramachandran outliers [%]	0.00
Rotamer outliers [%]	0.00

Clashscore	3.13
Average B-factor	17.34
macromolecules	15.69
ligands	22.44
solvent	32.06

10.2 List of Publications and Author's Contributions

J. Hochscherf, D. Lindenblatt, B. Witulski, R. Birus, D. Aichele, C. Marminon, Z. Bouaziz, M. Le Borgne, J. Jose and K. Niefind, "Unexpected Binding Mode of a Potent Indeno[1,2-b]indole-Type Inhibitor of Protein Kinase CK2 Revealed by Complex Structures with the Catalytic Subunit CK2α and Its Paralog CK2α'", Pharmaceuticals (Basel), vol. 10, no. 4, Dec 13 2017, doi: 10.3390/ph10040098

K.N. and J.H. designed the crystallographic experiments. **D.L.** and B.W. performed the crystallographic experiments. **D.L.**, B.W., J.H. and K.N. analyzed the crystallographic data. J.H. and **D.L.** purified the protein. D.A. and J.J. designed the cellular assay. D.A. and R.B. performed the cellular assay. C.M. and Z.B. managed the synthesis of compound 4p and performed purification and structural identification for all intermediates to generate the targeted compound; J.H. and K.N. wrote the paper. M.L.B. and J.J. designed the drug design project and revised the paper.

D. Lindenblatt, M. Horn, C. Götz, K. Niefind, I. Neundorf, and M. Pietsch, "Design of CK2β-Mimicking Peptides as Tools To Study the CK2α/CK2β Interaction in Cancer Cells", ChemMedChem, vol. 14, no. 8, pp. 833-841, Dec 17 2019, doi: 10.1002/cmdc.201800786

K.N., I.N., and M.P. conceived and designed the experiments. **D.L.** performed the solid-phase peptide synthesis and fluorescence anisotropy measurements. M.H. performed the cellular assays and toxicity studies, and C.G. contributed to the radioactive phosphorylation assays. **D.L.**, K.N., I.N., and M.P. wrote the manuscript.

D. Lindenblatt, A. Nickelsen, V. M. Applegate, J. Hochscherf, B. Witulski, Z. Bouaziz, C. Marminon, M. Bretner, M. Le Borgne, J. Jose and K. Niefind, "Diacritic Binding of an Indenoindole Inhibitor by CK2α Paralogs Explored by a Reliable Path to Atomic Resolution CK2α' Structures", *ACS Omega,* vol. 4, no. 3, pp. 5471-5478, Mar 31 2019, doi: 10.1021/acsomega.8b03415

D.L., V.M.A., J.H. and B.W. designed and performed the crystallographic experiments. **D.L.**, V.M.A., J.H. and K.N. analyzed the crystallographic data. A.N. performed the measurements of dose-response curves on isolated CK2. Z.B. and C.M. managed the synthesis and purification of

compounds 4p, THN27, and AR18. M.B. provided the compound MB002 for crystallographic experiments. K.N. wrote the manuscript which was revised by **D.L.**, V.M.A., M.B., M.L.B., and J.J.

K. Chojnacki, D. Lindenblatt, P. Wińska, M. Wielechowska, C. Toelzer, K. Niefind and M. Bretner, "Synthesis, biological properties and structural study of new halogenated azolo[4,5-b]pyridines as inhibitors of CK2 kinase", Bioorg Chem, vol. 106, p. 104502, Jan 2021, doi: 10.1016/j.bioorg.2020.104502

K.C., P.W., and M.W. synthesized all tested TBBt and TBBi derivatives and performed the radiometric phosphorylation assays and the cell viability experiments. **D.L.** and C.T. designed and performed the crystallographic experiments. **D.L.**, C.T., and K.N. analyzed the crystallographic data. M.B. and K.N. wrote the manuscript.

D. Lindenblatt, A. Nickelsen, V. M. Applegate, J. Jose, and K. Niefind, "Structural and Mechanistic Basis of the Inhibitory Potency of Selected 2-Aminothiazole Compounds on Protein Kinase CK2", J Med Chem, vol. 63, no. 14, pp. 7766-7772, Jul 23 2020, doi: 10.1021/acs.jmedchem.0c00587

D.L. designed the crystallographic experiments. **D.L.** and V.M.A. performed the crystallographic experiments. **D.L.**, V.M.A., and K.N. analyzed the crystallographic data. A.N. performed the measurements of dose-response curves on isolated CK2. A.N. and K.N. analyzed the collected kinetic data. K.N. wrote the manuscript, which was subsequently corrected and approved by all authors.

D. Lindenblatt, V, Applegate, A, Nickelsen, M, Klußmann, I, Neundorf, C. Götz, J. Jose and K. Niefind, "Molecular Plasticity of Crystalline CK2α' Leads to KN2, a Bivalent Inhibitor of Protein Kinase CK2 with Extraordinary Selectivity", J Med Chem, vol. 65, no. 2, pp. 1302-1312, Jan 27 2022, doi: 10.1021/acs.jmedchem.1c00063

The synthesis and purification of KN2 were managed by **D.L.** and V.M.A. The crystallization experiments were designed and performed by **D.L.** and V.M.A. The analysis of the structural data was done by **D.L.**, V.M.A., and K.N. Inhibition data were collected by A.N. using the capillary electrophoresis assay. M.K. determined the cytotoxic effect of KN2 and Silmitasertib. The Western blot analysis was performed by C.G. The kinase selectivity screening with KN2 was outsourced to

Carna Biosciences. **D.L.** and K.N. wrote the manuscript. All authors corrected and approved the manuscript.

10.3 Curriculum Vitae

CV – DIRK LINDENBLATT

PERSONAL INFORMATION

Name	Dirk Lindenblatt
Date of Birth	20.07.1990
Place of Birth	Dortmund, Germany
Nationality	German

UNIVERSITY EDUCATION

03/2017 - 03/2023	**Ph.D. studies** University of Cologne, Department of Biochemistry Supervisor: Prof. Dr. K. Niefind
04/2014 - 09/2016	**M.Sc. in Biological Sciences** University Hospital of Cologne, Department of Pharmacology Thesis supervisor: Dr. Markus Pietsch Thesis title: Synthesis of Chemically Optimized CK2β Mimicking Peptides and Their Pharmacological Characterization on Isolated CK2α and on HeLa Cells.
10/2014 - 03/2022	**B.Sc. in Chemistry** University of Cologne, Department of Chemistry Thesis supervisor: Prof. Dr. Stephanie Kath-Schorr Thesis title: Einbau eines 8-Methylguanosin-Phosphoramidit-Bausteins in RNA mittels Festphasensynthese zur Ausbildung von Z-Form Duplexstrukturen.
10/2010 - 09/2013	**B.Sc. in Biological Sciences** University of Cologne, Department of Biochemistry Thesis supervisor: Prof. Dr. K. Niefind Thesis title: Optimization of a Peptide Interfering with the Subunit Interaction of Human Protein Kinase CK2α to a Peptidomimetic Compound.

Unterschrift
(Dirk Lindenblatt)

10.4 Declaration

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

Teilpublikationen:

- D. Lindenblatt, M. Horn, C. Götz, K. Niefind, I. Neundorf, and M. Pietsch, “Design of CK2β-Mimicking Peptides as Tools To Study the CK2α/CK2β Interaction in Cancer Cells”, ChemMedChem, vol. 14, no. 8, pp. 833–841, Apr 17 2019, doi: 10.1002/cmdc.201800786.
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- D. Lindenblatt, A. Nickelsen, V. M. Applegate, J. Jose, and K. Niefind, “Structural and Mechanistic Basis of the Inhibitory Potency of Selected 2-Aminothiazole Compounds on Protein Kinase CK2”, J Med Chem, vol. 63, no. 14, pp. 7766–7772, Jul 23 2020, doi: 10.1021/acs.jmedchem.0c00587.
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Datum, Name und Unterschrift

16.12.22, Dirk Lindenblatt