Selective modulation of the Protein Kinase CK2:

discovery, syntheses and characterization

of non-ATP site inhibitors of CK2

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The present study was conducted from October 2011 to November 2015 under the supervision of Prof. Dr. Rolf W. Hartmann in the faculty of Pharmaceutical and Medicinal Chemistry of Natural Sciences and Technology of Saarland University, and under the supervision of Prof. Marc Le Borgne and Dr. Thierry Lomberget in the department of Medicinal Chemistry in the faculty of pharmacy of Lyon University.

Die vorliegende Arbeit wurde von Oktober 2011 bis November 2015 unter Anleitung von Herrn Prof. Dr. Rolf W. Hartmann in der Fachrichtung 8.2 Pharmazeutische und Medizinische Chemie der Naturwissenschaftlich-Technischen Fakultät III der Universität des Saarlandes angefertigt, und unter Anleitung von Herrn Prof. Marc Le Borgne und Herrn Dr. Thierry Lomberget in der Fachrichtung Medizinische Chemie der Pharmazeutische Fakultät der Universität des Lyon angefertigt.

Le travail présenté a été réalisé entre octobre 2011 et novembre 2015 sous la supervision du Prof. Dr. Rolf W. Hartmann au sein du département de pharmacie et de chimie médicinale de la faculté des sciences naturelles de l'Université de la Sarre, et sous la supervision du Prof. Marc Le Borgne et du Dr. Thierry Lomberget au sein du département de chimie thérapeutique de la faculté de pharmacie de l'Université de Lyon.

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"It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us, we were all going direct to Heaven, we were all going direct the other way […]"

A Tale of Two Cities (Charles Dickens)

English summary

Selective modulation the Protein Kinase CK2: discovery, syntheses and characterization of non-ATP site inhibitors.

The protein kinase CK2 is a tetrameric enzyme composed of a dimer of regulatory subunits (β) and two catalytic subunits, CK2a and/or CK2a'. The catalytic subunit of CK2 is constitutively active, while the regulatory subunit modulates the selectivity toward a subset of substrate proteins. CK2 is a ubiquitous Ser/Thr protein kinase involved in the control of various signaling pathways, and dysregulation of CK2 promotes cancer development. CK2 has been proved to be a valuable target in cancer treatment.

Our objective was to target CK2 outside the ATP-pocket. Two independent classes of compounds were studied:

- Based on a first hit with a low potency (IC_{50} = 30 µM) but a non-ATP competitive mechanism of action, several 2-aminothiazole derivatives were synthesized to lead to a potent (IC_{50} = 0.6 µM) and cell efficient allosteric inhibitor of CK2. Using single mutation scanning, CD spectrometry, STD-NMR and docking experiments, the binding site of our compounds was precisely defined outside the ATP-pocket, at the interface of the glycine-rich loop and the aC-helix.

- Inhibitors of the a/β interaction were studied from a small cyclic peptide to the development of small molecules through Virtual Ligand Screening. Structures Activity Studies were conducted on the synthesized derivatives and cellular based assays to evaluate the a/β inhibitors were set up.

The two classes of compounds developed herein are valuable tools to understand the physiological regulation of the protein kinase CK2, and potential new opportunities in cancer treatment.

Key words: Oncology, Medicinal chemistry, Biology, Protein Kinase

German summary

Selektive Modulation der Proteinkinase CK2: Entdeckung, Synthese und Charakterisierung von nicht ATP-kompetitiven Hemmstoffen.

Das Protein Kinase CK2 ist ein tetrameres Enzym, das aus einem Dimer von regulatorischen Untereinheiten (β) und zwei katalytischen Untereinheiten (CK2a und/oder CK2a') besteht. Die katalytische Untereinheit der CK2 ist konstitutiv aktiv, während die regulatorische Untereinheit die Auswahl einiger der durch CK2 phosphorylierten Substrate steuert. CK2 ist eine ubiquitäre Proteinkinase, die an der Kontrolle zahlreicher Signalwege beteiligt ist. Eine Fehlregulation der CK2 fördert die Tumorenstehung. Es konnte gezeigt werden, dass CK2 eine vielversprechende Zielstruktur für die Entwicklung neuer Therapeutika ist. Unser Ziel war es, neue Hemmstoffe der Proteinkinase CK2 zu entwickeln, die an anderen Stellen als dem aktiven Zentrum angreifen.

Zwei Serien von Verbindungen sind untersucht worden:

- Basierend auf einem ersten schwach aktiven "Hit" (IC_{50} = 30 µM), der einen nicht-ATP-kompetitiven Wirkmechanismus aufwies, wurden einige neue 2-Aminothiazol-Derivate synthetisiert. Dadurch wurden allosterische Inhibitoren mit einer deutlich gesteigerte Potenz (IC_{50} = 0,6 µM) und einer beachtlichen Zellaktivität erhalten. Mittels eines CK2-Punktmutanten-Screenings, Zirkulardichroismus-Spektrometrie, STD-NMR und molekularer Docking-Simulationen konnte die Bindestelle unserer Hemmstoffe außerhalb der ATP-Bindetasche, zwischen der Glycin-reichen Schleife und der aC-Helix, lokalisiert werden.

- Desweiteren wurden niedermolekulare Inhibitoren der α/β-Interaktion entwickelt, ausgehend von einem zyklischen Peptid sowie von Hitverbindungen aus einem virtuellen Screening. Neue Verbindungen wurden synthetisiert und die Struktur-Wirkungsbeziehungen analysiert; zusätzlich wurde ein Zellassay zur Überprüfung des postulierten Wirkmechanismus etabliert.

Die beiden entwickelten Verbindungsklassen sind interessante Werkzeuge, um die physiologische Regulation der Proteinkinase CK2 näher zu analysieren; überdies stellen sie Ausgangspunkte für die Entwicklung neuartiger Krebstherapeutika dar.

Stichworte: Onkologie, Medizinische Chemie, Biologie, Protein Kinase

French summary

Modulation sélective de la Protéine Kinase CK2 : Identification, synthèse et caractérisation d'inhibiteurs ne ciblant pas le site ATP.

La Protéine Kinase CK2 est une enzyme tétramérique composé d'un dimère de sous-unité régulatrice (β) et de deux sous-unités catalytiques, CK2a et/ou CK2a'. La sous-unité catalytique de CK2 est constitutivement active alors que la sous-unité régulatrice régule seulement la sélection des substrats phosphorylés par CK2. CK2 est une Ser/Thr protéine kinase ubiquitaire impliquée dans le contrôle de nombreuses voies de signalisations. La dérégulation de CK2 promeut le développement des cancers et il a été démontré que CK2 est une cible pertinente dans le traitement des cancers.

Notre objectif était de cibler la protéine kinase CK2 de manière indépendante du site actif. Deux séries de composés ont été étudiés :

- Basé sur un premier hit faiblement actif (CI_{50} = 30 μM) mais inhibant CK2 de manière non-ATP compétitive, des dérivés comportant le noyau 2-aminothiazole ont été synthétisés et un composé actif (CI_{50} = 0,6 μM) et efficace *in cellulo* a été obtenu. Grace à des expériences sur des mutants ponctuels de CK2, des expériences de dichroïsme circulaire, de la STD-RMN et de la modélisation moléculaire, le site de fixation de nos inhibiteurs a été précisément défini à l'interface de la boucle riche en glycine et de l'hélice-aC.

- Des inhibiteurs de l'interaction a/β ont été étudiés à partir d'un peptide cyclique jusqu'au développement de petites molécules via un screening virtuel. Des études de relations structure-activité ont été réalisé sur la série de composés synthétisées et des tests cellulaires ont été mis en place afin d'évaluer ces composés.

Les deux classes de molécules décrites sont des outils intéressants pour comprendre la régulation physiologique de la protéine kinase CK2 et des opportunités prometteuses dans le traitement de certains cancers.

Mots-clés: Oncologie, Chimie Médicinale, Biologie, Protéine Kinases

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Abbreviations

µM: Micromolar

3D: Three-Dimensional

ADMET: Absorption, Distribution, Metabolism, and Excretion - toxicity

AGC: protein kinase A, G and C (PKA, PKC, PKG)

Akt: Protein kinase B (PKB)

AMPPNP: Adenosine 5'-(β,γ-imido)triphosphate

ANS: 8-Anilinonaphthalene-1-sulfonic acid

ATP: Adenosine TriPhosphate

Bcr-Abl: breakpoint cluster region - Abelson murine leukemia oncogene homolog 1

Bn: Benzyl

Boc: tert-ButOxyCarbonyl

br s: broad singlet

CaM: Calmodulin

CAMK: Calmodulin/Calcium regulated Kinases

ccRCC: clear cell Renal Cell Carcinoma

CDK: Cyclin-dependent kinases

CFTR: Cystic fibrosis transmembrane conductance regulator

CK1: Cell Kinase 1 group

CK2: Casein Kinase 2

Clk: Cdc2-like kinase

CMGC: CDK, MAPK, GSK3 and CLK families

Cpd: compound

d: doublet

DBC1: Deleted in Breast Cancer 1

dd: doublet of doublet

DIPEA: DiIsoPropylEthylAmine

DLS: Dynamic Light Scattering

DMAP: 4-(Dimethylamino)pyridine

DMAT: 4,5,6,7-tetrabromo-N,N-dimethyl-1H-benzimidazol-2-amine

DMF: N,N'-dimethylformamide

DMSO: DiMethylSulfOxide

DRB: 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside

DSC: Differential Scanning Calorimetry

dt: doublet of triplet

DYRK: Dual specificity tyrosine-phosphorylation-regulated kinase

eg: examplia gratia

ELISA: Enzyme-Linked ImmunoSorbent Assay

EMA: European Medicines Agency

EMT: Epithelial–Mesenchymal Transition

ERK: Extracellular signal-regulated kinases

ESI: Electro Spray Ionization

Et: Ethyl

Fig: Figure

GBD: Global Burden of Diseases

GPCR: G protein–coupled receptor

GST: glutathion S-transférase

GTP: Guanosine TriPhosphate

HATU: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid
hexafluorophosphate

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC: High Performance Liquid Chromatography

HRMS: High Resolution Mass Spectrometry

HTRF: Homogeneous Time Resolved Fluorescence

HTS: High-throughput screening

IC_{50}: Concentration required for 50 % inhibition

ICM: International Computer Management

ie: id est

IQA: [5-oxo-5,6-dihydro-indolo(1,2-a)quinazolin-7-yl]acetic acid

ITC: isothermal titration calorimetry

K_A: Association constant

K_D: Dissociation constant

LC: Liquid Chromatography

LE: Ligand Efficiency

LogP: Octanol-water partition coefficient

m: multiplet

MDR: Multi-Drug Resistance

Me: Methyl

MEK: Mitogen/Extracellular signal-regulated Kinase

mM: Millimolar

MS: Mass Spectrometry

Ms: Mesylate (MeSO$_2$)

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MW: Micro-Wave

NBS: N- bromosuccinimide

n-Bu: n-Butyl

NCS: N-ChloroSuccinimide

nM: Nanomolar

NMR: Nuclear Magnetic Resonance

Pc: cyclic peptide

PCNA: proliferating cell nuclear antigen

PDB: Protein Data Bank

PEI: Percentage Efficiency Index;

Ph: Phenyl

PK: Protein Kinase

POM: PolyOxoMetalate

PP2I: Protein-Protein Interaction Inhibitors

q: quadruplet

RCC: Renal Cell Carcinoma

RGC: Receptor Guanylate Cyclases

RMSD: Root-Mean-Square Deviation

RT: Room Temperature

s: singlet

SAR: Structure Activity Relationship

SAXS: Small-Angle X-ray Scattering

SD: Standard deviation

SPR: Surface Plasmon Resonance

SR: serine/arginine

STD: Saturation-Transfer Difference

t: triplet

Tab: Table

TAT: Trans-Activator of Transcription

TBB: 4,5,6,7-Tetrabromo-2-azabenzimidazole

TBBi: 4,5,6,7-tetrabromobenzimidazole

TBCA: (E)-3-(2,3,4,5-Tetrabromophenyl)acrylic acid

TBS: Tris-Buffer Saline

TBST: Tris-Buffer Saline Tween

TCA: Trichloroacetic-acid

td: triplet of doublet

TdCD: Temperature-dependent circular dichroism

TFAA: TriFluoroAcetic Anhydride

THF: TetraHydroFuran

TIBI: 4,5,6,7-tetraiodobenzimidazole

TIBI: 4,5,6,7-tetraiodobenzimidazole

TK: Tyrosine Kinase

TKL: Tyrosine Kinase-Like

TLC: Thin Layer Chromatography

TMSA: TriMethylSilylAcetylene

TPSA: Topological Polar Surface Area

Ts: Tosyl

TSA: Thermal Shift Assay

UV: Ultra-Violet

VLS: Virtual Ligand Screening

Contents

1. Introduction

1.1   Cancer and cell signaling

1.1.2   Epidemiology

Cancers were responsible for 5660 thousands deaths in the world in 1990 and 8236 thousands in 2013, representing 15% of human mortality in 2013 (Fig 1). Deaths caused by cancer are generally increasing, mainly because of ageing of the world's population. Notably, death rates for cancers are not homogeneous, from cancer types but also from geographical area.[1,2]
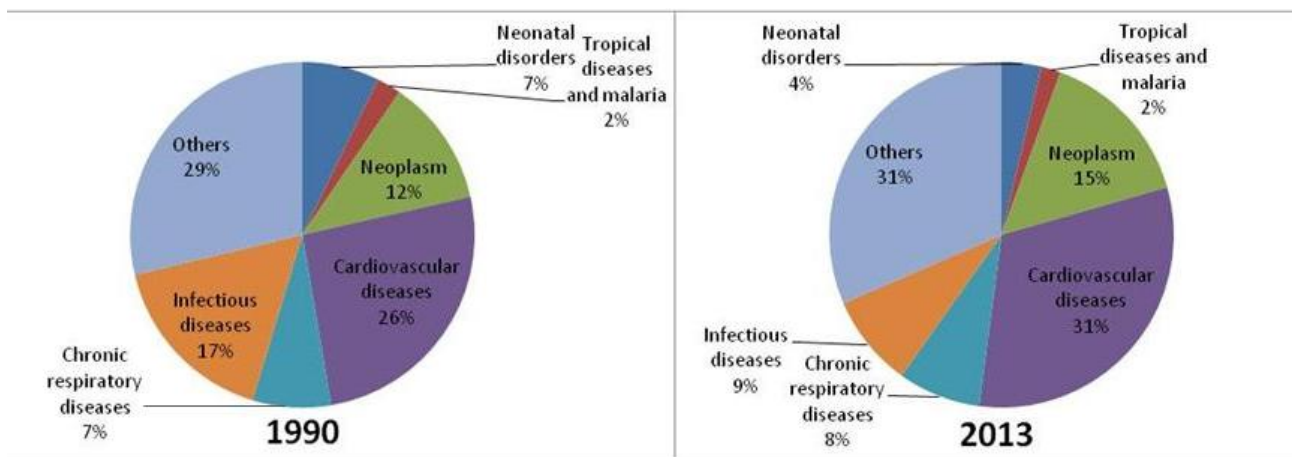
Fig 1: Percentages of death causes in the world in 1990 and 2013 (Reprinted from the Lancet GBD 2013[2], Copyright (2016), with permission from Elsevier).

In 2008, 12.7 million cancer cases and 7.6 million cancer deaths were estimated to have occurred. For females, the most frequent cancer is breast cancer with 23% of total cancer cases and 14% of cancer deaths. For males, lung cancer is the most frequent with 17% of the cases and 23% of deaths. Notably, mortality rates are similar in developing countries and in developed countries.[3]

1.1.3   Cancer definition

Sometimes, cancers are classified by the organ at the source of neoplasm but there are actually more than 100 distinct types of cancer and different types of cancer can exist in one organ. Nevertheless, all subtypes of tumors share six common hallmarks (Fig 2): (i) sustaining

proliferative signaling, (ii) inducing angiogenesis, (iii) insensitivity to anti-growth signals, (iv) evading apoptosis, (v) activating invasion and metastasis, (vi) enabling replicative immortality. These six characteristics are the common denominator of cancer cells.[4]
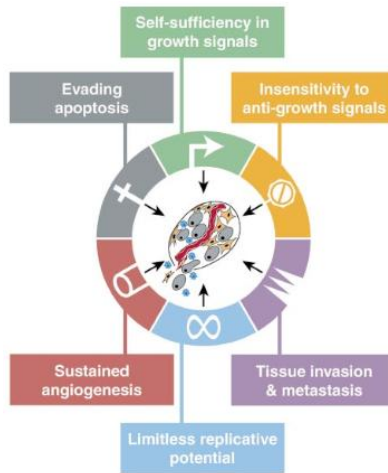
More than one deregulation is required to transform a normal human cell into cancer cells. Globally, a succession of genetic changes, each one providing a step promoting a growth advantage, is required for the acquisition of the six characteristics of cancer cells. Each genetic change is responsible of a small deregulation in cell signaling, leading to tumor development.[5]

1.2 Protein kinases in cell signaling

1.2.1 Cell signaling

Cell signaling is the global system that enables cells to react properly to environmental factors. Indeed, cells are controlled by various internal or external factors. In order to respond to these stimuli, cells have to be able to detect them and to react specifically to them. A network of proteins is responsible of the signal carrying from the environment into the cells. Each protein inside the cell signaling network controls a specific part of the signal transduction. Together, they control specific responses to one stimulus.

1.2.2 Deregulation in cancer development

Cell signaling is responsible of the control of the cellular functions. Cell signaling regulates cell proliferation, cell differentiation, apoptosis, cell survival, cell mobility, cell cycle, metabolism, transcription, translation... In consequence, any small deregulation of this machinery could be the starting point of a complete change in cell behavior. In some cases, pro-survival and anti-apoptotic features can be acquired in normal cells that could be then transformed into cancer cells. Thus, deregulation of cell signaling is a common feature of cancer cells.

1.2.3 Protein Kinases

Protein Kinases are one of the major classes of protein involved in cell signaling. Protein kinases are a group of enzymes able to transfer a phosphate group from a phosphate donor such as ATP or GTP to a protein substrate (Fig 3). In living cells, phosphorylation is a reversible mechanism as dephosphorylation is operated by another class of proteins named protein phosphatases. Phosphorylation is a very well controlled mechanism enabling cells to react in a time frame of milliseconds to seconds. Indeed, each protein kinase is able to phosphorylated specific substrates at specific amino acids. According to recent estimations, at least 30% of proteins in eukaryotic cells are phosphorylated.[6] Addition of phosphate groups can alter protein activity, stability, cellular localization and interactions. Consequently, protein phosphorylation is a key regulator of cellular physiology, and abnormal phosphorylation contributes to diseases such as cancer, diabetes and neurodegeneration.
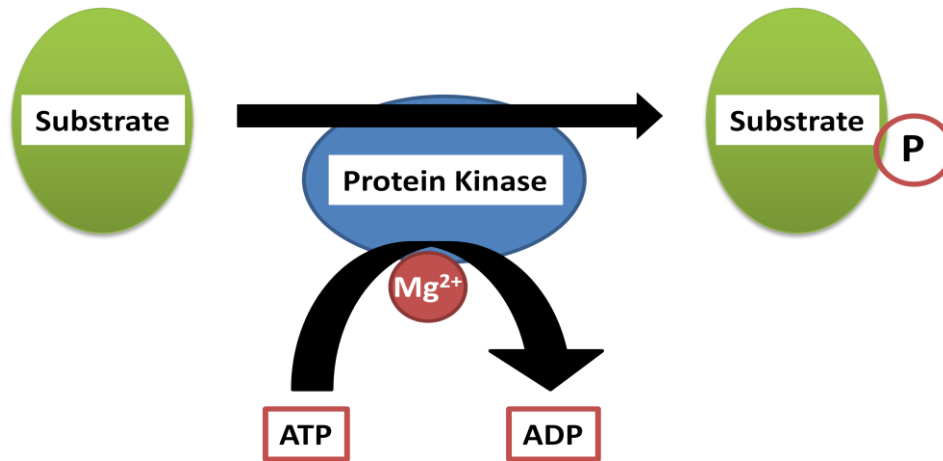
Fig 3: Schematic representation of protein phosphorylation.

1.2.4 Human kinome

The completion of the human genome sequence allows the classification of all the 518 kinases that represent about 1.7% of the human genome.[7] The entire set of human protein kinases are called human kinome and could be divided into eight major subfamilies (Fig 4):

- TK for Tyrosine Kinase which phosphorylates almost exclusively tyrosine residues,
- TKL for Tyrosine Kinase-Like which are similar to TK but phosphorylates serine/threonine substrates,
- STE that are homologs of the yeast STE genes,
- CK1 for Cell Kinase 1,
- AGC for protein kinase A, G and C (PKA, PKC, PKG),
- CAMK for Calmodulin/Calcium regulated Kinases,
- CMGC named after CDK, MAPK, GSK3 and CLK families,
- RGC for Receptor Guanylate Cyclases.

Notably, some protein kinases do not belong to any of the major branches of the human kinome. As an example, CK2a is phylogenetically close to the CMGC family with similarities with DYRK, CLK and CDK but CK2a is located on a different branch in the kinome (Fig 4).
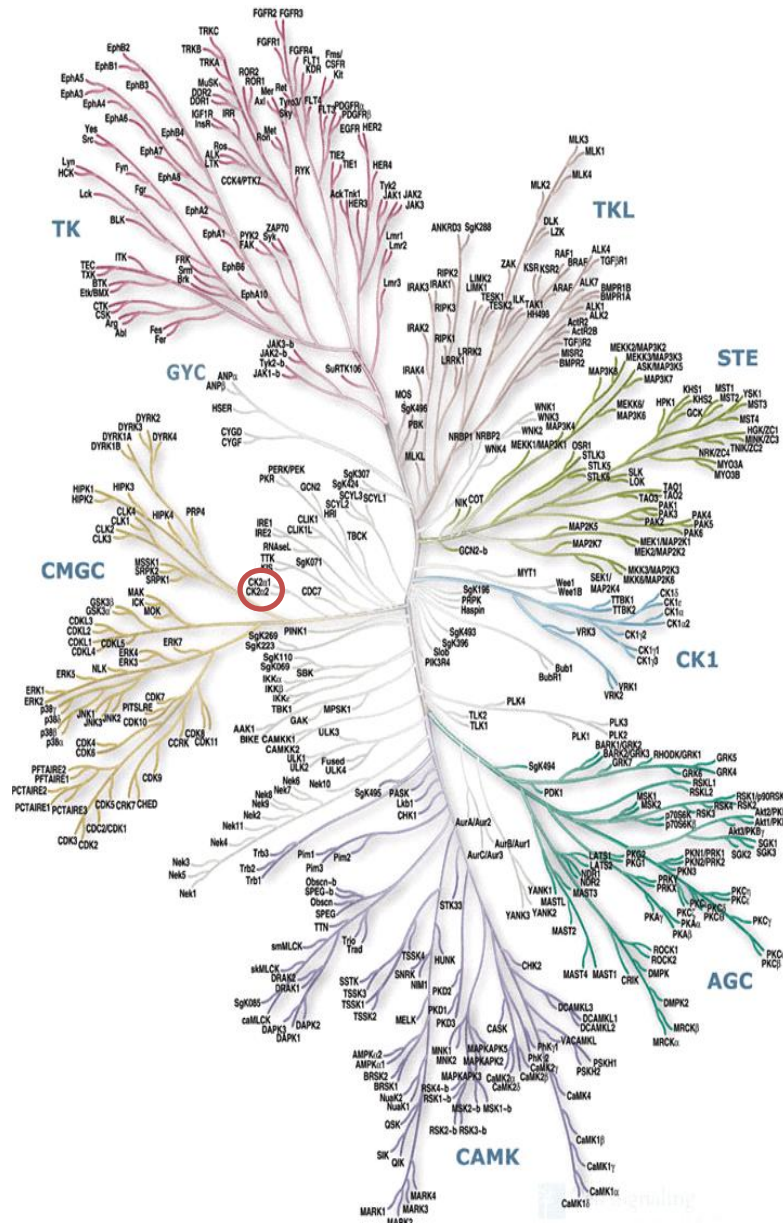
Fig 4: The Human Kinome (From Manning, G. *et al.*[7]**). Each kinase is at the tip of a branch and the similarity between various kinases is inversely related to the distance between their positions. CK2a and CK2a' are surrounded in red. Illustration reproduced with the courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).**

Notably, protein kinases are present in all living organisms but with structural differences, as examples:

- Eukaryotic Protein Kinases (EPK) in eukaryotes, probably more than 28,000 different EPK, exist,

- EPK-Like Kinase (ELK) are present in prokaryotes, 18,699 ELKs have been identified,
- Histidine kinases are also present in bacteria but they are structurally different to EPK or ELK.[8,9]

1.2.5 Protein Kinase structures

Protein kinase A (PKA) was the first protein kinase structure resolved by crystallization and X-ray diffraction in 1991.[10] Rapidly, a large number of protein kinase structures have been determined and all share the same structural core features (Fig 5a). All of them are composed of two lobes. The N-terminal lobes are mainly composed of five β strands, and one crucial and well conserved αC-helix, which is very important for the overall structure organization. Indeed, it has been reported that displacement of the αC-helix is the major way for allosteric kinase inhibition (see paragraph 1.4.3). The C-terminal lobe is mainly composed of helices rendering it more rigid than the N-terminal lobe. The ATP binding site is localized at the interface of the two lobes, in a very well conserved pocket. The adenosine moiety of ATP binds in a hydrophobic pocket and makes special H-bonds with the hinge region. Close to the adenosine binding site, a "gatekeeper" residue controls the access to a second pocket used by several marketed inhibitors to improve their selectivity (Fig 5b,c). The triphosphate moiety of ATP binds with the help of two magnesium ions between the glycine-rich loop (also called P-loop) and the magnesium-binding loop. Therefore, the third phosphate group is exposed and ready to be transferred to a protein substrate. The catalytic loop is an important regulator of kinase activity and, in the majority of kinases, this loop can adopt two conformations: open or closed. The closed conformation is the basal and inactive position. This conformation could be switched to the open or active conformation by a specific event, such as a phosphorylation in the catalytic loop or an activating-protein binding.[11]

(a)
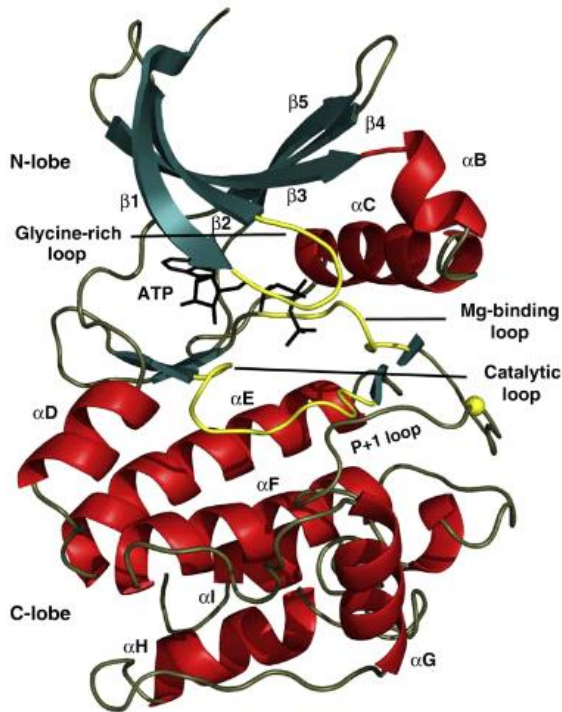
Study of the spatial conservation of residues among kinases revealed that two groups of amino acid are spatially very well conserved. Due to their spine pattern going through the catalytic site and the key elements of kinase regulation, they were called catalytic spine and regulatory spine, respectively (Fig 6). Both of them are anchored on the αF-helix. Based on the protein kinase A sequence: the catalytic spine is composed of 8 amino-acids, V57, A70, M128, L172, L173, I174, L227, M231 and the regulatory spine is composed of 5 amino-acids, L95, L106, F185, Y164, D220.[15] In contrast to active kinase structures, these residues were not found to be well positionned in inactive kinase structures, underlining the relevance of these two spines in kinase regulation.[16]

1.2.6   Protein Kinases as therapeutic targets

The 518 protein kinases of the human kinome represent less than 2% of the whole human genome[7] but there are considered to represent 22% of the druggable genome.[17]

Because of their crucial roles in cell signaling, protein kinases are considered as the second major drug target after G-Protein-Coupled Receptors. However, since the seminal discovery of R. Erikson, showing that the transforming factor of Rous sarcoma virus was a protein kinase (v-Src),[18] it took more than twenty years for the approval in 2001 by the authorities of the first selective small molecule kinase inhibitor targeting Bcr-Abl and called imatinib (Fig 7).[19]

The first oncogene is shown to be a protein kinase.

Isoquinoline sulphonamides are shown to inhibit protein kinases.

Pharmaceutical companies start to develop inhibitors of protein tyrosine kinases.

PD98059 is shown to reverse the phenotype of RAS-transformed cell lines by preventing the activation of MKK1 by RAF.

The first nanomolar inhibitors of receptor protein tyrosine kinases are developed.

Cytokine-synthesis anti-inflammatory drugs are shown to inhibit p38 MAPK.

Gleevec (CGP57148, STI-571) enters human clinical trials for the treatment of CML.

Gleevec is approved for clinical use in the United States.

1978 1981 1984 1986 Late 1980s 1991 1994 1995 1996 1999 2001

Tumour-promoting phorbol esters are shown to activate PKC.

Staurosporine is shown to be a nanomolar inhibitor of PKC.

Three-dimensional structure of the first protein kinase is determined (PKA).

The immunosuppressant cyclosporin is shown to inhibit a protein phosphatase (PP2B).

The immunosuppressant rapamycin is shown to inhibit a protein kinase (Tor).

The first protein-kinase inhibitor (fasudil, HA1077) is approved in Japan for the treatment of cerebral vasospasm.

Rapamycin is approved for clinical use as an immunosuppressant in the United States.

CML, chronic myelogenous leukaemia; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; MKK1, mitogen-activated protein kinase kinase 1; PKA, protein kinase A; PKC, protein kinase C; Tor, target of rapamycin.

Fig 7: Key events in the development of protein-kinase inhibitors (Reprinted by permission from Macmillan Publishers Ltd: [Nat. Rev. Drug Discov.] from Cohen, P.[19] copyright (2016))

Imatinib (Gleevec®, Novartis) is an orally available inhibitor of Bcr-Abl and the first kinase inhibitor approved by the FDA and EMA for the treatment of chronic myelogenous leukaemia. Since, tremendous efforts of many laboratories have been focused on the identification of new kinase inhibitors and an increasing number of these inhibitors has been approved to treat several diseases (Table1). In 2013, imatinib was elected as the most innovative drug in oncology in the last 25 years, based on a physicians' survey.[20]

Table 1: Small molecule kinase inhibitors approved by EMA, classified by approval year until 2013.[21,22]

Target	Molecule name	Trade name	Year of EMA approval	Company
Bcr-Abl	imatinib	Gleevec®	2001	Novartis
EGFR	erlotinib	Tarceva®	2005	Roche
Bcr-Abl, Src, c-KIT	dasatinib	Sprycel®	2006	Bristol-Myers Squibb
VEGF	pegaptanib	Macugen®	2006	OSI/Pfizer
VEGFR, PDGFR, BRAF, c-Kit…	sorafenib	Nexavar®	2006	Bayer
VEGFR, PDGFR	sunitinib	Sutent®	2006	Pfizer
HER2	lapatinib	Tyverb®	2008	GlaxoSmithKline
Bcr-Abl	nilotinib	Tasigna®	2009	Novartis
EGFR	gefitinib	Iressa®	2009	AstraZeneca/Teva
VEGFR, PDGFR, c-Kit	pazopanib	Votrient®	2010	GlaxoSmithKline
VEGFR, PDGFR, c-KIT	axitinib	Inlyta®	2012	Pfizer
ALK, HGFR, c-MET	crizotinib	Xalkori®	2012	Pfizer
JAK	ruxolitinib	Jakavi®	2012	Novartis
VEGFR, EGFR, RET, BRK	vandetanib	Caprelsa®	2012	AstraZeneca
BRAF	vemurafenib	Zelboraf®	2012	Roche
Bcr-Abl	bosutinib	Bosulif®	2013	Pfizer
ErbB	afatinib	Giotrif®	2013	Boehringer Ingelheim
Bcr-Abl, BEGFR, PDGFR, FGFR, EPH, Src, c-KIT, RET, TIE2, FLT3	ponatinib	Iclusig®	2013	ARIAD Pharmaceuticals
RET, VEGFR, PDGFR	regorafenib	Stivarga®	2013	Bayer

1.3   Protein kinase CK2

1.3.1   Overview

Protein Kinase CK2 was discovered in 1954 during a study conducted by G. Burnett and E. Kennedy.[23] CK2 activity was revealed by the ability of the soluble extract of rat liver mitochondria to phosphorylate a protein, the casein, rather than small metabolites. In consequence, they suspected the presence of a possible protein phosphokinase.

In 1979, G. Hathaway and J.Traugh[24] purified two kinases from rabbit reticulocytes. Due to their ability to phosphorylate casein in vitro, they named them Casein Kinase 1 and Casein Kinase 2. Casein Kinase 2 was able to use either ATP (Km = 10 µM) or GTP (Km = 40 µM) as phosphate donors. Moreover, a highly purified preparation of CK2 analyzed by denaturing gel electrophoresis showed proteins of molecular weights of 42, 38 and 24 kDa. This finding suggests a heterogeneous subunit structure of CK2.

In the 80's, several groups worked on other kinases such as the dog cardiac Troponin-T kinase by C. Villar-Palasi and A. Kumon in 1981[25] or as the Glycogen Synthase Kinase 5 (GSK-5) by the group of P. Parker in 1982[26]. Later, researchers realized that these two supposed kinases were however one, the protein kinase CK2.

At that time, the confusion came from two points. First, based on its ability to phosphorylate α-casein *in vitro*, Casein Kinase 2 was misnamed at its discovery since casein is not an *in vivo* substrate of CK2. Second, CK2 phosphorylates a pleiotropic number of substrates and cumulative literature described more than 300 *in vivo* substrates of CK2, involved in all aspects of the cell signaling network.[27] Consequently, several questions rise from these findings: How CK2 could be a good therapeutic target with so many substrates? What does make protein kinase CK2 a "special" kinase?

1.3.2   Structural specificities

1.3.2.1 The holoenzyme (CK2$\alpha_2\beta_2$)
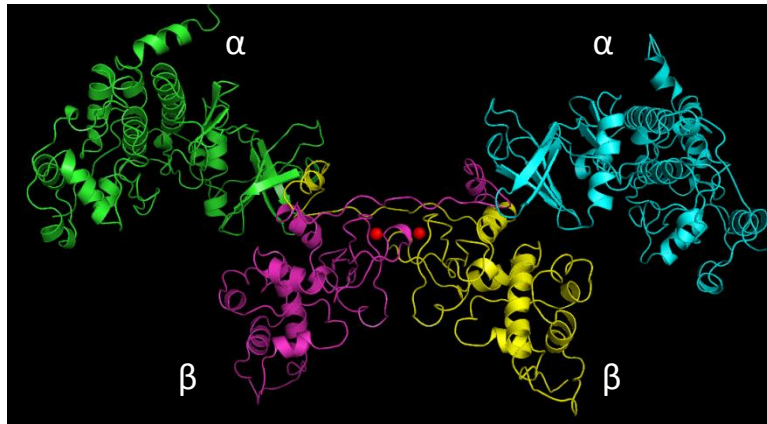
Fig 8: Structure of the holoenzyme form of the protein kinase CK2 (1JWH).[28,29] In green and in blue: the two catalytic subunits (α). In magenta and in yellow: the dimer of regulatory subunits (β) linked by a "zinc finger binding motif" (the two red spheres).

CK2 is a multimeric kinase (Fig 8). The CK2 holoenzyme complex (125 kDa) is composed of a dimer of two β-subunits (regulatory subunits) and two α- or α'-subunits (catalytic subunits). The α-subunit (40 kDa) shares general features of protein kinases but the β_2-subunit (45 kDa) is a unique protein with no sequence similarity with any other proteins.

In contrast to the majority of multimeric kinases like PKA or CDKs, that requires the binding/or unbinding of their regulatory subunit for activity, the β subunit does not turn on/off the kinase activity but instead, modulates its substrate selection of CK2 in a complex and not fully understood manner.

1.3.2.2 The CK2α catalytic subunit

The catalytic subunit shares general features of human kinases such as a large C-terminal lobe mainly composed of α-helix and a smaller N-terminal lobe mainly composed of β-sheets. Nevertheless, CK2α exhibits some structural specificities (Fig 9):[30,31]

- An activation segment locked in an active state, responsible of the constitutive activity of CK2α,
- A good plasticity of the hinge/αD region,
- An unusual "DWG" motif instead of the usual "DFG".
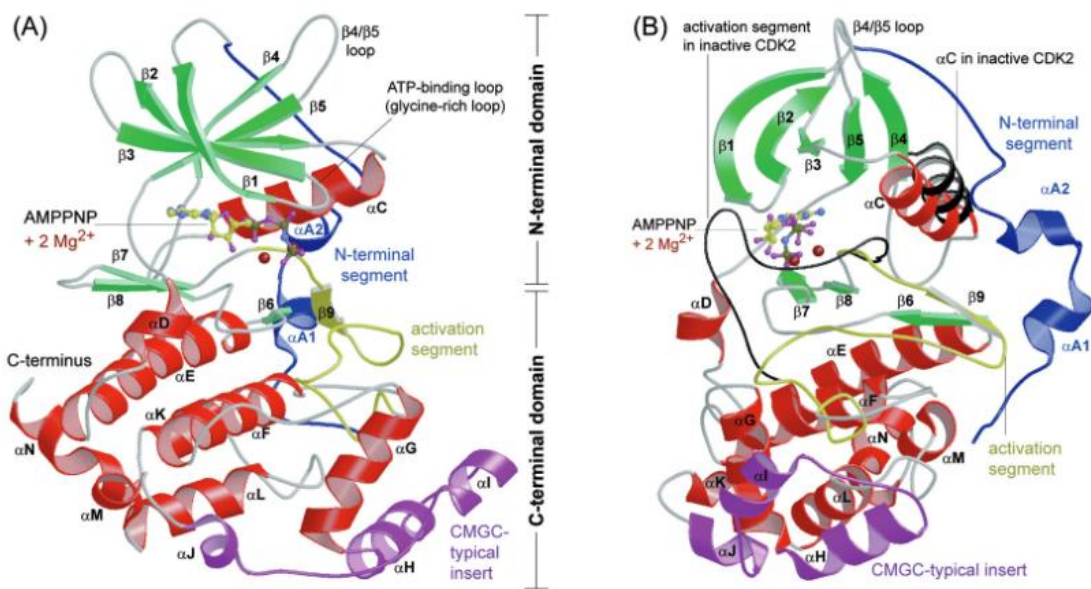
Fig 9: Structure of the catalytic subunit of CK2 from *Zea mays* in complex with AMPPNP and magnesium ions[31–33] (From Niefind, K. *et al.*[31], with permission of Springer). (B) represent the same than (A) but after a 90° rotation around a vertical axis. The activation segment and the αC-helix drawn in black are from the inactive conformation of CDK2 to show a possible inactive state of CK2.

A global comparison of X-ray structures of CK2 with the structures of CDK2 (Fig 10) revealed some interesting differences in their structural flexibility. Indeed, the N-terminal segment, the αC-helix, the catalytic loop and the activation segment of CK2α are rigid and always observed in the same conformation whereas flexibility of these regions was observed in CDK2 structures. In the other hand, the glycine-rich loop, the β4/β5 loop, the hinge region and the αI-helix are the main flexible part of CK2α whereas these regions are rigid in CDK2 structures. The

complete different flexibility profile obtained for CK2 and CDK2 supports the specificity of CK2 regulation, in comparison with other kinases.[31]

The activation segment (Mg-binding loop, β9, activation loop, P+1 loop) of CDK2 is very flexible. Upon cyclin binding, the positions of these key regulatory elements change and so, induce CDK2 activation. In the opposite, the activation segment of CK2a is rigid and is not modified upon CK2β binding. Indeed, in contrast to the majority of other protein kinases,[34] the activation segment of CK2a is locked in the active conformation. No phosphorylation event is required to switch this activation segment in the active state because specific interactions of the N-terminal segment with the activation loop lock it in the fully productive conformation.[30]
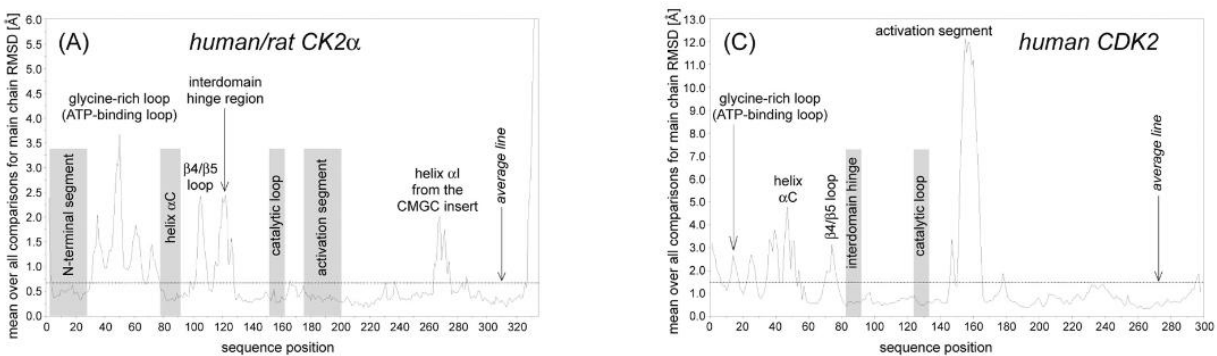
Fig 10: Representation of structural invariable and variable region for human CK2 in comparison with CDK2 (From Niefind, K. _et al._[31], with permission of Springer). All possible 3D structures were superposed and an average structure was determined. Then, average deviation represented by RMSD shows the structural variability of each part of the kinase structure. Specific rigid region are represented in grey and variable regions are represented by a peak.

The hinge region (H115-D120) connects the two lobes of CK2α. This region plays a central role in ATP/GTP binding by forming several hydrogen bonds with adenine or guanine.[32] The hinge region is closely connected to the aD-helix and two different conformations can be observed: open or closed and the amino acid Phe121 is the flag used to determine the conformation. Indeed, Phe121 can be observed "in" the C-spine making it complete (closed form) or "out" the C-spine (open form) (Fig 11). The equivalent residue of Phe121 in PKA catalytic spine is the residue Met128. But, on the contrary of PKA spines, in CK2 structures, ATP seems to favor the open conformation of the hinge/αD region, making the catalytic spines uncompleted. Therefore, based on X-ray structures, CK2α seems to be active with an uncomplete C-spine.[35–37]

The DFG motif is composed of three amino-acids (Aspartic acid 175, Phenylalanine 176, Glycine 177 in PKA). This motif is very well conserved in EPKs. The aspartic acid is crucial for magnesium binding but the most important residue of this motif is the central phenylalanine. Indeed, the phenylalanine can twist from the "in" or active conformation to the "out" or inactive conformation.[38] In drug design, this special feature is a frequent strategy to target the ATP pocket in an inactive state. For example, imatinib targets the ATP pocket of Bcr-Abl in the DFG-out conformation.[39,40] In the entire human kinome, CK2α (and α') is the only kinase that does not have the DFG motif but instead a DWG one. The presence of a large residue tryptophan in position 176 instead of a phenylalanine induces stronger contacts and interactions with other residues of the environment. In consequence, the DWG-motif is stabilized in the "in" (active) conformation. The "out" conformation was never observed in X-ray structures of CK2α. However, kinetic experiments with CK2α mutant (W176F) and CK2α wild type revealed that they both have the same V_{max} value. This result is not consistent with the fact that Trp176 is responsible of the fully active state of CK2α and that the mutated CK2α to Phe176 supports the release of ADP as expected.[41,42]

1.3.2.3 The CK2α' catalytic subunit isoform

A second isoform of CK2α was identified and named CK2α'. Both isoforms coexist in cells but CK2α' is present in a larger quantity in the brain and testicles.[43] On the contrary of CK2α, mice with CK2α' knockout are viable, but sterile.[44] Overall comparison of amino-acid sequences revealed only very few differences, mainly in the C-terminal segment (Fig 12).[45] The affinity between CK2α' and CK2β is, of note, weaker (about 12-fold) than the affinity between CK2α and CK2β.[46]

Bischoff *et al.*[45] shown that the differences in C-terminal segment and the affinity for CK2β are linked. In CK2α', the prolongation of the N-terminal β-sheet by a β-strand and the presence of a tryptophan residue (Trp34) in a conserved hydrophobic cavity (localized between the β4/β5 loop and the helix-αC) stabilized the β4/β5 loop in the open conformation. This conformation is required for CK2β binding but authors proposed that the rigidity of the β4/β5 loop in CK2α' could prevent adaptation under β-binding, and is consequently responsible for the lower affinity. The second important point underlined in their study is that the "in" conformation of the hinge/αD

region observed for CK2α' whereas the "out" conformation is observed in the large majority of CK2α structures. Nevertheless, more crystallization experiments are required to conclude if this observation is a generality or a rare case.[45]

```
numb. hsCK2α':    1        10          20          27 Pro32 Trp34  41      50          60          70
                  |         |           |           |        | |          |           |           |
hsCK2α'           mpgpaagSRA RVYaeVNslR sREYWDYEaH VpsWGNQDDY QLVRKLGRGK YSEVFEAINI TNNErVVVKI
hsCK2α            ~msgpvpSRA RVYtdVNthR pREYWDYEsH VveWGNQDDY QLVRKLGRGK YSEVFEAINI TNNEkVVVKI

numb. hsCK2α':   71        80          90         100         110         120         130         140
                  |         |           |           |           |           |           |           |
hsCK2α'           LKPVKKKKIK REvKILENLR GGtNIIkLiD tVKDPVSkTP ALVFEyiNNT DFKQLYQiLT DfDIRFYMYE
hsCK2α            LKPVKKKKIK REiKILENLR GGpNIItLaD iVKDPVSrTP ALVFEhvNNT DFKQLYQtLT DyDIRFYMYE

numb. hsCK2α':  141       150         160         170         180         190         200         210
                  |         |           |           |           |           |           |           |
hsCK2α'           lLKALDYCHS kGIMHRDVKP HNVMIDHqqk KLRLIDWGLA EFYHPaQEYN VRVASRYFKG PELLVDYQMY
hsCK2α            iLKALDYCHS mGIMHRDVKP HNVMIDHehr KLRLIDWGLA EFYHPgQEYN VRVASRYFKG PELLVDYQMY

numb. hsCK2α':  211       220         230         240         250         260         270         280
                  |         |           |           |           |           |           |           |
hsCK2α'           DYSLDMWSLG CMlASMIFRr EPFFhGqDNY DQLVRIAKVL GTEeLYgYlk KYhIdLDPhF NDILGqHSRK
hsCK2α            DYSLDMWSLG CMlASMIFRk EPFFhGhDNY DQLVRIAKVL GTEdLYdYid KYnIeLDPrF NDILGrHSRK

numb. hsCK2α':  281       290         300         310 C-terminus of 3E3B: Gln334 Cys336           350
                  |         |           |           |                                  | |          |
hsCK2α'           RWEnFiHSEN rHLVSPEALD lLDKLLRYDH QqRLTAkEAM EHPYFYpVVK eQsqpcadna vlssgltaar
hsCK2α            RWErFvHSEN qHLVSPEALD fLDKLLRYDH QsRLTArEAM EHPYFYtVVK dQarmgsssm pggstpvrsa

numb. hsCK2α':

hsCK2α'           ~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~
hsCK2α            nmmsgissv ptpsplgpla gspviaaanp lgmpvpaaag aqq
```

Fig 12: Sequence alignment of CK2α and CK2α'. Lower case letters are used to underline non-identical position and the C-term structurally non-determined region (Reprinted from Bischoff, N. et al. [45], Copyright (2016), with permission from Elsevier).

1.3.2.4 The CK2β regulatory subunit

The regulatory subunit of CK2 (β₂) is a unique protein composed of a dimer of β protein linked by the strong interaction of two zinc chelating fingers motif (Fig 13). The zinc-binding motif (F106-P146) is only composed of β-sheets and loops whereas the other part of the protein also harbors α-helix.[47] Each CK2β subunit of the dimer is able to bind to one α-subunit through an interaction with the loop (L187-H193) of the CK2β tail (Fig 13).
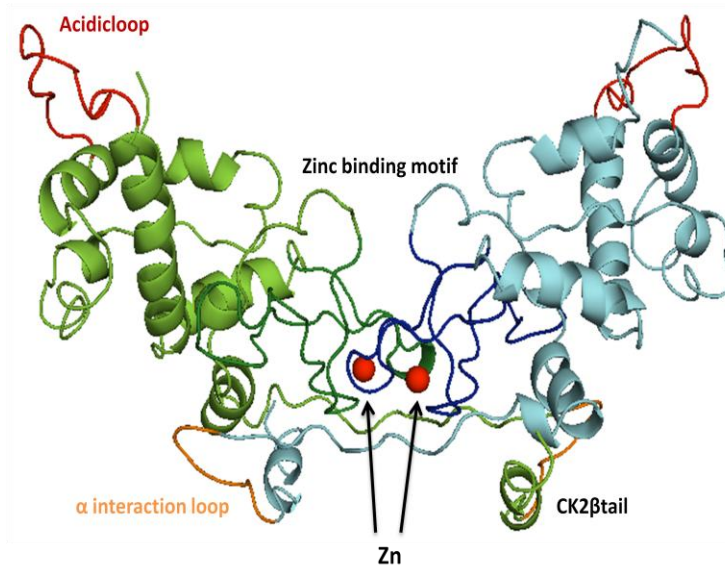
Fig 13: Schematic representation of a β-subunit dimer (1JWH).[28,29] One β monomer is represented in green and dark green for its zinc-binding motif and the second one in blue and dark blue, respectively. The two zinc ions are represented by red spheres. Acidic loop is represented in dark red. The α-interacting loops with the two α-interaction hot-spots (Y188 and F190) are represented in orange.

The α/β interface has been well studied to understand the mode of formation of the holoenzyme and, in particular, if it is a permanent or a transient complex. The identification of α/β interaction modulators would be the Grail that will enable scientists working on protein kinase CK2 to understand the cellular role of this protein/protein interaction.

Since the first crystal structure of CK2 holoenzyme was solved,[28] several studies have pointed out important features of the α/β interface. The size of this interface is between 771 Å² and 1099 Å², depending on the crystal structure and interface type (symmetric or asymmetric).[48] The affinity between CK2α and CK2β is very high, with a dissociation constant measured between 4 and 13 nM.[48,49]

CK2β stabilizes CK2α as shown by the Differential Scanning Calorimetry experiment (Fig 14, A). The CK2α melting point increased from 45.6°C to 54.7°C in presence of the β dimer. On the other side, the melting point of the β-subunit is only slightly decreased from 60.6°C to 60.2°C.[49]

The interaction between the two subunits of CK2 is mainly directed by the "interaction loop" or "CK2β tail" (L187-H193) of β-subunit and the "remote cavity" or "α/β interaction pocket" defined by the strands β1 to β5 of the α-subunit (Fig 14, B and D).

In the regulatory subunit, the two hot spots responsible for a substantial part of the affinity are Tyr188 and Phe190. Thus, mutations of these residues to alanine induce a strong decrease in α/β affinity.[50] CK2β tail is preformed to bind to CK2α, as the conformation of the β tail is identical in the bound or unbound state (Fig 14, C).[49]

In the catalytic subunit, the hot spots are Leu41 and Phe54, localized on the β1 and β2 strands. In the bound form, these residues are in close interaction with the β-hairpin loop of the β-subunit. The β4/β5 loop is another key element of the α/β interaction and this loop can adopt different conformations, depending of the α/β interaction pocket occupancy (Fig 14, D). The β4/β5 loop is closed in the absence of the β subunit but an "open" conformation was observed in presence of the β subunit[28,48] or in presence of a cyclic peptide that mimics the β tail.[51]
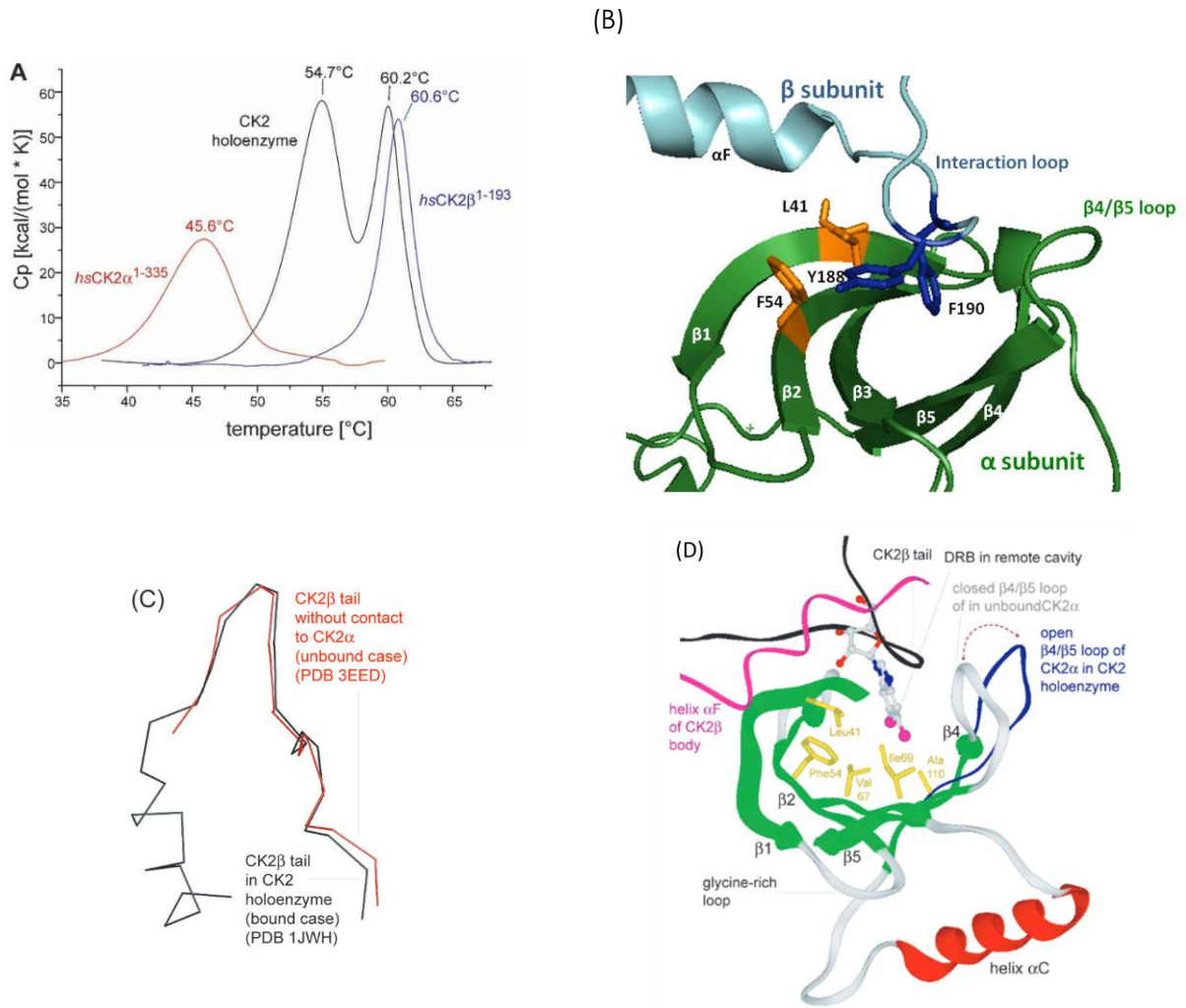
Fig 14: Key elements of the CK2 α/β interaction. (A) DSC curves of hsCK2α[1-335] (red), hsCK2β[1-193](blue), and the corresponding holoenzyme (black) (From Raaf, J. et al.[49], with the permission of John Wiley and Sons). (B) Structural overview of the α/β interface. CK2a is represented in green and the hot spots (L41 and F54) in orange. CK2β is colored in blue and the two hot spots (Y188 and F190) in dark blue (based on 4DGL[52]) (C) Superposition of the CK2β tail bound or unbound to CK2α (From Niefind, K. et al.[31], with permission of Springer). (D) Structural overview of the α/β interaction with a focus on the two conformations of the β4/β5 loop (From Niefind, K. et al.[31], with permission of Springer).

1.3.3 CK2 implication in pathophysiology

1.3.3.1 CK2α overexpression in cancers

As a multifunctional/multi-substrates enzyme, CK2 occupied a central position in cell signaling. Therefore, CK2 role in cancer development has been extensively investigated. The expression level and activity of CK2 have been widely studied in diverse tumor types and it has been determined that CK2 is globally a marker of poor survival prognosis for patients.

Indeed, in 2004, CK2α overexpression has been shown to be a marker of poor prognosis in squamous cell carcinoma of the lungs. A survey on 16 patients shown that after 90 months, only 10% of patients with a high CK2a level survive whereas 50% of patients with a normal CK2α expression were alive (Fig 15, A).[53]

In 2007, a survey on 131 patients with prostate cancer has shown than CK2α is overexpressed in prostate cancer cells in comparison with normal prostate cells. Moreover, nuclear localization of CK2α is correlated with high-grade tumors and poor prognostic factors.[54]

The same year, a second implication of CK2α was found in Acute Myeloid Leukemia (AML) as a survey on 48 patients has shown that CK2α overexpression is correlated with a poor survival prognosis (Fig 15, B).[55]

In 2010, CK2α expression was studied in breast carcinoma and revealed that CK2α is individually a poor prognosis marker. Moreover, CK2α was chosen together 11 other markers to bring an accurate classification for 86% of the tumors on 905 patients.[56]

In 2011, overexpression of CK2α in nuclei of colorectal tumor tissues have been correlated to bad prognosis for patients and as an independent prognostic marker for human colorectal cancer (Fig 15, C).[57]

In 2013, the analysis of 537 glioblastomas has demonstrated that CSNK2A1 gene encoding CK2α was overexpressed in 33.7% of glioblastomas and even in more than 50% of classical glioblastomas whereas CK2α' expression was not changed. Only a slight modification was observed in CK2β expression (7.3% of gene deletion). Moreover, downregulation of CK2α, by siRNA or inhibitors, counteracted glioblastoma development.[58]

In 2014, 187 patients with gastric carcinoma were studied and CK2α expression was a marker of survival prognosis (Fig 15, D). CK2α was involved in phosphorylation of DBC1 and the inhibition of both CK2α and DBC1 decreased proliferation of cancer cells.[59]

In 2015, a small cohort study revealed that CK2α was upregulated in Renal Cell Carcinoma tumor samples. Moreover, 786-O cells were used as model of RCC and CK2 inhibition by CX-4945 treatment underlined the potential of CK2 as a target to cure RCCs.[60]
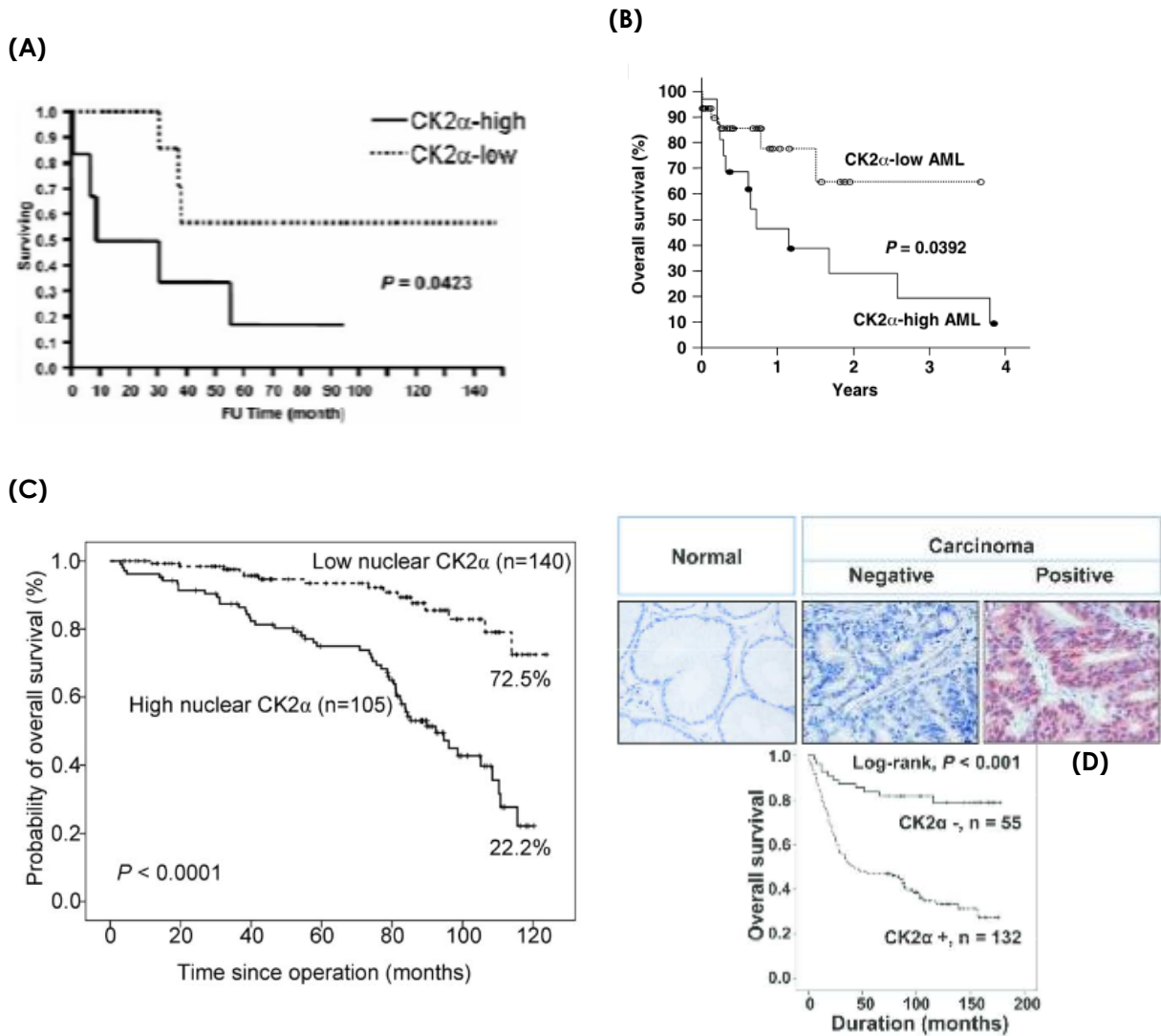
Fig 15: Clinical implication of the protein kinase CK2. (A) Survival rate in function of the expression level of CK2α in squamous cell carcinoma (Reprinted from O-charoenrat, P. *et al.*[53], with permission from AACR). (B) Survival rate of patient with Acute Myeloid Leukemia (AML) depending of their CK2α expression level (Reprinted from Kim, J. S. *et al.*[55], with permission from AACR). (C) Survival analysis of patients with human colorectal cancer. Low nuclear CK2α means labeling index ≤40% for nuclear CK2α by immunoreactivity and high nuclear CK2α means >40% (From Lin, K.-Y. *et al.*[57], with the permission of John Wiley and Sons). (D) Implication of CK2α in gastric carcinoma, immunohistochemical expression of CK2α in normal tissue, low and high

CK2α gastric carcinoma and patient survival depending of the expression level of CK2α (From Bae, J. S. *et al*.[59], with the permission of John Wiley and Sons).

1.3.3.2 CK2α implication in cell signaling

The correlation between CK2α overexpression and prognosis in a large number of cancers is in relation with the multiple consequences of this overexpression in cells (Fig 16).[61]
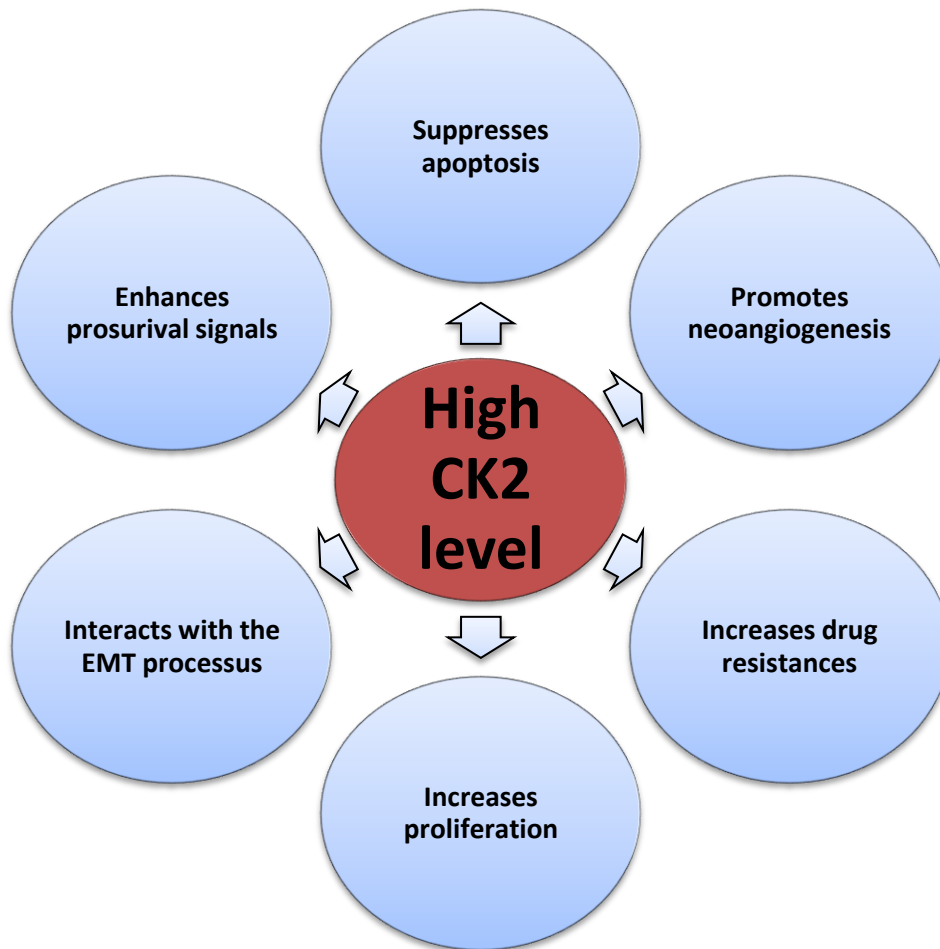
Fig 16: Consequences of a high CK2 expression.

CK2α overexpression induces favorable conditions for tumor development by acting on several cell signaling pathways implicated in cancer.

CK2 is involved in the regulation of NF-κB pathway at multiple levels. CK2 phosphorylates IκB leading to its degradation and the release of NF-κB.[62] Moreover, CK2 can directly

phosphorylate NF-κB p65 subunit, inducing the augmentation of the transcriptional activity of NF-κB *in vivo*.[63,64]

CK2 is able to promote the Wnt signaling by several actions. CK2 phosphorylates Disheveled (Dvl), β-catenin on Thr393 and LEF-1 on Ser42 and Ser61. These events lead to the stabilization of β-catenin and to the promotion of β-catenin/LEF-1 complex formation (Fig 17, B).[61,62] In parallel, CK2 phosphorylates a-catenin (Ser641), thus destabilizing the a-catenin/β-catenin complex that inhibits β-catenin. Therefore, CK2 favors the transcription of survival genes.[65]

CK2 activates the PI3K/Akt pathway by a direct phosphorylation of Akt in position Ser129 and Ser473. Morevover, CK2 also acts upstream by a negative control on PTEN, which is a natural blocker of the PI3K/Akt pathway. Laterally, CK2 enhances the protection of Akt by HSP90 activation/stimulation. Thus, CK2 favors survival signals.[61]

Consequenly, CK2 inhibition has been proved to be a valuable way to treat cancer cells. Indeed, CK2 acts as a major support to prosurvival and antiapoptotic signals by acting on the NF-κB, Wnt and Akt pathway (Fig 17). CK2 is not directly involved in these pathways but acts as a side regulator through activation or inhibition of key enzymes.[66]
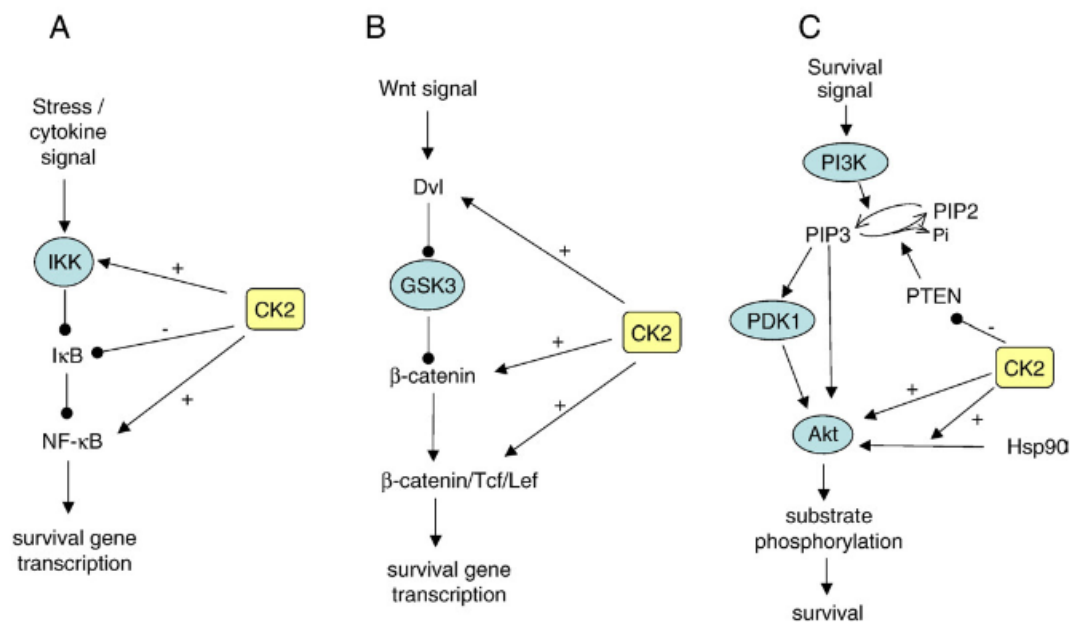
Fig 17: Role of CK2 in regulation of NF-κB (A), β-catenin (B) and Akt (C) signaling (Reprinted from Ruzzene, M. *et al.*[66], Copyright (2016), with permission from Elsevier).

In addition, CK2 is involved in the multidrug resistance phenomenon.[67] CK2 inhibition has been demonstrated to be efficient to restore sensitivity toward imatinib of imatinib-resistant LAMA84 cells[68], as well to induce apoptosis in various drug resistant cell lines.[69] Moreover, a large-scale proteomic study on the phosphorylation stoichiometries in lung cancer cells and gefitinib-resistant lung cancer cells has rencently demonstrated that CK2 and EGFR are the two kinases with wider implication in drug resistance pathway.[70] Thus, CK2 appears to be an interesting target to counteract drug resistance phenomena.

1.3.3.3 Functional role of CK2β

CK2β knockout experiments have been used to determine the role of the regulatory subunit in different models.

In *Saccharomyces cerevisiae*, the knockout of one or the two different regulatory subunits (CK2β1 and CK2β2) induces only salt sensitivity and a partial blockage in the adaptation to the G_2/M checkpoint arrest but no difference in growth or viability was observed, so showing that yeasts can survive in the absence of CK2β.[71,72]

CK2β deletion in mice leads to early embryonic lethality, leading to mutant embryos with a reduced size. A decrease in cells proliferation was observed but not in apoptosis markers. Thus, in contrast to yeast, mammals require a normal level of CK2β to survive.[73] Moreover, conditional CK2β knockout in embryonic central nervous system led to defects in proliferation and differentiation, the latter underlining the major role of CK2β in central nervous system development.[74]

CK2β deletion in mammary epithelial cells (MCF10A) leads to phenotypic changes. Indeed MCF10A epithelial cells transfection with siRNA targeting CSNK2B gene induces a mesenchymal phenotypes. Immunofluorescence images have shown a decrease in E-Cadherin expression, an increase in the N-Cadherin level and a reorganization of the actin filaments. These observations are characteristics of an increase in cell mobility (Fig 18).[75,76]

Moreover, unbalanced level of CK2 subunits (lower expression level of β-subunit) in breast tumour samples has been correlated with EMT markers such as decrease in E-Cadherin and Occludin expression and increase in N-Cadherin, Vimentin, αSMA and Snail1 expression.[76]
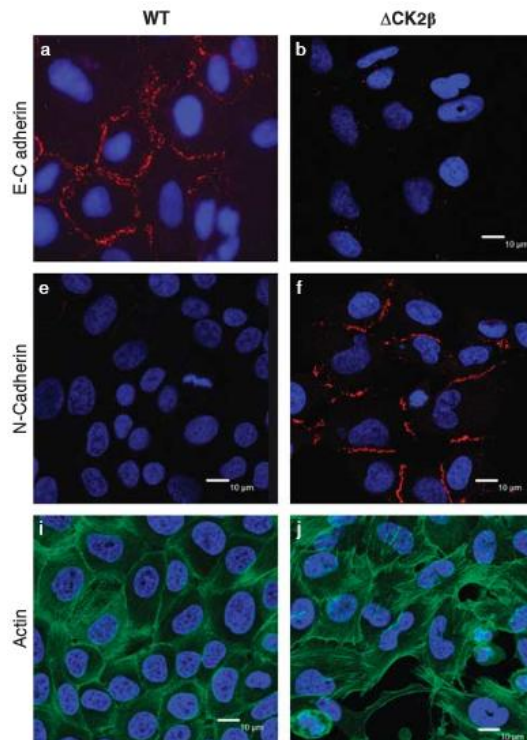
Fig 18: Immunofluorescence images of MCF10A WT and ΔCK2β with E-Cadherin, N-Cadherin or Phalloidin antibodies in combination with Hoechst-33342 (Reprinted by permission from Macmillan Publishers Ltd: [Oncogene] from Deshiere, A. *et al.*[76], copyright (2016)).

Biochemical approaches have been used to identify proteins interacting with CK2β. CK2β partners could be classified in two categories:

- CK2α dependent partners which bind specifically to CK2β,
- CK2α independent partners which bind specifically to CK2β only when CK2α is not bound to the regulatory subunit.

Several CK2α dependent binding partners of CK2β were characterized, some of them only *in vitro* such as p90[Rsk], PKCζ, topoisomerase II, p27[KIP1], CD5, FGF-2, Nopp 140 and L41 but few of them were also characterized as CK2β binding partners *in vivo* (Table 2). Interestingly, four serine/threonine kinases, c-Mos, Chk1, A-Raf and AMPKα2 were characterized as CK2a independent binding partners of CK2β. The β-subunit binding site on these kinases is localized in the same area than on CK2α. Moreover CK2β is able to modulate their activity like the one of CK2α.[77] As a remark, activation of AMPKα2 by CK2β suppressed EMT.[78]

Table 2: *In vivo* CK2β binding partners.

Interaction partner	Function	Reference
CK2α dependent binding partners of CK2β		
p53	Tumor supressor	[79–81]
p21[WAF1/CIP1]	CDK inhibitor	[81,82]
Cdc25B	Phosphatase, CDK activator	[83]
L5	Ribosomal protein	[81,84]
HHV-6IE2	Human herpesvirus 6 immediate-early protein	[85]
CK2α independent binding partners of CK2β		
c-Mos	Serine/Threonine protein kinase, MAPK activation	[81,86,87]
Chk1	Serine/Threonine protein kinase, regulator of DNA damage, induced G2 arrest	[88]
A-Raf	Serine/Threonine protein kinase, mitogenic signaling, cell proliferation	[81,84,89]
AMPKα2	AMP-activated protein kinase, heterotrimeric serine/threonine kinase, cellular energy homeostasis, EMT	[78]

A possible consequence of the multiple interactome of CK2β is proposed in Fig 19. If the amount of CK2α (or/and CK2α') in one cell compartment is equal or higher to the quantity of CK2β, the holoenzyme form ($\alpha_2\beta_2$) is predominant and CK2 mainly interacts with proteins that could bind to the holoenzyme form. In the contrary, if the regulatory subunit is present in excess, free β dimer could bind to other kinases such as c-Mos, Chk1, A-Raf and regulate them (Fig 19, b), or to other proteins such as p53, p21, p27 (Fig 19, c)... In consequence, the equilibrium between CK2α and CK2β expression has to be very well controlled in subcellular compartments and any dysregulation may lead to dramatical alteration in cell signaling pathways.[90]
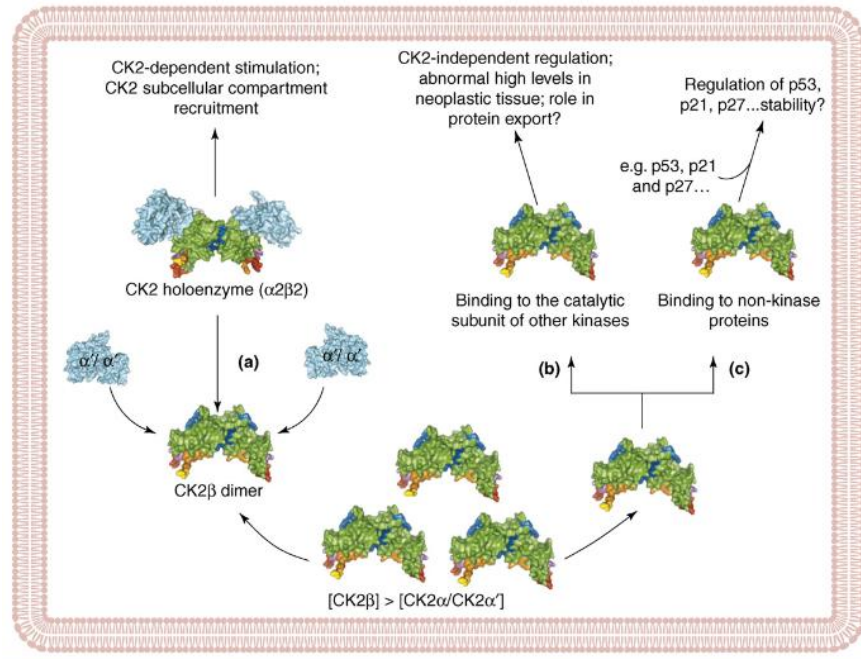
Fig 19: Possible mechanisms of cell signaling modulation by CK2 as a function of CK2β expression (Reprinted from Bolanos-Garcia, V. M. _et al._[90], Copyright (2016), with permission from Elsevier). (a) In the presence of an excess of CK2β, CK2 is preferentially in the holoenzyme form and interacts with holoenzyme's substrate. (b) Free CK2β could interact and modulate other kinases (such as c-Mos, A-Raf, Chk1...) in the same way than for CK2α. (c) CK2β could bind to other proteins independently of CK2α and maybe modulates them by activation/inactivation, stabilization...

1.3.4 Known CK2 inhibitors

For a long time, CK2 was considered as an "undruggable kinase".[91] Indeed, despite the long period since the discovery of CK2 in 1954[23], its full characterization in 80s and the first x-ray structure of CK2 solved in 1998[30], only one potent ATP competitive inhibitor, CX-4945, underwent clinical trials.[92]

Various chemical structures have been identified as CK2 inhibitors, through various approaches[93–96] and the majority of them could be classified in several general families (Fig 20):

- Benzimidazole derivatives including TBB, TBI and DMAT,
- Anthraquinone derivatives based on the natural product emodin,
- Coumarin derivatives such as ellagic acid,
- Indolo-quinazoline derivatives such as IQA,
- Benzo-naphthyridine derivatives, with CX-4945 in phase II clinical trial,
- Atypical inhibitors such as GIGB-300 (also in clinical trials), POMs, azonaphthalenes, peptide-like α/β interaction inhibitors and bi-substrate inhibitors.

Benzimidazole scaffold

IC$_{50}$ = 23 µM

(3R,4S,5R)-2-(5,6-dichloro-1H-benzo[d]imidazol-1-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol
(DRB)

Ki = 0.4 µM

4,5,6,7-tetrabromo-1H-benzo[d][1,2,3]triazole
(TBB)

Ki = 0.04 µM

4,5,6,7-tetrabromo-N,N-dimethyl-1H-benzo[d]imidazol-2-amine
(DMAT)

Ki = 0.3 µM

4,5,6,7-tetrabromo-1H-benzo[d]imidazole
(TBI)

Anthraquinone

Ki = 1.5 µM

1,3,8-trihydroxy-6-methylanthracene-9,10-dione
(Emodin)

Ki = 0.06 µM

1,2,5,8-tetrahydroxyanthracene-9,10-dione
(Quinalizarin)

Coumarin scaffold

Ki = 0.8 µM

7-hydroxy-3H-phenoxazin-3-one
(Resorufin)

Ki = 0.02 µM

2,3,7,8-tetrahydroxychromeno[5,4,3-cde]chromene-5,10-dione
(Ellagic acid)

Atypical inhibitors

IC$_{50}$ = 0.4 µM

(E)-3-hydroxy-4-((1-hydroxynaphthalen-2-yl)diazenyl)-7-nitronaphthalene-1-sulfonic acid **(Azonaphthalene 4)**

$[P_2Mo_{18}O_{62}]^{6-}$

Ki < 0.01 µM

(POM)

WMSPRHLGT-Tat

(GIGB-300)

GCRLYGFKIHGCG

(Cyclic peptide)

IC$_{50}$ = 0.025 µM

(K137-E4)

Indolo-quinazoline scaffold

IC$_{50}$ = 0.39 µM

2-(5-oxo-5,6-dihydroindolo[1,2-a]quinazolin-7-yl)acetic acid
(IQA)

Benzo-naphthyridine scaffold

IC$_{50}$ = 0.0015 µM

5-((3-chlorophenyl)amino)benzo[c][2,6]naphthyridine-8-carboxylic acid
(CX-4945)

IC$_{50}$ = 0.0023 µM

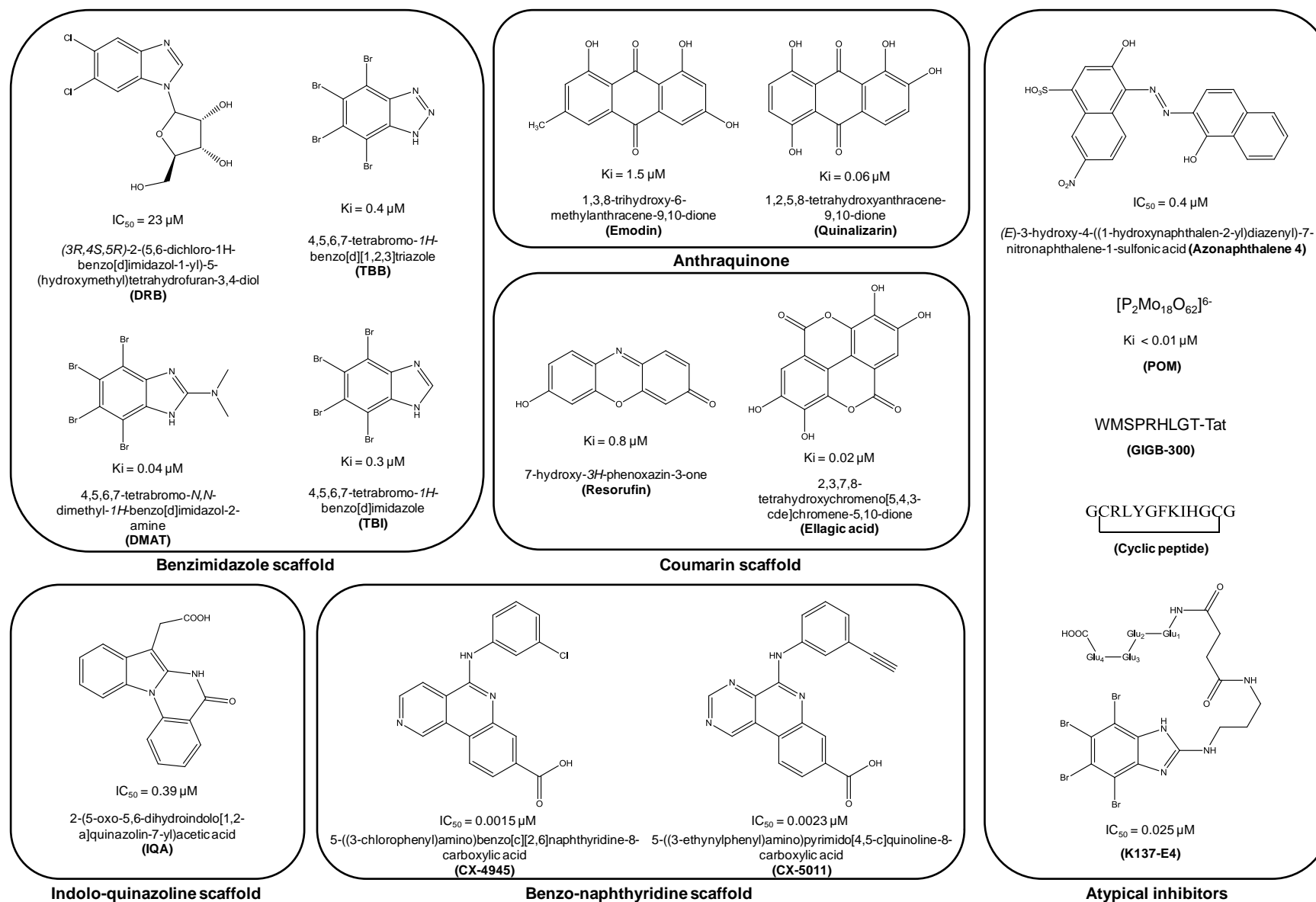5-((3-ethynylphenyl)amino)pyrimido[4,5-c]quinoline-8-carboxylic acid
(CX-5011)

Fig 20: Selected examples of CK2a modulators based on their chemical scaffold or mechanism of action.

The following discussion will focus on CX-4945, which is the most developed CK2 inhibitor and some of the atypical inhibitors will be discussed in paragraph 1.4.4 and 1.5.4.

1.3.4.1 CX-4945 and in vitro selectivity

CX-4945 is the prototype of CK2 inhibitor and is in clinical Phase II for the treatment of several cancers. This molecule, developed by Cylene Pharmaceuticals®, belongs to the family of benzo-naphthyridine. It is a very potent inhibitor of CK2 (IC_{50} = 14.7 nM) and one of the most selective.[97] Indeed, CX-4945 has a Gini coefficient[98] of 0.667 on a panel of 235 protein kinases. In comparison, other CK2 inhibitors have lower Gini coefficient, 0.612 for quinalizarin, 0.60 for TBCA, and even lower than 0.4 for TBB, DMAT or TIBI (Table 3).[97]

However, an off-target inhibitory activity of Cdc2-like kinases (Clks) was recently reported for CX-4945.[99] The Clk family of kinases is involved in the regulation of alternative splicing through the phosphorylation of serine/arginine rich (SR) proteins: CX-4945 is actually more potent on Clk-2 (IC_{50} = 3.8 nM) than on CK2α (IC_{50} = 14.7 nM). Furthermore, CX-4945 causes the inhibition of cellular SR protein phosphorylations at low micromolar concentrations in a CK2 independent manner.[99] Although the consequences of this off-target remain to be clearly understood, this recent result underlines the fact that potent and selective CK2α inhibitors are difficult to obtain through targeting the ATP binding pocket. Another study describes several CX-4945 off-targets with the respective IC_{50} of 1 nM on CK2a and CK2a', 17 nM on DAPK3, 35 nM on FLT3 and on TBK1, 41 nM on Clk3, 45 nM on HIPK3, 46 nM on Pim-1 and 56 nM on CDK1/cyclinB.[92]

Table 3: Selectivity of CK2 inhibitors expressed with Gini coefficients in comparison with some approved kinase inhibitors.[98]

Target	Inhibitor	Number of PK tested	Gini coefficient
CK2α	CX-4945 (0.5 µM)[100]	102	0.62
	CX-4945 (0.5 µM)[100]	235	0.67
	Quinalizarin (1 µM)[101]	78	0.62
	TBCA (0.5 µM)[102]	300	0.60
	TBB (10 µM)[103]	70	0.38
	DMAT (10 µM)[103]	70	0.34
	TIBI (10 µM)[103]	70	0.31
Bcr-Abl	Imatinib (0.5 µM)[102]	300	0.77
Bcr-Abl	Dasatinib (0.5 µM)[102]	300	0.74
PDGFR/VEGFR	Sunitinib (0.5 µM)[102]	300	0.52

1.3.4.2 Cellular effects of CX-4945

CX-4945 induces cell death in various cell lines with an average EC_{50} of 5.5 µM in 43 cell lines originated from different cancers (Fig 21). Moreover, extensive studies in breast cancer cell lines have shown an apparent correlation between the efficacy of CX-4945 and the level of CK2α expression. Cell lines with a higher level of CK2α (mRNA and protein) are more sensitive toward CX-4945:[92] this last point underlined the importance of CK2a inhibition to treat cancers that are dependent on abnormal high CK2α level.
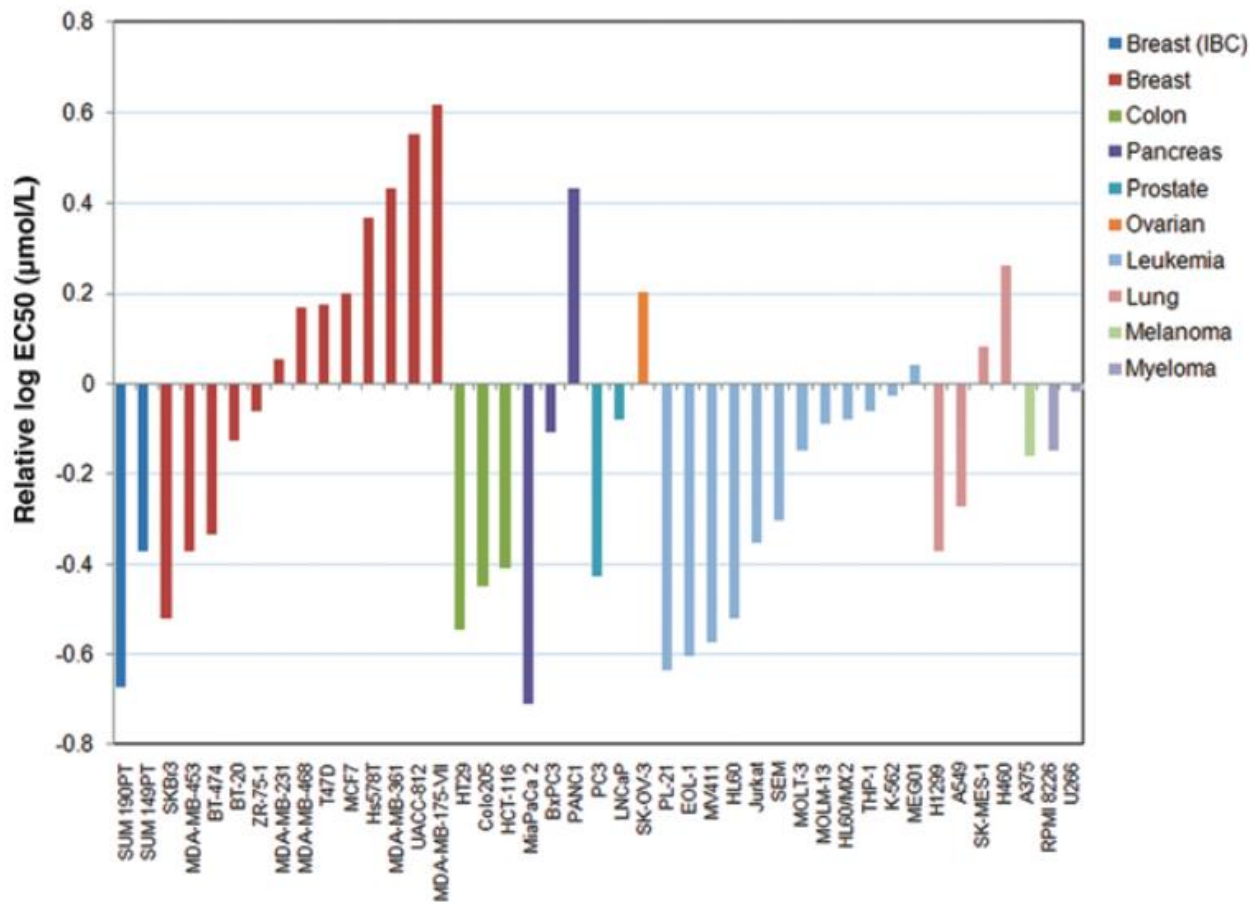
Fig 21: Cell viability assay results on a panel of 43 cancer cell lines. The baseline represents the average EC$_{50}$ (5.5 μM), cell lines with a negative logEC$_{50}$ are more sensitive than average, and positive less sensitive (Reprinted from Siddiqui-Jain, A. et al.[92], with permission from AACR).

CX-4945 inhibits CK2 activity in various cell lines in the micromolar range. A decrease of Akt phosphorylation (Ser129, specific to CK2) was observed. Moreover, CX-4945 induces a decrease in phosphorylation of p21 (Thr145), PTEN (Ser370 and Ser380) but at a concentration about 10-fold higher.[69,92,104]

Xenograft model experiments were conducted and showed CX-4945 efficiency to induce breast and pancreatic tumor regression without any weight loss for mice (Fig 22).
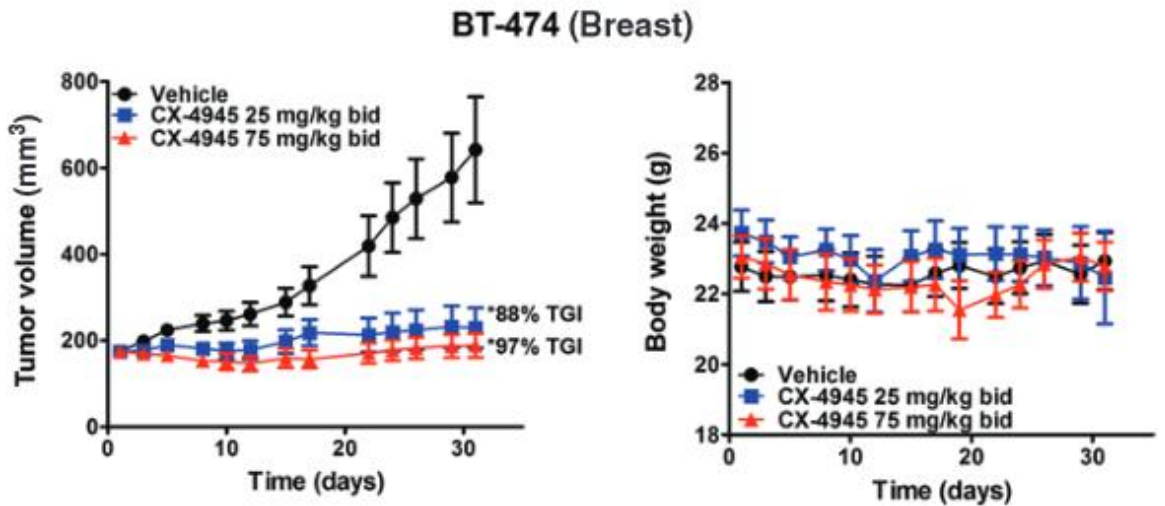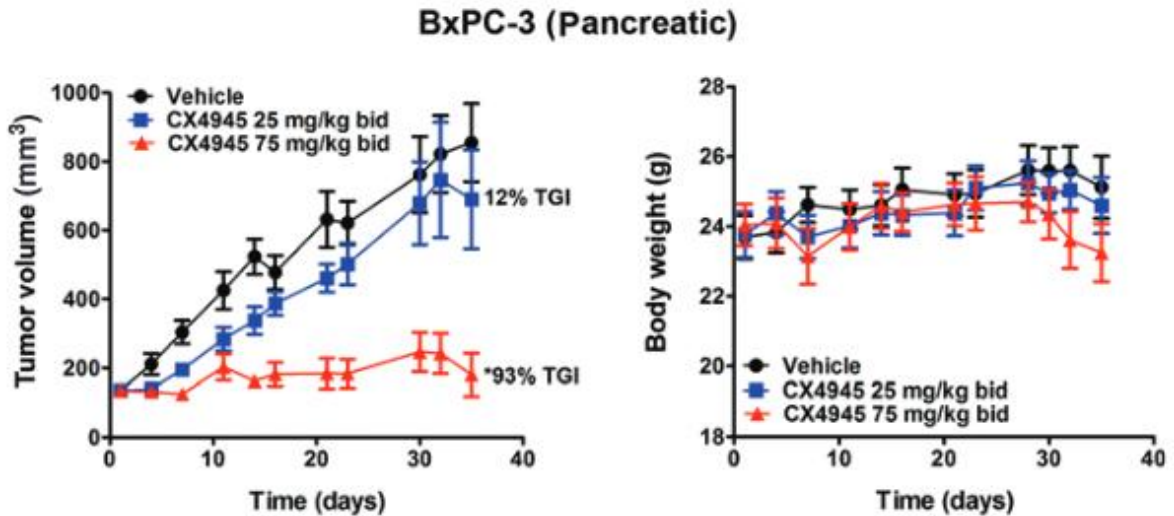
Fig 22: Effect of CX-4945 in BT-474 (A) and BxPC-3 (B) xenograft models. CX-4945 or Sodium Phosphate Buffer (vehicle) were administrated by oral gavage twice a day (Reprinted from Siddiqui-Jain, A. *et al.*[92], with permission from AACR).

Recently, a study dealing with human head and neck squamous cell carcinomas revealed a possible resistance mechanism to CX-4945 treatment, both in cellular-based assay and in xenograft models. Resistance to CX-4945 involved an increase in the reporter gene activity of p21, p53, AP-1 and IL-8, as well as an increase of Ki67. The resistance is, of note, clearly dependent on the cell lines.[105]

To date, CX-4945 remains the most promising CK2 inhibitor efficient *in vitro*, in cell-based assay and *in vivo*. Nevertheless, the recently described off-targets (Clk, for example) and resistance pathways could be a serious drawback in clinical development. Notwithstanding, CX-

4945 validates CK2 as a target of interest to treat several cancer types. Consequently, new potent and more selective CK2 inhibitors are still required to make CK2 inhibition a success story in cancer treatment.

1.3.4.3 Overview of CK2 inhibitors binding sites

The major part of the described CK2 modulators bind to the ATP cleft (Fig 23) but several compounds have been characterized as non-ATP competitive modulators of CK2 and they can be classified in four groups:

- Allosteric inhibitors of CK2α including azonaphthalene class of compounds, POMs, CFTR fragment peptide…
- α/β interaction inhibitors which are Protein Protein Interaction Inhibitors (PP2I) more than kinase inhibitors. This group includes DRB (5,6-dichlorobenzimidazole 1-β-D-ribofuranoside) and a cyclic peptide (Pc) derived from the C-terminal tail of CK2β.
- Substrate inhibitors represented by one small peptide named CIGB-300, nowadays in phase II clinical trials. CIGB-300 does not interact directly with CK2 but with its substrates. Indeed, this small peptide is able to bind selectively to a CK2 phosphorylation site of CK2 substrate, thus preventing CK2 phosphorylation *in vitro*. The main target of CIGB-300 *in vivo* is B23/nucleophosmin.[106–108]
- The last class aim to develop a dual site inhibitor, by coupling TBBi to an amino-acid sequence rich in aspartate residues. This molecule is able to bind simultaneously in the ATP pocket and in the substrate binding site, as shown by a very high affinity for CK2 (Ki = 0.5 nM).[109]
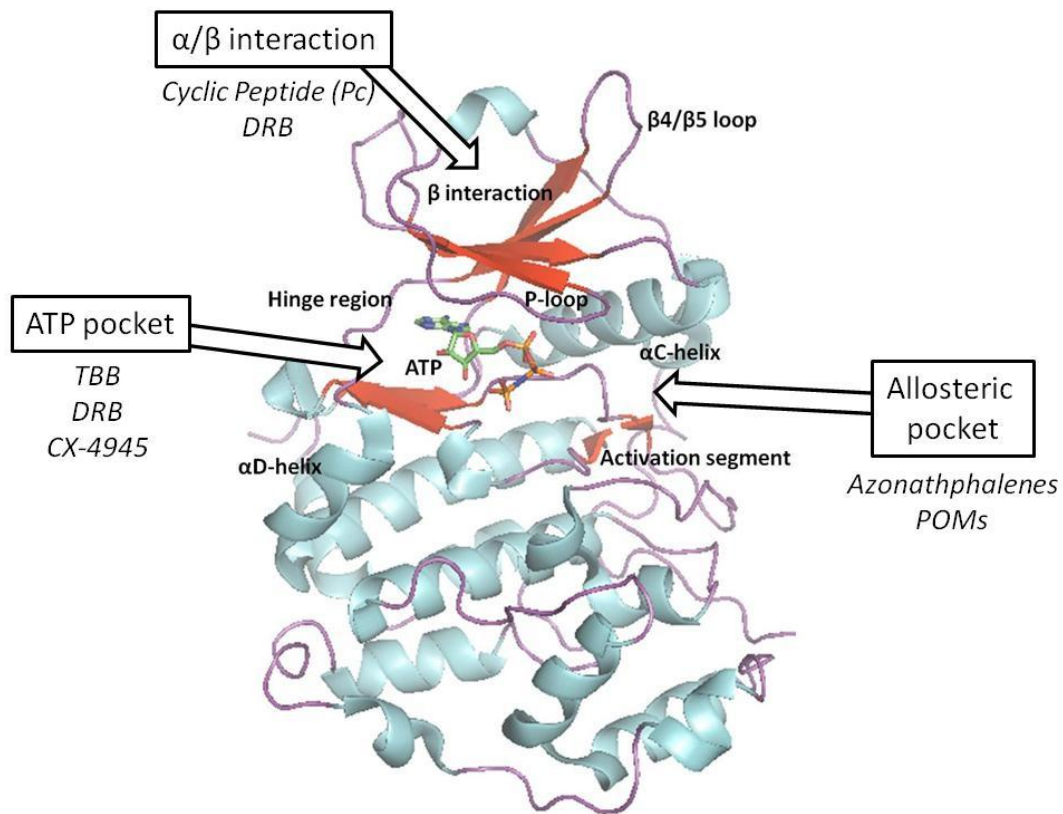
Fig 23: Schematic representation of the different inhibitors binding sites on CK2α structure (based on 2PVR cristallographic file[110]).

1.4 Allostery and non-ATP competitive inhibitors

1.4.1 Allostery definition

The word "allosteric" was first described in 1961, after the 26th Cold Spring Harbor Symposium on Quantitative Biology dedicated to "cellular regulatory mechanisms". The word allosteric was used in the report of this conference by Monod and Jacob.[111] This statement followed years of research on feedback inhibition of biosynthetic pathway in bacteria by the end product of the synthetic chain[112–115] and on the hemoglobin subunit cooperation to bind oxygen molecules. These two authors proposed to call "allosteric inhibition" the phenomenon of end-product inhibition "where the inhibitor is not a steric analogue of the substrate": they guessed that this statement implied a different binding site.

Following this seminal research, the first model describing the allostery concept was proposed in 1965 and was called Monod-Wyman-Changeux (MWC) by the name of its inventors.[116] The MWC model tried to define the main criteria of allosteric inhibition in multimeric proteins. A part of the new concepts focused on the change of protein conformation that induced the inhibition. They supposed that the protein could exist in both active and inactive form and that an allosteric inhibitor favors the inactive conformation whereas the natural substrate favors the active conformation.[117] One year later, a second model was proposed by Koshland, Nemethy and Filmer (KNF model). The major difference with the MWC model is that monomers of the oligomer structure are not connected to each other and so, each monomer could have a different conformation. The binding of one ligand induced a conformational modification favorable to the binding of the ligand in the adjacent subunit.[118] In 1984, the first model describing allosteric regulation without conformational change was proposed by Cooper and Dryden.[119]

Allosteric regulations of proteins are very interesting, due to the opportunity to modulate with increased selectivity previously targeted enzyme and to target some of the previously "non-druggable" proteins. This last point is very similar to what researchers try to achieve with Protein-Protein Interactions Inhibitors (PP2Is), as they wish to bring new targets in drug discovery.[120]

1.4.2 Kinase inhibitor classes and allostery

The first kinase inhibitors were ATP-competitive. They targeted the canonical ATP cleft but soon, the poor selectivity of these inhibitors was discovered (eg for staurosporine). Indeed, a good selectivity is very difficult to achieve through ATP-site targeting because of the numerous proteins that binds ATP molecules. On the other hand, high potency was obtained with ATP competitive inhibitors because of the easy targeting of the large hydrophobic ATP pocket. In addition, by exploiting specific features, several well potent and selective ATP-competitive inhibitors are already in clinical use, such as imatinib (Bcr-Abl)[121], gefitinib (EGFR)[122], sunitinib (VEGFR, PDGFR)[123] or dabrafenib (B-Raf)[124]. To efficiently target some refractory kinases, allosteric inhibitors were considered and several out-of-the-box inhibitors were identified and will be discussed thereafter.

Researchers defined four categories (Fig 24) to classify the different kinase inhibitors, depending on their mechanisms of action:

✓ Type I: ATP-competitive inhibitors that strictly bind into the ATP pocket and maintain the kinase in an active conformation but prevent the ATP-Mg complex binding are defined as class I inhibitor. Staurosporine and dasatinib are two examples of this class.

✓ Type II: encompass the inhibitors that bind to the ATP cleft and simultaneously to a hydrophobic pocket located between the DFG motif in the "out" position and the aC-helix. The binding to this double pocket enable a greater selectivity. Imatinib and sorafenib are type II inhibitors. Recently, a new special class named 1/2 was added for the molecules that bind to the same pockets than type II inhibitors but with the DFG in the "in" position.

✓ Type III: inhibitors that do not compete with ATP and bind to a true allosteric pocket, inducing an inactive conformation. Most of them act through aC-helix displacement or by locking the DFG motif in an inactive conformation.

✓ Type IV: compounds that are also "type III-like" allosteric inhibitors but which bind to a pocket distant from the ATP cleft.
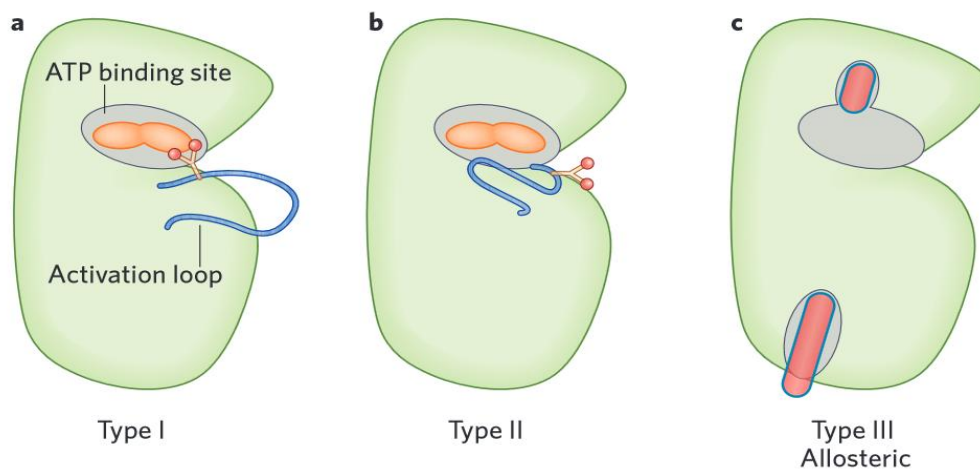
Fig 24: Schematic representation of kinase inhibitor types (Reprinted by permission from Macmillan Publishers Ltd: [Nat. Chem. Biol.] from Foda, Z. H. *et al.*[125], copyright (2016). (A) Type I: small molecule (orange) binds to ATP pocket (grey) with the DFG motif on the activation loop (blue) in the "in" position. (B) Type II: small molecule binds to the ATP pocket but with the DFG motif in the "out" position. (C) Type III (and IV): allosteric inhibitor (red) binds in a different pocket near the ATP pocket or far from it.

1.4.3   Examples of non-ATP competitive inhibitors of protein kinases

Fig 25: Few examples of kinase inhibitors in their binding sites (Reprinted from Nussinov, R. *et al.* [126]**, Copyright (2016), with permission from Elsevier). (A) Orthosteric inhibitor (Gefitinib in red) bound to active EGFR kinase (PDB ID: 3UG2). (B) Allosteric inhibitor (PD318088 in cyan) and ATP (in red) bound to inactive MEK1 (PDB ID: 1S9I). (C) ATP competitive inhibitor (imatinib in red) bound to inactive p38 (PDB ID: 3HEC). (D) Allosteric i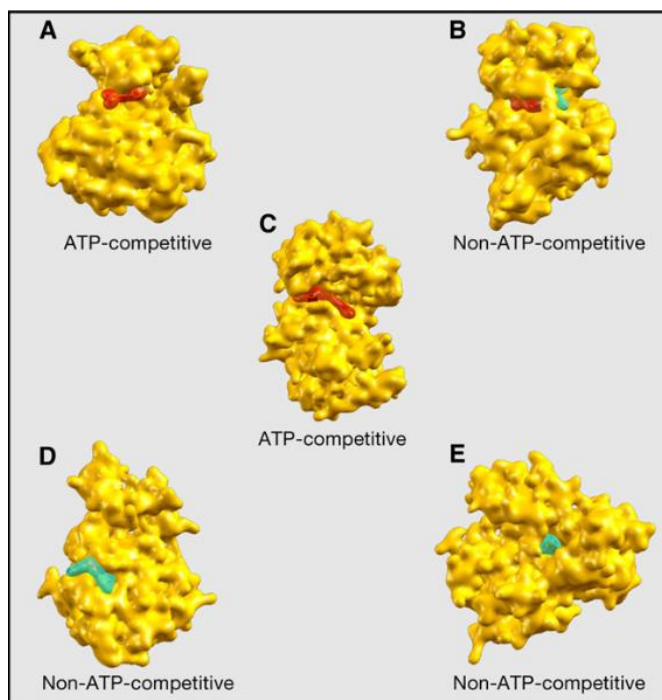nhibitor (in red) bound to CHK1 (PDB ID: 3JVR). (E) Allosteric inhibitor bound to the interface of Akt and PH domain (PDB ID: 3O96).**

1.4.3.1 ANS pocket

Cyclin-Dependent Kinases (CDKs) are key regulators of the cell cycle and they are deregulated in numerous pathologies. Despite numerous efforts to find good CDK inhibitors, and several of these molecules that underwent into clinical trials, research groups did not succeed to develop an efficient compound. All of the developed molecules were ATP-competitive inhibitors and one of their major drawbacks was their selectivity. Recently, a new pocket was identified on CDK2 and named ANS pocket, as this compound (8-anilino-1-naphtalene sulfonate) was the only molecule described to bind into this pocket.

ANS is a commonly used fluorescent probe. In the process of searching for new compounds able to compete with cyclins for CDK2 binding, Betzi *et al.* have set up an assay based on ANS displacement. They realized later that ANS specifically binds to CDK2 with a Kd of 37 µM and X-ray structures revealed a specific pocket able to bind two ANS molecules (Fig 26). ANS inhibits CDK2 through aC-helix displacement, thus locking the inactive state of CDK2.[127,128]
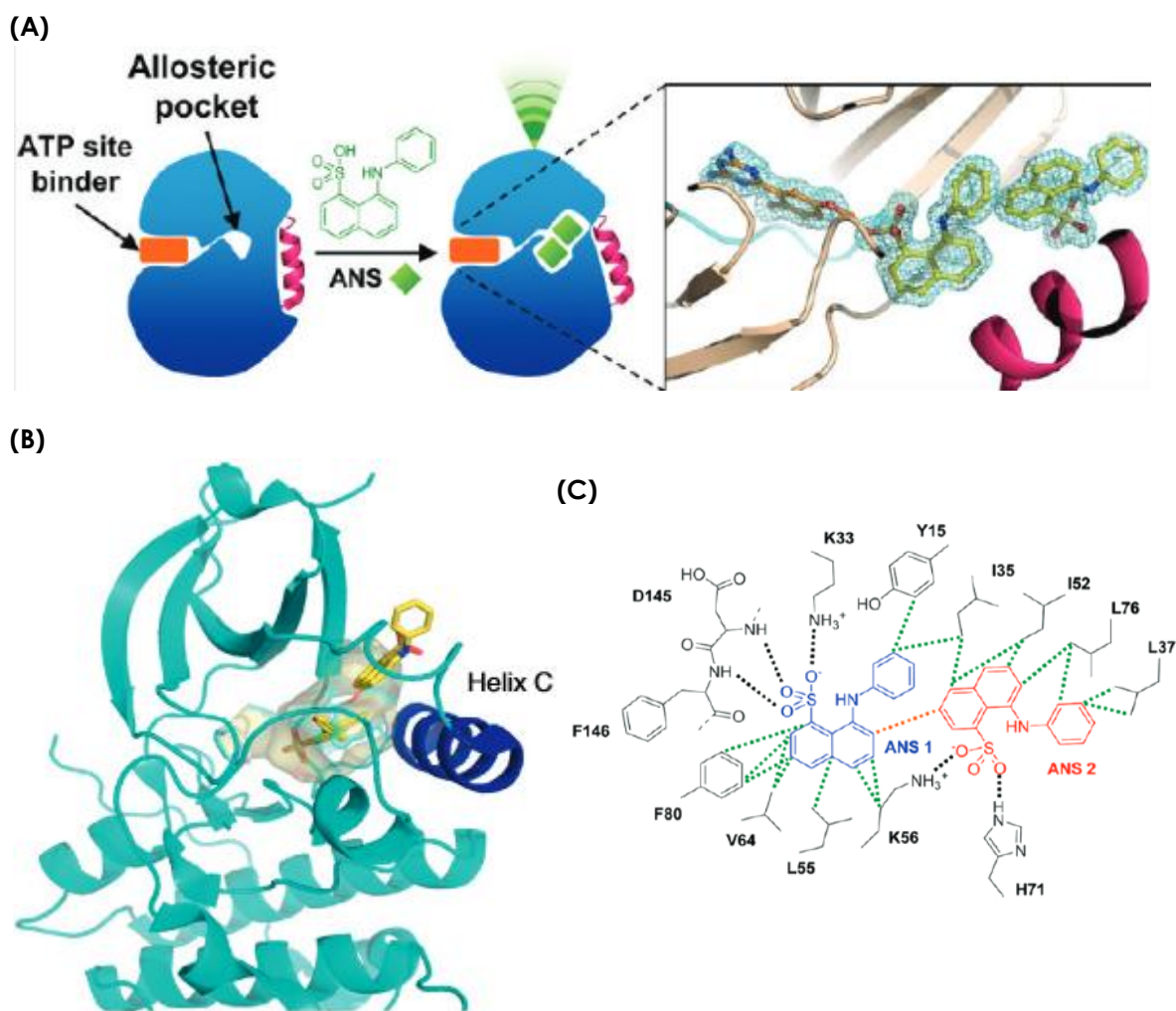
Fig 26: ANS pocket in CDK2. (A) Schematic representation of the ANS pocket without or in presence of the two ANS molecules. A small ATP competitive compound can bind in the same time than ANS without any competition (Reprinted with permission from Betzi, S. *et al.*[128], Copyright (2016) American Chemical Society). (B) CDK2 X-ray structure with two ANS molecules bound (PDB ID: 3PXF) (Reprinted from Palmieri, L. *et al.*[129], Copyright (2016), with permission from Elsevier). (C) Schematic representation of the ANS interactions into their binding sites in CDK2 (Reprinted with permission from Betzi, S. *et al.*[128], Copyright (2016) American Chemical Society).

The AGC protein kinase family is one of the most evolutionary conserved groups that represents 12% of human kinome (61 kinases and 2 pseudo kinases).[130] The C-terminal segment of AGC kinases presents a unique hydrophobic motif which fold back in a hydrophobic pocket localized between β4, β5 loop and α-B-, α-C-helix. This pocket, named PIF pocket, played a central role in AGC protein kinases regulation (Fig 27, A).[131] Despite the general presence of the PIF pocket in AGC kinases, PDK1 is the kinase in which the PIF pocket has the most crucial role. Indeed, in PDK1, binding of the C-terminal segment does not only turn on/off the kinase activity but modulates the protein kinase substrate selection.
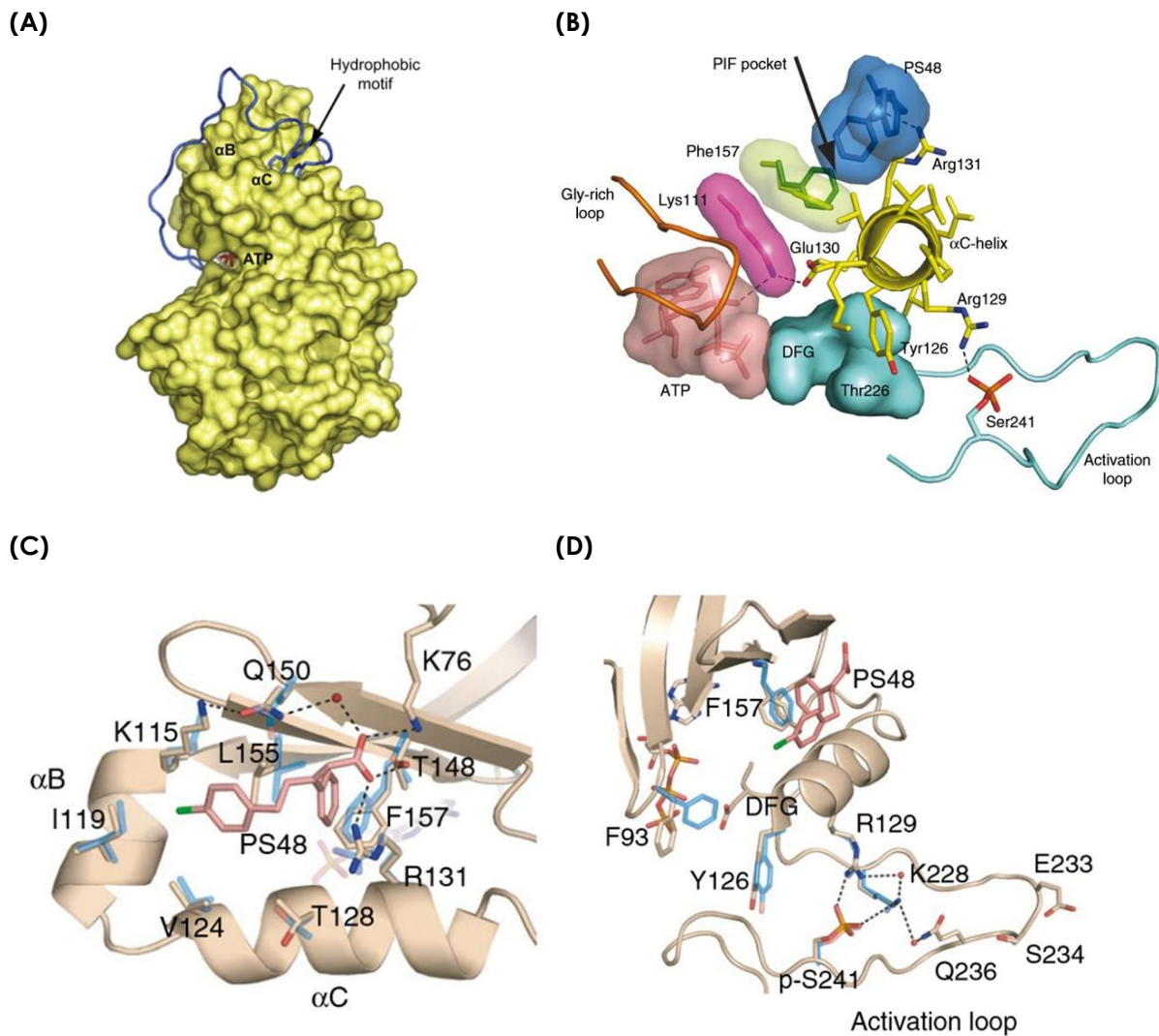
(A)

(B)

(C)

(D)

Fig 27: PIF pocket in PDK1. (A) General X-ray structure of AGC kinases (from PKA, PDB ID: 1ATP) with the C-terminal extension in blue and the two hydrophobic residues bound to the PIF pocket

The crucial role of the PIF pocket in PDK1 makes it a promising target for therapeutic drugs. 2-(3-Oxo-1,3-diphenylpropyl)malonic acid and its derivatives were identified as small molecules targeting the PIF pocket of PDK1. They act as *in vitro* activators of PDK1 and selective substrate inhibitors in cell assays.[133–135] These molecules selectively bind to the hydrophobic pocket localized behind the aC-helix and stabilize key residues implicated in kinase activity (Fig 27).

The PIF pocket is also present in PKCζ and 4-benzimidazolyl-3-(4-chloro-phenyl)butanoic acid family of compounds targets this pocket. Compounds of this class are allosteric inhibitors of PKCζ with a very good selectivity whereas they are weak activator of PDK1.[136]

1.4.3.3 Akt pocket

Another member of the AGC group has interesting features for allosteric inhibition, the protein kinase Akt (also named Protein Kinase B, PKB). Akt plays a key role in survival and proliferation regulation. All three human isoforms (Akt1, Akt2 and Akt3) of Akt share an N-terminal Pleckstrin Homology (PH) domain which takes part in Akt regulation. Indeed, the kinase in its PH "out" form is ready to be activated by phosphorylation whereas the PH "in" form is inactive.

The importance of Akt makes this kinase a promising target for cancer treatment. One of the compound screenings undergone to find Akt inhibitors revealed two molecules with a mixed-competitive effect toward ATP. These molecules inhibit Akt in a PH-dependent manner. Indeed, the IC_{50} of these compounds are in the micromolar range in the presence of the PH domain whereas they are completely inactive in the absence of the PH domain. This effect is opposite to the one observed with staurosporine, which is active in both cases but with preference for Akt in the absence of the PH domain.[137]

Medicinal chemistry and structural optimization drove researchers to find improved compounds with nanomolar potency and high selectivity (Fig 28, B and C).[138,139] X-ray structures revealed a new pocket formed in the presence of the PH domain. Furthermore, a disorganization of the αC-helix was observed in presence of small molecules bound in this pocket. The Akt pocket is localized at the interface of the kinase domain and of the PH-domain making the presence of the PH-domain a strict requirement for inhibitor binding (Fig 28, A).[140]

(A)

(B)

IC$_{50}$ (Akt1) = 58 nM
IC$_{50}$ (Akt2) = 210 nM
IC$_{50}$ (Akt3) = 2119 nM

(C)

IC$_{50}$ (Akt1) = 5 nM
IC$_{50}$ (Akt2) = 18 nM
IC$_{50}$ (Akt3) = 170 nM
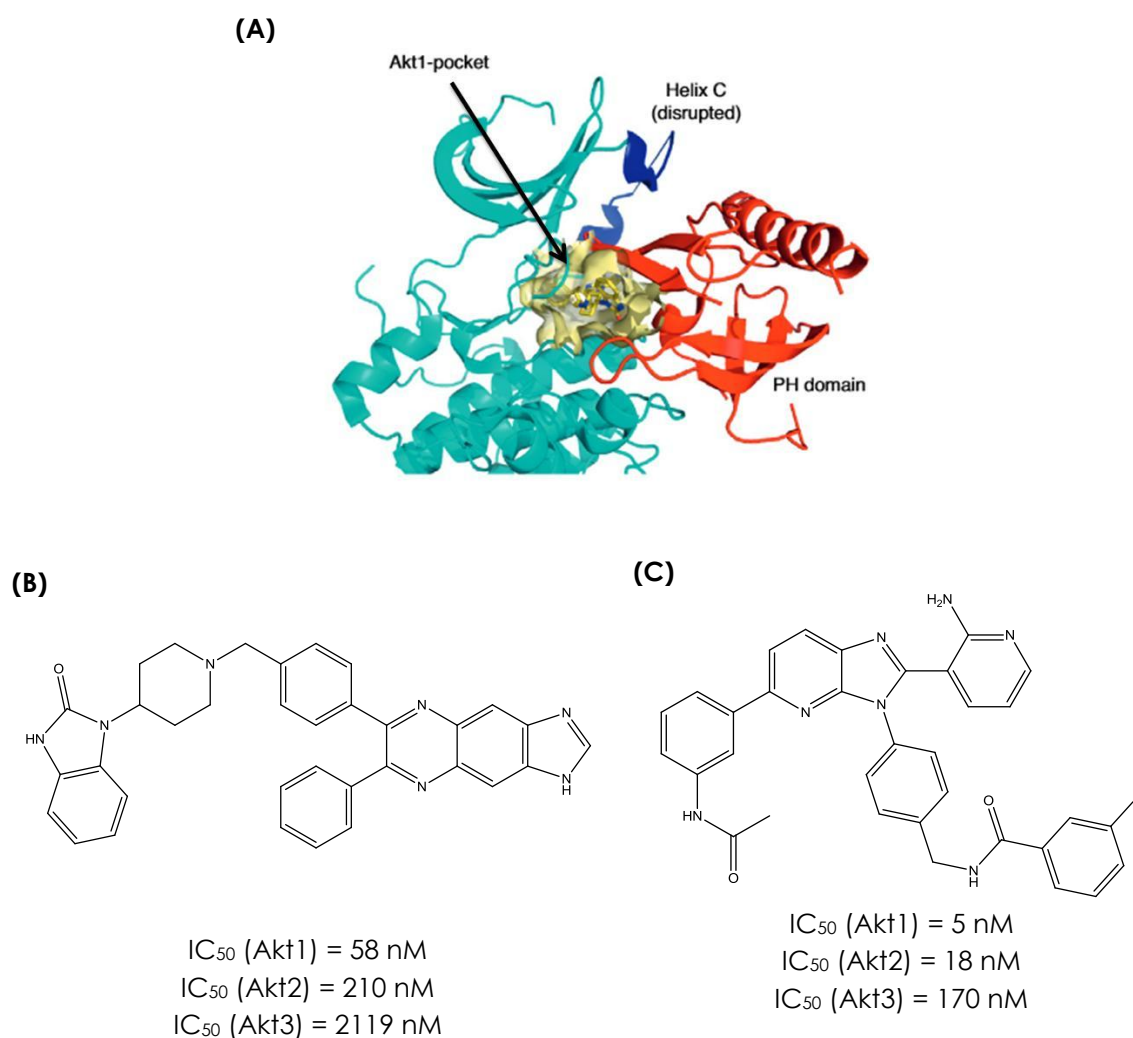
Fig 28: Overview of Akt1 pocket. (A) Structural overview of Akt1 pocket with the Pleckstrin Homology (PH) domain in red (Reprinted from Palmieri, L. *et al*.[129], Copyright (2016), with permission from Elsevier) (B) Chemical structure of the allosteric Akt inhibitor VIII[139]. (C) Chemical structure of the allosteric Akt inhibitor described by Ashwell *et al*[138].

1.4.3.4 P-loop pocket

The P-loop pocket (or MEK pocket) is localized between the P-loop (Mg-binding loop) and the αC-helix in Mitogen-activated protein kinase kinase (MEKs). Several small molecules have been found to bind to the MEK pocket: the first one PD184352 was described in 1999.[141] Based on mutation experiments, a putative binding site was considered[142] and later confirmed by X-ray structure determination (Fig 29).[143]

Interestingly, all of the type III inhibitors of MEKs have been crystallized in presence of an ATP analog. Hydrogen bonds could be observed between inhibitors and phosphates of the nucleotide and synergetic effect for binding was observed for some of these type III inhibitors.[144]

(A)   **(B)**
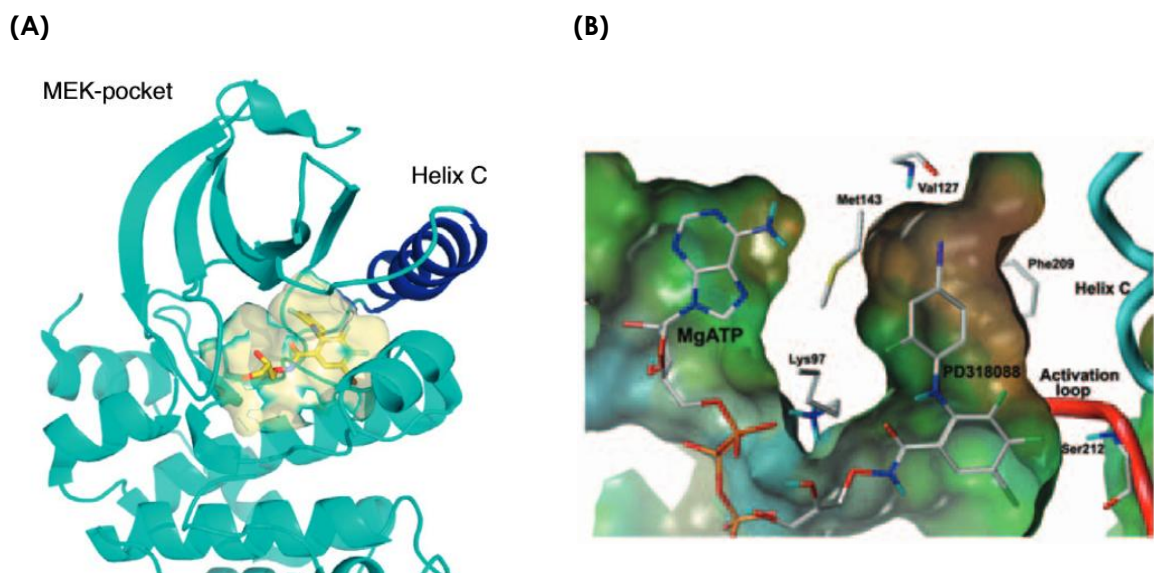
Fig 29: Structural view of the MEK-pocket localized between the DFG-motif and the αC-helix. (A) General 3D view of the MEK-pocket (PDB ID: 1S9J) (Reprinted from Palmieri, L. *et al*.[129], Copyright (2016), with permission from Elsevier). (B) View, from the *N*-terminal lobe, of the ATP molecule bound to the catalytic site and of PD318088 compound bound to the allosteric MEK pocket. Brown surface represents the most hydrophobic residues and green the most hydrophilic

Following these results, several highly selective MEK1/MEK2 inhibitors were discovered and one of them, trametinib[145](Fig 30) was approved by FDA in 2013 for the treatment of patients with BRAF V600E mutated metastatic melanoma. Nowadays, trametinib is used in combination with dabrafenib to overcome resistances.[146]

Trametinib (allosteric MEK1/MEK2 inhibitor)   **Dabrafenib** (B-Raf inhibitor)
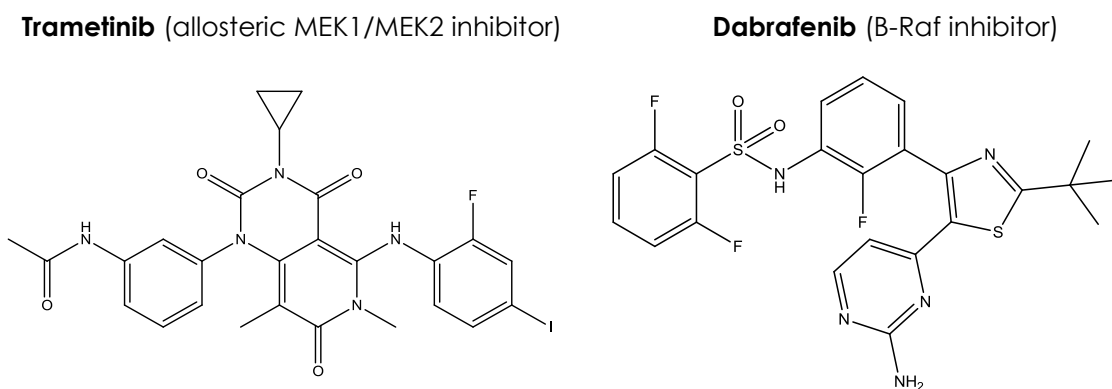
Fig 30: Chemical structure of trametinib and dabrafenib.

Recently, findings about the binding mode of an ERK inhibitor named SCH772984 revealed that it shares some features with MEK Type III inhibitors. SCH772984 was firstly described as a potent and selective ERK inhibitor but its mechanism of action remained unknown.[147] Then, Chaikuad *et al.* supposed that a Type II mechanism of action could be the reason of the overall good efficacy of this inhibitor. Experiments enabled them to determine the exact and unique binding mode of this molecule. Indeed, SCH772984 binds to the ATP pocket and, at the same time, to the P-loop pocket providing specific properties such as slow binding kinetics. Piperazine-phenyl-pyrimidine moiety of this compound binds to the specific P-loop pocket near the aC-helix with the P-loop Tyr36 shifted to the "in" position whereas the indazole moiety binds classically in the ATP pocket (Fig 31). Interestingly, SCH772984 binding mode is different in off-targeted kinases such as Haspin where it binds with a classical type I mechanism.[148]

(A)

(B)

(C)
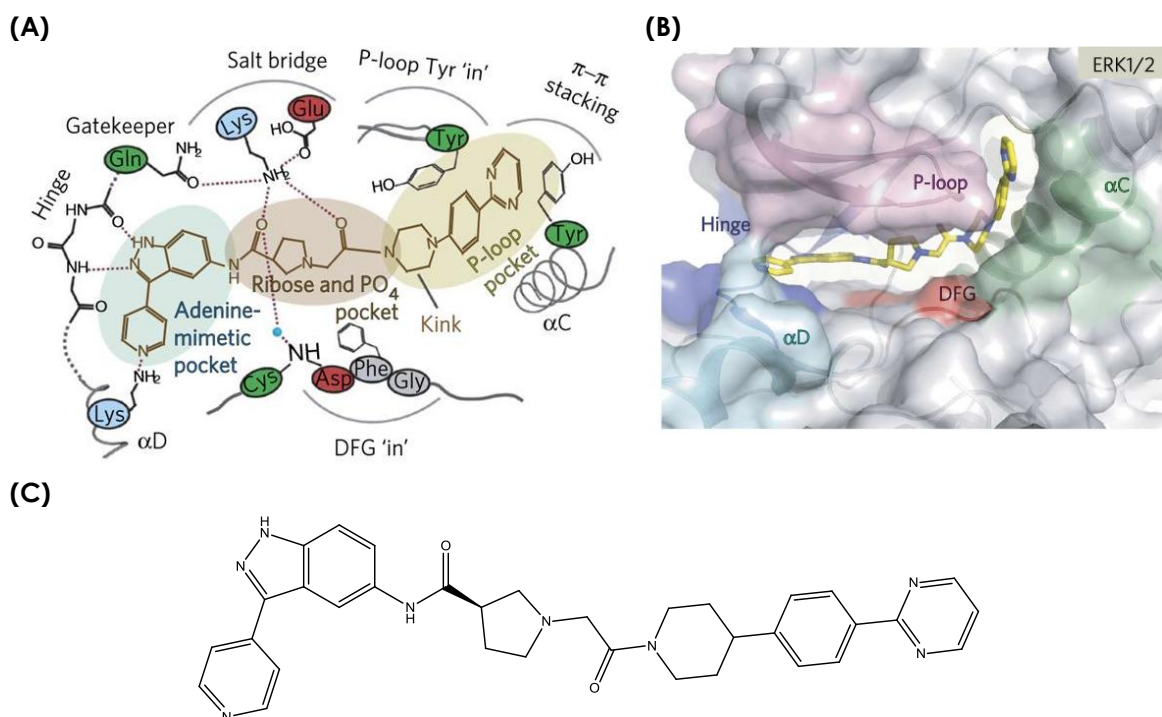
Fig 31: SCH772984 binding mode to ERK1/2 using the dual ATP/P-loop pocket. (A) Schematic representation of SCH772984 bound to ERK1/2. Adenine pocket is underlined with a blue shadow, ribose and phosphates pocket with a red shadow and the P-loop pocket with a brown shadow (Reprinted by permission from Macmillan Publishers Ltd: [Nat. Chem. Biol.] from Chaikuad, A. *et al.*[148], copyright (2016)). (B) ERK1/2 surface representation with SCH772984 (PDB ID: 4QTA) (Reprinted by permission from Macmillan Publishers Ltd: [Nat. Chem. Biol.] from Chaikuad, A. *et al.*[148], copyright (2016)). (C) Chemical structure of SCH772984.

1.4.4 Allosteric inhibitors and CK2

As described in paragraph 1.3.2.**2**, CK2 is considered as always active.[30] This statement is based on the observation of the same active conformation in all X-ray structures of CK2α. Moreover, no regulation mechanisms such as phosphorylation are required for CK2α activity. Nevertheless, during attempts to crystallized CK2α in the presence of small molecule (DRB or glycerol) in the α/β interaction pocket, Raaf *et al.* obtained the first CK2a structure in an inactive conformation. In this structure, the conformation of the ATP-binding loop (or glycine-rich loop, or P-loop) differs from the canonical one. Indeed, Tyr50 of the P-loop collapses in the ATP pocket, preventing ATP binding, instead of making interaction with Lys74 and Lys77 of the αC-helix (Fig 32).[149] This structure opens the question about the "always active conformation" and it is fair to expect that specifically designed small molecules could favor this inactive conformation and act as allosteric inhibitors. Moreover, by looking at the β-factor of the CK2α structures, it is clear that the ATP-binding loop is one of the most flexible parts of CK2α, together with the β4/β5 loop. This point is confirmed by the global overview of CK2α structures, already discussed in paragraph 1.3.2.**2** (Fig 10).[31] A displacement of the ATP-binding loop by a small molecule seems to be a likely event.
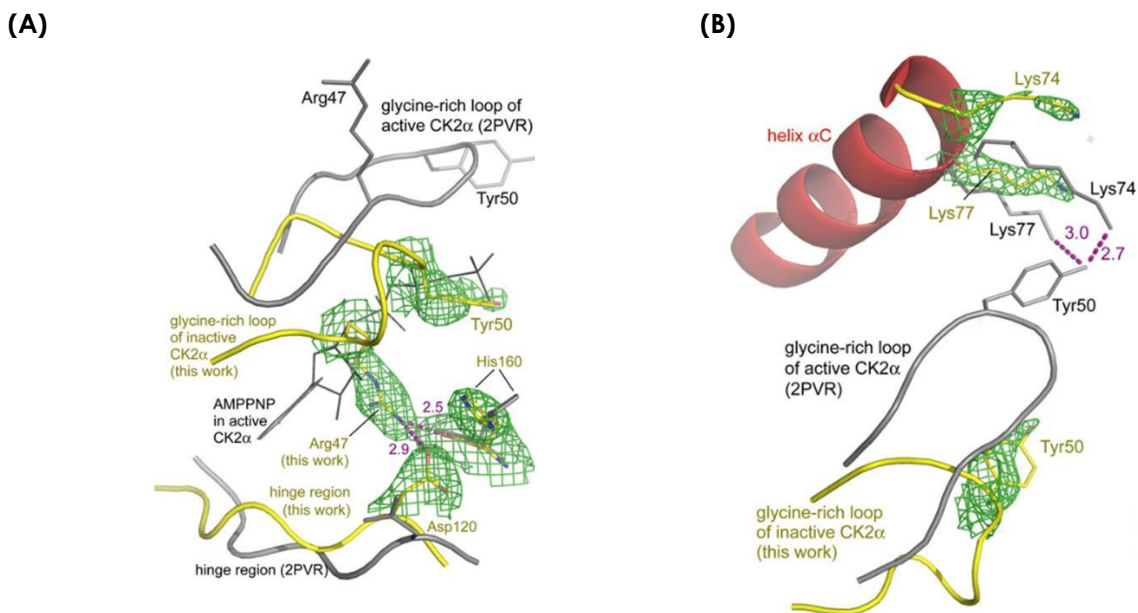
Fig 32: Glycine-rich loop conformation in active CK2α (PDB ID: 2PVR) and in inactive CK2α (PDB ID: 3FWQ) (Reprinted from Raaf, J. et al.[149], Copyright (2016), with permission from Elsevier). (A) Representation of the ATP binding pocket occupied by ATP (grey) in active CK2α and by Arg47 and Tyr50 (yellow) in inactive conformation. (B) Specific interactions of Tyr50 with Lys74 and Lys77 in active conformation (grey) and absence of interactions in inactive CK2α (yellow).

As already mentioned, almost all CK2 inhibitors described until now target the ATP pocket and are ATP-competitive inhibitors (TBB, TBI, CX-4945...). Nevertheless, one exception, the azonaphthalene class of compounds, is particularly interesting, even if the binding site was not precisely determined (Fig 33). Indeed, this family of small molecules has been described as non ATP-competitive inhibitors of CK2α. SAXS experiments disclosed that, in the presence of an azonaphthalene derivative (Fig 33, C), a striking deformation of the kinase structure takes place in the region near the P-loop, αC-helix and activation segment has been observed (Fig 33, A). Moreover, three CK2α mutants Lys71A, Lys76A and Leu178A were found less sensitive toward azonaphthalene inhibition than WT-CK2α (Fig 33, B).[150] Lys71 and Lys76 residues are localized in the αC-helix and they make interactions with the P-loop. Leu178 is located at the beginning of the activation segment, close to the ATP pocket. These results support the concept that the binding site of azonaphthalene series is localized in the area between αC-helix and the activation segment.
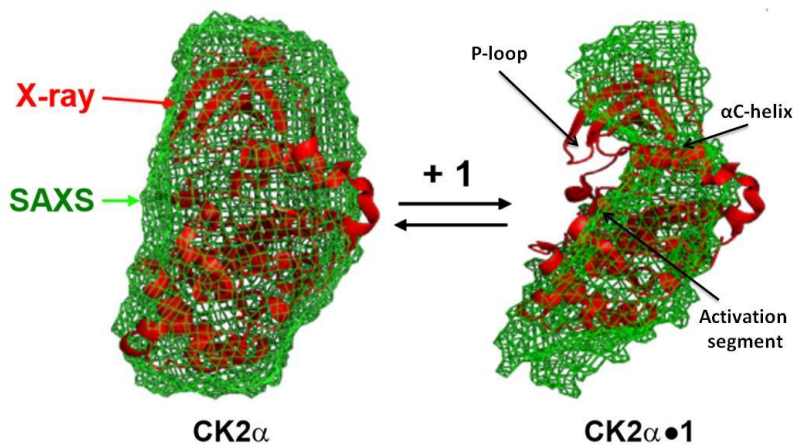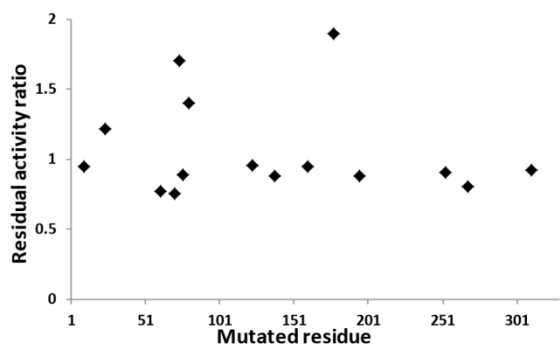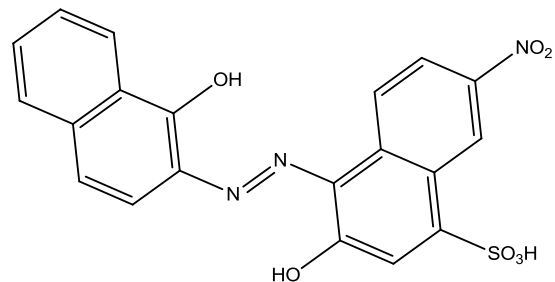
Fig 33: Overview of the azonaphthalene class of compounds. (A) Surimposition of X-ray structure (PDB ID: 1PJK) in red ribbon and SAXS ab initio shape restoration for CK2α and CK2α + azonaphthalene (From Moucadel, V. *et al.*[150], with the permission of Impact Journals, LLC). (B) Inhibitory effect of 0.4 µM azonaphthalene on CK2α mutated in the residues: 10, 24, 61, 71, 74, 76, 80, 123, 138, 160, 178, 195, 253, 268, 308, 311 to alanine (From Moucadel, V. *et al.*[150]). (C) Chemical structure of the lead compound azo of the azonaphthalene class.

Few other non ATP-competitive inhibitors have been described such as polyoxometalates (POMs).[151] Studies also supported their binding site to be located in the same region of CK2α than azonaphthalenes but, as POMs could not be classified as drug-like small molecules, the following discussion will not deal with this class of compounds.

A last class of dual site inhibitors is of particular interest. They were obtained by coupling the commonly used TBBi (ATP competitive inhibitor) to a small acidic peptide containing multiple aspartate residues. As the result, they were able to interact both with the ATP-site and with the substrate binding site of CK2α, leading to very potent compounds. The most potent inhibitor ARC-1502 showed a tremendous affinity for CK2a with a Ki of 0.5 nM, more than 2500-fold better than the initial ATP competitive inhibitor (Ki = 1.34 µM). As expected, the acidic peptide part of ARC-1502 binds to the substrate binding region of CK2α (PDB ID: 4FBX) but the low resolution of this part of the molecule in the X-ray structure prevents a better understanding of its the binding mode.[109] Following this work, novel dual-site inhibitors were obtained by coupling the ATP-competitive inhibitor K137 to small peptide sequences. The most potent compound (K137-E4) was linked to four glutamic acid residues and exhibited an IC$_{50}$ of 25 nM for CK2$\alpha_2\beta_2$. The glutamic acid residues interact with the residue involved in binding of n+1 (R191, R195, K198) and n+3 (K74, K77) of the substrate proteins. Interestingly, K137-E4 was lacking cell membrane permeability: this molecule was therefore able to inhibit selectively the ecto-CK2 activity. In consequence, K137-E4 appears to be a valuable tool to understand the physiological role of the ecto-CK2.[152]

In conclusion, previous studies support the potential interest of the P-loop, the aC-helix and the activation segment to be targeted by small molecules to induce CK2a inactivation. Nevertheless, up to now, no precise allosteric pocket has been defined on CK2a and allosteric drug-like inhibitors have not been identified yet.

In this part of the manuscript, α/β interaction inhibitors that could be considered as allosteric modulators of CK2 have not been discussed. Information about this strategy for CK2 modulation will be detailed in paragraph 1.5.4.

1.5   Protein-Protein Interaction Inhibitors

1.5.1   Definitions and importance

Protein-Protein Interactions (PPI) have been extensively studied because of their crucial role in protein regulation. Indeed, a simple interaction between two proteins is the most common way to regulate their activity through protection toward degradation, translocation, activation, inactivation, degradation… Human protein-protein interaction network, also called interactome, consists of the whole set of interactions between proteins that could occur in a given organism.[153] The size of the human interactome is estimated to have 130,000 binary interactions and, consequently, 130,000 possible targets to modulate cell signaling.[154] A web database called "Interactome Database"[155] reports all described binary interactions.

Protein-protein interaction enables scientists to target proteins that were considered undruggable such as p53/mdm2, EphA4/ephrinB, HPV E2/E1…

1.5.2   Specific difficulties in PP2I discovery

Protein-protein interaction inhibitors (PP2Is) faced different difficulties compared to "usual" orthosteric enzyme inhibitors. In contrast to enzyme inhibitors, there are no natural small molecule which could act as ligands as starting scaffolds, nor well-defined pockets, nor convenient enzymatic assays to evaluate these small molecules… Large and flat interfaces, lack of cavities, competition with large protein, smooth interface are the main difficulties for the design of new PP2Is.

Most of the proteins interact through the intermediate of a large surfaces,[156,157] with a common surface size of about 1,500 to 3,000 Å². To the opposite, interactions between proteins and small molecules are much smaller, with an average surface interaction equals from 300 to 1,000 Å². However, alanine scanning mutagenesis of residues located in protein interfaces revealed that, despite the large number of residues implicated in the interaction, only few of them are responsible of the major part of the affinity. Most of the time, one to three hydrophobic residues located at the center of the interactions are responsible of the majority of the affinity, and so, they are called hot spots.[158] In consequence, small molecules targeting only these key elements could be sufficient to modulate the protein-protein interaction.[159–161]

Another important feature of protein-protein interfaces is their global flatness. Nevertheless, surface flexibility allows adaptation to small molecule binding. Indeed, side chain or loop movements can reveal small pockets that are not observable at a first glance on the protein structure. Transient pockets could be revealed in flat protein surface during 10 ns molecular dynamic simulation, as shown with $Bcl-X_L$, IL-2 and MDM2. In these three examples, transient pockets opened during simulation, confirming the situation obtained in crystal structures of these proteins with small molecules inhibitors. This statement underlined the drugability of the "appearantly flat" protein interfaces.[162]

Most of PP2Is come from compound library screenings. Nevertheless, screenings for PP2Is are considered arduous, due to the low hit rate that is generally obtained from large library screening. One source of this problem could be that compound libraries have been designed to target classical enzyme active sites whereas protein-protein interfaces required different chemical structures properties. In consequence, specific libraries have been designed to target protein interactions.[163,164] In addition to screenings, fragment-based approaches using various methods such as NMR, SPR or enzymatic assays are often used to identify PP2Is.[165] Since the success of the molecule ABT-737, discovered by NMR fragment screening and now in clinical trials,[166,167] several other protein-protein interactions such as ZipA/FtsZ,[168] pVHL/HIF-1a[169] and Bcl-X_L[170] have been studied by NMR. Elisa Barile *et a*l. have recently published a very interesting review which described in details all the NMR methods used for the identification and optimization of PP2Is.[171]

Lipinski's "rule of five" (H_{Donor}< 5, $H_{Acceptor}$< 10, MW < 500, LogP< 5) is a global feature leading to a good absorption and so, to a good *in vivo* efficiency. These rules are commonly accepted as general guidelines to follow in drug discovery programs.[172] However, for PP2Is, a consensus is generally accepted: due to the size of protein-protein interfaces, larger molecules are required. A comparison between molecular descriptors of PP2Is and a subset of classical drugs enables a verification of this statement (Fig 34):[173]

- Molecular weight: the average molecular weight of PP2Is is bigger than for other drugs but most of PP2Is have a molecular weight under 500 g/mol.
- logP: none of the described PP2Is has a negative logP and they have a general logP greater than classical drugs (75% of PP2Is have a logP under 5).
- Hydrogen bond formation: both subsets of molecules respect Lipinski's rules concerning the number of hydrogen bond acceptors and the number of hydrogen bond donors.

- Topological Polar Surface Area (TPSA): TPSA is considered to be less than 140 Å² and both families of molecules respect this statement, even if PP2Is share a larger TPSA than classical drugs.

This comparison confirms the global need of larger molecules to address protein-protein interfaces but most of the PP2Is still respect the Lipinski's rule of five and in consequence could be promising candidates.
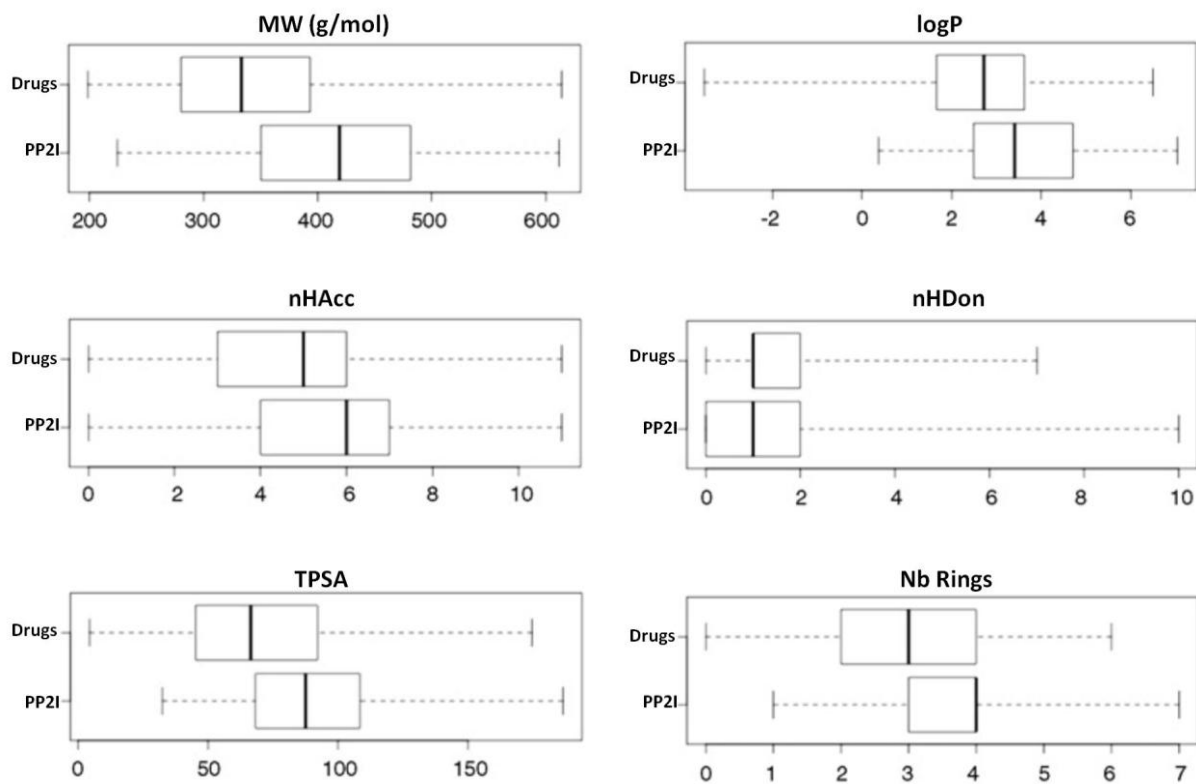
Fig 34: Distribution of several molecular descriptors calculated on a family of 66 PP2Is and 557 drugs. Minimum value, first quartile, median, third quartile and maximum values are represented (Reprinted from Sperandio, O. et al.[173], Copyright (2016), with permission from Elsevier).

1.5.3 Successful stories

Protein-protein interactions can be classified in two subtypes depending of the natural secondary structure type:

- Helix-mediated interaction: an α-helix is the natural binder of the targeted protein,

- Loop-mediated interaction: a loop, often more flexible and related to macrocycles, is the natural binder of the protein.

1.5.3.1 Helix mediated interaction inhibitors

A survey on all PDB structures shows that only 15% of PDB entries consist of multiprotein complexes and, in this subgroup, 62% of the interactions have an helix at the interface.[174] Naturally, helix mimetics are the best option to find inhibitors of such interactions[175] and this could be obtained:

- From helix stabilization, using side-chain cross links by covalent bonds or hydrogen bonds,
- From β-foldamers, composed of amino-acid analogs able to adopt an helix conformation,
- From small molecule scaffolds which are helical surface mimetics. This category is advantageous because they are composed of small organic molecules and not of peptide derivatives.[176]

Several successful molecules have been developed as helix competitors. One of the most famous cases of protein-protein interaction, the p53/MDM2 interaction, is mediated by an helix. Indeed, one α-helix of the N-terminal transactivation domain of p53 binds to MDM2, thus preventing p53 tumor suppressor effect. In 2004, Vassilev *et al.* published the first *in vivo* effective small molecule inhibitor of the p53/MDM2 interaction: Nutlin-3a.[177] These results were the proof-of-concept that inhibition of this interaction in a murine model restored the p53 tumor suppressor activity. Following this result, researchers focused their works to find other efficient chemical structures targeting this interaction.[178,179] Nowadays, 5 molecules are already in clinical trials and some more are going to enter such trials (Fig 35).[180]
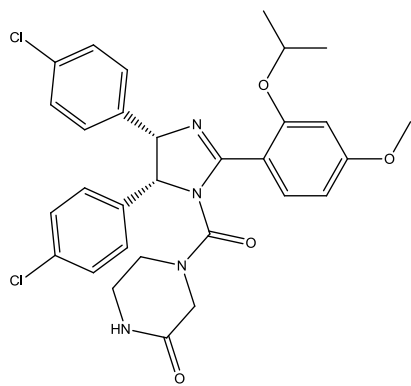
Nutlin-3a[177]
HTRF IC_{50} = 88 nM
MTT IC_{50} = 1.5 μM
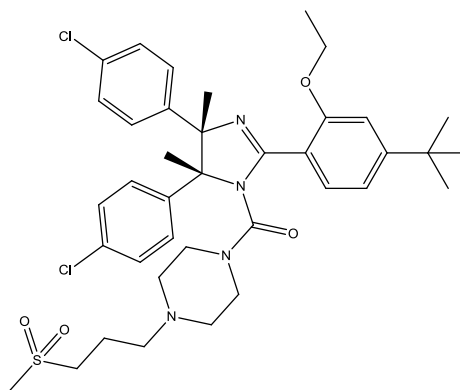
RG7172[181]
HTRF IC_{50} = 18 nM
MTT IC_{50} = 0.4 μM
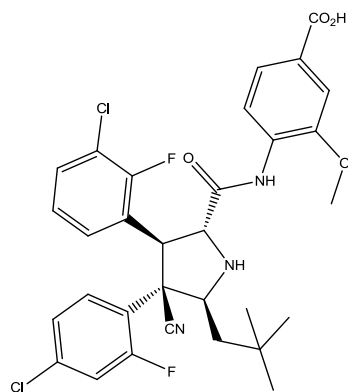
RG7388[182]
HTRF IC_{50} = 6 nM
MTT IC_{50} = 0.03 μM
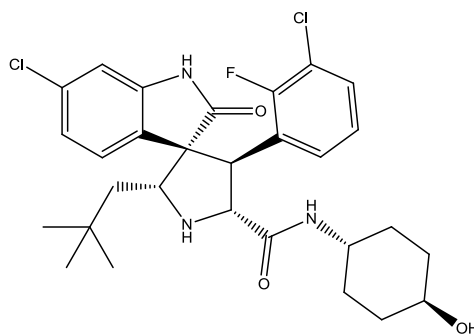
SAR405838[183]
Ki = 0.88 nM
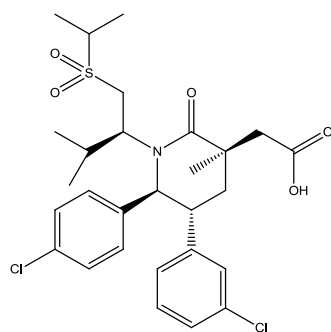
AMG-232[184]
SPR Kd = 0.045 nM
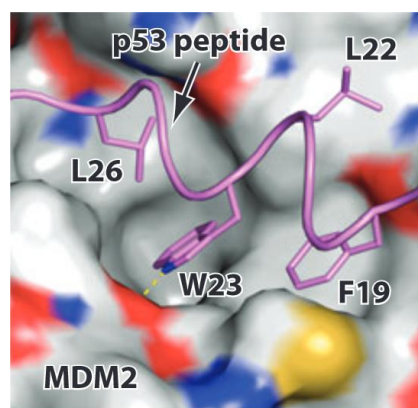
p53 peptide bound to Mdm2[179] (PDB ID: 1YCR) Kd = 60 nM[185]

Fig 35: Examples of p53/MDM2 inhibitors and representation of the natural residues involved in the interaction (From Shangary, S. *et al.*[186], with the Annual Review of Pharmacology and Toxicology).

Small molecule inhibitors of p53/MDM2 interaction mimic the key residue (Phe19, Trp23, Leu26) of the p53 helix which binds to MDM2 (Fig 36). Medicinal chemistry optimization has led to small molecules with stronger affinities for MDM2 than p53 (60 nM).
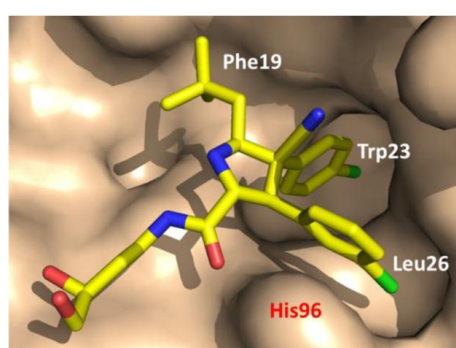
RG7388
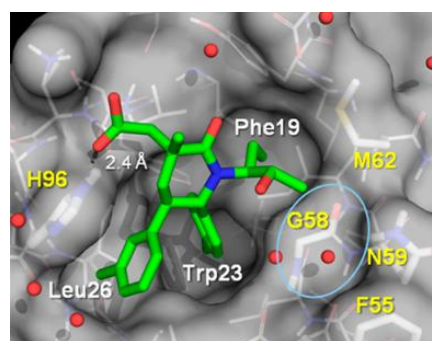
AMG-232

Fig 36: Structure of RG7388 (Reprinted with permission from Ding, Q. *et al.*[182], Copyright (2016) American Chemical Society) and AMG-232 (Reprinted with permission from Sun, D. *et al.*[184], Copyright (2016) American Chemical Society) bound to MDM2 protein. Amino-acid names written in white correspond to the position of the key residue of p53 bounds to MDM2.

Even if p53/MDM2 is probably the most well studied protein-protein interaction and with the greatest number of drugs in development, some other inhibitors have been successfully developed for other interactions (Bcl-X$_L$/BAK or BAD, HPV E2/E1, ZipA/FtsZ, IL-2, α-tubulin/β-tubulin…). Extensive and recent reviews concerning PP2Is have been published by Tracy L. Nero *et al.*[187], Madhu Aeluri *et al.*[178], Lech-Gustav Milroy *et al.*[188]

1.5.3.2 Loop-mediated interaction inhibitors

Despite the fact than α-helix interactions composed the major part of protein-protein interactions described in the PDB databank, non-helical and non-strand loops are the drivers of many protein-protein interactions.[189] A computational approach was described by Jason

Gavenonis *et al.* to identify loops ("hot loops") which are important for affinity in protein interfaces. Among all identified loops, computational alanine scanning was used to determined hot spots and loops that include more than one hot spot. Using this method, 1407 hot loops were identified in the PDB databank, which represents 5.6% of all interfaces loops. Among this subgroup, 19% of the hot loops represent more than 75% of the predicted energy of the all interface, 36% more than 50% of the predicted energy interface and 67% more than 25%. This study highlights the importance of these hot loops in protein-protein interactions. Interactions mediated by loops can also be targeted for drug development[190] and some examples are presented below.

EphA4 protein belongs to the Eph family of tyrosine kinase, composed of 16 members, which can bind to different glycosylphosphatidylinositol-anchored ephrin-A or transmembrane ephrin-B to regulate various biological responses.[191] Modulation of EphA4/ephrin interaction is a promising target to cure several diseases such as cancers or amyotrophic lateral sclerosis.

The interaction between EphA4 and ephrin-B is mediated by a loop of ephrin-B that binds to a groove of EphA4.[192] As frequently observed, the first modulator of EphA4/ephrin-B interaction was a small peptide. Actually, three different peptides were discovered by phage display screening as EphA4 binders.[193] Further investigations led to the identification of the binding site of these peptides and revealed their competition with ephrin for EphA4 binding. Moreover, these peptides are selective for EphA4 among Eph receptors (Fig 37, A and B).[194]

Two small molecules were later identified as inhibitors of EphA4/ephrin interaction by using an ELISA-based screening for the interaction between EphA4 and Biot-KYL peptide.[195] These small molecules have moderate affinity (about 20 µM) for EphA4, probably because of the absence of an interaction with the complete interface of EphA4/ephrin. Nevertheless, they remain good starting point for further drug optimization. Competition experiments, combined with NMR experiments and docking simulations, enabled the determination of a putative binding site consistent with affinity and ability to prevent ephrin binding (Fig 37, C and D).[196]

By using the same screening approach, another structurally unrelated compound was discovered as EphA4/ephrin inhibitor: lithocholic acid. This compound has a moderate affinity for EphA4 ($Ki = 49$ µM), despite a higher molecular weight. Other bile acids such as cholic acid, deoxycholic acid and chenodeoxycholic acid did not show any effect on EphA4/ephrin interaction. However, lithocholic acid showed an effect on all EphA and EphB proteins, which was consistent with a lack of selectivity and a common mechanism to bind ephrins (Fig 37, E).[197]

Another example is brought by compound 76D10, which was characterized as an inhibitor of EphA/ephrin interaction with a better potency (4.4 µM) but with an irreversible mechanism. Moreover, the instability of 76D10 prompted Roberta Noberini *et al.*[195] to suppose that the degradation product of 76D10 was the true active compound. They were not able to identify this product but speculated about an oxidation mechanism (Fig 37, F).

Bainan Wu *et al.*[198] used a high-throughput screening by using a NMR approach to find inhibitors of the EphA4/ephrin-B interaction. From a library of fragments related to peptide structure, few weak inhibitors were identified. Afterwards, structural improvements through medicinal chemistry strategies led them to a potent and specific inhibitor of EphA4/ephrin-B interaction. Compound 22 is able to inhibit the interaction between EphA4 and ephrin-B with an IC_{50} of 3.7 µM and a good selectivity among Eph receptors. They speculated that a smaller molecule even less potent than compound 22 could be obtained by setting aside amino acid derivatives for more effective compound.[198]

All the studies dealing with EphA4/ephrin-B interaction represent a good overview of the strategy to find potent and selective PP2Is, from the characterization of the interaction (the first active peptides) and the difficult translation to small molecules by classical screening. The use of innovative methods, adapted to the identification of potent and active PP2I, was most of the time necessary.

(A)

KYL peptide

KYLPYWPVLSSL

K_D = 0.8 μM (ITC)

APY peptide

APYCVYRGSWSC

K_D = 1.5 μM (ITC)

VTM peptide

VTMEAINLAFPG

K_D = 4.7 μM (ITC)

(B)

(C)

Compound 1

K_D = 20 μM (ITC)

Compound 2

K_D = 26 μM (ITC)

(D)

EphA4/Compound 1   EphA4/Compound 2

(E)

Lithocholic acid (LCA)

Ki = 49 μM

(F)

76D10

IC_{50} = 2.6 µM

(G)

ephrin-B2: EFSPNLWGL

EphA4

Compound 22

K_D = 1.2 µM (ITC)
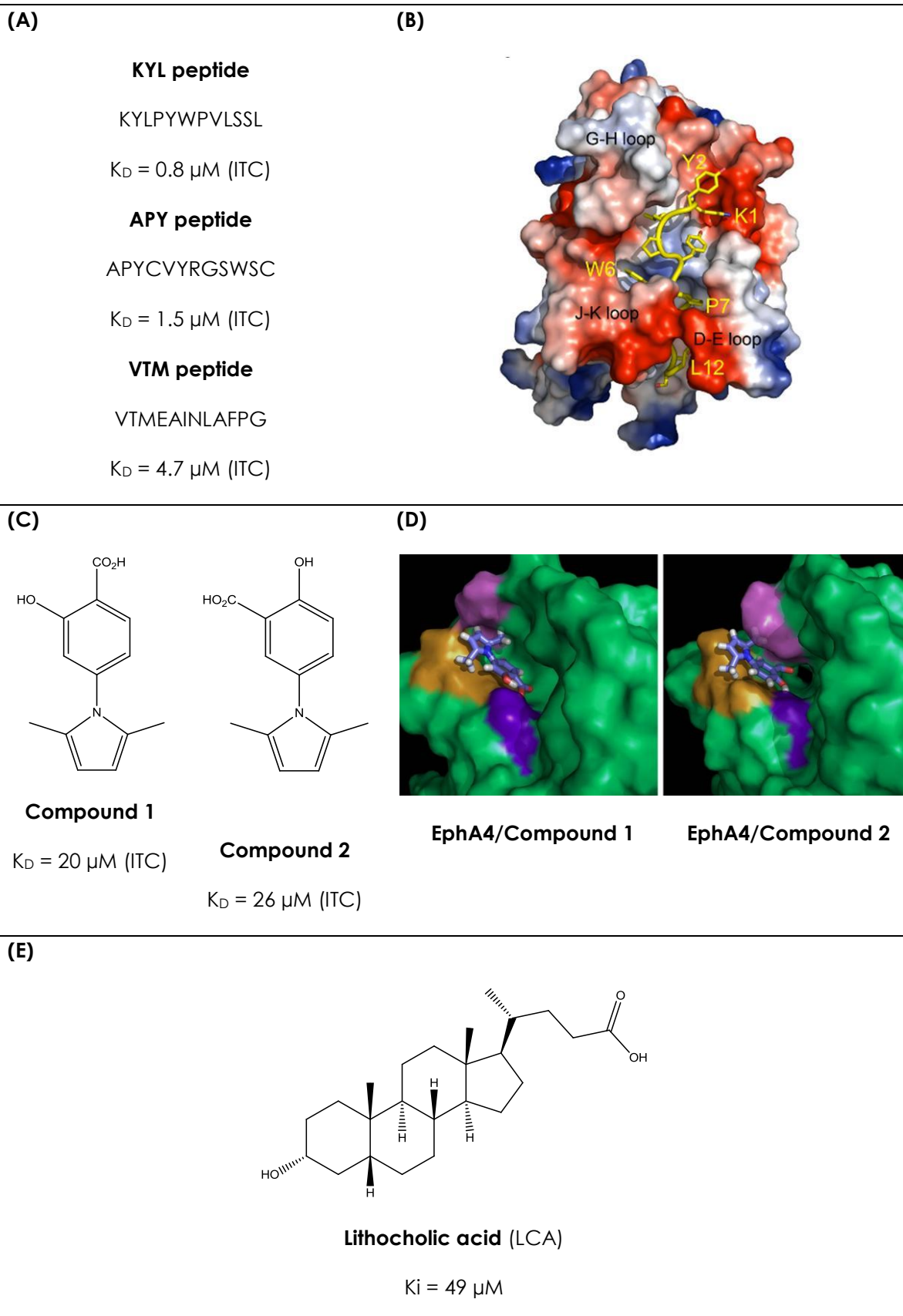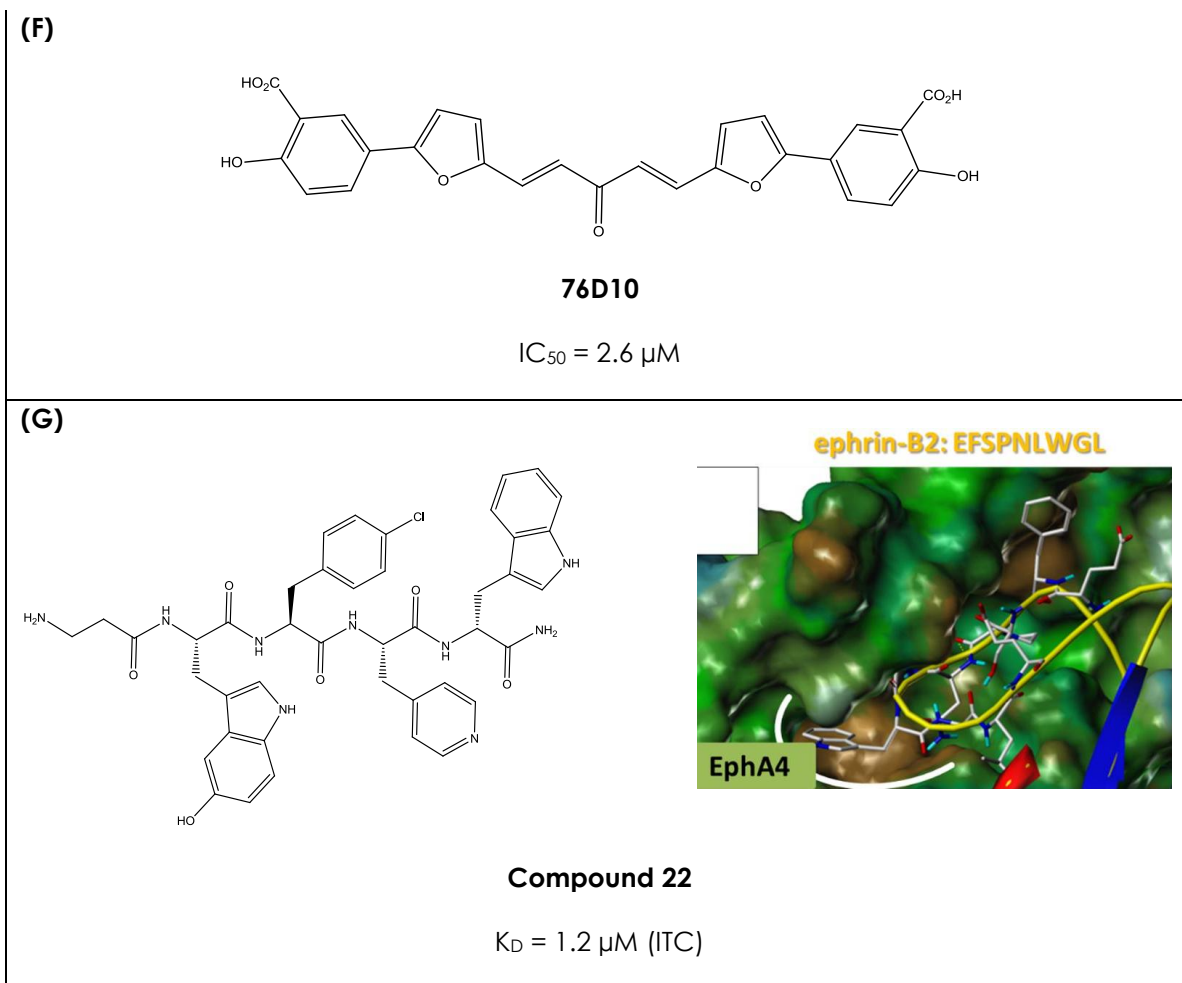
Fig 37: EphA4 interaction. (A) Amino acid sequences of the three peptides active on EphA4/ephrin-B interaction. (B) Docking model of KYL peptide in EphA4, based on NMR and mutagenesis experiments (From Lamberto, I. *et al.*[194], with the permission of Portland Press journals). (C) Chemical structures of two small molecule inhibitors of the EphA4/ephrin-B interaction. (D) Docking model of EphA4 binding site of the two small molecules (from (C)), based on NMR experiments. Residues Ile31 & Met32 in the D-E loop are represented in brown, Ile131 & Gly132 of the J-K loop are represented in violet and Gln43 in the β-strand is colored in blue (From Qin, H. *et al.*[196] with the permission of Portland Press journals). (E) Chemical structure of lithocholic acid, a moderate inhibitor of Eph/ephrin interaction. (F) Chemical structure of a putative irreversible inhibitor of EphA4/ephrin interaction. (G) Chemical structure of the peptide derivative 22, inhibitor of the EphA4/ephrin-B interaction and docking model of compound 22 and EphA4. Ephrin-B2 loop is represented by a yellow ribbon (From Barile, E. *et al.*[171], with the permission of ACS Publication).

XIAP antagonists target another loop-mediated interaction and exhibited interesting results. Inhibitors of Apoptosis Proteins (IAPs) are overexpressed in many cancers and they prevent apoptosis by caspase inhibition. XIAP antagonists could reverse caspases inhibition and induce apoptosis. Smac is a natural XIAP antagonist and the nature of the interaction between Smac and XIAP was determined by NMR.[199] Later, synthetic molecules with nanomolar inhibitory activities were obtained, based on tripeptide derivatives (Fig 38).[200] A combinatorial screening strategy based on tri-phenylurea scaffold was also used to successfully find cellular effective XIAP antagonists.[201]
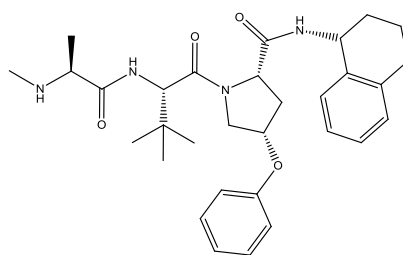
XIAP antagonist

$K_D = 5$ nM

Fig 38: Structure of tripeptide-based small molecule with XIAP antagonist properties.

Menin/MLL interaction is also a successful example to find potent inhibitors of loop-mediated interactions. Mixed Lineage Leukemia (MLL) fusion protein requires the interaction with Menin protein to promote oncogenic activity. Inhibitors of this interaction will induce apoptosis and block proliferation. Initial PP2I hits were found by HTS and then developed by medicinal chemistry optimization to lead to several compounds with sub-micromolar IC_{50} (Fig 39).[202]

MI-2
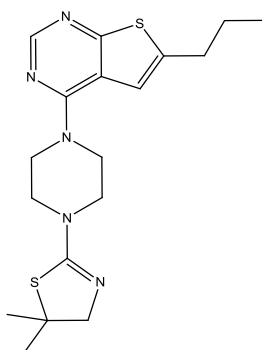
IC_{50} = 0.45 µM

LE = 0.35

Fig 39: Chemical structure of a small molecule inhibitor of Menin/MLL interaction.

Research firstly focused on α-helix mediated interaction and a lot of protein-protein interaction inhibitors which mimic α-helix have been identified.[175,178,188] However, a large proportion of protein-protein interaction is mediated by loops and only several examples of inhibitors targeting loop mediated interaction have been identified. Without any doubt, the number of PP2Is that interact with loop interactions will increase in the near future.

1.5.4 PP2I and CK2

As described in paragraph 1.3.2.1, CK2 is a multimeric protein, composed of a dimer of regulatory subunit (β subunit) that could bind two catalytic subunits (α and/or α' subunit). The regulatory subunit changes the substrate selection of CK2α. Moreover, *in vitro* experiments have shown that the regulatory subunit activates and stabilizes CK2α. Association and dissociation of the catalytic subunit to beta dimer (β₂) is an *in vivo* dynamic process but the driver of this phenomenon has not been identified yet.[203]

Interactions between CK2α and CK2β have been well characterized and already described in paragraph 1.3.2.4. The first crystal structure of CK2 holoenzyme (PDB ID: 1JWH) was published in 2001. The interface between α and β subunit was described as asymmetric, *id* the two α/β interactions in the holoenzyme are not identical, with an average interaction surface of

860Å².[28] More recently, a new structure of α/β interaction was resolved (PDB ID: 4DGL), showing a symmetric interaction with a surface about 1000Å² and a K_D of 4 nM.[48] The interaction is mediated by the interaction loop of the regulatory subunit which binds to the N-terminal lobe of CK2α. The main two hot spots of the interaction loop are Tyr188 and Phe190.[50] In the other side, the two corresponding hot spots of the α-subunit for β binding are Leu41 and Phe54 (Fig 40, A).[204] Despite the strong affinity between CK2α and CK2β, the fact that α/β complex is transient make it a good target for protein-protein interaction inhibitors design.

(A)

(B)

GCRLYGFKIHGCG
Cyclic Peptide (Pc)

Pc:	GCRLYGFKIHGCG	IC_{50} = 3 μM
Pc-R3A:	GCALYGFKIHGCG	IC_{50} = 16.5 μM
Pc-L4A:	GCRAYGFKIHGCG	IC_{50} = 22 μM
Pc-Y5A:	GCRLAGFKIHGCG	IC_{50} = 54 μM
Pc-G6A:	GCRLYAFKIHGCG	IC_{50}> 100 μM
Pc-F7A:	GCRLYGAKIHGCG	IC_{50}>> 100 μM
Pc-K8A:	GCRLYGFAIHGCG	IC_{50} = 6.5 μM
Pc-I9A:	GCRLYGFKAHGCG	IC_{50} = 85 μM
Pc-H10A:	GCRLYGFKIAGCG	IC_{50} = 15 μM

(C)

(D)   **W16**

(E)

DRB

(F)

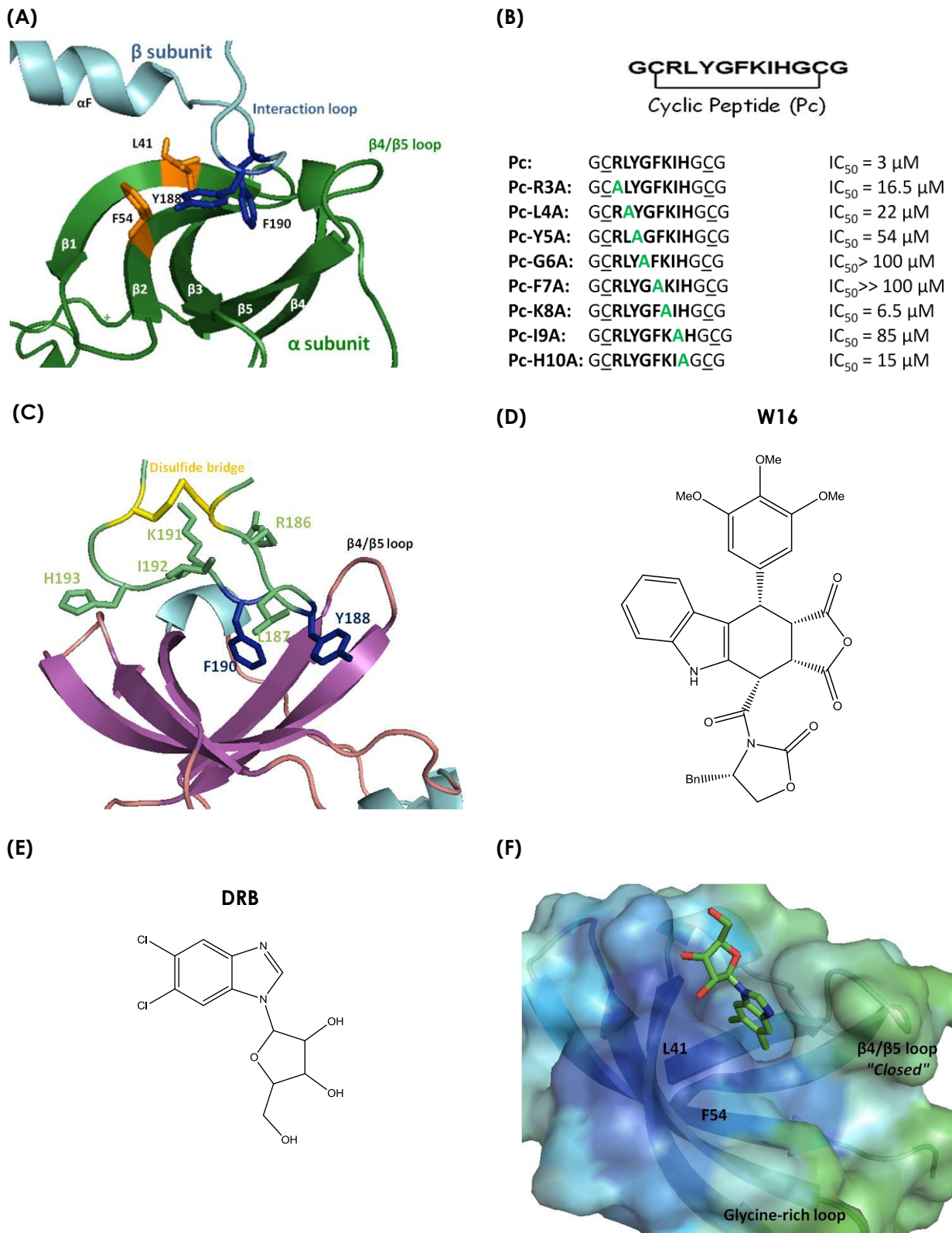**Fig 40: Modulators of CK2α/β interaction. (A) Structural overview of the α/β interface. CK2α is represented in green and the two hot spots (L41 and F54) in orange. CK2β is colored in blue and**

In 2007, Béatrice Laudet *et al.*[50] described a small cyclic peptide derived from a loop localized in the C-terminal part of CK2β. This peptide competes with CK2β to bind on CK2α. This small peptide, called Pc, was designed after mutagenesis experiments that revealed the key residues (Tyr188 and Phe190) involved in α/β interaction. These two hot spots are located at the extremity of a 90° β-hairpin loop formed by residues $R^{186}LYGFKIH^{193}$: Pc mimics this sequence. Then, glycine and cysteine residues were added to cyclize the peptide, and by this way, to lock the peptide conformation. The resulting cyclic peptide inhibited the phosphorylation of CK2β-dependent peptide substrate with an IC_{50} of 3 µM whereas no inhibition was observed on CK2β-independent substrates. Moreover, Pc is able to disrupt the $CK2\alpha_2\beta_2$ complex with the same potency. Alanine scanning mutagenesis experiments were conducted with Pc and confirmed the crucial role of YGF motif, which binds deeper inside the α/β pocket (Fig 40, B).[50] In 2013, Jennifer Raaf *et al.* were able to co-crystallize CK2α and Pc (PDB ID: 4IB5). The structure confirmed the expected binding mode and underlined the importance of the YGF motif in Pc binding.[51] Recently, during the development of an HTS assay to identify inhibitor of α/β interaction, the Pc peptide was improved. Based on docking experiments, central phenylalanine was replaced by *para*-iodo-phenyl alanine and the K_D of the novel I-Pc was improved: 0.24 µM, measured by ITC, instead of 0.56 µM for the initial Pc.[205]

In 2008, two small molecules were published as α/β interaction modulators. Béatrice Laudet *et al.* published indolo analogs of podophyllotoxine, also named the W family of compounds, with the W16 as the hit compound. This molecule was described as an α/β interaction inhibitor but CK2α is inhibited by W16 with the same potency, probably by protein aggregation. In consequence, this compound was not suitable for further optimizations (Fig 40, D).[206] In 2008, Jennifer Raaf *et al.*[207] published the first crystal structure of CK2α with a small molecule in the α/β interaction pocket (also called remote pocket). In attempts to crystallize CK2α and 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB, Fig 40, F) known as CK2α

inhibitor, they obtained a crystallographic structure with two molecules of DRB: one in the ATP pocket and one in the α/β interaction pocket (Fig 40, F). Biochemical assays have confirmed the dual binding mode of DRB but despite the binding of DRB in the α/β interaction pocket, no significant effect on the association or dissociation of the holoenzyme was observed in presence of DRB. This lack of efficiency is probably due to the weak affinity of DRB for CK2α (Fig 40, E, F).[207]

Several groups have put a lot of efforts to find a cellular efficient inhibitor of CK2α/CK2β interaction that will enable a deeper understanding of the role of this interaction in living organism. Nonetheless, such an inhibitor has not been published yet.

2. Aim of the thesis

Cancer cell growth relies on activated oncogenes as well as on deregulated non-oncogenes.[208] The Protein Kinase CK2 is one of the deregulated non-oncogenic proteins which supports cancer development through activation of pro-survival, anti-apoptotic and proliferative signals (See Introduction 1.3.3.1-2. for further details).[61,66] CK2 is an ubiquitous Ser/Thr protein kinase, composed of a dimer of regulatory subunit (β) and two catalytic subunits (α and/or α').[28] The catalytic subunit of CK2 is constitutively active, while the regulatory subunit modulates the selectivity toward a subset of substrate proteins.[77] The central position of CK2 in the control of various cell signaling pathways requires a fine internal control of CK2 activity. However, little is known about the cellular regulation of CK2 and the role of the regulatory subunit in the substrate selection. Additionally, previous studies promoted the interest of targeting CK2 to counteract cancer development, and one ATP-competitive inhibitor is currently in clinical trial, CX-4945, (5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid, silmitasertib).[209] Nevertheless, off-targets and resistances to CX-4945 have been recently revealed (see Introduction 1.3.4.1-2. for further details).[99]

In contrast to ATP-competitive inhibitors, only few small molecules were reported to target allosteric binding sites on the catalytic domain of kinases, which can be explained by the fact that these allosteric inhibitors exploit unique binding sites and isoform–specific regulatory mechanisms.[210] Besides the fact that allosteric kinase inhibitors usually present higher selectivity, these molecules do not compete with the high cellular ATP concentration. Indeed, for kinases with relatively high ATP binding affinities (Km = 1–20 µM), it could be difficult to develop ATP-competitive inhibitors, which could be selective and, at the same time, potent enough to exhibit cellular activity.[211]

The aim of this work was:

A) to develop novel non ATP-competitive inhibitors of CK2. Two distinct hit compounds from previous *in silico* screening campaigns (Fig 41) had to be optimized using

different medicinal chemistry approaches. Series of analogs had to be designed, also supported by molecular docking. Finally the inhibitory activity and binding affinity toward CK2 had to be determined;

Class 1

Allosteric modulators of CK2α

Class 2

Protein –protein interaction inhibitors (PP2Is) targeting the CK2α/CK2β interaction
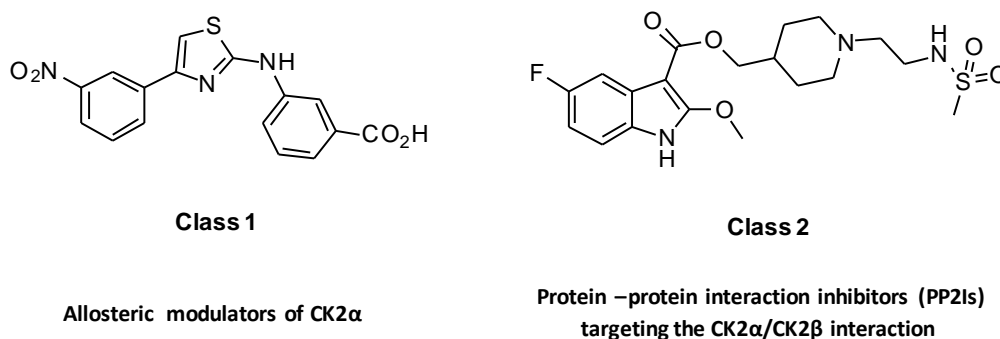
Fig 41: Class 1 and class 2 hit compounds from previous *in silico*-screening campaigns.

B) to confirm and further investigate the mechanism of action of the optimized non-ATP competitive inhibitors. Based on the original hits, two distinct chemical classes (1 & 2) of compounds had been developed in part A, and were supposed to modulate CK2 activity by different mechanisms. The following modes of action were postulated and had to be investigated using diverse cell-free and cell-based assays:

i. **Allosteric modulators of CK2a** could overcome the selectivity issues observed in the case of CX-4945 and we believed that they could exert a better *in vivo* potency than ATP-competitive inhibitors. It was hypothesized that compounds deriving from class 1 (Fig 41) might bind to a novel allosteric site, outside the ATP binding pocket and could lock the "always active" CK2α conformation into an inactive state. The goal of our study was to verify the allosteric mechanisms of our optimized inhibitors and eventually identify the binding site using a combination of single-alanine mutagenesis, temperature-dependent circular dichroism, saturation-transfer difference NMR experiments and molecular

modeling. Finally, consequences of the novel mechanism of action on the regulation of CK2 in cells were to be investigated.

ii. **Protein–protein interaction inhibitors (PP2Is) targeting the CK2α/CK2β interaction**: compounds deriving from class 2 (Fig 41) were supposed to block the interaction between the regulatory subunit (β) and the catalytic subunit (α) because the β binding pocket had been used as a template for the *in silico* screening from which the class 2 secondary hit was identified. Since the α/β subunit interaction is dynamic[212], optimized derivatives of this compound might be useful to obtain a deeper understanding of the mechanisms that control this interaction and their physiological consequences in cells and whole organisms. The goal was to synthesize a small library of class 2 analogs and to evaluate their activity in biochemical and cell-based assays. Another objective was the characterization of the effects of optimized compounds. Moreover, a complementary study of the CK2 activity in cells of a previously described cyclic peptide was planned, in order to assess whether the novel and unique approach of modulating CK2 by small PP2Is could have potential therapeutic applications.

3. Results

3.1 Modulation of protein kinase CK2 activity in cells through novel non-ATP competitive inhibitors

CK2 is an ubiquitous Ser/Thr protein kinase involved in the control of various signaling pathways and is thought to be constitutively active. In the present study, we identified a novel class of CK2 inhibitors, which displayed a non ATP-competitive mode of action and stabilized an inactive conformation of CK2 in solution. Single mutation scanning, CD spectrometry and STD-NMR experiments demonstrated that the compounds bind in an allosteric pocket outside the ATP-binding site. Our data strongly suggested that the putative binding site was located at the interface between the aC helix and the flexible glycine-rich loop. The optimized compound **7** showed a micromolar potency to reduce CK2 substrate phosphorylation both in enzymatic assays and in cell–based experiments. Furthermore, a substrate–dependent CK2 inhibition as well as modulation of the CK2a subcellular localization was observed upon compound **7** treatment. Thus, compound **7** is a novel tool to investigate conformation–dependent mechanisms of CK2 regulation in cells and may be further developed into anti-cancer drugs exploiting a novel mode of CK2 inhibition.

3.1.1 Scientific rationale

The protein kinase CK2 has an exceptional position within the human kinome due to the significant proportion of the cellular phosphoproteome that can be attributed to this kinase.[27] This ubiquitously expressed protein kinase is involved in the activation of several pro-oncogenic pathways that are critical for cell proliferation, differentiation and survival.[213] Numerous reports underlined the relationship between CK2 overexpression and poor survival rates in lung carcinoma, prostate cancer, acute myeloid leukemia, breast carcinoma, colorectal tumors, glioblastomas and gastric carcinoma.[67] The crucial role of CK2 as a cancer driver makes it an interesting target in cancer therapy, and CK2 inhibition proved to be an effective method to induce tumor regression.[92]

CK2 is a tetrameric enzyme composed of a dimer of regulatory subunits (β) and two catalytic subunits, CK2a and/or CK2a'.[28] The catalytic subunit of CK2 is considered as constitutively active[30] and can phosphorylate more than 300 substrates[27] while the regulatory subunit modulates the selectivity toward a subset of substrate proteins.[77]

Most of the known CK2 inhibitors bind to the ATP pocket and show an ATP-competitive behavior in enzymatic assays[91] CX-4945 (5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-

carboxylic acid, silmitasertib) is one of the most potent CK2 inhibitors (IC_{50} = 14 nM) and is currently in phase II clinical trials.[209] X-ray crystallographic analysis revealed that CX-4945 binds to the ATP-binding pocket of CK2a in the active conformation.[214] Although CX-4945 is rather selective, it was recently reported to be actually more potent against Clk (Cdc2-like kinase) 2 (IC_{50} = 4 nM), a kinase involved in the regulation of alternative splicing.[99] This recent result underlines the fact that potent and selective CK2a inhibitors are difficult to obtain through targeting the ATP binding pocket. Therefore, research efforts are spent to identify inhibitors that exploit specific regulatory mechanism to suppress the catalytic activity of the kinase. Besides the potential pharmacological benefit, such allosteric ligands are often invaluable tools to investigate the underlying allosteric mechanism that allows the catalytic domain to switch between the active and the inactive state. It was previously reported that azonaphthalene dye–derived compounds inhibit the catalytic activity of CK2a in a non-ATP-competitive manner, however, the exact binding site and a potential mechanism of inhibition remained elusive [150]. Besides, inorganic PolyOxoMetalate complexes (POMs), e. g., $[P_2Mo_{18}O_{62}]^{6-}$, were described as potent allosteric inhibitors of CK2a, exhibiting nanomolar IC_{50}s and great selectivity in a panel of 29 kinases.[151] However, POMs lack cellular activity and there is little perspective to develop them further as *in vivo* active agents.

In the present study, we describe the identification of a novel, cellularly active class of non ATP-competitive CK2 inhibitors. Using different methodologies, we provide evidence that the compounds target an alternative binding pocket distinct from the ATP binding site.

3.1.2 Hit compound 1 is a non-ATP competitive inhibitor of CK2

1
$IC_{50} = 7.0 \ \mu M$
$K_i{}^a = 1.6 \ \mu M$

2
9 (±7) %

3
27 (±10) %

4
0 (±6) %

5
$IC_{50} = 9.0 \ \mu M$
$K_i{}^a = 1.1 \ \mu M$

6
61 (±3) %

7
$IC_{50} = 3.4 \ \mu M$
$K_i{}^a = 0.7 \ \mu M$
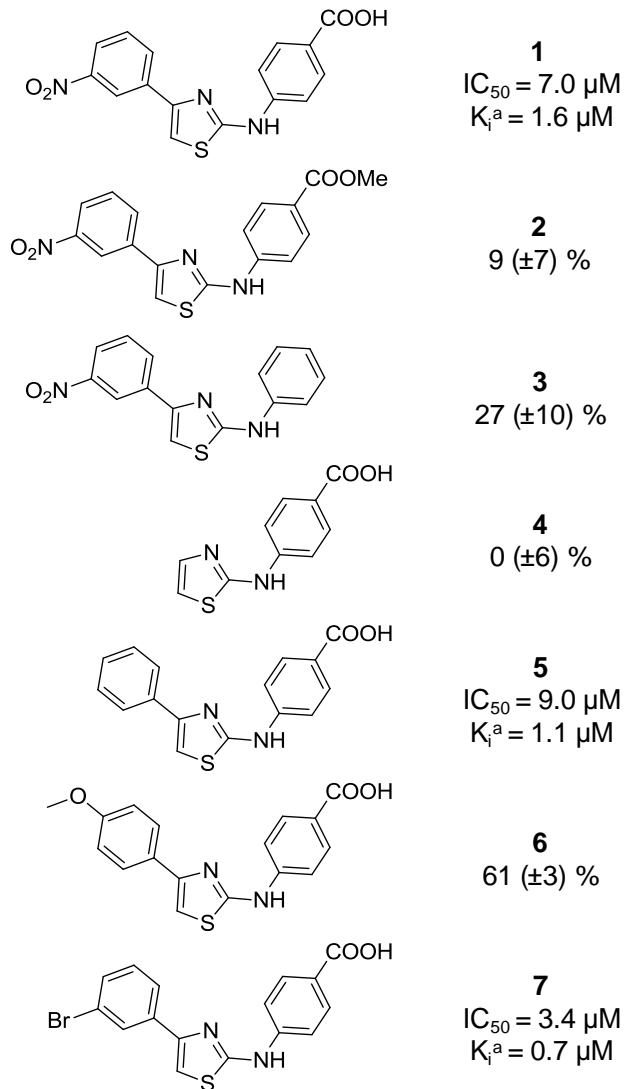
Figure 1. Chemical structures of compounds **1-7**. IC_{50} (S.D. < 20 %) and K_i are depicted for compound **1**, **5** and **7**. For the others, CK2a inhibition percentages in presence of 10 μM of respective compound are depicted with S.D. values given in brackets. ^{a}K_i values were determined from graphical plotting of the Lineweaver-Burk plot slopes.

Compound **1** (Figure 1) was identified as a part of a Virtual Ligand Screening (VLS) campaign against non-ATP binding sites on CK2.[215,216] Michaelis-Menten kinetic experiments were performed on top ranking VLS hits in order to confirm the non-ATP-competitive mechanism of their action. Despite its relatively weak potency ($IC_{50} = 7$ μM on CK2a), this evaluation sparked

our interest in compound **1** because it exhibited an unusual mixed-type mechanism of inhibition (Figure 2A) and a constant potency in the presence of increasing ATP concentrations (Figure S1A). Moreover, raising the concentration of the peptide substrate did not impair CK2 inhibition by **1**, ruling out that the compound competed with substrate binding (Figure S1B). Furthermore, monomeric CK2a showed the same sensitivity toward inhibition by compound **1** as the preformed tetrameric complex CK2$a_2\beta_2$ (Figure 2B). In addition, CK2 inhibition by compound **1** was not affected by increasing concentrations of CK2β (Figure 2C). These results clearly demonstrated that the regulatory β-subunit did not influence the inhibition by compound **1**, suggesting that **1** does not bind in the a/β interaction pocket on the a-subunit. Next, the selectivity of **1** was evaluated against a panel of 44 selected kinases. The selectivity profile was encouraging: besides CK2a, only three other kinases from the panel were inhibited by more than 50 % in the presence of 50 µM of compound **1**: EGFR (74 % inhibition), EphA4 (55 %) and Pim-1 (54 %). Two additional kinases, MuSK (38 %) and PDGFRa (35 %) were inhibited by more than 30 % (Table S1).
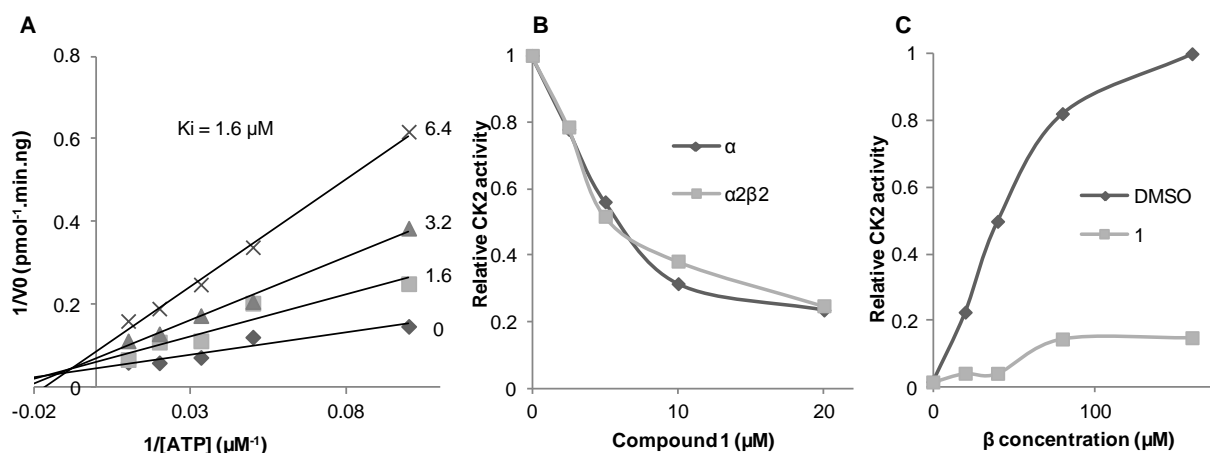
Figure 2. Analysis of the mode of inhibition of compound **1**. **A.** Lineweaver-Burk inhibition plot of human recombinant CK2a by compound **1** at various ATP concentrations. The K_i was determined by plotting the slopes at varying inhibitor concentrations. **B.** CK2a and CK2$a_2\beta_2$ activity in the presence of increasing concentrations of compound **1**. **C.** Effect of increasing CK2β concentrations on the inhibition of fixed amounts of CK2a by compound **1** (20 µM).

3.1.3 Structural requirements of the new inhibitors for CK2α affinity

Based on these promising results, a small set of compound **1** derivatives was synthesized (Scheme S1) to establish preliminary structure–activity–relationships (SAR) and possibly optimize the potency toward CK2α. The compound structures are depicted in Figure 1. Conversion of the carboxylic acid function in compound **1** to an ester, or deletion of it, resulted in a strong decrease in affinity for CK2α (Figure 1, compounds **2** and **3**), suggesting that the binding involved ionic interactions with a basic residue. The presence of a phenyl group at position 4 of the aminothiazole core was also found to be crucial, as indicated by the complete lack of activity of the truncated analog **4** (Figure 1). While modulation of the electron density of this 4-phenyl did not change the inhibitory activity compared with that of the unsubstituted congener **5** (cf. **1, 2** and **6** in Figure 1), increasing the lipophilicity by a bromo-substituent significantly enhanced the affinity to reach the sub-micromolar range (K_i = 0.7 µM, compound **7**, Figure 1). Thus, the SAR obtained with the 4-phenyl ring suggested that binding of this moiety to CK2α was driven by hydrophobic interaction with a lipophilic pocket (Figure 1).

The selectivity of the most potent compound **7** was then evaluated against a panel of 32 selected kinases, specifically enriched by closely related kinases from the CMGC family but also including kinases from other branches of the kinome (Table S2). In addition to CK2a, six others kinases were inhibited by more than 60 % in the presence of 50 µM of compound **7** EphA4 (73 %), GSK3β (71 %), CK1γ1 (69 %), ACVR1 (66 %), Clk4 (61 %) and MLCK (61 %). Notably, Clk2, the main off-target of the ATP-competitive compound CX-4945, was inhibited by only 33 %.

3.1.4 Identification of a putative allosteric pocket

Enzyme kinetic experiments (Figure 2 and Figure S1) suggested an alternative mechanism of action relying on a potential binding site outside the ATP pocket. To find a clue about the potential location of an alternative binding pocket for small molecules, we analyzed the available CK2a crystal structures. A study by Niefind *et al.*[31] suggested that the glycine-rich loop is one of the most variable regions in X-ray structures of CK2a, suggesting a putative flexibility of this loop in solution. This feature was also supported by metadynamics studies simulating conformational transitions [217] and by recent structural studies.[218] In most CK2a crystal structures, the glycine-rich loop conformation is maintained by two hydrogen bonds linking the Tyr50 of this loop and two lysine residues (Lys74, Lys77) of the basic cluster (compare e.g., PBD entries 3PE1 and 3Q9Z). In order to find alternative conformations of the glycine-rich loop, we analyzed all 72

crystal structures (and individual chains within these structures) of the human enzyme available in the PDB as of November 2014. Two crystal structures (PDB entries: 3FWQ and 3JUH) were identified, in which the key hydrogen bonds fixing Tyr50 are lacking. In consequence, Tyr50 has fully collapsed in the ATP binding site of the 3FWQ structure.[149] The second structure (PDB code: 3JUH), also presents Tyr50 turned away from its usual location to an intermediate position toward the ATP binding site (Figure 3A).[33] Thus, it can be assumed that CK2 exists in conformational states that allow a movement of the glycine-rich loop. Further examination of the X-ray structures in PDB entries 3FWQ and 3JUH revealed a hydrophobic pocket, large enough to accommodate a small molecule, which, in the active conformation, is partially occluded by Tyr50 and the glycine-rich loop (Figure 3B). This putative allosteric pocket was located between the glycine-rich loop and the αC-helix, delimited by residues Tyr50, Lys71, Val73, Lys74 and Lys77. To investigate if this region might be targeted by our compounds, nine GST-CK2α single-point alanine mutants covering this area were tested in enzymatic assays in the presence of a fixed concentration of **7**. All CK2α mutants displayed reasonable catalytic activity, which was at least 30 % of that of the wild type. Three mutants (Lys74Ala, Lys77Ala and His160Ala) were found to be more resistant to inhibition by compound **7** than the wild type, and therefore selected for dose–response experiments. These studies showed that the IC_{50} values of compound **7** were significantly increased for the Lys74Ala (1.8-fold), the Lys77Ala (3.2-fold), and the His160Ala mutant (2.3-fold) (Figure 4). It is reasonable to assume that the lysine residues interact with the carboxylate of **7**, and that in each lysine mutant, the remaining nearby lysine residue can compensate in part for the loss of electrostatic interactions, thus attenuating the drop in binding affinity. Taken together, our results from enzyme inhibition kinetics, site–directed mutagenesis and SAR analysis were consistent with the binding of our new compounds to an allosteric site, composed of a carboxylate coordination site formed by the basic cluster containing Lys74/77, and an adjacent hydrophobic cavity which accommodates the 4-phenyl moiety. As suggested by the 3D structures (PDB entries 3JUH and 3FWQ, see above), the hydrophobic pocket was most likely located between the αC helix, 3rd β-strand of the N-terminal lobe, the activation loop and the glycine-rich loop of the kinase (Figure 3).
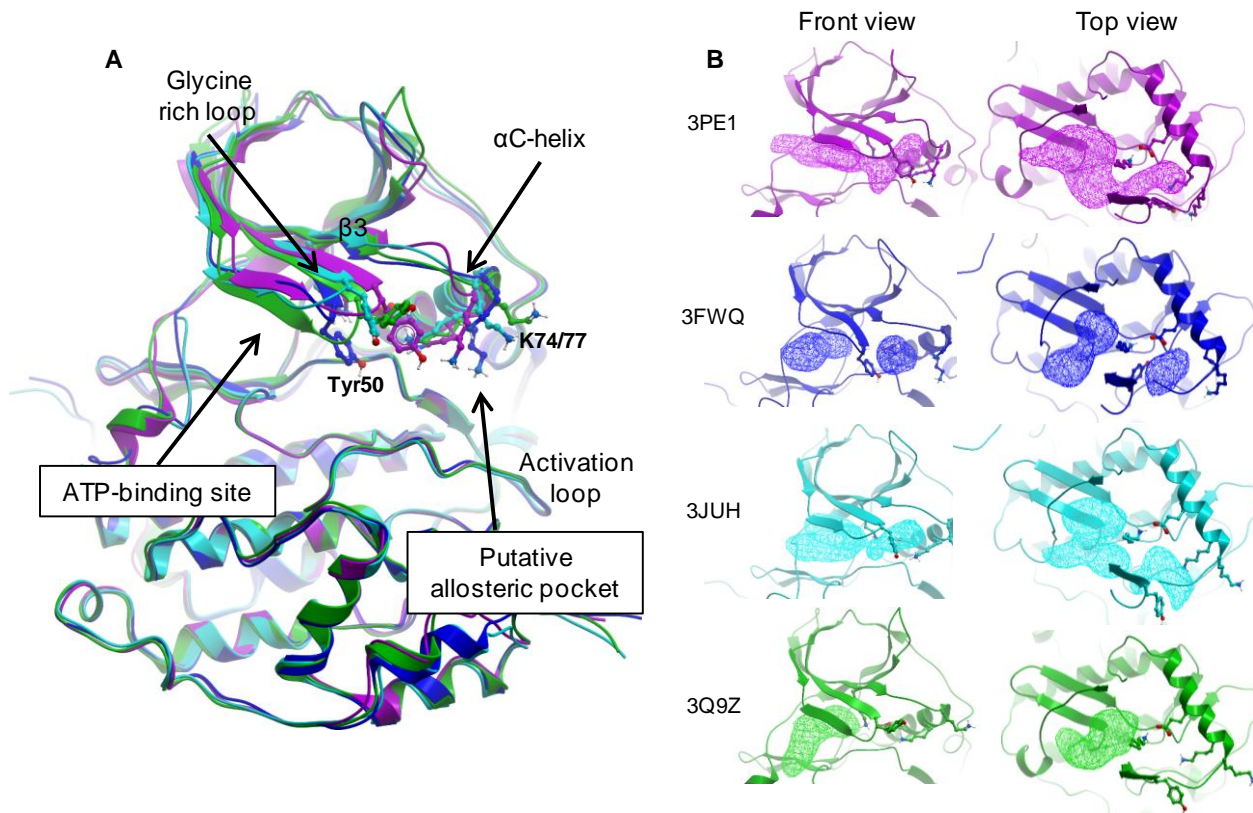
Figure 3. A. Superposition of four CK2α structures (3PE, magenta; 3FWQ, blue; 3JUH, cyan; 3Q9Z; green). The ATP-binding site is occupied by CX-4945 in 3PE1 and by AMPPNP in 3JUH and 3Q9Z (not shown for clarity). **B.** A separated view from front or top of each structure is depicted. The pockets were identified using ICM pocket finder and represented with a mesh surface. The putative allosteric pocket is partially available in 3PE1, fully accessible in 3FWQ and 3JUH but completely absent in the conformation crystallized in 3Q9Z.

The chart shows "CK2 inhibition (%) by 7 (4 µM)" with y-axis from 0% to 90%.

	WT	Y26A	K71A	V73A	K74A	K76A	K77A	I78A	H160A	E180A
IC_{50} (µM)	3.4	nd	nd	4.0	6.0	nd	10.9	nd	7.7	nd
IC_{50} fold increase	1			1.2	1.8		3.2		2.2	

Figure 4. Percentage of inhibition of GST-CK2α wild-type and single alanine mutants in the presence of compound **7** (4 µM). IC_{50}s were determined for GST-CK2a wild-type and mutants with reduced sensitivity toward inhibition (S.D. < 20 %). The fold increase in the respective IC_{50} over that of the wild-type is indicated at the bottom. nd: not determined.

3.1.5   Compounds 5 and 7 induce a thermal destabilization of CK2a

Binding of the allosteric inhibitor was expected to stabilize a conformation in which the interactions between Tyr50 and Lys74/77 do no longer link the glycine-rich loop with the aC-helix, thereby leading to an increase in the glycine-rich loop flexibility. Hence we hypothesized that the inactive conformation stabilized by our inhibitors might be less protected against thermal denaturation than the apo enzyme or the complex with ATP binding site–directed compounds. Indeed, using CD spectroscopy to monitor the transition to the unfolded state, w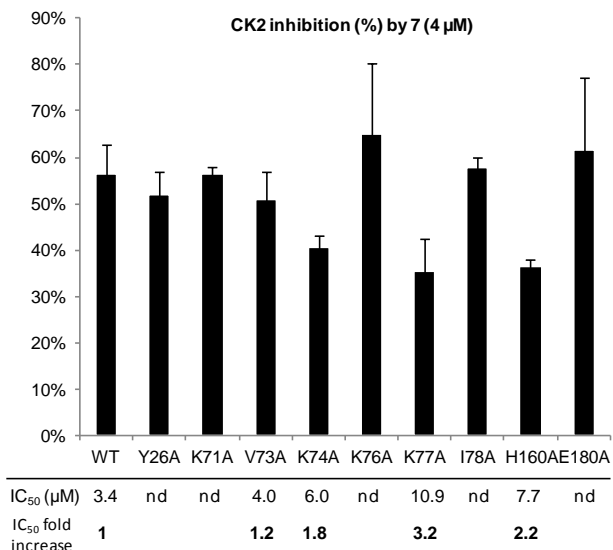e observed a clear reduction of the melting temperature (Tm) in the presence of compounds **5** or **7** compared with the enzyme alone (Table 1). In contrast, the ATP-competitive reference compound CX-4945 stabilized the protein structure, resulting in an increased Tm. An enhancement of the thermal stability was consistently reported for small ligands targeting the ATP binding site, comprising the classical ATP-competitive inhibitors but also the so-called type II inhibitors, which induce a local conformational change involving the DFG-motif – although not many thermal shift assay data are available in the latter case.[219,220] Hence, the differential effect of our compounds was a further hint that they were not directed to the ATP-binding site.

Table 1. Unfolding temperatures of CK2α in the presence of compound **5**, **7** or CX-4945 as monitored by TdCD.

Sample	Tm (°C)
CK2α	53.2 (± 0.3)
CK2α + **5** (50 µM)	50.3 (± 0.3)
CK2α + **7** (6.25 µM)[a]	52.0 (± 0.2)
CK2α + CX-4945 (6.25 µM)[a]	55.4 (± 0.2)

The midpoint unfolding temperatures (Tm) shown are averages of three independent experiments (± S.D.). The CK2α concentration was 5 µM. [a]Concentrations were limited by the compound solubility in methanol.

3.1.6 The binding sites of compound 5 and the ATP-competitive inhibitor CX-4945 do not overlap

To provide further evidence for a binding of our compounds to an alternative, allosteric pocket, we performed binding NMR experiments, which permit to analyze binding events in solution. Compound **5** was chosen for Saturation Transfer Difference (STD) experiments[221] due to its good solubility in water. Competition experiments between compounds **5** and CX-4945 revealed that they could bind simultaneously to CK2α, proving that they were addressing two distinct binding sites (Figure 5A). By comparison, further STD experiments showed that the 7-azaindole, a fragment substructure known to bind the ATP-site of kinases, did not interact with CK2α in the presence of CX-4945 (Supplementary Figure S2).

STD experiments can also discriminate protons of the inhibitor buried in the protein structure from protons of the compound exposed to the solvent, permitting the so-called STD–based epitope mapping.[221] Hence, STD factors were calculated for the different protons of compound **5**. Clearly, the benzoic acid moiety was found to be exposed to the solvent, as indicated by weak STD factors, while the remaining proton of the thiazole and the proton at position 10 of the adjacent phenyl ring displayed larger STD factors, indicating that these molecule parts were fully buried in the CK2α structure (Figure 5B-C).
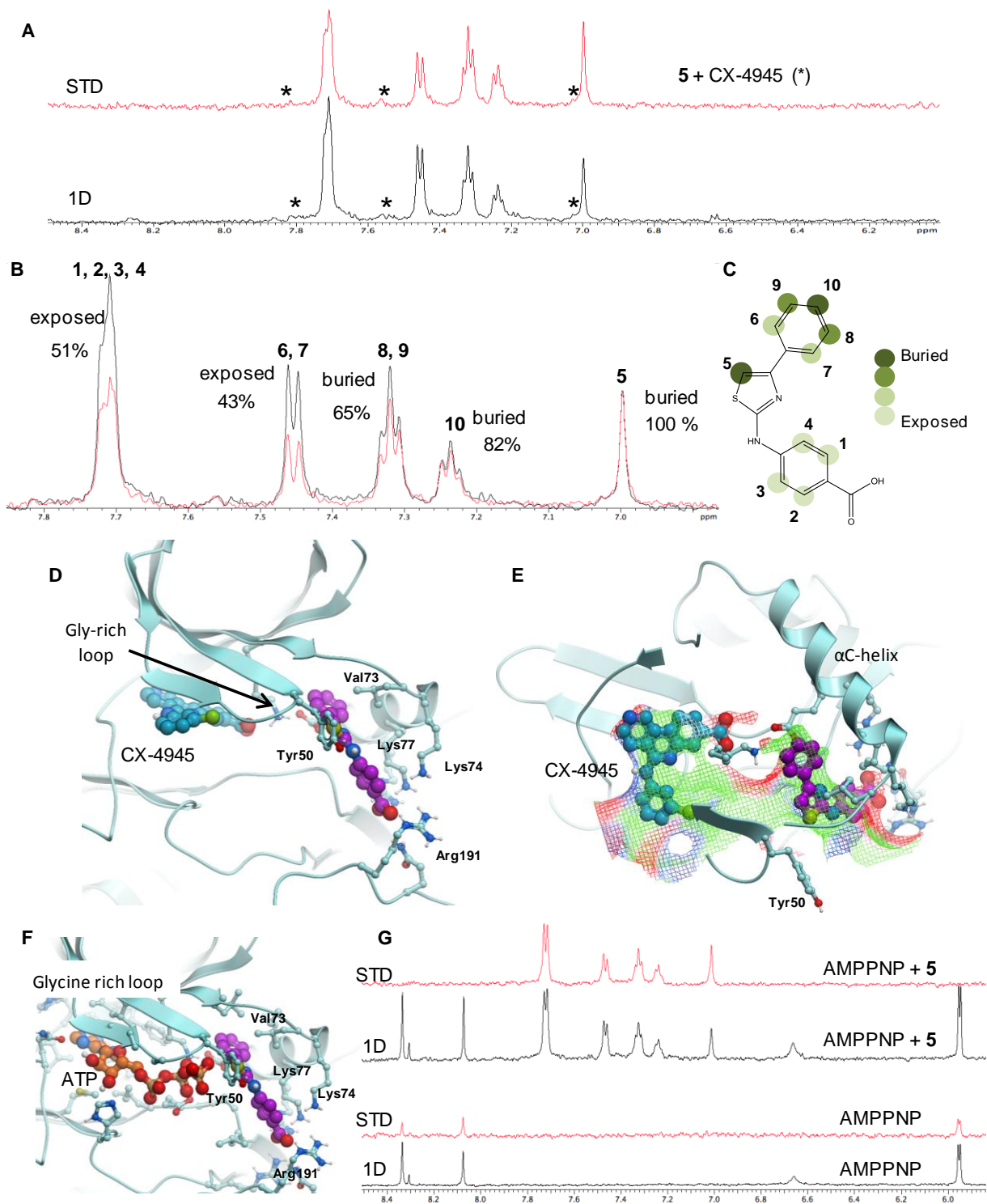
Figure 5. STD–based evidence for the simultaneous binding of compounds **5** and CX-4945, epitope mapping for compound **5** and correlation with the docking model. **A.** 1D (black) and STD (red) NMR experiments of compound **5** (500 μM, approx. 700 x K_i) recorded in the presence of the kinase inhibitor CX-4945 (100 μM, approx. 10^5 x K_i) with 5 μM CK2α, showing that CX-4945

does not displace compound **5** binding. **B.** The STD factors measured for compound **5** upon binding to CK2α are indicated for the different protons as observed in the ¹H-NMR spectrum. **C.** Representation of the STD factors with a green scale on the chemical structure of **5**. **D.** Double docking poses of compound **5** (magenta, in the putative allosteric pocket) and CX-4945 (blue, in the ATP-binding pocket). Residues interacting with compound **5** are depicted and named, except for Arg80, which is hidden by compound **5**. **E.** Top view of the double docking poses of compound **5** and CX-4945. The mesh represents the so-called Lee-Richards surface [222]: the imaginary surface where ligand atom centers should be placed for optimal interactions with the receptor; it is colored by preferred ligand atom properties, with green, white, red, and blue standing for aliphatic, aromatic, H-bond acceptor and H-bond donor, respectively. **F.** 3D docking model of ATP and **5** simultaneously bound to CK2α (PDB: 1JUH). The proximity between ligands is energetically unfavorable. Accordingly, the docking score for compound **5** in the presence of bound ATP (-28.64 ICM score units) was much less favorable than its score in the absence of ATP (-37.42 ICM score units). **G.** Binding of AMP-PNP to CK2α. 1D (black) and STD (red) NMR experiments of AMP-PNP (500 µM) is shown in the absence and in the presence of compound **5** (500 µM) with 5 µM CK2α. The binding of compound **5** prevents the interaction of AMP-PNP with CK2α.

3.1.7   *In silico* prediction of the binding mode

To obtain more detailed insight in the binding mode of the CK2 inhibitors, we performed docking simulations using the coordinates of PBD entries 3JUH and 3FWQ. The backbone conformation of the CK2α structure in 3JUH[33] was found to be most compatible with the proposed mode of interaction. Hence, the final docking model was prepared from this structure by reverting Val66Ala and Met163Leu mutations in the ATP binding pocket of the crystal structure back to their wild-type amino-acids, followed by a conformational refinement of the side chains near the proposed pocket in the presence of the docked compounds.

Compounds **1**–**7** were docked into this final model, alone and in combination with either ATP or CX-4945. A representative docking pose for compound **5** is depicted in Figure 5D-E. Interestingly, the predicted binding modes of all compounds **1**–**7** did not differ between the apo- and CX-4945–bound CK2α. In all cases, basic residues in the αC-helix (Lys74, Lys77 and Arg80) and the activation segment (Arg191) were found to coordinate the carboxyl–substituted phenyl ring of the compounds *via* a network of favorable hydrogen bonds and salt bridges. The overall binding model was consistent with the SAR and the pattern of exposed/buried protons as determined by the STD-NMR (cf. Figure 5B-C). Furthermore, the decreased sensitivity of the K74A

and K77A mutants toward the inhibitor (Figure 4) was in full accordance with a role for the αC-helix and the activation segment in binding the carboxylate; however, the decreased sensitivity of the H160A mutant could not be explained by a direct contact with the inhibitor. It is conceivable that, in this mutant, the formation of the allosteric pocket might be impeded, thus increasing the energetic barrier for inhibition by our compound.

While the conformation of the protein structure model based on PDB entry 3JUH was compatible with a simultaneous docking of CX-4945 and either of the compounds **1-7**, docking of ATP was only successful with the apo-model; when attempted in the presence of either of the docked compounds **1-7**, a minor steric clash occurred with the terminal phosphate group of ATP (Figure 5F). The latter was consistent with the prevention of AMP-PNP binding in the presence of **5** as observed in the STD-NMR (Figure 5G).

3.1.8   Compound 7 retains its cell-free potency in cell–based CK2 inhibition assays

As mentioned above, dysregulated CK2 is considered as pro-oncogenic and was validated as a pharmaceutical target for anti-cancer therapies [66]. Specifically, the importance of CK2 activity for the development of clear-cell renal cell carcinomas (ccRCCs) has been described recently [60]. Therefore, the ccRCC cell line 786-O was chosen as an *in vitro* model to evaluate the anti-cancer activity of compounds **1-7.** In the cytotoxicity assay, all of the compounds affected the cell viability after a 24 h treatment. The most potent inhibitor **7** also effected the strongest reduction in 786-O cell viability (EC_{50}: 25 µM). This cellular efficacy was remarkable since in the same assay, the canonical ATP-competitive inhibitor CX-4945 exhibited an EC_{50} of 6.1 µM, reflecting only a 4-fold higher activity than **7** (Figure S3), in spite of its much higher cell-free potency (IC_{50}: 5 nM).

Next, we performed Western Blot analyses to investigate whether CK2α activity was indeed inhibited by our compounds in living cells. CK2 is responsible for the phosphorylation of a-catenin on residue Ser641,[223] and Akt1 on residue Ser129.[224] Therefore, these two phospho-acceptor sites were chosen as selective reporters of CK2 cellular activity. After a 24 h treatment of 786-O renal carcinoma cells with compound **7**, a strong decrease in both α-catenin (Ser641) and Akt1 (Ser129) phosphorylation was observed (Figure 6A), confirming that CK2 was a cellular target of this inhibitor. Subsequent dose–response experiments showed that **7** inhibited the phosphorylation of a-catenin (Ser641) and Akt1 (Ser129) with EC_{50}s of 6 µM and 16 µM, respectively (Figure 6B). These EC_{50}s were in good agreement with the concentration required to reduce the viability of 786-O cells by half (25 µM), also arguing that the inhibition of the CK2–dependent signaling was functionally linked to the observed cell death induction. Interestingly,

we noted only a modest 5-fold drop in potency for **7** between the cell-free assay (IC$_{50}$: 3.4 μM) and the suppression of Akt phosphorylation in living cells (EC$_{50}$: 16 μM). In comparison, the reference compound CX-4945 exhibited a cell-free potency of 5 nM and a cellular efficacy of 1 μM for both substrates (Figure S6), indicating a striking 200-fold drop in potency. This finding was in accordance with the distinct mechanism of action identified for **7**. As indicated by its observed mixed-type of mechanism for CK2 inhibition, this compound did not directly compete with ATP. Therefore the high cellular ATP concentration should not affect the binding of **7** to CK2α to the same degree as would be expected for CX-4945.
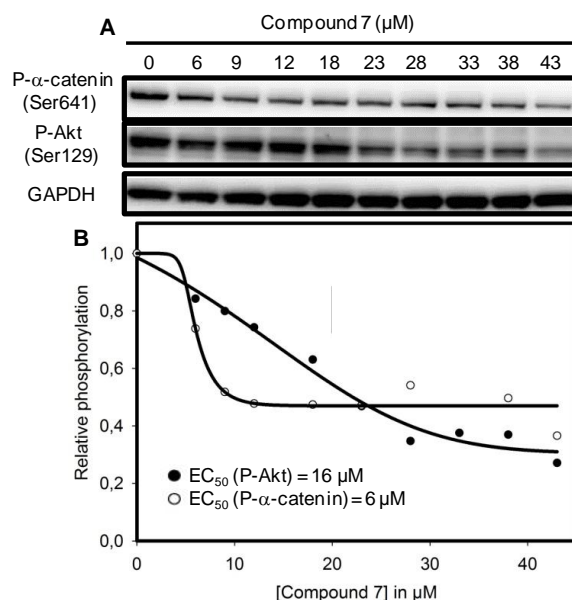
Figure 6. Inhibition of cellular CK2 activity by compound **7**. **A.** 786-O cells were incubated with various concentrations of **7** for 24h, and the phosphorylation status of two protein substrates of CK2 was measured by Western Blot analysis of the cell extracts. GAPDH was probed as loading control. **B.** Western Blot quantification was performed using ImageJ and fitted to a sigmoid equation using SigmaPlot to determine the EC$_{50}$s.

3.1.9 The inhibition of CK2 activity by compound 7 is substrate–dependent

It was intriguing that compound **7** exhibited a small but reproducible difference in the potency to inhibit the substrate phosphorylations of α-catenin (IC$_{50}$: 6 μM) *vs.* Akt1 (IC$_{50}$: 16 μM), a feature which was not seen in the case of CX-4945. Hence we aimed at corroborating this observation by analyzing the phosphorylation of further known CK2 protein substrates, using defined cell-free conditions (Figure 7A). Compound **7** inhibited Six1 phosphorylation and CK2β

autophosphorylation with the same efficacy as the canonical peptide substrate used in the previous dose–response assay (IC_{50}: 3.4 μM). However, a striking 10-fold difference was observed for the phosphorylation of nucleolin (IC_{50}: 42 μM). In contrast, CX-4945 inhibited the phosphorylation of nucleolin with about the same potency as that of other protein substrates and the peptide substrate (IC_{50}: 5 nM). This result suggested that the efficacy of compound **7** to inhibit CK2 activity depends on the protein substrate.

Nucleolin is an abundant nucleolar protein known to bind to CK2α with very high affinity.[225] Due to this complex formation, a significant portion of CK2α in growing cells is usually concentrated in the nucleoli [226]. CK2 phosphorylates the bipartite nuclear localization signal near the N-terminus of nucleolin, thus promoting its nuclear translocation.[227] In consequence, the phosphorylation status of the CK2 phospho-acceptor site in nucleolin is expected to determine the subcellular localization of the high-affinity complex, which can be monitored by a fluorescent label. Using an EGFP-CK2α construct, we analyzed whether compound **7** and CX-4945 differently influence the subcellular localization of CK2α in 786-O renal cancer cells. Indeed, we found that CX-4945 induced a significant translocation of EGFP-CK2α from the nucleoli to the nuclear matrix and the cytoplasm compartments (Figure 7B), whereas compound **7** was without visible effect. These results were consistent with the observed potency differences of compound **7** and CX-4945 with respect to the inhibition of nucleolin phosphorylation. Thus, the alternative mode of action of **7**, that rendered its potency substrate–dependent, further translated to a different impact on the subcellular localization pattern of high affinity CK2α/substrate complexes when compared with the ATP-competitive inhibitor CX-4945.
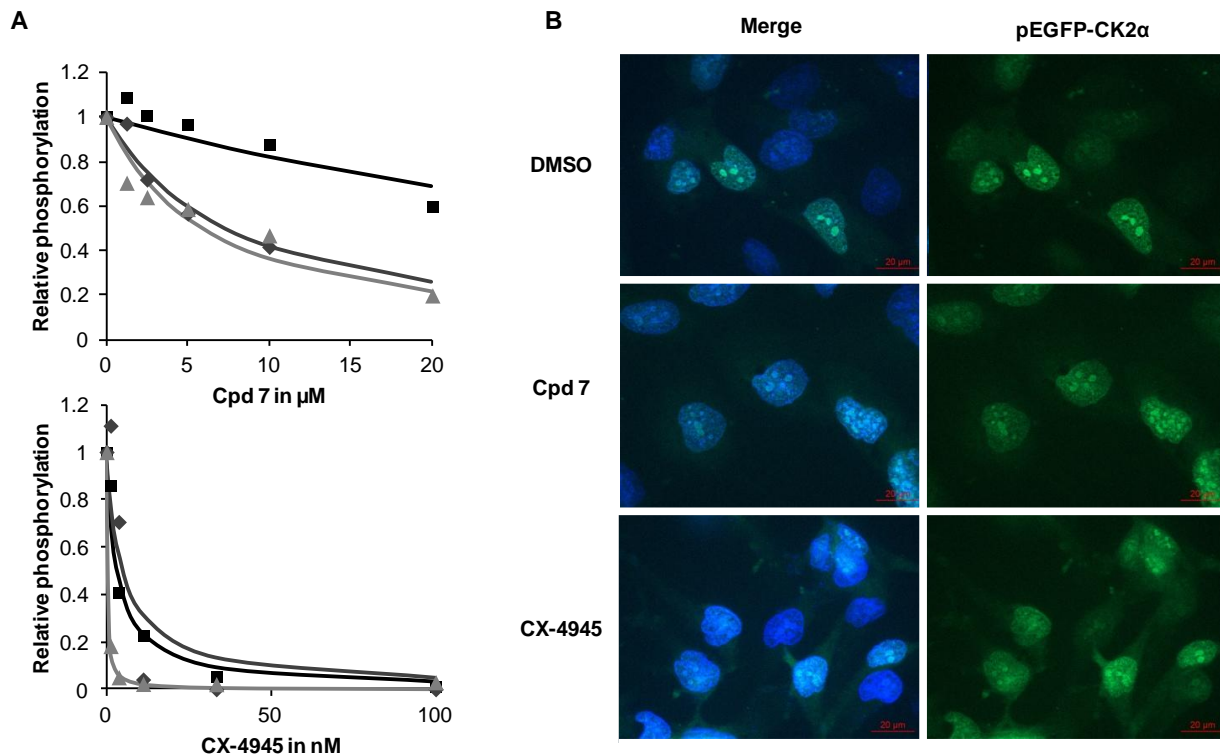
Figure 7. Cellular consequences of the stabilization of the CK2α inactive conformation. **A.** Protein substrates phophosphorylation (■ nucleolin, ▲ Six1, ◆ CK2β auto-phosphorylation) by CK2α₂β₂ in presence of various concentration of compound **7** (upper panel) or the ATP-competitive inhibitor, CX-4945 (under panel). Graphs represent the average of two measurements. **B.** 786-O pEGFP-CK2α cell imaging showing the subcellular localization of CK2α after 12h treatment with DMSO, compound **7** (40 μM) or CX-4945 (8 μM). Nuclei were stained with Hoechst-33342 and merged images as well as GFP single channel were depicted for clarity.

3.1.10 Conclusion

CK2 is of main interest in cancer treatment because of its implication in promoting cancer development,[66] in drug resistances[70] and in epithelial plasticity.[228] However, only one CK2 inhibitor, CX-4945, entered clinical trials up to now.[92] The vast majority of CK2 inhibitors developed so far was directed against the ATP binding site, entailing insufficient selectivity and cellular activity in many cases.[103,229] Even with CX-4945, selectivity issues arise[99]. To date, very few studies described alternative strategies for the pharmacological inhibition of CK2, and none of them presented drug-like small molecules.[150–152,230] Herein, we have provided first evidence for the existence of an alternative binding pocket, distinct from the conserved ATP-site, which can

accommodate small drug-like molecules. We have also proven that binding affinities sufficient to impart efficient CK2 inhibition can be achieved. Based on single mutation scanning and STD-NMR experiments, also supported by *in silico* studies, the small molecules described in this paper were predicted to bind to an allosteric pocket localized at the interface between the glycine-rich loop and the aC-helix. Notably, the position of the allosteric pocket described here partially superimposes with that of the previously discovered alternative binding pocket in the MAP kinases MEK1 and -2 (data not shown)[143]. However, an aC-helix displacement was clearly observed upon allosteric inhibitor binding in the MEK pocket whereas the rigidity of this helix in CK2a prevents a significant displacement. In the case of CK2a, it is more likely that the glycine-rich loop is the mobile part during pocket opening. Nevertheless, based on structural similarities, the identification of this allosteric pocket on the constitutively active CK2a supports the possible existence of a comparable pocket in other related kinases.[148,231]

Our STD-NMR experiments revealed that binding of the ATP analog AMP-PNP was prevented in the presence of the 2-aminothiazole derivative **5**. This might be explained by a partial direct competition of AMP-PNP – and analogously of ATP as well – with the small ligand. The docking result depicted in Figure 5F demonstrates that compound **5** would come rather close to the terminal phosphate moiety of theoretically bound ATP. Alternatively, the available crystal structures 3JUH and 3FWQ support the idea that upon binding of the 2-aminothiazole derivatives, the glycine-rich loop might switch to a conformation not compatible with binding of the terminal ATP phosphate moiety.[149,218] The resulting allosteric down-regulation of ATP binding is also in keeping with the data from the STD-NMR and, importantly, also from the enzyme kinetics analysis. The latter showed a mixed-type of inhibition toward ATP with an increase in K_m and a decrease in V_{max}, suggesting that the inhibitors stabilize a less productive conformation, thus causing a decrease in the apparent affinity for ATP. In consequence, a truly allosteric mechanism of inhibition is more likely to be the prevailing mechanism of action of our compounds (Figure S5). This view also supported by the cellular experiments, which revealed for compound **7** only a low drop of potency in cells (EC$_{50}$ (a-catenin phosphorylation): 6 µM) compared with the cell-free assay (IC$_{50}$: 3.4 µM). Considering the high intracellular levels of ATP in the mM range, the noticeable retention of activity of **7** suggests that at least in cells, (partial) ATP competitive mechanisms cannot play a major role. It is noteworthy that a similar outcome had been observed with CI-1040, a highly selective and non-ATP competitive inhibitor of MEK1/2, that showed a ratio of about 5 to 6-fold between enzymatic potency (IC$_{50}$ = 17 nM) and cellular efficacy (EC$_{50}$ = 100 nM).[141,232] For classical ATP-competitive inhibitors, the commonly observed ratio is much higher (in the 100-fold range), depending on the Km of the respective kinase for

ATP.[11] Hence, the alternate mechanism of inhibition may provide an advantage for *in vivo* applications of future optimized derivatives of **7**.

Proteins and among them protein kinases are regulated through various conformational changes. In solution, an equilibrium between various conformations is observed and some of them could be stabilized by ligand binding, leading to a conformational selection.[233,234] CK2a was considered as a constitutively active protein kinase since decades.[30] Given that more than 300 proteins were identified as potential substrates of this kinase, it is fair to ask how the catalytic activity towards these numerous substrates is regulated.[27] Although CK2β was found to modulate substrate specificity of the catalytic subunit upon complex formation,[77] it is unlikely that this is the only physiological mechanism of controlling the CK2 activity.[212] Raaf et al. (2009) solved by X-ray crystallography an inactive conformation of CK2a, which displayed a putative allosteric pocket in the region we mapped as the interaction site; however, no evidence could be provided that such an inactive state exists *in vivo*.[149] Altogether, the results presented herein provide evidence that CK2a does not exclusively exist in a "switch on" state but can adopt an inactive conformation, which can be stabilized by binding of our compounds, and which may resemble the inactive state of CK2a observed in the mentioned crystal structure. Furthermore, our results obtained both in cell-free and cellular assays demonstrated that the inhibitory efficacy of our allosteric modulators was substrate-dependent, suggesting that some substrates may affect - by physical interaction with CK2a - the compound´s ability to stabilize a catalytically inactive conformation. Therefore, the compounds described herein could be considered as the first substrate-selective modulators of cellular CK2 activity, clearly differing from the common total CK2 inhibitors. Nevertheless, compound **7** effected growth inhibition of tumor cells *in vitro*, even though - in contrast to CX-4945 - its potency to suppress specific phosphorylations may vary depending on the protein substrate.

In conclusion, our compounds are useful as probes to investigate how the modulation of CK2 conformational states may contribute to the regulation of the kinase´s activity in specific contexts. Also supported by the results from such experiments, our inhibitors may be further developed into drugs targeting pathogenic signaling pathways in specific tumor entities.

Supplementary Figures, Tables and Scheme

Figure S1. A. Effects of increasing ATP concentrations on the inhibition of CK2a activity by compound **1**. CK2a (20 ng) was incubated with or without 15 μM of **1** after which its activity was assayed with 200 μM of CK2β–independent pepti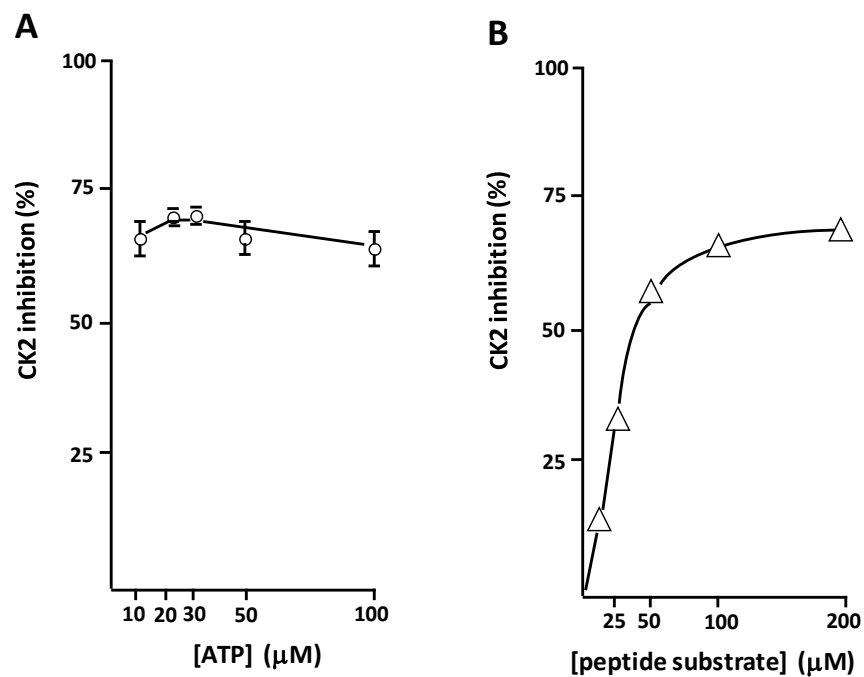de substrate in the presence of increasing ATP concentrations. **B**. Effects of increasing peptide substrate concentrations on the inhibition of CK2 activity by **1**. CK2a (20 ng) was incubated with or without 15 μM of **1** after which its activity was assayed with 100 μM ATP in the presence of increasing concentrations of CK2β–independent peptide substrate.
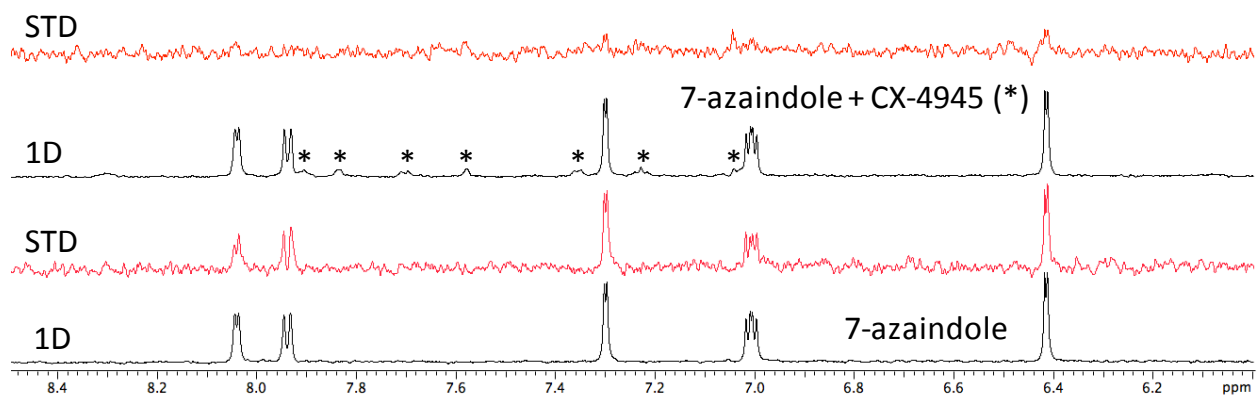
Figure S2. STD NMR experiments for CK2a. 1D (black) and STD (red) NMR experiments of the compound 7-azaindole (500 µM), a small molecule known to target the ATP-binding site of kinases (STD signals with CK2a alone are displayed at the bottom), is displaced by the kinase inhibitor CX-4945 (100 µM) (STD spectrum at the top).
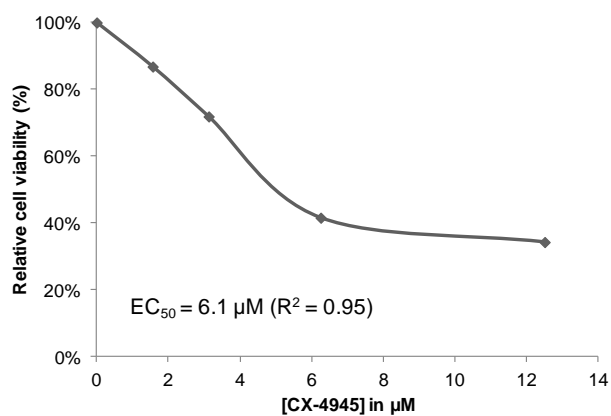
Figure S3. 786-O cells viability in presence of various CX-4945 concentrations. EC_{50} was determined by linear interpolation after transformation to log[c] scale.

A CX-4945 (µM)

	0	0.5	1	3	6
P-α-catenin					
P-Akt-S129					
GAPDH					

B

Relative phosphorylation

- ● P-Akt(Ser129)
- ○ P-α-catenin(S641)

EC_{50} (P-Akt) = 0.90 µM
EC_{50} (P-α-catenin) = 0.95 µM

[CX-4945] in µM
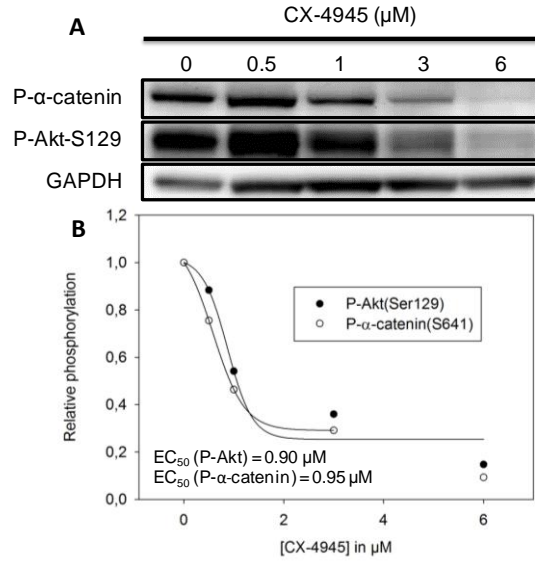
Figure S4. Inhibition of cellular CK2 activity by CX-4945. **A**. 786-O cells were plated and incubated for 24 h with various concentrations of CX-4945. The phosphorylation status of two protein substrates of CK2 was then measured by Western Blot analysis of the cell extracts. Anti-GAPDH was used as loading control. **B**. Western Blot quantification was performed using ImageJ and fitted to a sigmoid equation using SigmaPlot to determine the EC_{50}s.
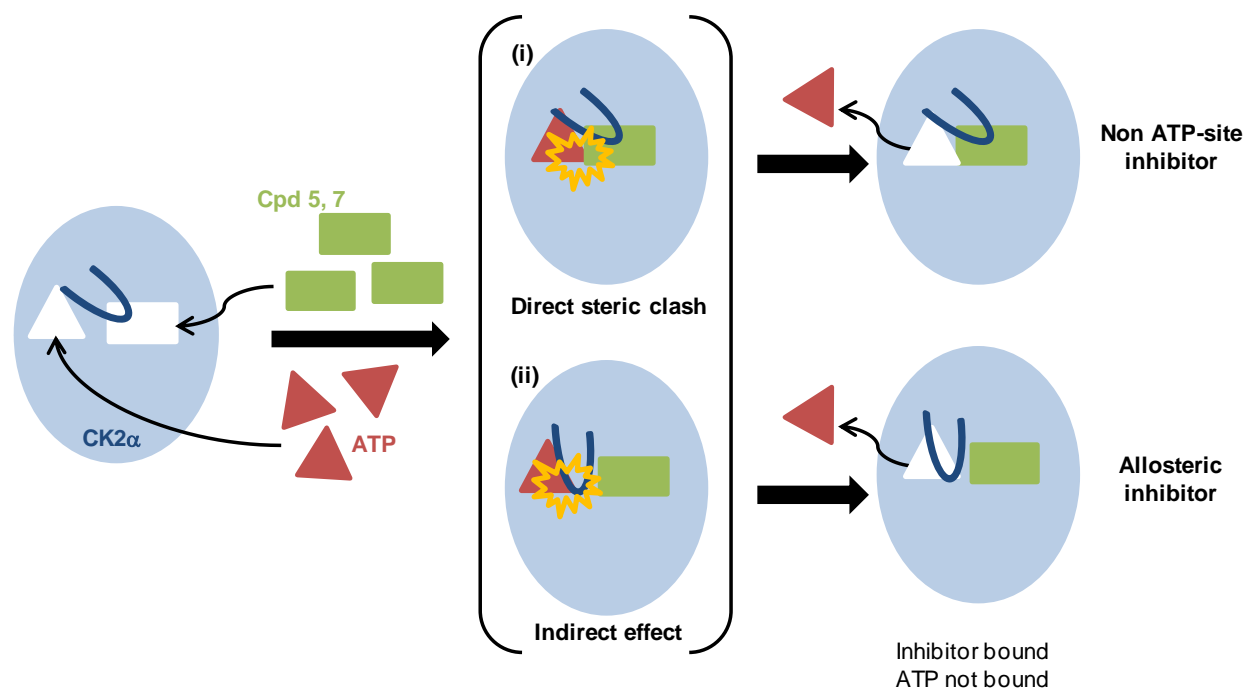
Figure S5. Proposed models of the mechanism of CK2a inhibition by compounds **5** and **7**. On the left, compounds **5** and **7** bind to CK2a prior to or after ATP binding. Binding of the small ligands induces a decrease in the Vmax and an increase in the Km for ATP (cf. Lineweaver-Burk plot, Figure 2). Also considering the results from the STD-NMR experiments (cf. Figures 5), two alternative mechanisms are consistent with this mixed-type inhibition, as illustrated in the middle: (i) a direct, but limited steric clash between ATP and the new compounds, adding a minor competitive proportion to the non-ATP competitive mechanism ("non ATP-site inhibitor"); (ii) binding of new compounds stabilize a conformational deformation of the ATP-binding pocket, which involves a larger movement of the glycine-rich loop (in blue), inducing a decrease in affinity for ATP (indirect effect); this represents a truly allosteric mechanism.

Table S1. Residual kinase activity in the presence of compound **1** at 50 µM.

| Protein Kinase	Residual activity (%)	Protein Kinase	Residual activity (%)	Protein Kinase	Residual activity (%)	Protein Kinase	Residual activity (%)
CK2α(h)	0	HIPK2(h)	83	IR(h), activated	93	CaMK1(h)	101
EGFR(h)	26	MAPK1(h)	84	CDK6/cyclinD3(h)	95	CDK2/cyclinE(h)	101
EphA4(h)	45	LKB1(h)	85	cSRC(h)	97	Met(h)	102
Pim-1(h)	46	PKBα(h)	85	FGFR1(h)	97	PAK2(h)	102
MuSK(h)	62	CDK2/cyclinA(h)	86	Fyn(h)	97	IKKα(h)	103
PDGFRα(h)	65	PKCα(h)	86	HIPK1(h)	97	MEK1(h)	103
AMPKα1(h)	71	cKit(h)	87	HIPK3(h)	98	Abl (h)	104
Aurora-B(h)	74	PKA(h)	87	CDK9/cyclinT1	99	CDK7/cyclinH(h)	104
DYRK2(h)	78	CDK1/cyclinB(h)	89	Rsk1(h)	99	JAK2(h)	104
Aurora-A(h)	79	CHK2(h)	89	ROCK-1(h)	100	Lyn(h)	104
GSK3β(h)	79	c-RAF(h)	92	Yes(h)	100	mTOR(h)	104

Results were obtained from kinase profiler service offered by Life Technologies with ATP concentration adjusted to the respective Km of each kinase. Duplicate values did not differ by more than 15 % in all assays.

Table S2. Residual kinase activity in presence of compound **7** at 50 µM.

Protein Kinase	Residual activity (%)	Protein Kinase	Residual activity (%)	Protein Kinase	Residual activity (%)
EphA4(h)	27	CLK2(h)	67	PKCα(h)	92
GSK3β(h)	29	Abl(h)	69	MEK1(h)	95
CK1γ1(h)	31	CHK2(h)	72	JAK2(h)	98
ACVR1 (ALK2)	34	TBK1	75	MKNK1 (MNK1)	101
CLK4(h)	39	CDK1/cyclinB(h)	78	PDGFRα(h)	102
MLCK (MLCK2)	39	DYRK1a	79	DAPK3	104
STK17A (DRAK1)	48	CLK1(h)	80	EGFR(h)	106
CK2a2	48	HIPK3	82	CDK2/cyclinE(h)	107
GSG2 (HASPIN)	55	CLK3(h)	86	ROCK-1(h)	107
Pim-1(h)	57	RIPK2	86	cKit(h)	116
HIPK2	57	MuSK(h)	90		

Results were obtained from kinase profiler service offered by Life Technologies with ATP concentration adjusted to the respective Km of each kinase. Duplicate values did not differ by more than 17 % in all assays.
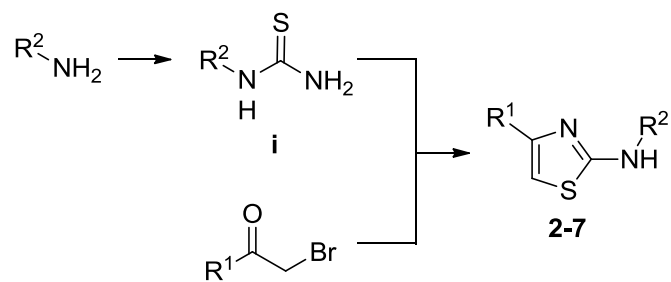
Scheme S1. Synthesis of the aminothiazole **2-7** compounds. Reagents and conditions: a) CS_2, Et_3N in THF/H_2O, RT, 24 h, then I_2 in THF, 0 °C to RT, 3 h b) NH_4OH 30 %, RT, 6 h c) EtOH, reflux, 3-12 h.

3.2 2-Aminothiazole derivatives as potent and selective allosteric modulators of the protein kinase CK2

Protein kinase CK2 is an ubiquitous protein Ser/Thr kinase involved in cancer development. Based on the 4-(4-phenylthiazol-2-ylamino)benzoic acid scaffold, extensive Structure Activity Relationship studies were carried out and led to significant improvement of potency. The non-ATP competitive mechanism of action of the leading compound, 2-hydroxy-4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzoic acid (**8e**) was confirmed. In addition, cellular experiments have shown interesting effects for cellular CK2 inhibition and apoptosis induction, in comparison with CX-4945, a phase II clinical trial ATP-competitive CK2 inhibitor. Thus, targeting CK2 in a non-ATP competitive manner leads to *in cellulo* highly efficient compounds with interesting perspectives for further *in vivo* developments.

3.2.1 Scientific rationale

Tumor development could rely on deregulated oncogenes but also on deregulated non-oncogenes.[208] Indeed, several proteins sustain tumor development, even if they are not the primary cause of the cancer. In this context, CK2 plays a central role as several studies revealed its implication as a cancer driver.[66] CK2 overexpression has been widely correlated with a poor prognosis for patient in prostate cancer[54], breast carcinoma[56], glioblastoma[58], gastric carcinoma[235] or renal cell carcinoma.[60] Indeed, CK2 acts as a major support to prosurvival and antiapoptotic signals by interfering with NF-κB, Wnt and Akt pathways.[66] Thus, CK2 overexpression supports a favorable environment for cancer development described as the "hallmarks of cancer".[4,5,236]

CK2 is a ubiquitous Ser/Thr protein kinase composed of a dimer of regulatory subunits (β) and two catalytic subunits, CK2α and/or CK2α'.[28] The catalytic subunit of CK2 is constitutively active, while the regulatory subunit modulates the selectivity toward a subset of substrate proteins.[77]

CK2 inhibition has been proved to be a valuable strategy to fight cancer and several inhibitors have been developed, among which CX-4945 (5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid, silmitasertib).[209] This ATP-competitive inhibitor of CK2 is the most promising molecule. Developed by Cylene Pharmaceuticals, this compound is a highly potent (IC_{50} = 14 nM) and selective (Gini coefficient[98]

= 0.67) inhibitor of CK2α with a good efficiency in cellular experiments. Besides, in mice xenografts models, tumor regression was observed upon CX-4945 treatment.[92,97] Looking for splicing regulation through phosphorylation of serine/arginine rich (SR) proteins, Kim *et al*. revealed a special CK2-independent implication of CX-4945 in cellular regulation of SR protein phosphorylation.[99] Indeed, this compound was found to be a strong inhibitor of Cdc2-like kinase-2 (Clk-2) (IC_{50} = 4 nM), almost four-fold more potent than on CK2α (IC_{50} = 14 nM).[99] Although the consequences in splicing regulation remain elusive, this off-target activity of silmitasertib underlines the need for potent and more selective CK2 inhibitors.

Recently, 2-aminothiazole derivatives **1-3** (Figure 1) were identified as non-ATP competitive inhibitors of CK2a. A complete study allowed us to determine the allosteric mechanism of action of these molecules: (i) A simultaneous binding of compound **2** and of the ATP-competitive inhibitor CX-4945 was observed by STD-NMR experiments; (ii) The unfolding temperature of GST-CK2α monitored by CD spectrometry decreased upon compound **2** or **3** binding whereas an increase was observed in the presence of CX-4945; (iii) Single mutation scanning coupled with extensive molecular modeling studies have led to the identification of an 2-aminothiazole binding site, at the interface of the flexible glycine-rich loop, the αC-helix, the third β-strand of the N-terminal lobe and the activation loop. The allosteric pocket was defined by residues Tyr50, Leu70, Val73, Lys74, Lys77, Arg80 and Arg191. These findings emphasize the specificity of such mechanism of CK2 inhibition by this class of compounds.
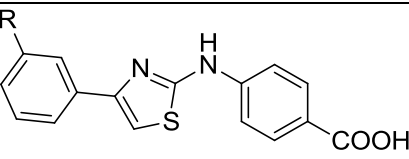

Cpd	R	IC_{50} (CK2a) in μM
1	NO$_2$	7
2	H	9
3	Br	3.4

Figure 1. Chemical structures of three allosteric inhibitors previously described (S.D. < 20 %). (chapter 3.1)

3.2.2 Chemistry

Aminothiazoles core were prepared according to Hantzsch-type syntheses from 2-bromoacetophenones and thiourea intermediates (Scheme 1). Most of the 2-bromoacetophenones **4** were commercially available or easily prepared (**4a-c**) from commercially available acetophenones through a bromination step, as described in the literature.[237,238] Thioureas **5** were obtained after reaction of isothiocyanate intermediates with ammonia solution. The latter were synthesized after reaction of the primary amine of aniline derivatives with carbon disulfide, in presence of triethylamine, followed by iodine oxidation.[239] Hantzsch syntheses were carried out in ethanol and the resulting products were directly obtained as solids, after their precipitation with water. Semi-preparative HPLC, recrystallization, or washing procedures led to the final products with a LC-UV (254 nm) purity higher than 95%. In some cases, ethyl ester derivatives were obtained instead of the free carboxylic acids. Then, a saponification step with NaOH was carried out to obtain the desired products **6c** and **7g**.

Compound **9** could not be obtained through Hantzsch-type synthesis, maybe due to the spatial proximity between the primary amine and the carboxylic acid of 2-aminobenzoic acid, which prevents the formation of the isothiocyanate intermediate. Consequently, a nucleophilic substitution between 2-bromo-4-(3-nitrophenyl)thiazole and 2-aminobenzoic acid in hydrochloric acid was performed to obtain the desired 2-((4-(3-nitrophenyl)thiazol-2-yl)amino)benzoic acid (**9**) albeit in a low 2% yield (Scheme 2).

Several modifications were considered on the carboxylic acid function (Scheme 3). Methyl ester derivative **10** was easily obtained through a classical acidic esterification step. Sulfonamide derivatives **11a-b** were obtained according to amino-acid coupling procedures whereas acetoxymethyl prodrugs **12a-c** were accessed from carboxylic acid derivatives (**1** or **7i-j**) and bromomethyl acetate through a previously described method.[240]
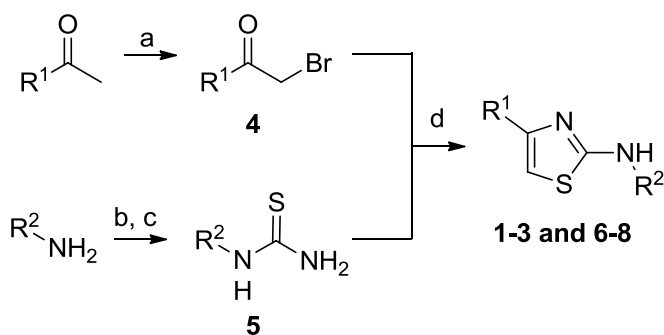
Scheme 1. Hantzsch-type synthesis. Reagents and conditions: a) Br$_2$, CHCl$_3$, 40°C, 5 min or Br$_2$, HBr 32% in AcOH, MeOH, 60°C, 4h. b) CS$_2$, Et$_3$N in THF/H$_2$O 1/1, RT, 24h, then I$_2$ in THF, 0°C to RT, 3h. c) NH$_4$OH 30%, RT, 6h. d) EtOH, reflux, 3-12h.
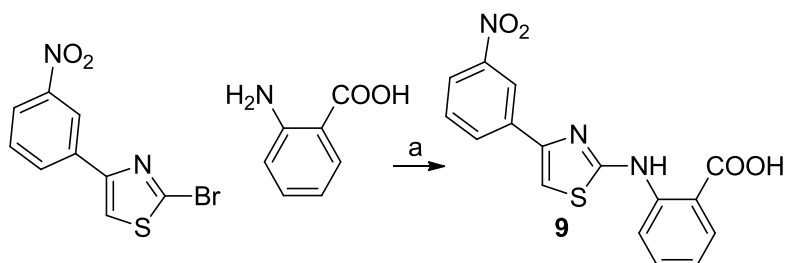
Scheme 2. Preparation of compound **9** through aromatic nucleophilic substitution. Reagents and conditions: a) HCl$_{(aq)}$ 37%, 1,4-dioxane, 90°C, 48h.
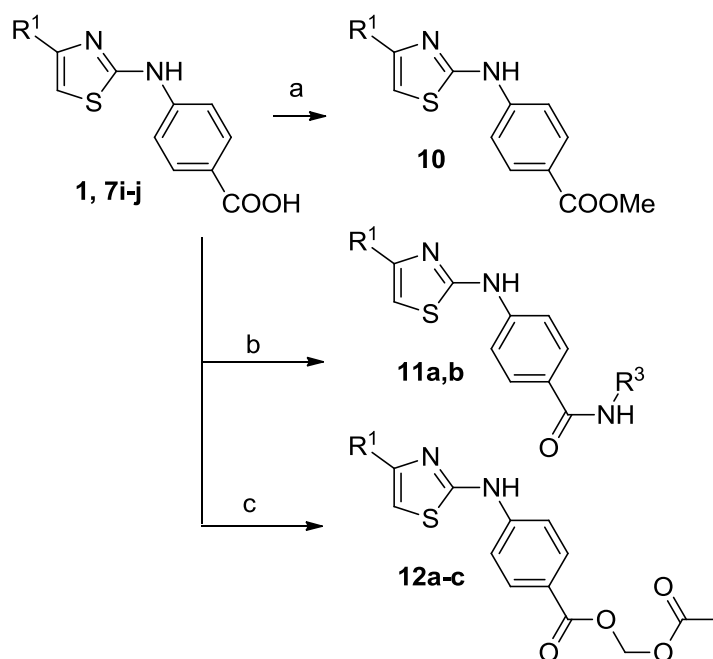
Scheme 3. Syntheses of esters and amides **10**-**12**. Reagents and conditions: a) H$_2$SO$_4$ 95% 3 drops, MeOH, reflux, 2h. b) **11a**: HATU, DIPEA, dry DMF, 30 min; NaH, RSO$_2$NH$_2$, dry THF, RT, 30 min; then RT, 4h. **11b**: EDC, HCl, DMAP, RSO$_2$NH$_2$, CH$_2$Cl$_2$, RT, 36h. c) Bromomethyl acetate, Et$_3$N, dry DMF, RT, 12h.

3.2.3   Structure-Activity Relationships

The preliminary Structure-Activity Relationships (SAR) revealed the importance of key features of our diaryl aminothiazole derivatives for CK2α inhibition (chapter 3.1). Indeed, attempts to remove or to hide (as a methyl ester) the acid function led to totally inactive compounds (Table 1; compounds **6a** and **10**, respectively), underlying the importance of such functional group on the aromatic ring. Consequently, we envisaged the evaluation of several analogs sharing the 3-nitrophenyl moiety but with variations of the carboxylic acid part (Table 1). Compound **6b** having this group at position 3 was 4-fold less potent than its *para* regioisomer **1**. However, when the carboxylic acid functional group was displaced in position 2 (derivative **9**) we observed a similar range of efficiency than the parent compound **1**. Compound **6c** with two carboxylic acid functions in *meta* positions was also prepared but its very low solubility prevented any biological evaluation. Compound **6d** with a phenyl acetic acid moiety was, by far, less efficient on CK2a than compound **1**, underlining the fact that benzoic acid in position 4 is the best moiety for protein inhibition.

We tried thereafter various modulations on the second aromatic ring while conserving the 4-benzoic acid group for the first one (Table 2). Starting from compound **1** with a 3-nitro substitution on the phenyl moiety, we modulated the electron density of this ring using various substitutions: 3-bromo **3**, 3-methoxy **7a**, 4-methoxy **7b**, 2,4-methoxy **7c**, 2,4-hydroxy **7d**, 2-hydroxy-4-bromo **7e**, pyridin-2-yl **7f** and without any substitution **2**. In these cases, no significant improvement of IC_{50} was observed, even though hydrophobic substituent such as a bromine atom at position 3 or 4 improved CK2a inhibition, with IC_{50} values of 3.4 µM and 5 µM (**3** and **7e**, respectively).

The small differences in IC_{50} values of compounds **1**-**3** and **7a**-**f** with these various substitutions prompted us for more drastic structural modifications of the phenyl moiety. Removal of the phenyl ring (**7g)** or its replacement by a non-aromatic bulky *tert*-butyl moiety **7h** resulted in a strong decrease of CK2a inhibition. On the other hand, replacement of the phenyl ring by a 3-thiophene **7i** improved twice the inhibition of CK2a (IC_{50}= 5 µM for **7i**). Moreover, we have observed that a naphthyl ring substitution resulted in a better protein inhibition, with an IC_{50} value of 3 µM for compound **7j**. This latter finding is consistent with the requirement of a hydrophobic moiety in this part of the inhibitors, as already noticed with compound **3**.
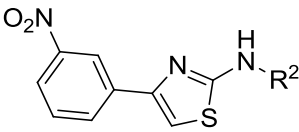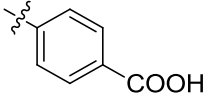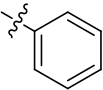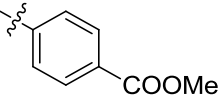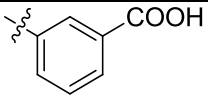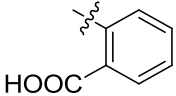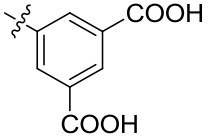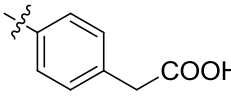
In consequence, several benzoheterocyclic derivatives **7k-m** were synthesized and evaluated. Compound **7k** with a chromene instead of the naphthyl group and compound **7l** containing a 2-benzofuran moiety were less potent than compound **7j.** The promising result obtained with a thiophene substitution (**7i)** suggested us to introduce a 5-benzothiophene substituent, but in this case, no significant improvement of the inhibition of CK2a was noticed.

Preliminary docking experiments conducted on compounds **1** and **7j** suggested that the size of the naphthyl moiety induces a more constraint binding mode in the allosteric pocket than for compound **1**. In consequence, it seems promising to have a new look on the carboxylic acid substitution on a 4-naphthalen-2-yl-thiazol-2-yl-arylamines. Several derivatives **8a**-**e** were synthesized (Table 3) and evaluated for their CK2a inhibitory properties. Compounds **8a** without acid function and **8b** with a 4-cyano substitution definitely lacked for CK2a inhibition. However, with the naphthyl substitution in R[1], the carboxylic acid function appears to better increase the potency in position 3 (**8c**) than in position 4 (**7j**). The opposite result was observed in the presence of the 3-nitrophenyl group for R[1] (Table 1; compounds **1** and **6b**). The 6-methoxy-3-benzoic acid analog **8d** exhibited a potency on CK2a similar than the parent compound **8c**. So, results from the SAR studies underlined the need of an acid group in position 3/4 of the phenyl ring and docking results suggested that more interactions between the basic residues (Lys74, Lys77, Arg80 and Arg191) of CK2a and this acidic function could be possible. To increase these interactions, we tried to add on this ring another functional group and a salicylic acid derivative **8e** was considered. This structural modification provided a very good result as the inhibitory properties was five-fold higher (compound **8e** compared to **7j**), as shown by the sub-micromolar IC_{50} value (IC_{50} = 0.6 µM for compound **8e**, see Table 3).

Carboxylic acid derivatives could sometimes be a source of troubles for *in vivo* experiments with cell permability issues. To continue the structural exploration, we considered bioisosteric modifications and a prodrug approach. Several compounds were synthesized, deriving from **1**, **7i,j** (Table 4) and compared with *para*-substituted acids **7j** and **8e**. These derivatives might not exhibit better *in vitro* potency but better physicochemical properties, suitable with cellular experiments. Thereby, cell viability experiments were considered as the easiest way to evaluate compound efficiency as CK2 inhibitors induce a decrease in cell viability.[92,101] Acylsulfonamide derivatives are well known carboxylic acid bioisosteres as they preserve the acidic properties (pKa ≈ 4-5) but with improved bioavailability.[241] In our case, two acylsulfonamide derivatives were synthesized and evaluated: methylsulfonamide **11a** and 4-methylbenzenesulfonamide **11b** both exhibited similar *in vitro* potency with a respective 41% and 54% inhibition of CK2a at 10 µM. However, looking at their ability to reduce cell viability, compound **11b** (87% decrease at 25 µM) was by far better than compound **11a** (23% decrease at 25 µM). Moreover, tosyl derivative **11b** was more efficient than the parent compound **7j** (43% decrease in cell viability at 25 µM) and as efficient as the best compound of this series (**8e**, 87% decrease at 25 µM). In order to discriminate these compounds, the Percentage Efficiency Index
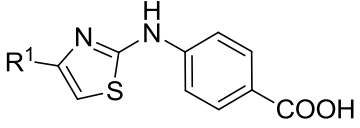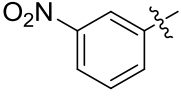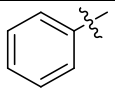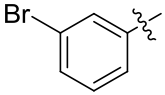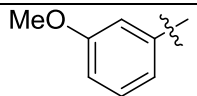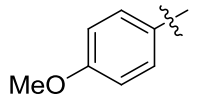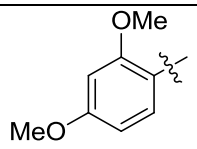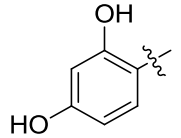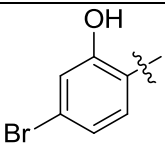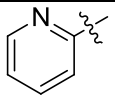
(PEI), which render ligand efficiency as a function of molecular weight,[242] was calculated for each molecule (Table 4).This index underlined the predominance of compound **8e** (PEI = 2.6) in comparison with compound **11b** (PEI = 1.1). For two structures having the same cellular efficiency, the one with the lowest molecular weight (**8e**) was considered as the best candidate for further developments. A prodrug strategy using acetoxymethyl ester groups was considered.[240] Among the three evaluated derivatives **12a-c**, none of them led to improvement in cellular efficiency (Table 4). Further experiments evaluating the stability of these prodrugs have to be carried out before envisaging new derivatives.
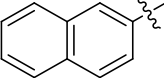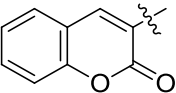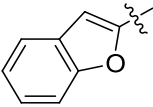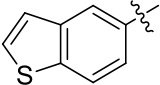
Table 1. Importance of the benzoic acids function on R^2.

Cpd	R^2	IC_{50}^{a} (μM)
1		7
6a		>25
10		>25
6b		30
9		8
6c		b
6d		>50

[a]IC_{50} values are the mean of ≥ 2 experiments with errors within 40% of the mean. [b]Solubility troubles prevent IC_{50} determination.
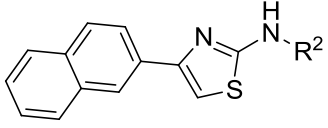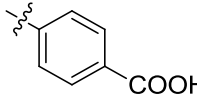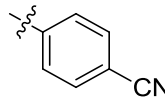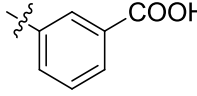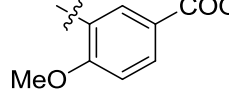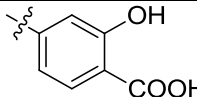
Table 2. SAR studies on hydrophobic ring R^1.

Cpd	R^1	IC_{50}^a (μM)
1	O_2N-phenyl	7
2	phenyl	9
3	Br-phenyl	3.4
7a	MeO-phenyl	12
7b	MeO-phenyl	6
7c	OMe, MeO-phenyl	13
7d	OH, HO-phenyl	7
7e	OH, Br-phenyl	5
7f	N-pyridyl	14
7g	H	>50

7h		>50
7i		5
7j		3
7k		8
7l		14
7m		4
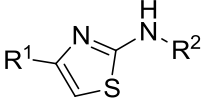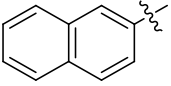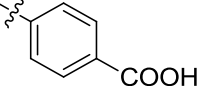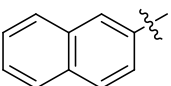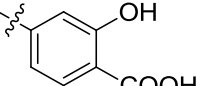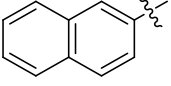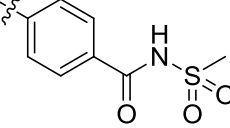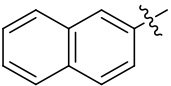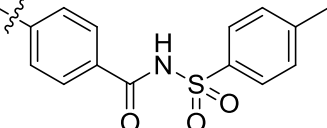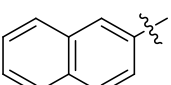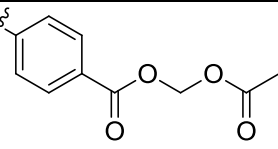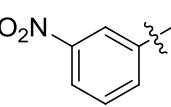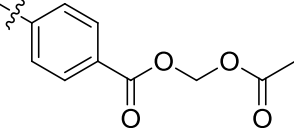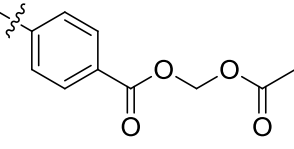
[a]IC_{50} values are the mean of ≥2 experiments with errors within 40% of the mean.

Table 3. Pharmacomodulation studies on 4-naphthalen-2-yl-thiazol-2-yl-arylamine derivatives.

Cpd	R^2	IC_{50}^a (μM)
7j		3
8a		>50
8b		>50
8c		2
8d		8
8e		0.6

$^a IC_{50}$ values are the mean of ≥2 experiments with errors within 40% of the mean.

Table 4. *In vitro* and cellular effects of selected carboxylic acid bioisosteres and prodrugs.

R^1-thiazol-2-yl-NH-R^2 (2-amino-thiazole core)

Cpd	R^1	R^2	CK2a inhibition (%) at 10 µM[a] (IC$_{50}$[b])	PEI[c]	Cell toxicity (% from DMSO) at 25 µM[d]
7j	naphthalen-2-yl	4-(COOH)phenyl	67 (3)	1.9	43
8e	naphthalen-2-yl	2-OH-4-(COOH)phenyl	96 (0.6)	2.6	87
11a	naphthalen-2-yl	4-(C(O)NH-SO$_2$CH$_3$)phenyl	41	1	23
11b	naphthalen-2-yl	4-(C(O)NH-SO$_2$-(4-methylphenyl))phenyl	54	1.1	87
12a	naphthalen-2-yl	4-(C(O)O-CH$_2$-O-C(O)CH$_3$)phenyl	41	1	45
12b	3-(O$_2$N)phenyl	4-(C(O)O-CH$_2$-O-C(O)CH$_3$)phenyl	7	0.2	42
12c	thiophen-3-yl	4-(C(O)O-CH$_2$-O-C(O)CH$_3$)phenyl	37	1	48

[a]Recombinant CK2a inhibition percentage in presence of 10 µM of corresponding compound. [b]IC$_{50}$ values are the mean of ≥2 experiments with errors within 40% of the mean. [c]PEI = (%

inhibition as fraction (0 – 1.0) in presence of 10 μM of corresponding compound) / (molecular weight, kDa).[242] [d]786-O cell toxicity, 24h, 25 μM of respective compound.

3.2.4 Enzymatic characterization of compound 8e effects on CK2

With this efficient CK2α inhibitor **8e** in hand, we then wanted to know if its mechanism of action was similar to that of initial compounds described in Figure 1, ie through binding to an allosteric site.

So, to evaluate if this compound exhibits non-ATP competitive behaviors in enzymatic assays, we have performed a Lineweaver-burk plot analysis of CK2α inhibition by compound **8e**. As illustrated in Figure 2A, a non-ATP competitive mechanism of action, similar to compounds **1-3**, was observed, with a Ki value of 0.18 μM. Then, the effect of compound **8e** was evaluated on CK2α and on the holoenzyme CK2$\alpha_2\beta_2$, with a β-independent peptide substrate (peptide 29) and a β-dependent peptide substrate (peptide M) as well. These three experimental conditions displayed similar results, as the potency of **8e** remained constant (Figure 2B): this thereby underlines the ability of **8e** to inhibit CK2α and the holoenzyme complex with the same potency.
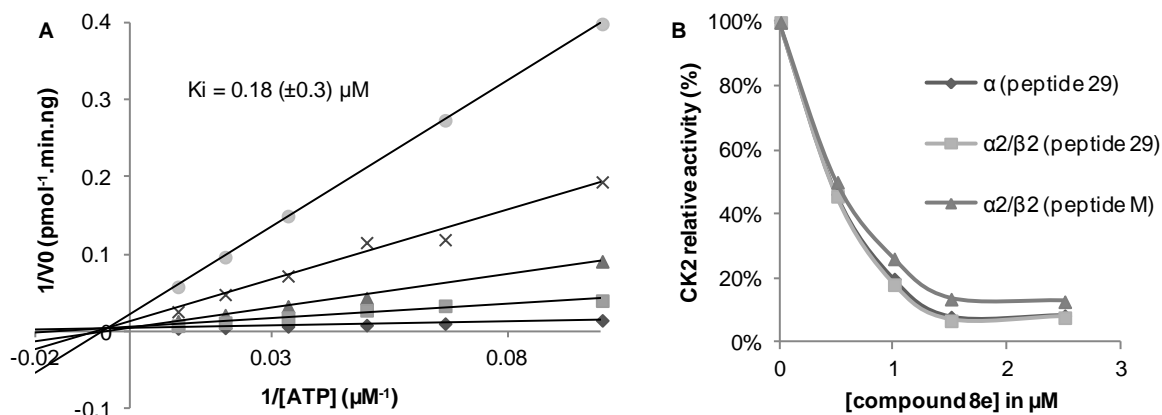
Figure 2. Enzymatic characterization of compound **8e**. **A**. Lineweaver-Burk inhibition plot of human recombinant CK2a by compound **8e** at various concentrations: 0, 0.5, 1, 1.5, 2.5 μM. Ki was determined by plotting the slopes at varying inhibitor concentration from three independent experiments. **B**. CK2a and CK2$a_2\beta_2$ activity with various peptide substrates in presence of increasing concentration of compound **8e**.

3.2.5   Compound 8e binds to an allosteric pocket of CK2α

Compound **8e** was screened on a panel of 13 GST-CK2α mutants, mainly located in the area of the putative binding site described in chapter 3.1. Three mutants were significantly less sensitive toward inhibition by **8e**: Val73Ala, Lys77Ala and His160Ala (Figure 3) and therefore selected for dose response experiments. Compound **8e** IC_{50} was strongly increased in comparison with the wild-type GST-CK2α (IC_{50} = 0.6 µM), a 2.8-fold increase for Val73Ala (1.7 µM) was observed, a 4.3-fold increase for Lys77Ala (2.6 µM) and a 1.7-fold increase for His160Ala (1 µM).
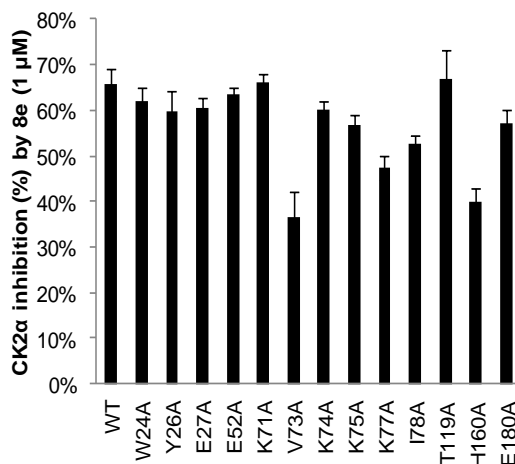
Figure 3. Percentage of inhibition of GST-CK2α (WT or single alanine mutant) in presence of compound **8e** (1 µM).

3.2.6   Compound 8e is a selective inhibitor of CK2

Selectivity among human kinome is an important feature for kinase inhibitors to avoid side effects.[102,243] Using the Gini coefficient as a reporter of the overall selectivity, it is clear that allosteric inhibitors such as PD184352 (MAPKK1 optimized inhibitor), is more selective (Gini coefficient = 0.91)[98] than quinalizarin, an optimized ATP-competitive inhibitor of CK2 (Gini coefficient = 0.61).[101] Targeting the less conserved allosteric binding site of kinases could be a solution to have a higher selectivity. For our study, compound **8e** was evaluated against a selected panel of 53 kinases (Supplementary Table S1) and only four other kinases, in addition to CK2α, were inhibited by more than 50%: ErbB2 (70%), Aurora-B (62%), Pim-1 (60%) and PI3K (53%). It was noteworthy that the main off-target of the CX-4945, Clk2, was not inhibited by **8e** and, among the four isoforms of cdc2-like kinases, only Clk4 (23%) was weakly inhibited. Moreover, compound **8e** overall selectivity (Gini coefficient = 0.78) was higher than for CX-4945 (Gini

coefficient = 0.67) and than for other CK2 inhibitors described in the literature.[97,103]

3.2.7   Inhibition of CK2 cellular activity and apoptosis induction by 8e

CK2 inhibitors are known to decrease the cell viability[92,101] and, consequently, the most active compound **8e** was selected for a cell viability screening on nine cell lines, originated from various tissues (Table 5). Among cancer cells, aminothiazole **8e** exhibited GI_{50} values from 5 μM in clear cell renal cell carcinoma (ccRCC) cell line 786-O to 20 μM in prostate cancer cell (DU145). Interestingly, compound **8e** is by far less toxic in non-cancerous (benign) kidney cells (RPTEC) with a GI_{50} 5-fold higher (27 μM) than in kidney cancer cells (786-O). This latter result is consistent with the dependence of cancer cells to a high level of CK2a expression for their development, as already described.[66] Of note, CX-4945, the canonical ATP-competitive inhibitor of CK2a exhibited a GI_{50} of 6 μM in 786-O cells (Supplementary Figure S1), so in the same range than **8e**.

786-O cells are known to be resistant to classical chemotherapy.[244] Moreover, the potential of CK2a as a relevant target to treat 786-O cells has been already described.[60] Therefore, the 786-O cell line was selected as a cellular model for further evaluation of compound **8e** efficiency, by using Western Blot analysis.

First, inhibition of CK2 activity within living cells was evaluated. Two protein substrates, Akt and a-catenin, known to be phosphorylated by CK2 on residue Ser129[224] and Ser641[223], respectively, were used as reporter of CK2 cellular activity. A strong decrease in phosphorylation of Akt (Ser129) and a-catenin (Ser641) was observed after a 24h treatment with compound **8e** (Figure 5A). We can conclude that compound **8e** inhibited CK2 cellular activity and decreased cell viability, also observed macroscopic observations during cell viability experiments (presence of numerous dead cells).

Then, we were interested in apoptosis markers. Two of them, the apparition of cleaved PARP and the decrease in survivin protein level, were investigated by Western Blot. Both apoptosis markers appeared simultaneously after 24h treatment by compound **8e** (Figure 5B). The concentration of **8e** needed for apoptosis induction (about 6 μM) is consistent with the GI_{50} (about 5 μM) and the cellular inhibition of CK2 activity (about 5 μM) supporting that CK2 inhibition is responsible for cell death.

Table 1. Effect of compound **8e** on various cell lines.

Tissue	Cell name	Cmpd **8e** GI$_{50}$ in µM
Kidney	786-O	5 (± 2)
	RPTEC	27 (± 5)
Breast	MCF7	10 (± 2)
	MCF10A	10 (± 3)
Lung	H1299	9 (± 4)
	A549	12 (± 3)
Prostate	PC3	7 (± 2)
	DU145	20 (± 6)
Glioblastoma	U138	13 (± 3)

Cells were grown as described in the experimental part and GI$_{50}$ values were determined by linear interpolation after transformation to log[c] scale. Standard deviations from minimum two independent experiments are depicted between brackets.
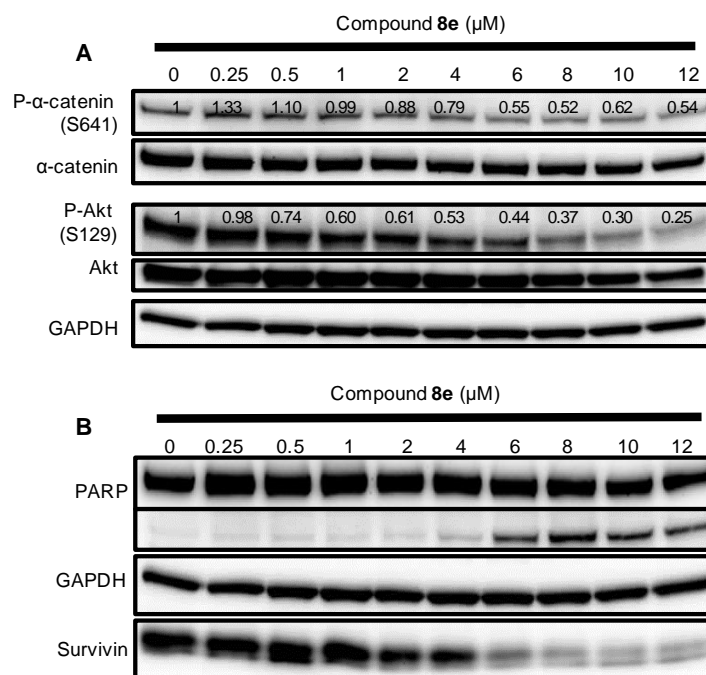
Figure 5. Cellular effect of compound **8e**. **A**. Cellular inhibition of CK2 by compound **8e**. 786-O cells were plated and incubated 24h in the presence of various concentrations of **8e**. Phosphorylation statuses of two protein substrates of CK2 were measured by Western Blot analysis

of cell extracts. Band intensities were quantified using ImageJ and Anti-GAPDH was used as loading control. **B**. Apoptosis induction upon compound **8e** treatment. 786-O cells were plated and incubated 24h in the presence of various concentrations of **8e**. Apparition of cleaved PARP and decrease of survivin was observed by Western Blot analysis of cell extracts. Anti-GAPDH was used as loading control.

3.2.8   Conclusion

From the allosteric inhibitor of CK2a described in chapter 3.1, compound **3**, we investigated new pharmacomodulation studies based on the 2-aminothiazole scaffold. SAR studies have led to potent and selective allosteric inhibitors of CK2a. The SAR demonstrated clearly that a hydrophobic and aromatic substitution in position 2 of the 2-aminothiazole core had a positive effect on CK2a inhibition; as well as an acid function at the opposite side, on the aromatic linked to the amino group of the thiazole. The binding site of the optimized compound **8e** was confirmed in the allosteric pocket by single mutation scanning and docking studies. The allosteric binding pocket of **8e** is located at the interface delimited by the flexible glycine-rich loop, the aC-helix, the 3rd β-strand of the N-terminal lobe and the activation loop. A good selectivity was achieved with a Gini coefficient of 0.78 against a panel of 53 selected kinases. In comparison, the clinical phase II inhibitor, CX-4945 exhibited a Gini coefficient of 0.67. Moreover, Clk2, the main off-target of CX-4945 was not inhibited by **8e**, neither the other kinase of the Clk family. In addition of an interesting selectivity, the mechanism of action of compound **8e** may be responsible for the advantageous efficiency in cellular-based assay. Indeed, **8e** exhibited an very good potency to inhibit CK2a cellular activity, in the same ranges than the clinical phase II inhibitor, CX-4945. This latter result has to be linked with the important difference of potency on recombinant CK2a (0.6 µM for **8e** and 0.014 µM for CX-4945). As a potent CK2a inhibitor, **8e** induced apoptosis after 24h treatment at a concentration of 6 µM and lead various cancer cell lines to death. In summary, the optimized allosteric inhibitor of CK2, **8e**, exhibited a cell efficiency comparable with the clinical phase II inhibitor, CX-4945 and it is a promising molecule for further *in vivo* studies.

3.3 Identification of the first cellular efficient modulator of the protein kinase CK2 subunit interaction

CK2 is a tetrameric protein kinase composed of two catalytic subunits (α and/or α') and a dimer of regulatory subunit (β). CK2β interaction changes the substrate selection of the catalytic moiety. Previous studies demonstrated that a deregulation of the dynamic interaction between CK2α and CK2β induced major alterations in cell signaling leading to phenotype modifications. However, cellular efficient CK2β-competitive inhibitors are required to understand this complex mechanism. In the present study, a cellular efficient inhibitor of the CK2 subunit interaction was obtained through biomolecular engineering of a small cyclic peptide previously described as a CK2α antagonist. This fusion peptide exhibited a micromolar potency to interfere with the CK2α/β interaction in cell-based assay leading to striking modifications of the CK2 subunits subcellular localization as well as to a significant modification in p21 phosphorylation, coupled with EGFR down-regulation. In addition of its potential to investigate the dynamic regulation of the CK2α/β interaction in living-cell, the remarkable decrease in cell viability observed upon disruption of α/β interaction makes it as a valuable cell death-inducing tool.

3.3.1 Scientific rationale

CK2 exhibits an heterotetrameric quaternary structure in which a central dimer of regulatory subunits (CK2β) bridges two catalytic subunits, CK2α and/or CK2α'.[28] The plethora of CK2 substrates[27] that are involved in the promotion of cell survival, proliferation and differentiation[213] shows the complexity of the CK2 cellular signaling network and supports the view that this enzyme participates to the regulation of many of the same cellular responses that characterize the ''hallmarks of cancer'' originally described by Hanahan and Weinberg[4,5]. Thus the crucial role of CK2 in tumorigenesis makes it an attractive pharmacological target in cancer therapy, and CK2 inhibition proved to be an effective method to induce tumor regression.[92] Most of chemically diverse CK2 inhibitors bind to the ATP pocket[91] such as, for example, CX-4945 (5-(3 chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid, silmitasertib, IC_{50} = 14 nM) which is currently in phase II clinical trials.[209]

Both the isolated CK2α subunits and the holoenzyme are endowed with constitutive activity. However, the regulatory subunit modulates the substrates preference of the catalytic subunit.[77] Previous studies demonstrated that the CK2 subunits interactions are dynamic and that each subunit could be independently found in different subcellular compartment.[203,245] It is

assumed that a dynamic rather than a static interaction of the CK2 subunits may help adjust the kinase specificity to ensure that the relevant form of the catalytic subunit is present at each of these locations. Moreover, several studies[75,228] revealed the role of the regulatory subunit in the control of the epithelial to mesenchymal transition (EMT). Indeed, CK2β down-regulation in epithelial breast cells is sufficient to promote EMT through Snail1 upregulation.[76] However, despite the great importance of the α/β interaction in the regulation of cell polarity and cell morphology, the cellular effectors responsible of its fine control remain unknown. Furthermore, perturbating the prominent CK2α/CK2β interaction with artificial compounds might suppress specific CK2 holoenzyme functions.

Very few potential inhibitors of this interaction have been published[50,51,206,207]. Using a structure-based design approach[50] and a random screening strategy,[206] we previously identified a CK2β-competitive lead compound: Pc, a cyclic peptide that could actually counteract the holoenzyme formation in cell-free assays.[50] In the CK2 holoenzyme structure, a segment located in the N-terminal region of CK2β (L187-H193) forms a β-hairpin loop which inserts deep into a remote hydrophobic groove present in the β4/β5 sheets of CK2α.[28] Two residues, Tyr188 and Phe190 were characterized as the hot-spots and therefore, the peptide Pc was designed from the structure of the CK2β-tail encompassing these two hot-spots. Thus, Pc represents a unique CK2β-based small molecule fully capable of interacting with CK2α and to disrupt the α/β interaction in cell-free assays at 3 µM concentration. Moreover, Pc was shown to disturb the CK2-holoenzyme-catalyzed phosphorylation of CK2β-dependent CK2-substrate proteins.[50] The hot-spots mutations (Tyr5Ala, Phe7Ala; equivalent to Tyr188 and Phe190 in CK2β structure) were sufficient to impair the *in vitro* efficiency of Pc.[50] Later on, both X-ray crystallography data and thermodynamic signatures demonstrated that Pc and CK2β binding to CK2α are similar.[51] Thus, Pc represents the first effective CK2β–competitive compound derived from the C-terminal CK2β segment. However, this peptide antagonist lacks cellular efficiency and its optimization through structural modifications, such as attachment to cell-permeable adducts was required.

In the present study, we described biomolecular engineering of the Pc peptide to promote its cellular activity. Using this peptidic tool, we provide the first evidence of the consequences of a specific disruption of CK2α/CK2β interaction in living cells.

3.3.2   Design of a cell permeable chimeric TAT-Pc

We synthesized a chimeric Pc analogue termed TAT-Pc composed of both active Pc in which the disulfide bond used for cyclization have been replaced by an amide linking the side chain of K1 and to the C-term of G13 (KGGRLYGFKIHGG) and a 13-mer peptide (GRKKRRQRRRPPQ) derived from TAT protein of the human immunodeficiency virus 1.[246,247] This

TAT sequence is a cell-penetrating peptide which can carry element such as drugs, small peptide or nucleic acid into the cells.[248] Thus, the covalently linked TAT-Pc chimeric construct in which the TAT sequence is placed at the N-terminal side of Pc was expected to be cell-permeable (Figure 1). In order to assess the cell penetration, a TAMRA fluorescent probe was added to the construct which could then be located by fluorescence microscopy. The TAT sequence enabled a remarkable uptake of the TAMRA-TAT-Pc construct into the cells as the majority of the peptide was located into the cells in less than 4h (data not shown). We also synthesized a cyclic peptide (TAT-Pc-rand) in which the Pc residues were in a random position was obtained as well as a cyclic peptide (TAT-Pc-mut) with the two Pc hot spots residues (Tyr5 and Phe7) mutated to alanine (Figure 1).

GCRL**YGF**KIHGCG   Pc

TAT KGGRL**YGF**KIHGG   TAT-Pc

TAT G---------------CG   TAT-Pc-rand

TAT GCRL**AGA**KIHGCG   TAT-Pc-mut

TAMRA TAT GCRL**YGF**KIHGCG   TAMRA-TAT-Pc

TAT sequence = GRKKRRQRRRPPQ-
TAMRA = 5-Carboxytetramethylrhodamine
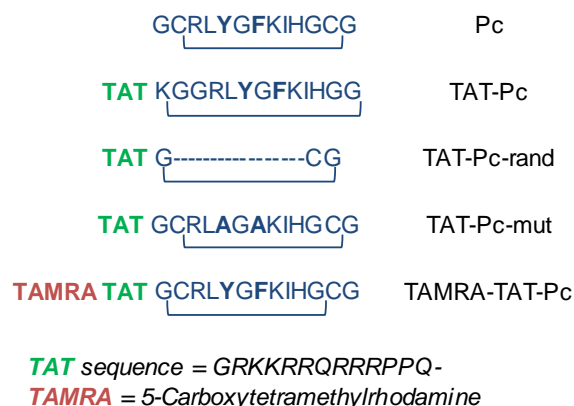
Figure 1: Sequences of the Pc, TAT-Pc, TAT-Pc-rand, TAT-Pc-mut, TAMRA-TAT-Pc. The two spots, Y5 and F7 (corresponding to Y188 and F190 in CK2β sequence) or mutated residues are depicted in bold for clarity.

3.3.3   TAT-Pc suppresses the CK2α/CK2β interaction in living cells

Previous studies have demonstrated that Pc peptide is an efficient CK2β-competitor in cell-free assay.[50,51] Consequently, we were interested in the cellular observation of the CK2α/CK2β interaction upon treatment with the cell-permeable TAT-Pc. Proximity ligation assay is a method of choice to evaluate a protein-protein interaction in living cells as it enables the direct observation of proteins interactions in unmodified cells.[249] Thus, we applied this methodology in HeLa cells. In untreated cells, CK2$\alpha_2\beta_2$ complexes were observable as dots with an average 5.5 interactions per cell (Figure 2). Confirming literature data,[245] the CK2$\alpha_2\beta_2$

complexes were transient as only few sporadic complexes were detected and as they did not overlap with the large CK2α and CK2β expression observed by immunostaining experiments (data not shown). In TAT-Pc treated cells, a striking decrease of the overall number of complex formation was observed with an average number of interactions lower than 2 per cell for a concentration of TAT-Pc equal or greater than 40 µM (Figure 2). These observations suggest that TAT-Pc translocates through cell membranes and behaves as a cell-efficient inhibitor of the CK2α/CK2β interaction.
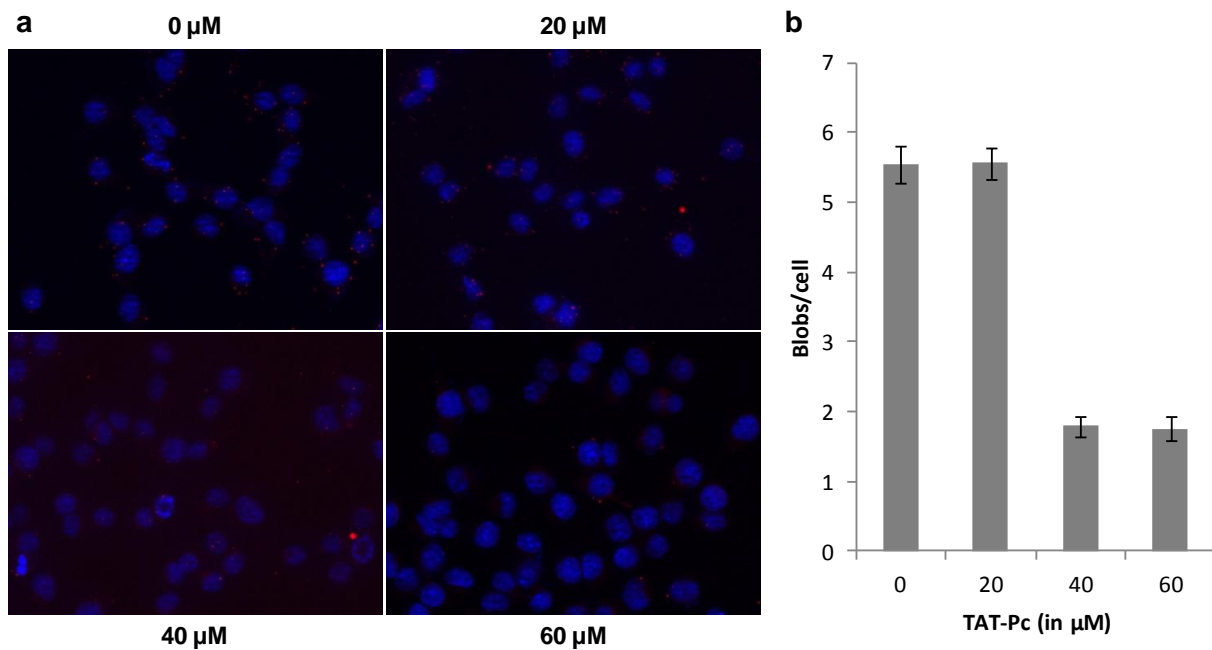
Figure 2: Inhibition of the CK2 subunit interaction in HeLa cells. **(a)** PLA images of HeLa cells treated for 6h with 0, 20, 40 or 60 µM of TAT-Pc. Red blobs represents CK2α/CK2β interaction. Nuclei were stained with Hoechst-33342. **(b)** Quantification of the average blobs per cell using BlobFinder.[250] More than 150 Cells were analyzed for each condition. Error bars represent S.E.M.

3.3.4   CK2 subunits subcellular localization is altered by TAT-Pc

Previous studies demonstrated that CK2 subunits have the capacity to translocate independently from the nucleus to the cytoplasm.[245] We hypothesized that the inhibition of the CK2α/CK2β interaction might affect/disturb the CK2 subunits localization. Indeed, we observed a strinking translocation of CK2α and CK2α' from the nucleus to the cytoplasm upon TAT-Pc

treatment (Figure 3), whereas the catalytic subunits remained mostly nuclear in presence of DMSO or in the presence of the ATP-competitive CX-4945 inhibitor. In contrast, CK2β was mainly located in the cytoplasm in controls as well as in the presence of TAT-Pc. These observations are in agreement with the independent and dynamic regulation of CK2 subunit subcellular localization and highlight the influence of the holoenzyme formation on CK2 subunit translocations.
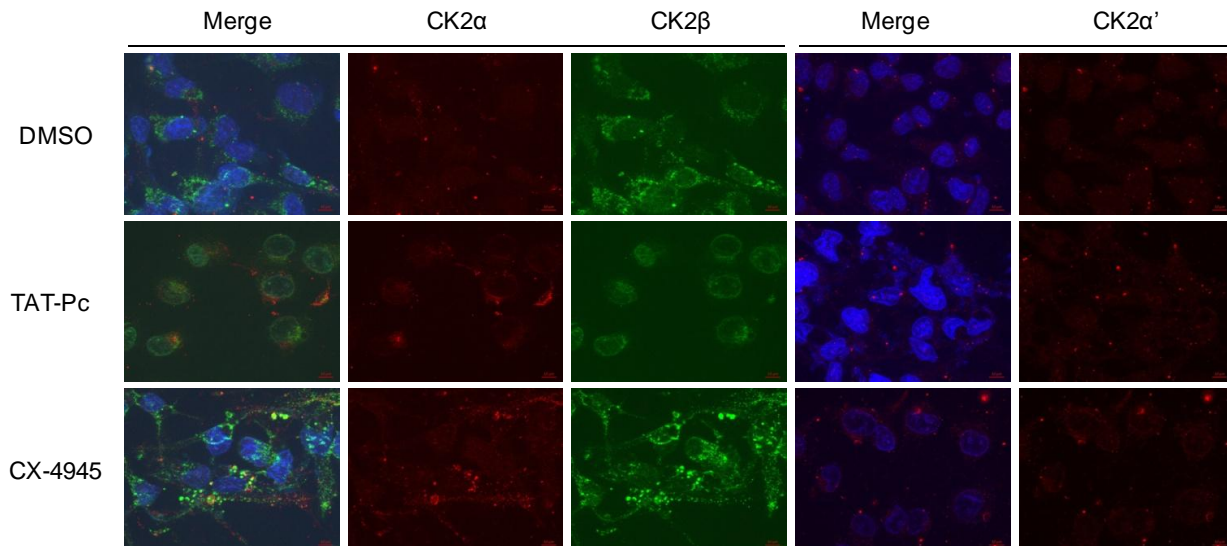
Figure 3: Subcellular localization of CK2 subunits in 786-O renal carcinoma cells. Cells were treated with DMSO as control, TAT-Pc (30 μM) or CX-4945 (12 μM) for 12h before fixation.

3.3.5   Epidermal growth factor receptor (EGFR) down-regulation upon TAT-Pc treatment

Upon stimulation, the EGFR can initiate several downstream signaling cascades leading to cell proliferation.[251] Autophagosome-mediated EGFR down-regulation induced by CK2 inhibition has been documented.[252] As we observed a decrease in cell proliferation after TAT-Pc treatment, we investigated the EGFR status in presence of TAT-Pc or CX-4945. Using immunostaining methods, we observed a striking translocation from the cell membrane to the cytoplasm associated with a down-regulation of EGFR after TAT-Pc or CX-4945 treatments in mammary epithelial cells (MCF10A) (Figure 4). Moreover, these observations were correlated with an increase of endosomal activity attested by the LAMP1 marker. Interestingly, stable silencing of CK2β expression in these epithelial cells (MCF10A-Δβ) led to a similar translocation of

EGFR from the cell membrane to the cytoplasm (Figure 4). Thus, the previously described implication of CK2 in EGFR regulation[252] is dependent on the CK2α/CK2β interaction and its perturbation could lead to a decrease in cell proliferation. This finding may provide a new strategy to target the EGFR signaling pathways and to overcome resistance to EGFR inhibitors.[253]
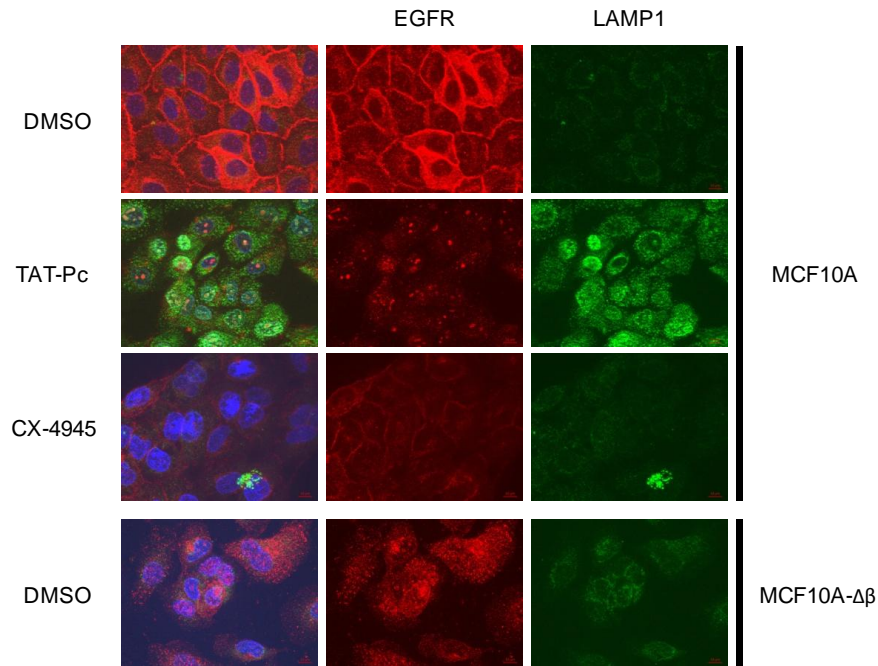
Figure 4: EGFR degradation through lysosomal activity upon TAT-Pc treatment. MCF10A cells were treated for 12h with DMSO as control, TAT-Pc (30 µM) or CX-4945 (12 µM). MCF10A-Δβ cells were used as additional reference. Cells were immunostained with anti-EGFR (red), anti-LAMP1 (green), chemically stained with DAPI (blue), and then analyzed by confocal microscopy.

3.3.6   p21 phosphorylation is increased by TAT-Pc

p21[CIP1/WAF1] is an universal inhibitor of cyclin-dependent kinases such as CDK1, CDK2 and CDK4/6 playing a key role in cell cycle regulation.[254,255] It has been reported that p21 is phosphorylated by CK2$\alpha_2\beta_2$ and interacts with the CK2β N-terminal end *in vitro*.[256] A decrease of p21 (T145) phosphorylation was systematically observed upon treatment with ATP-competitive inhibitors of CK2,[92,257] suggesting that status of p21 phosphorylation could be used as a reporter of CK2 activity. However, in contrast of these previously described results, we did not observe the phosphorylation of recombinant p21 protein by CK2$\alpha_2\beta_2$ in our *in vitro* assay (data not shown). Nevertheless, as p21 phosphorylation seems to be related to CK2 activity in cellular-based assays,

we evaluated this feature by Western Blot analysis of cell extracts. Indeed, a striking dose-dependent increase of p21 (T145) phosphorylation was observed upon treatment with TAT-Pc (Figure 5a-b). Additionally, this effect was not observed upon treatment with the cell-permeable but inactive versions of Pc (TAT-Pc-rand and TAT-Pc-mut) (Figure 5a-b). In contrast, the ATP-competitive inhibitor, CX-4945, promoted a decrease of p21 (T145) phosphorylation (Figure 5a). The TAT-Pc dependent increase of p21 phosphorylation was, of note, a general effect as it was observed in several cell lines (786-O, MCF10 and HeLA).

Interestingly, previous studies showed that the C-terminal segment of p21 (p21[(145-164)]) could adopt different secondary conformations regulating proteins PCNA, CaM and SET binding[258] and the phosphorylation of p21 on the residue T145 prevents PCNA binding.[259] Since the nuclear localization signal of p21 (RKR[(140-142)]) is located near T145,[260] we hypothesized that phosphorylation on T145 could impact the C-terminal structure leading to significant modification of protein binding and of the subcellular localization of p21. Indeed, TAT-Pc treatment triggered a striking nuclear localization of p21 whereas a homogeneous localization in the cytoplasm and in the nucleus was observed in DMSO controls or upon treatment with the ATP-competitive inhibitor, CX-4945 (Figure 5c-d).
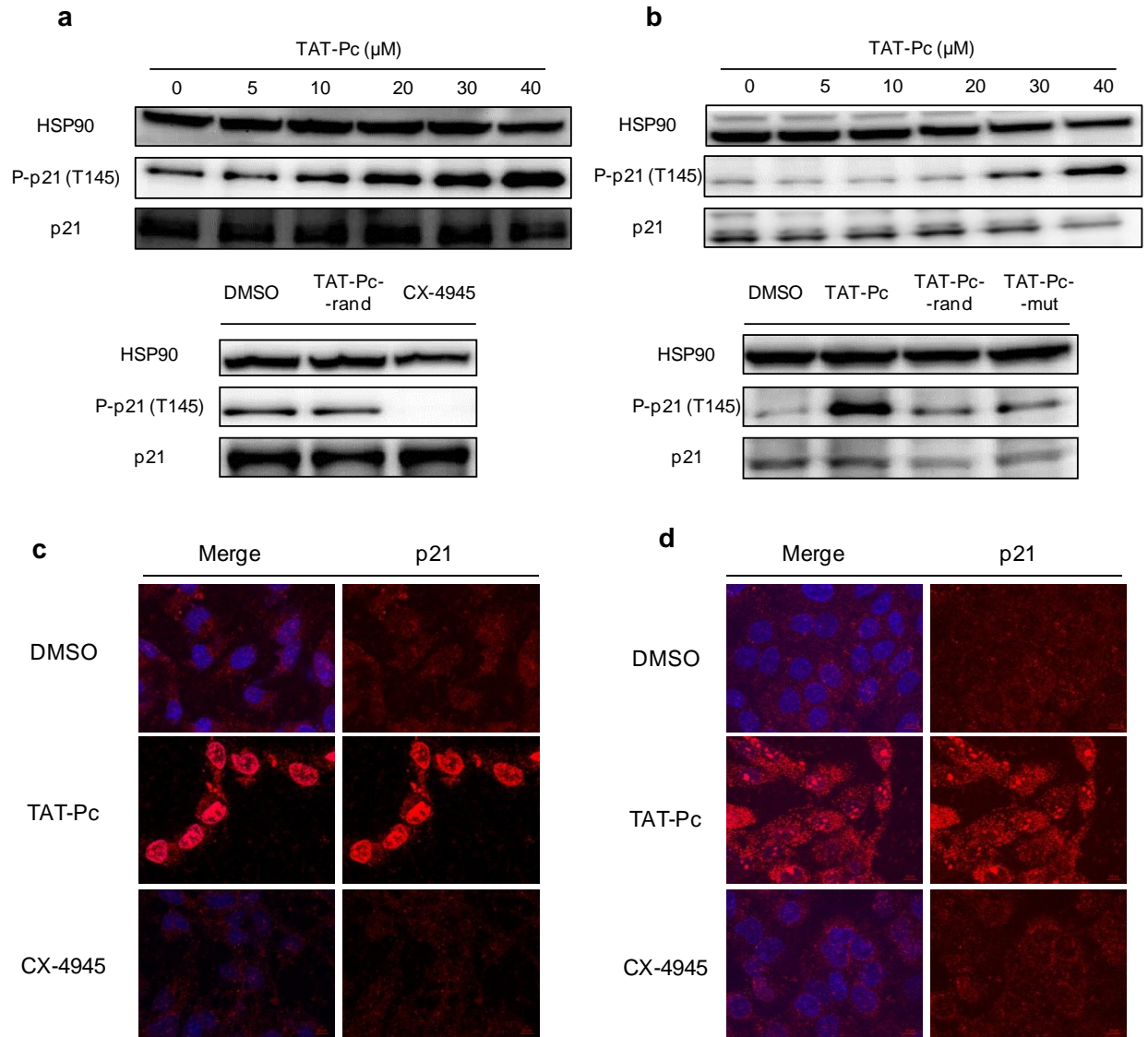
Figure 5: Effect on p21. **(a)** Phosphorylation status of p21 in 786-O renal carcinoma cells. Cells were treated for 48h with TAT-Pc (various concentrations, see upper panel), DMSO, TAT-Pc-rand (40 μM) or CX-4945 (6 μM) (see lower panel). **(b)** Phosphorylation status of p21 in MCF10A cells. Cells were treated for 48h with TAT-Pc (various concentration, upper panel), DMSO, TAT-Pc (40 μM), TAT-Pc-rand (40 μM) or TAT-Pc-mut (40 μM) in the lower panel. **(c)** Subcellular localization of p21 in 786-O renal carcinoma cells treated for 12h with DMSO as control, TAT-Pc (30 μM) or CX-4945 (12 μM). **(d)** Subcellular localization of p21 in MCF10A cells treated for 12h with DMSO as control, TAT-Pc (30 μM) or CX-4945 (12 μM).

3.3.7   Differential effects of CK2α/CK2β interaction inhibition and CK2β knockdown

Previous studies demonstrated that CK2β has a key role in epithelial plasticity and that shRNA-mediated silencing of CK2β expression in epithelial MCF10A led to Epithelial-to-Mesenchymal Transition (EMT) through upregulation of SNAIL1.[76] Therefore, we investigated the effect of TAT-Pc on SNAIL1 expression as well as on two CK2 activity reporters (α-catenin-phospo-S641 and Akt1-phopho-S129). Surprisingly, no changes in Snail1 expression or in reporter phosphorylation level were observed whereas p21 phophorylation was strongly increased upon TAT-Pc treatment (Figure 6). The latter support the concept that, in contrast to CK2β silencing, inhibition of CK2α/CK2β interaction alters CK2 functions in a different manner that conveys in different cell phenotypes. Indeed, both approaches induced th e degradation of EGFR (Figure 4) whereas EMT induction was only observable in the case of CK2β silencing.
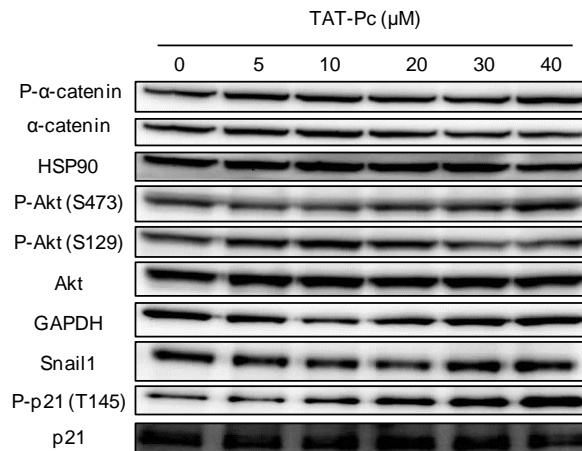
Figure 6: Western Blot analysis of TAT-Pc effects on CK2 activity markers (P-α-catenin, P-Akt), on EMT (Snail1 expression) and on p21 phosphorylation. 786-O renal carcinoma cells were treated with TAT-Pc for 48h prior to Western Blot analysis.

3.3.8   TAT-Pc decreases cancer cell viability

The observation that TAT-Pc induced the EGFR down-regulation and p21 translocation into the nucleus suggested that these two events could respectively lead to a decrease of cell viability and to cell cycle inhibition. Indeed, we observed a significant decrease of HeLa cell viability upon TAT-Pc treatment whereas the inactive TAT-Pc-rand did not exhibit any effect

(Figure 7). Therefore, this effect on cell viability as well as other cellular consequences triggered by TAT-Pc treatment are likely to be related to its ability to inhibit the CK2α/CK2β interaction.
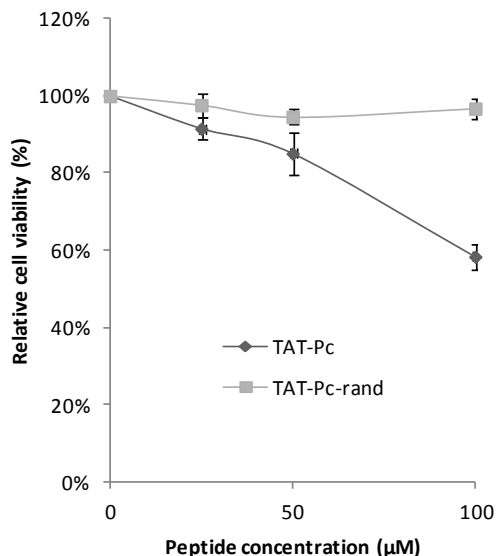
Figure 7: TAT-Pc decreased cell viability. HeLa cells were treated with TAT-Pc or TAT-Pc-rand for 48h before evaluation of cell viability in comparison with DMSO treatment.

3.3.9   Conclusion

Molecules that selectively inhibit the CK2 subunit interaction would be useful in determining the importance of CK2β in the control of the many cellular processes that are governed by this multifunctional kinase. In particular, such inhibitors would provide more rapid and reversible tools than siRNA or overexpression methods for correctly identifying relevant CK2β-dependent substrates. They will also serve as leads for the rational design of function-specific chemical drugs that disrupt some actions of CK2, but leave others intact, allowing the deregulation of specific intracellular pathways. Furthermore, selective disruption of the CK2α–CK2β interaction could find important applications to test pharmacologically the importance of this interaction in tumor cell growth.

3.4 Structure-Based discovery of a small chemical scaffold targeting the CK2 Subunits interface

The small cyclic peptide described in the chapter 3.3 demonstrated the therapeutic interest of the CK2α/CK2β interaction inhibition. However peptides are more difficult to transform into therapeutics than small molecules. Therefore, a virtual ligand screening was considered to identify small molecules able to bind in the α/β interaction pocket.

3.4.1 Virtual Screening and hit validation

A Virtual Ligand Screening directed on fumigated pocket at the interface of CK2α/CK2β subunit was realized by Irina Kufareva and Ruben Abagyan (Skaggs School of Pharmacy, La Jolla, USA). The hundred top scoring compounds were selected and their ability to inhibit the phoshorylation of CK2β-dependent substrate *in vitro* was evaluated. After hit validation and removal of the promiscuous binders, one compound remained, the hit compound **1** (Figure 1). A parent compound (**2**) was commercially available and thus, was purchased.
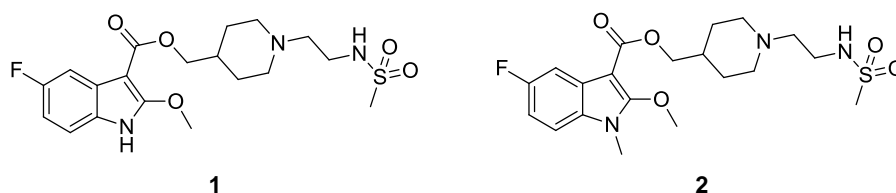
Figure 1. Chemical structures of the hit compound (**1**) from the in silico-screening and the parent inactive compound **2**.

Interestingly, compound **1** was able to inhibit the phosphorylation of CK2β-dependent peptide substrate whereas compound **2** was not efficient (Figure 2a). CK2β-independent peptide substrate phosphorylation was only slightly impacted by **1** and independently of CK2β concentration (Figure 2b) whereas the inhibition of the CK2β-dependent peptide substrate depends on CK2β.
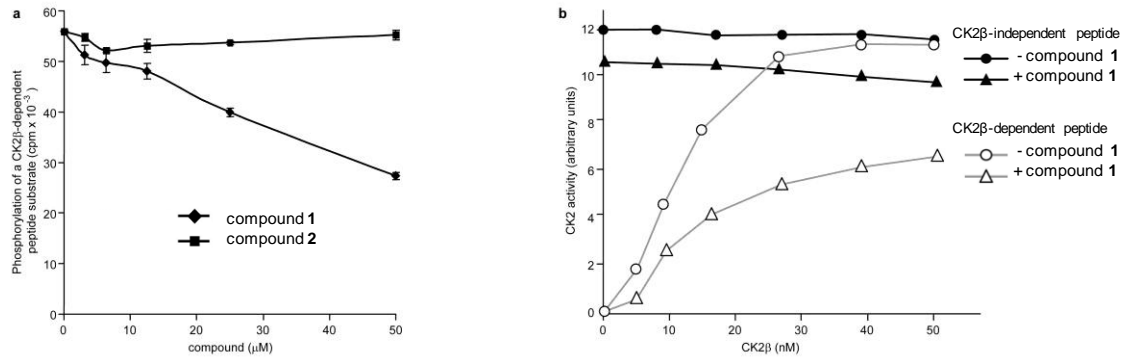
Figure 2. (**a**) Compound **1** inhibits phosphorylation of a CK2β-dependent substrate (eIF2-derived peptide) by the CK2 holoenzyme in a dose-dependent manner. Under the same conditions, compound **2** has no effect; (**b**) Compound **1** affects CK2β-independent peptide phosphorylation to a smaller degree than CK2β-dependent peptide phosphorylation. CK2α (40 nM) was incubated without or with 100 μM compound **1**, in the presence of increasing concentrations of CK2β and assayed for phosphorylation of CK2β-independent and CK2β-dependent peptide substrates (133 and 600 μM, respectively) and 100 μM ATP.

In addition, inhibition of CK2β-dependent phosphorylation by compound **1** can be antagonized by increasing CK2β concentrations (Figure 3a). This result indicates that **1** and CK2β are likely to bind to CK2a in a mutually exclusive mode. Steady-state kinetic analysis of compound **1** was performed by incubation of CK2a with increasing concentrations of CK2β in the presence of different concentrations of the compound (Figure 3b). This experiment suggests a mixed competitive inhibition towards CK2β: Ki was estimated to 66 μM.
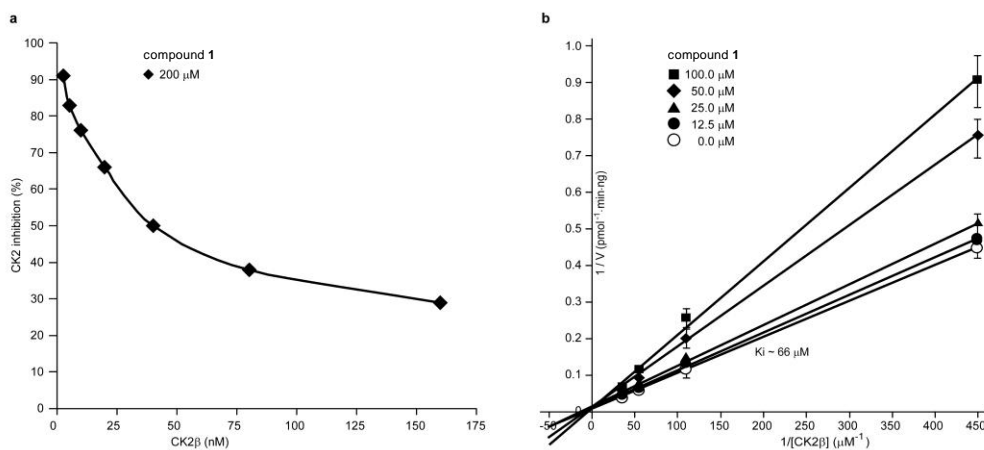
Figure 3. Inhibition of CK2β-dependent phosphorylation by compound 1 is reversed by CK2β. (**a**) CK2α (50 nM) was incubated in the presence of 200 μM compound **1**, saturating concentrations

145

of the CK2β-dependent peptide substrate (600 μM) and ATP (100 μM), and increasing concentrations of CK2β; (**b**) Steady-state kinetic analysis of CK2 complexation showed a Ki of 66 μM. The data represent the average of three experiments performed in duplicate.

Docking of compound **1** in the β-interaction pocket (Figure 4) is consistent with the preliminary results obtained. Moreover, the reason of the inactivity of compound **2** is easy to understand as the N-methyl substitution on the indole will prevent the hydrogen bond between the amino function and the backbone of Tyr39. Furthermore, we can postulate that the size of the methyl prevents the entry of the indole core into the hydrophobic pocket.
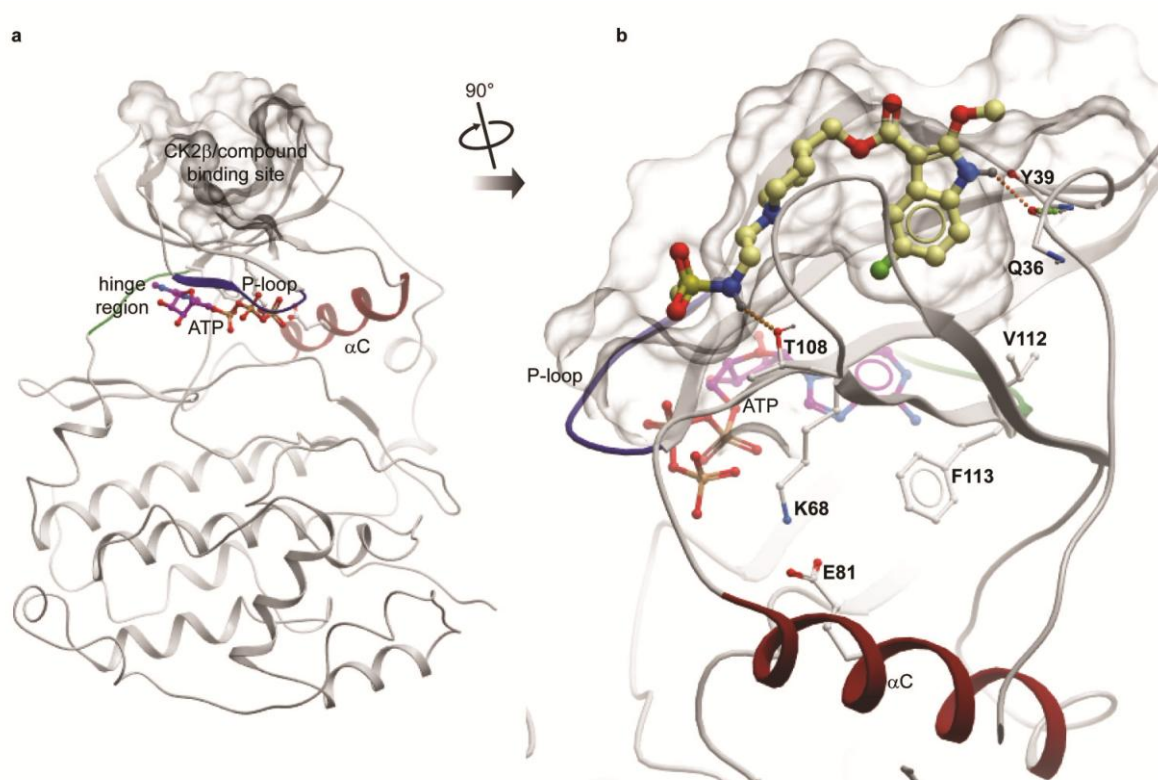
Figure 4. Compound binding mode predicted by ICM virtual docking: (**a**) Overall architecture and compound/CK2β binding site; (**b**) Predicted pose of compound **1**.

The two compounds described above exhibited interesting *in vitro* properties to inhibit the CK2α/CK2β interaction and they demonstrated the druggability of the CK2α/β interaction pocket. Therefore, we considered the development of a small library of compound 1 analogs to

improve their potency and study the structure-activity relationships. A small selection of compounds and their syntheses are presented in the next paragraphs.

3.4.2 Chemical synthesis

3.4.2.1 Linear synthetic approach for the grafting of the lateral chain

A linear synthetic approach was first investigated by Dr. Mohamed Ettaoussi (EA 4446 B2C): three intermediates were required for this strategy.

First of all, the acid function had to be introduced on the C3 indole position. Several methods have been tried and the most effective one had a global yield up to 80 % on 5-nitro-*1H*-indole, with an easy work up and no additional purification was needed (Scheme 1).[261,262]

3a-c   **4a-c**   **5a-c**
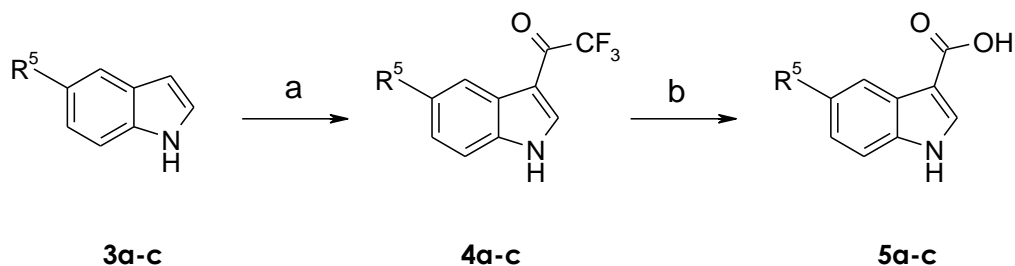
Scheme 1. Acid functionalization in position 3 of the indole. Reagents and conditions: a) TFAA (2.25 eq), anhydrous DMF, 0°C to 150°C, 12h; b) NaOH (10 eq), H_2O, 100°C, 2 h.

Compound **8** was also required and was obtained as described in Scheme 2 from the commercially available ethyl isonipecotate **6** in two steps. First, the amino function was protected with a benzyl group. The ester function was then reduced into an alcohol using lithium aluminum hydride to give compound **8** with an excellent yield over the two steps (Scheme 2).

6   **7**   **8**
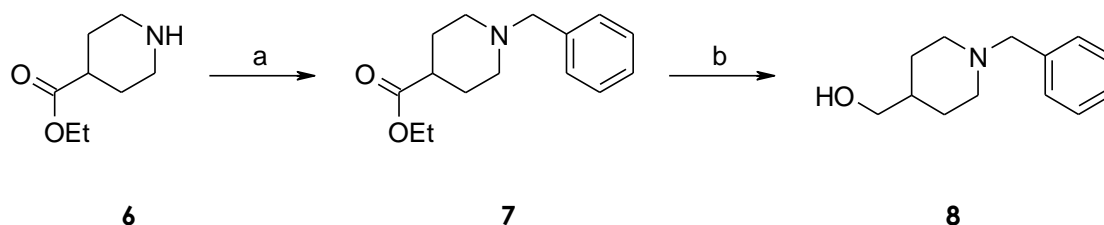
Scheme 2. Synthesis of (1-benzyl-piperidin-4-yl)methanol. Reagents and conditions: a) BnCl, K_2CO_3, EtOH, RT, 48h; b) $LiAlH_4$, THF, 0°C then reflux, 3h.

Chloro derivatives were used to graft the sulfonamide part of the target molecules. Compounds **10** (Scheme 3) were obtained by reaction between chloroethylamine **9** and methyl- or phenyl-sulfonylchloride, in the presence of triethylamine.

9

10a,b

$R^8 = CH_3$, Ph
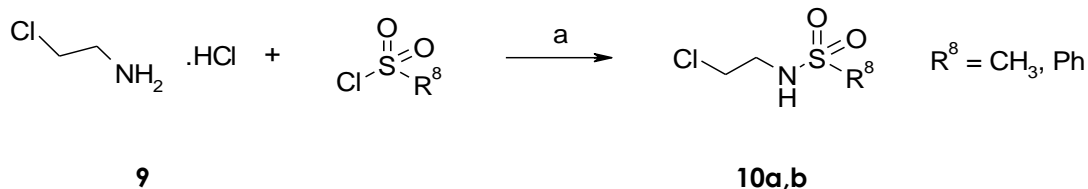
Scheme 3. Synthesis of *N*-(2-Chloroethyl)-methane/phenyl-sulfonylamide derivatives. Reagents and conditions: a) NEt$_3$, THF, 5-10°C then RT.

The linear synthesis consists of 3 steps. From 3-carboxylic-*1H*-indole acid, an esterification reaction was carried out throught acyl chloride intermediate to obtain compound **11** (Scheme 4). The second step was the deprotection of the amino function: the benzyl group was cleaved using a catalytic hydrogenation to give compound **12**. The last step was the amino alkylation with chloro derivatives **10**. The expected compounds were obtained and were isolated among a complex mixture of polysubstitued indoles. The purification of this crude mixture was very difficult to carry out (Scheme 4).

5   **11**   **12**
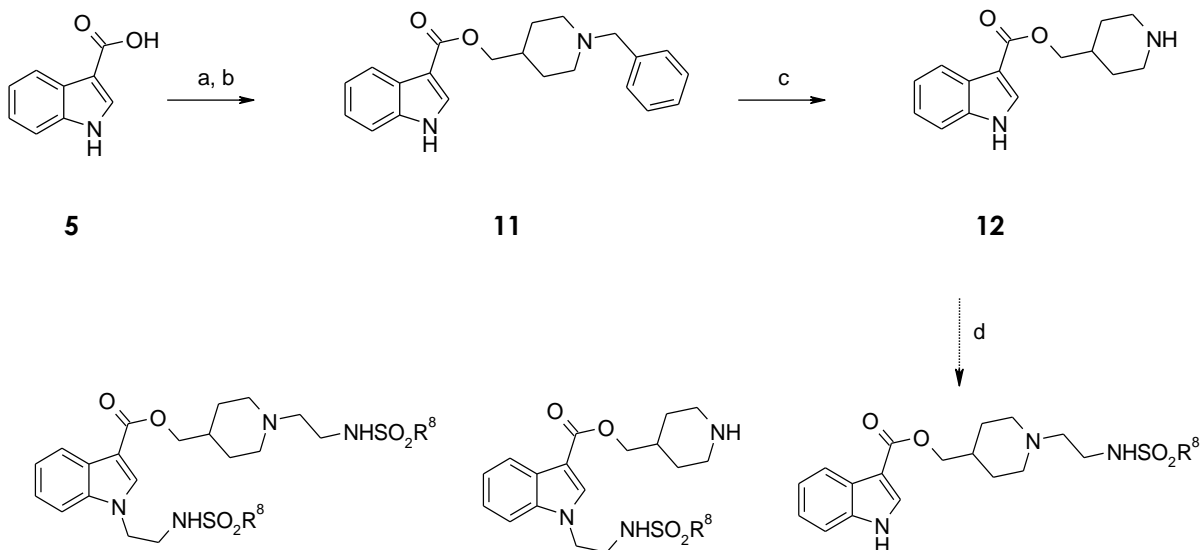
Scheme 4. Synthesis of *N*-(2-Chloroethyl)-methane/phenyl-sulfonylamide derivatives. Reagents and conditions: a) (COCl)$_2$, THF, RT, 24h; b) (1-Benzyl-piperidin-4-yl)methanol (**8**), CHCl$_3$, 0°C then

reflux for 24 h; c) H_2, Pd/C, MeOH, RT; d) *N*-(2-Chloroethyl)-methane or phenyl-sulfonylamide (**10**) K_2CO_3, acetone, reflux for 24 h.

Two main solutions were considered to circumvent this problem:

- The N1 indole protection with a benzenesulfonyl group, for example.
- A new and more convergent synthesis pathway, with the esterification at last step.

The second solution seemed to be the most reliable one because it was well described on similar structures.[263]

3.4.2.2 Convergent synthetic approach

To circumvent the poly alkylation problem at the last step, a new synthetic pathway was applied. The key step was the esterification between an indole 3-carboxylic acid and a functionalized piperidinyl (Scheme 5). Furthermore, these two acid and alcohol precursors were easily obtained in large amounts.

15a-e

5a-c + **14a-b**
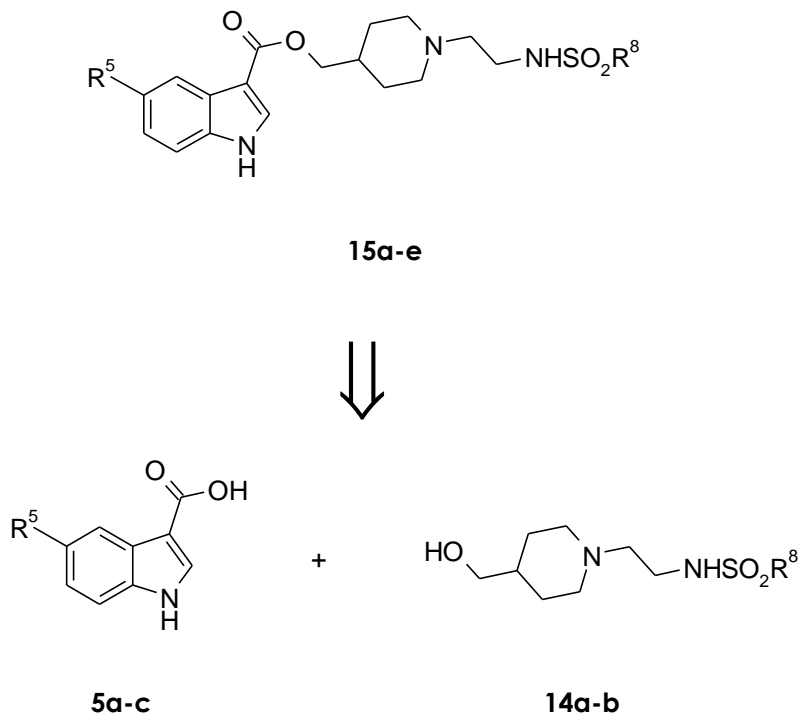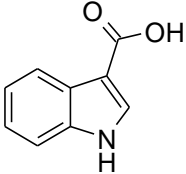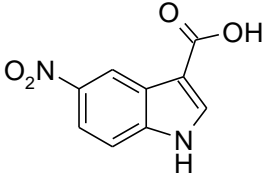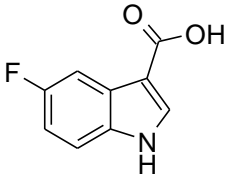
Scheme 5. Retrosynthetic convergent synthetic pathway.

a) Acid intermediates

Acid building blocks **5b-c** were obtained by the TFAA method (Scheme 1) or, in the case of 3-carboxylic-*1H*-indole acid **5a**, from the 1*H*-indole-3-carbonitrile intermediate. With these two methods, six compounds were obtained in good yields (Table 1).

Table 1: Acid derivatives.

Structure/Name	Yield
3-carboxylic-*1H*-indole acid - **5a**	69 % *From 1H-indole-3-carbonitrile*
5-nitro-*1H*-indole-3-carboxylic acid – **5b**	91 %
5-fluoro-*1H*-indole-3-carboxylic acid – **5c**	90 %

b) Alcohol intermediates

Alcohol derivatives were obtained in two steps by a different method than in the linear pathway (Scheme 6).[263–265] The difference in yields between the two analogues **14a** and **14b** (Table 2) could be explained by the products' solubility: precipitation in the last step of the sulfonamide with a phenyl group occurred, whereas it was not observed with the methyl sulfonamide. This precipitation facilitates the isolation of intermediate **14b**. The extraction of the methyl derivative (R^8 = Me) was more difficult leading to a lower yield (30%).
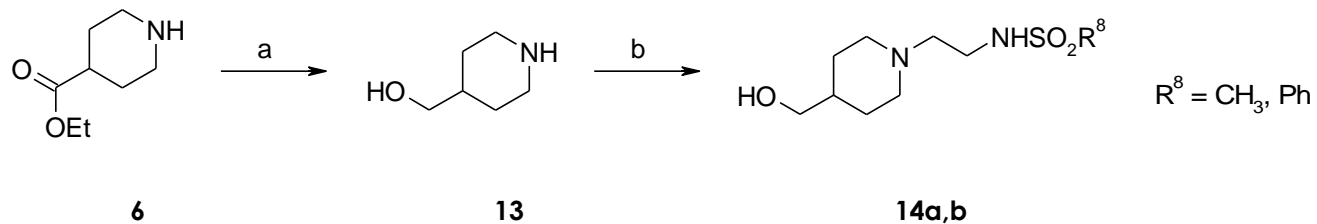
Scheme 6. Synthesis of alcohol intermediates. Reagents and conditions: a) LiAlH$_4$, anhydrous THF, 0°C to RT, 20h; b) *N*-(2-Chloroethyl)phenylsulfonylamide, DIPEA, MeCN, reflux, 2h; or *N*-(2-Chloroethyl)methylsulfonylamide, MeCN, RT, 12h.

Table 2: Alcohol intermediates.

Structure/Name	Yield
N-[2-[4-(hydroxymethyl)-1-piperidinyl]ethyl]methanesulphonamide - 14a	30 %
N-[2-[4-(hydroxymethyl)-1-piperidinyl]ethyl]phenylsulphonamide - 14b	82 %

c) Coupling step

The esterification step described in Scheme 7 is exactly the same for all derivatives, which allowed the use of a Radleys© parallel reaction device.

5a-c

14a,b

$R^5 = H, F, NO_2$

$R^8 = CH_3, Ph$
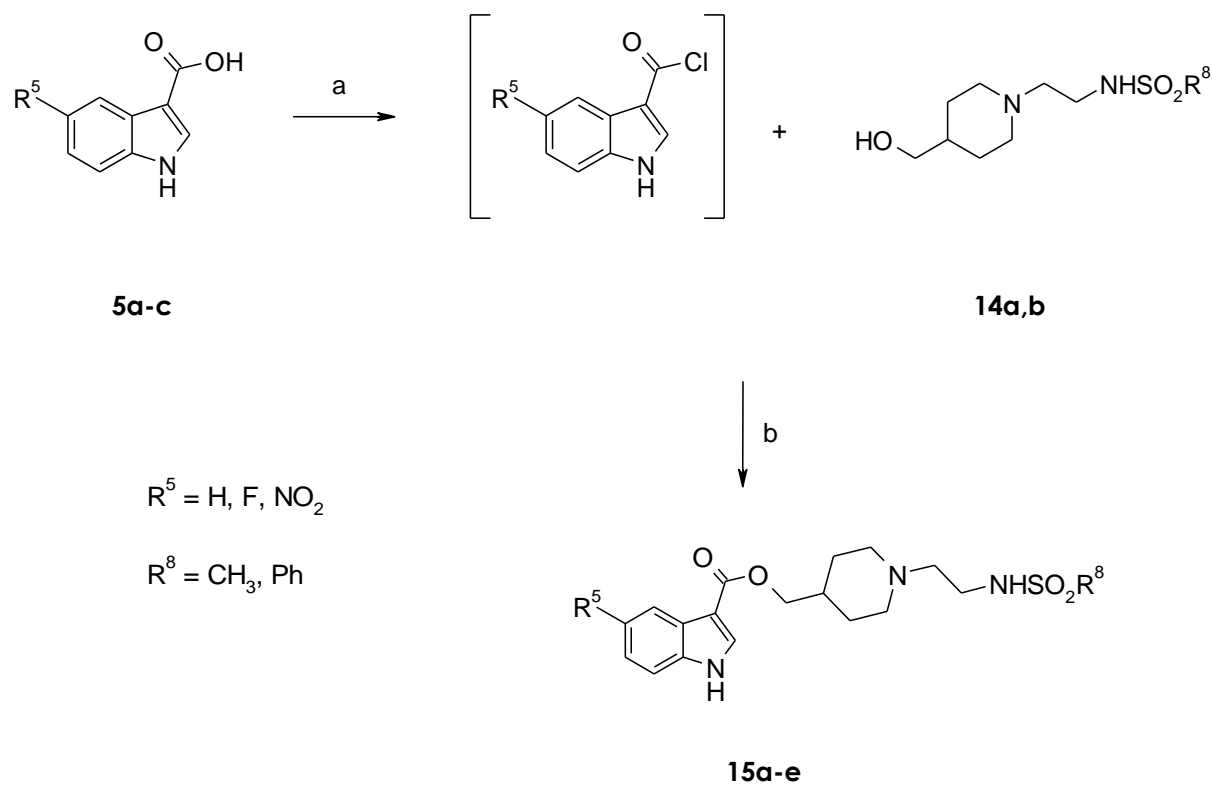
15a-e

Scheme 7. Coupling step through an esterification reaction. Reagents and conditions: a) SOCl$_2$, CH$_2$Cl$_2$, reflux, 24h; b) CH$_2$Cl$_2$, reflux, 24h.

With this method, the small library of compounds **15a-e** was synthesized within few steps and with easily accessible building blocks.

d) Substitution in position 2 of the indole

The insertion of the methoxy in position 2 was also a key reaction because it allowed an additional functionalization on the indole core at the last step.

The method described in Scheme 8 consists of a two-step reaction: first a chlorination with *N*-chlorosuccinimide in dichloromethane followed by addition of the reaction mixture over an acidic methanol solution. This protocol was successfully adapted from litterature[263,266] for the

preparation of all ester derivatives, except with the 5-nitroindole core as LC/MS analyses showed that the reaction did not occur at all.

15c → **16**
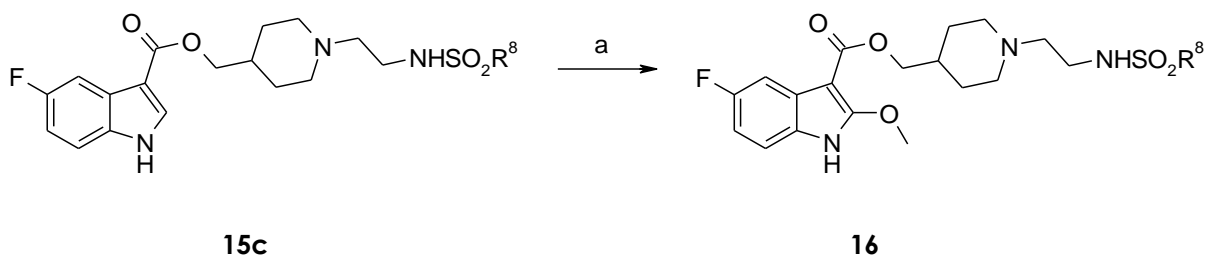
Scheme 8. Methoxy substitution at position 2 of the indole ring. Reagents and conditions: a) NBS, CH_2Cl_2, 0°C, 10 min and then $MeSO_3H$, MeOH, 0°C to RT, 2h.

3.4.3 Cell-free biological evaluation

Compounds were evaluated in kinase activity assays and in SPR dose-response experiments to measure the affinity for CK2a.

Two different peptide substrates were used in these assays:

- peptide 29, phosphorylated either by CK2a or by the holoenzyme (β-independent),
- peptide M, only phosphorylated by the holoenzyme (β-dependent).
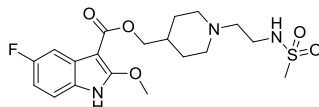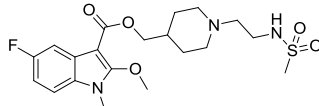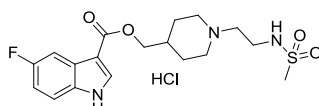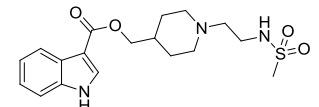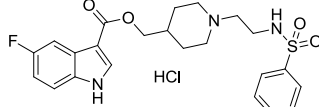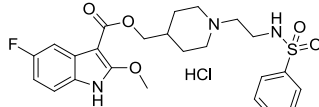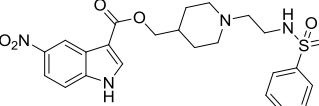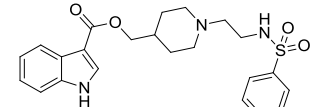
This assay was reliable and reproducible but it measured the kinase activity and not only the direct impact on the a/β interaction. It was performed in three different experimental conditions to evaluate the effect on the a/β interaction as well as the effects on a inhibition (Table 3):

- (a) with peptide 29 (β independent) and a 10 µM ATP concentration,
- (b) with peptide M (β dependent) and a 10 µM ATP concentration,
- (c) with peptide M and 100 µM ATP concentration.

Results obtained from Surface Plasmon Resonance (SPR) and enzymatic experiments were consistent, though the CK2 inhibition was observed in every conditions (a, b and c) of the enzymatic assays. The latter was in opposite of our expectations for a strong inhibition, only in the presence of the β-dependent peptide substrate and independently of the ATP concentration.

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A possible explanation relies on the close proximity of the CK2α/CK2β interaction with the ATP pocket. Indeed, the Tyr39 residue separates the ATP pocket to the bottom of the hydrophobic pocket, in which the indole core is binding. Therefore, binding of other compounds could partially impair the ATP binding according to an allosteric model. This hypothesis was supported by the mixed-type of inhibition observed toward ATP with compound **1**.

Table 3: Evaluation of CK2α affinity and of CK2 inhibition in presence of 50 µM of the corresponding inhibitor.

Compound	Structures	CK2 inhibition (%) in condition			SPR
		(a)	(b)	(c)	Kd (µM)
1		37	55	29	100 (20)
2		nd	nd	15	nd
15a		29	49	13	87 (8)
15b		9	29	25	100 (20)
15c		53	67	44	61 (2)
16		68	80	52	37 (1)
15d		72	79	41	14 (1)
15e		45	49	57	33 (5)

The percentage inhibition is the average of duplicate experiments, performed in the presence of the respective inhibitor at 50 µM and (a) peptide 29 (β-independent) (100

μM), ATP (10 μM); or (b) peptide M (β-dependent) (100 μM), ATP (10 μM); or (c) peptide M (100 μM), ATP (100 μM). Kd values represent the affinity for CK2α and were determined from dose-response curve from SPR experiments. The error in affinity determination is represented between brackets. nd: not determined.

3.4.4   Structure-Activity Relationship Studies

The preliminary SAR study with compound **1** and **2** revealed the importance of the free nitrogen atom on the indole core: this structural motif was thus conserved in all the compounds. The suppression of the methoxy group at position 2 of the indole (compound **15a**) did not impact the affinity for CK2a, the latter is consistent with the docking of compound **1**, in which the methoxy group was exposed to the solvent (Fig 4, b) and did not exhibit specific interaction with CK2a. Next, we studied the influence of the fluoro substitution at position 5 of the indole: our attempt to remove it (**15b**) demonstrated significant effect of this substituent.

At this point, all the compounds (**1**, **15a,b**) exhibited more or less the same affinity for CK2a. Consequently, we hypothesized that this absence of significant effects could be due to the too weak affinities of our compounds for CK2a, which could prevent a good ranking among them. Looking for substantial modifications that could improve the activity of our compound, we envisaged the addition of a phenyl group on the sulfonamide part. Indeed, preliminary docking studies suggested that the phenyl could make a π-cation interaction with the Lys71 and therefore be an asset for CK2a affinity. Indeed, compound **15c** exhibited a better inhibition of CK2 than the parent compound **15a**. Consequently, we studied further the effect of the methoxy group at position 2 of the indole as well as the substitution at position 5, while maintaining the phenylsulfonamide moiety. Interestingly, in this case, the methoxy group at position 2 (**16**) increased CK2a affinity as well as the substitution of the indole in position 5 with a nitro function (**15d**). The fluoro substitution at position 5 (**15c**) was, of note, negative for the activity in comparison with an unsubstituted compound (**15e**).

3.4.5   Biological evaluation in living cells

In the chapter 3.3, CK2a/CK2β interaction inhibition by TAT-Pc led to a decrease in cell viability. Consequently, we considered to begin our evaluation by a cell viability screening of our small set of compounds in MCF10A cells (Figure 5). In accordance with the *in vitro* evaluation,

compound **1**, **15a,b** did not exhibited a good efficiency. However, almost all the cells were dead after a 48h treatment with compounds **15c,d** and **16**. As a remark, precipitation occurred with compound **15d** at a concentration inferior to its cell efficiency concentration, thus preventing further evaluation of this derivative.
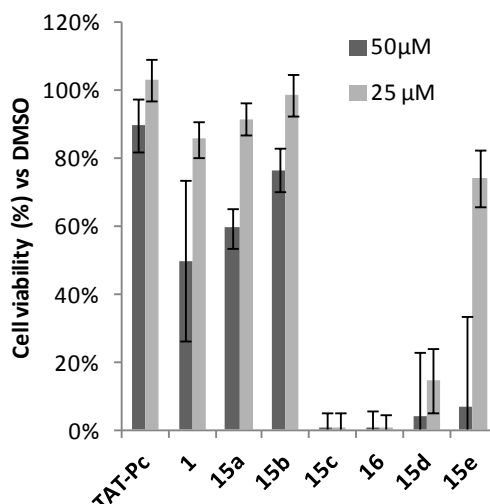
Figure 5. MC10A cell viability after 48h treatment with compounds **1**, **15a-e** and **16**.

In the chapter 3.3, p21 phosphorylation was proved to be a cellular marker of CK2α/CKβ interaction inhibition. Indeed, p21 phosphorylation was increased in a dose-dependent manner and correlated with the cell free potency of various TAT-Pc constructs. Therefore, after 48h cell treatment with compound **15b,e** and **16**, cell extracts were analyzed by western blot to evaluate the phosphorylation of p21 (Figure 6). The less potent compound **15b** exhibited a weak impact on p21 phosphorylation. Indeed, a concentration of 100 µM was necessary to increase p21 phosphorylation. No effect was observed in the presence of 50 µM of compound **15b**, the latter is consistent with the 80 % of living cell observed in the cell viability experiment at 50 µM (Figure 5) and the 25 % of CK2 inhibition at 50 µM measured in the enzymatic assay (Tab 3).

Whereas compound **15e** increased in a dose-dependent manner p21 phosphorylation in the same range than TAT-Pc, compound **16** exhibited a striking better efficiency than TAT-Pc to increase p21 phosphorylation. Indeed, a 20 µM concentration of **16** is enough to reach the maximum level of p21 phosphorylation.
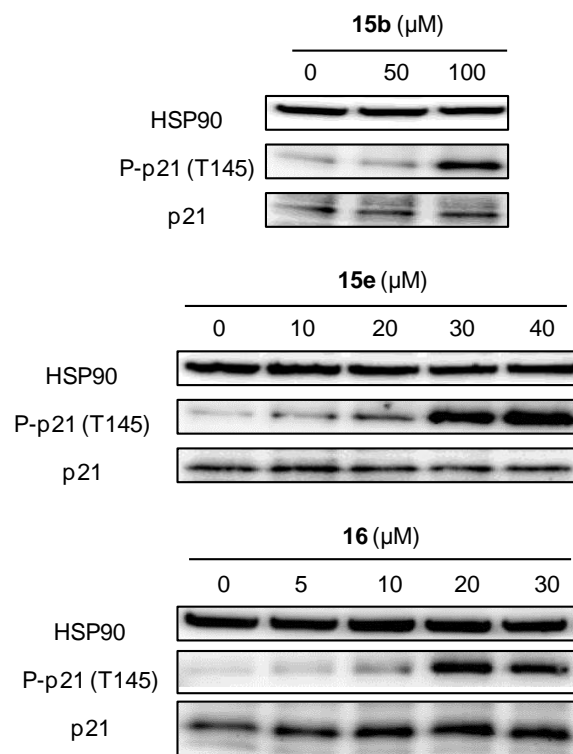
Figure 6. Phosphorylation status of p21 in MC10A after 48h treatment with compounds **15b**, **15e** and **16**.

3.4.6 Conclusion

In this chapter, we identified a small chemical scaffold which targets the CK2α/CK2β interaction after a virtual ligand screening. The effect of the hit compound **1** was *in vitro* characterized in several biochemical assays and demonstrated its ability to inhibit CK2β-dependent substrate phosphorylation and to bind to CK2α in a mutual exclusive mode with CK2β. Then, a small set of compounds was synthesized using a convergent synthesis pathway and led to a first SAR study. Consequently, three compounds were evaluated in a cell-based assay and their ability to inhibit the CK2α/CKβ interaction was assessed through the western blot analysis of a marker of this inhibition. The best compound **16** exhibited a better efficiency than the reference TAT-Pc and could therefore be used as a relevant starting point for the development of new structures aiming to disturb the CK2α/CK2β interaction.

4. Final discussion

Identifying new mechanisms of protein regulation is of great interest as it opens the door to innovative strategies and new targets in therapeutic area. Two hit compounds with atypical mechanisms of action on protein kinase CK2 were identified during *in silico* screening campaigns. Thus, the aim of the present study was to develop these two classes of compounds and to characterize their effects in biochemical as well as in cellular-based assay. Therefore, their novel mechanism of CK2 regulation could be described accurately and the relevance of such approaches to target CK2 could be evaluated. The two classes of compounds could be defined as:

(i)   allosteric modulators of CK2α (4.1)

(ii)   CK2α/CK2β interaction inhibitors (4.2)

4.1   Allosteric modulator of CK2α

A weak hit was identified in a screening process for CK2 inhibitors and further characterization revealed its non-ATP competitive mechanism of action. Therefore, we developed a library of small molecules and obtained several compounds with good overall properties, as described in chapters 3.1 and 3.2.

4.1.1   CK2 specificities and allosteric inhibitors

The discovery of allosteric modulator of CK2 is challenging as CK2 was always observed in an active state.[30] Indeed, in previous studies, only few small molecules were proposed to inhibit CK2α with a non-ATP competitive and non-β competitive mechanism (see Introduction of chapter 1.4.4).[230] Derivatives of the azonaphthalen class of dyes were described to modulate the CK2 activity in a non-ATP competitive manner. However, the precise binding site of these compounds was not characterized. Nevertheless, the use of CK2α mutants and SAXS experiments allowed to approximately localize the binding site near the aC-helix and the activation segment.[150] Unfortunately, these molecules could not be considered as drug-like and their development had to be stopped.

Besides small organic molecules, inorganic PolyOxoMetalates (POMs) have been described as potent allosteric inhibitors of CK2 and one of them, $[P_2Mo_{18}O_{62}]^{6-}$, exhibited a nanomolar IC_{50} and a great selectivity in a panel of 29 kinases.[151] POMs are anionic complexes of early-transition metal ions and oxo ligands that lack cellular activity. Thus, there is few perspectives to develop them further into *in vivo* active agents. Biochemical analyses revealed that the mode of inhibition was mixed-type with respect to ATP and non-competitive toward the

peptide substrate. Partial proteolysis by trypsin followed by amino acid sequencing showed that two distinct fragments were protected from degradation by POM binding. One comprised of the N-terminal domain and three β strands (β1, β2 and β3) encompassing the glycine-rich loop, whereas the other one consisted of the activation segment and the aF and aG-helices.[151]

Hence, the putative binding sites of the negatively charged azonaphthalenes and POMs molecules show an overlap in the region encompassed by αC-helix, glycine-rich loop and activation segment. This region plays a crucial role in kinase regulation, and several allosteric kinase inhibitors were demonstrated to act through αC-helix displacement.[129] The constitutive activity of Protein kinase CK2 is mainly due to the unique interaction between the N-terminal segment and the activation loop abolishing the necessity of any activating phosphorylation. In consequence, the activation loop is locked in the open-active conformation, and this region is one of the less flexible in CK2α crystal structures, suggesting that any displacement by small molecules might be a tricky challenge.[30] On the other hand, the glycine-rich loop is one of the most flexible regions of CK2α, which might facilitate a potential induced movement of this loop by small molecules.[31] In most of the CK2a crystal structures, the conformation of the glycine-rich loop is maintained by two hydrogen bonds linking the Tyr50 of the glycine-rich loop and two lysine residues (Lys74, Lys77) of the basic cluster. However, two crystal structures (PDB ID: 3FWQ[149], 4UB7[218]) make exception by the lack of these key hydrogen-bonds and the collapse of Tyr50 in the ATP binding site. No explanations such as small molecule presence or crystallographic conditions were proposed as probable reason of the inactive conformation observed in 3FWQ.[149] However, in the case of 4UB7, they proposed that a specific halogen bond between an ATP-competitive inhibitor and the Tyr50 in presence of high salt concentration could be responsible of the glycine-rich loop displacement.[218] All the hints from the literature described above let us believe that CK2 could harbor an allosteric pocket in the region encompassing the activation segment, the glycine-rich loop (P-loop) and the αC-helix (Fig 42a). Therefore, preliminary docking experiments as well as single-alanine mutagenesis experiments were focused on this area and led to the determination of the precise interactions between our compounds and CK2 (Fig 42b).
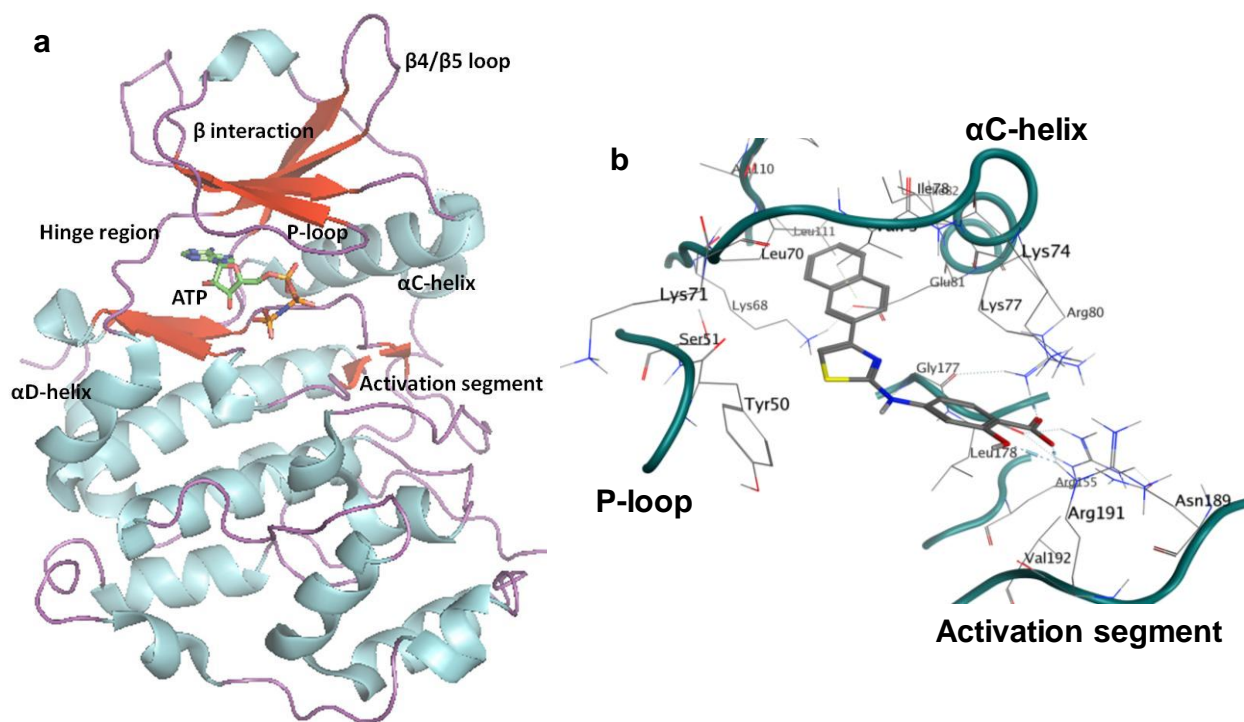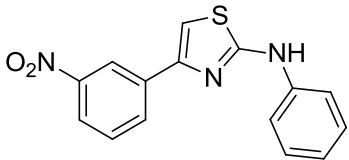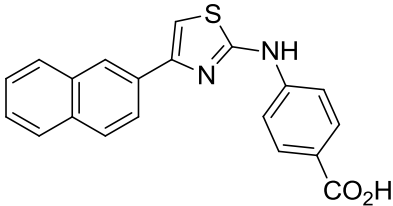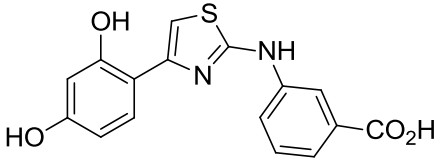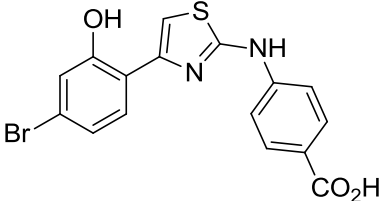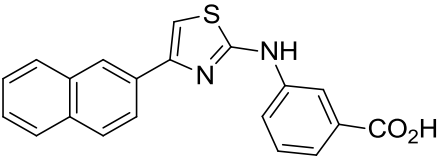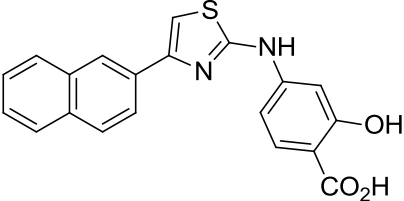
Fig 42: Key features of the structure of CK2 and of its allosteric pocket. (a) Key features of the structure of CK2 (based on 2PVR[110]). (b) Allosteric pocket of CK2 with 3.2-8e docked inside (based on PDB ID: 3JUH[33]).

4.1.2   X-ray structures

Co-crystallization experiments in collaboration with Jean-Baptiste Reiser (Institut de Biologie Structurale, Grenoble) were envisaged to identify the binding site of our compounds. After several campaigns, suitable co-crystallization conditions were identified (based on PEG3350, pH 5.5, ammonium sulfate), and after a scale-up, diffracting crystals of CK2α in the presence of several 2-aminothiazole derivatives were obtained (Table 4). Of note, the resolution obtained with the compound **3.1-3** was too low (superior to 3 Å) to lead to precise structure: its refinement was therefore not pursued. In addition, crystals obtained in the presence of compound **D3,35** (Table 4) were found without any ligand. However, electron density modification, representing compounds **3.2-7j**, **3.2-7e** and **3.2-8e** were identified: those compounds were located into the ATP pocket. The main interaction of our compounds with CK2 was between the carboxylic acid and the K68/D81 residues, with the cooperation of a water molecule (Fig 43). Of note, the same type of interaction is visible in CX-4945/CK2α co-crystal structures (PDB ID: 3NGA and 3PE1).[97,214] Moreover, the overall structure of CK2α in the presence of our inhibitors is similar to those obtained in the presence of ATP-competitive inhibitors or

AMPPNP (PDB ID: 2PVR, 3MB6, 3MB7, 3NSZ).[110,267] Therefore, based only on X-ray results, our class of compounds should exibit an ATP-competitive mechanism of action. Consequently, we tried to answer to the key question: "How can we link the X-ray diffraction results with the whole set of our data?"

Table 4: Main parameters of the CK2α X-ray structures obtained in the presence of various compounds.

Compound	Structure	Resolution	Electron density of the inhibitor
3.1-3		> 3 Å	-
3.2-7j		2.65 Å	Yes
D3,35		2.85 Å	No
3.2-7e		2.85 Å	Yes (partial)
3.2-8c		2.40 Å	partial
3.2-8e		2.20 Å	Yes
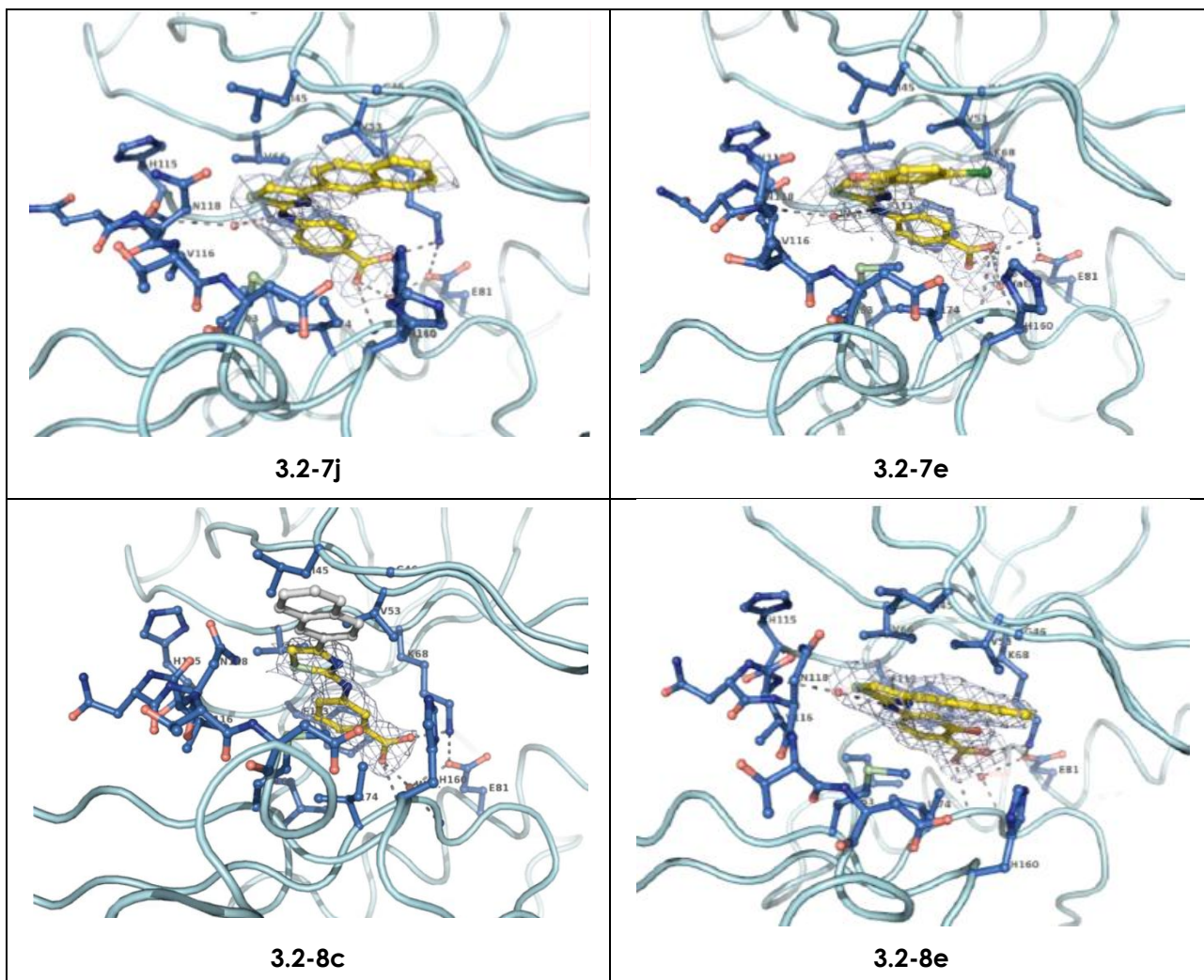
Fig 43: Representation of the ATP-binding pocket with several of our inhibitors, obtained from the X-ray structures.

The incompatibility of the results obtained from X-ray structures and the ones obtained from enzymatic kinetics, TdCD, STD-NMR and mutagenesis experiments (chapter 3.1) needed to be explained. Indeed, no information in the X-ray structures is in accordance with the mixed-type of inhibition, neither the lower thermal stability, nor the effects of V73A and K77A mutants. In addition, STD-NMR experiments clearly showed that 2-aminothiazole derivatives and CX-4945 could simultaneously bind to CK2α. The latter is obviously impossible if 2-aminothiazole derivatives would bind into the same pocket than CX-4945, ie the ATP-site.

Crystal packing or crystallization conditions could be the source of the obtained incoherent structures. Indeed, co-crystals were obtained under special salt and pH conditions (pH = 5.5) in which CK2α does not show any catalytic activity. In addition, X-ray structures are by definition obtained from a solid state which could block a specific conformation of the protein.

Due to their chemical structures, our compounds could certainly bind to the ATP-pocket, as seen in the X-ray structures. However, based on strong evidences, we have demonstrated that they could also bind to an allosteric pocket. Furthermore, the high cellular efficiency exhibited by compound **3.2-8e**, in comparison with ATP-competitive inhibitors, suggested that the cellular effects of our compounds relied on the allosteric mechanism of action.

Therefore, further co-crystallization campaigns are required to find the proper conditions to obtain new X-Ray structures consistent with the rest of our data. Additional attempts of co-crystallization, in presence of both CX-4945 and one of our inhibitor, will be considered.

4.1.3 Similarities and differences with others allosteric inhibitors

αC-helix displacement is a common method for allosteric inhibition of protein kinases[129] and several small molecules were described to bind near the aC-helix.[128,140,148,268] Interestingly, as in the case of our allosteric inhibitors of CK2, the allosteric inhibitor of MEK1, PD318088,[143] and the allosteric inhibitor of ERK2, SCH772984[148] (Fig 44), bind at the interface of the aC-helix and of the glycine-rich loop, also called P-loop (Fig 45a).

PD318088 (MEK1/2 inhibitor) **SCH772984** (ERK1/2 inhibitor)
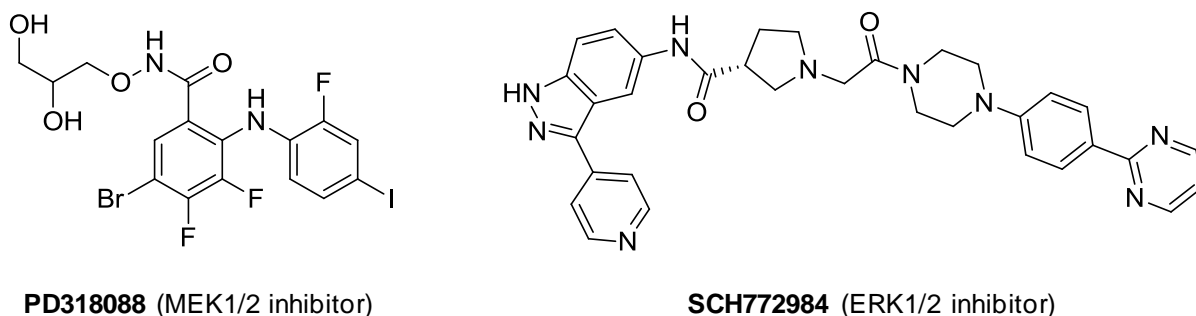
Fig 44: Chemical structures of PD318088 and SCH772984

The inactivation of MEK1 occurs through a small displacement (0.9 Å) of the αC-helix as well as a larger movement (2 Å) of the short helix present in the activation segment.[269] The allosteric inhibitors of MEK1 (*eg* PD318088) stabilize this inactive conformation[143] and, interestingly, they bind to the protein in the presence of an ATP molecule or an ATP analog, with a synergistic effect.[144] Structure superimposition showed that the binding site of the allosteric MEK1 inhibitors (PD318088) and the allosteric CK2 inhibitor **3.2-8e** are in close proximity and share some similarities (Fig 45a,b). Nevertheless, the presence of the small helix in the activation segment of MEK1, and its flexibility combined with the one of the αC-helix, enable a deeper binding of the

allosteric MEK1 inhibitors in comparison with CK2 inhibitor **3.2-8e**. Moreover, the basic cluster of residues located in the aC-helix and in the activation segment of CK2, and necessary for **3.2-8e** binding, is absent in MEK1. Additionally, allosteric CK2 inhibitors and the ATP-competitive inhibitor, CX-4945, could bind together to CK2 (see chapter 3.1) but they could not bind in the presence of ATP or ATP analogs whereas MEK1 inhibitor can. Therefore, despite some similarities in their binding site, allosteric CK2 inhibitors and allosteric MEK1 inhibitors are very different and **3.1-7** and **3.2-8e** did not inhibit MEK1 (see the kinase panel, chapter 3.1 and 3.2).

SCH772984 is a potent and selective ERK inhibitor,[147] which acts through a simultaneous binding in the ATP-site and in the P-loop pocket.[148] The piperazine-phenyl-pyrimidine moiety of SCH772984 binds into the P-loop pocket near the αC-helix, with the P-loop Tyr36 shifted to the "in" position (Fig 45). The latter is similar to the binding of the naphthyl moiety of **3.2-8e** which also requires the switch to the "equivalent of Tyr36" in the CK2 structure, *ie* Tyr50. Interestingly, CK2α and CK2α' are strongly inhibited by SCH772984 but their mechanism of inhibition is not described.[148] However, SCH772984 also inhibits the kinase Haspin but in an ATP-competitive manner and the same mechanism could be suspected for CK2 as the residue Tyr64 of ERK1 necessary for SCH772984 binding is replaced by a lysine in the case of CK2.

Despite strong differences which enable to design highly selective inhibitors, the similarities in the binding pocket of these three compounds open the possibility to have similar pockets in other related kinases.
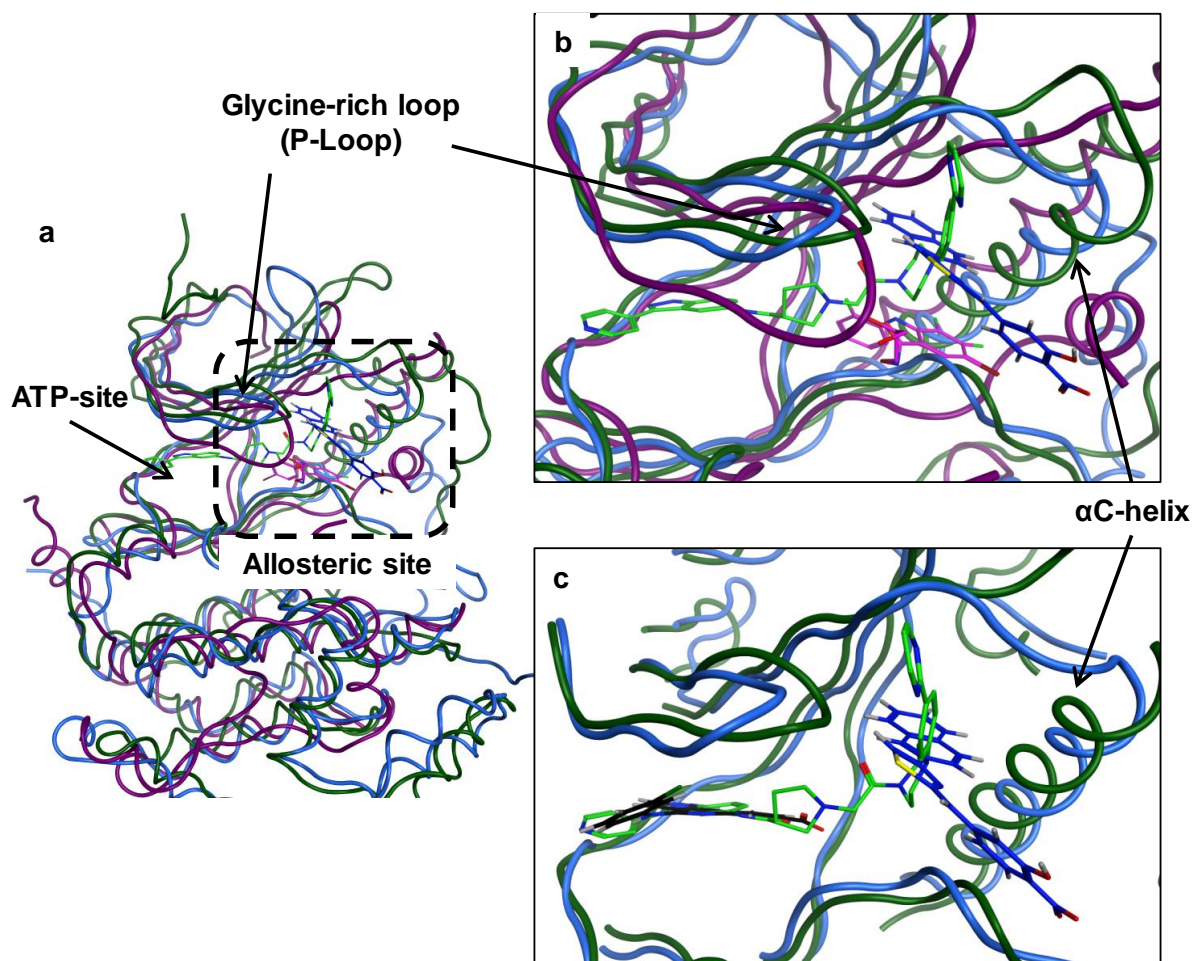
Fig 45: Allosteric binding site in CK2, MEK1 and ERK2. Structure from molecular modeling of CK2 (blue, based on PDB ID: 3JUH[33]) and X-ray structures of MEK1 (magenta, PDB ID: 1S9J[143]) and ERK2 (green, PDB ID: 4QTA[148]) were superimposed. Respective allosteric inhibitors for each kinase, 3.2-8e for CK2, PD318088 for MEK1 and SCH772984 for ERK2 were depicted in dark blue, pink and bright green. (a) Overview of the kinase structures. (b) Overview of the allosteric pocket located between the αC-helix and the glycine-rich loop. (c) For clarity, only CK2/3.2-8e and ERK2/SCH772984 were conserved. The ATP-competitive inhibitor of CK2, CX-4945 (black), was docked in the ATP-pocket.

4.1.4   Indirect effect in the single-alanine mutagenesis experiments

In the single-alanine mutagenesis described in the chapter 3.1 and 3.2, we noticed an impact of the His160Ala mutation: a 2.2-fold increase of the IC_{50} was observed with **3.1-7**, and 1.7-fold for **3.2-8e**. Nonetheless, the His160 was not in direct interaction with our compounds in

the 3D structure models. Interestingly, an hydrogen bonding between Tyr50 and His160 was observed in the inactive structure (4UB7) described by Guerra, B. *et al*. (Fig 46)[218] Thus, we could hypothesize that the His160 residue is necessary to stabilize Tyr50 in the inactive conformation, and so for the efficient binding of our compounds.
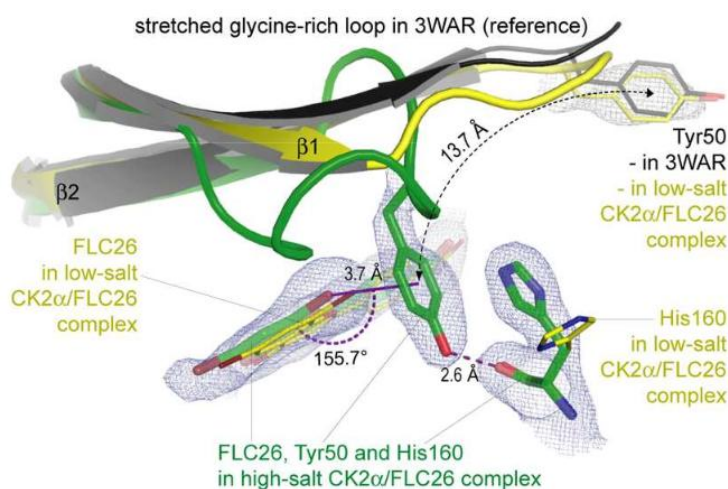
Fig 46: Glycine-rich loop displacement (from Guerra, B. *et al*.[218]).

4.2   CK2α/CK2β interaction inhibitors

Different effects than with "classical" CK2 inhibitors were expected, as the regulatory subunit modulates the selectivity of CK2 toward a subset of substrate proteins.[77]

Previously, a cyclic peptide (Pc) was identified as a potent *in vitro* modulator of CK2α but it was ineffective in cellular based assay.[50] Therefore, this peptide was conjugated to a viral TAT sequence and the resulting compound TAT-Pc was used in cellular-based assay. At the same time, a Virtual Ligand Screening (VLS) campaign was performed and several small molecules inhibitors of the α/β interaction were identified. After hit validation, one molecule **3.4-1** was selected as a true α/β interaction inhibitor. A small library of analogs was synthesized and then evaluated in biochemical assays and in cellular assays.

4.2.1   Structurale similarities with other related compound

Previously, 5,6-Dichlorobenzimidazole-1-β-D-ribofuranoside (DRB) was suggested to bind to two different sites: the ATP pocket and the "remote cavity" in the α/β interaction, although only CK2α inhibition through the interaction with the ATP pocket was proven.[207] However,

interestingly structural similarities could be observed between DRB and our class of compounds **3.4-1** (Fig 47). Indeed, both compounds share hydrophobic moiety (benzimidazole or indole) which could deeply bind into the hydrophobic pocket, occupied by the Phe190 of CK2β. In addition, the more polar moiety (ribofuranoside or ester/methoxy functions) are more exposed on the surface of CK2α. Of note, active compounds from our library have an additional side chain composed of a piperidine linked to a methyl- or phenyl-sulfonamide. The latter function is responsible of the strict binding into the α/β interaction pocket, thus leading to a better observed affinity for CK2.
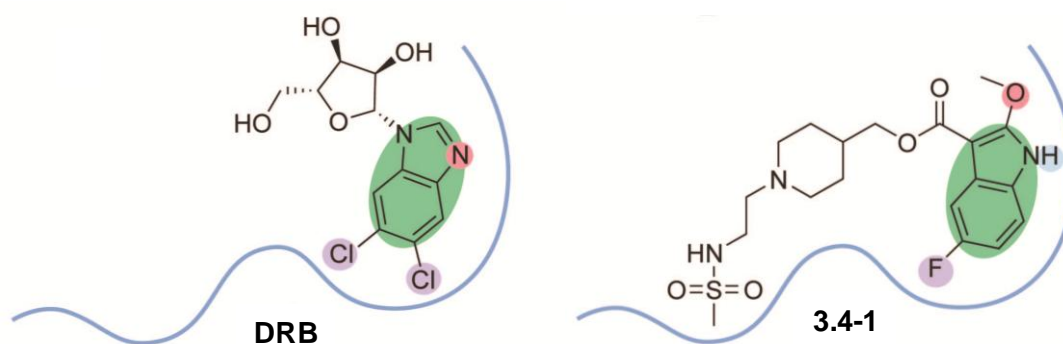
Fig 47: Schematic representation of the DRB (based on PDB ID: 3H30[207]) and compound 4.2-1 (based on docking experiments) in the α/β interaction pocket (blue line).

4.2.2   Effects in cell signaling

Both small molecules and the TAT-Pc construction induced different cellular effects than the ATP-competitive inhibitors or the allosteric inhibitors described in chapters 3.1 and 3.2. As described in Fig 48, CK2α/CK2β inhibitors led to degradation of EGFR which could block the MAPK, Akt and JNK pathways, therefore leading to a decrease of cell proliferation.[270] An increase of p21 phosphorylation correlated with its translocation to the nucleus was also observed, the latter is likely to induce cell cycle arrest.[260,271] In consequence, CK2α/CK2β interaction inhibitors seem to have a great potential in cancer therapy.
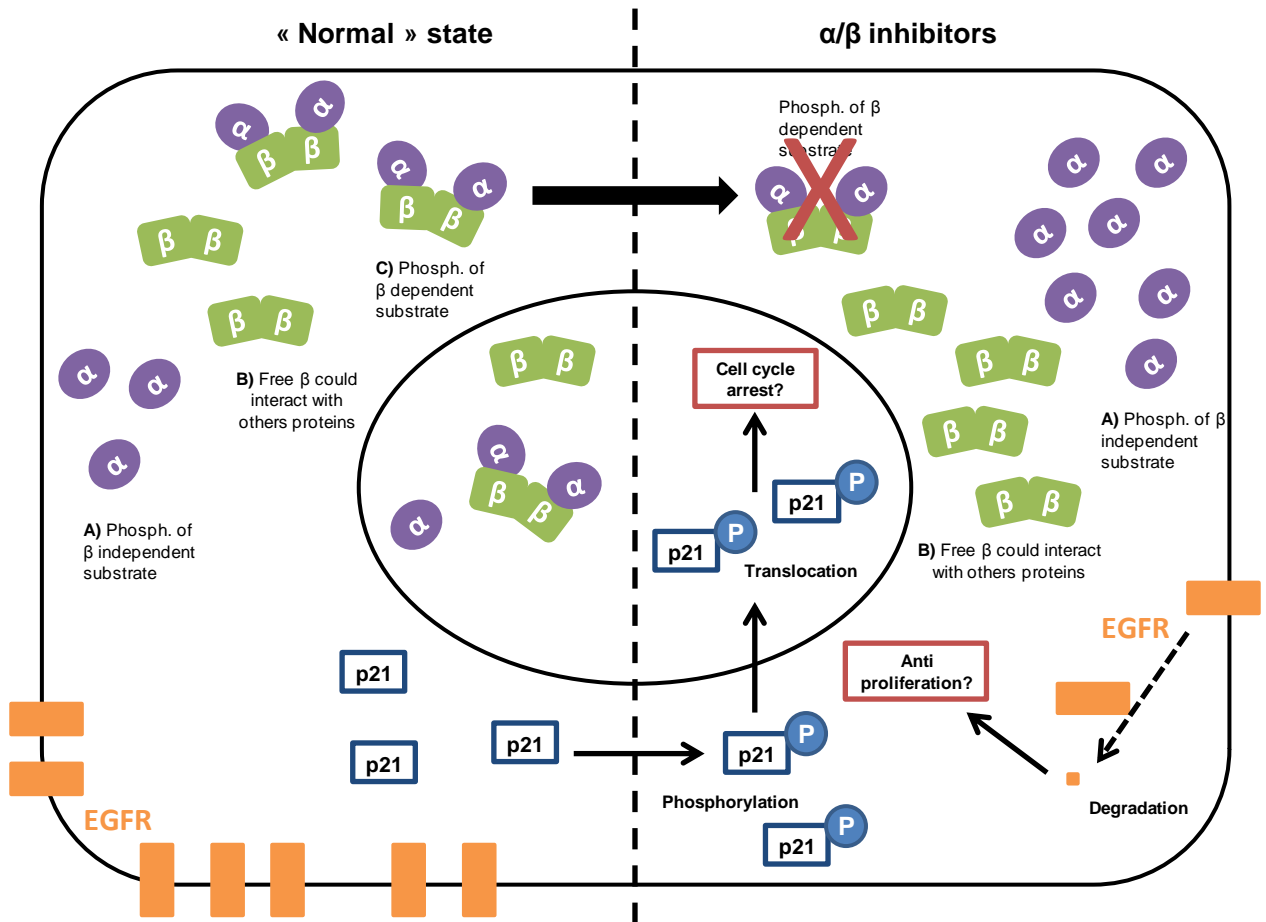
Fig 48: Schematic representation of the cellular consequences of the CK2α/CK2β interaction inhibition. "Normal" state represents the cell in absence of α/β interaction inhibitor and the dash line is the separation with the cell in presence of α/β interaction inhibitors.

4.3   Perspectives of further drug development

The two classes of compound described herein are of potential therapeutic interest. Therefore, the question of their further development into *in vivo* candidates emerged. Compound **3.2-8e** for the allosteric inhibitor and compound **3.4-16** for the protein-protein interaction inhibitor are the respective lead compound of each class (Fig 49).

Allosteric modulator of CK2α

Protein-protein interaction inhibitor (PP2I) targeting the CK2α/CK2β interaction

3.2-8e

3.4-16

362.4 g.mol^{-1}

489.6 g.mol^{-1} (free base)

EC$_{50}$: 3-6 µM (CK2 activity inhibition)

EC$_{50}$~ 15 µM (p21 phosphorylation)
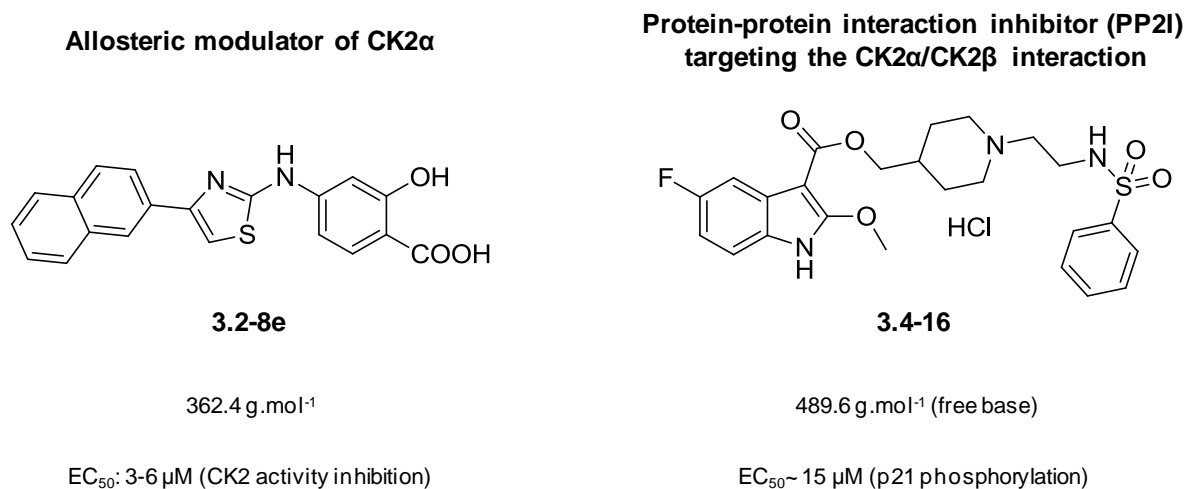
Fig 49: Overview of the lead compounds of each class.

4.3.1   Allosteric modulator of CK2α

Compound **3.2-8e** demonstrated acceptable cell efficiency despite a strong binding to bovine serum albumin. Indeed, decrease of the serum concentration in the cell culture medium led to significant improvement of its cellular efficiency. This feature is in accordance with the chemical structure of **3.2-8e** whose acid function attached to a strong hydrophobic naphthyl part is the good pharmacophore for albumin binding.[272] The structural optimization of this compound in order to decrease the albumin binding is tempting, especially to modify the naphthyl ring, which is not really drug-like. And this appear to be feasible, thanks to the low molecular weight (362.4 g.mol^{-1}) of compound **3.2-8e**. However, literature reviewing demonstrated that 24% of the recently approved drugs have a plasma protein binging higher than 99% and that the low intrinsic clearance is the most important parameter to reach an acceptable *in vivo* unbound level of drug.[273] Therefore, **3.2-8e** does not have to be optimized before further *in vivo* development in reason of its binding to albumin. Nevertheless, the sharp

SAR and the recent identification of the binding site could enable a quick optimization of compound **3.2-8e** in regard to potency and drug-like properties.

4.3.2   CK2α/CK2β interaction inhibitors

On the other hand, the optimized compound, **3.4-16**, belonging to the CK2α/CK2β interaction inhibitors is still in the preliminary development. Indeed, the SAR sample size appears to be relatively limited and further studies could lead to significant improvement of it affinity. Compound **3.4-16** demonstrated the feasibility of the CK2α/CK2β interaction inhibition and its therapeutic interest. Thus, the novelty of this class of compounds is remarkable. However, protein-protein interaction inhibition is highly challenging and requirement large and long studies.[178,187,188] Consequently, further STD-NMR, X-ray crystallography, SAR studies and cellular evaluations are necessary prior to consider *in vivo* development of this class of compounds.

4.4   Outlook

In conclusion, the present study aimed to describe two novel approaches to target CK2 (Fig 50):

- ▪ CK2 modulators which are able to block CK2a into an inactive state, through a binding in an allosteric pocket located at the interface of the αC-helix and the P-loop.
- ▪ CK2α/CK2β interaction inhibitors which are able to counteract the formation of the holoenzyme through binding in the β interaction pocket in the N-terminal lob of CK2α.

We believe that the two new strategies described herein are of main interest because they could lead to more selective inhibitors with better *in vivo* efficiency. Moreover, a fine substrate-dependent modulation of CK2 activity instead of a complete inhibition could be achieve through both strategy, thus they could decrease the side effect inherent to cancer treatment. A precise mapping of the *in vivo* consequences of CK2 modulation with the inhibitors described herein will be required to understand completely their implications in cell signaling modulation.

TAT GCRLYGFKIHGCG

TAT-Pc

III - 2

α/β interaction inhibitor

α

α

β   **β**

Allosteric modulator

I - 7

II – 8e

β4/β5 loop

β interaction

Hinge region   P-loop

ATP   αC-helix

αD-helix   Activation segment
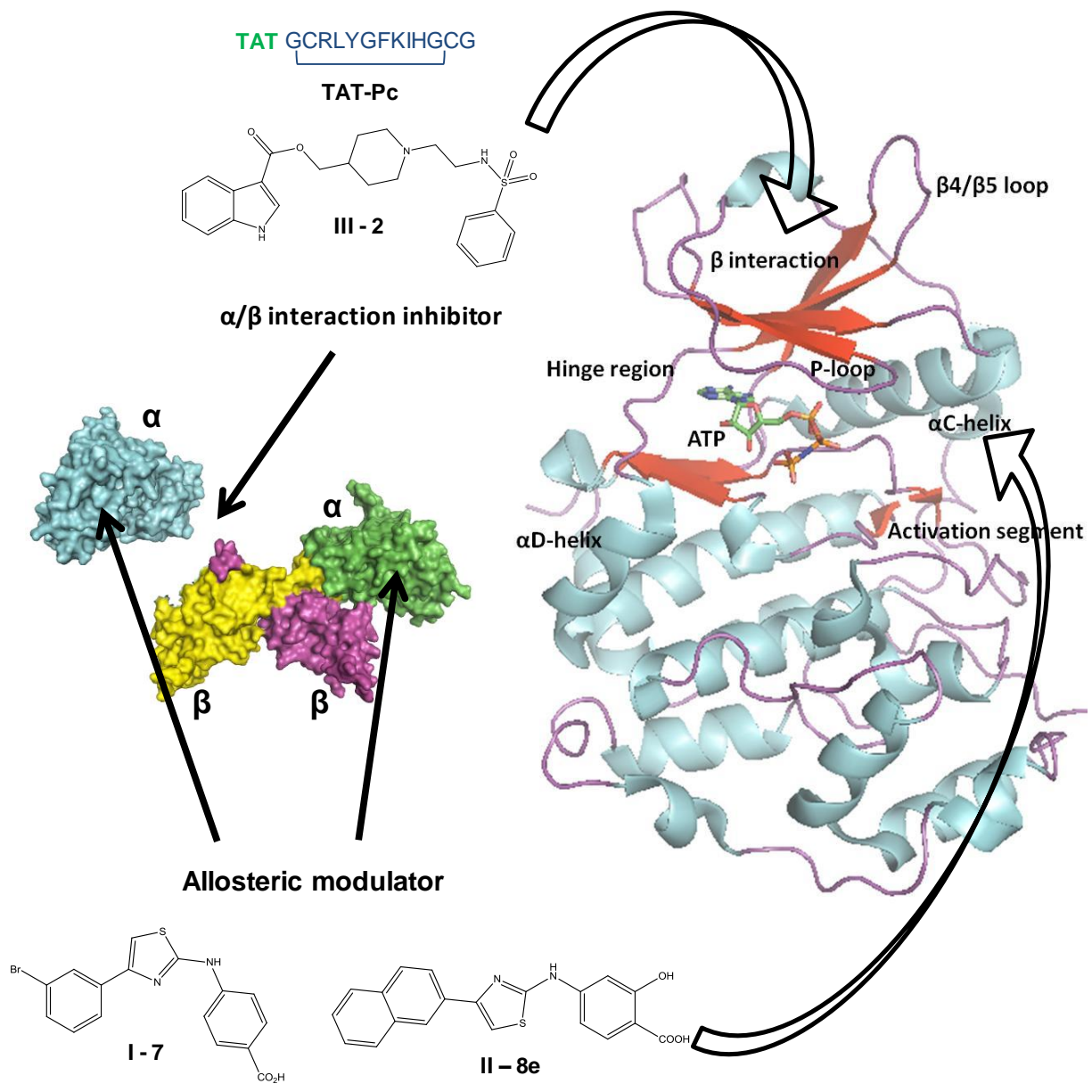
Fig 50: Schematic representation of the two novel strategies targeting CK2 presented in this work.

5. Experimental section

6.1 Chemistry

6.1.1   Synthesis of compounds of chapter 3.1

4-((4-(3-Nitrophenyl)thiazol-2-yl)amino)benzoic acid **(1)** was purchased from Sigma-Aldrich. All commercially available chemicals (phenylthiourea, 2-bromo-3'-nitro-acetophenone, 1,2-dichloro-1-ethoxyethane, 2-bromoacetophenone, 2-bromo-4'-methoxyacetophenone, 2-bromo-1-(3-bromophenyl)ethanone) and solvents were purchased from Sigma-Aldrich, Acros Organics, Fischer scientific or Alfa Aesar and were used without further purification. Reactions were monitored by Thin Layer Chromatography (TLC Silica gel 60 F254) purchased from Merck and observed under UV light (254 nM). Purification by semi-preparative HPLC was carried out on an Agilent 1200 series HPLC system (Agilent Technologies) using an Agilent C18 column (30 x 100 mm/10 μm) as stationary phase and a gradient of water and acetonitrile as eluent. ^1H and ^{13}C NMR spectra were obtained in dimethylsulphoxide-d_6 on a Bruker DRX-500 instrument, operating at 500 MHz for the ^1H and at 125 MHz for the ^{13}C at 300 K, using residual signal of deuterated NMR solvent as internal reference [274]. Chemical shifts are reported in parts per million (ppm), multiplicity of the signals are indicated by lower-case letters (singlet s, doublet d, triplet t, quadruplet q, multiplet m, broad singlet br s, or combination of letters). DEPT 135 was used to determine carbon valence. Analytical HPLC was performed using a SpectraSYSTEM™ (ThermoFisher) with a Macherey-Nagel C18 column (3 x 125 mm/5 μm). HPLC purities were determined by UV absorption at 254 nm. ElectroSpray Ionisation (ESI) mass spectra were measured on a Finnigan Surveyor MSQ Plus mass spectrometer (ThermoFisher). Melting points (mp) of the solids were determined by SMP3 Melting Point Apparatus from Bibby Sterling.

4-Thioureidobenzoic acid (i). 4-Thioureidobenzoic acid was prepared according to the procedure already described [239]. Carbon disulfide (22 mmol, 1.32 mL) was slowly added to a mixture of 4-aminobenzoic acid (7.3 mmol, 1 g), THF (5 mL), H_2O (5 mL) and Et_3N (18 mmol, 1.82 mL). The resulting mixture was stirred at RT for 24 h. Then iodine (7.7 mmol, 1.94 g) in THF (5 mL) was added dropwise at 0 °C and mixture was stirred for 3h. A 1 M HCl aqueous solution (7.5 mL) and Na_2SO_3 (1.46 mmol, 184 mg) were added and stirring was pursued for 15 min. The aqueous layer was extracted with EtOAc (3 x 50 mL). Combined organic layers were washed twice with brine, dried on Na_2SO_4, filtered and concentrated to give the isothiocyanate intermediate as a slightly yellow solid, which was converted to the thiourea derivatives by stirring it at RT in NH_4OH

(30 %) for 6 h. After removal of the insolubles by filtration on a sintered-glass funnel, filtrate was concentrated to afford the *title* product as an off-white solid (7.3 mmol, 1.43 g). Yield = quantitative. LC-UV purity = 80 %. The compound was not ionized by ESI. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ (ppm) 7.56 – 7.58 (m, 2H), 7.80 – 7.82 (m, 2H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ (ppm) 116.9, 120.9, 129.3, 130.6, 169.2, 181.0. mp = 189-191 °C (in accordance with literature: 194 °C) [275].

Methyl 4-((4-(3-nitrophenyl)thiazol-2-yl)amino)benzoate (2). Three drops of 95 % H$_2$SO$_4$ were added to a mixture of 4-((4-(3-nitrophenyl)thiazol-2-yl)amino)benzoic acid (0.14 mmol, 46 mg) in MeOH (5 mL) and the mixture was then heated at reflux for 2 h. Na$_2$CO$_3$ 1 M was added until pH = 10-11 and MeOH was removed under *vacuo*. The obtained solid was filtrated and washed with water (2 x 5 mL) to afford the *title* product as a yellow solid (0.14 mmol, 49 mg). Yield = quantitative. LC-UV purity = 97 %. LCMS (ESI$^+$) m/z = 356.01 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ (ppm) 3.82 (s, 3H), 7.79 (m, 4H), 7.96 (s, 2H), 8.17 (s, 1H), 8.40 (s, 1H), 8.71 (s, 1H), 10.84 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ (ppm) 51.7 (CH$_3$), 107.0 (CH), 116.1 (2 x CH), 119.9 (CH), 121.8 (C), 122.1 (CH), 130.3 (CH), 130.6 (2 x CH), 131.9 (CH), 135.7 (C), 144.9 (C), 147.8 (C), 148.3 (C), 162.6 (C), 165.8 (C). mp = 242-244 °C.

General procedure for the preparation of 2-aminothiazole analogs. An ethanolic solution of 2'-bromo-acetophenone derivative and aryl thiourea derivative at equimolar ratio adjusted with respect to LC-UV purity was heated at 80 °C for 3-12 h (reaction monitoring by TLC). The mixture was then cooled down to RT and water (twice more than EtOH v/v) was added. Mixture was stirred for 30 min and then filtrated. The obtained solid was washed twice with water to afford the product as a solid. When necessary, purification was performed (i) by recrystallization or washing with boiling MeCN (ii) by recrystallization or washing in H$_2$O/MeOH (iii) by HPLC preparative to obtain the product with LC-UV purity higher than 95 %.

4-(3-Nitrophenyl)-*N*-phenylthiazol-2-amine (3). The reaction was carried out according to the general procedure. Scale: 2-bromo-3'-nitro-acetophenone (0.66 mmol, 161 mg), phenylthiourea (0.66 mmol, 100 mg), absolute EtOH (5 mL). After removal of the solvents, water (10 mL) and MeOH (2 mL) were added. The pH of the solution was adjusted to 9 with solid Na$_2$CO$_3$ and then stirred for 1 h. After filtration and washing of the residual solid with water (3 x 5 mL), the product was obtained as a yellow solid (0.66 mmol, 196 mg). Yield = quantitative. LC-UV purity = 99 %. LCMS (ESI$^+$) m/z = 298.06 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ (ppm) 6.99 (t, J = 6.9 Hz, 1H), 7.36 (t, J = 6.6 Hz, 2H), 7.65 (s, 1H), 7.71 (m, 3H), 8.14 (d, J = 7.3 Hz, 1H), 8.35 (d, J = 6.9 Hz, 1H), 8.69 (s, 1H), 10.39 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ (ppm) 105.6 (CH), 116.9 (2 x CH), 119.8

(CH), 121.4 (CH), 121.9 (CH), 129.0 (2 x CH), 130.1 (CH), 131.7 (CH), 135.9 (C), 140.9 (C), 147.6 (C), 148.2 (C), 163.5 (C). mp = 136-138 °C. (in accordance with literature: 122-124 °C)[276].

4-(Thiazol-2-ylamino)benzoic acid (4). The reaction was carried out according to the general procedure. Scale: 1,2-dichloro-1-ethoxyethane (0.41 mmol, 50 µL), 4-thioureidobenzoic acid (0.41 mmol, 80 mg), absolute EtOH (5 mL). Purification by semi-preparative HPLC (H_2O/MeCN/TFA: from 79/20/1 to 0/99/1 in 40 min) to afford ethyl 4-(thiazol-2-ylamino)benzoate as a white solid (0.09 mmol, 23 mg). Yield = 23 %. LC-UV purity = 96 %. LCMS (ESI$^+$) m/z = 249.01 for [M+H]$^+$. The ethyl 4-(thiazol-2-ylamino)benzoate (0.085 mmol, 21 mg) obtained was directly hydrolyzed in a mixture of THF (2 mL) and aqueous 0.5 M NaOH (1.15 mmol, 2.3 mL) at reflux for 4 h. Aqueous 1 M HCl was added until pH = 1 and then solvents were removed *in vacuo*. Crude product was purified by semi-preparative HPLC (H_2O/MeCN + 0.1 % TFA: from 90/10 to 10/90 in 40 min) to afford the *title* product as a white solid (0.07 mmol, 16 mg). Yield = 86 %. LC-UV purity > 99 %. LCMS (ESI$^+$) m/z = 220.78 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d_6): δ (ppm) 7.02 (d, J = 3.8 Hz, 1H), 7.33 (d, J = 3.8 Hz, 1H), 7.72 (d, J = 8.5 Hz, 2H), 7.88 (d, J = 8.8 Hz, 2H), 10.59 (s, 1H). ^{13}C-NMR (127 MHz, DMSO-d_6): δ (ppm) 109.7 (CH), 115.7 (2 x CH), 122.5 (C), 130.7 (2 x CH), 138.8 (CH), 144.9 (C), 162.9 (C), 166.9 (C). mp = 270-272 °C (commercially available).[277]

4-((4-Phenylthiazol-2-yl)amino)benzoic acid (5). The reaction was carried out according to the general procedure. Scale: 2-bromo-acetophenone (0.51 mmol, 102 mg), 4-thioureidobenzoic acid (0.51 mmol, 100 mg), absolute EtOH (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford the *title* product as a grey solid (0.24 mmol, 70 mg). Yield = 58 %. LC-UV purity = 95 %. The compound was not ionized by ESI. ^{1}H-NMR (500 MHz, DMSO-d_6): δ (ppm) 7.33 (t, J = 6.9 Hz, 1H), 7.44 (t, J = 6.9 Hz, 3H), 7.84 (d, J = 8.5 Hz, 2H), 7.95 (d, J = 7.9 Hz, 4H), 10.70 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d_6): δ (ppm) 104.1 (CH), 115.9 (2 x CH), 122.8 (C), 125.7 (2 x CH), 127.7 (CH), 128.6 (2 x CH), 130.8 (2 x CH), 134.3 (C), 144.9 (C), 150.2 (C), 162.2 (C), 167.0 (C). mp = 256-258 °C (in accordance with literature: 252 °C).[278]

4-((4-(4-Methoxyphenyl)thiazol-2-yl)amino)benzoic acid (6). The reaction was carried out according to the general procedure. Scale: 2-bromo-4'-methoxy-acetophenone (0.71 mmol, 163 mg), 4-thioureidobenzoic acid (0.51 mmol, 100 mg), absolute EtOH (10 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford the *title* product as a grey solid (0.15 mmol, 49 mg). Yield = 29 %. LC-UV purity = 96 %. LCMS (ESI$^+$) m/z = 327.09 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d_6): δ (ppm) 3.80 (s, 3H), 7.00 (d, J = 7.3 Hz, 2H), 7.29 (s, 1H), 7.81 (d, J = 7.9 Hz, 2H), 7.87 (d, J = 7.9 Hz, 2H), 7.93 (d, J = 7.9 Hz, 2H), 10.65 (s, 1H), 12.54 (br s, 1H, O-H).

^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ (ppm) 55.1 (CH$_3$), 101.9 (CH), 114.0 (2 x CH), 115.9 (2 x CH), 122.7 (C), 127.1 (2 x CH), 127.2 (C), 130.8 (2 x CH), 145.0 (C), 150.1 (C), 158.9 (C), 162.1 (C), 167.0 (C). mp = 266-268 °C (commercially available).

4-((4-(3-Bromophenyl)thiazol-2-yl)amino)benzoic acid (7). The reaction was carried out according to the general procedure. Scale: 2-bromo-1-(3-bromophenyl)ethanone (0.36 mmol, 100 mg), 4-thioureidobenzoic acid (0.54 mmol, 106 mg), absolute EtOH (5 mL). Purification was performed by recrystallization in MeCN (5 mL) to afford the *title* product as a yellow solid (0.20 mmol, 75 mg). Yield = 56 %. LC-UV purity = 95 %. LCMS (ESI$^+$) m/z = 374.62/376.65 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ (ppm) 7.40 (t, J = 7.9 Hz, 1H), 7.51 (ddd, J = 7.9, 1.9, 0.9 Hz, 1H), 7.59 (s, 1H), 7.80 (d, J = 8.8 Hz, 2H), 7.94 – 7.97 (m, 3H), 8.11 (t, J = 1.9 Hz, 1H), 10.72 (s, 1H), 12.60 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ (ppm) 105.7 (CH), 115.9 (2 x CH), 122.1 (C), 122.9 (C), 124.7 (CH), 128.0 (CH), 130.3 (CH), 130.8 (2 x CH), 130.9 (CH), 136.5 (C), 144.7 (C), 148.4 (C), 162.4 (C), 166.9 (C). mp = 281-283°C.

6.1.2 Synthesis of compounds of chapter 3.2

4-((4-(3-Nitrophenyl)thiazol-2-yl)amino)benzoic acid (**1**) and 3-((4-(3-nitrophenyl)thiazol-2-yl)amino)benzoic acid (**6b**) was purchased from Sigma-Aldrich. All commercially available chemicals,2-bromo-1-(3-nitrophenyl)ethanone (**4d**), 2-bromo-1-phenylethanone (**4e**), 2-bromo-1-(3-bromophenyl)ethanone (**4f**), 2-bromo-1-(2,4-dimethoxyphenyl)ethanone (**4g**), 2-bromo-1-(2,4-dihydroxyphenyl)ethanone (**4h**), 2-bromo-1-(4-bromo-2-hydroxyphenyl)ethanone (**4i**), 1-bromo-3,3-dimethylbutan-2-one (**4j**), 2-bromo-1-(thiophen-3-yl)ethanone (**4k**), 2-bromo-1-(naphthalen-2-yl)ethanone (**4l**), 3-(2-bromoacetyl)-2H-chromen-2-one (**4m**), 1-(benzofuran-2-yl)-2-bromoethanone (**4n**), 1-(benzo[b]thiophen-5-yl)-2-bromoethanone (**4o**), other starting materials and solvents were purchased from Sigma-Aldrich, Acros Organics, Fischer scientific or Alfa Aesar and were used without further purification. Reactions were monitored by Thin Layer Chromatography (TLC Silica gel 60 F254) purchased from Merck and observed under UV light (254 nm). Purification by semi-preparative HPLC was carried out on an Agilent 1200 series HPLC system (Agilent Technologies) using an Agilent C18 column (30 x 100 mm/10 μm) as stationary phase and a gradient of water and acetonitrile as eluent. ^{1}H and ^{13}C NMR spectra were obtained in dimethylsulphoxide-d$_6$, acetone-d$_6$ or CDCl$_3$ on a Bruker DRX-500 instrument, operating at 500 MHz for the ^{1}H and at 125 MHz for the ^{13}C at 300K, using residual signal of deuterated NMR solvent as internal reference.[279] Chemical shifts are reported in parts per million (ppm), multiplicity of the signals are indicated by lower-case letters (singlet s, doublet d, triplet t, quadruplet q, multiplet m, broad singlet br s, or combination of letters). DEPT 135 was used to determine carbon multiplicity. Analytical HPLC was performed using a SpectraSYSTEM™ (ThermoFisher) with a Macherey-Nagel C18 column (3 x 125 mm/5 μm). HPLC purities were determined by UV absorption at 254 nm. ElectroSpray Ionisation (ESI) mass spectra were measured on a Finnigan Surveyor MSQ Plus mass spectrometer (ThermoFisher). Melting points (mp) of solids were determined by SMP3 melting point apparatus from Bibby Sterling.

General procedure 1 for the preparation of 2'-bromo-acetophenone analogs from acetophenones (method A):[237]

Bromine (1 eq) was added dropwise over 5 min to a stirred and warmed (40°C) solution of acetophenone derivative (1 eq) in CHCl$_3$. At the end of the addition, the mixture was cooled down at RT; Et$_2$O was then added, followed by a saturated aqueous solution of NaHCO$_3$ and the resulting mixture was then stirred for 30 min. The organic layer was separated, washed with a saturated aqueous solution of NaHCO$_3$, dried with Na$_2$SO$_4$ and filtered. The volatiles were removed under reduced pressure to afford the product as a solid, which was used in the next step without further purification.

General procedure 2 for the preparation of 2'-bromo-acetophenone analogs from acetophenones (method B):[238]

Bromine (1 eq) was added dropwise to a solution of acetophenone derivative (1 eq) in a mixture of MeOH and HBr 32 % (2 eq) in AcOH. The mixture was then heated at 60°C for 4h. After removal of the volatiles *in vacuo*, the solid residue was washed 5 times with Et_2O and filtered to afford the product as a solid, which was used in the next step without further purification.

2'-bromo-3-methoxy-acetophenone (4a)

The reaction was carried out according to general procedure 1, scale: 3-methoxyacetophenone (3.33 mmol, 459 µL), bromine (3.33 mmol, 172 µL), $CHCl_3$ (10 mL), Et_2O (100 mL), to afford the *title* product as a yellow solid (3.33 mmol, 762 mg). Yield = quantitative. LC-UV purity = 75%. The compound was not ionized by ESI[+]. ^{1}H-NMR (500 MHz, $CDCl_3$) δ = 3.70 (s, 3H, CH_3-O), 4.3 (s, 2H, CH_2-Br), 6.99 (ddd, J = 1.0; 2.5; 8.2 Hz, 1H), 7.24 (t, J = 8.2 Hz, 1H), 7.34 (dd, J = 1.6, 2.5 Hz, 1H), 7.39 (ddd, J = 1.0, 1.6, 7.6 Hz, 1H). mp = 52-54°C, spectral data and melting point are in accordance with lit. data (61-62°C).[280]

2'-bromo-4-methoxy-acetophenone (4b)

The reaction was carried out according to general procedure 1, scale: 4-methoxyacetophenone (3.33 mmol, 500 mg), bromine (3.33 mmol, 172 µL), $CHCl_3$ (10 mL), Et_2O (100 mL), to afford the *title* product as a grey solid (3.33 mmol, 765 mg). Yield = quantitative. LC-UV purity = 78%. LC-MS (ESI[+]) m/z = 229.31 for [M+H][+]. ^{1}H-NMR (500 MHz, $CDCl_3$) δ = 3.89 (s, 3H, CH_3-O), 4.40 (s, 2H, CH_2-Br), 6.95 – 6.97 (m, 2H), 7.96 – 7.98 (m, 2H). mp = 49-51°C, spectral data is in accordance with lit. data[281,282]

2-bromo-1-(pyridin-2-yl)ethanone hydrogen bromide (4c)

The reaction was carried out according to general procedure 2, scale: 2-acetylpyridine (8.2 mmol, 930 µL), bromine (8.2 mmol, 430 µL), HBr 32 % in AcOH (16.5 mmol, 2.98 mL), MeOH (1 mL), to afford the *title* product as a slightly yellow solid (8.2 mmol, 2.3 g). Yield = quantitative. LC-UV purity = 85%. LC-MS (ESI[+]) m/z = 200.05/202.08 for [M+H][+]. ^{1}H-NMR (500 MHz, $CDCl_3$) δ = 4.95 (s, 2H, CH_2-Br), 7.70 – 7.73 (m, 1H), 7.99 (td, J = 1.3, 7.9 Hz, 1H), 8.04 – 8.07 (m, 1H), 8.62 (br s, NH[+]), 8.70 – 8.71 (m, 1H). mp = 206-208°C, spectral data and melting point are in accordance with lit. data (200-205°C).[283]

General procedure 3 for the preparation of thiourea analogs from amines:

Carbon disulfide (3 eq) was slowly added to a mixture of aminobenzoic acid derivative (1 eq) in a 1/1 THF/H$_2$O (0.7 mol.L^{-1}) solvent mixture and Et$_3$N (2.5 eq). The resulting mixture was stirred at RT for 24h. Then iodine (1.05 eq) in THF (5 mL) was added dropwise at 0°C and the mixture was stirred for 3h. A 1M HCl aqueous solution (7.5 mL) and Na$_2$SO$_3$ (0.2 eq) were added and the stirring was continued for 15 min. The aqueous layer was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed twice with brine (25 mL) and dried on Na$_2$SO$_4$. After filtration, the volatiles were removed under reduced pressure to give the isothiocyanate intermediate as a slightly yellow solid, which was converted to the thiourea derivative by stirring at RT in NH$_4$OH (100 mL) for 6h. NH$_4$OH refers to a 35% w/w ammonia solution in water. After removal of the solids by filtration on a sintered-glass funnel, the filtrate was concentrated to afford the thiourea product, which was used in the next step without further purification.

4-thioureidobenzoic acid (5a)

The reaction was carried out according to the general procedure 3, scale: 4-aminobenzoic acid (7.3 mmol, 1 g), to afford the *title* product as an off-white solid (7.3 mmol, 1.43 g). Yield = quantitative. LC-UV purity = 80%. The compound was not ionized by ESI$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.56 – 7.58 (m, 2H), 7.80 – 7.82 (m, 2H). mp = 189-191°C, spectral data and melting point are in accordance with lit. data (194°C).[275]

3-thioureidobenzoic acid (5b)

The reaction was carried out according to the general procedure 3, scale: 3-aminobenzoic acid (7.3 mmol, 1 g), to afford the *title* product as an off-white solid (7.3 mmol, 1.43 g). Yield = quantitative. LC-UV purity = 96%. The compound was not ionized by ESI$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.28 (t, J = 7.8 Hz, 1H), 7.57 – 7.59 (m, 2H), 8.04 (d, J = 6.5 Hz, 1H), 8.92 (br s, 1H), 10.96 (br s, 1H). mp = 183-185°C, melting point is in accordance with lit. data (163°C).[284]

2-(4-thioureidophenyl)acetic acid (5d)

The reaction was carried out according to the general procedure 3, scale: 2-(4-aminophenyl)acetic acid (3.31 mmol, 500 mg), to afford the *title* product as an off-white solid (3.1 mmol, 651 mg). Yield = 93 %. LC-UV purity = 97%. LC-MS (ESI$^+$) m/z = 211.05 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 3.34 (s, 2H), 5.00 (br s, 3H), 7.15 (d, J = 8.5 Hz, 2H), 7.30 (d, J = 8.2 Hz, 2H), 7.58 (br s, 1H), 10.2 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 43.0, 122.7, 129.2, 133.4, 137.2, 173.9, 181.0. mp = 139-141°C (partially described).[285]

5-thioureidoisophthalic acid (5e)

The reaction was carried out according to the general procedure 3, scale: 5-aminoisophthalic acid (7.3 mmol, 1.32 g), to afford the *title* product as an off-white solid (7.3 mmol, 1.75 g). Yield = quantitative. LC-UV purity = 82%. The compound was not ionized by ESI+. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.53 (s, 1H), 8.09 – 8.18 (m, 3H), 9.18 (br s, 1H), 11.20 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 124.8, 125.5, 137.3, 139.4, 170.4, 181.6. mp = 246-248°C.

2-hydroxy-4-thioureidobenzoic acid (5f)

The reaction was carried out according to the general procedure 3, scale: 4-aminosalicylic acid (7.3 mmol, 1.12 g) to afford the *title* product as an off-white solid (7.3 mmol, 1.55 g). Yield = quantitative. LC-UV purity = 70%. The compound was not ionized by ESI+. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 6.73 (dd, J = 2.2, 8.2 Hz, 1H), 6.96 (s, 1H), 7.30 – 7.50 (br s, labile H), 7.60 (d, J = 8.2 Hz, 1H), 7.56 – 7.70 (br s, labile H), 10.00 (s, 1H). mp = 185-187°C, melting point is in accordance with lit. data (179-180°C).[286]

4-methoxy-3-thioureidobenzoic acid (5g)

The reaction was carried out according to the general procedure 3, scale: 3-amino-4-methoxybenzoic acid (7.3 mmol, 1.22 g) to afford the *title* product as an off-white solid (7.3 mmol, 1.65 g). Yield = quant. LC-UV purity = 97%. LC-MS (ESI+) m/z = 226.79 for [M+H]+. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 3.80 (s, 3H), 5.33 (br s, labile H), 6.97 (d, J = 8.8 Hz, 1H), 7.52 (br s, 1H), 7.70 (dd, J = 1.9, 8.5 Hz, 1H), 8.10 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 55.7, 110.4, 126.5, 127.5, 127.7, 129.6, 154.2, 169.3, 181.6. mp = 241-243°C.

Phenylthiourea (5i)

Phenylisothiocyanate (8.37 mmol, 1 mL) was added to a solution of NH$_4$OH$_{(aq)}$ (40 mL) and stirred at RT for 12h. After removal of volatile *in vacuo*, the white residue was triturated in water and filtered to obtain the *title* product as a white solid (8.15 mmol, 1.24 g). Yield = 97%. LC-UV purity > 99%. LC-MS (ESI+) m/z = 153.1 for [M+H]+. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.12 (t, J = 7.3 Hz, 1H), 7.32 (t, J = 7.3 Hz, 2H), 7.40 (d, J = 7.6 Hz, 2H), 9.70 (s, 1H). mp = 153-155°C, spectral data and melting point are in accordance with lit. data (153-154°C).[287]

1-(4-cyanophenyl)thiourea (5j)

A solution of 4-cyanophenyl isothiocyanate (6.24 mmol, 1 g) in $NH_4OH_{(aq)}$ (80 mL) was stirred at RT for 12h. After cooling at 0°C, the mixture was filtrated to afford the *title* product as an off-white solid (4.85 mmol, 860 mg). Yield = 78%. LC-UV purity > 99%. The compound was not ionized by ESI[+]. ^{1}H-NMR (500 MHz, DMSO-d6): δ = 7.75 (m, 4H), 10.07 (s, 1H). mp = 204-206°C, spectral data and melting point are in accordance with lit. data (239°C).[283,288]

General procedure 4 for the preparation of 2-aminothiazole analogs:

An ethanolic solution of 2'-bromo-acetophenone derivative and aryl thiourea derivative at eqimolar ratio, corrected with respect to LC-UV purity, was heated at 80°C for 3-12h (reaction monitoring by TLC). The mixture was then cooled down to RT and water (twice more than EtOH v/v) was added. The mixture was stirred for 30 min and then filtrated. The obtained solid was washed twice with water to afford the product as a solid. When necessary, purification was performed (i) by recrystallization or washing with boiling MeCN (ii) by recrystallization or washing in H_2O/MeOH (iii) by HPLC preparative to obtain the product with LC-UV purity higher than 95%.

4-((4-phenylthiazol-2-yl)amino)benzoic acid (2)

The reaction was carried out according to general procedure 4, scale: 2-bromo-acetophenone (0.51 mmol, 102 mg), 4-thioureidobenzoic acid (0.51 mmol, 100 mg), absolute EtOH (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford the *title* product as a grey solid (0.24 mmol, 70 mg). Yield = 58%. LC-UV purity = 95%. The compound was not ionized by ESI[+]. ^{1}H-NMR (500 MHz, DMSO-d6): δ = 7.33 (t, J = 6.9 Hz, 1H), 7.44 (t, J = 6.9 Hz, 3H), 7.84 (d, J = 8.5 Hz, 2H), 7.95 (d, J = 7.9 Hz, 4H), 10.70 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d6): δ = 104.1 (CH), 115.9 (2 x CH), 122.8 (C), 125.7 (2 x CH), 127.7 (CH), 128.6 (2 x CH), 130.8 (2 x CH), 134.3 (C), 144.9 (C), 150.2 (C), 162.2 (C), 167.0 (C). mp = 256-258 °C (in accordance with literature: 252 °C).[278]

4-((4-(3-bromophenyl)thiazol-2-yl)amino)benzoic acid (3)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(3-bromophenyl)ethanone (0.36 mmol, 100 mg), 4-thioureidobenzoic acid (0.54 mmol, 106 mg), absolute EtOH (5 mL). Purification was performed by recrystallization in MeCN (5 mL) to afford the *title* product as a yellow solid (0.20 mmol, 75 mg). Yield = 56%. LC-UV purity = 95%. LC-MS (ESI[+]) m/z = 374.62/376.65 for [M+H][+]. ^{1}H-NMR (500 MHz, DMSO-d6): δ = 7.42 (t, J = 7.9 Hz, 1H), 7.52 (d, J = 7.1 Hz, 1H), 7.62 (s, 1H), 7.79 (d, J = 8.8 Hz, 2H), 7.93 – 7.98 (m, 3H), 8.11 (t, J = 1.6 Hz, 1H), 10.73 (s,

1H), 12.60 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 105.7 (CH), 115.9 (2 x CH), 122.1 (C), 122.9 (C), 124.7 (CH), 128.0 (CH), 130.3 (CH), 130.8 (2 x CH), 130.9 (CH), 136.5 (C), 144.7 (C), 148.4 (C), 162.4 (C), 166.9 (C). mp = 281-283°C.

4-(3-nitrophenyl)-*N*-phenylthiazol-2-amine (6a)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-3'-nitro-acetophenone (0.66 mmol, 161 mg), phenylthiourea (0.66 mmol, 100 mg), absolute EtOH (5 mL). After removal of the solvents under reduced pressure, water (10 mL) and MeOH (2 mL) were added. The pH of the solution was adjusted to 9 with solid Na$_2$CO$_3$ and then stirred for 1h. After filtration and washing of the residual solid with water (3 x 5 mL), the product was obtained as a yellow solid (0.66 mmol, 196 mg). Yield = quantitative. LC-UV purity = 99%. LC-MS (ESI$^+$) m/z = 298.06 for [M+H]$^+$.^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 6.99 (t, J = 6.9 Hz, 1H), 7.36 (t, J = 6.6 Hz, 2H), 7.65 – 7.71 (m, 4H), 8.14 (d, J = 7.3 Hz, 1H), 8.35 (d, J = 6.9 Hz, 1H), 8.69 (s, 1H), 10.39 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 105.6 (CH), 116.9 (2 x CH), 119.8 (CH), 121.4 (CH), 121.9 (CH), 129.0 (2 x CH), 130.1 (CH), 131.7 (CH), 135.9 (C), 140.9 (C), 147.6 (C), 148.2 (C), 163.5 (C). mp = 136-138 °C (in accordance with literature: 122-124 °C)[276].

5-((4-(3-nitrophenyl)thiazol-2-yl)amino)isophthalic acid (6c)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(3-nitrophenyl)ethanone (0.42 mmol, 102 mg), 5-thioureidoisophthalic acid (0.63 mmol, 151 mg), EtOH absolute (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford a yellow solid (0.36 mmol, 140 mg). Yield = 87%. LC-UV purity = 97%. LC-MS (ESI$^+$) m/z = 385.59 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.71 (t, J = 7.6 Hz, 1H), 7.77 (s, 1H), 8.10 (s, 1H), 8.17 (d, J = 6.9 Hz, 1H), 8.38 (d, J = 6.3 Hz, 1H), 8.63 (s, 2H), 8.77 (s, 1H), 10.79 (s, 1H), 13.27 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 106.6 (CH), 120.0 (CH), 121.1 (2 x CH), 122.2 (CH), 122.6 (CH), 130.1 (CH), 131.6 (CH), 132.1 (2 x C), 135.8 (C), 141.3 (C), 147.6 (C), 148.4 (C), 162.9 (C), 166.6 (2 x C). mp > 410°C.

2-(4-((4-(3- amino)phenyl) nitrophenyl)thiazol-2-yl) acetic acid (6d)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-3'-nitro-acetophenone (0.48 mmol, 117 mg), 2-(4-thioureidophenyl)acetic acid (0.48 mmol, 100 mg), EtOH (5 mL) to afford ethyl 2-(4-((4-(3-nitrophenyl)thiazol-2-yl)amino)phenyl) (0.41 mmol, 158 mg). Then, a mixture of ethyl 2-(4-((4-(3-nitrophenyl)thiazol-2-yl)amino)phenyl)acetate in THF (5 mL) and NaOH 0.5 M (1.3 mmol, 2.6 mL) was heated at 80°C for 4h. After removal of THF under reduced pressure, HCl 1Mwas added until pH = 1. The product was obtained after filtration as a

black solid (0.22 mmol, 78 mg). Yield = 73%. LC-UV purity = 96%. LC-MS (ESI⁺) m/z = 355.74 for [M+H]⁺. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 3.53 (s, 2H), 7.24 (d, J = 8.2 Hz, 2H), 7.64 (d, J = 8.2 Hz, 2H), 7.69 (s, 1H), 7.73 (t, J = 7.9 Hz, 1H), 8.16 (d, J = 7.9 Hz, 1H), 8.37 (d, J = 7.9 Hz, 1H), 8.70 (s, 1H), 10.43 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 39.7 (CH$_2$), 105.5 (CH), 116.9 (CH), 119.8 (CH), 122.0 (CH), 128.0 (C), 129.0 (CH), 130.2 (CH), 131.7 (CH), 135.9 (C), 139.5 (C), 147.6 (C), 148.2 (C), 163.6 (C), 172.8 (C). mp = 205-207 °C.

4-((4-(3-methoxyphenyl)thiazol-2-yl)amino)benzoic acid (7a)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-3'-methoxy-acetophenone (0.66 mmol, 152 mg), 4-thioureidobenzoic acid (0.51 mmol, 100 mg), EtOH (10 mL). Purification was performed by washing the residue with boiling MeCN (5 mL) and hot filtration to give a crude solid which was then purified by flash chromatography (cyclohexane/Acetone 50/50) to afford the *title* product as a yellow solid (0.09 mmol, 29 mg). Yield = 17%. LC-UV purity = 96%. LC-MS (ESI⁺) m/z = 327.10 for [M+H]⁺. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 3.83 (s, 3H), 6.91 (dd, J = 8.2, 2.2 Hz, 1H), 7.36 (t, J = 8.2 Hz, 1H), 7.48 (s, 2H), 7.53 (d, J = 7.9 Hz, 1H), 7.81 (d, J = 8.8 Hz, 2H), 7.94 (d, J = 8.8 Hz, 2H), 10.69 (s, 1H), 12.56 (br s, 1H).^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 55.0 (CH$_3$), 104.5 (CH), 111.1 (CH), 113.2 (CH), 115.8 (CH), 118.1 (CH), 122.8 (C), 129.7 (CH), 130.8 (CH), 135.6 (C), 144.8 (C), 150.0 (C), 159.5 (C), 162.1 (C), 167.0 (C). mp = 246-248°C.

4-((4-(4-methoxyphenyl)thiazol-2-yl)amino)benzoic acid (7b)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-4'-methoxy-acetophenone (0.71 mmol, 163 mg), 4-thioureidobenzoic acid (0.51 mmol, 100 mg), absolute EtOH (10 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford the *title* product as a grey solid (0.15 mmol, 49 mg). Yield = 29%. LC-UV purity = 96%. LC-MS (ESI⁺) m/z = 327.09 for [M+H]⁺. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 3.80 (s, 3H), 7.00 (d, J = 7.3 Hz, 2H), 7.29 (s, 1H), 7.81 (d, J = 7.9 Hz, 2H), 7.87 (d, J = 7.9 Hz, 2H), 7.93 (d, J = 7.9 Hz, 2H), 10.65 (s, 1H), 12.54 (br s, 1H, O-H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 55.1 (CH$_3$), 101.9 (CH), 114.0 (2 x CH), 115.9 (2 x CH), 122.7 (C), 127.1 (2 x CH), 127.2 (C), 130.8 (2 x CH), 145.0 (C), 150.1 (C), 158.9 (C), 162.1 (C), 167.0 (C). mp = 266-268 °C (commercially available).

4-((4-(2,4-dimethoxyphenyl)thiazol-2-yl)amino)benzoic acid (7c)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(2,4-dimethoxyphenyl)ethanone (0.38 mmol, 100 mg), 4-thioureidobenzoic acid (0.58 mmol, 114 mg), EtOH (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to

afford an off-white solid (0.25 mmol, 89 mg). Yield = 66%. LC-UV purity = 97%. LC-MS (ESI$^+$) m/z = 356.78 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$) δ = 3.81 (s, 3H), 3.91 (s, 3H), 6.65 – 6.67 (m, 2H), 7.31 (s, 1H), 7.81 (td, J = 2.2, 9.1 Hz, 2H), 7.93 (td, J = 2.2 Hz, 8.83 Hz, 2H), 8.08 – 8.10 (m, 1H), 10.58 (s, 1H), 12.56 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d6): δ = 55.2 (CH$_3$), 55.5 (CH$_3$), 98.5 (CH), 105.2 (CH), 105.5 (CH), 115.7 (CH), 115.8 (C), 122.5 (C), 130.2 (CH), 130.7 (CH), 145.0 (C), 146.2 (C), 157.7(C), 159.8 (C), 160.3 (C), 167.0 (C). mp = 271-273°C.

4-((4-(2,4-dihydroxyphenyl)thiazol-2-yl)amino)benzoic acid (7d)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(2,4-dihydroxyphenyl)ethanone (0.43 mmol, 100 mg), 4-thioureidobenzoic acid (0.65 mmol, 127 mg), absolute EtOH (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford the *title* product as yellow solid (0.38 mmol, 125 mg). Yield = 89%. LC-UV purity = 95% LC-MS (ESI$^+$) m/z = 328.84 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 6.34 (dd, J = 8.4, 2.4 Hz, 1H), 6.36 (d, J = 2.3 Hz, 1H), 7.26 (s, 1H), 7.67 (d, J = 8.8 Hz, 2H), 7.76 (d, J = 8.2 Hz, 1H), 7.94 (d, J = 8.8 Hz, 2H), 9.48 (br s, 1H), 10.67 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 102.2 (CH), 102.8 (CH), 107.1 (CH), 111.6 (C), 116.0 (CH), 122.9 (C), 129.0 (CH), 130.8 (CH), 144.7 (C), 147.6 (C), 156.2(C), 158.1 (C), 161.2 (C), 166.9 (C). mp = 367-369°C.

4-((4-(5-bromo-2-hydroxyphenyl)thiazol-2-yl)amino)benzoic acid (7e)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(5-bromo-2-hydroxyphenyl)ethanone (0.34 mmol, 100 mg), 4-thioureidobenzoic acid (0.51 mmol, 100 mg), absolute EtOH (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford a yellow solid (0.31 mmol, 120 mg). Yield = 90%. LC-UV purity = 98%. LC-MS (ESI$^+$) m/z = 390.65/392.62 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$) δ = 6.91 (d, J = 8.5 Hz, 1H), 7.31 (dd, J = 2.5, 8.5 Hz, 1H), 7.64 (s, 1H), 7.68 (d, J = 8.8 Hz, 2H), 7.94 (d, J = 9.1 Hz, 2H), 8.12 (d, J = 2.5 Hz, 1H), 10.74 (s, 1H), 10.84 (s, 1H), 12.60 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 107.7 (CH), 110.3 (C), 116.0 (CH), 118.4 (CH), 122.1 (C), 123.1 (C), 130.3 (CH), 130.8 (CH), 130.9 (CH), 144.6 (C), 145.4 (C), 154.2 (C), 161.4 (C), 166.9 (C). mp = 312-314°C.

4-((4-(pyridin-2-yl)thiazol-2-yl)amino)benzoic acid (7f)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(pyridin-2-yl)ethanone hydrogen bromide (0.61 mmol, 171 mg), 4-thioureidobenzoic acid (0.51 mmol, 100 mg), absolute EtOH (10 mL). After water (5 mL) addition to the mixture, NH$_4$OH (30%) was added until complete dissolution. The pH of the solution was then adjusted to 2-3 by addition of aqueous HCl (1M) and the mixture was stirred for 30 min. The obtained solid was then filtrated and washed

with water (3 x 5 mL) to afford the *title* product as a yellow solid (0.28 mmol, 85 mg). Yield = 56%. LC-UV purity = 97%. LC-MS (ESI$^+$) m/z = 298.07 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.83 (t, J = 5.5 Hz, 1H), 7.95 (m, 4H), 8.35, (br s, 1H), 8.50 (m, 2H), 8.79 (d, J = 5.4 Hz, 1H), 11.24 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 114.8 (CH), 116.6 (2 x CH), 123.3 (CH), 124.7 (CH), 130.7 (2 x CH), 143.1 (CH), 144.2 (C), 144.6 (CH), 146.0 (C), 163.5 (C), 167.0 (C). mp = 274-276°C.

4-(thiazol-2-ylamino)benzoic acid (7g)

The reaction was carried out according to the general procedure 4, scale: 1,2-dichloro-1-ethoxyethane (0.41 mmol, 50 μL), 4-thioureidobenzoic acid (0.41 mmol, 80 mg), absolute EtOH (5 mL). Purification by semi-preparative HPLC (H$_2$O/MeCN/TFA: from 79/20/1 to 0/99/1 in 40 min) to afford ethyl 4-(thiazol-2-ylamino)benzoate as a white solid (0.09 mmol, 23 mg). Yield = 23%. LC-UV purity = 96%. LC-MS (ESI$^+$) m/z = 249.01 for [M+H]$^+$.The ethyl 4-(thiazol-2-ylamino)benzoate (0.085 mmol, 21 mg) obtained was directly hydrolyzed in a mixture of THF (2 mL) and aqueous 0.5 M NaOH (1.15 mmol, 2.3 mL) at reflux for 4h. Aqueous 1 M HCl was added until pH = 1 and then solvents were removed *in vacuo*. Crude product was purified by semi-preparative HPLC (H$_2$O/MeCN + 0.1% TFA: from 90/10 to 10/90 in 40 min) to afford the *title* product as a white solid (0.07 mmol, 16 mg). Yield = 86%. LC-UV purity > 99%. LC-MS (ESI$^+$) m/z = 220.78 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.02 (d, J = 3.8 Hz, 1H), 7.33 (d, J = 3.8 Hz, 1H), 7.72 (d, J = 8.5 Hz, 2H), 7.88 (d, J = 8.8 Hz, 2H), 10.59 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 109.7 (CH), 115.7 (2 x CH), 122.5 (C), 130.7 (2 x CH), 138.8 (CH), 144.9 (C), 162.9 (C), 166.9 (C). mp = 270-272 °C (commercially available).[277]

4-((4-(tert-butyl)thiazol-2-yl)amino)benzoic acid (7h)

The reaction was carried out according to the general procedure 4, scale: 1-bromo-3,3-dimethylbutan-2-one (0.45 mmol, 60 μL), 4-thioureidobenzoic acid (0.41 mmol, 80 mg), absolute EtOH(5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford the *title* product as a white solid (0.10 mmol, 28 mg). Yield = 25%. LC-UV purity = 96%. LC-MS (ESI$^+$) m/z = 277.04 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 1.28 (s, 9H), 6.53 (s, 1H), 7.70 (d, J = 8.5 Hz, 2H), 7.88 (d, J = 8.5 Hz, 2H), 10.48 (s, 1H), 12.49 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 29.5 (CH$_3$), 34.3 (C), 100.5 (CH), 115.5 (CH), 122.3 (C), 130.6 (CH), 145.1 (C), 161.5 (C), 161.6 (C), 166.9 (C). mp = 280-282 °C.

4-((4-(thiophen-3-yl)thiazol-2-yl)amino)benzoic acid (7i)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(thiophen-3-yl)ethanone (0.41 mmol, 84 mg), 4-thioureidobenzoic acid (0.41 mmol, 80 mg), absolute EtOH

(5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration by hot filtration to afford the *title* product as a brown solid (0.20 mmol, 59 mg). Yield = 48%. LC-UV purity = 96%.LC-MS (ESI+) m/z = 302.86 for [M+H]+. 1H-NMR (500 MHz, DMSO-d6): δ = 7.27 (s, 1H), 7.59 (m, 2H), 7.82 (m, 2H), 7.91 (m, 3H), 10.65 (s, 1H), 12.56 (br s, 1H). 13C-NMR (125.7 MHz, DMSO-d6): δ = 103.4 (CH), 115.9 (2 x CH), 121.8 (CH), 122.7 (C), 125.9 (CH), 126.8 (CH), 130.7 (2 x CH), 136.6 (C), 144.8 (C), 146.6 (C), 162.1 (C), 166.9 (C). mp = 278-280 °C.

4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzoic acid (7j)

The reaction was carried out according to the general procedure 4,scale: 2-bromo-1-(naphthalen-2-yl)ethanone (0.41 mmol, 102 mg), 4-thioureidobenzoic acid (0.41 mmol, 80 mg), absolute EtOH (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford the *title* product as a brown solid (0.17 mmol, 59 mg). Yield = 42%. LC-UV purity = 95%. LC-MS (ESI+) m/z = 346.57 for [M+H]+. 1H-NMR (500 MHz, DMSO-d6): δ = 7.53 (m, 2H), 7.60 (s, 1H), 7.89 (d, J = 8.8 Hz, 2H), 7.93 (d, J = 7.9 Hz, 2H), 7.98 (m, 3H), 8.04 (d, J = 8.5 Hz, 1H), 8.08 (dd, J = 8.5, 1.9 Hz, 1H), 8.50 (s, 1H), 10.73 (s, 1H). 13C-NMR (125.7 MHz, DMSO-d6): δ = 104.8 (CH), 116.0 (2 x CH), 122.8 (C), 123.9 (CH), 124.3 (CH), 126.0 (CH), 126.3 (CH), 127.5 (CH), 128.1 (CH),128.2 (CH), 130.8 (2 x CH), 131.7 (C), 132.4 (C), 133.1 (C), 144.8 (C), 150.1 (C), 162.3 (C), 167.0 (C). mp = 294-296 °C.

4-((4-(2-oxo-2H-chromen-3-yl)thiazol-2-yl)amino)benzoic acid (7k)

The reaction was carried out according to the general procedure 4, scale: 3-(bromoacetyl)coumarin (0.45 mmol, 120 mg), 4-thioureidobenzoic acid (0.54 mmol, 106 mg), EtOH absolute (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford the *title* product as a yellow solid (0.33 mmol, 120 mg). Yield = 73%. LC-UV purity = 96%. LC-MS (ESI+) m/z = 364.86 for [M+H]+. 1H-NMR (500 MHz, DMSO-d6): δ = 7.42 (t, J = 8.2 Hz, 1H), 7.46 (d, J = 8.5 Hz, 2H), 7.64 (t, J = 8.2 Hz, 1H), 7.88 (m, 3H), 7.99 (d, J = 8.8 Hz, 2H), 8.02 (d, J = 7.9 Hz, 1H), 8.75 (s, 1H), 10.75 (s, 1H). 13C-NMR (125.7 MHz, DMSO-d6): δ = 110.8 (CH), 115.8 (CH), 116.2 (CH), 119.2 (C), 120.1 (C), 122.9 (C), 124.6 (CH), 129.0 (CH), 130.9 (CH), 131.7 (CH), 138.9 (CH), 143.6 (C), 144.6 (C), 152.3 (C), 158.7 (C), 161.7 (C), 167.0 (C). mp = 343-345 °C.

4-((4-(benzofuran-2-yl)thiazol-2-yl)amino)benzoic acid (7l)

The reaction was carried out according to the general procedure 4, scale: 1-(benzofuran-2-yl)-2-bromoethanone (0.42 mmol, 100 mg), 4-thioureidobenzoic acid (0.50 mmol, 99 mg), absolute EtOH (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford a brown solid which was further purified by semi-preparative HPLC (H2O/MeCN + 0.1% TFA:

from 70/30 to 0/100 in 45 min) to afford the *title* product as a yellow solid (0.19 mmol, 63 mg). Yield = 45%. LC-UV purity = 99%. LC-MS (ESI+) m/z = 336.96 for [M+H]+. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.28 (m, 2H), 7.34 (dd, J = 8.2, 1.3 Hz, 1H), 7.45 (s, 1H), 7.62 (d, J = 7.6 Hz, 1H), 7.70 (d, J = 7.3, 1H), 7.84 (d, J = 8.8 Hz, 2H), 7.95 (d, J = 8.8 Hz, 2H), 10.83 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 103.0 (CH), 106.4 (CH), 110.9 (CH), 116.1 (CH), 121.4 (CH), 123.0 (CH), 123.2 (C), 124.7 (CH), 128.4 (C), 130.7 (CH), 141.4 (C), 144.6 (C), 151.6 (C), 154.1 (C), 163.0 (C), 166.9 (C). mp = 296-298°C.

4-((4-(benzo[b]thiophen-5-yl)thiazol-2-yl)amino)benzoic acid (7m)

The reaction was carried out according to the general procedure 4, scale: 1-(1-benzothiophen-5-yl)-2-bromo-1-ethanone (0.39 mmol, 100 mg), 4-thioureidobenzoic acid (0.59 mmol, 115 mg), absolute EtOH (5 mL). The obtained solid was stirred in boiling MeCN (20 mL) and filtrated. The filtrate layer was concentrated and the remaining solid was then dissolved in hot MeOH (10 mL). After cooling at RT, water (10 mL) was added and the obtained solid was filtrated and washed with water (2 x 5 mL) to obtain the *title* product as a yellow solid (0.15 mmol, 51 mg). Yield = 38%. LC-UV purity = 95%. LC-MS (ESI+) m/z = 352.76 for [M+H]+. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.48 (s, 1H), 7.56 (d, J = 5.4 Hz, 1H), 7.79 (d, J = 5.4 Hz, 1H), 7.88 (d, J = 8.8 Hz, 2H), 7.94 – 7.99 (m, 3H), 8.04 (d, J = 8.5 Hz, 1H), 8.48 (d, J = 1.6 Hz, 1H), 10.70 (s, 1H), 12.52 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 103.7 (CH), 115.9 (2 x CH), 120.7 (CH), 122.3 (CH), 122.7 (CH), 122.8 (C), 124.3 (CH), 128.0 (CH), 130.8 (3 x CH), 138.4 (C), 139.9 (C), 144.9 (C), 150.3 (C), 162.2 (C), 167.0 (C). mp = 353-355°C.

4-(naphthalen-2-yl)-N-phenylthiazol-2-amine (8a)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(naphthalen-2-yl)ethanone (0.66 mmol, 164 mg), phenylthiourea (0.66 mmol, 100 mg), absolute EtOH (15 mL). After water (25 mL) addition, the pH was adjusted to 9 with aqueous Na$_2$CO$_3$ (1M). The resulting mixture was cooled at 0°C and the precipitate was filtrated to afford the *title* product as a white solid (0.65 mmol, 195 mg). Yield = 98%. LC-UV purity> 99%.LC-MS (ESI+) m/z = 302.85 for [M+H]+. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.00 (t, J = 7.3 Hz, 1H), 7.39 (t, J = 8.2 Hz, 2H), 7.48 (s, 1H), 7.52 (qi, J = 8.2 Hz, 2H), 7.79 (d, J = 7.6 Hz, 2H), 7.91 (d, J = 7.9 Hz, 1H), 7.95 (d, J = 8.8 Hz, 1H), 8.00 (d, J = 7.9 Hz, 1H), 8.07 (dd, J = 8.5, 1.5 Hz, 1H), 10.32 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 103.6 (CH), 116.8 (CH), 121.2 (CH), 124.0 (CH), 124.1 (CH), 125.9 (CH), 126.3 (CH), 127.5 (CH), 128.0 (CH), 128.1 (CH), 129.0 (CH), 131.9 (C), 132.4 (C), 133.1 (C), 141.1 (C), 150.0 (C), 163.1 (C). mp = 148-150°C.

4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzonitrile (8b)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-2'acetonaphtone (1.41 mmol, 351 mg), 1-(4-cyanophenyl)thiourea (1.41 mmol, 250 mg), EtOH absolute (5 mL). The resulting mixture was cooled at RT and aqueous Na_2CO_3 (1M) was added dropwise until pH = 8.After cooling at 0°C,the precipitate was filtrated and washed with H_2O (2 x 5 mL) to afford the *title* product as a slightly yellow solid (462 mg, 1.41 mmol). Yield = quantitative. LC-UV purity = 99%. LC-MS (ESI$^+$) m/z = 327.94 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.51 (dt, J = 6.9, 1.3 Hz, 1H), 7.55 (dt, J = 6.9, 1.6 Hz, 1H), 7.61 (s, 1H), 7.81 (d, J = 8.8 Hz, 2H), 7.92 (d, J = 8.2 Hz, 1H), 7.96 (m, 3H), 8.01 (d, J = 7.9 Hz, 1H), 8.08 (dd, J = 8.5, 1.3 Hz, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 101.8 (C), 105.2 (CH), 116.8 (CH), 119.6 (C), 123.9 (CH), 124.3 (CH), 126.0 (CH), 126.4 (CH), 127.5 (CH), 128.1 (CH), 131.6 (C), 132.4 (C), 133.1 (C), 133.5 (2 x CH), 145.2 (C), 150.2 (C), 162.4 (C). mp = 228-230°C.

3-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzoic acid (8c)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(naphthalen-2-yl)ethanone (0.40 mmol, 100 mg), 3-thioureidobenzoic acid (0.52 mmol, 102 mg), EtOH absolute (5 mL). The *title* product was obtained pure after filtration as a yellow solid (0.34 mmol, 118 mg). Yield = 86%. LC-UV purity = 98%. LC-MS (ESI$^+$) m/z = 346.80 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.49 – 7.56 (m, 4H), 7.59 (d, J = 7.6 Hz, 1H), 7.90 – 7.98 (m, 4H), 8.08 (dd, J = 1.9, 8.5 Hz, 1H), 8.55 (s, 1H), 8.77 (s, 1H), 10.56 (s, 1H), 13.01 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 104.1 (CH), 117.7 (CH), 120.8 (CH), 121.9 (CH), 123.8 (CH), 124.5 (CH), 126.0 (CH), 126.4 (CH), 127.5 (CH), 128.0 (CH), 128.1 (CH), 129.2 (CH), 131.5 (C), 131.8 (C), 132.4 (C), 133.2 (C), 141.3 (C), 149.8 (C), 162.7 (C), 167.4 (C). mp = 275-277°C.

4-methoxy-3-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzoic acid (8d)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(naphthalen-2-yl)ethanone (1 mmol, 250 mg), 3-methoxy-4-thioureidobenzoic acid (1.3 mmol, 294 mg), EtOH absolute (25 mL). Product was obtained pure after filtration as a yellow solid (0.94 mmol, 353 mg). Yield = 94%. LC-UV purity = 97%. LC-MS (ESI$^+$) m/z = 376.82 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$) δ = 3.97 (s, 3H), 7.14 (d, J = 8.5 Hz, 1H), 7.49 – 7.55 (m, 3H), 7.64 (dd, J = 2.2, 8.2 Hz, 1H), 7.91 – 7.96 (m, 3H), 8.07 (dd, J = 1.9, 8.5 Hz, 1H), 8.60 (d, J = 0.6 Hz, 1H), 9.70 (d, J = 2.2 Hz, 1H), 9.90 (s, 1H), 12.71 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 55.9 (CH$_3$), 104.7 (CH), 110.0 (CH), 118.8 (CH), 122.9 (C), 123.5 (CH), 123.6 (CH), 124.5 (CH), 125.9 (CH), 126.3 (CH), 127.5 (CH), 127.9

(CH), 128.0 (CH), 129.9 (C), 131.8 (C), 132.3 (C), 133.2 (C), 149.1 (C), 150.8 (C), 162.7 (C), 167.5 (C). mp = 256-258°C.

2-hydroxy-4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzoic acid (8e)

The reaction was carried out according to the general procedure 4, scale: 2-bromo-1-(naphthalen-2-yl)ethanone (0.50 mmol, 124 mg), 2-hydroxy-4-thioureidobenzoic acid (0.70 mmol, 149 mg), EtOH (5 mL). Purification was performed by washing with boiling MeCN (5 mL) and hot filtration to afford a yellow solid (0.37 mmol, 133 mg). Yield = 73%. LC-UV purity = 96%. LC-MS (ESI$^+$) m/z = 362.84 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.19 (dd, J = 1.9, 8.8 Hz, 1H), 7.49 – 7.57 (m, 3H), 7.61 (s, 1H), 7.80 (d, J = 8.5 Hz, 1H), 7.92 (d, J = 7.9 Hz, 1H), 7.98 (d, J = 8.2 Hz, 2H), 8.07 (d, J = 8.5 Hz, 1H), 8.46 (s, 1H), 10.75 (s, 1H), 11.8 (br s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 103.0 (CH), 105.2 (CH), 105.6 (C), 108.4 (CH), 124.0 (CH), 124.2 (CH), 126.0 (CH), 126.5 (CH), 127.6 (CH), 128.1 (CH), 128.2 (CH), 131.3 (CH), 131.7 (C), 132.5 (C), 133.1 (C), 147.0 (C), 150.2 (C), 162.1 (C), 162.6 (C), 171.7 (C). mp = 221-223°C.

2-((4-(3-nitrophenyl)thiazol-2-yl)amino)benzoic acid (9)

A solution of HCl 37% (1 mL) and 1,4-dioxane (5 mL) containing 2-bromo-4-phenylthiazole (2.08 mmol, 500 mg) and 2-aminobenzoic acid (2.08 mmol, 286 mg) was heated at reflux for 60h. After solvents removal, the residual solid was washed with water (5 mL) and then with cold acetone (5 mL) to obtain a white solid which was further purified by flash chromatography (cyclohexane/EtOAc, from 7/3 to 0/100) to obtain a second white solid containing 75% of product (by HPLC). After a second purification by preparative HPLC (H$_2$O/MeCN - 1% formic acid, from 70/30 to 0/100), the pure title product was obtain as a white solid (0.04 mmol, 12 mg). Yield = 2%. LC-UV purity = 97%.LC-MS (ESI$^+$) m/z = 297.08 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 7.07 (t, J = 7.9 Hz, 1H), 7.34 (t, J = 7.6 Hz, 1H), 7.45 (t, J = 7.6 Hz, 2H), 7.53 (s, 1H), 7.69 (td, J = 7.6, 1.6 Hz, 1H), 7.94 (d, J = 6.9 Hz, 2H), 8.02 (dd, J = 7.9, 1.6 Hz, 1H), 8.62 (d, J = 8.5 Hz, 1H), 11.48 (s, 1H).^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 104.6 (CH), 113.7 (C), 116.9 (CH), 120.4 (CH), 125.7 (CH), 127.8 (CH), 128.6 (CH), 131.4 (CH), 134.1, 134.7 (CH), 142.9 (C), 150.3 (C), 161.8 (C), 170.0 (C). mp = 95-97 °C.

Methyl 4-((4-(3-nitrophenyl)thiazol-2-yl)amino)benzoate (10)

Three drops of 95% H$_2$SO$_4$ were added to a mixture of 4-((4-(3-nitrophenyl)thiazol-2-yl)amino)benzoic acid (**1**) (0.14 mmol, 46 mg) in MeOH (5 mL) and the mixture was then heated at reflux for 2h. Na$_2$CO$_3$ 1 M was then added until pH = 10-11 and MeOH was removed under *vacuo*. The obtained solid was filtrated and washed with water (2 x 5 mL) to afford the *title*

product as a yellow solid (0.14 mmol, 49 mg). Yield = quantitative. LC-UV purity = 97%. LC-MS (ESI$^+$) m/z = 356.01 for [M+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d$_6$): δ = 3.82 (s, 3H), 7.74 – 7.84 (m, 4H), 7.96 (s, 2H), 8.17 (s, 1H), 8.40 (s, 1H), 8.71 (s, 1H), 10.83 (s, 1H). ^{13}C-NMR (125.7 MHz, DMSO-d$_6$): δ = 51.7 (CH$_3$), 107.0 (CH), 116.1 (2 x CH), 119.9 (CH), 121.8 (C), 122.1 (CH), 130.3 (CH), 130.6 (2 x CH), 131.9 (CH), 135.7 (C), 144.9 (C), 147.8 (C), 148.3 (C), 162.6 (C), 165.8 (C). mp = 242-244 °C.

N-(methylsulfonyl)-4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzamide (11a)

A solution of 4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzoic acid (**7j**) (0.23 mmol, 80 mg), HATU (0.28 mmol, 106 mg) and DIPEA (0.69 mmol, 120 µL) in dry DMF (2 mL) was stirred under N$_2$ for 30 min. And then added to a solution of NaH (0.92 mmol, 22 mg) and MeSO$_2$NH$_2$ (1.15 mmol, 110 mg) in dry THF (2 mL) beforehand stirred at RT under N$_2$ for 30 min. The resulting mixture was stirred at RT for 4h. After removal of solvents under vacuo, residue was purified by semi-preparative HPLC (H$_2$O/MeCN + 0.1% TFA: from 40/60 to 0/100 in 50 min) followed by a second purification by semi-preparative HPLC (H$_2$O/MeCN + 0.1% TFA: from 25/75 to 20/80 in 45 min) to obtain the *title* product as a white solid (0.026 mmol, 11 mg). Yield = 11%. LC-UV purity = 99%. LC-MS (ESI$^+$) m/z = 423.73 for [M+H]$^+$. ^{1}H-NMR (500 MHz, acetone-d$_6$) δ = 3.40 (s, 3H), 7.48 (s, 1H), 7.49 – 7.56 (m, 2H), 7.92 (d, J = 7.9 Hz, 1H), 7.96 (d, J = 8.6 Hz, 1H), 7.99 (d, J = 8.0 Hz, 1H), 8.01 – 8.04 (m, 2H), 8.08 - 8.12 (m, 3H), 8.56 (s, 1H), 9.89 (brs, 1H), 10.49 (brs, 1H). ^{13}C-NMR (125.7 MHz, acetone-d$_6$) δ = 42.8 (CH$_3$), 106.2 (CH), 118.3 (CH), 126.0 (CH), 126.2 (C), 126.8 (CH), 128.0 (CH), 128.3 (CH), 129.5 (CH), 130.1 (CH), 130.2 (CH), 131.8 (3 x CH), 134.0 (C), 135.1 (C), 135.7 (C), 153.1 (C), 167.1 (C). mp = 240-242°C.

4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)-*N*-tosylbenzamide (11b)

4-methylbenzenesulfonamide (0.23 mmol, 39.5 mg), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (0.46 mmol, 88 mg) and 4-dimethylaminopyridine (0.46 mmol, 56 mg) were added to a solution of 4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzoic acid (**7j**) (0.23 mmol, 80 mg) inCH$_2$Cl$_2$ (10 mL). The resulting mixture was stirred at RT under N$_2$ atmosphere for 36h. NH$_4$Cl$_{(aq)}$ (10 mL) was added, and then H$_2$O (10 mL). The aqueous layer was extracted with CH$_2$Cl$_2$ (2 x 20 mL). The combined organic layers were dried with Na$_2$SO$_4$, filtrated and concentrated to give a brown solid which was further purified by semi-preparative HPLC (H$_2$O/MeCN + 0.1% TFA: from 30/70 to 0/100 in 45 min) to obtain the *title* product as a white solid (0.026 mmol, 13 mg). Yield = 11%. LC-UV purity = 97%. LC-MS (ESI$^+$) m/z = 499.79 for [M+H]$^+$. ^{1}H-NMR (500 MHz, aceton-d$_6$) δ = 2.43 (s, 3H), 7.43 (dt, J = 7.9, 0.6 Hz, 2H), 7.52 (m, 2H), 7.91 (dd, J = 7.9, 0.6 Hz, 1H), 7.94 (d, J = 9.1 Hz, 1H), 7.99 (m, 8H), 8.09 (dd, J = 8.5, 1.9 Hz, 1H), 8.54 (s, 1H). ^{13}C-NMR (125.7 MHz, Acetone-d$_6$) δ = 22.5 (CH$_3$), 118.2 (CH), 125.9 (CH), 126.1 (C), 126.8 (CH), 127.9

(CH), 128.3 (CH), 129.5 (CH), 130.1 (CH), 130.2 (CH), 130.2 (CH), 131.2 (CH), 131.7 (CH), 133.9 (C), 135.0 (C), 135.6 (C), 139.2 (C), 146.3 (C), 147.3 (C), 152.8 (C), 164.2 (C), 165.9 (C). mp = 223-225°C.

General procedure 5 for the preparation of acetoxymethyl prodrug:

To a solution of the acid derivative (1 eq) in dry DMF (5 mL) under N_2 flow was added triethylamine (5 eq) and bromo-methylacetate (3 eq). After stirring for 12h, water (10 mL) was added and a precipitate occurred. The solid obtained after filtration was washed three times with water (5 mL) and further purified by semi-preparative HPLC (H_2O/MeCN: from 40/60 to 0/100 in 50 min) to obtain the desired product.

acetoxymethyl 4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzoate (12a)

The reaction was carried out according to the general procedure 5, scale: 4-((4-(naphthalen-2-yl)thiazol-2-yl)amino)benzoic acid (**7j**) (0.14 mmol, 50 mg), Et_3N (0.7 mmol, 98 µL), bromo-methylacetate (0.43 mmol, 42 µL), DMF (5 mL), then H_2O (10 mL). After purification by semi-preparative HPLC, the product was obtained as an off-white solid (0.098 mmol, 41 mg). Yield = 70%. LC-UV purity = 95%. LC-MS (ESI+) m/z = 418.61 for [M+H]+. 1H-NMR (500 MHz,aceton-d_6) δ = 2.11 (s, 3H), 5.98 (s, 2H), 7.47 (s, 1H), 7.49 – 7.55 (m, 2H), 7.91 (d, J = 8.2 Hz, 1H), 7.96 (d, J = 8.8 Hz, 1H), 8.03 – 8.06 (m, 5H), 8.12 (dd, J = 8.5, 1.9 Hz, 1H), 8.59 (s, 1H), 9.90 (s, 1H). 13C-NMR (125.7 MHz, aceton-d_6) δ = 21.6 (CH_3), 81.2 (CH_2), 106.2 (CH), 118.3 (CH), 123.3 (C), 125.9 (CH), 126.8 (CH), 127.9 (CH), 128.2 (CH), 129.5 (CH), 130.1 (CH), 130.3 (CH), 133.2 (CH), 134.04 (C), 135.1 (C), 135.7 (C), 148.0 (C), 153.2 (C), 164.3 (C), 166.2 (C), 171.1 (C). mp = 148-150°C.

acetoxymethyl 4-((4-(3-nitrophenyl)thiazol-2-yl)amino)benzoate (12b)

The reaction was carried out according to the general procedure 5, scale: 4-((4-(3-nitrophenyl)thiazol-2-yl)amino)benzoic acid (**1**) (0.26 mmol, 90 mg), Et_3N (1.3 mmol, 183 µL), bromo-methylacetate (0.79 mmol, 78 µL), DMF (5 mL), then H_2O (10 mL). After purification by semi-preparative HPLC, the product was obtained as an orange solid (0.11 mmol, 46 mg). Yield = 43%. LC-UV purity > 99%. LC-MS (ESI+) m/z = 413.60 for [M+H]+. 1H-NMR (500 MHz, DMSO-d_6): δ = 2.11 (s, 3H), 5.91 (s, 2H), 7.73 (t, J = 8.2 Hz, 1H), 7.80 (s, 1H), 7.86 (d, J = 8.8 Hz, 2H), 7.97 (d, J = 8.8 Hz, 2H), 8.16 (ddd, J = 8.2, 2.2, 1.0 Hz, 1H), 8.40 (dt, J = 8.2, 1.0 Hz, 1H), 8.70 (t, J = 1.9 Hz, 1H), 10.94 (s, 1H).13C-NMR (125.7 MHz, DMSO-d_6): δ = 20.5 (CH_3), 79.4 (CH_2), 107.2 (CH), 116.2 (CH), 119.9 (CH), 120.4 (C), 122.1 (CH), 130.3 (CH), 131.1 (CH), 131.9 (CH), 135.7 (C), 145.6 (C), 147.8 (C), 148.3 (C), 162.4 (C), 164.0 (C), 169.4 (C). mp = 169-171°C.

acetoxymethyl 4-((4-(thiophen-3-yl)thiazol-2-yl)amino)benzoate (12c)

The reaction was carried out according to the general procedure 5, scale: 4-((4-(thiophen-3-yl)thiazol-2-yl)amino)benzoic acid (**7i**) (0.25 mmol, 77 mg), Et$_3$N (1.25 mmol, 176 µL), bromo-methylacetate (0.76 mmol, 75 µL), DMF (5 mL), then H$_2$O (10 mL). After purification by semi-preparative HPLC, the product was obtained as an off-white solid (0.04 mmol, 14 mg). Yield = 16%. LC-UV purity = 96%. LC-MS (ESI$^+$) m/z = 374.66 for [M+H]$^+$. ^{1}H-NMR (500 MHz, aceton-d$_6$) δ = 2.10 (s, 3H), 5.96 (s, 2H), 7.15 (s, 1H), 7.51 (dd, J = 5.0, 3.2 Hz, 1H), 7.60 (dd, J = 5.0, 1.3 Hz, 1H), 7.93 (dd, J = 3.2, 1.3 Hz, 1H), 7.98 (d, J = 9.0 Hz, 2H), 8.03 (d, J = 9.0 Hz, 2H), , 9.83 (s, 1H).^{13}C-NMR (125.7 MHz, acetone-d$_6$) δ = 21.6 (CH$_3$), 81.2 (CH2), 105.0 (CH), 118.3 (CH), 123.2 (C), 123.8 (CH), 127.7 (CH), 128.1 (CH), 133.1 (CH), 139.0 (C), 148.0 (C), 149.5 (C), 164.2 (C), 166.2 (C), 171.7 (C). mp = 136-138°C.

6.1.3 Synthesis of compounds of chapter 3.4

All commercially available chemicals and solvents were purchased from Sigma-Aldrich, Acros Organics, Fischer scientific and Alfa Aesar. They were used without further purification, unless specified. ^{1}H NMR spectra were obtained on a Bruker ALS300 and DRX300 (300 MHz), and a DRX400 Bruker (400 MHz) using residual signal of deuterated NMR solvent as internal reference.[274] Chemical shifts are expressed in parts per million (ppm), multiplicity of the signals are indicated by lower-case letters (singlet s, doublet d, triplet t, quadruplet q, multiplet m, broad singlet br s, doublet of triplet dt, triplet of doublet td, triplet of triplet tt) coupling constants are expressed in Hz, and deuterated solvents are either dimethylsulphoxide-d_6 or $CDCl_3$. Analytical HPLC was performed on an Agilent Technology 1290 Infinity chromatographic system equipped with a 1260 Diode Array Detector and a single quadrupole 6120 Mass Spectrometer Detector (column: Zorbax Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm; temperature: 40°C). Solvent system was water/acetonitrile, each containing 0.1% of formic acid with an isocratic 90/10 elution during 0.5 min then a gradient till 10/90 over 5 min and kept during 1 min at 0.5 mL/min. Injection volume was typically 0.1 µL HPLC purities were measured at 254 nm. Electrospray ionization spectra (ESI) are obtained on an Agilent Technologies 6120 Quadrupole LC/MS. High Resolution Mass Spectrometry (HRMS) were performed on Bruker microTOF-Q II. Parallel syntheses were performed with a Radleys Tech system. Purifications are performed on silica gel 40-60 µm, 60A for normal phase. Thin Layer Chromatographies (TLC) (thickness: 200 µm, particle size: 25 µm) were purchased from Fluka Analytical. When specified, products were obtained in different batches according to their HPLC purity. In this case, analytical and biological data were obtained from the most pure batch.

_1H_-Indole-3-carboxylic acid (5a)
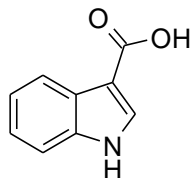
To a solution of _1H_-indole-3-carbonitrile (13.4 mmol, 1.9 g) in water (50 mL) was added a solution of NaOH (147 mmol, 5.9 g) in water (50 mL). The mixture was then heated at reflux under argon for 24h. This aqueous layer was washed with 3 x 20 mL of EtOAc and then acidified until pH = 1 with HCl 1M. A white precipitate occured and it was filtered, washed with HCl 1M to give the _title_ compound as a white solid (9.24 mmol, 1.49 g). Yield = 69 %. Chemical formula: $C_9H_7NO_2$.

Molecular weight: 161.16 g.mol^{-1}. LC/MS (ESI): 95 % pure, *m/z* [M + H]$^+$: 162. ^{1}H-NMR (300 MHz, DMSO-d$_6$) δ = 7.09 – 7.23 (m, 2H), 7.41 – 7.50 (m, 1H), 7.96 – 8.04 (m, 2H), 11.80 (s, 1H).[289]

5-Nitro-1*H*-indole-3-carboxylic acid (5c)
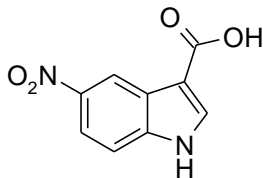
General procedure 1 for the preparation of indole-3-carboxylic acids

TFAA (42 mmol, 5.9 mL) was added dropwise to a solution of 5-nitro-*1H*-indole (18.5 mmol, 3 g) in dry DMF (25 mL) at 0°C under argon. After the end of the addition, the mixture was heated at reflux for 12h. Solvent was removed under reduced pressure and crude was taken off with water (50 mL) and a solution of NaOH (250 mmol, 10 g) in water (100 mL) was added. The resulting mixture was then heated at reflux for 12h. The solution was washed with Et$_2$O (4 x 50 mL) and then acidified with HCl 1M until pH = 2. The resulting solid was filtered and dried to afford the *title* compound (16.8 mmol, 3.46 g). Yield = 91 %. Chemical formula: C$_9$H$_6$N$_2$O$_4$. Molecular weight: 206.158 g.mol^{-1}. LC/MS (ESI): 97 % pure, *m/z* [M + H]$^+$: 207.4. ^{1}H-NMR (300 MHz, DMSO-d$_6$) δ = 7.68 (d, *J* = 9.0, 1H), 8.09 (dd, *J* = 2.4, 9.0, 1H), 8.27 (d, *J* = 2.9, 1H), 8.89 (d, *J* = 2.4, 1H), 12.52 – 12.58 (m, 2H). mp: 244-245°C. Spectral data and melting point are in accordance with literature reference.[290]

5-Fluoro-1*H*-indole-3-carboxylic acid (5b)
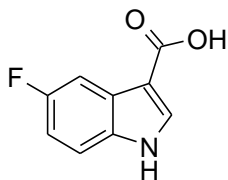
The reaction was carried out according to General procedure 1 with the corresponding scale: 5-fluoro-*1H*-indole (22.2 mmol, 3 g), TFAA (50 mmol, 7.1 mL), DMF (22 mL) then NaOH (300 mmol, 12 g), water (70 mL). The *title* compound (20.0 mmol, 3.59 g) was isolated as a grey solid. Yield = 90 %. Chemical formula: C$_9$H$_6$FNO$_2$. Molecular weight: 179.15 g.mol^{-1}. LC/MS (ESI): 95 % pure, *m/z* [M + H]$^+$: 180. ^{1}H-NMR (300 MHz, DMSO-d$_6$) δ = 7.03 (td, *J* = 2.6, 9.2, 1H), 7.48 (dd, *J* = 4.6, 8.9, 1H), 7.64 (dd, *J* = 2.7, 10.0, 1H), 8.05 (d, *J* = 3.0, 1H), 11.99 (s, 1H). mp > 410°C.[291]

N-(2-Chloroethyl)methanesulfonamide (10a)
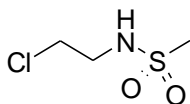
To a slurry of 2-chloroethylamine hydrochloride (70 mmol, 8.12 g) in MeCN (150 mL) at 5°C was added dropwise and simultaneously Et_3N (140 mmol, 19.7 mL) and $MeSO_2Cl$ (70 mmol, 5.42 mL) were also added during 30 min. After 30 min at 5°C, the mixture was slowly allowed to warm at RT and the stirring was pursued during 3h. The mixture became slightly orange. After removal of solvent, residue was taken off with water, the resulting aqueous phase was saturated with NaCl and extracted with CH_2Cl_2 (6 x 50 mL). The combined organic layers were dried on Na_2SO_4, filtered and the solvent was removed under vacuum to give a brown oil (5.87 g) which was purified on a normal phase silica gel column (cyclohexane/EtOAc: 6/4) to afford the *title* compound as a yellow oil which crystallized at -18°C (19.9 mmol, 3.13 g). Yield = 28 %. Chemical formula: $C_3H_8ClNO_2S$. Molecular weight: 157.62 g.mol^{-1}. LC/MS (ESI): not UV visible, *m/z* [M + H]$^+$: not ionizable. ^{1}H-NMR (300 MHz, $CDCl_3$) δ = 3.02 (s, 3H), 3.49 (td, *J* = 5.2, 6.2, 2H), 3.68 (dd, *J* = 5.17, 6.1, 2H), 4.80 (s, 1H).

N-(2-Chloroethyl)benzenesulfonamide (10b)
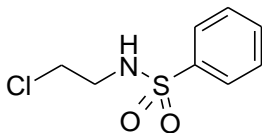
To a mixture of 2-chloroethylamine hydrochloride (43.1 mmol, 5 g) solubilized in CH_2Cl_2 (100 mL) and H_2O (50 mL) at 10°C was added K_2CO_3 (43.1 mmol, 5.7 g) followed by chlorosulfonylbenzene (43.1 mmol, 5.5 mL). The reaction was stirred during 3h, with a temperature kept at 10±3°C and the pH value was maintained around 7-8 with K_2CO_3. The mixture was then allowed to warm up at RT for 2h. Layers were partitioned and aqueous one was extracted with CH_2Cl_2 (2 x 100 mL). After recrystallization in *i*Pr_2O (50 mL, 70°C then RT), the *title* compound was obtained as a white solid (28.9 mmol, 6.36 g). Yield = 67 %. Chemical formula: $C_8H_{10}ClNO_2S$. Molecular weight: 219.69 g.mol^{-1}. LC/MS (ESI): 86 % pure at 254 nm, *m/z* [M + H]$^+$: not ionizable. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ

= 3.32 (q, J = 6.0, 2H), 3.56 (t, J = 6.0, 2H), 4.97 (br s, 1H), 7.51 – 7.64 (m, 3H), 7.87 – 7.89 (m, 2H). mp: 70-72°C. Melting point is in accordance with lit. data (70°C).[292]

(Piperidin-4-yl)methanol (13)
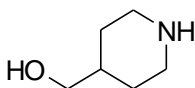
A solution of ethyl isonipecotate (77.9 mmol, 12.24 g) in anhydrous THF (80 mL) was added dropwise under argon to an ice-cooled suspension of LiAlH4 (97.3 mmol, 3.69 g) in anhydrous THF (250 mL). After 30 min, the mixture was allowed to warm at room temperature and stirred for 20h. After treatment with water (4.8 mL), NaOH$_{(aq)}$ 1M (4.8 mL), and water (9.6 mL) the solution was stirred with diethyl ether (200 mL) for 30 min. The slurry was then filtered and washed with diethyl ether (3 x 100 mL). Solvents were removed under vacuum to afford the *title* compound as a colorless oil (77.9 mmol, 8.97 g). Yield = 100 %. Chemical formula: $C_6H_{13}NO$. Molecular weight: 115.17 g.mol^{-1}. LC/MS (ESI): not UV visible, *m/z* [M + H]$^+$: 116.2. ^{1}H-NMR (300 MHz, CDCl3) δ = 1.06 – 1.22 (m, 2H), 1.61 (s, 1H), 1.67 – 1.77 (m, 2H), 2.00 (s, 3H), 2.60 (td, *J* = 2.6, 12.2, 2H), 3.03 – 3.16 (m, 2H), 3.44 – 3.47 (m, 2H). Spectral data are in accordance with lit. data.[264]

N-[2-[4-(Hydroxymethyl)-1-piperidinyl]ethyl]methylsulphonamide (14a)
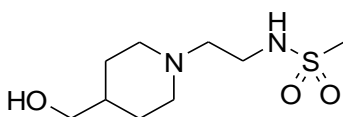
To an ice-cooled solution of (piperidin-4-yl)methanol (67.7 mmol, 7.8 g) in MeCN (70 mL) was added dropwise a N-(2-chloroethyl)methanesulfonamide (54.1 mmol, 8.45 g) solution in MeCN (50 mL). After the end of the addition, the mixture was allowed to warm at RT and stirred over 2 days. Then a 4 % aqueous solution of NaOH (200 mL) was added dropwise. The mixture was extracted with CH2Cl2 (6 x 100 mL) and the combined organic layers were dried on MgSO4 and then filtered. After solvent removal, a yellow oil was obtained and was further purified by normal phase silica gel column (CH2Cl2/EtOH: 5/5) to afford the *title* compound as a yellow oil (16.2 mmol, 3.8 g). Yield = 30 %. Chemical formula: $C_9H_{20}N_2O_3S$. Molecular weight: 236.34 g.mol^{-1}. LC/MS (ESI): not UV visible, *m/z* [M + H]$^+$: not ionizable. ^{1}H-NMR (300 MHz, CDCl3) δ = 1.20 (t, *J* = 7.0,

2H), 1.51 (s, 1H), 1.69 – 1.77 (m, 2H), 1.97 – 2.06 (m, 2H), 2.45 – 2.57 (m, 2H), 2.87 (d, J = 11.6, 2H), 2.96 (s, 3H), 3.10 – 3.25 (m, 2H), 3.49 (d, J = 6.3, 2H). Spectral data are in accordance with lit. data.[265]

N-[2-[4-(Hydroxymethyl)-1-piperidinyl]ethyl]phenylsulphonamide (14b)
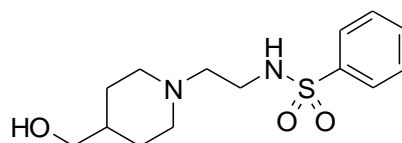
To a solution of piperidin-4-ylmethanol (17.4 mmol, 2 g) in MeCN (50 mL) were added DIPEA (35 mmol, 6.1 mL) and N-(2-chloroethyl)benzenesulfonamide (17.4 mmol, 3.82 g). The mixture was heated at reflux under argon for 3h. The solvent was evaporated under reduced pressure to give a white solid which was washed with water (2 x 75 mL) to give the *title* compound as a white solid (13.2 mmol, 3.95 g). Yield = 76 %. Chemical formula: $C_{14}H_{22}N_2O_3S$. Molecular weight: 298.41 g.mol^{-1}. LC/MS (ESI): 95 %, m/z [M + H]$^+$: 299.3. ^{1}H-NMR (300 MHz, DMSO-d$_6$) δ = 1.25 (d, J = 14.5, 2H), 1.50 – 1.62 (m, 2H), 1.69 – 1.97 (m, 2H), 2.33 (s, 2H), 2.73 (d, J = 9.6, 2H), 2.86 (t, J = 6.9, 2H), 3.19 (dd, J = 3.8, 6.2, 2H), 4.40 (d, J = 6.0, 1H), 7.61 (qdd, J = 1.9, 3.6, 8.8, 4H), 7.81 (dd, J = 1.7, 8.0, 2H).

(1-(2-(Methylsulfonamido)ethyl)piperidin-4-yl)methyl 5-fluoro-*1H*-indole-3-carboxylate hydrochloride (15a)
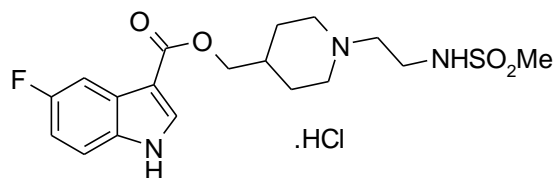
General procedure 2 for the preparation of (1-(2-(Phenyl-/methyl-sulfonamido)ethyl)piperidin-4-yl)methyl 1H-indole-3-carboxylate

Thionyl chloride (5.6 mmol, 406 μL) was slowly added to a suspension of 5-fluoro-*1H*-indole-3-carboxylic acid (1.12 mmol, 200 mg) in dry CH_2Cl_2 (10 mL) under argon, then the mixture was heated at reflux for 24h. After removal of the volatiles under reduced pressure, the residue was immediately put under argon atmosphere then taken off with dry CH_2Cl_2 (10 mL) and N-[2-[4-(hydroxymethyl)-1-piperidinyl]ethyl]methylsulphonamide (1.10 mmol, 260 mg) was added. The

resulting mixture was heated at reflux under argon for 24h. After removal of the solvent under vacuum, the resulting brown solid was taken off with water (50 mL) and then the aqueous layer was saturated with NaCl and extracted with CH_2Cl_2 (5 x 50 mL). The combined organic layers were washed with brine, dried on Na_2SO_4, filtered and solvents were removed under vacuum. The resulting grey solid was purified on a 20 g normal phase silica column with eluent (CH_2Cl_2/MeOH 90/10 to 75/25) to afford the product as brown foam (0.34 mmol, 134 mg). Yield = 30 %. The product was directly engaged in the next step.[293]

General procedure 3 for the preparation of hydrochloride acid salt

To a solution of (1-(2-(methylsulfonamido)ethyl)piperidin-4-yl)methyl 5-fluoro-*1H*-indole-3-carboxylate (0.16 mmol, 62 mg) in MeOH (3 mL) was slowly added acetyl chloride (0.48 mmol, 34 µL). After removal of solvent under reduced pressure, the residue was taken off with $CHCl_3$ (3 x 5 mL) and evaporated 3 times then with Et_2O (3 x 5 mL) to afford the *title* compound as a solid (0.16 mmol, 73 mg). Yield = quantitative. Chemical formula: $C_{18}H_{25}FClN_3O_4S$. Molecular weight: 433.93 g.mol^{-1}. LCMS (ESI): (2012/02/22) 96 % pure at 254 nm, *m/z* [M + H]$^+$: 398.2. HRMS (ESI) *m/z*: calcd. for $C_{18}H_{26}FClN_3O_4S$: 398.1544; [M+H]$^+$ found: 398.1536. ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ = 12.24 (s, 1H), 10.61 (s, 1H), 8.27 − 8.18 (m, 1H), 7.62 (dd, *J* = 2.8, 9.8, 1H), 7.51 (dd, *J* = 4.6, 9.1, 2H), 7.06 (td, *J* = 2.7, 9.2, 1H), 4.23 − 4.07 (m, 2H), 3.53 (s, 3H), 3.15 (s, 2H), 2.98 (s, 3H), 2.11 − 1.84 (m, 4H), 1.76 (d, *J* = 13.1, 2H), 1.35 (d, *J* = 5.8, 2H). ^{13}C NMR (101 MHz, DMSO-d$_6$) δ = 25.7, 26.7, 30.4, 33.0, 34.4, 37.2, 51.7, 55.7, 66.2, 105.2, 106.4, 110.8, 113.8, 126.3, 133.1, 157.1, 159.5, 164.0.

(1-(2-(Methylsulfonamido)ethyl)piperidin-4-yl)methyl *1H*-indole-3-carboxylate (15b)
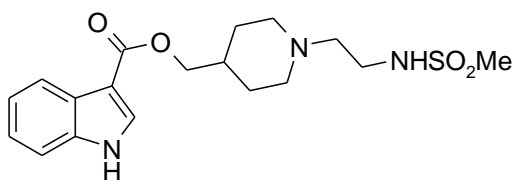
The reaction was carried out according to the general procedure 2 with the corresponding scale: *1H*-indole-3-carboxylic acid (1.67 mmol, 270 mg), $SOCl_2$ (8.35 mmol, 606 µL), CH_2Cl_2 (10 mL), then N-[2-[4-(hydroxymethyl)-1-piperidinyl]ethyl]methylsulphonamide (1.58 mmol, 373 mg), CH_2Cl_2 (10 mL). The crude was purified without work-up on a normal phase silica gel chromatography (CH_2Cl_2/MeOH 90/10) to afford the *title* compound as brown foam (0.83 mmol, 313 mg). Yield = 50 %. Chemical formula: $C_{18}H_{25}N_3O_4S$. Molecular weight: 379.48 g.mol^{-1}. LCMS (ESI): 98 % pure, *m/z* [M + H]$^+$: 380.2. HRMS (ESI) *m/z*: calcd. for $C_{18}H_{26}N_3O_4S$: 380.1639; [M+H]$^+$ found: 380.1629. ^{1}H NMR (400 MHz, DMSO-d$_6$) δ = 1.75 (s, 2H), 1.83 − 1.94 (m, 2H), 1.93 − 2.05 (m,

1H), 2.92 (s, 2H), 2.97 (s, 3H), 3.07 (q, J = 7.7, 8.1, 2H), 4.14 (d, J = 5.9, 2H), 7.14 – 7.26 (m, 2H), 7.41 – 7.54 (m, 2H), 7.96 – 8.02 (m, 1H), 8.15 (d, J = 3.0, 1H), 10.81 (s, 1H), 12.14 (d, J = 3.1, 1H).^{13}C NMR (101 MHz, DMSO-d$_6$) δ = 18.6, 33.2, 37.6, 38.9, 40.1, 40.2, 51.8, 56.0, 66.2, 106.2, 107.0, 112.4, 116.1, 120.4, 121.4, 122.4, 125.6, 132.8, 136.5, 164.4.

(1-(2-(Phenylsulfonamido)ethyl)piperidin-4-yl)methyl 5-fluoro-1H-indole-3-carboxylate hydrochloride (15c)
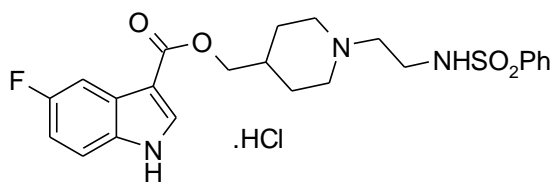
The reaction was carried out according to to the general procedure 2 with the corresponding scale: 5-fluoro-1H-indole-3-carboxylic acid (1.67 mmol, 300 mg), SOCl$_2$ (8.35 mmol, 605 µL), CH$_2$Cl$_2$ (10 mL), then N-[2-[4-(hydroxymethyl)-1-piperidinyl]ethyl]phenylsulphonamide (1.67 mmol, 498 mg), CH$_2$Cl$_2$ (10 mL). For the purification, a normal phase silica gel column (CH$_2$Cl$_2$/EtOH/NH$_{3(aq)}$ 95.5/4/0.5) was used to afford the product as a brown foam (0.65 mmol, 299 mg). Yield = 39 %. LC/MS (ESI): 86 % pure, m/z [M + H]$^+$: 460.2 The product was directly engaged in the next step.

The reaction was carried out according to the general procedure 3 with the corresponding scale: (1-(2-(phenylsulfonamido)ethyl)piperidin-4-yl)methyl 5-fluoro-1H-indole-3-carboxylate (0.28 mmol, 131 mg), MeOH (3 mL), acetyl chloride (0.86 mmol, 61 µL). After removal of solvent under reduced pressure, residue was washed with diethyl ether (3 x 3 mL). MeOH (3 mL) was added onto the resulting solid (120 mg) and heated at reflux for 15 min. The insoluble part was separated and washed with diethyl ether (2 x 3 mL). This operation was repeated two times to obtain the *title* compound as a white solid (64 mg, 0.13 mmol). Yield = 46 %. Chemical formula: C$_{23}$H$_{28}$FN$_3$O$_4$S. Molecular weight: 496.00 g.mol^{-1}. LC/MS (ESI): 98 % pure at 254 nm, m/z [M + H]$^+$: 460.2. HRMS (ESI) m/z: calcd. for C$_{23}$H$_{29}$FN$_3$O$_4$S: 460.1701 [M+H]$^+$; found: 460.1704. ^{1}H-NMR (400 MHz, DMSO-d$_6$) δ = 1.74 (tt, J = 7.1, 13.6, 2H), 1.89 (d, J = 13.6, 2H), 1.93 – 2.07 (m, 1H), 2.97 (q, J = 11.3, 2H), 3.07 – 3.27 (m, 5H), 3.40 – 3.54 (m, 2H), 4.13 (d, J = 5.9, 2H), 7.06 (td, J = 2.7, 9.2, 1H), 7.50 (dd, J = 4.6, 8.9, 1H), 7.58 – 7.72 (m, 4H), 7.85 (dd, J = 1.8, 7.0, 2H), 8.18 (d, J = 5.6, 1H), 8.23 (d, J = 3.1, 1H), 10.53 (s, 1H), 12.24 (d, J = 3.4, 1H). ^{13}C NMR (101 MHz, DMSO-d$_6$) δ = 25.5, 32.8, 37.1, 40.2, 48.6, 51.6, 55.3, 66.1, 105.0, 105.2, 106.3, 106.4, 110.6, 110.8, 113.7, 113.8, 126.2, 126.3, 126.6, 126.6, 129.4, 132.8, 133.1, 134.4, 139.7, 157.1, 159.4, 164.0. mp: 227-228°C

(1-(2-(Phenylsulfonamido)ethyl)piperidin-4-yl)methyl 5-nitro-*1H*-indole-3-carboxylate (15d)
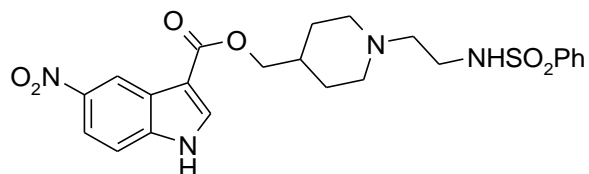
The reaction was carried out according to the general procedure 2 with the corresponding scale: 5-nitro-*1H*-indole-3-carboxylic acid (1.45 mmol, 300 mg), $SOCl_2$ (7.25 mmol, 526 µL), CH_2Cl_2 (10 mL), then *N*-[2-[4-(hydroxymethyl)-1-piperidinyl]ethyl]phenylsulphonamide (1.45 mmol, 432 mg), CH_2Cl_2 (10 mL). After the work-up, the resulting brown solid (512 mg) was taken off with MeOH (25 mL) and the insoluble part was removed by filtration. After removal of solvents, the resulting yellow solid was washed with EtOAc (3 x 20 mL) and Et_2O (2 x 20 mL) to afford the *title* compound as a yellow solid (0.385 mmol, 187 mg). Yield = 26 %. Chemical formula: $C_{23}H_{26}N_4O_6S$. Molecular weight: 486.55 g.mol^{-1}. LC/MS (ESI): 90 % pure, *m/z* [M + H]$^+$: 487.2. HRMS (ESI) *m/z*: calcd. for $C_{23}H_{27}N_4O_6S$: 487.1646 [M+H]$^+$; found: 487.1628. ^{1}H-NMR (300 MHz, DMSO-d$_6$) δ = 1.49 (s, 2H), 1.83 (d, *J* = 13.4, 3H), 2.73 (t, *J* = 2.0, 2H), 3.01 (s, 4H), 4.17 (d, *J* = 6.0, 2H), 7.57 – 7.72 (m, 4H), 7.83 (dd, *J* = 1.7, 8.0, 2H), 8.11 (dd, *J* = 2.4, 9.0, 1H), 8.40 (s, 1H), 8.86 (d, *J* = 2.3, 1H), 12.66 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d$_6$) δ = 26.4, 51.9, 67.0, 108.4, 113.3, 116.9, 117.8, 125.0, 126.6, 129.3, 132.6, 136.3, 139.6, 140.1, 142.4, 163.5. mp: 201-202°C.

(1-(2-(Phenylsulfonamido)ethyl)piperidin-4-yl)methyl *1H*-indole-3-carboxylate (15e)
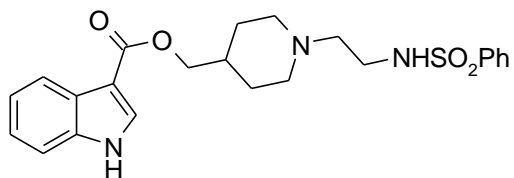
The reaction was carried out according to the general procedure 2 with the corresponding scale: $SOCl_2$ (4.65 mmol, 337 µL), *1H*-indole-3-carboxilic acid (0.93 mmol, 150 mg), dry CH_2Cl_2 (10 mL), *N*-{2-[4-(hydroxymethyl)piperidin-1-yl]ethyl}benzenesulfonamide (1.12 mmol, 333 mg). The resulting grey solid was purified on a 20 g normal phase silica column with eluent CH_2Cl_2/MeOH 95/5 to give oil (154 mg) containing the product. This fraction was further purified on a 40 g normal phase silica column with eluent CH_2Cl_2/MeOH 97/3 to give the *title* compound as a white solid (0.068 mmol, 30 mg). Yield = 7 %. Chemical formula: $C_{23}H_{27}N_3O_4S$. Molecular weight: 441.55

g.mol⁻¹. LC/MS (ESI): 80 % pure, *m/z* [M + H]⁺: 442.2. ¹H-NMR (300 MHz, DMSO-d₆) δ = 1.24 (d, *J* = 9.1, 2H), 1.66 (d, *J* = 11.4, 3H), 1.87 (t, *J* = 11.1, 2H), 2.28 (t, *J* = 6.8, 2H), 2.72 (d, *J* = 10.6, 2H), 2.85 (t, *J* = 6.9, 2H), 4.07 (d, *J* = 5.9, 2H), 7.15 – 7.22 (m, 2H), 7.45 – 7.50 (m, 1H), 7.52 – 7.68 (m, 3H), 7.77 – 7.85 (m, 2H), 7.94 – 8.00 (m, 1H), 8.07 (s, 1H), 11.94 (s, 1H).

(1-(2-(Phenylsulfonamido)ethyl)piperidin-4-yl)methyl 5-fluoro-2-methoxy-*1H*-indole-3-carboxylate hydrochloride (16)
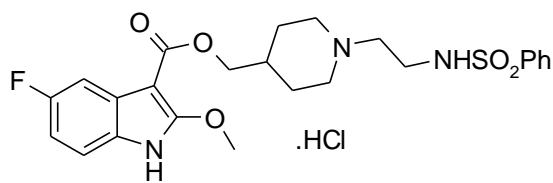
To a solution of (1-(2-(phenylsulfonamido)ethyl)piperidin-4-yl)methyl 5-fluoro-*1H*-indole-3-carboxylate (0.32 mmol, 148 mg) in dry CHCl₃ (7 mL) was added *N*-chlorosuccinimide (0.39 mmol, 52 mg). The mixture was heated at reflux under argon for 4h. After removal of solvent under reduced pressure, the residue was taken off with MeOH (10 mL) and heated at reflux under argon for 12h. After removal of solvent under vacuum, the resulting brown foam (207 mg) was purified on a normal phase silica gel column (CH₂Cl₂/MeOH 9/1) to afford the product as brown foam (0.22 mmol, 109 mg). Yield = 56 %. LC/MS (ESI): 99 % pure at 254 nm, *m/z* [M + H]⁺: 490.2. The product was directly engaged in the next step.

The reaction was carried out according to the general procedure 3 with the corresponding scale: (1-(2-(phenylsulfonamido)ethyl)piperidin-4-yl)methyl 5-fluoro-2-methoxy-*1H*-indole-3-carboxylate (0.22 mmol, 109 mg), MeOH (3 mL), acetyl chloride (0.70 mmol, 50 µL). The crude oil was dissolved in boiling methanol (3 mL) and then cooled to -18°C overnight. A white precipitate occurred. After removal of MeOH, the resulting solid was washed with methanol (1 x 2 mL) and with diethyl ether (3 x 2 mL). MeOH (3 mL) was added to the resulting solid and heated at reflux for 15 min. The insoluble part was separated and then washed with diethyl ether (2 x 3 mL). This purification was repeated once to give the *title* compound as a white solid (0.07 mmol, 38 mg). Yield = 33 %. Chemical formula: C₂₄H₂₉FClN₃O₅S. Molecular weight: 526.03 g.mol⁻¹. LC/MS (ESI): 99 % pure at 254 nm, m/z [M + H]⁺: 490.2. HRMS (ESI) m/z: calcd. for C₂₄H₃₀FClN₃O₅S: 490.1806; [M+H]⁺ found: 498.1800. ¹H-NMR (400 MHz, DMSO-d₆) δ = 1.54 – 1.69 (m, 2H), 1.88 (d, *J* = 12.9, 2H), 1.97 (s, 1H), 2.96 (q, *J* = 11.2, 2H), 3.11 (t, *J* = 5.6, 2H), 3.16 (s, 1H), 3.18 (d, *J* = 6.3, 2H), 3.40 (s, 2H), 3.48 (d, *J* = 12.0, 2H), 4.07 (d, *J* = 6.4, 2H), 4.11 (s, 3H), 6.88 (ddd, *J* = 2.7, 8.6, 9.6, 1H), 7.30 (dd, *J* = 4.7, 8.7,

1H), 7.45 (dd, J = 2.7, 10.2, 1H), 7.59 – 7.71 (m, 3H), 7.82 – 7.88 (m, 2H), 8.16 (t, J = 5.9, 1H), 10.42 (d, J = 67.8, 1H), 12.27 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ = 25.8, 33.0, 37.1, 38.9, 39.1, 39.3, 39.5, 39.6, 39.7, 39. 8, 39.9, 40.0, 40.2, 40.2, 48.6, 51.6, 55.4, 58.8, 65.6, 86.5, 86.5, 104.6, 104.9, 107.8, 108.1, 112.1, 112.2, 126.6, 126.9, 127.1, 127.2, 129.4, 132.8, 139.7, 157.2, 158.5, 159.5, 163.2. mp: 195-196°C.

6.2 Biology

6.2.1 Biology of the chapter 3.1

Radiometric kinase assays

CK2 radiometric kinase assay was performed as previously described in Prudent *et al*. [151]. Production and purification of GST-rhCK2a mutants were performed as described in the paper of Moucadel *et al*. [150] and inhibitors were tested following the CK2 radiometric kinase assay procedure. ATP concentrations were 100 µM if not stated otherwise. Substrate dependent phosphorylation assays on nucleolin, Six1 and CK2β auto-phosphorylation were performed following the CK2 radiometric kinase assay with CK2$a_2\beta_2$, the reaction was quenched by Laemmli buffer and samples were submitted to electrophoresis in NuPAGE buffer (150 V for 75 min) using pre-cast 4-12 % gradient gel (Bio-Rad). Proteins were stained using Coomassie blue (InstantBlue™, Expedeon, Cambridfgeshire, UK) and phosphorylation status measured using a phosphorimager PMI (BIO-RAD) and quantified with ImageJ.

Temperature-dependent circular dichroism (TdCD)

All single Far-UV-CD spectra were recorded using a Jasco-1500 spectropolarimeter (Gross-Umstadt, Germany). All spectra were recorded using a 1 mm path-length quartz cell, 1 nm bandwidth, 100 nm/min scan speed and 3 accumulations, within a wavelength range of 195 to 250 nm. GST-CK2a protein was diluted at 5 µM with 10 mM KH_2PO_4 and 50 mM Na_2SO_4, pH 7.4. Ligand binding was tested at 50 µM for compound **5**, 6.25 µM for compound **7** and 6.25 µM for CX-4945 with a final methanol concentration of 0.8 % (v/v). Compound **7** and CX-4945 concentrations were limited by their solubility in the methanol stock. Spectra in absence of protein were recorded and subtracted from the experimental spectra. Temperature ramping experiments were performed from 20 °C to 86 °C, and prior to each recording point, the sample was allowed to equilibrate for 30 sec at ± 0.1 °C from the desired temperature. Midpoint unfolding temperatures (Tm) were determined using Thermal Denaturation multi-Analysis software.

Protein-ligand interactions by NMR

NMR experiments were recorded on a 600 MHz spectrometer at 293 K in PBS pH 7.3, 10 mM NaCl, MgCl$_2$ 2 mM, 10 % D$_2$O. 1D experiments were performed using a excitation sculpting pulse sequence. Saturation Transfer Difference was performed with on-resonance protein saturation at 0.6 ppm using 2 s saturation time. 7-azaindole was used as a reference compound to test the STD-competition with CX-4945. STD experiments were performed using 5 µM CK2a protein in the presence of 4 mM MgCl$_2$. STD factors were measured as previously reported [221], and the values were normalized by assigning the value 100 % to the highest STD factor. Concentrations of the molecules AMP-PNP, 7-azaindole and compound **5** were 500 µM, and concentration of CX-4945 was 100 µM.

Molecular modeling and docking

Structures of the catalytic subunit of the human CK2 enzyme were retrieved from the Pocketome database [287]. Including the multiple chains within each structure, there was a total of 72 structures. Candidate ligand docking locations were delineated as shown in figure 3, one comprising the canonical ATP binding site, and another the proposed allosteric binding site. Prior to docking, the kinase structures were completed by rebuilding and optimization of the missing side-chains and the hydrogen atoms in the presence of co-crystallized ligands (if any). Compounds **1-7** presented here, as well as ATP and CX-4945 were docking to each of the two sites in each of the available structures using ICM (Molsoft L.L.C.) [288] (The ICM docking procedure and scoring are presented in the supporting procedures).

Top ranking predicted ligand poses were inspected manually, taking into account the pose consistency between multiple compounds in the series as well as interactions with the residues highlighted by site-directed mutagenesis. From this analysis, it became clear that the backbone conformation with the largest distance between the αC-helix and the middle β-strand of the N-terminal lobe is most compatible with the proposed model of binding. Based on this consideration, PDB 3JUH was selected as a backbone template for further studies. Val66Ala and Met163Leu mutations in the ATP binding pocket of this structure were reverted back to their wild-type amino-acids. Residue side-chains in the allosteric pocket were conformationally optimized in the presence of docked compounds to resemble pocket shape observed in PDB 3FWQ. The optimized model was used for the second round of compound docking using the ICM grid potential based protocol described in the supporting procedures. Top-ranking poses of compounds (including CX-4945 and ATP) from this round are presented.

ICM docking procedure

The ICM docking engine predicts ligand poses by stochastic global optimization of the flexible ligand molecule (represented in internal coordinates) inside a set of pre-calculated grid potential maps representing the binding pocket of the receptor [289–291] and including (i) van der Waals (represented as Lennard-Jones potential for hydrogen, carbon and "large atom" probes); (ii) electrostatic potential (calculated by the Coulomb formula with the distance-dependent dielectric constant of $4r$); (iii) hydrogen bonding potential combining the donor and acceptor fields; (iv) apolar surface energy. Prior to sampling, multiple starting poses of the ligand were generated by exhaustively sampling the ligand *in vacuo* and overlaying each of the resulting conformations onto the binding pocket in four principal orientations. The sampling phase was performed using biased probability Monte Carlo optimization [288] of rotational, translational, and torsional variables of the ligandin an attempt to achieve the global minimum of the objective energy function that combines ligand interactions with the receptor grid potentials and the explicit (full-atom) interactions within the ligand itself ("ligand strain"). Top-ranking predicted poses of the ligand were combined with the full-atom representation of the receptor pocket and evaluated with ICM ligand binding score [292] calculated as $S_{bind} = E_{int} + T\Delta S_{Tor} + E_{vw} + \alpha_1 \times E_{el} + \alpha_2 \times E_{hb} + \alpha_3 \times E_{hp} + \alpha_4 \times E_{sf}$. Here E_{vw}, E_{el}, E_{hb}, E_{hp}, and E_{sf} are Van der Waals, electrostatic, hydrogen bonding, non-polar and polar atom solvation energy differences between bound and unbound states, E_{int} is the ligand internal strain, ΔS_{Tor} is its conformational entropy loss upon binding, T = 300 K, and α_i are ligand- and protein-independent constants that have been previously optimized on a diverse screening benchmark.

Cell culture

The human kidney carcinoma cell line (786-O) was cultured in RPMI 1640 medium (Gibco) supplemented with 10 % (v/v) fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C in 5 % CO_2 atmosphere. Experiments were performed in RPMI 1640 (GIBCO) supplemented with 0.1 % or 0.5 % (v/v) fetal serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) 37 °C in 5 % CO_2 atmosphere.

Cell viability assay

Cells were plated into 96-well plates at 1 x 10^4 cells/well. The following day, the culture medium was replaced with fresh medium containing the inhibitors at various concentrations. DMSO (0.25 %) and cell free wells were used as control. After 24 h, 50 µL of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) solution in PBS (5 mg/mL) were added in each well and the

plate incubated for 2 h at 37 °C in 5 % CO_2 atmosphere. Then, 80 µL of SDS (10 %) containing 0.01 M HCl were added in each well and gently stirred at RT for 4 h. Absorbance was measured at 570 nm with a microplate reader (Infinite 200 Pro, Tecan). Results are the average of a triplicate experiment with a standard deviation lower than 10 %. EC_{50} was determined by linear interpolation after transformation to log[c] scale.

CK2 inhibition in cells

Cells were plated into 6-well plates at 3×10^5 cells/well. The following day, the culture medium was replaced with fresh medium containing the inhibitors at various concentrations or DMSO (0.5 %) as reference. After incubation for 24 h, medium was removed, cells were washed with cold PBS and frozen at -80°C. The phosphorylation status of two protein substrates of CK2 (□-catenin and Akt1) was measured by Western Blot analysis of cell extracts.

Western Blot analysis

Primary antibodies were a-catenin (phospho-Ser641) antibody from SAB Signalway Antibody, Akt1-(phosphor-Ser129) antibody from Abgent, anti-GAPDH mAb from Ambion. Secondary antibodies were peroxidase-conjugated affinity pure Goat anti-rabbit IgG (#111035003) and peroxidase-conjugated affinity pure Goat anti-mouse IgG (#115035003) from Jackson Immuno Research. Cells were lysed in RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 0.5 % DOC and 1 mM EDTA) containing both protease- and phosphatase-inhibitor cocktails (Sigma-Aldrich; P8340, P2850, P5726). Cell homogenates were quantified using BCA protein Assay kit (Thermo Scientific). SDS-PAGE was performed using pre-cast 4-12 % gradient gel (Bio-Rad) and submitted to electrophoresis in NuPAGE buffer (150 V for 75 min). Separated proteins at 20 µg/lane were transferred to PVDF membranes (100 V for 60 min). Blotted membranes were blocked during 1 h at room temperature with saturation buffer (1 % BSA in Tris Buffer Saline 10 mM, Tween 0.1 % (TBST)), and then incubated with primary antibody diluted in saturation buffer, for 2 h or overnight. After 3 washes with TBST, secondary antibodies were added for 1 h. Luminata Forte Western HRP substrate (Millipore) was added and membranes were read with Fusion Fx7 (PerkinElmer). Anti-GAPDH was used as loading control. Images were analyzed and band intensities were quantified using ImageJ software. EC_{50} were determined by fitting band intensities in function of inhibitor concentration to a sigmoid equation using SigmaPlot.

Cell transfection and imaging

The cDNA encoding GFP-CK2a was subcloned from pEGFP-CK2a vector [245] into pMSCV-puro plasmid. Viral particles were produced and stable expression of EGFP-CK2a was accomplished by transfection of 786-O cells followed by 1 µg/ml puromycin–mediated selection. 786-O pEGFP-CK2a cells were seeded on Lab-Tek™ slides. After treatment with DMSO, compound **7** (40 µM) or CX-4945 (8 µM) for 12 h, cells were washed twice with PBS and fixed with 4 % paraformaldehyde for 10 min at 20 °C. Nuclei were stained with Hoechst-33342 (Sigma-Aldrich) for 30 min and cells were washed with PBS (3 x 15 min). Images were acquired with a Zeiss ApoTome microscope and analyzed using Zen software. Fluorescence intensity, exposure times and post-acquisition modifications were kept identical for all the samples.

Screening of compound selectivity against a kinase panel

Kinase selectivity of compound **1** and **7** were assessed using the Kinase Profiler service offered by Life Technologies. The assays were performed with ATP concentration adjusted to the respective Km of each kinase in the presence of 50 µM inhibitor. Inhibition, expressed as the percent of activity determined in the absence of inhibitor, was calculated from the residual activity measured in the presence of 50 µM inhibitor.

6.2.2 Biology of the chapter 3.2

Radiometric kinase assays

CK2 radiometric kinase assay was performed as previously described in Prudent *et al*.[151] Production and purification of GST-rhCK2a mutants were performed as described in the paper of Moucadel *et al*.[150] and inhibitors were tested following the CK2 radiometric kinase assay procedure. ATP concentrations were 100 µM if not stated otherwise.

Cell culture

The human kidney carcinoma cell line (786-O) was cultured in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C in 5% CO_2 atmosphere. Experiments were performed in RPMI 1640 (GIBCO) supplemented with 0.1% or 0.5% (v/v) fetal serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) 37 °C in 5% CO_2 atmosphere.

Cell viability assay

Cells were plated into 96-well plates at 1×10^4 cells/well. The following day, the culture medium was replaced with fresh medium containing the inhibitors at various concentrations. DMSO (0.25%) and cell free wells were used as control. After 24 h, 50 µL of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) solution in PBS (5 mg/mL) were added in each well and the plate incubated for 2 h at 37 °C in 5 % CO_2 atmosphere. Then, 80 µL of SDS (10%) containing 0.01 M HCl were added in each well and gently stirred at RT for 4 h. Absorbance was measured at 570 nm with a microplate reader (Infinite 200 Pro, Tecan). Results are the average of a triplicate experiment with a standard deviation lower than 10%. EC_{50} was determined by linear interpolation after transformation to log[c] scale.

CK2 inhibition in cells

Cells were plated into 6-well plates at 3×10^5 cells/well. The following day, the culture medium was replaced with fresh medium containing the inhibitors at various concentrations or DMSO (0.5%) as reference. After incubation for 24 h, medium was removed, cells were washed with cold PBS and frozen at -80°C. The phosphorylation status of two protein substrates of CK2 (a-catenin and Akt1) was measured by Western Blot analysis of cell extracts.

Western Blot analysis

Primary antibodies were a-catenin (phospho-Ser641) antibody from SAB Signalway Antibody, Akt1-(phosphor-Ser129) antibody from Abgent, anti-GAPDH mAb from Ambion, anti-Akt1, anti-a-catenin, anti-PARP, anti-survivin. Secondary antibodies were peroxidase-conjugated affinity pure Goat anti-rabbit IgG (#111035003) and peroxidase-conjugated affinity pure Goat anti-mouse IgG (#115035003) from Jackson Immuno Research. Cells were lysed in RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% DOC and 1 mM EDTA) containing both protease- and phosphatase-inhibitor cocktails (Sigma-Aldrich; P8340, P2850, P5726). Cell homogenates were quantified using BCA protein Assay kit (Thermo Scientific). SDS-PAGE was performed using pre-cast 4-12% gradient gel (Bio-Rad) and submitted to electrophoresis in NuPAGE buffer (150 V for 75 min). Separated proteins at 20 µg/lane were transferred to PVDF membranes (100 V for 60 min). Blotted membranes were blocked during 1 h at room temperature with saturation buffer (1 % BSA in Tris Buffer Saline 10 mM, Tween 0.1% (TBST)), and then incubated with primary antibody diluted in saturation buffer, for 2 h or overnight. After 3 washes with TBST, secondary antibodies were added for 1 h. Luminata Forte Western HRP substrate (Millipore) was added and membranes were read with Fusion Fx7 (PerkinElmer). Anti-GAPDH was used as loading control. Images were analyzed and band intensities were quantified using ImageJ software.

6.2.3   Biology of the chapter 3.3

Cell culture

The human kidney carcinoma cell line (786-O) was cultured in RPMI 1640 medium (Gibco) supplemented with 10 % (v/v) fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C in 5 % CO_2 atmosphere. MCF10A and MCF10A-Δβ cells were cultured as described [293]. HeLa cells were cultured in DMEM with 10 % (v/v) fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C in 5 % CO_2 atmosphere.

Monitoring of the CK2α/CK2β interaction in cells

HeLa cells were seeded on Lab-Tek™. After treatment with DMSO, TAT-Pc (30 µM) or CX-4945 (12 µM) for 6h, cells were washed twice with PBS and fixed with 4 % paraformaldehyde for 10 min at 20° C, followed by permeabilization with 0.5% Triton X100. CK2α/CK2β interactions in cells were measured following the procedure of the DuoLink™ kit (Olink Bioscience). Images were acquired with a Zeiss ApoTome microscope and analyzed using Zen software. Quantification was performed using the BlobFinder software [250].

Cell immunostaining and imaging

786-O, MCF10A or MCF10A-Δβ cells were seeded on Lab-Tek™. After treatment with DMSO, TAT-Pc (30 µM) or CX-4945 (12 µM) for 12h, cells were washed twice with PBS and fixed with 4 % paraformaldehyde for 10 min at 20° C followed by permeabilization with 0.5% Triton X100. The following primary antibodies were diluted in TBS containing 0.1% Tween20. They were purchased from Santa Cruz Biotechnology for anti-CK2α sc6479 (1/200); anti-CK2β (6D5) sc-12739 (1/200); anti-CK2α' (C-20) sc-6481 (1/200); anti-EGFR (1005) sc-03 (1/200); anti-p21 (C-19) sc-397 (1/200); and from BD Pharmingen™ for Anti-Human CD107a (LAMP1) Clone H4A3 (RUO) (1/500). Cells were washed with PBS (3 x 15 min) and the secondary antibodies were Cy3-labeled donkey-anti-goat, Dy649-labeled donkey-anti-mouse IgG, and Cy3-labeled goat-anti-rabbit IgG. Nuclei were stained with Hoechst-33342 (Sigma-Aldrich) for 30 min and cells were washed with PBS (3 x 15 min). Images were acquired with a Zeiss ApoTome microscope and analyzed using Zen software. Fluorescence intensity, exposure times and post-acquisition modifications were kept identical for all the samples.

Cell viability assay

Cells were plated into 96-well plates at 1 x 10^4 cells/well. The following day, the culture medium was replaced with fresh medium (90 µL) containing the inhibitors at various concentrations. DMSO (0.25 %) and cell free wells were used as control. After 24 h, 10 µL of PrestoBlue® solution were added in each well and the plate incubated for 1 h at 37° C in 5 % CO_2 atmosphere. Results are the average of a triplicate experiment with a standard deviation lower than 10 %.

Western Blot analysis

Cells were plated into 6-well plates at 3 x 10^5 cells/well. The following day, the culture medium was replaced with fresh medium containing the inhibitors at various concentrations or DMSO (0.5 %) as reference. After incubation for 48 h, medium was removed, cells were washed with cold PBS and frozen at -80°C. Primary antibodies were a-catenin (phospho-Ser641) antibody from SAB Signalway Antibody, anti-a-catenin, Akt1-(phosphor-Ser129) antibody from Abgent, anti-Akt1-(phosphor-Ser473), anti-Akt1, anti-p21, anti-p21-(phosphor-Thr145), anti-GAPDH mAb from Ambion, anti-SNAIL1 from Cell signaling, anti-HSP90. Secondary antibodies were peroxidase-conjugated affinity pure Goat anti-rabbit IgG (#111035003) and peroxidase-conjugated affinity pure Goat anti-mouse IgG (#115035003) from Jackson Immuno Research. Cells were lysed in RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 0.5 % DOC and 1 mM EDTA) containing both protease- and phosphatase-inhibitor cocktails (Sigma-Aldrich; P8340, P2850, P5726). Cell homogenates were quantified using BCA protein Assay kit (Thermo Scientific). SDS-PAGE was performed using pre-cast 4-12 % gradient gel (Bio-Rad) and submitted to electrophoresis in NuPAGE buffer (150 V for 75 min). Separated proteins at 20 µg/lane were transferred to PVDF membranes (100 V for 60 min). Blotted membranes were blocked during 1 h at room temperature with saturation buffer (1 % BSA in Tris Buffer Saline 10 mM, Tween 0.1 % (TBST)), and then incubated with primary antibody diluted in saturation buffer, for 2 h or overnight. After 3 washes with TBST, secondary antibodies were added for 1 h. Luminata Forte Western HRP substrate (Millipore) was added and membranes were read with Fusion Fx7 (PerkinElmer). Anti-HSP90 and/or anti-GAPDH were used as loading control.

6.2.4 Biology of the chapter 3.4

CK2β-independent and CK2β(+)-dependent substrates

The following peptide substrates were employed: a canonical CK2 peptide substrate RRREDEESDDEE phosphorylated equally by CK2a and CK2a2β2 (CK2β-independent peptide substrate), and MSGDEMIFDPTMSKKKKKKKP (eIF2 peptide) exclusively phosphorylated by CK2a2β2 (CK2β-dependent peptide substrate.[294]

CK2 kinase assays were performed in a final assay volume of 18 mL containing 3 mL of compounds, 3 mL of CK2a (36 ng) and a mixture of 100 μM peptide substrate, 10 mM MgCl$_2$, and 1 μCi [γ^{32}P]-ATP. Final concentration of ATP was 100 μM if not specified otherwise. Assays were performed under linear kinetic conditions for 5 min at room temperature before termination by the addition of 60 μL of 4% TCA. ^{32}P incorporation in peptide substrate was determined as previously described.[295]

Surface Plasmon Resonance (SPR)

SPR binding studies were performed using a Reichert SR7000DC instrument optical biosensor (Reichert Technologies) equipped with a CMD500m sensor chip obtained from XanTec Bioanalytics. CK2α was immobilized by a capturing approach using a monoclonal anti-GST antibody (27 kDa, Clone GST-R 6G9 produced in rat, SAB4200055 Sigma Aldrich). Prior to use, the anti-GST antibodies were purified twice by micro dialysis at 4°C (Membrane: dialysis tubing benzoylated, 9 mm, D2272-5FT) in 10 mM sodium acetate, pH = 5. Anti-GST antibodies were immobilized using amine-coupling chemistry at 12°C. The surfaces of all two flow cells were activated for 7 min with a 1:1 mixture of 0.1 M NHS (N-hydroxysuccinimide) and 0.1 M EDC (3-(N,N-dimethylamino) propyl-N-ethylcarbodiimide) at a flow rate of 10 μl/min. The antibody at a concentration of 30 μg/ml in 10 mM sodium acetate, pH 5.0, was immobilized at a density of 2000 RU on flow cell 2; flow cell 1 was left blank to serve as a reference surface. All the surfaces were blocked with a 3 min injection at 10 μL/min of 1 M ethanolamine, pH 8.0. GST tagged CK2a at a concentration of 50 μg/mL in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20, pH = 7.4 was captured at a density of 900 RU on flow cell 2.

SPR Kd determination

To collect kinetic binding data, analytes in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005 % (v/v) polysorbate 20, 1% DMSO (v/v), pH = 7.4 were injected over the two flow cells at concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56 µM at a flow rate of 25 µl/min and at a temperature of 25°C. The complex was allowed to associate and dissociate for 120 s. Duplicate injections (in random order) of each sample and a buffer blank were flowed over the two surfaces. Affinities were obtained after treatments (DMSO calibration, blank and references subtractions) using the software Scrubber 2.0c.

Cell culture

MCF10 were cultured as described.[293]

Cell viability assay

Cells were plated into 96-well plates at 1 x 10^4 cells/well. The following day, the culture medium was replaced with fresh medium (90 µL) containing the inhibitors at various concentrations. DMSO (0.25 %) and cell free wells were used as control. After 24 h, 10 µL of PrestoBlue® solution were added in each well and the plate incubated for 1 h at 37° C in 5 % CO_2 atmosphere. Results are the average of a triplicate experiment with a standard deviation lower than 10 %. EC_{50} was determined by linear interpolation after transformation to log[c] scale.

Western Blot analysis

Cells were plated into 6-well plates at 3 x 10^5 cells/well. The following day, the culture medium was replaced with fresh medium containing the inhibitors at various concentrations or DMSO (0.5 %) as reference. After incubation for 48 h, medium was removed, cells were washed with cold PBS and frozen at -80°C. Primary antibodies were anti-p21, anti-p21-(phosphor-Thr145), anti-HSP90. Secondary antibodies were peroxidase-conjugated affinity pure Goat anti-rabbit IgG (#111035003) and peroxidase-conjugated affinity pure Goat anti-mouse IgG (#115035003) from Jackson Immuno Research. Cells were lysed in RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 0.5 % DOC and 1 mM EDTA) containing both protease- and phosphatase-inhibitor cocktails (Sigma-Aldrich; P8340, P2850, P5726). Cell homogenates were quantified using BCA protein Assay kit (Thermo Scientific). SDS-PAGE was performed using pre-cast 4-12 % gradient gel (Bio-Rad) and submitted to electrophoresis in NuPAGE buffer (150 V for 75 min). Separated proteins at 20 µg/lane were transferred to PVDF membranes (100 V for 60 min). Blotted membranes were blocked during 1 h at room temperature with saturation buffer (1

% BSA in Tris Buffer Saline 10 mM, Tween 0.1 % (TBST)), and then incubated with primary antibody diluted in saturation buffer, for 2 h or overnight. After 3 washes with TBST, secondary antibodies were added for 1 h. Luminata Forte Western HRP substrate (Millipore) was added and membranes were read with Fusion Fx7 (PerkinElmer). Anti-HSP90 was used as loading control.

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