



**UNIVERSITÀ
DEGLI STUDI
DI PADOVA**

Università degli Studi di Padova

Dipartimento di Scienze Biomediche

**SCUOLA DI DOTTORATO DI RICERCA IN
BIOSCIENZE E BIOTECNOLOGIE**

INDIRIZZO BIOCHIMICA E BIOFISICA

CICLO XXVI

EFFECTS OF DIFFERENT CK2 INHIBITION STRATEGIES IN TUMOR CELLS

DIRETTORE DELLA SCUOLA : CH.MO PROF. GIUSEPPE ZANOTTI

COORDINATORE D'INDIRIZZO: CH.MO PROF. FABIO DI LISA

SUPERVISORE: DOTT.SSA MARIA RUZZENE

DOTTORANDO: SOFIA ZANIN

Contents

Contents.....	I
Summary.....	III
Riassunto	VII
Abbreviations.....	XIII

INTRODUCTION.....	1
1. Protein phosphorylation.....	3
1.1. Protein kinases classification.....	5
1.2. Protein kinases structure and function.....	7
1.3 Protein kinase specificity.....	8
1.4. Ser/Thr kinases.....	10
2. Structural Biology of protein kinase CK2.....	12
2.1. CK2 substrate specificity.....	19
3. Physiopathological role of CK2.....	22
3.1. Role of CK2 in survival and apoptosis.....	22
3.2. CK2 role as a “later” player.....	27
3.3. CK2 involvement in regulating chaperone machinery.....	27
3.4. Role of CK2 in cancer.....	29
3.5. CK2 as an ectokinase.....	32
4. Inhibition of protein kinase CK2.....	33
4.1. Why and how to target CK2.....	33
4.2. Selectivity of the CK2 inhibitors.....	35
4.3. Potency of CK2 inhibitors	25
4.4. Classification of CK2 inhibitors.....	23
4.4.1. ATP-competitive inhibitors.....	37
4.4.2. ATP-noncompetitive inhibitors.....	47
4.4.3. Bi-substrates CK2 inhibitors.....	48
4.5. <i>In vivo</i> versus <i>in vitro</i> inhibitory activity.....	49
4.6. How druggable is CK2?.....	51
5. Multi-drug Resistance (MDR).....	53
5.1. CK2 and MDR.....	57

MATERIALS AND METHODS

1. Materials.....	61
2. Methods.....	63

RESULTS

1. Effects of CK2 inhibitors CX-4945 and CX-5011 in drug-resistant cells.....	73
1.1. Introduction.....	75
1.2. Aim of the study.....	79
1.3. Results.....	80
1.4. Discussion.....	96
2. Effects of CK2 bi-functional inhibitors in tumor cells.....	101
2.1. Introduction.....	103
2.2. Aim of the study.....	107
2.3. Results.....	108
2.4. Discussion.....	117
3. Peptides targeting CK2 substrates: in vivo and in cell effects.....	119
3.1. Introduction.....	121
3.2. Aim of the study.....	126
3.3. Results.....	127
3.4. Discussion.....	158
4. Inhibition strategies targeting a different kinase, the “Homodomain Interacting protein kinase 2” (HIPK2).....	163
4.1. Introduction.....	165
4.2. Aim of the study.....	171
4.3. Results.....	172
4.4. Discussion.....	174

GENERAL CONCLUSIONS.....	175
--------------------------	-----

REFERENCES.....	181
-----------------	-----

PUBLICATIONS.....	211
-------------------	-----

SUMMARY

Protein kinase CK2, constituted by two catalytic (α and/or α') and two regulatory (β) subunits, is a highly conserved kinase which phosphorylates a huge number of substrates and is involved in different cellular pathways and functions. It has a major anti-apoptotic role, it enhances the apoptosis resistance (MDR) phenotype, and it is abnormally expressed in a wide variety of cancers, where it operates as a “cancer driver” by creating a cellular environment favorable to the development, growth and propagation of malignancy. Since CK2 expression is not linked to a specific type of cancer, its targeting could be a successful strategy, because of its very general applicability and widespread effects. Importantly, tumor cells are more sensitive to CK2 inhibition than normal ones, probably because they rely more on CK2 for their survival, accordingly to the concept of cancer addiction to CK2 (Ruzzene and Pinna, 2010). Therefore, CK2 represents a suitable target for developing anticancer drugs.

Different compounds have been developed so far as CK2 inhibitors, useful not only for investigating CK2 physiological functions, but also as possible therapeutic tools. The majority of them act targeting the ATP-binding site of CK2; some compounds have shown high potency and specificity toward the kinase, and have disclosed a proapoptotic effect in cancer cells more evident than in normal ones.

The research presented in this thesis provides examples of different strategies to target protein kinase CK2, in order to selectively reduce its activity in tumor cells and to produce cellular effects which can counteract the malignant transformation. In particular, we used: 1) CX-4945 and CX-5011, ATP-competitive CK2 inhibitors (Pierre *et al.* 2011); 2) K137-E4, a bi-functional inhibitor designed to compete with both the ATP and the peptide binding sites of CK2; 3) a compound called CRIBI-300, derived from CIGB-300, designed to prevent the phosphorylation of specific CK2 substrates (Perea *et al.*, 2004). The three studies are summarized below. A fourth study is also described, which exploited a compound, TBID, developed from a CK2 inhibitor but modified to become selective toward another protein kinase, HIPK2.

Effects of the ATP-competitive CK2 inhibitors CX-4945 and CX-5011 in apoptosis-resistant cells

CX-4945 and CX-5011 inhibitors (developed by Cylene Pharmaceuticals) act competing with ATP for the binding to the catalytic pocket of the CK2. These compounds are very potent and selective in inhibiting CK2 (Pierre *et al.*, 2011; Battistutta *et al.*, 2012): CX-4945, in particular, has shown good pharmacological features and has advanced in clinical trials with promising outcomes. However, the CX compounds had never been used in apoptosis-resistant cells. In this work we demonstrate that CX-4945 and CX-5011 are efficient in reducing endogenous CK2 activity and in inducing apoptosis in several pairs of apoptosis-sensitive and resistant tumor cell lines. Furthermore, we also observed that resistant cells which express the drug-extruding pump P-gp are sensitized by CX-4945 towards conventional antitumor drugs. In fact, we found that doxorubicin uptake in resistant cells is increased, and that combined treatment with CX-4945 and vinblastine produces synergistic effects on cell death. In summary, our data suggest that CX inhibitors can be considered as a valid therapeutic strategy also in case of pharmacological resistance.

Effects of CK2 bi-functional inhibitors in tumor cells

A very new strategy to target CK2 is based on compounds denoted as bi-functional or bi-substrate inhibitors, designed to hit both the ATP and the substrate sites of the kinase at the same time, in order to improve selectivity toward CK2. Among these compounds, K137-E4 has been recently developed in our laboratory and characterized by *in vitro* studies; it was found to strongly inhibit recombinant CK2 ($IC_{50}=0.025\ \mu M$) and to be very selective. In the study presented in this thesis, we had the aim of investigating the employment of this compound in cells. We first demonstrated that it is also able to inhibit the activity of native CK2 towards cellular substrates. However, this was performed by means of *in vitro* phosphorylation assay, while, when we used K137-E4 for cell treatment we did not observed any effect on CK2 activity and cell viability. We therefore supposed that the compound, quite hydrophilic, is not cell permeable. Studies are under investigation to modify the molecule structure in order to improve its cell permeability, without significantly decreasing its inhibitory capacity. At present, we found that the K137-E4 molecule, in virtue of its impossibility to cross plasma membrane, can be useful to study CK2 ecto-kinase, that portion of this enzyme that resides at the external surface

of cells (Kubler *et al.*, 1989). We demonstrated that it is able to inhibit CK2 ecto-kinase, but this has not effect on cell viability of tumor and non-tumor cells. This finding, in conjunction with the observation that CK2 ecto-kinase activity seems to be similar in tumor and not-tumor cells, suggests that CK2 ecto-kinase is not implied in cell survival and tumor-related events.

In vitro and in cell effects of peptides targeting CK2 substrates

A recently introduced approach to target CK2 is based on the development of a cyclic peptide called CIGB-300, which interferes with the phosphorylation site of specific CK2 substrates, rather than inhibiting the enzyme *per se* (Perea *et al.*, 2004). CIGB-300 is already employed in clinical trials (Perea *et al.*, 2011), however, its mechanism of action is only partially understood. We therefore exploited a new synthesized peptide, denoted as CRIBI-300, with the same functional domain of the parental CIGB-300, and we performed *in vitro* and *in cell* studies, comparing it with CIGB-300 and several other derivatives. We found that CK2 activity *in vitro* is affected by CRIBI-300 peptide, but with different effects accordingly to the type of substrate used: inhibition is observed only towards substrates which are strictly dependent on the presence of CK2 β for their phosphorylation by CK2, while the activity towards substrates which are preferentially phosphorylated by the CK2 monomeric α is rather stimulated by CRIBI-300. This suggests that CRIBI-300 could interfere with the CK2 holoenzyme structure, producing an unstable and/or unfunctional tetrameric conformation which prevents the phosphorylation of β -dependent substrates.

When we performed cell treatment experiments, we also found that CRIBI-300 induces a strong reduction of endocellular CK2 activity, but this is accompanied by an early and rapid loss of CK2 protein amount. In parallel, cell death was also induced by CRIBI-300, but it was not due to apoptosis. Moreover, we observed a loss in the amount of also other proteins beside CK2; alteration in the activity of proteolytic systems occurs as well, with the activation of lysosomal proteases and the blockage of proteasome machinery. All the effects appear more evident in tumor than in non-tumor cells.

We then analyzed the effects of CRIBI-300 derived peptides, with single aminoacid substitutions or large sequence differences. Interestingly, we found that the Tat sequence (the portion of CRIBI-300 introduced to render it cell permeable (Holm *et al.*, 2006)) is

essential for its activity towards CK2 in cells, but not sufficient *per se*, being specific aminoacids in the remaining part of CRIBI-300, crucial for producing effects.

Of the many effects evoked by CRIBI-300, we wondered if and which ones are mediated by its action on CK2. We found that the reduction of CK2 expression (obtained by RNA-interference cellular experiments) also attenuates some of the CRIBI-300 effects, although not all. Moreover, those CRIBI-300 sequence alterations which prevent *in vitro* effects on CK2 are also able to reduce cellular effects. We therefore concluded that CK2 is indeed an early and important target of CRIBI-300. However, additional mechanisms of action of this compound should exist to explain all the observed effects, and this suggests that extreme caution is required for its employment at a clinical level.

Effects of TBID inhibitor on cellular HIPK2 activity

A collateral study presented in this thesis concerns a compound, TBID, derived from a CK2 inhibitor (TBI) but modified in structure to become selective for a different kinase, HIPK2 (homeodomain-interacting protein kinase 2), a Ser/Thr kinase controlling cell proliferation and survival (D'Orazi *et al.*, 2012), whose investigation has been hampered by the lack of specific inhibitors able to dissect its cellular functions. TBID is an ATP-competitive inhibitor very potent towards HIPK2 *in vitro* ($IC_{50} = 0.33 \mu M$) and very selective. Here we show that it is also cell permeable, being able to inhibit endocellular HIPK2 activity, and to reduce the phosphorylation state of a HIPK2 target, Ser46 of p53 protein. Since TBID is not cytotoxic under the conditions used to check its effects on cellular HIPK2 activity, we propose TBID as the only tool presently available to down regulate cellular HIPK2.

Altogether, the results presented in this thesis demonstrate that kinase inhibition is an extremely versatile tool to be used in cells, exploitable either to investigate on the role of a specific kinase or to block an unwanted hyperactivity. In particular, in the case of CK2 inhibitors, we analyzed different strategies. Although for the moment the more effective and convincing one is still that represented by the classical ATP-site directed compounds, other approaches are also presented, which need further investigation but could be promising in term of improved specificity or more targeted effects.

RIASSUNTO

La proteinchinasi CK2, costituita da due subunità catalitiche (α e/o α') e due subunità regolatorie (β), è un enzima altamente conservato, che fosforila un gran numero di substrati ed è coinvolto in diverse vie di segnale e funzioni cellulari. CK2 svolge un importante ruolo anti-apoptotico, è coinvolta nel fenomeno della farmacoresistenza all'apoptosi, ed è altamente espressa in un'ampia varietà di tumori, dove opera come un "promotore cancerogeno" creando un ambiente cellulare favorevole alla sviluppo, alla crescita, e alla propagazione della malignità. Poiché la sua sovraespressione non è direttamente connessa ad uno specifico tipo di tumore, colpire CK2 può diventare un'interessante strategia di successo data la sua generale applicabilità e suoi diffusi effetti. È importante sottolineare che le cellule tumorali sono più sensibili all'inibizione di CK2 rispetto a quelle normali, probabilmente perché sono più dipendenti da CK2 per la loro sopravvivenza, e questo va in accordo con il recente concetto di "*cancer addiction*" a CK2 (Ruzzene e Pinna, 2010). Pertanto, CK2 rappresenta un obiettivo adatto per lo sviluppo di farmaci antitumorali.

Finora sono stati sviluppati diversi inibitori di CK2, utili non solo per indagare il suo ruolo fisiologico, ma sfruttati anche come possibili strumenti terapeutici. La maggior parte di questi composti agisce bloccando il sito di legame per l'ATP dell'enzima; alcuni di questi hanno dimostrato elevato potere inibitorio e specificità verso la chinasi, e si sono rivelati più efficaci nell'indurre apoptosi in cellule tumorali rispetto a quelle normali. La ricerca presentata in questa tesi fornisce esempi di diverse strategie di inibizione mirate a bloccare CK2, al fine di ridurre selettivamente la sua attività nelle cellule tumorali e di produrre effetti cellulari che possono contrastare la trasformazione maligna. In particolare, abbiamo utilizzato: 1) CX-4945 e CX-5011, inibitori di CK2 ATP-competitivi (Pierre *et al.*, 2011); 2) K137-E4, un inibitore bifunzionale progettato per competere simultaneamente con ATP e con il sito di legame per il peptide substrato di CK2; 3) CRIBI-300, un composto derivato da CIGB-300, progettato per impedire la fosforilazione di substrati specifici da parte di CK2 (Perea *et al.*, 2004). Questi tre argomenti di ricerca sono riassunti di seguito. Viene presentato anche un quarto studio che ha riguardato un composto, TBID, sviluppato partendo da un inibitore di CK2, modificandolo per renderlo selettivo nei confronti di un'altra proteinchinasi, HIPK2.

Effetti degli inibitori di CK2 ATP-competitivi CX-4945 e CX-5011 in cellule resistenti all'apoptosi

Gli inibitori CX-4945 e CX-5011 (sviluppati dalla Cylene Pharmaceuticals) agiscono competendo con l'ATP per il legame nella tasca catalitica di CK2. Questi composti sono molto potenti e selettivi nell'inibire CK2 (Pierre *et al.*, 2011; Battistutta *et al.*, 2012), il CX-4945 in particolare ha mostrato buone proprietà farmacologiche ed è in sperimentazione clinica nell'uomo con promettenti risultati. Tuttavia, i composti CX non sono mai stati utilizzati in cellule resistenti all'apoptosi. In questo lavoro dimostriamo come CX-4945 e CX-5011 siano efficaci nel ridurre l'attività endogena di CK2 e nell'indurre apoptosi in diverse linee cellulari tumorali sensibili e resistenti all'apoptosi. Abbiamo inoltre trovato che le cellule resistenti che esprimono una pompa di estrusione dei farmaci, la P-gp, vengono rese sensibili a farmaci antitumorali convenzionali grazie all'uso di CX-4945. Infatti abbiamo trovato che l'accumulo di doxorubicina viene incrementato in cellule resistenti e che il trattamento combinato con CX-4945 e vinblastina produce effetti sinergici sulla morte cellulare. Pertanto, i nostri dati suggeriscono che gli inibitori CX possono essere considerati come una valida strategia terapeutica anche nei casi di resistenza farmacologica.

Effetti di inibitori di CK2 bifunzionali in cellule tumorali

Una nuova strategia per inibire CK2 è basata su composti definiti come inibitori bifunzionali o bisubstrato, disegnati per legarsi contemporaneamente ai siti di legame per l'ATP e per il substrato di CK2, con lo scopo di aumentarne la selettività per CK2. Tra questi composti il K137-E4 è stato recentemente sviluppato e caratterizzato *in vitro* nel nostro laboratorio: è stato dimostrato che è molto potente nell'inibire la CK2 ricombinante ($IC_{50} = 0,025 \mu M$) e che ha buona selettività. Nello studio presentato in questa tesi, ci siamo proposti di analizzare la possibilità di utilizzo di questo composto anche in cellule. Innanzitutto abbiamo dimostrato che il K137-E4 è in grado di inibire l'attività di CK2 nativa nei confronti di substrati cellulari. Tuttavia, questo si riferisce ad esperimenti *in vitro*, mentre quando abbiamo trattato le cellule con il K137-E4 non abbiamo osservato alcun effetto né sull'attività di CK2 né sulla vitalità cellulare. Abbiamo pertanto ipotizzato che il composto, essendo piuttosto idrofilico, non fosse in grado di attraversare la membrana cellulare. Sono tuttora in corso modifiche strutturali della molecola mirate ad aumentarne la permeabilità cellulare senza diminuirne

significativamente la sua capacità inibitoria. Ad oggi, abbiamo pensato che il K137-E4, in virtù della sua impossibilità di attraversare la membrana plasmatica, può essere utile per studiare l'attività ectochinasica di CK2, cioè di quella porzione di enzima che risiede sulla superficie esterna delle cellule (Kubler *et al.*, 1989). Abbiamo infatti dimostrato che questo composto è capace di inibire CK2 ectochinasica, ma che ciò non ha alcun effetto sulla vitalità cellulare né di cellule tumorali né di cellule non tumorali. Questa scoperta, assieme all'osservazione che l'attività ectochinasica di CK2 sembra essere simile in cellule tumorali e non, suggerisce che la CK2 ectochinasica non è implicata nella sopravvivenza cellulare e negli eventi correlati alla tumorigenesi.

Effetti in vitro e in cellule di peptidi mirati a legare substrati di CK2

Un nuovo approccio recentemente introdotto per colpire CK2 è basato sullo sviluppo di un peptide ciclico, chiamato CIGB-300, che interferisce con la fosforilazione di specifici substrati di CK2 piuttosto che inibire con l'enzima *per se* (Perea *et al.*, 2004). CIGB-300 è già impiegato in studi clinici (Perea *et al.*, 2011), tuttavia il suo meccanismo d'azione è solo parzialmente conosciuto. Abbiamo dunque sfruttato un nuovo peptide sintetico, chiamato CRIBI-300, con lo stesso dominio funzionale del CIGB-300, e abbiamo effettuato studi *in vitro* ed in cellule, comparandolo con il CIGB-300 e altri suoi derivati. Abbiamo scoperto che l'attività di CK2 *in vitro* può essere influenzata dal peptide CRIBI-300, ma con effetti differenti a seconda del tipo di substrato utilizzato: abbiamo osservato inibizione solo per quei substrati la cui fosforilazione da parte di CK2 è strettamente dipendente dalla presenza della subunità regolatoria β , mentre troviamo stimolazione da parte del CRIBI-300 nei confronti di substrati che sono fosforilati preferenzialmente dalla subunità catalitica α . Questi dati suggeriscono che il CRIBI-300 potrebbe interferire con la struttura dell'oloenzima CK2, dando luogo ad una conformazione tetramericamente instabile e non funzionale e impedendo la fosforilazione di substrati β -dipendenti.

Anche in esperimenti di trattamento cellulare, abbiamo trovato che CRIBI-300 induce una forte riduzione dell'attività cellulare di CK2, che è però accompagnata da una precoce e rapida perdita della quantità proteica di CK2. In parallelo, CRIBI-300 induce anche morte cellulare, ma non per apoptosi. Inoltre, abbiamo osservato diminuzione delle quantità di altre proteine, oltre a CK2, e un'alterazione dell'attività dei sistemi proteolitici con l'attivazione di proteasi lisosomiali e il blocco del proteasoma. Tutti questi effetti appaiono più evidenti in cellule tumorali che in cellule non tumorali.

Abbiamo poi analizzato gli effetti di derivati peptidici del CRIBI-300, con sostituzioni di singoli aminoacidi o con sequenze diverse. E' interessante il fatto che abbiamo trovato che la sequenza Tat (la porzione del CRIBI-300 introdotta per renderlo permeabile alla cellula (Holm *et al.*, 2006)) è essenziale per la sua attività su CK2 *in vitro* e in cellula, ma non è sufficiente di per se dal momento che specifici amminoacidi nella parte funzionale del CRIBI-300 (la sequenza non corrispondente a Tat) risulta essere cruciale per produrre gli effetti. Dei molti effetti causati dal CRIBI-300, abbiamo cercato di capire quali fossero mediati dalla sua azione su CK2. Abbiamo trovato che la riduzione dell'espressione di CK2 (ottenuta mediante esperimenti di "RNA interference") attenua alcuni degli effetti del CRIBI-300, sebbene non tutti. Inoltre, quelle modifiche della sequenza del CRIBI-300 che impediscono i suoi effetti *in vitro* su CK2 sono anche capaci di ridurre gli effetti a livello cellulare. Possiamo perciò concludere che CK2 è sicuramente uno dei più importanti bersagli di CRIBI-300. Tuttavia altri meccanismi d'azione di questo composto devono esistere per spiegarne tutti gli effetti osservati, e ciò suggerisce quindi che un'estrema cautela è richiesta per l'utilizzo di questo composto.

Effetti dell'inibitore TBID sull'attività cellulare di HIPK2

Uno studio collaterale presentato in questa tesi riguarda il composto TBID, che deriva da un inibitore di CK2, il TBI, ma che è stato modificato nella struttura per renderlo selettivo nei confronti di un'altra chinasi, HIPK2 (Homeodomain-interacting protein kinase 2), una Ser/Thr chinasi che controlla la proliferazione e sopravvivenza cellulare (D'Orazi *et al.*, 2012) il cui studio è stato rallentato dalla mancanza di inibitori specifici capaci di svelarne le funzioni cellulari. TBID è un inibitore ATP-competitivo molto potente nei confronti di HIPK2 *in vitro* ($IC_{50} = 0,33 \mu M$) e molto selettivo. In questa tesi mostriamo che TBID è anche in grado di entrare nelle cellule, dimostrando che inibisce l'attività di HIPK2 intracellulare e riduce la fosforilazione della Ser46 di p53, un sito fosforilato da HIPK2. Dal momento che TBID non è citotossico nelle condizioni utilizzate per analizzarne gli effetti sull'attività di HIPK2 cellulare, proponiamo TBID come l'unico strumento attualmente disponibile per inibire HIPK2 in cellule.

I risultati presentati in questa tesi dimostrano che l'inibizione di chinasi rappresenta uno strumento estremamente versatile per l'utilizzo nelle cellule, sia per analizzare il ruolo di una specifica chinasi, sia per bloccarne una non desiderata iperattività. In particolare, nel caso di inibitori di CK2 abbiamo analizzato diverse strategie. Sebbene al momento la più

efficace e convincente rimanga quella rappresentata dai classici composti ATP-competitivi, abbiamo presentato altri approcci che, pur necessitando di ulteriori studi, potranno in futuro dimostrarsi promettenti in termini di aumentata specificità o di maggiore selettività degli effetti prodotti.

ABBREVIATIONS

AMINOACIDS

Ala	Alanine	A
Arg	Arginine	R
Asn	Asparagine	N
Asp	Aspartic Acid	D
Cys	Cysteine	C
Gln	Glutamine	Q
Glu	Glutamic acid	E
Gly	Glycine	G
His	Histidine	H
Ile	Isoleucine	I
Leu	Leucine	L
Lys	Lysine	K
Met	Methionine	M
Phe	Phenylalanine	F
Pro	Proline	P
Ser	Serine	S
Thr	Threonine	T
Trp	Tryptophan	W
Tyr	Tyrosine	Y
Val	Valine	V

ACRONYMS

ATP	Adenosine 5'-triphosphate
DMSO	Dimethyl sulfoxide
DC ₅₀	Half maximal death concentration
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FBS	Fetal bovine serum
GTP	Guanosine 5'-triphosphate
HRP	Horseradish peroxidase
IC ₅₀	Half maximal inhibitory concentration
IgG	immunoglobulins
kDa	KiloDalton
K _d	Dissociation constant
K _i	Inhibition constant
MTT	Tetrazolium
M _w	Molecular weight
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TRIS	Tris (hydroxymethyl) aminomethane
WB	Western blot

INTRODUCTION

PROTEIN PHOSPHORYLATION

Protein phosphorylation is a post-translational biological event, which regulates any function of a eukaryotic cell life such as division, metabolism, movement, survival and apoptosis. Hence, any alteration of phosphorylation events will alter cell functions and may cause disease (Cohen *et al*, 2002). Phosphorylation reactions are catalyzed by enzymes known as protein kinases (PKs). In eukaryotic cells About one-third of the potential 30,000 proteins in the human proteome are estimated to be substrate for phosphorylation in their stage of life cycle (Cohen et al, 2000). The mechanism of action by which PKs act is very simple: they exploit the γ -phosphate of ATP (or rarely GTP) to generate phosphate monoesters using mainly protein alcohol groups on Ser and Thr residues and/or phenolic groups on Tyr residue as phosphate acceptors. The transfer of the phosphate group ($\gamma\text{-PO}_3^{2-}$) is generally facilitated by magnesium (Mg^{2+}) or manganese (Mn^{2+}) (in some class of kinases), which chelate the γ - and β -phosphate groups of ATP. This reaction is unidirectional, due to the high free energy that is released when the phosphate-phosphate bond of ATP is broken to form adenosine diphosphate (ADP). The transfer of the phosphoryl groups onto target proteins, as schematically illustrated in Fig.1 produces an alteration that can affect them in several ways.

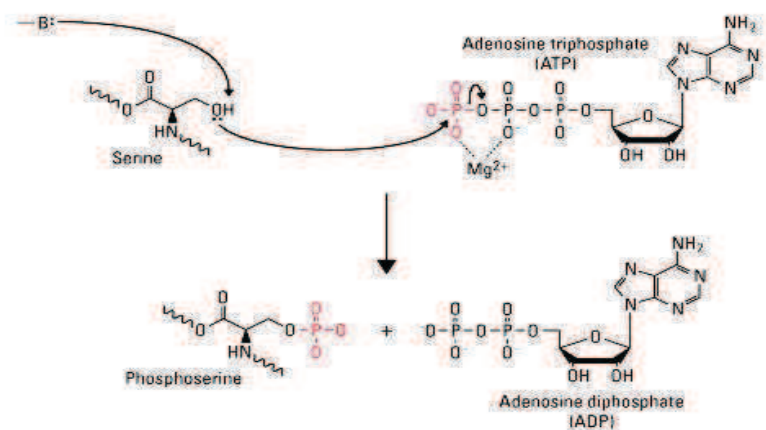


Figure 1. Schematic representation of serine phosphorylation.

Enzyme-catalyzed proton transfer from the (-OH) group on serine stimulates the nucleophilic attack of the γ -phosphate group on ATP, resulting in transfer of the phosphate group to serine to form phosphoserine and ADP. (-B:) indicates the enzyme base that initiates proton transfer (From Thermo Scientific site).

Firstly, proteins can undergo conformational changes that regulate their catalytic activity, activating or inactivating them (illustrated in the left panel of Figure 2). Secondly, phosphorylated proteins can recruit neighboring proteins having structurally conserved domains that recognize and bind to phospho-motifs. These domains show specificity for distinct amino acids and are critical for signal transduction, in which downstream effector proteins are attracted to phosphorylated signaling proteins.

Protein phosphorylation is reversed by the action of phosphatases, which remove phosphoryl groups from target proteins (Blagden and De Bono, 2005), as illustrated in Figure 2. Thus kinases and phosphatases play a crucial role for the conservation of the natural phosphorylation state of a protein in a cell. In fact, the cellular phosphoproteome depends on the temporal and spatial balance between kinase and phosphatase activities.

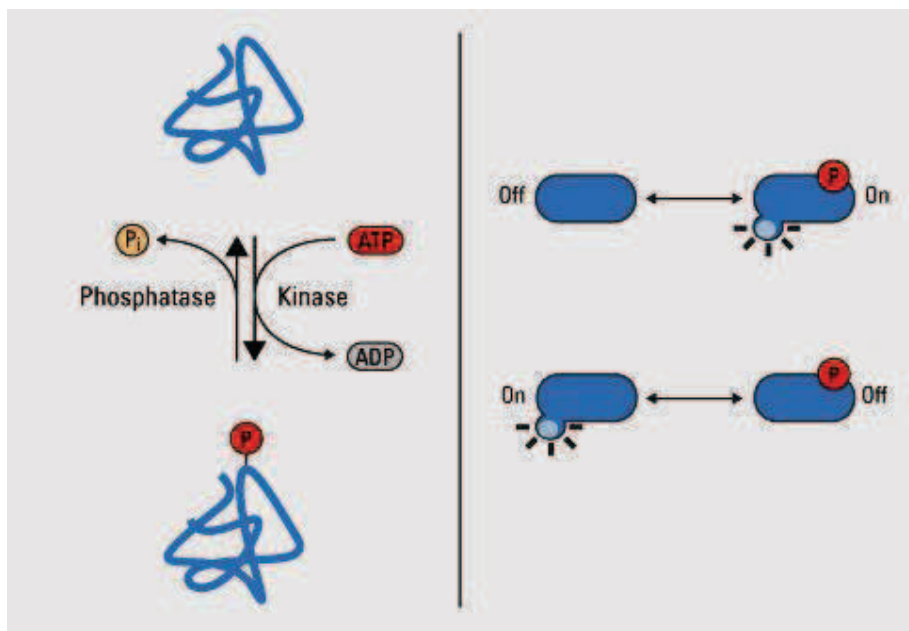


Figure 2. Phosphorylation is a reversible protein modification that regulates protein function. Left panel: Protein kinases mediate phosphorylation at serine, threonine and tyrosine residues, and phosphatases reverse protein phosphorylation by hydrolyzing the phosphate group. Right panel: Phosphorylation causes conformational changes in proteins that either activate (top) or inactivate (bottom) protein function. (From Thermo Scientific site).

Some PKs phosphorylate only one partner protein substrate while others, such as CK2 on which this thesis is focused can phosphorylate up to hundred targets.

Phosphorylation of specific proteins leads to the activation of signal-transduction pathways, and therefore it represents a fundamental mechanism by which intra and extra-cellular signals are transmitted throughout the cell and to the nucleus. For example, all phases of the cell cycle are driven by PKs, which undergo timely destruction and/or inactivation after they have performed their functions. This enables the cell to properly proceed through the life cycle (Blagden and de Bono, 2005).

In accordance with their role in signaling transduction, most protein kinases are inactive (“off”) in the basal conditions and they are switched “on” in response to one or more signals. CK2 is an important exception, being constitutively active, as it will be extensively describe above.

Manning *et al.* (2002) have defined the human “kinome space“, consisting of 518 protein kinase genes. It has been reported that about half of the protein kinases encoded by the human genome are expressed from loci associated with specific diseases or regions amplified in human cancer (Blume-Jensen and Hunter, 2001; Manning, 2009; Brognard and Hunter, 2010). Therefore, it is not surprising that the interest in elucidating protein phosphorylation machinery is always very high, and more and more protein kinases and phosphatases are becoming targets for drug development.

PROTEIN KINASES CLASSIFICATION

Protein kinases are among the largest families of genes in eukaryotes, and they can be classified in different manner. The two most conventional classifications are based on substrate specificity and amino acid sequences of the catalytic domains, respectively. The first one divides PKs into two subfamilies that show specificity for distinct catalytic domains and identifies tyrosine kinases (TKs) or serine/threonine kinases (S/TKs). More than 90% of the phosphoproteome consists of pSer and pThr. Indeed, studies have shown that the relative abundance ratio of pS:pT:pY in a cell is 1800:200:1 (Mann, 2002). Although pTyr is not as abundant as pSer and pThr, global tyrosine phosphorylation is particularly interesting from a clinical point of view because of its correlation to human disease caused by dysregulation of receptor tyrosine kinases (RTKs). In addition to these two classes there are protein kinases that phosphorylate histidine residues (Hanks and Hunter,

1995; Johnson and Lewis, 2001). The second classification takes into account the sequence comparison of the catalytic domains, and also other structure domain and known biological functions (Manning, 2002). In this classification, the total 518 human kinases can be subdivided into 478 eukaryotic protein kinases (ePKs) and 40 atypical protein kinases (aPKs), but also 106 pseudokinases have been identified among human kinome (Manning, 2002). The aPKs possess catalytic kinase activity even if they lack sequence similarity to the ePKs catalytic domain. Several more aPKs have been later identified and the number of human ePKs is now believed 480 (Braconi-Quintaje and Orchard, 2008). The schematic illustration in Figure 3 shows the major ePK groups: the tyrosine kinases (TK), cyclic nucleotide- and calcium-phospholipid-dependent kinases (AGC, which comprises PKA, PKG and PKC families), Ca^{2+} /calmodulin-regulated kinases (CAMK), the CMGC group (including cyclin-dependent kinase (CDKs), mitogen-activated protein kinase (MAPKs), glycogen synthase kinase (GSK), homeodomain-interacting protein kinases (HIPKs), and casein kinase 2 (CK2)), homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases (STE), tyrosine kinase-like (TKL) and casein kinase 1 family (CK1); in addition an eighth group, RGC (receptor guanylate cyclase kinases), was added.

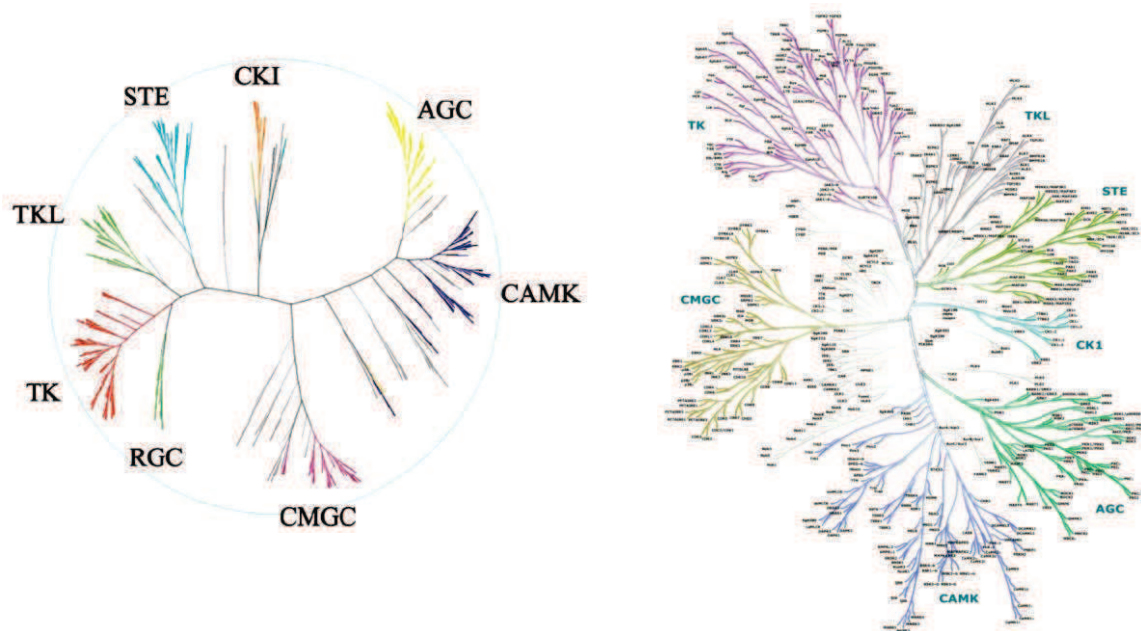


Figure 3. Dendrogram of the 478 human ePK. The left figure schematically shows the ePK groups and branches (From Manning et al, 2002) while in the right figure all the ePK known at 2002 are reported (from cell signaling site).

PROTEIN KINASE STRUCTURE AND FUNCTION

The architecture of a kinase can be partly predicted by its sequence, but only x-ray crystal structure can give precise information. The first structure to be solved was PKA in (Taylor *et al.*, 1993). Since then, many other kinase structures have been solved so far. The architecture of the kinase can reveal the way by which a kinase can recognize a peptide/protein substrate (Knighton *et al.*, 1991), can suggest potential inhibitor, and can disclose similarities between different enzymes. Comparing many protein kinase structures and looking for spatially conserved residues, the global architecture for an active protein kinase was revealed. In ePK the internal architecture permits distal parts of the enzyme to be linked by conserved hydrophobic elements in the active state, whose vicinity is instead disrupted upon inactivation. This complex regulatory machinery distinguishes the ePKs from most metabolic enzymes, which generally are highly regulated by allosteric mechanisms and by post-translational modifications. Indeed, ePKs have evolved not to efficiently turn over lots of product, but rather to be transiently activated (Taylor *et al.*, 2011).

The catalytic domain of all ePKs possesses three separated roles: (i) binding and orientation of the ATP (or GTP) phosphate donor as a complex with divalent cation (usually Mg^{2+} or Mn^{2+}); (ii) binding and orientation of the protein (or peptide) substrate; and (iii) transfer of the γ -phosphate from ATP (or GTP) to the acceptor hydroxyl residue (Ser, Thr, or Tyr) of the protein substrate.

In detail, the structure of each kinase consists of two structurally and functionally distinct lobes, N- and C-terminal that permit catalysis and possibly regulation. The N-terminal lobe is smaller, and is composed of five stranded β -sheet coupled to a α -helical subdomain known as C-helix (Figure 4, panel A). There are conserved motifs: the first motif GxGxxG, known as Glycine-rich Loop, is located over the nucleotide and responsible for the ATP (or GTP) phosphates orientation; it is the most flexible part of the N-lobe. Another important motif, AxK, in the β 3 strand, is represented by Lys72 that bind the phosphates of ATP to the C-helix. The C-helix is a unique, very dynamic regulatory element of each protein kinase, structurally belonging to the N-lobe, but occupying a strategically important position between the two lobes. In the contrary, the C-terminal lobe is larger, and rich, in α -helices. This domain, which is extremely stable, forms the core of the kinase and function as

a surface for protein/peptide substrates. Spatially conserved hydrophobic motifs, termed Spines, link the two lobes; they were found in every active kinase but not in inactive kinases (Figure 4, panel B).

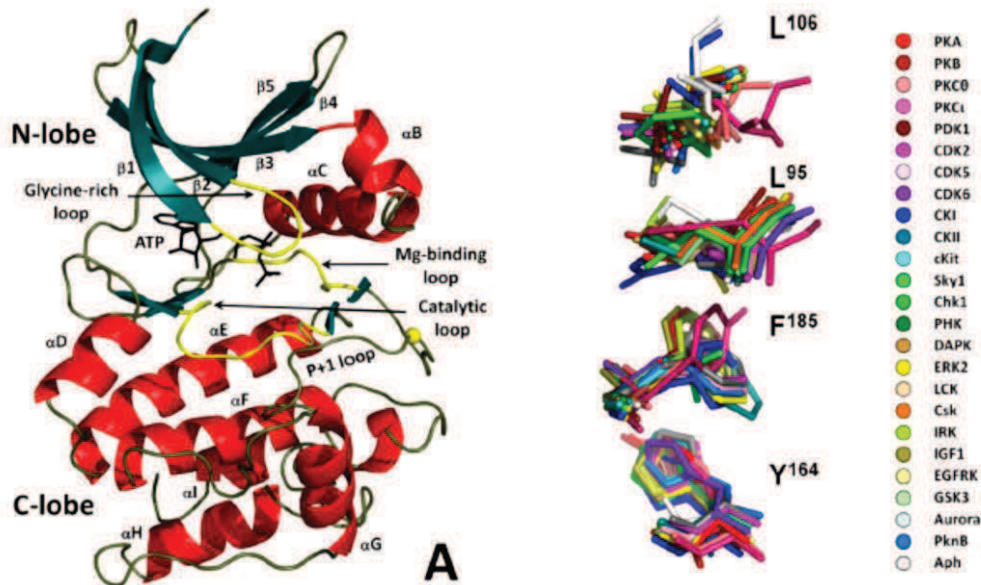


Figure 4. Structural conserved elements of a protein kinases. A. Protein kinase core is bilobed. The N-terminal lobe (N-lobe) contains five β -strands (colored teal) and a universally conserved α C-helix. The C-lobe is mostly helical (colored red). An ATP molecule is bound to a deep cleft between the lobes in black. Major catalytically important loops are colored yellow. B. Multiple alignments of four residues spines from 25 human kinases and two simpler eukaryotic-like kinases (eLKs), (PknB and Aph) is shown. All structures of active ePKs have conserved structural motifs in their core which form highly conserved spatial patterns. These patterns, termed “spines”, do not form sequential motifs coming from different parts of protein kinases sequence. (From Taylor et al, 2011).

PROTEIN KINASE SPECIFICITY

As protein kinases are implicated in a wide variety of cellular processes, but affect only one or few of the many potential phosphoacceptor residues in their targets, they must be endowed with very high selectivity (Pinna and Ruzzene, 1996). The sequence of the substrate close to the phosphorylatable Ser/Thr or Tyr residue is an important determinant of specificity and different protein kinases show different “consensus sequences” with respect to the neighboring sequence of the residues to be phosphorylated (Figure 5). Also co-localization of protein kinases and their substrates at specific subcellular compartments and particular association mediated

by targeting elements outside the catalytic domain, strongly enhance the specificity of the kinase reaction (Pinna and Ruzzene, 1996).

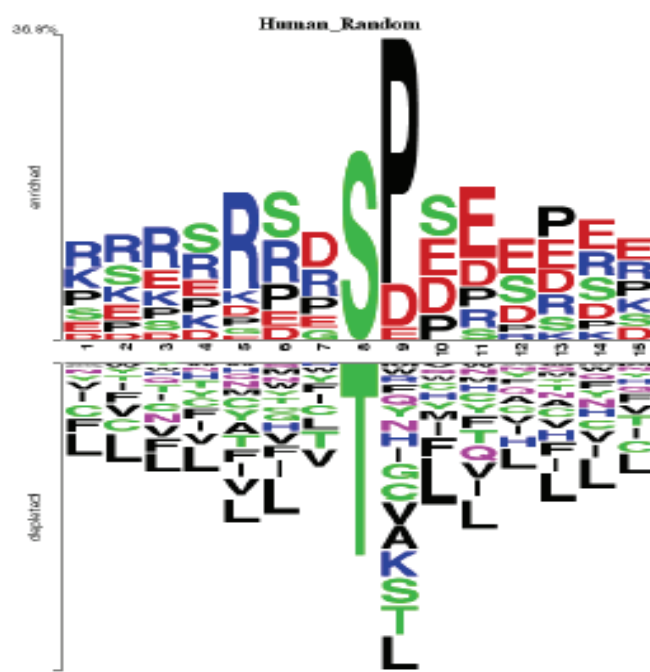


Figure 5. Two-sample logo based on 10,899 phospho-sites in humans. Upper, the most representative residues found in the neighbourhood of phosphorylated Ser/Thr in phosphopeptides; bottom, the most depleted. (from Pinna, 2009, Screening, MedChem and ADMET Europe, Berlin, Germany).

In addition to phosphorylating other proteins, many protein kinases are able autophosphorylate themselves. The phosphate of the phosphodonor may be incorporated either in the enzyme catalytic domain or in the regulatory domain(s). One of the most dynamic regions of the protein kinase core, the activation loop, which retains the substrate close to the ATP binding site, often contains one or more critical phosphorylation sites, and its phosphorylation represents a common tool regulating the activity of most protein kinases (Smith *et al.*, 1993).

Ser/Thr KINASES

About 400 of the total 518 eukaryotic kinases identified in mammals are Ser/Thr protein kinases, covering the 80% of the total kinome. Ser/Thr kinases are divided in many different subfamilies and all show a particular specific sequence required for phosphorylation. Among them, the following are included: protein kinases regulated by cyclic nucleotides (i.e. protein kinase A, PKA), diacylglycerol-regulated protein kinases (i.e. protein kinase C, PKC), Ca²⁺/calmodulin-regulated protein kinases, G-protein-coupled receptor protein kinases, protein kinase CK1, protein kinase CK2, glycogen synthase kinase (GSK), cyclin-dependent kinases (CDK), and mitogen-activated kinases (MAP kinases), and HIPKs family. There are many other protein kinases that do not show any close relationship to these subfamilies. It is worth to mention that among Ser/Thr protein kinases, enzymes with dual specificity are to be included, in that they can phosphorylate Ser/Thr and also Tyr residues. The only physiologically relevant example of dual specificity protein kinase is given by MAP kinase kinase (MEK) family (Pinna and Ruzzene, 1996). How and why Ser/Thr kinases prefer to target a Serine residue rather than Threonine remain obscure; however, in general, Serine is preferred respect to Threonine. According to the specific consensus determinants of the substrate recognition site, Ser/Thr protein kinases can be divided in three distinct groups: (1) basophilic protein kinases: they use basic and, often, also hydrophobic residues as specificity determinants, usually located in the N-terminal side of the target residue (this group includes the whole of the large ACG group, e.g. PKA, Akt, PKC, and all the members of CAMK group); (2) proline-directed protein kinases: they share the absolute requirement of the Ser/Thr-Pro motif and these comprise members of the CMGC group that belong to the families of cyclin-dependent kinases, MAP kinases and also HIPK kinases; (3) acidophilic and phosphate-directed Ser/Thr protein kinases: they use carboxylic and/or phosphorylated side chains as specificity determinants, among them there are CK2, GSK3, CK1 and the Golgi apparatus casein kinase G-CK (Ruzzene and Pinna, 1996). A recent study conducted by Salvi and co-workers, 2009, has highlighted that the majority of the phosphoproteome is not generated by basophilic protein kinase (the largest branch of Ser/Thr-specific protein kinases) but by proline-directed and by acidophilic protein kinases. This latter represents a small minority of the kinome but, among them, CK2 could be

responsible alone for the generation of a substantial proportion of the eukaryotic phosphoproteome.

Although some Ser/Thr-specific protein kinases are constitutively active in the cell (see CK2 above), most of them are tightly regulated. In accord with the general rule that a protein kinase can be in a “off” or a “on” state depending on which signaling stimulus is picked up, there are multiple modes for keeping Ser/Thr protein kinase domains in the inactive state at rest. One mechanism is to have regulatory domains as separate subunits which function as combinatorial platform on which protein-protein interactions, such as kinase with substrates and regulators, can be assembled (Newton, 2001). Moreover, regulatory domains or subunits can be autoinhibitory or they can be activating (Kobe and Kemp, 1999). An “autoinhibitory” domain can be located within the enzyme, in its catalytic site, blocking binding of substrates. It has been proposed that the binding of allosteric activators induces conformational changes in the autoinhibitory domain that disrupt its interaction with the catalytic domain, hence activating the kinase to phosphorylate exogenous substrates (Soderling, 1990). An example of this regulation is provided by protein kinase CK1, which displays an autoinhibitory autophosphorylation site that *in vivo* is maintained in the dephosphorylated, active state by cellular protein phosphatases (Rivers, 1998). The regulatory subunits can instead be activating if they allow productive orientation of ATP and protein substrate in the active site of the catalytic subunit.

STRUCTURAL BIOLOGY

CK2 is generally described as a stable tetramer composed of two catalytic (α and/or α') and two regulatory subunits (β). In the following paragraph the features of the single subunits and of the holoenzyme will be described.

The α and α' subunits

Two isoforms of CK2 catalytic subunit, (α and α') are coded by two distinct genes, CSNK2A1 and CSNK2A2 (Wirkner *et al.*, 1994; Yang-Feng *et al.*, 1994), respectively, and are related to the CMGC kinase subfamily group. Their eukaryotic primary sequences are well conserved among the species, presenting the canonical kinase structural motifs: (i) the P-loop (phosphate-binding loop, also known as glycine-rich loop, G-loop); (ii) the substrate binding site; (iii) the catalytic loop; (iv) the activation loop (also called T-loop).

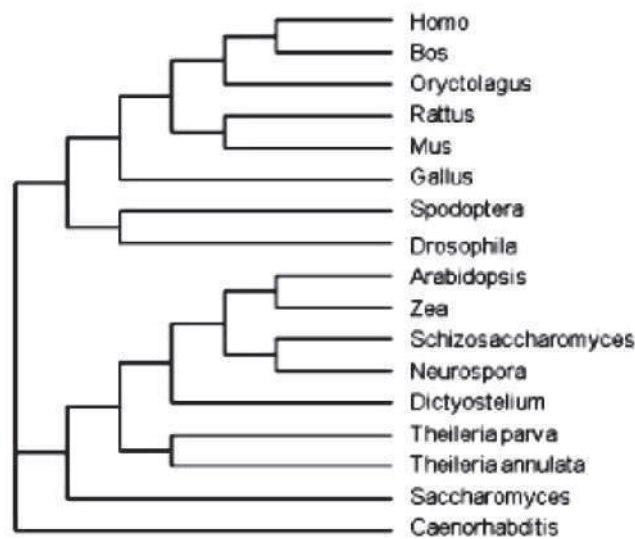


Figure 6. *Phylogenetic tree of eukaryotic CK2 (from Cozza et al., 2010).*

CK2 α is composed of 391 aminoacids, for a Mw of 45 kDa, while CK2 α' isoform is composed of 350 aminoacids, for a Mw of 41 kDa. CK2 α general architecture is shown below (in Figure 7); it presents two main topological determinants: a small β -strands-rich lobe and a large lobe dominated by α -helix. The ATP binding site is localized between these two lobes linked through a short loop, called “hinge region”. The CK2 N-terminal

region from Ser7 to Glu36 is very peculiar and unique, and it seems to play a key role in the CK2 “always active conformation”.

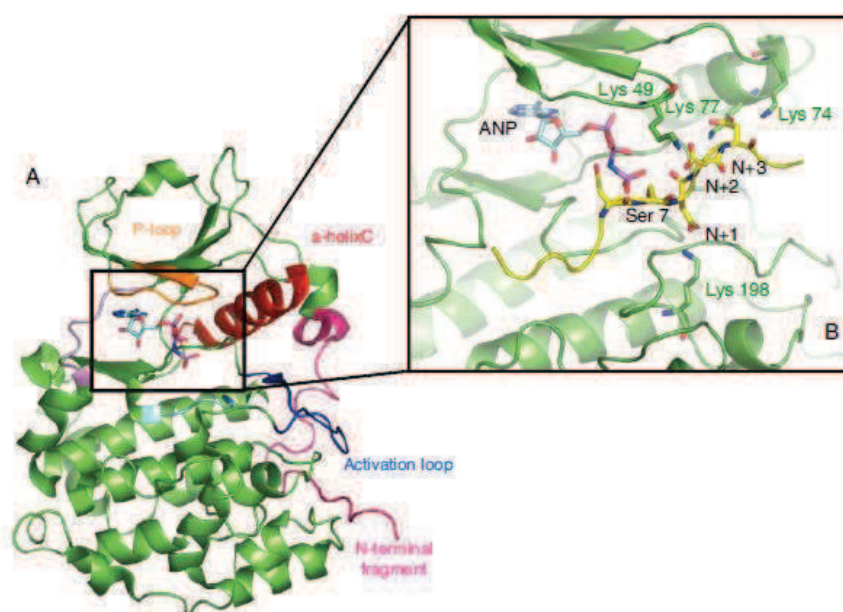


Figure 7. A: Representation of most important human CK2 subdomains B: Representation of CK2 substrate binding region. The most important interactions with the substrate peptide RRRADDSDDDDD (in yellow) are highlighted (from Cozza *et al.*, 2010).

Differently from almost all the other protein kinases, CK2 is able to use both ATP and GTP as phosphate donor due to its particular P-loop.

The global sequence similarity between α and α' is about 75%, and this value increases to 86% if the 1–329 sequence (1–330 in α') is considered. On the contrary, there are differences in C-terminal domain length (α' is 41 residues shorter than α) and sequence (only 38% similarity); however, this has no significant influence on the kinase activities. A specific role for the two catalytic isoforms has not been assigned until now; however there are evidences of implication of CK2 α' subunit in cell proliferation and spermatogenesis (Litchfield *et al.*, 2001; Lou *et al.*, 2008). Also specific partner substrates have been reported, such as Pin1, which interacts with only α (Messenger *et al.*, 2002) and caspase-3, specifically phosphorylated by α but not α' (Turowec *et al.*, 2013).

CK2 catalytic subunits show some unique sequence characteristics that differ from the other kinases of the CMGC group. First, both α and α' subunits present a considerable

number of basic residues around the substrate recognition site. In particular, there are ten basic amino acids available for the interaction with the acidic substrates of CK2, while no other kinase of the CMGC group displays such a number of basic residues in the same positions.

Another unique feature of CK2 is the absence of a typical phosphorylation site in the substrate recognition domain. In many kinases this phosphorylation site controls the conformational changes of the substrate-binding domain and represents an important regulatory mechanism of the substrate-phosphorylation process. On the contrary, unlike all the other CMGC and most of the other eukaryotic Ser/Thr kinases, the activation loop of CK2 does not require its phosphorylation to promote the maximal activity of the enzyme since it is maintained in the active conformation by the N-terminal region.

Also the CK2 glycine-rich loop displays some unique characteristics respect to the classical “GXGXXG” motif (present in the 84% of the CMGC kinases); in fact, it presents only two instead of three glycine residues (GXGXXS) (Sarno *et al.*, 1997). Finally, CK2 presents a particular stretch of four histidines at a distance of five residues each other (at the positions 148, 152, 154, 160, and 166 in the human CK2). Interestingly, while the His148 and His152 are conserved among almost all CMGC members, His160 and His166 are unique. The role of this stretch is still not clear, but it may contribute to the substrate recognition upstream to the phosphorylated residue (Dobrowolska *et al.*, 1994).

The β Subunit

CK2 β regulatory subunit has a sequence composed of 215 aminoacids, very conserved among the eukaryotic kingdom, with a number of peculiar features related to its function. Thanks to structural and biochemical data the functions of the N- and C-terminal regions have been explained. The sequence including residues 55-64 is an acid region exerting a negative regulation of CK2, whose effect can be partially removed, by polybasic proteins or polyamines (Meggio *et al.*, 1994). In fact, polyamines are able to bind to this acidic stretch of CK2 β and activate CK2. Interestingly, mutations of specific residues in the acidic region of the CK2 β subunit suppress stimulation by polyamines and augment the basal activity of the enzyme. Moreover CK2 β MSSSEE N-terminal sequence contains an

autophosphorylation site at Ser2. The CK2 β C-terminal region, in particular the sequence between 181-203 residues, is a positive regulatory domain, able to stabilize and improve the CK2 catalytic activity; moreover CK2 β C-terminal is necessary for the β - β dimerization, for the protection from proteolysis and unfolding, and for the association with the catalytic subunit α (Pinna *et al.*, 1997). The C-terminal region of CK2 β contains also another phosphorylation site, Ser209, which is phosphorylated in cell and *in vitro* by p34^{cdc2} in a cell cycle-dependent manner (Lithcfield *et al.*, 1995).

CK2 β contains a sequence very similar to the “destruction box”, a domain that confers mitosis-specific degradation to cyclin B (King *et al.*, 1996); it seems to regulate CK2 β stability and/or degradation during mitosis. (King *et al.*, 1996; Zhang *et al.*, 2002).

CK2 β sequence includes a zinc finger motif containing four cysteine residues (Cys109, Cys114, Cys137, and Cys140) responsible for the β - β dimerization (Figure 8). Mutations of Cys109 and Cys114 disrupt the zinc finger and prevent the interaction between the β subunits and between β and α subunits. This region is rich in β sheet strands, and its high hydrophobicity is responsible of the many hydrophobic interactions between monomers (Chantalat *et al.*, 1999). It has been proposed that CK2 β is synthesized in excess respect to α in cells, so that the β - β dimerization can occur without the presence of α (Gietz *et al.*, 1995; Graham *et al.*, 2000; Canton *et al.*, 2001; Chantalat *et al.*, 1999). CK2 β mutants unable to form dimers fail in the holoenzyme formation, indicating that the β - β dimerization is a prerequisite to form a stable complex with catalytic subunits.

It has been proposed that CK2 β is an independent entity, which functions as partner also for other kinases. This is suggested by its localization in cell compartments where CK2 α is not detected (Krek *et al.*, 1992). Authors reports that CK2 β interacts with c-Mos and A-Raf protein kinases, by means of its C-terminal region, as in the case of CK2 α interaction (Chen *et al.*, 1997). In the case of c-Mos, interaction with β has a negative effect, while in the case of A-Ras β induces an enhanced kinase activity. Other partner proteins have been reported to interact with CK2 β in an *in vitro* screening (Arrigoni *et al.*, 2008); other studies reported the tumor suppressor BRCA1 (O’Brien *et al.*, 1999), the traffic regulator LYST (Tchernev *et al.*, 2002) and the superoxide radical production enzyme p47-phox (Kim *et al.*, 2001).

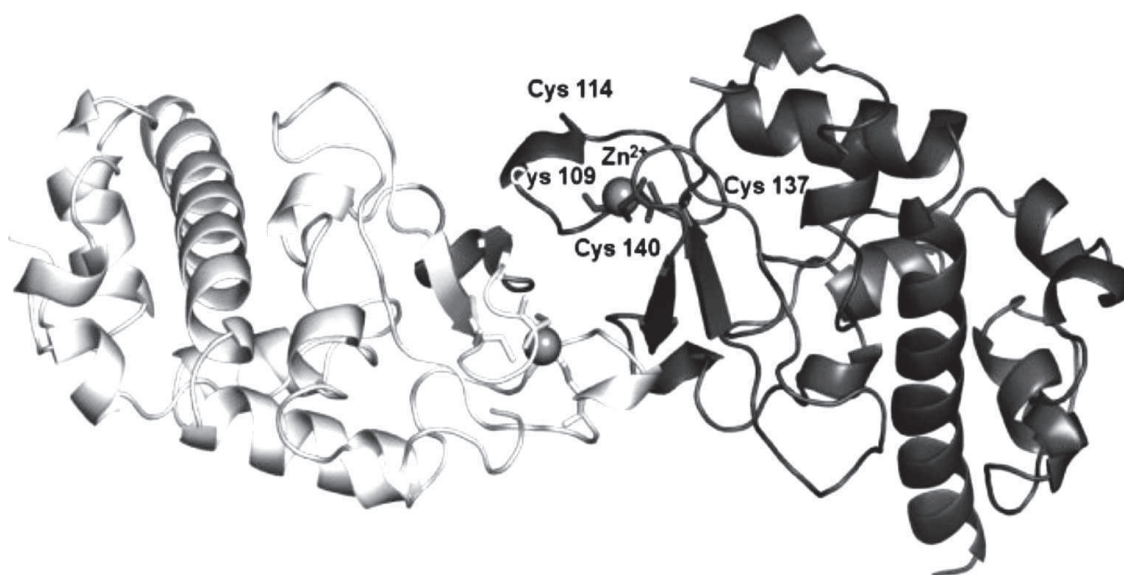


Figure 8. Molecular representation of β - β dimer, focusing on the Zinc finger domain (from Cozza *et al.*, 2010).

The CK2 Holoenzyme

The first crystal structure of the CK2 holoenzyme has published in 2001 (Niefind *et al.*, 2001). It emerged that the catalytic subunits (α and/or α') are linked to the β - β dimer by means of C-terminal domain of the regulatory subunits. The tetrameric complex is frequently defined as “butterfly”-shaped for its architecture, shown in Figure 9. It is generally more stable than the monomeric form and, its catalytic activity is usually higher than that of the catalytic α subunit alone (Antonelli *et al.*, 1996; Boldyreff *et al.*, 1993) although this depends on the substrate type (see below Figure 12). As reported in the previous paragraph, an autophosphorylation site exists in the N-terminal region of β subunit but it does not represent a regulatory mechanism, since mutants of the autophosphorylation site display activities similar to w.t. protein (Bodenbach *et al.*, 1994). However, β autophosphorylation could contribute to confer higher stability to the regulatory subunit itself. Structural data from CK2 crystallographic analysis have confirmed that the autophosphorylation event on β subunits occurs between two holoenzymes. In fact, the autophosphorylation site is more than 30 Å distant to the nearest catalytic site and it is inconsistent with an intra-molecular autophosphorylation unless conformational changes in the “butterfly”-shaped structure of the holoenzyme occur.

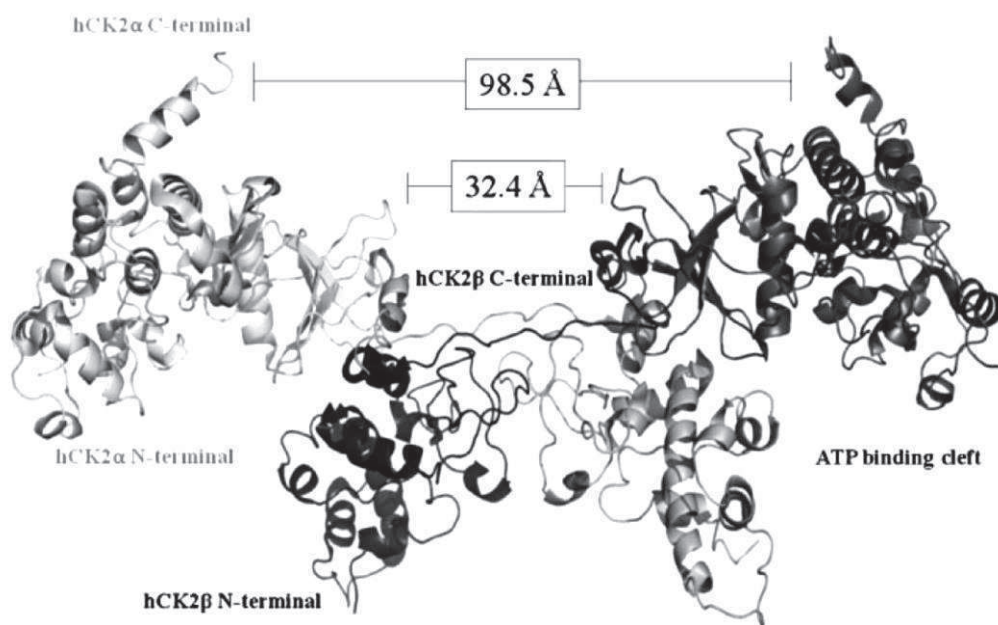


Figure 9. Molecular representation of the human CK2 tetramer (from Cozza *et al.* 2010).

Multimeric structures of CK2 tetramers have been frequently proposed: forms of CK2, at physiological ionic strength, were very early detected in *Drosophila*, by electron microscopy techniques, suggesting that this form might be present in cells (Glover *et al.*, 1986). Later, Valero and coworkers (Valero *et al.*, 1995) observed the formation of ring-shaped structures composed of four tetramers using recombinant *Drosophila* CK2. In both cases authors confirmed that the filamentous and ring-shaped forms of CK2 could be dissociated into the standard $\alpha_2\beta_2$ tetramers by the addition of high salt concentration. Moreover, a new hypothesis consistent with the existence of trimeric aggregate of three CK2 tetramer has been published (Olsen *et al.*, 2008). Very recently, Lolli *et al.* (2012 and 2013) demonstrated that the supramolecular CK2 structure would be a trimer of tetramers, and that polymers of these trimers can possibly form (Figure 10) with a regulatory valence. In the CK2 trimeric structure, the N-terminal domain of β subunit of a tetramer interacts with the catalytic α subunit of another one; in these forms the autophosphorylation of β in Ser2 (or Ser3) would represent an autoinhibitory mechanism of CK2, leading to a non-productive conformation. However, the occurrence of this structure *in vivo* is still to be demonstrated.

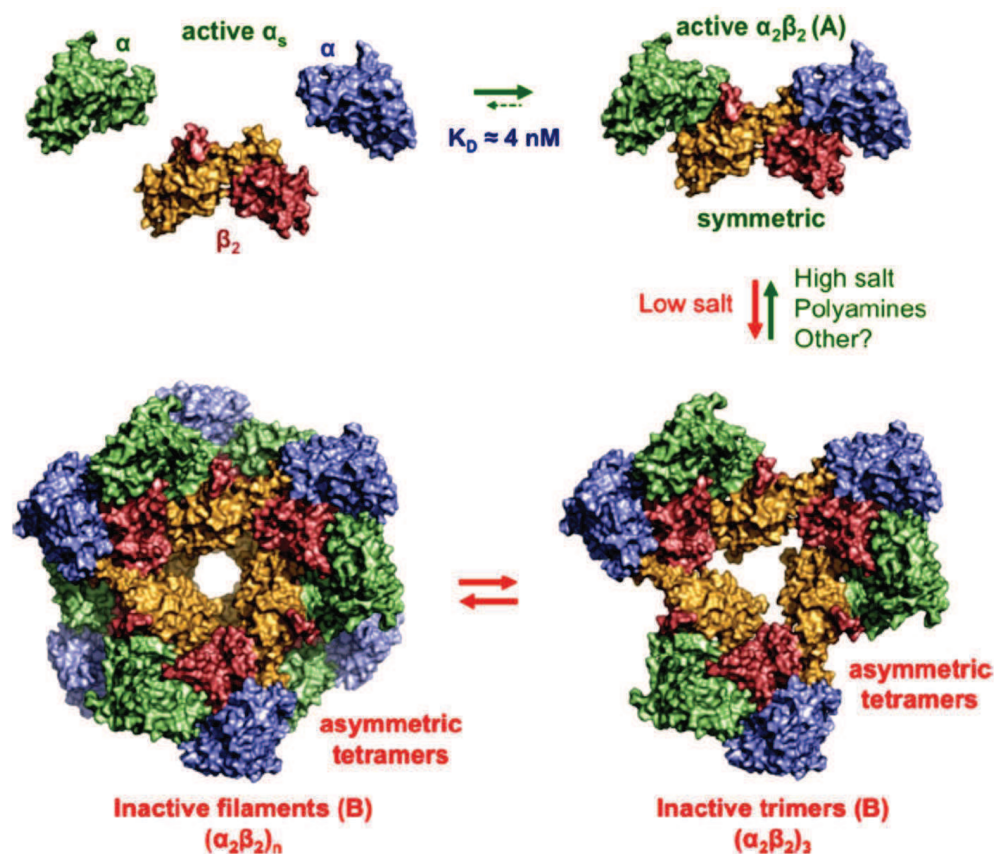


Figure 10. A functional model of CK2 holoenzyme supramolecular organization is presented. When co-localized in time and space, CK2 α and β subunits readily assemble into symmetric active tetramers (A). Activity is switched off through their organization (essentially via electrostatic interaction) in inactive trimers of tetramers and higher-order interactions generating filaments (B). Activity is restored upon a depolymerization process triggered by variations in the physical-chemical local environment (pH, ionic strength, etc.), action of substrates or effectors (e.g., polyamines), or other events (from Lolli et al., 2013).

CK2 SUBSTRATE SPECIFICITY

CK2 is an acidophilic kinase able to phosphorylate substrates in which the phosphoacceptor site is surrounded by acidic aminoacids. The minimum consensus sequence of CK2 requires an acidic residue at position $n+3$ downstream from the phosphoacceptor site; this acidic residue can be represented by glutamic or aspartic acid, but also by previously phosphorylated serine or tyrosine (S/T-x-x-D/E/pS/pY) (Marin *et al.*, 1999). The target aminoacid is a serine or a threonine, but also tyrosine can be exceptionally phosphorylated (Marin *et al.*, 1999). It is important to note that, in general, this minimal requirement is associated with additional acidic residues which play a positive role in any position between $n-7$ and $n+7$; moreover the absence of basic residues in the proximity of the target aminoacid is preferred (Meggio and Pinna, 2003; Salvi *et al.*, 2009). The presence of a proline residue in $n+1$ negatively affects phosphorylation. The CK2 residue preference around its known phosphoacceptor sites, has been recently reanalyzed using a CK2 bona fide substrates collection (533 phosphosites), generating the IceLogo (Colaert *et al.*, 2009) shown in Figure 11. It is a graphical representation of the different aminoacidic preference at each position in CK2 substrates compared to the total human phosphoproteome. From this analysis (Salvi *et al.*, 2009; Salvi *et al.*, 2010) it is immediately evident that CK2 displays a preference for acidic residues at all positions considered (from $n-7$ to $n+7$) with particular emphasis at position $n+3$ where D or E are present in about 71% of total sites, 77% considering the sequences S/TxxD/E/S, and $n+1$ where D or E are present in about 50% of total sites, while only 17% of CK2 phosphosites lack acidic residue at both positions. Basic residues are instead discarded at all positions, consistent with their role as negative determinants. It is important to note that P is also negatively selected at all positions; in particular, P+1, an essential requirement of Pro-directed kinases, has the most negative effect, as already known from experiments of peptide phosphorylation (Meggio *et al.*, 2003).



Figure 11. CK2 substrate specificity. IceLogo of bona fide CK2 substrates (upper part of the figure) versus human Ser/Thr phosphoproteome (lower part). 555 nonredundant sites of CK2 have been considered from PhosphositePlus (www.phosphositeplus.org) (from Pinna, 2012).

CK2 β subunit has an important role in modulating substrate selectivity. It has been demonstrated that for some CK2 substrates, including DNA topoisomerase II, Nopp140, FAF-1, p53, and eiF2 β (reviewed in Cozza *et al.*, 2010), CK2 β represents a docking platform influencing the access of the catalytic site for substrate binding. In the case of protein HIV-REV60 (Marin *et al.*, 2000) and the translation factor eiF2 β (Salvi *et al.*, 2006), which are both *in vivo* substrates, phosphorylation by CK2 occurs only when β subunit is present. On the contrary, another *in vivo* substrate, calmodulin, is phosphorylated by the CK2 α alone, while β subunit dramatically inhibits the reaction, except in the presence of polybasic regulators (Meggio *et al.*, 1992). For these reasons, a subdivision of CK2 specific substrates has been proposed (Figure 12): class I substrates, are phosphorylated with the same efficiency by both CK2 tetramer and α alone, class II substrates are phosphorylated only by monomeric CK2 (and by the holoenzyme only in the presence of polylysine or polybasic peptides), and class III substrates are phosphorylated only in the presence of CK2 β (Pinna, 2002).

It is important to observe that the effect of the β subunit on these particular substrates is not mediated by turning on or off the catalytic activity, which is constitutively ‘on’, but by allowing specific interactions with the protein substrates (Pinna, 2002). Since the α

catalytic subunit alone and the holoenzyme affect different substrates, it could be predicted that, if a balance exists *in vivo* between the two forms, this could represent a mechanism for a fine control of the cellular events governed by CK2.

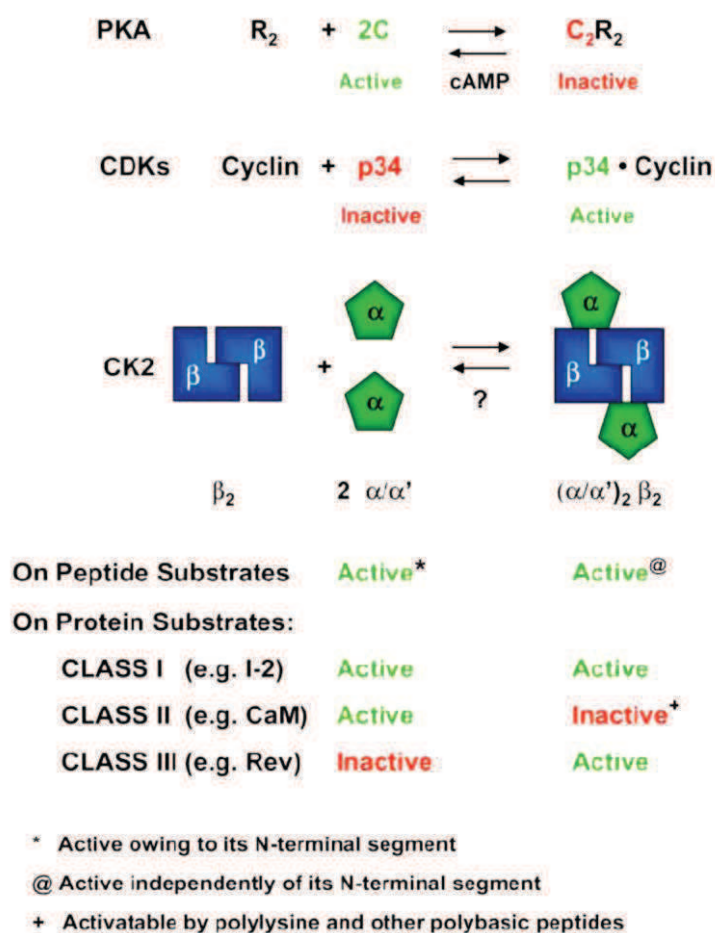


Figure 12. Comparison between the mechanism of activation of PKA (Protein kinase A), CDKs (cyclin-dependent kinases) and CK2. For PKA, whose heterotetrameric structure is similar to that of CK2, the binding of cyclic AMP to the inactive tetrameric form leads to the release of the free active catalytic subunits. In the case of CDKs, the isolated catalytic subunits are inactive until their heterodimeric binding to the cognate cyclin and the phosphorylation of the activation loop. α CK2 is constitutively active and this is due to the unique interaction between the activation loop and its N-terminal segment and the association with β CK2 whose presence indeed influences the substrate specificity, summarized in the three classes: class I substrates phosphorylated with the same efficiency by both CK2 forms, class II substrates phosphorylated only by monomeric CK2 and by the holoenzyme only in the presence of polylysine or polybasic peptides and class III substrates phosphorylated only in the presence of β CK2 (from Pinna, 2002).

PHYSIOPATHOLOGICAL ROLE OF CK2

CK2 is highly pleiotropic and more than 300 substrates have been identified so far, many of them also *in vivo* (Meggio and Pinna, 2003). This underlies the massive involvement of CK2 in many cellular processes. However, the CK2 recognized global function, its pro-survival and anti-apoptotic action, will be described in the following paragraph.

ROLE OF CK2 IN CELL SURVIVAL AND APOPTOSIS

CK2 phosphorylates a huge number of substrates related to cell life/death, thus the mechanisms by which it controls survival/proliferation and blocks apoptosis are complex. CK2 is essential for cell viability, as reported by various authors (Glover *et al.*, 1998; Kikkawa *et al.*, 1992). Observations from experiments performed in yeast indicated that the catalytic activity of CK2, just not only the presence of the protein, is required for viability (Birnbaum and Glover, 1991; Glover *et al.*, 1998). In mammal cells, recent and multiple studies always suggest the absolute requirement of α and/or α' for cell viability (Vilk *et al.*, 1999; Xu *et al.*, 1999; Ahmed *et al.*, 2001; Ruzzene *et al.*, 2002), and a deep connection between CK2 down-regulation and apoptosis has been disclosed. The mechanisms exploited by CK2 in preventing apoptosis are multiple. One is represented by the phosphorylation of proteins which are substrates of caspases, the proteases activated in response to apoptotic stimuli. It has been demonstrated that several caspases substrates, when phosphorylated by CK2, are protected from cleavage. The most important examples are Bid, which is a pro-apoptotic member of Bcl-2 family (Desagher *et al.*, 2001), Max, which is the partner of the c-Myc proto-oncogene product (Krippner-Heidenreich *et al.*, 2001), hematopoietic lineage cell-specific protein 1 (HS1) (Ruzzene *et al.*, 2002), Presenilin-2 (Walter *et al.*, 1999), Connexin 45.6 (Yin *et al.*, 2001), PTEN (Torres *et al.*, 2003) and caspase 9 (McDonnell *et al.*, 2008). Caspases themselves have been found to be phosphorylated by CK2 and thus protected from activatory cleavage. Clear examples are caspase-9 (McDonnell *et al.*, 2008) and caspase-2 (Turowec *et al.*, 2013).

A Specificity of Caspases vs CK2

Group 1 (Caspases 1,4,5)	W-E-H-D ↓
Group 2 (Caspases 2,3,7)	D-E-X-D ↓
Group 3 (Caspases 6,8,9)	(I/V/L)-E-X-D ↓
CK2	(S/T)-X-X-(D/E/pS/pY)

B Specific CK2-regulated Caspase Targets

Substrate	Sequence	Enzyme
Max	-I-E-V-E ↓ S-D-E-E-	Caspase 5
Bid	-E-D-E-L-Q-T-D ↓ G-S	Caspase 8
HS1	?	Caspase 3
Connexin 45.6	S-D-E-V-E ↓ G-P	Caspase 3

Figure 13. CK2 regulates the cleavage of caspase targets (A) Consensus sequences for caspases and for CK2 are illustrated. Acidic residues within these recognition sequences are highlighted in pink. Caspase cleavage sites are indicated with arrows. Residues phosphorylated by CK2 are boxed in green. (B) Specific examples of proteins whose phosphorylation by CK2 has been shown to inhibit caspase-mediated cleavage. Acidic residues, CK2 phosphorylation sites and caspase cleavage sites are highlighted as in (A). CK1 phosphorylation site that may act co-operatively with the CK2 phosphorylation site in Bid is highlighted in yellow. Note that Bid is cleaved at aspartic acid, whereas Max and connexin 45.6 are both cleaved at glutamic acid. The cleavage site in HS1 has not yet been determined. The specific caspase affected for each protein is indicated (from Litchfield *et al.*, 2003).

Indeed, it has been noticed that a strong similarity between the recognition sequence for degradation by caspases and the consensus motif for phosphorylation by CK2 exists (Litchfield *et al.*, 2010) (Figure 13). Furthermore, CK2 is also involved in the control of caspase effectors, such as ARC (apoptosis repressor with caspase recruitment domain), whose phosphorylation by CK2 inhibits caspase 8 activation (Li *et al.*, 2002) (Figure 14). Moreover the up-regulation of survivin (member of the inhibitors of apoptosis proteins, IAP) occurs in response to increased level of CK2 (Tapia *et al.*, 2006). We can conclude that the effects of CK2 on caspase machinery are multiple, with the global result that, when CK2 level/activity are high, caspase-mediate degradation is counteracted.

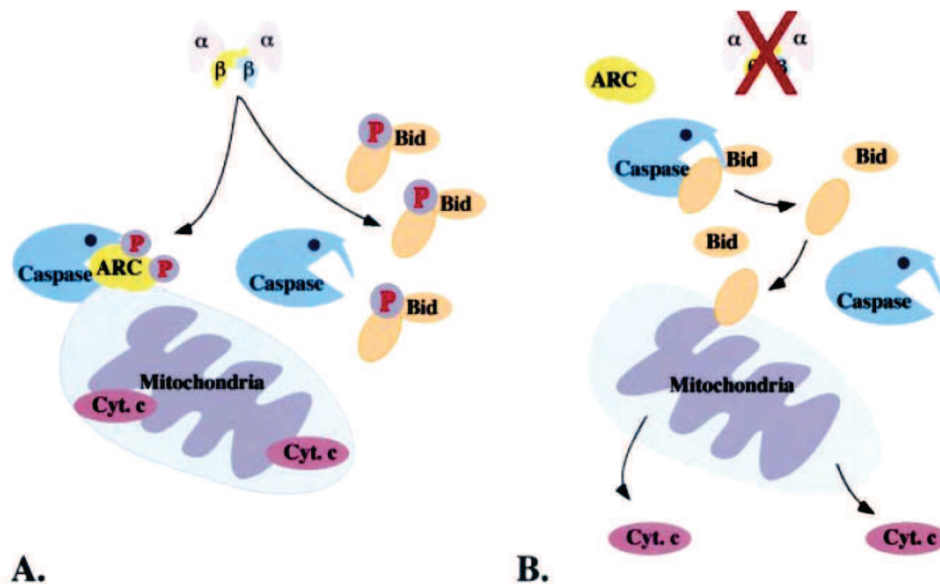


Figure 14. Role of CK2 in the regulation of apoptosis. (A) Under survival conditions, CK2 phosphorylates proteins such as ARC and Bid. When phosphorylated by CK2, ARC is targeted to mitochondria, where it inhibits caspase 8. Bid is resistant to cleavage by caspase 8 when it is phosphorylated by CK2. (B) Under apoptotic conditions and/or when CK2 is compromised, CK2 phosphorylation sites on proteins such as Bid and ARC are not phosphorylated. Under these conditions, ARC is not targeted to mitochondria and does not inhibit caspase 8. In the absence of phosphorylation, Bid is susceptible to cleavage by caspase 8. The subsequent translocation of Bid to the mitochondria is followed by release of cytochrome c (Cyt. c) that results in the amplification of caspase activation. (from Litchfield et al. 2003).

CK2 cooperates with other anti-apoptotic pathways and processes such as PI3K/Akt(PKB), NF- κ B and Wnt pathways, and DNA repair, all involved in sustaining cell survival. A brief description of how CK2 regulates these signalling pathways will be presented below and schematically illustrated in Figure 15:

- *NF- κ B*: as summarized by Dominguez and coauthors (Dominguez *et al.*, 2009), CK2 acts at multiple levels on NF- κ B signaling pathway, which is essential for development but also related to oncogenesis, in the case of untimely activation. NF- κ B is normally retained in cytosol, bound to its inhibitor I κ B, which prevents NF- κ B nuclear translocation. Once phosphorylated by IKK, I κ B undergoes proteolytic degradation, releasing NF- κ B, which translocates to the nucleus, where functions as transcription factor for anti-apoptotic and pro-proliferative genes. CK2 regulates IKK, whose expression is induced by CK2 (Eddy *et al.*, 2005), and directly phosphorylates I κ B, promoting an alternative degradation signal

(McElhinny *et al.*, 1996). Moreover, CK2, phosphorylating β -arrestin, a suppressor of NF- κ B, prevents NF- κ B association to I κ B (Luan *et al.*, 2005). Finally, CK2 also phosphorylates the p65 subunit of NF- κ B, thus increasing NF- κ B transactivation potential (Wang *et al.*, 2000).

- *Wnt/ β -catenin*: this proliferation signal is essential for embryonic development but is often up-regulated in cancer. It is supported by CK2 from several fronts: CK2 directly phosphorylates β -catenin (Song *et al.*, 2003) protecting it from proteasome degradation, at opposite to GSK3 which instead phosphorylates β -catenin and promotes its ubiquitination/degradation (Yost *et al.*, 1996). Other Wnt signaling players are CK2 targets, such as dishevelled (Dvl), whose phosphorylation is important to maintain a high protein level (Song *et al.*, 2000) but also to allow its CK1-dependent phosphorylation (Bernatik *et al.*, 2011). Moreover, LEF-1, a Wnt dependent DNA-binding protein, upon CK2 dependent phosphorylation, stimulates binding of β -catenin/LEF-1 complexes to Wnt-target genes (Wang and Jones, 2006).
- *PI3K/Akt*: CK2 operates as a multisite up-regulator in this pathway, again acting at different levels. The first step is represented by the tumor suppressor PTEN, the phosphatase which, dephosphorylating PIP3 (phosphatidylinositol 3,4,5 trisphosphate), maintain the PI3K/Akt signaling down-regulated. CK2 phosphorylates PTEN, enhancing its stability (Torres and Pulido, 2001) but reducing its activity (Arevalo and Rodriguez-Tebar, 2006) leading, as final effect, to Akt activation. PTEN level in many cancer cells has been demonstrated to be very low; on the contrary, in many primary T-ALL cells its amount is higher than in normal T-cell precursors, but interestingly, its phosphatase activity is reduced due to a high expression of CK2 (Silva *et al.*, 2008). Moreover, neprisylin (NEP) has been reported to be a CK2 substrate; it interacts directly with PTEN, and its CK2-dependent phosphorylation has been demonstrated to prevent its inhibitory action on PTEN (Siepmann *et al.*, 2010). The second level of CK2 intervention on this pathway involved directly Akt, whose CK2 dependent phosphorylation on Ser129 induces its up-regulation (Di Maira *et al.*, 2005). An indirect effect of this CK2-mediated phosphorylation has also been

documented since it maintains high level of phospho-Thr308, one of the two canonical phosphorylation sites on Akt, ensuring a stable association between Akt and the chaperone protein Hsp90, known to protect phospho-Thr308 from dephosphorylation (Di Maira et al., 2009). The final result of all of these interventions of CK2 on the PI3K/Akt pathway is a global up-regulation of the pro-survival functions of Akt on its multiple downstream effectors, such as the β -catenin/survivin signaling (Ponce et al., 2011). In this context, survivin is under the control of β -catenin, but its CK2 dependent phosphorylation (Barrett et al., 2011) is crucial for its anti-apoptotic and mitotic roles.

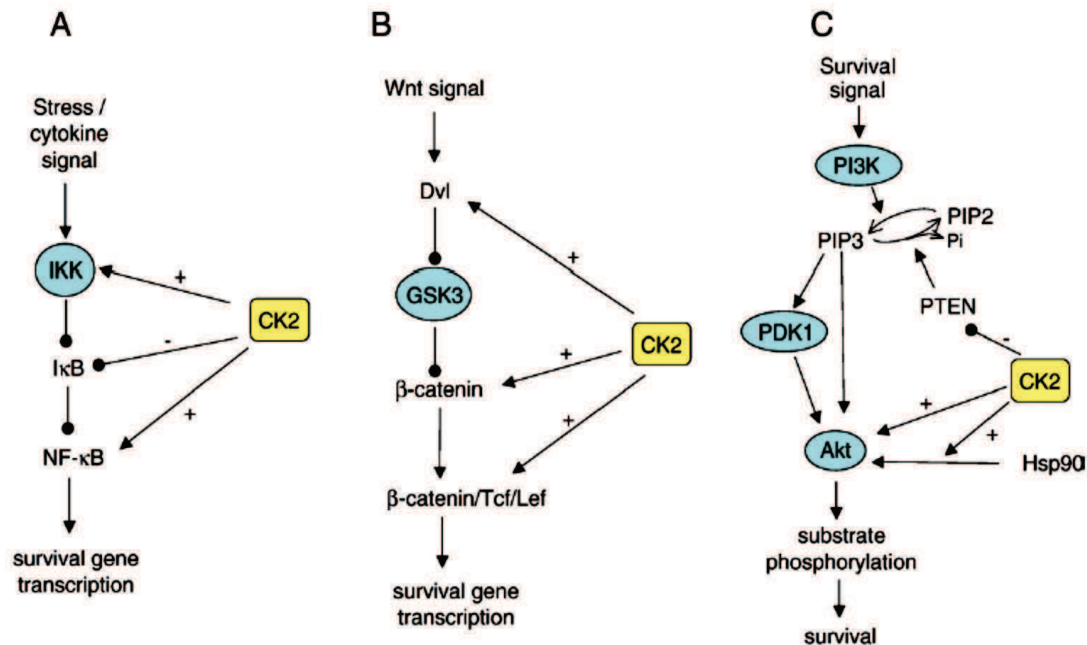


Figure 15. CK2-dependent multisite regulation of NF- κ B (A), β -catenin (B) and PI3K/Akt (C) signaling. A negative effect (–) is indicated by a dot-arrow, and means inhibition or increased degradation, while a positive effect (+), indicated by a normal arrow, means enhanced stability and/or activity. Circles, in blue, denote kinases located in “vertical pathways”, downstream from extra-cellular signals, while the CK2 “lateral role” is highlighted by the yellow rectangle (from Pinna and Ruzzene 2010).

Collectively, it is evident how CK2 acts at different levels, assuming multiple and overlapping roles in promoting cell survival and preventing apoptosis: this is certainly related to its oncogenic potential.

CK2 ROLE AS A “LATERAL PLAYER”

As schematized in Figure 20, CK2 behaves in an atypical way respect to the other protein kinases, in the signaling cascades where it is involved. It is important to stress that CK2 is a constitutively active enzyme and mechanisms able to switch on or off its activity do not exist. Indeed, CK2 can be seen as a “later player” of many survival pathways (Ruzzene and Pinna, 2010). Generally, a cell gets a signal from the outside environment and the information is delivered to the nucleus through a hierarchical mechanism in a figurative vertical direction; from this point of view, CK2 acts as a “horizontal” player, acting at different levels of the signaling cascade, hitting, at the same moment, multiple actors of the pathway. CK2 is always available and ready to act in the cell, but it better performs its functions only when the activation of a pathway occurs offering new substrates, now available for phosphorylation. Thus, a signal that stimulates a cellular response of growth, proliferation, and survival will be potentiated by the intervention of CK2, while a death message will be counteracted.

CK2 INVOLVEMENT IN THE REGULATION OF CHAPERONE MACHINERY

CK2 phosphorylates two important members of chaperone machinery, namely Hsp90 and Cdc37. Hsp90 is one of a major chaperone molecules required for the folding and maintenance of many proteins. Several observations sustain the connections between CK2 and Hsp90. CK2 was initially co-purified with a 90-kDa substrate that was later identified as Hsp90 (Meggio *et al.*, 1985; Dougherty *et al.*, 1987), and it co-immunoprecipitates with Hsp90 from cell lysates (Miyata and Yahara, 1995). CK2 phosphorylates both α and β isoforms of Hsp90 *in vitro* and *in vivo*, and this phosphorylation may influence Hsp90 and its clients activity (Szyszka *et al.*, 1989). Hsp90 forms two major chaperone complexes: one is specific for the steroid hormone receptors and in this case the co-chaperone of the immunophilin family FKBP52 is present; the other is specific for the folding of signaling protein kinases, and include the co-chaperone Cdc37 (Miyata *et al.*, 2009). Besides Hsp90, both co-chaperones are phosphorylated by CK2 (Cox *et al.*, 2007; Miyata *et al.*, 1997). In particular, Cdc37 is phosphorylated by CK2 at Ser13, and this

phosphorylation is essential for its stabilization and activation of many signaling protein kinases (Miyata *et al.*, 2009). It has also been proposed that CK2 is itself a Cdc37 client kinase (Kimura *et al.*, 1997; Bandhakavi *et al.*, 2003) and that a positive feedback loop can occur between the two proteins in promoting the folding of multiple client kinases (Miyata *et al.*, 2009). The chaperone systems have very important functions in controlling different signaling pathways. It is therefore evident that by affecting co-chaperones, and Cdc37 in particular, the CK2 action is greatly amplified, allowing the targeting of a huge number of proteins, including kinases and their downstream substrates.

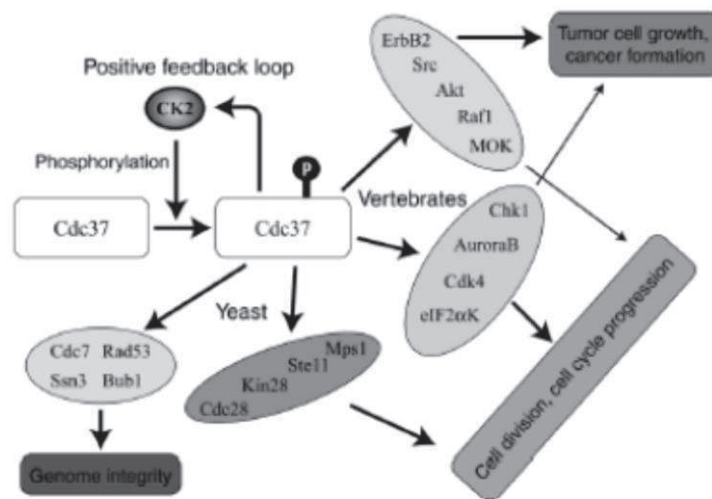


Figure 16. Connections between CK2 and Cdc37 protein. CK2 and Cdc37 constitute a positive feedback loop that results in promoting multiple client protein kinases in yeast and in vertebrates. CK2-dependent phosphorylation is essential for the role of Cdc37 in maintaining Hsp90-Cdc37-kinase heterocomplexes and thus the physiological function of multiple client protein kinases. In the picture many other Cdc37-dependent signaling client kinases are omitted for simplicity (from Miyata, 2009).

ROLE OF CK2 IN CANCER

The involvement of CK2 in cancer is well documented and a huge number of observations have confirmed its role in the sustaining malignant transformation. Indeed, CK2 level and activity have been found to be invariably higher in a wide variety of tumors respect to normal cells (Tawfic *et al.*, 2001; Unger *et al.*, 2004; Trembley *et al.*, 2009). Despite the abnormally high CK2 level found in the majority of cancer cells, CK2 is neither the cause nor the consequence of neoplastic transformation but rather it may well reflects the tendency of the tumor to preferentially settle in those cells where CK2 is higher, regardless of the genetic accidents responsible of transformation (Ruzzene *et al.*, 2011). As schematically shown in Figure 21, a high level of CK2 promotes neoplastic growth by means of several modes of action: the transforming potential of many oncogenes is increased by CK2 (Seldin and Leder, 1995; Channavajhala and Seldin, 2002); CK2 stabilizes most of the onco-kinases in their active conformation phosphorylating the co-chaperon Cdc37 (Miyata and Nishida, 2004); CK2 blocks the efficacy of antitumor drugs, i.e. imatinib (Mishra *et al.*, 2007) and melphalan (Piazza *et al.*, 2006); CK2 sustains neovascularization (Ljubimov, *et al.*, 2004); CK2 contributes to the multi-drug resistance phenotype (Di Maira *et al.*, 2007) and, most generally, CK2 creates a large number of pro-survival and anti-apoptotic signals. Persistence of growth signals, escape of apoptosis and sustainment of angiogenesis are fundamental events which together lead to malignant growth (Figure 17). Thus, it is conceivable that CK2 reaches a critical threshold and it may become a cancer driver creating an environment particularly susceptible to the development of tumor phenotype. As a possible consequence, the higher the CK2 level is, the worse the grading of the malignancy will be (Unger *et al.*, 2004).

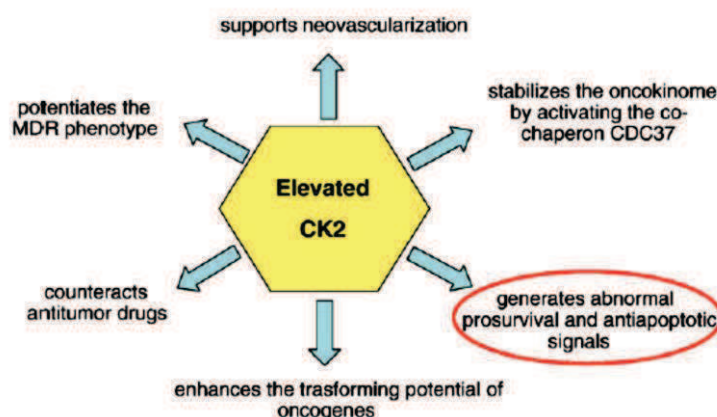


Figure 17. Effects promoted by abnormally high CK2 levels (from Ruzzene and Pinna, 2010)

ADDICTION TO CK2

The essential role demonstrated by CK2 in embryonic development and growth (Lou et al., 2008; Landesman-Bollag *et al.*, 2011) underlines how CK2 pro-survival and pro-proliferation functions are fundamental for viability of all cells. However, recent evidences have indicated that, although CK2 is necessary for the entire organism, it is “more necessary” for some cells than for others (Ruzzene and Pinna, 2010). It has been suggested that this reliance of cells on CK2 could represent an example of “non-oncogene addiction” (Solimini *et al.*, 2007), a term coined to denote situations where the tumor phenotype displays an overdue reliance on abnormally high level of proteins which are not subjected to genetic alterations as oncogenes are, but nevertheless generate a cellular environment which is particularly favorable to neoplasia. In a given population of normal cells, where the concentration of CK2 is variable, few cells will have a level of CK2 significantly higher than average, but this will not be critical to trigger a malignant phenotype. However, if an oncogenic mutation of one or more genes occurs, these cells are more resistant to apoptosis and are able to survive better, and will be gradually selected, becoming at the end the majority of the neoplastic tissue, since they are in some way “predisposed” to malignant transformation more than cells where CK2 level is normal (this concept is illustrated in Figure 18) (Ruzzene *et al.*, 2011). This goes in parallel with the fact that such “oncophilic” cells are more sensitive to

CK2 inhibitors and die for apoptosis when this powerful tool for tumor survival has been removed (see below).

The causes leading to CK2 accumulation in malignant cells are still obscure. Mutations have never been found, thus, excluding genetic alteration, and usually the over-expression of CK2 in malignant cells is not accompanied by a proportional increase of mRNA (Tawfic *et al.*, 2001; Di Maira *et al.*, 2007). Several hypothesis have been proposed (Ruzzene *et al.*, 2011) looking for causes correlated to epigenetic events. Enhanced translation could be possibly due to altered micro-RNAs regulation; an alternative mechanism could be represented by defective degradation of CK2, through down-regulation of proteolytic pathways, and/or hyper-protection of the CK2 molecule by the chaperone machinery, whose efficiency is largely dependent on CK2 (Miyata *et al.*, 2009) but which in turn could also prevent a regular CK2 protein turnover.

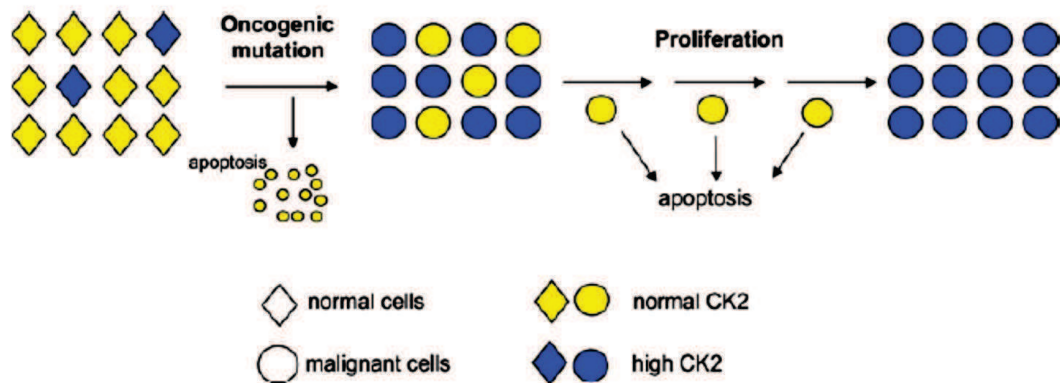


Figure 18. Non-oncogene addiction by CK2. Schematic representation of the mechanism by which cells with abnormally high CK2 are selected by malignancy, accounting for the phenomenon of “non-oncogene addiction” (from Ruzzene *et al.*, 2011).

CK2 AS AN ECTOKINASE

CK2 is present in all cell compartments. Early immunocytochemical studies showed that it is mostly detected in the cytoplasm and in the nucleus, but it is also associated to cellular membranes and organelles. In proliferating cells CK2 phosphorylates and alters the activity of a number of regulatory nuclear proteins, among them, DNA topoisomerase II (Escargueil *et al.*, 2000). Pools of cytoplasmic CK2 shuttling from the cytosol to the nucleus and/or *viceversa* in response to various stimuli such as cellular stress (Yamane *et al.*, 2005) and cell growth and apoptosis (Guo *et al.*, 2001), have been well described.

Moreover, CK2 has been also found on the external side of the cellular membrane where, acting as ectokinase, can phosphorylate extracellular proteins and external domains of membrane proteins. Originally it was found (Kubler *et al.*, 2002) that incubation of HeLa cells with CK2 model substrates, such as casein or phosvitin, resulted in the release of the enzyme activity into the medium. Later on, several physiological extracellular proteins have been identified as substrates of the CK2 ectokinase activity; one is vitronectin whose phosphorylation has been suggested to regulate the adhesion of cells to the extracellular matrix (Seger *et al.*, 2001; Stepanova *et al.*, 2002). More recently, it has been shown that CK2 phosphorylates the C9 component of the complement and it has been suggested that this phosphorylation might control cell lysis caused by this complement protein (Bohana and Kashtan, 2005). Another recent example of an extracellular protein domain phosphorylated by ecto-CK2 is the collagen XVII receptor. The phosphorylation may inhibit its degradation catalyzed by metalloproteases (Zimina *et al.*, 2007). However, studies on CK2 functions as ectokinase are still inconclusive, and many questions remain unanswered; moreover the mechanism by which CK2 is transported to the external surface of the cellular membrane is unknown. It is worth to note that phosphorylation of extracellular membrane proteins may have a role in the regulation of cell adhesion and cell migration, and this could be involved in the development/sustainment of metastasis. Therefore, a better understanding of the CK2 functions as ectokinase would be crucial also from a medical point of view. In this context, preliminary data will be presented in Chapter 2 of this thesis, where a CK2 not cell-permeable inhibitor, developed in our laboratory, has been exploited to selectively block ecto-CK2 kinase activity.

INHIBITION OF PROTEIN KINASE CK2

WHY AND HOW TO TARGET PROTEIN KINASE CK2?

CK2 is a constitutively active enzyme known for several years, but the understanding of its role inside the cell has been particularly difficult. Specific inhibitors, able to selectively and potently block CK2 activity, can be very useful in this context. Therefore, a huge number of chemical compounds able to inhibit CK2 activity have been developed to this purpose. There could be multiple reasons to develop CK2 inhibitors with therapeutic implications. First of all, CK2 is considered a potential drug target for cancer therapy. In fact, as described in paragraph 3.3, its amount and activity are elevated in various cancers (Guerra and Issinger, 2008), it suppresses apoptosis and strongly promotes cell survival and proliferation (Pinna and Ruzzene, 2010), and it reinforces the multi-drug resistant phenotype (Di Maira *et al.*, 2007). Therefore, it is considered a key player in the development of global favorable conditions for tumorigenesis, and all these observations support the rationale of inhibiting CK2 as a potential successful strategy for cancer therapy. Moreover, CK2 is not only correlated to cancer, but also implicated in viral and infective diseases, and several viral proteins essential for virus life cycle are among CK2 substrates (Meggio and Pinna, 2003). Thus, CK2 inhibitors with anti-viral and anti-infectious properties could be potentially interesting. Moreover, new roles for CK2 have been recently discovered in the molecular pathology of different neurodegenerative disorders, such as Alzheimer, Parkinson and amyotrophic lateral sclerosis (Perez *et al.*, 2011). Thus, CK2 can be considered a potential new target for the treatment of these diseases. CK2 is also reported to play an important role in many vascular system diseases, such as atherosclerosis (Harvey *et al.*, 2007), laminar shear stress (Widder *et al.*, 2007), and hypoxia (Hubert *et al.*, 2006), and it has also been recently connected to angiogenesis and related diseases (Ljubimov *et al.*, 2004). Finally it has been disclosed a possible functional link between CK2 and cystic fibrosis pathogenesis

(Pagano *et al.*, 2010; Venerando *et al.*, 2011). In this context, it is important to underline that a large number of CK2 inhibitors have shown a good correlation between the *in vitro* efficacy toward the kinase and the capacity to induce apoptosis in different tumor cell lines. A great impulse to the development of inhibitors comes from the resolution of CK2 crystal structure: the first one was maize α subunit (Niefind *et al.*, 1998), and then several other structures have been solved, allowing structure-based approach for developing CK2 inhibitors. Different molecular modeling techniques have been used to propose and analyze the essential pharmacophoric features for CK2 binding, and significant results have been obtained by molecular docking, a computational technique whose goal is the detection of the lowest free energy conformation of an inhibitor inside the protein active pocket, and the evaluation of the different chemical/physical interactions (Kitchen *et al.*, 2004). 22 crystal structures of CK2 kinase domains in complex with different ATP-competitive inhibitors are available on the Protein Data Bank (PDB) (Berman *et al.*, 2002). The compounds so far available for CK2 inhibition are usually small and hydrophobic molecules, very heterogeneous in structure.

Most CK2 inhibitors act as ATP-competitive molecules. Although the ATP binding site is highly conserved in all kinases, in the case of CK2 it presents peculiar features, and in particular it is much smaller if compared with the majority of the other kinases, thus representing the rationale for the selectivity of CK2 inhibitors. In detail, the pharmacophoric features for an inhibitory activity toward CK2 include an hydrophobic/aromatic moiety in the center of the molecule. This is essential and present in all the best CK2 inhibitors; in fact many apolar bulky aminoacids are present in the CK2 ATP-binding pocket, such as Met163, Ile66, and Ile174 (Niefind *et al.*, 1998). It is worth to note that the latter two residues are unique of CK2, being replaced by alanine or other small residues in almost all the other kinases. This aspect explains the CK2 specific inhibition by relatively small molecules (Battistutta *et al.*, 2001), and also the fact that CK2 has a very low responsiveness to staurosporine (Meggio *et al.*, 1995), an inhibitor of almost all the other kinases, composed of a large molecular mass, unable to access to the small active site of CK2.

SELECTIVITY OF CK2 INHIBITORS

The selectivity of the most promising CK2 inhibitors has been tested *in vitro* evaluating their activity against a panel of different protein kinases. The first compounds were assayed towards 25/50 kinases (Pagano *et al.*, 2007; Sarno *et al.*, 2003; Sandholt *et al.*, 2009) but recently larger panels (up to 200) are used (Pagano *et al.*, 2008; Battistutta *et al.*, 2011). From these studies, it has turned out that the selectivity for CK2 is usually high, but that other kinases are also often inhibited by CK2 inhibitors, such as DYRK1A, DYRK3, the members of the PIM family, HIPK2, ERK8, PKD, and in some cases also GSK3 β and CDK2 (Pagano *et al.*, 2008b). In some cases, the selectivity that was initially considered very high was reevaluated by screening the same compound toward a large panel of kinases (Pagano *et al.*, 2008); for example TBB, still widely used as a CK2 specific inhibitor, turned out to be much less selective than other compounds such as quinalizarin (Cozza *et al.*, 2009) or CX-4945 (Battistutta *et al.*, 2011). Interestingly, the kinases targeted by CK2 inhibitors are not randomly distributed throughout the kinome, but tend to belong to the same subset of enzymes. This suggests that important analogies in the ATP-binding sites of these kinases are present; for these reasons, it is possible to exploit the available huge repertoire of CK2 inhibitors as a source for the development of inhibitors that selectively blocks one or few other kinases beside CK2, for the so-called multi-target strategy. An example is provided by the serine/threonine kinases of the Pim family, which control both apoptosis and cellular metabolism and are believed to be potential targets for the treatment of various human diseases including cancer and ischemic diseases (Amaravadi *et al.*, 2005). From these considerations, it is evident that a compound simultaneously blocking both CK2 and PIM activity could represent an interesting drug candidate (Cozza *et al.*, 2014). Sometimes the incidental inhibition of other kinases by a CK2-directed compound has been exploited to develop new selective inhibitors. This is the case of TBID, the first specific HIPK2 inhibitor, developed in our laboratory by chemical modification of the tetrabromo-benzimidazole scaffold, initially studied for CK2 inhibition, and now established as a selective HIPK2 inhibitor, as it will be described in Chapter 4 of Results.

The strategy applied to obtain specific CK2 inhibitors as is based on the optimization of the interactions with the hydrophobic residues of the ATP-binding

pocket (Val45, Val53, Lys68, Val95, Phe113, Val116, and Met163) and in particular with the peculiar Ile66 and Ile174. However, the peculiarity of the CK2 ATP-binding pocket is not only due to the small size and the hydrophobic nature, but also to its electrostatic properties (Cozza *et al.*, 2010). Indeed, only PoloLike Kinase 1 (PLK1) shows charge distribution of the ATP pocket similar to that of CK2, while, interestingly, PIM1 and PIM2, often targeted by CK2 inhibitors, are quite different in this respect, suggesting a way to improve selectivity.

A further enhancement of the specificity toward CK2 is expected from a new strategy, represented by the so-called “bi-functional” compounds that are designed to bind simultaneously to the ATP and the phosphoracceptor site of CK2. This approach will be described in details in the Results (see Chapter 2).

POTENCY OF CK2 INHIBITORS

Beside the K_i , a common manner to define the potency of a kinase inhibitor is IC_{50} , which represents the concentration of the compound required to inhibit 50% of the kinase activity, many CK2 inhibitors display an IC_{50} in the nanomolar range. The most potent CK2 inhibitors have been studied have been co-crystallized in complex with the kinase and have shown common features: they all presents high hydrophobicity and thus are readily transferred from the aqueous phase to the hydrophobic active site; they have excellent shape complementarity with the small active site of CK2 (to maximize van der Waals contacts) and, most importantly, they are able to simultaneously establish electrostatic interaction and hydrogen bonds both with the hinge region and/or with the phosphate binding region. Crystal structures of very potent compounds indicate that, to reach efficacy in the very low nanomolar range, inhibitors must target both the hinge region and the positive area with polar interactions. The best characterized CK2 inhibitor carrying all these features is CX-4945 which displays an IC_{50} of 2 nM towards CK2 α (Battistutta *et al.*, 2011); a very strong binding to the enzyme, with a K_d <1 nM has been confirmed by crystallographic studies (Battistutta *et al.*, 2011). This compound will be described in details in the next paragraph and in Chapter 1 of the Results section. A different issue is represented by the potency of an inhibitor when it is used in biologic systems (in cells or even in animal). Considerations on this usage will be

done in the last paragraph of this Chapter. Here it is worth to mention that a frequently used parameter to give information on the potency of CK2 inhibitor in cells is its DC_{50} (concentration of the compound required to induce 50% of cell death). Since CK2 down-regulation is expected to produce cell death (at least in cancer cells), the efficacy of the inhibitor is proportional to the DC_{50} . Usually DC_{50} is significantly higher than IC_{50} (up two order magnitude), for reasons that will be described in last paragraph.

CLASSIFICATION OF CK2 INHIBITORS

Many inhibitors directed to several protein kinases have been developed until now and different strategies have been exploited, to target the ATP-binding site or an allosteric pocket. As far as CK2 inhibitors are concerned, research started in the 80s searching for ATP-competitive small molecules very potent and selective for this kinase. Although at that time it was not known, this was particularly successfully due to by the CK2 peculiar ATP-binding pocket of CK2, which, as already described, is smaller respect to the other kinases, and is able to use either ATP or GTP as phosphate donor.

We can roughly distinguish CK2 inhibitors between a large group of ATP-competitive inhibitors and a smaller group of compound able to target different domains of the kinase. In the following section a brief description is presented of all CK2 inhibitor families known so far with special refer to their selectivity, mechanism of action and potency in cells.

ATP-COMPETITIVE CK2 INHIBITORS

The majority of CK2 inhibitors are ATP-competitive compounds. Their mechanism of action is based on the binding to the active conformation of the enzyme, in the position normally occupied by an ATP (or GTP) molecule, thus affecting the global structure of the protein only marginally. Perturbations have been observed at the level of the phosphate-anchor loop in the complexes of quinalizarin with the maize and human enzymes (Cozza *et al.*, 2009; Papinutto *et al.*, 2012). In this context it is important to underline that fundamental apolar interactions occur between CK2 and CK2 inhibitors. In fact, for these compounds, the energetic contribution for the

binding is given by hydrophobic forces, in particular hydrophobic interactions and van der Waals contacts, which take place on the hydrophobic surface of the CK2 binding site. The residues involved are Leu85, Val95, Leu111, Phe113, and Ile174 that constitute the hydrophobic region I; Val53, Ile66, Val116 and Met163 that constitute the adenine region; and Val45 and Tyr115 for the hydrophobic region II (see Figure 19)

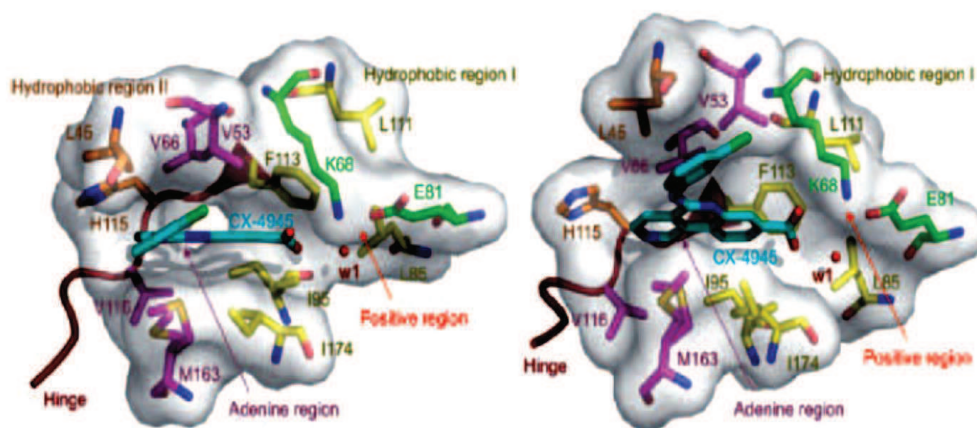


Figure 13. Main structural features of the CK2 α ATP-binding site shown in two different orientations . The active site is occupied by the CX-4945 inhibitor (cyan), belonging to the class of carboxyl acid derivatives CK2 inhibitors. Residues of the three hydrophobic regions common to the “kinase pharmacophore” are shown in yellow (hydrophobic region I, in the deepest part of the cavity), in orange (hydrophobic region II, at the entrance of the cavity), and in magenta (adenine region). Other important elements of the CK2 α pharmacophore are the hinge region (dark red) and the area with a positive electrostatic potential. These two regions are the main polar anchoring points for CK2 inhibitors (from Pinna, 2012).

Val66 and Ile174 are bulky residues not present in the majority of other kinases, reducing the space available in the CK2 molecule for ATP and inhibitors. This is an important point to be considered when an ATP-competitive CK2 inhibitor has to be designed. For instance, the active site of CDK2, another kinase belonging to CMGC group, is larger than in CK2 for the presence of Ala31 instead of Ile66 and of Ala144 instead of Ile174. As a consequence, the inhibitor TBB (4,5,6,7-tetrabromo-1-benzotriazole) binds differently to the two proteins, showing a remarkable selectivity for CK2 (De Moliner *et al.*, 2003). Inhibition data on CK2 α mutants confirmed the importance of Ile66 and Ile174; in the case of the single mutants Ile174Ala or Val66Ala and for the double mutant Ile174Ala/Val66Ala, the TBB IC₅₀ increases from 0.50 to 1.74, and 13.0 to 12.5 μ M, respectively (Sarno *et al.*, 2005). A reduced sensitivity of these mutants is observed toward several other CK2

inhibitors. Moreover, the smaller size of the CK2 α active site prevents the action of staurosporine, a promiscuous protein kinase inhibitor, against which CK2 has a striking modest sensitivity due to the large size of the molecule (Meggio *et al.*, 1995).

Another important peculiarity for the binding of ATP-competitive CK2 inhibitors is the presence of a positively charged region located in CK2 α between the hydrophobic region I and the salt bridge between Lys68 and Glu81 (see Figure 20). The electrostatic interaction with this area greatly contributes to the different orientation of the ligands in the active site of CK2. Some examples are available, regarding TBB, its benzoimidazole analog TBI and the carboxyl acid CX-4945, and they will be discussed in the respective sections.

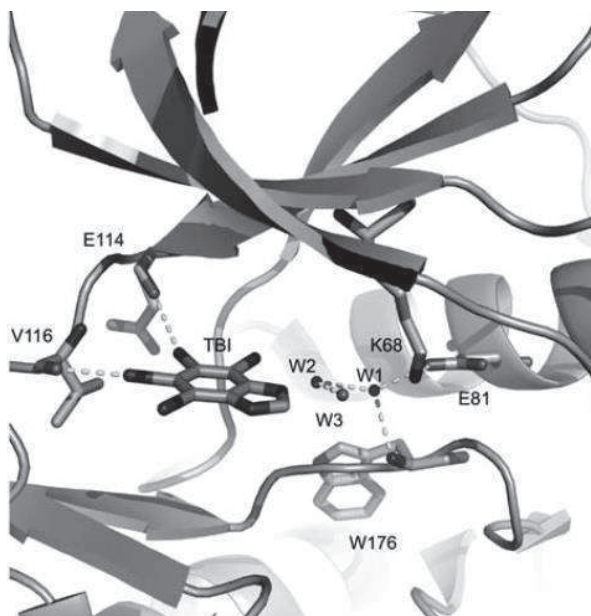


Figure 20. Three important water molecules reside in the positive area near Lys68. W1 is strongly anchored to the Lys68 side chain and to the Trp176 backbone amide nitrogen, and it is conserved in all CK2 structures, so it can be considered a “constitutive” component of the kinase. W2 is present in many structures but is displaced by inhibitors strongly interacting with W1 and Lys68, typically harboring a carboxylic function. W3 is present only in few complexes with small inhibitors such as TBI, a benzoimidazole derivate, shown in the figure. Note the two halogen bonds (dashed lines) between TBI bromines and carbonyls of Glu114 and Val116 from the hinge region (from Pinna, 2012).

Polyhalogenated Benzimidazole and Benzotriazole derivatives

Benzoimidazole derivatives constitute the first and the most abundant family of inhibitors developed to target protein kinase CK2. Many of these compounds have

been crystalized in complex with CK2 and structural data revealed that hydrophobic and van der Waals interactions are necessary for the binding to the kinase. Moreover, the presence and the nature of halogen atoms are fundamental for the inhibitory activity and potency. The first ATP-competitive CK2 inhibitor of this family was 5,6-dichloro-1-(-D-ribofuranosyl)benzimidazole (**DRB**), developed as a nucleoside analogue, which had an IC_{50} of 23 μ M. Authors reported (Lonn *et al.*, 1987; Dobrowolska *et al.*, 1991) that this molecule shows high cytotoxicity and is able to block also topoisomerase and RNA synthesis. Starting from this compound many others derivatives were designed and tested, looking for a better IC_{50} and a higher selectivity towards CK2. Among them, **TBB** (4,5,6,7-tetrabromobenzotriazole), a largely employed CK2 inhibitor, has shown an IC_{50} of 0.16 μ M and a good selectivity on a panel of 30 protein kinases (Sarno *et al.*, 2001). Moreover, when tested on a larger panel, it was found to inhibit several others PKs beside CK2 (Pagano *et al.*, 2008). TBB, as many other derivatives, presents four bromine atoms on the benzene ring, that are responsible for the hydrophobic interactions with bulky side chains inside the ATP binding site (Val 66 and Ile 174), and for a network of halogen bonds with the hinge region. The replacement of bromine atoms with chlorine and fluorine, that are less bulky, or their reduction in number, severely impairs the inhibitory efficiency. A class of benzimidazole derivatives with iodines atoms, bulkier than bromines, on the benzene ring was synthesized and they displayed IC_{50} values generally much lower than the brominated analogs. In particular 4,5,6,7-tetraiodo-1H-benzimidazole (**K88**), 4,5,6,7-tetraiodo-2-methyl-1H-benzimidazole, (**K92**), 2-ethyl-4,5,6,7-tetraiodo-1H-benzimidazole, (**K93**), and 2-[(4,5,6,7-tetraiodo-1H-benzimidazol-2-yl)amino]ethanol (**K100**), show K_i values around 20 nM (Gianoncelli *et al.*, 2009). More recently, a novel group of DRB-derived inhibitors of protein kinase CK2 has been described where methyl or ethyl substitutions at benzene ring have been introduced (Makowska *et al.*, 2011); they shows a high activity in the micromolar range, the best one being compound 40 (IC_{50} = 0.16 μ M) shown in Figure 21.

Benzofurane derivatives

Recently, a new class of benzofurane CK2 inhibitors, similar to the benzoimidazole derivatives, has been considered. Among these benzofurane molecules, four novel compounds with an IC_{50} from the micromolar to the nanomolar range have been

identified. In particular, **TF** compound (6,7-dichloro-1,4-dihydro-8-hydroxy-4-[(4methylphenylamino) methylen]dibenzo[*b,d*]furan-3(2H)-one) has been demonstrated to markedly inhibit recombinant CK2 ($IC_{50} = 29$ nM, $K_i = 15$ nM), and to be cell permeable. Moreover, a screen of TF compound against a panel of 61 human kinases, shows that seven of them (Aurora A, KDR/VEGFR2, SGK1, FLT4/VEGFR3, PIM1, PKD2, LCK) are sensitive to the inhibitor (inhibited >70%) (Gotz *et al.*, 2012).

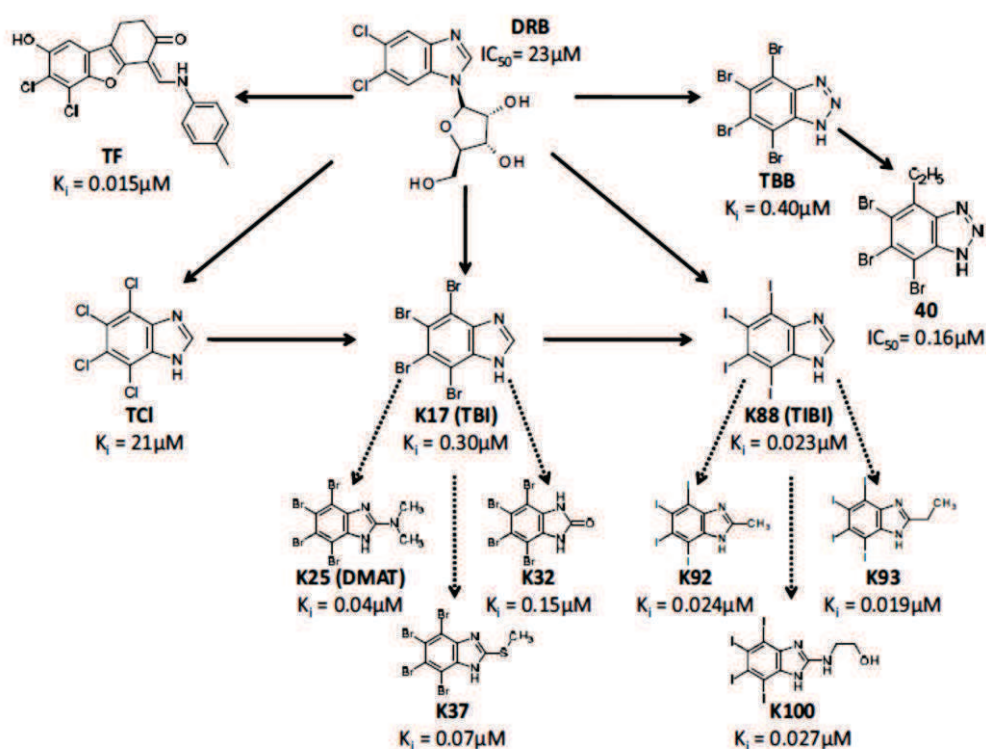


Figure 21. Schematic representation of the most active polialogenated benzimidazole and benzofurane derivatives as inhibitors of CK2 (from Cozza *et al.*, 2013).

Natural derived CK2 inhibitors

The progenitor of another class of ATP-competitive CK2 inhibitors is the natural anthraquinone **emodin** (Figure 22) identified from *Rheum palmatum*, displaying anti-inflammatory and anti-tumor properties (Sarno *et al.*, 2002). Its K_i for CK2 is 1.5 μ M, but it inhibits several other kinases (Sarno *et al.*, 2002). Starting from emodin, structure based improvements have been done leading to 1,8-dihydroxy-4-nitroanthraquinone (**MNA**), 1,8-dihydroxy-4-nitro-xanthen-9-one (**MNX**), and 1,4-

diamino-5,8-dihydroxy-anthraquin-one (**MNY**) (De Moliner *et al.*, 2003). Interestingly these compounds very similar in structure, except for a nitro-group, exert different effects toward monomeric CK2, while their activity on tetrameric CK2 is very similar.

The most potent and selective anthraquinone inhibitor of CK2 is 1,2,5,8-tetrahydroxy-anthraquinone (**quinalizarin**) (Figure 22). It displays a K_i value of 60 nM for CK2 and at 1 μ M concentration (inhibiting 93% of CK2 activity) none of the other 75 kinases tested is affected. Cell treatment experiments have revealed that quinalizarin is cell-permeable, able to inhibit more than 50% of endogenous CK2 activity at 5 μ M (Cozza *et al.*, 2009). Other compounds belonging to this class are based on fluorenone scaffold as **FL12** (2,7-dihydroxy-3,6-dinitrofluoren-9-one) which displays an IC_{50} of 1 μ M, and benzonaphtones, like **THN**, a tetra-hydroxy-benzonaphthone (K_i of 0.68 μ M) (Figure 22) (Sarno *et al.*, 2002; Meggio *et al.*, 2004).

Flavonoids, a class of polyphenolic compounds, are present in fruit, stems, flowers, roots, vegetables, wine and tea; they are very important for plants, being responsible of flowers pigmentation. Very common in the human diet, they play an important role as scavengers of oxygen-derived free radicals, and have been shown to exert anti-microbial, anti-inflammatory and anti-cancer activities (Middleton *et al.*, 1998). Several flavonoids have been related to inhibition of CK2; among them **fisetin** (3,3',4',7-tetrahydroxy-flavone), **quercetin** (3,3',4',5,7 penta-hydroxy-flavone), **apigenin** (4',5,7-tri-hydroxy-flavone) and **myrecitin** (3,3',4',5,5',7-hexahydroxy-flavone), whose IC_{50} are reported in Figure 22 (Sarno *et al.*, 2002). Their selectivity is usually poor, although systematic analysis has not been done. Recently the flavonol scaffold has being optimized leading to new derivates with IC_{50} in the nanomolar ranges (FLC21, IC_{50} = 40 nM, FLC26, IC_{50} =9 nM) (Golub *et al.*, 2011).

Another class of CK2-targeting natural compounds, largely distributed in plants (in particular Apiaceae and Rutaceae) is represented by coumarins, important for plant protection against other organisms and wounds. It has been reported that many polyhydroxylated coumarins possess anticancer properties (Lacy *et al.*, 2004; Kawase *et al.*, 2003). About 60 cumarines derivatives have been synthesized as CK2 inhibitors; the most active compounds are 3,8-dibromo-7-hydroxy-4-

methylchromen-2-one (**DBC**) and the 8-hydroxy-4-methyl-9-nitrobenzo(*g*)chromen-2-one (**NBC**) (Figure 22).

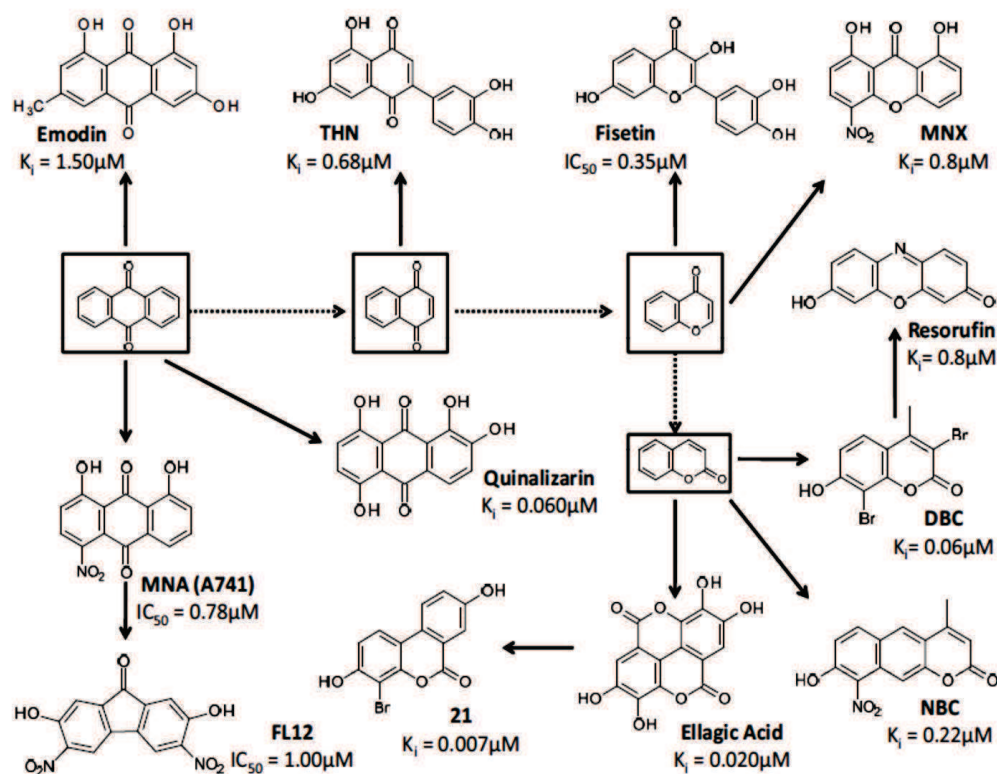


Figure 22. Schematic representation of anthraquinone, xanthenone, fluorenone and coumarin derivatives, inhibitors of CK2 (from Cozza *et al.*, 2013).

Another natural CK2 inhibitor is **ellagic acid**, whose structure corresponds to two fused coumarins. Ellagic acid has been demonstrated to be a very potent CK2 inhibitor, able to interact simultaneously with the phosphate binding region and with the hinge region (Cozza *et al.*, 2006) and it is reported to have antineoplastic and antioxidant properties (Cozza *et al.*, 2011). An optimization of ellagic acid scaffold was also performed (Cozza *et al.*, 2006). From this analysis, **urolithin A** moiety was suggested as a new scaffold linking ellagic acid and coumarin analogues. After structural optimization, a novel potent urolithin derivative has been identified as CK2 inhibitor: 4-bromo-3,8-dihydroxy-benzo[*c*] chromen-6-one (compound **21**, Ki = 7 nM, Figure 22).

Resorufin is another natural compound recently described as CK2 inhibitor (Figure 15). Its crystal structure in complex with human CK2 shows a direct interaction of

the compound with the phosphate binding site and, at the same time, with the carboxyl group of Val 116 in the hinge region, but its potency in cells is modest (Sandholt *et al.*, 2009).

Carboxyl acid derivatives

This class of ATP-competitive CK2 inhibitors includes different compounds which share the presence of a carboxyl group in their scaffolds. The first compound of this group was produced by Novartis, the indoloquinazoline **IQA** (5-oxo-5,6-dihydroindolo-(1,2-a) quinazolin-7-yl acetic acid, also known as CGP029482). It displays an IC_{50} of 0.39 μ M and a quite good specificity (Sarno *et al.*, 2003). From structural analysis it was possible to highlight the importance of the interaction between the carboxyl function of the inhibitor and Lys68 of CK2 α (Sarno *et al.*, 2003).

Another member of this group is the tetrabromocinnamic acid (**TBCA**), derived from the fusion between the tetrabromominated benzene ring of TBB and the carboxyl function of IQA. Also in this case an electrostatic interaction with Lys68 occurs, as well as hydrophobic interactions in the adenine binding region (Pagano *et al.*, 2007). TBCA was successfully used in *in vivo* study (Yao *et al.*, 2012). TBCA derivatives were obtained, such as the benzoic acid derivative 3,4,5-tribromobenzoic acid (compound **26**, IC_{50} = 0.64 μ M) and **TID46** (2-(4,5,6,7-tetraiodo-1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propanoic acid) with a IC_{50} of 0.15 μ M (Golub *et al.*, 2008) (Figure 23).

Another family of compounds belonging to the carboxyl acid group is represented by the 2,6-disubstituted pyrazines, whose best member is compound **1a** which shows an IC_{50} of 30 nM, and a remarkable selectivity against CK2 α' (IC_{50} = 7 nM) (Suzuki *et al.*, 2008).

A new class of 3-carboxy-4(1H)-quinolones derivatives has been developed, with four compounds with IC_{50} lower than 2 μ M; in particular compound **7** (5,6,8-trichloro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid) shows an IC_{50} of 0.3 μ M against CK2 and a good selectivity (Golub *et al.*, 2006) (Figure 23).

From TBCA scaffold, two novel carboxylated CK2 inhibitors have been produced. The first molecule is (*E*)-3-(3-stibonophenyl) prop-2-enoic acid (denoted as compound **10** in Figure 23) (IC_{50} = 0.15 μ M against CK2), the second one is

compound **35** (2,3,4,5-tetrabromo-6-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-benzoic acid) ($IC_{50} = 0.08 \mu M$) (Prudent *et al.*, 2008). However these compounds have poor specificity, since they affect also other signaling pathways, such as Bax-Bcl-X(L) (Tan *et al.*, 2003).

Another family of CK2 inhibitors belonging to this group presents a phenyl-azole scaffold (Hou *et al.*, 2012). In particular compound **10** shows an IC_{50} against CK2 α and α' of 32 and 46 nM respectively. The thiazole ring has been shown to interact with the hinge region (Val116, Glu114) and the carboxylic function with Lys68. It has been also assayed on cell lines, showing an IC_{50} in the low micromolar range (Hou *et al.*, 2012).

Finally, the most interesting compounds of this class include pyridoquinoline derivatives. **CX-4945** (5-(3-Chloro-phenylamino)-benzo[*c*][2,6]naphthyridine-8 carboxylic acid) (Figure 23 and 24), the first CK2 inhibitor in clinical trials developed by Cylene Pharmaceuticals, belongs to this group. CX-4945 displays an IC_{50} of 2 nM for CK2 α (Chua *et al.*, 2008) and the selectivity profile against a panel of over 145 protein kinases shows significant preference for CK2, being all the other kinases unaffected or inhibited with higher IC_{50} , such as DAPK3 ($IC_{50} = 17$ nM), FLT3 ($IC_{50} = 35$ nM), HIPK3 ($IC_{50} = 45$ nM), PIM1 ($IC_{50} = 46$ nM), CDK1/cyclin B ($IC_{50} = 56$ nM) and DYRK2 ($IC_{50} = 91$ nM).

Derivatives of CX-4945 (**CX-5011** and **CX-5279**) able to inhibit CK2 with similar potency (IC_{50} of 9 nM and of 3 nM respectively) were recently published (Pierre *et al.*, 2011). Consistent with their mechanism of action, all the CX compounds bind in the ATP active site of CK2 creating multiple hydrophobic interactions with the binding cavity. Moreover, the carboxylic group, present in all three compounds, penetrates into the deepest part of the active site making interactions with a conserved water molecule and Lys68, thus accounting for the crucial role of this negatively charged group in conferring high potency to this class of inhibitors. The presence of a pyrimidine in CX-5011 and in CX-5279 instead of a pyridine (as in CX-4945) confers higher specificity to these compounds in panels of 102 and/or 235 kinases, with the highest selectivity ever reported for CK2 inhibitors (Battistutta *et al.*, 2011). Since these compounds were specifically used to obtain the results presents in this thesis, a more detailed description of them will be given in the Introduction of Chapter 1 of the Result Section.

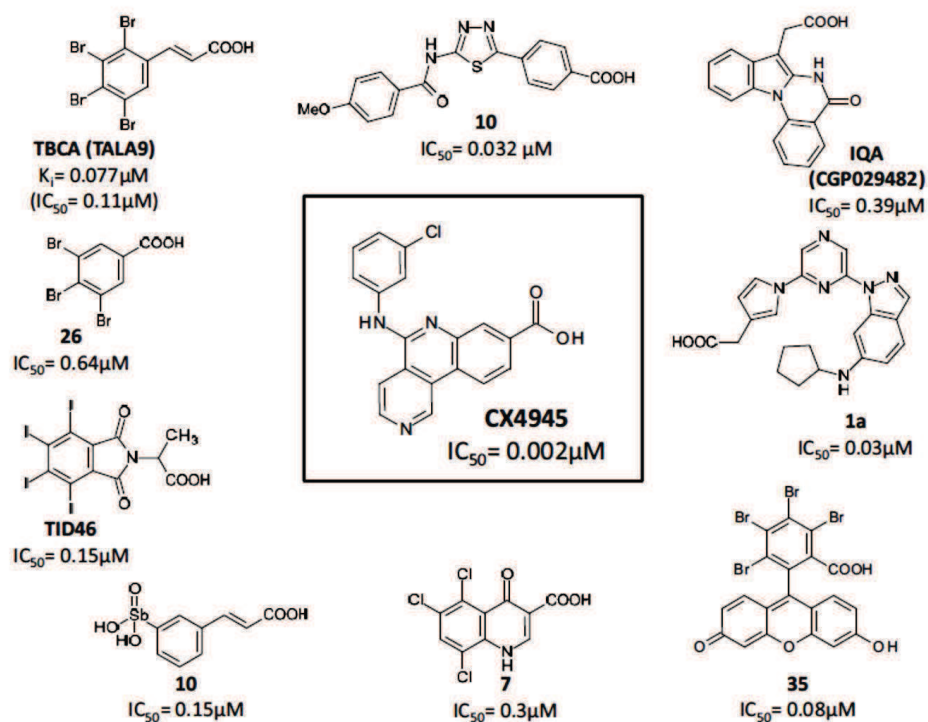


Figure 23. Example of carboxyl acid derivatives as inhibitors of CK2 (from Cozza et al., 2013).

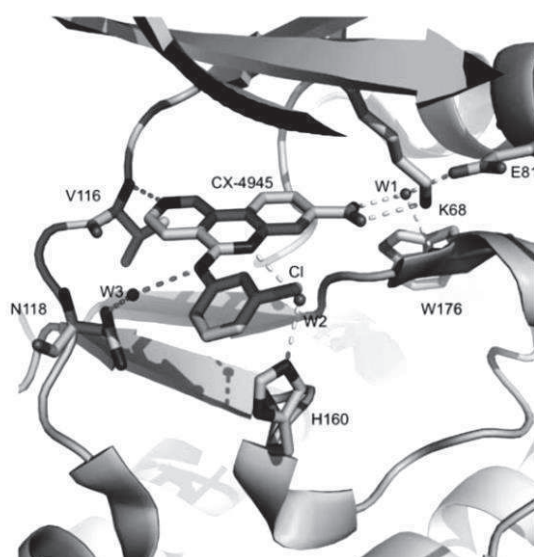


Figure 24. Inhibitor CX-4945 bound to the CK2 active site. CX-4945 is able to establish polar interactions with the two possible anchoring sites present in the CK2 ATP binding pocket, i.e., the N-terminal portion of the hinge region (Val116 backbone) and the positive area in the deepest part of the cavity (W1 and Lys68) (from Pinna, 2012).

NON ATP-COMPETITIVE CK2 INHIBITORS

A rational strategy to inhibit a kinase would be by modeling compounds competing with the protein kinase phosphoacceptor substrate site instead of the phosphodonor site: in fact while ATP is the phosphodonor substrate used by all the kinases, phosphoacceptor substrates are largely variable, and molecules competing with its binding would ensure higher specificity.

Besides compounds able to compete with the ATP, or peptide substrates, allosteric compounds can be considered, which bind to the kinase not at the catalytic site, but at a different site which interfere with the catalytic activity. An alternative site for the binding of a non-competitive inhibitor, correspond to the interface between CK2 α and CK2 β subunits, as suggested by structural information. In particular, a segment of the CK2 β subunit forms a β hairpin loop which is situated deep inside an hydrophobic groove present in CK2 α (Niefind *et al.*, 2001). The identification of this new “druggable” site, suggested the design of compounds able to disrupt or prevent the CK2 α /CK2 β assembly as a strategy to regulate CK2 activity. One of the oldest ATP-competitive CK2 inhibitor, DRB, can be considered also an inhibitor of this type. In fact, it has been found to bind not only to the ATP-binding site (competitive inhibition constant 29.2 μ M), but also an external hydrophobic cavity (“secondary or allosteric binding site”), which is the main anchoring point between α and β subunit in the holoenzyme formation, so interfering with the assembly of the tetrameric $\alpha_2\beta_2$ structure. As expected this binding shows a non-competitive inhibitory effect on CK2 activity (non-competitive inhibition constant 39.7 μ M) (Raaf *et al.*, 2008). Recently, another non-ATP-competitive class of inhibitors has been proposed, comprising the podophyllotoxine indolo derivatives. Also these small cyclic peptides block the interaction between the CK2 subunits, disrupting CK2 tetrameric architecture, and modulating CK2 substrate specificity (Laudet *et al.*, 2008). **W16** (IC₅₀ = 20 μ M) was indicated as an allosteric inhibitor devoid of ATP competitive action and able to selectively disrupt the CK2 α/β assembly (Laudet *et al.*, 2008).

A different and promising strategy for the development of allosteric CK2 inhibitors derives from CFTR (fibrosis cystic transmembrane regulator), whose fragment with

Phe508 deletion, peculiar of the fibrosis cystic pathology (CFTR Δ F508) seems to interfere with CK2 activity by an allosteric mechanism (Pagano *et al.*, 2010). This is not fully understood, yet, but, interestingly, inhibition occurs only of isolated CK2 α catalytic activity, and not in the case of CK2 $\alpha_2\beta_2$ holoenzyme, which is instead up-regulated. Since these CFTR-derived inhibitors are peptides, they are probably not easy permeable to cell membranes, but they have physiological relevance in the case of CFTR pathology, whose fragments formed from this channel might *in vivo* regulate CK2 activity.

A completely different approach for CK2 inhibition is provided by a cyclic peptide inhibitor, **CIGB-300** (also known as P15-Tat), introduced by Perea and colleagues (Perea *et al.*, 2008) which also entered clinical trials. It has been designed to target CK2 substrates instead of the kinase but its mechanism of action is still unclear. More detailed information of this peptide and the biochemical and cellular data obtained with it will be presented in Chapter 3 of Results section.

BI-SUBSTRATE CK2 INHIBITORS

A new family of CK2 inhibitors is that of the so-called bi-substrate compounds. These molecules have an ATP-competitive portion and a substrate mimetic tail by which they compete with the target protein; they therefore interfere with both the ATP and the protein substrate simultaneously. The greater advantage would be an extremely high specificity. The most potent inhibitor of CK2 belonging to this family is **ARC-1504**, which shows a remarkably good IC₅₀ (1.8 nM); however, its interference with the phosphoacceptor binding site is not fully understood (Enkvist *et al.*, 2012).

In our laboratory, a class of bi-substrate compounds derived from tetrabromoimidazole has been recently developed and they have been used in the experiments presented in this thesis. Therefore, they will be described in details in Chapter 2 of the Results section.

***In Vivo* VERSUS *In Vitro* INHIBITORY ACTIVITY**

To be useful in biological studies and to be considered as potential pharmacological tools, inhibitors must be cell permeable and active towards their targets inside the cell.

Most of the CK2 inhibitors presented in this chapter are small hydrophobic molecules, without any particular problem in crossing cellular membranes. Indeed, for many of them the action in cells has been confirmed. This can be easily done by analyzing the effects on cell viability, since, as already anticipated, the reduction of CK2 activity is usually associated to the induction of apoptosis. Moreover, the endogenous CK2 activity can be measured in treated cells by means of *in vitro* assays towards CK2-specific peptides, or by analyzing the phosphorylation level of known CK2 endogenous substrates in western blot experiments with phospho-specific antibodies (Ruzzene *et al.*, 2010). Of course, the concentrations required in cells are usually much higher (up to two orders magnitude) than *in vitro* (see recommendation in Davies *et al.*, 2000). This is probably due to the sequestration of lipophilic compounds by cellular structures and, in the case of ATP-competitive inhibitors, to the fact that they have to cope with a very high ATP concentration (in the mM range) inside the cell.

An important aspect that dictates the choice of an inhibitor to be used in cells is of course its specificity and the absence of side effects not related to the inhibition of CK2, such as targeting of different cellular structures.

All together these considerations allow to identify those inhibitors that are convenient for *in vivo* studies. TBB was one of the first compounds tested in cells (Ruzzene *et al.*, 2002), it is very effective and quite specific (Sarno *et al.*, 2001), and, also because it is commercially available, it is still one of the most widely used CK2 inhibitor. However, as already mentioned, its selectivity has been reevaluated (Pagano *et al.*, 2008) and it turned out that it is not the best choice for *in vivo* investigations.

On the other side, some inhibitors are not sufficiently stable in water, so their addition to the aqueous cellular medium can produce too low concentrations; this is the case of IQA (Sarno *et al.*, 2005). In other cases the availability in the organism is not optimal, and this should be considered, especially when animal treatments are

planned; for example, ellagic acid is not able to cross the intestinal barrier and it is converted to urolithin (Seeram *et al.*, 2006).

Several examples of usage of CK2 inhibitors *in vivo* are available. The first employment of a CK2 inhibitor in animal model was presented by Ljubimov and colleagues showing that CK2 inhibition prevented retinal neovascularization in a mouse model of oxygen-induced retinopathy (Ljubimov *et al.*, 2004). Another pioneering study of CK2 inhibitor administration to animals employed TBB and DRB in several pain models (Li *et al.*, 2005), while brain injections of TBCA allowed to disclose a relationship between CK2 inhibition and superoxide anion production (Kim *et al.*, 2009). However, the majority of the studies have been performed to demonstrate the efficacy of CK2 inhibition in murine xenograft cancer models. They were initially performed with TBB or its derivatives (Manni *et al.*, 2012; Sass *et al.*, 2011) or with different class of compounds such as ellipticine derivatives (Prudent *et al.*, 2010), or hematein (Hung *et al.*, 2013), but a great impulse to this field came from the discovery, and later the commercialization, of CX-4945 (Pierre *et al.*, 2011). Many reports have shown the efficacy of this compound in reducing tumor sizes, mostly in murine xenograft models (Zheng *et al.*, 2013).

At present, quinalizarin (Cozza *et al.*, 2009) and the CX-compounds (CX-4945, CX-5011 and CX-5279) (Pierre *et al.*, 2011) are the CK2 inhibitors with the highest selectivity and demonstrated effectiveness in cells. In particular, the effects of CX-4945 and CX-5011 in cells will be extensively described in Chapter 1 of Results.

HOW DRUGGABLE IS CK2?

As already mentioned, CK2 is involved in several diseases, such as cancer and neurodegenerative disorders, and can be therefore considered a potential drug target. Several potent, selective, and cell-permeable CK2 inhibitors are available as described above, and they could represent good from a therapeutic point of view. However, CK2 is an essential kinase for cell viability, involved in many physiological processes, with hundreds substrates; this raises the question if it is really an effective drug target for therapeutic intervention, and for many years research did not considered convenient the investments on this field. However, later it turned out that CK2 amount is much higher in tumor than in normal cells, and that it is involved in many of the processes associated to malignant transformation. Recently, the hypothesis of the addiction to CK2 of cancer cells has been proposed (Ruzzene and Pinna, 2010) as an example of "non-oncogene addiction" on abnormally high CK2 level (Ruzzene and Pinna, 2010). In this view, CK2 is able to generate an environment favorable to neoplasia consistent with its global antiapoptotic and prosurvival role, and tumor cells develop a reliance on particularly high level of CK2. CK2 is therefore not a real "promoter" of malignancy, but a "driver" of it, being able to support several other biological alterations, truly responsible of the malignancy. The hypothesis of this higher reliance of cancer cells to CK2 compared to normal cells is supported by several experimental data showing how tumor cells are more susceptible to apoptosis induced by CK2 inhibitors than their normal counterpart (Kim *et al.*, 2007; Piazza *et al.*, 2006; Silva *et al.*, 2008). In particular, Slaton and colleagues (2004) have reported that, in xenograft models, inhibition of CK2 activity by siRNA and antisense vectors is able to induce apoptosis in cancer cells with no or small effects on normal cells. All these results have allowed the beginning of animal treatments with CK2 inhibitors and also of clinical trials of experimentation in humans. In particular, encouraging data from Phase I clinical trial of CX-4945 have been reported in different kinds of cancer (Siddiqui-Jain *et al.*, 2011); the compound has demonstrated favorable safety, and pharmacokinetic and pharmacodynamics characteristics.

A new strategy, based on multitarget drugs, is presently under investigation, looking for additive synergistic effects between different drugs. This approach

should be successful to target a particular disease, improving antitumor activity and also overcoming drug-resistance. Good examples are already available for kinases different from CK2; among them Sorafenib and Sunitinib, approved by FDA, which target the vascular endothelial growth factor receptor, the platelet-derived growth factor receptor, the FMS-like tyrosine kinase 3 (FLT-3), and the stem cell factor receptor (c-Kit). Targeting CK2 would be particularly convenient because of its extreme pleiotropicity: by reducing its activity, especially high in cancer cells, other survival pathways, which are typically and specifically up-regulated in different type of cancers will be also affected.

MULTI DRUG RESISTANCE

Drug resistance is a phenomenon by which human tumors acquire resistance to a certain drug and often also to many other compounds, different in structure and mode of action; for this reason it is usually defined as multi-drug resistance, or MDR, and it results in a decreased drug activity. It is defined as primary resistance, if present prior to drug exposure, or as acquired resistance, if it appears during or after the course of treatment. In both cases this event critically limits treatment outcomes, and it is one of the major reasons for the failure of cancer therapy.

But where do resistant cells come from? The development of human cancers is a complex multistage process involving accumulation of both genetic and epigenetic alterations over time (Caulin and Maley, 2011). As a consequence, a single tumor is composed of heterogeneous cells with distinct genetic fingerprints (Heppner, 1978, Marusyk and Polyak, 2010, Michor and Polyak, 2010). As the tumor progresses, some cells undergo genetic alterations, with selection of those having a superior growth advantage in a given context. The innate heterogeneity has relevance in cancer resistance. Since cancer cell selection obeys the Darwinian law of evolution, under therapeutic pressure, those populations that are most adaptive or resistant to treatment will be selected. These clones will then dominate and populate the tumor rendering it highly resistant to a given therapy (Figure 24) (Breivik *et al.*, 2005; Crespi and Summers, 2005; Lichtenstein *et al.*, 2005; Moncevicute-Eringiene *et al.*, 2005; Greaves *et al.*, 2007).

At a cellular level, there are multiple active mechanisms of resistance, such as increased activity of efflux pumps, i.e. ATP-dependent transporters; reduced drug influx; activation of mechanisms that repair drug-induced DNA damage; deficiencies in apoptotic signaling pathways. Alteration in drug efflux mechanisms is one of the most frequent causes of MDR; in fact, transporters pump anticancer agents out of the intracellular environment into the extracellular matrix, thereby preventing the agents from reaching their minimally effective intracellular concentrations (Perez *et al.*, 2009).

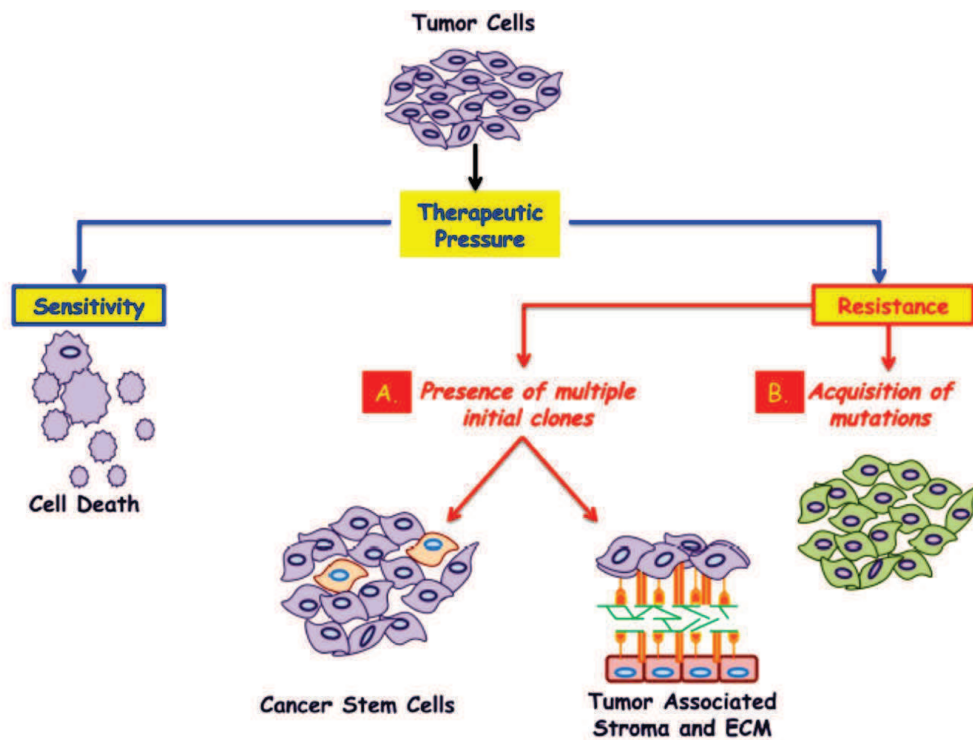


Figure 24. Development of cancer resistance. While most tumors initially respond to the given therapy, the majority will relapse following treatment, and in some cases resistance even predates clinical intervention. Cells can develop resistance by at least two mechanisms: (A) presence of multiple initial clones some of which emerge as dominant after treatment. These subpopulations possess stem-like characteristics and/or use their interactions with the surrounding microenvironment to enter into a dormant state, thus surviving the insult of therapy; (B) acquisition of alterations within the cancer cells per se. In all cases, the surviving population is less likely to respond to any further therapy and will be responsible for the minimal residual disease and cancer relapse (from Zahreddine and Borden, 2013).

Among the 48 known ATP binding cassette (ABC) human transporter proteins, at least three members can be involved in developing cancer chemoresistance (Gottesman *et al.*, 2002; Gottesman *et al.*, 2002). They are the P-glycoprotein (P-gp, MDR1 gene product), the multidrug-resistance-associated protein 1 (MRP1), and the mitoxantrone resistance protein (MXR; also known as breast cancer resistance protein (BCRP) or placenta ABC protein (ABC-P)). P-gp pump functions as an efflux pump with a broad specificity for hydrophobic anti-cancer drugs such as vinblastine, doxorubicin, vincristine, and taxol, and therefore its increased expression has been correlated with resistance to these compounds (Gottesman *et al.*, 2002). Studies conducted in 31 breast cancer clinical specimens demonstrated that overexpression of P-gp was associated with 3-fold increased risk

of failure to respond to chemotherapy and its expression was found to increase after chemotherapy exposure (Trock *et al.*, 1997). MRP1 instead transports negatively charged natural drugs and drugs modified by the conjugation of glutathione (GSH), glucuronic acid or sulfate (Jedlitschky *et al.*, 1996; Hipfner *et al.*, 1999; König *et al.*, 1999; Borst *et al.*, 2000). MXR altered expression has been correlated with resistance to topoisomerase I inhibitors, anthracyclines, and mitoxantrone (Gottesman *et al.*, 2002).

As already mentioned a drug transporter can recognize to resistance structurally different compounds, thus the therapeutic employment of a certain drug could influence the efficacy of many other compounds. For this reason, new strategies to overcome this problem are being proposed, such as the usage of drugs that are not recognized by transporters and therefore evade efflux, efflux inhibitors, drugs that are selectively lethal to P-gp expressing cells (Hall *et al.*, 2009; Nobili *et al.*, 2012). Drug inactivation can also play an important role in the development of resistance. These mechanisms include, for example, conjugation of the drug to glutathione (GSH), a powerful anti-oxidant that protects the cells against the damaging effects of reactive oxygen species. GSH conjugation to platinum drugs, such as oxaliplatin and cisplatin used in the treatment of various types of cancers, transforms them in optimal substrates for ABC transporters which enhance drug efflux (Ishikawa and Ali-Osman, 1993). Furthermore, the binding of platinum drugs, particularly cisplatin, to metallothionein (MT), a small cysteine-rich protein, is another mode of drug inactivation (Kasahara *et al.*, 1991). Interestingly, cancer cells can develop resistance through decreased drug activation mechanism for those agents that need of metabolic activation to show their cytotoxic effects. This occurs via the downregulation or mutation of enzymes involved in this metabolic pathway.

Although MDR mechanisms are well-characterized, agents targeted against multidrug resistance have produced poor results. Recent observations have highlighted that MDR phenotype of some cancer cells is by no means the result of a single gene aberration. Since it is recognized that multiple genetic defects, such as DNA mutations, translocations, truncations, deletions, or duplications, are present in cancer cells, the role of drug-induced epigenetic aberrations such as histone posttranslational modifications, DNA hypermethylation, and subsequent gene silencing is of increasing importance. Epigenetic changes can occur rapidly upon chemotherapy exposure.

Other mechanisms by which cancerous cells circumvent the effects of targeted inhibitor drugs have also been proposed, including amplification of alternative oncogenes or inactivation of alternative survival pathways (le Coutre *et al.* 2000). In some cases, targeting of one protein alone can become ineffective because another parallel pathway sustains tumor survival, resulting in the development of a lethal relationship between these two networks (Tucker and Fields, 2003). Thus, the loss/inactivation of one of these genes would be supported by the other pathway and, for the most effective treatment one would need to target both pathways (Nijman *et al.*, 2011).

Intrinsic or acquired resistance can occur also towards biologic agents, such as trastuzumab (Valabrega *et al.*, 2007) and hormonal therapies (Musgrove and Sutherland, 2009), not only through the specific pathways that underlie their respective mechanisms of action, but also through redundancy and cross-talk with other pathways that finally result in treatment failure (Leary *et al.*, 2010).

Strategies to Overcome Drug Resistance

Mechanisms of intrinsic drug resistance and the acquisition of resistance during the course of malignant progression are strongly influenced by the tumorigenic pathways. Both genetic and epigenetic changes are dynamic events which work together for the maintenance of the malignant “homeostasis”. Many attempts to overcome mechanisms of resistance to cytotoxic, hormonal, and biologic agents have failed. Some approaches have evaluated concomitant administration of agents that mislead resistance, but have produced limited satisfactory results for therapy (Fracasso *et al.*, 2000, Pusztai *et al.*, 2005). More recently, the incorporation of mTOR inhibitors or demethylation agents has had some success in preclinical and clinical studies (Nahta and O'Regan, 2010; Baselga *et al.*, 2012; Bachelot *et al.*, 2012). It is common for patients with resistant tumors to receive additional chemotherapy agents that demonstrate somewhat different mechanisms of action and/or biochemical structures respect to the canonical therapy for a given tumor type. These combinations may also show synergism with good efficacy on tumor progression since they impact on multiple intracellular escape pathways essential for tumor cell growth and survival. In recent years, an ever-growing group of

cytotoxic drugs with different mechanism of action has been considered in combination treatment strategies.

CK2 AND MDR

CK2 is dramatically involved in the sustainment of malignant transformation, and also involved in the MDR phenotype. It has been reported that CK2 phosphorylates P-gp (Glavy *et al.*, 1997). Matsumoto and co-workers (1999) have also highlighted that in a MDR cell lines, overexpressing P-gp, CK2 is also overexpressed at the mRNA level. Moreover, in a study conducted in our laboratory some years ago, it has been demonstrated that CK2 catalytic subunit is overexpressed in a MDR cell line compared to the non-MDR counterpart, and this contributes to the maintenance of the resistant phenotype, as demonstrated by the effects of selective down-regulation of CK2 activity, either pharmacologically or through siRNA technique (Di Maira, 2007). Moreover, CK2 positively controls the P-gp activity, since the accumulation of its substrate doxorubicine in P-gp expressing cells is increased in cell treated with CK2 inhibitors (Di Maira *et al.*, 2007). Recently, it has been reported that the activity of another efflux pump, MRP1, is regulated by the phosphorylation at its Thr249 by CK2 (Stolarczyk *et al.*, 2012). Therefore CK2 inhibition has been found to induce a higher accumulation of drugs in cells overexpressing these transporter proteins.

It is worth to note that CK2 overexpression is not an absolute requirement for the MDR phenotype, since several examples have been reported where its level is very similar in resistant cells and their apoptosis-sensitive counterparts (Di Maira *et al.*, 2007; Borgo *et al.*, 2013). However, CK2 inhibition has always been found to induce apoptosis in resistant cells, regardless of its overexpression; in several cases, interestingly, a synergistic effect has been also observed between CK2 inhibitors and conventional drugs, such as melphalan (Piazza *et al.*, 2006), vinblastine (Di Maira *et al.*, 2007) and imatinib (Borgo *et al.*, 2013). In this thesis, we will present an additional example of synergism between antitumor drugs and a new and prominent CK2 inhibition, CX-4945.

MATERIALS AND METHODS

MATERIAL AND METHODS

MATERIALS

Proteins

Recombinant human CK2 α , $\alpha_2\beta_2$ and GST-p53 were purified and kindly provided by Dr. Andrea Venerando (University of Padova, Italy). Native CK2 was purified from rat liver in our laboratory (Meggio et al., 1981). Recombinant His-B23 was from AbCam. Total casein and α -casein were from Sigma.

Peptides

The CK2 peptide substrates CK2-tide (RRRADDSDDDDD), eiF2 β -tide (MSGDEMIFDPTMSKKKKKKKKP) and HIPK2-tide (NKRRRSPTPPE) were synthesized and kindly provided by Dr. Oriano Marin (University of Padova, Italy).

Inhibitors

CK2 inhibitors

CX-4945 (5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid) and CX-5011 (5-(3-ethynylphenylamino)pyrimido[4,5-c]quinoline-8-carboxylic acid) were synthesized and kindly provided by Cylene Pharmaceuticals (San Diego, USA). Stock solutions were made in dimethylsulfoxide (DMSO) and stored at -20°C.

K137 and K137-E4 was synthesized and kindly provided by Prof. Giuseppe Zagotto (University of Padova, Italy). Stock solutions were made in dimethylsulfoxide (DMSO) and stored at -20°C.

CRIBI-300 and peptide derivatives (listed in Table 1, Chapter 3) were synthesized and kindly provided by Prof. O. Marin and Michele Sandre (University of Padova, Italy). Stock solutions were made in sterile water and aliquots were stored at -20°C.

HIPK2 inhibitors

TBID (4,5,6,7-tetrabromo-2-(1*H*-imidazol-2-yl)isoindoline-1,3-dione) was synthesized and kindly provided by Prof. Conrad Kunick (University of Braunschweig, Germany). Stock solution was made in DMSO and stored at -20°C.

Others

Staurosporine, Vinblastine and Doxorubicin were from Sigma.

Antibodies

CK2 α -subunit antisera were raised in rabbit against the sequence of the human protein at C-terminus [376–391] (Sarno *et al.*, 1996), total Akt, total Cdc37, LAMP and S6 antibodies were from Santa Cruz Biotechnology, β -actin and α -tubulin antibodies were from Sigma, PARP antibody was from Roche, caspase-3, total p53 and p53Sp46 were from Cell Signaling Technology, CK2 β and HIPK2 antibodies were from Epitomic; B23 antibody was from Invitrogen, P-gp monoclonal antibodies were from Calbiochem, Akt Sp129 phospho-specific antibodies were raised in rabbit and purified in our laboratory (Di Maira *et al.*, 2005); Cdc37 Sp13 phospho-specific antibodies were from Abcam; secondary antibodies towards rabbit and mouse IgG, conjugated to horse radish peroxidase, were from PerkinElmer.

Oligonucleotides

CK2 α specific siGENOME SMARTpool siRNAs and aspecific siRNA siCONTROL riscfree#1 were from Dharmacon (Lafayette, CO, USA). CK2 β siRNA (sense, 5'-GCCAUGGUGAAGCUCUACUdTdT-3'; antisense, 5'-AGUAGAGCUUCACCAUGGCdTdT-3') were from Dharmacon. Transfecting reagent INTERFER in was from Polyplus-transfection SA (Illkirch, France).

Cell lines

For these thesis the following cell lines were used: human T lymphoblastoid CEM cells (normally sensitive, S-CEM, and their MDR variant, R-CEM, selected with 0.1 μ g/ml vinblastine, Vbl (Di Maira *et al.*, 2007)); human osteosarcoma U2OS cells (normal sensitive, S-U2OS, and their MDR variant, R-U2OS (Cenni *et al.*,

2004)); human ovarian carcinoma 2008 cells (normally sensitive, S-2008, and their cisplatin-resistant variant, R-2008 (Marzano et al., 2007)); acute promyelocytic leukemia HL60 cells; acute T lymphoblastic Jurkat cells; human hepatocellular liver carcinoma HepG2 cells; human cervix adenocarcinoma HeLa cells; human embryonic kidney HEK-239T cells; human lung CCD34Lu cells; chronic myeloid leukemia (CML) LAMA84 (le Coutre *et al.*, 2000), KCL22 (Redaelli *et al.*, 2010), and K562 (Redaelli *et al.*, 2010) cells, in their sensitive (S) or resistant (R) to Imatinib, kindly supplied by Dr. C. Gambacorti-Passerini (University of Milano-Bicocca, Milano).

METHODS

Cell culture and treatments

Cells were cultured in an atmosphere containing 5% CO₂; CEM, LAMA84, K562, KCL22 and HL60 cell lines were maintained in suspension in RPMI 1640 medium (Sigma), U2OS, 2008, HepG2, HeLa, HEK293-T and CCD34LU cell lines were adherent cells maintained in D-MEM medium (Sigma); both media were supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cell treatments with inhibitors were performed in the culture medium at 3×10^6 cells/ml for cells that grow in suspension, and about 70% of confluence for adherent cells, but with 1% FCS, where not differently indicated. Control cells were treated with equal amounts of the inhibitor solvent.

Cell viability

Cell viability was detected by means of MTT (3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltriazoliumbromide) reagent, a tetrazolium salt that is metabolized by mitochondrial dehydrogenases and produces a violet precipitate (formazan) in viable cells. For cell viability experiments, cell treatments were performed in 96-well plates at a density of 10^5 cells/100 µl for cells in suspension; 2×10^4 adherent cells were seeded in 96-well plates in 100 µl medium, and left to adhere for one night before treatment. Cells were incubated for variable times under the conditions

indicated in the figure legends. 1 h before the end of the incubation, 10 μ l of MTT solution (5 mg/ml in PBS) was added to each well. Incubations were stopped by addition of 20 μ l of lysis solution at pH 4.7, containing 20% (w/v) SDS, 50% (v/v) N,N-dimethylformamide, 2% (v/v) acetic acid and 25mM HCl. Plates were read for OD at λ 590 nm, in a Titertek Multiskan Plus plate reader (Flow Laboratories). DC₅₀ (concentrations inducing 50% of cell death) values were calculated with Prism 4.0c software (GraphPad Software).

Combined treatments

Drug interactions were assessed by treating cells with increasing concentrations of two different compounds, at fixed concentration ratio, indicated in the figure legends. The combination index (CI) (Giovannetti *et al.*, 2007), was calculated with the software Calcsyn (Biosoft); CI<1, CI = 1, and CI>1 indicate synergistic, additive and antagonistic effects, respectively.

Doxorubicin accumulation measurement

About 100000 CEM cells were seeded in 96-well plates and treated with 25 μ M doxorubicin for 30 min. CK2 inhibitors, where present, were added 30 min before doxorubicin. After washing with phosphate-buffered saline, cells were lysed in the lysis buffer described above, supplemented with 1% (w/v) SDS. Doxorubicin content was determined fluorimetrically, by reading the plate at λ_{exc} .485 nm, λ_{em} .590 nm with Titertek Multiskan Plus plate reader (Flow Laboratories).

Apoptosis assay

Apoptosis was evaluated by detecting the cleavage of the caspase substrate PARP or the activation of pro-caspase 3 by WB. Apoptosis quantitative determination was performed by means of the Cell Detection Elisa kit (Roche), based on the quantification of nucleosomes present in the cytosol of the apoptotic cells, by measuring the absorbance at λ 405– λ 490, following the manufacturer's instructions. About 10 000 cells were used for each determination. Alternatively, DNA-laddering was performed starting from 10^7 cells, in the presence or absence of CK2 inhibitors. Cells were washed with PBS and pelleted by centrifugation. Pellets were resuspended in 1.5 mL of lysis buffer containing 10 mM Tris-HCl, pH 7.5, 400

mM NaCl, 2 mM EDTA, pH 8.2, 0.7% SDS and Proteinase K (2 mg/mL) and incubated overnight at 37°C. Proteins were then precipitated by addition of 0.5 mL of 5 M NaCl at room temperature for 1 hour. The precipitated proteins were pelleted by centrifugation at 3300 g for 30 minutes. The DNA, present in the supernatant, was transferred in a new tube and precipitated with 2.5 volumes of cold absolute ethanol and washed in 70% ethanol after centrifugation at 3300 g for 30 minutes. The DNA pellet was dissolved in 100 µL of TE buffer containing 0.5 mg/mL RNase and was allowed to dissolve for 2 hours at 37°C before quantitating. After quantification of the DNA content by Nanodrop, 10 µg of DNA per lane was separated on 1.7% agarose gel at 60V for 2 hours at 4°C. The staining was performed in ethidium bromide (1 µg/mL) for 15 minutes and washed for 30 minutes in water.

Cell lysis

Cells were collected by centrifugation at 1200 *rpm* for 5min, washed with cold PBS, and lysed to obtain a total lysate, by adding an ice-cold buffer consisting of 20mM Tris-HCl, pH 7.5, 150mM NaCl, 2mM EDTA, 2mM EGTA, 0.5% (v/v) Triton X-100, 2mM dithiothreitol (DTT), protease inhibitor cocktail Complete (Roche), 10 mM NaF, 1 µM okadaic acid, 1mM Na vanadate. After 30min incubation on ice, the lysates were centrifuged at 13200 *rpm* for 10 min, at 4°C. The supernatants contained the cell-soluble fraction, whose protein concentration was determined by the Bradford method.

Western blot analysis

Equal amounts of proteins were solubilized in Laemmli buffer, loaded on 11% SDS-PAGE (Laemmli, 1970), blotted on PVDF membranes (Immobilon-P Millipore) in a TE 22 Mini Tank Transfer Unit (GE Healthcare), at 60V for 75 min, using a buffer containing 10 mM CAPS-NaOH (3-(Cyclohexylamino)-1-propanesulfonic acid pH 10, DTT 3 mM and 1% (v/v) methanol. Dried PVDF membranes were then washed with TBS buffer (Tris-HCl 50 mM pH 7.5, NaCl 50 mM) with 1% (w/v) BSA (Sigma-Aldrich) and then incubated with the indicated antibodies diluted in the same buffer. The incubation times were variable, ranging from 2h to 16h, at room temperature (< 3h incubation) or at 4°C (longer

incubation). Then membranes were incubated with a secondary HRP-conjugated antibodies solution for 30 min in the same buffer and then washes three times with TBS buffer. Antibodies signals were detected by ECL solution (enhanced chemiluminescence, Amersham Biosciences). For the quantitation of the signal, this solution was left in contact with membrane for a time ranging from 2 sec to 4 min; a Kodak Image Station 4400MM PRO was used for detection and the analysis was performed with the Kodak 1D Image software.

Immunoprecipitation assays

Immunoprecipitation was performed incubating 100-200 µg of proteins from total cell lysates over night at 4°C, with the specific antibody for the interested protein or with an aspecific antibody as a negative control, in gently rocking . Then, protein A-Sepharose (Sigma-Aldrich) is added for 40 min at 4°C. Immunoprecipitates were washed twice with 50 mM Tris-HCl pH 7.5 and once with 5 mM Tris-HCl pH 7.5 and used for phosphorylation assays.

Immunofluorescences

About 200000 cells were seeded on glass cover-slips and allowed to adhere overnight. Cells were washed with warm PBS, fixed with 4% (w/v) paraformaldehyde (Sigma) for 20 minutes at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 10 min at 4°C, and incubated with the primary antibody anti-CK2 (1:50) from a time variable ranging from 2h at room temperature to overnight at 4°C. After 3 times washes with PBS, cells were incubated with Alexa Fluor 633 goat anti-rabbit IgG secondary with PBS, cells were incubated with the secondary antibody. Alexa Fluor 633 conjugated goat anti-rabbit IgG (Life Science) was used at 1:400 dilution, FITC conjugated secondary antibody (Dako) was used at 1:50 dilution for 1 hour at 37°C in the dark. Nuclei were stained with Hoechst 33342 (1:10000; Sigma) for 10 min at room temperature in the dark. The coverslips were mounted with fluoromount solution (Sigma) and the immunofluorescence images were acquired with LEICA TCS SP5 confocal microscope equipped with HCX PL APO lambda blue 63x1.4 oil immersion

objective. Argon laser emitting at 488 nm (for detection of LAMP1) and a HeNe laser emitting at 633 nm (for detection of CK2) were used. LAS IF software was used for image processing.

CK2 ACTIVITY ASSAYS

Activity assay of purified CK2

Recombinant CK2 $\alpha_2\beta_2$ (40ng), recombinant CK2 α (10-50ng) or native CK2 (0.005 U) were incubated with peptides or protein substrates in the presence of 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 μ M [γ -³³P] ATP (1000-2000 c.p.m/pmol) and 0.1 M NaCl, in a total volume of 20 μ l. When CK2 α was used, NaCl was omitted. Substrates concentrations were: CK2-tide 0.1mM, eiF2 β 0.1mM, α -casein 0.5 μ g, total casein 0.5 μ g, p53 0.5 μ g and calmodulin 2 μ g. When CK2 $\alpha_2\beta_2$ and α activities were compared, the same units were added (0.01), where 1 unit is defined as the amount of enzyme which catalyzed the introduction of 1nmol of Pi to CK2-tide/min under the experimental conditions. Incubation was carried out at 30°C for 10 min. Reaction of peptide phosphorylation were stopped by sample absorption on phospho-cellulose paper. Papers were washed three times with 75 mM phosphoric acid and counted in a scintillation counter (Perkin Elmer).

In the case of protein substrates reactions were stopped by the addition of Laemmli buffer (Laemmli, 1970) and proteins were separated in SDS-PAGE, stained with Coomassie blue and analyzed by digital autoradiography (CyclonePlus Storage Phosphor System, PerkinElmer).

CK2 activity assay in cell lysates

0.5-2 μ g of proteins from cell lysate were incubated in the presence of 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 μ M [γ -³³P] ATP (1000-2000 c.p.m/pmol) and 0.1 M NaCl, with 0.1mM CK2-tide in a total volume of 20 μ l. Incubation was carried out at 30°C for 10 min and stopped by sample absorption on phospho-cellulose P81 paper. Papers were washed three times with 75 mM phosphoric acid and counted in a scintillation counter. Control samples were performed in the absence of CK2-tide; they always turned out to display an activity lower than 5% of the same samples

incubated with CK2-tide. Phosphorylation of endogenous proteins was performed on 5 µg of lysate proteins under the same conditions for 20 min, but without the addition of the peptide substrate. The reactions were stopped by the addition of Laemmli buffer and the proteins were analyzed by SDS/PAGE/WB and digital autoradiography (CyclonePlus Storage Phosphor System, PerkinElmer).

CK2 ectokinase activity assay (from Kubler et al., 1989)

For cells growing in adhesion, 100000-200000 cells were used, seeded in a 96 well plate 24 h before the assay; for cells growing in suspension, 50000 cells were used and pelleted in sterile tubes. Cells were washed three times with an isotonic buffer containing 70 mM NaCl, 30 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 5 mM KHPO₄, 0.5 mM EDTA and 75 mM glucose and then preincubated with 50 µl of the same buffer for 3 min at 37°C. Reactions started when a mixture containing ATP to a final concentration of 10 µM, [γ -³³P] ATP (10000-20000 cpm/pmol) and the CK2-specific peptide for a final concentration of 1 mM, was added in each well for total volume of 56.6 µl. Samples incubated with the same mixture but without the peptide substrate were used to check non-specific phosphorylation. Controls of CK2-tide phosphorylation activity in cell supernatant were performed under the same condition using 50 µl of the cellular medium, in a total incubation volume of 56.6 µl. Plates or tubes were incubated at 37 °C, with gentle shaking (130-150 rpm) for variable times. Then 20 µl of the phosphorylation mixture are spotted on phosphocellulose paper and analyzed as described for purified CK2 at different times.

Cellular HIPK2 activity assay

Cellular HIPK2 activity was assayed on HIPK2 immunoprecipitation from 350 µg of total lysate proteins deriving from HepG2 cells, with 2.5 µl anti-HIPK2 or an aspecific antibody, as negative control (for details see above Immunoprecipitation assay) Immunoprecipitated HIPK2 activity was measured towards the specific peptide substrate NKRRRSPTPPE at 1.6 mM final concentration using a radioactive mixture composed of 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 µM [γ -³³P] ATP (1000-2000 c.p.m/pmol) and 0.1 M NaCl, for 10 min at 30 °C. Peptide radioactivity was measured after sample spotting on phospho-cellulose paper,

washing and scintillation counting, while the amount of HIPK2 immunoprecipitated was evaluated by WB.

RNA interference assay

LAMA84 (1.5×10^6) were transfected with 40 nM CK2 α specific siGENOME SMARTpool siRNAs or 30nM CK2 β siRNA (see above), or 40nM aspecific siRNA siCONTROL riscfree#1 as control, using the transfecting reagent INTERFER in (Polyplus-transfection SA, Illkirch, France), according to the manufacturer's recommendations. Transfection was performed for 72 h in RPMI supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Statistical analysis

All experiments were performed at least 3 times, each time in duplicate; graphs and statistical analysis were performed with Prism 4.0c software (GraphPad Software).

Peptide Synthesis

The peptides used for this work, provided by Prof. O. Marin, were synthesized by solid-phase technique with a multiple peptide synthesizer on a *p*-benzyloxybenzyl alcohol resin using the fluoren-9-ylmethoxycarbonyl (Fmoc) strategy (Marin *et al.*, 1994). In case of cyclic peptides, cyclization was achieved by oxidizing the two cysteines of the purified peptide (0.1 mg/ml) in 100mM NH_4HCO_3 buffer (pH 8.2) for 72 hours. The formation of disulphide bridges and each peptide mass were verified by mass spectrometry analysis performed by Michele Sandre (University of Padova).

RESULTS

**EFFECTS OF CK2
INHIBITORS
CX4945 AND CX5011
IN DRUG-RESISTANT CELLS**

INTRODUCTION

As largely presented in the Introduction of this thesis, CK2 has a central role in multiple pathways that support cancer, and it is generally overexpressed in cancer compared to the healthy cells. Therefore the development of selective CK2 inhibitors has emerged as an attractive approach for the treatment of cancers. Indeed, a compound, CX-4945, has been recently developed by Cylene Pharmaceuticals and entered human clinical trials.

CX-4945 is a potent and selective ATP-competitive CK2 inhibitor with a Mw of 350 (chemical structure in Figure 1) (Pierre *et al.*, 2011). It has shown the same inhibitory efficacy toward both isoforms of the CK2 catalytic subunits, CK2 α and CK2 α' , with IC₅₀ values close to 1 nM (Siddiqui-Jain *et al.*, 2010) (see Table 1). The crystal structure of CK2 α in complex with CX-4945 has been solved, and reveals that there are two major polar interactions in the ATP pocket: one hydrogen bound to Val116 amide in the hinge region and the salt-bridge with Lys68 (Figure 1). The removal of one of these interactions causes a dramatic decrease in the inhibitor affinity (Pierre *et al.*, 2011).

Kinase	IC ₅₀ (nmol/L)	Kinase	IC ₅₀ (nmol/L)
CK2 α	1	PI3K β	>500
CK2 α'	1	PI3K δ	>500
DAPK3	17	PI3K γ	>500
FLT3	35	PDK1	>500
TBK1	35	AKT1	>500
CLK3	41	AKT2	>500
HIPK3	45	AKT3	>500
PIM1	46	mTOR	>500
CDK1/cyclin B	56	p70S6K	>500

Table 1. IC₅₀ determinations for CX-4945 against selected kinases from the 238-kinase selectivity panel (from Siddiqui-Jain *et al.*, 2010).

CX4945 is very specific for CK2: when it was tested at 0.5 μ M, a concentration 500-fold greater than the IC₅₀ for CK2 α , on a panel of 238 kinases, only 7 of them were inhibited more than 90%.

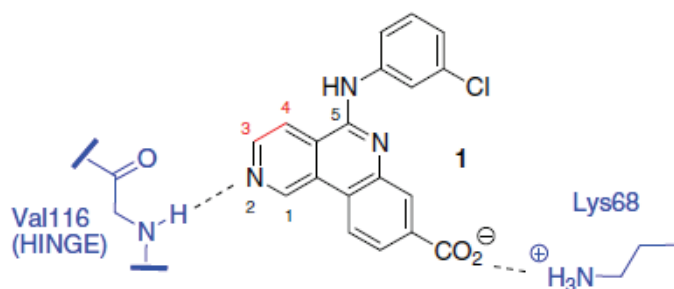


Figure 1. Schematic representation of CX-4945 binding model in ATP pocket of CK2. Binding resulted from interactions with hydrophobic residues (not shown), a ionic bridge with positively charged Lys68, and hydrogen bonding with the hinge Val116 amide (from Pierre *et al.* 2011).

CX-4945 has been also demonstrated to inhibit pro-survival and angiogenic signaling in cancer cell lines and it is active against a broad range of cancer cell types, including breast, lung, and prostate cancers, where CK2 is usually overexpressed (Siddiqui-Jain *et al.*, 2010; Pierre *et al.*, 2011).

The molecular mechanism by which CX-4945 acts in cells is essentially represented by the inhibition of CK2. This in turn affects several signaling pathways where CK2 is involved, as demonstrated in different *in cell* studies. For example, its effects has been checked on PI3K/Akt signaling, cell cycle arrest, and induction of apoptosis (Siddiqui-Jain *et al.*, 2010 and Figure 2).

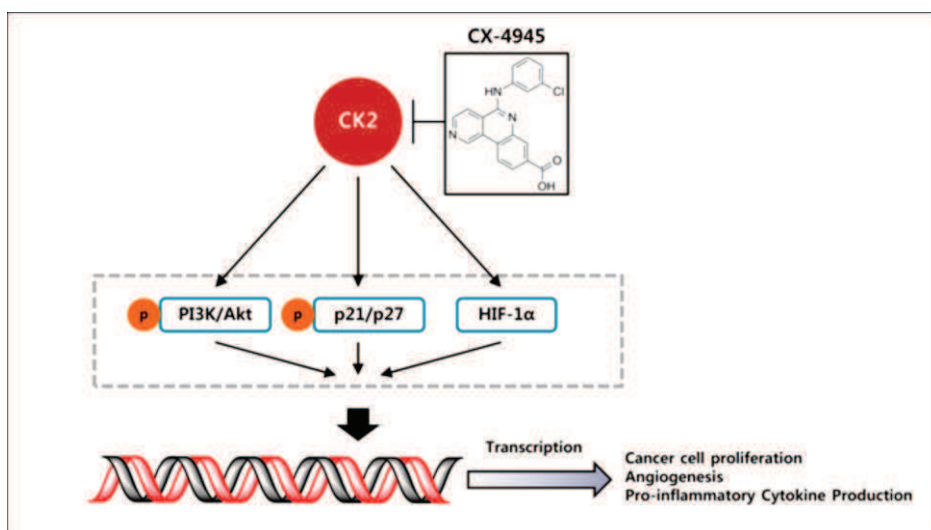


Figure 2. Schematic mode of action of CX-4945. CX-4945 inhibits the phosphorylation of PI3K/AKT and p21/p27 and transcriptional activation of HIF1α in the nucleus. These effects might influence cancer survival, angiogenesis, and pro inflammatory cytokine production (from Kim J. and S.H. Kim, 2012).

CX-4945 induces a reduced phosphorylation of Akt on Ser129, the direct CK2 phosphorylation site on Akt kinase, and a subsequent reduction in the canonical regulatory sites of Akt (Thr308 and Ser473). In addition, in cells treated with CX-4945, a decreased phosphorylation of the cell cycle inhibitor protein p21 at Thr145 is observed, and G2/M or G1 cell cycle arrest occurs. In parallel, an apoptotic response with the induction of caspase-3/7 activity in CX-4945–treated cancer cells is observed. In different studies, CX-4945 was used to induce apoptosis not as a single agent, but in combination with other anti-tumor drugs, after showing interesting synergistic effects (Quotti-Tubi *et al*, 2013; Buontempo *et al.*, 2013; Manni *et al.*, 2013).

After *in vitro* and *in cells* experiments, CX-4945 has been tested in murine xenograft models (Buontempo *et al.*, 2013; Zheng *et al.*, 2013; Siddiqui-Jain *et al.*, 2012). In these studies, CX-4945 exhibited dose-dependent antitumor activity with 86% tumor growth inhibition at the highest dose level; it was well tolerated at all doses as indicated by minimal changes in body weight (Figure 3). CX-4945 has also been found to exert an anti-angiogenic activity (Siddiqui-Jain *et al.*, 2010), as expected considering the demonstrated role of CK2 in angiogenesis and vascularization (Ljubimov *et al.*, 2004).

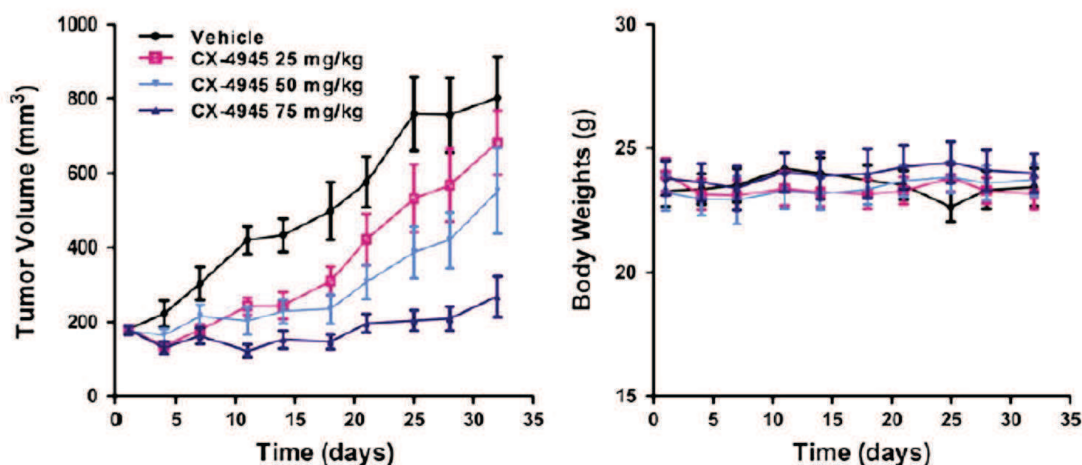


Figure 3. Antitumor efficacy of CX-4945 delivered orally, twice-a-day in PC3 prostate cancer xenograft model (from Pierre *et al.*, 2011.)

The promising results obtained in animal studies concerning safety, pharmacokinetics, and efficacy studies have led to the current clinical evaluation of CX-4945 for the treatment of cancer. The human experimentation started with

Cylene Pharmaceuticals, on relapsed or refractory multiple myeloma, breast cancer, advanced solid tumors and Castleman's Disease (www.clinicaltrials.gov). Encouraging preliminary results were reported (Pierre *et al.*, 2011) and the compound successfully reached the end of phase I of clinical trials. At present, Cylene Pharmaceuticals is no more active, and CX-4945 experimentation has been transferred to a new company Senhwa Biosciences (www.senhwabiosciences.com). Another molecule belonging to the class of ATP-competitive inhibitors of CK2 the CX-4945 analog CX-5011, is very potent (IC₅₀ for CK2 α of 2.3 nM) and selective (Battistutta, *et al.*, 2011). It has also been used in cellular assays, such as prostate cancer cells, where it is able to decrease endogenous CK2 activity (Pierre *et al.*, 2011). Studies in mouse model of formalin-induced pain have demonstrated that this compound induces a dose-dependent reduction of pain and its possible anti-inflammatory action has been suggested (Pierre *et al.*, 2011). The development of CX-4945 and its advancement into clinical trials has allowed, for the first time, the therapeutic targeting of CK2 with an ATP-competitive inhibitor for the treatment of cancer. However, this promising compound has never been tested in apoptosis-resistant cells. My research in this field has been prompted by this observation, as presented in the following paragraphs.

AIM OF THE STUDY

CK2 is deeply connected to the resistance to apoptosis, and evidences of its intervention in the MDR phenomenon have already been reported (Di Maira *et al.*, 2007); moreover, several inhibitors have been proposed as a useful tool to overcome drug resistance (Di Maira *et al.*, 2007). However, no study has been performed in drug-resistance cells with the only one CK2 inhibitor already in clinical trials, CX-4945. This part of the work was performed to assess if CX-4945 and its analog CX-5011 are able to induce apoptosis in drug-resistant cells. We therefore exploited different cell lines available in the apoptosis normally sensitive form (S) and in a variant resistant to apoptosis (R). In this pairs of cell lines, we analyzed if CX-4945 and CX-5011 are able to induce a reduction of CK2 endogenous activity, and if concomitantly cell viability is affected by apoptosis induction. Moreover, we wanted to assess if CX-4945 is able to increase the amount of drug retained inside in cells overexpressing an efflux pump, such as P-gp, which extrudes drugs outside from the cell, and if a consequent sensitization to the drug occurs. For this purpose we planned experiments of combined treatment with CX and conventional anti-tumor drugs.

RESULTS

For this study, several cancer cell lines have been exploited, available in the apoptosis sensitive (S) and resistant (R) variants. In particular we used: CEM (human T lymphoblastoid cells, S and the MDR variant R), U2OS (human osteosarcoma cells, S and the MDR variant R), 2008 (human ovarian carcinoma cells, S and R, resistant to cisplatin), and three pairs of chronic myeloid leukemia cells (CML), LAMA84, KCL22 and K562, whose R variants are resistant to imatinib. As reported in the Figure 4 A, R-CEM and R-U2OS cells express P-gp pump and are therefore considered MDR (multi-drug resistant) lines, while the R variant of 2008 cells and CML cells do not express Pg-P and are resistant toward specific drugs, CisPt in the case of R-2008, and imatinib in the case of CML; their resistance mechanisms are still largely unknown. CML is related to the expression of the chimeric fusion protein Bcr-Abl, which represents a hyperactive state of the Abl tyrosin kinase. Bcr-Abl is the target of imatinib, the first-choice drug for this pathology, and resistance to imatinib is sometimes due to Bcr-Abl mutations. However, in the CML cell lines used in this work, no mutation are reported in Bcr-Abl; while in the case of R-LAMA84, the fusion protein is overexpressed compared to S-LAMA84 (Figure 4 B).

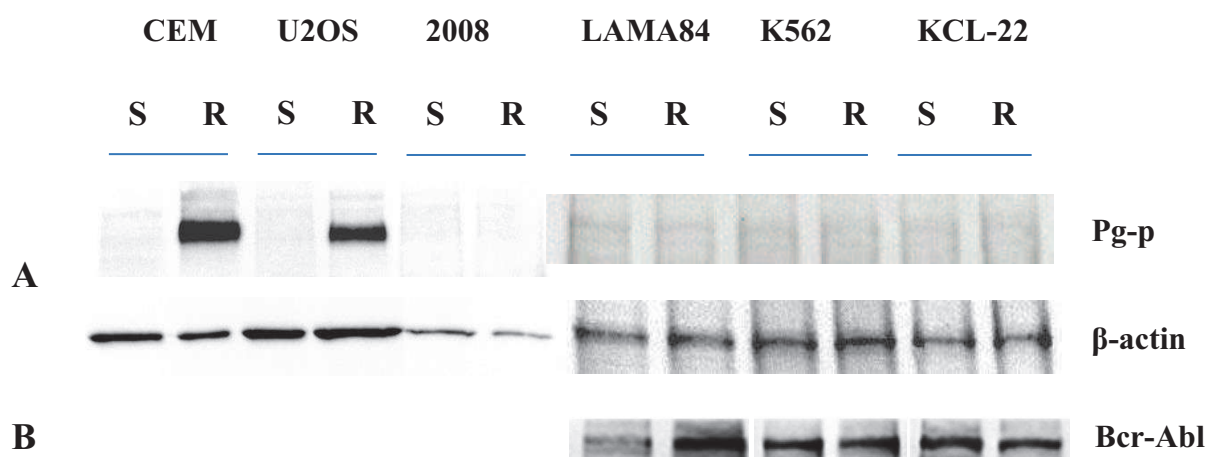


Figure 4. P-gp and Bcr-Abl expression. A. 10 μ g of proteins from total lysates of the indicated cells were loaded onto an SDS-PAGE, blotted and analyzed by WB with anti-P-gp or anti- β actin (from Zanin et al. 2012). B. 30 μ g of proteins from total lysates of the indicated cells were loaded onto an SDS-PAGE, blotted and analyzed by WB with anti-Bcr-Abl (from Borgo et al., 2013).

EFFECTS OF CX-4945 AND CX-5011 ON CK2 ACTIVITY IN S AND R CELLS

To verify that CX compounds are able to inhibit CK2 both in sensitive cells and resistant counterparts, we firstly measured CK2 activity in cells treated with increasing concentrations of CX-4945 and CX-5011 for different times. To this purpose we exploited *in vitro* assays of endogenous CK2 present in total lysates and western blot analysis of specific phosphorylation sites of CK2 substrates. The *in vitro* assays were performed by testing the activity of endogenous CK2 present in total lysates, measured towards a CK2 specific synthetic peptide: as shown in Figure 5 cell treatment with CX-4945 promptly inhibit CK2 in S and R cells, with similar efficacy, without affecting CK2 amount (Figure 6). For the evaluation of the phosphorylation state of CK2 intracellular substrates, phospho-specific antibodies were used directed to Ser129 of Akt and Ser13 of the co-chaperone protein Cdc37, which are two known key targets of CK2. In all cases, CK2 activity toward peptide is reduced more than 50% at submicromolar concentrations of CX-4945, and this data correlates with the reduction of phospho-Ser129Akt and phosphor-Ser13Cdc37. As shown in Figure 5 CK2 activity is almost totally reduced after only 6 h treatment with CX-4945, indicating that these compounds acts very rapidly on CK2; longer treatment times produce only a minimal increase of the inhibition degree. Interestingly, a marked dephosphorylation of Ser129Akt occurs after 6 h treatment at very low concentrations (0.5 μ M CX-4945), while a slower reduction of phosphorylation on Cdc37 Sp13 is observed, indicating that this CK2 site is more resistant to dephosphorylation, which is clear appreciable only after higher concentrations and longer times. It is worth to note that, as evident in Figure 6 and previously reported (Di Maira *et al.*, 2007) CK2 amount significantly differs in S and R CEM cell. Consequently, the level of phosphorylation of the analyzed sites Ser129Akt and Ser13Cdc37 in untreated cells are higher in R than in S cell, especially considering that the amount of both proteins is instead higher in S cells.

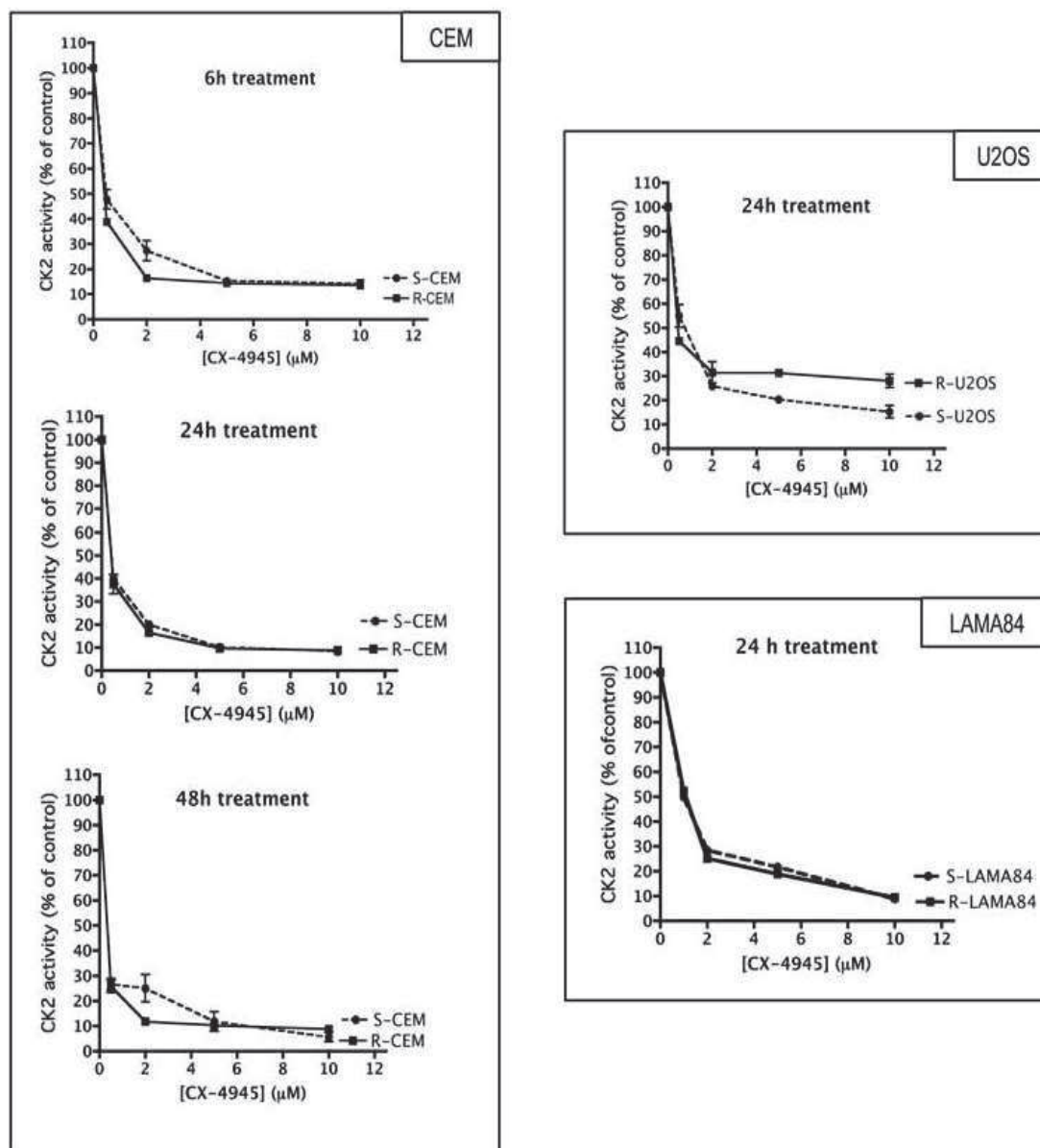


Figure 5. CK2 activity in CX-4945-treated cells. CK2 activity was measured towards a synthetic specific peptide. 1–2 μ g of proteins from total lysates of the indicated cells were incubated with the peptide and a radioactive phosphorylation mixture (see Materials and Methods). Activity is reported as percentage of that found in vehicle-treated control cells. Mean $\pm$ SE values of four independent experiments are shown (from Zanin et al., 2012).

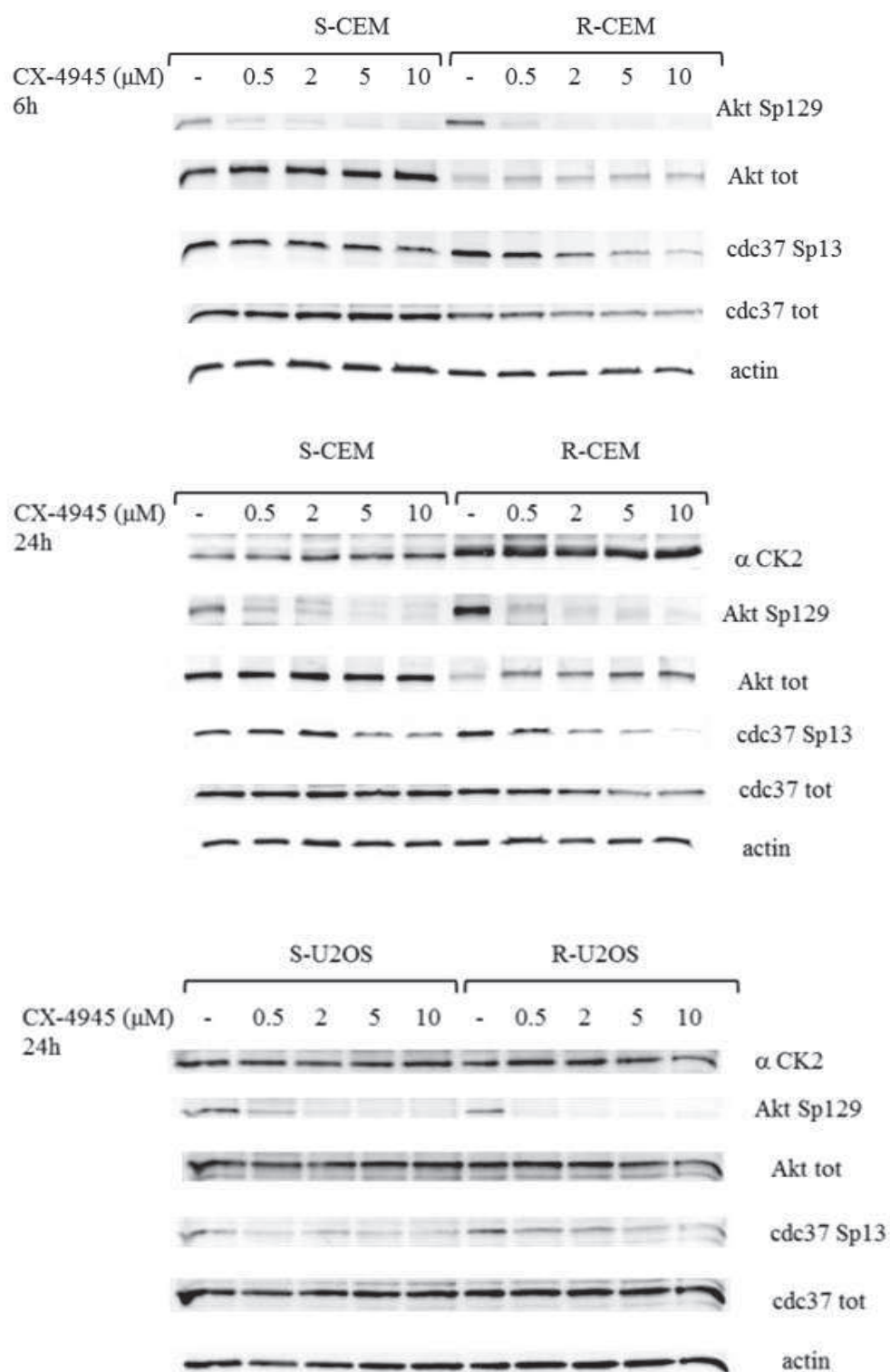


Figure 6. CK2 activity in CX-4945-treated cells. 10 μg of total proteins were analyzed with the indicated antibodies; actin was used to normalize the loading. Representative WB of three independent experiments are shown (from Zanin et al., 2012).

Similar data are observable in total lysates from cells treated with increasing concentration of CX-5011 and for different times (not shown and Figure 7 for 24h treatment in CEM cells).

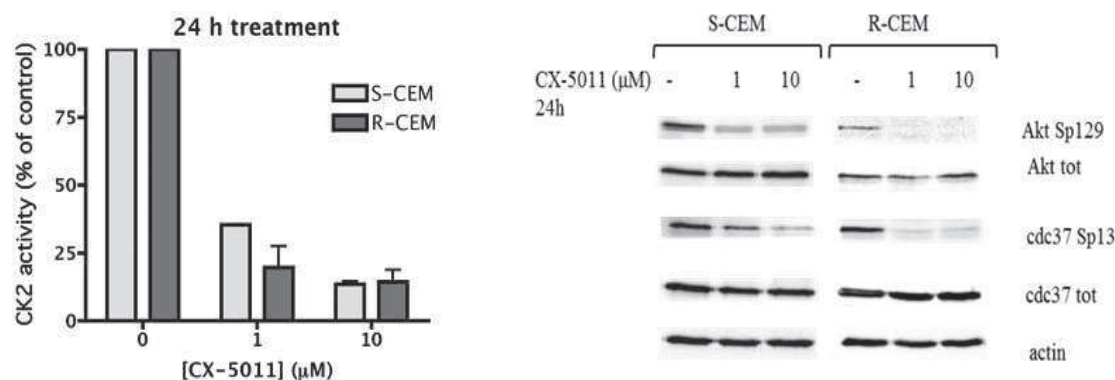


Figure 7. CK2 activity in CX-5011-treated cells. S-CEM and R-CEM cells were treated for 24 h with different concentrations of CX-5011. Cell lysates were analyzed for the activity of CK2 towards a synthetic specific peptide (on the left), or by WB with the indicated antibodies (on the right, separate development for S- and R-CEM cells). See details in the legend of Figure 5 (from Zanin *et al.*, 2012).

To further confirm CK2 inhibition, we analyzed the radioactive phosphorylation of endogenous proteins from total lysates derived from cell treated with CX-4945 or CX-5011 or vehicle. As Figure 8 shows, the phosphorylation level of some protein bands is lower in treated than in untreated cells. The effect is already evident when cells were treated with 1μM CX-4945 and CX-5011, which almost totally reduces the radioactivity incorporated in several bands, similar to what observed when the compounds are added in vitro. Even with this approach, we observed a very similar inhibition by CX4945 and CX-5011 in S and R cells. To exclude a non-specific effect, cells were treated with staurosporine at concentrations that inhibit the majority of protein kinases but no CK2 (Meggio *et al.*, 1995). Staurosporine, at the concentration used (1μM), induces a degree of cell death similarly to that observed with CX inhibitors (not shown), but is ineffective on endogenous protein phosphorylation. This confirms the specificity of CX-4945 and CX-5011 on CK2, and indicates that under the used conditions, CK2 is the major kinase responsible for the observed phosphorylated bands, as expected for such a pleiotropic and constitutively active enzyme.

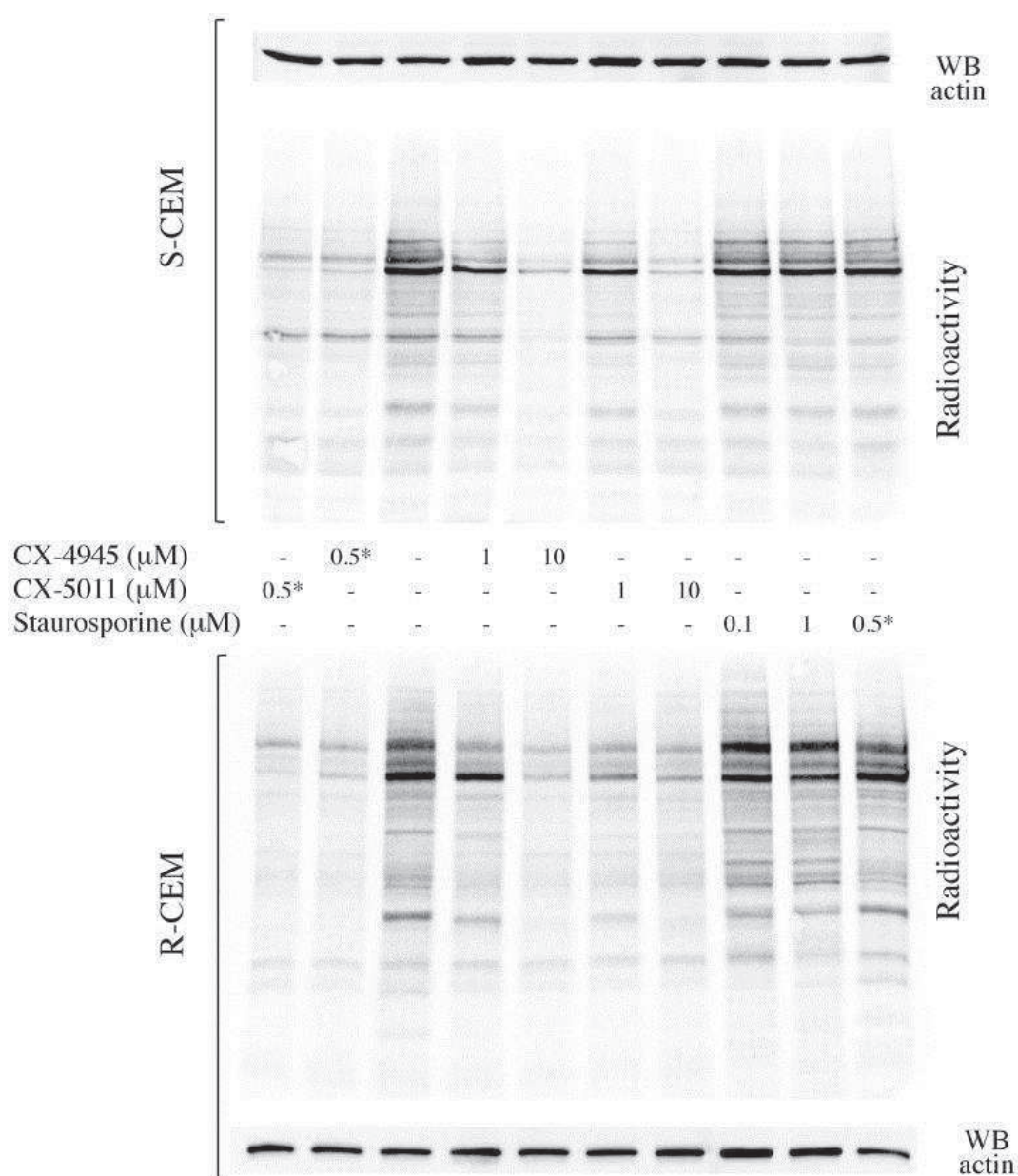


Figure 8. Protein phosphorylation in lysates from cells treated with CX-4945, CX-5011, or staurosporine. S-CEM (upper panel) or R-CEM (lower panel) were treated with the indicated concentrations of the CX inhibitors or staurosporine for 16 h, and then lysed. 5 μg of total proteins were incubated with a radioactive phosphorylation mixture, resolved by SDS-PAGE plus blotting, and analyzed by digital autoradiography and WB for actin, as loading control. Asterisk * denotes samples where the inhibitors were added in vitro during the phosphorylation assay. Representative results of five independent experiments are shown (from Zanin et al., 2012).

EFFECTS OF CX-4945 AND CX-5011 ON CELL VIABILITY IN S AND R CELLS

Then we moved to verify if CK2 inhibition by CX compounds induces cell death, as expected whenever this anti-apoptotic and pro-survival kinase is inhibited. For this purpose, we treated cells with increasing concentrations of CX-4945 and CX-5011 for 24 or 48 hours and we evaluated cell viability by the MTT method.

In Figure 9, 10, 11 and 12 some of the most representative results are reported, obtained under different experimental conditions and treatments times. We found that the U2OS and 2008 cell R variants show a very similar response to CX-4945 than the corresponding S variants (Figure 9 and 10). In the case of CEM cells, the R variant shows a slightly lower sensitivity to CX-4945 when the treatment were performed in the presence of only 1% (v/v) fetal calf serum (FCS) (Figure 11). However the effect is quite similar if cells are treated in the presence of a higher (10%) FCS concentration (Figure 11, lower panel). Also in the case of CX-5011 a similar effect on cell viability is observed in sensitive and resistant cells. An immediate outcome of these results, and in particular of those obtained with R-CEM and S-U2OS that express high level of the P-gp pump, is that both CK2 inhibitors are not P-gp substrates and can be used to induce cell death also in MDR cells. Figure 12 instead shows the effects of CX-4945 and CX-5011 on the viability of three CML S and R cell lines. All the R variants of these cells are resistant to imatinib (the well-known Bcr-Abl inhibitor used for CML treatment), although for different and only partially understood reasons. In all cases, we found that the CX inhibitors induce cell death in R cells to a very similar or even higher degree than in S cells. This finding has suggested the usage of CX inhibitors to sensitize these resistant cells to the effect of imatinib, as it was later successfully demonstrated (Borgo *et al.*, 2013). All the results on cell viability of the different lines analyzed are summarized in Table 2, where the DC_{50} (concentration required to induce 50% cell death respect to vehicle-treated cells) are reported. All values have been calculated from the results obtained at 48 h, shown in Figure 9, 10, and 12, except the values reported for CEM cells in the presence of 10% FCS, that are obtained by the 24h-treatment experiment (Figure 11). It is evident that in all cases the DC_{50} values are very similar in the R variant compared to the respective S counterpart. As anticipated, R-CEM, which seem less sensitive than S-CEM when 1% of FCS is

present, respond instead very well in the presence of 10% FCS. The reason of this result is presently unknown (see Discussion).

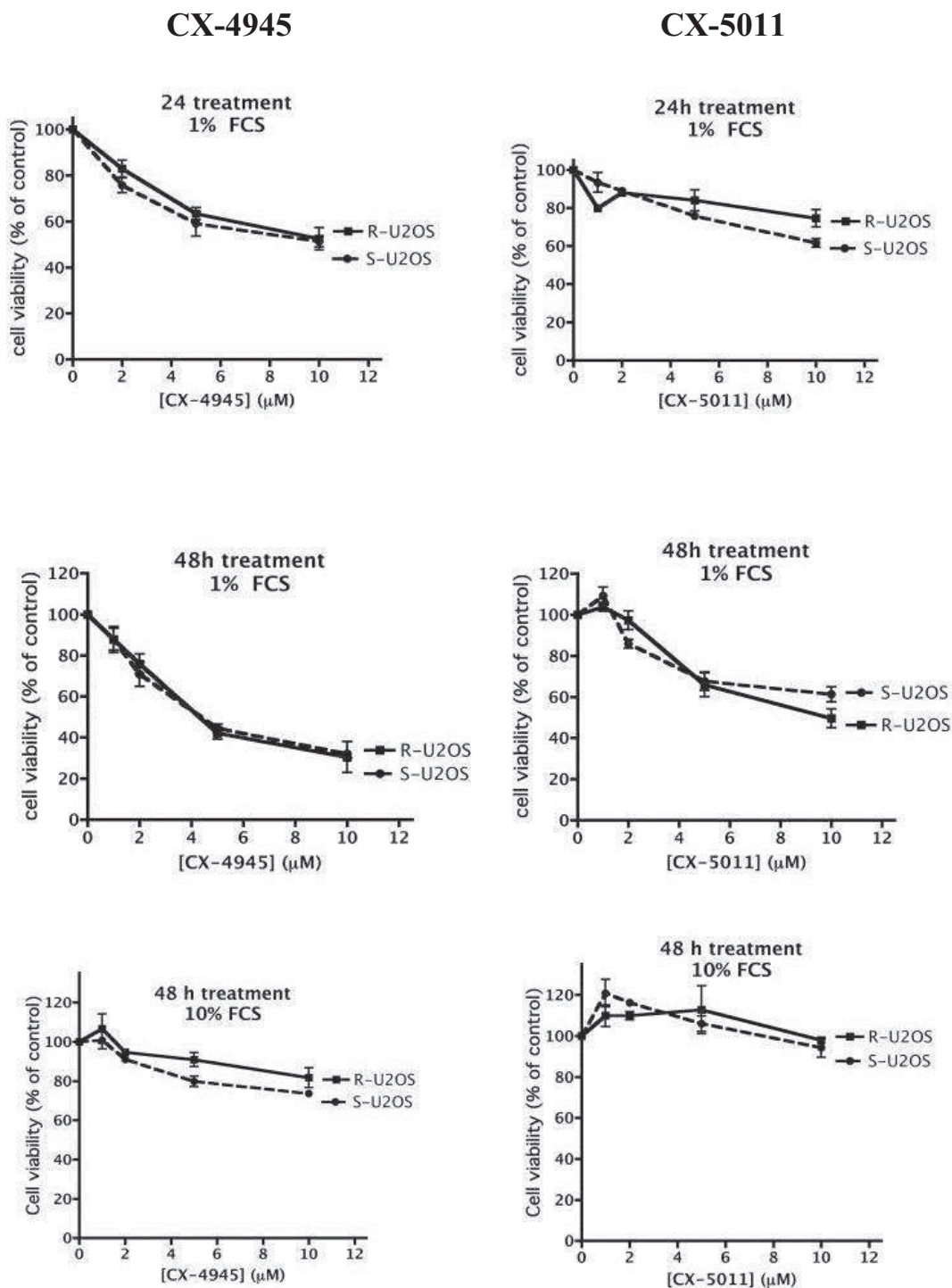


Figure 9. Cell viability of CX-4945 and CX-5011 treated U2OS cells. Cells were incubated with increasing concentrations of the CX compounds; the FCS concentration in the medium and the treatment time are indicated on each graph. Cell viability was assessed by the MTT method, assigning 100% value to the vehicle-treated control cells. Mean $\pm$ SE values of at least four independent experiments are reported (from Zanin et al., 2012).

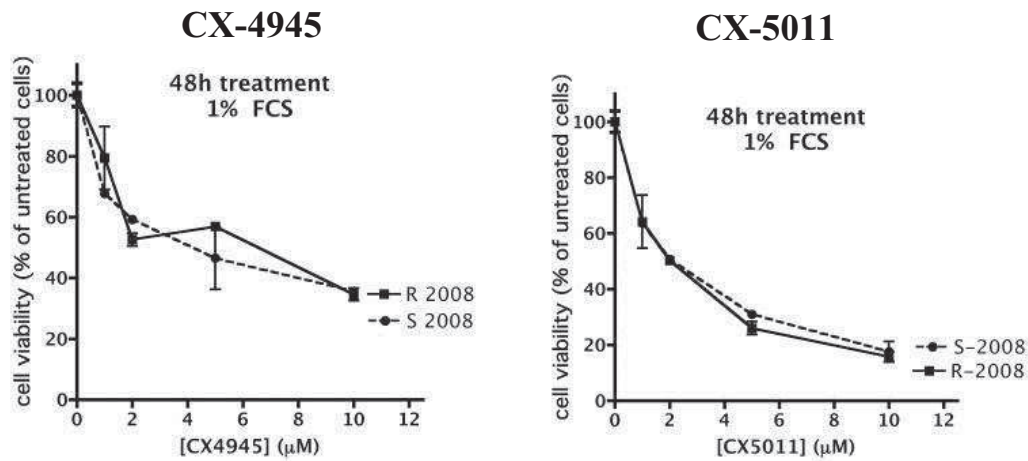


Figure 10. Cell viability of CX-4945 and CX-5011 treated 2008 cells. See legend of Figure 9 for details (from Zanin et al., 2012).

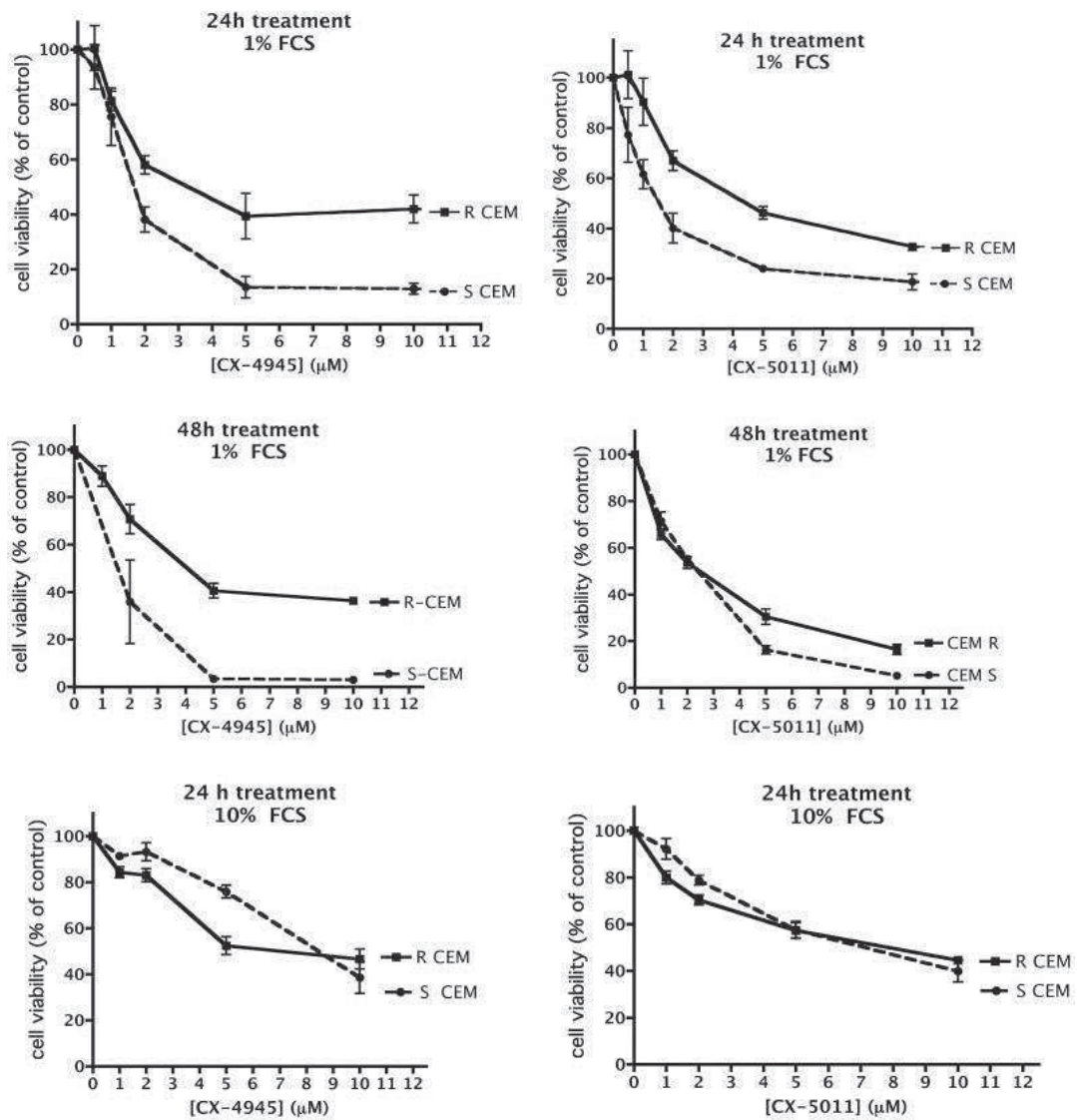


Figure 11. Cell viability of CX-4945 and CX-5011 treated CEM cells. See legend of Figure 9 for details (from Zanin et al., 2012).

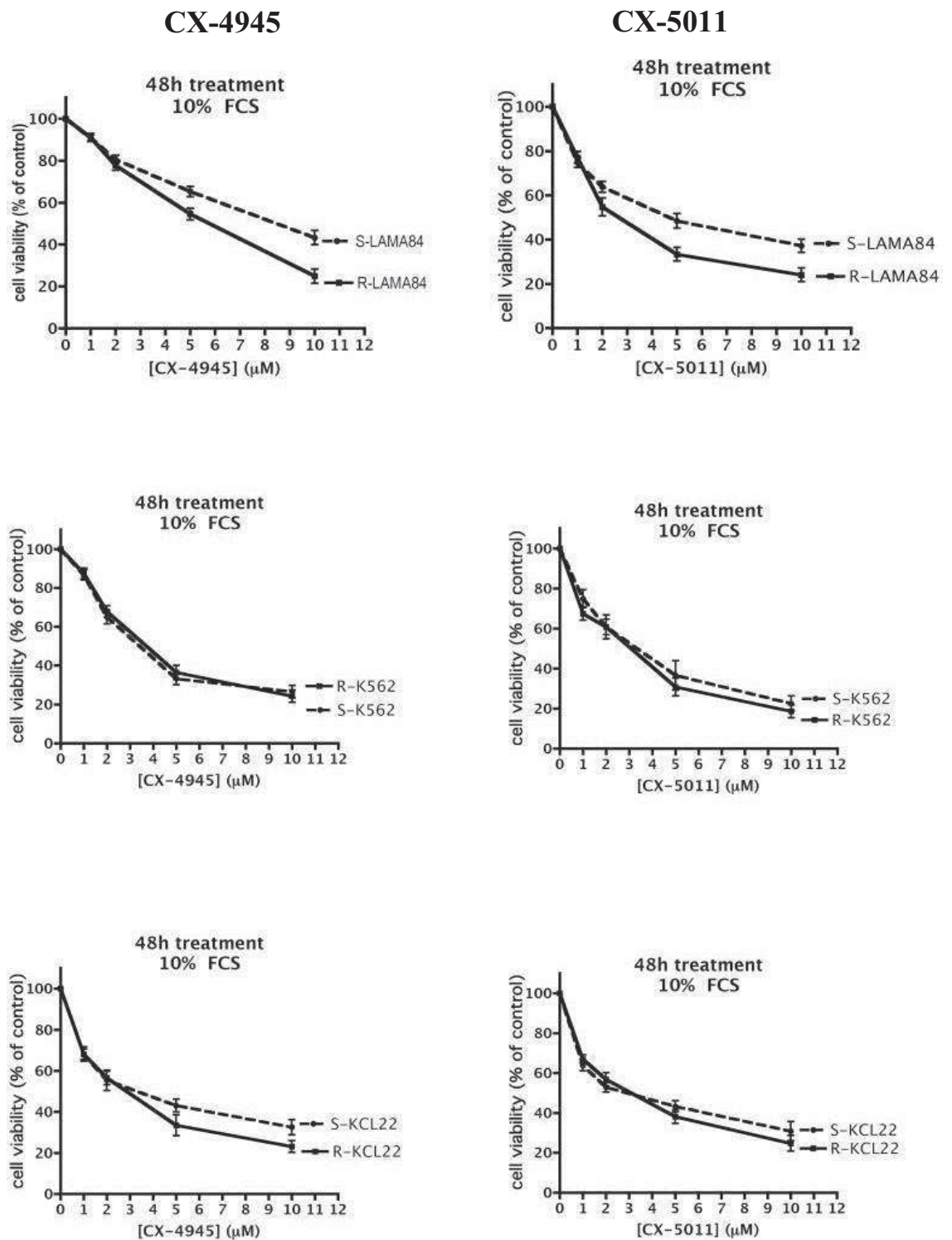


Figure 12. Cell viability of CX-4945 and CX-5011 treated Imatinib-resistant cells. See legend of Figure 9 for details (from Zanin et al., 2012).

	CEM		CEM*		U2OS		2008		LAMA84		K562		KCL22	
	1% FCS		10% FCS		1% FCS		1% FCS		10% FCS		10% FCS		10% FCS	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R
CX-4945	1.92	4.09	8.62	6.50	4.58	4.33	4.87	6.56	8.55	5.79	3.41	3.72	3.30	2.95
(μ M)	± 1.00	± 0.27	± 0.68	± 1.40	± 0.44	± 0.63	± 2.50	± 0.48	± 1.10	± 0.86	± 0.55	± 0.42	± 1.46	± 0.89
CX-5011	2.40	2.51	7.51	7.59	>10	8.59	2.13	2.01	5.07	2.68	3.58	3.05	3.01	3.10
(μ M)	± 0.07	± 0.60	± 2.33	± 1.35		± 0.62	± 0.02	± 0.21	± 1.42	± 0.81	± 1.18	± 0.78	± 1.39	± 0.86

Table 2. DC_{50} of CX-4945 and CX-5011. The values, calculated from 48 h MTT assays reported in Figures 9, 10, 11 and 12 (except for CEM* = 24 h treatment), are the mean of 4–6 independent experiments $\pm$ Standard Deviation. FCS concentration (v/v) during the treatment is also indicated.

APOPTOSIS OCCURS IN CELLS TREATED WITH CX-4945 AND CX-5011

The next step was to check if cell death induced by CX-4945 and CX-5011 in different cell lines was due to apoptosis. To this purpose we used two approaches: the evaluation of nucleosomes formation in the cell cytosol of treated cell (measured by a commercial kit), and the analysis of the caspase-dependent cleavage of PARP (detected by WB of total cell lysates). Figure 13 A shows that in CEM cells apoptosis-dependent release of nucleosome in the cytosol occurs in response to the treatment, in a dose-dependent manner, and with a similar efficacy in S and R cells. The results were confirmed by the finding of PARP cleavage, as shown in Figure 13 B also for U2OS cells, indicating that caspase activation has been caused. Similar results were obtained in all the cell lines analyzed (data not shown).

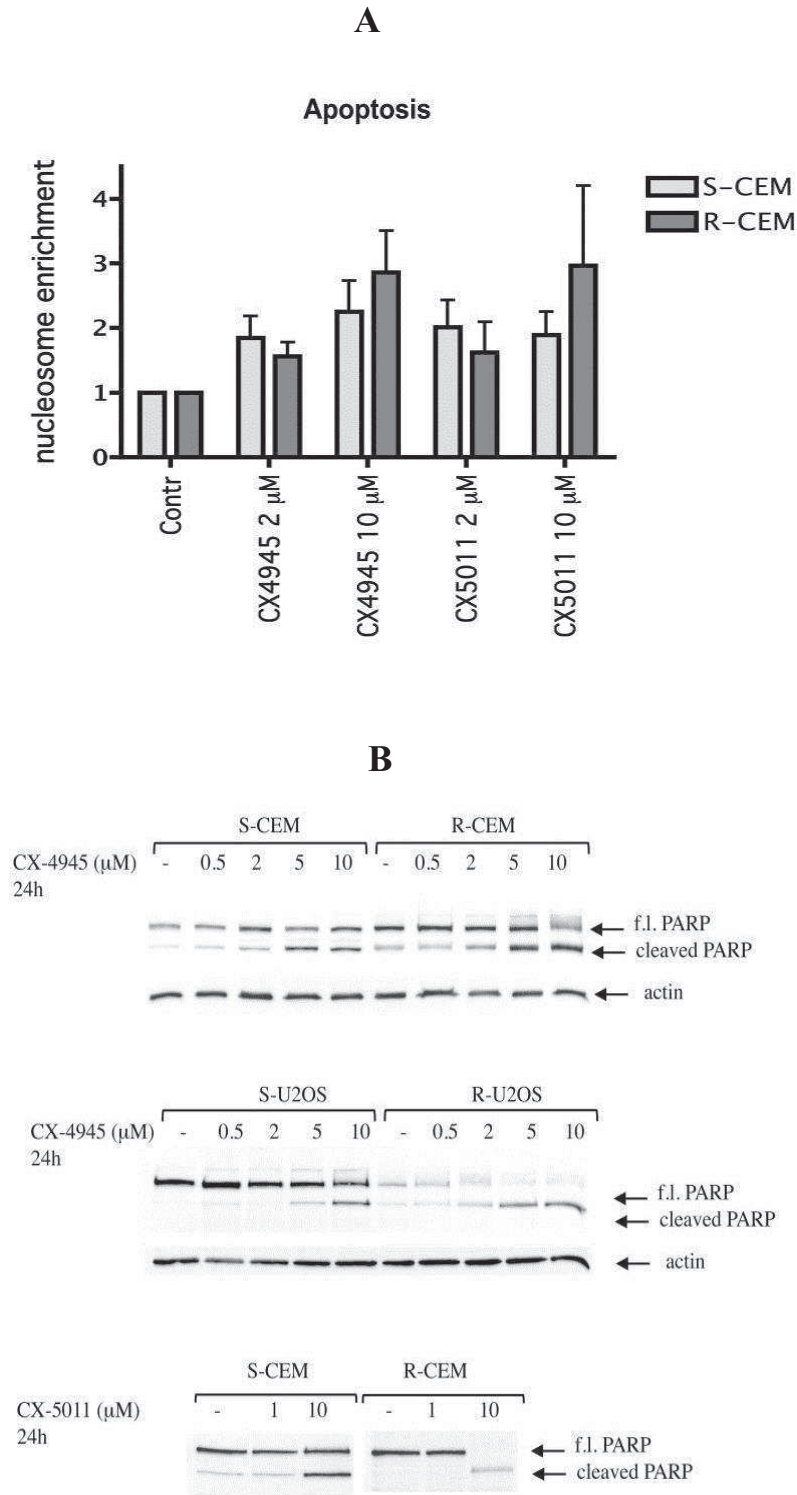


Figure 13. Apoptosis induction by CX-4945 and CX-5011. A. Apoptosis was assessed by evaluation of nucleosomes present in the cytosol, using the Cell Detection Elisa kit (Roche), after 8 h of CEM cell treatment as indicated. Nucleosome enrichment was calculated from the ratio between the signal in treated and untreated cells; reported values are the means $\pm$ SE of four independent experiments. B. Caspase-dependent PARP cleavage was analyzed by WB on 10 μ g proteins of lysate from CEM or U2OS cells treated as indicated. Actin WB was used as loading control. Representatives WB of three independent experiments are shown. f.l. PARP: full length PARP (from Zanin et al., 2012).

EFFECTS ON CEM CELL VIABILITY OF COMBINED TREATMENT WITH VINBLASTINE AND CX-4945

Since we have demonstrated that CX-4945 is not recognized by the P-gp pump, and considering that P-gp has been previously demonstrated to be regulated by CK2 (Glavy *et al.*, 1997; Di Maira *et al.*, 2007), we then tested if CX-4945 is also able to sensitize MDR cells to other conventional chemotherapeutic drugs. We focused on R-CEM cells (a line selected for the resistance to vinblastine (Di Maira *et al.*, 2007)) and performed combined treatments with vinblastine and CX-4945, looking for a synergistic effect in inducing cell death. As shown in Figure 13 the effect of vinblastine is significantly higher if it is administered in combination with CX-4945 even at very low concentration; it can be calculated that DC_{50} for vinblastine changes from 39.4 to 6.2 $\mu\text{g/ml}$. We calculated also the combination index (CI), a parameter which gives information on the possibility that two compounds exert a synergistic, additive or antagonistic effect, assuming values of <1 , $=1$ or >1 , respectively (Giovannetti *et al.*, 2004). We found that CI for CX-4945 plus vinblastine in R-CEM is indeed 0.66 ± 0.04 confirming a synergistic effect. Curiously, a synergistic effect occurs also in S-CEM occurs when Vbl and CX-4945 are administered together, with a $CI = 0.70 \pm 0.08$ (Figure 13, upper panel). The reason of this synergism is not obvious (see Discussion), since S-CEM cells do not express significative level of P-gp (see Figure 4), but in any case confirm that the simultaneous administration of the CK2 inhibitors in combining treatment can be applied to significantly reduce the lower doses of conventional drugs.

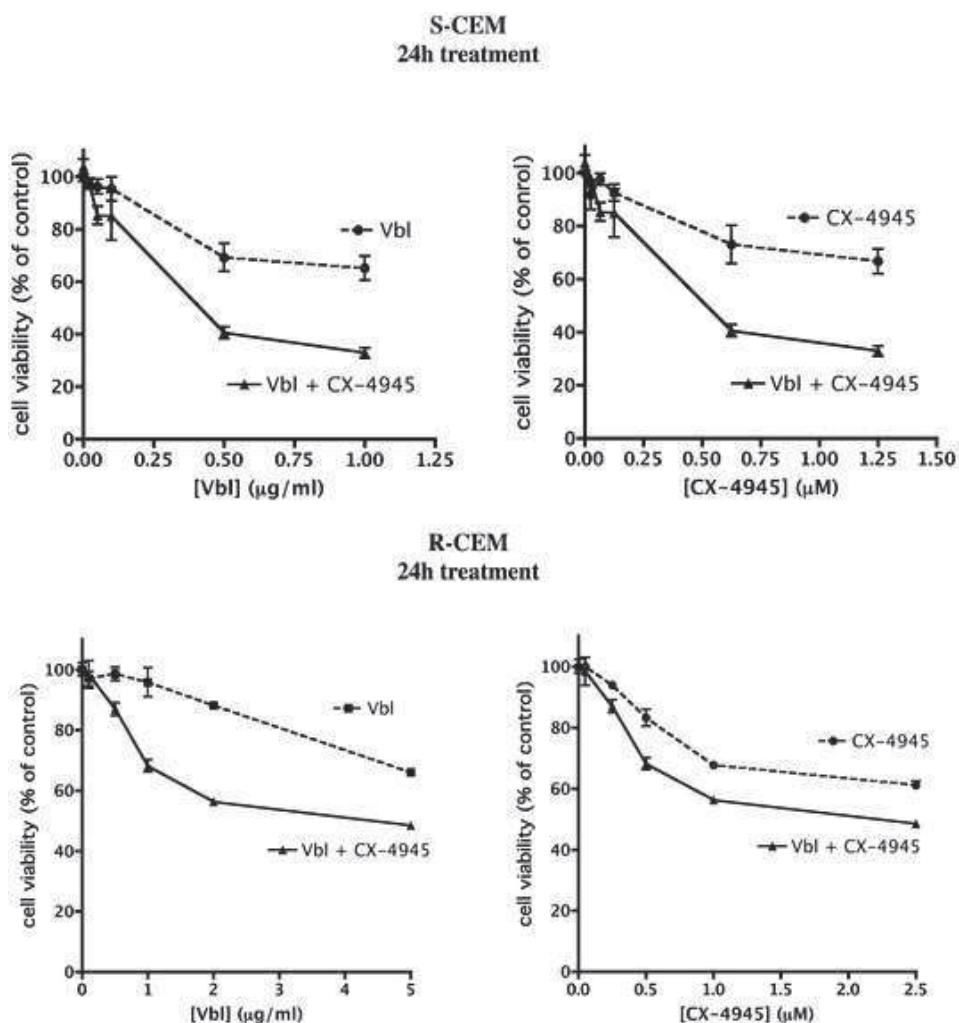


Figure 13. Effect of combined treatment with CX compounds and chemotherapeutic drugs. S-CEM and R-CEM viability was assessed by the MTT method after 24 h treatment with increasing concentrations of both CX-4945 and Vbl, administrated alone or in combination, at fixed ratio (1 μM CX-4945 : 0.8 μg/ml Vbl for S-CEM, 1 μM CX-4945 : 2 μg/ml Vbl for R-CEM, due to their different sensitivity to Vbl). Viability (mean values ± SE of four experiments) was plotted as function of Vbl concentrations (left panels), or CX-4945 concentrations (right panels) (from Zanin et al., 2012).

EFFECTS OF CX-4945 ON THE ACCUMULATION OF CHEMOTHERAPEUTIC DRUGS INSIDE RESISTANT CELLS

The synergistic effect observed in experiments of combined treatment with CX-4945 and vinblastine prompted us to check whether conventional drugs accumulate more abundantly inside resistant cells after CX-4945 treatment. To this purpose we exploited the fluorescence of doxorubicin and measured its uptake in CEM cells pre-treated with CX-4945, compared to untreated cells (Figure 14). We found that CK2 inhibitor markedly increases the amount of drug retained inside R-CEM cells in a dose dependent manner, while in S-CEM this effect is not observed.

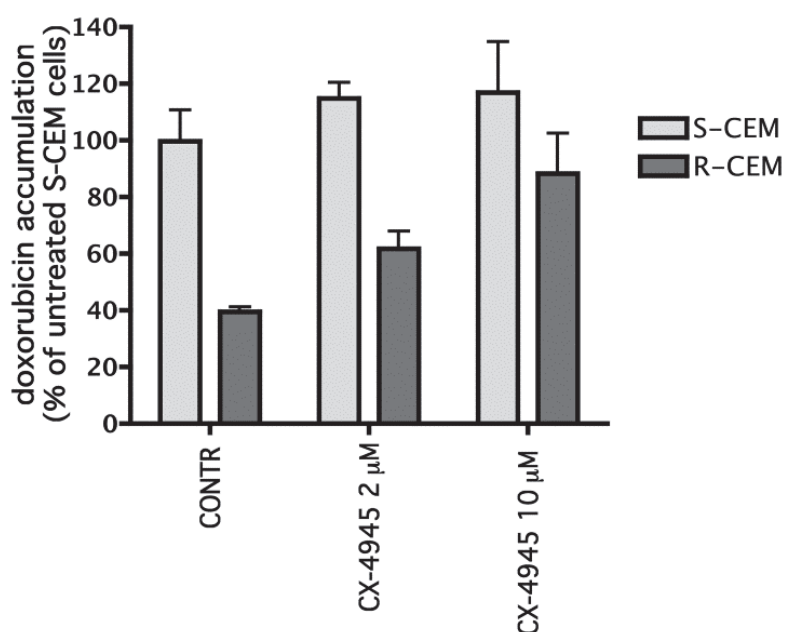


Figure 14. Effect of CX-4945 on doxorubicin accumulation. CEM cells were preincubated for 30 min with vehicle (Contr) or CX-4945 at the indicated concentrations, then further incubated for 30 min with 25 μ M doxorubicin, whose amount, detected fluorimetrically as described in Materials and Methods, is reported as percentage of the value found in control S-CEM (mean $\pm$ SE of three independent experiments) (from Zanin et al., 2012).

EFFECT OF COMBINED TREATMENT WITH CX-4945 AND CX-5011

Curiously, we found a synergistic effect between the two CX-compounds when simultaneously administered. As shown in Figure 15, the effect on cell viability of combining CX-4945 and CX-5011 concentration is higher than that observed with the individual inhibitors in CEM and U2OS cells, both S and R variants. Interestingly, when we performed the same combined treatments with CX compounds in non-tumor cell lines, such as 293T (human embryonic kidney cells) and CCD34LU (human neonatal lung fibroblasts) we did not observed any synergistic effect (Figure 15, right panel). We therefore hypothesized that the synergistic combination is a peculiarity of the cancer cells; conjectures can be done about the possibility of the two compounds have different and complementary side effects, or inhibit different CK2 pool, such as those located in specific subcellular compartments (see Discussion).

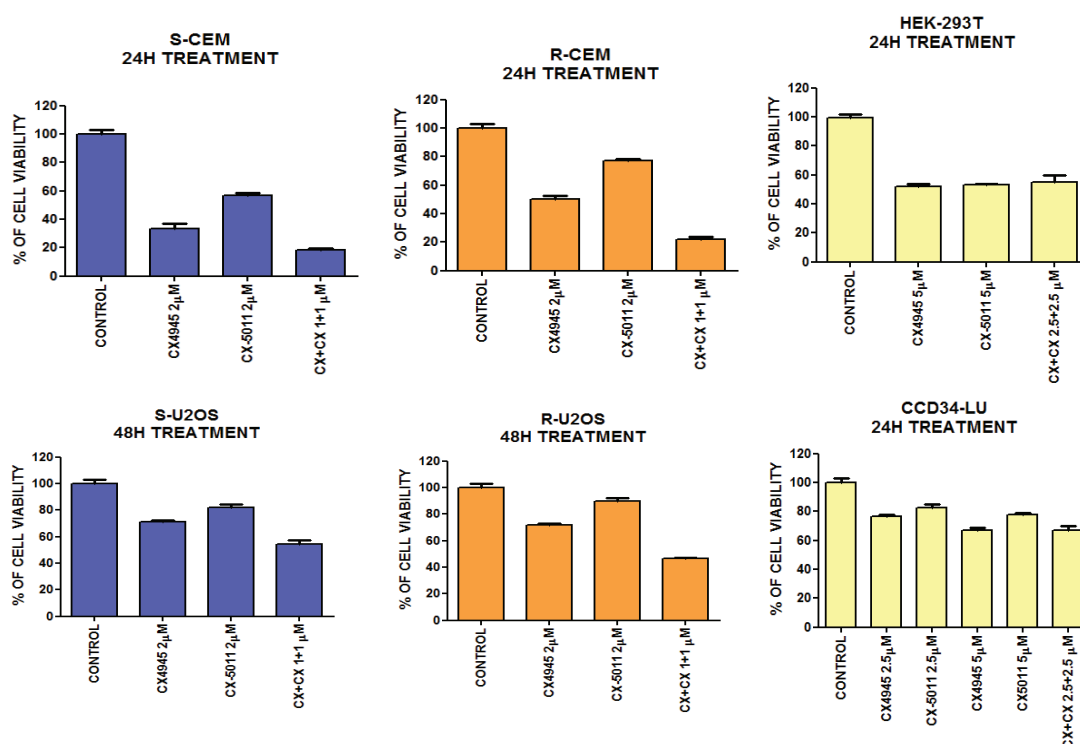


Figure 15. Effect of combined treatment with CX-4945 and CX-5011. Cell viability was assessed by the MTT method after treatment of the indicated cell lines with CX-4945, CX-5011 or both (CX+CX), as indicated (in blue sensitive tumor cells, in orange resistant tumor cells, in yellow non tumor cells). Mean $\pm$ SE values of at least three independent experiments are reported.

DISCUSSION

As widely presented in the general Introduction of this thesis, CK2 overexpression in cancer cells is important in tumorigenesis, and we have previously hypothesized that a high level of CK2 ensure a survival advantage under the pressure represented by treatment with pro-apoptotic drugs (Ruzzene *et al.*, 2011). CK2 is thus expected to play a major role in the apoptosis resistant phenotype, as also suggested by previous studies (Di Maira *et al.*, 2007; Di Maira *et al.*, 2008). Here we focused on CX-4945 (the first ATP-competitive CK2 inhibitor that has entered clinical trials) and on its analog CX-5011 since there were no previous evidences of their efficacy in apoptosis resistant cells.

In this work we demonstrate that the CX inhibitors are able to overcome apoptosis resistance, since they show similar efficacy in resistant cells (R) and their normally sensitive counterparts (S), in a panel of cell lines displaying different mechanism of apoptosis-resistance: R-2008 cells are resistant to cisplatin; R-LAMA84, R-KCL22 and R-K562 are resistant to imatinib, while R-CEM and R-U2OS are MDR cells, expressing the P-gp pump.

The first outcome of our data is that CK2 inhibition by CX compounds has a general effect on resistant cells, by reducing the efficacy of cellular equipment to escape apoptosis. Secondly, we can also conclude that these inhibitors are not recognized by the Pgp, since their effects are detectable in cells expressing this MDR pump.

In the case of CEM cells we observed that CX compounds are slightly less effective in the MDR resistant than in the sensitive lines. However, the differences between the two variants are detectable on cell viability and not on endogenous CK2 inhibition which is very similar in S and R-CEM (see Figure 5). Moreover the difference on cell viability is abrogated when assays are performed in the presence of 10% instead of 1% FCS (Figure 11). Although we do not know the exact reason of the results observed at different concentration of FCS used during treatments, our interpretation is that, at quite critical conditions (such as 1% FCS), the antiapoptotic machinery of R-CEM is more effective than that of S-CEM in protecting cells from the stress represented by CK2 inhibition; on the contrary, if cells are maintained in healthy conditions (10% FCS), the two cell variants are equally equipped to counteract apoptosis. In any case, these findings suggest that any observed

difference is not due to extrusion of the inhibitors by the P-gp, as also confirmed by the results obtained with other Pgp-expressing cells (compare S-U2OS and R-U2OS, Figure 9). As far as U2OS cells is concerned, CX compounds, especially CX-5011, seem to be less effective on this type of cancer cells than with the other cell lines (compare Figure 9 with Figures 10, 11 and 12); at the moment we have no explanations, however they are not related to the MDR phenomenon, being the response very similar in the S and R variants of this cell line.

Another observation from our results is that the CX compounds efficacy is independent of the level of CK2 expression. In fact previous data obtained in our laboratory demonstrated that R-CEM display a higher level of the CK2 catalytic subunit compared to S-CEM (Di Maira et al., 2007) and this is also confirmed by the results shown in Figure 6, where we can appreciate that the phosphorylation state of CK2-dependent sites (particularly of Ser129 Akt) in R-CEM is higher than in S-CEM. Interestingly, despite the different endogenous CK2 activity, the degree of inhibition induced by CX-4945 and CX-5011 is very similar in S- and R-CEM cells (Figure 5).

While our results obviously confirmed that CK2 inhibition leads to apoptosis, an interesting observation emerging from these data is that cell death is appreciable only when the degree of CK2 inhibition induced by the CX compounds is sufficiently high to ensure the dephosphorylation of major substrates. In fact we found that, while the endogenous CK2 activity towards the specific peptide substrate is already reduced more than 50% in cell treated with 0.5 μ M inhibitors (see for example 24 h treatment in CEM cells, Figure 5), significantly higher concentrations are required to induce 50% of cell death (see Figure 10). If we consider the phosphorylation state of endogenous CK2 substrates analyzed by phospho-specific antibodies, we notice that while Ser129 Akt is promptly reduced, Cdc37 Sp13 phosphorylation is much more stable. This observation may be ideally extended to the multitude of CK2 substrates, imaging that each one has its own susceptibility to CK2 inhibition that will mainly depend on the turnover of its phosphorylation state; since this is obviously the result of the balance between kinase and phosphatase activity, there will be variability depending on cell type and conditions. We therefore presume that the dephosphorylation of one or few specific CK2 substrates is required before cell death occurs, or, alternatively, that only when

a massive dephosphorylation of all CK2 substrates occurs, cell death is observed, whose degree, therefore, does not necessarily correlate with the decrease of CK2 catalytic activity.

Another important result from our data has demonstrated that CX-4945 is able to sensitize resistant cells to conventional chemotherapeutic drugs. It has already been reported that CX-4945 augments the anti-tumor efficacy of gemcitabine and cisplatin on ovarian cancers (Siddiqui-Jain *et al.*, 2012). Additionally, CX-4945 appears to enhance the efficacy of inhibitors which disrupt BCR-mediated signaling pathways as well as fludarabine in primary CLL cells (Prins *et al.*, 2013). It is also reported that the combination between CX-4945 and erlotinib (Bliesath *et al.*, 2012), an EGF-receptor inhibitor, produce cell death by reducing PI3K-Akt-mTOR pathway, with the final complete abrogation of Akt phosphorylation. Moreover, a parallel study conducted in our laboratory (Borgo *et al.*, 2013) has disclosed a synergistic effect between imatinib (the first choice drug for CML patients) and CX-4945 in imatinib-resistant cell lines. Our data extend the drug-combination experiments to MDR cells, showing that R-CEM are six fold more sensitive to Vbl when simultaneously treated with sub-lethal doses of CX-4945, compared to cells treated with Vbl alone. Interestingly, we also observed accumulation of doxorubicin in R-CEM (Figure 14), after treatment with CX-4945; this effect is probably due to the positive effect that the blockage of CK2 exerts on P-gp. As expected, the drug accumulation is unaffected by CX-4945 in S-CEM, not expressing P-gp. However, the synergistic effect between CX-4945 and the conventional anticancer drug is instead observable also in S-CEM (Figure 13), suggesting that additional mechanisms of increased cell death, other than the effect on the P-gp pump, are induced by the combined treatment.

Surprisingly we found a synergistic effect between CX-4945 and CX-5011; this was observed when the two compounds were simultaneously administered to both S and R cells, but was not evident in non-tumor cells (Figure 15). At the moment we have no explanation for these results but different speculations can be done. The first obvious hypothesis is that, despite their high specificity (Battistutta *et al.*, 2011), the two compounds hit different and complementary kinases, beside CK2. If these alternative targets are typical and/or expressed only in cancer cells, this would account for the tumor specific effect that we observed. Another possibility is that the two CX compounds, due to their differences in structure, are able to reach

different subcellular localization; in this case, the combined treatment would target additional pools of CK2, for a more complete inhibition than in the case of individual administration. However, preliminary experiments of cell fractionation have shown that CK2 localizes in different cell compartments, is similarly inhibited by CX-4945 and CX-5011 (not shown), although more experiments will be required to completely exclude this hypothesis. At present, we can only suggest that, independently of its reason, the combined treatment with CX-4945 and CX-5011 is an additional strategy that can be considered.

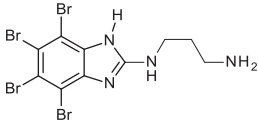
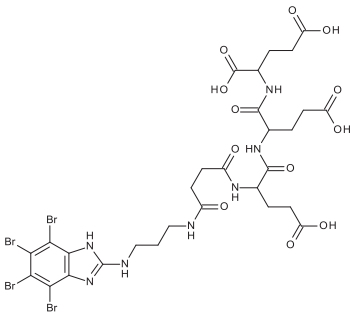
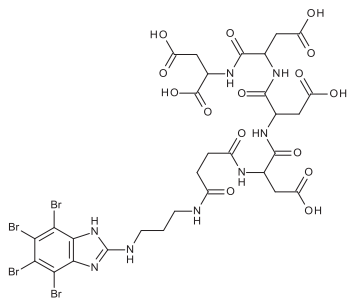
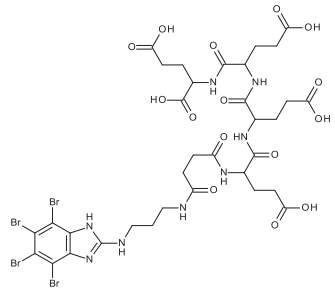
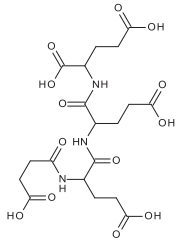
In summary, our results show that CX-4945 and CX-5011 are internalized in resistant cells, inhibit endogenous CK2, and, alone or in combination with other drugs, induce significant cell death, suggesting that they can be considered as a valid therapeutic strategy also in case of pharmacological resistance.

**EFFECTS OF
CK2 BI-FUNCTIONAL
INHIBITORS
IN TUMOR CELLS**

INTRODUCTION

In order to obtain CK2 inhibitors as more specific as possible, it was decided to *in silico* develop (Dr. G. Cozza) a new class of compounds capable to inhibit CK2 by simultaneously bind to the ATP and peptide substrate sites. For this purpose, suitable modifications have been introduced on an ATP-competitive molecule. The chosen inhibitor was K137 (chemical *formula* in Table 1), whose structure is very similar to that of TBB, presenting, however a benzimidazole ring instead of TBB benzotriazole scaffold; the carbon atom at position 2 of K137 benzimidazole is functionalized with a diamino propyl chain, whose N-terminal is suitable for a peptide derivatization. K137, indeed, was derivatized by linking its amino terminal group to a short peptide chain composed by three or four aspartic/glutamic acids (Prof. G. Zagotto, Padova). Since CK2 is an acidophilic kinase, recognizing serine or threonine surrounded by acidic residues, the compounds designed should be capable to bind both CK2 substrate site thorough their acidic peptide sequence, and the ATP site through their tetrabromo-benzimidazole portion. They are therefore called “bi-functional” or “bi-substrate” inhibitors.

The compounds obtained are listed in Table 1. The *in vitro* analysis on recombinant CK2, demonstrates that the molecular design of bifunctional inhibitor was successful, being the introduction of the acidic chain very effective in reducing the IC_{50} for CK2, compared to K137 (Table 2, Dr. G. Cozza, University of Padova, personal communication). The most effective compound is K137-E4, which presents a tail of four glutamic acid residues. The inhibition curve of recombinant CK2, assayed towards a synthetic peptide substrate, is reported in Figure 1 in comparison with the parental compound K137: it is evident that K137-E4 ($IC_{50}=0.025\mu M$) is about five-fold more efficient in inhibiting CK2 than K137 ($IC_{50}=0.13\mu M$). Two other compounds listed in Table 1, denoted as K137-D4 and K137-E3, are able to inhibit CK2 with an efficacy higher than that of K137. Interestingly, the oligo-glutamic or aspartic moieties alone, lacking of the ATP-mimetic part, are inefficient in inhibiting CK2 up to $40\mu M$, denoting a dramatic synergism between the two components of the bi-functional compounds (see Table 1).

Compound	Formula	IC ₅₀ for recombinant CK2 (μM)
K137		0.13
K137-D3		0.033
K137-D4		0.035
K137-E4		0.025
AS-D3		>40

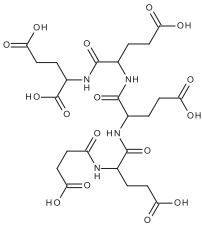
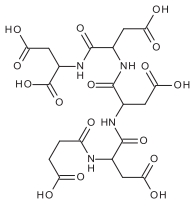
AS-E4		>40
AS-D4		>40

Table 1. Chemical structure, name and IC_{50} of K137-E4 and other potential bifunctional CK2 inhibitors (from Dr. G. Cozza).

Once established that the bi-functional inhibitors are able of potently inhibiting CK2, the selectivity of the most promising one, K137-E4, was evaluated, by testing its effect at 1 μ M concentration on a panel of 140 kinases. The results obtained (not shown) suggest that K137-E4 is a very selective inhibitor of CK2, with the activity of most other kinases left unchanged; only one kinase, ERK8 was inhibited more than 70%. Interestingly, K137-E4 was found ineffective also towards those kinases which are inhibited by K137 such as HIPK2 and PIM kinase (Dr. G. Cozza, personal communication) (Table 2).

Compound	CK2	PLK2	CK1 δ	HIPK2	PIM1	Aurora-A
K137	0.13	39	5.7	0.34	0.25	>40
K137-D3	0.033	2.34	34	>40	>40	>40
K137-D4	0.035	3.76	21	>40	>40	>40
K137-E4	0.025	0.400	16	>40	>40	>40
AS-D3	>40	>40	>40	>40	>40	>40
AS-E4	>40	>40	>40	>40	>40	>40
AS-D4	>40	>40	>40	>40	>40	>40

Table 2. IC_{50} (μ M) values of K137, bi-functional inhibitors and oligo-glutamyl or aspartyl moieties towards CK2 and other kinases (from Dr. G. Cozza).

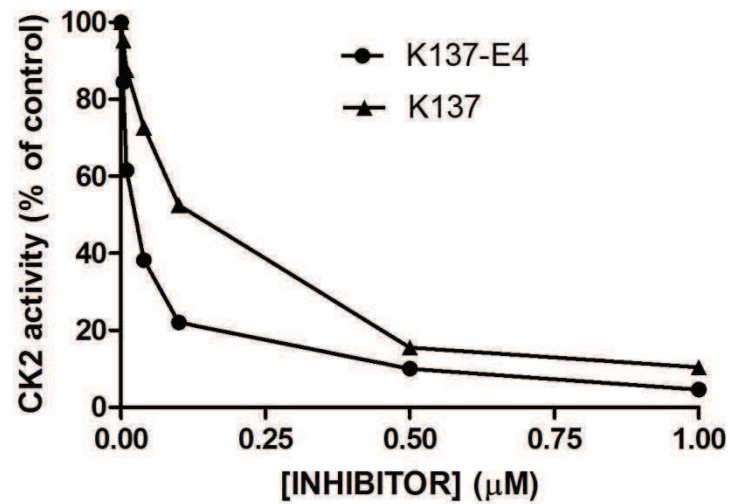


Figure 1. Inhibition curve of K137 and K137-E4 on the activity of recombinant CK2 holoenzyme measured against a CK2-specific synthetic peptide (from Dr. G. Cozza). Values $\pm$ SEM of three independent experiments are reported.

In summary, this new approach to develop CK2 specific inhibitors resulted successful as judged from *in vitro* studies. However, no data were available for their employment in cells.

AIM OF THE STUDY

In this work we focused on a the CK2 inhibitor K137-E4 belonging to the new class of CK2 inhibitors of the so called bi-functional inhibitors, able to bind to both the phosphate donor (ATP) site and the phosphate acceptor (protein) site of CK2. The compound was designed and developed in our laboratory with the final goal to obtain a highly potent and specific inhibitor of CK2. K137-E4 was indeed found to meet the expectations, but its inhibitory activity was assayed only toward recombinant CK2, using synthetic peptides as substrates. Therefore, the first aim of this work was to check whether K137-E4 is also able to inhibit native CK2 (not only the enzyme purified as a recombinant protein) and to reduce the phosphorylation state of cellular substrates, besides model peptides. Secondly, we wanted to verify if K137-E4 is cell-permeable and produces the effects expected when endocellular CK2 is blocked, apoptosis in particular. In case K137-E4 is not able to cross plasma membrane, an additional aim of this work is the investigation on its possible employment to CK2 ectokinase (CK2 present at the external side of the cell) and on the cellular effects of this inhibition.

RESULTS

EFFECTS OF K137-E4 ON THE ACTIVITY OF NATIVE CK2

Since K137-E4 has been shown to be a potent inhibitor of recombinant CK2, we started to check if it has the same efficacy also toward cellular CK2, evaluating its activity on its cellular substrates. For this purpose, we exploited cellular lysates as source of endogenous CK2 and substrates, and we performed *in vitro* phosphorylation assays in the presence of increasing concentrations of K137-E4. For the analysis, we used also the canonical ATP-competitive CK2 inhibitors CX-4945 and K137; this compounds had been added as a control, in order to distinguish the CK2-dependent protein bands from all the phosphorylated proteins that can be separated by SDS-PAGE/blot and detected by autoradiography. As shown in Figure 2 K137-E4 induces a marked decrease in the phosphorylation state of a large number of protein bands, in a dose-dependent manner, and particularly above all at the highest concentration used (5 μ M). The same bands are also affected by other CK2 inhibitors and can be therefore considered as CK2 substrates; they are highlighted by arrows in Figure 2. Interestingly, in accordance with the higher inhibition capacity of K137-E4 compared to K137 towards recombinant CK2, we also found a stronger effect of K137-E4 on endogenous CK2 as judged by the more marked reduction of band phosphorylation when K137-E4 is added (for example compared lane 4 and 7 of Figure 2 where 1 μ M K137-E4 and K137 were present, respectively).

It can be concluded that the inhibition properties of K137-E4 observed *in vitro* toward recombinant CK2 can be extended to cellular CK2. However, it can be noted that the effective concentrations are higher in assays exploiting cellular extracts. As it will be discussed, this is expectable, due to the sequestration of the compounds by the huge amount of cellular components cellular components present in these kinds of assays.

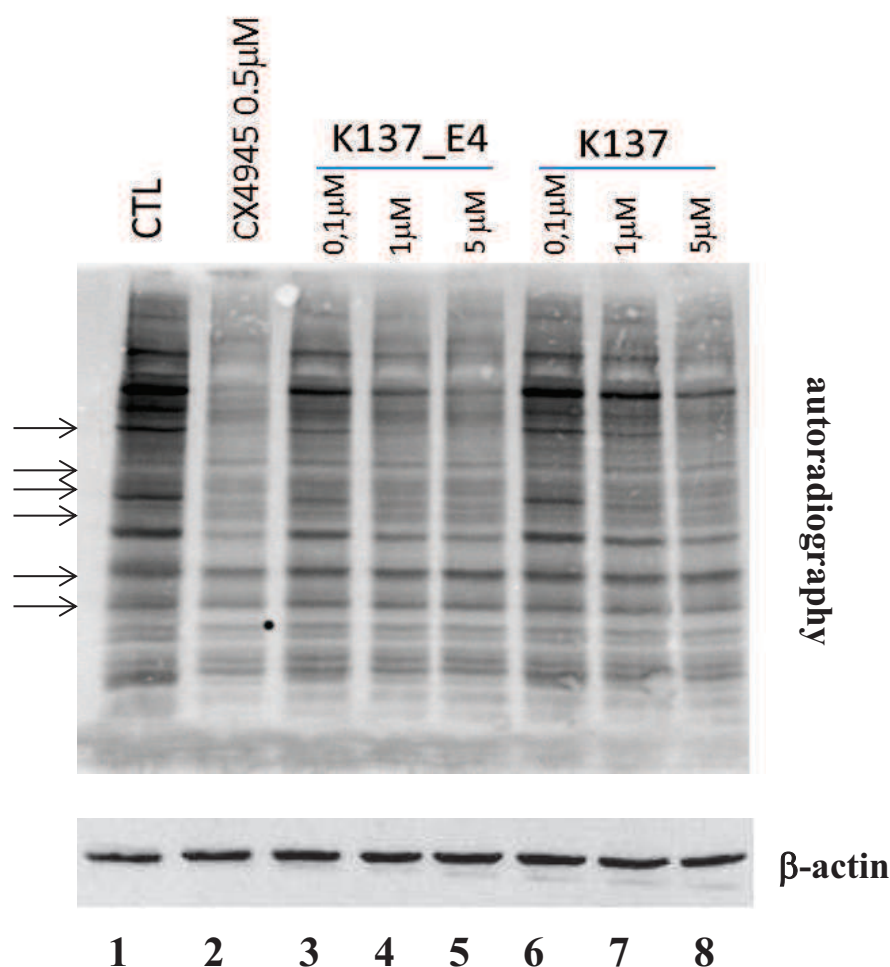


Figure 2. Effects of K137-E4 on the CK2-dependent protein phosphorylation in cell lysates. 5 μ g of proteins from total lysate (HepG2 cells), were incubated with a radioactive phosphorylation mixture (see “Methods” for details) and CX-4945, K137-E4 and K137 as indicated. Proteins were separated by SDS-PAGE, blotted and analyzed by autoradiograph. CTL=control (cells where no inhibitor was added). WB towards β -actin was used as loading control. Arrows indicate protein bands whose phosphorylation is reduced by the well-known CK2 inhibitors CX-4945 and K137. Three different experiments were performed and a representative result is shown.

EFFECTS OF K137-E4 ON CELL VIABILITY

The next step of our work was to analyze the effects of K137-E4 on cell viability, as an indicator of its ability to inhibit CK2 inside the cell. For this purpose we performed MTT assay to verify if K137-E4 provokes cell death when administered in intact cells. We treated Jurkat cells (human leukemia T lymphocytes) for 24 h with increasing concentrations of K137-E4 and in parallel with the canonical CK2 inhibitors, CX4945 and K137, as controls of cell death induced by CK2 inhibitors. As reported from the Figure 3, while CX4945 and K137 provoke a 50% of cell death at low concentrations

(lower than 5 μM), K137-E4 does not induce appreciable effects on cell viability. This result is the first indication that K137-E4 is not permeable to the plasma membrane and is not able to reach endocellular CK2.

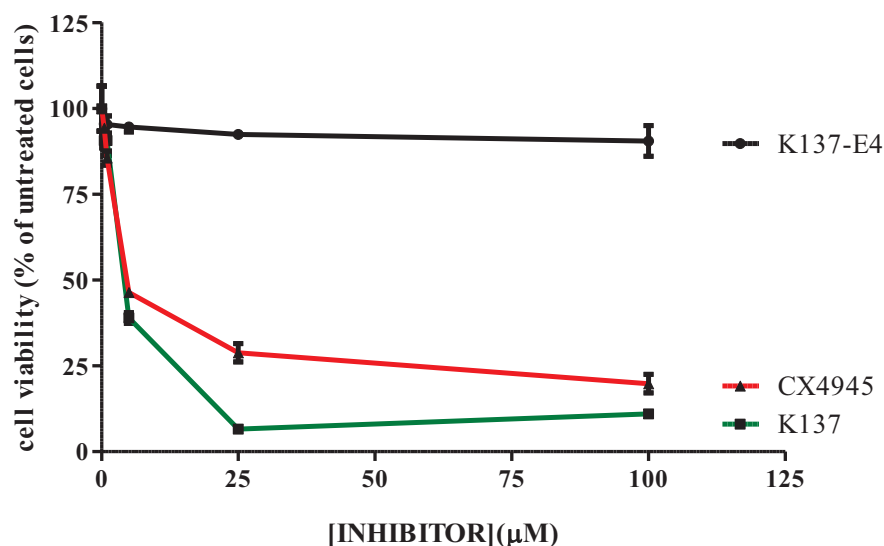


Figure 3. Cell viability of Jurkat cells treated with K137-E4, K137 and CX-4945. Cells were incubated for 24 h with increasing concentrations of K137-E4, K137 and CX-4945. Cell viability was assessed by the MTT method, and is reported as % of viable cells compared to the vehicle-treated cells. Values $\pm$ SEM of three independent experiments are reported.

To confirm this result, we performed WB analysis of total cell lysates from treated cells, and we analyzed the cleavage of PARP protein, known to occur in apoptotic cells. Figure 4 shows that, differently to CX-4945, which induces apoptosis (for a detailed description of CX-4945 cellular effects see Chapter 1 of Results), K137-E4 even at very high concentration has no effect on PARP protein.

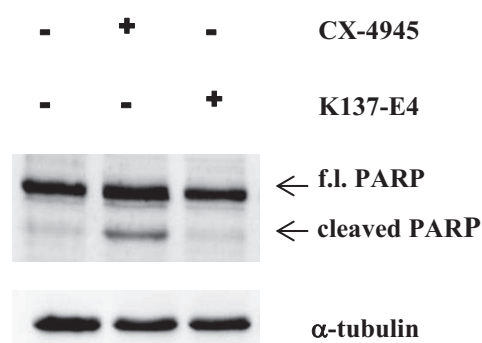


Figure 4. Analysis of apoptotic effects in Jurkat cells treated with K137-E4 or CX-4945. Apoptosis was assessed by PARP cleavage analyzed by WB on 10 μg lysate proteins from Jurkat cells treated with K137-E4 100 μM for 24 h. Treatments with CX-4945 2 μM was performed as control of cell permeable CK2 inhibitor. α -tubulin was used as loading control. Representatives WB of three independent experiments are shown. f.l. PARP: full length PARP.

EFFECTS OF K137-E4 ON CK2 ENDOCELLULAR ACTIVITY

To be completely sure that the absence of effect of K137-E4 on cell viability was due to its incapacity to reach endocellular CK2, we analyzed the phosphorylation state of the CK2 intracellular substrates by WB of total lysates. In particular, we checked Sp129 of Akt (Di Maira *et al.*, 2007) and Sp125 of B23 (Louvret *et al.*, 2006) (not shown) and we found that their phosphorylation was not prevented by cell treatment with K137-E4 (as shown by CX-4945 treatments) (Figure 5). Similarly CK2 endogenous activity from cell lysates, measured towards a CK2 specific peptide, is not altered (not shown).

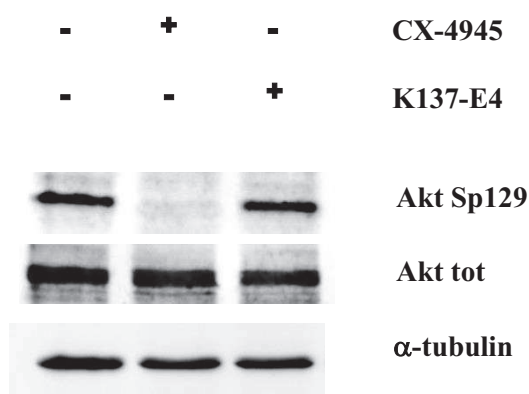


Figure 5. CK2 activity in K137-E4 or CX-4945-treated cells. 10 μ g of total proteins from Jurkat cells treated with CX-4945 2 μ M or K137-E4 100 μ M for 24 h were analyzed with the indicated antibodies; α -tubulin was used as loading control. Representative WB of three independent experiments are shown.

EFFECTS OF A K137-E4 MODIFIED COMPOUND ON CELL VIABILITY

Once we demonstrated that K137-E4 is not cell permeable, we thought that a chemical modification able to increase its hydrophobicity could enhance its capability to permeate through the plasma membrane. Our collaborating chemical synthesis group (Prof. G. Zagotto, Padova) produced a compound corresponding to K137-E4 with all carboxylic groups esterified by a methyl group; this compound was denoted as K137-E4Me, whose structure is illustrated in Figure 6.

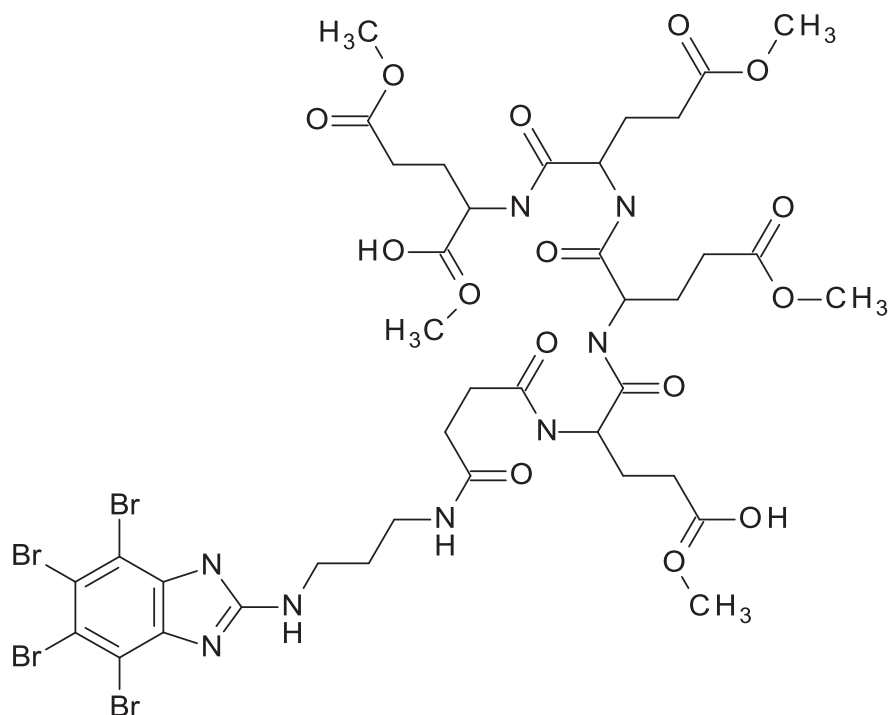


Figure 6. Chemical structure of K137-E4Me (from Dr.G.Cozza).

We performed cell treatments in Jurkat cells with increasing concentration of the compound for 24 h and 48 h and cell viability was tested with the MTT method (Figure 7). K137-E4Me used at a concentration of 100 μ M, leads to about 40% of cell death in 24 h and little more (50%) in 48 h. Moreover K137-E4Me has a significantly lower efficacy than K137 and CX-4945 (Figure 7). This could be due to a still very modest cell permeability, despite the chemical modification, or to a loss in efficacy towards CK2. To discriminate between the two hypothesis, *in vitro* kinase assays were performed with recombinant CK2 and a peptide substrate which showed that K137-E4Me IC_{50} is 40-fold higher than that of K137-E4 (Dr. G. Cozza, personal communication). We therefore concluded that a different strategy needs to be applied, since the charged groups are essential for the binding to the CK2 substrate binding site (see Discussion).

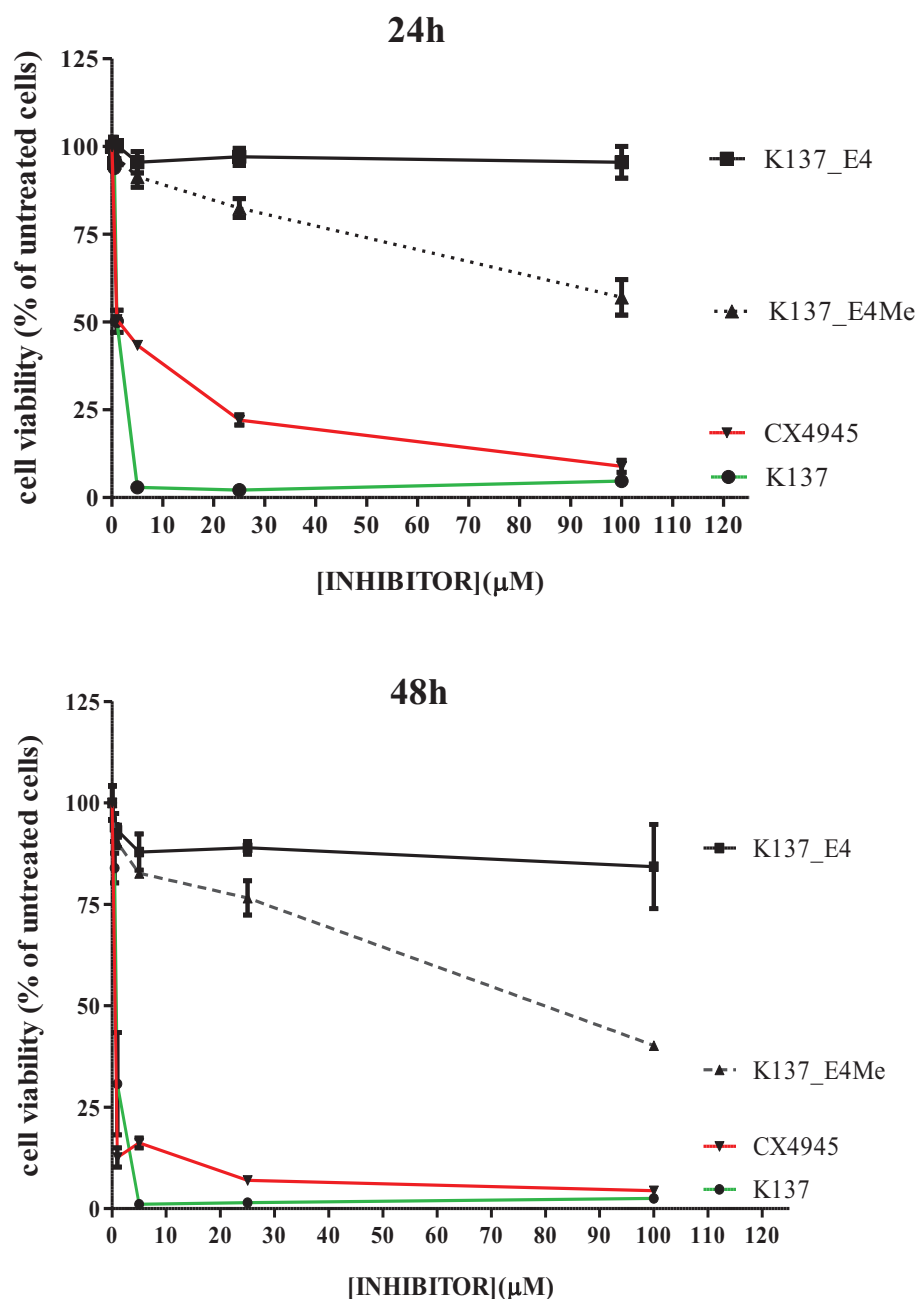


Figure 7. Effects of K137-E4Me on cell viability. Jurkat cells were incubated for 24 h (upper panel) or 48 h (lower panel) with increasing concentrations of K137-E4Me, K137-E4, K137 and CX-4945. Cell viability was assessed by the MTT method, and is reported as % of viable cells compared to the vehicle-treated cells. Values $\pm$ SEM of three independent experiments are reported.

EFFECTS OF K137-E4 ON CK2 ECTOKINASE

As described above, K137-E4 needs to be optimized for its usage in cells. However, actually, it could be useful for studies concerning CK2 ectokinase activity. In fact, CK2 can be exposed outside the plasma membrane of the cells (as described in the general

Introduction), even if its roles are only partially known. Despite other cell permeable inhibitors that could block both CK2 ectokinase activity and endocellular CK2 activity K137-E4 is a very specific and powerful tool that can be exploited to selectively inhibit only CK2 ectokinase and analyze the consequent effects.

Firstly, we analyzed the correct conditions to measure CK2 ectokinase activity *in cell*, performing phosphorylation kinase assays in several cell lines. We exploited non tumor cell (HEK-293T), and tumor cell (Jurkat and HeLa) lines. We measured CK2 ectokinase toward a CK2 specific peptide substrate at different times. The activity was measured on the cellular pellet and in the supernatant medium, after cell centrifugation. This was necessary to assess if contamination occurred from endocellular CK2 due to cell lysis. As reported in Figure 8, we found the conditions in which was detectable in intact cells and was instead very low in cell supernatant. Moreover, CK2 ectokinase activity was essentially due to the peptide phosphorylation (being the blanks without peptide very low), and it was roughly linear respect to the incubation times (Figure 8). We had therefore correct conditions to study the possible inhibition of CK2 ectokinase by K137-E4, and its effect.

The assays were initially performed on HeLa and HEK-293T, which both grow in adhesion on cell plate. Data obtained from these cell lines have shown a poor inhibitory effect of K137-E4 towards CK2 ectokinase (not shown). However, when the assays were repeated on HEK-293T keeping them in suspension, K137-E4 was found to exert a good inhibitory effect on the CK2, (Figure 9). The same study was then performed in Jurkat cells, which grow in suspension, and also in this case K137-E4 effectively inhibits CK2 ectokinase (Figure 9). We do not know the exact reason for the lack of effect of K137-E4 on adherent cells, although a reduced accessibility of the inhibitor can be speculated (see Discussion). However, from this studies performed on cells in suspension, we can concluded that K137-E4 inhibits CK2 ectokinase activity.

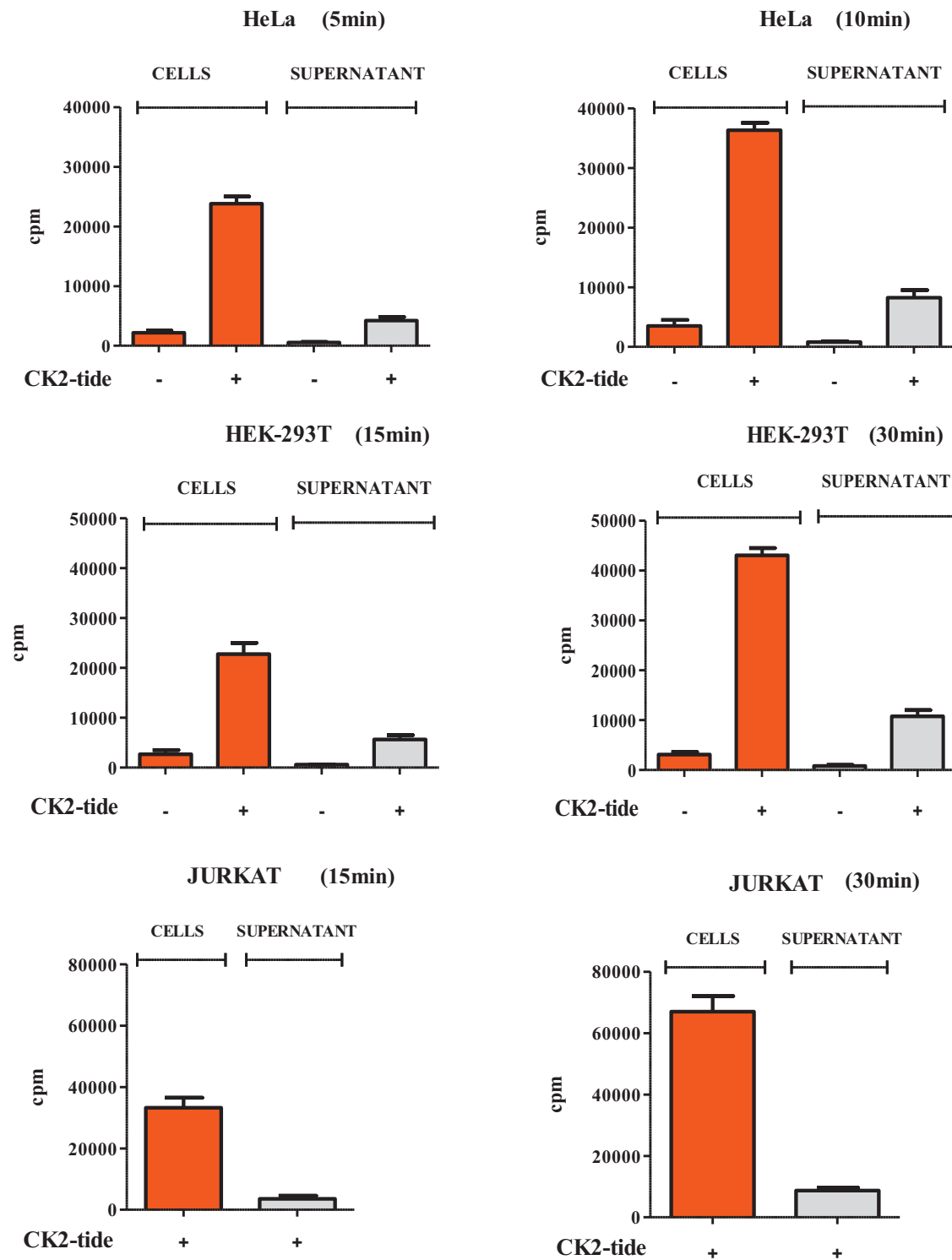


Figure 8. CK2 ectokinase activity in different cell lines. CK2 activity was measure in the presence (+) or in the absence (-) of the synthetic peptide CK2-tide (1mM) for the indicated times. Intact cells (as indicated), in isotonic buffer or supernatant, after 1000g cell centrifugation, were assayed. Cell preparation and conditions are detailed in the Methods section. Values $\pm$ SEM of four independents experiments are reported.

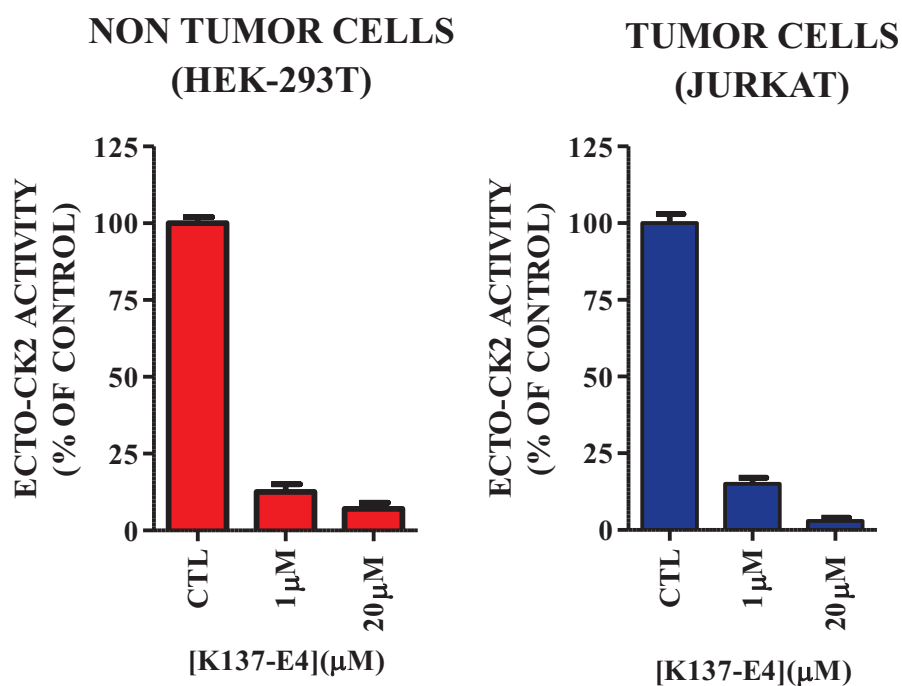


Figure 9. Effects of K137-E4 on CK2 ectokinase activity. CK2 activity towards the synthetic peptide CK-tide (1mM) was measured in intact HEK-293T (left) and Jurkat (right) cells for 30 min at 37°C, under conditions described in the Methods section. K137-E4 was added at the indicated concentrations before the adding of the radioactive mixture. CTL=control (cells incubated without the addition of K137-E4). Values $\pm$ SEM of three independent experiments are reported.

DISCUSSION AND PERSPECTIVES

In this study, we evaluated the possibility to use the CK2 inhibitor K137-E4 to inhibit cellular CK2. This compound, belonging to the new class of bi-functional inhibitors of CK2, had been tested only towards recombinant CK2. Here we show that it is able to block also the activity of native CK2, measured in cell lysate towards endogenous protein substrates (Figure 2). A precise IC_{50} of its efficacy in these conditions has not been determined, however, from our results we can suppose that it is significantly higher than that found with recombinant CK2 ($0.025\mu M$). In fact, in Figure 2 it is evident that the phosphorylation of most CK2-dependent bands is significantly reduced only by $1\mu M$ K137-E4. However, this is somehow expectable and often observed, since in this kind of assay a lot of lipophilic molecules and cellular structures are present which can trap/mask the inhibitor and reduce its effective concentration. Indeed, we can also do the same considerations for the canonical ATP-competitive inhibitor K137, whose effect in the experiment of Figure 2 is lower than that expected from its IC_{50} for recombinant CK2. We can therefore conclude that the efficacy of K137-E4 is higher than that of K137 both towards recombinant and native CK2.

The approach described above exploited cellular CK2 and relative substrates, but it was performed *in vitro*, by adding K137-E4 during the phosphorylation assay. On the contrary, when we tried to perform treatments of intact cells with K137-E4, we did not observe any appreciable effect: both cell viability and CK2 activity were unchanged (Figure 3, 4 and 5), allowing us to suppose that the compound is unable to cross plasma membrane. This is not surprising, considering that the chain of four Glu present in K137-E4 confers a negative charge and a partial hydrophilic nature to the molecule. For this reason, we tried to improve its hydrophobicity by methylating all the carboxyl groups. The resulting compound, K137-E4Me, produced modest effects in cells (Figure 7), most probably due to the loss of efficacy towards CK2 (not shown). Indeed, the structure of K137-E4 had been modeled to optimally fit to the peptide-binding site of CK2, which recognizes acidic residues in the vicinity of the target aminoacid. Therefore, a compound completely devoid of negative charges is unlikely to have high affinity for CK2. On this basis, future work will be necessary, to produce molecules still effective on CK2 but less charged than K137-E4. A possibility that we considered is a partial, instead of total, esterification of carboxyl group. Alternatively, different

techniques for cell delivery can be experimented, such as the conjugation of K137-E4 to the cell-penetrating HIV-derived Tat peptide (Holm *et al.*, 2006). In any case, the final goal will be a cell-permeable molecule which inhibits CK2 not only powerfully but also with high selectivity, the first reason for which these kind of inhibitors is under investigation.

At present, we have demonstrated that K137-E4 can be used as a not cell permeable inhibitor for studies related to CK2 ectokinase. In fact, this form of CK2 has been known for several years (Kubler *et al.*, 1989); a few studies have been reported, and some of its substrates have been identified as well (Stepanova *et al.*, 2002; Bohana-Kashtan *et al.*, 2005), but a precise role for a CK2 activity at the external side of the cell has not been determined yet. We reasoned that the employment of a classical inhibitor risks to fail in giving information, being able to enter cells and block also endocellular CK2 activity; in turn, the total absence of K137-E4 activity inside the cells can be particularly useful. We first found out the conditions for a reliable measurement of ecto-CK2 activity in intact cells, then we determined that K137-E4 is able to significantly inhibit it, with more than 80% inhibition already at 1 μ M (Figure 9). Surprisingly, we found that the inhibitory efficacy is much more evident if cells are kept in suspension, instead of adherent to the plates (not shown). We speculated that the surface reachable by the compound is probably higher in “round” suspended cells. We also tried to compared the efficacy of canonical ATP-competitive inhibitors in adhesion and in suspension cell cultures; however, their effects seem quite similar in the two conditions (not shown), suggesting that their entrance in cells makes negligible the availability of access surface. Future studies will be required to better understand this point.

From our ecto-CK2 studies, we have some preliminary but interesting results; in particular, since we found that K137-E4 effectively inhibits ecto-CK2 (Figure 9) but does not induce any effect on cell viability (Figure 7), we can suggest that ecto-CK2, at variance with endo-CK2, is not required for cell survival. This should be related to ecto-CK2 amount found in our cell models: the activity is well evident in tumor and non-tumor cells, and no significant differences are appreciable, although a correct comparison, normalized to cell surface and/or obtained by comparing cells from the same kind of tissue, has not been done yet. Our feeling is that, while tumor cells rely on endocellular CK2 for their survival and indeed express a higher amount of it compared to their normal counterpart, the CK2 ectokinase activity is not related to cancer cell survival and is probably not more expressed than in normal cells.

**PEPTIDES TARGETING
CK2 SUBSTRATES:
EFFECTS *IN VITRO* AND *IN CELL***

INTRODUCTION

Several approaches have been tested so far to directly inhibit protein kinase CK2, as largely described elsewhere in this thesis. However, some years ago, a research group from Havana introduced a new approach to target CK2, based on the development of a peptide interfering with the CK2 phosphorylation site on its substrates rather than hitting the enzyme *per se* (Perea *et al.*, 2004). This peptide, formerly termed as P15, was originally identified by screening a random cyclic peptide phage library using the human papillomavirus (HPV-16) E7 acidic domain as target (Perea *et al.*, 2004), and in fact *in vitro* experiments showed that P15 peptide abrogated the phosphorylation of E7-GST protein by CK2 (Figure 1).

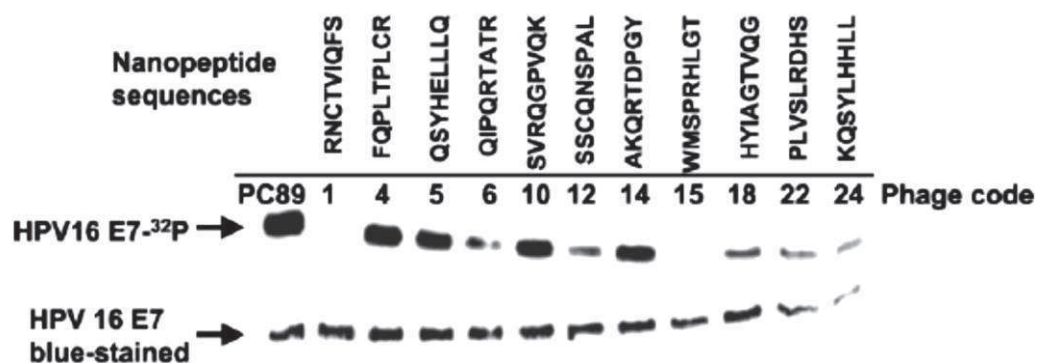
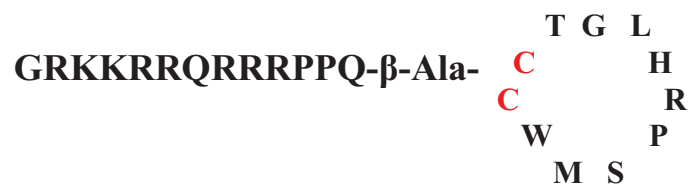


Figure 1. CK2 acidic domain binders abrogate the phosphorylation of HPV-16 E7 *in vitro*. The recombinant HPV16 E7-GST was expressed and further purified on Glutathione Sepharose beads. Ten micrograms of HPV-16 E7-GST were preincubated 1 hour at 37°C with the selected peptide-presenting phages. The gel was blue stained. Levels of the ³²P-HPV16 E7-GST protein were normalized with respect to levels of the Coomassie-stained HPV-16 E7-GST (from Perea *et al.*, 2004).

Later, the antitumor properties of the P15 peptide were examined; for this purpose modifications on the peptide were introduced. In particular, two cysteine residues were added at the C- and N-terminus to permit the formation of a sulfide bridge and generate a cyclic P15, expected to be more resistant to cellular protease action. Moreover, the cyclic P15 sequence was fused to the cell penetrating peptide Tat (48-60) (GRKKRRQRRRRPPQ) that has been extensively used for delivering peptide cargoes within cells (Wadia and Dowdy, 2005). Its effect of cargoes internalization is due to its aminoacidic composition; its positive charge, due to the

presence of arginine and lysine residues, allows the initial electrostatic interaction with polyanionic glycosaminoglycan present on the extracellular membrane (Ziegler and Seelig, 2004). Different mechanisms for Tat-cargoes internalization have been reported, such as clatrin-dependent endocytosis (Richard *et al.*, 2005), caveolin-dependent endocytosis (Ferrari *et al.*, 2003) or macropinocytosis (Kaplan *et al.*, 2005). Once inside the cell, Tat peptide conveys its cargoes to the nucleus, where it translocates passing through nuclear pores (Hauber *et al.*, 1989; Truant and Cullen, 1999).

The structure of P15-Tat molecule, now known as CIGB-300 (Perea *et al.*, 2008), is the following:



Proteomic studies have also been performed and different set of proteins up- and down-regulated by the peptide have been found (Rodríguez-Ulloa *et al.*, 2010). Moreover “pull-down” experiments were carried out to determine if the peptide was bound to CK2 substrates; among other target proteins, a predominance of the nucleophosmin/B23 was found. This is a nucleolar protein phosphorylated by CK2 at Ser125; it has a chaperone activity, regulated by phosphorylation, according to the model proposed in (Szebeni *et al.*, 2003) and illustrated in Figure 2. Interestingly, 13 structural ribosomal proteins from the small subunit and one from the large subunit were also identified as CIGB-300 targets (Perera *et al.*, 2009).

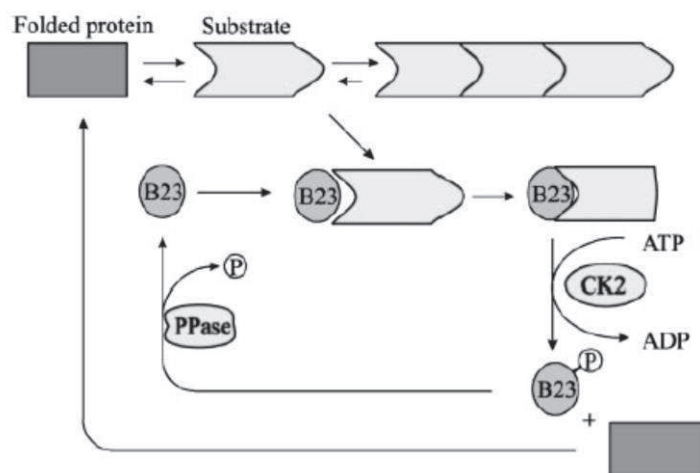


Figure 2. A model for the plausible involvement of phosphorylation of B23 protein in its molecular chaperone activity. Under the macromolecular crowding conditions of the nucleolus proteins would have a tendency to form aggregates when partially unfolded. B23 protein could recognize and bind to an exposed surface of the protein and temporarily remove it from the aggregation pathway. Refolding of the substrate protein could be accomplished either in the complex or after its release. Phosphorylation of the complex by casein kinase II (abundant in the nucleolus) would reduce the affinity of B23 for a substrate and promote dissociation of the complex. After release of substrate, a phosphoprotein phosphatase (PPase) could dephosphorylate protein B23. The latter step would restore the affinity of B23 for partially unfolded substrates and allow it to participate in additional cycles of binding and release of substrates (from Szebeni *et al.*, 2003).

After preliminary *in vitro* studies, CIGB-300 was employed in cells and reported to induce apoptosis in different tumor lines (Perea *et al.*, 2008). Moreover, it was applied *in vivo* and found effective in different cancer model. Intratumoral administration of CIGB-300 led to a significant reduction or complete inhibition of tumor growth in murine xenografts models, compared with the groups treated both with PBS and with a negative control peptide (Figure 3). Interestingly, the differences between CIGB-300 and the control groups were maintained after cessation of treatment and until the end of the analysis (Perea *et al.*, 2008). Moreover, systemic administrations of CIGB-300 in xenograft mice also inhibited the tumor growth (Perera *et al.*, 2008). Finally, to investigate the safety and tolerability of the CIGB-300 a clinical trial has started: the compound is so far reported to be safe, well tolerated and able to produce signs of clinical benefit, with a significant lesion reduction in 19.3% of the cases (Perera *et al.*, 2008; Solares *et al.*, 2009).

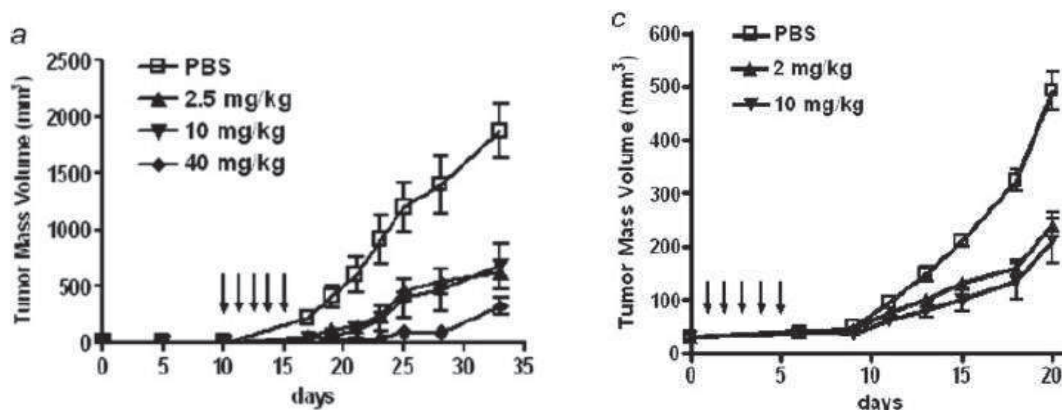


Figure 3. Systemic administration of CIGB-300 in cancer models. Different doses of CIGB-300 or PBS vehicle were systemically administered during 5 consecutive days (represented by arrows) (a) and (c) represent the mean of tumor mass volume and the respective standard error from the murine TC-1/C57/BL6 and human NCI-H125/nude mice tumor models respectively (from Perera et al., 2008).

Regarding the molecular mechanism of CIGB-300 action, based on what reported by Perea and co-workers, it is known to impair the phosphorylation of CK2 substrates, in particular of the nucleolar B23 protein. However, it is not clear if it is also a direct CK2 inhibitor, as frequently defined, and if it interacts with CK2 producing a global impairment of its catalytic activity. To investigate this point and to try to understand if CK2 is involved or even directly responsible of the cell death observed in CIGB-300 treated cells, in this work we used a new synthesized peptide, provided by Prof. O. Marin (Padova), denoted CRIBI-300. Its functional domain P15 is identical to the original peptide but a slightly different amino-acidic sequence in the Tat peptide is present; moreover, Tat is fused at the C-terminal of P15 in CRIBI-300, while in CIGB-300 it was at the N-terminal. The structure of CRIBI-300, compared to CIGB-300, and that of several other variants used in this study are reported in Table 1.

CIGB-300	GRKKRRQRRRPPQ-β-Ala-CWMSPRHLGTC	C
CRIBI-300	CWMSPRHLGTC-β-Ala-GYGRKKRRQRRRG-NH ₂	C
CRIBI-300LIN	CWMSPRHLGTC-β-Ala-GYGRKKRRQRRRG-NH ₂	L
P15	CWMSPRHLGTC	C
TAT	GYGRKKRRQRRRG-NH ₂	L
CRIBI-300 SCRAMBLE *	CHPGSTWMRLC-β-Ala-GYGRKKRRQRRRG-NH ₂	C
EMO-TAT **	CAGEYGAEALC-β-Ala-GYGRKKRRQRRRG-NH ₂	C
CRIBI-300ALA	<u>A</u> WMSPRHLGTA-β-Ala-GYGRKKRRQRRRG-NH ₂	L
CRIBI-300C1	<u>A</u> WMSPRHLGTC-β-Ala-GYGRKKRRQRRRG-NH ₂	L
CRIBI-300C11	CWMSPRHLGTA-β-Ala-GYGRKKRRQRRRG-NH ₂	L
CRIBI-300W	C <u>A</u> MSPRHLGTC-β-Ala-GYGRKKRRQRRRG-NH ₂	L
CRIBI-300S	CWM <u>A</u> PRHLGTC-β-Ala-GYGRKKRRQRRRG-NH ₂	L
CRIBI-300P	CWMS <u>A</u> RHLGTC-β-Ala-GYGRKKRRQRRRG-NH ₂	L
CRIBI-300R	CWMSPA <u>H</u> LGTC-β-Ala-GYGRKKRRQRRRG-NH ₂	L
CRIBI-300L	CWMSPRH <u>A</u> GTC-β-Ala-GYGRKKRRQRRRG-NH ₂	L
CRIBI-300-FAM***	CWMSPRHLGTC-β-Ala-GYGRKKRRQRRRGK-FAM	C
PENETRATIN	RQIKIWFQNRRMKWKK-NH ₂	L
CRIBI-300PEN	CWMSPRHLGTC-β-Ala- RQIKIWFQNRRMKWKK-NH ₂	L

Table 1. Sequences of the CIGB-300 derived peptides used this study, as synthesized and kindly provided by Prof. O. Marin (Padova). In the first column, the conventional name used in this thesis is reported. Tat sequence in green, P15 in pink, while aminoacid variants are bold and underlined. *The sequence between the two cysteines is the scramble sequence used in (Perera et al., 2012). **The sequence between the two cysteines is derived from Hemoglobin subunit Alpha 1[21-30]. *CRIBI-300 is conjugated to 5-6 carboxyfluorescein (FAM) and used for immunofluorescence assays. The right column denotes linear (L) or cyclic (C) peptides, referring to the P15 sequence.**

AIM OF THE STUDY

In this study, we wanted to investigate at a molecular level the mechanism of action of the compound CIGB-300, recently included in clinical trials in humans. In fact, it is frequently reported as a CK2 inhibitor, but the rationale of its design was the targeting of CK2 substrate, and not of the kinase itself. Therefore we wanted to shed light on a possible direct effect of this compound on CK2 and to understand if CK2 is directly involved in mediating the effects *in cells* and *in vivo*. To this purpose, beside CIGB-300, we exploited a new synthesized CIGB-300 derived cyclic peptide, called CRIBI-300, and performed *in vitro* and *in cell* experiments, to analyze the effects on CK2 and on different signaling pathways. We also aimed at identifying the specific aminoacidic position responsible of the CRIBI-300 activity. To this purpose, several single aminoacid modifications were introduced in the CRIBI-300 sequence; the resulting peptides were synthesized by Prof. Marin (Padova) and provided for the *in vitro* and *in cell* analysis. A list of the tested peptides and their sequences is presented in Table 1.

RESULTS

CRIBI-300 EFFECTS ON RECOMBINANT CK2 ACTIVITY DEPENDS ON THE PEPTIDE SUBSTRATES USED

To investigate if the CIGB-300 derived CRIBI-300 peptide directly inhibits CK2 activity, we performed *in vitro* kinase assays with recombinant CK2 $\alpha_2\beta_2$ in the presence of increasing concentrations of CRIBI-300. Two different CK2 peptides were used as substrates for the phosphorylation assay. One is the canonical CK2 substrate peptide, designed on the basis of its consensus (Meggio *et al.*, 1994), and here called “CK2-tide” (sequence: RRRADDSDDDDD); the other is a peptide derived from the CK2 substrate eiF2 β (sequence: MSGDEMIFDPTMSKKKKKKKKP), here called “eiF2 β -tide”, and has the peculiarity to be phosphorylated only by the tetrameric CK2 $\alpha_2\beta_2$, and not by the monomeric α alone (Salvi *et al.*, 2006).

Interestingly, the effects of CRIBI-300 on CK2 activity are different with the two substrates exploited. In the case of CK2-tide no inhibition was observed: as reported in Figure 4, panel A, CK2 activity was unaffected up to 50 μ M CRIBI-300. On the contrary, in the case of eiF2 β -tide, CK2 activity is strongly impaired by CRIBI-300 in a dose-dependent manner: at 0.5 μ M CRIBI-300 we already observe a 40% reduction in CK2 activity, and more than 60% at 5 μ M (Figure 4, panel B). Very similar effects were induced by CIGB-300, the original peptide developed by Perea and coworkers (Perea *et al.*, 2004) (Figure 4). Therefore, the position of the Tat sequence respect to P15 is not relevant, and hereafter we will use CRIBI-300 as our reference peptide.

We also tested other CK2-specific peptide substrates, such as a REV-derived peptide (Marin *et al.*, 2000) and a B23-derived peptide, but we did not find any significant effects (not shown). Therefore, the only case of CRIBI-300-induced inhibition was that of the peptide which strictly requires the presence of CK2 β for its phosphorylation. We wondered if the effects of CRIBI-300 on CK2 activity were due to the cyclic P15 functional domain or to the Tat sequence (see Table 1).

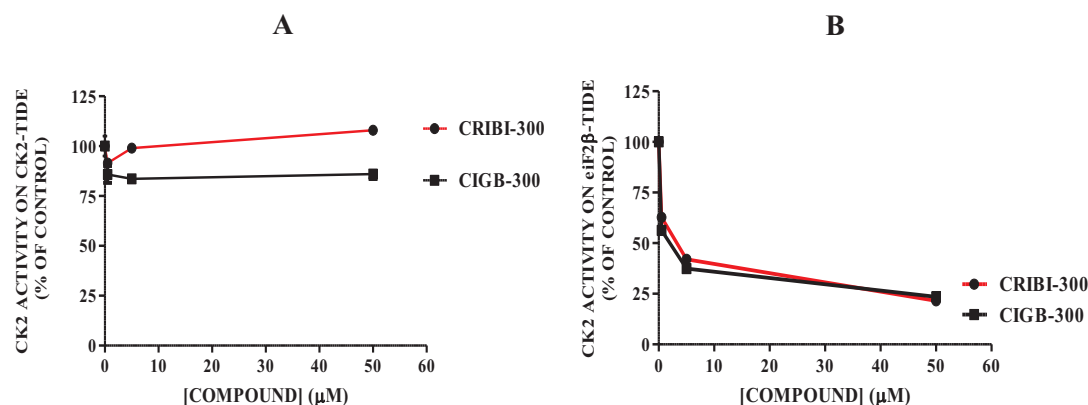
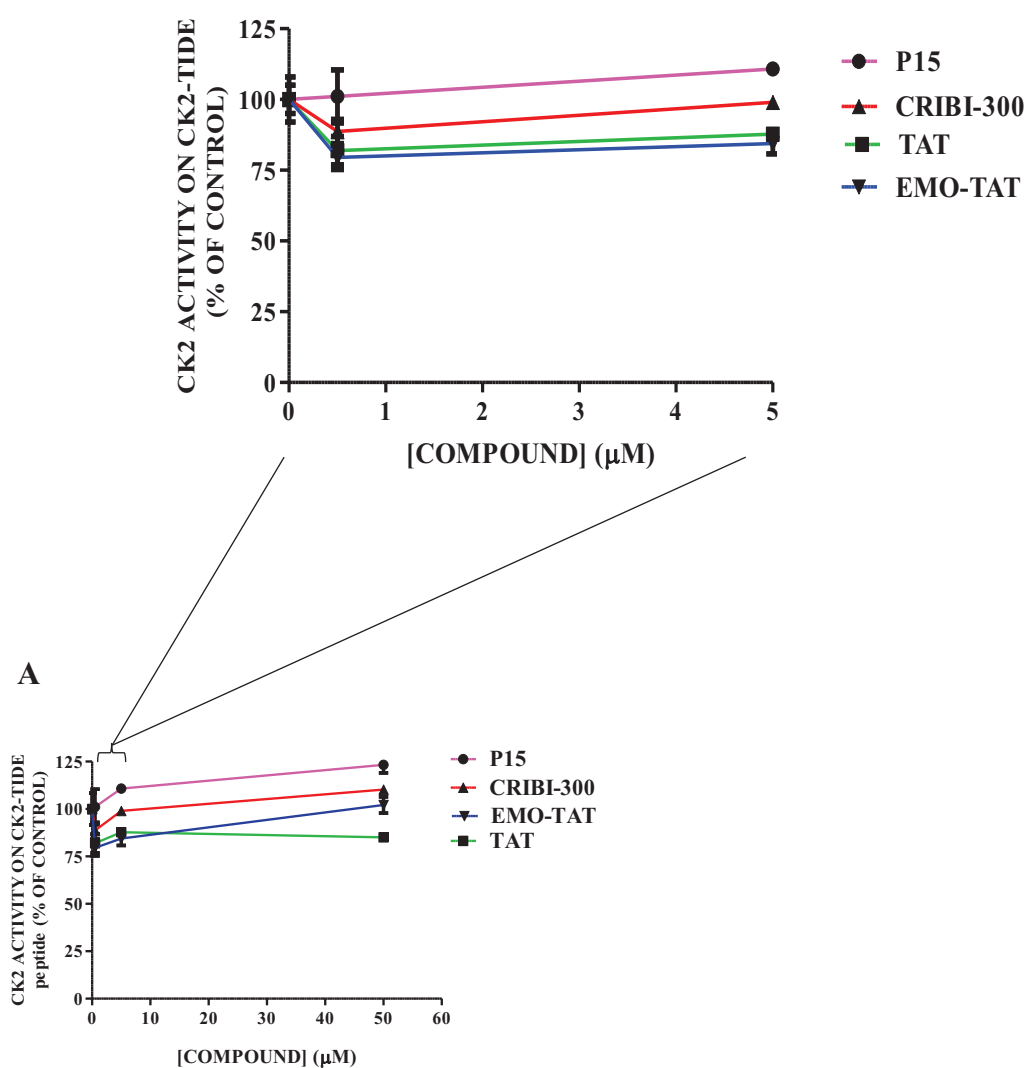


Figure 4. Effects of CRIBI-300 and CIGB-300 on CK2 activity towards peptide substrates. 40ng of recombinant CK2 $\alpha_2\beta_2$ were incubated at 30°C for 10 min with increasing concentration of CRIBI-300 (red) and CIGB-300 (black), in the presence of a radioactive phosphorylation mixture and the specific CK2-tide substrate (0.1mM)(A) or the eiF2 β -tide substrate (0.1mM) (B). Activity is reported as % of the control (CK2 incubated in the absence of CRIBI-300), corresponding to 100000 $\pm$ 5000 cpm in the case of CK2-tide and 110000 $\pm$ 7000 cpm in the case of eiF2 β -tide, respectively). Values $\pm$ SEM of three independent experiments are reported.

We therefore performed CK2 kinase assays with increasing concentration of P15 peptide or Tat peptide. As shown in Figure 5 A, we found that they do not affect CK2 activity towards the CK2-tide, up to 50 μ M, as observed for CRIBI-300. In the case of eiF2 β -tide, P15 alone do not affect CK2 activity, while Tat peptide is slightly inhibitory, with only a 50% reduction of CK2 activity at 50 μ M (Figure 5 B). Therefore, as also evident in Figure 5 B, we concluded that the entire CRIBI sequence, including P15 and Tat fragment, is necessary for the inhibition of CK2 activity towards the eiF2 β . We also tried the simultaneous addition of the two separated P15 and Tat peptides during the eiF2 β -peptide phosphorylation assay, but we did not find a synergistic effect compared to the individual additions (not shown). We then wondered if the CRIBI-300 effect was due to Tat peptide fused to any other cyclic sequence; for this reason, we analyzed the effect of a peptide in which Tat sequence has been fused to a completely aminoacidic sequence but with the same length of CRIBI-300. The effect of this compound, called EMO-Tat, is shown in Figure 5. It has no effect on CK2 activity toward CK2-tide, while the inhibition of eiF2 β -tide phosphorylation is quite similar to that exerted by Tat alone. This suggests that the effects induced by CRIBI-300 are mostly to be attributed to the cyclic P15 peptide fused to Tat. We also checked if the Tat sequence can be

substituted by a different peptide with the same cell-penetrating function. A compound was therefore synthesized, called CRIBI-300 PEN, composed of the P15 portion fused to the penetrating sequence (Jones *et al.*, 2005) (see Table 1). Its effects were very similar to those of CRIBI-300 (not shown), indicating that the sequence to be fused to P15 is not essentially that of Tat, and that probably a quite basic sequence is sufficient.



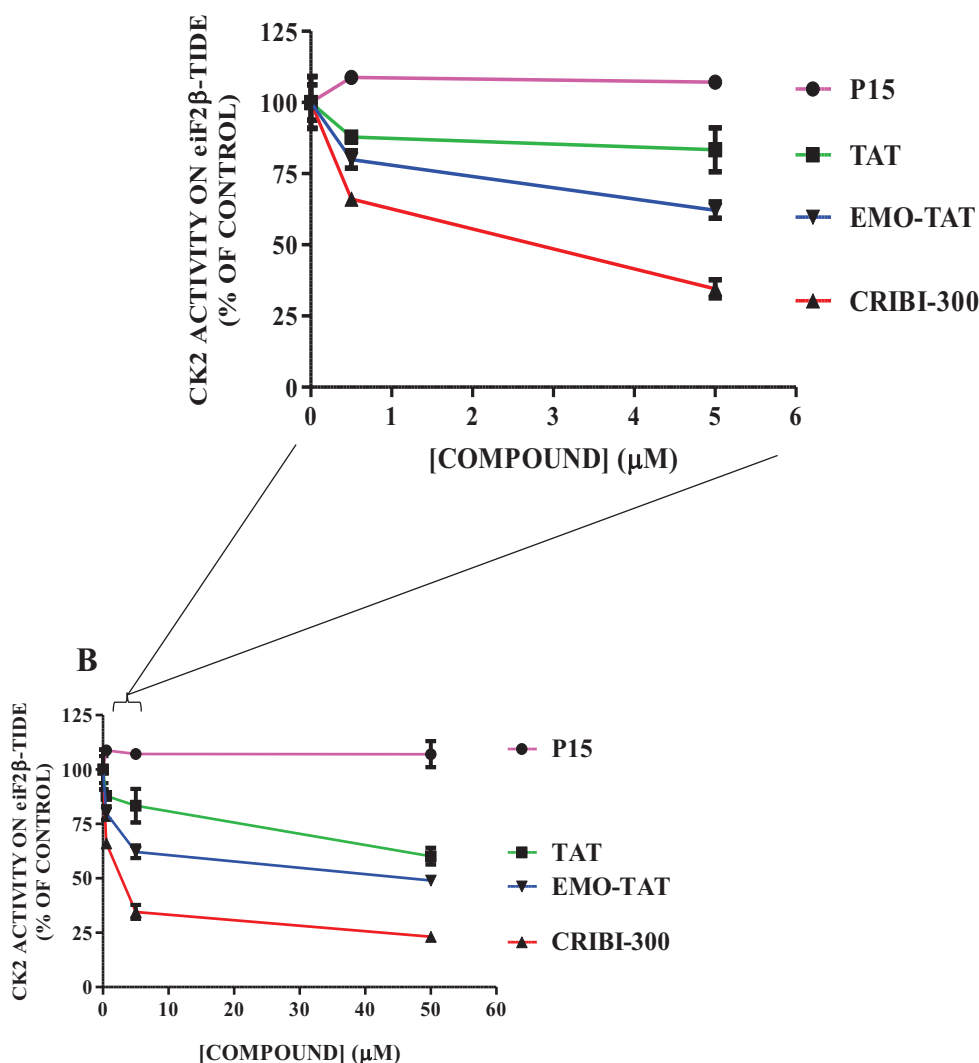


Figure 5. Effects of CRIBI-300 and derivatives on CK2 activity towards peptide substrates. Increasing concentrations of P15 (pink), Tat (green), EMO-Tat (blue) and CRIBI-300 (red) were used. For details and conditions, see Figure 4.

We then wanted to identify which specific aminoacidic residues of P15 are essential for the activity of CRIBI-300. For this purpose we used a panel of sequence-modified peptides which are listed in Table 1. Firstly we investigated the effects of CRIBI-300-ALA, where the two cysteines necessary for P15 cyclization have been substituted by two alanines. As shown in Figure 6, it induces much more modest effects on eiF2 β phosphorylation than CRIBI-300, with only 50% CK2 activity reduction at 50 μ M. Interestingly, this was not due to the absence of cyclization since the compound CRIBI-300 LIN, whose P15 sequence was left unchanged, but not induced to cyclization, had more pronounced effects than CRIBI-300 (Figure 7).

Moreover, we found that Cys or Trp substitutions on this linear peptide were not detrimental for its effect (Figure 7). Its effects are very similar to that observed in the case of CRIBI-300. We then investigated the effects of another compound,

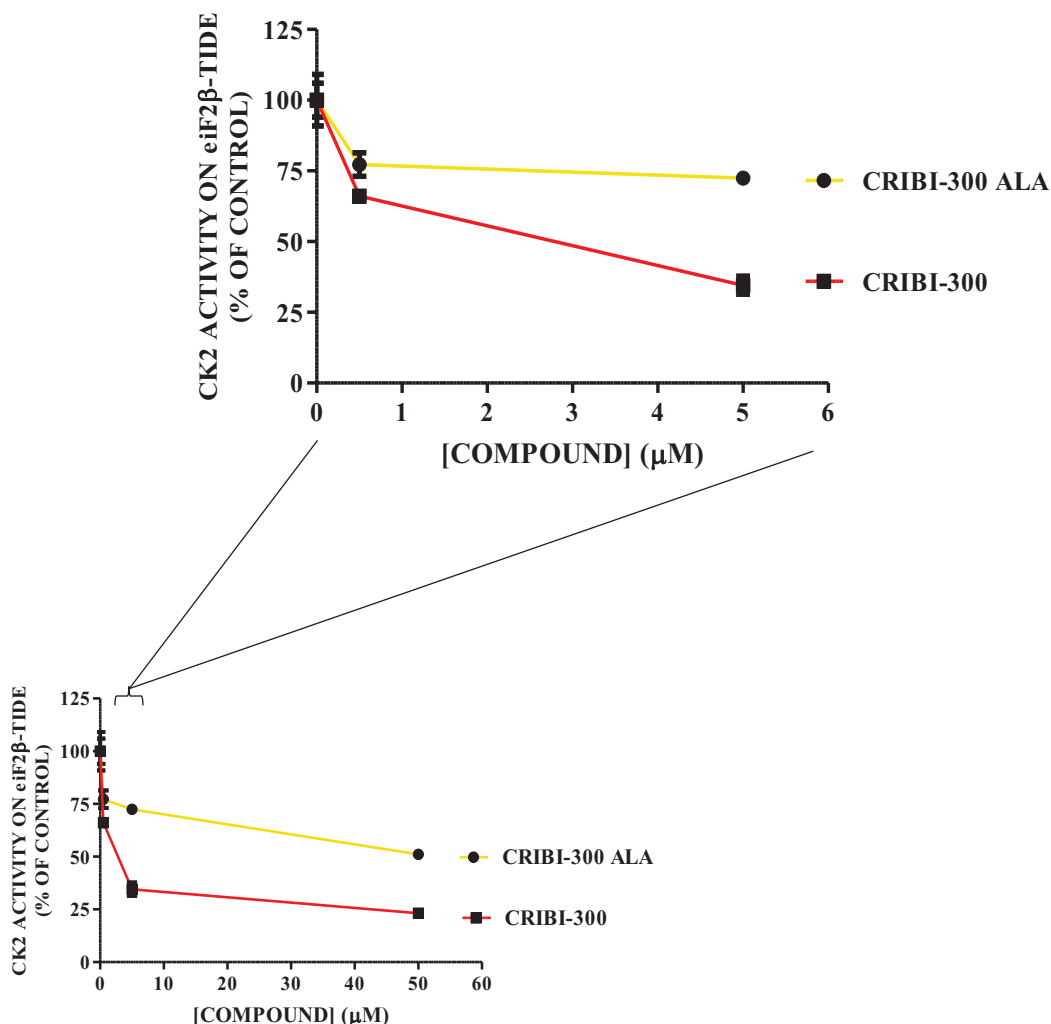


Figure 6. Effects of CRIBI-300 and CRIBI-300 ALA on CK2 activity towards eiF2β substrates. Increasing concentrations of CRIBI-300 ALA (yellow) and CRIBI-300 (i red) were used. For details and conditions, see Figure 4.

Surprisingly, when we tested a CRIBI-300 derived peptide containing a P15-scrambled sequence, where the aminoacidic composition was left unchanged but the sequence was completely shuffled, we found a still potent inhibition of eiF2β-tide phosphorylation by CK2 (Figure 8). The reasons for this result are presently unclear, but we hypothesized that some specific aminoacids need to be present, although not in a defined position.

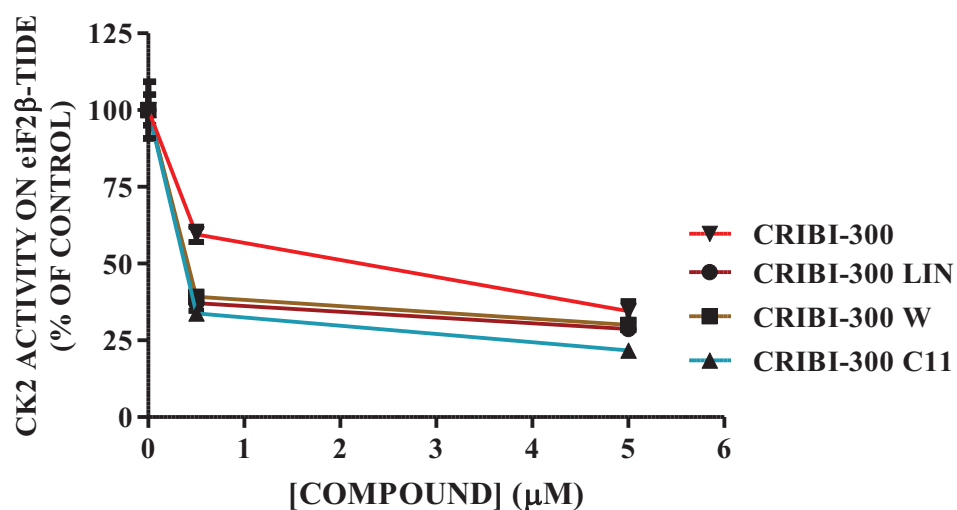


Figure 7. Effects of CRIBI-300 and derivatives on CK2 activity towards eiF2 β peptide. Increasing concentrations of CRIBI-300 (red), CRIBI-300 LIN (dark red), CRIBI-300 W (brown) and CRIBI-300 C11 (light blue) were used. For details and conditions, see Figure 4.

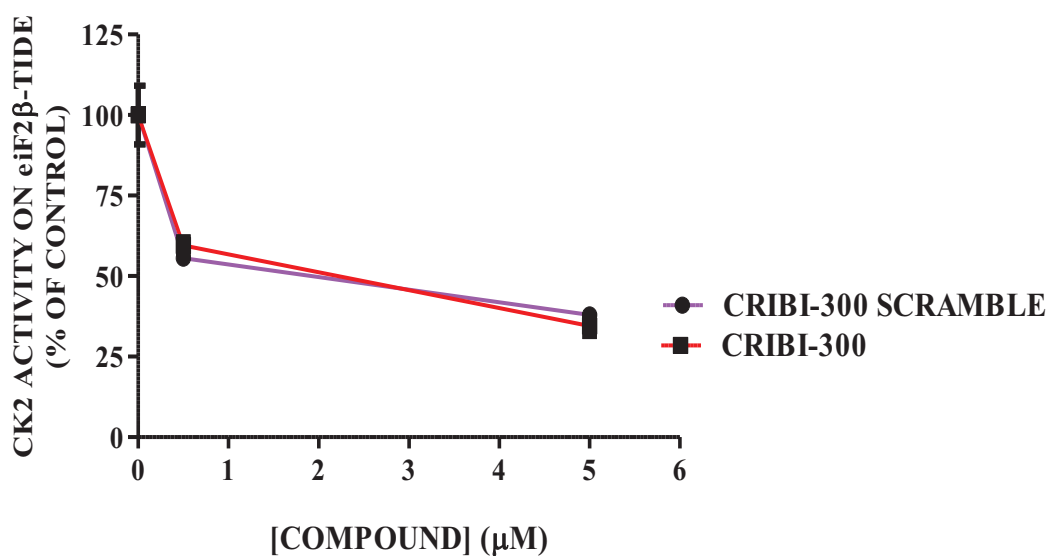


Figure 8. Effects of CRIBI-300 and CRIBI-300 SCRAMBLE on CK2 activity towards eiF2 β peptide. Increasing concentrations of CRIBI-300 (red) and CRIBI-300 SCRAMBLE (violet) were used. For details and conditions, see Figure 4.

EFFECTS OF CRIBI-300 ON RECOMBINANT CK2 ACTIVITY TOWARDS PROTEIN SUBSTRATES

The effects of CRIBI-300 peptide and derivatives above describe were observed on CK2 activity towards peptides. We then wanted to evaluate the effects on protein substrates. The CK2 protein substrates available in the laboratory for *in vitro* assays were α -casein, total casein, p53, and calmodulin. We first checked their phosphorylation by CK2 using both the monomeric α and the tetrameric CK2 $\alpha_2\beta_2$. As shown in Figure 9, when using 0.01 units of α and $\alpha_2\beta_2$ (measured towards CK2-tide, see Methods), we found that, under the chosen conditions, all these substrates were better phosphorylated by α than $\alpha_2\beta_2$. Calmodulin, as already known (Meggio *et al.*, 1994), was phosphorylated only by α (being $\alpha_2\beta_2$ able to recognize it only in the presence of polyamines or polybasic peptides).

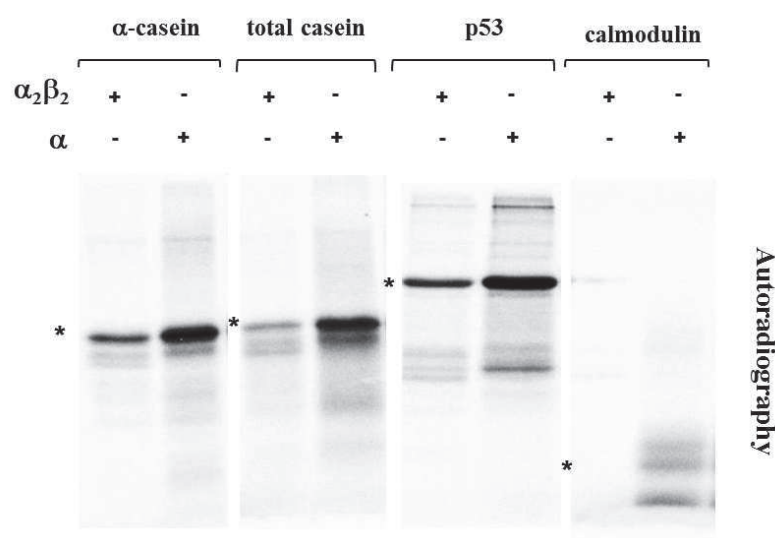


Figure 9. Phosphorylation of α -casein, total casein, p53 and calmodulin by CK2 α alone or CK2 $\alpha_2\beta_2$. CK2 α or CK2 $\alpha_2\beta_2$ (0.01 U, see Methods) were incubated with 0.5 μ g of α -casein, total casein, and p53 or 2 μ g of calmodulin with a radioactive mixture. Samples were resolved by SDS-PAGE and the radioactivity was detected by autoradiography. The position of α -casein, total casein, p53 and calmodulin are indicated by asterisk. Representative autoradiography of four independent experiments is shown.

Surprisingly, on all these substrates, CRIBI-300 induces an activatory effect on their phosphorylation by CK2 holoenzyme: in particular, at least 5-fold increased phosphorylation of α -casein, total casein and p53 was observed at 10 μ M CRIBI-300 concentration (Figure 10 A and B). In the case of p53 phosphorylation, different CRIBI-300 concentrations were applied, and a slight effect was observed

already at 1 μ M, while no further increase was induced passing from 10 to 100 μ M (Figure 10 B). Also in the case of calmodulin, CRIBI-300 has an activatory effect on CK2 holoenzyme. In fact, while its phosphorylation in the control lane is negligible, a weak phosphorylation is observed when CRIBI-300 is added at 10 μ M concentration (Figure 10 C), suggesting that CRIBI-300 mimic a polybasic effector.

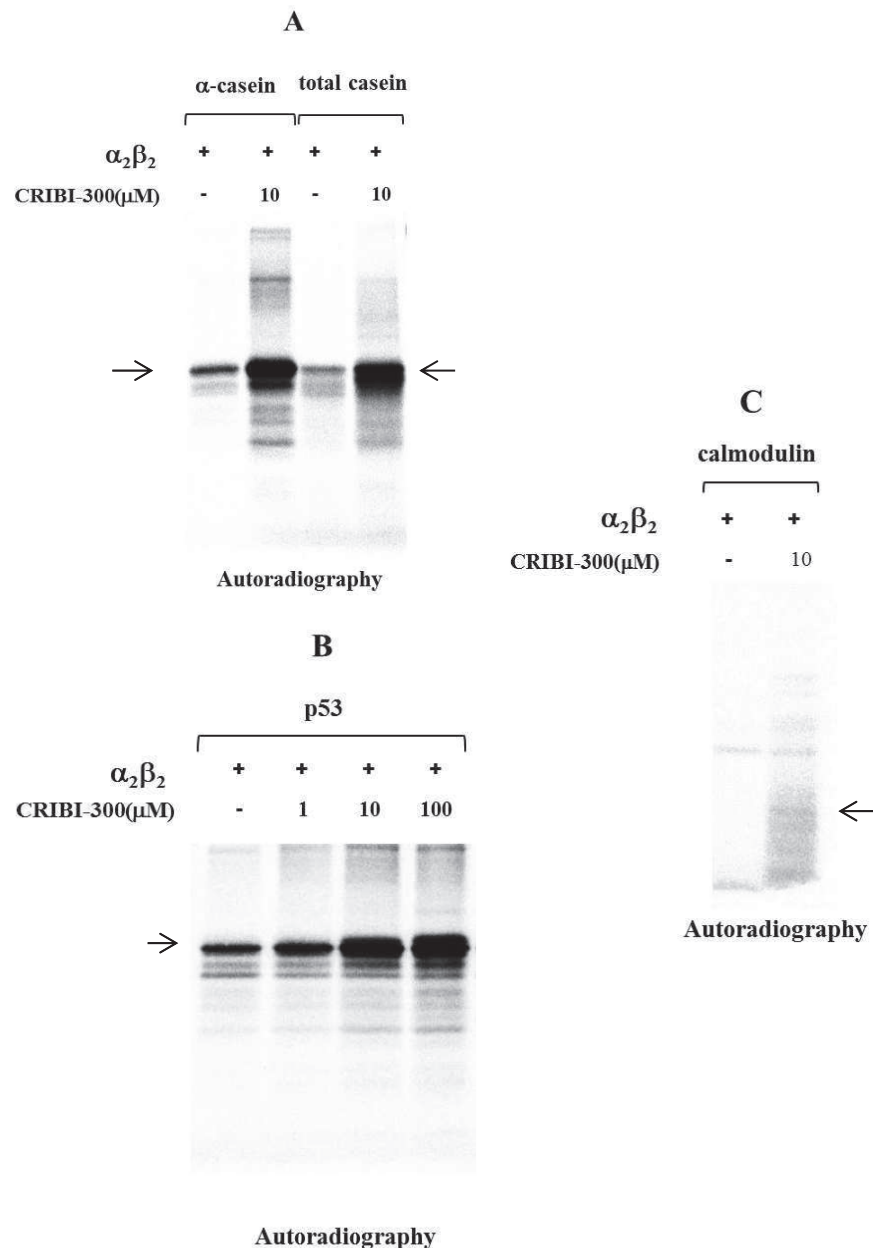


Figure 10. Effects of CRIBI-300 on the phosphorylation of α -casein, total casein, p53 and calmodulin by CK2 $\alpha_2\beta_2$. 40ng of CK2 $\alpha_2\beta_2$ were incubated with 0.5 μ g of α -casein, total casein and p53 or 2 μ g of calmodulin, with a radioactive mixture, resolved by SDS-PAGE and the radioactivity was detected by autoradiography. CRIBI-300 was added at the indicated concentrations before the addition of the radioactive mixture. The position of α -casein, total casein, p53 and calmodulin are indicated by arrows. Representative autoradiographies of three independent experiments are shown.

When we performed protein phosphorylation with CK2 α catalytic subunit, we do not observe any activation induced by CRIBI-300. In fact, as shown in the autoradiography of Figure 11, the phosphorylation level of α -casein and total casein by CK2 α is unchanged or even inhibited by CRIBI-300. Similar results were observed with p53 with higher concentrations (not shown).

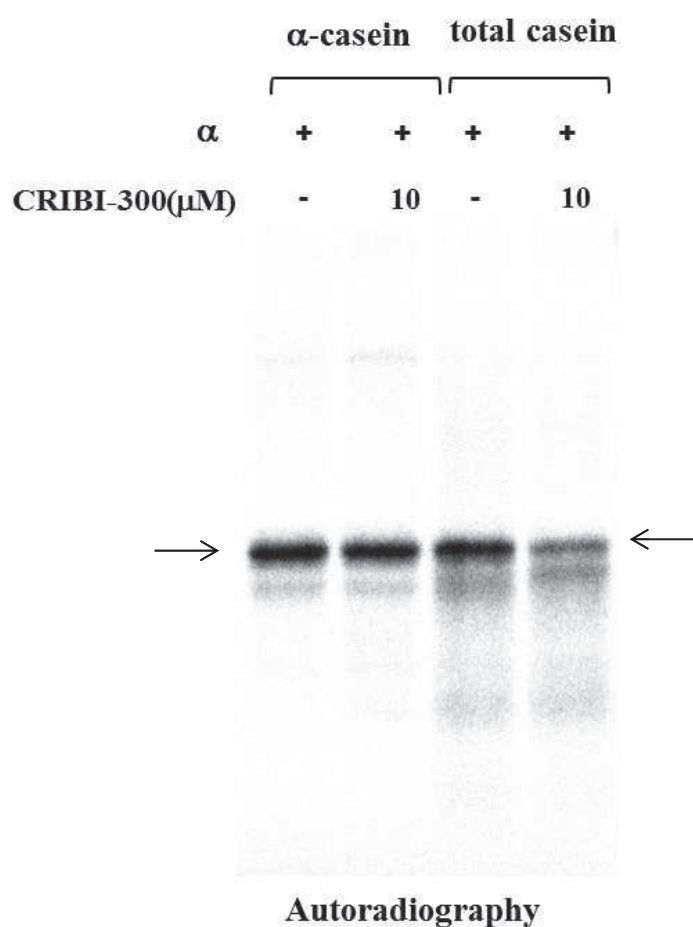


Figure 11. Effects of CRIBI-300 on the phosphorylation of α -casein and total casein by CK2 α . 30ng of CK2 α was incubated with 0.5 μ g of α -casein and total casein, with a radioactive mixture; samples were separated by SDS-PAGE and the radioactivity was detected by autoradiography. CRIBI-300 was added at the indicated concentrations before the addition of the radioactive mixture. The position of α -casein and total casein are indicated by arrows. Representative autoradiographies of three independent experiments are shown.

EFFECTS OF CRIBI-300 ON CK2 AUTOPHOSPHORYLATION

We then wondered if CRIBI-300 has also a direct effect on recombinant CK2 autophosphorylation activity, than usually occurs at the level of β subunit, as described in the general Introduction. We therefore incubated CK2 holoenzyme with a radioactive phosphorylation mixture, without any protein substrate, in the presence of increasing concentration of CRIBI-300 (Figure 12). We found that it induces a decrease in the autophosphorylation of β subunit, already visible at 1 μ M concentration, accompanied by concomitant induction of CK2 α autophosphorylation. This effect is similar to that induced by polyamines or polybasic peptides (Meggio *et al.*, 1994). This evidence, in addition to the effect of CRIBI-300 on calmodulin phosphorylation by CK2, suggests that it can behave as a polybasic effector. To exclude that this was due to the basic portion of CRIBI-300 corresponding to the Tat sequence, we performed autophosphorylation assays with increasing concentration of Tat peptide not conjugated to P15. However, the results demonstrate that it is almost ineffective in inhibiting CK2 β autophosphorylation and increasing CK2 α autophosphorylation (Figure 12). We therefore concluded that the all sequence of CRIBI-300 is required to produce polybasic-like effects on CK2. We also demonstrated that these effects were induced also on native CK2, deriving from rat liver, beside recombinant CK2 (Figure 12 B).

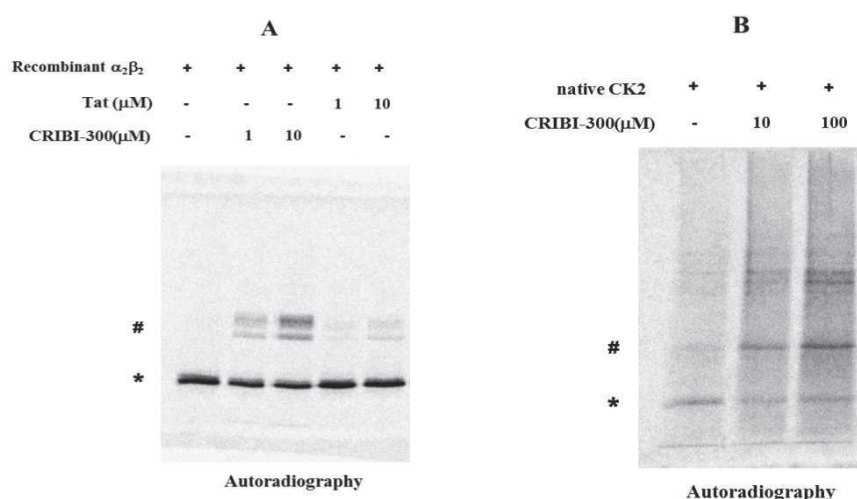


Figure 12. Effects of CRIBI-300 on the autophosphorylation of recombinant and native CK2. 40ng of recombinant CK2 $\alpha_2\beta_2$ (A) or 0.05 U of native CK2(B) (see Materials), were incubated with CRIBI-300 or Tat as indicated with the corresponding concentrations in the presence of a radioactive mixture, separated by SDS-PAGE and the radioactivity was detected by autoradiography. The position of CK2 α autophosphorylation and CK2 β autophosphorylation are indicated by # or *, respectively. Representative autoradiographies of four independent experiments are shown.

EFFECTS OF CRIBI-300 ON CK2 PHOSPHORYLATION OF B23

Special attention is deserved by the CK2 substrate nucleophosmin/B23, which has been demonstrated to be the major target of CIGB-300 (Perea *et al.*, 2008). Therefore, also *in vitro* CRIBI-300 was expected to bind to the phosphorylation site of B23 and prevent its phosphorylation by CK2. However, when we performed *in vitro* CK2 kinase assay towards B23, we had difficulties in obtaining reproducing results. In fact, with recombinant B23 we never observed inhibition by CRIBI-300 of the CK2-dependent phosphorylation, which instead was activated (Figure 13A). On the contrary, when we performed the CK2 assay on the B23 immunoprecipitated from a cell total lysate, we sometimes observed a strong inhibition (as shown in Figure 13 B). However, even this effect on immunoprecipitated B23 was poorly reproducible. At present we do not know the reasons of these inconsistent results. We can hypothesize (see Discussion) that a double effect is produced by CRIBI-300 in this kind of assay, being it able to bind to both the kinase and the substrate.

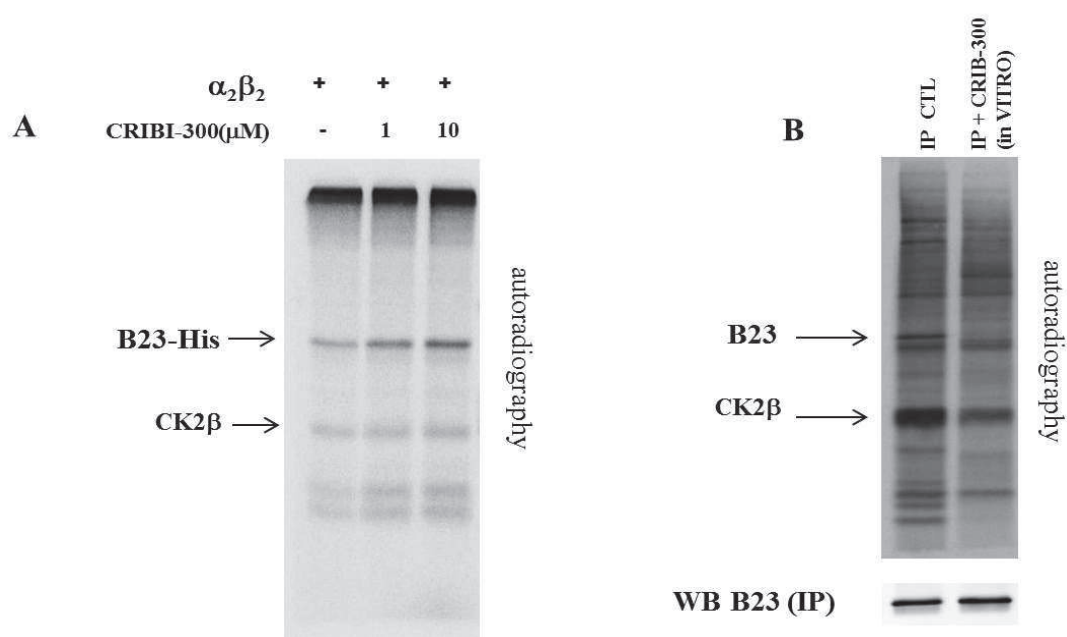


Figure 13. Effects of CRIBI-300 on the phosphorylation of B23 by CK2 $\alpha_2\beta_2$. 40 ng of CK2 $\alpha_2\beta_2$ was incubated with 1 μ g of recombinant B23 (A) or B23 immunoprecipitated from cell lysate as described in Methods section (B). CRIBI-300 was added at the indicated concentrations before the addition of the radioactive mixture. The position of B23 and CK2 β are highlighted by arrows. WB anti-B23 is also shown in (B) as a control of equal amount of B23 immunoprecipitation. Representative autoradiographies of three separated experiments are shown.

CRIBI-300 LOCALIZATION INSIDE THE CELL

We then passed to use CRIBI-300 for cell treatments. CRIBI-300 is provided of Tat sequence which allows it to enter cell and targets it to the nucleus. It has been reported that Tat peptide binds directly to the nucleolar protein B23 (Stauber and Pavlaskis, 1998), one of the major targets of CRIBI-300 (Perea *et al.*, 2008). Thus, in order to assess the cell permeability and subcellular localization of CRIBI-300, we exploited a CRIBI-300 peptide fused to fluorescein dye (CRIBI-300-FAM) and performed confocal microscopy immunofluorescence experiments. As evident from Figure 14, CRIBI-300 turned out to be cell-permeable, reaching the nucleus in a very short time (30 min). Moreover, its accumulation in the nucleolus was also observed (Figure 14).

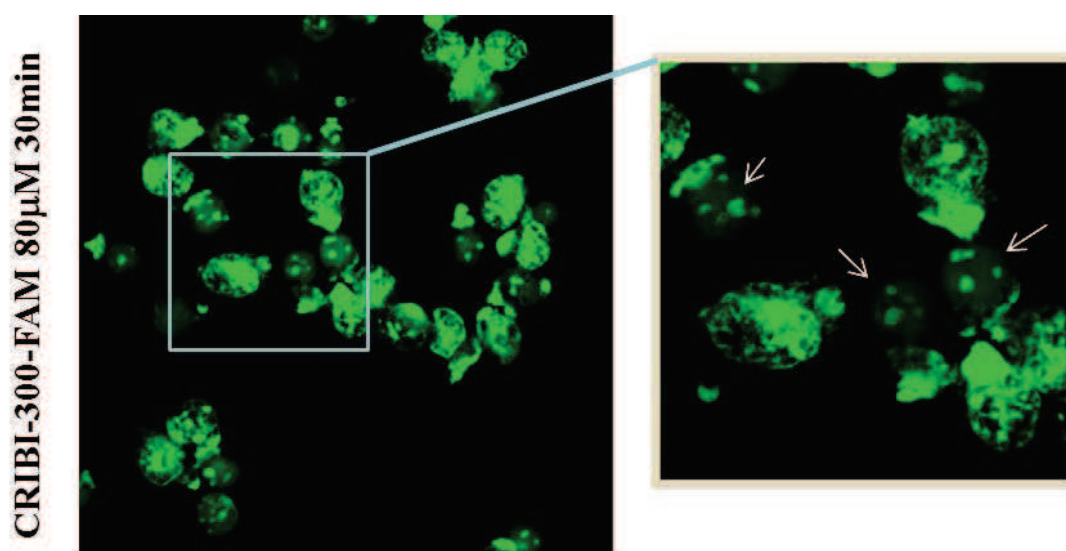


Figure 14. CRIBI-300 subcellular localization. R-CEM cells were treated with CRIBI-300-FAM (green) at 80 μ M for 30 min. Cells were then fixed and mounted on coverslip and the images were acquired with LEICA TCS SP5 confocal microscope. The figure is a representative picture of three independent experiments. The square of the left image is magnified on the right. Arrows indicate the nucleolar localization of CRIBI-300.

EFFECTS OF CRIBI-300 ON CK2 CELLULAR ACTIVITY AND AMOUNT

Data described so far show that CRIBI-300 can affect CK2 activity, with different effects in dependence of substrate, and it enters cells and localized mainly to the nucleolus. Thus, we decided to evaluate the effect of CRIBI-300 on CK2 endocellular activity. We performed cell treatments with increasing concentrations

of CRIBI-300 and for different times, and tested the endocellular CK2 activity in treated cells, compared to control cells, exploiting synthetic peptides as CK2 substrate. We observed a marked dose- and time-dependent reduction of CK2 activity. This was evident either using CK2-tide (Figure 15) or eiF2 β -tide (not shown), at variance with what observed *in vitro*. However, when we checked the CK2 amount by Western Blot analysis of cell lysates, we found that it dramatically decreases in cell treated with CRIBI-300 even after very short times (Figure 15). For example, CK2 activity in S-CEM is strongly reduced after 2 h treatment with 25 μ M CRIBI-300 and it is completely abolished after 2 h with 80 μ M CRIBI-300, but a parallel decrease of CK2 protein amount is observed. Similar results were observed in R-CEM although with a slower time-course.

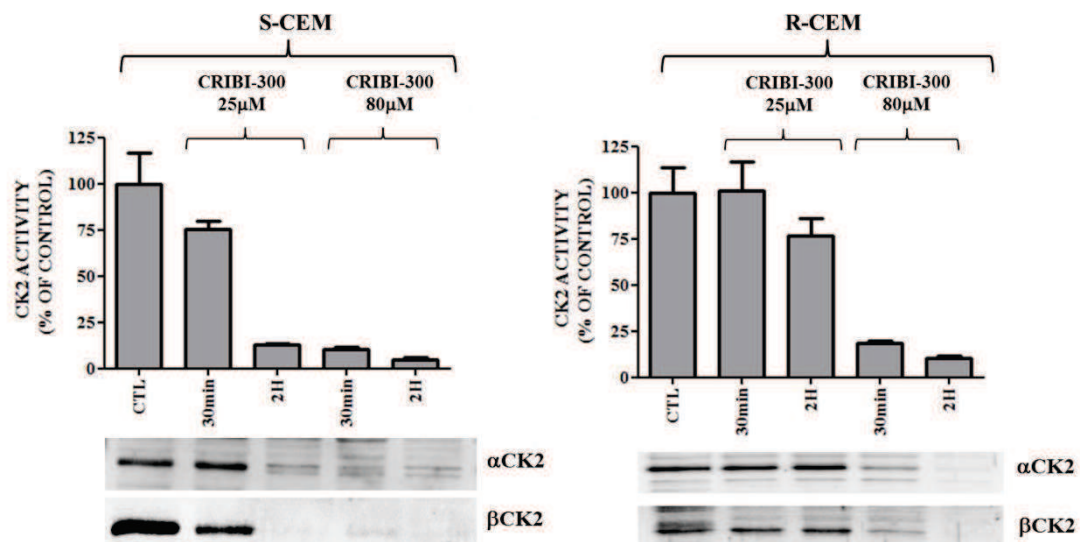


Figure 15. CK2 activity and amount in CRIBI-300-treated cells. S-CEM and R-CEM cells were treated for the indicated times and with the indicated concentrations of CRIBI-300. Cell lysates were analyzed for the activity of CK2 towards CK2-tide (graphs on the top). Mean $\pm$ SE values of four independent experiments are shown. 10 μ g of total proteins of the same cell lysates was analyzed by WB with the indicated antibodies for the amount of CK2 (on the bottom). Representatives WB of three independent experiments are shown. CTL=control (cells incubated 2 h in the absence of CRIBI-300).

These results were very surprising since CK2 is usually very resistant to proteolysis and it is not degraded after cytotoxic stimuli; even when cells are treated with CK2 canonical inhibitors, a reduction of its amount is never observed, up to 24-48 h (Di Maira *et al.*, 2007).

Experiments in LAMA84 and HL60 cells also confirmed the results (Figure 16); in this case, Western Blot analysis was performed by using either an antibody raised

against C-terminal or N-terminal CK2, and the results were very similar indicating that not only a partial proteolysis of CK2 occurs, but the entire protein is cleaved. Moreover, also CK2 β and the alternative isoform of the catalytic subunit, CK2 α' , are reduced in parallel with CK2 α (Figure 16). Whatever its causes, the prompt decrease in CK2 amount in response to CRIBI-300 treatment indicates that the reduction in CK2 activity is not a real inhibition, but simply reflects the amount of the active kinase present.

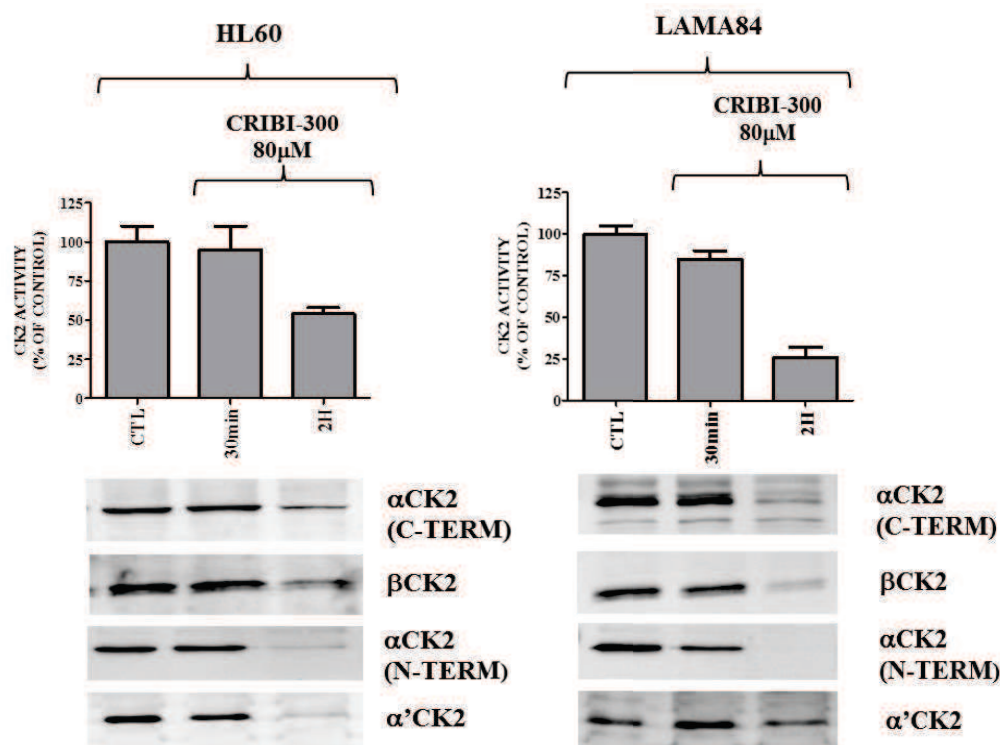


Figure 16. CK2 activity and amount in CRIBI-300-treated cells. HL60 and LAMA84 cells were treated for the indicated times and with the indicated concentrations of CRIBI-300. Cell lysates were analyzed for the activity of CK2 towards a synthetic specific peptide (graphs on the top). Mean $\pm$ SE values of four independent experiments are shown. 10 μ g of total proteins of the same cell lysates was analyzed by WB with the indicated antibodies for the amount of CK2 (on the bottom). Representatives WB of three independent experiments are shown. CTL=control (cells incubated 2 h in the absence of CRIBI-300).

We also tried to evaluate the endocellular CK2 kinase activity by WB analyses of its known phosphosites, such as Sp129 Akt. However this approach failed to give information, since, beside CK2 decrease, also other protein amounts are reduced (see below). Therefore, when we observed that phosphorylation decreased, this was

in parallel with the decrease of total protein amount. Figure 17 shows the results obtained in R-CEM cells.

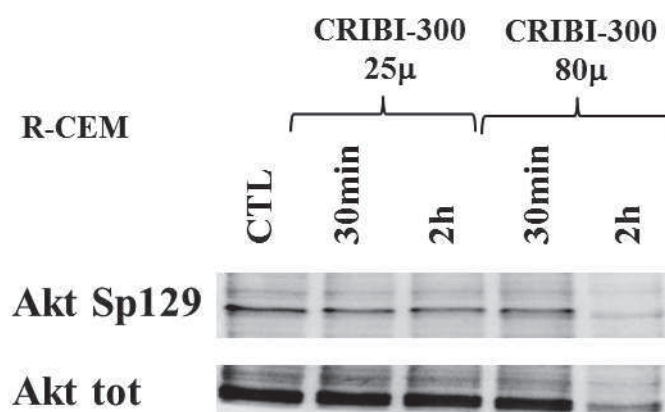


Figure 17. CK2 endogenous activity in CRIBI-300 treated cells. 10 µg of total proteins were analyzed by WB with the indicated antibodies. Representatives WB of three independent experiments are shown. CTL=control (cells incubated 2 h in the absence of CRIBI-300).

EFFECTS OF CRIBI-300 ON CELL VIABILITY

We then move to the cytotoxicity of CRIBI-300 on a panel of human cancer cell lines, namely Jurkat, HL-60, LAMA84, HeLa, HepG2, U2OS, S-CEM and their resistant variant, R-CEM. We also performed cell treatments in non tumor cells such as HEK-293T and CCD34Lu. We found variable sensitivity to CRIBI-300: we can distinguish a group of high-sensitive cells, essentially represented by blood tumor cells (Jurkat, HL-60, LAMA84, S-CEM and R-CEM), a middle-sensitive group corresponding to cells deriving from solid tumor (HeLa, HepG2 and U2OS), and a very low-sensitive group corresponding to non tumor cells (HEK-293T and CCD34Lu). In Figure 18 A, B and C some of these results are shown. Interestingly, we found that CRIBI-300 induces cell death in a similar manner in sensitive and resistant CEM cells (Figure 18 A). Table 2 summarizes the effects of CRIBI-300 in different lines by showing the DC₅₀ values (concentrations required to induce 50% of cell death in 24 h). Also from these values, it emerges the very low response to CRIBI-300 of the non tumor cell lines tested.

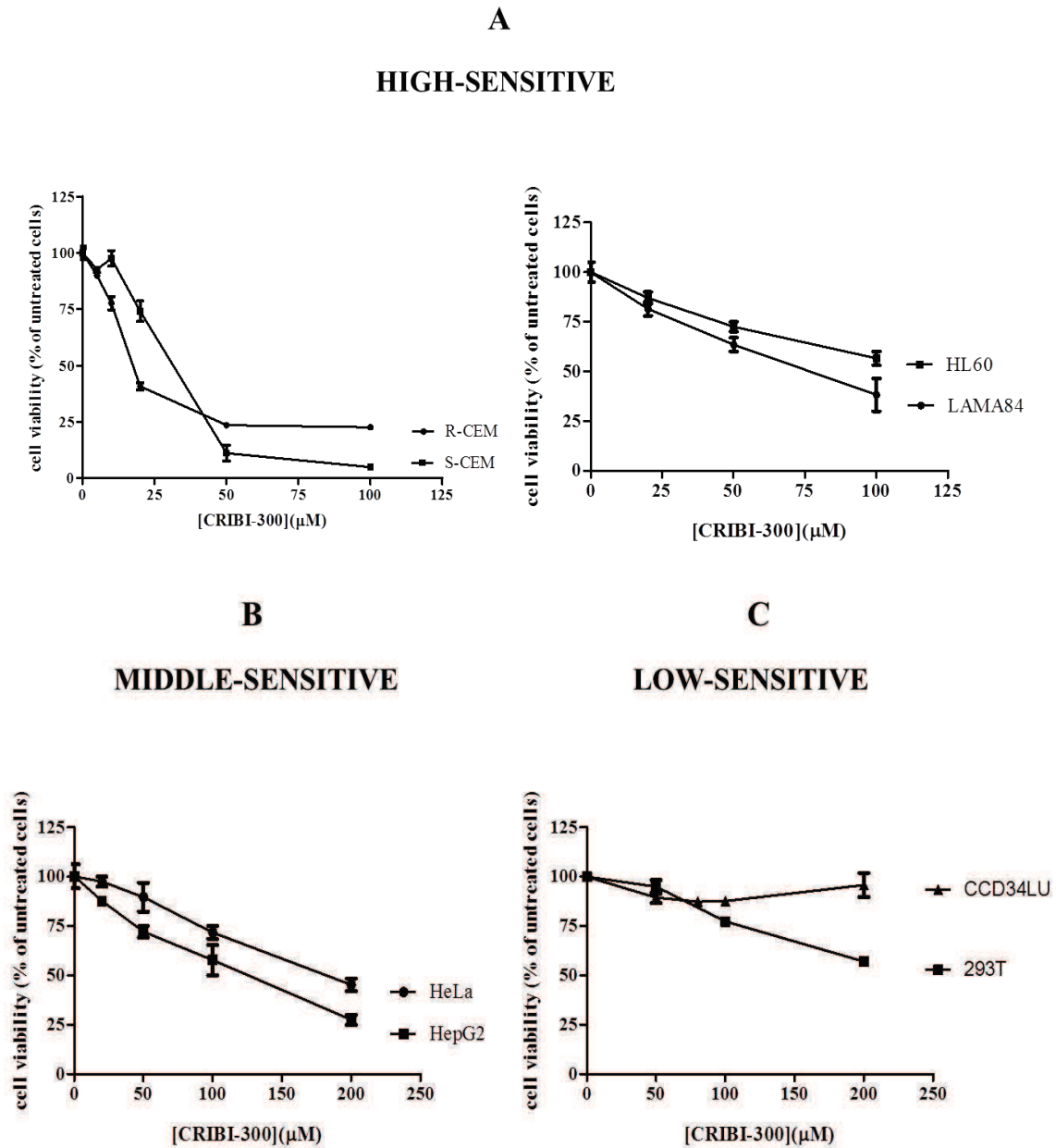


Figure 18. Different sensitivity on cell viability of CRIBI-300-treated cells. Cells were incubated with increasing concentrations of the CRIBI-300 for 24 h; cell viability was assessed by the MTT method, assigning 100% value to the vehicle-treated control cells. Panels A, B and C correspond to cells with similar sensitivity toward CRIBI-300. Mean $\pm$ SE values of at least four independent experiments are reported.

TUMOR CELL LINE	ORIGIN	DC ₅₀ (μM)	SD	
S-CEM	Human T lymphoblastoid	31.0	2.1	HIGH-SENSITIVE
R-CEM	Human T lymphoblastoid (apoptosis-resistant)	17.5	0.7	
LAMA84	Human chronic myeloid leukemia	79.2	17.1	
Jurkat	Human acute T lymphoblastic	64.6	6.3	
HL60	Human acute promyelocytic leukemia	95.1	3.2	
HeLa	Human cervix adenocarcinoma	182.1	17.2	MIDDLE-SENSITIVE
HepG2	Human hepatocellular liver carcinoma	120.6	22.1	
CCD34Lu	Human lung	>200	-	LOW-SENSITIVE
293T	human embryonic kidney	>200	-	

Table 2. DC₅₀ values of CRIBI-300. DC₅₀ values (concentration required to induce 50% of cell death in 24 h) were calculated from viability curves in Figure 18, by means of the MTT method (see Methods). The values are the mean of 4–6 independent experiments ± Standard Deviation (SD).

The next step was to evaluate the effects on cell viability of Tat peptide not conjugated to P15. In no cell line we have ever observed any signal of cell death in response to Tat (representative example are shown in Figure 19). Similarly, P15 peptide was ineffective on cell viability (not shown). Thus we concluded that similarity to what observed for the effects on CK2 activity, the whole CRIBI-300 structure, including the cyclic P15 sequence, is necessary to induce cell death.

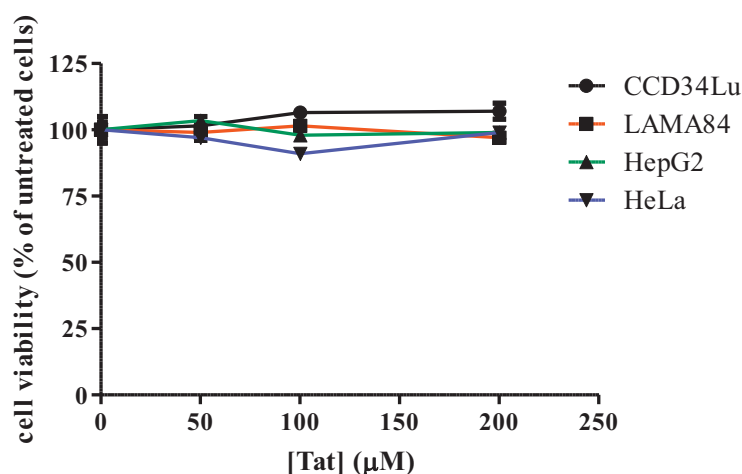
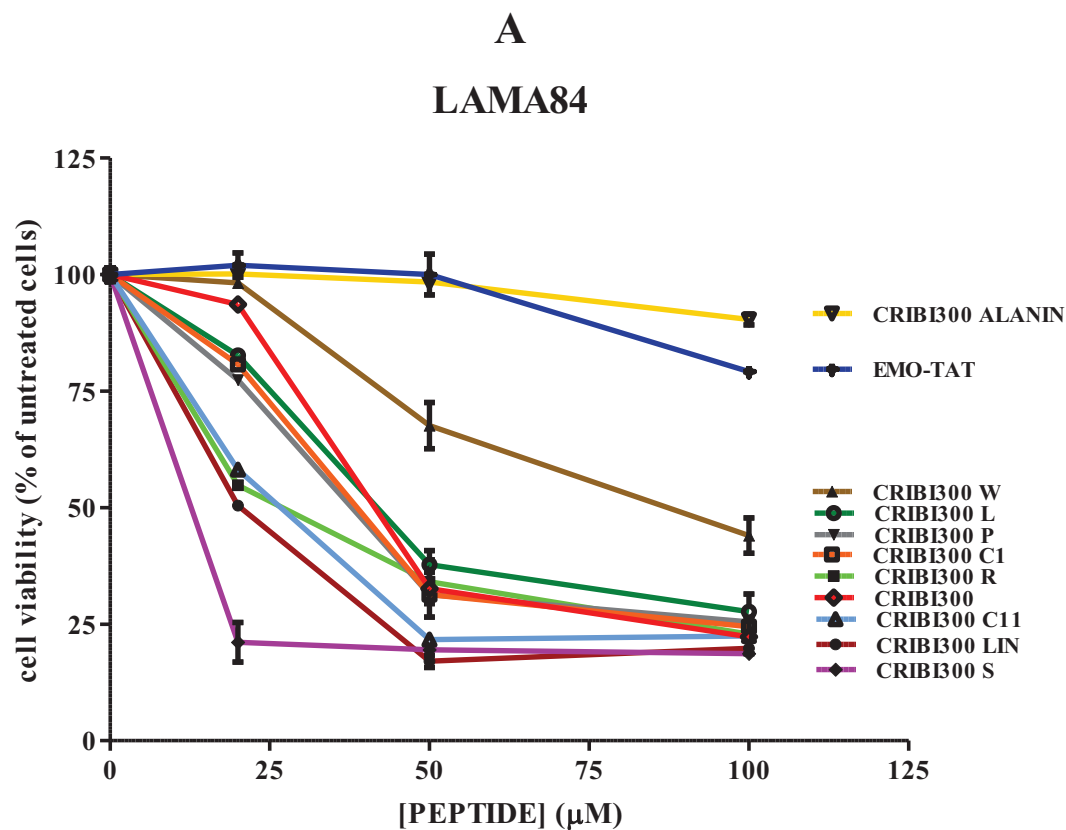


Figure 19. Cell viability of Tat peptide-treated cells. Cells were incubated with increasing concentrations of the Tat peptide for 24 h; cell viability was assessed by the MTT method, assigning 100% value to the vehicle-treated control cells. Mean ± SE values of at least three independent experiments are reported.

We performed a comparative analysis by assaying cell viability with several variants of CRIBI-300 available (see Table 1). First we found that EMO-Tat and CRIBI-300-ALA are almost ineffective as observed on the *in vitro* activity. Then we analyzed the effects of peptides presenting a point substitution of a single aminoacid in P15 domain, in order to assess which are the aminoacid essential for the CRIBI-300 effects. The results shown in Figure 20 and Table 3 for HL60 and LAMA84, revealed that some substitutions are indeed more detrimental, such as that of the tryptophan, at least in LAMA84 cells. On the contrary, some variants are even more effective than the parental peptide, such as those devoid of Ser or Cys of P15.



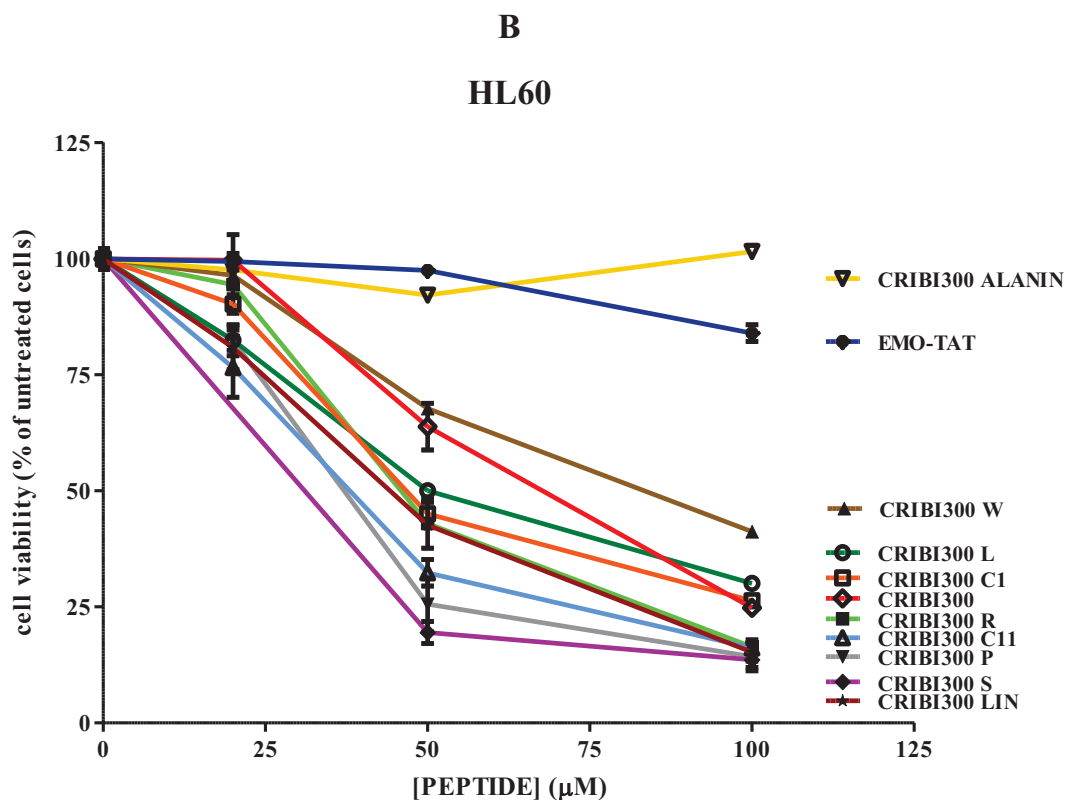


Figure 20. Cell viability of LAMA84 and HL60 cells treated with CRIBI-300 and CRIBI-300 derivatives peptide. LAMA84 (A) and HL60 (B) cells were incubated with increasing concentrations of the CRIBI-300 and CRIBI-300 derivatives for 2 h; cell viability was assessed by the MTT method, assigning 100% value to the vehicle-treated control cells. The colors assigned to each peptide correspond to those in Table 1. Mean $\pm$ SE values of at least three independent experiments are reported.

HL60			LAMA84		
PEPTIDE	DC ₅₀ (μM) (2h)	SD	PEPTIDE	DC ₅₀ (μM) (2h)	SD
CRIBI-300	67.6	5.6	CRIBI-300	55.0	8.0
CRIBI-300 ALA	n.d	-	CRIBI-300 ALA	n.d	-
EMO-TAT	n.d	-	EMO-TAT	n.d	-
CRIBI-300 LIN	44.2	3.6	CRIBI-300 LIN	25.5	6.7
CRIBI-300 C1	46.0	2,8	CRIBI-300 C1	46.0	2.8
CRIBI-300 R	45.8	0.6	CRIBI-300 R	32.3	4.7
CRIBI-300 C11	38.0	0.8	CRIBI-300 C11	26.1	1.6
CRIBI-300 P	37.1	1.9	CRIBI-300 P	38.0	2.9
CRIBI-300 S	31.1	2.0	CRIBI-300 S	12.7	1.1
CRIBI-300 L	51.6	2.8	CRIBI-300 L	41.9	1.8
CRIBI-300 W	83.3	0.7	CRIBI-300 W	86.9	12.2

Table 3. 2 h DC₅₀ of CRIBI-300 and CRIBI-300 derivatives. The values, calculated from 2 h MTT assays reported in Figures 20 are the mean of 4 independent experiments $\pm$ Standard Deviation (SD).

CELL DEATH INDUCED BY CRIBI-300 IS NOT APOPTOSIS

Once determined that CRIBI-300 induces very fast cell death in tumor cells, we wanted to investigate if this is due to apoptosis. For this purpose we exploited different techniques; firstly we checked caspase activation and the consequent caspase-dependent cleavage of PARP, by analyzing WB of total cell lysates. Figure 21 shows that PARP is not cleaved in response to CRIBI-300 treatments up to 80 μ M and 12 h. The same applies to pro-caspase-3, which is not degraded and activated to caspase-3. This was surprising since CK2 amount and activity reduction observed in the same experimental conditions (up to 50% after 2 h 80 μ M see Figure 16) should correlate with the occurrence of apoptosis.

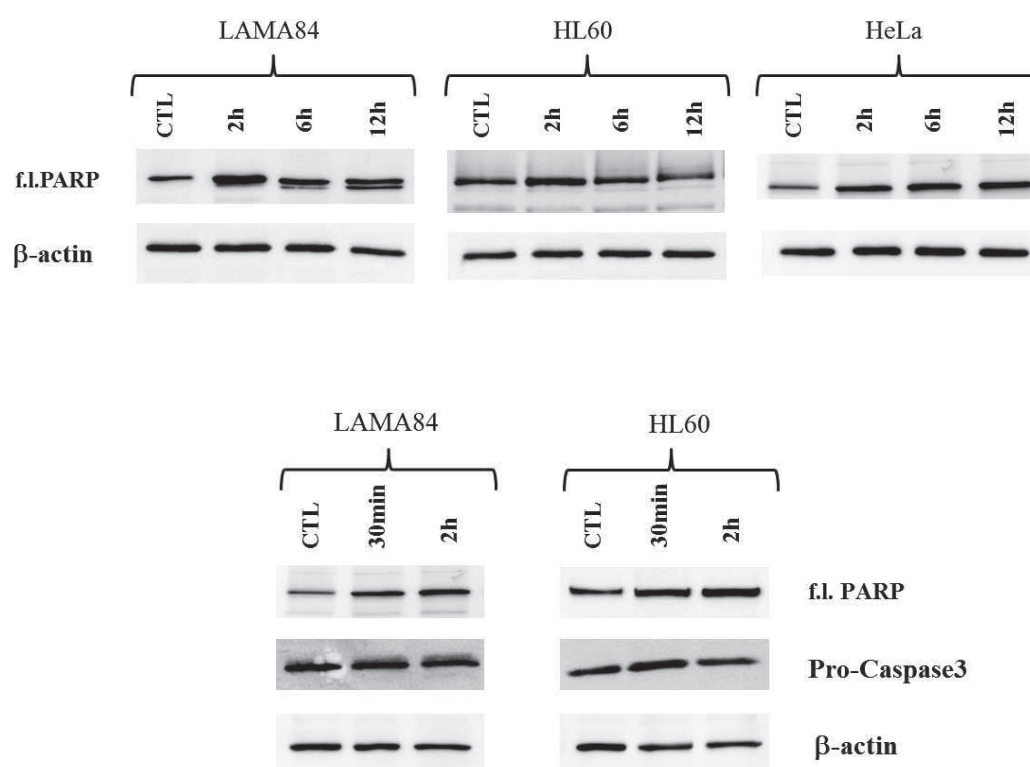


Figure 21. Detection of apoptosis in CRIBI-300 treated cells. Cells were treated with 80 μ M CRIBI-300 for the indicated times and 10 μ g of proteins from total lysates were analyzed by WB with the indicated antibodies. β -actin was used as loading control. Representatives WB of three independent experiments are shown. f.l. PARP: full length PARP.

We also tried to evaluate apoptosis by different methods, such as the detection of DNA-laddering (Figure 22) and of nucleosomes release (by a commercial kit) (not shown): in no cases we observed apoptotic events. Once excluded apoptotic cell death, we hypothesized that CRIBI-300 could induce necrotic death. In support of

this hypothesis, we observed lactate-dehydrogenase (LDH) release in the supernatant of treated cells and increase in LHD activity in a time-dependent manner after CRIBI-300 treatment (not shown). However, these are preliminary data that have to be confirmed and more deeply investigated.

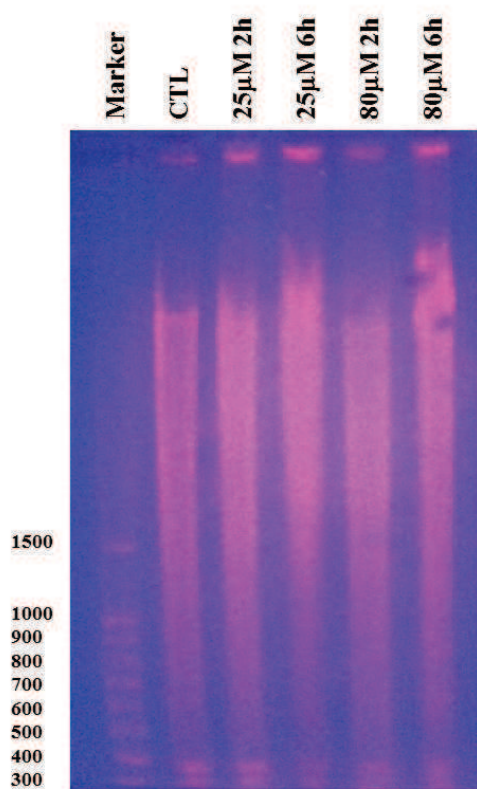


Figure 22. Effects of CRIBI-300 on DNA-laddering. 10 µg of extracted DNA from CRIBI-300 treated S-CEM for the times and concentrations indicated, were separated on a 1.7 % agarose gel as described in Methods. Marker: 100bp DNA Ladder (Promega) CTL=control (cells incubated in the absence of CRIBI-300).

CHANGES IN CK2 SUBCELLUAR LOCALIZATION UPON CRIBI-300 TREATMENT

In order to better understand the events involving CK2 in response to CRIBI-300, we decided to investigate if CK2 changes its subcellular localization after CRIBI-300 cell treatment. We performed confocal microscopy analysis; the immunofluorescence images obtained from CEM cells are shown in Figure 23. CK2 (red) is clearly detectable in the cytoplasm and also in the nucleus in control cells, but, interestingly, it moves out from the nucleus after a very short time of treatment

(30 min), and it almost completely accumulates in the cytoplasm, with a punctuate distribution after 2 h treatment. Again, as observed for the CK2 amount reduction, the effects are very similar in S-CEM and R-CEM, but slightly slower in R-CEM (Figure 24). Immunofluorescence experiments performed in other tumor cell lines confirmed this early change in CK2 subcellular localization (see U2OS cells in Figure 25).

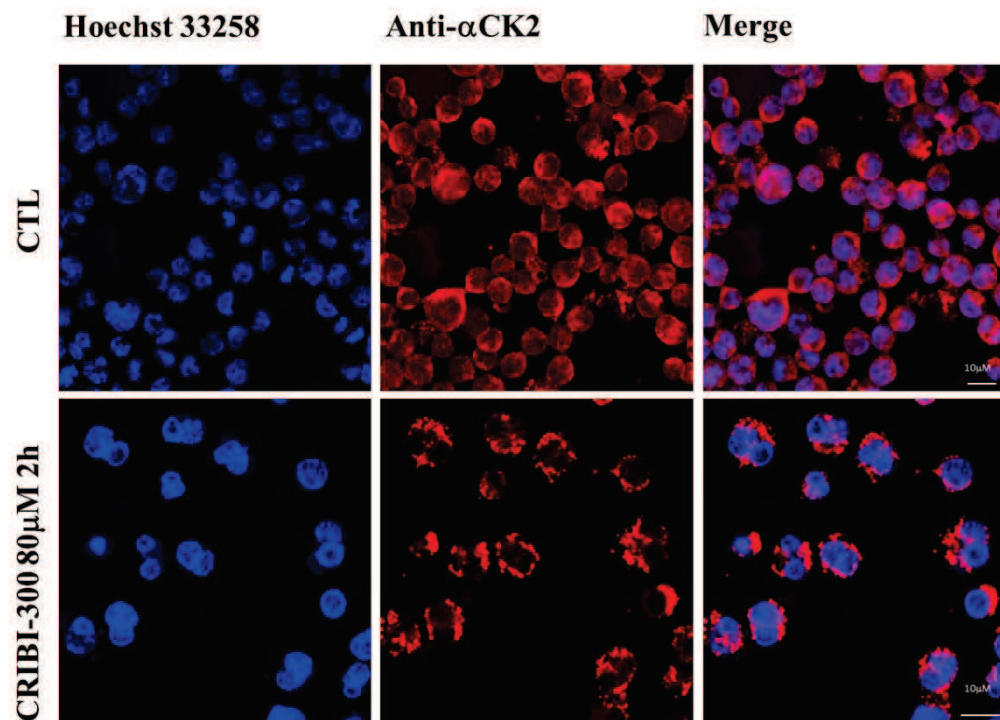


Figure 23. Effects of CRIBI-300 in CK2 subcellular localization in S-CEM cells. S-CEM cells were treated as indicated, then stained with anti- α CK2 (conjugated to Alexa 633) (red); nuclei were stained with Hoechst 33258 (blue). Fluorescence images were captured with confocal microscopy. Merge images are shown, where CK2 and DNA colocalization gives violet signal. Representative images of six independent experiments are shown.

CK2 confocal images of cells treated with apoptosis-inducers have never shown such a behaviors for CK2 (not shown). This suggests that this punctuated CK2 pattern is a specific response to CRIBI-300, not related to the cell death events; moreover, the CRIBI-300 induced CK2 localization may be related to its degradation (see above). Indeed, we hypothesized a possible localization of CK2 at level of proteolytic systems, such as lysosomes.

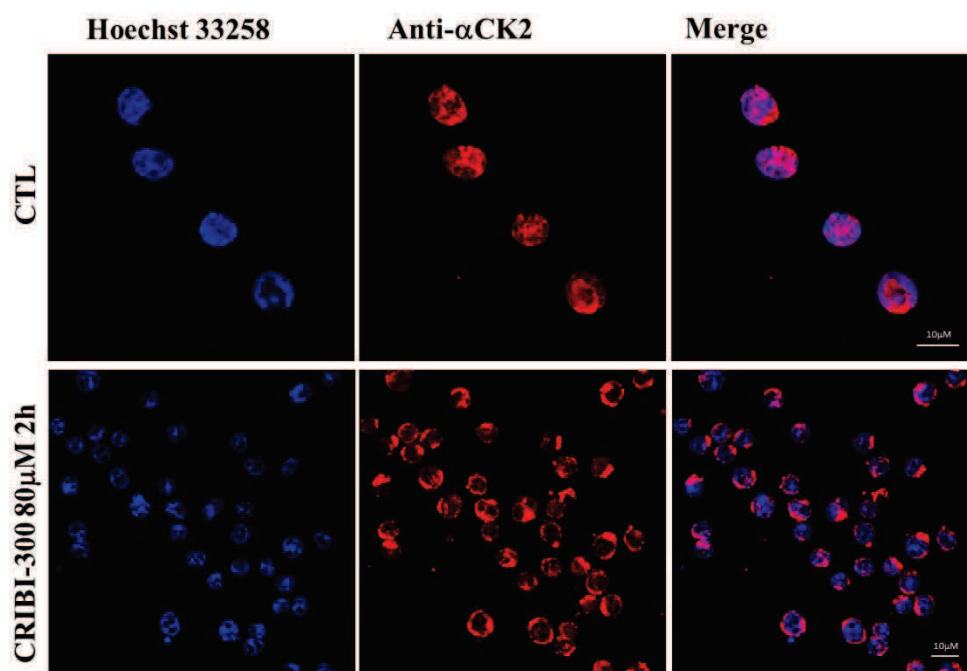


Figure 23. Effects of CRIBI-300 in CK2 subcellular localization in R-CEM cells. For details see Figure 24.

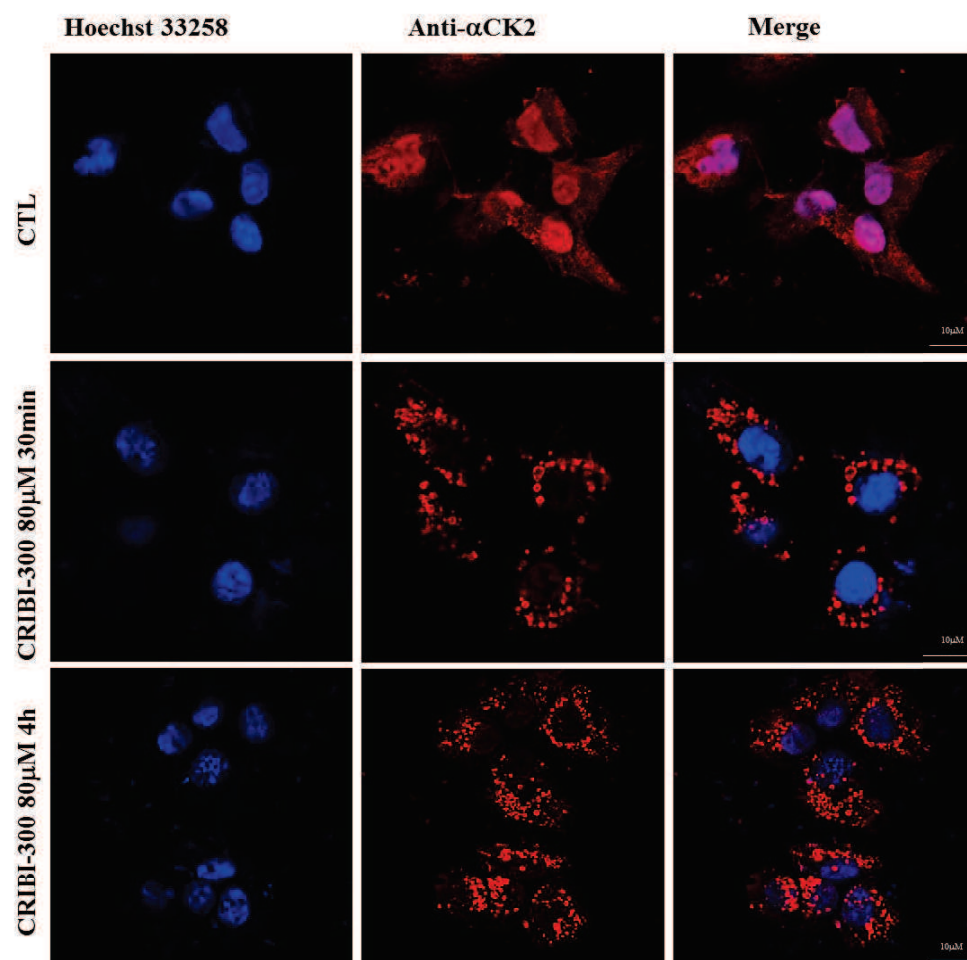


Figure 25. Effects of CRIBI-300 in CK2 subcellular localization in U2OS cells. For details see Figure 24.

To check this hypothesis we exploited co-localization confocal images of CK2 and LAMP1, a protein used as a lysosomal marker. Images are shown in Figure 26, for experiments performed in LAMA84 cells. Firstly they demonstrated that CRIBI-300 induces the activation of lysosomal system as judged from the strongly increased LAMP1 signal. Secondly, they show that CK2 partially co-localized with LAMP1 after 2 h CRIBI-300 treatment, confirming our hypothesis.

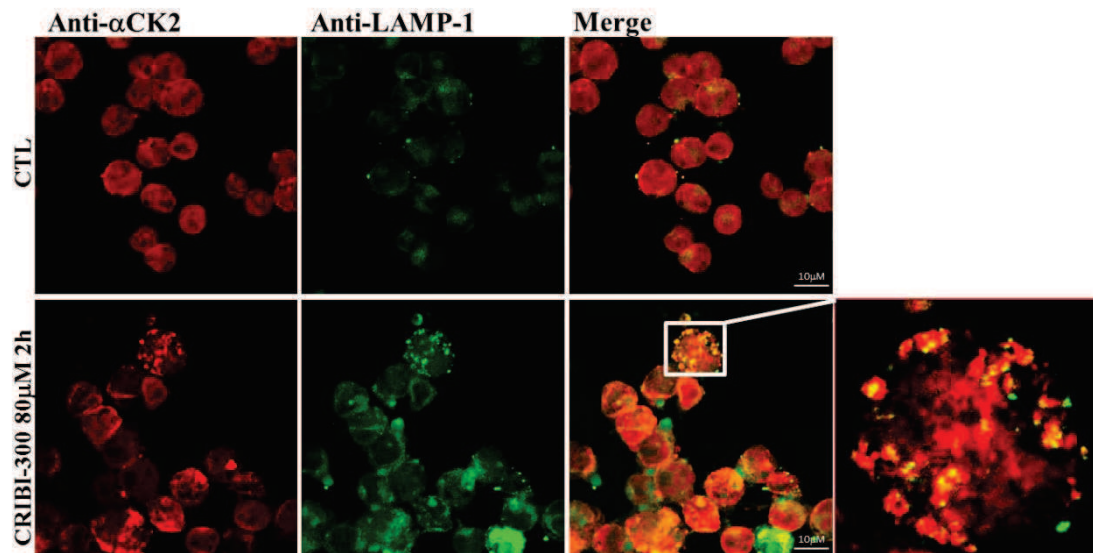


Figure 26. CK2 and LAMP1 co-localization. LAMA84 cells were stained with anti- α CK2 conjugated with Alexa Fluor 633 (red) and with anti-LAMP1 conjugated with FITC (green). Fluorescence images were captured using confocal microscopy. Merge images are shown, where CK2 and LAMP1 colocalization gives yellow signal. Representative images of three independent experiments are shown. Image in the square is magnified (5-fold) on the right.

CRIBI-300 seems also to affect the nuclear structure of the cells. From *in vivo* time-lapse analysis (see Figure 28), DNA signal gradually disappears over time, suggesting that possible side effects are triggered by CRIBI-300 in tumor cells after very short treatment times.

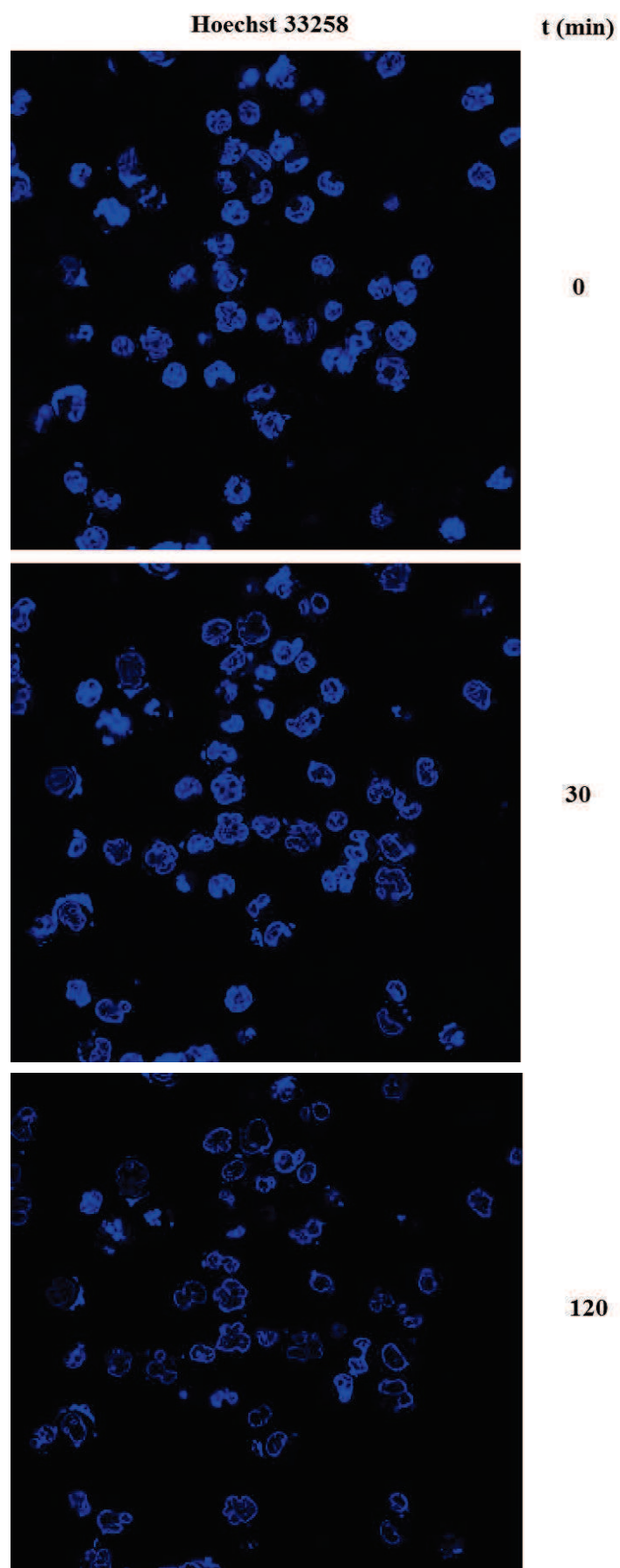


Figure 28. Nuclear changes after CRIBI-300 treatment. LAMA84 cells were stained with Hoechst 33258 for 15 min at 37°C and maintained in an isotonic buffer for 2h. Fluorescence images were captured in vivo every 2 min, using confocal microscopy. Representative images experiments are shown.

EFFECTS OF CRIBI-300 ON PROTEASOMAL AND LYSOSOMAL PROTELYTIC SYSTEMS.

The observation that CK2 amount decreases in cell treated with CRIBI-300, and that lysosomal activation seems to occur, as detected by immunofluorescence of LAMP1 protein, prompted us to investigate on a possible action of CRIBI-300 on proteolytic systems. First, we confirmed the increase in LAMP1 amount by means of WB analysis (Figure 29 A): in two different cell lines (LAMA84 and HL60) LAMP1 amount is dramatically higher after 2 h treatment with 80 μ M CRIBI-300 than in untreated cells. We also checked the possible involvement of the proteasome system, by analyzing the ubiquitination state of cellular proteins by means of an antibody recognizing polyubiquitinated proteins, a condition required for the recognition and subsequent degradation by proteasome (Andersen *et al.*, 2005). We observed an increase of poly-ubiquitinated proteins in a time-dependent manner in LAMA84 cells treated with CRIBI-300, with a maximal after 12 h (Figure 29 B). The accumulation of ubiquitinated proteins suggests a possible impairment of the proteasome activity, whose reason needs further investigation to be understood (see Discussion). Furthermore, the decrease of ubiquitinated signal observed at longer times (see 24 h in Figure 29 B) might indicate that the blockage of the proteasome could be counteracted by the activation of alternative pathways for protein degradation which intervene to solve proteasome engulfment. Of course more studies will be necessary; however, we have preliminary data that suggest that also the catabolic autophagic process is triggered by CRIBI-300. In fact, p62, a protein that normally is degraded in autophagy (Lin *et al.*, 2013), gradually disappears in cells treated with CRIBI-300 (Figure 29 B) indicating that its degradation occurs.

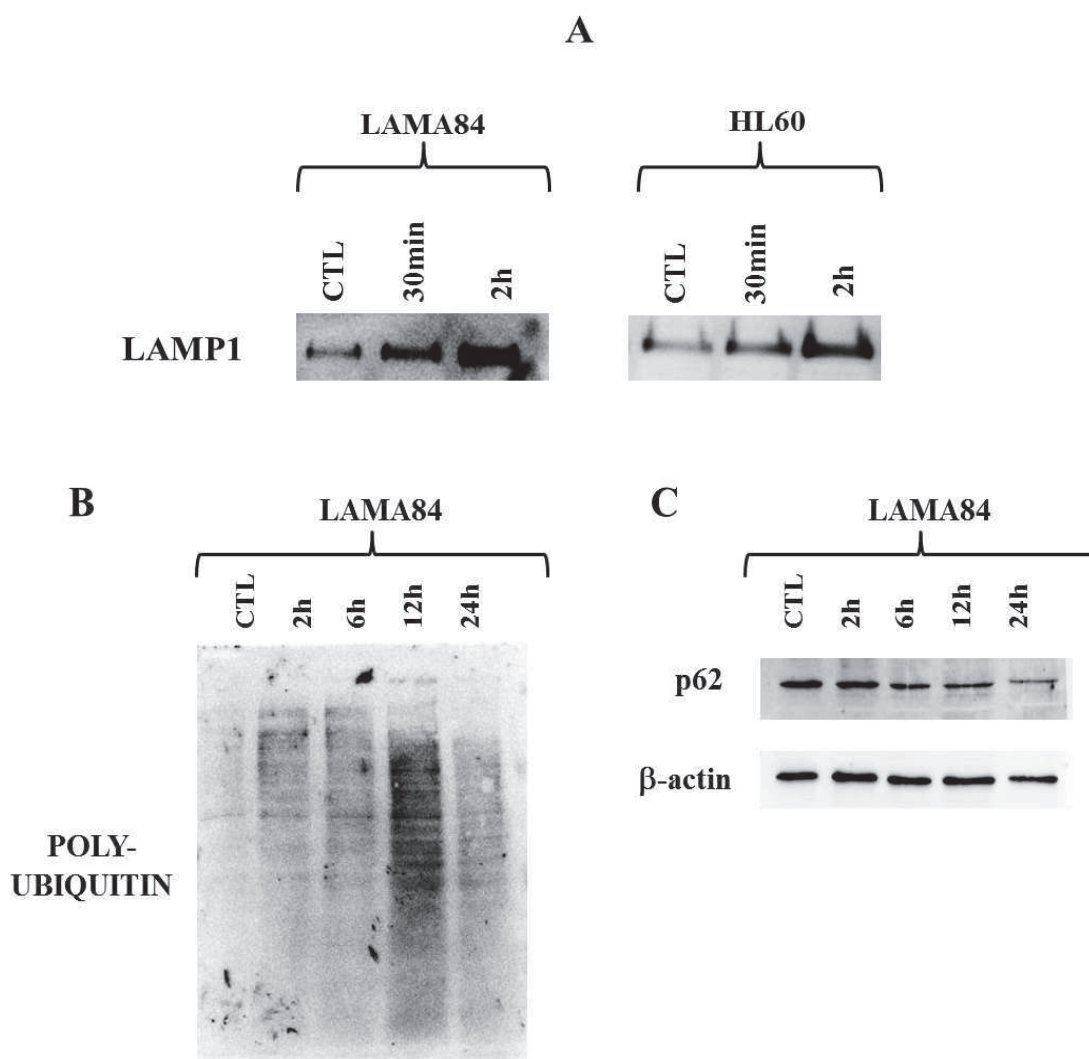


Figure 29. Effects of CRIBI-300 on cellular proteolytic systems. The indicated cells were treated with 80 μ M CRIBI-300 for the indicated times, then 20 μ g of cell lysate proteins was analyzed by WB for LAMP1 (whose amount correlates with lysosomal protease activity) (A), or poly-ubiquitin, whose accumulation indicates proteasome function defects (B), or p62, whose degradation is a marker of autophagy (C). β -actin was analyzed as loading control. CTL = cells incubated for 24 h in the absence of CRIBI-300.

EFFECTS OF CRIBI-300 ON THE AMOUNT OF OTHER CELLULAR PROTEINS

Since we have found that CK2 amount rapidly decreases after CRIBI-300 treatment and that proteolytic system activities are affected by the peptide, we verify if also the amount of other proteins is changed. We therefore exploited WB with available antibodies to check the amount of several proteins which are particularly relevant, being known as CIGB-300 targets (B23 and S6, Perea *et al.*, 2008) or involved in

CK2-dependent signaling (such as Akt, Hsp90 and Cdc37, Ruzzene and Pinna, 2010). From WB analyses, we observed that also the amount of B23, the major target of CIGB-300 (Perea *et al.*, 2008) and known substrate of CK2, decreases, although not as rapidly as CK2: figure 30 shows a B23 reduction of about 70% in LAMA84 cells after 2h treatment and an almost total disappearance in S-CEM and HL60. Another protein that we analyzed is the ribosomal protein S6 (rpS6), which was also reported to be a target of CIGB-300 (Perea *et al.*, 2008). Also rpS6 is rapidly degraded after CRIBI-300 treatment; for example, in S-CEM, it completely disappears after 30 min at 80 μ M treatment (Figure 30). Structural proteins, such as β -actin and α -tubulin seem to be more resistant to degradation, which however occurs after 2 h treatment in S-CEM (Figure 30). Other proteins such as Akt, Hsp90 and Cdc37 seem to be more stable (Figure 30). Since we observed a powerful effect of CRIBI-300 on nuclear structure (see above) we also analyzed its effect on histones, by exploiting an antibody toward H2B and H3 histones. We found that they totally disappear after 30 min in LAMA84 and HL60 (not shown). From our results, therefore, we can conclude that not only CK2 but several other proteins undergo a probable degradation in response to CRIBI-300. However, with the exception of histones, CK2 cleavage seem to be one of the earliest event, consistent with the hypothesis that CRIBI-300 directly binds to CK2, as suggested by *in vitro* experiments. We also checked the effects on the amount of CK2 and of other proteins in response to CRIBI-300-ALA and EMO-Tat treatments; in all cases, as observed for CK2 activity *in vitro* and for CK2 viability, they were inactive on the protein level amounts up to 24 h treatment (Figure 31).

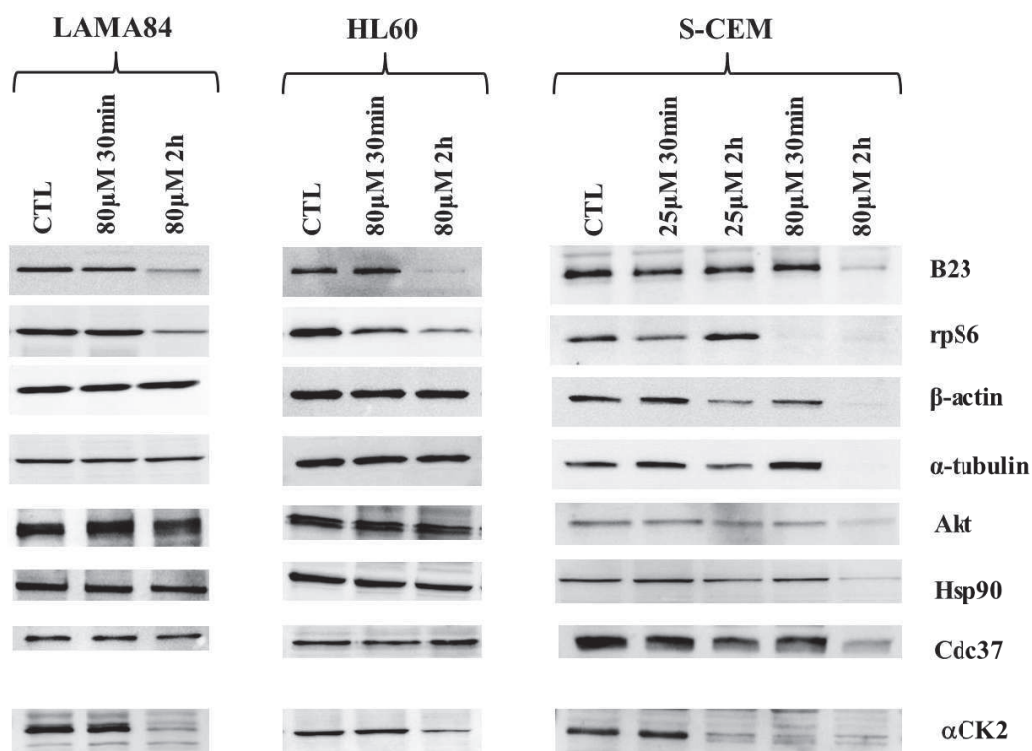


Figure 30. Effects of CRIBI-300 on the amount of other cellular proteins compared to that of CK2 α . Cells were treated with increasing concentrations of CRIBI-300 as indicated and for the indicated times. 10 μ g of total proteins was analyzed by WB with the indicated antibodies. Representative WB of three independent experiments are shown.

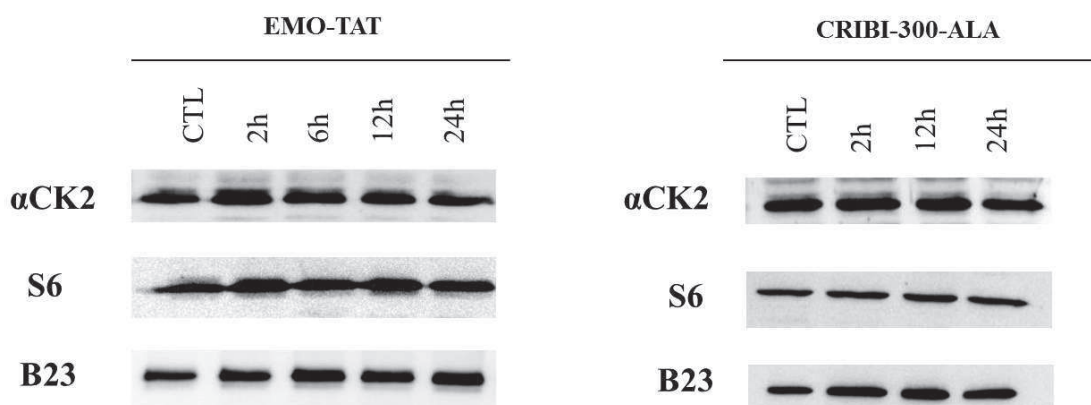


Figure 31. Effects of EMO-TAT and CRIBI-300 ALA on the protein amounts. LAMA84 were treated with the indicated CRIBI-300 derived peptides, EMO-Tat and CRIBI-300 ALA, respectively, at 80μM concentrations for the indicated times. 10 μ g of total proteins was analyzed by WB with the indicated antibodies. Representative WB of three independent experiments are shown.

EFFECTS OF CRIBI-300 IN CK2 KNOCKED-DOWN CELLS

In order to understand if CK2 degradation is an initial and essential cellular event which mediates the effect of CRIBI-300, we checked if it is less effective in cells where CK2 α and β amounts are reduced. We therefore performed RNA interference experiments to silence α and β CK2 expression with specific siRNA. Then cell treatments of LAMA84 with CRIBI-300 were performed. Firstly we checked the level of α and β amount to confirm silencing. As shown in Figure 32, the approach proved successful for diminishing the level of CK2 α expression of about 50%, while CK2 β expression was reduced only of about 20%. Then we analyzed the effect of a 2 h treatment with 80 μ M CRIBI-300. As shown in Figure 32, when CK2 α is partially silenced, the effect of CRIBI-300 on its amount is less dramatic if compared with cells where CK2 expression is normally high. Compared to the relative control (not CRIBI-300 treated cells), CRIBI-300 induced a 25% reduction of CK2 α in α knocked-down cells, while 70% reduction is observed in not knocked-down cells. The difference is even more dramatic when the effect of CRIBI on β is observed: CRIBI-300 is ineffective in α knocked-down cells (60% reduction in not α knocked-down). Also in the case of CK2 β knocked-down the effects of CRIBI-300 are reduced (α decrease of 10% instead of 80%, β of 10% instead of 70%). This suggests that knocking-down α or β CK2 partially removes from cells major CRIBI-300 targets and its effects are less dramatic. However, more work is necessary to better understand these results, since, when we analyzed the amount of other proteins, such as rpS6 (Figure 32) we found that it is strongly reduced even in cells where CK2 α or β are silenced.

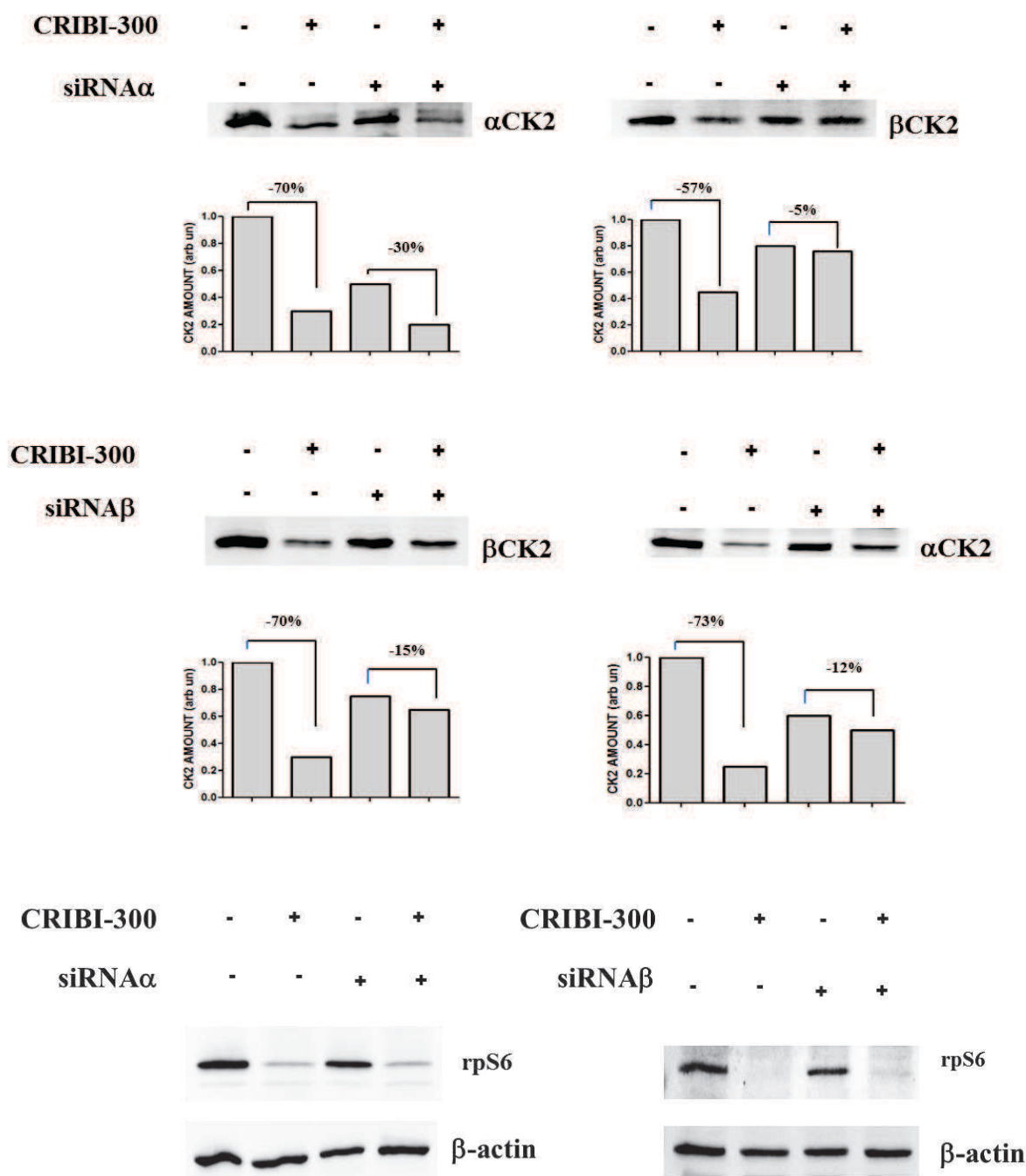


Figure 32. Effects of CRIBI-300 in CK2 silenced cells. LAMA84 cells were transfected for 72h with specific CK2α or β siRNA, as indicated. Control cells were transfected with aspecific siRNA. Then cells were incubated with or without 80μM CRIBI-300 for 2 h, lysed, and 10 μg of total protein samples was analyzed by WB as indicated. A quantitation of CK2 α and β bands is presented in the histogram, performed by Kodak 4400MM PRO Software. The % of band decrease induced by CRIBI-300 compared to each control is reported above the bands. Representative WB of three independent experiments are shown.

DISCUSSION

In this work we analyze the action *in vitro* and *in cell* of the CRIBI-300 cyclic peptide, whose structure resembles that of the compound CIGB-300 (Perea *et al.*, 2004) recently advanced in clinical trials (Perea *et al.*, 2011). Both peptides are composed of a functional part, called P15, fused to the cell-penetrating peptide Tat (Holm *et al.*, 2006). However, Tat sequence is at the C-terminal side of P15 in CIGB-300, at the N-terminal side in CRIBI-300. Our *in vitro* results are very similar for the two compounds, showing that they exert effects on recombinant CK2; therefore, most of the experiments were then performed only with CRIBI-300, whose sequence was also variably modified, in order to obtain information on the minimal requirements for the peptide activity.

Despite it was designed to interfere with the site of phosphorylation by CK2 in some of its substrates, CIGB-300 is frequently defined as a CK2 inhibitor (Perea *et al.*, 2004). However, we found that the effect of CRIBI-300 on CK2 activity were different depending on the type of substrate used. In fact, an activation by CRIBI-300 occurs towards substrates which are preferentially phosphorylated by the CK2 monomeric α catalytic subunit (α -casein, total casein, p53, calmodulin, see Figure 10), while the phosphorylation of substrates strictly dependent on the presence of CK2 β subunit is instead impaired (eiF2 β -tide, see Figure 4). More complex are the effects observed on the CK2 substrate nucleophosmin/B23 (Louvet *et al.*, 2006): we obtained poorly reproducible results (Figure 13). However this can be partially explained by a double effect, exerted by CRIBI-300 on both CK2 and B23, which is reported as the major target of CIGB-300 (Perea *et al.*, 2008). We have also to consider that different results were observed using recombinant B23 or immunoprecipitated B23: the recombinant protein could produce artifacts due to the presence of a poly(His) tag (which can also leads to the formation of B23 oligomers or aggregates, as sometimes detectable on the top of the gel after Coomassie blue staining or by anti-B23 WB (not shown)); on the other hands, B23 immunoprecipitated from cell lysate can be associated to cellular components producing a different action of CRIBI-300.

These first *in vitro* evidences suggest that CRIBI-300 interferes with CK2 and has a direct effect on the catalytic activity, beside of being able to bind to CK2 substrates.

This is also confirmed by the analysis of the CK2 autophosphorylation in the absence of any substrate. In particular, we observed that CRIBI-300 provokes a decrease in CK2 autophosphorylation that normally occurs on β subunit, but this is accompanied by a concomitant exceptional induction of CK2 autophosphorylation of α subunit. This is the well-known effect exerted by polybasic effector or polyamines (Meggio *et al.*, 1994) on CK2; we therefore hypothesized that, in the case of CRIBI-300, it was due to the Tat portion, whose aminoacidic composition is highly basic. However, the only Tat peptide was almost ineffective on CK2, excluding this possibility. Interestingly, also the mere P15 sequence was unable to produce any effect on CK2 autophosphorylation and *in vitro* activity, suggesting that the Tat sequence is necessary but not sufficient for CRIBI-300 binding to CK2 (Figure 12). As far as the importance of the exact sequence is concerned, we can say that a certain composition, more than the sequence, is required to induce the reduction of CK2 activity towards β -dependent substrates, since a scramble peptide (with P15 composition) is also effective, while a completely different sequence has only weak effects (Figure 5 and 8). Moreover, peptide cyclization (whose rationale is based on an expected higher resistance to cellular protease action) seems to be not required for the effects on CK2 (see Figure 7, where linear peptide is even more active than the cyclic one).

Altogether considered, our *in vitro* results suggest that CRIBI-300 could interfere with the CK2 holoenzyme structure, producing an unstable and/or unfunctional tetrameric conformation which prevents the phosphorylation of β -dependent substrates, increases that of α -preferred substrates, and induces a polybasic-like effect on autophosphorylation. This would also provoke a higher susceptibility to proteolytic cleavage (Marin *et al.*, 1995), and is in perfect accordance with our *in cell* observation (see below). Indeed, preliminary results suggest that *in vitro* digestion of CK2 α by recombinant proteases is accelerated by the presence of CRIBI-300 (not shown).

A compound that bind to CK2 by provoking β subunit removal or holoenzyme disruption could dramatically change the pattern of CK2-dependent phosphorylation since the free catalytic subunit and the holoenzyme have quite different substrate preferences (Pinna, 2002). Therefore, expanding our observations to a cellular environment, we can imagine that CRIBI-300 could

interfere with the crucial balance that regulates all the cellular processes governed by CK2. Indeed, when we moved to analyze the cellular effects of CRIBI-300 we observed a very fast induction of cell death in tumor cells, as already demonstrated for CIGB-300 (Perea *et al.*, 2004). However, at variance to what reported for CIGB-300, we have never found any symptom of apoptosis, such as PARP cleavage, caspase activation (Figure 21), DNA-laddering (Figure 22) or nucleosomes formation (not shown). We suppose that cell death occurs with the intervention of proteolytic system other than caspases (see below).

Interestingly, we observed that tumor cells are differently sensitive to CRIBI-300: leukemic cells seem to be more susceptible to the peptide than cells deriving from solid tumors (Figure 18 and Table 2). We have hypothesized that cells deriving from blood tumors, growing in suspension in the culture medium, expose entirely their plasma membrane surface to the compound; on the contrary, cells from solid tumors, which grow adherent to plates, offer a lower surface available to the interaction with the peptide. We tested this possibility performing cell treatments with CRIBI-300 with solid tumors cells, but maintaining them in suspension; however, we did not observed an increased sensitivity towards CRIBI-300, compared to adherent cells (not shown). Another possibility is that the differences in the sensitivity to CRIBI-300 between leukemic and solid tumor cell lines is due to more efficient mechanisms of Tat-cargoes internalization of plasma membrane in blood tumor cells. Future studies will be necessary to shed line on this point. We have also found that CRIBI-300 is almost ineffective in inducing cell death in non-tumor cells (Figure 18); this is in accordance with several other observations obtained by using canonical ATP-competitive CK2 inhibitors, and explained by the concept of the addiction of cancer cells to CK2 (Ruzzene and Pinna, 2010).

In accordance to the observations already discussed from *in vitro* assays on recombinant CK2 activity, we found that also the induction of cell death by CRIBI-300 requires the presence of both P15 and Tat sequence, but that a precise sequence is not essential, since the scramble peptide, containing the same aminoacidic composition of P15 but randomized compared to CRIBI-300, induces the same level of cell death then CRIBI-300. The two Cys of P15 (see Table 1) are essential, since their substitution to Ala completely abrogates CRIBI-300 effects in cells; however, their role in cyclization is not important, since a linear peptide where the two Cys are maintained is even more active than the cyclic peptide (Figure 20).

Furthermore, point substitutions on the hydrophobic aminoacids of P15 with alanines have shown that tryptophan could be particularly important for the action of CRIBI-300 on cell death, at least in LAMA84 cells; however more detailed analysis on the effects of CRIBI-300 derivatives on cell viability will be necessary to better understand which are the most important aminoacid residue responsible of CRIBI-300 action. When we treated tumor cells with CRIBI-300, cell death was accompanied by a marked reduction in the endocellular CK2 activity, which is in accordance with the pro-survival role of CK2. However, this reduction was accompanied by a dramatic loss in CK2 amount even after very short treatment times (Figure 15 and 16). This is not a common event, and is usually not observed when canonical CK2 inhibitors are administrated to cells. In the case of CRIBI-300, we conclude that the reduction of CK2 activity observed in treated cells is not a real inhibition but is due to a reduced enzyme amount.

Considering the very short time required for its occurrence, we suppose that this reduction of CK2 amount is due to an increased degradation and not to a block in synthesis. This is also in accordance with our hypothesis formulated from *in vitro* data, assuming that CRIBI-300 interferes with the interaction between α and β in the holoenzyme structure; in fact, it is known that β protects α from proteolytic cleavage (Meggio *et al.*, 1992). By immunofluorescence experiments we also demonstrated that CK2 subcellular localization changes in a very short time after treatment, becoming more abundant in the cytosol in a punctuate pattern and apparently concentrated in the lysosomes, which are in turn activated as confirmed by increased level of LAMP1 (Figure 26).

We then analyzed other proteins possibly involved in mediating the effect of CRIBI-300; we found that also the amount of some of them is rapidly reduced by CRIBI-300. Several signals of perturbation in the proteolytic pathways were found in CRIBI-300 treated cells: an increase in ubiquitinated protein signal, suggesting the alteration in the proteasome machinery (Figure 29 B), the degradation of p62 protein, an autophagy marker (Figure 29 C), and a general activation of lysosomal proteases (Figure 29 A). Our evidences led us to formulate the following speculation: we noticed that the Ubiquitin Interacting Motif (UIM), a stretch of about 20 amino acid residues present in 26S proteasome subunit but also in other proteins known to recognize ubiquitinated molecules, is very similar to the CK2

consensus sequence (Miller *et al.*, 2004). Since the P15 portion of CRIBI-300 was developed with the aim of binding to CK2 substrates (Perea *et al.*, 2004), it could be able to bind not only to CK2 consensus site, but also to UIM. By this way, beside interfering with CK2 substrates phosphorylation, it could also block the proteasome-mediated degradation of ubiquitinated proteins. The consequent engulfment of proteasome machinery and accumulation of ubiquitinated proteins would in turn activate alternative pathways for their degradation. Indeed, functional links between the proteasome system and the lysosomal autophagic pathway are nowadays recognized (Koga and Cuervo, 2011), supporting switching toward different mechanisms for protein clearance. In this context, p62 protein could play an important role, since it is normally degraded during autophagy but also interacting with polyubiquitin chains and driving polyubiquitinated proteins to proteolytic pathways (Shin, 1998).

From all our findings we assume that the molecular mechanisms by which CRIBI-300 acts inside the cell are multiple. First, as already known for CIGB-300 (Perea *et al.*, 2008), CRIBI-300 interacts with its main target B23 protein and probably other ribosomal proteins, blocking their phosphorylation by CK2; secondly, from our *in vitro* data we conclude that CRIBI-300 undoubtedly acts directly on CK2, interfering with the holoenzyme, probably disrupting its stability; finally, our cellular results suggest that CRIBI-300 could interfere with the proteasome activity, leading to a general perturbation of cellular proteolytic systems.

We also wondered how much CK2 is involved in mediating the observed CRIBI-300 effects. CK2 seems to be one of the earliest targets, being very promptly degraded (Figure 15 and 16); moreover, preliminary experiments of CK2 knocking-down demonstrated that when CK2 amount is reduced, some CRIBI-300 effects are less evident (Figure 32). We also observed parallel effects induced by CRIBI-300 derivatives on CK2 activity *in vitro* and on cell death induction (compare for example the effects of CRIBI-300 ALA or EMO-Tat *in vitro* and in cells, Figure 5 and 6 with Figure 20), suggesting that cell death is indeed directly correlated to the targeting of CK2. However, it is evident that the profound cellular perturbation induced by CRIBI-300 is the results of several events involving multiple targets. This study has started to shed light on its complex mechanism of action, and we suggest that particular caution is required for its clinical employment.

**INHIBITION STRATEGIES
TARGETING A DIFFERENT KINASE,
THE “HOMODOMAIN-INTERACTING
PROTEIN KINASE 2” (HIPK2)**

INTRODUCTION

Small cell permeable inhibitors of protein kinases have become very important molecules for dissecting signaling pathways mediated by each of them. Many compounds have been proposed as “specific” for a certain protein kinase. However, since the human kinome is composed of more than 500 members, the issue of selectivity is critical and only a limited number of inhibitors have been really shown to display a narrow selectivity window hitting only few or one individual protein kinases (Bain *et al.*, 2007). Data presented in this chapter are the result of a collateral study, on a kinase, HIPK2, which was among those inhibited by some CK2-directed inhibitors. In fact, during the development of CK2 inhibitors, the *in silico* modification of existing compounds was disclosed in order to obtain a molecule not effective on CK2, but selective for HIPK2. This kinase is a member of the so called “homeodomain-interacting protein kinases” (HIPKs) family, belonging to CMGC group of the kinome, and no specific inhibitor for this kinase was available.

HIPK2 has been identified for its interaction with the homeodomain transcription factor NK-3 (Kim *et al.*, 1998). It is a kinase conserved from flies to human, and plays a critical role in the regulation of cell growth, development, morphogenesis, transcription and cell death in multiple organisms and cell types (Hofmann *et al.*, 2002; D’Orazi *et al.*, 2002). It is considered as a potent growth suppressor and apoptosis activator (Calzado *et al.* 2007; Rinaldo *et al.*, 2007). The mechanism by which HIPK2 mediates apoptosis has been described; the phosphorylation of p53 Ser46 in the C-terminal domain stabilizes the binding between p53 and DNA (Puca *et al.*, 2008) and leads to p53-mediated transcriptional activation of proapoptotic factors such as Bax, Noxa, and Puma (D’Orazi *et al.*, 2002; Di Stefano *et al.*, 2004). As illustrated in Figure 1, the p53 Ser46 phosphorylation is considered to be a sensor for DNA-damage intensity that induces a fine change of p53 conformation varying the p53 affinity for different promoters with a shift from cell cycle arrest related genes (i.e. MDM2) toward proapoptotic genes (i.e. p53AIP1) (Oda *et al.*, 2000; Mayo *et al.*, 2005). Accordingly, HIPK2 was found to be activated in response to DNA-damage inducing stimuli, including UV or infrared

(IR) irradiation, or treatment with genotoxic chemotherapeutic drugs such as adriamycin and cisplatin (Hofmann *et al.*, 2002; Rouse *et al.*, 2002).

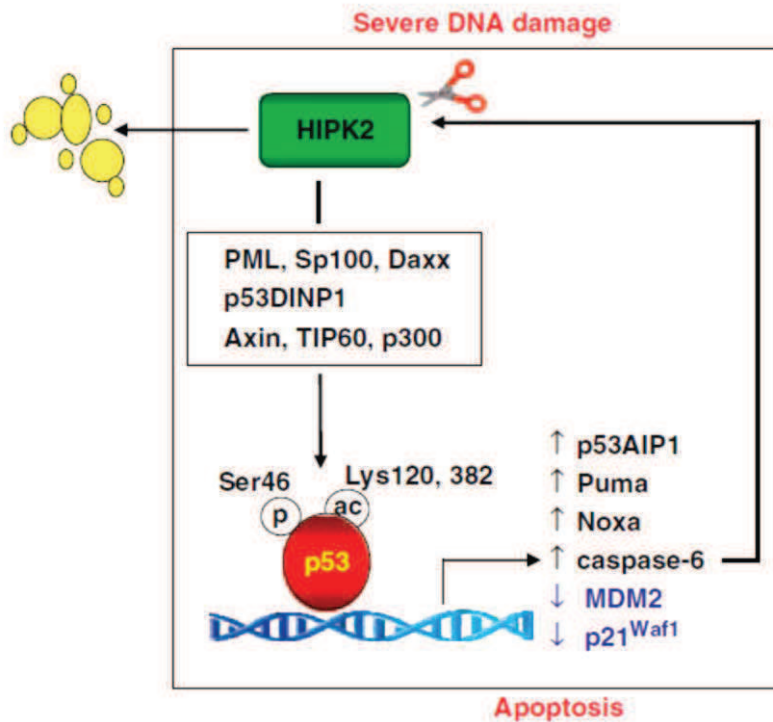


Figure 1. Schematic representation of p53 apoptotic regulation by HIPK2. HIPK2 phosphorylates p53 at Ser46 to direct p53 transcription toward proapoptotic genes rather than cell cycle (p21) or p53 regulatory (MDM2) genes. Important cofactors for HIPK2-induced p53 Ser46 phosphorylation (PML, Daxx, Axin and so on) and p53 acetylation (that is, p300, TIP60), which efficiently induce apoptosis, are reported in the box. The regulatory loop between p53 and HIPK2 leading to HIPK2 proteolysis during the termination phase of apoptosis, through p53-induced caspase-6 activation, is shown (from Puca *et al.*, 2010).

HIPK2 can be therefore considered as a tumor-suppressor element, and this correlates with the tendency of human cancer cells to inactivate HIPK2 through different mechanisms. For instance, HIPK2 is transcriptionally downregulated in thyroid and breast carcinoma specimens (Pierantoni *et al.*, 2002), and it was found to be mutated in a very small subset (2 out of 130 cases) of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) patient, (Li *et al.*, 2007), although these rare mutations do not clearly support a causative role of HIPK2 in the pathogenesis of these tumors. Interestingly, high-mobility group A1 protein (HMGA1), a proto-oncogene overexpressed in several cancer, (Fusco and Fedele,

2007), was shown to inactivate HIPK2 by removing it from the cell nucleus and retaining it in the cytoplasm.

However, recent reports have presented data in contrast with a HIPK2 role as a tumor suppressor. In fact, HIPK2 amplification occurs in pilocytic astrocytomas, the most common glia cell-derived brain tumor (Deshmukh *et al.*, 2008). Consistently, HIPK2 overexpression in a glioma cell line resulted in increased cell growth and not in growth arrest, as reported for several other tumors (D'Orazi *et al.*, 2002; Giraud *et al.*, 2004; Doxakis *et al.*, 2004), but the molecular mechanism by which HIPK2 can increase cell growth still needs to be clarified.

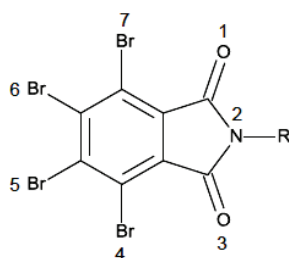
Therefore, for all its implications in signaling pathways and its double role in controlling cell death/cell growth, HIPK2 represents an interesting kinase whose functions have not been entirely characterized yet.

The only HIPK2 inhibitor mentioned in the literature, some years ago, was SB203580, a p38 MAP kinase inhibitor employed to block also HIPK2 (Di Stefano *et al.*, 2004) due to its similarity to p38. Later, several laboratories exploited SB203580 as a “HIPK2 inhibitor” (Roscic *et al.*, 2006; Yamada *et al.*, 2009), considering it a selective agent for this kinase, even if, from profiling studies of SB203580 on a panel of 71 protein kinases at 1 μ M concentration, inhibition of HIPK2 was negligible (14%) (Bain *et al.*, 2007). Moreover, the members of the HIPK family are not among the kinases inhibited by SB203580 (nor by any other compound examined) in a comprehensive profiling of kinase inhibitor selectivity (Anastassiadis *et al.*, 2011). This sheds doubts on the interpretation of the effects of SB203580 as really mediated by cellular HIPK2 blockage.

During studies conducted on CK2 in our laboratory, it has been observed that several and potent CK2 inhibitors such as TBB, TBI and other related tetrabromo-benzimidazole derivatives, also exert a drastic effect on HIPK2 (Pagano *et al.*, 2008; Sarno *et al.*, 2011) and a few other protein kinases. Therefore, modifications of the tetrabromo-benzimidazole scaffold were designed, focused on decreasing the efficacy toward CK2 and other kinases drastically inhibited by TBI and TBB, while maintaining or eventually improving that toward HIPK2. One of these derivatives, 4,5,6,7-tetrabromo-2-(1*H*-imidazol-2-yl)isoindoline-1,3-dione (TBID), has been found that selectively inhibit HIPK2 *in vitro*.

TBID STRUCTURE, *IN VITRO* ACTIVITY AND SELECTIVITY

Previous studies have shown that TBB and TBI, two commonly used CK2 inhibitors, also share the ability to inhibit HIPK2 besides CK2 (Pagano *et al.*, 2008). These compounds present a number of bromine atoms clustered on their benzene ring, which are essential for interaction with the kinase active site (Battistutta R *et al.*, 2001; Battistutta *et al.*, 2007). This is especially true for TBI whose IC₅₀ values with either HIPK2 or CK2 are nearly identical, 0.7 vs 0.6 μ M, (Pagano *et al.*, 2008 and Table 1). From these observations it has been decided to insert bulky side chains in order to reduce affinity toward CK2 as compared to that toward HIPK2. This goal was obtained by derivatizing a tetrabromoisindoline-1,3-dione scaffold (nearly super-imposable to that of TBI) with an imidazole group, to give rise to 4,5,6,7-tetrabromo-2-(1*H*-imidazol-2-yl)isindoline-1,3-dione (TBID, 5a). TBID (5a) inhibits HIPK2 with the same efficiency as TBI, while shows a more than 10-fold higher IC₅₀ value toward CK2 (see Table 1). Modifications in the position of basic nitrogen atom of the imidazole ring have disclosed the crucial role of position 3, as displayed by the loss of inhibitory activity toward HIPK2 of compound **5e** (Cozza *et al.*, 2014) (see Table 1).



Entry	R-	HIPK2	CK2	PIM1	CK1
TBID (5a)		0.33	5.50	>40.0	>40.0
5e		>40.0	23.0	>40.0	>40.0
TBB		5.3	0.15	1.04	>40.0
TBI		0.7	0.60	0.115	15
SB203580		>40.0	n.d.	n.d.	n.d.

Table 1. IC_{50} (μM) of TBID (**5a**) and compound **5e** for HIPK2, CK2, PIM1, CK. The scaffold structure of TBID-derived compound is shown in the upper part. R- is represented by the different moieties indicated in the table. TBB and TBI values are drawn from Pagano *et al.*, 2008. n.d.= not determined (from Cozza *et al.*, 2014).

The selectivity of the newly developed HIPK2 inhibitor TBID was tested by analyzing its effect at 10 μM concentration, on a panel of 70 protein kinases (Figure 2): the activity of HIPK2 was entirely suppressed while none of the other protein kinases underwent a similar inhibition; the residual activity of the second (BTK) and third (CAMKKb) most inhibited kinases were 29% and 34%, respectively. In addition, the activity of other kinases generally inhibited by CK2 inhibitors (i.e. PIM1 and ERK8) and CK2 itself, was less affected or almost totally unaffected. More detailed information about selectivity of TBID were obtained by profiling analysis at 1 instead of 10 μM concentration on a larger panel of 125 protein kinases, including other members of the HIPK sub-family and many protein tyrosine kinases which were scarcely represented in the smaller panel.. The data (Cozza *et al.*, 2014) have displayed that HIPK2 is the most susceptible kinase to TBID (31% residual activity). HIPK1 and HIPK3 however are also significantly inhibited, with residual activities of 39% and 53%, respectively. In contrast none of the protein tyrosine kinases tested is appreciably

affected by TBID with the only possible exception of IGF-IR (68% residual activity). This, together with CAMK1 and CAMKKb, are the only kinases inhibited more than 20%, a part from the HIPKs.

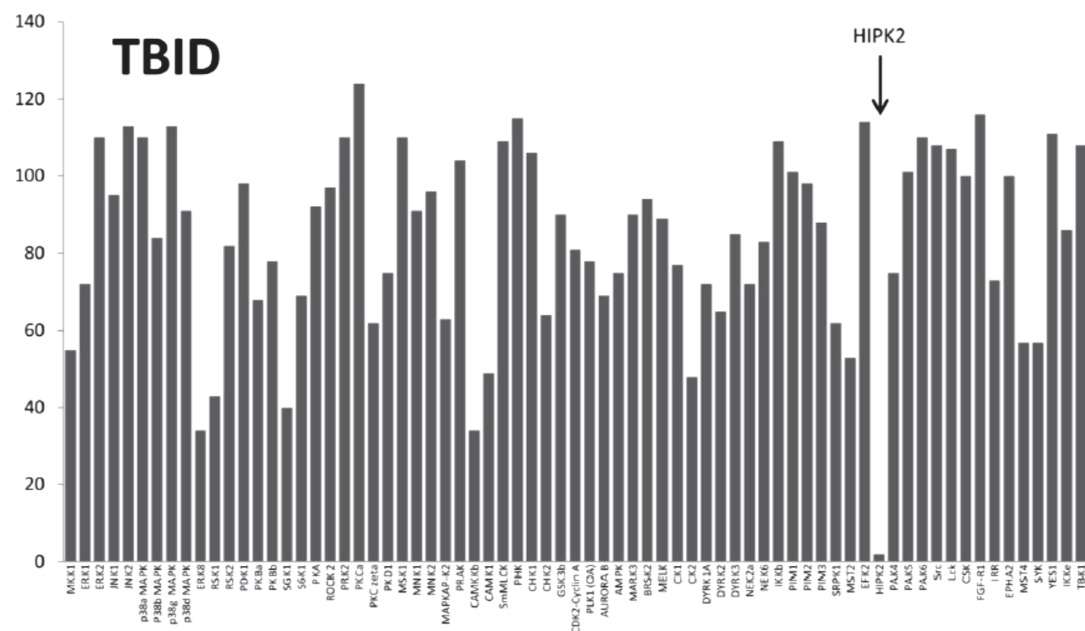


Figure 2. Selectivity profile of TBID (10 μM) against 70 kinases panel. Residual activity expressed as per cent of activity in the absence of TBID is shown (from Cozza et al., 2014).

AIM OF THE STUDY

The aim of my work in this study was to check the cell permeability of the compound TBID (4,5,6,7-tetrabromo-2-(1*H*-imidazol-2-yl)isoindoline-1,3-dione). This is the first specific inhibitor of HIPK2 ($IC_{50}=0.33\mu M$) but its properties have been analyzed only *in vitro* so far. The physiological role of HIPK2 is presently only partially understood, therefore the availability of a specific inhibitor for cellular studies would be of great advantage to shed light on it. We thus tested if TBID administrated to intact cells induces inhibition of endogenous HIPK2. For this purpose, we exploited phosphorylation assays of lysates from treated cells, using a synthetic peptide as specific substrate for HIPK2; otherwise we checked the phosphorylation level of p53 HIPK2 sites with a phosphor-specific antibody. We also wanted to verify the absence of cytotoxicity of TBID under the condition used for cell treatment.

RESULTS

INHIBITION OF ENDOGENOUS HIPK2 IN CELL TREATED WITH TBID

To check if TBID is cell permeable, we treated intact cells with increasing concentrations of TBID, then we lysed cells and measured HIPK2 activity by an immunoprecipitation assay. HIPK2 was pulled down with a specific antibody, that recognized only HIPK2 kinase and no other HIPK isoforms, and its activity was assayed using a HIPK2 specific peptide substrate, in the presence of a mixture containing radioactive ATP. As shown in Figure 3, endogenous HIPK2 activity is reduced in a dose dependent manner upon cell treatment with TBID, without any effects on the amount of HIPK2. As a control, we treated cells with the TBID analog 5e, which is inactive towards HIPK2 *in vitro* (see Table1). As evident in Figure 3, HIPK2 immunoprecipitated from cells treated with 5e does not display any reduction of catalytic activity.

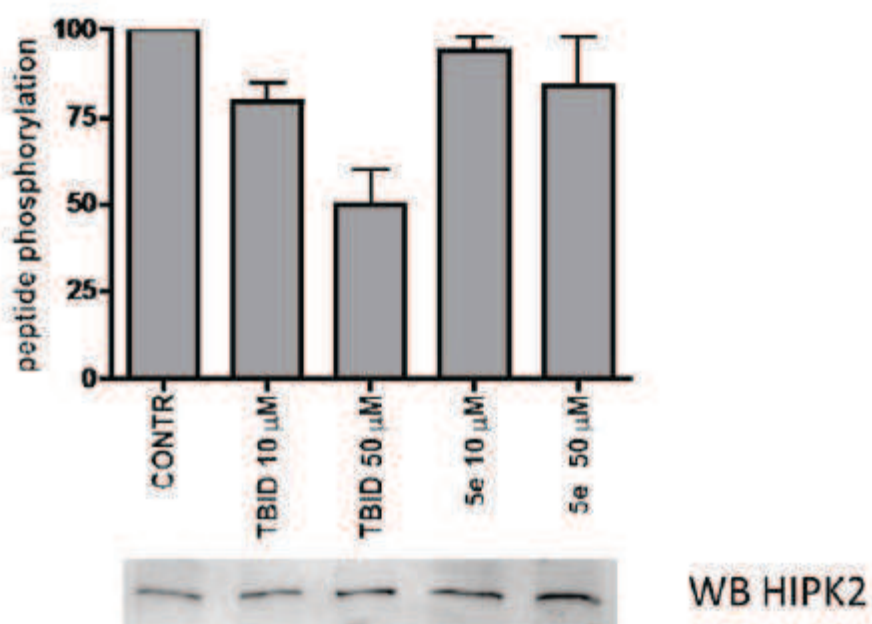


Figure 3. Cell treatment with TBID inhibits endogenous HIPK2. HIPK2 was immunoprecipitated from lysates of HepG2 cells treated with different concentrations of TBID or the inactive analog 5e, as indicated; HIPK2 activity was measured towards the specific peptide substrate, as detailed in Materials and Methods. The amount of immunoprecipitated HIPK2 is shown by WB (from Cozza et al., 2014).

To reinforce the analysis of HIPK2 inhibition in cells, we evaluate the phosphorylation state of a HIPK2 endocellular substrate in treated cells. We checked the phosphorylation state of p53 at Ser46, which is a known target of HIPK2 (Puca *et al.*, 2008). As shown in Figure 4, treatment with TBID markedly reduces the phosphorylation level of this residue, without affecting the amount of p53.

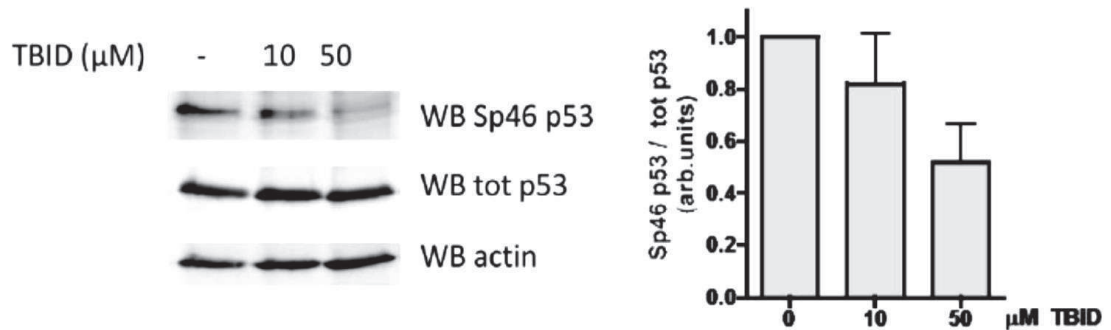


Figure 4. Phosphorylation level of p53ser46. CEM cells were treated as indicated, then 10 μg of cell lysate proteins was analyzed by WB with anti-Sp46 of p53, anti-total p53, or anti-actin, as loading control. A representative experiment is shown on the left, while a histogram is presented on the right where Sp46 p53 of three separate experiments has been quantified, normalized to the p53 total level, and reported as means + SEM (from Cozza *et al.*, 2014).

We finally wanted to exclude that TBID exerts a cytotoxic effect under the conditions used for the inhibition of endogenous HIPK2. The absence of cytotoxicity was demonstrated by checking cell viability with an MTT assay, and excluding that apoptosis occurs, as judged by the absence of any cleavage of the caspase substrate PARP, whose amount seems instead to increase in response to TBID.

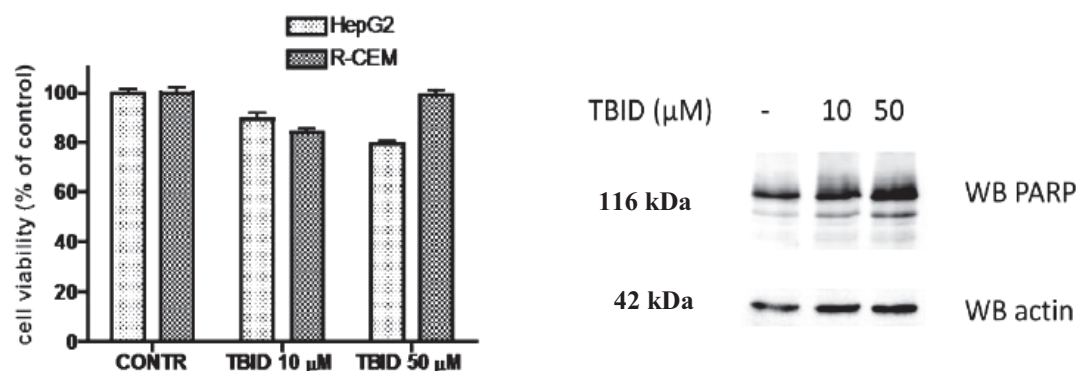


Figure 5. Effects of TBID on cell viability. Cell viability was assessed by the method of MTT, treating HepG2 or CEM cells as indicated (left panel) or by evaluating the amount of the caspase substrate PARP (HepG2 cells, right panel), whose reduction would denote apoptosis. The recognized band corresponds to the full length PARP protein of 116kDa (from Cozza *et al.*, 2014).

DISCUSSION

Data obtained by *in vitro* experiments had demonstrated that TBID is a very effective and selective inhibitor of HIPKs and of HIPK2 in particular. It displays an IC_{50} of 0.33 μ M towards HIPK2 and, tested at 1 μ M on 125 PKs, only HIPK family members are inhibited more than 50% (Cozza *et al.*, 2014). My work has focused on cell experiments, and has demonstrated that TBID is suitable for *in cell* studies where the role of HIPK kinases in signal transduction needs to be investigated. This availability is particular significant for a protein kinase whose role is emerging as regulator of cell growth/apoptosis, with implication in the mode of action of chemotherapeutic agents, but whose mechanism of action is still largely unknown. The only compound available until now as an HIPK2 inhibitor was SB203580, which however inhibits different classes of protein kinases and has a questionable efficacy in inhibiting HIPK2 activity (Bain *et al.*, 2007). On the other hand, a number of compounds are known which inhibit both protein kinase CK2 and HIPK2 (Sarno *et al.*, 2008), and indeed TBID is the result of modification operated on a CK2/HIPK2 inhibitor to render it selective for HIPK2. In this context, it should be noted that the concentrations required to inhibit HIPK2 in cells are much higher *in vitro*, with half maximal inhibition two orders magnitude higher (50 μ M vs 0.33 μ M). However, this is not unusual among protein kinase inhibitors (Davies *et al.*, 2000), and similar ratio has been frequently observed for other kinase inhibitors (e.g. Ruzzene *et al.*, 2002; Sarno *et al.*, 2012; Zanin *et al.*, 2012). This may be explained by possible adsorbance of lipophilic compounds to cellular structures and to the fact that ATP competitive inhibitors (such as TBID) have to overcome a very high ATP concentration (in the mM range) within the cell.

In conclusion, the TBID properties in conjunction with its ability to permeate cells, make TBID the first choice and for the time being the only tool to down regulate HIPK2 in cells.

GENERAL CONCLUSIONS

In this thesis we have analyzed different inhibition strategies to target protein kinase CK2, and their cellular effects on tumor and non-tumor cell lines. In accordance with its pro-survival and anti-apoptotic function and its overexpression in cancer cells, CK2 can be considered as a suitable pharmacological target. For this reasons we have analyzed three different approaches to hit CK2, all with the final goal to block its activity and induce cell death in tumor cells. We compare the cellular effects of two canonical ATP-competitive CK2 inhibitors, CX-4945 (at present in clinical trials) and its analog CX-5011 (Pierre *et al.*, 2011); a bi-functional CK2 inhibitor called K137-E4, very recently developed in our laboratory, designed to block both the ATP and the peptide sites of CK2; the cyclic peptide called CRIBI-300, derived from CIGB-300, which was designed to prevent the phosphorylation of some CK2 substrates (Perea *et al.*, 2004) and in clinical trials as well (Perea *et al.*, 2011).

From our results we conclude that compounds which act as ATP-competitive inhibitors remain, until now, the best choice to target CK2 in tumor cells. In fact, we demonstrated that ATP-competitive CX-4945 and CX-5011 inhibitors are able to efficiently induce apoptosis also in tumor resistant cells, overcoming the problem of multi-drug resistance. Moreover CX-4945 is able to sensitize resistant cells, expressing the P-gp pump, to the conventional chemotherapeutics, displaying a synergistic effect when co-administrated with vinblastine.

K137-E4 has been demonstrated to be the most specific and potent CK2 inhibitor presently available, but it is not exploitable for *in vivo* studies, since we found that it is not cell permeable; future improvements will be necessary to render it more hydrophobic, thus able to cross the plasma membrane.

On the other hand, alternative strategies to target CK2 without interfering with the ATP binding site have been also taken into account as they avoid some issues of most conventional ATP-competitive inhibitors: they overcome the problem of competition with intracellular high ATP concentration, and they are expected to be more specific, not relying on residues of the ATP binding site conserved in all the kinases. CRIBI-300, a cyclic peptide whose sequence has been modeled with the aim of interfering with the phosphorylation of some specific CK2 substrates, could display some of these advantages. From our findings, however, CRIBI-300 has proved to have multiple sites of action: besides targeting CK2 substrates, we found that it directly hits CK2 and produces its early degradation; moreover, it causes

perturbation in the proteolytic activity of the cells and other dramatic effects, with the final outcome of cell death, which however is not due to apoptosis. Thus, further studies at a molecular level are necessary, in order to rationalize and optimize its therapeutic employment.

One of the major aims of this work, and in general of the research on kinase inhibitors, was the finding of very selective compounds, able to block only one specific target. This would be of course a great advantage for the usage of the inhibitor in cell experiments aimed to investigating the role of the target kinase; in this case, collateral and often unknown targets can produce results difficult to be interpreted or even misunderstood. However, it is worth to note that, when the inhibitor has to be developed as a possible pharmacological tool, its absolute selectivity might not be really preferred. For example in cancer, redundancies of pathological signaling pathways very often occur, and they can not be effectively controlled by a too selective inhibitor. In this case, down regulating more than one target could be an alternative and fruitful strategy. In fact, the combination of two or more compounds is often shown to produce a synergistic instead of additive effect; moreover, agents that are able to address simultaneously two or more targets could be useful in this context. Research is moving on this direction, and dual inhibitors are now very promising compounds; also in the field of CK2, a dual inhibitor has been recently proposed, TDB (Cozza *et al.*, 2014), able to simultaneously block CK2 and another cancer related kinase, PIM1 (Brault *et al.*, 2010).

On this regard, in this thesis preliminary but interesting results are presented concerning the synergism between CX-4945 and CX-5011, both selective CK2 inhibitors, but for some reasons able to produce a higher degree of apoptosis (also in resistant cells) when administrated together instead than separately.

The results here presented of cell death induction by CK2-targeting compounds have confirmed that tumor cells are more sensitive respect to non-tumor cells. This is in accordance with other observations already done (e.g. Piazza *et al.*, 2006; Di Maira *et al.*, 2007), and it is explainable considering that cancer cells seem to be addicted to CK2: they display higher amount of CK2 if compared to normal cells, and they rely more on it for they survival; as a consequence, when CK2 is blocked, they are more susceptible and more inclined to die.

In this study, we had also the opportunity to investigate on a still poorly known form of CK2, the so called ecto-CK2, corresponding to the pool of this kinase present at the external side of the plasma membrane. In fact, the CK2 inhibitor K137-E4, not able to cross membranes has been exploited to this purpose. We demonstrated that ecto-CK2 is active both in tumor and non tumor cells, presumably at similar levels, and we provided the first evidence that its inhibition does not correlate with the induction of apoptosis. Thus we conclude that the CK2 ectokinase roles are probably not related to the well-established pro-survival and anti-apoptotic functions of endocellular CK2.

Considering the importance of inhibitor development for the investigation of a kinase function, the collateral study presented in this thesis is of great relevance, since it characterizes TBID, a very selective and cell-permeable HIPK2 kinase inhibitor, the only compound presently available to investigate the cellular physiological role, still poorly understood, of HIPK2 kinase. TBID was developed in our laboratory by a modification of an ATP-competitive CK2 inhibitor, and it is an example of how unexpected (or even unwanted) results, such as limited specificity, can be exploited and improved to obtain new and useful biochemical tools.

In summary, the study presented in this thesis has shown different strategies of kinase inhibition and possible employments of CK2 inhibitors as promising tools in cancer therapy.

Although in the past years the developing of CK2 inhibitors has been concentrated on ATP-competitive compounds, which still seem to be the first-choice strategy, we can imagine that alternative possibilities will become applicable in future, and will be optimized to block only those CK2-dependent events which are strictly related to cancer or other diseases.

REFERENCES

- Amaravadi, R., and Thompson, C.B. The survival kinases Akt and Pim as potential pharmacological targets. *J. Clin. Invest.* 2005; 115, 2618–2624.
- Anastassiadis T, Deacon S, Devarajan K, Ma H, Peterson J. Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. *Nature biotechnology.* 2011; 29 (11): 1039–1045.
- Andersen, K.M., Hofmann, K., and Hartmann-Petersen, R. Ubiquitin-binding proteins: similar, but different. *Essays Biochem.* 2005; 41, 49–67.
- Antonelli, M., Daniotti, J. L., Rojo, D., Allende, C. C. and Allende, J. E. Cloning, expression and properties of the alpha subunit of casein kinase 2 from zebrafish (*Danio rerio*). *European Journal of Biochemistry.* 1996; 241 (1), pp. 272–279.
- Aparicio, J.G., and Applebury, M.L. The photoreceptor guanylate cyclase is an autophosphorylating protein kinase. *J. Biol. Chem.* 1996; 271, 27083–27089.
- Arrigoni, G., Pagano, M. A., Sarno, S., Cesaro, L., James, P. and Pinna, L. A. Mass spectrometry analysis of a protein kinase CK2 beta subunit interactome isolated from mouse brain by affinity chromatography. *The Journal of Proteome Research*, 2008; 7 (3), pp. 990–1000.
- Bachelot T., C. Bourcier, C. Cropet et al., Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast cancer with prior exposure to aromatase inhibitors: a GINECO study. *Journal of Clinical Oncology*, 2012; vol. 30, no. 22, pp. 2718–2724
- Baker E. K., R. W. Johnstone, J. R. Zalcberg, and A. El-Osta. Epigenetic changes to the MDR1 locus in response to chemotherapeutic drugs. *Oncogene*, 2005; vol. 24, no. 54, pp. 8061–8075,
- Bain J, Plater L, Elliott M, Shpiro N, Hastie C, Mclauchlan H, Klevernic I, Arthur J, Alessi D, Cohen P. The selectivity of protein kinase inhibitors: a further update. *Biochem. J.* 2007; 408: 297–315.

- Baselga J., M. Campone, M. Piccart et al., Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *New England Journal of Medicine* 2012; vol. 366, no. 6, pp.520–529
- Battistutta R, Cozza G, Pierre F, Papinutto E, Lolli G, Sarno S, O'Brien S, Siddiqui-Jain A, Haddach M, Anderes K, Others. Unprecedented selectivity and structural determinants of a new class of protein kinase CK2 inhibitors in clinical trials for the treatment of cancer. *Biochemistry*. 2011; 50 (39): 8478–8488.
- Battistutta R, De Moliner E, Sarno S, Zanotti G, Pinna L. Structural features underlying selective inhibition of protein kinase CK2 by ATP site-directed tetrabromo-2-benzotriazole. *Protein Science*. 2001; 10 (11): 2200–2206.
- Battistutta R, Mazzorana M, Cendron L, Bortolato A, Sarno S, Kazimierczuk Z, Zanotti G, Moro S, Pinna L. The ATP-Binding Site of Protein Kinase CK2 Holds a Positive Electrostatic Area and Conserved Water Molecules. *Chembiochem*. 2007; 8 (15): 1804–1809.
- Berman H, Battistuz T, Bhat T, Bluhm W, Bourne P, Burkhardt K, Feng Z, Gillil, Iype L, Jain S, Others. The protein data bank. *Acta Crystallographica Section D: Biological Crystallography*. 2002; 58 (6): 899–907.
- Birnbaum M, Glover C. The phosphotransferase activity of casein kinase II is required for its physiological function in vivo. *Biochemical and biophysical research communications*. 1991; 181 (2): 524–528.
- Blagden S, Bono J. Drugging cell cycle kinases in cancer therapy. *Current drug targets*. 2005; 6 (3): 325–335.
- Bliesath, J., Huser, N., Omori, M., Bunag, D., Proffitt, C., Streiner, N., Ho, C., Siddiqui-Jain, A., O'Brien, S.E., Lim, J.K.C., et al. Combined inhibition of EGFR and CK2 augments the attenuation of PI3K-Akt-mTOR signaling and the killing of cancer cells. *Cancer Lett*. 2012 322, 113–118.
- Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature*. 2001; 411 (6835): 355–365.

- Bohana-Kashtan O, Pinna LA, Fishelson Z. Extracellular phosphorylation of C9 by protein kinase CK2 regulates complement-mediated lysis. *Eur J Immunol* 2005;35:1939–1948
- Boldyreff, B., Meggio, F., Pinna, L. A. and Issinger, O. G. Reconstitution of normal and hyperactivated forms of casein kinase-2 by variably mutated beta subunits. *Biochemistry*, 1993; 32 (47), pp. 12672–12677.
- Boldyreff, B., Meggio, F., Pinna, L. A. and Issinger, O. Efficient autophosphorylation and phosphorylation of the beta-subunit by casein kinase-2 require the integrity of an acidic cluster 50 residues downstream from the phosphoacceptor site. *Journal of Biological Chemistry*, 1994; 269 (7), pp. 4827–4831.
- Borgo, C., Cesaro, L., Salizzato, V., Ruzzene, M., Massimino, M.L., Pinna, L.A., and Donella-Deana, A. Aberrant signalling by protein kinase CK2 in imatinib-resistant chronic myeloid leukaemia cells: biochemical evidence and therapeutic perspectives. *Mol Oncol* 2013; 7, 1103–1115.
- Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. *Journal of the National Cancer Institute*. 2000; 92 (16): 1295–1302.
- Braut, L., Gasser, C., Bracher, F., Huber, K., Knapp, S., and Schwaller, J. PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers. *Haematologica* 2010; 95, 1004–1015.
- Breivik, J. The evolutionary origin of genetic instability in cancer development. *Semin. Cancer Biol.* 2005; 15, 51–60.
- Brognard J, Hunter T. Protein kinase signaling networks in cancer. *Current opinion in genetics & development*. 2011; 21 (1): 4–11.
- Buontempo, F., Orsini, E., Martins, L.R., Antunes, I., Lonetti, A., Chiarini, F., Tabellini, G., Evangelisti, C., Evangelisti, C., Melchionda, F., et al. Cytotoxic activity of the casein kinase 2 inhibitor CX-4945 against T-cell acute lymphoblastic leukemia: targeting the unfolded protein response signaling. *Leukemia*. 2013

- Calzado M, Renner F, Roscic A, Schmitz M. HIPK2, a versatile switchboard regulating the transcription machinery and cell death. *Cell Cycle*. 2007; 6 (2): 139–143.
- Canton, D., Zhang, C. And Litchfield, D. Assembly of protein kinase CK2: investigation of complex formation between catalytic and regulatory subunits using a zinc-finger-deficient mutant of CK2 beta . *Biochem. J*, 2001; 358 pp. 87–94.
- Caulin, A.F., and Maley, C.C. Peto's Paradox: evolution's prescription for cancer prevention. *Trends Ecol. Evol. (Amst.)* 2011; 26, 175–182.
- Channavajhala P., D.C. Seldin, Functional interaction of protein kinase CK2 and c-Myc in lymphomagenesis. *Oncogene* 2002; 21 5280–5288.
- Chantalat, L., Leroy, D., Filhol, O., Nueda, A., Benitez, M. J., Chambaz, E. M., Cochet, C. and Dideberg, O. Crystal structure of the human protein kinase CK2 regulatory subunit reveals its zinc finger-mediated dimerization. *The EMBO journal*, 1999; 18 (11), pp. 2930–2940.
- Chekhun V. F., N. Y. Lukyanova, O. Kovalchuk, V. P. Tryndyak, and I. P. Pogribny, Epigenetic profiling of multidrugresistant human MCF-7 breast adenocarcinoma cells reveals novel hyper- and hypomethylated targets. *Molecular Cancer Therapeutics*. 2007;vol. 6, no. 3, pp. 1089–1098
- Chen, M., Li, D., Krebs, E. G. and Cooper, J. A. The casein kinase II beta subunit binds to Mos and inhibits Mos activity. *Molecular and cellular biology*, 1997; 17 (4), pp. 1904–1912.
- Chua, P.C.P., F.; Whitten, J.P. , Serine-threonine protein kinase and PARP modulators. Patent number WO 2008/028168 A2, 2008.
- Cohen P. Protein kinases: the major drug targets of the twenty-first century?. *Nature Reviews Drug Discovery*. 2002; 1 (4): 309–315.
- Cohen P. The regulation of protein function by multisite phosphorylation: a 25 year update. *Trends in biochemical sciences*. 2000; 25 (12): 596–601.

- Colaert N, Helsens K, Martens L, V, Ekerckhove J, Gevaert K. Improved visualization of protein consensus sequences by iceLogo. *Nature methods*. 2009; 6 (11): 786–787.
- Cozza G, Bonvini P, Zorzi E, Poletto G, Pagano M, Sarno S, Donella-Deana A, Zagotto G, Rosolen A, Pinna L, Others. Identification of ellagic acid as potent inhibitor of protein kinase CK2: a successful example of a virtual screening application. *Journal of medicinal chemistry*. 2006; 49 (8): 2363–2366.
- Cozza, G., Bortolato, A. and Moro, S. How druggable is protein kinase CK2?. *Medicinal research reviews*, 2010; 30 (3), pp. 419–462.
- Cozza G, Gianoncelli A, Bonvini P, Zorzi E, Pasquale R, Rosolen A, Pinna L, Meggio F, Zagotto G, Moro S. Urolithin as a converging scaffold linking ellagic acid and coumarin analogues: design of potent protein kinase CK2 inhibitors. *ChemMedChem*. 2011; 6 (12): 2273–2286.
- Cozza G, Girardi C, Ranchio A, Lolli G, Sarno S, Orzeszko A, Kazimierczuk Z, Battistutta R, Ruzzene M, Pinna L. Cell-permeable dual inhibitors of protein kinases CK2 and PIM-1: structural features and pharmacological potential. *Cellular and Molecular Life Sciences*. 2014;: 1–13.
- Cozza G, Mazzorana M, Papinutto E, Bain J, Elliott M, Di Maira G, Gianoncelli A, Pagano M, Sarno S, Ruzzene M, Others. Quinalizarin as a potent, selective and cell-permeable inhibitor of protein kinase CK2. *Biochem. J*. 2009; 421: 387–395.
- Cozza G, Meggio F, Moro S. The dark side of protein kinase CK2 inhibition. *Current medicinal chemistry*. 2011; 18 (19): 2867–2884.
- Cozza G, Pinna L, Moro S. Kinase CK2 Inhibition: An Update. *Current medicinal chemistry*. 2013; 20 (5): 671–693.
- Crespi B, Summers K. Evolutionary biology of cancer. *Trends in ecology & evolution*. 2005; 20 (10): 545–552.
- D'orazi G, Cecchinelli B, Bruno T, Manni I, Higashimoto Y, Saito S, Gostissa M, Coen S,

- Marchetti A, Del Sal G, Others. Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nature cell biology*. 2001; 4 (1): 11–19.
- Davies S, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* 2000; 351: 95–105.
- De Moliner E, Moro S, Sarno S, Zagotto G, Zanotti G, Pinna L, Battistutta R. Inhibition of Protein Kinase CK2 by Anthraquinone-related Compounds a structural insight. *Journal of Biological Chemistry*. 2003; 278 (3): 1831–1836.
- Desagher, S., Osen-Sand, A., Montessuit, S., Magnenat, E., Vilbois, F., Hochmann, A., Journot, L., Antonsson, B. and Martinou, J. C. Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. *Mol. Cell* 2001;8, 601– 611
- Di Maira G, Brustolon F, Bertacchini J, Tosoni K, Marmiroli S, Pinna L, Ruzzene M. Pharmacological inhibition of protein kinase CK2 reverts the multidrug resistance phenotype of a CEM cell line characterized by high CK2 level. *Oncogene*. 2007; 26 (48): 6915–6926.
- Di Stefano V, Bl, Ino G, Sacchi A, Soddu S, D'orazi G. HIPK2 neutralizes MDM2 inhibition rescuing p53 transcriptional activity and apoptotic function. *Oncogene*. 2004; 23 (30): 5185–5192.
- Dobrowolska, G., Meggio, F., Szczegielniak, J., Muszynska, G. And Pinna, L. A. Purification and characterization of maize seedling casein kinase IIB, a monomeric enzyme immunologically related to the α subunit of animal casein kinase-2. *European journal of biochemistry*, 1992; 204 (1), pp. 299–303.
- Dobrowolska G, Muszynska G, Shugar D. Benzimidazole nucleoside analogues as inhibitors of plant (maize seedling) casein kinases. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*. 1991; 1080 (3): 221–226.
- Enkvist E, Viht K, Bischoff N, Vahter J, Saaver S, Raidaru G, Issinger O, Niefind K, Uri A. A subnanomolar fluorescent probe for protein kinase CK2 interaction studies. *Organic*

& Biomolecular Chemistry. 2012; 10 (43): 8645–8653.

- Escargueil, A. E., Plisov, S. Y., Filhol, O., Cochet, C. and Larsen, A. K. Mitotic phosphorylation of DNA topoisomerase II alpha by protein kinase CK2 creates the MPM-2 phosphoepitope on ser-1469. *J. Biol. Chem.* 2000; 275, 34710–34718
- Fracasso P. M., P. Westervelt, C. L. Fears et al., Phase I study of paclitaxel in combination with a multidrug resistance modulator, PSC 833 (Valspodar), in refractory malignancies, *Journal of Clinical Oncology*, 2000; vol. 18, no. 5, pp. 1124–1134
- Fritz, G., Issinger, O.-G., and Olsen, B.B. Selectivity analysis of protein kinase CK2 inhibitors DMAT, TBB and resorufin in cisplatin-induced stress responses. *Int. J. Oncol.* 2009; 35, 1151–1157.
- Fusco A, Fedele M. Roles of HMGA proteins in cancer. *Nature Reviews Cancer*. 2007; 7 (12): 899–910.
- Gianoncelli A, Cozza G, Orzeszko A, Meggio F, Kazimierczuk Z, Pinna L. Tetraiodobenzimidazoles are potent inhibitors of protein kinase CK2. *Bioorganic & medicinal chemistry*. 2009; 17 (20): 7281–7289.
- Gietz, R. D., Graham, K. C. and Litchfield, D. W. Interactions between the subunits of casein kinase II. *Journal of Biological Chemistry*, 1995; 270 (22), pp. 13017–13021.
- Glavy J, Horwitz S, Orr G. Identification of the in vivo phosphorylation sites for acidic-directed kinases in murine mdrlb P-glycoprotein. *Journal of Biological Chemistry*. 1997; 272 (9): 5909–5914.
- Glover C. A filamentous form of Drosophila casein kinase II. *Journal of Biological Chemistry*. 1986; 261 (30): 14349–14354.
- Glover III, C. V. On the physiological role of casein kinase II in *Saccharomyces cerevisiae*. *Prog. Nucleic Acid Res.* 1998; Mol. Biol. 59, 95– 133
- Golub A, Bdzhola V, Kyshenia Y, Sapelkin V, Prykhod'Ko A, Kukharenko O, Ostrynska O, Yarmoluk S. Structure-based discovery of novel flavonol inhibitors of human protein

- kinase CK2. *Molecular and cellular biochemistry*. 2011; 356 (1-2): 107–115.
- Golub A, Yakovenko O, Bdzhola V, Sapelkin V, Zien P, Yarmoluk S. Evaluation of 3-Carboxy-4 (1 H)-quinolones as Inhibitors of Human Protein Kinase CK2. *Journal of medicinal chemistry*. 2006; 49 (22): 6443–6450.
- Golub A, Yakovenko O, Prykhod'ko A, Lukashov S, Bdzhola V, Yarmoluk S. Evaluation of 4, 5, 6, 7-tetrahalogeno-isoindole-1, 3 diones as inhibitors of human protein kinase CK2. *Biochimica et Biophysica Acta (BBA)-Proteins & Proteomics*. 2008; 1784 (1): 143–149.
- Gotz C, Gratz A, Kucklaender U, Jose J. TF—A novel cell-permeable and selective inhibitor of human protein kinase CK2 induces apoptosis in the prostate cancer cell line LNCaP. *Biochimica et Biophysica Acta (BBA)-General Subjects*. 2012; 1820 (7): 970–977.
- Gottesman M. Cancer gene therapy: an awkward adolescence. *Cancer gene therapy*. 2003; 10 (7): 501–508.
- Greaves M. Darwinian medicine: a case for cancer. *Nature Reviews Cancer*. 2007; 7 (3): 213–221.
- Guerra B, Issinger O. Protein kinase CK2 in human diseases. *Current medicinal chemistry*. 2008; 15 (19): 1870–1886.
- Haddach, M., Pierre, F., Regan, C.F., Borsan, C., Michaux, J., Stefan, E., Kerdoncuff, P., Schwaebe, M.K., Chua, P.C., Siddiqui-Jain, A., et al. Synthesis and SAR of inhibitors of protein kinase CK2: novel tricyclic quinoline analogs. *Bioorg. Med. Chem. Lett*. 2012 22, 45–48.
- Hall M, H, Ley M, Gottesman M. Is resistance useless? Multidrug resistance and collateral sensitivity. *Trends in pharmacological sciences*. 2009; 30 (10): 546–556.
- Hanahan D., Weinberg, R.A. The hallmarks of cancer, *Cell* 2000; 100 57–70
- Hanks S, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *The FASEB Journal*. 1995; 9 (8):

576–596.

Harvey E, Li N, Ramji D. Critical Role for Casein Kinase 2 and Phosphoinositide-3-Kinase in the Interferon- gamma –Induced Expression of Monocyte Chemoattractant Protein-1 and Other Key Genes Implicated in Atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2007; 27 (4): 806–812.

Hauber, J., Malim, M.H., and Cullen, B.R. Mutational analysis of the conserved basic domain of human immunodeficiency virus tat protein. *J. Virol*. 1989 63, 1181–1187.

Heppner G, Dexter D, Denucci T, Miller F, Calabresi P. Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mammary tumor. *Cancer research*. 1978; 38 (11 Part 1): 3758–3763.

Hipfner D, Deeley R, Cole S. Structural, mechanistic and clinical aspects of MRP1. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 1999; 1461 (2): 359–376.

Hofmann T, Moller A, Sirma H, Zentgraf H, Taya Y, Droge W, Will H, Schmitz M. Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. *Nature cell biology*. 2001; 4 (1): 1–10.

Holm, T., Johansson, H., Lundberg, P., Pooga, M., Lindgren, M., and Langel, U. Studying the uptake of cell-penetrating peptides. *Nat Protoc* 2006 1, 1001–1005.

Hou Z, Nakanishi I, Kinoshita T, Takei Y, Yasue M, Misu R, Suzuki Y, Nakamura S, Kure T, Ohno H, Others. Structure-based design of novel potent protein kinase CK2 (CK2) inhibitors with phenyl-azole scaffolds. *Journal of Medicinal Chemistry*. 2012; 55 (6): 2899–2903.

Hubert, A., Paris, S., Piret, J.-P., Ninane, N., Raes, M., and Michiels, C. Casein kinase 2 inhibition decreases hypoxia-inducible factor-1 activity under hypoxia through elevated p53 protein level. *J. Cell. Sci*. 2006 119, 3351–3362.

Hung, M.-S., Xu, Z., Chen, Y., Smith, E., Mao, J.-H., Hsieh, D., Lin, Y.-C., Yang, C.-T., Jablons, D.M., and You, L. Hematein, a casein kinase II inhibitor, inhibits lung cancer

- tumor growth in a murine xenograft model. *Int. J. Oncol.* 2013. 43, 1517–1522.
- Ishikawa T, Ali-Osman F. Glutathione-associated cis-diamminedichloroplatinum (II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. *Journal of Biological Chemistry.* 1993; 268 (27): 20116–20125.
- Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Research.* 1996; 56 (5): 988–994.
- Johnson L, Lewis R. Structural basis for control by phosphorylation. *Chemical reviews.* 2001; 101 (8): 2209–2242.
- Jones, S.W., Christison, R., Bundell, K., Voyce, C.J., Brockbank, S.M.V., Newham, P., and Lindsay, M.A. Characterisation of cell-penetrating peptide-mediated peptide delivery. *Br. J. Pharmacol.* 2005 145, 1093–1102.
- Kaplan, I.M., Wadia, J.S., and Dowdy, S.F. Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J Control Release* 2005 102, 247–253.
- Kasahara K, Fujiwara Y, Nishio K, Ohmori T, Sugimoto Y, Komiya K, Matsuda T, Saijo N. Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. *Cancer research.* 1991; 51 (12): 3237–3242.
- Kawase M, Sakagami H, Hashimoto K, Tani S, Hauer H, Chatterjee S. Structure-cytotoxic activity relationships of simple hydroxylated coumarins. *Anticancer research.* 2003; 23 (4): 3243–3246.
- Kim, G.S., Jung, J.E., Niizuma, K., and Chan, P.H. CK2 is a novel negative regulator of NADPH oxidase and a neuroprotectant in mice after cerebral ischemia. *J. Neurosci.* 2009; 29, 14779–14789.
- Kim, J., and Kim, S.H. Druggability of the CK2 inhibitor CX-4945 as an anticancer drug and beyond. *Arch. Pharm. Res.* 2012; 35, 1293–1296.

- Kim, J.S., Eom, J.I., Cheong, J.-W., Choi, A.J., Lee, J.K., Yang, W.I., and Min, Y.H. Protein kinase CK2alpha as an unfavorable prognostic marker and novel therapeutic target in acute myeloid leukemia. *Clin. Cancer Res.* 2007; 13, 1019–1028.
- Kim Y, Choi C, Lee S, Conti M, Kim Y. Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. *Journal of Biological Chemistry.* 1998; 273 (40): 25875–25879.
- Kitchen, D.B., Decornez, H., Furr, J.R., and Bajorath, J. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nat Rev Drug Discov* 2004; 3, 935–949.
- Kikkawa U, Mann S, Firtel R, Hunter T. Molecular cloning of casein kinase II alpha subunit from Dictyostelium discoideum and its expression in the life cycle. *Molecular and cellular biology.* 1992; 12 (12): 5711–5723.
- Kobe B, Kemp B. Active site-directed protein regulation. *Nature.* 1999; 402 (6760): 373–376.
- Koga, H., and Cuervo, A.M. Chaperone-mediated autophagy dysfunction in the pathogenesis of neurodegeneration. *Neurobiol. Dis.* 2011; 43, 29–37.
- Konig J, Rost D, Cui Y, Keppler D. Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. *Hepatology.* 1999; 29 (4): 1156–1163.
- Knighton D, Zheng J, Ten Eyck L, Xuong N, Taylor S, Sowadski J. Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science.* 1991; 253 (5018): 414–420.
- Krek, W., Maridor, G. and Nigg, E. Casein kinase II is a predominantly nuclear enzyme. *The Journal of cell biology,* 1992; 116 (1), pp. 43–55.
- Krippner-Heidenreich, A., Talanian, R. V., Sekul, R., Kraft, R., Thole, H., Ottleben, H. and Luscher, B. Targeting of the transcription factor Max during apoptosis :

- phosphorylation-regulated cleavage by caspase-5 at an unusual glutamic acid residue in position P1. *Biochem. J.* 2001; 358, 705– 715
- Kubler D, Pyerin W, Burow E, Kinzel V. Substrate-effected release of surfacelocated protein kinase from intact cells. *Proc Natl Acad Sci USA* 1983; 80:4021–4025.
- Lacy, A., and O’Kennedy, R. Studies on coumarins and coumarin-related compounds to determine their therapeutic role in the treatment of cancer. *Curr. Pharm. Des.* 2004 10, 3797–3811.
- Laudet B, Moucadel V, Prudent R, Filhol O, Wong Y, Royer D, Cochet C. Identification of chemical inhibitors of protein-kinase CK2 subunit interaction. *Molecular and cellular biochemistry.* 2008; 316 (1-2): 63–69.
- Le Coutre J, Kaback H. Structure–function relationships of integral membrane proteins: membrane transporters vs channels. *Peptide Science.* 2000; 55 (4): 297–307.
- Leary A. F., S. Drury, S.Detre et al., Lapatinib restores hormone sensitivity with differential effects on estrogen receptor signaling in cell models of human epidermal growth factor receptor 2-negative breast cancer with acquired endocrine resistance. *Clinical Cancer Research*, 2010; vol. 16, no. 5, pp. 1486–1497
- Leroy, D., Herich'E, J., Filhol, O., Chambaz, E. M. and Cochet, C. Binding of polyamines to an autonomous domain of the regulatory subunit of protein kinase CK2 induces a conformational change in the holoenzyme A proposed role for the kinase stimulation. *Journal of Biological Chemistry*, 1997; 272 (33), pp. 20820–20827.
- Li P., Li J., Muller E., Otto A., Dietz R. and von Harsdorf, R. Phosphorylation by protein kinase CK2. A signaling switch for the caspase-inhibiting protein ARC. *Mol. Cell* 2002; 10, 247– 258
- Li, X., Shi, X., Liang, D.-Y., and Clark, J.D. Spinal CK2 regulates nociceptive signaling in models of inflammatory pain. *Pain*, 2005; 115, 182–190.
- Li X, Wu L, Ying S, Chang C, He Q, Pu Q. Differentiation and hematopoietic-support of

clonal cells in myelodysplastic syndromes. *Leukemia & lymphoma*. 2007; 48 (7): 1353–1371.

Lichtenstein, A.V. Cancer: evolutionary, genetic and epigenetic aspects. *Clin Epigenetics* 2010; 1, 85–100.

Lin, X., Li, S., Zhao, Y., Ma, X., Zhang, K., He, X., and Wang, Z. Interaction domains of p62: a bridge between p62 and selective autophagy. *DNA Cell Biol.* 2013; 32, 220–227.

Litchfield, D. W., Bosc, D. G., Canton, D. A., Saulnier, R. B., Vilks, G. and Zhang, C. Functional specialization of CK2 isoforms and characterization of isoform-specific binding partners. *Molecular and cellular biochemistry*, 2001; 227 (1-2), pp. 21–29.

Litchfield, D. W., Bosc, D. G., Slominski, E. The protein kinase from mitotic human cells that phosphorylates Ser-209 on the casein kinase II beta-subunit is p34-cdc2. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1995; 1269 (1), pp. 69–78.

Ljubimov A, Caballero S, Aoki A, Pinna L, Grant M, Castellon R. Involvement of protein kinase CK2 in angiogenesis and retinal neovascularization. *Investigative ophthalmology & visual science*. 2004; 45 (12): 4583-4591.

Lolli G, Pinna L, Battistutta R. Structural determinants of protein kinase CK2 regulation by autoinhibitory polymerization. *ACS Chemical Biology*. 2012; 7 (7): 1158–1163.

Lolli G, Ranchio A, Battistutta R. Active form of the Protein Kinase CK2 alpha-2 beta-2 holoenzyme is a strong complex with symmetric architecture. *ACS chemical biology*. 2013.

Lönn, U., and Lönn, S. 5,6-Dichloro-1-beta-O-ribofuranosylbenzimidazole induces DNA damage by interfering with DNA topoisomerase II. *Eur. J. Biochem.* 1987; 164, 541–545.

Lou, D. Y., Dominguez, I., Toselli, P., L, Esman-Bollag, E., O'brien, C. and Seldin, D. C. The alpha catalytic subunit of protein kinase CK2 is required for mouse embryonic

- development. *Molecular and cellular biology*, 2008; 28 (1), pp. 131–139.
- Louvet, E., Junéra, H.R., Berthuy, I., and Hernandez-Verdun, D. Compartmentation of the nucleolar processing proteins in the granular component is a CK2-driven process. *Mol. Biol. Cell* 2006; 17, 2537–2546.
- Makowska M, Lukowska-Chojnacka E, Winska P, Kus A, Bilinska-Chomik A, Bretner M. Design and synthesis of CK2 inhibitors. *Molecular and cellular biochemistry*. 2011; 356 (1-2): 91–96.
- Mann M, Ong S, Gronborg M, Steen H, Jensen O, P, Ey A. Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends in biotechnology*. 2002; 20 (6): 261–268.
- Manni, S., Brancalion, A., Tubi, L.Q., Colpo, A., Pavan, L., Cabrelle, A., Ave, E., Zaffino, F., Di Maira, G., Ruzzene, M., et al. Protein kinase CK2 protects multiple myeloma cells from ER stress-induced apoptosis and from the cytotoxic effect of HSP90 inhibition through regulation of the unfolded protein response. *Clin. Cancer Res.* 2012; 18, 1888–1900.
- Manning B. Challenges and opportunities in defining the essential cancer kinome. *Science signaling*. 2009; 2 (63): 15.
- Manning G, Whyte D, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. 2002; 298 (5600): 1912–1934.
- Marin, O., Meggio, F., Boldyreff, B., Issinger, O.G., and Pinna, L.A. Dissection of the dual function of the beta-subunit of protein kinase CK2 (casein kinase-2): a synthetic peptide reproducing the carboxyl-terminal domain mimicks the positive but not the negative effects of the whole protein. *FEBS Lett.* 1995; 363, 111–114.
- Marin O, Meggio F, Sarno S, Cesaro L, Pagano M, Pinna L. Tyrosine Versus Serine/Threonine Phosphorylation by Protein Kinase Casein Kinase-2 a study with peptide substrates derived from immunophilin Fpr3. *Journal of Biological Chemistry*. 1999; 274 (41): 29260–29265.

- Marin, O., Sarno, S., Boschetti, M., Pagano, M.A., Meggio, F., Ciminale, V., D'Agostino, D.M., and Pinna, L.A. Unique features of HIV-1 Rev protein phosphorylation by protein kinase CK2 (casein kinase-2). *FEBS Lett.* 2000; 481, 63–67.
- Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer.* 2010; 1805 (1): 105–117.
- Matsumoto Y, Kunishio K, Nagao S. Increased phosphorylation of DNA topoisomerase II in etoposide resistant mutants of human glioma cell line. *Journal of neuro-oncology.* 1999; 45 (1): 37–46.
- Mayo L, Seo Y, Jackson M, Smith M, Guzman J, Korgaonkar C, Donner D. Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified. *Journal of Biological Chemistry.* 2005; 280 (28): 25953–25959.
- McDonnell MA, Abedin MJ, Melendez M, Platikanova TN, Ecklund JR, Ahmed K, Kelekar A. Phosphorylation of murine caspase-9 by the protein kinase casein kinase 2 regulates its cleavage by caspase-8. *J Biol Chem.* 2008; 283(29):20149-58.
- Meggio, F., Boldyreff, B., Issinger, O.G., and Pinna, L.A. Casein kinase 2 down-regulation and activation by polybasic peptides are mediated by acidic residues in the 55-64 region of the beta-subunit. A study with calmodulin as phosphorylatable substrate. *Biochemistry* 1994a 33, 4336–4342.
- Meggio, F., Boldyreff, B., Marin, O., Issinger, O. and Pinna, L. A. Phosphorylation and activation of protein kinase CK2 by p34cdc2 are independent events. *European Journal of Biochemistry*, 1995; 230 (3), pp. 1025–1031.
- Meggio F, Boldyreff B, Marin O, Marchiori F, Perich J, Issinger O, Pinna L. The effect of polylysine on casein-kinase-2 activity is influenced by both the structure of the protein/peptide substrates and the subunit composition of the enzyme. *European Journal of Biochemistry.* 1992; 205 (3): 939–945.
- Meggio, F., Marin, O., and Pinna, L.A. Substrate specificity of protein kinase CK2. *Cell. Mol. Biol. Res.* 1994b 40, 401–409.

- Meggio F, Pagano M, Moro S, Zagotto G, Ruzzene M, Sarno S, Cozza G, Bain J, Elliott M, Deana A, Others. Inhibition of protein kinase CK2 by condensed polyphenolic derivatives. An in vitro and in vivo study. *Biochemistry*. 2004; 43 (40): 12931–12936.
- Meggio F, Pinna L. One-thousand-and-one substrates of protein kinase CK2?. *The FASEB Journal*. 2003; 17 (3): 349–368.
- Michor, F., and Polyak, K. The origins and implications of intratumor heterogeneity. *Cancer Prev Res (Phila)* 2010; 3, 1361–1364
- Miyata Y. Protein kinase CK2 in health and disease: CK2: the kinase controlling the Hsp90 chaperone machinery. *Cell Mol Life Sci* 2009; 66:1840–1849
- Miyata Y., E. Nishida, CK2 controls multiple protein kinases by phosphorylating a kinase-targeting molecular chaperone Cdc37. *Mol. Cell. Biol.* 24 2004; 4065–4074
- Miller, S.L.H., Malotky, E., and O'Bryan, J.P. Analysis of the role of ubiquitin-interacting motifs in ubiquitin binding and ubiquitylation. *J. Biol. Chem.* 2004; 279, 33528–33537.
- Mishra S., V. Pertz, B. Zhang, P. Kaur, H. Shimada, J. Groffen, Z. Kazimierczuk, L.A. Pinna, N. Heisterkamp, Treatment of P190 Bcr/Abl lymphoblastic leukemia cells with inhibitors of the serine/threonine kinase CK2, *Leukemia* 2007; 21 178–180.
- Moncevičiūtė-Eringiene, E. Neoplastic growth: the consequence of evolutionary malignant resistance to chronic damage for survival of cells (review of a new theory of the origin of cancer). *Med. Hypotheses* 2005; 65, 595–604.
- Musgrove E. A. and R. L. Sutherland, Biological determinants of endocrine resistance in breast cancer. *Nature Reviews Cancer*, 2009; vol. 9, no. 9, pp. 631–643
- Nagar B, Bornmann W, Pellicena P, Schindler T, Veach D, Miller W, Clarkson B, Kuriyan J. Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Research*. 2002; 62 (15): 4236–4243.
- Nahta R. and R. O'Regan. Evolving strategies for overcoming resistance to HER2-directed

therapy: targeting the PI3K/Akt/mTOR pathway. *Clinical Breast Cancer*, 2010; vol. 10, supplement 3, pp. S72–S78

Newton A, C R. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chemical Reviews*. 2001; 101 (8): 2353–2364.

Niefind K, Guerra B, Ermakowa I, Issinger O. Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme. *The EMBO journal*. 2001; 20 (19): 5320–5331.

Niefind K, Guerra B, Pinna L, Issinger O, Schomburg D. Crystal structure of the catalytic subunit of protein kinase CK2 from *Zea mays* at 2.1 Å resolution. *The EMBO journal*. 1998; 17 (9): 2451–2462.

Nijman S. Synthetic lethality: general principles, utility and detection using genetic screens in human cells. *FEBS letters*. 2011; 585 (1): 1–6.

Nobili S, L, Ini I, Mazzei T, Mini E. Overcoming tumor multidrug resistance using drugs able to evade P-glycoprotein or to exploit its expression. *Medicinal research reviews*. 2012; 32 (6): 1220–1262.

O'Brien, K. A., Lemke, S. J., Cocke, K. S., Rao, R. N. and Beckmann, R. P. Casein kinase 2 binds to and phosphorylates BRCA1. *Biochemical and biophysical research communications*, 1999; 260 (3), pp. 658–664.

Oda Y, Sakamoto A, Satio T, Kawauchi S, Iwamoto Y, Tsuneyoshi M. Molecular abnormalities of p53, MDM2, and H-ras in synovial sarcoma. *Modern Pathology*. 2000; 13 (9): 994–1004.

Olsen B, Rasmussen T, Niefind K, Issinger O. Biochemical characterization of CK2 alpha and alpha-paralogues and their derived holoenzymes: evidence for the existence of a heterotrimeric CK2 alpha-holoenzyme forming trimeric complexes. *Molecular and cellular biochemistry*. 2008; 316 (1-2): 37–47.

- Pagano, M.A., Bain, J., Kazimierczuk, Z., Sarno, S., Ruzzene, M., Di Maira, G., Elliott, M., Orzeszko, A., Cozza, G., Meggio, F., et al. The selectivity of inhibitors of protein kinase CK2: an update. *Biochem. J.* 2008; 415, 353–365.
- Pagano M, Marin O, Cozza G, Sarno S, Meggio F, Treharne K, Mehta A, Pinna L. Cystic fibrosis transmembrane regulator fragments with the Phe508 deletion exert a dual allosteric control over the master kinase CK2. *Biochem. J.* 2010; 426: 19–29.
- Pagano M, Poletto G, Di Maira G, Cozza G, Ruzzene M, Sarno S, Bain J, Elliott M, Moro S, Zagotto G, Others. Tetrabromocinnamic acid (TBCA) and related compounds represent a new class of specific protein kinase CK2 inhibitors. *Chembiochem.* 2007; 8 (1): 129–139.
- Pagano M, Sarno S, Poletto G, Cozza G, Pinna L, Meggio F. Autophosphorylation at the regulatory β subunit reflects the supramolecular organization of protein kinase CK2. *Molecular and cellular biochemistry.* 2005; 274 (1-2): 23–29.
- Papinutto E, Ranchio A, Lolli G, Pinna L, Battistutta R. Structural and functional analysis of the flexible regions of the catalytic α -subunit of protein kinase CK2. *Journal of Structural Biology.* 2012; 177 (2): 382–391.
- Pargellis C, Tong L, Churchill L, Cirillo P, Gilmore T, Graham A, Grob P, Hickey E, Moss N, Pav S, Others. Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nature Structural & Molecular Biology.* 2002; 9 (4): 268–272.
- Pierre F, Chua P, O'Brien S, Siddiqui-Jain A, Bourbon P, Haddach M, Michaux J, Nagasawa J, Schwaebe M, Stefan E, Others. Pre-clinical characterization of CX-4945, a potent and selective small molecule inhibitor of CK2 for the treatment of cancer. *Molecular and cellular biochemistry.* 2011; 356 (1-2): 37–43.
- Perea, S.E., Baladron, I., Garcia, Y., Perera, Y., Lopez, A., Soriano, J.L., Batista, N., Palau, A., Hernández, I., Farina, H., et al. CIGB-300, a synthetic peptide-based drug that targets the CK2 phosphoacceptor domain. Translational and clinical research. *Mol. Cell. Biochem.* 2011; 356, 45–50.

- Perea S, Reyes O, Baladron I, Perera Y, Farina H, Gil J, Rodriguez A, Bacardi D, Marcelo J, Cosme K, CIGB-300, a novel proapoptotic peptide that impairs the CK2 phosphorylation and exhibits anticancer properties both in vitro and in vivo. *Molecular and cellular biochemistry*. 2008; 316 (1-2): 163–167.
- Perea, S.E., Reyes, O., Puchades, Y., Mendoza, O., Vispo, N.S., Torrens, I., Santos, A., Silva, R., Acevedo, B., López, E., et al. Antitumor effect of a novel proapoptotic peptide that impairs the phosphorylation by the protein kinase 2 (casein kinase 2). *Cancer Res*. 2004; 64, 7127–7129.
- Perera, Y., Costales, H.C., Diaz, Y., Reyes, O., Farina, H.G., Mendez, L., Gómez, R.E., Acevedo, B.E., Gomez, D.E., Alonso, D.F., et al. Sensitivity of tumor cells towards CIGB-300 anticancer peptide relies on its nucleolar localization. *J. Pept. Sci*. 2012; 18, 215–223.
- Perera, Y., Farina, H.G., Gil, J., Rodriguez, A., Benavent, F., Castellanos, L., Gómez, R.E., Acevedo, B.E., Alonso, D.F., and Perea, S.E. Anticancer peptide CIGB-300 binds to nucleophosmin/B23, impairs its CK2-mediated phosphorylation, and leads to apoptosis through its nucleolar disassembly activity. *Mol. Cancer Ther*. 2009; 8, 1189–1196.
- Perera, Y., Farina, H.G., Hernández, I., Mendoza, O., Serrano, J.M., Reyes, O., Gómez, D.E., Gómez, R.E., Acevedo, B.E., Alonso, D.F., et al. Systemic administration of a peptide that impairs the protein kinase (CK2) phosphorylation reduces solid tumor growth in mice. *Int. J. Cancer* 2008; 122, 57–62.
- Perez D, Gil C, Martinez A. Protein kinases CK1 and CK2 as new targets for neurodegenerative diseases. *Medicinal research reviews*. 2011; 31 (6): 924–954.
- Perez E. A. Impact, mechanisms, and novel chemotherapy strategies for overcoming resistance to anthracyclines and taxanes in metastatic breast cancer, *Breast Cancer Research and Treatment*, 2009; vol. 114, no. 2, pp. 195–201,
- Piazza F, Ruzzene M, Gurrieri C, Montini B, Bonanni L, Chioetto G, Di Maira G, Barbon F, Cabrelle A, Zambello R, Others. Multiple myeloma cell survival relies on high activity

- of protein kinase CK2. *Blood*. 2006; 108 (5): 1698–1707.
- Pierantoni G, Bulfone A, Pentimalli F, Fedele M, Iuliano R, Santoro M, Chiariotti L, Ballabio A, Fusco A. The homeodomain-interacting protein kinase 2 gene is expressed late in embryogenesis and preferentially in retina, muscle, and neural tissues. *Biochemical and biophysical research communications*. 2002; 290 (3): 942–947.
- Pinna L. *Protein kinase CK2*. Ames, Iowa, USA: Wiley-Blackwell; 2013.
- Pinna L. Protein kinase CK2: a challenge to canons. *Journal of cell science*. 2002; 115 (20): 3873–3878.
- Pinna, L.A., and Ruzzene, M. How do protein kinases recognize their substrates? *Biochim. Biophys. Acta* 1996; 1314, 191–225.
- Prelle, A., Sciacco, M., Tancredi, L., Fagiolari, G., Comi, G.P., Ciscato, P., Serafini, M., Fortunato, F., Zecca, C., Gallanti, A., et al. Clinical, morphological and immunological evaluation of six patients with dysferlin deficiency. *Acta Neuropathol*. 2003; 105, 537–542.
- Prins, R.C., Burke, R.T., Tyner, J.W., Druker, B.J., Loriaux, M.M., and Spurgeon, S.E. CX-4945, a selective inhibitor of casein kinase-2 (CK2), exhibits anti-tumor activity in hematologic malignancies including enhanced activity in chronic lymphocytic leukemia when combined with fludarabine and inhibitors of the B-cell receptor pathway. *Leukemia* 2013; 27, 2094–2096.
- Prudent R, Moucadel V, Laudet B, Barette C, Lafanech`Ere L, Hasenknopf B, Li J, Bareyt S, Lac^Ote E, Thorimbert S, Others. Identification of polyoxometalates as nanomolar noncompetitive inhibitors of protein kinase CK2. *Chemistry & biology*. 2008; 15 (7): 683–692.
- Prudent, R., Moucadel, V., Nguyen, C.-H., Barette, C., Schmidt, F., Florent, J.-C., Lafanechère, L., Sautel, C.F., Duchemin-Pelletier, E., Spreux, E., et al. Antitumor activity of pyridocarbazole and benzopyridoindole derivatives that inhibit protein kinase CK2. *Cancer Res*. 2010; 70, 9865–9874.

- Puca R, Nardinocchi L, Gal H, Rechavi G, Amariglio N, Domany E, Notterman D, Scarsella M, Leonetti C, Sacchi A, Others. Reversible dysfunction of wild-type p53 following homeodomain-interacting protein kinase-2 knockdown. *Cancer research*. 2008; 68 (10): 3707–3714.
- Puca R, Nardinocchi L, Givol D, D'orazi G. Regulation of p53 activity by HIPK2: molecular mechanisms and therapeutical implications in human cancer cells. *Oncogene*. 2010; 29 (31): 4378–4387.
- Pusztai L., P. Wagner, N. Ibrahim et al., Phase II study of tariquidar, a selective P-glycoprotein inhibitor, in patients with chemotherapy-resistant, advanced breast carcinoma. *Cancer*, 2005; vol. 104, no. 4, pp. 682–691
- Quotti Tubi, L., Gurrieri, C., Brancalion, A., Bonaldi, L., Bertorelle, R., Manni, S., Pavan, L., Lessi, F., Zambello, R., Trentin, L., et al. Inhibition of protein kinase CK2 with the clinical-grade small ATP-competitive compound CX-4945 or by RNA interference unveils its role in acute myeloid leukemia cell survival, p53-dependent apoptosis and daunorubicin-induced cytotoxicity. *J Hematol Oncol* 2013; 6, 78.
- Quintaje S, Orchard S, Ra. The Annotation of Both Human and Mouse Kinomes in UniProtKB/Swiss-Prot One Small Step in Manual Annotation, One Giant Leap for Full Comprehension of Genomes. *Molecular & Cellular Proteomics*. 2008; 7 (8): 1409–1419.
- Raaf J, Brunstein E, Issinger O, Niefind K. The interaction of CK2-alpha and CK2-beta, the subunits of protein kinase CK2, requires CK2-beta in a preformed conformation and is enthalpically driven. *Protein Science*. 2008; 17 (12): 2180–2186.
- Richard, J.P., Melikov, K., Brooks, H., Prevot, P., Lebleu, B., and Chernomordik, L.V. Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. *J. Biol. Chem*. 2005; 280, 15300–15306.
- Rinaldo C, Prodosmo A, Siepi F, Soddu S. HIPK2: a multitalented partner for transcription factors in DNA damage response and development This paper is one of a selection of

papers published in this Special Issue, entitled 28th International West Coast Chromatin and Chromosome Conference, and has undergone the Journal's usual peer review process. *Biochemistry and Cell Biology*. 2007; 85 (4): 411–418.

Rivers A, Gietzen K, Vielhaber E, Virshup D. Regulation of casein kinase I epsilon and casein kinase I delta by an in vivo futile phosphorylation cycle. *Journal of Biological Chemistry*. 1998; 273 (26): 15980–15984.

Rodríguez-Ulloa, A., Ramos, Y., Gil, J., Perera, Y., Castellanos-Serra, L., García, Y., Betancourt, L., Besada, V., González, L.J., Fernández-de-Cossio, J., et al. Proteomic profile regulated by the anticancer peptide CIGB-300 in non-small cell lung cancer (NSCLC) cells. *J. Proteome Res*. 2010; 9, 5473–5483.

Roscic A, Moller A, Calzado M, Renner F, Wimmer V, Gresko E, Ludi K, Schmitz M. Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. *Molecular cell*. 2006; 24 (1): 77–89.

Ruzzene M, Penzo D, Pinna L. Protein kinase CK2 inhibitor 4, 5, 6, 7-tetrabromobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells. *Biochem. J*. 2002; 364: 41–47.

Ruzzene M, Pinna L. Addiction to protein kinase CK2: a common denominator of diverse cancer cells? *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*. 2010; 1804 (3): 499–504.

Salvi M, Cesaro L, Tibaldi E, Pinna L. Motif analysis of phosphosites discloses a potential prominent role of the Golgi casein kinase (GCK) in the generation of human plasma phospho-proteome. *Journal of proteome research*. 2010; 9 (6): 3335–3338.

Salvi M, Sarno S, Cesaro L, Nakamura H, Pinna L. Extraordinary pleiotropy of protein kinase CK2 revealed by weblogo phosphoproteome analysis. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 2009; 1793 (5): 847–859.

Salvi, M., Sarno, S., Marin, O., Meggio, F., Itarte, E., and Pinna, L.A. Discrimination

between the activity of protein kinase CK2 holoenzyme and its catalytic subunits. *FEBS Lett.* 2006; 580, 3948–3952.

Sandholt, I.S., Olsen, B.B., Guerra, B., and Issinger, O.-G. Resorufin: a lead for a new protein kinase CK2 inhibitor. *Anticancer Drugs* 2009; 20, 238–248.

Sarno S, Demoliner E, Ruzzene M, Pagano M, Battistutta R, Bain J, Fabbro D, Schoepfer J, Elliott M, Furet P, Others. Biochemical and three-dimensional-structural study of the specific inhibition of protein kinase CK2 by [5-oxo-5, 6-dihydroindolo-(1, 2-a) quinazolin-7-yl] acetic acid (IQA). *Biochem. J.* 2003; 374: 639–646.

Sarno S, Moro S, Meggio F, Zagotto G, Dal Ben D, Ghisellini P, Battistutta R, Zanotti G, Pinna L. Toward the rational design of protein kinase casein kinase-2 inhibitors. *Pharmacology & therapeutics.* 2002; 93 (2): 159–168.

Sarno S, Reddy H, Meggio F, Ruzzene M, Davies S, Donella-Deana A, Shugar D, Pinna L. Selectivity of 4, 5, 6, 7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 (casein kinase-2). *FEBS letters.* 2001; 496 (1): 44–48.

Sarno S, Ruzzene M, Frascella P, Pagano M, Meggio F, Zambon A, Mazzorana M, Di Maira G, Lucchini V, Pinna L. Development and exploitation of CK2 inhibitors. *Molecular and cellular biochemistry.* 2005; 274 (1-2): 69–76.

Sarno, S., Vaglio, P., Marin, O., Meggio, F., Issinger, O. and Pinna, L. A. Basic Residues in the 74–83 and 191–198 Segments of Protein Kinase CK2 Catalytic Subunit are Implicated in Negative but not in Positive Regulation by the beta-Subunit. *European Journal of Biochemistry*, 1997; 248 (2), pp. 290–295.

Sass, G., Klinger, N., Sirma, H., Hashemolhosseini, S., Hellerbrand, C., Neureiter, D., Wege, H., Ocker, M., and Tiegs, G. Inhibition of experimental HCC growth in mice by use of the kinase inhibitor DMAT. *Int. J. Oncol.* 2011; 39, 433–442.

Seeram N, Henning S, Zhang Y, Suchard M, Li Z, Heber D. Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 hours. *The Journal of nutrition.* 2006; 136 (10): 2481–2485.

- Seger D, Seger R, Shaltiel D. The CK2 phosphorylation of vitronectin: Promotion of cell adhesion via the 3-phosphatidylinositol 3-kinase pathway. *J Biol Chem.* 2001; 276:16998–17006
- Seldin D.C., P. Leder, Casein kinase II alpha transgene-induced murine lymphoma: relation to theileriosis in cattle, *Science* 1995; 267 894–897
- Shin, J. P62 and the sequestosome, a novel mechanism for protein metabolism. *Arch. Pharm. Res.* 1998; 21, 629–633.
- Siddiqui-Jain, A., Bliesath, J., Macalino, D., Omori, M., Huser, N., Streiner, N., Ho, C.B., Anderes, K., Proffitt, C., O'Brien, S.E., et al. CK2 inhibitor CX-4945 suppresses DNA repair response triggered by DNA-targeted anticancer drugs and augments efficacy: mechanistic rationale for drug combination therapy. *Mol. Cancer Ther.* 2012; 11, 994–1005.
- Silva A., J.A. Yunes, B.A. Cardoso, L.R. Martins, P.Y. Jotta, M. Abecasis, A.E. Nowill, N.R. Leslie, A.A. Cardoso, J.T. Barata, PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability, *J. Clin. Invest.* 2008; 118 3762–3774
- Soderling T. Protein kinases. Regulation by autoinhibitory domains. *J Biol Chem.* 1990; 265 (4): 1823–1826.
- Solares, A.M., Santana, A., Baladrón, I., Valenzuela, C., González, C.A., Díaz, A., Castillo, D., Ramos, T., Gómez, R., Alonso, D.F., et al. Safety and preliminary efficacy data of a novel casein kinase 2 (CK2) peptide inhibitor administered intralesionally at four dose levels in patients with cervical malignancies. *BMC Cancer* 2009; 9, 146.
- Solimini NL, Luo J, Elledge SJ Non-oncogene addiction and the stress phenotype of cancer cells. *Cell* 2007; 130:986–988
- Stauber, R.H., and Pavlakis, G.N. Intracellular trafficking and interactions of the HIV-1 Tat protein. *Virology* 1998; 252, 126–136.

- Stepanova V, et al. Urokinase-dependent human vascular smooth muscle cell adhesion requires selective vitronectin phosphorylation by ectoprotein kinase CK2. *J Biol Chem* 2002; 277:10265–10272.
- Stolarczyk E, Reiling C, Pickin K, Coppage R, Knecht M, Paumi C. Casein kinase 2- α regulates multidrug resistance-associated protein 1 function via phosphorylation of Thr249. *Molecular Pharmacology*. 2012; 82 (3): 488–499.
- Suzuki Y, Cluzeau J, Hara T, Hirasawa A, Tsujimoto G, Oishi S, Ohno H, Fujii N. Structure-Activity Relationships of Pyrazine-Based CK2 Inhibitors: Synthesis and Evaluation of 2, 6-Disubstituted Pyrazines and 4, 6-Disubstituted Pyrimidines. *Archiv der Pharmazie*. 2008; 341 (9): 554–561.
- Szebeni, A., Hingorani, K., Negi, S., and Olson, M.O.J. Role of protein kinase CK2 phosphorylation in the molecular chaperone activity of nucleolar protein b23. *J. Biol. Chem*. 2003 278, 9107–9115.
- Taylor S, Kornev A, P R. Protein kinases: evolution of dynamic regulatory proteins. *Trends in biochemical sciences*. 2011; 36 (2): 65–77.
- Taylor S, Radzio-Andzelm E, Knighton D, Ten Eyck L, Sowadski J, Herberg F, Yonemoto W, Zheng J. Crystal structures of the catalytic subunit of cAMP-dependent protein kinase reveal general features of the protein kinase family. *Receptor*. 1993; 3 (3): 165.
- Tan, Y.-J., Teng, E., and Ting, A.E. A small inhibitor of the interaction between Bax and Bcl-X(L) can synergize with methylprednisolone to induce apoptosis in Bcl-X(L)-overexpressing breast-cancer cells. *J. Cancer Res. Clin. Oncol*. 2003 129, 437–448.
- Tawfic S, Yu S, Wang H, Faust R, Davis A, Ahmed K. Protein kinase CK2 signal in neoplasia. *Histol Histopathol* 2001; 16:573–582
- Tchernev, V. T., Mansfield, T. A., Giot, L., Kumar, A. M., N, Abalan, K., Li, Y., Mishra, V. S., Detter, J. C., Rothberg, J. M., Wallace, M. R. and Others. The Chediak-Higashi protein interacts with SNARE complex and signal transduction proteins. *Molecular*

Medicine, 2002; 8 (1), p. 56.

Thomberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P. et al. A combinatorial approach of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* 1997; 272, 17907– 17911

Torres J, Rodriguez J, Myers MP, Valiente M, Graves JD, Tonks NK, Pulido R
Phosphorylation-regulated cleavage of the tumor suppressor PTEN by caspase-3: implications for the control of protein stability and PTEN-protein interactions. *J Biol Chem.* 2003; 278(33):30652-60.

Trock B, Leonessa F, Clarke R. Multidrug resistance in breast cancer: a meta-analysis of MDR1/gp170 expression and its possible functional significance. *Journal of the National Cancer Institute.* 1997; 89 (13): 917–931.

Truant, R., and Cullen, B.R. The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. *Mol. Cell. Biol.* 1999; 19, 1210–1217.

Tucker C, Fields S. Lethal combinations. *Nature genetics.* 2003; 35 (3): 204–205.

Unger G.M., A.T. Davis, J.W. Slaton, K. Ahmed, Protein kinase CK2 as regulator of cell survival: implications for cancer therapy, *Curr. Cancer Drug Targets* 2004; 4 77–84.

Valabrega G, Montemurro F, Aglietta M. Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Annals of oncology.* 2007; 18 (6): 977–984.

Valero E, De Bonis S, Filhol O, Wade R, Langowski J, Chambaz E, Cochet C. Quaternary Structure of Casein Kinase 2 characterization of multiple oligomeric states and relation with its catalytic activity. *Journal of Biological Chemistry.* 1995; 270 (14): 8345–8352.

- Vener, O A, Pagano M, Tosoni K, Meggio F, Cassidy D, Stobbart M, Pinna L, Mehta A. Understanding protein kinase CK2 mis-regulation upon F508del CFTR expression. *Naunyn-Schmiedeberg's archives of pharmacology*. 2011; 384 (4-5): 473—488
- Venerando, A., Pagano, M.A., Tosoni, K., Meggio, F., Cassidy, D., Stobbart, M., Pinna, L.A., and Mehta, A. Understanding protein kinase CK2 mis-regulation upon F508del CFTR expression. *Naunyn Schmiedebergs Arch. Pharmacol*. 2011; 384, 473–488.
- Vilk G, Saulnier R, Pierre R, Litchfield D. Inducible expression of protein kinase CK2 in mammalian cells. Evidence for functional specialization of CK2 isoforms. *Journal of Biological Chemistry*. 1999; 274 (20): 14406–14414.
- Wadia, J.S., and Dowdy, S.F. Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer. *Adv. Drug Deliv. Rev*. 2005; 57, 579–596.
- Wallace M, Adams M, Kanouni T, Mol C, Dougan D, Feher V, O'Connell S, Shi L, Halkowycz P, Dong Q. Structure-based design and synthesis of pyrrole derivatives as MEK inhibitors. *Bioorganic & medicinal chemistry letters*. 2010; 20 (14): 4156–4158.
- Widder J, Chen W, Li L, Dikalov S, Th"Ony B, Hatakeyama K, Harrison D. Regulation of tetrahydrobiopterin biosynthesis by shear stress. *Circulation research*. 2007; 101 (8): 830–838.
- Wirkner, U., Voss, H., Lichter, P. and Pyerin, W. Human protein kinase CK2 genes. *Cellular & molecular biology research*, 1994; 40 (5-6), p. 489.
- Yamada D, P'Erez-Torrado R, Filion G, Caly M, Jammart B, Devignot V, Sasai N, Ravassard P, Mallet J, Sastre-Garau X, Others. The human protein kinase HIPK2 phosphorylates and downregulates the methyl-binding transcription factor ZBTB4. *Oncogene*. 2009; 28 (27): 2535–2544.
- Yamane, K. and Kinsella, T. J. CK2 inhibits apoptosis and changes its cellular localization following ionizing radiation. *Cancer Res*, 2005; 65, 4362–4367.

- Yang-Feng, T. L., Naiman, T., Kopatz, I., Eli, D., Dafni, N. and Canaani, D. Assignment of the human casein kinase II [alpha][prime] subunit gene (CSNK2A1) to chromosome 16p13. 2-p13. 3. *Genomics;(United States)*, 1994; 19 (1).
- Yao K, Youn H, Gao X, Huang B, Zhou F, Li B, Han H. Casein kinase 2 inhibition attenuates androgen receptor function and cell proliferation in prostate cancer cells. *The Prostate*. 2012; 72 (13): 1423–1430.
- Yin, X., Gu, S. and Jiang, J. X. Regulation of lens connexin 45.6 by apoptotic protease, caspase-3. *Cell Adhesion Commun*. 2001; 8, 373– 376
- Xu, X., Toselli, P. A., Russell, L. D. and Seldin, D. C. Globozoospermia in mice lacking the casein kinase II alpha catalytic subunit. *Nat. Genet*. 1999; 23, 118– 121
- Zahreddine H, Borden K. Mechanisms and insights into drug resistance in cancer. *Frontiers in pharmacology*. 2013; 4.
- Zanin, S., Borgo, C., Girardi, C., O'Brien, S.E., Miyata, Y., Pinna, L.A., Donella-Deana, A., and Ruzzene, M. Effects of the CK2 inhibitors CX-4945 and CX-5011 on drug-resistant cells. *PLoS ONE*, 2012; 7, e49193.
- Zhang, C., Vilks, G., Canton, D. A. and Litchfield, D. W. Phosphorylation regulates the stability of the regulatory CK2beta subunit. *Oncogene*, 2002; 21 (23), pp. 3754–3764.
- Zheng, Y., McFarland, B.C., Drygin, D., Yu, H., Bellis, S.L., Kim, H., Bredel, M., and Benveniste, E.N. Targeting protein kinase CK2 suppresses prosurvival signaling pathways and growth of glioblastoma. *Clin. Cancer Res*. 2013; 19, 6484–6494.
- Ziegler, A., and Seelig, J. Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters. *Biophys. J*. 2004; 86, 254–263.
- Zimina EP, et al. Extracellular phosphorylation of collagen XVII by ecto-CK2 inhibits ectodomain shedding. *J Biol Chem*. 2007; 282:22737–22746.

PUBLICATIONS

Effects of the CK2 Inhibitors CX-4945 and CX-5011 on Drug-Resistant Cells

Sofia Zanin¹, Christian Borgo¹, Cristina Girardi¹, Sean E. O'Brien², Yoshihiko Miyata³, Lorenzo A. Pinna^{1,4}, Arianna Donella-Deana¹, Maria Ruzzene^{1,4*}

1 Department of Biomedical Sciences and National Research Council Institute of Neurosciences, University of Padova, Padova, Italy, **2** Cylene Pharmaceuticals, San Diego, California, United States of America, **3** Department of Cell and Developmental Biology, Graduate School of Biostudies, Kyoto University, Kyoto, Japan, **4** Venetian Institute of Molecular Medicine, Padova, Italy

Abstract

CK2 is a pleiotropic protein kinase, which regulates many survival pathways and plays a global anti-apoptotic function. It is highly expressed in tumor cells, and is presently considered a promising therapeutic target. Among the many inhibitors available for this kinase, the recently developed CX-4945 and CX-5011 have proved to be very potent, selective and effective in inducing cell death in tumor cells; CX-4945 has recently entered clinical trials. However, no data are available on the efficacy of these compounds to overcome drug resistance, a major reasons of cancer therapy failure. Here we address this point, by studying their effects in several tumor cell lines, each available as variant R resistant to drug-induced apoptosis, and normal-sensitive variant S. We found that the inhibition of endogenous CK2 was very similar in S and R treated cells, with more than 50% CK2 activity reduction at sub-micromolar concentrations of CX-4945 and CX-5011. A consequent apoptotic response was induced both in S and R variants of each pairs. Moreover, the combined treatment of CX-4945 plus vinblastine was able to sensitize to vinblastine R cells that are otherwise almost insensitive to this conventional antitumor drug. Consistently, doxorubicin accumulation in multidrug resistant (MDR) cells was greatly increased by CX-4945. In summary, we demonstrated that all the R variants are sensitive to CX-4945 and CX-5011; since some of the treated R lines express the extrusion pump Pgp, often responsible of the MDR phenotype, we can also conclude that the two inhibitors can successfully overcome the MDR phenomenon.

Citation: Zanin S, Borgo C, Girardi C, O'Brien SE, Miyata Y, et al. (2012) Effects of the CK2 Inhibitors CX-4945 and CX-5011 on Drug-Resistant Cells. PLoS ONE 7(11): e49193. doi:10.1371/journal.pone.0049193

Editor: Alexander V. Ljubimov, Cedars-Sinai Medical Center, United States of America

Received: August 9, 2012; **Accepted:** October 5, 2012; **Published:** November 8, 2012

Copyright: © 2012 Zanin et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants from University of Padova (Progetto Ateneo 2011) to MR, from Associazione Italiana per la Ricerca sul Cancro Project IG 10312 to LAP, and from Italian Miur PRIN 2008 to LAP. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: SOB is currently employed by Cylene Pharmaceuticals which holds United States patents for CX-4945 (United States 7,956,064) and CX-5011 (United States 7,910,600). This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials. The remaining authors have declared that no competing interests exist.

* E-mail: maria.ruzzene@unipd.it

Introduction

CK2 is a Ser/Thr protein kinase usually present in the cells as a tetrameric enzyme composed of two catalytic (α and/or α') and two regulatory (β) subunits. It is constitutively active and ubiquitously expressed, and phosphorylates such a striking number of substrates to be considered the most pleiotropic protein kinase [1]. It is involved in several cellular processes, such as cell cycle, gene expression, protein synthesis, signal transduction and metabolism; however, its hall-mark is considered its prosurvival and anti-apoptotic function [2–5]. This is supported by the observation that many CK2 substrates are proteins involved in cell death/survival, and, more importantly, that the reduction of CK2 activity or expression (induced by cell treatment with specific inhibitors or by RNA interference technology, respectively) is invariably followed by cell death, mainly due to apoptosis (reviewed in [6]).

Consistent with the anti-apoptotic function of CK2, cancer cells, which are characterized by rapid proliferation and defective apoptosis, express particularly high levels of CK2. It has a special role in tumorigenesis [7], potentiating pathways that are frequently

up-regulated or untimely activated in cancer [8], and it has consequently been defined as “a key player in cancer biology” [9]. Whenever comparison has been performed, CK2 has been shown significantly more abundant in tumor cells than in healthy counterparts. However, at the same time tumors rely more on CK2 for their survival, and this phenomenon, described as “addiction” to CK2 of cancer cells [6], explains why they are more sensitive to its inhibition or knocking-down, compared to normal cells.

On these bases, CK2 is presently considered a promising therapeutic target [7,10], also exploiting the fact that, due to the peculiar structure of the CK2 catalytic site [11,12], several very specific inhibitors are available (reviewed in [13]). Many of them have already proved to be able to kill cancer cells and in some cases also employed for successful animal treatment (e.g. [14–18]).

The two compounds CX-4945 and CX-5011 are among the most selective and effective CK2 inhibitors developed so far. They are tricyclic ATP-competitive compounds, displaying a K_i in vitro <1 nM [17,19], and an unprecedented selectivity for CK2, proved by profiling them against a panel of 235 protein kinases [19]. Both CX-4945 and CX-5011 are able to cause apoptosis in a

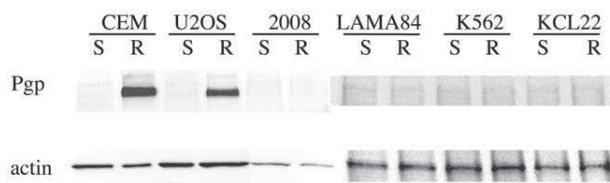


Figure 1. Pgp expression. 10 μ g of proteins from total lysates of the indicated cells were loaded onto an SDS-PAGE, blotted and analyzed by WB with anti-Pgp or anti-actin.
doi:10.1371/journal.pone.0049193.g001

number of cancer cell lines and are effective in reducing tumor size in animal models of cancer [17,20]; CX-4945 is orally bio-available, and is presently in clinical trial for treatment of different kinds of cancer [17]. However, CX-4945 and CX-5011 have never been tested in cells that are resistant to drug-induced apoptosis.

Apoptosis resistance is a major reason of cancer therapy failure; its mechanisms can be different and multifaceted, and is only partially understood. In many cases it is due to the (over)expression of extrusion pumps of the ABC-transporter family, such as Pgp, which drive drugs outside the cell and reduce their effective concentration [21]. Cells expressing these pumps are selected for their survival in response to treatment with a certain drug, but usually a cross-resistance occurs towards other compounds, even not structurally related; in these cases, cells are indicated as multidrug-resistant (MDR). Many other mechanisms have been reported to be involved in apoptosis resistance, including alteration in genetic features, DNA repair, drug target molecules, metabolic and growth pathways [22,23]. In some cases, specific resistance is observed, such as that towards Imatinib and its derivatives targeting Bcr-Abl tyrosine kinase, frequently due to kinase mutations, but also to epigenetic changes, alternative splicing or induction of compensatory signaling pathways [24].

CK2 has been already associated to the phenomenon of drug resistance: it phosphorylates Pgp [25] and another extrusion pump, MRP1 [26] and its inhibition allows a higher accumulation of drugs in Pgp [27] or MRP1 [26] expressing cells, suggesting that

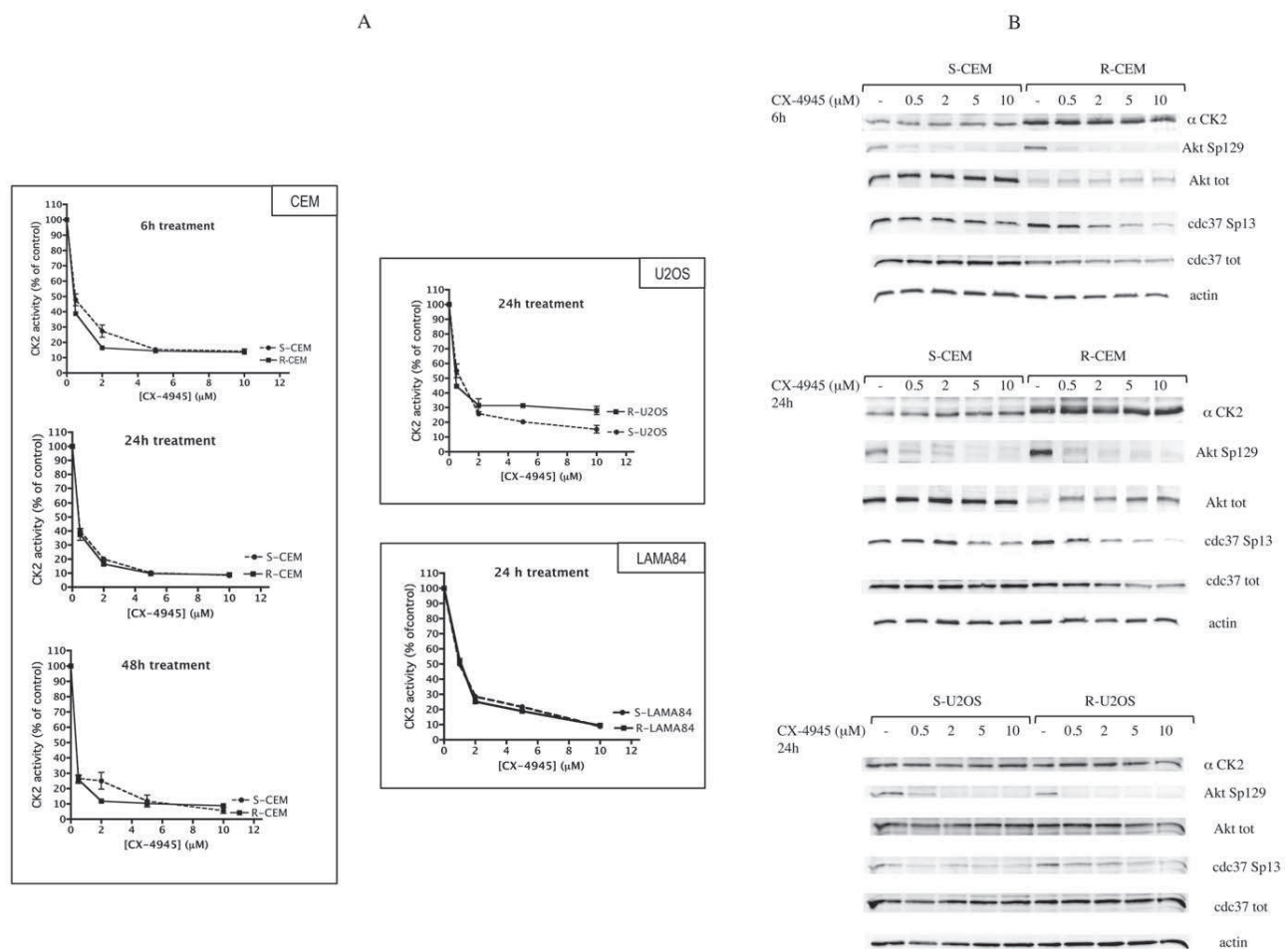


Figure 2. CK2 activity in CX-4945-treated cells. (A) CK2 activity was measured towards a synthetic specific peptide. 1–2 μ g of proteins from total lysates of the indicated cells were incubated with the peptide and a radioactive phosphorylation mixture (see Materials and Methods). Activity is reported as percentage of that found in vehicle-treated control cells. Mean $\pm$ SE values of four independent experiments are shown. (B) 10 μ g of total proteins were analyzed with the indicated antibodies; actin was used to normalize the loading. Representative WB of three independent experiments are shown.
doi:10.1371/journal.pone.0049193.g002

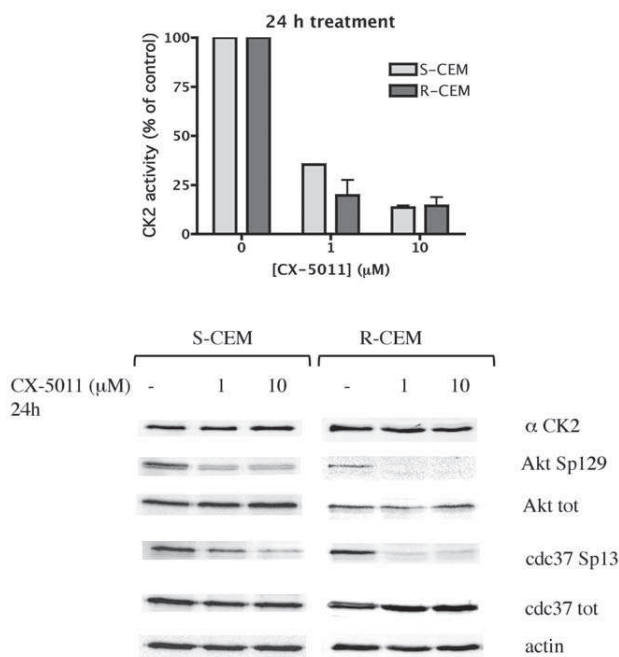


Figure 3. CK2 activity in CX-5011-treated cells. S-CEM and R-CEM cells were treated for 24 h with different concentrations of CX-5011. Cell lysates were analyzed for the activity of CK2 towards a synthetic specific peptide (upper graph), or by WB with the indicated antibodies (lower panel, separate development for S- and R-CEM cells). See details in the legend of Figure 2.

doi:10.1371/journal.pone.0049193.g003

CK2 can up-regulate the Pgp function. Moreover, we have previously found that the CK2 catalytic subunit is overexpressed in a MDR cell line compared to the non-MDR counterpart, and that its overexpression contributes to the maintenance of the resistant phenotype [27].

Here we evaluate the efficacy of the CK2 inhibitors CX-4945 and CX-5011 in a number of different cell lines, available as pairs, each pair containing a variant selected for resistance to drug-induced apoptosis, and we demonstrate that these compounds can overcome the problem of drug resistance.

Materials and Methods

Antibodies

CK2 α -subunit antisera were raised in rabbit against the sequence of the human protein at C-terminus [376–391], total Akt and total Cdc37 antibodies were from Santa Cruz Biotechnology, β -actin monoclonal antibodies were from Sigma, PARP antibodies were from Roche, Pgp monoclonal antibodies were from Calbiochem, Akt Sp129 phospho-specific antibodies were raised in rabbit and purified as elsewhere described [28]; Cdc37 Sp13 phospho-specific antibodies were from Abcam; secondary antibodies towards rabbit and mouse IgG, conjugated to horse radish peroxidase, were from PerkinElmer.

Cell lines

The following cell lines were used in this study: human T lymphoblastoid CEM cells (normal sensitive, S-CEM, and their MDR variant, R-CEM, selection with 0.1 μ g/ml vinblastine, Vbl [27]); human osteosarcoma U2OS cells (normal sensitive, S-U2OS, and their MDR variant, R-U2OS [29]); human ovarian

carcinoma 2008 cells (normal sensitive, S-2008, and their cisplatin-resistant variant, R-2008 [30]); chronic myeloid leukemia (CML) LAMA84 [31], KCL22 [32], and K562 [32] cell lines, sensitive or resistant to Imatinib, kindly supplied by Dr. C. Gambacorti-Passerini.

Inhibitors

CX-4945 (5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid) and CX-5011 (5-(3-ethynylphenylamino)pyrimido[4,5-c]quinoline-8-carboxylic acid) were synthesized by Cylene Pharmaceutical. Solutions were made in dimethylsulfoxide (DMSO).

Cell culture and treatment

Cells were cultured in an atmosphere containing 5% CO₂; CEM, LAMA84, K562 and KCL22 cell lines were maintained in RPMI 1640 medium (Sigma), U2OS and 2008 lines were maintained in D-MEM medium (Sigma); both media were supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cell treatments with inhibitors were performed in the culture medium, but with 1% FCS, where not differently indicated. Control cells were treated with equal amounts of the inhibitor solvent. At the end of the incubations, cells were harvested by centrifugation, washed, and lysed as indicated below.

Cell viability

Cell viability was detected by means of MTT (3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltriazolium bromide) reagent: cells (10⁵ cells/100 μ l) were incubated for variable times in a 96-well plate under the indicated conditions. 1 h before the end of the incubation, 10 μ l of MTT solution (5 mg/ml in PBS) were added to each well. Incubations were stopped by addition of 20 μ l of lysis solution at pH 4.7, as described elsewhere [33]. Plates were read for OD at λ 590 nm, in a Titertek Multiskan Plus plate reader (Flow Laboratories). DC₅₀ (concentrations inducing 50% of cell death) values were calculated with Prism 4.0c software (GraphPad Software).

Apoptosis assay

Apoptosis was evaluated by means of the Cell Detection Elisa kit (Roche), based on the quantification of nucleosomes present in the cytosol of the apoptotic cells, by measuring the absorbance at λ 405– λ 490, following the manufacturer's instructions. About 10 000 cells were used for each determination.

Cell lysis and western blot analysis

For lysate preparation, cells were lysed as described in [27]. Protein concentration was determined by the Bradford method. Equal amounts of proteins were loaded on 11% SDS-PAGE, blotted on Immobilon-P membranes (Millipore), and processed in western blot (WB) with the indicated antibody, detected by chemiluminescence. Quantitation of the signal was obtained by chemiluminescence detection on a Kodak Image Station 440MM PRO and analysis with the Kodak 1D Image software.

CK2 activity assay

1–2 μ g of lysate proteins were incubated for 10 min at 30°C with 0.1 mM CK2-specific peptide RRRADDSDDDDD, in the presence of phosphorylation reaction mixture [34]. Phosphorylation of endogenous proteins was performed on 5 μ g of lysate proteins under the same conditions, but without the addition of the

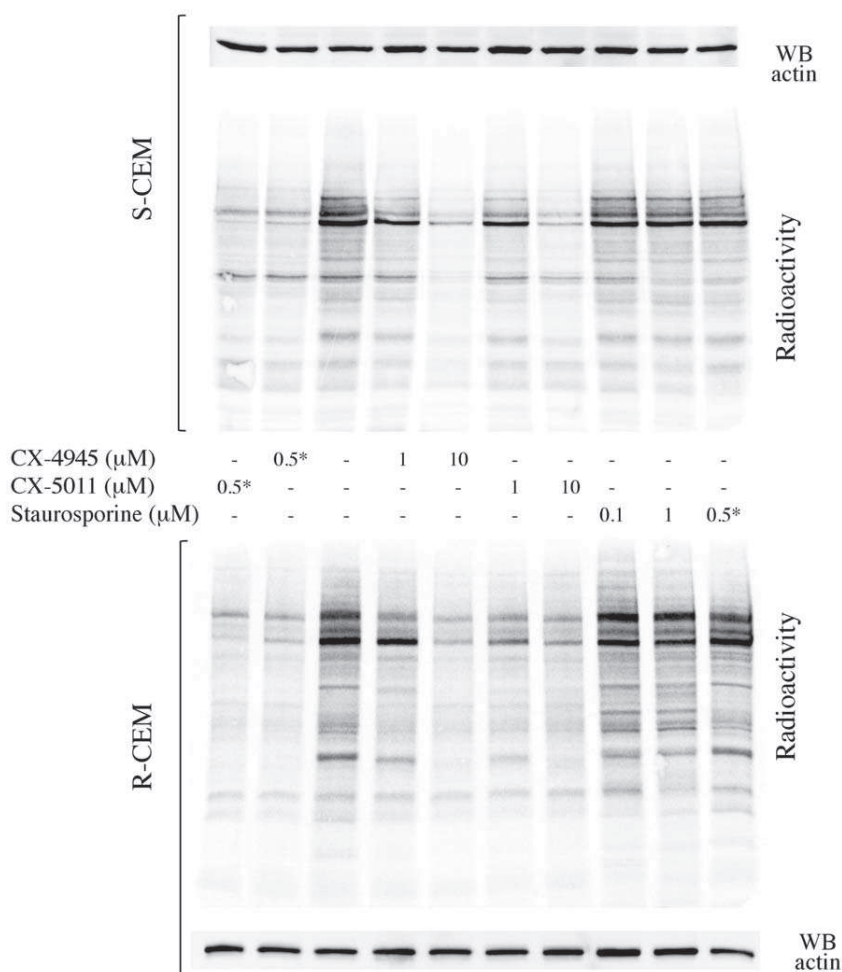


Figure 4. Protein phosphorylation in lysates from cells treated with CX-4945, CX-5011, or staurosporine. S-CEM (upper panel) or R-CEM (lower panel) were treated with the indicated concentrations of the CX inhibitors or staurosporine for 16 h, then lysed. 5 μg of total proteins were incubated with a radioactive phosphorylation mixture, resolved by SDS-PAGE, blotted, and analyzed by digital autoradiography (radioactivity). WB for actin was used as loading control. Asterisk * denotes samples where the inhibitors were added in vitro during the phosphorylation assay (not administrated to the cells). Representative results of five independent experiments are shown.
doi:10.1371/journal.pone.0049193.g004

peptide substrate, and analyzed by SDS/PAGE and digital autoradiography (CyclonePlus, PerkinElmer).

Combined treatments

Drug interactions were assessed by treating cells with increasing concentrations of both Vbl and CX-4945, at fixed concentration ratio, as indicated. The combination index (CI) [35], was calculated with the software Calcsyn (Biosoft); $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergistic, additive and antagonistic effects, respectively. Values are reported as mean of three separate experiments $\pm$ SE.

Doxorubicin accumulation measurement

Doxorubicin measurements were performed as previously described [27]. Briefly, CEM cells were treated with 25 μM doxorubicin (Sigma) for 30 min, after 30 min preincubation in the absence or in the presence of CX-4945. After washing with phosphate-buffered saline, cells were lysed in the same buffer, supplemented with 1% (w/v) SDS. Doxorubicin content was determined fluorimetrically (λ_{exc} 485 nm, λ_{em} 590 nm).

Statistical analysis

All experiments were performed at least 3 times, each time in duplicate; graphs and statistical analysis were performed with Prism 4.0c software (GraphPad Software).

Results

For this study, we used 6 different pairs of cell lines, each available in two variants: the S variant, corresponding to cells normally sensitive to drug-induced apoptosis, and the R variant, characterized by drug resistance due to different reasons. As shown in Figure 1, CEM and U2OS resistant cells express the Pgp pump, responsible for the extrusion of a variety of drugs from the cell (reviewed by Goda et al., [36]); thus R-CEM and R-U2OS are considered MDR (multi-drug resistant) lines. Pgp is not appreciably expressed by the R variants of the other cell lines, which instead display resistance for specific drugs: 2008 cells were selected for their resistance to cisplatin, while R-LAMA84, R-KCL22 and R-K562 are resistant to Imatinib [37].

CX-4945

CX-5011

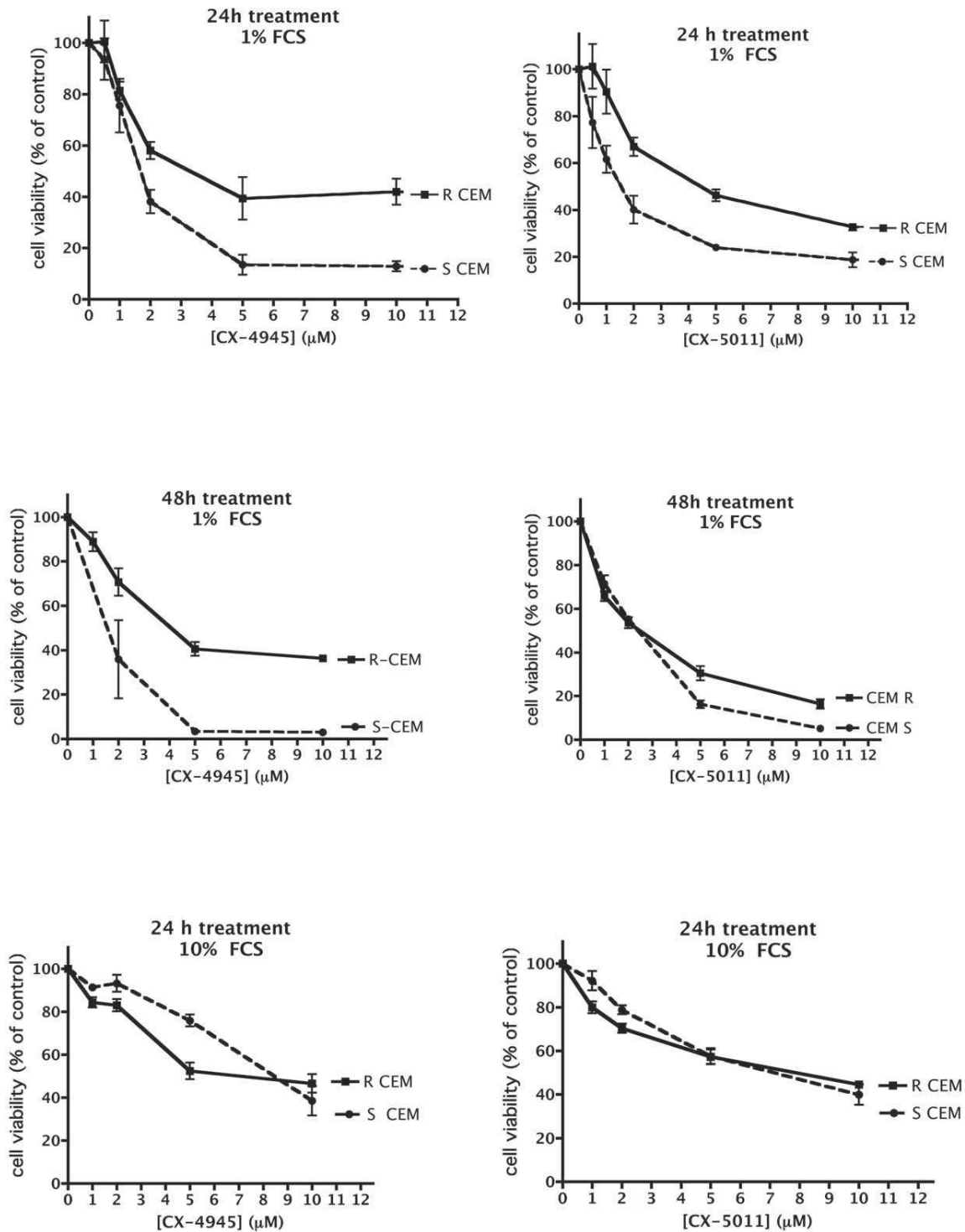


Figure 5. Cell viability of CX-4945 and CX-5011 treated CEM cells. Cells were incubated with increasing concentrations of the CX compounds; the FCS concentration in the medium and the treatment time are indicated on each graph. Cell viability was assessed by the MTT method, assigning 100% value to the vehicle-treated control cells. Mean $\pm$ SE values of at least four independent experiments are reported. doi:10.1371/journal.pone.0049193.g005

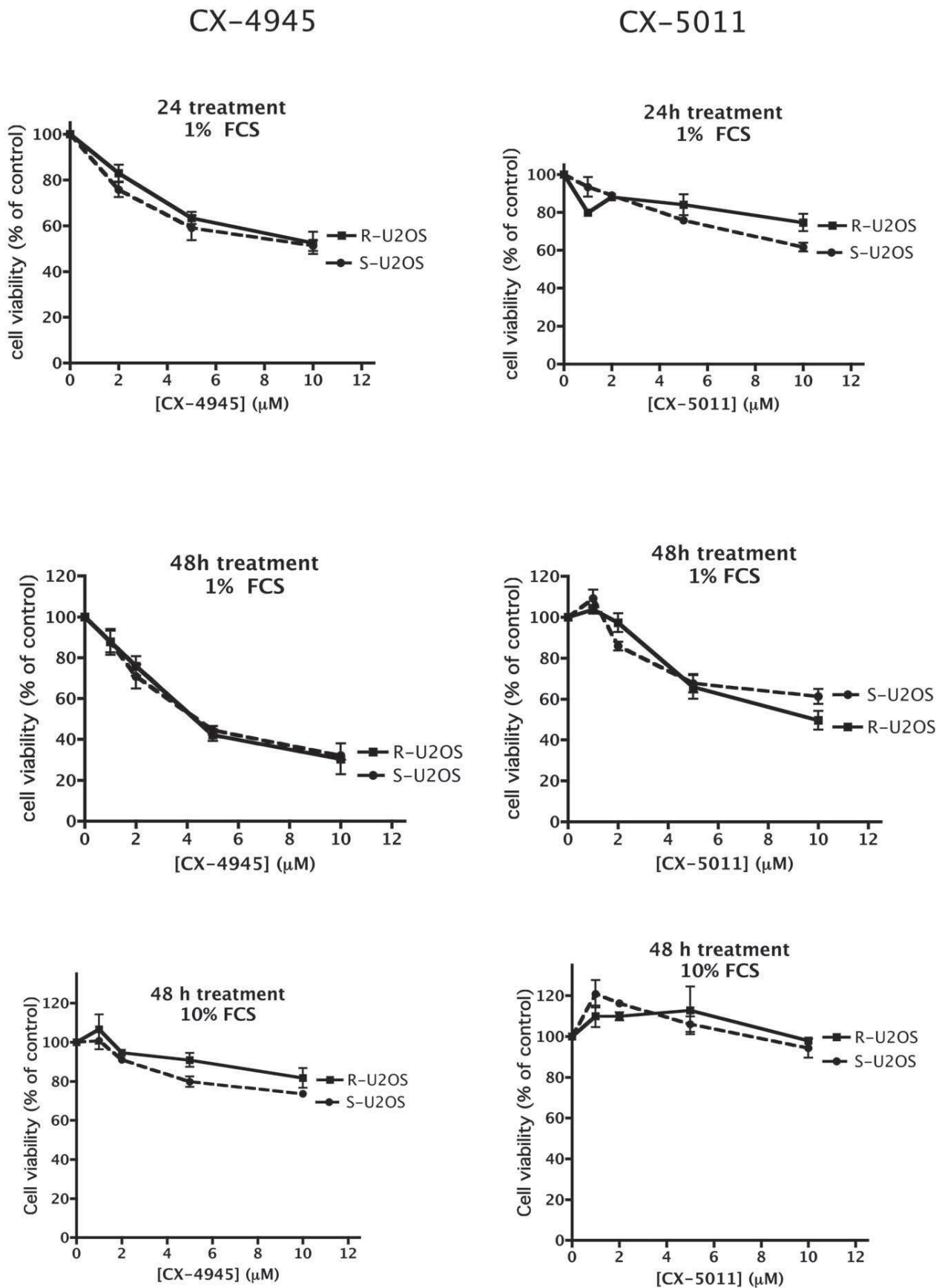


Figure 6. Cell viability of CX-4945 and CX-5011 treated U2OS cells. See legend of Figure 5 for details.
doi:10.1371/journal.pone.0049193.g006

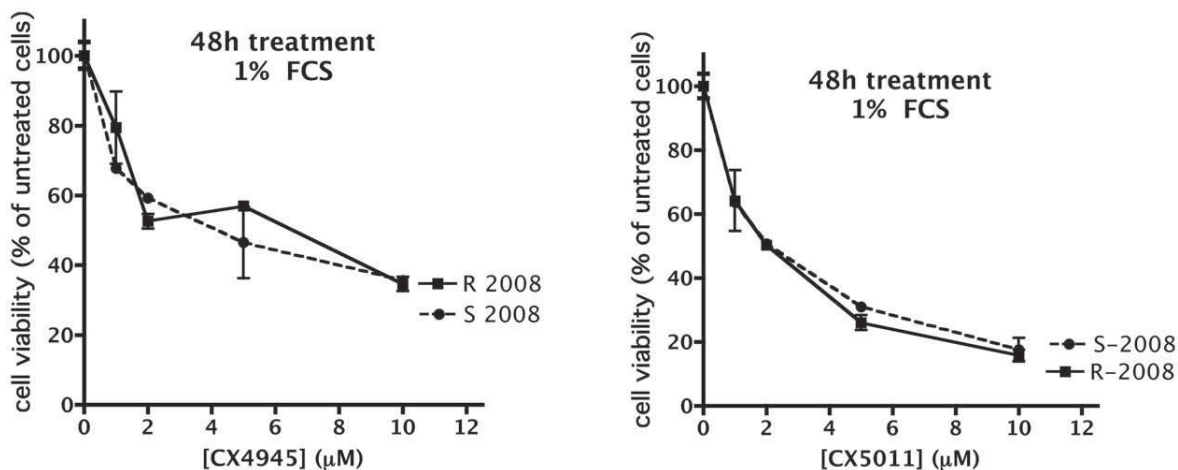


Figure 7. Cell viability of CX-4945 and CX-5011 treated 2008 cells. See legend of Figure 5 for details.
doi:10.1371/journal.pone.0049193.g007

We first measured CK2 activity in cells treated for different times with increasing concentrations of the compounds. To this purpose we exploited two strategies: in vitro assays of endogenous CK2 present in total lysates from treated cells [34], and evaluation of the phosphorylation state of CK2 specific intracellular substrates, by means of phospho-specific antibodies towards two key targets of CK2, namely Akt S129 [28] and Cdc37 S13 [38]. Both approaches indicated that the compounds promptly inhibit CK2 in S and R cells, with similar efficacy, without affecting the amount of CK2. Figure 2 shows the results obtained with CX-4945, in CEM, U2OS, and LAMA84 cells, while in Figure 3 the effects of CX-5011 on CEM cells is shown; similar results were observed in response to treatment of the other cell lines (not shown). As evident in Figure 2B, the expression of CK2 significantly differs in S- and R- CEM cells, as already reported [27], and, in untreated cells (see control lanes, marked as “-” in Figure 2B), it correlates with the phosphorylation level of endogenous substrates Cdc37 Sp13 (phospho-Ser13) and Akt Sp129 (phospho-Ser129), despite the low level of Akt in R-CEM. In all cases, at submicromolar concentrations of the compounds, CK2 activity is reduced by more than 50%, and a parallel dephosphorylation of endogenous substrates occurs. Interestingly, a 6 h treatment is sufficient to inhibit CK2 to almost the maximal level, and longer treatment times only minimally increase the degree of inhibition (Figure 2A). The reduction of Akt Sp129 is also promptly induced (6 h, 0.5 μM CX-4945), while the CK2 target site on Cdc37 seems to be more resistant to dephosphorylation, which is clearly observed only at higher concentrations and longer treatments (Figure 2B). Similar results are observable in response to CX-5011 (not shown and Figure 3 for 24 h treatment).

Inhibition of CK2 was also confirmed by analyzing the radioactive phosphorylation of endogenous proteins in treated cells. Figure 4 shows that several protein bands are less phosphorylated in S- and R-CEM cells treated with CX-4945 or CX-5011 (similarly to what is observable when the compounds are added in vitro, during the phosphorylation assay). To rule out the possibility of a non-specific effect, we treated cells with staurosporine, at concentrations which inhibit the majority of protein kinases but not CK2 [39]. This treatment, while inducing a high degree of cell death (not shown) similarly to what caused by the CK2 inhibitors (see below), it is almost ineffective on endogenous protein phosphorylations; this also suggests that,

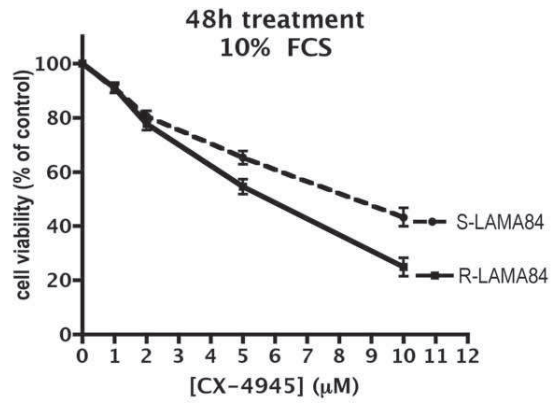
under the used conditions, CK2 is the major kinase responsible for the observed radioactivity, as expected for a highly expressed, pleiotropic and constitutively active enzyme.

To ascertain if CK2 inhibition by CX-4945 and CX-5011 is effective in inducing cell death also in case of drug resistance, we treated cells with increasing concentrations of the compounds, and measured cell viability by the MTT method. Figures 5, 6, 7, 8 show representative results obtained under different time and assay conditions. We found that both inhibitors are able to induce appreciable cell death also in R cells, in a manner quite similar to S cells (at least when 10% FCS is present, see Discussion). In particular, the responsiveness of MDR cells (R-CEM and R-U2OS) indicates that these compounds are not substrate of the Pgp. Table 1 shows the DC_{50} values calculated from the MTT assays shown in Figure 5, 6, 7, 8; the 48 h assays were used for calculation, except for CEM cells in the presence of 10% FCS, where 24 h assays were considered.

To understand if cell death induced by CX-4945 and CX-5011 in our cell lines was due to apoptosis, we evaluated the formation of nucleosomes in treated cells; the results, shown in Figure 9 for CEM cells, and confirmed for the other cell lines (not shown), indicated that apoptosis occurs to a similar degree in S and R cells, in response to these CK2 inhibitors. The results are also confirmed by the cleavage of the caspase substrate PARP (Figure 9).

Then we assessed if the treatment with CX compounds can sensitize resistant cells towards conventional antitumor drugs; in particular, we considered the Vbl-resistant R-CEM cells, and we evaluated if the combined treatment with CX-4945 induces a higher degree of cell death in response to Vbl. As shown in Figure 10 (lower panels), very low concentrations of CX-4945 are able to significantly reduce the DC_{50} of Vbl (from 39.4 to 6.2 $\mu\text{g}/\text{ml}$). The calculated Combination Index (CI) is indeed 0.66 ± 0.04 , where values <1 indicate synergism [35]. Interestingly, CX-4945 exerts a synergistic effect with Vbl also in S-CEM: as shown in Figure 10, upper panels, the effect of very low concentrations of Vbl on cell viability is increased by the simultaneous administration of the CK2 inhibitor ($\text{CI} = 0.70 \pm 0.08$), indicating that the combined treatment can be applied for therapy with significantly lower drug doses. An important synergistic effect is also observable between Imatinib and CX-4945 on Imatinib-resistant cells (manuscript in preparation).

CX-4945



CX-5011

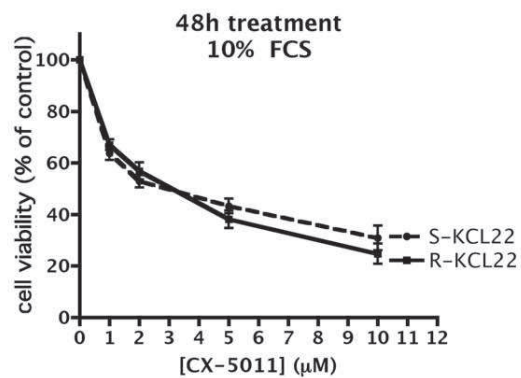
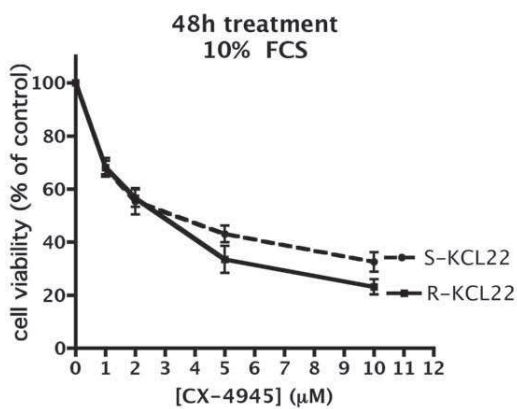
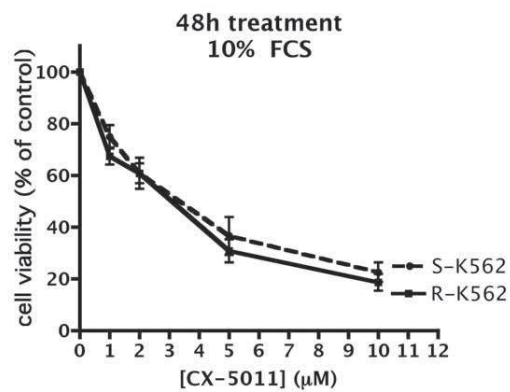
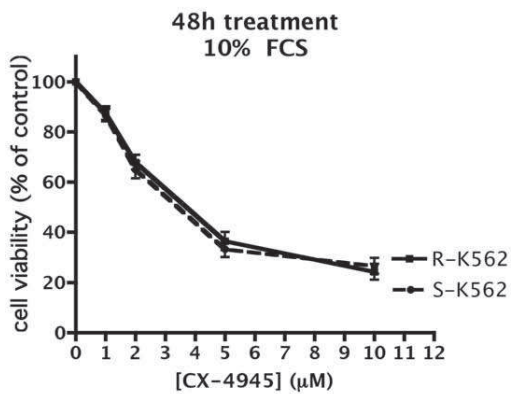
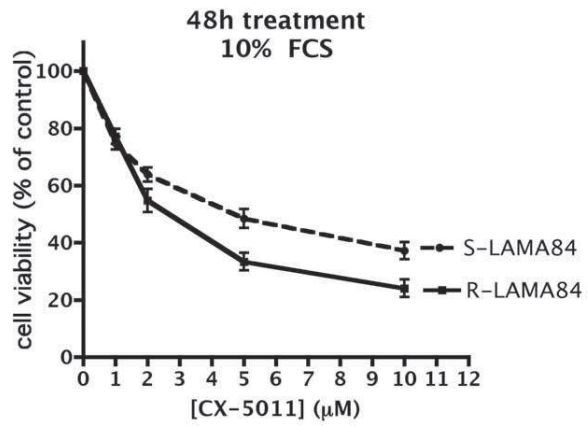


Figure 8. Cell viability of CX-4945 and CX-5011 treated Imatinib-resistant cells. See legend of Figure 5 for details.
doi:10.1371/journal.pone.0049193.g008

Table 1. DC₅₀ of CX-4945 and CX-5011.

	CEM		CEM*		U2OS		2008		LAMA84		K562		KCL22	
	1% FCS		10% FCS		1% FCS		1% FCS		10% FCS		10% FCS		10% FCS	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R
CX-4945	1.92	4.09	8.62	6.50	4.58	4.33	4.87	6.56	8.55	5.79	3.41	3.72	3.30	2.95
(μ M)	± 1.00	± 0.27	± 0.68	± 1.40	± 0.44	± 0.63	± 2.50	± 0.48	± 1.10	± 0.86	± 0.55	± 0.42	± 1.46	± 0.89
CX-5011	2.40	2.51	7.51	7.59	>10	8.59	2.13	2.01	5.07	2.68	3.58	3.05	3.01	3.10
(μ M)	± 0.07	± 0.60	± 2.33	± 1.35		± 0.62	± 0.02	± 0.21	± 1.42	± 0.81	± 1.18	± 0.78	± 1.39	± 0.86

The values, calculated from 48 h MTT assays reported in Figures 5, 6, 7, 8 (except for CEM* = 24 h treatment), are the mean of 4–6 independent experiments $\pm$ Standard Deviation. FCS concentration (v/v) during the treatment is also indicated.

doi:10.1371/journal.pone.0049193.t001

Since the extrusion pumps expressed in MDR cells are phosphorylated by CK2 [25,26], and an activation effect has been clearly demonstrated, at least in the case of MRP1 [26], we finally assessed whether a short treatment with CX-4945 allows a higher accumulation of conventional drugs inside resistant cells. To this purpose, exploiting the fluorescence of doxorubicin, we measured its uptake in CEM cells pre-treated with CX-4945, compared to untreated cells (Figure 11), and we found that the inhibitor markedly increases the amount of drug retained inside R-CEM cells, while it is almost ineffective in S-CEM cells.

Discussion

Although CK2 is not considered a direct cause of cancer and cannot be strictly defined as an oncogene, its high abundance in cancer cells is indicative of its importance in tumorigenesis. We have previously hypothesized [40] that, whenever, for any reason, a cell displays a higher level of CK2, that cell will have a survival advantage over the other cells, and will be selected to proliferate under the pressure represented by treatment with pro-apoptotic drugs. CK2 is thus expected to play a major role in the apoptosis resistant phenotype, as also suggested by previous studies [27,41]. Moreover, since CK2 expression is not linked to specific types of cancers, its targeting could be a successful strategy, because of its very general applicability and widespread effects.

Importantly, cancer cells are expected to be more sensitive to CK2 inhibition than normal cells, since they are addicted to CK2, strongly relying on it for their survival [6]. Accordingly, the CK2 inhibitors have proved to be more effective in tumor cell lines than in normal ones [17,42], and, on these bases, CX-4945 has entered clinical trials, with promising initial results. Here we demonstrate that the CX inhibitors are also able to overcome the problem of resistance to apoptosis, since they are similarly effective in resistant cells (R) and their normally sensitive counterparts (S). Interestingly, our R cell lines display different kind of apoptosis resistance: R-2008 cells are resistant to cisplatin; R-LAMA84, R-KCL22 and R-K562 are resistant to Imatinib, while R-CEM and R-U2OS are MDR cells, expressing the Pgp pump. Therefore, the first outcome of our data is that CK2 inhibition has a general effect on resistant cells, by reducing the efficacy of cellular equipment to escape apoptosis; secondly, we can conclude that CX-4945 and CX-5011 inhibitors are not recognized by the Pgp, since their effects are visible in cells expressing this MDR pump.

An observation from our results is that the CX compounds, especially CX-5011, seem to be less effective with U2OS cells than with the other cell lines (compare Figure 6 with Figures 5, 7 and 8); the reasons are presently unknown, however they are not related

to the MDR phenomenon, being the response very similar in the S and R variants of this cell line.

It can also be observed that the inhibitors are slightly less effective in R-CEM than in S-CEM; however the difference between the two variants is detectable on cell viability and not on endogenous CK2 inhibition, and is abrogated when cell viability assays are performed in the presence of 10% instead of 1% FCS (Figure 5). Although we do not know the exact reason of the different results observed at 10% or 1% FCS, our interpretation is that, at quite critical conditions (such as 1% FCS), the anti-apoptotic machinery of R-CEM is more effective than that of S-CEM in protecting cells from the stress represented by CK2 inhibition; on the contrary, under fully healthy conditions (10% FCS), the two cell variants are equally equipped to counteract apoptosis. In any case, these findings suggest that any observed difference is not due to extrusion of the inhibitors by the Pgp, as also confirmed by the results obtained with other Pgp-expressing cells (compare S-U2OS and R-U2OS, Figure 6).

We have previously found that R-CEM display a higher level of the CK2 catalytic subunit compared to S-CEM [27], and this is confirmed by the results here shown in Figure 2B, where it is also evident that the phosphorylation state of CK2-dependent sites (particularly of Akt Sp129) in R-CEM is higher than in S-CEM. Interestingly, despite the different endogenous CK2 activity, the degree of inhibition induced by CX-4945 and CX-5011 is very similar in S- and R-CEM cells (Figure 2A).

While it is obviously confirmed that CK2 blockade causes apoptosis, an interesting observation emerging from our results is that cell death is appreciable only when the degree of CK2 inhibition induced by the CX compounds is sufficiently high to ensure a dephosphorylated state of major substrates. In fact we found that, while the endogenous CK2 activity towards a peptide substrate is already halved in cell treated with $<0.5 \mu$ M inhibitors (see for example 24 h treatment in CEM cells, Figure 2), significantly higher concentrations are required to induce 50% cell death (see Figure 5). However, if we consider the phosphorylation states of the CK2 sites analyzed by phospho-specific antibodies, we observe that while Akt Sp129 is promptly reduced, Cdc37 Sp13 phosphorylation is much more stable. Of course, extending our considerations to the multitude of CK2 substrates, we can presume that each one has its own susceptibility to CK2 inhibition, that will mainly depend on the turnover of its phosphorylation state; since this is obviously the result of the balance between kinase and phosphatase activity, there will be a variability depending on cell type and conditions. We cannot exclude that the dephosphorylation of one or few specific CK2 substrates is required before cell death occurs; alternatively, we

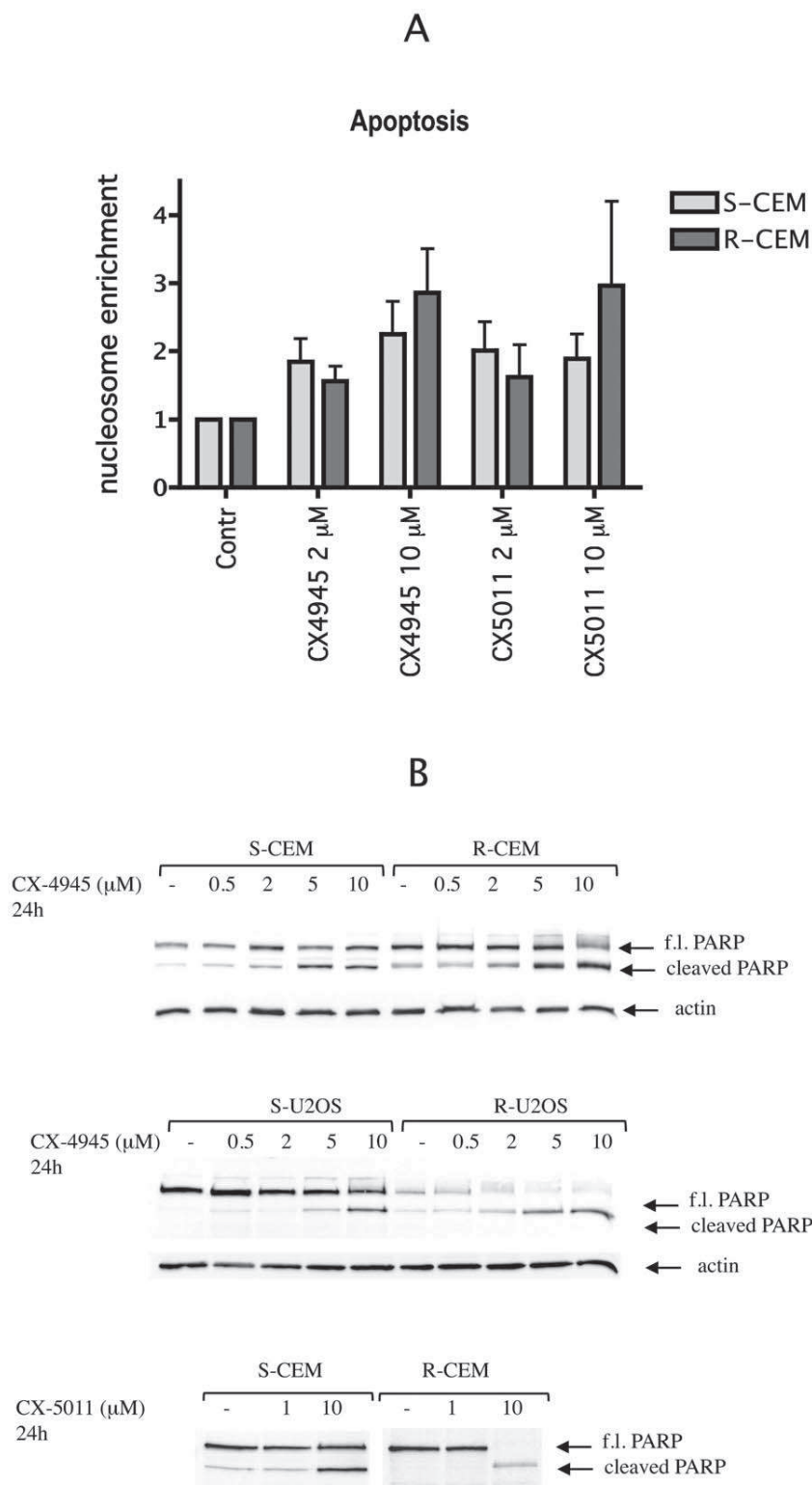
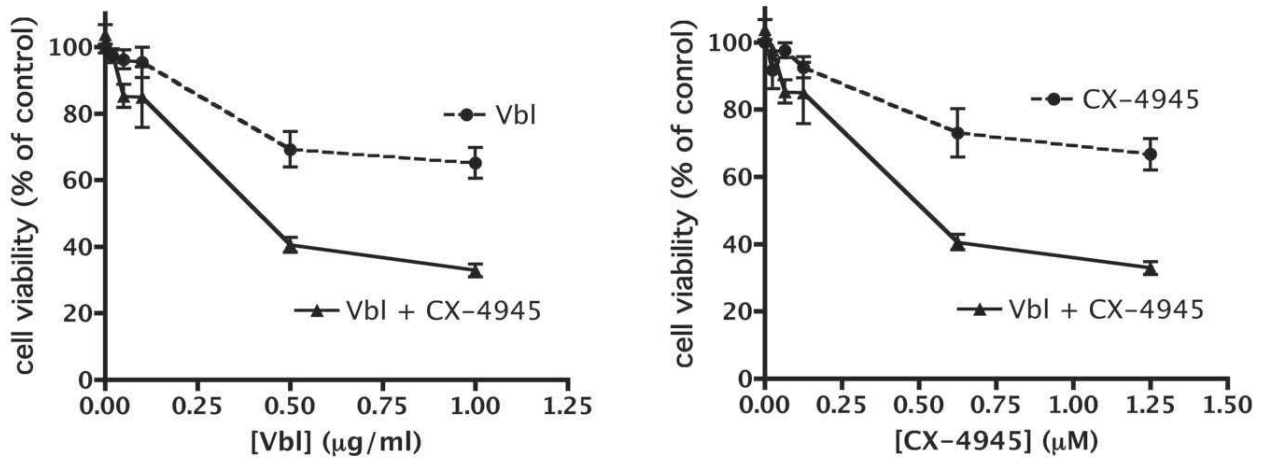


Figure 9. Apoptosis induction by CX-4945 and CX-5011. (A) Apoptosis was assessed by evaluation of nucleosomes present in the cytosol, using the Cell Detection Elisa kit (Roche), after 8 h of CEM cell treatment as indicated. Nucleosome enrichment was calculated from the ratio between the signal in treated and untreated cells; reported values are the means $\pm$ SE of four independent experiments. (B) Caspase-dependent PARP cleavage was analyzed by WB on 10 μ g proteins of lysate from CEM or U2OS cells treated as indicated. Actin WB was used as loading control. Representative WB of three independent experiments are shown. f.l. PARP: full length PARP.
doi:10.1371/journal.pone.0049193.g009

S-CEM 24h treatment



R-CEM 24h treatment

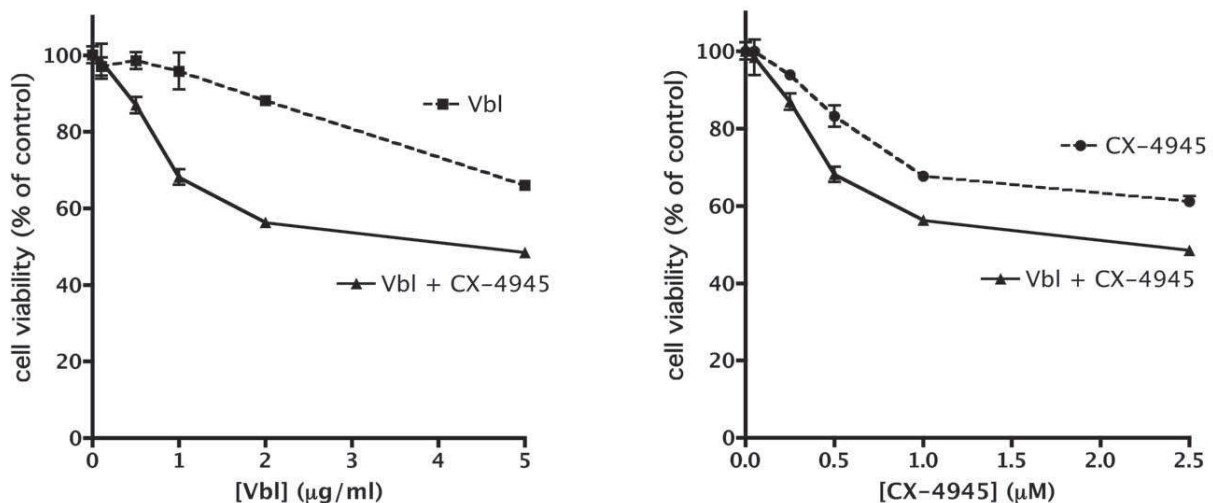


Figure 10. Effect of combined treatment with CX compounds and chemotherapeutic drugs. S-CEM and R-CEM viability was assessed by the MTT method after 24 h treatment with increasing concentrations of both CX-4945 and Vbl, administrated alone or in combination, at fixed ratio (1 µM CX-4945 : 0.8 µg/ml Vbl for S-CEM, 1 µM CX-4945 : 2 µg/ml Vbl for R-CEM, due to their different sensitivity to Vbl). Viability (mean values $\pm$ SE of four experiments) was plotted as function of Vbl concentrations (left panels), or CX-4945 concentrations (right panels).
doi:10.1371/journal.pone.0049193.g010

can assume that only a massive dephosphorylation of CK2 substrates will produce cell death, whose extent, therefore, does not necessarily correlate with the decrease of CK2 catalytic activity.

Another important outcome of our data is that CX-4945 can be useful to sensitize resistant cells to conventional chemotherapeutic drugs. It has already been reported that CX-4945 augments the anti-tumor efficacy of gemcitabine and cisplatin on ovarian cancers [43]. The association of CX-4945 with Erlotinib, an

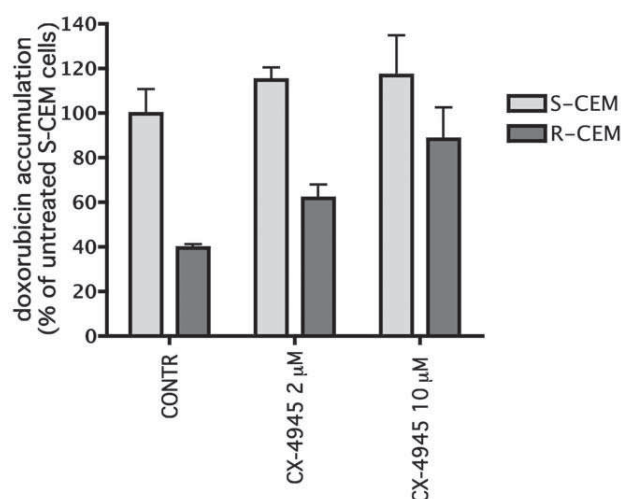


Figure 11. Effect of CX-4945 on doxorubicin accumulation. CEM cells were preincubated for 30 min with vehicle (Contr) or CX-4945 at the indicated concentrations, then further incubated for 30 min with 25 μ M doxorubicin, whose amount, detected fluorimetrically as described in Materials and Methods, is reported as percentage of the value found in control S-CEM (mean $\pm$ SE of three independent experiments).
doi:10.1371/journal.pone.0049193.g011

EGF-receptor inhibitor, has also been proposed [44]; in this case, interestingly, the enhanced killing effect of the combined treatment is accompanied by a reduction of the PI3K-Akt-mTOR pathway,

with the complete abrogation of Akt phosphorylation. Here we extend the drug-combination experiments to MDR cells, showing that R-CEM are six fold more sensitive to Vbl when simultaneously treated with sub-lethal doses of CX-4945, compared to when treated with Vbl alone. Moreover, we also found that the CK2 inhibition by CX-4945 allows an increased accumulation of doxorubicin in R-CEM (Figure 11), most probably blocking the positive effect that CK2 exerts on Pgp. As expected, the drug accumulation is unaffected by CX-4945 in S-CEM, not expressing Pgp; since the synergism between CX-4945 and chemotherapeutic drug is instead observable also in S-CEM (Figure 10), we have to assume that additional mechanisms of increased cell death, other than the effect on the Pgp pump, are induced by the combined treatment.

A parallel study has been undertaken for the synergistic effect of Imatinib and CX-4945 in Imatinib-resistant LAMA84 cell line (manuscript in preparation).

In summary, our results show that CX-4945 and CX-5011 are internalized in resistant cells, inhibit endogenous CK2, and, alone or in combination with other drugs, induce significant cell death. We suggest that they can be seriously considered as a valid therapeutic strategy also in case of pharmacological resistance occurrence.

Author Contributions

Conceived and designed the experiments: MR ADD LAP. Performed the experiments: SZ CB CG. Analyzed the data: MR SZ CB CG ADD. Contributed reagents/materials/analysis tools: SOB YM. Wrote the paper: MR.

References

- Salvi M, Sarno S, Cesaro L, Nakamura H, Pinna LA (2009) Extraordinary pleiotropy of protein kinase CK2 revealed by weblogo phosphoproteome analysis. *Biochim Biophys Acta* 1793: 847–859.
- Ahmed K, Gerber DA, Cochet C (2002) Joining the cell survival squad: an emerging role for protein kinase CK2. *Trends Cell Biol* 12:226–230.
- Litchfield DW (2003) Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem J* 369:1–15.
- Ahmad KA, Wang G, Unger G, Slaton J, Ahmed K (2008) Protein kinase CK2—a key suppressor of apoptosis. *Adv Enzyme Regul* 48:179–187.
- St-Denis NA, Litchfield DW (2009) Protein kinase CK2 in health and disease: From birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival. *Cell Mol Life Sci* 66:1817–1829.
- Ruzzene M, Pinna LA (2010) Addiction to protein kinase CK2: a common denominator of diverse cancer cells? *Biochim Biophys Acta* 1804: 499–504.
- Duncan JS, Litchfield DW (2008) Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. *Biochim Biophys Acta* 1784:33–47.
- Dominguez I, Sonenshein GE, Seldin DC (2009) Protein kinase CK2 in health and disease: CK2 and its role in Wnt and NF-kappaB signaling: linking development and cancer. *Cell Mol Life Sci* 66:1850–1857.
- Trembley JH, Wang G, Unger G, Slaton J, Ahmed K (2009) Protein kinase CK2 in health and disease: CK2: a key player in cancer biology. *Cell Mol Life Sci* 66:1858–1867.
- Trembley JH, Chen Z, Unger G, Slaton J, Kren BT, et al. (2010) Emergence of protein kinase CK2 as a key target in cancer therapy. *Biofactors* 36:187–195.
- Sarno S, Ghisellini P, Pinna LA (2002) Unique activation mechanism of protein kinase CK2. The N-terminal segment is essential for constitutive activity of the catalytic subunit but not of the holoenzyme. *J Biol Chem* 277: 22509–22514.
- Battistutta R (2009) Protein kinase CK2 in health and disease: Structural bases of protein kinase CK2 inhibition. *Cell Mol Life Sci* 66: 1868–1889.
- Sarno S, Papinutto E, Franchin C, Bain J, Elliott M, et al. (2011) ATP site-directed inhibitors of protein kinase CK2: an update. *Curr Top Med Chem* 11: 1340–1351.
- Ljubimov AV, Caballero S, Aoki AM, Pinna LA, Grant MB, et al. (2004). Involvement of protein kinase CK2 in angiogenesis and retinal neovascularization. *Invest Ophthalmol Vis Sci* 45:4583–4591.
- Mishra S, Pertz V, Zhang B, Kaur P, Shimada H, et al. (2007) Treatment of P190 Bcr/Abl lymphoblastic leukemia cells with inhibitors of the serine/threonine kinase CK2. *Leukemia* 21:178–180.
- Prudent R, Moucadel V, Nguyen CH, Barette C, Schmidt F, et al. (2010) Antitumor activity of pyridocarbazole and benzopyridoindole derivatives that inhibit protein kinase CK2. *Cancer Res* 70:9865–9874.
- Siddiqui-Jain A, Drygin D, Streiner N, Chua P, Pierre F, et al. (2010) CX-4945, an orally bioavailable selective inhibitor of protein kinase CK2, inhibits pro-survival and angiogenic signaling and exhibits antitumor efficacy. *Cancer Res* 70: 10288–10298.
- Manni S, Brancaloni A, Quotti Tubi L, Colpo A, Pavan L, et al. (2012) Protein kinase CK2 protects multiple myeloma cells from ER stress-induced apoptosis and from the cytotoxic effect of HSP90 inhibition through regulation of the unfolded protein response. *Clin Cancer Res* 18:1888–1900.
- Battistutta R, Cozza G, Pierre F, Papinutto E, Lolli G, et al. (2011) Unprecedented selectivity and structural determinants of a new class of protein kinase CK2 inhibitors in clinical trials for the treatment of cancer. *Biochemistry* 50: 8478–8488.
- Siddiqui-Jain A, Chua P, Darjanian L, Lim J, O'Brien S, et al. (2008) The discovery and characterization of CX-5011, a highly selective, potent inhibitor of Protein Kinase CK2. *AACR Meeting Abstracts* 2008: 4875.
- Fukuda Y, Schuetz JD (2012) ABC transporters and their role in nucleoside and nucleotide drug resistance. *Biochem Pharmacol* 83: 1073–1083.
- Sawicka M, Kalinowska M, Skierski J, Lewandowski W (2004) A review of selected anti-tumour therapeutic agents and reasons for multidrug resistance occurrence. *J Pharm Pharmacol* 56: 1067–1081.
- Tew KD (2012) Drug resistance in cancer. Editorial. *Biochem Pharmacol* 83:985–6.
- Rosenzweig SA (2012) Acquired resistance to drugs targeting receptor tyrosine kinases. *Biochem Pharmacol* 83: 1041–1048.
- Glavy JS, Horwitz SB, Orr GA (1997) Identification of the in vivo phosphorylation sites for acidic-directed kinases in murine mdr1b P-glycoprotein. *J Biol Chem* 272: 5909–5914.
- Stolarczyk EI, Reiling CJ, Pickin KA, Coppage R, Knecht MR, et al. (2012) Casein Kinase 2 α (CK2 α) Regulates Multidrug Resistance Associated Protein (MRP1) Function Via Phosphorylation of Thr249. *Mol Pharmacol*: Epub ahead of print.
- Di Maira G, Brustolon F, Bertacchini J, Tosoni K, Marmiroli S, et al. (2007) Pharmacological inhibition of protein kinase CK2 reverts the multidrug resistance phenotype of a CEM cell line characterized by high CK2 level. *Oncogene* 26: 6915–6926.
- Di Maira G, Salvi M, Arrigoni G, Marin O, Sarno S, et al. (2005) Protein kinase CK2 phosphorylates and upregulates Akt/PKB. *Cell Death Differ* 12: 668–677.

29. Cenni V, Maraldi NM, Ruggeri A, Secchiero P, Del Coco R, et al. (2004) Sensitization of multidrug resistant human osteosarcoma cells to Apo2 Ligand/TRAIL-induced apoptosis by inhibition of the Akt/PKB kinase. *Int J Oncol* 25: 1599–1608.
30. Marzano C, Gandin V, Folda A, Scutari G, Bindoli A, et al. (2007) Inhibition of thioredoxin reductase by auranofin induces apoptosis in cisplatin-resistant human ovarian cancer cells. *Free Radic Biol Med* 42: 872–881.
31. le Coutre P, Tassi E, Varella-Garcia M, Barni R, Mologni L, et al. (2000) Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood* 95: 1758–1766.
32. Redaelli S, Boschelli F, Perini P, Pirola A, Viltadi M, et al. (2010) Synergistic activity of the Src/Abl inhibitor bosutinib in combination with Imatinib. *Leukemia* 6: 1223–1227.
33. Ruzzene M, Penzo D, Pinna LA (2002) Protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells. *Biochem J* 364: 41–47.
34. Ruzzene M, Di Maira G, Tosoni K, Pinna LA (2010) Assessment of CK2 constitutive activity in cancer cells. *Methods Enzymol* 484: 495–514.
35. Giovannetti E, Mey V, Danesi R, Mosca I, Del Tacca M (2004) Synergistic cytotoxicity and pharmacogenetics of gemcitabine and pemetrexed combination in pancreatic cancer cell lines. *Clin Cancer Res* 10: 2936–2943.
36. Goda K, Bacsó Z, Szabó G (2009) Multidrug resistance through the spectacle of P-glycoprotein. *Curr Cancer Drug Targets* 9: 281–297.
37. Bixby D, Talpaz M (2009) Mechanisms of resistance to tyrosine kinase inhibitors in chronic myeloid leukemia and recent therapeutic strategies to overcome resistance. *Hematology Am Soc Hematol Educ Program*: 461–476.
38. Miyata Y, Nishida E (2005) CK2 binds, phosphorylates, and regulates its pivotal substrate Cdc37, an Hsp90-cochaperone. *Mol Cell Biochem* 274: 171–179.
39. Meggio F, Donella Deana A, Ruzzene M, Brunati AM, Cesaro L, et al. (1995) Different susceptibility of protein kinases to staurosporine inhibition. Kinetic studies and molecular bases for the resistance of protein kinase CK2. *Eur J Biochem* 234:317–322.
40. Ruzzene M, Tosoni K, Zanin S, Cesaro L, Pinna LA (2011) Protein kinase CK2 accumulation in “oncophilic” cells: causes and effects. *Mol Cell Biochem* 356: 5–10.
41. Di Maira G, Brustolon F, Tosoni K, Belli S, Krämer SD, et al. (2008) Comparative analysis of CK2 expression and function in tumor cell lines displaying sensitivity vs. resistance to chemical induced apoptosis. *Mol Cell Biochem* 316: 155–161.
42. Piazza FA, Ruzzene M, Gurrieri C, Montini B, Bonanni L, et al. (2006) Multiple myeloma cell survival relies on high activity of protein kinase CK2. *Blood* 108: 1698–1707.
43. Siddiqui-Jain A, Bliesath J, Macalino D, Omori M, Huser N, et al. (2012) CK2 inhibitor CX-4945 suppresses DNA repair response triggered by DNA-targeted anticancer drugs and augments efficacy: mechanistic rationale for drug combination therapy. *Mol Cancer Ther* 11: 994–1005.
44. Bliesath J, Huser N, Omori M, Bunag D, Proffitt C, et al. (2012) Combined inhibition of EGFR and CK2 augments the attenuation of PI3K-Akt-mTOR signaling and the killing of cancer cells. *Cancer Lett* 322:113–1188.

SYNTHESIS AND PROPERTIES OF A SELECTIVE INHIBITOR OF HOMEODOMAIN-INTERACTING PROTEIN KINASE 2 (HIPK2)

Giorgio Cozza¹, Sofia Zanin¹, Renate Determann², Maria Ruzzene^{1,3}, Conrad Kunick², Lorenzo A. Pinna^{1,3*}

¹ Department of Biomedical Sciences, University of Padova, and CNR Institute of Neurosciences, Viale G. Colombo 3, 35131 Padova, Italy

² Technische Universität Braunschweig, Institut für Medizinische und Pharmazeutische Chemie, Beethovenstraße 55, 38106 Braunschweig, Germany

³ Venetian Institute of Molecular Medicine (VIMM) Padova, Italy, Via G. Orus 2, 35129 Padova, Italy

***Correspondence:** Lorenzo A. Pinna Department of Biomedical Sciences, University of Padova, Viale G. Colombo 3 35131 Padova, Italy tel. +39 049 8276108, Fax. +39 049 8073310, email: lorenzo.pinna@unipd.it

ABSTRACT

Homeodomain-interacting protein kinase 2 (HIPK2) is a Ser/Thr kinase controlling cell proliferation and survival, whose investigation has been hampered by the lack of specific inhibitors able to dissect its cellular functions. SB203580, a p38 MAP kinase inhibitor, has been used as a tool to inhibit HIPK2 in cells, but here we show that its efficacy as HIPK2 inhibitor is negligible ($IC_{50} > 40 \mu M$). In contrast by altering the scaffold of the promiscuous CK2 inhibitor TBI a new class of HIPK2 inhibitors has been generated. One of these, TBID, displays toward HIPK2 unprecedented efficacy ($IC_{50} = 0.33 \mu M$) and selectivity (Gini coefficient 0.592 out of a panel of 76 kinases). The two other members of the HIPK family, HIPK1 and HIPK3, are also inhibited by TBID albeit less efficiently than HIPK2. The mode of action of TBID is competitive with respect to ATP, consistent with modelling. We also provide evidence that TBID is cell permeable by showing that HIPK2 activity is reduced in cells treated with TBID, although with an IC_{50} two orders of magnitude higher (about $50 \mu M$) than in vitro.

INTRODUCTION

The CMGC group of the human kinome is split into several branches, one of which, also including DYRKs and CLKs, gives rise to a sub-branch composed by so called “homeodomain-interacting protein kinases” (HIPKs). Four HIPKs are present in human, with HIPK2 attracting special attention for its role as a regulator of growth and apoptosis in various types of cells [1]. HIPK1/2 double deficient mice exhibit defects in hematopoiesis, vasculogenesis and angiogenesis [2]. HIPK2 was firstly recognized as a DNA damage responsive kinase exerting a tumor suppressor function by mediating p53 activation [3,4]. HIPK2 however can also mediate apoptosis in the absence of p53 [5,6,7] and a number of observations summarized in [1] strongly argue for additional non apoptotic roles of HIPK2, whose precise understanding will require the identification of new HIPK2 targets. These studies have been hampered by the lack of selective inhibitors of HIPK2. Small cell permeable inhibitors of protein kinases have become invaluable reagents for dissecting signaling pathways mediated by each of them. In recent years a huge repertoire of compounds purported to be “specific” toward a large number of protein kinases have become available. Since however the human kinome is composed by some 500 members the issue of selectivity is critical and only in a limited number of cases inhibitors have been shown to display a really narrow selectivity window hitting only few and in very rare cases one individual protein kinases [8,9].

In the case of HIPK2 the rational design of specific inhibitors has never been reported, the only HIPK2 inhibitor mentioned in the literature being SB203580, a compound firstly employed as HIPK2 inhibitor [10] because this kinase displays features similar to p38 like MAP kinase, whose susceptibility to SB203580 was already established. Consequently several laboratories exploited SB203580 as a “HIPK2 inhibitor” (e.g. [11,12]), based on the assumption that its targeting of HIPK2 is selective. However by profiling SB203580 on a panel of 71 protein kinases at $1 \mu M$ concentration, inhibition of HIPK2 was negligible (14%) as compared to that of 6 protein kinases which were inhibited $>60\%$, and it remained below the average inhibition of the whole panel (17.7%) [9]. Moreover the members of the HIPK family are not among the kinases inhibited by SB203580 (nor by any other compound examined) in a comprehensive profiling of kinase inhibitors selectivity [13]. This sheds doubts on the interpretation of the effects of SB203580 as really mediated by cellular HIPK2 blockage.

In the course of our studies aimed at the identification and development of compounds able to inhibit CK2, a highly pleiotropic kinase [14,15], playing a key role as an anti-apoptotic agent [16] and whose abnormally high level enhances the tumor phenotype through a non oncogene addiction mechanism [17,18], we observed that several potent CK2 inhibitors also exert a drastic effect on a few other protein kinases, notably DYRK1A, PIMs and HIPK2 [19,20]. This was especially true of the most common CK2 inhibitors, TBB (TBBt) and TBI (TBBz) and of related tetrabromo-benzimidazole derivatives.

These observations prompted us to design modifications of the tetrabromo-benzimidazole scaffold aimed at decreasing the efficacy toward CK2 and other kinases drastically inhibited by TBI and TBB, while maintaining or eventually improving that toward HIPK2.

Here we describe the properties of one of these derivatives, 4,5,6,7-tetrabromo-2-(1*H*-imidazol-2-yl)isoindoline-1,3-dione (TBID) which is able to inhibit HIPK-2 with a selectivity much higher than that of TBI, not to say of SB203580, whose ability to inhibit HIPK2 is in our hands negligible. These properties in conjunction with cell permeability, make TBID the first choice inhibitor of HIPK2 presently available for both in vitro and in cell studies.

MATERIALS AND METHODS

Chemistry

Synthesis and details concerning compounds **5a-5i** are provided in Supporting Information. Instruments were used and procedures for compound characterization were carried out as published before [21,22].

Source and purification of protein kinases

Native CK2 and CK1 were purified from rat liver [23].

The source of HIPK2 and of all of the other protein kinases used for specificity assays is as described elsewhere [9].

Kinase inhibition assays

HIPK2 (63 ng) was preincubated at 37 °C for 10 minutes either in the absence or in the presence of increasing amounts of each inhibitor in a final volume of 20 µl of solution containing 50 mM Tris-HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM EGTA, 10 mM magnesium acetate. The reaction was started by addition of 5 µl of a reaction mixture containing 20 µM [³³P-ATP] (500-1000 cpm/pmol), and the synthetic peptide substrate NKRRRSPTPPE [24] (600 µM, unless differently indicated). Similar results were obtained by replacing the peptide with MBP (0.33 mg/ml). The reaction was stopped by addition of 5 µl of 0.5 M orthophosphoric acid before spotting aliquots onto phosphocellulose filters. Filters were washed in 75 mM phosphoric acid (5-10 ml/each) four times and then once in methanol and dried before counting.

PIM1, CK2, CK1 phosphorylation assays were performed following similar procedures used for HIPK2 except that preincubation was omitted. In detail, PIM1 activity was determined at 100 µM ATP concentration and in the presence of 30 µM synthetic peptide substrate RKRRQTSMTD. CK2 and CK1 activities were assayed in a buffer containing 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 12 mM MgCl₂, 100 µM specific peptide substrate RRRADDSDDDDD (for CK2), or 200 µM IGDDDDAYSITA (for CK1) and 20 µM [γ -³³P]ATP (500–1000 c.p.m./pmol).

Conditions for the activity assays of all other protein kinases tested in selectivity experiments are as described or referenced in [9].

Kinetics

Initial velocities were determined at each of the ATP concentration tested, at 1 mM NKRRRSPTPPE peptide concentration. Km values were calculated either in the absence or in the presence of increasing concentrations of inhibitor, from Lineweaver-Burk double-reciprocal plots of the data. Inhibition constants were then calculated by linear regression analysis of Km/Vmax versus inhibitor concentration plots.

Selectivity parameters.

Lorenz curves were derived from the selectivity data. Gini coefficients and Hit Rates (expressing the percent of kinases inhibited >50% by a given compound) were calculated as described in [25].

Molecular modeling

Model Building: Human HIPK2 protein kinase was built using an homology modeling approach implemented into Molecular Operating Environment (MOE) [26] with DYRK1A as template (PDB code: 2WO6)[27]. All the ligands and cofactors were removed; hydrogen atoms were added using standard geometries to the protein structure with the MOE program. To minimize contacts between hydrogen's, the structures were subjected to Amber99 force field minimization until the *rms* of conjugate gradient was $<0.05 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ keeping all the heavy atoms fixed [26]. To strictly validate the model generated and to calibrate our high-throughput docking protocol, a small database of known HIPK2 inhibitors was built and a set of docking runs was performed.

Molecular docking: After the calibration phase, all compound structures were docked directly into the ATP binding site of the human HIPK2 model, by using the docking tool part of the GOLD suite [28]. Searching was conducted within a user-specified docking sphere (12 Å from the center of the binding cleft), using the Genetic Algorithm protocol and the GoldScore scoring function. GOLD performs a user-specified number of independent docking runs (50 in our specific case) and writes the resulting conformations and their energies in a molecular database file. Prediction of small molecule-enzyme complex stability (in terms of corresponding pKi value) and the quantitative analysis for non-bonded intermolecular interactions (H-bonds, transition metal, water bridges, hydrophobic, electrostatic) were calculated and visualized using several tools implemented in MOE suite.

Cell culture and treatment

HepG2 cells (human hepatocellular carcinoma) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 unit/ml penicillin and 100 µg/ml streptomycin; CEM cells (human T lymphoblastoid cells) were cultured in RPMI-1640 (Sigma) with the same supplements. Cells were cultured in an atmosphere containing 5% CO₂, at 37 °C. Treatments with TBID were performed in the same medium but with 1% fetal calf serum; control cells were treated with the solvent (DMSO). Total cell lysates were prepared as in [29].

Cellular HIPK2 kinase assays

Endogenous HIPK2 activity was evaluated by measuring the phosphorylation level of its target site Ser46 of p53: to this purpose, CEM cells were treated for 6 h as indicated, then lysed. 10 µg of total proteins were loaded on 11 % SDS-PAGE, blotted on Immobilon-P membranes (Millipore), and analyzed by western blot (WB) using an anti-phospho Ser46 p53 antibody (BD Biosciences); chemiluminescence signals were acquired with a Kodak 4000MM Pro Image Station. Bands were quantified by Carestream Molecular Imaging Software (Kodak) and the obtained values were normalized to total p53 signal with a Cell Signaling Technology antibody; anti-actin (Sigma) was used as loading control.

Alternatively, HIPK2 was immunoprecipitated with 2.5 µl anti-HIPK2 (Epitomics) from 350 µg of total lysate proteins deriving from HepG2 cells either treated or not with TBID following a protocol elsewhere described [30]. An aspecific antibody was used as negative control. Immunoprecipitated HIPK2 activity was measured towards the specific peptide substrate (NKRRRSPTPPE) at 1.6 mM concentration, for 10 min at 30 °C, under the same conditions described above for the in vitro kinase assay. Peptide radioactivity was measured after sample spotting on phospho-cellulose paper, washing and scintillation counting, as in [30], while the amount of HIPK2 immunoprecipitated was evaluated by WB.

Cell viability and apoptosis assays

Cell viability was evaluated by means of MTT (3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltriazolium bromide) reagent; 10⁵ cells/100 µl were incubated in a 96-well plate and treated for 6 h as indicated. 1 h before the end of the incubation, 10 µl of MTT solution (5 mg/ml in PBS) was added to each well. Incubations were stopped by addition of 20 µl of lysis solution at pH 4.7, as described elsewhere [30]. Plates were read for OD at λ 590 nm, in a Titertek Multiskan Plus plate reader (Flow Laboratories). Apoptosis was evaluated looking for the cleavage of the caspase substrate PARP with anti-PARP

antibody (Roche), recognizing both the full length (116 kDa) and the cleaved fragment (85 kDa) of PARP

RESULTS AND DISCUSSION

1. Synthesis of tetrabromoisindoline-1,3-dione derivatives that inhibit HIPK2.

The structures of two commonly used CK2 inhibitors, TBB and TBI are shown in Figure 1. These compounds share a number of bromine atoms clustered on their benzene ring which are essential for interaction with the kinase active site [31,32]; they also share the ability to inhibit HIPK2 besides CK2. This is especially true of TBI whose IC_{50} values with either HIPK and CK2 are nearly identical (0.7 vs 0.6 μ M, [19] and Table 1). Since these compounds have been shown to become entrapped in a hydrophobic cavity adjacent to the ATP binding site, whose size in CK2 is particularly small, owing to a number of bulky side chains which are replaced by smaller ones in the majority of protein kinases, HIPK2 included, we reasoned that a device to reduce affinity toward CK2 as compared to that toward HIPK2 could be to increase the size of the tetrabrominated ligand. This goal was attained by derivatizing a tetrabromoisindoline-1,3-dione scaffold (nearly super-imposable to that of TBI) with an imidazole group, to give rise to 4,5,6,7-tetrabromo-2-(1*H*-imidazol-2-yl)isindoline-1,3-dione (TBID, **5a**). The synthesis of TBID (**5a**) and analogs **5b-5i** was carried out following a published general synthesis protocol [33] by reacting tetrabromophthalic anhydride with suitable aminosubstituted hetarenes (See Supporting Information, Figure S1).

As shown in Table 1, TBID (**5a**) inhibits HIPK2 with the same efficiency as TBI, while displaying toward CK2 a more than 10-fold higher IC_{50} value.

Compared to TBID, all analogs **5b-5i** were less potent HIPK2 inhibitors (Table 1). The outcome of this analysis underscores the crucial role of the 5-membered imidazole ring to achieve high inhibitory efficiency toward HIPK2: in fact its replacement with 6-membered aromatic rings or even with 5-membered rings with different spacing between the nitrogen atoms promotes big rises in IC_{50} values. Especially telling in this respect is the loss of inhibitory efficacy underwent by compound **5e**, whereas compounds **5c** and **5d**, albeit less potently than TBID, still inhibit significantly HIPK2, highlighting the crucial role of a basic nitrogen atom at position 3.

Modeling accounts for the above observations disclosing the role of this nitrogen for binding. HIPK2 models were built and prepared as described in the experimental section; after a docking calibration phase, a small database of tetrabromo phthalimides was built and a series of docking experiments were performed. As shown in Figure 2 TBID interacts with the hinge region through hydrophobic interactions between Val 213, Val 261, Phe 277, Leu 280, Met 331, Ile 345, and the tetrabromine moiety, while the symmetric nitrogen atom at position 3 interacts with the catalytic Lys 228, thus playing a crucial role in the binding architecture. In fact compounds **5b**, **5c**, **5d**, **5g**, **5h** and **5i** still inhibit HIPK2 in the low micromolar range establishing the same electrostatic interaction found in the case of TBID. By contrast **5e**, a TBID isomer, presenting a pyrazol ring instead of the imidazole of TBID cannot perform the same interaction with Lys 228 due to the different position of the nitrogen atom.

As expected from modeling, the kinetics reported in Figure 3 show that inhibition of HIPK2 by TBID is competitive with respect to ATP. From these experiments a K_i value of 200 nM was calculated.

2. Selectivity of TBID

The selectivity of the newly developed HIPK2 inhibitor TBID was firstly tested at 10 μ M concentration on a panel of 76 protein kinases. As shown in Figure 4 the activity of HIPK2 was entirely suppressed while none of the other protein kinases underwent a similar inhibition, the

residual activity of the second (BTK) and third (CAMKKb) most inhibited kinases being 29% and 34%, respectively. To note in particular the modest inhibition of those kinases which generally tend to be susceptible to CK2 inhibitors, notably CK2 itself (48% residual activity), DYRK1A (72% residual activity) and PIM1 (entirely unaffected).

To gain more information about the selectivity of TBID the compound was profiled at 1 instead of 10 μ M concentration on a larger panel of 125 protein kinases, implemented with other members of the HIPK sub-family and many protein tyrosine kinases which were scarcely represented in the smaller panel. The data, shown in Supporting Information (Table S1), corroborate the concept that HIPK-2 is the kinase most susceptible to TBID (31% residual activity). HIPK1 and HIPK3 however are also significantly inhibited with residual activities of 39% and 53%, respectively. In contrast none of the protein tyrosine kinases tested is appreciably affected by TBID with the only possible exception of IGF-IR (68% residual activity). This together with CAMK1 and CAMKKb are the only kinases inhibited more than 20% a part from the HIPKs.

Collectively taken these data denote TBID as a very selective inhibitor of HIPKs in general and HIPK2 in particular, and they highlight the striking superiority of this new compound over both TBI and SB203580. To note that in our hands SB203580 is not appreciably affecting HIPK2 activity up to 40 μ M concentration (Table 1) consistent with previous reports [9,13]. In contrast the IC_{50} values with TBI (1 μ M) was only slightly higher than that with TBID, the latter however being much more selective as also highlighted by the observation that the number of kinases inhibited >90% by either 10 μ M TBID or TBI in the same panel is 1 (HIPK2, see Figure 4) and 10 (see ref. [20]) respectively. From the selectivity data of Figure 4 it was possible to draw a Lorenz curve (Figure 5) allowing to calculate a Gini coefficient (0.592) whose value denotes a remarkable selectivity, especially if compared to that of TBI (0.310). The difference in selectivity between TBID and TBI is also striking if their hit rates (0.10 vs 0.55) are compared.

3. Cell permeability of TBID

Dealing with protein kinase inhibitors, a crucial issue is their cell permeability which is essential to make these reagents useful for in vivo studies. Cell permeability of TBID was firstly assessed by treating HepG2 cells with increasing concentrations of either TBID or its very close analog **5e** almost devoid of inhibitory efficacy (see Table 1) and measuring HIPK2 activity in the cell lysate : HIPK2 was immunoprecipitated and then assayed for its activity using a specific peptide substrate. As shown in Figure 6A endogenous HIPK2 activity is reduced in a dose dependent manner upon cell treatment with TBID, but not with its inactive analog **5e**, providing the evidence that TBID is cell permeant. Incidentally this outcome places TBID in that category of protein kinase inhibitors whose efficacy persists after the kinase has been isolated from the treated cells. Such a behaviour is typical of many CK2 inhibitors [30,34,35], TBB and TBI included, but it has also been reported in the case of other kinases, e.g. PIM-1 [36]. The molecular features underlying persistent inhibition, suggestive of a very low K_{off} rate, are presently unclear, but it is plausible to assume that these compounds, once entrapped in the hydrophobic pocket of the kinase (see also Figure 2), undergo a thermodynamic advantage, hindering their release into the surrounding aqueous medium. We also considered the possibility that intracellular TBID could irreversibly inactivate HIPK-2 by preventing the phosphorylation of its up-regulatory tyrosine, an event occurring only during translation [37,38]. In our cell model, however, we couldn't detect any phospho-Tyr signal in HIPK-2 immunoprecipitated from either untreated or treated cells (not shown).

To reinforce the view that endogenous HIPK-2 is inhibited upon cell treatment with TBID, advantage has been also taken of p53 Ser46, a known target of the kinase [39]. As shown in Figure 6B, TBID treatment markedly reduces the phosphorylation level of this residue, without affecting the amount of p53, under conditions devoid of cell toxicity (Figure 6C). To note that, although p53 Ser46 is not targeted exclusively by HIPK2, other putative phosphorylating agents of this residue, notably DYRK2 [40] and PKC [41], are nearly unaffected by the inhibitor under conditions where HIPK2 is >70% inhibited (see Table S1). This observation, in conjunction with the similar dose dependency of HIPK2 activity inhibition and decrease of p53 Ser46 phosphorylation (compare the

histograms of panels A and B in Figure 6), support the view that the reduction of p53 Ser46 phosphorylation is mainly due to HIPK2 inhibition. It should be noted in this connection that the concentration required for half maximal inhibition is two orders of magnitude higher in cells than it is in vitro (50 μ M vs 0.33 μ M). This is not unusual among protein kinase inhibitors [42] as exemplified elsewhere [30,35,43] and may be accounted for by massive sequestration of lipophilic compounds to cellular structures and to the fact that ATP competitive inhibitors (such as TBID) have to cope with a very high ATP concentration (in the mM range) within the cell.

Collectively taken, the data presented fill a gap in the field of signal transduction mediated by protein phosphorylation by making available for the first time a specific and cell permeable inhibitor for HIPK2, a protein kinase whose emerging role as regulator of cell growth and apoptosis in various tissues and whose implication in the mode of action of chemotherapeutic agents is rising remarkable interest. The only compound used so far as an HIPK2 inhibitor in fact (SB203580) was developed to inhibit different classes of protein kinases and its efficacy to inhibit HIPK2 activity is questionable, as clearly shown here and elsewhere [9,13]. On the other hand a number of compounds able to drastically inhibit both protein kinase CK2 and HIPK2 [17,19] display a wide promiscuity, which hampers their usage as selective HIPK2 inhibitors. In contrast, the compound whose synthesis and characterization are described here, TBID, displays a good efficacy and a remarkable selectivity towards the members of the HIPK family, with special reference to HIPK2, as shown both by profiling it on large panels of kinases and by molecular modelling, accounting for its ATP competitive mode of action. These properties, in conjunction with ability to permeate cells, as judged from inhibition of endogenous HIPK2, make TBID the first choice and for the time being the only pharmacological tool to down regulate cellular HIPK2, with the caveat that the concentrations of the compound effective in cells are much higher than the IC₅₀ values calculated in vitro.

ACKNOWLEDGMENTS

We thank the Division of Signal Transduction Therapy and the International Centre for Kinase Profiling (www.kinase-screen.mrc.ac.uk), MRC Protein Phosphorylation Unit, University of Dundee (Scotland), for providing purified HIPK2 and for analysing the specificity of the inhibitor, respectively.

We thank the Molecular Modelling Section (MMS) coordinated by Professor S. Moro (Padova, Italy).

We thank Professor Oriano Marin (Padova, Italy) for providing the peptides used in this work.

REFERENCES

1. Sombroek D, Hofmann TG (2009) How cells switch HIPK2 on and off. *Cell Death Differ* 16: 187-194.
2. Aikawa Y, Nguyen LA, Isono K, Takakura N, Tagata Y, et al. (2006) Roles of HIPK1 and HIPK2 in AML1- and p300-dependent transcription, hematopoiesis and blood vessel formation. *EMBO J* 25: 3955-3965.
3. D'Orazi G, Cecchinelli B, Bruno T, Manni I, Higashimoto Y, et al. (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nat Cell Biol* 4: 11-19.
4. Mayo LD, Seo YR, Jackson MW, Smith ML, Rivera Guzman J, et al. (2005) Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified. *J Biol Chem* 280: 25953-25959.
5. Zhang Q, Yoshimatsu Y, Hildebrand J, Frisch SM, Goodman RH (2003) Homeodomain interacting protein kinase 2 promotes apoptosis by downregulating the transcriptional corepressor CtBP. *Cell* 115: 177-186.
6. Hofmann TG, Stollberg N, Schmitz ML, Will H (2003) HIPK2 regulates transforming growth factor-beta-induced c-Jun NH(2)-terminal kinase activation and apoptosis in human hepatoma cells. *Cancer Res* 63: 8271-8277.
7. D'Orazi G, Rinaldo C, Soddu S (2012) Updates on HIPK2: a resourceful oncosuppressor for clearing cancer. *J Exp Clin Cancer Res* 31: 63.
8. Bain J, McLauchlan H, Elliott M, Cohen P (2003) The specificities of protein kinase inhibitors: an update. *Biochem J* 371: 199-204.
9. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, et al. (2007) The selectivity of protein kinase inhibitors: a further update. *Biochem J* 408: 297-315.
10. Di Stefano V, Rinaldo C, Sacchi A, Soddu S, D'Orazi G (2004) Homeodomain-interacting protein kinase-2 activity and p53 phosphorylation are critical events for cisplatin-mediated apoptosis. *Exp Cell Res* 293: 311-320.
11. Roscic A, Moller A, Calzado MA, Renner F, Wimmer VC, et al. (2006) Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. *Mol Cell* 24: 77-89.
12. Yamada D, Perez-Torrado R, Filion G, Caly M, Jammart B, et al. (2009) The human protein kinase HIPK2 phosphorylates and downregulates the methyl-binding transcription factor ZBTB4. *Oncogene* 28: 2535-2544.
13. Anastassiadis T, Deacon SW, Devarajan K, Ma H, Peterson JR (2011) Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. *Nat Biotechnol* 29: 1039-1045.
14. Meggio F, Pinna LA (2003) One-thousand-and-one substrates of protein kinase CK2? *FASEB J* 17: 349-368.
15. Salvi M, Sarno S, Cesaro L, Nakamura H, Pinna LA (2009) Extraordinary pleiotropy of protein kinase CK2 revealed by weblogo phosphoproteome analysis. *Biochim Biophys Acta* 1793: 847-859.
16. Ahmed K, Issinger OG, Niefind K (2011) Protein kinase CK2: a catalyst for biology, medicine and structural biochemistry. *Mol Cell Biochem* 356: 1-3.
17. Ruzzene M, Pinna LA (2010) Addiction to protein kinase CK2: a common denominator of diverse cancer cells? *Biochim Biophys Acta* 1804: 499-504.
18. Ruzzene M, Tosoni K, Zanin S, Cesaro L, Pinna LA (2011) Protein kinase CK2 accumulation in "oncophilic" cells: causes and effects. *Mol Cell Biochem* 356: 5-10.
19. Pagano MA, Bain J, Kazimierczuk Z, Sarno S, Ruzzene M, et al. (2008) The selectivity of inhibitors of protein kinase CK2: an update. *Biochem J* 415: 353-365.
20. Sarno S, Papinutto E, Franchin C, Bain J, Elliott M, et al. (2011) ATP site-directed inhibitors of protein kinase CK2: an update. *Curr Top Med Chem* 11: 1340-1351.

21. Egert-Schmidt AM, Dreher J, Dunkel U, Kohfeld S, Preu L, et al. (2010) Identification of 2-anilino-9-methoxy-5,7-dihydro-6H-pyrimido[5,4-d][1]benzazepin-6-ones as dual PLK1/VEGF-R2 kinase inhibitor chemotypes by structure-based lead generation. *J Med Chem* 53: 2433-2442.
22. Brandt W, Mologni L, Preu L, Lemcke T, Gambacorti-Passerini C, et al. (2010) Inhibitors of the RET tyrosine kinase based on a 2-(alkylsulfanyl)-4-(3-thienyl)nicotinonitrile scaffold. *Eur J Med Chem* 45: 2919-2927.
23. Meggio F, Deana AD, Pinna LA (1981) Endogenous phosphate acceptor proteins for rat liver cytosolic casein kinases. *J Biol Chem* 256: 11958-11961.
24. Ferrari S, Marin O, Pagano MA, Meggio F, Hess D, et al. (2005) Aurora-A site specificity: a study with synthetic peptide substrates. *Biochem J* 390: 293-302.
25. Graczyk PP (2007) Gini coefficient: a new way to express selectivity of kinase inhibitors against a family of kinases. *J Med Chem* 50: 5773-5779.
26. Molecular Operating Environment (MOE 2009.10) CCG, Inc, 1255 University St., Suite 1600, Montreal, Quebec, Canada, H3B 3X3.
27. Cozza G, Gianoncelli A, Montopoli M, Caparrotta L, Venerando A, et al. (2008) Identification of novel protein kinase CK1 delta (CK1delta) inhibitors through structure-based virtual screening. *Bioorg Med Chem Lett* 18: 5672-5675.
28. Jones G, Willett P, Glen RC, Leach AR, Taylor R (1997) Development and validation of a genetic algorithm for flexible docking. *J Mol Biol* 267: 727-748.
29. Di Maira G, Salvi M, Arrigoni G, Marin O, Sarno S, et al. (2005) Protein kinase CK2 phosphorylates and upregulates Akt/PKB. *Cell Death Differ* 12: 668-677.
30. Ruzzene M, Penzo D, Pinna LA (2002) Protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells. *Biochem J* 364: 41-47.
31. Battistutta R, De Moliner E, Sarno S, Zanotti G, Pinna LA (2001) Structural features underlying selective inhibition of protein kinase CK2 by ATP site-directed tetrabromo-2-benzotriazole. *Protein Sci* 10: 2200-2206.
32. Battistutta R, Mazzorana M, Cendron L, Bortolato A, Sarno S, et al. (2007) The ATP-binding site of protein kinase CK2 holds a positive electrostatic area and conserved water molecules. *Chembiochem* 8: 1804-1809.
33. Pratt D.S.; Young CO (1918) Phthalic acid derivatives: constitutions and color, XIV. Some derivatives of tetrabromophthalimide. *J Am Chem Soc* 40: 1415-1425.
34. Ruzzene M, Di Maira G, Tosoni K, Pinna LA (2010) Assessment of CK2 constitutive activity in cancer cells. *Methods Enzymol* 484: 495-514.
35. Zanin S, Borgo C, Girardi C, O'Brien SE, Miyata Y, et al. (2012) Effects of the CK2 inhibitors CX-4945 and CX-5011 on drug-resistant cells. *PLoS One* 7: e49193.
36. Cozza G, Sarno S, Ruzzene M, Girardi C, Orzeszko A, et al. (2013) Exploiting the repertoire of CK2 inhibitors to target DYRK and PIM kinases. *Biochim Biophys Acta* 1834: 1402-1409.
37. Saul VV, de la Vega L, Milanovic M, Kruger M, Braun T, et al. (2013) HIPK2 kinase activity depends on cis-autophosphorylation of its activation loop. *J Mol Cell Biol* 5: 27-38.
38. Siepi F, Gatti V, Camerini S, Crescenzi M, Soddu S (2013) HIPK2 catalytic activity and subcellular localization are regulated by activation-loop Y354 autophosphorylation. *Biochim Biophys Acta* 1833: 1443-1453.
39. Hofmann TG, Moller A, Sirma H, Zentgraf H, Taya Y, et al. (2002) Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. *Nat Cell Biol* 4: 1-10.

40. Taira N, Nihira K, Yamaguchi T, Miki Y, Yoshida K (2007) DYRK2 is targeted to the nucleus and controls p53 via Ser46 phosphorylation in the apoptotic response to DNA damage. *Mol Cell* 25: 725-738.
41. Yoshida K, Liu H, Miki Y (2006) Protein kinase C delta regulates Ser46 phosphorylation of p53 tumor suppressor in the apoptotic response to DNA damage. *J Biol Chem* 281: 5734-5740.
42. Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351: 95-105.
43. Sarno S, Mazzorana M, Traynor R, Ruzzene M, Cozza G, et al. (2012) Structural features underlying the selectivity of the kinase inhibitors NBC and dNBC: role of a nitro group that discriminates between CK2 and DYRK1A. *Cell Mol Life Sci* 69: 449-460.

FIGURE LEGENDS

Figure 1. Structures of TBB and TBI

Figure 2. Molecular docking of TBID (yellow) bound to the active site of the human HIPK2 model (green).

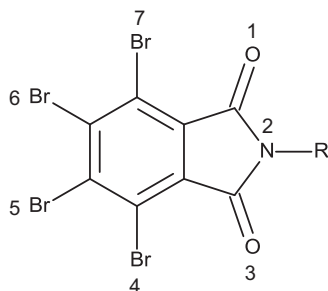
Figure 3. Inhibition of HIPK2 by TBID is competitive with respect to the phosphodonor substrate ATP. Kinetics were performed as described in Materials and Method either in the absence or in the presence of the indicated TBID concentrations. The data represent means of triplicate experiments with SEM never exceeding 15%.

Figure 4. Selectivity profile of TBID (10 μ M) against 70 kinases panel. Inhibition assays were performed with 10 μ M TBID under conditions described or referenced in the Experimental section. Residual activity expressed as per cent of activity in the absence of TBID is shown.

Figure 5. Lorenz Curves, Gini coefficients and Hit Rates for TBID and TBI. For details see experimental section and [25]. Lorenz Curves were drawn from the selectivity profile of Figure 4 and from analogous data published in ref. [19] for TBID and TBI, respectively.

Figure 6. Cell treatment with TBID inhibits endogenous HIPK. A. HIPK2 was immunoprecipitated from lysates of HepG2 cells treated with different concentrations of TBID or the inactive analog **5e**, as indicated; HIPK2 activity was measure towards the specific peptide substrate, as detailed in Materials and Methods. The amount of immunoprecipitated HIPK2 is shown by WB. B. CEM cells were treated as indicated, then 10 μ g of cell lysate proteins were analysed by WB with an antibody against pS46 of p53, anti-total p53, or anti-actin, as loading control. A representative experiment is shown on the left, while a histogram is presented on the right where Sp46 p53 of three separate experiments has been quantified, normalized to the p53 total level, and reported as means $\pm$ SEM. C. Cell viability was assessed by the method of MTT, treating HepG2 or CEM cells as indicated (left panel) or by evaluating the amount of the caspase substrate PARP (HepG2 cells, right panel), whose reduction would denote apoptosis. The recognized band corresponds to the full length PARP protein of 116 kDa.

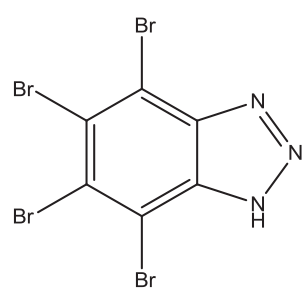
Table 1: IC₅₀ (μM) of 2-aryl-4,5,6,7-tetrabromoisindoline-1,3-diones **5** for HIPK2, CK2, PIM1, CK1; TBB and TBI (Figure 1) values are drawn from [19]. n.d.= not determined



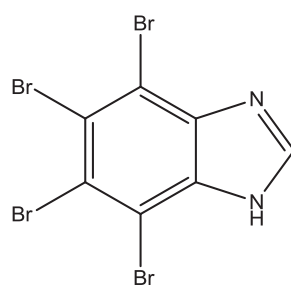
Entry	R-	HIPK2	CK2	PIM1	CK1
TBID (5a)		0.33	5.50	>40.0	>40.0
5b		0.72	16.0	40.0	9.3
5c		2.6	24.0	>40.0	>40.0
5d		2.5	35.0	>40.0	>40.0
5e		>40.0	23.0	>40.0	>40.0
5f		>40.0	>40.0	>40.0	>40.0
5g		22.7	25.0	>40.0	>40.0
5h		23.6	>40.0	>40.0	>40.0
5i		3.62	>40.0	>40.0	>40.0

TBB	5.3	0.15	1.04	>40.0
TBI	0.7	0.60	0.115	15
SB203580	>40.0	n.d.	n.d.	n.d.

Figure 1.



TBB (1)



TBI (2)

Figure 2.

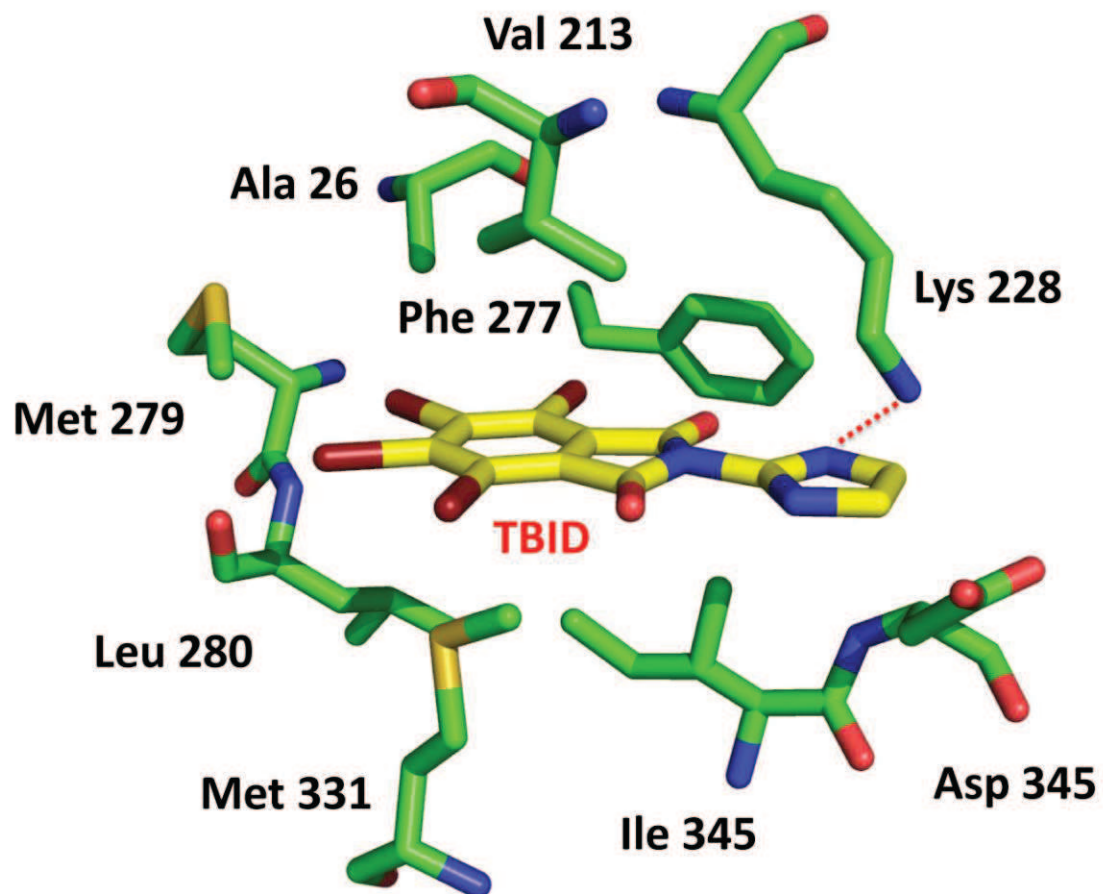


Figure 3.

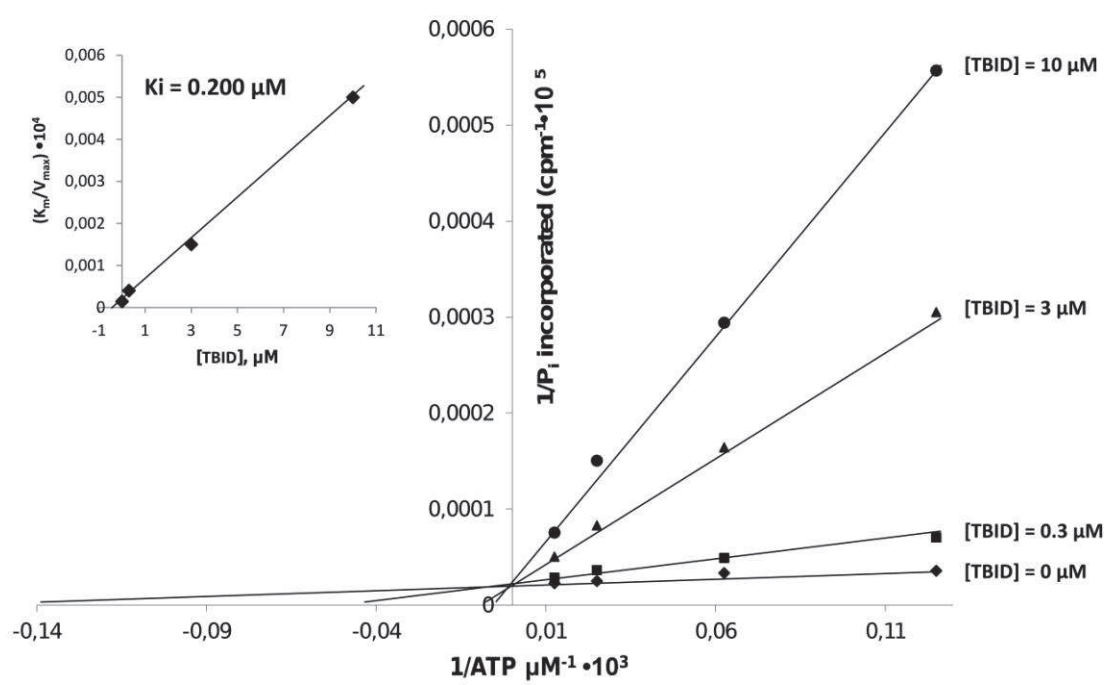


Figure 4.

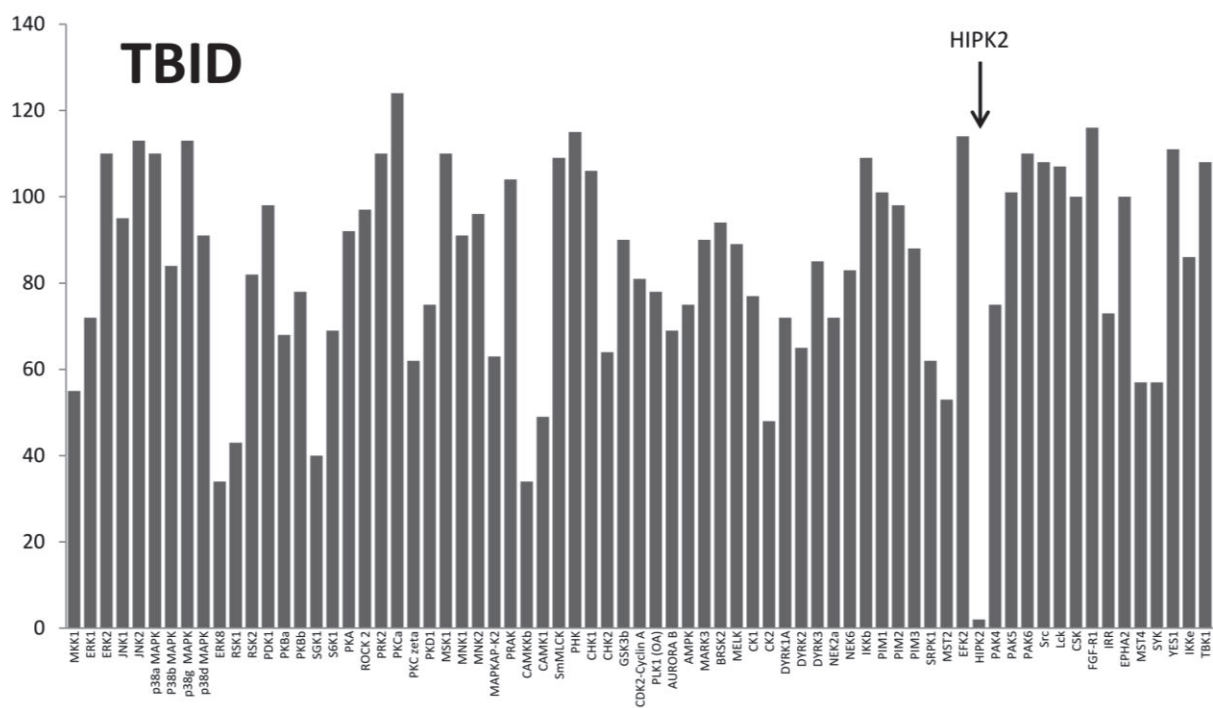


Figure 5.

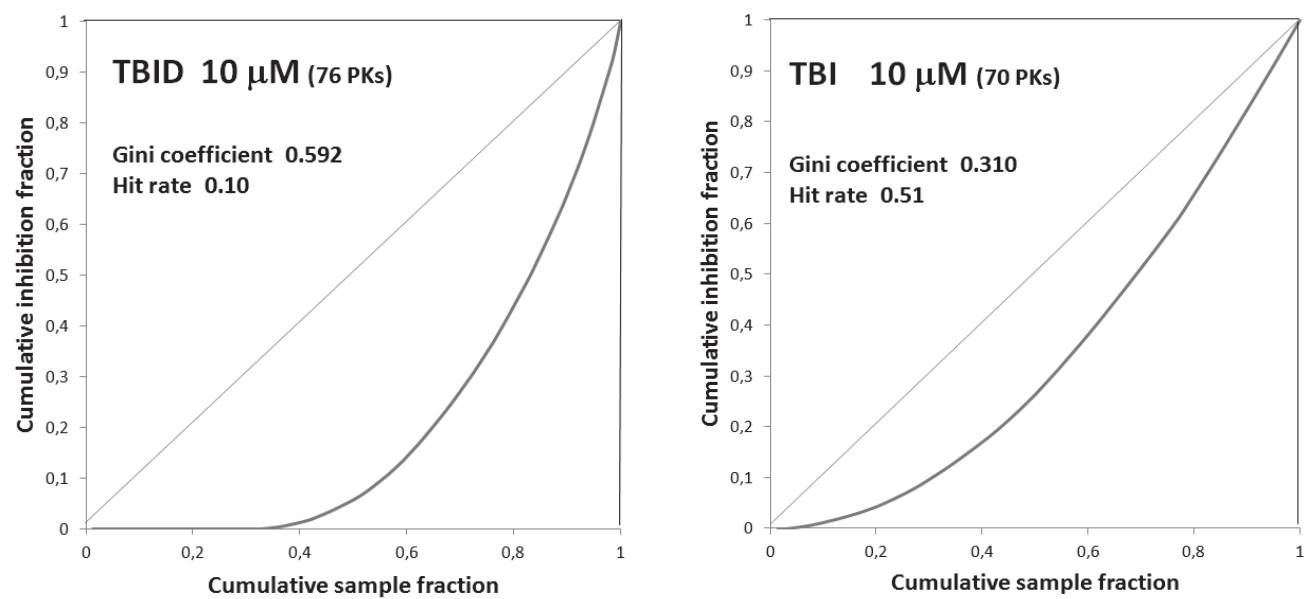


Figure 6.

