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PROTEIN KINASE CK2: A NEW TARGET TO OVERCOME IMATINIB-RESISTANCE IN CHRONIC MYELOID LEUKEMIA CELLS

PROTEIN CHINASI CK2: UN NUOVO BERSAGLIO CONTRO LA RESISTENZA ALL'IMATINIB NELLA LEUCEMIA MIELOIDE CRONICA

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SUMMARY

Chronic myeloid leukemia (CML), a malignant myeloproliferative disorder of hematopoietic system, is driven by the chromosomal translocation [t(9;22)(q34;q11)], yielding the Philadelphia chromosome and generating a fusion gene that encodes the Bcr-Abl protein, a constitutively active tyrosine kinase necessary and sufficient for the initiation, maintenance and progression of CML [Faderl S. *et al.*, 1999]. Despite the great efficacy of the Bcr-Abl-specific inhibitor imatinib, which represents the gold-standard drug of choice for CML patients, resistance to this drug is recognized as a major problem in CML therapy failure [Bixby D. and Talpaz M., 2009]. In this context, this work focuses on the analysis of the protein kinase CK2, a ubiquitous, pleiotropic and constitutively active Ser/Thr kinase, composed of two catalytic (α and/or α') and two regulatory (ß) subunits. CK2 is abnormally elevated in a wide variety of tumors, where it does not induce directly the cancer but it is critically required to create a cellular environment favourable to the development of neoplasia, mainly through its anti-apoptotic and pro-survival role [Ruzzene M. and Pinna L.A., 2010].

The goal of the research is to shed light on the role of the protein kinase CK2 in chronic myeloid leukemia oncogenic signaling, using two different CML cell lines, LAMA84 and KCL22, either sensitive (S) or resistant (R) to imatinib.

In my laboratory it had been previously observed that resistant-LAMA84 CML cells, which are characterized by *BCR-ABL1* gene amplification [Le Coutre P. *et al.,* 2000], contain a two-fold higher amount of CK2α and CK2ß, but not CK2α', subunits as compared to parental cells [Borgo C. *et al.,* 2013]. Consistently, the quantification of the CK2 subunits demonstrates that CK2 protein is expressed at very high levels compared to total cellular proteins in LAMA84 cells. Subcellular fractionation analysis shows that most CK2 is located in the cytoplasmic fraction of R-LAMA84 cells, where it co-localizes with Bcr-Abl. CK2 and Bcr-Abl are members of the same multi-protein complex(es) only in R-LAMA84 cells, as demonstrated by their co-sedimentation in glycerol-gradients and co-immunoprecipitation. Interestingly, while cell treatment with

imatinib does not affect the binding occurring between CK2 and Bcr-Abl, the CK2-specific inhibitor CX-4945 almost abrogates this interaction, suggesting that CK2 kinase activity plays a specific role in the binding.

In spite of the CK2 up-regulation occurring in imatinib-resistant LAMA84 cells, we also demonstrate that imatinib-resistant KCL22 cells express similar protein-level and activity of both CK2 and Bcr-Abl as compared to the sensitive counterpart. Moreover, CK2 co-immunoprecipitates with Bcr-Abl in both KCL22 cell variants. To assess whether CK2 might be a player in imatinib-resistant KCL22 cells, we investigated the complex Bcr-Abl oncogenic network dedicating particular attention to MAPK and PI3K/Akt/mTOR pathways, which have been frequently demonstrated to be up-regulated in cancer cells [Saini K.S. *et al.*, 2013]. We found that resistant KCL22 cells are characterized by a strikingly higher phosphorylation extent of ERK1/2 T202/Y204, as previously reported by Colavita I. *et al.* (2010), Akt S473 and ribosomal protein S6 (rpS6) S240/4-235/6 as compared to sensitive cells. In R-KCL22 cells, the treatment with high concentrations of imatinib causes a substantial inhibition of ERK1/2 and Akt S473 phosphorylation, while, unexpectedly, it only partially affects the phosphorylation of rpS6, the common downstream effector of MAPK and PI3K/Akt/mTOR pathways. Interestingly, rpS6 phosphorylation is almost abrogated by CK2 down-regulation, as judged by cell treatment with CX-4945, which does not affect ERK1/2 and Akt activities, and by CK2 knocking down by siRNA. Consistent with the down-regulation of rpS6, protein involved in translation initiation, the treatment of R-KCL22 cells with CX-4945 reduces the protein synthesis efficacy of about 50% as compared to the control.

To further assess the contribution of CK2 to chronic myeloid leukemia, the effect of CK2-inhibition on cell viability was examined. CX-4945 significantly reduces the cell viability and induces apoptosis in both LAMA84 and KCL22 cell lines, either sensitive or resistant to imatinib. However, CX-4945 concentrations required to induce apoptosis in imatinib-resistant cells are lower than those effective in sensitive cells, suggesting that resistant cells become more dependent on CK2 for their survival. Interestingly, CX-4945 added in combination with imatinib promotes a synergistic reduction of cell viability in imatinib-resistant CML cell variant, partially rescuing the response to imatinib.

In resistant KCL22 cells, we also show that CK2-inhibition sensitizes leukemic cells to the anticancer compounds U0126, an inhibitor of MAPK pathway, and rapamycin, the specific inhibitor of mTORC1 complex. Interestingly, the ternary association of CX-4945 with imatinib and U0126 represents the best effective combination of drugs to reduce the viability of R-KCL22 cells.

Taken together, our results identify CK2 as a pivotal player in CML imatinib-resistance and suggest that CK2 inhibitors might represent promising drugs for combined strategies to overcome CML imatinib-resistance.

RIASSUNTO

La leucemia mieloide cronica (LMC), una malattia mieloproliferativa maligna del sistema ematopoietico, è determinata dalla traslocazione cromosomica [t(9; 22)(q34, q11)], che causa la formazione del cromosoma Philadelphia e del gene di fusione *BCR-ABL1.* Tale gene codifica per la proteina Bcr-Abl, una tirosin chinasi costitutivamente attiva, necessaria e sufficiente per l'insorgere, il mantenimento e la progressione della patologia [Faderl S. *et al.*, 1999]. Nonostante la grande efficacia dell'imatinib, inibitore specifico di Bcr-Abl, che rappresenta il farmaco d'elezione per il trattamento dei pazienti affetti da LMC, la resistenza a questo farmaco è riconosciuta come uno dei maggiori problemi del fallimento chemioterapico [Bixby D. and Talpaz M., 2009]. In questo contesto, il lavoro della mia tesi è stato rivolto allo studio della protein chinasi CK2, una serin/treonin chinasi ubiquitaria, pleiotropica e costitutivamente attiva, composta da due subunità catalitiche (α e/o α') e due regolatorie (ß). Il livello proteico di CK2 è anormalmente elevato in un ampio numero di tumori, in cui tuttavia la chinasi non è mai riconosciuta come la causa che scatena la patologia ma risulta essere criticamente necessaria per l'instaurarsi di un ambiente cellulare favorevole allo sviluppo della neoplasia, principalmente grazie al suo ruolo anti-apoptotico e pro-sopravvivenza [Ruzzene M. and Pinna L.A., 2010].

L'obiettivo della ricerca è quello di far luce sul ruolo svolto dalla protein chinasi CK2 nelle vie oncogeniche di segnale che caratterizzano la LMC, utilizzando le due linee cellulari LAMA84 e KCL22, sia sensibili (S) che resistenti (R) all'imatinib.

Nel mio laboratorio era stato precedentemente osservato che le cellule LAMA84 resistenti all'imatinib, caratterizzate dell'amplificazione del gene *BCR-ABL1* [Le Coutre P. *et al.,* 2000], contengono una quantità proteica delle subunità CK2α e CK2ß, ma non CK2α', circa due volte superiore rispetto alle cellule sensibili all'imatinib [Borgo C. *et al.,* 2013]. In accordo con questo risultato, la quantificazione proteica delle subunità di CK2 dimostra che il livello della chinasi è marcatamente elevato nelle cellule LAMA84 rispetto alle proteine

totali. L'analisi del frazionamento subcellulare mostra che la maggior parte di CK2 si trova nella frazione citoplasmatica delle cellule R-LAMA84, dove co-localizza con Bcr-Abl. CK2 e Bcr-Abl sono membri dello stesso complesso multi-proteico ed interagiscono tra loro solo nelle cellule LAMA84 resistenti all'imatinib, come dimostrato dagli esperimenti di co-sedimentazione in gradienti di glicerolo e di co-immunoprecipitazione. È interessante notare che, mentre il trattamento cellulare con imatinib non influenza l'interazione tra CK2 e Bcr-Abl, il CX-4945, uno specifico inibitore di CK2, abolisce quasi interamente questo legame, suggerendo che l'attività chinasica di CK2 svolga un ruolo specifico nel legame tra le due proteine.

Diversamente da quanto descritto nelle cellule LAMA84, le cellule KCL22 resistenti all'imatinib esprimono un livello proteico, ed un'attività chinasica, sia di CK2 che di Bcr-Abl, simile in cellule sensibili e resistenti all'imatinib. CK2 risulta inoltre interagire con Bcr-Abl in entrambe le varianti cellulari di KCL22. Per valutare se CK2 potesse avere un ruolo nella resistenza all'imatinib anche nelle cellule KCL22, abbiamo studiato la complessa rete oncogenica regolata da Bcr-Abl, dedicando particolare attenzione alle seguenti due vie di trasduzione del segnale: MAPK e PI3K/Akt/mTOR, spesso iperattivate nelle cellule tumorali [Saini K.S. et al., 2013]. E' stato trovato che, rispetto alle cellule sensibili, le cellule KCL22 resistenti all'imatinib sono caratterizzate da un più elevato grado di fosforilazione delle seguenti proteine nei loro siti regolatori: ERK1/2 (T202/Y204), come precedentemente riportato da Colavita I. *et al.* (2010), Akt (S473) e rpS6 (S240/4-235/6). Nelle cellule R-KCL22, il trattamento con alte concentrazioni di imatinib riesce ad inibire drasticamente la fosforilazione di ERK1/2 (T202/Y204) e Akt S473, mentre, inaspettatamente, diminuisce solo in parte la fosforilazione di rpS6, l'effettore comune a valle delle vie di segnale MAPK e PI3K/Akt/mTOR. È interessante notare che la fosforilazione di rpS6 è invece praticamente abolita dall'inibizione dell'attività catalitica di CK2. Tale risultato è dimostrato sia dal trattamento cellulare con CX-4945, il quale non altera nè il grado di fosforilazione di ERK1/2 né quello di Akt S473, che dal silenziamento genico tramite interferenza dell'mRNA di CK2. In parallelo al diminuito grado di fosforilazione di rpS6, proteina coinvolta nella fase dell'inizio della traduzione, il trattamento delle cellule R-KCL22 con CX-4945

riduce l'efficacia della sintesi proteica cellulare di circa il 50% rispetto al controllo.

Per valutare ulteriormente il contributo di CK2 nella leucemia mieloide cronica, è stato esaminato l'effetto dell'inibizione dell'attività chinasica di CK2 sulla vitalità cellulare. Il trattamento cellulare con CX-4945 riduce in modo significativo la vitalità delle cellule e induce apoptosi in entrambe le linee cellulari LAMA84 e KCL22, sia nella variante sensibile che in quella resistente all'imatinib. Tuttavia, le concentrazioni di CX-4945 necessarie per indurre apoptosi nelle cellule resistenti sono inferiori rispetto a quelle efficaci nelle cellule sensibili, suggerendo come le cellule resistenti siano maggiormente dipendenti da CK2 per la loro sopravvivenza. È inoltre interessante notare che, il trattamento combinato di CX-4945 con imatinib promuove un effetto sinergico sulla riduzione della vitalità delle cellule resistenti all'imatinib in entrambe le linee cellulari di LMC, ripristinando parzialmente l'effetto dell'imatinib. Nelle cellule R-KCL22, è stato anche dimostrato che l'inibizione di CK2 rende le cellule leucemiche sensibili all'azione di altri composti come l'U0126, un inibitore della via di segnale MAPK, e la rapamicina, inibitore specifico del complesso mTORC1. L'associazione ternaria di CX-4945, imatinib e U0126 rappresenta la migliore associazione sinergica capace di ridurre la vitalità delle cellule R-KCL22.

Nel loro insieme, i nostri risultati mettono in luce come CK2 svolga un ruolo da protagonista nelle cellule LMC resistenti all'imatinib e suggeriscono come la chinasi possa rappresentare un promettente bersaglio per lo studio di strategie farmacologiche combinate per il trattamento della LMC nei pazienti resistenti all'imatinib.

ABBREVIATIONS

AMINO ACIDS

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

4E-BP1	Eukaryotic initiation factor 4E (eIF-4E) binding protein
c-Abl	Abelson murine leukemia viral oncogene homolog 1
Akt	Protein kinase B
ALL	Acute lympohoblastic leukemia
AML	Acute myeloid leukemia
AP	Accelerated phase
Bad	Bcl-2-associated death promoter
BCR	Breakpoint cluster region protein
Bid	BH3 interacting-domain death agonist
BP	Blastic phase
c-Cbl	Casitas B-lineage Lymphoma
Cdc34	Cell cycle division (Ubiquitin-conjugating enzyme E2 R1)
Cdc37	Cell cycle division 37 (Hsp90 co-chaperone)
Cdk1	Cyclin-dependent kinase 1
Chk1	Serine/threonine-protein kinase Chk1
CNL	Chronic neutrophil leukemia
CP	Chronic phase
CREB	cAMP response element-binding protein
CrkL	Crk-like protein
CSCs	Cancer stem cells
DC_{50}	Concentration inducing the 50% of cell death
DNA	Deoxyribonucleic acid
Dvl	Segment polarity protein dishevelled homolog
eEF2K	Eukaryotic elongation factor 2 kinase
eIF2β	Eukaryotic translation initiation factor 2 beta
eIF4A	Eukaryotic initiation factor 4A
eIF4B	Eukaryotic translation initiation factor 4B
eIF4E	Eukaryotic translation initiation factor 4E
eIF4G	Eukaryotic translation initiation factor 4G
eIF5	Eukaryotic translation initiation factor 5

Elk1	ETS domain-containing protein
ERK1/2	Extracellular-signal-regulated kinase
FOXO	Forkhead box O transcription factor
Gab2	Grb2 associated binding protein 2
Grb2	Growth factor receptor-bound protein 2
GSK3ß	Glycogen-synthase kinase 3ß
GTP	Guanosine Triphosphate
HS1	Hematopoietic lineage cell-specific protein 1
HSCs	Hematopoietic stem cells
Hsp90	Heat shock protein 90
IKK	IκB kinase
c-Kit	Mast/stem cell growth factor receptor (SCFR)
Ki	Inhibitor constant
JAK	Janus kinases
Lev1	Lymphoid enhancer-binding factor 1
LSCs	Leukemic stem cells
MAPK	Mitogen-activated protein kinase
Max	Myc-associated factor X
Mdm2	Mouse double minute 2 homolog
MEK	Mitogen-activated protein kinase kinase (MAP2K)
mLST8	Target of rapamycin complex subunit LST8
c-Mos	Proto-oncogene serine/threonine-protein kinase mos
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MW	Molecular weight
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
$p21^{WAF1/CIP1}$	Cyclin-dependent kinase inhibitor 1A
$p27^{KIP1}$	Cyclin-dependent kinase inhibitor 1B
$p34^{cdc2}$	Cell division cycle protein 2 homolog
p53	Tumor suppressor p53

PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphatidylinositol dependent kinase-1
P-gP	P-glycoprotein
Ph	Philadelphia chromosome
PI3K	Phosphoinositide 3-kinase
Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PIP2	Phosphatidylinositol 4,5 biphosphate
PIP3	Phosphatidylinositol 3,4,5 phosphate
PKCα	Protein kinase C alpha
PP2A	Protein phosphatase 2
PRAS40	Proline-rich AKT1 substrate
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase
A-Raf	Serine/threonine-protein kinase A-Raf
RB1	Retinoblastoma-associated protein
REV	Regulator of Expression of Virion Proteins
Rheb	Ras-homolog enriched in brain
rpS6	40S ribosomal protein S6
RSK	p90 ribosomal protein S6 kinase
S6K	Ribosomal protein S6 kinase
SFK	Src family kinase
SGK1	Glucocorticoid-induced protein kinase 1
SH	Src Homology
Six1	Homeobox protein SIX1
Src	Proto-oncogene tyrosine-protein kinase Src
SSRP1	Structure specific recognition protein 1
STAT	Signal transducer and activator of transcription
Tal-1	T-cell acute lymphocytic leukemia protein 1
TGFß	Transforming growth factor-beta receptor
TKIs	Multi-target kinase inhibitors
TSC2	Tuberous sclerosis 2 protein

INTRODUCTION

1. CHRONIC MYELOID LEUKEMIA

Chronic myeloid leukemia (CML) is a malignant myeloproliferative disorder of hematopoietic system characterized by a clonal expansion of primitive pluripotent stem cells that causes a greatly increase in the number of circulating granulocytes, even if monocytic, megakaryocitic, erithroid, B-lymphoid and occasionally T-lymphoid lineages could be affected [Faderl S. *et al.* 1999; Chen Y. *et al.* 2010].

1.1 - CLINICAL FEATURES

The disease represents about 15% of all adult leukemias with an annual incidence of 1-1.5 cases per 100,000 people. It is more frequent in male respect to female (1,3:1) with a median age at diagnosis of 45-55 years [Rumjanek V.M. *et al.*, 2013]. Based on clinical features, CML presents a tri-phasical clinical course: an initial benign chronic phase (CP) progresses into an accelerated (AP) and then blastic phase (BP) (see Fig.I). In 90% of cases CML is diagnosed in CP through routine blood testing; up to half of patients are asymptomatic but common symptoms are anorexia, fatigue, weight loss, bleeding, anemia, leukocytosis and thrombocytosis. At this stage physiological hematopoiesis coexists together with the leukemic clone, granulocytes are increased 10- to 100-fold in the blood but their differentiation and function is apparently still not altered. Over about 4-6 years, due to molecular abnormalities accumulation (secondary chromosomal changes), epigenetic alterations and genetic instability (alteration of different gene such as loss of p53 functions, RB1 rearrangement, c-Myc amplification), the disorder proceeds into BP, that is clinically similar to an acute leukemia. This aggressive and fatal phase starts when more than 30% immature blasts appear in bone marrow or peripheral blood; myeloid progenitor cells develop at different stages of maturation and are released prematurely in peripheral blood [Pasternak G. *et al.,* 1998].

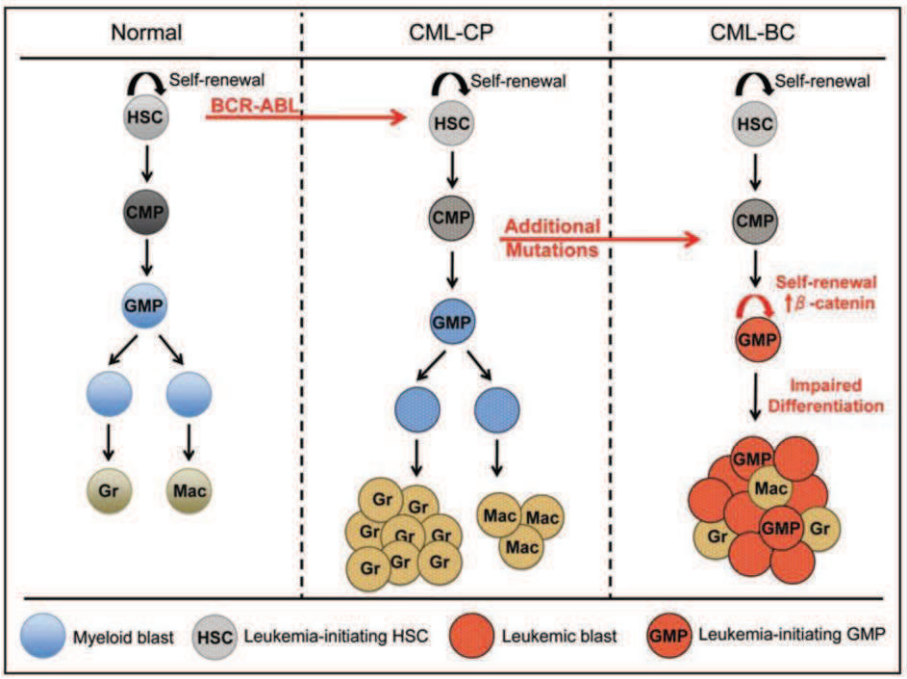


Figure I. Evolution of leukemic clone in CML progression. *The hematopoietic stem cells (HSCs), which are characterized by self-renewal capacity and multi-lineage potential, give rise to all differentiated, mature blood cells through successive stages. The production of BCR-ABL1 gene in the HSC compartment is sufficient to induce a clonal expansion resulting in a greatly increase of mature granulocytes (chronic phase). Additional mutations and epigenetic alterations lead to accumulation of immature blasts (blast crisis). (HSC, hematopoietic stem cell; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; Gr, granulocyte; Mac, macrophage)[Stuart S.A. et al., 2009].*

1.2 - CYTOGENETIC HALLMARK

CML is the best known leukemia at molecular level: its cytogenetic hallmark is the Philadelphia chromosome (Ph) that characterizes all hematopoietic cell lineages of about 90% of patients. For the first time, in 1960, Nowell and Hungerford correlated the leukemia with a specific karyotype abnormality, later shown arising from the reciprocal translocation [t(9;22)(q34;q11)] [Rowley J.D. *et al.*, 1973] (see Fig.II), in which the cellular proto-oncogene *c-ABL* moves from chromosome 9 [Bartram C.R. *et al.*, 1983] to the *BCR* (breakpoint cluster region) on chromosome 22 [Groffen J. *et al.,* 1984], thus yielding a shortened chromosome 22. The resulting fusion gene, *BCR-ABL1*, is an oncogene that codes for the tyrosine kinase Bcr-Abl, which is endowed with constitutive activity. Bcr-Abl protein production is necessary and sufficient for

the initiation, maintenance and progression of CML phenotype, since the retroviral insertion of a human *BCR-ABL1* gene into murine hematopoietic stem cells causes CML-like disease in mice [Daley G.Q. *et al.*, 1990].

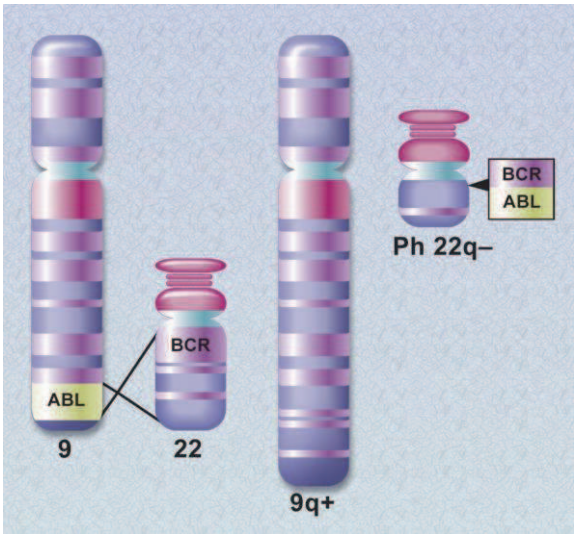


Figure II. Scheme of the reciprocal translocation yielding Ph chromosome. *Ph chromosome is a shortened chromosome 22 (22q-), which originates from the reciprocal translocation between the long arms of chromosomes 9 and 22 [Druker B.J., 2008].*

About 5-10% of CML patients are characterized by the absence of the Philadelphia chromosome (Ph–) as judged by cytogenetic analysis. However, at molecular level, the *BCR-ABL1* fusion gene is detectable. These patients present a clinical course and respond to therapy similarly to Ph+ cases [Bartman C.R. *et al.,* 1983]. At variance, a residual subset of patients are both Ph chromosome and *BCR-ABL1* negative but they represent a separate leukemic entity.

The hybrid gene is not restricted to CML, it is also present in 5% of childhood, 25% of ALL (acute lympohoblastic leukemia) and few cases of AML (acute myeloid leukemia) [Kurzrock R. *et al.*, 2003].

1.3 - MOLECULAR BASIS OF THE PHILADELPHIA CHROMOSOME

As above mentioned, *BCR-ABL1* fusion gene, arisen from the reciprocal exchange of DNA between the long arms of chromosome 9 and 22 [Kurzrock R. *et al.,* 2003], is sufficient to drive the malignant transformation of cells, thereby it represents the molecular fingerprint of CML.

1.3.1. *c-ABL* gene and its protein product

The proto-oncogene *c-ABL* is the human homologue of the viral *ABL* transforming gene carried by the Abelson murine leukemia virus (A-MuLV). It is a large gene, phylogenetically highly conserved, situated in the band 9q34 and made up of 12 exons and different introns. Based on an alternative splicing of the first exon (1a or 1b), the gene gives rise to two mRNAs which encode two similar proteins, with a MW of 145 kDa, belonging to the family of non-receptor tyrosine protein kinases [Pasternak G. *et al.,* 1998]. At its N-terminal domain, c-Abl contains (see Fig.III) a myristoylation sequence (only in Abl-1b), which connects the protein to the inner surface of the plasma membrane [Van Etten R.A., 1999]. Toward the N-terminus, c-Abl presents three Src-homology domains (SH1, SH2 and SH3). SH2 and SH3 domains allow the binding to protein ligands with phospho-tyrosine sites and proline motifs, respectively. SH1 carries the catalytic tyrosine kinase activity and plays a key role in the induction of CML. Its activity must be tightly controlled in cells. Auto-inhibition effect is achieved by intricate intra-molecular interactions of N-terminal sequences including the myristolyl group, the SH2 and SH3 domains, and the Cap region. Deletion of N-terminus results in aberrant constitutive enzymatic activity. In the central area are present three proline-rich binding sites (PxxP) capable of binding to adaptor proteins. The C-terminal segment shows three nuclear localization signals (NLSs) and one nuclear export signal, (NES) which are responsible for the different subcellular localizations of the protein. c-Abl physiologically shuttles between the nuclear and the cytoplasmic compartments in response to physiological stimuli. Moreover, C-terminus contains three DNA-

binding site as well G-(globular) and F-(filamentous) actin binding domains [Kurzrock R. *et al.,* 2003]. Normally c-Abl acts in the regulation of cell cycle [Sawyers C.L. *et al.,* 1994], in genotoxicity [Wang J.Y., 1998] and in cell motility [Hantschel O. and Superti-Furga G.., 2004].

1.3.2. *BCR* gene and its protein product

BCR gene is mapped to 22q11.23 and can be translated into two proteins that vary in size (130000 and 160000 kDa of MW) because of an alternative splicing [Laurent E. *et al.*, 2000]. Like Abl, Bcr protein is ubiquitously expressed and localized in both nucleus and cytoplasm [Wetzler M. *et al.*, 1993]. At its N-terminal domain (see Fig.III), Bcr has a coiled-coil oligomerization domain, two cyclic adenosine monophosphate kinase homologous domains and a catalytically active S/T kinase domain. At the center of the protein there is a specific guanine nucleotide exchange factor (GEF). The C-terminus presents a Rac-specific GTP-ase activating protein (RacGAP) domain and a putative calcium-dependent lipid binding site [Olabisi O.O. *et al.*, 2006]. Autophosphorylated Y177 residue exerts a crucial role for the binding to Grb2, the activatory upstream protein of multiple signaling pathways, as detailed in section 1.4. Bcr affects signal transduction, although its native functions and its role in hematopoiesis remain not yet clear. BCR knockout mice have a normal reproduction and viability [Pasternak G. *et al.*, 1998].

1.3.3. *BCR-ABL1* fusion gene and its protein product

Depending on the specific site of breakpoint in the translocation, *BCR-ABL1* fusion gene can be translate into several forms of Bcr-Abl protein with different molecular weights (see Fig.III). In CML patients the most common breakpoint occurs in the so-called major BCR (M-BCR) between exon 13 and 14 or 14 and 15 [De Breakeleer M. *et al.*, 1986], which binds to exon 2 of ABL1 gene. As transcriptional result, the p210 Bcr-Abl chimeric protein is originated. However, *BCR-ABL1* oncogene is not confined to CML. When the breakpoint happens in the minor BCR (m-BCR) or in the micro-BCR (μ-BCR) the fusion

gene encodes the p190 and p230 proteins [Groffen J. *et al.,* 1984; Fainstein E. *et al.,* 1987; Saglio G. *et al.*, 1990], respectively. p190 is responsible for adult acute lymphoblastic leukemia (ALL), p230 for the chronic neutrophil leukemia (CNL). In summary, while Abl portion is almost invariably constant and achieves the transforming principle, Bcr sequence varies in size and dictates the phenotype of leukemia.

p210 Bcr-Abl protein is composed of N-terminus sequences of Bcr fused to C-terminal motifs of Abl. Bcr-Abl is endowed with constitutively elevated levels of tyrosine kinase activity, which is located within Abl and is considered the critical factor for leukemogenesis. Bcr components are simultaneously required for the oncogenic transformation. The oligomerization domain and the autophosphorylated Y177 site of Bcr are essential to determinate the aberrant catalytic activity of Abl since their loss reduces dramatically the transforming potential of Bcr-Abl [Olabisi O.O. *et al.* 2006]. The extent of kinase activity correlates with the degree of induced transforming activity [Lugo T.G. *et al.*, 1990]. Because of the deletion of Abl C-terminal moiety containing nuclear localizing signals, Bcr-Abl is exclusively localized in the cell cytoplasm where it probably interacts with the majority of proteins involved in transforming activities and in oncogenic pathways [Cilloni D. and Saglio G., 2012].

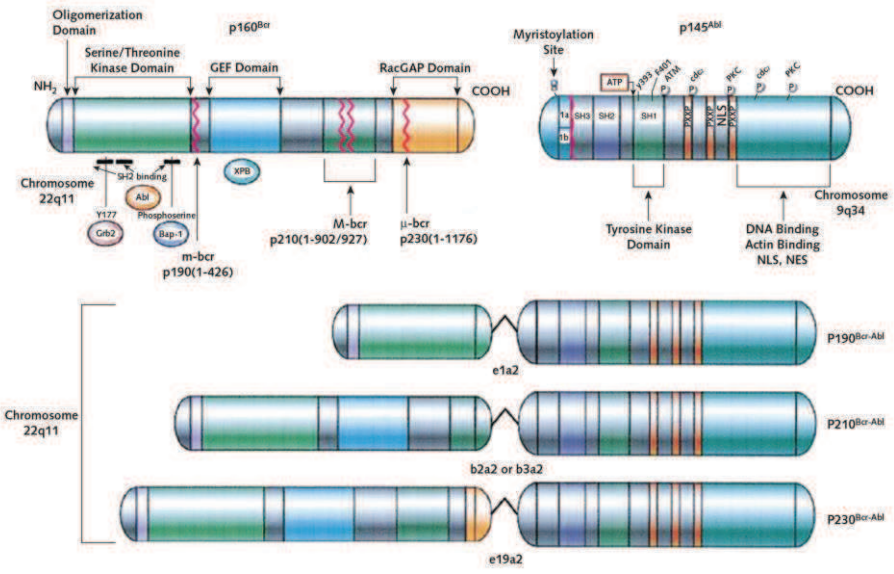


Figure III. Bcr and Abl proteins and the aberrant Bcr-Abl protein isoforms. *Representation of the functional sites of Bcr (upper panel, left) and c-Abl (upper panel, right) proteins. At the bottom of the Figure, schematic representation of different Bcr-Abl fusion proteins [From Kurzrock et al., 2003].*

1.4 - BCR-ABL ONCOGENIC SIGNALING

Bcr-Abl expression influences a complex network of survival signalings leading to enhanced proliferation, decreased apoptosis and reduced cell adhesion, which are responsible for the malignant transformation (see Fig.IV). Among the various pathways addressed by Bcr-Abl protein the Ras/MAPK, PI3K/Akt/mTOR and JAK/STAT cascades are the most important implicated in leukemogenesis. They present many cross connections with multiple points of convergence such as the perturbation of one affects the others [Rumpold H. and Webersinke G., 2011].

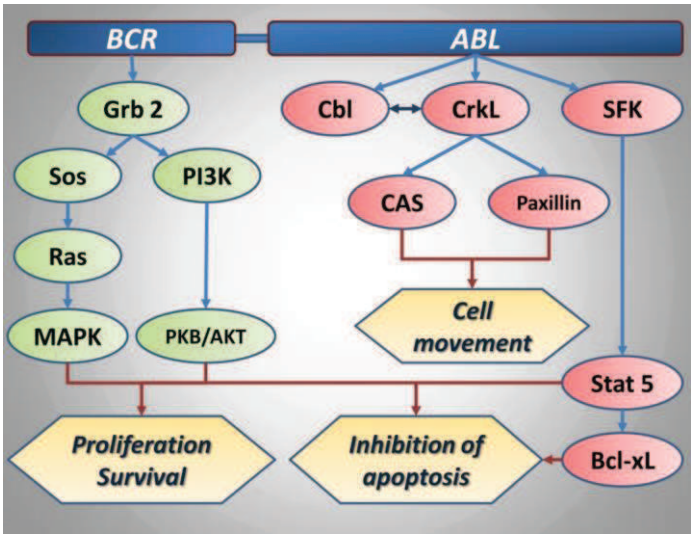


Figure IV. Schematic representation of Bcr-Abl-induced signaling pathways in CML. *Bcr-Abl expression leads to the activation of nuclear and cytoplasmic signal transduction pathways that influence cell survival of hematopoietic cells [Zaharieva M.M. et al., 2013].*

1.4.1. CrkL

The main substrate for the Bcr-Abl kinase is represented by the adaptor protein CrkL, which is involved in the regulation of cell motility [Uemura N. and Griffin J.D., 1999]. The Bcr-Abl catalyzed phosphorylation of CrkL Y207 is related to the development of leukemia since the protein was found to be phosphorylated in tissues of *BCR-ABL1* transgenic mice but not in normal mice. CrkL acts as a linker between the oncokinase and its downstream targets [De

Jong R. *et al.*, 1997]. In CML cells, CrkL recruits paxillin to Bcr-Abl protein sustaining, with other proteins, the deficient cell adhesion to stroma cells and extracellular matrix [Salgia R. *et al.,* 1995]. This molecular event could explain the clinically premature release of progenitors cells from the bone marrow [Kurzrock R. *et al.,* 2003]. Moreover, CrkL allows the binding between Bcr-Abl and c-CBL, a molecular complex able to activate aberrant signaling, as described below in detail.

1.4.2. Mitogen-activated protein kinase (MAPK) pathway

The mitogen-activated protein kinase pathway is physiologically stimulated by mitogens, cytokines or growth factors and can be activated pathologically by Bcr-Abl expression [Cilloni D. and Saglio G., 2012; Ahmed W. and Van Etten R.A., 2013] (see Fig.V). Autophosphorylation of Y177 within Bcr sequence represents a key event absolutely required for leukemogenesis. It acts as a docking site for the Grb2 adapter protein, which couples to Sos protein (son of sevenless) stabilizing the active GTP-bound state of Ras and activating the scaffold protein Gab2 [Deininger M.W.N. *et al.*, 2000]. Ras triggers a sequential activation of a series of protein kinases. First, it stimulates the recruitment of Raf to the plasma membrane, where it phosphorylates MEK. MEK phosphorylates, in turn, its predominant downstream target ERK1/2 that can enter into the nucleus and directly activates a plethora of transcription factors such as c-Myc and Elk1. Cytoplasmic ERK1/2 is able to phosphorylate and activate RSK, that, on one hand, regulates ribosome biogenesis, protein synthesis, cell size and cell cycle proliferation via 40S ribosomal protein S6 (rpS6) phosphorylation, and on the other hand, causes the activation of the transcription factor CREB [Steelman L.S. *et al.*, 2004].
MAPK cascade normally regulates gene expression, cell cycle, cell growth, differentiation and apoptosis. Its perturbation leads to enhanced cell proliferation and reduced programmed cell death. This pathway has been reported to be hyper-activated in many type of tumors including acute

myelogenous leukemia, acute lymphocytic leukemia, breast and prostatic cancers where some components, such as Ras, Raf, MEK and ERK, are mutated or aberrantly expressed [Steelman L.S. *et al.*, 2004]. The role of MAPK pathway in the deregulated cell proliferation and also in the drug resistance of hematopoietic cells has been well evidenced [McCubrey J.A. *et al.,* 2007].
MAPK cascade is also able to interact with other signal transduction pathways including the PI3K/Akt/mTOR pathway, as demonstrated by the ability of both ERK1/2 and RSK to phosphorylate TSC2 (see below and Fig.V).

1.4.3. PI3K/Akt/mTOR pathway

PI3K/Akt/mTOR pathway (see Fig.V) is normally stimulated by growth factors and regulates cell survival. Wide evidence demonstrates that up-regulation of PI3K cascade is implicated in oncogenesis and drug resistance [McCubrey J.A. *et al.,* 2007]. It has been demonstrated that in CML this pathway is activated by multiple mechanisms regulated by Bcr-Abl expression. The above described Grb2-Gab2-Sos complex or the binding of Bcr-Abl to CrkL and c-Cbl adaptor protein [Skorski T. *et al.*, 1995; Hochhaus A. *et al.*, 2002] activates PI3K, which converts phosphatidylinositol 4,5 diphosphate (PIP2) into phosphatidylinositol 3,4,5 phosphate (PIP3) in the lipid rich plasma membrane. PIP3 acts as a docking site to recruit PDK1 and its mediator Akt to the membrane through the pleckstrin homology (PH) domain. This reaction can be reversed by the phosphatase PTEN, a tumor suppressor protein which removes phosphate from PIP3, preventing Akt activation. Mutations of PTEN occur in many human cancers [Steelman L.S. *et al.*, 2004]. The serine-threonine protein kinase Akt becomes fully activated following the phosphorylation at T308 by PDK1 and at S473 by mTORC2. Akt can be further stimulated by the protein kinase CK2-mediated phosphorylation of S129, which prevents the de-phosphorylation of Akt T308 maintaining the kinase in its active conformation [Di Maira G. *et al.*, 2005]. On one hand, activated Akt triggers the apoptotic machinery through the phosphorylation of a wide variety of substrates including Bad, GSK3ß, caspase 9, FOXO and Mdm2 [Cilloni D. and Saglio G., 2012]. On the other

hand, Akt promotes cell proliferation mainly inducing, directly or indirectly, the mammalian target of rapamycin (mTOR) activation. mTOR is a S/T kinase present in two distinct complexes, mTORC1 and mTORC2, which differ in composition, substrate specificities, physiological functions and sensitivity to the potent macrolide-derived inhibitor rapamycin. mTORC1, sensitive to rapamycin, regulates positively ribosomal biogenesis and the initiation/progression of protein synthesis. It consists of mTOR, raptor, which acts as a scaffold for recruiting mTORC1 substrates, mLST8, a positive regulator of mTOR kinase activity and two negative regulators, named PRAS40 and deptor. mTORC2, insensitive to rapamycin, has a key role in cell survival and actin cytoskeleton organization by phosphorylating several substrates including Akt S473, SGK1 S422 and PKCα S657. mTORC1 can be directly activated by Akt through PRAS40 T246 phosphorylation or indirectly through TSC2/Rheb axis. mTORC1 downstream substrates are S6K and 4E-BP1. Full activation of S6K requires dual phosphorylation at T389 by mTORC1 and then at T229 by PDK1, which occurs in a manner independent on PIP3 binding. Targets of S6K are rpS6, eIF4B and eEF2K. In particular, rpS6, activated by the sequential phosphorylation of S235/236 and S240/244 catalyzed by S6K and RSK (MAPK pathways), regulates protein synthesis and cell size via unclear mechanisms [Foster K.G. and Fingar D.C., 2010; Magnuson B. *et al.*, 2012]. 4E-BP1 phosphorylation on multiple sites (T37-48, T70, S65) catalyzed by mTORC1 induces its dissociation from eIF4E and allows eIF4A and eIF4G to assemble to eIF4E initiating mRNA translation [Populo H. et al., 2012]. Mutations of mTOR gene and hyper-activation of mTOR signaling have been reported in many kinds of tumors [Populo H. et al., 2012]. In addition, the up-regulation of mTORC1 activity mediated by Bcr-Abl protein counteracts autophagy [Cilloni D. and Saglio G., 2012], a highly conserved homeostatic process that plays an important role in tumor development and progression [Chen P. *et al.*, 2013].
In summary, PI3K/Akt/mTOR pathway stimulates cell growth and proliferation, favors protein synthesis and blocks autophagy process.

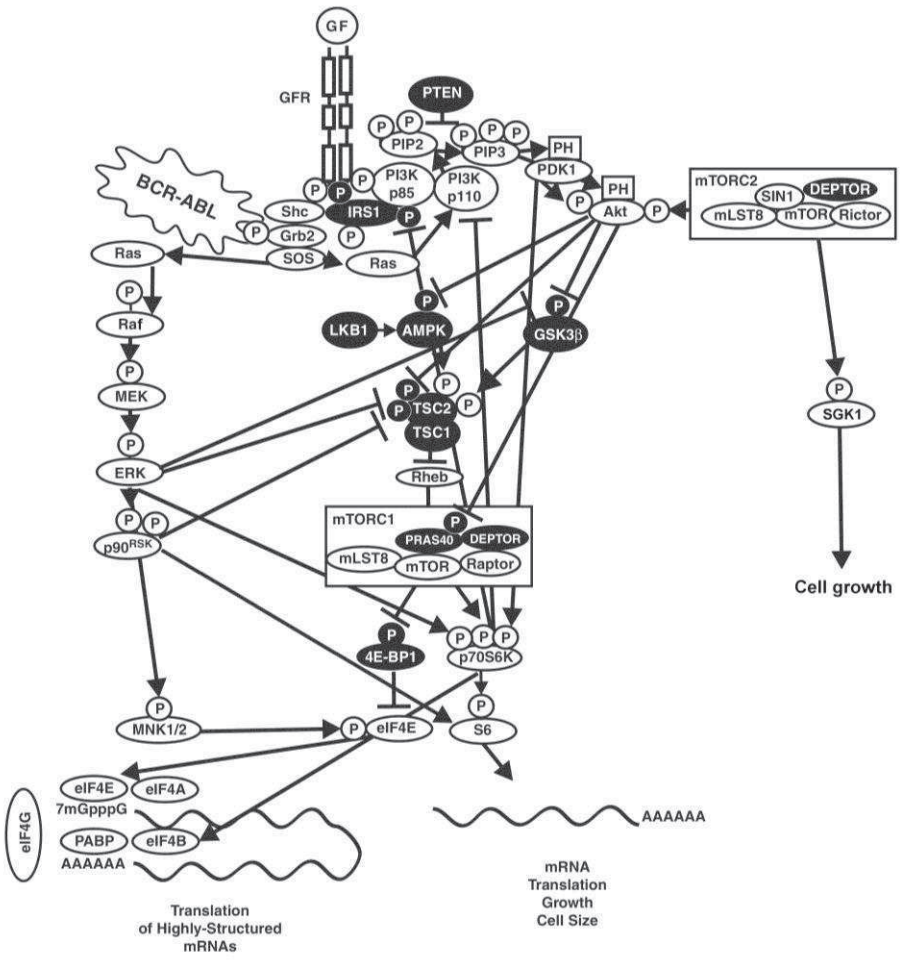


Figure V. Overview of the MAPK and PI3K/Akt/mTOR signaling pathways activated by Bcr-Abl oncoprotein. *Schematic representation of MAPK and PI3K/Akt/mTOR cascades and their interactions that result in the regulation of protein synthesis. Proteins in white ovals are positive regulators while molecules in black ovals are negative regulators [adapted from Martelli A.M. et al., 2011].*

1.4.4. JAK-STAT pathway

Among the multiple signalings initiated by Bcr-Abl it worth mentioning the JAK-STAT cascade, which is associated with cytokine and growth factor receptors. In normal cells the large family of Janus kinases (JAK) activates the signal transducer and transcription activator proteins (STATs) after stimulation of cytokine receptors, regulating the transcription of genes involved in cell growth control. In CML, STATs are directly activated by Bcr-Abl in a JAK-independent manner. The function of JAK kinases in the pathogenesis of CML is still not understood, however, they are interacting with and activated by Bcr-

Abl, thus stimulating the Src kinase Lyn [Ahmed W. and Van Etten R.A., 2013]. STAT1 and STAT5 have been reported to be up-regulated in Bcr-Abl positive cell lines and in primary cells from CML patients inducing cytokine independence. In CML, activated STAT5 regulates mainly the transcriptional activation of the anti-apoptotic protein Bcl-xL and the related cell survival induction [Deininger M.W. *et al.*, 2000; Cilloni D. and Saglio G., 2012].

1.4.5. Src family kinases

One of the numerous signal transduction pathways perturbed in CML is represented by the non-receptor kinases belonging to the Src-family, which affect cell growth, differentiation and survival. Src proteins exert a key role in both development and progression of CML and in the mechanisms of resistance induced by pharmacological CML treatment. In particular, Hck, Lyn and Fgr have been reported to be overexpressed and/or hyper-activated by Bcr-Abl in myeloid cells. In turn, Hck, Lyn and Fyn are able to phosphorylate Abl creating a complex bilateral regulation. Finally, highly activation of Lyn and Hck is observed in drug-resistant CML patients [Rumpold H. and Webersinke G., 2011].

Taken together, the described signaling pathways induce enhanced cell proliferation, altered cell adhesion and reduced apoptosis leading to the expansion of the leukemic clone.

1.5 - CML THERAPEUTIC APPROCHES

The CML therapeutic history has been deeply replaced since the first attempts with arsenicals (1856). Over many years, CML treatment was only palliative and did not improve the survival of patients. The chemicals used, busulfan (1950s) and then hydroxyurea (1970s), induced a partial hematological remission without a cytogenetic response. Allogenetic stem cell transplantation represented the therapeutic suitable alternative. However, age-limitation, donor-restriction, potentially complication as graft-versus-host disease and infections limited its applications. In the 1980s the introduction of interferon-α achieved, for the first time, complete hematological (50-80% of cases) and cytogenetic responses (30% of cases) in CP patients. [Faderl S. et al., 1999].

More than 10 years ago Bcr-Abl protein expression has been established as the crucial event in leukemogenesis, and since then a profound improvement in CML therapy occurred. In the late 1990s, therapy management has been revolutionized by the introduction of imatinib mesylate (Glivec®) [Baccarani M. et al., 2006]. To date it represents the gold-standard therapy of choice for CML patients in all the disease phases; in fact it is able to induce a complete hematologic (96%) and cytogenetic remission (70-90%) with minimal toxicity. Imatinib, an orally bioavailable 2-phenylamino pyrimidine, is a strong and highly specific inhibitor of Bcr-Abl. In cell assay, its DC_{50} values (concentration inducing the 50% of cell death) are about 0.1 to 0.5 μM [Gambacorti-Passerini *et al.*, 1997; Quintas-Cardama et al., 2009]. It acts as a competitive agent directed to the ATP binding pocket of the Abl kinase domain (see Fig.V). Interestingly, it only binds to the closed (inactive) conformation of Abl kinase domain stabilizing it and inhibiting, on one hand, its auto-phosphorylation and, on the other hand, the phosphorylation of its substrates (see Figure IV). As a consequence of the inhibition of Bcr-Abl catalytic activity, cell proliferation is abrogated and apoptosis is restored in CML cells [Aguilera D.G. and Tsimberidou A.M. 2009; Lamontanara A.J. et al., 2012].

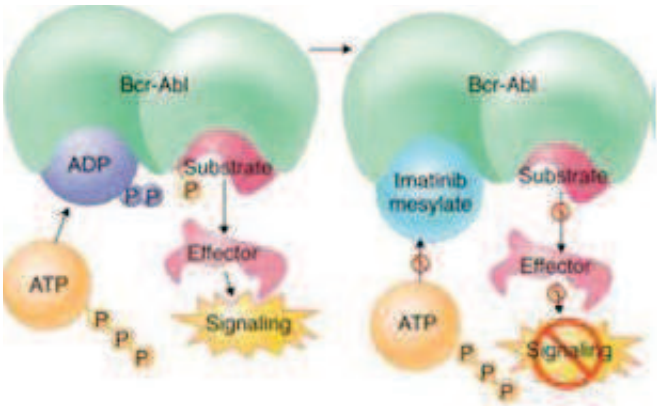


Figure VI. Model illustrating the inhibition of Bcr-Abl kinase activity by imatinib. *Under physiological conditions c-Abl binds ATP and transfers the terminal phosphate group from ATP to the substrate protein, resulting in activation of downstream pathways. Imatinib binds the ATP-binding site of Bcr-Abl preventing substrate phosphorylation and inhibiting downstream signalings. [Goldman L. and Ausiello D.A., 2008].*

The finding that imatinib is active also on the receptor tyrosine kinases c-Kit and PDGFR, validated its administration in other diseases, including Bcr-Abl positive ALL [Druker B.J. *et al.,* 2001], gastrointestinal stromal tumors [Joensuu H. *et al.,* 2001] and chronic eosinophilic leukemia [Cools J. *et al.,* 2004].

In spite of the potent effect of imatinib, up to one-third of patients develop either intolerance or resistance to the drug, requiring alternative strategies of cure [Bixby D. and Talpaz M., 2009]. The emergence of imatinib-resistance has provided the opportunity for combined therapies and a second generation of multi-target kinase inhibitors of Bcr-Abl (TKIs) aimed at circumventing the resistance and restoring the response to the drug. These agents, including dasatinib (Sprycel®) and nilotinib (Tasigna®), are dual-specific Src and Abl inhibitors. Even if less selective, TKIs are more potent than imatinib and well tolerated. Treatment with TKIs is able to eradicate resistant clones, but its success has been mainly hampered by its ineffectiveness towards T315I Bcr-Abl mutant [Aguilera D.G. and Tsimberidou A.M., 2009]. The recently approved third generation TKI ponatinib was designed to bind Bcr-Abl with high specificity and potency, and its efficacy in T315I-positive patients is impressive [Lamontanara A.J. et al., 2012; Frankfurt O. and Licht J.D., 2013].

1.6 MECHANISMS OF IMATINIB-RESISTANCE

Resistance to chemotherapy represents the major concern emerged in all types of cancers. Imatinib-resistance can be primary (innate), when patient has no response since initial treatment, or secondary (acquired) when patient initially responds to the drug but later relapses [Gorre M.E. et al., 2001]. Resistance to imatinib may arise from different mechanisms, which can be Bcr-Abl -dependent (intrinsic) or -independent (extrinsic). Bcr-Abl dependent mechanisms of resistance occur when Bcr-Abl become hyper-activated due to *BCR-ABL1* gene-amplification or point mutations. At variance, Bcr-Abl -independent mechanisms are associated with activation of alternative signaling pathways, CML stem cell quiescence, overexpression of the P-glycoprotein efflux pump or epigenetic modifications [Illmer T. *et al.*, 2004; Bixby D. and Talpaz M., 2009].

1.6.1. Bcr-Abl dependent mechanisms of resistance

- **Point mutations**

The predominant mechanism of imatinib-resistance is represented by the occurrence of point mutations in *BCR-ABL1* gene. In the majority of cases, point mutations lead to a single amino acid substitution in the Abl kinase domain. In particular, the single cytosine → thymine nucleotide exchange at position 944 of *ABL* gene causes the substitution of threonine to isoleucine at position 315, the gatekeeper residue of Abl sequence. This point mutation is critical because it compromises the imatinib binding and confers resistance also to second generation TKIs [Bixby D. and Talpaz M., 2009]. Gly-rich loop and activation loop represent other hotspots for mutations occurrence. Mutations that lead to amino acid substitutions in the Gly-rich loop (G250E, Q252H and others) interfere with the flexibility of the loop preventing the establishment of the protein conformation required to bind imatinib. Amino acid substitution in the activation loop (H396P) makes the activation loop to adopt the active

conformation. Other mutations are also identified far from the imatinib binding site and outside of the kinase domain [Lamontanara A.J. et al., 2012].

- *BCR-ABL1* gene amplification

In about 5-10% of cases, imatinib-resistance is caused by the genomic amplification of *BCR-ABL1* fusion gene [Gadzicki D. *et al.,* 2005]. The resulting overexpressed and hyper-activated Bcr-Abl protein decreases the imatinib sensitivity and increases the transforming potential. In has been described that imatinib-resistant CML LAMA84 cells contain more than 15 copies of the fusion gene and, in parallel, a 4- to 5-fold increase of protein level as compared to the sensitive counterpart. The duplication of *BCR-ABL1* gene was identified also in cells obtained from CML and ALL imatinib-resistant patients [Gorre M.E. et al., 2001].

1.6.2. Bcr-Abl independent mechanisms of resistance

- Drug efflux

P-glycoprotein (P-gP) efflux pump mediates multidrug resistance in several types of cancers regulating the efflux of different chemotherapeutic compounds. It has been reported that imatinib is a substrate for P-gP [Dai H. et al., 2003] and that P-gP, overexpressed in cells from imatinib-resistant BP CML patients, inhibits intracellular accumulation of the drug [Mahon F.X. et al., 2000]. However, the role of P-gP in imatinib-resistance is still unclear because of conflicting results obtained from other groups [Quintas-Cardama *et al.*, 2009].

- Leukemic stem cells (LSCs)

According to an emerging concept of cancer biology, cancer cells might contain a subpopulation of rare cancer stem cells (CSCs), characterized by self-renewal, ability to differentiate, capacity to become quiescent and resistance to apoptosis.

Leukemic stem cells (LSCs) share with hematopoietic stem cells (HSCs) common features. It is well accepted that LSCs are HSCs that have become capable of indefinite proliferation due to accumulated mutations and/or epigenetic changes over time [Bonnet D., 2005].
In CML, LSCs are resistant to current therapies and they could be responsible for relapse. Eradication of this niche of cells represents a critical element for the success of any therapy. It has been demonstrated that Hedgehog, Wnt, TGFß and Notch pathways play a pivotal role in LSCs survival. However, more studies aimed at elucidating diverse stemness factors between LSCs and HSCs are needed to develop new treatments to overcome drug-resistance [Cilloni D. and Saglio G., 2012].

- Activation of other pathways

Imatinib-resistance can be originated from the Bcr-Abl independent activation of several survival signalings including MAPK, PI3K/Akt/mTOR, JAK/STAT pathways, which are normally under the control of Bcr-Abl (see section 1.1.4). Moreover, overexpression of Src family kinases provides survival advantage against imatinib [Roychowdhury S. and Talpaz M., 2011].

2. PROTEIN KINASE CK2

Phosphorylation event is the most important and the best known reversible post-translational modification playing a critical role in signal transduction pathways. Phosphorylation is carried out by the large family of enzymes named protein kinases, often collectively referred to as "kinome" [Manning G. *et al.,* 2002]. Protein kinases are capable to transfer the terminal phosphate group from ATP (or rarely from GTP) to a substrate molecule, affecting its conformation and function. Based on the similarity of the catalytic domain, more than 500 putative protein kinases encoded by the human genome, are classified in different subfamilies [Manning G. *et al.,* 2002]. Protein kinase CK2 belongs to the big family of the eukaryotic protein kinases (EPKs) [Hanks S.K. and Hunter T. 1995].

The discovery of protein kinase CK2 dates back to 1954 [Burnett G. and Kennedy E.P., 1954], when it was misnamed "casein kinase II" due to its ability to phosphorylate casein *in vitro* but, as demonstrated only many years later, not *in vivo*. CK2 is evolutionary highly conserved and ubiquitously expressed: it is distributed across eukaryotes, almost present in every kind of tissue and located all over subcellular compartments. This enzyme is pleiotropic and phosphorylates more than 500 proteins (http://www.phosphosite.org), implicated in several physiological and pathological processes.

CK2 is an acidophilic kinase recognizing serine and threonine residues. The hallmark for CK2-dependent phosphorylation is represented by an acidic residue in position n+3 (X_{n-1}-S/T-X_{n+1}-X_{n+2}-E/D/Sp/Yp) downstream from the phosphor-acceptor site [Pinna L.A. and Ruzzene M., 1996; Meggio F. and Pinna L.A., 2003]. This minimum consensus sequence is commonly accompanied by acidic clusters from position n−1 to n+7, with preference for aspartic residue at n+1 and glutamic residue from n+2 to n+7. Basic residues around the target amino acid represent negative determinants, as well as proline, whose location at position n+1, is negatively selected.. It is interestingly to note that CK2 is endowed with the unusual ability to use, as phosphate donor, not only ATP but also GTP, with similar efficacy because of its singular binding cleft [Niefind K. *et al.,* 1998]. With respect to the most of protein kinases which become active

in response to specific stimuli like phosphorylation events or second messengers, CK2 is constitutively active [Pinna L.A., 2002]. Although the sophisticated regulation of CK2 activity is not clearly established yet, increasing evidence suggests that it might be controlled by different mechanisms like recruitment to complexes [Keller D.M. *et al.*, 2001; Allende-Vega N. *et al.,* 2008] and cell compartment shuttling [Filhol O. and Cochet C., 2009].

CK2 is essential for cell life and represents a "master regulator" of fundamental cellular processes such as cell cycle, gene expression, protein synthesis, proliferation, growth, survival, cytoarchitecture, migration, metabolism and apoptosis [Pinna L.A., 2002; Ahmed K. *et al.*, 2002; Litchfield D.W., 2003].

CK2 dysregulation is intimately related to cancer, viral infections, neurodegenerative syndromes, inflammatory and cardiovascular diseases and cystic fibrosis [Cozza G. *et al.*, 2012].

2.1 - STRUCTURE AND ENZYMATIC FEATURES

CK2 is composed of two catalytic (α and/or α') and a dimer of regulatory subunits (ß) and is normally expressed in mammalian cells as a tetramer, consisting in $\alpha_2ß_2$, $\alpha\alpha'ß_2$, $\alpha'_2ß_2$ configurations [Chantalat L. *et al.,* 1999]. However, *in vitro* assays have demonstrated that recombinant CK2 catalytic subunits are active either alone or associated with the ß subunits [Pinna L.A., 2002].

The CK2 catalytic subunits are present in two different isoforms, α and α', of 42 and 38 kDa molecular weights, respectively. They are encoded by distinct genes and show an overall sequence resemblance of 80%, except for the C-terminal portion which is completed unrelated and present only in the α subunit [Lozeman F.J. *et al.*, 1990]. Both catalytic subunits share the typical kinase structure, formed by two lobes, a small, ß-sheet rich one (called C-terminal lobe) and a large, α-helix rich one (called N-terminal lobe) [Cozza G. *et al.,* 2012]. As for the other protein kinases, the structural subdomains of CK2 catalytic subunits can be divided in: *i)* the P-loop (phosphate-binding loop, also known as glycine-rich loop): this small loop is located in the ATP binding zone,

and is fundamental for the correct orientation of ATP phosphates; in the case of CK2 the classical "G-X-G-X-X-G" motif is modified by the presence of a serine (S51) instead of the third glycine residue; *ii)* the catalytic loop: so called for the presence of the catalytic aspartate (D156). *iii)* the activation loop: in most protein kinases this loop is responsible for the full active state through a series of phosphorylation in its residues; this event blocks the activation loop in an "open" conformation, granting the access of both the ATP and the substrate. In the case of CK2, however, phosphorylations in the activation loop are not required for its full activity; on the contrary, CK2 displays a constitutively active state thanks to the interaction between the activation loop and the N-terminal domain [Sarno S. *et al.,* 2002]. *iiii)* The substrate binding site: characterized by the phosphorylation consensus motif X_{n-1}-S/T-X_{n+1}-X_{n+2}-E/D/Sp/Yp, as previously anticipated [Marin O. *et al.,* 1986]. Despite the specific role of each catalytic subunit has not yet been discovered, several studies have demonstrated some functional specializations. In yeast, simultaneous disruption of the genes encoding both CK2 catalytic subunits results lethal [Glover C.V. *et al.,* 1998]. While yeast with catalytically inactive CK2α presents defects of cell polarity, catalytic inactivation of CK2α' causes cell cycle arrest [Hanna D.E. *et al.*, 1995; Glover C.V. *et al.*, 1998]. Studies in mouse models have also demonstrated that while CK2α' knock-out mice causes infertility in male without affecting viability, CK2α (or CK2ß) knock-out results embryonic lethal [Lou D.Y. *et al.*, 2008], suggesting that α subunit is able to compensate functionally, even if not absolutely, the lack of α' in the context of viability [Xu X. *et al.,* 1999]. In mammals, CK2α is phosphorylated, without effecting its activity, at multiple sites of the C-terminus, which is not present CK2α', in a cell cycle dependent manner, suggesting a different functional specialization between the two catalytic subunits during the cell cycle [Litchfield D.W., 2003].

ß-subunit (25 kDa) has no extensive homology with other protein kinase regulatory subunits. It presents: *i)* a C-terminal region, responsible for both the ß-ß dimerization and the association with the catalytic subunit; *ii)* a N-terminal region which hosts the auto-phosphorylation site (MSSSEE) and the acidic

cluster involved in the positive/negative regulation of CK2 phosphorylation. Although CK2 is constitutively active and the definition of CK2ß as "regulatory" subunit does not appear really correct, it has been demonstrated that ß subunits regulate CK2 activity conferring CK2α/α' substrate specificity. Usually, the catalytic activity of the tetramer is higher than that of isolated catalytic subunits. However, while for most of substrates, such as p53 and REV, CK2ß is absolutely required for substrate phosphorylation [Theis-Febvre N. *et al.*, 2003], for other proteins, like calmodulin [Marin O. *et al.*, 1999] and HS1 [Ruzzene M. *et al.,* 2000], CK2ß plays an inhibitory effect since they are phosphorylated only by monomeric CK2α or α' form. Furthermore, over the last decade *in vitro* and *in vivo* studies have evidenced that CK2ß subunits exist and perform functions independently of CK2 holoenzyme, interacting with and modulating the kinase activity of several proteins like A-Raf, c-Mos and Chk1 [Guerra B. *et al.*, 1999; Guerra B. *et al.,* 2003]. To support this hypothesis, an unbalanced expression of catalytic and regulatory subunits and their independent subcellular mobility has been highlighted in several tissues and cell cancers [Bibby A.C. and Litchfield D.W., 2005].

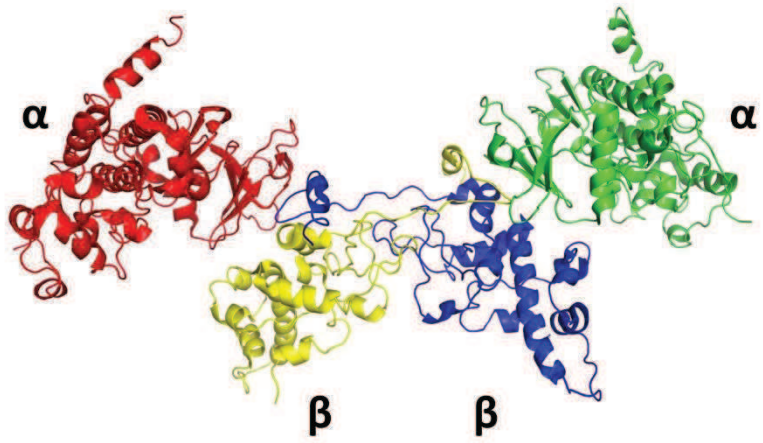


Figure VII. Molecular representation of CK2 holoenzyme. *CK2α (red and green) and CK2ß (yellow and blue) are highlighted.*

The association of CK2 catalytic and regulatory subunits in a tetrameric structure preserve the complex against unfolding and proteolysis. The crystallographic data of human CK2 tetramer reveal a "butterfly structure" (see Fig.VII), where the regulatory subunits form a stable dimer linking the two

catalytic subunits, which make no direct contact with one another. Each catalytic subunit interacts with both regulatory chains, through the ß subunit C-terminal tail. This interaction is mainly based on electrostatic contacts between α and ß subunits, since the presence of the isolated catalytic subunit or the tetrameric form of CK2 depends crucially on ionic strength. While different oligomerization models of the tetrameric structure have been proposed and the regulatory role of the ß subunit needs more clarification, it has been suggested that the ß subunit can provide a docking platform for substrates or potential positive/negative regulators [Cozza G. *et al.,* 2012]

2.2 - ROLE OF CK2 IN CELL SURVIVAL AND APOPTOSIS

As mentioned previously, CK2 is essential for cell life end exerts a vast array of cellular functions related to cell decision between death and life, promoting cell survival and protecting cells from apoptosis. CK2 is a kinase for cell survival at all costs [Ruzzene M., 2013].

CK2 and cell cycle

Mounting evidence demonstrates that CK2 plays an essential role in every stage of cell cycle progression. Studies in both yeast and mammalian cells indicate that CK2 is required for G0/G1, G1/S and G2/M transitions probably through the phosphorylation and regulation of many cell cycle regulatory proteins including Topoisomerase II [Daum J.R. and Gorbsky G.J., 1998; Escargueil A.E. *et al.,* 2000], Cdc34 [Block K. *et al.,* 2001], Cdk1 [Russo G.L. *et al.,* 1992] and Six1 [Ford H.L. *et al.*, 2000]; however, the specific functions of these phosphorylations are still largely unclear. CK2 is also able to modulate the activity of the master regulator of cell cycle CAK (Cdk-activating kinase) [Schneider E. *et al.,* 2002]. CK2 regulates G1/S signaling through interaction with and phosphorylation of p53, SSRP1, Mdm-2, Cdk inhibitory proteins

$p21^{WAF1/CIP1}$ and $p27^{KIP1}$ [St-Denis N.A. and Litchfield D.W., 2009]. Additional evidence shows that, in G2/M transition, CK2 co-localizes with the mitotic spindle and centrosomes [Yu I.J. *et al.,* 1991]. CK2 interacts with many proteins involved in mitosis including Pin1 [Messenger M.M. *et al.,* 2002], and PP2A [Heriche J.K. *et al.,* 1997]. Further evidence for the essential role played by CK2 in cell cycle comes from the observation that both CK2α and CK2ß are phosphorylated in mitotic cells: the former by CdK1 at multiple sites (T344, T360, S262, S370) within its unique C-terminal domain, while the latter by $p34^{cdc2}$ at site S209 [St-Denis N.A. and Litchfield D.W., 2009].
CK2 is implicated also in transcription control, regulating directly the activity of RNA polymerases I, II and III [Panova T.B. *et al.,* 2006; Lin C. *et al.*, 2006; Cabrejos M.E *et al.,* 2004; Johnston I.M. *et al.*, 2002] and phosphorylating many transcription-associated factors. CK2-catalyzed phosphorylation of eIF2ß [Llorens F. *et al.,* 2006] and eIF5 [Majumdar R. *et al.*, 2002] suggests also an involvement of CK2 in the translation machinery.

CK2 and apoptosis

Apoptosis is a form of programmed cell death, a mechanism tightly regulated that leads to cell elimination without damage in the surrounding cells [Gordeeva A.V. *et al.,* 2004]. Its perturbation has been linked to cancer [Vermeulen K. *et al.,* 2005].
The role of CK2 in preventing apoptosis has been deeply dissected. CK2 exerts its anti-apoptotic function impinging on apoptosis pathway either intrinsically, induced by DNA-damage [Yamane K. and Kinsella T.J., 2005], or extrinsically, induced by receptor stimulation [Izeradjene K. *et al.*, 2004]. In particular, CK2 counteracts directly and indirectly the action of caspases, the proteases activated in response to apoptotic stimuli. CK2 is well suited to act phosphorylating several caspase substrates, including Bid [Desagher S. et al., 2001], HS1 [Ruzzene M. *et al.*, 2002], connexin 45.6 [Yin X. *et al.*, 2001], presenilin-2 [Walter J. *et al.*, 1999], PTEN [Torres J. *et al.,* 2003] and Max [Krippner-Heidenreich A. *et al.*, 2001], rendering them refractory to caspase-

mediated cleavage, thus avoiding apoptosis. It is noteworthy that a similarity between CK2 consensus sequence for phosphorylation and the recognition sequence for caspase cleavage has been observed [Duncan J.S. *et al.,* 2010]. CK2 blocks caspase activity also directly by phosphorylating pro-caspase 2 [Shin S. *et al.,* 2005], pro-caspase 9 [McDonnell M.A. *et al.*, 2008] and the caspase inhibitor ARC [Li P.F. *et al.*, 2002] and preventing their activation.
Finally, CK2 overexpression correlates with the up-regulation of survivin, an inhibitor of apoptosis proteins [Tapia J.C. *et al.*, 2006].

2.3 - ONCOGENIC POTENTIAL OF CK2

The correlation between CK2 and neoplasia has been known for a long time. An elevated protein level and activity of CK2 has been observed in many human cancers including both solid tumors [Laramas M. *et al.,* 2007; Lin K.I. *et al.,* 2011] and hematological malignances [Kim J.S. *et al.*, 2007]. CK2 up-regulation correlates with aggressive cancer behavior, unfavorable prognosis and poor survival [Laramas M. *et al.*, 2007; Piazza F. *et al.*, 2012]. Studies in mouse have further reinforced the oncogenic potential imparted by CK2 dysregulation: targeted expression of CK2 in transgenic mice promotes tumorigenesis, also cooperating with several oncogenes, including Tal-1 and c-Myc, or with the loss of the tumor suppressor p53 [Kelliher M.A. *et al.*, 1996; Channavajhala P. and Seldin D.C., 2002; Landesman-Bollag E. *et al.,* 2001]. Interestingly, this increase in CK2 protein level is usually not accompanied by a parallel mRNA enhancement [Trembley J.H. *et al.*, 2009; Borgo C. *et al.*, 2013], suggesting a slower turnover of CK2 at the protein level [Di Maira G. *et al.*, 2007]. With regard to this, many studies have reported that in normal cells CK2 is distributed in all compartments, while in cancer cells it is more concentrated in the nuclear compartment [Faust R.A. *et al.,* 1999].
CK2 is not an oncogene in *sensu stricto*, in fact it is expressed in non-transformed cells and no gain-of-function mutations have ever been shown in cancer cells. Although CK2 is never the main driver of malignant

transformation, it is critically required to promote an environment particularly suited for the development of neoplasia. This view has raised to the concept of CK2 non-oncogenic addiction. According to this view, CK2 reduction is not dramatic for normal cells, which are already adapted to low levels of protein and less dependent on the kinase activity. On the contrary, cancer cells, characterized by an invariantly high CK2 amount, are more dependent on CK2 functions and strongly sensitive to CK2 down-regulation. Addiction to CK2 represents a common feature of different cancer cells, not a peculiarity of only one [Ruzzene M. and Pinna L.A., 2010]. The common idea is that CK2 is neither the cause nor the consequence of neoplastic transformation, but a cooperating partner of tumorigenic pathways, mainly through its pro-survival and anti-apoptotic role.

CK2 sustains the progression of tumorigenesis perpetuating abnormal pro-survival and anti-apoptotic signals by alternative approaches: 1) by supporting the transforming potential of various oncogenes [Seldin D.C. and Leder P., 1995]; 2) by stabilizing the onco-kinome through the activation of the co-chaperone Cdc37, which acts preserving the active conformation of many onco-kinases [Miyata Y. and Nishida E., 2004]; 3) by facilitating DNA repair [Loizou J.I. *et al.,* 2004]; 4) by promoting rRNA and tRNA biogenesis [Ghavidel A. and Schultz M.C., 2001]; 5) by counteracting the activation of caspases [Duncan J.S. *et al.,* 2010]; 6) by preventing the efficacy of anti-tumor drugs [Piazza F. *et al.,* 2006; Mishra S. *et al.,* 2007]; 7) by potentiating the multidrug resistance phenotype [Di Maira G. *et al.,* 2007; Borgo *et al.*, 2013]; 8) by favoring the neovascularization [Ljubimov A.V. *et al.,* 2004].

The number and type of signaling pathways regulated by CK2 is impressive. Among them, the following pathways are frequently up-regulated in cancers (see Fig.VIII):

a) PI3K/Akt/mTOR pathway. CK2 reinforces the survival signal dictated by PI3K cascade, operating as a multi-site up-regulator. First, CK2 phosphorylates the tumor suppressor PTEN, resulting in PTEN stabilization [Torres J. and Pulido R., 2001] and functional inactivation [Arevalo M.A. and Rodriguez-Tebar A., 2006] with consequent increase in PI3K/Akt/mTOR signaling. Not surprisingly PTEN is frequently mutated in human cancers. Secondly, CK2 up-regulates the

anti-apoptotic Akt kinase by a direct phosphorylation on S129 [Di Maira G. *et al.*, 2005], which fully activates the kinase preventing the de-phosphorylation of Akt T308 [Di Maira G. *et al.*, 2009], one of the two activatory sites of Akt, by ensuring a stable association with the chaperone protein Hsp90 [Ruzzene M. and Pinna L.A., 2010].

b) MAPK pathway. CK2 interferes with the regulation of cell proliferation operated by MAPK signaling. First, CK2ß has been shown to be a binding partner for A-Raf [Boldyreff B. and Issinger O.G.., 1997] and this interaction stimulates the A-Raf-mediated phosphorylation of MEK [Hagemann C. *et al.,* 1997]. Secondly, CK2-catalyzed phosphorylation of ERK1/2 at the residues S244 and S246 is sufficient to induce its translocation from the cytoplasm into the nucleus, where ERK1/2 may interact with different proteins implicated in cell proliferation and differentiation [Plotnikov A. *et al.*, 2011].

c) Wnt signaling: CK2 strengthens the proliferation signal transduced by Wnt/ ß-catenin pathway, which is essential for embryogenesis, generally silent in adult tissues but hyper-activated in up to 50% of human cancers. CK2 directly phosphorylates ß-catenin protecting it from proteolysis [Song D.H. *et al.*, 2003] and resulting in the activation of several pro-survival signals (c-Myc, c-Jun, cyclin D). CK2-catalyzed phosphorylation of Dvl [Song D.H. *et al.,* 2003] and LEF1 [Wang S. and Jones K.A., 2006], two Wnt signaling intermediates, further reinforces cell survival.

d) NF-κB: Similarly to Wnt pathway, NF-κB cascade is essential for the regular development and its dysregulation can lead to oncogenesis. CK2 phosphorylates IκB promoting its proteolytic degradation, an event that activates NF-κB which translocates into the nucleus, where it acts as transcription factor for anti-apoptotic and pro-survival genes [Dominguez I. *et al.*, 2009]. Moreover, CK2 induces also the expression of IKK [Eddy S.F. *et al.,* 2005], which normally induces IκB proteolysis. Finally, the p65 subunit of NF-κB itself is phosphorylated and regulated by CK2 [Wang G. *et al.*, 2000].

Interestingly, the participation of CK2 in each pathway is atypical with respect to the other protein kinases because it is not hierarchical. CK2 acts as a lateral player, with an horizontal role, impinging on different levels of the oncogenic pathways. On the contrary, canonical signaling cascades are activated by a

stimulus from outside the cells which achieves the membrane and transmits its message inside the cell in a figurative longitudinal direction [Ruzzene M. and Pinna L.A., 2010].

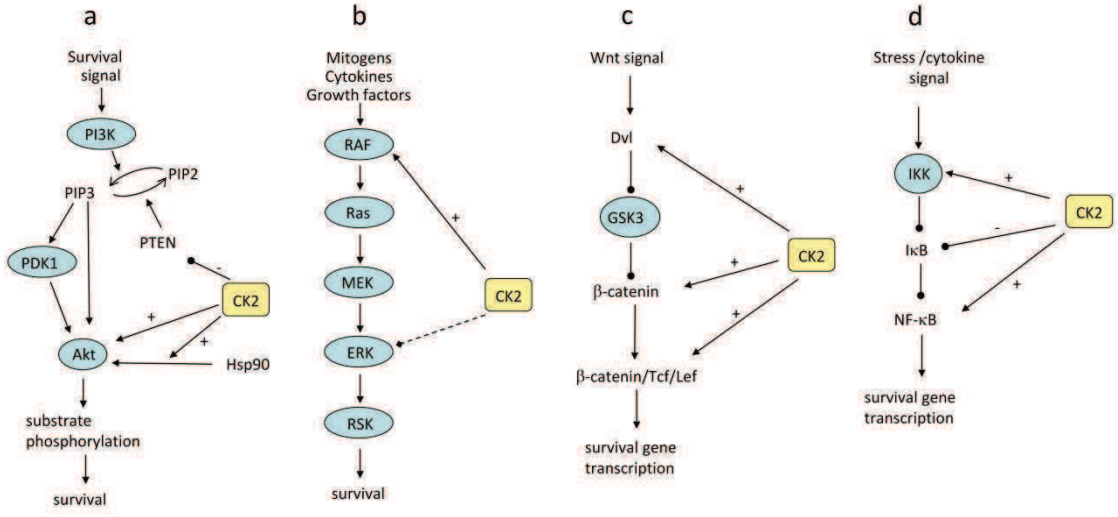


Figure VIII. CK2-dependent control of PI3K (a), MAPK (b), Wnt (c) and NF-κB (d) pathways.
Schematic representation of the multisite regulation played by CK2, a lateral player of "vertical signaling cascades". Normal arrows indicate a positive effect (+) , dot-ended lines indicate a negative effect (-). Dot-arrows indicate subcellular translocation induction.
[Adapted from Ruzzene M. and Pinna L.A., 2010]

CK2 and drug-resistance

The phenomenon of resistance to cancer therapy is a mechanism of paramount medical relevance being one of the major cause for cancer therapy failure, especially in the leukemic transformation [Suarez L. *et al.*, 2005; Di Maira G. *et al.*, 2007]. The molecular mechanisms referred to drug-resistance have been dissected for many years: it is well known that they are due to multiple factors, which range from development of pro-survival mutations in key signaling molecules, to acquisition of alternative survival signaling pathways, to the overexpression of efflux transporter proteins such as P-glycoprotein (P-gP), which extrudes cytotoxic molecules from the cells [Rosenzweig S.A., 2012]. As final result, in all these cases cancer cells become refractory to apoptosis ensuring cell survival. Many studies have highlighted an intimate implication between CK2 and drug-resistance. CK2 overexpression has been associated with drug resistance mechanisms, either related to multi drug resistance

phenotype or induced by single drug [Di Maira G. *et al.,* 2008; Matsumoto Y. *et al.,* 2001]. In particular, P-gP is a well known CK2-substrate [Glavy J.S. *et al.*, 1997]. Although the role of this phosphorylation has never been elucidated, it has been shown that in P-gP expressing cells the drug accumulation is enhanced in parallel with the degree of CK2 inhibition, reflecting a positive role carried out by CK2 on P-gP activity [Di Maira G. *et al.,* 2007; Di Maira G. *et al.,* 2008]. Furthermore, in multidrug resistant glioma cells the overexpression of CK2 has been recognized to be responsible for the high *in vivo* phosphorylation of the DNA topoisomerase II [Matsumoto Y. *et al.,* 2001]. Finally, it is noteworthy that cell treatment with inhibitors of CK2 induces significant cell death activating the apoptotic process in different cancer cells, either sensitive or resistant to conventional chemotherapy [Zanin S. *et al.,* 2012].

CK2: a logical target in cancer therapy

Considering the dual role of CK2 in cell proliferation and cell death, the two main deregulated features in cancers, it is presently considered a promising therapeutic target.

It has been observed that CK2 down-regulation, by pharmacological inhibition or by knocking down its expression, induces cell death mainly due to apoptosis. In particular, CK2 represents a promising pharmacological target to fight a tumor because it allows to overtake two major theoretical limits of cancer therapy: the lack of target selectivity and the inability to affect selectively cancer cells. First, CK2 has a peculiar structure of the catalytic site with respect to other kinases, which has allowed the development of specific and selective inhibitors. Secondly, the inhibitors affect more tumor cells than normal cells in agreement with the concept of addiction to CK2, that is not a peculiarity of some tumors but a common denominator of diverse cancer cells [Ruzzene M. and Pinna L.A., 2010]. On these bases, it has been developed a wide variety of cell permeable, potent and selective, ATP-site directed inhibitors belonging to different chemical classes [Sarno S. *et al.*, 2001; Cozza G. *et al.*, 2010] able to kill several cancer cell lines [Mishra S. *et al.*, 2007; Buontempo F. *et al.,* 2013].

Interestingly, CK2-inhibition may sensitize tumor cells to the activity of other chemotherapeutic agents such as melphalan [Piazza F. *et al.,* 2006], vinblastine [Di Maira G. *et al.,* 2007], cisplatin [Siddiqui-Jain A. *et al.,* 2012]. Even more interesting, CK2-inhibitors may be used to overcome multidrug resistance phenomenon. With respect to this, of especial interest is the CK2-specific inhibitor CX-4945, a small tricyclic compound displaying a Ki *in vitro* <1nM, that induces apoptosis selectively in tumor cells as compared with normal cells [Siddiqui-Jain A. *et al.,* 2010]. It has been also demonstrated that CX-4945 is effective in different cancer cell lines associated to pharmacological resistance occurrence, either related to the expression of multi-resistance phenotype or induced by specific drugs [Zanin S. *et al.*, 2012]. In murine xenograft models, CX-4945 shows a potent efficacy and is well tolerated. Finally, CX-4945 is orally bioavailable and it has recently entered clinical trials for the treatment of different kinds of cancers [Siddiqui-Jain A. *et al.,* 2010].

AIM OF THE STUDY

AIM OF THE STUDY

It has been described that cancer cells are addicted to protein kinase CK2, which is never the direct cause of tumor but is critically required to promote a more favorable environment for the development of neoplasia, mainly through its anti-apoptotic and pro-survival role [Ruzzene M. and Pinna L.A., 2010]. Aim of my PhD research was to shed light on the role of protein kinase CK2 in chronic myeloid leukemia (CML) pathology, which is driven by the constitutively active tyrosine kinase Bcr-Abl. Despite the great efficacy of the specific Bcr-Abl inhibitor imatinib, resistance to this drug represents the major problem in CML therapy. To highlight the role of CK2 in CML oncogenic signaling, my experiments were performed using two different CML cell lines, LAMA84 and KCL22, either sensitive (S) or resistant (R) to imatinib. Cells were examined to highlight *i)* the potential cross-talk occurring between CK2 and Bcr-Abl, *ii)* the possible implication of CK2 in the mechanism(s) of resistance induced by imatinib treatment, *iii)* the significance of CK2 as a potential target for CML treatment and of CK2-inhibitors as drugs for combined therapies to overcome CML imatinib-resistance.

It has been recently demonstrated that in resistant LAMA84 cells, characterized by *BCR-ABL1* gene amplification, CK2 is up-regulated as compared to the sensitive cells [Borgo C. *et al.,* 2013]. To analyze the properties of the protein kinase CK2 and its role in imatinib-resistant LAMA84 cells, I investigated the CK2 cellular protein level and its subcellular distribution looking for a possible co-localization and interaction with Bcr-Abl. On the other hand KCL22 cells, which show an expression of CK2 and Bcr-Abl similar in imatinib-sensitive and -resistant cells, were examined to gain insights into unknown mechanisms of imatinib-resistance related to CK2. The participation of CK2 in the complex oncogenic network was investigated at multiple levels since the kinase does not act in a hierarchical way, but as a lateral player in the oncogenic signal transduction [Ruzzene M. and Pinna L.A., 2010]. Particular attention was paid to two Bcr-Abl downstream signalings: MAPK and PI3K/Akt/mTOR pathways, which have been frequently demonstrated to be hyper-activated in cancer cells

[Britten C.D., 2013]. The role played by CK2, in the deregulated pathways evidenced in resistant KCL22 cells, was studied by treating cells with the potent and selective CK2-inhibitor, CX-4945, currently in clinical trials for the treatment of several kinds of tumors [Siddiqui-Jain A. *et al.,* 2010].

To further assess the contribution of CK2 in CML, the effect of CK2-inhibition was examined on the viability and apoptosis induction of LAMA84 and KCL22 cells. Finally, it was analyzed the possibility that CK2-inhibition might act synergistically with other anticancer agents counteracting the imatinib resistance.

MATERIALS AND METHODS

MATERIALS

1. *Antibodies*

Anti-c-Abl used for immunofluorescence was from Calbiochem (Darmstadt, Germany) while anti-c-Abl for western blot was from Santa Cruz Biotechnology (Santa Cruz, CA), such as antibodies raised against Anti-CK2α', Akt, lamin B, LDH, rpS6, phospho-SGK(S422). Anti-phospho-tyrosine was purchased from Millipore Corporation (Billerica, MA), anti-PARP from Roche (Basel, Switzerland) and anti-tubulin from Sigma-Aldrich (Dorset, U.K.). Anti-CK2ß, anti-CrkL, anti-phospho-CrkL(T207), anti-RSK and anti-phospho-RSK(S244) from Epitomics (Burlingame, CA). Antibodies against ERK1/2, phospho-ERK1/2(Y202-204), phospho-RSK(T573), phospho-p53(S15), phospho-Akt(S473), phospho-Akt(T308), phospho-mTOR(S2448), phospho-GSK3ß(S9), phospho-PDK1(S241), phospho-rpS6(S235/6-240/4), 4E-BP1, phospho-4E-BP1(T37/46) were form Cell Signalling Technology (Danvers, MA). Anti-CK2α against the sequence of the C-terminus (376-391) [Sarno S. *et al.*, 1996] and anti-phospho-Akt(S129) [Di Maira G. *et al.,* 2005] antibodies were raised in rabbit. Secondary antibodies: anti-rabbit and anti-mouse HRP-labeled were from Perkin-Elmer (Walthan, MA), anti-goat IgG biotinylated was from Sigma-Aldrich, streptavidin-horseradish peroxidase conjugate was from GE Healthcare Life Sciences (Milano, Italy).

2. *Inhibitors*

Imatinib mesylate was purchased from Cayman Chemicals (Ann-Arbor, MI) , CX-4945 was from AbMole BioScience (Hong Kong, China), U0126 and rapamycin from Selleck Chemicals (Houston, TX). All compounds were diluted in 100% dimethylsulphoxide (DMSO) and stored at -20°C.

3. *Recombinant CK2 and CK2-substrate*

Recombinant CK2 ($\alpha_2ß_2$) and recombinant His-tagged-CK2α' were kindly provided by Dr. Andrea Venerando (University of Padova, Italy). RRRADDSDDDDD peptide was kindly provided by Dr. Oriano Marin (University of Padova, Italy). ß-casein was from Sigma-Aldrich.

4. *Radioactive materials*

[γ^{33}P]ATP was purchased from Perkin-Elmer. [^{35}S]-L-methionine/cysteine protein labeling mix was from Hartmann Analytic (Braunschweig, Germany).

5. *Oligonucleotides*

CK2α specific siGENOME SMARTpool siRNAs and aspecific siRNA siCONTROL riscfree#1 were from Dharmacon (Lafayette, CO, USA). The used siRNAs are designed by Dr. Mauro Salvi (University of Padova, Italy) and correspond to the sequence CK2ß-siRNA (sense, 5'-GCCAUGGUGAAGC UCUACUdTdT-3'; antisense, 5'-AGUAGAGCUUCACCAUGGCdTdT-3') and to the sequence CK2α' -siRNA (sense, 5'-GCUGCGACUGAUAGAUUGGdTdT-3'; antisense 5'-CCAAUCUAUCAGUCGCAGCdTdT-3'). Transfecting reagent INTERFERin was from Polyplus-transfection SA (Illkirch, France).

6. *Other Chemicals*

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent, ColorBurst™ colored electrophoresis protein marker and BSA were from Sigma-Aldrich. Immobilon-P transfer membranes were from Millipore Corporation. Phosphatase inhibitor cocktails 2 and 3 were from Sigma-Aldrich while protease inhibitor cocktail Set III was from Calbiochem.

METHODS

1. *Cell culturing and treatment*

LAMA84 and KCL22 cell lines, either sensitive or resistant to imatinib, were kindly provided by Prof. C. Gambacorti-Passerini (University of Milano-Bicocca). Cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 2mM L-glutamine, 100U/ml penicillin and 100 mg/ml streptomycin at 37°C in an atmosphere containing 5% CO_2. Resistant LAMA84 and KCL22 cells were supplemented with 1.5 μM and 3 μM imatinib, respectively.
For the treatments, CML cells were seeded at 1 x 10^6/ml and treated for different times in the culture medium as indicated in the Figure legend. Imatinib, CX-4945, U0126 and rapamycin were dissolved in 100% DMSO. Control cells were treated with equal amount of the inhibitor vehicle. At the end of incubations, cells were harvested by centrifugation, washed with PBS and lysed as detailed below.

2. *Cell lysis*

Cells were lysed with ice-cold buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% (v/v) triton X-100, 1mM NaF, protease inhibitor cocktail Set III and phosphatase inhibitor cocktails 2 and 3. After 1h incubation on ice, samples were centrifuged at 13.2 rpm for 15 minutes, at 4°C. The supernatant represents the total soluble cell fraction. The lysate protein content was determined by the Bradford method.

3. *Western blot analysis*

Equal amounts of lysate proteins were loaded on 9%, 11% or 15% SDS-PAGE (Laemmli,1970) and blotted on Immobilon-P membranes (Millipore) in a TE 22 Mini Tank Transfer Unit (GE Healthcare), at 60 V for 90 or 120 min, using a buffer containing 10 mM CAPS-NaOH (3-(Cyclohexylamino)-1-propanesulfonic acid pH 10, 3 mM dithiothreitol (DTT) and 1% (v/v) methanol. Membranes were dried, washed with TBS buffer (50 mM Tris-HCl pH 7.5, NaCl 50 mM) with 1% (w/v) BSA for saturation, processed with the indicated antibodies and then developed using an enhanced chemiluminescence detection system (ECL). Immunostained bands were quantified by means of a Kodak Image Station 4000MM PRO and analysis with Carestream Health Molecular Imaging software (New-Haven, CT).

4. *Immunoprecipitation experiments*

Indicated lysate proteins or indicated volume of the pooled fractions of glycerol gradients were immunoprecipitated overnight with the specific antibody or with an aspecific antibody, used as a negative control. Protein A-Sepharose (Sigma-Aldrich) or Protein G Plus (Santa Cruz Biotechnology) was then added for 1 h at 4°C. The immunocomplexes, washed three times with 50 mM Tris-HCl pH 7.5, were analysed by western blot.

5. *Subcellular fractionation by differential centrifugation*

For the isolation of subcellular particles, a cell fractionation protocol by Kang and Welch [Kang H.S. and Welch W.J., 1991] was used with some modifications. Briefly, about 8 x 10^6 cells were resuspended in 400 µl of a hypotonic buffer (10 mM Tris/acetate pH 7.4 containing protease and phosphatase inhibitor cocktails), incubated for 5 min on ice and broken by 50 strokes in a dounce homogenizer with a tight pestle. The solution was immediately adjusted to 0.25 M sucrose, 1

mM $MgCl_2$, and centrifuged at 1000 g for 10 min at 4°C to remove nuclei. The supernatant was removed and centrifuged at 10000 g for 20 min to isolate the mitochondria. The supernatant was further centrifuged at 100000 g for 90 min to separate the cytosol from the microsomes. Each pellet was resuspended in an appropriate volume of lysis buffer. The same volume of the different fractions (the µl containing 10 ug of cytosol) was analyzed by Western blot with the indicated antibodies.

6. *In-gel kinase assay of CK2α*

The activity displayed by CK2α subunit alone was determined by running similar volumes of the subcellular fractions obtained as described in section 5 on a 11% SDS-PAGE including the CK2-substrate ß-casein (0.5 mg/ml). After electrophoresis, in order to remove SDS, the gel was washed twice in a buffer composed of 50 mM Tris-HCl pH 8 containing 20%(v/v) 2-propanol for 30 min at room temperature (RT). Gel was quickly rinsed out in 50 mM Tris-HCl pH 8 and then incubated progressively with the indicated buffers: 50 mM Tris-HCl pH 8 and 5 mM 2-mercaptoethanol for 1 h at RT; 50 mM Tris-HCl pH8, 5 mM 2-mercaptoethanol and 6 M guanidine for 1h at RT; 50 mM Tris-HCl pH8, 5 mM 2-mercaptoethanol and 0.04% (v/v) Tween-20 overnight at 4°C to renature proteins. The activity of CK2α toward the co-localized ß-casein was detected by incubating the gel with a phosphorylation medium containing 10 µM ATP, 4 µCi [γ^{33}P]ATP, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$. Subsequently the gel was washed with 5% trichloroacetic acid (TCA) and 1% sodium pyrophosphate in order to remove the ATP in excess. Each incubation was performed with gentle shaking. Radioactive ^{33}P-ß-casein was evidenced by analysing the dried gel with a Cyclone Plus Storage PhosphorSystem (PerkinElmer). Image was processed with the optiQuantTM image Analysis software and radioactivity was expressed in Digital Light Units (DLU).

7. *CK2 kinase activity assay*

Lysate proteins were incubated for 10 min at 30° C in a phosphorylation mixture containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1 M NaCl, 10 μM [γ-^{33}P]ATP and 400 μM CK2-specific peptide RRRADDSDDDDD. The reaction was stopped by absorption on phospho-cellulose P81 paper. Papers were washed three times for 10 minutes with 75 mM phosphoric acid, dried and the peptide radioactivity was counted in a Scintillation Counter (PerkinElmer).

8. *Separation of multi-protein complexes of cell extracts by glycerol-gradient sedimentation*

LAMA84 cells ($20x10^6$) were lysed with a buffer containing 20mM Tris-HCl, pH 7.5, 10mM KCl, 1mM EDTA, 10% glycerol, 0.2% triton X-100, protease and phosphatase inhibitors. Samples were centrifuged and supernatant fractions (400 μg) were layered on the top of a 3.6 ml of a glycerol linear gradient (10%-40%) made in 50 mM Hepes, pH 8, 1 mM EDTA, 1 mM DTT, protease and phosphatase inhibitors. After centrifugation at 100000 g in a SW60Ti rotor (Beckman) for 18 hours at 4°C. 20 fractions were collected from the bottom of the tube and 40 μl of each were immunoblotted with the indicated antibodies. Bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa) were run on separated tubes, as standards, for estimating the molecular weight of the complexes.

9. *Immunolocalization of CK2 and Bcr-Abl by confocal microscopy*

LAMA84 cells (5×10^5) were seeded on polylysine-coated glass coverslips and allowed to adhere overnight. Cells were fixed with 4% para-formaldehyde in PBS for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 10 min at 4°C. For dual labelling, cells were first incubated with

mouse anti-Abl (1:10) overnight at 4°C, followed by 1 h incubation with anti-mouse IgG/FITC conjugated (1:50) in a dark, humidified chamber at 37°C. Cells were then incubated with rabbit anti-CK2-alpha (1:50) for 1 h at 37°C, followed by Alexa Fluor 633 coniugated secondary antibody [goat anti-rabbit (1:500)] for 1 h at 37°C, as previously described. Nuclei were stained with Hoechst 33342 (Sigma-Aldrich). The coverslips were mounted with a drop of mounting medium (Fluoromount, Sigma-Aldrich). Fluorescence images were captured by means of LEICA TCS SP5 confocal microscopy (Wetzlar, Germany), equipped with HCX PL APO lambda blue 63 x 1.4 oil immersion objectives. The analysis was performed with Argon, HeNe and Diode 405 lasers, allowing separate collection of green fluorescence (Argon laser at 488 nm), red fluorescence (HeNe laser at 633 nm) and blue fluorescence (Diode 405 laser) of the same microscope field. Images were processed with the LAS AF software.

10. Cell viability assay

Cell viability was detected by the method of MTT [3-(4,5-dimethylthiazol-2yl)-3,5-diphenyltetrazolium bromide), incubating 4 x 10^4 cells/100 µl in a 96-well plate for 48 h under the indicated conditions. After 1 h of incubation 10 µl of MTT solution (5 mg/ml in PBS) were added to each well. Incubations were stopped by addition of 20 µl of a pH 4.7 solution containing 20% (w/v) SDS, 50% (v/v) N,N-dimethylformamide, 2% (v/v) acetic acid and 25 mM HCl. Plates were read for OD at λ 590, in a Titertek Multiskan Plus plate reader (Flow Laboratories). DC_{50} (concentration inducing 50% of cell death) values were calculated with Prism 4.0c software (GraphPad Software).

11. Combined treatments

Concomitant administration of two or more inhibitors (imatinib, CX-4945, U0126, rapamycin) was assessed by treating cells with increasing concentrations of inhibitors at fixed concentration ratio, as indicated in the

Figure legend. The combination index (CI) for the combined treatment with imatinib, CX-4945, U0126 and rapamycin was calculated with the software Calcusyn (Biosoft, Cambridge, U.K.) [Chou T.C., 2006].

12. ^{35}S-Methionine/Cysteine metabolic labelling

5 x 10^6 cells per well were seeded in six-well plates and pre-treated for 3 h with vehicle (DMSO) or 4 μM CX-4945 prior to add 100 μg/ml cycloheximide. After 2 h the medium was replaced by methionine- and cysteine-free RPMI supplemented with 2 mM L-glutamine and 100 μg/ml cycloheximide. Cells were incubated for 1 h. Medium was then removed and cells were pulsed in the presence of a methionine- and cysteine-free RPMI supplemented with 2 mM L-glutamine, 10 μCi/ml [^{35}S]-L-methionine/cysteine protein labeling mix. After 1 h radiolabeled medium was removed and cells were washed and lysed as previously described. Exposure to CX-4945 was maintained throughout the experiment. Labeled lysate proteins were subjected to SDS-PAGE, blotted and the intensity of the radioactive bands was measured using the Cyclone Plus Storage PhosphorSystem and expressed as Digital Light Units (DLU).

13. Apoptosis assay by nucleosome enrichment quantification

Apoptosis was determined using the Cell Death Detection Elisa kit (Roche), which quantify the nucleosomes present in the cytosol of the apoptotic cells by measuring the absorbance at λ 405 and λ 490 following the manufacturer's instructions.

14. RNA interference

Cells (1.5 x 10^6) were transfected with 50 nM CK2ß, CK2α' or CK2α specific siGENOME SMARTpool siRNAs or aspecific siRNA siCONTROL

riscfree#1 as control, using the transfecting reagent INTERFERin, according to the manufacturer's recommendations.

15. Statistical analysis

Prism 4.0c software was used for graphs and statistical analysis (GraphPad Software). Data are presented as means ± SD and mean differences were analyzed using *t-test*. A $p<0.05$ was considered as statistically significant.

RESULTS

CML LAMA84 cells

Over the last few years increasing evidence highlighted an involvement of protein kinase CK2 in different drug-resistance mechanisms [Di Maira G. *et al.*, 2008; Matsumoto Y. *et al.*, 2001]. With regard to this, in my laboratory it has been observed that resistant-LAMA84 CML cells, which are characterized by *BCR-ABL1* gene amplification [Le Coutre P. *et al.*, 2000], contain a 2-fold higher amount of CK2α catalytic and CK2ß regulatory subunits as compared to parental cells [Borgo C. *et al.*, 2013].

Protein quantification of CK2 in LAMA84 cells

To better characterize the anomalous properties of CK2 previously found in LAMA84 cells, we performed a relative quantification of cellular CK2α and ß subunits in LAMA84 cells by comparative analysis with recombinant CK2 holoenzyme $\alpha_2ß_2$. The comparison suggests that the two CK2 subunits are expressed at very high levels in both CML cell variants (Fig.1). In particular, the densitometric analysis shows that in imatinib-resistant cells the amount of CK2α subunit represents about 0.3% of total proteins, a concentration in the range of structural proteins. In parallel, this analysis confirms that cellular protein levels of CK2α and ß are about two-fold higher in resistant cells with respect to the sensitive counterpart (Fig.1).

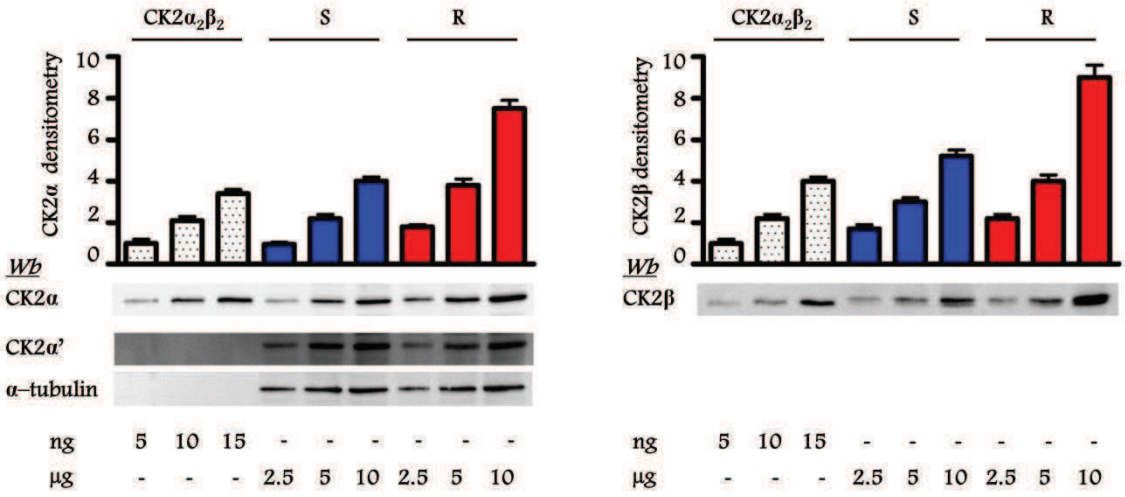


Figure 1. Relative quantification of CK2 α and ß subunits in LAMA84 cells. *The indicated quantities of recombinant CK2 $\alpha_2\beta_2$ and of lysate proteins obtained from S-LAMA84 and R-LAMA84 cells were analysed by wb with the indicated antibodies. α-tubulin is shown as a loading control. Means of densitometric values ± SD, expressed in arbitrary units, are reported above the relative subunit bands. Cellular CK2 subunit amounts were calculated by densitometric analysis and extrapolation from the calibration curve built on the signal of recombinant CK2 subunits.*

It has been previously shown that CK2α' protein level is similar in both imatinib-sensitive and -resistant LAMA84 cells. To compare the cellular expression of the two catalytic subunits, α and α', in these cells, the amount of cellular CK2α' was examined in parallel with known quantities of His-tagged recombinant CK2α'. The comparative analysis pointed out that the cellular level of CK2α' is comparable to that of CK2α present in imatinib-resistant cells (Fig.2), highlighting that also this kinase subunit is highly expressed in LAMA84 cells.

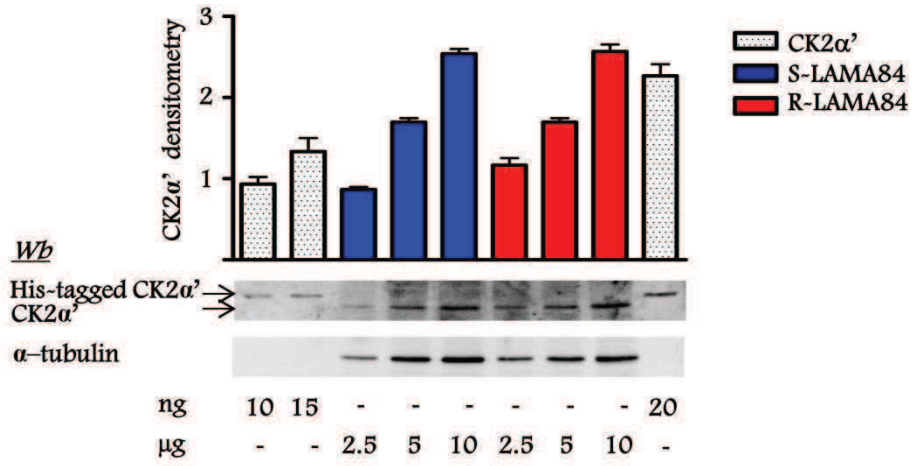


Figure 2. Relative quantification of CK2α' subunit in LAMA84 cells. *The indicated quantities of recombinant CK2 α' and of lysate proteins from S-LAMA84 and R-LAMA84 cells were analysed by wb with the indicated antibodies. α-tubulin is shown as a loading control. Means of densitometric values ± SD, expressed in arbitrary units, are reported above the relative subunit bands. Cellular CK2α' amount was calculated by densitometric analysis and extrapolation from the calibration curve built on the signal of His-tagged recombinant CK2.*

Subcellular distribution of protein kinase CK2 in LAMA84 cells

Since subcellular localization of a protein often suggests clues to its functions, we analysed the CK2 distribution in the subcellular compartments of LAMA84 cell lines (Fig.3). The comparison of CK2 subcellular localization in the two cell variants revealed that the amount of CK2α' is similar in the different subcellular compartments of S-LAMA84 and R-LAMA84 cells. In contrast, while CK2α level is comparable in nuclei and almost undetectable in mitochondria, it is overexpressed in the cytosolic and microsomal fractions of R-LAMA84 cells. Likewise, the protein-level of CK2ß is consistently higher in cytosol and microsomes.

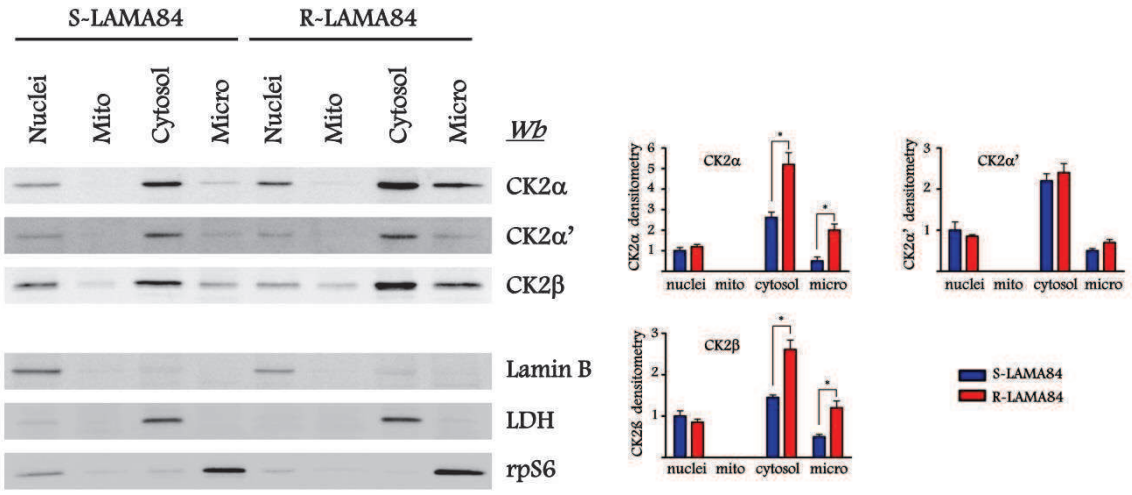


Figure 3. Analysis of the subcellular distribution of CK2 in LAMA84 cells. *The subcellular compartments of LAMA84 cells were separated as detailed in Material and Methods. The same volume of the different fractions (the µl containing 10 µg of cytosol) was immunoblotted with the indicated antibodies. The pureness of nuclei, cytosol and microsomes was verified using the specific organelle-marker antibodies lamin B, lactate dehydrogenase (LDH) and ribosomal protein S6 (rpS6), respectively. The Figure is representative of four separated experiments. Bars report the mean values ± SD of the densitometric analysis of the CK2-subunit bands obtained by wb analysis. Densitometric values are expressed in arbitrary units. *p<0.05.*

To assess whether a correlation occurs between protein-level and activation state of CK2 in R-LAMA84, we analysed the activity displayed by the kinase catalytic subunit α in the different subcellular fractions. To this purpose, the same volumes of each compartments were run on SDS-PAGE containing the CK2 substrate ß-casein and the activity of the α-subunit toward the co-localized substrate was determined by a radioactive in-gel kinase assay. Using this method the catalytic subunit α' is not detectable. In agreement with the different CK2α subcellular distribution, while ^{33}P-phosphorylation of ß-casein is similar in the nuclei, it results two-fold higher in the cytosol and microsomes of imatinib-resistant cells as compared to sensitive cells (Fig.4).

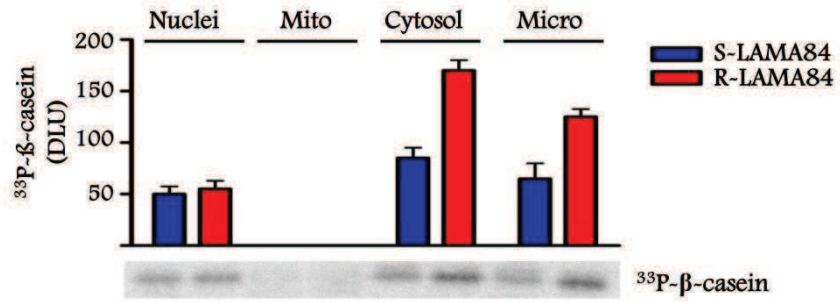
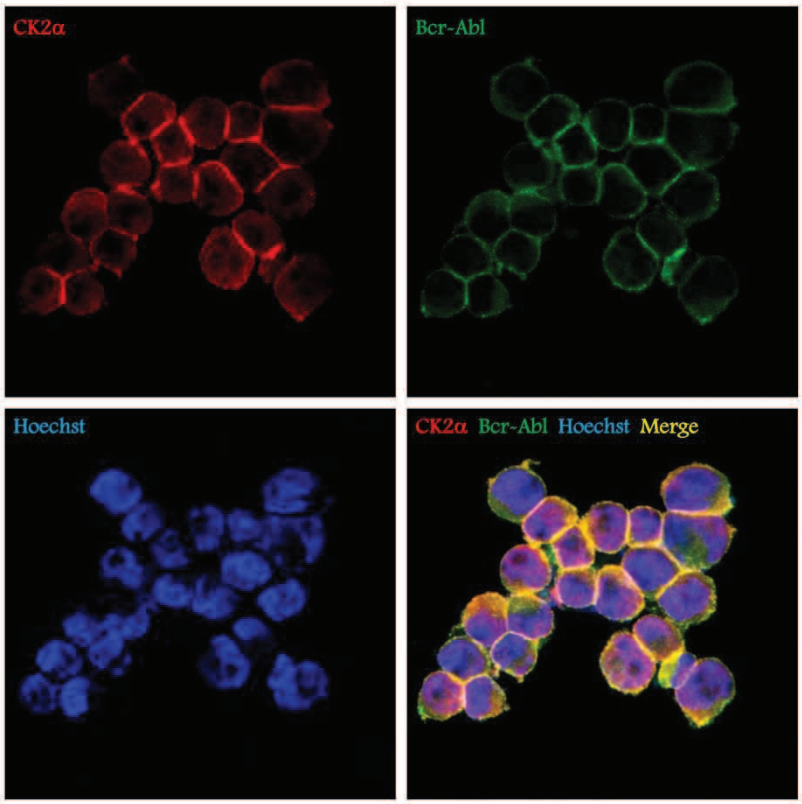


Figure 4. Analysis of the kinase activity of CK2α subunit in the subcellular fractions of LAMA84 cells. *The same volume of the different subcellular compartments of S- and R-LAMA84 cells were loaded on polyacrylamide gel containing the CK2-substrate ß-casein and the activity of the catalytic CK2α subunit was analysed as detailed in Materials and Methods. ^{33}P-phosphorylation of ß-casein was evidenced by Cyclone Plus Storage PhosphorSystem and expressed as Digital Light Units (DLU). Reported values are means ± SD of three separated experiments.*

CK2 and Bcr-Abl co-localize in imatinib-resistant LAMA84 cells

We have demonstrated that, in imatinib-resistant LAMA84 cells, CK2 is overexpressed in the cytoplasm (cytosol and microsomes), the compartment where Bcr-Abl is also retained in chronic myeloid leukemia cells and where it interacts with most proteins involved in its oncogenic pathway [Cilloni D. and Saglio G., 2012]. This observation led us to analyse whether the two kinases, which are overexpressed in resistant cells, could co-localize. To this purpose, we performed confocal microscopy immunofluorescence experiments. Fig.5 shows that LAMA84 cells present a circular shape and are characterized by a tight cytoplasm that is compressed between the big nucleus and the plasma membrane. CK2α red fluorescence is observable in the nucleus but it is mostly localized in the cytoplasm, where Bcr-Abl is exclusively visible and appears to co-localize with CK2 (Figs.5A,5B).

A



B

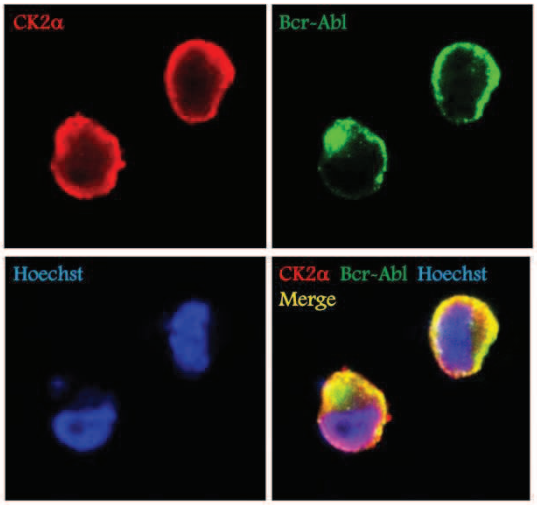


Figure 5. Immunolocalization of CK2 and Bcr-Abl by confocal microscopy. *(A,B) Confocal microscopy analysis of double immunofluorescence staining of R-LAMA84 cells with CK2α (red) and Bcr-Abl (green) antibodies. Nuclei were stained with Hoechst 33342 in blue. Merging of red and green fluorescence is visualized in yellow.*

Immunolocalization analysis performed in parallel with sensitive LAMA84 cells highlighted a similar distribution of CK2α fluorescence, which is more evident in the cytoplasm (data not shown), whereas Bcr-Abl localization was unfeasible because the oncokinase fluorescence was not detectable.

CK2 and Bcr-Abl are members of the same multi-protein complex(es) and co-immunoprecipitate in R-LAMA84 cells

CK2 and Bcr-Abl co-localization prompted us assess whether the two protein kinases are interacting proteins, a finding that has been previously evidenced in cells overexpressing the two kinases and in lymphoblastic cells obtained from Bcr-Abl transgenic mouse [Mishra S. *et al.*, 2003]. To this purpose, LAMA84 cells were lysed under mild conditions to preserve the multi-molecular complex(es) that were separated by ultracentrifugation on glycerol gradient (Fig.6). We found that in sensitive cells Bcr-Abl is distributed in a main peak (fractions 8-11), which is partially superimposed to that of CK2 (fractions 7-13) (Fig.6A). However, the two kinases are not interacting as demonstrated by CK2α-immunoprecipitation experiments performed using the pooled fractions 8-11 of the gradient (right panel of Figs.6A,6B). At variance, in imatinib-resistant LAMA84 cells, Bcr-Abl and CK2 co-migrate in several fractions (Fig.6B) and are members of the same complex(es) as highlighted by their co-immunoprecipitation assayed in the gradient fractions 8-11 (right panel of Figs.6A,6B). This finding suggests an involvement of CK2 in the molecular machinery that reinforces the imatinib-resistance.

To further analyse the CK2/Bcr-Abl interaction and to evaluate the role played by the activity of each kinase on the reciprocal binding, imatinib-resistant LAMA84 cells were treated with imatinib or CX-4945, a potent CK2-inhibitor currently in clinical trials. Cellular extracts were then subjected to glycerol gradient sedimentation. Cell treatment with imatinib does not change the sedimentation profile of both CK2 and Bcr-Abl (Fig.6C) or their binding as demonstrated by the relative co-immunoprecipitation experiments (left panel of Fig.6C). Interestingly, while CK2-specific inhibitor, CX-4945, does not significantly affect the distribution profile of Bcr-Abl along the gradient, it triggers the shift of CK2α towards fractions of lower molecular weights (Fig.6D), implying that CK2 dissociates from Bcr-Abl, as judged by the reduced immunoprecipitation of the two kinases (left panel of Fig.6D). These findings corroborate the hypothesis that CK2 catalytic activity is crucial for the

interaction between the two kinases as suggested by the disruption of the complex induced by CK2 inhibition. However, ongoing studies are necessary to understand whether the binding between the two kinases is direct or mediated by adaptor proteins.

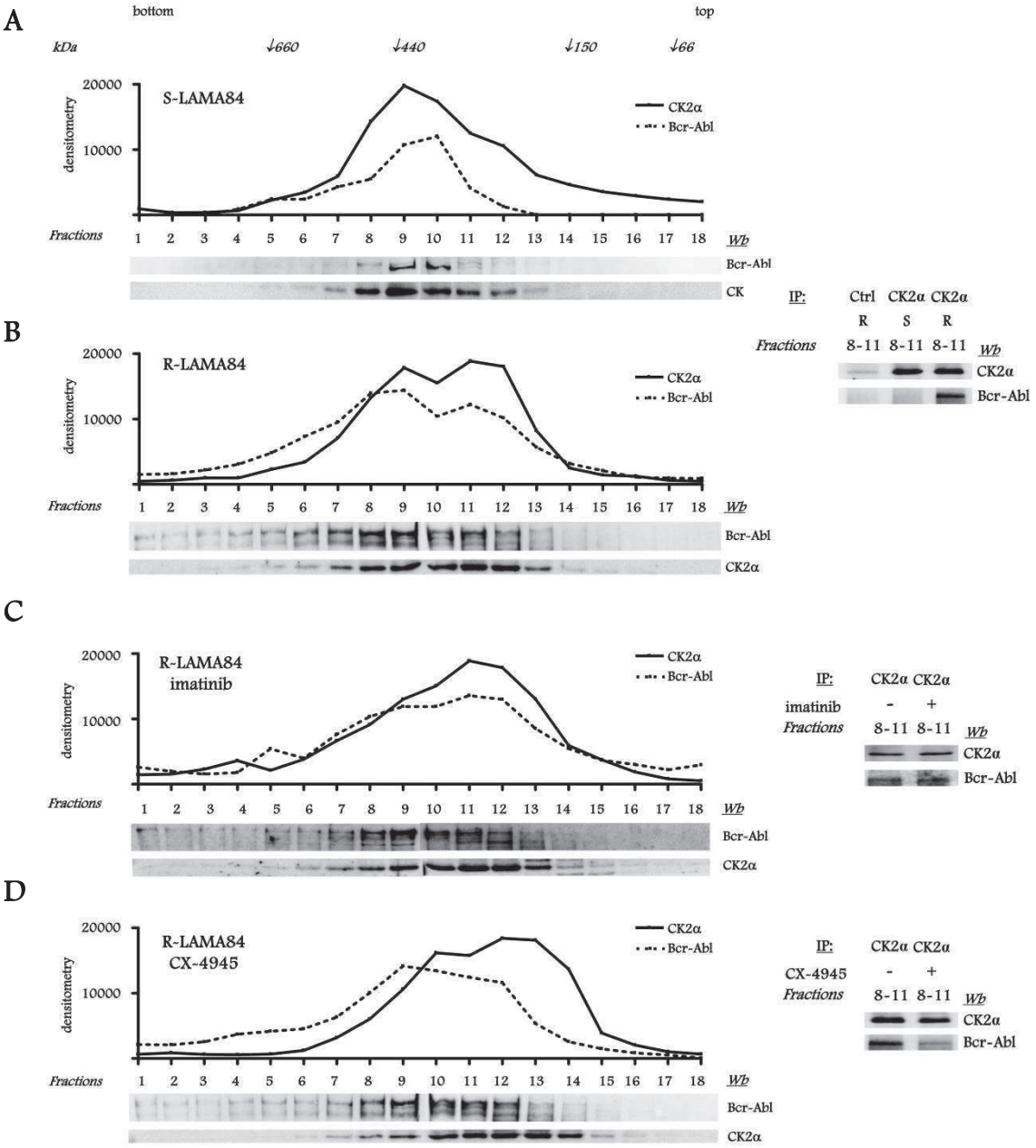


Figure 6. Analysis of CK2 and Bcr-Abl interaction in LAMA84 cells. *S-LAMA84 (A) and R-LAMA84 (B-D) cells were treated for 24h with vehicle or 5 μM CX-4945 (C) or 3 μM imatinib (D) for 24h. 400 μg of a mild cell extracts of cells were loaded on a linear glycerol gradient, as detailed in Materials and Methods, and 40 μl of each fraction were immunoblotted with the indicated antibodies. Bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa) were run on separated tubes as molecular weight standards. The densitometric analysis of the bands is reported above the relative gradient. (Right panels) Fractions 8-11 of each gradient were pooled, and 100μl were immunoprecipitated with an aspecific antibody (Ctrl) or anti-CK2α and analysed by wb with the indicated antibodies. Figure is representative of four different experiments.*

Effect of CK2-inhibition on LAMA84 CML cell viability

As previously demonstrated by my research group, CX-4945 is able to reduce the cell viability of different types of cancer cells including LAMA84 CML cell lines with DC_{50} (concentration inducing the 50% of cell death) values of about 8 μM and 5 μM in imatinib-sensitive and -resistant cells, respectively [Borgo C. *et al.*, 2013]. In parallel, the DC_{50} values calculated for imatinib are about 0.3 and 2.1 μM in sensitive and resistant cell lines, respectively [Borgo C. *et al.*, 2013]. These results prompted us to verify whether the cell death induced by the two inhibitors was mediated by apoptosis induction. Apoptosis occurrence was therefore analysed by comparing the cleavage of the caspase substrate PARP in the two cell variants. As expected, PARP is almost completely cleaved by treatment with 0.5 μM imatinib in sensitive LAMA84 cells, an event paralleled by the proteolysis of Bcr-Abl and α-tubulin (Fig.7A). On the contrary, as expected, treatment with up to 1 μM imatinib does not induce any appreciable effect in resistant cells (Fig.7A). The opposite is observable with CX-4945 which is not effective up to 5 μM concentration in S-LAMA84 cells, while the same concentration of inhibitor induces an almost complete cleavage of PARP and of the other analysed proteins in R-LAMA84 cells (Fig.7B). This outcome supports the hypothesis that imatinib-resistant cells are more dependent on CK2 activity for their survival than sensitive cells.

Apoptosis occurrence induced by CK2 in imatinib-resistant LAMA84 cells prompted us to examine whether CX-4945 might sensitize resistant-cells to imatinib. To this purpose, cells were treated with CX-4945 and imatinib either separately or in combination (Fig.8). We then examined if the combined treatment induced a higher degree of cell death compared to the separate treatments. Interestingly, low concentrations of CX-4945 are able to substantially increase the effect of imatinib on resistant LAMA84 cells (Fig.8). The value of the combination index, which denotes synergism if <1 [Chou T.C., 2006], is 0.57 for R-LAMA84, demonstrating that the combined treatment promotes a synergistic reduction of cell viability, partially rescuing the response to imatinib.

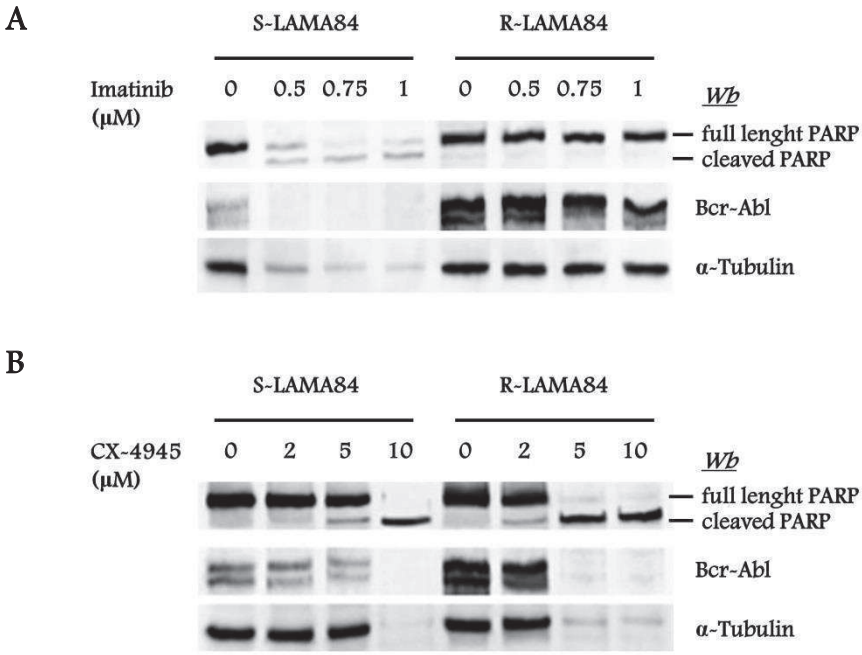


Figure 7. Cell death induction by imatinib and CX-4945 in LAMA84 cells. *S-LAMA84 and R-LAMA84 cells were treated with the indicated concentration of imatinib (A) or CX-4945 (B) for 48h. Lysate proteins (30 μg) were analysed by wb with the indicated antibodies. Figure is representative of five separate experiments.*

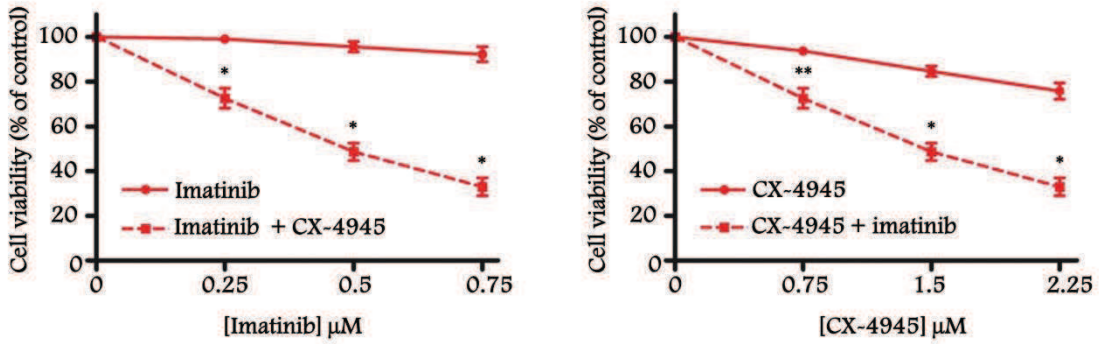


Figure 8. Synergistic effect of CX-4945 and imatinib treatment on R-LAMA84 cell viability. *Cell viability was analysed by the MTT method after 48h treatment with increasing concentration of CX-4945 and imatinib administrated alone or in combination at the fixed ratio 1:3. Viability was expressed as percentage of controls and the combination index was calculated with the software Calcusyn.*

The above described results are part of the data published in:

Borgo C., Cesaro L., Salizzato V., Ruzzene M., Massimino M.L., Pinna LA., Donella-Deana A. (2013) Aberrant signalling by protein kinase CK2 in imatinib-resistant chronic myeloid leukaemia cells. Biochemical evidence and therapeutic perspectives. *Mol.Onc.7(6):1103-15.*

CML KCL22 cells

In the first part of my thesis we have demonstrated that in imatinib-resistant LAMA84 cells CK2 is up-regulated as compared to imatinib-sensitive cells and that it co-operates with Bcr-Abl to maintain CML phenotype. We then investigated also in CML KCL22 cells the potential occurrence of a CK2 involvement in imatinib-resistance and of a cross-talk with Bcr-Abl.
Aim of my work was to gain insights into unknown mechanisms of drug-resistance related to CK2.

Analysis of Bcr-Abl and CK2 expression in KCL22 cell line

In KCL22 cells, the resistance to imatinib is not caused by *BCR-ABL1* gene amplification, mutations in the Bcr-Abl kinase domain [Le Coutre P. *et al.*, 2000; Redaelli S. *et al.*, 2010] or by expression of the efflux drug transporter P-glycoprotein [Le Coutre P. *et al.*, 2000; Zanin S. *et al.*, 2012].
First of all, we probed the protein levels and the activities of the two kinases under investigation, Bcr-Abl and CK2, in imatinib-sensitive and -resistant cells. Western blot analysis of equal amounts of proteins obtained from S- and R-KCL22 cell lysates showed that similar protein level of Bcr-Abl is present in parental and imatinib-resistant cell variants (Fig.9). We then analysed the extent of Bcr-Abl autophosphorylation, which is indicative of the kinase activation state. Fig.9 shows that the protein is constitutively active and similarly autophosphorylated in the two cell variants as also corroborated by the similar phosphorylation of the key Bcr-Abl substrate CrkL [De Jong R. *et al.,* 1997].

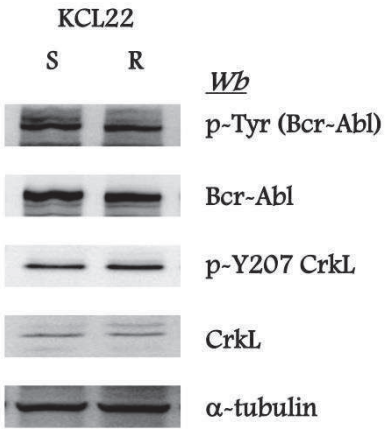


Figure 9. Analysis of Bcr-Abl activation in KCL22 cells. *30 μg of proteins from cellular lysates obtained from sensitive and resistant-KCL22 cells were analysed by wb with the indicated antibodies. α-tubulin was used as a loading control. Figure is representative of four separated experiments.*

CK2 expression was later examined by western blot analysis of lysate proteins obtained from KCL22 cells using antibodies raised towards the kinase catalytic (α and α') and regulatory (ß) subunits. CK2 subunits resulted similarly expressed in both cell variants (Fig.10A). In parallel, the catalytic activity of CK2 was tested in equal amounts of KCL22 cell lysates by *in vitro* kinase assays using the CK2 specific peptide-substrate $R_3AD_2SD_5$. Consistent with the protein level, also the cellular CK2 activity is not significantly different in sensitive and resistant cells (Fig.10B).

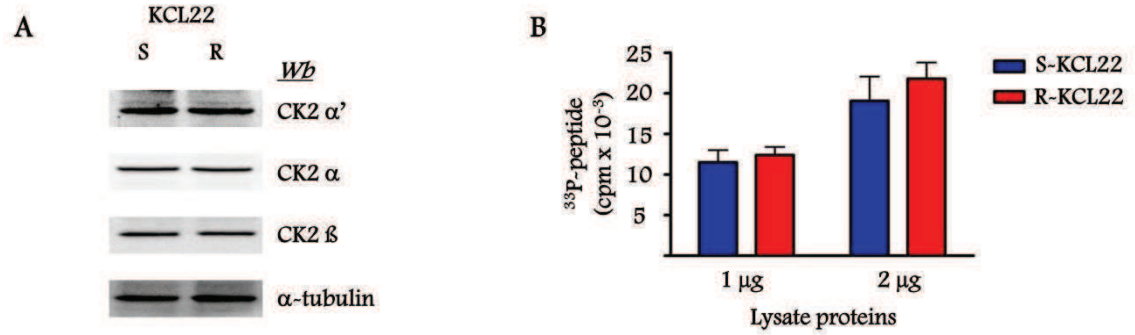


Figure 10. Analysis of protein level and activity of CK2 in KCL22 cells. *S- and R-KCL22 cells were lysed. (A) 10 μg of lysate proteins were analysed by wb with the indicated antibodies. α-tubulin was used as a loading control. Figure is representative of four separated experiments. (B) 1 or 2 μg of proteins from cell lysates were incubated with a CK2 specific peptide-substrate in a phosphorylation mixture containing [γ^{33}P]ATP as detailed in Materials and Methods. Reported values are means ± SD of at least three separated experiments.*

Bcr-Abl and CK2: are they interacting proteins?

In the first section of the results, we have demonstrated that the two kinases are interacting proteins only in imatinib-resistant LAMA84 cells. Therefore, we performed a similar analysis in KCL22 cells, which were treated with vehicle, imatinib or CX-4945, lysed and immunoprecipitated with anti-Abl antibody. Fig.11 shows that, differently from LAMA84 cells, a substantial interaction between Bcr-Abl and CK2 is detectable in both imatinib-sensitive and -resistant KCL22 cells. Interestingly, while imatinib does not affect the protein binding, CX-4945 greatly counteracts the interaction between the two kinases, as already shown in R-LAMA cells (Fig.6). This finding confirms that CK2 catalytic activity is involved in the CK2/Bcr-Abl association.

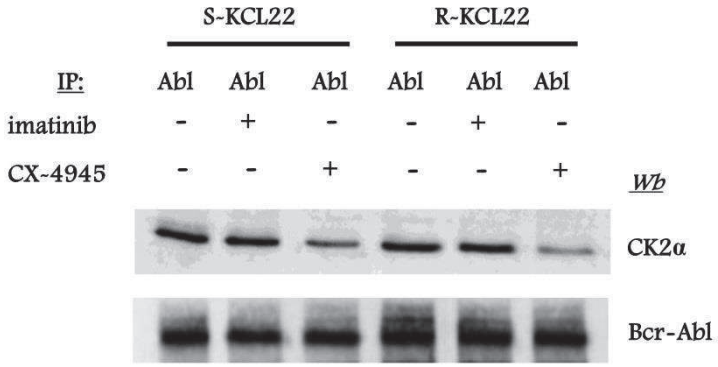


Figure 11. Analysis of CK2 and Bcr-Abl interaction in KCL22 cells. *S- and R-KCL22 cells were treated for 16h with vehicle, imatinib or CX-4945. 300 μg of lysate proteins were immunoprecipitated with anti-Abl antibody. The immunocomplexes were then analysed by wb with anti-CK2α and anti-Abl antibodies. Figure is representative of three separated experiments.*

Analysis of the potential signaling pathways deregulated in imatinib-resistant KCL22 cells

It has been described that imatinib-resistance may be originated from the constitutive and Bcr-Abl independent activation of survival pathways, which are also under the control of Bcr-Abl (see scheme of Fig.12). To identify additional targets involved in imatinib-resistance, we compared both expression and phosphorylation state of specific proteins in imatinib-resistant and

-sensitive KCL22 cells. The analysis of the tyrosine kinase Lyn, whose up-regulation has been described to be associated with imatinib-resistance in CML K562 cells [Ptasznik *et al.,* 2004], showed that the expression and the activity of this Src-kinase is similar in both variants of KCL22 cells (data not shown). Particular attention was paid to MAPK and PI3K/Akt/mTOR pathways, which are under the control of Bcr-Abl, related to CK2 and frequently demonstrated to be up-regulated in cancer cells [Britten C.D. 2013; Saini K.S. *et al.*, 2013].

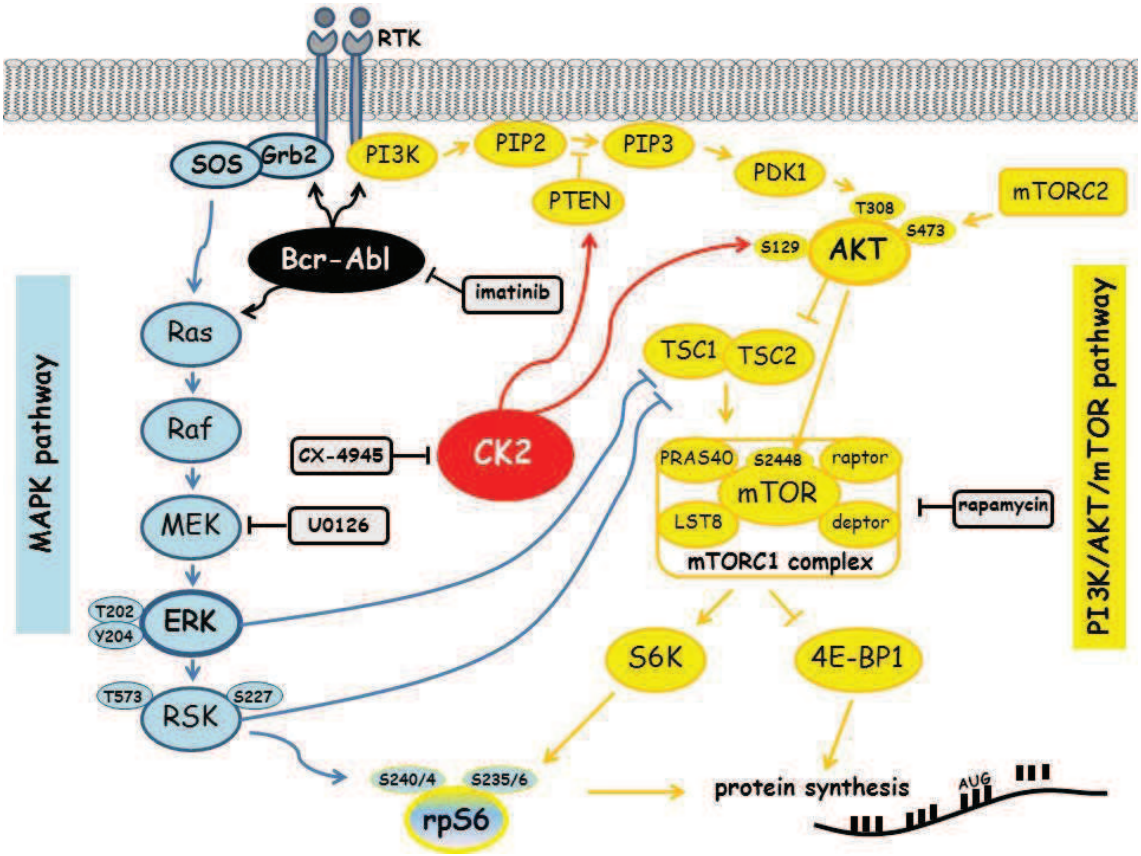


Figure 12. BCR-ABL1 signaling cascade*. Bcr-Abl induces activation of different signaling pathways, including MAPK and PI3K/Akt/mTOR cascades. rpS6 represents a downstream target of this two pathways.*

KCL22 cell lines were therefore lysed and probed with specific antibodies. We observed that whereas the level of total ERK1/2 is similar in R-KCL22 and S-KCL22 cells, imatinib-resistant KCL22 cells are characterized by a strikingly higher phosphorylation extent of ERK1/2 at the residues T202/Y204 (Fig.13), in agreement with results reported by Colavita *et al.* (2010). The finding that, the phosphorylation level of the ERK-targets RSK T573 and p53 S15 are similar

in imatinib-sensitive and -resistant cells (Fig.13), suggests that other proteins among the numerous ERK-substrates might be affected by the anomalous ERK hyper-activation induced by imatinib-resistance.

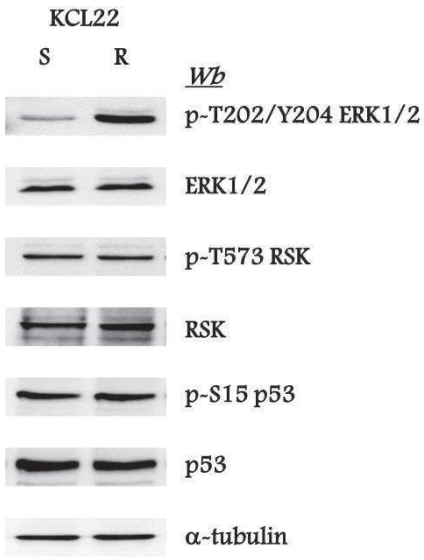


Figure 13. Analysis of the phosphorylation extent of MAPK/ERK signaling proteins. *20 μg of lysate proteins obtained from sensitive and resistant-KCL22 were analysed by wb with the indicated antibodies. α-tubulin was used as a loading control. Figure is representative of four separated experiments.*

As far as the PI3K/Akt/mTOR pathway is concerned, it is well known that Akt becomes active following the phosphorylation of T308 and S473, catalyzed by PDK1 and mTORC2, respectively. Akt can be further stimulated by the CK2-mediated phosphorylation of S129, which prevents the de-phosphorylation of Akt T308 maintaining the kinase in its active conformation. Our analysis of Akt at S473 revealed that this activatory residue is highly phosphorylated in R-KCL22 as compared to S-KCL22 cells (Fig.14A). Consistently, a great enhancement of the Akt-catalyzed phosphorylation of mTOR S2448 and a substantial increase of the Akt-target GSK3ß S9 is observed, revealing that the activity of this pro-survival kinase is highly up-regulated in resistant cells. To further investigate the Akt activation state, we examined mTORC2 and PHPPL2, the kinase and the phosphatase, respectively, responsible for the phosphorylation and de-phosphorylation of the Akt residue S473. Fig.14A shows that both the activation state of mTORC2, as judged by the phosphorylation of its substrate SGK1 at S422, and the protein level of PHPPL2 are similar in S- and R-KCL22 cells. Further experiments are needed to assess

whether the phosphatase activity of PHPPL2 might be different in the two cell variants or whether other factors are responsible for the striking phosphorylation of Akt S473 in imatinib-resistant cells. In contrast with Akt S473, the phosphorylation extent of Akt T308 is similar in the two different variants, consistent with the similar auto-phosphorylation and activation state of PDK1, the kinase responsible for this site phosphorylation (Fig.14B). In agreement with the comparable CK2 protein expression, the CK2-catalyzed phosphorylation of Akt S129 and of PTEN S370 is similar in sensitive and resistant cells (Fig.14B).

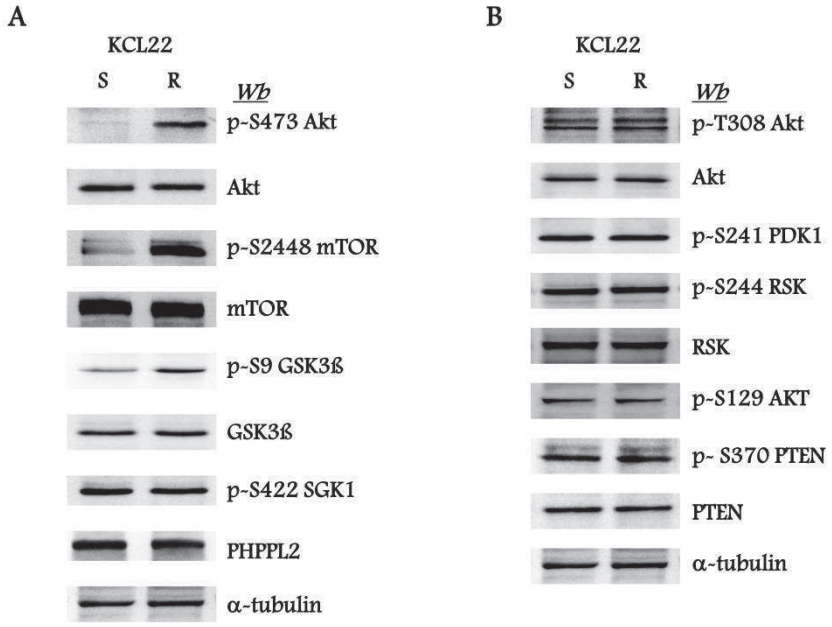


Figure 14. Analysis of the phosphorylation state of PI3K/Akt/mTOR signaling proteins. *(A,B) 20 μg of proteins from cellular lysates obtained from sensitive and resistant-KCL22 were analysed by wb with the indicated antibodies. α-tubulin was used as a control. Figure is representative of four separated experiments.*

Finally, we analysed the phosphorylation extent of S235/236 and S240/244 of the 40S ribosomal protein S6 (rpS6), a downstream effector of both MAPK/ERK and PI3K/Akt/mTOR signaling cascades. Interestingly, all the four phosphorylation residues of rpS6 appeared substantially more phosphorylated in R-KCL22 cells as compared to the parental cell line (Fig.15). At variance, the eukaryotic translational initiation factor 4E binding protein 1 (4E-BP1), another

PI3K/Akt/mTOR downstream effector, is similarly phosphorylated in the two cell variants (Fig.15).

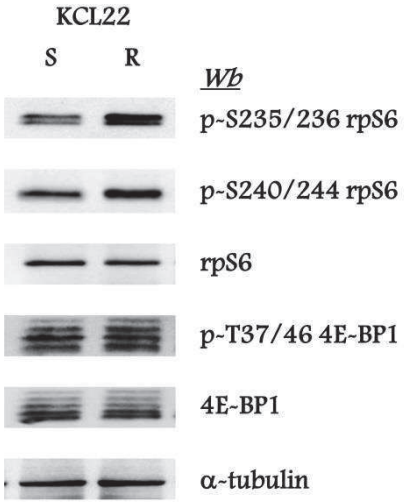


Figure 15. Phosphorylation analysis of rpS6 and 4E-BP1. *20 μg of proteins from cellular lysates obtained from sensitive and resistant-KCL22 were analysed by wb with the indicated antibodies. α-tubulin was used as a control. Figure is representative of four separated experiments.*

In summary, our western blot analyses, performed with phospho-specific antibodies, revealed that different key proteins become hyper-phosphorylated in resistant KCL22 cells, demonstrating that the imatinib-resistance is associated with an anomalous up-regulation of ERK1/2, Akt at S473 and rpS6. Differently from KCL22 cells, it is interesting to mention that in imatinib-resistant LAMA84 cells Akt is hyper-phosphorylated at S473, while ERK1/2 and rpS6 are not up-regulated in comparison with the sensitive counterpart (data not shown).

CK2 down-regulation: a strategy to counteract imatinib-resistance ?

Although protein kinase CK2 is not overexpressed in resistant KCL22 cells, as previously shown in resistant-LAMA84 cells, it is well known that: *i)* cancer cells are addicted to CK2 [Ruzzene M. and Pinna L.A., 2010], *ii)* CK2 acts as a cancer driver by creating a favorable environment to cancer progression [Ruzzene M. and Pinna L.A., 2010] *iii)* CK2 is involved in different kinds of drug-resistance [Di Maira G. *et al.*, 2008; Matsumoto Y. *et al.*, 2001]. To assess

whether CK2 might be involved in the Bcr-Abl-independent mechanisms of resistance induced by imatinib-treatment, R-KCL22 cells were treated with the CK2-specific inhibitor, CX-4945. In parallel, the effects of CK2-inhibition on Bcr-Abl signaling cascade were compared with those caused by other compounds that are known to counteract the activity of the proteins that we found to be anomalously up-regulated in imatinib-resistant KCL22 cells.

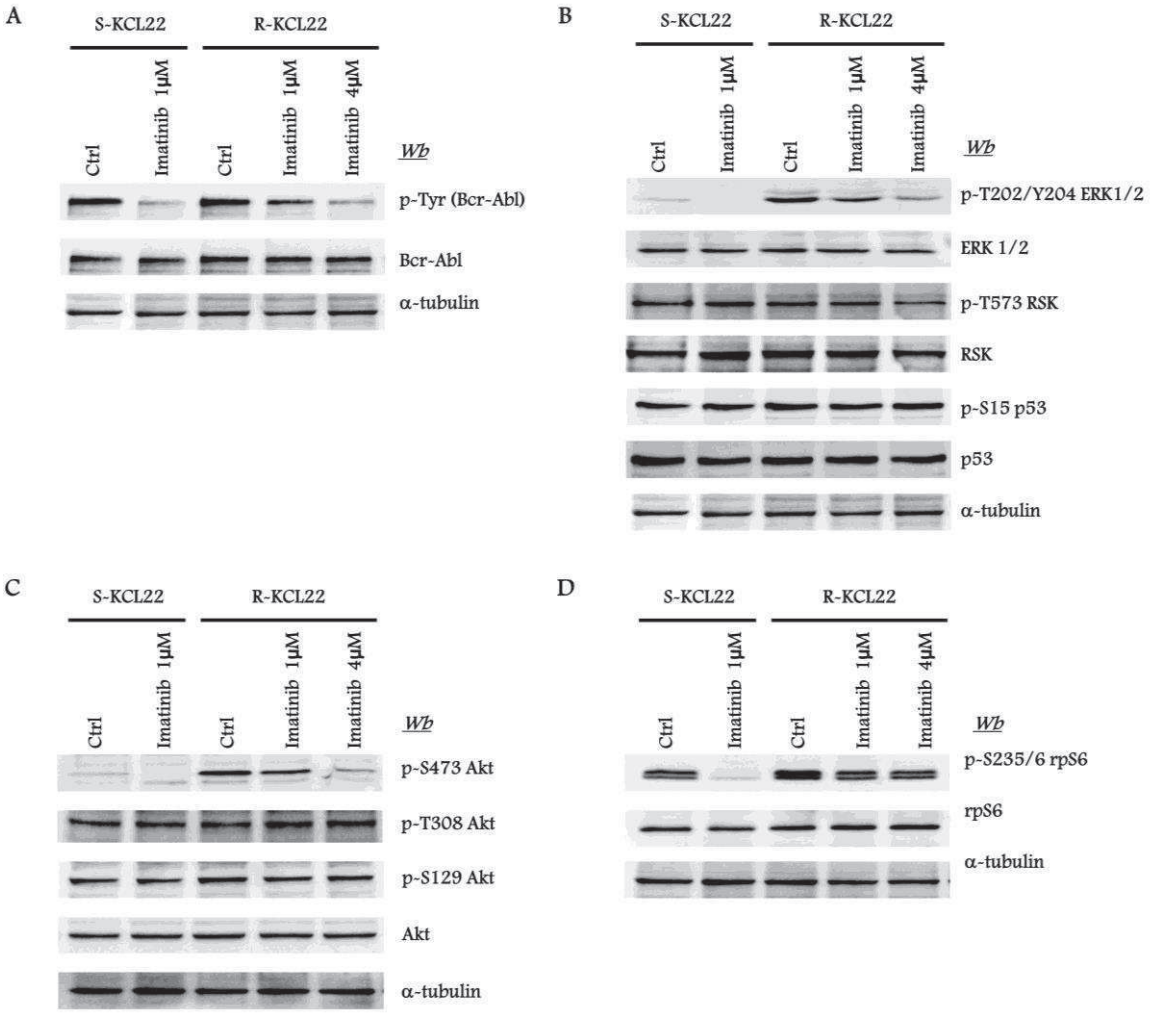


Figure 16. Effects of imatinib-treatment on Bcr-Abl signaling cascade in KCL22 cells imatinib-sensitive and -resistant. *KCL22 cells were treated for 4 hours with vehicle (DMSO) or imatinib at the indicated concentrations. Cell were lysed and 20μg of lysate proteins were analysed by wb with the indicated antibodies. α-tubulin was used as a loading control. Figure is representative of three separated experiments.*

We first compared the effect of imatinib in S- and R-KCL22 cells. In sensitive cells the treatment with 1 μM imatinib is sufficient to greatly counteract Bcr-Abl auto-phosphorylation (Fig.16A) and to almost abrogate its downstream

signaling as demonstrated by the inhibition of the phosphorylation of ERK1/2, Akt S473 and rpS6 (Figs.16B,16C and 16D, respectively). As expected, in resistant cells Bcr-Abl activation is strongly inhibited only when cells are treated with high concentration of imatinib (4 μM) (Fig.16A). The same drug concentration causes a substantial inhibition of ERK1/2 activity and of Akt phosphorylation at S473 (Figs.16A,16B). Interestingly, Bcr-Abl inhibition reduces only partially (about 35%) the phosphorylation extent of rpS6 (Fig.16D), the downstream effector of MAPK/ERK and PI3K/Akt/mTOR cascades (see scheme of Fig.12). This finding confirms that in imatinib-resistant cells additional mechanisms, that are Bcr-Abl independent, sustain cell survival and strengthen the CML resistant phenotype. Therefore, resistant cells were treated with the following inhibitors: 4 μM CX-4945 (CK2-inhibitor), 1 μM imatinib (Bcr-Abl inhibitor), 10 μM U0126 (MEK-inhibitor) or 20 nM rapamycin (mTORC1-inhibitor) (Fig.17). As already shown in Fig.16D, imatinib-treatment only slightly affects rpS6 phosphorylation. In parallel, cell treatment with U0126, which inhibits the ERK1/2 phosphorylation, affects neither Bcr-Abl nor Akt activities counteracting only partially rpS6 phosphorylation (Fig.17). On the contrary, as expected, the potent inhibitor of mTORC1 rapamycin abrogates the phosphorylation of rpS6 (Fig.17) [Jefferies H.B. *et al.,* 1994]. Interestingly, addition of 4 μM CX-4945 which reduces the phosphorylation of the CK2-catalyzed Akt S129, almost abrogates the rpS6 phosphorylation (Fig.17). Notably, CK2-inhibition does not affect the activities of Bcr-Abl, ERK1/2 and Akt as demonstrated by the unaffected phosphorylation extent of Akt regulatory sites S473 and T308, and of the Akt-substrate GSK3ß. These findings indicate that in imatinib-resistant KCL22 cells: *i)* rpS6 phosphorylation is only partially under the control of MAPK/ERK signaling, while it is strongly regulated by PI3K/Akt/mTOR pathway as indicated by the abrogation of rpS6 phosphorylation induced by the mTORC1 inhibitor rapamycin (see scheme of Fig.12); *ii)* the striking inhibition of rpS6 phosphorylation caused by CX-4945, which does not affect Akt activity, demonstrates that CK2 acts on downstream targets of Akt signaling.

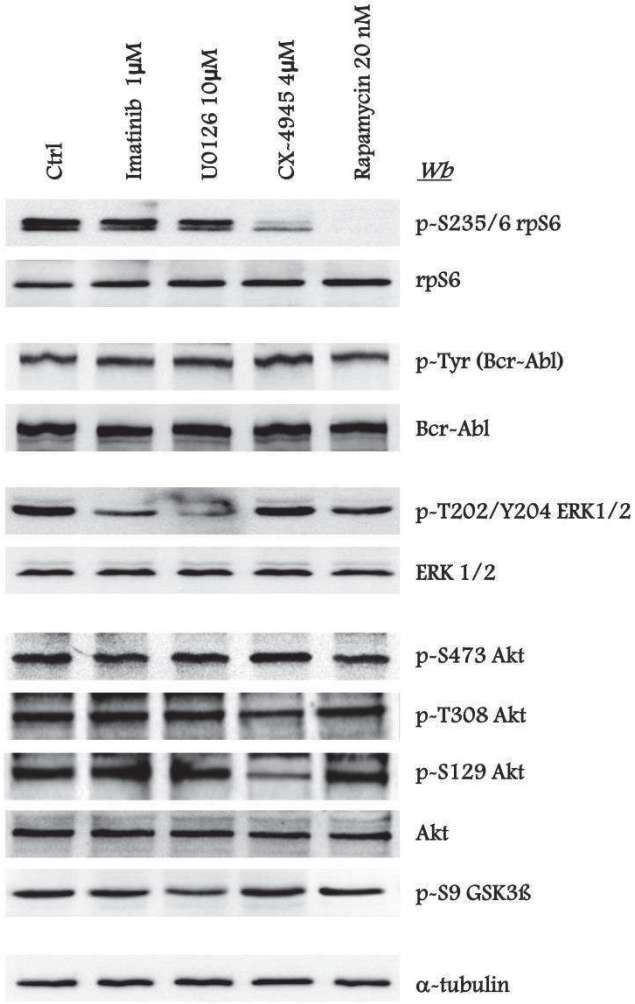


Figure 17. Effects of different inhibitors on Bcr-Abl signaling cascade in resistant-KCL22 cells. *Cells were treated for 4 hours with vehicle (DMSO), imatinib, U0126, CX-4945 or rapamycin at the indicated concentrations, lysed and analysed by wb with the indicated antibodies. α-tubulin was used as control. Figure is representative of three separated experiments.*

Although CX-4945 is highly selective for CK2, to further reinforce the specific role of CK2 in imatinib-resistance of CML KCL22 cells, we performed RNA-interference experiments knocking down the expression of the regulatory ß or the catalytic subunits (α or α') of CK2. Fig.18 shows that, while the decrease of CK2α amount does not affect rpS6 phosphorylation extent, an appreciable reduction of this phosphorylation can be observed when CK2α' or CK2ß are down-regulated confirming the specific involvement of CK2 in the regulation of rpS6 phosphorylation. Moreover, the finding that the down-regulation of CK2ß subunit correlates with a concomitant reduction of the expression of CK2α',

suggests that this specific CK2 catalytic subunit might be involved in events leading to rpS6 phosphorylation.

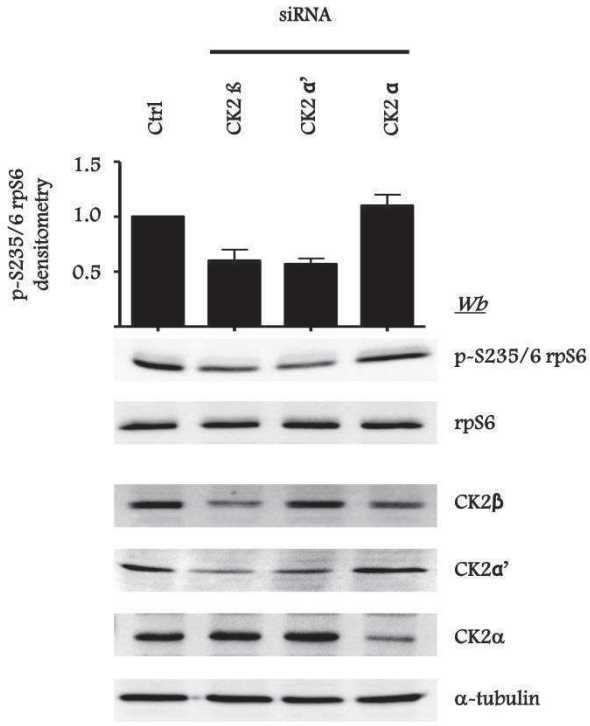


Figure 18. Effect of CK2 knocking down by siRNA on rpS6 phosphorylation extent. *R-KCL22 cells were transfected with aspecific siRNA (Ctrl) or CK2ß, CK2α' or CK2α specific siRNA. After 72 h, cells were lysed and 20μg of proteins were analysed by wb with the indicated antibodies. α-tubulin was used as control. Figure is representative of three separated experiments. Bars report the mean values ± SD of the densitometric analysis of the p-235/6 rpS6 bands obtained by wb analysis. Densitometric values are expressed in arbitrary units.*

To analyse whether rpS6 might be a substrate of CK2 we performed *in vitro* phosphorylation experiments adding recombinant CK2 holoenzyme to rpS6 immunoprecipitates obtained from R-KCL22 cell lysates (data not shown). The finding that rpS6 is not phosphorylated by CK2 *in vitro* suggests that the ribosomal protein is not a direct target of CK2 in cells.

Experiments aimed at elucidating the mechanism(s), by which CK2 is involved in mTOR signaling mediating the rpS6 phosphorylation, highlighted that CK2α and CK2α' co-immunoprecipitate with mTORC1 complex (mTOR and raptor) and p70S6K (Fig.19) (see scheme of Fig.12). Inhibition of CK2 activity by cell treatment with CX-4945 does not affect the binding between these proteins.

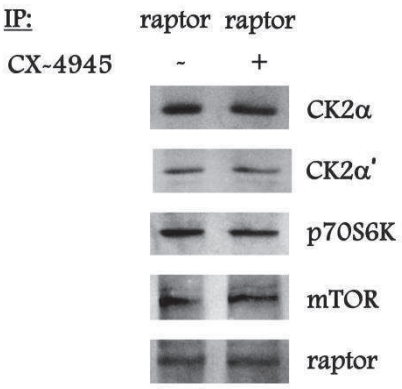


Figure 19. Analysis of CK2 interaction with mTOR signaling proteins*. R-KCL22 were treated with vehicle or 4μM CX-4945 for 3h. 300μg of lysate proteins were immunoprecipitated with anti-CK2α antibody. The immunocomplexes were then analysed by wb with the indicated antibodies. Figure is representative of three separated experiments.*

Since it has been demonstrated that rpS6 phosphorylation is involved in the regulation of translation initiation, the limiting step of protein synthesis [Holz M.K. *et al.*, 2005], we verified whether CK2-inhibition could affect the rate of the global protein synthesis by performing metabolic labeling. Resistant-KCL22 cells were pre-treated with vehicle or CX-4945 for 3h prior to be pulsed in the presence of a radiolabeled methionine and cysteine media mix before quantification of total protein labeling. Interestingly, CK2-inhibition reduces the protein synthesis efficacy of about 50% as compared to control (Fig.20A). In parallel, the presence of CX-4945 in the medium strongly decreases rpS6 phosphorylation, whereas the level of the total rpS6 is not affected (Fig.20B). These results confirm that CK2 is involved in the regulation of rpS6 phosphorylation and highlight a new protein that mediates the effect of CK2 on the protein synthesis.

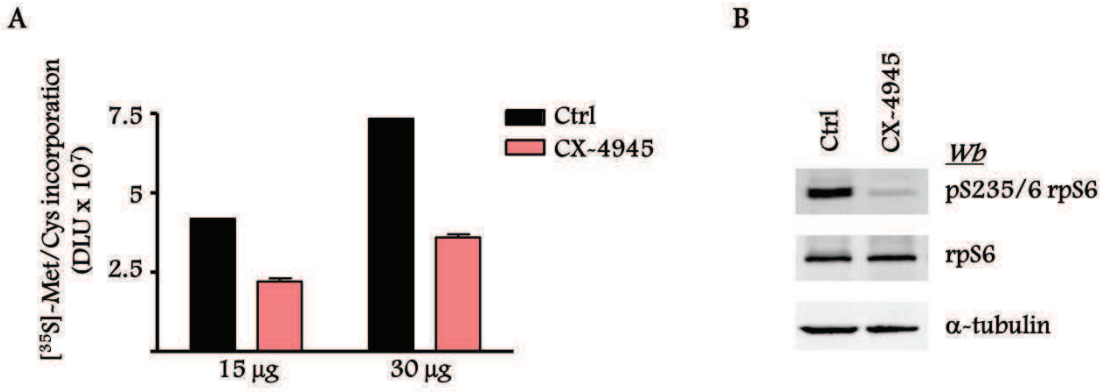


Figure 20. Effect of CK2-inhibition on protein synthesis. *(A,B) R-KCL22 cells were pre-treated with vehicle (Ctrl) or 4 µM CX-4945 for 3h prior to add 100mg/mL cycloheximide for 2h. Cells were then incubated in methionine- and cysteine-free RPMI media supplemented with 100mg/mL cycloheximide for 1h. Media were replaced with radiolabeled RPMI (10 µCi/mL of [35S]-Met/Cys protein labeling mix). After 1h the media were removed and cells were washed with PBS and lysed. Exposure to CX-4945 was maintained throughout the experiment. (A)15µg and 30µg of lysate proteins were subjected to SDS-PAGE, blotted, and the intensity of radioactive bands was measured using the Cyclone Plus Storage PhosphorSystem and expressed as Digital Light Units (DLU). Reported values are means ± SD of four separate experiments. (B) 15µg of lysate proteins were analysed by wb with the indicated antibodies. α- tubulin was used as a loading control.*

Effects of CK2-inhibition on KCL22 cell viability

Since it has been shown that cell treatment with specific CK2-inhibitors causes cell death by apoptosis in different types of tumor cells [Zanin S. *et al.*, 2012; Buontempo F. *et al*, 2013], we examined whether CK2-inhibition causes cytotoxicity in KCL22 cells. The effect of CX-4945 on cell viability was compared in parallel experiments with that caused by imatinib or by U0126 and rapamycin that counteract the pathways under the control of deregulated ERK1/2 and mTOR, respectively. To this purpose, cells were treated for 48h with increasing concentrations of inhibitors and cell viability was tested by the MTT assay. Results showed that imatinib-resistant KCL22 cells are sensitive to high concentrations of imatinib with a DC_{50} value of about 40 µM versus the value of 0.9 µM found with sensitive cells (Fig.21A). Otherwise, CX-4945 decreases significantly the cell viability of both S- and R-KCL22 cell lines with DC_{50} values of 5 µM and 3.8 µM, respectively, showing a slightly more efficacy on R- with respect to S-cells (Fig.21B). The treatment with U0126 is similarly effective toward S- and R-KCL22 cells with DC_{50} of about 15 µM (Fig.21C),

while the DC_{50} values for rapamycin are 130 nM and 90 nM in S- and R-cells, respectively (Fig.21D).

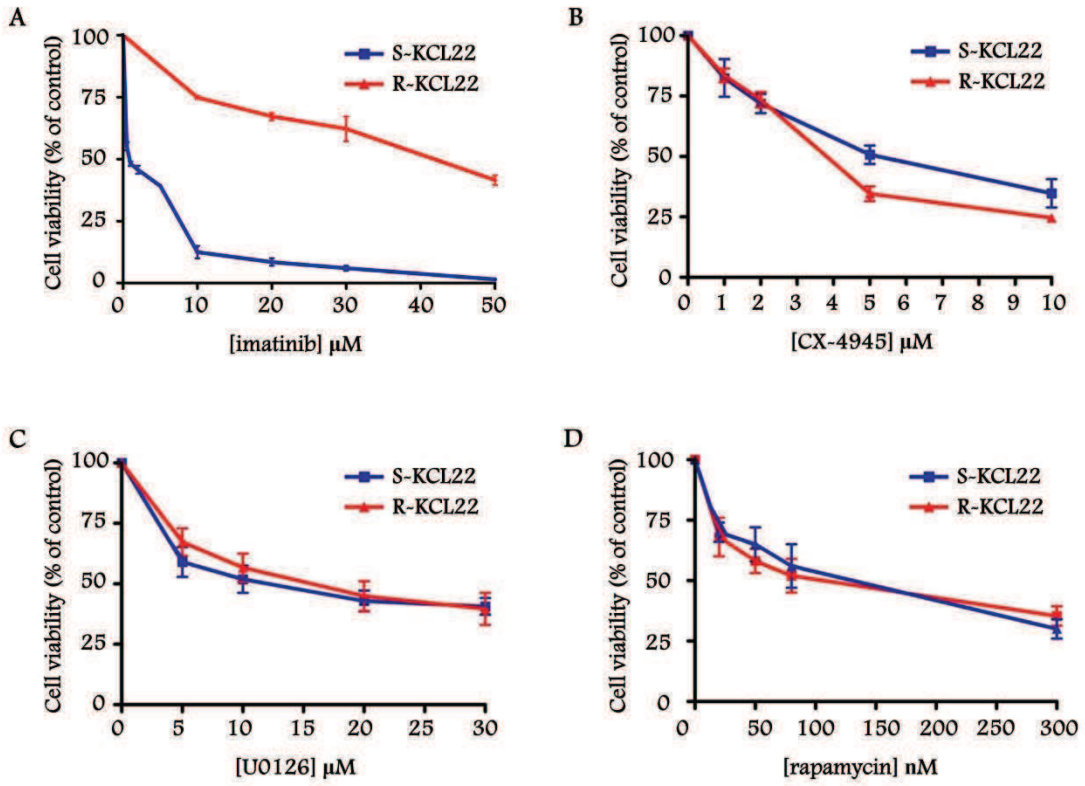


Figure 21. Effect of imatinib, CX-4945, U0126 and rapamycin on KCL22 cell viability. *KCL22 cell viability was analysed by the MTT method, and expressed as percentage of controls, after 48 hours treatment with increasing concentrations of CX-4945, U0126 or imatinib.Viability was expressed as percentage of control and plotted as function of each drug concentration. Mean ± SD values of at least five independent experiments are reported.*

It has been described that inhibitors of the ERK pathway, including U0126, and rapamycin, can be classified as cytostatic but not as cytotoxic anticancer drugs able to "kill" cells by apoptosis [Kohno M. and Pouyssegur J., 2006; Quentmeier *et al.,* 2011]. Therefore, we compared the induction of apoptosis following the treatment of KCL22 cells with CX-4945, imatinib, U0126 and rapamycin. As shown in Fig.22, 1 µM imatinib induces apoptotic cell death in sensitive cells, while, as expected, it is ineffective in imatinib-resistant cells (Fig.22, lane 2). On the contrary, while the treatment with CX-4945 up to 10 µM (Fig.22, lanes 3-5) only partially affects the PARP cleavage in sensitive cells, 2 µM concentration of the CK2-inhibitor is sufficient to induce, in resistant KCL22

cells, a complete cleavage of the protein and a parallel proteolysis of cellular proteins, including α-tubulin. As previously mentioned, the treatment with U0126 and rapamycin does not cause PARP cleavage in KCL22 cells (Fig.22, lanes 6-7). This finding demonstrates that imatinib-resistant cells become partially dependent on CK2 for their survival.

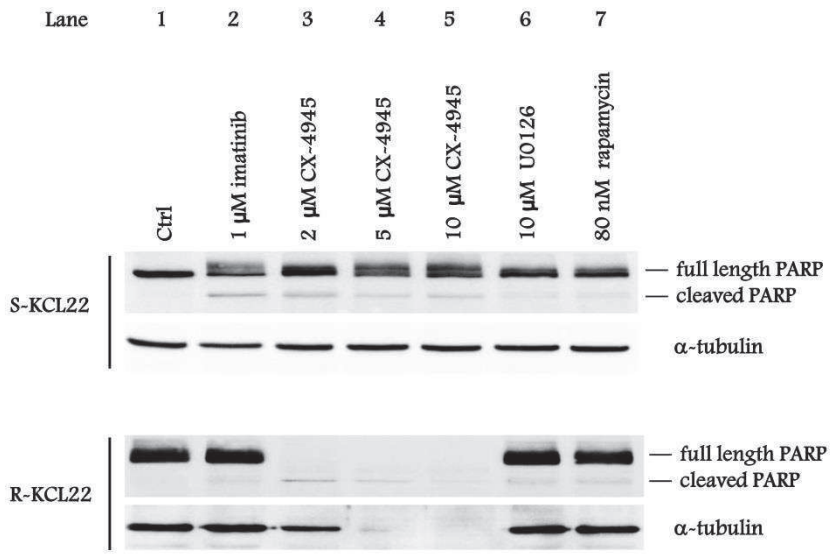
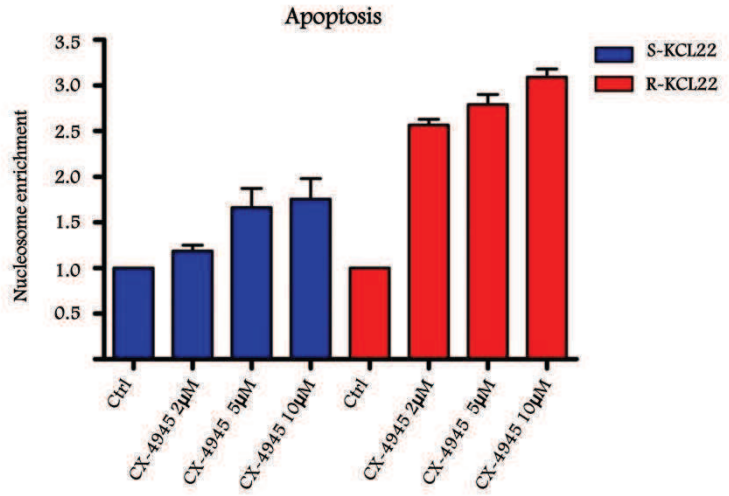


Figure 22. Cell death induction by imatinib, CX-4945, U0126 and rapamycin in KCL22 cells. *10μg proteins of lysates from KCL22 cells treated as indicated for 48 hours were analysed by wb using anti-PARP and anti-α-tubulin antibodies. Figure is representative of three separated experiments.*

To better understand the mechanism of cell death activated by CX-4945, we evaluated the nucleosome formation in both imatinib-sensitive and -resistant cells. Fig.23 shows that the treatment with CX-4945 causes apoptosis in both cell lines (A) while necrosis is not induced (B). Interestingly, the presence of nucleosomes in cell cytosol, which indicates apoptosis, is more pronounced in resistant cells than in the parental cell line (Fig.23A), confirming that resistant cells rely more on CK2 activity for their survival than the sensitive one.

A



B

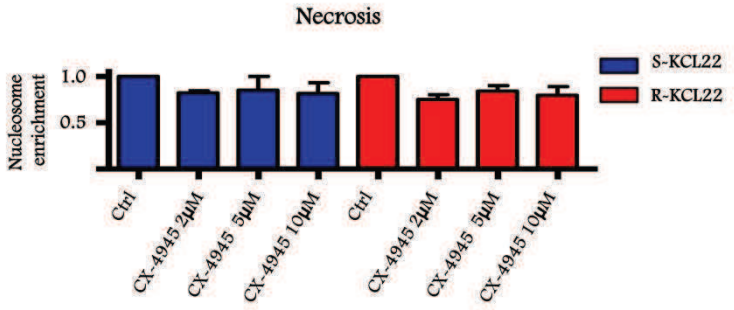


Figure 23. Apoptosis and necrosis induction by CX-4945 in KCL22 cells. *Apoptosis (A) and necrosis (B) were tested using the Cell Death Detection Elisa kit (Roche), following the manufacturer's instruction. Nucleosome enrichment was determined from the ratio between the signals obtained in treated and untreated cells. Reported values are means ± SD of four independent experiments.*

Combined strategies in CML therapy

Since imatinib-resistance CML cells evade the effect of Bcr-Abl inhibitors, alternative approaches are needed to treat this pathology. With respect to this, combined administration of two or more drugs, directed to different oncogenic targets activated by imatinib-resistance, can improve the therapeutic efficacy by means of synergistic effects. Based on the inhibitory effect on the viability of KCL22 cells demonstrated by imatinib, CX-4945, U0126 or rapamycin (Fig.21), we examined whether CX-4945 added in combination with the other agents could induce higher cytotoxicity in comparison with the single treatments.

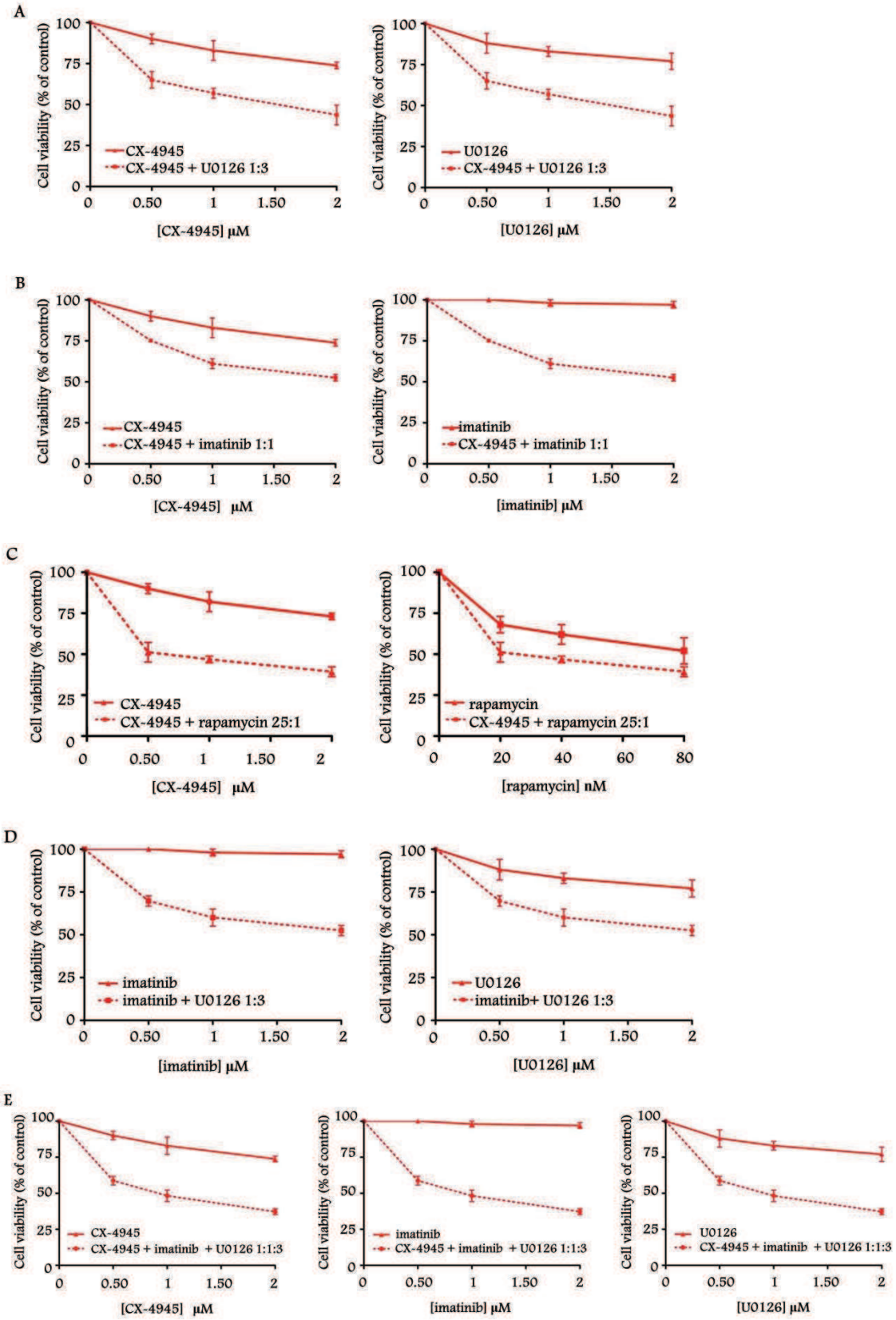


Figure 24. Effect of combined treatments with different drugs. *R-KCL22 viability was assessed by the MTT method after 48h treatment with increasing concentrations of the following drug combinations, used at the indicated fixed ratio: (A) CX-4945 + U0126, 1:3; (B) CX-4945 + imatinib, 1:1; (C) CX-4945 + rapamycin, 25:1; (D) imatinib + UO126, 1:3; (E) CX-4945 + imatinib + U0126, 1:1:3.*
Viability was expressed as percentage of control and plotted as function of each drug concentration. Mean ± SD values of at least five independent experiments are reported.

Combined treatment	C.I. (mean ± SD)
CX-4945 + U0126	0.53 ± 0.02
CX-4945 + imatinib	0.5 ± 0.03
CX-4945 + rapamycin	0.4 ± 0.06
Imatinib + U0126	0.6 ± 0.05
CX-4945 + imatinib + U0126	0.35 ± 0.04

Table 1. Combination index values obtained from the experiments shown in Fig.24.
Combination index < 1, combination index = 1, and combination index > 1 characterize synergism, additivity, and antagonism, respectively [Chou T.C., 2006]. Mean ± SD values of at least five independent experiments are reported.

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Different drug combinations were tested to determine synergism, additivity or antagonism. To this purpose, resistant KCL22 cells were treated for 48 hours with the inhibitors either alone or in binary or ternary association, by increasing simultaneously the concentration of the drugs added at fixed ratios. Cell viability was determined by the MTT method and the viability was plotted as function of the single inhibitor concentration (Fig.24). Intriguingly, all the combined treatments promote a synergistic reduction of cell viability, as judged by the combination index (C.I.), calculated at the 50% of cell lethality, which denotes synergism if < 1 [Chou T.C., 2006] (Table 1). When CX-4945 is used in association with U0126 (Fig.24A), imatinib (Fig.24B) or rapamycin (Fig.24C) a good synergistic effect was observed, with a C.I. value of 0.53, 0.5 and 0.40, respectively. CK2 inhibition causes a significant reduction of the DC_{50} values of imatinib and U0126. The combination of imatinib with U0126 (Fig.24D) showed that also these inhibitors act synergistically with a C.I. value of 0.6, consistent with results reported by Hentschel J. *et al.* (2011). Interestingly, the ternary association of CX-4945 with imatinib and U0126 (Fig.24E) represents the most effective synergistic combination to inhibit the viability of R-KCL22 cells (C.I. = 0.35).

These data suggest that the association of CX-4945 with imatinib and/or inhibitors of MAPK/ERK pathway might be a promising strategy for the treatment of the CML pathology overcoming the drug-resistance.

DISCUSSION

DISCUSSION

It has been recently demonstrated, in our laboratory, that in imatinib-resistant CML LAMA84 cell line, characterized by a *BCR-ABL1* gene amplification [Le Coutre P. *et al.*, 2000], the protein kinase CK2 is up-regulated in comparison with the parental cell line. In particular, while CK2α' is equally expressed, the level of CK2α and CK2ß subunits, and in parallel the CK2 catalytic activity, are about two-fold higher in R-LAMA84 than in sensitive cells. [Borgo C. *et al.*, 2013]. Consistently, in the present study we quantify the protein amount of the different CK2 subunits and demonstrate that in imatinib-resistant cells the level of CK2 is very high and may be considered in the range of structural proteins (Fig.1). With respect to this, it is interesting to mention that high intracellular level of CK2 has been shown to be associated with an environment favourable to cancer progression [Ruzzene M. and Pinna L.A, 2010]. It is noteworthy that CK2 has been found over-represented in highly proliferating myeloblastic cells from CML patients in blast crisis [Phan-Dinh-Tuy F. *et al.*, 1985], a phase in which Bcr-Abl overexpression has been associated with imatinib-resistance [Barnes D.J. *et al.*, 2005; Gorre M.E. *et al.*, 2001; Keeshan K. *et al.,* 2001; Virgili A. and Nacheva E.P., 2010]. Given the pro-survival role of CK2, it is conceivable that its increased level represents a device to escape apoptosis and potentiate the imatinib-resistant cell survival. It has been reported that, while CK2 is generally distributed in various subcellular compartments, its nuclear concentration is particularly high in cancer cells [Trembley J.H. *et al.,* 2009]. In contrast, in S-LAMA84 and R-LAMA84 cells CK2 is mainly present in the cytoplasm (Figs.3,5). We also show that CK2 and Bcr-Abl co-localize in the cytoplasm of R-LAMA84 cells (Fig.5), where Bcr-Abl is exclusively present and interacts with most proteins implicated in the oncogenic pathways [Cilloni D. and Saglio G., 2012] and where the CK2-targets related to imatinib-resistance are presumably placed. CK2 and Bcr-Abl co-localization reflects the finding that, in resistant LAMA84 cells, these two kinases are members of the same multi-protein complex(es) as demonstrated by their co-sedimentation in glycerol-gradients and co-immunoprecipitation (Fig.6B and right panel of

Figs.6A,6B). The occurrence of an interaction between CK2 and Bcr-Abl has been previously described in NHI3T3 fibroblasts overexpressing the two protein kinases and in lymphoblastic cells obtained from Bcr-Abl transgenic mouse [Hériché J.K. and Chambaz E.M., 1998; Mishra S. *et al.*, 2003]. The region responsible for CK2 interaction is localized to residues 242-413 of the Bcr moiety of Bcr-Abl [Mishra S. *et al.*, 2003]. Our results demonstrate that the two kinases do not apparently interact in S-LAMA84 cells, while they do in R-LAMA84 cells (Fig.6A and right panel of Figs.6A,6B). Consistent with the finding that CK2-alpha is an *in vitro* substrate of Bcr-Abl [Hériché J.K. and Chambaz E.M, 1998], a Bcr-Abl-dependent Tyr-phosphorylation of CK2α has been evidenced in R-LAMA84 cells [Borgo C. *et al.,* 2013]. However, here we demonstrate that the Bcr-Abl phosphorylation is not required for the interaction occurring between the two kinases, since the protein binding is not affected by cell treatment with imatinib (Fig.6C and right panel of Fig.6C). On the contrary, inhibition of CK2, which has been described to be unable to phosphorylate Bcr-Abl *in vitro* [Borgo C. *et al.,* 2013], almost abrogates the interaction occurring between the two enzymes (Fig.7D and right panel of Fig.7D), corroborating the hypothesis that CK2 activity plays a specific role in this binding. Our experiments further reinforce the notion that CK2 and Bcr-Abl are interacting proteins, however, additional studies are in progress to understand the physiological meaning of this binding in CML-resistance and its regulation by CK2 activity.

Our experiments in CML KCL22 cells demonstrate that protein-amount and activity of both Bcr-Abl (Fig.9) and CK2 (Fig.10) are similar in the two cell variants. CK2 up-regulation has been described in imatinib-resistant LAMA84 cells [Borgo C. *et al.,* 2013] and in cancer cell lines with other resistance mechanisms, either related to a multidrug resistance phenotype or induced by specific drugs [Di Maira G. *et al.*, 2008; Matsumoto Y. *et al.,* 2001]. Our results in KCL22 cells highlight that CK2 up-regulation does not represent an absolute requirement for the resistant phenotype as also shown in other resistant cells [Di Maira G. *et al.*, 2008].

The analysis in KCL22 cells evidences a novel Bcr-Abl-independent mechanism of resistance in agreement with the notion that imatinib-resistance can be

associated with the activation of oncogenic pathway(s) independently of Bcr-Abl catalytic activity [Tipping A.J. *et al.*, 2003]. On one hand, we identify new hyper-activated proteins in R-KCL22 versus S-KCL22 cells, and on the other, we highlight the important role played by CK2-dependent signalling in the resistant oncogenic network. Our results show that MAPK and PI3K/Akt/mTOR pathways are up-regulated in R-KCL22 cells as compared to the sensitive counterpart. While the hyper-activation of ERK1/2 has been previously highlighted [Fig.13; Colavita I. *et al.* 2010], we evidence, for the first time, an up-regulation of Akt as suggested by the hyper-phosphorylation of S473, one of the two canonical Akt activation sites, and of the Akt substrates mTOR and GSK3ß (Fig.14A). These findings demonstrate that, in KCL22 cells, imatinib-resistance escapes the drug effect highly potentiating the cell proliferation mediated by ERK1/2 activation [Deschênes-Simard X. *et al.,* 2014] and the pro-survival and anti-apoptotic functions of up-regulated Akt [Toker A. and Yoeli-Lerner M., 2006; Altomare D.A. and Testa J.R., 2005]. Interestingly, we also find that rpS6, a common downstream effector of both MAPK and PI3K/Akt/mTOR pathways, is substantially more phosphorylated in imatinib-resistant KCL22 cells as compared to the sensitive cells (Fig.15). Considering the role played by rpS6 in ribosome biogenesis and in translation initiation, the limiting step of protein synthesis [Holz M.K. *et al.*, 2005], it is conceivable to assume that its hyper-phosphorylation in R-KCL22 cells might strengthen the resistant leukemic phenotype, promoting cell proliferation.

To evaluate the role played by CK2 in imatinib resistance, KCL22 cells were treated with Bcr-Abl and CK2 specific inhibitors. Treatment with high concentrations of imatinib greatly reduces ERK1/2 and Akt anomalous activation induced by the drug resistance, while rpS6 phosphorylation is only partially (about 35%) inhibited (Fig.16). On the contrary CK2-inhibition by CX-4945 almost abrogates rpS6 phosphorylation (Fig.17) demonstrating that in imatinib-resistant KCL22 cells CK2 plays a role in rpS6 regulation. Our *in vitro* assays reveal that rpS6 is not a direct target of CK2 (data not shown). However, the finding that CX-4945 does not reduce the activity of ERK1/2 and Akt (Fig.17) suggests that CK2 modulates rpS6 pathway by a mechanism that lies downstream from mTORC1 complex (see scheme of Fig.12). Consistent with

this hypothesis, we provide the first evidence that CK2 interacts with mTOR and raptor, proteins of mTORC1 complex, and S6K (Fig.19). The finding that CK2 is involved in the regulation of rpS6 is also supported but the inhibition of the protein phosphorylation induced by knocking down the CK2α' expression by RNA interference experiments (Fig.18). With respect to this, it is interesting to mention that CK2 plays a "lateral" role impinging on the canonical "longitudinal" pathways at different levels [Ruzzene M. and Pinna L.A., 2010]. Although we have not yet clarified the specific mechanism(s) by which CK2 is involved in the events leading to rpS6 phosphorylation, it is significant that CK2-inhibition causes a reduction of the protein synthesis efficacy of about 50% as compared to the control (Fig.20). Interestingly, we identify a new pathway mediated by CK2 able to affect cellular protein synthesis in imatinib-resistant KCL22 cells.

The significant contribution played by CK2 in chronic myeloid leukemia is supported by the viability data obtained from cell treatments with the specific CK2-inhibitor CX-4945, which neither affects the protein level nor the activity of Bcr-Abl (Fig.17). Indeed, the viability of both LAMA84 and KCL22 cells, either sensitive or resistant to imatinib, is significantly reduced whenever CK2 catalytic activity is inhibited by CX-4945 (Fig.21B and Borgo C. *et al.,* 2013), consistent with the general anti-apoptotic and pro-survival role of CK2 in cancer cells [Zanin S. *et al.*, 2012; Buontempo F. *et al*, 2013]. Of especial interest is the effect of CX-4945 on resistant CML cells, where the reduction of cell viability and the induction of apoptosis are caused by lower concentration of inhibitor as compared to the sensitive cells (Figs.7B, 22 and 23A). This finding supports the hypothesis that imatinib-resistant cells are more dependent on CK2 activity for their survival than sensitive cells. Interestingly, CX-4945 added in combination with imatinib promotes a synergistic effect on cell viability of imatinib-resistant CML cells, partially rescuing the response to imatinib (Figs.8 and 24B). With regard to this, we can hypothesize that the interaction occurring between CK2 and Bcr-Abl, either direct or mediated by adaptor-protein(s), might be one of the molecular mechanisms reinforcing the imatinib-resistance but also offering the possibility to sensitize cells to imatinib by CK2 down-regulation and consequent binding disruption (right panel of

Figs.6D and 11). The increased evidence concerning the Bcr-Abl-independent mechanisms of imatinib-resistance that we report in KCL22 cells has provided the opportunity to analyse new drug combinations. Of note, we demonstrate that CX-4945 acts synergistically with U0126, an inhibitor of MAPK pathway, or with rapamycin, the potent inhibitor of mTORC1 complex, inducing a great decrease of the cell viability of imatinib-resistant KCL22 cells (Figs.24A,24C). Even more interesting, the treatment with the ternary association of CX-4945 with imatinib and U0126 causes a strong synergistic decrease of cell viability, reducing considerably the effective cellular dose of each drug (Fig.24E). Pertinent to this, it has been already described that CX-4945 increases the efficacy of other chemotherapeutic agents such as gemcitabine and cisplatin in models of ovarian cancers [Siddiqui-Jain A. *et al.*, 2012].

In conclusion, among to different adaptations associated with imatinib-resistance, we provide the first evidence that CK2-dependent signalling represents an additional mechanism that can be exploited to ensure survival to CML cells. In LAMA84 cells we have shown that CK2 up-regulation strengthens the CML oncogenic pathway(s) and sustains the imatinib-resistant phenotype. In KCL22 cells we have highlighted the CK2-dependent activation of the signalling downstream from mTORC1 complex, which cooperates to circumvent the imatinib inhibitory effect on Bcr-Abl pathways.

CONCLUSIONS

CONCLUSIONS

Although in the last decade imatinib has revolutionized the treatment of chronic myeloid leukemia, resistance to this drug remains one of the major reasons for the failure of cancer therapy and represents a problem of great interest. An improved understanding of the molecular mechanisms underlying imatinib-resistance is required to design novel strategies to treat this pathology.
In this study we compared the properties of the protein kinase CK2 in imatinib-sensitive and -resistant CML cell lines providing innovative evidence about the mechanisms that reinforce the imatinib-resistant phenotype. We identified CK2 as a player in CML imatinib-resistance and demonstrated, for the first time, that CK2-dependent signaling contributes to sustain the aberrant phenotype caused by Bcr-Abl expression. In particular, we found that Bcr-Abl-dependent and/or -independent oncogenic pathways are supported by CK2 activity, which provides survival advantage against imatinib through different mechanisms. In resistant-LAMA84 cells up-regulated CK2 co-operates with Bcr-Abl reinforcing its dependent-signaling pathways, which lead to proliferation and survival. In resistant-KCL22, on one hand, CK2 interacts with Bcr-Abl presumably supporting its functions, on the other hand, CK2 is implicated in a Bcr-Abl-independent signaling, downstream of mTORC1 complex, which affects protein synthesis and circumvents the imatinib inhibitory effect potentiating the pro-survival and anti-apoptotic signals. Finally, we found that the down-regulation of CK2 significantly reduces the viability of CML cells by apoptosis induction and rescues the response to imatinib. On these bases, we suggest that CK2 inhibitors, with special reference to CX-4945, a compound already in clinical trials for the treatment of different tumors, might represent promising drugs for combined strategies in imatinib-resistant CML therapy.

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Aberrant signalling by protein kinase CK2 in imatinib-resistant chronic myeloid leukaemia cells: Biochemical evidence and therapeutic perspectives

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ABSTRACT

Chronic myeloid leukaemia (CML) is driven by the fusion protein Bcr-Abl, a constitutively active tyrosine kinase playing a crucial role in initiation and maintenance of CML phenotype. Despite the great efficacy of the Bcr-Abl-specific inhibitor imatinib, resistance to this drug is recognized as a major problem in CML treatment. We found that in LAMA84 cells, characterized by imatinib-resistance caused by *BCR-ABL1* gene amplification, the pro-survival protein kinase CK2 is up-regulated as compared to the sensitive cells. CK2 exhibits a higher protein-level and a parallel enhancement of catalytic activity. Consistently, CK2-catalysed phosphorylation of Akt-Ser129 is increased. CK2 co-localizes with Bcr-Abl in the cytoplasmic fraction as judged by subcellular fractionation and fluorescence immunolocalization. CK2 and Bcr-Abl are members of the same multi-protein complex(es) in imatinib-resistant cells as demonstrated by co-immunoprecipitation and co-sedimentation in glycerol gradients. Cell treatment with CX-4945, a CK2 inhibitor currently in clinical trials, counteracts CK2/Bcr-Abl interaction and causes cell death by apoptosis. Interestingly, combination of CX-4945 with imatinib displays a synergistic effect in reducing cell viability. Consistently, knockdown of CK2α expression by siRNA restores the sensitivity of resistant LAMA84 cells to low imatinib concentrations. Remarkably, the CK2/Bcr-Abl interaction and the sensitization towards imatinib obtained by CK2-inhibition in LAMA84 is observable also in other imatinib-resistant CML cell lines.

These results demonstrate that CK2 contributes to strengthen the imatinib-resistance phenotype of CML cells conferring survival advantage against imatinib. We suggest that CK2 inhibition might be a promising tool for combined strategies in CML therapy.

1. Introduction

The cytogenetic hallmark of the chronic myeloid leukaemia (CML) is the chromosomal translocation t(9; 22)(q34; q11), yielding the Philadelphia chromosome and generating a fusion gene that encodes Bcr-Abl, a constitutively active protein tyrosine kinase. Signal transduction pathways activated by Bcr-Abl kinase activity promote cell survival and

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proliferation while protecting cells from apoptosis (Goldman and Melo, 2003). Since Bcr-Abl plays a critical role in the initiation and maintenance of the CML phenotype, targeting its tyrosine kinase activity is the therapeutic strategy of choice. Imatinib mesylate is a potent inhibitor selective for Bcr-Abl that has become the frontline therapy for CML patients. However, despite the high effectiveness of this therapeutic approach, up to one third of CML patients develop either resistance or intolerance to imatinib and require alternative therapy (Bixby and Talpaz, 2009; Roychowdhury and Talpaz, 2011). The various mechanisms of imatinib-resistance described up to now, either Bcr-Abl-dependent (gene amplification or mutation) or Bcr-Abl-independent (decreased imatinib bioavailability or activation of alternative signalling pathway) (Bixby and Talpaz, 2009; Roychowdhury and Talpaz, 2011) have provided the opportunity for second-generation or combination therapies aimed at preventing resistance or restoring response to the drug (Santos et al., 2011; Stegmeier et al., 2010; Roychowdhury and Talpaz, 2011).

Protein kinase CK2 is a ubiquitous, highly conserved and pleiotropic Ser/Thr kinase, endowed with constitutive activity, independent of any known second messenger or phosphorylation events. CK2 is usually present as a tetrameric holoenzyme composed of two catalytic subunits (α and/or α′) and a dimer of regulatory (β) subunits. It phosphorylates a huge number of protein substrates, implicated in fundamental cell processes and is essential for cell life (Meggio and Pinna, 2003; Ruzzene and Pinna, 2010; Salvi et al., 2009). CK2 is abnormally elevated in a wide variety of tumours, where it plays a global role as an anti-apoptotic and pro-survival agent (Ahmad et al., 2008; St-Denis and Litchfield, 2009) and there is strong evidence that it operates as a cancer driver by creating a cellular environment favourable to neoplasia (Ruzzene and Pinna, 2010). Different data suggest that CK2 may also have a significant role in the pathogenesis of haematopoietic tumours, including CML (Piazza et al., 2012), where a relationship between CK2 and Bcr-Abl has been suggested (Hériché and Chambaz, 1998; Mishra et al., 2003, 2007). The role of CK2 in imatinib-resistance, however, has never been explored.

In this study we compare the properties of the protein kinase CK2 in imatinib-sensitive and resistant LAMA84 cell lines. We also analyse the potential cross-talk between CK2 and Bcr-Abl and the possibility of using CK2-specific inhibitors for combined therapy to overcome the imatinib-resistance of CML cells.

2. Materials and methods

2.1. Materials and antibodies

[γ^{33}P]ATP was purchased from Perkin–Elmer (Waltham, MA). Protease inhibitor cocktail was from Calbiochem (Darmstadt, Germany), while phosphatase inhibitor cocktails and β-casein from Sigma–Aldrich (Dorset, U.K.). Imatinib mesylate was from Cayman Chemical (Ann-Arbor, MI), while CX-4945 was provided by Cylene-Pharmaceuticals (S. Diego, CA). Inhibitors GNF-2 and staurosporine, and other chemicals were from Sigma–Aldrich. RRRADDSDDDDD peptide (Ruzzene et al., 2010) and recombinant CK2 ($\alpha_2\beta_2$) (Lolli et al., 2012) were kindly provided by Dr. Oriano Marin and Dr. Andrea Venerando (University of Padova, Italy), respectively. Anti-CK2α (Sarno et al., 1996) and anti-phospho-Akt(Ser129) (Di Maira et al., 2005) antibodies were raised in rabbit. Anti-c-Abl was from Calbiochem, anti-CK2β, CrkL and phospho-CrkL(Tyr207) from Epitomics (Burlingame, CA), anti-CK2α′, Akt, phospho-Akt(Ser473), Lyn, lamin B, LDH and rpS6 from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-tyrosine from Millipore Corporation (Billerica, MA), anti-PARP from Roche (Basel, Switzerland) and anti-tubulin from Sigma–Aldrich.

2.2. Cell culture

KCL-22, K562 and LAMA84 cell lines, either sensitive or resistant to imatinib, were kindly supplied by Dr. C. Gambacorti-Passerini (le Coutre et al., 2000; Redaelli et al., 2010). Cells were maintained in RPMI 1640 supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100U/ml penicillin and 100 mg/ml streptomycin in the absence (sensitive) or presence (resistant) of imatinib (3 μM, 0.6 μM and 1.5 μM for KCL-22, K562 and LAMA84 cells, respectively).

2.3. Cell lysis and western blot analysis

Cells were lysed as previously described (Di Maira et al., 2007). Protein concentration was determined by Bradford method. Proteins were subjected to 9% or 11% SDS-PAGE, blotted on Immobilon-P membranes (Sigma–Aldrich), processed in western-blot with the indicated antibodies and developed using an enhanced chemiluminescent detection system (ECL). Immunostained bands were quantified by means of a Kodak-Image-Station 4000 MM-PRO and analysis with Carestream Molecular Imaging software (New-Haven, CT).

2.4. Immunoprecipitation experiments

Indicated lysate proteins were immunoprecipitated overnight with the specific antibody, followed by addition of protein A-Sepharose. The immunocomplexes, washed three times with 50 mM Tris–HCl, pH 7.5, were analysed by western-blot.

2.5. RNA extraction and real-time quantitative PCR

Total RNA from S- and R-LAMA84 cells was extracted using TRIzol reagent (Life-Technologies, Carlbad, CA) and 1 μg RNA was reverse transcribed with TaqMan® Reverse Transcription Reagents (Life-Technologies) according to the manufacturer's instructions and subsequently used for real-time quantitative PCR. Amplification and quantification was performed with Power SYBR® Green PCR Master Mix (Life-Technologies) and a Rotor-Gene 3000 system (Corbett Life Science, Concorde, NSW). The oligonucleotide primers (Sigma–Aldrich) used for CK2α were: 5′-GAGAGGAGGTCCCAACATCA-3′ (sense) and 5′-TGACATTATGGGGCTTGACA-3′ (antisense), and for β-actin: 5′-GGACTTCGAGCAAGAGATGG-3′ (sense) and 5′-AGCACTG TGTTGGCGTACAG-3′ (antisense). Expression levels were normalized to β-actin.

2.6. CK2 kinase activity assay

Lysate proteins were incubated for 10 min at 30 °C in 25 μl of a phosphorylation medium containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$, 400 μM synthetic peptide-substrate RRRADDSDDDDD and 20 μM [γ^{33}P]ATP (1000 cpm/pmol). Assays were stopped by absorption onto phosphocellulose filters. Filters were washed four times in 75 mM phosphoric acid (Ruzzene et al., 2010) and analysed by a Scintillation Counter (PerkinElmer).

2.7. In-gel kinase assay of CK2α

The activity displayed by CK2α subunit alone was determined by running cell lysates on an 11% SDS-PAGE containing the CK2-substrate β-casein (0.5 mg/ml). After electrophoresis, the activity of CK2α toward the co-localized β-casein was detected by incubating the gel with the above described phosphorylation medium containing 1 mM [γ^{33}P]ATP (Ruzzene et al., 2010). Radioactive ^{33}P-β-casein was evidenced by analysing the dried gel with a Cyclone Plus Storage PhosphorSystem (PerkinElmer).

2.8. Subcellular fractionation by differential centrifugation

Cells (8×10^6) were re-suspended in a hypotonic buffer (10 mM Tris/acetate, pH 7.4, containing protease and phosphatase inhibitor cocktails), incubated for 5 min on ice and broken by Dounce homogenization. The solution was immediately adjusted to 0.25 M saccharose, 1 mM $MgCl_2$ and subjected to differential centrifugation to separate nuclei, mitochondria, microsomes and cytosol (Kang and Welch, 1991). Pellets were re-suspended in a volume of lysis buffer corresponding to that of cytosol. Same volumes of different fractions were analysed by western-blot.

2.9. Immunolocalization of CK2 and Bcr-Abl by fluorescence microscopy

Cells (5×10^5) were seeded on polylysine-coated glass coverslips, allowed to adhere overnight, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilised with 0.1% Triton X–100 in PBS for 10 min at 4 °C. For dual labelling, cells were first incubated with mouse anti-Abl antibody (1:10) overnight at 4 °C, followed by 1 h incubation with anti-mouse IgG/FITC conjugated antibody (1:50) at 37 °C. Cells were then incubated with rabbit anti-CK2α antibody (1:50) for 1 h at 37 °C followed by goat anti-rabbit Alexa-Fluor 633 conjugated antibody (1:500) for 1 h at 37 °C. Nuclei were stained with Hoechst 33342. Fluorescence images were captured using a LEICA-TCS SP5 confocal microscopy (Wetzlar, Germany), equipped with HCX PL APO lambda blue 63 × 1.4 oil immersion objective. Images were processed with the LAS-AF software.

2.10. Glycerol gradient sedimentation

Cells (20×10^6) were lysed with the above-described lysis buffer containing 10 mM KCl and 0.2% triton X-100. 400 μg of lysates were layered on the top of a 3.6 ml of a glycerol linear gradient (10%–40%) in 50 mM Hepes, pH 8, 1 mM EDTA, 1 mM DTT, protease and phosphatase inhibitors. The tubes were centrifuged at 100 000× *g* for 18 h at 4 °C and fractionated from the bottom into 20 fractions.

2.11. RNA interference

Cells (1.5×10^6) were transfected with 30 nM CK2α specific siGENOME SMARTpool siRNAs (Dharmacon, Lafayette, CO, USA) or aspecific siRNA siCONTROL riscfree#1 (Dharmacon), as control, using the transfecting reagent INTERFERin (Polyplus-transfection SA, Illkirch, France), according to the manufacturer's recommendations.

2.12. Cell viability assay

Cell viability was detected by the method of MTT [3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide), incubating 15×10^3 cells/100 μl in a 96-well plate under different conditions. 1 h before the incubation end, 10 μl of MTT solution (5 mg/ml in PBS) was added to each well. Incubations were stopped by addition of 20 μl of a pH 4.7 solution containing 20% (w:v) SDS, 50% (v:v) N,N-dimethylformamide, 2% (v:v) acetic acid and 25 mM HCl. Plates were read at λ540 nm absorbance, in a Titertek Multiskan Plus plate reader (Flow Laboratories, Sutton, U.K.).

2.13. Combined treatments

The combination index (CI) (Chou, 2006) for the combined treatment with imatinib and CX-4945 was calculated with the software Calcusyn (Biosoft, Cambridge, U.K.).

2.14. Statistical analysis

Data are presented as means ± SD and mean differences were analysed using *t-test*. A $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Protein-level and activity of protein kinase CK2 in imatinib-sensitive and -resistant CML cells

The CML cell lines KCL-22, K562 and LAMA84, either sensitive (S) or resistant (R) to imatinib, were characterized with specific antibodies. In these cell lines the imatinib-resistance is neither due to Bcr-Abl mutations (le Coutre et al., 2000; Redaelli et al., 2010) nor to a multidrug resistance phenotype (le Coutre et al., 2000; Zanin et al., 2012). Western blot analysis of equal amounts of cell lysates shows that the protein-level of Bcr-Abl, while similar in parental and imatinib-resistant KCL-22 and K562 cell lines, is about four-fold higher in imatinib-resistant as compared to imatinib-sensitive LAMA84 cells (Figure 1A). This finding is consistent with the notion that in this cell line imatinib-resistance is associated with an overexpression of the oncokinase mediated by gene amplification (le Coutre et al., 2000). As expected, the parallel

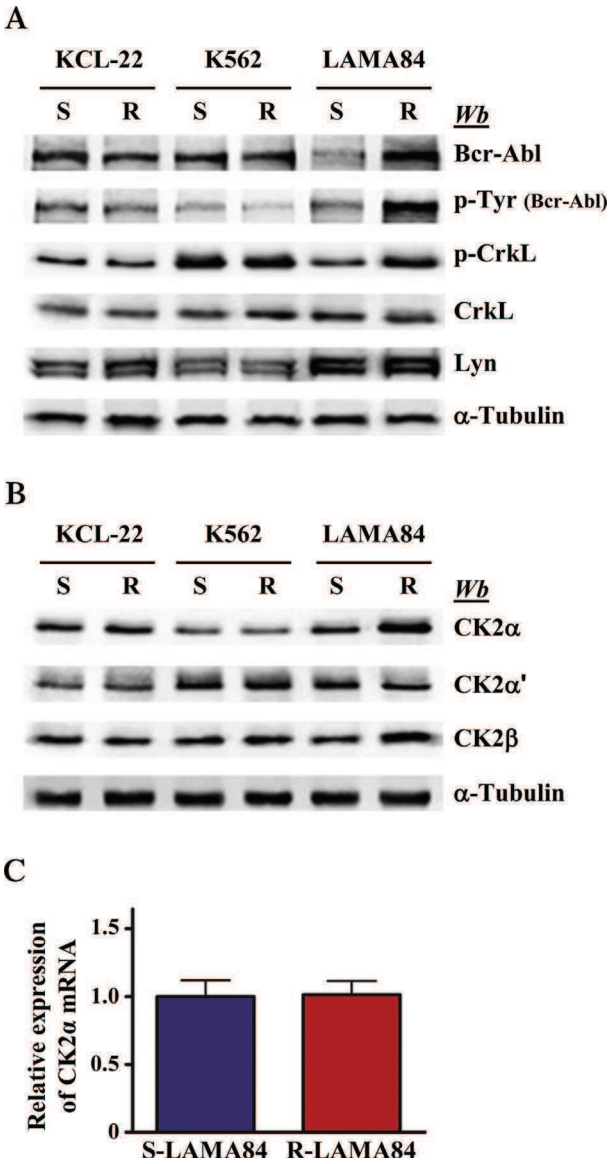

Figure 1 – Expression analysis of Bcr-Abl, Lyn and CK2 in different CML cell lines. (A,B) 30 μg (A) or 10 μg (B) of lysate proteins were analysed by Western blot. Anti-p-Tyr immunostaining was superimposed on Bcr-Abl band, which was detected by anti-Abl antibody. Anti-α-tubulin Western blot is shown as a loading control. Figure is representative of at least five separate experiments. (C) Real-time quantitative PCR analysis of CK2α gene expression was performed using cDNA obtained from reverse transcribed total RNA from S-LAMA84 and R-LAMA84 cells. The CK2α mRNA level was assessed by quantitative PCR analysis as described in Materials and methods. Results were normalized to β-actin mRNA used as internal standard and the expression level of CK2α gene in R-LAMA84 is normalized to that of S-LAMA84 cells. Reported values are means ± SD of four separate experiments performed in triplicate.

analysis with anti-phospho-tyrosine antibody shows that overexpressed Bcr-Abl is constitutively active as judged from its autophosphorylation and the phosphorylation of the key Bcr-Abl substrate CrkL (de Jong et al., 1997), which are higher in R-LAMA84 than in sensitive cells (Figure 1A).

Since up-regulation of the Src-kinase Lyn has been described to be associated with imatinib-resistance (Ptasznik et al., 2004), the protein-level of this Src family tyrosine kinase was also analysed in the leukaemic cells. In all CML cell lines Lyn is similarly expressed in imatinib-resistant and sensitive cells (Figure 1A).

The expression of the protein kinase CK2 was next examined using antibodies toward the kinase catalytic (α and α′) and regulatory (β) subunits (Figure 1B). While CK2 subunits are similarly expressed in both imatinib-sensitive and -resistant KCL-22 and K562 cell lines, the amount of CK2α and β subunits is approximately two-fold higher in R-LAMA84 cell line as compared to the sensitive counterpart. At variance, the expression of CK2α′ is similar in sensitive and resistant cells. The outcome that CK2 is more abundant in R-LAMA84 cells prompted us to further characterizes the two LAMA84 cell variants. To assess whether the high CK2α amount detected in resistant cells might be due to altered regulation at transcription level, CK2α mRNA amount was examined by means of relative semi-quantitative RT-PCR (not shown) and real-time quantitative PCR (Figure 1C). Both methods showed that comparable levels of mRNA are present in S-LAMA84 and R-LAMA84 cells.

The activity of cellular CK2 was then tested using increasing amounts of cell lysates by *in vitro* kinase assay toward a CK2-specific peptide-substrate. Consistent with the protein level, the cellular kinase activity is about two-fold higher in R-LAMA84 cells than in parental cell line (Figure 2A). To further characterize the detected kinase activity, a parallel analysis was performed using the lysates of cells treated with imatinib or CX-4945, a potent and selective CK2-inhibitor currently in clinical trials for the treatment of different tumours (Siddiqui-Jain et al., 2010). As expected, CX-4945 strongly reduces CK2 activity in the two cell lines, while imatinib treatment does not affect it (Figure 2B).

Cellular CK2 was also studied by analysing the activity displayed by its catalytic subunit α. To this purpose CK2α was separated on a polyacrylamide gel containing the CK2-substrate β-casein and the activity of the α-subunit toward the co-localized substrate was determined by a radioactive in-gel kinase assay. Equal amounts of cellular lysates show a higher ^{33}P-phosphorylation of β-casein in R-LAMA84 cells as compared to the parental counterpart (Figure 2C).

3.2. *Protein quantification and subcellular distribution of CK2 in LAMA84 cells*

CK2 level was found markedly increased in highly proliferating myeloblastic cells from CML patients in comparison with normal granulocytes (Phan-Dinh-Tuy et al., 1985). This finding prompted us to perform a relative quantification of cellular CK2α and β subunits in LAMA84 cells by comparative analysis with recombinant CK2 holoenzyme ($\alpha_2\beta_2$) containing equimolar amounts of the two subunits. The comparison suggests that the two CK2 subunits are expressed at very high levels in both CML cell lines and confirms that imatinib-resistant cells contain about twice as much CK2α and β subunits (Figure 3A). In particular, the densitometric analysis suggests that in imatinib-resistant cells the amount of CK2α represents about 0.3% of total proteins.

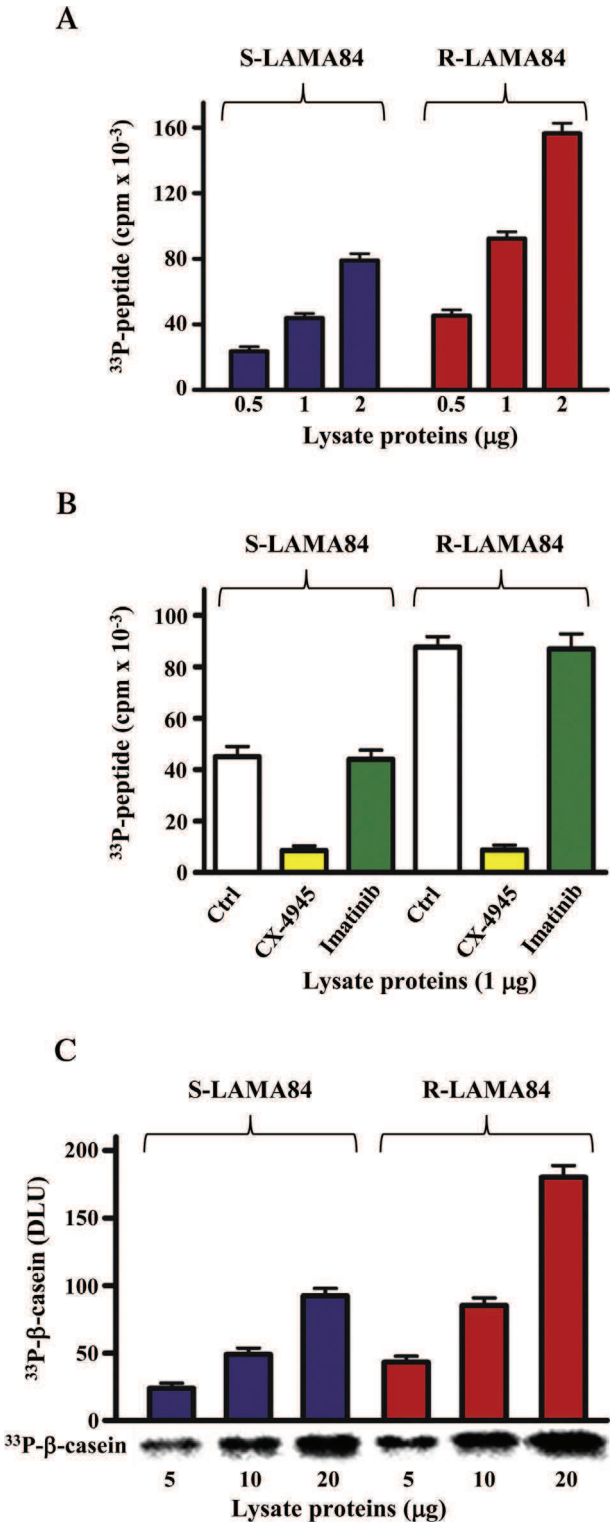

Figure 2 – Analysis of CK2 activity in S-LAMA84 and R-LAMA84 cells. (A) The kinase activity of cellular CK2 was tested, as detailed in Materials and methods, toward the peptide-substrate RRRADDSDDDDD in a phosphorylation medium containing the indicated micrograms of lysate proteins. Reported values are means ± SD of four separate experiments. (B) Cells, incubated for 24h with vehicle DMSO (Ctrl), CX-4945 (3 µM) or imatinib (0.5 µM in S-LAMA84 and 2 µM in R-LAMA84), were lysed and CK2 activity was tested in 1 µg of lysate proteins as described in (A). Reported values are means ± SD of four separate experiments. (C) The kinase activity of monomeric CK2α was analysed by an in-gel kinase assay. The indicated micrograms of lysate proteins were loaded on a polyacrylamide gel containing the CK2-substrate β-casein and CK2α activity was detected as detailed in Materials and methods. The ^{33}P-phosphorylation of β-casein, evidenced by a Cyclone Plus Storage PhosphorSystem is expressed in Digital Light Units (DLU). Reported values are means ± SD of four separate experiments.

There is ample evidence that CK2 is distributed in nearly every subcellular compartment, where it plays different functions, and that the subcellular localization of CK2 is tightly regulated (Filhol and Cochet, 2009). Therefore studying the distribution of this kinase may be a key to understand its function. The comparison of CK2 subcellular localization in the two cell variants (Figure 3B) reveals that the amount of CK2α′ is similar in the different subcellular compartments of S-LAMA84 and R-LAMA84 cells. In contrast, while CK2α level is comparable in nuclei and almost undetectable in mitochondria, it is overexpressed in the cytosolic and microsomal fractions of R-LAMA84 cells. Likewise, the protein-level of CK2β is consistently higher in cytosol and microsomes.

It is noteworthy that, in resistant cells, CK2 is overexpressed in the cytoplasm (cytosol and microsomes), where Bcr-Abl is also mainly retained in CML cells and where it interacts with most proteins involved in the oncogenic pathway (Cilloni and Saglio, 2012). This prompted us to perform a confocal microscopy immunofluorescence analysis looking for a possible co-localization of the two protein kinases, which are both overexpressed in R-LAMA84 cells. CK2 fluorescence is observable in the nucleus but is mostly localized in the cytoplasm, where Bcr-Abl is exclusively visible and appears to co-localize with CK2 (Figure 3C). Immunolocalization performed in parallel with S-LAMA84 cells showed a similar distribution of CK2 fluorescence, which is more evident in the cytoplasm (results not shown), while Bcr-Abl localization was unfeasible because the oncokinase fluorescence was not detectable (see also Figure 1A).

3.3. *CK2 and Bcr-Abl interact in CML cells*

CK2 and Bcr-Abl co-localization prompted us to check if the two protein kinases are interacting proteins. To this purpose, Bcr-Abl-immunoprecipitates obtained from LAMA84 cellular lysates were probed with anti-CK2 antibodies (Figure 4Aa). Interestingly, while CK2 does not co-immunoprecipitate with Bcr-Abl in S-LAMA84 cells, a substantial amount of both CK2α and β subunits is detectable in R-LAMA84 cells. Consistently, Bcr-Abl is present in CK2α immunocomplexes only in imatinib-resistant cells (Figure 4Ab). A parallel analysis was also performed to compare LAMA84 with K562 and KCL-22 cell lines, where Bcr-Abl and CK2 are similarly expressed in S and R variants (Figure 4Ac). Differently from LAMA84, in K562 and KCL-22 the interaction between Bcr-Abl and CK2 is detectable also in imatinib-sensitive cells. Interestingly, in K562 the association observed is higher in imatinib-resistant than in sensitive cells as in the case of LAMA84 cells (Figure 4Ac).

Since it has been shown that Abl tyrosine kinase phosphorylates CK2α *in vitro* (Hériché and Chambaz, 1998), the lysates of

the CML cell lines were immunoprecipitated with anti-CK2α antibody and analysed for the presence of phospho-tyrosine (p-Tyr) (Figure 4B). In LAMA84 cells CK2α is Tyr-phosphorylate d only in the imatinib-resistant counterpart, while in K562 and KCL-22 the subunit is Tyr-phosphorylated in both cell variants (Figure 4Ba). Interestingly, in the case of K562 the extent of Tyr-phosphorylation is higher in the imatinib-resistant than in the sensitive cells, as observed in LAMA84 cells suggesting a relationship between higher CK2 association to Bcr-Abl (Figure 4Bc) and higher CK2α Tyr-phosphorylation. Parallel

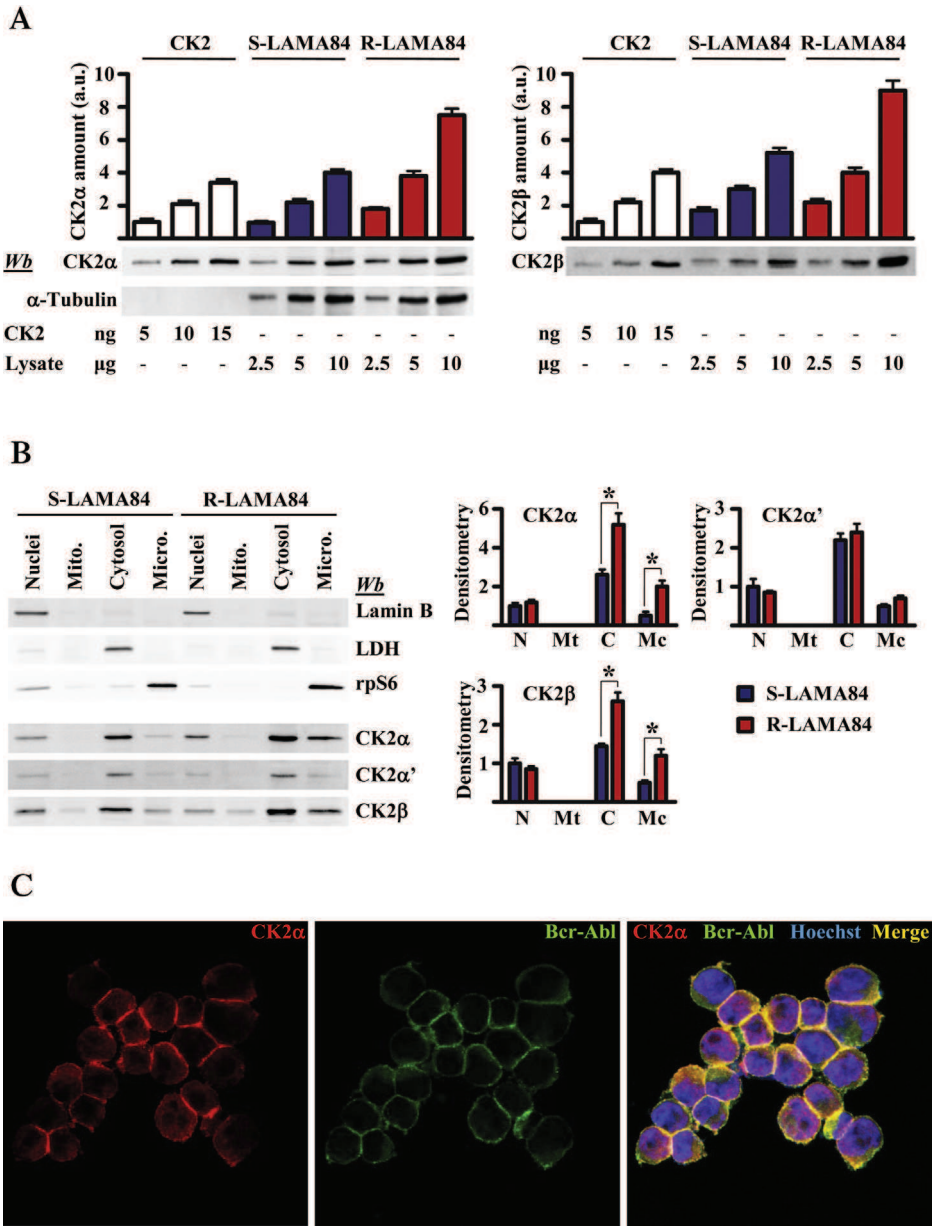

Figure 3 – Quantification and subcellular distribution of CK2 in S-LAMA84 and R-LAMA84 cells. (A) The indicated ng of recombinant CK2 holoform ($\alpha_2\beta_2$) and µg of lysate proteins from S-LAMA84 and R-LAMA84 cells were analysed by Western blot with anti-CK2α (left panel) and anti-CK2β (right panel) antibodies. Anti-α-tubulin Western blot is shown as a loading control. Means of densitometric values ± SD, expressed in arbitrary units (a.u.), are reported above the relative subunit bands. Cellular CK2 subunit amounts were calculated by densitometric analysis and extrapolation from the calibration curve built on the signal of recombinant CK2. (B) (Left panel) Cells were disrupted by Dounce homogenization and subcellular fractionation was performed by differential centrifugation as detailed in Materials and methods. Subcellular fractions (N, nuclei; Mt, mitochondria; C, cytosol and Mc, microsomes) were resuspended in an equal volume and the same volume of resulting fractions was immunoblotted with the indicated antibodies including the organelle-specific antibodies against lamin B (nuclei), lactate dehydrogenase (LDH) (cytosol) and S6 ribosomal protein (microsomes and nucleoli). The Figure is representative of five separate experiments. (Right panel) Bars report the mean values ± SD of the densitometric analysis of the CK2-subunit bands obtained as in left panel. Densitometric values are expressed in arbitrary units. *$p < 0.05$. (C) Confocal microscopy of double immunofluorescence staining of R-LAMA84 cells with anti-CK2α (red) and anti-Abl (green) antibodies. Nuclei were stained with Hoechst 33342. Co-localization of red and green fluorescences is visualized by the yellow fluorescence appearing after merging of both signals. Violet appears from the merging of nuclear staining and red fluorescence.

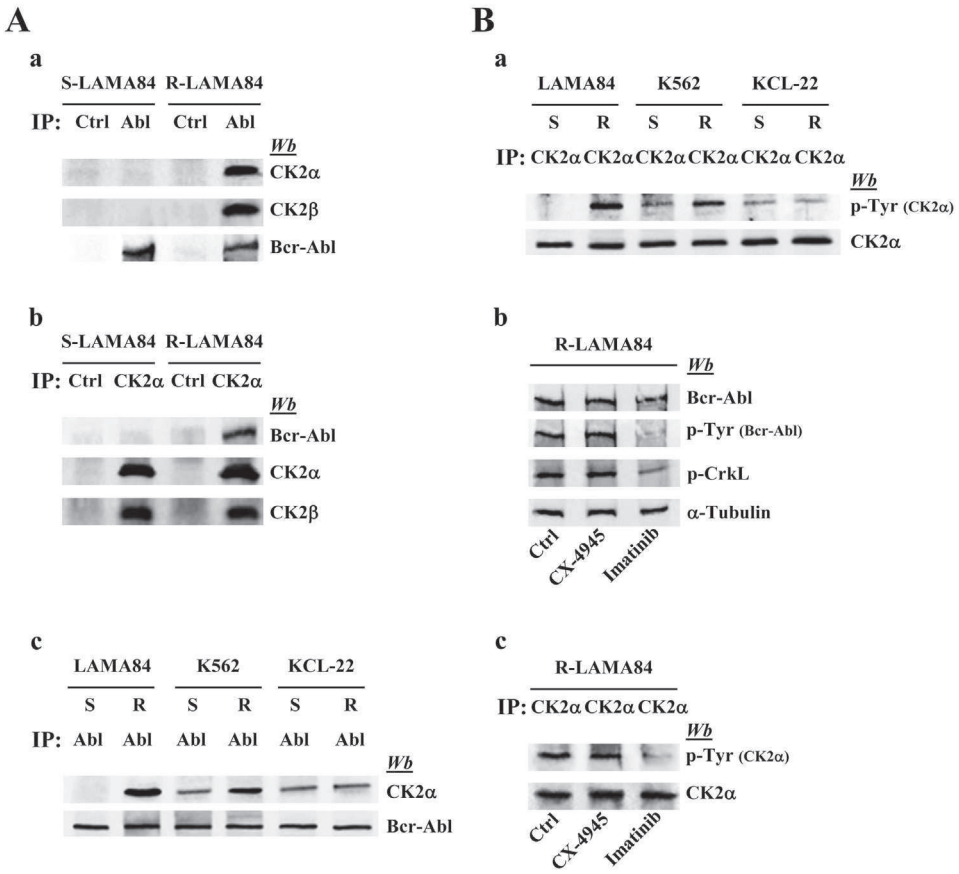

Figure 4 – Analysis of CK2 and Bcr-Abl interaction in S-LAMA84 and R-LAMA84 cells. (Aa, Ab) S- and R-LAMA84 cells were lysed and 300 μg of lysate proteins were immunoprecipitated with a control antibody from the same class (Ctrl) and anti-Abl antibody (Aa), or pre-immune serum (Ctrl) and anti-CK2α antibody (Ab). The immunocomplexes were then analysed by Western blot with the indicated antibodies. (Ac) LAMA84, K562 and KCL-22 cells were immunoprecipitated with anti-Abl antibody and immunocomplexes were analysed by anti-CK2α immunostaining. (Ba) LAMA84, K562 and KCL-22 cells were lysed and lysate proteins (300 μg) were immunoprecipitated with anti-CK2α antibody. The immunocomplexes were then analysed by Western blot with anti-phospho-tyrosine (anti-p-Tyr) followed by anti-CK2α antibodies. (Bb,Bc) R-LAMA84 cells were treated with vehicle, CX-4945 (5 μM) or imatinib (3 μM) for 24h and then lysed. (Bb) Cellular lysates were analysed by Western blot with the indicated antibodies. (Bc) 300 μg of cellular lysates were immunoprecipitated by anti-CK2α antibody and immunocomplexes were immunostained with anti-p-Tyr followed by anti-CK2α antibodies. Figure is representative of at least four separate experiments.

experiments demonstrated that Tyr-phosphorylation is undetectable in CK2β immunoprecipitates (data not shown).

To assess whether CK2 itself, or Bcr-Abl (or eventually both) might be responsible for this Tyr-phosphorylation, imatinib-resistant LAMA84 cells were treated for 24 h with vehicle, CX-4945 or imatinib. CX-4945, which neither affects the protein-level nor the activity of Bcr-Abl (Figure 4Bb), does not reduce the Tyr-phosphorylation extent of immunoprecipitated CK2α (Figure 4Bc), ruling out the possibility that CK2 catalytic subunit might undergo Tyr-autophosphorylation in R-LAMA84 cell line, as found in other mammalian cells (Vilk et al., 2008). On the contrary, imatinib greatly decreases both the Bcr-Abl activation state (Figure 4Bb) and the extent of CK2α Tyr-phosphorylation (Figure 4Bc), consistent with the concept that Bcr-Abl is the kinase responsible for this phosphorylation. Experiments aimed at highlighting the effect of this Tyr-phosphorylation on CK2 catalytic activity failed to show any significant difference between CK2α immunoprecipitated in comparable amounts from control or imatinib-treated R-LAMA84 cells (not shown).

To evaluate the role played by the activity of each kinase on the reciprocal binding, CK2/Bcr-Abl interaction was analysed in R-LAMA84 cells treated with different kinase inhibitors (Figure 5A). Intriguingly, the treatment with CX-4945 almost abrogates the interaction occurring between CK2 and Bcr-Abl, while imatinib and GNF-2, an allosteric non-ATP competitive inhibitor of Bcr-Abl (Adrián et al., 2006), do not affect this binding. The additional finding that staurosporine, added at a concentration ineffective toward CK2 but able to inhibit most protein kinases (Meggio et al., 1995) including Bcr-Abl (not shown), does not counteract the interaction between CK2 and Bcr-Abl (Figure 5A), corroborates the hypothesis that CK2 kinase activity plays a specific role in the binding. Consistently, a highly reduced amount of Bcr-Abl is detectable in CK2α immunoprecipitates from R-LAMA84 cells treated with CX-4945 (Figure 5B). This finding prompted us to assess whether CK2-catalysed phosphorylation of Bcr-Abl might be a prerequisite for the interaction of the two kinases. However, phosphorylation assays performed *in vitro* by adding

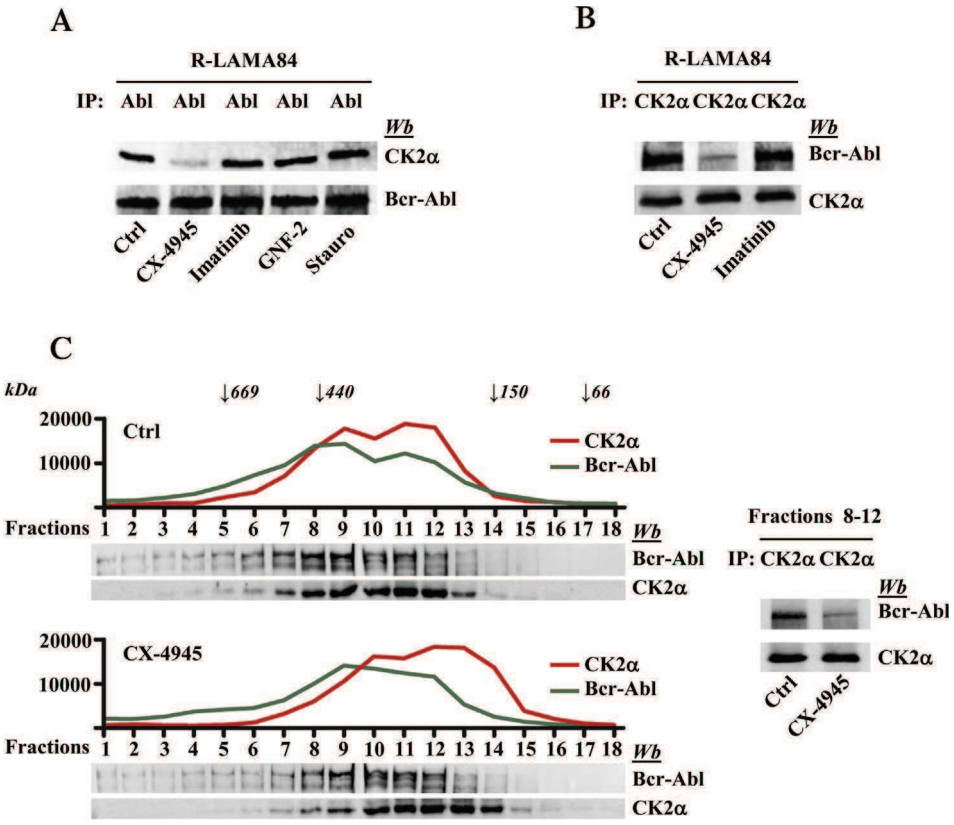

Figure 5 – Effect of CX-4945 on CK2/Bcr-Abl interaction. (A) R-LAMA84 cells were treated with vehicle, CX-4945 (5 μM), imatinib (3 μM), GNF-2 (10 μM) or staurosporine (1 μM) for 24 h. Lysate proteins (300 μg) were immunoprecipitated by anti-Abl antibody and immunocomplexes were analysed by Western-blot. (B) R-LAMA84 cells were treated with the indicated inhibitors as in (A). Lysate proteins (300 μg) were immunoprecipitated with anti-CK2α antibody and then probed with the indicated antibodies. (C) R-LAMA84 cells, treated for 24 h with vehicle (Ctrl) or 5 μM CX-4945, were lysed and lysate proteins were separated on glycerol gradient as detailed in Materials and methods. Molecular weight standards were run on separated tubes: bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa). 40 μl of the resulting fractions were analysed by Western blot. The densitometric analysis of CK2α and Bcr-Abl bands is reported above the relative gradient. (Right panel) Fractions 8–12 of each gradient were pooled and immunoprecipitated with anti-CK2α antibody. The immunocomplexes were analysed by Western blot. Figure is representative of four separate experiments.

recombinant CK2 holoenzyme to Bcr-Abl immunoprecipitated from R-LAMA84 lysates, do not support the hypothesis that Bcr-Abl might be a target–substrate of CK2 (not shown).

To further analyse the CK2/Bcr-Abl interaction, R-LAMA84 cells treated with vehicle or CX-4945 were lysed under mild conditions and subjected to glycerol gradient sedimentation (Figure 5C). In control cells CK2α subunit co-migrates with most Bcr-Abl (fractions 8–12), suggesting that they are partners of the same complex(es) as confirmed by their co-immunoprecipitation observed using the pooled fractions 8–12 of the gradient (Figure 5C, right panel). Interestingly, CX-4945-treatment, which does not significantly change the sedimentation profile of Bcr-Abl, makes CK2α to shift towards fractions containing complexes displaying lower molecular weights (fractions 10–14), implying that CK2 dissociates from Bcr-Abl as corroborated by the reduced co-immunoprecipitation of the two oncokinases (Figure 5C, right panel).

3.4. *Effect of CK2 down-regulation on CML cell viability*

The effect of imatinib on LAMA84 cell viability was compared with that of CX-4945. As expected, the DC_{50} values (concentration inducing the 50% of cell death) calculated for imatinib are about 0.3 and 2.1 μM, in sensitive and resistant cell lines, respectively (Figure 6A). Treatment with CX-4945 reduces the viability of both S-LAMA84 and R-LAMA84 cells with DC_{50} values of about 8 and 5 μM, respectively (Figure 6B).

Apoptosis occurrence was then analysed by comparing the cleavage of the caspase substrate PARP in the two cell variants. As expected, PARP is almost completely cleaved by treatment with 0.5 μM imatinib in sensitive LAMA84 cells, an event parallelled by the proteolysis of Bcr-Abl, Akt and α-tubulin (Figure 6C). On the contrary, treatment with up to 1 μM imatinib does not induce any appreciable effect in resistant cells (Figure 6C). The opposite is observable with CX-4945, which is not effective up to 5 μM concentration in S-LAMA84 cells, while the same concentration of inhibitor induces an almost complete cleavage of PARP and of the other analysed proteins in R-LAMA84 cells (Figure 6D). This outcome supports the hypothesis that imatinib-resistant cells are more dependent on CK2 activity for their survival than sensitive cells.

Since CK2 phosphorylates Akt at Ser129 inducing an increased activity of this pro-survival kinase (Di Maira et al., 2005), the phosphorylation state of this residue was evaluated

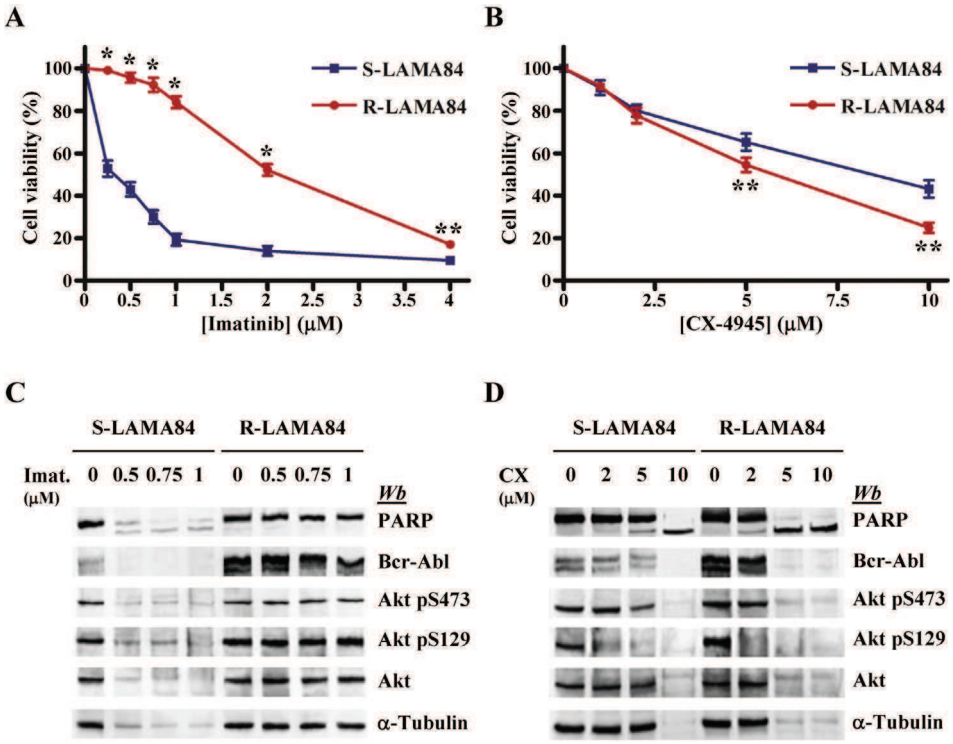

Figure 6 – Cell death induction by imatinib and CX-4945 in LAMA84 cells. (A–D) S-LAMA84 and R-LAMA84 were treated with the indicated concentration of imatinib (A,C) or CX-4945 (B,D) for 48 h. (A,B) Cell viability was assessed by MTT method and expressed as percentage of controls. *$p < 0.01$, **$p < 0.05$ *vs* S-LAMA84 cells. (C,D) Cellular lysates (30 μg) were analysed by Western blot. Anti-PARP antibody recognizes the full length protein and its p85 fragment. Figure is representative of five separate experiments.

upon treatment with the two inhibitors. Consistent with the higher CK2 activity, the extent of Akt Ser129 phosphorylation is higher in imatinib-resistant than in sensitive cells under basal conditions (Figure 6C and D) suggesting an up-regulation of Akt signalling in R-LAMA84 cells. Moreover, while imatinib treatment does not affect Ser129 phosphorylation (Figure 6C), CX-4945 strongly reduces the phosphorylation of this Akt residue, which, in both cell variants, is almost abrogated by 2 μM CX-4945, a concentration not affecting the total Akt amount (Figure 6D).

We have recently found that inhibition of CK2 by CX-4945 reduces also the viability of the CML cell lines K562 and KCL-22 (Zanin et al., 2012). In all the tested CML lines, CK2 inhibition induces cell death also in the imatinib-resistant variants (see Figure 6B and Zanin et al., 2012), independently of the CK2 expression level (Figure 1B); this prompted us to investigate whether CX-4945 might sensitize resistant cells to imatinib. To this purpose, cells were treated with CX-4945 and imatinib either separately or in combination. We then examined if the combined treatment induced a higher degree of cell death compared to the separate treatments. Interestingly, low concentrations of CX-4945 are able to significantly increase the effect of imatinib on all the resistant CML cell lines analysed (Figure 7). The values of the combination index (which denotes synergism if <1) (Chou, 2006) are 0.57 for R-LAMA84, 0.75 for R-K562, and 0.87 for R-KCL-22, demonstrating that the combined treatment promotes a synergistic reduction of cell viability more pronounced in R-LAMA84 and R-K562 cells.

To further support a specific role of CK2 in imatinib-resistance, we knocked down the expression of CK2α in LAMA84 cells by performing RNA-interference experiments. A decrease of CK2α protein-level of about 52% and 80% was obtained in S- and R-LAMA84 cells, respectively (Figure 8A). Also CK2 activity was reduced, although to a lesser extent (about 30% and 56% in S- and R-LAMA84 cells, respectively) (Figure 8B), due to the contribution of the other catalytic subunit (α′) not affected by the silencing procedure. Moreover, CK2α down-regulation by siRNA greatly reduces the interaction occurring between CK2 and Bcr-Abl (Figure 8C) as previously shown in LAMA84 cells treated with the CK2-inhibitor CX-4945 (Figure 5A,B). When we treated control and CK2α down-regulated cells with increasing concentration of imatinib, we found that no significant effect on cell viability was induced in sensitive cells by CK2α silencing (Figure 8D). On the contrary, in imatinib-resistant cells, CK2α down-regulation promotes a higher sensitivity to low imatinib concentrations (Figure 8E), confirming the data obtained with the pharmacological blockade of CK2 (Figure 7A).

4. Discussion

In this study, we provide the first evidence that, among different adaptations described to be associated with imatinib-resistance, CK2-dependent signalling represents an additional mechanism that can be exploited to ensure survival

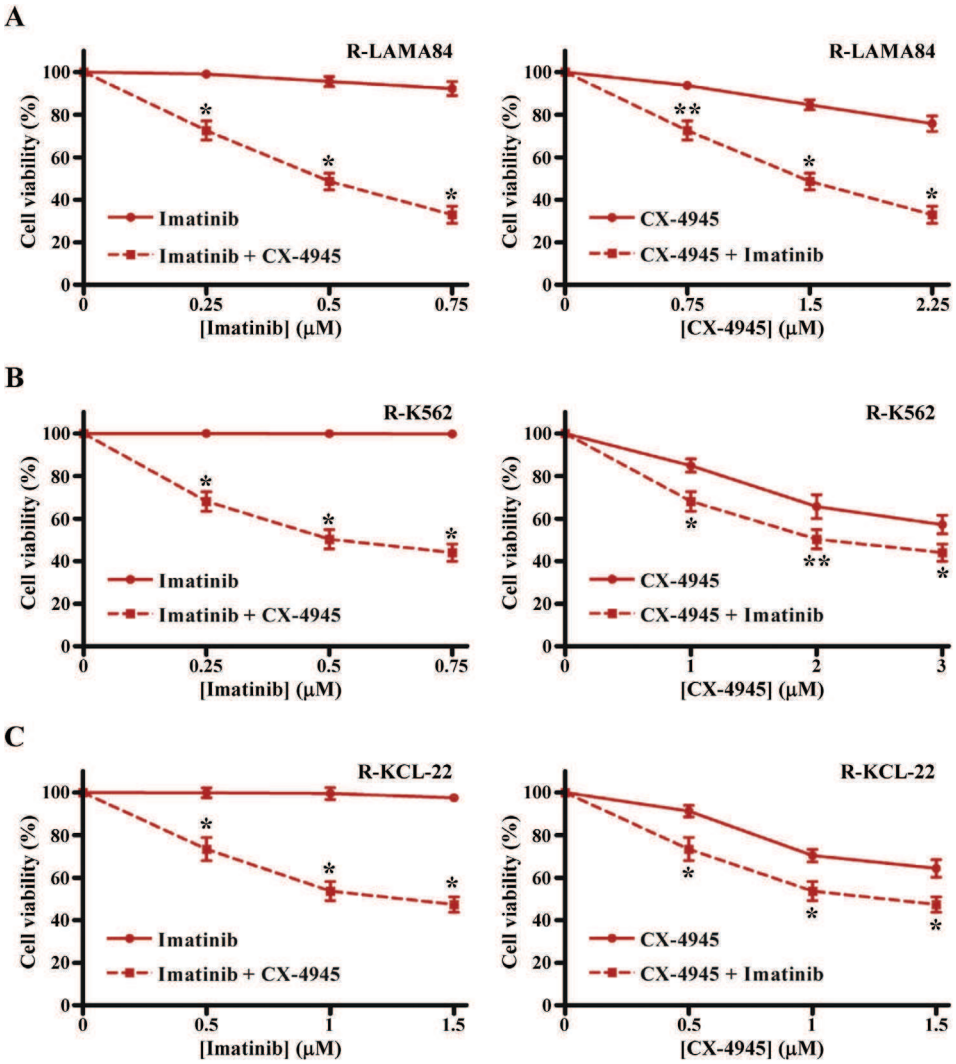

Figure 7 – Synergistic effect of CX-4945 and imatinib treatment on CML cell viability. (A) R-LAMA84, (B) R-K562 or (C) R-KCL-22 were treated for 48 h with the indicated concentration of imatinib, CX-4945 or with the two drugs in combination by increasing simultaneously the concentration of both compounds added at 1:3 (A), 1:4 (B), and 1:1 (C) imatinib:CX-4945 ratio. Viability, assessed by MTT method and expressed as percentage of controls, was plotted as function of imatinib concentration (left panel), or CX-4945 concentration (right panel). *$p < 0.01$, **$p < 0.05$ *vs* cells treated with a single inhibitor.

to CML cells. In particular, we found that in imatinib-resistant LAMA84 cell line, characterized by a *BCR-ABL1* gene amplification, CK2 is upregulated in comparison with the parental cell line. While CK2α′ is equally expressed, the level of CK2α and β subunits is about two-fold higher in R-LAMA84 than in sensitive cells (Figure 1A and B). CK2 protein increase, which is accompanied by a parallel increase of cellular CK2 catalytic activity (Figure 2), appears related to an altered regulation at protein level since the mRNA amount of CK2α is very similar in the two cell variants (Figure 1C). These results are in agreement with studies where abnormally high level of CK2 protein and activity in cancer cells is not accompanied by a parallel mRNA increase (Di Maira et al., 2007; Trembley et al., 2009).

It is noteworthy that CK2 has been found overrepresented in highly proliferating myeloblastic cells from CML patients in blast crisis (Phan-Dinh-Tuy et al., 1985), a phase in which Bcr-Abl overexpression has been associated to imatinib-resistance (Barnes et al., 2005; Gorre et al., 2001; Keeshan et al., 2001; Virgili and Nacheva, 2010). Considering the pro-survival function of CK2, it is conceivable that its increased level represents a device to escape apoptosis. Although CK2 up-regulation is not an absolute requirement for the resistant phenotype (Figure 1B and Di Maira et al., 2008), overexpression of CK2α, either alone or in combination with the β subunit has been already associated in other cancer cell lines with resistance mechanisms, either related to a multidrug resistance phenotype or induced by specific drugs (Di Maira et al., 2008; Matsumoto et al., 2001).

CK2 nuclear concentration has been reported to be particularly high in cancer cells (Trembley et al., 2009). In contrast, in S-LAMA84 and R-LAMA84 cells CK2 is mainly present in the cytoplasm (Figure 3B,C). We also show that CK2 and Bcr-Abl co-localize in the cytoplasm, where the CK2-targets related to imatinib-resistance are presumably placed.

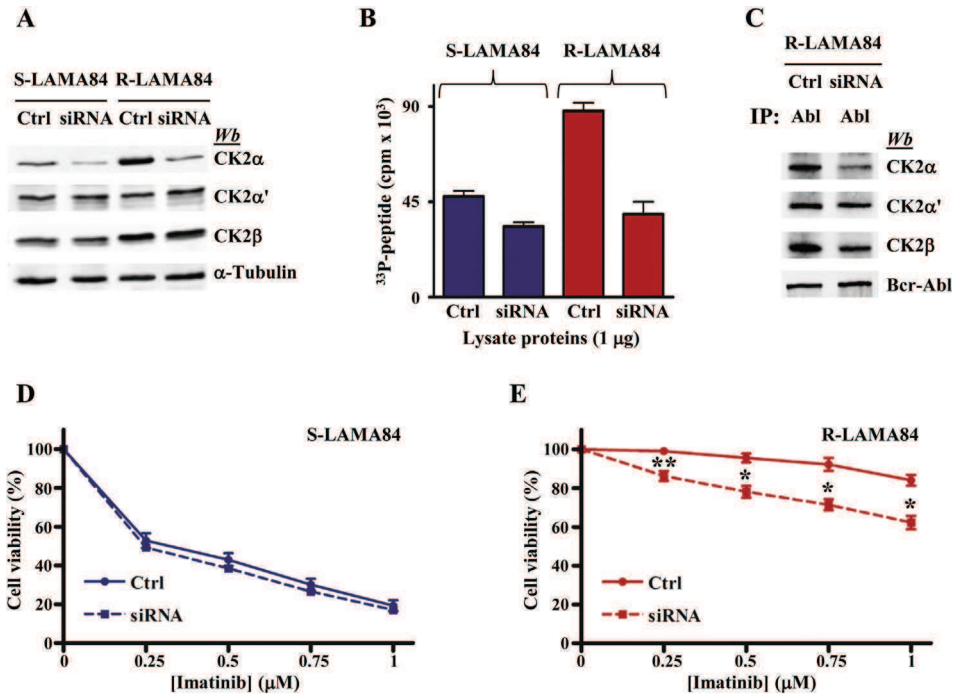

Figure 8 – Effect of CK2α knocking down by siRNA on LAMA84 sensitivity to imatinib. S-LAMA84 and R-LAMA84 cells were transfected with aspecific siRNA (Ctrl) or CK2α specific siRNA. (A, B and C) After 96 h, cells were lysed, and (A) 10 μg of lysate proteins were analysed by Western blot, (B) 1 μg of lysate proteins was tested for CK2 activity toward the specific peptide RRRADDSDDDDD, and (C) 300 μg of R-LAMA84 cell proteins were immunoprecipitated with anti-Abl antibody and then analysed by Western blot with the indicated antibodies. Panels A–C are representative of four separate experiments (D, E) 48 h after transfection, S-LAMA84 (D) or R-LAMA84 (E) cells were treated for 48 h with the indicated imatinib concentrations and cell viability was analysed by MTT method. *$p < 0.01$, **$p < 0.05$ *vs* control cells. Panels D and E are representative of five separate experiments performed in triplicate.

Phospho-proteomic analyses are in progress to identify the proteins whose phosphorylation, sensitive to CX-4945-inhibition, is evoked/increased in R-LAMA84 as compared to sensitive cells.

CK2 and Bcr-Abl co-localization reflects the finding that, in resistant LAMA84 cells, these two oncokinases are members of the same multi-protein complex(es) as demonstrated by their co-immunoprecipitation and co-sedimentation in glycerol-gradients (Figures 4A, 5C). CK2α and Bcr-Abl interaction is also detectable in the other CML cell lines analysed, K562 and KCL-22 (Figure 4Ac). Our results demonstrate that in the case of LAMA84 and K562 cells the two oncokinases interact more in imatinib-resistant than in sensitive cells (Figure 4Ac). The occurrence of an interaction between CK2 and Bcr-Abl has been previously described in cells overexpressing the two protein kinases and in lymphoblastic cells obtained from Bcr-Abl transgenic mouse (Hériché and Chambaz, 1998; Mishra et al., 2003). The region responsible for CK2 interaction was localized to residues 242–413 of the Bcr moiety of Bcr-Abl (Mishra et al., 2003).

In the attempt to detect reciprocal phosphorylation of the two protein kinases in CML cell lines we disclosed a Bcr-Abl-dependent Tyr-phosphorylation of CK2α, which is more evident in R-LAMA84 and R-K562 cells (Figure 4Ba), where also Bcr-Abl/CK2 interaction is higher (Figure 4Ac). The Tyr-phosphorylation of CK2α, however, is not required for the interaction occurring between the two kinases, which is not affected by imatinib. On the contrary, inhibition of CK2 almost abrogates the binding between the two enzymes (Figure 5).

A significant contribution of CK2 to chronic myeloid leukaemia is supported by data obtained from cell treatments with the highly selective CK2 inhibitor CX-4945, which affects neither the amount nor the activity of Bcr-Abl (Figure 4Bb). Indeed the viability of both imatinib-sensitive and -resistant CML cells is significantly reduced whenever CK2 activity is inhibited by CX-4945, consistent with the general anti-apoptotic and pro-survival role played by CK2 in cancer cells (Figure 6B and Zanin et al., 2012). Interestingly, CX-4945 added in combination with imatinib promotes a synergistic effect on the cell viability of imatinib-resistant CML variants, partially rescuing the response to imatinib. The synergism is especially evident in R-LAMA84 and R-K562 cells (Figure 7), where Bcr-Abl/CK2 interaction is also higher (Figure 4Ac). In this respect, we can hypothesize that the interaction occurring between CK2 and Bcr-Abl might be one of the molecular mechanisms reinforcing the imatinib-resistance but also offering the possibility to sensitize cells to imatinib by CK2 down-regulation and consequent binding disruption (Figure 5 for CK2 inhibition by CX-4945, and Figure 8C for CK2 knock-down by siRNA).

The hypothesis that imatinib-resistant cells become partially dependent on CK2 for their survival was confirmed by the observation that the CX-4945 concentrations required to induce apoptosis in R-LAMA84 cells are lower than those effective in S-LAMA84 cells (Figure 6B,D).

It has been proposed that the high CK2 level observed in cancer cells may generate an environment, which favours cancer progression by promoting/fostering multiple oncogenic pathways (Ruzzene and Pinna, 2010). Since some of these deregulated pathways are also under the control of Bcr-Abl (Perrotti et al., 2010; Quintás-Cardama and Cortes, 2009), CK2 might, on one hand, potentiate the Bcr-Abl oncogenic signalling and, on the other, strengthen the imatinib-resistant phenotype by activating key molecular events able to circumvent the drug inhibitory effects on Bcr-Abl pathways. Pertinent to this, we have found that Akt-signalling is reinforced in R-LAMA84 cells by the increased phosphorylation of the CK2 target-residue Ser129 (Figure 6C,D).

Imatinib is the first-line therapy for chronic myeloid leukaemia, but resistance to this drug frequently occurs and causes therapy failure. This study identifies the protein kinase CK2 as a player in CML imatinib resistance, where it supports the Bcr-Abl oncogenic potential conferring survival advantage against imatinib. Down-regulation of CK2 rescues the response to imatinib. We suggest that CK2 inhibitors, with special reference to CX-4945, a compound already in clinical trials for the treatment of different tumours, might represent promising drugs for combined strategies in CML therapy.

Conflict of interest

The authors declare no conflict of interest.

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