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CICLO: XXV

"OVEREXPRESSION OF THE PROTEIN KINASE CK2 INCREASES THE SURVIVAL AND RESISTANCE TO CHEMOTHERAPY OF ACUTE MYELOID LEUKEMIA CELLS"

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ABBREVIATIONS

AML	acute myeloid leukemia
CEBPA	CCAAT/enhancer-binding protein alpha
CK2	casein kinase 2
DAUNO	daunorubicin
DOX	doxorubicin
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELN	European leukemia network
FAB	French-American-british
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLT3	Fms-like tirosinKinase 3
NPM1	nucleophosmin 1
PCR	plymerase chain reactio
PARP	poly(ADP-ribose) polymerase.
TNFα	tumor necrosis factor α
WB	Western Blot
WHO	World Health Organization

ABSTRACT

Background: The critical role of protein kinase CK2 in the regulation of cellular apoptosis suggests its may be involvement in tumor cell resistance to both conventional and unconventional therapies.

Aim of the study: To study the role of CK2 in acute myeloid leukemia (AML) cell survival and response to chemotherapeutic agents.

Methods: Apoptotic pathways were evaluated by AnnexinV/Propidium Iodide staining, Western Blot analysis of Caspase3 and Parp cleavage and Real Time PCR of antiapoptotic gene expression.

Results: CK2 α catalytic subunit expression level and activity were increased in AML cells as compared to normal $CD34^+$ hematopoietic cells. CK2 inactivation with the synthetic chemical inhibitors K27, CX-4945 or RNA interference induced AML cell apoptosis of p53 wild-type but not of p53-null cells, suggesting that the apoptosis triggered by CK2 inhibition needs the presence of functional p53. Inhibition of CK2 activity with K27 or CX-4945 was associated to an increased sensitivity of the cytotoxic effects of doxorubicin and daunorubicin. Cells were also nucleofected with siRNA oligos directed against CK2 α catalytic, β regulatory or both subunits. Interestingly RNA interference of CK2 β□□ reduced cell viability and enhanced apoptosis induced by daunorubicin, indicating a prominent role of this subunit in the resistance to chemotherapy.

Based on our *in-vitro* results we have then examined the expression level of CK2 α and β in 32 newly diagnosed AML patients (excluding acute promyelocitic leukemia) from 2008 to 2011. Preliminary data shows a higher expression of the CK2 α subunit in the two AML subgroups with the highest risk based on cytogenetic characteristics. These laters nowadays represent the strongest prognostic factor predicting chemoresistance. Interestingly we did not find a correlation with expression of the regulatory subunit CK2 β.

Conclusions: These data highlight the relevance of CK2 in AML cell survival for the influence of this kinase on the activation of anti-apoptotic pathways implicated in AML cell resistance to chemotherapy.

A study to establish wether the CK2 expression at diagnosis represents an independent prognostic factor in the progression free survival and overall survival for these patients is needed.

RIASSUNTO

Numerose evidenze supportano un ruolo fondamentale della protein chinasi CK2 nella regolazione dell'apoptosi e della proliferazione cellulare nell'ambito dei tumori solidi. Ad oggi, tuttavia, un possibile ruolo di CK2 anche nella patogenesi della leucemia mieloide acuta (LMA) risulta ancora incerto. Nel presente lavoro sperimentale abbiamo testato l'ipotesi che CK2 potesse avere un ruolo nel modulare la resistenza all'apoptosi e quindi la sopravvivenza delle cellule di LMA sia in condizioni basali sia in risposta ad agenti chemioterapici. Abbiamo dimostrato che i livelli di espressione ed attività di CK2 sono aumentati nelle cellule di LMA rispetto alla controparte normale costituita dalle cellule staminali $CD34^+$. Studi di localizzazione intracellulare ci hanno consentito di osservare una concentrazione preferenziale di CK2 a livello citoplasmatico. Abbiamo dimostrato che il blocco dell'attività chinasica determina un aumento di espressione della proteina p53, la mancanza della quale rende le cellule di LMA resistenti all'apoptosi indotta dagli inibitori di CK2. Ipotizzando un ruolo di CK2 nella chemioresistenza delle cellule di LMA, abbiamo dimostrato che il blocco dell'attività di CK2 incrementa la sensibilità di tali cellule al trattamento con chemioterapici quali doxorubicina e daunorubicina.

Sulla base dei risultati ottenuti *in vitro* abbiamo analizzato l'espressione di CK2 α e β in 32 pazienti con leucemia mieloide acuta di nuova diagnosi (ad esclusione della leucemia promielocitica) dal 2008 al 2012. Risultati preliminari mostrano come CK2 α sia più elevata nei pazienti appartenenti a gruppi a piu' alto rischio in base alla classificazione dell' European Leukemia Network (ELN) e la sua aumentata espressione, seppur non in modo statisticamante significativo, appaia correlata ad una peggior overall survival nel gruppo omogeneo di pazienti sottoposto a chemoterapia. Per la subunità β al contrario non è risultata alcuna correlazione.

Possiamo concludere che CK2 interviene nella sopravvivenza delle cellule di LMA modulandone la resistenza all'apoptosi in maniera p53 dipendente e l'aumentata espressione della subunità catalitica α alla diganosi puo' rappresentare un nuovo fattore prognostico in questa patologia così eterogenea. Il suo ruolo all'interno delle attuali classificazioni dovrà essere validato in futuro su un'ampia casisitica di pazienti.

INTRODUCTION

Acute myeloid leukemia

Leukemia is a heterogenous group of diseases characterized by clonal cells that exhibit maturation defects that correspond to stages in hematopoietic differentiation. Hematopoietic stem cells are multipotent and have the capacity to differentiate into the cells of all 10 blood lineages — erythrocytes, platelets, neutrophils, eosinophils, basophils, monocytes, T and B lymphocytes, natural killer cells, and dendritic cells. In order to sustain hematopoiesis, stem cells are part of a developmental hierarchy capable of three basic functions:

- Maintenance in a non-cycling state (ie, not actively progressing through the cell cycle)
- Self-renewal, allowing production of additional stem cells
- Production of committed progenitor cells

These progenitor cells commit to subsets of myeloid and lymphoid lineages, and ultimately to single developmental pathways, resulting in the expression of the terminally differentiated stage of each cell type

Acute myeloid leukemia (AML) develops as the consequence of a series of genetic changes in a hematopoietic precursor cell. These changes alter normal hematopoietic growth and differentiation, resulting in an accumulation of large numbers of abnormal, immature myeloid cells in the bone marrow and peripheral blood. These cells are capable of dividing and proliferating, but cannot differentiate into mature hematopoietic cells.

Incidence

Acute myeloid leukemia represents the commonest acute leukemia in adults; 3 per 100.000 annually. The frequency increases with the age (median 64; incidence 35/100.000 at age 90). 66% are > 60 years of age.

Classification

The FAB classification

In the 1970s, a group of French, American, and British leukemia experts divided acute myeloid leukemias into subtypes, M0 through M7, based on the type of cell from which the leukemia developed and how mature the cells are (1). This was based largely on the microscope analysis after routine staining.

Subtypes M0 through M5 all start in precursors of white blood cells. M6 AML starts in very early forms of red blood cells, while M7 AML starts in early forms of cells that make platelets.

The WHO classification

The current WHO classification (*table 1*) established in 2008 reflects the fact that an increasing number of acute leukemias can be categorized based upon their underlying cytogenetic or molecular genetic abnormalities, and that these genetic changes form clinico-pathologic-genetic entities (*Table 2*) (2,3).The subgroup "AML with recurrent genetic abnormalities" comprises several primary AML entities. "AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*" and "AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*" are considered as AML regardless of bone marrow blast counts. In "APL with t(15;17)(q22;q12); *PML-RARA*," *RARA* translocations with other partner genes are recognized separately. The former category "AML with 11q23 (*MLL*) abnormalities" was redefined in that "AML with t(9;11)(p22;q23); *MLLT3-MLL*" is now a unique entity; balanced translocations other than that involving *MLLT3* should be specified in the diagnosis. Three new cytogenetically defined entities were incorporated: "AML with t(6;9)(p23;q34); *DEK-NUP214*"; "AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*"; and "AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*," a rare leukemia most commonly occurring in infants. Two new provisional entities defined by the presence of gene mutations were added, "AML with mutated *NPM1* [nucleophosmin (nucleolar phosphoprotein B23, numatrin)]," and "AML with mutated *CEBPA* [CCAAT/enhancer binding protein (C/EBP), alpha]." There is growing evidence that these 2 gene mutations represent primary genetic lesions (so-called class II mutations)(4) that impair hematopoietic differentiation. Mutations in the fms-related tyrosine kinase 3 (*FLT3*) gene are found in many AML subtypes and are considered class I mutations conferring a proliferation and/or survival advantage. AML with *FLT3* mutations are not considered a distinct entity, although determining the presence of such mutations is recommended by WHO because they have prognostic significance. The former subgroup termed "AML with multilineage dysplasia" is now designated "AML with myelodysplasia-related changes." Dysplasia in 50% or more of cells, in 2 or more hematopoietic cell lineages, was the diagnostic criterion for the former subset. AMLs are now categorized as "AML with myelodysplasia-related changes" if (1) they have a previous history of myelodysplastic syndrome (MDS) or myelodysplastic/myeloproliferative neoplasm (MDS/MPN) and evolve to AML with

a marrow or blood blast count of 20% or more; (2) they have a myelodysplasia-related cytogenetic abnormality or (3) if 50% or more of cells in 2 or more myeloid lineages are dysplastic. “Therapy-related myeloid neoplasms” has remained a distinct entity; however, since most patients have received treatment using both alkylating agents and drugs that target topoisomerase II for prior malignancy, a division according to the type of previous therapy is often not feasible. Therefore, therapy-related myeloid neoplasms are no longer subcategorized. Myeloid proliferations related to Down syndrome are now listed as distinct entities.

Categories
Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
APL with t(15;17)(q22;q12); *PML-RARA**
AML with t(9;11)(p22;q23); *MLLT3-MLL*†
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
Acute myeloid leukemia with myelodysplasia-related changes‡
Therapy-related myeloid neoplasms§
Acute myeloid leukemia, not otherwise specified (NOS)
Acute myeloid leukemia with minimal differentiation
Acute myeloid leukemia without maturation
Acute myeloid leukemia with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis (syn.: acute myelofibrosis; acute myelosclerosis)
Myeloid sarcoma (syn.: extramedullary myeloid tumor; granulocytic sarcoma; chloroma)
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (syn.: transient myeloproliferative disorder)
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); *BCR-ABL1*‖
Mixed phenotype acute leukemia with t(v;11q23); *MLL* rearranged
Mixed phenotype acute leukemia, B/myeloid, NOS
Mixed phenotype acute leukemia, T/myeloid, NOS
Provisional entity: Natural killer (NK)–cell lymphoblastic leukemia/lymphoma

Table 1: The WHO CLASSIFICATION 2008

European leukemia network (ELN) classification

In 1990, 2003 and more recently in 2010 expert working groups published recommendations for diagnosis, standardization of response criteria and treatment outcomes and reporting standards for clinical trials in AML (24,25,23). These are widely adopted in general practice, within clinical trials and by regulatory agencies. In 2010 the panel proposed a standardized reporting system for genetic abnormalities presenting data correlating genetic findings with clinical outcome allowing for a better comparison of data among studies (*table 2*). This standardized report includes data from cytogenetic analysis and from mutation analysis of the NPM1, CEBPA and FLT3 genes.

Genetic group	Subsets
Favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD (normal karyotype) Mutated *CEBPA* (normal karyotype)
Intermediate-I*	Mutated *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); *MLLT3-MLL* Cytogenetic abnormalities not classified as favorable or adverse†
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* t(6;9)(p23;q34); *DEK-NUP214* t(v;11)(v;q23); *MLL* rearranged −5 or del(5q); −7; abnl(17p); complex karyotype‡

Table 2: AML classification propsed by the European Leukemia network (ELN classification)

Prognostic factors

Prognostic factors may be subdivided into those related to patient characteristics and general health condition and those related to characteristics particular to the AML clone. The former subset usually predicts treatment-related mortality (TRM) and becomes more important as patient age increases while the latter predicts resistance to, at least, conventional therapy.

Patient-related factors

Increasing age is an adverse prognostic factor (5). Even after accounting for risk factors, such as cytogenetics, molecular genetics, type of AML (ie, de novo AML; AML with previous history of MDS or MDS/MPN; therapy-related AML), and performance status, older patients have worse outcomes than younger patients, suggesting the effect of unknown age-related factors. Nonetheless, age is not the most important prognostic factor for either TRM or resistance to therapy. Attention should be given to a careful evaluation and documentation of comorbidities. In a recent study of patients older than 60 years of age receiving induction therapy (idarubicin 12 mg/m^2 for 3 days, cytarabine 1.5 g/m^2 for 3 days), scoring of baseline comorbidities using the hematopoietic cell transplantation comorbidity index (HCTCI) was predictive of early death rates and overall survival (OS) (6).

AML-related factors

AML-related prognostic factors includes white blood count (WBC), existence of prior MDS, previous cytotoxic therapy for another disorder and cytogenetic and molecular genetic changes in the leukemic cells at diagnosis. Various other factors, such as splenomegaly and elevated serum lactate dehydrogenase (LDH) levels, have been reported to confer some prognostic effect but with variable consistency among studies. The significance of a prognostic factor is always dependent on the therapy given to a patient.

Cytogenetics

The karyotype of the leukemic cells is the strongest prognostic factor for response to induction therapy and for survival (7,8). Younger adult patients are commonly categorized into 3 risk groups, favorable, intermediate or adverse (9). The most appropriate risk group assignment for a number of the rarer cytogenetic abnormalities, for example, del(7q), isolated trisomy 8, del(9q), t(v;11)(v;q23) other than t(9;11), and del(20q), remains uncertain due to limitations of sample size and differences in treatment schedule among studies. Complex karyotype, which occurs in 10% to 12% of patients, has consistently been associated with a very poor outcome (10). Complex karyotype has been defined as the presence of 3 or more (in some studies ≥ 5) chromosome abnormalities in the absence of t(8;21), inv(16) or t(16;16), and t(15;17), because in most studies increased karyotype complexity in these subgroups did not

adversely affect outcome. As indicated in the new WHO classification, cases with other recurring genetic abnormalities, such as AML with t(9;11) or t(v;11), AML with inv(3) or t(3;3), and AML with t(6;9) should also be excluded, because these groups constitute separate entities (3). Recently, a new cytogenetic category has been proposed that distinguishes AML of particularly unfavorable risk, that is, the monosomal karyotype (11). In this study, the monosomal karyotype was defined by the presence of one single monosomy (excluding isolated loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding core-binding factor [CBF] AML). One striking observation is the increasing incidence of adverse versus favorable cytogenetic abnormalities with increasing age. This, at least in part, contributes to the poorer outcome of AML in older adults (5,12).

Molecular genetics

Gene mutations and deregulated gene expression have been identified that allow us to decipher the genetic diversity within defined cytogenetic groups, in particular the large and heterogeneous group of patients with CN-AML (*Fig.1*) (13,14,15). Prognostic significance within CN-AML has consistently been shown for mutations in the *NPM1*, *CEBPA*, and *FLT3* genes alone or in combination in younger adult patients. CN-AML patients harboring internal tandem duplication (ITD) of the *FLT3* gene have an inferior outcome compared with cases without *FLT3*-ITD (15,16,17). In several, but not all studies, the presence of *NPM1* mutation in CN-AML has been associated with higher CR rates and better RFS and event-free survival (EFS) (18,19). Of note, approximately 40% of patients with *NPM1* mutations also carry *FLT3*-ITD, and multiple studies have shown that the genotype "mutated *NPM1* without *FLT3*-ITD" represents a favorable prognostic marker, with higher CR rates, and better RFS and OS that is reminiscent of that seen in patients with inv(16) or t(8;21).(15,18-20). CN-AML with mutations in *CEBPA* is another subset that has been associated with a favorable prognosis (15,21). The survival data are very similar to those of AML patients with mutated *NPM1* without *FLT3*-ITD. Two recent studies suggest that there is heterogeneity among mutated *CEBPA* cases, in that only cases with double mutations, usually biallelic, have a favorable outcome (22). It remains an open question whether the presence of a *FLT3*-ITD impacts on prognosis in patients with mutant *CEBPA* (15). In cytogenetically favorable CBF AML [ie, AML with t(8;21) or inv(16)/t(16;16)], the presence of a *KIT* mutation has been shown to have an unfavorable

influence on outcome in retrospective studies. There is a growing list of genetic abnormalities that are being investigated: these include mutation analyses of the *WT1*, *RUNX1*, *TET2*, and *IDH1* genes, and the analyses of gene expression signatures, or of deregulated expression of single genes, such as *EVI1*, *ERG*, *M N1*, and *BAALC* genes (23).

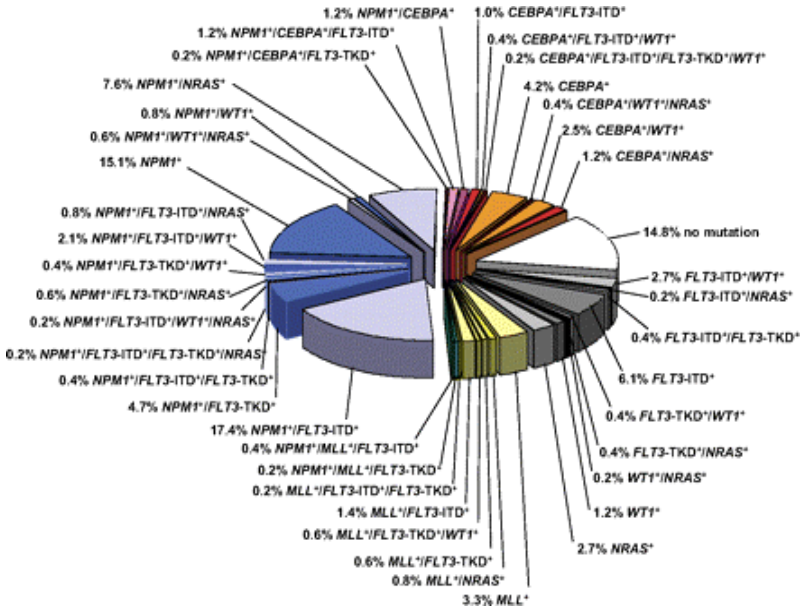
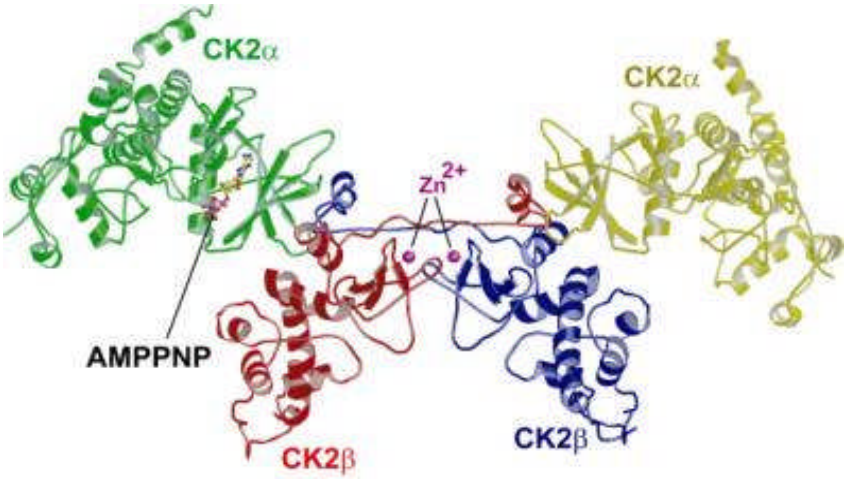


Figure 1: **Pie chart illustrating the molecular heterogeneity of cytogenetically normal AML based on mutations in the *NPM1*, *CEBPA*, *MLL*, *FLT3* (ITD and TKD mutations at codons D835 and I836), *NRAS*, and *WT1* genes**.
The bluish colors denote *NPM1*-mutated subsets, the orange/red colors *CEBPA*-mutated subsets, and the yellow/green colors *MLL*-mutated subsets. The gray colors depict subsets without hypothetical class II mutations, and the white sector shows the subset without any mutation in the above-mentioned genes. Data are derived from mutational analysis of 485 younger adult patients with cytogenetically normal AML from AMLSG.

TP53 mutations in acute myeloid leukemia

In AML TP53 alterations (mutations and/or losses) have been closely associated with AML with complex karyotype (CK-AML), occurring in approximately two-thirds of the cases. Clinically TP53 alterations appear to be associated with inferior outcome. In a recent analysis of 234 AML patients, cases with TP53-altered were characterized by a higher degree of genomic complexity TP53-altered more frequent exhibited a monosomal cariotype. Patients with TP53 alterations were older and had a significantly lower complete remission rates, inferior event-free and overall survival (26).

Protein kinase CK2



Protein kinase CK2 is a ubiquitous serine-threonine kinase involved in a multitude of cellular processes. CK2 is a tetramer enzyme composed most often by two catalytic subunits (α or α', encoded by separate genes) and two molecules of the regulatory subunit (β), so that the possible species in the cells are $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ (27). CK2 phosphorylates a great number of substrates with disparate functions (28). Deletion of CK2 α and β in mice is embryonic lethal (29) and knock out of CK2 α' results in globozoospermia and other defects (30).

A remarkable feature of CK2 is the frequent overexpression and high kinase activity displayed in several types of malignant solid tumors. Indeed, CK2 has been demonstrated to contribute to the malignant phenotype and tumor progression in mouse models as well as in human cancer cells (31). In this regard, a peculiar property of CK2 is the ability to protect cells from apoptosis (32). This action is believed to be achieved through several mechanisms. For instance, CK2 interferes with tumor suppressor Promyelocytic leukemia (PML) and PTEN protein stability and function by phosphorylating critical serine residues on these proteins and rendering them less active: in the case of PML through enhanced proteasome-mediated degradation, in the case of PTEN through the stabilization of a less active form of the molecule (33,34).

CK2 phosphorylation of anti-apoptotic molecules also contributes to protection from apoptosis. CK2 targets Apoptosis Repressor with Caspase Recruiting domain (ARC), shifting the molecule to the mitochondria where it inhibits caspase 8 (35). In addition CK2 phosphorylation of BID protects it from caspase 8 cleavage and cell death (36) and positively regulates growth-promoting cascades, such as the

PI3K/AKT (37), the NF-kB and the Wnt/β-catenin signaling pathways with the result of strongly directing cell fate towards survival and against programmed cell death (38).

Interestingly, a recently proposed unifying model for CK2 function relies on the regulation of the Cdc37/Hsp90 chaperone complex through S13 phosphorylation on Cdc37 (39). This modification is essential for the chaperoning activity of Hsp90 directed towards an array of client protein kinases, many of which are oncogenic. CK2 has also been involved in the cellular DNA damage response, since it was shown that this kinase can regulate both single strand and double strand DNA break repair, by facilitating the XRCC1 function (40) and the UV light response by activating the NF-kB pathway and phosphorylating the high mobility group protein SSRP1 (41,42).

Taken together, the established role played by CK2 in tumorigenesis, could rely on the extraordinary property of this kinase to "addict" cells towards an apoptosis-resistant, proliferation and DNA damage repair-prone-phenotype (42). Whereas CK2 expression and activity of in a number of solid tumors are more defined, its function in blood cancers is less understood.

The JAK STAT pathway

The JAK-STAT pathway is crucial in transmitting signals from many cytokines and growth factors into the nucleus, regulating gene expression. Cytokines of the IL-6 family, type I and II IFNs, and growth factors such as growth hormone (GH) activate the JAK-STAT signaling pathway. Oncostatin M (OSM), a cytokine belonging to the IL-6 family, is a potent activator of the JAK-STAT signaling pathway (43). Binding of OSM induces heterodimerization of its receptors, gp130 and OSMRβ, and the receptor-associated JAKs, JAK1 and JAK2, become activated, leading to phosphorylation of gp130 tyrosine residues. The phosphorylated residues direct the recruitment of STAT proteins, including STAT-3, STAT-1, and STAT-5, which in turn become JAK substrates. Activated tyrosine–phosphorylated STATs form homodimers or heterodimers, translocate to the nucleus, and bind to consensus sequences in the promoters of OSM-responsive genes, inducing transcription (44). The JAK tyrosine kinase family comprises 4 mammalian members: JAK1, JAK2, JAK3, and TYK2. JAK2 is essential in erythropoiesis (45), and its dysfunction has been implicated in myeloproliferative disorders (MPDs) and leukemias (46). Both CK2 and JAK-STAT signaling play pivotal roles in cell survival, proliferation, and antiapoptotic

mechanisms, and their dysregulation is associated with human malignancies. However, little is known of potential cross-talk between CK2 and the JAK-STAT pathway. Recently Zheng et al demonstrated in primary or immortalized mouse embryonic fibroblasts (MEFs) that silencing of CK2α or CK2β expression led to reduced OSM-induced STAT-3 activation. The inhibitory effect was most pronounced when both CK2α and CK2β were diminished in expression. The same effect was shown using CK2 chemical inhibitors such as TBB, whereas the inhibitory effect of TBB on STAT-3 activation was abolished by introducing HA-CK2α-inhibitor resistant concluding that CK2 expression and activity is required for OSM-induced STAT-3 tyrosine phosphorylation (47).

METHODS

Primary AML blasts, AML cell lines and cultures

Patients were charged to the University of Padova Hospital. Informed consent was obtained from patients according to the declaration of Helsinki. AML blasts from peripheral blood (PB) and bone marrow (BM) were obtained from PB and BM aspirates of patients and processed as described (48). Acute myeloid leukemia NB4 (acute promyelocytic leukemia, FAB M3), HL60 (acute myeloblastic leukemia, FAB M2), ML2 (acute myelo-monocytic leukemia, FAB M4), K562 (Philadelphia-positive chronic myeloid leukemia in blast crisis), human osteosarcoma Saos-2 and human embryo kidney HEK293 and human bone marrow HS-5 cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) and the American Type Culture Collection (Rockville, USA). Normal $CD34^+$ haematopoietic stem cells (HSC) were obtained from PB or BM healthy donors. In co-culture experiments, AML blasts from patients were seeded on top of a monolayer of HS-5 stromal cells. After the indicated time points and treatments, AML blasts were gently removed from the HS-5 cells by washing with PBS and subsequently processed for downstream analysis. Cell lines were maintained in RPMI 1640 or DMEM medium, both supplemented with 10% fetal bovine serum, L-glutamine, antibiotics (Gibco Laboratories, Grand Island, NY, USA) under controlled-atmosphere in incubators at 37 °C in the presence of 5% CO_2.

Western blot (WB) and antibodies

Whole cell extracts were prepared by lysis with 20 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% Triton X-100 supplemented with complete protease inhibitor cocktail (Complete Mini; Roche, Basel, Switzerland), 1 mM dithiothreitol (DTT; Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom), 1 mM phenyl-methyl-sulfonyl fluoride (PMSF; Sigma-Aldrich), 10 μM sodium fluoride (Sigma-Aldrich), 1 μM okadaic acid, and 1 mM sodium orthovanadate (Calbiochem, San Diego, CA). 40 to 60 μg of whole cell extracts (WCE) or nuclear and cytoplasmic sub-fractions were subjected to SDS-PAGE and processed by immuno-blot. Detection was performed using chemiluminescence reaction (Pierce, USA). Proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the following primary antibodies: anti-CK2 α-subunit

rabbit antiserum raised against the [376-391] region of human protein[38]; anti-CK2β (BD Bioscences, USA); anti-PARP and anti-SMAC/DIABLO, anti-STAT3, phospho-Tyr702-STAT3, and phospho-Ser727-STAT3 (Cell Signaling, Beverly, MA); anti-caspase-3, anti-caspase-9, and anti-caspase-8 (Calbiochem-Merck Biosciences, Bad Soden, Germany); anti-SOCS3 (Cell Signaling Technology, Beverly, MA, USA) and anti-p65 (CT) (Upstate Biotechnology, Lake Placid, NY). As protein normalization:GAPDH (Ambnon) and β-actina (Sigma-Aldrich, Germania) were used.

Detection was performed by ECL chemiluminescence reaction according to the manufacturer's instructions (Amersham Biosciences). When indicated, densitometry was performed with ImageJ 1.34 S software (Apple, Cupertino, CA).

Cytokines and chemicals

- Daunorubicin, Doxorubicin and MG132 were respectively purchased from Pfizer and Sigma-Aldrich, Italy.
- CK2 inhibitors: K27 purchased from Dott. Z. Kazimierczuk (Warsaw, Poland) and CX-4945 (Activate Scientific GmbH).

CK2 activity in cell lysates

CK2 activity was determined as previously described (48). The CK2 activity was in a linear range according to the lysate protein amount used for the assay.

Evaluation of growth and apoptosis

AML cell growth was monitored using the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide)-based assay, according to the manufacturer’s protocol (Roche, Monza, Italy) and as described elsewhere (48). Apoptosis was assessed by Annexin V/Propidium Iodide staining (BD Pharmingen) as per manufacturer’s instructions using a FACScalibur cytofluorometer with the CellQuest® analytic software (Becton Dickinson).

Immunofluorescence and confocal microscopy

Preparation of cell samples was done as described (48). For confocal imaging, a Nikon Eclipse TE300 inverted microscope equipped with a PerkinElmer Ultraview LCI confocal system was employed; excitation was performed using the appropriate laser lines. Magnification was set at 600x, using an oil immersion objective. Antibodies used were: ant-CK2α described above, Alexa Fluor® 594 goat anti-rabbit (Molecular Probes Europe, The Netherlands).

Transient transfection and luciferase assays

For luciferase assay experiments, HEK293 cell lines at 3 x 10^5 cells/well were transfected with an NF-κB luciferase reporter plasmid (Stratagene, La Jolla, CA) using the FuGENE transfection reagent (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's instructions and as described elsewhere (48). The Dual Luciferase Reporter System (Promega, Madison, WI) was used according to the supplier's protocol. Experiments were performed in triplicate or quadruplicate. In p53 overexpression experiments p53 null Saos-2 cells were transfected with the empty pCMV or pCMV-p53WT vectors (kind gift of Dr. P.P. Pandolfi, Harvard University, MA, USA) using the Lipofectamine reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions.

Quantitative RT-polymerase chain reaction

Total RNA was extracted using the RNAeasy Mini Kit Protocol (Qiagen, Hamburg, Germany) and complementary-DNA was synthesized using the Reverse Trascription System (Promega Corporation, Madison, WI, USA), according to the manufacturers' instructions. Real time PCR reactions were carried out on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed with SYBR Green PCR Master Mix (Invitrogen Corporation, Carlsbad, CA, USA). β-actin was used as reference gene for the adjustment of relative expression data. All assays were performed at least twice to ensure their reproducibility, and a negative control was included in each run. Oligonucleotides used are the following: β-actin Forward 5'-CCAGCTCACCATGGATGATG-3' and

Reverse 5'-ATGCCGGAGCCGTTGTC-3', CK2α Forward 5'-TCTGAAGGCCCTGGATTATTGT-3' and Reverse 5'-TCGAGAGTGTCTGCCCAAGATAT-3'.

RNA interference (RNAi)

For RNA interference the electroporator Nucleofector (Amaxa Biosystems) and the kit (Lonza, Switzerland) for electroporation was used. For transfection of specific-CK2α smart pool interfering RNA (siRNA: GCAUUUAGGUGGAGACUUC; GGAAGUGUGUCUUAGUUAC; GCUGGUCGCUUACAUCACU; AACAUUGUCUGUACAGGUU) and of CK2β (siRNA: CAACCAGAGUGACCUGAUU; GCAAGGAGACUUUGGUUAC; GCAAUGAAUUCUUCUGUCA; CCAAGUGCAUGGAUGUGUA) were purchased from Thermo Scientific. The cell suspension (2×10^6 leukemia cells) was immediately electroporated by according to the manufacturer's instructions. Immediately after electroporation, the cells were suspended in the complete medium and incubated in a humidified 37°C/5% CO_2 incubator. The cells were harvested after variable times and used for the experiments.

Drug Combination Studies

To study the effect of the use of chemotherapeutic agents and CK2 inhibitors the combination index (CI) theorem of Chou Talalay that offers quantitative definition for additive effect (CI=1), synergism (CI < 1) and antagonism (CI > 1) in drug combinations was applied (49). This method also provides algorithms for automated computer simulation for synergism and/or antagonism at any effect and dose level. To assess cell growth the method of tritiated thymidine was used.

Patients

A total of 32 primary untreated AML patients were included in this study. The diagnosis was made in our Unit between January 2008 and July 2012. Pretreatment cytogenetic analysis of bone marrow was done in all patients considering at least 25 metaphases. The analysis of NPM1 and FLT3 was performed in 23 patients (Dott.sse Bonaldi e Bertorelle, Istituto Oncologico Veneto). The expression of CK2 α and CK2 β

in the bone marrow or in the peripheral blood was analysed at diagnosis as above described. AML samples selected for expression profile studies presented a blasts percentage of at least 80%. All patients were evaluated for CK2 β expression, 27 patients for CK2 α. The caracteristics of patients are described in the *table 3*:

Patient	M/F	age at diagnosis	Pretreatment cytogenetic analysis	NPM1/FLT3 mutation	ELN prognostic group	Chemotherapy
1	M	59	46,XY	mut NPM1	fav	yes
2	F	45	46,XX	mut NPM1	fav	yes
3	F	61	46,XX	mut NPM1	fav	yes
4	M	21	46,XY inv 16	nd	fav	yes
5	M	44	46,XY	mut CEBPA	fav	yes
6	M	44	46,XY	mut NPM1	fav	yes
7	F	64	46,XX, t(8,21)	no mutation	fav	yes
8	M	59	46,XY	mutNPM1/FLT3 ITD	int-1	yes
9	M	66	46,XY	mutNPM1/FLT3 ITD	int-1	yes
10	M	21	46,XY	no mutation	int-1	yes
11	F	76	46,XX	mutNPM1/FLT3 ITD	int-1	no
12	M	51	46,XY	FLT3 ITD	int-1	yes
13	M	71	46,XY	FLT3 ITD	int-1	no
14	F	60	46,XY	mutNPM1/FLT3 ITD	int-1	yes
15	M	63	46,XY	no mutation	int-1	yes
16	F	34	46,XX	FLT3 ITD	int-1	yes
17	F	60	46,XX	no mutation	int-1	yes
18	M	77	46,XY	nd	int-1	no
19	M	62	46,XY	mutNPM1/FLT3 ITD	int-1	yes
20	M	55	46,XY	no mutation	int-1	yes
21	F	80	46,XX	nd	int-1	yes
22	F	81	46,XX	nd	int-1	no
23	M	73	46,XY,t(12;17)(p13;q21)[9]/46,XY[1]	FLT3 ITD	int-2	yes
24	F	77	46,XX,del(1)(p33)[6]/46,XX[1]	mut NPM1	int-2	no
25	M	41	46,XY,+21[9]/46,XY[16]	no mutation	int-2	yes
26	M	60	47,XY,+19[13]/47,XY,+del(1)(p13)[3]/46,XY[9]	nd	int-2	yes
27	M	41	46,XY,der(8)t(8;?)(p11;?)[20]	no mutation	int-2	yes
28	M	84	46,XY,del(7)(q22)[18]/46,XY[7]	nd	unfav	no
29	F	20	>3 alterations	nd	unfav	yes
30	M	50	>3 alterations	no mutation	unfav	yes
31	F	77	46,XX, -7,+8[22]/46,XX[2]	nd	unfav	no
32	F	86	>3 alterations	nd	unfav	no

Table 3: Patients' characteristics

Statistical analysis

Data obtained were evaluated for their statistical significance with the two-tail paired Student's *t* test. Values were considered statistically significant at *p* values below 0.05. Patients were divided into two groups (CK2α-high versus CK2α-low) according to the levels of constitutive expression of CK2α protein in pretreatment AML samples. The CK2α-high cases were arbitrary defined as cases showing the ratio of (CK2α absorbance/GAPDH absorbance) > 0,75. The same arbitrary cut off was adopted for the β subunit. The overall survival probability was calculated using the Kaplan-Meier method. Log-rank statistics were used to test the difference in survival times between groups.

RESULTS

Expression levels and kinase activity of CK2 are increased in AML cells

Since CK2 is critically involved in cell survival and its protein levels have been found increased in an array of solid tumor cells, we asked whether a common hematologic malignancy, like AML, could also present alterations in CK2 protein levels and activity. We first analyzed CK2α and CK2β expression in different AML cell lines, including K562, NB4, HL60 and ML2. Western blot (WB) analysis was performed on total protein lysates using a rabbit polyclonal antibody recognizing the CK2α C-terminal region, previously characterized (50). As control, $CD34^+$ healthy hematopoietic stem cells were used. All AML cell lines analyzed showed significantly higher levels of CK2α protein as compared to $CD34^+$ control cells. Among the different AML cell lines, the K562 displayed the highest CK2α levels (up to fourteen-fold more as compared to $CD34^+$ cells); NB4 and ML2 showed intermediate (up to seven-fold more as compared to $CD34^+$ cells) and HL60 cells the lowest CK2α levels (*Fig. 1A*). We next looked at whether CK2α mRNA levels paralleled the trend of the protein levels. Indeed, CK2α mRNA expression, as evaluated by qRT-PCR, displayed a trend fairly reproducing that showed by the protein (*Fig. 1B).*

We then analyzed CK2α subcellular localization in AML cells. To this aim confocal microscopy immunofluorescence experiments were performed using the ML2 AML cell line. CK2α was detectable mostly in the cytoplasm of AML cells, in which high fluorescence intensity was observed *(Fig.1C).*

To test whether cellular levels of CK2α paralleled its kinase activity, we measured CK2α-directed kinase activity using a radioactive kinase assay against a specific synthetic peptide, in AML and control cells *(Fig. 1D*). Rather surprisingly, however, we could not detect a correlation between the observed CK2α protein levels and its kinase activity. In fact, when normalized against the total CK2α protein levels (*Fig. 1E*), our data showed that cells with the highest CK2 protein levels, namely K562, displayed a CK2α-specific kinase activity comparable with that of control $CD34^+$ cells, whereas significantly higher CK2α-specific kinase activity was detected in protein extracts from NB4 and ML2 cells ($p<0.01$, n=3 experiments). HL60 cells showed intermediate to low kinase activity levels.

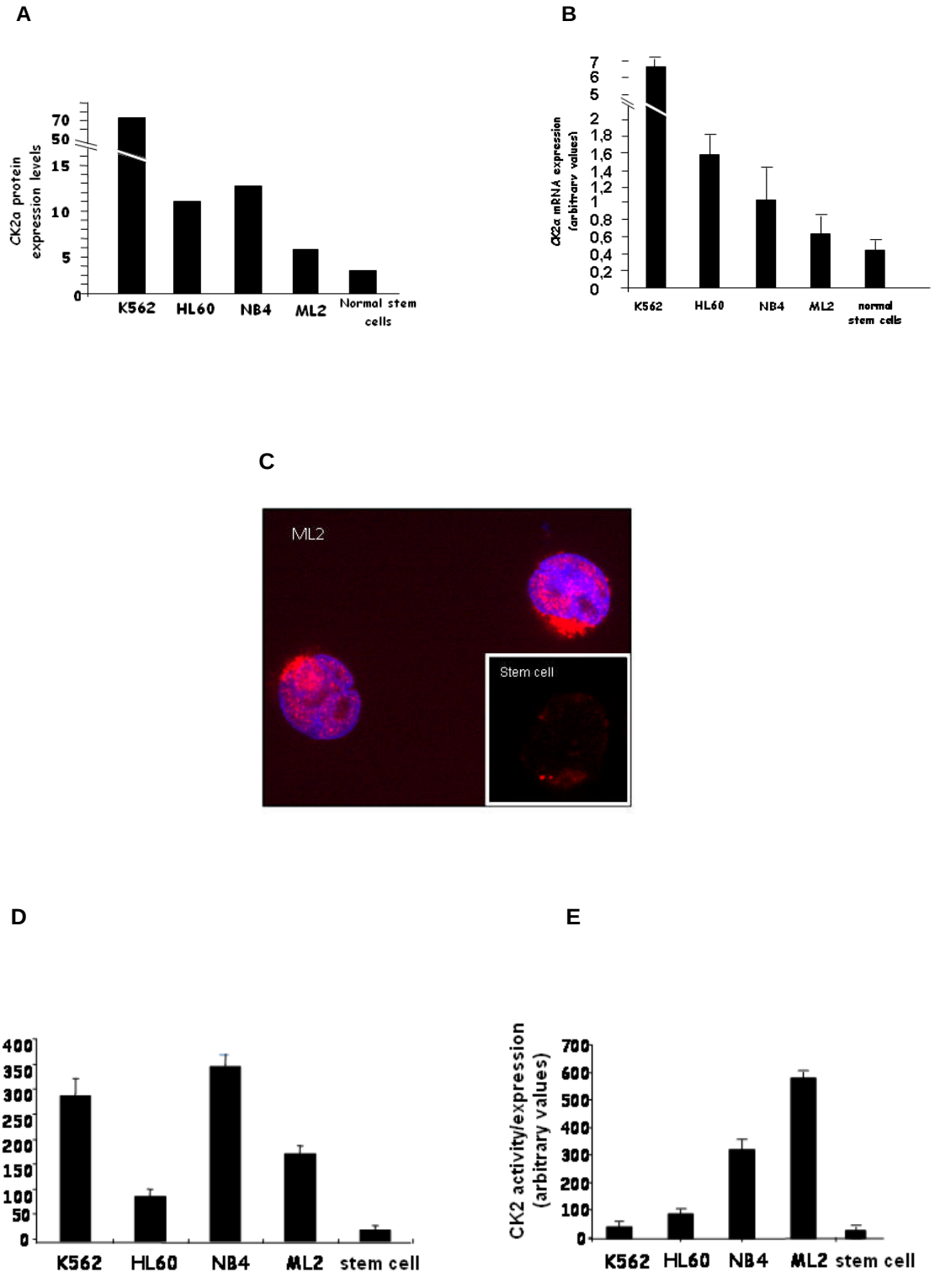


Figure 1: Expression of CK2 in cell lines of acute myeloid leukemia

A. Densitometric values of CK2α expression obtained by Western blot of total protein lysates belonging to the cell lines K562, HL60, NB4 and ML2. The control is represented by CD34 + stem cells

B. Expression of CK2α determined by real-time PCR performed on cDNA cell lines K562, HL60, NB4 and ML2. The control is represented by stem cells CD34 + ($p < 0.05$)

C. Location of CK2: Immunofluorescence conducted on cells ML2 using an antibody directed against CK2α (red). Nuclei were marked with DAPI (blue).

D. CK2α activity determined by radioactive kinase assay of total cell lysates (K562, HL60, NB4, ML2). The normal control is represented by CD34 + stem cells. The results are representative of 3 separate experiments $p < 0.01$.

E. Relationship between the kinase activity of CK2α and its protein expression levels (arbitrary units) in the various cell lines of acute myeloid leukemia (K562, HL60, NB4, ML2) and normal CD34 + hematopoietic stem cells. The results are representative of 3 separate experiments $p < 0.01$

Inhibition of CK2 activity causes AML cell apoptosis

To investigate the CK2 role in AML cell survival, different AML cell lines were treated with the CK2 inhibitors, K27 and CX-4945. After eighteen-hours exposure of ML2, HL60 and NB4 cells to increasing concentration of the inhibitor, cell survival was analyzed by annexin V/propidium iodide (AV/PI) staining and FACS analysis. AML cells were treated with the vehicle (DMSO 0.1% in medium), as a control. Each cell line displayed a different sensitivity to CK2 inhibition. ML2 and NB4 cells resulted extremely sensitive to CK2 inhibitors, while HL60 cells showed a remarkable resistance to the treatment, being refractory to inhibitors used K27 (*Fig. 2A*) and CX-4945 (*Fig 2B*) at all the concentrations tested.

Moreover, immunoblot analysis of PARP cleavage also revealed that the treatment of AML cells with CK2 inhibitors was able to trigger the apoptotic pathways, in a dose-dependent fashion, in ML2, NB4, but not in HL60 cells (*Fig. 2C and 2D*).

To confirm that inhibition of CK2 results also in apoptosis of primary AML blast from patients, we treated with 5μM K27 freshly isolated AML blasts from 4 different patients and analyzed AV staining by flow cytometry after 8 hours. CK2 inhibition indeed caused a statistically significant induction of apoptosis (AV positive cells) also of primary AML cells, supporting the results obtained with the AML cell lines ($p<0.01$, n=4) (*Fig. 3*).

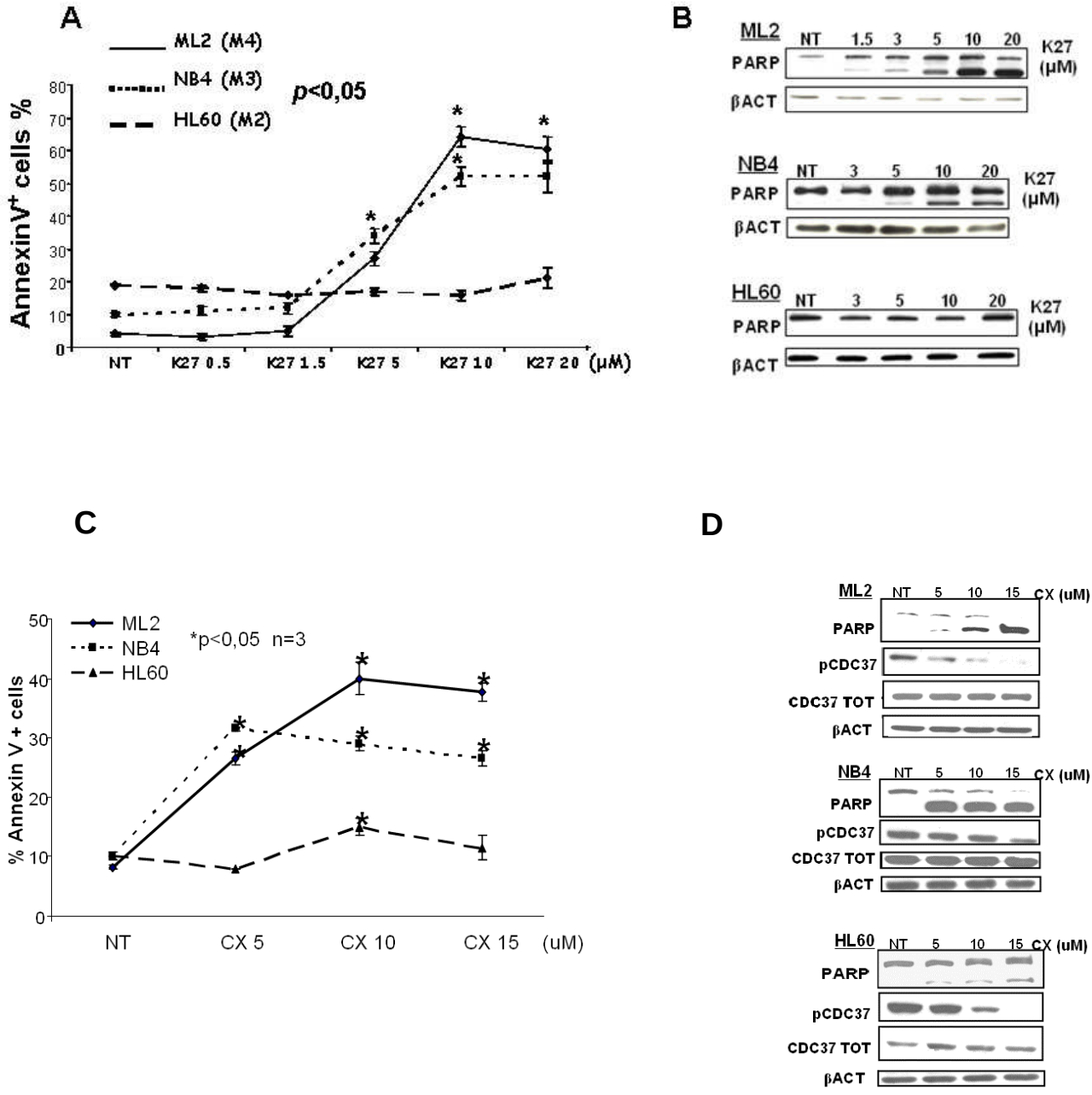


Figure 2: Response of apoptotic cells of acute myeloid leukemia after inhibition of CK2A with K27 and CX-4945.

A. In different cell lines of acute myeloid leukemia (ML2, NB4 and HL60) CK2 was inhibited by treatment with K27 at increasing concentrations (0.5-1.5-5-10-20µM) for 18 hours. The extent of apoptosis was assessed by annexin V test. The abscissa shows the different concentrations of K27 used, on the ordinate the percentage of cells positive for annexin V (apoptotic). The results shown are representative of 3 separate experiments ($p < 0.05$).

B. The expression of total and cleaved PARP was determined by Western-blot analysis of protein lysates of NB4 cells, ML2 and HL60 following treatment for 18 hours with increasing doses of K27 and respective normalization with β-actin

C. In different cell lines of acute myeloid leukemia (ML2, NB4 and HL60) CK2 was inhibited by treatment with CX-4945 at increasing concentrations (5-10-15 µM) for 18 hours. The extent of apoptosis was assessed by annexin V test. The abscissa

shows the different concentrations of CX-4945 used, on the ordinate the percentage of cells positive for annexin V (apoptotic). The results shown are representative of 3 separate experiments ($p < 0.05$)

D. The expression of total and cleaved PARP was determined by Western-blot analysis of protein lysates of NB4 cells, ML2 and HL60 following treatment for 18 hours with increasing doses of CX-4945 and respective normalization with β-actin.

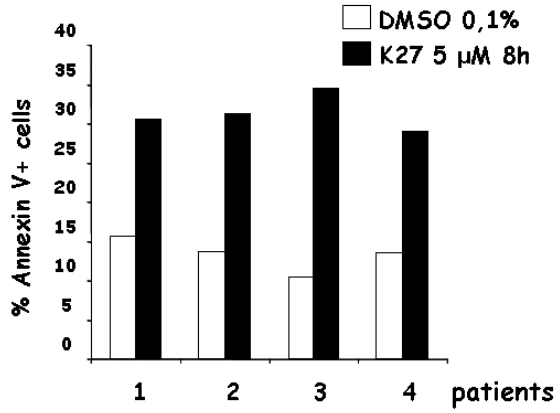


Figure 3: The pharmacological inhibition of CK2 causes apoptosis in AML Blasts isolated from 4 different patients with AML and treated with the inhibitor of CK2α K27 (black bars). As a control DMSO (white bars), the medium in which K27 was dissolved, was used. Apoptosis was analyzed by flow cytometry using Annexin V staining. The histograms show separately the results obtained in 4 patients.

CK2 inhibitors-induced apoptosis is p53-dependent

The refractoriness of HL60 cells to the CK2 blockade-induced apoptosis prompted us to test whether this process was dependent on an intact p53 tumor suppressor function. In fact, HL60 are p53 null cells, due to a gene deletion (51) and this suggested that the apoptosis consequent to CK2 inhibition could rely on p53. To test this hypothesis we first analyzed p53 levels in wild-type p53 ML2 cells in different conditions. After 6 hours of treatment with 5µM K27, p53 levels were investigated by WB and densitometric analysis (*Fig.4A*). K27-treated cells displayed a marked increase of p53 levels as compared to vehicle-treated control cells. Since one major mechanism regulating p53 protein turnover is its proteasome mediated degradation, as a control of our experiments we checked whether inhibition of the proteasome could give the same p53 increased levels in AML cells. Indeed, treatment of ML2 cells with 10µM of proteasome inhibitor MG132 resulted in an accumulation of p53 in the cells (*Fig.4A*). This result indicates that p53 expression and/or stability are likely regulated by CK2.

To further support our hypothesis that CK2 inhibition causes apoptosis through p53, we next made use of another cellular model. We treated the p53-null human osteosarcoma cell line Saos-2 (52) with 10µM K27 and analyzed cell viability by AV/PI staining and FACS analysis. We could confirm that also the Saos-2 cells were resistant to CK2-inhibitors induced apoptosis. However, Saos-2 cells transfected with a p53 wild-type expressing vector, but not Saos-2 cells transfected with an empty vector, displayed a

significantly increased sensitivity to apoptosis induced by eighteen-hour treatment with 10µM K27($p<0.05$,$n=3$) These results were confirmed by experiments of gene silencing of CK2α by RNA interference in the same cell line (*Fig. 4B*). The cells were transfected with the plasmid vector p-CMV-p53 or p-CMV control, selected on the basis of their neomycin resistance after a week of culture and subsequently electroporated with an oligonucleotide inert (no target) or with an oligo-silencing for CK2α. After 72 hours, the analysis of apoptosis using Annexin V was made. The recovery of the expression of p53 has, as expected, increased the percentage of apoptotic cells compared to the conditions in which it is not expressed (cells not transfected or transfected with empty vector) (AV + 35.5% ± 0.7%), but especially made the cells sensitive to apoptosis induced by silencing of CK2α (AV +43% ± 1.4) (*Fig. 4C*). The total protein lysates were assayed by Western Blot analysis, which confirmed the silencing of CK2α and the presence of p53 in transfected cells. The levels of cleaved PARP were increased in cells in which it has been restored the expression of p53 was restored and CK2α inhibited (*Fig. 4D*).

These results were next confirmed in HL60 cells transfected with the plasmid vector p-CMV-p53 or p-CMV control, selected on the basis of their neomycin resistance after a week of culture and subsequently treated with the inhibitor CX-4945 at 7 µM. After 18 hours of treatment the analysis of apoptosis by Annexin V staining was assessed. As in Saos cells rescue of p53 expression sensitized HL60 cells to apoptosis induced by CK2 inhibition . In particular , as expected, the recovery of the expression of p53 has increased the percentage of apoptotic cells compared to the conditions in which it is not expressed (cells not transfected or transfected with empty vector) (AV + 61.1% ± 1.6%), but especially made the cells sensitive to apoptosis induced by CK2 inhibitors (AV +67% ± 0.9%) (*Fig 4E and 4F*).

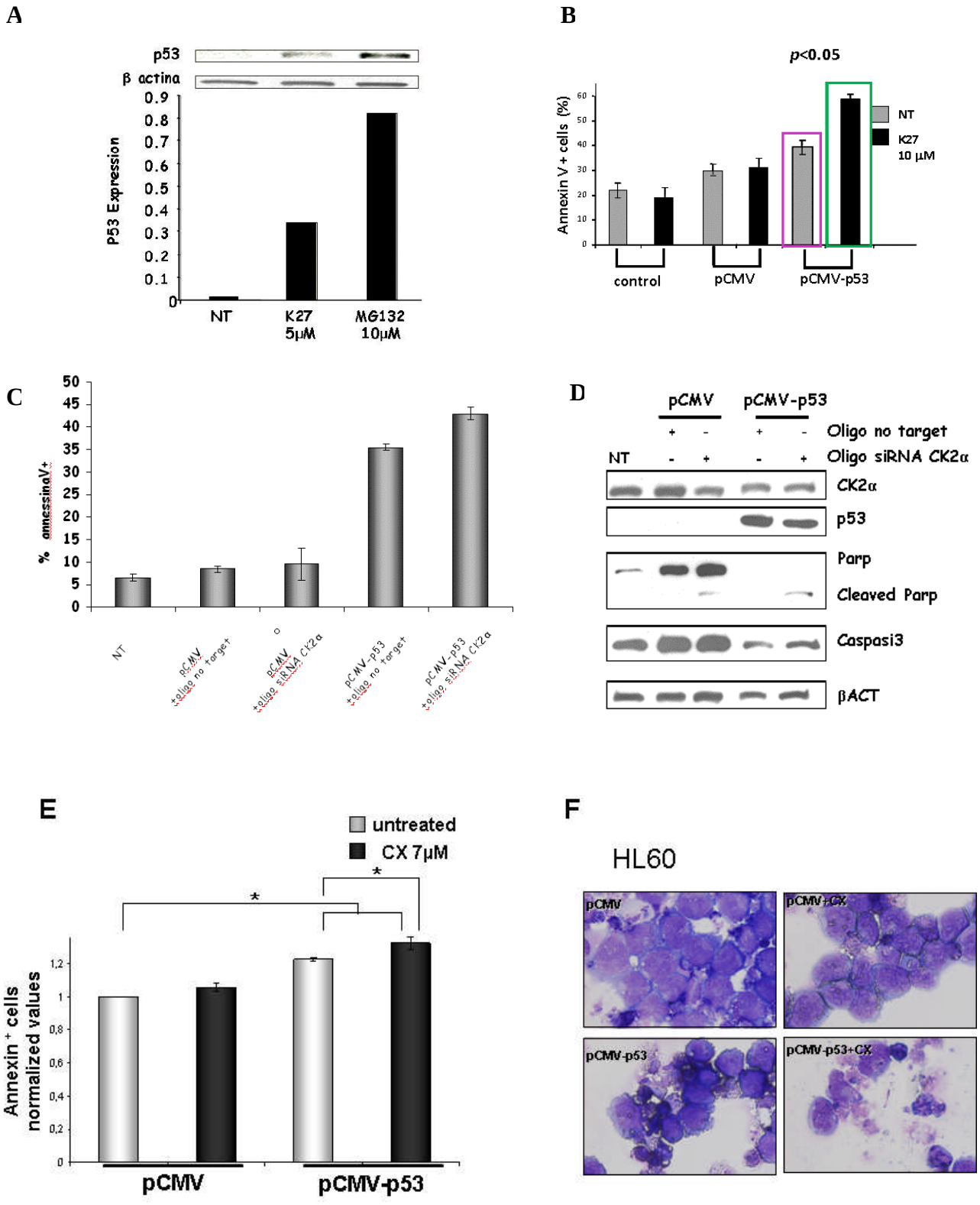


Figure 4: Expression of p53 protein and effect on the apoptosis induced by inhibition of CK2

A. Western-Blot analysis and relative densitometric values of the expression of p53 in total protein lysates of cells treated or not with K27 ML2 5μM and MG132 (10μM) for 6 hours and relative normalization with β-actin.

B. SAOS-2 transfected with an expression vector for p53; after 48 hours of transfection cells were subjected or not to treatment with K27 (10μM) for 18 hours. The extent of apoptosis was assessed by the test of the AV. As a control cells

untransfected and transfected with a vector lacking an insert for p53 (pCMV) were used. The results are representative of 3 separate experiments, $p < 0.05$.

C. SAOS-2 cells transfected with an expression vector for p53 and subsequently transfected with an oligonucleotide short interfering RNA to silence the gene of CK2α. The extent of apoptosis was assessed by the test of the AV. As a control cells untransfected and transfected with a vector lacking an insert for p53 (pCMV) in combination with an inert oligonucleotide (oligo no target) were used. The results are representative of 2 separate experiments (* = $p < 0.05$).

D. Western blot analysis of the expression of CK2α, p53, total and cleaved PARP, Caspase 3 total, phospho p65 and p65 total, total protein lysates experiment silencing of Saos-2 and its normalization with β-actin.

E. HL60 cells transfected with an expression vector for p53; after 48 hours of transfection cells were subjected or not to treatment with Cx-4945 (7μM) for 18 hours. The extent of apoptosis was assessed by the test of the AV. As a control cells untransfected and transfected with a vector lacking an insert for p53 (pCMV) were used. The results are representative of 3 separate experiments, $p < 0.05$.

F. Morphological data before and after transfection and after treatment with CX-4945

AML cells show increased sensitivity to doxorubicin and daunorubicin after CK2 inhibition

Since in AML cells CK2 protein activity is increased as compared to normal CD34+ cells and its inhibition sensitizes cells towards p53 partially-dependent apoptosis, we decided to investigate whether hampering of CK2 activity rendered AML cells more susceptible to the cytotoxic effect of chemoterapeutic drugs.

To this aim, ML2 cells were incubated for 18 hours with a combined treatment with subtoxic (4μM) K27 concentrations and increasing doses of daunorubicin (0.05-0.1-0.15 μM), an agent used in chemotherapy protocols for the treatment of AML. AML cells were treated with the same concentrations of daunorubicin and vehicle (DMSO 0.1%), as a control and analyzed with flow cytometry for the expression of AV. A significant synergy of the chemotherapic agent and the inhibitor of CK2α in inducing apoptosis was found (AV + cells with K27 + dauno 0.05: 28.5% ± 1.7%; K27 + dauno 0.1: 29% ± 5.4, K27 + dauno 0.15: 35.75% ± 8.1%) (*fig 5A*) Also WB analysis of PARP cleavage confirmed the cumulative effect of K27 and daunorubicin in inducing apoptosis (*Fig5B*). Next, treatment with daunorubicin was combined with another CK2 inhibitor, the CX4945. ML2 cells were exposed for 18 hours to increasing doses of daunorubicin (0.05-0.1-0.15 μM) in the presence of a fixed dose of CX4945 4 μM. Cells were then analyzed by flow cytometry for the expression of AV. As for K27 treatment a significant synergy between the chemotherapy agent and─CX-4945 in inducing apoptosis was

demonstrated (AV + cells with CX-4945 + dauno 0.05: 16.6% ± 1.5%; CX-4945 + dauno 0.1: 21,8% ± 5.0, CX-4945 + dauno 0.15: 23.5% ± 8 (*Fig 5C* and *5D*).

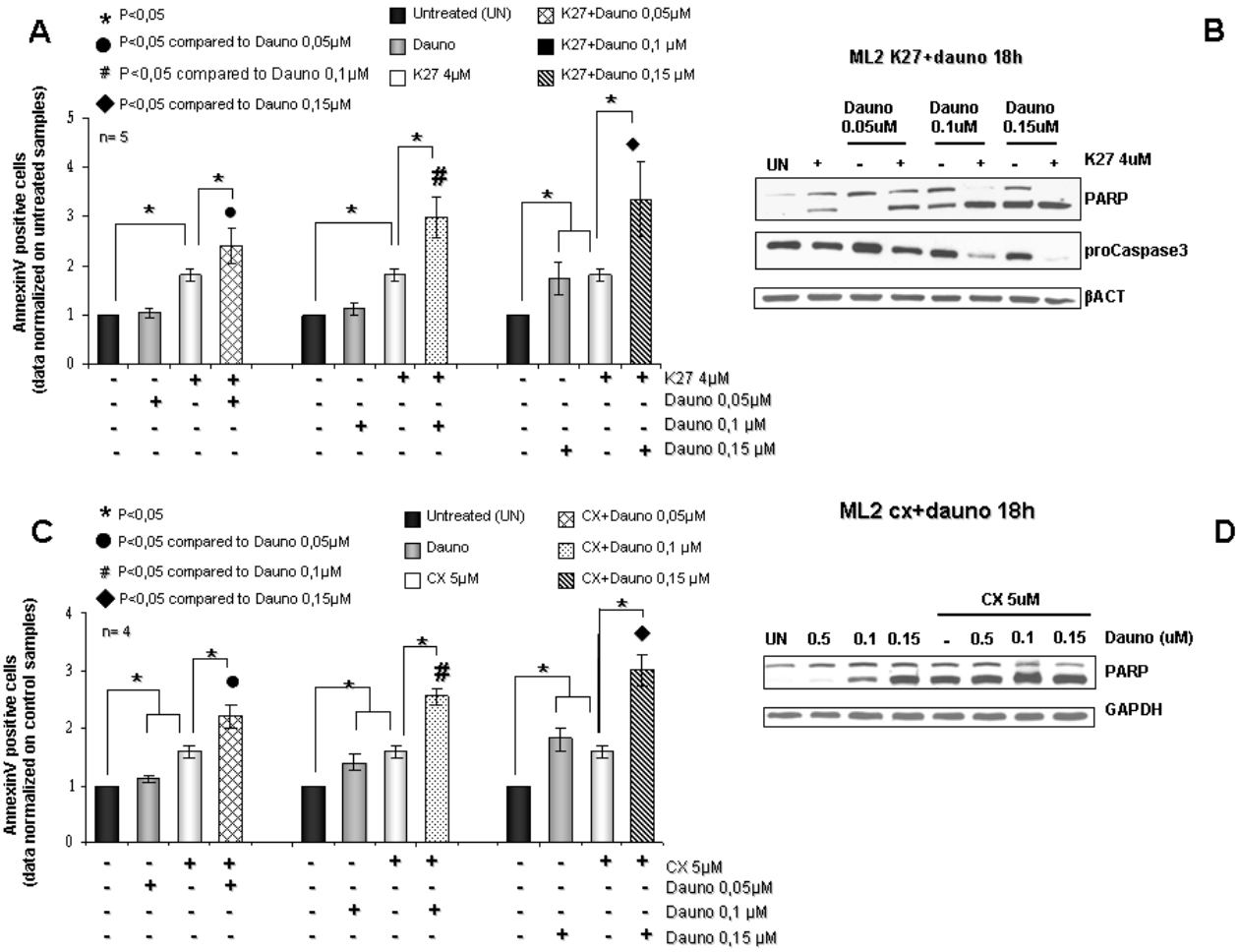


Figure 5: Cytotoxic effect of combined treatment with daunorubicin and inhibitors of CK2

ML2 cells were treated for 18 hours with increasing doses of daunorubicin (0.05-0.1-0.15 mM) and with a constant dose of K27 (4 mM) (**A**) or CX-4945 (5 mM) (**C**). The apoptosis was assessed by staining with AV. treatments used are reported In the abscissa with the respective concentrations, on the ordinate the percentage of annexin V-positive cells. The control consists of untreated ML2 cells. The results shown for the combination of daunorubicin and K27 are representative of five separate experiments, P <0.05, • p <0.05 compared to daunorubicin 0.05µM; # p <0.05 compared to daunorubicin 0.1µM; ♦ p <0.05 compared to daunorubicin 12:15 µM. Results reported for the association of daunorubicin and CX are representative of four separate experiments, P <0.05, • p <0.05 compared to daunorubicin 0.05µM; # p <0.05 compared to daunorubicin 0.1µM; ♦ p <0.05 compared to daunorubicin 0.15µM. The expression of total and cleaved PARP was analyzed by Western Blot. The normalization was performed with β-actin (**B**) and GAPDH (**D**).

Lastly, the apoptotic response to the combined treatment was tested also on freshly isolated AML blasts cultured onto a layer of HS-5 bone marrow stromal cells in order to mimic the patho-physiological microenvironemt present *in vivo*. AML blasts were cultured in DMSO 0.1%, DMSO plus daunorubicin

0.05 μM, K27 3 μM or both agents. AV staining and FACS analysis were then performed on cells harvested from the co-cultures after 18 hours. By flow cytometric and western blot analysis (*Fig. 6A* and *6B*) an increased apoptosis with the combination of the two agents was shown. Another series of experiments on AML blasts patients was conducted using CX 4945 5 μM combined with daunorubicin, as shown in *Fig 6C* and *6D*.

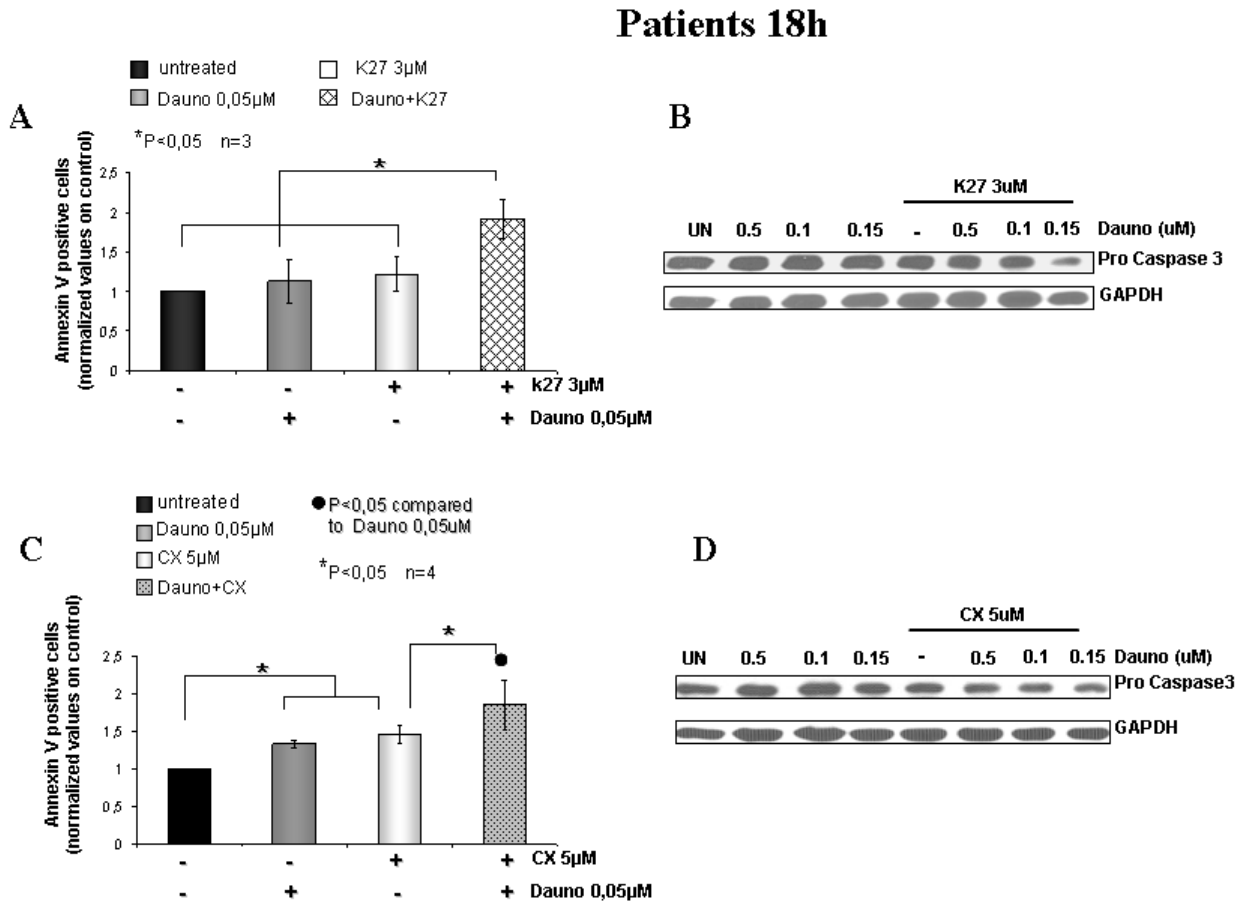


Figure 6: Effect of anthracycline and CK2 inhibitors in cells of acute myeloid leukemia

Blasts isolated from a patient with AML and treated for 18 hours with increasing concentrations of daunorubicin (0,5-1 mM) and K27 (3 uM) above (**A**-**B**) or with CX-4945 (5 uM) below (**C**-**D**). The extent of apoptosis was assessed by an assay of AV and WB.

CK 2 silencing by RNA interference causes apoptosis and empowers daunorubicin-dependent citotoxicity in AML cells

To further analyze the role of CK2 in AML cell survival and resistance to apoptosis we performed RNA interference experiments to knock down CK2 protein expression levels in ML2 cells. ML2 cells were transfected with oligonucleotides directed against the α catalytic subunit, the regulatory subunit β or a combination of both. As control cells transfected with oligonucleotides lacking specific target (no target)

were used. The efficiency of transfections was verified by parallel electroporations of inert oligonucleotides marked with green fluorophore (siglo green). Silencing of both CK2 subunits was confirmed respectively by real-time PCR (Figure 7A) and by Western blot analysis. The efficiency of transfection was high (transfected cells ranged from 80% to > 90%) in all the experiments performed (not shown). As shown in Figure 7B we could achieve a significant reduction of CK2 α and CK2 β expression at both mRNA and protein levels, peaking 48 hours and lasting as long as 72 hours. Knock down of CK2 α and CK2 β was associated to a significant increase in apoptosis as shown by AV staining and PARP cleavage (Figure 7b and 7c), along the same line of CK2 chemical inhibition experiments.

48 hours after transfection ML2 cells were treated with two different doses of daunorubicin (0.15 uM and 0.25 uM) for additional 18 hours and then evaluated for apoptotic response by PARP cleavage and AV staining analysis (*Fig. 7B and 7C*). Interestingly, the protein expression of CK2 β may be reduced in the presence of down-modulation of single catalytic subunit α (1.37 for the β subunit, 1.07 for the α subunit, normalized data on the value of the control transfected with no oligonucleotide target after densitometric analysis). The apoptotic cells, however, further increase after association with daunorubicin, giving statistically significant results in the presence of down-regulation of both subunits and treatment with the lower dose of chemotherapeutic (0.15 uM) (siRNA CK2β + daunorubicin 0.15μM: 1.98, siRNA CK2β + daunorubicin 0.25μM: 2.38, CK2α siRNA + daunorubicin 0.15μM: 1.38, CK2α siRNA + daunorubicin 0.25μM: 2.07; siRNA CK2α and CK2β + daunorubicin 0.15μM: 1.93, CK2α and CK2β siRNA + daunorubicin 0.25μM: 2.75: normalized data on value of the control transfected with no target oligonucleotide in the absence of daunorubicin, after densitometric analysis) (*Fig. 7C*).In fact the Western blot analysis shows an increase of the sensitivity of the cells to daunorubicin: it an increase of the cleavage of PARP at the lowest dose of the chemotherapeutic agent was observed when the single β subunit or both β and α were silenced (*Fig. 7B*)

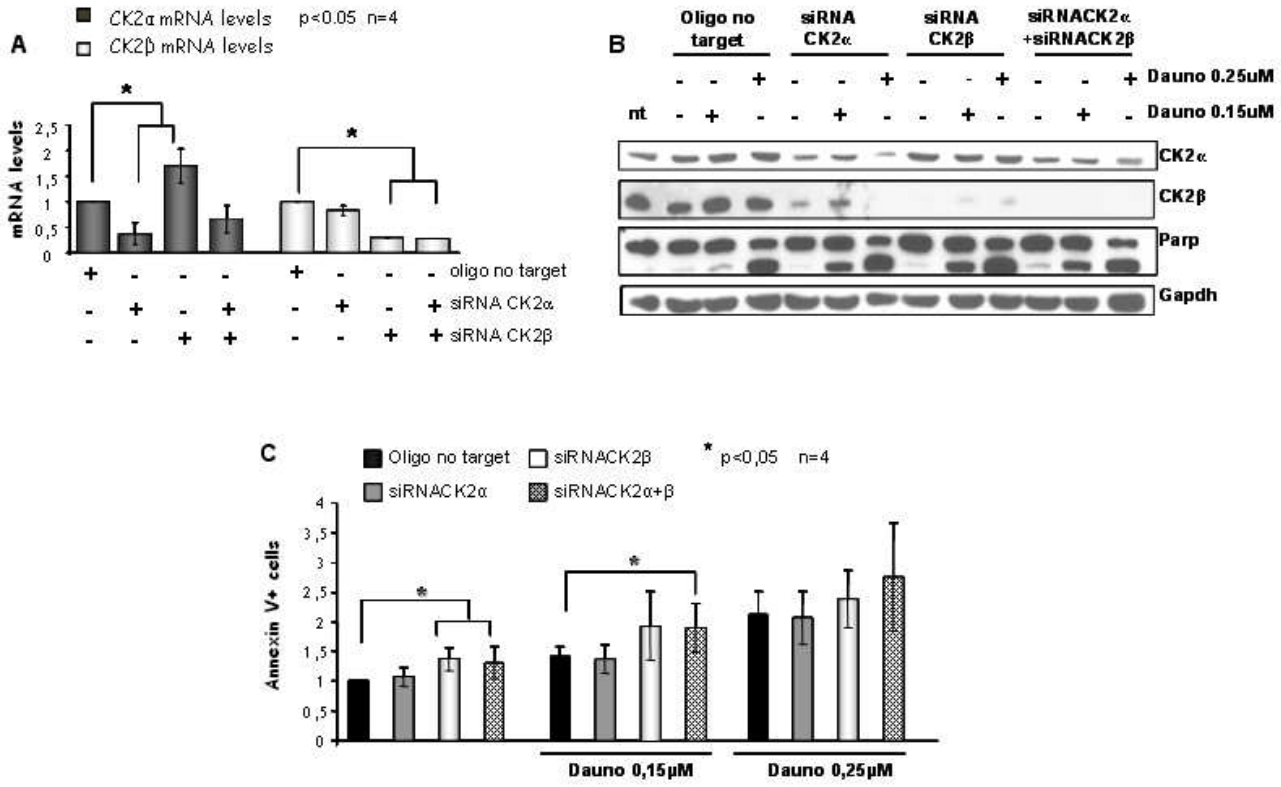


Figure 7: Response of the line ML2 to treatment with daunorubicin following the silencing of CK2

A. Expression of CK2α and CK2β determined by real-time PCR performed on cDNA ML2 cells transfected with oligonucleotides directed against the mRNA of the β subunit and α of CK2 (CK2β siRNA and siRNA CK2α respectively). The control is represented by cells transfected with oligonucleotides without target (no target oligo). The results were obtained from 4 independent experiments, P <0.05.

B. Protein levels at 72 hours of CK2α, CK2β and cleaved PARP and total lysates of total protein of the cell line ML2, treated with daunorubicin (0.15 uM and 0.25 uM) after silencing by RNA interference (siRNA) of the subunits α and β. Below normalization with GAPDH.

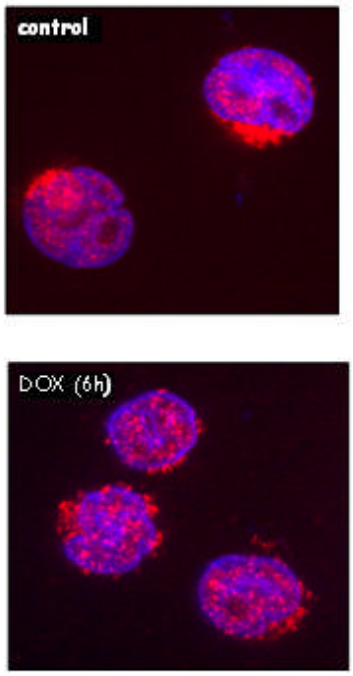
C. Percentage of apoptosis (normalized data compared to untreated control) of ML2 cells after transfection with siRNA CK2α, CK2β siRNA and siRNA CK2α + CK2β and treatment with daunorubicin (0.15 uM, 0.25 uM). The abscissa shows the combinations of different treatments. The results obtained are representative of 4 independent experiments. p <0.05 * p <0.05

CK2 subcellular localization is modulated by doxorubicin in AML cells

Since inhibition of CK2 renders AML cells more susceptible to doxorubicin-induced cell death, we next sought to determine whether there could be a cross-talk between doxorubicin-induced pathways and CK2. To this aim, we analyzed CK2α expression levels and kinase activity in ML2 cells exposed for 6 hours to doxorubicin at increasing concentrations (0.4-0.8µM). CK2 α protein expression levels were found slightly increased after 6 hours upon doxorubicin treatment. Also CK2 kinase activity increased after doxorubicin treatment (data not shown). We analyzed CK2α cellular distribution upon doxorubicin

treatment in AML cells. IF microscopy experiments of AML cells exposed to 0.8μM doxorubicin for as long as 6 hours revealed that CK2α redistributed from cytoplasm into the nuclear compartment (*Fig. 8A*). This result was confirmed in WB experiments, in which CK2α protein expression was investigated in the cytoplasmic and nuclear cellular protein fractions: while in untreated AML cells CK2α was predominantly present in the cytoplasmic fraction, after doxorubicin treatment for 6 hours it was detectable mostly in the nuclear fraction (*Fig. 8B*). Thus, CK2α changes its intracellular localization upon exposure to a chemotherapeutic drug in AML cells.

A **B**



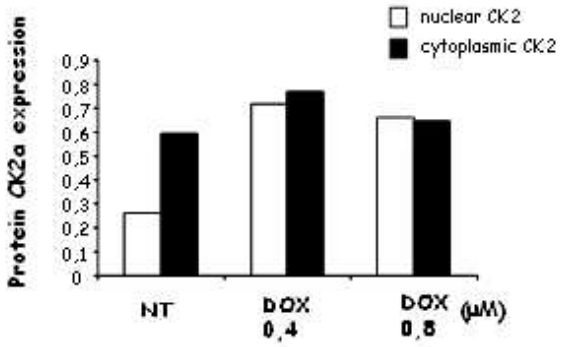


Figure 8: Relocalization of CK2α following treatment with CK2α.

A. Immunofluorescence performed on cells ML2. CK2α is red, the nucleus is blue (DAPI). In the upper box untreated cells are shown, in the box below cells treated with doxorubicin (0.8μM) for 6 hours.
B. The expression of CK2α was evaluated by Western-blot analysis of cytoplasmic and nuclear protein lysates of cells ML2 treated for 6 hours with doxorubicin (0.4 and 0.8μM); densitometric values normalized on the β-actin are shown.

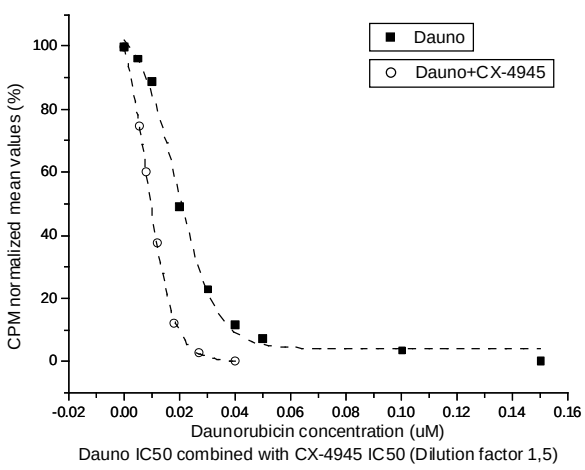
The combination of CK2 inhibitors and daunorubicin induces apoptosis of AML cells with a synergic effect

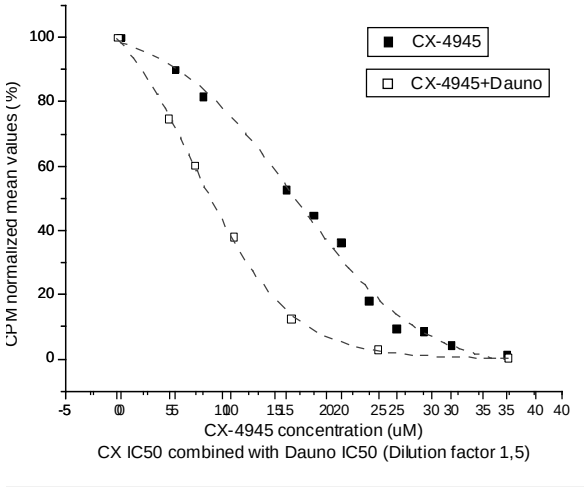
The observation that the cytotoxic effect of daunorubicin is enhanced by CK2 blockade prompted us to further investigate the basis of this cooperation using the Combination Index (CI). ML2 cells were treated with sequential doses of K27, CX 4945 and daunorubicin, after 18 hour. After 48 hours the rate of cell proliferation was assessed by tritiated thymidine proliferation assay (counts per minute). First, for each drug was determined the IC 50 (concentration of the drug that is required for 50% inhibition in vitro): *IC_{50} daunorubicin = 0.0188 μM; IC_{50} CX-945 = 16.65925 μM; IC_{50} K27 = 5.7 μM* ant then was

determined the IC50 for the combination of the two drugs. The concentrations obtained for each combination were lower than the IC50 obtained in the single treatment (daunorubicin IC50 after combination with CX-4945: 0.008 M; IC50 CX-4945 after combination with daunorubicin: 7.48911 M; daunorubicin IC50 after combination with K27: 0.0058 M; IC50 K27 after combination with daunorubicin: 2.28uM).

It was then possible to relate the different IC50 values acquired for each pair of treatment, obtaining for both associations a Combination Index less than 1 (CI daunorubicin + CX-4945: 0.86; CI daunorubicin + K27: 0.7). A synergistic proapoptotic effect of the combined treatment of daunorubicin with both inhibitors of CK2 could be demonstrated (*Fig. 9*).

A





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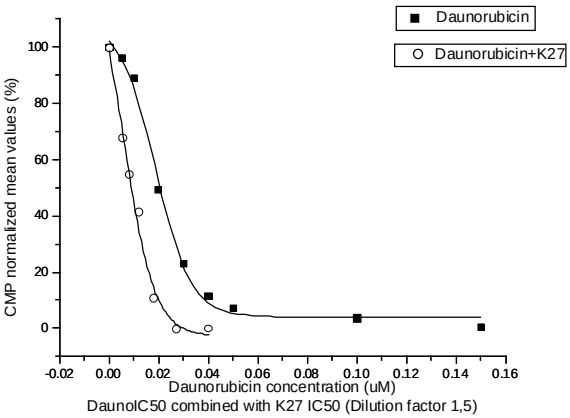

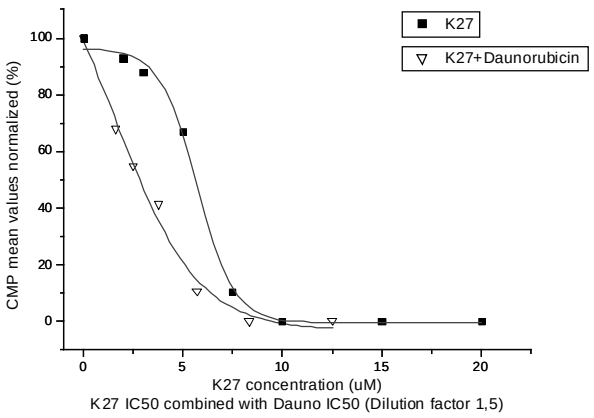

Fig 9: Combination Index of the combination daunorubicin/CK2 inhibitors CX-4945 or K27

The cell proliferation and therefore the incorporation of tritiated thymidine incorporation is expressed as counts per minute (CPM), reported on the ordinate. The concentrations of the different agents used are reported in abscissa. The results are representative of 3 independent experiments after 48 hours of treatment.

A. Dose-response curve with IC50 values of daunorubicin alone or in combination with CX (left) and the corresponding curve after treatment with IC50 values of CX alone or combined with daunorubicin (right)

B. Dose-response curve with IC50 values of daunorubicin alone or in combination with K27 (left) and of IC50 values of K27 alone or in combination with daunorubicin (right).

CK2 modulates daunorubicin-induced activation of JAK-STAT pathway

To unravel molecular mechanisms underlying the synergistic proapoptotic effect of daunorubicin and CK2 blockade ~~we~~ focused on signalling pathways modulated by doxorubicin, genotoxic stress and CK2. Among many signalling cascades, we first look at the JAK-STAT pathway, a critical mediator of the DNA damage response (DDR) and doxorubicin resistance (53). In fact, very recent studies have shown that it is activated by CK2 (47).

ML2 cells were treated with K27 4 μM or CX-4945 5 μM plus different concentrations of daunorubicin (0.05 μM, 0.1 μM, 0.15 μM).

We evaluated the activation of JAK/STAT pathway after 18 hours by assessing expression levels of two target genes of STAT such as mcl-1, anti-apoptotic gene, and SOCS3, suppressor of JAK receptor by RT-PCR and Western Blot analysis (*Fig. 10*). The state of phosphorylation in serine 727 of STAT3, active form of the transcription factor, was also investigated. As expected STAT3 was found to be constitutively phosphorylated in ML2 cells. The treatment with daunorubicin induced a slight increase of phosphorylated STAT3 and STAT3-dependent transcription, as shown by increased expression of mcl-1 and SOCS3~~.~~

Then association of the daunorubicin treatment was combined with the CK2 inhibitors: the block of the pathway activation yet at daunorubicin subtoxic dose (0.05 mM) was demonstrated by the reduction of phosphorylated STAT3 and reduced levels of mcl-1 and SOCS3 (*Fig. 10*).

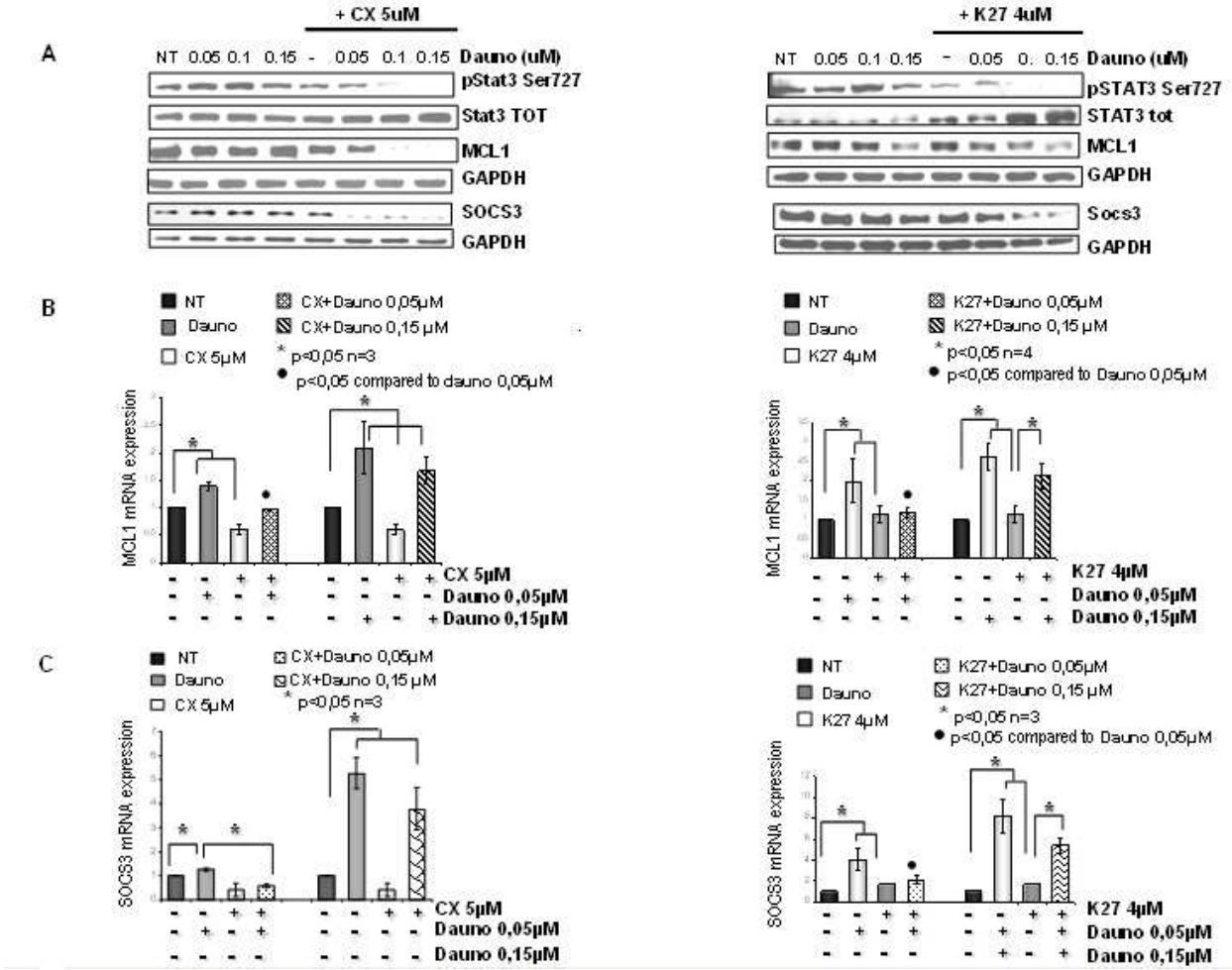

Figure 10: Analysis of the state of activation of the JAK/STAT pathway after treatment with daunorubicin in the presence or absence of K27 or CX-4945

A. Western Blot analysis after treatment with daunorubicin and K27 (on the left) or CX-4945 (on the right). Normalization with GAPDH.

B-C. Real-time PCR for genes mcl-1 (on the top) and SOCS3 (on the bottom) following treatment with daunorubicin and CX-4945 (on the left) or daunorubicin and K27 (on the right). The ordinate shows the levels of expression normalized on the untreated control (NT) and the abscissa the different treatments used. The data obtained are derived from three different independent experiments. * $p < 0.05$, • $p < 0.05$ compared to daunorubicin 0,05 uM

CK2 α and β expression analysis of in primary AML cells

In order to translate our in vitro studies into a clinical setting, CK2 espression levels were then sistematically assessed in AML primary cells. Patients were divided into two groups (CK2α-high versus CK2α-low) according to the levels of constitutive expression of CK2α protein in pretreatment AML samples as described in the statistical analysis (ratio CK2α absorbance/GAPDH absorbance > or < 0,75). Western blot analysis revealed that the expression of CK2α protein was elevated in 17 out of the 27 AML samples. Considering the ELN prognostic classification a high expression was found: in 2 (29%) of the 7 patients with a favorable risk class, in 8 (61%) of the 13 patients with an INT-1 risk class,4 (100%) of the 4 patients with an INT-2 risk class and 2 (100%) of the 2 patients with a high risk class. Representative Western blot analysis is shown in *Fig. 11*.

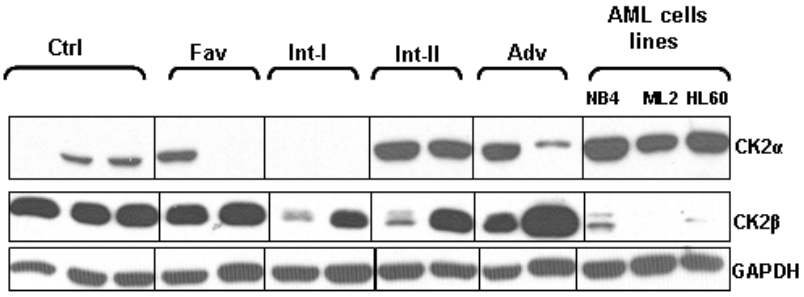

Fig.11: Representative Western Blot Analysis of expression of CK2α and CKβ in AML patients classified on the basis of ELN prognostic classification.

While high CK2α expression levels associated with highest risk classes, interestingly AML samples belonging to INT-1 risk class showed a bimodal expression profile (*Fig.12*).

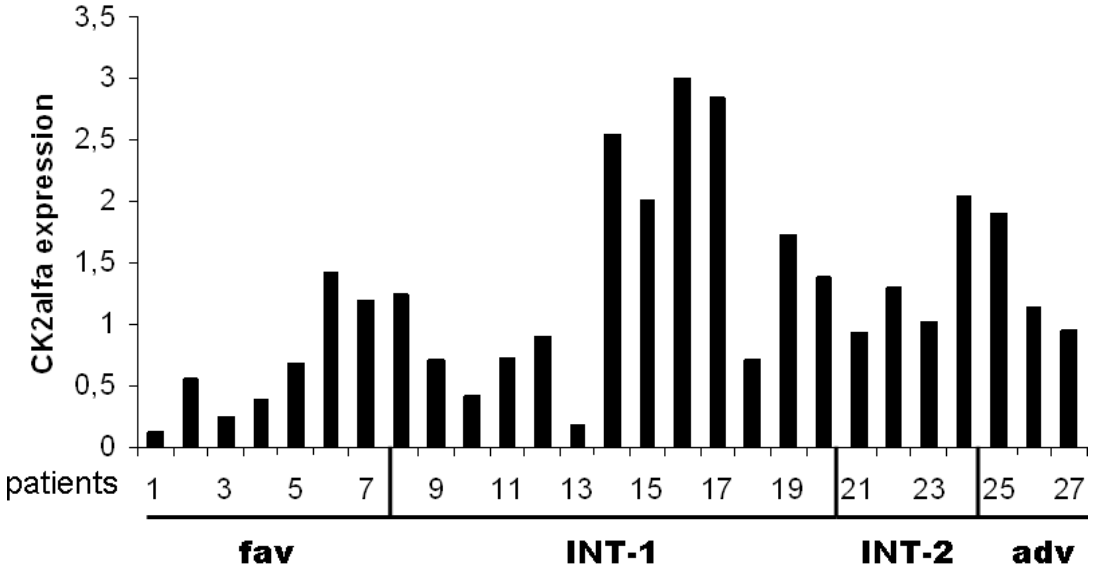

Fig 12: CK2α expression (densitometric values) of 27 patients with AML. Patients are divided in the 4 prognostic ELN groups.

The same arbitrary cut off was adopted for the β subunit. Western blot analysis revealed that the constitutive expression of CK2β protein was high in 21 out of the 27 patients evalauted for the expression of this subunit. Considering the ELN prognostic classification a high CK2β expression was found: in 6 (86%) out of the 7 patients with a favorable risk class ; in 8 (61%) out of the 13 patients with an INT-1 risk class; 3 (60%) out of the 5 patients with an INT-2 risk class and 4 (80%) out of the 5 patients with a high risk class. Representative Western blot analyses are shown in *Fig.13.*

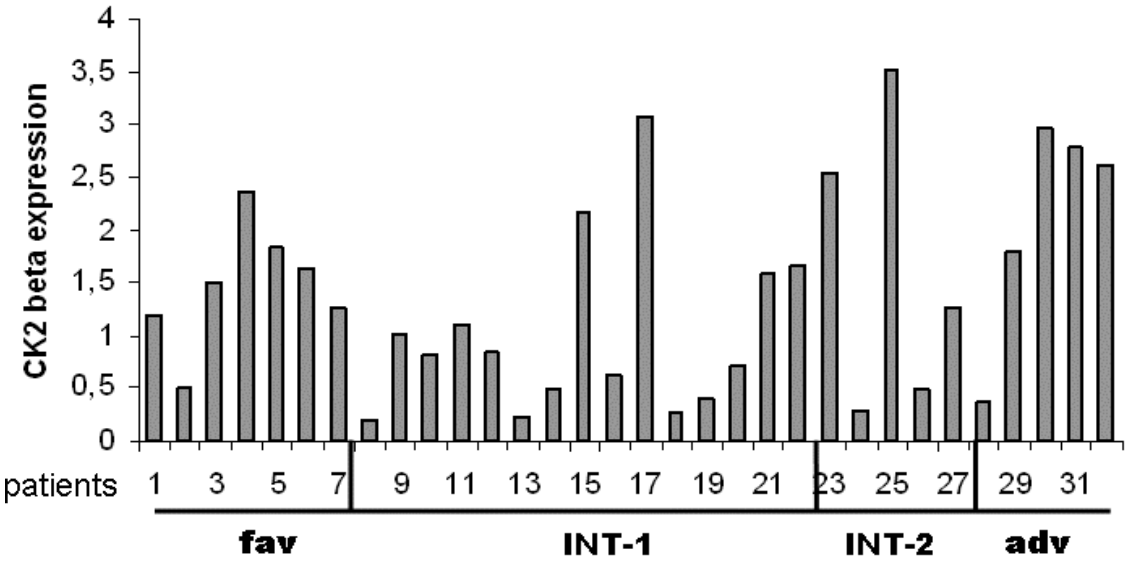

Fig 13: CK2β expression (densitometric values) of 32 patients with AML. Patients are divided in the 4 prognostic ELN groups.

Prognostic significance of CK2α expression in AML

The association of CK2α expression with various clinical variables was then evaluated. There was no significant difference in the patient age and sex in relation to CK2α expression. Kaplan-Meier survival curves showed that overall survival rate was lower in CK2α-high AML compared with the CK2α-low AML cases, though it did not reach statistical significance given the low number of samples analyzed *(Fig 14).* Along the same line Kaplan-Meier survival curves in the group of patients who underwent chemotherapy showed a better outcome, yet not statistical significant as above, in patients displaying low expression of CK2 α *(Fig 15).* Kaplan Meier survival analysis showed that there was not difference on overall survival depending on the CK2β levels (data not shown).

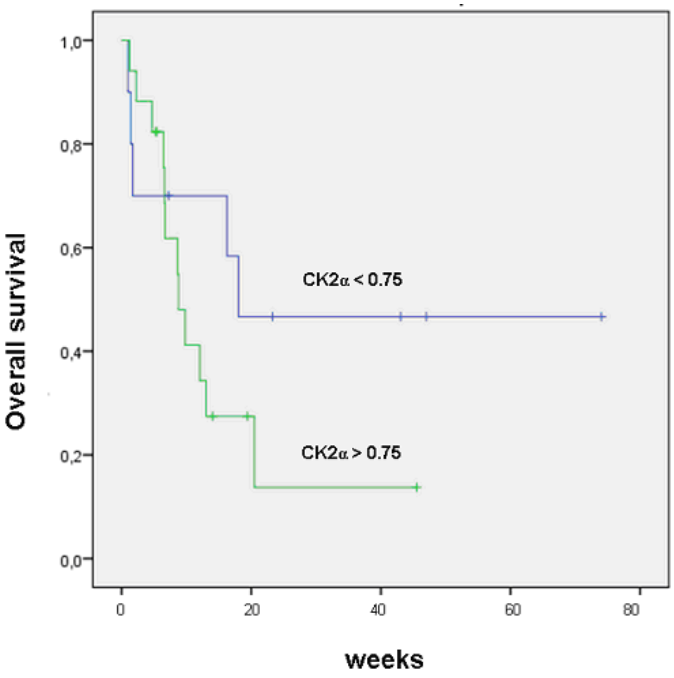

Fig. 14.: Kaplan Meier survival curves in Ck2 α high risk AML compared with the CK2α-low AML (p=0.2)

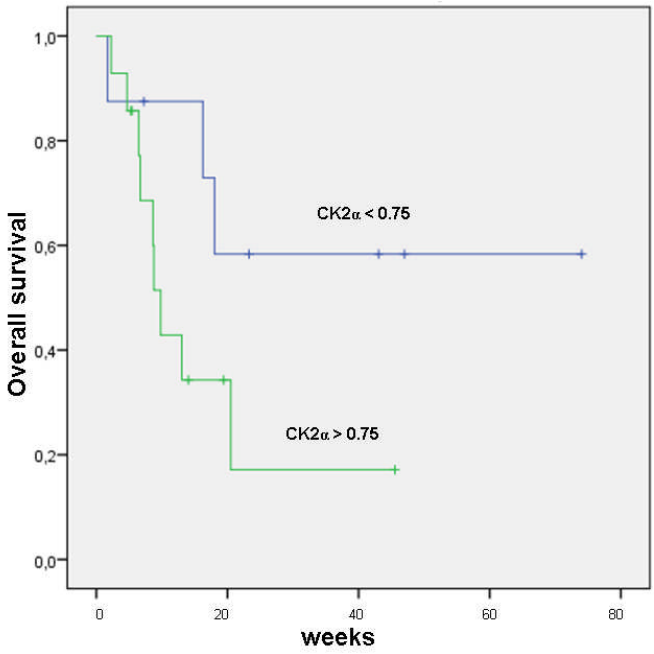

Fig 15.: Kaplan Meier survival curves in Ck2α high risk AML compared with the CK2α-low AML (p=0.1) in patients who underwent chemotherapy

DISCUSSION

Acute myeloid leukemia is the most common acute leukemia diagnosed in adults patients. Although most patients achieve complete remission with induction chemotherapy, the majority of patients relapse after achieving clinical remission.

The use of consolidation therapy with high dose cytarabine or stem cells transplantation has improved outcomes in adult AML patients; however despite the use of intensive consolidation strategies outcomes for AML patients remain poor. Nowadays the most important prognostic factors to predict response to chemotherapy is the cytogenetic profile together with molecular markers such as NPM1 and FLT3. As recently proposed and validated by the European Leukemia Network (23), AML patients can be stratified into different prognostic risk groups according to their chromosomal abnormalities. However, 45% of adults with *de novo* AML have normal karyotype and therefore lack chromosomal markers. This heterogeneous cohort of patients has variable clinical outcomes. Given that intensive treatments, such as allogeneic hematopoietic stem cell transplantation, are associated with high treatment-related mortality, the elucidation of novel prognostic markers can lead to the development of risk-adapted therapeutic strategies in karyotypically normal AML patients. We have shown here that CK2 is highly expressed in AML cells, regulates AML cell survival and sensitivity to doxorubicin and daunorubicin by modulation of the Jak-Stat signaling pathway.

CK2 is emerging as a critical cellular serine-threonine kinase that regulates a large array of processes related to cell survival and proliferation (31). The role of CK2 in sustaining cell growth is markedly exploited by malignant cells that seemingly rely on CK2 activity in a “non-oncogene addicted” fashion to keep oncogenic pathways constitutively activated (48). Most of the AML cell lines used in this study displayed increased levels and activity of CK2. Our data confirmed that CK2 upregulation accompanies the transformed phenotype in malignant hematopoietic cells and are consistent with what has been found in several types of solid tumor cell lines and tissues. Remarkably, the cytotoxic effect of the CK2 inhibitors was also evident in freshly isolated AML blasts from patients. Thus, the efficacy of inducing AML cell apoptosis by the CK2 inhibitors is similar both in AML cell lines and primary AML cells.

Compelling evidence was also provided that CK2 inhibition at least in part leads to AML apoptosis in a p53-dependent fashion. In fact, the p53-mutated HL60 AML, as well as Saos osteosarcoma cell lines, displayed refractoriness to the cytotoxic effect triggered by CK2 inhibitors K27 and CX 4945. Most importantly, restoring a normal p53 function in HL60 and Saos cells was associated with the acquisition of sensitivity to CK2 inhibitors and rendered these cells prone to apoptosis induced by these compounds. The molecular relationships between CK2 and p53 are complex and not fully understood. CK2 can phosphorylate p53 in S392 upon UV light exposure and this would increase p53 transcriptional activity (54,55). However, in *in vivo* mouse models CK2 over-expression synergized with p53 loss in inducing lymphomas (56). Other studies also provided evidence that CK2 antagonizes p53 tumor suppressor activity [reviewed in (28). The results obtained in our study suggest that AML in which p53 function is lost are resistant to CK2 inhibition-induced cell death and might not present CK2 overexpression or increased function. Indeed, HL60 did not display these features (*Fig. 1D, E*), while the most CK2-inhibition sensitive AML cell lines, NB4 and ML2, did. We also demonstrated that in p53 wild-type expressing AML cells, CK2 inhibition was accompanied to an accumulation of p53, suggesting that CK2 activity would be important for the regulation of p53 protein turnover in AML cells.

Most importantly, we established that the anti-apoptotic role of CK2 in AML cells is pivotal in protecting AML cells against drug-induced apoptosis. We have shown that doxorubicin and daunorubicin, ~~a~~ widely used drugs for the therapy of both solid tumors and hematological malignancies, induces AML cell apoptosis at a higher rate in the abscence of CK2 function, suggesting that CK2 regulates anti-apoptotic signaling pathways involved in doxorubicin and daunorubicin-incuced cell death (*Fig.4A, B*). The protective effect of CK2 against doxorubicin was also evident both in AML cell lines and in AML blasts freshly isolated and grown onto a layer of stromal cells (*Fig. 4C, D*). This latter effect suggests that the cooperation between the chemotherapic agents and the CK2 inhibitor is effective also when mimicking the AML malignant microenvironment. To note, we demonstrated that doxorubicin triggers the nuclear shift of CK2, without significant changes in its protein levels and kinase activity. CK2 nuclear functions are largely unknown, however, it is believed that in the nucleus CK2 might influence the activity of transcription factors and of the DNA-damage molecular machineries, thus regulating cell life fate (28). In this regard, we demonstrated that one of the major anti-apoptotic cascade elicited by the exposure of

tumor cells to chemotherapeutics, the Jak Stat pathway, is strongly activated by daunorubicin in AML cells. The use of the CK2 inhibitors-concomitant to the chemotherapy is able to reduce this activation as demostrated by the reduction of Stat phosphorylation as well as of Mcl-1 and Socs3 transcription. The prognostic role of CK2a in AML has been so far explored only by single study (57) focused only patients with normal karyotype without stratification into the cytogenetic risk groups. In our study we analyzed CK2α in representative samples from the different AML cytogenetic risk groups and demonstrated that CK2α–high expression levels associate with higher risk groups. Moreover, INT1 risk group could be stratified in two subgroups based on CK2α–high and -low expression levels suggesting that CK2 may play a role as prognostic factor together with well-known molecular markers such as FLT3 and NPM. Survival analysis resulted statistically not significant because of the low number of samples analyzed. However being overall survival rate lower in CK2α-high AML compared to the CK2α-low AML cases, especially in patients treated with chemotherapy, this pattern suggests a prognostic significance of CK2 expression. On the other hand, Ck2β expression resulted to be extremely variable and not related to different prognostic groups

In summary, this study provides experimental evidence that AML cell survival relies at least in part on an intact CK2 function. CK2 might protect against chemotherapy-induced cell death through inhibition of p53 and activation of Jak-Stat. Therefore, the pharmacological disruption of CK2 could achieve the goal of restoring p53 function while simultaneously inhibiting Jak-Stat activity and could be envisioned as a complementary therapeutic strategy in the management of p53 wild-type/Stat overexpressing AML. Moreover our study suggests a role for CK2α expression as prognostic marker in AML further contributing to the class risk profiling.

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