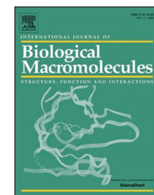
International Journal of Biological Macromolecules 286 (2025) 138305

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Critical role of protein kinase CK2 in chronic myeloid leukemia cells harboring the T315I BCR::ABL1 mutation

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ARTICLE INFO

Keywords:
Casein kinase 2
CSNK2
CK-II
Thr315Ile
CML
Phosphorylation
Signaling

ABSTRACT

Chronic myeloid leukemia (CML) is characterized by the fusion protein BCR::ABL1, a constitutively active tyrosine kinase. The frontline treatment, represented by tyrosine kinase inhibitors (TKIs), has dramatically improved the clinical outcomes of patients. However, TKI resistance through various mechanisms has been reported. In particular, the BCR::ABL11 T315I mutation is associated with resistance to first- and second-generation TKIs and poor survival outcomes. For patients harboring this mutation, treatments with third generation TKIs are indicated, which are however accompanied by adverse events.

Protein kinase CK2 is implicated in several human diseases. Although its role in CML has already been proven, its essentialness in T315I-mediated TKI resistance has yet to be investigated. Here we show that CK2 contributes to the aberrantly high signaling pathways in T315I-cells, and that its pharmacological or genetic targeting diminishes those signals, induces apoptosis, and reduces the proliferation and clonogenic potential of T315I-cells. The effects of CK2 inhibition are also observed in the presence of bone marrow stromal cells and under hypoxic conditions, and, remarkably, in patient-derived cells. Moreover, CK2 inhibition or genetic ablation of the CK2α catalytic subunit sensitizes T315I-cells towards TKIs.

Collectively, our results suggest the potential benefit of inhibiting CK2 in CML characterized by T315I-dependent resistance.

1. Introduction

Chronic myeloid leukemia (CML) is a malignant myeloproliferative disorder of primitive pluripotent stem cells caused by the chromosomal translocation [t(9;22)(q11;q34)], which generates a fusion gene encoding BCR::ABL1, a constitutively active protein tyrosine kinase, which plays a crucial role in initiation and progression of the CML-phenotype [1]. BCR::ABL1 activates multiple signal pathways [2–4], including the Src family kinases, PI3K/AKT, MAPK/ERK and STATs, protecting cells from apoptosis and promoting aberrant survival and proliferation. Imatinib mesylate is a potent BCR::ABL1-specific inhibitor that has become the frontline therapy for CML patients. However, despite the high effectiveness of this therapeutic approach, up to 35 % of CML patients exhibit primary or acquired resistance, or intolerance to this drug [5–7]. The various mechanisms of imatinib-resistance described up to now are Both BCR::ABL1-dependent (gene amplification or mutation) and BCR::ABL1-independent (decreased imatinib bioavailability or activation of alternative signaling pathways) resistance mechanisms have been described [8,9]. To counteract imatinib-resistance, in addition to exploring combination therapies, second-

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https://doi.org/10.1016/j.ijbiomac.2024.138305
Received 26 July 2024; Received in revised form 6 November 2024; Accepted 1 December 2024
Available online 3 December 2024
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and third-generation tyrosine kinase inhibitors (TKIs) have been developed, which include nilotinib, dasatinib, bosutinib, and ponatinib [10–13]. These more recently developed TKIs are extremely active against all the imatinib resistance *BCR*::*ABL1* mutations with the exception of the particularly frequent "gate-keeper" mutation T315I [14]. Only ponatinib, a multi-target third generation TKI, is active against BCR::ABL1 harboring this mutation. Indeed, ponatinib is widely used for resistant patients treated with previous therapies [15,16]. Unfortunately, in one-third of patients it induces a broad spectrum of severe adverse events (in particular cardiovascular reactions, especially arterial occlusive events, as well as skin rash, myelosuppression and pancreatitis) [17–20], which significantly limit its therapeutic potential. A response-based, dose-reduction strategy in OPTIC trial demonstrates to mitigate the toxicity but at 2 years 20 % of patients still experience progression of disease [21,22]. Asciminib, a STAMP (Specifically Targeting the ABL1 Myristoyl Pocket) inhibitor, and olverembatinib, in phase I and I/II trial respectively, are promising in terms of their safety and efficacy in T315I-mutated CML [23,24], but longer follow-up in larger patient cohorts are necessary to consolidate these data. Therefore, T315I-mediated CML resistance remains largely an unmet clinical need and alternative therapies are urgently necessary [25].

The protein kinase CK2 is a ubiquitous, pleiotropic and constitutively active Ser/Thr kinase, usually present in cells as a tetramer composed of two catalytic (α and/or α′) and two regulatory (β) subunits [26]. It not only plays crucial functions in many physiological processes, but also is involved in several diseases [27], notably in cancer, where it is frequently overexpressed and contributes to the malignant phenotype through multiple mechanisms [28,29]. We have previously reported that, in CML cell lines characterized by imatinib-resistance unrelated to *BCR*::*ABL1* mutations, CK2 is overexpressed [30] and contributes to the resistant phenotype by a BCR::ABL1-independent activation of rpS6 signaling [31]. Notably, the CK2-inhibitor CX-4945, which is currently in clinical trials for the treatment of different tumors [32], and its derivative CX-5011 [33], markedly activate the apoptotic pathways and restore the sensitivity to low TKI doses in all the analyzed imatinib-resistant CML cells [30,31].

The potential benefit of CK2 targeting in T315I-mediated TKI resistance has never been investigated so far. Mitrovský and colleagues recently established six novel imatinib- and dasatinib-resistant CML cell lines [34], and found high CK2 activity in resistant compared to sensitive cells, thus confirming our previous finding [30,35]. Although JURL-MK1 cells with the T315I mutation was one of the newly established resistance cell lines, a detailed analysis of the response of the T315I cells to CK2 inhibitors, alone or in combination with other agents, has not been performed.

Here, we dissect the roles of CK2 in the BCR::ABL1 T315I mutation-mediated TKI-resistance through both pharmacological and genetic approaches, and investigate potential novel therapeutic strategies to overcome this resistance.

Our data suggest that CK2 plays essential roles in the molecular phenotype induced by the BCR::ABL1 T3151 mutation, and that CK2 targeting represents a promising strategy to induce apoptosis of T315I-CML cells and to sensitize them to TKI drugs.

2. Materials and methods

2.1. Materials and antibodies

Protease inhibitor cocktail was from Calbiochem (Darmstadt, Germany), while phosphatase inhibitor cocktails from Sigma-Aldrich (Dorset, U.K.). Imatinib mesylate was from Cayman Chemical (Ann-Arbor, MI, USA). Ponatinib, bosutinib, CX-4945, GO289 and SGC-CK2–1 were from MedChemExpress (Monmouth Junction, NJ, USA). Anti-CK2α/α′ antibody was from Biorad Laboratories (Hercules, CA, USA). Anti-CK2β, anti-phospho-LYN(Y396), anti-LYN, anti-caspase 3 were from Epitomics (Burlingame, CA, USA). Anti-phospho-ABL1(Y245) was from Calbiochem. Anti-c-ABL1, anti-CK2α′, anti-AKT, anti-rpS6, anti-LDH (lactate dehydrogenase), anti-phospho-p65 NF-kB (S529), anti-p65 NF-kB, anti-HSP70, anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PARP was from Roche (Basel, Switzerland). Anti-ERK1/2, anti-phospho-ERK1/2(T202/Y204), anti-phospho-AKT(S473), anti-phospho-rpS6(S235–6), anti-STAT5, anti-STAT3, anti-phospho-STAT3 (Y705), were from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-AKT(S129), anti-phospho-STAT5 (Y694) were from Abcam (Cambridge, U.K.). Anti-tubulin was from Sigma-Aldrich (MI, USA).

2.2. Cells and cell culture

Human CML cell lines KBM5 and KBM5-T315I [36,37], and MS5 (murine stromal cells) were grown in RPMI 1640 supplemented with 10 % (v/v) fetal bovine serum (FBS) and PSG (1 % penicillin and streptomycin and 2 mM L-Glutamine) (Sigma). KBM5-T315I cells were grown in the presence of 1 μM imatinib, which, before experiments, was removed followed by a wash-out period of 2–3 days.

All cells were maintained at 37 °C in a humidified atmosphere with 21 % O_2, 5 % CO_2 (normoxia, see below for hypoxia).

Peripheral blood sample were obtained from a patient with diagnosis of chronic phase CML and T315I mutation after written informed consent. Analysis on patient-derived cells were performed in line with the principles of the Declaration of Helsinki. Approval was granted by Ethics Committee (Istituto Oncologico Veneto n.2021–102 date 19/07/2021). Leukocytes were purified from whole blood, which were treated twice with 155 mM NH_4Cl, 10 mM $KHCO_3$, and 100 mM EDTA at pH 7.4 for 5 min at room temperature (RT) to lyse erythrocytes followed by a centrifugation at 800 xg for 5 min (RT). Leukocytes were then washed twice with PBS and then cultured in RPMI 1640 supplemented with 10 % (v/v) fetal bovine serum (FBS) and PSG (1 % penicillin and streptomycin and 2 mM L-Glutamine) (Sigma) at 37 °C in the presence of 5 % CO_2.

2.3. Generation of KBM5-T315I knockout cells by CRISPR/Cas9 technology

CK2α subunit knockout was performed as previously detailed [38]. Briefly, all-in-one plasmids expressing Cas9-DasherGFP and the sgRNA guide (CMV-Cas9-2A-GFP, Cas9-ElecD) to target CK2α were purchased from ATUM (Newark, CA, USA). The sgRNA guide sequence for *CSNK2A1*, 5′-CCTGGATTATTGTCACAGCA-3′, was chosen using the CRISPR MultiTargeter tool [39] and off targets were excluded using GT-Scan tools [40].

KBM5-T315I cells were cultured in 6-well dishes to 70–80 % confluence and then co-transfected with 2 μg of sgRNA plasmid and jetOPTIMUS (Polyplus, France) according to the manufacturer's instructions. Forty-eight hours post-transfection, cells were pelleted in PBS and GFP-positive cells were sorted in 96-well plates using fluorescence-activated cell sorting (FACS) with a FACSAria II cell sorter (BD Bio-Sciences, San Jose, CA, USA). Sorted cells were expanded to obtain individual clones, which were selected by immunoblotting analysis using a CK2α specific antibody in order to verify the absence of target-protein expression.

2.4. Western blot analysis

Cells were lysed by suspension (1 h at 4° C) in the lysis buffer A containing 20 mM Tris-HCl (pH 7.5), 1 % Triton X-100, 10 % glycerol, 1 mM EDTA, 150 mM NaCl and protease and phosphatase inhibitor cocktails. Protein concentration was determined by Bradford method. Proteins from cell lysates (50 μg for BCR::ABL1 detection, 20–30 μg for the other proteins) were subjected to 11 % SDS-PAGE (or 7.5 % in the case of BCR::ABL1), blotted on Immobilon-P membranes (Sigma-

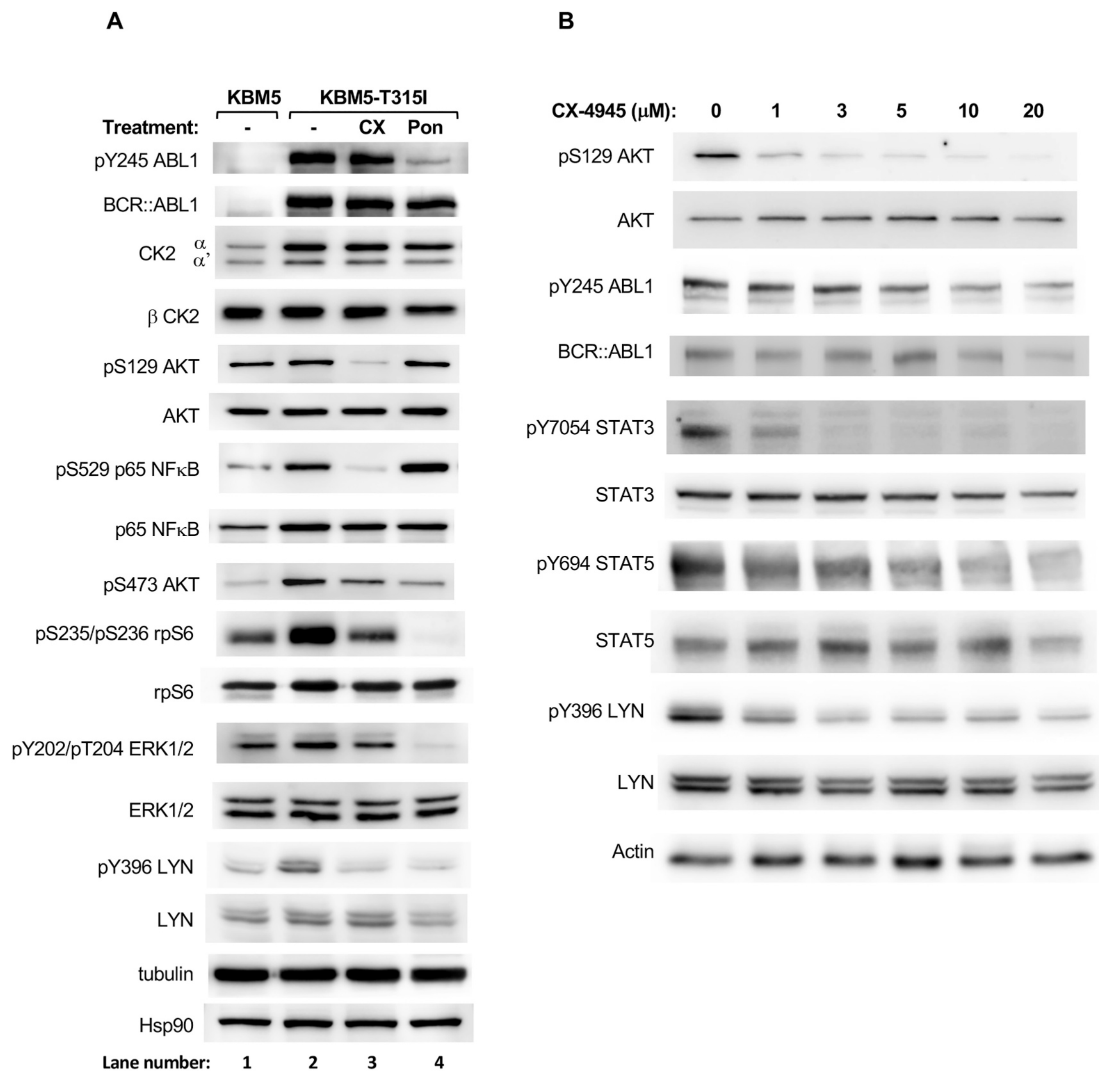


Fig. 1. Analysis of BCR::ABL1 and CK2 signaling in KBM5 and KBM5-T315I CML cells, and effects of CK2 inhibition.
20–50 μg proteins from KBM5 cells and KBM5-T315I cell lysates were analyzed by WB with the indicated antibodies. Tubulin, HSP90, or actin were used as loading control. (A) KBM5 and KBM5-T315I cells were treated for 5 h with vehicle (DMSO) (−, lane 2), 3 μM CK-4945 (CX, lane 3), or 2 nM ponatinib (Pon, lane 4), as indicated. Representative WB of at least five separate experiments are shown, while quantification of the bands is shown in Supplementary Fig. 1A. (B) KBM5-T315I cells were treated for 24 h with vehicle (DMSO) (−), or increasing concentrations of CX-4945. Representative WB of at least three separate experiments are shown, while quantification of the bands is shown in Supplementary Fig. 1A.

Aldrich), processed in western blot with the indicated antibodies and developed using an enhanced chemiluminescent detection system (ECL). Immunostained bands were detected by chemiluminescence on Image-Quant LAS 500 (GE Healthcare Life Sciences) and quantified with Carestream Molecular Imaging software (Carestream, Rochester, NY, USA).

2.5. RNA interference

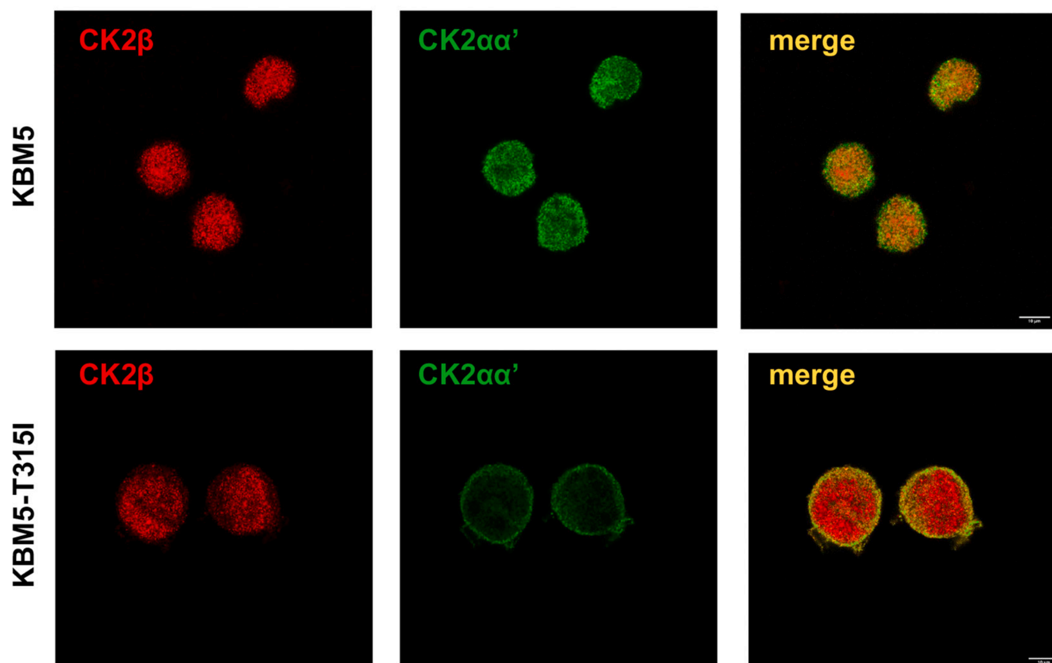
Cells (1.5×10^6) were transfected for 48 h with 50 nM CK2α specific siRNAs as described in [41]. Unspecific siRNA siCONTROL riscfree#1 (Dharmacon) was used as a control. Cells were transfected using the transfecting reagent INTERFERin (Polyplus, Illkirch, France), according to the manufacturer's recommendations.

2.6. Immunofluorescence microscopy

Cells (5×10^5) were seeded on polylysine-coated glass coverslips, fixed with 4 % para-formaldehyde in PBS for 20 min at room temperature (RT), then permeabilized and blocked with a solution containing 0.1 % Triton X–100 and 5 % BSA in PBS for 1 h at RT. For dual labeling cells were firstly incubated with mouse rabbit anti-CK2αα′ antibody (1:50) overnight at 4 °C followed by anti-mouse Alexa Fluor 488 conjugated antibody (1:500) for 1 h at 37 °C. Cells were then incubated with rabbit anti-CK2β antibody (Thermofisher, 1:100) followed by goat anti-rabbit Alexa-Fluor 555 conjugated antibody (1:500) for 1 h at 37 °C. Between each incubation, three washes in PBS were performed. Coverslips were mounted on microscope slides using Mowiol (Sigma Aldrich) and images were captured using a LEICA-TCS SP5 confocal microscopy (Wetzlar, Germany), equipped with HCX-PL-APO 100×/1.4 oil immersion objective. Images were processed with the LAS-AF software.

2.7. Cell fractionation

Cells (7×10^6) were washed twice with PBS and then resuspended in 300 μl of ice-cold hypotonic buffer containing 20 mM Tris-HCl, pH 7.8, 1 % (v/v) Nonidet P-40, and protease and phosphatase inhibitor cocktails. After 5 min, samples were diluted 1:1 with water and the solution passed through a 23 G needle 10 times using a 2 ml syringe. Samples were immediately centrifuged at 700 rpm for 10 min at 4 °C.



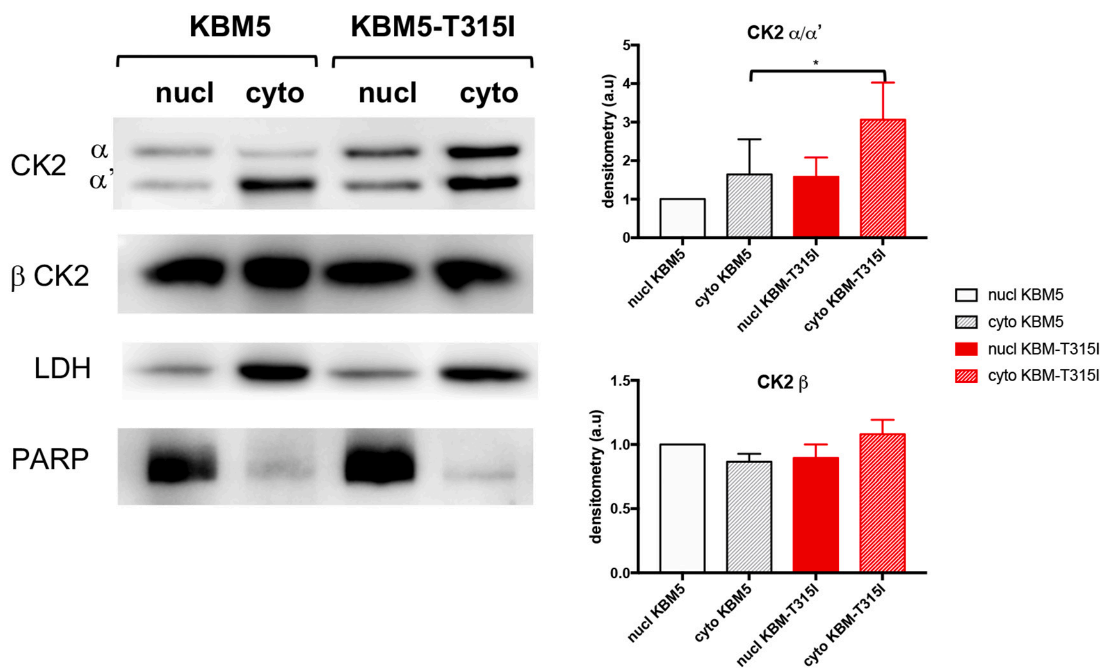
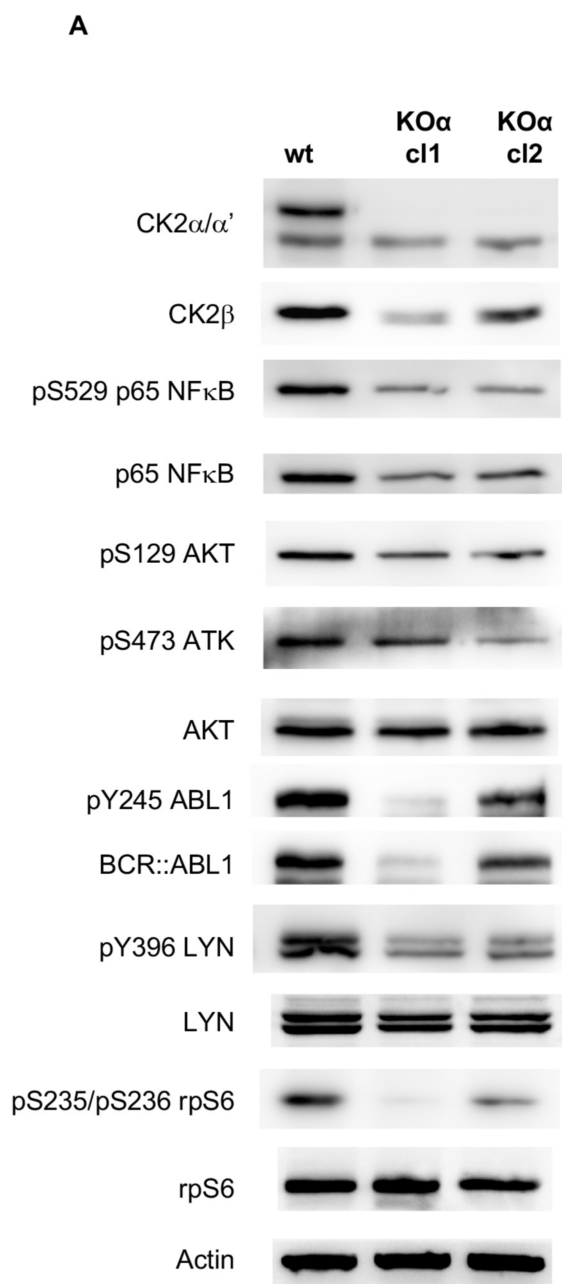


Fig. 2. Subcellular distribution of CK2 subunits in KBM5 and KBM5-T315I cells.
(A) Representative microphotographs of KBM5 or KBM5-T315I cells is immunostaining for CK2αα′ (green) and CK2β (red). Merging of both signals is visualized in yellow. Images were captured with confocal microscopy (zoom 2×). (B) 20 μg of nuclear or cytoplasmic protein fractions were analyzed by WB with the indicated antibodies. LDH or PARP were used as cytosolic or nuclear fraction markers, respectively. Representative WB of at least three experiments are shown, while quantification of the bands is shown in bar graphs (means ± SD).



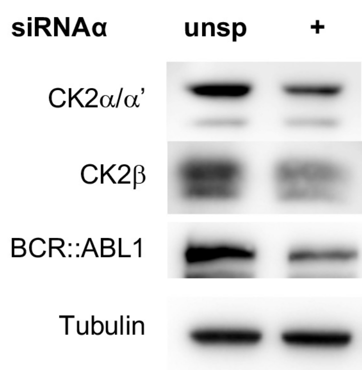


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Fig. 3. Analysis of BCR::ABL1 and CK2 protein kinase signaling in KBM5-T315I cells knockout and knockdown for CK2α.
(A) 20–50 μg of cell lysates from KBM5-T315I cells wild type (wt) or two different clones knocked out for *CSNK2A1* (KOα, cl1 and cl2) were analyzed by WB with the indicated antibodies. Actin was used as loading control. Representative images of at least four experiments are shown. (B) KBM5-T315I cells were transfected with unspecific (unsp) or CK2α specific siRNA, as indicated. After 48 h, lysate proteins (20 μg) were analyzed by WB with the indicated antibodies. Tubulin was used as loading control. Representative images of two experiments are shown.

Supernatants were collected as cytoplasmic fractions, while the pellets containing the nuclear fractions were extracted with 100 μl of lysis buffer A.

2.8. Cell viability assay

Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide] assays, incubating 3×10^4 cells/100 μl in a 96-well plate under different conditions, in triplicate. 2 h before the total incubation time, 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 λ 540 nm absorbance, in a Tecan spectrophotometer plate reader (Tecan Group Ltd., Switzerland). DC_{50} (concentration inducing 50 % of cell death) values were calculated with Prism 7 software (GraphPad Software, MA, USA).

2.9. Combination treatments

Effect of combined inhibitors was assessed by treating cells with increasing concentrations of drugs, added at fixed ratio, as indicated. The combination index (CI) [42] for the combination treatments were calculated with the software Compusyn (Biosoft, Cambridge, U.K.); CI $<$ 1, CI $=$ 1 and CI $>$ 1 indicate synergistic, additive and antagonistic effects, respectively.

2.10. Hypoxic treatments

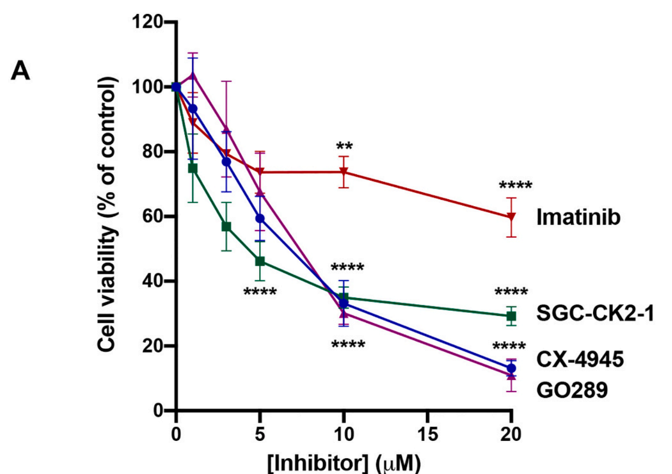
For hypoxia, cells were kept for 24 h in an incubator chamber (STEMCELL technologies, Cologne, Germany) purging the chamber at a rate of 20 l/min for 4–5 min with a gaseous mixture composed by O_2 1% - CO_2 5 % - N_2 94%.

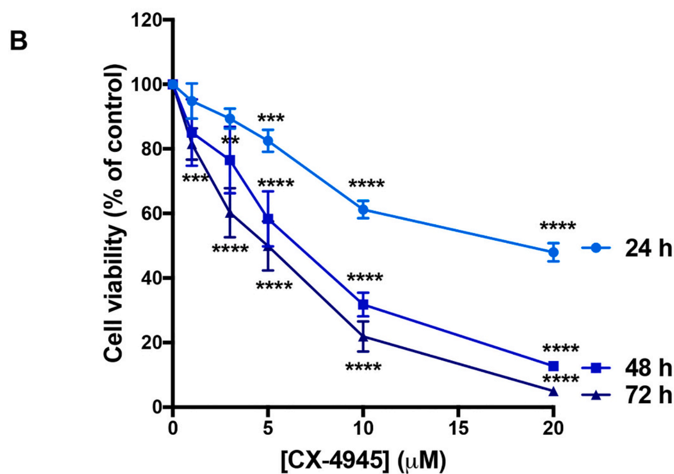
2.11. Clonogenic survival assays

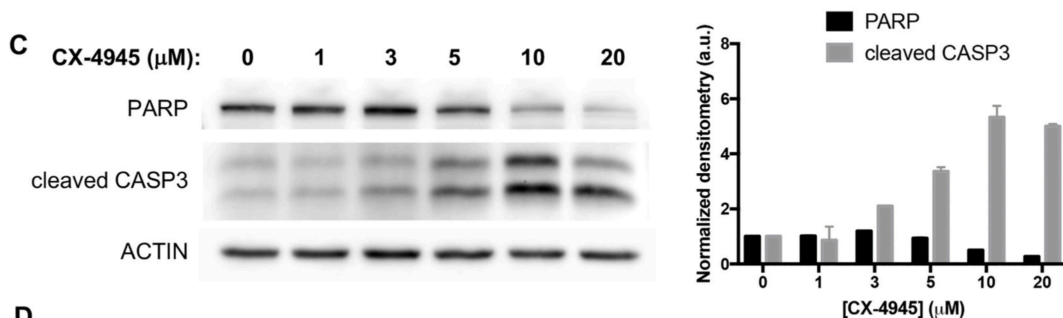
4.5×10^3 cells were suspended in 1 ml of RPMI medium with 0.3 % (w/v) agar (Sigma, USA) and then seeded over a 1 ml base layer of RPMI medium with 0.6 % (w/v) agar in 12-well plates. Media were replaced by fresh culture medium every 3 days. After 14 days, liquid media was removed and cells were stained with 0.45 mg/ml MTT to count active colonies. Digital images of cell colonies were obtained using a DCC1645C camera (ThorLabs, Newton, NJ, USA) equipped with a 109/0.25 objective. Quantification was performed with ImageJ software ("ColonyArea" plugin) [43] to determine the area covered by colonies in the well and the colony intensity percentage.

2.12. Flow cytometry (FACS) analysis

MS5 cells were seeded at 8×10^4/ml in a 12-well plate and allowed to grow for 24 h. Next, for co-culture condition KBM5 cells were seeded at $0{,}56 \times 10^6$/ml on top of MS5 cells. After 48 h, cells were treated with CX-4945 at 5, 10 or 20 μM for 24 or 48 h. Non-adherent cells were collected by pipetting, washed with PBS w/o Ca^{2+} and resuspended in 200 μl antibody staining mix (Annexin V PE and 7-AAD) (Invitrogen by







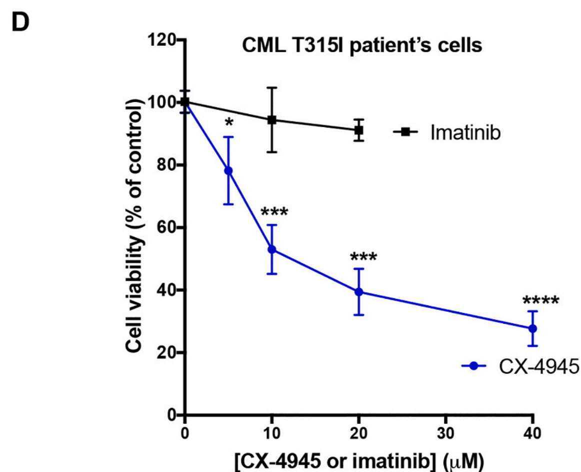


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Fig. 4. Effects of CK2 inhibition or CK2α knockout on KBM5-T315I cell viability.
(A) KBM5-T315I cells were treated for 48 h with increasing concentrations of the indicated inhibitors. Cell viability, analyzed by MTT, is expressed as % of the vehicle (DMSO)-treated controls (means of at least three experiments). (B) KBM5-T315I cells were treated for the indicated times with increasing concentrations of CX-4945. Cell viability, analyzed by MTT, is expressed as % of the vehicle (DMSO)-treated controls (means of at least three experiments). (C) KBM5-T315I cells were treated with the indicated concentrations of CX-4945 for 24 h, then cell lysate proteins (20 μg) were analyzed by WB for PARP and the cleaved forms of caspase 3 (CASP3), as indicated. Actin was used as loading control. WBs are representative of four separated experiments, while quantification of the bands (PARP and lower CASP3 bands) is shown in the bar graph (means ± SD) in arbitrary units (a.u.) assigning 1 to the corresponding band in untreated cells. (D) Leukocytes from a CML patient harboring the BCR::ABL1 T315I mutation were treated with increasing concentrations of CX-4945 for 48 h. Cell resistance to high concentrations of imatinib was also confirmed. Cell viability, analyzed by MTT, is expressed as percentage of the controls (treated with vehicle, DMSO).
Statistical significance refers to the vehicle-treated controls.

Thermo Fisher, Carlsbad, CA, USA) for 10 min on ice in the dark before analysis with the Attune NxT® Acoustic Focusing Cytometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with blue excitation laser (488 nm).

2.13. Statistical analysis

Data were analyzed using Prism Software (GraphPad, Boston, USA), and are presented as means ± SD. The number of experiments is indicated in each figure legend.

The statistical significance was evaluated by Student's *t*-test for the comparison of two groups, or one-way ANOVA followed by Bonferroni's multiple comparison test for more than two groups, setting (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$. Where no asterisks are indicated, not significance was found.

3. Results

3.1. CK2 contribution to pro-survival signaling pathways in KBM5-T315I cells

To investigate the role of CK2 in the T315I-dependent imatinib resistance of CML, we performed a comparison between KBM5 cells (harboring wild-type BCR::ABL1) and KBM5-T315I imatinib-resistant cells harboring T315I BCR::ABL1. We analyzed the BCR::ABL1 activation state and the CK2-dependent signaling, as well as other pro-survival and oncogenic pathways (Fig. 1A, lanes 1 and 2, and Supplementary Fig. 1A), and we assessed the effects of CK2 inhibition on these signals (Fig. 1A, and B), also in comparison to BCR::ABL1 inhibition.

As previously described [44], in KBM5-T315I cells the $p210^{BCR/ABL1}$ oncokinase is not only mutated, but also overexpressed and hyper-activated, as evidenced by the autophosphorylation site Y245 (Fig. 1A, lanes 1 and 2, and Supplementary Fig. 1A). Interestingly, the protein levels of both the CK2 catalytic subunits (CK2α and CK2α′) are higher in KBM5-T315I compared to those in imatinib-sensitive cells, without a concomitant increase of the regulatory β-subunit (Fig. 1A, lanes 1 and 2, and Supplementary Fig. 1A).

Since CK2 phosphorylates and activates the pro-survival kinase AKT1 at Ser129 [45,46] and the anti-apoptotic transcription factor NF-kB (nuclear factor kappa B) at Ser529 of the p65 subunit [47], we analyzed the phosphorylation levels of these sites as reporters of the CK2 activity and of its function in potentiating oncogenic pathways. We found that they are higher in KBM5-T315I as compared to KBM5 cells (Fig. 1A, and Supplementary Fig. 1A), with an increase also of p65 NF-kB protein amount. Then we examined other pro-survival pathways, which are considered downstream to BCR::ABL1 [2,3] (Fig. 1A and B, and Supplementary Fig. 1). We observed hyper-phosphorylation of AKT (activation site Ser473) and up-regulation of the AKT downstream-target ribosomal protein rpS6. Additionally, the SRC-family tyrosine kinase LYN is strongly activated in KBM5-T315I as compared to control cells. On the contrary, we found that ERK1/2 activation, expected to be dependent on BCR::ABL1 [2], is not significantly different in KBM5 parental or TKI-resistant cells (Fig. 1A, lanes 1 and 2, and Supplementary Fig. 1A).

The effects of CK2 inhibition on these signals were analyzed by treating KBM5-T315I cells for 5 h with CX-4945 [48], a selective and potent CK2 inhibitor currently under clinical trials [49], and were compared with those induced by ponatinib, the only TKI active against T315I-BCR::ABL1 [16].

As expected, CX-4945 is ineffective on BCR::ABL1, while it almost abrogates the phosphorylation of the CK2-dependent sites; concomitantly, it strongly reduces all the signaling pathways found higher in KBM5-T315I than in parental cells (Fig. 1 A, and Supplementary Fig. 1A). In contrast, the ERK1/2 activity state, which is similar in sensitive and resistant cells, is not affected by the CK2 inhibitor. On the other hand, as expected ponatinib almost abrogates the BCR::ABL1 and ERK1/2 activation, and reduces the AKT, rpS6, LYN signaling pathways, without affecting the CK2 protein-amount and its activity towards its specific targets (Fig. 1A, and Supplementary Fig. 1A). Supplementary Fig. 2 shows that the analyzed survival pathways were similarly affected by the CK2 inhibition also in the parental imatinib-sensitive KBM5 cells, with the notable exception of LYN, whose phosphorylation, strongly reduced by CX-4945 in KBM5-T315I cells, was instead affected only by Ponatinib in KBM5 cells (see Discussion for comments).

To better evaluate the response to CK2 inhibition of KBM5-T315I cells, we performed cell treatment with various concentrations of CX-4945, and for a longer time (24 h). Fig. 1B shows that a strong inhibition of CK2 is already present at 1 μM CX-4945, as indicated by the pS129 AKT level, a concentration already effective in reducing LYN signaling. Moreover, with 24 h treatment, the inhibition of phospho-BCR::ABL1 was also observed. Furthermore, Fig. 1B shows that CX-4945 is effective also on the two oncogenic transcription factors STAT3 and STAT5 [50], whose phosphorylation at crucial activation sites (pTyr705-STAT3 [51] and pTyr694-STAT5 [52]) is already affected at 1 μM CX-4945. All quantifications are shown in Supplementary Fig. 1B.

These results demonstrate that the aberrant activation of signaling pathways crucial for the KBM5-T315I TKI-resistance are under the control of CK2, besides BCR::ABL1.

3.2. CK2 catalytic subunits display a cytosolic localization and interact with BCR::ABL1 in KBM5-T315I cells

We performed confocal microscopy immunofluorescence experiments to compare the subcellular localization of the CK2 subunits in the two KBM5 cell variants. As shown in Fig. 2A, while the regulatory β subunit is similarly localized in the KBM5-T315I and the parental KBM5 cells, both in the cytoplasm and the nucleus, the α and α′ catalytic subunits display a different distribution, much more evident in the cytoplasm in KBM5-T315I cells. The preferred cytoplasmic localization of the CK2 catalytic subunits (especially CK2α) in KBM5-T315I cells is also supported by the WB analysis of cytoplasmic and nuclear fractions obtained by means of subcellular fractionation by differential centrifugation (Fig. 2B). BCR::ABL1 is mainly retained in the cytoplasm of CML cells, where most proteins involved in its oncogenic pathway are located [53], thus the higher cytosolic amount of CK2 in the resistant cells, along with the already reported association of CK2 with BCR::ABL1 [30], further support the crosstalk between the two signaling molecules.

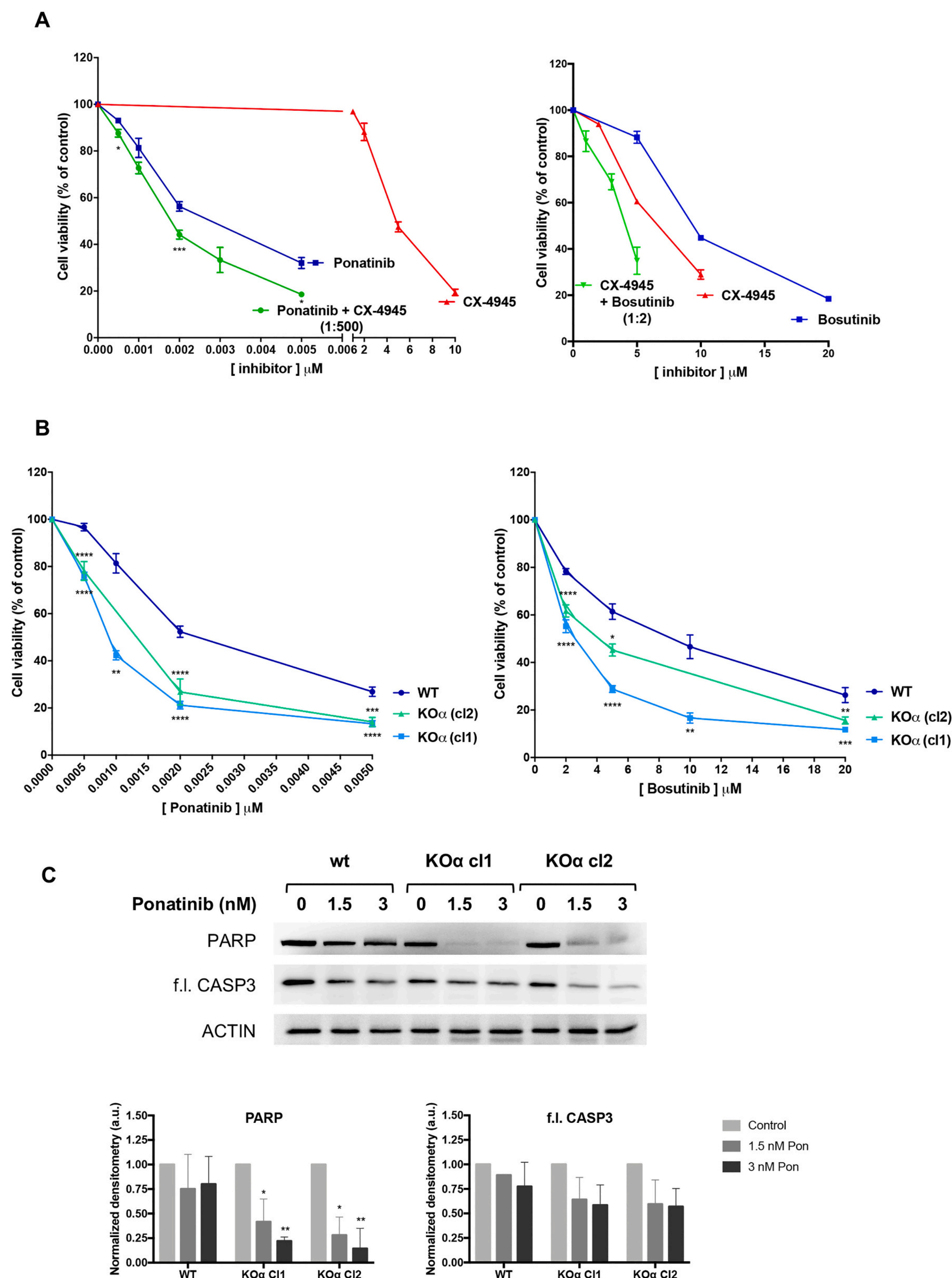


Fig. 5. Effect of CK2 targeting in combination with ponatinib or bosutinib on KBM5-T315I cell viability.
(A) KBM5-T315I cells were treated with the indicated concentrations of CX-4945, ponatinib or bosutinib, or with two combined drugs by simultaneously increasing their concentration at the ratio of 1:500 for ponatinib + CX-4945 (left panel) or 2:1 for bosutinib + CX-4945 (right panel). Viability was assessed by the MTT method after 48 h treatment and expressed as percentage of vehicle-treated controls. Statistical significance was calculated comparing TKI alone vs TKI + CX-4945 treatments (B) KBM5-T315I cells, either wt or KOα (two clones), were treated for 48 h with increasing concentrations of ponatinib (left panel), or bosutinib (right panel). Viability was assessed by the MTT method, and is expressed as percentage of wt cells. Mean ± SD values of at least four independent experiments are reported. Statistical significance refers to the comparison between each KOα clone and wt cells. (C) KBM5-T315I cells, either wt or KOα (two clones), were treated with the indicated concentrations of ponatinib. Cell lysates (20 μg) were analyzed by WB with the indicated antibodies (f.l. CASP3: full length caspase 3). Actin was used as loading control. Quantification of the bands (means ± SD of two independent experiments) is shown in the bar graphs.

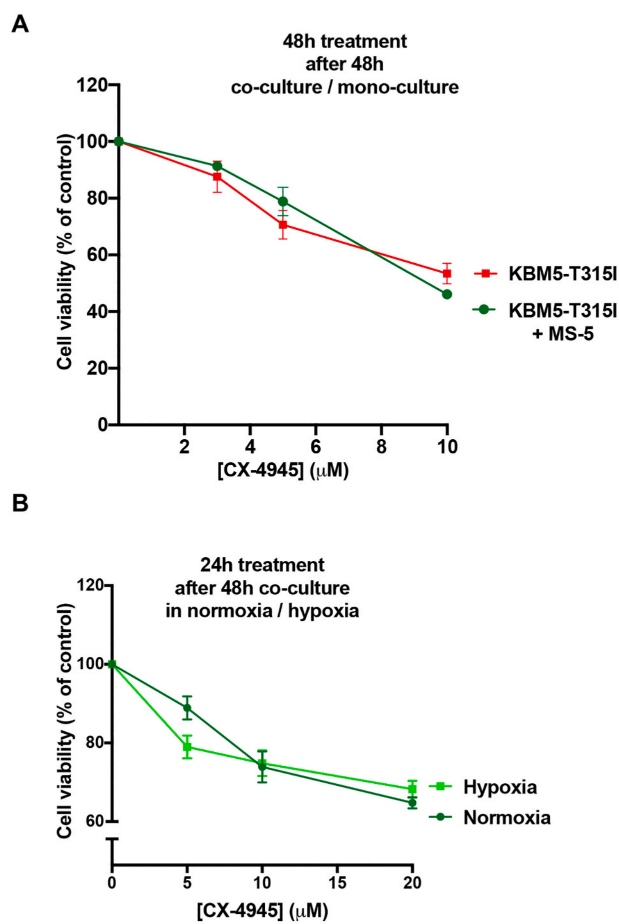


Fig. 6. Effect of CX-4945 on KBM5-T315I cell viability in the presence of stromal cells, and under hypoxic conditions.
(A) KBM5-T315I cells were treated for 48 h with increasing concentrations of CX-4945, either in mono-culture or in co-culture with MS5 cells. (B) KBM5-T315I cells, in co-culture with MS5 cells, were treated for 24 h with increasing concentrations of CX-4945, either in normoxic or hypoxic conditions. In (A) and (B), cell viability, assessed by the MTT method, is expressed as a percentage of vehicle-treated controls, as mean ± SD of two independent experiments (each one in triplicate). No statistical significance was found by comparing mono-culture with co-culture (A) or normoxic with hypoxic (B) conditions.

3.3. CK2α knockout significantly decreases the amount of BCR::ABL1 and down-regulates survival signaling pathways peculiarly hyperactivated in KBM5-T315I cells

To further confirm the results obtained with the CK2 inhibitor CX-4945, supporting the role of CK2 in the imatinib-resistance of KBM5-T315I cells, we generated two stable CK2α knockout clones (KOα), by CRISPR/Cas9 deletion of the CK2α coding gene *CSNK2A1*. Fig. 3A shows the characterization of two KOα clones in comparison to the control cells (indicated as wild type (wt), meaning the parental KBM5-T315I cells, with no CK2α ablation). The ablation of CK2α does not affect the amount of the other catalytic isoform, CK2α′, while significantly down-regulates the CK2β regulatory subunit (as expected, due to its instability when not associated with the catalytic subunits) [54,55]. In the KOα cells, CK2 activity is reduced, as judged by the phosphorylation level of the CK2 targets AKT Ser129 and p65 NF-kB Ser529 (with a remarkable decrease of the p65 NF-kB protein level as well) with residual phosphorylation most probably due to the unchanged expression of the CK2α′ isoform (Fig. 3A and Supplementary Fig. 3A). As in CX-4945-treated cells, in both CK2α KO clones we observed a reduction of the signaling pathways up-regulated in the resistant cells, namely LYN, rpS6, and AKT. Interestingly, the KOα cells display a reduced level of the CML-causative protein BCR::ABL1 (Fig. 3A and Supplementary Fig. 3A). To rule out the possibility that the BCR::ABL1 down-regulation detected in KOα cells is due to compensative effects in stably CK2α-deleted cells during clone selection, we performed transient CK2α silencing in KBM5-T315I cells. We found that also the partial and transient reduction of CK2α level (which, as expected, is accompanied by CK2β reduction) [54,55], is associated with a decrease of BCR::ABL1 protein level (Fig. 3B and Supplementary Fig. 3B), confirming the close interplay occurring between CK2 and BCR::ABL1 in these imatinib-resistant cells.

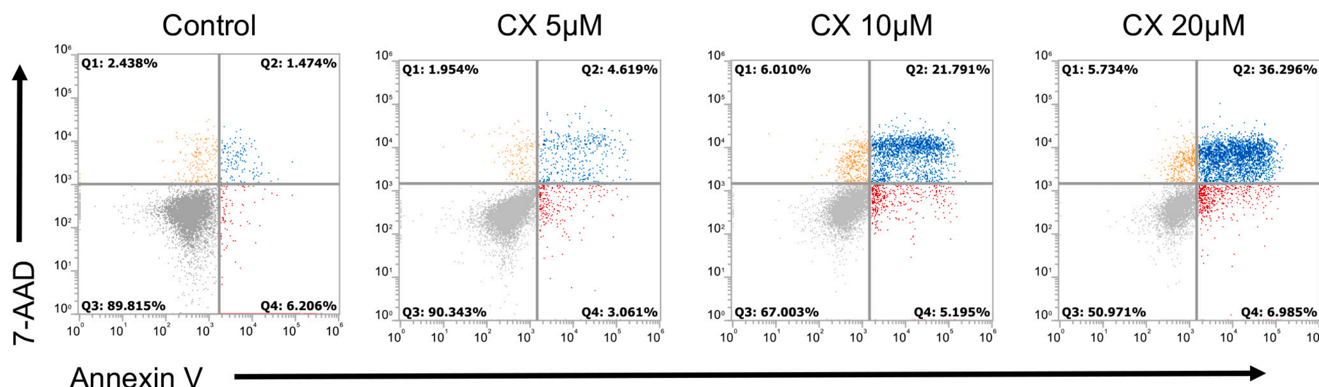
3.4. Pharmacological or genetic targeting of CK2 promotes apoptosis and TKI sensitization of KBM5-T315I cells

Since our results indicate that CK2 targeting attenuates signaling pathways that are crucial for KBM5-T315I cell survival and their TKI resistant phenotype, we next evaluated cell survival in response to treatments with different and structurally unrelated CK2 inhibitors, including the most commonly used CX-4945 [48], and two more recently developed compounds, GO289 [56] and SGC-CK2–1, this latter representing the most selective CK2 inhibitor known so far [57]. Fig. 4A shows MTT survival assays of KBM5-T315I cells treated for 48 h with increasing concentrations of the inhibitors, which are all effective in reducing viable cells in the low μM range (while, as expected, imatinib, used for comparison, is ineffective, even if used at very high concentrations compared to those usually applied). The effects of the different CK2 inhibitors on crucial signaling pathways are shown in the Supplementary Fig. 4. Given the similar efficacy of the CK2 inhibitors, and the clinical trials already ongoing with CX-4945 (https://classic.clinicaltrials.gov/ct2/results?cond=&term=CX-4945&cntry=&state=&city=&dist=), this compound was chosen to further explore the sensitivity of the KBM5-T315I cells, that was concentration- and time-dependent (Fig. 4B). The strongest effect requires at least 48 h treatment, in agreement with a doubling time between 24 and 60 h reported for KBM5 cells [36]. The cleavage of the apoptosis-associated proteins PARP and caspase 3 (Fig. 4C) indicates that CK2 inhibition induces apoptotic cell death. As expected, sensitivity to CK2 inhibitors was also observed in TKI-sensitive KMB5 cells, as shown in Supplementary Fig. 5.

Of great importance to the significance of our investigation, we also had the opportunity, despite the rarity of the cases, to analyze the efficacy of CX-4945 on cells derived from a patient with the T315I mutation of BCR::ABL1. The patient experienced refractoriness to imatinib treatment and failure to dasatinib at 12 months, consequently, as per clinical guidelines [58], he was investigated for BCR::ABL11 kinase domain-mutations with Sanger sequencing, that demonstrate the T315I mutation [59]. As shown in Fig. 4D, the patient cells were sensitive to the treatment with the CK2 inhibitor, which decreased viable cells in the same range of concentrations effective in KBM5-T315I cells. The expected resistance to imatinib, due to the T315I mutation, was also confirmed and is shown in Fig. 4D.

We also explored the efficacy of CX-4945 in combination with ponatinib or bosutinib (a very active and safe second-generation dual Src/ABL1 inhibitor [13,60]) by treating KBM5-T315I cells for 48 h with the inhibitors either alone or in combination. We found (Fig. 5A) that CX-4945 administrated with the TKIs lowered their effective doses in reducing cell viability, with a synergistic effect as confirmed by a combination index (C.I.) of 0.38 (calculated at the 50 % of cell death) in the case of ponatinib (synergism is attested by C.I. < 1 [42]).

Similarly, the sensitivity to TKIs was higher in KBM5-T315I CK2α KO clones, compared to control KBM5-T315I cells. MTT assays (Fig. 5B) revealed a shift of the DC50 (dose inducing 50 % cell death) for ponatinib from 2.3 nM of the wt cells to 0.9 and 1.3 nM of the KOα clones 1 and 2, respectively, and for bosutinib from 8.8 μM of the control cells to 2.6 (KOα clone 1) and 4.1 (KOα clone 2) μM. The increased sensitivity to TKIs of the KOα clones is also documented by the cleavage of PARP and caspase 3 induced by ponatinib at doses almost completely ineffective in



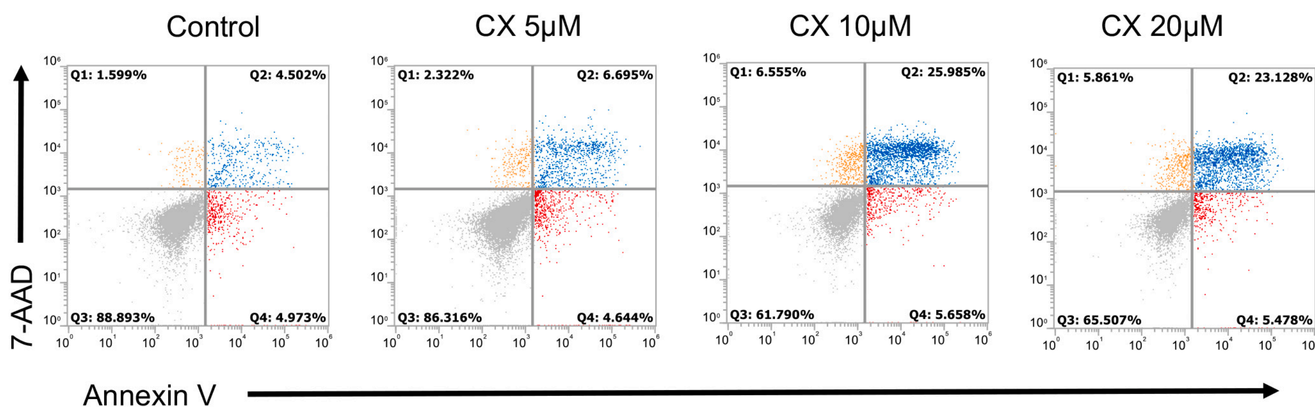


Fig. 7. Apoptosis FACS analysis of KBM5-T315I cells, in mono-culture or in the presence of stromal cells, in response to 48 h CX-4945 treatment.
After 48 h of mono-culture or co-culture with MS5 cells, KBM5-T315I cells were treated for 48 h with the indicated concentrations of CX-4945 (or vehicle, DMSO, for the controls), then stained with Annexin V PE (for apoptotic cell detection) and 7-AAD (for cell death detection) and analyzed by FACS.

control cells (Fig. 5C).

3.5. *The CK2 inhibitor CX-4945 is effective on KBM5-T315I cells also in the presence of stromal cells and under hypoxic conditions*

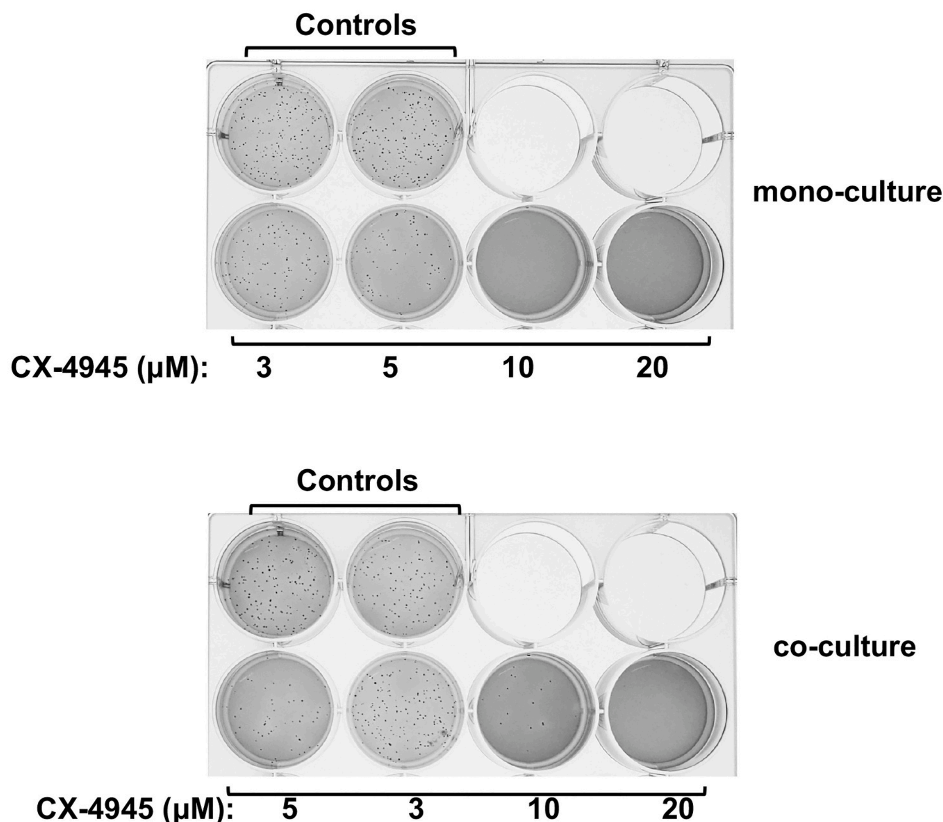
The bone marrow microenvironment (BMM) is known to protect leukemia cells against death inducing drugs [61]. To partially mimic the protective functions of BMM, we co-cultured KBM5-T315I cells with MS5 stromal cells, and evaluated the effects of CX-4945 firstly by MTT survival assays (exploiting the growth in suspension of the KBM5 cells, compared to the adhesion growth of MS5 cells), compared to those of the cells without MS5 co-culture. As shown in Fig. 6A, a 48 h CX-4945-treatment has similar efficacy without or with stromal cell co-cultures. Since the protection by the BMM is especially exerted under hypoxic conditions [62], we analyzed the potential protective effect of the stromal cells after 48 h of hypoxia (1 % O_2). We observed high spontaneous cell death after long exposure to hypoxic conditions (not shown), we therefore reduced the CX-4945 treatment to 24 h. We found that the inhibitor has a similar effect under hypoxia or normoxia conditions (Fig. 6B).

A FACS analysis (Fig. 7) showed that the death was mainly due to apoptosis. The percentages of apoptotic cells induced by 48 h treatment with increasing concentrations of CX-4945 were very similar in KBM5-T315I cultured alone (Fig. 7A) or co-cultured with MS5 (Fig. 7B). Similarity was also observed in the earlier events in response to the treatment (see results at 24 h, Supplementary Fig. 6).

To further assess the effect of CK2 inhibition on the survival and proliferation rate of KBM5-T315I cells, and the potential protective effect of stromal cells, we performed soft-agar colony assays (Fig. 8). We found that CX-4945 strongly reduces the clonogenic potential of KBM5-T315I cells, and MS5 cells prevents its effect only at the lower concentrations.

4. Discussion and conclusions

Despite the great clinical improvements since the introduction of the TKIs, CML patients with the T315I mutation in BCR::ABL1 still pose a therapeutic challenge, since they do not respond to first- and second-generation TKIs, and the treatment with the third-generation TKI ponatinib is unfortunately accompanied by important side effects, so that a shorter overall and failure-free survival is associated to this mutation [25]. Hence, CML is by no means a solved problem, and the



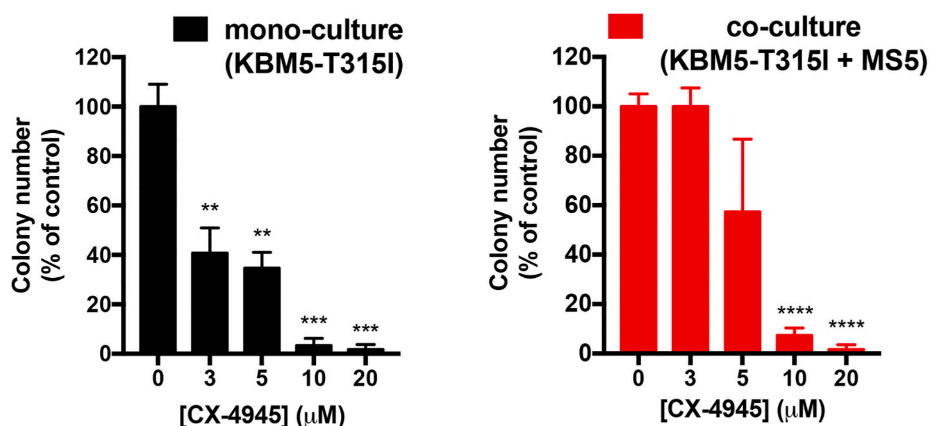


Fig. 8. Clonogenic assay of KBM5-T315I cells, in mono-culture or in the presence of stromal cells, in response to CX-4945.
KBM5-T315I cells were cultured alone or with MS-5 stromal cells 48 h before treatment, then treated for 48 h with CX-4945 at the indicated concentrations. Later, the KBM5-T315I cells were collected and seeded in semi-solid soft agar. Cells were cultured for 14 days and then treated with MTT to count live colonies. Representative images from 2 independent experiments are shown (A). Quantification is shown in (B), with data presented as mean ± SD. Statistical significance refers to the vehicle-treated controls.

identification of new therapeutic strategies is still urgently needed.

In this study, we suggest that protein kinase CK2 can indeed represent a valuable drug target in case of T315I mutation. This is based on several observations.

First, CK2 boosts survival pathways (such as AKT, NF-kB, LYN, STAT3, STAT5, rpS6) which are hyperactivated in T315I imatinib-resistant compared to imatinib-sensitive cells. The same signaling are reduced by CK2 inhibition, which is instead ineffective on those pathways that are equally active in resistant and in sensitive cells (see ERK signaling, Fig. 1), suggesting a quite specific function of CK2 on the resistant molecular phenotype. The effect of CK2 inhibition on LYN deserves special attention: to our knowledge, while the dependence of CK2 by LYN or other Src-related kinases has been already considered [63,64], our results are the first evidence of LYN affected by CK2

targeting. However, interestingly, this is observed only in Imatinib-resistant (Fig. 1 and Fig. 3) but not in Imatinib-sensitive (Supplementary Fig. 2) cells. This observation, along with the LYN hyperactivation in KBM5-T315I cells compared to the parental KBM5 cells, allows to speculate that a CK2/LYN axis could play a special role in supporting resistance to TKI in general, or, possibly, in the specific case of T315I-driven resistance, an aspect that could deserve future investigation.

The different CK2 inhibitors that we employed have very similar effects on KBM5-T315I signaling pathways (see Fig. 1 and Supplementary Fig. 4).

Our results obtained by pharmacological inhibition of CK2 were confirmed by genetic targeting of the CK2 α catalytic subunit. Two CK2α KO clones were analyzed, both confirming the downregulation of major signaling pathways hyperactive in resistant cells (although with slight differences between clones, see below). Among other signals, also BCR::ABL1 activation is sustained by CK2, whose inhibition reduces the level of the activating phospho-site Y245 in KBM5-T315I. Interestingly, the CK2 genetic targeting (CK2α knock-out by CRISPR-Cas9 or knock-down by siRNA) reduces also the BCR::ABL1 protein amount (Fig. 3), what was only minimally observed upon CK2 pharmacological inhibition. This suggests a role for the physical CK2/BCR::ABL1 interaction [30] which can be enhanced by the largely cytosolic localization of CK2α in the resistant cells (Fig. 2). It is worth noting that this peculiar localization of CK2α, observed in imatinib-resistant compared to -sensitive cells, together with the almost equal expression of CK2β in the two variants, implies that a certain amount of CK2α in the cytosol is not combined in the tetrameric holoenzyme. This is quite relevant considering that the presence of the isolated CK2α subunit has been already reported to have a role in drug resistance [65]. On the other hand, in the nucleus, a certain amount of CK2β appear to be free of catalytic subunits, as judged by the lower α/β colocalization signal observed in KBM5-T315I (Fig. 2A). Indeed, in our cellular model, the amount of CK2β seems to be important, as inferred by some differences that we observed between the two CK2α KO clones: clone 1, where the deletion of α is accompanied by a more pronounced reduction of the CK2β expression, is in general more effective than clone 2 in diminishing the oncogenic features of KBM5-T315I cells (see most signaling pathways of Fig. 3, but also sensitivity to ponatinib, Fig. 5). This could depend on holoenzyme functions (more impaired when CK2β is more reduced), or to specific functions of CK2β, considering that it has been found associated to other proteins and hypothesized to have also CK2-independent roles [66]. Future studies will be necessary to understand if a specific role of CK2β can be envisaged in this kind of TKI resistance.

A second important observation supporting our conclusions is that CK2 inhibitors induce apoptosis of T315I CML cells. Several CK2 inhibitors are available [67]. The recently discovered SGC-CK2–1 [57] is one of the most selective, and, although its efficacy in cancer cells has been questioned [68], it looks very promising [69]. However, the most widely used compound is still CX-4945, which is already in clinical trials. For this reason, while we confirmed the sensitivity of the T315I cells to different inhibitors, including SGC-CK2–1 (Fig. 4), we decided to focus our study mostly on CX-4945 employment. Importantly, we were able to perform CX-4945 treatment experiments also in cells derived from a CML patient in chronic phase with the T315I mutation, thus confirming the efficacy of the inhibitor at the same concentration range applied to KMB5 cells (Fig. 4). Of note, the pro-apoptotic action of CX-4945 occurs even in the presence of protective stromal cells and under hypoxic conditions (Figs. 6 and 7), where the effects of most drugs are dampened [61,70–72]. CK2 inhibition also reduces the proliferation and the clonogenic potential of the CML cells (Fig. 8). In this case, differently from the MTT assay (and probably due to the different type of assay and parameters evaluated), a protection by the stromal cells is observed at 3 and 5 μM CX-4945, which is however abrogated at the highest inhibitor concentrations.

Finally, and very importantly, the effects of CK2 targeting seem to be synergistic with TKIs, allowing to reduce the cell death-inducing doses of drugs normally used for CML, namely ponatinib and bosutinib (Fig. 5). This opens new perspectives on possible combination therapies based on CK2 inhibitors and TKIs, with the important consequence to reduce, together with the TKI effective doses, the side effects that they can cause, while maintaining both activity and efficacy of the administered therapy.

Collectively, our results demonstrate critical roles of CK2 in CML with BCR::ABL1 T325I mutation and suggest that protein kinase CK2 can represent a valuable drug target in T315I mutant CML. As multiple agents targeting CK2 are under development, this approach has the potential to rapidly move to clinic and enhance TKIs.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.138305.

CRediT authorship contribution statement

Camila Paz Quezada Meza: Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Valentina Salizzato:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Elisabetta Calistri:** Supervision, Resources. **Marco Basso:** Writing – review & editing, Resources. **Manuela Zavatti:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Sandra Marmiroli:** Writing – review & editing, Supervision, Resources, Conceptualization. **Mauro Salvi:** Resources, Methodology, Investigation, Funding acquisition. **Bing Z. Carter:** Writing – review & editing, Resources. **Arianna Donella-Deana:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Christian Borgo:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Maria Ruzzene:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding

This work was supported by University of Padova [grants RUZZ_BIRD23_01 to M.R., SALV_BIRD2222_01 to M.S., BORG_BIRD2121_01 to C.B.].

Declaration of competing interest

The authors have nothing to declare.

Acknowledgements

We thank Dr. Luca Cesaro for technical assistance.

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