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ORIGINAL ARTICLE

Cytotoxic activity of the casein kinase 2 inhibitor CX-4945 against T-cell acute lymphoblastic leukemia: targeting the unfolded protein response signaling

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Constitutively active casein kinase 2 (CK2) signaling is a common feature of T-cell acute lymphoblastic leukemia (T-ALL). CK2 phosphorylates PTEN (phosphatase and tensin homolog) tumor suppressor, resulting in PTEN stabilization and functional inactivation. Downregulation of PTEN activity has an impact on PI3K/Akt/mTOR signaling, which is of fundamental importance for T-ALL cell survival. These observations lend compelling weight to the application of CK2 inhibitors in the therapy of T-ALL. Here, we have analyzed the therapeutic potential of CX-4945—a novel, highly specific, orally available, ATP-competitive inhibitor of CK2α. We show that CX-4945 treatment induced apoptosis in T-ALL cell lines and patient T lymphoblasts. CX-4945 downregulated PI3K/Akt/mTOR signaling in leukemic cells. Notably, CX-4945 affected the unfolded protein response (UPR), as demonstrated by a significant decrease in the levels of the main UPR regulator GRP78/BIP, and led to apoptosis via upregulation of the ER stress/UPR cell death mediators IRE1α and CHOP. *In vivo* administration of CX-4945 to a subcutaneous xenotransplant model of human T-ALL significantly delayed tumor growth. Our findings indicate that modulation of the ER stress/UPR signaling through CK2 inhibition could be exploited for inducing apoptosis in T-ALL cells and that CX-4945 may be an efficient treatment for those T-ALLs displaying upregulation of CK2α/PI3K/Akt/mTOR signaling.

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INTRODUCTION

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic tumor resulting from the malignant transformation of T-cell progenitors. T-ALL accounts for 10–15% of pediatric and 25% of adult ALL cases.[1,2] Although originally associated with high relapse rates, the prognosis of T-ALL has gradually improved with the introduction of intensified chemotherapy, with cure rates reaching over 75% in children and about 50% in adults.[3] However, the outcome of T-ALL patients with primary resistant or relapsed leukemia remains poor, highlighting the need to develop novel targeted therapeutic strategies.[4–12] Casein kinase 2 (CK2) is an ubiquitously expressed and constitutively active serine/threonine kinase that phosphorylates an impressive array of substrates[13] and is involved in disparate cellular processes. However, the most prominent role of CK2 is the global promotion of cell growth and prevention of apoptosis.[14] There is compelling evidence that CK2 operates as a 'cancer driver' by creating a cellular environment positive to neoplastic cells.[15] Overexpression and hyperactivation of CK2 have mostly been observed in solid tumors[16] but also in some blood malignancies,[17] including multiple myeloma (MM),[18] acute myeloid leukemia[19] and T-ALL.[20] CK2 is therefore considered a promising target for innovative cancer treatment and CK2 inhibitors display cytotoxic effects against cancer cells.[21–23] CX-4945 is a potent CK2 inhibitor[24] that has recently entered phase I clinical trials in patients with advanced solid cancers (NCT00891280) and in MM patients (NCT01199718). Regarding T-ALL, the functions of CK2 are intimately linked with the inositol lipid phosphatase PTEN (phosphatase and tensin homolog), a negative regulator of the PI3K/Akt/mTOR pathway, which is frequently upregulated in T-ALL patients.[20] In primary T-ALL cells, CK2-mediated phosphorylation of PTEN at its C-terminus resulted in PTEN stabilization and functional inactivation, with ensuing increase in PI3K/Akt/mTOR signaling.[25] In the scenario of T-ALL, another major signaling pathway, the endoplasmic reticulum (ER) stress/unfolded protein response (UPR) pathway, is gaining increasing recognition.[26,27] Tumor cells are often exposed to hypoxia, oxidative stress and metabolic dysregulation that cause ER stress and activation of the UPR.[28] UPR signaling is an important mechanism required by cancer cells to maintain malignancy and gaining therapy resistance. In fact, cancer cells depend on ER integrity to maintain the correct structure of oncokinases. For this reason, whereas UPR is involved in drug resistance, cancer cells are 'paradoxically' more sensitive than healthy cells to UPR changes, and pharmacologically sustained induction or repression of UPR may have therapeutic effects against cancer cells.[29] In this context, it was recently documented

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that CK2 inhibition caused MM cell apoptosis through alterations of the UPR signaling pathway,[30] whereas in T-ALL, it was shown that the downregulation of UPR drives apoptotic cell death.[26,27] However, it remains to be determined whether a link between CK2 and regulation of UPR exists in T-ALL. In the present study, we report the cytotoxic effects induced by the inhibition of CK2 with CX-4945 on a panel of T-ALL cell lines and primary cells from T-ALL patients. CK2 inhibition by CX-4945 potently downregulated UPR signaling. We also observed that a combination of CX-4945 with thapsigargin, an ER stress inducer, displayed significant synergistic cytotoxic activity. Moreover, CX-4945 was efficacious in a subcutaneous human T-ALL xenograft mouse model. Overall, our findings establish a link between CK2 activity and UPR signaling in T-ALL cells and could lead to the design of more effective therapeutic protocols for this disorder based on CK2 inhibition.

MATERIALS AND METHODS

Materials

CX-4945 used *in vivo* studies was kindly provided by Cylene Pharmaceuticals (San Diego, CA, USA). CX-4945 and temsirolimus for *in vitro* studies were from Selleck Chemicals (Houston, TX, USA). Allophycocyanin-conjugated antibody to CD45 was from Beckman Coulter (Miami, FL, USA). Antibody to Ser129 p-Akt, geldanamycin, okadaic acid and insulin-transferrin-sodium selenite were from Sigma-Aldrich (St Louis, MO, USA). All of the other primary and secondary antibodies for western blotting and flow cytometry analyses were from Cell Signaling Technology (Danvers, MA, USA). Thapsigargin was from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture and primary T-ALL samples

T-ALL cell lines were grown in RPMI 1640, supplemented with either 10 or 20% heat-inactivated fetal bovine serum, depending on the cell line. MS-5 mouse stromal cells were grown in MEM Alpha medium supplemented with 10% fetal bovine serum. Patient samples were obtained after informed consent according to the Institutional guidelines and isolated by Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden). T-ALL lymphoblasts were cultured in RPMI 1640 containing 20% fetal bovine serum and insulin-transferrin-sodium selenite.

Cell viability analysis

MTT (3-[4,5-Dimethylthythiazol-2-yl]-2,5-Diphenyltetrazolium Bromide) assays were performed as previously reported.[31] For drug-combination experiments, a combination index (CI) number was calculated using the CalcuSyn software (BioSoft, Cambridge, UK) based on the Chou and Talalay method.[32] CI values between 0.1 and 0.9 define different grades of synergism: values between 0.9 and 1.1 are additive, whereas values >1.1 are antagonistic.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining and cell cycle analysis

To determine the extent of apoptosis induction after drug treatment, flow cytometric analysis of Annexin V-FITC/PI-stained samples was performed as previously reported.[31] Cell cycle analysis was carried out using a PI/RNaseA staining according to standard procedures. All the flow cytometric analyses were performed using an FC500 flow cytometer (Beckman Coulter).

Flow cytometric analysis of p-PTEN (Ser380), IRE1α and CHOP levels

This was performed as reported elsewhere.[31]

Western blot analysis and immunoprecipitation

This was performed as previously detailed.[33] Cells were lysed using the M-PER Mammalian Protein Extraction Reagent, supplemented with the Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc, Rockford, IL, USA). Akt immunoprecipitation was carried out as described elsewhere.[34]

Jurkat cells coculture with MS-5 mouse stromal cells

Jurkat cells were seeded at 2.5×10^5/ml and, after an overnight incubation, cell suspension was transferred on the top of MS-5 mouse stromal cells (at $\sim$70% confluence) for 3 h before the addition of 5 μM CX-4945. After 48 h, Jurkat cells were harvested, washed and incubated with allophycocyanin-conjugated anti-CD45 antibody or with an irrelevant isotypic control antibody. After a 30-min incubation, cells were resuspended in binding buffer containing Annexin V-FITC and analyzed using flow cytometry after electronic gating on $CD45^+$ leukemic cells.

Scanning electron microscopy (SEM) analysis of cocultured Jurkat and MS-5 cells

After 48 h of incubation at 37 °C, cocultured cells were processed for morphological studies using SEM, as reported elsewhere.[35] Samples were sputter-coated with 20-nm-thick palladium-gold film and examined under a Philips SEM 515 operating at 15 kV.

Small interfering (siRNA) knockdown of CK2α

CEM-S cells were transfected with the Amaxa T cell Nucleofector kit (Lonza, Basel, Switzerland) according to the manufacturer's optimized protocol kit for this cell line. CEM-R cells (2×10^6 cells) were transfected with 300 nM of siRNA CK2α-specific siGENOME SMARTpool siRNAs or aspecific (scrambled) siRNA siCONTROL riscfree no. 1 (Dharmacon, Lafayette, CO, USA). Cells were diluted to between 2 and 3×10^5/ml and incubated for further 48 h.

In vivo subcutaneous human T-ALL mouse model

Mice were housed and bred in a specific pathogen-free animal facility, treated in accordance with the European Union guidelines and approval by the Institutional Ethical Committee of Instituto de Medicina Molecular. Eight weeks old nonobese diabetic/severe combined immunodeficient mice were subcutaneously injected in both flanks with 10×10^6 MOLT-4.Luc.GFP cells, generated as described,[36] and resuspended in 100 μl of phosphate-buffered saline. On day 5, mice were injected with luciferin to assess tumor burden by whole-body bioluminescence imaging and were equally distributed in two groups to receive CX-4945 dissolved in 25 mM disodium hydrogen phosphate (Na_2HPO_4) or vehicle control. Tumor growth was monitored weekly by bioluminescence and caliper measurements, and mice were weighed frequently to determine treatment-induced toxicity. For bioluminescence imaging, mice were anaesthetized, intraperitoneally injected with 150 mg D-luciferin (Caliper Life Sciences, Hopkinton, MA, USA) per kg body weight and scanned with an IVIS Lumina bioimaging device (Caliper Life Sciences) after 15 min, as described.[36] Total flux (photons per second) was calculated using the Living Image software (Caliper Life Sciences).

Statistical analysis

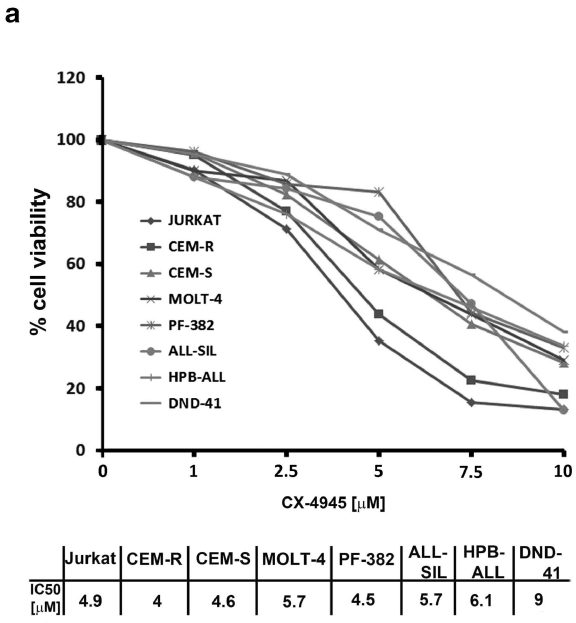
The data are presented as the mean values from three separate experiments $\pm$ s.d. *In vivo* differences in tumor development were determined by two-way analysis of variance (ANOVA). Differences were considered significant for $P<0.05$.

RESULTS

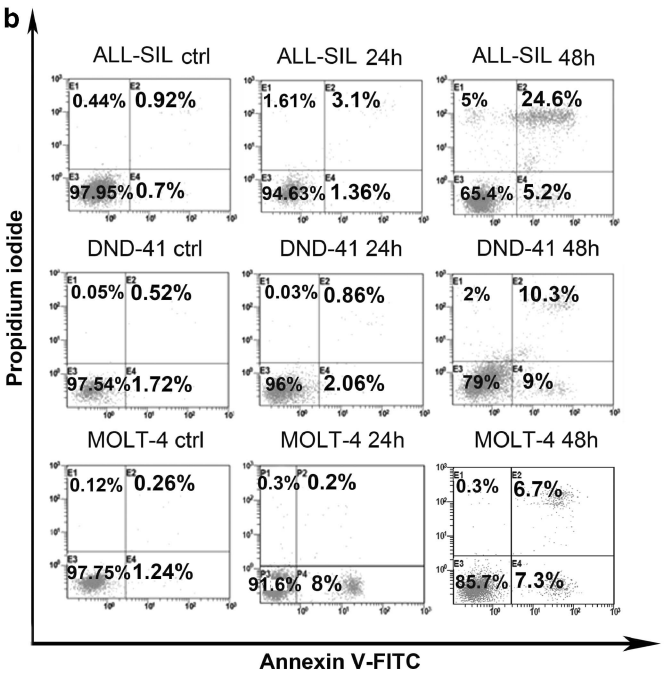
CX-4945 induces both cytotoxic and cytostatic effects on T-ALL cell lines

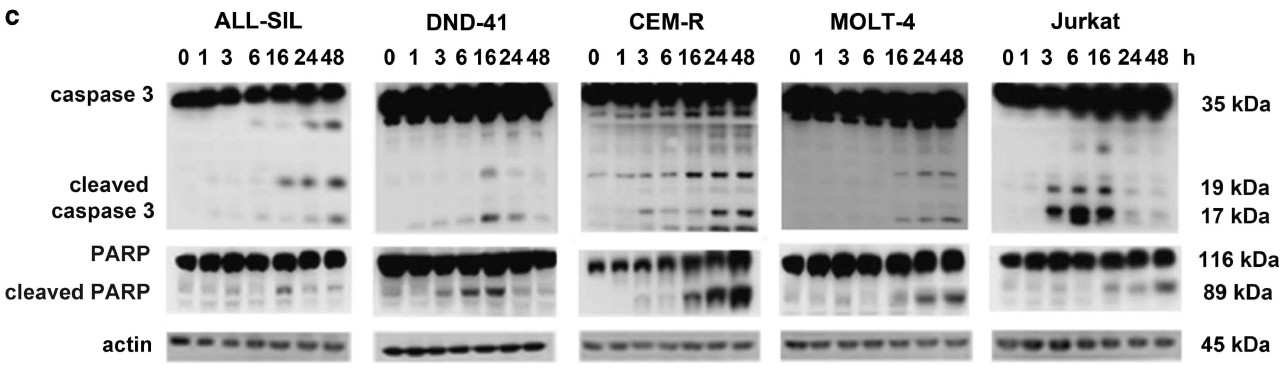
The effects of CX-4945 on a panel of T-ALL cell lines were tested by incubating the cells for 48 h with increasing concentrations of the drug and then analyzing the rates of viability using MTT assays. T-ALL cell lines displayed IC_{50s} to CX-4945 ranging from 4.0 μM for CEM-R (drug-resistant) cells to 9.0 μM for DND-41 cells (Figure 1a). To evaluate whether the effects of CX-4945 on cell viability could be related to apoptosis, flow cytometric analysis was performed. In response to treatment with 5 μM CX-4945, we detected an increase in the percentage of early apoptotic (positive for Annexin V) and/or late apoptotic (positive for both Annexin V and PI) cells after either 24 or 48 h of treatment of T-ALL cell lines (Figure 1b).

Apoptosis was further investigated by western blotting, which documented a time-dependent cleavage of caspase-3 and poly (ADP-ribose) polymerase in response to CX-4945 (Figure 1c).



	Jurkat	CEM-R	CEM-S	MOLT-4	PF-382	ALL-SIL	HPB-ALL	DND-41
IC50 [μM]	4.9	4	4.6	5.7	4.5	5.7	6.1	9





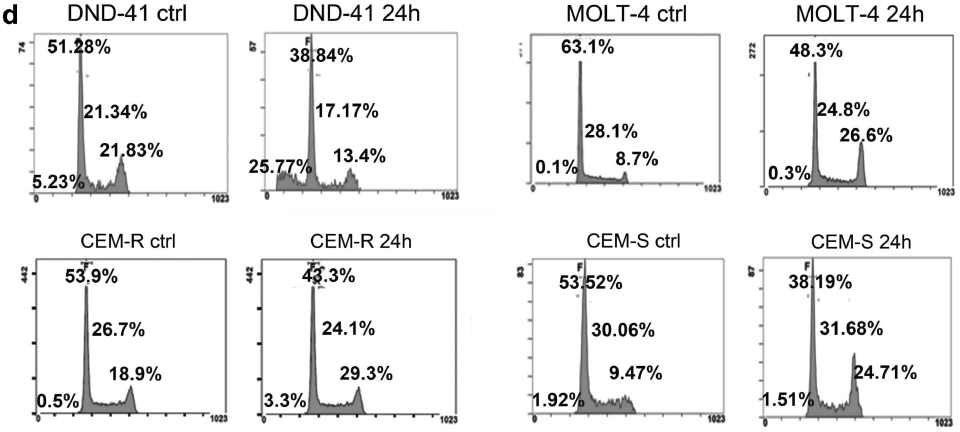


Figure 1. CX-4945 induces cytotoxic and cytostatic effects in T-ALL cell lines. (**a**) MTT assays of T-ALL cell lines treated with CX-4945 for 48 h. Corresponding IC_{50} values are indicated for each cell line in the table. Data are representative of three independent experiments and s.d. was $<10\%$. (**b**) Flow cytometric analysis of Annexin V-FITC/PI-stained T-ALL cells treated with CX-4945 for different times. The percentages of early apoptotic cells (Annexin V $FITC^+/PI^-$; bottom right quadrant) and late apoptotic/necrotic cells (Annexin V $FITC^+/PI^+$; top right quadrant) are indicated. ctrl, untreated cells. (**c**) Western blot analysis documenting a time-dependent activation of caspase-3 and cleavage of poly (ADP-ribose) polymerase by CX-4945 (5 μM). Fifty micrograms of protein were blotted to each lane. Antibody to β-actin served as a loading control. Molecular weights are indicated at right. (**d**) Flow cytometric analysis of cell cycle distribution in T-ALL cell lines treated with CX-4945 (5 μM) for 24 h. The histograms are representative of three separate experiments performed in duplicate. ctrl, control cells.

The effects of CX-4945 on cell cycle progression were also studied. Flow cytometric analysis of PI-stained T-ALL cells treated with CX-4945 for 24 h revealed a block in the G_2/M phase of the cell cycle in MOLT-4, CEM-R and CEM-S (parental) cell lines. In contrast, in DND-41 cells, we only detected an increase in sub-G1 cells, corresponding to apoptotic cells (Figure 1d). Taken together, our findings demonstrated that CX-4945 had both cytostatic and cytotoxic effects on T-ALL cell lines by promoting cell cycle arrest at the G_2/M phase of the cell cycle and by inducing apoptosis.

PI3K/PTEN/Akt/mTOR signaling pathway modulation by CX-4945

To study CK2 expression levels in T-ALL cell lines and to demonstrate that CX-4945 effectively inhibited CK2 activity, we incubated cells with 5 μM CX-4945 at different time points, and then performed the analysis of protein expression using western blot. CK2 protein levels were variable among T-ALL cell lines. However, they did not change after CX-4945 treatment. PTEN is one of the best known targets of CK2.[25] Western blot analysis of PTEN-positive T-ALL cell lines (HBP-ALL, ALL-SIL, DND-41) using an antibody to a CK2-targeted amino-acidic residue (Ser380)[37] demonstrated time-dependent PTEN dephosphorylation in response to CX-4945, whereas the total levels of PTEN did not change (Figure 2a). As expected, PTEN-deleted T-ALL cell lines (Jurkat, CEM-S, PF-382) did not display any PTEN and p-PTEN expression.

Next, we performed a concentration-dependent study of PTEN dephosphorylation by CX-4945 in PTEN-expressing T-ALL cell lines. Western blot analysis demonstrated that PTEN dephosphorylation was already detectable at 2.5 μM CX-4945 after 24 h of treatment with the inhibitor (Figure 2b). Furthermore, CX-4945 down-regulated Akt phosphorylation at Ser129, which is targeted by CK2,[38] in both DND-41 and CEM-R cell lines (Figure 2c). Thus, our data indicate that CK2 inhibition using CX-4945 induced time- and/or dose-dependent Ser380 PTEN and Ser129 p-Akt dephosphorylation in T-ALL cell lines.

Given that T-ALL cells frequently display hyperactivation of PI3K/Akt/mTOR signaling as a consequence of functional inhibition of PTEN mediated by CK2,[25] the activation status of this pathway was further evaluated. Western blot analysis documented a time-dependent decrease in Thr308 p-Akt in T-ALL cell lines exposed to the CX-4945, whereas total Akt levels were unaffected by the inhibitor. Moreover, the mTOR complex 1 (mTORC1) downstream substrate S6RP was efficiently dephosphorylated by CX-4945 already after 1–3 h of treatment, whereas its total level of expression did not change (Figure 2d).

Since we observed dephosphorylation of p-Akt at Thr308 in both PTEN-expressing and PTEN-deleted cell lines, we sought an

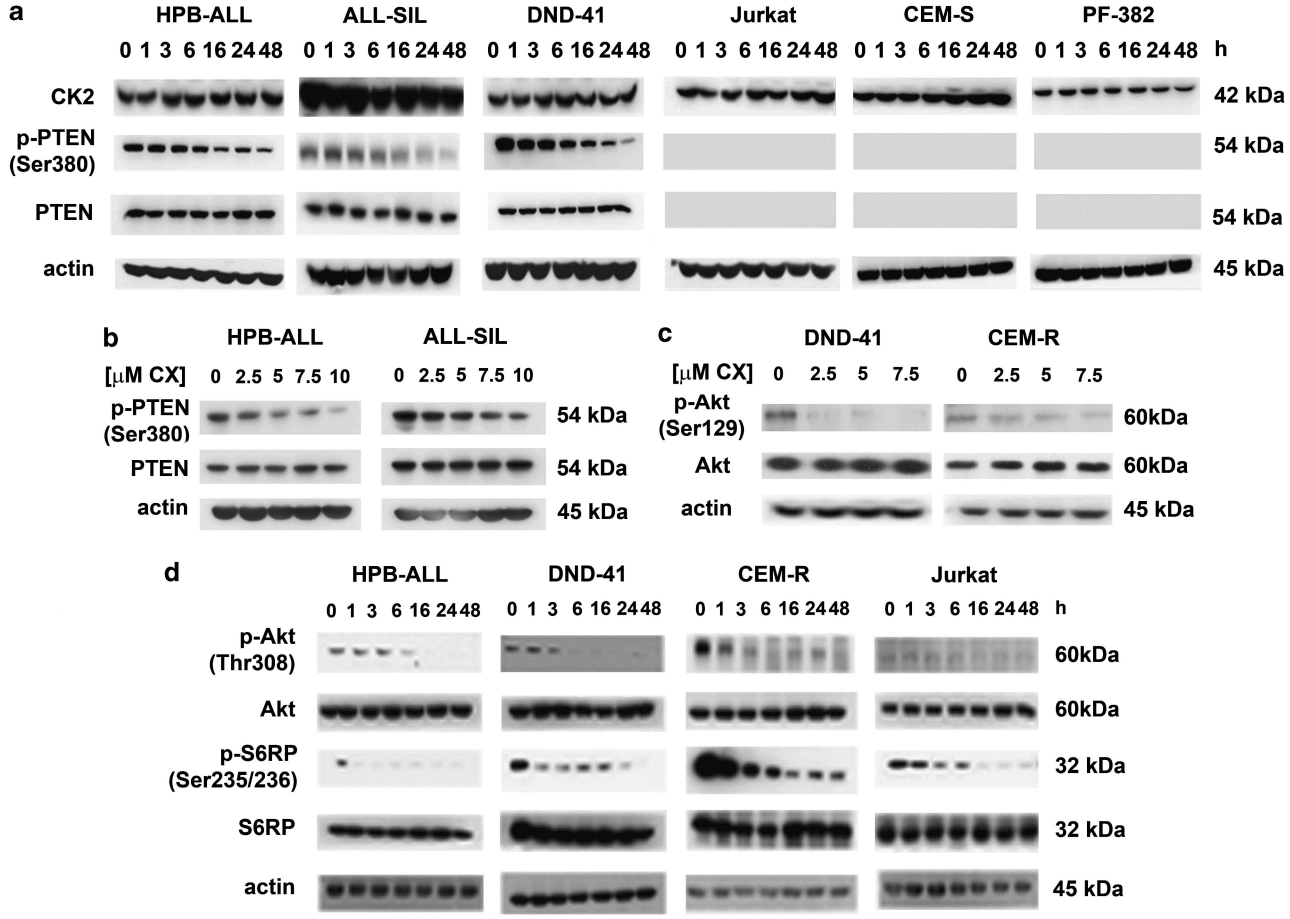


Figure 2. Molecular effects of inhibition of CK2 after treatment with CX-4945 and modulation of the activation status of the PI3K/PTEN/Akt/mTOR signaling pathway in T-ALL cell lines. (**a**) Cells were cultured for different times in the presence of 5 μM of CX-4945, and western blot analysis was then performed. CK2 protein levels did not change after CX-4945 treatment. CX-4945 induced a time-dependent dephosphorylation of PTEN (at Ser380). (**b**) Western blot analysis documenting the concentration-dependent effects of CX-4945 on Ser380 p-PTEN. (**c**) Western blot analysis documenting the concentration-dependent effects of CX-4945 on Ser129 p-Akt. In **b** and **c** treatment time with the drug was 24 h. (**d**) CX-4945 (5 μM) induced a time-dependent dephosphorylation of Thr308 p-Akt and Ser235/236 p-S6RP. In **a–d**, 50 micrograms of protein were blotted to each lane. Antibody to β-actin served as a loading control. Molecular weights are indicated at right.

explanation for this phenomenon. It has been documented that CK2-mediated phosphorylation of Akt at Ser129 increased Akt association with the chaperone protein HSP-90 and protected Akt from PP2A phosphatase activity on Thr308.[39] Using CEM-R cells, we immunoprecipitated Akt and we analyzed its association with HSP-90 using western blot. Whereas the total levels of immunoprecipitated Akt did not change in response to CX-4945 treatment, those of HSP-90 did indeed decrease (Supplementary Figure S1A). Moreover, when CEM-R cells were treated with geldanamycin, an inhibitor of HSP-90,[40] we observed a reduction in Thr308 p-Akt levels, whereas the levels of Ser473 p-Akt, Ser129 p-Akt, Akt, and HSP-90 did not change (Supplementary Figure S1B). Furthermore, the PP2A inhibitor, okadaic acid, prevented dephosphorylation of Thr308 p-Akt caused by CX-4945 (Supplementary Figure S1C). Overall, these findings indicated that CX-4945 not only downregulated PTEN phosphorylation but also disrupted the interactions between Akt and HSP-90 independently of PTEN, thus rendering the Thr308 residue of Akt more susceptible to the action of protein phosphatase PP2A.

CX-4945 affects viability of Jurkat cells even in the presence of MS-5 stromal cell support

As interactions between bone marrow stromal cells and leukemic cells are thought to be responsible for decreased anti-leukemic drug sensitivity,[41] we investigated whether CX-4945 could bypass the support of stromal cells and induce apoptotic cell death in a coculture system. We used the murine stromal cell line MS-5, which is known to provide long-term support for primitive hematopoietic progenitors and to mimic the bone marrow microenvironment.[42] Coculture with stromal cells decreased but did not abolish the sensitivity of Jurkat cells to treatment with CX-4945, when compared with Jurkat cells growing as suspension cultures (Supplementary Figure S2A).

The effects of CX-4945 on Jurkat cells cocultured with MS-5 stromal cells were studied also by SEM. Untreated Jurkat cells displayed typical microvillous expansions evenly distributed over the intact cell membrane (Supplementary Figure S2B, left). Jurkat cells treated with CX-4945 displayed cell shrinkage, loss of surface microvilli and disappearance of cell-to-cell contacts. Many detached apoptotic-like bodies were observed (Supplementary Figure S2B, center). Moreover, leukemic cells exhibited plasma membrane alterations, including perforation and blebbing (Supplementary Figure S2B, right), suggestive of apoptosis.

CK2 inactivation by CX-4945 or siRNA affects ER stress/UPR signaling

It has been documented, in different tumor models, that CK2 inhibition induces apoptosis through inhibition of UPR mechanisms.[43,44] These observations extend to T-ALL, where down-regulation of UPR was shown to drive apoptotic cell death.[26,27,45]

To better understand the molecular mechanisms involved in CX-4945-mediated T-ALL cell death, we studied the effect of CK2 inhibition on ER stress/UPR signaling. We found a decrease in the expression of GRP78/BIP (a marker of UPR activation[26]) starting after 3–6 h of drug treatment, implying that CK2 inactivation led to UPR inhibition (Figure 3). In contrast, the expression of IRE1α, p-EIF2α and CHOP increased after exposure to CX-4945, indicating concomitant induction of significant stress in the ER lumen

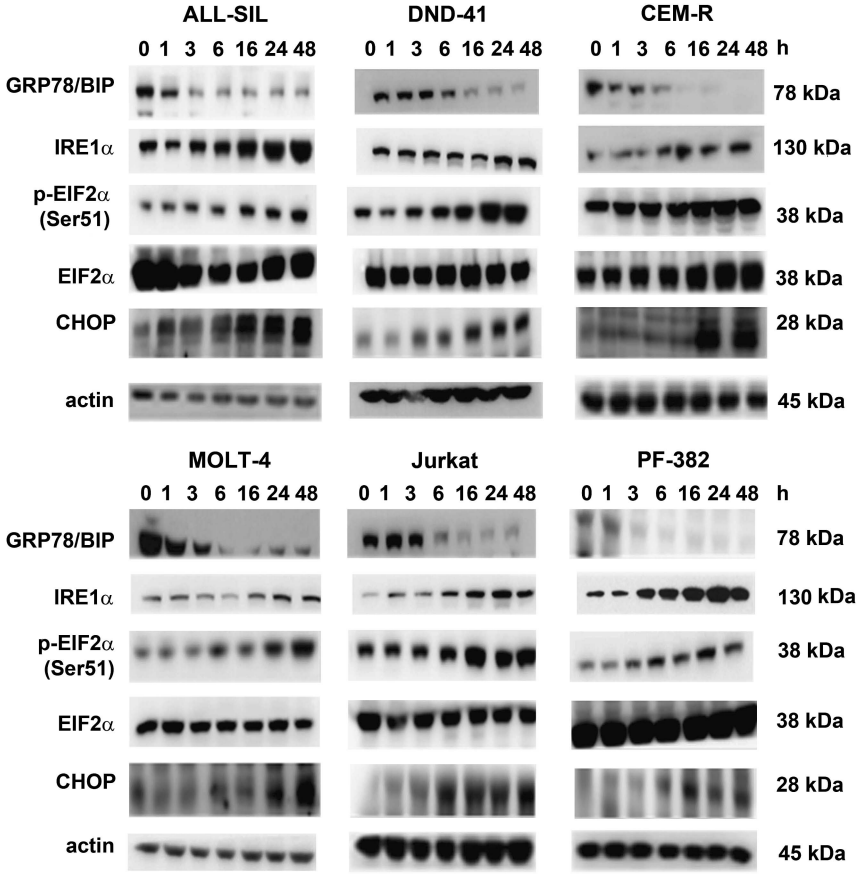


Figure 3. CX-4945 modulates ER stress/UPR signaling in T-ALL cell lines. Western blot analysis documenting the time-dependent modulation of ER stress/UPR signaling markers induced by CX-4945 (5 μM) in T-ALL cell lines. Fifty micrograms of protein were blotted to each lane. Antibody to β-actin served as a loading control. Molecular weights are indicated at right.

(Figure 3). Indeed, IRE1α, p-EIF2α and CHOP are well-established markers of ER stress/UPR-mediated apoptosis.[46] Therefore, our findings suggested that a potential mechanism by which CX-4945 induced T-ALL cell death involved ER stress induction and the inability of leukemic cells to adequately respond to ER stress by upregulating the UPR.

We next employed an siRNA strategy to downregulate CK2α in CEM-S cells (Figure 4a) and analyze the effects on cell viability and ER stress/UPR signaling response. MTT assays demonstrated a significant reduction in cell viability of CEM-S cells treated for 48 h with siRNA specific to CK2α in comparison to control siRNA (Figure 4b). Western blot analysis documented a decrease in GRP78/BIP and an increase in IRE1α and p-EIF2α in cells with downregulated CK2α expression (Figure 4c). The results obtained with siRNA downregulation of CK2α in CEM-S cells were similar to those obtained through CK2 chemical inhibition by CX-4945 (Figure 4d).

CX-4945 synergizes with temsirolimus, an allosteric mTORC1 inhibitor

The relationship between protein translation and UPR activation has been recently highlighted. Hyperactivation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), resulting from the inhibition of mTORC1 signaling, was shown to prevent GRP78/BIP induction and consequently led to the failure of UPR.[47] CX-4945 was able to inhibit UPR while causing p-4E-BP1 dephosphorylation in T-ALL cell lines (data not shown). Thus, we investigated whether CX-4945 synergized with the mTORC1 inhibitor, temsirolimus, in T-ALL cell lines.

MOLT-4 and Jurkat cells were incubated for 48 h with either drug alone or with their combination at a fixed ratio. We found a strong synergism (CI $\leqslant$0.3) in both MOLT-4 and Jurkat cells at a CX-4945 concentration (1 μM) clearly below the respective IC_{50} in these cells (Supplementary Figure S3A). The effects of the drug combination on apoptosis were determined with Annexin V-FITC/PI staining. After 48 h, the combined treatment was more effective in inducing apoptosis than single treatments (Supplementary Figure S3B). To study the effects of the drug combination on ER stress/UPR signaling, western blot analysis was performed after 24 h of treatment. CX-4945 and temsirolimus synergized in decreasing GRP78/BIP and p-4E-BP1 levels, and in increasing IRE1α and CHOP expression (Supplementary Figure S3C). These findings confirmed that the drug combination induced apoptotic cell death through ER stress/UPR signaling modulation.

CX-4945 synergizes with the ER stress inducer thapsigargin

The therapeutic potential of targeting UPR signaling in cancer mainly involves two approaches: inhibition of UPR to hit tumors with a highly stressed ER that are strongly dependent on an activated UPR for their survival,[30] or induction of accumulation of misfolded proteins in ER to overload restoration capacity of tumors with a previously compromised UPR.[48] As we demonstrated that, in T-ALL cells, CX-4945 treatment inhibited UPR, we next studied the existence of a potential synergism between CX-4945 and thapsigargin, a sarcoplasmic Ca^{2+} ATPase inhibitor, which is a powerful inducer of both ER stress and UPR.[48] MOLT-4 and PF-382 cells were incubated for 48 h either with each drug alone or with a combination of the two drugs at a fixed ratio. Using MTT assays, we observed a moderate (MOLT-4 cells) or strong (PF-382 cells) synergism at CX-4945 concentrations (2.5 and 1 μM, respectively) clearly below their respective IC_{50} in these cells (Figure 5a). Flow cytometric analysis of Annexin V-FITC/PI-stained cells confirmed that the drug combination was more effective than each single agent in inducing apoptosis (Figure 5b). Moreover, CX-4945 and thapsigargin synergized in enhancing IRE1α and CHOP expression, thus suggesting that thapsigargin increased ER stress induced by CX-4945. As expected, thapsigargin activated UPR and this was documented by the strong enhancement of GRP78/BIP expression, also in combination with CX-4945. p-4E-BP1 behaved as GRP78/BIP (Figure 5c). Overall, these findings suggested that the blockage of UPR induction observed in T-ALL

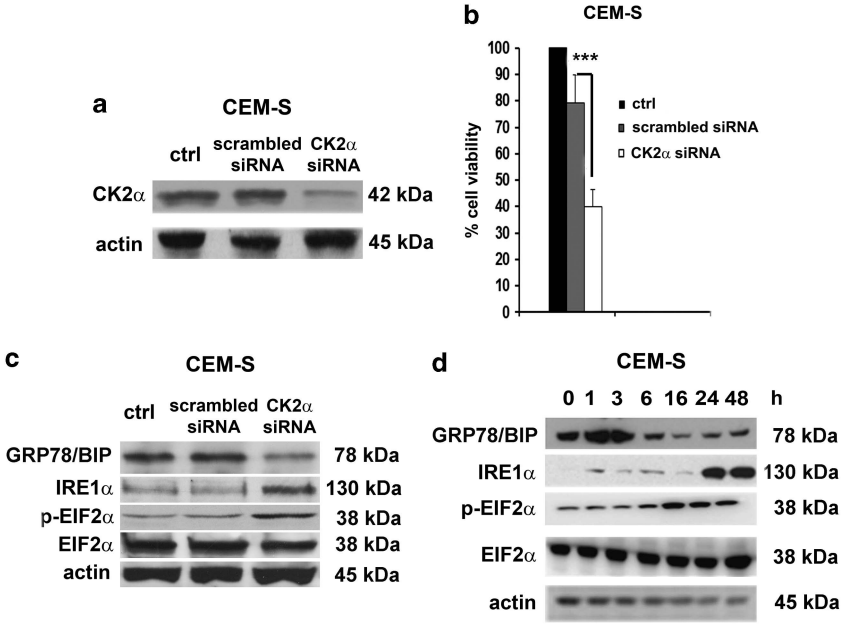


Figure 4. CK2α downregulation by siRNA affects CEM-S cell viability and modulates ER stress/UPR signaling. (**a**) Western blot analysis confirmed that CK2α was efficiently down-modulated upon nucleofection of CEM-S cells with specific siRNA, whereas scrambled (control) siRNA had no effect. (**b**) MTT analysis of CEM-S cells nucleofected with 300 nM of siRNA to CK2α documented a significant reduction in cell viability when compared with scrambled siRNA-transfected cells ($P<0.05$). Results are the mean of three different experiments ± s.d. (**c**) Western blot analysis demonstrated that CK2α downregulation affected the expression levels of ER stress/UPR signaling markers as chemical treatment with 5 μM CX-4945 for different time periods (**d**).

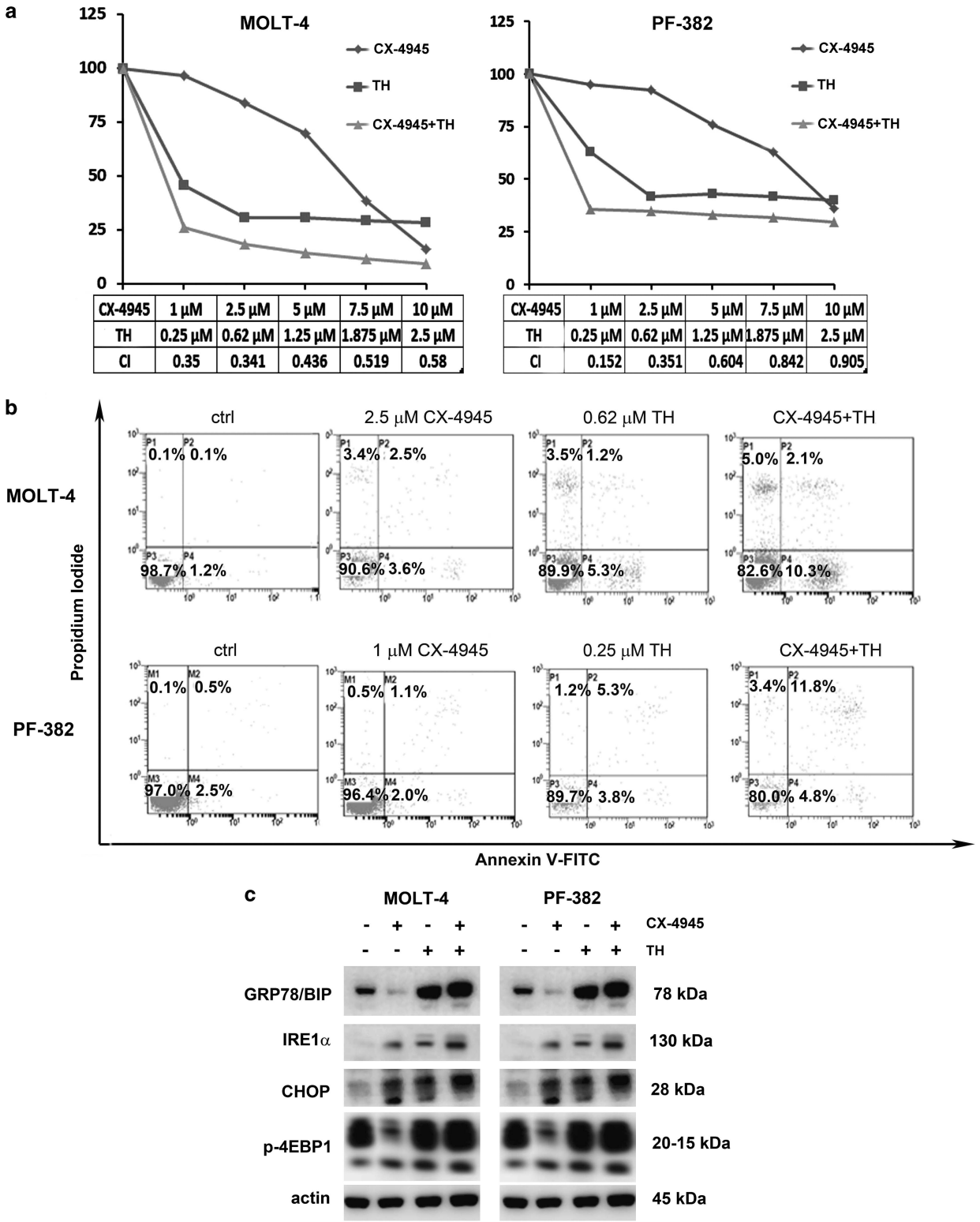


Figure 5. CX-4945 synergizes with thapsigargin in T-ALL cells. (**a**) MTT assays of MOLT-4 and PF-382 cell lines treated for 48 h with CX-4945 alone or in combination with thapsigargin (TH) at a fixed ratio. The combined treatments resulted in a moderate synergism (CI: 0.3–0.7) in MOLT-4 cells and in a strong synergism (CI: 0.1–0.3) in PF-382 cells. Data are representative of three independent experiments and s.d. was $<10\%$. (**b**) Flow cytometric analysis of Annexin V-FITC/PI-stained MOLT-4 and PF-382 cells treated for 24 h with CX-4945 and thapsigargin either alone or in combination documented that the combined treatment was more effective in inducing apoptosis than single treatments. Ctrl, untreated cells. (**c**) Western blot analysis of cells treated for 24 h with CX-4945 and thapsigargin, either alone or in combination, showed that drugs synergized in enhancing IRE1α and CHOP expression, suggesting that thapsigargin increased ER stress induced by CX-4945 treatment. In contrast to CX-4945, thapsigargin activated UPR and this was documented by the strong enhancement of GRP78/BIP expression, also in combination with CX-4945. p-4E-BP1 behaved as GRP78/BIP. Fifty micrograms of protein was blotted to each lane. Antibody to β-actin served as a loading control. Molecular weights are indicated at right. In **a**–**c**, TH: thapsigargin.

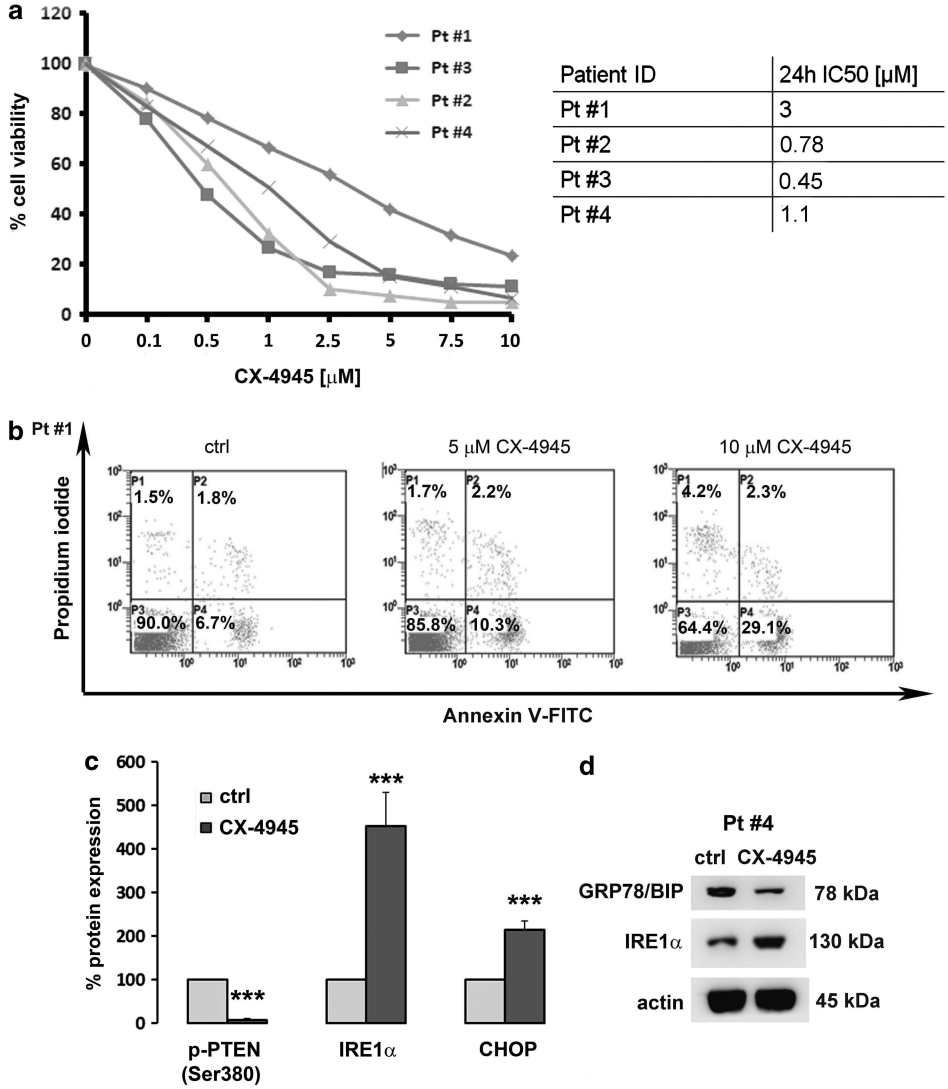
cells treated with CX-4945 was not able to switch off the ER stress induced by thapsigargin, thus leading to leukemic cell apoptosis.

CX-4945 is effective in primary T-ALL blasts

To better assess the effectiveness of CX-4945 as a potential therapeutic agent in T-ALL, we studied four pediatric T-ALL patient samples isolated from the bone marrow. Cells were treated with increasing concentrations of the drug for 24 h, and the rates of survival were analyzed by MTT assays. The CX-4945 IC_{50} for patient samples ranged from 0.45 to 3.0 μM (Figure 6a). CX-4945 induced apoptosis in T-ALL lymphoblasts, as documented using flow cytometric analysis of Annexin V-FITC/PI-stained samples (Figure 6b). Consistent with findings obtained in T-ALL cell lines, CX-4945 downregulated Ser380 p-PTEN and upregulated both IRE1α and CHOP, as documented using flow cytometric analysis (Figure 6c). Western blot analysis demonstrated inhibition of UPR signaling (reduction of the GRP78/BIP level) and induction of ER stress (increase in the levels of IRE1α) in patient samples (Figure 6d).

CX-4945 delays T-ALL tumor growth *in vivo*

The significant *in vitro* antitumor activity of CX-4945 on T-ALL cells led us to further explore its therapeutic potential by investigating the anti-leukemic efficacy of CX-4945 in an animal model of human T-ALL. Nonobese diabetic/severe combined immunodeficient mice were injected subcutaneously with MOLT-4.Luc.GFP cells. On day 5 after cell transfer, mice were equally distributed according to tumor burden in two groups to receive CX-4945



Patient ID	24h IC50 [μM]
Pt #1	3
Pt #2	0.78
Pt #3	0.45
Pt #4	1.1

Figure 6. CX-4945 is cytotoxic to primary lymphoblasts from T-ALL patients (Pt) and affects ER stress/UPR signaling in T-ALL patients. (**a**) MTT assays of T-ALL blasts from four pediatric patients treated with CX-4945 for 24 h and their corresponding IC_{50} values. Data are representative of three independent experiments and s.d. was $<10\%$. (**b**) Flow cytometric analysis of Annexin V-FITC/PI-stained T lymphoblasts, treated with CX-4945, documented a significant and a dose-dependent increase in apoptotic cells. A representative patient is shown. Ctrl, untreated cells. (**c**) Flow cytometric analysis demonstrated a significant decrease in the expression levels of Ser380 p-PTEN, and an increase in the levels of both IRE1α and CHOP proteins after 10 μM CX-4945 treatment for 24 h. Data are the mean from three patients. Asterisks indicate statistically significant differences with respect to untreated cells, considered as 100% (***$P<0.0005$). Ctrl, untreated cells. (**d**) Western blot analysis of proteins extracted from T-ALL lymphoblasts confirmed an increase in the quantity of IRE1α and a decrease in GRP78/BIP expression levels. A representative patient is shown. Fifty micrograms of protein were blotted to each lane. Antibody to β-actin served as a loading control. Molecular weights are indicated at right.

(75 mg/kg, orally, twice a day) or vehicle control. We observed a significant delay in tumor growth in the CX-4945-treated group at 3 and 4 weeks (Figures 7a and b, $P = 0.015$; two-way ANOVA) without significant toxicities (not shown). Taken together, our data indicate that inhibition of CK2 activity using CX-4945 may be an useful therapeutic strategy for T-ALL.

DISCUSSION

The clinical-stage CK2α inhibitor CX-4945 has been tested in hematological malignancies that include chronic myeloid leukemia[49] and B-cell chronic lymphocytic leukemia.[50,51] The findings that emerged from these studies have provided preclinical evidence that CK2α could be a valuable therapeutic target in blood cancers and supported the possible initiation of clinical trials using CK2α antagonists in the context of hematological malignancies. These may potentially include also CIGB-300, the only other currently available clinical-grade CK2 inhibitor, which has shown significant antitumor effects in murine cancer models and was safe and well tolerated in phase I clinical trials in cervical cancer that demonstrated tumor reduction.[52]

In this study, we have evaluated the therapeutic potential of CX-4945 in preclinical settings of T-ALL. There is a strong rationale for targeting CK2α in T-ALL, as this kinase phosphorylates and inactivates the inositol lipid phosphatase PTEN,[20] which results in downregulation of PTEN function and in enhanced activity of the PI3K/Akt/mTOR signaling network, that has a key role in proliferation, survival and drug resistance of T-ALL cells.[53] We showed that CX-4945 reduced cell viability in a concentration-dependent manner in all the tested T-ALL cell lines and primary patient samples and was effective in both PTEN-expressing and PTEN-deleted T-ALL cell lines, suggesting that CK2 inhibition targets both PTEN-dependent and PTEN-independent T-ALL cell viability.

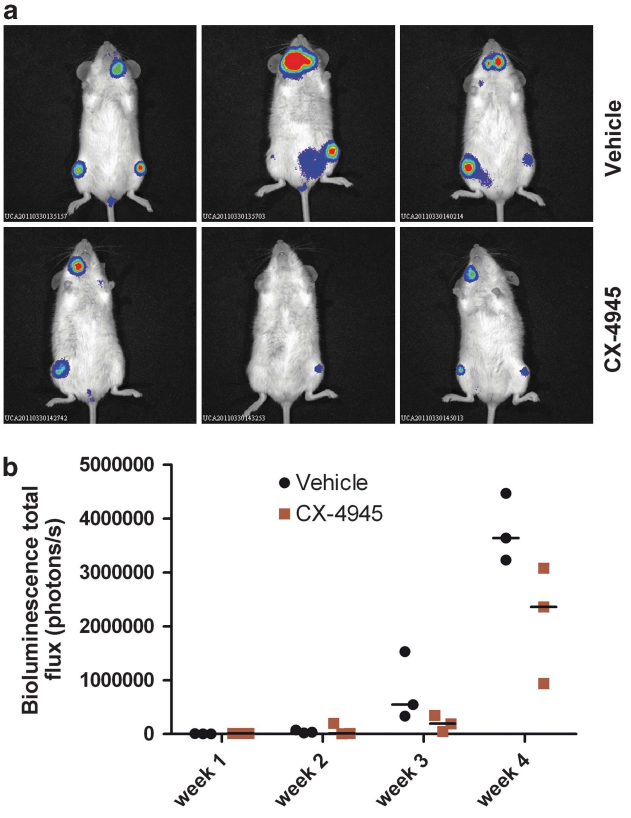


Figure 7. CX-4945 delays T-ALL tumor growth *in vivo*. Nonobese diabetic/severe combined immunodeficient mice were injected subcutaneously with 10×10^6 MOLT-4.Luc.GFP cells in each flank. Five days after cell transfer, mice were equally distributed according to tumor burden in 2 groups ($n = 3$ per group): Control (vehicle control) and CX-4945 (CX-4945, 75 mg/Kg, orally, twice a day). Tumor burden was assessed at 3 weeks (**a**) or the indicated time points (**b**) after cell transplantation ($P = 0.015$; two-way ANOVA).

The cytotoxic effects of CX-4945 were accompanied by evidence of CK2α activity inhibition, as documented by the concentration- and/or time-dependent dephosphorylation of two main direct targets of CK2α, Ser380 p-PTEN and Ser129 p-Akt. The outcome of Akt phosphorylation at Ser129 by CK2α is still debated. However, it has been proposed that this phosphorylative event enhances Akt interactions with the chaperone protein HSP-90,[39] which protects Akt from protein phosphatase PP2A acting on the Thr308 residue of Akt.[54] In agreement with this theory, we observed that CX-4945 dephosphorylated Thr308 p-Akt in both PTEN-expressing and PTEN-deleted T-ALL cell lines. Accordingly and in agreement with previous findings,[39] the amount of Akt associated with HSP-90 was markedly decreased by CX-4945. Moreover, geldanamycin, a drug that disrupts the interactions between Akt and HSP-90, reduced the levels of Thr308 p-Akt (but not those of Ser473 p-Akt), thus mimicking the effects of CX-4945, while okadaic acid, a PP2A inhibitor,[54] opposed the effects of CX-4945 on Thr308 p-Akt. It is worth remembering here that the Ser473 residue of Akt is dephosphorylated by the PHLPP protein phosphatase.[55]

The CX-4945 cytotoxic effects correlated with a significant induction of apoptosis and cleavage of caspase-3 and poly (ADP-ribose) polymerase. Moreover, treatment with CX-4945 induced cell cycle arrest in the G_2/M phase of the cell cycle in most of T-ALL cell lines. These findings suggest that CK2 inhibition resulted in deregulation of mechanisms involved in cell cycle progression and mitosis regulation and are in line with the well-documented roles played by CK2α during the G_2/M phase of the cell cycle.[56]

As survival signals generated by cytokines secreted by cells of the bone marrow microenvironment have an important role in the emergence of drug resistance, we evaluated whether CX-4945 could diminish these survival signals. Interestingly, CX-4945 retained most of its pro-apoptotic activity even when Jurkat cells were cocultured with MS-5 murine bone marrow stromal cells. Of note, we extended our observations on the therapeutic potential of CX-4945 in T-ALL by showing that *in vivo* administration of CX-4945 to a subcutaneous xenotransplant model of human T-ALL significantly delayed tumor growth.

It is emerging that downregulation of UPR signaling pathway drives apoptotic cell death in preclinical models of T-ALL.[26,27] Moreover, Manni *et al.*[30] recently reported that CK2 inhibition caused a strong apoptotic response through alterations of the UPR pathway in MM cells. CX-4945 was able to inhibit UPR signaling in T-ALL cell lines and primary samples, as evidenced by downregulation of GRP78/BIP, a marker of UPR activation. However, CX-4945 also induced ER stress as demonstrated by upregulation of IRE1α, p-EIF2α and CHOP expression levels. Thus, we hypothesize that a potential mechanism by which CX-4945-induced cell death in T-ALL cells involved the induction of ER stress and the concomitant inability of leukemic cells to adequately cope with the ER stress by properly activating UPR signaling.[45] Modulation of ER stress/UPR signaling markers, through CK2 inhibition, was also confirmed by experiments in which CK2α expression was down-modulated by siRNA. It should be emphasized that changes in ER stress/UPR signaling were detected in both PTEN-expressing and PTEN-deleted cell lines. We have demonstrated that CK2 treatment downregulated Thr308 p-Akt; however, it did not affect Ser473 p-Akt levels (data not shown). Nevertheless, it has been established that decreased Akt phosphorylation at Thr308 is sufficient to inhibit mTORC1 activity.[57] Consistently with mTORC1 inhibition, CX-4945

decreased the Ser235/236 p-S6RP levels. Therefore, down-regulated mTORC1 activity could be the driving force for inhibition of the UPR signaling in response to CX-4945 in T-ALL cells,[47,58] as we have demonstrated by the use of temsirolimus. It remains to be established how CX-4945 induces ER stress in T-ALL, as ER stress can be generated by the accumulation of unfolded proteins, by depletion of amino acids or by oxidative stress.[59]

However, it should be underlined that T-ALL, unlike certain secretory tumor types (MM, pancreatic adenocarcinoma), does not rely on a high basal UPR activity,[48] and in fact CX-4945 treatment further depressed UPR signaling. In this scenario, treatment with an ER stress inducer, thapsigargin, together with CX-4945, resulted in synergistic killing of leukemic cells through modulation of the ER stress/UPR signaling pathway, as reported in other tumors.[60] Although at present existing ER stress inducers could not be used *in vivo* because of their side effects, a combined treatment consisting of CX-4945 and novel nontoxic ER stress inducers could be considered as a therapeutic opportunity for future treatment of T-ALL.

In conclusion, our results indicate, for the first time, that modulation of the ER stress/UPR signaling through CK2 inhibition could be exploited for inducing T-ALL cell death and that CX-4945 may be an efficient treatment for T-ALLs that have aberrant upregulation of the CK2/PI3K/Akt/mTOR signaling pathway, independently from PTEN expression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)