Selinexor (KPT-330) induces tumor suppression through nuclear sequestration of IκB and down-regulation of survivin

Jayasree S. Nair[*1], Elgilda Musi[1], Gary K. Schwartz[1]

[1]Columbia University Medical Center, Herbert Irving Comprehensive Cancer Center, New York, USA

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[*]To whom correspondence should be addressed: Jayasree Nair, PhD, Columbia University, New York, USA. jayasree_nair@hotmail.com

Statement of Translational Relevance

Last year, approximately 15,000 people were diagnosed with sarcoma in the United States, 20% of whom died from this disease. Therefore, the need to identify novel treatment strategies for sarcomas is of utmost importance. Selinexor, an inhibitor of XPO1, has shown preclinical and clinical activity in sarcoma. However, the mechanism of action of this drug in sarcoma is not well understood. In this study, we show that selinexor induces nuclear localization of IκB. Pretreatment with the proteasome inhibitor carfilzomib can further stabilize IκB, which is sequestered in the nucleus to inhibit transcriptional activity of NFκB and suppresses expression of survivin thereby inducing apoptosis. Thus, this novel mechanism by which selinexor suppresses NFκB signaling provides unique opportunities for cancer therapy, especially when this drug is combined with a proteasome inhibitor.

Abstract

Purpose: Selinexor, a small molecule that inhibits nuclear export protein XPO1 has demonstrated efficacy in solid tumors and hematologic malignancies with the evidence of clinical activity in sarcoma as a single agent. Treatment options available are very few and hence the need to identify novel targets and strategic therapies is of utmost importance.

Experimental design: The mechanistic effects of selinexor in sarcomas as a monotherapy and in combination with proteasome inhibitor, carfilzomib, across a panel of cell lines *in vitro* and few in xenograft mouse models were investigated.

Results: Selinexor induced IκB nuclear localization as a single agent and the effect was enhanced by stabilization of IκB when pretreated with the proteasome inhibitor carfilzomib. This stabilization and retention of IκB in the nucleus resulted in inhibition of NFκB and transcriptional suppression of the critical anti-apoptotic protein, survivin. Treatment of carfilzomib followed by selinexor caused selinexor-sensitive and selinexor-resistant cell lines to be more sensitive to selinexor as determined by an increase in apoptosis. This was successfully demonstrated in MPNST xenograft model with enhanced tumor suppression.

Conclusions: The subcellular distributions of IκB and NFκB are indicative of carcinogenesis. Inhibition of XPO1 results in intra-nuclear retention of IκB which inhibits NFκB and thereby provides a novel mechanism for drug therapy in sarcoma. This effect can be further enhanced in relatively selinexor-resistant sarcoma cell lines by pre-treatment with proteasome inhibitor, carfilzomib. Because of these results, a human clinical trial with selinexor in combination with a proteasome inhibitor is planned for the treatment of sarcoma.

Introduction

XPO1, the main nuclear export protein, transports cargo proteins containing nuclear localization signals out of the nucleus (1-3). Export of proteins such as survivin (4,5), inhibitor of NFκB (IκB), p53 (6,7), Rb (8) and topo II α (9) from the nucleus through nuclear pore complex constitutes a key component of intracellular signalling which regulates cell proliferation and apoptosis (10-13). Malfunctioning of the nuclear pore complex can cause cancer by stimulating tumor growth and inhibiting apoptosis (2,14) and overexpression of XPO1 has been reported in many types of cancers, including sarcomas(15). Because its expression level and activity perpetuate tumor growth, XPO1 is an important target for the development of small molecule inhibitors to treat cancer (10).

Survivin is a member of the inhibitors of apoptosis protein family and is overexpressed in most common human cancers including breast, prostate, pancreatic, and haematological malignancies (16-21). It plays a vital role in oncogenesis by being involved in both cell cycle control and resistance to apoptosis (22). As a predominantly cytoplasmic protein, survivin is exported to the cytoplasm through an exclusively XPO1-dependent pathway (5). In malignant peritoneal mesothelioma, survivin was shown to rely on XPO1 to be shuttled into the cytoplasm for its antiapoptotic function (23). While expression of survivin has been shown to be regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) in breast cancer (24), activated NFκB pathway has also been shown to contribute to malignant progression of adult T-cell leukemia (ATL) through survivin (25). When in the nucleus, NFκB binds to DNA and activates a pro-oncogenic transcriptional program. However, NFκB can be inhibited from binding to DNA by IκB which controls the transcriptional activity of NFκB. XPO1 transports

IκB out of the nucleus thereby preventing it from inhibiting NFκB (26). However, when XPO1 is inhibited, IκB export to the cytoplasm is blocked and instead accumulates in the nucleus where it binds to NFκB and blocks its pro-oncogenic activity (27).

Sarcoma is a rare cancer with many subtypes that are genetically and biologically distinct (28-30). Molecular heterogeneity and resistance to chemotherapy make sarcoma a rather useful model to study mechanisms of tumorigenesis and drug resistance (31,32). Surgery is the only curative method for resectable disease and chemotherapy and radiation have only limited ability to control inoperable or metastatic disease (33). Thousands of people are diagnosed with sarcoma in the United States every year and the mortality rate is also relatively high. Due to the lack of treatment options for patients with metastatic sarcomas the need to identify novel targets and strategic therapies for sarcomas is essential.

Selinexor, a small molecule inhibitor of XPO1, has been reported to have potent *in vitro* and *in vivo* effects against a broad panel of sarcoma models (34). In the present study, we have set out to define the molecular mechanism underlying the anti-proliferative effects of selinexor. We show that selinexor treatment results in increased nuclear localization of IκB in sarcoma cell lines. Selinexor then sequesters IκB in the nucleus where it binds to and inhibits NFκB transcriptional activity. Furthermore, pre-treatment with a proteasome inhibitor stabilizes IκB and enhances this effect even in selinexor-resistant sarcoma cell lines. In vitro assays show decreased binding of NFκB to the survivin promoter resulting in decreased survivin expression and increased apoptosis. Down regulation of survivin using specific siRNA against survivin made the cells more sensitive to selinexor and transient overexpression of survivin made

sensitive LS141 cells more tolerant towards selinexor. This treatment strategy was tested effectively in a xenograft sarcoma model which was resistant to single agent, selinexor.

Materials and Methods

Cell culture

Sarcoma cell lines A673 and CHP100 (35) (Ewing), MPNST and ST8814 (malignant peripheral nerve sheath) (36)), LS141 and DDLS (dedifferentiated liposarcoma, (37)), WDD (liposarcoma), SK-UT1, SK-UT1B and SK-LMS (uterine leiomyosarcoma), HSSY and SYO (synovial), SaOs2 (osteosarcoma), were maintained in RPMI with 50 U/ml each of penicillin and streptomycin, and 10% heat-inactivated fetal bovine serum, and incubated at 37°C in 5% CO_2 and cultured not more than 4 months. Malignant peripheral nerve sheath tumor cell lines (MPNST, ST8814) were supplied by Dr. Jonathan Fletcher (Dana Farber Cancer Institute, Boston, MA). MPNST and ST8814 cell lines were authenticated as previously described (37). Ewing sarcoma (CHP100, A673) cell lines were obtained from Dr. Melinda S. Merchant (Center for Cancer Research, NCI/NIH, Bethesda, MD). Dedifferentiated liposarcoma cell lines (LS141, DDLS, WDD) were obtained from Dr. Samuel Singer [Memorial Sloan Kettering Cancer Center (MSKCC), New York, NY], and were authenticated by gene expression profiling before distribution. Synovial sarcoma cell lines (SYO-1 and HSSY-II) were obtained from Dr. Marc Ladanyi (MSKCC). Osteosarcoma cell line (Saos2) and uterine leiomyosarcoma cell lines SK-UT1, SK-UT1B and SK-LMS were obtained from ATCC. Initial stocks of all cell lines were received from their sources within the past 3 years. Cell lines CHP100 and A673 were authenticated using RT-PCR, and found to have their expected characteristic chromosomal translocations. SYO-1 and HSSY-II cell lines were authenticated by confirming the expression of the pathognomonic SYT-SSX fusion gene by RT-PCR. ASKSPY cell line was obtained from Karyopharm. All cell lines were determined to be mycoplasma free via testing using biochemical assay MycoAlert.

Reagents

Selinexor was provided by Karyopharm Therapeutics (USA) **(**85 Wells Ave., 2nd floor, Newton, MA 02459). Carfilzomib was purchased from Selleckchem.com.

Colorimetric cell proliferation assay

The assay was performed as per the manufacturer's protocol (Dojindo Molecular Technologies, Inc.). Briefly, 2,000 cells were plated in 100 µL volume per well of a 96-well plate. Cells were pretreated with vehicle, selinexor or carfilzomib for an hour followed by vehicle or selinexor for a period of 3 days. Then the media was replaced with MEM without phenol red with 10% serum and 10% CCK-8 solution, and were incubated at 37°C for an additional 1 to 4 h. Optical density was measured at 450 nm to determine the cell viability using EMax Plus Microplate Reader (Molecular Devices Corp).

Flow cytometry

For flow cytometry (38), the cells were washed and fixed in 70% ice-cold ethanol before staining with propidium iodide (50 µg/mL) containing RNase (5 µg/mL) to measure DNA content. Mitotic population was determined by labelling with the phospho MPM-2 monoclonal antibody (Millipore), which recognizes specific phosphorylated epitopes present only in mitosis followed by Alexa Flour 488 anti-mouse secondary antibody (Invitrogen, Oregon, USA). Samples were analysed on a FACScan (Becton Dickinson) for cell cycle distribution and mitotic index (fraction of cells positive for phospho MPM-2) using the Cell Quest software. 10,000 events were examined per sample.

siRNA transfection

Cells were plated on 60-mm plates, and transfections using lipofectamine RNAiMAX (Invitrogen) were performed according to the manufacturer's protocol. The siRNA sequences for XPO1 and survivin were purchased from Santa Cruz Biotechnology and Cell Signaling Technology respectively. Cells were harvested 48 hours after transfection for Western Blot analysis or FACScan analysis.

Survivin Overexpression

Cells were transiently transfected with a survivin expression plasmid, pcDNA 3HA Survivin (CH3 BioSystems) as previously described (39)

Cell lysate extraction, immunoblotting and immunoprecipitation

Cell were lysed in whole-cell lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 1 mmol/L EDTA, 0.25% sodium deoxy cholate, 0.1 mmol/L Na_3VO_4, with protease inhibitor cocktail tablet (Roche)), allowed to incubate on ice for 10 min, homogenized by syringe and cleared by centrifugation at 13,000 rpm for 10 min at 4°C. Thirty micrograms of protein were fractionated by SDS-PAGE and transferred onto Immobilon membranes (Millipore). After blocking with 5% nonfat milk, membranes were probed with primary antibodies. The following antibodies were used in this study: mouse monoclonal to cleaved poly (ADP-ribose) polymerase (PARP), rabbit polyclonal to survivin, rabbit polyclonal to cleaved caspase 3, rabbit polyclonal to NFκB, mouse monoclonal to IκB and tubulin, rabbit monoclonal to XIAP, rabbit monoclonal to IAP, mouse monoclonal to Bcl-xL, rabbit polyclonal to RelA and mouse monoclonal to Ku70 (Cell Signaling, Danvers, MA 01923, USA). Bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies or IRDye@800CW secondary

antibodies (LiCOR) and visualized by enhanced chemiluminescence reagent (both from GE Healthcare UK Limited) or using the Odyssey scanner (LiCOR Biosciences). Immunoprecipitation was performed from lysates by using 500 μg of soluble protein incubated with 2 μg of rabbit NFκB polyclonal antibody overnight at 4°C followed by incubation with 50 μL of protein A-agarose beads (Upstate Biotechnology). Immunocomplexes were washed five times in lysis buffer and fractionated by SDS-PAGE. Western blot analysis was performed as previously described.

Caspase Glo assay

Induction of Caspase 3/7 activity was determined using CaspaseGlo (Promega) assay according to manufacturer's instructions.

NFκB Transcription Factor assay

NFκB transcription activity was determined as per the manufacturer's protocol (Thermo Scientific).

Chromatin Immunoprecipitation Assay (ChIP)

Cells were cross-linked with 1% formaldehyde, quenched by 0.125 M glycine, washed, and nuclear extraction was performed using the Simple Chip Enzymatic ChIP Kit (Cell Signaling) following the manufacturer's instructions. Equivalent amounts of chromatin from each sample were immunoprecipitated with the NFκB antibody overnight at 4°C. Antibody-protein complexes were then collected using Protein G agarose beads (Cell Signaling) pre-blocked with salmon sperm. Eluted DNA was reverse cross-linked, treated with proteinase K, and purified.

Immunoprecipitated DNA and input controls were analyzed on an Applied Biosystems 7500 real-time PCR machine using Taq SYBR Green (Life Technologies) and primer sets for survivin (Hs_BIRC5_2_SG QuantiTect Primer Assay, Qiagen).

Nuclear extraction

Nuclear extraction was performed as per manufacturer's protocol (Thermo Scientific, Rockford, IL, USA) with the following changes. Five million cells were resuspended in 100 µL CERI, vortexed for 15 seconds and incubated on ice for 10 minutes. Samples were centrifuged at maximum speed for 5 minutes after adding 5.5 µL of CERII followed by vortexing and incubating for 1 minute on ice. The insoluble pellet was dissolved in NER and the nuclear extract was prepared by centrifuging the samples for 10 minutes at the highest speed after vortexing every 10 minutes while incubating on ice for 40 minutes.

Quantitative fluorescent microscopy (QFM)

Cells were collected after drug treatment and fixed in 3% paraformaldehyde. The nuclear morphology of cells was examined using fluorescence microscopy after staining with 4′, 6-diamidino-2-phenylindole (DAPI) at a concentration of 25 µg/ mL. Number of cells with decondensed, fragmented chromatin was counted as a measure for apoptosis. A minimum of 400 cells were counted for each sample and calculated as a percentage of untreated cells.

Xenograft studies

Athymic mice bearing LS141 (7 mice/cohort) of 150 mm^3 diameters were treated with either vehicle control or 15 mg/kg of selinexor p.o. once daily 5x for 3 weeks. Twenty-four hours after

the last treatment, one animal from each cohort was sacrificed and the tumors examined for XPO1 and survivin by western blot analysis. Tumors were measured every 2 to 3 days with calipers, and tumor volumes were calculated by the formula 4/3 x r3 [r = (larger diameter + smaller diameter)/4]. The percentage of tumor regression was calculated as the percentage ratio of difference between baseline and final tumor volume to the baseline volume. Toxicity was monitored by weight loss. These studies were done in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, released 1985).

Statistical analysis

All *in vitro* experiments were carried out at least three times. The statistical significance of the experimental results was determined by the two-sided *t* test. We chose $P = 0.05$ as statistically significant in individual comparisons. For *in vivo* studies, the two-sided *t* test was used as a summary measure for each mouse. Tumor volume was compared between groups of mice. *P* values were calculated using the Wilcoxon Rank Sum test.

Results

Selinexor is active in sarcoma cell lines and suppresses survivin expression

Selinexor was tested for its ability to inhibit cell growth in a panel of sarcoma cell lines. Table 1 depicts the effect of selinexor on proliferation of sarcoma cell lines of different lineages. The selinexor IC_{50} values for the cell lines varied, ranging from 50 nM for LS141 to 1 μM for CHP100 cells. As shown in the Table, sensitivity towards selinexor was independent of the p53 status of the cell lines. Cells were then treated with 250 nM of selinexor for 48 hours and tested for induction of apoptosis. As shown in Figure 1A, liposarcoma (LS141, DDLS, WDD), synovial cells (SYO and HSSY) and uterine leiomyosarcoma (SK-UT1, SK-UT1B) cell lines showed increased sensitivity with a pronounced cleavage of PARP and caspase-3 when treated with selinexor. Other sarcoma cell lines including Ewing's sarcoma (CHP100) exhibited essentially no PARP cleavage or caspase-3 activation upon exposure to selinexor. All the cell lines in which there was induction of apoptosis showed a down regulation in survivin protein expression. XPO1, the target for selinexor, was down regulated in all cell lines confirming target inhibition. Using siRNA specific for XPO1, these effects on protein expression could be recapitulated in LS141, SYO and UT1 cell lines (Figure 1B). This effect of apoptosis upon inhibition of XPO1 was independent of p53 status of the cell line. The ability of selinexor to inhibit cancer cell growth was further tested as a monotherapy in a xenograft mouse model of liposarcoma, LS141. As shown in figure 1C, selinexor induced statistically significant tumor growth suppression ($p = 0.0425$) without weight loss, and in lysates prepared from tumors collected after 14 days of treatment down regulation of both survivin and XPO1 protein expression was observed (Figure 1D).

The role of survivin in selinexor-induced apoptosis

Because treatment with a single dose of 250 nM selinexor induced high levels of PARP cleavage in LS141 cells but not in CHP100 cells, these cells lines were selected for mechanistic studies. Cell cycle analysis by flow cytometry was performed on cells that were treated with 100 and 1000 nM selinexor for 24 hours (Figure 2A and B). Sub-G1 peak analysis in LS141 showed 13 and 25% apoptosis with 100 and 1000 nM of selinexor, respectively (Figure 2A), while in CHP100 cells minimal apoptosis was detected at either drug concentrations (Figure 2B). Western blot analysis of cells treated with increasing concentrations of selinexor (up to 10 μM) showed prominent apoptosis in LS141 cells at a concentration as low as 100 nM (Figure 2C) while again there was no evidence of apoptosis in CHP100 by cleaved PARP (Figure 2D). Selinexor suppressed survivin in the sensitive LS141 cells but induced survivin in resistant CHP100 cells.

Because survivin transcription is regulated by NFκB, we sought to determine whether the regulator of NFκB, IκB, was affected by selinexor treatment. Activation of NFκB requires the ubiquitination and 26S proteasome mediated degradation of IκB leading to the release of NFκB (25). Despite changes in survivin protein levels, selinexor did not affect the levels of IκB protein in either LS141 or CHP100 cell line (Figure 2C and D). Apoptotic proteins whose expressions shown to be regulated by NFkB, such as Bcl-xL, XIAP and IAP, were unaffected upon treatment with selinexor. Next we tested whether the differences in the sensitivity to selinexor in the two cell lines could be attributed to differences in the subcellular localization of IκB. Localization of IκB was tested by immunofluorescence and as shown in Figure 2E. LS141 cells showed a rapid shift in localization of IκB to the nucleus upon selinexor treatment while the efficiency of

nuclear localization of IκB in CHP100 cells (Figure 2F) was less than in LS141 cells. The difference in nuclear localization of IκB between the two cell lines upon treatment with selinexor was further confirmed by probing the nuclear fraction of cell lysates for IκB by western blot analysis. In LS141 cells IκB efficiently moves to nucleus as early as 4 hours of treatment with selinexor (Figure 2G) while in CHP100 cells IκB shows partial localization to the nucleus only at 24 hours of treatment (Figure 2H).

Inhibition of proteasome pathway potentiates the effect of selinexor *in vitro* and *in vivo*

NFκB activation occurs when its regulator, IκB, is ubiquitinated and then degraded via the proteasome (40). To determine whether the efficacy of selinexor can be enhanced by inhibiting proteasome activity and thereby stabilize IκB, LS141, MPNST and CHP100 cells were exposed to 10 nM of the proteasome inhibitor carfilzomib for one hour followed by 100, 500 and 1000 nM selinexor, respectively, then tested for cell viability. As shown in Figure 3, carfilzomib enhanced the effect of selinexor in all three cell lines. LS141 cells were not sensitive to carfilzomib alone but showed increased sensitivity to selinexor in the presence of carfilzomib (Figure 3A). MPNST cells were equally sensitive to either drug alone but showed enhanced selinexor sensitivity in the presence of carfilzomib (Figure 3B). CHP100 cells showed the greatest enhancement of selinexor activity in the presence of carfilzomib with a 40% decrease in viability compared to either drug alone (Figure 3C). In MPNST and LS141 the combination effect was more than additive (80% to 40% and 70% to 40%, respectively), however in CHP100 viability effect was synergistic (80% to 20%).

Additional analyses were performed to determine the mechanism by which carfilzomib potentiates the efficacy of selinexor. Cells were tested for apoptosis first by using flow cytometry to analyse the sub-G1 cell population. Figure 3D shows a significant potentiation in the apoptotic cell population with the combination of selinexor and carfilzomib across a panel of sarcoma cell lines compared to either drug alone. Apoptosis was then measured by detecting caspase 3 activity from the panel of cell lines (Figure 3E). Lastly, apoptosis was measured from lysates from the panel of cells exposed to combination therapy apoptosis by detecting cleaved PARP by western blot analysis (Figure 3F). In all these assays selinexor concentrations used were; 1 μM for Ewing sarcoma cell lines A673 and CHP100, 100 nM for LS141 and 500 nM for the rest of the cell lines. When selinexor was combined with carfilzomib, both the Ewing sarcoma cell lines CHP100 and A673 had a 2-3 fold increase in apoptosis by all three assays. The drug combination had an additive effect on apoptosis in the liposarcoma cell line LS141. Although either drug alone had no effect on apoptosis in DDLS, the combination did have a synergistic effect in DDLS while on MPNST and ST88 the combination had an additive effect. All the cell lines, independently of their p53 status responded efficiently to selinexor combination therapy with proteasome inhibitor.

To test whether proteasome inhibition enhances the efficacy of selinexor *in vivo*, selinexor with and without carfilzomib was evaluated in the relatively less selinexor sensitive (IC_{50} 250 nM) malignant peripheral nerve sheath tumor cell line, MPNST, xenograft mouse model (Figure 4). Selinexor (15 mg/kg) inhibited growth by 30% compared to vehicle *in vivo* against MPNST xenografts after 2 weeks of treatment, and inhibition was increased to 65% when mice were dosed with 5 mg/kg of carfilzomib prior to receiving selinexor (Figure 4A and B). Carfilzomib

alone at 5 mg/kg did have a growth inhibitory effect. Analysis of lysates from the xenograft tumor after 10 days of treatment show the induction of apoptosis as indicated by PARP cleavage that was only present in the combination therapy (Figure 4C).

Effects of carfilzomib and selinexor on NFκB activity

Finally, we sought to understand the mechanism of action of the combination of selinexor and carfilzomib and its impact on survivin expression via NFκB transcriptional activity (Figure 5). In LS141 cells treated with selinexor alone and in combination with carfilzomib, PARP and caspase 3 cleavage were induced and survivin expression repressed, with the markers of apoptosis appearing to be further induced with combination treatment (Figure 5A). CHP100 cells treated with selinexor and carfilzomib together showed increased PARP and caspase-3 cleavage while survivin protein levels were decreased in the combination treatment, as compared to selinexor alone where there was no apoptosis or change in survivin levels (Figure 5B). Down regulation of survivin was a direct effect of selinexor and not a secondary effect of apoptosis because pre-treatment with caspase inhibitor Z-Vad had no effect on the selinexor induced down regulation of survivin protein (Supplement 1). Pre-treatment with carfilzomib followed by selinexor enhanced the immunofluorescent nuclear detection of IκB in both cell lines (Figure 5C and D), though nuclear IκB was apparent with selinexor alone in the LS141 cell line. To test whether IκB localized to the nucleus was bound to NFκB, an immunoprecipitation assay was performed in the nuclear extract using an antibody for NFκB. Selinexor alone induced nuclear localization as well as binding of IκB to NFκB in LS141 cell lysate (Figure 5E). However, in CHP100 cells nuclear localization and binding of IκB to NFκB occurred predominantly after stabilization of IκB with the proteasome inhibitor (Figure 5F).

Lastly, we tested whether the carfilzomib and selinexor-mediated increase in IκB in the nucleus was able to suppress the transcriptional activity of NFκB. A functional assay of the combination treatment in LS141 and CHP100 cells showed an additive effect on inhibition of NFκB activity (Figure 5G &H). Next, we tested whether the reduced NFκB activity was responsible for the down regulation of survivin expression by performing the CHIP assay (Figure 5I). Selinexor concentration used in these experiments is 100 nM in LS141 and 1000 nM in CHP100 and duration of exposure is 1 hour carfilzomib followed by 18 hours of selinexor. As shown in Figure 5I, a reduction in promoter occupancy of survivin gene upon treatment with selinexor in LS141 or selinexor followed by carfilzomib in both LS141 and CHP100 is observed.

Finally to confirm survivin is downregulated at the mRNA level, RT-PCR assay was performed using primers for survivin (Figure 6A). The concentrations of Selinexor used were 100 nM, 500 nM and 1000 nM respectively for LS141, MPNST and CHP100 cells and carfilzomib exposure for 1 hour followed by 18 hours of selinexor in the combination therapy. These results show that the combination of carfilzomib and selinexor in both MPNST and CHP100 cells resulted in the down regulation of survivin at the mRNA level, and while this was also observed in LS141 cells treated with selinexor alone, the effect was enhanced with the combination. The down regulation of survivin upon treatment with selinexor therefore appears to be due to inhibition of NFκB transcriptional activity. Thus, stabilization of IκB by proteasome inhibition in conjunction with selinexor-mediated IκB nuclear retention serves to further inhibit NFκB, resulting in the down regulation of expression of survivin with the net result being increased apoptosis and cancer cell death.

Downregulation of survivin is critical for Selinexor induced growth suppression

To further explore the role of survivin in the apoptosis induced by selinexor, survivin was ectopically overexpressed in selinexor sensitive LS141 cells and exposed to selinexor. As shown in Figure 6B, transient overexpression of survivin and treatment with selinexor, increased the cell viability from 15% to 50% and this corresponded to a reduction in PARP cleavage, indicating a significant role for survivin in the selinexor induced apoptosis in LS141 cells. Survivin was down regulated in CHP100, LS141 and MPNST cells with specific siRNA and their sensitivity towards selinexor was tested. As shown in Figure 6C, except in MPNST cells, down-regulation of survivin alone caused a decrease in cell viability and this could be further enhanced by treatment with selinexor in all the cell lines. This was associated with an increase in PARP cleavage. Survivin's role was tested in a resistant clone of LS141 ($LS141^{R}$) which was developed with continuous exposure of the parental cell line to increasing doses of selinexor. As shown in Figure 6D, survivin expression was higher in the resistant clone compared to the parental LS141. Despite increasing concentrations of the drug, the resistant cell line was "resistant" to survivin suppression and showed no activation of caspase 3 or cleavage of PARP. These experiments confirm our findings that survivin plays a significant role in the selinexor induced cell growth suppression.

Discussion

In this study we show that sarcoma cell lines of different lineages have a wide distribution of sensitivity to selinexor with IC_{50} values ranging from 50 nM to 2.5 μM. Selinexor suppressed growth in the sensitive cell lines by inducing apoptosis as well as down regulating survivin protein. Since survivin has been shown to be inhibitor of apoptosis and is transcriptionally regulated by NFκB, we tested and confirmed that survivin is downregulated through the NFκB pathway. Stabilizing IκB, the regulator of NFκB, by pretreatment with a proteasome inhibitor, induced even the relatively selinexor-resistant cell lines to become sensitive to selinexor.

Our results show that selinexor effectively inhibits sarcoma tumor growth in *vivo*; however selinexor sensitivity varies across sarcoma cell lines in vitro. Our findings support a recent report which has indicated the activity of selinexor against a panel of sarcoma cell lines, but the study did not report the mechanism of action (34). Among the sarcoma cell lines we tested, liposarcoma (LS141) shows the greatest sensitivity to selinexor and this appears to be due to the induction of apoptosis as well as the down regulation of survivin protein expression, both *in vitro* and *in vivo*. Selinexor has completed Phase I and Phase 1b studies (41,42) and antitumor activity was noted in patients, among them patients with dedifferentiated liposarcoma (DDLPS), with 40% of the 15 patients showing reduction in target lesions and 47% showing stable disease for 4 months or longer (41). Based on these clinical results, a phase II randomized study of selinexor versus placebo in patients with advanced liposarcoma is under way **(**NCT02606461).

Sarcomas are rare tumors with genetically and biologically distinct types. The majority of these tumors do not respond to chemotherapy. Survivin has been shown to be overexpressed in soft tissue sarcomas (15) and plays a vital role in oncogenesis via its involvement in cell cycle control

(22). In order to execute its anti-apoptotic function, survivin protein must be exported by XPO1 to the cytoplasm (5). However, upon inhibition of XPO1, survivin protein remains in the nucleus and enhances apoptosis (23). XPO1 inhibitor with proteasome inhibitor combination therapy was recently reported to be efficient in human multiple myeloma (43)

We report for the first time in sarcoma that selinexor alone, by inhibiting XPO1 mediated nuclear export, results in increased nuclear localization of IκB and inhibition of NFκB transcriptional activity. This single-agent effect is specific to sarcoma cell lines that are most sensitive to selinexor treatment and was not observed in the relatively selinexor-resistant cell lines. The inhibition of NFκB transcriptional activity is achieved in the relatively selinexor-resistant cell lines only when selinexor was combined with the proteasome inhibitor, carfilzomib, which promotes stabilization of IκB. Stabilized IκB in the presence of selinexor binds to and sequesters NFκB in the nucleus and inhibits it from binding to the survivin promoter and mRNA expression. The net effect is to suppress the expression of survivin. The previous finding that NFκB signaling prevents apoptosis through activating the expression of survivin (24,25) supports our finding in sarcoma. Similar results were obtained with another proteasome inhibitor bortezomib (data not shown).

The findings in this study suggest that inhibition of NFκB signaling resulting from the combination of selinexor and a proteasome inhibitor has therapeutic implications and could provide a means to expand the therapeutic index of selinexor across sarcoma subtypes. Based on these mechanistic results, a Phase I study of a proteasome inhibitor and selinexor is now being planned for patients with advanced sarcomas.

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Table 1: IC_{50} values of selinexor in sarcoma cell lines

Cell line	$IC_{50\ in\ nM}$	p53 status
LS141	50	WT
DDLS	70	WT
WDD	60	WT
HSSY	75	WT
SaOs2	100	Null
SK-LMS	200	WT
MPNST	250	MT
ST88	250	MT
SK-UT1	500	MT
SK-UT1B	2500	WT
CHP100	1000	Null
A673	1500	Null

Figure Legends

Selinexor, a molecule that inhibits the nuclear export protein XPO1, suppresses sarcoma cancer cell growth

A. Western blot detection of c-PARP, XPO1, c-Caspase 3, survivin and tubulin in sarcoma cell lines treated with selinexor (250 nM) for 48 hours. B. Western blot detection of XPO1, c-PARP, survivin, and tubulin in LS141, SYO and SK-UTI cells that were either treated with selinexor (250 nM) for 24 hours or transfected with siRNA specific for XPO1 for 48 hours. Results shown are representative of the mean of three or more independent experiments. C. Athymic female nude mice were implanted with LS141 tumors and were treated with selinexor (15 mg/kg) or vehicle (*n*=7, as described in the Materials and Methods). Tumor volume was measured every 2 to 3 days and the mean tumor volume was plotted against time in days. D. Western blot analysis of XPO1, survivin and tubulin in lysates prepared from tumors excised 24 hours after the final treatment.

Figure 2: Selinexor induces IκB nuclear localization

A. Cell cycle distribution was determined by FACScan analysis in LS141 (A) and CHP100 (B) cells treated with 0, 100 and 1000 nM selinexor for 24 hours. Western blot analysis of total protein lysates from LS141 (C) and CHP100 (D) cells treated with 0-10 μM of selinexor for 24 hours and probed for c-PARP, IκB, survivin, Bcl-xL, IAP, XIAP and tubulin. Immunofluorescence assay of LS141 (E) and CHP100 (F) cells after treatment with 100 nM selinexor for 0, 4, 18 and 24 hours. IκB localization is shown in green and nuclei are stained with

DAPI (blue). Western blot analysis of nuclear extracts from LS141 (G) and CHP100(H) cells treated with 100 nM selinexor for 0, 4, 18 and 24 hours probed for IκB and Ku70.

Figure 3: Proteasome inhibition potentiates the effect of selinexor in vitro

Viability measurements in LS141 (A), MPNST (B), CHP100 (C) cells treated with 100, 500, and 1000 nM selinexor, respectively, with and without carfilzomib pretreatment. D. Flow cytometry analysis of a panel of sarcoma cell lines treated with either vehicle, selinexor (Sel), Carfilzomib (C) followed by vehicle or carfilzomib followed by selinexor (C-Sel). (E) Activated caspase 3 was measured in the treated cell lysates and plotted as fold change compared to control. F. Western blot analysis for cleaved PARP in total protein lysates from sarcoma cells treated with either vehicle, selinexor (Sel), carfilzomib followed by vehicle (C-ND) or carfilzomib followed by selinexor (C-Sel). All experiments were done at least three times and mean values are plotted.

Figure 4: Proteasome inhibitor potentiates the effect of selinexor in vivo

A. MPNST xenografts in athymic mice were treated with vehicle, 15 mg/kg selinexor (Sel), 5 mg/kg carfilzomib followed by vehicle (C-ND) or 5 mg/kg carfilzomib followed by selinexor (C-Sel). Tumor volume after two weeks of treatment was plotted with vehicle control as 100%. B. Comparison of tumor volumes between four treatment conditions in two different mice per condition. C. Western blot analysis for cleaved PARP and tubulin in xenograft tumor lysates 24 hours after the final treatment.

Figure 5: Carfilzomib and selinexor-mediated stabilization and nuclear localization of IκB leads to inhibition of NFκB transcriptional activity

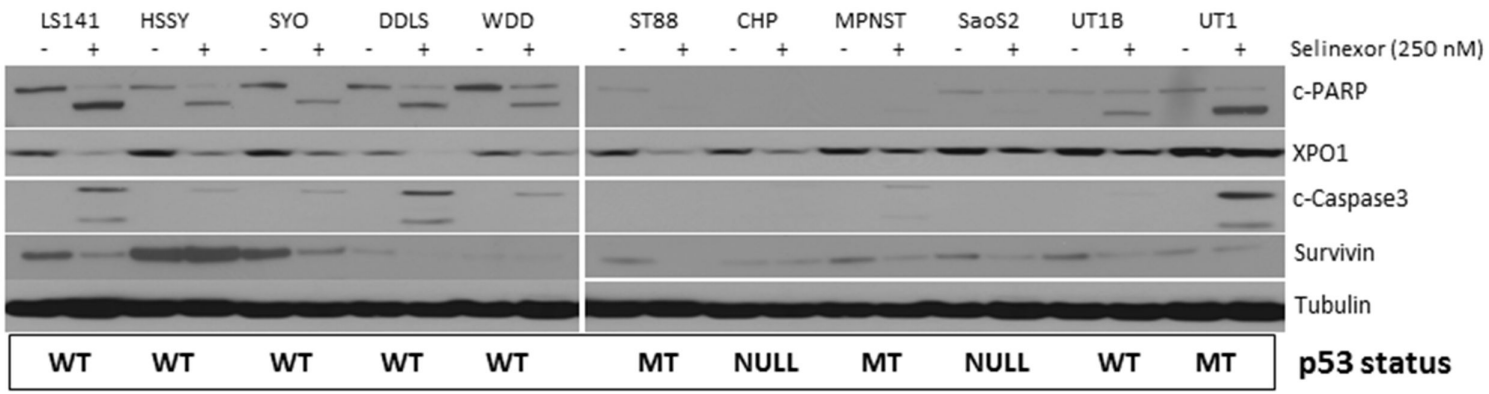
Western blot analysis of LS141 (A) and CHP100 (B) cells were treated with vehicle, selinexor (Sel), carfilzomib followed by vehicle (C-ND) or carfilzomib followed by selinexor (C-Sel) for 24 hours and the total protein lysates were probed for cleaved-PARP, Cl-caspase, survivin, IκB, Bcl-xL, Mcl-1 and tubulin. Immunofluorescence assay of (C) LS141 and (D) CHP100 cells treated with vehicle (ND), selinexor (Sel), carfilzomib followed by vehicle (C-ND) or carfilzomib followed by selinexor (C-Sel) for 4 hours were performed with IκB antibody (green) and nuclei stained with DAPI (blue). Immunoprecipitation assay was performed with the lysates from (E) LS141 and (F) CHP100 cells treated with vehicle (ND), selinexor (Sel), carfilzomib followed by vehicle (C-ND) or carfilzomib followed by selinexor (C-Sel) for 18 hours with NFκB antibody followed by western blot using IκB. NFκB transcription activity was determined and plotted with vehicle as 100% control of LS141 (G) and CHP100 (H) cells treated with vehicle (ND), selinexor (Sel), carfilzomib followed by vehicle (C-ND) or carfilzomib followed by selinexor (C-Sel) for 18 hours. I. ChIP assay was performed in LS141 and CHP100 cells treated with vehicle (ND), selinexor (Sel), carfilzomib followed by vehicle (C-ND) or carfilzomib followed by selinexor (C-Sel) for 12 hours. Cell lysates were immunoprecipitated with NFκB and the survivin promoter region was amplified using oligo primer. The results were plotted with vehicle control as 100%. All experiments were done at least three times and the results shown are average of all.

Figure 6: Downregulation of survivin is critical for Selinexor induced growth suppression

A. mRNA expression of survivin in CHP100, LS141 and MPNST cells treated with vehicle, selinexor (Sel), carfilzomib followed by vehicle (C-ND) or carfilzomib followed by selinexor (C-Sel) for 24 hours by RT-PCR. B. LS141 cells were transiently transfected with pcDNA 3HA survivin or plasmid vector and treated with vehicle (ND) or selinexor (Sel) for 48 hours for cell viability assay and 24 hours for Western blot analysis to check survivin, cleaved PARP in total protein lysates with GAPDH as control. C. Survivin down regulation using specific siRNA in CHP100, LS141 and MPNST and treated with vehicle (ND), selinexor (Sel), carfilzomib followed by vehicle (C-ND) or carfilzomib followed by selinexor (C-Sel) for 48 hours for cell viability assay and 18 hours for Western blot analysis to check survivin, cleaved PARP. D. Selinexor resistant clone of LS141 and parental LS141 cells treated with vehicle (ND), 0.01, 0.1 or 1 μM selinexor (Sel) for 48 hours for cell viability and 18 hours for Western blot analysis for survivin, cleaved caspase3, cleaved PARP and IκB. All experiments were done at least three times and the results shown are representative of all.

Figure 1

A.



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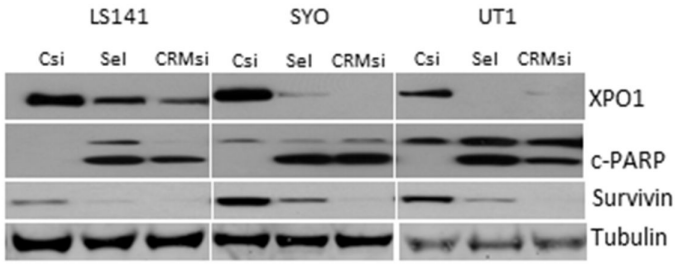

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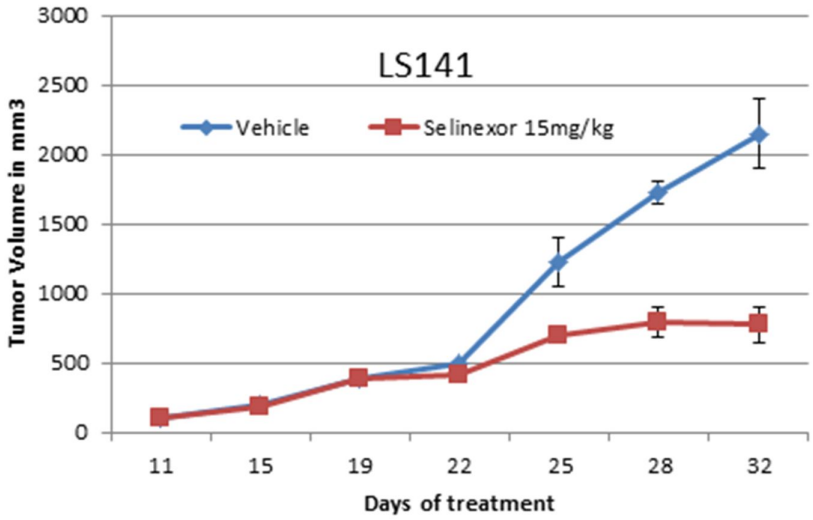

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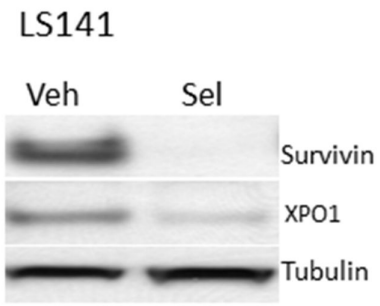
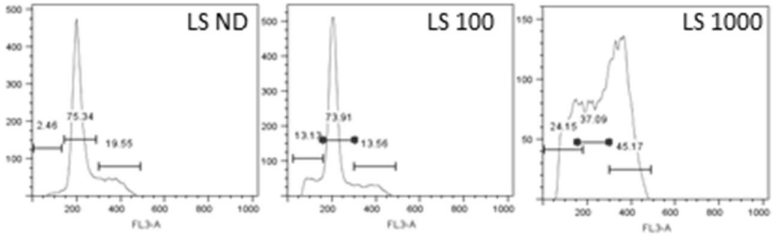


Figure 2

A. LS141



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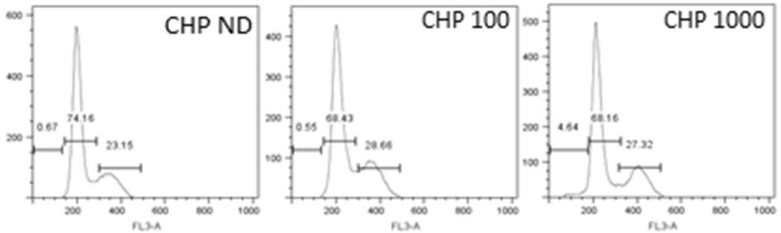
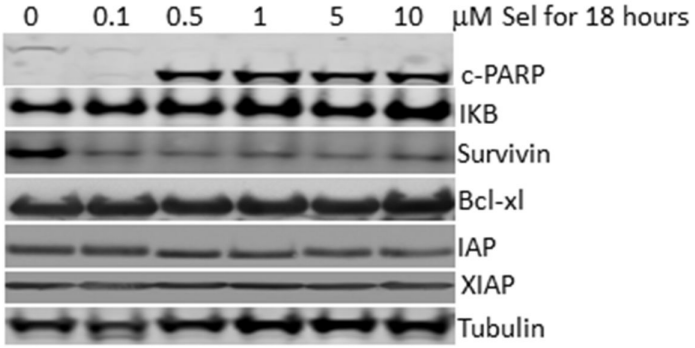
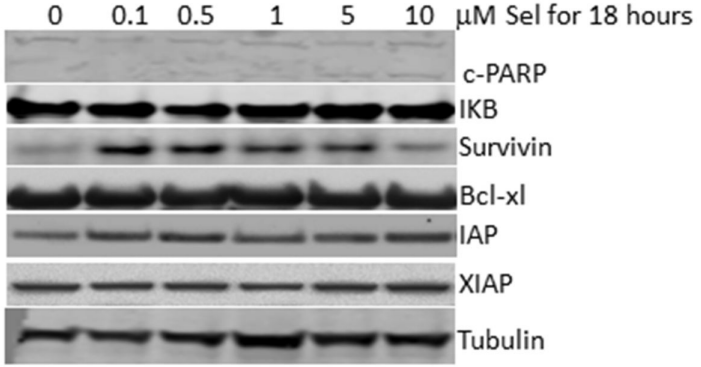


C. LS141

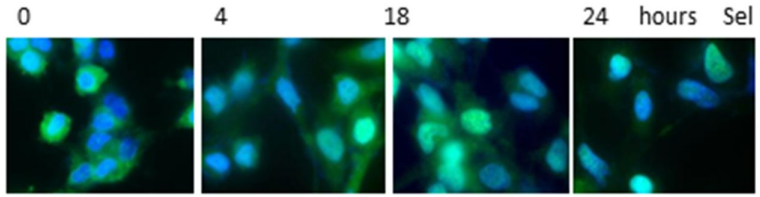


D. CHP100

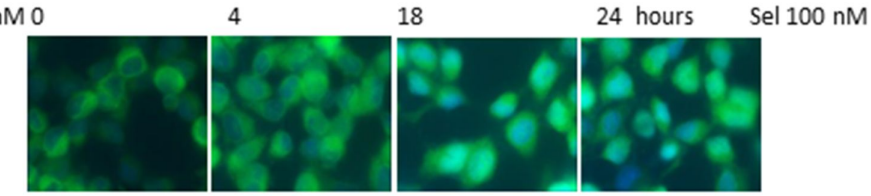


E. LS141

Immunofluorescence iκB

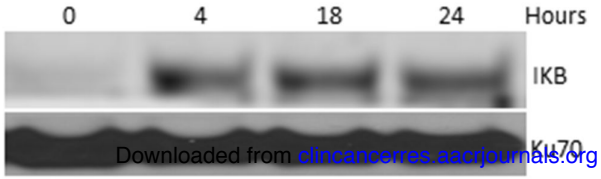


F. CHP100



G. LS141

Nuclear Fraction



H. CHP100

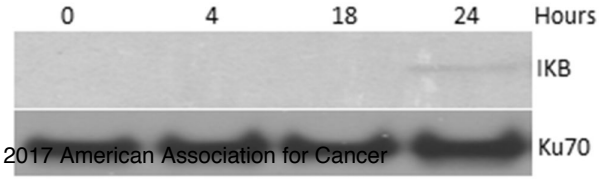


Figure 3

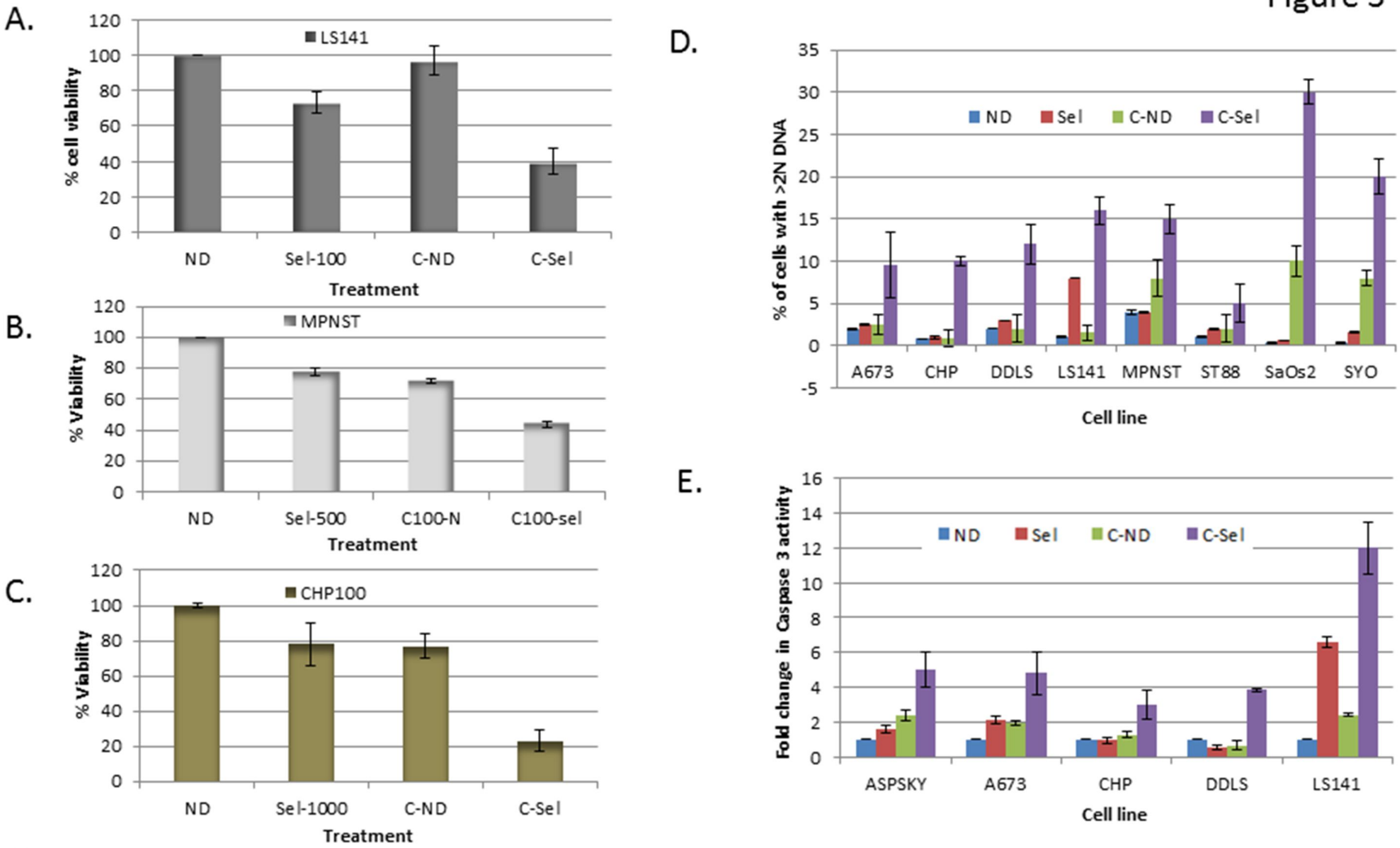
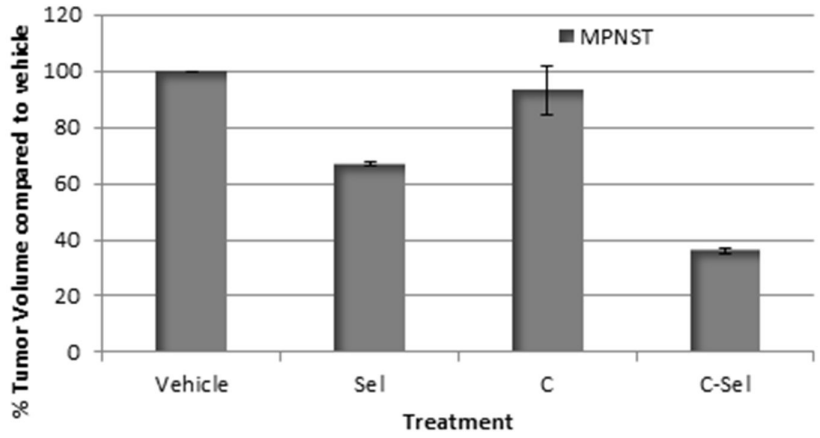


Figure 4

A.



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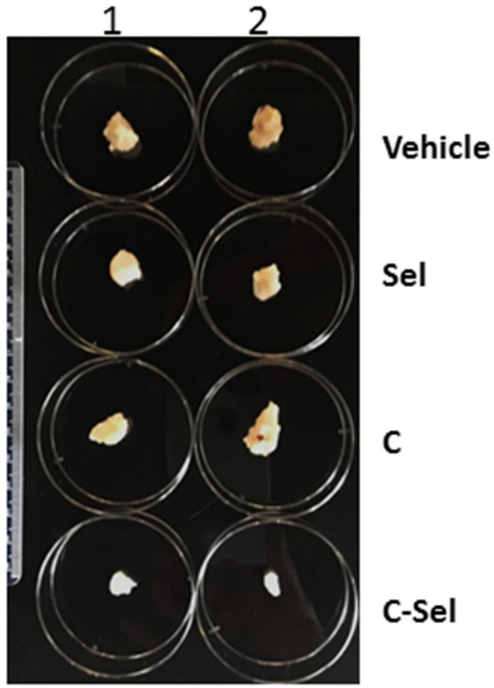

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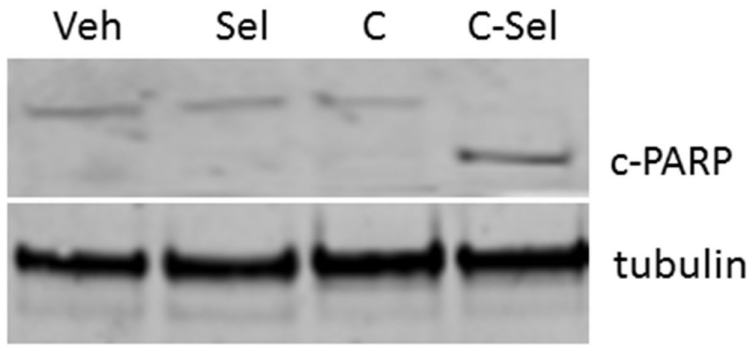


Figure 5

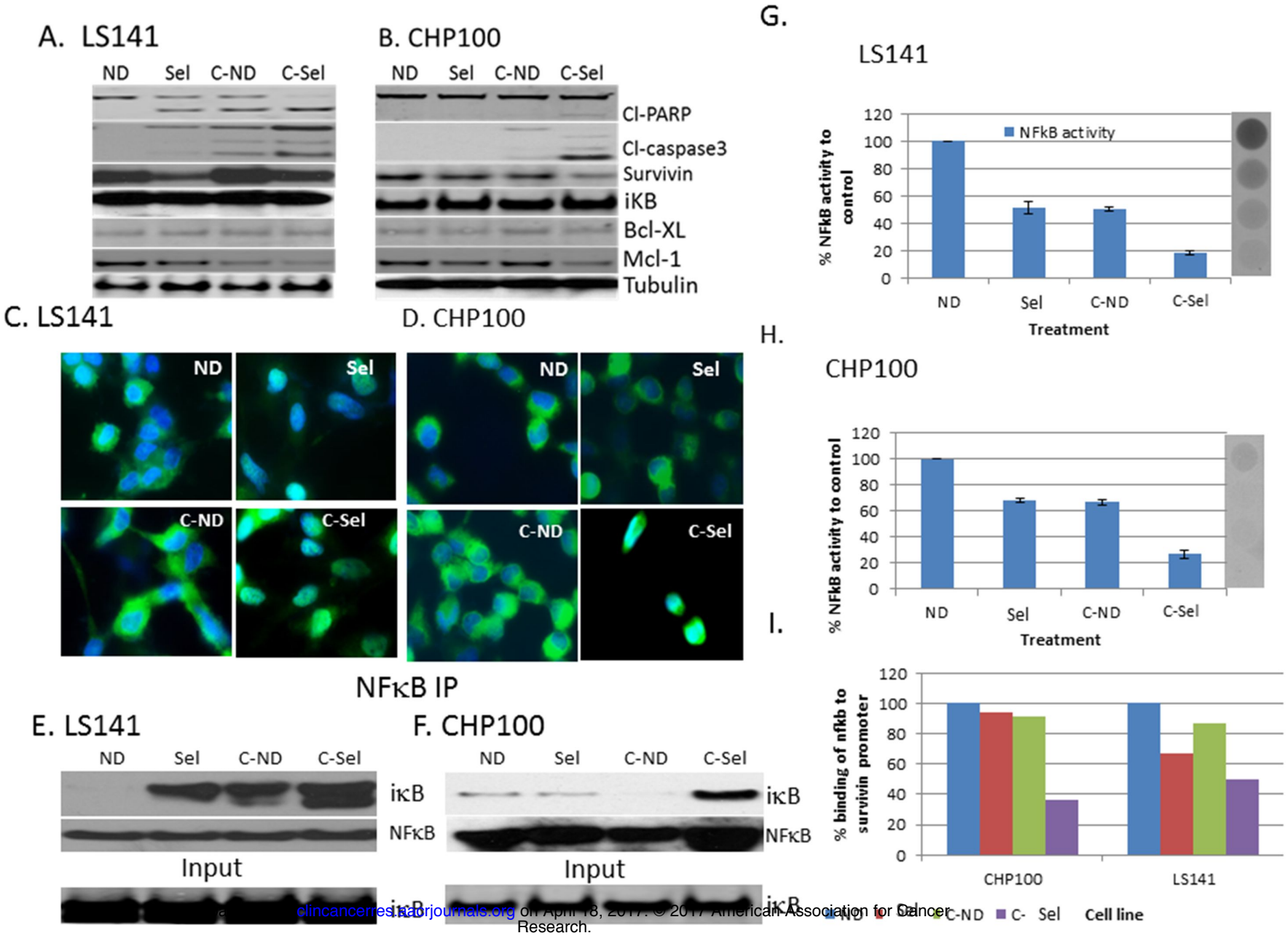
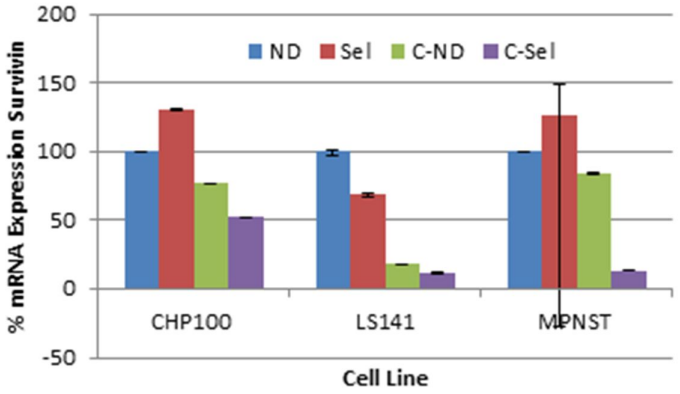


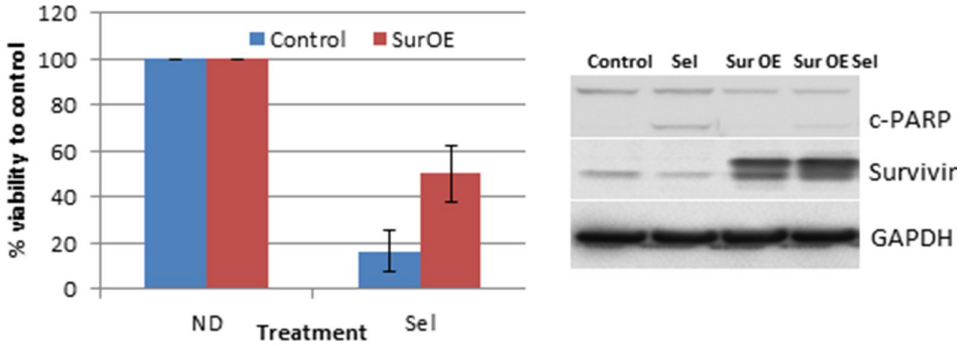
Figure 6

A. mRNA expression of survivin by RT-PCR



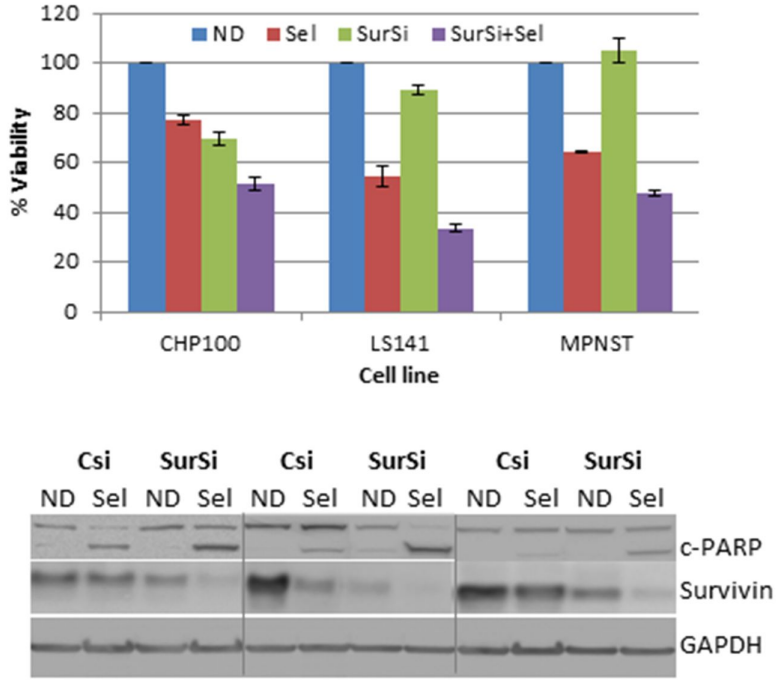
B.

Ectopic overexpression of Survivin increases cell viability

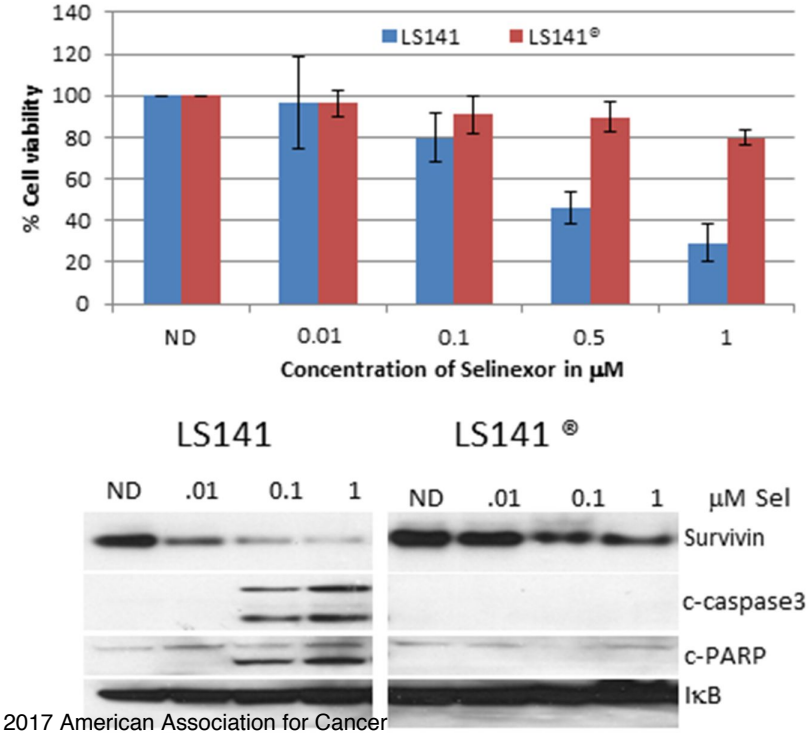


Down regulation of survivin sensitizes cells to selinexor

C.



D. Selinexor resistant LS141 shows increased Survivin level



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Selinexor (KPT-330) induces tumor suppression through nuclear sequestration of IkappaB and down-regulation of survivin

Jayasree S Nair, Elgilda Musi and Gary K Schwartz

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