Clinical Cancer Research

XPO1 Inhibition using Selinexor Synergizes with Chemotherapy in Acute Myeloid Leukemia by Targeting DNA Repair and Restoring Topoisomerase IIα to the Nucleus

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Abstract

Purpose: Selinexor, a selective inhibitor of XPO1, is currently being tested as single agent in clinical trials in acute myeloid leukemia (AML). However, considering the molecular complexity of AML, it is unlikely that AML can be cured with monotherapy. Therefore, we asked whether adding already established effective drugs such as topoisomerase (Topo) II inhibitors to selinexor will enhance its anti-leukemic effects in AML.

Experimental Design: The efficacy of combinatorial drug treatment using Topo II inhibitors (idarubicin, daunorubicin, mitoxantrone, etoposide) and selinexor was evaluated in established cellular and animal models of AML.

Results: Concomitant treatment with selinexor and Topo II inhibitors resulted in therapeutic synergy in AML cell lines and patient samples. Using a xenograft MV4-11 AML mouse model, we show that treatment with selinexor and idarubicin significantly prolongs survival of leukemic mice compared with each single therapy.

Conclusions: Aberrant nuclear export and cytoplasmic localization of Topo IIα has been identified as one of the mechanisms leading to drug resistance in cancer. Here, we show that in a subset of patients with AML that express cytoplasmic Topo IIα, selinexor treatment results in nuclear retention of Topo IIα protein, resulting in increased sensitivity to idarubicin. Selinexor treatment of AML cells resulted in a c-MYC–dependent reduction of DNA damage repair genes (*Rad51* and *Chk1*) mRNA and protein expression and subsequent inhibition of homologous recombination repair and increased sensitivity to Topo II inhibitors. The preclinical data reported here support further clinical studies using selinexor and Topo II inhibitors in combination to treat AML. *Clin Cancer Res; 22(24); 6142–52. ©2016 AACR.*

Introduction

Exportin 1 (XPO1) is a nuclear receptor exporter involved in the active transport of a number of cargo proteins, including transcription factors (i.e., FOXO3A), tumor suppressor proteins (TSP; i.e., TP53 and CDKN1A), cell-cycle regulators (i.e., CDKN1A), and RNA molecules (1–4). XPO1 overexpression has been reported in several solid tumors and in hematologic malignancies, including acute myeloid leukemia (AML) and it is associated with poor prognosis (5–7). Targeting nuclear exporter receptors such as XPO1 is a novel approach to restore tumor suppressor function in AML. We and others have shown that small inhibitors of XPO1 have potent antileukemic activity *in vitro* and *in vivo* in preclinical models of AML (8, 9). Selinexor, a selective inhibitor of XPO1, is currently being tested in a phase I clinical trial in AML. Preliminary data indicate that selinexor is well-tolerated, safe, and active in primary refractory and relapsed patients with AML (10). However, considering the molecular complexity of AML (11, 12), it is unlikely that this disease can be cured with monotherapy and therefore we asked whether adding already established effective drugs such as topoisomerase (Topo) II inhibitors to selinexor will enhance or improve its antileukemic effects in AML. The rationale for exploring an interaction between Topo II inhibitors and XPO1 inhibition is based on the interplay between Topo II and XPO1. Patients with primary refractory or relapsed AML after induction therapy with cytarabine and Topo II inhibitors have a poor prognosis (13–15). It has been shown in some diseases such as lung cancer, a mutation in Topo IIα results in a shift in Topo IIα localization from the nucleus to the cytoplasm, potentially leading to resistance to Topo II inhibitors (16); however, in the case of hematologic malignancies such as AML, the mechanism behind aberrant cytoplasmic localization of Topo IIα remains to be discovered. When Topo IIα is exported to the cytoplasm, it is not in contact with DNA,

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Translational Relevance

The standard treatment for acute myeloid leukemia (AML) in the United States is induction chemotherapy with anthracycline and cytarabine followed by post-remission consolidation chemotherapy or/and allogeneic stem cell transplants. However, the prognosis of AML is poor, with only approximately 40% of younger (<60 years) and 10% older (>60 years) patients surviving at 5 years. This highlights the urgent need for novel therapeutic approaches and individualized therapies beyond "one-fits-all" chemotherapy regimens. Here, we show that concomitant treatment with selinexor and topoisomerase II inhibitors resulted in therapeutic synergy in AML *in vitro* and *in vivo*. Selinexor mediated downregulation of DNA damage repair genes and inhibition of homologous recombination sensitizes AML cells to anthracycline therapy. On the basis of our data, we have initiated 2 clinical trials enrolling primary refractory and relapsed patients with AML to selinexor in combination with anthracyclines.

and Topo II inhibitors such as anthracyclines are unable to induce DNA cleavage complexes and cell death. It is known that Topo IIα is exported from the nucleus by XPO1 (1, 17, 18). Thus, we hypothesize that increasing Topo II nuclear accumulation, by using a selective XPO1 inhibitor (selinexor), may sensitize primary refractory and relapsed AML blasts to Topo II inhibitors. Here, we first tested whether there is synergism between selinexor and Topo II inhibitors by performing *in vitro* and *in vivo* studies and subsequently dissected possible mechanisms responsible for such interaction.

Materials and Methods

Cell culture

MV4-11 cells (# CRL-9591) were purchased from ATCC, and MOLM-13 cells (#ACC554) were purchased from DSMZ. MV4-11 cells were cultured in Iscove Modified Dulbecco Medium (# 10-016-CV; Corning), and MOLM-13 cells were cultured in RPMI-1640 medium (# 10-040-CV; Corning). Both the mediums were supplemented with 10% FBS and (100 U/mL) penicillin and (100 μg/mL) streptomycin (# 15140-122; Gibco). MV4-11 cells resistant to idarubicin (MV4-11 Ida^R) were generated by culturing MV4-11 cells with low dose of idarubicin (3 times below IC_{50}) for several weeks.

Primary AML samples and culture

Primary refractory ($n = 2$), relapsed ($n = 1$), and newly diagnosed untreated ($n = 6$) frozen bone marrow AML patient samples were obtained from the Ohio State University Leukemia Tissue Bank after getting informed consent approved by the Cancer Institution Review Board. Primary cells were thawed and death cells were removed using dead cell removal kit (Miltenyi Biotec) according to manufacturer's instructions. The cells were allowed to recover overnight (16–18 hours) after which drug treatment studies were carried out. Primary cells were cultured in StemSpan SFEM (STEMCELL Technologies) supplemented with StemSpan CC100 cytokine cocktail (STEMCELL Technologies) and 20% FBS.

Compounds

Selinexor was obtained from Karyopharm Therapeutics. Idarubicin, mitoxantrone, and etoposide were purchased from Selleckchem. Daunorubicin was purchased from Sigma.

TaqMan gene assays and antibodies

All the real-time PCR TaqMan gene assays were purchased from Life Technologies (MSH2: Hs00953523_m1; MLH1: Hs00179866_m1; MSH6: Hs00264721_m1; PMS2: HS00241053_m1; Rad51: Hs00153418_m1; Chk1: Hs00967506_m1). The antibodies to caspase-3 (#9662), Rad51 (#8875), MLH1 (#3515), Chk1 (#2360), γH2AX (#9718), MSH2 (#2850), and topoisomerase IIα (D10G9; #12286) were purchased from Cell Signaling Technology; PMS2 (#2251.00.02) and MSH6 (#2203.00.02) were purchased from Sdix; α-tubulin was from Abcam (ab15246); Lamin A/C was from Cell Signaling Technology (#2032); and β-actin (#sc-81178) was purchased from Santa Cruz Biotechnology. The secondary antibodies for Western blotting were purchased from LI-COR and for immunofluorescence were purchased from Invitrogen (#A11008).

Real-time quantitative reverse transcription PCR

Cells were treated with the indicated selinexor concentrations and cells were collected at different time points. RNA was extracted from cells using RNeasy Kit (#74106, Qiagen) and reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (#4368813, Applied Biosystems). mRNA for the indicated genes was quantified using ViiA7 Real-Time PCR system and analyzed by the V1.2 software (Life Technologies). TRIzol/chloroform extraction step was performed for the primary AML samples prior to the actual RNA extraction step.

Immunofluorescence

Cells were exposed to the indicated treatment regimen. About 100 to 200 μL of the cell suspension from each treatment condition was loaded onto a cytospin cuvette with a coverslip and was spun at 800 rpm for 5 minutes using a Cytospin. The adhered cells were fixed using ice-cold 100% methanol for 15 minutes, washed with 1× PBS, and then blocked/permeabilized using a solution containing 0.1% Tween-20, 0.3 mol/L glycine, 1% BSA in 1× PBS. The cells were incubated with the primary antibody overnight at 4°C. The cells were washed 3 times with 1× PBS and incubated with 1:2,000 of the secondary antibody for 1 hour. The cells were washed with 1× PBS, treated with 1:1,000 1 μg/mL DAPI for 5 minutes and then mounted to a glass slide using Vectashield mounting medium (# H-1400, Vector Laboratories).

Western blotting

Cells were washed with 1× PBS and then lysed with RIPA buffer (#89901, Thermo Scientific) supplemented with protease inhibitor (# 05892791001, Roche) and phosphatase inhibitor (# 04906837001, Roche). The nuclear and cytoplasmic fractions were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (#78833, Thermo Scientific) according to manufacturer's protocol. The protein level of each sample was quantified and normalized using BCA assay (#23225, Thermo Scientific). Twenty micrograms of each sample was run in 4% to 12% Bis–Tris Gel (Life Technologies) and later transferred to nitrocellulose membrane using iBlot Gel Transfer Kit (Life Technologies). The membranes were blocked using LI-COR blocking buffer

(#927-40000, LI-COR), probed with the indicated antibodies, and analyzed using LI-COR Odyssey.

WST-1 assay and calculation of combination index

Cells were seeded into 96-well plates (50,000 cells per well) and treated for 48 hours with individual drug selinexor, idarubicin, daunorubicin, mitoxantrone, etoposide, or the combination of selinexor with one other individual Topo II inhibitor drug. Cell viability was evaluated using the cell proliferation reagent WST-1 (Roche) according to manufacturer's protocol. The absorbance of wells at 450 nm (reference wavelength, 650 nm) was measured with a microplate reader (SoftMax Pro, Molecular Devices). The doses for each drug were chosen according to their individual IC_{50} (2-fold dilutions) that was determined previously by WST-1 assay. For sequential treatments, the second drug was added 24 hours after the first drug treatment without washing. Plates were read 48 hours after second drug was added. The effects of the combinations were calculated using CalcuSyn software, where $CI < 1$ indicates synergy, $CI = 1$ is additive, and $CI > 1$ is antagonistic.

Homologous recombination assay

Homologous recombination (HR) was assessed using a direct repeat GFP (DR-GFP) assay essentially as previously described (19). The HeLA-DR cells possess an integrated DR-GFP construct, whose expression is prevented by an insert with the I-*Sce*I restriction site in the reading frame, whereby transfection of I-*Sce*I endonuclease creates a DSB, which when repaired by error-free HR leads to GFP-expressing cells (19). Briefly, HeLA-DR cells were transiently transfected with either the negative control phCMV-1 I-*Sce*I, the functional endonuclease pCMV3xnlsI-*Sce*I, or pGFP (as control for transfection efficiency) and treated with DMSO (control) or selinexor at indicated concentrations. Cells were trypsinized 72 hours after transfection and assessed for GFP expression with FACS Calibur flow cytometer and CellQuest Pro software. The percentage of GFP-positive cells in 50,000 to 100,000 events was normalized to the negative control and corrected for transfection efficiency. There were no significant differences in transfection efficiencies between treatments.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed according to the protocol of the EpiTect Kit (Qiagen). MV4-11 cells (about 5×10^6) were treated with DMSO or selinexor for 24 hours and then fixed with 1% formaldehyde. The cross-linked DNA complexes were sheared to 500 to 1,000 base pair fragments and immunoprecipitated with either c-Myc (Santa Cruz Biotechnology) or IgG control antibody. The immunoprecipitated DNA was then purified and amplified by qPCR using SYBR green. Primers sequences are as follows: RAD51 (CACGTTGGCCAGGTTTATCT, GGGGGCATTGAATTTCATAA) and CHK1 (GATCCATACGCCTCAGCTTC, AGGCC AAGCAGAACAATCG).

Mice

Female nonobese diabetic SCID γ (NSG) mice that lack mature T cells, B cells, or functional natural killer (NK) cells and are deficient in cytokine signaling were purchased from Jackson ImmunoResearch Laboratories. All mice used in the experiments were between 6 and 10 weeks of age. All animal studies were conducted in accordance to the rules and regulations of the Institutional Animal Care and Use Committee at the Ohio State University (Columbus, OH).

MV4-11 xenograft mouse model

Spleen cells (5×10^4) from MV4-11 transplanted NSG mice were intravenously injected into NSG mice via tail vein. One week after leukemia cell injection, the mice were given either vehicle control or idarubicin alone (1.5 mg/kg, i.v., on days 7, 8. and 9 only), selinexor alone (10 mg/kg, oral gavage, twice a week, on days 7, 10, 14, 17, 21, 24, 28), or idarubicin (1.5 mg/kg, i.v., on days 7, 8, and 9 only) and selinexor (10 mg/kg, oral gavage, twice a week, on days 7, 10, 14, 17, 21, 24, 28). Mice were monitored closely for clinical signs of leukemia such as weight loss and hind limb paralysis. Expected median survival for untreated animals in this model is 30 days. Blood was drawn for blood counts analysis that allowed for confirmation of leukemia. All animal studies were conducted in accordance to the rules and regulations of the Institutional Animal Care and Use Committee at the Ohio State University.

Statistical analysis

Survival data were analyzed using Kaplan–Meier and log-rank test methods (GraphPad Prism). Differences between continuous variables were analyzed using the Student t tests. $P < 0.05$ was considered significant.

Results

Combination of selinexor and Topo II inhibitors results in synergistic inhibition of proliferation and induction of apoptosis in AML cells *in vitro*

To evaluate the combinatorial effect of selinexor and Topo II inhibitors (idarubicin, daunorubicin) on cell proliferation and apoptosis, 2 AML cell lines (MV4-11 and MOLM-13) were treated concomitantly with both drugs at fixed ratios of their respective IC_{50} values (2-fold dilutions, Supplementary Table S1) for 48 hours. Cell proliferation was measured by WST-1 assay, and the combination index (CI) was calculated according to the Chou–Talalay method (20). As shown in Fig. 1A and Supplementary Table S2, synergism was observed in both MV4-11 and MOLM-13 cell lines ($CI < 1$). The synergistic effect of selinexor and Topo II inhibitors (idarubicin, daunorubicin) was also validated in five primary refractory/relapsed or newly diagnosed AML blast samples (Fig. 1B and Supplementary Table S3). In addition, we tested the combinatorial effect of selinexor with 2 other Topo II inhibitors (etoposide, mitoxantrone) in MV4-11 and MOLM-13 cell lines and observed similar synergistic effects (Fig. 1C and Supplementary Table S4). Induction of apoptosis was measured by Annexin V and PI staining of MV4-11 and MOLM-13 cell lines 48 hours after drug treatment showing increased apoptosis in the combinatorial treatment versus either drug alone (Fig. 1D and Supplementary Fig. S1A–S1C). To evaluate whether the order of drug treatment affected synergy, we performed WST-1 assays in AML cell lines MV4-11 and MOLM-13 testing both concomitant and sequential treatment of daunorubicin with selinexor. The cells were treated with the individual drugs alone, combination of drugs either concomitantly or sequentially. In all the combinations tested, the concomitant treatment resulted in lower CI values (indicating better synergy) compared with sequential treatment (Supplementary Fig. S2A and S2B).

Selinexor restores nuclear localization of Topo IIα

Topo IIα is a nuclear enzyme involved in relieving the torsional stress created during DNA replication and transcription (21, 22).

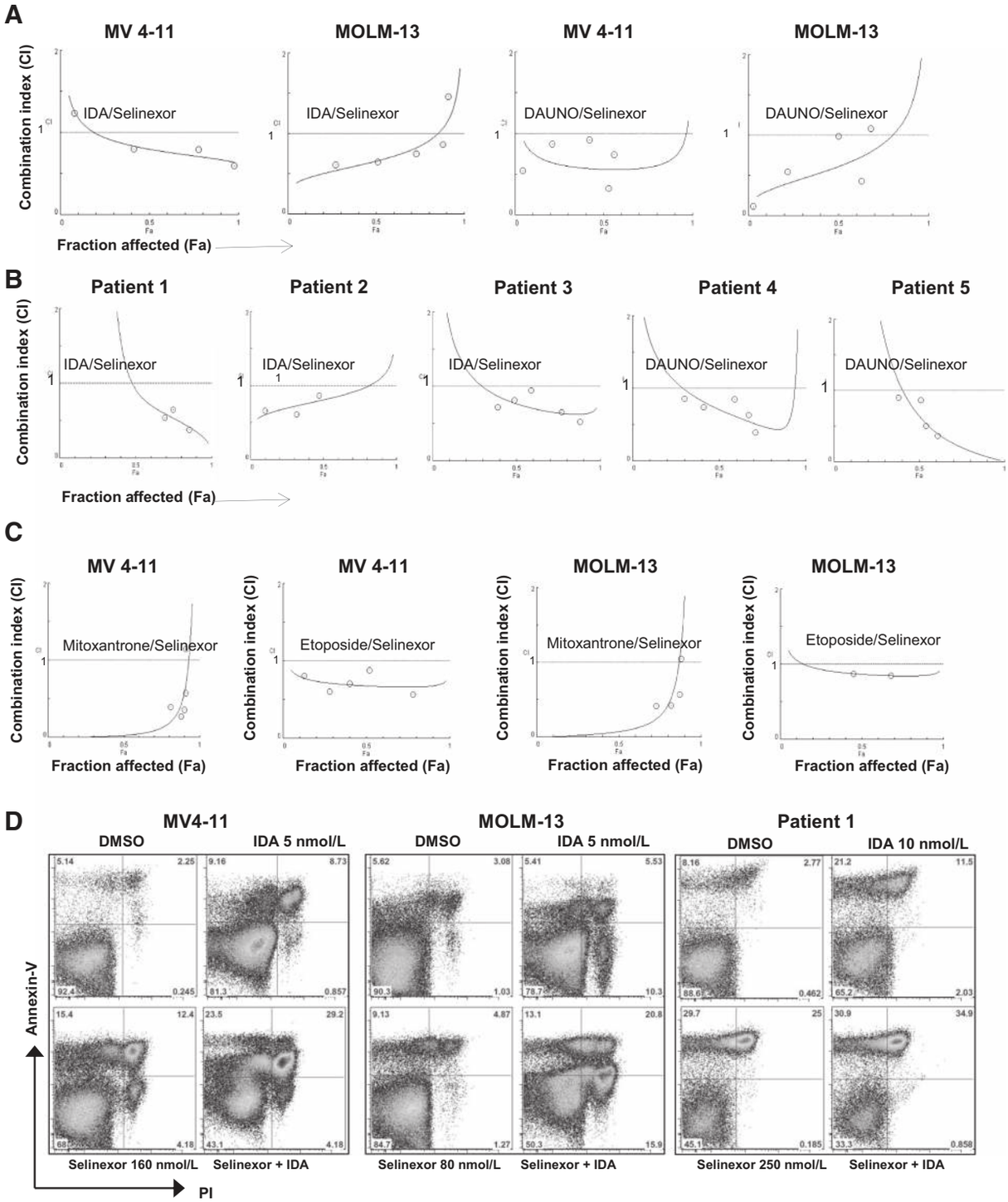


Figure 1.

Combination of selinexor and idarubicin or Topo IIα inhibitors results in synergistic inhibition of proliferation and induction of apoptosis in AML cells *in vitro*. **A,** CI plots of selinexor with idarubicin (IDA) and daunorubicin (DAUNO) concomitant treatment in AML cell lines MV4-11 and MOLM-13 and patient blasts (**B**). The effect of the combinations was assessed by WST-1 assay after 48 hours of concomitant drug treatment. The doses for both drugs were chosen according to their individual IC_{50} (2-fold dilutions) that were determined by using WST-1 assay (Supplementary Table S1). **C,** CI plots of selinexor with Topo IIα inhibitors, etoposide and mitoxantrone, in MV4-11 and MOLM-13 AML cell lines. The effects of the combinations were calculated using CalcuSyn software, where CI < 1 indicates synergy, CI = 1 is additive, and CI > 1 is antagonistic. The results of the WST-1 assays are representative of at least 2 independent experiments performed in quadruplicate. **D,** Apoptosis in MV4-11, MOLM-13, and AML primary patient blast was measured by Annexin-V/PI staining 48 hours after drug treatment at indicated concentrations.

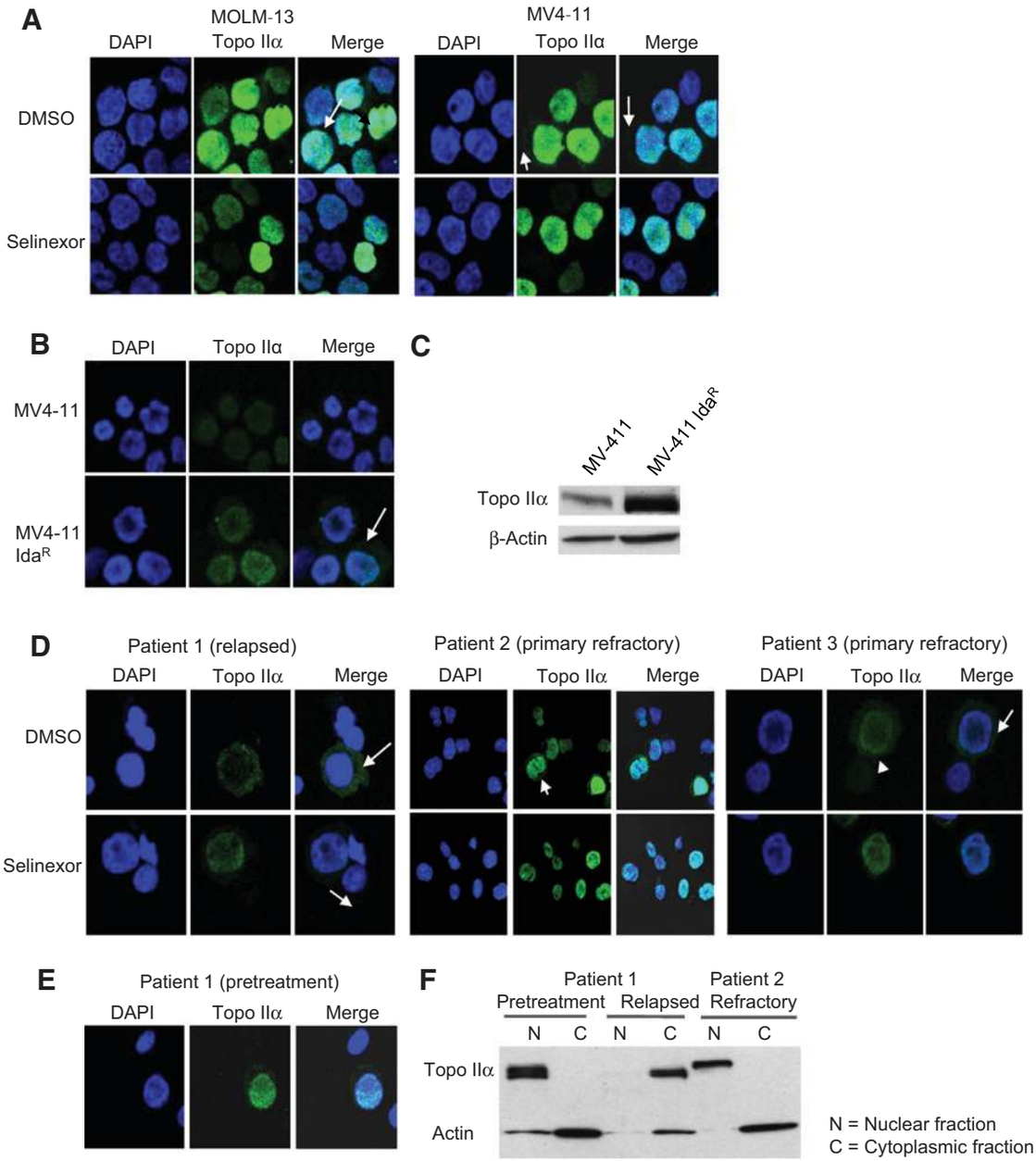


Figure 2.

Selinexor restores nuclear localization of Topo IIα **A,** Topo IIα cellular localization assessed by confocal microscopy in MV4-11 and MOLM-13 cells after treatment with DMSO (control) or selinexor for 24 hours. At least 500 cells were counted and one representative experiment of 3 is shown. Arrows pointing to cytoplasmic distribution of Topo IIα (**B**). Topo IIα cellular localization and protein expression (**C**) measured by confocal microscopy and Western blotting of whole-cell lysate in MV4-11 cells resistant to idarubicin (MV4-11 R). **D,** Confocal microscopy of Topo IIα in 2 primary refractory and 1 relapsed AML patient samples after treatment with DMSO (control) or selinexor for 24 hours and in a pretreatment sample from patient 1 (**E**). Left, DAPI staining (cell nucleus). Middle, Topo IIα staining; right, merged image of DAPI and Topo IIα staining. **F,** Topo IIα expression measured by Western blotting in pretreatment and relapsed AML samples from patient 1 and from primary refractory AML samples (patient 2).

Topo IIα shuttles between the nucleus and the cytoplasm, with the equilibrium tending toward nuclear localization. The nuclear export of Topo IIα is XPO1-dependent through the interaction with 2 functional leucine-rich nuclear export signal sequences (NES; refs. 16–18, 21, 23, 24). Aberrant nuclear export and cytoplasmic localization of Topo IIα has been identified as one of the mechanisms that lead to drug resistance in cancers such as multiple myeloma (1, 25–27). When Topo IIα is exported to the cytoplasm, it is not in contact with DNA, and Topo II inhibitors such as anthracyclines are unable to induce DNA cleavage

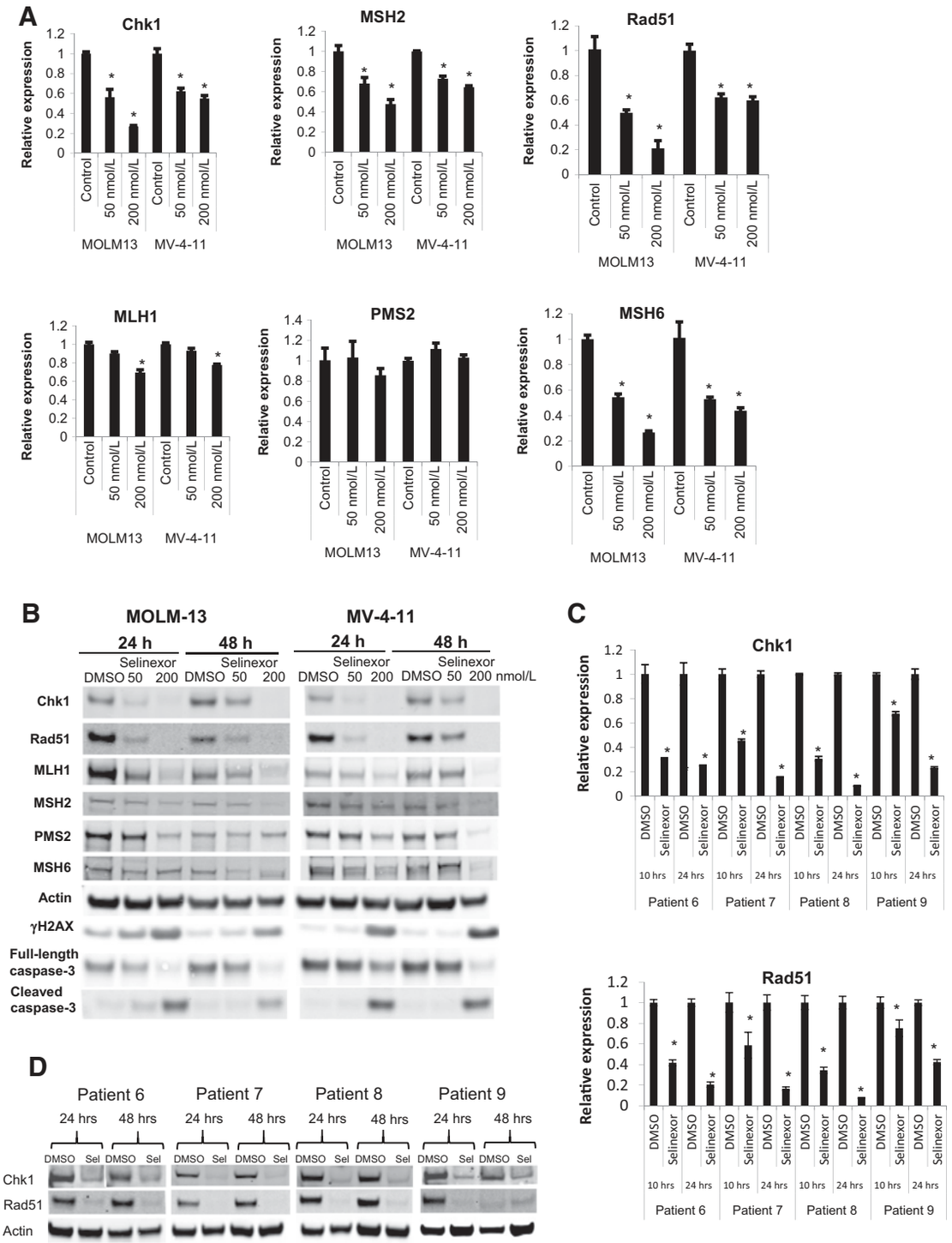
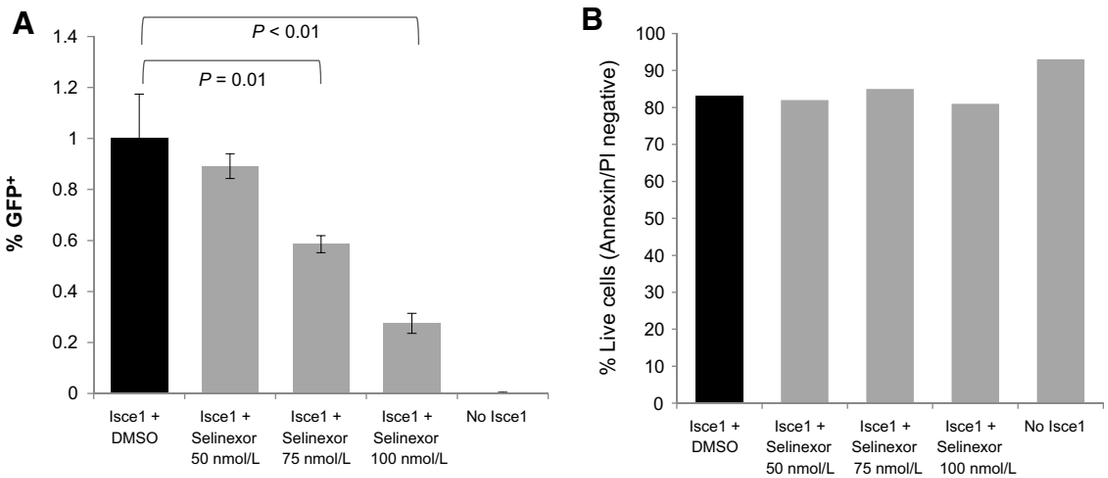


Figure 3.

Selinexor reduces expression of DNA damage repair genes. **A,** Expression levels of *Chk1, MSH2, Rad51, MLH1, PMS2*, and *MSH6* were measured by quantitative PCR from total mRNA extracted from AML cell lines 6 hours after selinexor treatment. The average relative expression and SD of 2 independent experiments is shown. Selinexor-treated versus untreated. *, $P < 0.05$. **B,** Immunoblots of whole proteins from MOLM-13 and MV4-11 cell lines after treatment with DMSO or selinexor at the indicated doses and time points. Increased γH2AX concurrently with increased caspase-3 cleavage are apoptosis indicators. One representative experiment of two is shown. Total mRNA (**C**) and protein expression (**D**) of Chk1 and Rad51 measured by real-time PCR and Western Blotting after DMSO or selinexor treatment in primary AML blasts. Quantification of RNA expression was done by quantitative PCR from whole RNA patient samples treated with selinexor for 10 and 24 hours, and protein expression was analyzed by immunoblots of whole protein extracts treated with selinexor for 24 and 48 hours. Selinexor-treated versus untreated. *, $P < 0.05$.



C

2 Hours of 10 nmol/L Idarubicin	–	+ Visualize 2hrs	+ Washout	+ Washout
48 Hours of 100 nmol/L selinexor	–	–	–	+
48 Hours of recovery	–	–	+	–

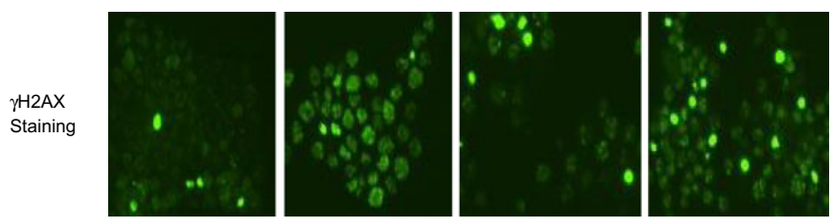


Figure 4.

Selinexor blocks HR after DNA damage and prevents recovery from DNA damage caused by idarubicin treatment. **A,** Percentage of GFP positivity in HeLa DR cells after ISCE1 cleavage and DMSO or selinexor treatment. HeLa DR cells carry 2 copies of inactive GFP genes integrated into the genome. The cells were treated with the ICSE1 enzyme that cuts within the specific DNA sequence of the GFP gene. If HR occurs, there is repair of the DSBs and GFP fluorescence is observed. **B,** Percentage of viable cells after DMSO and selinexor treatment showing no difference, evidence that lack of GFP repair was due to inhibition of HR and not toxicity or cell death from drug treatment. **C,** Immunofluorescent staining of γH2AX, a marker of DNA damage in MV-4-11 cells, treated with 10 nmol/L idarubicin for 2 hours. Idarubicin was washed out and cells were either allowed to recover or treated with 100 nmol/L selinexor for 48 hours.

complexes and cell death (1, 25–27). Using confocal immunofluorescence, we confirmed that Topo IIα is predominantly expressed in the nucleus of the AML cell lines MV4-11 and MOLM-13 (Fig. 2A). However, some degree of Topo IIα expression could also be observed in the cytoplasm. Interestingly, cytoplasmic Topo IIα expression increases in MV4-11 cells resistant to idarubicin (MV4-11 Ida^R; Fig. 2B and C). We also determined Topo IIα expression in 3 primary refractory or relapsed AML patient samples using confocal microscopy and found variable cytoplasmic Topo IIα expression (Fig. 2D). Treatment of AML cell lines or patient AML samples with selinexor restores Topo IIα exclusively to the nucleus, supporting that nuclear export of Topo IIα is mediated via XPO1 in AML (Fig. 2A and D and Supplementary Fig. S3A–S3D). Restoration of Topo IIα to the nucleus was associated with strong synergism with idarubicin in AML patient samples (Fig. 1B). In our study, pretreatment and relapsed samples for one patient (Patient 1) were available. While in the pretreatment sample, Topo IIα was exclusively nuclear (Fig. 2E and F); in the relapse sample, Topo IIα was mostly localized in the cytoplasm (Fig. 2D and F). Interestingly, this patient initially received anthracycline-based induction therapy and achieved complete remission. However, when the AML relapsed 9 months later, his leukemia was refractory to an anthracycline-based intensive induction regimen. Interestingly, treatment with selinexor of this patient's blasts *in vitro* restored Topo IIα nuclear localization and strongly synergized with idarubicin (Figs. 2D and 1B).

Selinexor reduces expression of DNA damage repair genes

In addition to Topo IIα nuclear restoration, we hypothesized that other mechanisms may explain the synergistic effect of selinexor and idarubicin. High-throughput studies on protein expression in tumor cells after selinexor treatment indicated that several DNA damage repair proteins are downregulated (28). We confirmed this in AML by showing that selinexor treatment of AML cell lines MV4-11 and MOLM-13 resulted in significant downregulation of DNA damage repair proteins. These include the DNA damage response protein Chk1 (29, 30) and DNA damage repair protein Rad51 (31–34) that assist in double-strand

DNA repair by HR as well as MSH2, MLH1, PMS2, and MSH6 that assist in mismatch repair. Selinexor inhibited the expression of DNA damage repair genes *MSH2*, *MLH1*, and *MSH6* but not *PMS2* at the mRNA (Fig. 3A) and downregulated the protein levels of all genes MSH2, MLH1, MSH6, and PMS2 (Fig. 3B) before apoptosis was observed. Of note is that the depletion of DNA damage repair proteins did not induce DNA damage in itself, as increased Ser 139-phosphorylated H2AX histone (γH2AX), a marker of DNA damage, is only seen concurrently with increased caspase-3 cleavage, indicating the initiation of cell death by apoptosis. Similar reduction of Rad51 and Chk1 mRNA and protein level was observed in 4 primary AML patient samples (Fig. 3C and D). On the basis of these data, we reasoned that the synergistic effects of selinexor with Topo II inhibitors could be explained, in part, by selinexor induced downregulation of DNA repair proteins thus preventing leukemia cells from repairing chemotherapy-induced DNA damage.

Selinexor blocks HR after DNA damage and blocks DNA damage repair caused by idarubicin treatment

Topo II inhibitors induce DNA double-strand breaks (DSB) that can be repaired by the HR pathway (35, 36). Rad51 recombinase plays a central role in governing HR (31–34). To measure the ability of cells to carry out HR in the presence of selinexor, we used the HeLa DR cells that express 2 copies of inactive GFP genes integrated into their genome. The cells were treated with the ICSE1 enzyme that cuts DNA in a specific site within the GFP gene. If HR occurs, then it repairs the DSB and GFP fluorescence is observed (19). Using this assay, when HeLa DR cells were treated with selinexor, we observed a significant dose-dependent decrease in GFP expression compared with control cells (Fig. 4A) before any significant apoptosis was detected (Fig. 4B). To assess whether selinexor treatment of AML cells prevents recovery from DNA damage caused by idarubicin treatment, MV4-11 cells were treated for 2 hours with 10 nmol/L idarubicin followed by washing out of idarubicin. The cells were then either allowed to recover or were treated with 100 nmol/L of selinexor for 48 hours. The cells were fixed with methanol and stained for γH2AX (37, 38). Two-hour treatment of idarubicin induced DNA damage confirmed by increased staining of γH2AX (Fig. 4C). The staining faded when the cells were allowed to repair their DNA damage. However, incubation of these cells with selinexor after idarubicin washout maintained γH2AX staining, suggesting that the cells did not recover from the idarubicin-induced DNA damage. Incubation with selinexor at doses lower than 200 nmol/L did not induce H2AX phosphorylation (Fig. 3B).

Selinexor downregulates c-Myc expression and binding to DNA damage repair gene promoters in AML

Next, we investigate the possible mechanisms by which selinexor may regulate Rad51 and Chk1. It has been reported that c-Myc is a positive regulator of Rad51 and Chk1 (39, 40). We also have shown previously that c-Myc is a target of selinexor in multiple cancers including AML, multiple myeloma, and hepatocellular carcinoma (41–43). Thus, we reasoned that selinexor may downregulate Rad51 and Chk1 by targeting c-Myc. Here, we show that selinexor treatment reduces c-Myc protein level in AML cell lines (Fig. 5A). Furthermore, using ChIP assay, we demonstrated that binding of c-Myc to Rad51 and Chk1 promoters is also significantly decreased by selinexor treatment in MV4-11 cells (Fig. 5B).

Idarubicin enhances selinexor antileukemic activity *in vivo*

Last, we tested the efficacy of the selinexor–idarubicin combination *in vivo* using an established xenograft mouse model of AML. MV4-11 cells obtained from spleens of primary MV4-11 xenografts were transplanted into nonobese diabetic-SCID (NOD-SCID) gamma (NSG) mice via tail vein. Mice were monitored closely for clinical signs of leukemia as described in methods. One week after leukemia cell injection, the mice were given either vehicle control or idarubicin alone (1.5 mg/kg, i.v., on days 7, 8, and 9 only), selinexor alone (10 mg/kg, oral gavage, twice a week on days 7, 10, 14, 17, 21, 24, 28), or idarubicin (1.5 mg/kg, i.v., on days 7, 8, and 9 only) and selinexor (10 mg/kg, oral gavage, twice a week on days 7, 10, 14, 17, 21, 24, 28). All treatments were given for 3 weeks after leukemia cell injection. Single-agent selinexor at the low dose of 10 mg/kg had no effect in prolonging the survival of mice with respect to the control mice

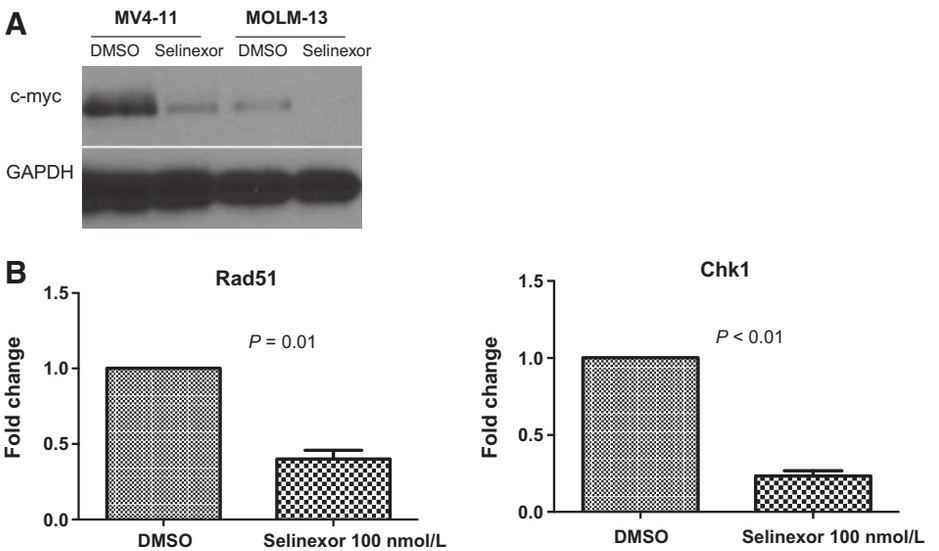


Figure 5.
Selinexor downregulates c-Myc expression and binding to DNA damage repair gene promoters in AML. **A,** c-Myc protein expression in AML cell lines MV4-11 and MOLM-13 treated with selinexor for 24 hours. One representative Western blot analysis of 3 experiments is shown. **B,** ChIP assays of c-Myc on the Rad51 and Chk1 promoter regions in MV4-11 cells after treatment with DMSO or selinexor for 24 hours.

(vehicle control), corroborating our previous reports (44). Idarubicin at 1.5 mg/kg increases median survival of mice slightly. In contrast, the combination treatment of idarubicin and selinexor significantly increased mice survival compared with selinexor alone (31 vs. 38 days, $P < 0.001$; Fig. 6A) and compared with idarubicin alone (33 vs. 38 days, $P < 0.001$; Fig. 6A). On day 25, separate cohorts of vehicle and drug-treated mice were sacrificed and blood drawn for white blood cell counts (WBC) comparison. The combination therapy of idarubicin with selinexor resulted in the lowest blast counts ($P < 0.01$, Fig. 6B). In addition, leukemic spleens were harvested and weighed. The idarubicin–selinexor combination–treated mice exhibited smaller and lighter spleens than the other groups and controls ($P = 0.01$, Fig. 6C). It should be noted that the dose of selinexor (10 mg/kg) used for this study has been shown to be ineffective when used as a single agent (44). However, the combination treatment of idarubicin with selinexor at low dose enhanced the antileukemic activity of selinexor. This is relevant to patients because it is now possible to use lower doses of selinexor to increase tolerability without compromising the antileukemic activity.

Discussion

We show that concomitant treatment with selinexor and Topo II inhibitors (idarubicin, mitoxantrone, and etoposide) resulted in therapeutic synergy in AML cell lines and patient AML samples. Using a xenograft AML mouse model, we show that *in vivo* treatment of leukemic mice with selinexor and idarubicin significantly prolongs survival of these mice and reduces leukemic burden as compared to each single therapy alone.

Topo IIα is an important enzyme involved in DNA replication, and chemotherapeutic agents inhibiting Topo II such as idarubicin, daunorubicin, mitoxantrone, and etoposide are used to treat a wide variety of hematologic malignancies including AML (3, 14, 15, 21, 45, 46). In normal cells, Topo IIα is constantly shuttling between the nucleus and the cytoplasm via the 3 nuclear

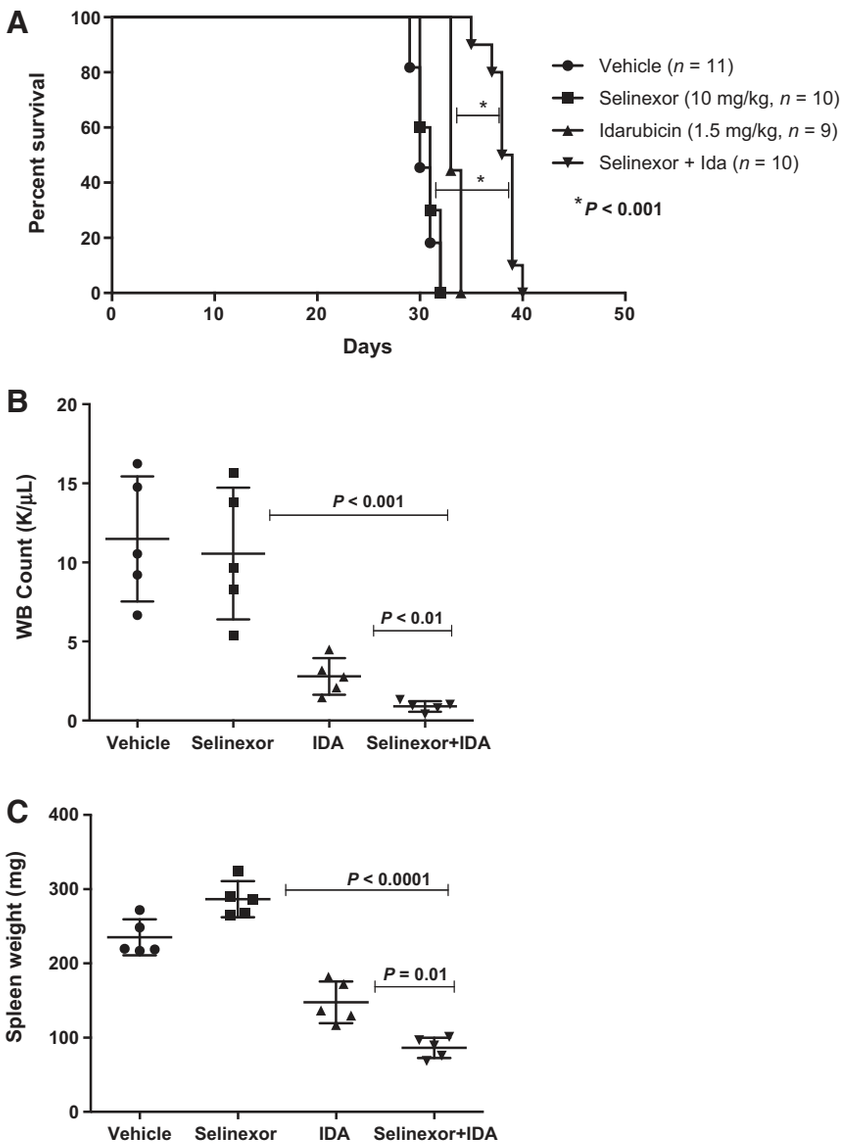


Figure 6.
Idarubicin enhances selinexor antileukemic activity *in vivo*. **A,** Survival curve of NSG injected with MV4-11 xenografts and treated with indicated drugs. Survival comparison was made with log-rank test. **B,** WBC count on day 25 ($n = 5$ per group). *P* values obtained using *t* test. **C,** Spleen weights (mg) on day 25 ($n = 5$ per group). *P* values obtained using *t* test.

localization signals (NLS) at the COOH end responsible for nuclear import and 2 leucine-rich NES that mediate XPO1 dependent nuclear export, with the equilibrium tending toward nuclear localization (16–18, 23, 24). It has been reported that aberrant cytoplasmic localization of Topo IIα results in resistance to Topo II inhibitors due the loss of contact of Topo IIα with DNA (1, 2, 27). In our study, we report the Topo IIα localization is mostly nuclear; however, there are patients with AML where Topo IIα is found in the cytoplasm as well. In particular, we reported a patient with AML where Topo IIα was found exclusively in the nucleus in the pretreatment AML blasts. However, in the bone marrow sample obtained when the leukemia relapsed, Topo IIα was found mostly localized in the cytoplasm. Remarkably, treatment of this patient's blast *in vitro* with selinexor restored Topo IIα nuclear localization and strongly synergized with idarubicin. Likewise, we show that treatment of AML cell lines and patient blasts with selinexor restores nuclear expression of Topo IIα, sensitizing the cells to idarubicin therapy. It has been reported in multiple myeloma that blocking Topo IIα nuclear export using XPO1 inhibitors increase sensitivity of myeloma cells to anthracyclines (26, 27). Thus, our data are consistent with similar data reported in other malignancies and provide a rationale to treat patients who express cytoplasmic Topo IIα with selinexor to sensitize them to anthracycline therapy.

In addition, we identified that selinexor treatment of AML cells results in significant reduction in the transcription and translation of the DNA damage repair genes, among them Rad51 and Chk1, and subsequent inhibition of HR repair. We reasoned that this could be another mechanism to explain the synergism observed between selinexor and idarubicin. The anthracycline idarubicin is a Topo II that induces DNA DSBs, which are highly toxic to the cell. AML blasts can evade cell death following Topo II inhibitors by repairing the DSBs induced by chemotherapy through many mechanisms, including upregulation of DNA repair proteins such as Rad51 and Chk1. By downregulating Rad51 and Chk1, selinexor prevents blasts recovery from idarubicin-induced DNA damage. Our results also support previous research that show that suppression of DDR genes *BRCA1*, *CHK1*, and *RAD51* by other drugs such as histone deacetylase inhibitors (47) or inhibition of Chk1 by a selective inhibitor (48) sensitizes AML cells to chemotherapy. Targeting DNA repair mechanisms in cancer cells is currently being developed for many cancers with the goal to increase chemotherapy sensitivity.

We further show that selinexor effects on Rad51 and Chk1 regulation are likely due to the targeting of c-Myc by the drug. It has been reported that c-Myc, one of the major oncogenes that is upregulated in AML through multiple pathways, is binding to Rad51 and Chk1 promoters and positively regulate their expression (40, 49, 50). Selinexor treatment of AML cell lines significantly decreases c-myc protein levels and consequently reduced its association with the promoter of Rad 51 and Chk1.

In summary, here we report the synergistic activity of the XPO1 selective inhibitor, selinexor with Topo II inhibitors in AML cells, primary AML blasts and in a murine AML xenograft model. The preclinical data reported here support further clinical studies using selinexor and Topo II inhibitors in combination to treat AML. On the basis of our results, there are currently 2 clinical trials enrolling primary refractory and relapsed patients with AML to selinexor in combination with anthracyclines; selinexor plus standard cytarabine and idarubicin (7+3; NCT02249091), and selinexor plus mitoxantrone, etoposide and cytarabine (MEC; NCT02299518).

Disclosure of Potential Conflicts of Interest

M. Kauffman is an employee of and is a consultant/advisory board member for Metamark and Verastem. No potential conflicts of interest were disclosed by the other authors.

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References

1. Mutka SC, Yang WQ, Dong SD, Ward SL, Craig DA, Timmermans PB, et al. Identification of nuclear export inhibitors with potent anticancer activity *in vivo*. Cancer Res 2009;69:510–7.
2. Turner JG, Dawson J, Sullivan DM. Nuclear export of proteins and drug resistance in cancer. Biochem Pharmacol 2012;83:1021–32.
3. Fukuda M, Asano S, Nakamura T, Adachi M, Yoshida M, Yanagida M, et al. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. Nature 1997;390:308–11.
4. Kanai M, Hanashiro K, Kim SH, Hanai S, Boulares AH, Miwa M, et al. Inhibition of Crm1-p53 interaction and nuclear export of p53 by poly (ADP-ribosyl)ation. Nat Cell Biol 2007;9:1175–83.
5. Shen A, Wang Y, Zhao Y, Zou L, Sun L, Cheng C. Expression of CRM1 in human gliomas and its significance in p27 expression and clinical prognosis. Neurosurgery 2009;65:153–9; discussion 159–60.
6. van der Watt PJ, Maske CP, Hendricks DT, Parker MI, Denny L, Govender D, et al. The Karyopherin proteins, Crm1 and Karyopherin beta1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. Int J Cancer 2009;124:1829–40.
7. Kojima K, Kornblau SM, Ruvolo V, Dilip A, Duvvuri S, Davis RE, et al. Prognostic impact and targeting of CRM1 in acute myeloid leukemia. Blood 2013;121:4166–74.

8. Etchin J, Sun Q, Kentsis A, Farmer A, Zhang ZC, Sanda T, et al. Antileukemic activity of nuclear export inhibitors that spare normal hematopoietic cells. Leukemia 2013;27:66–74.
9. Ranganathan P, Yu X, Na C, Santhanam R, Shacham S, Kauffman M, et al. Preclinical activity of a novel CRM1 inhibitor in acute myeloid leukemia. Blood 2012;120:1765–73.
10. Garzon R, Flinn I, Berdeja J, Yee KWL, Savona M, Kauffman M, et al. A phase 1 dose escalation study of the oral selective inhibitor of nuclear export (SINE) selinexor (KPT-330) in patients (pts) with relapsed/refractory acute myeloid leukemia (AML) [abstract]. Haematologica 2014;99:Abstract S1353.
11. Dohner K, Dohner H. Molecular characterization of acute myeloid leukemia. Haematologica 2008;93:976–82.
12. Mrozek K. Cytogenetic, molecular genetic, and clinical characteristics of acute myeloid leukemia with a complex karyotype. Semin Oncol 2008;35:365–77.
13. Estey E, Dohner H. Acute myeloid leukaemia. Lancet 2006;368:1894–907.
14. Kuendgen A, Germing U. Emerging treatment strategies for acute myeloid leukemia (AML) in the elderly. Cancer Treat Rev 2009;35:97–120.
15. Robak T, Wierzbowska A. Current and emerging therapies for acute myeloid leukemia. Clin Ther 2009;31:2349–70.
16. Mirski SE, Cole SP. Cytoplasmic localization of a mutant M(r) 160,000 topoisomerase II alpha is associated with the loss of putative bipartite nuclear localization signals in a drug-resistant human lung cancer cell line. Cancer Res 1995;55:2129–34.
17. Mirski SE, Sparks KE, Friedrich B, Kohler M, Mo YY, Beck WT, et al. Topoisomerase II binds importin alpha isoforms and exportin/CRM1 but does not shuttle between the nucleus and cytoplasm in proliferating cells. Exp Cell Res 2007;313:627–37.
18. Turner JG, Engel R, Derderian JA, Jove R, Sullivan DM. Human topoisomerase IIalpha nuclear export is mediated by two CRM-1-dependent nuclear export signals. J Cell Sci 2004;117:3061–71.
19. Cuozzo C, Porcellini A, Angrisano T, Morano A, Lee B, Di Pardo A, et al. DNA damage, homology-directed repair, and DNA methylation. PLoS Genet 2007;3:e110.
20. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 2010;70:440–6.
21. Pendleton M, Lindsey RHJr, Felix CA, Grimwade D, Osheroff N. Topoisomerase II and leukemia. Ann N Y Acad Sci 2014;1310:98–110.
22. Champoux JJ. DNA topoisomerases: structure, function, and mechanism. Annu Rev Biochem 2001;70:369–413.
23. Mirski SE, Gerlach JH, Cole SP. Sequence determinants of nuclear localization in the alpha and beta isoforms of human topoisomerase II. Exp Cell Res 1999;251:329–39.
24. Mirski SE, Gerlach JH, Cummings HJ, Zirngibl R, Greer PA, Cole SP. Bipartite nuclear localization signals in the C terminus of human topoisomerase II alpha. Exp Cell Res 1997;237:452–5.
25. Gravina GL, Senapedis W, McCauley D, Baloglu E, Shacham S, Festuccia C. Nucleo-cytoplasmic transport as a therapeutic target of cancer. J Hematol Oncol 2014;7:85.
26. Turner JG, Dawson J, Emmons MF, Cubitt CL, Kauffman M, Shacham S, et al. CRM1 inhibition sensitizes drug resistant human myeloma cells to topoisomerase II and proteasome inhibitors both *in vitro* and *ex vivo*. J Cancer 2013;4:614–25.
27. Turner JG, Marchion DC, Dawson JL, Emmons MF, Hazlehurst LA, Washausen P, et al. Human multiple myeloma cells are sensitized to topoisomerase II inhibitors by CRM1 inhibition. Cancer Res 2009; 69:6899–905.
28. Kashyap T, Crochiere ML, Friedlander S, Klebanov B, Senapedis W, Baloglu E, et al. Selective inhibitors of nuclear export (SINETM) block the expression of DNA damage repair proteins and sensitize cancer cells to DNA damage inducing therapeutic agents. In: Proceedings of the 26th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics; 2014 Nov 18–21.
29. Karran P. DNA double strand break repair in mammalian cells. Curr Opin Genet Dev 2000;10:144–50.
30. Sorensen CS, Hansen LT, Dziegielewski J, Syljuasen RG, Lundin C, Bartek J, et al. The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. Nat Cell Biol 2005; 7:195–201.
31. Baumann P, Benson FE, West SC. Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions *in vitro*. Cell 1996;87:757–66.
32. Baumann P, West SC. Role of the human RAD51 protein in homologous recombination and double-stranded-break repair. Trends Biochem Sci 1998;23:247–51.
33. Benson FE, Baumann P, West SC. Synergistic actions of Rad51 and Rad52 in recombination and DNA repair. Nature 1998;391:401–4.
34. Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, Ogawa H, et al. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J 1998;17:598–608.
35. Chapman JR, Taylor MR, Boulton SJ. Playing the end game: DNA double-strand break repair pathway choice. Mol Cell 2012;47:497–510.
36. Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double-strand break repair pathway choice. Cell Res 2008;18:134–47.
37. Mah LJ, El-Osta A, Karagiannis TC. gammaH2AX: a sensitive molecular marker of DNA damage and repair. Leukemia 2010;24:679–86.
38. Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K, Bonner W. Histone H2A variants H2AX and H2AZ. Curr Opin Genet Dev 2002; 12:162–9.
39. Luoto KR, Meng AX, Wasylishen AR, Zhao H, Coackley CL, Penn LZ, et al. Tumor cell kill by c-MYC depletion: role of MYC-regulated genes that control DNA double-strand break repair. Cancer Res 2010;70:8748–59.
40. Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat Rev Cancer 2008;8:976–90.
41. Lee KWL, Savona M, Sorensen M, Brown P, Blum WG, Garzon R, et al. A phase 1 dose-escalation study of the oral selective inhibitor of nuclear export (SINE) KPT-330 (selinexor) in patients (pts) with relapsed/refractory acute myeloid leukemia (AML). J Clin Oncol 32:25s, 2014 (suppl; abstr 7032).
42. Schmidt J, Braggio E, Kortuem KM, Egan JB, Zhu YX, Xin CS, et al. Genome-wide studies in multiple myeloma identify XPO1/CRM1 as a critical target validated using the selective nuclear export inhibitor KPT-276. Leukemia 2013;27:2357–65.
43. Zheng Y, Gery S, Sun H, Shacham S, Kauffman M, Koeffler HP. KPT-330 inhibitor of XPO1-mediated nuclear export has anti-proliferative activity in hepatocellular carcinoma. Cancer Chemother Pharmacol 2014;74: 487–95.
44. Ranganathan P, Yu X, Santhanam R, Hofstetter J, Walker A, Walsh K, et al. Decitabine priming enhances the antileukemic effects of exportin 1 (XPO1) selective inhibitor selinexor in acute myeloid leukemia. Blood 2015;125:2689–92.
45. Kaufmann SH, Gore SD, Miller CB, Jones RJ, Zwelling LA, Schneider E, et al. Topoisomerase II and the response to antileukemic therapy. Leuk Lymphoma 1998;29:217–37.
46. Fortune JM, Osheroff N. Topoisomerase II as a target for anticancer drugs: when enzymes stop being nice. Prog Nucleic Acid Res Mol Biol 2000;64:221–53.
47. Xie C, Drenberg C, Edwards H, Caldwell JT, Chen W, Inaba H, et al. Panobinostat enhances cytarabine and daunorubicin sensitivities in AML cells through suppressing the expression of BRCA1, CHK1, and Rad51. PLoS One 2013;8:e79106.
48. Karp JE, Thomas BM, Greer JM, Sorge C, Gore SD, Pratz KW, et al. Phase I and pharmacologic trial of cytosine arabinoside with the selective checkpoint 1 inhibitor Sch 900776 in refractory acute leukemias. Clin Cancer Res 2012;18:6723–31.
49. Hoffman B, Amanullah A, Shafarenko M, Liebermann DA. The proto-oncogene c-myc in hematopoietic development and leukemogenesis. Oncogene 2002;21:3414–21.
50. Renneville A, Roumier C, Biggio V, Nibourel O, Boissel N, Fenaux P, et al. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. Leukemia 2008;22:915–31.