XPO1 Inhibition Using Selinexor Synergizes With Chemotherapy in Acute Myeloid Leukemia (AML) by Targeting DNA Repair and Restoring Topoisomerase IIα to the Nucleus

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Statement of Translational relevance

The standard treatment for acute myeloid leukemia (AML) in the US 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. Based on our data, we have initiated two clinical trials enrolling primary refractory and relapsed AML patients to selinexor in combination with anthracyclines.
ABSTRACT

Purpose: Selinexor, a selective inhibitor of \textit{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 to 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 AML patients 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 (\textit{Rad51} and \textit{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.
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., p53 and p21), cell-cycle regulators (i.e., Cdkn1a) and RNA molecules (1-4). XPO1 over-expression has been reported in several solid tumors and in hematological malignancies, including 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 anti-leukemic 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 1 clinical trial in AML. Preliminary data indicate that selinexor is well tolerated, safe and active in primary refractory and relapsed AML patients (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 anti-leukemic 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, 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.

**Methods**

**Cell culture**

MV4-11 cells (# CRL-9591) was purchased from ATCC and MOLM-13 cells (#ACC554) were purchased from DSMZ. MV4-11 cells were cultured in Iscove’s 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 (100U/mL) penicillin and (100ug/mL) streptomycin (# 15140-122;Gibco). MV4-11 cells resistant to idarubicin (MV4-11 Ida<sup>R</sup>) were generated by culturing MV4-11 cells with low dose of idarubicin (3 times below IC<sub>50</sub>) for several weeks.

**Primary AML samples and culture**

Primary refractory (n=2), relapsed (n=1) and newly diagnosed untreated (n=6) frozen BM 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 hrs) 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 Caspase3 (#9662), Rad51 (#8875), MLH1 (#3515), Chk1 (#2360), Gamma H2A.X (#9718), MSH2 (#2850), Topoisomerase IIα (D10G9) (#12286) were purchased from Cell Signaling; PMS2 (#2251.00.02), MSH6 (#2203.00.02), were purchased from Sdix, Alpha tubulin - Abcam (ab15246); Lamin A/C -Cell Signaling (#2032) and beta-Actin (#sc-81178) was purchased from Santa Cruz Biotechnology. The secondary antibodies for Western Blotting were purchased form 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. 100-200uL of the cell suspension from each treatment condition was loaded onto a cytospin cuvette with a coverslip and was spun at 800 rpm for 5mins using a Cytospin. The adhered cells were fixed using ice-cold 100% methanol for 15mins, washed with 1XPBS and then blocked/permeabilized using a solution containing 0.1% Tween-20, 0.3M Glycine, 1%BSA in 1XPBS. The cells were incubated with the primary antibody overnight at 4°C. The cells were washed 3 times with 1XPBS, incubated with 1:2000 of the secondary antibody for 1hr. The cells were washed with 1XPBS, treated with 1:1000 1ug/mL Dapi for 5mins and then mounted to a glass slide using Vectashield mounting medium (# H-1400, Vector Laboratories).

**Western Blotting**

Cells were washed with 1X PBS and then lysed with RIPA buffer (#89901, Thermo Scientific) supplemented with protease inhibitor (# 05892791001, Roche) and phosphatase inhibitor (# 04906837001, Roche). The nuclear and cytoplasmatic 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). 20ug of each sample were run in 4-12% 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 Licor 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 hrs 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, Germany) 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 was 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 hrs after the first drug treatment without washing. Plates were read 48 hrs 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.

**HR Assay**

HR was assessed using a direct repeat green fluorescent protein (DR-GFP) assay essentially as previously described (36). The HeLA-DR cells possess an integrated DR-GFP construct, whose expression is prevented by an insert with the I-SceI restriction site in the reading frame, whereby transfection of I-SceI endonuclease creates a DSB, which when repaired by error-free HR leads to GFP-expressing cells (36). Briefly, HeLA-DR cells were transiently transfected with either the
negative control phCMV-1 I-SceI, the functional endonuclease pCMV3xnlsl-SceI, 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 (ChIP) Assay

The ChIP assay was performed according to the protocol of the EpiTect kit (Qiagen). MV4-11 cells (about $5 \times 10^6$) were treated with DMSO or selinexor for 24 hrs, and then fixed with 1% formaldehyde. The cross-linked DNA complexes were sheared to 500–1000 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 follow: RAD51 (CACGTTGGCCAGGTTTATCT, GGCGAGTGAATTTCATAA); CHK1 (GATCCATACGCCTCAGCTTC, AGGCCAAGCAGAACAATCG).

Mice

Female non-obese diabetic severe combined immunodeficient gamma (NSG) mice that lack mature T cells, B cells, or functional NK cells, and are deficient in cytokine signaling, were purchased from Jackson Immuno Research laboratories (Ban Harbor, ME). All mice used in the experiments were between 6-10 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.

**MV4-11 xenograft mouse model**

Spleen cells ($5 \times 10^4$) 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 (1.5mg/kg, i.v., on days 7, 8 and 9 only), selinexor alone (10mg/kg, oral gavage, twice a week – on days 7, 10, 14, 17, 21, 24, 28) or idarubicin (1.5mg/kg, i.v., on days 7, 8 and 9 only) and selinexor (10mg/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, 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 long-rank test methods (Graph Pad Prism). Differences between continuous variables were analyzed using Student’s t-tests. All $p$ values lesser than 0.05 were 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, two 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, Supplemental Table 1) for 48 hrs. Cell proliferation was measured by WST-1 assay and the combination index (CI) was calculated according to the Chou-Talalay method (19). As shown in Figure 1A and Supplemental Table 2, 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, Supplemental Table 3). In addition, we tested the combinatorial effect of selinexor with two other Topo II inhibitors (etoposide, mitoxantrone) in MV4-11 and MOLM-13 cell lines and observed similar synergistic effects (Fig. 1C, Supplemental Table 4). Induction of apoptosis was measured by Annexin-V and PI staining of MV4-11 and MOLM-13 cell lines 48 hrs post drug treatment showing increased apoptosis in the combinatorial treatment versus either drug alone (Figure 1D and Supplementary Figure 1A-C).

To evaluate if 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 to sequential treatment (Supplementary Figure 2 A-B).
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 (20, 21). Topo IIα shuttles between the nucleus and the cytoplasm, with the equilibrium tending towards nuclear localization. The nuclear export of Topo IIα is XPO1 dependent through the interaction with two functional leucine rich nuclear export signal sequences (NESs) (16-18, 20, 22, 23). 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, 24-26). 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 complexes and cell death (1, 24-26). 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 IdaR) (Fig. 2B and C). We also determined Topo IIα expression in three 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, Supplementary Figure 3 A-D). 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 anthracyclines 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 (Fig. 2D and Fig. 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 (27). We confirmed this in AML by showing that selinexor treatment of AML cell lines MV4-11 and MOLM-13 resulted in significant down-regulation of DNA damage repair proteins. These include the DNA damage response protein Chk1 (28, 29) and DNA damage repair protein Rad51 (30-33) that assist in double strand DNA repair by homologous recombination; 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, since increased Ser 139-phosphorylated H2AX histone (γ-H2A.X), 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 four primary AML patient samples (Fig. 3C and D). Based on these data, we reasoned that the synergistic effects of
Selinexor with Topo II inhibitors could be explained in part by selinexor induced down-regulation of DNA repair proteins thus preventing leukemia cells from repairing chemotherapy-induced DNA damage.

**Selinexor blocks homologous recombination 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 homologous recombination (HR) pathway (34, 35). Rad51 recombinase plays a central role in governing HR (30-33). To measure the ability of cells to carry out HR in the presence of selinexor, we used the HeLa DR cells that express two copies of inactive Green Fluorescent Protein (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 (36). Using this assay, when HeLa DR cells were treated with selinexor, we observed a significant dose dependent decrease in GFP expression compared to 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 hrs with 10nM idarubicin followed by washing out of idarubicin. The cells were then either allowed to recover or were treated with 100nM of selinexor for 48 hrs. The cells were fixed with methanol and stained for γ-H2A.X (37, 38). Two hours treatment of idarubicin induced DNA damage confirmed by increased staining of γH2A.X (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 γH2A.X staining.
suggesting that the cells did not recover from the idarubicin induced DNA damage. Incubation with selinexor at doses lower than 200nM did not induce H2A.X 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 down-regulate 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 chromatin immunoprecipitation (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 anti-leukemic 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 non-obese diabetic/severe combined immunodeficient (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 (1.5mg/kg, i.v., on days 7, 8 and 9 only), selinexor alone (10mg/kg, oral gavage, twice a week –on days 7, 10, 14, 17, 21, 24, 28) or idarubicin (1.5mg/kg, i.v., on days 7, 8 and 9 only) and selinexor (10mg/kg, oral gavage, twice a week – on
days 7, 10, 14, 17, 21, 24, 28). All treatments were given for three weeks after leukemia cell injection. Single agent selinexor at the low dose of 10mg/kg had no effect in prolonging the survival of mice with respect to the control mice (vehicle control), corroborating our previous reports (44). Idarubicin at 1.5mg/kg increase median survival of mice slightly. In contrast, the combination treatment of idarubicin and selinexor significantly increased mice survival compared to selinexor alone (31 vs. 38 days, p<0.001; Fig. 6A) and compared to 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 (10mk/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 antileukemia 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 hematological malignancies including AML (3, 14, 15, 20, 45, 46). In normal cells, Topo IIα is constantly shuttling between the nucleus and the cytoplasm via the three nuclear localization signals (NLS) at the COOH end responsible for nuclear import, and two leucine rich NES that mediate XPO1 dependent nuclear export, with the equilibrium tending towards nuclear localization (16-18, 22, 23). 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, 26). In our study, we report the Topo IIα localization is mostly nuclear; however there are AML patients where Topo IIα is found in the cytoplasm as well. In particular we reported an AML patient 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 (25, 26). Thus our data is consistent with similar data reported in other malignancies and provides a rationale to treat patients who express cytoplasmic Topo IIα with selinexor to sensitize them to anthracycline therapy.
Additionally, 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 homologous recombination 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 double stranded breaks, which are highly toxic to the cell. AML blasts can evade cell death following Topo II inhibitors by repairing the double stranded breaks induced by chemotherapy through many mechanisms, including up-regulation 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. Based on our results there are currently two clinical trials enrolling primary refractory and relapsed AML patients to selinexor in combination with anthracyclines; selinexor plus standard cytarabine and idarubicin (7+3) (NCT02249091), and selinexor plus mitoxantrone, etoposide and cytarabine (MEC) (NCT02299518).

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Conflict of Interests

T.K, Y.L, M.K and S.S are employees of Karyopharm therapeutics, a clinical stage biopharmaceutical company that develops selective inhibitors of nuclear export-targeted therapeutics.

The online version of this article contains a data supplement.
REFERENCES


FIGURE LEGENDS

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) Combination index (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 hrs 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 (Supplemental Table 1). (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 two 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 hrs 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 hrs. At least 500 cells were counted and one representative experiment of three 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 two primary refractory and one relapsed AML patient samples after treatment with DMSO (control) or selinexor for 24 hrs and
in a pretreatment sample from patient 1 (E). The left panel shows the DAPI staining (cell nucleus). The center panel is Topo IIα staining and the right panel is the merged image of DAPI and Topo IIα staining. (F) Topo IIα expression measured by western blotting in a pretreatment and relapsed AML samples from patient 1 and from a primary refractory AML samples (patient 2).

**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 hrs after selinexor treatment. The average relative expression and standard deviation of two 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 γH2A.X 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 hrs and protein expression was analyzed by immunoblots of whole protein extracts treated with selinexor for 24 and 48 hrs. Selinexor treated versus untreated, * p<0.05.

**Figure 4. Selinexor blocks homologous recombination 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 two 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 double strand breaks 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) Immunofluorescence staining of γH2A.X, a marker of DNA damage in MV-4-11 cells treated with 10nM idarubicin for 2 hours. Idarubicin was washed out and cells were either allowed to recover or treated with 100nM selinexor for 48 hrs.

**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 hrs. One representative western blot of three experiments is shown. (B) Chromatin immunoprecipitation (ChIP) assays of c-Myc on the Rad51 and Chk1 promoter regions in MV4-11 cells after treatment with DMSO or selinexor for 24 hrs.

**Figure 6. Idarubicin enhances selinexor anti-leukemic 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) White blood cell 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.
Figure 1

A

Combination Index (CI)

Fraction Affected (Fa)

MV 4-11

MOLM-13

MV 4-11

MOLM-13

B

Patient 1

Patient 2

Patient 3

Patient 4

Patient 5

Combination Index (CI)

Fraction Affected (Fa)

IDA/Selinexor

IDA/Selinexor

IDA/Selinexor

IDA/Selinexor

DAUNO/Selinexor

DAUNO/Selinexor

DAUNO/Selinexor

DAUNO/Selinexor

C

MV 4-11

MV 4-11

MOLM-13

MOLM-13

Combination Index (CI)

Fraction Affected (Fa)

Mitoxantrone/Selinexor

Etoposide/Selinexor

Mitoxantrone/Selinexor

Etoposide/Selinexor

D

MV4-11

MOLM-13

Patient 1

DMSO

IDA 5nM

IDA 10nM

Annexin-V

Selinexor 160nm

Selinexor + IDA

Selinexor 80nm

Selinexor + IDA

Selinexor 250 nM

Selinexor + IDA

PI
Figure 2

A. DAPI, MOLM-13 Topo IIα, Merge

DMSO
Selinexor

B. DAPI, Topo IIα, Merge

MV4-11
MV4-11 Ida^R

C. Topo IIα, β actin

D. Patient 1 (Relapsed) DAPI, Topo IIα, Merge

Patient 2 (Primary refractory) DAPI, Topo IIα, Merge

Patient 3 (Primary Refractory) DAPI, Topo IIα, Merge

DMSO
Selinexor

E. Patient 1 (Pretreatment) DAPI, Topo IIα, Merge

Patient 2

F. Patient 1

Patient 2

N = nuclear fraction
C = Cytoplasmic fraction

N= nuclear fraction
C= Cytoplasmic fraction

MV4-11 Ida^R
Figure 3

A. Relative Expression of Chk1, MSH2, Rad51, MLH1, PMS2, MSH6 in MOLM13 and MV-4-11 cells.

B. Western Blot analysis of Chk1, Rad51, MLH1, MSH2, PMS2, MSH6, Actin, γH2A.X, Full length Caspase 3, Cleaved Caspase 3 in MOLM-13 and MV-4-11 cells.

C. Relative Expression of Chk1 and Rad51 in patient samples.

D. Relative Expression of Chk1, Rad51, Actin in patient samples.
Figure 4

A

![Graph showing % GFP positive cells](attachment:Figure_A.png)

B

![Bar graph showing % live cells (Annexin/PI negative)](attachment:Figure_B.png)

C

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<td>48hrs of Recovery</td>
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γH2A.X Staining

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Figure 5

A

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B

**Rad51**

- DMSO
- Selinexor 100 nM

- Fold change: 1.5
- p-value: 0.01

**Chk1**

- DMSO
- Selinexor 100 nM

- Fold change: 1.5
- p-value: <0.01
**Figure 6**

A

![Percent Survival Graph](image)

- **Vehicle (n=11)**
- **Selinexor (10mg/kg, n=10)**
- **Idarubicin (1.5 mg/kg, n=9)**
- **Selinexor + Ida (n=10)**

* p<0.001

B

![WBC Count Graph](image)

- **Vehicle**
- **Selinexor**
- **IDA**
- **Selinexor + IDA**

p<0.001

p<0.01

C

![Spleen Weight Graph](image)

- **Vehicle**
- **Selinexor**
- **IDA**
- **Selinexor + IDA**

p<0.0001

p=0.01
XPO1 Inhibition Using Selinexor Synergizes With Chemotherapy in Acute Myeloid Leukemia (AML) by Targeting DNA Repair and Restoring Topoisomerase IIα to the Nucleus

Parvathi Ranganathan, Trinayan Kashyap, Xueyan Yu, et al.

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