XPO1/CRM1 Inhibition Causes Antitumor Effects by Mitochondrial Accumulation of eIF5A

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Abstract

Purpose: XPO1 inhibitors have shown promise for cancer treatment, and yet the underlying mechanisms for the antitumor effects are not well understood. In this study, we explored the usefulness of selective inhibitors of nuclear export (SINE) compounds that are specific inhibitors of XPO1.

Experimental Design: We used proteomic analysis in XPO1 inhibitor–treated ovarian cancer cell lines and examined antitumor effects in ovarian and breast cancer mouse models. We also studied the effects of XPO1 inhibitor in combination with chemotherapeutic agents.

Results: XPO1 inhibitor treatment substantially increased the percentage of apoptotic cells (60%) after 72 hours of incubation. XPO1 inhibitor promoted the accumulation of eIF5A in mitochondria, leading to cancer cell death. Topotecan showed the greatest synergistic effect with XPO1 inhibitor. XPO1 inhibitors prevented the translocation of IGF2BP1 from the nucleus to the cytoplasm, thereby permitting the localization of eIF5A in the mitochondria. This process was p53, RB, and FOXO independent. Significant antitumor effects were observed with XPO1 inhibitor monotherapy in orthotopic ovarian (P < 0.001) and breast (P < 0.001) cancer mouse models, with a further decrease in tumor burden observed in combination with topotecan or paclitaxel (P < 0.05). This mitochondrial accumulation of eIF5A was highly dependent on the cytoplasmic IGF2BP1 levels.

Conclusions: We have unveiled a new understanding of the usefulness of selective inhibitors of nuclear export (SINE) compounds against ovarian and breast cancer. Our data also ascertain the combinations of XPO1 inhibitors with specific chemotherapy drugs for therapeutic trials. Clin Cancer Res; 21(14); 3286–97. ©2015 AACR.

Introduction

Transport of macromolecules between the nuclear and cytosolic compartments is an essential process in all eukaryotic cells, allowing them to regulate fundamental processes such as degradation of p53 (1). Exportin 1 (XPO1), the key nuclear export protein more commonly called chromosome region maintenance 1 (CRM1), is required for transporting cargo proteins with leucine-rich nuclear export sequences from the nucleus to the cytoplasm (2). An association between increased XPO1 expression and poor prognosis has been reported in osteosarcoma and pancreatic, lung, and ovarian cancers (3–6). Leptomycin B (LMB) was the first specific XPO1 inhibitor shown to inhibit XPO1 binding to the leucine-rich nuclear export sequence of the cargo protein substrate (7). LMB was found not to be clinically useful owing to toxicity (8). Derivatives of LMB with improved pharmacokinetic (drug-like) properties showed antitumor activity with improved tolerability in animal models (9). Given that inhibition of nuclear export is a potent yet unachieved therapeutic strategy, more specific XPO1 inhibitors with less toxicity are needed. Moreover, the underlying mechanisms are not well understood.

Here, we investigated the efficacy of selective inhibitors of nuclear export (SINE) compounds that inhibit XPO1 by binding to Cys528, located in the vicinity of the NES-binding domain. Unlike LMB that forms an irreversible covalent bond with Cys528, SINE compounds bind in a slowly reversible manner (10, 11). The efficacy of SINE compounds against ovarian and breast cancer was evident in mouse models, and we observed a synergistic effect of SINE in combination with specific chemotherapeutic agents. Initial experiments pointed toward a mechanism involving mitochondrial function. Among the many proteins that showed increased expression after treatment with SINE, we focused on eukaryotic translation initiation factor 5A (eIF5A; refs. 12, 13). Further investigation revealed that SINE compounds inhibit nuclear export of insulin-like growth factor 2 mRNA binding.
**Translational Relevance**

In this study, we investigated the efficacy of a novel inhibitor of XPO1 in cancer. We uncovered the important roles of elf5a and IGF2BP1 in XPO1 inhibitor-mediated cell death. These findings provide a new understanding of the mechanisms by which XPO1 inhibitor mediates its antitumor effects and facilitates its future clinical development.

protein 1 (IGF2BP1), a protein first reported here to bind and inhibit elf5a-mediated apoptotic effects.

**Materials and Methods**

**Cell lines and culture conditions**

Sources of the human epithelial ovarian cancer cell lines that were used in this study [A2780, A2780CP20, IGROV-1, SKOV3, HeyA8, HeyA8MDR, RMG2, HeC265, SPEC2, Ishikawa, MDA-MB-231, MDA-MB-157, H1299, OVCA2429, and luciferase-transfected SKOV3ip1 (SKOV3-Luc)] have been described previously (14–17). All cultures were used when cells were 70% to 80% confluent.

**Drugs and reagents**

The SINE compounds KPT-185 and selinexor (KPT-330, molecular weight 443.31, chemical formula C17H11F6N7O) were obtained from Karyopharm Therapeutics Inc. KPT-185 is suitable for in vitro use, and selinexor is suitable for in vivo use. Primary antibodies used included anti-p53 (Cell Signaling), anti-lamin B1 (Life Technologies), anti-tubulin (Cell Signaling), anti-COX IV antibody, anti-IGF2BP1 (Abcam), anti-Ki67 (Thermo Lab Vision), and anti-β-actin (Sigma-Aldrich), anti-eIF5A (Abcam), anti-IgG2BP1 (Abcam), anti-Ki67 (Thermo LabVision), and anti-cleaved caspase 3 (Cell Signaling). The following secondary antibodies were used: horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G2a (Serotec Harlan Bioproducts for Science, Inc.), and fluorescent Alexa 594 immunoglobulin G (Life Technologies).

**Cytotoxicity assay for cancer cell lines**

The cytotoxicity of KPT-185 and topotecan was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake assay, as described previously (18). After 72 hours of incubation with DMSO (control) or various concentrations of CRM1 inhibitor, growth was assessed by adding 50 μL of 0.15% MTT (Sigma-Aldrich) to each well. After incubating for 2 hours at 37°C, medium was removed from each well, and 100 μL of DMSO was added. Each sample at each condition was assayed in triplicate. For the combination assay, cytotoxic agents were added as indicated by the ratios in Supplementary Table S1. The combination index was calculated as previously reported (19) using the Chou and Talalay method. A combination index of less than 1 indicates synergy; equal to 1, additivity; and more than 1, antagonism.

**Apoptosis assays**

The relative percentage of apoptotic cells was assessed at 0, 30, and 40 hours after treatment with 0.25 μmol/L KPT-185 using an Annexin V-coupled fluorescein isothiocyanate (FITC) apoptosis detection kit-1 (BD Pharmingen) as manufacturer's protocol.

**In vitro gene silencing**

The siRNAs against Rb, FoxO3, and elf5a were purchased from Sigma-Aldrich (sequences are listed in Supplementary Table S5). A nonsilencing siRNA that did not share sequence homology with any known human mRNA from a BLAST search was used as a control for target siRNA. Briefly, A2780 and HeyA8 ovarian cancer cells were transfected with siRNA (20 nmol/L) using Lipofectamine 2000 transfection reagent (Invitrogen Corporation) according to the manufacturer's instructions. Cells were collected as lysates after 48 and 72 hours, and expression of Rb, FoxO3, and elf5a was determined using Western blot analysis. For the cytotoxicity assay of KPT-185 in Rb-, FoxO3-, and elf5a-knockdown cells, the cells were reverse transfected with the same siRNA (second transfection) 48 hours after the first transfection, and DMSO or KPT-185 was added 6 hours after the second transfection. Cell viability was determined after 72 hours using MTT.

**1D Gel/LC-MS/MS**

The cytoplasmic fraction of A2780 cells treated with or without 0.25 μmol/L KPT-185 was immunoprecipitated with anti-elf5a antibody in lysis buffer. Immunoprecipitated proteins were resolved by 4% to 12% SDS-PAGE and stained using the Pierce Silver Stain Kit for Mass Spectrometry (Thermo Fisher Scientific). Six bands that showed different intensities were excised, digested in the gel with 200 ng of modified trypsin (sequencing grade; Promega) for 18 hours at 37°C, extracted, and analyzed by high-sensitivity LC-MS/MS and an orbital ion-trap mass spectrometer (Orbitrap Elite; Thermo Scientific). Proteins were identified by searching the fragment spectra against the Swiss-Prot protein database (EBI) using Mascot (Matrix Science) or Sequest (Thermo Fisher Scientific).

**Animal care**

Female athymic nude mice (8–12 weeks old) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center. The mice were quarantined, housed, and maintained under specific pathogen-free conditions in an animal facility that is approved by the American Association for Accreditation of Laboratory Animal Care in agreement with the current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH. The study protocols were approved and supervised by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center.

**Orthotopic implantation of tumor cells in mice**

To produce ovarian cancer orthotopic models, we injected mice intraperitoneally with 1 × 106 A2780, A2780CP20, SKOV3, or SKOV3-Luc cells in 0.2 mL of Hanks balanced saline solution (HBSS; Life Technologies Invitrogen). To produce the breast cancer orthotopic model, we injected mice with 4 × 106 MDA-MB-231 cells in 0.05 mL of HBSS through the mammary fat pad. Mice were monitored daily for adverse effects of therapy and were killed by cervical dislocation on days 35 to 42 after tumor cell injection or when any of the mice appeared moribund. The tumor weight from each mouse was determined by weighing all of the tumor nodules together.
Luminescence imaging
Mice were injected intraperitoneally with $1 \times 10^6$ SKOV3-Luc cells per mouse ($n = 5$ mice per group). After 18 days, mice were randomly assigned to one of the four treatment groups: vehicle, KPT-330 (20 mg/kg, twice per week) alone, topotecan (5 mg/kg, once per week) alone, or KPT-330 (20 mg/kg, twice per week) plus topotecan (5 mg/kg, once per week). Bioluminescence imaging and data acquisition were performed with the IVIS 100 imaging system coupled to the Living Image Software (Xenogen), as reported previously (20, 21).

Fluorescence microscopy and image analysis
For immunofluorescence analysis of p53 subcellular localization in cells treated with KPT-185, $5 \times 10^4$ A2780 cells were plated in an 8-well chamber slide (BD Biosciences) in complete growth medium, and 24 hours after plating, medium was replaced with fresh medium with dimethyl sulfoxide for the control group or 0.25 μmol/L KPT-185 for the treatment group. Six hours after incubation, cells were fixed with 4% cold acetone, blocked with 4% fish gelatin, and then stained with rabbit-anti-p53 antibody and labeled with goat-anti-rabbit Alexa Fluor 547 secondary antibodies. Nuclear staining was performed using Hoechst (Life Technologies) for 10 minutes. Slides were covered with propylgallate and coverslips for microscopic evaluation.

Nuclear and cytoplasmic fractionation of cultured cells
Nuclear and cytoplasmic fractions were obtained using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical) according to the manufacturer's protocol.

Mitochondria isolation
Mitochondria isolation from control A2780 cells and A2780 cells treated with KPT-185 was performed using the mitochondria isolation kit for cultured cells (Pierce Chemical) according to the manufacturer's protocol.

Mitochondrial membrane potential
The change in mitochondrial transmembrane potential ($\Delta m$) induced by KPT-185 was observed using flow cytometry by, using the Mito Probe JC-1 Assay Kit for Flow Cytometry (Life Technologies) according to the manufacturer's protocol.

Proteomic analysis by two-dimensional gel electrophoresis and MALDI TOF/TOF MS
Proteins were be extracted from samples in destreak Buffer (GE Healthcare Lifesciences) as and analyzed by 2D gel electrophoresis (2DGE) as described previously (22–24). After electrophoresis, the gels were fixed (10% methanol, 7% acetic acid in ddH2O), and stained with SYPRO-Ruby (Bio-Rad) overnight, and destained in Fix Buffer. The destained gels were scanned at a 100-μm resolution using the GE Healthcare Typhoon Trio System using 460/80 nm excitation and 620 nm (long pass) emission filters. The SYPRO Ruby staining is linear with respect to input protein over a large dynamic range spanning the spot intensities of the analyzed proteins (25). The 2D gel images were analyzed using Progenesis/SameSpots version 4.1 software (Nonlinear Dynamics, Ltd.). Spot matching between gels was manually reviewed and adjusted as necessary. The spot volumes were normalized using a bias value calculated by the software based on the assumption that the great majority of spot volumes represent no change in abundance (ratio control to experimental = 1.0) (personal correspondence, D. Bramwell, Nonlinear Dynamics, 2008). A change in normalized spot volumes greater than ±2.5-fold was considered significantly changed, and these spots were subsequently robotically picked, trypsin-digested, and peptide masses identified by MALDI TOF/TOF. The 4800 MALDI TOF/TOF (AB Sciex) was used for protein identification. MS/MS was performed in a data-dependent mode and the data processed using the manufacturer's software (23, 24). The significance of a protein match, based on the peptide ions score from MS/MS data from several precursor ions, is calculated as $-10\log(P)$, where $P$ is the absolute probability of the match occurring due to a random event. The significance level (MASCOT score indicating identity) is the score at which an identified protein has 0.1% chance or less of being a random event (assuming $P < 0.001$). These peptide scores are further combined into a "protein score," which is the sum of the highest ions score for each distinct sequence. That is, excluding the scores of duplicate matches and is deemed a high confidence identification if $P > 62$. All protein scores reported herein exhibited protein scores $> 62$.

Immunohistochemical staining
For mouse tissues, cleaved caspase 3 and Ki67 were evaluated using formalin-fixed, paraffin-embedded tumors; 5-μm thick sections were deparaffinized and rehydrated. Antigen retrieval was performed using Berg Decloaker (BioCare Medical) with a pressure cooker for anti-cleaved caspase 3 or Diva (BioCare Medical) with a steamer for anti-Ki67. Endogenous peroxidase and nonspecific epitopes were blocked with 3% H2O2 (Thermo Fisher Scientific) in PBS for 12 minutes at room temperature; nonspecific protein binding was blocked with 5% normal horse serum and 1% normal goat serum for anti-Ki67 antibody or with 4% coldwater fish skin gelatin (Electron Microscopy Sciences) for anti-cleaved caspase 3. Sections were incubated with primary antibodies in blocking solution overnight at 4°C. For negative control, sections were incubated without a primary antibody and with human immunoglobulin G antibody (Jackson Immuno-Research Laboratories). After washing with PBS, we applied the appropriate secondary antibody, and visualization was performed using the Vectastain ABC detection Kit (Vector Labs) according to the manufacturer's instructions. The chromogenic reaction was performed with 3, 3'-diaminobenzidine (DAB; Phoenix Biotechnologies), and counterstaining was performed with Gill's no. 3 hematoxylin (Sigma-Aldrich).

Statistical analysis
Continuous variables were compared with the two-sample t test (between two groups) or with ANOVA (for all groups) if normally distributed (as determined by the Kolmogrov–Smirnov test), and the Mann–Whitney test was used if distributions were nonparametric. A P value of less than 0.05 from a two-tailed statistical test was considered statistically significant.

Results
KPT-185 shows tumor suppressor protein–independent cytotoxic effects
We first examined the antitumor effects of KPT-185, a SINE compound suitable for in vitro use (26), in ovarian cancer cell
Cell viability assays after 72 hours of incubation with KPT-185 showed that IC50 concentrations ranged from 0.1 μmol/L to 0.96 μmol/L (Fig. 1A). The IC50 concentrations of KPT-185 in normal hepatocytes, peripheral blood mononuclear cell, and mouse embryo fibroblasts are 10 μmol/L, 20 μmol/L, and 20 μmol/L, respectively (data not shown). To investigate whether there is an association between XPO1 expression and the efficacy of KPT-185, we determined XPO1 expression of these cell lines (Supplementary Fig. S1A). To investigate the antitumor effects of KPT-185 in other cancer types, we further tested cell viability in uterine and breast cancer cell lines (Supplementary Fig. S1B). IC50 concentrations of KPT-185 after 72 hours of incubation ranged from 0.11 μmol/L to 0.5 μmol/L for uterine cancer cell lines, and 0.5 μmol/L for MDA-MB-231 cells.

To investigate possible mechanisms of KPT-185 activity, we first examined expression of caspases 3 and 9 in A2780 cells. After 12 hours of incubation with KPT-185, there was a significant increase in cleaved caspase 3 and 9 expression (Fig. 1B). The percentage of apoptotic cells was 2% in the control cells (no treatment) and increased to 60% after KPT-185 treatment (Fig. 1C). Because XPO1 is the only nuclear exporter of the majority of tumor suppressor proteins (27), we examined the effect of KPT-185 on the subcellular localization of p53 in A2780 cells. Untreated cells expressed p53 mainly in the cytoplasm, but nuclear localization of p53 was observed after 4 hours of incubation with KPT-185 in H1299 and MDA-MB-157 cells. The mean percentages of apoptotic cells were 8.3% in untreated and 27% in treated H1299 cells, and 14.1% in untreated and 36.2% in treated MDA-MB-157 cells. (data not shown). To study the antitumor effect of KPT-185 in other cancer types, we further tested cell viability in uterine and breast cancer cell lines (Supplementary Table S1). In addition, an increased percentage of apoptotic cells was observed in these p53-null cell lines after 48 hours of incubation with KPT-185 (Fig. 1E). We also tested the effect of KPT-185 on cell viability in Rb- or FoxO3-depleted A2780 cells (using siRNA transfection). The antitumor effect of KPT-185 was observed in both Rb- and FoxO3-depleted A2780 cells (Fig. 1F; Supplementary Fig. S1C and S1D) and in HeyA8 cells (Supplementary Fig. S1E–S1G). These results...
further suggest that the antitumor effects of KPT-185 are independent of these major tumor suppressor proteins.

**Treatment with KPT-185 results in mitochondrial accumulation of eIF5A and cell death**

We next investigated the therapeutic effect of KPT-185 in combination with commonly used chemotherapeutic agents for ovarian cancer. Analysis of combination indexes showed that paclitaxel, cisplatin, topotecan, and liposomal doxorubicin had a synergistic effect when combined with KPT-185, and topotecan showed the highest synergistic effect (Supplementary Fig. S2A–S2E; Supplementary Table S2). Given that topotecan has been reported to mainly localize in mitochondria (30), we asked whether KPT-185 affects mitochondrial function. We used mitochondrial membrane potential (MMP) to determine the mitochondrial function and observed a significant increase in cells with low MMP following treatment with 0.2 μmol/L KPT-185 for 24 hours (Fig. 2A).

To investigate the mechanism of KPT-185–induced MMP decrease, we separated mitochondrial proteins from cells incubated with dimethyl sulfate (DMSO; control) or KPT-185 for 6 hours using 2DGE. Quantitative analyses of these samples, followed by mass spectrometry analysis, identified 71 of 1,423 proteins with altered expression levels after treatment with KPT-185 in excess of 2.5-fold (Supplementary Table S3). Because eIF5A showed the highest fold change (7.7-fold increase) after incubation with KPT-185 among these proteins, we chose eIF5A for further investigation. We subjected mitochondrial protein from A2780 cells incubated with DMSO or KPT-185 for 6 hours to immunoblot analysis, which confirmed the increased level of eIF5A in mitochondria after incubation with KPT-185 (Fig. 2B). To determine whether these mechanisms are specific to XPO1 inhibition, we silenced XPO1 using siRNA. The level of eIF5A in mitochondria was increased 72 hours after XPO1 silencing (Supplementary Fig. S2F and S2G).

Recent findings suggest that eIF5A plays a role in translation elongation, but the subcellular localization of eIF5A has remained controversial (31). Therefore, we next examined whether eIF5A localization was affected by XPO1 inhibition. Nuclear and cytoplasmic proteins obtained from A2780 cells treated with DMSO or KPT-185 were subjected to immunoblot analysis with the anti-eIF5A antibody, which showed that eIF5A localization was not affected by XPO1 inhibition (Fig. 2C). We also investigated whether eIF5A accumulation in the mitochondria led to cell death using bongkrekic acid, which is a mitochondrial uptake inhibitor. Mitochondrial and cytosolic proteins obtained from A2780 cells treated with DMSO or KPT-185 with or without bongkrekic acid were subjected to immunoblot analysis with the anti-eIF5A antibody. The mitochondrial fraction of eIF5A was lower after treatment with KPT-185 and bongkrekic acid than after treatment with KPT-185 alone (Fig. 2D). Incubation with bongkrekic acid reduced the cytotoxic effect of KPT-185 in A2780 cells (Fig. 2E). Similar results were observed in p53-null H1299 cells and patient-derived ovarian cancer cells (OVCA2429; Fig. 2F; Supplementary Fig. S2H). Interestingly, the cytotoxic effect of KPT-185 was decreased in eIF5A-depleted A2780 cells (Fig. 2G; Supplementary Fig. S2I). These results suggest that the cytotoxic effect of KPT-185 is eIF5A-dependent and results from eIF5A accumulation in the mitochondria.

**IGF2BP1 binds to eIF5A in the cytoplasm and inhibits accumulation of eIF5A in mitochondria**

Next, we sought to determine the mechanism by which eIF5A accumulation in the mitochondria is prevented. Cytoplasmic proteins obtained from A2780 cells treated with DMSO or KPT-185 for 6 hours were immunoprecipitated with the anti-eIF5A antibody, separated using electrophoresis, and stained for analysis. Mass spectrometry was carried out on the unknown bands around 20, 35, 38, 45, 70, and 90 kDa, which showed decreased intensity in cells treated with KPT-185 (Supplementary Fig. S3). Proteins were identified by searching for the fragment spectra in the Swiss-Prot protein database (EBI) using Mascot (Matrix Science) or Sequest (Thermo Fisher Scientific; Supplementary Table S4). In the mass spectrometry analysis, a total of 60 proteins were identified to be expressed at different levels between A2780 cells treated with DMSO or KPT-185. Out of these proteins, we focused on IGF2BP1, as it has been previously reported to have a nuclear export sequence and can bind to XPO1 (32). We obtained nuclear and cytoplasmic proteins from A2780 cells treated with DMSO or KPT-185 for 4 hours and determined IGF2BP1 level. After treatment with KPT-185, IGF2BP1 level in the cytoplasm was significantly decreased (Fig. 3A).

We next examined for direct binding between eIF5A and IGF2BP1. Cytoplasmic proteins obtained from A2780 cells treated with DMSO or KPT-185 for 6 hours were immunoprecipitated with the anti-eIF5A antibody and subjected to immunoblotting with the anti-IGF2BP1 antibody; eIF5A binding with IGF2BP1 was decreased after treatment with KPT-185 (Fig. 3B). To determine whether binding with IGF2BP1 prevents eIF5A from accumulating in the mitochondria, we examined IGF2BP1-depleted A2780 cells (siRNA transfection; Fig. 3C). Mitochondrial and cytosolic proteins were obtained from A2780 cells 72 hours after transfection with scramble or IGF2BP1 siRNA, and the proteins were subjected to immunoblotting with the anti-eIF5A antibody. Mitochondrial eIF5A level was 2.8-fold higher in the IGF2BP1-depleted compared with control-treated A2780 cells (Fig. 3D). To determine the cytotoxic effect of IGF2BP1 depletion, we performed apoptosis assays in A2780 cells transfected with scramble or IGF2BP1 siRNA. The percentage of apoptotic cells increased following IGF2BP1 silencing (Fig. 3E).

**Selinexor (KPT-330) increases tumor apoptosis and decreases tumor proliferation**

Our discovery that KPT-185 induces apoptosis led us to explore whether SINE may represent a candidate therapeutic agent for ovarian cancer. To determine treatment dosage and schedule, we first conducted a dose-finding *in vivo* experiment in the A2780 orthotopic ovarian cancer mouse model with selinexor, which is an orally bioavailable SINE XPO1 antagonist suitable for *in vivo* use (26) that is currently studied in phase 1 clinical trials in patients with advanced solid tumor and hematologic malignancies (11, 33–35). Selinexor was administered at 3 different dosages: 10, 20, and 30 mg/kg. Mice were sacrificed 12, 48, or 96 hours after treatment with selinexor. Tumors were collected and subjected to immunofluorescence staining with the anti-p53 antibody to find the optimal dosing regimen. Because nuclear localization of p53 was observed after 48 hours and lasted for 96 hours with 20 and 30 mg/kg of selinexor (Supplementary Fig. S4A), the 20 mg/kg twice-weekly regimen (similar to that
Because topotecan showed the best synergistic effect when combined with KPT-185 in vitro, we examined the in vivo therapeutic effect of the topotecan–selinexor combination. A2780 tumor–bearing mice were randomly assigned to one of the following groups (n = 10 mice per group): vehicle (control), selinexor (20 mg/kg, twice per week), topotecan (5 mg/kg, once per week), or selinexor plus topotecan. Selinexor was administered orally, and topotecan was administered intravenously. After
were the same as in the A2780 tumor model \((n = 10\) mice per group\), and treatment was initiated 7 days after tumor cell injection. After 4 weeks of treatment, the mean tumor weight in mice treated with selinexor plus topotecan was significantly lower than in mice treated with vehicle or topotecan alone (Fig. 4C). Although the number of tumor nodules did not significantly differ among the groups, the number of tumors in mice treated with selinexor plus topotecan tended to be lower than in the other groups (Fig. 4D).

Because A2780 cells are reported to contain wild-type p53, we next used a p53-mutant ovarian cancer cell line, SKOV3, to investigate the cytotoxicity of selinexor in p53-mutant cells (36–38). Four different treatment groups were used \((n = 10\) mice per group\): vehicle; selinexor \((20\ \text{mg/kg, twice per week})\) alone, paclitaxel \((75\ \mu\text{g, once per week})\) alone, and selinexor plus paclitaxel. As paclitaxel also showed the second highest synergistic effect in combination with KPT-185 in vitro (after topotecan), we examined the therapeutic effect of selinexor in combination with paclitaxel. After 4 weeks of treatment, mean tumor weight and the number of tumor nodules in mice treated with selinexor alone or selinexor plus paclitaxel were significantly lower than in mice treated with vehicle; mean tumor weight in mice treated with selinexor in combination with paclitaxel was significantly lower than in mice treated with paclitaxel alone (Fig. 4E and F). These in vivo experiments showed a significant difference in mean tumor weight between topotecan monotherapy and selinexor plus topotecan (Fig. 4A and C) or paclitaxel monotherapy and selinexor plus paclitaxel (Fig. 4E), and suggest that selinexor has at least additive effects in combination with chemotherapy agents.

To investigate the biologic effects of selinexor, we used the A2780 tumor model to examine apoptosis (caspase 3) and proliferation (Ki67) of tumors. The percentage of caspase 3–positive cells was higher in mice treated with selinexor alone, topotecan alone, and selinexor plus topotecan than in mice treated with vehicle (Fig. 4G). Fewer Ki67-positive cells were observed in tumors from mice treated with selinexor alone, topotecan alone, and selinexor plus topotecan than in mice treated with vehicle (Fig. 4H). A similar pattern was observed in the A2780CP20 and SKOV3 tumor models (Supplementary Fig. S4D and S4E).

To investigate the therapeutic effect of selinexor in other cancer types, we used the MDA-MB-231 triple-negative breast cancer orthotopic mouse model (39). In this model, selinexor alone showed the same antitumor effect as paclitaxel alone, and an increased antitumor effect was observed with selinexor plus paclitaxel (Fig. 4I). Immunohistochemical staining of caspase 3 and Ki67 showed that MDA-MB-231 tumors treated with selinexor alone, paclitaxel alone, and selinexor plus paclitaxel had a higher percentage of apoptotic cells and a lower percentage of cell proliferation than tumors treated with vehicle (Supplementary Fig. S4F). Moreover, tumors treated with selinexor plus paclitaxel showed less cell proliferation than tumors treated with paclitaxel alone.

As many patients with ovarian cancer have a large tumor burden, we conducted in vivo therapeutic experiments using bioluminescence imaging to determine whether treatment with selinexor can reduce the volume of large tumors. For these experiments, we initiated treatment 18 days after SKOV3-Luc tumor cell injection, after confirming the tumor signal using bioluminescence. Compared with tumors in the control group,
tumors treated with selinexor alone, topotecan alone, and selinexor plus topotecan showed a significantly lower photon count at 43 days after tumor cell injection (Fig. 5A and B). Interestingly, the photon counts of tumors from mice treated with topotecan alone started increasing at day 43. Mice were sacrificed at day 43 owing to tumor burden in the control group, and the mean tumor weight and number of tumors in mice treated with selinexor alone, topotecan alone, and selinexor plus topotecan were lower than in mice treated with vehicle (Fig. 5C and D). Increased percentage of apoptotic cells and decreased percentage of proliferative cells were seen in tumors from mice treated with selinexor alone, topotecan alone, and selinexor plus topotecan than in mice treated with vehicle (Supplementary Fig. S5A and S5B). Interestingly, tumors treated with topotecan alone showed a higher percentage of proliferative cells than tumors treated with selinexor alone and selinexor plus topotecan (Supplementary Fig. S5B). These results suggest that some tumor cells may have become resistant to topotecan in mice treated with topotecan alone at day 43, but tumors in mice treated with selinexor plus topotecan were still sensitive to treatment.

Next, to investigate the efficacy of selinexor on survival, we used the A2780 mouse model. After 7 days, mice were randomized to

Figure 4. Selinexor (KPT-330) monotherapy and combination therapy with cytotoxic agents show therapeutic effects in ovarian and breast cancer orthotopic mouse models. A–F, mean tumor weight (A, C, E) and number of tumor nodules (B, D, F) in mice treated with vehicle (Control), selinexor (KPT), topotecan (TOPO), or KPT plus TOPO (KPT + TOPO) in A2780 (A and B) and A2780CP20 (C and D) models, and in mice treated with Control, KPT, paclitaxel (PTX), or KPT plus PTX (KPT + PTX) in a SKOV3 (E and F) model 7 days after tumor cell injection. Error bars, SEM; *, P < 0.05; ***, P < 0.001; n = 10. G and H, immunohistochemical staining for caspase 3 (G) and Ki67 (H) in tumor specimens obtained from the A2780 orthotopic model (A and B). Error bars, SEM; ***, P < 0.001; n = 10. I, mean tumor weight in mice treated with vehicle, KPT, PTX, or KPT + PTX 7 days after tumor cell injection in a breast cancer model (MDA-MB-231). Error bars, SEM; *, P < 0.05; ***, P < 0.001; n = 10.
Figure 5.
Effects of selinexor (KPT-330)-based therapy in ovarian cancer models. A, bioluminescence images of tumors in mice treated with vehicle (Control), selinexor (KPT), topotecan (TOPO), or KPT plus TOPO (KPT + TOPO) at days 18, 25, 32, 39, and 43 after tumor cell injection. B, photon measurements from bioluminescence in the mice shown in A. Error bars, SEM; ***, P < 0.001; n = 10. C and D, tumor weight (C) and number of tumor nodules (D) 43 days after tumor cell injection. Error bars, SEM; **, P < 0.01; ***, P < 0.001; n = 10. E, survival curves of A2780 model mice treated with vehicle (Control), topotecan (TOPO), selinexor or selinexor plus TOPO (n = 7/group). The log-rank test was used to calculate P value. F, the mean number of tumor nodules at the primary (ovary) and metastatic sites.
treatment with selinexor, topotecan, or combination of selinexor and topotecan. We found that selinexor + topotecan treatment resulted in significantly prolonged survival (Fig. 5E). We also examined the effects of selinexor-based therapy on tumor metastasis using the A2780 model in which the cancer cells are injected directly into the ovary. We found that mice treated with selinexor-based therapy had significant reduction in metastases (Fig. 5F and Supplementary Fig. S5C).

**Discussion**

In this study, we observed that SINE compounds KPT-185 and selinexor (KPT-330, a closely related, orally bioavailable drug candidate in clinical trials) showed antitumor effects in ovarian and breast cancer models in vivo and in vitro. The SINE compounds were also useful when combined with other chemotherapeutic agents, such as topotecan and paclitaxel, in terms of synergistic efficacy. Selinexor is currently being evaluated in three phase I studies in patients with advanced solid tumor and hematologic malignancies. Therefore, our results suggest that XPO1 inhibition by SINE might provide a novel therapy for patients with ovarian cancer as single agent or in combination with other standard therapies. The inhibition of nuclear export of tumor suppressor proteins, which leads to the accumulation of high nuclear levels and reduced cytoplasmic degradation of these proteins, has been thought to be a major mechanism of the antitumor effect of SINE compounds. In contrast, our in vitro data suggest that the antitumor effects of SINE compounds might be tumor suppressor protein independent. Our proteomic data showed that eIF5A expression was markedly increased in the mitochondria of ovarian cancer cells after treatment with KPT-185 (Fig. 6). eIF5A is known to be an essential protein for cell survival and proliferation, and it is posttranslationally modified by hypusination (13, 40–44). Modification of eIF5A by hypusination has been well studied, but other regulatory mechanisms of eIF5A, as well as how eIF5A induces apoptosis, remain unclear. To our knowledge, this is a previously unrecognized mechanism where inhibition of XPO1 causes eIF5A accumulation in the mitochondria and leads to the induction of apoptosis.

"IGF2BP1 Regulates eIF5A and Apoptosis"

Figure 6. Model of eIF5A regulation in response to inhibition of the nuclear export system. Under normal conditions, IGF2BP1 is exported by XPO1 and binds to eIF5A in cytoplasm (A). The binding with IGF2BP1 prevents eIF5A from accumulating in mitochondria. SINE inhibits the nuclear export of IGF2BP1, resulting in decreased cytoplasmic IGF2BP1 (B). Unbound eIF5A accumulates in mitochondria and induces apoptosis.
binding protein of eIF5A and regulates eIF5A function in apoptosis (Fig. 6). Our data represent a new understanding of eIF5A regulation in response to inhibition of the nuclear export system, providing a biologically plausible and potent novel potential therapeutic strategy supporting the further exploration of XPO1 inhibition for the treatment of ovarian and other cancers. Moreover, the data presented here show that the mechanism of antitumor effect of XPO1 inhibitor differs from chemotherapeutic agents that are commonly used in ovarian cancer treatment. Our data demonstrated that XPO1 inhibitor is a potent therapeutic agent, especially in combination with topotecan or paclitaxel. Furthermore, selinexor treatment can prolong survival in combination with chemotherapy and inhibit metastasis. These results identify opportunities for future clinical trials for cancer patients.

Disclosure of Potential Conflicts of Interest

D. McCaulley holds ownership interest (including patents) in Karyopharm Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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