XPO1 Inhibition Enhances Radiation Response in Preclinical Models of Rectal Cancer

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Abstract

Purpose: Combination of radiation with radiosensitizing chemotherapeutic agents improves outcomes for locally advanced rectal cancer. Current treatment includes 5-fluorouracil–based chemoradiation prior to surgical resection; however pathologic complete response varies from 15% to 20%, prompting the need to identify new radiosensitizers. Exportin 1 (XPO1, also known as chromosome region 1, CRM1) mediates the nuclear export of critical proteins required for rectal cancer proliferation and treatment resistance. We hypothesize that inhibition of XPO1 may radiosensitize cancer cells by altering the function of these critical proteins resulting in decreased radiation resistance and enhanced antitumor effects.

Experimental Design: To test our hypothesis, we used the selective XPO1 inhibitor, selinexor, to inhibit nuclear export in combination with radiation fractions similar to that given in clinical practice for rectal cancer: hypofractionated short-course radiation dosage of 5 Gy per fraction or the conventional long-course radiation dosage of 1 Gy fractions. Single and combination treatments were tested in colorectal cancer cell lines and xenograft tumor models.

Results: Combination treatment of radiotherapy and selinexor resulted in an increased of apoptosis and decrease of proliferation compared with single treatment, which correlated with reduced tumor size. We found that the combination promoted nuclear survivin accumulation and subsequent depletion, resulting in increased apoptosis and enhanced radiation antitumoral effects.

Conclusions: Our findings suggest a novel therapeutic option for improving radiation sensitivity in the setting of rectal cancer and provide the scientific rationale to evaluate this combination strategy for clinical trials. Clin Cancer Res; 22(7); 1663–73. ©2015 AACR.

Introduction

Rectal cancer is a major health problem around the world, representing about one-third of the total colorectal cancer cases (1). The lack of serosa covering the rectum and the proximity of the rectum to other pelvic organs commonly leads to locally advanced disease. Moreover, due to their anatomic location, surgical resections are more challenging for rectal cancer compared with colon cancer, increasing the risk of local recurrence (2, 3). The modern treatment strategies for stage II to III of rectal cancer involve the combination of neoadjuvant radiation therapy with concurrent 5-fluorouracil (5-FU)-based chemotherapy to shrink the tumor and to improve local control without increasing morbidity (4, 5). There is, however, in need to improve on this approach as the pathologically complete response (pCR) varies from 15% to 20% after completion of 5-FU–based neoadjuvant chemoradiotherapy, and one-third of the patients die within 5 years (6–8). The expression of antiapoptotic proteins in response to radiation, the normal tissue toxicity, and the formation of toxic byproducts are significant problems that limit the clinical use of the radiosensitizers (9, 10). For these reasons, it is important to identify new radiosensitizers that are able to counteract the antiapoptotic proteins upregulated by radiation with minimal toxicity to normal tissues to enhance the therapeutic use of radiation.

Protein transport between the nucleus and the cytoplasm is critical for normal cell homeostasis. Exportin 1 (XPO1, also known as chromosome region 1, CRM1) is required for transporting cargo proteins with nuclear export sequences (NES) from the nucleus to the cytoplasm (11–13). Specifically, the XPO1–cargo complex is transported through the nuclear pore complex to the cytoplasm, where the cargo is released after Ran–GTPase–activating protein (GAP)–catalyzed GTP hydrolysis (14, 15). XPO1–mediated shuttling of their cargo proteins is normally tightly controlled, but it is often deregulated in cancer cells (16). XPO1 transports over 200 cargo proteins including tumor suppressor (TSP) and different regulatory proteins such as survivin, etc (12–14, 17). Most of these proteins are only functional, capable of preventing cancer initiation and progression, when they are properly localized inside the nucleus. However, they are often mutated, deleted, or aberrantly located within the cytosol in cancer cells; therefore, changes in the nuclear/cytoplasmic transport of these proteins can modify the survival and proliferation of...
Translational Relevance

This report evaluates the radiosensitizing effects of XPO1 inhibitor (selinexor, KPT-330) in preclinical models of rectal cancer. Selinexor is a first-in-class SINE XPO1 antagonist being evaluated in multiple registration-directed and other later-stage trials in patients with relapsed and/or refractory hemato logic and solid tumor malignancies. We and others have previously reported that XPO1 inhibition has antiproliferative and proapoptotic activity in several cancer types. The data presented show that combining selinexor with radiation greatly reduces cancer cell viability, increases apoptosis, and slows tumor growth. Mechanistically, we show that selinexor synergizes with radiation by promoting nuclear accumulation and subsequent depletion of survivin. These findings provide a novel therapeutic option for improving radiation sensitivity in the setting of rectal cancer. On the basis of these findings, our co-authors from our radiation oncology department are committed to evaluate this combination in a phase I/II clinical trial.

cancer cells (18–21). The XPO1 target survivin (also known as baculoviral IAP repeat containing 5, BIRC5) is a member of the inhibitor of apoptosis family (22), and it is considered as an important radiation resistance factor in rectal cancer (23–28). The expression of survivin is associated with an unfavorable local control rate after radiotherapy (29, 30). XPO1 plays an essential role in the function of survivin, as its cytoplasmic localization is critical to survivin nuclear export and subsequent depletion of survivin. These findings provide a novel therapeutic option for improving radiation sensitivity in the setting of rectal cancer. On the basis of these findings, our co-authors from our radiation oncology department are committed to evaluate this combination in a phase I/II clinical trial.

Materials and Methods

Cell lines and reagents

As there is not a specific human rectal cancer cell line, we used the human colorectal cancer cell lines LoVo, HT29, HCT116, and SW620 as in vitro models. LoVo and HT29 cell lines were purchased from and tested by the ATCC in July 2013. HCT116 and SW620 cell lines were purchased and tested by the ATCC in 2011. All cell lines were expanded, frozen, and used for experiments within 4 months of cell culture after receiving them and their authentication was performed by ATCC. The cell lines were grown in different conditions recommended by ATCC: McCoy (HCT116 and HT29), Leibovitz (SW20), and F-12K (LoVo) medium, supplemented with 10% FBS (Gibco), 100 U/mL penicillin, and 10 μg/mL streptomycin (Invitrogen). LoVo, HT29, and HCT116 cell lines were maintained in a humidified atmosphere of 5% CO2 at 37°C, while SW620 was kept without CO2. Selinexor was obtained from Karyopharm Therapeutics, Inc. A 10-nmol/L stock solution dissolved in DMSO was diluted in medium to the final concentrations indicated. Cells were transfected with siRNA to survivin (#6546) or two different siRNA controls using Lipofectamine 2000. 6MYC-survivin was a kind gift from H. Cheung (University of Ottawa, Ottawa, Ontario, Canada).

Cell viability and isobologram analysis

A total of 1 × 104 cells/well were seeded in 96-well plates and incubated overnight. Cells were treated with fixed doses of selinexor ranging below and above their IC50 (concentration of a compound where 50% of its maximal effect is observed): 100, 350, 500, 750, 1,000, 1,500, 1,750, 2,500, and 4,000 nmol/L and/or fixed doses of radiation: 1, 5, and 10 Gy and further incubated for 72 hours. Thereafter, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, 5 mg/mL) was added to each well, and the cells were incubated for another 4 hours at 37°C. After the removal of the culture medium, the cells were lysed in 200 μL of DMSO, and the optical density (OD) was measured at 570 nm using a microplate reader (Bio-Rad). To calculate cell viability, the following formula was used: cell viability = (OD of the experimental sample/OD of the untreated group) × 100%. Assay values obtained in eight replicates were used to derive the IC50 values by curve fitting to the Hill equation using the GraphPad Prism software. To compare the antitumor effect of single-agent treatments with the combination treatment, synergy was determined by the isobologram analysis derived from the median-effect principle of Chou and Talalay (37) using the Compusyn software. Compusyn calculates a combination index (CI) at different concentrations, using the formula for mutually nonexclusive mechanisms: CI = [(D1/Dx1) + (D2/Dx2) + (D1 × D2/Dx1 × Dx2)], where Dx1 and Dx2 are selinexor and radiation doses, respectively, that are required to achieve a particular fraction affected, and D1 and D2 are the doses of the two agents (combined treatment) required for achieving the same fraction affected. CI values 1, more than 1, and less than 1 indicate additive, antagonistic, and synergistic interactions, respectively. CI = 1 is represented with a line in the graph, and the distribution of the experimental combination data points below the expected additive line indicates that the combination results synergistic.

Caspase-3/7 activity

A total of 1 × 104 cells/well were seeded in 96-well plates and incubated overnight. Cells were treated with serially diluted selinexor ranging from 0 to 3,000 nmol/L and further incubated for 48 hours. Caspase-3/7 activity was determined using the Caspase-Glo 3/7 Assay (Promega), and luminescence was measured on a Wallac Victor2 multi-label plate reader (Perkin-Elmer) according to the manufacturer’s instructions.

Annexin V–FITC/PI assay

For Annexin V–FITC/PI (propidium iodide) assay, we used the Apoptosis Detection Kit (BD Biosciences). Cells were treated with 0.9 μmol/L of selinexor in HT29 and 0.4 μmol/L in LoVo, exposed to a single dose of radiation (0–5 Gy) or both for 48 hours. Both adherent and floating cells were collected in PBS for the assay. The...
cells were suspended in 1 mL of FITC–Annexin V solution, and PI was added to a final concentration of 1 mg/mL. The samples were analyzed by flow cytometry (Becton Dickinson) using blue light excitation. The experiment was done in triplicate.

Clonogenic survival

Sensitivity to selinexor or radiotherapy was assessed using clonogenic survival assays. Controls were treated with DMSO or mock irradiated. For combination studies, the cells were seeded at a density of approximately $1 \times 10^5$ cells per well in 6-well plates and left to adhere before being exposed to a single dose of radiation (0–5 Gy) and/or treatment with selinexor (0–1.5 μmol/L) for 24 hours. After treatment, cells were washed twice with PBS before being replenished with fresh drug-free medium and incubated for approximately 10 to 14 days to allow colonies to form. Colonies were fixed, stained [0.5% (w/v) crystal violet in 5% acetic acid, 20% H2O, and 75% methanol], and (≥50 cells) counted. Replica dishes usually containing 50 to 200 colonies per well were manually counted for each treatment. The surviving fraction (SF) was calculated as mean colonies/[cells inoculated × plating efficiency]. Survival curves for each experiment were constructed by plotting the mean surviving fractions semilogarithmically as a function of irradiation dose. The data were analyzed, and survival curves were plotted following the linear quadratic (LQ) model $SF = e^{-aD^{0.5}+bD^2}$ using GraphPad Prism 5.0 software, where SF is the surviving fraction and D is the radiation dose. At least three parallel samples were scored in three to five repetitions performed for each treatment condition. All data described apply to exponentially growing cells. Cell survival at each dose of each irradiation protocol was determined by dividing the plating efficiency of the irradiated cells by that of the untreated control.

Protein extraction and cell fractionation

Whole-cell lysates were prepared using RIPA lysis buffer, and separation of nuclear and cytoplasmic protein fraction was prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) following the manufacturer’s instructions.

Western blot analysis

Protein (25 μg) was separated by 4–12% Bis-Tris NuPAGE MES Precast Gel (Invitrogen), transferred to polyvinylidene difluoride membranes, and blocked in 5% BSA for 45 minutes at room temperature. After blocking, the membrane was incubated overnight at 4°C with primary antibodies for survivin (#2808), Gapdh (sc-25778), β-actin (sc-4778), anti-Myc (46-0603), Parp (#9542), cParp (#9541S), and p53 (sc-98). The membranes were washed in TBS-T and incubated with appropriate secondary antibodies: anti-mouse (sc-2371), anti-rabbit (sc-2313), or anti-goat (sc-2020) at room temperature for 1 hour. Protein–antibody complexes were visualized using the enhanced chemiluminescence kit (Thermo Fisher Scientific). Densitometry was done using ImageJ software (NIH, Bethesda, MD).

mRNA extraction and quantitative RT-PCR

Total mRNA was isolated from HT29 and LoVo cells using RNeasy Mini Kit (Qiagen). CDNA synthesis was performed with 2 μg of total RNA using ThermoScript RT-PCR System (Invitrogen). Quantitative PCR was performed using Fast SYBR Green Master Mix (Invitrogen) with the use of the Light Cycler 96 (Roche). Reactions were run in triplicate in three independent experiments. Expression data were normalized to the geometric mean of housekeeping gene GAPDH to control the variability in expression levels and were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Xenograft mouse models

Because of the lack of rectal cancer cell lines, we used the colorectal cancer cell line LoVo for the xenograft tumor model. A total of $2 \times 10^6$ of LoVo cells were injected into subcutaneous tissue of right side of flank of 6-week-old female Foxn1 (nude) mice (Taconic), and the treatment was initiated when tumor volume reached around 150 to 200 mm³. Tumor-bearing mice were randomly assigned ($n = 7$ per each group). Once tumors were established, mice were divided into six different cohorts and treated with clinically relevant radiation fractions similar to that given in clinical practice for rectal cancer and/or selinexor. The treatments were the following: (i) vehicle alone, (ii) selinexor (10 mg/kg), (iii) hypofractionated short-course radiation dosage of 5 Gy per fraction for 5 consecutive days (M, T, W, T, and F), (iv) conventional long-course radiation dosage of 1 Gy fractions for 5 consecutive days (M, T, W, T, and F), (v) combination of short-course radiation with selinexor, (vi) or long-course radiotherapy with selinexor. Selinexor (10 mg/kg) was delivered by oral gavage every other day for 1 week (M, W, and F). Selinexor was formulated in aqueous 0.5% w/v Pluronic F-68 and 0.5% w/v polyvinyl pyrrolidone K-29/32 diluents. Vehicle was used for the corresponding control group. Mice were irradiated using X-Rad 320 machine. Tumor dimensions were measured every 2 days for 60 days and tumor volume was estimated using the following formula: volume = (length x width x width)/2. Mice were euthanized when the largest tumor diameter reached 15 mm, or when mice fell into euthanasia criteria. All experiments were done in full compliance with institutional guidelines and with the approval of the Massachusetts General Hospital (MGH, Boston, MA) Subcommittee on Research Animal Care.

Statistical analysis

Comparisons between two groups were performed using a Student t test. P values less than 0.05 were considered statistically significant. Calculations were performed using GraphPad Prism software.

Results

XPO1 inhibition synergizes with radiation in in vitro models of colorectal cancer

To evaluate the efficacy of the XPO1 inhibitor, selinexor, as a radiosensitizer in rectal cancer, we first assessed its effect alone on cell viability by MIT assay in a panel of four colorectal cancer cell lines: HT29, LoVo, HCT116, and SW620. We observed that selinexor potently inhibited the growth of the four different cell lines, HT29, LoVo, HCT116, and SW620 with IC50 values of 0.9, 0.4, 0.27, and 0.30 μmol/L, respectively, after 72 hours of treatment (Fig. 1A). Next, we sought to determine whether the observed decrease in cell viability by selinexor treatment was attributable to the induction of the apoptotic pathway. Consequently, we measured caspase-3/7 activity in LoVo and HT29 cell lines treated with selinexor for 48 hours as an apoptosis surrogate marker. Selinexor treatment, indeed, increased caspase-3/7 activity in both cell lines in a
dose-dependent manner ($P < 0.05$; Supplementary Fig. S1A). Moreover, Western blot analysis showed that selinexor induced cleavage of Parp, a well-established biochemical marker of apoptosis, in both HT29 and LoVo cells in a time-dependent manner (Supplementary Fig. S1B). Therefore, selinexor has an important proapoptotic and antiproliferative effect in colorectal cancer cell model systems.

To evaluate whether the combination of selinexor and radiation results synergistic, additive, or antagonistic, we performed isobologram analysis using CompuSyn software (37). HT29, LoVo, HCT116, and SW620 colorectal cancer cell lines were treated with varying doses of selinexor, and cell viability was assessed by MTT assay at 72 hours after treatment. B, normalized isobolograms at nonconstant combination ratio of HT29, LoVo, HCT116, and SW620 cells are shown. Each data point represents the mean of three separate experiments.

![Figure 1. XPO1 inhibition synergizes with radiation in *in vitro* models of colorectal cancer. A, HT29, LoVo, HCT116, and SW620 colorectal cancer cell lines were treated with varying doses of selinexor, and cell viability was assessed by MTT assay at 72 hours after treatment. B, normalized isobolograms at nonconstant combination ratio of HT29, LoVo, HCT116, and SW620 cells are shown. Each data point represents the mean of three separate experiments.](image)

Combination of selinexor and radiation results in an increase of apoptosis

We then examined the effects of the combination treatment of selinexor and radiation by their ability to form colonies using clonogenic assay (Fig. 2A and Supplementary Fig. S2). Selinexor is metabolized in 24 hours in the human body; therefore, for further translational application of our *in vitro* data, HT29 and LoVo cells were treated with selinexor or DMSO for 24 hours, subjected to one single dose of radiation (1 or 5 Gy), and cultured in new media for approximately 2 weeks. The results from the clonogenic assays are represented by kill curves that show the combination of radiation with XPO1 inhibition synergistically decreases cell proliferation in HT29 and LoVo cell lines in a dose-dependent manner (Fig. 2A). To study whether the radiation-enhancing effects of XPO1 inhibitor are due to an increase of apoptosis, we evaluated the onset of apoptosis in HT29 and LoVo cell lines using higher doses of drug (2.5–5 μmol/L), indicating that therapeutic doses of selinexor have a strong radiosensitizing effect on different colorectal cancer cell lines.
three different assays: Annexin-V levels, caspase-3/7 activation, and measurement of cParp levels by Western blot analysis (Fig. 2B). In all experiments, HT29 and LoVo cells were treated with selinexor or DMSO for 24 hours, 5 Gy, or concurrent combination. We observed that the combination of selinexor and radiation resulted in a greater increase in apoptosis in the two colorectal cancer cell lines compared with either single treatments of selinexor or radiation alone, as measured by Annexin-V FACS and caspase-3/7 activation. An increase in cParp was also observed in cell lines treated with concurrent combination of selinexor and radiation versus single treatments. Therefore, selinexor appears to have a radiosensitizing effect on colorectal cancer cell lines possibly through activation of the apoptotic pathway.

Combination of XPO1 inhibition and radiation slows colorectal cancer tumor growth in vivo

To test the effects of the combination of selinexor and radiation in tumor growth in vivo, we used the colorectal cancer cell line, LoVo, to generate human xenograft tumors in nude mice. Once tumors were established, mice were divided into six different cohorts and treated with clinically relevant radiation doses and/or selinexor. The treatments were the following: (i) vehicle alone, (ii) selinexor (10 mg/kg), (iii) hypofractionated short-course radiation dosage of 5 Gy per fraction, (iv) conventional long-course radiation dosage of 1 Gy fractions, (v) combination of short-course radiation with selinexor, (vi) or long-course radiation with selinexor. Treatment schedule is represented in
Supplementary Fig. S3A. Treatment was stopped after 5 days, and tumor growth was observed in all treatment groups (Fig. 3A). Changes in tumor volume from day 1 to day 40 of treatment are shown in Fig. 3B. Statistically significant differences were observed between the single-treatment groups and the groups treated with selinexor and radiation independently of the combination. Survival for the combination group was statistically longer than for either single therapy. Of note, 20% of the mice treated with 5 Gy and selinexor showed complete disappearance of the tumor. As single therapy and in combination, both inhibitors were well tolerated with no significant effect on animal weight (Supplementary Fig. S3B). Together, these data show that selinexor in combination with radiation results in a decrease of tumor size.

Combination of radiation and XPO1 inhibitor results in nuclear retention of survivin promoting survivin loss

Survivin is a well-known XPO1 target that results upregulated in response to radiation, and it mediates resistance (25, 30). Its nuclear localization correlates with enhanced survival in colorectal cancer patients, whereas its cytoplasmic localization is responsible for the resistance to radiation due to its antiapoptotic and tumor-promoting functions (28). Impairing the export of survivin to the cytoplasm by the inhibition of XPO1 with Leptomycin or other SINE compounds results in survivin loss (28, 38). On the basis of these studies, we hypothesize that the inhibition of XPO1 with selinexor will abrogate the upregulation of survivin cytoplasmic levels in response to radiation and, therefore, it will decrease the resistance to radiation in the radiated tumors. To test our hypothesis, we evaluated the impact of the combination treatment of 0.4 μmol/L of selinexor at different time points and/or a single dose of radiation of 5 Gy for 24 hours in LoVo cells at three different levels: total survivin protein (Fig. 4A), survivin mRNA (Fig. 4B), and the nuclear and cytoplasmic survivin protein levels (Fig. 4C and D). We first observed that 5 Gy of radiation provoked an increase of total survivin levels, but the combination of selinexor and radiation resulted in a depletion of total survivin protein over time (Fig. 4A), starting at 8 hours. This result indicates that selinexor is able to counteract the increased total protein levels of survivin after radiation. Moreover, the reduction of total survivin correlated with an increase of cParp protein levels, indicating that apoptosis is enhanced in the combination treatment. To better understand if XPO1 inhibition regulates the transcription of survivin or if its regulation takes place through a posttranscriptional mechanism, we examined the effect of selinexor combined with radiation on survivin mRNA levels. Single treatment with selinexor induced an initial increase of survivin mRNA at 15 hours, followed by a decrease over time as it is observed at 24 and 48 hours (Fig. 4B). The accumulation of nuclear survivin protein after treatment with selinexor may induce the production of mRNA survivin initially (until 15 hours after treatment), but it decreased dramatically at 24 and 48 hours due to transcription repression (38). On the other hand, selinexor combined with radiation significantly decreased the levels of survivin mRNA at 24 and 48 hours after treatment compared with radiation alone (Fig. 4B), indicating that treatment with selinexor impairs the transcription of survivin over time. Selinexor specifically inhibits XPO1, resulting in nuclear accumulation of XPO1 targets such as survivin [17]. To get a further insight into the effect of selinexor on nuclear and cytoplasmic levels of survivin over time, we prepared nuclear and cytosolic protein fractions in LoVo cells treated with or without 0.4 μmol/L of selinexor. A decrease in cytosolic survivin protein levels was observed as early as 3 hours and an increase in nuclear protein by 3 hours after treatment. Survivin continued to accumulate within the nucleus for 15 hours after treatment, and after this time point, it decreased dramatically both within this compartment and within the cytosol (Fig. 4C). These results suggest that XPO1 inhibition initially promotes survivin nuclear localization but at later time points leads to a reduction in total survivin protein levels in vitro, indicating that the nuclear accumulation of survivin may promote further survivin loss. To study the effect of the combination...
Figure 4.
Combination of radiation and XPO1 inhibitor results in nuclear retention of survivin promoting survivin loss. A, LoVo cells were treated with selinexor (0.4 μmol/L), RT (5 Gy), or combination after 24 hours. Protein lysates were analyzed by Western blot analysis for the indicated proteins. Below the immunoblot analysis, the quantification of the survivin protein levels normalized with Gapdh for each time point is shown. B, LoVo cells were treated with selinexor (0.4 μmol/L), RT (5 Gy), or combination, and survivin mRNA levels (mean ± SD of three independent experiments) relative to Gapdh were assessed by real-time analysis. C, subcellular fractionation and Western blot analyses of LoVo cells were performed after treatment with selinexor for the indicated times. Quantification of the survivin levels in the nucleus and cytoplasm after selinexor treatment over time is shown. D, subcellular fractionation and Western blot analyses of cells treated with selinexor (0.4 μmol/L), RT (5 Gy), or combination after 24 hours are shown. Lysates were immunoprecipitated with anti-survivin and β-actin and the immune-complexes were resolved by SDS-PAGE. The quantification of the nuclear and cytoplasmic survivin levels normalized with Gapdh is shown below the blot. Each data point represents the mean of three experiments.
treatment of selinexor and radiation on survivin nuclear export over time, we treated the LoVo cells with or without 0.4 μmol/L of selinexor, 5 Gy, or the combination and prepared nuclear and cytosolic protein fractions (Fig. 4D). The combination of XPO1 inhibitor with radiation provokes an increase of nuclear and a decrease of cytoplasmic survivin at 8 hours, which results in complete survivin loss at 24 hours (as we also observed in Fig. 4A). Importantly, the decrease in survivin levels observed in the combination treatment after 24 hours correlates with the timing of the cellular antitumor effects of this combination and therefore supports a hypothesis that XPO1 inhibition in combination with radiation leads to a loss of survivin protein, which then leads to inhibition of tumor cell growth and enhanced tumor cell apoptosis.

**Combination of radiation and XPO1 inhibitor results in decrease of total survivin in vivo**

To evaluate whether survivin plays a similar role in in vivo models of rectal cancer as we have observed in vitro in Fig. 4, tumors from mice xenografts that were treated with single or combination therapy were extracted, and total protein and mRNA levels were measured. Total survivin and cParp protein levels were analyzed by Western blot analysis (Fig. 5A). We observed that the mice treated only with radiotherapy (both 1 Gy and 5 Gy) showed higher levels of total survivin compared with those treated with selinexor alone or selinexor combined with radiation. Importantly, reduced levels of survivin correlated with high levels of cParp, which indicates an enhancement of apoptosis. To identify whether the reduced levels in total survivin protein observed in Fig. 5A resulted from a decrease of survivin transcription or a loss in the survivin protein stability, we measured the mRNA levels of survivin and Gapdh from the tumor sample at 24 hours and 48 hours after treatment. We observed that 24 hours and 48 hours after treatment, the mice treated with the combination of selinexor and radiation showed lower levels of survivin mRNA when compared with radiation alone (Fig. 5B and Supplementary Fig. S4). Importantly, 24 hours after treatment with selinexor, there is an increase of mRNA survivin levels relative to control mice, but it decreased dramatically at 48 hours (Figs. 4B and 5B and Supplementary Fig. S4). The accumulation of nuclear survivin protein after treatment with selinexor may induce the production of mRNA survivin initially, until 15 hours after treatment in vitro and until 24 hours after treatment in vivo (Figs. 4B and 5B, respectively). This result shows that selinexor initially induces survivin transcription at early time points, but over time impairs the transcription of survivin, which results in the decrease of protein expression.

![Graph](image-url)
Apoptosis resulting from the combination of XPO1 inhibition and radiation is dependent on survivin.

To determine whether the increase of apoptosis observed in the combination treatment of XPO1 inhibition and radiation was mediated by the survivin loss, we treated LoVo cells with two different siRNA survivin, 0.4 μmol/L selinexor, 5 Gy, or combination, and we measured apoptosis. Survivin knockdown resulted in an increase of cParp levels (Fig. 6A) and caspase-3 levels (Fig. 6B), indicating that the lack of survivin induces apoptosis in LoVo cells. Importantly, the combination of selinexor, 5 Gy, and siRNA-survivin showed a significant increase of apoptosis over the cells treated with selinexor, 5 Gy, and control siRNA, suggesting either a synergistic or additive effect of survivin loss on apoptosis mediated by XPO1 inhibition and radiotherapy (Fig. 6A and B).

To determine whether selinexor antagonizes the survivin-mediated antiapoptotic pathway activated by radiation, we performed experiments to assess whether survivin could rescue LoVo cells from selinexor- and selinexor and radiation-mediated apoptosis. To this end, we overexpressed a MYC-tagged survivin expression construct in LoVo cells and treated the cells with or without 0.4 μmol/L of selinexor, 5 Gy, or the combination of both. Exogenous expression of survivin protected the selinexor-treated cells or radiation-treated cells from apoptosis compared with the single treatments, as measured by cParp (Fig. 6C) and caspase-3 levels (Fig. 6D). Together, these results suggest that survivin plays a functional role in the increase of apoptosis mediated by selinexor combined with radiation in colorectal cancer cell lines.

**Discussion**

Our study provides a strong clinical rationale for the use of the XPO1 inhibitor, selinexor, as a novel radiosensitizer for rectal cancer. Selinexor, the first-in-class selective inhibitor of nuclear export to be developed for clinical use, is currently in phase I/II clinical trials. Preliminary results suggest that it is generally well-tolerated, with minimal side effects, and reversible and manageable with supportive care. Selinexor is metabolized through the liver in 24 hours. For that reason, our *in vitro* experiments using selinexor were generally performed after 24 hours of...
treatment. Marked synergy was observed when selinexor was used with a variety of chemotherapies and targeted therapies including platinum, B-Raf inhibitors, topoisomerase II inhibitors, proteasome inhibitors, etc (34, 39, 40). However, whether selinexor synergizes with radiation and its underlying mechanism has not been studied yet. In this study, we show that selinexor synergizes with clinically relevant doses of radiation in colorectal cancer cell lines and xenograft models. Selinexor likely synergizes with radiation by promoting nuclear accumulation of the antiapoptotic survivin and its subsequent depletion. Selective knockdown of endogenous survivin using specific siRNA resulted in an increase of apoptosis when combined to radiation compared with the siRNA control. On the other hand, overexpression of survivin by transient transfection resulted in dramatic resistance to radiation-induced apoptosis. These data strongly suggest that nuclear survivin plays a significant role in lethality induced by radiation.

While survivin is highly expressed during fetal development and is downregulated in most terminally differentiated normal tissues, the protein is found expressed in almost every human malignancy examined so far (41, 42). In patients with rectal cancer treated with the preoperative 5-FU-based radiochemotherapy, the expression of survivin protein was associated with unfavorable local control rates, increased risk of recurrences, lymph node metastases, and worse overall survival (25, 30, 43). It is well known that ionizing radiation significantly elevates survivin levels in malignant cells, and its expression has been correlated with elevated resistance to radiation and reduced frequencies of apoptosis (24–26). Several preclinical studies have suppressed survivin expression by the use of different approaches: antisense oligonucleotides, small-interfering RNAs, ribozymes, the application of dominant negative mutants, and the use of inhibitors of transcriptional regulators of survivin (44–47). They showed that survivin suppression increases apoptosis, diminishes tumor cell survival, and reduces tumor growth potential (25). Although survivin is an excellent candidate for anticancer therapies and efforts have been made to develop strategies that can functionally interfere with this molecule, the structural properties of survivin makes this protein "nondruggable", and additional efforts are currently underway (45).

Survivin is present in different subcellular compartments, in the cytosol, the mitochondria, and the nucleus, where it has distinct cellular functions (45). Cytoplasmic survivin predominantly mediates the antiapoptotic function, whereas nuclear survivin mediates the mitotic function and is significantly less stable (32, 48). Cytoplasmic survivin has been shown to be particularly high in rectal tumors and to be an independent predictor of poor prognosis, whereas nuclear survivin has been a favorable factor (38, 49, 50). As there is a need to decrease the cytoplasmic surviving levels resulted from radiation, and because nuclear survivin is suppressive for tumor growth, targeting the cytoplasmic (antiapoptotic) fraction of survivin would be an ideal therapeutic option.

In this study, we show that radiation increases the survivin cytoplasmic levels, and as survivin requires XPO1 for its nuclear exit, inhibiting the activity of this complex can directly address this therapeutic need by increasing the tumor-suppressive nuclear survivin and reducing the radiation-induced levels of cytoplasmic survivin. In line with that, this study shows that the accumulation of nuclear survivin in response to the XPO1 inhibitor correlates with higher levels of apoptosis. Moreover, we show that selinexor reduces tumor growth and it shows low toxicity when combined with therapeutic doses of radiation in in vivo models of rectal cancer.

Although other XPO1 targets may be involved in the synergy observed in the combination of XPO1 inhibitor and radiation, their role would be secondary, as we demonstrate the key role of nuclear survivin in the enhancing effects of selinexor as a radiosensitizer. Taken together, our results show that selinexor could be a useful adjunct for the treatment of rectal cancer, particularly for cancer patients that show resistance to radiation, and provide a scientific rationale to evaluate this combination strategy for clinical trials.

Disclosure of Potential Conflicts of Interest

V. Landesman and W. Senapedis have ownership interest (including patents) in Karyopharm Therapeutics, Inc. J.C. Cusack reports receiving commercial research grants from Karyopharm Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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References

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