XPO1 (CRM1) inhibition represses STAT3 activation to drive a survivin-dependent oncogenic switch in triple negative breast cancer

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Running Title: XPO1 inhibition represses STAT3. Key Words: CRM1, XPO1, SINE, survivin, STAT3. Abbreviations: CRM1/XPO1, exportin 1; SINE, selective inhibitors of nuclear export; STAT3, signal transducer and activator of transcription; CBP, CREB-binding protein; TNBC, triple negative breast cancer; XIAP, X-linked inhibitor of apoptosis protein; NES, nuclear export sequence.

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Abstract

Inhibition of XPO1 (CRM1)-mediated nuclear export of multiple tumor suppressor proteins has been proposed as a novel cancer therapeutic strategy to turn off oncogenic signals and enhance tumor suppression. Survivin is a multifunctional protein with oncogenic properties when expressed in the cytoplasm that requires the XPO1-RanGTP complex for its nuclear export. We investigated the anti-tumor mechanisms of the drug-like selective inhibitors of nuclear export (SINE) XPO1 antagonists, KPT-185, KPT-251 KPT-276, and KPT-330 in estrogen-receptor positive and triple negative breast cancer (TNBC) cell lines and xenograft models of human breast tumors. KPT compounds significantly inhibited breast cancer cell growth and induced tumor cell death, both in vitro and in vivo. These drugs initially promoted survivin accumulation within tumor cell nuclei. However, their major in vitro effect was to decrease survivin cytoplasmic protein levels, correlating with the onset of apoptosis. XPO1 inhibition repressed Survivin transcription by inhibiting CBP-mediated STAT3 acetylation, and blocking STAT3 binding to the Survivin promoter. Additionally, caspase-3 was activated to cleave survivin, rendering it unavailable to bind XIAP and block the caspase cascade. Collectively, these data demonstrate that XPO1 inhibition by SINE compounds represses STAT3 transactivation to block the selective oncogenic properties of survivin and supports their clinical use in triple negative breast tumors.
Introduction

XPO1, one of seven nuclear exportin proteins required for all mammalian cells, shuttles ~220 diverse proteins and some RNAs from the nucleus to the cytoplasm (1-4). XPO1 interacts with cargo proteins through a nuclear export sequence (NES) that is dependent on the small GTPase protein Ran in its GTP-bound form, which binds to XPO1 together with export cargos (2). NES-containing cargo proteins include multiple transcription factors and tumor suppressor proteins that play key roles in the growth and survival of cancer cells. Interestingly, XPO1 is the sole nuclear export protein for the major tumor suppressor proteins [e.g. p53 (5), STAT3 (6), survivin (7), FOXO3 (8)]. Many of these proteins lose their tumor suppressor function when they are exported out of the nucleus. Therefore, inhibiting XPO1, leading to forced nuclear localization, accumulation and activation of tumor suppressor proteins, is considered a potential therapeutic target for anti-cancer drug development.

Survivin is a multi-functional protein with its major oncogenic property being inhibition of caspase-dependent apoptosis that it accomplishes, in part, by stabilizing XIAP in the cytoplasm of tumor cells (9). Survivin is highly expressed in breast tumor cells and is one of the genes profiled on OncoDx and Mammoprint as a predictor of clinical response to therapy (10). Survivin export from the nucleus to the cytoplasm is mediated by the XPO1-Ran-GTP complex (7, 11); cytoplasmic localization is required for survivin’s anti-apoptotic and tumor-promoting functions (12-14). Disruption of the survivin NES leads to enhanced susceptibility to anti-cancer treatments (12, 14). One mechanism of targeting survivin’s selective cytoplasmic function without affecting its anti-tumor nuclear effects could be to inhibit its cytoplasmic export.
STAT3 is a member of the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) that is constitutively activated in multiple cancer types (15), including triple-negative breast cancer (TNBC). STAT3 activation in these tumors leads to increased expression of anti-apoptotic proteins, including survivin (16, 17), and other proteins to enhance cell proliferation, induce angiogenesis, and suppress immune responses. Thus, STAT3 is a potential high-yield target for drug development to treat TNBC for which there are no currently approved molecular therapies. Although several small-molecule STAT3 inhibitors have been reported (18-20), thus far none are in clinical trials due to pharmacokinetic and other problems. Interestingly, STAT3 has at several NES elements through which it binds to XPO1 for its nuclear export (21).

Leptomycin B (LMB) was the first natural XPO1 inhibitor discovered that was shown to be a potent anti-cancer agent (22). This agent failed in an early clinical trials due to off-target side effects predictable from animal toxicity testing, prompting the development of more selective and less toxic XPO1 inhibitors (23). The recent crystal structure of LMB and XPO1 shows that LMB binds covalently to Cys-528 in the XPO1 NES-binding groove, occupying the majority of the groove and undergoing hydrolysis by XPO1(24). Newer small-molecule, Selective Inhibitors of Nuclear Export (SINE) XPO1 antagonists developed by Karyopharm Therapeutics bind similarly in the NES groove, however due to their smaller size, these molecules occupy less space and are more specific for XPO1, with no detectable binding to other proteins (24). X-ray crystal structures of SINEs bound to XPO1 have been published and confirm covalent modification of Cys528 (24). SINEs have been demonstrated to reduce tumor growth with good tolerability in several pre-clinical models of hematologic cancers and solid
tumors (25, 26). The exact molecular mechanism of their anti-tumor effects in different cancer subtypes is not yet well-defined.

We sought to characterize the effects of XPO1 inhibition on survivin using a breast cancer model and the SINE compounds. In vitro and in vivo assays demonstrated that these drugs potently inhibit breast cancer growth by blocking proliferation and enhancing cell death pathways, as demonstrated for other tumor types. Importantly, here we show that these compounds can dramatically repress Survivin transcription by blocking STAT3 transactivation and that they enhance survivin degradation through cleavage by caspase-3. These results identify survivin- and STAT3-dependent mechanisms for the XPO1 inhibitors that may explain their in vivo anti-tumor effects. We also note that XPO1 inhibitors force the nuclear retention of tumor suppressor proteins p53, FOXO and others (25). Together, these data show that XPO1 inhibition confers very different effects on oncogenic drivers (i.e., cytoplasmic survivin, survivin degradation) compared with growth regulatory proteins (i.e., nuclear p53, growth suppressive nuclear survivin). These findings suggest that nuclear export could represent an important switch between oncogenesis and tumor suppression.
Materials and Methods

Cell lines and reagents

All tumor cell lines were purchased from and tested by the American Type Culture Collection. Cell lines were passaged less than six months for these studies. XPO1 inhibitors (KPT-185cis, KPT-185trans, KPT-276, KPT-330) were obtained from Karyopharm Therapeutics, Inc. A 10mM stock solution dissolved in DMSO was diluted in medium to the final concentrations indicated. To inhibit protein translation, cells were treated with 20μg/ml cyclohexamide (Sigma-Aldrich). To inhibit proteasome-mediated degradation, cells were treated with 2μM MG132 (SelleckBio.com) for 18h or 40μM MG132 for 6h. To inhibit caspase-3 and caspase-9, cells were treated with 20 μM of inhibitors for 4 hours. 6× MYC-survivin was a gift from H. Cheung. Cells were transfected with siRNA to XPO1 (Ambion/Invitrogen), survivin (Cell Signaling), XIAP (Thermo Scientific Dhharmacon) or control (Thermo Scientific Dhharmacon).

Cell viability and apoptosis assays

Cells were seeded at a density of 5000 cells/well then treated at the doses indicated. 72 hours later, cells were incubated with CellTiter 96 Aqueous One Solution Reagent (Promega), and viability was assessed using a Synergy Mx Monochromator (BioTek Instrument Inc.). Flow cytometry-based apoptosis assays were performed with the Annexin V-FITC Apoptosis Kit (Biovision), following the manufacturer’s instructions. Multiwell plate apoptosis assays were performed with the Annexin V FITC Assay Kit (Cayman Chemical).
IC50 determination

Subconfluent cultures were grown in 96-well plates and treated with KPT compounds for 72 hours before performing MTT assays with CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). Data was graphed and analyzed using Kaleidagraph software (Synergy Software). Assay values obtained in triplicate were used to derive the IC50 by curve fitting to the Hill equation.

Subcellular fractionation, immunoprecipitation, western blot, and tricine-SDS-PAGE

Subcellular fractionation was performed as previously described (27). Immunocomplexes were resolved by SDS-PAGE. Tricine-SDS-PAGE was carried out as described (28). Anti-XPO1 (H-300): sc-5595, anti-survivin (D-8), anti-survivin (FL-142), anti-c-Myc (9E11), anti-caspase-3 (H-277), anti-STAT3 (C-20, sc-482), anti-Sp1 (PEP 2) were obtained from Santa Cruz Biotechnology. Anti-Cleaved PARP (Asp214) (D64E10) XP™ and anti-Acetylated-STAT3 (Lys685, 2523) were obtained from Cell Signaling Technology. Anti-γ-Tubulin (GTU-88), anti-α-Tubulin (B-5-1-2) were obtained from Sigma Aldrich Inc.

Immunohistochemistry and TUNEL

Immunohistochemistry (IHC) was performed as described (13). Tumor sections were deparaffinized and antigen retrieval was performed prior to primary antibody incubation. Primary antibodies were anti-survivin (Abcam, ab24479), anti-STAT3 (Abcam, ab325015), and anti-Ki67 (Cell Marque, 275R-18). TUNEL staining was performed on paraffin sections using Apoptag Kit (Millipore, S71003).
Cell-free proteolysis assay

Total protein was extracted from HEK293T cells, sonicated for 30 secs to shear DNA, then centrifuged (12,000 rpm, 15 min, 4°C). Supernatants were collected for protein quantitation and proteolysis assay. 50 μg of protein was incubated with 1 Unit of activated recombinant Caspase 3 (EMD Millipore) for various time points at 37°C. Proteins were then resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

Luciferase assays

HEK293T cells were transfected with a luciferase reporter construct containing 2 STAT3-binding SIE fragments derived from the promoter region of mouse IRF1, together with cDNAs encoding STAT3 and CBP, using Lipofectamine 2000. Twenty-four hours after transfection, KPT-276 was added for 48 hr or cells were treated with 5μM KPT-276 over a time course. Equal volumes of PBS and Dual-GloTM Luciferase Reagent (Promega) were added and absorbance of firefly luminescence measured. Dual-GloTM Stop&Glo Reagent was added, and absorbance of Renilla luminescence measured. The experiments were performed in triplicate a minimum of three independent times. Two-tailed Student t tests were performed to assess significance.

Quantitative RT-PCR

PCR (qRT-PCR) for Survivin and Gapdh was performed as described (13). Results were analyzed using the ABI HT7900 Sequence Detection System. Relative quantification was performed using the standard curve method.
In vivo tumor models

Fifty female SCID mice (Charles River Labs), aged 5 to 6 weeks were used. Mice were inoculated s.c. in the left flank with $5 \times 10^6$ MDA-MB-468 cells. When tumors reached a mean size of 100-200 mm$^3$, mice were randomly and prospectively divided into a vehicle control group (10 mice) and five treatment groups (8 mice per group). Mice were treated with vehicle (0.1 ml/10kg PO of 1% Pluronic F-68 and 1% PVP K29/32 in sterile water), standard of care control drug (5-FU, 40 mg/kg IP) or KPT-330. KPT-330 was given on either a MWF schedule beginning on Day 0 (dose range 5 to 25 mg/kg PO, by gavage) or on a Monday/Thursday schedule (25 mg/kg PO, by gavage) for a total of 42 days. Animals were fed with sterile Labdiet® 5053 (pre-sterilized) rodent chow and sterile water was provided ad libitum. Animals’ weights and condition were recorded daily, and tumors were measured on MWF with micro-calipers, and tumor volume was calculated as $(\text{length} \times \text{width} \times \text{width})/2$. Statistical differences between groups were determined using Mann-Whitney Rank Sum or ANOVA tests with a critical value of 0.05. The study was performed at Biomodels AAALAC accredited facility in Watertown, MA. IACUC approval for this study was obtained from Biomodels IACUC.
Results

Inhibition of XPO1 inhibits tumor cell growth and enhances apoptosis in breast cancer cells

The panel of SINE reagents used here (KPT-185\textit{cis}, KPT-185\textit{trans}, KPT-251, KPT-276, KPT-330) are similar drug-like small molecules that share a trifluoromethyl phenyl triazole scaffold (Supplementary Fig. S1)(24). KPT-185\textit{trans}, the \textit{trans} isomer of KPT-185\textit{cis}, has been reported to elicit 10-50 fold reduced XPO1 inhibition and apoptosis induction and serves as a negative control (29). To determine the effects of these XPO1 inhibitors on the growth of breast cancer cell lines, we performed dose- and time-dependent studies on cells representing several different molecular sub-types of breast tumors, including an estrogen-receptor/progesterone receptor positive cell line (MCF7), two ER-negative/PR-negative/Her2-negative/EGFR+/basal cell lines (MDA-MB-231 and MDA-MB-486), and a Her2+ cell line (SKBR3) with the KPT drugs. To examine the dose-dependent effects of these compounds, we treated cells with varying doses of the compounds (0, 0.01, 0.1, 1, 10, 100 \textmu M) or vehicle alone and assessed viability at 72 hours after treatment (Fig. 1A). The IC50 for each compound was then calculated (Table 1). The KPT-330 compound (currently in Phase 1 clinical trials) was the most potent growth inhibitor in these cell lines, with IC50 values of 5-21 nM, while the KPT-185\textit{trans} drug had significantly higher IC50 values (893-6200 nM), consistent with its altered structure that inhibits binding to the XPO1 NES site, and with the dependence of growth inhibition on XPO1 antagonism (29). To determine the time course of growth inhibition, we performed cell viability assays at 0, 24, 48, and 72 hours after treatment using 1\textmu M of each agent. KPT-185\textit{cis}, KPT-251, and KPT-276 markedly reduced cell viability in all three breast cancer cells evaluated, while the KPT-185\textit{trans} compound had minimal effects on viability in MCF7 and MDA-MB-231 cells (Fig.1B).
To determine the effects of the SINE compounds on apoptosis, we treated SKBR3 and MDA-MB-486 cells with varying doses of KPT-276 and KPT-330 for 24 hours. Cells treated with 10 μM KPT-276 showed a 5-fold increase in apoptosis over untreated control cells and a 2-fold increase in apoptosis over cells treated with the pro-apoptotic drug staurosporine (Fig. 1C). This indicates that these compounds can induce apoptosis at higher doses than required to just inhibit cell growth. To determine whether apoptosis was induced in a caspase-dependent manner, we treated SKBR3 and MDA-MB-468 cells with varying doses of KPT-276 and KPT-330 for 24 hours then performed western blots for caspase-3, cleaved caspase-3, PARP and cleaved PARP. KPT-276 induced PARP and caspase-3 cleavage at doses greater than 1 μM (Fig. 1D), while KPT-330 induced PARP and caspase-3 cleavage at doses greater than 0.01 μM, indicating that apoptosis is mediated at least in part by caspase-3-dependent mechanisms and that KPT-330 is more potent at inducing apoptosis.

XPO1 inhibition retards breast tumor growth in vivo

To determine the effects of XPO1 inhibition on TNBC growth in vivo, we used MDA-MB-468 cells to create human xenograft tumors in SCID mice. Once tumors were established (100-200m³ in size), mice were divided into 6 different groups (8-10 mice/group) and treated with varying doses of KPT-330 (5-25 mg/kg/dose per orum), 5-FU (40 mg/kg/dose i.p.) or vehicle alone for 42 days. The mean tumor volume for each treatment group is shown in Figure 2A. Changes in tumor volume from Day 1 to Day 42 of treatment are shown in Figure 2B. Statistically significant differences were observed between the vehicle control groups and the groups treated with KPT-330 at 5 mg/kg on Mondays, Wednesdays and Fridays (p=0.020), KPT-
KPT-330 at 15 mg/kg on Mondays, Wednesdays and Fridays (p=0.011), KPT-330 at 25 mg/kg on Mondays, Wednesdays and Fridays (p=0.008), and KPT-330 at 25 mg/kg on Mondays and Thursdays (p=0.011). The data suggest that twice weekly dosing with KPT-330 significantly reduces growth of breast tumors that is more than two-fold greater than vehicle alone or standard treatment with 5-FU.

To determine whether the tumor cells showed evidence of changes in proliferation and/or apoptosis after treatment in vivo, we dissected tumors from animals after 42 days of treatment and stained sections with the proliferation marker, Ki67 and the marker for apoptosis, TUNEL. Results showed a decrease in the number of Ki67-positive cells remaining after treatment with KPT-330, and an increase in TUNEL-positive cells in the remaining tumor tissue (Fig. 2C). To correlate the drug levels achieved in vivo with our in vitro experiments, we performed limited pharmacokinetic studies, measuring plasma concentration of KPT-330 over the first 24 hours after IV and po drug administration (Supplementary Fig. S2). The serum concentration of KPT-330 was ~1000ng/ml 4 hours after treatment. This is approximately the same concentration achieved in vitro at the measured apoptotic time point of 24 hours (Fig. 1C) therefore they are relatively similar in concentration. Together, these data show that KPT-330 reduces proliferation and induces apoptosis in breast tumor cells.

**Nuclear retention of survivin promotes survivin loss**

In previous work, we determined that survivin acetylation at Lys-129 enhances its stability as a homodimer within the nucleus of breast cancer cells where it interacts with the chromosomal passenger complex during mitosis (13). We also showed that survivin
deacetylation by HDAC6 facilitates monomeric survivin to bind XPO1 for nuclear export which is required for its anti-apoptotic function (27). We hypothesized that inhibition of XPO1 by the SINE compounds, as with the natural product XPO1 inhibitor LMB (13), would block survivin nuclear export. To determine the effects of XPO1-inhibition by these compounds on survivin nuclear export, we treated 2 different breast tumor cell lines (MCF7 and SKBR3) with or without 10 μM KPT-276 and prepared nuclear and cytosolic protein fractions. Cells were lysed and proteins analyzed by western blot. A decrease in cytosolic survivin protein was observed as early as 4 hours and an increase in nuclear protein by 4 hours after treatment (Fig. 3A and 3B). Survivin continued to accumulate within the nucleus until 12 hours after treatment, after which time it decreased dramatically both within this compartment and within the cytosol. 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. This decrease in survivin protein levels correlates with the timing of the cellular anti-tumor effects of these compounds and supports a hypothesis that XPO1 inhibition leads to a loss of survivin protein which then leads to inhibition of tumor cell growth and enhanced tumor cell apoptosis.

To validate whether the decrease in survivin protein after KPT treatment is specific to its inhibition of XPO1, we treated MCF7 and SKBR3 cells with two different XPO1 siRNAs and measured survivin protein by western blot. XPO1 knockdown decreased survivin protein levels, similar to that observed with SINE treatment alone (Fig. 3C), supporting the XPO1-specificity of the SINE-induced decrease in survivin levels. To determine if the SINE compounds inhibit the protein-protein interaction between survivin and XPO1, we treated SKBR3 cells with or without 5 μM KPT-185cis, KPT-185trans, or KPT-276 then immunoprecipitated survivin with anti-XPO1 antibodies. Survivin was still capable of binding XPO1 in the presence of all the SINE
compounds (Fig. 3D), suggesting that loss of survivin-XPO1 binding is not required for the observed effects on the decrease in survivin protein levels.

To determine the contribution of the loss of survivin to apoptosis following XPO1 inhibition, we used siRNA to knockdown Survivin in SKBR3 cells then treated these cells with or without 5 μM KPT-276. Survivin knockdown resulted in a two-fold increase in apoptosis over control siRNA-treated cells, as measured by Annexin V FACS (Fig. 4A), suggesting either a synergistic or additive effect of survivin loss on cell death. To determine whether the SINE compounds antagonize the survivin-mediated anti-apoptosis pathway, we performed experiments to assess whether survivin could rescue SKBR3 cells from SINE-mediated apoptosis. To this end, we overexpressed a MYC-tagged survivin expression construct in SKBR3 cells then treated the cells with or without 5 μM KPT-276. Exogenous expression of survivin protected SINE-treated cells from apoptosis, as measured by Annexin V FACs (Fig. 4B). Together, these results suggest that survivin plays a functional role in the apoptosis pathway, as stimulated by XPO1 inhibition in breast cancer cells.

**XPO1 inhibition promotes survivin degradation through caspase 3 activation**

To further characterize the observed effects on survivin protein loss after nuclear retention, we performed a dose-response and time course of survivin protein expression with KPT-276 in SKBR3 cells. Results showed that survivin protein decreased after 12 hours and was undetectable by 72 hours after treatment (Fig. 5A). Doses of 100nM of drug led to a 10-fold decrease in survivin protein, while 10 μM of drug resulted in near disappearance of the protein (Fig. 5B). To determine whether the observed decrease in protein levels could occur as a result of
changes in protein stability, we examined survivin protein levels after inhibiting protein synthesis in the presence and absence of the drug. SKBR3 cells were treated with the protein synthesis inhibitor cyclohexamide (CHX) then with or without 5 μM of KPT-276. Results showed that survivin protein levels decreased further after treatment with KPT-276, indicating that inhibition of XPO1 and survivin nuclear export accelerates survivin protein degradation (Fig. 5C).

Survivin is known to be degraded by the ubiquitin/proteasomal degradation pathway which is stimulated when tumor cells are arrested in the G1 phase of the cell cycle (30). Therefore we examined whether XPO1 inhibition could stimulate this pathway. To this end, we blocked ubiquitin-mediated proteasome degradation by treating SKBR3 cells with the 26S-proteosome inhibitor, MG132. MG132 had an effect on protecting cells from KPT-mediated survivin degradation, shown as a small increase in survivin protein levels at 12 and 24 hours after treatment with both MG132 and KPT-276 compared with KPT-276 alone (Fig. 5D and Supplementary Fig. S3A). The X-linked inhibitor of apoptosis (XIAP) contains a RING domain possessing E3 ligase activity that participates in the ubiquitin-proteasome pathway (31) and has been demonstrated to be one of the mechanisms of ubiquitin-proteasomal degradation of survivin (32). To examine whether XIAP was required for ubiquitin-proteasomal degradation of survivin in response to its nuclear retention, we treated SKBR3 cells with XIAP siRNA then examined survivin levels after XPO1 inhibition. Downregulation of XIAP did not restore survivin levels after KPT treatment (Supplementary Fig. S4), suggesting that other proteins are likely involved in regulating survivin degradation in the nuclear compartment.

To investigate the potential requirements for survivin degradation in response to KPT drugs, we used the pan-caspase inhibitor Z-VAD to block the penultimate proteolytic pathway.
Treatment of SKBR3 cells with Z-VAD in the presence of KPT-276 inhibited survivin degradation (Fig. 5E), suggesting that caspase proteins are required for this process. To uncover the specific caspase protein(s) responsible for this phenotype, we used inhibitors to the caspase 3 and caspase 9 proteins. Treatment with either inhibitor significantly blocked survivin degradation by KPT-276 (Fig. 5F and Supplementary Fig. S3B), supporting a role for these proteases in survivin cleavage after XPO1 inhibition. To determine whether caspase-mediated survivin degradation was dependent on XPO1, we depleted MCF7 cells of XPO1 using XPO1 siRNA in the presence and absence of Z-VAD. Inhibition of caspase activity with Z-VAD partially restored survivin levels from XPO1 siRNA-treated cells (Fig. 5G), supporting that XPO1 blocks caspase-mediated survivin degradation. To further examine survivin cleavage by caspase 3 we used a cell-free system. Whole cell lysates were treated with recombinant active caspase 3 protein over a time course at 37 C. After 2 hours, we detected a large amount of cleaved survivin protein (Fig. 5H). Together, these data support a role for caspase-3 in cleaving survivin in response to SINE-mediated survivin nuclear retention.

**XPO1 inhibition represses Survivin transcription by inhibiting STAT3 transactivation**

To gain further insight into a mechanism whereby XPO1 inhibition and survivin nuclear retention results in loss of survivin, we determined the dose and time-dependent effects of the SINE drugs on Survivin mRNA. We treated SKBR3 cells with 5μM KPT-276 over a time course of 24 hours then prepared cDNA and performed quantitative qRT-PCR for Survivin and GAPDH. KPT-276 significantly decreased the levels of Survivin mRNA at 12 and 24 hours after treatment (Fig. 6A). To determine a potential mechanism for the observed decrease in Survivin

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mRNA levels, we tested the effects of the SINEs on known transcriptional activators of *Survivin*. Signal transducer and activator of transcription 3 (STAT3) is a latent cytosolic transcription factor that stimulates *Survivin* transcription in the nucleus in response to growth factors and cytokines such as epidermal growth factor (EGF) and interleukin-6 (IL-6)(15). For its activation, it requires either phosphorylation by the Janus kinase proteins or acetylation by CREB-Binding-Protein (CBP)(33). In prior work, we showed that nuclear survivin binds the transactivation domain of STAT3 and inhibits STAT3 enhancesome activity (13).

To establish a potential effect of the SINE compounds on STAT3 transactivation, we co-transfected HEK293T cells with a STAT3-luciferase construct (STAT3-luc) along with STAT3 and CBP expression constructs, then treated the cells with varying concentrations of KPT-276 for 48 hours. KPT-276 inhibited STAT3 transactivation at all three drug concentrations tested (Fig. 6B). To determine the time course for inhibition of STAT3 transactivation by KPT-276, we treated the transfected cells with 5 μM KPT-276 and quantified luciferase activity at 12 and 24 hours after treatment. STAT3 transactivation was inhibited approximately 2-fold after treatment at 24 hours (Fig. 6B). To determine whether STAT3 binding to the STAT3 promoter consensus binding sequence was affected, we transfected cells with MYC-STAT3 and CBP then immunoprecipitated STAT3 with an oligonucleotide-bound SIE (STAT3 promoter sequence) and immunoblotted with anti-MYC. KPT-276 inhibited STAT3 binding to its consensus sequence (Fig. 6C). Together, these results suggest that KPT-276 can block the ability of STAT3 to transcriptionally activate some of its target genes.

To determine whether KPT-276 directly affects endogenous STAT3 protein levels, we treated SKBR3 cells with or without KPT-276 and examined endogenous STAT3 levels. Although a small decrease in STAT3 protein was observed after treatment, this was of much
lower magnitude than that observed for the effect on survivin protein levels (Fig. 6D), suggesting that the mechanism of STAT3 repression by SINEs may not be directly through a decrease in native STAT3 protein levels. To examine whether the observed SINE-mediated repression of STAT3 transactivation inhibits CBP-mediated STAT3 acetylation, we cotransfected SKBR3 cells with a MYC-tagged STAT3 expression construct along with Flag-tagged-CBP then treated the cells with or without KPT-276. KPT-276 significantly decreased acetylated STAT3 levels in the presence of CBP (Fig. 6E), suggesting that it may repress STAT3 transactivation by inhibiting CBP-mediated acetylation. To determine the effects of XPO1 inhibition on STAT3-mediated Survivin transcription, we treated 293T cells transfected with Survivin-reporter, STAT3, and CBP with or without KPT-276. KPT treatment led to a decrease in STAT3 mediated transactivation of the Survivin promoter (Fig. 6F and 6G). These data confirm that KPT inhibition of XPO1 regulates Survivin transcription through inhibition of STAT3 transactivation. To examine the in vivo effects of the SINES on survivin and STAT3 we immunostained tumor tissue isolated from mice that had been treated with KPT-330 for 42 days (Fig. 6H). As expected, XPO1 inhibition resulted in sequestration of survivin within the nucleus of most, if not all cells. STAT3 labeling decreased dramatically in treated tumor cells with no evidence of nuclear STAT3 remaining after treatment with inhibitor.
Inhibition of nucleo-cytoplasmic transport by natural and synthetic products has been pursued as a therapeutic avenue in cancer based on a number of biological observations (34). Multiple tumor suppressor proteins, as well as XPO1 itself, are mislocalized or expressed at supraphysiologic levels within cancer cells (35, 36). Empiric evidence also shows that derivatized natural product or drug-like small molecule SINE XPO1 inhibitors (29) are highly efficacious at functioning as anti-cancer agents in several in vitro and in vivo models of human cancer. The focus of many recent studies of these inhibitors, including the SINES, has been to demonstrate efficacy in different tumor types and to define the pharmacokinetic profiles of these different drugs. Additionally, detailed in vitro binding and protein structural studies have shown that the SINES have exquisite specificity for the XPO1-NES binding groove and that unlike other XPO1 inhibitors they do not bind cysteine proteases, thus limiting their off-target effects (24, 25). Although many of these drugs have been demonstrated to inhibit tumor cell growth and have some effects at enhancing programmed cell death in cancer cells, the signaling pathways by which they convey these effects have not been extensively explored.

Breast cancer is the most common cancer in women and carries a 15% mortality rate (37). Improvements in personalized medicine with molecular characterization of breast tumor subtypes and the availability of biological agents have enhanced survival rates for many patients with the exception of triple negative (ER-, PR-, Her2-) and basal breast tumors for which biological therapies are not yet available. Very high levels of survivin expression are independent risk factors for poor prognosis in several clinical breast cancer studies (38, 39). Cytoplasmic survivin has been shown to be particularly high in breast tumors and to be an independent predictor of poor prognosis (40), while nuclear survivin has been a favorable factor.
(39) in some studies. These clinical results support the notion that nuclear survivin is suppressive for tumor growth, and further that targeting the cytoplasmic, anti-apoptotic fraction of survivin would be an ideal therapeutic avenue, if feasible. As survivin requires XPO1-RanGTP for its nuclear exit, inhibiting the activity of this complex could directly address this therapeutic need by increasing the tumor suppressive nuclear survivin and reducing the anti-apoptotic cytoplasmic survivin.

The results of this study demonstrate that the SINES are potent anti-cancer agents in an in vivo breast tumor model of basal breast cancer. Twice-weekly dosing with the clinical candidate KPT-330 (25 mg/kg for 42 days) significantly reduced tumor cell growth to approximately one-third the volume of tumors observed in 5-FU treated animals. Reductions in tumor growth were associated with a reduced percentage of cellular proliferation and an increase in markers for apoptosis which correlated with the in vitro findings demonstrating that these compounds activate PARP and caspase-3. In vitro expression assays also demonstrated that survivin plays a role in the cell death pathway activated by SINEs, with modulations in survivin expression levels by siRNA or exogenous survivin expression altering the potency of the KPT drugs.

Our results suggest that survivin is an essential component of the downstream signaling pathway of XPO1 inhibition in breast cancer cells and, intriguingly, that an increase in the total concentration of survivin can interfere with these drugs’ anti-tumor effects. KPT-276 treatment led to an initial decrease in cytoplasmic survivin protein levels with a corresponding increase in its nuclear expression. This result was transient however, as the major effect was to deplete total survivin levels. Interestingly, other reports have shown a decrease in total survivin following treatment with LMB (41) and more recently it was reported that the NF-κB-dependent survival factor, Mcl1, is depleted in response to KPT-185 (25). Here, we show that this effect is
independent of survivin binding to XPO1 since KPT-276 does not abrogate this protein-protein interaction. Survivin is the first protein to demonstrate this specific characteristic that may occur as a consequence of its smaller size (16.5 kd) and occupancy of the XPO1 binding site, relative to the other XPO1 binding proteins studied to date.

As reported in our study, KPT-276 represses STAT3 transactivation to inhibit Survivin transcription as a major mechanism by which it acts to deplete total survivin protein levels. STAT3 is constitutively activated in breast cancer and has been heavily pursued as a drug-treatment target in clinical trials of this disease (42, 43). STAT3 phosphorylation by growth factor receptors, Janus kinase (Jak) and Src kinase families or by acetylation by CBP induces its homodimerization and activates target gene transcription (33). Several dimerization-disrupting STAT3 inhibitors have been reported though none has entered clinical trials due to pharmacokinetic problems (20, 44, 45). KPT-276 inhibits CBP-mediated STAT3 acetylation which leads to a reduction in STAT3 binding to the Survivin promoter and a decrease in Survivin transcription. Additionally, STAT3 expression was undetectable in the nuclei of treated tumor cells in vivo. This expands the potential use of the SINE XPO1 antagonists to include their application as inhibitors of STAT3 transactivation in breast cancer therapy and suggests that this function should be explored further with other STAT3 targets.

Activation of the ICE/CED-3 family of cysteine proteases (caspases) is a requirement for the execution phase of apoptosis in both healthy and malignant cells (46). This pathway is frequently disrupted in tumor cells due to dysregulation of one or more of its components. Following a variety of toxic stimuli, programmed cell death is initiated by cytochrome c release and formation of the Apaf-1/caspase 9 complex (47). Activation of the downstream effector caspases, including caspase 3, then occurs. Many in vivo caspase substrates exist, including
transcription factors, kinases, enzymes involved in DNA repair, and cytoskeletal proteins. In addition, several proteins essential for cell cycle regulation, such as pRb (48), MDM2 (49), p21WAF1/CIP1, p27Kip1 (50), and STAT3 (51) are also caspase targets. Prior to our study, survivin was not a known caspase target. An algorithm for prediction of caspase substrate cleavage sites identifies 5 potential caspase cleavage sites in the human survivin protein (52, 53). One such site, at the N-terminal region of the human survivin protein (FLKD, position 13-18) would be consistent with the observed cleavage pattern identified here (Fig. 5H). These results add to the repertoire of caspase-substrates and provide a biochemical mechanism for the critical role for survivin in the function of the SINE XPO1 antagonists. Together, this study expands the application of these agents in the treatment of breast cancer and other tumors. As the SINE KPT-330 is currently in Phase 1 clinical studies in humans (NCT01607905), these results are directly applicable to patients with advanced solid tumors.
Author’s Contributions

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**Analysis and interpretation of data** (e.g., statistical analysis, pharmacokinetic analysis): Rachel A. Altura, Yan Cheng, Michael P. Holloway, Dilara McCauley

**Writing, review, and/or revision of the manuscript:** Rachel A. Altura, Michael G Kauffman, Sharon Shacham, Yan Cheng, Michael P. Holloway, Kevin Nguyen, Dilara McCauley, Yosef Landesman

**Study supervision:** Rachel A. Altura, Michael G Kauffman, Sharon Shacham
References

Table 1. IC50 (µM) of the KPT-SINE compounds in breast cancer cells.

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<th>KPT-185cis</th>
<th>KPT-185trans</th>
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<td>MCF7</td>
<td>79</td>
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<td>577</td>
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<td>SKBR3</td>
<td>284</td>
<td>893</td>
<td>874</td>
<td>670</td>
<td>0.21</td>
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</table>
Figure Legends

Figure 1. XPO1 inhibition by the KPT-SINES represses breast cancer cell growth and enhances apoptosis. **A**, MCF7, MDA-MB-231, and SKBR3 cells representing hormone receptor positive, hormone receptor negative, and Her2+ cells, respectively, were treated with varying doses of KPT-185, 251, 276, and 330 compounds and cell viability was assessed at 72 hours. **B**, MCF7, MDA-MB-231 and SKBR3 cells were treated with the KPT compounds and cell viability was assessed at the indicated times. **C**, SKBR3 cells were treated with KPT-276 or staurosporine (STS) and apoptosis was assessed by Annexin V FACS at 24 hours. **D**, SKBR3 and MDA-MB-468 cells were treated with varying doses of KPT-276 and KPT-330, respectively for 24 hours. Protein lysates were analyzed by western blot for the indicated apoptosis proteins.

Figure 2. XPO1 inhibition by the KPT-SINES blocks in vivo breast tumor growth. **A**, Mice harboring MDA-MB-468 xenografts were treated with the oral KPT-330 compound, 5-FU, or vehicle alone at the indicated times and schedules. Mean tumor volumes were calculated from the length and width measurements. Group means were calculated and are shown with error bars representing SEM for each group. **B**, Mean tumor volumes at Day 1 and Day 42 for each treatment schedule (MWF= Monday, Wednesday, Friday; MTh = Monday, Thursday; 5, 15, 25=mg/kg dose). Significant differences from vehicle control (p< 0.05) are indicated. **C**, At the 42 day time point, the remaining tumor tissue was dissected, paraffin embedded and stained for markers for proliferation with Ki67 and apoptosis with TUNEL.

Figure 3. XPO1 inhibition blocks survivin nuclear export and induces survivin loss.

**A and B**, Subcellular fractionation and western blots of MCF7 and SKBR3 cells after treatment with KPT-276 for the indicated times. Shown below each immunoblot are the survivin protein...
levels normalized with tubulin for each time point. C, Western blot showing XPO1 and survivin protein levels after treatment with two different siRNAs to XPO1. UT = untreated, C = control siRNA. D, SKBR3 cells were treated with the indicated compounds for 24 hours. Lysates were immunoprecipitated with anti-XPO1 and survivin and XPO1 immune complexes were resolved by SDS-PAGE. Total survivin and XPO1 (input) was determined by western blot.

**Figure 4. Apoptosis by XPO1 inhibition is dependent on survivin.** A, SKBR3 cells were transfected with Survivin siRNA or control then treated with KPT-276 for 24 hours. Apoptosis was assessed by Annexin-V FACS. Western blot shows the survivin knockdown. B, SKBR3 cells were transfected with a MYC-tagged survivin construct or control vector then treated with KPT-276 for 24 hours. Apoptosis was assessed by Annexin-V FACS. Western blot shows MYC-survivin expression.

**Figure 5. Inhibition of XPO1 activates caspase-dependent cleavage of survivin.**

A and B, SKBR3 cells were treated with KPT-276 for the indicated times and doses. Western blots were performed and blotted with the indicated antibodies. C, SKBR3 cells were treated with or without the protein synthesis inhibitor cyclohexamide (CHX) and KPT-276. Western blots were performed and blotted with the indicated antibodies. D, SKBR3 cells were treated with or without the proteasome inhibitor MG132 and KPT-276. Western blots were performed and blotted with the indicated antibodies. E, SKBR3 cells were treated with or without the pan-caspase inhibitor, Z-VAD and KPT-276. Western blots were performed and blotted with the indicated antibodies. F, SKBR3 cells were treated with or without the pan-caspase inhibitor, Z-VAD, the specific caspase inhibitors to caspase 3 (casp3i) or caspase 9 (casp9i) and KPT-276. Western blots were performed and blotted with the indicated antibodies. G, MCF7 cells were...
transfected with XPO1 siRNA or control then treated with or without the pan-caspase inhibitor Z-VAD. Western blots were performed and blotted with the indicated antibodies. 

**Figure 6. Inhibition of XPO1 represses STAT3 transactivation.**

A, SKBR3 cells were treated with KPT-276 and RNA was prepared at the indicated time periods. Survivin mRNA levels were determined by quantitative RT-PCR. B, 293 cells were co-transfected with STAT3 and CBP expression constructs, along with a STAT3-luciferase reporter construct. Luciferase activity was determined after treatment with KPT-276 at the indicated doses and times. C, 293 cells were co-transfected with a MYC-tagged STAT3 and a Flag-tagged CBP expression construct followed by treatment with or without KPT-276. STAT3 complexes were affinity purified using agarose beads conjugated with an oligonucleotide containing the STAT3 consensus DNA binding sequence (SIE-oligo). D, SKBR3 cells were treated with or without KPT-276 and lysates were separated by SDS-PAGE and blotted with the indicated antibodies. E, SKBR3 cells were co-transfected with MYC-tagged STAT3 and Flag-tagged CBP or empty vector then treated with or without KPT-276. Lysates were separated by SDS-PAGE and blotted with the indicated antibodies. F and G, 293 cells were co-transfected with Survivin-reporter, STAT3, and CBP expression constructs. Luciferase activity was determined after treatment with KPT-276 at the indicated doses and times. H, Tumor sections from MDA-MB-468-xenograft mice treated with KPT-330 or control for 42 days were immunostained with anti-survivin and anti-STAT3.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
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XPO1 (CRM1) inhibition represses STAT3 activation to drive a survivin-dependent oncogenic switch in triple negative breast cancer

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