Anti-tumor efficacy of Selinexor (KPT-330) in gastric cancer is dependent on nuclear accumulation of p53 tumor suppressor

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Exportin-1 (XPO1) controls the nucleo-cytoplasmic trafficking of several key growth regulatory and tumor suppressor proteins. Nuclear export blockade through XPO1 inhibition is a target for therapeutic inhibition in many cancers. Studies have suggested XPO1 upregulation as an indicator of poor prognosis in gastric cancer. In the current study, we investigated the anti-tumor efficacy of selective inhibitors of nuclear export (SINE) compounds KPT-185, KPT-276 and clinical stage selinexor (KPT-330) in gastric cancer. XPO1 was found to be overexpressed in gastric cancer as compared to adjacent normal tissues and was correlated with poor survival outcomes. Among the 3 SINE compounds, in vitro targeting of XPO1 with selinexor resulted in greatest potency with significant anti-proliferative effects at nanomolar concentrations. XPO1 inhibition by selinexor resulted in nuclear accumulation of p53, causing cell cycle arrest and apoptosis. Also, inhibition of XPO1 lead to the cytoplasmic retention of p21 and suppression of survivin. Orally administered selinexor caused significant inhibition of tumor growth in xenograft models of gastric cancer. Furthermore, combination of selinexor with irinotecan exhibited greater anti-tumor effect compared to individual treatment. Taken together, our study underscores the therapeutic utility of XPO1 targeting in gastric cancer and suggests the potential benefits of XPO1 inhibition in combination with chemotherapy.

Intracellular location of tumor suppressor proteins (TSPs) and growth regulatory proteins (GRPs) is critical to cancer cells for proliferation and survival1. Many of these proteins must localize to the cell nucleus to prevent cancer initiation, progression and resistance to chemotherapy. During malignant transformation or in response to the tumor environment, cancer cells appear to acquire intracellular mechanisms for nuclear exclusion of tumor suppressor proteins2. In this regard, therapeutic targeting of the nucleo-cytoplasmic shuttling of macromolecules has emerged as a promising approach in cancer treatment3. Exportin1 (XPO1) also called chromosome region maintenance 1 (CRM1), is a key nuclear export protein that mediates protein export from the nucleus to the cytoplasm through the nuclear pore complex. It is a member of the karyopherin β protein family of transport receptors that can recognize cargo proteins with leucine-rich nuclear export signals (NESs)4. XPO1 is the primary nuclear exporter of a number of tumor suppressor and cell cycle regulatory proteins such as p53, p21, FOXO1, cyclin B1/D1 and chemotherapeutic targets such as DNA topoisomerases I and II alpha5,6. Overexpression of XPO1 was shown to be associated with poor prognosis or resistance to chemotherapy in various cancers7. XPO1 upregulation also results in enhanced nuclear-cytoplasmic transport. This causes mislocalization, inactivation or...
aberrant activation of tumor suppressor proteins (TSPs), thereby resulting in oncogenesis. In gastric cancer, high expression of XPO1 was shown to have a significant correlation with advanced tumor stage, distant metastasis and poor prognosis.

Leptomycin B was the first XPO1 inhibitor shown to efficiently inhibit nuclear export in various cancer cell lines in vitro. However, its clinical development was subsequently limited due to severe toxicities without significant efficacy in human phase I trial. Selective inhibitor of nuclear export (SINE) compounds (KPT-185, KPT-276, and KPT-330) were developed based on the fact that Leptomycin B specifically binds to the Cys528 residue in the cargo-binding groove of XPO1 to restore the function of TSPs in the nucleus, leading them to induce cancer-specific apoptosis. SINE compounds have shown anti-tumor activity in several solid and hematological malignancies in preclinical in vitro and/or in vivo studies, including renal cancer, pancreatic cancer, prostate cancer, breast cancer, melanoma, multiple myeloma, mantle cell lymphoma, chronic lymphocytic leukemia, acute myeloid leukemia, and non-Hodgkin lymphoma. Further to the Phase 1 clinical trials of oral KPT-330 (selinexor, Karyopharm Therapeutics) for both solid tumors (NCT01607905, NCT01896505) and advanced hematologic malignancies (NCT01607892), the compound is currently undergoing Phase 2/3 trials. In addition, given the noted synergistic effects on cytotoxicity in neoplastic cells, the potential for combining XPO1 inhibition with conventional chemotherapy, such as topoisomerase inhibitors, is also being explored in recent clinical trials (NCT02283359). A recently concluded Phase II trial that tested selinexor in combination with dexamethasone showed an overall response rate (ORR) of 21% in patients with heavily pretreated, refractory myeloma. In the present study, we investigated the therapeutic efficiency of XPO1 inhibition with SINE compounds KPT-185, KPT-276 and KPT-330 in gastric cancer. The mechanism of XPO1 inhibitor-mediated proliferation inhibition and apoptosis in gastric cancer cells is also determined. Furthermore, the anti-tumor efficacy of KPT-330 is also characterized using a xenograft model of gastric cancer.

Methods

Patient tissue samples and genomic profiling. An independent patient cohort including 153 tumors and 100 normal samples were profiled for gene expression using Affymetrix human genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA). Gene expression microarray data is available under GEO accession number GSE15460. All primary gastric tissues were obtained from the National University Hospital, Singapore or National Cancer Centre, Singapore tissue repositories with approvals from the Research Ethics Review Committee, National University Hospital and Institutional Review Board, National Cancer Centre. All methods were performed in accordance with the relevant guidelines and regulations and signed patient informed consent. Tumor samples were histologically confirmed to contain cancer cells, with an average tumor cellularity of 40%. Non-malignant samples (normal tissue) were harvested from stomach tissue distant from the tumor and exhibiting no visible evidence of tumor or intestinal metaplasia/dysplasia upon surgical assessment. Histopathological data and patient characteristics of the cohort is provided in a previous publication.

Cell lines and reagents. Gastric cancer cell lines, AGS and NCI-N87 were purchased from ATCC, USA. IM95, NUGC-3 and NUGC-4 were purchased from Health Science Research Resources Bank, Japan. MKN1, MKN45, TMK1 and YCC10 were obtained from DUKE NUS, Singapore. HGC-27 was purchased from Public Health England, UK. All cell lines except YCC10 cell lines were cultured in RPMI 1640 medium (Gibco; Grand Island, NY) containing 10% heat inactivated fetal bovine serum (Gibco; Grand Island, NY) and 1% penicillin/streptomycin (Gibco; Grand Island, NY). YCC10 cell lines was cultured in MEM supplemented with 20% heat inactivated fetal bovine serum (Gibco; Grand Island, NY), 1% penicillin/streptomycin (Gibco; Grand Island, NY) and 1% sodium pyruvate (Gibco; Grand Island, NY). All cell lines were maintained at 37°C in humidified atmosphere containing 5% CO₂.

Reagents, antibodies and materials. KPT-185, KPT-276 and KPT-330 were provided by Karyopharm Therapeutics and dissolved in DMSO for in vitro studies. KPT-330 was diluted in 0.6% Pluronic® F68 and PVP K-29/32 solution for the in vivo study. Irinotecan was purchased from Pfizer (Pfizer; NY, USA) and stored at 4°C. Primary antibodies including anti-caspase-3, anti-caspase-9, anti-p53, anti-p21, anti-survivin, anti-ß-tubulin, anti-lamin, anti-p27, GAPDH were purchased from Cell Signaling Technology (Cell Signaling; MA, USA). Primary anti-XPO1 was purchased from Proteintech (Proteintech; IL, USA). Secondary antibodies (anti-rabbit IgG, HRP-Linked and anti-mouse IgG, HRP-Linked) were purchased from Cell Signaling Technology (Cell Signaling; MA, USA). Alexa Fluor® 568 Goat Anti-Rabbit IgG and ProLong® Gold Antifade Reagent with DAPI were purchased from Molecular Probes (Life Technologies; CA, USA).

Quantitative PCR (qPCR) analysis. Total RNA was extracted from cultured cells with RNeasy Mini kit (Qiagen, CA, USA) with the use of Qiashredder spin column for homogenization and an on-column DNase digestion. 2 μg of the total RNA was reversely transcribed using M-MLV reverse transcriptase enzyme (Promega, WI, USA). The cDNA obtained was analysed quantitatively using Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) on an ABI7300 Real-time PCR system. Primers used are listed in Table 1. Cycling conditions were 95°C for 15 min, 40 cycles of 15 s at 94°C, 30 s at 55°C and 30 s at 72°C. Ct values were generated using default analysis settings. Relative quantification (RQ) was calculated using 2−ΔΔCT method.

Cell viability and proliferation assays. To assess the chemo sensitivity of tumor cells to SINE compounds, cell viability was measured by CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega; WI, USA). Cell suspension was cultured in 96-well flat-bottomed micro titer plates at seeding density of 2 × 10³ cells/well and incubated overnight. Drugs were tested at concentrations ranging from 0.1 μM to 10.0 μM. Micro titer wells containing tumour cells without drug treatment served as controls, and wells containing complete medium...
served as blank controls. Each drug was tested in triplicate. Cells were incubated for 72 hours before the addition of the assay reagent (1 mg/mL per well) and absorbance was read at 550 nm using a spectrophotometric micro plate reader (Tecan; Männedorf, Switzerland). The percentage cell viability to different drug concentrations was calculated as the inhibition rate of (mean absorbance of treated wells/mean absorbance of control wells) × 100%.

IC50 was calculated by GraphPad Prism v4.0 (GraphPad Software, Inc; CA, USA).

Cell cycle analysis. Cell cycle distribution of cells was analyzed using flow cytometry. Cells were fixed with ice-cold 70% ethanol at 4 °C overnight, and washed in PBS. Cells were then resuspended in PBS RNase (Qiagen, CA, USA) and propidium iodide (BD Pharmingen ™, CA, USA) and incubated for 15 min at room temperature. Cell cycle analysis was performed on a BD LSRII Analyzer equipped with FlowJo software (version vX 0.7). A total of 10,000 cells were analyzed for each sample.

Apoptosis assay. Apoptosis was detected by Annexin V-FITC (fluorescein isothiocyanate) kit (BD Pharmingen) according to manufacturer's instructions. Briefly, the cells (1 × 105 cells/ml) were grown to 80% confluency in 25 cm² flasks in F12K supplemented with 10% fetal calf serum. After 24 hours of drug treatment, cells were harvested, washed thrice with cold PBS and resuspended in 1x binding buffer. An aliquot of 100 µl of the cell suspension was transferred into a microfuge tube and mixed with equal volumes (5 µl) of Annexin V-FITC and Propidium Iodide (PI). The cells were gently vortexed and incubated for 15 minutes at 37 °C in the dark, before the addition of 400 µl of 1x binding buffer in each tube. The cell samples were then analyzed using a flow cytometer BD™ LSR II (BD biosciences, CA, USA) equipped with FlowJo software (version vX 0.7).

Western blot and protein analysis. Cells treated with SINE compounds were washed with ice cold PBS and resuspended in lysis buffer (Cellytic; Sigma-Aldrich; St Louis, MO) containing protease inhibitor cocktail (Roche; Mannheim, Germany). Lysates were sonicated, incubated on ice for 20 min and centrifuged at 14000 rpm for 20 min at 4 °C. Protein concentrations were determined using Bradford assay (Bio-Rad, CA, USA). Nuclear and cytoplasmic extracts were prepared with NE-PER Kit (Pierce; IL, USA). 20 µg of protein samples were electrophoretically separated on 12% SDS-PAGE and electro-transferred to a PVDF membrane (Immun-Blot PVDF;
Membranes were blocked for an hour at room temperature in 5% non-fat dry milk (Bio-Rad; CA, USA) and subsequently incubated with primary antibodies overnight at 4 °C. After three washes with Tween 20 in PBS, membranes were incubated for 1 h at room temperature with corresponding hors eradish peroxidase conjugated anti-rabbit and anti-mouse secondary antibodies. The membranes were washed four times with PBS/Tween 20, and the signals were visualized by ECL reagent (AmershamTM ECL Plus Western Blotting Detection System; GE Healthcare; Buckinghamshire, UK), followed by exposure to chemiluminescence film (Amersham HyperfilmTM ECL; GE Healthcare; Buckinghamshire, UK). Immunoblot analyses were repeated at least twice for each protein tested.

**Immunofluorescence Staining.** Cells were seeded onto the 8 wells chamber slide and allowed to grow until 70% confluence. The cells were fixed with 4% formaldehyde for 10 minutes and permeabilised by 0.1% Triton X100 in PBS (PBST) for 10 minutes. The slides were blocked with 1% Bovine Serum Albumin (BSA) in PBST for an hour at room temperature. The cells were incubated overnight with primary antibody p53 and p21 (Cell Signaling, MA, USA) at 4 °C and subsequently incubated with secondary antibody conjugated with Alexa Fluor 568 for an hour in the dark at room temperature. Prolong Gold with DAPI was added into each wells and air-dried in the dark to detect nuclei. The slides were sealed with coverslips for imaging using a confocal laser microscope (Olympus FluoViewTM FV1000).

**GC xenograft murine model.** 4–6 week old NOD-SCID mice (n = 50) were injected subcutaneously with AGS gastric cell lines (5 × 10^6) suspended in Matrigel (BD biosciences). Six animals were randomly assigned to each treatment group (1) Vehicle (0.6% pluronic F-68 and 0.6% Plasdone K-29/32) (2) Irinotecan (20mpk, i.v) (3) KPT-330 (10mpk, oral-gavage) (4) combination of irinotecan and KPT-330 (intra venous/ oral-gavage). Effects of XPO1 inhibition on proliferation in GC cell lines. 6–8 week old NOD-SCID mice (n = 6) were randomly assigned to each treatment group (1) Vehicle (0.6% pluronic F-68 and 0.6% Plasdone K-29/32) (2) Irinotecan (20mpk, i.v) (3) KPT-330 (10mpk, oral-gavage) (4) combination of irinotecan and KPT-330 (intra venous/ oral-gavage). KPT-330 showed the highest expression (Fig. 1c,d).

**Apoptosis induction and cell cycle effects in GC cells treated with KPT-330.** The effect of XPO1 inhibition in cell cycle regulation and apoptosis was determined in AGS cells that showed high XPO1 expression as the only independent prognostic factors (Table 1). Expression levels of XPO1 were also determined in a panel of 10 gastric cancer cell lines. All cell lines showed detectable expression levels of XPO1, among which AGS and YCC10 showed the highest expression (Fig. 1c,d).
in drug treated cells. As shown in Fig. 3c, AGS cells treated with KPT-330 showed a dose dependent increase in expression of cleaved caspase-3 and caspase-9 (active form). The expression of cleaved forms of caspase-3 & 9 was detectable at dosing concentrations as low as 100 nM, hence suggesting a caspase dependent induction of apoptosis in KPT-330 treated cells.

KPT-SINE induced apoptosis in GC cells was mediated by p53 and CRM1. To further investigate the mechanism of apoptosis induced by the KPT-330, we examined the expression profiles of apoptotic proteins in drug treated AGS cells (Fig. 4a). Expectedly, treatment of cells with KPT-330 lead to a dose-dependent decrease in XPO1 expression levels. A resultant effect of this was observed with marked upregulation of p53 and its transcriptional target p21. On the other hand, a decrease in expression of anti-apoptotic survivin was also observed hence suggesting a KPT-330 triggered induction of cell death. The subcellular localization of p53 and p21 was determined by immunofluorescence analysis. As shown in Fig. (4b), treatment with KPT-330 resulted in a dose-dependent upregulation of p53 and p21 expression in cell nuclei, hence suggesting a gain in apoptotic function.

The KPT-330 induced effects in the intracellular localization of other oncogenic determinants were also evaluated. The cytoplasmic and nuclear fractions of KPT-330 treated AGS cells were extracted and subjected to immunoblot analysis (Fig. 5). Consistently, KPT-330 led to the nuclear retention and upregulation of p53 expression in AGS cells. In contrary, the expression of p27, a non p53 regulated protein, remained unchanged in the nucleus, whereas an increase in its cytoplasmic expression was observed. Interestingly, a decrease in expression of survivin was observed both in the cytoplasmic and nuclear fractions of KPT-330 treated cells.

KPT-330 inhibits growth of gastric cancer cells in a xenograft model. The effect of XPO1 inhibition in tumor growth was determined in vivo using a mouse xenograft model. NOD-SCID mice transplanted with AGS cells showed a remarkable decrease in tumor growth after treatment with KPT-330 as compared to the mice treated with vehicle controls. Since preclinical evaluation of XPO1 inhibition in combination with chemotherapy yielded promising results in other cancers, we evaluated the combinatorial effect of KPT-330 with irinotecan in gastric cancer (Fig. 6). The xenografts showed significant anti-tumor effects can be achieved by irinotecan monotherapy. Strikingly, a synergistic effect of KPT-330 was observed in combination of KPT-330 with irinotecan, causing greater appreciable anti-tumor effects. The SINE compound was well tolerated with no toxic deaths and minimal weight loss. In addition, no gross pathology was observed at necropsy carried out at the end of the experiment. Consistent with the in vitro data, KPT-330 treatment induced an upregulation of P53 and p21 expression in in vivo xenografts tissues. An increase in p21 expression was also seen in irinotecan and combinatorial treatment samples, while p53 showed a modest increase as compared to vehicle controls. Furthermore, immunohistochemical analysis revealed a decrease in ki67 expression in all treated samples as against the vehicle controls (Supplementary Fig. S3).
Discussion

The requirement for nucleo-cytoplasmic export of key proteins in normal cells is suggested to be less stringent than that in malignant cells. This "Achilles heel" of malignant cells could be targeted using nuclear export inhibitors to achieve therapeutic benefits. In this regard, selective killing of tumor cells could be achieved through restoration and reactivation of tumor suppressors. XPO1 is highly expressed in many tumor types and coordinates the nucleo-cytoplasmic export of ~220 proteins. This includes several tumor suppressors and mediators of proliferative and pro-survival signaling pathways. The current study identified XPO1 as an independent prognosis indicator, as its overexpression correlates with poor survival in gastric cancer patients. XPO1 expression was shown to be significantly high in diffuse type of GC, however no association was observed between XPO1 expression with stage and grade of cancer (Supplementary Table S1). The three SINE compounds (KPT-185, KPT-276 and KPT-330) presented in our study were previously shown to induce covalent modification of the Cys528 residue at the NES groove of XPO1 protein. They are structurally similar, but differ in the pharmacokinetic properties. KPT-185 was shown to have poor PK properties, hence making it unsuitable for in vivo study. On the other hand, KPT-276 and KPT-330 exhibit optimal PK properties. Here, we establish the anti-tumor efficacy of XPO1 inhibitor KPT-330, both in in vitro and in vivo models of gastric cancer. Our findings fall in-line with the previous reports that suggested anti-tumor efficacy of XPO1-targeting drugs in various hematological malignancies and solid tumors.

Figure 2. IC_{50} of KPT-SINE analogs to 10 gastric cancer cell lines. Mean IC_{50} ± Standard Error (SE) of at least two independent experiments were performed in triplicates. Cells were treated with KPT-SINE analogs for 72 h and cell proliferation was assessed using the MTS assay.

Figure 3. AGS cells treated with IC_{50} concentrations of KPT-330 for 24 hours was analysed for cell cycle profiles and apoptosis (a) Cell cycle analysis (b) Annexin-FITC apoptosis assay. (c) Immunoblots of apoptotic protein expressions in AGS cells treated with varying doses of KPT-330 for 24 hours. The values reported under each blot indicates the percentage expression of cleaved caspase 3 & 9 relative to controls taken as 1 after normalization by GAPDH (Image J quantification). The results shown are representative of two independent experiments. *p-value of less than 0.05 was considered statistically significant.
Since XPO1 inhibition results in nuclear retention and activation of multiple TSPs, its anti-tumor effects could largely be independent of underlying oncogenic drivers that are key in maintaining a neoplastic state. Although the exact mechanism underlying the selective anti-tumor efficacy of KPT-330 remain uncharacterized, a major mediatory role of p53 is assumed. As a nuclear export mediator, XPO1 channels the nuclear export of p53. This leads to the cytoplasmic degradation and functional inactivation of p53 as a tumor suppressor. XPO1 inhibition by KPT-330 abrogates p53 degradation through its nuclear retention. When p53 is activated in the nucleus, it could promote either cell cycle arrest or apoptotic cell death. In our study, the activation of caspase-3 and 9 in KPT-330 treated cells is a clear indication of induction of apoptosis. Other proteins that were found to be affected by XPO1 inhibition include tumor suppressor protein p21 and survivin. The upregulation of p21, a transcription target of p53, is suggestive of the restoration/reactivation of p53 tumor suppressor function. Of note, the molecular function of p21 depends on its cellular localization as it plays different roles in nucleus and in cytoplasm.

Figure 4. (a) Immunoblots of tumor suppressor protein expression in AGS cells treated with KPT-330 for 24 hours. The values reported under each blot indicates the percentage expression relative to controls taken as one after normalization by GAPDH (Image J quantification). The results shown are representative of two independent experiments. (b) Immunofluorescence of p53 and p21 expression in KPT-330 treated AGS cells. P53 and p21 stained with Alexa Flour568 (red) and cell nuclei stained with DAPI (Blue).

Figure 5. Immunoblot of protein expression in cytoplasmic and nuclear fractions of AGS cells treated with KPT-330. Lamin B1 and β-tubulin serve as loading controls for nuclear and cytoplasmic fractions, respectively. The values reported under each blot indicates the percentage expression relative to controls taken as 1 after normalization by GAPDH (Image J quantification). The results shown are representative of at least two independent experiments.
of survivin has been reported to promote tumorogenesis and confer chemotherapy resistance. Our study adds cell cycle arrest and apoptosis is consistent with the earlier studies. No changes in expression of p27, a nucleus upon CRM1 inhibition (Fig. 5). Our finding that XPO1 inhibition restored p53 nuclear function, caus-

The continued evaluation of KPT-330 to establish the safety of targeting nuclear export through XPO1 would should explore if XPO1 inhibition induces a shift in balance of neoplastic cells from a hyper proliferative state to an anti-apoptotic role. As shown by immunofluorescence microscopy, p21 expression was retained in the nucleus upon CRM1 inhibition (Fig. 5). Our finding that XPO1 inhibition restored p53 nuclear function, causing cell cycle arrest and apoptosis is consistent with the earlier studies. No changes in expression of p27, a non-p53-regulated cell cycle inhibitor, was observed. Intriguingly, XPO1 inhibition by KPT-330 also lead to a decrease in survivin expression levels. Survivin constantly shuttles between the nucleus and the cytoplasm and plays a major role in apoptosis regulation and control of cell cycle progression at the G2/M phase. Elevated level of survivin has been reported to promote tumorogenesis and confer chemotherapy resistance. Our study adds further value to a synergistic interaction that could be utilized in the treatment of refractory gastric cancers.

The combination of molecularly targeted agents with chemotherapy has shown the potential of improving the outcomes of cancer patients. In earlier studies, the ability of XPO1 inhibitors to induce apoptosis or anti-proliferative effects in cell lines was shown to have improved synergistically when combined with chemo-

The present study strongly correlates XPO1 expression with poor prognosis in gastric cancer. Future studies should explore if XPO1 inhibition induces a shift in balance of neoplastic cells from a hyper proliferative state to an apoptotic state that could further be enhanced by concomitant targeting of other crucial cellular processes. The combined evaluation of KPT-330 to establish the safety of targeting nuclear export through XPO1 would augment the clinical translation of XPO1 inhibitors for the treatment of gastric cancer. Nonetheless, the enhanced efficacy and low toxicity profiles of KPT-330 in combination with conventional chemotherapy is promising and adds value to a synergistic interaction that could be utilized in the treatment of refractory gastric cancers.

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Author Contributions
V.V.S., M.S.Y., L.W., F.Y.W. and Y.W.P. designed and performed experiments, analyzed data and wrote the manuscript. S.H.T., N.P., M.Y.S. analyzed the data. W.L.T. (W.L. Thuya & W.L. Tan) assisted in performing the experiments. P.C.P. helped to perform in-vivo mouse studies. D.S.T., E.B., S.S., P.T. and H.P.K. helped with intellectual contributions.

Additional Information
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