Selective inhibitors of nuclear export for the treatment of non-Hodgkin's lymphomas

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ABSTRACT

The nuclear export protein chromosome maintenance region 1, found to be elevated in non-Hodgkin's lymphomas, controls localization of critical tumor suppressor proteins. Nuclear localization of tumor suppressor proteins is necessary for their cell surveillance function. However, their nuclear exclusion by chromosome maintenance region 1 renders them ineffective making this nuclear transporter an attractive therapeutic target. We have identified selective inhibitors of nuclear export that lock tumor suppressor proteins in the cell nucleus leading to apoptosis of lymphoid but not normal cells. Our inhibitors induce tumor suppressor protein nuclear retention-dependent growth inhibition and apoptosis in a panel of non-Hodgkin's lymphoma cell lines. Western blot of nuclear protein fraction and confocal microscopy analysis demonstrated retention of major tumor suppressor proteins in the cell nucleus. Co-immunoprecipitation studies showed disruption of the tumor suppressor protein-chromosome maintenance region 1 interaction. Small inhibitor RNA knockdown of two major tumor suppressor proteins, p53 in wild-type protein-53 and protein 73 in mutant-protein-53, abrogated inhibitor activity. Oral administration of related inhibitor at 75 and 150 mg/kg resulted in 65 and 70% tumor reduction, respectively and subcutaneous injections of inhibitor (25 and 75 mg/kg) resulted in 70 and 74% suppression of non-Hodgkin's lymphoma tumor growth with no toxicity; residual tumors showed activation of the protein 73 pathway. Our study verifies chromosome maintenance region 1 as a therapeutic target in non-Hodgkin's lymphoma, indicating that this nuclear export protein warrants further clinical investigations.

Introduction

Despite the advancements in our understanding and classification of non-Hodgkin's lymphomas (NHL), as well as the introduction of the R-CHOP regimen, these lymphomas remain deadly diseases, with ~200,000 deaths globally each year.[1] These statistics show that newer, molecular-based therapeutic modalities are urgently needed. Most anti-cancer drugs target nuclear retention of tumor suppressor proteins (TSP) such as p53 family proteins,[2] FOXO[3] and p27.[4] However, mislocalization of these and other TSP by over-expression of the nuclear export protein chromosome maintenance region 1 (CRM1) in cancer cells leads to their functional inactivation.[5] Nuclear exclusion of TSP, mediated by CRM1, is now appreciated as a significant mechanism of therapy resistance by malignant cells.[6] Here, we report a novel strategy to overcome these CRM1-mediated effects in NHL.

CRM1 is a member of the importin β superfamily of nuclear transport receptors, recognizing proteins bearing a leucine-rich nuclear export sequence (NES).[7] There are seven known nuclear export proteins, but CRM1 mediates the export of nearly all major TSP out of the nucleus. Nuclear exclusion of p53 family proteins, FOXO, p27, and other TSP by CRM1 renders cancer cells resistant to apoptosis by different therapies.[8] Forced nuclear retention of TSP by inhibition of CRM1 (without affecting their nuclear import) leads to restoration of their tumor-suppressing activities and prevents their proteasome-mediated degradation in the cytoplasm.[9] Nuclear localization with functional activation of TSP has been shown to lead to selective elimination of tumor cells.[10] Inhibition of CRM1 is one approach to restore nuclear localization and activation of multiple TSP, allowing them to function properly and induce cancer-specific apoptosis.

Earlier approaches to target CRM1 led to the development of leptomycin B (LMB)[11] which proved to have limited clinical applicability because of associated toxicity and minimal efficacy.[12] Semi-synthetic derivatives of LMB with improved pharmacological properties had better therapeutic indices in animals indicating that the side effects of LMB were due to off-target effects;[13] these agents have not entered clinical studies. A novel small molecule reversible inhibitor of CRM1 was also reported to have activity against multiple myeloma.[14] This suggests that newer CRM1 inhibitors with high specificity, cancer cell selectivity and low toxicity are needed.

Using high throughput screening and structure-based drug design, we have developed a highly specific small molecule inhibitor of CRM1 that irreversibly binds to the putative target protein NES recognizing the Cys-528 residue (*Online*

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Supplementary Figure S1 and Figure 1A). This results in locking of TSP in the nucleus of cancer cells leading to selective apoptosis in solid tumors[15,16] and hematologic malignancies.[17,18] In this proof-of-concept study, we investigated the anti-cancer potential of selective inhibitors of nuclear export (SINE) against NHL cell lines and corresponding xenograft models. Our findings can potentially be translated towards clinical application of SINE against NHL.

Design and Methods

WSU-FSCCL, representing follicular small cleaved cell lymphoma (wt-p53), WSU-DLCL2 (mut-p53), representing diffuse large cell lymphoma, and WSU-WM representing Waldenstrom's macroglobulinemia (mut-p53), were developed and characterized in our laboratory at Wayne State University.[19,20] Peripheral blood lymphocytes (PBL) were obtained from normal non-smoking healthy donors at Karmanos Cancer Institute. Primary antibodies for FOXO3a, p27, p21, p53, p73, Bax, β-actin and lamin were purchased from Cell Signaling (Danvers, MA, USA). All secondary antibodies were obtained from Sigma (St. Louis, MO, USA).

Cell growth inhibition determined by the trypan blue assay

Cells were seeded at a density of 2×10^5 viable cells/mL in 24-well or 6-well culture plates (Costar, Cambridge, MA, USA), or 10-cm cell culture dishes (Corning Inc., Corning, NY, USA). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), at 37°C in a humidified incubator with 5% CO_2. The number of viable cells was determined by a trypan blue exclusion test [trypan blue (0.4%), Sigma Chemical Co. St. Louis, MO, USA]. KPT SINE were added at indicated concentrations (0-150 nM) diluted from a 10 μM stock. The results were plotted as means ± SD of three separate experiments using three determinations per experiment for each experimental condition.

Quantification of apoptosis by histone DNA enzyme-linked immunosorbent assay and annexin V-FITC assay

Cell apoptosis was detected using an annexin V-FITC assay (Biovision, Danvers, MA, USA) and a Histone DNA ELISA Detection Kit (Roche, Life Sciences) according to the manufactur-

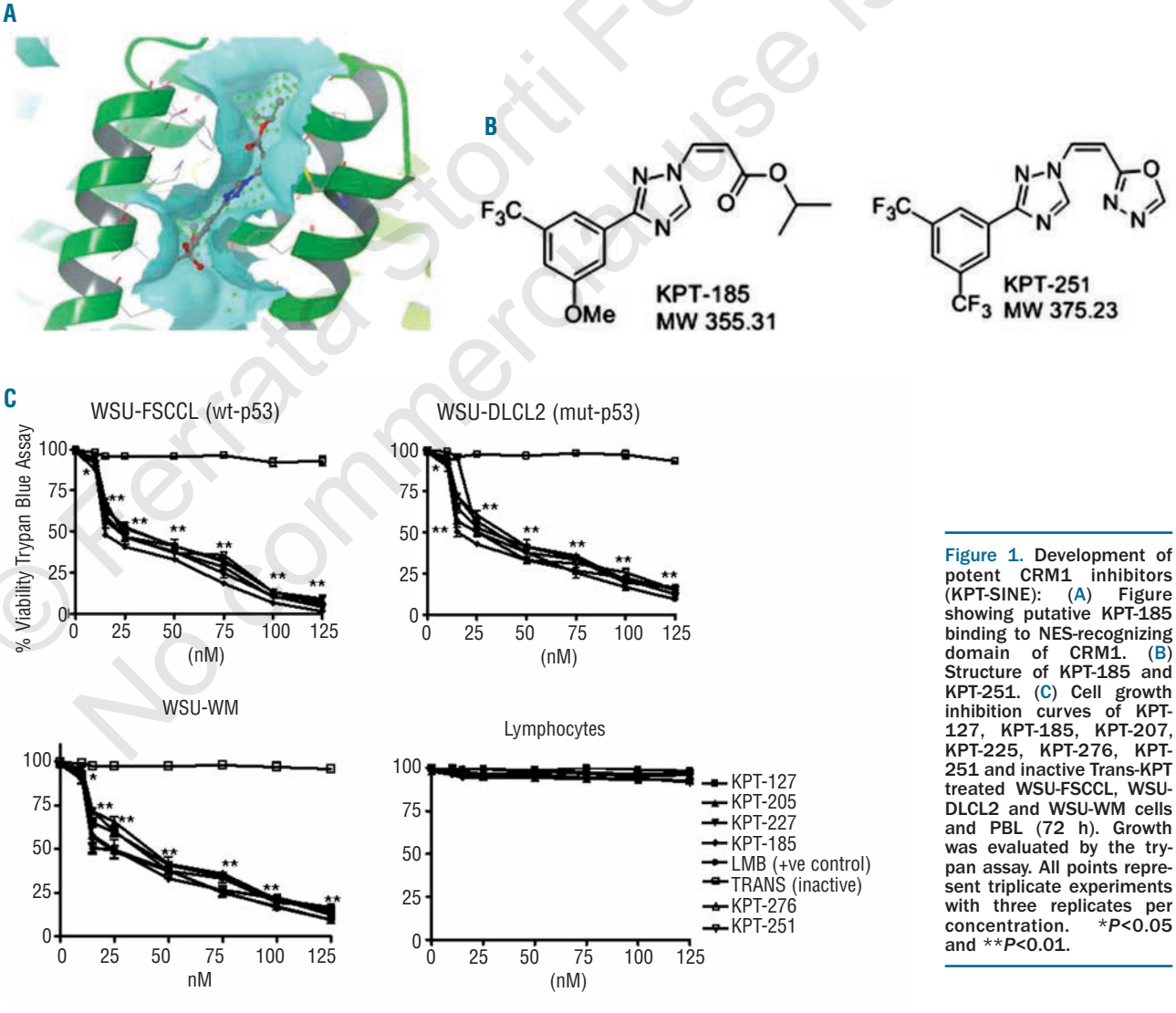

Figure 1. Development of potent CRM1 inhibitors (KPT-SINE): (A) Figure showing putative KPT-185 binding to NES-recognizing domain of CRM1. (B) Structure of KPT-185 and KPT-251. (C) Cell growth inhibition curves of KPT-127, KPT-185, KPT-207, KPT-225, KPT-276, KPT-251 and inactive Trans-KPT treated WSU-FSCCL, WSU-DLCL2 and WSU-WM cells and PBL (72 h). Growth was evaluated by the trypan assay. All points represent triplicate experiments with three replicates per concentration. **P*<0.05 and ***P*<0.01.

ers' protocols. NHL cells were seeded as described previously and treated with KPT-SINE for 72 h. All procedures were performed according to our previously published protocols.[21]

Western blot and immunoprecipitation analysis

Cells (1×10^6) were grown in 6-well petri plates and exposed to indicated concentrations of KPT-185 for 24 h followed by extraction of nuclear and cytosolic proteins for western blot analysis and immunoprecipitation assays using previously described methods.[21]

Immunofluorescence assay for p53 and p73 cellular staining

For protein localization experiments, 1×10^6 cells were grown in 24-well plates and exposed to KPT-185 at indicated concentrations for 24 h. At the end of the treatment the cells were mounted on glass slides using cytospin (2500 rpm for 10 s twice) followed by fixing with 10% paraformaldehyde for 20 min. The fixed slides were permeabilized using 0.5% Triton (Sigma, St Louis, USA) and were blocked in 0.2% horse serum for 45 min. The slides were probed with primary and secondary antibodies according to our previously published methods.[22] The slides were then dried and mounting medium was added; the slides were covered with coverslips before analysis under an inverted three-color (DAPI, GFP and RFP) fluorescent microscope.

Small interfering RNA and transfections

To study the effect of p53/p73 silencing on the activity of KPT-185, we utilized small interfering RNA (siRNA) silencing technology. p53 siRNA, p73 siRNA and control siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with either control siRNA or p53/p73 siRNA for 24 h using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. All procedures were standardized and have been published previously.[23] After the siRNA treatment, cells were further treated with KPT-185 in 24-well plates for the trypan blue and annexin V-FITC assays.

p53-DNA binding and transcriptional activation by KPT-185

Electromobility shift assay (EMSA) was performed using the Odyssey Infrared Imaging System with p53 IRDye labeled oligonucleotide from LI-COR, Inc. (Lincoln, NE, USA). The DNA binding reaction included 5 μg of the nuclear extract mixed with oligonucleotide and gel shift binding buffer consisting of 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.25 mg/mL poly(dI): poly(dC). The reaction was incubated at room temperature in the dark for 30 min. Next, 2 μL of 10× Orange G loading dye was added to each sample and loaded on the pre-run 8% polyacrylamide gel and run at 30 mA for 1 h. Specific transcription factor DNA binding in nuclear extract after treatments was detected using the sensitive non-radioactive "Cayman's p53 Transcription Factor Assay kit" (Ann Arbor, MI, USA). The procedure for nuclear extract preparation and transcription activity was performed according to the manufacturer's protocol.

Development of animal xenografts and pre-clinical efficacy trial

Mouse xenografts were established as described previously.[24] The maximum tolerated dose (MTD) of KPT-276 and KPT-251 in severe combined immunodeficient mice was determined to be 150 mg/kg PO (every other day x 3/week or every day x 5/week for 2 weeks) and subcutaneous (s.c.) injection of 75 mg/kg, respectively. Once the MTD had been determined, mice were treated with KPT-276 orally at doses of 75 and 150 mg/kg or KPT-251 at doses of 25 and 75 mg/kg s.c. daily for 2 weeks. The CHOP regimen was used as the positive control at the MTD, as described previously.[25] Mice in the control and KPT-SINE treated groups were followed for measurement of s.c. tumors, changes in body weight, and other side effects of the drugs. Tumors were measured twice weekly. Tumor weight (mg) was calculated using the formula: $(A \times B^2) / 2$, where A and B are the tumor length and width (in mm), respectively. To avoid discomfort and in keeping with our IACUC procedures, animals were euthanized when their total tumor burden reached 2,000 mg. All studies involving mice were done under Animal Investigation Committee-approved protocols.

Immunohistochemical determination of tumor markers

The expression of p73 was detected in histological sections of tumor xenografts. Sections were cut from formalin-fixed, paraffin-embedded tissue blocks, collected on 3-ethoxy-aminoethyl-silane-treated slides, and allowed to dry overnight at 37°C. Sections were dewaxed in xylene, rehydrated through graded concentrations of ethanol to distilled water, immersed in 10 mmol/L citrate buffer (pH 6.0), and processed in a thermostatic water bath for 40 min at 98°C for antigen retrieval. The sections were incubated with anti-p73 and ki67 overnight at room temperature in a humidified atmosphere followed by a 30-min incubation with secondary antibody. Finally, the slides were incubated with streptavidin peroxidase and visualized using the 3,3′-diaminobenzidine chromogen (Lab Vision).

Isolation of tumor tissue proteins and western blot analysis

At the end of the treatment period tumors were excised and one part was minced in protein isolation buffer using our well established methods.[26] Fifty micrograms of tumor lysates were resolved using a western blotting assay. The membranes were probed for p73, Bax and β-actin. All tumor tissue western blots experiments were performed in triplicate.

Results

KPT-SINE induces growth inhibition in non-Hodgkin's lymphoma cell lines irrespective of p53 function

Based on the crystal structure of human and mouse CRM1, we have designed various SINE as a new class of drug-like, small molecule inhibitors of CRM1 (Figure 1A shows the drug binding to CRM1 and Figure 1B illustrates the structures of two SINE, KPT-185 and KPT-251). KPT-185 induces a covalent modification of Cysteine 528 in the NES-recognizing domain of CRM1, and consequently restricts its ability to interact with cargo proteins including TSP. Six analogs KPT-185, KPT-127, KPT-205, KPT 251, KPT-276 and KPT-227, the *trans*-isomer of KPT-TRANS which has minimal CRM1 inhibitory activity as a negative control, and LMB (as a positive control) were investigated for their growth inhibitory and apoptotic potential against a panel of NHL cell lines and a normal human PBL using the trypan blue assay. As can be seen from Figure 1C, exposure of different NHL cell lines to sub-micromolar concentrations of KPT SINE for 72 h resulted in a dramatic loss of viability (IC_{50} ~25 nM in the three NHL cell lines). Moreover, SINE have broad specificity against different tumor types with IC_{50} values generally in the sub-micromolar range (*Online Supplementary Figure S2*). Most significantly the drugs showed negligible cytotoxicity to PBL (IC_{50}>20 μM). Reduction in viability was associated with CRM1 inhibito-

ry activity, as KPT-TRANS, which lacks significant SINE activity, did not induce statistically significant growth inhibition. LMB had a somewhat similar growth inhibitory profile as the SINE compounds.

KPT SINE induce apoptosis in lymphoma but not normal cells

We investigated whether inhibition of CRM1 induces apoptosis in the NHL cell lines by using an annexin V-FITC assay and histone DNA ELISA. As expected and in line with the growth inhibition results, SINE induced apoptosis in WSU FSCCL (>80% at 100 nM), WSU-DLCL2 (>75% at 100 nM) and WSU-WM (>50% at 100 nM) cell lines while sparing PBL, which showed no apoptosis up to 2 μM (Figure 2A). The results of the histone DNA ELISA were comparable to those of the annexin V-FITC assay (Figure 2B). The inactive analog KPT-TRANS did not induce apoptosis in the tested cell lines. Collectively, the data presented thus far demonstrate the anti-cancer potential of various SINE as a new class of compounds which are cytotoxic to NHL cells but not to PBL.

Major tumor suppressor proteins are targets of KPT-SINE

In order to delineate the molecular mechanisms of cancer cell apoptosis induction by CRM1 inhibitors in greater detail, nuclear localization of different TSP was investigated using western blot analysis and fluorescence microscopy. As shown in Figure 3A, exposure of NHL cells to increasing concentrations of KPT-185 (0-50 nM) resulted in a progressive increase in the nuclear fraction of major TSP (FOXO3a, p53, p73, p27 and p21) in both WSU-FSCCL (wt-p53) and WSU-DLCL2 (mut-p53). Similary, we observed activation of both p53 and p73 protein and mRNA expression in WSU-FSCCL and WSU-DLCL2 cell lines, respectively (*Online Supplementary Figure S3*). In contrast, KPT-185 exposure did not result in any significant activation of p53/p73 in normal PBL at similar concentrations (Figure 3B). We also evaluated p53 and p73 nuclear retention using an immunofluorescence assay. As can be seen in Figure 3C, DMSO control WSU-DLCL2 or WSU-FSCCL cells showed minimal p73 or p53 nuclear staining. However, exposure to 50 nM KPT-185

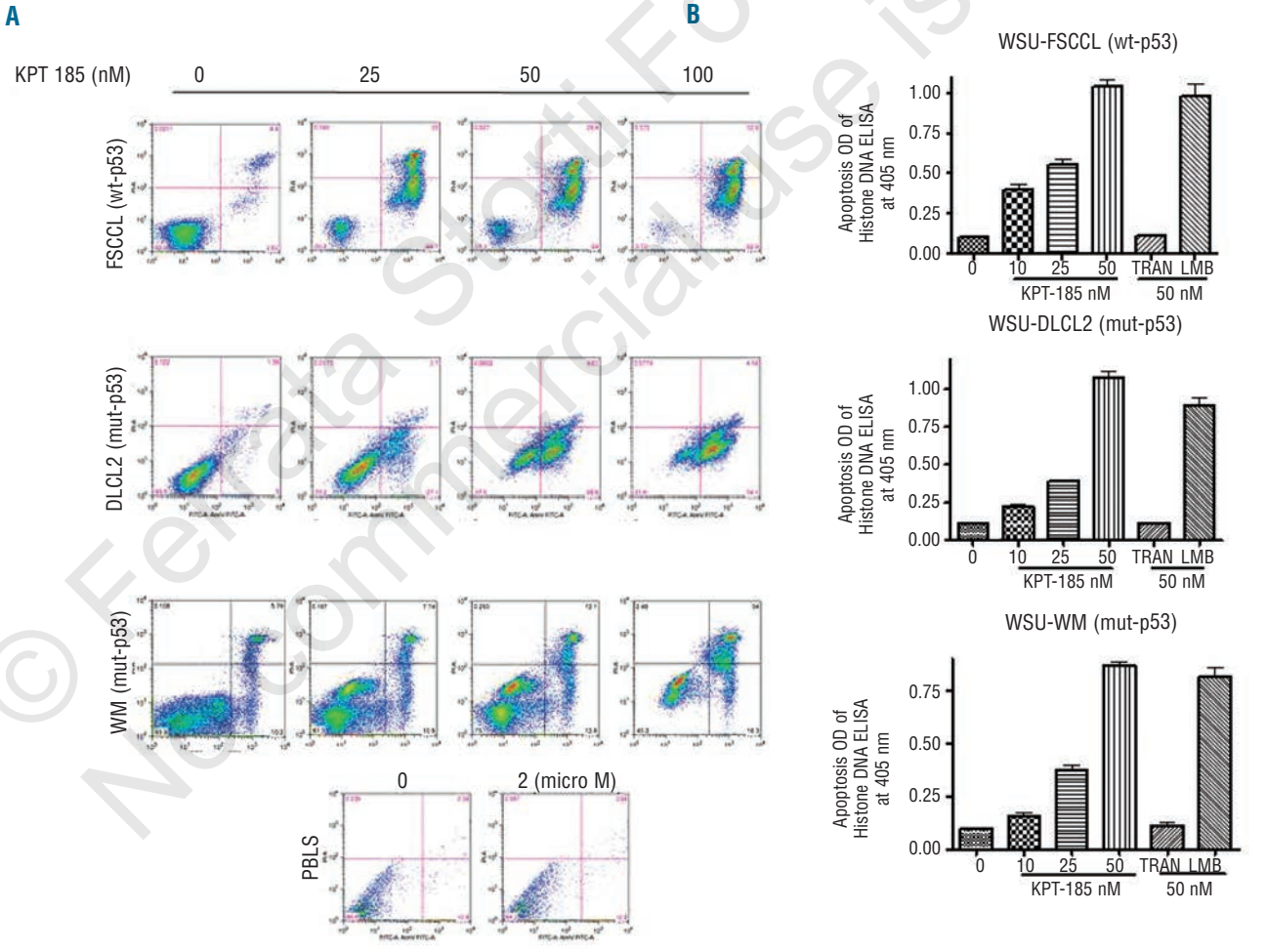

Figure 2. KPT SINE induce apoptosis: NHL cells were exposed to indicated concentrations of KPT-185 for 72 h. (A) Apoptosis was analyzed using annexin V FITC and (B) histone DNA ELISA according to the manufacturer's protocol (Biovision Danvers and Roche Paolo Alton, respectively). Note: apoptosis was induced by KPT-185 in NHL cells only and not in PBL. The inactive analog KPT-TRAN did not induce apoptosis. Results are representative of three independent experiments. **P*<0.05 and ***P*<0.01.

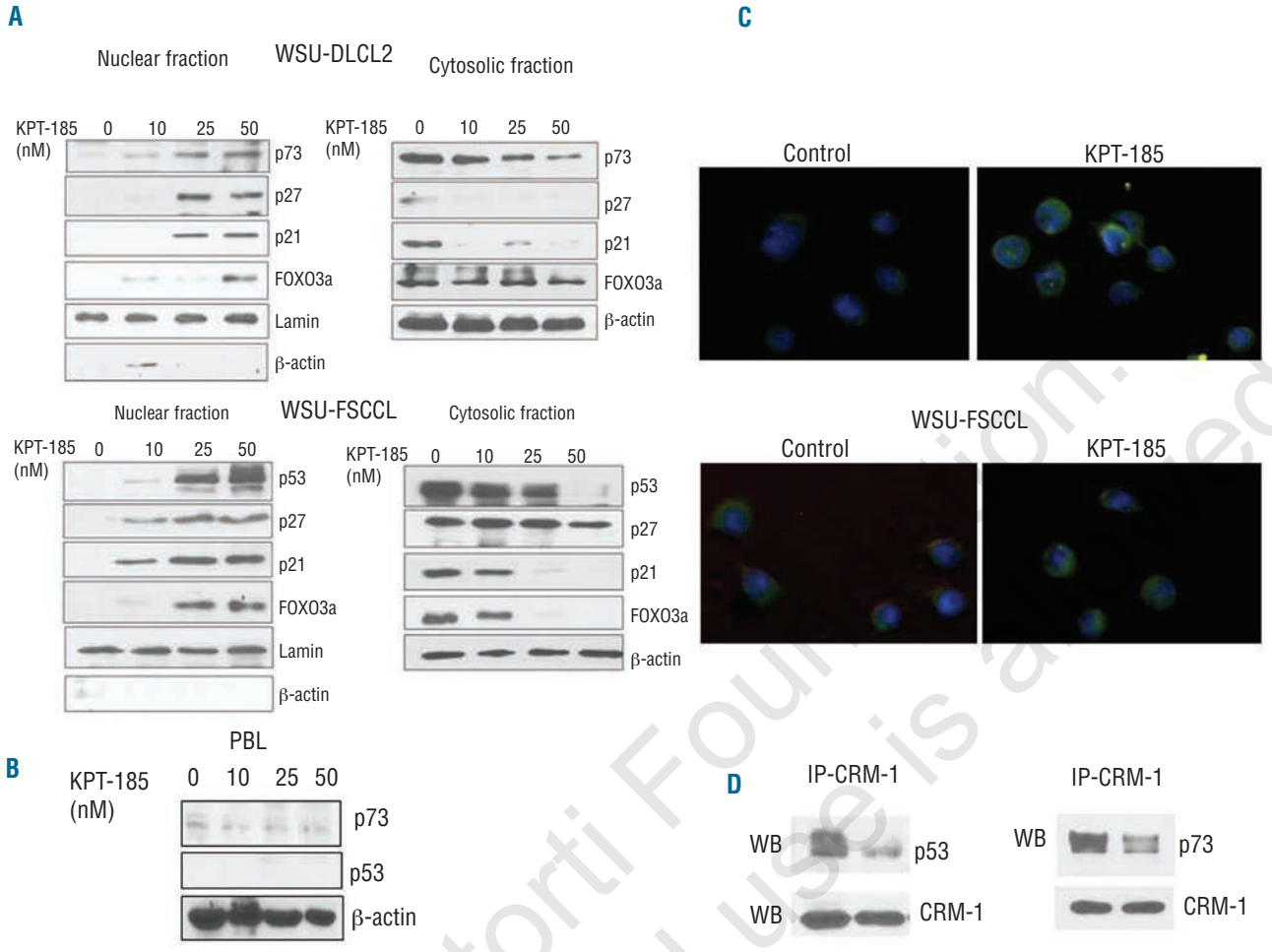

Figure 3. KPT-185 induces nuclear retention of major TSP and disrupts the CRM1-p53/p73 interaction in NHL cells. (A) Nuclear and cytosolic lysates (50 μg) from KPT-185-treated WSU-FSCCL and WSU-DLCL2 cells were resolved using western blotting. The membranes were probed with FOXO3a, p27, p21, p53, p73, lamin and β-actin (Cell Signaling, Danvers, MA, USA). β-actin was used as a loading control for whole cell lysates and lamin for nuclear lysates. Blots are representative of three independent experiments. (C) Immunofluorescence assay demonstrating p53 localization by KPT-185 in WSU-FSCCL and p73 nuclear localization in WSU-DLCL2 cells. Images are representative of three independent experiments. (D) Immunoprecipitation demonstrating disruption of the CRM1-p53/p73 interaction. Two hundred micrograms of whole cell lysates were immunoprecipitated using CRM1 antibody with the SIGMA immunoprecipitation assay kit (Sigma, St Louis). The samples were resolved using western blotting and probed with p53 and p73. Blots are representative of two independent experiments.

resulted in distinct nuclear accumulation of p73. Direct evidence that KPT-185 can disrupt the CRM1-p53/p73 interaction came from results of immunoprecipitation assays in which lysates from the DMSO control and KPT-185 treated cells were pulled down with CRM1 antibody and probed with p53 and p73, respectively, using western blotting. As can be seen from Figure 3D (left and right panels), KPT-185 treatment resulted in a reduced interaction between p53-CRM1 in WSU-FSCCL and p73-CRM1 in WSU-DLCL2 as little protein precipitated in the treated samples.

Tumor suppressor knockdown by RNA interference abrogates KPT SINE activity

In order to determine whether SINE-induced apoptosis was mediated primarily through p53 or p73, we investigated the effect of either p53 siRNA in wild-type and p73 siRNA in mutated cell lines on the activity of KPT-185. As can be seen in Figure 4A, siRNA knockdown of p53 and/or p73 markedly reduced apoptosis by KPT-185 in WSU-FSCCL and WSU-DLCL2 cell lines, respectively. Western blotting experiments on siRNA-treated cells showed down-regulation of p53 and p73. Of paramount significance is the observation that in addition to p53 and/or p73, siRNA silencing showed downregulation of common downstream effector pathway molecules such as cell cycle regulator p21 and pro-apoptotic Bax (Figure 4B). We also evaluated the effect of p53 and p73 siRNA on cell cycle regulation (a target of activated p53 and p73) by KPT-SINE. As shown in Figure 4C, in the absence of siRNA, KPT-185 treatment resulted in enhancement of G0-G1 cell cycle accumulation (Trans-KPT and LMB were used as negative and positive controls, respectively). However, in the presence of p53 siRNA in WSU-FSCCL and p73 siRNA in WSU-DLCL2, the KPT-815 was not effective in inducing G0-G1. These results provide further support for the primary role of p53 family members in KPT-SINE-mediated activity.

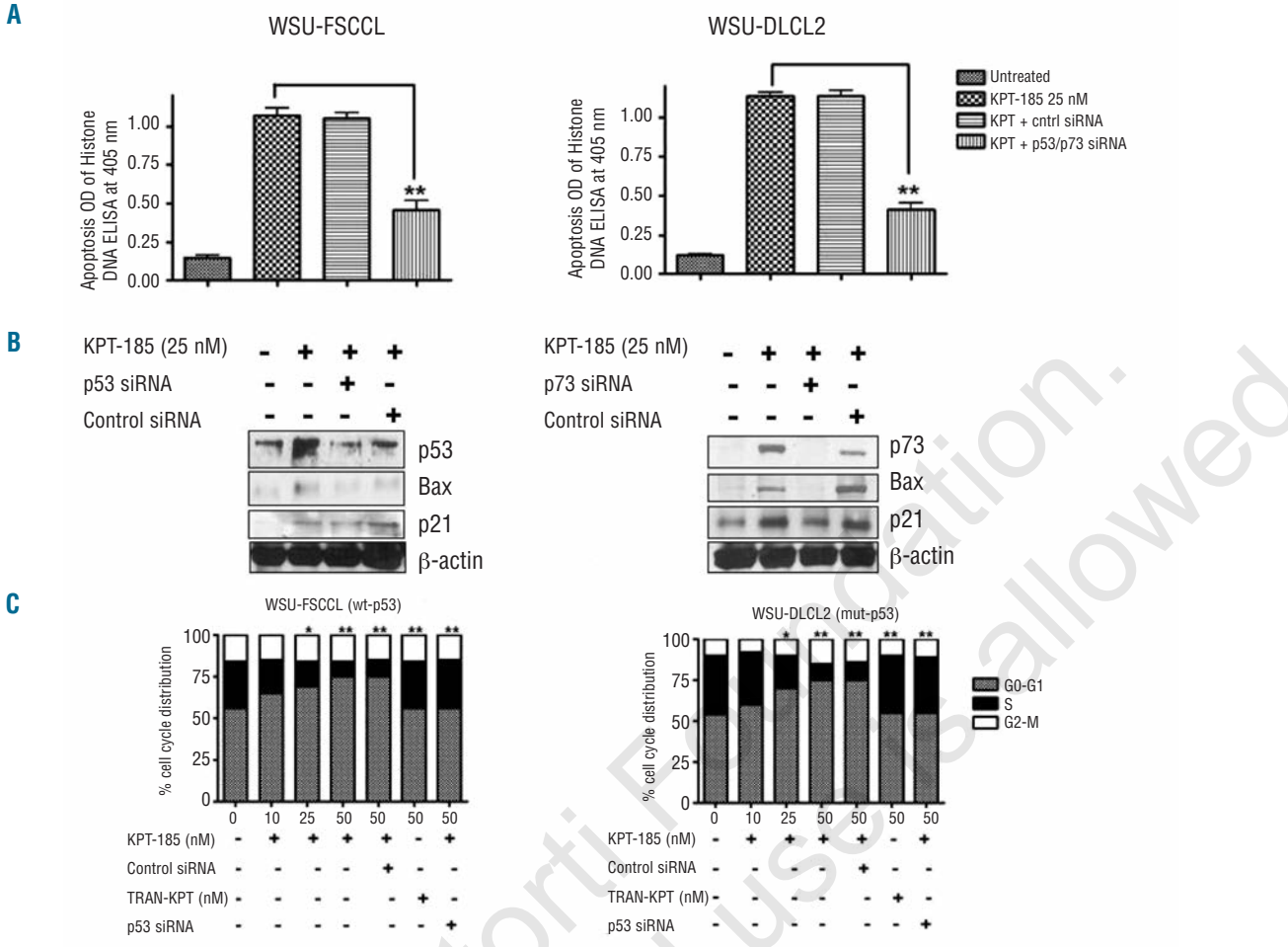

Figure 4. siRNA against p53 and p73 abrogates KPT SINE activity: (A) WSU-FSCCL and WSU-DLCL2 cells were exposed to 25 nM of KPT-185 in the absence or presence of control siRNA, p53 or p73 siRNA. Details of siRNA treatment are provided in the *Design and Methods* section. Apoptosis was evaluated using a histone DNA ELISA as described in the *Design and Methods* section. Note that KPT-185 activity is suppressed in the presence of p53/p73 siRNA (**P<0.01) while control siRNA groups show no statistically significant effect. Results are representative of three independent experiments. (B) siRNA suppresses activation of p53 or p73 pathways (including downstream p21 and Bax). Blots are representative of three independent experiments. (C) Cell cycle analysis showing G0-G1 arrest after KPT-SINE treatment in both cell lines. Trans-KPT was ineffective. siRNA against p53 or p73 abrogates KPT-185 cell cycle regulatory activity in WSU-FSCCL and WSU-DLCL2 cell lines, respectively. Data are representative of three independent experiments. *P<0.05 and **P<0.01 compared to control. **P<0.01 in p73 siRNA or p53 siRNA treated group in comparison to the group treated with KPT-185 alone.

KPT-SINE promote cancer cell-selective p53 DNA binding

In order to verify whether SINE can promote nuclear localization of p53 in NHL cells, EMSA p53-DNA binding assays, and p53 transcription activation assays were performed. As shown in Figure 5A, SINE treatment resulted in enhancement of p53-DNA binding. At similar doses of SINE, there was no statistically significant p53 DNA binding in PBL, highlighting an NHL-specific p53 effect. Furthermore, a highly specific p53 transcription activation assay was utilized to validate p53 transcriptional activation in WSU-FSCCL and PBL. As shown in Figure 5B, KPT-185 but not the inactive trans-isomer KPT-TRANS, induced p53 transcriptional activation in WSU-FSCCL cells. Intriguingly, unlike Nutlin-3 and LMB (used as a positive control), which induced p53 activation in both WSU-FSCCL and PBL, KPT-185 showed p53 activation only in NHL cells but not in PBL.

KPT SINE shows potent in vivo *antitumor activity*

We evaluated the anti-tumor activity of KPT-276 (given orally) and KPT-251 (subcutaneously). KPT-276 administered orally up to a dose of 150 mg/kg and KPT-251 administered subcutaneously up to 75 mg/kg did not cause any visible signs of toxicity or weight loss in the tested animals (Figure 6B). Oral administration of KPT-276 at 75 and 150 mg/kg for 2 weeks resulted in statistically significant tumor growth inhibition (65% and 70%, respectively) in the WSU-DLCL2 xenograft model. On the other hand, s.c. administration of KPT-251 at doses of 25 and 75 mg/kg daily for 2 weeks showed 70% and 74% tumor growth inhibition. Most significantly, the anti-tumor activity of these KPT-SINE was equivalent to that of the standard, four-drug CHOP chemotherapy regimen (used as a positive control) (Figure 6A). At the end of the treatment period, tumors were excised for additional molecular analyses. In line with our *in vitro* results, histological examination of mut-p53 cell line-derived tumor sections revealed enhancement of p73 upon KPT-276 treatment and this was coupled with suppression of Ki67 (an index of proliferation) (Figure 6C). Additionally, western blot expression analysis of tumor tissue lysates showed similar trends with emergence

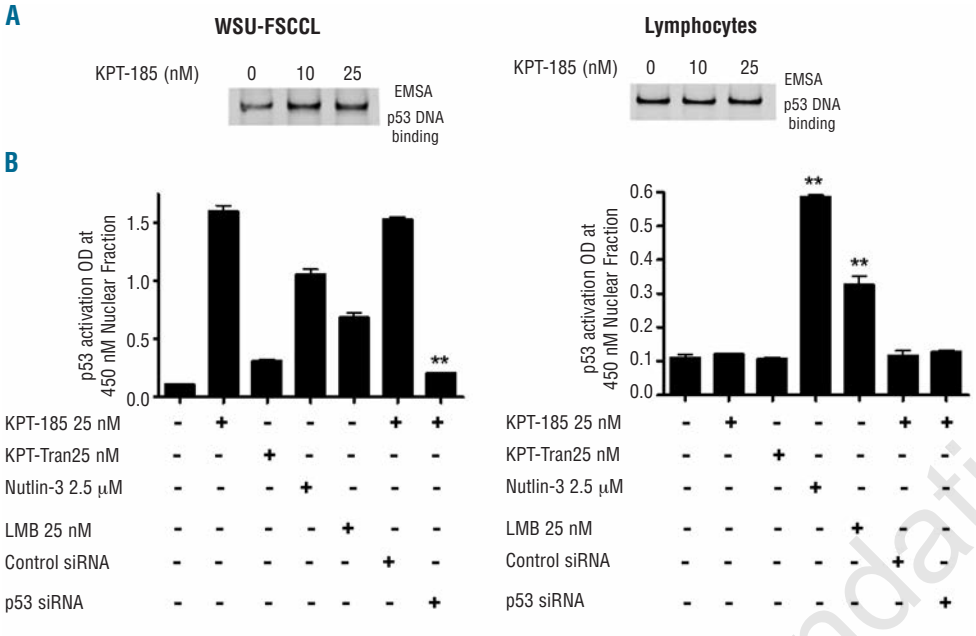

Figure 5. p53 DNA binding and transcriptional activation by KPT-185: (A) EMSA demonstrating enhancement in p53 DNA binding on treatment with increasing concentrations of KPT-185 in WSU-FSCCL but not normal lymphocytes. (B) Nuclear lysates were isolated and a p53 transcription assay was performed in 96-well plates according to the manufacturer's protocol (Cayman Chemicals Ann Arbor, MI, USA). Nutlin-3 (2.5 μM) and LMB (25 nM) were used as positive controls. In the presence of siRNA against p53 the DNA binding capacity is lost. ***P*<0.01.

of p73 and pro-apoptotic Bax (Figure 6D). Taken together, these results fortify our hypothesis that the anti-cancer activity of SINE is due at least in part to the intra-tumoral activation of p53 family proteins.

Discussion

In this report we show the anti-tumor potential of a new class of small molecule CRM1 inhibitors in models of both indolent and aggressive NHL. The data highlight that SINE activity involves nuclear retention and functional activation of major TSP and more specifically p53 family proteins. The pre-clinical evidence presented here forms a solid platform that could accelerate the clinical translation of SINE CRM1 antagonists for the treatment of NHL.

A common mechanism controlling gene expression involves alterations in the subcellular localization of transcriptional regulators. Many transcription factors and co-factors possess nuclear localization sequences and NES that co-ordinate entry into and exit from the nucleus, respectively. For proteins over ~40 kDa, passage into and out of the nucleus is governed by the nuclear pore complex, a multi-subunit structure embedded in the nuclear envelope.[27] Positively charged nuclear localization sequences are bound by importins α and β, which promote binding of cargo proteins to the cytosolic face of the nuclear pore complex and facilitate translocation of proteins into the nucleus.[28] There are seven known nuclear export proteins (XPO1 through XPO7).[29] The CRM1 protein, also referred to as exportin 1 (XPO1), mediates the transit of proteins with a canonical leucine-rich hydrophobic NES out of the nucleus.[30] CRM1 binds hydrophobic NES together with the small GTP binding protein Ran, occasionally requiring adapter proteins such as 14-3-3, and these complexes are shuttled out of the nucleus through a series of interactions with the nuclear pore complex.[31] Chemotherapy and targeted therapies work, at least in part, by forcing nuclear accumulation of TSP which initiate cascades of pathways resulting in cell death. However, CRM1 over-expression (which is common in NHL) results in constitutive nuclear export of TSP thereby suppressing apoptosis. This in turn translates into low efficacy of different anti-lymphoma regimens such as CHOP and R-CHOP. Targeted inhibition of CRM1 is, therefore, an attractive strategy against lymphoid tumors.

Apart from LMB, there have not been any serious attempts to develop targeted inhibitors against CRM1. LMB is a *Streptomyces* metabolite that covalently attaches to the sulfhydryl group of cysteine at position 528 in CRM1 and thereby prevents CRM1 from associating with NES-containing cargo.[32] As a natural product, LMB has very poor pharmaceutical properties, must be given parenterally, and has poor selectivity for cancer *versus* normal cells. It showed marked toxicity in mice, and similar toxicities in humans. Such promiscuity of this drug may be through weak associations with secondary proteins other than CRM1, resulting in unwanted side effects. This is a common phenomenon observed with most drugs derived from natural agents. The semi-synthetic, parenteral derivatives of LMB with improved pharmacological properties have better therapeutic windows in mouse cancer models. However, their clinical utility has yet to be verified. A reversible, orally bioavailable CRM1 inhibitor CBS9106 has been reported to have anti-myeloma activity but its developmental status is unknown.[14]

Our drug-like, small molecule SINE CRM1 antagonists have shown high specificity towards CRM1, can be administered orally, and show good tolerability. *In vitro*, they show killing of solid tumors and malignant lymphocytes with minimal toxicity (e.g., reversible, transient cell cycle arrest) to normal lymphocytes. Along these lines, data presented here provide a strong rationale supporting SINE-mediated localization and activation of apoptosis-inducing functions of the guardian protein p53 and its family member p73.

Although initially responsive, NHL tumors become resistant to immuno-chemotherapy such as R-CHOP. NHL has a complex genetic makeup and lacks clear drug targets.[33] While identifying new avenues and prognostic markers, studies from our laboratory and those of others have demonstrated that down-regulation of p53 (in the setting of

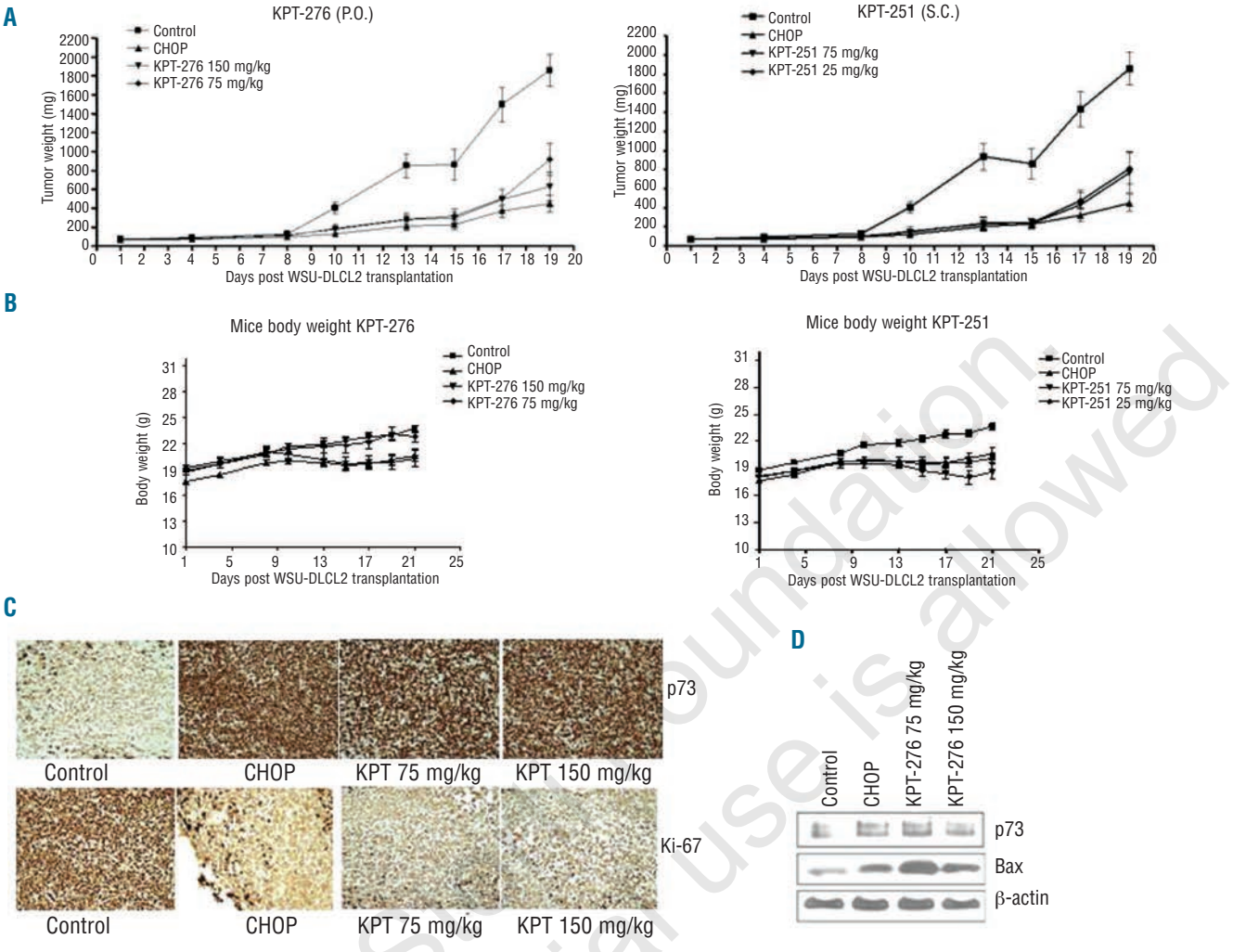

Figure 6. Pre-clinical anti-tumor efficacy trial of KPT-276 and KPT-251: (A) Efficacy trial of KPT-251 and KPT-276 treated at indicated doses twice a week for 2 weeks in comparison to CHOP (given i.v.). Note: both KPT-276 and KPT-251 show similar anti-tumor effects compared to the CHOP chemotherapeutic regimen. (B) ICR SCID animal body weight loss evaluation after KPT-SINE treatment. Note that there is no statistical difference compared to the untreated control group in body weight of animals given up to 150 mg/kg for KPT-276 (orally) and 75 mg/kg KPT-251 (s.c.) indicating that the drug is well tolerated. (C) Tumor tissue histology showing enhancement of p73 and suppression of the proliferation index Ki67. (D) Western blot analysis of tumor tissue lysates showing enhancement of p73 and Bax in the treated group compared to vehicle control. β-actin was used as a loading control.

wt p53) is directly correlated to therapy resistance and poor overall survival in NHL.[34] This led to our proposed hypothesis that p53 and its relative p73 could be a possible therapeutic target in NHL. Interestingly, most of the key TSP, including p53 and p73, carry a NES and are substrates for CRM1. Supporting these observations, our results show that SINE induced p53 family protein expression and nuclear localization in malignant but not normal lymphocytes. Enforced nuclear localization of p53, p73 and other TSP may prevent their (cytoplasmic) degradation by the proteasome, even in the presence of oncogenic signals that typically facilitate their nuclear export.

As CRM1 inhibition forces the nuclear localization of many TSP, delineation of which specific growth regulators may be involved in SINE-induced cytotoxicity is important. Here, we showed that in addition to FOXO, p27 and p21, siRNA silencing of p53 in wt- or p73 in mut-p53 cell lines abrogates SINE CRM1 antagonist functional TSP activation and downstream cytotoxicity *in vitro*. These findings indicate that in NHL, forced nuclear retention of TSP in general and p53 and/or p73 in specific may be critical to the anti-lymphoma activity of SINE compounds. Moreover, these data show that SINE compounds should have activity across multiple genotypes, including NHL with p53 mutations. Our data in both WSU-DLCL2 and WSU-WM confirm this broad activity, and implicate p73 in apoptosis induction in tumors harboring p53 mutations. As nuclear export is essential for normal cell homeostasis, it was critical to assess the tolerability of systemic administration of SINE CRM1 antagonists in animals. Along these lines, we observed that oral or s.c. administration of SINE is well tolerated at doses that have significant anti-tumor activity. Moreover, the efficacy of SINE was associated with activation of p73 signaling in the tested tumors *in vivo* (Figure 6A-C) confirming the *in vitro* findings.

In conclusion, we have rationally designed small molecule, drug-like SINE CRM1 antagonists, and performed *in vitro* and *in vivo* evaluations to demonstrate their anti-lymphoma potential using multiple NHL models. Based on these provocative pre-clinical studies, we anticipate that SINE will be evaluated in the clinic against NHL and other hematologic malignancies in the near future.

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