Pre-clinical Activity of a novel CRM1 inhibitor in Acute Myeloid Leukemia

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Abbreviations Used: CRM1= Chromosome maintenance protein 1; SINE= selective inhibitors of nuclear export; NPM1 = nucleophosmin 1.
ABSTRACT

CRM1 is a nuclear export receptor involved in the active transport of tumor suppressors [e.g. p53 and nucleophosmin] whose function is altered in cancer due to increased expression and overactive transport. Blocking CRM1 mediated nuclear export of such proteins is a novel therapeutic strategy to restore tumor suppressor function. Orally bioavailable selective inhibitors of nuclear export (SINE) that irreversibly bind to CRM1 and block the function of this protein have been recently developed. Here, we investigated the anti-leukemic activity of KPT-SINE (KPT-185 and -276) in vitro and in vivo in acute myeloid leukemia (AML). KPT-185 displayed potent anti-proliferative properties at submicromolar concentrations (IC50 values; 100-500nM), induced apoptosis (average 5 fold increase), cell-cycle arrest and myeloid differentiation in AML cell lines and patient blasts. A strong down-regulation of the oncogene FLT3 after KPT treatment in both FLT3-ITD and wild type cell lines was observed. Finally, using the FLT3-ITD positive MV4-11 xenograft murine model, we show that treatment of mice with oral KPT-276 (analog of KPT-185 for in vivo studies) significantly prolongs survival of leukemic mice (P<0.01). In summary, KPT-SINEs are highly potent in vitro and in vivo in AML. The preclinical results reported here support clinical trials of KPT-SINE in AML.
INTRODUCTION

The nucleocytoplasmic exchange of proteins (macromolecules bigger than 40kDa) is a spatially and temporally regulated process that involves a number of nucleocytoplasmic shuttling proteins\(^1\). CRM1 (Exportin-1, XPO1), a member of the karyopherin \(\beta\) family of transport receptors, is an important nuclear protein export receptor, that recognizes hydrophobic, leucine rich nuclear export signal (NES) and transports target proteins across a Ran-GTP gradient\(^2-5\). CRM1 is involved in the active transport of a number of cargo proteins, including transcription factors, tumor suppressors proteins (TSP) and cell-cycle regulators such as p53\(^6\), p21, p27\(^7\), nucleophosmin\(^8,9\) (NPM1) as well as RNA molecules\(^2\). Recent data indicate that TSP such as p53 can be excluded from the nucleus and thereby inactivated in cancer by hyperactive nuclear export\(^10-12\). Over-expression of CRM1 protein has been described in several cancers (glioblastoma, ovarian and cervical cancer) and it has been associated with worse outcome\(^13-15\). In addition, deregulated oncogenic pathways in cancer, such as aberrant \(AKT\) or \(BCR/ABL\) signaling, have been shown to cause post-transcriptional changes (in particular phosphorylation) of TSP such as p27 and FOX03, promoting their nuclear export through CRM1\(^16,17\). Thus, prevention of CRM1 mediated nuclear export of TSP presents itself as an attractive anti-neoplastic therapeutic modality. However progress in this direction has been limited due to the severe clinical toxicity of the anti-CRM1 drugs developed so far, the most well-known of which is leptomycin B\(^18\). Karyopharm Therapeutics has developed novel, oral bioavailable small molecule Selective Inhibitors of Nuclear Export (KPT-SINEs) that specifically and irreversibly bind to CRM1 and block the function of this protein by binding to the reactive site Cys 528 residue\(^19,21\). These compounds have been shown to
induce apoptosis and block proliferation in several cancer cell lines, including colon\textsuperscript{19,22}, pancreas\textsuperscript{22} and breast cancer\textsuperscript{23} as well as chronic myeloid\textsuperscript{24} and lymphocytic leukemias\textsuperscript{25}.

AML is a heterogeneous clonal disorder characterized by the accumulation of immature myeloid progenitors (blasts) in the bone marrow and peripheral blood\textsuperscript{26}. Non-random chromosomal abnormalities (e.g., deletions, translocations, duplications and inversions) are identified in approximately 55% of all adult primary AML patients\textsuperscript{26}. In contrast, about 40-50% of all AML cases are cytogenetically normal (CN-AML) when assessed using conventional banding analysis\textsuperscript{26}. Recent work has identified novel molecular abnormalities in CN-AML that has improved the classification and risk stratification of this large subgroup of patients\textsuperscript{27}. Among them, mutations of the nucleophosmin (\textit{NPM1}) gene, usually occurring at exon-12 and more rarely at exon-11 represent the most common genetic alteration in CN-AML (50 to 60 % of cases) and account for about one-third of all adult AML\textsuperscript{26,27}. This gene encodes for a ubiquitously expressed nucleolar protein that shuttles between the nucleus and cytoplasm in a CRM1 dependent manner\textsuperscript{28,29}. NPM1 protein is implicated in multiple functions, including ribosomal protein assembly and transport, control of centrosome duplication and regulation of \textit{Arf} tumor suppressor gene integrity\textsuperscript{29-32}. \textit{NPM1} mutations specifically result in the inappropriate re-localization of NPM1 from the nucleus into the cytoplasm\textsuperscript{29,33,34}, hence the term NPMc+ (cytoplasmic-positive) AML. Over-expression of NPMc+ in mice progenitors induces myeloid proliferation supporting a critical role in leukemogenesis\textsuperscript{35}. Remarkably, the aberrant nuclear export of NPM1 mutants is dependent on CRM1, as the NPM1 mutations lead to the creation of a novel CRM1 binding site in the NPM1.
cytoplasmic accumulation of NPM1 mutants is blocked by specific but toxic CRM1 inhibitors such as Leptomycin-B. In addition to *NPM1*, internal tandem duplications in the juxta-membrane domain or mutations in the second tyrosine kinase domain (TKD) of the *FLT3* gene have been found in 30 to 45% of CN-AML and frequently co-exist with *NPM1* mutations. Both types of mutations constitutively activate *FLT3* and *FLT3-ITD* mutations have been associated with increased risk of relapse. Therefore, therapies that restore mutant NPMc+ to the nucleus and/or suppress *FLT3* abnormalities should be highly active in AML.

In this work, we characterize the biological and pharmacological activity of KPT-CRM1 inhibitors in acute myeloid leukemia (AML) cell lines, patient blasts and in a xenograft AML model. KPT-SINE show potent anti-proliferative and pro-apoptotic properties against AML cell lines and patient blasts, including those from patients with *NPM1* and *FLT3-ITD* mutations. As expected, KPT-SINE treatment restored the localization of cytoplasmic mutant NPM1 into the nucleus. Furthermore, KPT treatment results in cell cycle arrest and blast differentiation. Finally we also show in-vivo anti-leukemia activity of KPT-SINE in a murine AML xenograft model bearing the *FLT3-ITD* abnormality.

**MATERIAL AND METHODS**

**Cell lines**

AML cell lines, MV4-11, Kasumi-1, MOLM-13, THP-1 and KG-1a were purchased from American Type Culture Collection. OCI-AML3 was purchased from DSMZ, Germany.
All cell lines were cultured in RPMI supplemented with 10% FBS and 100U/mL penicillin and 100μg/mL streptomycin.

**Primary AML samples**

Newly diagnosed untreated and frozen bone marrow AML patient samples were obtained from the Ohio State University Leukemia Tissue Bank after getting informed consent approved by the cancer institution review board in accordance with the Declaration of Helsinki. Primary cells were cultured in StemSpan SFEM supplemented with 20% FBS and StemSpan CC100 cytokine cocktail (STEMCELL Technologies).

**Cell Viability assay and establishment of IC50 values and growth curves**

Cells were seeded into 96-well plates and treated for 24, 48 and 72 h with KPT-SINE at various concentrations ranging from 10nM to 10μM. Cell viability was evaluated using the cell proliferation reagent WST-1 (Roche, Germany) according to manufacturer’s protocol. The absorbance of wells at 450 nm (reference wavelength 650 nm) was measured with a micro-plate reader (SoftMax Pro, Molecular Devices).

**Cell cycle analysis**

Cells were treated with indicated KPT-SINE for 24 hrs, harvested, washed in PBS, and fixed at a final concentration of 70% ice-cold ethanol. Cells were stored at −20°C, washed in PBS, and then stained with propidium iodide/RNase A/ 0.01% Trizol buffer for 40 minutes at 37°C. Cells were washed again and resuspended in PBS, for analysis by
flow cytometry with a Becton Dickinson FACS Calibur. Cell cycle events were analysed by incorporation of propidium iodide. Results are representative of four independent experiments performed in duplicate.

**Apoptosis assay**

Cells were treated with KPT-SINE for 24, 48 and 72 hrs. Annexin V staining was done using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions. Analysis by flow cytometry was done with a Becton Dickinson LSRII and data analysis was performed using FlowJo (Treestar).

**Western Blot**

Western blot was performed according standard protocol. Briefly, Cells were collected by centrifuge and then rinsed with ice cold PBS, and lysed in protease inhibitor containing buffer for 30 mins at 4°C. Total cell lysates were centrifuged and the soluble supernatant was collected. Protein concentration was quantified by BCA microprotein assay kit (Pierce). Protein lysates (~40 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P PVDF membrane (Millipore), and membranes were blocked for 1 hr with TBS containing 5% non-fat dry milk and 0.5% Tween 20 (TBST). Membranes were incubated with TBST containing 5% non-fat dry milk and primary antibodies against CEBPA (Cell Signaling Technology, 2295), FLT3 (sc-480, Santa Cruz Biotechnology), CRM1 (sc-5595, Santa Cruz Biotechnology), p53 (sc-126, Santa Cruz Biotechnology), p21 (sc-817, Santa Cruz Biotechnology), c-Kit (sc-168, Santa Cruz Biotechnology) and β-actin (Cell Signaling).
After washing with TBS, membranes were probed with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody (Cell Signaling) for 1 hr at room temperature. Membranes were developed using ECL (Pierce) or ECL plus chemiluminescence detection reagent (GE Healthcare). Results are representative of 3 independent experiments.

**Colony Formation Assay**

Cells were treated with KPT-SINE for the indicated specified time, after which they were washed with PBS. About 3000 cells were mixed with Methocult medium (Stem Cell Technology) and plated into 30mm dish. Colonies (>20 µm) were counted under microscope after 14 days.

**siRNA transfection**

p53 or scramble siRNA transfections were performed using the Amaxa Nucleofector (Amaxa, Germany). Briefly, OCI-AML3 cells were suspended in Amaxa Nucleofector Solution T supplemented with 100 pmol p53 or scramble ON_TARGETplus Smartpool siRNAs (Dharmacon) and the nucleofection was performed using cell-type specific protocol (X-001). KPT was introduced to cell culture 3 hours after transfection. Assays were performed at indicated time points.

**Immunofluorescent Staining**

Cells were first counted and diluted to 1X10^6/ml. Approximately 200, 000 cells were cytopspinned to slide. The cells were then fixed in 3.7% formaldehyde for 15 min
followed by permeabilization with 0.25% Triton. The slides were blocked by 1% goat
serum for 30 min. The cells were incubated with primary antibody NPM1 (sc-56503,
Santa Cruz Biotechnology) for 1 hour at room temperature. After washing, the sample
was incubated with fluorochrome conjugated secondary antibody for 1 hr at room
temperature. Nucleus was stained by DAPI. Samples were analyzed by confocal
microscope (OSU Image facility).

**Flow Cytometry**

Cell lines MV4;11, Kasumi-1 and OCI-AML3 were treated with DMSO control or KPT-
SINE at the predetermined IC50 concentration for 24, 48 and 72 hrs and stained with
CD11b antibody (eBioscience, San Diego, CA, USA). Cells were analysed on LSR II
(BD Bioscience) and data analysis was performed using FlowJo (Treestar).

**Morphological Examination**

Cells were treated with KPT-SINE or DMSO for indicated time points and cytopinned
onto glass sildes. Cells were fixed with methanol and Wright-Giemsa stained for
morphological examination.

**Real-time PCR**

Cellular RNA was extracted using TRIzol (Invitrogen) and reverse transcribed to cDNA
using superscript III first-strand synthesis system for RT-PCR (Invitrogen). Gene
expression levels of *CEBPA*, *G-CSFR* and *lysosome* were detected using TaqMan Gene
expression assays (Applied Biosystems). Normalization was performed using 18s RNA
expression levels. Comparative real-time qPCR was performed in triplicate and relative expression was calculated using the comparative Ct method.

**Mice**

Female non-obese diabetic severe combined immunodeficient gamma (NSG) mice that lack mature T cells, B cells, or functional NK cells, and are deficient in cytokine signaling, were purchased from Jackson ImmunoResearch laboratories (Ban Harbor, ME). All mice used in the experiments were between 4-6 weeks of age. All animal studies were conducted in accordance to the rules and regulations of the Institutional Animal Care and Use Committee at the Ohio State University.

**MV4-11 xenograft mouse model**

Spleen cells (0.3X10^6) from MV4-11 transplanted NSG mice were intravenously injected into NSG mice via tail vein. One week after tumor inoculation, the mice were given either vehicle control or KPT-276 (analog of KPT-185 with adequate oral bioavailability and pharmacokinetics for in vivo use) at 150mg/kg via oral gavage, three times a week. Mice were monitored closely for clinical signs of leukemia such as weight loss, hindlimb paralysis. Expected median survival for untreated animals in this model is 28 days. Blood was drawn for CBC analysis that allowed for confirmation of leukemia. On day 23 separate cohorts of vehicle and drug treated mice were sacrificed; spleens harvested, weighed and picture taken for comparative study of spleen enlargement due to tumor. Blood was drawn and CBC analysis performed to confirm leukemia.
Statistical analysis

Survival data were analyzed using Kaplan Meier and long-rank test methods (GraphPad Prism). Differences between continuous variables (e.g. RNA expression, spleen weights) were analyzed using t-tests. All P values are two sided.

RESULTS

KPT-SINE significantly inhibits proliferation and induces cell cycle arrest and apoptosis of AML cell lines and primary AML blasts.

In order to assess the biological activity of KPT-SINE CRM1 inhibitors, we treated a panel of AML cell lines (MV4-11, Kasumi-1, OCI/AML3, MOLM-13, KG1a and THP-1, well-characterized for cytogenetic and molecular features (Table 1). Submicromolar concentrations of KPT-185 inhibited leukemia cell proliferation with IC50 values ranged from 100nM to 500nM (Table 1 and Fig.1a).

Since CRM1 is involved in the export of several proteins involved in cell cycle control (e.g. p21) to the cytoplasm, we assessed the impact of KPT-185 treatment on the cell cycle of AML cells. KPT-185 at the predetermined IC50 value induced cell-cycle arrest at G1 with respect to vehicle-treated-control (DMSO) in MV4-11 (82.2±3.69% vs. 71.55±0.21%, P<0.01), OCI/AML3 (83.05±6.84% vs. 55.1±2.26% (P<0.01), and MOLM-13 (82.72±1.14% vs. 57.55±3.46%, P<0.01) cells at 24 hours (Fig.1b). A
concomitant decrease in the percentage of KPT-185-treated cells with respect to controls in S phase and G2/M phase was observed at 24 hours (Fig. 1b). Interestingly, treatment of Kasumi-1 cells with KPT-185 did not cause an arrest at G1, rather there was a significant accumulation of apoptotic cells in sub-G1 (24.32±6.01% vs. 5.44±2%, KPT-185 vs. DMSO, P<0.01), with a decrease in percentage of cells in the S (7.67±1.21% vs. 18.6±1.2%, P<0.01) and G2M phase (6.35±2.79 vs. 15.91±4.44%, P<0.01).

Treatment with KPT-185 at the predetermined IC50 value induced apoptosis in AML cell lines compared to DMSO treated controls at 48 hours as follows: MV4-11, 5.7 fold increase (P=<0.01); Kasumi-1, 5.41 fold increase (P<0.01); OCI-AML3, 4.91 fold (P<0.01) and MOLM-13, 4 fold increase (P<0.01) (Fig. 1c). Cell colony formation was reduced significantly after KPT-185 treatment at the IC50 for 72 hours in AML cell lines (Kasumi-1: 44.67±4.16 vs. 11.33±2.08, MV4-11: 29±8.88 vs. 6±3, OCI/AML3: 28.66±3.05 vs. 2±1, respectively, P<0.01, Fig.1d). Next, we validated our findings using primary AML blasts (see table 2 for cytogenetic and molecular abnormalities). KPT-185 inhibited cell proliferation and induced apoptosis in primary AML blasts across a variety of genotypes (Table 2 and Fig.1e and f). The IC50 values were in the 500 nM range, except for CN-AML patients with NPM1 mutations that were more sensitive to the KPT-SINE (IC50 100nM) (Table 2).

KPT-SINE treatment causes decrease of CRM1 protein level and accumulation of CRM1 cargo proteins in the nucleus.
The effect of CRM1 inhibitor, KPT-185, on the level of CRM1 protein was investigated in AML cell lines and primary AML blasts. We observed a significant decrease in the level of CRM1 protein by western blot as shown in Fig. 2a. Similar findings have been observed in multiple myeloma cell lines after using a different CRM1 inhibitor. In addition, we also measured the expression levels of two known CRM1 cargo proteins; p53 and NPM1, after treatment with KPT-SINE. A significant accumulation of p53 in the nucleus of MV4-11 and OCI-AML3 were observed after treatment with KPT-185 (Fig. 2b). This was confirmed by Western blotting in both cell lines (Fig. 2c, upper panel) and in primary AML blasts (Fig. 2c, lower panel). KPT-185 treatment of the NPM1 mutated OCI/AML3 cell line (which exhibits cytoplasmic localization of the protein) and of primary AML blasts from a patient with NPM1 mutation resulted in the accumulation of NPM1 into the nucleolus with respect to the controls (Fig. 2d).

**FLT3 and KIT oncogene proteins are down-regulated after CRM1 inhibition at the post-transcriptional level.**

Next, we asked whether critical oncogenic proteins involved in myeloid leukemogenesis are affected by CRM1 inhibition. In particular, we were interested in FLT3 since this TKR is over-expressed in the majority of AML patients and mutated (FLT3-ITD) frequently in patients with CN-AML and NPM1 mutations. Remarkably, we found a strong down-regulation of total FLT3 protein expression in AML cell lines and primary AML samples (Fig. 2e and f), irrespective of the mutation status. To dissect the possible mechanism of such effect, we measured FLT3 mRNA expression after CRM1 inhibition.
and we found no changes in FLT3 mRNA levels after KPT-185 treatment (Data not shown). This result suggests that FLT3 protein expression down-regulation occurs at the posttranscriptional level. Next we asked whether c-KIT (another tyrosine kinase receptor), which is also up-regulated in the great majority of AML cases and mutated in a fraction of patients with core binding factor leukemia, could be a target for CRM1 inhibition\(^{38,39}\). Interestingly, similarly to FLT3, we found that KIT protein but not mRNA expression levels were decreased in Kasumi-1 and OCI-AML-3 cells after KPT-185 treatment (Fig. 2g). Thus, our data indicate that KPT-SINE decreases FLT3 and KIT protein expression post-transcriptionally in AML blasts.

**CRM1 inhibition induces differentiation of AML cell lines**

Next, we investigate whether CRM1 inhibition could result in AML blasts differentiation. Indeed, we observed a significant increase in the myeloid differentiation marker CD11b expression as determined by FACS in MV4-11, Kasumi-1 and OCI-AML-3 after KPT-185 treatment with respect to controls (Fig. 3a). In addition, morphological changes characteristic of differentiation, such as the appearance of granules and condensation of the nucleous, were confirmed by Giemsa staining of cytoplasm preparations of KPT-185 treated cells (Fig. 3b).

To obtain insights about the mechanisms by which KPT-SINE induces blast differentiation we measured the expression of CEBPA, a member of the basic region leucine zipper (bZIP) family of transcription factors, which has been shown to be indispensable for granulocytic differentiation of myeloid progenitors\(^{40,41}\). Remarkably,
we observed a significant increase in the protein levels of CEBPA, with little change in mRNA levels, in MV4-11 and OCI-AML3 cell lines (Fig. 3c). We did not see any changes in the CEBPA protein levels when we treated the Kasumi-1 cell line with the CRM1 inhibitor (Data not shown). This result could be explained by the fact that the AML1-ETO fusion protein encoded by the t(8;21) is known to suppress the both CEBPA mRNA and protein levels\(^{42}\). Since CEBPA is known to induce the transcription of differentiation-related genes such as the \(G\text{-CSFR}\), and \(lysozyme\)\(^{41,43}\), we measured the mRNA expression of these genes by RT-PCR in the MV4-11 and the OCI-AML3 cell lines and confirmed that both genes were induced after KPT-185 treatment with respect to control (Fig.3d).

Based on previous reports that indicate that CEBPA is a p53 regulated DNA damage inducible gene\(^{44}\) and that p53 induction is involved in myeloid differentiation\(^{45}\), we asked whether the CEBPA up-regulation observed after KPT-185 treatment is mediated through p53 and/or whether p53 increased expression is responsible the phenotypic effects observed. In order to answer this question, we repressed endogenous p53 expression using siRNA in OCI-AML3 cells, treated the cells with KPT-185 or DMSO for 24 hours and measured CEBPA, p53 protein, CD11b and apoptosis overtime. As shown in Fig.3e, dampening p53 expression in OCI-AML 3 cells abrogates CEBPA up-regulation induced by KPT-185, decreases the expression of CD11b and induces less apoptosis (Fig. 3f and g). These results suggest that in this model, CEBPA is a p53 regulated gene\(^{44}\) that may be involved along with other p53 target genes in the differentiation program activated by CRM1 inhibition.
CRM1 inhibitor increases survival in a human leukemia xenograft model

To establish the activity of KPT-SINE in AML in vivo we used a xenograft human AML murine model (MV4-11). In this model, NOD/SCID gamma mice were intravenously inoculated (tail vein) with human MV4-11 AML cells which carry FLT3-ITD. One week after leukemic cell inoculation, the mice were given KPT-276 at 150mg/kg via oral gavage, three times a week or vehicle control. KPT-276 has the identical CRM1 Binding warhead and specificity as KPT-185, similar biological activity in vitro, but superior oral bioavailability and pharmacokinetics which allow it to be used in vivo. Mice were monitored for survival. Some mice were sacrificed at day 21 to assess the effects of KPT-276 on leukemia burden by measuring spleen weight and white blood cell count (WBC). KPT-SINE treatment significantly increased the survival of the mice (median survival vehicle vs. drug treated, 27 vs. 39.5 days, respectively, P<0.01, log-rank test, n=12 per group, **Fig. 4a**). The average WBC was 19,950 ±3.7 in the control group vs. 2,120 ±0.48 in the KPT-276 group, P=0.03 (**Fig. 4b**) at 21 days. The average spleen weight was also significantly higher in the control group vs. KPT-276 arm at 21 days (191.7 mg ± 25.3 vs. 22.81 mg ± 1.9, respectively, P<0.01) (**Fig.4c and d**). FLT3 protein expression was significantly lower in the spleen of leukemic mice treated with KPT-276 with respect to the vehicle controls at 21 days (**Fig.4d**). Altogether, our data shows that KPT-276 is active in vivo and prolongs the survival of the leukemic mice.

**DISCUSSION**
In this manuscript we show the in vitro and in vivo anti-tumor efficacy of KPT-SINE CRM1 antagonists in AML. Our results indicate that KPT-185 inhibits cell proliferation and induces cell cycle arrest and apoptosis of AML cell lines and primary blasts. Treatment of AML blasts with KPT-185 causes a reduction in the amount of CRM1 protein, and shows a significant nuclear accumulation of CRM1 cargo proteins such as p53 and NPM1. Using a xenograft AML mouse model, we show that in vivo treatment of leukemic mice with oral KPT-276 (analog of KPT-185 for in vivo studies) significantly prolongs survival of leukemic mice and reduces leukemic burden.

Interestingly, we found that primary AML blasts harboring *NPM1* mutations were very responsive to CRM1 inhibition. NPM1 is a nucleolar tumor suppressor phosphoprotein that continuously shuttles between the nucleolus and cytoplasm and regulates the p53-*ARF* tumor suppressor pathway. Mutations in the exon 12 of *NPM1* have been described in 25-35% of AML and are one of the most frequent mutations described up to date in this disease. These mutations alter the NPM1 protein at the C-terminus, leading to the formation of a novel and efficient CRM1 binding site, thus causing its aberrant cytoplasmic localization through a CRM1 dependent transport. Supporting an oncogene role for mislocalized mutant NPM1, it has been reported that over-expression of a human NPMc+ mutation in murine myeloid progenitors resulted in cytoplasmic NPM1 localization and myeloproliferation in the bone marrow and spleen. Based on these data, we reasoned that blocking NPMc+ nuclear export by inhibiting CRM1, may restore NPM1 tumor suppressor functions and prevent leukemogenesis. Here we have shown that blocking CRM1 does in fact restore nuclear localization of NPM1 mutants.
and induces potent antileukemic effects in cell lines and primary human AML blast. In fact, NPMc+ AML blasts from patients were the most sensitive to KPT inhibition (IC50 100nm) among all primary samples tested. Altogether, our results indicate that redirecting mutated NPM1 to the nucleus through CRM1 inhibition is a potential targeted therapy for this frequent subtype of AML. However, the fact that other AML cytogenetics groups harboring wild type \textit{NPM1} were also sensitive to KPT-SINEs indicate that others TSP and in particular p53, are involved in the antileukemic effects of the KPT-SINEs.

Disruption of terminal differentiation is a salient feature in the pathogenesis of AML\textsuperscript{48}; and differentiation-based anti-cancer treatments such as all-trans retinoic acid have been developed to overcome this block, thereby inducing apoptosis\textsuperscript{49}. Here we show that treatment of AML cancer cells with CRM1 inhibitors induces blast differentiation, as shown by the surface expression of the myeloid differentiation marker CD11b, as well as morphological changes associated with differentiation. To investigate further possible mechanisms by which CRM1 inhibition using KPT-SINEs resulted in blast differentiation we measured the expression of proteins known to be involved in myeloid differentiation such as CEBPA. Interestingly, we found a significant increase in the protein levels of CEBPA in MV4-11 and OCI-AML3 cells, while the levels of CEBPA mRNA were mildly increased only for MV4-11 cells. \textit{CEBPA} has been shown to induce myeloid differentiation via transcriptional activation of several genes that are critical for myeloid granulocytic differentiation\textsuperscript{41,43}. Based on work by Yoon et al, who reported that \textit{CEBPA} expression is up-regulated by p53 induced by ultraviolet radiation in keratinocytes\textsuperscript{44}, we reasoned that \textit{CEBPA} up-regulation after CRM1 inhibition could be
mediated by p53, a direct CRM1 target. Indeed, we show that blocking p53 expression using siRNA in OCI-AML3 cell lines abrogates CEBPA protein induction and differentiation by KPT-185. It is though important to mention that the fact that CEBPA protein expression did not change in Kasumi-1 cells despite exhibiting blast differentiation after KPT treatment argues that CEBPA alone is unlikely to be the primary mechanism. Our results point out to p53 as a critical mediator not only for the KPT induced differentiation effects but also for apoptosis.

In this work we also show that growth promoting c-KIT and FLT-3 tyrosine kinase receptor (TKR) proteins are down-regulated after KPT-185 treatment of AML cell lines and primary samples. This is relevant to AML, since both TKR are over-expressed in the majority of AML and/or mutated in some cases\(^\text{26,27,38,39}\). KIT gain of function mutations appear to be restricted to core binding factor AML which confers poor prognosis\(^\text{38,39}\). In contrast, activating mutations in the JM domain (FLT3-ITD) and in the tyrosin kinase domain (TKD) of FLT3 are found in 30–35% of patients with AML and represent the most frequent genetic alterations in AML\(^\text{26,50}\). Aberrant activation of either KIT or FLT3 signaling due to mutations and/or over-expression promotes cell proliferation and contributes to leukemogenesis\(^\text{27,38,39}\). Thus, the ability of KPT-SINE CRM1 inhibitors to downregulate KIT and FLT3 protein expression in AML constitute an important antileukemic mechanism for these compounds. Since patients with NPM1 mutations frequently have FLT3-ITD mutations\(^\text{27}\), CRM1 inhibitors have the potential to target two critical mutations signaling pathways simultaneously, while increasing levels and nuclear localizations of TSP. Last, our discovery has an immediate translational impact since
FLT3 expression could be used as a novel pharmacodynamic endpoint for testing of CRM1 inhibitors in Phase 1 clinical trials for AML.

In summary, here we report the biologic and pharmacologic activity of KPT-SINEs CRM1 inhibitors in both AML cells, primary AML samples and in a murine AML xenograft model. Our results indicate that KPT-SINE CRM1 inhibitors dampen cell proliferation and induce cell cycle arrest, apoptosis and cell differentiation through $p53$-$CEBPA$ pathway. We have identified that both KIT and FLT3 protein are downregulated after KPT-CRM1 inhibitors treatment. The preclinical in vitro and in vivo results reported here support further study of KPT-SINE CRM1 inhibitors as a novel therapeutic strategy for AML.

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Competing Interests: M.K and S.S are employees of Karyopharm therapeutics, a clinical stage biopharmaceutical company that develops selective inhibitors of nuclear export-targeted therapeutics.

Author contributions: P.R, X.Y, S.R and CN performed all the experiments; P.R and X.Y contributed equally; S.S, M.K, C.M.C, M.C, G.M, and R.G designed, supervised and analyzed research performed in their laboratories; R.K, W.B, A.W. collected primary AML samples; R.G and P.R wrote the manuscript.

References


Table 1. IC50 values for AML cell lines.

<table>
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<tr>
<th>Cell Line</th>
<th>Relevant Cytogenetic/molecular Data</th>
<th>IC 50</th>
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<tr>
<td>MV4-11</td>
<td>XY, +8, +18, +19, -21, t(4;11)(q21;q23). <strong>FLT3-ITD+</strong></td>
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<td>MOLM-13</td>
<td>XY, +13,del(8),ins(11;9)(q23;p22p23). <strong>FLT3-ITD+</strong></td>
<td>100nM</td>
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<td>OCI-AML3</td>
<td>X/XY, +1, +5, +8, t(1;18)(p11;q11), i(5p), del(13) (q13q21), dup(17)(q21q25). <strong>NPM1 mutation</strong> (type A) and the DNMT3A R882C mutation</td>
<td>250nM</td>
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<tr>
<td>Kasumi-1</td>
<td>X, -Y, -9, -13, -16, t(8;21)(q22;q22). <strong>KIT mutation</strong> (Asn822Lys)</td>
<td>500nM</td>
</tr>
<tr>
<td>KG1a</td>
<td>X/XY, -4, +8, +8, -12, -17, -20, del(5)(q?11q?13), dup(7)(q12q33), del(7)(q22q35).</td>
<td>250nM</td>
</tr>
<tr>
<td>THP-1</td>
<td>XY/XXY, -Y, +1, +3, +6, -8, -13, -19, -22, -22, , del(1)(q42.2), del(6)(p21), t(9;11)(p22;q23).</td>
<td>250nM</td>
</tr>
</tbody>
</table>
### Table 2. IC50 for primary AML patient samples.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>WHO</th>
<th>WBC*</th>
<th>Cytogenetics</th>
<th>NPM1</th>
<th>FLT3</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td>Acute myelomonocytic leukemia</td>
<td>39</td>
<td>46,XX(20)</td>
<td>Mut</td>
<td>WT</td>
<td>100nM</td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>AML with maturation</td>
<td>26</td>
<td>46,XY(20)</td>
<td>Mut</td>
<td>WT</td>
<td>100nM</td>
</tr>
<tr>
<td>3</td>
<td>62</td>
<td>AML without maturation</td>
<td>199</td>
<td>46,XX(20)</td>
<td>Mut</td>
<td>WT</td>
<td>100nM</td>
</tr>
<tr>
<td>4</td>
<td>77</td>
<td>AML with maturation</td>
<td>85</td>
<td>46,XY(20)</td>
<td>Mut</td>
<td>WT</td>
<td>50nM</td>
</tr>
<tr>
<td>5</td>
<td>62</td>
<td>AML with MDS related changes</td>
<td>8.8</td>
<td>46,XY(20)</td>
<td>WT</td>
<td>WT</td>
<td>500nM</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td>AML with maturation</td>
<td>75</td>
<td>46,XY(20)</td>
<td>WT</td>
<td>WT</td>
<td>500nM</td>
</tr>
<tr>
<td>7</td>
<td>45</td>
<td>Acute myelomonocytic leukemia</td>
<td>53</td>
<td>46,XX(20)</td>
<td>WT</td>
<td>WT</td>
<td>500nM</td>
</tr>
<tr>
<td>8</td>
<td>56</td>
<td>Acute myelomonocytic leukemia</td>
<td>69</td>
<td>46,XX(20)</td>
<td>WT</td>
<td>ITD+</td>
<td>500nM</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>AML with inv(16)</td>
<td>45</td>
<td>46,XX.inv(16)</td>
<td>WT</td>
<td>WT</td>
<td>500nM</td>
</tr>
<tr>
<td>10</td>
<td>53</td>
<td>Acute myelomonocytic leukemia</td>
<td>79</td>
<td>46,XX(20)</td>
<td>WT</td>
<td>ITD+</td>
<td>500nM</td>
</tr>
<tr>
<td>11</td>
<td>85</td>
<td>AML without maturation</td>
<td>66</td>
<td>46,XY(20)</td>
<td>WT</td>
<td>ITD+</td>
<td>500nM</td>
</tr>
<tr>
<td>12</td>
<td>52</td>
<td>AML with t(8;21)</td>
<td>2.9</td>
<td>45,X,-X,t(8;21)</td>
<td>WT</td>
<td>WT</td>
<td>500nM</td>
</tr>
<tr>
<td>13</td>
<td>50</td>
<td>AML with t(8;21)</td>
<td>15.6</td>
<td>45,X,-Y,t(8;21)</td>
<td>WT</td>
<td>WT</td>
<td>500nM</td>
</tr>
</tbody>
</table>

* x10^5

WT: wild type  
Mut: mutated

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**Figure Legends**
Figure 1. KPT-SINE significantly inhibits proliferation and induces cell cycle arrest and apoptosis of AML cell lines and primary AML blasts. (a) WST-1 assays in MV4-11, Kasumi-1, MOLM-13 and OCI-AML-3; (b) Cell cycle assessment using propidium iodine (PI) detection by flow cytometry at 24 hours; (c) Apoptosis as measured by Annexin V/PI staining using FACS at 48 hours; (d) Colony assays in Kasumi-1, MV4-11 and OCI-AML3 cells after KPT-185 treatment at 24, 48 and 72 hours; (e) Apoptosis as measured by Annexin V/PI staining using FACS at 48 hours in primary AML samples; (f) WST-1 assays in primary AML samples.

Figure 2. KPT-SINE treatment causes decrease of CRM1 protein level, accumulation of CRM1 cargo proteins in the nucleus and down-regulation of FLT3 and KIT oncoproteins. (a) Upper Panel: CRM1 protein expression as measured by Western Blotting in MV4-11, Kasumi-1 and OCI-AML3 cells after KPT-185 treatment or control (DMSO) after 24 hours. Loading control is Actin; (a) Lower panel: CRM1 protein expression as measured by Western Blotting in 3 primary AML blasts after KPT-185 treatment or control; (b) Confocal microscopy of p53 in MV4-11 and OCI-AML3 cells treated with KPT-185 or control at 24 hours. The left panel shows the DAPI staining (cell nucleus). The middle panel is p53 staining and the right panel is the merger of p53 and DAPI staining. Note the increase in the p53 expression in the nucleolus. (c) Whole cell p53 protein expression in MV4-11 and OCI-AML3 cells (upper panel) or primary AML blats (n=3) after KPT-185 or control treatment at 2 and 24 hours; (d) Confocal microscopy of NPM1 in OCI-AML3 cells and in a primary AML blasts from a patient with CN-AML and NPM1 mutation treated with KPT-185 or control at 24 hours. The left
panel shows the DAPI staining (cell nucleus). The middle panel is NPM1 staining and the right panel is the merger of NPM1 and DAPI staining. The arrows indicate the localization of NPM1 in the cytoplasm (NPMc+) in the untreated samples and the elimination of the cytoplasmic signal upon treatment with the drug, being detected exclusively in the nucleus; (e) FLT3 protein expression in MV4-11, Kasumi-1 and OCI-AML-3 cells as measured by Western Blotting after KPT-185 treatment or control at 24 hours; (f) FLT3 protein expression in primary AML blasts as measured by Western Blotting after KPT-185 treatment or control at 24 hours (Patient 1: CN-AML NPM1 WT, FLT3 ITD+; Patient 2: CN-AML, NPM1 mutated, FLT3 WT; Patient 3: CN-AML, NPM1 mutated, FLT3 WT); (g) c-KIT expression in Kasumi-1 and OCI-AML3 cells after KPT-185 treatment or control at 24 hours.

**Figure 3. CRM1 inhibition induces differentiation of AML cell lines.** (a) CD11b measurement by FACS in AML cell lines after KPT-185 treatment at 72 hours; (b) Giemsa stain of cytospins of Kasumi-1 and MV4-11 cells treated with KPT-185 or controls. Arrows shows nuclear condensation; (c) CEBPA protein expression in MV4-11 cells and OCI-AML3 cells after KPT-185 treatment or controls for 24 and 48 hours. Loading control is actin; (d) GCSFR and lysozyme mRNA expression after KPT-185 treatment or controls in MV4-11 cells and OCI-AML-3 cells. Results are shown as fold change after normalization with 18s and 2ΔCt calculations; (e) CEBPA protein expression after p53 siRNA or scramble oligonucleotide transfection in OCI-AML3 cells subsequently treated with KPT-185 or controls for 24 hours. Protein expression of p21 was measured as control for successful p53 inhibition; (f) CD11b expression in OCI-
AML3 cells after transfection with scramble or p53 antisense oligonucleotides and treatment with DMSO or KPT-185 at 72 hours; (g) Apoptosis as measured by Annexin V/PI staining using FACS at 48 hours after scramble or p53 antisense oligonucleotides and treatment with DMSO or KPT-185.

**Figure 4. CRM1 inhibitor increases survival in a human leukemia xenograft model.**

(a) Survival of MV4-11 xenograft mice after treatment with KPT-276 150 mg/kg (n=12) or vehicle control (n=11). Survival comparison was made with log-rank test; (b) White blood cell count in KPT treated mice vs. vehicle control (n=8) at 21 days, P-values obtained using t-test; (c) Spleen weights (mg) in KPT treated mice vs. vehicle control (n=7), P-values obtained using t-test; (d) Spleen photographs of three representative cases (KPT-276 n=3; vehicle n=3) at 21 days; (e) FLT3 protein expression in mouse spleen cells as measured by Western Blotting after KPT-276 treatment or vehicle control at 21 day
Fig. 1

(a) Bar graphs showing the absorbance at 450nm of MV4-11, IC50=100nM, Kasumi-1, IC50=500nM, and OCI-AML3, IC50=250nM, treated with KPT-185 at different concentrations over 24, 48, and 72 hours.

(b) Flow cytometry plots showing the distribution of MV4-11 and OCI-AML3 cells treated with KPT-185 for 24 hours, with G1, S, and G2/M stages labeled.

(c) Annexin-V staining of MV4-11, Kasumi-1, OCI-AML3, and MOLM-13 treated with DMSO or KPT-185, showing the percentage of early and late apoptosis.

(d) Bar graphs showing the number of colonies for Kasumi-1, MV4-11, and OCI-AML3 treated with DMSO or KPT-185 for 24, 48, and 72 hours.
Fig. 2

(a) Western blot analysis of p53 and Actin expression in MV4-11, Kasumi-1, and OCI-AML3 cells treated with DMSO or KPT-185 for 2hrs and 24hrs.

(b) Immunofluorescence staining of DAPI, p53, and merge in MV4-11 cells treated with DMSO or KPT-185.

(c) Western blot analysis of FLT3 and Actin expression in MV4-11, Kasumi-1, and OCI-AML3 cells treated with DMSO or KPT-185 for 24hrs.

(d) Immunofluorescence staining of DAPI, NPM1, and merge in OCI-AML3 cells treated with DMSO or KPT-185.

(e) Western blot analysis of FLT3 and Actin expression in MV4-11, Kasumi-1, and OCI-AML3 cells treated with DMSO or KPT-185 for 24hrs.

(f) Immunofluorescence staining of DAPI, p53, and merge in MV4-11 cells treated with DMSO or KPT-185.

(g) Western blot analysis of C-KIT and Actin expression in Kasumi-1 and OCI-AML3 cells treated with DMSO or KPT-185.

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Fig. 3

a) CD11b expression in MV4-11, Kasumi-1, and OCI-AML3 cells treated with DMSO or KPT-185 for 24 or 48 hours.

b) Morphological changes observed in Kasumi-1 cells treated with DMSO or KPT-185 for 72 hours.

MV4-11 DMSO 96hrs MV4-11 KPT-185 96hrs

MV4-11 DMSO 96hrs MV4-11 KPT-185 96hrs

C) Western blot analysis of CEBPA and Actin expression in MV4-11 and OCI-AML3 cells treated with DMSO or KPT-185 for 24 or 48 hours.

d) Quantitative analysis of GCSFR and Lysozyme expression in MV4-11 cells treated with DMSO or KPT-185 for 24 or 48 hours.

f) Quantitative analysis of CEBPA expression in OCI-AML3 cells treated with DMSO or KPT-185 for 24 or 48 hours.

g) Flow cytometry analysis of Annexin-V and PI staining in OCI-AML3 cells treated with DMSO or KPT-185, with or without p53 knockdown.
Fig. 4

a  

Percent survival

Days

P<0.001

b  

WBC

Vehicle  
KPT-276

Day 21

P<0.01

c  

Spleen weight (mg)

Vehicle  
KPT-276

Day 21

P<0.01

d  

1 cm

Day 21

Vehicle  
KPT-276

e  

Day 21

Vehicle  
KPT-276

FLT-3  
Actin
Pre-clinical activity of a novel CRM1 inhibitor in acute myeloid leukemia

Parvathi Ranganathan, Xueyan Yu, Caroline Na, Ramasamy Santhanam, Sharon Shacham, Michael Kauffman, Alison Walker, Rebecca Klisovic, William Blum, Michael Caligiuri, Carlo M. Croce, Guido Marcucci and Ramiro Garzon