The Second-Generation Exportin-1 Inhibitor KPT-8602 Demonstrates Potent Activity against Acute Lymphoblastic Leukemia

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Abstract

Purpose: Human exportin-1 (XPO1) is the key nuclear-cytoplasmic transport protein that exports different cargo proteins out of the nucleus. Inducing nuclear accumulation of these proteins by inhibiting XPO1 causes cancer cell death. First clinical validation of pharmacological inhibition of XPO1 was obtained with the Selective Inhibitor of Nuclear Export (SINE) compound selinexor (KPT-330) demonstrating activity in phase-II/III clinical trials when dosed 1 to 3 times weekly. The second-generation SINE compound KPT-8602 shows improved tolerability and can be dosed daily. Here, we investigate and validate the drug target interaction of KPT-8602 and explore its activity against acute lymphoblastic leukemia (ALL).

Experimental Design: We examined the effect of KPT-8602 on XPO1 function and XPO1-cargo as well as on a panel of leukemia cell lines. Mutant XPO1 leukemia cells were designed to validate KPT-8602’s drug-target interaction. In vivo, anti-ALL activity was measured in a mouse ALL model and patient-derived ALL xenograft models.

Results: KPT-8602 induced caspase-dependent apoptosis in a panel of leukemic cell lines in vitro. Using CRISPR/Cas9 genome editing, we demonstrated the specificity of KPT-8602 for cysteine 528 in the cargo-binding groove of XPO1 and validated the drug target interaction. In vivo, KPT-8602 showed potent anti-leukemia activity in a mouse ALL model as well as in patient-derived T- and B-ALL xenograft models without affecting normal hematopoiesis.

Conclusions: KPT-8602 is highly specific for XPO1 inhibition and demonstrates potent anti-leukemic activity supporting clinical application of the second-generation SINE compound for the treatment of ALL. Clin Cancer Res; 1–14. ©2016 AACR.

Introduction

Exportin-1 (XPO1), also known as chromosome region maintenance 1 (CRM1), is the best-characterized nuclear transporter that mediates the export of a wide range of different proteins, including transcription factors, tumor-suppressor proteins, and cell-cycle regulators from the nucleus to the cytoplasm. In recent years, XPO1 overexpression has been observed in different types of cancer and is related to tumor size and patient outcome (1–6). In addition, a recurrent mutation in codon 571 of XPO1 (p.E571K/G), located in the hydrophobic cargo-binding pocket has been reported in CLL (7) and AML (8).

XPO1 has been considered a desirable molecular target for cancer treatment for over 20 years and has resulted in several studies of diverse structurally unique small molecule inhibitors. Until recently, Leptomycin B (LMB) was the only compound that was investigated in the clinic. However, LMB was discontinued due to toxic side effects including nausea, gastrointestinal effects and fatigue (9). More recently, orally bioavailable small-molecule XPO1 inhibitors called Selective Inhibitors of Nuclear Export (SINE) compounds have been developed, with KPT-330 (Selinexor, Karyopharm Therapeutics) as the lead clinical compound. Selinexor is being investigated in multiple phase 1 and 2 clinical studies in various solid and hematologic malignancies. SINE compounds are optimized derivatives of the N-azolylacrylate class of small-molecule XPO1 inhibitors (10, 11), which prevent nuclear export of cargo proteins. At the molecular level these compounds covalently bind to the reactive cysteine residue at position 528 in the hydrophobic-binding groove of XPO1 (12, 13). SL-801 (Stemline Therapeutics), another XPO1 inhibitor, has recently been approved for clinical investigation in patients with advanced solid tumors.

Selinexor shows promising results as single agent in patients with heavily pre-treated relapsed/refractory hematologic or solid cancers (14, 15), and is currently being evaluated in combination
with other chemotherapeutic agents. As of today, >1,400 patients have been treated with selinexor. Although activity for selinexor is evident from these clinical trials, its dosing is limited to twice weekly to minimize any potential adverse events. It was hypothesized that increasing the reversibility of the covalent binding to XPO1 may increase tolerability. Therefore, KPT-8602 (Fig. 1A) was designed to be a Michael acceptor activated with an additional electron withdrawing group at the C1 position (16). KPT-8602 has reduced brain penetration (17) and displays improved tolerability as compared to selinexor allowing daily dosing. KPT-8602 has been introduced to human clinical trials in January 2016 to study its safety, tolerability and efficacy. KPT-8602 displays potent anti-leukemia activity both in ALL cell lines as well as in a mouse ALL model and in patient-derived xenograft T- and B-ALL models, supporting further clinical development of this drug for the treatment of ALL.

Translational Relevance
The article describes the drug-target validation and acute lymphoblastic (ALL) activity of the second-generation XPO1 inhibitor KPT-8602, which has been introduced to human clinical trials in January 2016 to study its safety, tolerability and efficacy. KPT-8602 displays potent anti-leukemia activity both in ALL cell lines as well as in a mouse ALL model and in patient-derived xenograft T- and B-ALL models, supporting further clinical development of this drug for the treatment of ALL.

Materials and Methods
Cell lines and reagents
T-ALL cells Jurkat, MOLT-4, ALL-SIL, DND41, and HPB-ALL; B-ALL cells BV173, EHEB, and REH; and myeloid leukemia cells MV4-11, MOLM13, K-562, and HL-60 were acquired from the ATCC or DSMZ and cultured in RPMI-1640 medium supplemented with 10% v/v FBS and 20 μg/mL gentamicin. The haploid HAP-1 cells, derived from KBM-7 CML cells (Horizon Discovery), were maintained in IMDM supplemented with 10% FBS and 20 μg/mL gentamicin. All cell lines were thawed upon receipt from the distributor, they were amplified and subsequently frozen in batches. After thawing of these lots, cell lines were kept no longer than 6 months in culture. Identity of leukemia cell lines was confirmed by karyotyping and full exome sequencing. Transfected HeLa NLSv40-AcGFP-NES/PIK were maintained in DMEM supplemented with 10% v/v FBS and 20 μg/mL gentamicin. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation (Lymphoprep). Cell viability was measured using a 3-day MTS assay (Promega), AlexaFluor488 Annexin V-Dead Cell Viability flow cytometry (Life Technologies), and/or the Acridine Orange (AO)/Propidium Iodide (PI) Cell Viability Kit (Westburg). CC50 values were calculated by fitting the obtained MTS cell viability data in GraphPad Prism using a 4-parameter log-based fit for inhibitors.

XPO1 phenotypic reporter assay and colocalization with Rev-BFP
To study the XPO1-mediated nuclear export, a stably transfected HeLa SV40-NLS-AcGFP-NES/PIK1 reporter cell line was used. To knock-out XPO1 protein expression by indel formation in the XPO1 gene, cells were transfected with a plasmid expressing the Cas9 endonuclease N-terminally fused to the NLS of SV40 and the P2A-mCherry domain to visualize transfection, and a plasmid expressing an sgRNA-targeting XPO1 (target sequence 5’-GGATTATGTGAACAGAAAAG-3’) using a Neon Transfection System (10 μL, 1475V, 10 ms, 3x, buffer R). Transfected and untransfected cells were then plated into an 8-well glass bottom μ-slide (IBIDI) containing DMEM. Two days later, the untransfected cells were treated with either DMSO or 1 μM KPT-8602 for 3 hours and then all cells, including the XPO1 knockout cells, were visualized for AcGFP localization using confocal fluorescence microscopy.

For colocalization of Rev-BFP and XPO1-mNeonGreen, HAP-1 cells stably expressing XPO1 fused 3-c terminally to mNeonGreen were electroporated with a plasmid encoding for HIV-1 Rev-BFP using the Neon Transfection System (10 μL, 1475V, 10 ms, 3x, buffer R) and plated into an 8-well glass bottom μ-slide (IBIDI) containing DMEM. Cells were then imaged the next day and treated with either DMSO or 2 μM KPT-8602 for 2 hours before two doses of treatment, Rev-BFP and XPO1-mNeonGreen were visualized by confocal fluorescence microscopy.

Microscopy
Transfected HeLa cells were imaged with a Leica TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany), employing a HCK PL APO 63x (NA 1.2) water immersion objective. Different fluorescentes were detected sequentially using excitation lines of 405 nm (BFP), 488 nm (AcGFP, mNeonGreen), or 500 nm (YFP). Emission was detected between 410–480 nm (BFP), 493–565 nm (AcGFP, mNeonGreen, Alexa Fluor 488), or 500–580 nm (YFP).

High-content imaging and analysis
Stably transfected HeLa SV40-NLS-AcGFP-NES/PIK1 reporter cells seeded in a 96-well imaging plate (Falcon), were treated with different doses of compound or solvent (DMSO) for 4 hours. After treatment, the cells were fixed and their nuclei counterstained with DAPI or Hoechst 33342. Fluorescence in the blue and green channel was read on an ArrayScan XTI High Content Reader (Thermo Fisher Scientific). Nuclear and cytoplasmic compartments were segmented and their average pixel intensities were quantitated employing the HCS Studio software. GraphPad Prism software was used for calculation of EC50 values based on the relative change (as compared with the DMSO control) of the percentage nuclear AcGFP signal for the different treatments. Experiments were repeated six times on three different dates.
Figure 1.
KPT-8602 inhibits XPO1. A, Chemical structure of KPT-8602. B, XPO1 reporter assay. Confocal fluorescence microscopy of HeLa cells stably expressing the NLS-GFP-NESPKI reporter after CRISPR/Cas9 knock-out of XPO1, or treatment with 1 μmol/L KPT-8602. C, Quantification of increase in nuclear GFP signal in HeLa NLS-GFP-NESPKI cells treated with different concentrations of selinexor (KPT-330) and KPT-8602. For each concentration, a minimal number of 400 cells were analyzed. Data are presented as mean ± SD. D, Confocal fluorescence microscopy of HeLa cells immunostained for XPO1 (left) and of CRISPR/Cas9 tagged HAP-1 XPO1endo-mNeonGreen cells (right). E, Confocal fluorescence microscopy of HAP-1 XPO1endo-mNeonGreen cells expressing Rev-BFP two hours after treatment with DMSO or 2 μmol/L KPT-8602. F, Simple western for XPO1 in Jurkat cells treated with KPT-330 or KPT-8602 in the absence or presence of bortezomib. For each condition the ratio of XPO1/β-tubulin levels was quantified and normalized to untreated control. Data are presented as mean ± SD (n = 2).
Figure 2. Drug-target validation. A, Sequence chromatogram of genomic DNA from CRISPR/Cas9 genome edited Jurkat cells. Upper case indicates mutated bases, underlined bases are missense mutations and other upper case bases are silent mutations, italic cases indicate the PAM site. B, Confocal immunofluorescence imaging of RanBP1 cargo protein in wild-type and mutant JurkatC528S cells. C, Confocal fluorescence microscopy of HeLa cells co-expressing Rev-BFP and mutant XPO1C528S-YFP 2 hours after treatment with DMSO or 2 μmol/L KPT-8602. D, Chemical structure of biotinylated KPT-8602 (KPT-9511). E, Pull-down of KPT-9511-bound XPO1 protein from Jurkat cells. Cells were incubated with DMSO or 1 μmol/L KPT-9511 for 3 hours and streptavidin affinity chromatography was used to pull down XPO1 protein out of wild-type or mutant XPO1C528S Jurkat cells. F, Quantification of pulled down XPO1 protein. G, KPT-330 and KPT-8602 bind to equal amounts of XPO1 in cells. Wild-type Jurkat cells were treated with either KPT-330 or KPT-8602 at different concentrations for 2 hours and subsequently incubated overnight with the KPT-9058 (biotinylated KPT-276). Streptavidin affinity chromatography was used to pull down KPT-9058-bound XPO1 and analyzed by simple western. The ratio of pulled down XPO1 over XPO1 input was quantified and normalized to untreated control (n = 2). Data are presented as mean ± SD (n = 2).
Immunofluorescence staining

For immunostaining of endogenous XPO1 in HAP-1 wild-type cells, cells were plated into a Lab-Tek 8-well chambered cover glass, pretreated with 0.1% (w/v) poly-L-lysine (Sigma). Cells were allowed to adhere to the slides and were fixed in 4% paraformaldehyde (PFA) in PBS. Following fixation, cells were washed with PBS and then permeabilized using 0.2% Triton X-100 in PBS. After permeabilization, cells were washed and further immunofluorescence staining was performed according to standard procedure. Antibodies included the primary XPO1 antibody and a secondary Alexa Fluor 488–conjugated antibody. Stained cells were then analyzed by confocal fluorescence microscopy.

For the p53 staining, MOLT-4 cells were treated with 10 μmol/L of compound or solvent (DMSO) for 3 hours. Cells were harvested, washed in PBS and then transferred into a Lab-Tek 8-well chambered cover glass, pretreated with 0.1% (w/v) poly-L-lysine (Sigma). Cells were allowed to adhere to the slides and were then fixed and then sequentially washed using 4% PFA in PBS, washed and permeabilized (0.2% Triton X-100 in PBS). Further immunofluorescence staining was then performed according to the standard procedure. Employed antibodies included mouse monoclonal anti-p53 (DO-1; sc-126, Santa Cruz Biotechnology) at a 1:100 dilution and secondary Alexa Fluor 488 goat anti-mouse antibody (A11001, Molecular Probes). Cell nuclei were counterstained with DAPI and the samples were imaged by confocal microscopy. The fluorescence in the green channel was quantitated on a per cell basis employing CellProfiler analysis software, nuclei were segmented based on the DAPI fluorescence staining and the cytoplasm was de-automated by expanding the nuclei by a distance of N = 6 pixels. A total of approximately 200 cells were analyzed for each condition. For each cell the ratio of the mean pixel intensities in nucleus vs. cytoplasm was calculated, as well as the mean pixel intensity of the entire cell. Results were analyzed using a two-sided unpaired t-test with Welch’s correction (*, P < 0.0001 or **, P < 0.0005) and are depicted as mean ± 95% confidence interval (CI).

CRISPR/Cas9 knock-in

CRISPR/Cas9 genome editing of HL-60, Jurkat, K-562, and MOLT-4 cell lines was performed as described in (13). Briefly, cells were transfected with plasmids expressing Cas9-NLSsv40 single guide RNA (target sequence 5’-GGACATTATGCAACA-GAAAAG-3’) targeting XPO1 and the ssDNA donor oligonucleotide (IDT) containing the Cys528Ser missense mutation. Following electroporation with a Neon Electroporation system (Thermo Fisher Scientific; 10 μL, 1475V, 10 ms, 3x, buffer R) and plated immediately in 1 mL prewarmed, antibiotic-free RPMI-1640 medium supplemented with 10% v/v FBS. Five days after transfection, cells were diluted (0.5 cell/well) into 96-well plates and scored for the formation of single colonies after 3 days. Single colonies were grown for a period of 2 weeks and the DNA was extracted as described above. The initial PCR and Sanger Sequencing was performed with the same primers as described above for the Cys528Ser knock-in.

For C-terminal knock-in of mNeonGreen into XPO1, the Cas9-NLSsv40 was again transfected together with an sgRNA-targeting XPO1 in the C-terminal region, just before the stop codon (target sequence: 5’-TTTTAATCCACACTTCTTC-3’). HAP-1 wild-type or XPO1 C528S cells were electroporated using the same settings as described above and an additional donor repair plasmid containing the sequence for mNeonGreen-T2A-PuroR was added to facilitate insertion of mNeonGreen C-terminally to XPO1 (21). Two days after electroporation, cells were selected for 5 days with 1 μg/ml puromycin and surviving cells were screened for the expression of XPO1-mNeonGreen. As almost all of the surviving cells showed green fluorescence with a typical pattern reminiscent of the endogenous XPO1 distribution, the polyclonal mixture was used for further experiments.

XPO1 pull down and competition assay

For the initial pulldown of XPO1 with biotinylated KPT-8602 (KPT-9511), 10 million wild-type or XPO1 C528S Jurkat cells were incubated at 37°C with 1 μmol/L of KPT-9511 or DMSO in 1 mL of RPMI medium supplemented with 10% v/v FBS for 2 hours. Following incubation, cells were washed in ice-cold PBS and then lysed 20 minutes on ice in RIPA buffer (R0278, Sigma-Aldrich) supplemented with 1x HALT protease inhibitors (Thermo Fisher Scientific). After lysis, the debris was collected by centrifugation (10 minutes, 20,000 g at 4°C). A fraction was taken for the total lystate and the rest of the lysates were allowed to bind to Dynabeads MyOne Streptavidin T1 (Life Technologies) by rotating overnight at 4°C. The supernatant was removed the following day and the beads were extensively washed in modified RIPA buffer (50 mmol/L Tris-HCl [pH 7.8], 150 mmol/L NaCl, 1% NP-40 [IPGEPAL CA-630], 0.1% sodium deoxycholate, 1 mmol/L EDTA) using a magnetic separator. Finally, the bead-bound proteins were eluted for 10 minutes at 95°C with 1% SDS containing sample buffer (Protein Simple). Total protein concentrations were determined by the BCA assay (Thermo Fisher Scientific).
Scientific) and the samples were then prepared for Simple Western analysis according to protocol.

The competition experiment resembles a modified XPO1 pull down experiment. For this experiment, 5 million wild-type Jurkat cells were incubated for 2 hours with different concentrations of KPT-330 or KPT-8602 as measured by MTS assay. Data are presented as mean ± SD (n = 3). B, Cell viability as measured by annexin V/PI staining flow cytometry at 16 hours after treatment. Data are presented as mean ± SD (n = 3). C, Lysates from Jurkat and MOLT-4 T-ALL, and BV173 B-ALL cells treated with 1 μmol/L KPT-8602 were assessed for cleavage of PARP by simple western. *, Lysate from cells treated with 1 μmol/L KPT-8602 in the presence of the pancaspase inhibitor Q-VD-OPh. D, Viability of normal PBMC treated with KPT-330 and KPT-8602 as measured by acridine orange/PI cell viability counting. Data are presented as mean ± SD (n > 2). E, Immunostaining of p53 in MOLT-4 cells treated with DMSO, 10 μmol/L KPT-330 or 10 μmol/L KPT-8602 for 3 hours. For each condition a total of approximately 200 cells were analyzed. For each cell the ratio of the mean pixel intensities in nucleus versus cytoplasm was calculated, as well as the mean pixel intensity of the entire cell. Results were analyzed using a two-sided unpaired t test with Welch’s correction (***, P < 0.0001 or ****, P < 0.0005) and are depicted as mean ± 95% CI. F, Simple western for phospho-p53 (Ser15) in MOLT-4 and BV173 whole-cell extracts treated with 1 μmol/L KPT-8602 for different times. G, Sequence chromatogram of genomic DNA from CRISPR/Cas9 genome edited leukemia cells. Upper case indicates mutated bases, underlined bases are missense mutations and other upper case bases are silent mutations; italic cases indicate the PAM site. (Continued on the following page.)

Figure 3.
KPT-8602 induces potent cytotoxicity in T-ALL and B-cell leukemia cell lines. A, Viability of T-ALL, B-cell and AML leukemia cell lines treated with different concentrations of KPT-330 or KPT-8602 as measured by MTS assay. Data are presented as mean ± SD (n = 3). B, Cell viability as measured by annexin V/PI staining flow cytometry at 16 hours after treatment. Data are presented as mean ± SD (n = 3). C, Lysates from Jurkat and MOLT-4 T-ALL, and BV173 B-ALL cells treated with 1 μmol/L KPT-8602 were assessed for cleavage of PARP by simple western. *, Lysate from cells treated with 1 μmol/L KPT-8602 in the presence of the pancaspase inhibitor Q-VD-OPh. D, Viability of normal PBMC treated with KPT-330 and KPT-8602 as measured by acridine orange/PI cell viability counting. Data are presented as mean ± SD (n > 2). E, Immunostaining of p53 in MOLT-4 cells treated with DMSO, 10 μmol/L KPT-330 or 10 μmol/L KPT-8602 for 3 hours. For each condition a total of approximately 200 cells were analyzed. For each cell the ratio of the mean pixel intensities in nucleus versus cytoplasm was calculated, as well as the mean pixel intensity of the entire cell. Results were analyzed using a two-sided unpaired t test with Welch’s correction (***, P < 0.0001 or ****, P < 0.0005) and are depicted as mean ± 95% CI. F, Simple western for phospho-p53 (Ser15) in MOLT-4 and BV173 whole-cell extracts treated with 1 μmol/L KPT-8602 for different times. G, Sequence chromatogram of genomic DNA from CRISPR/Cas9 genome edited leukemia cells. Upper case indicates mutated bases, underlined bases are missense mutations and other upper case bases are silent mutations; italic cases indicate the PAM site. (Continued on the following page.)
Protein quantification and visualization was performed by Simple Western size-based protein assay (WES, ProteinSimple) a non-gel based and Western blot–like substitute following manufacturer’s protocol. For PARP and XPO1 protein quantification, cells were seeded at 0.5 × 10⁶ cells/mL in RPMI with 10% FBS. Compounds were added and incubated for 0 to 16 hours, washed with ice-cold PBS and lysed in RIPA buffer (Cat no: R0278, Sigma) and 1x HALT protease inhibitor cocktail (Thermo Fisher Scientific) for 15 minutes on ice. Lysates were cleared by centrifugation and total protein concentrations were determined by BCA assay (Thermo Fisher Scientific). Protein lysates (~1–2 µg per lane) were loaded into each capillary and proteins of interest were identified using specific primary antibodies raised against PARP (#9542, Cell Signaling Technology; 1:100), CRM1/XPO1 (NB100-79802, Novus; 1:12,500), phospho-p53(Ser15; #9284, Cell Signaling Technology; 1:50) and β-Tubulin (NB600-936, Novus; 1:3,000) and probed with HRP conjugated secondary antibodies against mouse or rabbit (ProteinSimple). Chemiluminescence signals were exported from the Compass software (ProteinSimple).

In vivo drug formulation

KPT-8602 (Karyopharm Therapeutics Inc.) was formulated in 0.5% methylcellulose plus 1% Tween-80 and given by daily oral gavage at a concentration of 10 to 15 mg/kg. Placebo consisted of vehicle without KPT-8602.

Bone marrow transplantation assay

We transduced lineage negative mouse bone marrow cells with retroviral vectors containing cDNA of the JAK3 M511I mutant (Degryse and colleagues, ref. 24). Transduced cells were injected into 4 irradiated BALB/c female mice of 6- to 8-weeks-old. Blood samples were drawn every 2 weeks and measured with the Vet ABC Hematology Analyzer (SCIL), while transduced cells were tracked by their expression of GFP⁺/mCherry⁺ on the MACSQuant VYB (Miltenyi Biotec). Treatment was initiated when white blood cell counts exceeded 10,000/µL, with >50% mCherry⁺ leukemic cells.

Treatment of normal BALB/c mice

Five female BALB/c mice were randomly divided into 2 groups, with 3 animals receiving KPT-8602 and 2 animals receiving placebo. Mice were bled every week to determine the effect of...
Results

KPT-8602 inhibits XPO1-mediated nuclear cargo export

To assess the effect of KPT-8602 on XPO1-mediated nuclear export, it was tested in a phenotypic reporter assay based on a GFP reporter protein that is fused to the NES from the cAMP-dependent protein kinase inhibitor (PKI) and to the SV40 NLS, which both cause the GFP reporter to actively shuttle between nucleus and cytoplasm. In steady state, this GFP fusion protein is mainly localized to the cytoplasm in a XPO1-dependent manner as knock out of XPO1 resulted in accumulation in the nucleus due to decreased nuclear export (Fig. 1B). HeLa stably expressing this GFP reporter protein were treated with increasing concentrations of KPT-8602 and the localization of GFP was determined by imaging (Fig. 1C and Supplementary Fig. S1). KPT-8602 inhibited the XPO1-dependent nuclear export with an EC₅₀ value of 60.9 ± 3.6 nmol/L, which is similar to the EC₅₀ values for the first-generation XPO1 inhibitor selinexor (55.7 ± 6.5 nmol/L). Nuclear accumulation of the GFP reporter was observed as early as 4 minutes after treatment with 1 μmol/L KPT-8602 (Supplementary Movie S1). To investigate whether KPT-8602 effectively blocks the interaction of XPO1 with cargo protein in cells, we tagged cellular XPO1 protein with mNeonGreen in the genome of HAP-1 cells using CRISPaint gene tagging (21). The cellular labeled XPO1-mNeonGreen fusion protein was found at the nuclear membrane and throughout the nucleoplasm in agreement with the localization of untagged protein (Fig. 1D). When these cells were transfected with a plasmid expressing a Rev–BFP fusion, a prototype XPO1 cargo protein, a substantial fraction of the endogenous XPO1-mNeonGreen protein was co-localized with Rev–BFP in the nucleoli as a result of interaction between both proteins. Upon treatment of the cells with 2 μmol/L KPT-8602 the Rev-dependent nucleolar co-localization was abolished demonstrating that KPT-8602 is effectively inhibiting the XPO1 cargo interaction (Fig. 1E and Supplementary Movie S2). In addition, KPT-8602 also caused significantly decreased levels of XPO1 protein after overnight treatment, which was prevented by the proteasome inhibitor bortezomib suggesting a proteolytic degradation mechanism (Fig. 1F).

KPT-8602 directly binds XPO1

To further show that KPT-8602 is inhibiting the XPO1-mediated nuclear export by directly targeting XPO1, we applied CRISPR/Cas9 genome editing to introduce a resistance Cys528Ser point mutation in the XPO1 gene of Jurkat leukemia cells (Fig. 2A; ref. 13). The effect of the mutation on KPT-8602 activity was assessed by the visualization of the subcellular localization of RanBP1, another XPO1 cargo (Fig. 2B). In both wild-type and mutant Jurkat cells RanBP1 localized in the cytoplasm. Upon treatment of wild-type cells with KPT-8602, RanBP1 accumulated in the nucleus. In contrast, KPT-8602 was unable to induce nuclear accumulation of the RanBP1 cargo in the XPO1C528S mutant Jurkat cells, demonstrating that this mutation confers resistance to KPT-8602. This is supported by the observation that KPT-8602 was unable to inhibit the interaction between XPO1C528S with its prototypical cargo protein, Rev (Fig. 2C).

To further demonstrate the direct binding of KPT-8602 to the XPO1 cysteine 528 residue, Jurkat cells were treated with biotinylated KPT-8602 (KPT-9511; Fig. 2D) allowing for the pull down of the compound with its interacting factors from the lysate using streptavidin. KPT-9511 was able to pull down XPO1 protein from

Patient derived xenograft models

Human leukemia bone marrow cells from NOTCH1 mutant T-ALL patient 389E and BCR-ABL1 positive B-ALL patient XC51 were injected into the tail vein of 6-to-12-week old NOD.Cg–Prkdcm1 (Iitg2tm1Wyi)/Sld (NSG) mice. After successful engraftment, splenocytes were harvested and reinjected at a concentration of 1–2 × 10⁶ cells into 30 NSG mice to create secondary transplants. Blood samples were taken once a week and measured on the Vet ABC Hematology Analyzer (SCIL). Leukemic engraftment was determined with flow cytometry (FACS Canto II, BD) using anti-human CD45 (APC, eBioscience) as marker for the leukemic cells.

Treatment started when the CD45-percentage in the peripheral blood was respectively <0.5% and >5%. Mice were divided into groups of 4 to 5 mice, each treated animal matching a control mouse in both gender and CD45%. At the end of treatment, mice were sacrificed and spleen and bone marrow harvested for single cell isolation and histopathologic examination. All experiments were conducted on protocols approved by the ethical committee of the University of Leuven.

Immunohistochemistry

Spleen tissue and sternum were collected and fixed in 10% neutral buffered formalin (Sigma) for 24 hours and then processed for paraffin embedding (Thermo Scientific Excelsior AS Tissue Processor and HistoStar Embedding Workstation). Sections of 5 μm were mounted on Superfrost Plus Adhesion slides (Thermo Fisher Scientific) and stained with hematoxylin and eosin (Diapath). The degree of splenic or bone marrow colonization by leukemic cells was determined by staining with human HLA-A (Abcam) with a digital image analysis algorithm created on the ImageJ software platform.
Figure 5.
KPT-8602 shows potent anti-lymphoblastic leukemia activity in vivo. A, MOLT-4 subcutaneous xenografted mice were treated with vehicle (n = 8), doxorubicin at 5 mg/kg IP on days 1 and 15 (n = 8), or KPT-8602 given PO at 5 (n = 8) or 15 mg/kg (n = 8) daily. B, Total white blood cell counts and absolute lymphocyte and granulocyte count in the peripheral blood of a mouse bone marrow transplant JAK3(M511I) T-ALL model, placebo- (n = 2) versus KPT-8602 (n = 2)-treated animals. C, Total white blood cell, lymphocyte, granulocyte, red blood cell (rbc) and platelet counts in wild-type BALB/c mice, treated for 14 days with KPT-8602 (n = 3) versus placebo (n = 2). Data in A-C data are presented as mean ± SD and were analyzed using the Mann-Whitney U test. ns: not significant.
wild-type Jurkat cells, but not from Jurkat cells containing the homozygous XPO1 C528S mutation (Fig. 2E and F). To further investigate whether the binding of KPT-8602 to XPO1 has increased reversibility as compared to selinexor, we used these compounds in a competition assay with a biotinylated analogue of KPT-276 (KPT-9058), which can also covalently interact with cysteine 528 of XPO1 and can pull down XPO1 from cell lysates (13). When cells were pre-treated with selinexor, no further binding of KPT-9058 was detected, as the covalent interaction of selinexor to XPO1 prevented the binding of KPT-9058 (Fig. 2G; refs. 13, 22). If the interaction of KPT-8602 with XPO1 had increased reversibility compared to selinexor, KPT-9058 would outcompete KPT-6602 more efficiently than selinexor, resulting in increased amount of XPO1 being pulled down in the assay. However, at similar compound concentrations we did not observe a significant difference in the amount of pulled down XPO1 between KPT-8602 and selinexor treated cells.

KPT-8602 induces cytotoxicity and apoptosis in leukemia cell lines
To evaluate the anti-leukemic activity of KPT-8602, several human leukemia cell lines (T-ALL, B-ALL, and AML) were cultured in the presence of increasing concentrations of KPT-8602 and cell viability was assessed after 72 hours of treatment (Fig. 3A). Cell viability was reduced in all KPT-8602-treated leukemia cell lines with EC50 values ranging from 25 to 145 μmol/L (Supplementary Table S1). Decrease in leukemia cell viability was further confirmed by annexin V/PI staining of Jurkat and MOLT-4 T-ALL cells (Fig. 3B). Both cell lines demonstrated pronounced levels of apoptosis measured 16 hours after treatment. Induction of apoptosis was prevented by the pan-caspase inhibitor Q-VD-OPh, suggesting a caspase dependent mechanism. This was confirmed by the appearance of cleaved caspase-3 substrate PARP as early as 6 hours after treatment with 1 μmol/L KPT-8602 (Fig. 3C). Normal PBMCs were unaffected up to 10 μmol/L KPT-8602 (Fig. 3D).

Because the pro-apoptotic protein p53 is an important cargo of XPO1, we investigated whether p53 localization was affected by KPT-8602 treatment. Treatment of MOLT-4 cells with KPT-8602 resulted in an increased p53 nuclear as well as absolute staining (Fig. 3E). Upregulation and stabilization of p53 in MOLT-4 (p53 wild-type as measured by exome seq) and BV173 (p53 wild-type; ref. 23) was also evident by western blotting of phosphorylated Ser15 p53 as early as 5 hours post-treatment (Fig. 3F). This correlated with the observed induction of apoptosis evident from the caspase dependent cleavage of PARP as early as 6 hours after treatment (Fig. 3C).

Cytotoxic effects of KPT-8602 are completely attributed to XPO1 inhibition
To determine whether the observed anti-leukemic activity of KPT-8602 was exclusively caused by its inhibition of XPO1 and not by off-target effects we tested the activity of KPT-8602 on 4 leukemia cell lines (Jurkat, MOLT-4, HL-60 and K562) in which we engineered the XPO1 C528S mutation by CRISPR/Cas9 genome editing (Fig. 3G). For Jurkat, HL-60, and K562, we were able to select monoclonal homozygous mutants and for MOLT-4 we used polyclonal cells. Mutant leukemia cells containing the single XPO1 C528S mutation were 60 to 150 times more resistant to KPT-8602 than the parental cells (Fig. 3H and Supplementary Table S2), demonstrating that the C528S mutation confers resistance to the drug. This was confirmed by the absence of PARP cleavage in mutant Jurkat and MOLT-4 cells in the presence of micromolar concentrations KPT-8602 (Fig. 3I) and by the absence of decreased XPO1 protein levels after treatment (Fig. 3J). Altogether these results demonstrate that XPO1 is the target for KPT-8602 and that its anti-leukemic activity is completely caused by inhibition of XPO1-mediated nuclear protein export.

The XPO1 E571K/G mutation does not influence KPT-8602 activity
A recurrent mutation at codon 571 (p.E571K/G) in the XPO1 gene was recently described in some leukemia patients (7, 8). Because this mutation is located inside the hydrophobic cargo-binding pocket near residue cysteine 528 of XPO1, we evaluated whether or not this mutation could affect KPT-8602 activity. Therefore, we edited the XPO1 gene of HAP-1 cells (CML) using CRISPR/Cas9 to introduce the E571K mutation. Sensitivity of these mutant cells to KPT-8602 treatment was measured and compared to wild-type cells (Fig. 4). The dose-dependent inhibitory curves of wild-type and mutant cells were identical for KPT-8602, demonstrating that the C528S mutation confers resistance to KPT-8602 in mice.

KPT-8602 shows potent anti-lymphoblastic leukemia activity in vivo
To test the in vivo efficacy of KPT-8602, we first used the MOLT-4 T-ALL cell line in a subcutaneous murine xenograft model. KPT-8602 demonstrated potent anti-leukemia activity at daily doses between 5 and 15 mg/kg (Fig. 5A). Next, we used a mouse bone marrow transplant T-ALL model with the JAK3 (M511I) mutation, as previously described (24). Mice developed T-ALL after 100 to 200 days, characterized by elevated white blood cell (WBC) counts (>10 000 cells/μL) in the peripheral blood (PB). Mice received KPT-8602 at a dose of 15 mg/kg or placebo by daily oral gavage for 12 days and PB samples were taken at day 2 and at the end of the treatment period. After 2 days of treatment, animals already showed a marked reduction in total WBC counts compared to placebo-treated animals and the WBC counts continued to drop until they reached normal levels (<10 000 cells/μL) by day 12 (Fig. 5B). This decrease in WBC was mostly due to loss of leukemic lymphocytes, however, a minor decrease in normal granulocyte numbers was also observed (Fig. 5B). To further investigate the effect of KPT-8602 on normal hematopoietic cells, we treated healthy Balb/c mice with KPT-8602 for 14 days, showing no or minimal changes in the normal blood cells or platelets during treatment (Fig. 5C).

KPT-8602 prolongs survival of patient derived xenograft models of ALL
To determine the activity of KPT-8602 against primary human leukemia cells, we used a patient derived xenograft (PDX) model of a NOTCH1 mutant T-ALL sample and a BCR-ABL1 positive B-ALL sample with an IKZF1 deletion. Treatment consisted of KPT-8602 10 mg/kg or placebo once a day and was initiated when human leukemia cells in the PB of the mice ranged from 0% to 0.5%. After 3 to 4 weeks of treatment, all mice were sacrificed and their spleen, tibia and femur harvested for single cell isolation and histopathologic examination. Total WBC count of placebo- and drug-treated animals remained normal during treatment, as did...
T-ALL Patient derived xenograft model

A. Leukemic invasion in the peripheral blood

B. Bone marrow invasion at sacrifice

C. Spleen weight at sacrifice

D. Spleen invasion at sacrifice

Placebo

KPT-8602

E. Survival analysis

F. Leukemic invasion in the blood

*Endpoint criteria reached for all placebo mice

B-ALL Patient derived xenograft model

G. Leukemic invasion in the peripheral blood

H. Bone marrow invasion at sacrifice

I. Spleen weight at sacrifice

J. Spleen invasion at sacrifice

www.aacrjournals.org Clin Cancer Res; 2017 OF11

Published OnlineFirst October 25, 2016; DOI: 10.1158/1078-0432.CCR-16-1580

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Comparison of spleen weights in KPT-8602 versus placebo-treated T-ALL PDX-mice at the time of sacrifice. As new targeted chemotherapeutic regimens, whereas all treated patients face the risk of developing secondary malignancies (20). Because leukemia patients usually present with high WBC counts and severe organ invasion, we also tested the efficacy of KPT-8602 treatment in xenograft animals with high leukemic cell burden. Using the T-ALL PDX-model, we waited until mice had minimum 5% human leukemia cells in their PB before starting treatment. To determine the survival benefit, mice were sacrificed when 50% or more human leukemia cells were detected in the PB, or if they fulfilled other ethical endpoint criteria. By the end of the third week, 4 out of 5 untreated animals were still alive, compared to none of the placebo treated animals (P = 0.0041; Fig. 6E). KPT-8602 was able to reduce the number of human leukemic cells in some animals, while stabilizing the disease in other animals (Fig. 6F).

**Discussion**

ALL is the most common childhood cancer. Although survival rates are high (>90%) with current treatment protocols, subgroups of patients still face unfavorable outcome, including infants, adults and relapse patients (18). In addition, survivors of ALL are often confronted with severe side effects of chemotherapy both on the short and long term. Especially in children, cardiomyopathy and osteopenia are well-known consequences of chemotherapeutic regimens, whereas all treated patients face the risk of developing secondary malignancies (20). As new targeted therapies become available for specific subgroups of patients, these will help to improve outcome and reduce toxic side effects. However, targeted inhibitors are typically very specific and can only be used for the treatment of a small number of patients, making both drug development and clinical evaluation of efficacy difficult. A more general targeted approach such as proteasome and now XPO1 inhibitors can overcome patient specificity problems and are likely to be effective for all leukemia subtypes, regardless of mutational status.

The lead clinical SINE compound, selinexor, is now in phase II/IIb clinical trials for several solid and hematological tumors. However, selinexor dosing is restricted to 2 to 3 times per week due to adverse effects; primarily nausea, anorexia, and fatigue (25). Here, we tested a second-generation clinical stage SINE compound, KPT-8602, with improved tolerability allowing for daily dosing. We demonstrate that KPT-8602 is a potent XPO1 inhibitor with similar potency to selinexor in vitro. EC_{50} values were much below mean plasma concentrations reached (3 μmol/L) in primates orally dosed at 10 mg/kg (17). KPT-8602 binds directly to XPO1, inhibits cargo interaction and subsequent cargo protein export from the nucleus resulting in nuclear accumulation of tumor suppressors. Indeed, KPT-8602 treatment caused nuclear accumulation of the tumor-suppressor p53 resulting in caspase-dependent apoptosis induction. Introducing a serine mutation at cysteine 528 in multiple leukemia cell lines conferred drug resistance, providing ultimate proof for the drug-target interaction for KPT-8602. This cysteine mutation was experimentally identified as resistance mutation and has not been monitored in ongoing clinical trials. Although this cysteine is highly conserved amongst higher eukaryotes, mutations of this residue should be monitored as a mechanism for resistance in patients.

*In vivo* experiments showed potent anti-leukemic activity of KPT-8602 in both a mouse T-ALL model and two PDX ALL models, consistent with results from selinexor in preclinical and clinical phase I trials in patients with relapsed or refractory ALL and AML (NCT02091243). We did not observe evidence for toxic side effects of KPT-8602 dosed on a daily base at a concentration of 10 mg/kg. KPT-8602 had no effect on normal platelet, granulocyte or red blood cell counts in the PB of treated animals. This is important since decreases in normal hematopoietic cell numbers can lead to transfusion dependency or failed immune responses. As with selinexor, KPT-8602 may spare normal hematopoietic stem and progenitor cells (26). When treatment was started early on in ALL development, KPT-8602 was able to completely inhibit human leukemic cell growth in two PDX-models. However, to mimic the clinical situation of patients at diagnosis or relapse, we also set up a survival experiment starting therapy at a more advanced leukemia stage. As single agent, KPT-8602 was still able to reduce the leukemia cell numbers in 2 out of 5 mice, and stabilized the disease in the remaining animals. These findings together with its mode of action supports further investigation of KPT-8602 for the treatment of ALL in combination with standard therapy.

**Figure 6.** KPT-8602 shows potent activity in patient-derived ALL xenograft models. **A,** Percentage of human leukemia cells (human CD45-positive cells) in the blood of mice injected with human T-ALL cells during treatment with placebo or KPT-8602. Start of the treatment is indicated (arrow). **B,** Percentage of human leukemia cells (human CD45-positive cells) infiltrated in the bone marrow at the time of sacrifice in placebo versus KPT-8602 treated T-ALL PDX-animals. **C,** Comparison of spleen weights in KPT-8602 versus placebo-treated T-ALL PDX-mice at the time of sacrifice. **D,** Human HLA-staining of spleen slides taken at sacrifice from a representative placebo-treated T-ALL PDX-mouse and KPT-8602-treated animal (brown = infiltrating human leukemia cells, blue = normal mouse spleen tissue). **E,** Survival analysis of 5 KPT-8602 and 5 placebo-treated T-ALL PDX-mice. Treatment started at day 28 after injection when the percentage of human leukemia cells (human CD45-positive cells) in the blood was >5%. Animals were sacrificed when they reached ethical endpoints or the percentage of human CD45-positive cells in the blood was 50% or more. **F,** The percentage of human leukemia cells (human CD45-positive cells) in the blood of mice injected with human T-ALL cells during treatment with placebo or KPT-8602. Treatment started when human CD45% in the blood reached >5% (indicated by the arrow). **J,** There are no error bars at day 39 in the placebo treated group because at this time point the last remaining placebo mouse was sacrificed. **G,** The percentage of human leukemia cells (human CD45-positive cells) in the blood of mice injected with human BCR-ABL1-positive B-ALL cells during treatment with placebo or KPT-8602. Start of the treatment is indicated (arrow). **H,** Percentage of human leukemia cells (human CD45-positive cells) infiltrated in the bone marrow at the time of sacrifice in placebo versus KPT-8602 treated B-ALL PDX-animals. **I,** Comparison of spleen weights in KPT-8602 versus placebo treated B-ALL PDX-mice at the time of sacrifice. **J,** The percentage of human leukemia cells (human CD45-positive cells) infiltrated in the spleen tissue at the time of sacrifice in placebo versus KPT-8602-treated B-ALL PDX-animals. Data in A–C and F–J data are presented as mean ± SD; P values are calculated using the Mann–Whitney U test.
Especially the observation that KPT-8602 shows potent activity against BCR-ABL1 positive ALL, which is a poor prognostic subtype of B-cell ALL, warrants further investigation of KPT-8602 for the treatment of such poor prognostic subgroups.

In conclusion, our data validate the second-generation SINE compound KPT-8602 as a potent XPO1 inhibitor that can effectively inhibit leukemia growth in vivo with minimal effect on normal hematopoiesis.

Disclosure of Potential Conflicts of Interest

E. Baloglu, Y. Landesman, and S. Shacham are employees of Karyopharm. W. Senapedis holds ownership interest (including patents) in Karyopharm Therapeutics. D. Daelemans has a license agreement on XPO1 SINE inhibitors. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

We thank Lotte Brl, Bob Massant and Astrid D’Hoore for excellent technical help.

Grant Support

This work was supported by grants from Kom op Tegen Kanker (to J. Cools), Stichting Tegen Kanker (to J. Cools), the European Research Council (to J. Cools) and the IAP network (to J. Cools). Part of this research work was performed using the “Caps-It” research infrastructure (project ZW13-02, to P. Leyssen and J. Neyts) financially supported by the Hercules Foundation and Rega Foundation, KU Leuven; J. De Bie is supported by FWO-Vlaanderen, A. Dagklis is supported by Kom op Tegen Kanker.

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Received July 4, 2016; revised September 14, 2016; accepted October 10, 2016; published OnlineFirst October 25, 2016.

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Clinical Cancer Research

The Second-Generation Exportin-1 Inhibitor KPT-8602 Demonstrates Potent Activity against Acute Lymphoblastic Leukemia

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Clin Cancer Res Published OnlineFirst October 25, 2016.

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Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-16-1580

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