Nuclear–Cytoplasmic Transport Is a Therapeutic Target in Myelofibrosis

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Abstract

Purpose: Myelofibrosis is a hematopoietic stem cell neoplasm characterized by bone marrow reticulin fibrosis, extramedullary hematopoiesis, and frequent transformation to acute myeloid leukemia. Constitutive activation of JAK/STAT signaling through mutations in JAK2, CALR, or MPL is central to myelofibrosis pathogenesis. JAK inhibitors such as ruxolitinib reduce symptoms and improve quality of life, but are not curative and do not prevent leukemic transformation, defining a need to identify better therapeutic targets in myelofibrosis.

Experimental Design: A short hairpin RNA library screening was performed on JAK2V617F-mutant HEL cells. Nuclear–cytoplasmic transport (NCT) genes including RAN and RANBP2 were among top candidates. JAK2V617F-mutant cell lines, human primary myelofibrosis CD34+ cells, and a retroviral JAK2V617F-driven myeloproliferative neoplasms mouse model were used to determine the effects of inhibiting NCT with selective inhibitors of nuclear export compounds KPT-330 (selinexor) or KPT-8602 (eltanexor).

Results: JAK2V617F-mutant HEL, SET-2, and HEL cells resistant to JAK inhibition are exquisitely sensitive to RANKL or pharmacologic inhibition by KPT-330 or KPT-8602. Inhibition of NCT selectively decreased viable cells and colony formation by myelofibrosis compared with cord blood CD34+ cells and enhanced ruxolitinib-mediated growth inhibition and apoptosis, both in newly diagnosed and ruxolitinib-exposed myelofibrosis cells. Inhibition of NCT in myelofibrosis CD34+ cells led to nuclear accumulation of p53. KPT-330 in combination with ruxolitinib-normalized white blood cells, hematocrit, spleen size, and architecture, and selectively reduced JAK2V617F-mutant cells in vivo.

Conclusions: Our data implicate NCT as a potential therapeutic target in myelofibrosis and provide a rationale for clinical evaluation in ruxolitinib-exposed patients with myelofibrosis.

Introduction

Myelofibrosis has the most profound impact on quality of life and survival among the classic BCR-ABL–negative myeloproliferative neoplasms (MPN; ref. 1). Myelofibrosis can present de novo (primary myelofibrosis) or as secondary arising from polycythemia vera (post-PV myelofibrosis) or essential thrombocythemia (post-ET myelofibrosis). Cytopenias, thromboembolic complications, and transformation to acute myeloid leukemia (AML) cause excess mortality compared with age-matched controls, as well as patients with PV or ET (2, 3). Morbidity is profound due to debilitating constitutional symptoms such as fatigue, anorexia, night sweats, and weight loss (4). Constitutive activation of JAK/STAT signaling through mutations in JAK2 (JAK2V617F, 50%–60%), calreticulin (CALR, 20%–30%), or MPL (MPLV654S and others, 5%–7%) is characteristic of myelofibrosis (5–10). Most patients have additional mutations, commonly involving genes associated with epigenetic regulation, such as ASXL1, EZH2, TET2, IDH1/2, and DNMT3A (11–15). ASXL1 mutations are associated with inferior overall survival, while patients with CALR mutations exhibit a more indolent clinical course (16).

For many years, myelofibrosis treatment was limited to cytotoxic chemotherapy to control myeloproliferation, and supportive care, for example, cytokines, to improve cytopenias. Immuno-modulatory drugs such as thalidomide in combination with prednisone were used with modest success (17). The discovery of JAK2V617F in myelofibrosis led to the clinical development of the...
Myelofibrosis is a fatal hematopoietic stem cell neoplasm characterized by constitutive activation of JAK/STAT signaling. JAK kinase inhibitors such as ruxolitinib reduce myelofibrosis symptoms, but like all other drugs used in myelofibrosis are not curative, with persistence of mutant cells and prompt symptom rebound upon discontinuation. Therefore, more effective drug therapies are needed to improve survival in myelofibrosis. In this study, using a lentiviral short hairpin RNA screen, we have discovered that HEL and SET-2 cell lines and primary myelofibrosis cells are exquisitely dependent on nuclear–cytoplasmic transport (NCT). Pharmacologic inhibition of NCT by KPT-330 or KPT-8602 selectively suppressed colony formation by myelofibrosis compared with normal CD34+ cells and enhanced ruxolitinib-mediated growth inhibition and apoptosis. In a myeloproliferative neoplasms model addition of KPT-330 to ruxolitinib improved responses compared with monotherapy, including reductions of mutant cells and restoration of splenic architecture. Our results warrant clinical trials of KPT-330 and/or KPT-8602 in ruxolitinib-exposed patients with myelofibrosis.

**Materials and Methods**

**Cell lines**

We used human leukemia cell lines HEL (homozygous for JAK2V617F), SET-2 (heterozygous for JAK2V617F), TF1, UT7, and HEL-R (HEL resistant to JAK inhibition, described below). HEL, SET-2, TF1, and UT7 cells were obtained from DSMZ (Braunschweig), cultured in RPMI1640 medium supplemented with 10% FBS (Sigma-Aldrich), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL of streptomycin. TF1 and UT7 cells were cultured in 10 ng/mL GM-CSF. Cell lines were authenticated (GenePrint 24 Kit, Promega), and compared with the DSMZ online STR database. Cells were screened for Mycoplasma with the MycoAlert detection kit and determined to be negative. HEL cells resistant to JAK inhibition (HEL-R) were generated by long-term culture in increasing concentrations of momelotinib (CYT387), starting at 0.05 μmol/L with increases at 2-week intervals up to 7 μmol/L (>4-fold above the reported IC50; ref. 25). For this study, HEL-R cells were maintained in 5 μmol/L ruxolitinib. See Supplementary Methods.

**Primary samples**

Blood or bone marrow was subjected to Ficoll separation followed by red blood cell lysis. An AutoMACS Pro Separator (Miltenyi Biotec) was employed to purify CD34+ cells from myelofibrosis patient samples and cord blood (St. Louis Cord Blood Bank, SSM Health Cardinal Glennon Children's Hospital, St. Louis, MO). Primary cells were cultured in RPMI1640 medium supplemented with 10% FBS and cytokines (CC100: SCF, FLT3L, IL3, and IL6; StemCell Technologies). We typically culture cells for 24 hours prior to experiments to minimize the influence of drugs present in the plasma. "Ruxolitinib exposed" refers to the status of the patient from which the sample was collected, and is defined by the patient's physician, with adherence to published guidelines for diagnosis and disease monitoring (26–29). Except where noted as "responsive," this term includes primary and secondary occurrences of relapse, refractory, and resistant disease, collectively known as failure. Written informed consent was obtained from all donors (The University of Utah IRB #45880, Salt Lake City, UT). Patient information is summarized in Supplementary Table S1.

**shRNA library screen**

Cellecta, Inc. provided Human Module 1 (HM1) lentiviral shRNA library, containing 27,239 noncontrol shRNAs targeting 5,034 genes involved in cell signaling, with 5–6 shRNAs per gene. Each shRNA is identifiable by a unique 18-bp barcode. HEL cells were subjected to a library screen in 3 independent experiments using previously described methods (24). Briefly, HEL cells were infected with packaged library to yield a transduction rate of approximately 30% (monitored by flow cytometry for red fluorescent protein 72 hours postinfection), equivalent to multiplicity of infection of ≤1. Transduced cells were then selected with puromycin (2 μg/mL) for 9 days, as per manufacturer’s protocol (Cellecta). Medium changes and volume expansions were done as necessary to maintain exponential growth. After selection, DNA was extracted, and individual shRNA barcodes were amplified as recommended by the manufacturer (Cellecta; Pooled Barcoded Lentiviral shRNA library v5, http://www.cellecta.com/resources/protocols) with ≤14 cycles for each step to minimize biased
barcode amplification. Amplicon sizes were confirmed on a 3% agarose gel. PCR products were purified (PCR Clean-Up Kit, Qiagen), and subjected to next-generation sequencing (NGS; Illumina HiSeq 2500, rapid mode).

Bioinformatic analysis of the shRNA library
We previously described the algorithm used for bioinformatics analysis (24). Fastq files were deconvoluted by Cellecta, providing the frequency that reads matched a barcode from the HM1 library for each sample, as well as gene mapping. A median depth of 237–495 reads across the samples was obtained for identified barcodes. Barcode frequencies for each sample were normalized to a total of 20 million reads. The fold change for each shRNA was calculated as the ratio of observed reads to the Cellecta–provided HiSeq2000 plasmid counts after adjusting numerator and denominator by the addition of 10 to reduce potentially inflated estimates for low counts. Zero-read shRNAs and shRNAs present in only 1 out of 3 replicates were excluded, and the median fold-depletion of those remaining was analyzed. Candidate genes were selected on the basis of a 10-fold depletion in ≥3 shRNAs (Supplementary Table S2). All shRNA plasmids were made by Cellecta (pRSIT12-U6Tet-(sh)-CMV-TetRep-2A-TagRFP-2A-Puro).

Inhibitors
Karyopharm Therapeutics provided KPT-330 (selinexor) and KPT-8602 (eltanexor); CRM1 inhibitors known as SINE compounds (30, 31). Ruxolitinib was purchased from Chemietek. For in vitro analysis, inhibitors were dissolved in DMSO at 10 mmol/L and stored at −20°C. For in vivo use, see Supplementary Methods.

Viable cell assay (MTS)
Cells were seeded in triplicate in 96-well plates (cell lines, 5 × 10^3 cells/well; primary CD34+ cells, 1–2 × 10^4 cells/well) without or with 100 ng/mL doxycycline or indicated inhibitors. Viable cells were measured using CellTiter 96 AQueous One Solution MTS Reagent (Promega) on an Epoch Microplate Spectrophotometer (BioTek Instruments).

Apoptosis assay
Apoptosis was assayed with aliphophocyatin-conjugated annexin V in combination with 7-aminoactinomycin D (BD Biosciences) or DAPI (Sigma-Aldrich) on a Guava HT6 (Millipore) or a BD FACSCanto flow cytometer.

PCR and shRNA protocols and sequences
For details, see Supplementary Methods and Supplementary Tables S3 and S4.

Nucleocyttoplasmic fractionation and immunoblot analysis
Nucleocyttoplasmic fractionation was performed as described previously (24). For antibody information, see Supplementary Table S5.

 Colony formation assay
HEL or SET-2 shRAN cells (200 cells/dish) were plated in duplicate in MethoCult H4230 (StemCell Technologies) without or with 100 ng/mL doxycycline. Colonies were scored 7 days after plating. Primary myelofibrosis or cord blood CD34+ cells (1 × 10^5 cells/dish) were seeded in duplicate in MethoCult H4230 with CC100 and indicated inhibitors. Colonies were counted after 10–15 days (Supplementary Table S6).

Bone marrow transduction/transplant and drug delivery
Myelofibrosis-like disease was induced in Balb/c mice as described previously (25, 32, 33). Briefly, Balb/CJ mice were purchased from Jackson Laboratory. Donors (6 to 8 weeks old) were primed with 5-fluorouracil (100 mg/kg, Feseniux Kabi) by intravenous injection. Recipients were 7 to 9 weeks old at the time of irradiation and transplant. Complete blood counts were acquired on a Heska HemaTrue instrument and GFP was analyzed on a Guava HT6 flow cytometer. All drugs and vehicles were administered by oral gavage. Details of the dosing schedule are shown in Supplementary Tables S7 and S8. Bone marrow fibrosis was scored as described previously (33, 34). Bone marrow cellularity was estimated by a hematopathologist on a hematoxylin and eosin (H&E)–stained femur marrow cavity. Cellularity was variable within specimens, so an overall average was provided on the basis of multiple medium-power fields encompassing the whole specimen. The myeloid-erythroid (M:E) ratio was estimated on Periodic acid–Schiff-stained femur marrow cavity. A minimum of 5 high-power fields were evaluated within the more cellular areas to produce an overall average estimate rounded to the nearest integer.

Mouse procedures were performed according to guidelines approved by the Institutional Animal Care and Use Committee at The University of Utah (Salt Lake City, UT).

Statistics and IC50 calculations
Results are provided as mean ± SEM. Data were analyzed by 1-way ANOVA or a 2-tailed Student t test using Prism 7.02 (GraphPad). To compare single agent to combination treatment, we performed 1-way ANOVA (uncorrected Dunn test, Kruskal–Wallis test). P < 0.05 was considered to be statistically significant. For HEL and SET-2 cells, a 4-parameter variable-slope regression analysis was used to calculate IC50 values; for HEL-R cells, where 50% inhibition was not reached, IC50 values were determined by variable-slope regression analysis.

Synergy analysis used the "response surface" approach and formula (5) of Greco, Bravo, and Parsons (35) for 2-drug interaction. We set parameters m that were used to calculate the response surface. For HEL and SET-2 cells, a 4-parameter variable-

\[
E = E_c \left(1 - \left(1 + \frac{D_1}{IC_{50,1}} + \frac{D_2}{IC_{50,2}} + \alpha D_1 D_2 \left(\frac{IC_{50,1}}{IC_{50,2}}\right)^m\right)^{-1}\right)
\]

\[
D_1 \text{ and } D_2 \text{ are concentrations of Drugs 1 and 2 (ruxolitinib and KPT-330, or ruxolitinib and KPT-8602).}
\]

\[
E = \text{effect; } E_c = \text{Effect of control}
\]

The parameters to be estimated are:

\[
IC_{50,1} \text{ and } IC_{50,2} \text{ defined as concentrations of drugs 1 and 2 resulting in 50% inhibition.}
\]

m, a shape parameter. (Note again that there could be separate shape parameters for each drug. We have set them equal.)

α, a measure of interaction or synergy, with α > 0 representing positive interaction and α < 0 representing negative interaction.

Because of the normalization setting control equal to 100, for this data E_c = 100.

Synergy analysis was performed using the nonlinear mixed effects modeling package "nlme" in "R" statistical software language. The models had random patient effects for IC50,1 and IC50,2 corresponding to differential effectiveness for each patient. All models were fit by maximum likelihood. There was difficulty in fitting the models, most likely due to the small...
number of drug combinations (due to the limited number of cells available from any given myelofibrosis patient sample). For this reason, we first fit a model with $\alpha = 0$, fixed $m$, and then fit the model to estimate $\alpha$. We used a likelihood ratio test to evaluate $\alpha$.

**Results**

A functional genetic screen identifies NCT as essential for survival of JAK2V617F-mutant HEL cells

Aberrant JAK/STAT pathway activation is central to the pathogenesis of myelofibrosis. However, JAK inhibitors, such as ruxolitinib, only reduce symptoms, but are not curative. To identify novel vulnerabilities in myelofibrosis, irrespective of somatic mutation status, a shRNA library screen was performed on JAK2V617F-mutant HEL cells as a model of JAK/STAT-driven myeloid neoplasia. The shRNA library screen protocol is outlined in Supplementary Fig. S1. Barcode abundance in the final population and the original library were compared by NGS. Significant depletion of shRNAs are those targeting genes with a potentially critical role for HEL cell survival. Candidate genes were prioritized based on 2 criteria: (i) reduction of barcode abundance ≥10-fold, and (ii) reduction in ≥3 shRNAs targeting the same gene. This customized algorithm identified 72 genes putatively critical to HEL cell survival and/or proliferation. NCT-related genes RAN and RANBP2 were among the top 20 candidates (Table 1; Supplementary Table S2), suggesting that HEL cells are dependent on NCT for survival and/or proliferation.

**Cell lines expressing JAK2V617F are dependent on NCT**

To validate our screen results, we transduced HEL and another JAK2V617F-mutant, SET-2 cells with 4 different (3 from library, 1 new) doxycycline-inducible shRNAs targeting RAN and confirmed knockdown at the mRNA and protein level (Fig. 1A and B). RAN knockdown was associated with a time-dependent reduction of viable cells (Fig. 1C and D), and nearly abolished colony formation (Fig. 1E and G). RAN knockdown also strongly increased apoptosis at 72 hours after doxycycline addition (Fig. 1F, H–J). We also evaluated 2 shRNAs targeting RANBP2 (Fig. 2; 1 from library, 1 new) and observed reductions in viable cells with both, albeit along a slower timeline than with RAN knockdown. HEL and SET-2 cells transduced with the empty vector (pRSIT12) did not respond to doxycycline (colony assay vs. no doxycycline: HEL 103.3%, $P = 0.56$; SET-2 101.3%, $P = 0.8245$; Supplementary Fig. S2A). These data demonstrate that MPN cells expressing JAK2V617F are dependent on NCT, confirming the results of the shRNA library screen.

To further validate dependence of JAK2V617F-mutant HEL and SET-2 cells on NCT, we used the SINE compounds KPT-330 and KPT-8602 to inhibit NCT. KPT-330 covalently binds cysteine-528 of the chromosome region maintenance 1 protein (CRM1), a key component of NCT, thereby preventing cargo binding and consequently NCT. KPT-8602 is a second-generation inhibitor of CRM1, with similar pharmacokinetics to KPT-330. Unlike KPT-330, KPT-8602 has substantially lower brain penetration, resulting in improved tolerability (31). Both inhibitors are being evaluated in clinical trials for various malignancies. KPT-330 or KPT-8602 treatment (72 hours) reduced viability of HEL and SET-2 cells in a dose-dependent fashion, with $IC_{50}$ values approximately 100 nmol/L (Fig. 3A and B). Similarly, TF1 and UT7 cells, JAK2 WT myeloid leukemia lines, were also sensitive to these SINE compounds, with $IC_{50}$ values between 30 and 98 nmol/L (Supplementary Fig. S2B), similar to other malignant, nonleukemic cell lines (36–38), demonstrating that these inhibitors function through a JAK2-independent mechanism.

JAK2V617F-expressing cells acquire resistance to ruxolitinib and other JAK1,2 inhibitors through reactivation of JAK/STAT signaling, and the same mechanism is operational in vivo (39). We generated HEL cells resistant to JAK1,2 inhibitors (HEL-R) that maintain JAK2, STAT5, and STAT3 phosphorylation despite JAK1,2 inhibition (Supplementary Fig. S3A). To determine whether NCT remains a critical target in JAK inhibitor–resistant JAK2V617F-expressing cells, we first assessed the effects of RAN knockdown on HEL-R cells and found a significant increase in apoptosis (Supplementary Fig. S3B). We next added SINE

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Abbreviation: M, metabolism (1); N, nuclear–cytoplasmic transport (2); O, other (3); P, proteasome (7); T, transcription, translation (7).
Figure 1.
Knockdown of RAN inhibits growth and survival of cells expressing JAK2V617F. HEL cells (left) and SET-2 cells (right) were engineered to express doxycycline (Dox)-inducible shRNAs specifically targeting RAN. RAN expression was assessed by qRT-PCR and immunoblot (A and B) at 72 hours after adding doxycycline. Effects of RAN knockdown were measured by MTS assay (C and D), colony formation assay (E and G), and Annexin V flow cytometry (F, H, I, and J). Data are from 3 independent experiments (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
compounds to HEL-R cells growing in ruxolitinib. While the IC$_{50}$ of ruxolitinib in HEL-R exceeded 20 μmol/L, 45-fold higher than that in the parental cells, the IC$_{50}$ for KPT-330 or KPT-8602 in HEL-R was comparable (<100 nmol/L) with the parental cells, indicating HEL-R cells remain sensitive to NCT and that this dependence extends to JAK1,2 inhibitor-resistant cells.

Inhibition of NCT selectively suppresses primary myelofibrosis over normal stem/progenitor cells

We next investigated the NCT dependence of primary myelofibrosis and normal hematopoietic stem/progenitor cells.

Figure 2.
Knockdown of RANBP2 inhibits growth and survival of cells expressing JAK2V617F. HEL cells (A and B) and SET-2 cells (C and D) were engineered to express doxycycline (Dox)-inducible shRNAs specifically targeting RANBP2. RANBP2 expression was assessed by immunoblot (A and C) at 72 hours after adding doxycycline. Effects of RANBP2 knockdown were measured by viable cell counting (B and D). Data are from 2 to 3 independent experiments (*, P < 0.01; **, P < 0.001).

Figure 3.
Inhibition of NCT by KPT-330 or KPT-8602 reduces survival of cells expressing JAK2V617F. IC$_{50}$ of ruxolitinib, KPT-330, and KPT-8602 in HEL (A) and SET-2 (B) cells following 72 hour treatment. C, IC$_{50}$ of ruxolitinib, KPT-330, and KPT-8602 in HEL-R cells following 72 hour treatment. IC$_{50}$ values were measured by MTS assay in triplicate 72 hours after addition of inhibitors and data are from 2 to 3 independent experiments.
CD34⁺ cells were isolated from patients with myelofibrosis (for clinical details, see Supplementary Table S1) or from cord blood, and treated with vehicle (DMSO), KPT-330, or KPT-8602 in medium containing cytokines. KPT-330 at 100 nmol/L and KPT-8602 at 50 and 100 nmol/L induced significant reductions in viable cells in myelofibrosis compared with cord blood, and this was associated with much stronger induction of apoptosis in myelofibrosis than in cord blood CD34⁺ cells (Fig. 4A–C). Inhibition of NCT, especially by KPT-8602, also inhibited colony formation by myelofibrosis cells, with minimal reductions in cord blood cells, demonstrating selectivity toward myelofibrosis cells (Fig. 4D).

Inhibition of NCT enhances the effect of ruxolitinib on primary myelofibrosis cells

We next performed a series of experiments to investigate the effects of combining ruxolitinib with KPT-330 or KPT-8602 in myelofibrosis patient samples with different genotypes, including patients who failed ruxolitinib (Supplementary Table S1). We initially incubated primary myelofibrosis CD34⁺ cells with graded concentrations of either ruxolitinib alone or in combination with KPT-330 (Fig. 5A) or KPT-8602 (Fig. 5B). In all 6 samples tested, inhibition of NCT enhanced the effect of ruxolitinib. We tested for synergy for each KPT inhibitor in combination with ruxolitinib with the response surface approach and formula (5) of Greco, Bravo, and Parsons (35) for 2-drug interaction. The value of α for KPT-330 was 1.265 and the value for KPT-8602 was 8.003. This analysis revealed alpha values greater than 0 for both inhibitors, indicated that KPT-330 and KPT-8602 are synergic with ruxolitinib against myelofibrosis cells. Apoptosis induced by 100 nmol/L ruxolitinib was increased by adding 50 nmol/L KPT-330 or KPT-8602, resulting in a significant difference compared with cord blood cells (Fig. 5C and D). Similarly, 1.5 μmol/L ruxolitinib alone reduced myelofibrosis colony formation by approximately 70%, compared with approximately 40% for cord blood. Single-agent KPT-330 and KPT-8602 reduced myelofibrosis colony formation by approximately 50%, and approximately 85%, respectively, compared with approximately 25% and approximately 10% for cord blood (Fig. 5E and F). KPT-330 combined with ruxolitinib reduced myelofibrosis colony formation by >80%, compared with 60% for cord blood. Profound differential effects were observed for the KPT-8602/ruxolitinib combination, which reduced cord blood colonies by approximately 70%, but abolished myelofibrosis colony formation (Fig. 5E and F; Supplementary Table S6). In each annexin V or colony assay (Fig. 5C–F), the combination treatment was more effective against the myelofibrosis cells than was the ruxolitinib treatment alone (P < 0.002 in all tests). Altogether, these data show that KPT-330 or KPT-8602 enhance the effect of ruxolitinib on myelofibrosis CD34⁺ cells, with relative preservation of normal CD34⁺ cells.

p53 is retained in the nucleus following inhibition of NCT

Several studies have analyzed the effects of SIN1 compounds on the nuclear–cytoplasmic distribution of tumor suppressor proteins and associated their nuclear retention with effects on cell growth and viability (40–44). To determine the effect of KPT-330 and KPT-8602 on major NCT cargo proteins in JAK2V617F-expressing cells, we treated HEL cells with either vehicle, KPT-330, or KPT-8602 followed by nucletocytoplasmic fractionation and immunoblot analysis for tumor suppressor proteins previously identified as NCT substrates (Fig. 6A and B; Supplementary Fig. S4A) (45). Proper fractionation was confirmed by immunoblot for lamin B (nuclear protein marker) and tubulin (cytoplasmic protein marker). NPM1, p53, and IκBα were also analyzed in myelofibrosis CD34⁺ cells treated with KPT-8602 (Fig. 6C; Supplementary Fig. S4B and S4C). Nuclear localization of p53 was consistently increased, while results for NPM1 varied by sample. IκBα was not detected in the nucleus of any of the patient samples analyzed. These data suggest that KPT-330 and KPT-8602 promote nuclear retention of p53 and certain other tumor suppressor proteins in MPN, consistent with other malignancies, with considerable variations according to sample types and inhibitors.

Inhibition of NCT induces hematologic responses in an MPN mouse model

To evaluate the role of NCT in vivo, we tested KPT-330 in a mouse model of JAK2V617F-driven MPN that resembles PV progressing to myelofibrosis (32). MPN was induced in Balb/c mice by transplanting bone marrow infected with JAK2V617F-GFP retrovirus. On day 21 posttransplant, mice were randomized to vehicles, KPT-330 (initial dose: 20 mg/kg, 3× weekly, orally), ruxolitinib (initial dose: 50 mg/kg, twice daily, orally), or ruxolitinib plus KPT-330 using 13–14 mice/group. Blood GFP⁺ cells ranged from 7%–23%, with a median of 14%–16% in each of the treatment groups at this point (Supplementary Fig. S5). Over the course of the experiment, mice in all groups experienced weight loss and lethality, including the vehicle controls, implicating the vehicles as the cause of toxicity. As a result, KPT-330 was reduced to 10 mg/kg twice weekly, followed by a reduction of ruxolitinib to 50 mg/kg daily (Supplementary Tables S7 and S8). On treatment day 15, KPT-330 significantly reduced white blood cells (WBC) and granulocytes (both P < 0.05), with a trend toward significant reduction in blood GFP⁺ cells (P = 0.12), while hematocrit was unchanged (Fig. 7A–D). The KPT-330/ruxolitinib combination significantly reduced WBCs, hematocrit, granulocytes, and blood GFP⁺ cells (all P < 0.05). On treatment day 28, the KPT-330 single-agent group showed significantly reduced spleen GFP⁺ cells compared with controls (P < 0.05), while ruxolitinib-treated mice developed resistance, as shown by increasing WBC counts and GFP⁺ cells in peripheral blood, consistent with a previous study (46). In contrast, combination treatment significantly reduced WBCs, granulocytes, spleen GFP⁺ cells, and spleen weight (P < 0.05; Fig. 7A–G). Histopathology revealed that combination treatment also partially restored splenic architecture (Fig. 7H), while bone marrow fibrosis persisted in all treatment groups (Supplementary Fig. S6A–S6C). The myeloid-erythroid ratio and overall cellularity were reduced in the KPT-330 and combination groups (Supplementary Fig. S6D and S6E). Taken together, these data suggest that NCT inhibition, alone and in combination with JAK inhibitors, can reduce disease burden, attenuate myelofibrosis features, and suppress/delay resistance to JAK inhibitors in vivo.

Discussion

Ruxolitinib, the first approved JAK kinase inhibitor, is the drug therapy standard for intermediate-2 and high-risk myelofibrosis. Ruxolitinib ameliorates splenomegaly, improves quality of life, and may prolong survival, but its utility is frequently limited by myelosuppression that necessitates dose reduction or discontinuation (20, 21). Moreover, ruxolitinib
Figure 4.
Inhibition of NCT by KPT-330 or KPT-8602 selectively inhibits growth and survival of primary myelofibrosis compared with cord blood (CB) CD34+ cells.
A, KPT-330 (left, n = 3 primary myelofibrosis samples) or KPT-8602 (right, n = 3 primary myelofibrosis samples) treatment significantly reduced viable cells of primary myelofibrosis compared with cord blood CD34+ cells (n = 3 samples) at indicated concentrations as measured in triplicate by MTS assays after 72 hours.
B and C, KPT-330 or KPT-8602 treatment significantly induces apoptosis in primary myelofibrosis over cord blood CD34+ cells (n = 3 each, 72 hours). Representative flow plots are shown (B), and bar graphs show the data from 3 independent experiments (C). D, KPT-330 (n = 7) or KPT-8602 (n = 3) at indicated concentrations inhibited colony formation of primary myelofibrosis cells, with minimal effects on cord blood CD34+ cells (n = 3). Statistical comparisons were made between myelofibrosis versus cord blood (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
does not typically reduce mutant allele burden and clonal hematopoiesis persists despite clinical responses (22). Alternative JAK1/2 inhibitors in clinical development may overcome some of ruxolitinib’s shortcomings. Pacritinib, a selective JAK2/FLT3 inhibitor with less myelosuppressive effects than ruxolitinib, was found to be superior to best available therapy in 2 phase III randomized studies that included patients with thrombocytopenia (47, 48). Deaths related to bleeding and cardiovascular events lead to temporary full clinical hold by the FDA; additional studies are underway (49). Another JAK1/2 inhibitor, momelotinib, is no longer being developed due to lack of superiority over best available therapy in phase III studies, adding to a considerable list of JAK kinase inhibitors that failed in clinical trials (33, 50–52). In contrast to ruxolitinib and other clinical candidates, CHZ868 is a type II inhibitor that binds an inactive JAK2 conformation, and a reduction of mutant allele burden was demonstrated in mouse models, but clinical development of this molecule is uncertain (53). The limited disease-modifying ability of ruxolitinib monotherapy led to numerous trials of ruxolitinib-based combinations, some of which have shown activity, for example, the combination of ruxolitinib and 5-azacitidine (54, 55). Thus far however, JAK1/2 inhibitors have not solved the clinical challenge of progressive myelofibrosis.

To identify potential therapeutic targets in myelofibrosis other than JAK/STAT pathway, we performed a lentiviral shRNA library screen on HEL cells, a myeloid cell line homozygous for JAK2V617F. Figure 5. Inhibition of NCT by KPT-330 or KPT-8602 enhances effect of ruxolitinib on primary myelofibrosis CD34+ cells. Primary myelofibrosis CD34+ cells were treated with either ruxolitinib alone or in combination with a fixed concentration of KPT-330 at 50 or 100 nmol/L (A) or KPT-8602 at 50 or 100 nmol/L (B) for 72 hours. Viable cells were quantified by MTS assay. Each plot represents 1 sample from 1 patient. When known, genotypes and treatment status are shown. Inhibition of NCT by KPT-330 at 50 nmol/L (C) or KPT-8602 at 50 nmol/L (D) increased ruxolitinib (100 nmol/L)-induced apoptosis (n = 5 each, 72 hours). Primary myelofibrosis or cord blood CD34+ cells were treated with indicated concentrations of either ruxolitinib alone or in combination with KPT-330 or KPT-8602 for 72 hours, and apoptosis was measured with Annexin V. KPT-330 at 100 nmol/L (E) or KPT-8602 at 50 nmol/L (F) enhanced ruxolitinib (1.5 μmol/L)-induced inhibition in colony formation of primary myelofibrosis versus cord blood CD34+ cells (n = 5–7). Statistical significance was compared between myelofibrosis versus cord blood (*, P < 0.05; **, P < 0.01). Purple asterisks represent combination groups that are statistically distinct (P < 0.002 for each) from the ruxolitinib group.
of primary cells, their rapid proliferation in vitro allows large fold changes to occur over a limited period of time, enabling the identification of vulnerabilities (24). To minimize false-positive results, we developed a set of stringent criteria to prioritize candidates and identified genes related to proteasome, transcription, and translation, as well as NCT as potentially critical for the survival and/or proliferation of HEL cells. Despite the strong representation of proteasome components among the top hits, this lead was not followed, because proteasome inhibitors such as bortezomib were ineffective or even associated with increased disease activity in myelofibrosis clinical trials (56, 57). Given the strong and consistent reduction of shRNAs targeting NCT-related mRNAs, we hypothesized that NCT may represent a hitherto unrecognized vulnerability in myelofibrosis amenable to targeting with pharmacologic inhibitors (SINE compounds).

In initial experiments using shRAN, we demonstrated that HEL and SET-2 cells are highly dependent on RAN and that myelofibrosis CD34+ cells are much more sensitive to NCT inhibitors than cord blood CD34+ cells. This was consistent with our previous observations that cord blood CD34+ cells are less dependent on RAN than chronic myeloid leukemia CD34+ cells and suggested a therapeutic window for targeting NCT in myelofibrosis (24). KPT-330 was equally potent against myelofibrosis cells from newly diagnosed and ruxolitinib-exposed patients, including different genotypes, suggesting that targeting NCT in myelofibrosis would have broad applicability. The difference between cord blood and myelofibrosis seemed to be even greater for the next-generation compound KPT-8602, which may be clinically relevant, given the reduced central nervous system penetration and reduced side effects of KPT-8602 (31). Although more toxicity toward normal cells was observed with combinations of KPT-330 or KPT-8602 with ruxolitinib, selectivity toward myelofibrosis versus cord blood was consistently maintained. For clinical use, it will be important to optimize dosing and carefully monitor hematologic toxicity. The efficacy of NCT inhibition was confirmed in a mouse model of JAK2V617F-induced MPN. Compared with single-agent KPT-330 or ruxolitinib, KPT-330 combined with ruxolitinib appeared to have the most profound effects, including a reduction of JAK2V617F-expressing cells in the blood (day 15) and spleen (day 28), and delayed/suppressed emergence of resistance. A limitation of these experiments is the attrition of the cohorts due to toxicity, which was identical across treatment groups, implicating the vehicles in toxicity. The loss of animals and statistical power likely accounts for the relative inconsistency over time. Future optimization in vehicles, dosing, and treatment periods will be required to conclusively assess the efficacy of KPT-330 in this MPN model. Altogether, these data show that the selective ex vivo activity of SINE compounds against myelofibrosis over normal progenitors is reproducible in vivo. Given the efficacy of NCT inhibition against JAK inhibitor–exposed cells in primary myelofibrosis samples and in vivo mouse model, evaluation of SINE compounds in relapse and ruxolitinib-exposed patients with myelofibrosis is warranted.

Export of proteins from the nucleus to the cytoplasm is an energy-dependent process that involves the formation of a multimeric complex including RAN, RANBP2, and CRM1 (58). A nuclear export signal in cargo proteins promotes formation of a trimeric complex with CRM1 and the GTP-bound form of the small GTPase RAN that shuttles nuclear cargos through the nuclear pore complex into the cytoplasm. The RAN-GTP/RAN-GDP gradient provides the energy for directional cargo transport (59). Overexpression of CRM1 correlates with poor prognosis and reduced survival in many solid and hematologic malignancies, implicating NCT as a potential cancer therapy target (60, 61). CRM1 cargos include a number of tumor suppressor proteins, including p53, NPM1, p21, p27, fororkhead box O (FOXO) transcription factors, inhibitor of b (Ib), PP2A, and survivin (reviewed by Tan and colleagues; ref. 45). As these tumor suppressor proteins require nuclear localization to exert their functions, nuclear export leads to their functional inactivation and/or proteasome-dependent cytoplasmic degradation. We observed that p53 is consistently retained in the nucleus following treatment with SINE compounds, with variable results for other tumor suppressors such as NPM1, potentially implicating the NCT of these tumor suppressors as a SINE target in myelofibrosis, similar to other malignancies (45). Differences in the cargos may be related to the genetic heterogeneity of myelofibrosis.

In summary, our results are the first to demonstrate selective activity of SINE compounds against myelofibrosis progenitors, including cells from patients who failed ruxolitinib, over normal controls. This provides a strong rationale for testing this therapeutic concept in a clinical trial.

Disclosure of Potential Conflicts of Interest

M.W. Deininger is a consultant/advisory board member for Blueprint, Pfizer Inc., and Ascenage Pharma, and is part of the study management committee for Takeda. No potential conflicts of interest were disclosed by the other authors.
Figure 7. Inhibition of NCT attenuates MPN and reduces mutant allele burden in a JAK2V617F-driven MPN mouse model. Mice with established MPN were treated with vehicles, KPT-330, ruxolitinib (RUX), and combination KPT-330 and ruxolitinib (Combination). For each of the treatments, the left data set represents the results after 14 days, and the right data set after 28 days of treatment. Gray horizontal bars represent the range from 2 healthy mice for each parameter. White blood cells (WBC; A), hematocrit (HCT; B), and granulocytes (C) were measured with a Heska HemaTrue. D, GFP-positive cells were measured in the peripheral blood. At 4 weeks (end of treatment) GFP+ cells were measured in spleen (SP; E) and bone marrow (BM; F). G, Spleen weights were determined at the end of treatment. H, H&E stains of representative spleen sections are shown. The bar represents 1 millimeter. Data were analyzed with 1-way ANOVA (Kruskal–Wallis and uncorrected Dunn test, with nonparametric analysis) in GraphPad Prism 7.02. *P < 0.05 when compared with Vehicles.
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