Preclinical and clinical efficacy of XPO1/CRM1 inhibition by the karyopherin inhibitor KPT-330 in Ph+ leukemias

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Running Title: KPT-330 in Ph+ acute leukemias

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KEY POINTS

- XPO1/CRM1 is upregulated in a BCR-ABL1 kinase-dependent and -independent manner and negatively controls PP2A tumor suppressor activity.

- The XPO1 inhibitor KPT-330 antagonizes survival of TKI-resistant Ph⁺ acute leukemias in vitro, in CML-BC animals and in a CML-AP patient.

ABSTRACT

As tyrosine kinase inhibitors (TKIs) fail to induce long-term response in blast crisis chronic myelogenous leukemia (CML-BC) and Philadelphia chromosome-positive (Ph⁺) acute lymphoblastic leukemia (ALL), novel therapies targeting leukemia-dysregulated pathways are necessary. Exportin-1 (XPO1/CRM1) regulates cell growth and differentiation by controlling the nucleocytoplasmic trafficking of proteins and RNAs, some of which are aberrantly modulated in BCR-ABL1⁺ leukemias. Using CD34⁺ progenitors from CML, B-ALL and healthy individuals, we found that XPO1 expression was markedly increased, mostly in a TKI-sensitive manner, in CML-BC and Ph⁺ B-ALL. Notably, XPO1 was also elevated in Ph⁻ B-ALL. Moreover, the clinically-relevant XPO1 inhibitor KPT-330 strongly triggered apoptosis and impaired clonogenic potential of leukemic but not normal CD34⁺ progenitors, and increased survival of BCR-ABL1⁺ mice, 50% of which remained alive and, mostly, became BCR-ABL1-negative. Moreover, KPT-330 compassionate use in a TKI-resistant CML patient undergoing disease progression significantly reduced WBC count, blast cells, splenomegaly, LDH levels, and bone pain. Mechanistically, KPT-330 altered the subcellular localization of leukemia-regulated factors including RNA-binding protein hnRNP A1 and oncogene SET thereby inducing re-activation of PP2A tumor suppressor and inhibition of BCR-ABL1 in CML-BC cells. Because XPO1 is important for leukemic cell survival, KPT-330 may represent an alternative therapy for TKI-refractory Ph⁺ leukemias.
INTRODUCTION

While the success of TKIs as first line therapy for CML in chronic phase (CML-CP) is fully justified by the BCR-ABL1 kinase-dependence of leukemic progenitors, the etiopathogenesis of Ph+ acute leukemias is still unclear\textsuperscript{1-3}. In fact, the presence of BCR-ABL1 mutations and non-random secondary genetic abnormalities can only partially explain the lack of long-term response and/or development of resistance to TKIs (including ponatinib) and other therapeutic options\textsuperscript{1,4-8}. Thus, the biological processes underlying emergence and maintenance of CML-BC and Ph+ B-cell ALL likely involve different combinations of BCR-ABL1-independent genetic or epigenetic (cell-autonomous and microenvironment-induced) molecular events in addition to BCR-ABL1 oncogene-driven mechanisms occurring in a kinase-dependent and –independent manner\textsuperscript{1,9,10}. Post-transcriptional control of gene expression (mRNA processing, stability, export and translation) plays an essential role in the emergence, maintenance and/or progression of different types of cancer including Ph+ acute leukemias\textsuperscript{1,11-15}. In these hematologic malignancies, altered expression and activity of the nucleocytoplasmic shuttling heterogeneous ribonuclear proteins (hnRNPs) results in aberrant metabolism of their mRNA cargo that in most cases encompasses oncogenes, tumor suppressor proteins (TSPs) and growth/survival- or differentiation-regulating factors\textsuperscript{11,15}. Karyopherins also function to mediate the nucleocytoplasmic exchange of proteins and RNA through nuclear pore complexes\textsuperscript{14,16-18}. Specifically, the karyopherin β family member XPO1 (exportin-1, also called CRM1) is a critical regulator of cell proliferation and survival\textsuperscript{19-22} that is overexpressed in several hematologic and non-hematologic malignancies in some of which it was described as a poor prognostic factor\textsuperscript{22-30}. Different inhibitors of XPO1-mediated export through the nuclear pore complex have been developed\textsuperscript{31}; among these, the selective inhibitors of nuclear export (SINE, Karyopharm Therapeutics Inc.) are small molecules based on leptomycin B (LMB) that irreversibly bind to Cys528 in the cargo-binding groove of XPO1 to prevent XPO1-cargo interaction\textsuperscript{22,24-26,32}. Preclinical in vitro and/or in vivo studies have shown that the closely-related SINE compounds KPT-251, KPT-276 and KPT-330 have strong anti-leukemic activity in AML, T-cell ALL, mantle-
cell lymphoma and CLL, likely through signals mediated by altered subcellular localization of p53, IκBα and/or FoxO3α²²,²⁴-²⁶,³². Notably, the SINE KPT-330 is currently in clinical trials for advanced hematologic malignancies and solid tumors (NCT01607892 and NCT01607905). Here we report that XPO1 is also overexpressed in Ph⁺ acute leukemias, and that SINE-mediated XPO1 inhibition decreases survival of leukemic but not normal CD34⁺ progenitors, thereby impairing leukemogenesis both in vitro and in an animal model of Ph⁺ acute leukemia. Mechanistically, KPT-330-induced inhibition of XPO1-mediated nuclear export not only altered subcellular localization of p53, IκBα and FoxO3α but, importantly, it directly subverted the BCR-ABL1-hnRNP A1-SET network³³ thereby restoring the activity of the protein phosphatase 2A (PP2A) tumor suppressor, an event sufficient to selectively kill CML-BC and Ph⁺ ALL blasts³⁴.
MATERIALS AND METHODS

Cell cultures and primary cells. Parental, BCR-ABL-expressing 32Dcl3 and BaF3 cells and primary CD34+ bone marrow (BM) progenitors were maintained and used in clonogenic and apoptosis assays as reported in Supplemental Methods. Frozen samples of mononuclear hematopoietic cells from bone marrow (BM) of unidentifiable CML and ALL patients were obtained from The OSU Leukemia Tissue Bank, Columbus OH; the Division of Hematology; Maisonneuve-Rosemont Hospital, Montréal QC; the Hammersmith Hospital, Imperial College, London UK; and from the Department of Hematology, Aarhus University Hospital, Denmark. BM mononuclear cells from different healthy donors (NBM) were purchased from Cincinnati Children’s Hospital or The Ohio State University. All experiments with human specimens were carried out with approval from The OSU Institutional Review Board. All experiments were conducted in accordance with the Declaration of Helsinki. Infections with the SV40 small T antigen (small-t) and BCR-ABL-expressing retroviruses in 32Dcl3 and/or Ba/F3 cells were performed as described34. 32D-BCR/ABL cells expressing the shuttling-deficient hnRNP A1 mutants have been already described35. Where indicated cells were treated with the BCR-ABL1 kinase inhibitor imatinib (Novartis); src inhibitor PP2 (Calbiochem); mTORC inhibitor rapamycin, PKC inhibitor PKC-412, and PI-3K inhibitor LY294002 (Sigma-Aldrich); Jak2 inhibitor TG101348 (SAR302503, TargeGen/Aventis), MEK1 inhibitor U0126 (Promega), or the SINE compounds KPT-330 (MW: 443.31, chemical formula: C17H11F6N7O), KPT-185 and KPT-207 (Karyopharm Therapeutics, Inc.) used at time and concentrations indicated in Results.

Western blot analysis, subcellular fractionation and PP2A assay: Whole cell lysates and subcellular fractions were subjected to SDS-PAGE followed by immunoblot with the antibodies: anti-XPO1/CRM1, anti-SET, anti-p53 and anti-p21 (Santa Cruz Biotechnology); anti-GRB2, anti-pSTAT5 Y694, anti-pAkt S473, anti-p42/44MAPK T202/Y204 (Cell Signaling Technology); anti-ABL (BD Translab); anti-HA (Covance); and anti-hnRNP A1 (Sigma-Aldrich). Densitometric analysis was
performed using Image J software (NIH). PP2A phosphatase activity was quantitated as previously described\textsuperscript{34}.

**Confocal microscopy:** Cells were cytospun onto glass-slides, Triton X-100 (0.1% vol/vol) permeabilized, incubated for 10 min with 4% normal goat serum, immunostained with the anti-hnRNP A1 (Sigma-Aldrich), anti-SET, anti-CIP2A and anti-FoxO3a (Santa Cruz Biotechnology), anti-HA (Covance), anti-PP2Ac (Millipore) or anti-I\(\kappa\)B\(\alpha\) (Cell Signaling Technology), and then incubated with AF647-conjugated rabbit or mouse secondary antibodies (Invitrogen). Confocal micrographs were taken using the FV1000 Confocal Laser Scanning Microscope (Olympus) with a PLAPONSC 60x/1.4 Oil lens. Microscope settings remained unchanged between treated and untreated slides, and images were processed using FluoView software (Olympus).

**CML-BC mouse model:** Six-week-old immunocompromised ICR-SCID mice (n=30) were intravenously-injected with 3x10\textsuperscript{5} 32D-BCR/ABL cells (n=20) or used as controls (n=10). After one week, engraftment was confirmed by \textit{BCR-ABL1} nested RT-PCR performed on total RNA from peripheral blood (PB) of cell-injected animals as described\textsuperscript{34}. Thereafter, KPT-330 (15 mg/kg in 0.6% Pluronic F-68 and 0.6% Plasdone K-29/32)\textsuperscript{25} was administered by oral gavage to leukemic (n=12) and age-matched (n=7) mice twice per week. As controls, 8 leukemic mice were left untreated and 3 age-matched SCID mice received neither cells nor treatment. At 5 weeks post-transplant, 2 mice/group were sacrificed and spleens were subjected to macroscopic and microscopic (H&E staining) evaluation of leukemic-cell infiltration. The remaining mice were used for Kaplan-Meier survival analysis. At 16 weeks post-transplant, surviving KPT-330-treated animals were sacrificed, and spleen, liver, BM and PB were isolated and subjected to histopathologic examinations together with the organs collected from untreated leukemic mice immediately post-mortem. PB samples were used for RNA isolation and PCR-mediated detection of \textit{BCR-ABL1} transcripts (at 10 and 16 weeks post-transplant), and cytospun on glass-slides for morphologic evaluation after Wright-Giemsa staining. Cytospins and tissue sections were visualized with a Zeiss Axioskope 2 Plus; Achromplan (Zeiss) lenses
used were: 40x/0.65 (PB and BM), 10x/0.25 (liver) and 4x/0.10 (spleen). Images were taken with a QICLI-FM-12 CCD camera (QIImaging) equipped with an RGB liquid crystal color filter module for capturing color images.

**Patient:** A 37-year-old male with hyper-leukocytosis and severe bone pain was diagnosed at the Gabrail Cancer Center (Canton, OH) with accelerated phase CML (CML-AP). Patient was refractory to 9 prior therapies, including different TKIs and several investigational agents. After refusing BM transplant, the patient received KPT-330 for compassionate use. The regimen scheme was: run-in dose (12 mg/m$^2$ p.o.) on day 1, 3 and 5, followed by three administrations of KPT-330 at the therapeutic dose of 16.5 mg/m$^2$ on day 1, 3 and 5 of the second week. White blood cell (WBC) count, presence of immature cells in PB smears, serum levels of lactate dehydrogenase (LDH), and splenomegaly were monitored. One-week after KPT-330 administration, the patient declined dose escalation.

**Statistic Analysis:** One-tailed unpaired Student’s $t$ test was used for the *in vitro* studies, except colony forming assays that were analyzed by one-tailed paired Student’s $t$ test. EC$_{50}$ values were calculated by nonlinear regression curves fit using the Prism software (GraphPad). Log-rank (Mantel-Cox) test was used for evaluating differences in survival of treated vs. untreated mice. $P <0.05$ was considered statistically significant.
RESULTS

XPO1 expression is enhanced in Ph+ acute leukemia (CML-BC and B-ALL) progenitors.

Leukemic transformation of 32Dcl3 myeloid precursors by forced p210 BCR-ABL1 expression results in a dramatic induction of XPO1 protein levels (Figure 1A). Similarly, Western blot analysis showed that XPO1 expression was markedly higher in CML-CP (n=3) than NBM (n=7) CD34+ BM progenitors (Figure 1A), suggesting that BCR-ABL1, which drives CML-CP emergence and maintenance\(^1\), is responsible for increased XPO1 expression. Accordingly, CD34+ CML-BC (n=7) and CD34+/CD19+ t(9;22)(q34;q11) B-ALL (n=5) progenitors, both expressing BCR-ABL1 at levels higher than CML-CP\(^1\), also present significantly higher XPO1 expression (Figure 1A). However, XPO1 levels were further increased in Ph+ B-ALL (n=4), suggesting that BCR-ABL1 kinase-dependent and -independent molecular mechanisms may cooperate to increase XPO1 expression also in Ph+ acute leukemias. Indeed, suppression of BCR-ABL1 kinase activity by imatinib (1 µM, 72 hours) (Figure 1B right) only partially decreased XPO1 expression, which remained at levels significantly higher in CD34+ CML-BC (n=3) and CD34+/CD19+ Ph+ ALL (n=3) than in CD34+ NBM cells (n=3) (Figure 1B left). XPO1 protein expression was also significantly downmodulated upon LY294002 and PKC412 but not U0126, PP2 and TG101348 treatment (Figure 1C, left). Similarly, \textit{XPO1} mRNA levels were decreased upon BCR-ABL1 and PI-3K inhibition and further reduced upon inhibition of PKC and non-related kinases (e.g. c-Kit) by PKC-412 treatment (Figure 1C, right). Thus, BCR-ABL1 might induce XPO1 transcription through modulation of PI-3K/Akt- but not MAPK-, Src-, Jak2- and S6K-mediated signals. Furthermore, the marked effect of PKC-412 indicates that conventional PKCs or other PKC-412-sensitive kinases might be involved in the BCR-ABL1 kinase-independent regulation of XPO1 expression.

XPO1 is important for Ph+ leukemia cell survival: \textit{in vitro} KPT-330 anti-leukemic activity.

To determine the importance of XPO1 in BCR-ABL1 leukemogenesis, the clinically relevant SINE, KPT-330, was used to specifically inhibit XPO1 activity. CD34+ BM progenitors from
CML-CP (n=3) and CML-BC (n=4) patients, CD34+/CD19+ progenitors from Ph+ (n=3) and Ph- ALL (n=3) patients, and NBM CD34+ progenitors (n=4) were treated with KPT-330 (0-8 µM, 72 hours) and assayed for drug-induced apoptosis. Annexin-V/7AAD staining revealed that KPT-330 triggered apoptosis in CD34+ leukemic (CML-BC) and normal (NBM) hematopoietic progenitors with an EC50 equal to 0.39 µM and 1.23 µM, respectively (Figure 2A). Notably, KPT-330 (1 µM) treatment induced apoptosis in ~75% CML-CP and ~95% CML-BC CD34+ BM cells, with no significant induction of apoptosis in KPT-330-treated CD34+ NBM samples (Figure 2B). Similarly, KPT-330 treatment (0.5-1µM) of CD34+/CD19+ leukemic blasts isolated from B-ALL patients also induced apoptosis in ~85% of Ph+ and nearly 95% of Ph- leukemic progenitors (Figure 2B).

Accordingly, the cytokine-dependent colony forming ability of CML (CP and BC) CD34+ progenitors was severely impaired in a dose-dependent manner by exposure to KPT-330. In fact, KPT-330 treatment (0.5-1 µM) significantly reduced the clonogenic potential of CML-CP by ~45% (0.5 µM) and ~95% (1 µM), and that of CML-BC by ~65% (0.5 µM) and ~100% (1 µM), with less substantial effects on the clonogenic potential of progenitors isolated from healthy donors, which was reduced 3% (0.5 µM) and 35%-40% (1 µM) by KPT-330 treatment (Figure 2C). Notably, detrimental effects on survival of BCR-ABL1+ cells were also observed upon treatment of 32D-BCR/ABL (p210) and Ba/F3-BCR/ABL (p190) with two other SINE compounds, KPT-207 (150nM) and KPT-185 (150nM) (Supplemental Figure 1A), which are structurally similar to KPT-330, but with poor pharmacokinetic properties making them unsuitable for in vivo use22,32. This impaired survival induced by SINEs may not only result from inhibition of XPO1 activity but also from their reported negative effect on XPO1 expression36,37.

Indeed, XPO1 levels were found decreased in KPT-330-treated CML (CP and BC) and Ph+ ALL CD34+ progenitors (Figure 2A). Interestingly, 24 hour treatment with KPT-330 and imatinib (1 µM each) markedly potentiated apoptosis of IL-3-cultured BCR-ABL1+ cells, which was nearly 95% compared to the ~30% and ~50% observed in imatinib- and KPT-330-treated IL-3-cultured cells, respectively (Figure 2D), suggesting that KPT-330 inhibits both XPO1-mediated BCR-
ABL1-dependent and –independent leukemogenic signals. This is also supported by the evidence that KPT-330 specifically binds only XPO1, and interacts but not inhibits Aurora A and p70S6 kinases at 10 μM concentration in functional LANCE® TR-FRET assays (data not shown).

**Molecular mechanisms of KPT-330 anti-leukemic activity in BCR-ABL1+ cells.**

Because XPO1 is predicted to target hnRNP A138, and has been shown to directly interact with both BCR-ABL139 and the PP2A inhibitors SET40 and CIP2A41, we sought to investigate whether KPT-330-induced apoptosis of Ph+ leukemic blasts depends, at least in part, on interference with the BCR-ABL1/Jak2-induced hnRNP A1/SET pathway responsible for inhibition of PP2A tumor suppressor activity in Ph+ acute leukemias15,33,34 (Figure 3). Confocal microscopy and Western blot of subcellular fractionated lysates revealed that treatment of 32D-BCR/ABL cells with KPT-330 (1µM, 12h) sequestered the SET and CIP2A proteins in the nucleus, without altering the subcellular localization of PP2Ac (Figure 3A-B). Similarly, KPT-330 treatment also resulted in altered subcellular localization of hnRNP A1 that, surprisingly, accumulated in the cytoplasm rather than in the nucleus of 32D-BCR/ABL cells (Figure 3A-B). Interestingly, localization of HA-tagged shuttling-deficient hnRNP A1 mutants35 carrying either the G274A mutation in the nuclear import/export M9 domain (hnRNP A1-G274A-HA), which makes hnRNP A1 protein cytoplasmic, or a bipartite-basic-type nuclear localization signal (NLS) together with the G274A mutation, which has a defined nuclear localization, was not altered upon inhibition of XPO1 activity by treatment with KPT-330 (Supplemental Figure 2), suggesting that the integrity of the M9 domain is important for the effect of XPO1 on both hnRNP A1 localization and nuclear export of hnRNP A1 mRNA cargo. Moreover, consistent with the effect of KPT-SINE compounds in other hematologic malignancies22,24-30,32, confocal microscopy (Figure 3A) and subcellular fractionation (Figure 3C) analyses revealed that KPT-330-treatment (1 µM, 72 hours) induced a nuclear accumulation of IkBα, FoxO3a and p53, and enhanced levels of the p53-target p21. Conversely, we did not observe a nuclear accumulation of either p210 or p190 BCR-
ABL1 (not shown); however, both p190 and p210 BCR-ABL1 decreased in amount and activity in KPT-330-treated (1 µM, 72 hours) CD34+ CML-BC (n=3) and CD34+/CD19+ B-ALL (n=3) progenitors (Figure 4A), likely due to the nuclear accumulation of SET and CIP2A, and subsequent restoration of PP2A activity as was observed in KPT-330-treated (250 nM, 48 hours) 32D-BCR/ABL cells, which presented levels of active PP2A similar to parental 32Dcl3 cells (Figure 4B).

To determine the relative contribution of PP2A in mediating the effects of KPT-330 on BCR-ABL1 activity/expression and survival of CML-BC progenitors, PP2A activity was constitutively suppressed in 32D-BCR/ABL cells upon expression of the SV40 small T antigen (small-t). Annexin V/7AAD-staining revealed that KPT-330 (1µM, 36h) induced apoptosis in ~60% and ~35% of parental and small-t-expressing 32D-BCR/ABL cells, respectively (Figure 4C, left), indicating that ~50% of KPT-330-induced apoptosis is mediated by activation of PP2A. Accordingly, small-t expression prevented the KPT-330-induced downregulation of BCR-ABL1 expression and activity (Figure 4C, right). Consistent with the existence of a KPT-330-induced PP2A-dependent post-translational control of BCR-ABL1 activity/expression, BCR-ABL1 mRNA levels were not significantly changed in KPT-330 (1µM, 36h)-treated cells compared to untreated and imatinib (1µM, 36h)-treated 32D-BCR/ABL cells (Figure 4D). Nonetheless, short exposure to KPT-330 (1µM, 16h) did not reduced BCR-ABL1 activity while it impaired that of STAT5, Akt and p42/44 MAPKs (Figure 4E), suggesting that the pro-apoptotic activity of KPT-330 does not totally rely on inhibition of BCR-ABL1 kinase activity.

Similar to KPT-330, treatment with KPT-185 and/or KPT-207 also resulted in altered expression of the BCR-ABL1 downstream effectors hnRNP A1, hnRNP E2 and hnRNP K, inactivation and downregulation of BCR-ABL1 itself, activation of PP2A, and downregulation of the BCR-ABL1- and hnRNP K-target c-Myc, likely as a result of both hnRNP K downregulation and PP2A activation (Supplemental Figure 1). Thus, KPT-330 triggers apoptosis of BCR-ABL1-expressing cells by activating TSPs like PP2A and p53, and inhibiting the activity of oncogenes and proliferation/survival factors (e.g. BCR-ABL1, Akt, STAT5, MAPK, c-Myc, NF-kB) (Figure 3D).
KPT-330 exerts strong anti-leukemic activity in a CML-BC mouse model.

To evaluate the therapeutic relevance of KPT-330 in Ph+ acute leukemias in vivo, we employed an allograft model of CML-BC in which ICR-SCID mice were intravenously injected with 32D/BCR-ABL cells. After one week, nested RT-PCR-mediated detection of BCR-ABL1 transcripts in PB of cell-injected animals (n=20) confirmed engraftment of BCR-ABL1-expressing cells (Figure 5A). RNA extracted from a 1:10^6 mixture of BCR-ABL1+ vs. parental 32Dcl3 cells, and from PB of age-matched mice was used as positive and negative controls, respectively (Figure 5A). Treatment with KPT-330 (15mg/kg; twice per week by oral gavage) was thereafter started in leukemic mice (n=12). As controls, a group of leukemic mice (n=8) received vehicle only whereas a group of healthy animals (n=7) received KPT-330 at the same regimen. When half of the vehicle-treated mice died (5 weeks post-cell injection), two moribund untreated, two KPT-330-treated, and one age-matched healthy animals were sacrificed and evaluated for signs of leukemia. While vehicle-treated animals displayed massive splenomegaly (Figure 5B) due to marked infiltration of immature myeloid cells (see also Figure 5D), gross (Figure 5B) and histopathologic examination (not shown) of spleens from KPT-330-treated mice appeared similar to that of the age-matched animal (Figure 5B). Untreated leukemic mice all died with a median survival time of 5 weeks whereas 50% of KPT-330-treated animals died of leukemia mostly between week 8 and 11 post cell-injection (Figure 5C). At week 10 and 16 post-cell injection, nested RT-PCR on PB revealed that 3 of the living KPT-330-treated leukemic mice were negative for BCR-ABL1 (Figure 5A). Because after 15 weeks of drug treatment (16 weeks post cell-injection), 50% of the treated mice were still alive (Figure 5C) with no external signs of disease, all surviving KPT-330-treated animals (cell-injected and control drug-treated mice) and two age-matched healthy mice were sacrificed, and hematopoietic and non-hematopoietic organs were macro- and microscopically inspected for signs of leukemia. Gross examination revealed no differences between the two KPT-330-treated groups and age-matched mice, including absence of splenomegaly in drug-treated cell-injected mice (Figure 5B bottom panel). Furthermore, Wright-Giemsa-stained PB cytopsins and H&E-stained sections of
BM, spleen and liver from KPT-330-treated (16 weeks) leukemic animals showed organ architecture and cellularity similar to those of drug-treated or age matched control animals with no infiltration of leukemic blasts, which were readily detectable in PB and organs of untreated leukemic animals (Figure 5D). Overall, KPT-330 treatment significantly increased survival ($P=0.002$) of leukemic mice by antagonizing disease evolution, and, as expected without inducing either toxicity or abnormal behavior in non-leukemic KPT-330-treated animals, which were all alive at the end of the study (Figure 5C).

**Anti-leukemic activity of KPT-330 in a TKI-resistant CML-AP patient.**

A 37 year-old male with CML-AP, hyperleukocytosis (WBC count >300x10$^3$/µL), splenomegaly (13 cm below costal margin, BCM) and bone pain, whose disease was refractory to all available TKIs (including ponatinib) and to interferon, omacetaxine and azacitidine, received run-in dose of KPT-330 (12 mg/m$^2$) for one week and, thereafter, three oral therapeutic doses of KPT-330 (16.5 mg/m$^2$) on a compassionate use protocol after refusing BM transplant. After the first therapeutically effective dose (Table 1), the patient experienced a significant reduction in bone pain, splenomegaly (from 13 cm to 4 cm BCM), WBC count (from 3x10$^5$/µL to 7x10$^3$/µL) and serum LDH (from 513 to 264 IU/L). Blood smears revealed a dramatic reduction, albeit not a complete disappearance, of immature myeloid blasts. Following one week from the third KPT-330 dose, WBC (37x10$^3$/µL) and LDH (640 IU/L) increased. At this time the patient declined to undergo KPT-330 dose-escalation. Thus, the therapeutic efficacy of higher KPT-330 doses in this patient remains unknown, although the effect of KPT-330 dose-escalation is currently being evaluated in other patients.
DISCUSSION

Despite BCR-ABL1 playing a pivotal role in the emergence and maintenance of CML-BC and Ph⁺ B-ALL, the genetic heterogeneity of these malignancies may account for the lack of long-term response to TKI-based therapies. In fact, high levels of BCR-ABL1 expression/activity in Ph⁺ acute leukemias aberrantly enhances genomic instability that, together with unfaithful DNA repair machinery, leads to an accumulation of molecular and chromosomal abnormalities, some of which result in the inactivation of tumor suppressors and/or activation of oncogenic factors acting in a BCR-ABL1-independent fashion. Thus, a successful therapy of Ph⁺ acute leukemias has to rely on the simultaneous targeting of BCR-ABL1 and other pathways playing an important role in the survival of leukemic stem/progenitor cells.

Indeed, we showed that BCR-ABL1 kinase-dependent and -independent signals, likely involving PI-3K and PKCs, contribute to enhance transcription of the nuclear export regulator XPO1 that, in turn, controls the survival of Ph⁺ acute leukemia progenitors. Although this is not surprising given the involvement of XPO1 in several malignancies and its pleiotropic activity on many cell functions, the availability of specific inhibitors of XPO1 (e.g. KPT-330) with an acceptable safety profile make XPO1 an attractive target for the therapy of these unmanageable leukemias. In fact, KPT-330, a small molecule already in Phase I clinical trials for solid tumors and hematologic malignancies, not only efficiently and selectively impaired the leukemogenic potential of CML-BC and Ph⁺ B-ALL BM progenitors and/or BCR-ABL1⁺ cell lines in both ex vivo and in an animal model, but also markedly reduced disease burden (e.g. WBC counts and splenomegaly) in a CML-AP patient. Notably, the KPT-330 concentrations used in all the ex vivo and in vivo studies were achieved in the current Phase I trials with a dose of 30 mg/m², which is below the maximum tolerated dose.

This, together with the evidence showing that the increased survival of long-term (15 weeks) KPT-330-treated leukemic animals, which was associated with the absence of BCR-ABL1⁺ circulating cells in 60% of treated mice, suggests that KPT-330 may safely be used in those Ph⁺ acute leukemia patients who become refractory to TKIs-based therapies. Moreover, we also...
provide evidence that KPT-330 potentiates the effect of imatinib, suggesting that KPT-330 might
be used in combination with TKIs especially in those cases in which TKI resistance is due to
BCR-ABL1 amplification and blastic transformation; in fact, the first XPO1 inhibitor
discovered LMB, which shares the same XPO1-interacting surface with the SINEs but due to its
lack of specificity is severely toxic when used in cancer patients, was elegantly shown to
induce apoptosis of BCR-ABL1+ cells when used in combination with imatinib through a
mechanism involving the LMB-induced nuclear-entrapment and inactivation/degradation of
BCR-ABL1. In KPT-330-treated BCR-ABL1+ cells, although we did not observe a BCR-ABL1
nuclear relocation (not shown), BCR-ABL1 expression/activity was markedly reduced, likely
through post-translational and not transcriptional mechanisms, in primary CD34+ CML-BC and
Ph+ B-ALL BM cells, suggesting that other XPO1 targets might account for BCR-ABL1
downregulation. Similar effects on other oncogenic tyrosine kinases including Flt3 and BTK
have been reported in other hematologic malignancies.
Among the numerous XPO1 targets there are the endogenous negative regulators of PP2A,
SET and CIP2A, which were described as BCR-ABL1-regulated factors active in Ph+ leukemias
(Figure 3D). Here we reported that KPT-330 treatment led to nuclear accumulation of SET and CIP2A proteins and, unexpectedly, of cytoplasmic relocation of the
nucleocytoplasmic shuttling RNA binding protein hnRNP A1. Notably, hnRNP A1 is an important
regulator of BCR-ABL1 leukemogenesis and controls SET mRNA nuclear export (Figure
3). Specifically, hnRNP A1 expression and nuclear export activity is enhanced in a dose- and
kinase-dependent manner by BCR-ABL1 that, in turn, positively regulates the nuclear export of
hnRNP A1 target mRNAs (e.g. SET, E2F3, and Bcl-XL), and subsequently their protein
levels. Because KPT-330 counteracts the RanGTP-mediated XPO1 transport of its
cargo out of the nucleus and not vice versa, it is unlikely that hnRNP A1 is directly exported by XPO1 even if in silico analysis predicts hnRNP A1 to be an XPO1 target.
Nonetheless, we cannot exclude the possibility that KPT-330 may interfere with the hnRNP A1
M9-mediated nuclear export/import that is facilitated by transportin 1 (importin β2), a protein

similar to the XPO1 targets importin α1, α3 and α719,44,50. Indeed, we provided evidence that shuttling-deficient M9 mutant hnRNP A1 proteins35 are insensitive to KPT-330-mediated XPO1 inhibition.

Consistent with the effect of SET shRNA and with that of PADs (PP2A Activating drugs) that prevent SET-PP2A interaction32,51, the reduction of cytoplasmic SET and, likely, CIP2A41 levels induced not only by KPT-330 but also by the other structurally-related SINEs (KPT-185 and KPT-207) resulted in reactivation of the PP2A tumor suppressor in BCR-ABL1+ myeloid precursors. Supporting the possibility that PP2A may play an important role as a mediator of KPT-330 anti-leukemic activity in Ph+ acute malignancies stands also the evidence gained by using BCR-ABL1+ cells engineered to express the PP2A inhibitor small-t antigen. In fact, it appears that PP2A accounts for roughly 50% of the KPT-330-induced apoptosis. Importantly, rescue of PP2A activity in primary CML-BC and Ph+ B-ALL progenitors as well as in animal models of TKI-sensitive and-resistant Ph+ acute leukemias, is an event that, per se, is sufficient to catalyze a cascade of anti-mitotic and pro-apoptotic signals (including the SHP-1-mediated inactivation and proteasome-dependent degradation of BCR-ABL1) leading to killing of leukemic but not normal hematopoietic stem/progenitor cells1,10,51. Thus, it is not surprising that KPT-330 and other SINEs induced a marked PP2A-dependent (small-t sensitive) BCR-ABL1 inactivation/downregulation that, in turn, might also be responsible for the observed downregulation of hnRNP K and its target c-Myc, and of the differentiation-inhibitory factor hnRNP E215.

Although important, KPT-330-induced PP2A reactivation is likely, not the only mechanism responsible for apoptosis of drug-treated Ph+ acute leukemic progenitors; in fact, KPT-330 treatment of BCR-ABL1+ myeloid precursors induced nuclear accumulation of the p53 tumor suppressor and its transcriptional target p21, the ROS detoxifier and antagonist of cell cycle progression FoxO3a, and the negative NF-κB regulator IκBα, in agreement with the effect of SINEs on these XPO1 targets in other myeloid and lymphoid malignancies22,26,28,32,52. Likewise, it seems that KPT-330 negatively also regulates the activity of BCR-ABL1 downstream effectors
like STAT5, Akt and MAPK independently from downregulation of BCR-ABL1 kinase activity. Note that, binding and kinase assays exclude a direct effect of KPT-330 on these molecules and other factors regulating cell proliferation and survival (not shown). Although further data on KPT-330’s effectiveness in metastatic solid tumors and advanced hematological malignancies as well as its safety, tolerability and adverse effects will be soon be available through the two Phase I clinical trials, our preclinical and preliminary clinical data strongly support the immediate evaluation of this drug in CML-BC and Ph⁺ B-ALL patients refractory to the available TKI-based therapies.

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REFERENCES


### Table 1: Patient response to KPT-330

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<th>CML-AP</th>
<th>Pre-treatment</th>
<th>KPT-330 (16.5 mg/m²)</th>
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<tr>
<td>White blood cell count (Cells/µL)</td>
<td>&gt;300,000</td>
<td>7,000</td>
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<td>Spleen size (cm below costal margin)</td>
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Figure Legends:

**Figure 1. XPO1 expression is enhanced in Ph* acute leukemia (CML-BC and B-ALL) progenitors.** (A) Top Right Panel: XPO1 and BCR-ABL1 protein levels in BCR-ABL1- or empty vector-transduced 32Dcl3 myeloid cells were determined by immunoblot. Left Panel: Protein levels of XPO1 expressed as mean ± SEM of densitometric units after normalization with Grb2 levels, were determined by immunoblot of NBM (n=7), CML-CP (n=3) and CML-BC (n=7) CD34+ progenitors, and Ph+ B-ALL (n=5) and Ph− B-ALL (n=4) CD34+/CD19+ progenitors. Bottom Right Panel: Sample of immunoblots used to determine XPO1 protein levels used for quantification. (B) Left Panel: XPO1 protein levels expressed as mean ± SEM in vehicle- or imatinib-treated (1µM, 12h) CML-BC CD34+ cells and Ph+ B-ALL CD34+/CD19+ cells. Right Panel: Representative immunoblot of XPO1 protein levels and BCR-ABL1 activity (anti-PY) in vehicle- or imatinib-treated (1µM, 12h) Ph+ B-ALL CD34+/CD19+ (lanes 1 and 2) and CML-BC CD34+ (lanes 3 and 4) cells. (C) Left Panel: XPO1 protein levels in 32D-BCR/ABL cells treated (24h) with the indicated kinase inhibitors. Right Panel: XPO1 mRNA levels assessed by qRT-PCR in 32Dcl3 and untreated and kinase inhibitor-treated (24h) 32D-BCR/ABL cells. Asterisks indicate P values versus NBM; *P<0.05, **P<0.01.

**Figure 2. KPT-330 decreased survival and clonogenic potential in CML-BC and Ph* B-ALL cells.** (A) Top Panel: Representative (n=3) Western blot showing XPO1 protein levels in vehicle- or KPT-330 treated (1µM, 72h) CD34+ CML-CP and CML-BC, and CD34+/CD19+ Ph+ ALL progenitor cells. Numbers above the blots indicate relative densitometric units. Bottom Panel: Graph shows percentage of apoptosis (Annexin V+) in vehicle- or KPT-330-treated (0-8 µM, 72h) NBM–and CML-BC CD34+ cells. EC50 was calculated as described in Methods. (B) Graph shows percentage of Annexin V+ cells (mean ± SEM) in vehicle- and KPT-330 (0.5-1 µM, 72h)-treated NBM (n=3), CML-CP (n=3) and CML-BC (n=3) CD34+ BM cells, and CD34+/CD19+ Ph+ B-ALL (n=3) and Ph− B-ALL (n=3) BM cells. (C) Colony forming ability of vehicle- or KPT-330-treated (0.5-1 µM, 72h) NBM (n=3), CML-CP (n=3), and CML-BC (n=3) CD34+ BM cells. Clonogenic potential shown as mean ± SEM was normalized to the respective untreated
sample. (D) Annexin V\(^*\) cells (mean ± SEM) in 32D-BCR/ABL cells treated with KPT-330 (1 µM, 24h) and imatinib (1 µM, 24h) used alone or in combination. Significance was determined using \(t\) test of three identical experiments. Asterisks indicate \(P\) values versus NBM; *\(P<0.05\), **\(P<0.01\) ***\(P<0.001\).

**Figure 3. KPT-330 treatment alters the subcellular localization of tumor suppressors and negative regulators of PP2A.** (A) Single channel and merged confocal micrographs of 32D-BCR/ABL cells treated with vehicle or KPT-330 (1µM, 12h) and stained with anti-SET, anti-PP2Ac, anti-hnRNP A1, anti-CIP2A, anti-IkB\(\alpha\) or anti-FoxO3a antibody (left panels; green or red), DAPI (middle; blue) and merged (right). SET and hnRNP A1 (B), and p21 and p53 (C) protein levels in nuclear and cytoplasmic subcellular fractionated extracts from vehicle- and KPT-330-treated (1µM, 12h) 32D-BCR/ABL cells. Histone H1 and Grb2 levels were used as control for purity of nuclear and cytoplasmic fractions, respectively. (D): KPT-330 mediated XPO1 inhibition abrogates leukemogenesis by altering nuclear/cytoplasmic shuttling. In Ph\(^+\) acute leukemia progenitors, XPO1 expression is increased at least in part through a BCR-ABL1 kinase-dependent mechanism, and is responsible for nuclear export of the SET oncogene and CIP2A, and for the nucleocytoplasmic shuttling activity of hnRNP A1, a regulator of SET mRNA metabolism. SET and CIP2A are BCR-ABL1/Jak2 and BCR-ABL1, respectively, -regulated inhibitors of the PP2A tumor suppressor. In these cells, XPO1 activity also controls the subcellular localization of important regulators of cell survival as p53, p21, IkB\(\alpha\), and FoxO3a. Bottom Panel: Upon inhibition of XPO1 activity with the SINE KPT-330, the SET and CIP2A proteins are sequestered in the nucleus, which leads to activation of PP2A that, in turn, triggers inhibition/degradation of BCR-ABL1 contributing to cell death. Cytoplasmic accumulation of hnRNP A1 also contributes to decrease SET levels. In addition, nuclear accumulation of p53, p21, IkB\(\alpha\), and FoxO3a also likely contribute to impair leukemogenesis of Ph\(^+\) acute leukemia progenitors.
**Figure 4.** KPT-330 treatment increases PP2A activity and downregulates BCR-ABL1 expression and activity. (A) Representative (n=3) Western blot showing BCR-ABL1 activity (anti-PY) and expression (anti-ABL) in vehicle- and KPT-330-treated CD34⁺ CML-BC cells and CD34⁺/CD19⁺ Ph⁺ ALL cells. (B): PP2A activity in 32Dc13 (positive control), vehicle- and KPT-330-treated (250 nM; 48h) 32D-BCR/ABL cells. PP2A activity was normalized to 32Dc13 cells. (C) Left Panel: Graph shows percentage of apoptotic cells (Annexin V⁺) in vehicle- or KPT-330-treated (1 µM, 36h) parental and small-t-expressing 32D-BCR/ABL cells. Significance was determined using t test of three identical experiments. Asterisks indicate P values versus untreated; *P<0.05. Right Panel: BCR-ABL1 activity (anti-PY) and expression (anti-ABL) in vehicle and KPT-330 (1µM, 24h)-treated parental and small-t-expressing 32D-BCR/ABL cells. (D) qRT-PCR shows BCR-ABL1 mRNA levels in untreated, imatinib (1µM, 24h) and KPT-330 (1µM, 24h)-treated 32D-BCR/ABL1 cells. (E) Western blots shows effect of KPT-330 (1µM, 16h) on the activity of BCR-ABL1 (anti-PY); STAT5 (anti-pSTAT5\textsuperscript{Y694}); Akt (anti-pAkt\textsuperscript{S473}), and p42/44 MAPK (anti-pMAPK\textsuperscript{T202/Y204}). HSP90 was used as control for equal loading.

**Figure 5.** KPT-330 treatment increases survival of leukemic mice. (A) Nested RT-PCR for BCR-ABL1 mRNA in the peripheral blood (PB) measured one, ten and sixteen weeks after injection. PB of age-matched mice (NC) and a 1:10\textsuperscript{6} dilution of 32D-BCR/ABL cells with 32Dc13 cells were used as negative and positive controls, respectively. GAPDH mRNA levels were used as a control. (B) Gross anatomy of spleens isolated from vehicle- and KPT-330-treated (15mg/kg, 2x weekly) mice injected with 32D-BCR/ABL cells, age-matched controls and/or KPT-330 only-treated mice at 5 (top panel) and 16 (bottom panel) weeks after cell-injection. (C) Kaplan-Meier curve shows effect of KPT-330-treatment (15mg/kg, 2x weekly) on survival of SCID mice injected with 32D-BCR/ABL cells (n=10, red line). Untreated mice injected with cells (n=8, blue line) or KPT-330-treated mice that did not receive cells (n=7, green line) were used as controls. Survival was calculated by the Kaplan-Meier method, and the log-rank test evaluated the differences among survival distributions: P=0.002 (32D-BCR/ABL untreated vs. KPT-330-treated).
32D-BCR/ABL KPT-330-treated mice). (D) Wright/Giemsa staining of PB and H&E staining of sections from bone marrow (BM), spleen, and liver of untreated and KPT-330-treated control and cell-injected mice.
Preclinical and clinical efficacy of XPO1/CRM1 inhibition by the karyopherin inhibitor KPT-330 in Ph+ leukemias

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