Combinatorial targeting of XPO1 and FLT3 exerts synergistic anti-leukemia effects through induction of differentiation and apoptosis in *FLT3*-mutated acute myeloid leukemias: from concept to clinical trial

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ABSTRACT

Targeted therapies against *FLT3*-mutated acute myeloid leukemias have shown limited clinical efficacy primarily because of the acquisition of secondary mutations in *FLT3* and persistent activation of downstream pro-survival pathways such as MEK/ERK, PI3K/AKT, and STAT5. Activation of these additional kinases may also result in phosphorylation of tumor suppressor proteins promoting their nuclear export. Thus, co-targeting nuclear export proteins (e.g., XPO1) and FLT3 concomitantly may be therapeutically effective. Here we report on the combinatorial inhibition of XPO1 using selinexor and FLT3 using sorafenib. Selinexor exerted marked cell killing of human and murine *FLT3*-mutant acute myeloid leukemia cells, including those harboring internal tandem duplication and/or tyrosine kinase domain point mutations. Interestingly, selinexor treatment of murine *FLT3*-mutant acute myeloid leukemia cells activated FLT3 and its downstream MAPK or AKT signaling pathways. When combined with sorafenib, selinexor triggered marked synergistic pro-apoptotic effects. This was preceded by elevated nuclear levels of ERK, AKT, NFκB, and FOXO3a. Five days of *in vitro* combination treatment using low doses (i.e., 5 to 10 nM) of each agent promoted early myeloid differentiation of MOLM13 and MOLM14 cells without noticeable cell killing. The combinatorial therapy demonstrated profound *in vivo* anti-leukemia efficacy in a human *FLT3*-mutated xenograft model. In an ongoing phase IB clinical trial the selinexor/sorafenib combination induced complete/partial remissions in six of 14 patients with refractory acute myeloid leukemia, who had received a median of three prior therapies (ClinicalTrials.gov: NCT02530476). These results provide pre-clinical and clinical evidence for an effective combinatorial treatment strategy targeting XPO1 and FLT3 in *FLT3*-mutated acute myeloid leukemias.

Introduction

Acute myeloid leukemia (AML) is a molecularly heterogeneous hematologic disease defined by the accumulation of immature myeloid cells in blood and bone marrow, which results from a dysregulation of normal proliferation, differentiation, and apoptosis in these cells.[1] Mutations of the fms-like tyrosine kinase-3 (*FLT3*) gene, including internal tandem duplication (ITD) and the tyrosine kinase domain (TKD) mutations, are common in patients with AML with approximately one-third of newly diagnosed AML patients carrying these mutations. These gain-of-function

mutations constitutively activate FLT3 and its downstream effectors MEK/ERK, PI3K/AKT, and STAT5 by phosphorylation. The latter effectors also activate target genes such as p21, p53, and cyclin D1.[2,3] Thus, aberrant FLT3-mediated activation of effector proteins leads to uncontrolled proliferation, inhibition of differentiation and reduction of apoptosis in transformed hematopoietic blasts, and is also associated with poor prognosis in AML.[4]

Targeted therapies against FLT3 in *FLT3*-mutated AML using small molecule inhibitors such as sorafenib, quizartinib, midostaurin, crenolenib, and gilteritinib have shown clinical activity by reducing circulating leukemic blasts, and achieving temporary remission. However, these effects are apparently ineffective against leukemic stem cells in the bone marrow microenvironment and therefore the basis for temporary remission.[5-10] In fact, we have reported marked upregulation of MAPK signaling following treatment with FLT3 inhibitors in AML/stroma co-culture, hypoxia, and in clinical samples *ex vivo*; this upregulation could be partially overcome by the novel dual *FLT3*-ITD/MAPK inhibitor E6201.[11] However, recent studies indicate that mutations of FLT3 are late events in leukemogenesis, suggesting that they are acquired rather than founder mutations in leukemia-initiating cells.[12] Targeting FLT3 alone is, therefore, unlikely to be sufficient to eradicate leukemia-initiating cells.

Recently, exportin 1 (XPO1), also known as the nuclear export protein (CRM1), has been identified.[13] XPO1 is a nuclear receptor involved in the active transport of a large number of cargo proteins, including Foxo3A, p53, p21 and NPM1, across the nuclear membrane[14] along with microRNAs.[15,16] XPO1 overexpression is common in hematologic malignancies including AML, and it was reported by us to be associated with poor disease prognosis.[17]

Leukemic cells depend on the continuous nuclear export of one or more oncoproteins, and the removal of tumor suppressor proteins, which require nuclear localization for their functions.[18] Targeting nuclear membrane proteins such as XPO1 could, therefore, restore tumor suppressor function in AML. The small molecule XPO1 inhibitor, selinexor (KPT-330) is a first–in-class, orally bioavailable selective inhibitor of nuclear export compound that has shown promising anti-leukemia activity *in vitro* and *in vivo*.[19,20] Selinexor was effective in inducing apoptosis in cells from established AML cell lines that are in the G0/G1 phase of the cell cycle,[21] and targeting it also abrogated hypoxia-induced drug resistance in multiple myeloma cells.[22] These results suggest that targeting XPO1 with selinexor may have potent anti-proliferative effects against non-proliferating or slowly proliferating leukemia-initiating cells in primary AML unlike the limitation observed when using FLT3 inhibitors.[19] In addition, the recent results of phase I/II trials using selinexor as monotherapy (e.g. NCT02091245 and NCT02088541) or in combinations with conventional chemotherapeutic drugs (e.g. NCT02249091), have shown promising anti-leukemia activity with a high rate of blast clearance and complete remissions.[20,23-28] Initial problems with gastrointestinal toxicities and anorexia have largely been overcome by dose reduction without loss of clinical efficacy.[23,24] However, the anti-leukemia activity of selinexor in AML patients with FLT3 mutations, including those with acquired secondary mutations found in relapsed/refractory disease following FLT3-targeted therapy, has not been established.

In this study, we report that selinexor has marked pro-apoptotic effects against AML cells harboring *FLT3*-ITD and/or TKD mutations. However, compensatory upregulation of phosphorylated FLT3 and its downstream signaling pathways was observed in most of the FLT3-mutated cell lines tested *in vitro*. We, therefore, combined selinexor with sorafenib. This combinatorial drug regimen achieved markedly synergistic leukemia cell killing in cells harboring ITD and/or TKD mutations, which usually show resistance to FLT3-targeted therapy.[29,30] Of note, the combinatorial regimen also achieved encouraging clinical efficacy including molecular complete responses in an ongoing phase IB/II clinical trial of selinexor plus sorafenib in patients who were refractory to FLT3 inhibitor therapy. Thus, this combinatorial approach may abrogate selinexor-mediated FLT3 activation, resulting in abrogation of resistance to FLT3 inhibitors and induction of durable remissions in patients with additional acquired FLT3 mutations.

Methods

Reagents and antibodies

Selinexor was provided by Karyopharm Therapeutics (Newton, MA, USA). Sorafenib was purchased from Selleckchem (Houston, TX, USA). Their molecular structures are shown in *Online Supplementary Figure S1*. The antibodies against human phosphorylated (p)-p44/42 MAPK (ERK1/2)(Thr202/Tyr204), phospho-AKT(Ser473), phospho-FLT3(Tyr589/591), phospho-S6K(Ser240/244), AKT, S6K, Bcl-xL, C/EBPα, PU.1, STAT3, c-Myc and cleaved caspase-3 were purchased from Cell Signaling Technology (Danvers, MA, USA), against Bcl-2 from Dako (Carpinteria, CA, USA), against phospho-STAT5 A/B from Upstate (Lake Placid, NY, USA), against total STAT5A/B from R&D Systems Inc. (Minneapolis, MN, USA), against ERK2, FLT3, p53, IκB alpha, phospho-Stat3, and Mcl-1 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), against Bim and Puma from CalBiochem (San Diego, CA, USA), against HIF1α from BD Biosciences (San Diego, CA, USA), and against phospho-IκB alpha (ser32/36) from Novus (Littleton, CO, USA). The anti-luciferase antibody was purchased from Promega (Madison, WI, USA).

Acute myeloid leukemia cell lines and patients' samples

The Baf3/FLT3, Baf3/ITD, and Baf3/D835Y cell lines were kindly provided by Dr. Donald Small (Department of Pediatric Oncology, Johns Hopkins University, Baltimore, MD, USA) and Baf3/ITD+D835Y and Baf3/ITD+D835H cells by Dr. Neil Shah (Department of Medicine, The University of California at San Francisco, San Francisco, CA, USA). The FLT3-inhibitor-resistant cells Baf3/ITD+F691 and Baf3/ITD+Y842, which harbor *FLT3*-ITD plus F691L and Y842C mutations, respectively, were established by us as described previously.[30] The human AML cell lines THP-1, Kasumi-1, and MV4-11 were obtained from the American Type Culture Collection (Manassas, VA, USA), and MOLM13 and MOLM14 from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (Braunschweig, Germany). All cell lines were validated by short tandem repeat DNA fingerprinting using the AmpFISTR Identifiler kit according to manufacturer's instructions (Applied Biosystems cat. n. 4322288). All cells were maintained in RPMI medium supplemented with 10% fetal bovine serum, and interleukin-3-dependent murine Baf3/FLT3 cells were maintained in the presence of 2 ng/mL of interleukin-3. The FLT3 status of the AML cell lines used in this study is shown in Table 1.

Table 1. The half maximal responding concentrations (EC_{50s} and IC_{50} values)* of selinexor in leukemia cell lines.

	Cell lines	*FLT3* status	EC_{50} (μm)	95% confidence interval (lower/upper)	IC_{50} (μm)	95% confidence interval (lower/upper)
Murine cells	Baf3-FLT3	Wildtype	2.55	0.66/9.73	2.89	0.36/23.4
	Baf3-ITD	ITD mut**	0.48	0.30/0.77	0.03	0.01/0.16
	Baf3-D835Y	D835Y mut	0.65	0.40/1.04	0.06	0.02/0.17
	Baf3-ITD+D835Y	ITD+D835Y mut	0.32	0.07/1.50	0.09	0.06/0.12
	Baf3-ITD+D835H	ITD+D835H mut	0.22	0.04/1.30	0.08	0.05/0.13
	Baf3-ITD+Y842	ITD+Y842C mut	0.56	0.16/2.01	0.08	0.02/0.38
	Baf3-ITD+F691	ITD+F691L mut	0.61	0.17/2.23	0.06	0.02/0.25
Human cells	THP-1	Wildtype	20	5.02/48.19	0.12	0.04/0.25
	Kasumi-1	Wildtype	9.2	2.87/29.38	9.18	2.87/29.4
	MOLM13	ITD mut	0.74	0.21/1.53	0.03	0.01/0.09
	MV4-11	ITD mut	0.35	0.21/0.61	0.03	0.01/0.10

* EC_{50}: the half maximal responding concentration to induce apoptosis; IC_{50}: the half-maximal concentration to inhibit cell growth. ** mut: mutations

AML patients' samples with *FLT3*-ITD mutations were obtained after written informed consent following institutional guidelines of the University of Texas MD Anderson Cancer Center and in accordance with the principles of the Declaration of Helsinki. The mononuclear cells in these samples were purified by Ficoll-Hypaque (Sigma-Aldrich) density-gradient centrifugation, and the cells were cultured in RPMI 1640 culture medium supplemented with 10% fetal calf serum, as described above, prior to treatment.

Cell viability and apoptosis assays

The number of viable cells was determined using a Vi-CELL XR Cell Counter (Beckman Coulter Inc., Indianapolis IN, USA) with the trypan blue dye exclusion method, and apoptosis was determined via fluorescence-activated cell sorting (FACS) by annexin V positivity and propidium iodide positivity, as described previously.[31] The 50% inhibitory concentration (IC_{50}) for inhibition of cell growth and the 50% effective concentration (EC_{50}) for induction of apoptosis were calculated using CalcuSyn software (BioSoft, Cambridge, UK).

Immunoblot analyses

Protein levels in treated cells were determined by western blot analysis, as described previously.[6] Briefly, the treated cells were collected for preparation of cell lysates which were then resolved by electrophoresis on 12% precast sodium dodecyl sulfate-polyacrylamide gels, and transferred to Hybond-P membranes. After immunoblotting with antibodies, signals were detected by using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and semi-quantitatively assessed using the Scion Imaging system and software (beta version 4.03; Scion, Frederick, MD, USA).

Cellular morphology and assessment of differentiation

FLT3-ITD mutated AML cell lines MOLM13, MOLM14 and primary AML cell samples were plated at an initial density of 1.0×10^5 cells/mL in the presence of the indicated agents or combinations. Following 5 to 6 days of culture, cellular morphology was assessed after cytospinning onto slides and staining with Giemsa solution. Expression of the myeloid differentiation marker CD11b was determined by staining with anti-CD11b antibody (cell lines). The stained cells were washed twice with phosphate-buffered saline containing 2% bovine serum albumin. Morphology was evaluated by light microscopy; the percentage of CD11b cells and fluorescence intensity were determined using a FACS Calibur flow cytometer (Becton Dickinson).

Animal studies

The animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas, MD Anderson Cancer Center. NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NOG) mice (8-week old females; n=40; The Jackson Laboratory, Bar Harbor, ME, USA) were injected intravenously with $0.5x10^6$ of MOLM13-Luci-GFP cells that were lentivirally infected with firefly luciferase.[32] Mice (10 for each group) were treated with selinexor (15 mg/kg, dissolved in 0.6% Pluronic F-68 and 0.6% Plasdone K-29/32) or sorafenib [10 mg/kg, dissolved in cremophor EL/ethanol/water (12.5/12.5/75)] alone, or in combination (n=10); starting on day 4 after leukemia cell injection when an unambiguous luciferase signal was recorded. Animals injected with vehicle (only the solvents mentioned above in the same ratios without drugs) via gavage, at a once daily x 5/week schedule, served as controls. Mice were noninvasively imaged in a Xenogen-200 *in vivo* bioluminescence imaging system (Xenogen, Hopkinton, MA, USA) after injection with luciferin substrate (D-luciferin, GoldBoi, St Louis, MO, USA) at a concentration of 4 mg/mouse. Bioluminescence images were obtained and quantitated as described in detail previously.[6] Three mice for each group were sacrificed on day 18 after tumor cell injection, and spleen, liver, lung, and bone marrow samples were collected for immunohistochemical analysis. Briefly, the collected tissues were fixed in 10% neutral buffered formalin solution at 4°C overnight, then dehydrated, embedded in paraffin, and sectioned. After antigen retrieval, the slides were incubated with anti-luciferase antibodies.

Clinical trial

We initiated a phase IB/II clinical study of selinexor in combination with sorafenib in relapsed/refractory patients with *FLT3*-ITD or FLT3-D835 mutations (NCT02530476). The study included a dose-escalation phase IB portion in which sorafenib 400 mg BID continuously was combined with escalating doses of selinexor at a dose of 40 mg twice/week, 60 mg twice/week, and 80 mg twice/week to identify the recommended phase II dose of the combination. The selinexor was given twice/week for 3 weeks with 1 week off per 28-day cycle. Response to therapy was defined according to the International Working Group criteria.[33] Briefly, a complete remission (CR) was defined as ≤5% bone marrow blasts, a neutrophil count ≥1.0×10^9/L, and platelet count

≥100×10⁹/L. Briefly, a complete remission (CR) was defined as ≤5% bone marrow blasts, a neutrophil count ≥1.0×10⁹/L, and a platelet count ≥100×10⁹/L. CRi was defined as meeting all CR criteria except residual neutropenia (<1.0×10⁹/L) and/or thrombocytopenia (<100×10⁹/L). CRp (complete remission with incomplete platelet recovery) was defined as meeting all CR criteria except thrombocytopenia (<100 ×10⁹/L).

Statistical analyses

The Student *t*-test was used to analyze immunoblot and cell apoptosis data. A *P*-value ≤0.05 was considered statistically significant. All statistical tests were two-sided and the results are expressed as the mean of triplicate samples/experiments ± standard deviation/95% confidence intervals (error bars). The efficacy of selinexor on survival was estimated by the Kaplan–Meier method,[34] with log-rank statistics used to test for differences in survival.

Results

Selinexor, alone, or in combination with sorafenib, exerts marked pro-apoptotic effects in human and murine *FLT3*-mutated acute myeloid leukemia cells

We first investigated anti-leukemia effects of selinexor on AML cells with different *FLT3* mutational status. Selinexor triggered profound induction of apoptosis and inhibition of cell growth, at sub-micromolar concentra-

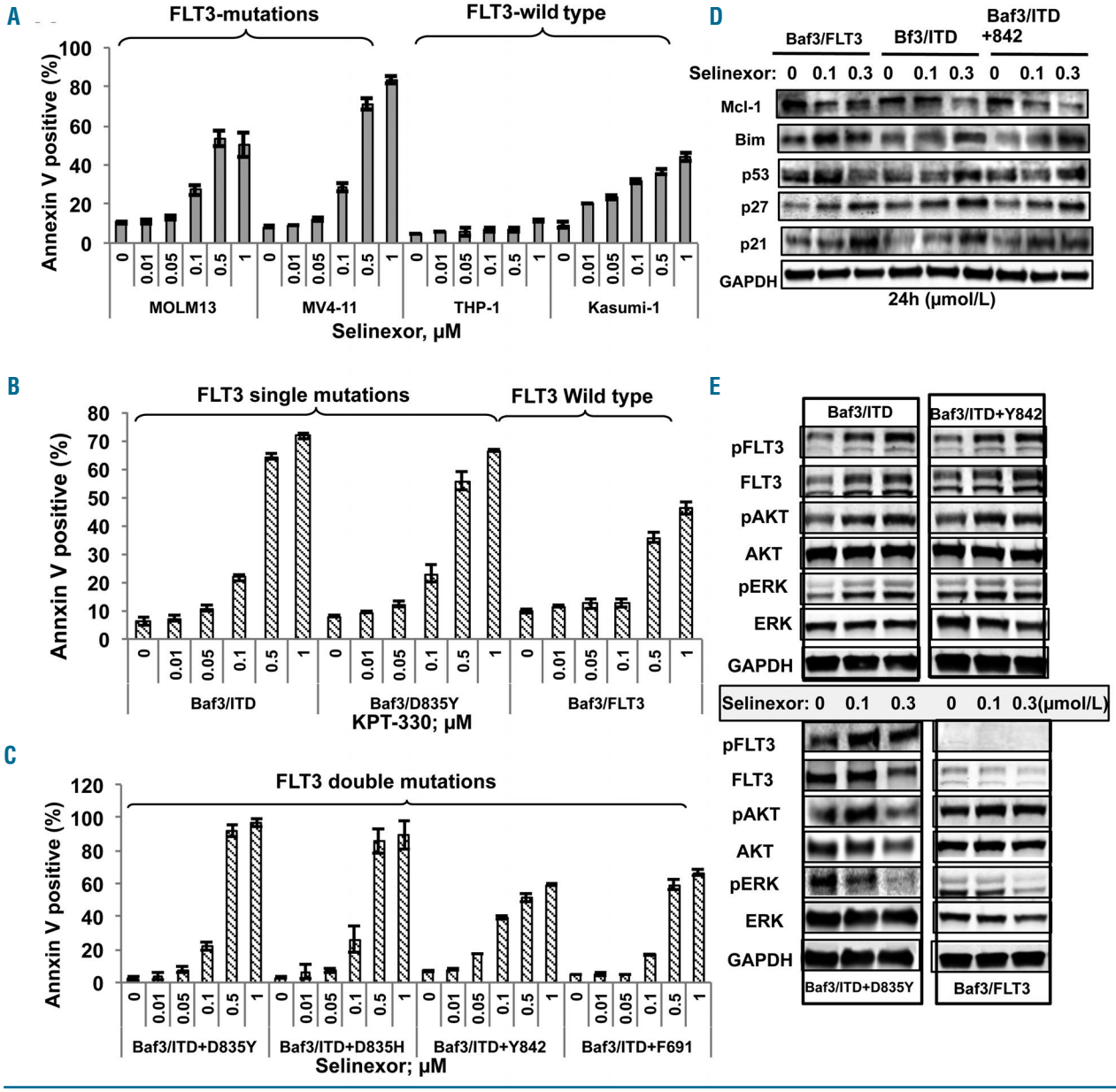

Figure 1. Targeting XPO1 with selinexor induces profound apoptosis in FLT3-mutated leukemic cells. Human (A) and murine (B, C) AML cell lines were treated with selinexor at the indicated concentrations for 72 h. Apoptosis induction was assessed as the percentage of annexin V–positive cells by flow cytometry. Data are the mean of three independent determinations. Error bars correspond to 95% confidence intervals. The protein levels of correlated signaling pathways (D) and phosphorylation status (E) were determined by immunoblotting after treatment with selinexor for 24 h. GAPDH was used as a loading control.

tions, in all human and murine AML cell lines that harbor ITD, TKD, or dual mutations of *FLT3*. The agent was much less effective in this regard in *FLT3* wildtype Baf3/FLT3, THP-1, and Kasumi-1 cell lines (FLT3 mutant cells, regardless of whether they had single or dual mutations of ITD and TKD, showed 5- to 10-fold lower EC_{50} values than those of FLT3-wildtype ones) (Figure 1A-C, Table 1 and *Online Supplementary Figures S2* and *S3*).

We next evaluated effects of selinexor on protein expression using immunoblot analysis after 24 h of exposure to selinexor. Selinexor inhibited expression of the anti-apoptotic protein Mcl-1 and upregulated the pro-apoptotic protein Bim. The tumor suppressor proteins p53, p21, and p27 were upregulated as well (Figure 1D). Unexpectedly, activations of FLT3 and its downstream signaling pathways were upregulated, as evidenced by increasing levels of phosphorylated FLT3, -ERK, and -AKT after exposure to selinexor for 24 h (Figure 1E), which were observed only in the *FLT3*-mutated cells. In addition, total FLT3 levels were also upregulated in *FLT3*-ITD and -ITD plus Y842 mutated cells, but not in ITD plus D835Y cells: this was true for both protein levels (Figure 1E) and mRNA transcriptional levels (*Online Supplementary Figure S4*). Kinetic analysis revealed that the upregulation of phospho-FLT3 was observable at 1 h, and phospho-ERK and -AKT at 6 h, after selinexor treatment (*Online Supplementary Figure S5*). These findings suggest that co-targeting FLT3 signaling, to suppress its downstream signaling pathways, simultaneously with nuclear export may potentially trigger synergistic cytotoxic effects in these cells.

We tested this hypothesis using combinatorial treatment with selinexor and sorafenib. The combinatorial regimen did indeed trigger synergistic pro-apoptotic effects in murine *FLT3*-ITD mutated, ITD plus Y842C and ITD plus

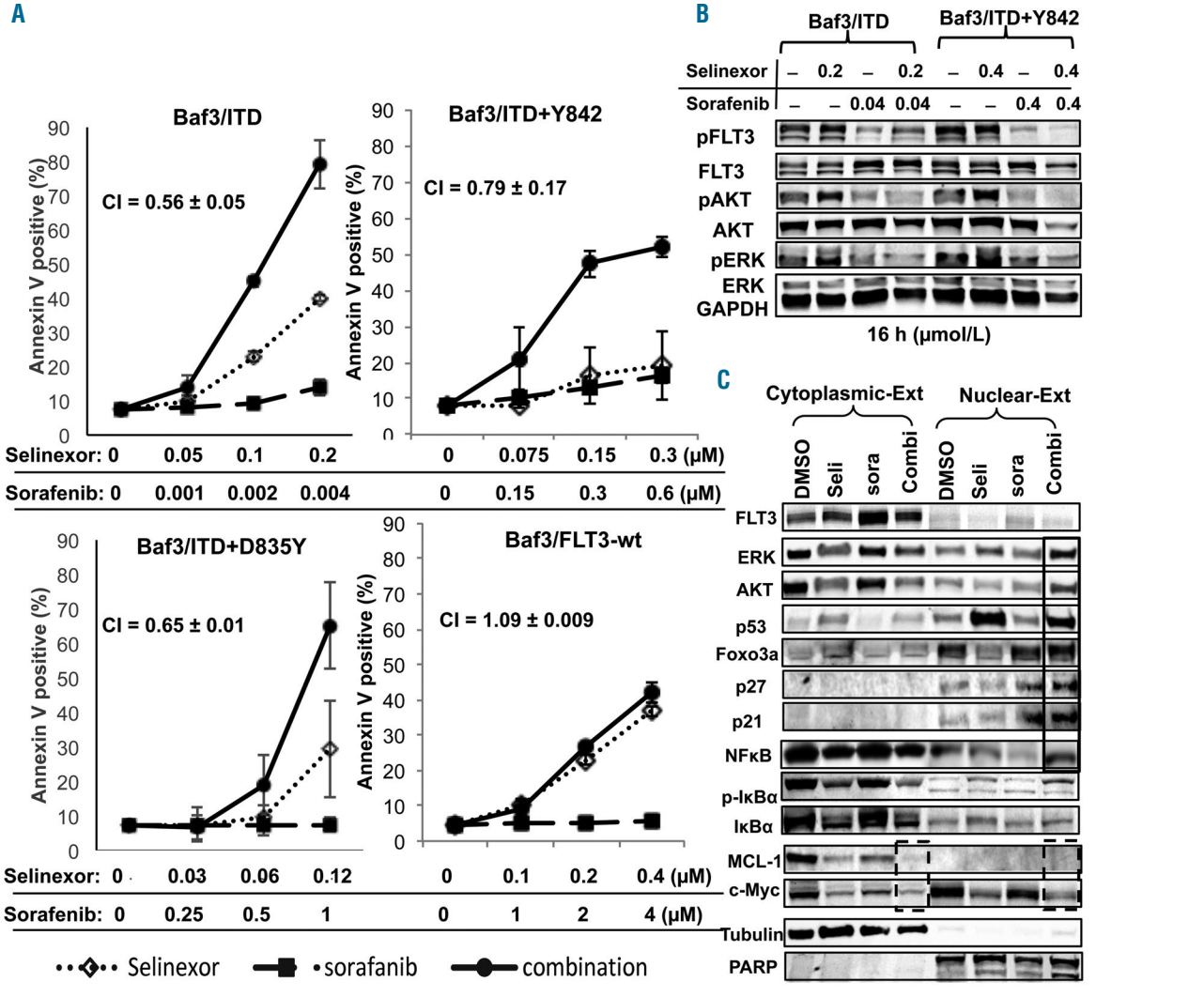

Figure 2. Combination treatment with selinexor and sorafenib triggers synergistically pro-apoptotic effects in murine cells with ITD or ITD plus TKD2 point mutations. (A) FLT3 mutated cells (Baf3/ITD, Baf3/ITD+Y842 and Baf3/ITD+D835Y) and wildtype cells (Baf3/FLT3-wt) were treated with either selinexor or sorafenib alone or the combination for 48 h, and examined for apoptosis induction (annexin V positivity) as described in Figure 1. (B) FLT3-mutated cells were treated with single agent(s) alone or the combination for 16 h, and levels of correlated phosphorylated proteins were measured by immunoblotting. GAPDH was used as a loading control. (C) MOLM13 cells were treated with the indicated concentrations of agent(s) for 16 h. Cytosolic and nuclear fractions were separated using a nuclear extract kit (Active Motif) following the manufacturer's instructions. The correlated protein levels were determined with immunoblotting. Poly (ADP-ribose) polymerase (PARP) was used as a loading control of the nuclear fraction and tubulin was used as a loading control of the cytosolic fraction. Dimethylsulfoxide (DMSO) was used as a control. Seli: selinexor; Sora: sorafenib; Combi: combination. Solid line box indicates upregulation and dotted line box indicates downregulation.

D835Y mutated cells (Figure 2A) and triggered additive pro-apoptotic effects in human FLT3-ITD-mutated MOLM13 and MV4-11 cells (*Online Supplementary Figure S5*). Of note, the combination regimen also demonstrated a synergistic pro-apoptotic effect in a primary AML sample with FLT3-ITD plus D839G dual mutations (*Online Supplementary Figure S6*). Immunoblot analysis showed that the combinatorial treatment markedly abrogated the upregulation of phospho-FLT3, -ERK and -AKT (Figure 2B) that was observed in the experiments of selinexor treatment alone. In fact, all phospho-proteins were suppressed far below basal levels by the combination treatment. Interestingly, we also observed that ERK, AKT, FOXO3a, NF-kB, p53, p27, and p21 were preferentially retained in the AML cell nuclei after 16 h of treatment with selinexor and sorafenib. This treatment also completely abrogated anti-apoptotic Mcl-1 expression and decreased c-Myc levels (Figure 2C), suggesting greater AML cell sensitivity to apoptosis.

Co-targeting XPO1 and FLT3 partially abrogates hypoxia-mediated chemoprotection

The hypoxic bone marrow microenvironment is a reservoir for leukemia-initiating cells, and it is associated with resistance to AML chemotherapy.[30,35] We examined apoptosis induction using the indicated concentrations of selinexor and/or sorafenib for a 68-h exposure under normoxic and hypoxic conditions. The combination partially abrogated hypoxia-mediated chemoprotection and induced synergistic apoptotic effects compared to those observed following treatment with either agent alone in *FLT3*-ITD-mutated MOLM13 and MOLM14 cells (Figure 3A,B). Immunoblot analysis indicated that the combination treatment profoundly suppressed the hypoxia-mediated upregulation of CXCR4 and HIF1α, suppressed phospho-FLT3, -ERK and -AKT, decreased Mcl-1, and increased the cleavage of caspase-3 (Figure 3C).

Co-targeting XPO1 and FLT3 enhances myeloid differentiation of *FLT3*-ITD-mutated human acute myeloid leukemia cells and human primary acute myeloid leukemia samples

Since selinexor and sorafenib treatments alone have been reported to induce differentiation of leukemic cells,[21,36] we investigated if the combination could enhance the differentiation of *FLT3*-ITD-mutated leukemic cells *in vitro*. MOLM13 and MOLM14 cells were exposed to nanomolar concentrations of either agent alone or the combination for 5 days. At these doses only growth arrest was observed. Morphological changes were observed, including indentation and bending of the nuclei and a decrease of nuclear/cytoplasmic ratio, with single-agent treatment in both ITD-mutated AML cell lines (Giemsa staining) suggesting a metamyelocyte stage of granulocytic differentiation. The combination treatment markedly enhanced these morphological changes. In addition, the myeloid differentiation marker $CD11b^{+}$ significantly increased in the cells following the combination treatment (Figure 4A,B).

We used the same regimen to treat two primary, *FLT3*-ITD-mutated, human AML samples (*Online Supplementary Table S1*) *in vitro* for 6 days. Enhanced morphological myeloid differentiation described above was observed fol-

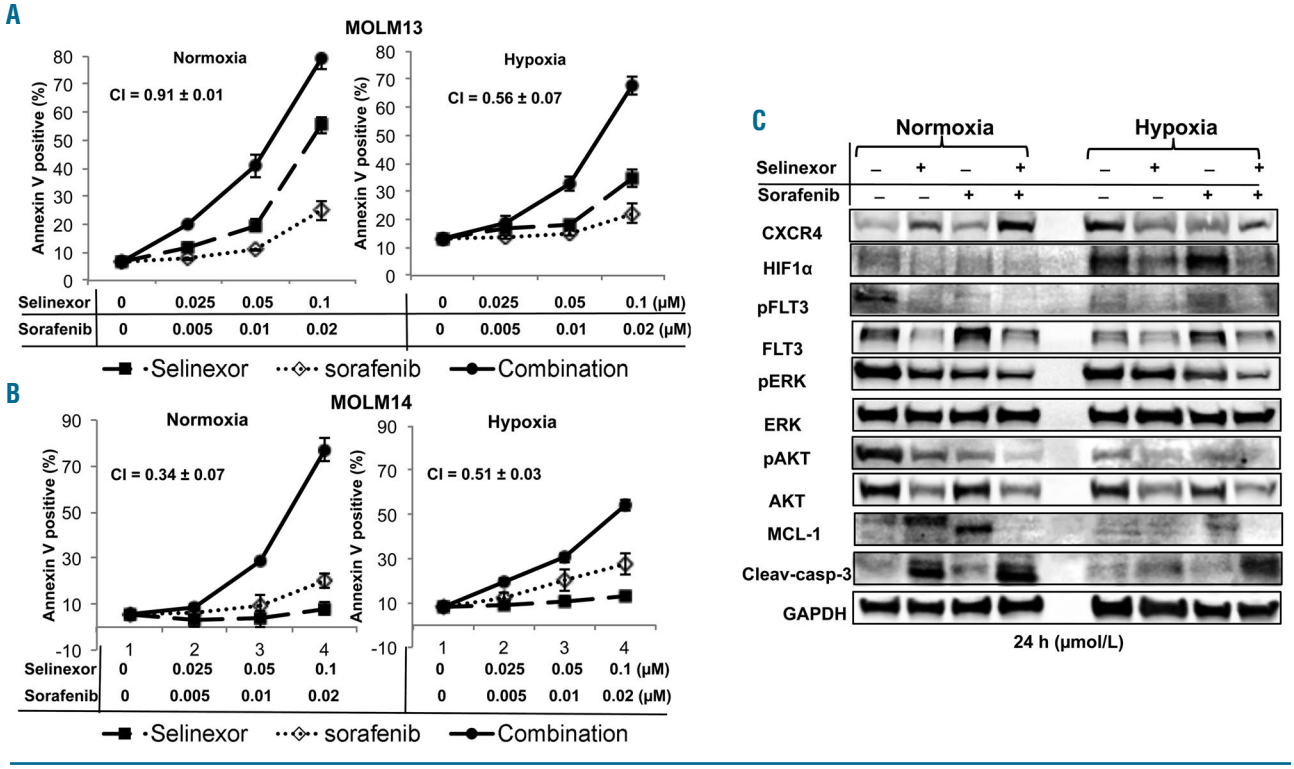

Figure 3. Combination treatment partially abrogates hypoxia protection and triggers synergistic apoptosis induction. MOLM13 (A) and MOLM14 (B) cells were treated with selinexor and/or sorafenib for 68 h. Apoptosis induction (annexin V positivity) was determined by flow cytometry. (C) MOLM13 cells were treated with the combination for 24 h and correlated protein levels were determined by immunoblotting.

lowing the combination treatment, as was a profound increase of the CD11b$^+$ population, which was more significant in the ITD-mutated AML sample (AML #2) than in the D835 TKD-mutated AML sample (AML #1) (Figure 4C). In addition, a decrease of the CD34$^+$ population was observed in both tested primary AML samples (*Online Supplementary Figure S8*). Of note, the doses of either drug alone, or in combination, were not enough to kill the primary AML cells, but were sufficient for growth arrest of the CD34$^+$ cells, implying that the combinatorial regimen could be beneficial by impairing the self-renewal capacity of the leukemic CD34$^+$ compartment. Mechanistically, marked upregulation of CCAAT/enhancer binding protein α (C/EBPα), one of the leucine zipper transcription factors that is important for normal myeloid cell differentiation, was observed in the cells following the combination treatment. This was accompanied by an increase in the C/EBPα/PU-1 ratio (Figure 4D), which has been reported to be associated with granulocytic myeloid cell differentiation.[37] Interestingly, upregulation of cell proliferation-related proteins, such as phospho-ERK, -STAT5, -STAT3, and tumor suppressor proteins, including p53 and p21, was also observed after exposing cells to low doses of both drugs for 5 days (Figure 4D).

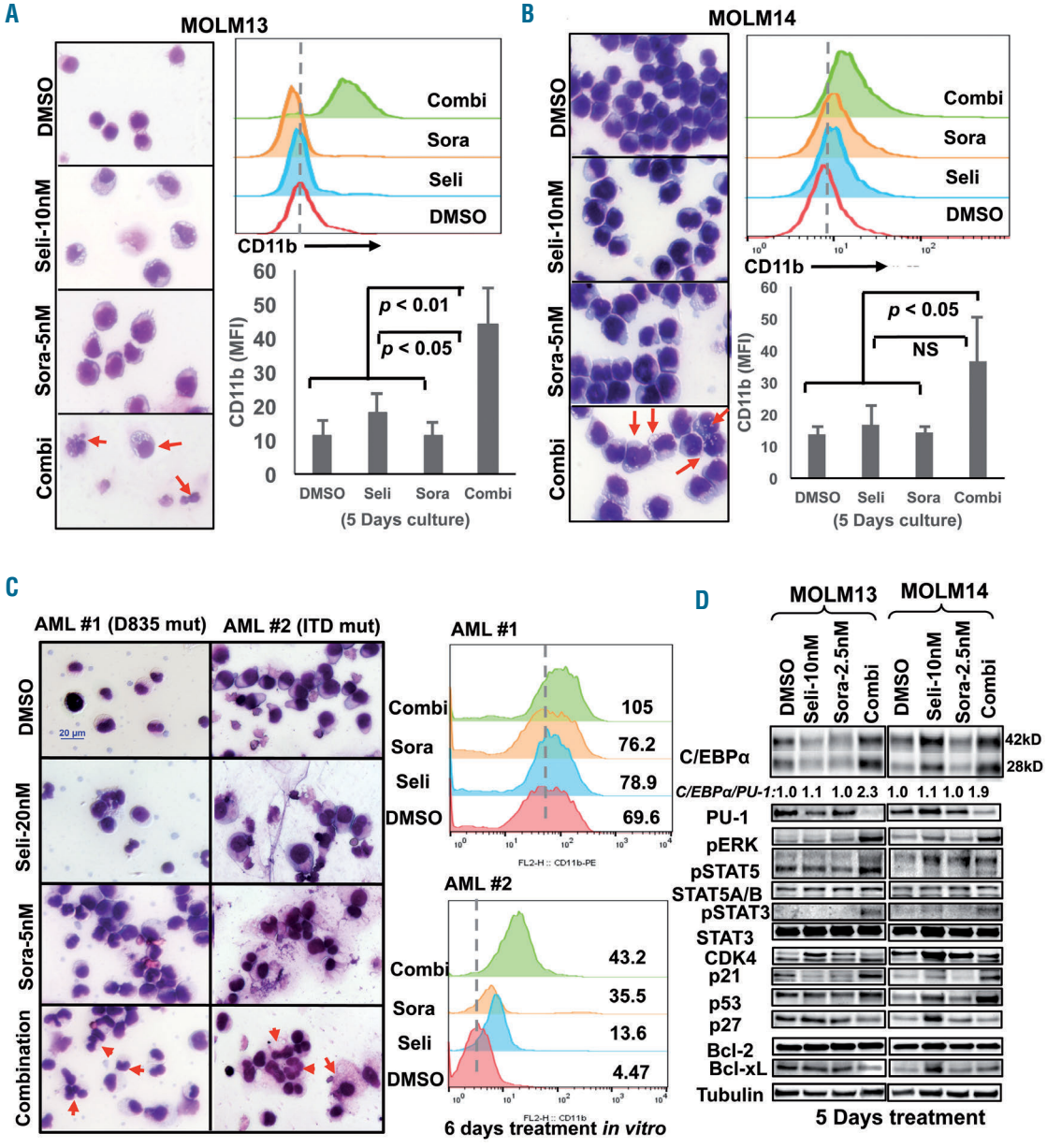

Figure 4. Combination treatment triggers myeloid differentiation of leukemic cells. FLT3-ITD mutated cell lines MOLM13 (A), MOLM14 (B) and primary AML patients' samples (C) were treated with the indicated concentrations of selinexor and/or sorafenib for 5 or 6 days *in vitro*; morphological changes of the cells were checked with Giemsa staining. Expression of the myeloid differentiation marker CD11b was determined using flow cytometry. The histograms present individual assays which measured fluorescent intensity of CD11b. Each column was generated from four individual assays. (D) Differentiation-related proteins were evaluated using immunoblotting. The expression levels and ratios of C/EBPα/PU-1 were measured (first lane which was defined as 1). Dimethylsulfoxide (DMSO) was used as a control. Seli: selinexor; Sora: sorafenib; Combi: combination.

The selinexor and sorafenib combination has marked anti-leukemia efficacy in a mouse xenograft model of human *FLT3*-ITD-mutated acute myeloid leukemia

We next assessed *in vivo* efficacy of the selinexor and sorafenib combination in a murine leukemia model. NOG mice bearing xenografts of MOLM13-Luc-GFP cells were treated with either selinexor or sorafenib alone or the drug combination. The vehicle served as a control. The mice received 39 days of treatment starting from day 4 after injection of leukemia cells. The median survival in the vehicle, sorafenib, selinexor, and combination treatment groups was 16, 23, 31, and 51 days, respectively ($P<0.001$) (Figure 5A). In addition, the combination significantly reduced leukemia burden compared with that in the control mice after 10 days of treatment (day 14). The mean luminescence was ~$1x10^7$ photons/s in the vehicle-treated group *versus* ~$2x10^5$ photons/s in the group treated with the combination (Figure 5B,C). The mice tolerated the individual drugs and the combination well, without signs of anorexia, weight loss, or other signs/symptoms of distress. One week after treatment cessation (i.e., day 49), the mice in the combination group developed increased leukemia burden and succumbed to AML (Figure 5D).

Further analysis revealed that the infiltration of leukemic cells was significantly reduced in peripheral blood after receiving 14 days of either single-agent treatment or combination treatment (Figure 6A). However, the bone marrow environment protected against sorafenib treatment-mediated leukemia cell killing, while selinexor alone had anti-leukemia efficacy. Impressively, the combination treatment almost eliminated leukemic cells from the bone marrow (i.e., approximately 100-fold lower levels than following treatment with the vehicle, $P<0.001$) (Figure 6B), which suggests potent killing of leukemia cells by this combinatorial regimen in the bone marrow environment. Immunohistochemical analysis further confirmed the profound reduction of leukemia cells in the bone marrow and other organs (Figure 6C).

The selinexor and sorafenib combination exerted anti-leukemia effects in patients with *FLT3*-mutated acute myeloid leukemia in a phase IB clinical trial

The preclinical synergy of selinexor and sorafenib led to the initiation of a phase IB/II study of the combination in relapsed/refractory patients harboring FLT3 mutations. Fourteen patients enrolled in this clinical trial were eligible for analysis. Remarkably, four of the 14 patients (29%) achieved a sustained CRp/CRi and two others (14%) had >50% blast reduction (Table 2). Six of the 11 (55%) patients treated previously with FLT3 inhibitors achieved responses. These patients comprised: (i) two *FLT3*-ITD mutated patients, previously treated with sorafenib, who achieved CRp with molecular CR with selinexor plus sorafenib; (ii) one *FLT3*-ITD and FLT3-D835 dual mutated

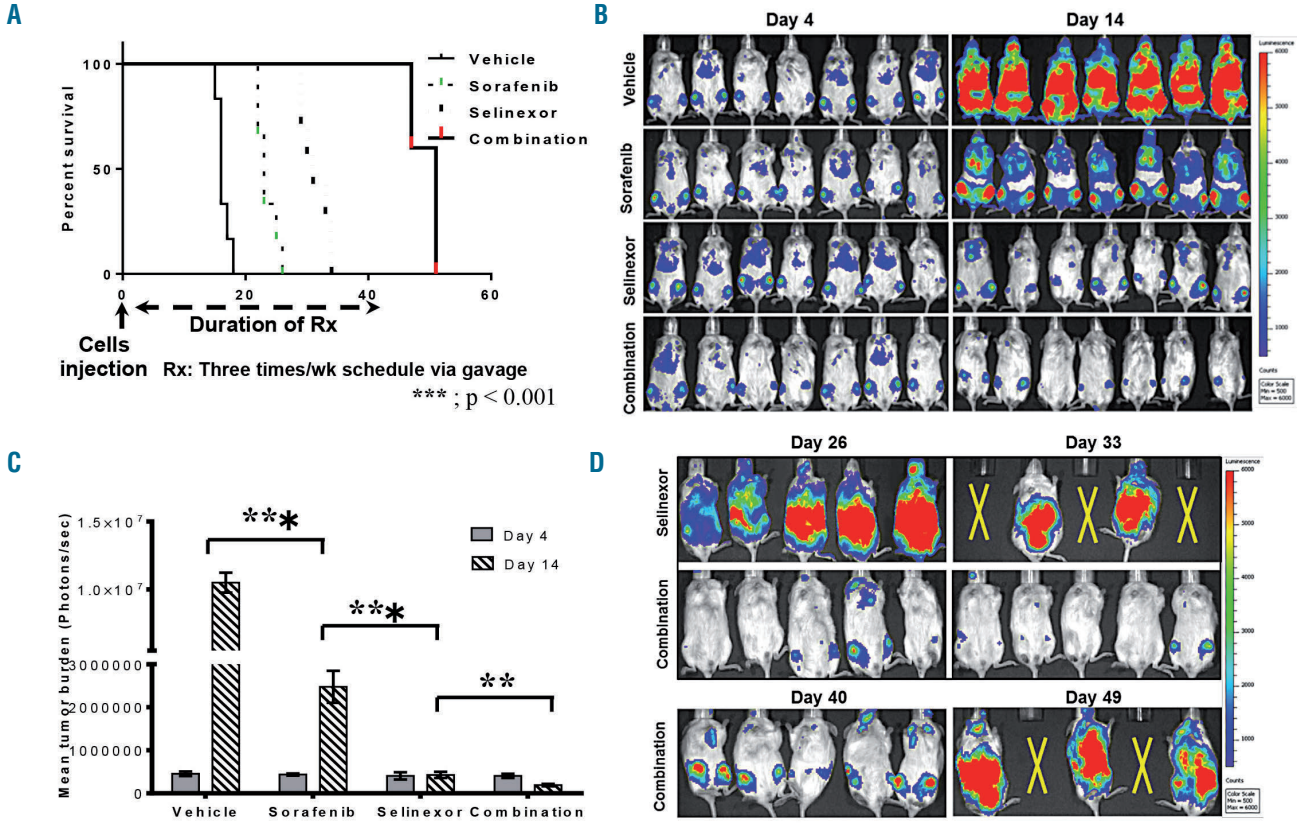

Figure 5. Combination treatment significantly improves mice survival and reduces leukemia burden in a MOLM13-engrafted murine leukemia model. (A) The median survival were assessed for each group by the Kaplan–Meier method, and log-rank statistics applied to test for differences in survival. (B) Serial bioluminescence images of representative mice at day 4 and day 14 after injection of leukemic cells in the groups treated with selinexor or sorafenib alone or the combination. (C) Quantitative analysis of the leukemia burden. (D) Serial bioluminescence images of representative mice at 26, 33, 40 and 49 days after leukemia cell injection.

patient, previously treated with quizartinib and idarubicin with cyatarabine plus crenolanib, who achieved CRi with molecular CR for both *FLT3*-ITD and *FLT3*-D835 with selinexor plus sorafenib; (iii) a FLT3-mutated patient previously treated with crenolanib, sorafenib and allogeneic stem cell transplantation who achieved CRi without molecular CR with selinexor plus sorafenib; and (iv) two *FLT3*-ITD and *FLT3*-D835 dual mutated patients who had received prior sorafenib therapy and achieved PR + >50% blast reduction with selinexor plus sorafenib (Daver *et al.*, ASH annual meeting, 2017, Abstract #1344). We have no information regarding differentiation induction in these patients at this time.

Discussion

A previous study using the XPO1 inhibitor KPT-185 demonstrated strong post-transcriptional downregulation of total FLT3 protein expression in AML cell lines and primary AML samples, which was associated with anti-leukemia efficacy.[21] We confirmed the suppression of phospho-FLT3 and accompanying downregulation of total FLT3 protein in human MOLM13 and MV4-11 cells after 24 h of selinexor treatment (*Online Supplementary Figure S9*). Unexpectedly, however, we observed marked upregulation of phospho-FLT3 in murine AML cells, including those harboring ITD, ITD plus D835Y or Y842C dual mutations, after exposure to the same concentrations of selinexor. Furthermore, total FLT3 protein and mRNA levels were upregulated in addition to the upregulation of FLT3 downstream phospho-ERK and -AKT levels in cells with these mutations, but not in the ITD plus D835Y mutants (Figure 1E, *Online Supplementary Figure S4*), suggesting that a possible transcriptional mechanism was involved in FLT3 upregulation in these murine cells. The modulation profiling suggests that selinexor-induced apoptosis is independent of the suppression of FLT3 and its downstream pathways. In fact, cells with the dual Baf3/ITD+D835 mutation showed greater sensitivity to selinexor-induced apoptosis compared with other dual-mutated cells, Baf3/ITD+Y842 and Baf3/ITD+F691 (EC_{50} values were 0.2 and 0.3 μM *versus* 0.56 and 0.6 μM, respectively, Baf3/ITD+D835H and Baf3/ITD+D835Y *versus* Baf3/ITD+Y842 and Baf3/ITD+F691 cells), and suppressed phospho-ERK and –AKT, but upregulated phospho-FLT3 levels. These results strongly support that selinexor induces apoptosis in an FLT3-independent manner in *FLT3*-mutated AML cells. In addition, we observed upregulation of XPO1 cargo proteins p53, p21 and p27, as well as downregulation of pro-survival Mcl-1 after 24 h of selinexor treatment in both human and murine FLT3-mutated AML cells (Figures 1D and 2C). We, therefore, postulated that the modulation of pro- and anti-apoptotic regulators was the driving force behind selinexor-triggered apoptosis in the *FLT3*-mutated AML cells. These results extend our previous observations in wild-type *FLT3* AML cells, which are likely also dependent on p53.[17]

We further postulated that the selinexor-induced upregulation of phospho-FLT3 and its downstream components may provide a rationale for the combinatorial treatment with FLT3 inhibitors. Concomitantly targeting XPO1 and FLT3 triggered profound synergistic pro-apoptotic effects in murine *FLT3*-mutated AML cells. However, only additive induction of apoptosis was observed in human *FLT3*-mutated AML cells (*Online Supplementary Figure S5*). Of note, we have reported that FLT3-targeted therapy upregulates FLT3 and its downstream proteins in human clinical samples and in most sorafenib-resistant AML cells *in vitro*.[5,11,30] Thus, it is reasonable to speculate that co-targeting FLT3 and XPO1

Table 2. Characteristics of patients who responded to the combination treatment.

Patients	Age (years)	FLT3 Status	Additional mutations[a]	Karyotypes	Blasts (%)	Dose level	Best response	Response duration	Prior therapies
Case 1	81	ITD mut[b]	TP53, IDH2, RUNX1, WT1	Diploid	67	-Selinexor 80 mg twice per week -Sorafenib 400 mg BID	CRp with undetectable FLT3-ITD by PCR	180 days	Aza+sorafenib, Anti-CD25 trial
Case 2	63	ITD mut	HRAS. WT1	t(6;9)	10	-Selinexor 60 mg twice per week -Sorafenib 400 mg BID	CRp, FLT3 PCR pending	75 days	7+3, CLIA+sorafenib
Case 3	24	ITD + D835 mut	NRAS, TET2, IDH1	Diploid	68	-Selinexor 80 mg twice per week -Sorafenib 400 mg BID	CRi with undetectable FLT3-ITD and FLT3-D835 by PCR	60 days then to autologous SCT	7+3, MEC, quizartinib, IA+crenolanib
Case 4	38	ITD mut	RUNX1	Miscellaneous	80	-Selinexor 60 mg twice per week -Sorafenib 400 mg BID	CRi with FLT3 positive by PCR	60 days	7+3+sorafenib, GCLAC +sorafenib, SCT
Case 5	78	ITD + D835 mut	DNMT3A, RUNX1	Diploid	66	-Selinexor 60 mg twice per week -Sorafenib 400 mg BID	Hi-blast reduction	35 days	Aza, aza+sorafenib
Case 6	50	ITD + D835 mut	WT1	Miscellaneous	94	-Selinexor 60 mg twice per week -Sorafenib 400 mg BID	Hi-blast reduction	35 days	7+3, MEC, MUD, SCT, Dac+sorafenib,

[a]Based on screening of bone marrow samples with a 28-gene mutation panel. [b]mut: mutations. PCR: polymerase chain reaction; Aza: azacytidine; CLIA: cladribine idarubicin, cytarabine; IA: idarubicin, cytarabine; MEC: mitoxantrone, etoposide, cytarabine; GCLAC: granulocyte colony-stimulating factor, cladribine, cytarabine; SCT: stem cell transplantation; MUD: matched unrelated donor; Dac: decitabine.

could achieve synergistic efficacy by overcoming the resistance to FLT3-targeted therapy. Our data demonstrate a synergistic anti-leukemia efficacy of the drug combination *in vivo*. In addition, the combination regimen produced a profound reduction of CXCR4 and HIF1α levels in hypoxia *in vitro* (Figure 3C), and also marked elimination of leukemic cells in a bone marrow *in vivo* murine AML model (Figure 6B), suggesting a potential benefit for overcoming resistance provided by the microenvironment. Remarkably, the combination regimen has achieved complete remissions, including ongoing complete molecular remissions, in 30% (3 of the first 10) relapsed/refractory AML patients in our on-going phase IB/II clinical trial of combinatorial therapy of selinexor and sorafenib. Of note, the three patients with CRp/CRi, who were previously clinically refractory to quizartinib/sorafenib monotherapy or to combinations

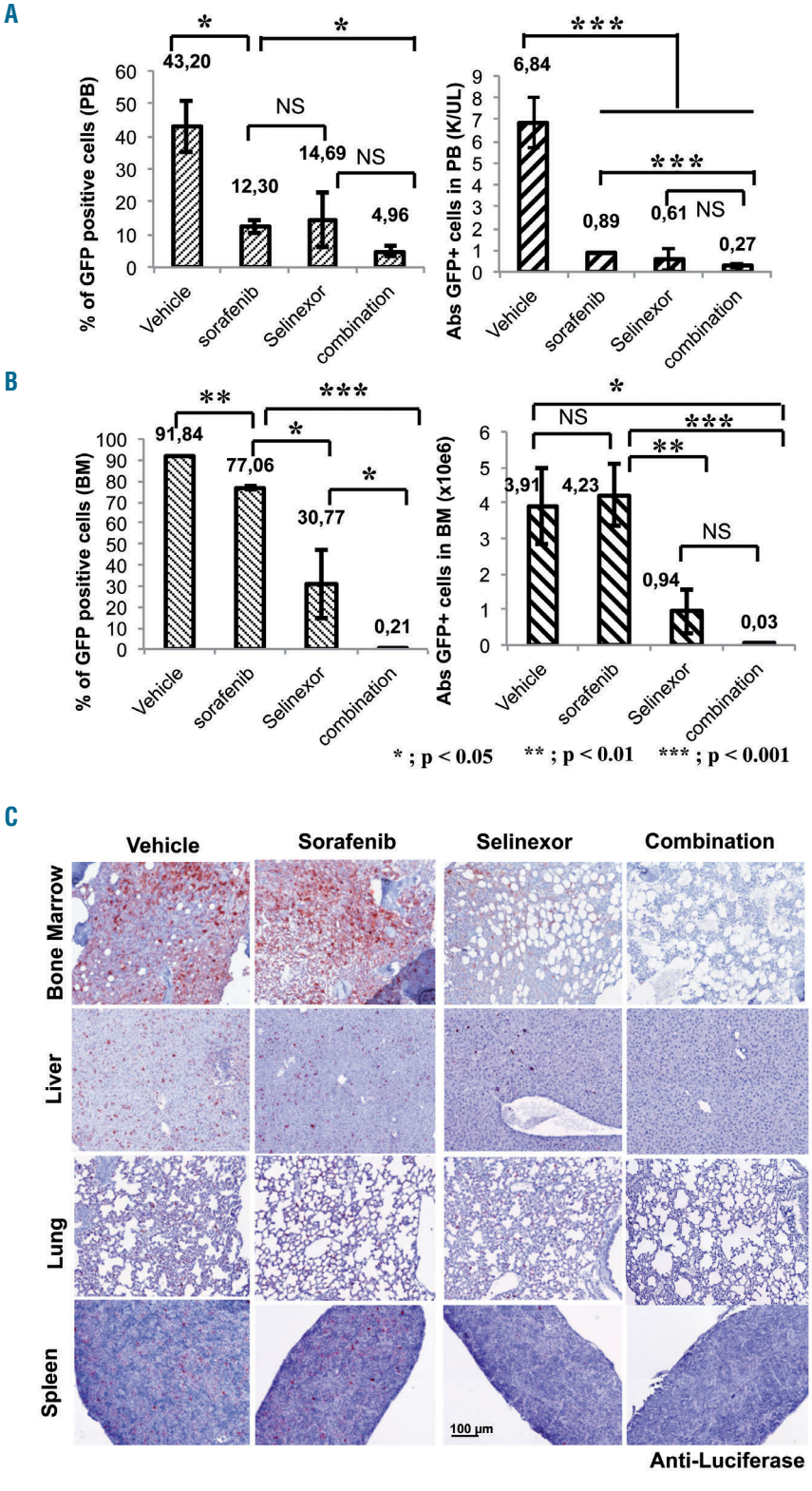

Figure 6. Combination treatment significantly eliminates leukemia cell engraftment in bone marrow and other organs. Leukemia cell infiltration was evaluated in (A) blood circulation and (B) bone marrow (BM) at day 18 by flow cytometry after gating GFP-positive cells, and in (C) other soft organs using immunostaining with anti-luciferase antibody in paraffin sections. The bar represents 100 µm.

with conventional chemotherapeutic drugs, achieved *FLT3*-ITD negativity as determined by the real-time polymerase chain reaction assay.

One hallmark of AML is the differentiation arrest of leukemic blasts. Promoting differentiation may therefore be beneficial for achieving and maintaining remissions in leukemias. Targeting *FLT3*-mutated AML cells with FLT3 inhibitors has been reported to induce cell-cycle arrest and differentiation, rather than apoptosis, which is reportedly driven by overexpression of *C/EBPα* and PU.1.[36,38] Schepers *et al.* described that upregulation of *C/EBPα* led to growth arrest of CD34$^+$ leukemia cells, which impaired the self-renewal capacity of the leukemic CD34$^+$ cells, and corresponded with enhanced myeloid differentiation as well.[39] Also, KPT-185 induced cell-cycle arrest and myeloid differentiation in AML cells, including patients' samples, which increased *C/EBPα*.[21] In fact, *C/EBPα* is a p53-regulated DNA damage-inducible gene,[40] and p53 induction is involved in myeloid differentiation.[41] Our data indicate that selinexor deactivated the nuclear export of a number of cargo proteins including p53. Furthermore, sorafenib mediated the upregulation of p21, and its combination with selinexor markedly enhanced levels of p53, p21, and *C/EBPα* (Figure 4D) as noted above, the last being one of the key hematopoietic-specific transcription factors mediating myeloid differentiation of leukemia cells. However, we did not observe an increase in another transcription factor, PU.1, which is reportedly an upregulated effector of *C/EBPα*. The precise function of PU.1 is still unclear. Dahl *et al.* suggested that lower levels of PU.1 direct granulocyte differentiation, whereas higher levels are required for macrophage differentiation.[37] Nevertheless, our data imply that an increase of C/EBPα levels was sufficient to induce myeloid differentiation of *FLT3*-ITD-mutated leukemic cells and decrease the CD34$^+$ population, especially for the combination of sorafenib and selinexor. This treatment restores nuclear p53 level by blocking XPO1, and then upregulates C/EBPα to enhance *C/EBPα/PU-1* and granulocyte differentiation as shown in Figure 4 and *Online Supplementary Figure S8*.

Signal transducer and activator of transcription (STAT) family proteins are reportedly involved in regulation of myeloid progenitor cell differentiation.[42] In fact, STAT5 plays an important role in early myeloid differentiation, and lacking expression of STAT5 reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice.[43] STAT3 activation has also been reported to be a critical step in terminal differentiation of myeloid cells.[44] On the other hand, upregulation of MAPK has also shown to be critical in both monocytic and granulocytic differentiation of myeloid cell lines, which can be abrogated by using the MEK inhibitor U0126.[45] All of these lines of evidence imply that high activation of these proteins may contribute to myeloid differentiation of leukemia cells. Of note, we observed profound upregulation of phosphorylated STAT3, STAT5 and ERK levels after combination treatment with low doses of sorafenib and selinexor in FLT3-mutated MOLM13 and MOLM14 cells (Figure 4D), suggesting that upregulation of STATs and/or MAPK signaling pathways may also contribute to differentiation induction of the combination regimen in FLT3 mutated AML cells.

In summary, our combinatorial strategy targeting FLT3 and XPO1 showed synergistic anti-leukemia effects in FLT3 inhibitor-resistant cells *in vitro* and *in vivo*. The combination of XPO1 and FLT3 inhibitors may also be able to eliminate leukemia-initiating cells by arresting cell growth and impairing the self-renewal capacity of leukemic CD34$^+$ cells. These results should provide a solid basis for examining these agents further in patients with FLT3-mutated AML, including those who have acquired resistance to FLT3-targeted therapy.

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