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MYELOID NEOPLASIA

Tyrosine kinase inhibitor–induced defects in DNA repair sensitize FLT3(ITD)-positive leukemia cells to PARP1 inhibitors

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KEY POINTS

- FLT3 inhibitor AC220 caused DNA repair defects and sensitized FLT3(ITD)-positive AML stem and progenitor cells to PARP1 inhibitors.
- Quiescent and proliferating FLT3(ITD)-positive AML cells were eliminated by the combination of FLT3 and PARP1 inhibitors.

Mutations in FMS-like tyrosine kinase 3 (FLT3), such as internal tandem duplications (ITDs), can be found in up to 23% of patients with acute myeloid leukemia (AML) and confer a poor prognosis. Current treatment options for FLT3(ITD)-positive AMLs include genotoxic therapy and FLT3 inhibitors (FLT3i's), which are rarely curative. PARP1 inhibitors (PARP1i's) have been successfully applied to induce synthetic lethality in tumors harboring BRCA1/2 mutations and displaying homologous recombination (HR) deficiency. We show here that inhibition of FLT3(ITD) activity by the FLT3i AC220 caused downregulation of DNA repair proteins BRCA1, BRCA2, PALB2, RAD51, and LIG4, resulting in inhibition of 2 major DNA double-strand break (DSB) repair pathways, HR, and nonhomologous end-joining. PARP1i, olaparib, and BMN673 caused accumulation of lethal DSBs and cell death in AC220-treated FLT3(ITD)-positive leukemia cells, thus mimicking synthetic lethality. Moreover, the combination of FLT3i and PARP1i eliminated FLT3(ITD)-positive quiescent and proliferating leukemia stem cells, as well as leukemic progenitors, from human and mouse leukemia samples. Notably, the combination of AC220 and BMN673 significantly delayed disease onset and effectively reduced leukemia-initiating cells in an FLT3(ITD)-positive primary AML xenograft mouse model. In conclusion, we postulate that FLT3i-induced deficiencies in DSB repair pathways sensitize FLT3(ITD)-positive AML cells to synthetic lethality triggered by PARP1i's. Therefore, FLT3(ITD) could be used as a precision medicine marker for identifying AML patients that may benefit from a therapeutic regimen combining FLT3 and PARP1i's. (*Blood*. 2018;132(1):67-77)

Introduction

Acute myeloid leukemia (AML) represents the deadliest form of acute leukemia among adults. Treatment involves chemotherapy and/or stem cell transplantation (for those who are eligible); however, the strategies are only curative in a fraction (30% to 40%) of younger patients and in <10% of patients older than 65 years. More specific therapies have been developed against AMLs carrying internal tandem duplications (ITDs) in FMS-like tyrosine kinase 3 (FLT3). Combination of chemotherapy with midostaurin, a tyrosine kinase inhibitor, which, among other targets, inhibits FLT3 activity, has shown efficacy in FLT3-mutant AML and has recently been approved by the US Food and Drug Administration.[1] However, other FLT3 activity inhibitors (FLT3i's), such as quizartinib (AC220) or sorafenib, rarely produced remissions when administered alone or in combination with

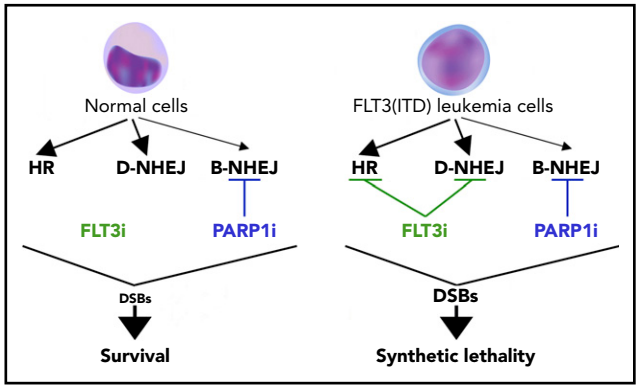

Figure 1. Proposed model of FLT3i-guided synthetic lethality triggered by PARP1i in FLT3(ITD)-positive AML cells. Synthetic lethality arises when a combination of deficiencies in the expression of 2 or more genes leads to cell death, whereas a deficiency in only 1 of these genes does not. FLT3i downregulates the expression of multiple genes involved in DSB repair causing HR and D-NHEJ deficiency in FLT3(ITD)-positive leukemia cells but not in normal counterparts. This effect causes PARP1i-triggered accumulation of toxic DSBs and synthetic lethality in leukemia cells, whereas normal cells are spared.

cytotoxic drugs, and these remission are often short-lived and followed by early relapse in almost all cases.[2]

Leukemia stem cells (LSCs) have a dual role as tumor-initiating and therapy-refractory cells.[3,4] Therefore, even if antitumor treatment clears a disease burden consisting mostly of leukemia progenitor cells (LPCs), it usually fails to eradicate LSCs and therapy-refractory residual LPCs. Several experimental approaches have been developed to eradicate LSCs, such as targeting of BCL2,[5] glutathione metabolism,[6] BCL6,[7] mTOR,[8] SDF-1,[9] HDAC,[10] and Wnt[11] or involving granulocyte-colony stimulating factor (G-CSF)[12] have recently been tested against LSCs. However, their clinical application may produce adverse events, because these proteins/mechanisms are also important in normal cells.[13,14] Therefore, it is imperative to identify new therapies that, alone or in combination with traditional treatments, will cure or prolong the remission time and/or be used in refractory AML patients.

Numerous reports indicated that AML cells accumulate high levels of spontaneous and drug-induced DNA lesions, including highly lethal DNA double-strand breaks (DSBs), but they survive because of enhanced/altered DNA repair activities.[15-22] DSBs are repaired by 2 major mechanisms: BRCA1/2-mediated homologous recombination (HR) and DNA-PK–mediated nonhomologous end-joining (D-NHEJ).[23] In addition, PARP1 plays a central role in preventing/repairing lethal DSBs by activation of base excision repair/single-stranded DNA break repair, by stimulation of fork repair/restart, and by mediating the back-up nonhomolopgous end-joining (NHEJ) repair.[24-27]

Accumulation of potentially lethal DSBs in AML cells creates an opportunity to eradicate these cells by targeting DNA repair mechanisms. The success of the PARP1 inhibitor (PARP1i) olaparib in BRCA1/2-mutated breast and ovarian cancers established a proof-of-concept for personalized cancer therapy utilizing synthetic lethality to target DNA repair mechanisms.[28] Because BRCA1/2 mutations are rare in AMLs,[29] markers predicting their sensitivity to DNA repair inhibitors need to be identified. Unfortunately, The Cancer Genome Atlas (TCGA) database analysis did not reveal whether AML-related mutations were associated with specific DSB repair deficiencies (supplemental Figure 1, available on the *Blood* Web site).

Given the high frequency and poor prognosis of FLT3(ITD) mutations, as well as the cellular stress induced by these mutations,[30] therapies targeting FLT3(ITD) mutations may leave AML cells vulnerable to DSB-inducing therapies. In particular, we hypothesized that FLT3i causes inhibition of HR and D-NHEJ ("BRCAness/DNA-PKness" phenotype), which, in combination with PARP1i, causes synthetic lethality in FLT3(ITD)-positive AML cells due to accumulation of lethal DSBs beyond the reparable threshold (Figure 1).

Materials and methods

Primary human cells

Peripheral blood and bone marrow samples from patients with newly diagnosed AMLs were obtained from the Department of Internal Medicine III, University of Ulm; the Department of Internal Medicine (Hematology, Oncology, Hemostaseology, and Stem Cell Transplantation), RWTH Aachen University; and the Department of Internal Medicine I, Medical University of Vienna. Clinically relevant mutations for these samples are listed in supplemental Table 1. Samples of normal hematopoietic cells were purchased from Cambrex Bio Science (Walkersville, MD). Lin^-CD34^+ cells were obtained from mononuclear fractions by magnetic sorting using EasySep negative selection human progenitor cell enrichment cocktail, followed by human CD34 positive selection cocktail (STEMCELL Technologies, Vancouver, BC, Canada), as described previously.[31]

Primary murine cells

Evi1-GFP–transgenic mice (GFP knockin to Evi1 locus does not affect the function of Evi1) were generously provided by Mineo Kurokawa (University of Tokyo),[32] and *FLT3(ITD)*-knockin mice[33] were obtained from The Jackson Laboratory. *FLT3(ITD)*-knockin mice were crossed to *Evi1-GFP* mice to generate the *FLT3(ITD);Evi1-GFP* compound mouse. Mice were genotyped in-house by polymerase chain reaction. As expected, *FLT3(ITD);Evi1-GFP* mice contained enlarged spleens compared with their *Evi1-GFP* counterparts. In addition, although spleen and bone marrow organ architecture and cellular composition remained preserved in the *Evi1-GFP* mice, they were essentially completely replaced by infiltrate of cells with large nuclei and scant occasionally granulated cytoplasm, consistent with AML, in *FLT3(ITD);Evi1-GFP* mice (supplemental Figure 2A-D). Moreover, we observed an approximately twofold increase in the number of $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells and a modest increase in $Lin^-Sca\text{-}1^-c\text{-}Kit^+$ cells in bone marrow of *FLT3(ITD);Evi1-GFP* mice over *Evi1-GFP* mice,[33] whereas >70% of LSK cells were GFP^+ (supplemental Figure 2E-F). The detection of LSK GFP^+ cells was performed by incubating with PE anti-mouse Lineage Cocktail antibody, Brilliant Violet 421–conjugated antibody to Sca1 (Ly6A/E; D7), and allophycocyanin-conjugated antibody to c-Kit (ACK2). LSK cells were also obtained from $FLT3(ITD)^{+/+}$ mice, $FLT3(ITD)^{+/-}$ mice, and their wild-type (wt) littermates.[33] All antibodies were purchased from BioLegend and eBioscience. Cells were sorted with a FACSAria (Becton Dickinson) automated cell sorter at the Temple University Lewis Katz School of Medicine flow cytometry core.

Cell lines

Human acute leukemia cell lines carrying homozygous *FLT3(ITD)* allele (MV-4-11)[34] and *FLT3*(wt) (HL60, REH)[35] were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotic cocktail. Murine BaF3 parental cell and clones overexpressing wt FLT3 and those expressing various FLT3 mutants[36,37] were cultivated in Iscove modified Dulbecco medium supplemented with 10% FBS, interleukin-3 (IL-3), and antibiotic cocktail. Expression of constitutively active FLT3 was confirmed by western blot analysis.

Inhibitors/drugs

The following compounds were used: the FLT3 activity inhibitors AC220, gilteritinib, and crenolanib, the PARP1i's BMN673 and olaparib (all from Selleckchem), mutT homolog 1 inhibitor (MTH1i) SCH51344 (Tocris), and reactive oxygen species (ROS) scavenger vitamin E (Sigma).

Western blot analyses

Nuclear cell lysates and total cell lysates were obtained and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, as previously described.[31] Protein expression was analyzed using primary antibodies detecting BRCA1 (MAB22101; R&D Systems), BRCA2 (MAB2476; R&D Systems), RAD51 (sc-6862; Santa Cruz Biotechnology), DNA-PKcs (A300-518A; Bethyl Laboratories), Ku70 (A302-623A; Bethyl Laboratories), Ku80 (MA5-15873; Thermo Fisher Scientific), PARP1 (sc-7150; Santa Cruz Biotechnology), PALB2 (A302-627A; Bethyl Laboratories), Lig3 (GTX70147; GeneTex), Lig4 (ab26039; Abcam), phospho-Y694/T699 STAT5 (sc-11761; Santa Cruz Biotechnology), STAT5 (sc-28685; Santa Cruz Biotechnology), cleaved caspase-3 (9661; Cell Signaling Technology), and β-actin (A5316; Sigma).

Flow cytometry

DSBs were detected by γ-H2AX immunofluorescence (fluorescein isothiocyanate–conjugated anti–γ-H2AX antibody; 580445; BD Pharmingen), and cell viability was determined using fixable dead cell stain (65-0865-14; eBioscience), as described previously.[38]

Comet assay

The neutral comet assay was performed as described previously.[39]

Examination of HR and D-NHEJ

HR and D-NHEJ activities in BRCA/DNA-PK–deficient (blue) and -proficient (red) primary AML samples were detected as described previously.[40] Briefly, 2-5 × 10^6 cells were nucleofected with 5 μg of I-SceI–linearized HR or NHEJ reporter plasmid and 2.5 μg of pDsRed plasmid (transfection efficiency control) using Nucleofector (program U-008, Human CD34 Cell Nucleofector Kit; Lonza). HR or D-NHEJ event restores functional GFP expression. After 72 hours, the percentage of GFP^+/$DsRed^+$ cells in $DsRed^+$ cells was analyzed by flow cytometry to assess HR and D-NHEJ activity.

In vitro treatment

Lin^-CD34^+ human AML primary cells and human bone marrow cells from healthy donors were cultivated in StemSpan SFEM (STEMCELL Technologies) in the presence of recombinant human growth factors (100 ng/mL stem cell factor [SCF]; 10 ng/mL Flt3 ligand; 20 ng/mL IL-3, IL-6, G-CSF, and granulocyte-macrophage colony-stimulating factor [GM-CSF]; 2.5 ng/mL TPO). Murine FLT3(ITD)-positive cells were cultured in Iscove modified Dulbecco medium supplemented with 20% FBS and recombinant growth factors (15 ng/mL mouse [m]IL-3, 20 ng/mL mSCF, 12.5 ng/mL human [h]IL-6). Cells were incubated with AC220, gilteritinib, crenolanib, olaparib, and/or BMN673 for 3 days, followed by counting in trypan blue or fixable dead cell stain. To examine clonogenic activity, 10^4 cells per 0.1 mL were incubated with the inhibitor(s) or were left untreated for 3 days, followed by plating in MethoCult (STEMCELL Technologies) in the presence of growth factors. Colonies were counted after 7-10 days. For quiescent/proliferating cells, Lin^- cells were stained with CellTrace Violet (CTV; eBioscience) and incubated for 5 days in StemSpan SFEM (STEMCELL Technologies) supplemented with the cocktail of growth factors (15 ng/mL [m]IL-3, 20 ng/mL mSCF, 12.5 ng/mL hIL-6) and inhibitors when indicated. Quiescent (CTV^{max}) and proliferating (CTV^{low}) leukemia cells were detected by flow cytometry using fluorochrome-conjugated anti-Lin (340546), anti-CD34 (347203), and anti-CD38 (555460) antibodies (all from BD Biosciences), as described previously.[41] Lin^-CD34^+ AML primary cells were also cocultured on HS-5 human bone marrow stromal cells (American Type Culture Collection), seeded at a 1:1 ratio under hypoxia (1% O_2), and treated with AC220 and/or olaparib for 3 days, followed by plating in MethoCult in the presence of growth factors.

In vivo treatment

NOD.*Rag1*$^{-/-}$;*γc*^{null} mice expressing human IL-3, GM-CSF, and SCF (NRGS mice,[42] The Jackson Laboratory) were sublethally irradiated (600 Gy) and injected with 1 × 10^6 Lin^-CD34^+ primary AML cells expressing FLT3(ITD) (10KM1950 sample; supplemental Table 1). Three weeks later, mice were treated with vehicle, AC220 (10 mg/kg),[43] BMN673 (0.33 mg/kg),[40] and AC220 + BMN673 for 7 days. $hCD45^+$ AML cells were detected in bone marrow cells, splenocytes, and/or peripheral blood leukocytes at the end of treatment, as described previously.[40,41] Moreover, 1 × 10^6 bone marrow cells were transplanted into the sublethally irradiated secondary recipients to assess the effect of treatment on LSCs. Median survival time of the primary and secondary recipients was determined. To assess the toxicity of the treatment, C57BL/6 mice were treated with vehicle or AC220 + BMN673, as described above. Peripheral blood, bone marrow, and other organs were tested to assess potential toxicity.

Statistical analyses

Data are presented as mean ± standard deviation (SD) from 3 independent experiments and were compared using the unpaired 2-tailed Student *t* test; *P* values <.05 were considered significant. The response additivity approach was used to study synergistic effects.[44] Median survival time of the mice ± standard error was calculated using Kaplan-Meier log-rank survival analysis. *P* values <.05 were considered significant.

Study approval

Human studies were approved by the appropriate Institutional Review Boards and met all requirements of the Declaration of Helsinki. Animal studies were approved by the Temple University Institutional Animal Care and Use Committee.

Results

FLT3i inhibited DSB repair activity in FLT3(ITD)-positive leukemia cells

To determine whether AC220-mediated inhibition of FLT3(ITD) activity is associated with inhibition of DSB repair activity, we performed a western blot array to assess expression of key proteins in DSB repair pathways (Figure 2A). FLT3(ITD)-positive BaF3 cells and their parental counterparts were treated with AC220 for 24 hours in the presence of IL-3 to inhibit FLT3(ITD) activity, as documented by downregulated phosphotyrosine-STAT5 content in total cell lysates (Figure 2B). At the same time, caspase-3 activation was minimal, PARP1 was not cleaved, and AC220-treated cells were alive in the trypan blue exclusion test. Selected key proteins in HR (BRCA1, BRCA2, PALB2, and RAD51) and D-NHEJ (LIG4) were downregulated in nuclear lysates from AC220-treated FLT3(ITD)-positive cells (Figure 2B).

Next, we examined whether AC220-induced downregulation of HR and D-NHEJ proteins caused reduction of DSB repair activities. Using specific reporter plasmids whereby restoration of GFP expression depends on HR or D-NHEJ, we determined that both DSB repair pathways were inhibited in AC220-treated FLT3(ITD)-positive MV-4-11 cells but not in REH cells expressing FLT3(wt) (Figure 2C-D).

These collective results strongly suggest that inhibition of FLT3 (ITD) activity resulted in induction of early defects in DSB repair pathways.

FLT3i enhanced PARP1i-dependent accumulation of DSBs and elimination of leukemia cells bearing FLT3 mutants

BaF3 cells expressing FLT3-activating mutations in juxtamembrane domain (ITD, delEY, delIns) and tyrosine kinase domain 1 (E611V), but not BaF3 cells transfected with empty plasmid and these overexpressing FLT3(wt), were sensitive to the FLT3i's AC220 and crenolanib and the PARP1i's olaparib and BMN673 used individually (Figure 3A; supplemental Figure 3). However, the strongest anti-leukemia effect was exerted by a combination of FLT3i + PARP1i selectively in BaF3 cells expressing FLT3 activating mutations.

It has been reported that FLT3(ITD)-positive leukemia cells accumulate ROS-induced DSBs.[17] To determine whether oxidative stress–induced DSBs contribute to PARP1i-mediated cytotoxicity, leukemia cells were incubated with BMN673 and ROS scavenger vitamin E or MTH1i SCH51344 (MTH1 sanitizes oxidized 2′-deoxynucleoside 5′-triphosphate pools to prevent incorporation of damaged bases during DNA replication).[45] Vitamin E diminished, whereas SCH51344 enhanced, the toxic effect of BMN673 in FLT3(ITD)-positive cells (Figure 3B).

DSBs were examined by neutral comet assay and by detection of Ser139 H2AX phosphorylation (γ-H2AX), which have been shown to give a sensitive and accurate estimation of the number of DSBs within DNA.[46,47] The combination of AC220 and BMN673 induced accumulation of DSBs in FLT3(ITD)-positive MV-4-11 cells, but not in FLT3(wt) HL60 and REH cells, 24 hours after the treatment (Figure 3C-D), which was associated with enhanced death of MV-4-11 cells detected 96 hours after treatment (Figure 3D).

Altogether, we postulate that AC220 sensitized FLT3(ITD)-positive leukemia cells to PARP1i's by promoting accumulation of lethal DSBs, while sparing cells carrying FLT3(wt).

FLT3i's enhanced the sensitivity of FLT3(ITD)-positive proliferative and quiescent LSC-enriched primary cells to PARP inhibitors

To test the effect of the combination of FLT3i and PARP1i on FLT3(ITD)-positive primary cells, LSK bone marrow cells from *FLT3(ITD)*$^{+/+}$, *FLT3(ITD)*$^{+/-}$, and *FLT3(wt)* mice were incubated with AC220 and olaparib, followed by γ-H2AX immunostaining and plating in methylcellulose. The combination of AC220 and olaparib resulted in enhanced accumulation of γ-H2AX–positive *FLT3(ITD)*$^{+/+}$ and *FLT3(ITD)*$^{+/-}$ cells but not *FLT3(wt)* cells (Figure 4A). This effect was associated with AC220 dose-dependent reduction of clonogenic potential of olaparib-treated *FLT3(ITD)*$^{+/+}$ and *FLT3(ITD)*$^{+/-}$ cells compared with their *FLT3(wt)* counterparts (Figure 4B).

To test the effect of FLT3i combined with PARP1i on FLT3(ITD)-positive stem cells, we employed hematopoietic precursors harvested from *FLT3(ITD)*$^{+/+}$;Evi1-GFP$^{+/-}$ mice. Using Evi1-GFP reporter mice, Kataoka et al had demonstrated that EVI1 is expressed exclusively in the hematopoietic stem cell (HSC) population in the bone marrow and that its expression marks hematopoietic cells with long-term multilineage repopulating activity,[32] which was recently reproduced by us.[48] We treated HSCs-enriched mouse FLT3(ITD)-positive GFP$^+$ cells with AC220 and olaparib, and found that FLT3(ITD)-positive GFP$^+$ LSK cells were synergistically sensitive to the combination of AC220 and olaparib (Figure 4C). At the same time, FLT3(wt) counterparts were approximately 7-times less sensitive to AC220 + olaparib.

We reported previously that D-NHEJ–deficient quiescent and HR/D-NHEJ–deficient proliferating tumor cells were sensitive to dual cellular synthetic lethality exerted by PARP1i.[40] Therefore, we tested whether FLT3i-induced downregulation of D-NHEJ and HR sensitizes FLT3(ITD)-positive quiescent and proliferating AML primary cells, respectively, to PARP1i-mediated synthetic lethality. AC220 caused downregulation of BRCA1, BRCA2, RAD51, and LIG4 in Lin$^-$CD34$^+$ primary AML cells (supplemental Figure 4). AC220, as well as the more recently developed FLT3i's gilteritinib and crenolanib, enhanced the sensitivity of LSC/LPC-enriched Lin$^-$CD34$^+$ cells to olaparib and BMN673 (Figure 4D). Moreover, AC220 increased the effect of olaparib against LSC-enriched Lin$^-$CD34$^+$CD38$^-$CTV^{low} proliferating and Lin$^-$CD34$^+$CD38$^-$CTV^{max} quiescent cells (Figure 4E-F). Under the same conditions, the combination of AC220 and olaparib exerted only a modest effect against normal hematopoietic cells (supplemental Figure 5A).

It has been reported that the bone marrow microenvironment induced resistance of FLT3(ITD)-positive AML cells to the kinase inhibitors and cytotoxic drugs.[49] To test the impact of the bone marrow microenvironment on the effectiveness of AC220+/− olaparib, FLT3(ITD)-positive Lin$^-$CD34$^+$ AML primary cells were cocultured under hypoxic conditions (1% O_2) with the HS-5

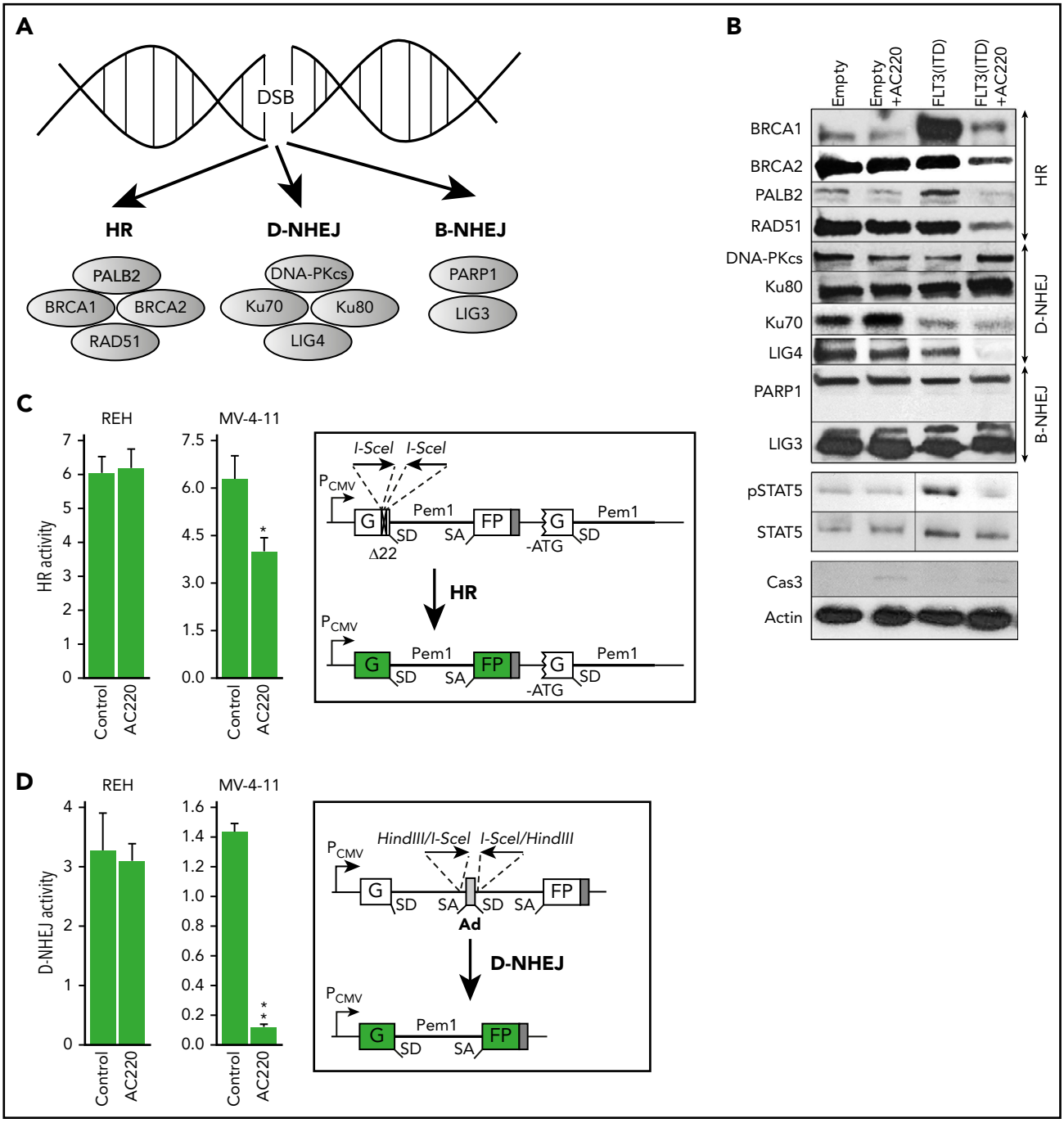

Figure 2. FLT3i AC220 downregulated HR and D-NHEJ proteins and inhibited HR and D-NHEJ activity. (A) DSB repair pathways and some key proteins. (B) Western blot analysis of the indicated proteins in parental BaF3 cells transfected with empty plasmid (Empty) and in those expressing FLT3(ITD) after a 24-hour incubation with 10 nM AC220 or vehicle in the presence of IL-3. Cells were nucleofected with linearized plasmids containing HR (C) or D-NHEJ (D) reporter cassette and pDsRed plasmid (transfection efficiency control). HR or D-NHEJ event restores functional GFP expression. After 72 hours, $GFP^+/DsRed^+$ cells in $DsRed^+$ cells were analyzed by flow cytometry to assess HR and D-NHEJ activity in untreated (Control) and AC220-treated (10 nM) REH cells and MV-4-11 cells. Results represent mean (percentage) ± SD of $GFP^+/DsRed^+$ cells in $DsRed^+$ cells from triplicates per sample. $^*P = .02$, $^{**}P < .001$, 2-tailed Student *t* test.

stromal cell line, which secrete significant levels of G-CSF, GM-CSF, macrophage colony-stimulating factor, SCF, macrophage inflammatory protein-1α, IL-6, IL-8, and IL-11 to support the growth of HSCs.[50] Under these conditions, olaparib reduced the number of clonogenic leukemia cells not eliminated by the high concentration (100 nM) of AC220 (Figure 4G).

Altogether, these results support the conclusion that inhibition of FLT3(ITD) activity enhanced the sensitivity of proliferating and quiescent LSCs to PARP1i's in vitro, while sparing significant numbers of normal counterparts.

AC220 enhanced the effect of BMN673 against FLT3(ITD)-positive primary AML xenografts in humanized immunodeficient mice

NRSG mice bearing FLT3(ITD)-positive AML primary cells were treated with vehicle (control), AC220, BMN673, or AC220 + BMN673

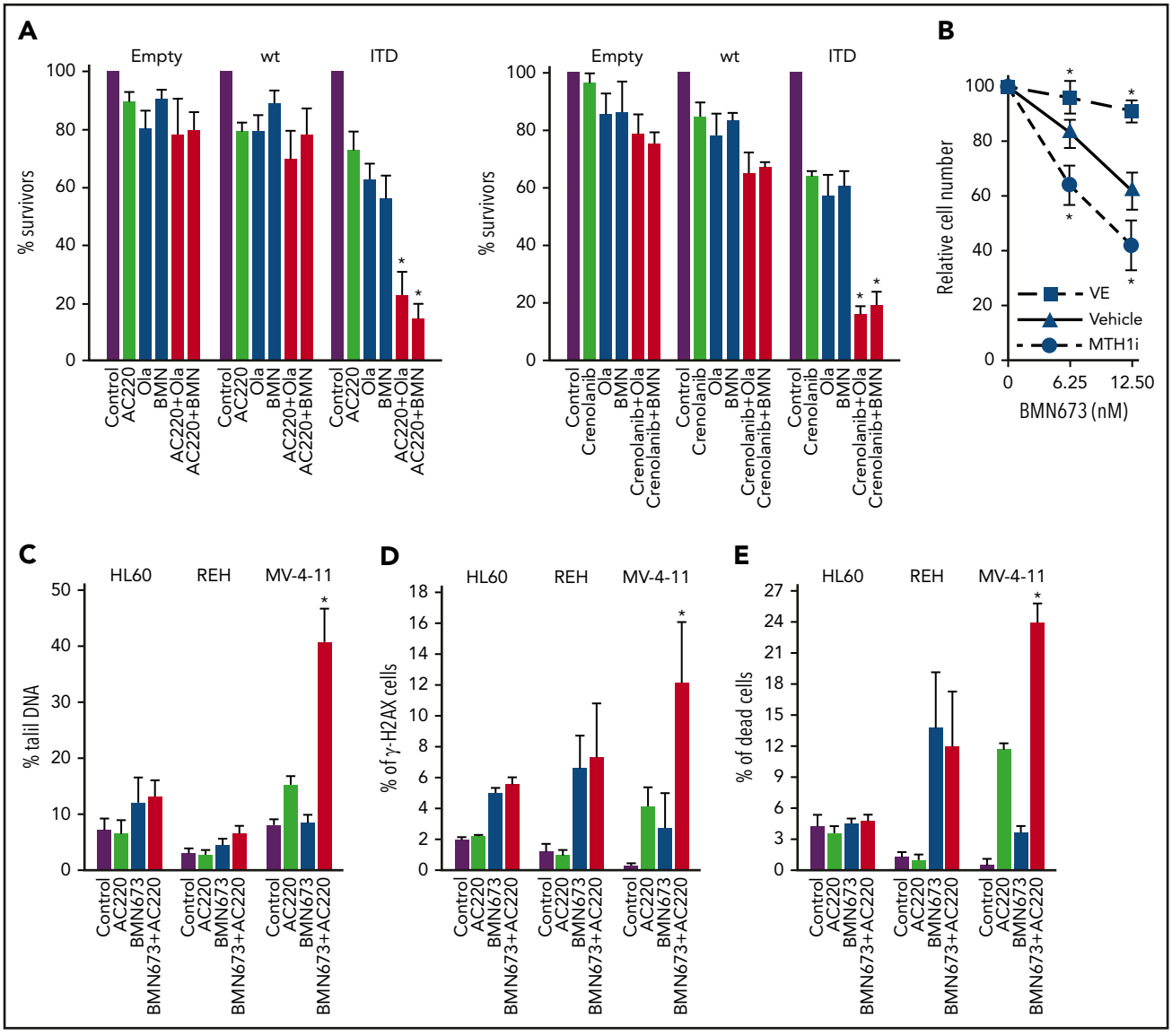

Figure 3. FLT3i's enhanced the antileukemia effect of PARP1i's in FLT3(ITD)-positive cell lines. (A) BaF3 parental cells transfected with empty plasmid (Empty) and those expressing FLT3(wt) or FLT3(ITD) mutant (ITD) were left untreated (Control) or were incubated with PARP1i [1.25 μM olaparib (Ola), 12.5 nM BMN673 (BMN)], FLT3i (10 nM AC220, 10 nM crenolanib), and FLT3i+PARP1i for 96 hours in the presence of IL-3. Results from 3 experiments are represented by the mean (percentage) ± SD of living cells detected by trypan blue exclusion compared with untreated counterparts. *$P < .001$ vs individual treatment, 2-tailed Student *t* test. (B) FLT3(ITD)-positive BaF3 cells were incubated with the indicated concentrations of BMN673, alone or in combination with 200 μM vitamin E (VE) or 3 μM MTH1i SCH51344, for 96 hours in the presence of IL-3. Results represent mean (percentage) ± SD of living cells detected by trypan blue exclusion in comparison with untreated control from 3 independent experiments. *$P < .05$ vs BMN673 treatment, 2-tailed Student *t* test. (C-E) MV-4-11 [FLT3(ITD)-positive] and HL60 and REH [both FLT3(ITD)-negative] human leukemia cell lines were left untreated (Control) or were treated with 5 nM BMN673, 10 nM AC220, or the combination. DSBs were detected after 24 hours by neutral comet assay (C) and γ-H2AX immunofluorescence (D). (E) Dead cells were determined by fixable dead cell stain after 96 hours. Results represent mean ± SD from 3 experiments. *$P < .02$ vs individual treatment, 2-tailed Student *t* test (C-E).

(Figure 5A). The therapeutic effect was examined by detection of hCD45$^+$ cells in peripheral blood and bone marrow, and by determination of median survival time (MST) of the treated mice. In addition, 1 × 10^6 bone marrow cells were retransplanted into secondary recipients to examine LSCs.

Leukemic mice treated with AC220 + BMN673 contained significantly fewer hCD45$^+$ leukemia cells in peripheral blood and bone marrow than did those treated with individual drugs (Figure 5B). Moreover, the combination of AC220 + BMN673 exerted a synergistic effect against hCD45$^+$ cells in bone marrow. In addition, MST of the leukemia-bearing mice was significantly prolonged by the combination of AC220 + BMN673 in comparison with mice treated with AC220 or BMN673 (Figure 5C). Importantly, secondary recipients injected with bone marrow cells harvested at the end of AC220 + BMN673 treatment survived longer than did mice injected with cells from individually treated animals (Figure 5D), suggesting that the combination targeted LSCs in vivo.

AC220 + BMN673 treatment did not appear to be significantly toxic for normal cells and tissues, except for the transient reduction (by ~43%) in the number of white cells in bone marrow (supplemental Table 2; supplemental Figure 6). Because the pharmacokinetic parameters of FLT3i and PARP1i may differ in humans and mice, we examined the prolonged in vitro effect of clinically relevant concentrations of AC220 combined with olaparib

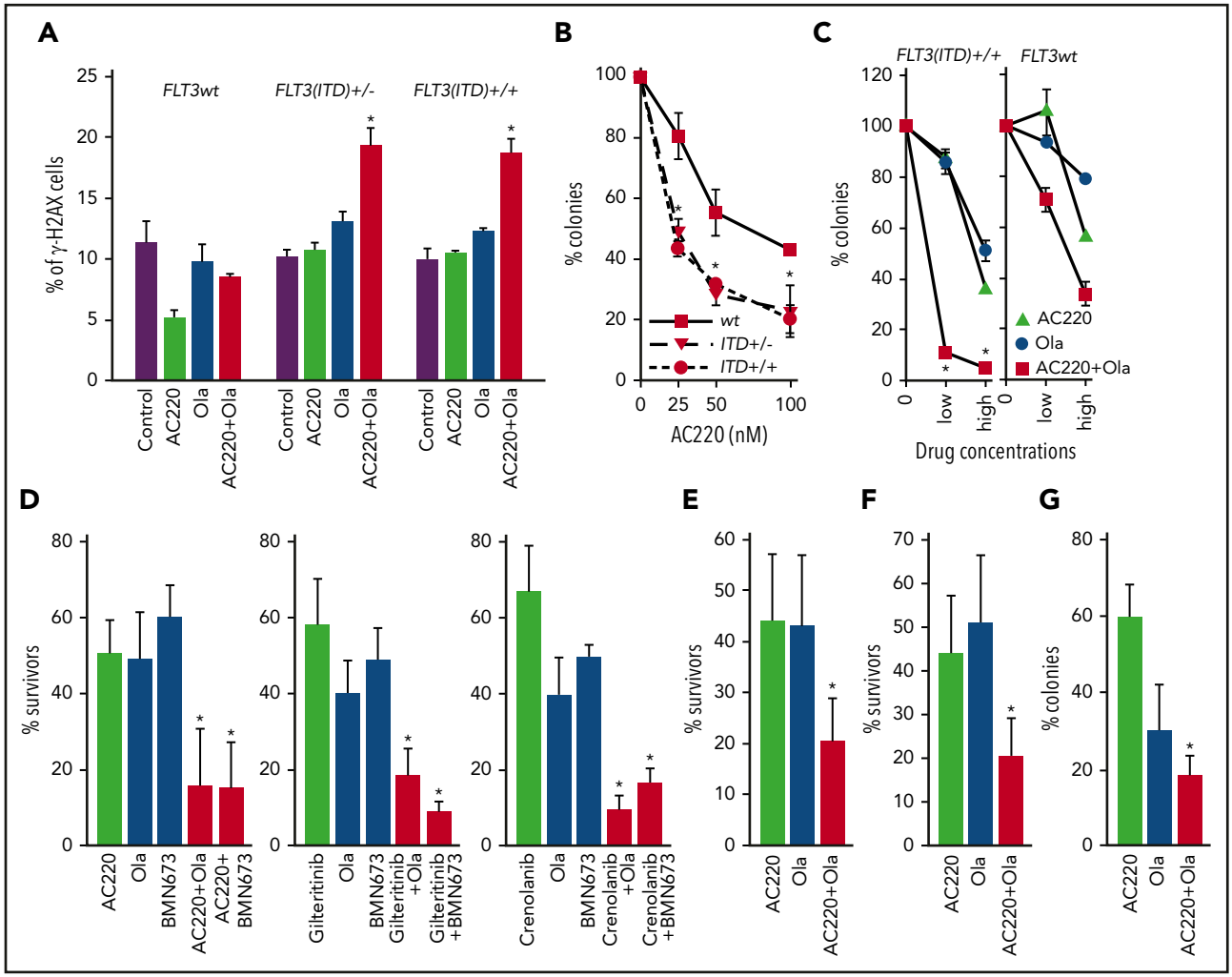

Figure 4. FLT3i enhanced the antileukemia effect of PARP1i's against proliferating and quiescent primary FLT3(ITD)-positive cells. (A-B) LSK bone marrow cells from *FLT3(ITD)*$^{+/+}$, *FLT3(ITD)*$^{+/-}$, and *FLT3(wt)* mice (n = 3 per group) were incubated for 24 hours with 100 nM AC220 and/or 2.5 μM olaparib (Ola), followed by γ-H2AX immunostaining, and for 4 days with 2.5 μM Ola and the indicated concentrations of AC220, followed by plating in methylcellulose. Results represent mean (percentage) ± SD of γ-H2AX positive cells (A) and surviving clonogenic cells (B) compared with their Ola-treated counterparts. *$P < .05$, 2-tailed Student *t* test. (C) GFP$^+$ FLT3(ITD)-positive LSK murine bone marrow cells [*FLT3(ITD)*$^{+/+}$] and GFP$^+$ LSK normal counterparts [*FLT3(wt)*] obtained from 2 or 3 mice per group were left untreated (0) or were treated with AC220 (low: 20 nM; high: 100 nM), Ola (low: 1 μM; high: 2.5 μM), and the combination of AC220 + Ola in the presence of growth factors for 4 days, followed by plating in methylcellulose. Results represent mean (percentage) ± SD of colonies in comparison with untreated group from triplicate experiments. *$P < .001$ vs individual treatments using response additivity approach. Lin$^-$CD34$^+$ FLT3(ITD)-positive cells from 5 AML patients (188, 194, 499, 9KM3949, 10KM1950) were stained with CTV and left untreated or were treated with the FLT3i's AC220 (10 nM), crenolanib (10 nM), and gliteritinib (10 nM), the PARP1i's Ola (2.5 μM) and BMN673 (25 nM), and the combination of FLT3i + PARP1i in the presence of growth factors for 3 days, followed by assessment of Lin$^-$CD34$^+$ clonogenic cells (D) and immunofluorescent detection of Lin$^-$CD34$^+$CD38$^-$CTV^{low} cells (E) and Lin$^-$CD34$^+$CD38$^-$CTV^{max} cells (F). Results represent mean (percentage) ± SD of clonogenic/surviving cells in comparison with untreated cells from triplicate experiments. *$P < .05$ vs individual treatments, 2-tailed Student *t* test. (G) Lin$^-$CD34$^+$ FLT3(ITD)-positive cells from 4 AML patients (188, 194, 499, 10KM1950) were left untreated or were treated with AC220 (100 nM), Ola (2.5 μM), and the combination of AC220 + Ola on HS-5 cell monolayers in 1% O_2 for 3 days, followed by plating in methylcellulose. Results represent mean (percentage) ± SD of clonogenic cells in comparison with untreated cells from triplicate experiments. *$P < .05$ vs individual treatment, 2-tailed Student *t* test.

on human Lin$^-$CD34$^+$ bone marrow cells obtained from healthy donors. Seven-day treatment with AC220 (535 nM)[51] + olaparib (6 μM)[52] in 100% FBS inhibited their clonogenic activity by ~40%, and olaparib did not significantly increase the toxicity of AC220 (supplemental Figure 5B).

Discussion

Although tyrosine kinase inhibitors, such as AC220 (quizartinib), target FLT3(ITD) activity, which functions as a driver oncogene in a large subgroup of AMLs, the treatment, even if combined with cytotoxic drugs, is not curative in the vast majority of cases because leukemia cells are able to deal with DNA damage.[2,53-55] Therefore, we postulated that specific inhibitors of DNA repair may dramatically improve the therapeutic outcome of patients with FLT3(ITD)-positive AML. The success of PARP1i's in patients with BRCA1/2 mutated breast and ovarian carcinomas supported our hypothesis.[28]

Although inactivating mutations in genes whose products are responsible for DNA repair are rather infrequent in leukemias,[29] we and other investigators reported that the presence of certain leukemia-driving oncogenes (eg, AML1-ETO, BCR-ABL1, PML-RARA) is associated with BRCA and/or DNA-PK–deficient status and sensitivity to synthetic lethality induced by PARP1i's.[31,40,56-58] On the other hand, because FLT3(ITD) activity was associated with activation of BRCA1-RAD51–dependent HR, it was unlikely that FLT3(ITD)-positive AML cells will be highly sensitive

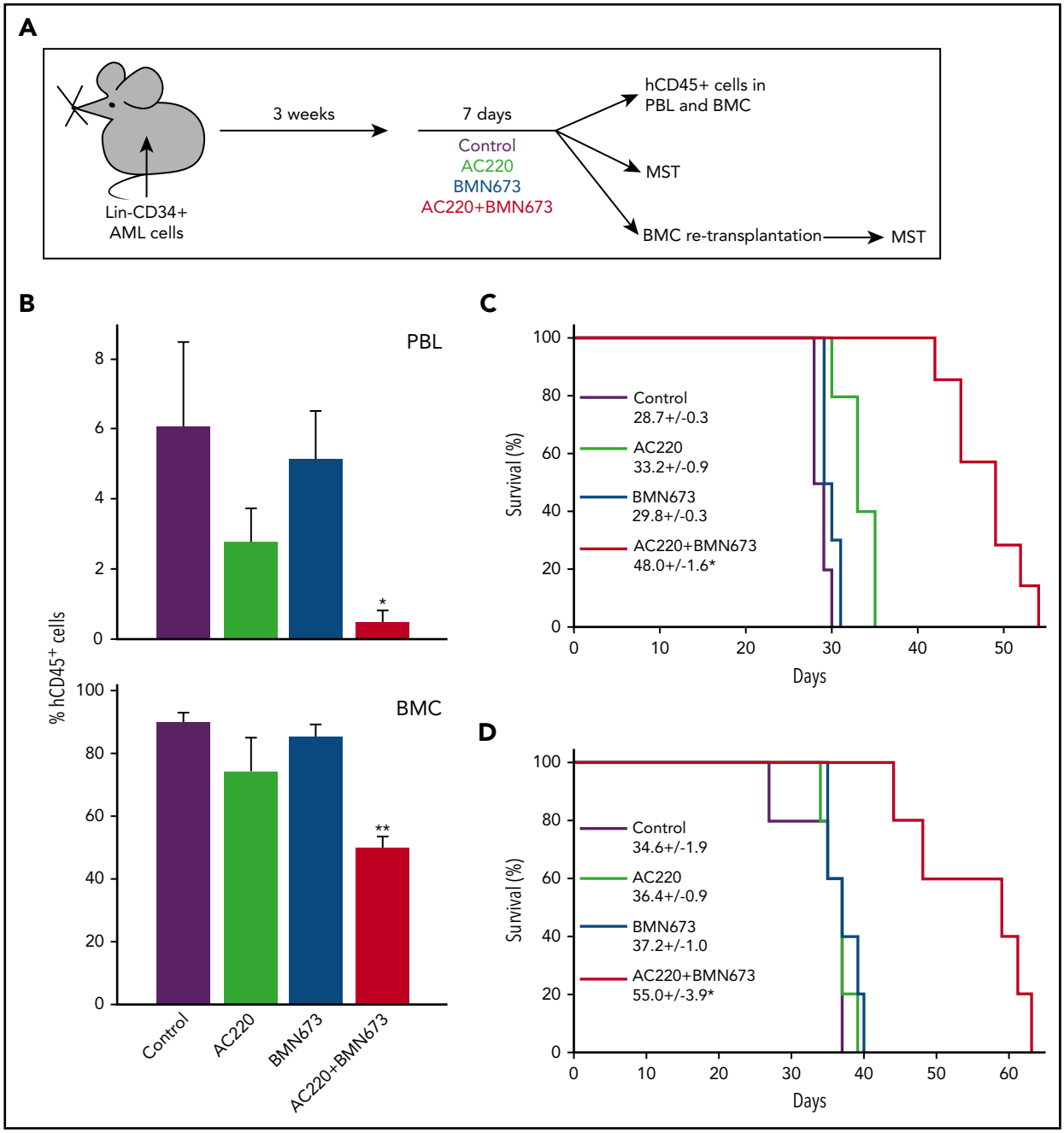

Figure 5. PARP1i BMN673 combined with FLT3i AC220 inhibited the growth in FLT3(ITD)-positive AML primary cells in mice and reduced the number of LSCs. (A) Experimental design. (B) Mean percentage of hCD45$^+$ cells ± SD in peripheral blood cells (PBL, n = 5-6) and bone marrow cells (BMC, n = 3-5). *$P < .01$, **$P = .002$ vs individual treatment, Student *t* test and response additivity approach, respectively. (C) Survival curves and MST of mice bearing a primary AML xenograft and treated as indicated (n = 6-10 mice per group). (D) Survival curves and MST of secondary transplant mice. *$P \leq .002$ vs individual treatments, Kaplan-Meier log-rank test.

to PARP1i's.[55,59] In concordance, we detected that FLT3(ITD)-positive cells displayed only modest sensitivity to PARP1i's, which is probably due to the accumulation of ROS-mediated DNA damage and/or inhibition of Ku70 protein involved in D-NHEJ.[17,35]

Because AC220 promotes quiescence rather than cytotoxicity,[60] it seemed important to combine AC220 with a drug that would be able to eliminate proliferating and nonproliferating cells. Remarkably, inhibition of FLT3(ITD) activity by AC220 caused early and dramatic downregulation of selected proteins in HR (BRCA1, BRCA2, PALB2, and RAD51) and D-NHEJ (LIG4), which resulted in simultaneous inhibition of HR and D-NHEJ activities ("BRCAness/DNA-PKness" phenotype). Numerous major intracellular signaling pathways, including PI3K-AKT, RAF1-MEK, and JAK2-STAT5, are activated in FLT3(ITD)-positive AML cells.[61,62] Results obtained with the use of specific inhibitors suggest that JAK2 kinase stimulates expression of BRCA1, RAD51, and LIG4; PI3K promotes expression of RAD51 and LIG4; and RAF1 kinase does not

regulate expression of these proteins in FLT3(ITD)-positive cells (supplemental Figure 7). The mechanisms responsible for FLT3i-mediated downregulation of BRCA2 and PALB2 are not known.

We have reported previously that HR- and D-NHEJ–deficient proliferating and quiescent leukemia cells were sensitive to synthetic lethality triggered by PARP1i's.[40] Therefore, PARP1i's should eliminate proliferating, as well as quiescent, leukemia cells. In concordance, we observed that FLT3i-treated FLT3(ITD)-positive AML cells, including LSC-enriched $Lin^{-}CD34^{+}CD38^{-}$ quiescent and proliferating cells, were extremely sensitive to PARP1i's in vitro and in vivo. This effect was associated with accumulation of DSBs and induction of cell death. The antileukemic effect of FLT3i AC220 + PARP1i olaparib was enhanced by addition of a standard cytotoxic drug, such as doxorubicin (supplemental Figure 8).

It has been reported that G_0-arrested FLT3(ITD)-positive leukemia cells display inappropriate RAD51 expression and HR activity.[55] Therefore FLT3i-mediated inhibition of RAD51-mediated HR in these cells may cause the "BRCAness" phenotype and also contribute to PARP1i-mediated synthetic lethality in FLT3(ITD)-positive quiescent AML cells.

We also showed that PARP1i used as a single agent was able to reduce the number of AML-initiating FLT3(ITD)-positive cells, as well as clonogenic cells, in bone marrow, including the hypoxic conditions mimicking the bone marrow microenvironment. This observation is in concordance with another report demonstrating hypoxia-induced HR deficiency and enhanced synthetic lethality triggered by PARP1 inhibition.[63] Moreover, combination of FLT3i + PARP1i was more effective than individual treatment. This is particularly important because FLT3(ITD)-positive leukemia cells expand in the bone marrow niche, which also promotes their survival under FLT3i treatment.[64,65] Altogether, we postulate that the presence of FLT(ITD) creates a window of opportunity to induce the FLT3i-mediated "BRCAness/DNA-PKness" phenotype promoting a profound PARP1i-mediated synthetic lethal effect in AML quiescent and proliferating stem and progenitor cells. This effect most likely depends on inhibition of FLT3(ITD) activity, because we showed that the FLT3i-mediated "BRCAness/DNA-PKness" phenotype was selectively induced in FLT3(ITD)-positive cells and that 3 FLT3i's displaying various target repertoires (AC220-FLT3, c-KIT, and PDGFRα; gilteritinib-FLT3 and AXL; and crenolanib-FLT3 and PDGFR)[66] enhanced the PARP1i-mediated anti-AML effect.

The degree of sensitivity of FLT3(ITD)-positive AML cells to PARP1i and to a combination of FLT3i + PARP1i could be affected by additional mutations (eg, AML1-ETO and MLL-AF90) and/or overexpression of transcription factors HOXA9 + MEIS1 (supplemental Figure 9). AML1-ETO–driven exceptional sensitivity and HOXA9 + MEIS1–mediated resistance to olaparib could be explained by BRCA deficiency and BRCA proficiency, respectively, as reported by us and other investigators.[40,58] On the other hand, strong sensitivity of BRCA-proficient MLL-AF9–expressing cells to AC220 + olaparib may be due to their enhanced overall dependence on PARP1-mediated DNA repair to promote survival under genotoxic stress and on FLT3i-mediated BRCA deficiency.[67]

In conclusion, we postulate that FLT3i-induced deficiencies in DSB repair pathways sensitize FLT3(ITD)-positive AML cells to synthetic lethality triggered by PARP1i's. Therefore, FLT3(ITD) could be used as a precision medicine marker to identify patients with AMLs who may benefit from a therapeutic regimen combining FLT3 and PARP1i's. Moreover, TKI-mediated inhibition of DSB repair, which sensitizes malignant cells to PARP1i-triggered synthetic lethality, may have a more broad application in tumors expressing oncogenic tyrosine kinases. This speculation is supported by our recent study indicating that the JAK2 inhibitor ruxolitinib caused the "BRCAness/DNA-PKness" phenotype, resulting in PARP1i-mediated synthetic lethality in myeloproliferative neoplasms.[68]

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Authorship

Contribution: S.M., M.N.-S., Y.D., K.S.-R., B.V.L., Z.L., P.P.-B., E.A.B., M.S., A.N., and M.T. performed experiments; M.M.M. and M.R. prepared next-generation sequencing (NGS) libraries and performed NGS of several patient samples; H.Z. performed statistical analyses; J.J. analyzed The Cancer Genome Atlas (TCGA) database; K.P. supervised B.V.L., M.A.W. supervised E.A.B., and T. Sliwinski supervised M.T.; T. Stoklosa analyzed NGS data and supervised M.M.M.; R.P. analyzed NGS data and supervised M.R.; T.F., S.M.S., N.C., S.K., L.B., P.V., and J.H. provided essential cell lines and primary cells and contributed to the final version of the manuscript; and T. Skorski conceived the idea, supervised the project, and wrote the final version of the manuscript.

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Footnotes

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