

Inhibition of menin, BCL-2, and FLT3 combined with a hypomethylating agent cures *NPM1/FLT3-ITD/-TKD* mutant acute myeloid leukemia in a patient-derived xenograft model

Menin inhibition has antileukemia activity in both, mixed-lineage leukemia-rearranged (*MLL-r*) and nucleophosmin (*NPM1*)-mutant (*NPM1c*) acute myeloid leukemia (AML)¹⁻⁵ by suppressing the menin-dependent *HOX/MEIS* gene signature.⁶ Menin inhibitors are in clinical development in hematological malignancies with these genomic alterations. While initial clinical results indicate efficacy in *MLL-r* and *NPM1c* AML, menin inhibition combined with other targeted agents has potential to enhance efficacy. *FLT3* is frequently mutated in *MLL-r* and *NPM1c* AML. Co-inhibition of menin and FLT3 preclinically showed enhanced activity in *MLL-r/FLT3-* and *NPM1/FLT3-*mutant AML.^{7,8} We recently reported that menin inhibition by SNDX-50469 synergized with BCL-2 inhibition by venetoclax *in vitro* in *NPM1/FLT3-*mutant primary AML cells and *in vivo* in a patient-derived xenograft (PDX) model of AML with *NPM1/FLT3-ITD/FLT3-TKD* mutations, resulting in significant survival extension.⁹ Others have since reported that menin and BCL-2 inhibitor combinations synergize against *MLL-r* and *NPM1c* AML.¹⁰ In agreement with reports that menin inhibition targets FLT3 in *NPM1/FLT3-*mutated AML, we previously showed that SNDX-50469 reduces p-FLT3 and that the combination of SNDX-50469 and venetoclax enhances this reduction in *NPM1/FLT3-*mutant patient cells *in vitro*. Interestingly, we observed that cells surviving the SNDX-50469/venetoclax combination at the end of treatment had increased p-FLT3 signaling *in vivo* in a *NPM1c/FLT3-ITD/FLT3-TKD* PDX model.⁹ This increase in p-FLT3 likely increased MCL-1 and contributed to leukemia outgrowth. We therefore investigated whether adding FLT3 inhibitor gilteritinib could enhance the activity of SNDX-50469 and venetoclax combination. We here report that combined inhibition of menin, BCL-2, and FLT3 has superior antileukemia activities in *NPM1/FLT3-*mutated AML *in vitro* and *in vivo* and that the combination effectively reduces BCL-2 and *HOX9/MEIS* expression and is potentially curative in a *NPM1/FLT3-*mutated AML model, in which efficacy is further enhanced in combination with 5-azacitidine.

Mononuclear cells from *NPM1-/FLT3-*mutant AML patients (all had additional co-mutations and 3/4 were resistant to venetoclax and FLT3 inhibitors; *Online Supplementary Table S1*) co-cultured with bone marrow (BM)-derived stromal cells were treated with two doses of SNDX-50469,

gilteritinib, venetoclax, or combinations. SNDX-50469/gilteritinib/venetoclax combination at one- or two-dose levels significantly increased apoptosis (Figure 1A) and decreased viable cell numbers (Figure 1B) compared to control, single-drug, or two-drug combinations in blasts and CD34⁺ AML stem/progenitor cells. SNDX-50469/gilteritinib/venetoclax/5-azacitidine was equally and possibly more effective than the triple-drug combination (Figure 1). The experiment in mice was conducted using the same PDX model (*NPM1c/FLT3-ITD/FLT3-TKD*) employed in our prior SNDX-50469/venetoclax combination study.⁹ When circulating human (hu) CD45⁺ cells reached 2.6%, the PDX-bearing NSG mice were treated: i) vehicle, ii) SNDX-50469 (0.1% in chow), iii) gilteritinib (35 mg/kg), iv) SNDX-50469/gilteritinib, v) gilteritinib/venetoclax (50 mg/kg), vi) SNDX-50469/gilteritinib/venetoclax, or vii) SNDX-50469/gilteritinib/venetoclax/5-azacitidine (2.5 mg/kg) (Figure 2A). Due to weight loss in mice treated with the triple-drug (1 died on treatment day 8) and quadruple-drug combinations (2 died on treatment day 7) (excluded in subsequent analysis), gilteritinib was reduced from 35 to 25 mg/kg and venetoclax was reduced from 50 to 35 mg/kg in these two groups beginning on treatment day 10, which prevented further weight loss (*Online Supplementary Figure S1*).

Disease progression and treatment response were assessed by flow cytometric measurement and/or immunohistochemical staining of huCD45⁺ cells in peripheral blood or tissues collected at the end of the treatment (31 days) or at the moribund stage. In order to assess the treatment effects on leukemia blasts and phenotypically-defined leukemia stem/progenitor cells and proteins in BM leukemia cell populations, we performed cytometry by time of flight (CyTOF) single-cell proteomic analysis¹¹ using an antibody panel described previously⁹ with additional antibodies against *HOXA9*, *MEIS1*, and *PBX3*.

At 2 weeks, all treatment arms significantly lowered the circulating huCD45⁺ cells compared to vehicle controls, and gilteritinib and gilteritinib/venetoclax greatly enhanced the activity of SNDX-50469 (Figure 2B). At 4 weeks, all treatment groups had significantly reduced circulating blasts compared to controls; no significant differences between treatment groups were observed (Figure 2C). Post-treatment assessments showed that all treatment groups had significantly lower splenic leukemia

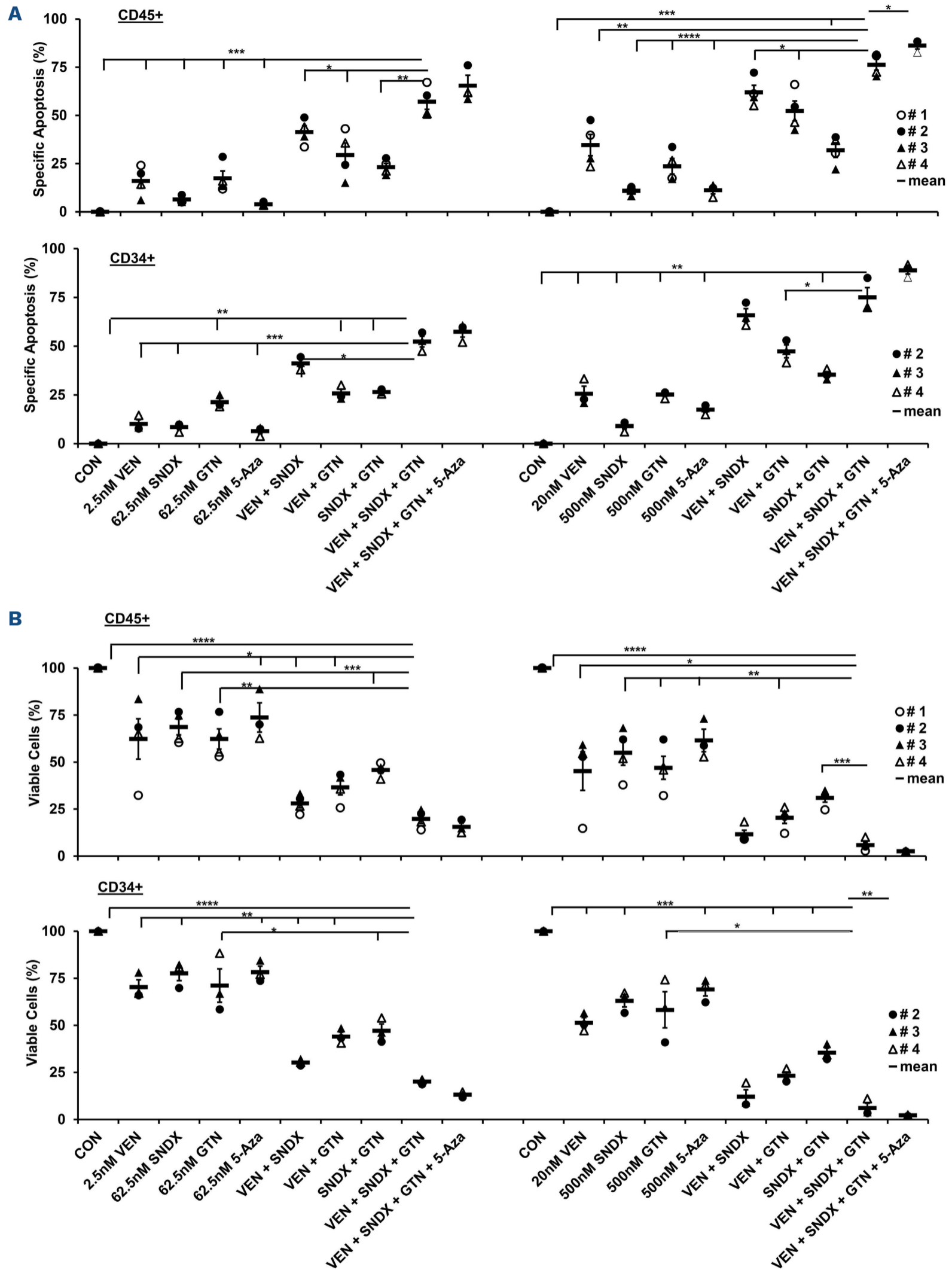


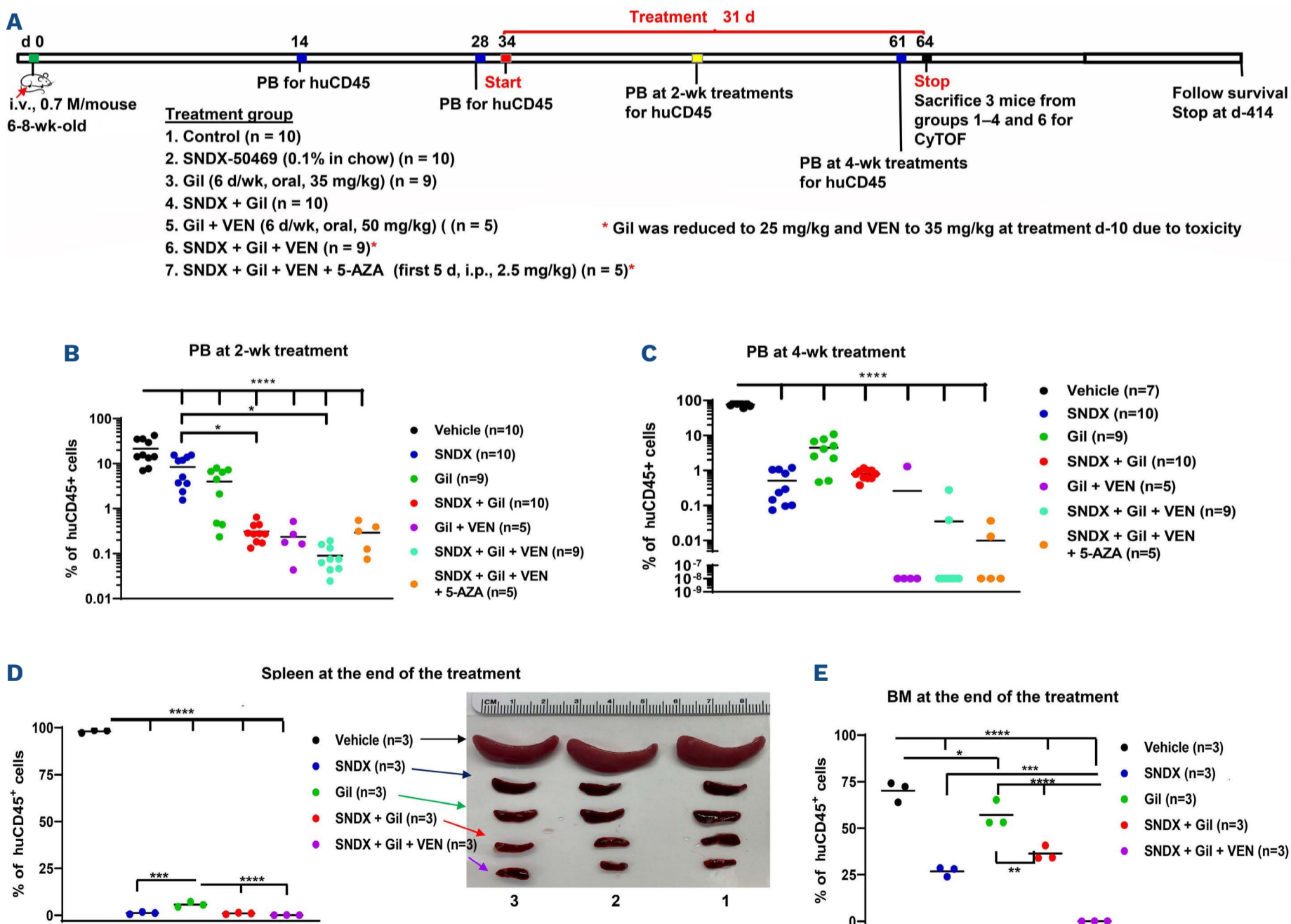
Figure 1. The combined inhibition of menin, BCL-2, and FLT3 exerts strong antileukemia activity in acute myeloid leukemia cells and stem/progenitor cells from patients with *NPM1*-/*FLT3*-mutant acute myeloid leukemia. Peripheral blood cells from patients with *NPM1*-/*FLT3*-mutant acute myeloid leukemia (AML) were co-cultured with bone marrow (BM)-derived stromal cells and treated with venetoclax (VEN), SNDX-50469 (SNDX), gilteritinib (GTN), 5-azacytidine (5-Aza), or various combinations for 24 hours (h). Apoptosis (Annexin V⁺) (A) and viable cells (B) in CD45⁺ and CD34⁺ cell populations were determined by flow cytometry.¹¹ Samples were obtained after acquiring written informed consent following MD Anderson Cancer Center Institutional Review Board approved protocol and in accordance with the Declaration of Helsinki. CON: control.

burdens than controls; SNDX-50469, SNDX-50469/gilteritinib, and SNDX-50469/gilteritinib/venetoclax were significantly more active than gilteritinib; and SNDX-50469/gilteritinib/venetoclax was more effective than SNDX-50469 and SNDX-50469/gilteritinib but did not reach statistical significance (Figure 2D). These results were consistent with the observed reduction in spleen sizes. All treatment groups had significantly lower BM leukemia burden than controls; of these treatment groups, gilteritinib was least effective and did not enhance the activity of SNDX-50469, which was significantly more active than gilteritinib. The percentage of BM leukemia cells in the SNDX-50469/gilteritinib/venetoclax group was significantly lower than those in all other treatment groups (Figure 2E).

All treatments significantly extended survival (Figure 2F) compared with controls (median survival duration 62 days). SNDX-50469 (survival duration 128 days) was significantly more effective than gilteritinib (survival duration 90.5 days). Control and SNDX-50469-treated mice had survival durations similar to those observed in our previous study.⁹ The

survival duration of the gilteritinib/venetoclax (121 days) and SNDX-50469/gilteritinib groups did not differ in this model. Even with reduced gilteritinib and venetoclax doses, the SNDX-50469/gilteritinib/venetoclax combination extended survival (survival duration >235 days) significantly longer than SNDX-50469, gilteritinib, SNDX-50469/gilteritinib, or venetoclax/gilteritinib, which was also superior than survival benefit achieved with the SNDX-50469/venetoclax combination (survival duration 143 days).⁹ The survival benefit is closely reflected in reductions observed in the BM leukemia burden, which supports the importance of BM blast reduction/elimination in this model.

Venetoclax as a single agent has limited clinical activity in resistant/relapsed AML.¹² Elderly AML patients have high response rates to venetoclax/hypomethylating agent combinations.¹³ In order to determine if hypomethylating agents further improve survival, we also treated mice with SNDX-50469/gilteritinib/venetoclax plus 5-azacytidine. The median survival duration had not been reached when the experiment was terminated on day 414. One mouse treated with the quadruple-drug combination (*, Figure 2F) survived



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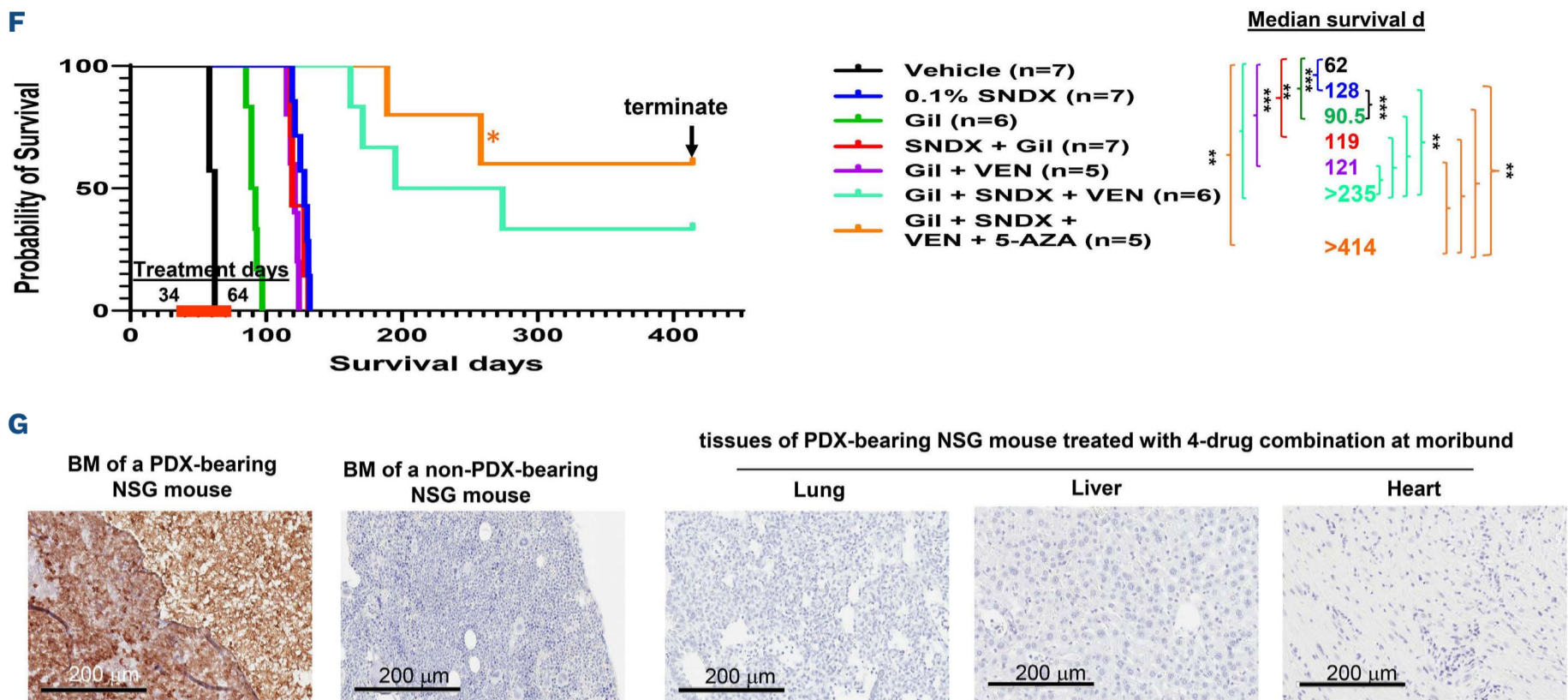
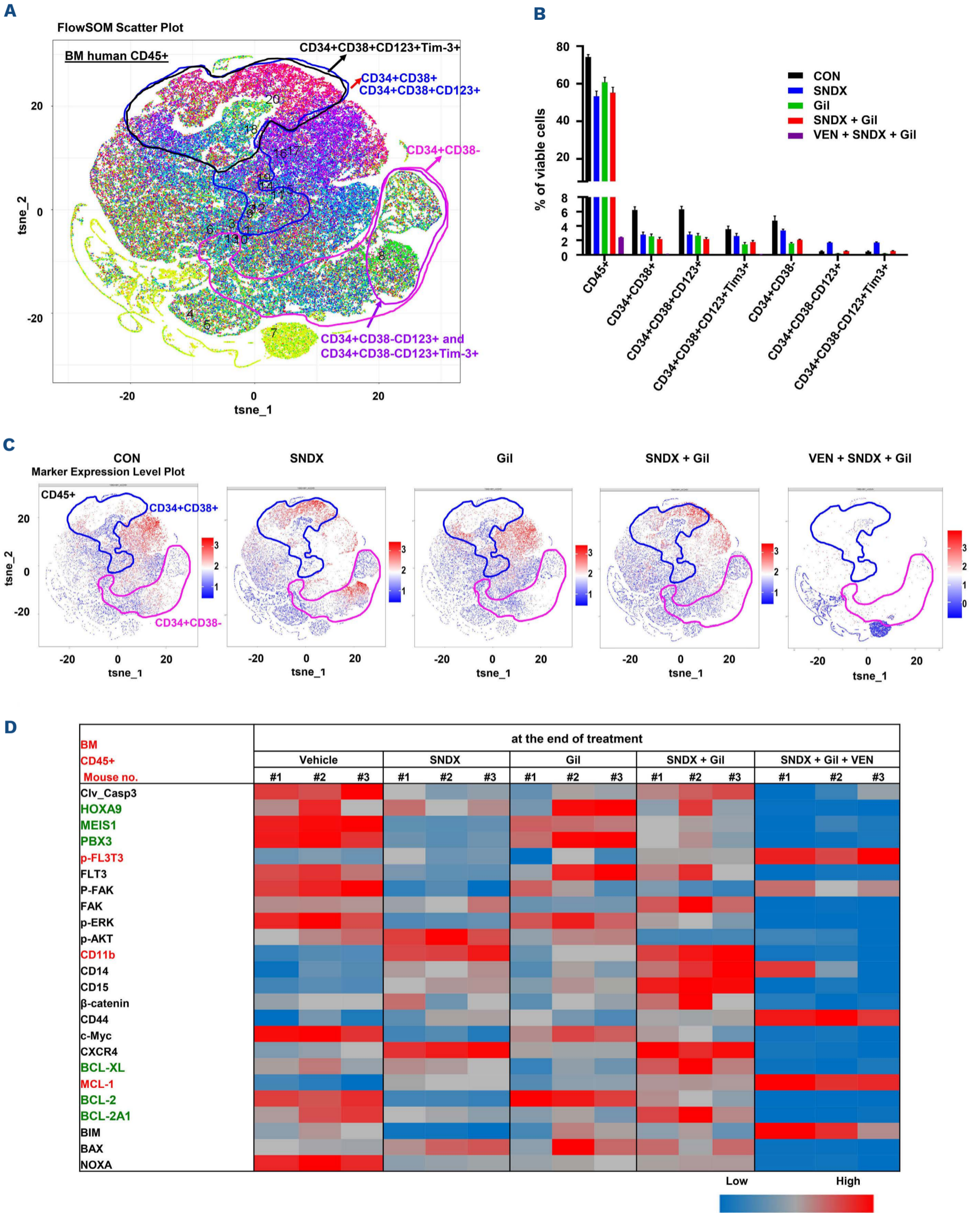


Figure 2. The combined inhibition of menin, BCL-2, and FLT3 exerts strong antileukemia activity and prolongs survival in an *NPM1c/FLT3-ITD/TKD* acute myeloid leukemia patient-derived xenograft model. Experiments in mice were conducted in accordance with Institutional Animal Care and Use Committee approved protocols. (A) The experimental scheme. (B-E) Percentages of human (hu) CD45⁺ in the peripheral blood at 2 weeks (B) and 4 weeks (C) and in the spleen (D) and bone marrow (BM) (E) at the end of treatment, as determined by flow cytometry. Spleens harvested at the end of the treatment are also shown in (D). (F) Survival by treatment type. Mouse survival was estimated using the Kaplan-Meier method, and survival data were analyzed using the log-rank test. (G) Immunohistochemical staining for huCD45. Left, immunohistochemical staining for huCD45 in BM cells from a patient-derived xenograft (PDX)-bearing NSG mouse (positive control) and BM cells from a non-PDX-bearing NSG mouse (negative control). Right, immunohistochemical staining for huCD45 in lung, liver, and heart tissues from a mouse treated with the quadruple-drug combination (marked * in [F]). Differences between groups were determined using the Student *t*-test. *P* values ≤ 0.05 were considered statistically significant. **P* ≤ 0.05 ; ***P* ≤ 0.01 ; ****P* ≤ 0.001 ; *****P* ≤ 0.0001 . M: million; d: day; wk: week; PB: peripheral blood; SNDX: SNDX-50469; Gil: gilteritinib; VEN: venetoclax; 5-AZA: 5-azacitidine.

258 days with a minimal leukemia burden in the BM (0.06%) and spleen (0.15%) and no huCD45⁺ cells were detected in the lungs, liver, or heart (Figure 2G). Importantly, two of six mice in the triple- and three of five mice in the quadruple-drug treatment groups lived close to the life expectancy of normal NSG mice with no detectable leukemia cells in the spleen or BM (*Online Supplementary Table S2*) when the experiment was terminated suggesting that these combinations eliminated all leukemia cells resulting in cure. CyTOF analysis clustered BM leukemia cells at the end of therapy based on cell surface antigen expression patterns (Figure 3A). The percentages of viable leukemia blasts and phenotypic stem/progenitor cells in the treatment groups and the cell populations in representative mice from each group are shown in Figure 3B, C, respectively. As we reported previously,⁹ SNDX-50469 is more active in CD34⁺CD38⁺ and CD34⁺CD38⁺CD123⁺ cell populations, except for CD34⁺CD38⁺CD123⁺Tim3⁺ cells, than CD34⁺CD38⁻CD123⁺, or CD34⁺CD38⁻CD123⁺Tim3⁺ populations, which are more sensitive to gilteritinib. The SNDX-50469/gilteritinib combination did not exhibit enhanced activity compared to either agent alone, but the triple-drug combination greatly reduced the number of leukemia blasts and stem/progenitor cells.

Protein expression data of BM leukemia cells at the end of treatment are shown in Figure 3D. Consistent with studies showing the effects of SNDX-50469 on RNA levels in Molm13 cells,⁵ CyTOF analysis revealed that cells treated with SNDX-50469 and SNDX-50469/gilteritinib exhibited reduced expression of MEIS1 and PBX3 proteins and to a lesser degree of HOXA9, *in vivo*. Cells treated with SNDX-50469/gilteritinib/venetoclax showed markedly reduced expression of HOXA9, and more profound reductions of MEIS1, PBX3, BCL-2, BCL-2A1, and BCL-XL than cells treated only with SNDX-50469, consistent with the efficacy of the triple-drug combination. As expected, SNDX-50469 increased CD11b expression, indicative of differentiation. Enhanced *in vivo* efficacy of combining menin and FLT3 inhibitors was observed in MLL-r/FLT3 mutant AML^{7,8} and to a lesser degree in *NPM1/FLT3* mutant AML.⁸ SNDX-50469/gilteritinib was more effective than SNDX-50469 in reducing circulating blast numbers at week 2, but it neither reduced the BM leukemia burden nor did it improve survival compared to SNDX-50469. It is unclear whether this discrepancy is due to different models. One possibility is that cells became less sensitive to SNDX-50469/gilteritinib over time. Indeed, at the end of treatments, SNDX-50469/gilteritinib-treated cells showed increased β -cate-



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Figure 3. Menin, FLT3, and/or BCL-2 inhibition targets leukemia cells and stem/progenitor cells and modulates HOX/MEIS/PBX3 and BCL-2 protein levels. PhenoGraph was used to cluster cell populations according to cell surface marker expression. Cisplatin-low viable single cells were gated with FlowJo software (version 10.7, FlowJo LLC) and exported as flow cytometry standard (FCS) data for subsequent analysis in Cytokit.¹⁴ Cell populations identified and embedded by PhenoGraph in the “Cytokit_analyzedFCS” files were gated in FlowJo to quantify marker expression. ArcSinh-transformed counts for each protein expression in desired cell populations were visualized with heat maps. (A) Clusters of leukemia cells and leukemia stem/progenitor cells. (B) Percentages of viable leukemia cells and leukemia stem/progenitor cells in each treatment group. (C) Human (hu) CD45 cells in the treatment groups. (D) Protein expression in huCD45⁺ cells in the treatment groups. Antibodies against PBX3, MEIS1, or HOXA9 were obtained from Proteintech (12571-1-AP), Origene (CF809622, clone OT1B4), and Abcam (ab191178), respectively. The other antibodies are as previously described.⁹ Cells were collected at the end of treatment from mouse BM. CON: control; SNDX: SNDX-50469; Gil: gilteritinib; VEN: venetoclax.

nin and BCL-XL expression compared to SNDX-50469 or gilteritinib, which were largely eliminated in the triple-drug-treated group (Figure 3). Our findings demonstrate that co-inhibition of menin, BCL-2, and FLT3 has profound activity against AML and AML stem progenitor cells with *NPM1c/FLT3-ITD/-TKD* mutations *in vitro* and *in vivo*. This combination reduces the HOX/MEIS signature and anti-apoptotic BCL-2 proteins, resulting in major survival benefit. The quadruple combination of SNDX-50469/gilteritinib/venetoclax/5-azacytidine resulted in cures of >50% of mice carrying triple-mutant human AML cells. Note, the triple-drug and quadruple-drug combination groups were treated with decreased doses of gilteritinib and venetoclax compared to single- or double-agent treatment groups. These combinations were well tolerated for extended time periods, after dose reductions, attesting to their tolerability and efficacy.

In the triple-drug combination group, residual leukemia cells were still characterized by increased p-FLT3/MCL-1 levels, although total FLT3 levels were decreased. Whether p-FLT3/MCL-1 was inhibited in the quadruple-drug combination group was not determined. Nevertheless, three of five mice in the quadruple-, and two of six mice in the triple-drug combination group lived close to the life expectancy of normal NSG mice with no detectable leukemia cells at termination of the experiment after >400 days suggesting, but not fully establishing a potential benefit of added 5-azacytidine to the triple-drug combination. Results warrant the clinical development of concomitant inhibition of menin, BCL-2, and FLT3 combined with or without a hypomethylating agent in *NPM1-/FLT3*-mutated AML.

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BZC received research funding from Syndax. GM and PO are employees of Syndax. MA is a consultant for Syndax.

Contributions

BZC conceptualized the study, analyzed data, and wrote the manuscript. PYM, WT and LBO performed experiments and analyzed data. DHM and BK performed experiments. GMM and PO discussed the study concept, provided materials, and edited the manuscript. MA conceptualized the study, interpreted the data, and edited the manuscript.

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Data-sharing statement

Materials described in the manuscript, including all relevant raw data, is freely available to any researcher wishing to use them for non-commercial purposes, without breaching participant confidentiality.

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