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A phase I study of pevonedistat, azacitidine, and venetoclax in patients with relapsed/refractory acute myeloid leukemia

Short title: Pevonedistat in relapsed acute myeloid leukemia

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G.M., E.A., A.S, S.H.K conceived and designed the study; G.M., E.A., A.S, M.L, A.N.S, S.H.K collected and assembled the data, performed data analysis and wrote the manuscript; all authors performed interpretation of data and provided final approval of the manuscript.

G.M and E.A had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest

Dr. Guru Subramanian Guru Murthy reports the following outside the submitted work - Cardinal Health (Honoraria), BMS (Advisory board), BeiGene (Advisory board), Pfizer (Advisory board), Gilead/Kite (Advisory board), Amgen (Speaker Bureau), Rigel (Speaker Bureau), Cancerexpert now (Consultancy), Qessential (Consultancy), Techspert (Consultancy), DAVA Oncology (Honoraria), Aptitude Health (Honoraria) and Curio science (Honoraria), all outside the submitted work.

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Dr. Ehab Atallah reports the following outside the submitted work – Abbvie (Speaker Bureau, consultant, research support), Novartis (Consultant, research support), BMS (Speaker Bureau, Consultant), Takeda (Research support), all outside the submitted work.

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Abstract

Azacitidine/venetoclax is an active regimen in patients with newly diagnosed AML. However, primary or secondary resistance to azacitidine/venetoclax is an area of unmet need and overexpression of MCL-1 is suggested to be a potential resistance mechanism. Pevonedistat inhibits MCL-1 through activation of NOXA, and pevonedistat/azacitidine has previously shown activity in AML. To assess the tolerability and efficacy of adding pevonedistat to azacitidine/venetoclax in relapsed/refractory AML, we conducted a phase I multicenter open-label study in 16 adults with relapsed/refractory AML. Patients were treated with azacitidine, venetoclax along with pevonedistat intravenously on days 1, 3 and 5 of each 28-day cycle at 10, 15 or 20 mg/m^2 in successive cohorts in the dose escalation phase. The impact of treatment on protein neddylation as well as expression of pro-apoptotic BCL2 family members was assessed. The recommended phase II dose of pevonedistat was 20 mg/m^2. Grade 3 or higher adverse events included neutropenia (31%), thrombocytopenia (13%), febrile neutropenia (19%), anemia (19%), hypertension (19%) and sepsis (19%). The overall response rate was 46.7% for the whole cohort including complete remission (CR) in 5 of 7 (71.4%) patients who were naïve to the hypomethylating agent/venetoclax. No measurable residual disease (MRD) was detected in 80.0% of the patients who achieved CR. The median time to best response was 50 (range: 23 – 77) days. Four patients were bridged to allogeneic stem cell transplantation. The combination of azacitidine, venetoclax and pevonedistat is safe and shows encouraging preliminary activity in patients with relapsed/refractory AML. (NCT04172844).

Introduction

Outcomes of patients with relapsed/refractory acute myeloid leukemia (AML) have remained poor (1). While salvage cytotoxic chemotherapy is commonly used, the availability of targeted agents is changing the treatment landscape of relapsed/refractory AML. Currently, gilteritinib (FLT3 inhibitor), ivosidenib and olutasidenib (IDH1 inhibitors) and enasidenib (IDH2 inhibitor) are approved for the management of subsets of patients with relapsed/refractory AML (2-4). In addition, the B-cell lymphoma 2 (BCL2) pathway has been explored as a therapeutic target in AML, with encouraging efficacy of venetoclax (BCL2 inhibitor) in combination with hypomethylating agents or cytarabine in the frontline management of AML (5-8). However, in contrast to the high efficacy of venetoclax-based therapy in newly diagnosed AML, the response rates with venetoclax remain low in the relapsed/refractory setting (9, 10). Pre-clinical studies investigating the mechanisms of BCL2 inhibitor resistance suggest overexpression of the anti-apoptotic protein MCL1 as a potential mechanism blocking the downstream effects of BCL2 inhibition (11, 12).

Pevonedistat (also known as TAK-924 and MLN4924) is a first-in-class, small-molecule inhibitor of the neural precursor cell expressed, developmentally downregulated 8 (NEDD8)-activating enzyme (NAE) that is important in the regulated turnover of proteins by Cullin RING ligases (12-14). As a consequence of its impact on protein turnover, pevonedistat upregulates the pro-apoptotic protein NOXA, leading to neutralization of MCL1 and facilitating apoptosis (15, 16). In preclinical models, the combination of pevonedistat and venetoclax has shown a synergistic effect in leukemia cell lines and clinical AML isolates (16). In addition, prior clinical trials have also investigated the safety and efficacy of pevonedistat in the setting of newly diagnosed as well as relapsed/refractory AML and myelodysplastic syndrome (17-20). Because

primary or secondary resistance to the hypomethylating agent/venetoclax combination can result from MCL1 overexpression and pevonedistat was shown to contribute to MCL1 neutralization through NOXA upregulation, we designed a clinical trial to assess the safety and preliminary efficacy of adding pevonedistat to the azacitidine/venetoclax backbone in patients with relapsed/refractory AML.

Methods

Patients

We conducted a phase I, multicenter, open-label study to determine the safety and recommended phase II dose (RP2D) of the combination of pevonedistat, venetoclax and azacitidine in patients with relapsed/refractory AML. The study enrolled patients from two US centers (Medical College of Wisconsin, Milwaukee and Mayo Clinic, Rochester). Key inclusion and exclusion criteria are included in **Supplemental Table 1**. Notably, patients with prior exposure to a hypomethylating agent and/or venetoclax therapy were eligible for the study. The study was approved by the institutional review board of each participating institution and was conducted according to the International Council for Harmonisation Good Clinical Practice guidelines and the ethical principles of the Declaration of Helsinki. All patients provided written informed consent. The study included a dose escalation phase (to determine the RP2D) and a dose expansion phase (with the RP2D) (**Supplemental Figure 1**). The primary endpoint was to determine the RP2D and toxicity profile of pevonedistat, azacitidine and venetoclax. Secondary endpoints included response rate (21), survival and correlative studies (**Supplemental Methods**).

Treatment

Pevonedistat was administered by intravenous (IV) infusion on days 1, 3 and 5 of each 28-day cycle along with standard doses of azacitidine (75 mg/m^2 IV daily for 7 days) and venetoclax [400 mg orally (PO) daily x 28 days]. During the dose escalation phase, pevonedistat was given in escalating doses in successive treatment cohorts (10 mg/m^2, 15 mg/m^2 and 20 mg/m^2) without intrapatient dose escalation. Dose escalation was overseen by the data safety monitoring committee of the Medical College of Wisconsin. Dose-limiting toxicities (DLT) were assessed during cycle 1 of therapy. Definitions of DLT are included in **Supplemental Table 2**. Dose expansion was conducted with the RP2D of pevonedistat. We did not attempt to go beyond 20 mg/m^2 as this was the RP2D in the azacitidine/pevonedistat study (20).

Correlative studies

Correlative studies included (i) examination of NAE inhibition; (ii) correlation of pretreatment levels of BCL2, BCLXL, MCL-1, BAX or BAK with response; (iii) determination of levels of NOXA (PMAIP1) mRNA and protein expression pre-and post-pevonedistat treatment; (iv) evaluation of BH3 mimetic profiling on bone marrow samples by flow cytometry and (v) assessment of pevonedistat sensitivity of leukemia and leukemic stem/progenitor cells. Details regarding the methods for these assays can be found in the supplement (16, 22) (**Supplemental Methods**).

Statistical analyses

The study followed a 3 + 3 design for the dose-escalation phase. The dose expansion phase utilized the RP2D of pevonedistat determined at the dose escalation phase. For the subjects in each dose level, their baseline characteristics, toxicity profile, and response rate were analyzed using descriptive statistics. The Kaplan-Meier method was used to estimate survival. Event free

survival was defined from the time of achievement of CR, CRi or CRh to the time of relapse/progression/death. OS was defined from the time of initiation of treatment until death due to any cause. Maximum tolerated dose was the highest dose level at which 0 of the first 3 patients treated or ≤1 of the first 6 patients treated had a DLT during cycle 1 of the dose escalation phase. Statistical analysis was conducted with a level of significance set at $p<0.05$.

Results

Baseline characteristics

Sixteen patients with relapsed/refractory AML participated in the study (15 evaluable for response), 13 in the dose escalation phase and 3 in the dose expansion phase (reported together with the RP2D of dose escalation phase). Baseline characteristics of the study cohort are presented in **Table 1**. Median age was 73 (61 – 91) years, 7 patients (43.8%) had secondary or therapy-related AML, 11 (68.7%) had adverse risk, 9 (56.3%) had received prior therapy with venetoclax/hypomethylating agent and 3 (18.8%) had relapse after prior allogeneic HSCT.

Safety and RP2D

The triplet combination of pevonedistat, azacitidine and venetoclax was well tolerated by participants. A summary of the common adverse events is provided in **Table 2**. The most common grade 3 or higher adverse events included neutropenia (31%), thrombocytopenia (13%), febrile neutropenia (19%), anemia (19%), hypertension (19%) and sepsis (19%). Atrial fibrillation was the only DLT that occurred in a patient who received pevonedistat at the 10 mg/m^2 dose. This cohort was subsequently expanded to a total of 6 patients, and no further DLT was observed. Pevonedistat 20 mg/m^2 was established as the RP2D. The regimen was also well tolerated beyond the DLT period, and no unexpected toxicities were observed when the regimen

was continued beyond the first cycle. Patients received a median 2 cycles of therapy (range 1-6 cycles).

Efficacy:

Fifteen patients were evaluable for response (one patient dropped out of the study prior to completion of cycle 1 therapy due to active CNS disease). Among patients who were evaluable, the ORR was 46.7% (**Table 3**). The CR rate was 33.3% for the overall cohort and 71.4% in patients with venetoclax/hypomethylating agent naïve relapsed/refractory AML (**Supplemental Table 3**). Among 9 patients with prior exposure to venetoclax/hypomethylating agent, one patient achieved morphological leukemia free status (MLFS). The median time to achieve best response was 50 (23 – 77) days. In patients achieving CR, 80.0% were MRD negative by flow cytometry. Among patients who achieved treatment response, 1-year OS was 80.0% (vs. 0% in those who did not achieve CR) (**Supplemental Figure 3**). For the overall population, the median progression free survival was 2.4 months, and median OS was 6.3 months (**Supplemental Figures 4 and 5**). Four patients subsequently underwent allogeneic HSCT.

Correlative studies:

Several studies were conducted to examine pharmacodynamic biomarkers of drug action (**Figures 1-3 and Supplemental Figures 6-8**). Serial samples of bone marrow or peripheral blood were available from eight patients to assess the impact of treatment on protein neddylation as well as expression of pro-apoptotic BCL2 family members. Immunoblotting showed that CUL1 neddylation was diminished, indicative of pevonedistat action, at 6 h after pevonedistat administration in five of eight patients. Importantly, cells from the two patients who achieved a CR displayed decreased CUL1 neddylation that persisted at 24 hours, whereas cells from those

who did not achieve CR had recurrent CUL1 neddylation by 24 hours (**Figure 1A**). Despite preclinical data suggesting that mRNA encoding PMAIP1 (NOXA) is upregulated by pevonedistat in AML cells *ex vivo*, quantitative RT-PCR on RNA isolated from serial samples failed to demonstrate a consistent pattern of changes in mRNA encoding proapoptotic BCL2 family members after therapy initiation (**Figure 1B**). Nonetheless, immunoblotting revealed treatment-associated upregulation of two proteins that bind MCL-1 and promote apoptosis, i.e., p53 upregulated modulator of apoptosis (PUMA) at 24 hours for two patients who achieved a CR (patients 021 and 022) and NOXA at 6 hours for patient M002 who achieved MLFS (**Figure 1A**). One patient (PAVE 20) whose leukemia was studied at the time of regrowth had significant upregulation of BCL2 and less prominent upregulation of MCL-1 and $BCLX_L$ (**Figure 1A and Supplemental Figure 6**). PLA assays indicated the presence of preformed complexes of BAK with various anti-apoptotic family members, indicative of a "primed for apoptosis" state that was reflected in BH3 mimetic profiling assays (**Supplemental Figure 7**). In the one patient whose samples were able to be studied at progression, there was a decrease in the preformed BAK/MCL-1 and BAK/BCL2 complexes compared to baseline (**Supplemental Figure 8**), suggesting a less primed state.

Additional studies were undertaken to search for potential predictive biomarkers in the pretreatment samples. Samples from four patients were treated with pevonedistat and venetoclax *ex vivo* for 24 h and examined for survival of leukemic stem and progenitor cells by 10-color flow cytometry (22). This assay demonstrated that leukemia stem-like cells from a patient achieving CR (PAVE 021) were the most sensitive to pevonedistat at 50-100 nM and the pevonedistat/venetoclax combination (**Figure 2**). In contrast, BH3 mimetic profiling of the bulk leukemia showed variable baseline sensitivity to BH3 mimetics (**Supplemental Figure 7**).

Pretreatment samples from 13 patients of the 16 treated patients were also examined by immunoblotting. Results of this assay demonstrated that levels of DNMT1 and DNMT3A were generally higher in samples from patients with CR or MLFS compared to samples from patients who did not respond, whereas pretreatment levels of pro- or anti-apoptotic proteins did not correlate with response (**Figure 3 and Supplemental Figure 6B**).

Genomic mutations:

Baseline mutation status at the time of study entry was known in 13 patients. Most commonly mutated genes included *DNMT3A*, *TP53*, *ASXL1*, *RUNX1*, *JAK2*, and *NRAS* (**Table 1**). One patient had an *IDH2* mutation, and none of the patients had a *NPM1* mutation. *TP53* mutation was seen in 18.7% of patients. Despite the limited sample size, no significant association was noted between baseline mutation profile and treatment response.

Discussion

The optimal salvage strategy for patients with relapsed/refractory AML remains unclear. While cytotoxic chemotherapy and targeted agents remain as tools in the armamentarium, there are limitations to the application of these strategies. For example, intensive cytotoxic salvage chemotherapy regimens such as CLAG-M (cladribine, cytarabine, G-CSF and mitoxantrone) or MEC (mitoxantrone, etoposide and cytarabine) are most useful in fit patients with adequate performance status (23). Targeted agents such as IDH1 inhibitors, IDH2 inhibitors and FLT3 inhibitors are most effective in patients harboring mutations in the targeted proteins (3, 24, 25). This leaves a large group of AML patients who are older, have comorbidities and lack targetable mutations in need of better salvage therapy. While hypomethylating agent/venetoclax-based salvage therapy has been investigated in patients with AML, the limited efficacy of this regimen

in the relapsed/refractory setting underscores the need for further research to improve long-term outcomes. Toward this end, we investigated the potential role of adding pevonedistat to the azacitidine/venetoclax backbone in patients with relapsed/refractory AML, and our results demonstrate a potentially important role for this combination in the management of these patients.

The present study found that the addition of pevonedistat to azacitidine and venetoclax was safe and well tolerated in patients with relapsed/refractory AML. When administered with a standard 7-day azacitidine and 28-day venetoclax regimen, pevonedistat 20 mg/m^2 was established as the recommended dose. Most patients included in our study were older adults (median age 73 years) with AML harboring poor risk features, and over half of the participants had been previously treated with hypomethylating agent/venetoclax combinations before study enrollment. The side effect profile of pevonedistat was consistent with that reported in prior clinical trials and no new safety concerns were noted when pevonedistat was added to azacitidine + venetoclax and given as a continued therapy (26). Because of the small sample size, the clinical efficacy data of this combination needs to be confirmed in larger studies. We noted a variability in response based on prior exposure to venetoclax and hypomethylating agents. In patients with relapsed/refractory AML who did not have prior exposure to venetoclax therapy, CR was observed in 5/7 (71.4%) patients treated with the triplet combination, numerically higher than previously reported for the azacitidine/venetoclax doublet, albeit with a small sample size. For example, a large retrospective series reported a CR/CRi of 24% with venetoclax-based salvage therapy (37% with azacitidine/venetoclax) in relapsed/refractory AML patients who were venetoclax-naïve (9). In contrast, among patients who had prior venetoclax exposure, one patient out of nine in the present study achieved MLFS with the addition of pevonedistat to azacitidine/venetoclax. While

the results are limited by the small size of this study, these observations suggest that this strategy might work best with the upfront addition of pevonedistat to azacitidine/venetoclax rather than sequential addition of pevonedistat after the onset of resistance to azacitidine/venetoclax.

Based on preclinical data (15, 16), several correlative studies were performed in serial samples to assess the impact of this regimen on apoptotic pathways. Immunoblotting demonstrated that CUL1 neddylation was inhibited at 6 and 24 h in a subset of cases (**Figure 1A**), as would be expected if NAE were inhibited by pevonedistat. However, upregulation of PMAIP1/NOXA at the mRNA and protein levels was not routinely observed in these cases (**Figure 1A and 1B**). While this might reflect loss of cells with the greatest PMAIP1/NOXA upregulation due to rapid killing, it is also possible that preclinical studies have failed to uncover important aspects of the antileukemic mechanism of pevonedistat.

Additional studies were performed to identify potential biomarkers that are predictive of response. No association was noted between genomic mutations and response; and the cohort was not enriched for venetoclax-sensitive mutations (27). Analysis of drug sensitivity *ex vivo* indicated that higher sensitivity to pevonedistat and pevonedistat + venetoclax was observed in cells from a patient who achieved CR (**Figure 2**). Examination of immunoblotting data indicated that sustained inhibition of CUL1 neddylation at 24 h and induction of BBC3/PUMA or PMAIP1/NOXA were associated with CR or prolonged MLFS, respectively (**Figure 1A**). Moreover, pretreatment levels of DNMT1 and DNMT3A tended to be higher in cases that achieved response (**Figure 3**), consistent with previous suggestions that pretreatment DNMT1 levels might be a potential harbinger of HMA response in AML (28, 29). All of these correlative studies were limited to AML cases with sufficient bone marrow cellularity or enough circulating blasts to permit completion of the various assays. Moreover, immunoblotting to complete proof

of mechanism studies and allow evaluation of potential predictive biomarkers at the protein level was prioritized over more exploratory biomarkers such as 10-color flow cytometry after drug exposure *ex vivo* and PLAs to assess the presence of constitutively activated BAK. Accordingly, the resulting observations require further assessment in future studies because of the small sample sizes.

In addition to the encouraging results with this combination in venetoclax-naïve patients with relapsed/refractory AML, prior studies have also demonstrated the efficacy of pevonedistat in myeloid malignancies. A phase I study by Swords *et al* in patients with MDS and oligoblastic treatment-naïve AML showed that the combination of pevonedistat and azacitidine was able to produce 50% overall response in newly diagnosed patients, including those with *TP53* mutations (20). More recently, two randomized clinical trials in patients with treatment naïve high-risk MDS and oligoblastic AML showed significantly higher response rates with azacitidine/pevonedistat as compared to azacitidine monotherapy, although this finding did not translate into a significant improvement in overall survival (18, 30). In addition, a phase 1/2 study of pevonedistat/azacitidine/venetoclax in patients with newly diagnosed secondary AML showed 64% CR/CRi (31). Together with our study, these results indicate a potential for improvement in response rates and good tolerability with the addition of pevonedistat to the azacitidine/venetoclax backbone. Achievement of CR is an important goal in patients with relapsed/refractory AML given the potential for then moving forward with potentially curative treatments such as allogeneic HSCT. Although limited by a small sample size and lack of planned cohort stratification based on prior venetoclax exposure, our study demonstrates the feasibility, safety and encouraging early efficacy of a potential triplet for the management of these patients.

Conclusion

The addition of pevonedistat to a backbone of venetoclax and azacitidine is safe and well tolerated in patients with relapsed/refractory AML. Dose escalation yielded encouraging efficacy specifically in patients with relapsed/refractory AML not previously treated with the hypomethylating agent/venetoclax combination. Given this encouraging clinical activity, these results suggest that further study of NAE inhibitors in this setting is warranted. Future efforts to investigate novel agent combinations, including triplet therapy options, could potentially improve the response rates and outcomes of patients with relapsed/refractory AML, particularly those without a targetable mutation.

References

1. DeWolf S, Tallman MS. How I treat relapsed or refractory AML. Blood. 2020;136(9):1023-1032.
2. DiNardo CD, Stein EM, de Botton S, et al. Durable Remissions with Ivosidenib in IDH1-Mutated Relapsed or Refractory AML. N Engl J Med. 2018;378(25):2386-2398.
3. Perl AE, Martinelli G, Cortes JE, et al. Gilteritinib or Chemotherapy for Relapsed or Refractory FLT3-Mutated AML. N Engl J Med. 2019;381(18):1728-1740.
4. de Botton S, Montesinos P, Schuh A, et al. Enasidenib vs conventional care in mutant-IDH2 relapsed/refractory acute myeloidleukemia: a randomized, phase 3 trial. Blood. 2023;141(2):156-167.
5. Konopleva M, Letai A. BCL-2 inhibition in AML: an unexpected bonus? Blood. 2018;132(10):1007-1012.
6. Pan R, Hogdal LJ, Benito JM, et al. Selective BCL-2 inhibition by ABT-199 causes on-target cell death in acute myeloid leukemia [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. Cancer Discov. 2014;4(3):362-375.
7. DiNardo CD, Jonas BA, Pullarkat V, et al. Azacitidine and Venetoclax in Previously Untreated Acute Myeloid Leukemia. N Engl J Med. 2020;383(7):617-629.
8. Wei AH, Strickland SA, Jr., Hou JZ, et al. Venetoclax Combined With Low-Dose Cytarabine for Previously Untreated Patients With Acute Myeloid Leukemia: Results From a Phase Ib/II Study. J Clin Oncol. 2019;37(15):1277-1284.
9. Stahl M, Menghrajani K, Derkach A, et al. Clinical and molecular predictors of response and survival following venetoclax therapy in relapsed/refractory AML. Blood Adv. 2021;5(5):1552-1564.
10. Aldoss I, Yang D, Aribi A, et al. Efficacy of the combination of venetoclax and hypomethylating agents in relapsed/refractory acute myeloid leukemia. Haematologica. 2018;103(9):e404-e407.
11. Konopleva M, Contractor R, Tsao T, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. Cancer Cell. 2006;10(5):375-388.
12. Carter BZ, Mak PY, Tao W, et al. Targeting MCL-1 dysregulates cell metabolism and leukemia-stroma interactions and resensitizes acute myeloid leukemia to BCL-2 inhibition. Haematologica. 2022;107(1):58-76.
13. Soucy TA, Smith PG, Milhollen MA, et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature. 2009;458(7239):732-736.
14. Lin JJ, Milhollen MA, Smith PG, Narayanan U, Dutta A. NEDD8-targeting drug MLN4924 elicits DNA rereplication by stabilizing Cdt1 in S phase, triggering checkpoint activation, apoptosis, and senescence in cancer cells. Cancer Res. 2010;70(24):10310-10320.
15. Cojocari D, Smith BN, Purkal JJ, et al. Pevonedistat and azacitidine upregulate NOXA (PMAIP1) to increase sensitivity to venetoclax in preclinical models of acute myeloid leukemia. Haematologica. 2022;107(4):825-835.
16. Knorr KL, Schneider PA, Meng XW, et al. MLN4924 induces Noxa upregulation in acute myelogenous leukemia and synergizes with Bcl-2 inhibitors. Cell Death Differ. 2015;22(12):2133-2142.
17. Swords RT, Erba HP, DeAngelo DJ, et al. Pevonedistat (MLN4924), a First-in-Class NEDD8-activating enzyme inhibitor, in patients with acute myeloid leukaemia and myelodysplastic syndromes: a phase 1 study. Br J Haematol. 2015;169(4):534-543.
18. Sekeres MA, Watts J, Radinoff A, et al. Randomized phase 2 trial of pevonedistat plus azacitidine versus azacitidine for higher-risk MDS/CMML or low-blast AML. Leukemia. 2021;35(7):2119-2124.
19. Saliba AN, Kaufmann SH, Stein EM, et al. Pevonedistat with azacitidine in older patients with TP53-mutated AML: a phase 2 study with laboratory correlates. Blood Adv. 2023;7(11):2360-2363.
20. Swords RT, Coutre S, Maris MB, et al. Pevonedistat, a first-in-class NEDD8-activating enzyme inhibitor, combined with azacitidine in patients with AML. Blood. 2018;131(13):1415-1424.
21. Dohner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129(4):424-447.

22. Knorr KL, Finn LE, Smith BD, et al. Assessment of Drug Sensitivity in Hematopoietic Stem and Progenitor Cells from Acute Myelogenous Leukemia and Myelodysplastic Syndrome Ex Vivo. Stem Cells Transl Med. 2017;6(3):840-850.
23. Mushtaq MU, Harrington AM, Chaudhary SG, et al. Comparison of salvage chemotherapy regimens and prognostic significance of minimal residual disease in relapsed/refractory acute myeloid leukemia. Leuk Lymphoma. 2021;62(1):158-166.
24. Stein EM, DiNardo CD, Pollyea DA, et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. Blood. 2017;130(6):722-731.
25. Montesinos P, Recher C, Vives S, et al. Ivosidenib and Azacitidine in IDH1-Mutated Acute Myeloid Leukemia. N Engl J Med. 2022;386(16):1519-1531
26. Swords RT, Watts J, Erba HP, et al. Expanded safety analysis of pevonedistat, a first-in-class NEDD8-activating enzyme inhibitor, in patients with acute myeloid leukemia and myelodysplastic syndromes. Blood Cancer J. 2017;7(2):e520.
27. DiNardo CD, Tiong IS, Quaglieri A, et al. Molecular patterns of response and treatment failure after frontline venetoclax combinations in older patients with AML. Blood. 2020;135(11):791-803.
28. Yu J, Qin B, Moyer AM, et al. DNA methyltransferase expression in triple-negative breast cancer predicts sensitivity to decitabine. J Clin Invest. 2018;128(6):2376-2388.
29. Ueda M, El-Jurdi N, Cooper B, et al. Low-Dose Azacitidine with DNMT1 Level Monitoring to Treat Post-Transplantation Acute Myelogenous Leukemia or Myelodysplastic Syndrome Relapse. Biol Blood Marrow Transplant. 2019;25(6):1122-1127.
30. Adès L, Girshova L, Doronin VA, et al. Pevonedistat plus azacitidine vs azacitidine alone in higher-risk MDS/chronic myelomonocytic leukemia or low-blast-percentage AML. Blood Adv. 2022;6(17):5132-5145.
31. Short NJ, Montalban-Bravo G, Alvarado Y, et al. Azacitidine, Venetoclax and Pevonedistat As Frontline Therapy for Patients with Secondary Acute Myeloid Leukemia Who Are Unfit for Intensive Chemotherapy: Results from a Phase I/II Study. Blood. 2021;138(Supplement 1):2349.

Table 1. Baseline Demographic and Clinical Characteristics of the Patients.

Characteristic	**N=16**
Age (median and range) in years	73 (61-91)
Gender	
Male	7 (43.8%)
Female	9 (56.3%)
AML subtype	
Denovo	9 (56.3%)
Secondary	5 (31.3%)
Therapy related	2 (12.5%)
ELN 2017 risk status	
Favorable	0(0%)
Intermediate	5 (31.3%)
Adverse	11(68.7%)
Number of prior lines of therapy	2 (1-6)
Prior Hypomethylating agents/venetoclax	
No	7 (43.8%)
Yes	9 (56.3%)
Prior allogeneic stem cell transplant	
No	13 (81.3%)
Yes	3 (18.8%)
Gene mutations	
DNMT3A	6 (37.5%)
TP53	3 (18.7%)
ASXL1	3 (18.7%)
RUNX1	3 (18.7%)
NRAS	2 (12.5%)
JAK2	2 (12.5%)
IDH2	1 (6.2%)
FLT3	1 (6.2%)
BCOR	1 (6.2%)
BCORL1	1 (6.2%)
KMT2A	1 (6.2%)
SRSF2	1 (6.2%)
ETV6	1 (6.2%)
NF1	1 (6.2%)
PTPN11	1 (6.2%)
CHEK2	1 (6.2%)
TET2	1 (6.2%)
CSF3R	1 (6.2%)
PHF6	1 (6.2%)
DDX41	1 (6.2%)
SMC1A	1 (6.2%)
MUTYH1	1 (6.2%)
EZH2	1 (6.2%)
SETBP1	1 (6.2%)
SMC1A	1 (6.2%)
PASK	1 (6.2%)
ZNF703	1 (6.2%)

Table 2. Adverse Events.

Cohort	Overall		Pevonedistat 10 mg/m^2		Pevonedistat 15 mg/m^2		Pevonedistat 20 mg/m^2	
Toxicity	All N=16	Grade 3+ N=16	All N=7	Grade 3+ N=7	All N=3	Grade 3+ N=3	All N=6	Grade 3+ N=6
Diarrhea	6 (38%)	0	3(43%)	0	1 (33%)	0	2(33%)	0
Hypotension	5(31%)	1 (6%)	2(29%)	1 (14%)	1 (33%)	0	2 (33%)	0
Neutropenia	5 (31%)	5(31%)	2(29%)	2 (29%)	1 (33%)	1 (33%)	2 (33%)	2 (33%)
Anemia	4(25%)	3(19%)	1(14%)	1 (14%)	0	0	3 (50%)	2 (33%)
Constipation	4 (25%)	0	1(14%)	0	1(33%)	0	2(33%)	0
Fatigue	4 (25%)	0	1(14%)	0	0	0	3(50%)	0
Hypertension	4 (25%)	3 (19%)	2(29%)	2(29%)	1(33%)	0	1 (17%)	1 (17%)
Dizziness	3(19%)	0	2(29%)	0	0	0	1(17%)	0
Febrile neutropenia	3(19%)	3(19%)	2(29%)	2(29%)	0	0	1(17%)	1(17%)
Fever	3(19%)	0	1(14%)	0	0	0	2(33%)	0
Hyperglycemia	3(19%)	0	0	0	0	0	3(50%)	0
Mucositis oral	3(19%)	0	0	0	0	0	3(50%)	0
Nausea	3(19%)	0	1(14%)	0	0	0	2(33%)	0
Sepsis	3(19%)	3 (19%)	2(29%)	2(29%)	0	0	1(17%)	1(17%)
Abdominal pain	2(13%)	0	0	0	0	0	2(33%)	0
Anorexia	2(13%)	0	0	0	0	0	2(33%)	0
Back pain	2(13%)	0	1(14%)	0	0	0	1(17%)	0
Dyspepsia	2(13%)	0	2(29%)	0	0	0	0	0
Dyspnea	2(13%)	1(6%)	1(14%)	1(14%)	0	0	1(17%)	0
Flatulence	2(13%)	0	0	0	0	0	2(33%)	0
Headache	2(13%)	0	1(14%)	0	0	0	1(17%)	0
Hyperuricemia	2(13%)	0	0	0	1(33%)	0	1(17%)	0
Hypoalbuminemia	2(13%)	0	0	0	0	0	2(33%)	0
Hypocalcemia	2(13%)	1(6%)	0	0	0	0	2(33%)	1(17%)
Hypokalemia	2(13%)	0	0	0	1(33%)	0	1(17%)	0
Hyponatremia	2(13%)	1(6%)	1(14%)	1(14%)	0	0	1(17%)	0
Oral pain	2(13%)	0	1(14%)	0	0	0	1(17%)	0
Pain	2(13%)	0	0	0	1(33%)	0	1(17%)	0

Cohort	Overall		Pevonedistat 10 mg/m^2		Pevonedistat 15 mg/m^2		Pevonedistat 20 mg/m^2	
Toxicity	All N=16	Grade 3+ N=16	All N=7	Grade 3+ N=7	All N=3	Grade 3+ N=3	All N=6	Grade 3+ N=6
Platelet count decreased	2(13%)	2(13%)	1(14%)	1(14%)	0	0	1(17%)	1(17%)
Sinus tachycardia	2(13%)	0	2(29%)	0	0	0	0	0
Skin infection	2(13%)	0	1(14%)	0	0	0	1(17%)	0

Table 3. Response Assessment.

Variables	Total N=16* (%)	Dose group: Pevonedistat 10 mg/m^2 N=7 (%)	Dose group: Pevonedistat 15 mg/m^2 N=3 (%)	Dose group: Pevonedistat 20 mg/m^2 N=6 (%)
Best response				
Complete remission (CR)	5 (33.3)	1 (16.7)	2 (66.7)	2 (33.3)
Morphologic leukemia free state (MLFS)	1 (6.7)	0 (0.0)	0 (0.0)	1 (16.7)
Partial remission marrow (PR)	1 (6.7)	0 (0.0)	0 (0.0)	1 (16.7)
Treatment failure	8 (53.3)	5 (83.3)	1 (33.3)	2 (33.3)
Time for start of treatment to best response, days				
N (responders)	7	1	2	4
Median (min - max)	50.0 (23.0 - 77.0)	52.0 (52.0 - 52.0)	45.0 (23.0 - 67.0)	38.5 (27.0 - 77.0)
Overall response				
No response	8 (53.3)	5 (83.3)	1 (33.3)	2 (33.3)
Response (CR/CRi/CRh/PR/MLFS)	7 (46.7)	1 (16.7)	2 (66.7)	4 (66.7)
CR/CRi				
No	11 (68.8)	6 (85.7)	1 (33.3)	4 (66.7)
Yes	5 (31.3)	1 (14.3)	2 (66.7)	2 (33.3)
MRD among CR/CRi				
CR/CRi with MRD-	4 (80.0)	1 (100.0)	2 (100.0)	1 (50.0)
CR/CRi with MRD+	1 (20.0)	0 (0.0)	0 (0.0)	1 (50.0)

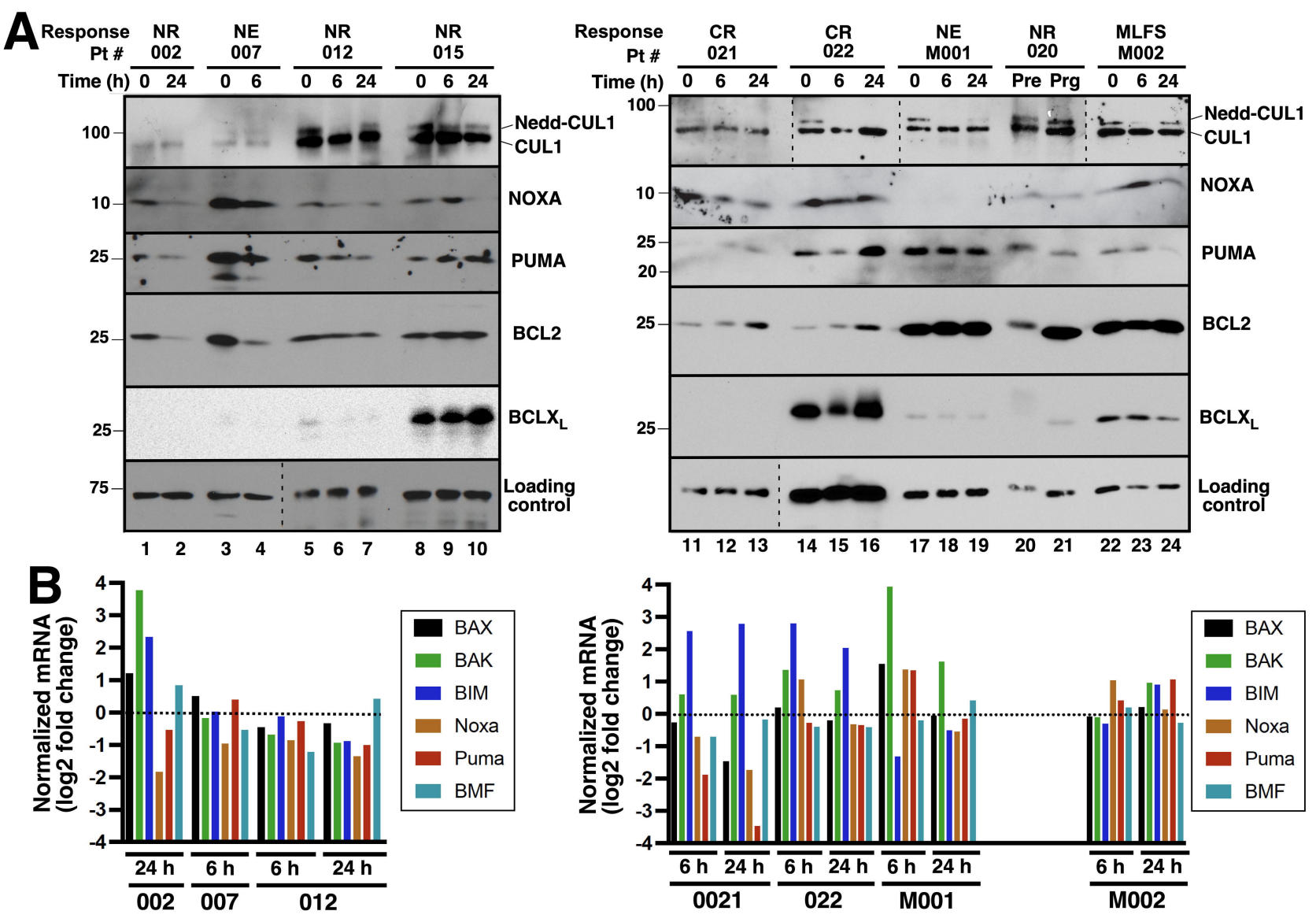
* 15 patients were evaluable for response

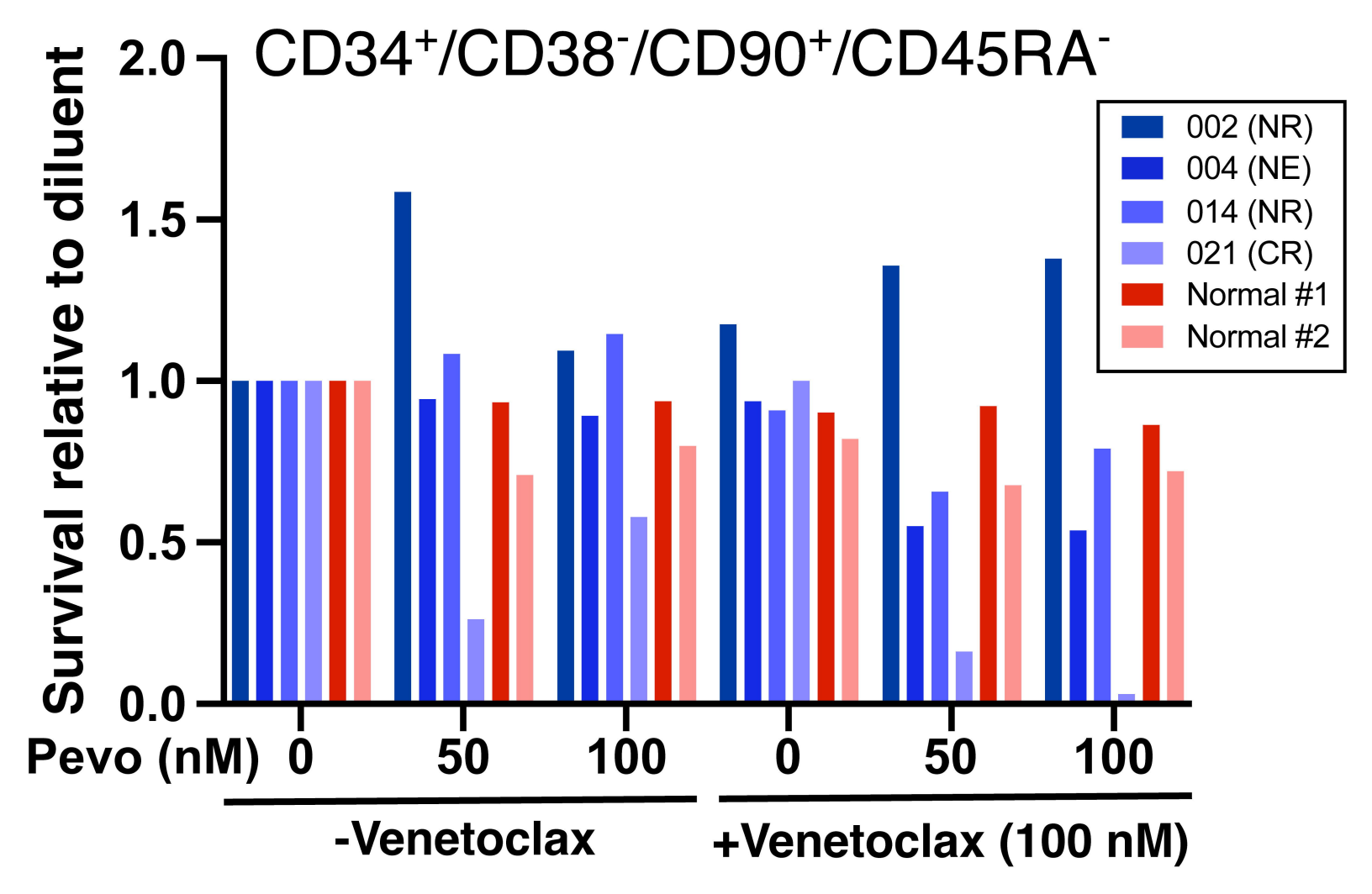
Figure Legends

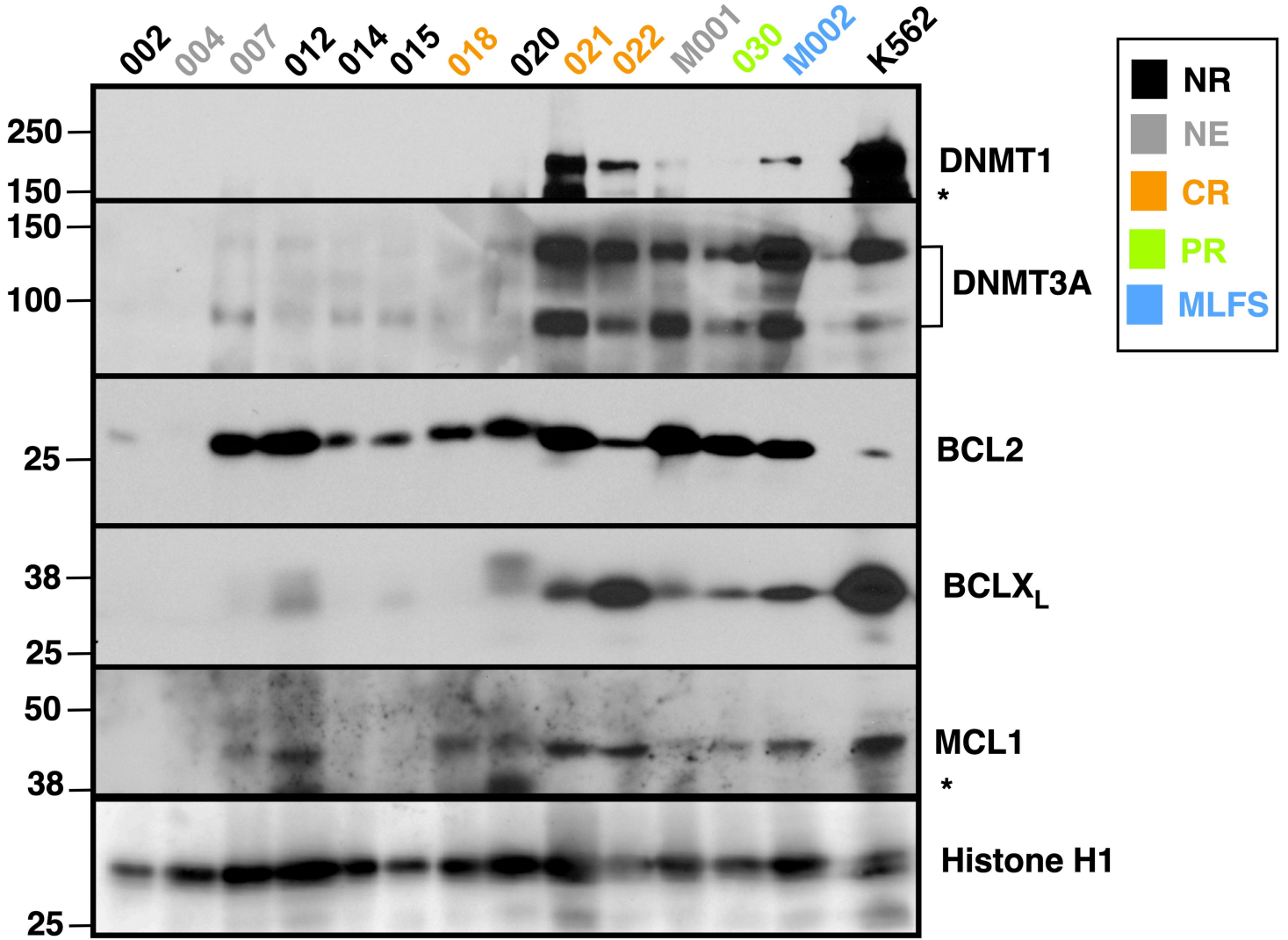
Figure 1. Impact of treatment on protein and mRNA levels. A, whole cell lysates harvested before (0 h) or at the indicated time after chemotherapy administration on Day 1 or on progression (Prg) were subjected to immunoblotting for the indicated antigens. LMNB1 or GAPDH served as a loading control. Response is indicated above each patient number. Dashed lines indicate juxtaposition of different exposures of the same blot (upper panels) or a different loading control (bottom panels). **B,** mRNA levels determined by qRT-PCR at the indicated time points were compared to mRNA levels in the pretreatment samples. Color coded responses are abbreviated as: NR, no response; NE, not evaluable; CR, complete response; PR, partial response; MLFS, morphological leukemia free state.

Figure 2. Relative survival of cells with a hematopoietic stem cell-like immunophenotype after a 24 h exposure to the indicated treatments *ex vivo*. Marrow mononuclear cells isolated prior to treatment of the indicated study patient (various shades of blue) or from normal individuals (red) were incubated for 24 h with the indicated concentrations of pevonedistat (pevo) and/or venetoclax and subjected to multiparameter flow cytometry as described in the Supplemental Methods. Results in each patient were normalized to the diluent control.

Figure 3. Potential predictive markers of response. Whole cell lysates harvested prior to therapy from the indicated patients were subjected to immunoblotting for the indicated antigen. Response of each patient is color coded. Responses are abbreviated as indicated in the legend to Figure 1. Additional correlative studies are found in **Supplemental Figures S2 and S6-8**.


A
Response
Pt #
Time (h)
NR 002
NE 007
NR 012
NR 015
0 24
0 6
0 6 24
0 6 24
CR 021
CR 022
NE M001
NR 020
MLFS M002
Pre Prg
Nedd-CUL1
CUL1
NOXA
PUMA
BCL2
BCLX$_L$
Loading control
100
10
25
20
75
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20 21 22 23 24
B
Normalized mRNA (log2 fold change)
BAX
BAK
BIM
Noxa
Puma
BMF
24 h
6 h
002
007
012
0021
022
M001
M002


CD34+/CD38-/CD90+/CD45RA-
Survival relative to diluent
2.0
1.5
1.0
0.5
0.0
002 (NR)
004 (NE)
014 (NR)
021 (CR)
Normal #1
Normal #2
Pevo (nM)
0
50
100
0
50
100
-Venetoclax
+Venetoclax (100 nM)


002
004
007
012
014
015
018
020
021
022
M001
030
M002
K562
NR
NE
CR
PR
MLFS
250
150
150
100
25
38
25
50
38
25
DNMT1
*
DNMT3A
BCL2
BCLX$_L$
MCL1
*
Histone H1

Supplemental Material

to

A PHASE I STUDY OF PEVONEDISTAT, AZACITIDINE AND VENETOCLAX IN PATIENTS WITH RELAPSED/REFRACTORY ACUTE MYELOID LEUKEMIA

SUPPLEMENTAL METHODS

Measurable residual disease (MRD) analysis

To assess the depth of response, MRD was assessed by 8-color flow cytometry in patients who achieved remission. Briefly, EDTA-anticoagulated bone marrow aspirates were lysed, and cell suspensions were prepared for incubation with 8 different fluorochrome-labeled antibodies per tube. Antibodies analyzed across multiple tubes include: CD7, CD11b, CD13, CD14, CD15, CD22, CD33, CD34, CD36, CD38, CD45, CD56, CD64, CD117, and HLA-DR. At least 200,000–500,000 events were collected per tube on a FACS CANTO cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed with Paint-A-Gate software (BD Biosciences, Franklin Lakes, NJ). Blasts were identified using cluster analysis, based on reproducible forward and light scatter properties and CD45 staining across tubes, along with the identification of other cell populations. Aberrant blast immunophenotypes were identified based on comparison with reproducible, known blast antigen expression patterns. Comparisons were also made to previous leukemic blast immunophenotypes when available. MRD was defined as at least a 0.01% population of aberrant myeloblasts in the absence of morphologic evidence of disease.

BH3 Mimetic Sensitivity Assays

To assess differential sensitivity to different BH3 mimetic agents, mononuclear cells from the peripheral blood (PBMCs) or bone marrow (BMMCs) were resuspended in RPMI 1640 medium containing 100 units/mL penicillin G, 100 µg/mL streptomycin, 2 mM glutamine, and 10% heat-inactivated fetal bovine serum at a concentration of 5 x 10^5 cells/mL, incubated for 4 hours at 37°C with the diluent dimethyl sulfoxide (0.1%), 1 µM venetoclax, 1 µM A1155463, or 1 µM S63845 (Chemietek, Indianapolis, IN) and assayed for apoptotic DNA fragmentation. Cells were sedimented at 150 x g for 5 min, stained with ice cold 50 µg/ml propidium iodide in 0.1% (w/v) sodium citrate containing 0.1% (w/v) Triton X-100, subjected to flow microfluorimetry using a Becton Dickinson FACSCanto II flow cytometer (Becton Dickinson, Mountain View, CA, USA), and analyzed using Becton Dickinson CellQuest software to quantitate subdiploid events as previously described (1). Diluent-induced cell death was subtracted from each sample and drug-induced cell death was calculated using the formula:

$$(\text{death}_{\text{observed}} - \text{death}_{\text{control}})/(100 - \text{death}_{\text{control}}) \times 100\%.$$

Leukemia stem and progenitor cell sensitivity *ex vivo*

Pretreatment BMMCs were treated for 24 h with diluent (0.1% DMSO) or 50 or 100 nM pevonedistat in the absence or presence of 100 nM venetoclax, stained with fluorochrome-coupled antibodies and subjected to 10-color flow cytometry as previously described.(2)

Proximity Ligation Assays (PLAs)

PLAs were used to assess the interactions between pro- and anti-apoptotic BCL2 family members. Cytospins of PBMCs or BMMCs were fixed with -20° C methanol and stored at -80 °C. Before processing for PLAs, slides were thawed at room temperature (20-22 °C), then washed 3 times in

1 x PBS for 5 minutes per wash. The manufacturer's Duolink (MilliporeSigma, St. Louis, MO) PLA protocol was used with modifications. Cells were permeabilized with 1 x PBS containing 0.25% (w/v) Triton X-100 for 10 minutes at room temperature. Cells were washed again 3 times in 1 x PBS. Cells were incubated with 2-4 drops of blocking buffer from the Duolink kit at 37 ^{o}C for 70 minutes in a heated humidity chamber. The blocking buffer was tapped off the slides, and 40 µL of primary antibody solution (in Duolink® Antibody Diluent from the MilliporeSigma PLA kit) were applied to each slide. Slides were stained using the following monoclonal antibodies: rabbit anti-BAK (Abcam, ab220790, 1:200), mouse anti-BCL2 (Thermo Fischer Scientific, Invitrogen, MA5-11757, 1:50), mouse anti-$BCLX_L$ (Santa Cruz, sc-8392, 1:100), or mouse anti-MCL1 (Thermo Fischer Scientific Invitrogen, MA5-15236, 1:100). The slides were mounted with Duolink In Situ Mounting Medium with DAPI and visualized using a Zeiss AxioObserver microscope. Cells were scored in a dichotomous fashion as positive or negative for the presence of BAK/BCL2, BAK/$BCLX_L$, and BAK/MCL1 complexes. Positive and negative controls for BAK/BCL2, BAK/$BCLX_L$, and BAK/MCL1 complexes were set up using RL, K562, and Jurkat cells, respectively, based on previous work showing constitutive binding of BAK to these anti-apoptotic proteins in the respective cell lines (**Supplemental Figure 2**).(3) Cells with fluorescent PLA puncta in a perinuclear arrangement were considered positive. Scoring was performed in triplicate, and results were summarized using a mean percentage of positive cells (+/- SD).

Immunoblotting

Immunoblotting was performed on whole cell lysates(4) prepared from pretreatment BMMC or, when circulating blast counts exceeded 2000/mm3, from PBMC harvested pretreatment, at approximately 6 h after initiation of chemotherapy, and just before administration of day 2 azacitidine at approximately 24 h after the start of therapy. Samples were subjected to electrophoresis on SDS-polyacrylamide gels containing a 5-20% acrylamide gradient to permit resolution of proteins from 8 kDa to > 250 kDa. Aliquots containing 5 x 10^5 K562 cells were included on both ends of the gels of pretreatment samples as a positive control for transfer and blotting. After transfer to nitrocellulose, samples were probed using previously published methods.(5) Primary antibodies were purchased from the following suppliers: rabbit monoclonal antibodies to DNMT1 (cat. # 5032), DNMT3A (# 3598), GAPDH (# 2118), BIM (# 2933) and BCL_XL (# 2764) as well as rabbit polyclonal anti-MCL-1 (# 4572) from Cell Signaling Technology (Danvers, MA); murine monoclonal anti-PUMA (cat #sc-374223) and Lamin B1 (cat # Cat # sc-377000) from Santa Cruz Biotechnology (Dallas, TX); murine monoclonal antibody to PMAIP1/Noxa (cat # ALX-804-408-C100) from Enzo Life sciences (New York, NY); murine monoclonal antibody to CUL1 (clone 2H4C9) from ThermoFisher (Waltham, MA), and rabbit polyclonal anti-Histone H1 (# 39707) from Active Motif (Carlsbad, CA). Rat monoclonal anti-BID was a kind gift from David Huang (Walter and Eliza Hall Institute, Melbourne, Australia).

SUPPLEMENTAL REFERENCES

1. Meng XW, Lee SH, Dai H, et al. Mcl-1 as a buffer for proapoptotic Bcl-2 family members during TRAIL-induced apoptosis: a mechanistic basis for sorafenib (Bay 43-9006)-induced TRAIL sensitization. J Biol Chem. 2007 Oct 12;282(41):29831-29846. eng. Cited in: Pubmed; PMID 17698840.
2. Knorr KL, Finn LE, Smith BD, et al. Assessment of Drug Sensitivity in Hematopoietic Stem and Progenitor Cells from Acute Myelogenous Leukemia and Myelodysplastic Syndrome Ex Vivo. Stem Cells Transl Med. 2017 Mar;6(3):840-850. doi:10.5966/sctm.2016-0034. Cited in: Pubmed; PMID 28297583.
3. Dai H, Ding H, Meng XW, et al. Constitutive BAK activation as a determinant of drug sensitivity in malignant lymphohematopoietic cells. Genes Dev. 2015 Oct 15;29(20):2140-2152. eng. Epub 2015/10/24. doi:10.1101/gad.267997.115. Cited in: Pubmed; PMID 26494789.
4. Patel AG, Flatten KS, Peterson KL, et al. Immunodetection of human topoisomerase I-DNA covalent complexes. Nucleic Acids Res. 2016 Apr 7;44(6):2816-2826. Epub 2016/02/27. doi:10.1093/nar/gkw109. Cited in: Pubmed; PMID 26917015.
5. Dai H, Meng XW, Lee SH, Schneider PA, Kaufmann SH. Context-dependent Bcl-2/Bak interactions regulate lymphoid cell apoptosis. J Biol Chem. 2009 Jul 3;284(27):18311-18322. eng. Epub 2009/04/09. doi:10.1074/jbc.M109.004770. Cited in: Pubmed; PMID 19351886.
6. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A Rapid and Simple Method for Measuring Thymocyte Apoptosis by Propidium Iodide Staining and Flow Cytometry. Journal of Immunological Methods. 1991;139:271-279.
7. Meng X, Chandra J, Loegering D, et al. Central role of FADD in Apoptosis Induction by the Mitogen Activated Activated Protein Kinase Kinase Inhibitor CI1040 (PD184352) in Acute Lymphocytic leukemia Cell Lines in Vitro. Journal of Biological Chemistry. 2003;278:47326-47339.

Supplemental Table 1

Study inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
1. Voluntary written consent given before performance of any study-related procedure not part of standard medical care, with the understanding that consent may be withdrawn by the subject at any time without prejudice to future medical care. 2. Male or female subjects 18 years or older. 3. A diagnosis of morphologically documented AML, secondary AML [from prior conditions, such as myelodysplastic syndrome (MDS)], or therapy-related AML (t-AML), as defined by World Health Organization (WHO) criteria. 4. During the dose-escalation phase, only subjects with relapsed/refractory AML (patients who have received prior AML therapy and have ≥ 5% blasts) will be eligible. 5. During the expansion phase, subjects with relapsed/refractory AML or newly diagnosed AML with adverse risk will be eligible. Adverse risk is defined as: - Adverse risk according to ELN criteria - Secondary AML defined as therapy-related AML or AML arising from MDS, myeloproliferative neoplasm or aplastic anemia. 6. Eastern Cooperative Oncology Group (ECOG) performance status of 0–2. 7. Clinical laboratory values within the following parameters: a. Albumin >2.7 g/dL. b. Total bilirubin ≤ institutional upper limit of normal (ULN). Patient with total bilirubin > ULN may enroll if direct bilirubin ≤1.5 x institutional ULN of the direct bilirubin. c. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) ≤3 × institutional ULN.	1. Acute promyelocytic leukemia. 2. Extramedullary ONLY relapse AML. 3. For the dose-expansion phase, newly diagnosed AML patients who are suitable for and agreeable to receive intensive induction chemotherapy. 4. Treatment with systemic antineoplastic therapy within 14 days or five half-lives from the last dose (whichever is sooner) before cycle 1 day 1 of therapy. Radiation within 14 days before cycle 1 day 1 of therapy. The use of hydroxyurea for leukoreduction is permitted. Subjects must have recovered from the side effects of prior therapy per treating physician discretion. 5. Hematopoietic Stem Cell Transplantation (HCT) within 100 days of enrollment, or evidence of veno-occlusive disease (VOD) at any time post-transplant, or active acute graft-versus-host disease requiring systemic immunosuppressive therapy. 6. Any serious medical or psychiatric illness that could, in the investigator's opinion, potentially interfere with the completion of study procedures. 7. Current systemic treatment with strong or moderate CYP3A inducers within seven days prior to cycle 1 day 1 of therapy. 8. Any evidence of spontaneous tumor lysis syndrome (TLS). 9. Active, significant, uncontrolled infection. Subjects with infections that are controlled by antibiotics, antiviral or antifungal therapy can be enrolled in the study. 10. Presence of another active malignancy (requiring treatment) diagnosed within 12 months with the exception of a. adequately treated non-melanoma skin cancer. b. adequately treated melanoma grade 2 or less.

d. Creatinine clearance ≥ 30 mL/min (calculated by Cockcroft-Gault formula, using actual weight).
e. White blood cell (WBC) count < 25,000/μL before administration of pevonedistat on cycle 1 day 1. (Note: hydroxyurea may be used to meet this criterion.)
f. PT and PTT < 1.5 x institutional ULN.

8. Female subjects who
   a. Are postmenopausal (see Appendix 9 for definition) for at least one year before the screening visit, OR
   b. Are surgically sterile, OR
   c. If they are of childbearing potential:
      i. Agree to practice one highly effective method and one additional effective (barrier) method of contraception, at the same time, from the time of signing the informed consent through four months after the last dose of study drug (female and male condoms should not be used together), OR
      ii. Agree to practice true abstinence, when this is in line with the preferred and usual lifestyle of the subject. (Periodic abstinence [e.g., calendar, ovulation, symptothermal, post-ovulation methods] withdrawal, spermicides only, and lactational amenorrhea are not acceptable methods of contraception).
9. Male subjects, even if surgically sterilized (i.e., status postvasectomy), who:
   a. Agree to practice effective barrier contraception during the entire study treatment period from the time of signing the informed consent through and through four months after the last dose of study drug (female and male condoms should not be used together), OR
   b. Agree to practice true abstinence, when this is in line with the preferred and usual lifestyle of the subject. (Periodic abstinence [e.g., calendar, ovulation,

c. cervical intraepithelial neoplasia.
d. adequately treated in situ carcinoma of the cervix uteri or carcinoma in situ of breast.
e. basal cell carcinoma of the skin or localized squamous cell carcinoma of the skin.
f. adequately treated prostate cancer.

11. Life-threatening illness with life expectancy < 6 months unrelated to cancer.
12. Known HIV positive patients who DO NOT meet the following criteria:
    • CD4 count > 350 cells/mm3
    • Undetectable viral load.
    • Maintained on modern therapeutic regimens utilizing non-CYP-interactive agents.
    • No history of AIDS-defining opportunistic infections.
13. Known hepatitis B surface antigen seropositive or known or suspected active hepatitis C infection. Note: Patients who have isolated positive hepatitis B core antibody (i.e., in the setting of negative hepatitis B surface antigen and negative hepatitis B surface antibody) must have an undetectable hepatitis B viral load. Patients who have positive hepatitis C antibody may be included if they have an undetectable hepatitis C viral load.
14. Known hepatic cirrhosis or severe pre-existing hepatic impairment.
15. Known cardiopulmonary disease defined as:
    a. Unstable angina.
    b. Congestive heart failure (New York Heart Association [NYHA] Class III or IV; see Appendix 7).
    c. Myocardial infarction (MI) within six months prior to enrollment. Subjects who had ischemic heart disease such as acute coronary syndrome (ACS), MI, and/or revascularization greater than six months before screening and who

symptothermal, post-ovulation methods for the female partner] withdrawal, spermicides only, and lactational amenorrhea are not acceptable methods of contraception.)	are without cardiac symptoms may enroll. d. Symptomatic cardiomyopathy. e. Clinically significant pulmonary hypertension requiring pharmacologic therapy. f. Clinically significant arrhythmia. i. History of polymorphic ventricular fibrillation or torsade de pointes. ii. Permanent atrial fibrillation (a fib), defined as continuous a fib for ≥ 6 months. iii. Persistent a fib, defined as sustained a fib lasting > 7 days and/or requiring cardioversion in the four weeks before screening. iv. Grade 3 a fib defined as symptomatic and incompletely controlled medically, or controlled with device (e.g., pacemaker), or ablation and v. Patients with paroxysmal a fib or < grade 3 a fib for period of at least six months are permitted to enroll provided that their rate is controlled on a stable regimen. 16. Chronic respiratory disease that requires continuous oxygen, or significant history of renal, neurologic, psychiatric, endocrinologic, metabolic, immunologic, hepatic, cardiovascular disease, or any other medical condition that in the opinion of the investigator would adversely affect his/her participating in this study. 17. Treatment with any investigational products, other than the study drugs, within 14 days before the study enrollment or during the study period. 18. Uncontrolled high blood pressure (i.e., systolic blood pressure > 180 mm Hg, diastolic blood pressure > 95 mm Hg). 19. Prolonged rate corrected QT (QTc) interval ≥ 500 msec, calculated as per institutional guidelines.

	20. Left ventricular ejection fraction (LVEF) < 50% as assessed by echocardiogram or radionuclide angiography. 21. Uncontrolled coagulopathy or bleeding disorder. 22. Known moderate-to-severe chronic obstructive pulmonary disease, interstitial lung disease, and pulmonary fibrosis. 23. Major surgery within 14 days before the enrollment or a scheduled major surgery during study period. 24. Known central nervous system (CNS) involvement with AML at time of study enrollment. Patients with previous treatment for CNS involvement who are neurologically stable and without evidence of disease are eligible. 25. Gastrointestinal (GI) tract disease that causes an inability to take oral medications, malabsorption syndrome, prior surgical procedures affecting absorption, uncontrolled inflammatory GI disease (e.g. Crohn's disease, ulcerative colitis). 26. Female subjects who are both lactating and breastfeeding or of childbearing potential who have a positive serum pregnancy test during screening 27. Female subjects who intend to donate eggs (ova) during the course of this study or within four months after receiving their last dose of study drug(s). 28. Male subjects who intend to donate sperm during the course of this study or within four months after receiving their last dose of study drug(s). 29. Has consumed grapefruit, grapefruit products, Seville oranges (including marmalade containing Seville oranges) or star fruit from three days prior to cycle 1 day 1 to throughout the study treatment.

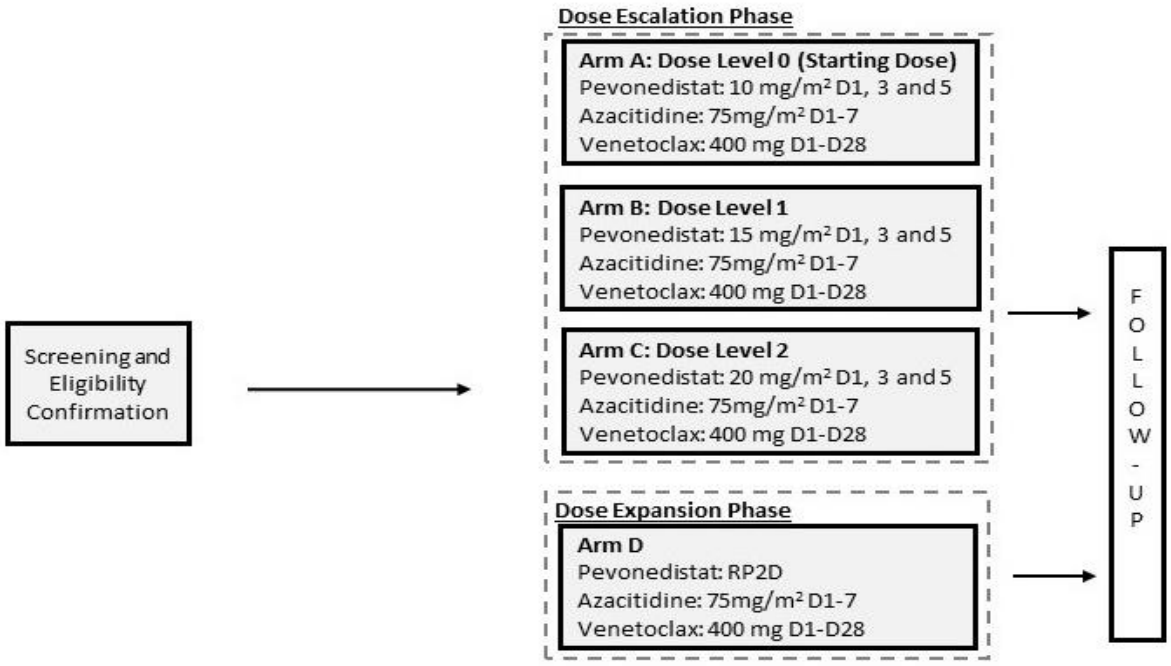
Supplemental Table 2
Definitions of dose limiting toxicity (DLT)

Hematological DLT	**Non-Hematological DLT**
Grade 4 neutropenia (ANC<500) lasting 42 days from the start of the cycle, in the absence of evidence of active AML and a pretreatment ANC of ≥ 500. Grade 3 or 4 thrombocytopenia that fails to recover to at least grade 2 at 42 days post-treatment in the absence of evidence of active AML, and a pretreatment platelet count of > 50,000. Neutropenic fever and/or infection is an expected complication in this subject population and will not be considered a DLT	DLT for non-hematological toxicity will be defined as any of the following events that are not attributable to underlying leukemia or an alternative cause: • Grade 3 or greater PT or aPTT elevation in the absence of anticoagulation therapy. • Grade 2 or greater elevation of the PT or aPTT that is associated with clinically significant bleeding (CNS, GI, etc.). • Grade 3 or greater nausea and/or emesis that lasts more than 48 hours despite use of optimal antiemetic prophylaxis. Optimal anti-emetic prophylaxis is defined as an antiemetic regimen that employs a 5-hydroxytryptamine 3 serotonin receptor antagonist given in standard doses and according to standard schedules. Grade 3 nausea and vomiting may not be excluded as a DLT if they require hospitalization, TPN or tube feeding. • Grade 3 or greater diarrhea that lasts more than 48 hours despite maximal supportive therapy. Grade 3 diarrhea may not be excluded as a DLT if it requires hospitalization, TPN or tube feeding. • Grade 3 arthralgia/myalgia that lasts more than 48 hours despite the use of optimal analgesia. • Any other grade 3 or greater non-hematologic toxicity with the following exceptions: o Brief (< 1 week) grade 3 fatigue. o Grade 3 or 4 electrolyte/metabolic abnormalities including, but not limited to abnormalities in sodium, albumin, glucose, potassium, magnesium, phosphorus, chloride and/or bicarbonate that are not corrected with adequate medical intervention (per institutional requirements) within 72 hours. o Grade 3 TLS

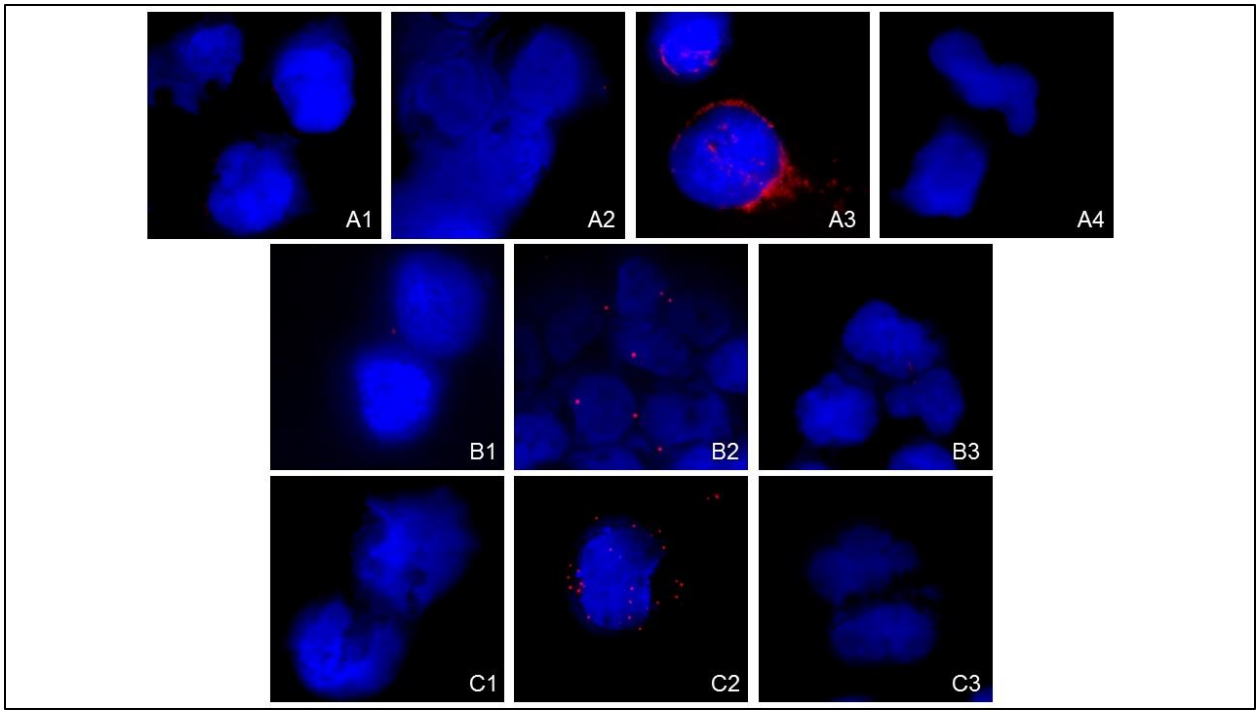
	• Persistent elevations of transaminases and bilirubin > grade 3 for > 2 days between doses. • Other non-hematologic toxicities grade 3 or greater that require a dose reduction (not related to concomitant medication) or discontinuation of therapy. • A delay in the initiation of cycle 2 due to a lack of adequate recovery from treatment-related toxicity (recovery to ≤ grade 1 or to subject's baseline values) of more than two weeks due to non-hematologic toxicities.

Supplemental Table 3
Response based on prior hypomethylating/venetoclax exposure

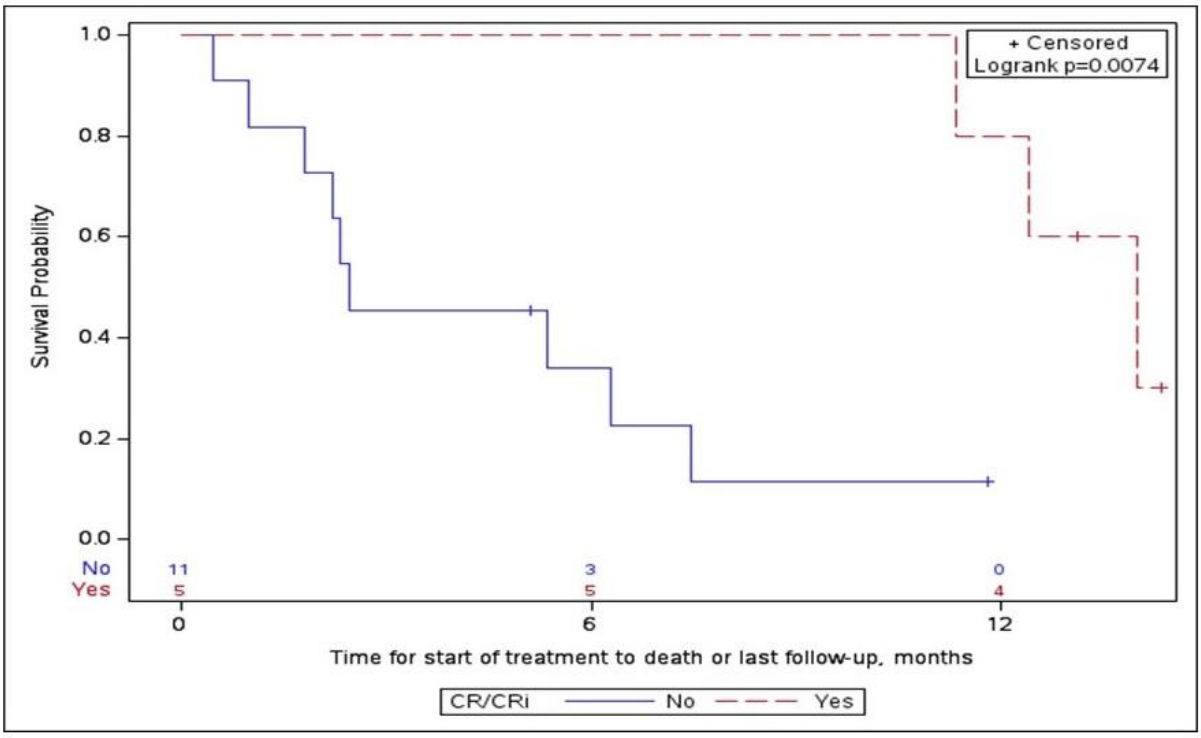
Variables	Total N=15 (col %)	Prior Venetoclax/hypomethylating: No N=7 (col %)	Prior Venetoclax/hypomethylating: Yes N=8 (col %)
Best response			
Complete remission (CR)	5 (33.3)	5 (71.4)	0 (0.0)
Morphologic leukemia free state (MLFS)	1 (6.7)	0 (0.0)	1 (12.5)
Partial remission marrow (PR)	1 (6.7)	1 (14.3)	0 (0.0)
Treatment failure	8 (53.3)	1 (14.3)	7 (87.5)
Overall response			
No response	8 (53.3)	1 (14.3)	7 (87.5)
Response (CR/CRi/CRh/PR/MLFS)	7 (46.7)	6 (85.7)	1 (12.5)
CR/CRi			
No	11 (68.8)	2 (28.6)	9 (100.0)
Yes	5 (31.3)	5 (71.4)	0 (0.0)
MRD among CR/CRi			
CR/CRi with MRD-	4 (80.0)	4 (80.0)	0 ()
CR/CRi with MRD+	1 (20.0)	1 (20.0)	0 ()
Missing (not in CR/CRi)	10	2	8



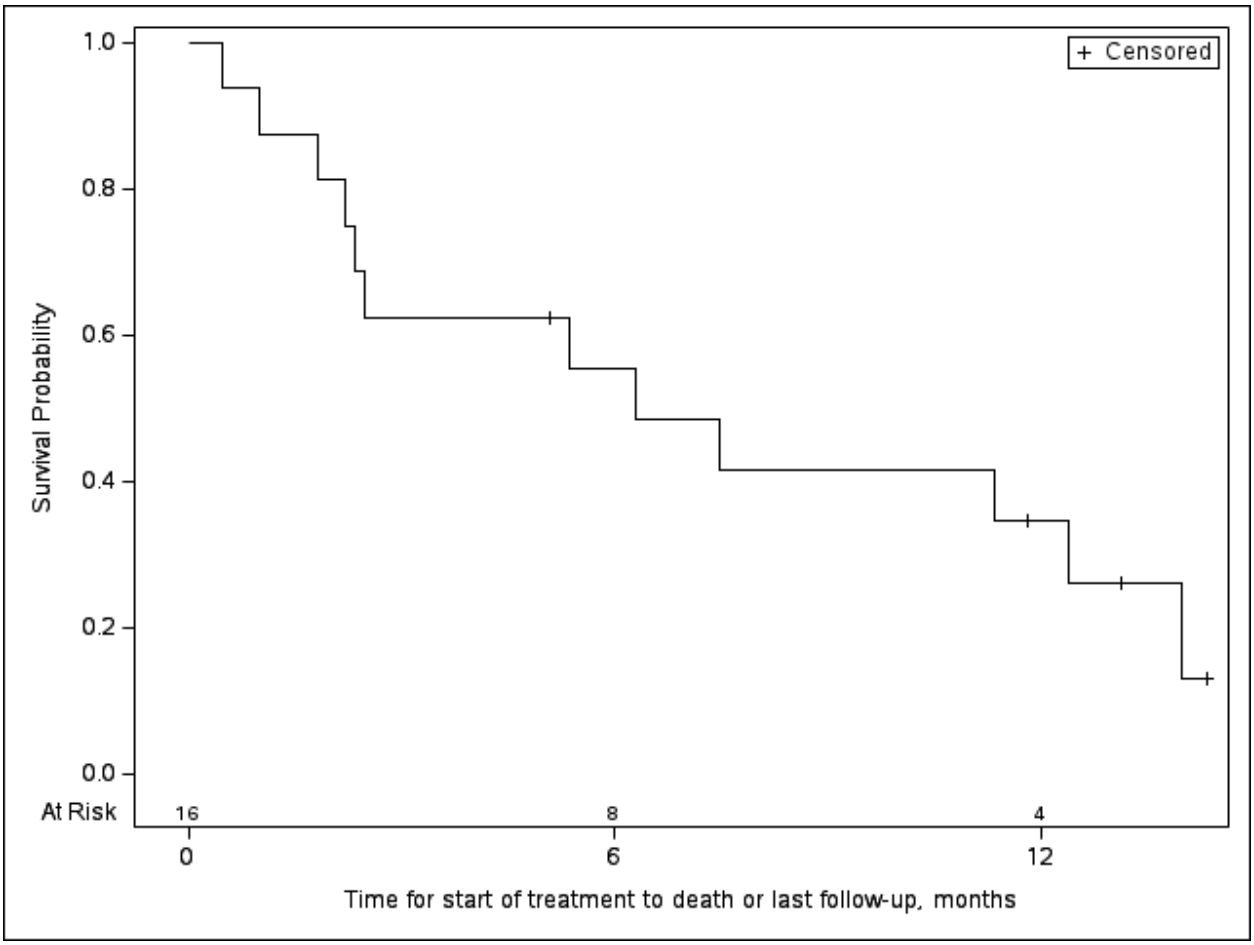
Supplemental Figure 1 – Study Design



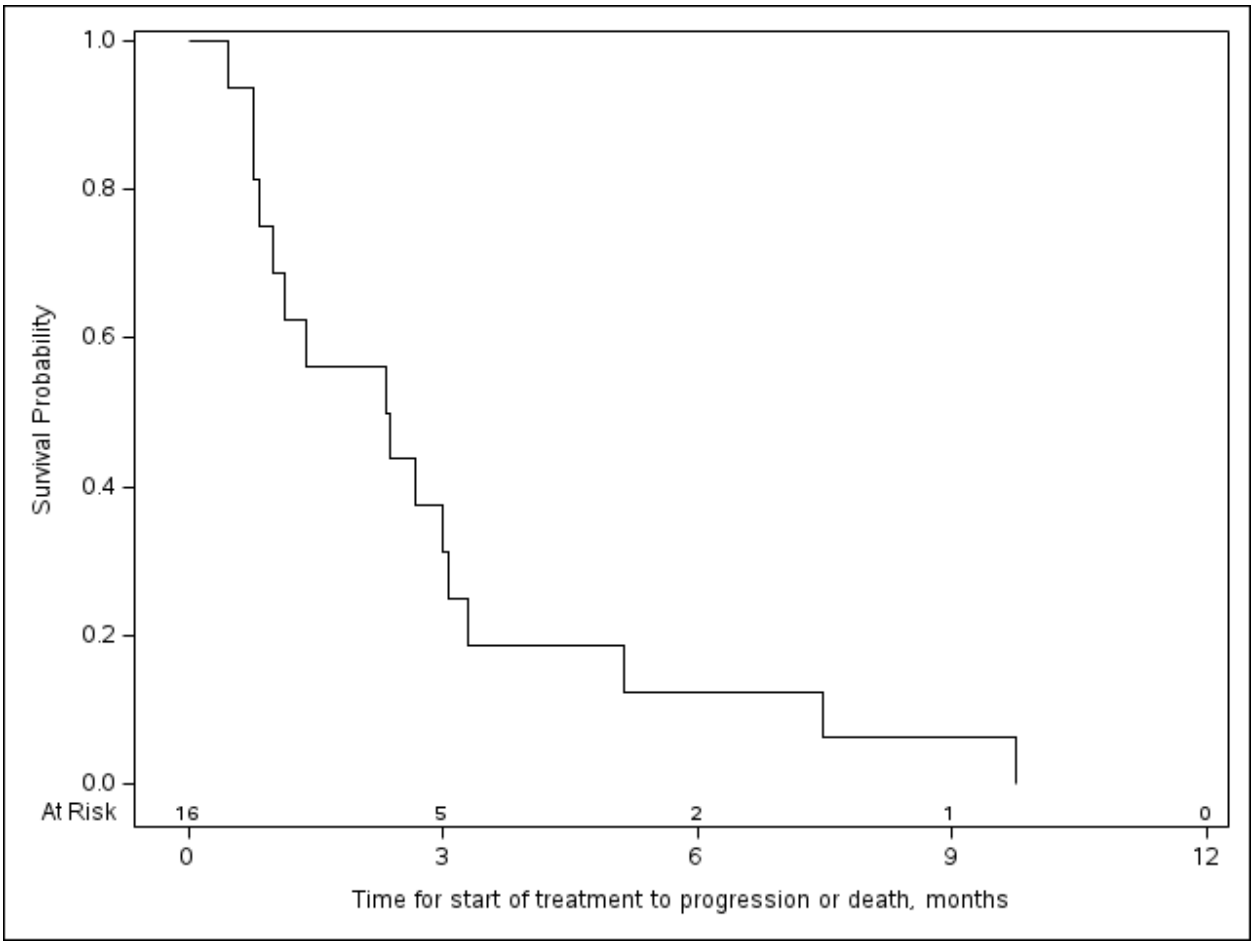
Supplemental Figure 2. Controls for Proximity Ligation Assays. A. Before being processed for PLA, parental K562 cells were incubated with BAK antibodies only (A1). Alternatively, *BAK*$^{-/-}$ K562 cells (A2) and parental K562 cells (A3) were incubated with BAK and BCLXL antibodies. Parental K562 cells were incubated with BAK and BCLXL antibodies after being pretreated with 5 µM of the pan-caspase inhibitor QVDOPH and the selective BCLXL inhibitor A1155463 at 7.5 µM for 2 hours (A4). **B.** Before being processed for PLA, Jurkat cells were incubated with BAK antibodies only (B1). Jurkat cells were incubated with BAK and MCL1 antibodies (B2). Jurkat cells were incubated with BAK and MCL1 antibodies after being pretreated with 5 µM of QVDOPH and the selective MCL1 inhibitor S63835 at 7.5 µM for 2 hours (B3). **C.** Before being processed for PLA, RL cells were incubated with BAK antibodies only (C1). RL cells were incubated with BAK and BCL2 antibodies (C2). RL cells were incubated with BAK and BCL2 antibodies after being pretreated with 5 µM of QVDOPH and the selective BCL2 inhibitor ABT-199 (venetoclax) at 7.5 µM for 2 hours (C3).



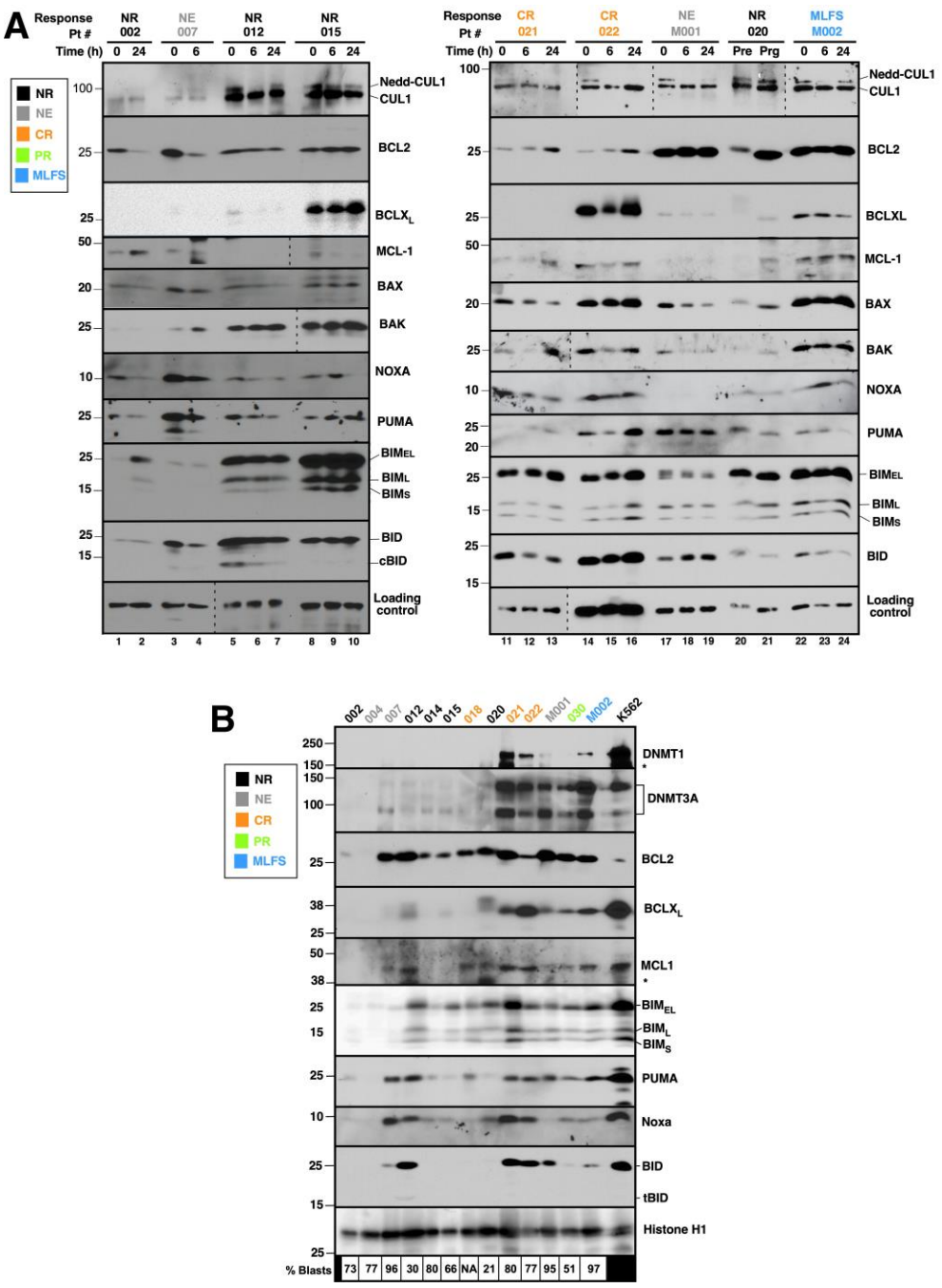
Supplemental Figure 3. Overall survival based on response to therapy



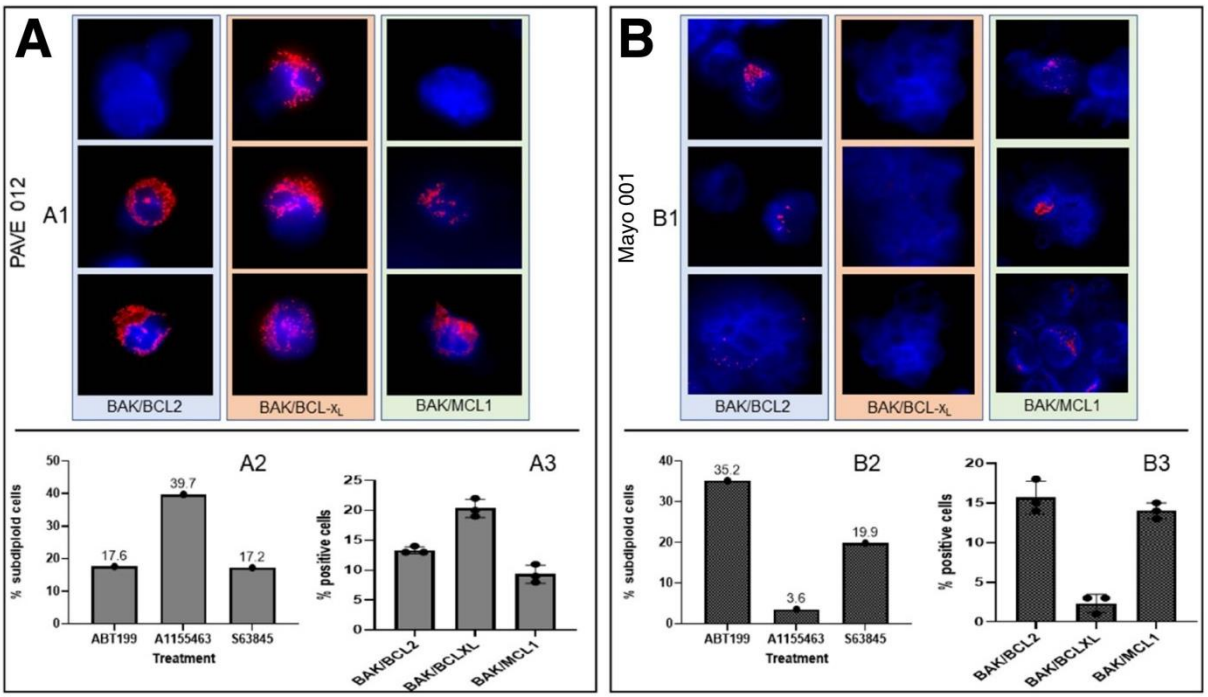
Supplemental Figure 4. Overall survival for the entire cohort



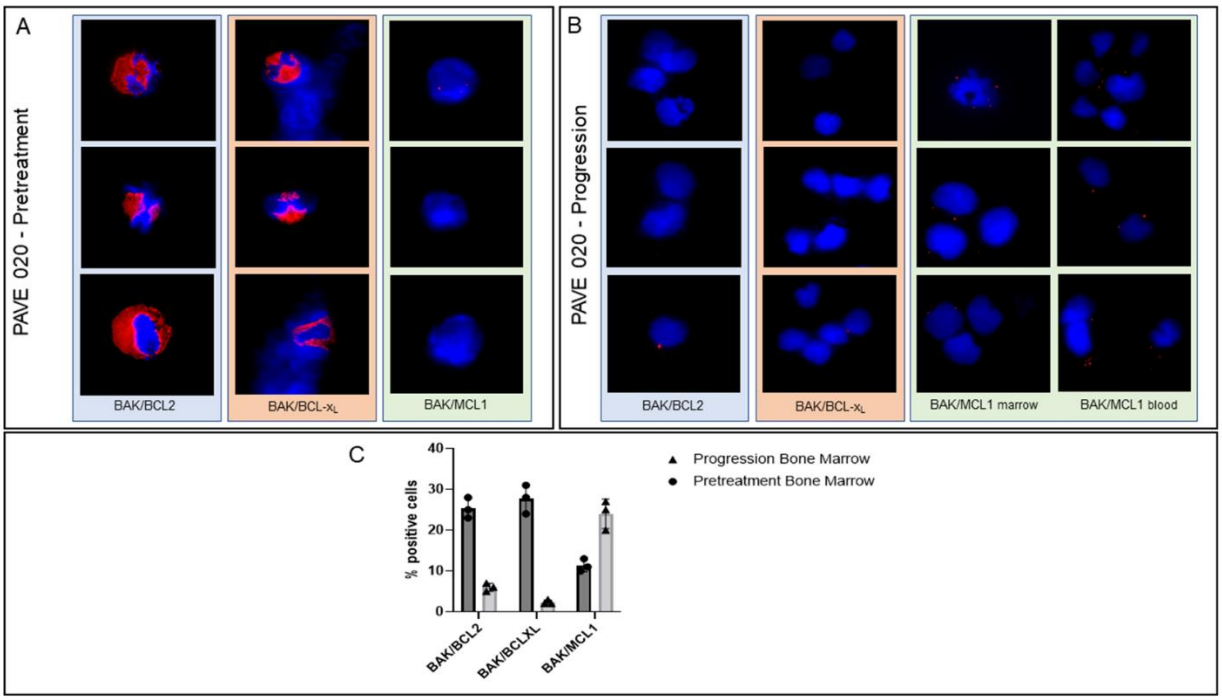
Supplemental Figure 5. Progression free survival for the entire cohort



Supplemental Figure 6 (related to Figure 1). A, aliquots of samples collected from peripheral blood prior to treatment (0 or pre), at 6 or 24 h after pevonedistat administration, and at progression (Prg) were subjected to immunoblotting for a more extended panel of antigens than shown in Fig. 2A. Lamin B or GAPDH served as a loading control. Dashed lines indicate that different exposures of the same blot (upper panels) or different loading controls (lower panel) have been juxtaposed. **B,** aliquots of pretreatment bone marrow aspirates were subjected to immunoblotting for a more extended panel of antigens, particularly BCL2 family members, compared to Fig. 2D. Numbers at bottom indicate the percentage of blasts in the sample. NA, not available.



Supplemental Figure 7. Proximity ligation assays and BH3 mimetic sensitivity. Pretreatment bone marrow samples from patients PAVE 012 and Mayo 001 were subjected to PLAs for preformed complexes of BAK with BCL2, $BCLX_L$ and MCL1 (illustrated in A1 and B1, summarized in A3 and B3). The same samples were treated for 4 h with 1 μM venetoclax (ABT199), A1155463 ($BCLX_L$ inhibitor) or S63845 (MCL1 inhibitor) and subjected to flow microfluorimetry for DNA fragmentation (panels A2 and B2) as previously described.(6, 7) Note that the pattern of BH3 mimetic sensitivity roughly parallels the pattern of preformed BAK complexes as previously described in tissue culture cell lines.(3)



Supplemental Figure 8. PLAs at diagnosis and progression. Bone marrow samples harvested from the same patient prior to treatment and at the time of progression were subjected to PLAs for preformed complexes of BAK with BCL2, $BCLX_L$ and MCL1 (panels A and B, respectively). Results are summarized in panel C. Note that complexes of BAK with BCL2 and $BCLX_L$ decreased at the time of relapse, consistent with the elevated levels of BAX (a tighter binder to BCL2 and $BCLX_L$) detected at progression (Supplemental Figure 6A, 5th panel), whereas complexes of BAK with MCL1 increased, consistent with the elevated levels of MCL1 at relapse (Supplemental Figure S6A, 4th panel).