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

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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).

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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.

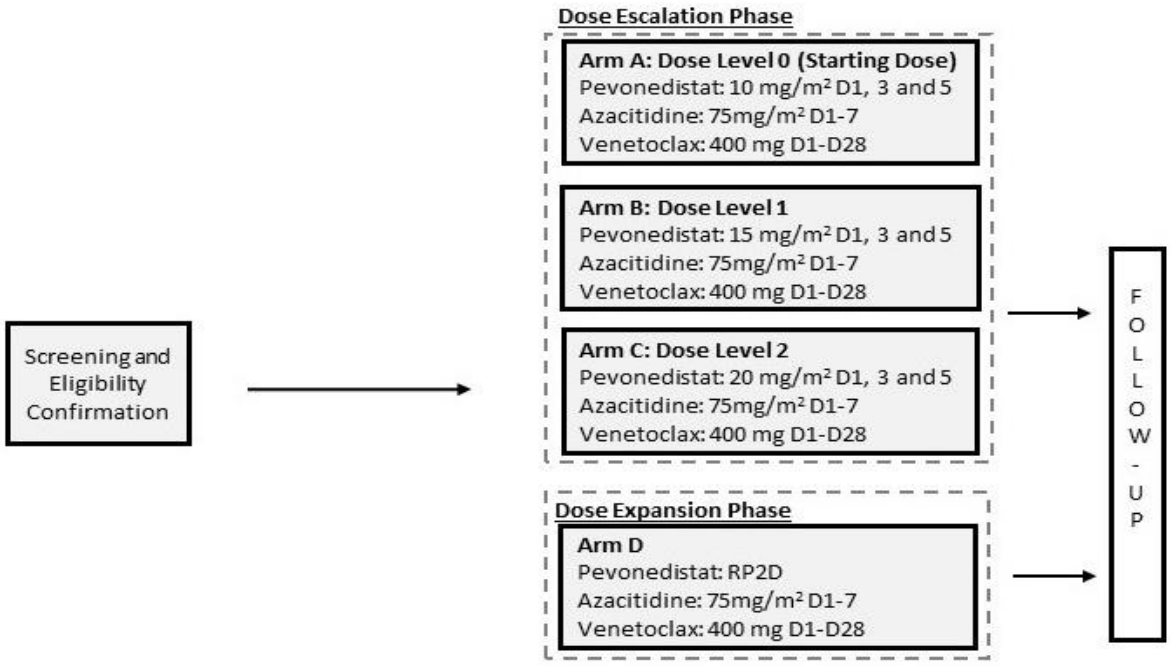
Supplemental Table 2
Definitions of dose limiting toxicity (DLT)

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

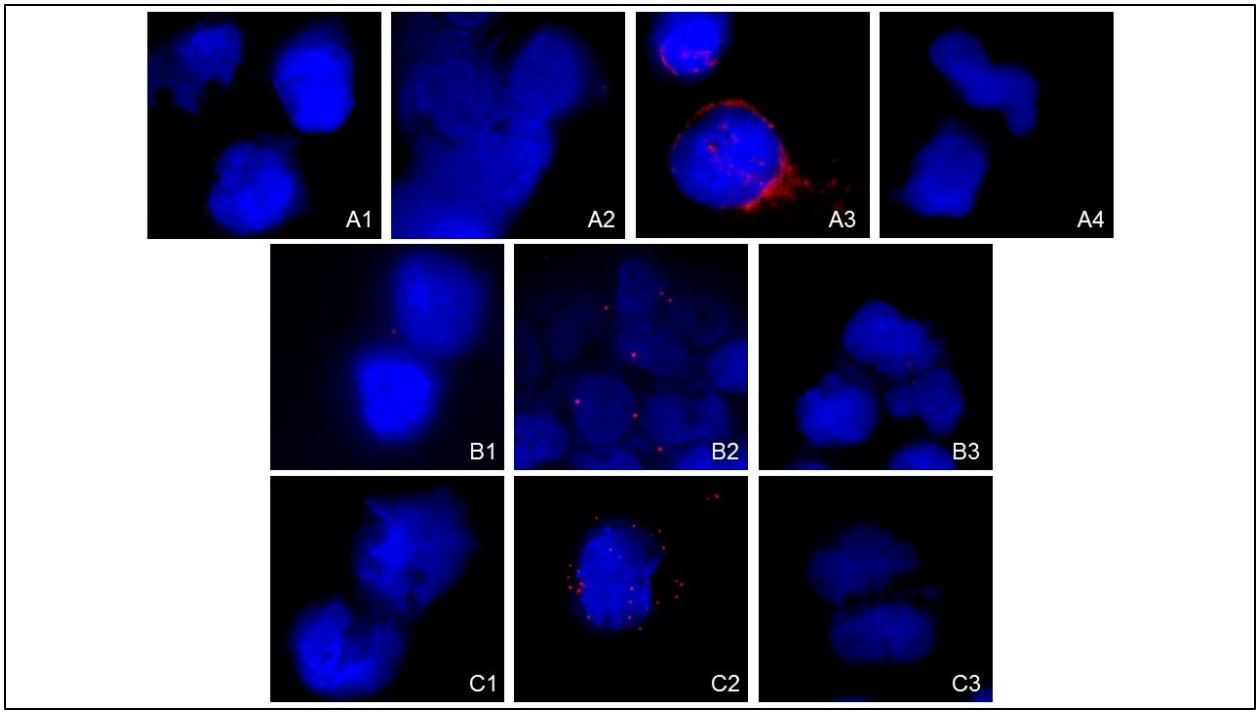
	• Persistent elevations of transaminases and bilirubin > grade 3 for > 2 days between doses. • Other non-hematologic toxicities grade 3 or greater that require a dose reduction (not related to concomitant medication) or discontinuation of therapy. • A delay in the initiation of cycle 2 due to a lack 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

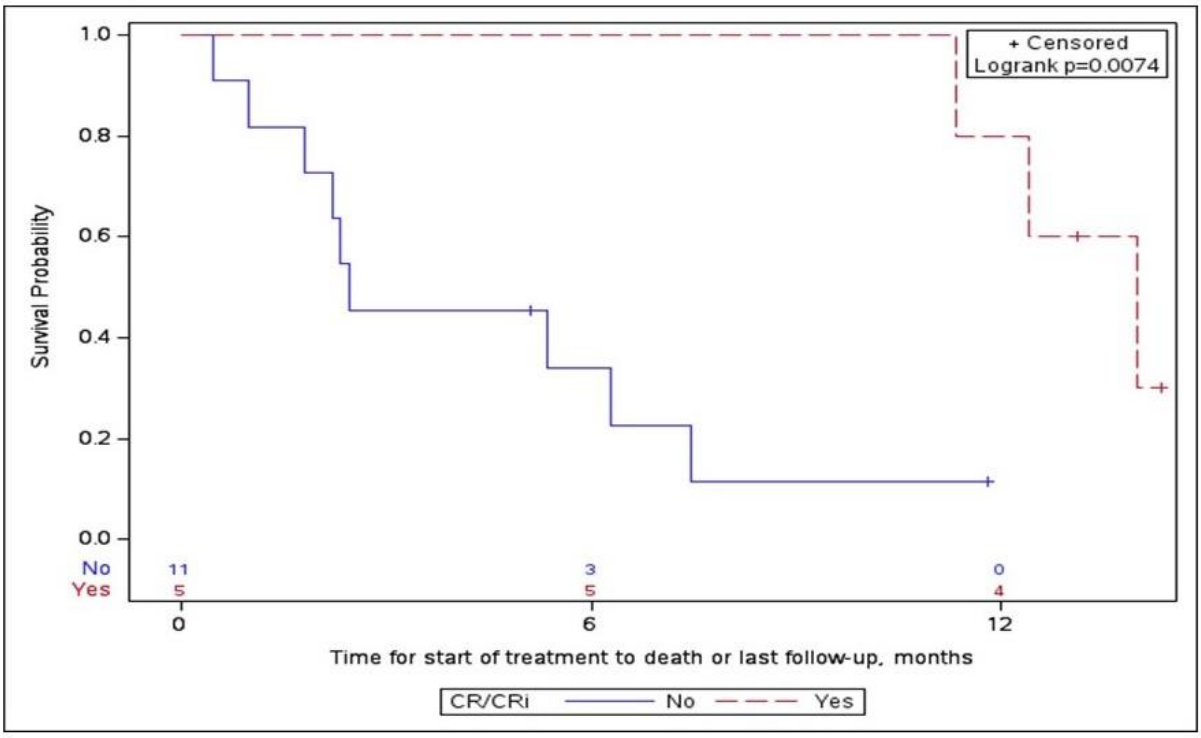
Variables	Total N=15 (col %)	Prior Venetoclax/hypomethylating: No N=7 (col %)	Prior Venetoclax/hypomethylating: Yes N=8 (col %)
Best response			
Complete remission (CR)	5 (33.3)	5 (71.4)	0 (0.0)
Morphologic leukemia free state (MLFS)	1 (6.7)	0 (0.0)	1 (12.5)
Partial remission marrow (PR)	1 (6.7)	1 (14.3)	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



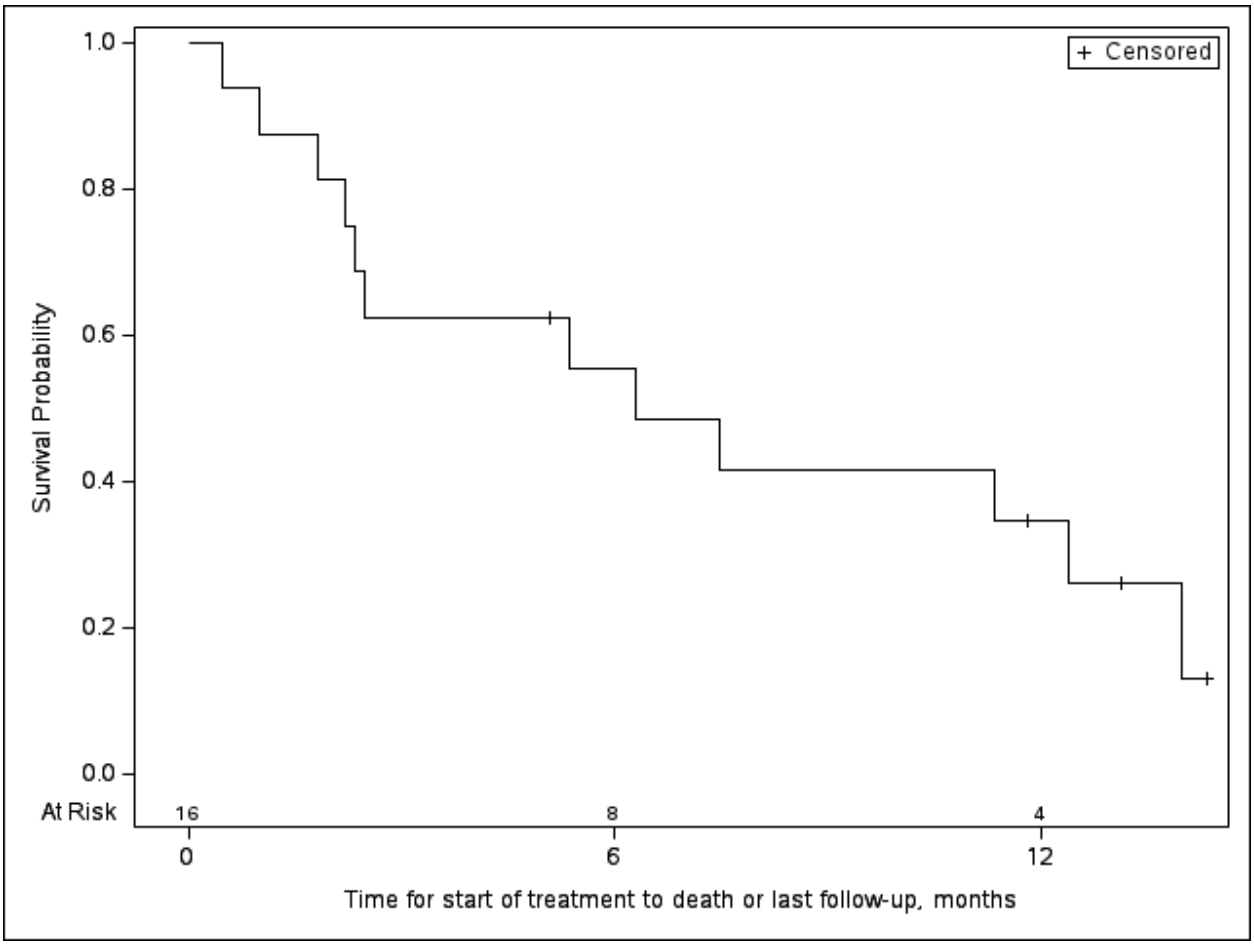
Supplemental Figure 1 – Study Design



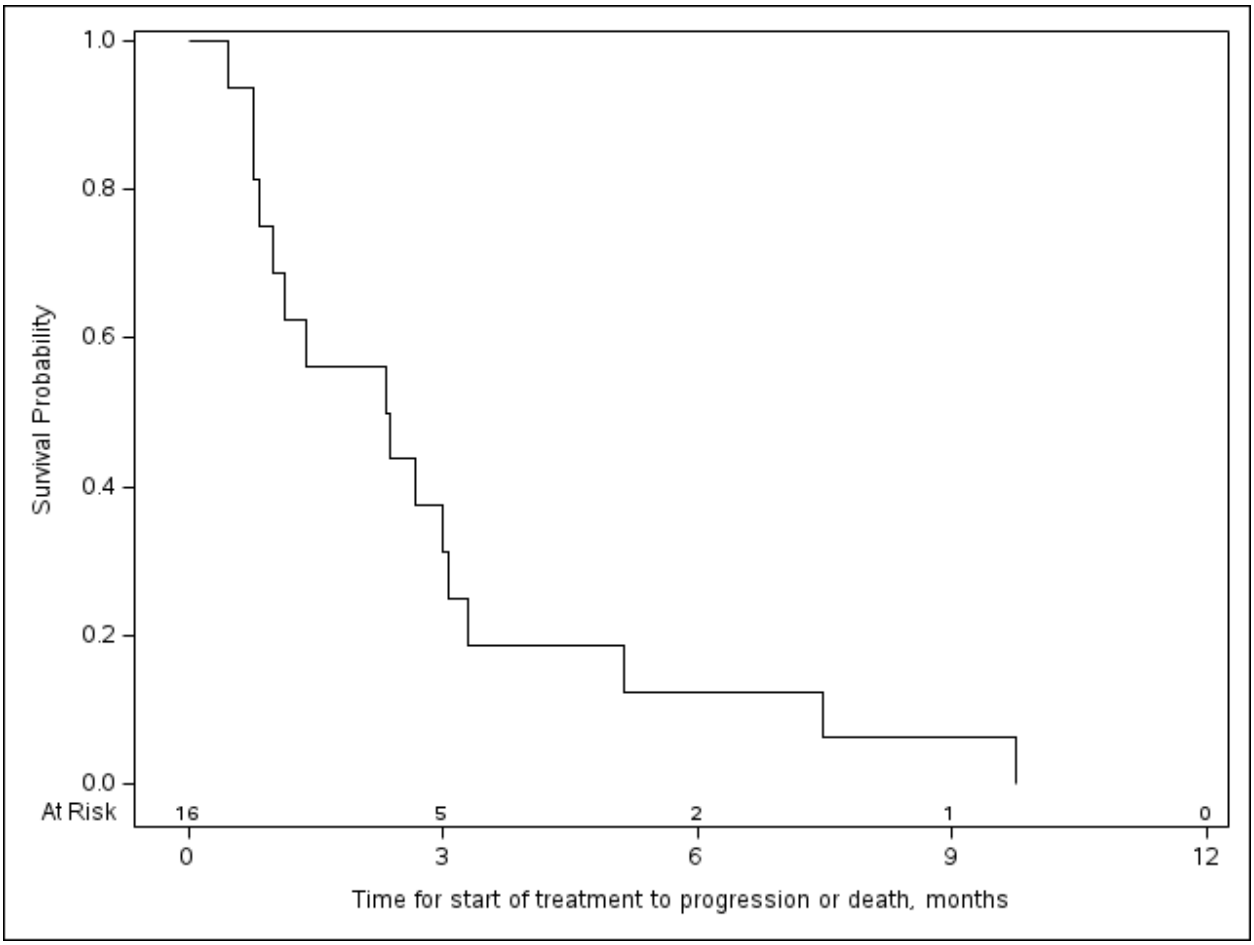
Supplemental Figure 2. Controls for Proximity Ligation Assays. A. Before being processed for PLA, parental K562 cells were incubated with BAK antibodies only (A1). Alternatively, *BAK*$^{-/-}$ K562 cells (A2) and parental K562 cells (A3) were incubated with BAK and BCLXL antibodies. Parental K562 cells 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).



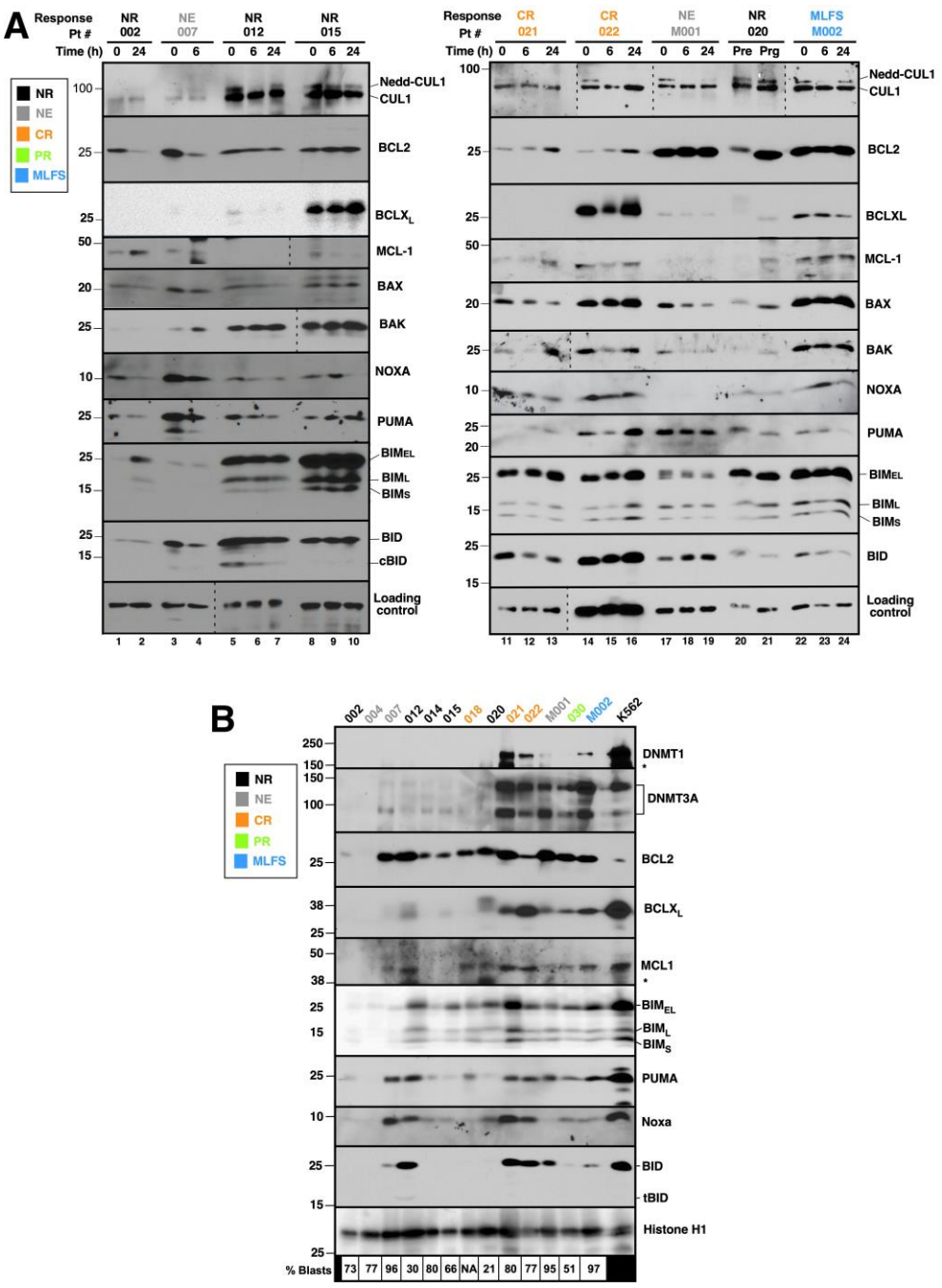
Supplemental Figure 3. Overall survival based on response to therapy



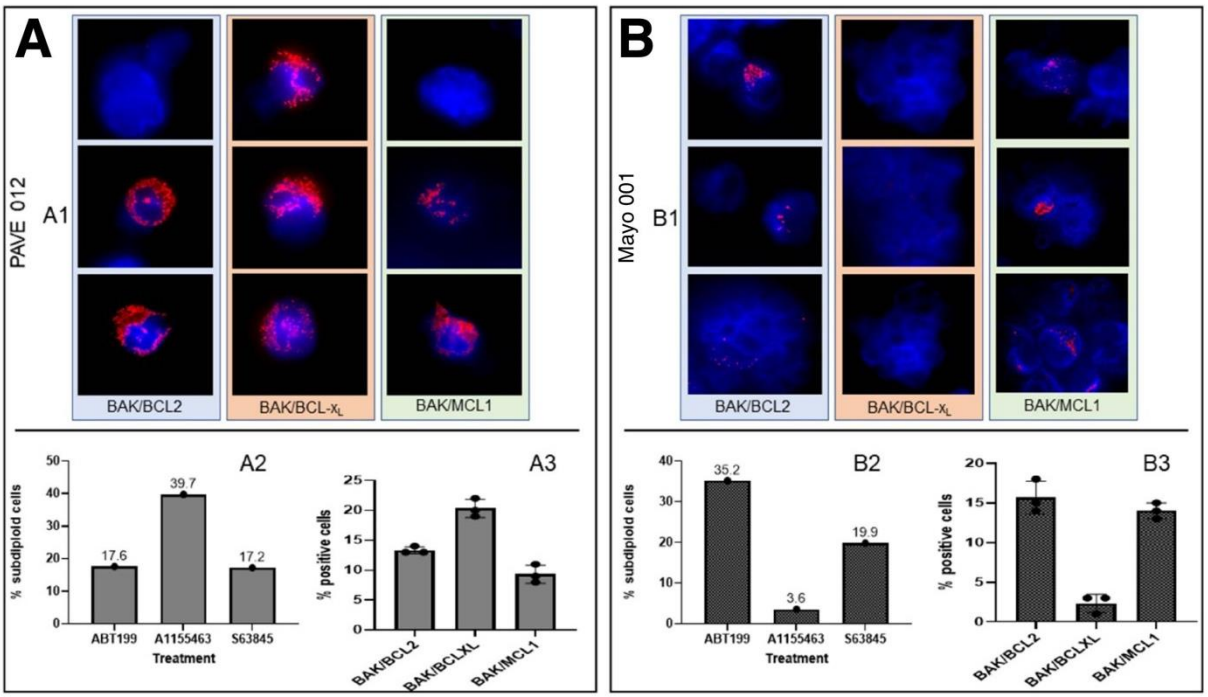
Supplemental Figure 4. Overall survival for the entire cohort



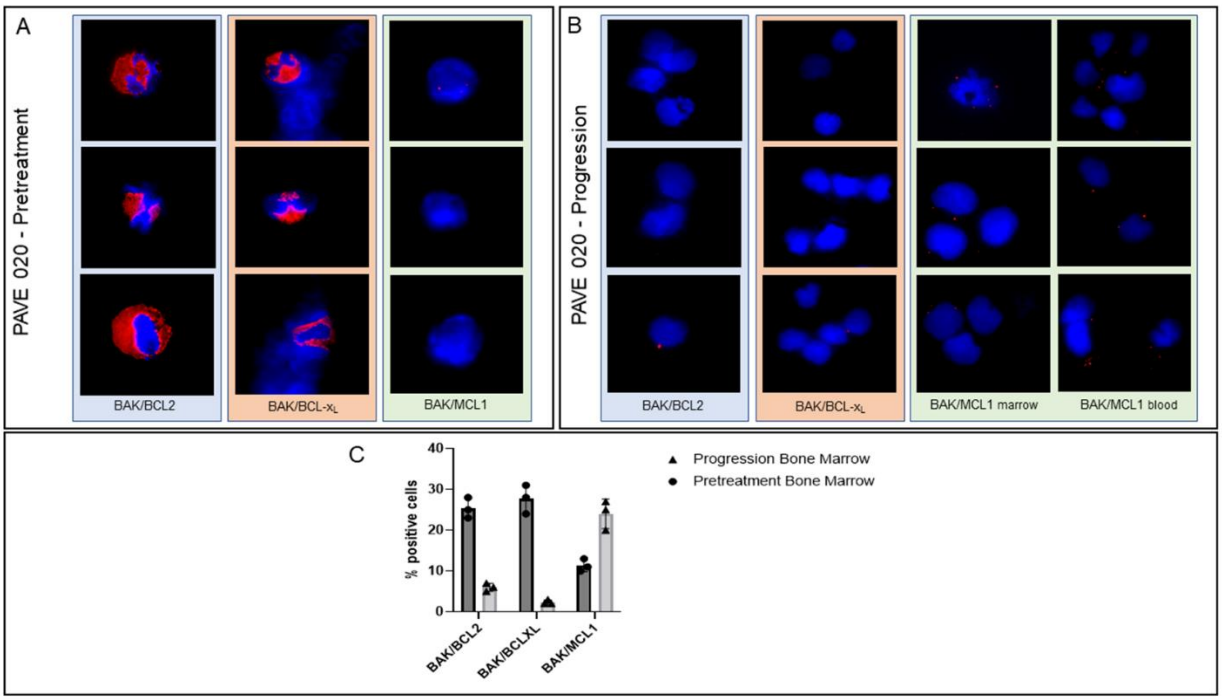
Supplemental Figure 5. Progression free survival for the entire cohort



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).