

# A Novel MCL1 Inhibitor Combined with Venetoclax Rescues Venetoclax-Resistant Acute Myelogenous Leukemia

Haley E. Ramsey<sup>1</sup>, Melissa A. Fischer<sup>1</sup>, Taekyu Lee<sup>2,3</sup>, Agnieszka E. Gorska<sup>1</sup>, Maria Pia Arrate<sup>1</sup>, Londa Fuller<sup>1</sup>, Kelli L. Boyd<sup>4</sup>, Stephen A. Strickland<sup>1,5</sup>, John Sensintaffar<sup>2</sup>, Leah J. Hogdal<sup>2</sup>, Gregory D. Ayers<sup>5,6,7</sup>, Edward T. Olejniczak<sup>2,3</sup>, Stephen W. Fesik<sup>2,3,5</sup>, and Michael R. Savona<sup>1,5</sup>

## ABSTRACT

Suppression of apoptosis by expression of antiapoptotic BCL2 family members is a hallmark of acute myeloblastic leukemia (AML). Induced myeloid leukemia cell differentiation protein (MCL1), an antiapoptotic BCL2 family member, is commonly upregulated in AML cells and is often a primary mode of resistance to treatment with the BCL2 inhibitor venetoclax. Here, we describe VU661013, a novel, potent, selective MCL1 inhibitor that destabilizes BIM/MCL1 association, leads to apoptosis in AML, and is active in venetoclax-resistant cells and patient-derived xenografts. In addition, VU661013 was safely combined with venetoclax for synergy in murine models of AML. Importantly, BH3 profiling of patient samples and drug-sensitivity testing *ex vivo* accurately predicted cellular responses to selective inhibitors of MCL1 or BCL2 and showed benefit of the combination. Taken together, these data suggest a strategy of rationally using BCL2 and MCL1 inhibitors in sequence or in combination in AML clinical trials.

**SIGNIFICANCE:** Targeting antiapoptotic proteins in AML is a key therapeutic strategy, and MCL1 is a critical antiapoptotic oncoprotein. Armed with novel MCL1 inhibitors and the potent BCL2 inhibitor venetoclax, it may be possible to selectively induce apoptosis by combining or thoughtfully sequencing these inhibitors based on a rational evaluation of AML. *Cancer Discov*; 8(12); 1566-81. ©2018 AACR.

See related commentary by Leber *et al.*, p. 1511.

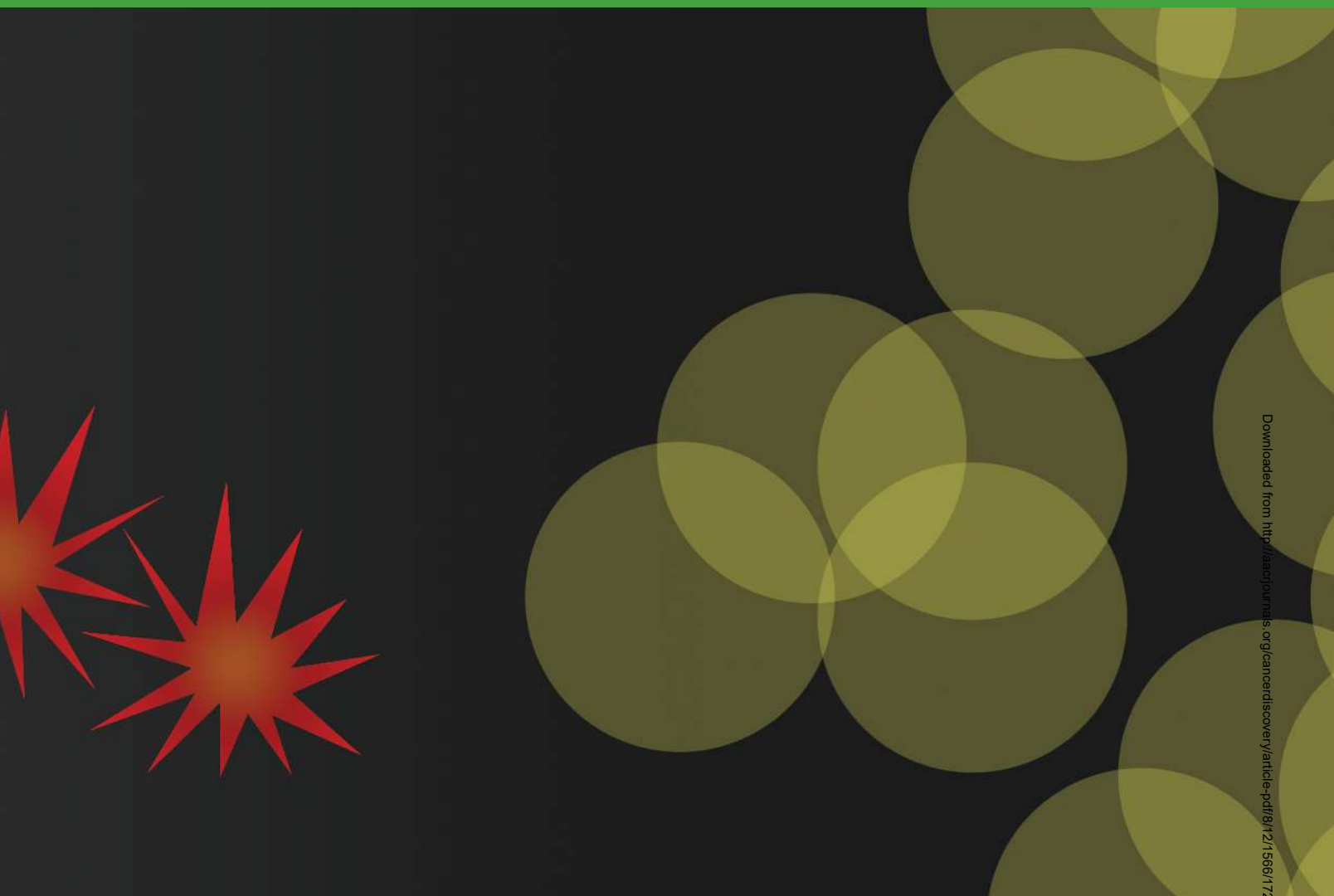
<sup>1</sup>Department of Internal Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee. <sup>2</sup>Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee. <sup>3</sup>Vanderbilt Institute for Chemical Biology, Vanderbilt University, Nashville, Tennessee. <sup>4</sup>Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee. <sup>5</sup>Vanderbilt-Ingram Cancer Center, Nashville, Tennessee. <sup>6</sup>Department of Biostatistics, Vanderbilt University School of Medicine, Nashville, Tennessee. <sup>7</sup>Vanderbilt Center for Quantitative Sciences, Vanderbilt University, Nashville, Tennessee.

**Note:** Supplementary data for this article are available at Cancer Discovery Online (<http://cancerdiscovery.aacrjournals.org/>).

**Corresponding Author:** Michael R. Savona, Vanderbilt University School of Medicine, 777 Preston Research Building, 2200 Pierce Avenue, Nashville, TN 37232. Phone: 615-936-3321; Fax: 615-343-7602; E-mail: michael.savona@vanderbilt.edu

**doi:** 10.1158/2159-8290.CD-18-0140

©2018 American Association for Cancer Research.



## INTRODUCTION

Acute myeloid leukemia (AML) is characterized by the block of differentiation and clonal proliferation of myeloid precursor cells resulting in the failure of normal hematopoiesis. Despite recent advances, mortality remains high, with most patients succumbing to their disease in less than 5 years (1–4). Clonal expansion in AML often occurs as a series of somatic mutations in a relatively small number of genes required for transcription, cell signaling, epigenetic modification, methylation, DNA repair, or other key cellular processes. These collude to provide survival advantages to subpopulations of neoplastic cells (5). This expansion of abnormal cells often coincides with dysregulation of cellular apoptotic machinery that regularly maintains healthy cell populations in homeostasis with a balance of proapoptotic and antiapoptotic proteins that control cell fate. As cells often become dependent on specific antiapoptotic proteins in malignancy, the development of small-molecule “BH3 mimetics” to selectively target these key proteins is of interest in AML (6–8).

The BCL2 family of proteins are important regulators of apoptosis and are engaged in normal homeostasis (9). The

elegant juxtaposition between proapoptotic and antiapoptotic BCL2 family proteins occurs at the mitochondrion where the effectors of apoptosis, BAK and BAX, promote mitochondrial outer membrane permeabilization (MOMP) and apoptotic cell death when activated by BH3-only activators BIM, BID, and PUMA. Antiapoptotic proteins (BCL2, MCL1, BCL-xL, and BCL-w) derail this process by sequestering the effector and activator proteins and preventing activation of MOMP (8, 10–15). Elucidation of this process has led to considerable efforts for discovering small molecules designed to occupy the hydrophobic BH3 binding site of antiapoptotic proteins freeing effector molecules BAX/BAK to oligomerize and to initiate apoptosis (16–22). Initially, fragment-based methods and structure-based design led to the discovery of ABT-737, an inhibitor of BCL2, BCL-xL, and BCL-w, and subsequently an orally bioavailable analogue, ABT-263 [navitoclax (NAV); refs. 16, 23–27]. In clinical trials, navitoclax induced the death of platelets culminating in severe thrombocytopenia (28) due to the inhibition of BCL-xL. To circumvent this thrombocytopenia, ABT-199 [venetoclax (VEN)], which selectively binds BCL2, was discovered using structure-based design (29).

Responses with VEN in clinical trials have validated the potential of this line of therapy in a wide variety of hematologic malignancies (6, 30, 31) and more recently in AML (32, 33). However, treatment with VEN can lead to the development of resistance largely through the upregulation of alternative antiapoptotic proteins. Follicular lymphoma cells treated with VEN developed resistance associated with the upregulation of MCL1 and, conversely, loss of MCL1 sensitized non-Hodgkin lymphoma to BCL2 inhibition with VEN (34, 35). AML cell lines conditioned for VEN resistance developed a dependence for MCL1 and, to a lesser degree, BCL-xL (36). Elevated MCL1 protein levels, specifically, have been noted in the setting of resistance to cytotoxic therapies and to therapies targeting BCL2/BCL-xL in myelodysplastic syndrome (MDS; ref. 37), and VEN treatment failure most strongly correlated with elevated MCL1 protein in a clinical trial of relapsed and refractory AML (32). This could explain the relatively modest clinical efficacy in trials of the BCL2/BCL-xL inhibitor ABT-263 in untreated, “BH3-unclassified” MDS and in monotherapy trials of VEN in relapsed/refractory AML (32, 38), and may emerge in resistant cases treated more effectively with VEN together with either DNA methyltransferase inhibitors (DNMTi; ref. 33) or low-dose cytarabine (NCT: 00287233). Although dependence on specific antiapoptotic proteins is not necessarily mutually exclusive in cells, it is discoverable, and mutable in the face of treatment. The use of combinatory, and perhaps even sequential, inhibition of antiapoptotic proteins holds promise.

Overexpression of the antiapoptotic gene *MCL1* is one of the most common aberrations in human cancer, and it is overexpressed in many types of hematologic malignancies where it is associated with high tumor grade and poor survival (39–42). Although hematologic malignancies such as chronic lymphocytic leukemia (CLL) escape apoptosis exclusively via *BCL2* overexpression and dysregulation, myeloid malignancies, particularly AML and MDS, tend to display heterogeneous expression of antiapoptotic proteins with common reliance on myeloid cell leukemia-1 (MCL1; refs. 6, 43, 44). Although MCL1 was considered a particularly difficult protein to target with small molecules (45), several groups have recently discovered potent MCL1 inhibitors (46–48). Using fragment-based methods and structure-based design, we have discovered a series of potent (sub nmol/L), selective, small-molecule MCL1 inhibitors (46). One of these molecules, VU661013, is a potent inhibitor of MCL1 *in vitro* and *in vivo* in a variety of tumors.

Here, we show that the targeting of MCL1 with the small-molecule BH3 mimetic VU661013 drastically reduces AML cell viability, and that responsiveness of cells can be predicted reliably via a dynamic *in vitro* bioassay consistent with antiapoptotic dependence as demonstrated by cytochrome C release assay (49). Strategic employment of MCL1 and BCL2 inhibition in sequence or in combination can dramatically enhance and extend duration of response in AML cells. Characterizing the BH3 mimetic dependence of patient cells *ex vivo*, prior to therapy or at relapse, could be used to design better treatment strategies to suppress AML. The introduction of potent MCL1 inhibitors into the clinic should lead to the rational deployment of BH3 mimetic therapy resulting in a more stable disease regression.

## RESULTS

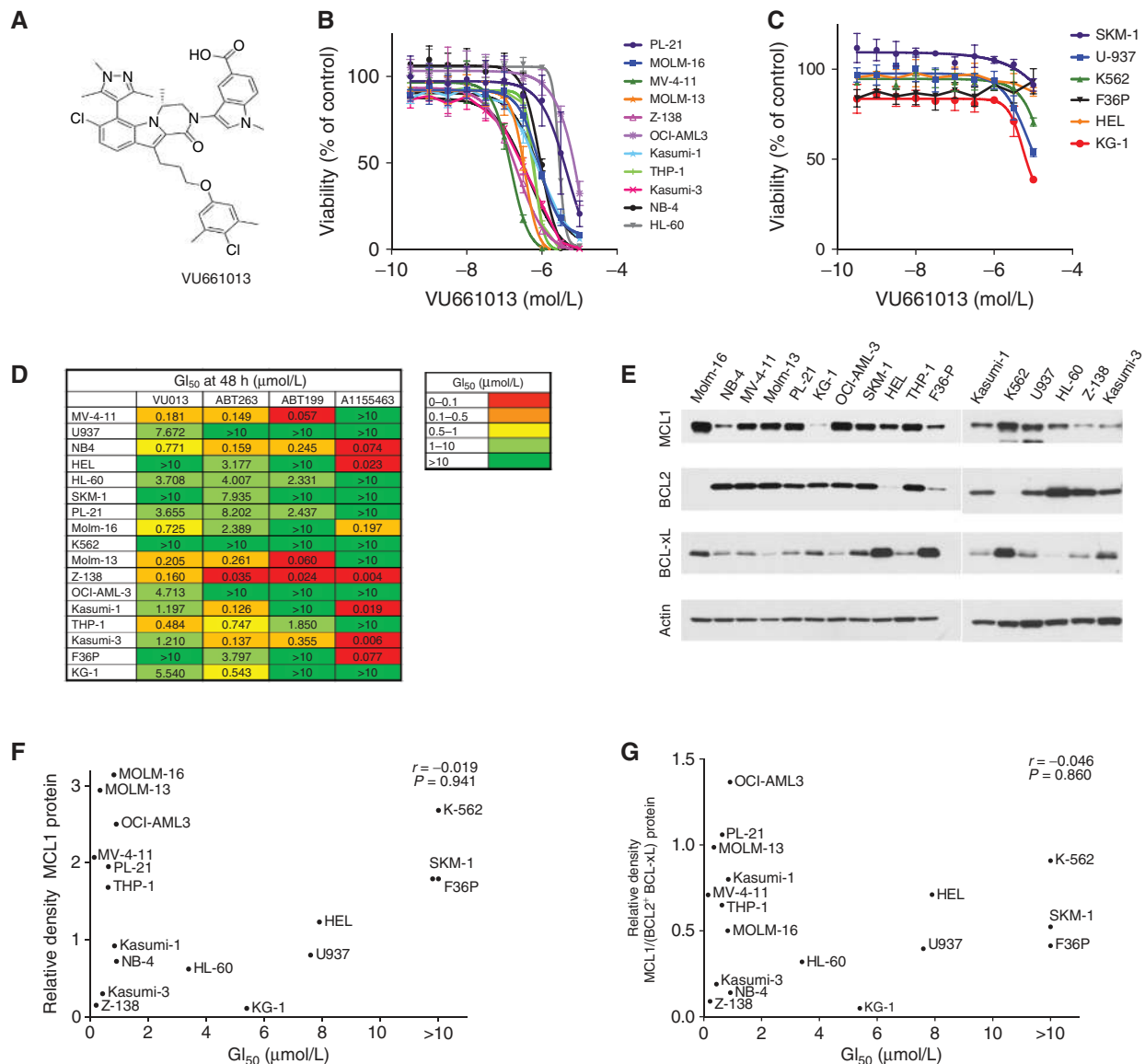
### VU661013 Is a Potent and Selective MCL1 Inhibitor That Reduces Expansion of AML Cell Lines

VU661013 (Fig. 1A) was discovered by potency optimization of our previously reported MCL1 inhibitors using structure-based design (46). VU661013 exhibited a  $K_i$  of  $97 \pm 30$  pmol/L to human MCL1 in a TR-FRET assay by displacing a fluorescently labeled peptide derived from the proapoptotic protein BAK. However, VU661013 did not significantly inhibit BCL-xL ( $K_i > 40$   $\mu$ mol/L) or BCL2 ( $K_i = 0.73$   $\mu$ mol/L; Supplementary Fig. S1).

In order to test the activity of VU661013 in cells, we treated AML cell lines with VU661013 and observed growth inhibition in a majority of the tested cell lines while few failed to respond (Fig. 1B and C). Additionally, we screened a variety of AML cell lines with specific inhibitors of both BCL2 and BCL-xL (NAV), BCL2 (VEN), and BCL-xL (A1155463). Multiple cell lines displayed a high sensitivity to both VEN and VU661013 as single agents. A small number of cell lines, including K562, showed resistance to all inhibitors, but most lines responded to one or more of the specific BCL2 family inhibitors with variable sensitivity (Fig. 1D). Protein levels of MCL1, BCL2, and BCL-xL in these cell lines were assessed via western blot (Fig. 1E). The quantity of antiapoptotic BCL2 family member protein detected in cell lines varied significantly, consistent with previous analyses describing the heterogeneity of BCL2 family protein levels and antiapoptotic dependence in AML (44, 50). The responsiveness of cells to VU661013 did not correlate with either protein levels of MCL1 (Fig. 1F) or the ratio of MCL1/(BCL2+BCL-xL) in the majority of cell lines (Fig. 1G). Although a selection of resistant cell lines revealed a strong correlation between sensitivity and protein content, this occurred only at extraordinarily high doses of VU661013 and may represent off-target effects (Supplementary Fig. S1B). Further associative analyses of the ratios of BCL2, BCL-xL, and MCL1 protein levels versus AML cell line sensitivity to the MCL1 inhibitor also failed to demonstrate a relationship between BCL2 family protein levels and response to MCL1 inhibition (Supplementary Fig. S2). These findings are consistent with previously published data noting poor correlation between protein level and response to other BH3 mimetics (51, 52).

### VU661013 Ameliorates Tumor Burden in a Disseminated Model of AML

Based on the significant potency observed for VU661013 *in vitro* (Fig. 1B; Supplementary Fig. S3A) and its favorable murine pharmacokinetic profile (Supplementary Fig. S3B), we tested our MCL1 inhibitor in a xenograft transplantation model of MV-4-11 cell line in NSGS mice (NOD/SCID IL2R $\gamma$ null-3/GM/SF). After establishing disseminated leukemia, NSGS mice were dosed intraperitoneally with 10, 25, or 75 mg/kg of VU661013 daily for 21 days. Weekly chimerism analyses were conducted, and the percentage of MV-4-11 cells was quantified in murine peripheral blood using antihuman CD45 (hCD45) and anti-hCD33 monoclonal antibodies. Twenty-eight days after transplant, vehicle-treated mice had developed large leukemia burdens, and thus mice were sacrificed and

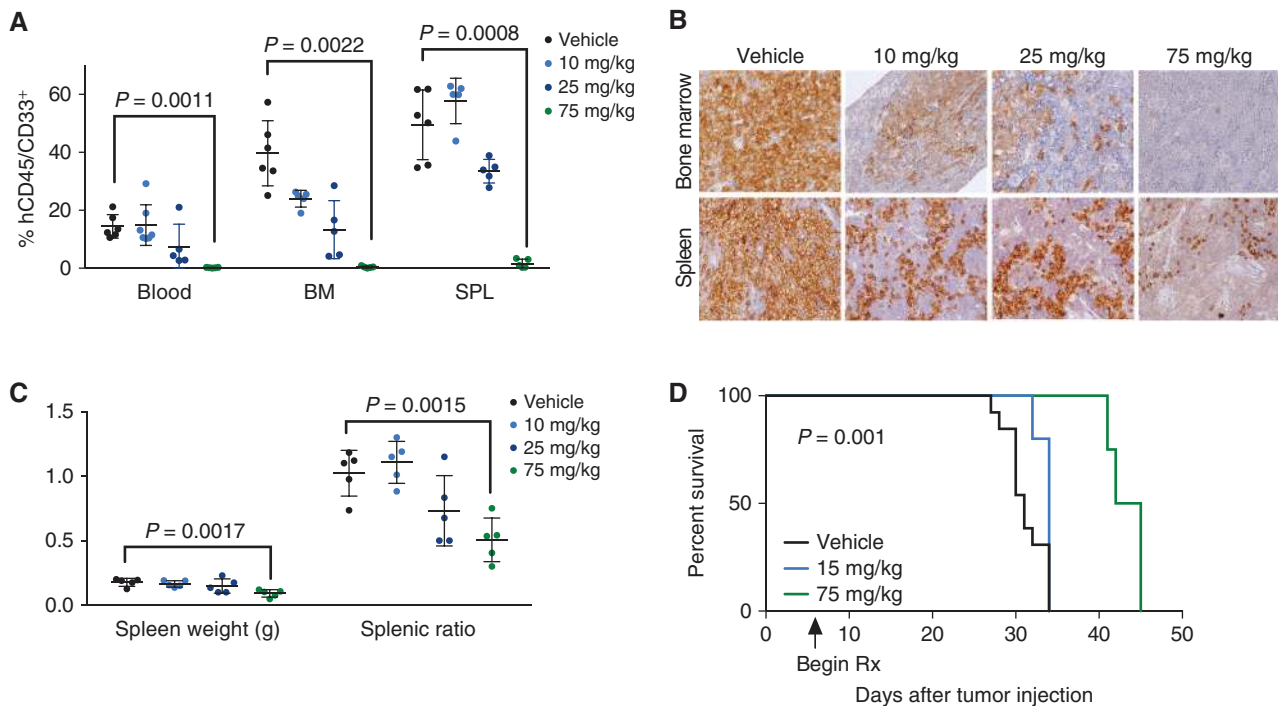


**Figure 1.** VU661013 blocks expansion of AML cell lines *in vitro*. **A**, The chemical structure of VU661013. **B**, Measurement of growth sensitivity to inhibition of MCL1 with VU661013 in several cell lines, **C**, with resistance in some lines (mean  $\pm$  SEM). **D**, AML cell lines were subjected to inhibition of MCL1 (VU661013), BCL2/BCL-xL/BCL-w [navitoclax (NAV)], BCL2 (venetoclax), or BCL-xL (A1155463), and GI<sub>50</sub> at 48 hours was calculated. Cell lines with GI<sub>50</sub> values above 10  $\mu$ mol/L are listed as >10  $\mu$ mol/L as a specific value is unknown above our maximum concentration tested. **E**, Western blot analysis of AML cell lines reveals a wide variation in the protein content of BCL2 family members. **F** and **G**, Correlation of MCL1 protein content to growth inhibition in response to VU661013 treatment.

their organs were harvested for analysis. Vehicle-treated mice died of xenografted AML, but VU661013-treated mice had no evidence of VU661013-related toxicity in nontarget organs (Supplementary Fig. S3C). VU661013 treatment of disseminated human AML resulted in a dose-dependent decrease in tumor burden, nearly eliminating the hCD45<sup>+</sup> MV-4-11 cells at the 75 mg/kg dose in the blood (mean, 13.0%  $\pm$  2.2% in vehicle vs. 7.4%  $\pm$  7.2% in 25 mg/kg vs. 0.17%  $\pm$  0.12% in 75 mg/kg treated mice), bone marrow (mean, 40.7%  $\pm$  13.9% in vehicle vs. 33.46%  $\pm$  4.0% in 25 mg/kg vs. 0.384  $\pm$  0.345 in 75 mg/kg treated mice), and spleen (mean, 46.22%  $\pm$  13.3% in vehicle vs. 13.31%  $\pm$  10.0% in 25 mg/kg vs. 1.588%  $\pm$  1.51% in 75 mg/kg treated mice) as noted in Fig. 2A. Evidence for this significant decrease in tumor burden was

also observed in the IHC analysis of hCD45 cells in bone marrow and splenic tissue of treated mice (Fig. 2B). Congruently, treatment with VU661013 reduced disease-associated splenomegaly (mean, vehicle vs. 75 mg/kg, 0.17  $\pm$  0.02 vs. 0.09  $\pm$  0.01 g), and amended spleen-to-body-weight ratio (vehicle vs. 75 mg/kg, 0.99 vs. 0.50; Fig. 2C). In a second MV-4-11 xenograft study, mice were followed until death, and survival was evaluated by Kaplan-Meier analysis. In this study, NSGS mice were treated daily (starting 7 days after transplant) with vehicle only, 15 mg/kg or 75 mg/kg of VU661013. Analysis revealed an increase in survival in mice treated with the 75 mg/kg dose (vehicle-treated mice = 31 days, vs. 15 mg/kg = 32 days, vs. 75 mg/kg treated mice = 43 days; Fig. 2D).





**Figure 2.** Inhibition of MCL1 reduces AML in an *in vivo* murine model. **A**, NSGS mice were engrafted with MV-4-11 human leukemia cells and were then treated with either vehicle ( $n = 6$ ) or 10 ( $n = 5$ ), 25 ( $n = 5$ ), or 75 ( $n = 5$ ) mg/kg of VU661013. Peripheral blood, bone marrow (BM), and spleen (SPL) were harvested for tricompartmental chimerism analysis. A nonparametric, unpaired, two-tailed *t* test was used to calculate significance. **B**, IHC of femurs and spleen (20 $\times$ ) stained with monoclonal antibody for hCD45 reveal AML cells left within the bone marrow and spleen of experimental mice at each dose level. **C**, Ratio of spleen to total body weight measurements from above-mentioned experiments. **D**, Kaplan-Meier analysis. Statistical significance was calculated using log-rank (Mantel-Cox) test ( $P = 0.001$ ;  $n = 5$  per arm).

### Cells Adaptively Resistant to MCL1 Inhibition Display an Increased BCL2 Dependency

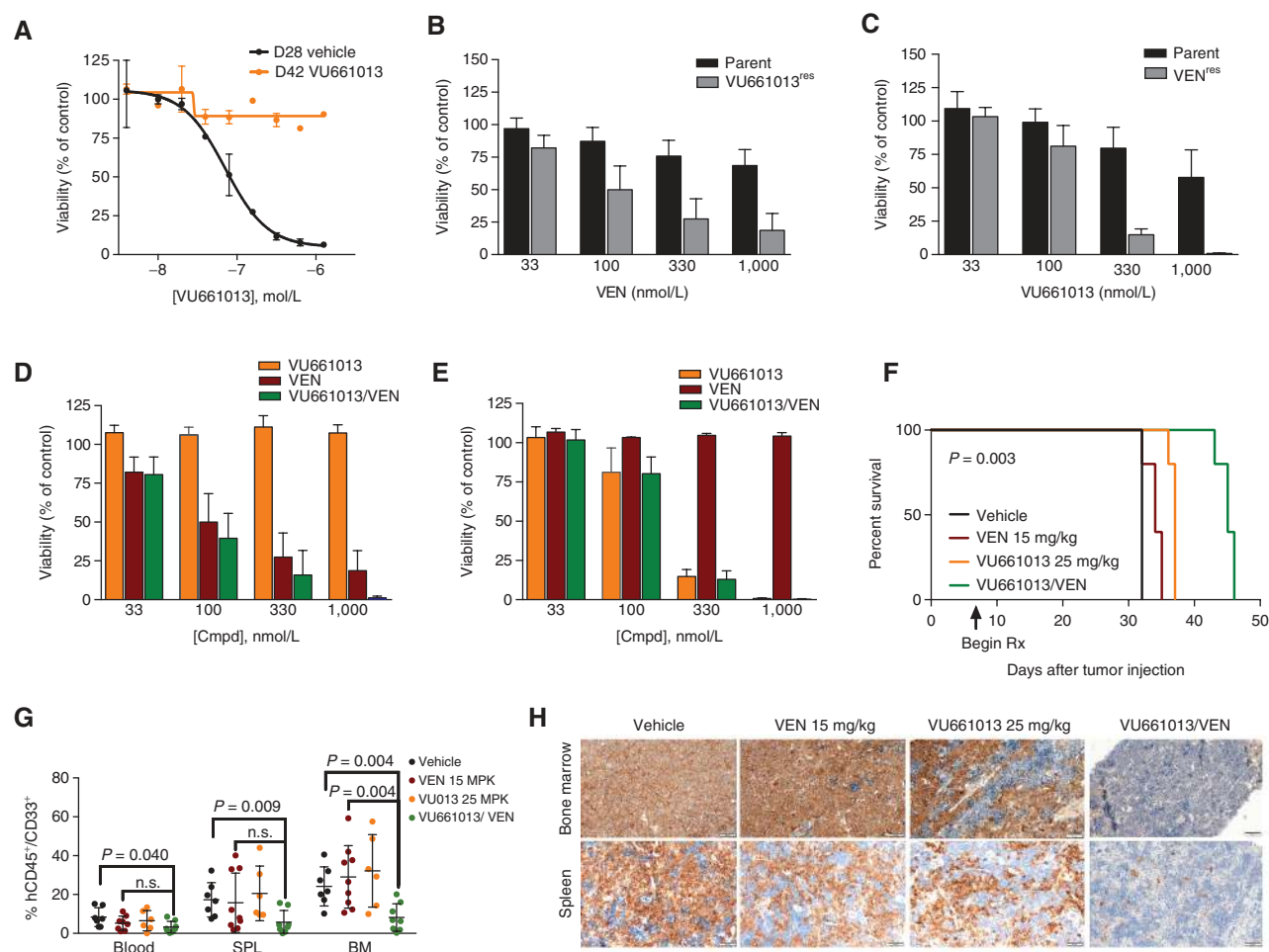
Despite initial dramatic reduction in MV-4-11 AML cell numbers after 3 weeks of treatment (Fig. 2A), NSGS mice treated daily with VU661013 75 mg/kg eventually succumbed to AML after ~42 days of treatment (Fig. 2D). When moribund, these mice had florid leukemia in the bone marrow ( $55.13\% \pm 10.69\%$  after 5–6 weeks of treatment vs.  $0.48\% \pm 0.29\%$  after 3 weeks of treatment; Supplementary Fig. S4). To investigate this finding, we harvested bone marrow and isolated the hCD45 cells from moribund mice for *ex vivo* growth inhibitor screening to determine if they had acquired a different antiapoptotic protein dependence. AML cells isolated from vehicle-treated mice (harvested at 28 days) retained their sensitivity to inhibition of MCL1 with VU661013; however, AML cells harvested from VU661013-treated mice (harvested after 42 days) showed signs of developing resistance to VU661013 ( $GI_{50}$  vehicle-treated 75 nmol/L vs. VU661013-treated  $>1.25 \mu\text{mol/L}$ ; Fig. 3A).

Given the resistance to VU661013 discovered in the xenograft studies, we sought to further understand the role of BCL2 in AML cells resistant to VU661013 and to characterize MCL1 inhibition in AML cells resistant to VEN. To this end, we developed MV-4-11 cell lines resistant to either VU661013 or VEN by growing naïve cells at various sublethal concentrations of VU661013 or VEN. After optimizing the dosage, cells were chronically treated to establish specific drug-resistant versions of the cell line. As predicted, AML cells selected

for resistance to the MCL1 inhibitor VU661013 (up to  $2.5 \mu\text{mol/L}$ ) gained a marked sensitivity to VEN compared with their naïve response (Fig. 3B). Conversely, we found that cells resistant to VEN (up to  $5 \mu\text{mol/L}$ ) exhibited drastic growth inhibition with single-agent MCL1 inhibitor (Fig. 3C).

### VU661013 and Venetoclax Have a Synergistic Effect in AML Cell Lines and Do Not Affect Human Hematopoietic Cells in Xenografts

We next aimed to determine if VU661013 has synergistic effects with other inhibitors of antiapoptotic BCL2 family proteins. In an effort to overcome resistance and to determine minimally active synergistic doses, we conducted combination studies in our panel of cell lines. VEN and VU661013 exhibited favorable synergy in several of these cell lines (Supplementary Fig. S5A–S5C), but a small number of lines maintained a resistant phenotype (Supplementary Fig. S5D). Given that MV-4-11 cells treated *in vitro* and *in vivo* with VU661013 ultimately developed resistance (Figs. 2D and 3A), we used our VU661013 or VEN-resistant MV-4-11 cells (Fig. 3B and C) to determine if the combination of VU661013 and VEN could overcome resistance to MCL1 or BCL2 inhibition. In this experiment, combination therapy effectively overcame VU661013 resistance and was more potent than VEN alone at higher doses (Fig. 3D). Conversely, VEN-resistant MV-4-11 cells were sensitive to VU661013, but there was no added benefit of VEN together with VU661013 (Fig. 3E).



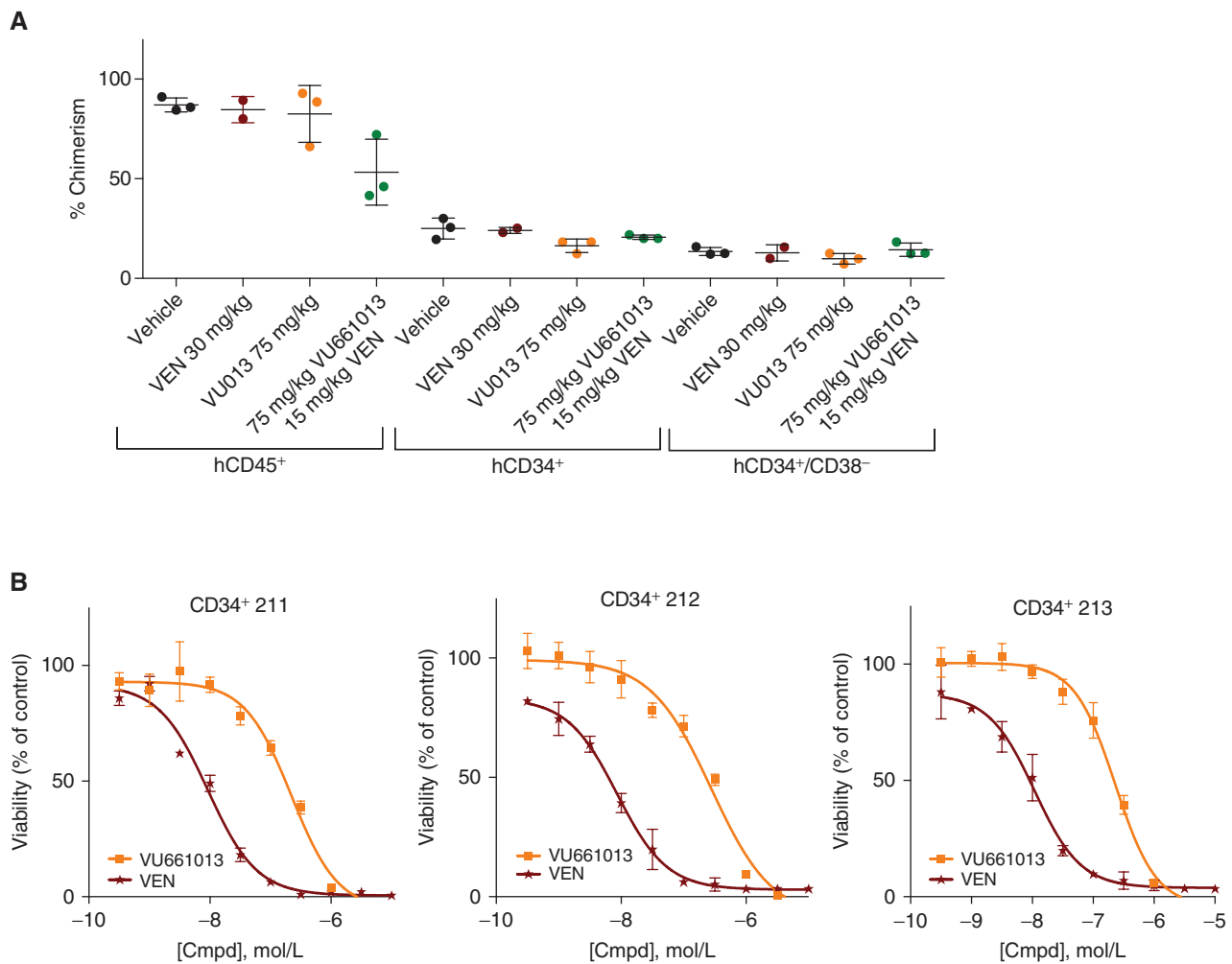
**Figure 3.** BH3-targeted inhibitors drive specific resistance in human cell lines, which can be overcome with alternating or combining inhibitors. **A**, Human MV-4-11 cells were isolated from the bone marrow of pre-morbid vehicle-treated mice at D28, and VU661013-treated mice at D42 and were tested *ex vivo* with VU661013 (mean  $\pm$  SEM;  $n = 3$ ). **B**, Naïve MV-4-11 cells (parent) and cells made resistant to VU661013 (VU661013-resistant) or venetoclax (VEN-resistant) were tested in growth inhibition assays with VEN and **(C)** VU661013 treatment. **D**, VU661013-resistant MV-4-11 cells treated with VU661013, VEN, or a combination of VU661013 and VEN, concentrations of each compound (Cmpd) are noted on the x-axis; **E**, VEN-resistant MV-4-11 cells treated with VU661013, VEN, or a combination of VU661013 and VEN, concentrations of each compound (Cmpd) are noted on the x-axis. For **B–E**, data shown as mean  $\pm$  SEM ( $n = 3$ ). **F**, The combination of VEN and VU661013 *in vivo* resulted in a survival benefit in an MV-4-11 AML model mice via Kaplan–Meier analysis. Statistical significance was calculated using log-rank (Mantel–Cox) test ( $P < 0.001$ ;  $n = 5$  per arm). **G**, The combination of VEN and VU661013 *in vivo* significantly decreased tumor burden in an MOLM-13 AML xenograft. Per arm vehicle ( $n = 7$ ), VEN ( $n = 9$ ), VU661013 ( $n = 6$ ), and VU661013/VEN ( $n = 8$ ). A nonparametric, unpaired, two-tailed *t* test was used to calculate significance. Data are combined from two independent experiments. **H**, IHC of bone marrow (femur) and spleen (20 $\times$ ), stained with monoclonal antibody for hCD45 in experimental mice. Scale bars, 50  $\mu$ m.

To determine if inhibition of BCL2 and MCL1 concurrently would be beneficial, we coadministered VEN and VU661013 in our MV-4-11 xenograft model at doses previously found to be ineffective when used as single agent (Fig. 2A–D). NSGS mice were transplanted with MV-4-11 cells and were treated with either VEN 15 mg/kg, VU661013 25 mg/kg, or a combination of both of these agents at these doses from 7 days after transplant until death. Kaplan–Meier survival analysis revealed *in vivo* activity between VEN and VU661013 (Fig. 3F;  $P = 0.003$ ).

In a second cell line combination therapy experiment, we transplanted NSGS mice with MOLM-13 AML cells, and again treated the mice with either 15 mg/kg VEN, 25 mg/kg VU661013, or a combination of both of these agents at the same doses from 7 days after transplant. Mice were treated until vehicle-treated mice became moribund (~20 days after

transplant, in this model), and cells were harvested. Again, the combination of VEN and VU661013 resulted in decreases of tumor burden in the blood (mean, vehicle  $8.34\% \pm 4.82\%$  vs.  $3.22\% \pm 2.97\%$  in VEN/VU661013 treated), bone marrow (mean, vehicle  $24.4 \pm 10.0\%$  vs.  $8.13 \pm 7.10\%$  in VEN/VU661013 treated), and spleen (mean, vehicle  $17.82\% \pm 8.82\%$  vs.  $5.66\% \pm 6.06\%$  in VEN/VU661013 treated; Fig. 3G). IHC staining of hCD45 cells in bone marrow and splenic tissue of treated mice also revealed effective depletion of human leukemia (Fig. 3H).

As with single-agent VU661013 therapy (Supplementary Fig. 3C), treatment with combination therapy did not display any evidence of organ toxicity or drug-associated death in the mice. To further define the potential toxicity of VU661013 on human hematopoietic cells, we engrafted sublethally irradiated mice with human CD34<sup>+</sup> (hCD34<sup>+</sup>) umbilical cord blood



**Figure 4.** **A**, Human UCB-derived CD34<sup>+</sup> cells were transplanted in NSGS mice. After confirmation of chimerism with notation of hCD45<sup>+</sup> cells in the peripheral blood at 2 weeks, mice were treated with vehicle, 30 mg/kg VEN, 75 mg/kg VU661013, or VEN 15 mg/kg and 75 mg/kg VU661013 in combination. Mice were sacrificed at D42, and chimerism in bone marrow was assessed. Human chimerism is measured by hCD45<sup>+</sup>. Progenitor cells are noted by hCD34<sup>+</sup>, and human hematopoietic stem cell-enriched cells are noted by hCD34<sup>+</sup>CD38<sup>-</sup>. Per arm: vehicle ( $n = 3$ ), VEN ( $n = 2$ ), VU661013 ( $n = 3$ ), and VU661013/VEN ( $n = 3$ ). Data, mean  $\pm$  SEM. **B**, hCD34<sup>+</sup> cells from three normal bone marrow samples were treated for 48 hours *in vitro* with VEN or VU661013 (mean  $\pm$  SEM;  $n = 3$ ).

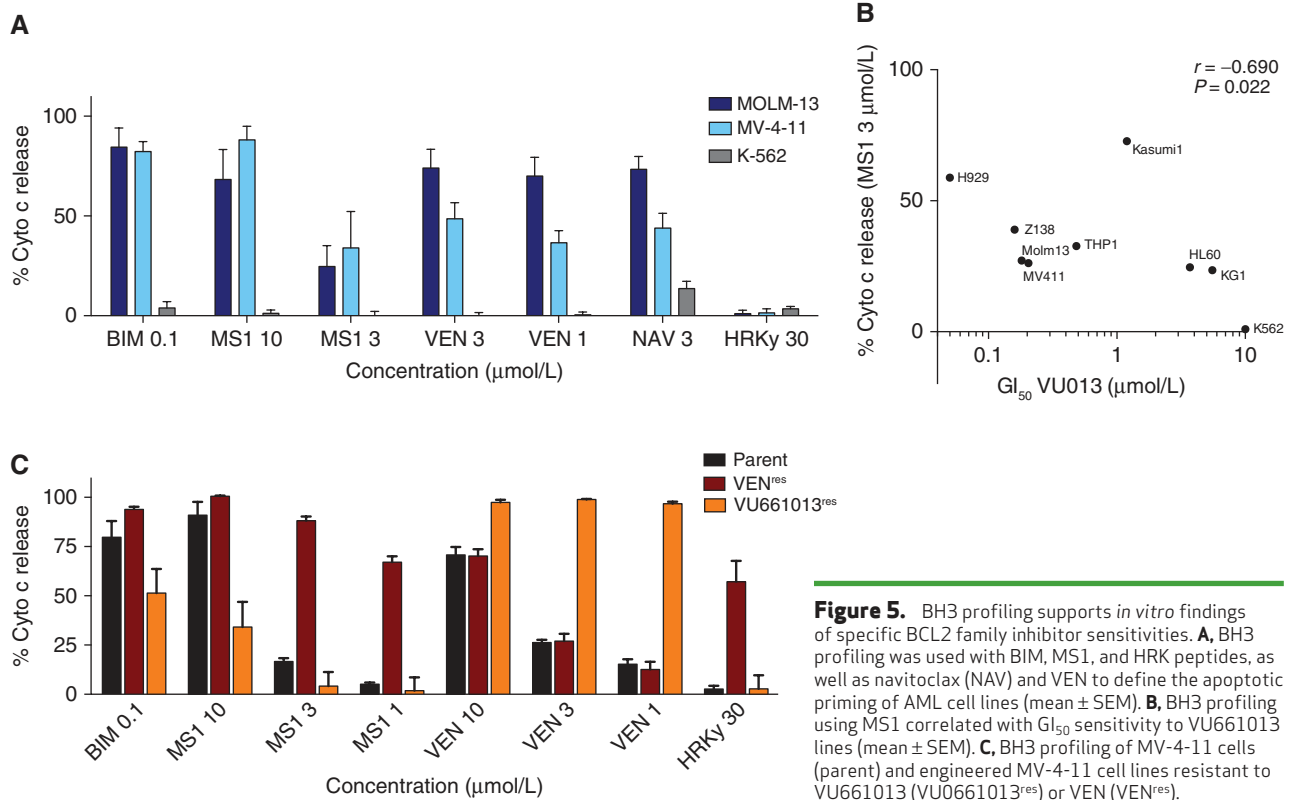
(UCB)-derived cells. Two weeks after transplant, mice began with monotherapy treatment of high-dose VEN (30 mg/kg), VU661013 (75 mg/kg), or combination (VEN 15 mg/kg and VU661013 75 mg/kg) beyond what was used in the MV-4-11 and MOLM-13 xenografts. Despite a decrease in overall hCD45 cells, hCD34 stem and progenitor cell populations remained unaffected in number (Fig. 4A). Furthermore, we subjected healthy human bone marrow-derived CD34<sup>+</sup> cells to VU661013, revealing less toxicity to normal stem and progenitor populations than with VEN (Fig. 4B).

### BH3 Profiling Predicts Response to Inhibition of MCL1

After demonstrating potent inhibition of MCL1 in AML, we set out to define the tumor cell dependence on specific BCL2 family members in AML cell lines using BH3 profiling (49, 52, 53). As previously shown, cell sensitivity to therapeutic intervention can be reliably forecasted by BH3 profiling, which

measures the dependence of a cancer cell on particular antiapoptotic proteins (49). Understanding which cells may be susceptible to inhibition of MCL1, BCL2, or BCL-xL pretreatment would help predict more efficient and tailored therapy, but also is critical to determine that a BH3 mimetic is selective for its intended target. To determine tumor cell dependence on specific BCL2 family members, we used BH3 profiling on a panel of myeloid tumor cell lines to reveal MOMP in a cytochrome C release assay. Cell lines that were sensitive to MCL1 inhibition showed increases in cytochrome C released when exposed to an MCL1-specific MS1 peptide (54). Conversely, resistant cell line K652 released less cytochrome C in the presence of MS1 peptide, representing a decreased dependence on MCL1 in these cell lines (Fig. 5A).

We questioned whether growth inhibition via MCL1 inhibition correlated with cytochrome C release induced by MS1 in a panel of AML cell lines. These data showed that although protein quantity was not a reliable predictor of function and BH3



dependence, BH3 profiling, indicated by MCL1 dependence, and growth inhibition to VU661013 were negatively correlated (Fig. 5B;  $r = -0.661$ ,  $P = 0.03$ ). In our resistant cell line assays (Fig. 3B and C), greater resistance to inhibition of MCL1 led to greater sensitivity to BCL2 inhibition, and vice versa. To assure cytochrome C release in these resistant MV-4-11 cells was consistent with drug inhibition assays, we performed BH3 profiling on these resistant lines. Here, overwhelming cytochrome C release occurred in the VU661013-resistant cell line with lower concentrations of VEN, and conversely treatment of the VEN-resistant cell line led to MOMP, even at concentrations of MS1 that had no effect on parental cell line (Fig. 5C). This underscores the predictive value of *in vitro* growth inhibition assays and targeted effect as measured by BH3 profiling.

### AML Patient Samples Have Variable BH3 Dependence and Response to VU661013 after Treatment with Venetoclax

In an attempt to further validate the correlation of BH3 profiling with *in vitro* growth inhibition, primary patient AML cells were analyzed. Mononuclear bone marrow cells from three different patients with AML were tested with the cytochrome C release assay, which revealed interpatient variability in BH3 dependence. Although all samples revealed at least a modicum of MCL1 dependence, AML 002 and AML 003 appeared more dependent on MCL1 than AML 001 (Fig. 6A). We treated AML 001 and AML 002 with a combination of VU661013 and VEN, which triggered apoptosis, congruent with BH3 profiling; VU661013 showed greater potency in AML 002 than AML 001 (Fig. 6B and C), and suggestion of synergy between VU661013 and VEN was noted in AML 002 (Supplementary Fig. S6A). To

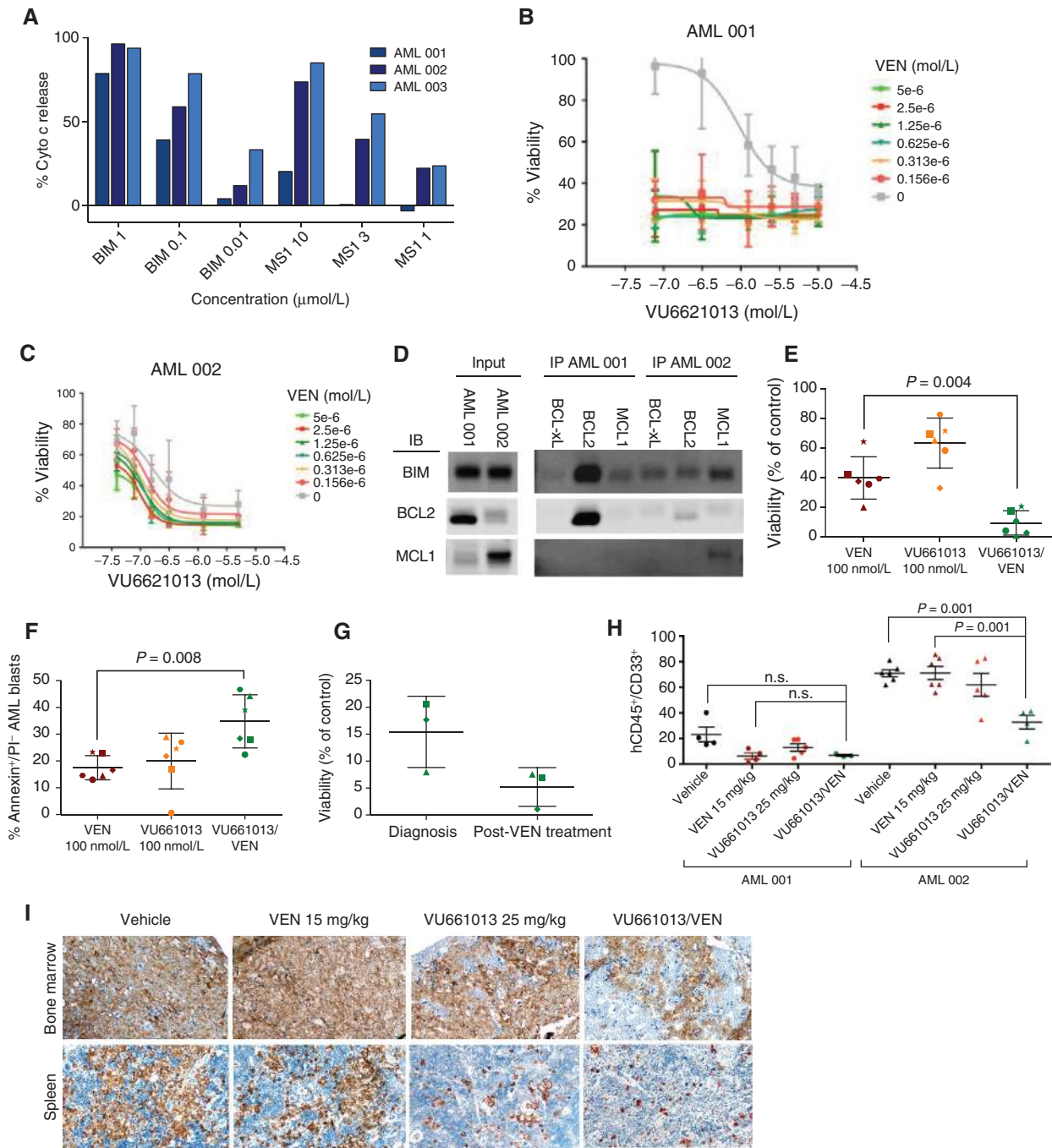
confirm our results, we performed a coimmunoprecipitation (co-IP) experiment to determine the levels of BCL2 and MCL1 in the patient samples, and the degree of heterodimerization of these proteins with BIM (55). A predominance of BCL2 was observed in a cell lysate from patient AML 001, although MCL1 was abundant in AML 002, as shown in the input samples. The BCL2 from patient AML 001 and the MCL1 from patient AML 002 were dimerized with BIM as shown from the immunoprecipitation samples (Fig. 6D).

Given these findings from AML 001 and AML 002, several additional samples were acquired from patients who were treated with VEN + low-dose cytarabine (LDAC) off study, at our institution, who were then found to be either refractory to or relapsed after this treatment (Supplementary Table S1). We treated the pretreatment AML samples from these patients *ex vivo* with VU661013, VEN, or VU661013/VEN to determine the relative sensitivity to combination therapy. After 48 hours of treatment, viability was noted to be lowest in the combination arm in all samples (Fig. 6E), with the highest rates of apoptotic AML blasts, likewise, seen in cells treated with the combination therapy (Fig. 6F). We then treated the post-VEN + LDAC treatment failure samples (when available) with VU661013/VEN. Although combination VEN/VU661013 therapy was effective in the initial diagnosis samples for these patients, it was more effective treatment in two of three of samples after clinical exposure to VEN and the onset of treatment failure (Fig. 6G).

### VU661013 and Venetoclax Can Be Synergistic in Patient-Derived Xenograft Transplantation Models

Following combination treatment and BH3 profiling on patient samples and given the synergy noted between





**Figure 6.** BH3 profiling of patient samples and improvement in disease control with combination therapy in a PDX model. **A**, AML patient samples analyzed using BH3 profiling were then (**B** and **C**) treated with dose titrations of VEN and VU661013 (mean  $\pm$  SEM). **D**, Coimmunoprecipitation (IP) experiment of patient samples AML 001 and AML 002 illustrate that AML 001 predominantly expressed high levels of BCL2, and AML 002 expressed high levels of MCL1 [input samples; immunoblot (IB)]. The BCL2 from the AML 001 patient sample and the MCL1 from AML 002 were dimerized with BIM. In patient sample AML 002, BCL2 and BCL-xL are also associated with BIM to a lesser degree. **E**, Samples from patients with AML who later failed VEN + LDAC treatment. Overall viability after VU661013/VEN treatment showed significant decreases in viability. **F**, After early during treatment (24 hours), blast cells from these samples began to undergo apoptosis with decreases in viability shown by Annexin V/PI staining. **G**, Comparison of pretreatment and posttreatment sensitivity to VU661013 + VEN combination therapy in samples from patients with AML who were treated with VEN + LDAC and relapsed. For **E–G**, individual patients are represented by shapes; in **G**, relative viability after *ex vivo* exposure to VU661013 + VEN in samples taken from patients prior to therapy and after therapy with VEN + LDAC in the clinic is noted;  $P = \text{n.s.}$  **H**, In patient-derived xenografts, VEN and VU661013 were given concomitantly at low doses with bone marrow harvested at day 42. For AML 001 [vehicle ( $n = 4$ ), VEN ( $n = 4$ ), VU661013 ( $n = 5$ ), and VU661013/VEN ( $n = 3$ )], there was no significant difference between treatments. For AML 002 [vehicle ( $n = 6$ ), VEN 15 ( $n = 6$ ), VU661013 ( $n = 5$ ), and VU661013/VEN ( $n = 4$ )], VU661013/VEN combination treatments led to reduction in engrafted human leukemia (mean  $\pm$  SEM). A nonparametric, unpaired, two-tailed *t* test was used to calculate significance. **I**, Posttreatment, reduction of human leukemia was noted through IHC staining of bone marrow for hCD45 in MCL1-dependent AML 002 (20 $\times$ ).

VU661013 and VEN in various human cell lines and patient samples, we proceeded to test dual inhibition of MCL1 and BCL2 in a patient-derived xenograft (PDX) transplantation assay. NSGS mice were transplanted with cells from patient AML 001 or AML 002. Two weeks after transplant, after chimerism was established, we began treating the mice with VEN or VU661013, or both agents at single-agent-subtherapeutic doses of 25 mg/kg of VU661013 and 15 mg/kg of VEN for 28 days. PDX leukemia accelerated by 6 weeks after transplant in the vehicle mice, and at 42 days after transplant the experiment was terminated to allow for analysis of bone marrow AML infiltration (Fig. 6H). Consistent with the BH3 profiling data and the *in vitro* bioassays, treatment of mice engrafted with disseminated AML from BCL2-dependent AML 001 did not benefit significantly from the addition of VU661013 to VEN (Fig. 6H; vehicle  $23.16\% \pm 5.7\%$  vs.  $6.83\% \pm 0.70\%$  in VEN/VU661013 treated). However, the combination of VEN and VU661013 in AML 002 (MCL1-dependent patient sample) resulted in decreases of tumor burden in the bone marrow beyond VEN alone (Fig. 6H; mean, vehicle  $72.5\% \pm 7.8\%$  vs.  $33.2\% \pm 11.2\%$  in VEN/VU661013 treated). Congruently, combination treatment reduced disease-associated splenomegaly (Supplementary Fig. S6B; mean,  $0.27 \pm 0.09$  in vehicle vs.  $0.07 \pm 0.02$  g in VEN/VU661013 treated). Staining with anti-hCD45 antibody not only revealed the extent of AML cell reduction but showed marrow architecture to have been significantly maintained during therapy (Fig. 6I). These findings suggest that in some AML, VEN and VU661013 could be combined to treat AML to achieve a synergistic reduction of tumor burden. Non-target tissues were unaffected in this experiment, and increased doses of both agents (4 weeks daily treated of VU661013 75 mg/kg combined with VEN 25 mg/kg) in separate experiments yielded no deleterious effect on spleen, kidney, liver, or heart tissue of the mice (Supplementary Fig. S6C).

Interestingly, AML cells harvested from the bone marrow of mice treated with 75 mg/kg of VU661013 for over 48 days showed signs of developing resistance to VU661013 *ex vivo* ( $GI_{50}$  naïve 0.7 nmol/L vs. VU661013 treated 2.4  $\mu$ mol/L; Supplementary Fig. S6D) and growing sensitivity to VEN, whereas the naïve AML 002 was resistant to VEN and sensitive to VU661013 (Supplementary Fig. S6E). In line with our previous findings, resistance to MCL1 inhibition may lead to greater sensitivity to VEN treatment (Fig. 3A–C; Supplementary Fig. S4).

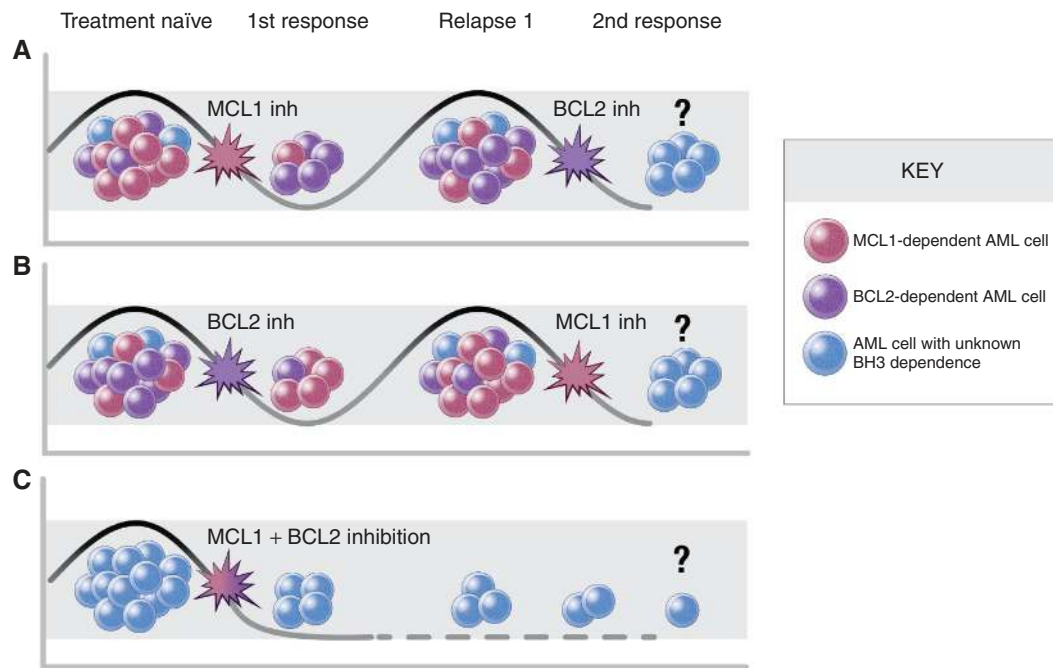
## DISCUSSION

We identified a potent and selective MCL1 inhibitor and showed how inhibition of MCL1 may be useful in the treatment of AML. We determined the MCL1 dependency in several AML cell lines and patient samples and showed that this MCL1 inhibitor selectively inhibited MCL1 and triggered cell death in myeloid leukemia cells, which were disseminated throughout xenografted mice—something not previously demonstrated with other MCL1 inhibitors (46–48, 56). Selective inhibitors have been used previously to define BCL2 family reliance in AML cells and other tumors (57, 58), and we used a similar *ex vivo* cytotoxicity bioassay to reliably predict the response to MCL1 inhibition. AML cells most sensitive to VU661013 were cells primed for MCL1 inhibition, as shown via BH3 profiling.

Recently, BH3 profiling was found to predict single-agent responses to VEN in patients with AML, yet the negative correlation between response to VEN and MCL1 dependence as per BH3 profiling was the most accurate predictor of response (6, 32, 59). Here, we found the inverse to be true with an MCL1 inhibitor, which led us to pursue the hypothesis that AML may be treated dynamically based on the changing reliance on antiapoptotic family members. In these experiments with an array of potent and selective BH3 mimetics available, we were able to treat multiple AML cell lines and patient samples and compare the relative dependence on MCL1, BCL2, or BCL-xL through BH3 profiling. BH3 profiling strongly supported our *in vitro* results of BH3 mimetic drug-sensitivity testing. Indeed, over time, leukemia treated with either VEN or VU661013 became preferentially responsive to the other BH3 mimetic after failure of the initial BH3 mimetic in our experiments, and patient samples primed for MCL1 inhibition evolved from VEN resistant to VEN sensitive in the face of treatment with VU661013.

This will undoubtedly be far more complicated in the clinic. BH3 profiling and viability assays conducted on samples from patients who failed VEN therapy did not yield definitive results—potentially a function of cotreatment with priming chemotherapy, and/or the heterogeneity of the tested samples. Nonetheless, the direct evidence of improvement in blast amelioration with VEN + VU661013 combination treatment in samples from patients who relapsed on VEN, and the exquisite sensitivity to combination therapy after relapse in representative samples of these patients, indicates potential to kill VEN-resistant AML via BCL2 + MCL1 inhibition. In multiple AML models, we found synergy, *in vitro* and *in vivo*, even at doses of both VEN and VU661013 that were ineffective as single agents. Indeed, VEN and VU661013, when used in combination, even at significantly reduced doses, resulted in increased lifespan of immunocompromised mice with human AML, and a considerable delay of sick phenotype prior to AML resurgence. Taken together, this edifies recent success in eradicating AML in experiments that dual-targeted BCL2 and MCL1 using VEN with lentiviral vectors expressing BH3-only proteins or using nonselective MCL1 inhibitors (51, 60–62).

MCL1 is a necessary protein for many normal cell types, yet reduced MCL1 (via *Mcl1*<sup>-/-</sup> murine model) in combination with standard chemotherapy does not increase toxicity in mice (63–68). We revealed that monotherapy with VU661013 and coadministration of MCL1 and BCL2 inhibitors has a reasonable safety profile in these models. Doses of VU661013 used in mice were enough to drive  $C_{max}$  and AUC to inhibit murine MCL1, and normal murine cells fared well, but it is important to remember the differences in murine to human homology of the MCL1 sequence. Whereas the VEN and VU661013 combination slightly reduced hCD45<sup>+</sup> cells in normal UCB xenografts, there was no effect of either drug or the combination on the number of CD34<sup>+</sup> or CD34<sup>+</sup>/CD38<sup>-</sup> cells in the engrafted mice. Further, VU661013 was less potent in the direct treatment of normal hCD34<sup>+</sup> cells than VEN, and given the reasonable safety profile VEN has acquired already in the clinic in AML (32, 33), this is reassuring. We have shown here a wide range of doses of VU661013 that exhibit no deleterious effects to normal hematopoietic stem and progenitor populations. Future experimentation to further



**Figure 7.** MCL1 and BCL2 inhibitors (inh) in the treatment of AML. Although antiapoptotic dependence is heterogeneous across patients and intrapatient with AML, individual patients may have greater MCL1 or BCL2 antiapoptotic dependence at diagnosis, and this may be interrogated to guide initial treatment. Resistance to BH3 mimetics may arise from upregulation of another antiapoptotic protein family member, and a patient may switch selective BH3 mimetic at that time. **A**, In some patients, this sequential targeting of antiapoptotic family members (with or without chemotherapy priming agent) may continue to provide disease remissions and clinical benefit. This may occur in a tumor that is initially MCL1 dependent. **B**, Or initially BCL2-dependent AML. **C**, Combination therapy with MCL1 inhibition and BCL2 inhibition at diagnosis has not been tested in patients, but may be tolerable and lead to tumor involution by targeting two important antiapoptotic proteins heterogeneously upregulated in AML.

define the therapeutic window for the combination or the potential eradication of the malignant clone is imperative.

Successfully treating samples from patients who ultimately failed BCL2 inhibition with combination therapy further illustrates a clear role in the combination. Pretreatment samples were far less likely to respond to MCL1/BCL2 inhibitor combination therapy than samples from the same patients after VEN failure. This signals a hope to rescue VEN resistance with the combination of VEN and MCL1 inhibitor. The use of potent MCL1 inhibitors in combination with chemotherapy and in combination with potent BCL2 inhibition in the clinic is not known, and priming with chemotherapy may very well remain part of this therapeutic approach. Co-administration with priming agents such as LDAC or DNMTi greatly increases the responses to VEN (33), and monitoring for mechanisms of resistance will be more of a challenge in the setting of these responses. The antiapoptotic dependence of any minimal residual disease (MRD) AML cells in responders is difficult to ascertain as the relative number of leukemic cells is low, and testing BH3 dependence of the MRD clone remains a technical challenge with traditional methods.

The ability to screen patients with AML for specific signatures of apoptotic priming with selective inhibitors has the potential to become a clinically relevant assay in the near future. However, strategies to use these tools in the clinic still need to be developed. AML is heterogeneous, and supplying BH3 mimetic-based therapy in the clinic based on the most susceptible antiapoptotic protein seems logical, but although

the possibility to alter therapy during disease surveillance is appealing, it has not yet been tested in clinical studies. When these techniques are used in patient care, it is worthwhile to consider sequential therapy or combination therapy with these inhibitors, as there are potential merits to either approach (Fig. 7). Single-agent therapy with VEN has been underwhelming in the clinic (32), but preselecting by profiling cells prior to treatment to determine the most important influence on apoptosis (e.g., MCL1 or BCL2) and then treating accordingly may likely improve upon this. Adding a priming agent such as LDAC or DNMTi to VEN has already been shown to vastly improve responses to VEN (33), and this technique could be used even more efficiently with BCL2-dependent AML. Perhaps the same concept will apply to MCL1 inhibition.

We have also shown that treatment of AML cell lines and patient samples with VEN leads to MCL1-driven resistance, which is then sensitive to VU661013, and the converse with using VU661013 as the initial treatment. In this vein, tumor profiling followed by sequential therapy could be pursued clinically. If a patient is found to have an MCL1-dependent AML, MCL1 inhibition could be used through remission (with or without a priming agent such as decitabine), and a BCL2 inhibitor could be used if/when the AML developed MCL1 resistance and, consistent with our results, sensitivity to BCL2 inhibitors (Fig. 7A). Alternatively, if BCL2 more dominantly drove AML, BCL2 inhibition (with or without a priming chemotherapy) may be an initial therapy followed by MCL1 inhibition at relapse (Fig. 7B).



Practically, changing BH3 mimetics at signs of resistance in the clinic, with or without chemotherapeutic combinations, may be feasible, as our *ex vivo* assays provide a simple test of this concept. There may be doubt that continued alternation of monotherapy could continue beyond one relapse, but this has yet to be illustrated one way or another.

An alternative approach may be to treat *de novo* AML with combination therapy. Our work illustrates that this has the potential to have greater effect on reducing the malignant clone given targeting of both MCL1 and BCL2, and the capacity to eradicate the malignant clone with dual targeting of MCL1 and BCL1 seems conceivable, but remains unclear (Fig. 7C). We further illustrate that this will be complicated, as PDX experiments using an AML sample found to be disproportionately BCL2-dependent revealed no benefit of combination therapy with VEN + VU661013 over VEN alone (whereas benefit from the combination was seen in samples shown to have reliance on both MCL1 and BCL2). Perhaps BH3 profiling will be best used to select less heterogeneously dependent patients who are best treated with one inhibitor over another (Fig. 7A and B).

Although there are some examples of non-chemotherapy-induced treatment-free remissions in hematologic malignancies that remain dormant after control of frank disease (presumably kept in check by immunosurveillance; refs. 69, 70), altering the apoptotic machinery in AML without chemotherapy may not be sufficient to alter gene mutations which are commonly found in AML and often drive the disease. Mutational burdens of AML samples in this study were variable, and no correlation to either MCL1 dependence or response to therapy was present in this small sample. Large cohorts and more extensive testing of these compounds in common myeloid clones will help determine if some clones are more susceptible to BH3 mimetic therapy, as has been suggested in the laboratory by others (6, 32, 52, 53).

Experience with VEN in relapsed and refractory CLL in clinical studies has been extraordinary, analogous to the near-universal dependence of CLL, and some lymphomas, on only BCL2 (34, 35, 43). AML is exceptionally heterogeneous with respect to antiapoptotic protein dependence and represents a different clinical challenge (71). Priming techniques combining BCL2 inhibition with LDAC or DNMTi have already seen success in the clinic based on significant synergy in preclinical models (refs. 33, 72, 73; NCT: 00287233). Still, there will be treatment failures, and the assembled evidence of MCL1-mediated resistance in AML and other cancers (32, 34, 36, 37, 59) makes the use of MCL1 inhibitors an attractive treatment approach.

## METHODS

### Patient Samples

Experiments were conducted on primary patient samples which were provided by the Vanderbilt-Ingram Cancer Center Hematopoietic Malignancies Repository, after acquisition of written informed consent, and in accordance with the tenets of the Declaration of Helsinki and approved by the Vanderbilt University Medical Center Institutional Review Board.

### Cell Lines

AML cell lines MV-411, Kasumi-1, K-562, HL-60, U-937 Kasumi-3, KG-1, NB4, SKM-1, PL-21, MOLM-16, and THP-1, and leukemia

mantle cell lymphoma cell line Z-138 were purchased from the ATCC. OCI-AML3, HEL, F36P, and MOLM-13 cell lines were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen. ATCC and DSMZ cell bank cell lines were authenticated by short tandem repeat profiling and cytochrome c oxidase gene analysis. Cultured cells were split every 3 days and maintained in exponential growth phase. Cell lines were tested for *Mycoplasma* in 2017 using the Universal Mycoplasma Detection Kit (ATCC). Cells were used for the experiments presented here within 10 to 20 passages from thawing. MV-411 cell line was grown in Iscove's modified Dulbecco's medium (IMDM), and all other cell lines were cultured in RPMI and supplemented with 10% to 20% fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were kept at 37°C in a 5% CO<sub>2</sub> incubator.

### Cell Proliferation Assay

Compounds were diluted in DMSO (<0.05% DMSO) and dispensed into a 384-well plate using the Echo 555 liquid handler (Labcyte). Following the addition of compounds, cells were pipetted into the 384-well plates at a concentration of between 2,000 and 8,000 cells per well in IMDM or RPMI media, as noted above, supplemented with 10% FBS and incubated at 37°C, 5% CO<sub>2</sub> in a tissue culture incubator. Plates were incubated for 48 hours, and cell viability was measured using the CellTiter-Glo reagent (Promega). Percent viability was defined as relative luminescence units (RLU) of each well divided by the RLU of cells in DMSO control. Dose-response curves and GI<sub>50</sub> values were determined using linear regression of double-log transformed data (GraphPad Prism version 6.0 h). Control bone marrow-derived CD34<sup>+</sup> cells were purchased from STEMCELL Technologies.

### Culturing of Resistant Cells

To generate cells that were resistant to BCL2 or MCL1 inhibition, MV-411 cells were treated over the course of 3 months with gradually increasing concentrations of VEN (5 nmol/L to 2.5 µmol/L) or VU661013 (100 nmol/L to 5 µmol/L). Cells were declared to be VEN- or VU661013-resistant when they were able to maintain 100% viability in the presence of these high concentrations (5 µmol/L of VU661013 and 2.5 µmol/L of VEN) of inhibitors.

### Quantitative Western Blot

Cells were grown in their respective media before total protein lysates were extracted in Laemmli sample buffer (Bio-Rad), sonicated, and boiled at 95°C for 10 minutes. The samples were loaded in a 10% sodium dodecyl sulfate polyacrylamide gel (1.67 × 10<sup>5</sup> cells/well). Western blot analysis was performed according to standard protocol with antibodies to MCL1, BCL2, BCL-xL, and Actin. Membranes were imaged and band densitometry was performed using ImageJ. The ratio of band intensity of MCL1, BCL2, and BCL-xL was calculated relative to loading control (Actin). Antibodies were obtained from the following sources: MCL1 (Cell Signaling Technology), BCL2 (BD Bioscience, R&D Systems), BCL-xL (Cell Signaling Technology, R&D Systems), and Actin (Sigma-Aldrich).

### Competitive Binding Assays

A TR-FRET-based competitive binding assay was used to measure compound affinity for MCL1 and fluorescence polarization (FP)-based assays were used to measure binding affinity to BCL2 and BCL-xL. A recombinant human MCL1 Maltose Binding Protein (MBP) fusion protein was expressed and purified in the laboratory of Stephen Fesik, and BCL2 and BCL-xL were purchased from R&D Systems. A fluorescein isothiocyanate (FITC)-labeled BH3 peptide derived from Bak (FITC-Bak-BH3; FITC-AHx-GQVGRQLAIIGDDINR-NH2) was purchased from GenScript and used without further purification.



MCL1 TR-FRET assay condition: 300 nmol/L FITC-BAK-BH3 peptide, 1 nmol/L MCL1-MBP fusion protein, and 1 nmol/L anti-MBP-terbium (Cisbio) were added to a buffer containing 0.5 mmol/L monobasic potassium phosphate, 15.5 mmol/L dibasic potassium phosphate, 1 mmol/L sodium EDTA, 50 mmol/L sodium chloride, 1 mmol/L DTT, and 0.05% Pluronic F-68 (Sigma-Aldrich) adjusted to pH 7.5. Compound was incubated with the protein peptide mixture in 384-well plates for 3 hours at room temperature. The final DMSO concentration was 1%. TR-FRET activity was measured on a Biotek Cytation 3 equipped with a filter cube containing an Ex 340/30 nmol/L Em 620/10 filter and an Ex 340/30 Em 520 filter. The change in TR-FRET signal ( $\Delta F$ ) was measured and used to calculate an  $IC_{50}$  (inhibitor concentration at which 50% of bound probe is displaced) by fitting the  $\Delta F$  values using XLFit (IDBS) to a four-parameter dose-response (variable slope) equation. This was converted into a binding dissociation constant ( $K_i$ ) according to the formula:  $K_i = [I]_{50}/([L]_{50}/K_d + [P]_0/K_d + 1)$ , where  $[I]_{50}$  is the concentration of the free inhibitor at 50% inhibition,  $[L]_{50}$  is the concentration of the free labeled ligand at 50% inhibition,  $[P]_0$  is the concentration of the free protein at 0% inhibition and  $K_d^{pep}$  represents the dissociation constant of the FITC-labeled peptide probe. Compounds were evaluated using replicate measurement, in duplicate;  $K_i$  values shown are the average of duplicate values.

### BCL2 and BCL-xL Fluorescence Polarization Assay Condition

The assay was carried out in 20 mmol/L TRIS pH 7.5 buffer containing 50 mmol/L NaCl, 3 mmol/L DTT, 0.01% CHAPS, containing either 50 nmol/L BCL2 and 10 nmol/L FITC-Bak-BH3 peptide, or 12.5 nmol/L BCL-xL and 12.5 nmol/L FITC-Bak-BH3. The final DMSO concentration was 5%. Compound was incubated with the protein peptide mixture in 384-well plates for 1.5 hours at room temperature. The FP signal (anisotropy) was measured on the Biotek Cytation 3 at an excitation wavelength of 480 nm and an emission wavelength of 535.  $IC_{50}$  and  $K_i$  values were calculated as described above.

### Coimmunoprecipitation of Patient Samples

Two samples containing 5e6 cells per vial were resuspended in nondenaturing Cell Lysis Buffer (Cell Signaling Technology) containing phenylmethylsulfonyl fluoride. Fifty micrograms of clarified total cell protein in 120  $\mu$ L volume was incubated with 2  $\mu$ g of Biotin-conjugated antibodies directed against MCL1 (clone RC13; Thermo Fisher), BCL2 (Clone 8C8; Thermo Fisher), or BCL-xL (Clone 7B2.5; Abcam) for 3 hours at 4°C. Thirty microliters of streptavidin-coated beads were washed in lysis buffer and added to each sample for 30 minutes at room temperature. Beads were washed 3 times in lysis buffer and protein was eluted at 95°C in RIPA and 1 $\times$  LI-COR SDS buffer. BCL2, BCL-xL, and MCL1 levels in immunoprecipitates, total cell lysates (input), and depleted lysates were performed using standard western blotting techniques. Blots were probed using antibodies directed against BIM (Y-36; Abcam), MCL1 (S-19; Santa Cruz Biotechnology), BCL2 (D55G8; Cell Signaling Technology), and BCL-xL (54H6; Cell Signaling Technology).

### In Vivo Murine Modeling

All animal experiments were conducted in accordance with guidelines approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. Female NSGS mice, 6 to 8 weeks old, were irradiated with 1 Gy microwave radiation. Twenty-four hours later, mice were transplanted intravenously with cells of interest. For cell line models,  $1 \times 10^6$  MV-4-11 or  $3 \times 10^3$  MOLM-13 cells were used. For patient sample xenografts,  $7 \times 10^4$  to  $4 \times 10^6$  AML mononuclear cells (MNC) were used. In humanized mouse studies,  $7.4 \times 10^3$  UCB-derived CD34<sup>+</sup> cells (Lonza) were used. To eliminate concern for bias in

these experiments, mice were randomized post-cell injection into cages of 5. Prior to treatment, peripheral microchimerism was documented at week 1 in all cell line (MV-4-11 and MOLM-13) models. For AML patient PDX models (AML 001 and AML002) and humanized UCB CD34<sup>+</sup> models, peripheral chimerism was established by 2 weeks. Mice showing no peripheral chimerism by 2 weeks in cell line, or 3 weeks in AML patient PDX models, were removed from the study. Upon establishing microchimerism, mice were treated with either venetoclax (Selleckchem) by daily gavage, VU661013 (Fesik Laboratory) by daily i.p. injection, or vehicle. VU661013 was dissolved in dimethylsulfoxide and diluted in ethanol, polyethylene glycol (PEG), and saline. Venetoclax was dissolved in PEG and ethanol, and diluted with Phosal 50 PG. Peripheral blood was assessed weekly for human chimerism. Spleen/body ratio was calculated as organ weight (gram) per gram of body weight.

### Ex Vivo Cell Studies

For *ex vivo* analysis from PDX-derived cells, bone marrow was flushed from treated mice and subjected to a red blood cell lysis (EL Buffer; Qiagen) for 15 minutes on ice before undergoing enrichment of human cells using a mouse cell depletion kit (Miltenyi). After depletion, cells were tested for purity staining for human CD45-APC (Clone 2D1; BioLegend) and murine CD45-PE (Clone 30-F11; BioLegend) and subjected to flow-cytometric analysis using a 3-laser LSRII (Becton Dickinson). All MNCs used in *ex vivo* assays were >96% purity for hCD45.

### Flow Cytometry

For flow cytometry, red blood cells were lysed with EL Buffer on ice (Qiagen), with remaining cells washed and resuspended in 1 $\times$  PBS with 1% BSA and stained for 15 minutes with the following antibodies: human CD45-APC (clone 2D1; BioLegend), human CD33-PE-Cy7 (clone P67.6; BioLegend), murine CD45-PE (clone 30-F11; BioLegend), and DAPI (BioLegend). For detection of human stem and progenitor cells, additional human CD34 (clone 561; BioLegend) and CD38 (clone HIT2; BioLegend) antibodies were used. Cells were washed and submitted for flow-cytometric analysis using a 3-laser LSRII (Becton Dickinson).

### Assessment of Apoptosis

For Annexin/propidium iodide staining, an Annexin V apoptosis kit was used as per the manufacturer's instructions (BD Pharmingen). For patient samples, mononuclear cells were subjected to 24 hours of drug treatment and stained for flow cytometry. AML blast cells were gated as CD45<sup>lo-mid</sup>/CD33<sup>hi</sup>/SSC-A<sup>lo</sup>.

### IHC

Tissues were fixed in 4% paraformaldehyde for 48 hours and stored in 70% ethanol before being embedded in paraffin and sectioned at 5  $\mu$ m. The bone tissue was decalcified prior to being embedded in paraffin. Sections were dewaxed in Xylene and rehydrated in successive ethanol baths. Standard Mayer's hematoxylin and eosin staining was performed. Antigen retrieval using a standard pH 6 sodium citrate buffer (BioGenex) was performed, and sections were stained with Monoclonal Mouse Anti-Human CD45 (Dako, M0701, dilution 1:200) using the M.O.M. Kit (Vector).

### BH3 Profiling/Mitochondrial Depolarization

We used a flow cytometry-based iBH3 profiling analysis following similar protocols as previously described (74, 75). Synthetic peptides for MS1, HRK, and BIM were purchased (GenScript). For this, cell lines were incubated with BIM, MS1 peptide (binds to MCL1), venetoclax (potent specific binding of BCL2), HRK (binds

to BCL-xL), DMSO (negative control), or Alamethicin (ALAM; positive control). Briefly, peptides were diluted to 2× in 0.002% digitonin (D5628; Sigma-Aldrich) in MEB2-P25 buffer (150 mmol/L mannitol [M9647; Sigma-Aldrich], 1 mmol/L EDTA [E6758; Sigma-Aldrich], 1 mmol/L EGTA [E3889; Sigma-Aldrich], 5 mmol/L succinate [S3674; Sigma-Aldrich], 0.1% IgG-free BSA [001-000-162; Jackson ImmunoResearch, PA], 10 mmol/L HEPES [H4034; Sigma-Aldrich], 50 mmol/L potassium chloride [P9541; Sigma-Aldrich], 2.5 g/L Polaxamer 188 [MT61161RM; Fisher], adjusted to pH 7.5 with potassium hydroxide [P5958; Sigma-Aldrich]). Cells were centrifuged at  $500 \times g$  for 5 minutes and suspended in MEB2-P25 at a density of  $3.0 \times 10^6$  per mL. Fifty microliters of cell suspension and 50  $\mu$ L of peptide/profiling solution was added to wells to give  $1.5 \times 10^5$  cells per well and incubated at room temperature for 60 to 75 minutes in the dark. To stop the reaction, 33  $\mu$ L of 4% paraformaldehyde (15710; Electron Microscopy Sciences) in PBS was added and incubated at room temperature for 10 minutes. To neutralize the fixation, 33  $\mu$ L of neutralization buffer (1.7 mol/L Tris base [T60040; Research Products International], 1.25 mol/L glycine [G36050; Research Products International] [pH 9.1]) was added for 5 to 10 minutes. Intracellular levels of cytochrome c were probed by adding 20  $\mu$ L of 10× Tween20 Intracellular Staining Buffer staining buffer (2% Tween20 [P9416; Sigma-Aldrich], 10% BSA in PBS) with 1:100 anti-human cytochrome c antibody (612310; BioLegend). Samples were stained overnight and then transferred into polystyrene tubes for flow-cytometric analysis the next day. For Fig. 5, DMSO (D8418; Sigma-Aldrich) was used as a negative cytochrome c release control, and ALAM (Alamethicin [BML-A150; Enzo]) at 15  $\mu$ M was used as a positive cytochrome c release control. Using these controls, the % cytochrome c released in each sample was calculated as  $100 \times [1 - ((MFI_{\text{sample}} - MFI_{\text{ALAM}}) / (MFI_{\text{DMSO}} - MFI_{\text{ALAM}}))]$ . For Fig. 6A, frozen bone marrow cells were washed 1× with PBS and stained with 1:100 Zombie Aqua Dye (423101; BioLegend) for viability, washed with PBS, and subsequently stained with 1:100 CD45-BV421 (clone HI30, 563879; BD Biosciences) and 1:100 CD33-PE (clone WM53, 561816; BD Biosciences) in FACS buffer (2% FBS in PBS). Cells were iBH3 profiled as described above, using the MS1 peptide, BIM, DMSO, and ALAM. AML blasts were identified by CD45<sup>lo-mid</sup>/CD33<sup>mid-hi</sup>/SSC-A<sup>lo</sup>.

### Next-Generation Sequencing

For next-generation sequencing (NGS), bone marrow aspirates were obtained from patients, and DNA was isolated using a DNA midi-prep (Qiagen) for NGS in a panel of commonly mutated regions of myeloid neoplasia-associated genes across the genome. The analytic targets included in the TruSight Myeloid Sequencing Panel (Illumina) include exonic regions across each of the following genes: *SRSF*, *U2AF1*, *TET2*, *IDH2*, *DNMT3A*, *RUNX1*, *TP53*, *BCOR*, *BCORL1*, *ETV6*, *NPM1*, *GATA2*, *WT1*, *ASXL1*, *EZH2*, *JAK2*, *FLT3*, *FBXW7*, *CBL*, *KRAS*, *NRAS*, *SETBP1*, *ABL1*, *CSF3R*, *PTEN*, *PTPN11*, *SRSF2*, *TP53*, *ZRSR2*, *PHF6*, *MYD88*, *IDH1*, *HRAS*, *CALR*, *BRAF*, and *CDKN2A*. The panel of validated genes consisted of therapeutic markers, as well as genes with diagnostic and prognostic utility in myeloid and other hematologic tumors.

### Statistical Analysis

Unless otherwise noted, data were summarized using the mean ( $\pm$  standard deviation). Per-group sample sizes are presented in figures and results reported from three separate experiments, unless stated otherwise. To avoid normality assumptions, pairwise group comparisons were made using the nonparametric Mann-Whitney *U* test. The distributions of survival were estimated using the method of Kaplan-Meier, and group comparisons of survival were conducted using the log-rank test. The nonparametric Spearman correlation was used to assess pairwise variable associations. Synergy was summarized using the combination index based on the median effect principle with

confidence intervals (76, 77). Data were analyzed using GraphPad Prism 6.0 for Windows (GraphPad Software; www.graphpad.com) and R [R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/].

### Disclosure of Potential Conflicts of Interest

T. Lee reports receiving commercial research support from Boehringer Ingelheim and has ownership interest (including stock, patents, etc.) in the same. K.L. Boyd has given expert testimony for the Hollingsworth Law Firm. J. Sensintaffar reports receiving a commercial research grant from, has ownership interest (including stock, patents, etc.) in, and has received other remuneration from Boehringer Ingelheim. E.T. Olejniczak reports receiving a commercial research grant from Boehringer Ingelheim and has received other remuneration from the same. S.W. Fesik reports receiving commercial research support from Boehringer Ingelheim. M.R. Savona reports receiving commercial research support from Boehringer Ingelheim, has ownership interest (including stock, patents, etc.) in Karyopharm Therapeutics, and is a consultant/advisory board member for Karyopharm Therapeutics, Celgene Corporation, Astex Pharmaceuticals, Gilead, Incyte Corporation, TG Therapeutics, Takeda Corporation, and Merck. No potential conflicts of interest were disclosed by the other authors.

### Authors' Contributions

**Conception and design:** H.E. Ramsey, T. Lee, S.W. Fesik, M.R. Savona

**Development of methodology:** H.E. Ramsey, M.A. Fischer, A.E. Gorska, M.P. Arrate, J. Sensintaffar, M.R. Savona

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** H.E. Ramsey, M.A. Fischer, A.E. Gorska, M.P. Arrate, L. Fuller, K.L. Boyd, L.J. Hogdal, M.R. Savona

**Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis):** H.E. Ramsey, M.A. Fischer, A.E. Gorska, M.P. Arrate, K.L. Boyd, L.J. Hogdal, G.D. Ayers, M.R. Savona

**Writing, review, and/or revision of the manuscript:** H.E. Ramsey, M.A. Fischer, T. Lee, A.E. Gorska, M.P. Arrate, L. Fuller, K.L. Boyd, S.A. Strickland, J. Sensintaffar, L.J. Hogdal, G.D. Ayers, E.T. Olejniczak, S.W. Fesik, M.R. Savona

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** H.E. Ramsey, M.A. Fischer, A.E. Gorska, M.P. Arrate, L. Fuller, K.L. Boyd, S.W. Fesik

**Study supervision:** T. Lee, S.W. Fesik, M.R. Savona

### Acknowledgments

Thank you to Scott Hiebert, PhD, for review of the manuscript and thoughtful insight. The Vanderbilt-Ingram Cancer Center (VICC) Hematopoietic Malignancies Tissue Repository, the Vanderbilt University Medical Center Translational Pathology Shared Resource, and other VICC shared Core Services were critical in completion of this work. This study was supported by the E.P. Evans Foundation Discovery Research Grant (M. Savona), the Adventure Allie Discovery Research Grant (M. Savona), and the Biff Ruttenberg Foundation (M. Savona). This project has been funded in part with federal funds from the NCI, NIH, under Chemical Biology Consortium Contract No. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government. The Biomolecular NMR Facility at Vanderbilt University is supported in part by NIH SIG grant 1S-10RR025677-01 (S. Fesik) and Vanderbilt University matching funds. The VICC is supported by NIH P30 CA068485-19. The REDCap database tool is supported by grant UL1 TR000445 from NCATS/NIH.

Received February 14, 2018; revised July 14, 2018; accepted August 28, 2018; published first September 5, 2018.

## REFERENCES

- Cortes JE, Goldberg SL, Feldman EJ, Rizzeri DA, Hogge DE, Larson M, et al. Phase II, multicenter, randomized trial of CPX-351 (cytarabine:daunorubicin) liposome injection versus intensive salvage therapy in adults with first relapse AML. *Cancer* 2015;121:234–42.
- Lancet JE, Cortes JE, Hogge DE, Tallman MS, Kovacsics TJ, Damon LE, et al. Phase 2 trial of CPX-351, a fixed 5:1 molar ratio of cytarabine/daunorubicin, vs. cytarabine/daunorubicin in older adults with untreated AML. *Blood* 2014;123:3239–46.
- Shah A, Andersson TM, Ratchet B, Bjorkholm M, Lambert PC. Survival and cure of acute myeloid leukaemia in England, 1971–2006: a population-based study. *Br J Haematol* 2013;162:509–16.
- Ravandi F, Ritchie EK, Sayar H, Lancet JE, Craig MD, Vey N, et al. Vosaroxin plus cytarabine versus placebo plus cytarabine in patients with first relapsed or refractory acute myeloid leukaemia (VALOR): a randomised, controlled, double-blind, multinational, phase 3 study. *Lancet Oncol* 2015;16:1025–36.
- Walter MJ, Shen D, Ding L, Shao J, Koboldt DC, Chen K, et al. Clonal architecture of secondary acute myeloid leukemia. *N Engl J Med* 2012;366:1090–8.
- Pan R, Hogdal LJ, Benito JM, Bucci D, Han L, Borthakur G, et al. Selective BCL-2 inhibition by ABT-199 causes on-target cell death in acute myeloid leukemia. *Cancer Discov* 2014;4:362–75.
- Keith FJ, Bradbury DA, Zhu YM, Russell NH. Inhibition of bcl-2 with antisense oligonucleotides induces apoptosis and increases the sensitivity of AML blasts to Ara-C. *Leukemia* 1995;9:131–8.
- Niu X, Wang G, Wang Y, Caldwell JT, Edwards H, Xie C, et al. Acute myeloid leukemia cells harboring MLL fusion genes or with the acute promyelocytic leukemia phenotype are sensitive to the Bcl-2-selective inhibitor ABT-199. *Leukemia* 2014;28:1557–60.
- Vaux DL. Immunology. Ways around rejection. *Nature* 1998;394:133.
- Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 1993;74:597–608.
- Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* 2001;8:705–11.
- Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. *Mol Cell* 2010;37:299–310.
- Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* 2002;2:183–92.
- Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996;86:147–57.
- Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001;292:727–30.
- Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 2005;435:677–81.
- Kvansakul M, Yang H, Fairlie WD, Czabotar PE, Fischer SF, Perugini MA, et al. Vaccinia virus anti-apoptotic F1L is a novel Bcl-2-like domain-swapped dimer that binds a highly selective subset of BH3-containing death ligands. *Cell Death Differ* 2008;15:1564–71.
- Sattler M, Liang H, Nettessheim D, Meadows RP, Harlan JE, Eberstadt M, et al. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* 1997;275:983–6.
- Liu X, Dai S, Zhu Y, Marrack P, Kappler JW. The structure of a Bcl-xL/Bim fragment complex: implications for Bim function. *Immunity* 2003;19:341–52.
- Lessene G, Czabotar PE, Sleebs BE, Zobel K, Lowes KN, Adams JM, et al. Structure-guided design of a selective BCL-X(L) inhibitor. *Nat Chem Biol* 2013;9:390–7.
- Czabotar PE, Lee EF, van Delft MF, Day CL, Smith BJ, Huang DC, et al. Structural insights into the degradation of Mcl-1 induced by BH3 domains. *Proc Natl Acad Sci U S A* 2007;104:6217–22.
- Czabotar PE, Westphal D, Dewson G, Ma S, Hockings C, Fairlie WD, et al. Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. *Cell* 2013;152:519–31.
- Wang JL, Liu D, Zhang ZJ, Shan S, Han X, Srinivasula SM, et al. Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. *Proc Natl Acad Sci U S A* 2000;97:7124–9.
- Zhou H, Chen J, Meagher JL, Yang CY, Aguilar A, Liu L, et al. Design of Bcl-2 and Bcl-xL inhibitors with subnanomolar binding affinities based upon a new scaffold. *J Med Chem* 2012;55:4664–82.
- Zheng CH, Yang H, Zhang M, Lu SH, Shi D, Wang J, et al. Design, synthesis, and activity evaluation of broad-spectrum small-molecule inhibitors of anti-apoptotic Bcl-2 family proteins: characteristics of broad-spectrum protein binding and its effects on anti-tumor activity. *Bioorg Med Chem Lett* 2012;22:39–44.
- Degterev A, Lugovskoy A, Cardone M, Mulley B, Wagner G, Mitchison T, et al. Identification of small-molecule inhibitors of interaction between the BH3 domain and Bcl-xL. *Nat Cell Biol* 2001;3:173–82.
- Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, et al. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature* 1996;381:335–41.
- Wilson WH, O'Connor OA, Czuczman MS, LaCasce AS, Gerecitano JF, Leonard JP, et al. Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumor activity. *Lancet Oncol* 2010;11:1149–59.
- Souers AJ, Levenson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves anti-tumor activity while sparing platelets. *Nat Med* 2013;19:202–8.
- Roberts AW, Davids MS, Pagel JM, Kahl BS, Puvvada SD, Gerecitano JF, et al. Targeting BCL2 with venetoclax in relapsed chronic lymphocytic leukemia. *N Engl J Med* 2016;374:311–22.
- Seymour JF, Ma S, Brander DM, Choi MY, Barrientos J, Davids MS, et al. Venetoclax plus rituximab in relapsed or refractory chronic lymphocytic leukaemia: a phase 1b study. *Lancet Oncol* 2017;18:230–40.
- Konopleva M, Pollyea DA, Potluri J, Chyla B, Hogdal L, Busman T, et al. Efficacy and biological correlates of response in a phase II study of venetoclax monotherapy in patients with acute myelogenous leukemia. *Cancer Discov* 2016;6:1106–17.
- DiNardo CD, Pratz KW, Letai A, Jonas BA, Wei AH, Thirman M, et al. Safety and preliminary efficacy of venetoclax with decitabine or azacitidine in elderly patients with previously untreated acute myeloid leukaemia: a non-randomised, open-label, phase 1b study. *Lancet Oncol* 2018;19:216–28.
- Phillips DC, Xiao Y, Lam LT, Litvinovich E, Roberts-Rapp L, Souers AJ, et al. Loss in MCL-1 function sensitizes non-Hodgkin's lymphoma cell lines to the BCL-2-selective inhibitor venetoclax (ABT-199). *Blood Cancer J* 2015;5:e368.
- Bodo J, Zhao X, Durkin L, Souers AJ, Phillips DC, Smith MR, et al. Acquired resistance to venetoclax (ABT-199) in t(14;18) positive lymphoma cells. *Oncotarget* 2016;7:70000–10.
- Lin KH, Winter PS, Xie A, Roth C, Martz CA, Stein EM, et al. Targeting MCL-1/BCL-XL forestalls the acquisition of resistance to ABT-199 in acute myeloid leukemia. *Sci Rep* 2016;6:27696.
- Jilg S, Reidel V, Muller-Thomas C, Konig J, Schauwecker J, Hockendorf U, et al. Blockade of BCL-2 proteins efficiently induces apoptosis in progenitor cells of high-risk myelodysplastic syndromes patients. *Leukemia* 2016;30:112–23.
- Arellano ML, Borthakur G, Berger M, Luer J, Raza A. A phase II, multicenter, open-label study of obatoclax mesylate in patients with previously untreated myelodysplastic syndromes with anemia or thrombocytopenia. *Clin Lymphoma Myeloma Leuk* 2014;14:534–9.



39. Wertz IE, Kusam S, Lam C, Okamoto T, Sandoval W, Anderson DJ, et al. Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. *Nature* 2011;471:110–4.
40. Rochaix P, Krajewski S, Reed JC, Bonnet F, Voigt JJ, Brousset P. In vivo patterns of Bcl-2 family protein expression in breast carcinomas in relation to apoptosis. *J Pathol* 1999;187:410–5.
41. Zhang B, Gojo I, Fenton RG. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. *Blood* 2002;99:1885–93.
42. Beroukhi R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, et al. The landscape of somatic copy-number alteration across human cancers. *Nature* 2010;463:899–905.
43. Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J Clin Invest* 2007;117:112–21.
44. Invernizzi R, Pecci A, Bellotti L, Ascarelli E. Expression of p53, bcl-2 and ras oncoproteins and apoptosis levels in acute leukaemias and myelodysplastic syndromes. *Leuk Lymphoma* 2001;42:481–9.
45. Arkin MR, Wells JA. Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. *Nat Rev Drug Discov* 2004;3:301–17.
46. Lee T, Bian Z, Zhao B, Hogdal LJ, Sensintaffar JL, Goodwin CM, et al. Discovery and biological characterization of potent myeloid cell leukemia-1 inhibitors. *FEBS Lett* 2017;591:240–51.
47. Kotschy A, Szlavik Z, Murray J, Davidson J, Maragno AL, Le Toumelin-Braizat G, et al. The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. *Nature* 2016;538:477–82.
48. Levenson JD, Zhang H, Chen J, Tahir SK, Phillips DC, Xue J, et al. Potent and selective small-molecule MCL-1 inhibitors demonstrate on-target cancer cell killing activity as single agents and in combination with ABT-263 (navitoclax). *Cell Death Dis* 2015;6:e1590.
49. Montero J, Letai A. Dynamic BH3 profiling-poking cancer cells with a stick. *Mol Cell Oncol* 2016;3:e1040144.
50. Raza A, Alvi S, Borok RZ, Span L, Parcharidou A, Alston D, et al. Excessive proliferation matched by excessive apoptosis in myelodysplastic syndromes: the cause-effect relationship. *Leuk Lymphoma* 1997;27:111–8.
51. Bogenberger J, Whatcott C, Hansen N, Delman D, Shi CX, Kim W, et al. Combined venetoclax and alvocidib in acute myeloid leukemia. *Oncotarget* 2017;8:107206–22.
52. Ishizawa J, Kojima K, McQueen T, Ruvolo V, Chachad D, Noguera-Gonzalez GM, et al. Mitochondrial profiling of acute myeloid leukemia in the assessment of response to apoptosis modulating drugs. *PLoS One* 2015;10:e0138377.
53. Grundy M, Seedhouse C, Jones T, Elmi L, Hall M, Graham A, et al. Predicting effective pro-apoptotic anti-leukaemic drug combinations using co-operative dynamic BH3 profiling. *PLoS One* 2018;13:e0190682.
54. Foight GW, Ryan JA, Gulla SV, Letai A, Keating AE. Designed BH3 peptides with high affinity and specificity for targeting Mcl-1 in cells. *ACS Chem Biol* 2014;9:1962–8.
55. Morales AA, Kurtoglu M, Matulis SM, Liu J, Siefker D, Gutman DM, et al. Distribution of Bim determines Mcl-1 dependence or codependence with Bcl-xL/Bcl-2 in Mcl-1-expressing myeloma cells. *Blood* 2011;118:1329–39.
56. Akgul C. Mcl-1 is a potential therapeutic target in multiple types of cancer. *Cell Mol Life Sci* 2009;66:1326–36.
57. Levenson JD, Phillips DC, Mitten MJ, Boghaert ER, Diaz D, Tahir SK, et al. Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and define improved strategies for cancer therapy. *Sci Transl Med* 2015;7:279ra40.
58. Waibel M, Solomon VS, Knight DA, Ralli RA, Kim SK, Banks KM, et al. Combined targeting of JAK2 and Bcl-2/Bcl-xL to cure mutant JAK2-driven malignancies and overcome acquired resistance to JAK2 inhibitors. *Cell Rep* 2013;5:1047–59.
59. Konopleva M, Contractor R, Tsao T, Samudio I, Ruvolo PP, Kitada S, et al. Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* 2006;10:375–88.
60. Teh TC, Nguyen NY, Moujalled DM, Segal D, Pomilio G, Rijal S, et al. Enhancing venetoclax activity in acute myeloid leukemia by co-targeting MCL1. *Leukemia* 2018;32:303–12.
61. Dey J, Deckwerth TL, Kerwin WS, Casalini JR, Merrell AJ, Grenley MO, et al. Voruciclib, a clinical stage oral CDK9 inhibitor, represses MCL-1 and sensitizes high-risk diffuse large B-cell lymphoma to BCL2 inhibition. *Sci Rep* 2017;7:18007.
62. Zhou L, Zhang Y, Sampath D, Levenson J, Dai Y, Kmiecik M, et al. Flavopiridol enhances ABT-199 sensitivity in unfavourable-risk multiple myeloma cells in vitro and in vivo. *Br J Cancer* 2018;118:388–97.
63. Brinkmann K, Grabow S, Hyland CD, Teh CE, Alexander WS, Herold MJ, et al. The combination of reduced MCL-1 and standard chemotherapeutics is tolerable in mice. *Cell Death Differ* 2017;24:2032–43.
64. Opferman JT, Iwasaki H, Ong CC, Suh H, Mizuno S, Akashi K, et al. Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* 2005;307:1101–4.
65. Peperzak V, Vikstrom I, Walker J, Glaser SP, LePage M, Coquery CM, et al. Mcl-1 is essential for the survival of plasma cells. *Nat Immunol* 2013;14:290–7.
66. Murphy MP, Caraher E. Mcl-1 is vital for neutrophil survival. *Immunol Res* 2015;62:225–33.
67. Masuda H, Hirose J, Omata Y, Tokuyama N, Yasui T, Kadono Y, et al. Anti-apoptotic Bcl-2 family member Mcl-1 regulates cell viability and bone-resorbing activity of osteoclasts. *Bone* 2014;58:1–10.
68. Malone CD, Hasan SM, Roome RB, Xiong J, Furlong M, Opferman JT, et al. Mcl-1 regulates the survival of adult neural precursor cells. *Mol Cell Neurosci* 2012;49:439–47.
69. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet* 2015;385:517–28.
70. Hochhaus A, Masszi T, Giles FJ, Radich JP, Ross DM, Gomez Casares MT, et al. Treatment-free remission following frontline nilotinib in patients with chronic myeloid leukemia in chronic phase: results from the ENESTfreedom study. *Leukemia* 2017;31:1525–31.
71. Kumar S, Kaufman JL, Gasparetto C, Mikhael J, Vij R, Pegourie B, et al. Efficacy of venetoclax as targeted therapy for relapsed/refractory t(11;14) multiple myeloma. *Blood* 2017;130:2401–9.
72. Bogenberger JM, Delman D, Hansen N, Valdez R, Fauble V, Mesa RA, et al. Ex vivo activity of BCL-2 family inhibitors ABT-199 and ABT-737 combined with 5-azacytidine in myeloid malignancies. *Leuk Lymphoma* 2015;56:226–9.
73. Bogenberger JM, Kornblau SM, Pierceall WE, Lena R, Chow D, Shi CX, et al. BCL-2 family proteins as 5-azacytidine-sensitizing targets and determinants of response in myeloid malignancies. *Leukemia* 2014;28:1657–65.
74. Deng J, Carlson N, Takeyama K, Dal Cin P, Shipp M, Letai A. BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* 2007;12:171–85.
75. Ryan J, Montero J, Rocco J, Letai A. iBH3: simple, fixable BH3 profiling to determine apoptotic priming in primary tissue by flow cytometry. *Biol Chem* 2016;397:671–8.
76. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984;22:27–55.
77. Lee JJ, Kong M, Ayers GD, Lotan R. Interaction index and different methods for determining drug interaction in combination therapy. *J Biopharm Stat* 2007;17:461–80.