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Targeting MCL-1 dysregulates cell metabolism and leukemia-stroma interactions and re-sensitizes acute myeloid leukemia to BCL-2 inhibition

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Abstract: MCL-1 and BCL-2 are both frequently overexpressed in acute myeloid leukemia and critical for the survival of acute myeloid leukemia cells and acute myeloid leukemia stem cells. MCL-1 is a key factor in venetoclax resistance. Using genetic and pharmacological approaches, we discovered that MCL-1 regulates leukemia cell bioenergetics and carbohydrate metabolisms, including the TCA cycle, glycolysis and pentose phosphate pathway and modulates cell adhesion proteins and leukemia-stromal interactions. Inhibition of MCL-1 sensitizes to BCL-2 inhibition in acute myeloid leukemia cells and acute myeloid leukemia stem/progenitor cells, including those with intrinsic and acquired resistance to venetoclax through cooperative release of pro-apoptotic BIM, BAX, and BAK from binding to anti-apoptotic BCL-2 proteins and inhibition of cell metabolism and key stromal microenvironmental mechanisms. The combined inhibition of MCL-1 by MCL-1 inhibitor AZD5991 or CDK9 inhibitor AZD4573 and BCL-2 by venetoclax greatly extended survival of mice bearing patient-derived xenografts established from an acute myeloid leukemia patient who acquired resistance to venetoclax/decitabine. These results demonstrate that co-targeting MCL-1 and BCL-2 improves the efficacy of and overcomes pre-existing and acquired resistance to BCL-2 inhibition. Activation of metabolomic pathways and leukemia-stroma interactions are newly discovered functions of MCL-1 in acute myeloid leukemia, which are independent from canonical regulation of apoptosis by MCL-1. Our data provide new mechanisms of synergy and rationale for co-targeting MCL-1 and BCL-2 clinically in patients with acute myeloid leukemia and potentially other cancers.
Introduction

Acute myeloid leukemia (AML) is a highly heterogeneous and aggressive hematological malignancy with dismal treatment outcomes. The survival of AML cells, including stem/progenitor cells, depends on deregulated apoptosis, which partially results from the altered expression of BCL-2 family proteins. Thus, BCL-2 family proteins are promising therapeutic targets in AML (1, 2). While combined inhibition of BCL-2 and BCL-XL caused profound thrombocytopenia (3), a highly selective BCL-2 inhibitor, venetoclax, demonstrated potent preclinical activity, but only marginal efficacy in clinical trials in AML (2, 4). Conversely, combinations of venetoclax with hypomethylating agents have yielded complete response (CR) or CR with incomplete cell count recovery rates of >65% in elderly AML patients, but overall survival is only 10 to 17 months due to the development of resistance (5-7).

Unlike other BCL-2 proteins, MCL-1 is short-lived and highly regulated at the transcriptional, post-transcriptional, and post-translational levels (8) in response to various stimuli. MCL-1 is required for the sustained growth of diverse oncogene-driven hematological malignancies (9, 10) and essential for AML development and AML stem cell self-renewal and survival (11-13). MCL-1 is upregulated in about half of resistant/relapsed AML patients and associated with poor prognosis (14). Importantly, high levels of MCL-1 expression are associated with resistance to venetoclax (2, 15, 16). Furthermore, MCL-1 was reported to increase the generation of reactive oxygen species (ROS) in lung cancer cells (17), and to positively regulate mitochondrial oxidative phosphorylation (OxPhos) in breast cancer, inducing the breast cancer stem cells, and promoting tumor chemoresistance (18). These studies suggest that, in addition to its anti-apoptotic function, MCL-1 is coupled to tumor cell metabolism to promote cell survival. Indeed, AML cells and stem cells exhibit increased mitochondrial OxPhos (19-21), suggesting that MCL-1 has a role in regulating mitochondrial OxPhos and altering AML cell metabolism.
Several specific MCL-1 inhibitors have been developed recently. S63845 (Servier/Novartis) was very effective and well tolerated in in vivo models (22). Others such as AZD5991 (AstraZeneca) (23) and AMG176/AMG397 (Amgen) (24) have entered clinical trials. Additionally, inhibitors of CDK9 that transcriptionally regulates short-lived proteins like MCL-1 are under development as a strategy to target MCL-1, and the CDK9 inhibitor AZD4573 (25) has entered clinical trials.

Rationales supporting BCL-2 and MCL-1 co-targeting have focused on apoptotic mechanisms. Targeting MCL-1 with a BIM transgene enhanced venetoclax activity against AML (26). We reported a combinatorial regimen of BCL-2 inhibition and MDM2 inhibition-mediated p53-activation that targeted MCL-1 activity and stability. This combination was synthetically lethal to AML cells (27) and in early clinical trials elicits 40-50% CR rates among relapsed/refractory AML patients (28). Recent studies in murine models of AML showed that the combined inhibition of BCL-2 and MCL-1 has potent antitumor activity, enhances venetoclax activity, and kills venetoclax-resistant disease (23, 24, 29, 30). However, the mechanisms underlying this synergism, including its effects on AML stem and venetoclax-resistant cells, have not been fully investigated.

Here, we determine the effects of MCL-1 inhibition with the novel MCL-1 inhibitor AZD5991 or the CDK9 inhibitor AZD4573 alone and in combination with venetoclax on multiple phenotypic AML stem/progenitor cell populations in vitro and in an in vivo PDX model. Additionally, our in vitro study was conducted with bone marrow (BM)-derived mesenchymal stromal cell (MSC) co-culture, which mimics the BM stromal microenvironment (31). In the in vivo study, for the first time, we utilized a PDX model derived from a patient that acquired resistance to venetoclax/decitabine treatment thus revealing a novel mechanism of synergy involving potential non-apoptotic, metabolic and microenvironmental functions of MCL-1.
**Methods**

*Cells and treatments.* AML cell lines (Molm13, MV4-11, and OCI-AML3) and primary AML cells (Supplementary Table 1) were obtained as described in supplemental materials. MCL-1-overexpressing (OE), MCL-1-knockdown (KD), and acquired venetoclax resistance AML cells were generated as described previously (27). BAX, BAK, or BAX/BAK double knockdown OCI-AML3 cells using siRNAs (control or ON-TARGETplus SMARTpool siRNAs for each target: Dhharmacon, Chicago, IL) were generated as previously described (32). Human BM-derived MSCs were isolated as described previously (33). Cells, cultured under the conditions previously described (34) were treated with venetoclax, AZD5991, AZD4573, IACS-10759 (Institute for Applied Cancer Science, MD Anderson, Houston, TX), BL-8040 (BioLineRx Ltd., Israel), and various combinations without or with MSCs (AML-to-MSC=4:1). For AZD4573 treatments, cells were exposed to AZD4573 for 6 h, and then the drug was removed.

*Cell viability assay.* Viable cells and apoptosis were assessed as previously described (34). For leukemia cells co-cultured with MSCs, CD45+ cells were counted. Apoptotic cells were defined as annexin V+ and/or 7-aminoactinomycin D+ (AnnV+/7AAD+) CD45+ cells. For patient samples, annexin V positivity was determined in bulk (CD45+), CD34+CD38+/CD38- and CD34+CD38+/CD38-CD123+ cells.

*Western blot analysis and co-immunoprecipitation.* Western blot analysis was conducted as described previously (34). Antibodies used are shown in supplemental materials.

*Protein determination by flow cytometry.* MCL-1 and cell surface CD44 and CXCR4 were also determined by flow cytometry as shown in supplemental materials.
Adhesion and migration assay. Leukemia cells’ migration and adhesion to MSCs were assessed as described previously (35).

Mitochondrial respiration assay. Mitochondrial respiration was measured using a Seahorse XF extracellular flux analyzer (Agilent Technologies, Inc., Santa Clara, CA) following the manufacturer’s instructions. The oxygen consumption rate (OCR) was expressed as pmol/min/1000 cells or relative to a control.

ROS and GSH measurement. Cellular and mitochondrial ROS were determined by flow cytometry after cells were stained with CellROS deep red or MitoSOX red (ThermoFisher Scientific), for 30 min at 37°C and expressed as mean fluorescence intensity shift between stained and unstained live cells (AnnV-/DAPI-).

Total Glutathione and glutathione disulfide (GSSG) were determined using a Glutathione (GSH) assay kit (Cayman Chemical; Ann Arbor, MI) following the manufacturer’s instructions. Reduced GSH was calculated by subtracting the amount of GSSG from total GSH.

$^{13}$C$_2$-1,2-glucose and $^{13}$C$_5$-glutamine tracing and ion chromatography-mass spectrometry metabolite analysis. Tracing and subsequence metabolite analysis are detailed in supplemental materials.

In vivo experiments. Mouse care and experiments were performed in accordance with institution Animal Care and Use Committee approved protocols. See supplemental materials for detailed experimental procedures.

CyTOF mass cytometry. Cells were stained with metal-tagged antibodies (Supplementary Table 2) against cell surface markers, barcoded, pooled, then stained with metal-tagged antibodies.
against intracellular proteins and subjected to CyTOF analysis as described previously (34, 36, 37). Briefly, viable single cells (cisplatin-low) were gated with FlowJo (software v10.7, FlowJo LLC) and exported as flow cytometry standard (FCS) data for subsequent analysis in Cytofkit (38). Cell populations identified and embedded by RPhenoGraph in the “Cytofkit_analyzedFCS” files were gated in FlowJo to quantify marker expression. ArcSinh-transformed counts for each protein expression in desired cell populations were visualized with heat maps.

**Statistical analyses.** Cell line experiments were performed in triplicates. Results were expressed as the mean±SEM. The Student t-test was used to assess differences between groups; \( P \) values \( \leq 0.05 \) were considered statistically significant. The combination index (CI) (39), determined using CalcuSyn software, was expressed as mean CI values at ED\(_{50}\), ED\(_{75}\), and ED\(_{90}\). A CI<1 was considered synergistic; CI=1, additive; and CI>1, antagonistic. EC\(_{50}\) and IC\(_{50}\) values were calculated using CalcuSyn. Mouse survival was estimated using the Kaplan-Meier method. Survival data were analyzed using the log-rank test.

**Results**

**MCL-1 regulates metabolic functions in AML cell lines and its inhibition decreases AML metabolism**

MCL-1-KD or -OE did not alter viability of AML cell line Molm13, MV4-11, or OCI-AML3 *in vitro* (Supplementary Figure S1A). However, NSGS mice harboring MCL-1-OE Molm13 cells exhibited higher leukemia burden and succumbed sooner to AML, while mice harboring MCL-1-KD OCI-AML3 cells had decreased leukemia burden and survived longer compared with their respective controls (Supplementary Figure S1B), suggesting that MCL-1 levels affect leukemia expansion. To determine whether this effect requires the modulation of bioenergetic/metabolic activity, we assayed mitochondrial respiration and found that MCL-1-OE increased, whereas
MCL-1-KD or inhibition with AZD5991 (at doses not affecting cell viability) decreased, OCR and ATP production in OCI-AML3 cells (Figure 1A). Compared with parental MV4-11 (MV4-11P), the venetoclax-resistant MV4-11 (MV4-11R) cells that expressed increased MCL-1 (27) had higher OCR and ATP production. In both cell types, AZD5991 inhibited OCR and ATP generation (Supplementary Figure S1C). In addition, compared with controls, MCL-1-OE OCI-AML3 cells exhibited higher levels of cellular and mitochondrial ROS, lower GSH levels, and lower GSH/GSSG ratios, whereas MCL-1-KD OCI-AML3 cells displayed lower ROS, higher GSH levels, and higher GSH/GSSG ratios (Figure 1B, C).

To further explore metabolic functions of MCL-1, we traced $^{13}$C incorporation from $^{13}$C$_2$-1,2-glucose and $^{13}$C$_5$-glutamine into downstream metabolites (40) using an MCL-1-KD clone (shC16) with an approximately 65% MCL-1 reduction (Supplementary Figure S1D), selected by limiting dilution of MCL-1-KD OCI-AML3 cells and OCI-AML3 cells treated with 50 nM AZD5991 (Supplementary Figure S1E). Metabolomic analysis revealed markedly lower overall incorporations of $^{13}$C from both glucose and glutamine into key TCA cycle intermediates including citrate, fumarate, and malate, in MCL-1-KD and AZD5991-treated OCI-AML3 cells compared to their respective controls (Figure 1D). An examination of the patterns of fractional $^{13}$C enrichment from $^{13}$C$_5$-glutamine into citrate, malate, and fumarate demonstrated increased M+4, but decreased M+3 fractional enrichment, in both AZD5991-treated and MCL-1-KD OCI-AML3 cells (Figure 1E), suggesting decreased glutamine entry into the TCA cycle accompanied by a smaller fraction of glutamine-derived carbon being used for anaplerotic reactions that fuel biosynthesis.

In both $^{13}$C$_2$-1,2-glucose and $^{13}$C$_5$-glutamine tracing experiments, the total amount of lactate secreted by AZD5991-treated or MCL-1-KD cells was statistically significantly lower than that secreted by their respective controls (Figure 1F). Notably, statistically significant $^{13}$C enrichment (M>0) in the secreted lactate of both the AZD5991-treated and MCL-1-KD OCI-AML3 cells, was observed from $^{13}$C$_2$-1,2-glucose, but not $^{13}$C$_5$-glutamine (Figure 1G). Glycolysis
of $^{13}$C$_2$-1,2-glucose generates lactate with two $^{13}$C labels (M+2 lactate). Thus, a reduction in M+2 lactate secretion suggests MCL-1 inhibition decreases glycolytic flux.

Next, we assessed whether the decrease in glycolytic flux was accompanied by a decrease in flux through the oxidative branch of the pentose phosphate pathway (oxPPP), a major pathway involved in the generation of the reducing equivalent NADPH that is used for ROS neutralization. When $^{13}$C$_2$-1,2-glucose is metabolized through the oxPPP and re-enters glycolysis via the non-oxPPP, the elimination of one $^{13}$C carbon through decarboxylation yields lactate containing only one $^{13}$C. The relative oxPPP flux, which we calculated by multiplying the ratio of [M+1]/([M+1]+[M+2]) from extracellular lactate with lactate secretion rate during $^{13}$C$_2$-1,2-glucose tracing, was lower in both AZD5991-treated and MCL-1-KD OCI-AML3 cells than in their respective controls (Figure 1H).

6-Phosphogluconic acid levels were also greatly decreased under MCL-1 inhibition (Figure 1H), in accordance with oxPPP modulation (41). Consistent with the aforementioned extracellular flux assay results, AZD5991-treated, and MCL-1-KD OCI-AML3 cells, exhibited decreased ATP levels (Figure 1I). These findings indicated that MCL-1 inhibition downregulates key pathways that support cellular energetics in AML cells as summarized in Figure 1J.

Furthermore, MCL-1 modulated the level of c-MYC in OCI-AML3 cells (Supplementary Figure S1F), which is a well-known metabolic regulator in malignant cells.

**MCL-1 regulates adhesion molecule expression in AML cell lines and AML-stroma interactions**

Since MSCs in the BM microenvironment upregulate MCL-1, we investigated whether MCL-1 had a role in leukemia-stroma interactions. MCL-1-OE OCI-AML3 cells exhibited increased surface expression of CXCR4 and CD44, both of which participate in leukemia-MSC interactions and drug resistance, and increased OCI-AML3 migration towards, and adhesion to, MSCs (Figure 2A). Conversely, MCL-1-KD OCI-AML3 exhibited decreased surface expression
of CXCR4 and CD44 and decreased OCI-AML3 migration toward, and adhesion to, MSCs (Figure 2B). Consistently, inhibition of MCL-1 with AZD5991 decreased OCI-AML3 surface CXCR4 and CD44 expression and interaction with MSCs (Figure 2C). Like other MCL-1 inhibitors, AZD5991 increased levels of MCL-1 (Figure 2C). BCL-2 inhibition with venetoclax in OCI-AML3 cells did not appreciably decrease CD44 surface expression, AML cell migration, or adhesion to MSCs. However, it decreased CXCR4 surface expression (Figure 2D). These results suggest that MCL-1 enhances surface adhesion molecule expression and promotes leukemia-stroma interactions.

**MCL-1 mediates venetoclax resistance, and its inhibition sensitizes AML cell lines to BCL-2 inhibition**

Molm13 and MV4-11 cells are relatively sensitive, whereas OCI-AML3 cells are resistant, to venetoclax (Supplementary Figure S2A). Acquired venetoclax-resistant AML cell lines have increased MCL-1, but they likely also have altered levels of many other proteins. To determine whether MCL-1 played a key role in venetoclax resistance and whether co-targeting BCL-2 and MCL-1 overcomes this resistance, we treated MCL-1-KD OCI-AML3 and MCL-1-OE Molm13 and MV4-11 cells with venetoclax or AZD5991. Whereas MCL-1-KD greatly sensitized OCI-AML3 cells to venetoclax (Figure 3A), MCL-1-OE Molm13 (Figure 3B) and MV4-11 cells (Supplementary Figure S2B) were largely insensitive to the drug. Responses to AZD5991 were similar, though to a lesser degree (Figure 3A, B; Supplementary Figure S2B). We then treated OCI-AML3 and MCL-1-OE Molm13 and MV4-11 cells with venetoclax plus AZD5991. The combination synergistically induced apoptosis and decreased viable cells in the intrinsically venetoclax-resistant OCI-AML3 (Figure 3C), MCL-1-OE Molm13 (Figure 3D), and MV4-11 cells (Supplementary Figure S2C). This was also the case in MV4-11R cells (Figure 3E).

We then tested CDK9 inhibitor AZD4573. AZD4573 decreased MCL-1 expression and synergized with venetoclax targeting OCI-AML3 cells (Supplementary Figure S2D). This
combination was also highly synergistic against MV4-11 and MV4-11R cells (Supplementary Figure S2E). To demonstrate that MCL-1 is indeed the critical target regulated by CDK9, we treated MCL-1-OE Molm13 and MV4-11 cells with AZD4573 and found that, similar to the MCL-1 inhibitor AZD5991, MCL-1 OE cells were more resistant than control cells to AZD4573. Furthermore, the combination of venetoclax and AZD4573 was highly synergistic in MCL-1-OE Molm13 and MV4-11 cells (Supplementary Figure S2F).

We next treated OCI-AML3 cells with venetoclax, AZD5991, or the combination and determined interactions between anti-apoptotic BCL-2, MCL-1, and BCL-XL with the pro-apoptotic activator BIM and effectors BAX and BAK by co-immunoprecipitation to potentially understand the mechanisms of synergy. We observed that BCL-2 was bound to BIM and BAX (Figure 4A), MCL-1 was bound to BIM and BAK (Figure 4B), and BCL-XL was bound to all three (Figure 4C). Venetoclax treatment decreased BCL-2/BIM and BCL2/BAX interactions, but increased MCL-1/BIM, BCL-XL/BAX, and BCL-XL/BAK interactions. AZD5991 treatment decreased MCL-1/BIM interaction, but increased BCL-2/BIM and MCL-1/BAK interactions. When the two drugs were combined, BCL-2/BIM and BCL2/BAX interactions were largely diminished, MCL-1/BIM interaction decreased, and the single agent treatment-mediated increases of BCL-XL/BAX, BCL-XL/BAK, and MCL-1/BAK interactions were abrogated (Figure 4A-C), suggesting that the combinatorial cooperative release of activator and effector BCL-2 family proteins likely contributed to the synergy in apoptosis induction (Figure 4D).

To determine the contribution of metabolism and leukemia-stroma interactions on the observed synergism, we treated OCI-AML3 cells with venetoclax in the presence of the OxPhos inhibitor IACS-10759 (42) or the CXCR4 inhibitor BL-8040 (4F-benzoyl-TN14003) (43). As expected, MSC co-culture protected AML cells from venetoclax-, AZD5991-, or IACS-10759-induced apoptosis (Figure 4E). While suppressing OCI-AML3 migration and adhesion to MSCs, BL-8040 did not affect cell viability (Figure 4E, 4F). Under MSC co-culture, venetoclax activity was minimally enhanced by OxPhos or CXCR4 inhibition, but markedly augmented by
combinatorial OxPhos and CXCR4 inhibition. Maximal apoptosis induction of OCI-AML3 was observed by combined MCL-1 and BCL-2 inhibition (Figure 4E). These results support that cooperative release of pro- from anti-apoptotic BCL-2 family proteins and inhibition of cell metabolism and key stromal microenvironmental mechanisms all contributed to the synergism of co-targeting MCL-1 and BCL-2 in AML cells.

**Combined BCL-2 and MCL-1 inhibition synergistically induces apoptosis in primary AML cells and AML stem/progenitor cells**

Primary AML cells were cultured with BM-derived MSCs and treated with venetoclax, AZD5991, AZD4573, venetoclax plus AZD5991, or venetoclax plus AZD4573. Apoptosis and viable cell counts of AML blast cells and CD34+ stem/progenitor cells were assessed and expressed as EC\textsubscript{50} (Figure 5A) or IC\textsubscript{50} (Supplementary Figure S3) for each agent used alone or in combination, as indicated. The patient characteristics, including mutations, venetoclax treatment status, and cytogenetics/risk categories, are shown in Figure 5B and Supplementary Table 1. Responses to venetoclax, AZD5991, or AZD4573 varied across the patient samples. Compared with the single-agent treatments, the venetoclax plus AZD5991 and venetoclax plus AZD4573 combinations were markedly more effective in inducing apoptosis and decreasing viable cell numbers (markedly lower EC\textsubscript{50} and IC\textsubscript{50} values, respectively) in not only blasts, but also CD34+, CD34+CD38+/CD34+CD38- and CD34+CD38+CD123+/CD34+CD38-CD123+ stem/progenitor cells in all primary AML samples, including those from venetoclax-resistant/relapsed patients (Figure 5A, B; Supplementary Figure S3, Supplementary Table 1) regardless of mutational status and cytogenetics.

Among samples 7, 8, 11, and 12 from venetoclax or venetoclax/decitabine resistant/relapsed patients, three (7, 8, and 12) had relatively high venetoclax EC\textsubscript{50} values. Samples 3 and 10 that exhibited relatively high venetoclax EC\textsubscript{50} values were from resistant/relapsed patients who received venetoclax after sampling and were resistant or
relapsed (Figure 5A, B; Supplementary Table 1), suggesting that our in vitro system mirrored clinical responses. Patient samples with high venetoclax EC$_{50}$ values tended to have high AZD5991 EC$_{50}$ values, suggesting that AZD5991 monotherapy would be less effective in venetoclax-resistant patients. However, more samples are needed to confirm our conclusion.

We observed that samples with WT1 (3 and 12) or BCORL1 (4 and 7) mutations were the most resistant to AZD5991 and AZD4573, respectively (Figure 5A, B). Experiments with primary AML cells treated without MSC co-culture yielded similar results (Supplementary Figure S4), but these cells were generally more sensitive to the treatments supporting the protective role of MSCs. The CI values of the combinations for each AML cell population are shown in Supplementary Table 3.

**Co-targeting BCL-2 and MCL-1 exerts pronounced anti-leukemia activity in a PDX model of clinical venetoclax/decitabine-relapsed AML**

To determine whether co-inhibition of BCL-2 and MCL-1 could overcome venetoclax resistance in vivo, we developed an AML PDX model using cells from a patient who initially responded to venetoclax/decitabine therapy, but relapsed after three cycles (Figure 6A and Supplementary Table 1). PDX-bearing mice were treated with venetoclax, AZD5991, AZD4573, venetoclax plus AZD5991, or venetoclax plus AZD4573 (Figure 6A). All treatments statistically significantly decreased circulating blasts compared to controls ($P \leq 0.0001$, treatment d 18). The venetoclax plus AZD5991 or venetoclax plus AZD4573 group had statistically significantly fewer circulating blasts than the venetoclax ($P < 0.01$) and AZD5991 ($P < 0.0001$) groups or the venetoclax ($P = 0.0001$) and AZD4573 ($P < 0.001$) groups, respectively (Figure 6B). Analyses of BM samples (treatment d 25) yielded similar results (Figure 6C). Spleens from AZD4573- or venetoclax plus AZD4573-treated mice had a statistically significantly lower leukemia burden compared to controls, which were similar for spleens from the mice treated with venetoclax plus AZD5991 compared to the untreated or AZD5991-treated groups (treatment d 25, Figure 6D).
Although all the treatments statistically significantly extended survival in the PDX model, venetoclax (54 d, $P=0.0035$), as expected, and AZD5991 (53 d, $P=0.0081$) showed minimal effects similar to that presented for the in vitro data. AZD4573, and to greater degrees the combinations, were more effective (AZD4573: 60 d, $P=0.0002$; venetoclax plus AZD4573: 71 d, $P=0.0005$; venetoclax plus AZD5991: 76.5 d, $P=0.0005$ compared to controls 51 d, Figure 6E). The median survival of the venetoclax plus AZD4573 treatment group was statistically significantly longer than that of the venetoclax or AZD4573 group ($P=0.0004$ for both), and the venetoclax plus AZD5991 group survival was statistically significantly longer than that of venetoclax ($P=0.0004$) or AZD5991 group ($P=0.0012$) (Figure 6E). No obvious weight loss was observed during the various treatments. The mice only began to lose weight several days before they succumbed to the disease (Supplementary Figure S5). Of the 60 mice, one in AZD5991 group, one in venetoclax plus AZD5991 group, and two in venetoclax plus AZD4573 group died of treatment procedures and were not included in the survival analyses.

To elucidate the treatment effects on phenotypically-defined cell populations and on protein expression in these populations, we performed CyTOF analysis on BM samples (treatment d 25). Cells were clustered based on expressions of cell surface markers. While venetoclax had no, and AZD5991 and AZD4573 had minimal, effects in several AML cell populations, venetoclax plus AZD4573 and, to a greater degree, venetoclax plus AZD5991 had profound anti-leukemia activity in all cell populations including AML stem/progenitor cells (Figure 6F).

Compared to the vehicle treated controls, the single-agent treatments increased BCL-2, MCL-1, and c-MYC levels in most of the AML cell populations, whereas the combinations, particularly venetoclax plus AZD5991, decreased c-MYC in all populations, decreased BCL-2 in all but the CD34+CD38 population, decreased MCL-1 in the CD45+, CD34+CD38+CD123+, and CD34+CD38CD123+ populations (Figure 7A). Interestingly, AZD5991, AZD4573, and, to a greater degree, venetoclax plus AZD5991 and venetoclax plus AZD4573 greatly decreased cell
surface CXCR4 levels in all cell populations. AZD5991 and AZD4573 also decreased CD44 levels in all populations (Figure 7B). These results paralleled our *in vitro* findings suggesting that the inhibition of MCL-1 decreases CXCR4 and CD44 expression to suppress leukemia-stroma interactions. We also measured several signaling proteins and found that the combination treatments greatly decreased β-CATENIN and p-AKT in several AML cell populations (Supplementary Figure S6A), particularly in CD45\(^+\) and CD34\(^+\)CD38\(^{-}\)CD123\(^+\) cells (Figure 7C). Because mouse BM samples were collected 1, 3, or 2 d after venetoclax, AZD5991, or AZD4573 administration, our analyses may underestimate the treatment effects on proteins/phosphor-proteins in leukemia cells.

We also performed CyTOF analysis on BM cells collected from moribund mice. Compared with those in controls, BCL-2, MCL-1, and c-MYC levels in all leukemia cell populations, with the exception of c-MYC in CD34\(^+\)CD38\(^-\) cells, were higher in venetoclax, AZD5991, and the combination treatment groups (Figure 7D). Cell signaling protein analysis (Supplementary Figure S6B) showed that in all but the AZD4573 treatment CXCR4 was suppressed (Figure 7E), whereas p-AKT was greatly induced especially in the combination treatment groups (Figure 7F). Although p-AKT was not induced in the AZD4573 group, the CXCR4 level recovered compared with that observed in the controls (Figure 7E, F).

**MCL-1 regulates metabolic activity in AML cell lines and leukemia-stromal interactions**

To determine if MCL-1 regulates AML cell metabolic activity and leukemia-stromal interactions independent of its anti-apoptotic functions, we knocked down BAX, BAK, or both with siRNAs in OCI-AML3 cells. Suppression of BAX and BAK levels were confirmed by western blot 48 h after siRNA transfection (Figure 8A). siRNA transfected cells were then treated with AZD5991 (50 nM), a dose that did not affect viable cell count (Supplementary Figure S1E). Apoptosis, mitochondrial respiration, and cell migration/adhesion to MSCs were determined. As shown in
Figure 8B, at the 50 nM dose of AZD5991, control siRNA-transfected cells showed no increase in apoptosis at 1 and 4 h, and only 5% more apoptotic cells over baseline at 24 h. The Bax, Bak, and Bax plus Bak siRNA-transfected cells were more resistant to AZD5991-induced apoptosis, as expected. However, inhibition of MCL-1 demonstrated the identical pattern of inhibition of mitochondrial respiration, detectable at 1 h after AZD5991 treatment, in all siRNA-transfected cells (Figure 8B). Furthermore, although AZD5991 treatment (24 h) induced low levels of apoptosis in control siRNA-transfected, but not in Bax and/or Bak siRNA-transfected OCI-AML3 cells, no apparent differences were observed in OCI-AML3 cell migration and adhesion inhibition to MSCs in control, Bax, and/or Bak siRNA-transfected and AZD5991 treated cells (Figure 8C). These results suggest that MCL-1 regulates AML metabolic activity and leukemia-stromal interactions independent of its functions as an apoptosis regulator.

Finally, we treated AML patient samples (n = 4) with low dose venetoclax (2.5 nM) or AZD5991 (12.5 nM) that induced low level apoptosis after 24 h (Supplementary Figure S7A) and found that AZD5991, but not venetoclax, effectively inhibited cellular and mitochondrial ROS production, reduced CXCR4 and CD44 levels, and diminished cell migration and adhesion to MSCs (Supplementary Figure S7B, C, D), further supporting that MCL-1 regulates mitochondrial respiration and leukemia-stromal-interactions in AML.

Discussion
Here, we demonstrate that co-targeting BCL-2 and MCL-1 is highly effective in AML cell lines and primary patient samples, which is consistent with recent reports (23, 24, 26, 29, 30). Importantly, combined MCL-1 and BCL-2 inhibition was highly synergistic against AML stem/progenitor cells and exerted pronounced anti-leukemia activity in venetoclax-resistant AML patient samples in vitro, even under conditions mimicking the BM stromal microenvironment. Furthermore, similar findings were obtained in a unique PDX model of clinical
venetoclax/decitabine-relapsed AML. Hence, the combination is not just more effective than each agent given alone, but has the potential to overcome intrinsic and acquired venetoclax resistance. In exploring the mechanisms underlying the combination’s efficacy, we demonstrated that targeting MCL-1 sensitizes to BCL-2 inhibition in AML through not only cooperative release of pro-apoptotic from binding to anti-apoptotic BCL-2 proteins but also inhibition of cell metabolism and key stromal microenvironmental mechanisms. The metabolic and stromal functions of MCL-1 can apparently act independently of the protein’s anti-apoptotic activity. These findings provide rationale for the clinical development of combined BCL-2 and MCL-1 targeting in resistant/relapsed AML patients, particularly in patients who were resistant/relapsed from venetoclax-based therapies.

We observed that WT1- or BCORL1-mutated primary AML samples were particularly resistant to AZD5991 and AZD4573, respectively. WT1 mutation in AML is associated with poor outcomes and chemoresistance (44), and BCORL1 encodes a transcription co-repressor, opposite to CDK9. We speculate that BCORL1-mutated cells are less transcriptionally suppressed, and therefore more resistant to CDK9 inhibition. However, the sample size for either WT1- or BCORL1-mutated cells was too small to draw definitive conclusions. Additional studies are needed to confirm our finding, which may help guide patient selection.

In contrast to a recent study showing that venetoclax-resistant cells are more sensitive to the MCL-1 inhibitor VU0661013 (30), we found that intrinsic or acquired venetoclax resistant AML cells were less responsive to the MCL-1 inhibitor AZD5991, but the combination of venetoclax and AZD5991 or AZD4573 synergistically induced cell death regardless of the single-agent response, both in vitro and in vivo. These data suggest that patients whose disease is resistant to, or relapses from, venetoclax therapy may potentially benefit from co-targeting MCL-1 and BCL-2.

To understand the mechanisms of synergy, especially in cells insensitive to BCL-2 or MCL-1 inhibition alone, we examined the interactions of anti- and pro-apoptotic BCL-2 proteins
in OCI-AML3 cells treated with venetoclax, AZD5991, or both. Targeting either BCL-2 or MCL-1 decreased the interactions of one of them with pro-apoptotic BCL-2 proteins, but increased the interactions of the other with pro-apoptotic proteins. Only when both are inhibited, maximal amounts of unbound activator protein BIM and effector proteins BAX/BAK become available, and more effectively induce apoptosis.

Interestingly, Pollyea et al. recently attributed the effectiveness of the venetoclax plus azacitidine combination in its ability to suppress OxPhos, disrupt the TCA cycle, and perturb cell energy metabolism, thereby efficiently targeting leukemia stem cells, an effect that was not achieved with the venetoclax treatment alone (7). We found that MCL-1 regulates redox and metabolic functions in AML cells and that genetic or pharmacological inhibition of MCL-1 suppressed several cellular energetic and metabolic pathways, including TCA cycle, glycolysis, and PPP. We further demonstrated that inhibiting this function of MCL-1 contributed to the enhanced activity of venetoclax against AML cells.

ROS stabilize HIF1 thereby activating hypoxia signaling (45, 46). Although MCL-1 alteration in AML cells did not elicit detectable changes in HIF1α levels, it elicited changes in CXCR4, a HIF1α target, and CD44. Both, the CXCR4/CXCL12 axis and CD44 are critical for leukemia-BM stromal microenvironment interactions. Indeed, we found that genetic or pharmacological manipulation of MCL-1, but not BCL-2, in AML cells altered cell migration and adhesion to MSCs. In confirmation, in vivo MCL-1, but not BCL-2, inhibition decreased CXCR4 and CD44 levels in BM leukemia cells from PDX mice suggesting a novel function of MCL-1, specifically the regulation of leukemia-stroma interactions. The cell intrinsic anti-apoptotic function of MCL-1 may therefore be complemented and enhanced by the cell-extrinsic enhanced adhesion to the BM stroma.

In addition to MCL-1, other mechanisms of intrinsic and acquired venetoclax resistance were reported in recent years (47-50). Although highly effective and statistically significantly
extending survival, our combination-treated mice eventually died of leukemia. We observed increased p-AKT levels in leukemia cells collected from these mice, which warrants further investigation.

Collectively, we demonstrated that MCL-1 regulates cell metabolism, leukemia-stroma interactions, and protects leukemia cells from BCL-2 inhibition. MCL-1 inhibition targets multiple cancer cell characteristics and therefore has multifaceted effects in AML. The MCL-1 inhibition-mediated suppression of metabolic activity and inhibition of CXCR4 and CD44 may contribute to its efficacy against AML stem cells in the BM stromal microenvironment. Treatment strategies involving combined MCL-1 and BCL-2 inhibition could improve the outcomes in AML patients for whom BCL-2-targeted therapy has failed, which warrants further clinical evaluation.

**Author contributions:** B.Z.C. conceptualized the study, designed the experiments, and wrote the manuscript; P.Y.M. and W.T. performed the experiments and analyzed the data; M.W. performed the experiments, analyzed the data, and wrote the paper; P.L.L. analyzed the data and edited the paper; D.M., V.R., and LT. performed experiments; J.C. and L.D. provided materials, supported study, and edited paper; M.A. contributed to the concept development and the experimental design and edited the manuscript.

**Competing Interests:** B.Z.C. and M.A. received research funding from AstraZeneca. J.C. and L.D. are employees of AstraZeneca.

**References**


**Figure Legends**

**Figure 1.** MCL-1 regulates cellular metabolic functions, and genetic or pharmacological inhibition of MCL-1 decreases mitochondrial respiration and metabolism in AML cells. A. Seahorse analysis of mitochondrial respiration in MCL-1-OE, MCL-1-KD, and AZD5991-treated (24 h) OCI-AML3 cells. For MCL-1-OE and –KD cells, bulk populations were used unless indicated otherwise. B. Cellular ROS (CellROS) and mitochondrial ROS (MitoSOX) in MCL-1-OE and MCL-1-KD OCI-AML3 cells. C. GSH and GSH/GSSG ratios in MCL-1-OE and MCL-1-KD OCI-AML3 cells. D-I: OCI-AML3 MCL-1-KD clone (shC16) with an approximately 65% MCL-1 reduction (Supplementary Figure S1D) and OCI-AML3 cells treated with 50 nM AZD5991 were used for metabolomics experiments. WT, untreated control. D. Relative enrichment of $^{13}$C from $^{13}$C$_2$-1,2-glucose or $^{13}$C$_5$-glutamine (M>0) into key TCA cycle intermediates. E. Fractional enrichment of $^{13}$C from $^{13}$C$_5$-glutamine for citrate, fumarate, and malate. F. Relative lactate levels in medium after 6 h of incubation with new medium. Lactate levels were determined by summing MS1 ion intensities for all isotopes in the $^{13}$C$_2$-1,2-glucose (left) and the $^{13}$C$_5$-glutamine tracer experiment (right). G. Fractional enrichment in extracellular lactate after 6 h of incubation in the $^{13}$C$_2$-1,2-glucose (left) and $^{13}$C$_5$-glutamine (right) tracer experiments. H and I. Relative oxPPP flux (left) and 6PG levels (right; H) and relative levels of unlabeled ATP and $^{13}$C enrichment into ATP (I). Average WT and shVec M+0 levels were set to a relative level of 1.

WT, parental OCI-AML3 cells; AZD5991, AZD5991-treated OCI-AML3 cells; shVec, vector control OCI-AML3 cells; shC16, MCL-1-KD OCI-AML3 cells. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. Experiments were performed in triplicates (for seahorse analysis: duplicates for each experiment). J. Schematic illustration of carbohydrate metabolic pathways decreased by MCL-1 inhibition. Cit, citrate; Fum, fumarate; Mal, malate; αKG, α-ketoglutarate; Oaa, oxaloacetic acid; 6PG, 6-phospho-gluconic acid.
Figure 2. MCL-1 regulates cell surface expression levels of CXCR4 and CD44 in AML cells and leukemia-stroma interactions. A and B. Cell surface CXCR4 and CD44 levels and migration and adhesion of vector control and MCL-1-OE OCI-AML3 cells (A) or vector control and MCL-1-KD OCI-AML3 cells (B) to MSCs. Blue bars represent the migration of OCI-AML3 cells to CXCL12 (positive control) or randomly (negative control). C and D. Cell surface CXCR4 and CD44 levels and migration and adhesion of OCI-AML3 cells treated with AZD5991 (C) or venetoclax (D) for 24 h to MSCs. Cell surface CXCR4 and CD44 and cellular MCL-1 levels were determined by flow cytometry. Migration was performed for 6 h and adhesion for 24 h. Experiments were performed in triplicates. Results are expressed as the mean±SEM. MFI, mean fluorescent intensity.

Figure 3. MCL-1 expression contributes to venetoclax resistance, and the combined inhibition of BCL-2 and MCL-1 synergistically induces apoptosis in AML cells with intrinsic or acquired resistance to venetoclax. A and B. Control (con) and MCL-1-KD OCI-AML3 cells (A) or control and MCL-1-OE Molm13 cells (B) were treated with venetoclax (VEN) or AZD5991 for 48 h. C-E. OCI-AML3 (C), MCL-1-OE Molm13 (D), and control parental MV4-11 cells (MV4-11P) or venetoclax-resistant MV4-11 cells (MV4-11R) (E) were treated with venetoclax and/or AZD5991 for the indicated times. Apoptosis and viable cell numbers were assessed by flow cytometry. Experiments were performed in triplicates. Results are expressed as the mean±SEM.

Figure 4. Mechanisms of synergy. Interactions of anti-apoptotic and pro-apoptotic BCL-2 proteins in control and BH3 mimetic-treated cells (A-D). OCI-AML3 cells were treated with venetoclax, AZD5991, or both (dose 1: venetoclax, 100 nM and AZD5991, 10 nM; dose 2: venetoclax, 250 nM and AZD5991, 25 nM) for 24 h. A-C. Interactions of BCL-2 (A), MCL-1 (B), or BCL-XL (C) with BIM, BAX, or BAK were determined by co-immunoprecipitation and western
blot analysis. D. Summary of the interactions. Roles of metabolic function and leukemia-stroma interactions (E, F). E. OCI-AML3 cells were treated with venetoclax alone or in combination with IACS-10759, BL-8040, or AZD5991 with or without MSC co-cultures for 48 h. Cell death in CD45⁺ cells were determined by flow cytometry. F. Migration (6 h) and adhesion (24 h) of BL-8040 treated-OCI-AML3 cells to MSCs were measured. Migration to CXCL12 was used as a positive control and random migration as a negative control. Experiments were performed in triplicates. Results are expressed as the mean±SEM.

Figure 5. Combined inhibition of BCL-2 and MCL-1 synergistically induces apoptosis in primary AML cells and AML stem/progenitor cells under MSC co-culture conditions. Primary AML cells co-cultured with MSCs were treated with venetoclax (VEN), AZD5991, AZD4573, VEN plus AZD5991, or VEN plus AZD4573 for 48 h. The apoptosis of blast cells and stem/progenitor cells were assessed by flow cytometry. A. EC₅₀ values of venetoclax alone or combined with AZD5991 or AZD4573, of AZD5991 alone or combined with venetoclax and of AZD4573 alone or combined with venetoclax in blast cells and stem/progenitor cells from individual patient samples are shown. Samples lacking the indicated cell populations or that had excessively high spontaneous apoptosis are not represented. *, >3000 nM. B. Mutations, venetoclax treatment status, and cytogenetics/risk category of each patient from whom primary AML samples were obtained. HR, complex cytogenetics and high risk; IR, intermediary risk.

Figure 6. Combined inhibition of BCL-2 and MCL-1 has strong anti-leukemia activity and prolongs survival in a PDX model of acquired venetoclax/Decitabine-resistant AML. A. PDX model and experimental scheme. B. Circulating human CD45⁺ (hCD45⁺) cells in mouse peripheral blood (PB) samples obtained from each treatment group on treatment d 18. C. hCD45⁺ cells in mouse BM samples obtained from each treatment group on treatment d 25. D. hCD45⁺ cells in mouse spleens and spleen sizes of each treatment group on treatment d 25.
hCD45+ cells were measured by flow cytometry. E. Survival curves. F. BM leukemia cell clusters based on the expression of cell surface markers and viable leukemic cells in various cell populations on treatment d 25 in each treatment group as determined by CyTOF. con, control; VEN, venetoclax; 5991, AZD5991; 4573, AZD4573 *. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001. Results are expressed as the mean±SEM. Survival data were analyzed using the log-rank test.

**Figure 7.** Protein levels in various leukemia cell populations in mouse BM as determined by CyTOF. BM cells were collected from the mice on 25 d of treatment (A-C) or from moribund mice (D-F) from each treatment group (n=2 or 3 mice/group). A and D. BCL-2, MCL-1, and c-MYC levels in various leukemia cell populations in each treatment group. B and E. Cell surface expression levels of CXCR4 and CD44 (B) or CXCR4 (E) in various cell populations in each treatment group. C. β-CATENIN and p-AKT levels in CD45+ and CD34+CD38−CD123+ cells. F. p-AKT levels in various cell populations. The protein levels of individual samples are presented as heat maps. After stained with antibodies against cell surface markers, all BM samples collected at d 25 treatment were barcoded, pooled into the same tube, stained, and run concomitantly and all BM samples collected from moribund mice were barcoded, pooled into the same tube, stained, and run concomitantly.

**Figure 8.** Effects of BAX and/or BAK knockdown on apoptosis, mitochondrial respiration, and migration/adhesion to MSCs of AML cells in responses to MCL-1 inhibition. OCI-AML3 cells were transfected by electroporation with control, Bax, Bak, or Bax and Bak siRNA (4 μM) for 48 h and then treated with AZD5991 (50 nM) for 24 h. A. BAX and BAK levels in siRNA-transfected OCI-AML3 cells at 48 h, determined by western blot. The left panel is the result of a representative experiment and the right is the quantitative results of three independent
experiments. B. Apoptosis by flow cytometry and mitochondrial respiration by Seahorse of siRNA-transfected OCI-AML3 cells treated with AZD5991 at various time points. C. Apoptosis and migration (6 h) and adhesion (24 h) of siRNA-transfected OCI-AML3 cells treated with AZD5991 for 24 h compared with untreated controls. The experiments were performed in triplicates (with duplicates for each experiment in the Seahorse experiment). The results are expressed as mean±SEM.
Supplemental Materials

Methods

**Cells.** AML cell lines Molm13 and MV4-11 were purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and American Type Culture Collection (Manassas, VA), respectively and OCI-AML3 was kindly provided by Dr. M. Minden (the Ontario Cancer Institute, Toronto, ON, Canada). AML patient samples (Supplementary Table 1) were obtained after acquiring written informed consent following MD Anderson Cancer Center IRB approved protocol and in accordance with the Declaration of Helsinki. Mononuclear cells were isolated from patient samples by density-gradient centrifugation using Lymphocyte Separation Medium (Corning; Manassas, VA).

**Western blot analysis and co-immunoprecipitation.** Antibodies against MCL-1 (Santa Cruz Biotechnology; Dallas, TX), and c-MYC (Cell Signaling Technology; Danvers, MA) were used for western blot analysis. For co-immunoprecipitation, BCL-2 (#15071s), MCL-1 (#94296), or BCL-XL (#2764) (all from Cell Signaling Technology) was pulled down with the respective antibody using the Dynabeads™ Protein G Immunoprecipitation Kit following the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA). IgG was used as a control. BCL-2 (#M0887, Dako/Agilent or #4223, Cell Signaling Technology), MCL-1 (#sc-12756, Santa Cruz Biotechnology), BCL-XL (#NBP1-28559, Novus Biologicals; Centennial, CO), BIM (#ab32158, Abcam; Cambridge, UK), BAX (#B8554, Sigma; St. Louis, MO), and BAK (#NBP1-28559, Novus Biologicals or #12105, Cell Signaling Technology) levels were determined by western blot analysis. β-ACTIN was used as a loading control.

**Protein determination by flow cytometry.** Cells were incubated with Fc Blocker (Miltenyi Biotec), stained with Ghost Dye Violate 510 (Tonbo Biosciences; San Diego, CA), then with PE-labeled anti-CD44 antibody or APC-labeled anti-CXCR4 antibody (both from BioLegend; San Diego, CA)
for 30 min. Cells stained with the anti-CXCR4 antibody were fixed with 4% paraformaldehyde/PBS, permeabilized with 100% methanol at 20°C overnight, and then stained with PE-labeled anti–MCL-1 antibody (Cell Signaling Technology) for 1 h. The stained cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter Life Science) and quantified using the FlowJo analytic platform (BD Bioscience). Protein levels were presented as mean fluorescence intensity differences between antibody-stained and IgG-stained cells.

$^{13}$C$_2$-1,2-glucose and $^{13}$C$_5$-glutamine tracing. MCL-1–KD or vector control OCI-AML3 cells and OCI-AML3 cells treated with AZD5991 (50 nM) or vehicle (DMSO) were cultured in a custom formulation of glucose- and glutamine-free RPMI-1640 medium (based on cat#SH30096, Hyclone, Logan, UT) containing 10% dialyzed fetal bovine serum (ThermoFisher Scientific) plus 10 mM $^{13}$C$_2$-1,2-glucose or 2 mM $^{13}$C$_5$-glutamine, respectively, for isotopic tracing. $^{13}$C$_2$-1,2-glucose and $^{13}$C$_5$-glutamine were purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Cells were seeded by adding 3 ml of cell suspension (0.8x10$^6$ cells/ml) to 6-cm-diameter plates. After a 6-h incubation for drug treatment and isotopic tracing, cells were isolated by centrifugation at 250 g for 2 min at 4°C, and washed with PBS. After the removal of PBS by centrifugation, polar metabolites were extracted by adding 500 μl of a 4:1 mix of LC-MS grade methanol (ThermoFisher Scientific) and water. For measuring extracellular lactate, 400 μl of methanol was added to 100 μl of media. Media and cell extracts were vortexed for 2 min using a Disruptor Genie (Scientific Industries Inc., Bohemia, NY). Proteins were removed by centrifugation at 20,000 g for 5 min at 0°C. The supernatants were dried and stored at -80°C until further processing.

Ion chromatography-mass spectrometry metabolite analysis. Samples were analyzed with ion chromatography-mass spectrometry (IC-MS) as described previously(1) to broadly assess
metabolites in cellular energetic pathways, including glycolysis, PPP, the TCA cycle, and nucleotide metabolism. Dried polar fractions were reconstituted in 50 μl of ultrapure water (Barnstead Nanopure Diamond, ThermoFisher Scientific), and 5 μl of the reconstituted fractions were subjected to IC-MS analysis with a Dionex ICS-5000+ system interfaced with an Orbitrap Fusion Tribrid mass spectrometer (both from ThermoFisher Scientific) operating at a resolution of 240,000 (FWHM at m/z 200) for MS1 acquisition (m/z range of 80–800). Peak areas were integrated using the TraceFinder 4.1 software package (ThermoFisher Scientific) and exported to a tabular format for further analysis. Peak areas and fractional enrichments were corrected for natural abundance using the ElemCor software tool (2). Replicates were normalized using summed intensity across all metabolites and adjusted for the amount of cells during cell harvest. Differences in metabolites between AZD5991-treated or MCL-1–KD cells and their respective control cells were assessed with a two-tailed equal variance Student t-test in Excel 2013 (Microsoft, Redmond, WA). Relative PPP flux was calculated as the lactate secretion rate \( \times \frac{[M+1 \text{ lactate}]}{([M+2 \text{ lactate}]+[M+1 \text{ lactate}])} \), where \([M+1 \text{ lactate}]\) and \([M+2 \text{ lactate}]\) are fractional \(^{13}\text{C}\) enrichments in secreted lactate and the lactate secretion rate is the total cell normalized lactate ion intensity after 6 h of cell culture. Flux was normalized to that of control cells (i.e. untreated parental cells or vector control cells) by setting the control average as 1.

**In vivo experiments.** Mouse care and mouse experiments were performed in accordance with protocols approved by MD Anderson Cancer Center Animal Care and Use Committee. For the AML xenograft model, MCL-1–OE and vector control Molm13 cells (0.4x10^6 cells/mouse, female) or MCL-1–KD and vector control OCI-AML3 cells (1x10^6 cells/mouse, male) were injected via tail vein into 10- to 12-wk-old NSGS mice (n=5/group; The Jackson Laboratory, Bar Harbor, ME). The PDX model was developed in NSGS mice using a BM sample (73% blasts) from an AML patient (Supplementary Table 1) whose disease initially responded to venetoclax plus...
decitabine but relapsed after 3 cycles of therapy. The PDX cells (0.5x10^6 cells/mouse) collected from the mouse spleen (>90% human CD45+) were injected into 6- to 8-wk-old male NSGS mice (The Jackson Laboratory). After engraftment, the mice (10 mice/group) were treated for 6 wk with vehicle; venetoclax (50 mg/kg daily by gavage); AZD5991 (60 mg/kg, once/wk by intravenous injection); AZD4573 (15 mg/kg twice daily by intraperitoneal injections [2 h apart], for 2 consecutive d/wk); venetoclax+AZD5991; and venetoclax+AZD4573. In the cases of drug combinations, venetoclax was given first. Leukemia engraftment and progression were assessed by flow cytometric measurement of human CD45+ cells in the peripheral blood, BM, or spleen. BM cells were collected for CyTOF analyses. Survival was followed.
**Supplementary Table 1. Patient Characteristics**

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<td>75</td>
<td>Relapsed AML. Subsequently was treated with venetoclax. Responded, then relapsed</td>
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<td>90</td>
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<td>Newly diagnosed. Subsequently was treated with venetoclax+decitabine and responded</td>
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**Supplementary Table 2. Antibody Panel for CyTOF Analysis**

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**Supplementary Table 3.** CI Values of Combination Treatment of Venetoclax with AZD5991 or AZD4573 in Various Cell populations of AML Patient Samples. VEN, venetoclax.

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**Supplementary Figure S1.** MCL-1 regulates metabolic activity and affects c-MYC levels in AML cells. A. Spontaneous apoptosis of MCL-1–OE and –KD AML cells. MCL-1 levels were determined by Western blot. B. Survival curves of NSGS mice harboring vector control or MCL-1–OE Molm13 cells (left) or vector control or MCL-1–KD OCI-AML3 cells (right) (n=5/group). Survival data were analyzed using the log-rank test. C. Seahorse analysis of mitochondrial respiration in parental MV4-11 cells (MV4-11P) and venetoclax-resistant MV4-11 cells (MV4-11R) untreated or treated with AZD5991 (5991). All Seahorse experiments were performed in triplicates, with duplicates for each experiment. The results are expressed as mean±SEM of the 6 repeats. D. Western blot analysis of MCL-1 expression in control (shVec) and MCL-1–KD (shC16) OCI-AML3 cells. E. Viability of OCI-AML3 cells used in the $^{13}$C$_2$-1,2-glucose and $^{13}$C$_5$-glutamine tracing experiments. F. Representative Western blotting (left) and quantitative analysis (n=5) of c-MYC levels in MCL-1–OE and MCL-1–KD OCI-AML3 cells. Results are expressed as the mean±SEM.
Supplementary Figure S2. MCL-1 expression contributes to venetoclax resistance, and BCL-2 inhibition with venetoclax plus MCL-1 inhibition with AZD5991 or AZD4573 synergistically induces apoptosis in AML cell lines with intrinsic or acquired resistance to venetoclax. A. EC$_{50}$ and IC$_{50}$ values of venetoclax (VEN) treatment for 48 h in leukemia cell lines. B. MCL-1–OE and control MV4-11 cells were treated with venetoclax or AZD5991 for 48 h. C. MCL-1–OE MV4-11 cells were treated with venetoclax and/or AZD5991 for 48 h. D–F. OCI-AML3 cells (D), control parental MV4-11 cells (MV4-11P) and venetoclax-resistant MV4-11 cells (MV4-11R) (E), and vector controls and MCL-1–OE Molm13 and MV4-11 cells (F) cells were treated with AZD4573 or with venetoclax and/or AZD4573 for the indicated times. For the AZD4573 treatments, cells were treated for 6 h, and then the drug was washed out. Cells were continuously cultured until they were analyzed. Apoptosis and viable cell counts were assessed by flow cytometry. Protein levels were measured by Western blotting. con, control; VEN, venetoclax. All experiments were performed in triplicates. Results are expressed as the mean±SEM.
Supplementary Figure S3. Combined inhibition of BCL-2 and MCL-1 synergistically decreased cell viability in primary AML cells and AML stem/progenitor cells under MSC co-culture conditions. Primary AML cells co-cultured with MSCs were treated with venetoclax, AZD5991, AZD4573, venetoclax+A AZD5991, or venetoclax+AZD4573 for 48 h. The viable cell numbers of blast cells and stem/progenitor cells were assessed by flow cytometry. IC50 values of venetoclax
alone or combined with AZD5991 or AZD4573, of AZD5991 alone or combined with venetoclax, and of AZD4573 alone or combined with venetoclax in blast cells and stem/progenitor cells from individual patient samples are shown. Samples lacking the indicated cell populations or that had excessively high spontaneous apoptosis are not represented.

Supplementary Figure 4

A

B
Supplementary Figure S4. Combined inhibition of BCL-2 and MCL-1 synergistically induces apoptosis and decreases viable cells in primary AML cells and AML stem/progenitor cells. Primary AML cells were treated with venetoclax (VEN), AZD5991, AZD4573, venetoclax+AZD5991, or venetoclax+AZD4573 for 48 h. The apoptosis and viable cell numbers of blast cells and stem/progenitor cells were determined by flow cytometry. EC$_{50}$ (A) and IC$_{50}$ (B) values of venetoclax alone or combined with AZD5991 or AZD4573, of AZD5991 alone or combined with venetoclax, and of AZD4573 alone or combined with venetoclax in blast cells and stem/progenitor cells from individual patient samples are shown. VEN, venetoclax; *, >3000 nM. Samples lacking the indicated cell populations or that had excessively high spontaneous apoptosis are not represented.

Supplementary Figure S5. Body weight of the PDX cell-bearing mice during treatments with venetoclax (VEN), AZD5991, AZD4573, and the combinations.
### Supplementary Figure 6

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**Supplementary Figure S6.** The expression of signaling proteins in various leukemia cell populations isolated from mouse BM as determined by CyTOF. A. Cells from BM samples collected on treatment d 25. B. Cells from BM samples of moribund mice. After stained with antibodies against cell surface markers, all BM samples collected at d 25 treatment were barcoded, pooled into the same tube, stained, and run concomitantly and all BM samples collected from moribund mice were barcoded, pooled into the same tube, stained, and run concomitantly.
Supplementary Figure S7. Inhibition of MCL-1, but not BCL-2 suppresses cellular and mitochondrial ROS generation, the expression of stromal microenvironmental factors, and migration and adhesion of AML cells to MSCs in primary AML samples. A. Primary AML patient
Peripheral blood samples (n = 4) were treated with venetoclax (2.5 nM) or AZD5991 (12.5 nM) for 24 h. Apoptosis in CD45+ cells were determined by flow cytometry. B. Cellular and mitochondrial ROS of live cells (CD45+AnnV-/DAPI-) and C. Cell surface expression of CXCR4 and CD44 were determined by flow cytometry and compared with untreated cells. D. Migration and adhesion of AML patient cells to MSCs were determined 24 h after BH3 mimetic-treated cells were incubated with MSCs and compared with untreated cells.

References
