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Venetoclax with Azacitidine Disrupts Energy Metabolism and Targets Leukemia Stem Cells in Acute Myeloid Leukemia Patients

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Acute myeloid leukemia (AML) is the most common acute leukemia in adults. Leukemia stem cells (LSCs) drive the initiation and perpetuation of AML, are quantifiably associated with worse clinical outcomes, and often persist after conventional chemotherapy resulting in relapse[1–5]. In this report, we show that treatment of older AML patients with the B-cell lymphoma 2 (BCL-2) inhibitor venetoclax in combination with azacitidine results in deep and durable remissions and is superior to conventional treatments. We hypothesized that these promising clinical results were due to targeting LSCs. Analysis of LSCs from patients undergoing treatment with venetoclax + azacitidine showed disruption of the TCA cycle manifested by decreased alpha-ketoglutarate and increased succinate levels, suggesting inhibition of electron transport chain complex II. In vitro modeling confirmed inhibition of complex II via reduced glutathionylation of succinate dehydrogenase. These metabolic perturbations suppress oxidative phosphorylation (OXPHOS), which efficiently and selectively targets LSCs. Our findings show for the first time that a therapeutic intervention can eradicate LSCs in AML patients by disrupting the metabolic machinery driving energy

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Author Contributions

D.A.P., B.M.S., C.L.J., A.W., M.M., R.C.-H. designed and performed the research; collected, analyzed, and interpreted the data; performed the statistical analysis; and wrote the manuscript. S.P., A.D., K.A.R., A.E.G., J.R.H., D.A., and D.S. analyzed and interpreted data, performed statistical analysis, and wrote the manuscript. J.A.G., E.P., C.S. designed the research and wrote the manuscript. C.T.J. designed and directed the research, analyzed and interpreted data, and wrote the manuscript.

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Competing interests Statement

The authors declare no competing interests.

metabolism, resulting in promising clinical activity in a patient population with historically poor outcomes.

BCL-2 is an anti-apoptotic member of the BCL-2 family of proteins[6]; it is overexpressed in LSCs, and pre-clinical studies suggest targeting BCL-2 can result in the preferential elimination of this population[7–10]. Our previous work demonstrated LSCs to be uniquely dependent on OXPHOS; BCL-2 inhibition impedes OXPHOS[7], suggesting BCL-2 inhibition may target LSCs in AML patients. Venetoclax is an orally bioavailable BH3 mimetic protein that selectively inhibits BCL-2[11]. Pre-clinical testing showed activity in AML, as did a single-agent clinical trial in relapsed or refractory patients[12,13]. This prompted a dose escalation study of venetoclax with a hypomethylator backbone of azacitidine or decitabine for newly diagnosed older AML patients ()[14], which revealed it to be an active, well-tolerated therapy with an overall response rate of 78%, median response duration of 11 months and overall survival (OS) of 15 months[14]. In the present study, we explored the LSC-directed mechanism of venetoclax + azacitidine in newly diagnosed older AML patients using clinical outcomes and pre/post-treatment samples from 33 patients from our institution enrolled on the clinical trial[14].

Baseline characteristics of our patients are summarized in Table 1 and supplemental Table 1. The overall response rate (complete remission [CR] + CR with incomplete hematological recovery [CRi] + partial response [PR] + morphologic leukemia free state [MLFS]) was 91% (30/33). Twenty (61%) had a CR, eight (24%) had a CRi and one each had a PR and MLFS. Median time to response was 34.5 days (25–62), and 19 patients (58%) achieved their best response after the first cycle. Three had no response; one was refractory after two cycles, and two discontinued the study before the first week of treatment completed for personal reasons. This regimen was well tolerated; the toxicity profile was consistent with what has been previously reported[14].

Nine responders (30%) relapsed; three relapsed after discontinuing therapy for reasons unrelated to toxicity, 44–165 days after discontinuation. Six relapsed while on therapy, 168–420 days after first response. Median follow up time is 580 days (95% CI 377, 713). Median response duration, progression free survival (PFS) and OS have not been reached (Figure 1a–c).

We compared these outcomes to control patients treated at our institution. For the control population, 285 AML newly-diagnosed patients consecutively treated with any therapy besides venetoclax + azacitidine at our institution over a seven-year period were screened; we selected those >59 years with intermediate to adverse cytogenetics (N=88; Supplemental Table 2). The CR/CRi rate for controls was 51%, inferior compared to the 85% that achieved CR/CRi with venetoclax + azacitidine (Z score=−3.102, p=0.0019). In addition, the median OS for venetoclax + azacitidine was superior to controls (not reached vs 341.5 days, p=0.0003, Figure 1d). See Supplemental Figure 1 for additional analyses stratified by treatment and age.

Univariate logistic regression did not reveal any relevant variables as response predictors (Supplemental Table 3). Prior to initiation of therapy, patient specimens were genotyped

using the TruSight myeloid panel (Supplemental Table 4) to assess patient-specific mutations. Of patients who completed more than one week of therapy, 3/4 with *FLT3* internal tandem duplications, 2/2 with *TP53* mutations and 5/5 with monosomal karyotypes experienced a CR/CRi. BH3 profiling of seven patients showed no correlation between response and degree of BCL-2 priming (Supplemental Figure 2). *Isocitrate dehydrogenase* (*IDH*) isoform 1 or 2 predicted longer responses (hazard ratio= 0.119 [0.015, 0.923], p=0.042), while *PTPN11* and other *RAS* pathway mutations predicted shorter responses (hazard ratio= 10.22 [2.36, 43.48]; p=0.0019).

Measurable residual disease (MRD) was evaluated using droplet digital PCR (ddPCR) in 22 responders for whom detectable mutations were available (Figure 1e; see Supplemental Figure 3 for baseline mutational profiles). Of the nine patients who relapsed, seven were analyzable for MRD by ddPCR; 0/7 had achieved MRD negativity. Eleven patients are in sustained remission with MRD positivity. Four patients achieved MRD negativity, defined as a lack of detectable evidence of all analyzed mutations; 0/4 have relapsed with a median follow up time of 823 days (623–1093), and two (patients 1 and 2), who opted to come off all treatment after achieving a remission after 1 and 3 cycles, remain MRD negative with no clinical signs of disease after 983 and 1093 days, respectively.

We examined peripheral blood from patients with circulating disease to investigate cellular events that accompanied responses to venetoclax + azacitidine. We noted rapid decreases in peripheral blood blasts in patients with circulating disease, with significant reduction in as little as 24 hours and complete eradication in all patients within six days of treatment (Figure 2a and Supplemental Table 5). Selected pre- and post-treatment patient samples were then interrogated using mass cytometry. As shown in Figure 2b and supplemental Figure 4a, the effects of treatment were highly selective for the phenotypically-defined AML blast cell population, with a dramatic and rapid reduction in tumor burden, consistent with the clinical experience with this regimen. Importantly, examination of phenotypically-defined LSCs (CD34+, CD38-, Lin-, CD123+) also demonstrated rapid eradication (Figure 2c). By comparison, analysis of patients undergoing treatment with standard induction chemotherapy showed strikingly different results, with no significant reduction in blasts during the first four days of treatment; this was observed regardless of whether patients ultimately responded to this therapy (supplemental Figure 4b). The venetoclax + azacitidine regimen also showed high specificity for malignant versus non-malignant cells. As shown in supplemental Figure 4c, no reduction in normal hematopoietic lineages CD3+, CD7+, or CD19+ subpopulations were evident after four days of therapy with this regimen.

To further investigate the molecular characteristics of cells targeted during the initial stages of treatment, we performed single cell RNA-seq studies at baseline and after two and four days of therapy (Figure 2d–e). In this analysis, various cell populations were identified by expression of lineage specific transcripts (see supplemental Figure 5 for annotation). As observed for the immunophenotypic analysis in Figure 2, we saw no changes in normal hematopoietic cells, while cells identified as blasts were rapidly depleted. Gene set enrichment analysis (GSEA) comparing global expression changes between leukemic and normal cells pre- and post-treatment demonstrated the population eradicated by venetoclax + azacitidine strongly expressed molecular signatures identified in primary human LSCs

(Figure 2f)[15,16]. These data, combined with the phenotypic findings (Figure 2c), suggest targeting of primitive AML populations is preferentially achieved with venetoclax + azacitidine.

We have previously reported that ROS-low AML cells are highly enriched in functionally-defined LSCs[7,17] and because ROS labeling is based on metabolic properties and not immunophenotypic markers, this approach circumvents the antigenic heterogeneity commonly observed in primary human AML specimens[18–20] (see supplemental Figure 6 for ROS-low isolation method). Notably, RNA-seq analysis of ROS-low specimens isolated six hours after the initiation of treatment with venetoclax + azacitidine showed that pathways related to OXPHOS were strongly down-regulated (Supplemental Figure 7), a significant finding as it is known that OXPHOS is a critical pathway for LSC maintenance and survival[7].

Preclinical studies have previously shown that BCL-2 inhibition decreases OXPHOS, leading to death of LSCs but not normal hematopoietic stem cells, as normal stem cells compensate for reduced OXPHOS through increased glycolysis[7]. Based on our clinical findings of frequent, deep and durable responses with venetoclax + azacitidine, we hypothesized that this regimen targets LSCs in patients by decreasing OXPHOS. We tested this hypothesis by performing metabolomic analyses on tumor specimens obtained from patients pre- and 24 hours post-treatment with venetoclax + azacitidine. Analysis of ROS-low cells showed that patient 7 had a significant decrease in basal OXPHOS post treatment (Figure 3a), and that patient 9 had a significant decrease in OXPHOS spare capacity post treatment (Figure 3b and supplemental Figure 8a). Importantly, decreased OXPHOS observed upon treatment with venetoclax + azacitidine was not observed in patients treated with standard induction chemotherapy (“7+3”) (Supplemental Figure 8b). Furthermore, two of three patients receiving azacitidine alone did not have alterations in OXPHOS levels upon azacitidine treatment (Supplemental Figure 8c).

Next, we confirmed these results *in vitro* by treating LSCs isolated from patients for four hours with venetoclax + azacitidine. We found a reduction in OXPHOS in 4/4 AML patient samples tested (Figure 3c and supplemental Table 6). Notably, venetoclax + azacitidine did not significantly reduce OXPHOS in ROS-high AML blasts (Supplemental Figure 8d). The expected consequence of decreased OXPHOS in LSCs is reduced ATP levels, which was indeed detected in treated specimens (Figure 3d). Overall, these data are consistent with our preclinical findings and demonstrate venetoclax + azacitidine decreases OXPHOS and energy production in AML patients.

To determine how venetoclax + azacitidine decreases OXPHOS we measured changes in metabolite abundance pre- and 24 hours post-treatment. Of particular interest were TCA cycle intermediates, as these metabolites supply substrate for OXPHOS directly through electron transport chain (ETC) complex II. As shown in Figure 3e, LSCs isolated from three patients displayed striking decreases in α-ketoglutarate, malate, and fumarate, and an increase in succinate, 24 hours after treatment. Succinate is the metabolic substrate for ETC complex II, and increased succinate levels indicate a defect in ETC complex II activity. We also observed a significant decrease in glutathione levels in 3 of 3 patients post-treatment

(Figure 3f), an event that correlates with increased cellular ROS (Figure 3g). Interestingly, patients receiving “7+3” also had increased levels of ROS (supplemental Figure 8e) suggesting that elevated ROS alone is not sufficient to target LSCs in patients. Glutathione has multiple crucial cellular functions, including regulation of the activity of succinate dehydrogenase A (sdhA)[21], a component of ETC complex II. Glutathionylation, a post translation modification, has been shown to increase ETC complex II activity[21]. Therefore, we hypothesized that venetoclax + azacitidine results in decreased glutathione and therefore reduced sdhA glutathionylation, events that would diminish ETC complex II activity and inhibit OXPHOS. To test this hypothesis, we directly measured ETC complex II activity and sdhA glutathionylation in primary AML specimens upon venetoclax + azacitidine treatment *in vitro*. In addition, we assessed the importance of glutathione in ETC complex II activity, as well as OXPHOS. First, we confirmed our *in vivo* observation that venetoclax + azacitidine decreases glutathione levels *in vitro* (Figure 4a). Next, we showed decreased glutathionylation of sdhA after treatment with venetoclax + azacitidine (Figure 4b), and then showed that this correlated with reduced ETC complex II activity (Figure 4c). Furthermore, pretreatment of leukemic cells with cell permeable glutathione increased intracellular glutathione even in the presence of venetoclax + azacitidine (Figure 4d). Cell permeable glutathione also induced a partial rescue of ETC complex II activity upon venetoclax + azacitidine treatment (Figure 4c). We next measured succinate levels and observed that upon treatment with venetoclax + azacitidine, intracellular succinate was elevated (Figure 4e), consistent with a loss of glutathione. Pretreatment with cell permeable glutathione completely restored succinate to normal levels demonstrating a reciprocal relationship between glutathione and succinate in cells treated with venetoclax + azacitidine. These data suggest decreased glutathione levels from venetoclax + azacitidine are at least partially responsible for reduced ETC complex II activity.

To determine the impact of glutathione reduction on OXPHOS, we measured OXPHOS in ROS-low LSCs after pretreatment with cell permeable glutathione followed by venetoclax + azacitidine. As shown in Figure 4c, venetoclax + azacitidine decreased OXPHOS in LSCs. However, pretreatment with cell permeable glutathione partially rescued OXPHOS levels upon venetoclax + azacitidine treatment (Figure 4f). Furthermore, pretreatment with cell permeable glutathione partially rescued the reduction in ATP levels after venetoclax + azacitidine treatment in LSCs (Figure 4g). Finally, we determined the functional role of decreased glutathione on LSC viability by showing that cell permeable glutathione pretreatment partially rescued cell viability upon venetoclax + azacitidine treatment (Figure 4h). Interestingly, the decreases in sdhA glutathionylation, ETC complex II activity, OXPHOS, and ATP levels only occurred upon treatment with both venetoclax + azacitidine, and not with either agent individually (Supplemental Figure 9 a–f). Furthermore, venetoclax + azacitidine resulted in synergistic cell killing in 5 of 6 primary AML specimens tested (Supplemental Figure 9g)[22,23]. However, venetoclax or azacitidine alone can increase ROS levels and the ROS levels induced by venetoclax + azacitidine are not significantly higher than that seen with the individual agents (Supplemental Figure 9h). Overall, these findings support the hypothesis that the combination of venetoclax + azacitidine decreases OXPHOS via disruption of ETC complex II in a glutathione-dependent fashion, resulting in the selective targeting of the LSC population.

To date, treatment options for older patients with AML have been limited. Single agent azacitidine in untreated older patients results in a CR/CRi rate of 28% with a response duration of 10 months[24]. Venetoclax + azacitidine showed greater activity with more durable responses; our single institution data were comparable to the clinical results of the larger study in which we participated[14], and when compared to a single-institution internal control population, were superior with respect to response rates and OS to all other therapies.

Durability is related to the depth of response, which can be measured by MRD[25]. Using ddPCR we showed MRD negativity can be achieved with a non-intensive regimen and correlates with sustained remissions. Larger numbers and longer follow up will be needed to fully recognize the clinical significance of responses of this depth, and to determine baseline characteristics that may predict MRD negative responses.

We previously hypothesized that an effective LSC-directed therapy should result in deep remissions and have curative potential[26,27]; two patients described here have achieved sustained MRD negative responses off all therapy for nearly three years, and others who remain on therapy have achieved similar response depth and durability, suggesting this regimen may be curative in some settings. However, while nearly all patients responded, some progressed. Consistent with prior pre-clinical and clinical observations[10,13], we report patients with *IDH* mutations had longer remission durations. Additionally, we report here that *RAS* pathway mutations predicted shorter remissions, which may help elucidate mechanisms of resistance to this regimen. We previously predicted that LSC-directed treatment may be effective irrespective of traditional biological risk factors[26,27]. This concept is supported by the results of the present study, in which no traditional adverse disease features associated with lack of response to the venetoclax + azacitidine regimen. Lastly, LSC-directed therapies may be expected to be more effective in the up-front treatment setting, based on the finding that LSCs are substantially fewer and more homogenous prior to relapse[20].

Perturbing the metabolic environment to target cancer has been an appealing strategy since it was first recognized that some cancer cells favor glycolysis over oxidation, known as the Warburg effect[28]. It has recently been reported in preclinical models that a “reverse Warburg” effect, involving reliance on OXPHOS for metabolism, is observed in several cancer stem cell subtypes, including LSCs[7,29,30]. Here we show that disruption of energy metabolism can be a therapeutic strategy for AML. Specifically, reduction in OXPHOS resulting in targeting of LSCs was achieved through reduced ETC complex II activity and induction of ROS within 24 hours of treatment with venetoclax + azacitidine. *In vitro* modeling suggests that decreased glutathione, which reduces ETC complex II activity, is at least partially responsible for the reduced OXPHOS and LSC targeting. Importantly, the combination of venetoclax + azacitidine was needed to decrease glutathione levels, suggesting future venetoclax-containing treatment regimens should continue to include azacitidine. We note that contrary to our previous in vitro studies[7], the experiments presented in the current study show that venetoclax alone is not sufficient to mediate OXPHOS inhibition in vivo. Rather, the combination of venetoclax + azacitidine is necessary to down-regulate OXPHOS (Supplemental Figure 9e), which perhaps at least partially explains the efficacy of the drug combination in patients. These findings

complement and extend several recent preclinical studies that demonstrate an important role for mitochondrial metabolism in LSC survival[7,10,31–33].

Although we believe the depth and durability of responses seen here is due to selective LSC targeting, the ability of this regimen to eradicate bulk tumor is impressive, with rapid reduction of peripheral blasts and a high remission rate observed after the first cycle of therapy. This would be an unexpected finding for a pure LSC-directed mechanism and is likely due to the well-described promotion of apoptosis via targeting BCL-2[12,34]. While our de novo specimens did not consistently show a high level of BH3 priming, we speculate that azacitidine may induce a physiological state that makes AML cells more susceptible to BCL-2 inhibition[35,36].

In conclusion, venetoclax + azacitidine is highly active in older, previously untreated patients with AML; responses are deep and durable, and outcomes are superior compared to historical controls. Mechanistic experiments done with specimens obtained from patients early during the course of therapy indicate that the LSC compartment can be effectively eradicated by targeting LSC-specific metabolic properties.

Methods

Trial design:

The dose escalation and expansion clinical trial () was conducted in compliance with the Declaration of Helsinki and was approved by all relevant institutional review boards; all patients gave written informed consent. The regimen, described in detail elsewhere, was comprised of azacitidine 75 mg/m^2 on days 1–7 of each 28-day cycle, with concomitant daily venetoclax taken orally for the entire cycle; cycles could be repeated indefinitely in the absence of toxicity or disease progression[1]. During dose escalation patients were assigned to receive 400, 800 or 1200 mg venetoclax; during dose expansion, all patients received 400 mg. Eligible patients were ≥60 with newly diagnosed AML without favorable cytogenetics[2], had received no prior treatment, and had adequate organ function. Response assessments were made according to the International Working Group (IWG) for AML[3]. Patients treated at our institution who achieved a MLFS or CRi had their response upgraded to a CRi or CR if a break in therapy and/or the use of growth factor support allowed for count recovery within 14 days of the bone marrow biopsy, without any other intervening treatment.

Clinical statistics:

Median follow up was calculated using the reverse Kaplan-Meier method. OS and PFS were calculated from the date of study entry to the date of death from any cause (OS) or the date of relapse or death (PFS). Response duration was calculated from the date of initial response to relapse. Living patients who had not progressed on or before February 28, 2018 were censored. Median OS was calculated using Kaplan-Meier product-limit estimates. Univariate logistic regression analysis was used to assess the effects of covariates on response status; p-values are two-sided, and a p-value threshold of 0.05 was used to indicate significance. Response rates between venetoclax + azacitidine and control cohorts were compared using the Z-score in a difference in proportions test, and OS between these groups was compared

using a Mantel-Cox logrank test. Analyses were performed using SAS version 9.4 (SAS Institute).

Next generation mutational sequencing:

Mutational analysis using a targeted sequencing panel of 49 AML-associated genes was performed on diagnostic bone marrow from all but one patient (Supplemental Table 3); in most cases, germline DNA extracted from fingernails was analyzed to ensure the somatic origin of detected mutations. Sequencing was done using the Raindance Myeloid Thunderbolt panel per the standard protocol. Briefly, genomic DNA extracted from the specimens was used for multiplex PCR amplification of 548 amplicons that target mutation hot spots in 49 genes recurrently mutated in AML. Next generation sequencing (NGS) was performed on Illumina MiSeq and analyzed with SoftGenetics NextGENe data analysis software. The UCSC hg19 and GRCh 37 reference genome were used as the reference sequence. As many disease-associated mutations as possible from the maximum number of patients were selected for subsequent MRD analysis using ddPCR (Supplemental Table 6) and quantified at various time points during therapy. The limit of detection was 0.02–0.05%.

Human Specimens:

AML specimens were obtained from bone marrow and peripheral blood of patients who gave informed consent for tissue banking at the University of Colorado (COMIRB #12–0173 and 06–0720). Total mononuclear cells were isolated from specimens by standard Ficoll procedures as previously done[4] (GE Healthcare).

Cell culture and media:

Primary cells were cultured as previously done in Pei et. al.[5]. Primary human AML cells were resuspended at about 100–200 e^6 cells per ml in freezing media composed of 50% FBS (GE Healthcare), 10% DMSO (Sigma) and 40% IMDM media (Gibco) and then cryo-preserved in liquid nitrogen. Cells were thawed in 37°C water bath, washed twice in thawing media composed of IMDM (Gibco), 2.5% FBS (GE Healthcare) and 10 ug/ml DNAse (Sigma). Cells were cultured in serum-free media (SFM) in 37°C, 5% CO_2 incubator. SFM is composed of IMDM (Gibco), 20% BIT 9500 (STEMCELL Technologies), 10ug/ml LDL (Low Density Lipoprotein, Millipore), 55uM 2-Mercaptoethanol (Gibco) and 1% Pen/Strep (Gibco). Complete SFM were made by supplementing the normal SFM with FLT-3, IL-3 and SCF cytokines (PeproTech), each at 10 ng/ml.

Droplet digital PCR Methods:

Bone marrow or peripheral blood samples were either lysed whole or Ficoll-separated and the mononuclear cell population lysed for DNA. Genomic DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen), and quantity and purity were measured via Nanodrop. DdPCR assays for AML-associated mutations were designed either by our lab or obtained pre-designed from RainDance Technologies (Supplemental Table 7). These assays utilize common forward and reverse primers which span a single nucleotide polymorphism of interest, as well as competitive TaqMan probes for wild-type and mutant alleles (labeled with VIC or FAM fluorophores, respectively). All assays were validated via quantitative

PCR as well as ddPCR to ensure optimal annealing temperatures and minimal false positive reads, using no template controls and known wild-type DNA samples for reference. 100 ng of DNA was added to a PCR master mix containing gene-specific primers and probes, droplet stabilizer, and ABI TaqMan Genotyping Master Mix (catalog number 4371355). PCR samples were loaded onto a RainDance Source chip, which was then inserted into the RainDance Source instrument to generate picoliter-size droplets containing individual molecules of DNA template. Dropletized samples were subjected to standard PCR using assay-optimized annealing temperatures. PCR-amplified samples were then placed into a droplet reader (RainDance Sense instrument) which counts individual droplets as "negative", "FAM-positive," or "VIC-positive." Data was generated as .fcs files, which were then analyzed with RainDrop Analyst II software. Variant allele frequencies (VAFs) are expressed as the percentage of mutant (FAM)-positive droplets relative to the total number of positive droplets (FAM + VIC). In some cases, VAF's determined via the RainDance ddPCR platform were confirmed using an alternative ddPCR platform, the BioRad QX200.

Mass cytometry:

These samples were processed and stained as described in Amir *et al.*[6]. Briefly, frozen aliquots of cells were thawed in 98% IMDM + 2% FBS + DNase I. Thawed cell pellets were immediately re-suspended in PBS containing 5 μM Cell-ID Cisplatin (Fluidigm) and incubated for 5 minutes at room temperature. Cells were washed one time with cell staining buffer and fixed using 2% formaldehyde for 10 minutes at room temperature. Fixed cells were washed with cell staining media (low barium PBS + 0.5% bovine serum albumin + 0.02% sodium azide) and incubated for 30 minutes at room temperature in surface epitope metal conjugated antibodies (see Supplemental Table 8 for panel of reagents). Surface stained cells were washed twice and re-suspended in cold methanol for 30 minutes at 4°C. Methanol permeabilized cells were washed one time and re-suspended in intracellular epitope metal conjugated antibody mix) for 1 hour at room temperature. Stained cells were washed once before incubation in 0.5 μM Cell-ID Intercalator-Ir (Fluidigm). Fixed and stained cells were washed two times in cell staining media and re-suspended at 2.5–5 $\times 10^5$ /ml in water before being run on CyTOF instrument. Data was analyzed utilizing the viSNE algorithm which was used to visualize and interpret data[7].

Single cell RNA-seq:

Single cell RNA sequencing data was initially processed by the Cell Ranger pipeline (v2.0.1) from 10x Genomics. The generated count matrices were then analyzed using the Seurat package (v2.2.1) in R. Read counts were normalized to library size, scaled by 10,000, log-transformed, and filtered based on the following criteria: cells with less than 250 genes detected or with a proportion of UMIs mapped to mitochondrial genes over 0.15 were excluded from analysis; genes detected in less than 5 cells were also excluded. Cell-to-cell variation in gene expression driven by the number of detected molecules and mitochondrial gene expression was regressed out using linear regression. Dimensionality reduction was performed with PCA followed by t-SNE projection and graph-based clustering using the first 15 principal components. Data is available in GEO (accession number: GSE116481).

RNA-seq:

The TruSeq RNA Sample Preparation Kit V2 (Illumina) was used for next generation sequencing library construction per the manufacturer's protocols. Amplicons are ~200–500 bp in size. The amplified libraries were hybridized to the Illumina single end flow cell and amplified using the cBot (Illumina). Single end reads of 100 nucleotides were generated for each sample and aligned to the organism specific reference genome. Raw reads generated from the Illumina HiSeq2500 sequencer were de-multiplexed using configurebcl2fastq.pl version 1.8.4. Quality filtering and adapter removal was performed using Trimmomatic version 0.32 with the following parameters: "SLIDINGWINDOW:4:20 TRAILING:13 LEADING:13 ILLUMINACLIP:adapters.fasta:2:30:10 MINLEN:15". Processed/cleaned reads were then mapped to the UCSC hg19 genome build with SHRiMP version 2.2.3 with the following setting: "–qv-offset 33 -all-contigs. Data is available in GEO (accession number: GSE116567).

Global Metabolomics:

Metabolomic analyses were performed via ultra-high pressure-liquid chromatography-mass spectrometry (UHPLC-MS – Vanquish and Q Exactive, Thermo Fisher, Bremen, Germany) as previously reported[8], and quantification of compounds of interest was performed against ^{13}C or D stable isotope labeled standards (Sigma Aldrich, St. Louis, MO and Cambridge Isotopes), as described[8]. A detailed description of this method is included in the supplemental materials.

Cell Sorting:

Primary AML specimens were thawed, stained with CD45 (BD, 571875) to identify the blast population, CD19 (BD, 555413) and CD3 (BD, 557749) to exclude the lymphocyte populations, DAPI (EMD Millipore, 278298), and CellROX deep red (Thermo Fisher, C10422), and sorted using a BD FACSARIA. ROS-low LSCs were identified as the cells with the 20% lowest ROS levels and the ROS-high blasts were identified as the cells with the highest 20% ROS levels, as previously described[9].

Viability Assays:

Patient samples were sorted and cultured without indicated metabolites or drugs for 24–72 hours. Viability was assessed by trypan blue (Gibco, 15250–071) staining and manual cell counting.

Seahorse Assays:

XF24 (Agilent Technologies, 100850–001) or XF96 (Agilent Technologies, 102417–100) extracellular flux assay kits were used to measure oxygen consumption (OCR) and glycolytic flux (ECAR). ROS-low LSCs or ROS-high blasts were sorted, drug treated for four hours, or directly plated in a XF24 or XF96 cell culture microplate. OCR and ECAR was measured according to the manufacture protocol and as previously described 9. Briefly, five replicate wells of 5×10^5 ROS-low LSCs, ROS-high blasts, or total AML blasts per well were seeded in a Cell-Tak (Corning, 324240) coated 24-well XF24 well plate. Thirty minutes prior to analysis, the medium was replaced with Seahorse XF media (Agilent

Technologies, 102353–100) and the plate was incubated at 37°C. Analyses were performed both at basal conditions and after injection of 5 μg/ml oligomycin (Sigma-Aldrich, 871744), 2 μM FCCP (Sigma-Aldrich, C2920), 5 μM Antimycin A (Sigma-Aldrich, A8774), and 5 μM rotenone (Sigma-Aldrich, R8875).

Immunoprecipitation and Immunoblotting:

Total cell lysates from venetoclax with azacitidine treated cells were precleared with protein A/G magnetic beads (Thermo Fisher Scientific) at room temperature for 1 hour. After preclearing cell lysates were incubated with sdhA antibody (cell signaling) overnight at 40C. Protein-antibody complexes were then bound to protein A/G magnetic beads (Thermo Fisher Scientific) for 1 hour at room temperature. After incubation, beads were sequentially washed 3 time with lysis buffer. Finally, the protein-antibody-bead complexes were boiled for 10 min in non-reducing SDS-PAGE sample buffer to elute all pulldown products for Western blot analysis. Lysates were loaded on a 10% polyacrylamide gel. Proteins were transferred to a PDVF membrane using the mini trans-blot transfer system (Biorad). To detect specific antigens, blot were probed with primary antibodies Glutathione (Invitrogen), GAPDH (Santa Cruz), and sdhA (Cell Signaling) on a shaker at 4 °C, overnight, followed by 1 h of room temperature incubation with HRP-conjugated secondary antibodies (Santa Cruz). Chemoluminescence was recorded using the automated Gel Doc XR system (Bio-Rad).

Complex II Activity Assay:

AML cells were treated with cysteinase or control inhibitors for 4 hours, samples were prepared, and enzyme activity was quantified according to the manufactures protocol (Abcam, ab109908).

In Vivo Treatment of Xenografts:

Animal experiments were conducted in accordance with an approved protocol (IACUC #0308) at the University of Colorado. NSG-S mice were conditioned 24 hours prior to transplant with 25 mg/kg busulfan via IP injection. AML patient bone marrow mononuclear cells ($1.0e^6$) were transplanted via tail vein in a final volume of 0.1 ml of PBS with 0.5% FBS. After 6 weeks, animals were separated to treatment and control groups (4 animals per group) and received either ABT-199 (100 mg/kg), azacitidine(3 mg/kg IP), vehicle or combination of these drugs once. After the 24-hour treatment, mice were sacrificed and human engrafted cells isolated by flow cytometry.

Data Availability Statement:

Patient-related clinical data not included in the paper were generated as part of a multicenter clinical trial (). A detailed description of the dose escalation portion of the study has been published (Dinardo et al)[1]. The dose expansion portion of the study is now complete and the manuscript describing these data is currently under preparation.

All DNA and RNA raw and analyzed sequencing data can be found at the GEO database and is available via accession number GSE116481(single cell RNA-seq) and accession number GSE116567(bulk RNA-seq).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This paper is dedicated to the memory of Richard Berger, a warrior in all aspects of life, who bravely confronted every obstacle and brought courage and inspiration to us, his family and all who were privileged enough to know him. We also gratefully acknowledge all of our patients and their families for their participation in this study. Grant support: D.A.P is supported by the University of Colorado Department of Medicine Outstanding Early Career Scholar Program. B.M.S and C.T.J are supported by a pilot grant provided by the University of Colorado RNA Bioscience Initiative. C.L.J. was supported by the American Cancer Society 25A5072 and the Colorado Clinical and Translational Sciences Institute AEF CCTSI YR9 CO 2301425. AD is supported by the Webb-Waring Early Career award 2017 sponsored by the Boettcher Foundation.

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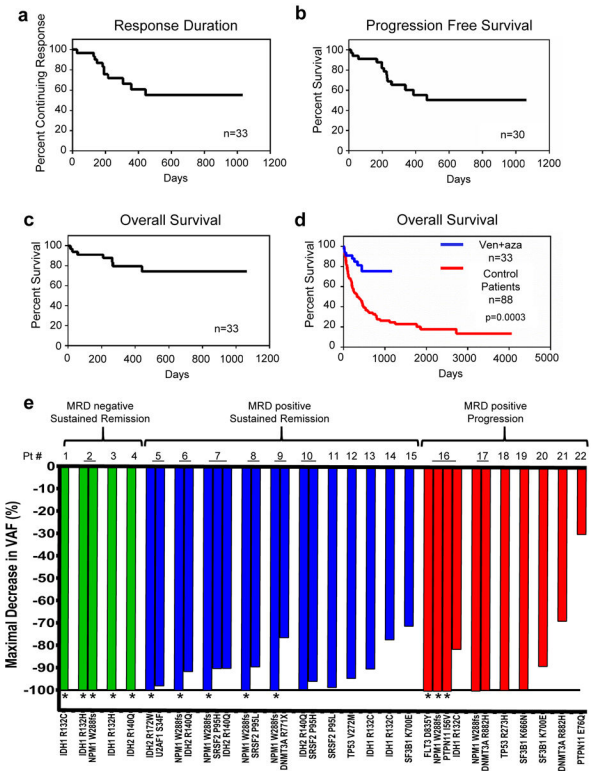

Figure 1: Clinical responses for the 33 patients treated with venetoclax + azacitidine at a single institution. Median follow up time was 580 days

a) Duration of response for the 33 responding patients. Median response duration was not reached. b) Progression free survival for the 30 responding patients. Median time to progression or death was not reached. c) Overall survival for all treated patients. Median overall survival was not reached. d) Overall survival comparison of 33 treated patients with venetoclax + azacitidine (blue line, venetoclax + azacitidine) versus any other treatment in 88 older previously untreated AML patients treated consecutively at a single institution (red line, control patients). Control patients had a significantly worse survival in comparison to venetoclax + azacitidine patients. Log-rank (Mantel-Cox) test p=0.0003 e) Measurable residual disease (MRD) monitoring of 22 patients who had a clinical response and an amenable mutation for droplet digital PCR performed with each bone marrow biopsy. Each patient had one to four mutations monitored; each number represents a patient, each gene monitored is shown as a bar beneath the patient number and annotated along the X-axis. The length of the bar represents the maximal decrease in variant allele frequency (VAF); 100% is equivalent to MRD negativity. Genes that achieved MRD negativity are annotated with an asterisk. Green bars represent patients who achieved MRD negativity and have not progressed. Blue bars represent patients who have not achieved MRD negativity and have not progressed. Red bars represent patients who did not achieve MRD negativity and subsequently progressed. No patients who achieved MRD negativity in all genes analyzed have progressed. All patients who have progressed did not achieve MRD negativity in at least one measurable gene.

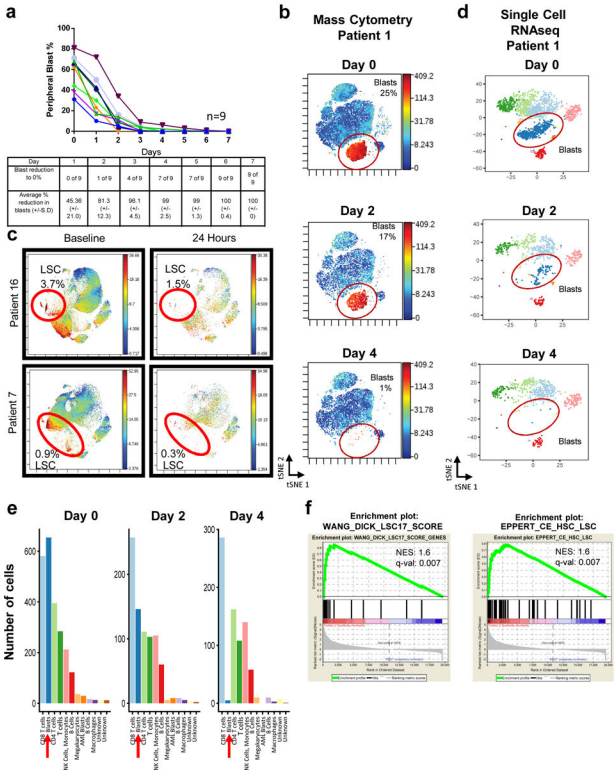

Figure 2: Mass cytometry and single cell transcriptomics at early time points show significant decreases in blasts and LSCs.

a) Temporal blast counts during the first week of therapy for nine patients who began treatment with >20% peripheral blasts at diagnosis (assessed by complete blood count with manual differential). Table below figure shows the proportion of patients at 0% and the average percent reduction of blasts for each day of analysis. For summary of blast counts on all available patients, please see supplemental Table 5. b) Mass cytometry analysis of peripheral blood at Day 0, 2, and 4 for a representative patient. AML blasts are indicated by the red shaded area, with blast percentages shown (n=1) . c) Mass cytometry analysis of peripheral blood at baseline and 24 hours for patients 7 and 16. Red circles indicate the phenotypically-defined LSC population (CD34+, CD38-, Lin-, CD123+) (n=1) . d) tSNE plots of single cell transcriptomics measured at baseline, day two and four post-treatment with blasts as defined by gene signature in red circle(n=1) . e) Number of cells in clusters of tSNE plot by time point. Blast cluster (indicated by red arrow) shows a decrease at day two and disappearance of cells by day four (n=1) . f) GSEA analysis of blast cluster compared to all other clusters in baseline sample tSNE plots shows the cluster is significantly enriched for two different LSC gene signatures.

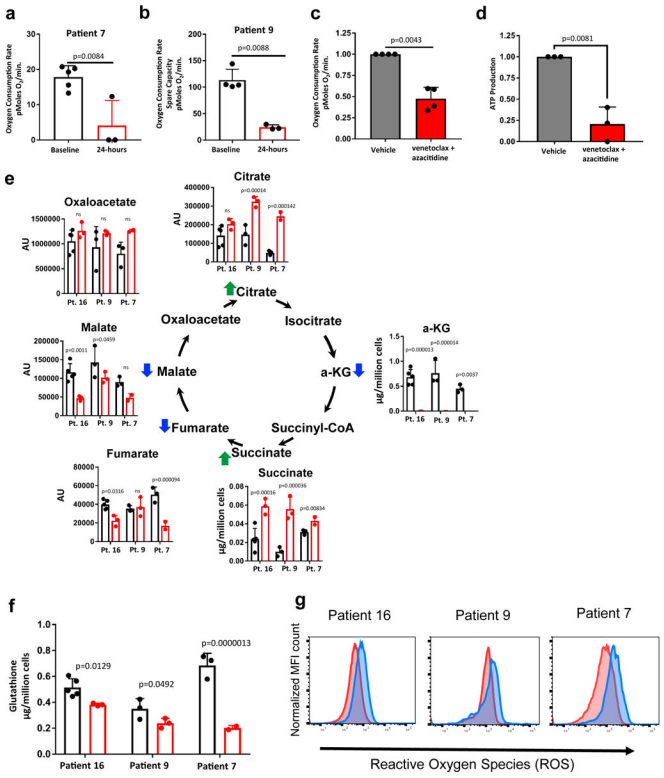

Figure 3: Venetoclax + Azacitidine reduces oxidative phosphorylation in AML LSCs.

a) Oxygen consumption rate as a measure of OXPHOS was quantified pre and 24 hours post venetoclax + azacitidine treatment in the leukemia cells isolated from an AML patient, revealing a significant reduction in OXPHOS levels. N=3–5, technical replicates. Significance was measure by unpaired two-tailed Student's t-test. b) Using FCCP to uncouple the electron transport chain and maximize oxidative phosphorylation, the oxygen consumption rate was measured in the leukemia cells of one patient before and 24 hours after treatment, revealing a significant decrease in the ability of the post-treatment leukemia cells to maximize oxygen consumption. N=3–4 technical replicates. Significance was measure by unpaired two-tailed Student's t-test. c) Oxygen consumption levels were measured in ROS-low LSCs isolated from 4 primary AML specimens A, B, C, and D treated with 500nM venetoclax + 2.5 µm azacitidine in vitro for 4 hours. n=4 (biological)/n=5 (technical) replicates. Significance was measure by paired two-tailed Student's t-test. d) ATP production was measured in ROS-low LSCs isolated from 3 primary AML specimens A, B, and C treated with 500nM venetoclax + 2.5 µm azacitidine in vitro for 4 hours. n=3 (biological)/ n=5 (technical) replicates. Significance was measure by paired two-tailed Student's t-test. (e-g) Three patients with circulating blasts sampled from the peripheral blood at baseline and 24 hours after treatment with venetoclax + azacitidine show evidence of decreased electron transport chain complex II activity. e) Levels of TCA cycle intermediates, alpha-ketoglutarate, succinate, citrate, fumarate, malate, oxaloacetate in patients pre and 24 hours post venetoclax + azacitidine treatment. n=3 (biological)/ n=2–5 (technical) replicates. Significance was measure by unpaired two-tailed Student's t-test. f) Levels of glutathione in patients pre and 24 hours post venetoclax + azacitidine treatment. n=3 (biological)/ n=2–5 (technical) replicates. Significance was measure by unpaired two-

tailed Student's t-test. g) Cellular reactive oxygen species (ROS) in leukemia cells from patients pre (red shaded histogram) and 24 hours post (blue shaded histogram) venetoclax + azacitidine treatment (as determined by labeling with CellROX™). ROS was measured in each individual patient once. Bars represent the mean of the replicates and error bars represent standard deviation for each bar graph. Statistical tests were performed on technical replicates only when assay was measuring metabolic responses of individual patients on therapy. NS= not significant, AU= arbitrary units

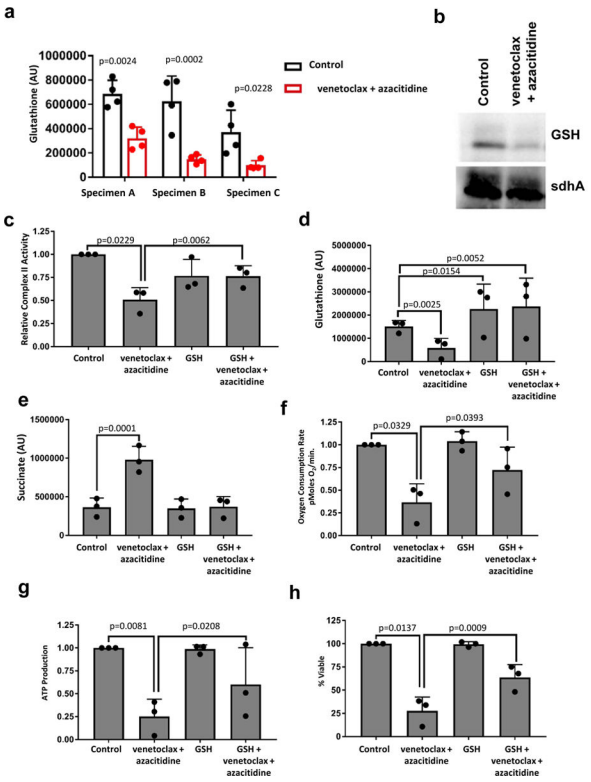

Figure 4: Venetoclax + azacitidine reduces complex II activity through reduction in sdhA glutathionylation.

a) Glutathione levels were measured in ROS-low LSCs isolated from 3 primary AML specimens A, B, and C treated with 500nM venetoclax + 2.5 μm azacitidine in vitro for 4 hours. n=3 (biological)/n=4 (technical) replicates. Significance was measure by paired two-tailed Student's t-test. b) Immune precipitation of succinate dehydrogenase (sdhA) followed by western blot for GSH modification of sdhA isolated from leukemia cells treated with 500nM venetoclax + 2.5 μm azacitidine in vitro for 4 hours. Blot shown here is a representative image using specimen A (n=3). c) Relative complex II activity measured in 3 AML specimens A, B, and C pretreated with cell permeable glutathione or vehicle and then treated with 500nM venetoclax + 2.5 μm azacitidine in vitro for 4 hours. N=3 (biological)/3 (technical) replicates. Significance was measure by multiple paired two-tailed Student's t-test. d) Levels of glutathione measured in LSCs isolated from 3 AML specimens A, B, and C, pretreated with cell permeable glutathione or vehicle and then treated with 500nM venetoclax + 2.5 μm azacitidine in vitro for 4 hours. N=3 (biological)/n=4 (technical) replicates. Significance was measured using multiple two-tailed paired Student's t-test. e) Levels of succinate measured in LSCs isolated from 3 AML specimens A, B, and C, pretreated with cell permeable glutathione or vehicle and then treated with 500nM venetoclax + 2.5 μm azacitidine in vitro for 4 hours. N=3 (biological)/4 (technical) replicates. Significance was measure by multiple paired two-tailed Student's t-test. f) Relative oxygen consumption measured in 3 AML specimens A, B, and C pretreated with cell permeable glutathione or vehicle and then treated with 500nM venetoclax + 2.5 μm azacitidine in vitro for 4 hours. N=3 (biological)/5 (technical) replicates. Significance was measure by multiple paired two-tailed Student's t-test. g) Relative ATP production measured

in 3 AML specimens A, B, and C pretreated with cell permeable glutathione or vehicle and then treated with 500nM venetoclax + 2.5 μm azacitidine in vitro for 4 hours. N=3 (biological)/5 (technical) replicates. Significance was measure by multiple paired two-tailed Student's t-test. h) Relative cell viability measured in 3 AML specimens A, B, and C pretreated with cell permeable glutathione or vehicle and then treated with 500nM venetoclax + 2.5 μm azacitidine in vitro for 4 hours. N=3 (biological)/3 (technical) replicates. Significance was measure by multiple paired two-tailed Student's t-test. Bars represent the mean of the replicates and error bars represent standard deviation for all bar graphs. Statistical tests were performed on technical replicates only when assay was measuring metabolic responses of individual patients. AU= arbitrary units

Table 1:

Baseline characteristics of the 33 older, previously untreated AML patients enrolled at a single center in the context of a larger multi-institutional clinical trial.

Characteristic	Value
Median age, years	75 (65–89)
Median baseline blasts, %	40 (20–95)
Cytogenetic Category	
Unknown	N=1 (3%)
Intermediate	N=20 (61%)
Unfavorable	N=12 (36%)
European LeukemiaNet Risk Group	
Favorable	N=3 (9%)
Intermediate	N=7 (21%)
Adverse	N=23 (70%)
400 mg venetoclax	N=23 (70%)
800 mg venetoclax	N=9 (27%)
1200 mg venetoclax	N=1 (3%)