Hypomethylating agent decitabine sensitizes diffuse large B-cell lymphoma to venetoclax

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Received: March 29, 2023.
Accepted: July 25, 2023.
Early view: August 3, 2023.

https://doi.org/10.3324/haematol.2023.283245

Abstract

Despite recent advances in the therapy of diffuse large B-cell lymphoma (DLBCL), many patients are still not cured. Therefore, new therapeutic strategies are needed. The anti-apoptotic B-cell lymphoma 2 (*BCL2*) gene is commonly dysregulated in DLBCL due to various mechanisms such as chromosomal translocation t(14;18)(q32;q21) and copy number alterations; however, targeting BCL-2 with the selective inhibitor, venetoclax, led to response in only a minority of patients. Thus, we sought to identify a rational combination partner of venetoclax to improve its activity against DLBCL cells. Utilizing a functional assay, dynamic BH3 profiling, we found that the DNA hypomethylating agent decitabine increased mitochondrial apoptotic priming and BCL-2 dependence in DLBCL cells. RNA-sequencing analysis revealed that decitabine suppressed the pro-survival PI3K-AKT pathway and altered the mitochondria membrane composition in DLBCL cell lines. Additionally, it induced a DNA damage response and increased BAX and BAK activities. The combination of decitabine and venetoclax synergistically suppressed proliferation of DLBCL cells both *in vitro* and *in vivo* in a DLBCL cell line-derived xenograft mouse model. Our study suggests that decitabine plus venetoclax is a promising combination to explore clinically in DLBCL.

Introduction

With recent advances in treatment, approximately two thirds of patients with diffuse large B-cell lymphoma (DLBCL) can be cured with currently available therapies; however, new treatment strategies are needed to improve outcomes for those who are not cured with existing regimens. Recent comprehensive genomic analyses[1–3] have identified genomic heterogeneity in DLBCL beyond the traditional activated B-cell-like DLBCL (ABC DLBCL) and germinal center B-cell-like DLBCL (GCB DLBCL) genotypes.[4,5] Notably, some of these newly identified genomic subtypes of DLBCL involve apoptotic signaling, suggesting that it may be promising to target this pathway therapeutically.[1–3,6]

The intrinsic (mitochondrial) pathway of apoptosis is regulated by the interaction between the proteins in the B-cell lymphoma/leukemia 2 (BCL-2) family including a group of pro-apoptotic proteins (e.g., multidomain effectors [BAK, BAX], BH3 only activators [BIM, BID], BH3 only sensitizers [BAD, BIK, BMF, HRK, PUMA, NOXA]) and anti-apoptotic proteins such as BCL-2, BCL-xL, MCL-1, BCL-w and BFL-1.[7,8] The ratio and interactions among these BCL-2 family proteins determine whether cancer cells survive or proceed to apoptosis. In GCB DLBCL, the *BCL2* gene is often subjected to chromosomal translocation t(14;18)(q32;q21) which places the *BCL2* gene under the control of immunoglobulin heavy chain (IgH) enhancer,[6] leading to high BCL-2 expression. Increased expression of BCL-2 can also be seen in ABC DLBCL, likely due to copy number alterations.[1–3] Thus, BCL-2 is a potential therapeutic target in several genetic subtypes of DLBCL.

Preclinical studies have investigated anti-growth effects of the BCL-2-selective inhibitor, venetoclax, in DLBCL cell lines and cell line-derived xenograft mouse models.[9–11] Notably, DLBCL cell lines required a higher dosage of venetoclax to kill the cells than other types of lymphoid malignancies.[9,10] In a phase I trial evaluating venetoclax in patients with relapsed or refractory non-Hodgkin lymphoma, although single-agent activity of the drug in

DLBCL was observed, the complete response rate was only 18%.[12] Given the modest single-agent activity of venetoclax in DLBCL, we sought to identify a rational combination partner that could increase the activity of venetoclax in this disease by specifically shifting the balance of BCL-2 family proteins toward a more BCL-2-dependent state.

In order to identify such a combination partner, we utilized BH3 profiling, an assay that can assess the mitochondrial apoptotic priming of cells, a measure of how close tumor cells are to the threshold of apoptosis.[13] The profile also helps to assess the functional dependence of cells on the various anti-apoptotic BCL-2 family proteins. A variation of this assay, known as dynamic BH3 profiling (DBP), is capable of assessing the change in mitochondrial apoptotic priming and dependence induced by *ex vivo* drug treatments of interest.

With DBP, we found that the hypomethylating agents azacitidine and decitabine could increase mitochondrial apoptotic priming and BCL-2 dependence in DLBCL, suggesting the combination of hypomethylating agents with venetoclax could be a potential therapeutic strategy worth exploring in DLBCL. Of note, the combination of venetoclax and a hypomethylating agent has been approved for use in acute myeloid leukemia (AML), where mechanistic studies demonstrated that azacitidine can downregulate MCL-1 and induce NOXA expression.[14,15] Decitabine functions differently from azacitidine, as decitabine is a more specific DNA hypomethylating agent. In addition, decitabine was shown to be more potent than azacitidine in suppressing certain cancer cells at equimolar concentrations[16] and outperformed azacitidine in anti-leukemic action in a rat model of myeloid leukemia.[16] However, mechanistic studies evaluating the combination of hypomethylating agents and venetoclax have been mostly focused on azacitidine. Thus in this study, we focused on decitabine and investigated whether decitabine could sensitize DLBCL cells to venetoclax, and we explored the mechanisms underlying the activity of this combination in DLBCL.

Methods

Cell lines and culture

The DLBCL cell lines (TMD8, OCI-Ly3, OCI-Ly1, OCI-Ly7, SUDHL4) were kindly provided by the laboratory of Dr. Anthony Letai (Dana-Farber Cancer Institute) and grown in RPMI 1640 media (Gibco 11875119, Billings, MT, USA) supplemented with 10% fetal bovine serum (Gibco 26140079), 100 U/mL penicillin-streptomycin (Gibco 15140163), 2 mM L-glutamine (Gibco 25030081). The venetoclax-resistant OCI-Ly1 cell line (OCI-Ly1R) was obtained from Dr. Catherine Wu's laboratory[17] and maintained in the above medium with 0.5 μM of venetoclax. All cell lines were authenticated using STR profiling at the Molecular Diagnostics Laboratory in January, 2023. Cell lines were routinely tested for mycoplasma contamination. Double-hit lymphoma (DHL) patient-derived xenograft (PDX) cell lines DW19 and DW20 were obtained from Dr. David Weinstock's laboratory and cultured in X-VIVO 15 media (Lonza, BE02-060Q, Portsmouth, NH, USA) supplemented with 20 ng/mL Fungizone (Invitrogen 15290018, Waltham, MA, USA). They were isolated from PDX mice available from the Public Repository of Xenografts (PRoX). All cell lines were cultured at 37°C in a 5% CO_2 atmosphere.

BH3 profiling

BH3 profiling was performed by flow cytometry as described previously.[13] Cells were incubated with antibodies for at least 24 hours before being analyzed by flow cytometry. Baseline BH3 profiling data were presented as percent cytochrome C loss. DBP data were presented as δ percent priming (cytochrome C loss in response to drug treatment minus cytochrome C loss in response to dimethyl sulfoxide [DMSO]).

Xenografts

All murine studies were performed according to DFCI Institutional Animal Care and Use Committee approved protocol. NOD/SCID/Il2R$\gamma^{-/-}$ (NSG) mice were purchased from the Jackson Laboratory; $5x10^6$ of luciferase-labeled OCI-Ly1 cells were resuspended in 100 μL of phosphate-buffered saline (PBS) and injected via the lateral tail veins of 7-week-old male mice. Five days following tumor inoculation, animals with established disease determined by bioluminescent imaging were divided into four cohorts and treated with: (i) vehicle control; (ii) 0.25 mg/kg decitabine in PBS, intraperitoneal, 5 consecutive days per week; (iii) 50 mg/kg venetoclax in 60% Phosal 50PG, 30% PEG400, 10% ethanol, orally, daily; (iv) both drugs at the indicated doses. Imaging was performed once every week. Treatments lasted for 21 days. Afterwards, the mice were observed for changes in total-body bioluminescence and survival. Detailed information can be found in the *Online Supplemental Appendix*.

Results

Diffuse large B-cell lymphoma cells have heterogenous dependence on anti-apoptotic BCL-2 family proteins

As DLBCL is a genetically heterogeneous disease, we used a panel of genetically diverse DLBCL cell lines to explore their dependence on various anti-apoptotic BCL-2 family proteins. We first examined the protein expression level of the anti-apoptotic proteins BCL-2, MCL-1, BFL-1 and BCL-xL in a group of DLBCL cell lines including both ABC DLBCL and GCB DLBCL. We found that more than one

anti-apoptotic protein was expressed in each cell line (Figure 1A). Of note, one GCB DLBCL cell line, OCI-Ly7, was BCL-2-deficient while the other cell lines had comparable BCL-2 expression level (Figure 1A; *Online Supplementary Figure S1*).

In order to investigate the dependence of DLBCL on the

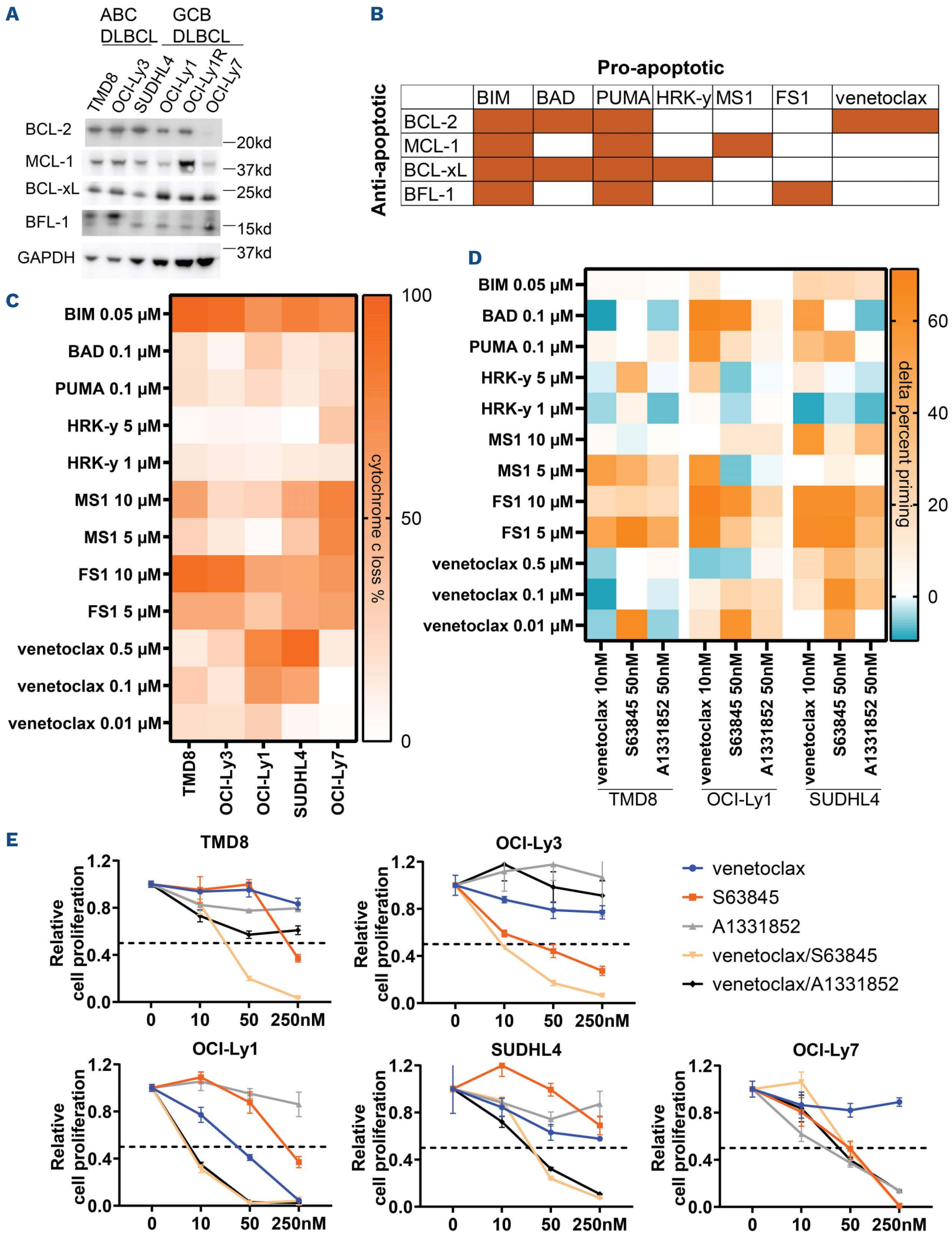

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Figure 1. Diffuse large B-cell lymphoma displays dependence on multiple BCL-2 family members. (A) Expression of the indicated BCL-2 family proteins in diffuse large B-cell lymphoma (DLBCL) cell lines by western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. (B) Interaction map between anti-apoptotic proteins and the BH3 peptides and mimetic used in the BH3 profiling assay. Orange color indicates high affinity interaction. (C) Baseline BH3 profiling of DLBCL cell lines. (D) Dynamic BH3 profiling (DBP) of DLBCL cell lines treated with single BH3 mimetic for 24 hours, data were presented as δ percent priming. (E) Relative cell proliferation of DLBCL cell lines treated with indicated BH3 mimetics alone or in combination for 24 hours. Cell proliferation was measured by CellTiter-Glo assay. Dashed line indicates 50% inhibition of cell proliferation. Data were normalized to dimethyl sulfoxide-treated cells. Data were presented as mean ± standard deviation, N=4. ABC: activated B-cell-like; GCB: germinal center B cell.

anti-apoptotic BCL-2 family members, we next performed baseline BH3 profiling on DLBCL cell lines utilizing a panel of BH3 mimetic peptides or inhibitors that have affinity for either one or multiple anti-apoptotic proteins (Figure 1B). Baseline BH3 profiling revealed that DLBCL cell lines were responsive to more than one BH3 peptide or BH3 mimetic (Figure 1C), suggesting dependence on multiple anti-apoptotic proteins.

Cytochrome C loss in response to venetoclax indicates cellular survival dependence on BCL-2, and the variable responses we observed indicate heterogenous dependence on BCL-2 among DLBCL cell lines. Cell lines that showed strong dependence on BCL-2 (OCI-Ly1, SUDHL4) were also dependent on other anti-apoptotic proteins, such as BFL-1 and MCL-1. Utilizing DBP, we treated DLBCL cells with venetoclax and other BH3 mimetics including S63845 (an MCL-1 inhibitor) and A1331852 (a BCL-xL inhibitor), and found that single BH3 mimetic treatment increased overall mitochondrial apoptotic priming and dependence on other anti-apoptotic proteins (Figure 1D). This finding suggests that some DLBCL cells may not be sensitive to venetoclax alone, as they can switch dependence and rely on other anti-apoptotic proteins for survival.

Since S63845 or A1331852 treatment can increase BCL2 dependence in DLBCL cells, we hypothesized that combining venetoclax with S63845 or A1331852 may enhance the cytotoxicity of venetoclax. In order to test that hypothesis, we treated multiple DLBCL cell lines with the indicated BH3 mimetics alone or in combination (Figure 1E), and measured cell proliferation by CellTiter-Glo assay. The response to venetoclax, S63845, and A1331852 was heterogenous, with OCI-Ly1 being most sensitive to venetoclax, and the remaining cell lines (TMD8, OCI-Ly3, SUDHL4 and OCI-Ly7) were naturally resistant to venetoclax. In BCL-2-expressing cell lines, combining venetoclax with S63845 or A1331852 had the most significant inhibitory effects on cell proliferation. Together, these data demonstrate heterogenous dependence of DLBCL cells on the anti-apoptotic BCL-2 family members and suggest that these cells utilize multiple anti-apoptotic proteins for survival. These data are consistent with clinical trials data suggesting that venetoclax monotherapy is not effective for most patients with relapsed/refractory DLBCL.[12]

Decitabine primes diffuse large B-cell lymphoma cells for apoptosis and synergizes with venetoclax in inducing apoptosis

We used DBP to identify therapeutic agents that can enhance response to venetoclax in DLBCL. Both hypomethylating agents azacitidine and decitabine increased mitochondrial apoptotic priming and BCL-2 dependence in DLBCL cells (Figure 2A; *Online Supplementary Figure S2*). Given that azacitidine and decitabine function via different mechanisms and decitabine was shown to be more potent than azacitidine in preclinical studies,[16,18] we decided to focus our study on decitabine. In order to mimic acquired venetoclax resistance that could occur in patients receiving venetoclax treatment, we included in our study a venetoclax acquired-resistant cell line OCI-Ly1R which was generated from the venetoclax-sensitive DLBCL cell line OCI-Ly1.[17] We found that decitabine could increase BCL-2 dependence in both venetoclax naturally resistant and acquired-resistant DLBCL cells (Figure 2A). Next, we investigated cell proliferation in response to the combination of decitabine and venetoclax in various DLBCL cell lines. Across all the cell lines tested, decitabine consistently sensitized DLBCL cells to venetoclax, including the venetoclax acquired-resistant cell line OCI-Ly1R that had minimal decrease in proliferation in response to venetoclax treatment alone (Figure 2B-D). The effects of this decitabine plus venetoclax combination were synergistic in reducing cell proliferation (Figure 2E). This combination also significantly induced apoptosis (*Online Supplementary Figure S3A*) and enhanced caspase 3 cleavage (*Online Supplementary Figure S3B*).

Since our DBP data had also revealed increased BCL-xL dependence in some of the cell lines and increased MCL-1 dependence in all the cell lines tested (Figure 2A), we also evaluated whether combining decitabine with A1331852 or S63845 could also achieve synergistic cell killing in certain cell lines. For these experiments, we tested one ABC DLBCL (TMD8), two GCB DLBCL (OCI-Ly1, SUDHL4) and one DHL-PDX cell line DW20. When we treated these cell lines with the combination of decitabine and A1331852, we found synergistic cell killing only in OCI-Ly1 and DW20 (*Online Supplementary Figure S4A*). The combination of decitabine and S63845 induced synergistic cell killing in OCI-Ly1, TMD8 and DW20 but not SUDHL4 (*Online Supplementary Figure S4B*).

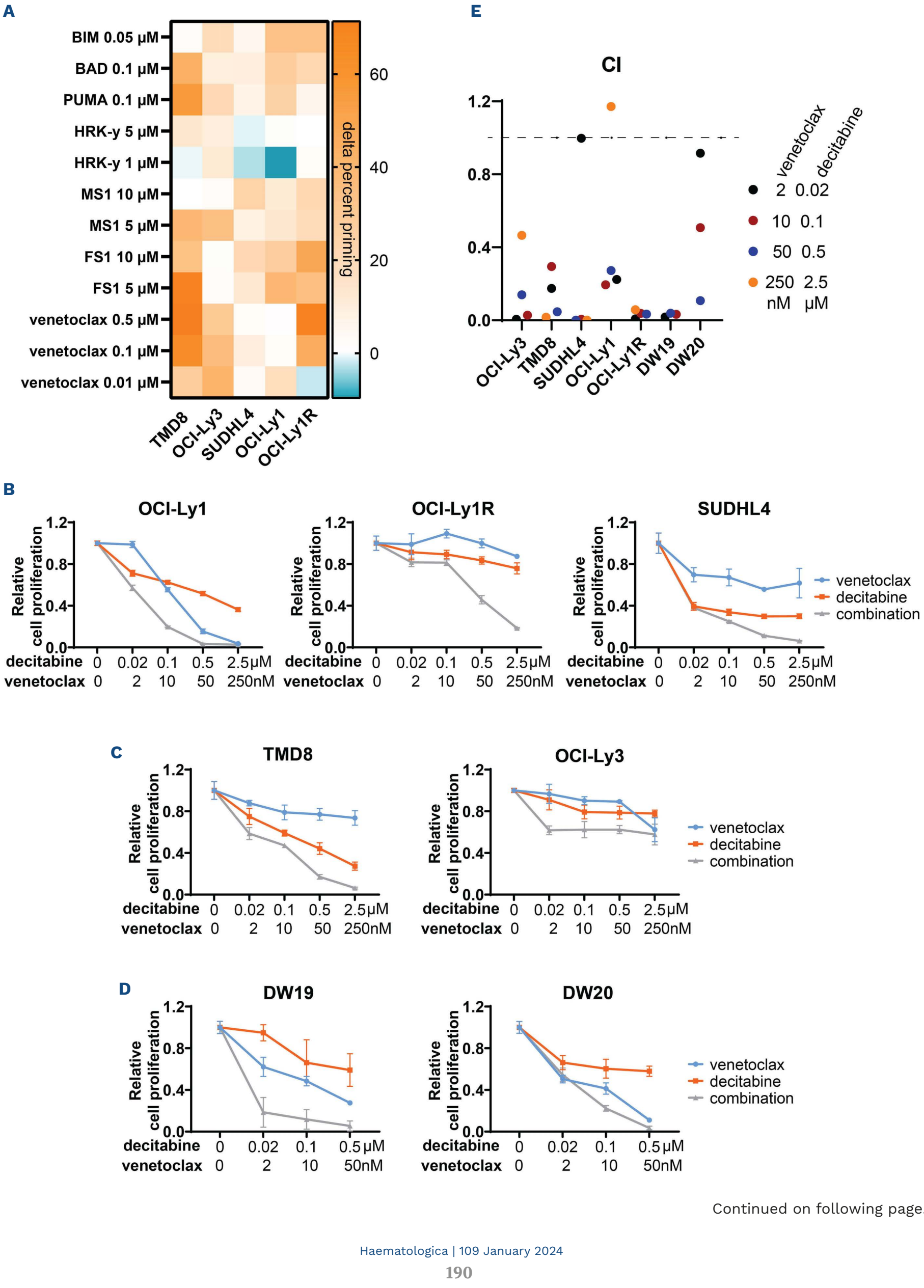

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Figure 2. Decitabine increases mitochondrial apoptotic priming and synergizes with venetoclax in suppressing the growth of diffuse large B-cell lymphoma cells. (A) Dynamic BH3 profiling (DBP) of diffuse large B-cell lymphoma (DLBCL) cells treated with dimethyl sulfoxide (DMSO) control or 2.5 μM of decitabine for 2 days. Data were presented as δ percent priming. (B-D) Germinal center B cell (GCB) DLBCL cell lines including venetoclax-sensitive parental OCI-Ly1 cell line, experimentally created venetoclax-resistant cell line OCI-Ly1R and venetoclax naturally resistant SUDHL4 cell line (B), venetoclax naturally resistant activated B-cell-like (ABC) DLBCL cell lines (C) and double-hit lymphoma-patient-derived xenograft (DHL-PDX) cell lines (D) were treated with indicated dosage of decitabine for 3 days, and venetoclax was added on the last day. Cell proliferation was determined by CellTiter-Glo assay. Data were normalized to DMSO-treated cells. Data were presented as mean ± standard deviation, N=4. (E) Combination index (CI) was calculated by Compusyn.

Taken together, the combination of venetoclax and decitabine strongly induced apoptotic cell death and suppressed cell proliferation in DLBCL cells, leading us to further explore the mechanisms underlying this synergy.

Decitabine induces DNA damage and regulates the expression and activity of BCL-2 family proteins

An established role of decitabine is to induce DNA damage.[19] When we treated DLBCL cell lines with decitabine, we observed a depletion of DNMT1 and an increase in phospho-H2AX (s139) level, indicating increased DNA damage formation (*Online Supplementary Figure S5A*). A previous study suggested a link between DNA damage induction and apoptotic priming in hematopoietic stem cells.[20] Therefore, we asked whether the DNA damage response is associated with apoptotic priming in DLBCL cells. We tested a chemotherapy drug doxorubicin, which is known to induce DNA damage but lacks the epigenetic functions of decitabine, in three DLBCL cell lines. By DBP, we observed increases both in mitochondrial apoptotic priming and BCL-2 dependence in response to doxorubicin treatment (Figure 3A), suggesting that induction of DNA damage in DLBCL is sufficient to increase apoptotic priming.

With cell cycle analysis, we found cell cycle arrest at the G2/M phase upon decitabine treatment, suggesting the formation of DNA damage during S phase (*Online Supplementary Figure S5B*). Nevertheless, apoptosis was not effectively induced in these cells. Next, we examined the expression level of BCL-2 family proteins in response to decitabine treatment in OCI-Ly1, SUDHL4 and TMD8 cells. We found downregulation of BFL-1 in all three cell lines and downregulation of BCL-xL in OCI-Ly1 and TMD8 cells. The expression level of BCL2, MCL1, BIM and PUMA remained almost the same (Figure 3B). The activity of BAX and/or BAK markedly increased upon decitabine treatment even though their expression level didn't change (Figure 3B, C). With a co-immunoprecipitation assay, we observed decreased interaction between BCL-2 and BAX upon decitabine treatment, which may account for the increased activity of BAX (Figure 3D). As DLBCL cells are not sensitive to apoptosis induced by decitabine alone, a threshold of BAK/BAX activity may exist that controls the cells' commitment to apoptosis. Thus, decitabine may increase apoptotic priming in DLBCL cells through DNA damage induction and regulating the expression and activity of BCL-2 family proteins.

Decitabine differentially alters gene expression in venetoclax-sensitive and -resistant diffuse large B-cell lymphoma cells

Decitabine regulates gene transcription through DNA hypomethylation.[19] In order to investigate the epigenetic role of decitabine in venetoclax-sensitive and -resistant contexts, we performed RNA-sequencing analysis with venetoclax-sensitive OCI-Ly1 parental and -resistant OCI-Ly1R cells. We treated both cell lines with decitabine (1 μM) for 3 days to ensure at least two rounds of replication, and analyzed their transcriptomes by RNA sequencing. Interestingly, we found that there was only about 10% overlap between OCI-Ly1 parental and OCI-Ly1R cells in terms of the significantly upregulated or downregulated genes (Figure 4A). In order to identify significantly altered gene sets, we performed gene set enrichment analysis (GSEA). The significantly altered gene signatures by decitabine were also quite different between the two cell lines (*Online Supplementary Figure S6A*). Nonetheless, there were still a few commonly deregulated gene signatures between parental and resistant cells. Notably, decitabine led to significant downregulation of the PI3K-AKT-mTOR1 pathway (Figure 4B), which generates a prosurvival signal in multiple genetic subgroups of DLBCL.[11] Glycogen synthase kinase 3β (GSK3β) is among one of the first identified substrates of AKT,[21] and its phosphorylation by AKT leads to its inactivation. A recent study found that active mTOR1 prevented nuclear localization of GSK3β,[22] leading to the stabilization of the nuclear substrates of GSK3β. Thus, the downregulation of PI3K-AKT-mTOR1 pathway by decitabine could potentially activate GSK3β. Indeed, we found decreased phosphorylation of GSK3β serine 9 in both cell lines following decitabine treatment, indicating increased GSK3β activity (Figure 4C). Additionally, we noted decreased expression of c-Myc, one of the substrates of GSK3β (Figure 4C). c-Myc downregulation in response to decitabine treatment has been reported in other types of cancer as well.[23,24] However, the underlying mechanism is not well understood. Our data suggest that decitabine could downregulate c-Myc expression via inhibition of the PI3K-AKT-mTOR1 pathway. Decitabine also led to upregulation of the intrinsic component of mitochondrial mem-

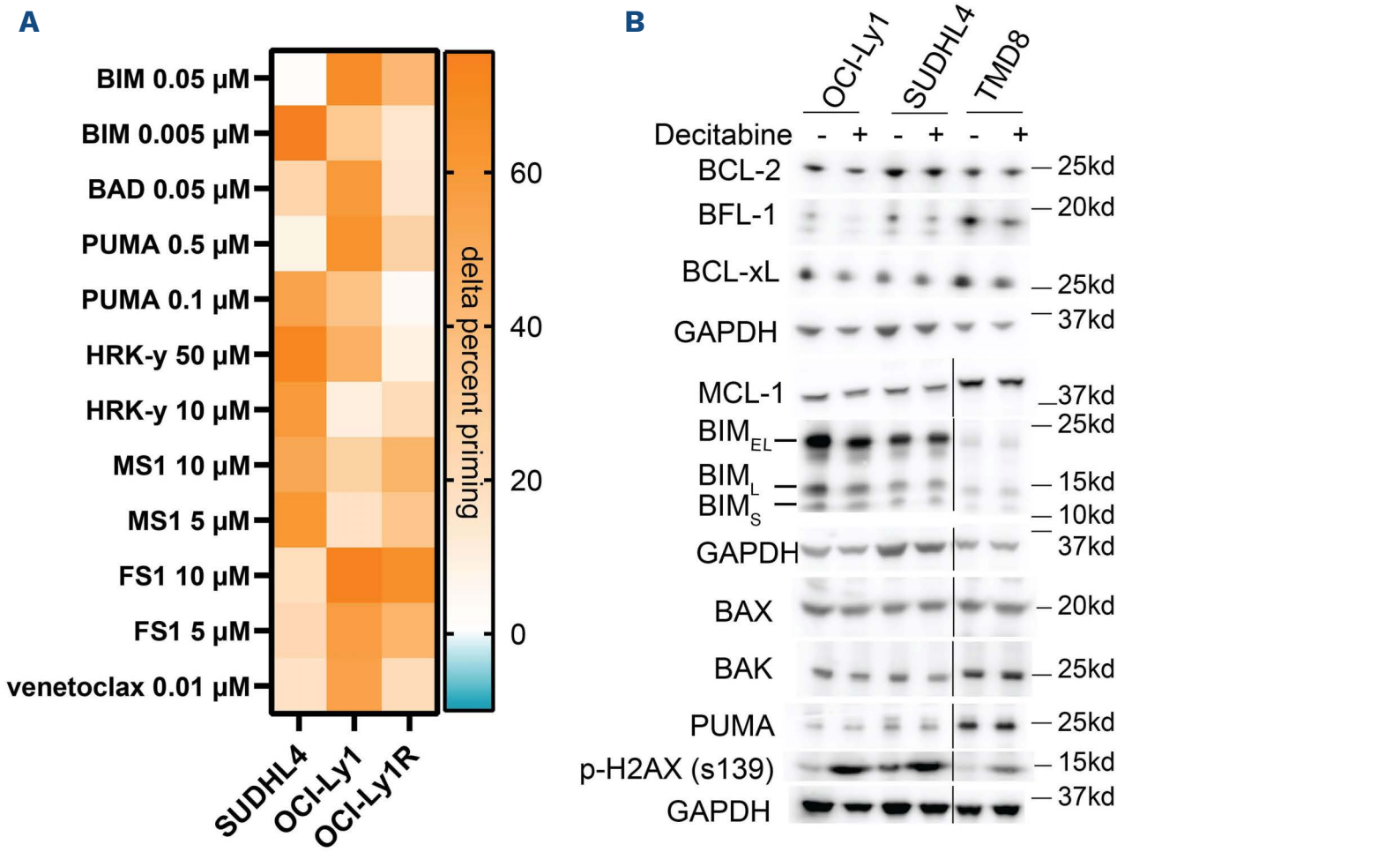

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Figure 3. Decitabine induces DNA damage and regulates the expression and activity of BCL-2 family proteins. (A) Dynamic BH3 profiling (DBP) of diffuse large B-cell lymphoma (DLBCL) cells treated with dimethyl sulfoxide (DMSO) control or 1 μM of doxorubicin for 2 days. Data were presented as δ percent priming. (B) DLBCL cells were treated with DMSO or 2.5 μM of decitabine for 3 days, cells were lysed for western blotting analysis of indicated proteins. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. For protein bands with dividing lines, images were from different parts of the same gel. (C) DLBCL cells were treated with DMSO or 2.5 μM of decitabine for 3 days, BAX (top) and BAK (bottom) activation were measured with conformation-specific antibody by flow cytometry. Median fluorescence intensity (MFI) was used for quantification, N=3. (D) DLBCL cell lines treated with DMSO or 2.5 μM of decitabine for 3 days. Anti-BAX antibody was used for immunoprecipitation (IP). The bound proteins were analyzed by western blotting with indicated antibodies. Protein band intensity was quantified by Image J. The ratio of BCL2 and BAX in the anti-BAX IP product was calculated and indicated in the figure.

brane-related gene signature in both the venetoclax-sensitive and -resistant cell lines (Figure 4D). The electron transport chain (ETC) complexes are important components of the inner mitochondria membrane and essential for energy metabolism. We assessed their formation using an antibody cocktail which contains five antibodies, detecting complex I subunit NDUFB8, complex II subunit SDHB, complex III subunit UQCRC2, complex IV subunit MTCO1 and complex V subunit ATP5A. These subunits are unstable when its complex is not assembled. We found upregulation of NDUFB8 (Figure 4E), suggesting increased formation of complex I. It has been reported that the combination of azacitidine and venetoclax reduced oxidative phosphorylation in leukemia stem cells (LSC) in AML, leading to selective targeting of LSC and durable disease remission.[25] We asked if decitabine and venetoclax would have similar effect on DLBCL cells. In order to evaluate this, we measured the oxygen consumption rate (OCR) in four DLBCL cell lines in response to decitabine. Decitabine alone did not change the OCR in OCI-Ly1 parental or OCI-Ly1R cells (Figure 4F), suggesting that protein expression level does not necessarily correlate with enzymatic activity of the ETC complex, and thus should not be used to predict functional change.[26] Decitabine significantly changed basal OCR in only one cell line (TMD8) (Figure 4F). Interestingly, the combination of venetoclax and decitabine significantly reduced basal OCR (Figure 4F) in all these cell lines, suggesting inhibition of OxPhos by decitabine and venetoclax. In addition, when comparing the gene expression signatures between OCI-Ly1 parental and OCI-Ly1R cells, we found that the resistant cells had downregulation in genes involved in several death pathways, including apoptosis, and senescence and autophagy (*Online Supplementary Figure S6B*). Taken together, our data suggest that decitabine suppresses PI3K-AKT pathway and the combination of decitabine and venetoclax disrupts energy metabolism in DLBCL.

Restoration of TGF-β signaling contributes to the cytotoxicity of decitabine in diffuse large B-cell lymphoma

It has been previously reported that the tumor suppressive TGF-β/TGF-βR2/SMAD1 signaling axis is frequently inactivated in DLBCL due to promoter hypermethylation of *SMAD1* gene.[27] Decitabine has been found to decrease tumor burden in DLBCL cell lines and primary cell-derived xenograft mouse models in part due to restoration of SMAD1 expression.[27] We confirmed upregulation of *SMAD1* mRNA levels following decitabine treatment in three DLBCL cell lines (Figure 5A). Even though SMAD1 protein level remained largely unaffected, the level of phospho-SMAD1 (s463, s465) increased (Figure 5B; *Online Supplementary Figure S7A*) in response to decitabine treatment, indicating increased SMAD1 activity. Next, we asked whether SMAD1 activation underlies the synergism observed between decitabine and venetoclax. In order to investigate this, we used a TGF-β receptor type I/II (TβRI/II) dual inhibitor, LY2109761, that can suppress decitabine induced SMAD1 phosphorylation (Figure 5B; *Online Supplementary Figure S7A*). However, when looking at cell proliferation, adding LY2109761 to decitabine only partially antagonized the toxicity of decitabine in OCI-Ly1 cells (Figure 5C). Adding LY2109761 to the combination of decitabine and venetoclax also partially rescued proliferation and apoptotic cell death of OCI-Ly1 cells (Figure 5C, D). Knockdown of *SMAD1* similarly made the cells less sensitive to the combination of decitabine and venetoclax (Figure 5E, F). In another two cell lines, TMD8 and SUDHL4, inhibition of TGF-β signaling had partial or no rescue on cell proliferation and apoptosis (*Online Supplementary Figure S7B, C*). Thus, the DNA damage response, regulation of BCL-2 family proteins and the other epigenetic targets are likely to also contribute to decitabine-induced cytotoxicity. Together, these data suggest that restoration of SMAD1 expression by decitabine only partially accounts for the synergism between decitabine and venetoclax.

Combination of decitabine and venetoclax is synergistic *in vivo* in a diffuse large B-cell lymphoma xenograft mouse model

In order to examine the *in vivo* activity of decitabine and venetoclax, we established a xenograft mouse model using a luciferase-labeled OCI-Ly1 cell line. Five days after tail vein injection of OCI-Ly1 cells, mice with successful engraftment of cancer cells as documented by bioluminescent imaging were divided into four groups of six to seven mice each. Each group then received one of the following treatments: i) vehicle control; ii) decitabine 0.25

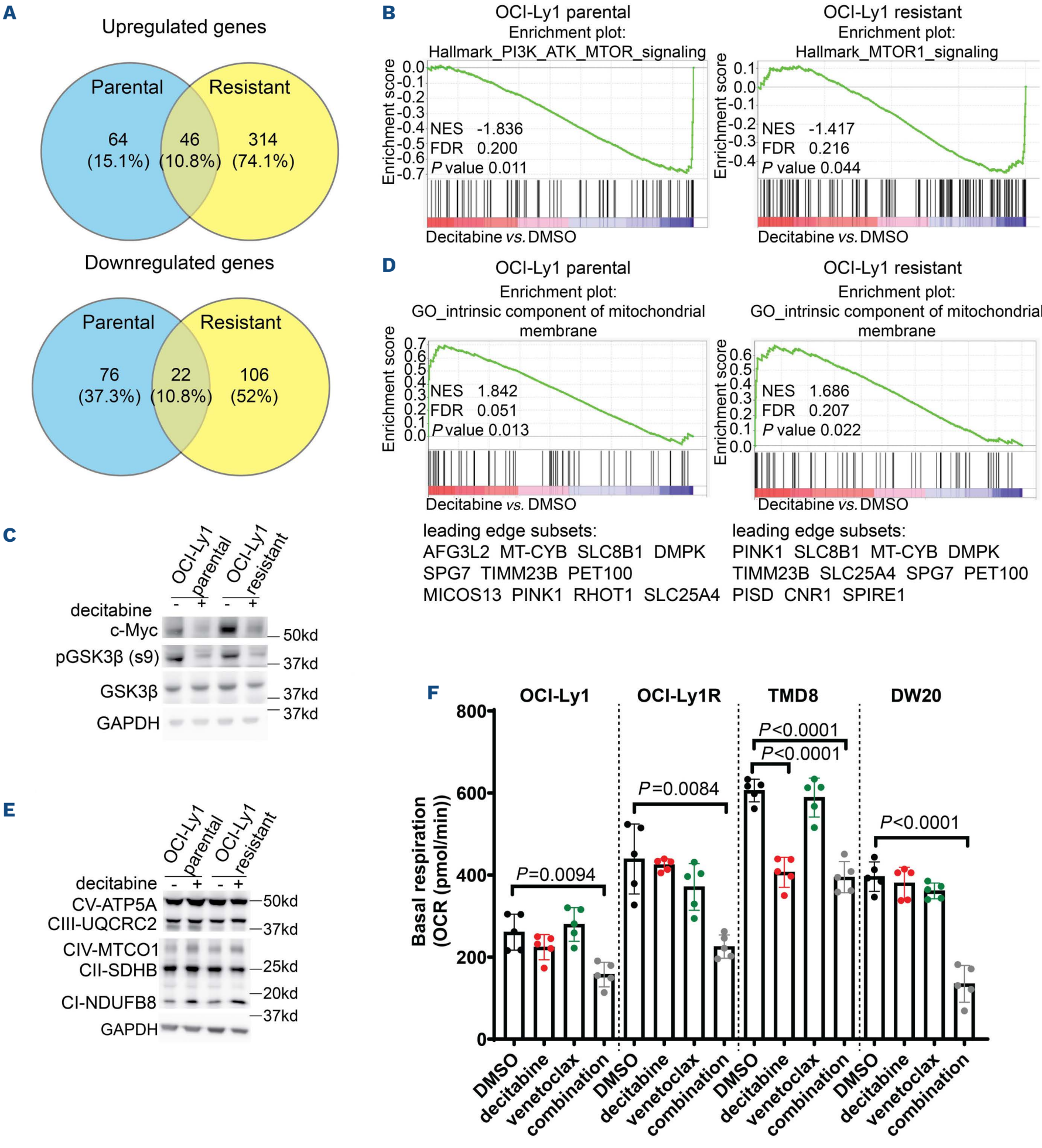
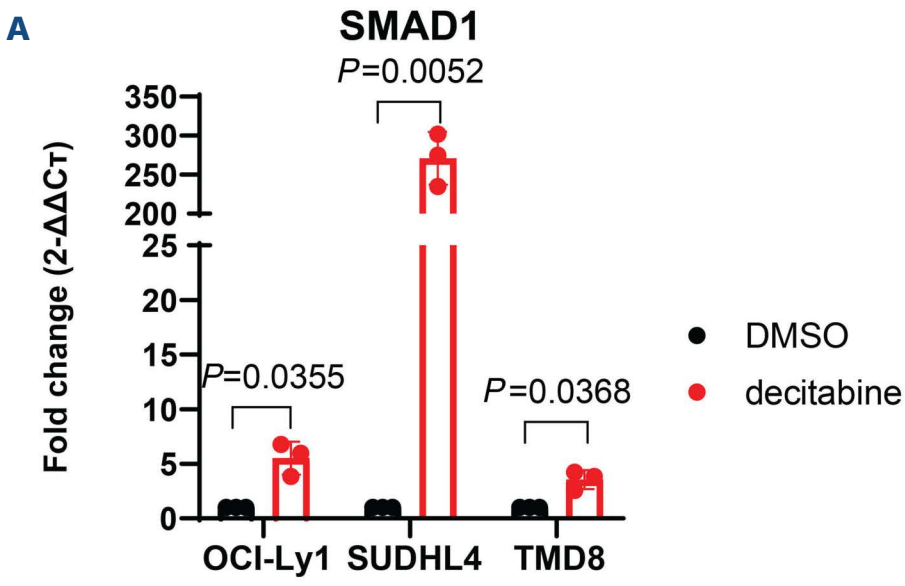
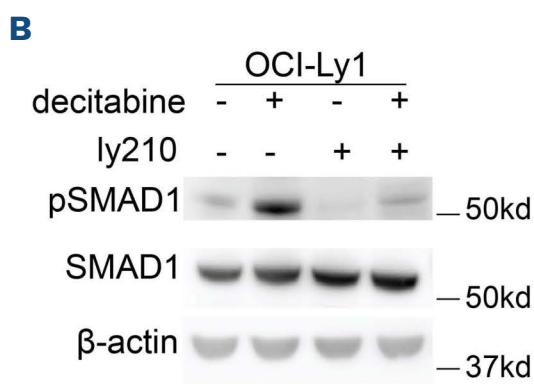
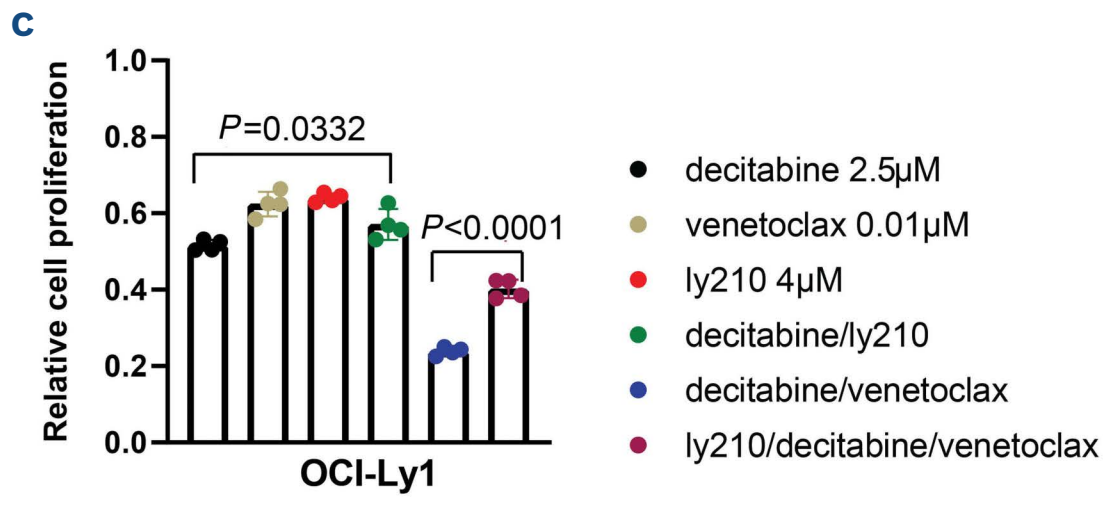
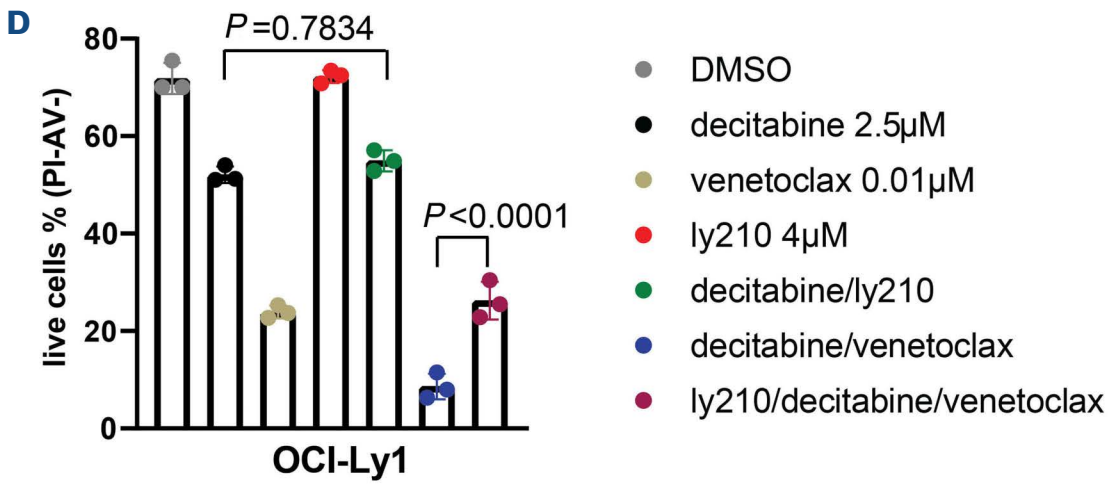


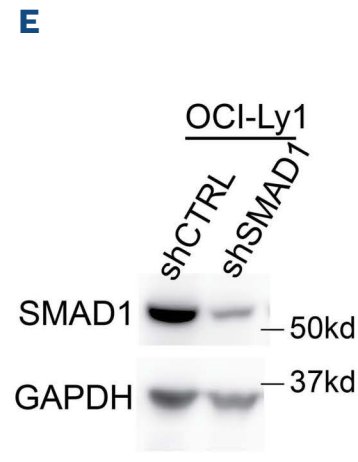
Figure 4. Decitabine regulates gene expression in diffuse large B-cell lymphoma. (A) Venn diagram of upregulated and downregulated genes in venetoclax-sensitive (OCI-Ly1 parental) and -resistant (OCI-Ly1R) cells treated with 1 μM of decitabine for 3 days. Cutoff threshold: adjusted *P* value ≤0.05, absolute (log_2 fold change [FC]) ≥1, N=3. (B) Decitabine significantly downregulates PI3K-AKT-MTOR pathway as revealed by gene set enrichment analysis (GSEA). (C) Expression of indicated proteins in cells treated with dimethyl sulfoxide (DMSO) or 0.5 μM of decitabine for 4 days. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. (D) Decitabine significantly upregulates mitochondrial membrane component-related gene signature as revealed by GSEA. Leading edge genes in each subset were shown. (E) Expression of indicated proteins in cells treated with DMSO or 0.5 μM of decitabine for 4 days. (F) Basal oxygen consumption rate (OCR) was measured by Seahorse XFe96 analyzer in 3 diffuse large B-cell lymphoma (DLBCL) cell lines (OCI-Ly1, OCI-Ly1R, TMD8) and 1 double hit lymphoma-patient-derived xenograft (DHL-PDX) cell line (DW20) treated as indicated. Decitabine 0.5 μM 3 days; venetoclax 1 nM for OCI-Ly1, 5 nM for DW20, 50 nM for OCI-Ly1R and TMD8, 1 day. Data were presented as mean ± standard deviation, N=5. *P* value was calculated by one-way ANOVA with multiple comparisons test. GO: gene ontology; NES: normalized enrichment score; FDR: false discover rate.











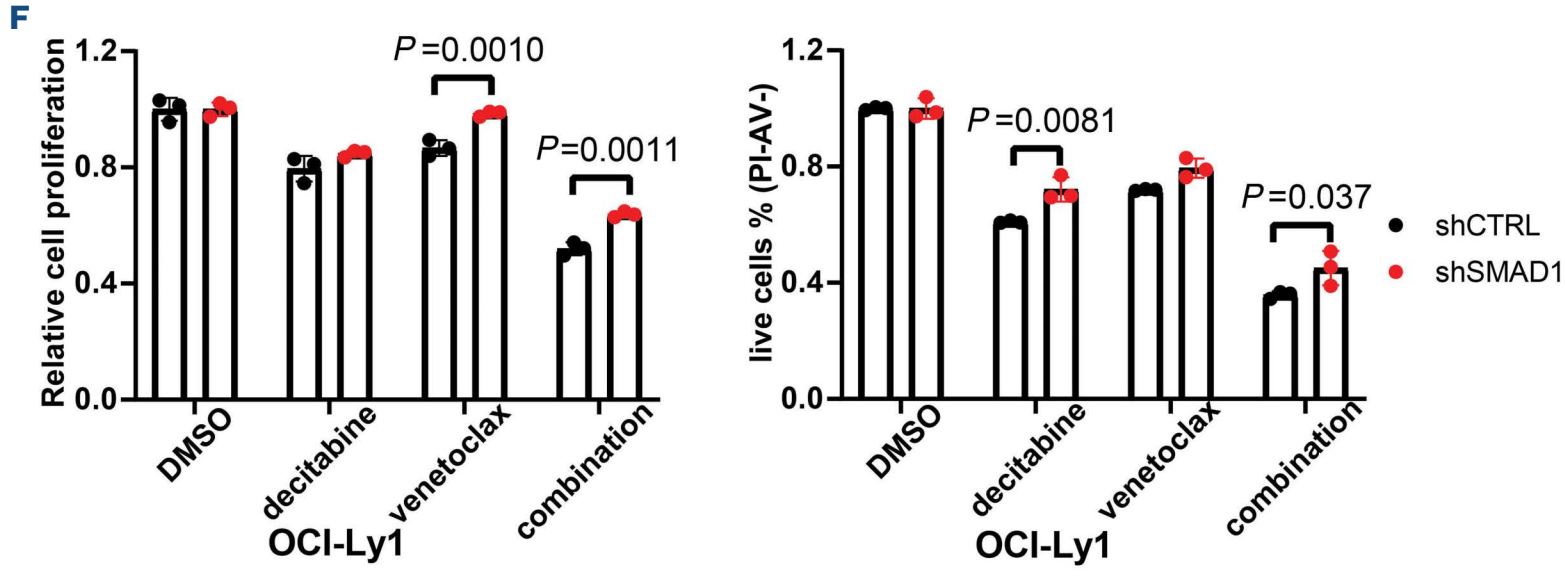


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Figure 5. Inhibition of TGF-β signaling partially blocks the synergism between decitabine and venetoclax. (A) Diffuse large B-cell lymphoma (DLBCL) cell lines were treated with dimethyl sulfoxide (DMSO) or 2.5 μM of decitabine for 3 days. SMAD1 transcript level was measured by quantitative polymerase chain reaction. Data were presented as mean ± standard deviation (SD), N=3. *P* value was calculated by student's *t* test. (B) OCI-Ly1 cells were treated with 2.5 μM of decitabine, 4 μM of TGF-β receptor type I/II (TβRI/II) dual inhibitor, LY2109761 (ly210), alone or in combination for 3 days, phospho-SMDA1 (s463/s465) and total SMAD1 were measured by western blotting. β-actin was used as loading control. (C, D) OCI-Ly1 cells were treated with indicated drugs alone or in combination. Decitabine and ly210 were added to cells for 3 days, venetoclax was added on the last day. Cell proliferation was determined by CellTiter-Glo assay. Data were normalized to DMSO-treated cells. Data were presented as mean ± SD, N=4 (C). Cell viability was measured by propidium iodide/Annexin V assay. Data were presented as mean ± SD, N=3 (D). *P* value was calculated by one-way ANOVA with multiple comparisons test. (E) Small hairpin RNA (shRNA)-mediated knockdown of *SMAD1* in OCI-Ly1 cells. Knockdown efficiency was confirmed by western blotting with anti-SMAD1 antibody, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. (F) OCI-Ly1 cells expressing control shRNA (shCTRL) or shSMDA1 were treated with 2.5 μM of decitabine and 0.01 μM of venetoclax alone or in combination. Decitabine was added to cells for 3 days, venetoclax was added on the last day. Left, cell proliferation was determined by CellTiter-Glo assay. Right, cell viability was measured by propidium iodide/Annexin V assay. Data were presented as mean ± SD, N=3. *P* value was calculated by one-way ANOVA with multiple comparisons test.

mg/kg, intraperitoneal, 5 days on/2 days off; iii) venetoclax 50 mg/kg, orally, daily; iv) the combination of decitabine and venetoclax. After 21 days, all treatments were stopped, and the status of the remaining mice was monitored.

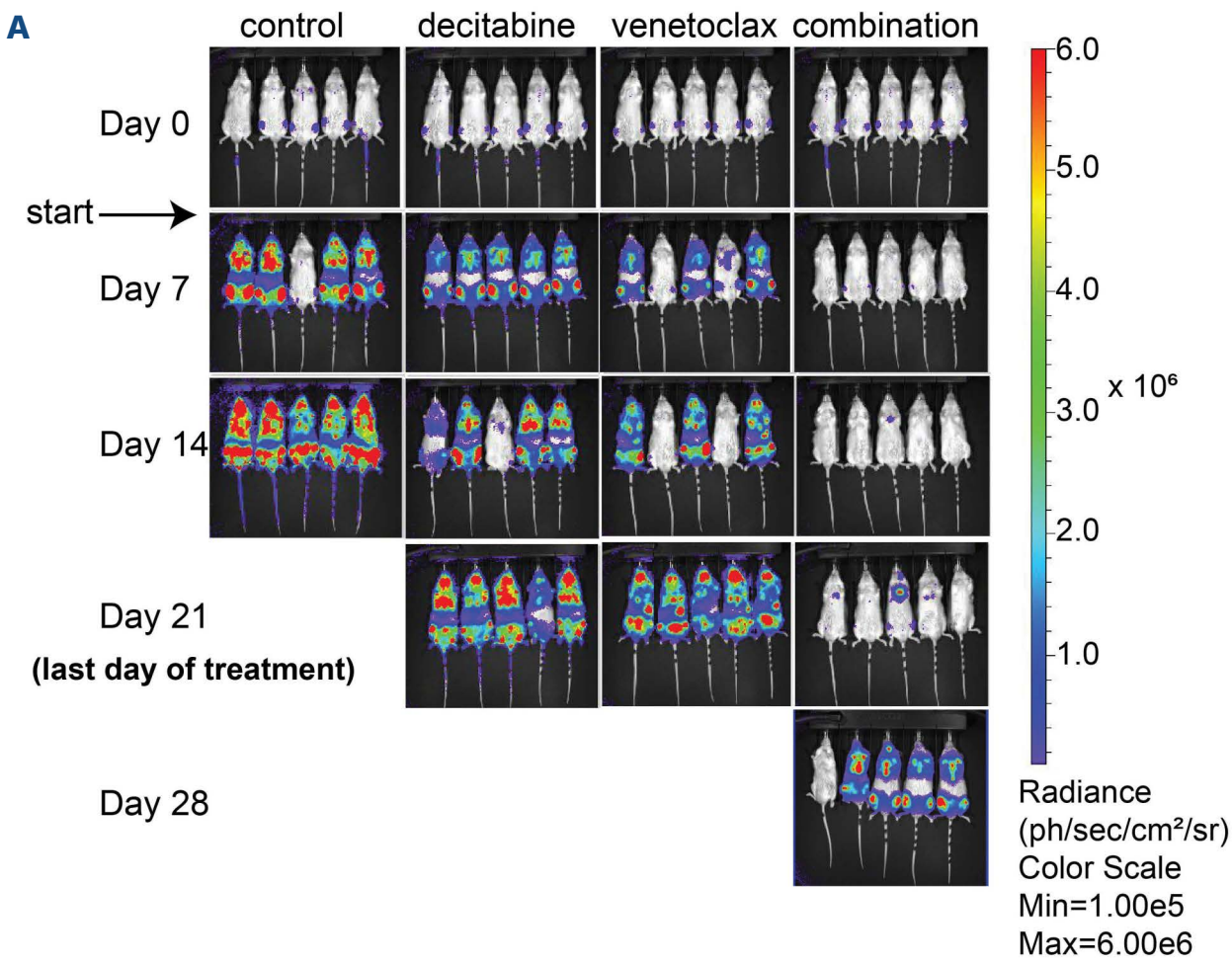
In this model, single-agent treatment with decitabine or venetoclax significantly delayed tumor growth (Figure 6A, B) and extended survival (Figure 6C) compared to vehicle control treatment. The combination of decitabine and venetoclax was able to maintain the tumor burden at a very low level during the treatment period. None of the mice receiving the combination treatment were euthanized due to poor body condition. After treatment was withdrawn, the disease progressed rapidly and all the mice receiving single-drug treatment were found paralyzed and had to be euthanized within 1 week. Mice receiving combination therapy survived longer than single-agent-treated mice did. These data suggest that combination of decitabine and venetoclax effectively inhibited DLBCL growth *in vivo*.

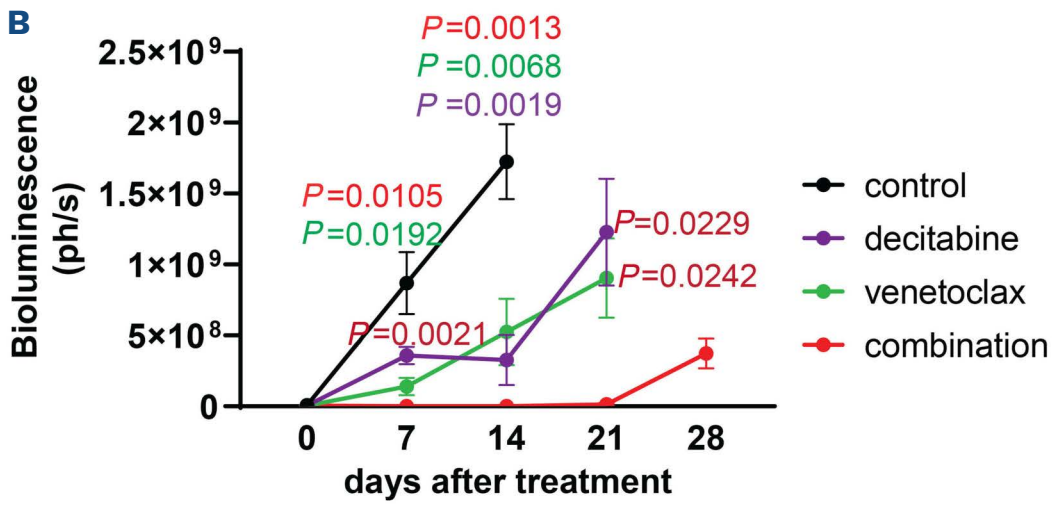
Discussion

Using DBP, we have demonstrated a DNA hypomethylating drug, decitabine, as an agent that can increase the mitochondrial apoptotic priming in DLBCL cells. Although decitabine alone induced DNA damage and cell cycle arrest in DLBCL, the cells were resistant to apoptotic cell death (*Online Supplementary Figures S3A* and *S5*) likely due to the expression of multiple anti-apoptotic proteins. In contrast, the combination of decitabine and venetoclax synergistically inhibited the proliferation of DLBCL cells including DHL-PDX cell lines (Figure 2), consistent with our finding using DBP that decitabine increased BCL-2 dependence, together with various other dependences that drive overall priming (Figure 2A). In a DLBCL cell line-derived xenograft mouse model, this combination also significantly suppressed tumor growth and extended survival *in vivo* (Figure 6). The combined use of a hypomethylating agent with venetoclax has been approved in AML. Our study provides a rationale for exploring a similar combination therapy in the clinic for patients with DLBCL.

In this study, we did not observe a correlation between BCL-2 expression level and venetoclax sensitivity in these cell lines, highlighting the limitations of using expression of a single anti-apoptotic protein alone as a way to predict response to BH3-mimetic drugs. As a cell line usually concurrently expresses more than one anti-apoptotic BCL-2 family protein, it is likely that upon inhibition of BCL-2 by venetoclax, the anti-apoptotic function of BCL-2 can be performed by other proteins such as MCL-1, BFL-1 or BCL-xL. Consistent with this hypothesis, targeting BCL-2 concomitantly with another anti-apoptotic protein dramatically inhibited proliferation of BCL-2-expressing DLBCL cells (Figure 1E). Thus, functional measurement of apoptotic proximity through DBP may be able to better guide rational selection of BH3 mimetic-based combination therapy. For example, if DBP of primary DLBCL cells following *ex vivo* decitabine treatment indicates increased BCL-2 dependence, this patient is likely to benefit from the combined use of decitabine and venetoclax. While a lack of BCL-2 dependence would suggest a different treatment plan.

In DLBCL, an increased dependence on MCL-1 or BCL-xL following decitabine treatment was not always accompanied by synergistic cell killing by combined use of decitabine and BH3 mimetics targeting MCL-1 or BCL-xL. The inconsistent degree of inhibition by these BH3-mimetic drugs might be explained by i) non-apoptotic activity of the BCL-2 family members. Non-apoptotic roles have been reported for BCL-2, BCL-xL and MCL-1.[28–32] When the BH3 domain is required for non-apoptotic functions, BH3 mimetics could induce non-apoptotic changes in the cell. ii) proliferation is measured by CellTiter-Glo assay which reflects total ATP amount inside the cells. Besides cell apoptosis, other cellular statuses such as cell cycle arrest and senescence can slow down cell proliferation and thereby influence the ATP level. Taken together, our data suggest that combining decitabine with venetoclax more





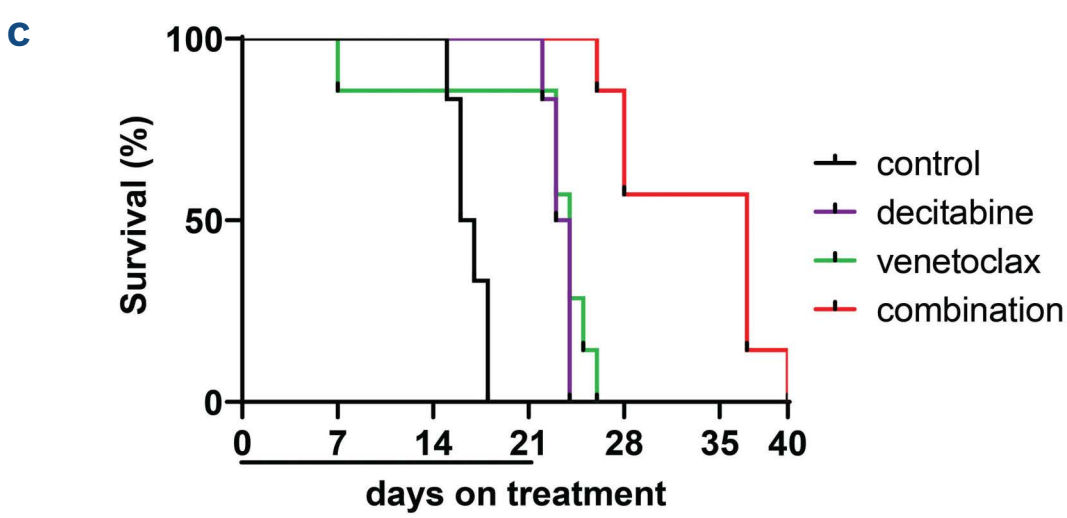


Figure 6. Combination of decitabine and venetoclax is synergistic in diffuse large B-cell lymphoma cell line-derived xenograft model. NSG mice transplanted with luciferized mCherry⁺ OCI-Ly1 cells were treated with vehicle control (N=6), decitabine (0.25 mg/kg intraperitoneal, 5 days on/2 days off, N=6), venetoclax (50 mg/kg, orally, daily, N=7) or decitabine + venetoclax (N=7). Day 21 was the last day of drug treatment. (A) Representative images from each group. Day 0 is 5 days after injection of tumor cells and the day before treatment starts. (B) Quantification of bioluminescence signal over time. Graph shows mean ± standard errorof the mean. *P* value was calculated using one-way ANOVA with multiple comparisons test. (C) Kaplan-Meier survival curve showing the percentage of mice without symptoms of disease (weight loss, paralysis). Control *versus* venetoclax, *P*=0.0216; control *versus* decitabine, *P*=0.0015; control *versus* combination, *P*=0.0006; venetoclax *versus* combination, *P*=0.0007, decitabine *versus* combination, *P*=0.0007. *P* value was calculated by log-rank test. Min: minimum; Max: maximum.

consistently kills a wide range of DLBCL cells compared to the more selective killing we observed with A1331852 or S63845.

A well-established role of hypomethylating agents, including decitabine, is to induce the DNA damage response, which may lead to cell cycle arrest and/or cellular apoptosis. In hematopoietic stem cells, a diminished apoptotic priming in response to DNA damage induction was associated with deficits in ATM activity which can be read by the level of γH2AX.[20] In DLBCL, we found that decitabine induced DNA double strand breaks as revealed by increased γH2AX, suggesting activation of ATM. However, the mech-

anism by which ATM activity contributes to apoptotic priming is not fully understood, especially in cells with mutant p53. A recent study has uncovered a role of ATM as a bridge for PP2A-dependent AKT dephosphorylation and subsequent activation of GSK3β, leading to mitochondria-dependent apoptosis.[33] Notably, PP2A plays a major role in the phosphorylation status of anti-apoptotic proteins and affects apoptotic priming in DLBCL cells.[34,35] Thus, ATM may regulate apoptotic priming through indirectly regulating the post-translational modification of BCL-2 family proteins. While the decitabine-induced DNA damage response appears to be consistent across different cell types, its function as an epigenetic regulator could be more dependent on the cellular context. By comparing the transcriptional changes in paired venetoclax-sensitive and -resistant OCI-Ly1 cell lines, we found downregulation of PI3K-AKT-mTOR1 pathway and significant changes in mitochondrial membrane-associated gene signatures in both cell lines. Other than that, the genes deregulated by decitabine are different between the two cell lines.

In conclusion, our study found that the DNA hypomethylating agent decitabine sensitizes DLBCL cells to venetoclax by increasing mitochondrial apoptotic priming and BCL-2 dependence. This combination significantly inhibited DLBCL growth both *in vitro* and *in vivo* and is worthy of clinical evaluation in patients with DLBCL.

Disclosures

MSD has received consulting fees from AbbVie, Adaptive Biosciences, Ascentage Pharma, AstraZeneca, BeiGene, BMS, Celgene, Eli Lilly, Genentech, Janssen, Merck, Ono Pharmaceuticals, Secura Bio, TG Therapeutics and Takeda; and research support from AbbVie, AstraZeneca, Ascentage Pharma, Genentech, MEI Pharma, Novartis, Surface Oncology and TG Therapeutics. JLC has received consulting fees from Incite, MorphoSys, Kite, ADC Therapeutics and Genmab; and research funding from Bayer, Abbvie, Genentech, and Merck.

Contributions

FZ, JLC and MSD developed the study concept. FZ, WN and SG acquired data. FZ, NMH and LR analyzed and interpreted data. SG, LH, SJFC, MCC, and JG provided technical assistance and discussion. MSD supervised the study. FZ wrote the original draft. All authors reviewed and edited the manuscript.

Funding

This work was funded in part through a PMC FLAMES FLAIR award (to JC). MSD is supported by the National Institutes of Health, National Cancer Institute grant R01CA266298-01A1 and is a clinical scholar of the Leukemia and Lymphoma Society. SJFC is a scholar of the Simeon J. Fortin Charitable Foundation postdoctoral fellowship and acknowledges funding support from the Simeon J. Fortin Charitable Foundation, Bank of America Private Bank co-trustee.

Data-sharing statement

All the data is available from the corresponding author upon request. RNA-sequencing data has been deposited under GEO accession number GSE223598. The remaining data are included in this published article and its Online Supplemental Appendix. The Online Supplementary Appendix is available at Haematologica's website.

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