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Running heads: decitabine increases BCL-2 dependence

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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 (DBP), 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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 \textit{BCL2} gene is often subjected to chromosomal translocation t(14;18)(q32;q21) which places the \textit{BCL2} gene under the control of immunoglobulin heavy chain (IgH) enhancer\textsuperscript{8}, leading to high BCL-2 expression. Increased expression of BCL-2 can also be seen in ABC DLBCL, likely due to copy number alterations\textsuperscript{1–3}. Thus, BCL-2 is a potential therapeutic target in several genetic subtypes of DLBCL.

Pre-clinical studies have investigated anti-growth effect of the BCL-2 selective inhibitor, venetoclax, in DLBCL cell lines and cell line-derived xenograft mouse models\textsuperscript{9–11}. Notably, DLBCL cell lines required a higher dosage of venetoclax to kill the cells than other types of lymphoid malignancies\textsuperscript{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%\textsuperscript{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.

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\textsuperscript{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 \textit{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\textsuperscript{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\textsuperscript{16} and outperformed azacitidine in antileukemic action in a rat model of myeloid leukemia\textsuperscript{16}. However, mechanistic studies evaluating 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 (DFCI)) and grown in RPMI 1640 media (Gibco 11875119, Billings, MT, USA) supplemented with 10% FBS (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 and maintained in the above medium with 0.5µM of venetoclax. All cell lines were authenticated using STR profiling at the Molecular Diagnostics Laboratory (DFCI) 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 20ng/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% CO₂ atmosphere.

BH3 profiling. BH3 profiling was performed by flow cytometry as described previously. 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 delta percent priming (cytochrome c loss in response to drug treatment minus cytochrome c loss in response to DMSO).
Xenografts. All murine studies were performed according to DFCI Institutional Animal Care and Use Committee approved protocol. NOD/SCID/Il2R-/- (NSG) mice were purchased from the Jackson Laboratory. 5 x 10^6 of luciferase-labeled OCI-Ly1 cells were resuspended in 100µl of 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 4 cohorts and treated with: (1) vehicle control; (2) 0.25 mg/kg decitabine in PBS, IP, 5 consecutive days per week; (3) 50 mg/kg venetoclax in 60% Phosal 50PG, 30% PEG400, 10% ethanol, orally, daily; (4) 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 supplemental file.

**Results**

**DLBCL 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, 1 GCB DLBCL cell line, OCI-Ly7, was BCL-2 deficient while the other cell lines had comparable BCL-2 expression level (Figure 1A, S1).

To investigate the dependence of DLBCL on the 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. 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.

Decitabine primes DLBCL 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, S2). Given that azacitidine and decitabine function via different mechanisms and decitabine was shown to be more potent than azacitidine in pre-clinical studies, we decided to focus our study on decitabine. 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. 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 (Figure S3A) and enhanced caspase 3 cleavage (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 1 ABC DLBCL (TMD8), 2 GCB DLBCL (OCI-Ly1, SUDHL4) and 1 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 (Figure S4A). The combination of decitabine and S63845 induced synergistic cell killing in OCI-Ly1, TMD8 and DW20 but not SUDHL4 (Figure S4B).

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 (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 (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 3 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, 3C). 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 DLBCL cells**

Decitabine regulates gene transcription through DNA hypomethylation. 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 (1µM) 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). 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 (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 pro-survival signal in multiple genetic subgroups of DLBCL\textsuperscript{11}. Glycogen synthase kinase 3β (GSK3\textbeta) is among one of the first identified substrates of AKT\textsuperscript{21}, and its phosphorylation by AKT leads to its inactivation. A recent study found that active mTOR1 prevented nuclear localization of GSK3\textbeta\textsuperscript{22}, leading to the stabilization of the nuclear substrates of GSK3\textbeta. Thus, the downregulation of PI3K-AKT-mTOR1 pathway by decitabine could potentially activate GSK3\textbeta. Indeed, we found decreased phosphorylation of GSK3\textbeta serine 9 in both cell lines following decitabine treatment, indicating increased GSK3\textbeta activity (Figure 4C). Additionally, we noted decreased expression of c-Myc, one of the substrates of GSK3\textbeta (Figure 4C). c-Myc downregulation in response to decitabine treatment has been reported in other types of cancer as well\textsuperscript{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 membrane 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 5 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\textsuperscript{25}. We asked if decitabine and venetoclax would have similar effect on DLBCL cells. To evaluate this, we measured the oxygen consumption rate (OCR) in 4 DLBCL cell lines in response to decitabine. Decitabine alone did not change OCR in OCI-Ly1 parental or OCI-Ly1R cells (Figure 4F), suggesting that protein expression level does not necessarily correlate with enzymatic activity of
ETC complex, and thus should not be used to predict functional change. 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 (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 DLBCL

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. 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. We confirmed upregulation of SMAD1 mRNA levels following decitabine treatment in 3 DLBCL cell lines (Figure 5A). Even though SMAD1 protein level remained largely unaffected, the level of phospho-SMAD1 (s463, s465) increased (Figure 5B, S7A) in response to decitabine treatment, indicating increased SMAD1 activity. Next, we asked whether SMAD1 activation underlies the synergism observed between decitabine and venetoclax. 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, 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, 5D). Knockdown of SMAD1 similarly made the cells less sensitive to the combination of decitabine and venetoclax.
(Figure 5E, 5F). In another two cell lines, TMD8 and SUDHL4, inhibition of TGF-β signaling had partial or no rescue on cell proliferation and apoptosis (Figure S7B, S7C). 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 DLBCL xenograft mouse model**

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 4 groups of 6~7 mice each. Each group then received one of the following treatments, 1) vehicle control; 2) decitabine, 0.25mg/kg, IP, 5 days on/2 days off; 3) venetoclax, 50mg/kg, orally, daily; 4) 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, 6B) 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 one 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 (Figure S3A, 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 drives 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 way to predict response to BH3-mimetic drugs. As a cell line usually concurrently expresses more than one anti-apoptotic BCL-2 family proteins, 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 mimetics-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 1) 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. 2) 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 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 \(\gamma\)H2AX\(^{20}\). In DLBCL, we found that decitabine induced DNA double strand breaks as revealed by increased \(\gamma\)H2AX, suggesting activation of ATM. However, the mechanism 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 GSK3\(\beta\), leading to mitochondria-dependent apoptosis\(^{33}\). Notably, studies from our group found that PP2A plays a major role in the
phosphorylation status of anti-apoptotic proteins and affects apoptotic priming in DLBCL cells. 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.
References


Figure Legends

Figure 1. DLBCL displays dependence on multiple BCL-2 family members. (A) expression of the indicated BCL-2 family proteins in DLBCL cell lines by western blotting. 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) DBP of DLBCL cell lines treated with single BH3 mimetic for 24 hours, data were presented as delta 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 DMSO treated cells. Data were presented as mean ± SD, n=4. DBP, dynamic BH3 profiling.

Figure 2. Decitabine increases mitochondrial apoptotic priming and synergizes with venetoclax in suppressing the growth of DLBCL cells. (A) DBP of DLBCL cells treated with DMSO control or 2.5µM of decitabine for 2 days. Data were presented as delta percent priming. (B-D) 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 ABC DLBCL cell lines (C) and 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 ± SD, n=4. (E) Combination index (CI) was calculated by Compusyn. DBP, dynamic BH3 profiling. DHL-PDX, double hit lymphoma-patient derived xenograft.
Figure 3. Decitabine induces DNA damage and regulates the expression and activity of BCL-2 family proteins. (A) DBP of DLBCL cells treated with DMSO control or 1µM of doxorubicin for 2 days. Data were presented as delta percent priming. (B) DLBCL cells were treated with DMSO or 2.5µM of decitabine for 3 days, cells were lysed for western blotting analysis of indicated proteins. 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 2.5µM 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 2.5µM 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. DBP, dynamic BH3 profiling.

Figure 4. Decitabine regulates gene expression in DLBCL. (A) Venn diagram of upregulated and downregulated genes in venetoclax sensitive (OCI-Ly1 parental) and resistant (OCI-Ly1R) cells treated with 1µM of decitabine for 3 days. Cut-off threshold: p-adjusted value ≤ 0.05, absolute (log2FC) ≥1, n=3. (B) Decitabine significantly downregulates PI3K-AKT-MTOR pathway as revealed by GSEA. (C) Expression of indicated proteins in cells treated with DMSO or 0.5µM of decitabine for 4 days. 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 0.5µM of decitabine for 4 days. (F) Basal OCR was measured by Seahorse XFe96 analyzer in 3 DLBCL cell lines (OCI-Ly1, OCI-Ly1R, TMD8) and 1 DHL-PDX cell line (DW20) treated as indicated. Decitabine, 0.5µM 3 days. Venetoclax,
1nM for OCI-Ly1, 5nM for DW20, 50nM for OCI-Ly1R and TMD8,1 day. Data were presented as mean ± SD, n=5. P value was calculated by one-way ANOVA with multiple comparisons test. GSEA, gene set enrichment analysis. OCR, oxygen consumption rate. DHL-PDX, double hit lymphoma-patient derived xenograft.

Figure 5. Inhibition of TGF-β signaling partially blocks the synergism between decitabine and venetoclax. (A) DLBCL cell lines were treated with DMSO or 2.5µM of decitabine for 3 days. SMAD1 transcript level was measured by qPCR. Data were presented as mean ± SD, n=3. P value was calculated by student’s T-test. (B) OCI-Ly1 cells were treated with 2.5µM of decitabine, 4µM 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 PI/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) shRNA-mediated knockdown of SMAD1 in OCI-Ly1 cells. Knockdown efficiency was confirmed by western blotting with anti-SMAD1 antibody, GAPDH was used as loading control. (F) OCI-Ly1 cells expressing control shRNA (shCTRL) or shSMAD1 were treated with 2.5µM of decitabine and 0.01µM 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 PI/Annexin V assay. Data were presented as mean ± SD, n=3. P value was calculated by one-way ANOVA with multiple comparisons test.
Figure 6. Combination of decitabine and venetoclax are synergistic in DLBCL cell line-derived xenograft model. NSG mice transplanted with luciferized mCherry+ OCI-Ly1 cells were treated with vehicle control (n=6), decitabine (0.25mg/kg IP, 5 days on/2 days off, n=6), venetoclax (50mg/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 ± SEM. 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). P value, control vs venetoclax 0.0216, control vs decitabine 0.0015, control vs combination 0.0006, venetoclax vs combination 0.0007, decitabine vs combination 0.0007. P value was calculated by log-rank test.
Supplemental Information

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

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BH3 profiling. BH3 profiling was performed by flow cytometry, as described previously. Briefly, DLBCL cells were exposed to 0.002% digitonin and BH3-only peptides for 60 min in MEB2 buffer (150 mM Mannitol, 10 mM HEPES-KOH pH 7.5, 150mM KCl, 1mM EGTA, 1mM EDTA, 0.1% BSA, 5mM Succinate, Polaxamer188 2.5g/L). After formaldehyde fixation and neutralization, fluorescent antibodies Alexa Fluor 488 anti-cytochrome c (Biolegend 612310, San Diego, CA, USA), and Hoechst 33364 (Invitrogen H3570, Waltham, MA, USA) were added. Cells were incubated with antibodies for at least 24 hours before being analyzed by flow cytometry.

Western blotting. Cells were lysed in CelLytic Cell Lysis Reagent (Sigma C2978, Burlington, MA, USA). The following antibodies were used: BCL-2 (Biolegend, 658702), MCL-1 (Cell Signaling Technology (CST), 94296, Danvers, MA, USA), BFL-1 (Abcam, ab45413, Waltham, MA, USA), BCL-xL (CST, 2764), BIM (CST, 2933), BAK (BD Biosciences, 556382, NJ, USA), BAX (Proteintech,50599-2-Ig, Rosemont, IL, USA), PUMA (CST, 12450), c-Myc (CST, 5605), phospho-GSK3β (CST, 9336), GSK3β (CST, 9832), total OXPHOS rodent WB antibody cocktail (Abcam, ab110413), DNMT1 (CST, 5032), γH2AX (CST, 2577), β-actin (CST, 5125), GAPDH (Proteintech, HRP-60004), SMAD1 (CST, 6944), phospho-SMAD1 (Abcam, ab214423). After blotting with the HRP-conjugated secondary antibody, the membrane was developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, 34580, Waltham, MA, USA). Position of protein marker (Bio-Rad, 1610375, Hercules, CA, USA) was indicated in the figure. Image J was used to quantify protein bands.

Drug treatments. Cells were treated with decitabine (Sigma, A3656), venetoclax (Selleckchem, S8048, Houston, TX, USA), S63845 (Selleckchem, S8383), A1331852 (Selleckchem, S7801),
LY2109761 (Selleckchem, S2704) and doxorubicin (Sigma, D1515) when needed. Combination indexes for drug treatment were calculated by CompuSyn (ComboSyn Inc, Paramus, NJ, USA) using the Chou-Talalay method\(^3\).

Cell cycle analysis. Cell cycle was analyzed with BD Pharmingen BrdU Flow Kits (552598). Briefly, cells were pulsed with BrdU for 3 hours, followed by fixation and permeabilization procedures. Then, the cells were stained with APC-conjugated anti-BrdU antibody, followed by 7-AAD staining for the total amount of DNA. Cells were then analyzed by flow cytometry. DNA contents were analyzed using FlowJo software (FlowJo, LLC).

Apoptosis. Cell apoptosis was measured using Propidium Iodide (PI) (invitrogen, P1304MP), and Alexa Fluor 488 anti-Annexin V (Invitrogen, A13201) staining following manufacturer’s protocol. Cells were then analyzed by flow cytometry.

Cell viability assay. Cell viability was measured using CellTiter-Glo 2.0 Assay (Promega, G9242, Madison, WI, USA) following manufacturer’s instructions. Luminescence signal was read using SpectraMax M3.

SMAD1 knockdown. Short hairpin shRNA in the pLKO.1 lentiviral vector targeting SMAD1 was purchased from Horizon Discovery (RHS3979-201752806, Cambridge, UK).

Immunoprecipitation. 20 million DLBCL cells were lysed with 1ml of CHAPS lysis buffer (40mM Hepes, pH 7.5, 120mM NaCl, 1mM EDTA, 0.3% CHAPS). CHAPS-containing lysates were incubated with 5µg of BAX antibody (Proteintech, 50599-2) or IgG isotype control (CST, 2729) overnight before adding Dynabeads Protein G (Invitrogen, 10003D) for another 3 hours. After washing the beads with lysis buffer, bound proteins were analyzed by western blotting.
BAX and BAK activity. To assess the activity of BAX and BAK, cells were fixed and permeabilized using BD Fixation/Permeabilization Kit (554714). Then cells were stained with conformation specific mouse-anti BAX (invitrogen, MA5-14003), mouse-anti BAK (BD Biosciences, 556382), or mouse IgG1 isotype control (Invitrogen, MA1-10407). Staining was visualized by adding Alexa Fluor 488 anti-mouse IgG (Invitrogen, A-11029) and flow cytometry.

Oxygen consumption rate. Oxygen consumption rate was measured by Seahorse bioscience extracellular flux XFe96 using Agilent (Santa Clara, CA, USA) Seahorse XF Cell Mito Stress Test Kit (103015-100). 1μM of oligomycin, 1μM of Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), 0.5μM of rotenone/antimycin A were used for OCR measurements.

RNA-seq analysis. Total RNA was extracted using GeneJET RNA Purification Kit (ThermoFisher Scientific, K0731) according to the manufacturer’s protocol. DNBSEQ stranded mRNA library was prepared and sequenced using service from BGI Genomics. For RNA-seq analysis, mapping, alignment, and differential expression were analyzed on the Galaxy public server (usegalaxy.org)⁴. Briefly, raw reads were aligned to the human reference genome (UCSC hg38) with HISAT2. Transcripts genes were assembled with StringTie. Differential expressions were analyzed with DESeq2. GSEA was performed locally by GSEA software (V4.0.3) (http://software.broadinstitute.org/gsea/index.jsp). For the GSEA analysis, molecular signatures databases h.all.v5.2 symbols.gmt was used. Additional 17 gene sets of mitochondrial functions, biogenesis, and electron transport chain were downloaded from the molecular signatures databases (available upon request). RNA-sequencing data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE223598.


Figure S1. Quantification of BCL2 expression level in DLBCL cell lines. Experiments were performed as indicated in figure 1A. Image J was used to quantify protein band intensity. In each experiment, BCL2 level was normalized to GAPDH loading control first, then normalized to BCL2 expression in TMD8 cell line. Data were presented as mean ± SD, n=3.
Figure S2. DBP of DLBCL cells treated with DMSO control or azacitidine at indicated dosages for 18 hours. Data were presented as delta percent priming.
Figure S3. Combination of decitabine and venetoclax enhances apoptosis of DLBCL cells. (A) TMD8 and SUDHL4 cells were treated with indicated dosages of decitabine for 3 days, and venetoclax was added on the last day. Cell viability was determined by PI/Annexin V assay. Data were presented as mean ± SD, n=4. P value was calculated by two-way ANOVA. (B) DLBCL cell lines were treated with 0.5µM of decitabine for 3 days and/or 50nM of venetoclax for 1 day (10nM of venetoclax for OCI-Ly1 cells), and cells were lysed for western blotting analysis of cleaved caspase 3. GAPDH was used as loading control.
Figure S4. Combination of decitabine and A1331852 (A) or S63845 (B) in DLBCL cells. DLBCL cell lines were treated with indicated dosages of decitabine for 3 days, and A1331852 or S63845 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=3. Combination index (CI) was calculated by Compusyn.
Figure S5. Decitabine induces DNA damage and leads to cell cycle arrest in DLBCL cells. (A) DLBCL cells were treated with DMSO or 0.5µM of decitabine for 3 days, and lysed for western blotting analysis of DNMT1 and phosphorylation of H2AX. β-actin was used as loading control. (B) Cell cycle analysis of DLBCL cells treated with DMSO or 0.5µM of decitabine for 3 days. Data were presented as mean ± SD, n=2. P value was calculated by student’s T-test, * p<0.05, ** p<0.01.
Figure S6. Decitabine regulates gene expression in DLBCL cells. (A) Significantly upregulated and downregulated gene signatures revealed by GSEA in venetoclax sensitive (OCI-Ly1 parental) and resistant (OCI-Ly1R) cells treated with 1µM of decitabine for 3 days. Normalized enrichment score (NES) was calculated for each gene set and shown in the figure. (B) Significantly downregulated gene signatures in OCI-Ly1R cells compared to OCI-Ly1 parental cells.
Figure S7. Inhibition of TGF-β signaling partially blocks the synergism between decitabine and venetoclax. (A) DLBCL cell lines TMD8 and SUDHL4 were treated with 2.5μM of decitabine, 4μM of TGF-β receptor type I/II (TβRI/II) dual inhibitor, LY2109761 (ly210), alone or in combination for 3 days, phospho-SMAD1(s463/s465) and total SMAD1 were measured by western blotting. * non-specific band. β-actin was used as loading control. (B-C) DLBCL cell lines 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 (B). Cell viability was determined by PI/Annexin V assay. Data were presented as mean ± SD, n=3 (C). P value was calculated by one-way ANOVA with multiple comparisons test.