Strategic therapeutic targeting to overcome venetoclax resistance in aggressive B-cell lymphomas

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Abstract

**Purpose:** B-cell lymphoma-2 (BCL-2), an anti-apoptotic protein often dysregulated in B-cell lymphomas, promotes cell survival and provides protection from stress. A recent Phase I first-in-human study of the BCL-2 inhibitor venetoclax in non-Hodgkin lymphoma showed an overall response rate of 44%. These promising clinical results prompted our examination of the biological effects and mechanism of action underlying venetoclax activity in aggressive B-cell lymphoma, including mantle cell lymphoma (MCL) and diffuse large B cell lymphoma (DLBCL).

**Experimental Design:** MCL and DLBCL cell lines, primary patient samples, and *in vivo* patient-derived xenograft (PDX) models were utilized to examine venetoclax efficacy. Furthermore, the mechanisms underlying venetoclax response and the development of venetoclax resistance were evaluated using proteomics analysis and Western blotting.

**Results:** Potential biomarkers linked to venetoclax activity and targeted combination therapies that can augment venetoclax response were identified. We demonstrate that DLBCL and MCL cell lines, primary patient samples, and PDX mouse models expressing high BCL-2 levels are extremely sensitive to venetoclax treatment. Proteomics studies showed that venetoclax substantially alters the expression levels and phosphorylation status of key proteins involved in cellular processes, including the DNA damage response, cell metabolism, cell growth/survival, and apoptosis. Short- and long-term exposure to venetoclax inhibited PTEN expression, leading to enhanced AKT pathway activation and concomitant susceptibility to PI3K/AKT inhibition. Intrinsic venetoclax-resistant cells possess high AKT activation and are highly sensitive to PI3K/AKT inhibition.

**Conclusions:** These findings demonstrate the on-target effect of venetoclax and offer potential mechanisms to overcome acquired and intrinsic venetoclax resistance through PI3K/AKT inhibition.
Translational Relevance

Aggressive B-cell lymphomas result in morbidity and mortality worldwide, primarily due to therapeutic resistance; therefore, the identification of novel treatment strategies is necessary to address this clinical need. Here, we demonstrate the efficacy of the BCL-2 inhibitor venetoclax in mantle cell lymphoma (MCL) and diffuse large B-cell lymphoma (DLBCL) in both in vitro and in vivo patient-derived cancer models. Furthermore, we elucidate the mechanisms underlying venetoclax resistance and identify therapeutic combinations that can be utilized to treat this resistance by targeting the PTEN/PI3K/AKT/mTOR pathway, which we found to be upregulated in venetoclax-resistant cells. Ultimately, this work demonstrates a significant potential therapeutic treatment option for patients with aggressive B-cell lymphoma who are venetoclax-resistant.
Introduction

Impaired apoptosis has been shown to play an important role in the tumorigenesis of a large number of cancers (1,2). Apoptosis is closely regulated by the B-cell lymphoma 2 (BCL-2) family of proteins, and BCL-2, one of the first identified members of this family, plays an anti-apoptotic role by binding and neutralizing BAX and BAK as well as other pro-apoptotic proteins, including the cellular stress sensors BIM, BID, Puma, BAD, and Noxa (3-5). Downregulation or inhibition of BCL-2 allows pro-apoptotic proteins to permeabilize the mitochondrial membrane, releasing cytochrome c, a hallmark of mitochondria-controlled apoptosis. In cancers, the ratio of BCL-2 expression to pro-apoptotic proteins such as BAX, BAK, and BIM can determine the sensitivity of the cancer cells to therapy (6), suggesting that high BCL-2 expression confers drug resistance and provides a survival advantage to the tumor cells. In addition, BCL-2 is commonly overexpressed in a variety of human neoplasms via diverse genetic and epigenetic mechanisms (7).

This study focused on two types of B-cell lymphoma, diffuse large B-cell lymphoma (DLBCL) and mantle cell lymphoma (MCL). DLBCL is a molecularly heterogeneous disease in which approximately 30-40% of DLBCL cases are characterized by the BCL-6/3q27 translocation. Furthermore, approximately 20% and 10% of DLBCL cases are characterized by translocations involving BCL-2/t(14;18) and MYC/8q24, respectively (8,9). Moreover, 5% of DLBCL cases have both BCL-2 and MYC translocations and are referred to as double hit lymphomas (DHL), which are clinically difficult to treat (10,11). MCL is a rare but distinct subset of B-cell non-Hodgkin lymphoma that is primarily characterized by a t(11;14) chromosomal translocation that results in cyclin D1 overexpression and cell cycle dysregulation (12). However, additional pathways regulate MCL progression, including the BCL-2-mediated anti-apoptotic pathway (12). Furthermore, BCL-2 inhibition has demonstrated efficacy in MCL (12,13). Overall, BCL-2 may be an effective target to reduce the tumorigenicity of both DLBCL and MCL.

Venetoclax (ABT-199), a small molecule oral drug that selectively targets BCL-2, was recently approved by the United States Food and Drug Administration for the treatment of patients with chronic lymphocytic leukemia (CLL) (14). Venetoclax was derived from the first-generation BH3 mimetic navitoclax (ABT-263), known to inhibit many BCL-2 family members, including
BCL-2, BCL-xL, and BCL-w (15). ABT-263 displayed anti-tumor activity in various relapsed/refractory B-cell malignancies, including CLL and non-Hodgkin lymphomas (NHL); however, inhibiting BCL-xL caused severe thrombocytopenia and prevented this agent from being optimized for clinical use (16). Preclinically, venetoclax has been shown to have greater specificity and selectivity for BCL-2 compared with BCL-xL (14). This selectivity has been supported by the results of an ongoing Phase I clinical trial showing that venetoclax has high activity rates in relapsed/refractory CLL and NHL without thrombocytopenia (17). The promising preclinical and clinical activity of venetoclax led us to hypothesize that targeting BCL-2 with this compound would be effective in DLBCL and MCL. We assessed the anti-tumor activity of venetoclax in DLBCL and MCL cell lines and primary patient cell cultures, correlated BCL-2 and related protein levels with venetoclax activity, analyzed the effects of venetoclax in vivo in our primary MCL or DLBCL-bearing patient-derived xenograft (PDX) models, and elucidated the potential mechanisms associated with acquired venetoclax resistance.

Materials and methods

Cells and reagents

DLBCL cell lines (MS, DS, DBr, JM (McA), FN, HF, HB, MZ, LR, CJ, LP, LVP-03, and RC) and MCL cell lines (Mino, JMP-1, and PF-1) were established in our laboratory and have been previously characterized (18-21). The Pfeifer, Maver-1, Jeko-1, DB-sp53, JVM-13, Z-138, REC-1, and Granta cell lines were purchased from ATCC (Manassas, VA, USA). The DLBCL cell lines U-2932, OCI-LY19, DOHH2, Toledo, SUDHL-4, SUDHL-10, HBL-1, TMD-8, DB, HT, OCI-LY10, and OCI-LY3 were obtained from Drs. Michael Rosenblum and R. Eric Davis (UT MD Anderson Cancer Center) (22,23). All cell lines were routinely tested for mycoplasma using a MycoSEQ™ Mycoplasma Detection kit (Invitrogen, Carlsbad, CA, USA) and were validated by short tandem repeat DNA fingerprinting at the Characterized Cell Line Core Facility at The University of Texas MD Anderson Cancer Center. Stocks of authenticated cell lines were stored in liquid nitrogen, and all cell lines used in these studies were obtained from these authenticated stocks and thawed within six months of the performed experiments.

Primary MCL and DLBCL cells were isolated from patient samples obtained through a protocol approved by the Institutional Review Board at MD Anderson Cancer Center after the obtainment of written informed consent. All studies utilizing patient samples followed the ethical guidelines.
put forth in the Declaration of Helsinki. The cells were cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA) containing 15% fetal calf serum (Gibco) and 1% penicillin/streptomycin (HyClone, Logan, UT, USA). Venetoclax, idelalisib, and MK-2206 drug stocks were purchased from Selleckchem (Houston, TX, USA). KA2237 was provided by Karus Therapeutics, Oxfordshire, UK. ACP-319 was provided by Acerta Pharma, Redwood City, CA.

Viability assays and apoptosis measurement
Cells from representative DLBCL and MCL cell lines were plated at 5,000 cells per well. The cells were incubated for 72 hours in 20 µL medium with 10% FBS and the compounds or dimethylsulfoxide (DMSO) at various concentrations. The assays were performed using the Celltiter-Glo Luminescent Cell Viability Assay according to the manufacturer’s instructions (Promega, Madison, WI, USA). To determine the half maximal inhibitory concentration (IC\textsubscript{50}), 6-8 concentration points (two-fold increase) were chosen for each cell line so that the IC\textsubscript{50} point was approximately in the middle of the concentration range. Experiments were performed 2-3 times independently, and each concentration was tested in triplicate. For the apoptosis assays, the cells were incubated for 24 or 48 hours with the appropriate venetoclax dilution or DMSO was added. The assay was performed using Annexin V/PI staining followed by flow cytometry analysis. The experiment was performed 3 times, and every concentration point was tested in duplicate.

Establishment of venetoclax-resistant lymphoma cell lines
We generated venetoclax-resistant (VR) MCL and DLBCL cell lines (Mino-VR, Rec-1-RV, and RC-VR) from the parental cell lines (Mino, Rec-1, and RC) by multistep exposures of cells to increasing doses (up to 100 nM) of venetoclax for 8 weeks. Briefly, cells were initially cultured at a low drug concentration for 1 week and then maintained in drug-free medium for 1 week to stabilize the cells. Medium with increasing drug concentration was changed every other week during the selection, and subsequently, the cells became resistant to venetoclax. The resistant clones were expanded in drug-free media. The parental cell lines were also cultured simultaneously in the absence of venetoclax to control for any effects of long-term culturing. Expanded clones were retested for drug resistance before any further studies.
Western blot analysis
Whole cell or nuclear extracts were solubilized with 1% sodium dodecyl sulfate (SDS) buffer and were subjected to SDS polyacrylamide gel electrophoresis on a 4%–15% gel (Bio-Rad, Hercules, CA, USA). We transferred proteins onto PVDF membranes and probed with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using the ECL system (Amersham, Little Chalfont, United Kingdom). Antibodies against BCL-2, caspase 3, PARP, γH2AX, AKT, PTEN, and pAKT were purchased from Cell Signaling Technology (Danvers, MA, USA).

Transient transfection and siRNA
Transient transfections in cultured lymphoma cells were conducted using the Neon Transfection System (Thermo Fisher Scientific) and representative DLBCL cells as previously described. (19) Pre-designed and validated AKT siRNA (S659, S660, S661) and control siRNA were purchased from ThermoFisher Scientific (Waltham, MA, USA).

Reverse-phase Protein Array (RPPA)
The MD Anderson Cancer Center RPPA Core Facility performed the RPPA analysis and antibody validation (24). In brief, cells were washed twice with ice-cold phosphate-buffered saline supplemented with complete protease and PhosSTOPphosphatase inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany) and 1 mM Na$_3$VO$_4$ to prepare the total protein lysates. Next, the cells were lysed, and the cell lysates were vortexed on ice, centrifuged, and adjusted to a 1 μg/μL concentration. A serial dilution of 5 concentrations was printed, with 10% of the samples replicated for quality control (2470 Arrayer; Aushon Biosystems, Billerica, MA, USA) on nitrocellulose-coated slides (Grace Bio-Labs, Bend, OR, USA). Immunostaining was performed using a DakoCytomation-catalyzed system and diaminobenzidine colorimetric reaction. Overall, 285 antibodies and 4 secondary antibody negative controls were analyzed (Supplemental table or website).

In vivo venetoclax treatment in PDX models
Six- to eight-week-old female NOD SCID IL2Rγ null (NSG) mice (Jackson Laboratory, Bar Harbor, ME, USA) were used to create the PDX models, and all experimental procedures and protocols were approved by The University of Texas MD Anderson Institute Animal Care Committee. As described previously(25), fresh human fetal bones of 17-19 gestational weeks (Advanced Bioscience Resources, Alameda, CA, USA) were subcutaneously implanted into (PT 2) SCID or NSG mice (SCID-hu or NSG-hu). Approximately 4 to 6 weeks following implantation, one injection of $5 \times 10^6$ freshly isolated tumor cells from MCL or DLBCL patient samples were administered directly into the human fetal bone implants within the SCID-hu NSG-hu hosts. Mouse serum was collected, and via a human $\beta_2$-microglobulin ($\beta_2$M) ELISA kit (Abnova Corporation, Walnut, California, USA), circulating human $\beta_2$M levels in the mouse serum were used to monitor tumor engraftment and burden. Once tumor growth was detected in the mice, the mice were randomized into groups of 5 mice each for drug treatment, and after treatment, tumor burden was evaluated by measuring either tumor volume or human $\beta_2$M levels to determine therapeutic efficacy. After sacrifice, the tumor masses were collected and subjected to H&E staining and anti-human BCL-2 (Abcam, Cambridge, MA, USA) immunohistochemical staining (IHC).

**Statistical analysis**

The IC$_{50}$ values were calculated using CalcuSyn software (Biosoft, Cambridge, MA, USA) for each cell line. Spearman’s rank correlation coefficient with t-test was used to evaluate the correlation between venetoclax IC$_{50}$ values and the indicated protein expression levels. The Cochran-Mantel-Haenszel chi-squared test was applied to examine the correlation between venetoclax sensitivity and dichotomized BCL-2 expressing conditional tumor types.

Relative protein levels for each sample in the RPPA analysis were determined by interpolation of each dilution curve from the “standard curve” using R package SuperCurve. All data points were normalized for protein loading and transformed to linear values. Normalized linear values were transformed to log2 values, and then median-centered for hierarchical cluster analysis and heatmap generation. The heatmap was generated in Cluster 3.0 (http://cluster2.software.informer.com/3.0/) as a hierarchical cluster using Pearson Correlation.
and a center metric. The resulting heatmap was visualized in Treeview (http://rana.lbl.gov/EisenSoftware.htm) and presented as a high resolution bmp format. GraphPad Prism 5.04 (GraphPad Software, Inc., La Jolla, CA, USA), R 3.1.2 with packages SuperCurve v1.4.4, BioNet v1.26.0 and nlme v3.1-120 were used for statistical analyses. \( P \) values of < 0.05 were considered significant.

**Results**

**Venetoclax sensitivity in aggressive B-cell lymphomas correlates with BCL-2 protein expression levels**

To determine the therapeutic efficacy of the BCL-2 inhibitor venetoclax in aggressive B-cell lymphomas, we exposed 36 representative aggressive B-cell lymphoma cell lines (26 DLBCL and 10 MCL) to increasing drug concentrations of venetoclax for 72 hours and measured cell viability (Supplementary Fig. S1). The IC\textsubscript{50} values of venetoclax in these cell lines ranged from 1 nM-10 \( \mu \text{M} \) (Table 1). Cell lines with IC\textsubscript{50} values of 100 nM (achievable concentration *in vivo*) and below were considered sensitive to venetoclax, and cell lines with IC\textsubscript{50} values greater than 100 nM were considered resistant. Overall, 8/15 (53%) GCB-DLBCL, 3/8 (38%) non-GCB-DLBCL, and 8/10 (80%) MCL cell lines were sensitive to venetoclax (IC\textsubscript{50} \( \leq \) 100 nM), whereas the other cell lines were more resistant to venetoclax (IC\textsubscript{50} > 100 nM) (Fig. 1A). Notably, 3/3 (100%) DHL cell lines (RC, U-2932, and CJ) were highly sensitive to venetoclax (IC\textsubscript{50} < 25 nM). No significant differences in venetoclax sensitivity were observed between the GCB and non-GCB subtypes of DLBCL (\( p = 0.4382; \) Fig. 1B, first panel) or between DLBCL and MCL (\( p = 0.5722; \) Fig. 1B, second panel). Next, we tested whether a correlation exists between venetoclax sensitivity and BCL-2 expression that can support the on-target action of killing via competition for the BCL-2 BH3-binding site. We used protein expression profiling by RPPA to detect BCL-2 expression (validated by Western blotting [Supplementary Fig. S1A]) in representative DLBCL and MCL cell lines, and correlated BCL-2 expression to venetoclax sensitivity. Most DLBCL and MCL cell lines (14/17 and 8/9, respectively) that expressed BCL-2 protein were sensitive to venetoclax; however, all of the DLBCL and MCL cell lines with low or no BCL-2 expression were highly resistant to venetoclax, showing a significant correlation between BCL-2 expression and venetoclax sensitivity (Table 1, \( p = 0.0008 \)). A significant correlation was also found
between BCL-2-positive vs. BCL-2-negative DLBCL and MCL cell lines (p = 0.0015; Fig. 1B, third panel) as well as between MYC/BCL-2 double hit (DH)/double expressor (DE) lymphoma vs non-DH/DE lymphoma groups (p = 0.0308; Fig. 1B, fourth panel). Three DLBCL cell lines (HBL-1, DB, and MZ) and one MCL cell line (Maver-1) expressed BCL-2 protein but were venetoclax-resistant (IC$_{50}$ > 500 nM; Fig. 1C).

Correlation between venetoclax sensitivity and the protein expression of BCL-2 family members in DLBCL cell lines

To determine whether additional BCL-2 family members play a role in venetoclax sensitivity, we used Spearman’s rank correlation coefficient to evaluate the correlation between venetoclax sensitivity (IC$_{50}$) and the protein expression levels of BCL-2 and related family members in 26 representatives DLBCL cell lines. Our results show that the protein expression levels of BCL-2, BIM, and BAK were significantly positively correlated with sensitivity to venetoclax, whereas the BCL-xL and BAD levels were negatively correlated with venetoclax sensitivity (all p < 0.05) (Fig. 1D). Protein levels of other related BCL-2 members such as BAX, BID, and MCL-1 were not statistically correlated with venetoclax sensitivity (Fig. 1D).

Venetoclax sensitivity in primary aggressive B-cell lymphoma cells and in vivo DLBCL and MCL PDX models

Venetoclax sensitivity was evaluated in 13 fresh primary samples collected from patients with DLBCL (n=4) and MCL (n=9) to validate the cell line findings. We observed considerable variation in venetoclax sensitivity among patient samples (Fig. 2A). Similar to the cell line findings, primary lymphoma cells with high BCL-2 expression were significantly more sensitive to venetoclax in comparison to cells with low/negative BCL-2 protein expression (Fig. 2B). We then evaluated the ability of venetoclax to suppress tumor growth in vivo in DLBCL and MCL PDX models established in NSG and SCID mice. In one MCL PDX model, the primary MCL cells were confirmed to be ibrutinib-sensitive by in vitro testing of the primary tumor cells (Supplementary Fig. S2; PT-2), and its PDX model was established after the primary tumor cells were injected into SCID-hu mice. These mice were then randomly divided into two groups (each n=5), with one group treated with venetoclax (50 mg/kg by daily oral gavage for 3 weeks) and the other treated with vehicle control. Mouse serum was collected and circulating human
β2M levels in the mouse serum were used to monitor tumor burden. As shown in **Fig. 2C upper panel**, venetoclax did not significantly lower the human β2M levels compared with the vehicle control group (p = 0.52), implying that this MCL PDX mouse model was venetoclax-resistant. The body weight was not different between the vehicle and venetoclax-treated groups (**Fig. 2C lower panel**, p > 0.05). We then found that the PDX tumor tissue did not significantly express human BCL-2 (**Fig. 2F, left panel**), indicating that low BCL-2 expression may underlie this venetoclax resistance as observed in the venetoclax-negative cell lines and patient samples. In another MCL PDX mouse model that was determined to be ibrutinib-resistant (**Supplementary Fig. S2; PT-6**), venetoclax significantly inhibited tumor growth (**Fig. 2D upper panel**, p = 0.028), showing that venetoclax can act lethally against ibrutinib-resistant MCL cells and may be an effective agent to treat ibrutinib-resistant MCL patients. Furthermore, IHC staining of PDX tumor tissue indicated high human BCL-2 expression in this PDX model (**Fig. 2F, middle panel**), again demonstrating that the presence of BCL-2 confers sensitivity to venetoclax. Finally, venetoclax had the same therapeutic effect against a DLBCL PDX mouse model (**Fig. 2E upper panel**, p = 0.04) that highly expressed human BCL-2 (**Fig. 2F, right panel**). The body weight was not different between the vehicle group and venetoclax-treatment group in venetoclax-sensitive MCL and DLBCL PDX mice (**Fig. 2D-E lower panel**, p > 0.05). These *in vivo* results show the potent activity of venetoclax in BCL-2-expressing DLBCL and MCL PDX mouse models.

### Venetoclax alters the expression levels of proteins related to apoptosis, DNA damage response, and metabolic processes in aggressive B-cell lymphomas

To identify a core venetoclax protein signature associated with sensitivity to venetoclax, we performed RPPA analysis of three representative DLBCL cells that were highly sensitive to venetoclax. As shown in the Venn Diagram (**Fig. 3A**) and heat map analysis (**Fig. 3B**), we identified a venetoclax signature that overlaps among three venetoclax-sensitive DLBCL cell lines (RC, U-2932, and HF) that is comprised of 30 commonly upregulated proteins and 60 commonly downregulated proteins. This signature consisted of expression changes in various proteins involved in myriad cellular processes, including apoptosis (caspases 3 and 7), the DNA damage response/repair (histone 3, H2AX, CHK2, ATM), growth/survival signaling pathways (Src, MAPK, AKT), metabolism (Glutamate-D1,2, HK2, PDK1, PKM2, Tigar, GCLM),
mitochondria (TFAM, SOD2) and ubiquitination (UBAC1, TRIM25) (Fig. 3C). To further determine the mechanisms underlying the cellular response to venetoclax, we examined the protein expression patterns between untreated and venetoclax-treated (24 hr) MCL cell line (Mino; Supplementary Table S1) compared with the venetoclax-treated DLBCL cell lines. The commonly altered proteins between the DLBCL subset and the Mino cell lines are bolded and underlined in Fig. 3C, and these proteins suggest that the activation of apoptotic and DNA damage/repair processes as well as key growth and survival signaling pathways occurs due to venetoclax treatment. For controls, venetoclax-resistant MCL and DLBCL cell lines (Maver, JMP-1, and HT) were also analyzed by RPPA with similar venetoclax drug concentrations but did not show significant protein alterations (data not shown).

The RPPA data showed upregulation of apoptotic-related proteins in venetoclax-treated cells; therefore, we tested whether venetoclax induces apoptosis in B-cell lymphoma cells. We selected three representative cell lines, two with high BCL-2 expression (U-2932 and RC) and one with no BCL-2 expression (HT), and a venetoclax-sensitive primary DLBCL case (PT-12) to examine apoptosis. As shown in Fig. 3D, apoptosis was induced after venetoclax treatment in both a time- and dose-dependent manner. However, apoptosis of the BCL-2-negative cell line (HT) was not affected after venetoclax treatment (Fig. 3D). Using Western blotting, we further confirmed that venetoclax induces caspase 3, caspase 7, and PARP cleavage (Fig. 3E). Venetoclax-induced apoptosis was further validated in vivo, showing increased caspase-3 activation in tumor tissue sectioned from a venetoclax-treated MCL PDX mouse with sensitivity to venetoclax in comparison to tumor tissue sectioned from a vehicle-treated mouse, indicative of cells undergoing apoptosis in vivo after venetoclax treatment (Fig. 2G upper panel). In addition, venetoclax induced γH2AX expression (Fig. 3E), suggesting that venetoclax is capable of stimulating a DNA damage response in aggressive B-cell lymphomas, further supporting our RPPA findings.

**Short- and long-term exposure to venetoclax resulted in loss of PTEN and upregulation of the AKT pathway, sensitizing lymphoma cells to selective PI3K-AKT inhibition**

AKT was induced in venetoclax-treated cells as shown both in vitro (Fig. 3B) and in vivo (Fig. 2G bottom panel); therefore, we determined whether targeting the PI3K-AKT pathway in combination with venetoclax would produce a more robust effect than venetoclax alone or
sensitize venetoclax-resistant cells to venetoclax. Venetoclax synergized with PI3K inhibitors and an AKT inhibitor in two representative cell lines, RC (DLBCL) and Mino (MCL) (Fig. 4A). The inhibitors used were the PI3K-p110δ-targeted idelalisib and ACP-319, and the novel, dual, selective PI3K-p110β/δ-targeted KA2237, currently in Phase I clinical studies in patients with B-cell lymphoma; the AKT inhibitor employed was MK-2206, which targets AKT1, 2 and 3. In cell lines that do not express BCL-2 and are resistant to venetoclax [BJAB and HT (Fig. 1C)], the PI3K and AKT inhibitors did not therapeutically sensitize these cells to venetoclax (Supplementary Fig. S3A). However, PI3K inhibitors and the AKT inhibitor suppressed venetoclax-induced pAKT in venetoclax-sensitive cell lines (Fig. 4B), subsequently leading to robust synergistic apoptosis induction after treatment with the drug combinations (Fig. 4C). Notably, the dual, selective PI3K-p110β/δ inhibitor KA2237 showed the most synergistic effects in the cell line models; therefore, we selected KA2237 to test its synergistic activity with venetoclax in primary DLBCL cells (n=2) and found highly synergistic activity between the two agents (Fig. 4D). To demonstrate the critical role of AKT in conferring venetoclax resistance, AKT was knocked down in two venetoclax-sensitive cell lines (RC and Mino) and two venetoclax-resistant cell lines (BJAB and HT) using siRNA. Knock down of AKT sensitized venetoclax-sensitive cells but not venetoclax-resistant cells to venetoclax (Fig. 4E). Notably, knock down of AKT significantly reduced cell viability in venetoclax-resistant in comparison to venetoclax-sensitive lymphoma cells.

To examine whether acquired venetoclax resistance is also associated with the activation of the AKT pathway, we generated three venetoclax-resistant lymphoma cell lines by chronic exposure to venetoclax at increasing drug concentrations over time. The resistant lines were at least 10-fold more resistant to venetoclax than the parental cell lines (Fig. 5A). We conducted RPPA analysis of a representative venetoclax-resistant and parental cell line pair (Mino) and found that the phosphorylation of AKT was induced and the level of PTEN was reduced in the venetoclax-resistant Mino cell line compared with the parental line (Supplementary Fig. S4), and these findings were also confirmed in the three venetoclax-resistant cell lines (Fig. 5B). The levels of the PI3K isoforms, which are key molecules that function upstream of PTEN and AKT, were not significantly changed in the venetoclax-resistant cell lines. Finally, AKT activation in the venetoclax-resistant cell lines was inhibited by treatment with two PI3K inhibitors idelalisib and
KA2237 (Fig. 5C). Since KA2237 appeared to be more active against activated AKT, we tested whether the acquired venetoclax-resistant cell lines were more sensitive to KA2237 compared with the parental cell lines. Indeed, the acquired venetoclax-resistant cell lines were more sensitive to KA2237 treatment (Fig. 5D).

**Intrinsic venetoclax-resistant cells possess high AKT activation and are highly sensitive to PI3K inhibition**

Several cell signaling proteins involved in the PI3K/AKT signaling pathway were induced (Src, MEK1, MAPK, AKT, and JNK) or inhibited (PTEN) after short-term treatment with venetoclax, suggesting that venetoclax-treated cells activate compensatory pathways that may lead to the development of venetoclax resistance. To examine whether these proteins are potential predictors of intrinsic venetoclax resistance, we used Spearman’s rank correlation coefficient to evaluate the correlation between venetoclax sensitivity (IC50) and the protein expression levels of pSrc, pMEK1, pMAPK, pAKT, and pJNK in 26 representative DLBCL cell lines. Our results showed that the levels of Src and AKT phosphorylation were significantly positively correlated with venetoclax sensitivity (Fig. 6A), whereas the pMEK1, pMAPK, and pJNK levels were not significantly correlated with venetoclax sensitivity (Supplementary Fig. S4B), suggesting that intrinsic venetoclax resistant cells possess high AKT activation. Next, we used Spearman’s rank correlation coefficient to evaluate the correlation between the PI3K inhibitor KA2237 sensitivity (IC50) and the protein expression levels of AKT/mTOR pathway components in 26 representatives DLBCL cell lines. Our results show that the PTEN protein expression level was significantly positively correlated with sensitivity to KA2237, whereas the phosphorylated levels of AKT, mTOR, and p70-S6K were negatively correlated with KA2237 sensitivity (all p < 0.05) (Fig. 6B). Treatment with KA2237 in two representative DLBCL cell lines (BJAB and HT) with high AKT activation showed down-regulation of pAKT and activation of cleaved caspase 3 (Fig. 6C), indicating that single agent KA2237 can overcome intrinsic venetoclax resistance.

**Discussion**

Aggressive B-cell lymphomas cause substantial morbidity and mortality worldwide, primarily due to therapeutic resistance (26,27). Thus, there is a clear clinical need to identify novel treatment strategies for patients with refractory aggressive B-cell lymphomas. Targeted therapy
offers an exciting opportunity to extend the life of cancer patients. One excellent example is targeting the oncogenic protein BCL-2 with the BH3 mimetic venetoclax. Although venetoclax has shown promising preclinical and clinical efficacy in various cancers, its biological effect and mechanism of action are not fully elucidated.

We demonstrate that targeting BCL-2 with venetoclax is efficacious in aggressive B-cell lymphomas, including DLBCL and MCL, in both *in vitro* and *in vivo* models. Our findings strongly suggest that the presence of the anti-apoptotic BCL-2 primarily dictates cellular sensitivity to venetoclax in aggressive B-cell lymphomas. These data also show that the sensitivity of venetoclax in lymphoma cell lines is significantly correlated with BCL-2, BIM, and BAK protein expression levels, where cells with high expression of these proteins are more sensitive to venetoclax. BIM/BAK may bind to BCL-2, neutralizing BCL-2 through the BH3 binding site; therefore, targeting BCL-2 with venetoclax may dissociate BIM/BAK from BCL-2, allowing mitochondria-induced apoptosis via the release of cytochrome C (*Fig. 6D*). We also observed that cells with high BCL-xL and BAD protein levels are more venetoclax-resistant. One possible mechanism is the replacement of BCL-2/BIM/BAK complexes with BCL-xL/BAD complexes, preventing cells from undergoing apoptosis even in the presence of venetoclax. Therefore, targeting venetoclax-resistant cells with BCL-xL inhibitors, such as navitoclax, may potentially overcome venetoclax resistance.

We also showed that AKT was activated after short-term venetoclax treatment and in acquired/intrinsic venetoclax-resistant cells, suggesting that venetoclax-exposed cells may activate this pathway to promote survival and resistance. For instance, in some cancers, venetoclax resistance may occur via the upregulation of anti-apoptotic proteins such as BCL-xL and MCL-1(28), and the AKT signaling pathway has been shown to regulate BCL-xL and BAD expression (29). Indeed, recent studies have shown a synergistic effect of targeting BCL-2 with venetoclax in combination with an mTOR (a down-stream molecule of the AKT pathway) inhibitor in DLBCL cells (30). These findings demonstrate the importance of AKT activation in venetoclax-exposed cells and provide a strong rationale for combining an AKT or PI3K inhibitor – notably a selective PI3K-p110β/δ inhibitor – with venetoclax in aggressive B-cell lymphomas that are responding to venetoclax (31). Intrinsic venetoclax-resistant cells also acquired
activation of the AKT pathway, most likely via genetic defect and/or loss of PTEN, suggesting that in cells with no BCL-2 expression, the AKT pathway may activate BCL-xL and BAD to compensate for BCL-2 absence (Fig. 6D). However, AKT pathway inhibition does not sensitize these cells to venetoclax, indicating that targeting the AKT pathway in combination with a BCL-xL inhibitor may be an optimal therapeutic option (32,33). The mechanism(s) mediating AKT activation in venetoclax-treated cells may rely on PTEN inhibition; however, the mechanism mediating venetoclax downregulation of PTEN expression remains unknown and requires investigation. These studies also underscore the importance of PTEN in aggressive B-cell lymphomas, as its expression suppresses AKT activation, presumably through blocking upstream signals from chronic/tonic BCR-PI3K, found to be constitutively activated in a subset of aggressive B-cell lymphomas (34).

If venetoclax specifically targets BCL-2, then this agent should be effective in cancer cells that express BCL-2; however, this activity does not always occur. For example, follicular lymphoma expresses high BCL-2 due to the hallmark t(14;18) translocation; yet, the venetoclax clinical response rate is quite low in patients with follicular lymphoma, suggesting that BCL-2 expression alone is not sufficient to predict BCL-2 dependence (35). We identified a small subset of lymphoma cell lines that do express BCL-2 but are also resistant to venetoclax, which may be due to acquired mutations in BCL-2 and related family members or post-translational modifications of BCL-2, such as phosphorylation, preventing venetoclax from displacing BIM and BAX from BCL-2, thereby blocking the mitochondrial-mediated apoptosis pathway (36,37).

In addition to inducing apoptosis, venetoclax can also induce a DNA damage response, a mechanism that has not been described previously. Activation of a DNA damage response may be a consequence of cells undergoing apoptosis or a result of BCL-2 inactivation as BCL-2 has been shown to suppress DNA repair mechanisms (38,39). We also discovered that venetoclax treatment can downregulate key members of the glycolytic pathway, suggesting that BCL-2 has a metabolic function independent of its anti-apoptotic properties or that this downregulation may simply be a consequence of the altered cell proliferation exerted by venetoclax. BCL-2 has been shown to play a role in cell biology beyond apoptosis and has been linked to the regulation of energy metabolism (40). Recent studies also have shown that targeting glutamine metabolism
has synergistic activity with BCL-2 inhibition in leukemia and myeloma cells, further indicating that BCL-2 is linked to cancer cell metabolism (41,42). Clearly, further studies are required to address whether DNA damage activation and suppression of metabolic pathways are attributable to venetoclax-mediated BCL-2 inhibition or effects that are independent of BCL-2.

Approximately 15% of patients with relapsed DLBCL have double hit lymphoma (DHL) (MYC/BCL-2 or c-MYC/BCL-6) and an additional subset of these patients have double expressor lymphoma (DEL) with high expression of MYC and BCL-2 (8,43). These patients experience an aggressive clinical course, high chemorefractoriness and low overall survival when treated with R-CHOP (8,44). The data presented in this study show that venetoclax is highly effective in DHL as well as DEL, suggesting that treatment with venetoclax may be useful for patients who have poor prognostic factors. Predictive biomarkers for venetoclax response in the clinic can potentially be identified by: 1) BCL-2 and pAKT protein levels (IHC staining); 2) BCL-2 and BCL-xL mRNA levels (RT-PCR); and 3) conventional cytogenetics of fluorescence in situ hybridization analysis.

Our pre-clinical evaluation of venetoclax in both in vitro cell lines and in vivo PDX mouse models showed promising efficacy in aggressive B-cell lymphomas. Furthermore, our studies identified a novel mechanistic basis for venetoclax activity, not only specific targeting of BCL-2, but also activation of the AKT pathway, DNA damage induction, and metabolic demand inhibition in aggressive B-cell lymphomas. These findings suggest that venetoclax may be of therapeutic value in specific subsets of patients with aggressive B-cell lymphomas and further supports the rationale for a clinical evaluation of venetoclax plus targeting the PI3K/AKT pathway in aggressive B-cell lymphomas.

**Authorship Contributions**

Acknowledgements
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References


Table 1. DLBCL and MCL cell line characterization and venetoclax sensitivity.

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MCL cell lines

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Cell viability assays were performed to identify the venetoclax IC₅₀ values. BCL-2 and MYC protein expression was analyzed by reverse-phase protein array. ABC, activated B cell-like;
GCB, germinal center derived B cell; DHL, double hit lymphoma; PM-BCL, primary mediastinal large B-cell lymphoma; STR, short-tandem repeats; wt, wild-type; mut, mutant; ND, no data.

Figure Legends

Figure 1. *In vitro* venetoclax response in representative human patient-derived DLBCL (26) and MCL (10) cell lines. (A) The effect of 72 hours of venetoclax treatment on the viability of human patient-derived MCL and DLBCL cell lines relative to cells treated with dimethylsulfoxide. Waterfall graph shows the IC$_{50}$ value of venetoclax for each cell line. (B) Comparison of venetoclax IC$_{50}$ values between non-GCB (n=9) and GCB (n=17) DLBCL subtypes (first panel); between DLBCL (n=26) and MCL (n=10) cell lines (second panel); and between BCL2-positive (n=26) and BCL2-negative (n=10) DLBCL and MCL cell lines (third panel). Comparison of venetoclax IC$_{50}$ values between MYC/BCL2 DHL/DEL (n=11) and non-DHL/DEL (n=15) DLBCL cells (fourth panel). (C) BCL-2 protein expression in DLBCL and MCL cell lines was measured by RPPA analysis. Gray bars indicate venetoclax sensitivity and black bars indicate venetoclax resistance. The asterisk (*) indicates cell lines that express BCL-2 protein but were resistant to venetoclax. (D) RPPA analysis was assessed in 26 representative DLBCL cell lines, and the expression level of BCL-2 and its family members was plotted against the corresponding venetoclax IC$_{50}$ for each cell line. Spearman’s rank correlation coefficient and $p$ values were determined based on the above analysis. $P$ values less than 0.05 indicate significant correlations. Solid line indicates the linear regression line, and the dotted line depicts the 95% confidence interval.

Figure 2. Venetoclax response in fresh primary human aggressive B-cell lymphoma patient samples and *in vivo* efficacy of venetoclax in MCL and DLBCL PDX models. (A) Cell viability of freshly isolated tumor cells from 9 MCL and 4 DLBCL primary samples after treatment with increasing concentrations of venetoclax for 48 hours. (B) BCL-2 protein expression in primary MCL and DLBCL samples analyzed by Western blotting. Actin served as a loading control. R, resistant (IC$_{50}$ > 100 nM); S, sensitive (IC$_{50}$ < 100 nM). (C) PT-2 PDX mice were randomly divided into two groups (n=5) and once human β$_2$M was detectable, mice were administered venetoclax 50 mg/kg or vehicle control by daily oral gavage for 3 weeks. Mouse
serum was collected, and the circulating human β2M levels in the mouse serum were used to monitor tumor burden (upper panel). The body weight was calculated at the endpoint of treatment (lower panel). (D) PT-6 MCL PDX mice were randomized into groups, and once the tumor reached to 3 mm$^3$ in size, the mice were administered either venetoclax 50 mg/kg or vehicle control by daily oral gavage for 3 weeks. Tumor burden was evaluated by measuring tumor volume to determine therapeutic efficacy (upper panel; p = 0.028, venetoclax versus vehicle control, n = 5). The body weight was calculated from day 0 to endpoint of treatment (lower panel). (E) The DLBCL PDX mice (PT-12) were randomized into groups, and once the tumor reached to 3 mm$^3$ in size, the mice were treated with venetoclax 50 mg/kg or vehicle control by daily oral gavage for 3 weeks (upper panel; p = 0.04, venetoclax versus vehicle control, n = 5). The body weight was calculated from day 0 to endpoint of treatment (lower panel). (F) The body weight changed from day 0 to endpoint of treatment. (I) H&E staining and anti-human BCL-2 IHC staining of tumor masses from 3 PDXs after mice were humanely euthanized at the endpoint.

**Figure 3. Mechanisms underlying venetoclax activity in aggressive B cell lymphomas.** (A) The Venn diagrams show common upregulated and downregulated proteins in venetoclax-treated RC, U-2932 and HF cells. (B) Representative heatmaps showing the common up- and downregulated proteins in venetoclax-treated RC, U-2932 and HF cells. (C) Table showing the venetoclax up- and downregulated proteins in different cellular processes. (D) Representative DLBCL cell lines with high BCL-2 [RC and U-2932] or low BCL-2 (HT) were treated with venetoclax in a dose- (0-10 nM) and time- (24 and 48 hours) dependent manner and then assessed for apoptosis by Annexin V staining. DMSO treatment serves as a control. (E) RC, U-2932, HT, and a primary DLBCL sample cells were treated with venetoclax (Ven) in a dose-dependent manner. Whole cell extracts were subjected to Western blot analysis for expression of cleaved caspase 3 (c-casp3), PARP, γH2AX, and actin (loading control). The PI3K inhibitor KA2237 (KA) was used as a control agent to induce apoptosis in HT cells.

**Figure 4. Venetoclax treatment activates the AKT compensatory pathway and novel agents targeting PI3K or AKT synergize with venetoclax in aggressive B cell lymphomas.** (A) Representative venetoclax-sensitive (RC and Mino) cell lines were treated with venetoclax in a
1:1 ratio drug combinations with PI3K inhibitors (ACP-319, idelalisib, and KA2237) or AKT inhibitor (MK-2206) in concentration-dependent manner for 72 h, and cell viability was assessed. The highest starting concentration for each drug was 20 µM. The following are the drug combinations: 100 nM: 20 µM; 50 nM:10 µM; 25 nM:5 µM; 12.5 nM:2.5 µM; 6.25 nM:1.25 µM; 3.1 nM:0.61 µM; 1.5 nM:0.3 µM. Data from two independent experiments performed in triplicate are shown. (B) RC and Mino cells were treated with venetoclax (5 nM) alone, PI3K inhibitors or AKT inhibitor (10 µM) alone, or combination of both drugs for 24 h. Protein extracts from the cells were subjected to western blot analysis to detect AKT, pAKT, cleaved caspase3, cleaved PARP, and actin (protein loading control). (C) Apoptotic cells were also detected by annexin V/propidium iodide staining of the above experiments. Data shown represent means ± SD from three independent experiments. (D) Two representative DLBCL primary cells (PT-12 and PT-13) were treated with venetoclax in 1:1 ratio drug combinations with the dual, selective PI3K-p110β/δ inhibitor, KA2237, in concentration-dependent manner for 72 h, and cell viability was assessed. The highest starting concentration for KA2237 was 20 µM. (E) RC, Mino, BJAB, and HT cells were transiently transfected with control or 3 different validated AKT siRNAs. At 48 h post-transfection, protein purified from the transfected cells was subjected to western blotting for AKT and actin (loading control). A small portion of the transfected cells were treated with increasing concentrations of venetoclax for an additional 72 h, and viability was assessed. Data are expressed as the mean ± standard deviation of at least 2 independent experiments with triplicate samples.

Figure 5. Mechanism of acquired venetoclax resistance in lymphoma B cells. (A) The effect of 72 hours of venetoclax treatment on the viability of parental (Mino-P, Rec-1-P and RC-P) and venetoclax-resistant (VR)(Mino-VR, Rec1-VR, and RC-VR) cell lines. (B) Protein extracts from the parental lines and venetoclax-resistant cell lines were subjected to western blot analysis to detect pAKT, AKT, PI3K, PTEN, and GAPDH (loading control). (C) Parental and venetoclax-resistant cells were treated with PI3K inhibitors (KA2237 or idelalisib) for 24 hrs. Protein extracts were subjected to western blot analysis to detect for pAKT, AKT, and GAPDH (loading control). (D) Viability assays were assessed to measure the growth inhibitory effect of the PI3K inhibitor KA2237 in parental and venetoclax-resistant cell lines.
Figure 6. Intrinsic venetoclax resistant cells possess high AKT activation and are highly sensitive to PI3K inhibition. (A) Src-pY527, AKT-pS473, and AKT-pT308 protein levels were plotted against the corresponding venetoclax IC\(_{50}\) for each cell line. Spearman’s rank correlation coefficient and \(p\) values determined based on the above analysis. \(P\) values less than 0.05 indicate significant correlations. (B) RPPA analysis was assessed in 26 representative DLBCL cell lines, and the expression level of PTEN, AKT, pAKT, p-mTOR, p70-S6K, PRAS40, and S6 was plotted against the corresponding KA2237 IC\(_{50}\) for each cell line. Spearman’s rank correlation coefficient and \(p\) values were determined based on the above analysis. \(P\) values less than 0.05 indicate significant correlations. (C) (Left panel) Cells with high protein levels of BCL-2, BIM, and BAK are highly sensitive to venetoclax treatment, presumably through the release of BIM and BAK to cause BAX-mediated cytochrome c release, and subsequently apoptosis. Short- and long-term exposure of venetoclax to lymphoma cells inhibits PTEN, leading AKT pathway activation, for maintenance of cell survival, presumably through upregulation of BCLx/L/BAD complex. (Right panel) Cells with low or no BCL-2 protein expression are intrinsically resistant to venetoclax and tend to acquire high AKT pathway activation, as well as BCL-xL and BAD protein expression. PI3K or AKT inhibitors are amenable to overcome both acquired and intrinsic venetoclax resistance.
Figure 1
Figure 3

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D.

E.

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Figure 4.

(A) PI3K inhibitor (ACP-319), PI3K inhibitor (Idelalisib), PI3K inhibitor (KA2237), AKT inhibitor (MK-2206).

(B) Venetoclax, ACP-319, Idelalisib, KA-2237, MK-2206.

(C) % of Apoptosis.

(D) PT-12, PT-13.

(E) RC, Mino, BJAB, HT.

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Figure 5

A. Viability (% of control) plotted against Venetoclax (nM) for different cell lines: RC-P, RC-VR, Mino-P, Mino-VR, Rec-1-P, and Rec-1-VR.

B. Western blot analysis showing PI3K-110β, PI3K-110δ, PTEN, p-AKT, AKT, and GAPDH levels for Mino, Mino-VR, Rec-1-P, Rec-1-VR, RC, and RC-VR.

C. Table summarizing drug treatments for Mino and Rec-1 cell lines:

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<th>Drug</th>
<th>Mino</th>
<th>Parental</th>
<th>Resistant</th>
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<td>Mino-P</td>
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<td>Mino-VR</td>
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<tr>
<td>Rec-1-P</td>
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<tr>
<td>Rec-1-VR</td>
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D. Viability (% of control) plotted against KA2237 (μM) for different cell lines: Mino-P (IC_{50}: 5 μM), Mino-VR (IC_{50}: 3.5 μM), Rec-1-P (IC_{50}: 26 μM), Rec-1-VR (IC_{50}: 0.96 μM), RC-P (IC_{50}: 3.7 μM), and RC-VR (IC_{50}: 0.7 μM).
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Strategic therapeutic targeting to overcome venetoclax resistance in aggressive B-cell lymphomas

Lan V Pham, Shengjian Huang, Hui Zhang, et al.

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