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Targeting BCL2 with venetoclax is a promising therapeutic strategy for "double-protein-expression" lymphoma with *MYC* and *BCL2* rearrangements

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ABSTRACT

The so-called "double-hit" and "double-protein-expression" lymphoma with *MYC* and *BCL2* rearrangements is a rare, mature B-cell neoplasm characterized by a germinal center B-cell phenotype, abundant protein expression of MYC and BCL2, rapid disease progression, and a poor prognosis. In this study, we showed the potential benefit of the BCL2 inhibitor venetoclax in the treatment of this disease. Immunohistochemical studies of the lymphoma tissues confirmed that overexpression of MYC and BCL2 was observed more frequently in this subtype than in other germinal center B-cell-like diffuse large B-cell lymphomas. In contrast, another pro-survival protein MCL1 was less expressed in this subtype, even when compared with its expression in the non-"double-hit" and "double-protein-expression" type. Furthermore, *in vitro* studies using two "double-hit" and "double-protein-expression" lymphoma-derived cell lines, Karpas231 and OCI-Ly8, clearly showed that a low concentration of venetoclax, but not the MCL1 inhibitor S63845, was sufficient to induce apoptosis in the two lines, compared with in other germinal center B-cell-derived cell lines, BJAB and SU-DHL10. These results indicate that the survival of this type of lymphoma depends predominantly on BCL2 rather than on MCL1. Unexpectedly, we found that venetoclax not only disrupts the interaction between BCL2 and the pro-apoptotic protein BIM, but also leads to dephosphorylation of BCL2 and further downregulates MCL1 protein expression, probably through modulation of the protein phosphatase 2A B56α activity in Karpas231 and OCI-Ly8. Indeed, a low concentration of venetoclax induced substantial apoptosis in the primary lymphoma cells, regardless of high protein expression of MCL1 associated with venetoclax resistance. Venetoclax clearly triggers the signal transduction related to BCL2 and MCL1 in "double-hit" and "double-protein-expression" lymphoma cells.

Introduction

Aggressive mature B-cell lymphomas harboring concurrent translocations of 8q24/*MYC* mainly with 18q21/*BCL2* are called "double-hit lymphomas (DHL)" now referred to as "high grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements (DH-HGBL)" according to the current World Health Organization (WHO) classification of lymphoid neoplasms.[1] The concurrent translocations of 8q24/*MYC* and 18q21/*BCL2* usually lead to overexpression of both proteins, and DH-HGBL clinically forms a specific group among "double-protein-expression lymphomas (DPL)".[1-3] The most common histological type of DH-HGBL is diffuse large B-cell lymphoma (DLBCL), which has heterogeneous clinicopathological,

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immunophenotypic, and genetic features.[1,4] Gene expression signatures have stratified DLBCL into germinal center B-cell (GCB)-like, activated B-cell (ABC)-like, and other subtypes, each of which results from different pathogenic mechanisms.[1,5,6] DH-HGBL cases with DLBCL morphology frequently result in disastrous consequences in spite of showing the GCB phenotype, which is regarded as a relatively favorable marker for survival.[1,2,4] Thus, to be DHL and DPL (DH-DPL) seems to have a negative impact on survival, especially in GCB-like DLBCL cases.[1-3]

MYC is a powerful transcriptional activator, target genes of which are associated with cell proliferation, DNA replication, protein synthesis, and cell metabolism, and its overexpression is a hallmark of tumor aggressivity.[7,8] In contrast, BCL2 is the first identified anti-apoptotic regulator that contributes to the survival of lymphoma cells.[9,10] Dysregulation of both genes likely generates aggressive lymphoma cells showing a fast growth rate and resistance to apoptotic stimuli. Clinically, DH-DPL has a poor prognosis when treated with the standard rituximab-combined cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) regimen, with a median survival of around 20 months.[2,11] Until now, optimal therapeutic strategies against DH-DPL remain to be determined.

Recent reports suggest that targeting MYC and BCL2 may be a promising strategy to control DH-DPL.[12-15] BRD4, a member of the bromodomain and extra-terminal domain (BET) family, is considered to be a convenient target for MYC-driven lymphomas.[16,17] BET family proteins recognize acetylated chromatin and act as transcription co-factors.[18] BRD4 is upregulated in DLBCL and Burkitt lymphoma cells, and its inhibition leads to a strong downregulation of MYC and its regulating genes, resulting in suppression of their cell growth.[16,17] Meanwhile, the selective BCL2 inhibitor venetoclax demonstrated excellent antitumor effects in chronic lymphocytic leukemia.[19,20] BCL2 and its family proteins function as inhibitors and activators of the intrinsic apoptotic pathway at the mitochondrial membrane level.[10,21] They contain at least one of four BCL2 homology (BH) domains (BH1-4) and are classified into three groups based on their structure and function: i.e., the pro-survival proteins (BCL2, BCL-xL, MCL1, BFL1, and BCLw) sequester the pro-apoptotic BH3-only proteins (BID, BIM, BAD, NOXA, PUMA, BMF, HRK, and BIK), which in turn activate the pore-forming proteins (BAX and BAK).[10,21] Oligomerization of BAX/BAK permeabilizes the mitochondrial membrane, resulting in cytochrome c release and apoptosis.[10,21] The BH3 mimetic venetoclax binds to the BH3 domain of BCL2, releases BH3-only proteins, and induces apoptosis.[10,21] Although short exposure to venetoclax can trigger significant antitumor effects in DLBCL cells,[12-15,19,22-24] this drug's clinical efficacy in DLBCL is less promising,[25] probably because the apoptotic sensitivity to venetoclax is influenced not only by total amounts of BCL2, but also by its phosphorylation status, especially at serine 70 (Ser70), and the further presence of other pro-survival proteins.[14,15,22-24,26-28] Among the pro-survival proteins, MCL1 is considered the major determinant of resistance to venetoclax.[22-24,28] Therefore, the therapeutic application of venetoclax to DH-DPL needs further investigation. In this study, we examined the apoptotic sensitivity of GCB-like DLBCL cells to the BRD4 inhibitor JQ-1 and BH3 mimetics, focusing on the association of BCL2 with MCL1.

Methods

Reagents

The BCL2 inhibitor venetoclax (Selleck Chemicals, Houston, TX, USA), MCL1 inhibitor S63845 (ApexBio, Houston, TX, USA), BCL-xL inhibitor A-1155463 (Selleck Chemicals), and BRD4 inhibitor JQ-1 (Sigma-Aldrich, St. Louis, MO, USA) were dissolved with dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) and added to the culture medium.

Cell lines

We used four GCB-like DLBCL cell lines: BJAB,[29] SU-DHL10 (ATCC, Manassas, VA, USA), Karpas231 (ECACC, Salisbury, UK), and OCI-Ly8 (kindly provided by Dr. Masao Seto, Kurume University, Kurume, Japan). They were maintained as described previously.[29]

Fluorescence *in situ* hybridization analyses

Fluorescence *in situ* hybridization analyses were performed using a Vysis LSI MYC dual-color, break-apart rearrangement probe, a Vysis LSI IGH/MYC/CEP8 tri-color dual-fusion probe, and a Vysis LSI *IGH/BCL2* dual-color, dual-fusion translocation probe (Abbott Molecular, Des Plaines, IL, USA).

Clinical samples and immunohistochemistry

Clinical samples were obtained at biopsy performed for initial diagnosis at our institution between September 2012 and October 2018. Diagnoses were made according to the current WHO classification,[1] and GCB-like DLBCL was defined using the Hans criteria.[30] A bone marrow specimen from one patient (UPN3) was subjected to *in vitro* susceptibility testing using venetoclax after having obtained written informed consent. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded specimens using standard techniques. The primary antibodies used are listed in the *Online Supplementary Methods*. The percentages of stained lymphoma cells were evaluated by visual estimation and recorded in 10% increments by at least two observers. The study protocol was approved by the Ethics Review Board of St. Marianna University.

Cell proliferation and annexin V-binding assays

We performed direct cell counting using a trypan blue-exclusion test (Thermo Fisher Scientific, Carlsbad, CA, USA) and annexin-V binding/7-amino-actinomycin D (7-AAD) rejection assays (Beckman Coulter, Brea, CA, USA) with a FACSCalibur flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Acquired data were analyzed using FlowJo software (BD Bioscience).

Western blot analysis

Cell lysates were prepared as described previously.[29] Equal amounts of protein (30 μg/well) were separated on a discontinuous sodium dodecyl sulfate-10% polyacrylamide gel and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The primary antibodies used are listed in the *Online Supplementary Methods*. Antibody signals were visualized using Western blue (Promega, Madison, WI, USA).

Immunoprecipitation

Whole cell lysate (100 μg of total protein) was added to 1/200 volume of each rabbit antibody and adjusted to 680 μL of volume with the lysis buffer. The antibodies used are listed in the *Online Supplementary Methods*. Rabbit serum-saturated protein G-Sepharose beads (20 μL) (GE Health Care, Uppsala, Sweden) were added to each lysate, and immunoprecipitation was performed for 1 h while rotating at 4°C. After incubation, the beads were

washed three times with ice-cold lysis buffer and boiled for 3 min with 20 μL/tube of loading buffer. The supernatant was subjected to western blot analysis.

Statistical analysis

Differences in positive rates of MYC and BCL2 family proteins between DH-HGBL and other GCB-like DLBCL were calculated using a two-tailed Mann-Whitney U test and Student *t* test. *P* values <0.05 were considered statistically significant. All analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, v.1.33).[31]

Results

MCL1 overexpression is less frequently observed in DH-HGBL

We initially evaluated the protein expression of MYC and BCL2 family members in clinical samples. A total of 27 cases were analyzed because they had minimal data and specimens for immunohistochemical and cytogenetic evaluations (*Online Supplementary Table S1*). Immunohistochemistry and fluorescence *in situ* hybridization analyses using *MYC* split and *IGH-BCL2* fusion probes confirmed that these clinical samples comprised eight DH-HGBL cases harboring both *MYC* and *BCL2* gene rearrangements and 19 GCB-like DLBCL cases. Of course, all DH-HGBL cases showed the GCB phenotype, and seven of the eight cases corresponded to DH-DPL (*Online Supplementary Table S1*). Among the 19 GCB-like DLBCL cases, five showed overexpression of both MYC and BCL2 proteins and were regarded as non-DH DPL (*Online Supplementary Table S1*). Although immunohistochemistry clearly detected overexpression of both MYC and BCL2 proteins more frequently in DH-HGBL than in other GCB-like DLBCL (Mann-Whitney U test, *P*=0.028 in MYC and *P*=0.013 in BCL2), differences between DH-HGBL and non-DH DPL were not seen (Figure 1A). Among the BCL2 family proteins, immunhistochemistry clearly confirmed the expression of BCL2, MCL1 and BIM, but rarely detected BAX regardless of the groups (Figure 1A,B, and *Online Supplementary Figure S1*). There were no differences in the rates of positivity for BIM and BAX among the three groups, whereas MCL1 overexpression was detected significantly more frequently in other GCB-like DLBCL than in DH-HGBL (Mann-Whitney U test, *P*=0.005) (Figure 1A). This significance was also observed between DH-HGBL and non-DH DPL (Student *t* test, *P*=0.019) (Figure 1A). Concerning the staining pattern for MCL1, we found differences between DH-HGBL and other GCB-like DLBCL including non-DH DPL. All DH-HGBL cases showed the cytoplasmic staining pattern, whereas two of five non-DH DPL (40%) and six of 14 other GCB-like DLBCL cases (43%) exhibited the nuclear staining pattern (Figure 1B, *Online Supplementary Table S1*). These results suggest that the intrinsic anti-apoptotic activities in DHL-HGBL or DH-DPL may be dependent on BCL2 rather than MCL1.

Karpas231 and OCI-Ly8 are DH-DPL cells

We next performed *in vitro* investigations to characterize DH-DPL cells. Fluorescence *in situ* hybridization analyses confirmed that all four GCB-like DLBCL-derived cell lines have the 8q24/*MYC* translocation (Figure 2A). *MYC* was fused to *IGH* in BJAB, SU-DHL10, and OCI-Ly8 (Figure 2A). In addition to the rearrangement, *IGH-BCL2* fusion resulting from t(14;18)(q32;q21) was detected in SU-DHL10, Karpas231, and OCI-LY8 (Figure 2A). Western blot analysis showed that the four lines express BCL6, MYC, BRD4, MCL1, BCL-xL, BIM, BAD, BAK, and BAX at a variety of levels (Figure 2B). Among the pro-apoptotic BH3-only proteins, BIM was abundantly expressed in each cell line (Figure 2B). The results were consistent with those from clinical samples. In contrast, protein expression levels of BCL-xL and MCL1 were relatively low in SU-DHL10 and OCI-Ly8, respectively (Figure 2B). Although SU-DHL10 has the *IGH-BCL2* fusion, we failed to detect BCL2 protein expression in this line (Figure 2B). Also, NOXA was not detected in BJAB cells (Figure 2B). These results indicate that BJAB represents non-DH DPL cells and that SU-DHL10, Karpas231 and OCI-Ly8 are regarded as DH-HGBL cells. Furthermore, Karpas231 and OCI-Ly8 correspond to DH-DPL cells having relatively abundant BCL2 proteins, a considerable part of which is phosphorylated at Ser70.

Exposure to 200 nM of venetoclax effectively induces apoptosis in DH-DPL cells

We subsequently evaluated the growth inhibitory effect and apoptotic sensitivity to JQ-1 and three BH3 mimetics in each cell line. Except for the BJAB cell line, JQ-1 similarly suppressed cell proliferation in a dose-dependent manner, while the inhibitory effects of BH3 mimetics were different among the four lines (Figure 3A). Venetoclax effectively suppressed proliferation of Karpas231 and OCI-Ly8 cells even at low concentrations (20 nM and 200 nM), whereas this agent had no effect on either BJAB or SU-DHL10 cells (Figure 3A). In contrast, S63845 clearly showed an inhibitory effect at low concentrations (10 nM and 100 nM) only in SU-DHL10 cells (Figure 3A). Although A-1155463 inhibits cell growth at levels less than 1 μM in sensitive cells,[28,32] at nanomolar levels this agent failed to suppress the proliferation of any cell line (Figure 3A).

Flow cytometry detected apoptotic changes 24 h after exposure to these drugs. Although 50 μM of JQ-1 efficiently suppressed proliferation of SU-DHL10, Karpas231, and OCI-Ly8 cells, the exposure was insufficient to induce apoptosis in the two DH-DPL-cell lines. Approximately 70% of SU-DHL10 cells were positive for annexin V, whereas the positive rates were limited to around 18~40% in Karpas231 and OCI-Ly8 cells (Figure 3B). Exposure to 200 nM of venetoclax effectively led to apoptotic changes in Karpas231 and OCI-Ly8 cells. More than 80% of the cells were positive for annexin V in both DH-DPL-cell lines (Figure 3B). Conversely, exposure to 100 nM of S63845 induced cell death only in SU-DHL10. Although around 60% of the cells were annexin V-positive, more than 90% of SU-DHL10 cells were stained with 7-AAD (Figure 3B). In this study, exposure to 1 μM of A-1155637 did not induce even modest cell death in each cell line (Figure 3B). These results indicate that anti-apoptotic activities in the four lines were less related to BCL-xL.

We further evaluated the alteration of apoptotic sensitivity to venetoclax in combination with S63845. In BJAB cells, the addition of S63845 to venetoclax led to a clear increase in annexin V-positive cells, indicating that S63845 and venetoclax had a synergic effect on inducing apoptosis (Figure 4A). In contrast, S63845 did not show even an additive effect in Karpas231, OCI-Ly8, and SU-DHL1 cells

(Figure 4B). Although the expression levels of MCL1 were nearly equal between BJAB and Karpas231 cells, S63845 failed to exert additive pro-apoptotic effects in Karpas231 cells. These results indicate that the intrinsic anti-apoptotic activities in BJAB are equally dependent on BCL2 and MCL1, but those in the two DH-DPL-cell lines and SU-DHL10 cells depend mainly on BCL2 and MCL1, respectively. We, therefore, focused on the biological effects of venetoclax especially in the two DH-DPL cell lines.

Venetoclax leads to dephosphorylation of BCL2 and downregulates MCL1 expression in DH-DPL cells

We then examined alterations of protein levels after exposure to venetoclax. Western blot analysis showed that expression levels of BCL2 were unchanged at least 6 h after exposure to venetoclax in Karpas231 and OCI-Ly8 cells (Figure 5A,B). In contrast, phosphorylation of BCL2 was clearly downregulated, and expression levels of BIM were also decreased 3 h after exposure to venetoclax in both DH-

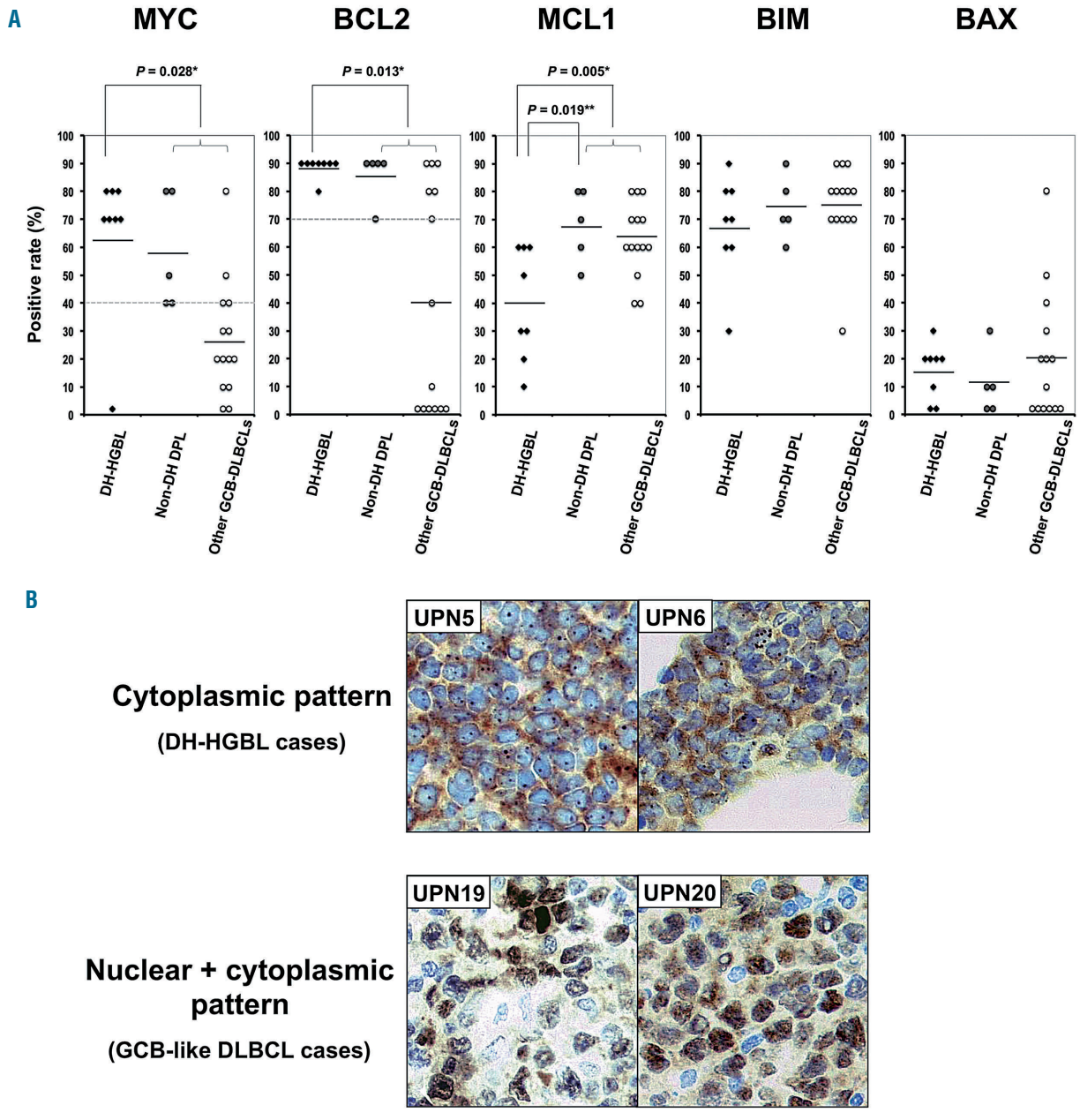


Figure 1. Expression of MYC and BCL2 family proteins in germinal center B-cell-like diffuse large B-cell lymphoma. (A) Immunohistochemistry-detected protein expression of MYC, BCL2, MCL1, BIM and BAX in clinical samples of double-hit (DH) high grade B-cell lymphoma with *MYC* and *BCL2* rearrangements (HGBL) and other germinal center B-cell (GCB)-like diffuse large B-cell lymphomas (DLBCL) including non-DH double-protein-expression lymphoma (DPL). Straight bars represent the mean of each positive rate. The differences in positivity were calculated using the Mann-Whitney U test* and Student *t* test**. The positive rates of MYC and BCL2 were significantly higher in DH-HGBL than in other GCB-like DLBCLs (P=0.028 and P=0.013, respectively). In contrast, MCL1 was significantly less expressed in DHL-HGBL than in other GCB-like DLBCL (P=0.005). This significance was also observed between DH-HGBL and non-DH DPL (P=0.019). P values are described only when each statistical power was above 0.8. (B) MCL1 was stained mainly in the cytoplasm in all eight cases with DH-HGBL, whereas eight of 19 (42%) other GCB-like DLBCL cases, including non-DH DPL, showed the nuclear and cytoplasmic staining pattern.

DPL cell lines (Figure 5A,B). In contrast, although the total protein levels of BCL2 were slightly decreased, the proportion of phosphorylated BCL2 was increased 6 h after exposure to venetoclax in BJAB cells (Figure 5C). Unexpectedly, MCL1 showed decreased expression levels in both Karpas231 and OCI-Ly8 cells within 3 h after exposure to venetoclax (Figure 5A,B). This phenomenon was not observed in the BJAB and SU-DHL10 lines (Figure 5C, D). Furthermore, the expression of protein phosphatase 2A (PP2A) B56α, which has a critical role in dephosphorylation of BCL2,[33] was decreased within 3 h after exposure to venetoclax in both DH-DPL cell lines (Figure 5A,5B). The expression levels of another regulatory subunit, PP2A B56δ,[34] were unchanged in each cell line (Figure 5A-D). These results confirmed that venetoclax clearly changes the biological behavior of BCL2 and MCL1 in the two DH-DPL cell lines.

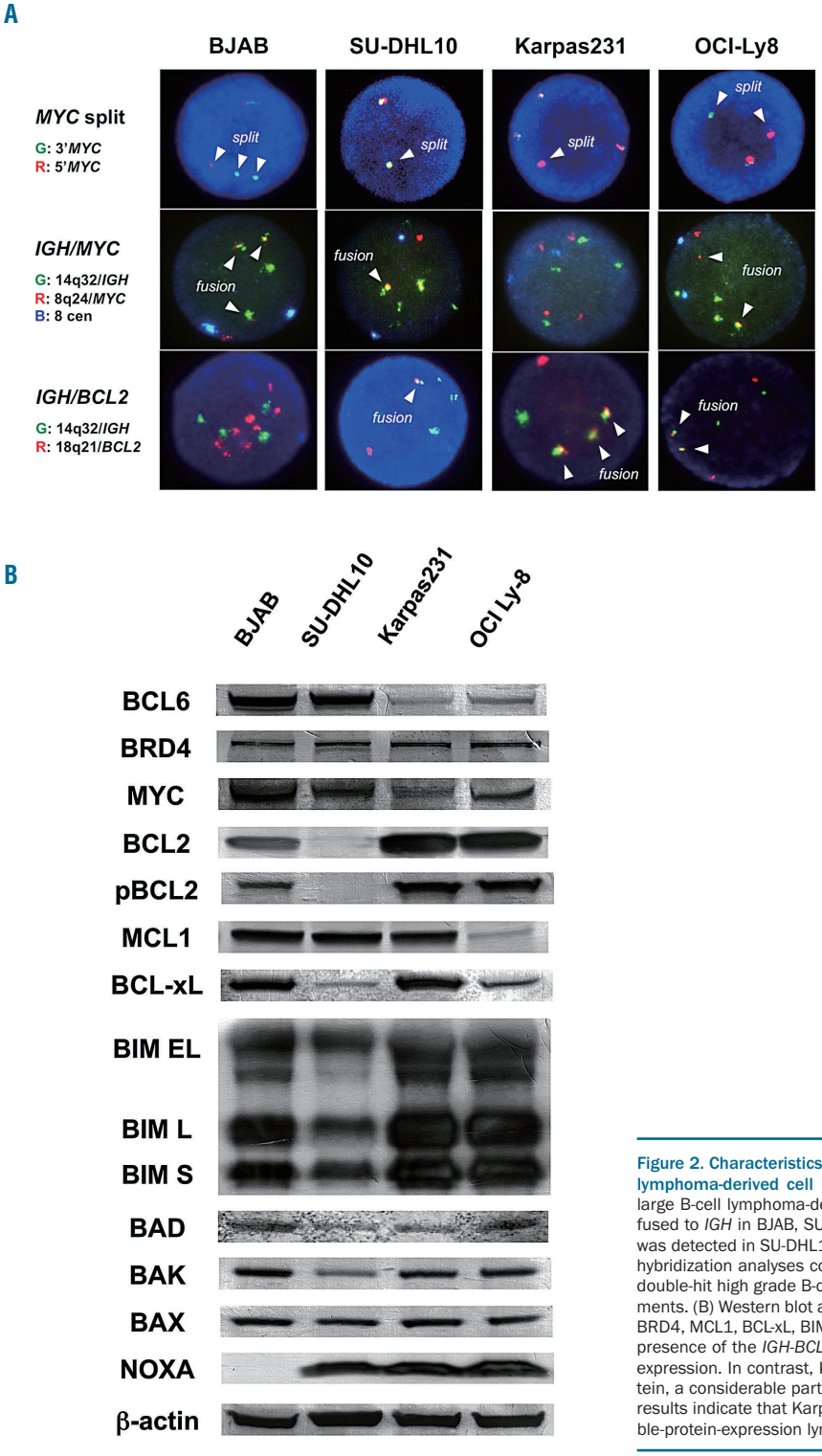
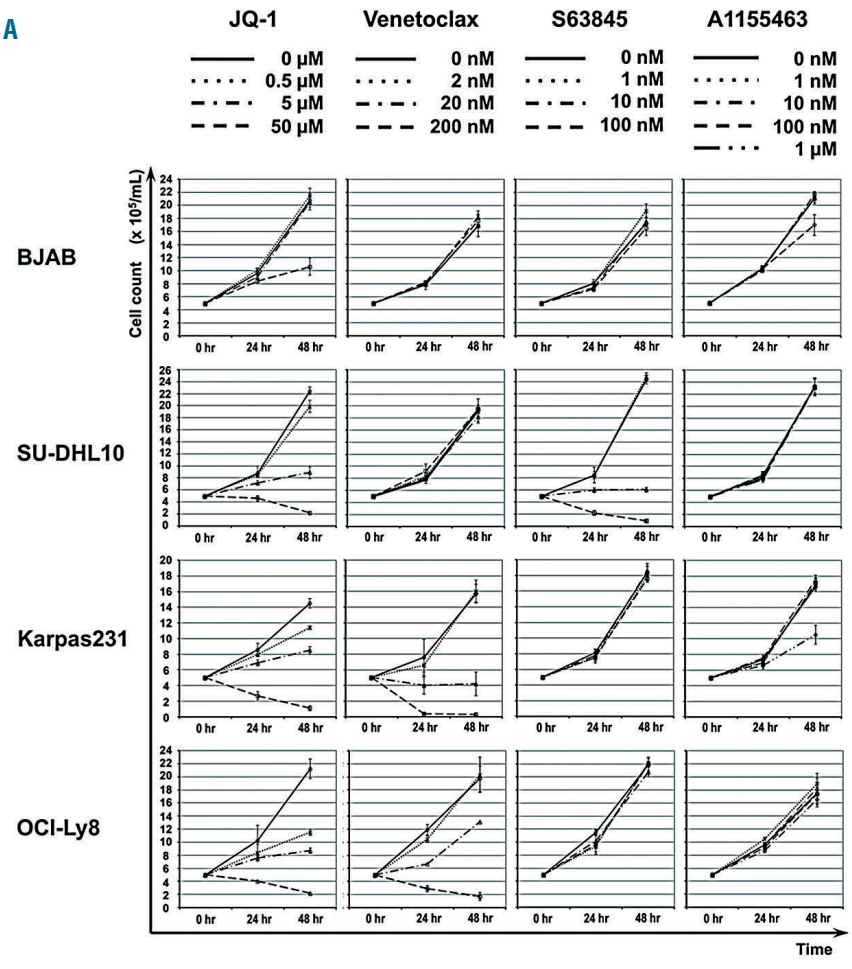


Figure 2. Characteristics of four germinal center B-cell-like diffuse large B-cell lymphoma-derived cell lines. (A) The four germinal center B-cell-like diffuse large B-cell lymphoma-derived cell lines have the *MYC* rearrangement. *MYC* is fused to *IGH* in BJAB, SU-DHL10, and OCI-Ly8 cells, while the *IGH-BCL2* fusion was detected in SU-DHL10, Karpas231, and OCI-Ly8 cells. Fluorescence *in situ* hybridization analyses confirmed that SU-DHL10, Karpas231, and OCI-Ly8 are double-hit high grade B-cell lymphoma cell lines with *MYC* and *BCL2* rearrangements. (B) Western blot analysis showed that the four lines express BCL6, MYC, BRD4, MCL1, BCL-xL, BIM, BAD, BAK, and BAX at a variety of levels. Despite the presence of the *IGH-BCL2* fusion, SU-DHL10 cells failed to show BCL2 protein expression. In contrast, Karpas231 and OCI-Ly8 cells had abundant BCL2 protein, a considerable part of which is phosphorylated (pBCL2) at serine 70. The results indicate that Karpas231 and OCI-Ly8 correspond to double-hit and double-protein-expression lymphoma cells.



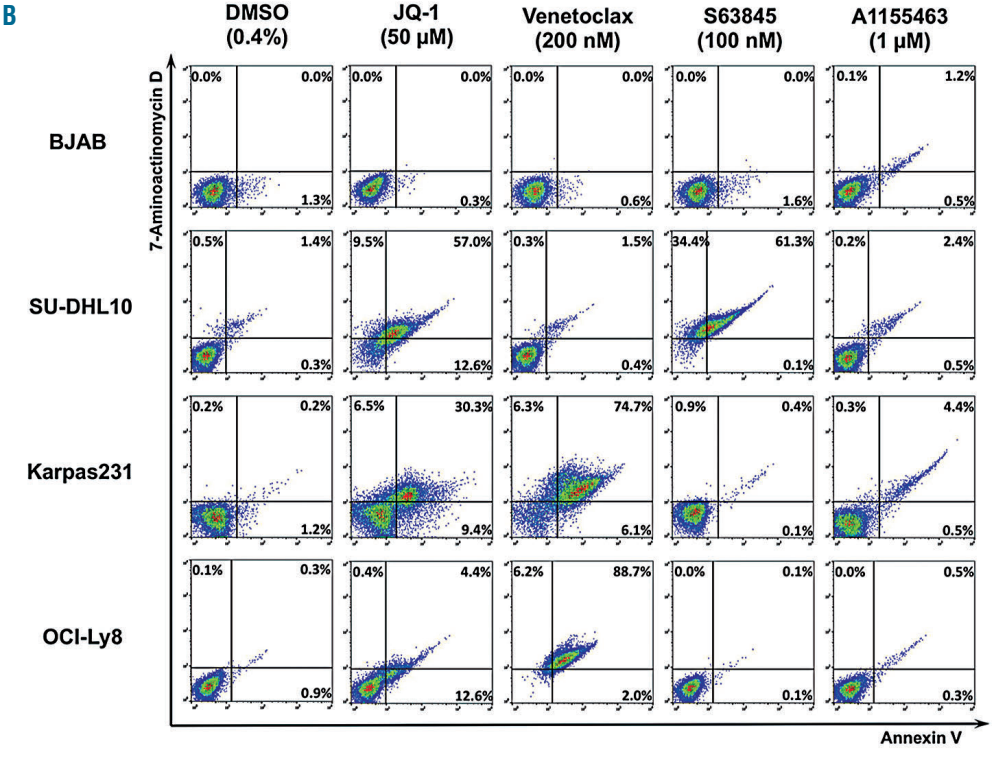


Figure 3. Growth inhibitory effect and apoptotic sensitivity of the four cell lines to JQ-1 and three BH3 mimetics. (A) JQ-1 suppressed proliferation in three cell lines, but not in BJAB, in a dose-dependent manner. Venetoclax inhibited proliferation of Karpas231 and OCI-Ly8 cells even at a concentration of 20 nM, but had no effect on either BJAB or SU-DHL10 cells. S63845 showed the inhibitory effect only in SU-DHL10 cells. At nanomolar concentrations, A-1155463 failed to suppress the proliferation of any cell lines. (B) Although 50 μM of JQ-1 suppressed cell proliferation in the SU-DHL10, Karpas231, and OCI-Ly8 lines, the exposure was insufficient to induce apoptosis in Karpas231 (annexin V^+ 7-aminoactinomycin D^+ 30.3%) and OCI-Ly8 (annexin V^+ 7-aminoactinomycin D^+ 4.4%) cells. Exposure to 200 nM of venetoclax effectively led to apoptotic changes (annexin V^+) in more than 80% of both Karpas231 and OCI-Ly8 cells. Exposure to 100 nM of S63845 induced cell death only in SU-DHL10 cells. Consistent with the results in cell proliferation assays, 1 μM of A-1155463 did not induce even modest cell death in any cell lines. DMSO: dimethyl sulfoxide.

Treatment with venetoclax augments the accumulation of PP2A B56α in BCL2 in DP-DHL cells

Immunoprecipitation and western blot analyses of Karpas231 and OCI-Ly8 cells showed that BCL2 was substantially associated with BIM at steady state (Figure 6A). Likewise, BCL2 was bound to PP2A B56α (Figure 6A). We also confirmed that PP2A B56α was bound to MCL1 in both DH-DPL cell lines (*data not shown*). We then evaluated alterations of these associations after exposure to venetoclax. Within 60 min after treatment with venetoclax, total protein levels of BIM bound to BCL2 were decreased similarly in Karpas231 and OCI-Ly8 cells (Figure 6B). In contrast, the proportion of PP2A B56α bound to BCL2 was slightly increased (Figure 6B). Although BIM protein levels decreased gradually following exposure to venetoclax, total levels of BCL2 bound to BIM were decreased at the early phase of venetoclax treatment in both DH-DPL cell lines (Figure 6B). These results indicate that venetoclax not only disrupts the association between BCL2 and BIM, but also augments the accumulation of PP2A B56α in BCL2.

Exposure to 200 nM of venetoclax induces apoptosis in primary DH-DPL cells

We also evaluated the apoptotic sensitivity of primary DH-DPL cells to venetoclax *in vitro*. The fresh bone marrow specimen was obtained from one patient with DH-DPL (UPN3) and exposed to 200 nM of venetoclax. Examination of the bone marrow revealed massive infiltration of blastoid cells with a mature B-cell phenotype [terminal deoxynucleotidyl transferase (TdT)-negative and BCL6-positive] (Figure 7A). Immunohistochemistry confirmed that MYC, BCL2, and MCL1 were highly expressed in the neoplastic cells (Figure 7A). Flow cytometry clearly detected the apoptotic change 24 h after exposure to venetoclax (Figure 7B). Almost all the blastoid DH-DPL cells were positive for annexin V (Figure 7B). Consistent with the results in Karpas231 and OCI-ly8 cells, the primary DH-DPL cells were highly susceptible to low concentrations of venetoclax.

Discussion

Since DH-DPL is a rapidly progressing disease and often refractory to even intensive therapies, patients with this lymphoma have dismal survival outcomes unlike those with any other type of DLBCL.[2,3,11] Hence, it is urgently necessary to develop effective treatments for DH-DPL. Appropriate balances between pro-survival and pro-apoptotic BCL2 family proteins are usually disrupted in GCB-like DLBCL including DH-DPL, in which the overexpression of pro-survival proteins, including BCL2, MCL1, and BCL-xL, likely confers resistance to chemotherapeutic agents.[10,12,15] In this study, we found that BCL2 seems to play crucial roles in anti-apoptotic activities in DH-DPL, and further verified that venetoclax is still a promising agent for this type of lymphoma. The present DH-HGBL cases, mostly corresponding to DH-DPL, showed lower levels of MCL1 expression, associated with resistance to venetoclax, compared with other GCB-like DLBCL cases. In addition, the frequent nuclear localization of MCL1 in

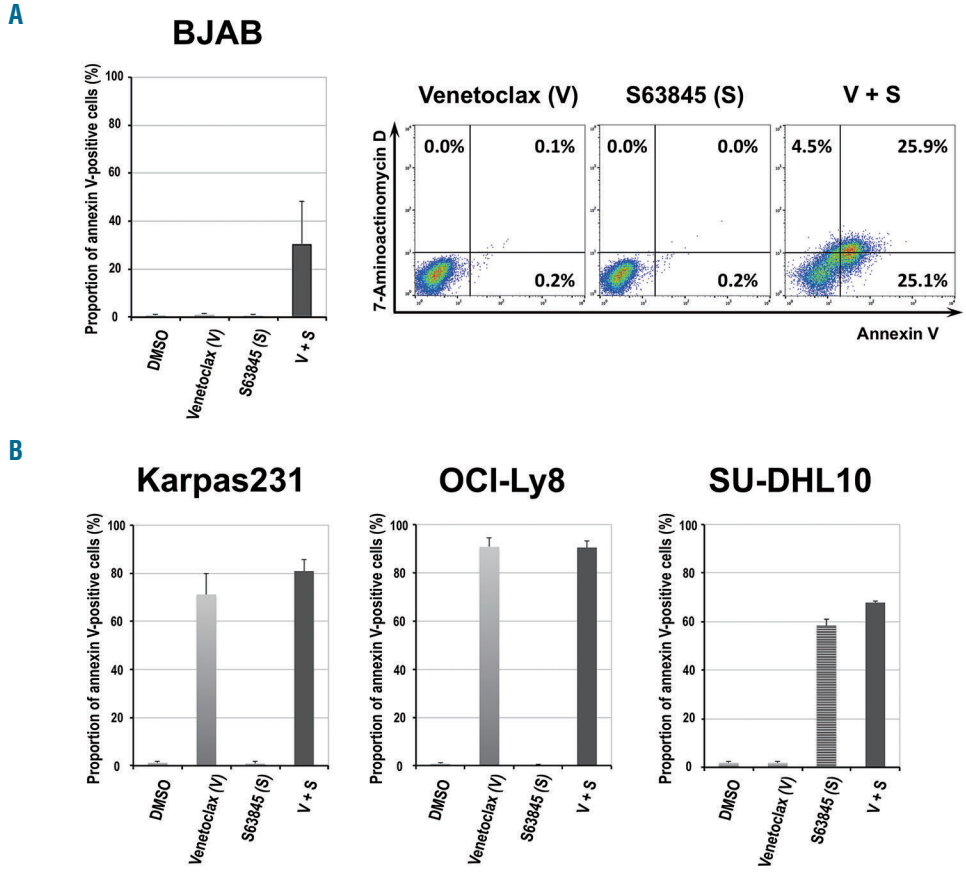


Figure 4. Alteration of apoptotic sensitivity to venetoclax in combination with S63845. (A) The proportion of annexin-V-positive cells was counted using flow cytometry in triplicate. Addition of 100 nM of S63845 to 200 nM of venetoclax led to a clear increase of annexin V-positive cells in the BJAB line, indicating that S63845 and venetoclax had a synergic effect on inducing apoptosis. (B) In contrast, 100 nM of S63845 or 200 nM of venetoclax did not show even an additive effect in Karpas231, OCI-Ly8, and SU-DHL1 cells. DMSO: dimethyl sulfoxide.

non-DH DPL and other GCB-like DLBCL cases not only indicates the abundant expression, but also implies the presence of distinct functions from its anti-apoptotic activity in these groups.[28,35] Furthermore, our *in vitro* study confirmed that the inhibition of MCL1 by S63845 did not have any additive pro-apoptotic effect to that of venetoclax in the two DH-DPL cell lines, whereas S63845 restored the apoptotic sensitivity to low concentrations of venetoclax in the non-DH DPL-cell line BJAB. A previous study showed that overexpression of MCL1 is observed more frequently in ABC-like DLBCL than in GCB-like DLBCL.[36] Our observations indicate that the anti-apoptotic activity of MCL1 for DH-DPL seems to be less limited than that for any other type of GCB-like DLBCL. DH-DPL seems to be dependent mainly on BCL2 for survival, and the pro-apoptotic action of venetoclax in DH-DPL cells may be less affected by intrinsic MCL1 expression.

The BRD4 inhibitor JQ-1 is considered a promising drug for the treatment of MYC-driven lymphomas.[16,17] Although JQ-1 suppressed cell proliferation, this agent did not lead to adequate apoptosis in the two DH-DPL-cell lines. Since BET inhibitors can induce cell-cycle arrest,[37] this raises concerns that JQ-1 might fail to exterminate malignant cells. Although the combination with BH3 mimetics is supposed to be a promising strategy, antagonistic effects have been observed.[15] BET inhibitors are effective at inhibiting tumor expansion, but might be inappropriate for eradicating of residual lymphoma cells.

The antitumor effect of venetoclax has mainly been discussed with regard to its physiological association with BCL2.[21,22,24] In the present study, we demonstrated for the first time that venetoclax not only disrupts the association between BCL2 and BIM, but also modulates signal transduction related to BCL2 and MCL1 in DH-DPL cells. Phosphorylation at Ser70 and its mutant in the BCL2 loop domain has been shown to enhance binding to BIM and BAK, and confirmed to inhibit the effects of BH3 mimetics on its replacement of BIM in leukemia cells.[27] Although protein levels of phosphorylated BCL2 are relatively high, venetoclax clearly led to dephosphorylation of BCL2 and effectively induced apoptosis in Karpas231 and OCI-Ly8 cells. In both DH-DPL cell lines, the dephosphorylation was probably mediated through accumulation of PP2A B56α in BCL2. The increased binding of PP2A B56α to BCL2 is likely to promote dephosphorylation of this protein. Consequently, a considerable part of phosphorylation at Ser70 seemed to be removed within 6 h after exposure to venetoclax. Dephosphorylation of BCL2 should

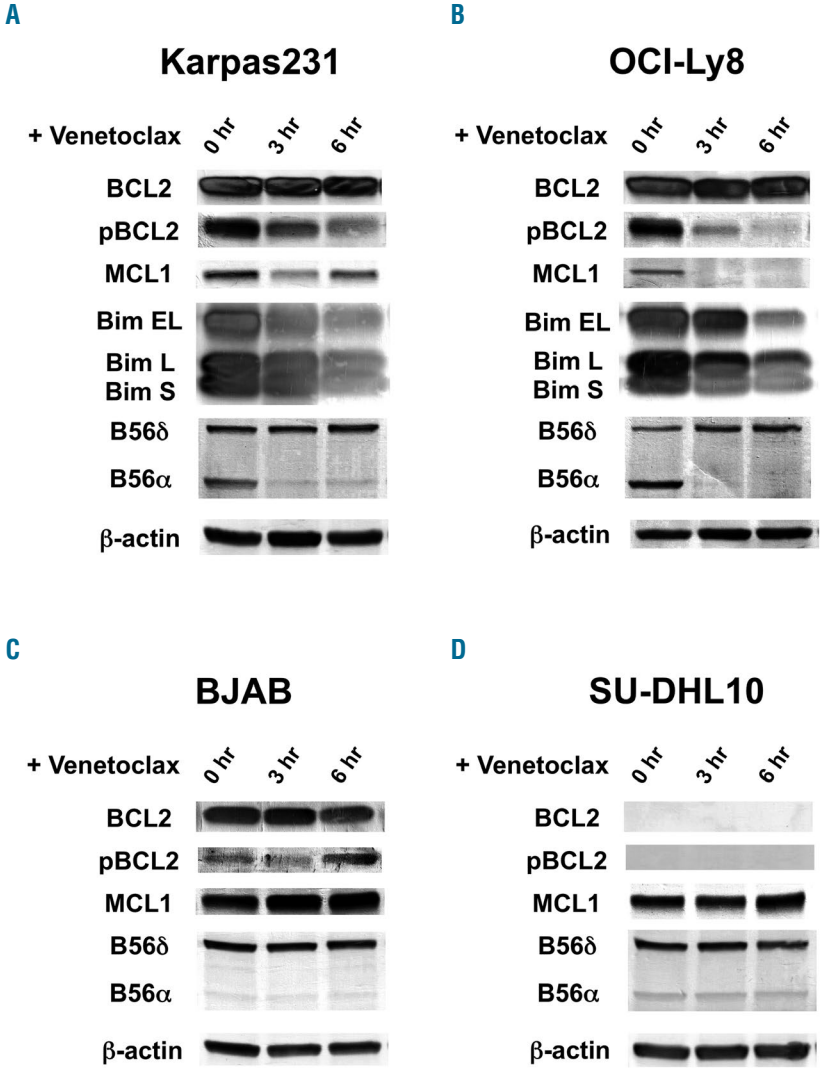


Figure 5. Venetoclax leads to dephosphorylation of BCL2 and downregulates MCL1 expression in the two double-hit and double-protein-expression lymphoma cell lines. (A-D) Although BCL2 protein levels were unchanged at least 6 h after exposure to venetoclax in Karpas231 (A) and OCI-Ly8 (B) cells, the proportion of phosphorylated BCL2 (pBCL2) was clearly decreased, and expression levels of BIM were also decreased 3 h after exposure to venetoclax in both double-hit and double-protein-expression lymphoma (DH-DPL) cell lines (A, B). Although the BCL2 protein levels were slightly decreased, the proportion of pBCL2 was increased 6 h after to venetoclax in BJAB cells (C). Unexpectedly, MCL1 showed decreased protein expression within 3 h after exposure to venetoclax in Karpas231 (A) and OCI-Ly8 (B) cells, but not in BJAB (C) and SU-DHL10 (D) cells. PP2A B56α showed decreased expression within 3 h after exposure to venetoclax in both DH-DPL cell lines (A, B), but not in BJAB (C) and SU-DHL10 (D) cells. In contrast, protein expression levels of PP2A B56δ were unchanged in all cell lines (A-D).

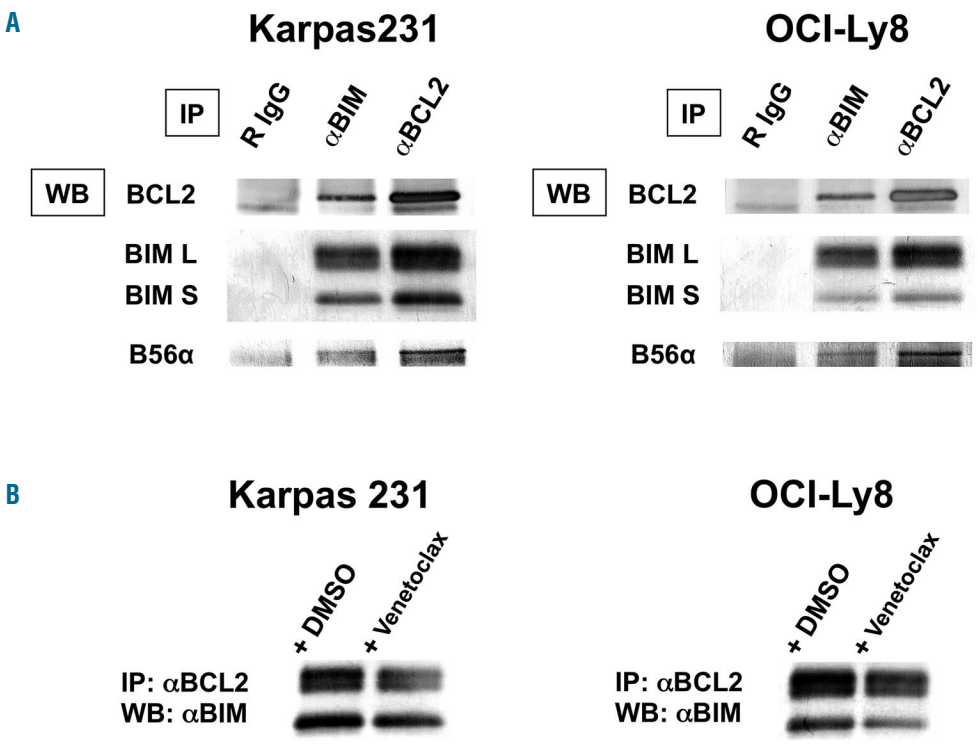


Figure 6. Treatment with venetoclax augments the binding of PP2A B56α to BCL2 in the double-hit and double-protein-expression lymphoma cells. (A) Immunoprecipitation (IP) and western blot (WB) analyses showed that BCL2 was bound to BIM and PP2A B56α at steady state. Rabbit (R) IgG was used as a negative control for the IP studies. (B) Although total BIM proteins bound to BCL2 were decreased similarly in Karpas231 and OCI-Ly8 cells within 60 min after exposure to venetoclax dissolved in dimethyl sulfoxide (DMSO), the proportion of B56α bound to BCL2 was slightly increased. Total BCL2 proteins bound to BIM were also decreased in the early phase of venetoclax treatment in both double-hit and double-protein-expression lymphoma cell lines.

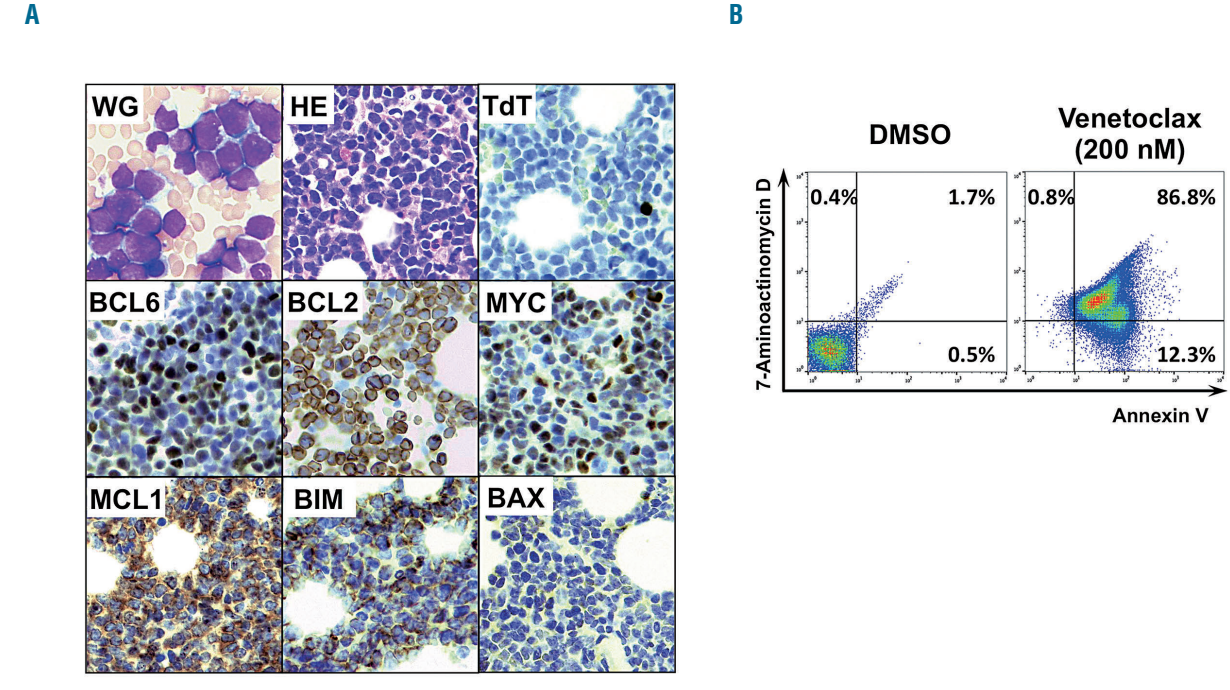


Figure 7. A low concentration of venetoclax induces apoptosis in primary double-hit and double-protein-expression lymphoma cells. (A) The bone marrow examination of one patient (UPN3) revealed massive infiltration by blastoid double-hit and double-protein-expression lymphoma (DH-DPL) cells with a mature B-cell phenotype terminal deoxynucleotidyl transferase (TdT)-negative and BCL6-positive [Wright-Giemsa (WG) stain x1000, hematoxylin-eosin (HE) stain x600]. Immunohistochemistry confirmed that the cells expressed high levels of MYC, BCL2, MCL1 and BIM (x600). (B) Flow cytometry clearly detected apoptotic changes 24 h after exposure to venetoclax dissolved in dimethyl sulfoxide (DMSO). More than 99% of blastoid DH-DPL cells were positive for annexin V.

cause increased sensitivity to venetoclax in DH-DPL cells. Therefore, phosphorylation at Ser70 in BCL2 at steady state is less likely to be a common mechanism of resistance to venetoclax in these DH-DPL cells. We further found that venetoclax altered the levels of expression of MCL1 in both DH-DPL cell lines. The stability of MCL1 is mainly controlled by its phosphorylation status.[38,39] Phosphorylated MCL1 can be an easy target of an E3 ubiquitin ligase complex and is mostly driven to proteasomal degradation.[38,39] PP2A dephosphorylates MCL1 and prevents interaction between MCL1 and the complex.[38,39] A previous study showed that PP2A inhibition dramatically decreased the protein levels of MCL1 within 2 h in Burkitt lymphoma cells.[39] Although we confirmed that PP2A B56α is bound to MCL1 in both Karpas231 and OCI-Ly8 cells, the association was unchanged after exposure to venetoclax (*data not shown*). However, total amounts of PP2A B56α, which should be bound to MCL1, were clearly decreased 3 h after exposure to venetoclax. Therefore, a considerable part of MCL1 protein may be phosphorylated and disassembled within 3 h after exposure to venetoclax in the two DH-DPL cell lines. We further verified that 200 nM of venetoclax was sufficient to induce cell death even in primary DH-DPL cells, despite these cells showing relatively abundant MCL1 expression. Venetoclax achieves and maintains plasma exposure levels of approximately 4 μM at daily doses ranging from 400 mg to 1200 mg in patients with chronic lymphocytic leukemia or lymphomas.[25,40] Prolonged exposure to very low concentrations of venetoclax may raise new issues about resistance due to the expression of other BCL2 family proteins such as BFL1.[14] However, we believe that venetoclax should kill primary DH-DPL cells at daily doses recommended in patients with chronic lymphocytic leukemia.

In conclusion, DH-DPL cells seem to be dependent mainly on BCL2 for survival and relatively low concentrations of venetoclax effectively induced apoptosis regardless of MCL1 expression. Venetoclax not only disrupts the BCL2-BIM interaction, but also leads to dephosphorylation of BCL2 and further downregulates MCL1 expression, probably through modulation of PP2A B56α activity in DH-DPL cells. Although further investigation is needed for clinical application, targeting BCL2 with venetoclax is a promising strategy for the treatment of DH-DPL.

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