Title: Co-targeting of BCL2 with venetoclax and MCL1 with S63845 is synthetically lethal in vivo in relapsed mantle cell lymphoma

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Running Title: Targeting BCL2 and MCL1 in mantle cell lymphoma

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Translational Relevance:
BCL2-targeting agent venetoclax (VTX) has promising anticancer activity in mantle cell lymphoma (MCL), but remissions tend to be short, which calls for rational drug combinations. We demonstrated that MCL1 and NOXA play important roles in mediating resistance to VTX. Consequently, we proposed an experimental treatment strategy based on co-targeting BCL2 with VTX and MCL1 with a highly specific small molecule MCL1 inhibitor S63845,. The combination of VTX and S63845 demonstrated synthetic lethality in vivo on a panel of five patient-derived xenografts established from patients with relapsed MCL with adverse cytogenetics.
Abstract

**Purpose:** Mantle cell lymphoma (MCL) is an aggressive subtype of B-cell non-Hodgkin lymphomas characterized by (over)expression of BCL2. A BCL2-targeting drug venetoclax (VTX) has promising anticancer activity in MCL. We analyzed molecular mechanisms of VTX resistance in MCL cells, and tested strategies to overcome it.

**Experimental Design:** We confirmed key roles of proapoptotic proteins BIM and NOXA in mediating VTX-induced cell death in MCL. Both BIM and NOXA are, however, differentially expressed in cell lines compared to primary cells. First, NOXA protein is significantly overexpressed in most MCL cell lines. Second, deletions of BIM gene harbored by three commonly used MCL cell lines (JEKO-1, MINO and Z138) were not found by array comparative genomic hybridization using a validation set of 24 primary MCL samples.

**Results:** We demonstrated that MCL1 and NOXA play important roles in mediating resistance to VTX. Consequently, we tested an experimental treatment strategy based on co-targeting BCL2 with VTX and MCL1 with a highly specific small molecule MCL1 inhibitor S63845. The combination of VTX and S63845 demonstrated synthetic lethality in vivo on a panel of five patient-derived xenografts established from patients with relapsed MCL with adverse cytogenetics.

**Conclusions:** Our data strongly support investigation of VTX in combination with S63845 as an innovative treatment strategy for chemoresistant MCL patients with adverse cytogenetics in the clinical grounds.

Introduction

Mantle cell lymphoma (MCL) is an incurable subtype of B-NHL(1). Currently, the front-line treatment of MCL is based on conventional chemotherapy and anti-CD20 monoclonal antibody rituximab. Relapsed or refractory patients usually receive either different conventional chemotherapy agents (e.g. bendamustine, cisplatin etc.), or they are offered innovative non-chemotherapy molecules including B-cell receptor inhibitor ibrutinib or immunomodulatory agent lenalidomide(2-4). After failure of a Bruton tyrosine-kinase (BTK) inhibitor ibrutinib, however, patients’ prognosis is usually dismal regardless of the subsequent...
treatment. Complex karyotypes at diagnosis or at disease relapse are associated with especially dismal prognosis due to frequent chemoresistance(5).

BCL2, one of the key anti-apoptotic and pro-survival proteins, is overexpressed virtually in all MCL tumors that are considered BCL2-dependent. Venetoclax (VTX), a BCL2 specific BH3 mimetic, kills MCL cells indirectly, by displacing proapoptotic effectors including BIM (and other BH3-only proteins), from BCL2, and by blocking BCL2 from its inhibitory interaction with proapoptotic BAX/BAK1 proteins. Unbound BIM is then available to bind and activate BAX/BAK1, which in turn disrupts mitochondrial outer membrane thereby triggering programmed cell death independent of TP53-regulated genotoxic pathway(6). So far, VTX has been approved for the patients with chronic lymphocytic leukemia / small lymphocytic lymphoma and showed promising anti-lymphoma activity in MCL and acute myelogenous leukemia patients(7-11).

Despite promising data from early clinical trials, resistance sooner or later develops in majority of MCL patients on VTX monotherapy(8). Mechanisms of constitutive or acquired VTX resistance remain poorly understood. It was repeatedly reported that similarly to approx. one third of currently available MCL cell lines (JEKO-1, Z138, MINO and REC-1) also primary MCL cells frequently harbor deletions of BCL2-like11/BIM(12-14). It was even speculated that the homozygous deletion of BIM plays an important role during MCL lymphomagenesis(13). Because BIM represents a key mediator of VTX proapoptotic activity its homozygous deletion might represent a valuable marker of VTX resistance. Upregulation of MCL1, another key anti-apoptotic regulator besides BCL2, was also reported as a plausible mechanistic rationale for acquired resistance to VTX(15-17). Therefore, several groups including our own employed diverse strategies to indirectly inhibit MCL1 protein (in combination with VTX) including cyclin-dependent kinase inhibitor dinaciclib, plant alkaloid homoharringtonine or anthracycline daunorubicine(16,18,19). In 2017, Kotchny et al. reported single-agent anti-tumor activity of a novel, highly specific MCL1 inhibitor S63845 in many cancers including hematologic malignancies(20). However, anti-lymphoma activity of S63845 in MCL (considered a BCL2-dependent malignancy) was not studied. Anti-tumor activity of the combination of VTX and S63845 has been studied in acute leukemias, and there is one active trial testing the combination of BCL2- and MCL1-inhibitors (VTX and S64315) clinically in patients with acute myeloid leukemias (GovTrial Number NCT03672695)(9-11,21,22).
Methods

Cell lines, patient-derived xenografts, and primary lymphoma samples

MCL cell lines were purchased from DSMZ or ATCC with the exception of HBL2, which was a kind gift of prof. Martin Dreyling. The cell lines were authenticated in July 2016 by Multiplexion. UPF1G and UPF1H cell lines and all patient-derived lymphoma xenografts (PDXs) were derived in our laboratory from patients with treatment-refractory MCL as previously described (23). The cell lines were tested for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza). All PDXs were confirmed by next-generation exome sequencing to keep majority of somatic mutations with the primary MCL cells from which they were derived (Supplemental Figure 1, Supplemental Table 1, Supplemental Table 2). Primary lymphoma cells were obtained from patients with MCL according to the Declaration of Helsinki. Informed written consent' was obtained from each subject. The experimental design was approved by the Ethics Committee of the General University Hospital Prague under number 63/16.

Next generation exome sequencing

Samples were sequenced by our facility on the NextSeq 500 (Illumina, San Diego, CA) instrument according to manufacturer's protocols and sequencing libraries were prepared using SureSelectXT Human All Exon V6+UTR kit (Agilent Technologies, Santa Clara, CA). Sequence reads from PDX samples were first aligned against the mouse reference genome mm10 combined with the human reference genome hg19 and murine reads were filtered out from further analysis by a custom script to reduce risk of contamination. Remaining reads were then aligned against the human reference genome hg19. All alignments were performed by BWA(24). Genomic variants were called with samtools and VarScan 2(25,26). Variant annotation was performed using SnpEff(27). Only nonsynonymous variants in the gene coding regions with coverage of at least 10 reads with mapping quality and base quality higher than 20 in all related samples were compared together based on their frequency. Variants present in patient’s germline DNA at frequency higher than 0.05 were excluded from analysis in all cases. We compared variants with an allele fraction ≥0.2 in at least one of the compared samples that were present in at least 3 reads in both relapsed sample and derived PDX samples. All variant filtering was done in RStudio and frequencies and counts of variants were plotted using the ggplot2 library (http://www.R-project.org; http://www.rstudio.com). These variants were then manually reviewed in Integrative
Genomics Viewer (http://www.broadinstitute.org/igv) and clear sequencing artefacts or variants present but not called in the germline sample were also excluded. List of 122 genes of special interest was created based on recent publications of frequently mutated genes in MCL samples and variants present in these genes were specifically selected and marked in resulting diagrams and tables (28-33). Copy number variants were predicted using CNVkit with normalization to pooled normal samples sequenced on the same instrument using the same library preparation kits (34). Inferred segmental changes were calculated using the fused lasso method and plotted in diagrams for relapse and PDX samples (35).

**Fluorescence in situ hybridization (FISH)**

Interphase FISH analyses were performed on fixed cell suspensions using commercially available probes from Abbott Molecular, USA (Vysis LSI MYC BA, LSI IGH/MYC/CEP8 TC DF, LSI CDKN2A/CEP 9, LSI ATM/LSI TP53, LSI IGH/CCND1 DF, LSI 13 RB1/LSI 13q34 and LSI BCL2 BA). FISH assays were performed according to the manufacturers’ protocols. At least 200 interphase nuclei were analyzed by two independent observers.

**Real-time RT-PCR**

Total RNA was isolated from cell lines in Ribozol (Amresco) using phenolchloroform extraction. Complementary DNA synthesis was carried out from 1 μg of total RNA with High-Capacity cDNA Reverse Transcription Kit (random primers) (Applied Biosystems). Real-time RT-PCR was performed using TaqMan Gene Expression Assays (MCL1: Hs01050896_m1, NOXA: Hs00560402_m1, GAPDH: Hs02758991_g1) on ABI 7900HT detection system (Applied Biosystems).

**Western blotting (WB)**

WB was performed as previously described (19). The antibodies were from Cell Signaling: BIM (C34C5), BCL-XL (2764), Santa Cruz Biotechnology: BCL2 (C21), MCL1 (S-19), Enzo: NOXA (114C307.1) and Abcam: beta-Actin (AC15).

**Immunoprecipitation**

Cells were lysed in CHAPS lysis buffer (0.3% CHAPS, 1 mM EDTA, 40 mM HEPES pH 7.5 and 120 mM NaCl) supplemented with protease and phosphatase inhibitor cocktail (Sigma)) for 30 minutes. Protein concentrations of cell extracts were determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and equal amounts of protein samples...
were incubated with BCL2 or MCL1 antibodies listed above or an isotype control immunoglobulin (rabbit IgG (Santa Cruz)) for 1 hour at 4°C. Consequently, G-protein beads were added for overnight incubation. Immunoprecipitates were washed in CHAPS lysis buffer, resuspended in 2x Laemmli buffer (Bio-Rad) and analyzed by western blotting.

*Array comparative genomic hybridization / single-nucleotide polymorphism microarray analysis*

A microarray analysis (array-comparative genomic hybridization/single-nucleotide polymorphism [aCGH/SNP]) was performed with SurePrint G3 Cancer CGH+SNP Microarray, 4x180K (Agilent technologies, USA) to detect unbalanced chromosomal changes and copy number neutral loss of heterozygosity. The final product was scanned with the Agilent G2565CA Microarray Scanner System (Agilent technologies, USA) and analyzed with Agilent Cytogenomics v4.0.3.12 (Agilent technologies, USA).

*Apoptosis measurement*

Number of apoptotic and/or necrotic cells was determined by flow cytometry (BD FACS Canto II) using Annexin V FITC (Apronex, Czech Republic) and propidium iodide (Sigma). Percentage of apoptotic and/or necrotic cells was calculated using the following formula: 

\[
\text{Percentage of apoptosis} = \frac{\text{measured apoptosis} - \text{basal apoptosis}}{100 - \text{basal apoptosis}} \times 100 \%
\]

Drug concentrations that induced apoptosis in 50% cells after 24 hours (IC50) were determined by nonlinear regression algorithms using Graph Pad Prism software.

*Immunohistochemistry*

Sections from FFPE blocks from patients and from murine DLBCL xenografts were cut and stained by Hematoxylin & Eosin and Giemsa stains. Immunohistochemistry was performed using BCL2 (clone 124, Dako), BCL-XL (clone B4H6, Cell Signaling), MCL1 (clone S-19, Santa Cruz), BIM (clone H-191, Santa Cruz) and NOXA (clone 114C307, Santa Cruz) as previously described(19). Heat induced pretreatment of deparaffinized tissue sections in buffer pH 9.0 (Dako, S2367) was applied. After blocking of endogenous peroxidase activity (3% solution of hydrogen peroxide), samples were incubated overnight at 4°C with primary antibodies anti- MCL1 (1:200) and anti-BCL-XL (1:200). Detection of primary antibody binding was performed for anti-MCL1 with polymer system (secondary antibody and peroxidase; N-Histofine Simple Stain MAX PO, Nichirei Biosciences) and for anti-BCL-XL with biotinylated secondary antibody and avidine-peroxidase complex (LSAB+, Dako...
REAL™ Detection Systems, HRP/DAB+, Rabbit/Mouse, Dako), followed by incubation with solution of hydrogen peroxide and chromogen substrate DAB (3,3’-diaminobenzidine tetrahydrochloride, Dako). Nuclei were counterstained with Harris hematoxylin. After dehydration and clearing in xylene, slides were mounted in organic-solvent-based medium and evaluated under light microscope. To differentiate BCL2, BCL-XL, MCL1, BIM and NOXA-positive and -negative MCL cases, we applied the commonly used cutoff value of 30% cells.

Establishment of MCL clones with knock-down or transgenic overexpression of MCL1, BIM and NOXA

**MCL1:** MCL1 cDNA was cloned into Sleeping beauty plasmids pSBtet Pur (https://www.addgene.org/60507/) published in PMID: 25650551 and then electroporated together with transposase coding plasmid (pCMV(CAT)T7-SB100) for insertion into genome. Control plasmid (pSBtet Pur empty) had all inducible elements, but was empty and did not produce any protein upon Dox addition. Three days after electroporation of cells of interest, puromycin was added in final concentration of 2 μg/mL and the cells selected for 6 days. Cells were then re-suspended in media with Dox at final concentration 200 ng/mL and MCL1 protein expression was measured after 48 hours by western blotting.

**BIM:** Cell lines with stably integrated shRNA-gene/cDNA were prepared as previously described(19). Briefly, packaging lentiviral vectors pMD2.G (Addgene, plasmid 12259), psPAX2 (Addgene, plasmid 12260) together with pLKO.1 (Sigma Aldrich)/pCDH-neo (SBI)/pLVX TetONE-puro vector containing the gene of interest were transfected into HEK 293T/17. Conditioned medium was harvested 36 hours later, centrifuged and precipitated using PEG-it (System Biosciences) according to manufacturer’s instructions. Precipitated particles were resuspended in PBS and stored in -80°C. Target cells were infected with equivalent multiplicity of infection (MOI) for 24 hours and the transductants were selected in the growth medium containing 2-3 μg/ml puromycin (LKO1-shRNAs, LVX TetONE-puro) or 2 mg/ml G-418 (CDH-cDNAs).

**NOXA:** For siRNA-mediated silencing, we used NOXA duplex (GGUGCACGUUUCAAUUTT) and a negative control siRNA as previously described (Eurogentec)(36,37). MCL cells were electroporated with Amaxa nucleofector system using Solution V (Lonza) using a program number 0-017. Twenty-four hours after nucleofection the knock-down efficiency was validated by western blotting and the cells were exposed to venetoclax or S63845.
Experimental therapy of lymphoma-bearing mice

The experimental design was approved by the Institutional Animal Care and Use Committee (MSMT-11255/2015-4; 592/15). NOD.Cg-Prkdcscid II2rgtm1Wjl/SzJ mice (referred to as NSG mice) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). All animals were maintained in a pathogen-free environment in individually ventilated cages and provided with sterilized food and water. Adult female NSG mice were used for all experiments. NSG mice were subcutaneously (SC) inoculated with 10 x 10^6 lymphoma cells. Therapy was initiated when all mice developed palpable tumors (= day 1, D1). At D1 all mice were stratified so that all cohorts contained mice with comparable calculated tumor volumes. VTX and S63845 were purchased from MedchemExpress. VTX (50 mg / kg, by oral gavage) was first diluted in ethanol and then mixed with phosal-G / PEG-400 (1 : 3 : 6). VTX was given on days 1, 2, 3, 6 and 7. S63845 (25 mg / kg) was diluted in 50mM HCL and 20% hydroxypropyl-beta-cyclodextrin, and administered intravenously (IV) on days 1, 2, 3, 6 and 7. In case of damaged veins, S63845 was administered intraperitoneally (IP). Tumor growth was recorded daily using three perpendicular dimensions (in millimeters) with a digital caliper. Tumor volumes were calculated using the following formula: \( \pi \times \text{length} \times \text{width} \times \text{height} \). Observation was terminated (and experimental mice euthanized) when grown SC tumors exceeded 2 cm in the largest diameter. Tumors were excised and weighed, and the euthanized mice were dissected in search for any signs of advanced (disseminated) lymphoma (e.g. splenomegaly, abdominal lymphoma spread etc.).

Statistical analysis

Data from five in vivo experiments were analysed, each experiment covering different time periods with different numbers of known data points (see Figure 5). For the purpose of assessing the statistical significance of treatment effectiveness, we made an assumption that the calculated differences between mean tumor sizes in the control group and groups treated with monotherapies as well as between the latter groups and those treated with combined therapies were generated by a process which includes a deterministic linear trend in the form of \( y_t = \beta_0 + \beta_1 t + \epsilon_t \), where \( y_t \) denotes the data-generating stochastic process of the analyzed differences, \( t = 1, 2, …T \) is a time variable, \( T \) signifies the length of the experiment in days and \( \epsilon_t \) is the Gaussian IID white noise. Having concurrently performed 34 statistical hypothesis tests about the zero value of \( \beta_1 \), the Bonferroni correction of 5% and 1%
simultaneous significance levels was utilized, resulting in individual significance levels of 0.1471% and 0.0294%, respectively. The results are shown in Table 1.

Results

Sensitivity of established MCL lines and primary MCL cells to VTX and S63845

Cytotoxic effects of VTX and S63845 was analyzed using a panel of nine MCL cell lines (Figure 1A-C) and nine freshly isolated primary MCL cells (Figure 1D, E). Patients’ baseline characteristics are displayed in Supplemental Table 3. Cells were arbitrarily considered as sensitive, if they displayed > 50% apoptosis 24 hours after exposure to 1 μM VTX or 1 μM S63845. Using these criteria five out of nine cell lines were sensitive to VTX (Figure 1A, C). Only MINO cell line was sensitive to S63845 (Figure B, C). In contrast to the cell lines, tested primary MCL samples were all sensitive to VTX, and five out of nine were sensitive to S63845 (Figure 1D-F).

Prevalence and impact of BIM deletion in established MCL cell lines and primary MCL cells

Protein expression of BCL2, MCL1, BIM and NOXA was analyzed by WB using a panel of 9 MCL cell lines, 9 primary MCL samples and 5 PDXs (Figure 2A-C). In addition, protein expression of BCL2, MCL1, BIM and NOXA was evaluated semi-quantitatively in formalin-fixed paraffin-embedded (FFPE) tissue samples obtained from MCL cell line-xenografted mice (Figure 2D) and from patients with MCL at diagnosis or disease relapse (Figure 2E).

As previously reported, three commonly used MCL cell lines (JEKO-1, MINO and Z138) lack BIM protein expression as a consequence of biallelic BIM gene deletion (Figure 2A). We confirmed that lack of BIM protein observed in these cell lines is associated with resistance (JEKO-1, Z138) or decreased sensitivity (MINO) to VTX (Figure 1A, C). First, we functionally analyzed the role of BIM in VTX-triggered apoptosis by transgenic BIM overexpression and shRNA-mediated BIM knock-down in selected MCL cell lines. We confirmed that ectopic (over)expression of BIM in JEKO-1 and Z138 partially restored VTX-sensitivity of these cell lines (Figure 3A, D). In analogy, knock-down of BIM expression in the highly VTX-sensitive cell lines HBL2 and MAVER-1 partially inhibited VTX-induced apoptosis (Figure 3B, E).

Some groups reported that deletions of BIM gene are frequently seen in primary MCL samples (12-14). We hypothesized that a deletion of BIM might predict VTX resistance in
MCL patients in the clinical grounds. Unexpectedly, analysis of BIM protein expression either by WB using a panel of nine primary MCL samples and five PDX cells, or by IHC using a panel of 37 FFPE tissue samples obtained from patients with MCL did not reveal a single sample with undetectable protein expression (Figure 2B, C, E, Supplemental Table 4). To confirm our findings at the genomic level, we implemented aCGH on a validation set of 24 primary MCL samples obtained from peripheral blood of leukemized patients and on two MCL cell lines with previously reported deletion of BIM (JEKO-1 and MINO). While deletion of BIM gene was confirmed in both tested MCL cell lines, only one out of 24 analyzed primary MCL cell samples had detectable monoallelic loss of BIM (in this case the whole long arms of chromosome 2 were deleted within a complex karyotype) (Supplemental Figure 3, Supplemental Table 3).

Expression of NOXA in MCL cells and its functional impact on MCL1-mediated VTX resistance

Discrepancies in the protein expression of NOXA between primary MCL cells and established cell lines have been reported by other groups(38). Here we confirmed that not only primary MCL cells, but also PDXs express significantly lower levels of NOXA protein compared to majority of the established MCL cell lines (Figure 2, Supplemental Table 4). NOXA is a BH3 only protein that specifically binds and blocks MCL1. We assumed that relative overexpression of NOXA observed in the majority of MCL cell lines compared to primary MCL cells and PDXs might render MCL cell lines (that do not harbor deletions of BIM) hypersensitive to VTX. Indeed, siRNA-mediated NOXA gene and protein knock-down decreased VTX-triggered apoptosis (Figure 3C, F).

Mechanisms of resistance of MCL cells to VTX

Besides biallelic deletions of BIM (which plausibly represent very rare events in MCL) upregulation of MCL1 was associated with VTX resistance in diffuse large B-cell lymphoma and acute myeloid leukemia cells(16,39). Mechanistically, MCL1 may serve as a buffer for BIM released upon binding of VTX to BCL2(16). Indeed, immunoprecipitation of REC-1 cells before and after VTX treatment showed not only expected great reduction of BIM bound to BCL2, but also substantial increase of BIM bound to MCL1, both as a consequence of VTX treatment (Figure 4A-D). This molecular mechanism (i.e. buffering of BIM by MCL1) also provides a plausible explanation for the observed VTX resistance of REC-1 cells. Next, we established MAVER-1 and HBL2 venetoclax-resistant clones by long-term cultivation
with gradually increasing VTX concentrations up to 1 μM. HBL2 VTX-R cells had significant upregulation of both MCL1 and NOXA proteins, but only MCL1 mRNA was significantly upregulated, specifically 22.6-times more MCL1 mRNA in VTX-R compared to CTRL. NOXA mRNA remained unchanged (i.e. < 2-fold total mRNA change) indicating posttranscriptional mechanism of NOXA protein deregulation in HBL2 VTX-R cells. MAVER-1 VTX-R had marked downregulation of NOXA with unchanged MCL1 protein. Messenger RNA levels of NOXA and MCL1 were not significantly (> 2-fold) changed between VTX-R and CTRL MAVER-1 cells (data not shown), again suggesting posttranslational mechanism of NOXA dysregulation.

Interestingly, transgenic overexpression of MCL1 was accompanied by a similar pattern of NOXA protein level deregulation as in the respective HBL2 and MAVER-1 VTX-R clones (compare Figures 4E and 4G). MAVER-1 MCL1-UP clone displayed upregulation of MCL1 accompanied by decreased levels of NOXA protein, which was associated with significant inhibition of both VTX and S63845-triggered apoptosis (Figure 4E-F). In contrast, HBL2 MCL1-UP clone displayed upregulation of NOXA and its sensitivity to VTX and S63845 compared to empty vector transfected HBL2 cells was only mildly attenuated (Figure 4E-F).

**Co-targeting of BCL2 and MCL1 on a panel of five PDX murine models of aggressive MCL**

MCL1 protein appears to be a critical molecule that attenuates VTX-induced apoptosis with MCL1 overexpression leading to VTX-resistance (Figure 4). Concurrent blockage of BCL2 with VTX and MCL1 with a highly-specific small molecule inhibitor S63845 might thus represent an effective treatment strategy in MCL.

**In vivo** experiments were implemented on a panel of five PDX models derived from patients with treatment-refractory MCL (Supplemental Figure 1, Supplemental Table 1, and Supplemental Table 2). First, single-agent S63845 (25 mg/kg) showed promising **in vivo** efficacy in all five PDX models. In the case of VFN-M3, efficacy of S63845 was even significantly higher than that of VTX (Figure 5, Supplemental Table 5). Second, the combination of VTX and S63845 was in all five PDX models associated with excellent anti-MCL synergy compared to single-agent approaches.

**Discussion**
In the current study we evaluated molecular mechanisms of sensitivity of MCL cells to BCL2-targeting agent VTX. We demonstrated critical role of the functional status of anti-apoptotic MCL1 protein in conferring both inherited and acquired resistance to VTX. As MCL1 and its interactions with NOXA appear as principal molecules that attenuates VTX-induced apoptosis, we proposed a treatment strategy aimed at concurrent inhibition of BCL2 by VTX and MCL1 by S63845, which proved to be highly synergistic in MCL in vivo.

From our data as well as from other published communications there is an apparent bias in the expression of BCL2 and MCL1-targeting BH3-only proteins BIM and NOXA between established cell lines and primary MCL cells. At the genomic level, biallelic loss of BIM gene was repeatedly reported in MCL cell lines, but never in primary MCL samples(12-14). In the original paper by Tagawa et al only heterozygous (not homozygous) deletions of BIM were found in as few as 5 of 27 (18.5%) MCL patients(12). In another study, Mestre-Escorihuela et al focused mainly on analysis of cell lines and the only information provided about BIM status in primary MCL samples was based on IHC analysis by tissue microarray with loss of BIM protein expression detected in 7 out of 22 (33%) patient samples(14). Katz et al. focused on genetic proof of concept of biallelic BIM gene deletion during MCL lymphomagenesis, however, did not analyze primary MCL cells(13).

In our study, aCGH analysis of 24 primary MCL samples obtained from patients with high-risk disease according to MCL international prognostic index (MIPI) identified only a single patient with a monoallelic BIM deletion (in the context of loss of entire long arms of chromosome 2 within a complex karyotype) (Supplemental Table 3, Supplemental Figure 3). Similarly to the genomic analysis, IHC analysis of 37 FFPE tissue samples obtained from MCL patients did not identify a single case with undetectable BIM protein (Supplemental Table 4). Based on our results, and other published data, it appears that biallelic deletions of BIM or loss of BIM protein expression are extremely rare events in primary MCL cells. It might be further speculated that MCL cell lines with loss of BIM might be derived from such rare cases reflecting extremely aggressive, highly-rearranged diseases. On the other hand, all five PDX murine models of MCL derived from patients with relapsed / refractory disease in our laboratory retained BIM protein expression by western blotting. Therefore, one should consider that loss of BIM in MCL cell lines might be a consequence of in vitro-induced changes associated with a cell line derivation.

Another molecule with markedly different protein expression between primary MCL samples and MCL cell lines is NOXA(38). As few as 4 of 72 primary MCL samples were reported to
express NOXA compared to ubiquitous overexpression of NOXA detectable in most (if not all) MCL cell lines(14). In our study, NOXA protein expression was markedly lower in primary MCL samples (and in PDX cells) in comparison to MCL cell lines, as analyzed by both approaches, IHC and western blotting (Figure 2, Supplemental Table 3).

In summary, the two critical proapoptotic proteins, BIM and NOXA, are differentially expressed between MCL primary samples and established cell lines (Figure 2). Using clones with transgenic (over)expression or shRNA/siRNA-mediated knock-down of BIM and NOXA we confirmed that they are indeed key mediators of VTX proapoptotic activity (Figure 3). As a consequence, MCL cell lines unfortunately represent unreliable models for assessing proapoptotic activity of BH3 mimetics including VTX. Loss of BIM makes some cell lines (JEKO-1, Z138, MINO) “falsely” VTX resistant, and conversely, overexpression of NOXA makes other cell lines (HBL2, MAVER1, GRANTA-519) “falsely” hypersensitive to VTX. From this perspective, PDX models, which both retain BIM expression, and do not overexpress NOXA, represent better models than cell lines, more closely reflecting primary MCL samples in this context. In addition, it was repeatedly demonstrated that microenvironmental factors may induce resistance to VTX, namely by PI3K-AKT-mTOR pathway-mediated upregulation of BCL-XL and MCL1, further suggesting that PDX models likely represent the most relevant preclinical models for evaluation of anticancer activity of BH3 mimetics(39-41).

Molecular mechanisms of acquired resistance to VTX in MCL remain poorly understood. Upregulations of MCL1 and BCL-XL were reported as plausible mechanisms in diffuse large B-cell lymphoma(39). Our data pinpointed MCL1 as a principal mediator of VTX resistance (VTX-R). First, MCL1 upregulation was observed in both MCL clones (HBL2-VTX-R and MAVER1-VTX-R) derived from two most VTX-sensitive cell lines HBL2 and MAVER1 (Figure G-H). Second, we confirmed that MCL1 can act as a buffer for BIM (and potentially other BH3-only proteins) released from BCL2 by VTX (Figure 4A-C)(16). And third, stable overexpression of MCL1 in HBL2 and MAVER1 cell lines was associated with significantly decreased sensitivity to VTX (Figure 4E-F). The data thus provided sound mechanistic explanation for potential synthetic lethality between VTX and S63845 in MCL. We have confirmed that both VTX- and S63845-induced cytotoxicity is a caspase-dependent apoptotic process (Supplemental Figure 4A-B). Interestingly, VTX-resistant HBL2 clones were “cross”-resistant not only to S63845, but also to two different BCL-XL inhibitors WEHI-539 and A-1155463 (Figure 4H, Supplemental Figure 4E-F). The data thus suggest a more
complex disruption of mitochondrial apoptosis (in addition to MCL1-NOXA deregulation) as a result of in vitro acquired venetoclax resistance.

Even though MCL is generally considered a BCL2-dependent malignancy, we showed very promising anti-lymphoma efficacy of S63845 monotherapy (Figure 5, Supplemental Table 4). Concurrent inhibition of MCL1 and BCL2 with S63845 and VTX, respectively, was associated with significantly increased anti-lymphoma efficacy compared to the single-agent approaches. The combination of VTX and S63845 was well tolerated, and induced long-term lymphoma-free survival of MCL xenografts in five different PDX models derived from patients with chemotherapy-refractory diseases.

In conclusion, the data strongly support investigation of S63845 in combination with VTX for targeted eradication of chemotherapy-resistant MCL cells including patients with adverse cytogenetics in the clinical grounds.

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Legends:

Figure 1. Sensitivity of MCL cell lines and primary cells to VTX and S63845

Legend:
A, B, D, E. Cytotoxic activity of VTX and S63845 toward established MCL cell lines (A-B) and primary MCL cells (D-E). Y axes show numbers of Annexin-V+/PI+ cells 24 hours after exposure to 1 μM VTX or S63845. PT stands for “patient”. Bars represent means ± standard deviations of two independent experiments. C, F. Calculated IC50 for the cell lines (C) and primary lymphoma samples (F). Basal apoptosis of unexposed patients’ cells for PT1-PT9 was 40%, 24%, 37%, 26%, 50%, 15%, 40%, 36%, and 44%.

Figure 2. Expression of BIM and NOXA in MCL cell lines and primary cells

Figure 2 Legend:
A-C. Western blot analysis of BCL2, MCL1, BIM and NOXA on a panel of established MCL cell lines (A), primary lymphoma samples (B) and PDXs (C). MAVER-1 was used as inter-assay control. D-E. Semi-quantitative intensity (Y axes) of staining of BCL2, MCL1, BIM and NOXA on a panel of formalin-fixed paraffin-embedded sections of MCL cell line-based subcutaneous xenografts (D) and patient (PT) primary samples (E). For detail see Supplemental Methods.

Figure 3. BIM and NOXA regulate sensitivity to VTX

Figure 3 Legend:
A. Transgenic overexpression of BIM in JEKO-1 and Z138 cell lines. “CTRL” stands for the original cell lines with BIM gene deletion. B. shRNA-mediated BIM gene knock-down in HBL2 and MAVER-1 cell lines. “Empty” stands for empty vector-transfected cells. C. siRNA-mediated NOXA gene knock-down in HBL2 and MAVER-1 cell lines. “siCTRL” stands for cells transfected with non-coding siRNA. D. Cytotoxic activity of VTX (1 μM, 24 hours) in JEKO-1 and Z138 cell clones with transgenic overexpression of BIM (JEKO-1:BIM, Z138:BIM). “DOX” stands for doxycycline. E. Cytotoxic activity of VTX (25 nM, 24 hours) in HBL2 and MAVER-1 cell clones with shRNA-mediated knock-down of BIM expression. F. Cytotoxic activity of VTX (25 nM, 24 hours) in HBL2 and MAVER-1 cell clones with siRNA-mediated knock-down of NOXA expression. Y axes show numbers of
Annexin-V+/PI+ cells. Bars represent means ± standard deviations of two independent experiments.

Figure 4. Molecular mechanisms of resistance to VTX

Figure 4 Legend:
A. Immunoblot of the total REC-1 protein lysate 24 hours after exposure to VTX (1 \( \mu \text{M} \)) compared to untreated REC-1 cells (CTRL) shows unchanged levels total BIM and NOXA proteins. B. Immunoprecipitation of REC-1 cells with BCL2 antibody 24 hours after exposure to VTX (1 \( \mu \text{M} \)) compared to untreated REC-1 cells (CTRL) shows decrease of BCL2-bound BIM after exposure to VTX. C. Immunoprecipitation of REC-1 cells with MCL1 antibody 24 hours after exposure to VTX (1 \( \mu \text{M} \)) compared to untreated REC-1 cells (CTRL) shows increase of MCL1-bound BIM (released from BCL2) after exposure to VTX. D. Numbers of Annexin-V+/PI+ cells 24 hours after exposure of REC-1 cells to VTX (1 \( \mu \text{M} \)), S63845 (5 \( \mu \text{M} \)) and the combination of both agents. Y axes show numbers of Annexin-V+/PI+ cells 24 hours after exposure to 1 \( \mu \text{M} \) VTX, or the combination of both agents. E. Immunoblots of HBL2 and MAVER-1 clones with transgenic overexpression of MCL1 (MCL1-UP) compared to cells transfected with an empty vector (Empty). F. Numbers of Annexin-V+/PI+ cells 24 hours after exposure of the MCL1-UP clones to VTX (100 nM) or S63845 (5 \( \mu \text{M} \)). G. Immunoblots of HBL2 and MAVER-1 clones with in vitro acquired resistance to VTX (VTX-R) compared to the original cell lines (CTRL). H. Numbers of Annexin-V+/PI+ cells 24 hours after exposure of the VTX-R clones to VTX (100 nM) or S63845 (5 \( \mu \text{M} \)) (Y axes).

Downregulation of MCL1 and BCL-XL in HBL2, as well as downregulation of MCL1 and BIM in MAVER-1 are the most plausible consequence of the apoptotic process.

Figure 5. Experimental therapy of MCL-bearing mice

Figure 5 Legend:
Growth curves of the individual treatment cohorts constructed from the calculated tumor volumes (means ± standard deviations). For details see Methods. X are days, where day 1 (D1) marks initiation of the therapy, Y shows calculated tumor volumes from three perpendicular tumor dimensions. CTRL= untreated controls, VTX = venetoclax. * The mice were euthanized despite small subcutaneous tumors because they developed generalized inability to thrive. Postmortem examination demonstrated spleen enlargement in a majority of mice- see Supplemental Table 4.
Table 1. Statistical analysis of *in vivo* experiments

**Table 1 Legend:** P-values of partial t-tests about zero slope of mean tumor size differences (statistical significance: * 5% and *** 1% simultaneous significance level; N indicates a negative slope). Except for the linear trend of mean tumor size differences between treatments with CFZ and VTX+CFZ combination in the VFN_M2 experiment, all slopes were positive, suggesting that each monotherapy was effective in reducing the tumor size, each combination being more efficient than the corresponding monotherapies. 26 out of 34 p-values in Table 1 show statistical significance at 1% simultaneous significance level, four p-values indicating statistical significance at 5% simultaneous significance level. These results suggest a statistically significant difference between the development of tumor size in control groups and those treated with monotherapies as well as between the groups treated with combined therapies and the one treated with corresponding monotherapies. In other words, there is not only practically significant but also statistically significant difference in the effectiveness of different treatments.
Table 1

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Figure 1

A. VTX 1 μM

B. S63845 1 μM

C. IC50 (nM)

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<th>MCL - cell line</th>
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<td>UPF1G</td>
<td>&gt;10000</td>
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</table>

D. VTX 1 μM

E. S63845 1 μM

F. IC50 (nM)

| PT 1 | 3   | 355  |
| PT 2 | 37  | 398  |
| PT 3 | 62  | 2495 |
| PT 4 | 244 | 6319 |
| PT 5 | 86  | 169  |
| PT 6 | 23  | 913  |
| PT 7 | 119 | 974  |
| PT 8 | 117 | 1991 |
| PT 9 | 982 | >10000 |
Figure 2

A. Cell line-based xenografts

B. PT samples

D. Cell line-based xenografts

E. PT samples

Semi-quantitative staining intensity

BCL2 MCL1 BIM NOXA
Co-targeting of BCL2 with venetoclax and MCL1 with S63845 is synthetically lethal \textit{in vivo} in relapsed mantle cell lymphoma

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