Cotargeting of BCL2 with Venetoclax and MCL1 with S63845 Is Synthetically Lethal In Vivo in Relapsed Mantle Cell Lymphoma

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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, has promising antitumor activity in MCL. We analyzed molecular mechanisms of venetoclax resistance in MCL cells and tested strategies to overcome it.

Experimental Design: We confirmed key roles of proapoptotic proteins BIM and NOXA in mediating venetoclax-induced cell death in MCL. Both BIM and NOXA are, however, differentially expressed in cell lines compared with 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 venetoclax. Consequently, we tested an experimental treatment strategy based on cotargeting BCL2 with venetoclax and MCL1 with a highly specific small-molecule MCL1 inhibitor S63845. The combination of venetoclax 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 venetoclax 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 first-line treatment of MCL is based on conventional chemotherapy and anti-CD20 mAb rituximab.

Relapsed or refractory patients usually receive either different conventional chemotherapy agents (e.g., bendamustine, cisplatin etc.), or they are offered innovative nonchemotherapy 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 antiapoptotic and prosurvival proteins, is overexpressed virtually in all MCL tumors that are considered BCL2-dependent. Venetoclax, 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 prosurvival 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, venetoclax has been approved for the patients with chronic lymphocytic leukemia/small lymphocytic lymphoma and showed promising antilymphoma activity in MCL and acute myelogenous leukemia patients (7–11).

Despite promising data from early clinical trials, resistance sooner or later develops in a majority of patients with MCL on venetoclax monotherapy (8). Mechanisms of constitutive or
acquired venetoclax resistance remain poorly understood. It was repeatedly reported that similarly to approximately one third of currently available MCL cell lines (JEKO-1, Z138, MINO, and REC-1), primary MCL cells also frequently harbor deletions of BCL2-like1/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 venetoclax proapoptotic activity, its homozygous deletion might represent a valuable marker of venetoclax resistance. Upregulation of MCL1, another key antiapoptotic regulator besides BCL2, was also reported as a plausible mechanistic rationale for acquired resistance to venetoclax (15–17). Therefore, several groups including our own employed diverse strategies to indirectly inhibit MCL1 protein (in combination with venetoclax) including cyclin-dependent kinase inhibitor dinaciclib, plant alkaloid homoharringtonine or anthracycline daunorubicin (16, 18, 19). In 2017, Kotchny and colleagues reported single-agent antitumor activity of a novel, highly specific MCL1 inhibitor S63845 in many cancers including hematologic malignancies (20). However, antilymphoma activity of S63845 in MCL (considered a BCL2-dependent malignancy) was not studied. Antitumor activity of the combination of venetoclax and S63845 has been studied in acute leukemias, and there is one active trial testing the combination of BCL2- and MCL1-inhibitors (venetoclax and S63845) clinically in patients with acute myeloid leukemias (GovTrial Number NCT03672093; refs. 9–11, 21, 22).

**Materials and 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 from Prof. Martin Dreyling. The cell lines were authenticated in July 2016 by Multiplexion. UPF1G and UPF1H cell lines and all patient-derived lymphoma xenografts (PDX) were derived in our Mycoplasma: using 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 (Supplementary Fig. S1; Supplementary Table S1; Supplementary Table S2). 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 (Illuma) instrument according to the manufacturer’s protocols and sequencing libraries were prepared using SureSelectXT Human All Exon V6 + UTR Kit (Agilent Technologies). 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 SnvEff (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 on the basis of 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).

**FISH**

Interphase FISH analyses were performed on fixed cell suspensions using commercially available probes from Abbott Molecular (Vysis LSI MYC BA, LSI IGH/MYC/CEP8 TC DF, LSI CDKN2A/CEP 9, LSI ATM/LSI TP53, LSI IGH/CCND1 DF, LSI CDKN2A/CEP 9, 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 phenol–chloroform 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).

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**Translational Relevance**

BCL2-targeting agent venetoclax 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 venetoclax. Consequently, we proposed an experimental treatment strategy based on cotargeting BCL2 with venetoclax and MCL1 with a highly specific small-molecule MCL1 inhibitor S63845. The combination of venetoclax and S63845 demonstrated synthetic lethality in vivo on a panel of five patient-derived xenografts established from patients with relapsed MCL with adverse cytogenetics.
Western blotting

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

Immunoprecipitation

Cells were lysed in CHAPS lysis buffer (0.3% CHAPS, 1 mmol/L EDTA, 40 mmol/L HEPES pH 7.5 and 120 mmol/L 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 Biotechnology)] for 1 hour at 4°C. Consequently, G-protein beads were added for overnight incubation. Immunoprecipitates were washed in CHAPS lysis buffer, resuspended in 2× Laemmli buffer (Bio-Rad) and analyzed by Western blotting.

Array comparative genomic hybridization/SNP microarray analysis

A microarray analysis [array-comparative genomic hybridization/SNP (aCGH/SNP)] was performed with SurePrint G3 Cancer CGH+SNP Microarray, 4 × 180K (Agilent Technologies) 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) and analyzed with Agilent Cytogenomics v4.0.3.12 (Agilent Technologies).

Apoptosis measurement

Number of apoptotic and/or necrotic cells was determined by flow cytometry (BD FACS Canto II) using Annexin V FITC (Apropox) and propidium iodide (Sigma). Percentage of apoptotic and/or necrotic cells was calculated using the following formula: (measured apoptosis − basal apoptosis)/(100 − basal apoptosis) × 100 (%). Drug concentrations that induced apoptosis in 50% cells after 24 hours (IC50) were determined by nonlinear regression algorithms using Graph Pad Prism software.

IHC

Sections from formalin-fixed paraffin-embedded (FFPE) blocks from patients and from murine DLBCL xenografts were cut and stained by hematoxylin & eosin and Giemsa stains. Cut and stained by hematoxylin & eosin and Giemsa stains. Blocks from patients and from murine DLBCL xenografts were IHC stained with the Agilent CGH microarray scanner system and analyzed by Western blotting.

BIM.

Cell lines with stably integrated shRNA-gene/cDNA were prepared as described previously (19). Briefly, packaging lentiviral vectors pMD2.G (Addgene, plasmid 12259), psPAX2 (Addgene, plasmid 12260) together with pLKO.1 (Sigma Aldrich)/pCDH-neo (SBI)/pLVX TetOPE-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 the manufacturer’s instructions. Precipitated particles were resuspended in PBS and stored in −80°C. Target cells were infected with equivalent multiplicity of infection for 24 hours and the transductants were selected in the growth medium containing 2 to 3 μg/mL puromycin (LK01-shRNAs, LVX TetOPE-puro) or 2 mg/mL G418 (CDH-cDNAs).

NOXA.

For siRNA-mediated silencing, we used NOXA duplex (GGUUGACUGUUAUCUAGUUU) and a negative control siRNA as described previously (Eurogentec; refs. 36, 37). MCL cells were electroporated with Amaza nucleofector system using Solution V (Lonza) using a program number 0-017. Twenty-four hours after nucleofection the knockdown 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-Pkdcre1-/-R2hx1-Ong7/W1 SCID mice (referred to as NSG mice) were purchased from The Jackson Laboratory. 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 inoculated with 10 × 10⁶ lymphoma cells. Therapy was initiated when all mice developed palpable tumors.
Statistical analysis

Data from five in vivo experiments were analyzed, each experiment covering different time periods with different numbers of known data points (see Fig. 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 combined therapies were generated by a process as a consequence of biallelic BIM gene deletion (Fig. 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 venetoclax (Fig. 1A and C). First, we functionally analyzed the role of BIM in venetoclax-triggered apoptosis by transgenic BIM overexpression and shRNA-mediated BIM knockdown in selected MCL cell lines. We confirmed that ectopic (over) expression of BIM in JEKO-1 and Z138 partially restored venetoclax-sensitivity of these cell lines (Fig. 3A and D). In analogy, knockdown of BIM expression in the highly venetoclax-sensitive cell lines HBL2 and MAVER-1 partially inhibited venetoclax-induced apoptosis (Fig. 3B and 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 venetoclax resistance in MCL patients in the clinical grounds. Unexpectedly, analysis of BIM protein expression either by Western blotting 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 (Fig. 2B, C, and E; Supplementary Table S4). 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 deleted BIM (JEKO-1 and MINO).
Although deletion of \textit{BIM} gene was confirmed in both tested MCL cell lines, only one of 24 analyzed primary MCL cell samples had detectable monoallelic loss of \textit{BIM} (in this case, the whole long arms of chromosome 2 were deleted within a complex karyotype; Supplementary Fig. S3; Supplementary Table S3).

Expression of NOXA in MCL cells and its functional impact on MCL1-mediated venetoclax 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 (Fig. 2; Supplementary Table S4). 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 with primary MCL cells and PDXs might render MCL cell lines (that do not harbor deletions of \textit{BIM}) hypersensitive to venetoclax. Indeed, siRNA-mediated NOXA gene and protein knockdown decreased venetoclax-triggered apoptosis (Fig. 3C and F).

Mechanisms of resistance of MCL cells to venetoclax

Besides biallelic deletions of \textit{BIM} (which plausibly represent very rare events in MCL) upregulation of MCL1 was associated with venetoclax 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 venetoclax to BCL2 (16). Indeed, immunoprecipitation of REC-1 cells before and after venetoclax 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 venetoclax treatment (Fig. 4A–D). This molecular mechanism (i.e., buffering of BIM by MCL1) also provides a plausible explanation for the observed venetoclax resistance of REC-1 cells. Next, we established MAVER-1 and HBL2 venetoclax-resistant clones by long-term cultivation with gradually increasing venetoclax concentrations up to 1 \( \mu \text{mol/L} \). HBL2 venetoclax-resistant (VTX-R) cells had significantly upregulated MCL1 mRNA in VTX-R compared with 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 different between unexposed patients’ cells for PT1-PT9 was 40%, 24%, 37%, 26%, 50%, 15%, 40%, 36%, and 44%, respectively.


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Figure 1.

Sensitivity of MCL cell lines and primary cells to venetoclax and S63845. \textbf{A}, \textbf{B}, \textbf{D}, and \textbf{E}, Cytotoxic activity of venetoclax and S63845 toward established MCL cell lines (\textbf{A} and \textbf{B}) and primary MCL cells (\textbf{D} and \textbf{E}). Y-axes show numbers of Annexin-V+/PI+ cells 24 hours after exposure to 1 \( \mu \text{mol/L} \) venetoclax or S63845. Bars represent means \pm SDs of two independent experiments. \textbf{C} and \textbf{F}, Calculated \( IC_{50} \) for the cell lines (\textbf{C}) and primary lymphoma samples (\textbf{F}). Basal apoptosis of unexposed patients’ cells for PT1-PT9 was 40%, 24%, 37%, 26%, 50%, 15%, 40%, 36%, and 44%, PT, patient.

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

Cotargeting 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 venetoclax-induced apoptosis with MCL1 overexpression leading to venetoclax resistance (Fig. 4). Concurrent blockage of BCL2 with venetoclax 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. MAVER-1 MCL1-UP clone displayed upregulation of MCL1 accompanied by decreased levels of NOXA protein, which was associated with significant inhibition of both venetoclax and S63845-triggered apoptosis (Fig. 4E and F). In contrast, HBL2 MCL1-UP clone displayed upregulation of NOXA and its sensitivity to venetoclax and S63845 compared with empty vector transfected HBL2 cells was only mildly attenuated (Fig. 4E and F).

Discussion

In the current study, we evaluated molecular mechanisms of sensitivity of MCL cells to BCL2-targeting agent venetoclax. We demonstrated critical role of the functional status of anti-apoptotic MCL1 protein in conferring both inherent and
acquired resistance to venetoclax. As MCL1 and its interactions with NOXA appear as principal molecules that attenuates venetoclax-induced apoptosis, we proposed a treatment strategy aimed at concurrent inhibition of BCL2 by venetoclax 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 article by Tagawa and colleagues, 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 and colleagues 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 of 22 (33%) patient samples (14). Katz and colleagues 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 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; Supplementary Table S3; Supplementary Fig. S3). Similarly to the genomic analysis, IHC analysis of 37 FFPE tissue samples.

**Figure 3.** BIM and NOXA regulate sensitivity to venetoclax. **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 knockdown in HBL2 and MAVER-1 cell lines. "Empty" stands for empty vector–transfected cells. **C,** siRNA-mediated NOXA gene knockdown in HBL2 and MAVER-1 cell lines. "siCTRL" stands for cells transfected with noncoding siRNA. **D,** Cytotoxic activity of venetoclax (1 μmol/L, 24 hours) in JEKO-1 and Z138 cell clones with transgenic overexpression of BIM (JEKO-1:BIM, Z138:BIM). DOX, doxycycline. **E,** Cytotoxic activity of venetoclax (25 nmol/L, 24 hours) in HBL2 and MAVER-1 cell clones with shRNA-mediated knockdown of BIM expression. **F,** Cytotoxic activity of venetoclax (25 nmol/L, 24 hours) in HBL2 and MAVER-1 cell clones with siRNA-mediated knockdown of NOXA expression. Y-axes show numbers of Annexin-V+/PI+ cells. Bars represent means ± SDs of two independent experiments.
obtained from MCL patients did not identify a single case with undetectable BIM protein (Supplementary Table S4). On the basis of 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 with 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 with MCL cell lines, as analyzed by both approaches, IHC and Western blotting (Fig. 2; Supplementary Table S3).

In summary, the two critical proapoptotic proteins, BIM and NOXA, are differentially expressed between MCL primary samples and MCL cell lines.
and established cell lines (Fig. 2). Using clones with transgenic (over)expression or shRNA/siRNA-mediated knockdown of BIM and NOXA, we confirmed that they are indeed key mediators of venetoclax proapoptotic activity (Fig. 3). As a consequence, MCL cell lines unfortunately represent unreliable models for assessing proapoptotic activity of BH3 mimetics including venetoclax. Loss of BIM makes some cell lines (JEKO-1, Z138, MINO) "falsely" venetoclax resistant, and conversely, overexpression of NOXA makes other cell lines (HBL2, MAVER1, GRANTA-519) "falsely" hypersensitive to venetoclax. 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 venetoclax, 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 venetoclax 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-R. First, MCL1 upregulation was observed in both MCL clones (HBL2-VTX-R, and MAVER1-VTX-R) derived from two most venetoclax-sensitive cell lines HBL2 and MAVER1 (Fig. 4G and H). Second, we confirmed that MCL1 can act as a buffer for BIM (and potentially other BH3-only proteins) released from BCL2 by venetoclax (Fig. 4A–C) (16). And third, stable overexpression of MCL1 in HBL2 and MAVER1 cell lines was associated with significantly decreased sensitivity to venetoclax (Fig. 4E and F). The data thus provided sound mechanistic explanation for potential synthetic lethality between venetoclax and S63845 in MCL. We have confirmed that both venetoclax- and S63845-induced cytotoxicity is a caspase-dependent apoptotic process (Supplementary Fig. S4A and S4B). Interestingly, venetoclax-resistant HBL2 clones were "cross"–resistant not only to S63845, but also to two different BCL-XL inhibitors WEHI-539 and A-1155463 (Fig. 4H; Supplementary Fig. S4E and S4F). 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 antilymphoma efficacy of S63845 monotherapy (Fig. 5; Supplementary Table S4). Concurrent inhibition of MCL1 and BCL2 with S63845 and venetoclax, respectively, was associated with significantly increased antilymphoma efficacy compared with the single-agent approaches. The combination of venetoclax 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 venetoclax for targeted eradication of chemotherapy-resistant MCL cells including patients with adverse cytogenetics in the clinical grounds.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: D. Prukova, O. Havranek, P. Klen.
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References
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