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Blockage of BCL-XL overcomes venetoclax resistance across BCL2-positive lymphoid malignancies irrespective of BIM status

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Abstract:
Venetoclax, a BCL2 inhibitor, has a promising single-agent activity in mantle cell lymphoma (MCL), acute lymphoblastic leukemia (ALL), and large B-cell lymphomas (LBCL), but remissions were generally short, which calls for rational drug combinations. Using a panel of 21 lymphoma and leukemia cell lines and 28 primary samples we demonstrated strong synergy between venetoclax and A1155463, a BCL-XL inhibitor. Immunoprecipitation experiments, and studies on clones with knockout of expression, or transgenic expression of BCL-XL confirmed its key role in mediating inherent and acquired venetoclax resistance. Of note, the venetoclax and A1155463 combination was synthetically lethal even in the cell lines with lack of expression of the pro-apoptotic BCL2L11/BIM, and in the derived clones with genetic knockout of BCL2L11/BIM. This is clinically important because BCL2L11/BIM deletion, downregulation, or sequestration results in venetoclax resistance. Immunoprecipitation experiments further suggested that the pro-apoptotic effector BAX belongs to principal mediators of the venetoclax and A1155463 mode of action in the BIM-deficient cells. Lastly, the efficacy of the new pro-apoptotic combination was confirmed in vivo on a panel of 9 PDX models including MCL (n = 3), B-ALL (n = 2), T-ALL (n = 1), and DLBCL (n = 3). Because continuous inhibition of BCL-XL causes thrombocytopenia, we proposed and tested an interrupted 4 days ON / 3 days OFF treatment regimen, which retained the desired anti-tumor synergy with manageable platelet toxicity. The proposed VEN and A1155463 combination represents an innovative chemotherapy-free regimen with significant preclinical activity across diverse BCL2-positive hematologic malignancies irrespective of the BCL1L11/BIM status.

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Title: *Blockage of BCL-XL overcomes venetoclax resistance across BCL2-positive lymphoid malignancies irrespective of BIM status*

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39

40 **KEY POINTS**

41 • BCL-XL belongs to key factors responsible for inherent and acquired resistance to venetoclax
42 across diverse BCL2-positive lymphoid neoplasms.
43 • Interrupted targeting of BCL-XL with A1155463 induces synergy with venetoclax even in BIM-
44 deficient cells, and diminishes platelet toxicity.

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45 **ABSTRACT**

46

47 Venetoclax, a BCL2 inhibitor, has a promising single-agent activity in mantle cell lymphoma (MCL),
48 acute lymphoblastic leukemia (ALL), and large B-cell lymphomas (LBCL), but remissions were generally
49 short, which calls for rational drug combinations. Using a panel of 21 lymphoma and leukemia cell lines
50 and 28 primary samples we demonstrated strong synergy between venetoclax and A1155463, a BCL-
51 XL inhibitor. Immunoprecipitation experiments, and studies on clones with knockout of expression, or
52 transgenic expression of BCL-XL confirmed its key role in mediating inherent and acquired venetoclax
53 resistance. Of note, the venetoclax and A1155463 combination was synthetically lethal even in the cell
54 lines with lack of expression of the pro-apoptotic BCL2L11/BIM, and in the derived clones with genetic
55 knockout of BCL2L11/BIM. This is clinically important because BCL2L11/BIM deletion, downregulation,
56 or sequestration results in venetoclax resistance. Immunoprecipitation experiments further suggested
57 that the pro-apoptotic effector BAX belongs to principal mediators of the venetoclax and A1155463
58 mode of action in the BIM-deficient cells. Lastly, the efficacy of the new pro-apoptotic combination
59 was confirmed in vivo on a panel of 9 PDX models including MCL (n = 3), B-ALL (n = 2), T-ALL (n = 1),
60 and DLBCL (n = 3). Because continuous inhibition of BCL-XL causes thrombocytopenia, we proposed
61 and tested an interrupted 4 days ON / 3 days OFF treatment regimen, which retained the desired anti-
62 tumor synergy with manageable platelet toxicity. The proposed VEN and A1155463 combination
63 represents an innovative chemotherapy-free regimen with significant preclinical activity across diverse
64 BCL2-positive hematologic malignancies irrespective of the BCL1L11/BIM status.

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65 **INTRODUCTION**

66

67 B-cell lymphoma 2 (BCL2) inhibitor venetoclax (VEN) revolutionized the therapy of chronic lymphocytic
68 leukemia (CLL) and acute myeloid leukemias (AML).[6-8]. In contrast, the efficacy of single-agent VEN in
69 aggressive lymphoid tumors, e.g., mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL),
70 and acute lymphoblastic leukemia (ALL) was rather disappointing[9-13]. Despite promising overall
71 response rates, remissions induced with single-agent VEN were generally short, underlining the need
72 for rational drug combinations[14, 15].

73 Several cell-intrinsic and cell-extrinsic mechanisms of VEN resistance have been reported in the
74 literature, including overexpression of anti-apoptotic BCL2 proteins MCL1, and BCL-XL, deletion of pro-
75 apoptotic *BIM*, mutations of *BCL2*, *BAX*, *TP53*, *SMARCA4*, and other genes, deregulated oxidative
76 phosphorylation, as well as multiple microenvironmental factors[16-22]. We and others have reported a
77 strong synergy between VEN, and diverse MCL1 inhibitors[2, 23, 24]. Development of clinical-grade MCL1
78 inhibitors, however, has been hampered by adverse side effects, including cardiotoxicity, and
79 hepatotoxicity[25-29]. Another pivotal anti-apoptotic protein of the BCL2 family, BCL-XL, has been
80 repeatedly associated with VEN resistance in several cancer types[30, 31]. A new generation of dual
81 BCL2/BCL-XL inhibitors has demonstrated promising pre-clinical and clinical activity in hematologic and
82 solid cancers[32-34].

83 In this study, we bring conclusive evidence that concurrent blockage of BCL-XL and BCL2 with specific
84 nanomolar inhibitors A1155463 and venetoclax, respectively, induces synthetic lethality across a wide
85 range of BCL2-positive aggressive lymphoproliferative neoplasms including MCL, DLBCL and ALL.

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86 **METHODS**

87

88 **Cell lines, primary lymphoma samples and patient-derived xenograft models**

89 UPF1H, UPF29TO, UPF24F, UPF25R, UPF26K, UPF27L, UPF31A, UPF34N, and UPF35S cell lines were
90 derived in our laboratory from patients with treatment refractory MCL (UPF1H), double-hit DLBCL
91 (UPF29TO), B-ALL (UPF24F, UPF25R, UPF26K, UPF27L, UPF31A, UPF35S) and T-ALL (UPF34N). These
92 patient-derived lymphoma cell lines (PDCL) were cultured in IMDM supplemented with 20% human
93 plasma (Macopharma, France), 1% penicillin/streptomycin and 0.4% heparin. Commercially available
94 cell lines were obtained from DSMZ or ATCC cell banks and authenticated by Multiplexion. Patient-
95 derived lymphoma xenografts (PDXs) were established in our laboratory from patients with treatment-
96 refractory MCL, DLBCL or B-T-/ALL as described previously[1-3].
97 HS5 CD40L cell line was provided by M. Mraz (MU), and generated by transduction of HS5 cells (DSMZ)
98 by CDS for human CD40L and blasticidin resistance cloned into the pEZ-Lv197 lentiviral vector (plasmid
99 EX-G0117-Lv197, Genecopoeia).

100

101 **Establishment of lymphoma cell clones with knockout (*K/O*) of expression and transgenic**
102 **(re)expression of selected BCL2 genes**

103 Lymphoma cell clones with knockout of *BCL2L1* gene (coding for anti-apoptotic BCL-XL) were
104 generated using CRISPR/Cas12a system. A synthetic oligonucleotide containing Cas12a direct repeats
105 and crRNA-encoding DNA sequences targeting exon 2 and surrounding introns
106 (GATGCCCGGGAGGTGATCCCCAT – exon 2, TACCCCCGTCTTCTCCGAAATGC – intron 1-2,
107 GCCTCTGGTCAGAGATCCCCAAC – intron 2-3) was cloned into Cas12a-expressing plasmid pX AsCpf1-
108 Venus-NLS and electroporated into B-cell lymphoma cell lines. 24 h post-transfection, the cells were
109 single-cell sorted, and fluorescent Venus-expressing cells were cultivated further. Elimination
110 of BCL-XL in the clonal cultures was assessed by Western blotting using anti-BCL-XL antibody (#2764,
111 Cell Signaling Technology, MA, USA). Inducible (re)expression of BCL-XL in the derived clones with K/O

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112 of *BCL-XL* gene (designated BCL-XL K/O R clones) was achieved using the Sleeping Beauty transposon
113 system[4]. *BCL2L1* gene coding for BCL-XL was PCR-amplified from pCDH-puro-BCL-XL plasmid (#46972,
114 Addgene, MA, USA) using primers with SfiI overhangs
115 (F:TAGCGGCCTCTGAGGCCACCATGTCTCAGAGCAACCGGGAGC,R:ATGCGGCCTGACAGGCCTCATTTCCGA
116 CTGAAGAGTGAGCC) and cloned into pSBtet-Pur plasmid (#60507, Addgene, MA, USA). Resulting
117 pSBtet-Pur-BCL-XL plasmid was co-electroporated together with transposase-carrying plasmid
118 pCMV(CAT)T7-SB100 (#34879, Addgene, MA, USA) and pmaxGFP (2 μg, 3 μg and 1 μg, respectively)
119 into 1.5 million cells per sample. After 96 h the cells were FACS-sorted for GFP expression and selected
120 under 2 μg/ml puromycin (Serva, Germany). The transduced cells were incubated with 0.01 – 0.2 μg/ml
121 doxycycline (Duchefa Biochemie, Netherlands), and BCL-XL induction was confirmed by Western
122 blotting.
123 Lymphoma cell clones with a knockout of *BCL2L11* gene (coding for pro-apoptotic inducer BIM) and/or
124 knockout of *BAK1* gene (coding for pro-apoptotic effector BAK) were generated using CRISPR/Cas9
125 according to methodology described by Cong *et al*.[5]. Specific sgRNA were cloned into a pX330
126 recombinant plasmid (#42230, Addgene, MA, USA) carrying Cas9 nuclease. We targeted exon 4 of
127 *BCL2L11* gene with two guide sequences (GTTCTGATGCAGCTTCCATGAGG and
128 TCCTTGCATAGTAAGCGTTAGGG) and exon 3 of *BAK1* gene (CGTTTTTTACCGCCATCAGCAGG and
129 GCAGGTGAGCTACAACCGCTGGG). 6 μg of each pX330 plasmid was electroporated into the cells using
130 Neon transfection system together with 5 ug of pcDNA-EmGFP reporter plasmid. GFP-positive cells
131 were sorted using BD FACS Aria (NJ, USA) and seeded into 96-well plate (1 cell per well). After
132 establishing clonal cultures gDNA and protein lysates were isolated to confirm the deletion by both
133 PCR and Western blotting.
134

135 **Apoptosis measurement**

136 Venetoclax and A1155463 were purchased from MedChemExpress (NJ, USA), dissolved in
137 dimethylsulfoxid (DMSO, Carl Roth, Germany) at 10 mM concentration and stored at - 20°C. A

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138 percentage of apoptotic and necrotic cells was determined after 24 h incubation with the indicated
139 treatment by flow cytometry (BD FACS Canto II, NJ, USA) using Annexin V FITC (EXBIO, Czech Republic)
140 and propidium iodide (Sigma-Aldrich, MO, USA). Percentage was calculated using the following
141 formula: (measured apoptosis − basal apoptosis)/(100 − basal apoptosis) * 100 (%). Drug
142 concentrations that induced apoptosis in 50% of cells after 24 h (LD_{50}) were determined by nonlinear
143 regression algorithms using Graph Pad Prism software. CompuSyn version 1.0 software (ComboSyn)
144 was used to assess drug synergism between venetoclax and A1155463. The combination index (CI) for
145 two-drug combination was calculated for different concentrations of drugs considering various cell
146 sensitivity.
147

148 **Western blotting**

149 Cells were lysed in RIPA lysis buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 50 mM
150 Tris-HCl pH 7.4) supplemented with 1% protease (Protease Inhibitor Cocktail, Sigma-Aldrich, MO, USA)
151 and 10% phosphatase inhibitors (PhosSTOP, Roche, Switzerland). Protein concentration in the
152 collected supernatants was determined by the BCA protein assay (Pierce™ BCA Protein Assay, Thermo
153 Scientific™, MA, USA) according to the manufacturer's protocol. Lysate samples (25 µg) were combined
154 with SDS loading buffer containing 2-mercaptoethanol and boiled for 5 min. Samples were separated
155 on 10-15% SDS-PAGE minigels in Tris-glycine buffer (Bio-Rad, CA, USA). Proteins were transferred onto
156 PVDF membranes (Bio-Rad, CA, USA). Membranes were washed in 1x phosphate-buffered saline buffer
157 (PBS, VWR®, PA, USA) containing 0.1% Tween-20 and incubated in 10% non-fat dried milk (Carl Roth,
158 Germany) for 30 min. β-Actin was used as the loading control (1:10 000, Abcam, UK). Primary
159 antibodies were purchased from Cell Signaling Technology (anti-BAK, anti-BCL-XL, anti-BIM, anti-MCL1)
160 or BD Biosciences (anti-BCL2) and diluted 1:1000 or 1:2000, respectively (summarized in
161 Supplementary Table 1). After thorough washing in blocking buffer, a secondary horseradish
162 peroxidase-conjugated anti-mouse and/or anti-rabbit antibody (both from Jackson ImmunoResearch
163 Laboratories, Inc., PA, USA) was added (concentration 1:10 000). The signal was detected using

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164 Western blotting detection kit (WesternBright™ ECL, Advansta Inc., CA, USA) and membranes were
165 visualized by ChemiDoc Imaging System (Bio-Rad, CA, USA).

166

167 **Protein co-immunoprecipitation**

168 Cell pellets (10^7) were lysed at 4°C for 25 min in a non-denaturing lysis buffer [1% (w/v) Triton X-100,
169 50 mmol/L Tris-HCl (pH 7.4), 300 mmol/L NaCl, 5 mmol/L EDTA, 0.02% (w/v) sodium azide
170 supplemented with protease inhibitor] and centrifuged (16,000 × g, 4°C, 15 min). Protein
171 concentrations of cell extracts were measured as outlined above, using the BCA protein assay (Thermo
172 Scientific™, MA, USA). First, protein samples were precleared with Protein A/G Agarose bead slurry
173 (Pierce™ Protein A/G Agarose, Thermo Scientific™, MA, USA) which was incubated with anti-IgG
174 antibody (Normal Rabbit IgG, EMD Millipore Corp., MA, USA) for 30 min at 4°C, followed by
175 centrifugation (16,000 × g, 4°C, 2 s). Preclearing was repeated two more times. The cell lysates were
176 split and incubated with 10% BSA (Sigma-Aldrich, MO, USA) and Protein A/G Agarose beads with either
177 a specific antibody or a corresponding isotype control immunoglobulin bound to them. Incubation took
178 1 hour at 4°C. Immunocomplexes were then centrifuged (16,000 × g, 4°C, 2 s), washed three times in
179 ice-cold wash buffer [0.1% Triton X-100, 50 mmol/L Tris-HCl (pH 7.4), 300 mmol/L NaCl, 5 mmol/L
180 EDTA, 0.02% sodium azide)] and once more in ice-cold 1% PBS. Subsequently, the samples were
181 fractionated by 15% SDS-PAGE, followed by Western blotting detection, in which proteins in the gel
182 were transferred to PVDF membrane (Bio-Rad, CA, USA). To detect BIM interaction with BCL2 or BCL-
183 XL, anti-BIM (Cell Signaling Technology, MA, USA) antibody was used for the immunoprecipitation, and
184 the precipitates were subjected to immunoblot analysis using anti-BCL2 (BD Biosciences, NJ, USA) or
185 anti-BCL-XL (Abcam, UK) antibodies (summarized in Supplementary Table 1).

186

187 **Real-time PCR**

188 Total RNA was isolated from cell pellets (5 × 10^6) using RNeasy® Mini Kit (QIAGEN, Germany) by
189 following the manufacturer's instructions. Reverse transcription and PCR were performed in a single

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190 well provided by two-phase hot-start mechanism with the QuantiNova Probe RT-PCR Kit (QIAGEN,
191 Germany) in combination with TaqMan Gene Expression Assays (*BCL2L1*: Hs00236329_m1, *B2M*:
192 Hs00187842_m1) on QuantStudio 7 Pro Real-Time PCR System (Applied Biosystems, MA, USA). The
193 following qPCR conditions were used: initiation of cDNA synthesis - 10 min at 45°C, activation of DNA
194 Polymerase - 5 min at 95°C and amplification - 40 cycles of 5 s at 95°C and 35 s at 60°C. Quantification
195 cycle (Cq) values of *BCL2L1* gene were normalized to the reference gene *B2M*.
196

197 **Experimental therapy of lymphoma-bearing mice**

198 The experimental design was approved by the Institutional Animal Care and Use Committee (MSMT-
199 1712/2021-2). NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (referred to as NSG mice) were purchased from The
200 Jackson Laboratory (ME, USA). Adult female NSG mice were used for all experiments. NSG mice were
201 subcutaneously inoculated with $\sim 10 \times 10^6$ lymphoma or leukemia cells. Therapy was initiated when
202 all mice developed palpable tumors (designated as a day 1, D1). At D1 all mice were stratified so that
203 all cohorts contained mice with comparable calculated tumor volumes. Each cohort of mice contained
204 6 animals. Venetoclax (100 mg/kg, once daily by oral gavage), and A1155463 (10 mg/kg, once daily
205 intraperitoneally) were administered on days 1-4, and 8-11. Tumor volumes were calculated using the
206 following formula: $\pi/6 \times$ length $\times$ width $\times$ height. Experimental mice were euthanized when size of
207 subcutaneous tumors exceeded 2 cm in the largest diameter. The data were analyzed using the Graph
208 Pad Prism software.
209

210 **Evaluation of mouse platelet counting with an automated hematology**

211 The common site for blood collection in mice was retro-orbital sinus, and blood was collected into
212 EDTA capillary tubes (Vitrex, Denmark). Blood cell counts in mice on A1155463 therapy were analyzed
213 using the BC-5300 Auto Hematology Analyzer (Mindray, China).
214

215 **Statistical analyses**

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216 To assess the “*practical significance”* of treatment effectiveness, the charts with growth curves were
217 plotted indicating group mean tumor volumes accompanied by expert opinion on the differences
218 observed. For the purpose of attaching the label of “*statistical significance*” on treatment
219 effectiveness, we made an assumption that the calculated differences (between mean tumor volumes
220 in the compared groups) were generated by a process which includes a deterministic linear trend in
221 the form of: $Y_t = \beta_0 + \beta_1 t + \varepsilon_t$, where Y_t denotes random variables in the data-generating stochastic
222 process of the analyzed differences, t = 1, 2, …T is a time variable, T signifies the length of the
223 experiment (not the same for all experiments) in days and ε_t is the Gaussian IID white noise. For the
224 daily time series of these differences, statistical hypothesis tests of linear trend slopes equality to zero
225 were carried out. The Bonferroni correction was used to smooth the significance level for multiple
226 simultaneous statistical hypothesis tests.
227
228 **Patient consent and Ethics approval statement:** Primary lymphoma and leukemia cells were obtained
229 from 28 patients with MCL, DLBCL, CLL, or B-/T-ALL according to the Declaration of Helsinki. Informed
230 written consent was obtained from each subject. The experimental design was approved by the Ethics
231 Committee of the General University Hospital Prague under number 60/20.
232
233

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234 **RESULTS**

235

236 ***Co-targeting BCL-XL with A1155463 and BCL2 with VEN is synthetically lethal across BCL2-positive***
237 ***lymphoproliferative malignancies***

238

239 Several studies demonstrated a critical role of BCL-XL in mediating inherent and acquired resistance to
240 VEN[20, 31, 35-37]. Using a panel of 21 cell lines, MCL (n=7), DLBCL (n=7) and ALL (n=7), we demonstrated
241 strong *in vitro* synergy between VEN and BCL-XL inhibitor A1155463 (Table 1). The synergy was also
242 confirmed on *ex vivo* cultured primary lymphoma and leukemia cells obtained from patients not only
243 with newly diagnosed or treatment-refractory MCL (n=7), DLBCL (n=4), and ALL (n=2), but also other
244 BCL2-positive hematologic malignancies including CLL (n=7), follicular lymphoma (FL, n=3), AML (n=2),
245 and chronic myeloid leukemia (CML, n=3) (Supplementary Table 4). In all the tested primary cell
246 samples, the combination index between VEN and A1155463 was below 1, indicating a synergistic
247 effect (Supplementary Tables 2 and 3).

248

249 ***BAX is a key apoptotic trigger in BIM-deficient lymphoma and leukemia cells exposed to the VEN and***
250 ***A1155463 combination***

251

252 The pro-apoptotic inducer BCL2L11/BIM (referred to as BIM) has been repeatedly reported as the key
253 mediator of VEN anti-tumor activity[38, 39]. In line with this, BIM deletion, sequestration, or loss of
254 expression have been associated with VEN resistance[35, 40, 41]. In this study, the cytotoxic synergy of the
255 VEN and A1155463 combination was observed even in BIM-deficient lymphoma and leukemia cell
256 lines, which are inherently resistant or less sensitive to VEN (JEKO-1, Z-138, MINO, UPF26K) (Figure 1A,
257 Table 1). Similarly, lymphoma clones with knockout of *BIM* (BIM K/O clones) became VEN resistant (or
258 significantly less sensitive) but retained sensitivity to the tested pro-apoptotic VEN and A1155463
259 combination (Figure 1B, C). The data clearly suggested that other pro-apoptotic BCL2 molecule(s)

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260 contribute(s) to the synthetic lethality between VEN and A1155463. Immunoprecipitation experiments
261 in BIM-deficient Z-138 and MINO cells (characterized by strong synthetic lethality between VEN and
262 A1155463) detected pro-apoptotic effectors BAX, and BAK bound to BCL-XL. Both BAX and BAK were
263 released from BCL-XL upon exposure to A1155463. However, only BAX was enriched on BCL2 upon
264 exposure of the cells to A1155463, while BAK binding to BCL2 was not detected (Figure 1D). In addition,
265 VEN-induced apoptosis was more suppressed in HBL-2 *BAX* compared to *BAK* K/O cells. Last but not
266 least, *BAX* knockout, but not *BAK* knockout, partially block apoptosis triggered by the VEN and
267 A1155463 combination (Figure 1E, F).
268
269 ***BCL-XL is a critical modulator of VEN sensitivity***
270
271 To better understand the mechanistic role of BCL-XL in mediating susceptibility to VEN, we generated
272 clones with *BCL-XL* knockout (BCL-XL K/O) from two lymphoma cell lines (HBL-2 and MAVER-1) using
273 the CRISPR/Cas9 technology. Both clones became significantly more sensitive to VEN compared to the
274 parental cell lines (Figure 2A, B). In addition, transgenic (re)expression of BCL-XL in the HBL-2 BCL-XL
275 K/O cells decreased their sensitivity to VEN. The extent of doxycycline-induced transgenic
276 (re)expression of BCL-XL negatively correlated with the extent of sensitivity to VEN (Figure 2C, 2D).
277 Although *BCL-XL* knockout did not influence engraftment and growth of lymphoma cells *in vivo*, the
278 therapy of the mice bearing HBL-2 BCL-XL K/O tumors with single-agent VEN was significantly more
279 effective compared to the combinatorial therapy (i.e., VEN and A1155463) of the mice xenografted
280 with the wild-type HBL-2 parental lymphoma cells. Of note, 3 out of 6 mice remained without any signs
281 of lymphoma 6 months after initiation of therapy (Supplementary Figure 1).
282
283 ***Microenvironmental factors are critical triggers of BCL-XL expression***
284

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285 Multiple studies have shown that B-T cell interactions in the lymphoma/leukemia microenvironment
286 support the surivival of malignant B cells[42] including the CD40L-triggered NFkappaB activity leading to
287 overexpression of BCL-XL[36, 37]. Indeed, we confirmed that co-cultures of VEN-sensitive lymphoma cells
288 with CD40L-expressing stromal cells (HS5-CD40L) resulted in overexpression of BCL-XL mRNA and
289 protein leading to VEN resistance (Figure 3A, B, C). In contrast, the co-culture of BCL-XL K/O HBL-2 and
290 MAVER-1 clones with HS5-CD40L did not change their sensitivity to VEN. Co-treatment of the
291 unmanipulated (parental) lymphoma cell lines co-cultured with HS5-CD40L with the combination of
292 VEN and A1155463 overcame the observed microenvironment-mediated VEN resistance (Figure 3D).
293
294 ***In vivo acquired resistance to VEN is associated with upregulation of BCL-XL and increased binding***
295 ***of BIM to BCL-XL***
296
297 VEN-sensitive cell lines (HBL-2, MAVER-1, UPF1H) and PDX cells (VFN-M1, VFN-ALL6) were
298 subcutaneously xenografted into immunodeficient NSG mice and treated with VEN (100 mg/ kg/ daily)
299 until the development of VEN-resistant (VEN-R) tumors. The most frequently observed changes in the
300 expression of BCL2 family of proteins comprised upregulation of anti-apoptotic proteins BCL-XL, MCL1,
301 and BCL2 and downregulation of the pro-apoptotic protein BIM (Figure 4A-E). Real-time PCR analysis
302 showed upregulation of BCL-XL mRNA in all tested VEN-R samples (Supplementary Figure 2).
303 Immunoprecipitation experiments confirmed that BCL-XL serves as a buffer for the BIM protein
304 released from BCL2 after exposure of lymphoma cells to VEN (Figure 4F-H).
305
306 ***Combined inhibition of BCL2 and BCL-XL is strongly synergistic in vivo on a panel of 4 CDX and 9 PDX***
307 ***models of BCL2-positive aggressive lymphoid neoplasms***
308
309 Clinically, a blockage of BCL-XL with navitoclax, a dual BCL2/BCL-XL inhibitor, led to thrombocytopenia[6].
310 Similarly, A1155463, a specific BCL-XL inhibitor, induced thrombocytopenia in mice after 4 consecutive

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311 days of therapy with A1155463 (Table 2). Importantly, after 3 days of therapy cessation, the levels of
312 platelets rebounded to normal range. This led us to use an interrupted "4 days ON / 3 days OFF" dosing
313 schedule in all *in vivo* experiments implemented in this study.
314 *In vivo*, the combination of VEN and A1155463 more effectively inhibited tumor growth compared to
315 respective monotherapies, as demonstrated on a panel of 4 CDXs (HBL-2, MAVER-1, UPF1H and MINO),
316 and 9 PDX models obtained from patients with MCL (VFN-M1, M5R1, and M16), BCL2-positive DLBCL
317 (VFN-D1, D9, and D20), B-ALL (VFN-ALL1, VFN-ALL6), and T-ALL (VFN-ALL7). Of note, the combination
318 was effective even in the tumors inherently resistant to both VEN and A1155463 monotherapies (VFN-
319 M16, VFN-D1, VFN-D9), and in the tumors with acquired VEN-resistance (VEN-R, including HBL-2,
320 MAVER-1, UPF1H, VFN-M1, and VFN-ALL6) (Figure 5, Supplementary Figure 3).

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321 **DISCUSSION**

322

323 In recent years, a large body of evidence has suggested that BCL-XL belongs to the pivotal factors of
324 both inherent and acquired VEN resistance[20, 31, 35-37]. Microenvironmental factors, such as the CD40L-
325 induced NFkappaB signaling, and hypoxia-induced HIF1-alpha have been reported to induce BCL-XL
326 expression[37]. Although the precise mechanisms of BCL-XL upregulation during the establishment and
327 regrowth of VEN-R tumors upon or after VEN therapy remain largely elusive, we suppose that survival,
328 overgrowth and expansion of BCL-XL (over)expressing cells (VEN resistant) is a result of selective
329 pressure of VEN in a Darwinian way.
330 The tested combination of VEN and A1155463 might appear as a return to the old agent navitoclax, a
331 dual, BCL2 and BCL-XL inhibitor, which had failed to demonstrate better anti-tumor activity compared
332 to the BCL2-specific VEN[43]. However, navitoclax was a weak inhibitor of BCL2, and a strong inhibitor of
333 BCL-XL, thereby inducing deep thrombocytopenia while eliciting only moderate anti-leukemia effects.
334 In contrast, the combined strong inhibition of both BCL2 and BCL-XL leads to significant anti-tumor
335 synergy, which was confirmed in our study on a large panel of diverse PDX models derived from various
336 BCL2-positive hematologic malignancies. Curiously, the combination of VEN and low-dose navitoclax
337 had promising anti-tumor activity in patients with ALL / lymphoblastic lymphoma[44]. A new generation
338 of dual BCL2/BCL-XL inhibitors have also demonstrated pre-clinical and clinical activity[32-34]. In contrast
339 to the dual inhibitors, a combination of two separate highly specific nanomolar inhibitors would enable
340 a continued BCL2 blockage with an interrupted blockage of BCL-XL to mitigate thrombocytopenia, as
341 well as separate management of potential adverse side effects of either agent. The interrupted dosing
342 strategy proposed here was inspired by previous studies with navitoclax, in which not only a ramp-up
343 dosing of BCL-XL, but also an interrupted dosing schedule blunted the depth of thrombocytopenia[43].
344 Moreover, sequential administration of the two inhibitors (e.g., first single-agent VEN, followed by the
345 VEN+A1155463 combination) might substantially decrease the risk of tumor lysis syndrome, a known
346 life-threatening side effect of VEN.

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347 It was reported that the affinity of BIM follows the order BCL2 > BCL-XL > MCL1[20, 31]. This observation
348 underlined the importance of BCL-XL in mediating VEN resistance compared to MCL1. Mechanistically,
349 our data confirmed that BCL-XL indeed serves as a buffer for BIM released from the BCL2 protein upon
350 exposure to VEN. Knock-out of *BCL-XL* significantly increased sensitivity to VEN, while transgenic re-
351 expression of BCL-XL rendered lymphoma cells VEN resistant. This is in line with evidence provided by
352 previous studies[37, 45]. Furthermore, acquired resistance to VEN in PDX models caused by prolonged *in*
353 *vivo* therapy with single-agent VEN resulted in overexpression of BCL-XL and/or increased binding of
354 BIM to BCL-XL, which rendered VEN-R leukemia and lymphoma cells primed for the BCL-XL targeting
355 BH3-mimetic A1155463. Consequently, the VEN-R tumors could be effectively re-treated with the
356 combination of A1155463 and VEN, even though the combo was less effective compared to the original
357 (VEN sensitive) tumors. Of note, the therapy of mice xenografted with the HBL-2 BCL-XL K/O clone with
358 single-agent VEN was significantly more effective compared to the combinatorial therapy (i.e., VEN +
359 A1155463) of mice xenografted with the parental HBL-2 cells. Three out of six mice remained without
360 any signs of lymphoma 6 months after initiation of therapy. This not only underscores key importance
361 of BCL-XL in mediating susceptibility to VEN, but also suggests that next-generation, more specific BCL-
362 XL targeting agents might be associated with even better synthetic lethality.
363 BIM was repeatedly reported as the principal mediator of VEN mode-of-action[38]. BIM deletion, its
364 sequestration or decreased expression were all associated with VEN resistance[41, 46-49]. Indeed, our
365 lymphoma clones with *BIM* knockout became significantly less sensitive to VEN. Importantly, the
366 tested VEN and A1155463 combination was effective even in the BIM deficient cell lines, and the
367 derived clones with *BIM* knockout, which were all resistant to single-agent VEN. This observation
368 suggested that other pro-apoptotic BCL2 protein(s) (than BIM) can drive the pro-apoptotic activity of
369 the VEN and A1155463 combination. Our immunoprecipitation data demonstrated that both BAX and
370 BAK effectors are bound on BCL-XL in the BIM-deficient cell lines, but only BAX was detected enriched
371 on BCL2 upon exposure to A1155463. The data from the *BAX* and *BAK* knockout clones further
372 supported key roles of BAX, and to a lesser extent BAK as important mediators/effectors of VEN pro-

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373 apoptotic activity. In BIM-proficient cells, BIM-activated BAX/BAK pore formation can be initiated after
374 VEN-mediated release of these proteins from BCL2 only. In BIM-deficient cells, however, BAX and BAK
375 release from both BCL2 and BCL-XL is requisite for triggering the mitochondrial apoptosis.
376 Besides the upregulation of BCL-XL, other mechanisms might contribute to the acquired VEN
377 resistance. Although this was not the focus of this study, some of these mechanisms, in particular
378 overexpression of MCL1 might cause resistance even to the combination of VEN and A1155463.
379 Indeed, the combination of VEN and A1155463, despite proving more effective than the respective
380 monotherapies was not capable of long-term eradication of the engrafted PDX tumors. We can only
381 speculate that prolonged treatment schedules, continued exposure to VEN, or increased dosing of one
382 or both agents might be more effective compared to the tested "4 days ON- 3 days OFF" regimen. It
383 is, however, plausible that the observed molecular changes would eventually lead to drug resistance
384 to the combination of both BH-3 mimetics. There exist several potential three-drug combinations to
385 avoid the expected development of drug resistance mediated by MCL1 overexpression. Agents that
386 block cell cycle progression are known to downregulate MCL1 protein, including cyclin-dependent
387 kinase inhibitors (palbociclib), or antibody-drug conjugates (polatuzumab vedotin, inotuzumab
388 ozogamicin) with small molecule mitotic poisons attached to the antibody carriers as toxic payloads.
389 In addition to MCL1-downregulating agents, drugs with known synergy with VEN (e.g., azacytidine), or
390 agents with non-overlapping modes of action and non-overlapping adverse side effects including
391 therapeutical monoclonal antibodies (e.g., anti-CD20 rituximab or obinutuzumab), bispecifics (e.g.,
392 glofitamab, or epcoritamab) or targeted agents (e.g., Bruton tyrosin kinase inhibitors) might be
393 effective components of such experimental multi-agent chemo-free regimen.
394 In summary, co-targeting BCL-XL and BCL2 with A1155463 and VEN appears as a universal anti-
395 mitochondrial treatment strategy not only for MCL, ALL, and BCL2-positive DLBCL, but potentially for
396 most BCL2-positive hematologic malignancies (including CLL, FL, AML, and CML). Our data provide a
397 sound pre-clinical rationale for early clinical trials in patient subjects.
398

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413 analysis, E.H. and M.M. prepared the CD40L expressing fibroblasts, K.H. did statistical analyses, N.C.,
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416
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Sample	A1155463 (nM)	VEN (nM)	CI	Sample	A1155463 (nM)	VEN (nM)	CI	Sample	A1155463 (nM)	VEN (nM)	CI
MCL				DLBCL				ALL			
	1000	1	**0.150**		100	100	**0.214**		1000	100	**0.128**
HBL-2	1000	2.5	**0.284**	**UPF29TO**	1000	100	**0.151**	**UPF24F**	1000	500	**0.083**
	1000	5	**0.521**		1000	1000	**0.547**		1000	1000	**0.103**
	1000	5	**0.266**		1000	100	**0.211**		1000	50	**0.046**
MAVER-1	1000	10	**0.294**	**DB**	1000	500	**0.156**	**UPF25R**	1000	100	**0.031**
	1000	20	**0.347**		1000	1000	**0.099**		1000	500	**0.093**
	1000	50	**0.009**		1000	100	**0.113**		1000	10	**0.147**
UPF1H	1000	100	**0.005**	**OCI-LY19**	1000	500	**0.242**	**UPF26K**	1000	50	**0.071**
	1000	200	**0.003**		1000	1000	**0.177**		1000	100	**0.065**
	1000	10	**1.46E-04**		1000	100	**0.076**		1000	50	**0.568**
MINO	1000	50	**5.61E-04**	**SU-DHL4**	1000	500	**0.023**	**UPF27L**	1000	100	**0.429**
	1000	100	**8.62E-04**		1000	1000	**0.019**		1000	500	**0.649**
	100	100	**5.52E-04**		1000	100	**0.033**		1000	100	**0.142**
JEKO-1	1000	100	**3.56E-04**	**HBL-1**	1000	500	**0.125**	**UPF31A**	1000	500	**0.052**
	1000	1000	**1.15E-03**		1000	1000	**0.156**		1000	1000	**0.048**
	100	100	**0.264**		1000	100	**0.145**		1000	10	**0.350**
REC-1	1000	100	**0.559**	**OCI-LY3**	1000	500	**0.075**	**UPF34N**	1000	20	**0.229**
	1000	1000	**0.185**		1000	1000	**0.145**		1000	40	**0.252**
	100	100	**3.01E-09**		1000	100	**0.532**		1000	10	**0.068**
Z-138	1000	100	**9.90E-10**	**U2932**	1000	500	**0.401**	**UPF35S**	1000	50	**0.046**
	1000	1000	**4.96E-08**		1000	1000	**0.541**		1000	100	**0.066**

567

568 **Table 1. *In vitro* cytotoxic synergy induced by the combination of VEN and A1155463.**

569

570

571 **Table 2. Assessment of A1155463 platelet toxicity *in vivo* on a mouse model.**

572

A1155463 (10 mg/ kg)	**PLT** ref. value = 400 - 1600 (x 10^9/ l)							
Treatment regimen	**4 DAYS ON**				**3 DAYS OFF**			
	DAY1	**DAY2**	**DAY3**	**DAY4**	**DAY5**	**DAY6**	**DAY7**	**DAY8**
Mouse 1	784	145	371	74	21			1100
Mouse 2	641	257	317	78	12	-		1043
Mouse 3	644	164	330	180	353			1236

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576 **Figure Legends:**

577 **Figure 1. BAX belongs to key mediators of cytotoxicity triggered by the combination of VEN and**
578 **A1155463 in BIM-deficient lymphoma and leukemia cells.**

579 **A** Western blot analysis of selected BCL2 family proteins in the tested lymphoma and leukemia cell
580 lines; **B** Western blot confirmation of absence of BIM and/or BAK proteins in the HBL-2 and MAVER-1
581 lymphoma cell clones with knockout (K/O) of *BIM* and *BAK* genes; WT= wild-type (parental) lymphoma
582 cell lines; **C** Knockout of *BIM* in lymphoma cell lines (HBL-2 and MAVER-1 BIM K/O clones) resulted in
583 increased VEN resistant, while sensitivity to the combination was largely retained; sensitivity of WT
584 cells is shown for comparison; **D** Immunoprecipitations (IP) of two BIM-deficient cell lines (MINO and
585 Z-138) exposed for 3 hours to A1155463 (1000 nM) or medium only with anti-BCL-XL, and anti-BCL2
586 antibodies and subsequent western blot detection of BAX and BAK proteins bound on BCL-XL and BCL2;
587 **E** Western blot confirmation of absence of BAX protein expression in HBL-2 cell clone with knockout
588 (K/O) of *BAX* gene; **F** Apoptotis essays after exposure to the defined VEN and A1155463 concentrations
589 reveal that HBL-2 cells with *BAX* knockout are significantly more resistant to VEN and to the
590 VEN+A1155463 combination compared to HBL-2 cells with *BAK* knockout.

591

592 **Figure 2. BCL-XL belongs to key mediators of sensitivity / resistance to VEN-induced apoptosis.**

593 **A** Representative Western blot analysis confirms absence of BCL-XL protein expression in the HBL-2
594 and MAVER-1 lymphoma clones with knockout of BCL-XL gene; WT= wild-type (parental) lymphoma
595 cell lines; **B** Calculated lethal dose 50 (LD_{50}) for A1155463 and VEN in the HBL-2 and MAVER-1 cell
596 clones with BCL-XL knockout compared to the WT lymphoma cell lines; **C** Western blot analysis
597 confirms doxycycline-induced dose-dependent re-expression of BCL-XL protein in HBL-2 BCL-XL K/O
598 clone with transgenic (re)expression of BCL-XL (designated BCL-XL K/O R); expression of BCL-XL in the
599 WT HBL-2 cell line is shown for comparison; **D** Extent of the doxycycline-induced transgenic
600 (re)expression of BCL-XL in the HBL-2 BCL-XL K/O R clone under doxycycline promotor negatively

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601 correlates with sensitivity to VEN; sensitivity to HBL-2 WT cell line and HBL-2 BCL-XL K/O clone is shown
602 for comparison.
603
604 **Figure 3. Upregulation of BCL-XL induced by microenvironmental factors belongs to key mediators**
605 **of VEN resistance *in vitro***. **A** Western blot analysis of BCL-XL protein after 24-hour co-culture of the
606 tested lymphoma cell lines (HBL-2, MAVER-1, UPF1H and MINO) with HS5 CD40L feeder cells with
607 stable transgenic expression of human CD40 ligand (HS5 CD40L); **B** Bar charts showing the differences
608 of normalized expression levels of *BCL2L1/BCL-XL* in the tested WT lymphoma cell lines (white bars)
609 and after 24-hour co-culture with HS5 CD40L feeder cells (dark bars); **C** Co-culture of HBL-2 and
610 MAVER-1 WT lymphoma cell lines with HS5 CD40L feeder cells for 24 hours resulted in VEN resistance
611 as measured by numbers of apoptotic cells after exposure to VEN (10 and 100 ng/mL); in contrast, co-
612 culture of HBL-2 and MAVER-1 BCL-XL K/O clones with HS5 CD40L did not change their sensitivity to
613 VEN; **D** Combined treatment of the WT lymphoma cell lines co-cultured for 24 hours on HS5 CD40L
614 feeder cells with the combination of VEN (10, 100 nM), and A1155463 (1000 nM) overcame the
615 microenvironment-induced VEN resistance.
616
617 **Figure 4. Sequestration of BIM to upregulated BCL-XL belongs to hallmarks of acquired VEN**
618 **resistance.**
619 **A-E** Western blot analysis of selected BCL2 family proteins (MCL1, BCL-XL, BCL2, BAK, and BIM) in cell
620 lysates isolated from the subcutaneous cell line-based xenograft (CDX) tumors (HBL-2, MAVER-1,
621 UPF1H), and subcutaneous patient-derived xenograft (PDX) tumors (VFN-M1, VFN-ALL6) obtained
622 from the untreated animals (CTRL) and from the animals with acquired resistance to VEN (VEN-R); **F-H**
623 Immunoprecipitation (IP) of the selected CDX or PDX tumors with anti-BIM antibody and subsequent
624 detection of BCL-XL and BCL2 bound on BIM; one CDX model (UPF1H) and two PDX models (VFN-M1,
625 and VFN-ALL6) were analyzed; CTRL= tumors obtained from untreated mice; VEN-R= mice with
626 acquired resistance to VEN were treated with VEN daily until the respective CDX or PDX tumors grew

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627 on the continued VEN therapy; the mice were euthanized and tumors analyzed 24 hours after the last
628 dose of VEN.
629
630 **Figure 5. Co-targeting of BCL2 and BCL-XL with VEN and A1155463 is synthetically lethal on a panel**
631 **of PDX models derived from patients with MCL, ALL, and DLBCL.**
632 **A** Co-targeting of BCL2 and BCL-XL with VEN and A1155463 was tested on a panel of nine PDX models
633 including MCL (VFN-M1, VFN-M5R1, VFN-M16), ALL (VFN-ALL1, VFN-ALL6, VFN-ALL7), and DLBCL (VFN-
634 D1, VFN-D9, VFN-D20); x axis shows days from initiation of therapy; y axis shows calculated tumor
635 volumes ± standard deviations of PDX tumors; therapy was administered on days 1-4 and 8-12 (for
636 details see Methods section of the manuscript); **B** Co-targeting of BCL2 and BCL-XL with VEN and
637 A1155463 was effective even in the PDX models with acquired VEN resistance (VFN-M1 VEN-R and
638 VFN-ALL6 VEN-R); VEN-R PDX tumors were derived from animals, in which originally VEN-sensitive PDX
639 tumors grew on continued VEN therapy- such VEN-R PDX tumors were re-engrafted into secondary
640 recipients and subjected to re-treatment; each cohort of mice comprised 6 animals.
641

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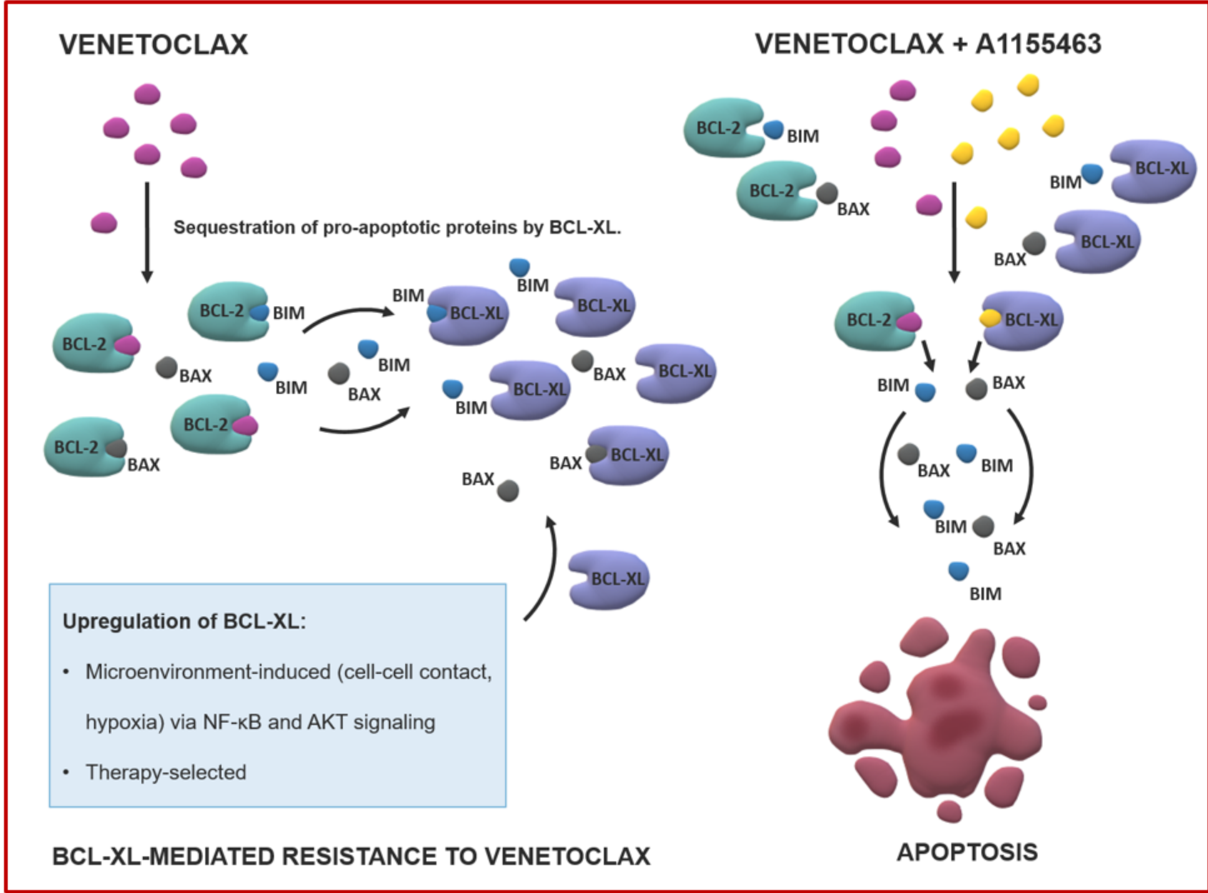
642 **Visual Abstract:**

Co-targeting BCL-XL and BCL2 is a universal anti-mitochondrial treatment strategy for most BCL2-positive hematologic malignancies



Conclusion: BCL-XL plays a pivotal role in venetoclax resistance across diverse BCL2-positive lymphoid tumors. Co-targeting BCL2 and BCL-XL is synergistic even in BIM-deficient cells.

Dolnikova et al.

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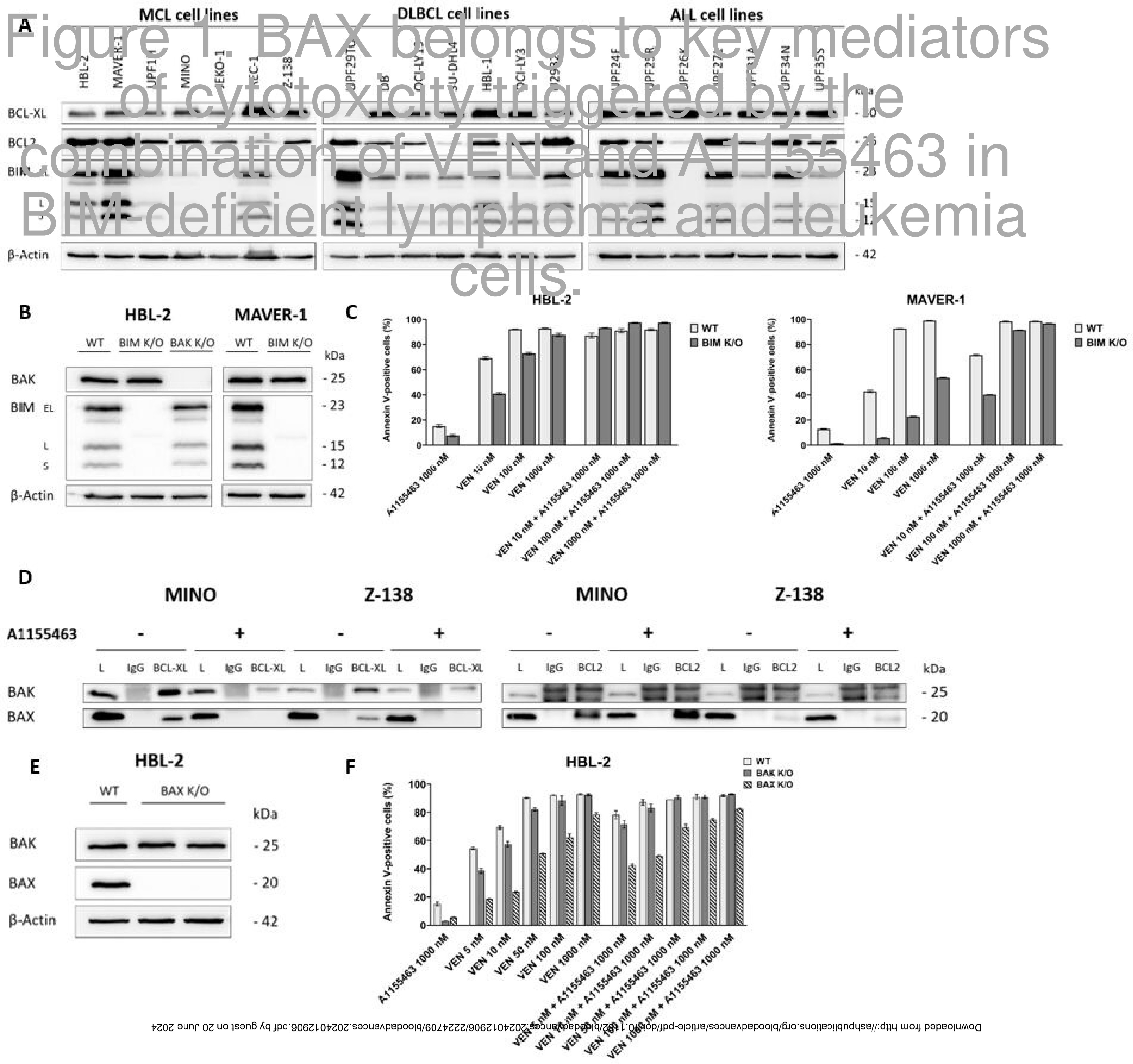

Figure 1. BAX belongs to key mediators of cytotoxicity triggered by the combination of VEN and A1155463 in BIM-deficient lymphoma and leukemia cells.

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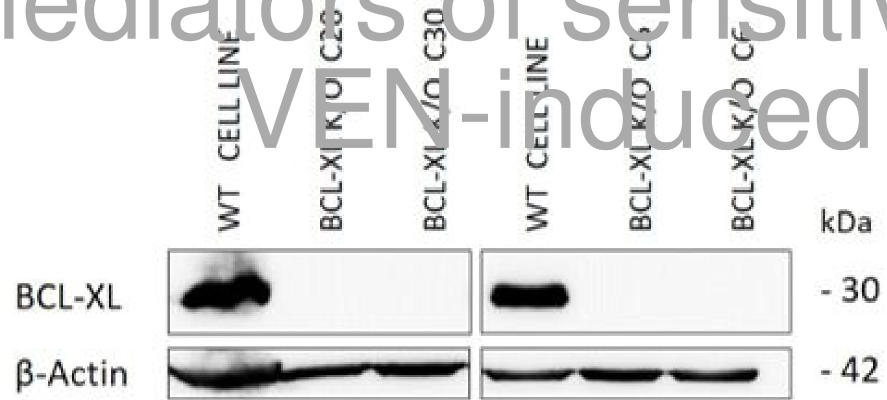
Figure 2. BCL-XL belongs to key mediators of sensitivity / resistance to VEN-induced apoptosis.

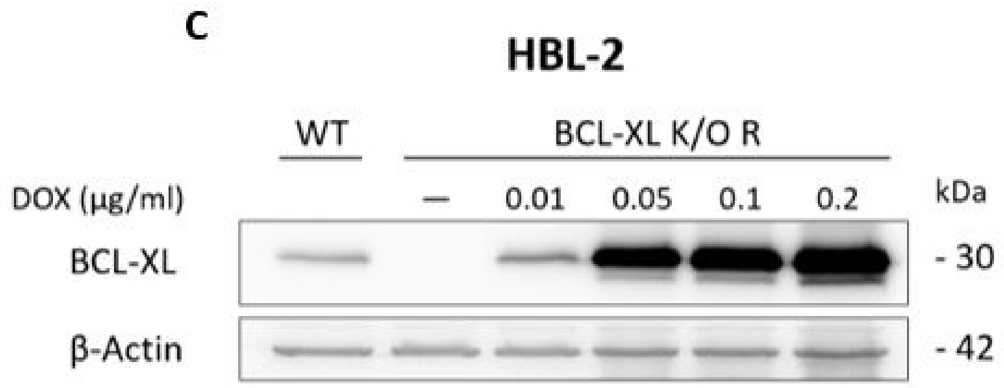
A



B

Cell line	A1155463 LD50 (μM)	VEN LD50 (nM)
HBL-2	7.6	5
HBL-2 BCL-XL K/O	> 30	< 2.5
MAVER-1	23.2	19
MAVER-1 BCL-XL K/O	> 30	5

C



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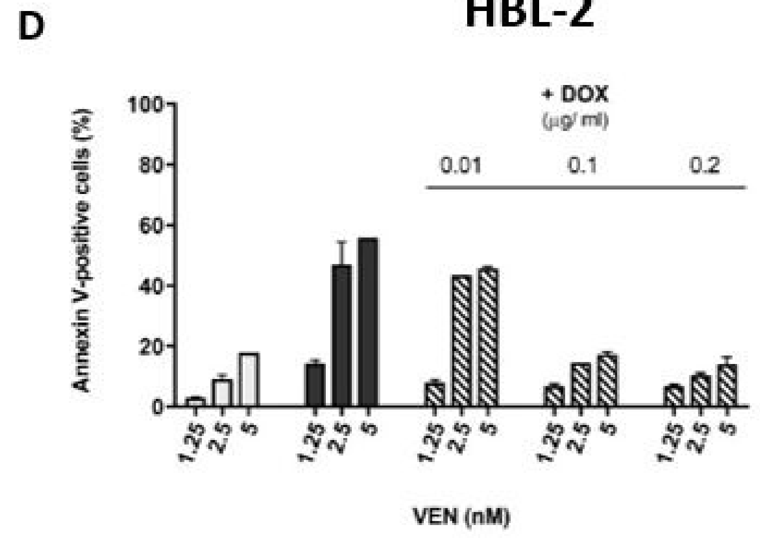

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Figure 3. Upregulation of BCL-XL induced by microenvironmental factors belongs to key mediators of VEN resistance in vitro.

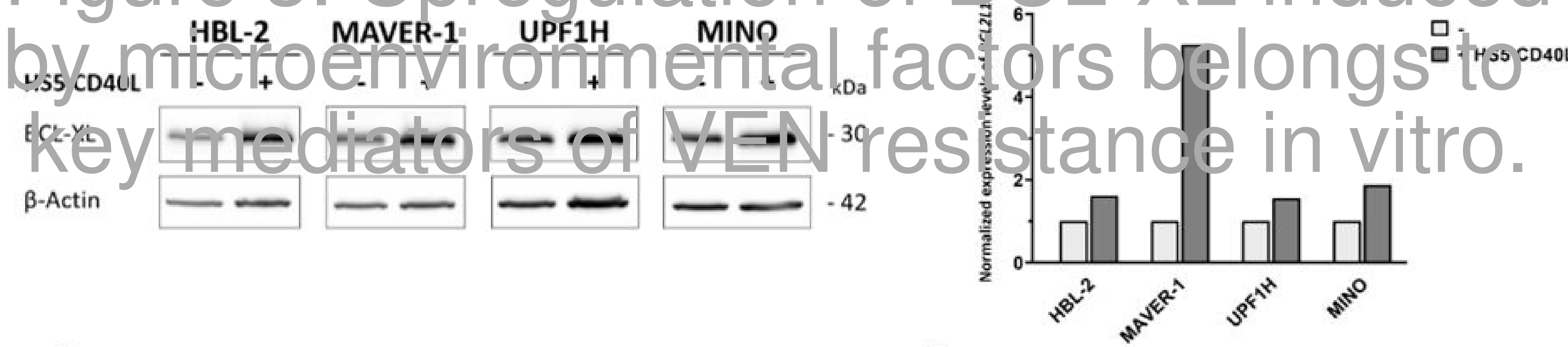

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Figure 4. Sequestration of BIM to upregulated BCL-XL belongs to hallmarks of acquired VEN resistance.

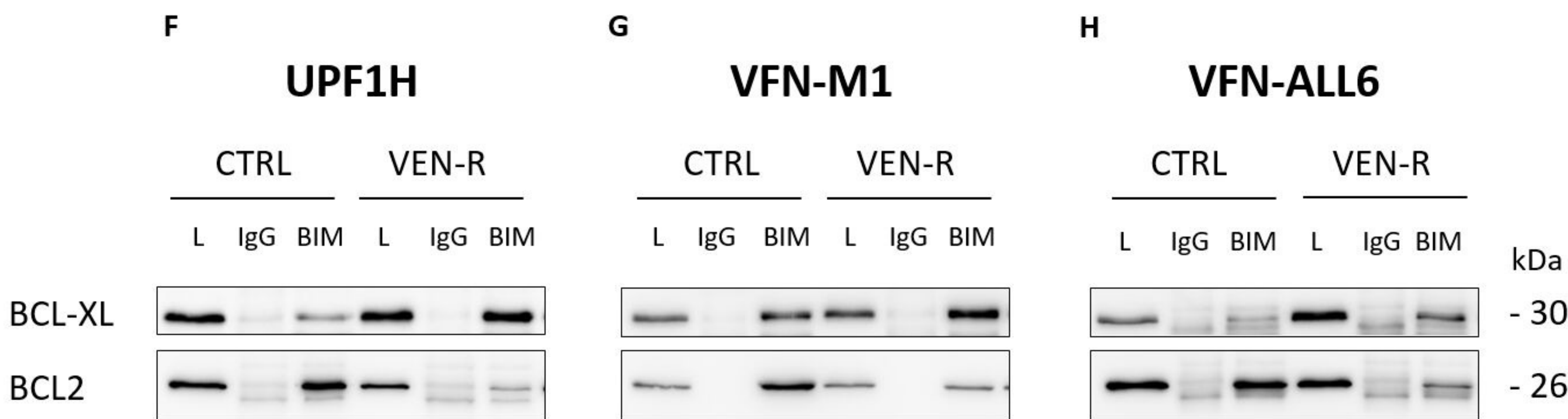

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Figure 5. Co-targeting of BCL2 and BCL-XL with VEN and A1155463 is synthetically lethal on a panel of PDX models derived from patients with MCL, ALL, and DLBCL.

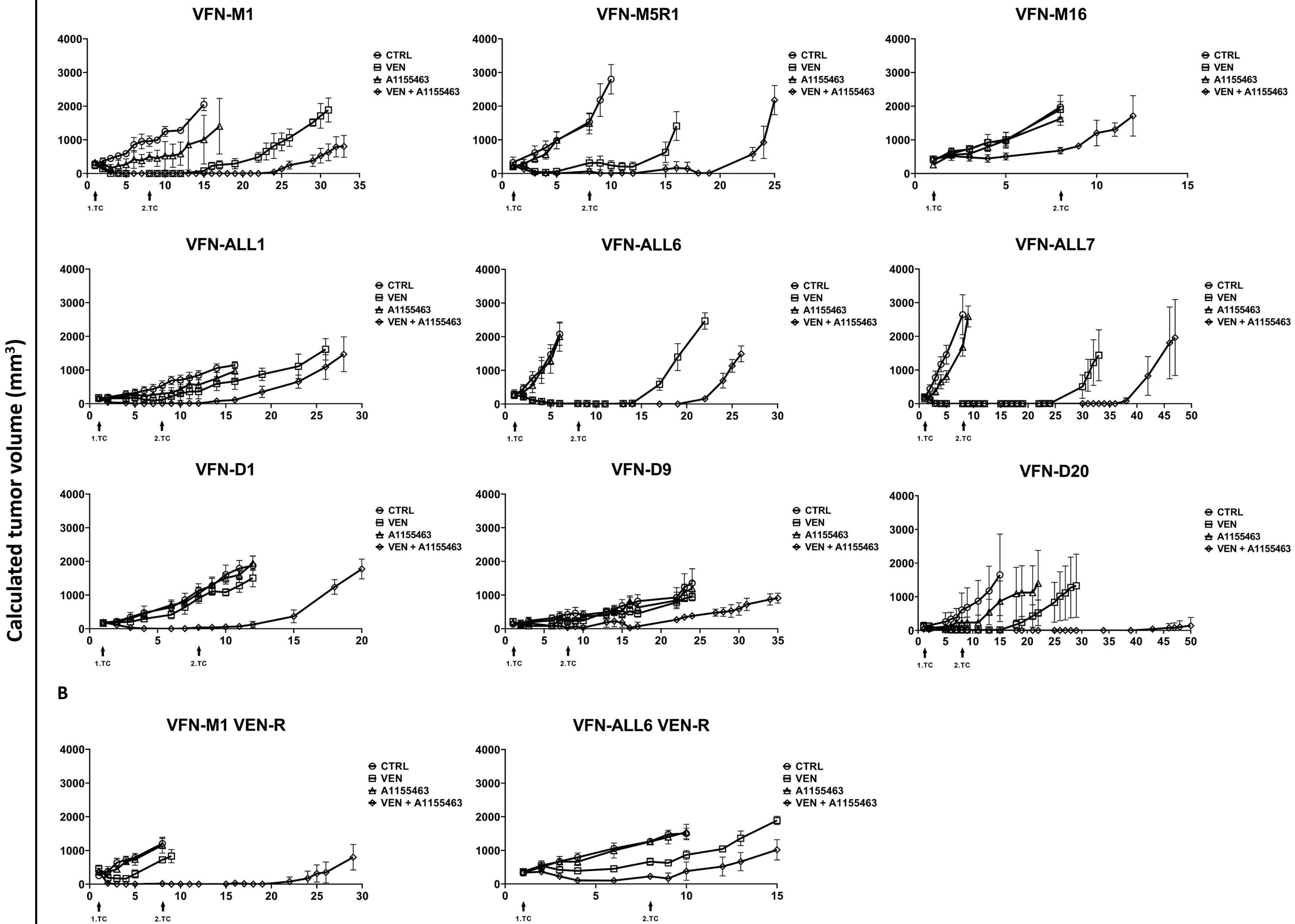

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Table 1. In vitro cytotoxic synergy induced by the combination of VEN and A1155463.

Sample	A1155463 (nM)	VEN (nM)	CI	Sample	A1155463 (nM)	VEN (nM)	CI	Sample	A1155463 (nM)	VEN (nM)	CI
MCL				DLBCL				ALL			
HBL-2	1000	1	0.150	UPF2[illegible]TO	100	100	0.214	UPF24F	1000	100	0.128
	1000	2.5	0.284		1000	100	0.151		1000	500	0.083
	1000	5	0.521		1000	1000	0.547		1000	1000	0.103
MAVER-1	1000	5	0.266	DB	1000	100	0.211	UPF25R	1000	50	0.046
	1000	10	0.294		1000	500	0.156		1000	100	0.031
	1000	20	0.347		1000	1000	0.099		1000	500	0.093
UPF1H	1000	50	0.009	OCI-LY19	1000	100	0.113	UPF26K	1000	10	0.147
	1000	100	0.005		1000	500	0.242		1000	50	0.071
	1000	200	0.003		1000	1000	0.177		1000	100	0.065
MINO	1000	10	1.46E-04	SU-DHL4	1000	100	0.076	UPF27L	1000	50	0.568
	1000	50	5.61E-04		1000	500	0.023		1000	100	0.429
	1000	100	8.62E-04		1000	1000	0.019		1000	500	0.649
JEKO-1	100	100	5.52E-04	HBL-1	1000	100	0.033	UPF31A	1000	100	0.142
	1000	100	3.56E-04		1000	500	0.125		1000	500	0.052
	1000	1000	1.15E-03		1000	1000	0.156		1000	1000	0.048
REC-1	100	100	0.264	OCI-LY3	1000	100	0.145	UPF34N	1000	10	0.350
	1000	100	0.559		1000	500	0.075		1000	20	0.229
	1000	1000	0.185		1000	1000	0.145		1000	40	0.252
Z-138	100	100	3.01E-09	U2932	1000	100	0.532	UPF35S	1000	10	0.068
	1000	100	9.90E-10		1000	500	0.401		1000	50	0.046
	1000	1000	4.96E-08		1000	1000	0.541		1000	100	0.066

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Table 2. Assessment of A1155463 platelet toxicity in vivo on a mouse model.

A1155463 (10 mg/ kg)	ref. value = 400 - 1600 ($x10^9$/ l)							
Treatment	4 DAYS ON				3 DAYS OFF			
regimen	DAY1	DAY2	DAY3	DAY4	DAY5	DAY6	DAY7	DAY8
Mouse 1	784	145	371	74	21			1100
Mouse 2	641	257	317	78	12	-		1043
Mouse 3	644	164	330	180	353			1236

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