

Expression Profile of BCL-2, BCL-X_L, and MCL-1 Predicts Pharmacological Response to the BCL-2 Selective Antagonist Venetoclax in Multiple Myeloma Models

Elizabeth A. Punnoose¹, Joel D. Levenson², Franklin Peale³, Erwin R. Boghaert⁴, Lisa D. Belmont⁵, Nguyen Tan⁵, Amy Young⁶, Michael Mitten⁴, Ellen Ingalla⁶, Walter C. Darbonne¹, Anatol Oleksijew⁴, Paul Tapang⁴, Peng Yue⁵, Jason Oeh⁶, Leslie Lee⁶, Sophie Maiga⁷, Wayne J. Fairbrother⁸, Martine Amiot⁷, Andrew J. Souers⁴, and Deepak Sampath⁶

Abstract

BCL-2 family proteins dictate survival of human multiple myeloma cells, making them attractive drug targets. Indeed, multiple myeloma cells are sensitive to antagonists that selectively target prosurvival proteins such as BCL-2/BCL-X_L (ABT-737 and ABT-263/navitoclax) or BCL-2 only (ABT-199/GDC-0199/venetoclax). Resistance to these three drugs is mediated by expression of MCL-1. However, given the selectivity profile of venetoclax it is unclear whether coexpression of BCL-X_L also affects antitumor responses to venetoclax in multiple myeloma. In multiple myeloma cell lines ($n = 21$), BCL-2 is expressed but sensitivity to venetoclax correlated with high BCL-2 and low BCL-X_L or MCL-1 expression. Multiple myeloma cells that coexpress BCL-2 and BCL-X_L were resistant to venetoclax but sensitive to a BCL-X_L-selective inhibitor (A-1155463). Multiple myeloma xenograft models that coexpressed BCL-X_L or MCL-1 with BCL-2 were

also resistant to venetoclax. Resistance to venetoclax was mitigated by cotreatment with bortezomib in xenografts that coexpressed BCL-2 and MCL-1 due to upregulation of NOXA, a proapoptotic factor that neutralizes MCL-1. In contrast, xenografts that expressed BCL-X_L, MCL-1, and BCL-2 were more sensitive to the combination of bortezomib with a BCL-X_L selective inhibitor (A-1331852) but not with venetoclax cotreatment when compared with monotherapies. IHC of multiple myeloma patient bone marrow biopsies and aspirates ($n = 95$) revealed high levels of BCL-2 and BCL-X_L in 62% and 43% of evaluable samples, respectively, while 34% were characterized as BCL-2^{High}/BCL-X_L^{Low}. In addition to MCL-1, our data suggest that BCL-X_L may also be a potential resistance factor to venetoclax monotherapy and in combination with bortezomib. *Mol Cancer Ther*; 15(5); 1132–44. ©2016 AACR.

Introduction

Multiple myeloma is a clonal malignancy of B cells (plasma cells) that accumulate primarily in the bone marrow. These cells can exhibit a variety of different cytogenetic lesions, the nature of which can impact prognosis and inform strategies

for therapy (1, 2). Standard-of-care drugs for the treatment of multiple myeloma include proteasome inhibitors (bortezomib and carfilzomib) and immunomodulatory agents such as lenalidomide and pomalidomide, which target the multiple myeloma-supportive bone marrow microenvironment (2). Although these drugs are effective and are often combined together to serve as a backbone for additional therapies, multiple myeloma remains a largely incurable malignancy. Moreover, efficacy can be limited due to poor tolerability and patients frequently relapse while on therapy as a result of acquired drug resistance. Thus, there remains an unmet medical need to identify novel targets based on disease pathobiology that will be efficacious as single agents or in combination with standard-of-care treatments for multiple myeloma.

Programmed cell death is governed by a complex network of interactions between prosurvival (BCL-2, BCL-X_L, and MCL-1) and pro-death (BIM, BAD, BAK, and BAX) BCL-2 family proteins, all of which possess 1-4 BCL-2 homology (BH) motifs (reviewed in refs. 3, 4, and 5). Prosurvival BCL-2 family proteins bind the BH3 motifs of pro-death counterparts, thereby sequestering them in a neutralized state. In human myeloma cell lines (HMCL), overexpression of prosurvival proteins, in particular MCL-1, has been observed and maintains survival

¹Oncology Biomarkers, Genentech, South San Francisco, California.

²Oncology Development, AbbVie, Inc, North Chicago, Illinois.

³Research Pathology, Genentech, South San Francisco, California.

⁴Oncology Discovery, AbbVie, Inc, North Chicago, Illinois.

⁵Discovery Oncology, Genentech, South San Francisco, California.

⁶Translational Oncology, Genentech, South San Francisco, California.

⁷INSERM UMR892, CNRS UMR6299, University of Nantes, Nantes, France.

⁸Early Discovery Biochemistry, Genentech, South San Francisco, California.

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E. Punnoose and J.D. Levenson contributed equally to this article.

Corresponding Author: Deepak Sampath, Genentech, 1 DNA Way, South San Francisco, CA 94080. Phone: 650-225-7786; Fax: 650-225-6240; E-mail: dsampath@gene.com

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(6–9). In addition, high-affinity small-molecule BH3 mimetics such as ABT-737 and navitoclax (ABT-263), that selectively inhibit BCL-2 and BCL-X_L but not MCL-1, induce apoptosis in multiple myeloma cells indicating dependencies on BCL-2 or BCL-X_L (10–15). However, due to their selectivity profiles, resistance to both ABT-737 and navitoclax is associated with the overexpression of MCL-1 in multiple tumor types, including multiple myeloma (16–18). The latter has been corroborated by sensitizing HMCLs to ABT-737 or navitoclax by knockdown of MCL-1 using siRNAs or pharmacologically with a selective MCL-1 inhibitor (15, 19).

Although navitoclax has demonstrated clinical efficacy in chronic lymphocytic leukemia (CLL), thrombocytopenia resulting from BCL-X_L inhibition limited its dosing (20, 21). The next-generation BH3 mimetic venetoclax (ABT-199/GDC-0199) is a BCL-2-selective inhibitor that retains robust activity against hematologic tumor cells (i.e., CLL) but spares platelets, resulting in a wider therapeutic index (22). Similar to ABT-737, proapoptotic effects have been observed with venetoclax in HMCLs and primary patient samples demonstrating that selective inhibition of BCL-2 can be sufficient for cell death (15, 23). For example, HMCLs harboring *t*(11;14) translocation and expressing higher ratios of *BCL2* relative to *MCL1* mRNA were more sensitive to venetoclax *in vitro* than cells with other cytogenetic backgrounds (23). However, given the heterogeneous expression of BCL-2, BCL-X_L, and MCL-1 in HMCLs, the antitumor activity of venetoclax in the context of BCL-X_L expression remains to be defined. Herein, we report that, in addition to *t*(11;14) status and the expression of BCL-2 relative to MCL-1, two additional biomarkers predict response to venetoclax in HMCLs. These include levels of BCL-2 complexed with BIM as a predictor of sensitivity to venetoclax and, notably, BCL-X_L as a predictor of resistance to venetoclax in HMCLs that coexpress BCL-X_L along with BCL-2. In addition, venetoclax enhances the *in vivo* efficacy of bortezomib in a multiple myeloma xenograft model that coexpresses MCL-1 but low levels of BCL-X_L. The mechanism of action of the venetoclax and bortezomib drug combination *in vivo* is in part due to bortezomib-induced neutralization of MCL-1 resulting in increased cell death. Our results indicate that relative expression of BCL-X_L and MCL-1 in HMCLs dictates pharmacologic responses to venetoclax as a monotherapy and in combination with standard-of-care drugs such as bortezomib.

Materials and Methods

Materials and Materials

Venetoclax, navitoclax, and BCL-X_L-selective inhibitors (A-1155463 and A-1331852) were manufactured or synthesized at AbbVie as previously described (24, 25). Bortezomib was purchased from Sigma. Pan-Caspase inhibitor, Z-VAD-FMK, was purchased from Promega. Human myeloma cell lines (*n* = 21: AMO-1, EJM, JIN-3, KMM-1, KMS-11, KMS-12BM, KMS-12PE, KMS-26, KMS-27, KMS-28BM, KMS-28PE, KMS-34, L-363, LP-1, MOLP-2, MOLP-8, MM.1S, NCI-H929, OPM-2, RPMI-8226, U266) were obtained from the ATCC or Deutsche Sammlung von Mikroorganismen und Zellkulturen in January, 2014, expanded and stored at early passage in a central cell bank. All cell lines were authenticated by short tandem repeat (STR) and genotyped upon re-expansions and tested within 6 months of authentication. Cell lines were grown in RPMI1640 medium supplemented with 10% FBS and 2 mmol/L glutamine (Invitrogen).

Cellular viability of HMCLs and primary multiple myeloma patient samples

HMCLs were seeded in 384-well plates at 2,000 cells per well. After 24 hours, cells were treated with drug concentrations ranging from 0.001 to 3.0 μmol/L. Cells were treated for 72 hours and cell viability determined using CellTiter-Glo (Promega) to measure ATP levels. Bone marrow samples were obtained from multiple myeloma patients at relapse from the Department of Hematology at University Hospital of Nantes (Nantes, France) after informed consent. Purified CD138-positive plasma cells were isolated from the bone marrow of 7 primary multiple myeloma patient samples as described previously (15) and cultured with increasing doses of venetoclax (100–500 nmol/L) for 18 hours. Cell death in primary myeloma cells was determined by flow cytometry using a combined analysis for the loss of CD138 surface expression and the alteration of cellular morphology (lower forward cell scatter indicative of dead cells). Fluorescence was analyzed on a FACSCalibur instrument.

Immunoblotting

Two million cells were lysed in ice-cold RIPA buffer (Cell Signaling Technology) containing 1 mmol/L Peflabloc, Phosphatase Inhibitor Cocktail 1 and 2 (Sigma-Aldrich), and complete EDTA-free protease inhibitor tablet (Roche). Equal amounts of protein were subjected to SDS-PAGE (4%–20% Tris-Glycine; Invitrogen) and probed with antibodies against BCL-2, BCL-X_L, MCL-1, BIM, NOXA, cleaved PARP and cleaved caspase-3 (Cell Signaling Technologies) or β-actin (Sigma). Specific antigen-antibody interaction was detected with a secondary antibody labeled with either IRDye800 (Rockland Immunochemicals) or Alexa Fluor 680 (Molecular Probes) and was visualized by the LI-COR Odyssey Imaging System. Immunoblot signal intensities were quantified with Odyssey software (LI-COR).

Immunoprecipitation

Cells were washed twice in ice-cold PBS and lysed in 1% CHAPS lysis buffer (50 mmol/L Tris HCl, pH 7.4, 110 mmol/L NaCl, 5 mmol/L EDTA, 1% CHAPS) supplemented with a protease inhibitor cocktail (Roche), phosphatase inhibitor cocktails (Sigma), and 1 mmol/L PMSF. Protein levels were quantified by the BCA Protein Assay Kit (Pierce Biotechnology), and normalized to equal concentrations. Equal amounts of protein from each sample were precleared with streptavidin agarose resin for 30 minutes at 4°C, then incubated with 4 μg biotinylated BCL-X_L antibody (Novus Biologicals), and 10 μL packed streptavidin agarose resin overnight at 4°C. Beads were washed three times with ice-cold 1% CHAPS lysis buffer and boiled in lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) supplemented with dithiothreitol for 10 minutes at 70°C.

Caspase-3/7 activation

For detection of caspase activation, cells were seeded at 2,500 cells per well in 384-well plates and incubated overnight before adding compounds to quadruplicate wells. After 24 hours, caspase activity was measured by luminescence using Caspase-Glo 3/7 reagent (Promega) according to the manufacturer's instructions.

Gene expression analysis

Gene expression analysis was performed on isolated total RNA using the BioMark 96.96 Dynamic Array platform (Fluidigm). Total RNA was reverse-transcribed into cDNA and

preamplified in a single reaction using gene-specific primers, Superscript III/Platinum Taq (Invitrogen), and 2× Reaction Mix (Invitrogen). The thermal cycling conditions were as follows: 1 cycle of 50°C for 15 minutes, 1 cycle of 70°C for 2 minutes, then 14 cycles of 95°C for 15 seconds, and 60°C for 4 minutes. Preamplified cDNA was diluted 1.94-fold and then amplified using TaqMan Universal PCR Master Mix (Applied Biosystems) on the BioMark platform (Fluidigm) according to the manufacturer's instructions. Both samples and assays were run in duplicate. Two custom-designed assays targeting the reference genes, TMEM55B and VPS33B, were included in the expression panel. The geometric mean of the C_t values for the two reference genes was calculated for each sample, and expression levels for *BCL2* and other apoptotic genes of interest were determined using the ΔC_t (ΔC_t) method as follows: average C_t (target gene) – Geomean C_t (reference genes). Gene list and TaqMan primers and probes are provided in Supplementary Table S1. In addition, the relative expression of *BCL2*, *BCL2L1*, and *MCL1* mRNA was defined on purified CD138-positive plasma cells. TaqMan gene expression assays for *BCL2* (Hs00608023_m1), *MCL1* (Hs00172036_m1), *BCL2L1*; Hs00236329_m1), and *RPL37a* (Hs01102345_m1) were purchased from Applied Biosystems. The following thermal cycling parameters were used: 50°C for 2 minutes for optimal AmpErase UNG activity and then 40 cycles at 95°C for 30 seconds and 60°C for 1 minute. Amplification of the housekeeping gene *RPL37a* was conducted for each sample as an endogenous control and used to normalize levels of *BCL2*, *BCL2L1*, and *MCL1*.

BCL-2:BIM complex evaluation

BCL-2:BIM complexes were measured using an immunoassay platform. Briefly, 15 million multiple myeloma cells were exposed to DMSO vehicle or 1 μ mol/L venetoclax for 24 hours and then processed for evaluation of the disruption of BCL-2:BIM complexes. Streptavidin-coated plates were blocked for 16 to 24 hours with 40 to 150 μ L blocking buffer (MesoScale Discovery) while protein concentrations of cell lysates were adjusted to 4 mg/mL using cell lysis buffer. Anti-BCL-2 mAb (Invitrogen) was labeled with 6:1 molar challenge ratio with ruthenium. Anti-BIM mAb (Epitomics) was labeled with biotin at a 20:1 molar challenge ratio. Ruthenium-tagged anti-BCL-2 antibody was diluted to 1 μ g/mL in incubation buffer and added to streptavidin-coated plates. An equal volume of cell lysates or BCL-2:BIM standard protein complexes were added to the plates and incubated at room temperature. Plates were washed with 0.5% polysorbate 20 in PBS followed by incubation with 1 μ g/mL biotinylated anti-BIM antibody for 90 minutes at room temperature. Electrochemiluminescent signal intensity was measured on the MesoScale Discovery SECTOR Imager 6000. Concentration of BCL-2:BIM complexes in samples treated with DMSO and venetoclax was determined by four-parameter fit logistic regression analysis based on the respective standard curves.

In vivo efficacy

All *in-vivo* studies were approved by Genentech's and AbbVie's Institutional Animal Care and Use Committee and adhere to the NIH Guidelines for the Care and Use of Laboratory Animals. Tumor xenografts were established by subcutaneous injection of human multiple myeloma cell lines in female SCID.beige mice (Charles River Laboratories). Animals were distributed into treatment groups when tumors reached a mean volume of approxi-

mately 150 to 250 mm³. Venetoclax (100 mg/kg), navitoclax (100 mg/kg), or BCL-X_L-selective inhibitor, A-1331852 (25 mg/kg), was administered by oral gavage (PO) in 60% phosal 50PG, 30% PEG 400, 10% ethanol while bortezomib was injected intravenously in saline. Venetoclax, navitoclax, or A-1331852 was dosed daily (QD) over a period of 21 days, whereas bortezomib was dosed Q4D x 3 (days 1, 5, and 9). Body weights and tumor volumes [caliper-based ellipsoid model: $L \times W^2/2$, where the larger (L) and smaller (W) perpendicular dimensions are measured] were recorded twice weekly. Percent tumor growth inhibition (TGI) was calculated at the end of drug treatment using the following formula: %TGI = 100 × (mean tumor volume in the vehicle treated group – mean tumor volume of drug treated group)/mean tumor volume of vehicle treated group. A partial response (PR)/animal was defined as a reduction of greater than 50% but less than 100% in tumor volume, compared with the starting tumor volume, observed during the course of treatment. A complete response (CR)/animal was defined as a 100% reduction in tumor volume, compared with the initial tumor volume, observed during the course of treatment. Overall response rate (ORR) is the sum of CRs and PRs.

IHC and FISH

Formalin-fixed, paraffin-embedded samples of multiple myeloma patient samples of decalcified bone marrow biopsies and aspirates ($n = 95$) were acquired from ProteoGenex and the MT Group following Institutional Review Board approval and informed consent of all subjects contributing specimens. Samples were sectioned at 4 μ m, stained with hematoxylin and eosin, and with antibodies to BCL-2 (clone 124, DAKO), BCL-X_L (clone 54H6, Cell Signaling Technologies), MCL-1 (clone 29H1L1, Spring Bioscience) or CD138 using conditions noted in Supplementary Information. Positive control samples of formalin-fixed, paraffin-embedded cell pellets were prepared from HMCLs or other cell lines such as A549 (lung adenocarcinoma) and U-698-M (B-cell lymphoma). BCL-2, BCL-X_L, and MCL-1 tumor cell staining was evaluated on a qualitative intensity scale of 0 to 3+. Scores of 0/1+ (weak), 1+/2+ (moderate), and 2+/3+ (strong) were assigned to samples with tumor cells of intermediate or heterogeneous staining intensities. Control HMCL samples for BCL-2 included KMS-34 (1+), KMS-11 (2+), and AMO-1 (3+). In addition, for BCL-2, an IHC score of 2+ was assigned if $\geq 50\%$ of tumor cells in the multiple myeloma patient specimen had a staining intensity equal to the predominant intensity of cytoplasmic staining in the mantle zone B cells and paracortical T cells found in tonsils, which also served as a positive control tissue. Specimens with signal weaker or stronger than the latter 2+ score were assigned an intensity of 1+ and 3+, respectively. For BCL-X_L, a score of 2+ was assigned to the predominant cytoplasmic intensity of megakaryocytes. Tumor cells contained within the multiple myeloma specimen with signal significantly weaker or stronger score than 2+ were assigned an intensity of 1+ and 3+, respectively. Control HMCL samples for BCL-X_L included AMO-1 (0/1+), KMS-26 (2+), and MM.1S (3+). For MCL-1, multiple myeloma patient specimens were scored based on comparable staining intensity of positive control tumor cell lines such as A549 (1+), LP-1 (2+), and U-698-M (3+). Translocation $t(11;14)$ status was determined using the Vysis *IGH/CCND1* DF FISH Probe Kit from Abbott Molecular in 26 multiple myeloma patient samples.

Statistical analysis

A heteroscedastic unpaired Student *t*-test was used to compare two groups. For three or more groups, a comparison to control using Dunnett method was used. To compare pretreatment to posttreatment data within a group, a matched paired *t*-test was used. Statistical significance was defined as $P < 0.05$.

Results

A composite of BCL-2, BCL-X_L and MCL-1 expression profiles predicts sensitivity to venetoclax in HMCLs *in vitro* and multiple myeloma patient samples *ex vivo*

To determine dependency of HMCLs on antiapoptotic proteins BCL-2 and BCL-X_L for survival, we evaluated sensitivity of 21 HMCLs to navitoclax, venetoclax, and a potent BCL-X_L-selective inhibitor, A-1155463 (Fig. 1A). A heterogeneous response to these inhibitors was observed. For example, seven HMCLs were sensitive to venetoclax based on a mean IC₅₀ ≤ 1 μmol/L, whereas six of these lines were also sensitive to navitoclax, suggesting that they were dependent on BCL-2 for survival (Fig. 1A). Four of the seven venetoclax-sensitive HMCLs harbored *t*(11;14) translocations: a cytogenetic subgroup of multiple myeloma that has been shown previously to express high levels of BCL-2 relative to MCL-1, which favors sensitivity to venetoclax (23). Four HMCLs that were sensitive to navitoclax but resistant to venetoclax were sensitive to A-1155463, suggesting that these lines were dependent on BCL-X_L rather than BCL-2 for survival (Fig. 1A).

To further define predictive markers of sensitivity and resistance to venetoclax in HMCLs, expression of a panel of 18 pro- and antiapoptotic genes in the intrinsic apoptosis pathway, including members of the *BCL2* family, were evaluated by qRT-PCR (primer sequences provided in Supplementary Table S1). This qRT-PCR panel of apoptosis genes is hereon referred to as Apopanel. As part of our analysis, we determined the ratios of *BCL2*:*BCL2L1* (encodes BIM), *BCL2*:*BCL2L1* (encodes BCL-X_L), and *BCL2*:*MCL1* mRNA transcripts. In order to identify the top correlates for venetoclax sensitivity and expression profiles of genes in the apopanel, sensitive and resistant lines were compared and statistical significance determined by an unpaired *t*-test (Supplementary Table S2). Sensitivity to venetoclax was inversely proportional to *BCL2L1* mRNA levels and directly proportional to the ratio of *BCL2*:*BCL2L1* (Fig. 1B and Supplementary Table S2). In comparison, sensitivity to navitoclax was inversely proportional to *MCL1* mRNA levels and directly proportional to the ratio of *BCL2*:*MCL1* (Fig. 1C and Supplementary Table S2). On the basis of the results from the Apopanel analysis, we determined individual cutoffs for *BCL2*, *BCL2L1*, and *MCL1* mRNA expression levels that would predict sensitivity to venetoclax in HMCLs. Establishing cutoffs for all three genes in composite and not just *BCL2* increased the distinction between venetoclax-sensitive versus -resistant HMCLs while minimizing false-positive and false-negative rates. For example, in HMCLs evaluated in which *BCL2*, *BCL2L1*, and *MCL1* mRNA levels were defined by ΔC_t values of > 2.7, <5, and <4.8, respectively, a 5% false-positive and 0% false-negative rate for venetoclax sensitivity was determined. However, by establishing a cutoff of > 2.7 for only *BCL2* expression, the false-positive rate for venetoclax sensitivity increased to 48% (i.e., 10 of 21 lines were falsely predicted to be sensitive to venetoclax). Therefore, the rank order of expression levels that best predicted sensitivity to venetoclax in HMCLs was defined as *BCL2* > *BCL2L1* > *MCL1*.

With the exception of two cell lines (MOLP-8 and KMS-34), all HMCLs evaluated had relatively high levels of BCL-2 protein by western blot analysis (Fig. 1D). Quantification of protein levels determined a significant correlation between mRNA levels for *BCL2* ($r^2 = 0.73$, $P < 0.01$), *BCLX_L* ($r^2 = 0.69$, $P < 0.05$), and *MCL1*, ($r^2 = 0.65$, $P < 0.05$). Similar to the observations determined by analysis of the apopanel, BCL-X_L protein levels correlated with resistance to venetoclax ($P = 0.018$) and sensitivity to the BCL-X_L-selective inhibitor A-1155463 ($P = 0.012$). Indeed, treatment of HMCLs that expressed BCL-X_L (KMS-28PE, KMS-34, RPMI-8226, and MM.1S) with A-1155463 resulted in increased levels of cleaved PARP and caspase-3 (Fig. 2A). In KMS-34, A-1155463 treatment induced caspase-3/7 activities to a greater extent than navitoclax with EC₅₀ values of 10 nmol/L versus 1 μmol/L, respectively (Fig. 2B). Moreover, A-1155463 effectively disrupted BCL-X_L:BIM complexes in the KMS-28PE and KMS-34 HMCLs at concentrations that induced cell death (Fig. 2C and D). The latter confirms that these cell lines are dependent on BCL-X_L for survival despite coexpression of BCL-2.

In addition, we analyzed CD138⁺ myeloma plasma cells purified from seven individual multiple myeloma patient samples for *BCL2*, *BCL2L1*, and *MCL1* mRNA expression levels and sensitivity to venetoclax *ex vivo*. A LD₅₀ (lethal dose that causes 50% cell death) of < 500 nmol/L was used as a threshold for venetoclax sensitivity. In four out of seven patient samples in which the LD₅₀ for venetoclax was > 500 nmol/L, the *BCL2L1*:*BCL2* mRNA ratio ranged from 2.03- to 15.195-fold (Supplementary Table S3). In the remaining three patient samples in which the LD₅₀ < 500 nmol/L, the ratio of *BCL2L1*:*BCL2* was 1 to 1 with the exception of one sample in which the ratio was 3.51 and the ratio of *MCL1*:*BCL2* was 0.46 to 1.0 (Supplementary Table S3). Analysis of this small set of multiple myeloma patient samples suggests that expression of *BCL2L1* relative to *BCL2* may be associated with decreased sensitivity to venetoclax *ex vivo*. Consistent with our previous findings (23), a higher ratio of *BCL2* to *MCL1* was observed in samples sensitive to venetoclax.

Venetoclax treatment results in disruption of BCL-2:BIM complex in both sensitive and resistant cell lines

Cells primed for death are defined by increased levels of prosurvival proteins bound to their cognate BH3 ligand resulting in a lower threshold for sensitivity to intrinsic apoptosis-inducing agents and cell-death signals (26). Indeed, levels of BCL-2:BIM complexes are correlated with sensitivity to venetoclax in AML cell lines or patient samples (27). Therefore, we quantified the levels of BCL-2 complexed with BIM in HMCLs to determine whether increased levels correlated with venetoclax sensitivity. BCL-2:BIM complexes were detectable in HMCL cell lines (range: 500–42,000 units; Fig. 3A). In the six cell lines with the highest BCL-2:BIM signal intensities (>10,000 units), five were sensitive to venetoclax based on a cellular viability IC₅₀ ≤ 1.0 μmol/L (Fig. 3A). Venetoclax treatment in these lines, such as KMS-12PE, resulted in a concentration-dependent disruption of BCL-2:BIM complexes (Fig. 3B and Supplementary Fig. S1) consistent with the molecule's binding mode and biochemical mechanism of action. Interestingly, venetoclax-resistant cell lines such as KMS-28PE and OPM-2, which coexpressed *BCL2L1* and *MCL1* mRNA at levels that correlated with resistance to venetoclax, also had relatively high levels of BCL-2 bound to BIM (Fig. 3A). In KMS-28PE cells, venetoclax and navitoclax were equally effective at disrupting BCL-2:BIM

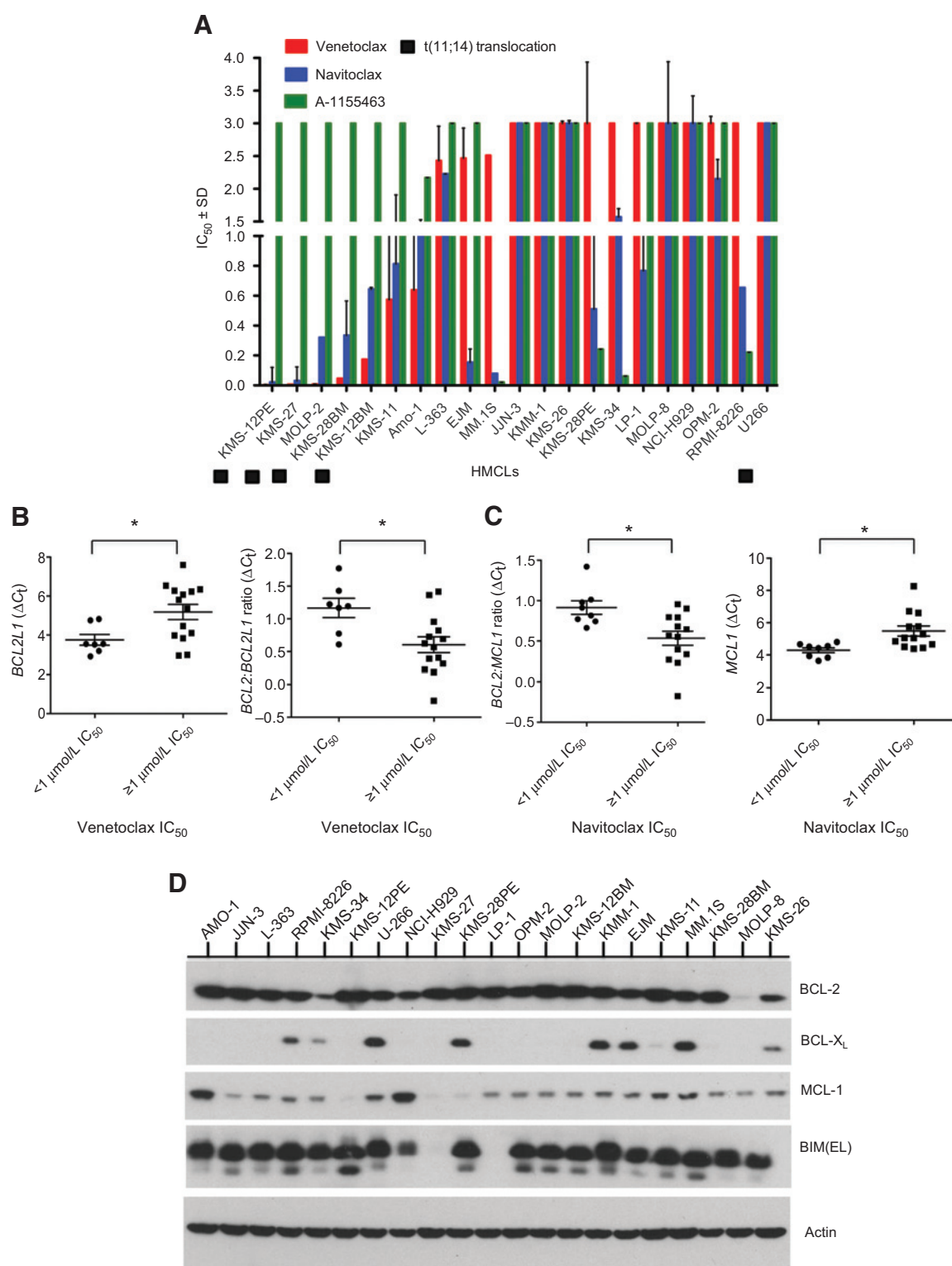


Figure 1. Sensitivity of HMCLs to venetoclax and expression of BCL-2, BCL-X_L, and MCL-1. A, cell viability of HMCLs as measured by Cell-Titer Glo (Promega) following treatment with 0.01 to 3.0 μmol/L of venetoclax, navitoclax, or A-1155463 for 72 hours. B and C, RNA expression levels of genes most strongly correlated with *in vitro* drug sensitivity in HMCLs. B, RNA expression levels (based on log₂-transformed delta C_t values and normalized to housekeeping genes) of *BCL2L1* (left) and ratio of *BCL2* to *BCL2L1* (right) in HMCLs binned on the basis of sensitivity to venetoclax. D, ratio of *BCL2* to *MCL1* (left) and RNA expression levels of *MCL1* (right) in HMCLs sorted on the basis of *in vitro* sensitivity to navitoclax. *, *P* < 0.05 by Student *t*-test. D, western blot analyses of BCL-2, BCL-X_L, MCL-1, and BIM (EL) protein levels in HMCLs were generated as described in Materials and Methods.

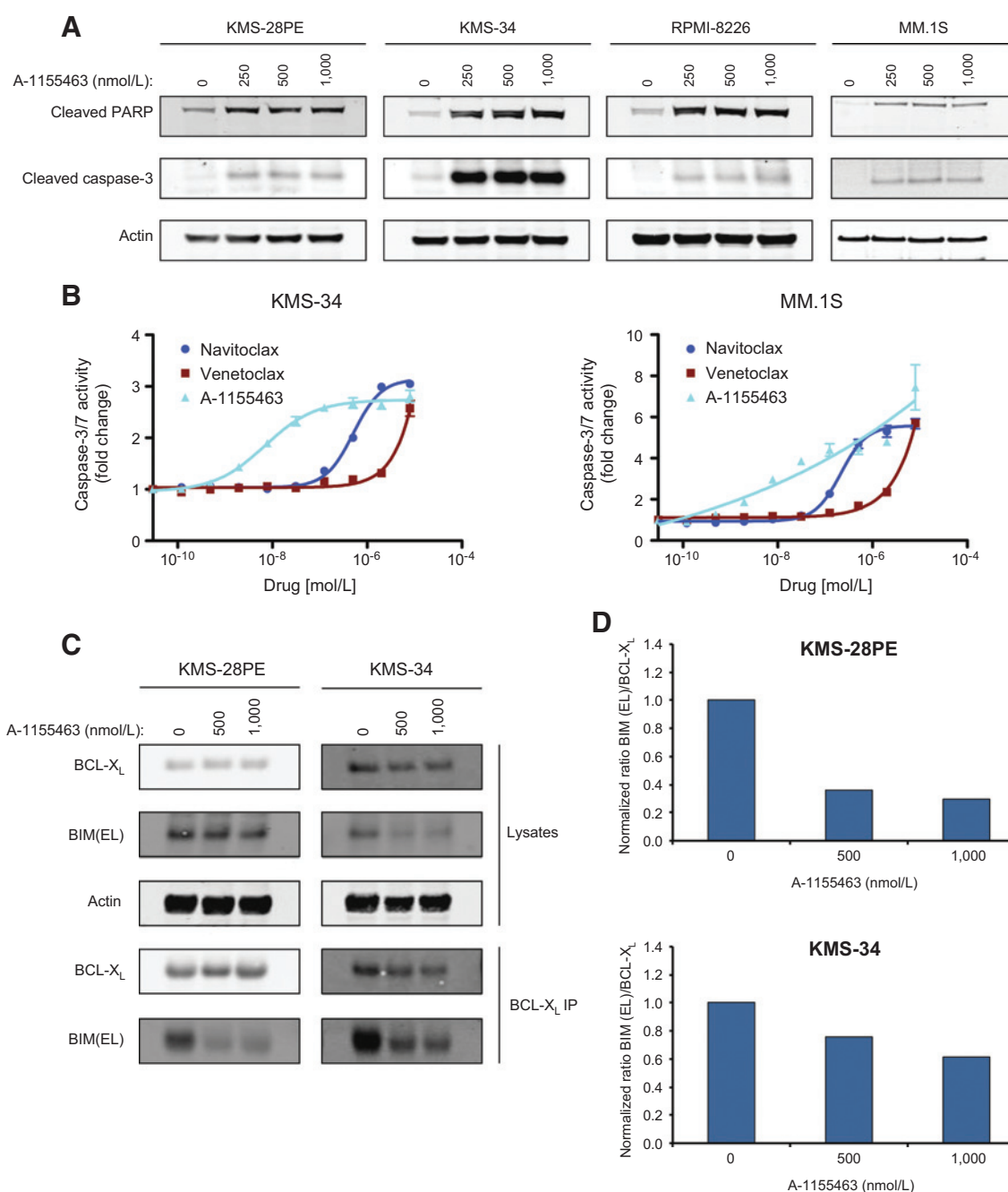


Figure 2. Induction of cell death and disruption of BCL-X_L:BIM complexes by a BCL-X_L-selective inhibitor, A-1155463. A, KMS-28PE, KMS-34, RPMI-8226, and MM.1S HMCLs were treated with the indicated concentrations of A-1155463 or vehicle control (DMSO) for 24 hours. Lysates were subjected to western blot analysis with the indicated antibodies as described in Materials and Methods. B, KMS-34 and MM.1S cells were treated with serial dilutions of the indicated compounds or vehicle control (DMSO) in quadruplicate. Caspase-3/7 activity was measured 24 hours after drug treatment. C, KMS-28PE and KMS-34 cells were treated with the indicated concentrations of A-1155463 or vehicle control (DMSO) for 24 hours. Lysates were subjected to immunoprecipitation with a BCL-X_L antibody followed by western blot analysis as described in Materials and Methods. D, the normalized ratios of BIM (EL) bound to BCL-X_L were quantified from the co-immunoprecipitation and western blot analyses shown in C and are depicted as histograms.

complexes, indicating that resistance to venetoclax was not necessarily due to ineffective drug binding to BCL-2 but a result of coexpression of BCL-X_L (Fig. 3C). The latter was consistent with our observation that A-1155463 effectively dissociated BCL-X_L from BIM in the KMS28-PE line (Fig. 2C).

***In vivo* efficacy of venetoclax in multiple myeloma tumor xenografts as a single agent and in combination with bortezomib**

We next determined the efficacy of venetoclax in HMCLs grown as subcutaneous xenografts that expressed high levels

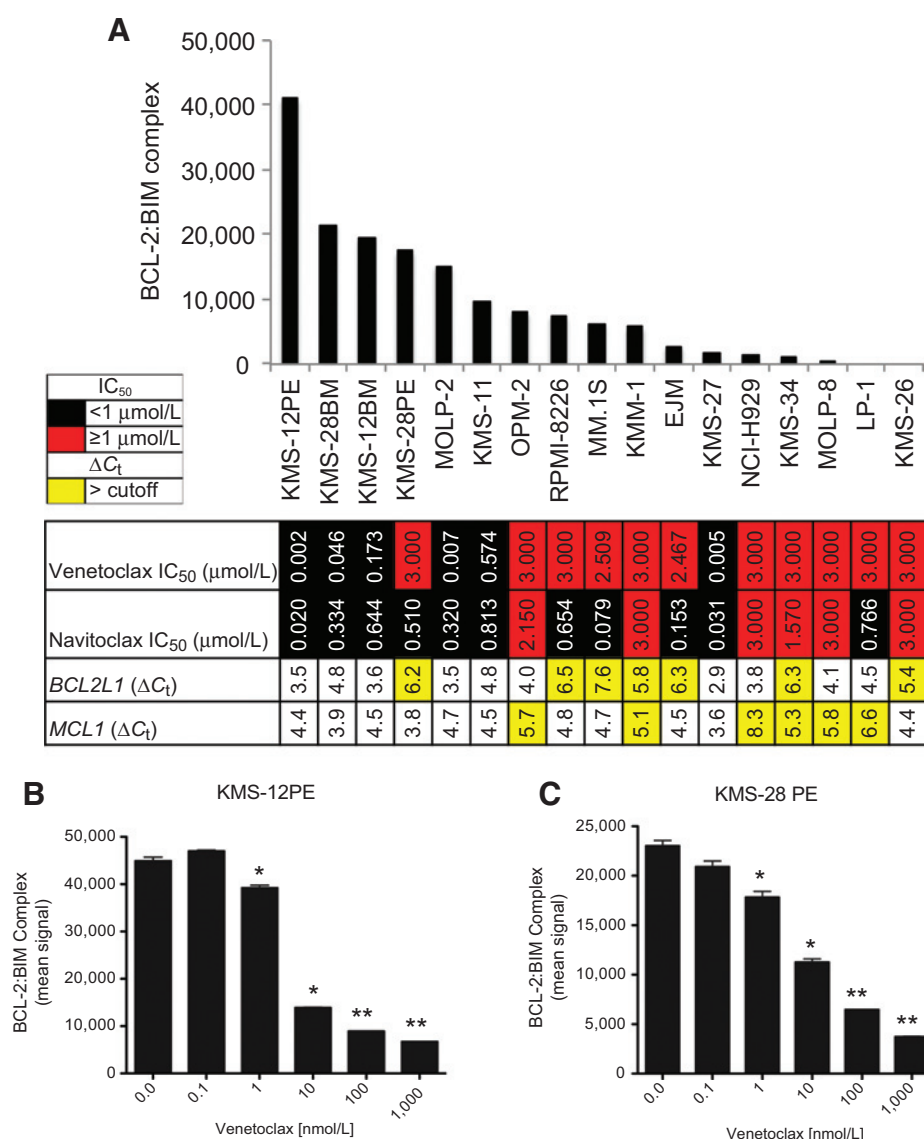


Figure 3. Evaluation of BCL-2:BIM complexes in HMCLs before and after treatment with venetoclax. A, baseline levels of BCL-2: BIM complexes were measured in untreated HMCL lysates as described in Materials and Methods. Mean signal intensity was determined by electrochemiluminescence. Cell lines are ordered from high to low based on levels of BCL-2:BIM complexes. Cell line characteristics are listed in the table below including mean intensity values for BCL-2:BIM complexes, IC₅₀ values of venetoclax and navitoclax as well as RNA expression levels of *BCL2L1* (encodes BCL-X_L) and *MCL1*. Yellow boxes identify cell lines with *BCL2L1* or *MCL1* RNA expression levels that are above the predictive cutoff for venetoclax sensitivity. B and C, levels of BCL-2:BIM complexes following treatment with venetoclax at a concentration range from 0.1 to 1000 nmol/L in the venetoclax-sensitive KMS-12PE cell line (B) and venetoclax-resistant KMS-28PE cell line (C). *, *P* < 0.01 and **, *P* < 0.001 versus untreated control as determined by Dunnett *t* test.

of BCL-2 and MCL-1 but variable levels of BCL-X_L to determine whether resistance to venetoclax was maintained *in vivo*. Similar to navitoclax, a dose and schedule of venetoclax (100 mg/kg/d) that are efficacious in Non-Hodgkin's Lymphoma xenograft models (22) demonstrated modest single-agent tumor growth inhibition (TGI) in OPM-2 xenografts (Fig. 4A; 59% TGI, *P* < 0.001 vs. vehicle treated). However, venetoclax was only marginally efficacious in the NCI-H929 and MM.1S xenograft models (Fig. 4B–D). BCL-2, BCL-X_L, and MCL-1 were expressed in all three xenograft models but to varying degrees (Supplementary Fig. S2). Therefore, our hypothesis was that reduced sensitivity to venetoclax *in vivo* may be due to coexpression of BCL-X_L and/or MCL-1, and that selective inhibition of these prosurvival proteins may overcome resistance to venetoclax upon cotreatment. Because selective MCL-1 inhibitors that are efficacious *in vivo* are unavailable, we tested the combination of venetoclax with bortezomib to determine whether increased tumor growth inhibition can be observed. Bortezomib induces the expression of the BH3-only protein NOXA, which selec-

tively neutralizes MCL-1 and induces degradation (14, 28–30). Compared with bortezomib treatment administered at a MTD, the combination of venetoclax and bortezomib increased TGI from 89% to 97% and the overall response rate (ORR) from 55% to 100%, respectively, in the OPM-2 xenograft model (Fig. 4A). The increase in ORR in the combination treatment group relative to bortezomib monotherapy was due to an increase in CRs from 11% to 80% relative to bortezomib treatment alone. Moreover, the coadministration of venetoclax and bortezomib resulted in a more durable therapeutic response based on delayed tumor growth when compared to bortezomib monotherapy. (Fig. 4A). However, as previously reported, navitoclax also enhanced the efficacy of bortezomib (31) suggesting that efficacy of both drugs in combination may be primarily due to BCL-2 inhibition. Both combinations were tolerated on the basis of minimal changes in animal body weights (data not shown).

The combination of venetoclax and bortezomib was also evaluated in the NCI-H929 xenograft model, which displayed

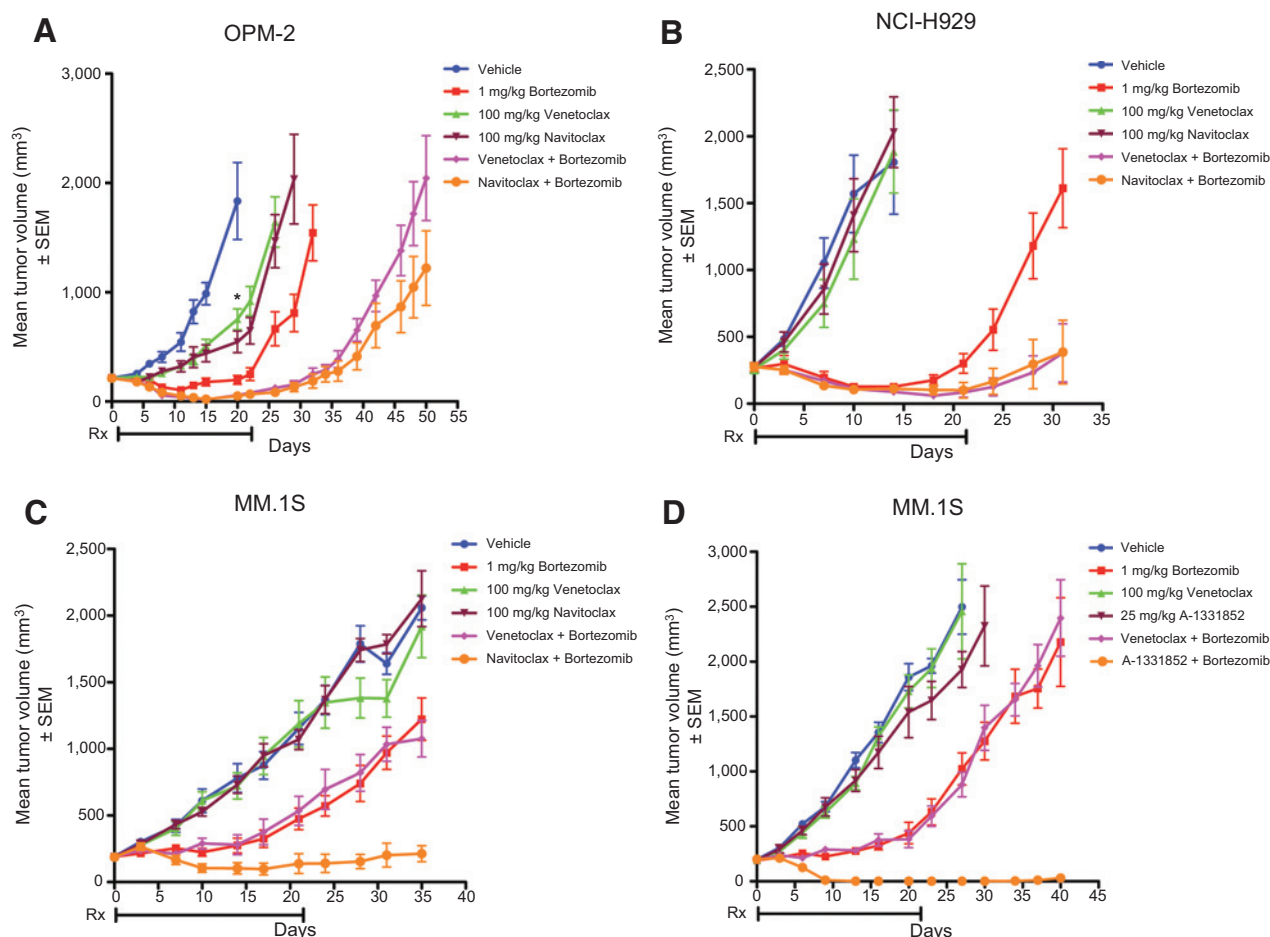


Figure 4. *In vivo* efficacy of venetoclax in combination with bortezomib in HMCL xenograft models: OPM-2 (A), NCI-H929 (B) and MM.1S (C and D). Venetoclax (100 mg/kg), navitoclax (100 mg/kg), or BCL-X_L-selective inhibitor A-1331852 (25 mg/kg) was administered by oral gavage (PO) in 60% phosal 50PG, 30% PEG 400, 10% ethanol while bortezomib was injected intravenously in saline. Venetoclax, navitoclax, or A-1331852 was dosed daily (QD) over a period of 21 days while bortezomib was dosed Q4D × 3 (days 1, 5, and 9). Rx denotes total treatment period. *, *P* < 0.001 vs. vehicle control-treated mice as determined by Dunnett *t* test.

higher BCL-2 and MCL-1 protein levels but low BCL-X_L (Supplementary Fig. S2). Moreover, NCI-H929 cells have been demonstrated to be dependent on MCL-1 for survival (19). Indeed, bortezomib treatment alone resulted in 90% TGI during the treatment period when compared with the vehicle control group while venetoclax was not efficacious (Fig. 4B). However, venetoclax enhanced the durability of the therapeutic response induced by bortezomib by significantly delaying tumor regrowth when both drugs were combined (Fig. 4B; *P* < 0.0001 for venetoclax plus bortezomib vs. bortezomib alone). Moreover, treatment with the combination of bortezomib and venetoclax increased the overall response from 29% with bortezomib treatment alone to 100%, including 6 complete responses. Similarly, navitoclax enhanced the efficacy of bortezomib when both drugs were combined to a similar degree as venetoclax suggesting that BCL-X_L inhibition may not be required for the observed effect. Thus, in NCI-H929 xenografts that express BCL-X_L at lower levels relative to BCL-2, the combination of venetoclax and bortezomib results in greater tumor growth inhibition and duration of response than bortezomib monotherapy.

The latter results suggest that BCL-X_L levels may also be a predictive biomarker for sensitivity to venetoclax when combined with bortezomib. To test this hypothesis *in vivo*, we evaluated the MM.1S xenograft model that expresses relatively higher levels of BCL-X_L (Supplementary Fig. S2) and is sensitive to the BCL-X_L-selective inhibitor A-1155463 *in vitro* (Fig. 1A). As observed in the NCI-H929 xenograft model, venetoclax was neither efficacious in MM.1S tumor xenografts nor did it increase the efficacy of bortezomib when compared with bortezomib alone (Fig. 4C). However, when compared with bortezomib treatment alone, the combination of navitoclax with bortezomib resulted in sustained tumor regressions and increased the overall response rate from 20% to 100%, respectively (Fig. 4C; *P* < 0.001 for navitoclax plus bortezomib vs. bortezomib). Similarly, A-1331852, a potent and selective BCL-X_L-selective inhibitor that is orally bioavailable in mice (25), was not efficacious as a single agent, but when combined with bortezomib resulted in durable tumor regressions and no regrowth when compared with bortezomib alone (Fig. 4D; *P* < 0.0001 for A-1331852 plus bortezomib vs. bortezomib). Moreover, compared with bortezomib monotherapy, the

combination of A-1331852 with bortezomib increased the ORR from 20% to 100% including 90% CRs (Fig. 4D). The latter confirmed that BCL-X_L inhibition was sufficient to enhance the efficacy of bortezomib in tumors that expresses high levels of BCL-X_L relative to BCL-2. The combination of navitoclax or A-1331852 with bortezomib was also well tolerated on the basis of minimal changes in animal body weights (data not shown).

Given enhanced antitumor activity of venetoclax in combination with bortezomib in NCI-H929 tumor xenografts, we sought to determine the mechanism of action of the dual combination. A single efficacious dose of bortezomib (1 mg/kg) resulted in an increase in NOXA protein levels and a decrease in MCL-1 levels within 24 hours following treatment (Fig. 5A). As shown previously in MDN myeloma cells *in vitro* (29), the decrease in MCL-1 protein levels in NCI-H929 cells following bortezomib treatment is likely due to caspase-dependent cleavage since in the presence of the pan-Caspase inhibitor, Z-VAD-FMK, decreased MCL-1 or cleaved caspase-3 was not observed (Supplementary Fig. S3A and S3B). The combination of venetoclax with bortezomib resulted in an increase in apoptotic markers (cleaved caspase-3 and cleaved PARP) in comparison with venetoclax alone within 8 to 24 hours (Fig. 5A). Importantly, treatment of NCI-H929 tumor xenografts with 0.5 mg/kg bortezomib, a dose that did not increase NOXA expression or reduce MCL-1 protein levels and induce apoptosis, was not efficacious as a single agent or in combination with venetoclax (Fig. 5B–C). These data suggest that increase in NOXA levels, which neutralizes MCL-1, and caspase-mediated degradation of MCL-1 may contribute to increased efficacy when bortezomib is combined with venetoclax *in vivo*.

BCL-2 is strongly expressed in multiple myeloma patient tissue specimens by IHC

Given that expression patterns of BCL-2, BCL-X_L, and MCL-1 in HMCLs predict sensitivity to venetoclax, IHC assays were optimized for detection of all three prosurvival proteins in patient bone marrow biopsies and aspirates to estimate prevalence. A semiquantitative scoring system was compared with the *in vitro* sensitivity of HMCLs to venetoclax as well as mRNA expression levels of *BCL2*, *BCL2L1*, and *MCL1* that were associated with sensitivity to venetoclax (Supplementary Fig. S4). Expression of BCL-2 and BCL-X_L in cell lines detected by IHC correlated with expression by western blot analysis and qRT-PCR analysis (Supplementary Figs S5A and S5B). On the basis of these results, IHC cutoffs for BCL-2 and BCL-X_L "high" and "low" expression were established similar to those used to predict venetoclax sensitivity by qRT-PCR (Fig. 1B).

Multiple myeloma tissue specimens ($n = 95$), including both bone marrow core biopsies and bone marrow aspirates, were evaluated for BCL-2 family expression by IHC (Fig. 6A). Evaluation of H&E and CD138-stained sections revealed 95 samples with adequate tumor cell content (>5% of cells). Of these, 59 (62%) were found to be BCL-2^{High} (score $\geq 2+$) and 54 (57%) were BCL-X_L^{Low} (score < 2+). In samples evaluable for MCL-1 tumor staining ($n = 77$), 81% (62/77) of the specimens were scored a 1+ or weaker. Thirty-two samples (34%) had a favorable combination of BCL-2^{High} and BCL-X_L^{Low} (Fig. 6B) that would predict sensitivity to venetoclax as a single agent (based on the HMCL cell viability data) or in combination with bortezomib. Given that HMCLs that harbored *t*(11;14) translocations were sensitive to venetoclax *in vitro*, we also evaluated *t*(11;14) trans-

location status by FISH in a subset of multiple myeloma patient samples. Translocations in *t*(11;14) were found in 20% of patients and distributed across BCL-2^{High} and BCL-2^{Low} patients (data not shown).

Discussion

BCL-2 family proteins are crucial regulators of multiple myeloma cell survival making them attractive therapeutic targets. Indeed, targeting the prosurvival proteins with dual BCL-2 and BCL-X_L inhibitors such as ABT-737 or navitoclax or the BCL-2 selective inhibitor, venetoclax, can induce apoptosis in HMCLs *in vitro* and isolated multiple myeloma patient samples *ex vivo* (10–15, 23). However, a subset of HMCLs is resistant to ABT-737 and venetoclax as a result of MCL-1 expression and dependency (15, 18, 23). Moreover, HMCLs and multiple myeloma patient samples that express lower levels of MCL-1 are sensitive to venetoclax (23). However, given the heterogeneous expression of BCL-2, BCL-X_L, and MCL-1 in HMCLs and multiple myeloma patient samples, it has yet to be determined whether coexpression or codependency on BCL-X_L is also a predictor of sensitivity to venetoclax. Thus the primary aim of our study was to further define expression patterns of BCL-2, BCL-X_L, and MCL-1 as biomarkers in HMCLs and multiple myeloma patient samples to refine predictors of response to venetoclax as a single agent and in combination with standard-of-care drugs such as bortezomib.

Consistent with previous reports, the majority of cell lines (19 out of 21) we tested expressed high levels of BCL-2 and those that coexpressed MCL-1 were less sensitive to venetoclax and the dual BCL-2/BCL-X_L inhibitor navitoclax. Interestingly, 4 HMCLs that coexpressed BCL-X_L and BCL-2 were resistant to venetoclax, but sensitive to navitoclax or the BCL-X_L-selective inhibitor A-1155463. Importantly, A-1155463 effectively disrupted BCL-X_L:BIM complexes in these HMCLs at concentrations that induced apoptotic cell death, indicating their dependence on BCL-X_L rather than BCL-2 for survival. Moreover, in HMCLs coexpression of BCL-X_L and the ratio of BCL-2/BCL-X_L were strong predictors of venetoclax sensitivity, whereas MCL-1 coexpression and the ratio of BCL-2/MCL-1 were better predictors of navitoclax sensitivity in HMCLs. The latter is consistent with previous reports regarding ABT-737 resistance in HMCLs that coexpress MCL-1 (15). Despite BCL-2 expression detected in the majority of HMCLs evaluated, our results demonstrate that a subset of multiple myeloma cell lines is primarily dependent on BCL-X_L for survival. In addition, in three out of seven CD138⁺ primary multiple myeloma patient samples in which sensitivity to venetoclax was lower, expression of BCL-X_L relative to BCL-2 was 2- to 15-fold higher. Our preliminary analysis suggests that increased expression of BCL-X_L relative to BCL-2 may be associated with decreased sensitivity to venetoclax but evaluation of a larger patient sample set will be needed for further confirmation.

Decreased sensitivity to venetoclax due to BCL-X_L coexpression or upregulation has been observed in other hematologic malignancies such as CLL. For example, primary CLL cells become resistant to venetoclax *ex vivo* upon stimulation with CD40 ligand (CD40L) or by cytokines (IL4 and IL21) that mimic a microenvironment containing activated T cells and follicular T cells (32). CD40L or cytokine treatment upregulated BCL-X_L and MCL-1 in a NF- κ B-dependent manner and siRNA knockdown of BCL-X_L restored sensitivity to venetoclax but

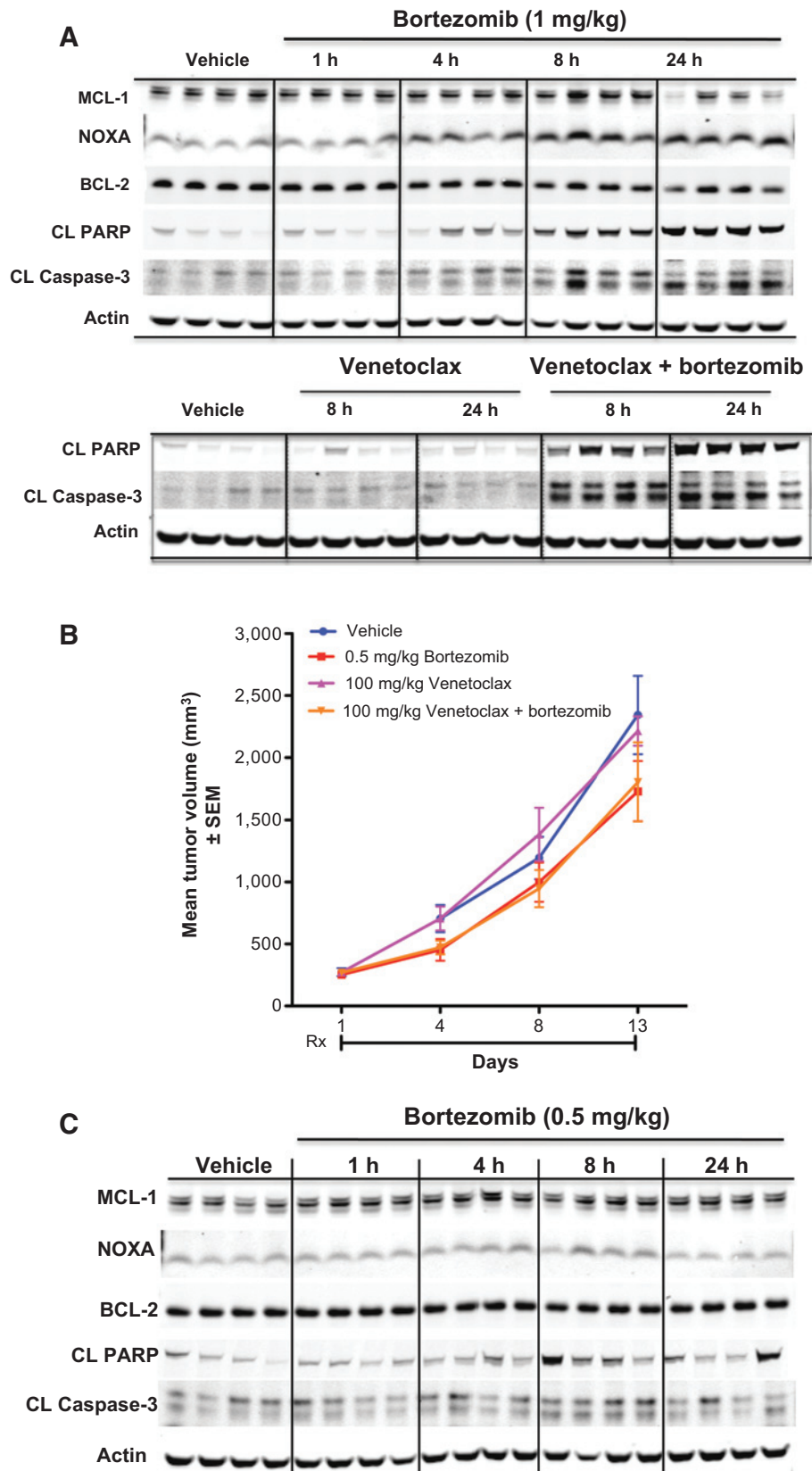


Figure 5. Bortezomib induces NOXA and MCL-1 degradation in the NCI-H929 xenograft model. A, immunoblot analyses of prosurvival proteins (MCL-1 and BCL-2), proapoptotic proteins (NOXA), and cell death markers [cleaved (CL) caspase-3 and PARP] in NCI-H929 xenografts after a single dose of bortezomib (1 mg/kg), vehicle (saline), venetoclax (100 mg/kg), or the combination of venetoclax plus bortezomib. Bortezomib was administered once intravenously while venetoclax was dosed orally for 13 days as indicated. Tumors ($n = 4$) were harvested at the time points indicated and processed for western blotting as described in Materials and Methods. B, efficacy of venetoclax in combination with a low dose of bortezomib in the NCI-H929 xenograft model. Venetoclax (100 mg/kg) was dosed orally and daily in vehicle (60% phosal 50PG, 30% PEG 400, 10% ethanol) for 13 continuous days while bortezomib (0.5 mg/kg) was dosed on days 1, 4, and 7. Rx, total treatment period. C, immunoblot analyses of prosurvival proteins (MCL-1 and BCL-2), proapoptotic proteins (NOXA), and cell death markers (cleaved caspase-3 and PARP) in NCI-H929 xenografts after a single intravenous dose of bortezomib (0.5 mg/kg), vehicle (saline). Tumors ($n = 4$) were harvested at the time points indicated and processed for western blotting as described in Materials and Methods.

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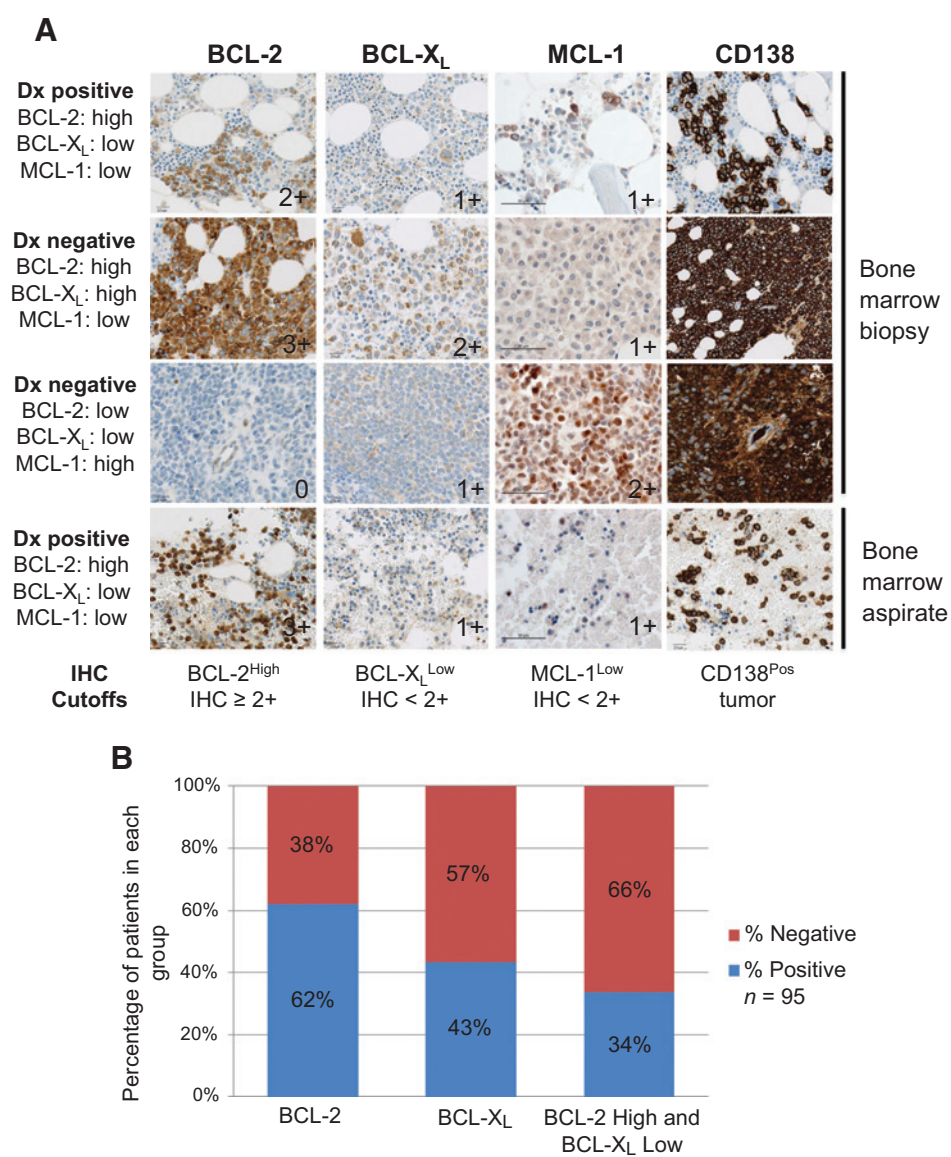


Figure 6. Prevalence of BCL-2, BCL-X_L, and MCL-1 in multiple myeloma patient samples (n = 95). A, representative photomicrographs of BCL-2, BCL-X_L, MCL-1, and CD138 IHC for patient bone marrow biopsy and aspirate samples. Multiple myeloma tumor cells are strongly CD138 positive. Multiple myeloma patient samples defined as diagnostically (Dx) positive and expected to be sensitive to venetoclax treatment were based on a BCL-2 signal intensity of ≥ 2+, with BCL-X_L and MCL-1 signal < 2+ in tumor cells. Diagnostically (Dx)-negative samples either have a BCL-2 signal intensity of ≥ 2+ but with BCL-X_L signal ≥ 2+, or a BCL-2 signal < 2+ in combination with any level of BCL-X_L and MCL-1 signal. IHC scores for BCL-2, BCL-X_L, and MCL-1 were based on staining intensities of positive control samples and defined in Materials and Methods. B, prevalence of BCL-2, BCL-X_L, and BCL-2+BCL-X_L based on percentage of positive and negative IHC staining of all evaluable multiple myeloma patient samples (n = 95).

silencing of MCL-1 did not (32). Similarly, MINO and MAVER-1 mantle cell lymphoma (MCL) cell lines that are cocultured with CD40L-overexpressing fibroblasts are resistant to venetoclax due to upregulation of BCL-X_L (33). Sensitivity of MINO cells to venetoclax when cocultured in the presence of CD40L was restored upon silencing BCL-X_L expression by siRNA (33). Knockdown of BCL-X_L by siRNA in Z138 and JeKo-1 MCL cells in the absence of CD40 stimulation also rescued resistance to venetoclax (33). Bogenberger and colleagues also demonstrated that siRNA silencing of BCL-X_L or MCL-1 restored sensitivity to venetoclax in a panel of AML cell lines in which BCL-X_L and MCL-1 was coexpressed with BCL-2 (34). Thus, emerging data suggest that in the context of BCL-2 expression, upregulation of BCL-X_L and/or MCL-1 can induce resistance to venetoclax in models of hematologic malignancies. Our findings demonstrate that BCL-X_L is a potential resistance factor for venetoclax in HMCLs based on expression levels and sensitivity to selective BCL-X_L inhibitors that induce apoptosis *in vitro* and *in vivo*.

The concept of tumor cells being primed for cell death is based on increased levels of prosurvival proteins bound to their cognate prodeath partners, thus resulting in a lower threshold for sensitivity to intrinsic apoptosis-inducing agents such as BCL-2 inhibitors (26). Indeed, levels of BCL-2:BIM complexes predict sensitivity to ABT-737 in HMCLs (12). Increased BCL-2:BIM complexes also correlated with venetoclax sensitivity in HMCLs with the exception of those lines that coexpressed BCL-X_L or MCL-1. Venetoclax treatment effectively disrupted BCL-2:BIM complexes in these resistant cell lines in a dose-dependent manner indicating that neither ineffective drug binding to BCL-2 nor lack of disruption of BCL-2:BIM complexes were the cause of resistance but likely due to BCL-X_L dependent survival. For example, the BCL-X_L selective inhibitor, A-1155463, effectively disrupted BCL-X_L:BIM complexes and induced apoptosis in the venetoclax-resistant KMS-28PE line, which also had relatively high levels of BCL-2:BIM complexes. Thus, in addition to overall expression levels of BCL-2

proteins, the priming state of HMCLs, based on association of BIM with either BCL-2 or BCL-X_L, may also be a predictive biomarker of response to venetoclax.

BH3 profiling is a functional methodology that can determine cellular fate and BCL-2 family dependencies by exposing mitochondria to known concentrations of BH3 domain peptides and measuring permeabilization of the outer mitochondrial membrane release of cytochrome *c* (35). Using this approach, Touzeau and colleagues recently determined that the dependency of 8 HMCLs on BCL-2, BCL-X_L, and MCL-1 was heterogeneous (36). Notably, one of these HMCLs (MM.1S) was found to be entirely dependent on BCL-X_L, which is consistent with our observations that this line is resistant to venetoclax but sensitive to the BCL-X_L-selective inhibitor, A-1155463.

To date, *in vivo* efficacy of venetoclax in multiple myeloma models has not been reported. We observed modest or marginal efficacy of venetoclax as a single agent in three xenograft models in which BCL-2, BCL-X_L, and MCL-1 were variably expressed. We hypothesized that the lack of single-agent efficacy was due to coexpression of either BCL-X_L and/or MCL-1 and therefore focused on combination regimens with therapeutic agents that may inhibit their activity. One such agent is the proteasome inhibitor, bortezomib, which induces caspase-dependent degradation of MCL-1 via upregulation of NOXA, a BH3-only protein that selectively neutralizes MCL-1 prosurvival activity in HMCLs (14, 28–30). Indeed, bortezomib treatment of NCI-H929 xenografts increased NOXA and decreased MCL-1 protein levels resulting in increased cell death when combined with venetoclax, thereby confirming our initial hypothesis. More importantly, coadministration of venetoclax and navitoclax enhanced the *in vivo* efficacy of bortezomib in OPM-2 and NCI-H929 xenografts. Notably, durable tumor regressions were observed in the NCI-H929 xenografts when venetoclax was combined with bortezomib. The latter suggests that inhibition of BCL-2 by venetoclax and downmodulation of MCL-1 by bortezomib is sufficient to induce synthetic lethality *in vivo*. In contrast in MM.1S xenografts, which expressed higher levels of BCL-X_L protein relative to levels observed in the NCI-H929 and OPM-2 xenograft models, the combination of navitoclax or a BCL-X_L-selective inhibitor (A-1331852) enhanced the efficacy of bortezomib resulting in durable tumor regressions while venetoclax did not. Collectively, our *in vivo* efficacy data demonstrates that venetoclax can enhance the efficacy of bortezomib and provides a mechanistic rationale for combination therapy. However, our data suggest that the combination of venetoclax with bortezomib is most likely to be efficacious in multiple myeloma patients that coexpress BCL-2 and MCL-1 but not BCL-X_L.

Similar to our observations in HMCLs, IHC analysis of 95 multiple myeloma bone marrow or aspirate samples revealed heterogeneous expression of BCL-2, BCL-X_L, and MCL-1. Accordingly, our aim was to correlate tumor sensitivity to venetoclax, initially in cell lines followed by human tumor samples, with the IHC signal intensities observed for BCL-2, BCL-X_L, and MCL-1. Our approach differs from some applications of BCL-2 IHC by focusing on intensity, rather than on the presence or absence of a detectable signal. A critical result of this analysis is that there is a reliably detectable level of BCL-2 IHC signal that is nevertheless below the threshold correlating with venetoclax sensitivity. Our approach shares the technical challenges associated with all IHC endpoints, including controlling vari-

ables in tissue preparation, staining, and interpretation. The IHC signal thresholds we chose, based on *in vitro* HMCL sensitivity to BCL-2 inhibition, were applied to archival myeloma patient samples. The results revealed that 62% of patient samples scored as BCL-2 positive, whereas 43% were BCL-X_L positive. Therefore, a subpopulation comprising about a third (34%) of the patient samples evaluated would be considered diagnostically positive based on an IHC profile of BCL-2^{High}/BCL-X_L^{Low} and may be likely to respond to treatment with venetoclax as a single agent or in combination with bortezomib.

In conclusion, our data indicate that, in addition to MCL-1, BCL-X_L is heterogeneously expressed in HMCLs and patient samples. The expression profile of BCL-X_L relative to BCL-2 and MCL-1 may be an important predictor of response to venetoclax sensitivity as a monotherapy and in combination with bortezomib. To determine the latter, we have developed robust IHC assays for evaluating BCL-2, BCL-X_L, and MCL-1 expression, and cutoffs for evaluating these potential predictive biomarkers in multiple myeloma patient samples. Venetoclax is currently in phase I/II clinical trials both as monotherapy and in combination with bortezomib plus dexamethasone in relapsed multiple myeloma patients (NCI.gov NCT identifier NCT01794520, NCT01794507). Initial results from these clinical trials demonstrate significant anti-myeloma activity of venetoclax including those patients harboring *t*(11;14) translocations (37 and 38). Exploratory analysis in these trials will evaluate if there is enrichment in venetoclax activity in BCL-2^{High}/BCL-X_L^{Low} multiple myeloma patients, using the IHC assays and scoring criteria described in this report.

Disclosure of Potential Conflicts of Interest

J.D. Levenson has ownership interest (including patents) in AbbVie. L. Belmont has ownership interest in Roche stock. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: E. Punnoose, J.D. Levenson, E.R. Boghaert, L. Belmont, W.J. Fairbrother, A.J. Souers, D. Sampath

Development of methodology: E. Punnoose, F. Peale, W. Darbonne, D. Sampath

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Punnoose, F. Peale, L. Belmont, N. Tan, A. Young, M.J. Mitten, E. Ingalla, W. Darbonne, A. Oleksijew, J. Oeh, L. Lee, S. Maiga

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Punnoose, J.D. Levenson, F. Peale, E.R. Boghaert, L. Belmont, N. Tan, W. Darbonne, P. Yue, J. Oeh, L. Lee, A.J. Souers, D. Sampath

Writing, review, and/or revision of the manuscript: E. Punnoose, J.D. Levenson, F. Peale, E.R. Boghaert, L. Belmont, M.J. Mitten, A. Oleksijew, P. Tapang, W.J. Fairbrother, M. Amiot, A.J. Souers, D. Sampath

Study supervision: J.D. Levenson, E.R. Boghaert, J. Oeh, A.J. Souers, D. Sampath

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