Clinical Cancer Research

Targeting of BCL2 Family Proteins with ABT-199 and Homoharringtonine Reveals BCL2- and MCL1-Dependent Subgroups of Diffuse Large B-Cell Lymphoma

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Abstract

Purpose: To investigate the roles of BCL2, MCL1, and BCL-XL in the survival of diffuse large B-cell lymphoma (DLBCL).

Experimental designs: Immunohistochemical analysis of 105 primary DLBCL samples, and Western blot analysis of 18 DLBCL cell lines for the expression of BCL2, MCL1, and BCL-XL. Pharmacologic targeting of BCL2, MCL1, and BCL-XL with ABT-199, homoharringtonine (HHT), and ABT-737. Analysis of DLBCL clones with manipulated expressions of BCL2, MCL1, and BCL-XL. Immunoprecipitation of MCL1 complexes in selected DLBCL cell lines. Experimental therapy aimed at inhibition of BCL2 and MCL1 using ABT-199 and HHT, single agent, or in combination, *in vitro* and *in vivo* on primary cell-based murine xenograft models of DLBCL.

Results: By the pharmacologic targeting of BCL2, MCL1, and BCL-XL, we demonstrated that DLBCL can be divided into BCL2-dependent and MCL1-dependent subgroups with a less pronounced role left for BCL-XL. Derived DLBCL clones with manipulated expressions of BCL2, MCL1, and BCL-XL, as well as the immunoprecipitation experiments, which analyzed MCL1 protein complexes, confirmed these findings at the molecular level. We demonstrated that concurrent inhibition of BCL2 and MCL1 with ABT-199 and HHT induced significant synthetic lethality in most BCL2-expressing DLBCL cell lines. The marked cytotoxic synergy between ABT-199 and HHT was also confirmed *in vivo* using primary cell-based murine xenograft models of DLBCL.

Conclusions: As homoharringtonine is a clinically approved antileukemia drug, and ABT-199 is in advanced phases of diverse clinical trials, our data might have direct implications for novel concepts of early clinical trials in patients with aggressive DLBCL. *Clin Cancer Res; 22(5); 1138–49. ©2015 AACR.*

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma subtype in the Western hemisphere. According to the cell of origin (COO) determined by gene expression profiling (GEP) or several immunohistochemistry (IHC)-based algorithms, DLBCL can be divided into two prognostically different groups: germinal center B-cell–like (GCB) DLBCLs and activated B-cell–like (ABC) DLBCLs, where ABC-DLBCLs are associated with more adverse prognosis (1–3).

Defects in apoptotic signaling represent one of the hallmarks of lymphoid malignancies (4). BCL2 belongs to key regulators of intrinsic apoptosis triggered in response to severe DNA damage or other cellular stresses. Overexpression of BCL2 provides aberrant survival advantage for lymphoma cells and is believed to play one of the key roles in chemoresistance (5–6). Overexpression of BCL2 was repeatedly associated with more adverse outcome to standardly used chemotherapy (7–10). Recently, a phase I dose-escalation study with a highly specific BCL2 inhibitor ABT-199 demonstrated excellent antitumor efficacy in mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL), both of which are known to ubiquitously overexpress BCL2 protein. In contrast, its efficacy in DLBCL was less obvious, as four of eight enrolled patients experienced progression on study treatment (11). Possible therapeutic application of ABT-199 in the treatment of DLBCL thus needs further investigation.

Significance of the other two BCL2 family members, MCL1 and BCL-XL, for the survival of DLBCL remains less clear. Wenzel and colleagues reported higher expression of MCL1 in ABC compared with GCB-DLBCLs (12). The authors suggested that MCL1 might

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Translational Relevance

Defects in apoptotic signaling including overexpression of BCL2 antiapoptotic proteins contribute to increased survival of diffuse large B-cell lymphoma (DLBCL) and might result in selection of resistant clones. Agents specifically blocking the aberrant antiapoptotic signals might result in effective elimination of lymphoma cells. By targeting BCL2 and MCL1 with ABT-199 and homoharringtonine, we demonstrated that DLBCL can be divided into BCL2- and MCL1-dependent subgroups with a less pronounced role for BCL-XL. Derived DLBCL clones with manipulated expressions of BCL2, MCL1, and BCL-XL, and immunoprecipitation experiments confirmed these findings at the molecular level. We demonstrated that concurrent inhibition of BCL2 and MCL1 by ABT-199 and homoharringtonine was highly synergistic *in vitro* and *in vivo* using primary cell-based murine xenograft models. As homoharringtonine is a clinically approved drug and ABT-199 in advanced clinical trials, our data might have direct implications for novel concepts of early clinical trials in patients with DLBCL.

represent a new drug target in this subgroup of DLBCL, which was partially demonstrated in the recent publication by Li and colleagues (13).

Homoharringtonine (HHT, Synribo), originally a plant alkaloid, was recently approved for the therapy of chronic myeloid leukemia (CML) resistant to tyrosine-kinase inhibitors (14). Molecular mechanisms of antitumor activity of HHT appear to be multifactorial, and include downregulation of short-lived proteins, including MCL1 (15). Currently, there is no knowledge on its antitumor activity in lymphomas.

In this study, we investigated the significance of BCL2, MCL1, and BCL-XL for the survival of DLBCL and the outcome of their targeting by various approaches.

Materials and Methods

DLBCL cell lines

Cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% FBS and 1% penicillin/streptomycin. With the exception of HBL-1, TMD8, and OCI-Ly2 (kindly provided by Dr. Francisco Hernandez-Ilizaliturri (Roswell Park Cancer Institute, Buffallo, NY), all the remaining cell lines were purchased from the cell banks (DSMZ, ATCC) and were passaged for fewer than 6 months since their receipt. According to the information provided by the cell banks, the cell lines were authenticated by PCR of short tandem repeat markers confirming unique DNA profiles. HBL-1 and TMD8 cells were confirmed to carry heterozygous missense mutations of *CD79B* gene Y196F and Y196H, respectively. OCI-Ly2 cell line has not been authenticated for lack of relevant molecular markers. COO of HBL1 and OCI-Ly2 murine xenografts was confirmed to be of ABC and GCB immunophenotype, respectively (Hans algorithm, data not shown). UPF4D cell line was derived in our laboratory from a patient with treatment-refractory GCB-DLBCL. Sequencing of IGHV confirmed clonal identity of UPF4D with the primary DLBCL cells, from which it was established (data not shown).

Reagents

ABT-199, ABT-737, and HHT were purchased from Selleck Chemicals and R&D Systems.

Cell proliferation/survival assays and apoptosis measurement

DLBCL cells were plated at a cell density of 5,000 cells/0.3 mL and exposed to ABT-199 or HHT. WST-8-based Quick Cell Proliferation Assay Kit (BioVision) was used according to the manufacturer's instructions. Number of apoptotic cells was determined by flow cytometry (BD FACS Canto II) using Annexin V (BD Biosciences). CompuSyn version 1.0 software (ComboSyn) was used to assess drug synergism between ABT-199 and HHT. Combination index (CI) was calculated for different drug combinations (16).

Real-time RT-PCR

Total RNA was isolated from cell lines in Ribozol (Amresco) using phenol–chloroform extraction and from formalin-fixed paraffin-embedded (FFPE) tissue samples using High Pure RNA Paraffin Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. cDNA synthesis was carried out from 1 μg of total RNA with High-Capacity cDNA Reverse Transcription Kit (random primers; Applied Biosystems). Real-time RT-PCR was performed using TaqMan Gene Expression Assays (*BCL2*, Hs00608023_m1, *MCL1*: Hs01050896_m1, *BCL-XL*: Hs00236329_m1) on ABI 7900HT detection system (Applied Biosystems). The reference gene was *GAPDH* (Hs02758991_g1).

Western blotting

Western blotting (WB) was performed as previously described (17). Antibodies were obtained from Cell Signaling: BAK (3814), BAX (2774), BCL-XL (2764), BID (2002), BIM (2933), PUMA (4976), cleaved PARP (9541), BCL-6 (4242); ENZO LS/Alexis: CASP3 (ALX-804-305), cFLIP (ALX-804-961); Santa Cruz: Actin-HRP (sc-1616), BAD (sc-8044), MCL1 (sc-819, sc-12756), NOXA (sc-56169), Actin (sc-1615), cMYC (sc-40); or BD Pharmingen: BCL2 (610539). Another MCL1 antibody used for confirmation of WB data was from BioVision (3035-100).

Immunoprecipitation

Cells were lysed in non-denaturating lysis buffer [1% (w/v) Triton X-100], 50 mmol/L Tris-HCl (pH 7.4), 300 mmol/L NaCl, 5 mmol/L EDTA, 0.02% (w/v) sodium azide supplemented with protease inhibitor cocktail, 2 μmol/L DTT, and 1 mmol/L PMSF for 20 minutes and centrifuged (16,000 × *g*, 4°C, 15 minutes). Protein concentrations of cell extracts were determined by Pierce BCA Protein Assay Kit and equal amount of protein samples were precleared with protein A-Sepharose bead slurry (Sigma-Aldrich) for 3 × 30 minutes at 4°C, split in half, and incubated with a specific antibody (MCL1 (Santa Cruz, sc-819), BIM (Cell Signaling, 2933), or an isotype control (rabbit polyclonal IgG, Calbiochem) bound to protein A-Sepharose beads for 1 hour at 4°C. Immunoprecipitates were centrifuged (16,000 × *g*, 4°C, 5 seconds), washed three times in wash buffer [0.1% Triton X-100, 50 mmol/L Tris-HCl (pH 7.4), 300 mmol/L NaCl, 5 mmol/L EDTA, 0.02% sodium azide), resuspended in sample buffer, denaturated for 5 minutes at 95°C, resolved on a 10%, 12%, or 15% SDS-PAGE and analyzed by immunoblotting.

Experimental therapy of DLBCL xenografts

In vivo studies were approved by the institutional Animal Care and Use Committee. Immunodeficient NOD.Cg-*Prkdc*^{scid}

Il2rg^{tm1Wjl}/SzJ mice (Jackson Laboratory) were maintained in individually ventilated cages. Primary DLBCL-based mouse models designated KTC (treatment refractory DLBCL) and NOVA-313 (DLBCL transformed from CLL) were established in the same way as previously described (18). KTC or NOVA-313 cells were chosen for *in vivo* experiments, because they coexpressed BCL2 and MCL1 (Fig. 5C). The cells were injected intravenously (10×10^6/mouse) into the tail vein of 8- to 12-week-old female mice on day 1. Therapy with once-weekly intraperitoneal administrations of ABT-199 (500 μg/dose), HHT (50 μg/dose), and/or PBS was initiated on day 5. Each treatment group comprised six animals. Animals were euthanized after they developed hind-leg paralysis or generalized inability to thrive (slow movement, tremor, tachypnea, progressive wasting, and other symptoms of advanced disease). Differences in survival between treatment groups were evaluated using Kaplan–Meier survival estimates with Graph-Pad Prism software.

Immunohistochemistry

Sections from FFPE blocks from patients and from murine DLBCL xenografts were cut and stained by hematoxylin and eosin and Giemsa stains. Immunohistochemistry was performed using BCL2 (clone 124; Dako), BCL-XL (clone B4H6; Cell Signaling), MCL1 (clone S-19; Santa Cruz), and in case of primary DLBCL samples also with following antibodies CD20 (clone L26; Dako), CD5 (clone 4C7; Biogenex), CD10 (clone 56C6; Novocastra), CD23 (clone SP23; Labvision), BCL6 (clone PG-B6p; Dako), MUM1 (clone 1p; Dako), Ki-67 (clone MIB-1; Dako). Heat-induced pretreatment of deparaffinized tissue sections in buffer pH 9.0 (Dako, S2367) was applied. After blocking of endogenous peroxidase activity (3% solution of hydrogen peroxide), samples were incubated overnight at 4°C with primary antibodies anti-MCL1 (1:200) and anti-BCL-XL (1:200). Detection of primary antibody binding was performed for anti-MCL1 with polymer system (secondary antibody and peroxidase; N-Histofine Simple Stain MAX PO; Nichirei Biosciences) and for anti-BCL-XL with biotinylated secondary antibody and avidine–peroxidase complex (LSAB+, Dako REAL Detection Systems, HRP/DAB+, Rabbit/Mouse; Dako), followed by incubation with solution of hydrogen peroxide and chromogen substrate DAB (3,3′-diaminobenzidine tetrahydrochloride; Dako). Nuclei were counterstained with Harris hematoxylin. After dehydration and clearing in xylene, slides were mounted in organic-solvent-based medium and evaluated under light microscope. To differentiate BCL2, BCL-XL, and MCL1-positive and -negative DLBCL cases, we applied the commonly used cutoff value of 30% cells (12). In case of positive samples, the staining intensity was scored by two expert hematopathologists as 1 (low expression), 2 (intermediate expression), and 3 (high expression; Supplementary Fig. S1).

Establishment of DLBCL clones with knockdown or transgenic overexpression of BCL2, MCL1, or BCL-XL

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

Statistical analysis

The strength of linear relationship between the expression levels of BCL2, BCL-XL, and MCL1 proteins (evaluated by densitometry of Western blots) and the sensitivity to ABT-199 and HHT (level of apoptosis after 24 hours) was evaluated by Pearson's correlation coefficients (rP). Because our data do not follow the bivariate normal distribution and scatter plot analysis indicated that some of the analyzed relationships are nonlinear (but monotone), Spearman's rank correlation coefficients (rS) were used to assess the statistical significance of the relationships (i.e., to test null hypotheses about no relationship). We also report 95% bootstrap confidence intervals for the Spearman's rank correlation coefficient (1,000 bootstrap replications being employed). Fisher exact test was conducted to analyze the relationship between two categorical variables in a 2×2 contingency table. The significance level of 5% was selected.

Results

DLBCL cell lines and primary samples show similar patterns of expression of BCL2, MCL1, and BCL-XL

Initially, we aimed to determine whether established DLBCL cell lines represent relevant models for the study of BCL2 family proteins. Implementing semiquantitative approaches (western blot and IHC), we analyzed the expression patterns of BCL2, MCL1, and BCL-XL in 18 established DLBCL cell lines (GCB = 12, ABC = 6) and 105 primary DLBCL samples obtained from patients at diagnosis (GCB = 48, ABC = 57). BCL-XL protein was abundantly expressed in all cell lines (Fig. 1A). BCL-XL protein expression in primary samples was also high with 99 of 105 samples (=94.3%) showing intermediate to high expression, with only six samples showing low expression (=5.7%). There were no primary samples negative for BCL-XL in our cohort. Similar to BCL-XL, MCL1 protein was also expressed in all cell lines, however, in two of them (NU-DHL-1 and HBL-1, = 11.1%) its expression was very low (Fig. 1A). Expression of MCL1 in primary DLBCL samples was variable. Although no MCL1 expression was detected in 13 of 105 samples (=12.4%), low, intermediate, and high MCL1 expression was detected in 36 (=34.3%), 32 (=30.4%), and 24 (=22.9%) samples, respectively (Fig. 1B). BCL2 protein was not expressed in 6 of 18 (=33.3%) cell lines, and very low expression was observed in NU-DUL-1 cell line (Fig. 1A). In the remaining 11 cell lines, BCL2 protein was well expressed. BCL2 protein was negative in 21 of 105 (=20%) primary samples, whereas low, intermediate, and high BCL2 protein was expressed in 15 (=14.3%), 24 (=22.8%), and 45 (42.9%) primary samples (Fig. 1B). In the case of BCL2, Fisher exact test was performed (cell lines and primary samples vs. positive and negative BCL2 expressions), resulting in $P = 0.209$ and thus not rejecting the null hypothesis about no differences in proportions at 5% significance level. The data thus confirmed that DLBCL cell lines do represent relevant models for the investigation of the roles of BCL2, MCL1, and BCL-XL in the survival of DLBCL. Interestingly, real-time qRT-PCR analyses documented that all DLBCL cell lines and primary samples (including those with undetectable BCL2 or MCL1 protein expression) expressed

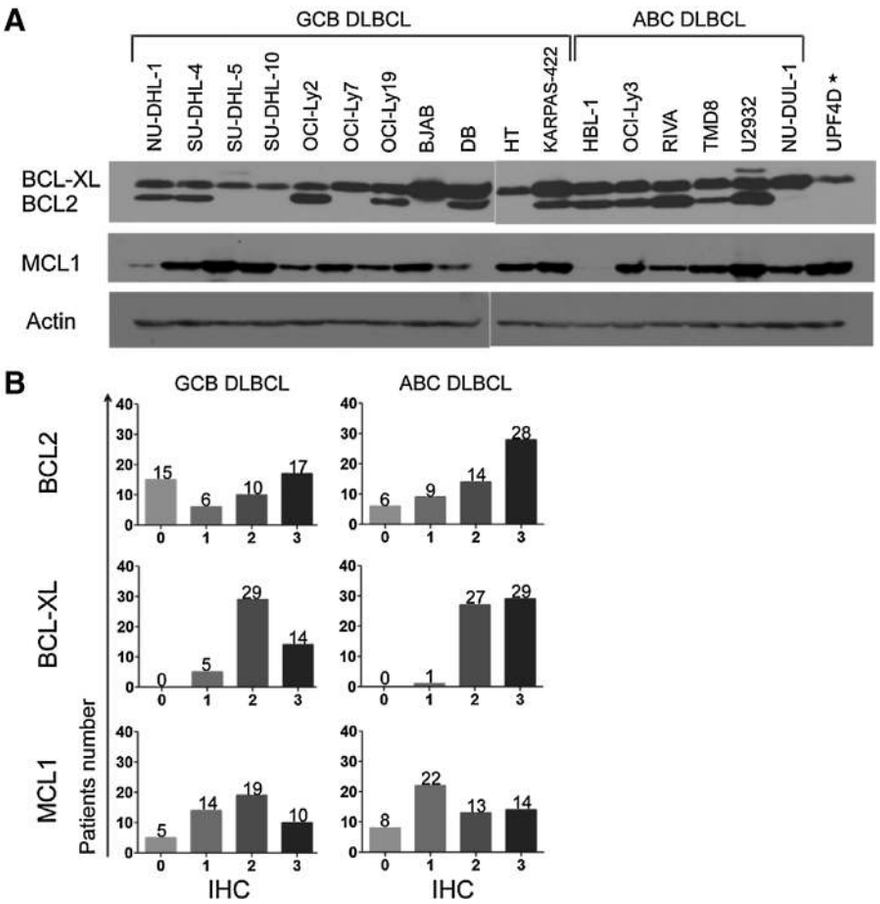

Figure 1.
The expression profiles of BCL2 family proteins in DLBCL cell lines (A) and primary DLBCL samples (B). A, Western blot analysis of BCL-XL, BCL2, and MCL1 proteins in 18 DLBCL cell lines [12 germinal center B-cell-like (GCB) and 6 activated B-cell-like (ABC) DLBCL cell lines]. *, UPF4D cell line is of GCB origin and was derived in our laboratory. B, immunohistochemical analysis of fresh frozen and formalin-fixed paraffin-embedded DLBCL samples obtained from 105 patients (48 GCB and 57 ABC-DLBCL) at diagnosis showing level of expression of BCL2, BCL-XL, and MCL1 protein. The extent of protein expression was evaluated semiquantitatively showing either no expression (0) or various intensity of staining (1–3; see Materials and Methods).

BCL2 or *MCL1* mRNA suggesting that posttranscriptional regulation of BCL2 and MCL1 expressions might in DLBCL represent another important regulatory node (data not shown). The protein expression profiles of additional selected regulators of apoptosis in the 18 cell lines are shown in Supplementary Fig. S2.

BCL2-negative DLBCL cell lines are resistant to ABT-199

Significant differences in BCL2 expression in the analyzed DLBCL cell lines prompted us to examine whether they are also reflected in the sensitivity of these cells to ABT-199, a potent BCL2 inhibitor. Using WST-8 cell proliferation/survival assay we indeed confirmed that all sis BCL2-negative cell lines (SU-DHL-5, SU-DHL-10, OCI-Ly7, BJAB, HT, and UPF4D), and interestingly also 4 of 12 (=33.3%) BCL2-positive cell lines (SU-DHL-4, DB, Karpas-422, and NU-DUL-1) were resistant to ABT-199 (i.e., survived and proliferated in the medium supplemented with 1 μmol/L ABT-199 considered a clinically relevant plasma concentration; ref. 19). The remaining eight BCL2-positive cell lines (NU-DHL-1, OCI-Ly2, OCI-Ly19, HBL-1, OCI-Ly3, RIVA, TMD8, and U-2932) were sensitive to ABT-199 (Supplementary Fig. S3).

In addition to proliferation assays, we also analyzed ABT-199-triggered activation of apoptosis in all 18 cell lines. Moreover, we also analyzed ABT-737-triggered activation of apoptosis, as ABT-737 is an inhibitor of BCL-XL, BCL2, and BCL-W (Supplementary Fig. S4). The almost identical sensitivity of DLBCL cell lines to ABT-199 compared with ABT-737 suggested, in correlation with previously published data (20), that inhibition of BCL2, and not BCL-XL, was the principal molecular mechanism of cell death in the sensitive DLBCL cell lines.

We demonstrated statistically significant positive correlation between the level of expression of BCL2 and sensitivity to ABT-199 (sensitivity = the extent of apoptosis after a 24-hour incubation with ABT-199 1 μmol/L, $r_P = 0.667$; $r_S = 0.716$; $P = 0.001$; 95% CI from 0.305 to 0.963), irrespective of the expression levels of BCL-XL or MCL1 proteins. Our results also suggest a weak negative correlation between the expression levels of MCL1 and the sensitivity to ABT-199 ($r_P = -0.11$; $r_S = -0.284$; $P = 0.254$; 95% CI from −0.699 to 0.261). Nonrejection of the null hypothesis at 5% significance level is presumably caused by the low power of the test (small sample size) rather than the absence of an association between the analyzed variables.

MCL1-targeting agent HHT is antiproliferative and proapoptotic in most DLBCL cell lines

In their recent communication, Wenzel and colleagues showed that MCL1 protein is deregulated in a subset of DLBCLs, might contribute to therapy resistance, and thus could represent a relevant druggable target in DLBCL (12). To our best knowledge, there is currently no specific MCL1 inhibitor in clinical practice or advanced clinical development. Among currently approved antitumor agents, HHT was persuasively shown to trigger efficient

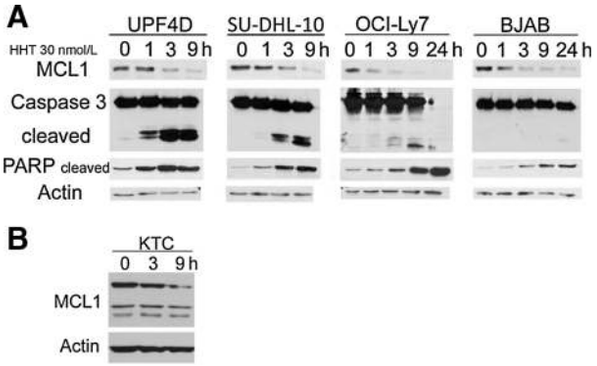

Figure 2.
HHT downregulates MCL1 protein and induces apoptosis in DLBCL cell lines (A) and primary cells (B). A, Western blot analysis of MCL1 protein and selected markers of apoptosis [cleaved caspase-3, cleaved poly(ADP ribose) polymerase (PARP)] in 4 selected DLBCL cell lines after exposure (0, 1, 3, 9, and 24 hours) to HHT (30 nmol/L). B, Western blot analysis of KTC primary cells isolated from murine spleen 3 and 9 hours following i.p. administration of a single dose of 50 mg HHT.

downregulation of MCL1 protein (15). We confirmed the decreased expression of MCL1 protein after exposure to 30 nmol/L HHT (=a steady-state plasma level in CML patients treated with Synribo) in four HHT-sensitive DLBCL cell lines (Fig. 2A; ref. 21). In these cells, HHT also triggered proapoptotic signaling including processing of CASP3 and its target PARP. In addition to *in vitro* experiments, the downregulation of MCL1 as a result of treatment with HHT was also confirmed *in vivo* using a primary cell-based mouse xenograft model of DLBCL (KTC model; Fig. 2B). Besides downregulation of MCL1, we identified other molecular changes associated with exposure of DLBCL cell lines to HHT including downregulation of MYC, BCL6, or cFLIP (Supplementary Fig. S5). These changes, however, were not detected in all analyzed cell lines (unlike downregulation of MCL1). Nevertheless they might contribute to the cytotoxicity of HHT toward DLBCL cells at least in some cell lines. Similarly as for ABT-199 we also determined long-term survival of HHT-treated DLBCL cells. As HHT-resistant were taken those cells that survived/proliferated in the medium supplemented with 30 nmol/L HHT (Supplementary Fig. S3). With a borderline exception of DB, all other DLBCL cells were HHT sensitive. These HHT-prone cells also demonstrated increased apoptosis as documented in the Supplementary Fig. S4. Cell cultures containing >50% apoptotic cells following their 24-hour exposure to 30 nmol/L HHT were defined as HHT-high sensitive, whereas those with less than 50% apoptotic cells as HHT-low sensitive.

We demonstrated statistically significant negative correlation between the expression levels of BCL2 protein and the sensitivity to HHT (sensitivity = the extent of apoptosis after 24 hours of incubation with HHT 30 nmol/L; $r_P = -0.562$; $r_S = -0.674$; $P = 0.003$; 95% CI from −0.862 to -0.311). A weak negative correlation with the sensitivity to HHT was also found for BCL-XL ($r_P = -0.365$; $r_S = -0.35$; $P = 0.168$; 95% CI from −0.763 to 0.223). Nonrejection of the null hypothesis at 5% significance level may be caused by the low power of the test (small sample size) rather than the absence of an association between the analyzed variables. The expression levels of MCL1 protein did not correlate with the sensitivity to HHT.

Transgenic overexpression or targeted knockdown of BCL2 proteins impacts viability and sensitivity of DLBCL cells to ABT-199 and/or HHT

The results obtained from the treatment of the cell lines with ABT-199 and HHT suggested that DLBCL can be divided into different BCL2- and/or MCL1-dependent categories (Fig. 6 and Supplementary Table S2). To further investigate the significance of BCL2, MCL1, and BCL-XL for the survival of DLBCL, we established DLBCL clones with targeted downregulation or transgenic overexpression of these proteins and corresponding control cells [expressing either nonsilencing (NS) shRNAs or an empty pCDH vector; Fig. 3A, D, G, J, M, and P]. In all apoptosis assays, the clones with manipulated expression of BCL2 proteins were compared with the corresponding controls.

BCL2

Knockdown of BCL2 expression in OCI-Ly2 cells that are highly sensitive to ABT-199 was lethally toxic (repeated attempts, data not shown). U2932^{shBCL2} clone derived from ABT-199–sensitive cell line demonstrated increased sensitivity to ABT-199 compared with U2932^{shNS}. Response of DB^{shBCL2} clone derived from ABT-199 resistant to ABT-199 was not changed. Both these clones, however, became sensitized to HHT (Fig. 3B and C). Resistance of SU-DHL-5$^{BCL2\text{-}cDNA}$ and UPF4D$^{BCL2\text{-}cDNA}$ clones with the increased BCL2 expression to ABT-199 did not change compared with corresponding controls, but they became markedly resistant to HHT (Fig. 3E and F). To elucidate the molecular mechanisms responsible for the acquired resistance of SU-DHL-5$^{BCL2\text{-}cDNA}$ to HHT compared with SU-DHL-5$^{empty\text{-}vector}$ cells, we performed immunoprecipitation of both cell populations with anti-BIM antibody in unexposed cells and in cells exposed to HHT. We detected BCL2 protein bound on BIM in SU-DHL-5$^{BCL2\text{-}cDNA}$ cells. Importantly, higher amounts of BCL2 were bound on BIM after exposure of the cells to HHT compared with HHT-unexposed cells (2.45-fold higher according to densitometry; Fig. 4B).

MCL1

Knockdown of MCL1 expression in the HHT-high–sensitive UPF4D cell line was lethally toxic (repeated attempts, data not shown). Karpas-422^{shMCL1} clone derived from the HHT-high–sensitive cell line had increased sensitivity to HHT, but unchanged sensitivity to ABT-199. U2932^{shMCL1} clone derived from the HHT-low–sensitive cell line demonstrated mildly decreased sensitivity to HHT, and increased sensitivity to ABT-199 (Fig. 3H and I). NU-DHL-1$^{MCL1\text{-}cDNA}$ ectopically expressing MCL1 became more resistant to HHT, but demonstrated no significant change in sensitivity to ABT-199. HBL-1$^{MCL1\text{-}cDNA}$ clone showed no change in susceptibility to HHT or ABT-199 (Fig. 3K and L).

BCL-XL

Knockdown of BCL-XL expression was achieved in all tested cell lines. BJAB$^{shBCL\text{-}XL}$ and DB$^{shBCL\text{-}XL}$ clones demonstrated significantly increased sensitivity to HHT, and mildly increased sensitivity to ABT-199 (Fig. 3N and O). SU-DHL-5$^{BCL\text{-}XL\text{-}cDNA}$ and UPF4D$^{BCL\text{-}XL\text{-}cDNA}$ clones had decreased sensitivity to HHT, and remained resistant to ABT-199 (Fig. 3Q and R). Moreover, both UPF4D and SU-DHL-5 cells with acquired resistance to HHT demonstrated increased BCL-XL expression (Supplementary Fig. S6).

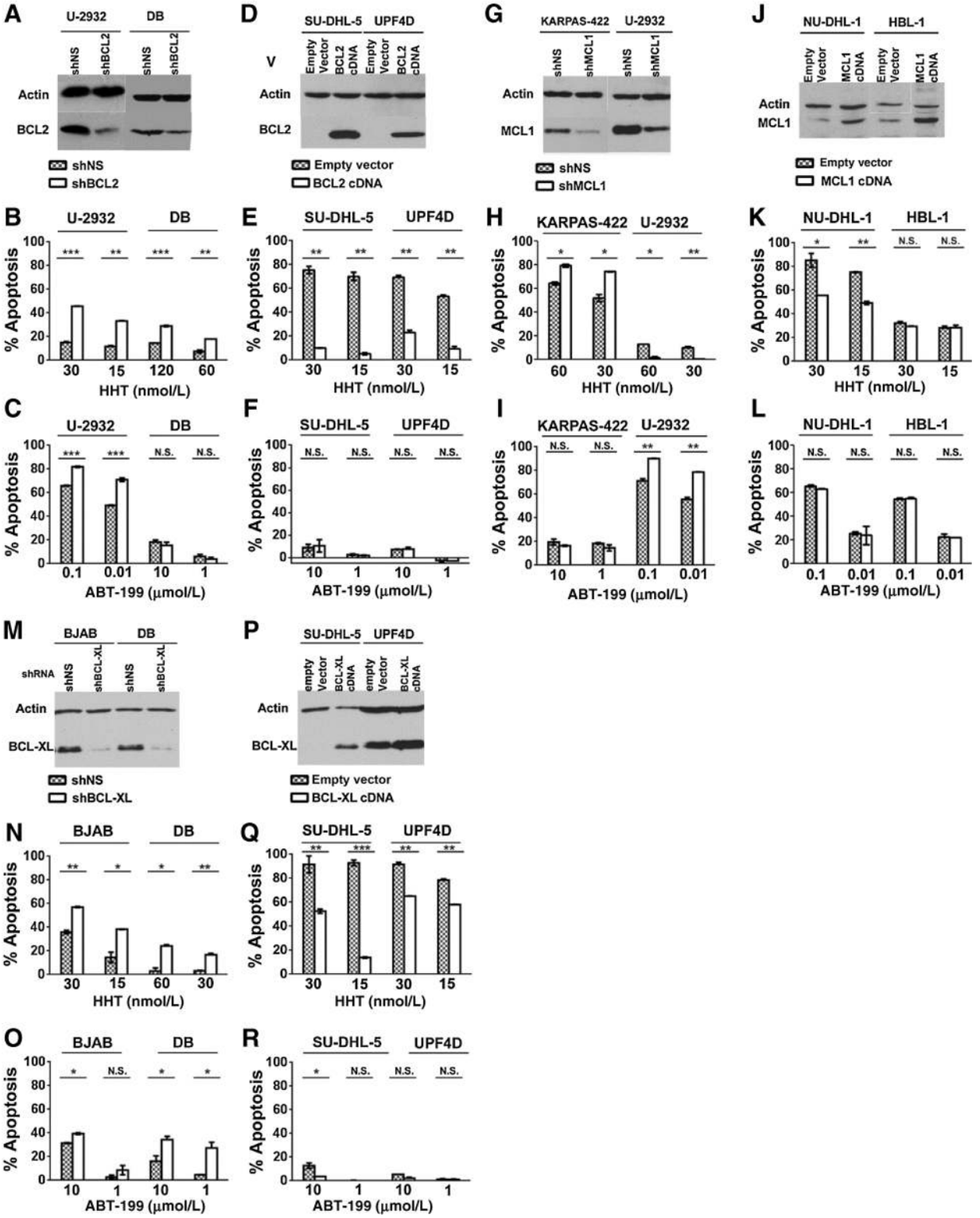

Figure 3.

Transgenic overexpression or targeted knockdown of BCL2 (A–F), MCL1 (G–L), and BCL-XL (M–R) proteins impact sensitivity of DLBCL cells to ABT-199 and HHT. Western blot analysis of BCL2 (A, D), MCL1 (G, J), and BCL-XL (M, P) proteins in DLBCL clones and their corresponding controls. The extent of apoptosis following 24-hour exposure to HHT or ABT-199 is shown. N.S., not significant; *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

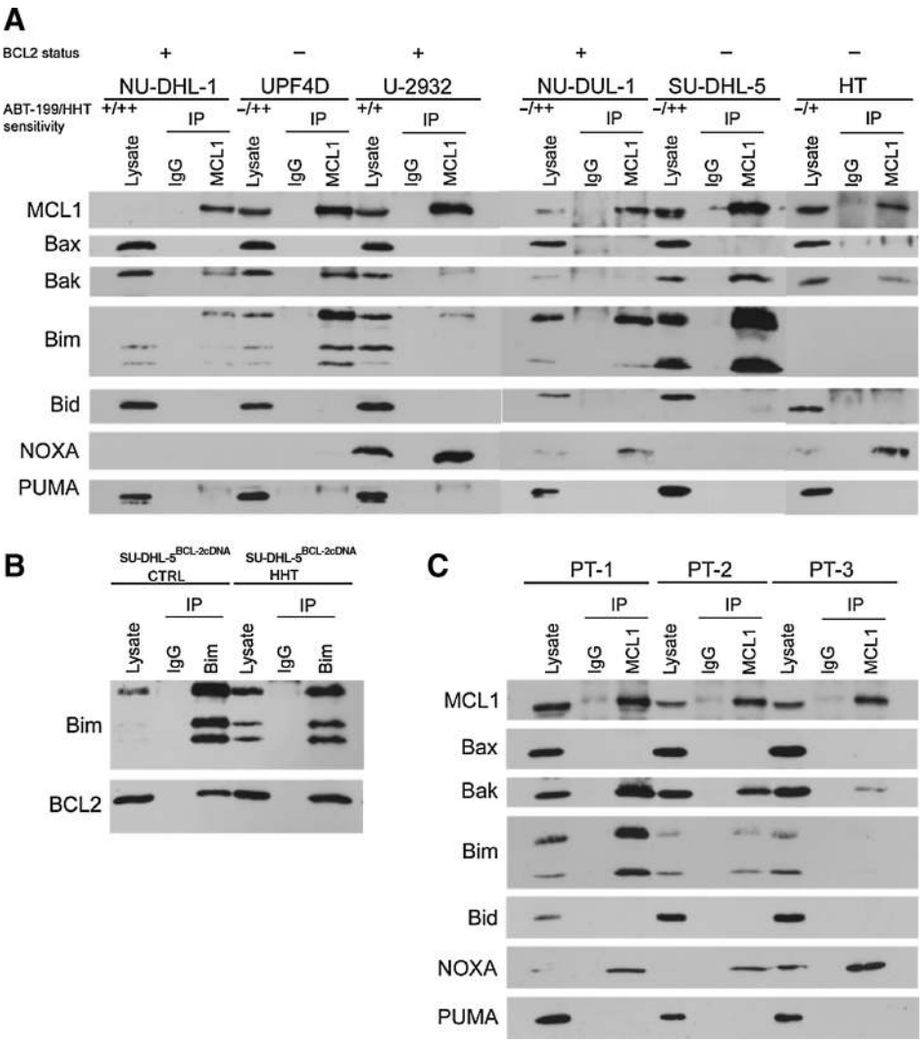

Figure 4.
BH3-only proteins bound to MCL1 determine MCL1-dependence of DLBCL cells. A, immunoprecipitation (IP) of MCL1 protein and protein analysis (by Western blot) of MCL1 binding partners in six selected DLBCL cell lines with different sensitivities to HHT. High amount of BIM bound to MCL1 protein was detected in HHT-high–sensitive cell lines NU-DHL-1, UPF4D, NU-DUL-1, and SU-DHL-5. An interaction of MCL1 protein with NOXA and in much lesser extent with BAK or BIM is seen in 2 HHT-low–sensitive cell lines U2932 and HT. BCL2 status and sensitivity of DLBCL cell lines to ABT-199 (+/−, sensitive/resistant) or HHT (++/+/− high-sensitive/low-sensitive/resistant) are depicted with symbols. B, IP of BIM and protein analysis of BIM binding partners in SU-DHL-5$^{BCL2\text{-}cDNA}$ clone with ectopic expression of BCL2 before and after exposure to HHT demonstrates higher amounts of BCL2 protein bound to BIM after exposure to HHT. C, analysis of MCL1 complexes in primary DLBCL cells obtained from three patients (PT) at diagnosis of DLBCL. Interactions of MCL1 protein with BIM, BAK, or NOXA are shown.

BH3-only proteins bound to MCL1 determine MCL1 dependence of DLBCL cell lines

MCL1 and BCL2 proteins protect cells from apoptosis by sequestering diverse proapoptotic BH3-only proteins and by blocking proapoptotic BAX/BAK proteins (22–23). Thus, the analysis of their binding partners could provide additional information on their cell-specific function. The immunoprecipitation of MCL1 complexes uncovered high amounts of BH3-only protein BIM bound to MCL1 protein in the HHT-high–sensitive cell lines UPF4D, NU-DHL-1, SU-DHL-5, and NU-DUL-1 (Fig. 4A). In concordance with Wenzel and colleagues, we confirmed attenuated interaction of MCL1 with BIM (but increased amount of NOXA) in the HHT-low–sensitive U2932. In the other HHT-low–sensitive, BIM-nonexpressing HT we also co-immunoprecipitated NOXA (Fig. 4A; Supplementary Fig. S2; ref. 12). In contrast to BAX and BID, BAK co-immunoprecipitated with MCL1 in majority of analyzed DLBCL cell lysates (Fig. 4A). Analogical MCL1 complexes were formed in three analyzed primary DLBCL cells—depending on the level of expression they contained BIM, NOXA, and BAK, but not BID, BAX, or PUMA. (Fig. 4C).

HHT synergizes with ABT-199 in eliminating BCL2-positive DLBCL cells

HHT and ABT-199 target different key molecules involved in the survival of DLBCL. We therefore asked, whether combining HHT and ABT-199 in the 18 DLBCL cell would elicit additive or even synergistic cytotoxic effect. We demonstrated cytotoxic synergy between HHT and ABT-199 in most of the tested BCL2-positive cell lines (8 of 12, 66.7%; see Fig. 5A and B), whereas in the remaining 4 of 12 BCL2-positive cell lines (OCI-Ly3, TMD8, RIVA, and Karpas-422) additive cytotoxic effect was observed (data not shown). With the exception of OCI-Ly7 no benefit of combining HHT and ABT-199 was observed in BCL2-negative cell lines (5 of 6, 83.3%, data not shown). Cytotoxic synergy between ABT-199 and HHT was confirmed in selected DLBCL cell lines using the CI (in HBL-1 cell line: $0.0131 < CI < 0.02683$; in SU-DHL-4 cell line: $0.03612 < CI < 0.25985$; ref. 16).

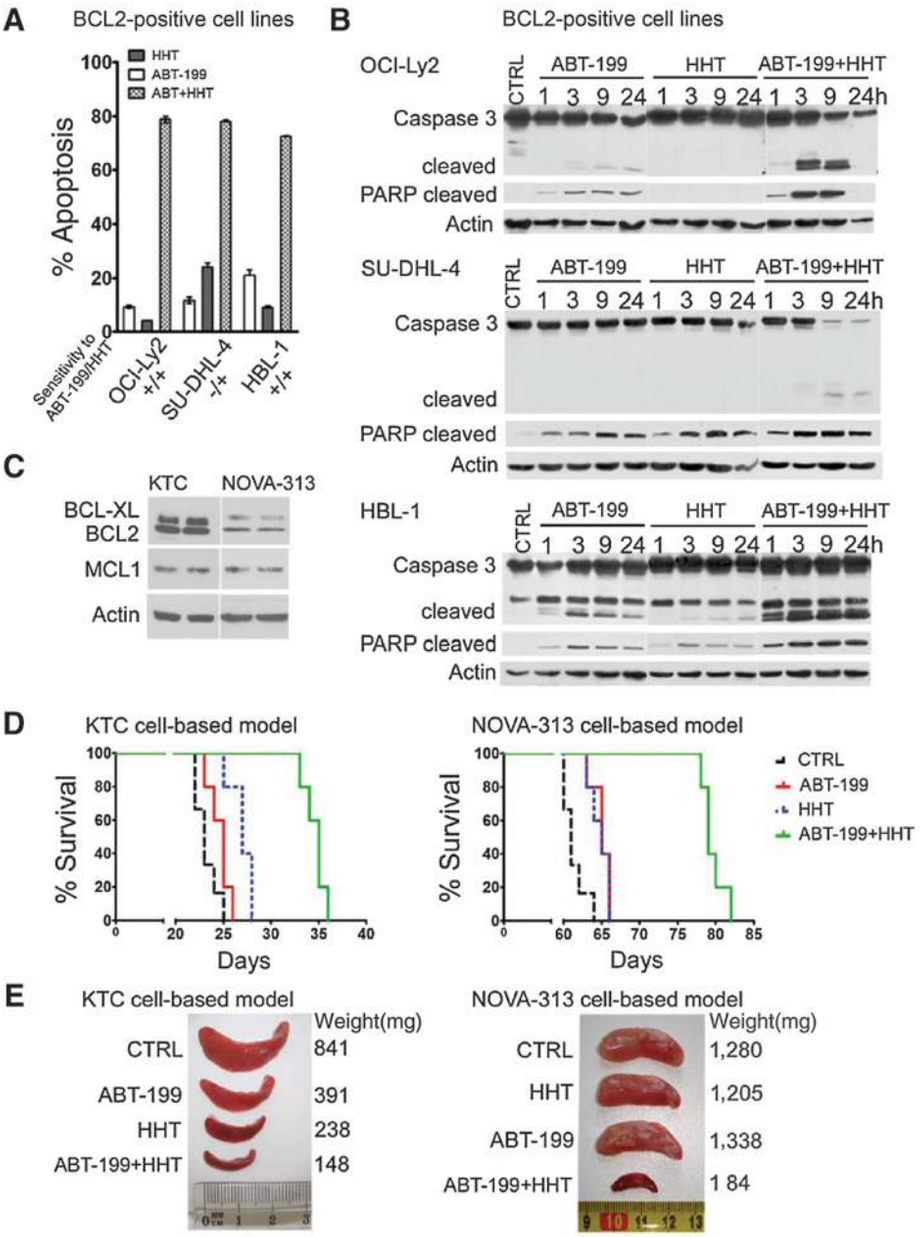

Figure 5.
HHT in combination with ABT-199 is highly synergistic *in vitro* and *in vivo*. A, the extent of apoptosis following 24-hour exposure to ABT-199, HHT, or combination of both agents in OCI-Ly2, SU-DHL-4, and HBL-1 cell lines demonstrates marked synergism of the combination compared with single-agent approaches. Cytotoxic synergy between ABT-199 and HHT was confirmed in selected DLBCL cell lines using the CI (in HBL-1 cell line: 0.0131 < CI < 0.02683; in SU-DHL-4 cell line: 0.03612 < CI < 0.25985). The doses of ABT-199 and HHT were chosen to maximize the synergistic effect of the drug combination and were different for particular cell lines: OCI-Ly2, SU-DHL-4 (ABT-199 0.1 μmol/L, HHT 30 nmol/L), HBL-1 (ABT-199 0.01 μmol/L, HHT 15 nmol/L). Sensitivity of OCI-Ly2, SU-DHL-4, and HBL-1 cell lines to ABT-199 (+/−, sensitive/resistant) or HHT (++/+/− high-sensitive/low-sensitive/resistant) is depicted with symbols. B, Western blot analysis of selected markers of apoptosis in OCI-Ly2, SU-DHL-4, and HBL-1 cells after exposure (1, 3, 9, and 24 hours) to ABT-199, HHT, or combination of both agents. C, Western blot analyses of KTC and NOVA-313 primary DLBCL cells in duplicate demonstrate coexpression of BCL2 and MCL1 in both cell types. D, Kaplan–Meier survival curves demonstrate statistically significant prolongation of survival of the mice treated with the combinatorial therapy using ABT-199 and HHT compared with the corresponding monotherapies or untreated controls. E, spleen weights obtained from one mouse from each treatment cohort (compared to untreated CTRL) at the time of lymphoma-specific demise of the first untreated control confirms the synergistic efficacy of ABT-199 and HHT compared with monotherapies.

HHT in combination with ABT-199 significantly prolongs overall survival of primary DLBCL xenograft-bearing mice compared with single-agent approaches

To confirm the observed anti-DLBCL synergism between HHT and ABT-199 *in vivo*, we used two primary cell-based mouse xenograft models of DLBCL known to coexpress BCL2 and MCL1 proteins (established in our laboratory and designated KTC and NOVA-313; Fig. 5C). It must be emphasized that the primary endpoint of these *in vivo* experiments was not single-agent efficacy of ABT-199 or HHT monotherapies given at maximum tolerated doses (which would had required higher dosing and more dense administrations of either agent), but the confirmation of the *in vitro* shown synergism at relatively low, nontoxic doses (Fig. 5A and B). In the KTC model (derived from a patient with treatment refractory DLBCL), HHT in combination with ABT-199 significantly prolonged overall survival (OS) compared with untreated animals ($P = 0.0012$) or compared with HHT ($P = 0.0026$) or ABT-199 monotherapy ($P = 0.0021$; Fig. 5D). In the NOVA-313 model (derived from a patient with the transformation of CLL into DLBCL), HHT in combination with ABT-199 significantly prolonged OS compared with the untreated controls ($P = 0.0012$) or both single-agent approaches ($P = 0.0026$ and 0.0027 for ABT-199 and HHT, respectively; Fig. 5D). Spleen weights obtained from mice treated with the combination of both agents were

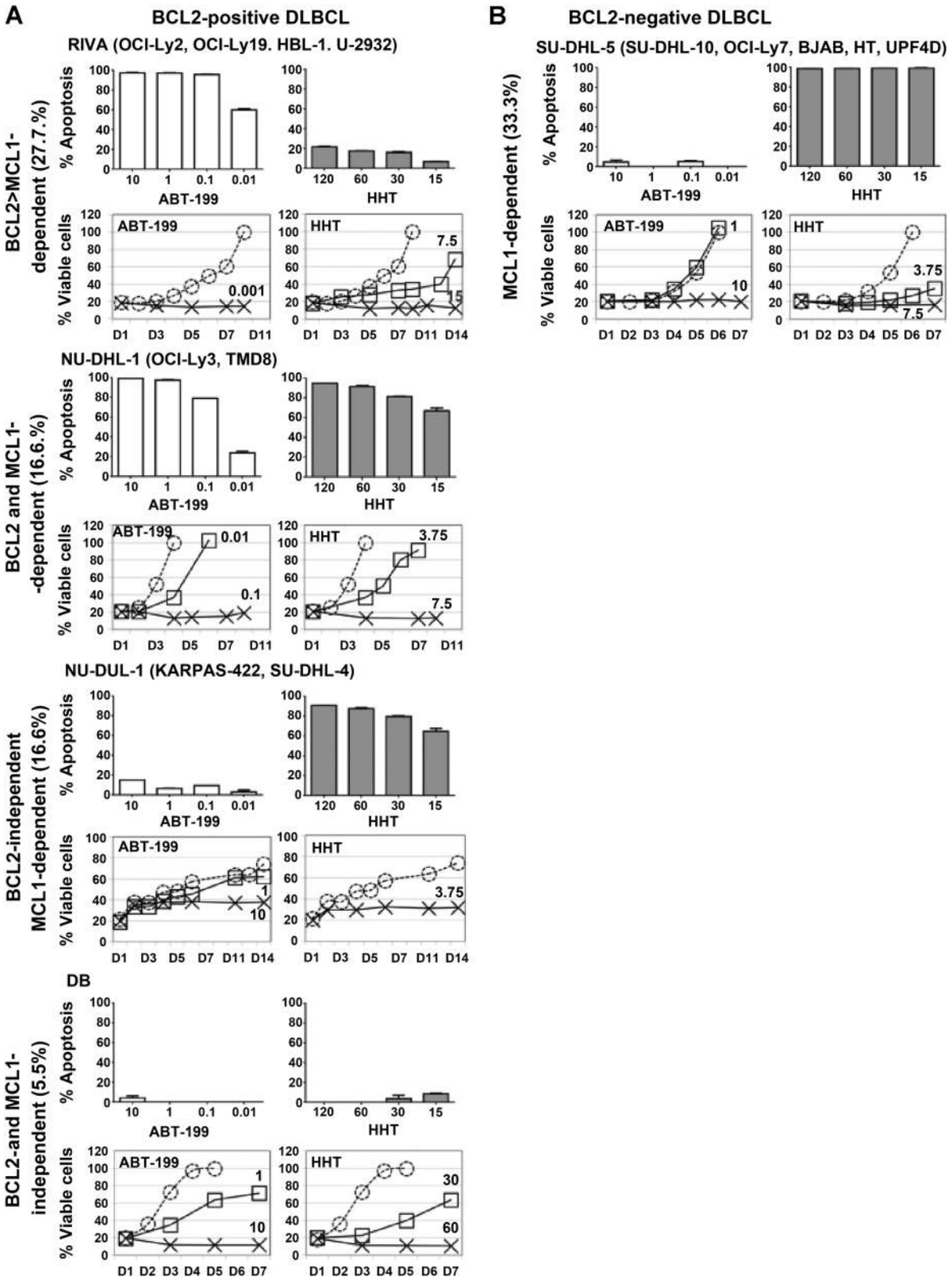

Figure 6.
Suggested division of DLBCL into BCL2-dependent and/or MCL1-dependent subgroups. Based on the sensitivity to ABT-199 and HHT (determined either by the extent of apoptosis following 24-hour exposure to ABT-199 or HHT, or assessed by cell survival/proliferation assay), the 18 tested DLBCL cell lines can be divided into BCL2- and/or MCL1-dependent categories. Although BCL2-positive DLBCL cell lines are BCL2-dependent and/or MCL1-dependent (A), BCL2-negative DLBCL cell lines appear exclusively MCL1 dependent (B). Only 1 DLBCL cell line was resistant to both agents (DB) indicating its independence to inhibition of both BCL2 and MCL1.

significantly smaller compared with spleens obtained from untreated controls or mice treated with single-agent approaches (Fig. 5E).

Discussion

Targeted inhibition of BCL2 with its highly selective inhibitor ABT-199 recently emerged as a promising treatment strategy for some B-cell malignancies such as CLL and MCL, but its possible application in DLBCL seems to be less obvious (24). It must be emphasized that a substantial part of DLBCL cases lack BCL2 by standard IHC analysis (10). Moreover, the expression status of BCL2 in the reported clinical trials with ABT-199 was not specified (11).

We analyzed 18 established DLBCL cell lines as relevant substitutes for primary DLBCL samples to evaluate the role of major antiapoptotic proteins BCL2, MCL1, and BCL-XL in the survival of DLBCL. As previously suggested, we also demonstrated that the lack of BCL2 protein expression was associated with resistance to ABT-199 (ref. 25; Fig. 1; and Supplementary Fig. S3). In translation, the data indicate that ABT-199-based therapy should be avoided in BCL2-negative DLBCL patients. However, ABT-199 might prove effective (or even highly effective) in a subgroup of BCL2-positive DLBCL, the larger part of which appears to depend (at least partially) on BCL2 for survival. The almost identical sensitivity of DLBCL cell lines to ABT-199 compared with ABT-737 (Supplementary Fig. S4) suggested, in agreement with previously published data (20), that inhibition of BCL2, and not BCL-XL, was the principal molecular mechanism of cell death in the sensitive cell lines.

IHC analysis of 105 FFPE primary DLBCL samples and Western blot analysis of 18 DLBCL cell lines demonstrated high ubiquitous expression of BCL-XL protein in DLBCL. Compared with previously published data, our own results demonstrated somewhat higher expression of MCL1 protein in DLBCL cell lines and primary samples (Fig. 1A and B; refs. 12–13). To confirm our data, the protein expression of MCL1 in the cell lines was verified using another anti-MCL1 antibody with the same results (data not shown). We also confirmed that IHC analysis of subcutaneous murine xenografts of selected DLBCL cell lines for MCL1 protein expression corresponded well with Western blot analysis of the cell lysates (Supplementary Table S1). We must, however, emphasize that both Western blot and IHC are semiquantitative methods, and potential differences in the technical aspects and interpretation of the data might play central roles in the observed differences between our results and the work of other groups.

Based on the sensitivity to ABT-199 and HHT, the 18 tested DLBCL cell lines could be divided into three categories (Fig. 6 and Supplementary Table S2). Nine DLBCL cell lines (SU-DHL-4, SU-DHL-5, SU-DHL-10, OCI-LY-7, BJAB, HT, Karpas-422, NU-DUL-1, and UPF4D) were sensitive to HHT, but resistant to ABT-199 suggesting their predominant MCL1 dependence. From the eight DLBCL cell lines sensitive to both agents, five cell lines (RIVA, OCI-Ly2, OCI-Ly19, HBL-1, and U-2932) were significantly more susceptible to ABT-199 compared with HHT, suggesting their predominant BCL2 dependence. The remaining three DLBCL cell lines (NU-DHL-1, OCI-Ly3, and TMD8) appeared markedly sensitive to both agents, suggesting their parallel BCL2 and MCL1 dependence. Interestingly, only a single DLBCL cell line (DB) was resistant to both agents.

Results from the DLBCL clones with manipulated expression of BCL2 family members confirmed the suggested division of DLBCL into predominantly BCL2-dependent and/or MCL1-dependent subgroups with a minor role left for BCL-XL. Downregulation and upregulation of BCL-XL increased and decreased sensitivity to HHT, respectively (Fig. 3), suggesting that BCL-XL could function as general or background anti-apoptotic cushion in DLBCL cells. In addition, acquired resistance of DLBCL cells to HHT was associated with upregulation of BCL-XL protein (Supplementary Fig. S6). We could deduce from these results that acquired overexpression of BCL-XL might suppress MCL1 dependence of DLBCL cells.

Overexpression of BCL2 in BCL2-negative DLBCL cell lines (UPF4D, SU-DHL-5) did not affect resistance of the derived clones to ABT-199, which is in concordance with the previous communication by Deng and colleagues for ABT-737 (20). However, the upregulated BCL2 protein significantly decreased apoptosis induced by HHT (Fig. 3). We proved that transgenic expression of BCL2 can serve as a buffer for apoptosis-triggering protein BIM after it is released from MCL1 as a result of MCL1 downregulation upon exposure of the cells to HHT (Fig. 4B). In addition, overexpression of BCL2 might also buffer the additional proapoptotic activities induced by HHT (specified in Supplementary Fig. S5). The data thus at least partially explain molecular basis of the observed trend to increased sensitivity of BCL2-negative DLBCL cell lines to HHT compared with BCL2-positive cell lines ($61.9\pm28.7\%$ vs. $38.2\pm32.3\%$ average level of apoptosis after 24 hours of incubation with 30 nmol/L HHT, $P = 0.07$).

Although BCL2 dependence of DLBCL can conclusively be determined by the highly-specific BCL2-inhibitor ABT-199, MCL1-dependence is more difficult to assess due to the lack of MCL1-specific inhibitors. Based on the data obtained from the clones with manipulated MCL1 expression derived from the HHT-high–sensitive cell line Karpas-422 and the HHT-low–sensitive cell line U2932, it appears that only HHT-high–sensitive cell lines are predominantly ("truly") MCL1 dependent (Fig. 3), whereas cell death observed in HHT-low–sensitive cell lines might be a result of additional antitumor activities of HHT (Supplementary Fig. S5). This hypothesis was further confirmed by immunoprecipitation experiments, which demonstrated that only in HHT-high–sensitive DLBCL cell lines (UPF4D, SU-DHL-5, NU-DHL-1, NU-DUL-1) MCL1 protein sequesters apoptosis-activator BIM. In contrast, MCL1 complexes isolated from the HHT-low–sensitive cell lines U-2932 and HT bound predominantly high amounts of the apoptosis-sensitizer NOXA (Fig. 4A). It has been previously shown that only cells harboring antiapoptotic BCL2 proteins (such as MCL1) that are occupied by apoptosis-activator BH3-only proteins were "primed for death" and specifically depend on the antiapoptotic BCL2 proteins for survival (22).

Our data thus indicate that DLBCL can be divided into BCL2-dependent and/or MCL1-dependent subgroups (Fig. 6). Although the BCL2-positive subgroup comprises both BCL2- and MCL1-dependent cells that might be targeted by ABT-199, HHT, or the combination of both agents (with a marked synergistic efficacy), the BCL2-negative DLBCL subgroup appears to be predominantly (if not exclusively) MCL1 dependent. As previously proposed by others, our data thus confirmed that MCL1 indeed is a promising druggable target in DLBCL. In our study we used HHT to target MCL1. In their recent publication in Leukemia, Li and colleagues used other anticancer agents to downregulate MCL1 including cyclin-dependent kinase inhibitor dinaciclib and standardly used cytostatics (doxorubicin, etoposide, cytarabine; ref. 13). However,

all these agents (like HHT used in our study) have broad spectra of anticancer activities that go far beyond downregulation of MCL1. Nevertheless our data suggest that HHT works specifically through inhibition of MCL1 at least in the HHT-high–sensitive DLBCL cells.

In conclusion, ABT-199 might still prove effective anti-DLBCL agent, but only in the subgroup of BCL2-positive DLBCL. However, HHT appears to be a promising antilymphoma agent for DLBCL irrespective of BCL2 expression status, despite the fact that it after all appears more effective in the BCL2-negative DLBCL. Most importantly, the concurrent inhibition of BCL2 and MCL1 (in our hands by ABT-199 and HHT) induces significant synthetic lethality in most BCL2-positive DLBCL cell lines (Fig. 5A and B), and might represent a new treatment strategy for this subgroup of DLBCL as confirmed also *in vivo* on murine xenograft models based on xenotransplantation of primary DLBCL cells coexpressing BCL2 and MCL1 (Fig. 5C–E). As HHT is a clinically approved antitumor drug and ABT-199 is in advanced phases of clinical testing in diverse malignancies, our data might have direct implications for novel concepts of experimental therapy of DLBCL targeted at BCL2 and/or MCL1 using HHT and ABT-199, single-agent, or in combination.

Disclosure of Potential Conflicts of Interest

L. Andera is an employee of Apronex Ltd. and Institute of Biotechnology AS CR. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: L. Andera, D. Prukova, M. Trneny, P. Klener

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