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.

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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...
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 apoptotic 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 cells were con

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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 (CompuSyn) 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 LifeSciences: CASP3 (ALX-804-305), eFluor (ALX-804-961); Santa Cruz: Actin- HRP (sc-1616), RAD (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-denaturing 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 precluded 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-Pkdcrad-1

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1139
Mouse; Dako), followed by incubation with solution of hydrogen

PBS and stored in

manufacturer's instructions. Precipitated particles were resuspended in

precipitated using PEG-it (System Biosciences) according to man-

tioned medium was harvested 36 hours later, centrifuged, and

the gene of interest were transfected into HEK 293T/17. Condi-

with pLKO.1 (Sigma Aldrich)/pCDH-neo (SBI) vector containing

plasmid 12259), psPAX2 (Addgene, plasmid 12260) together

overexpression of BCL2, MCL1, or BCL-XL

samples, the staining intensity was scored by two expert hema-

monly used cutoff value of 30% cells (12). In case of positive

MCL1-positive and -negative DLBCL cases, we applied the com-

under light microscope. To differentiate BCL2, BCL-XL, and

ABT-199 (500 μg/dose), HHT (50 μg/dose), and/or PBS was

for

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Statistical analysis

The strength of linear relationship between the expression levels of BCL2, BCL-XL, and MCL1 proteins (evaluated by densi-

metry of Western blots) and the sensitivity to ABT-199 and

HHT (level of apoptosis after 24 hours) was evaluated by Pear-

son’s correlation coefficients (rP). Because our data do not follow

the bivariate normal distribution and scatter plot analysis indi-

cated 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 rela-

tionship between two categorical variables in a 2 x 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 (GCR = 12,

ABC = 6) and 105 primary DLBCL samples obtained from pa-

tients 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, samples

were expressed very low (Fig. 1A). Expression of MCL1 in primary

DLBCL samples was variable. Although no MCL1 expres-

sion was detected in 13 of 105 samples (12.4%), low, interme-

tiate, 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 investiga-

tion 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
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 six BCL2-negative cell lines (SU-DHL-5, 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 MCL1 proteins. The almost identical sensitivity of DLBCL cell lines to ABT-199 compared with ABT-737 suggested, in correlation with previous 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, rS = 0.667; rP = 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 (rP = −0.11; rS = −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 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.

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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). U2932shBCL2 clone derived from ABT-199–sensitive cell line demonstrated increased sensitivity to ABT-199 compared with U2932shNS. Response of DBshBCL2 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-5BCL2-cDNA and UFP4DHL-5-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-5BCL2-cDNA to HHT compared with SU-DHL-5empty-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-5BCL2-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 UFP4D cell line was lethally toxic (repeated attempts, data not shown). U2932shMCL1 clone derived from the HHT-sensitive cell line had increased sensitivity to HHT, but unchanged sensitivity to ABT-199. U2932shNS and DBABT199–sensitive cell line demonstrated increased sensitivity to HHT, and increased sensitivity to ABT-199 (Fig. 3H and I). NU-DHL-1MCL1-cDNA ectopically expressing MCL1 became more resistant to HHT, but demonstrated no significant change in sensitivity to ABT-199. HBL-1MCL1-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. BIABshBCL-XL and DBshBCL-XL clones demonstrated significantly increased sensitivity to HHT, and mildly increased sensitivity to ABT-199 (Fig. 3N and O). SU-DHL-5BCL-XL-cDNA and UFP4DHL-5BCL-XL-cDNA clones had decreased sensitivity to HHT, and remained resistant to ABT-199 (Fig. 3Q and R). Moreover, both UFP4D 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.
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 NU-DHL-1, UPF4D, NU-DUL-1, and SU-DHL-5 (Fig. 4A). In concordance with Wenzel and colleagues, we confirmed attenuated 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-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.

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).
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 have required higher dosing and more dense administrations of either agent), but the confirmation of the in vitro shown synergy 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.
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, NIM-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 (NIM-DUL-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. 4). 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.±28.7% vs. 38.2±32.3% 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 UI2932, 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, NIM-DUL-1, NIM-DUL-1) MCL1 protein sequesters apoptosis-activator BIM. In contrast, MCL1 complexes isolated from the HHT-low–sensitive cell lines UI-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 antitumor 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 antitumor 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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Correction: Targeting of BCL2 Family Proteins with ABT-199 and Homoharringtonine Reveals BCL2- and MCL1-Dependent Subgroups of Diffuse Large B-Cell Lymphoma

In this article (Clin Cancer Res 2016;22:1138–49), which was published in the March 1, 2016, issue of Clinical Cancer Research (1), an additional affiliation for two authors was omitted. The byline and affiliation list, with the additional affiliation for Ladislav Andera and Simona Benesova, should read as follows:

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The authors regret this error.

Reference

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

Magdalena Klanova, Ladislav Andera, Jan Brazina, et al.


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