

Variable Responses of MYC Translocation Positive Lymphoma Cell Lines To Different Combinations of Novel Agents: Impact of BCL2 Family Protein Expression



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Abstract

Several newly developed drugs including JQ1 (BET inhibitor), ABT199 (BCL2 inhibitor), and bortezomib (proteasome inhibitor) may offer novel therapeutic strategies for aggressive diffuse large B-cell lymphoma (DLBCL). We tested these drugs together with doxorubicin in a series of combinations in 16 DLBCL cell lines including 4 ABC-DLBCL (OCI-Ly3, OCI-Ly10, SUDHL2, RIVA) and 12 GCB-DLBCL lines (OCI-Ly4, OCI-Ly18, BJAB, SUDHL4, SUDHL6, SUDHL10, DB, PR1, VAL, SC1, Karpas-231, Karpas-422). Among these cell lines, ABT199 and doxorubicin, and to a lesser extent JQ1 and bortezomib, showed high variations in their ED50 values. Of the six cell lines showing high ABT199 ED50 values, four (SUDHL10, OCI-Ly4, SUDHL2, and BJAB) had no or little BCL2 expression, and SUDHL6 also displayed a low BCL2 expression. There was no association between the ED50 value of doxorubicin, JQ1 and bortezomib, and TP53/MYC/BCL2 genetic abnormalities or cell of origin subtype. A synergistic effect in all or the majority of drug combinations was seen in 11 cell lines, while an antagonistic effect in a high proportion of drug combinations was observed in the remaining 5 cell lines including the 3 (SUDHL10, OCI-Ly4, and SUDHL2) with little BCL2 expression, and additionally OCI-Ly18 and RIVA. Extensive Western blot analyses revealed high MCL1 expression in SUDHL10 and OCI-Ly4 but no apparent alterations in other cell lines. The molecular mechanism underlying the antagonistic effect of drug combinations in DLBCL is heterogeneous with the altered BCL2 family protein expression (absent BCL2, but high MCL1) in some cell lines.

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Introduction

Diffuse large B-cell lymphomas (DLBCLs) account for 30%–45% of non-Hodgkin's lymphomas in adult and are highly heterogeneous in their genetic makeup and molecular mechanisms. Clinically, DLBCLs show variable responses to standard immunochemotherapy, typically R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone). A high proportion of DLBCLs show primary treatment failure (~10%), partial response (~15%), or relapse after initial response (20%–30%) [1]. The refractory and relapsed DLBCLs respond poorly to any of the available second-line therapies, thus representing a huge clinical challenge. The genetics and molecular mechanisms underlying the refractory/relapsed DLBCLs are highly heterogeneous albeit not yet fully characterized. Several biomarkers have been shown to be associated with poor overall

survival by independent studies from various centers. *MYC* translocation, particularly when concurring with *BCL2* translocation and/or *TP53* mutation, is associated with very poor clinical outcome, and these cases are known as double-hit lymphoma [2]. The activated

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B-cell like DLBCL (ABC-DLBCL) shows significantly poorer overall survival than the germinal center B-cell like DLBCL (GCB-DLBCL).

There are several drugs that have shown promising results in the treatment of various non-Hodgkin's lymphomas in animal models and/or clinical trials, providing novel therapeutic strategies for treatment of aggressive DLBCL.

JQ1 is a small-molecule inhibitor of the bromodomain and extra-terminal (BET) family proteins that interact with acetylated chromatin, particularly in the region of lineage-specific super-enhancers such as IGH enhancer, and facilitate active transcription [3,4]. In view of this, JQ1 might be particularly suited for treatment of lymphoma bearing a chromosome translocation involving the *IG* gene such as lymphoma carrying *MYC* and *BCL2* translocation. Indeed, JQ1 treatment was highly toxic to a range of leukemia and lymphoma cell lines including those harboring *MYC* or *MYC/BCL2* translocation, and significantly suppressed *MYC* expression under both *in vitro* and *in vivo* experimental conditions [3,5,6].

ABT199 is a highly potent and specific inhibitor of BCL2, selectively disrupting the BCL2-BIM complex and triggering the activation of proapoptotic proteins BAX and BAK, thus causing apoptosis [7]. It has been shown that ABT199 is active in treatment of a range of hematological malignancies including chronic lymphocytic leukemia, refractory or relapsed DLBCL and follicular lymphoma, and acute myeloid leukemia in phase I and II clinical trials [8]. *In vitro* studies also demonstrate that ABT199 is active in killing double-hit lymphoma cells, particularly in combination with other agents [7,9,10].

Bortezomib is a 26S proteasome inhibitor and its therapeutic effect is thought to be largely due to inhibition of NF- κ B activation and induction of ER stress. In a phase II nonrandomized clinical trial, Dunleavy and colleagues showed that addition of bortezomib to chemotherapy significantly improved the treatment response and overall survival of the patients with refractory/relapsed ABC-DLBCL that is characterized by enhanced NF- κ B activities, but not those with refractory/relapsed GCB-DLBCL [11]. In the prospective randomized REMoDL-B trial, the benefit of bortezomib appeared to be mainly in ABC-DLBCL with low IPI and those with a Burkitt lymphoma-like expression signature [12].

The use of the above novel agents alone in treatment of aggressive DLBCL is most likely of limited value due to development of eventual drug resistance [10,13]. Combined use of these agents has been investigated *in vitro* but largely restricted to a few DLBCL-derived cell lines and not yet comprehensively studied [9,10]. Given the diverse genetic changes and molecular mechanisms underlying aggressive DLBCL, it is important to systematically test various drug combinations and examine their combined effect in a large cohort of cell lines with defined genetic changes and molecular subtypes. In this study, we have investigated the therapeutic potential of JQ1, ABT199, bortezomib, and doxorubicin in a series of systematic combinations in 16 DLBCL cell lines.

Materials and Methods

Cell Lines

A total of 17 human aggressive B-cell lymphoma cell lines were included in this study, and they were 4 ABC-DLBCL (OCI-Ly3, OCI-Ly10, SUDHL2, RIVA) and 13 GCB-DLBCL lines (OCI-Ly4, OCI-Ly18, OCI-Ly19, BJAB, SUDHL4, SUDHL6, SUDHL10, DB, PR1, VAL, SC1, K231, Karpas-422). The culture condition for

these cell lines and their *MYC* and *BCL2* translocation, and *TP53* mutation status are summarized in Supplementary Table 1.

Drugs

Doxorubicin was purchased from Sigma-Aldrich (Sigma, UK), bortezomib from AdooQ Bioscience (York, UK), bromodomain inhibitor JQ1 from APExBio (York, UK), and ABT199 from Active Biochemicals (Hong Kong, China).

Interphase Fluorescence In Situ Hybridization (FISH)

Where indicated, cell clot was prepared from the cultured DLBCL cells and formalin fixed and paraffin embedded. The cell clots were used for investigation of *MYC* and *BCL2* translocation with *MYC*, *BCL2* dual-color break-apart probe and *MYC-IGH* and *BCL2-IGH* dual-color dual-fusion rearrangement probes (Vysis/Abbott Laboratories, UK) [2].

Mutation Analysis by Targeted Sequencing

TP53 mutation along with another 21 lymphoma-associated genes in DLBCL cell lines was investigated by Fluidigm Access Array PCR and Illumina MiSeq sequencing as described previously [14].

Cell Viability Assessment

For each DLBCL cell line, cells were plated at 4×10^4 cells in 100- μ l culture medium per well in a 96-well plate and exposed to a series of drug dilutions in 3 replicates at 37°C in an incubator. At the end of 48-hour exposure, the cells were assessed for viability using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Southampton, UK) according to the manufacturer's protocol. Data analyses and calculation of median effective dose (ED50) were carried out using GraphPad Prism 5 (GraphPad software Inc, La Jolla, CA).

Measurement of Synergy, Additivity, or Antagonism in Drug Combination

Cells were treated with two or three drugs combined at seven different concentrations based on serial fixed ratios of their ED50 values, i.e., 0-, 0.25-, 0.5-, 1-, 2-, and 4-folds of the ED50 value of each drug. As above, each treatment was performed in triplicate. The data were analyzed using Calcsyn software (Biosoft), and the combination index (CI), a measure of drug combination effect, was calculated from the drug cytotoxicity curves using the Chou-Talalay method [15]. The effect (synergistic, additive, and antagonistic) of drug combination can be different at different dose or effect levels. In general, for cancer therapies, synergism at high effect levels (e.g., at ED90, ED95) is much more therapeutically relevant than at low effect levels (e.g., ED50). The weighted average CI value [$CI_{wt} = (CI_{50+} + 2CI_{75+} + 3CI_{90+} + 4CI_{95})/10$] was calculated and used to assign the final effect of drug combination (synergy: $CI_{wt} < 0.90$, additivity: $CI_{wt} = 0.90-1.10$, and antagonism: $CI_{wt} > 1.10$) as described previously by Chou [16].

For OCI-Ly18, two independent experiments on evaluation of the effect of drug combination were carried out, and similar results were obtained.

Western Blotting

Total cell lysate protein (25 μ g) from cultured DLBCL cells that were not subjected to any treatment was separated by electrophoresis on precast NuPAGE 4%-12% gels and then transferred to a polyvinylidene difluoride membrane (GE Healthcare). The membranes were probed with an appropriate primary antibody (source and

specificity summarized in Supplementary Table S2) and then detected using the Millipore HRP detection kit according to the manufacturer's protocols. The signals were visualized using Fujifilm LAS 4000 image reader and quantified using Image J.

Short Tandem Repeat (STR) Genotyping

Selected cell lines were verified by STR genotyping using the PowerPlex 16 HS kit (Promega Corporation, Madison, WI), with the PCR products analyzed on an ABI Genetics analyzer. The data were analyzed and allele assigned using the GeneMapper ID software.

Statistical Analysis

Data are shown as means \pm SE. Statistical calculations were performed using Student's *t* test. Statistical significance was accepted as *P* values less than .05.

Results and Discussion

Among the 17 DLBCL cell lines investigated, 4 were purchased with authentication from DSMZ or American Type Culture Collection (Table S1). A further five cell lines that were from other research laboratories but uncertain on their definite source were investigated by STR genotyping, and three were authenticated (Table S1). Of the remaining two cell lines, OCI-LY4 did not have any published STR genotyping profile online; nonetheless, it carried the two *TP53* mutations (M237I; R248Q) as described originally (Table S1) {Chang, 1995 11,189 /id}. "OCI-LY19" had a distinct STR profile not matching to that documented in Cellosaurus or any cell line in online databases and thus was excluded from the study. *MYC* and *BCL2* translocation, and *TP53* mutation status in the 16 DLBCL cell lines investigated in the present study were collated from published works. These genetic changes were further confirmed by interphase FISH and PCR/sequencing in the present study and are summarized in Supplementary Table S1.

Responses to Treatment with Single Agent

Doxorubicin Treatment. Doxorubicin mediates its cytotoxic effect largely through the disruption of topoisomerase-II-mediated DNA repair and generation of free radicals that cause damage to DNA and cellular proteins. There were remarkable variations in the ED50 concentration among the 16 DLBCL cell lines investigated, ranging from 26 nM for OCI-Ly18 to 2290 nM for DB (mean \pm SD = 590 \pm 639 nM, Figure 1A, Fig. S1). There was no apparent association between the doxorubicin ED50 values and *TP53* mutation or *MYC* and *BCL2* translocation status, or cell of origin (COO) molecular subtype.

Bortezomib Treatment. Among the 4 drugs investigated, bortezomib showed the least variation in the ED50 concentrations among the 16 DLBCL cell lines investigated, ranging from 2.1 nM for SUDHL6 to 10 nM for BJAB (mean \pm SD = 4.8 \pm 2.0 nM). Although GCB BJAB had the highest ED50 dosage, there was no significant difference in ED50 value between the 4 ABC and 12 GCB DLBCL cells (Figure 1B, *P* = .30). There was also no apparent association between ED50 value and *TP53* mutation or translocation status.

JQ1 Treatment. JQ1 treatment may inhibit the transcriptional circuits that are deregulated in tumor cells and critical for their proliferation and survival, such as *MYC* and *BCL2* translocation [3,4]. There were considerable variations in the JQ1 ED50 values among the 16 DLBCL cell lines investigated, ranging from 283 nM for OCI-Ly4 to 1923 nM for BJAB (mean \pm SD = 1051 \pm 560 nM). However, there was

no apparent association between the ED50 values, and *MYC* and *BCL2* translocation or *TP53* mutation status (Figure 1C).

ABT199 Treatment. The ED50 concentration varied hugely among the 16 cell lines investigated, with 6 cell lines, namely, SUDHL10, OCI-Ly4, SUDHL2, BJAB, Kappas-422, and SUDHL6, showing a high ED50 value above 1500 nM, while the remaining 10 cell lines displayed an ED50 value less than 150 nM (mean \pm SD = 1995 \pm 3209 nM, Figure 2). There was no apparent association between the ED50 values and *BCL2* and *MYC* translocation. However, Western blot analysis of cells naive to any drug treatment revealed absent or little *BCL2* expression in SUDHL10, OCI-Ly4, SUDHL2, and BJAB and relatively low levels of *BCL2* expression in SUDHL6 in comparison with the cell lines that showed low ED50 values (Figure 2). Interestingly, with the exception of SUDHL2, all other 5 cell lines with high ED50 values were GCB subtype.

BCL2 gene is frequently mutated as a result of "off target" by the somatic hypermutation machinery in lymphomas derived from the germinal center B cells [17]. The mutation may involve coding sequence, alter amino acid sequence, and affect the antibody recognition site, thus leading to the false-negative detection of *BCL2* protein [17–19]. To investigate this, we performed Western blot analyses using two alternative *BCL2* antibodies (SP66 and E17), which recognize different regions of *BCL2* (Table S2). SUDHL10 was consistently shown to be absent for *BCL2* expression by Western blot with these additional *BCL2* antibodies, while OCI-Ly4, SUDHL2, and BJAB displayed a faint band with these alternative *BCL2* antibodies (Fig. S2). These findings confirmed the absent or little *BCL2* expression in these cell lines. Interestingly, SUDHL10 that showed no *BCL2* expression had the highest ED50 value (Figure 2).

Taken together, the above findings demonstrate that *BCL2* expression status is a major factor determining whether lymphoma cells are sensitive to ABT199 treatment. These findings are consistent with the recent observations that *BCL2*-negative DLBCL cell lines are resistant to ABT199 at the clinically relevant plasma concentration [20].

Responses to Treatment with Combined Agents

We systematically tested the cytotoxic effect of drug combination at serial fixed ratios of ED50, and the weighted CI was calculated and used to measure the effect of the drug combination [15]. Overall, 11 of the 16 cell lines showed a synergistic effect in all or the majority of the drug combinations (Figure 3, Fig. S3, Table S3). Among the remaining five cell lines, five (SUDHL10, OCI-Ly4, SUDHL2, OCI-Ly18, and RIVA) displayed an antagonistic effect in the majority or a high proportion of drug combinations (Figure 3).

SUDHL10, OCI-Ly4, and SUDHL2 showed no or little *BCL2* expression as detailed above, and the overwhelming antagonistic effect among various drug combinations that include ABT199 in these cell lines is likely to be the consequence of ineffective killing by the *BCL2* inhibitor. However, this did not explain why other drug combinations without ABT199 also displayed antagonistic effect in these cell lines. We reasoned that in the absence or very low level of *BCL2* expression, the lymphoma cells may acquire aberrant expression of other *BCL2* family proteins, thus evading apoptosis in a *BCL2*-independent manner. To investigate this, we analyzed MCL-1, BCL-XL (*BCL2-L1*), BAX, and BIM expression by Western blot. These additional analyses revealed high MCL-1 expression but low BIM expression in SUDHL10 and OCI-Ly4, and high BCL-XL expression in BJAB in comparison with *BCL2*-positive cell lines (Figure 2).

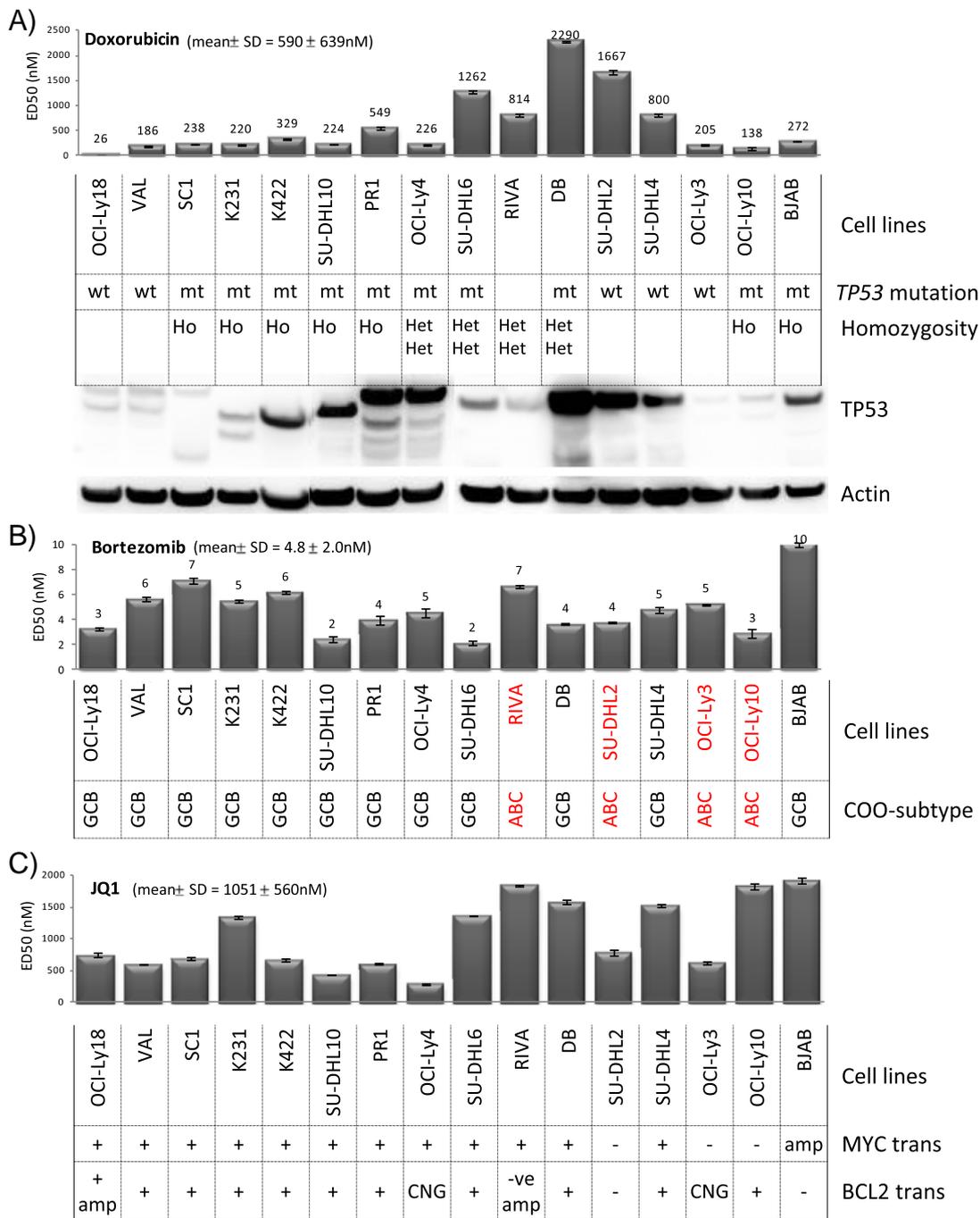


Figure 1. ED50 values of various B-cell lymphoma lines with treatment by the indicated drug. For each DLBCL cell line, cells were exposed to a series of drug dilutions for 48 hours in triplicates, and their viability was measured. Data analyses and calculation of median effective dose (ED50) were carried out using GraphPad Prism 5. wt: wild type; mt: mutant type; Ho: homozygous mutation; Het: heterozygous mutation with two “het” indicating that there are two mutations and each of these mutations is heterozygous, thus potential inactivation of both TP53 alleles. In panel A, the slice on Western blot between K422 and SU-DHL10 was due to removal of a cell line. In panel B, the ABC-DLBCL cell lines are highlighted in the red text. COO: cell of origin; ABC: activated B-cell; GCB: germinal center B cell; amp: amplification; CNG: copy number gain.

SUDHL2 showed no apparent aberrant expression of these proteins, and interestingly, this cell line demonstrated antagonistic effect in only one of the four drug combinations without ABT199 (Figures 2 and 3).

In support of the above observations, a recent study also showed no benefit of combining ABT199 and HHT (homoharringtonine) in five of six BCL2-negative DLBCL cell lines including SUDHL10 and BJAB investigated in this study, and all the five lines had enhanced

MCL1 or BCL-XL expression [20]. Taken together, these findings indicate that lack of BCL2 expression is not only associated with ABT199 resistance but also linked to an adverse effect of drug combinations, potentially due to overexpression of MCL-1 and/or BCL-XL expression.

MCL1 inhibits apoptosis by sequestering the proapoptotic BH3-only proteins and by blocking the essential apoptosis effectors BAX

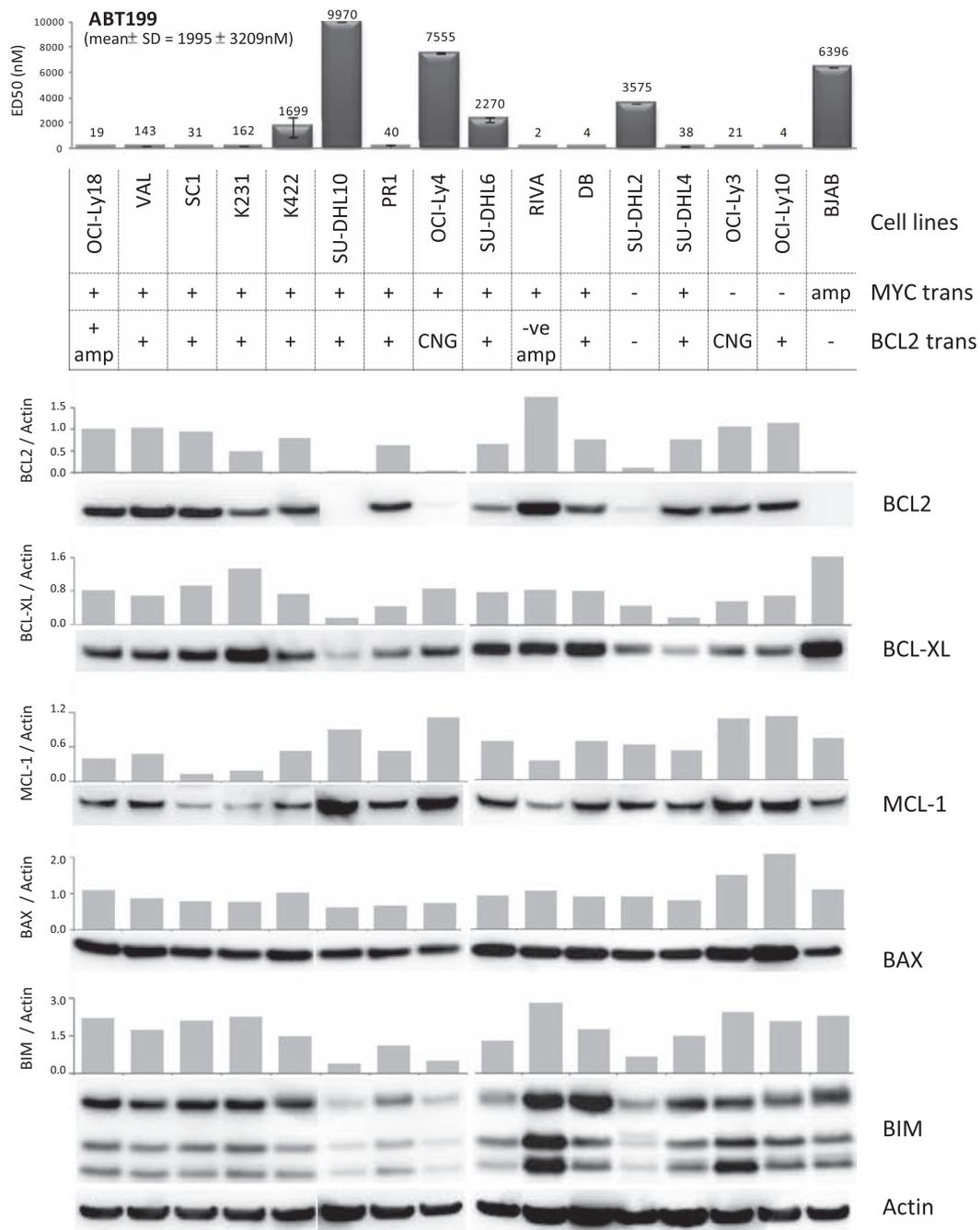


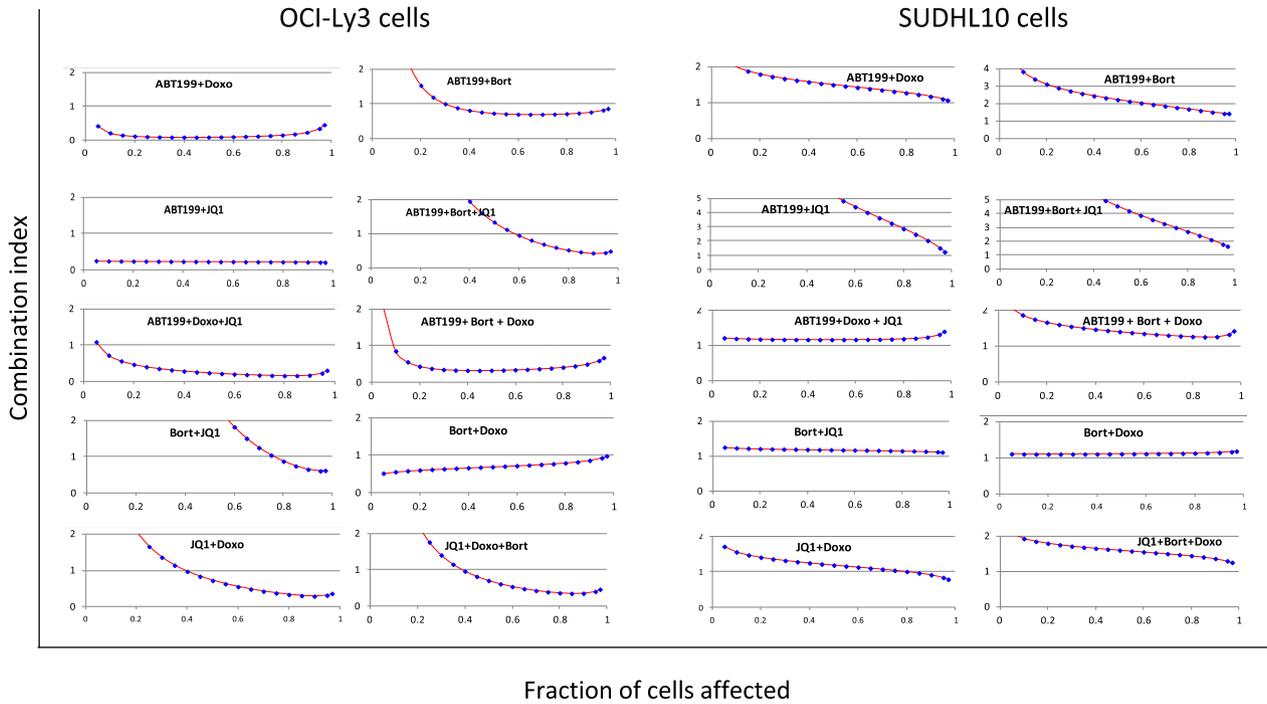
Figure 2. ED50 values of various B-cell lymphoma lines with treatment by ABT199 and its relationship with expression of BCL2 and its family members. For each DLBCL cell line, cells were exposed to a series of ABT199 dilutions for 48 hours in triplicates, and their viability was measured and ED50 calculated using GraphPad Prism 5. The expression level of BCL2 and its family members was investigated by Western blot analysis and further quantified in relative ratio to the loading control and presented as a histogram panel. The slice on Western blot between K422 and SU-DHL10 was due to removal of a cell line. Amp: amplification; CNG: copy number gain.

and BAK. Previous studies by immunohistochemistry show MCL1 expression in a high proportion of primary DLBCL, particularly those of ABC subtype, with strong expression in over 10% of cases irrespective of BCL2 expression status [21,22]. MCL1 overexpression confers resistance to both chemotherapeutic drugs as well as ABT199 [10,20–22]. Similarly, BCL-XL is another member of the BCL2 family of apoptosis inhibitors, and its overexpression is associated with resistance to ABT199 and also an antagonistic effect in combined ABT199 and radiotherapy [10,20,23]. In a similar manner, overexpression of MCL1 and BCL-XL

was also linked to decreased sensitivity of DLBCL lines to histone deacetylase inhibitor [24].

The molecular mechanisms underlying the antagonistic effect of drug combination in OCI-Ly18 and RIVA cell lines are not clear. Interphase FISH showed evidence of BCL2 translocation and also amplification of the translocated BCL2 allele in OCI-Ly18, and BCL2 gene amplification but not translocation in RIVA cells (Table S1). Both cell lines express high levels of BCL2 protein and are sensitive to ABT199 treatment (Figure 2). Nonetheless, RIVA cells

A) Example of responses of DLBCL cell lines to combined drug treatments



B) Summary of responses of DLBCL cell lines to combined drug treatments

	OCI-LY18	VAL	SC1	K231	K422	SUDHL10	PR1	OCI_LY4	SUDHL6	RIVA	DB	SUDHL2	SU-DHL4	OCI-LY3	OCI-LY10	BJAB
<i>COO subtype</i>	GCB	GCB	GCB	GCB	GCB	GCB	GCB	GCB	GCB	ABC	GCB	ABC	GCB	ABC	ABC	GCB
<i>MYC translocation</i>	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	amp
<i>BCL2-translocation*</i>	+	+	+	+	+	+	+	CNG	+	-ve amp	+	-	+	CNG	+	-
<i>BCL2 expression</i>	+	+	+	+	+	-ve	+	little	+	+	+	little	+	+	+	little
<i>TP53 mutation</i>	wt	wt	mt	mt	mt	mt	mt	mt	mt	mt	mt	wt	wt	mt	mt	mt
<i>TP53 homozygosity</i>			homo	homo	homo	homo	homo	homo	homo		homo				homo	homo
ABT/Doxo	syn	syn	syn	syn	syn	ant	syn	add	syn	ant	syn	syn	syn	syn	syn	syn
ABT/Bort	add	syn	syn	syn	syn	ant	syn	ant	ant	add	syn	add	syn	syn	add	add
ABT/JQ1	add	syn	syn	syn	syn	ant	syn	add	syn	syn	syn	n/a	syn	syn	syn	syn
ABT/Doxo/JQ1	add	syn	syn	syn	syn	ant	syn	ant	syn	syn	ant	ant	syn	syn	syn	syn
ABT/Doxo/Bort	syn	syn	syn	syn	syn	ant	syn	ant	ant	add	ant	ant	syn	syn	syn	add
ABT/Bort/JQ1	ant	syn	syn	syn	syn	ant	syn	ant	syn	syn	syn	ant	syn	syn	syn	syn
Bort/JQ1	ant	syn	syn	syn	syn	ant	ant	ant	syn	syn	syn	syn	syn	add	syn	ant
Bort/Doxo	add	add	ant	ant	ant	ant	add	ant	add	ant	syn	syn	syn	syn	syn	ant
JQ1/Doxo	ant	ant	add	ant	syn	add	syn	ant	syn	ant	syn	syn	syn	syn	syn	syn
JQ1/Doxo/Bort	ant	add	ant	add	add	ant	add	ant	syn	ant	syn	ant	syn	syn	syn	n/a

COO: cell of origin; ABT: ABT199; Doxo: doxorubicin; Bort: bortezomib; wt: wild type; mt: mutant; -ve: negative; +: positive; amp: amplification; CNG: copy number gain; ant: antagonistic effect; syn: synergistic effect.

Figure 3. Effect of drug combination in DLBCL cell lines. (A) Example of responses of DLBCL cell lines to combined drug treatments. (B) Summary of responses of DLBCL cell lines to combined drug treatments. Cells were treated with two or three drugs combined at serial fixed ratios of their ED50 values in triplicate. The weighted average CI value $[CI_{wt} = (CI_{50} + 2CI_{75} + 3CI_{90} + 4CI_{95})/10]$ was calculated (Table S3) and used to define the effect of drug combination (synergy: $CI_{wt} < 0.90$, additivity: $CI_{wt} = 0.90-1.10$, and antagonism: $CI_{wt} > 1.10$) as described previously by Chou [16]. *OCI-Ly18 cells harbor BCL2 translocation and also amplification of the translocated BCL2 allele, while RIVA cells have BCL2 gene amplification but not translocation.

were rather resistant to bortezomib and JQ1 treatments (Figure 1). RIVA is an ABC subtype and has been shown to be insensitive to a range of drugs including BTK inhibitor (ibrutinib), PI3K inhibitor (LY294002) and PDK1 inhibitor (BX-912) [25], and CDK inhibitor (dinaciclib or flavopiridol) [22], while OCI-Ly18 is a GCB subtype and has been shown to be resistant to Src family kinase inhibitor (dasatinib) [26], a dual PI3K and mTOR inhibitor (PQR309) [27], dual SYK/JAK inhibitor (cerdulatinib) [28], and temozolomide [29]. To further probe the potential mechanism underlying the antagonistic effect of drug combination in these cell lines, we investigated AKT (including pAKT-T308 and pAKT-S473), FOXO1 (including pFOXO1-T24 and pFOXO1-S256), PTEN, and c-MYC expression by Western blot analysis but did not find any clue (data not shown).

In summary, the majority of the DLBCL cell lines investigated in this study show synergistic responses to various combinations of the doxorubicin, bortezomib, JQ1, and ABT199. Those that do not show any beneficial effect of combined treatment are associated with aberrant expression of BCL2 family proteins (absent/low BCL2 expression, high MCL1 and/or BCL-XL expression). Clearly, DLBCL cell lines, even among those with the same COO subtype, are highly variable in their responses to treatment with these drugs either alone or in combination. These variable responses most likely reflect their intrinsic differences in their genetic makeup and molecular mechanisms. Thus, it is crucial to test a sufficient number of DLBCL cell lines in preclinical assessment of any potential therapeutic drugs and identify the biomarkers that could predict treatment responses.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2018.07.007>.

Author Contributions

Experimental data collection and analysis: W. D., A. C., H. L., M. W., Y. H., and R. D.; study design and research funding: M. Q. D. and P. J. Manuscript writing and preparation: M. Q. D. and W. D. All authors have reviewed and commented on the manuscript and approve its submission.

Conflict of Interest

The authors declare no conflict of interest.

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