Combination of Ibrutinib and ABT-199 in Diffuse Large B-Cell Lymphoma and Follicular Lymphoma

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Abstract

Diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma are the most prevalent B-lymphocyte neoplasms in which abnormal activation of the Bruton tyrosine kinase (BTK)–mediated B-cell receptor signaling pathway contributes to pathogenesis. Ibrutinib is an oral covalent BTK inhibitor that has shown some efficacy in both indications. To improve ibrutinib efficacy through combination therapy, we first investigated differential gene expression in parental and ibrutinib-resistant cell lines to better understand the mechanisms of resistance. Ibrutinib-resistant TMD8 cells had higher BCL2 gene expression and increased sensitivity to ABT-199, a BCL-2 inhibitor. Consistently, clinical samples from ABC-DLBCL patients who experienced poorer response to ibrutinib had higher BCL2 gene expression. We further demonstrated synergistic growth suppression by ibrutinib and ABT-199 in multiple ABC-DLBCL, GCB-DLBCL, and follicular lymphoma cell lines. The combination of both drugs also reduced colony formation, increased apoptosis, and inhibited tumor growth in a TMD8 xenograft model. A synergistic combination effect was also found in ibrutinib-resistant cells generated by either genetic mutation or drug treatment. Together, these findings suggest a potential clinical benefit from ibrutinib and ABT-199 combination therapy. Mol Cancer Ther; 16(7); 1246–56. ©2017 AACR.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL), accounting for roughly 30% of newly diagnosed cases in the United States. DLBCL is a heterogeneous lymphoma consisting of activated B-cell (ABC) and germinal center B-cell-like (GCB) subtypes that have different gene expression profiles, oncogenic aberrations, and clinical outcomes (1, 2). Compared with the GCB subtype, ABC-DLBCL has a significantly lower survival rate after multiagent chemotherapy (3) and is characterized by chronically active B-cell receptor (BCR) signaling (4), which is required for cell survival. Therefore, components of the BCR signaling pathway are emerging as attractive therapeutic targets in the ABC subtype of DLBCL.

Bruton’s tyrosine kinase (BTK), pivotal to BCR signaling, is covalently bound by ibrutinib with high affinity. Ibrutinib is a first-in-class, once-daily, oral inhibitor of BTK that is approved by the FDA for the treatment of patients with chronic lymphocytic leukemia (CLL), including those with deletion 17p, patients with mantle cell lymphoma (MCL) who have received at least one prior therapy, and those with Waldenström’s macroglobulinemia. With respect to DLBCL, a phase I/II clinical trial showed an overall response rate of 37% in the ABC subtype (5) with single-agent ibrutinib therapy.

Tumor responses to single-agent kinase inhibitor therapies are often limited by the cell’s ability to bypass the target via alternative pathways or acquired mutations in the target or its pathway (6, 7). It has been shown that a small number of CLL patients acquire resistance to ibrutinib through mutations in BTK and its substrate phospholipase C gamma 2 (PLCG2) following prolonged treatment (8, 9). In addition to acquisition of these mutations, other mechanisms of resistance, such as upregulation of potentially druggable survival pathways or clonal evolution of other genetic alterations, have been reported for ABC-DLBCL (10) and CLL (11). Such mechanisms may be overcome by combinations of targeted agents that block pathways that cooperate in resistance. Through screening of parental and acquired ibrutinib-resistant cell lines, we have identified and report here that a B-cell lymphoma 2 (BCL-2) inhibitor, ABT-199, synergizes with ibrutinib in vitro and in vivo and is able to overcome the ibrutinib-resistant phenotype in tumor cells overexpressing BCL-2.

Materials and Methods

Cell culture

The TMD8 and HBL1 cell lines were gifts from Dr. Daniel Krappmann in 2011 (German Research Center for Environmental Health, Neuherberg, Germany). The OCI-LY10 cell line was a gift from Dr. Richard Davis in 2010 (MD Anderson Cancer Center, Houston, TX). The WSU-DLCL-2, RL, SU-DHL-4, DoHH2, and...
WSU-FSCCL cell lines were purchased from ATCC or DSMZ in 2014. CellCheck service by IDEXX was used to provide cell line authentication. Cell lines were grown to log phase at 37°C in the presence of 5% CO₂. TMD8 and HBL1 cells were cultured in RPMI1640 medium (Life Technologies) with 10% FBS (Atlanta Biologicals), 1 mmol/L sodium pyruvate (Life Technologies), and 1% penicillin/streptomycin (Life Technologies). OCI-LY10 cells were cultured in IMDM medium (Life Technologies) with 20% heparinized normal human plasma (Equitech-Bio), 55 μmol/L 2-mercaptoethanol (Life Technologies), and 1% penicillin/streptomycin. WSU-DLCL-2, RL, SU-DHL-4, DoHH2, and WSU-FSCCL cells were cultured in RPMI1640 medium with 10% FBS, and 1% penicillin/streptomycin. Ibrutinib-resistant HBL1, TMD8, and DoHH2 cells were generated by in vitro culture of the parental cell lines for prolonged periods of time with progressively increasing concentrations of ibrutinib.

**Generation of BTK-WT and BTK-C481S cell lines**

Custom BTK-plasmid constructs expressing either the wild-type BTK (BTK-WT) or BTK genes containing a single mutation C481S (BTK-C481S) were obtained from GeneCopoeia with the Lv201 lentiviral backbone. Plasmids were amplified, purified, and sequenced by System Biosciences. Plasmids (5.0 μg) were then used to transfect 293T cells in a 10-cm dish using a lentiviral packaging kit (GeneCopoeia) following the manufacturer’s instructions. Media were removed 16 hours after transfection, and fresh media were added to the dish and incubated for 24 hours. Virus-containing supernatants were harvested and filtered (0.2-μm filter) and used for TMD8 transduction.

TMD8 cells were plated in 6-well plates in 2-mL medium at the concentrations of 1 × 10^5 cells/mL. Virus supernatants (500 μL) were added to wells followed by DOTAP Liposomal Transfection Reagent (final concentration 10 μg/mL, Sigma). Plates were centrifuged for 1 hour at 2,000 rpm and kept in culture at 37°C overnight. Supernatants were removed and cells were resuspended in fresh medium and incubated for 2 days. Cells were selected using 0.2 μg/mL puromycin to generate stable cell lines.

OCI-LY10 (BTK-C481S) was generated by introducing mutant HIS6/Strep-tagged BTK (C481S) under control of the EF1α promoter into the OCI-LY10 cell line using lentiviral transduction. Transduced cells were selected using blasticidin as an antibiotic resistance marker.

**Cell viability assays**

CellTiter-Glo (Promega) luminescent cell viability assay was performed according to the manufacturer’s instructions. Briefly, cells were seeded at 8,000 to 25,000 cells/well in a 96-well plate in the presence of single drugs or drug combinations for 3 or 5 days. The number of viable cells in culture was determined by quantification of ATP present, which was proportional to luminescent signal detected. Combination index (CI), a drug interactivity measurement, was calculated with CalcuSyn (Biosoft). Chalice Analyzer (Horizon CombinatoRx) was used to calculate the Loewe excess values, which were commonly used to indicate the excess percent inhibition. Excess percent inhibition was calculated by deducting the expected percent inhibition values of various combinations from the experimental percent inhibition values. These data allowed us to generate the isobolograms and synergy scores. In general, synergy scores >1 and CI <1 indicate a synergistic combination effect (12).

**qRT-PCR assays**

The TaqMan Fast Cells-to-Cytoplasmic Kit (Life Technologies) was used to extract total RNA and reverse transcribe RNA to cDNA according to the manufacturer’s specifications. Four microtiter of cDNA from the RT reaction was used to set up TaqMan qRT-PCR on a QuantStudio 7 Flex Real-Time PCR System (Life Technologies). The TaqMan Gene Expression Assays used for this study separation and subsequently transferred onto a polyvinylidene difluoride membrane (PVDF). The membranes were incubated with Odyssey Blocking Buffer (927-40000, LI-COR Biosciences) for 1 hour and probed overnight at 4°C with rabbit anti-BCL-2 (ab182858, Abcam), mouse anti-β-actin (3700S, Cell Signaling Technology). After washing with 0.1% Tween-TBS, the membranes were incubated with IRDye800CW- or IRDye680RD–conjugated secondary antibodies for 1 hour in the dark and detected using the Odyssey Imaging System (LI-COR Biosciences).

**siRNA transfection**

Accell human SMARTpool siRNAs targeting MAP3K7 (E-003790-00-0005) or IRAK4 (E-003302-00-0005) were purchased from Dharmacon. TMD8 cells (1 × 10^7/mL) were incubated with the Accell delivery medium (1% FBS) containing 1 μmol/L of siRNAs at 37°C according to the manufacturer’s instructions. After overnight incubation, fresh delivery medium (1% FBS) was added to each well. Cells were harvested 3 days after transfection and used for qRT-PCR.

**Western blot analysis**

Cells were washed twice with ice-cold PBS and lysed with RIPA buffer (R0278, Sigma-Aldrich) supplemented with 1× protease/phosphatase inhibitor. Cell lysates were subjected to SDS-PAGE separation and subsequently transferred onto a polyvinylidene difluoride membrane (PVDF). The membranes were incubated with Odyssey Blocking Buffer (927-40000, LI-COR Biosciences) for 1 hour and probed overnight at 4°C with rabbit anti-BCL-2 (ab182858, Abcam), mouse anti-β-actin (3700S, Cell Signaling Technology). After washing with 0.1% Tween-TBS, the membranes were incubated with IRDye800CW- or IRDye680RD–conjugated secondary antibodies for 1 hour in the dark and detected using the Odyssey Imaging System (LI-COR Biosciences).

**Xenograft study**

All animal studies were completed under the Institutional Animal Care and Use Committee (IACUC)–approved protocols for animal welfare. CB17 SCID mice (Charles River Laboratories) were subcutaneously inoculated with 1 × 10^7 TMD8 cells in a suspension containing Matrigel (Corning). When tumors reached approximately 100 mm^3 (16 days after tumor inoculation), mice were randomly assigned and treated once daily with ibrutinib (12 mg/kg), ABT-199 (40 mg/kg), or the combination of both by oral gavage, with 10 mice per group. Tumor volume was measured twice a week and calculated as tumor volume = (length × width^2) / 2.

**Apoptosis assays**

The ApoDETECT Annexin V-FITC Kit (Life Technologies) was used to quantify the apoptotic cell population according to the manufacturer’s specifications. Briefly, cells were washed with ice-cold PBS and resuspended in 1× binding buffer at a concentration of 5 × 10^5 cells/mL. Annexin V-FITC (10 μL) was added to 190 μL of cell suspension and incubated at room temperature for 10 minutes. After being washed with 1× binding buffer, cells were resuspended in 190 μL of binding buffer with 10 μL of 20 μg/mL propidium iodide (PI) and analyzed by flow cytometry.
Colony formation assays

HL-1 cells (1000 cells per well) were suspended in 0.9% methylcellulose (Methocult H1100, StemCell Technologies) containing culture medium with vehicle, ibrutinib, ABT-199, or an ABT-199/ibrutinib combination, and 0.3 ml of the mixture was plated in each well of 24-well culture plates. The colonies were counted on day 7.

Microarray data analyses and statistics

The GeneChip Human Transcripome Array 2.0 (HTA 2.0, Affymetrix) was used to analyze gene expression in TMD8 parental and ibrutinib-resistant cell lines and the work was done at Open Medicine Institute. A heatmap of apoptosis-related gene expression was generated using Transcriptome Analysis Console v2.0 (Affymetrix).

Gene expression of formalin-fixed paraffin-embedded (FFPE) specimens from the phase II PCYC-1106 trial (NTCT1825701) was analyzed using the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix), and data were normalized using the robust multiaffy array average (RMA) algorithm. Subtypes of DLBCL were identified on the basis of the classification algorithm (3). For the analysis restricted to ABC-DLBCL subtype, only the samples having a gene expression profiling call of ABC-DLBCL were used and normalized separately. A test for differential expression of genes between ABC-DLBCL responders [complete response (CR) + partial response (PR)] and nonresponders [stable disease (SD) + progressive disease (PD)] to ibrutinib was performed using the rank product statistic (RankProd R package). For the ABC-DLBCL versus GCB-DLBCL comparison plot and heatmap, all subtypes were normalized together. The data were plotted in linear scale.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE93986 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE93986).

Results

Ibrutinib-resistant TMD8 cells had higher BCL2 gene expression and were more sensitive to ABT-199

Ibrutinib-resistant TMD8 and DoHH2 cells were generated by culturing the parental cell lines in vitro with progressively increasing concentrations of ibrutinib. The EC_{50} of ibrutinib-resistant TMD8 and DoHH2 cells were 1.061 and 210 nmol/L, compared with 13 and 4 nmol/L for TMD8 and DoHH2 parental cells. BCL2, among other apoptosis-related genes, showed increased expression in ibrutinib-resistant TMD8 cells compared with TMD8 parental cells in our microarray data analysis (Fig. 1A). In addition, BCL2 is the only gene in the BCL-2 family with an anti-apoptotic function that was significantly increased (P < 0.05; Supplementary Table S1). The upregulation of BCL2 gene expression was confirmed by qRT-PCR (Fig. 1B). An increase in BCL2 gene expression was also observed in ibrutinib-resistant DoHH2 cells (Fig. 1C). Consistent with its increased gene expression, a higher level of BCL-2 protein was detected in ibrutinib-resistant cells (Fig. 1D). In addition to increased BCL2 expression, our microarray analyses showed reduced levels of MCL1 and BCL2A1 in ibrutinib-resistant cells. Reduction of both MCL1 and BCL2A1 may contribute to higher ABT-199 sensitivity in the ibrutinib-resistant cells (13–15). Indeed, ibrutinib-resistant TMD8 cells were much more sensitive to ABT-199 than TMD8 parental cells (Fig. 1E).

The identification of MYD88 L265P mutations in ABC-DLBCL suggests the importance of Toll-like receptor (TLR) signaling in this malignancy (16). ABC-DLBCL cells that are most sensitive to ibrutinib harbor both CD79A/B and MYD88 L265P mutations with chronically active BCR signaling in addition to TLR pathways (5). Similar to chronically active BCR signaling, TLR signaling also contributes to the activation of the NF-kB pathway (17), which is involved in the transcriptional regulation of BCL2 (18). Stimulation with a TLR9 agonist (ODN 2216) resulted in a 20-fold increase in the EC_{50} of ibrutinib in TMD8 cells. Intriguingly, we observed a 1.42-fold increase in MAP3K7 (P = 0.028) and a 1.83-fold increase in IRAK4 (P = 0.004) in ibrutinib-resistant TMD8 cells. We additionally observed a 6.69-fold reduction in the negative regulator of TLR signaling, IRAK3 (P = 0.052) in ibrutinib-resistant TMD8 cells. To investigate the role of TLR signaling in regulating BCL2 gene expression, we stimulated TMD8 cells with a TLR9 agonist, Cpg ODN 2216, and detected an increased BCL2 level compared with the control ODN treated cells (Fig. 1F). Consistently, knockdown of MAP3K7 or IRAK4 using siRNA reduced BCL2 gene expression (Fig. 1G and H). Therefore, we postulate that resistant cells have upregulated TLR pathways, which lead to an increase in BCL2 gene expression. However, we believe that further mechanistic characterization of this interesting question is beyond the scope of this manuscript.

In addition to BCL2, we identified several genes with 3- to 10-fold increases in expression in the ibrutinib-resistant TMD8 cells (P < 0.05; Supplementary Table S2) and another subset of genes with 3- to 10-fold reductions in expression (P < 0.05; Supplementary Table S3).

Figure 1.
Ibrutinib-resistant TMD8 cells had higher BCL2 gene expression and were more sensitive to ABT-199. A, Heatmap presentation of normalized log_{2}-transformed apoptosis-related gene expression profiles in TMD8 parental versus ibrutinib-resistant TMD8 cells (red, high; green, low). B, BCL2 gene expression increased in ibrutinib-resistant TMD8 cells. Gene expression levels of BAX, BCL2, and MCL1 were determined by qRT-PCR, with GAPDH and ACTB used as reference genes. All data are presented as fold change over TMD8 parental cells. C, BCL2 gene expression increased in ibrutinib-resistant DoHH2 cells. BCL2 gene expression was determined by qRT-PCR, with GAPDH used as a reference gene. Data are presented as fold change over DoHH2 parental cells. D, BCL2 protein level in parental and ibrutinib-resistant TMD8 or DoHH2 cells was determined by immunoblot analysis. E, Ibrutinib-resistant TMD8 cells were more sensitive to ABT-199 than TMD8 parental cells. Cells were treated with ABT-199 for 3 days, and the drug effect on cell growth was determined by CellTiter-Glo luminescent cell viability assay. F, TMD8 cells were stimulated with 1 pmol/L of the TLR9 agonist ODN 2216 for 3 days, and the gene expression level of BCL2 was determined by qRT-PCR, and GAPDH was used as the reference gene. Gene expression was also observed in ibrutinib-resistant DoHH2 cells. G, TMD8 cells were transfected with nontargeting control siRNA or siRNAs targeting MAP3K7 and gene expression levels of BCL2 and MAP3K7 were determined by qRT-PCR, and GAPDH was used as the reference gene. Data are presented as fold change over control siRNA transfected cells. H, TMD8 cells were transfected with nontargeting control siRNA or siRNAs targeting IRAK4 and gene expression levels of BCL2 and IRAK4 were determined by qRT-PCR, and GAPDH was used as the reference gene. Data are presented as fold change over control siRNA transfected cells.
Higher BCL2 gene expression was observed in tumors from ABC-DLBCL patients with poorer responses to ibrutinib

Gene expression was analyzed in clinical pretreatment FFPE specimens from the PCYC-1106 study, a phase II trial testing single-agent ibrutinib in patients with DLBCL. BCL2 was found to be differentially expressed in ABC-DLBCL and GCB-DLBCL patient samples, with ABC-DLBCL samples having higher BCL2 gene expression than those from patients with GCB-DLBCL (Fig. 2A). Three of 18 (17%) GCB-DLBCL versus 20 of 28 (71%) ABC-DLBCL patients had BCL2 expression higher than the median level. Interestingly, within the ABC subtype (n = 28), patients who experienced objective response to ibrutinib (CR + PR) had lower BCL2 gene expression (Fig. 2B). In addition, ABC-DLBCL patients with lower BCL2 gene expression had a longer median progression-free survival (PFS) after ibrutinib therapy (Fig. 2C).

Ibrutinib and ABT-199 synergistically inhibited cell growth of ABC-DLBCL, GCB-DLBCL, and follicular lymphoma cells

Previous work (19) identified ABT-199 as a compound with potential synergistic effects when combined with ibrutinib in ABC-DLBCL cells. Consistent results were observed when combining these two compounds in the TMD8 and HBL1 cell lines (Fig. 3A). To illustrate the synergistic effects of this combination, we normalized viability data relative to the effect of 199 as a single agent. In these plots, the dose response of ABT-199 combined with ibrutinib shifted toward a lower EC50 compared with the ibrutinib-only dose response, indicating that the combination of these agents induced greater toxicity than either agent alone and suggesting that addition of ABT-199 is able to overcome BCL-2-associated ibrutinib resistance. Chalice Analyzer was used to analyze the drug dose matrix and obtain the percentage of growth suppression for each of the combinations (Fig. 3B). The isobologram shows how much less drug is required when it is used in combination compared with the single-agent doses needed to achieve a desired effect. Synergy between ABT-199 and ibrutinib was confirmed using isobologram analyses, synergy scores, and the CI obtained for each of the cell lines tested (Fig. 3C and D). The combination of ibrutinib and ABT-199 was also confirmed to have synergistic growth-suppressive effects in GCB-DLBCL (WSU-DLCL-2, RL, and SU-DHL-4) and follicular lymphoma (DoHH2 and WSU-FScCL) cell lines, as shown by our cell viability results revealing a dose response shift toward a lower EC50 (Fig. 4A and B) and by our CI analyses (Fig. 4C).

Ibrutinib and ABT-199 synergistically suppressed cell growth in ibrutinib-resistant ABC-DLBCL and follicular lymphoma cells

Given the synergy of ibrutinib and ABT-199 in parental ABC-DLBCL cells, we explored the effects of this combination in ibrutinib-resistant cells generated by genetic mutation [TMD8 (BTK-C481S) and OCI-LY10 (BTK-C481S)] or drug treatment (ibrutinib-resistant HBL1 and TMD8). Relative gene expression of total BTK in TMD8 (BTK-WT) and in TMD8 (BTK-C481S) was confirmed by qRT-PCR (Supplementary Fig. S1A). ABT-199 increased the sensitivity of both cell lines to ibrutinib treatment (Fig. 5A). We observed that the effect of ABT-199 on ibrutinib sensitivity may be treatment time-dependent as evidenced by the reduced effect of ABT-199 after a 3-day treatment in TMD8 (BTK-WT) cells compared with a 5-day treatment in the parental TMD8
Figure 3.
Ibrutinib and ABT-199 synergistically suppressed cell growth in ABC-DLBCL cells. A, TMD8 and HBL1 cells were treated with the indicated concentrations of ibrutinib combined with ABT-199 (10, 30, and 100 nmol/L) or vehicle for 5 days, and drug effect on cell growth was determined using CellTiter-Glo Luminescent Cell Viability Assay. B, Drug dose matrix data in TMD8 and HBL1 cells. The numbers indicate the percentage of growth inhibition of cells treated with the corresponding compound combination relative to vehicle control-treated cells. The data are visualized over the matrix using a color scale. C, Isobologram analyses and synergy scores of the data in B indicate synergy for the combination of ibrutinib and ABT-199. D, CI of ibrutinib and ABT-199 at indicated concentrations in TMD8 and HBL1 cells. Boxes represent median values with the first and third quartiles. Whiskers represent the maximum and minimum values.
We further generated OCI-LY10 (BTK-C481S) cells, which were confirmed to display approximately 300-fold resistance to ibrutinib compared with parental OCI-LY10 cells (Supplementary Fig. S1B). Consistently, ABT-199 sensitized OCI-LY10 (BTK-C481S) cells to ibrutinib (Fig. 5B and C). A strong synergistic toxicity of ibrutinib and ABT-199 was confirmed by isobologram analysis of viability data (Fig. 5D), as well as the CI obtained (Fig. 5E). Consistent results were obtained in ibrutinib-resistant HBL1 and TMD8 cells (Fig. 5F). In addition to ibrutinib-resistant ABC-DLBCL cells, the combination of ABT-199 and ibrutinib enhanced the sensitivity of ibrutinib-resistant DoHH2 cells to ibrutinib (Fig. 5G), and synergy between these two compounds was demonstrated by the CI obtained (Fig. 5H).

Combining ibrutinib and ABT-199 increased apoptosis, inhibited colony formation, and suppressed tumor growth in ABC-DLBCL cells

In addition to the effects on cell growth, treatment of TMD8 cells with a combination of ibrutinib and ABT-199 resulted in increased cellular apoptosis (Fig. 6A). We also evaluated the effect of this combination on the clonogenicity of HBL1 cells. While single-agent ibrutinib and ABT-199 significantly reduced the colony number, the combination of both compounds completely abrogated colony formation in the methylcellulose medium (Fig. 6B).

We next investigated the effect of this drug combination in a xenograft model of ABC-DLBCL. As a single agent, 12 mg/kg of...
Figure 5.
Ibrutinib and ABT-199 synergistically suppressed cell growth in ibrutinib-resistant ABC-DLBCL and follicular lymphoma (FL) cells. A, TMD8 (BTK-WT) and TMD8 (BTK-C481S) cells were treated with indicated concentrations of ibrutinib combined with ABT-199 (100 and 300 nmol/L) or vehicle for 3 days, and drug effect on cell growth was determined using CellTiter-Glo Luminescent Cell Viability Assay. B, OCI-LY10 (BTK-C481S) cells were treated with indicated concentrations of ibrutinib combined with ABT-199 (10, 30, and 100 nmol/L) or vehicle for 5 days, and drug effect on cell growth was determined using CellTiter-Glo Luminescent Cell Viability Assay. C, Drug dose matrix data from OCI-LY10 (BTK-C481S) cells. D, Isobologram analysis and synergy score of the data shown in (B). E, CI of ibrutinib and ABT-199 at the indicated concentrations in OCI-LY10 (BTK-C481S) cells. F, Ibrutinib-resistant HBL1 and TMD8 cells were treated with indicated concentrations of ibrutinib combined with ABT-199 (10 nmol/L) or vehicle for 3 days, and drug effect on cell growth was determined using CellTiter-Glo Luminescent Cell Viability Assay. G, Ibrutinib-resistant DoHH2 cells were treated with the indicated concentrations of ibrutinib and ABT-199 at the indicated concentrations in ibrutinib-resistant DoHH2 cells. H, CIs of ibrutinib and ABT-199 at the indicated concentrations in ibrutinib-resistant DoHH2 cells.
ibrutinib or 40 mg/kg of ABT-199 only partially suppressed TMD8 tumor growth, whereas the combination of these agents produced full growth inhibition (Fig. 6C). Notably, the apoptotic cell population significantly increased in the drug combination–treated tumors (P < 0.05, unpaired t test; Fig. 6D), consistent with our in vitro observation (Fig. 6A).

**Discussion**

Although ibrutinib shows significant promise in ABC-DLBCL, it has limited efficacy as a single agent (5). Fortunately, potential mechanisms by which to bypass this limitation are quickly being discovered (9, 20). One approach to overcoming these obstacles is the use of combination therapy. BCL-2 inhibition in combination with other targeted therapies has demonstrated efficacy in several hematologic malignancies (21, 22). Consistent with previous high-throughput analyses (19), we showed that ibrutinib and ABT-199 synergistically suppressed cell growth, reduced colony formation, increased apoptotic cell death, and inhibited tumor growth in an ABC-DLBCL mouse model. The combination effect of ibrutinib and ABT-199 is not limited to ABC-DLBCL. We also identified synergy between these two agents in both GCB-DLBCL and follicular lymphoma. Similar efforts have been utilized and promising results have been obtained for this combination strategy in other B-cell malignancies, including CLL (23), MCL (24, 25), and Waldenström’s macroglobulinemia (26). Surprisingly, we also observed synergy between ibrutinib and ABT-199 in ibrutinib-resistant cell lines, both in cells selected in vitro for ibrutinib resistance and in those carrying a C481S-mutated form of BTK.

Deregulation of the antiapoptotic protein BCL-2 has been associated with resistance to targeted therapy and chemotherapy in cancers (27, 28). We show in this study that response to ibrutinib correlates with expression of BCL-2 in both cell lines and patient tissues, with higher expression of BCL-2 associated with a more limited response. Segregating ABC-DLBCL patients by BCL2 expression appears to identify a subpopulation of patients with worse PFS, consistent with our finding that patients

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Figure 6.
Combination of ibrutinib and ABT-199 increased the apoptotic cell population, inhibited colony formation, and suppressed tumor growth. A, TMD8 cells were treated for 1 day with ibrutinib (300 nmol/L), ABT-199 (1 μmol/L), or the combination and analyzed for Annexin V binding as well as for PI uptake. The percentage of Annexin V-positive, PI-positive or both Annexin V- and PI-positive cells is indicated. B, HBL1 cells were plated in 0.9% MethoCult (1,000 cells/well) with vehicle, ibrutinib (10 nmol/L), ABT-199 (50 nmol/L), or the combination, and colony formation was scored after 7 days. Graph represents quantifications of 3 wells expressed as the mean ± SD. C, TMD8 tumor cells were implanted into CB17 SCID mice, and the indicated drugs were orally administered daily when the tumors reached 100 mm³. Tumors were measured twice a week. Graph represents quantifications of 10 mice expressed as the mean ± SE. D, The apoptotic cell population (Annexin V-positive and PI-negative) of TMD8 tumor cells from CB17 SCID mice treated with indicated drugs were analyzed by flow cytometry. Data are expressed as the mean ± SE.
with SD and PD during ibrutinib treatment have higher BCL2 expression than those with PR and CR. These data suggest the existence of an antiapoptotic mechanism that limits the impact of BTK inhibition.

Early studies indicated that ibrutinib interferes with the homing process of MCL cells and increases circulating tumor cells (29). These circulating cells are more sensitive to ABT-199 than those attached to fibroblast cells (13), providing the rationale for combining ibrutinib and ABT-199 in MCL. The finding discussed here that ibrutinib-resistant cells may have elevated sensitivity to ABT-199 due to compensatory upregulation of antiapoptotic proteins such as BCL-2 provides another molecular mechanism underlying the synergy between ibrutinib and ABT-199 in ABC-DLBCL cells. Whether there are other disease-dependent mechanisms accounting for this combination effect requires further investigation.

Several genes that were upregulated in ibrutinib-resistant cells according to our microarray analyses have been previously linked to ibrutinib resistance and development of lymphomas and leukemias. In a phase I/II clinical trial, ABC-DLBCL patients with tumors harboring mutations in both CD79B and MYD88 had a much higher ibrutinib response rate (4/5; 80%) than those with tumors containing WT CD79B and mutant MYD88 (0/7; 0%) (5). Interestingly, recent work from Kim and colleagues showed upregulation of CD79B expression in three distinct ibrutinib-resistant DLBCL cell lines (30). In addition, this study convincingly demonstrated that overexpression of WT CD79B induced tumor cell resistance to ibrutinib while depletion of CD79B sensitized tumor cells to ibrutinib. Consistently, our microarray analyses revealed that CD79B, among others, was significantly increased in ibrutinib-resistant TMD8 cells. Interestingly, TMD8 cells are known to be heterozygous for a CD79B mutation (4). Whether the increase in CD79B gene expression is of the WT or mutant form and whether these different forms play distinct functions in the cells requires further investigation. In addition to CD79B, we identified an increase in the BCR signaling–related gene PLCG2 in ibrutinib-resistant cells. This upregulation is consistent with previous findings identifying PLCG2 mutations in clinical samples from CLL patients with ibrutinib resistance and the role of PLCG2 in the formation of a BTK-bypass pathway (11, 31). Several additional genes that were upregulated in ibrutinib-resistant cells are known to be involved in the development of lymphomas or leukemias, including FOXP1 (32), IGF1R (33), and KDM1A (34). Combining ibrutinib treatment with inhibitors targeting these proteins may prevent the formation of ibrutinib resistance.

Collectively, these data indicate that the combination of ibrutinib with ABT-199 may be highly effective in treating DLBCL and follicular lymphoma. Our data also implicate BCL-2 in resistance to single-agent ibrutinib therapy and suggest that disruption of this compensatory mechanism can shift the cell toward an apoptotic fate. On the basis of our findings in both in vitro and in vivo models, the combination of ibrutinib with ABT-199 appears promising and is currently being tested in clinical trials.

Disclosure of Potential Conflicts of Interest
H.P. Kuo, S. Haieh, M. Sirisawad, K. Eckert, K.J. Schweighofer, and B.Y. Chang have patents with Pharmacyclics LLC, an AbbVie Company and ownership interest (equity ownership) with AbbVie. S.A. Ezell has had travel accommodations from Pharmacyclics LLC, an AbbVie Company, was employed with AstaZeneca and Amgen, and has ownership interest (equity ownership) with AbbVie. L.W.K. Cheung has patents with Pharmacyclics LLC, an AbbVie Company and ownership interest (equity ownership) with AbbVie, was employed by and has ownership interest (equity ownership) with Eli Lilly & Co. M. Apaita has ownership interest (equity ownership) with AbbVie and her daughter is employed with Kaiser Permanente. S.J. Hsu is employed and has patents with Astellas and has patents and ownership interest (equity ownership) with Pharmacyclics LLC, an AbbVie Company. C.T. Chen has patents with MD Anderson Cancer Center and ownership interest (equity ownership) with AbbVie. D.M. Beaupre has leadership, research funding, patents, and travel accommodations from Pharmacyclics LLC, an AbbVie Company and ownership interest (equity ownership) with AbbVie. M. Verese has patents with Janssen and ownership interest (equity ownership) with Johnson & Johnson.

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