Sequential Transcription Factor Targeting for Diffuse Large B-Cell Lymphomas

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Abstract

Transcription factors play a central role in malignant transformation by activating or repressing waves of downstream target genes. Therapeutic targeting of transcription factors can reprogram cancer cells to lose their advantages in growth and survival. The BCL6 transcriptional repressor plays a central role in the pathogenesis of diffuse large B-cell lymphomas (DLBCL) and controls downstream checkpoints, including the p53 tumor suppressor gene. We report that a specific inhibitor of BCL6 called BPI can trigger a p53 response in DLBCL cells. This was partially due to induction of p53 activity and partially due to relief of direct repression by BCL6 of p53 target genes. BPI could thus induce a p53-like response even in the presence of mutant p53. Moreover, sequential BCL6 peptide inhibitors followed by p53 peptide or small-molecule activators provided a more powerful antilymphoma effect than either treatment alone by maximally restoring p53 target gene expression. Therefore, tandem targeting of the overlapping BCL6 and p53 transcriptional programs can correct aberrant survival pathways in DLBCL and might provide an effective therapeutic approach to lymphoma therapy. [Cancer Res 2008;68(9):3361–9]

Introduction

Diffuse large B-cell lymphomas (DLBCL) are the most common type of B-cell lymphomas. The BCL6 (B-cell lymphoma 6) proto-oncogene is frequently constitutively expressed in DLBCL due to translocation of heterologous promoters to the BCL6 coding region, or point mutations of negative regulatory elements due to misdirected somatic hypermutation (1). Constitutive expression of BCL6 in mouse models that mimic human BCL6 translocations can induce formation of DLBCL (2, 3). Many DLBCLs express BCL6 even in the absence of genetic lesions, suggesting that other factors that drive BCL6 expression could also be oncogenic hits. The lymphomagenic effects of BCL6 may be related to its ability to directly repress critical cell cycle checkpoint genes, including TP53 (tumor protein p53), ATR (ataxia telangiectasia and Rad3 related), CHEKI (CHK1 checkpoint homologue), and p21<sup>cDKN1A</sup> [cyclin-dependent kinase inhibitor 1A (p21, Cip1); refs. 4–7]. DLBCL cells become dependent on BCL6 because its continued presence is required to maintain proliferation and survival of DLBCL cells (6, 8).

BCL6 is a transcriptional repressor and member of the BTB/POZ (bric-a-brac-tramtrack-broad complex/pox virus zinc finger) family of proteins. The BCL6 BTB domain plays a key role in repression by recruiting the SMRT (silencing mediator of retinoid and thyroid receptors), N-CoR (nuclear receptor corepressor), and BCoR (BCL6 corepressor) corepressors (9, 10). These three corepressors can bind to a groove located on the surface of BCL6 BTB homodimers (11). We designed a BCL6 peptide inhibitor (BPI), which mimics the 18-amino-acid region of SMRT that binds to BCL6. BPI readily penetrates DLBCL cells and blocks BCL6 from recruiting N-CoR, SMRT, and BCoR, resulting in chromatin remodeling and reactivation of BCL6 target genes (8). BPI specifically inhibited BCL6 and not other transcriptional repressor proteins (8). BPI specifically killed DLBCL cells that express BCL6 but had no effect on BCL6-negative DLBCL cells (6–8, 12). Finally, BPI abrogated the biological activity of BCL6 on B-cells <em>in vivo</em>, demonstrating that effective levels of this inhibitor can be readily achieved in an animal model (8).

Targeting of oncogenic transcription factors like BCL6 is a feasible strategy for cancer therapy (13, 14). However, a single targeted agent is unlikely to be curative because tumors typically contain multiple oncogenic “hits.” One way to overcome this obstacle is to combine a transcription therapy agent with chemotherapeutic drugs, as routinely used in the treatment of acute promyelocytic leukemia (15). We propose another more specific route toward enhancing the activity of specific transcription therapy agents. In particular, we hypothesized that it is possible to enhance antitumor effects by combining agents that target successive waves of transcriptional programming. In the case of BCL6, an attractive downstream network for therapeutic targeting is that of the p53 tumor suppressor, which regulates genes involved in cell death and proliferation (16). Restoration of defective p53 function could potentially facilitate induction of cell death and growth arrest in tumor cells. Recent studies carried out in lymphoma and other tumor cells showed that p53 function could be enhanced or restored by specific peptides or small molecules, which rescue certain types of mutant as well as wild-type (WT) p53 proteins (reviewed in ref. 17). Herein, we show that BPI can induce p53 expression and biological functions in DLBCL cells, including those with certain p53 mutations. These effects were due both to induction of <em>TP53</em> and to loss of BCL6 repression on p53 target genes. Sequential administration of BPI followed by a p53-activating peptide or a small molecule cooperatively enhanced p53 activity and killed DLBCL cells. p53 and BCL6 are thus intricately functionally linked in DLBCL and can be therapeutically harnessed as a form of tandem transcription therapy with potent antilymphoma activity.
Materials and Methods

Cells, peptides, and drugs. The DLBCL cell lines Ly1, Ly4, and Ly10 were grown in Iscove’s medium with 10% FCS and penicillin G/ streptomycin. The Farage, Ly3, SU-DHL6, and SU-DHL4 cell lines were grown in RPMI with 10% FCS, penicillin G/streptomycin, and HEPES. p53C-TAT (GSRASSHLKKKGGQSTSRHKKGYGRKKRRQRRR), p53-control peptide (CP2; GSRASSHLHHAEQGTSRHKKGYGRKKRRQRRR), and control peptide (CP; GYGRKKRRQRRR) were obtained from Biosynthesis, Inc. The purity determined by high-performance liquid chromatography–mass spectrometry was 98% or higher for each peptide. Unless noted, peptides were used at the following concentrations: BPI and CP 5 μmol/L, five times per day, and p53C-TAT and CP 10 μmol/L once per day. The Peptide-NN-(His)8-pTAT-HA (18) was expressed in Escherichia coli cells BL21 (DE3; EMD Biosciences, Inc.) and purified by affinity purification with Ni-NTA Hi-Trap column (APBiotech) using an AKTA Purifier 10 (APBiotech). Cyclic-pthitrrh-α [2-(4-methylphenyl)-imidazo[2,1-b]-5,7,8-tetrahydrobenzoazolone; EMD Biosciences, Inc.] was reconstituted in DSMS0 immediately before use. For combination experiments, pthitrhrh-α 20 μmol/L (or DSMS0) was added 12 h before the BPI or CP and every 12 h thereafter until analysis. PRIMA-1 [2,2-bis[(hydroxymethyl]-1-azabicyclo(2,2,2)octan-3-one] was obtained from Tocris Cookson, Inc.

Growth inhibition determination. Ly1, Ly3, Ly4, Ly10, SU-DHL4, SU-DHL6, and Farage cells were grown at concentrations to maintain exponential growth over the 48 h drug/peptide exposure time. We determined cell viability using a MTS tetrazolium-based method (Cell Titer 96 Aqueous One, Promega Corp.) and a Polystar Optima microplate reader (BMG Labtechnologies). We performed six-point standard curves for each cell line (correlating the number of viable cells by trypan blue with absorbance). For combination studies, viability was determined using a fluorometric resazurin reduction method (CellTiter-Blue, Promega Corp.) and the Polystar Optima microplate reader. The number of viable cells in each treatment was well tested by using the linear least-squares regression of the standard curve. Absorbance or fluorescence was determined for four replicates per treatment condition or standard. We verified cell viability by the sulforhodamine B assay (Sigma) with minor modifications for suspended cells. Experiments were carried out in triplicates and data are represented as percentage of growth inhibition to respective control. We used the CalcuSyn software (Biosoft) to determine the concentration of peptides and PRIMA-1 that inhibits 50% the growth of cell lines compared with control treated cells (GI50) and determine the combination index (CI). For the CI experiments, cells were treated with BPI at 5, 15, and 25 μmol/L and/or PRIMA-1 at 25, 75, and 100 μmol/L (1:5 constant ratio). We assume a mutually exclusive effect for the combination of BPI and PRIMA-1.

Cell cycle analysis. Cells were fixed with a formaldehyde-based fixation buffer (Santa Cruz Biotechnology), reconstituted in permeabilization buffer (Santa Cruz Biotechnology), and stained with anti–Ki-67 antibody (Santa Cruz Biotechnology) and 50 μl propidium iodide 1 mg/mL (Sigma) or 7-amino-actinomycin D (BD Biosciences) with 20 μl RNase A 10 μg/mL (Sigma). Samples were analyzed by flow cytometry in a FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences). Detection of G0-G1, Ki-67 negative), and S (intermediate DNA content, Ki-67 positive). Stained cell suspensions (107) were transferred to a microscope slide and viewed under a fluorescent light microscope (Axioskop 2, Carl Zeiss AG) with ×100, ×200, and ×400 magnifications. A total of 100 cells were counted in triplicates per treatment condition.

Caspase-7/3 activity. The activity of caspase-7 and caspase-3 was determined using the Apo-ONE caspase-3/7 assay (Promega). DLBCL cell lines were treated for 6 and 18 h with drug/peptides followed by 1-h exposure to the pro-fluorescent Z-DEVD-R110 substrate. Activation of Z-DEVD-R110 by the activity of caspase-3/7 allows the R110 group to become intensely fluorescent (Ex490nm/Em520nm), which was measured using the Polystar Optima microplate reader in four replicates. Caspase-7/3 activity was related to the cell number determined by CellTiter-Blue (Promega) in a multiplex assay.

DNA fragmentation. An ELISA kit (Cell death detection ELISA, Roche Diagnostics) was used to determine the presence of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of cell lysates. DLBCL cells were exposed to BPI and/or p53C-TAT and/or control peptides for 24 h. Following the manufacturer’s instructions, the cell lysates were applied in triplicates onto an anthistone-coated plate. After saturation of nonspecific binding with a blocking solution, the remaining immobilized complexes were incubated with a peroxidase-conjugated anti-DNA antibody. The amount of peroxidase retained was colorimetrically determined by using ABTS as substrate (absorbance A405nm = A630nm - A750nm, Polystar Optima microplate reader). The enrichment of nucleosomes in the cytoplasm of treated cells was calculated by comparing the absorbance of the treated cells with the corresponding control.

Tissue microarrays, chromatin immunoprecipitation analysis, Annexin V/propidium iodide staining, Western blotting, quantitative real-time PCR (qRT-PCR), and TP53 sequencing are provided as Supplementary Data.

Results

BPI induces p53 expression in DLBCL cells. Because BPI can reactivate BCL6 target genes and kill DLBCL cells (6–8), and p53 is a BCL6 target gene (4), we wondered whether BPI could induce p53 expression in DLBCL cells. For this analysis, we selected three representative DLBCL cell lines: Ly1, which expresses BCL6 and has biallelic point mutations in the p53 DNA-binding region (21–23); Ly10, which expresses BCL6 and has WT p53 (23); and Ly4, which expresses little BCL6, is BCL6 independent and has only one allele of p53 which is inactivated by two mutations (refs. 22, 23; see also Supplementary Table S1). We examined the kinetics of BPI effects on p53 mRNA abundance by qRT-PCR over a time course of 48 hours. BPI caused rapid 3.5-fold induction of p53 transcription in Ly10 (p53 WT) cells, peaking at 6 hours, but had different kinetics in Ly1 cells, reaching a maximum of 2-fold increase at 24 hours (Fig. 1A). There was no induction of p53 in the BCL6-independent Ly4 cell line. p53 mRNA was baseline expressed at similar levels (ratio to glyceraldehyde-3-phosphate dehydrogenase, Ly4: 0.63, Ly1: 0.74, and Ly10: 0.68) in the three cell lines regardless of mutational status or BCL6 expression. BPI also induced p53 protein levels (Fig. 1B) with a 1.7 peak fold increase in Ly10 cells at 12 hours followed by a decline in protein abundance at 48 hours, whereas in Ly1 cells the protein steadily accumulated during the observation time, peaking at 24 hours, possibly because the mutated protein may not as efficiently induce its own degradation. In contrast, p53 protein was not increased in Ly4 cells.

BPI induces a p53 response in DLBCL cells. To determine the functional effect of BPI-mediated induction of p53, we examined the kinetics of p53 target gene expression in DLBCL cells exposed to BPI versus control peptide by qRT-PCR. In Ly10 cells, BPI induced the p53 target genes p21, NOXA, PUMA (p53-up-regulated modulator of apoptosis), and GADD45A (growth arrest and DNA
damage–inducible 45α) within 6 to 12 hours and PIG3 (p53 induced gene 3) within 24 hours (Fig. 2A). p21 induction was slightly biphasic, peaking first at 6 hours and increasing again at 24 hours. Because p21 is also a direct target of BCL6 (5), it is possible that its induction by BPI was partially independent of p53. Accordingly, BPI induced p21 in Ly1 (p53 mutant) cells with a similar biphasic pattern and level of expression (Fig. 2A). In contrast, PUMA, NOXA, and PIG3 were unaffected by BPI in Ly1 cells. Like p21, GADD45A was also induced in Ly1 cells to a 2-fold maximum at 24 hours. We wondered whether GADD45A is also a BCL6 target gene. The GADD45A promoter contains a BCL6 consensus sequence located within a highly conserved region at −500. Chromatin immunoprecipitation assays confirmed strong enrichment of BCL6 to this site (Supplementary Fig. S1). None of these genes were induced by BPI in Ly4 cells. These results indicate that BPI can increase p53 transcriptional function in DLBCL cells and show that the BCL6 and p53 transcriptional programs are at least partially overlapping.

We next examined DLBCL cells for cell cycle progression 24 hours after BPI exposure (at which point many BPI-treated cells are still viable). The Ly10 cell line displayed a reproducible doubling in G2-M phase cells from 8% to 15%, with a corresponding reduction in S-phase (consistent with biological effects of p53; ref. 24; Fig. 2B). In contrast, Ly1 cells showed a small but reproducible increase in the S-phase fraction of cells from 50% to 60% with a corresponding reduction in G2-M (consistent with our work showing that BCL6 suppresses an ATR-dependent S-phase checkpoint; ref. 7). Ly4 cells were not affected by BPI. BPI impaired viability of both the Ly10 and Ly1 DLBCL cells within 48 hours, as shown in metabolic assays (Fig. 2C). In contrast, Ly4 cell viability and survival were unaffected. The cellular effects of BPI in DLBCL cells thus vary with the mutational status of p53.

**p53 induction contributes to BPI-mediated cell death.** To determine the contribution of p53 to effects of BPI, we used pifithrin-α, an inhibitor of p53 (25). We first examined the effect of pifithrin-α on expression of p21, GADD45A, and PIG3. BPI induced p21 and GADD45A in a pifithrin-α–independent manner in (p53 mutant) Ly1 cells, consistent with their direct regulation by BCL6, but not PIG3 (Fig. 3A). In contrast, pifithrin-α markedly impaired the induction of all three of these genes in p53 WT Ly10 cells, suggesting that the mechanism controlling expression of these genes is dependent on whether p53 was WT or mutated (Fig. 3A).

As expected, BPI and pifithrin-α had no effect in Ly4 cells. Pifithrin-α rescued Ly10 cells from BPI-mediated loss of viability, but had no such effect on Ly1 cells (Fig. 3B). To confirm the specificity of these results, we repeated these experiments using a dominant negative form of p53 delivered by TAT protein transduction, or a mutant inactive form of p53 as a control (18). Like pifithrin-α, dominant negative p53 could at least partially overcome the effects of BPI in Ly10 but had no effect on Ly1 cells (Fig. 3C). Dominant negative p53 had a mild viability effect in Ly4 cells, the reason for which is unknown but could be related to interference with gain of function effects of the mutant p53 variants expressed in these cells. Taken together, these data suggest that BPI-mediated release of the p53 pathway from BCL6 repression plays an important role in its ability to kill DLBCL cells with WT p53. Release of p53 targets also regulated by BCL6 may contribute in part to BPI effects on DLBCL cells with mutant p53.

**BCL6 inhibition plus p53 activation cooperate to kill DLBCL cells.** A TAT-p53 COOH-terminal peptide (p53C–TAT) was shown to enhance the transcriptional activity of WT p53 (18) and to rescue the activity of certain p53 DNA contact mutants (26), without significantly increasing the p53 protein levels. We tested the effect of p53C–TAT on p53 target gene expression in DLBCL cells. In Ly10 cells, p53C–TAT induced p21, GADD45A, and PIG3 by 2.5-fold within 6 hours, and PUMA and NOXA at later time points (Fig. 4A). p53C–TAT also induced these target genes in Ly1 cells, although with generally delayed kinetics (Fig. 4A) but had negligible activity in Ly4 cells. Consequently, p53C–TAT modestly reduced the number of viable cells in Ly1 (by 16%), Ly10 (by 22%), and Ly4 cells (by 4%) after 48 hours of exposure compared with control (Supplementary Fig. S2). Because BPI can induce p53 expression, and considering that the downstream genes induced by BPI and p53 partially overlap, we asked whether BPI and p53C–TAT might cooperate in a transcriptional response that could lead to enhance antilymphoma effects. DLBCL cells were exposed to BPI to release p53 from BCL6-mediated suppression, followed 24 hours later by p53C–TAT to further induce the transcriptional activity of

![Figure 1.](https://www.aacrjournals.org) BPI induces p53 expression in DLBCL cells. A, Ly4, Ly1, and Ly10 DLBCL cells were exposed to 5 μmol/L BPI for 48 h and mRNA was collected for qPCR determination of p53 transcript abundance at the indicated time points. Results are expressed as fold of induction relative to control peptide and normalized by GADPH. Results represent the mean of biological triplicates, each of which was performed in experimental triplicates. B, abundance of p53 protein in Ly4, Ly1, and Ly10 cells exposed to CP or BPI for 12, 24, and 48 h. The band intensities for p53 and actin were determined by densitometry and the ratio of p53 to actin in cells treated with BPI versus control peptide is shown on the right. The experiment shown is representative of three replicates.
p53. We first measured the fold induction of p53 target gene mRNA transcripts relative to their respective controls. Remarkably, BPI and p53C\(^{-}\)-TAT mediated supra-additive induction of p21, PUMA, and NOXA in both Ly10 and Ly1 cells and of PIG3 in Ly1 cells (Fig. 4B; Supplementary Fig. S3). Induction of GADD45A was additive in both cell lines. None of the p53 target genes were additively activated in Ly4 cells. Based on these results, we wondered whether the combination of BPI and p53C\(^{-}\)-TAT might also cooperate in killing lymphoma cells. DLBCL cells were exposed to BPI or control peptide followed 24 hours later by p53C\(^{-}\)-TAT or its control peptide for another 24 hours. BPI alone mediated 40% loss of viability in Ly1 cells and 50% loss of viability in Ly10 cells whereas it had no effect in Ly4 cells. The sequential administration of p53C\(^{-}\)-TAT after BPI resulted in markedly enhanced antilymphoma activity to a roughly 80% and 70% loss of viability in Ly1 and Ly10 cells, respectively (Fig. 4C).

**BPI and p53 activation can induce different types of cell death.** To better understand BPI and p53 antilymphoma effects, we explored death mechanisms triggered by these agents alone or in combination. For example, in addition to apoptosis, p53 can also activate a necrosis-like cell death program in distinct tumor cell types (27). In the search for more effective therapies, the induction of alternative death pathways may be important to bypass resistance mechanisms associated with a particular type of cell death (27, 28). To determine whether 24-hour exposure to BPI and p53C\(^{-}\)-TAT induce the same or alternative p53-dependent cell death pathways, Ly4, Ly1, and Ly10 were examined microscopically by ethidium bromide/acridine orange staining, and by flow cytometry using Annexin V and propidium iodide (Fig. 5; Supplementary Fig. S4). Cells undergoing necrosis can be distinguished from apoptotic cells based on morphologic criteria, including cellular shape, cellular volume, chromatin condensation, nuclear fragmentation, and status of the plasma membrane (29).

In accordance with a recent report on p53 COOH-terminal peptide effects in prostate cancer cells (27), we found that p53C\(^{-}\)-TAT induced mostly a necrotic-type cell death in DLBCL cells.
regardless of the mutation status of p53 (Fig. 5A first and second row; Supplementary Fig. S4). Ly1 cells exposed to BPI for 24 hours showed a morphologically apoptotic type of death, whereas Ly10 cells exhibit a necrotic type cell of death similar to that induced by p53C−TAT (Fig. 5A third row; Supplementary Fig. S4). The concurrent administration of BPI and p53C−TAT increased both types of death, with a higher proportion of necrosis in Ly10 cells than in Ly1 cells (Fig. 5A fourth row; Supplementary Fig. S4). Moreover, BPI alone or in combination with p53C−TAT strongly induced DNA fragmentation and caspase-7 and caspase-3 activity in Ly1 cells, consistent with death by apoptosis (Fig. 5B and C). In contrast, Ly10 and Ly4 cells displayed minimal changes in DNA fragmentation or caspase activation (Fig. 5B and C). Apoptosis is an energy/ATP–dependent process, whereas, in contrast, ATP levels dissipate rapidly in cells undergoing necrosis (30), perhaps due to cellular depletion of NAD+ by poly(ADP)ribose polymerase (PARP; refs. 31, 32), with consequent depletion of ATP. On the contrary, in most apoptotic cells, PARP is inactivated by caspase-dependent cleavage (33) and the cellular ATP pool is not depleted. We found that after 18 hours of exposure of Ly4, Ly1, and Ly10 cells to p53C−TAT, there is a sharp decline in the cellular levels of ATP whereas the PARP protein remains intact (Fig. 5D). A similar effect was observed after BPI exposure in Ly10 cells. However, Ly1 cells treated with BPI maintained levels of ATP similar to control-treated cells whereas the PARP protein was cleaved in this cell line (Fig. 5D). Concomitant administration of BPI and p53C−TAT yielded a mixed result with partial ATP depletion and a lesser extent of PARP cleavage. Taken together, these results indicate that the type of cell death induced depends on the mechanism of action of the therapeutic agent (p53C−TAT induces mostly necrosis) and on the genetic background of the tumor cell.

**Cotargeting of BCL6 and p53 enhances antilymphoma activity.** Recent screening efforts identified small molecules that reactivate a greater spectrum of p53 mutants and with higher efficiency than p53-derived COOH-terminal peptides. One such molecule is called PRIMA-1 (34). The mechanism of action of PRIMA-1 is not yet fully understood, but may involve the promotion of refolding of mutant forms of p53 into active conformations (35). Our data suggest that combinatorial targeting of BCL6 and p53 could provide enhanced antilymphoma activity by inducing either apoptosis or necrosis in DLBCL cell lines. To confirm and expand these results, we examined BPI and PRIMA-1 effects in a larger panel of DLBCL cell lines, including one BCL6-independent (Ly4) and six BCL6-dependent cell lines. We analyzed, by direct sequencing, the TP53 mutational status in this panel of cells and found that on top of the reported mutations, Ly4 and Ly1 have additional mutations affecting the DNA binding region of p53 (G245D and R249K, respectively; Supplementary Table S1). The SU-DHL4 and Farage cell lines contained R273C and E285Q mutations, respectively, affecting the DNA binding region of p53. Ly10, Ly3, and SU-DHL6 had WT p53 (Supplementary Table S1). PRIMA-1 has been reported to preferentially reactivate and induce cell death in tumor cells with mutant p53 proteins (34). We determined the GI50 for PRIMA-1 in our panel of DLBCL cell lines (Fig. 6A) and found that in fact the GI50 for cell lines with mutated p53 were significantly lower than for those cell lines with WT p53 (P = 0.0004, Wilcoxon’s rank sum test; Fig. 6A; Supplementary Table S2).

To determine whether PRIMA-1 cooperates with BPI in killing lymphoma cells, we exposed BPI-responsive DLBCL cells to each drug alone or in combination. The dose of BPI used was the median of the GI50 values of the cell lines (for a single dose exposure) and the dose of PRIMA-1 was the median of the GI50 of the most responsive cell lines. We found that the concurrent administration of BPI and PRIMA-1 decreased the viability for all the cell lines to a higher degree than either compound alone (Fig. 6B). With the exception of Ly10, this effect was preceded by an increase in the activity of the executioner caspases, caspase-7 and caspase-3, suggesting that apoptosis contributes to the antilymphoma effect of combined therapy in most cell lines (Fig. 6C). Because BPI can induce p53 expression in DLBCL cells, which is the therapeutic target of PRIMA-1, we predicted that the most effective sequencing strategy would consist of first treating with BPI followed by
Our study addresses the therapeutic targeting and functional interaction between BCL6 and p53 in the setting of DLBCL. Previous reports have shown that p53 expression is relatively common in DLBCL, and that its presence is not necessarily correlated with mutations of its coding sequence (reviewed in ref. 36). Although p53 is a BCL6 target gene, a recent report showed that p53 was expressed in BCL6 positive germinal center B-cell type DLBCLs (37). Similarly, we found that median p53 transcript abundance was greater in the BCL6-positive B-cell receptor–type DLBCL and that BCL6 and p53 proteins can be coexpressed in DLBCL (Supplementary Fig. S5). p53 was also expressed in all DLBCL cell lines examined, whether it was mutated or not. However, inhibition of BCL6 in DLBCL cells induced a higher expression of p53. These results suggest that p53 expression is maintained below a critical threshold by BCL6 and/or that p53 is functionally impaired in the presence of BCL6, as otherwise p53 would be expected to counteract BCL6-mediated survival and proliferation. Our data support this scenario because BPI induced the amount and activity of p53 as judged by transcriptional activation of target genes and biological effects. Many mechanisms could account for the functional impairment of p53 in the presence of BCL6 in DLBCLs. One of them could be a BCL6-dependent repression of genes upstream of p53 that control its activity through posttranslational modifications. We have recently shown that BCL6 directly represses ATR, a protein kinase and master regulator of DNA damage sensing and genomic integrity (7). Accordingly, BPI could induce ATR expression and activity, resulting in phosphorylation and activation of p53 (7). Our most recent preliminary data suggest that BCL6 may also impair p53 acetylation (38).

Another mechanism through which BCL6 might attenuate the activity of p53 is by downstream interference with the p53 transcriptional program. It was reported that BCL6 can directly repress p21 (5) and we find that BPI can induce p21 in a p53-independent manner. BCL6 disruption of the p53 program is even more extensive,
Figure 5. BPI and p53 restoration can induce different types of cell death. A, Ly4, Ly1, and Ly10 cells treated for 24 h with control peptides (first row), 10 μmol/L p53C-TAT (second row), 5 μmol/L BPI (third row), or the combination of BPI and p53C-TAT (fourth row) were examined (>400 is shown) by ethidium bromide/acridine orange staining to categorize the morphologic aspect of dead cells. In the ethidium bromide/acridine orange staining method, live cells exhibit a morphologically normal green nucleus; early apoptotic cells are smaller and have a bright green nucleus (indicating condensed or fragmented chromatin with intact plasma membrane); late apoptotic cells are smaller and display bright orange fragmented nucleus (indicating condensed chromatin and affected plasma membrane); whereas necrotic cells are larger and have a structurally normal orange nucleus (indicating cytoplasm swelling, noncondensed chromatin, and rupture of the plasma membrane that can be lost in the late necrosis). EA, early apoptosis; LA, late apoptosis; N, necrosis; LN, late necrosis; V, viable; BN, binucleated cell; D, cell debris. Percentages for each type of dead (necrotic or apoptotic-like in red and yellow, respectively) and viable cells (green) from triplicate experiments are shown in the bars. For each triplicate, we categorized at least 300 cells per experimental condition. B, DNA fragmentation was measured by mononucleosome and oligonucleosome enrichment [(treated A_405nm-A_490nm/control A_405nm-A_490nm) × 100] in the cytoplasmic fraction of cells treated as above. Results represent the mean of biological triplicates, each of which was performed in experimental triplicates. C, caspase-7/3 activity measured by the cleavage of a specific profluorescent substrate in cells treated as above. Y-axis, caspase-7/3 activity (Ex499nm/Em521nm) over cell number determined by multiplexing with a metabolic assay (Ex560nm/Em590nm). Results represent the mean of biological triplicates, each of which was performed in four experimental replicates. D, level of ATP in cells treated for 18 h with control peptides (white columns), 10 μmol/L p53C-TAT (light gray columns), 5 μmol/L BPI (dark gray columns), and the combination of BPI and p53C-TAT (black columns). Results represent the mean of biological triplicates, each of which was performed in four experimental replicates. Western blot showing PARP (113 kDa) and the major fragment of PARP (89 kDa) resulting from caspase activity in cells treated as before.
Figure 6. Cotargeting of BCL6 and p53 enhances antilymphoma activity. A, PRIMA-1 dose-response curves for a panel of seven DLBCL cell lines. The effect (Y-axis) was measured as percentage of cell number in PRIMA-1–treated cells compared with vehicle-treated cells (plotted on the left). The mean of biological triplicates, each of which was performed in four experimental replicates, was plotted in three dose-response curves, one of which is shown on the left. The r values, which represent the goodness of fit of the experimental data with the median-effect principle, were >0.94 for all the cell lines tested. The GI50 values (bar, 95% CI) obtained from the dose-effect curves of PRIMA-1 are shown on the right. The cell lines are represented according to whether TP53 is mutated (closed symbols) or WT (open symbols). B, BCL6-dependent cells were exposed to a single dose of BPI 10 μmol/L or PRIMA-1 25 μmol/L or both drugs for 48 h. Y-axis, cell number measured by metabolic assay comparing treated cells with control cells. Results represent the mean of biological triplicates, each of which was performed in four experimental replicates. C, caspase-7/3 activity was determined in the same panel of cells after 6 and 18 h of exposure to BPI, PRIMA-1, or the combination. Results represent the mean of biological triplicates, each of which was performed in four experimental replicates. D, representation of the combination index values for the effect at 48 h for the sequence of BPI administered once at time 0 h followed by PRIMA-1 administered once at 24 h (●) or the sequence of PRIMA-1 administered once at time 0 h followed by BPI administered once at 24 h (○). CI = 1 ± 0.1 was considered as additive, CI > 1.1 was considered as infra-additive, and CI < 0.9 was considered as supra-additive. The mean of biological triplicates, each of which was performed in four experimental replicates, was plotted in three combinatorial dose-response curves. Points, mean of three CI values for a particular effect; bars, SE. Values are also shown in Supplementary Table S3.
because we find that BCL6 can directly repress GADD45a and reported previously that BCL6 directly regulates additional DNA damage checkpoint genes, including GADD45B and CHEK1 (6). BCL6 can thus suppress genotoxic integrity checkpoints at multiple levels, an effect that is presumably necessary for survival of GC B-cells but that also contributes to lymphomagenesis.

Our current data help to link the biochemical mechanism of action of BCL6 with its biological effects in lymphoma cell survival. DLBCL survival requires binding of the SMRT and N-CoR corepressors to the BCL6 BTB domain, which is the target of BPI (6, 8). The fact that BPI up-regulates p53 indicates that repression of p53 is dependent on SMRT and N-CoR and explains in part how blockade of the BCL6 BTB domain can kill lymphoma cells. In contrast, BPI has no effect on BCL6 target genes involved in differentiation such as PRDM1 (8). Instead, BCL6 represses PRDM1 through the MTA3/NuRD corepressor complex (12, 39). Opposite to BPI, MTA3/BPI has no effect on BCL6 target genes involved in differentiation and of the BCL6 BTB domain can kill lymphoma cells. In contrast, BPI is dependent on SMRT and N-CoR and explains in part how blocking the BCL6 BTB domain can kill lymphoma cells. Therefore, these results show the functional separation of BCL6-controlled pathways due to differential corepressor requirements.

Taken together, our data reveal a complex functional relationship between p53 and BCL6 in DLBCL and show that although p53 is often expressed in association with BCL6, it is in a relatively inactive state that can be switched on by therapeutically targeting BCL6.

Restoration of p53 function in tumor cells has been the focus of intense research, either through gene therapy approaches or through small-molecule or peptide therapies aiming to stabilize p53 protein or enhance its transcriptional activity (40). The fact that p53 is usually WT in BCL6-positive DLBCL and can be switched on by blocking BCL6 can provide an excellent opportunity for harnessing the therapeutic potential of p53. The ability to sequentially target successive waves of transcription factors introduces the concept of tandem transcription therapy. In this case, inhibiting BCL6 makes available a second transcription factor (p53), which can also be therapeutically targeted by enhancing its activity. Because blocking BCL6 and enhancing p53 is harmless to normal tissues (8, 18), it may be possible to extensively reprogram death pathways in lymphoma cells with minimal effects on the host. Thus, given sufficient information on the transcriptional networks that sustain specific tumor types, it could be possible in the future to deliver combinatorial therapy that would eliminate or reduce the need for chemotherapy drugs.

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