Pharmacological and Protein Profiling Suggests Venetoclax (ABT-199) as Optimal Partner with Ibrutinib in Chronic Lymphocytic Leukemia

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Abstract

Purpose: Bruton’s tyrosine kinase (BTK) is a critical enzyme in the B-cell receptor pathway and is inhibited by ibrutinib due to covalent binding to the kinase domain. Though ibrutinib results in impressive clinical activity in chronic lymphocytic leukemia (CLL), most patients achieve only partial remission due to residual disease. We performed a pharmacologic profiling of residual circulating CLL cells from patients receiving ibrutinib to identify optimal agents that could induce cell death of these lymphocytes.

Experimental Design: Ex vivo serial samples of CLL cells from patients on ibrutinib were obtained prior and after (weeks 2, 4, and 12) the start of treatment. These cells were incubated with PI3K inhibitors (idelalisib or IPI-145), bendamustine, additional ibrutinib, or BCL-2 antagonists (ABT-737 or ABT-199), and cell death was measured. In vitro investigations complemented ex vivo studies. Immunoblots for BTK signaling pathway and antiapoptotic proteins were performed.

Results: The BCL-2 antagonists, especially ABT-199, induced high cell death during ex vivo incubations. In concert with the ex vivo data, in vitro combinations also resulted in high cytotoxicity. Serial samples of CLL cells obtained before and 2, 4, 12, or 36 weeks after the start of ibrutinib showed inhibition of BTK activity and sensitivity to ABTs. Among the three BCL-2 family antiapoptotic proteins that are overexpressed in CLL, levels of MCL-1 and BCL-XL were decreased after ibrutinib while ABT-199 selectively antagonizes BCL-2.

Conclusions: Our biologic and molecular results suggest that ibrutinib and ABT-199 combination should be tested clinically against CLL. Clin Cancer Res. 21(16): 3705–15. ©2015 AACR.

Introduction

Chronic lymphocytic leukemia (CLL) is a neoplastic disorder characterized by mature B lymphocytes that accumulate due to impaired programmed cell death (1). These cells reside in micro-environmental niches, such as peripheral blood, bone marrow, and lymph nodes (2). The gene expression profile differs between compartments; however, current treatments do not target the unique biology of this disease (3, 4). In fact, the standard of care for CLL is a cytotoxic therapy that includes clonal deletion, apoptosis, and lymph node destruction (2). The BCL-2 family of proteins has provided a targeted nonchemotherapy alternative, as this pathway is a primary requisite for development and maintenance of both normal and malignant B cells (11, 12). This network is important for B-cell proliferation, differentiation, and survival as well as cell migration and tissue homing (13). Bruton’s tyrosine kinase (BTK) is a pivotal enzyme in the BCR pathway (14). Ibrutinib (PCI-32765) is a highly selective and irreversible inhibitor of BTK (14); this oral agent binds covalently to cysteine-481, which is located in the active site of this enzyme, thus inhibiting its activity (with an IC50 = 0.5 nM). These results underscore the need for a regimen that targets the biology of CLL disease.

Studies of the B-cell receptor (BCR) axis and its downstream kinases have provided a targeted nonchemotherapy alternative, as this pathway is the primary requisite for development and maintenance of both normal and malignant B cells (11, 12). This network is important for B-cell proliferation, differentiation, and survival as well as cell migration and tissue homing (13). Bruton’s tyrosine kinase (BTK) is a pivotal enzyme in the BCR pathway (14). Ibrutinib (PCI-32765) is a highly selective and irreversible inhibitor of BTK (14); this oral agent binds covalently to cysteine-481, which is located in the active site of this enzyme, thus inhibiting its activity (with an IC50 = 0.5 nM). These results underscore the need for a regimen that targets the biology of CLL disease.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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signals from the microenvironment, downregulated survival
and proliferative pathways, and lacked cytotoxicity toward T
cells (12, 18–21). Importantly, a phase I trial (14), phase II
trial (22), and subsequent trial of ibrutinib in elderly patients
with CLL (23) demonstrated high tolerability and an overall
response rate of >70%, with a 26 month progression-free and
overall survival rate of >75% (22).

Although ibrutinib results in impressive clinical outcomes,
it has limitations. First, most responses have been partial remis-
sions, and continuous use of the drug is required. For patients
with 17p abnormalities, even ibrutinib as a front-line therapy did
not produce any complete remissions (24). Second, recent genomic
profiling studies of CLL patients who acquired resistance to
ibrutinib identified resistance mutations in BTK and phospholi-
pase C\(^{\text{2}}\) kinase, as well as genetic alterations unrelated to the BCR
pathway (25–28). Third, although lymph nodes shrink after
ibrutinib therapy, the disease is not cleared efficiently from the
bone marrow (22).

To overcome these limitations, we performed a pharmacolog-
ics profiling in residual circulating CLL cells after ibrutinib
therapy to identify agents that could induce cell death of these
lymphocytes. These post-ibrutinib CLL cells were incubated with
phosphatidylinositol-3 kinase (PI3K) inhibitors (idelalisib
or IPI-145), a chemotherapeutic agent (bendamustine),
additional ibrutinib, BCL-2 antagonist (venetoclax, ABT-
199), or BCL-2 and BCL-X\(_{\text{L}}\) antagonist (ABT-737). The BCL-2
antagonists (especially ABT-199) most effectively induced
cell death during ex vivo incubations. In accordance with these
results, the in vitro combination of ibrutinib and a BCL-2
antagonist showed additive or more than additive cytotoxicity.
Serial samples of CLL cells obtained from patients on clinical
trial, before (baseline) and after (at 2, 4, 12, and 36 weeks)
ibrutinib therapy initiation, showed inhibition of BTK activity,
decreased MCL-1 protein, and increased sensitivity to the BCL-2
antagonists. Collectively, among the agents tested, our results
identified ABT-199 as an ideal partner to be combined with
ibrutinib.

**Translational Relevance**

Ibrutinib has revolutionized the way CLL is treated due to
its impressive clinical activity in this disease. Despite its
clinical effectiveness, the drug does not result in deep
remission and has not been able to cure this malignancy.
Recent studies have also suggested an emerging mechanism
of resistance found in some patients on ibrutinib treatment,
^difficult prompting the development of combination stra-
tegies. Using residual CLL cells after ibrutinib therapy in
our pharmacologic profile, we identified that the BCL-2
antagonist ABT-199 was able to augment ibrutinib’s effects
making these two drugs a potential combination to use in
the clinic. *In vitro* combination studies further confirmed
the synergy.

At the molecular level, our results show ibrutinib therapy
reduced MCL-1 and BCL-X\(_{\text{L}}\) protein levels, whereas ABT-199
targeted BCL-2; hence, this combination targets the three
antiapoptotic BCL-2 family proteins that drive the survival
and proliferation of CLL cells.

**Materials and Methods**

**Drugs and reagents**

Ibrutinib and ABT-199 were respectively purchased from Sell-
eckhem and Xecbio, whereas ABT-737 was provided by Abbott.
Goat F(ab’\(^{\text{2}}\))2 fragments to human IgM were purchased from MP
Biomedicals.

**Isolation of lymphocytes**

All experiments were carried out using freshly isolated cells
from peripheral blood of patients with CLL. After isolation, cells
were immediately suspended in warm medium; there was no
interval freezing. Patients gave written informed consent to par-
ticipate in this laboratory protocol, which was approved by the
Institutional Review Board (IRB) of MD Anderson Cancer Center.
Cells were isolated using Ficoll–Hypaque (Life Technologies)
as described (18). The isolated lymphocytes were resuspended
(1 × 10\(^{7}\) cells/mL) in RPMI-1640 medium supplemented with
10% human AB serum (Cambrex Biosciences). The cell number
and mean cell volume were determined using a Coulter Channe-
lyzer (Coulter Electronics).

**Sample collection during clinical trial**

For *ex vivo* incubations and for serial sampling, blood samples
were obtained from patients enrolled in ibrutinib trials. All patients
received 420 mg of ibrutinib per day, and samples were collected
before and/or at 2, 4, and 12 weeks after start of ibrutinib treat-
ment. Collection of blood sample at baseline and at 36 weeks after
ibrutinib therapy (used for Supplementary Fig. S3) was also done
at Ohio State University using an IRB-approved clinical protocol.

**Cell death assessment**

A *in vitro* or *ex vivo* treatments, CLL cells were resuspended in
binding buffer (Roche) and stained with Annexin V (BD Phar-
mingen) plus propidium iodide (PI; Sigma-Aldrich). Cells were
measured using a Becton Dickinson FACSCalibur flow cytometer.
A time-matched control was treated with DMSO alone, and % cell
death obtained in this control was subtracted from drug-treated
value.

**Immunoblot assays**

Treated or untreated cells were lysed using Complete Mini
Protease Inhibitor Cocktail (Roche) in RIPA buffer (Bio-Rad).
Protein content was measured using a DC protein assay kit (Bio-
Rad). Protein samples were electrophoresed on Criterion bis-Tris
gels using XT MOPS buffer (Bio-Rad) and transferred to nitrocel-
lulose membranes. Multiple proteins of similar size were analyzed
using stripping and reprobing of the membrane. Primary anti-
odies to detect total and phosphorylated proteins and their
sources are listed (Supplementary Table S1).

**Gene expression assay**

RNA was isolated using the RNAeasy Kit (Qiagen), and relative
transcript levels of gene expression were assessed using TaqMan
One Step RT-PCR (Applied Biosystems). Predesigned primers and
probes are listed (Supplementary Table S1). Experiments were
done in triplicate, and the results were plotted as fold change
compared with control.

**Reverse-phase protein array**

Protein lysates from CLL cells from patients on clinical trials
were arrayed on nitrocellulose coated slides and were probed with
antibodies. The signal obtained was amplified using a Dako Cytomation–catalyzed system (Dako). Relative protein levels were determined by interpolation of each dilution curve, and all data were normalized for protein loading. Linear mixed-effect modeling was used to assess the differences in protein expression. This model includes the fixed effect of time point (2 levels: pre- vs. posttreatment) and the random effect of patient. To account for multiple testing, we estimated the false discovery rates of the F-tests of the time point effect using the Benjamini–Hochberg method (29).

TCL1 CLL adoptive transfer mouse model
We used a TCL1 adoptive transfer CLL mouse model with a TCL1 leukemic clone (TCL1-192) that expresses BCR-reactive malignant cells (30). Spleen cells were provided by Dr. Chiorazzi (Feinstein Institute for Medical Research, Manhasset, NY). Once mice developed the disease, CLL cells were obtained from mouse blood and incubated with no drug, ibrutinib, ABT-199, and ibrutinib plus ABT-199. Flow cytometry analyses were done for Annexin V/PI staining to quantitate percent apoptosis and for the CLL B-cell markers CD5 and B220.

Statistical analysis and determination of drug interactions
Statistical tests were done using GraphPad Prism (GraphPad Software). For calculation of ibrutinib and second drug interaction, we used the fractional product method described by IL Webb. The fractional product was used to determine whether the effect on cell viability and apoptosis induced by the combinations was additive/synergistic/antagonistic. This method is appropriate when nonexclusive drugs (agents with independent mechanisms of action) are combined.

Results
Ex vivo studies of postibrutinib residual circulating CLL cells
Blood from patient #595, who had been treated with ibrutinib for 4 weeks, was isolated and incubated with no drug, ibrutinib, ABT-199, and ibrutinib plus ABT-199. Flow cytometry analyses were done for Annexin V/PI staining to quantitate percent apoptosis and for the CLL B-cell markers CD5 and B220.

Figure 1.
Ex vivo studies in circulating residual CLL cells from patients enrolled in an ibrutinib clinical trial. A, lymphocytes isolated from 1 ibrutinib-treated CLL patient (#595) were incubated ex vivo for 24 hours with therapeutic agents currently in clinical trials or approved for CLL: inhibitors of PI3K (idelalisib (GS1101; GS) and IPI-145), BTK inhibitor (ibrutinib (IB)), BCL-2 antagonists (ABT-737 and ABT-199), and an alkylating agent [bendamustine (Benda)]. The concentrations of the drugs are shown on the abscissa. After the incubation period, cell death was determined by Annexin V/PI staining using flow cytometry. Untreated (U) and vehicle-treated (V) cells were also included for each experiment. The level of endogenous cell death, determined in vehicle control (DMSO)-treated cultures, was subtracted from all treatments in each sample to determine the cytotoxicity exerted only by the agents. B, additional CLL cells were isolated from peripheral blood obtained from patients (n = 10) who had received ibrutinib for 4 weeks and were treated ex vivo under the same conditions as in A. C and D, lymphocytes were isolated from the blood of patients who had received ibrutinib for 2, 4, and 12 weeks and were treated with ABT-737 (C) and ABT-199 (D) ex vivo for 24 hours. Cell death was measured and plotted as described for A.
ABT-737 (Fig. 1B; P value < 0.004). Still, excluding ABT-199, when compared with the other agents, ABT-737 also induced significant cell death. Altogether, these data suggest that BCL-2 inhibition is effective in ibrutinib-treated lymphocytes.

To determine if the ABTs’ cytotoxicity could be replicated at earlier and later time points, we isolated lymphocytes from peripheral blood collected at shorter and longer times after patients began ibrutinib treatment. The rates of ABT-737–mediated cell death were a median 47%, 33%, and 38% in samples collected at 2 (n = 2), 4 (n = 9), and 12 weeks (n = 7) after ibrutinib, respectively (Fig. 1C; Supplementary Table S4). Comparison of week 4 data with week 12 data suggested similar

Figure 2. In vitro studies in CLL lymphocytes obtained from blood of ibrutinib-naive CLL patients. A–C, IC50 values were calculated for ibrutinib (A), ABT-737 (B), and ABT-199 (C). For this, CLL cells were incubated with the individual drugs at various concentrations, and cell viability was then tested using Annexin V/PI staining. D–G, a second IC50 assessment was performed for the combinations using 2 ibrutinib concentrations, 5 and 10 μmol/L, with ABT-737 (D and E) and ABT-199 (F and G). Cells were incubated with escalating concentrations of ABT-737 and a fixed concentration of ibrutinib, 5 μmol/L (D) or 10 μmol/L (E), or with escalating doses of ABT-199 and a fixed concentration of ibrutinib, 5 μmol/L (F) and 10 μmol/L (G). Cell death was measured as described before. H and I, assessment of combination. H, lymphocytes were left untreated (Unt) or incubated with the vehicle DMSO (0), ABT-737, ibrutinib, or the combination of ABT-737 plus ibrutinib for 24 hours. A parallel experiment of the same 4 treatments was done in cells stimulated with 10 μg/mL anti-IgM 30 minutes after vehicle, ibrutinib, or ABT-737 was added to the culture. I, conditions similar to those described in H were followed, except ABT-737 was replaced with ABT-199. Cell death was measured as described before. J, changes in levels of phosphorylated and total protein after in vitro incubations. Immunoblots were obtained after 24 hours of incubation of CLL cells from patient 424 with single-agent ibrutinib in the absence (left) or presence (right) of anti-IgM (added 30 minutes after ibrutinib); analyzing for targets in the BTK signaling pathway and BCL-2 family proteins. Only one GAPDH was needed because all proteins were done from the same gel and membrane by cutting in several sections and using antibodies from different species. We use a fluorescence-based imaging system, LiCor Odyssey system to analyze our immunoblots. With this system, our secondary antibodies against primary antibodies derived from different species are tagged with different fluorescence colors.
extent of cell death ($P = 0.98$). Similar to ABT-737, ex vivo treatment with ABT-199 resulted in median 49%, 62%, and 58% cell death in week 2 ($n = 2$), week 4 ($n = 9$), and week 12 samples ($n = 8$; Fig. 1D; Supplementary Table S4), and was similar at weeks 4 and 12 ($P = 0.84$). Comparison of data with ABT-737 and ABT-199 demonstrated that ABT-199 is more potent. Collectively, these data suggest that CLL lymphocytes are sensitive to BCL-2 inhibition regardless of ibrutinib treatment duration or patient characteristics (Supplementary Table S3) and that ABT-199 is the more potent agent under these conditions.

### In vitro studies in lymphocytes from ibrutinib-naïve CLL patients

To determine if the ex vivo results could be recapitulated in vitro, we studied lymphocytes from ibrutinib-naïve CLL patients. To determine optimal concentrations to use, we calculated the IC$_{50}$ values of ibrutinib and the BCL-2 antagonists alone. To determine optimal concentrations to use, we calculated

\[
\text{IC}_{50} = \frac{\text{free concentration of the drug}}{\text{total concentration of the drug}}
\]

Below are the IC$_{50}$ values of ibrutinib and the BCL-2 antagonists alone.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC$_{50}$ Value (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ibrutinib</td>
<td>0.15</td>
</tr>
<tr>
<td>ABT-737</td>
<td>0.05</td>
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<tr>
<td>ABT-199</td>
<td>0.02</td>
</tr>
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</table>

With these concentrations, we tested the effects of ibrutinib, ABT-737, and ABT-199 in combination with IgM stimulation. The molecular impact of ibrutinib on CLL cells in the absence or presence of anti-IgM was analyzed by immunoblots evaluating for targets in the BTK signaling pathway and BCL-2 family proteins prevalent in CLL cells. IgM stimulation resulted in phosphorylation (activation) of p-BTK-Y223, p-AKT-S473, and other proteins.

### Table 1. In vitro evaluation of ABT-199 and ABT-737 in combination with ibrutinib using previously untreated lymphocyte samples

<table>
<thead>
<tr>
<th>CLL patient</th>
<th>ABT-737 1 nmol/L + ibrutinib</th>
<th>ABT-199 1 nmol/L + ibrutinib</th>
</tr>
</thead>
<tbody>
<tr>
<td># 403</td>
<td>Expected % Cell death</td>
<td>Observed % Cell death</td>
</tr>
<tr>
<td>87</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>592</td>
<td>39</td>
<td>42</td>
</tr>
<tr>
<td>270</td>
<td>2</td>
<td>4</td>
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<tr>
<td>284</td>
<td>38</td>
<td>47</td>
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<td>868</td>
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<td>103</td>
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<td>279</td>
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<td>835</td>
<td>21</td>
<td>25</td>
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<td>189</td>
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<td>33</td>
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<td>424</td>
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<td>256</td>
<td>37</td>
<td>45</td>
</tr>
<tr>
<td>625</td>
<td>13</td>
<td>15</td>
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\[
P = 0.04; n = 9\]

We then investigated the role of ibrutinib when the BCR pathway is active, and conducted a parallel experiment of the same 4 treatments in cells stimulated with 10 µg/mL anti-IgM. This stimulation always resulted in less cell death. Still, both combination treatments (ibrutinib plus ABT-737 or ABT-199) were more cytotoxic than the single agents (Fig. 2H and I). Similar studies were performed in additional primary CLL samples (Table 1). The combination of ABT-737 and ibrutinib in the absence of anti-IgM stimulation resulted in a cytotoxic range of 15% to 45% similar to the expected cell death range (13%–44%). While in the presence of IgM, the observed range of cell death was 4% to 47%, which was higher than the expected value (2% to 38%). The combination of ABT-199 and ibrutinib in the absence of anti-IgM stimulation resulted in 36% to 63% cell death, which was higher than expected (23% to 53%). Similar results were observed in the stimulation treatments (8% to 60%), with rates of cell death higher than expected (3% to 50%). These data suggest that the combination of ibrutinib and an ABT is more cytotoxic against CLL cells than any of the agents alone, regardless of whether BCR is active and independent of the patients’ cytogenetics (Supplementary Table S3); the combined level of cytotoxicity was either additive or synergistic.

The molecular impact of ibrutinib on CLL cells in the absence or presence of anti-IgM was analyzed by immunoblots evaluating for targets in the BTK signaling pathway and BCL-2 family proteins prevalent in CLL cells. IgM stimulation resulted in phosphorylation (activation) of p-BTK-Y223, p-AKT-S473, and other proteins.

To determine if the ex vivo results could be recapitulated in vitro, we studied lymphocytes from ibrutinib-naïve CLL patients. To determine optimal concentrations to use, we calculated the IC$_{50}$ values of ibrutinib and the BCL-2 antagonists alone. To determine optimal concentrations to use, we calculated

\[
\text{IC}_{50} = \frac{\text{free concentration of the drug}}{\text{total concentration of the drug}}
\]
Cervantes-Gomez et al. showed heterogeneity in MCL-1 and BCL-XL protein levels at gated in ibrutinib-incubated CLL cells. Interestingly, our results Fig. 3A). ABT-737 and especially ABT-199 resulted in increased

**Ex vivo studies of pre- and postibrutinib residual circulating CLL cells**

To establish if lymphocytes differed in drug sensitivity before and after ibrutinib treatment, we collected a sample (from patient #775) at weeks 0 (pre-ibrutinib), 4, and 12 (post-ibrutinib; Fig. 3A). ABT-737 and especially ABT-199 resulted in increased cell death compared with the other agents regardless of ibrutinib treatment time. To confirm these results, we collected 5 additional patient samples for the same weeks and tested them under similar conditions with all 6 drugs as well as with vehicle (DMSO) control. Endogenous cell death (in vehicle control sample) was higher in pre-ibrutinib lymphocyte sample (median endogenous cell death, 29%; range, 5%–57%) than in sample 4 weeks after ibrutinib (median, 10%; range, 10%–36%) and sample 12 weeks after ibrutinib (median, 12%; range, 4%–13%; Fig. 3B). The level of endogenous cell death was not associated with the increase or decrease in lymphocyte count after ibrutinib therapy (Fig. 3C). Except in 1 patient (#327) treated with bendamustine, all drugs elicited <40% cytotoxicity (Fig. 3D). ABT-737- and ABT-199-induced cytotoxicity was plotted for the same 6 CLL samples at weeks 0, 4, and 12 (Fig. 3E). ABT-737-mediated median cell death was similar in pre-ibrutinib and post-ibrutinib CLL samples (Supplementary Table S5). ABT-199 treatment yielded higher levels of cell death overall, but the values were similar in preibrutinib samples (median, 53%) and samples obtained at 4 weeks (57%) and 12 weeks (59%) after the start of ibrutinib.
therapy (Supplementary Table S5). Samples obtained at 36 weeks and treated at a separate site (Ohio State University) also showed similar level of sensitivity to ABT-199 (median, 58% apoptosis; Supplementary Fig. S3). These data suggest that lymphocytes before ibrutinib or residual CLL cells after ibrutinib are similarly sensitive to ABT-induced cytotoxicity. Furthermore, both the in vitro and ex vivo studies demonstrated that ibrutinib is more potent than ABT-737.

Immunoblots were performed in lymphocytes obtained from patients on ibrutinib treatment. Ibrutinib administration resulted in inhibition of BTK activity in primary CLL cells, as shown by the decrease in autophosphorylation at p-BTK-Y223 (Fig. 4A and B). To evaluate the impact of this inhibition on changes in the levels of BCL-2 family proteins, we performed reverse-phase protein array (RPPA) in the same freshly obtained CLL cells isolated from peripheral blood of patients receiving ibrutinib. There was a consistent and significant decrease in MCL-1 protein levels, which is consistent with human CLL cell studies (Table 1). Bendamustine, an approved and well-tolerated alkylating agent for CLL, also was not a stimulator for cell death (34). In contrast with these above tested agents, inhibition of BCL-2 consistently resulted in induction of cell death. Furthermore, between the two BH3 mimetics, ABT-199 unfailingly produced the higher percentage of cell death.

For the current investigation, we selected 6 drugs based on their current use for treatment of CLL and mechanistic rationale to combine with ibrutinib. Earlier preliminary investigations in ibrutinib-treated CLL patient samples identified transcription inhibitor, dinaciclib, that targets cyclin-dependent kinase (CDK) inhibitor as a potent cell death inducer in these lymphocytes (35). In contrast, similar to present investigation, kinase inhibitors that impact components of BCR pathway did not induce apoptosis. Recent investigations using similar model system suggested carfilzomib, an endoplasmic reticulum (ER) stress agent, as a potential partner with ibrutinib (36). The positive interaction between these drugs was prevalent at different times after start of ibrutinib. For example in our studies, cell obtained after weeks 2, 4, 12 (Fig. 1C and D), and 36 (Supplementary Fig. S3) showed similar cell death with ABT-199. Because ABT-199–mediated cytotoxicity was similar in pre- and post-ibrutinib samples, these data suggest that most of the cell death response is

**Discussion**

Ibrutinib has revolutionized how we treat CLL; it targets the very pathway that is involved in production and maintenance of B lymphocytes. Ibrutinib covalently binds and inhibits BTK, which is critical in the BCR pathway. Recent investigations have identified that signaling proteins in the BCR axis not only play a pivotal role in the development of normal and malignant B cells but also become mutated or modified in CLL cells from patients whose disease is becoming resistant to ibrutinib (25–28). Hence, targeting additional elements in the BCR pathway could result in increased cytotoxicity. Idelalisib (GS1101) targets the p110 delta isoform of PI3K, while IPI-145 targets both delta and gamma isoforms (31, 32). These PI3K subunits are expressed at high levels primarily in lymphocytes, providing selectivity with these inhibitors. Because these kinases dominate the BCR pathway, they were primary molecules for our testing. Consistent with the data with idelalisib and IPI-145, additional ibrutinib also did not provide increased cytotoxic benefit (Figs. 1B and 3D). Although these inhibitors may provide an advantage in the clinic by mobilizing CLL cells from the lymph nodes, our primary goal was to induce cell death in cells that are either mobilized or residual after ibrutinib. Furthermore, the kinase inhibitors may be effective for ibrutinib-resistant CLL clone (33). Bendamustine, an approved and well-tolerated alkylating agent for CLL, also was not a stimulator for cell death (34). In contrast with these above tested agents, inhibition of BCL-2 consistently resulted in induction of cell death. Furthermore, between the two BH3 mimetics, ABT-199 unfailingly produced the higher percentage of cell death.

**In vitro studies in lymphocytes from TCL1 mouse model**

To determine if the human CLL cell studies could be recapitulated in lymphocytes derived from a CLL mouse model, cells from the TCL1 mouse were isolated and incubated with ABT-199 (0.5 and 1 nmol/L) and ibrutinib (10 μmol/L) for 24 hours. These data resulted in an additive or more than additive cell death as assessed by Annexin V/PI staining (Supplementary Fig. S5), which is consistent with human CLL cell studies (Table 1).

**Figure 4.** Total and phospho BTK protein levels in cells obtained from the blood of CLL patients before and after ibrutinib therapy. A, immunoblots analyzing for p-BTK-Y223 and total BTK protein levels in untreated lymphocytes isolated from patients before (week 0) and after ibrutinib (weeks 2, 4, and 12) initiation. Both total and phospho-BTK were from the same gel. Because the antibodies were from the same species, the membrane was stripped and reprobed. We use a fluorescence-based imaging system, LiCor Odyssey system, to analyze our immunoblots. B, quantitation was performed for each band, and results plotted as change compared with control (week 0) for each post-ibrutinib sample (weeks 2, 4, or 12) and plotted for p-BTK.
Similar to CLL model system, major efforts have been utilized in other B-cell malignancies where ibrutinib and BCL-2 antagonists have shown promising activity in B-cell diseases, such as mantle cell lymphoma (37), Waldenstrom’s macroglobulinaemia (38), and diffuse large B-cell lymphoma (39). Ibrutinib’s primary action on cell proliferation is in concert with co-operation with transcription inhibitor dinaciclib, ER stressor carfilzomib, and BCL-2 antagonist, ABT-199. These reports direct us to recognizing best small molecule combination drug partner with ibrutinib. However, combination strategies that pair ibrutinib with rituximab, GA101, or ofatumumab warrant preclinical testing especially because efficacy with antibodies to treat CLL has been shown in the clinic, and these agents are FDA approved (40).

While detailed mechanistic studies were not performed, CLL biology, characteristics of these agents, and the results obtained with these combinations provide clues to explain these cell death differences. Among the 6 BCL-2 family survival proteins, BCL-2, BCL-X<sub>L</sub>, and MCL-1 are the most abundant in CLL cells (41). In addition, among these 3 proteins, BCL-2 and BCL-X<sub>L</sub> are present at
levels 4 to 12 times higher than MCL-1 (42). Although ABT-737 targets and binds with high affinity (Ki ≤ 1 nmol/L) to the hydrophobic BH3-binding groove of BCL-XL, BCL-2, and BCL-w (43). ABT-199 targets only BCL-2 with much higher potency (Ki ≤ 0.01 nmol/L; ref. 44). Prolonged and potent perturbation of either BCL-2 or BCL-Xl should result in apoptosis, as the overall stoichiometry of these molecules is a determinant of CLL cell survival or death (37). Ibrutinib and ABT-737 cooperated well in DLBCL (39). Finally, our data suggest that ibrutinib therapy constantly causes an intracellular MCL-1 decrease (Fig. 5A, D, and E), which may further augment ABT-199–induced apoptosis. Although detailed time kinetics were not captured in our investigations, at 4 and 12 weeks after ibrutinib initiation, CLL cells consistently showed a decline in MCL-1 protein levels in RPPA and immunoblot assays (Fig. 5A, D, and E). This result however was not observed during in vitro incubations, which can be attributed to limited incubation time (24 hours for in vitro assays vs. minimum 2 weeks for in vivo studies) with ibrutinib (Supplementary Fig. S1). MCL-1 protein decrease or induction of proapoptotic protein Noxa by dinaciclib and car

Our data also suggest that ibrutinib may add benefit to ABT-737 (and ABT-737) is beneficial to ibrutinib therapy. First, among the tested agents, addition of ABT-199 was most conducive in inducing apoptosis. Second, residual or lymphocytosed cells were sensitive to ABT-199–mediated cytotoxicity. Third, compared with baseline (before ibrutinib) CLL cells, post-ibrutinib mobilized CLL cells were relatively resistant to cell death (data not shown). However, they were equally sensitive to ABT-199 (Supplementary Table S5). Fourth, at the systemic level, CLL cells reside in 3 different niches in the human body: lymph nodes, bone marrow, and peripheral blood. Ibrutinib preferentially targets the lymph node reservoir, whereas ABT-199 addresses CLL cells residing in blood and marrow. Early preliminary data (44) and data from a subsequent phase 1 study of ABT-199 in patients with high risk relapsed or refractory CLL (49) reported an overall response rate of 84%, with a complete remission rate of 23%. Notably, ABT-199 resulted in the clearance of leukemia cells from the blood and marrow. In fact, no or low minimum residual disease was observed in 8 patients treated (49). Consequently, these 2 drugs would be beneficial in removing malignant cells from all three compartments.

Our data also suggest that ibrutinib may add benefit to ABT-737 or ABT-199. First, when 2 different doses of ibrutinib were added to ABT-737, the IC50 of ABT-737 changed from 6 nmol/L to 2 nmol/L to 1 nmol/L (Fig. 2B, D, and F). Such reduction in IC50 demonstrates the advantage of adding ibrutinib, and it also indicates that in the clinic we can do a dose reduction for ABT-263 which will alleviate drug-induced thrombocytopenia.
which was problematic for its use. Second, when two different doses of ibrutinib were combined with ABT-199, the IC50 of ABT-199 was reduced from 2 nmol/L to 1 nmol/L to 0.5 nmol/L (Fig. 2C, F, and G). Hence, in clinic, ibrutinib may be combined with lowest dose of ABT-199 as suggested in the current clinical trial in high-risk patients with relapsed/refractory CLL (49). Third, data presented in Table 1 provide cytotoxic basis of synergy when ibrutinib is added to ABT-199 or ABT-737 in IgM-stimulated cells (P values = 0.0002 and 0.0004). Fourth, decrease in MCL-1 protein level by ibrutinib (Fig. 5A, D, and E) provides another advantage to ABT-737 and ABT-199 as these BCL-2 antagonists do not target MCL-1, and this has been shown to be one of the mechanisms for resistance to ABT-263 (50). At the cellular and molecular level, our data suggest that by combining these two agents (ibrutinib and ABT-199 or ABT-737), we will target all 3 BCL-2 survival proteins, MCL-1, BCL-XL, and BCL-2. CLL malignant cells have been shown to be addicted to these proteins for their maintenance and survival; for example, an ABT-199–resistant cell line was sensitized to ABT-199 by BCR nexus (such as PI3K, AKT, and mTOR) inhibitors (51). Hence, we postulate that the combination approach described in this study has the potential to eradicate CLL cells, and a clinical trial combining these 2 agents is needed to validate this combination.

Disclosure of Potential Conflicts of Interest

M.J. Keating is a consultant/advisory board member for Genentech. V. Gandhi reports receiving commercial research grants from Pharmacyclics. No potential conflicts of interest were disclosed by the other authors.

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