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 after 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-X_L 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.

Introduction

Chronic lymphocytic leukemia (CLL) is a neoplastic disorder characterized by mature B lymphocytes that accumulate due to impaired program 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 fludarabine plus cyclophosphamide with the monoclonal antibody rituximab (FCR; refs. 5–7). In newly diagnosed patients, FCR results in a 95% overall response rate, with a 70% rate of complete remissions, and about 1/3 of patients have event-free survival for approximately 10 years (i.e., cure). Yet, the FCR regimen has many limitations. For example, fludarabine-based regimens were not associated with improved outcomes in older (age ≥65 years) patients, who make up the majority of the CLL population (8). In addition, patients with markers of poor prognosis, such as 11q del, 17p del, or IgVH unmutated gene or mutated TP53, remain high risk with shorter progression-free survival even with FCR combination therapy (9). Moreover, recent long-term results in first salvage FCR-treated CLL patients (n = 300) emphasize alternative therapeutic strategies. In these individuals, 33% maintained remission after 12 years, whereas about 50% developed relapsed/refractory disease, 8% died in remission, and 7% developed second and aggressive malignancies, such as acute leukemias or Richter's transformation (10). These statistics 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 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 relatively 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 nmol/L, and t1/2 > 24 hours; refs. 15, 16). Lymph nodes and bone marrow provide tumorigenic protective microenvironments for CLL cells via the BCR network, and consequently, BCR kinase inhibitors disrupt this pathway (11, 17). In in vitro, ex vivo, and in vivo settings, ibrutinib inhibited the cytotoxic
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, hence prompting the development of combination strategies. 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\textsubscript{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.

Although ibrutinib results in impressive clinical outcomes, it has limitations. First, most responses have been partial remissions, 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 phospholipase C\textsubscript{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 pharmacologic 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 IP\textsubscript{1}-145), a chemotherapeutic agent (bendamustine), additional ibrutinib, BCL-2 antagonist (venetoclax, ABT-199), or BCL-2 and BCL-X\textsubscript{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 and ABT-199 were respectively purchased from Selleckchem and Xcellbio, whereas ABT-737 was provided by Abbott. Goat F(\text{ab}')\textsubscript{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 participate 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\textsuperscript{6} 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 Channelizer (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 treatment. 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

For in vitro or ex vivo treatments, CLL cells were resuspended in binding buffer (Roche) and stained with Annexin V (BD Pharmingen) 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 nitrocellulose membranes. Multiple proteins of similar size were analyzed using stripping and reprobing of the membrane. Primary antibodies to detect total and phosphorylated proteins and their sources are listed (Supplementary Table S1).

Gene expression assay

RNA was isolated using the RNeasy 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 obtained, and lymphocytes were isolated and incubated ex vivo for 24 hours with 6 drugs at the indicated concentrations and cell death was measured. The level of endogenous cell death was subtracted from each sample. ABT-737 and ABT-199 (5 and 10 nmol/L) resulted in >60% apoptosis, while a low cytotoxic effect was observed for the other agents (Fig. 1A). In order to confirm this pattern of cytotoxicity, we obtained blood samples from 10 additional patients who had received ibrutinib for 4 weeks and incubated their lymphocytes in the same manner (Fig. 1B). Overall, ABT-199 was the most potent cytotoxic agent (median cell death: 61%), followed by ABT-737 (median cell death: 33%; Fig. 1B and Supplementary Table S2), regardless of the patients' cytogenetics, disease stage, and previous treatment (Supplementary Table S3). Significant cytotoxicity was determined for ABT-199 when it was compared with the rest of agents, including

![Graph](image_url)

**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
extent of cell death ($P = 0.98$). Similar to ABT-737, ex vivo treatment with ibrutinib 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 ibrutinib demonstrated that ibrutinib 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 ibrutinib 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 IC50 values of ibrutinib and the BCL-2 antagonists alone. To determine optimal concentrations to use, we calculated in vitro 

$$279 37 40 28 38 51 50 60$$

$$202 37 45 30 43 51 50 48 52$$

$$256 24 26 23 27 34 41 31 35$$

$$424 25 33 25 32 44 47 38 50$$

$$189 48 63 44 53$$

$$75 23 21 16 23 45 41 38 39$$

$$625 13 15 12 13 23 36 14 26$$

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.

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

<table>
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<th>CLL patient</th>
<th>Nonstimulated ABT-737 1 nmol/L + ibrutinib 10 μmol/L</th>
<th>igM stimulated ABT-737 1 nmol/L + ibrutinib 10 μmol/L</th>
<th>Nonstimulated ABT-199 1 nmol/L + ibrutinib 10 μmol/L</th>
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$P = 0.004; n = 9$  
$P = 0.0004; n = 10$  
$P = 0.05; n = 11$  
$P = 0.0002; n = 12$

NOTE: CLL cells were obtained from several patients and were treated with DMSO (vehicle), 1 nmol/L ABT-737 or ABT-199, 10 μmol/L ibrutinib, or a combination of ibrutinib and an ABT for 24 hours in the absence or presence of IgM. Total cell death was determined by flow cytometry, and endogenous cell death observed in DMSO control was subtracted from the drug-treated conditions. The expected percentage of cells surviving after treatment with the combination was calculated using fractional two-drug combinational analysis. $P$ values compare expected versus observed cell death for each of the combinations with or without IgM stimulation.

To compare the expected and observed (Annexin V/PI staining) levels of cell death for the combination treatments, we first calculated the expected level of cell survival for each combination. This was done by multiplying the percentage of cells surviving ibrutinib treatment (100% − X% Annexin V/PI staining) by the percentage of cells surviving ABT-199 or ABT-737 treatment (100% − X% Annexin V/PI staining) and then dividing the result by 100.
p-ERK\textsuperscript{T102/Y204} (Fig. 2I). Activation of these kinases was abrogated in ibrutinib-incubated CLL cells. Interestingly, our results showed heterogeneity in MCL-1 and BCL-X\textsubscript{L} protein levels at 24 hours of ibrutinib treatment in 5 nonstimulated CLL samples (Supplementary Fig. S1). As expected, BCL-2 protein levels did not change in any sample (Supplementary Fig. S1). Similarly, mRNA levels were determined for MCL-1, BCL-X\textsubscript{L}, and BCL-2 (Supplementary Fig. S2A–S2C). Real-time RT-PCR data showed no consistent change in MCL-1, BCL-X\textsubscript{L}, and BCL-2 mRNA levels in the same samples. Collectively, these data suggest that ibrutinib can abrogate BTK activation when stimulatory antigens are present; however, in the absence of IgM, ibrutinib has a heterogeneous effect on the antiapoptotic proteins that drive CLL pathogenesis.

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

To establish if lymphocytes differed in drug sensitivity before and after ibrutinib therapy, 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 treatment. To further confirm these results, we collected and analyzed 24-hour incubations in lymphocytes isolated from the blood of a CLL patient (#775) collected at weeks 0 (pre-ibrutinib; top), 4 (post-ibrutinib; middle), and 12 (post-ibrutinib; bottom). Cells were left untreated (U) or treated with vehicle (V), ABT-199, ABT-737, ibrutinib (IB), IPI-145, GS1101 (GS), and bendamustine (Benda) at the indicated concentrations for 24 hours. Annexin V/PI staining was performed by flow cytometry to determine cell death level. B, endogenous cell death in CLL cells before and after ibrutinib therapy was assessed after 24 hours in vitro incubations in lymphocytes isolated from 6 patient samples on pre- (week 0) and postibrutinib therapy (weeks 4 and 12). C, peripheral blood was obtained from CLL patients (n = 13) before ibrutinib treatment (pre-IB) and 4 weeks after ibrutinib administration was initiated (post-IB). The absolute lymphocyte count for each sample determined from clinical blood count was determined and is plotted as 1,000 lymphocytes per microliter (μL) of blood. D, six CLL patient samples were collected at weeks 0, 4, and 12 of ibrutinib treatment, and lymphocytes isolated and treated under the same conditions described for A, E, cytotoxicity exerted by ex vivo treatment with ABT-737 and ABT-199 on lymphocytes isolated at weeks 0, 4, and 12 of ibrutinib treatment.
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 ABT-199 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 decline in MCL-1 protein levels (Fig. 5A; \( P = 0.0042 \)), with some changes in BCL-XL and BCL-2 (Fig. 5B and C, respectively). Immunoblot assays followed by quantitation validated the RPPA results. For example, levels of antiapoptotic proteins MCL-1 and BCL-X\(_i\) (Fig. 5D) declined significantly after 4 and 12 weeks of ibrutinib therapy (Fig. 5E and F, respectively). In contrast, BCL-2 (Fig. 5D and G) levels showed no consistent decrease or increase after ibrutinib therapy on the 6 analyzed CLL samples. This was not related to cytogenetics, prognostic factor, or disease state. Levels of BIM, total and p-ERK1/2-T202/Y204, and total and p-AKT-S473 (Supplementary Fig. S4) remained unchanged or showed heterogeneity in response to ibrutinib therapy (quantitation of phosphorylated vs. total AKT and ERK proteins are provided below the immunoblot).

RT-PCR analyses for the antiapoptotic proteins MCL-1, BCL-X\(_i\), and BCL-2 were performed to determine if the decrease observed at the protein level was due to diminished mRNA levels. Only 4 of 6 samples showed a decline in MCL-1 and BCL-X\(_i\) transcript levels; BCL-2 results were heterogeneous (Fig. 5H–J, respectively).

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).

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 beta 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.

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, cells 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...
generated by ABT-199. 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 macroglobulaenia (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 carfilzomib, respectively, may be the primary mechanisms for increased cell death. The decline in MCL-1 protein level was partly (4 of 6 samples) associated with a decrease in MCL-1 transcript level in post-ibrutinib samples (Fig. 5H). The BCR pathway, including the PI3K/AKT axis, has been shown to impact transcriptional, translational, and posttranslational modifications of MCL-1, resulting in increased transcription, overexpression of protein, and longer maintenance of protein (45–47). Both during in vitro incubations (Fig. 2I) and especially during clinical trials (Fig. 4A and B), ibrutinib reliably inhibited BTK activity (measured as p-BTK). Our results are in accordance with a previous study showing a decrease in BTK phosphorylation in circulating CLL cells during therapy and consistent with the observation that at a dose of 420 mg/day, ibrutinib completely occupied the BTK-binding pocket (14, 35). Hence, it can be presumed that the BTK pathway would be abrogated in the leukemia cells of these CLL patients, affecting transcriptional and translational modifications of MCL-1 and other proteins.

Inhibition of BTK pathway resulted in unexpected increase in circulating leukemia cells that were due to mobilization of lymphocytes from lymph nodes (22). It has been established that at the molecular level, there are differences in malignant CLL lymphocytes from peripheral blood, bone marrow, and lymph nodes. An increased BCR signature, proliferation markers, and NF-kB activity were identified in gene expression array analyses in cells from lymph node compared with those from marrow or blood (4). Furthermore, transcripts such as STAT-1, MYC, FOS, AKT, and BCL-2 were also several fold higher in lymph node CLL cells compared with those from other compartments (48). Increased expression of BCL-2 transcript is of specific interest because this protein assists CLL cells to survive longer. Compared with the baseline samples, in the ibrutinib–mediated lymphocytosed cells, we observed either similar or an increased expression of BCL-2 protein measured by RPPA assay (Fig. 5C) and validated by immunoblot assay (Fig. 5G).

To mimic these ex vivo studies, we performed in vitro combination cytotoxicity assays by combining BH3 mimetics with ibrutinib. To model BCR stimulation, parallel cultures were anti-igM stimulated. Compared with expected cytotoxicity, calculated based on single-agent response in each sample, the observed cytotoxicity was significantly higher. The extent of this difference was strongest when cells were stimulated for the BCR pathway (P values = 0.0004 and 0.0002 with ABT-737 and ABT-199, respectively; Table 1). Collectively, these in vitro and ex vivo results demonstrate the utility and benefit of combining ABT-199 with ibrutinib in the clinic. Translation of these preclinical results to clinic should be feasible. The clinically decided dose of ibrutinib for CLL (420 mg) is well-tolerated and is actually below the MTD. At this dose, the peak plasma concentration of free drug is around 80 ng/mL; equivalent to 0.2 µmol/L. While in the present work, ibrutinib is used at 10 µmol/L, due to serum/plasma protein binding, the free drug is less than 3% (i.e., 0.3 µmol/L) which is similar to what is acquired during therapy (22). Similarly, the concentration of ABT-199 in our experiments was 1 to 10 nmol/L, which is easily achievable in the clinic (44, 49).

Although these ex vivo investigations of CLL cells during ibrutinib therapy provide a novel and unique cell population to identify small-molecule drugs that collaborate well with ibrutinib, there are limitations of this system. First, we only used Annexin/PI positivity to identify partner agent for ibrutinib. It is highly likely that agents, which may not induce profound CLL lymphocyte death, will be missed during such interrogations. Second, CLL cells pre- and post-ibrutinib are nonproliferative during ex vivo or in vitro culturing. Needless to say that in such a model system, agents that are tackling cell proliferation would not be identified. Third, pathways such as PI3K, AKT, mTOR, and NF-kB that may be critical in CLL biology were not tested during these incubations. Investigations on impact of these networks may provide other molecules to be added to ibrutinib. Finally, animal model system that can recapitulate CLL cells in different niches and microenvironment (blood, bone marrow, and lymph node) may provide selective targeted agents to be coupled with ibrutinib.

Even with these shortcomings, our data and prior investigations provide clear rationale that ABT-199 (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-373 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.

References


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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.A. Woyach, W.G. Wierda, M.J. Keating, V. Gandhi
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Combination of ABT-199 and Ibrutinib for CLL


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