The Proteasome Inhibitor Carfilzomib Functions Independently of p53 to Induce Cytotoxicity and an Atypical NF-κB Response in Chronic Lymphocytic Leukemia Cells


Abstract

Purpose: The proteasome consists of chymotrypsin-like (CT-L), trypsin-like, and caspase-like subunits that cleave substrates preferentially by amino acid sequence. Proteasomes mediate degradation of regulatory proteins of the p53, Bcl-2, and nuclear factor-κB (NF-κB) families that are aberrantly active in chronic lymphocytic leukemia (CLL). CLL remains an incurable disease, and new treatments are especially needed in the relapsed/refractory setting. We therefore investigated the effects of the proteasome inhibitor carfilzomib (CFZ) in CLL cells.

Experimental Design: Tumor cells from CLL patients were assayed in vitro using immunoblotting, real-time polymerase chain reaction, and electrophoretic mobility shift assays. In addition, a p53 dominant-negative construct was generated in a human B-cell line.

Results: Unlike bortezomib, CFZ potently induces apoptosis in CLL patient cells in the presence of human serum. CLL cells have significantly lower basal CT-L activity compared to normal B and T cells, although activity is inhibited similarly in T cells versus CLL. Co-culture of CLL cells on stroma protected from CFZ-mediated cytotoxicity; however, PI3K inhibition significantly diminished this stromal protection. CFZ-mediated cytotoxicity in leukemic B cells is caspase-dependent and occurs irrespective of p53 status. In CLL cells, CFZ promotes atypical activation of NF-κB evidenced by loss of cytoplasmic IκBα, phosphorylation of IκBα, and increased p50/p65 DNA binding, without subsequent increases in canonical NF-κB target gene transcription.

Conclusions: Together, these data provide new mechanistic insights into the activity of CFZ in CLL and support phase I investigation of CFZ in this disease.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the growth and accumulation of malignant B-lymphocytes in the blood, bone marrow, lymph nodes, and spleen. Despite the introduction of new therapies, most CLL patients eventually relapse and succumb to their disease. Identification of new strategies for relapsed and refractory CLL is essential to make a positive impact on patient survival.

Protein homeostasis is critical for the processes that govern cell survival, and the multicatalytic 26S proteasome regulates the turnover of key proteins in these processes. Proteasomes consist of a catalytic 20S core capped by regulatory 19S subunits (1). There are 2 forms of the 20S core: c20S and i20S. The former is ubiquitously expressed in all cells, and the latter specifically in hematopoietic cells and cells exposed to inflammatory cytokines (2). The active site in the c20S and i20S core has 6 N-terminal threonine proteases consisting of chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L) subunits (3, 4). Because selective inhibition of the CT-L site has a minor effect on total cellular protein turnover, proteasome inhibitors that block only the CT-L subunit are considered optimal (5). Proteasomes mediate degradation of a variety of key regulatory factors including cell-cycle control proteins (e.g., cyclins (6), p21 (7) and p27 (8), p53 (9), p53 target proteins Puma, Noxa, and Bax of the Bcl-2 family (10), and the inhibitor of nuclear factor-κB (NF-κB; IκB, ref. 11). Imbalanced expression of Bcl-2 family proteins, constitutive NF-κB activation, and variable p53 function are hallmarks of CLL cells (12–14).
Translational Relevance

Standard therapies for chronic lymphocytic leukemia (CLL) are often ineffective in p53-mutated cases and detrimental to existing T cells, leaving patients at risk of opportunistic infections. A major focus of therapeutic development has been the investigation of agents that are cytotoxic to B cells independent of p53 but have minimal effects on T cells. Proteasome inhibitors have known activity in multiple myeloma, but to date have not shown clinical efficacy in CLL. This manuscript shows B-cell selective efficacy of the proteasome inhibitor carfilzomib (CFZ) that is independent of p53, and provides new mechanistic insights to its mode of action in CLL patient cells. This work supports an ongoing phase I clinical trial of CFZ in CLL.

Bortezomib (BTZ, Velcade) is a proteasome inhibitor developed for the treatment of multiple myeloma and mantle cell lymphoma (15). Concentrations of BTZ that produce an antitumor response in vitro inhibit activities of the CT-L and C-L subunits of the proteasome (2). In spite of a high degree of cytotoxicity in vitro in CLL cells, BTZ failed to produce objective responses in CLL patients in a phase II clinical trial at the achieved doses (16). The lack of BTZ efficacy in vitro has been attributed to the inactivation of its boronate moiety by dietary flavonoids in human plasma (17). Carfilzomib (CFZ, PR-171) is a novel proteasome inhibitor that specifically and irreversibly inhibits the CT-L activity of the proteasome (18). Unlike BTZ, CFZ has minimal activity against off-target enzymes including serine proteases, while at the same time inhibiting the C-L subunit of the proteasome more potently (19–21). Importantly, CFZ lacks the boronate moiety of BTZ that is potentially responsible for that agent’s inactivity in CLL patient cells. Here, we investigate the effects of CFZ on CLL patient cells. This work shows that CFZ irreversibly inhibits the C-L activity, promotes an antiproliferative response in CLL including cases with del(17p13.1), and promotes an atypical activation of NF-kB that may lack the classical prosurvival effect of this pathway.

Materials and Methods

Reagents

CFZ was provided by Onyx Pharmaceuticals. Boc-D-FMK (Enzyme Systems Products) was used at 100 μmol/L. BTZ was obtained from Millennium Pharmaceuticals Inc., and TNF from R&D Systems. CD40L was purchased from PeproTech. 2-Fluoro-ara-A (active metabolite of fludarabine), G418, doxycycline, and puromycin were purchased from Operon. CpG DSP30 (22) was purchased from Eurofins Operon.

Cells and cell lines

Blood was obtained from patients following written, informed consent under a protocol approved by the Institutional Review Board of The Ohio State University. All patients examined had immunophenotypically defined CLL as outlined by IWCLL criteria (23) and were newly diagnosed or without treatment for a minimum of 30 days at time of collection. The occurrence of del(17p13.1) was determined in CLL patient samples by fluorescence in situ hybridization as described (24), and in each positive case at least 30% of cells showed this deletion. Normal cells were obtained from partial leukocyte preparations from the American Red Cross. B- or T-lymphocytes and CLL cells were negatively selected using RosetteSep reagents (Stem-Cell Technologies). The HS-5-GFP stromal cell line was provided by Dr. Beverly Torok-Storb (Fred Hutchinson Cancer Research Center; ref. 25). 293 cells were obtained from American Type Culture Collection (Manassas, VA) and 697 lymphoblastic cells were obtained from DSMZ. Cells were incubated at 37°C and 5% CO2 in AIM-V medium (Invitrogen) or in RPMI 1640 with 10% FBS or 10% human serum (HS) supplemented with penicillin, streptomycin, and t-glutamine (Sigma).

Viability assays

Cell viability was monitored by flow cytometry using annexin V/propidium iodide (BD Biosciences) on a FC500 instrument (Beckman Coulter). CellTiter 96 (MTS) assays were conducted to monitor growth inhibition per manufacturer’s instructions (Promega). LIVE/DEAD (Invitrogen) staining was done to monitor cytotoxicity with drug treatments using the manufacturer’s instructions.

Immunoblot analyses

Nuclear and cytoplasmic lysates were prepared with NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce). Antibody to polyADP-ribose polymerase (PARP) was from EMD Biosciences, p21 (OP64) and p53 (OP43) from Calbiochem, p27 (89264) from Abcam, and pIκBα from Cell Signaling Technologies. Remaining antibodies were from Santa Cruz Biotechnology. Bands were quantified on an AlphaImager system (Proteinsimple).

Electrophoretic mobility shift assay

A probe containing an NF-kB consensus binding site (5’ AGTTGAGGGCAGTCTCCAGGC 3’; Santa Cruz Biotechnology) was 32P-labeled using the Nick Translation System (Invitrogen). Five micrograms of nuclear protein was incubated 30 minutes at room temperature in binding buffer (10 mmol/L Tris-HCl, pH 7.5, 1.0 mmol/L ethylenediaminetetraacetic acid (EDTA), 4% Ficoll, 1.0 mmol/L dithiothreitol, 75 mmol/L KCl) plus 250 ng poly dI-dC (Sigma). Complexes were separated on polyacrylamide gels in Tris-borate EDTA buffer (89 mmol/L Tris-base, 89 mmol/L boric acid, 2.0 mmol/L EDTA), dried, and autoradiographed. For supershift experiments, antibodies to p65, p50, or c-Rel (Santa Cruz Biotechnology) were incubated with nuclear extract for 10 minutes before addition of probe.

Real-time reverse transcription-PCR

RNA was extracted using TRIzol (Invitrogen). cDNA was prepared with SuperScript First-Strand Synthesis System.
Real-time reverse transcription PCR (RT-PCR) was done on an ABI 7900 (Applied Biosystems) using TaqMan Universal Master Mix, primers, and labeled probes (Applied Biosystems) and TBP as an endogenous control. Mean threshold cycle (Ct) values were calculated by PRISM software (Applied Biosystems) to determine fold differences according to manufacturer’s instructions.

CT-L activity assay
Whole cell extracts without protease inhibitors were analyzed using a CT-L–specific 20S proteasome assay kit (Chemicon) following the manufacturer’s instructions. The assay is based on detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate. Free AMC was measured using a 380/460 nm fluorometer filter set. Relative activity was standardized by protein concentration, determined by BCA assay.

Gamma irradiation
Cells were irradiated using 5 or 8 Gy as indicated. RNA and lysates were collected from a subset of cells after 4 hours, and viability was determined in remaining cells at 24 hours by annexin V/propidium iodide or LIVE/DEAD staining.

Retroviral vectors and generation of cell line with inducible dominant-negative (DN) p53 activity
The retroviral construct pRetroX-Tight-Puro (pRetro; Clontech) was used to stably transfect 697 cells with the p53DN system. Mutations in the p53 DNA-binding domain at codon 273 (Arg→His, p53DN818) and codon 281 (Asp→Glu, p53DN843) were selected to generate 2 different p53DN constructs using the QuikChange mutagenesis kit (Stratagene). DNA was sequenced to confirm mutations. Using primer sequences provided by Clontech, the presence and orientation of p53DN818 and p53DN843 sequences in the pRetro vector were similarly confirmed. Retrovirus particles were produced by cotransfecting plasmid DNA and ecotropic helper plasmids (pVSV and pGPZ) into the 293 cell line using calcium phosphate precipitation. The inducible Tet activator 697 cell line (pRetrox-tet-on) was established according to the manufacturer’s protocol (Clontech). 697pTet-on cells were infected with retrovirus by culturing for 10 hours in conditioned media with 8 µg/mL polybrene. Cells were then washed and incubated 48 hours before selection with 1 µg/mL puromycin and 100 µg/mL G418.

Statistics
For all experiments, linear mixed effects models were used to account for dependencies among the data. From the models, estimated differences between experimental conditions were calculated along with 95% confidence intervals (CI). Log transformations of the data were applied when necessary to stabilize variances. Data from real-time RT-PCR experiments were first normalized to internal controls, and then linear mixed models were applied to the log-transformed fold changes. All analyses were done using SAS/STAT software, v9.2 (SAS Institute).

Figure 1. T cells express more chymotrypsin-like (CT-L) proteasome activity than B cells. A, basal CT-L activity in T cells (n = 10) is higher than B cells (n = 11) from healthy donors and in primary CLL cells (n = 10; *, P < 0.0001 for both). CT-L activity in B cells was also higher in in CLL cells (**, P = 0.0003). Results are displayed in relative fluorescence units (RFU). B, T and B cells from healthy donors (n = 8 each) and CLL patient samples (n = 10) were incubated in serum-free AIM-V media with various concentrations of CFZ for 1 hour. Mitochondrial activity as a surrogate for cell viability was determined by MTS assay at 48 hours, and is shown relative to time-matched untreated controls. Horizontal lines represent the mean. CFZ across all doses is more cytotoxic to CLL cells than T cells (*, P < 0.0001). CFZ is also more cytotoxic to CLL cells than to B cells at 33 and 100 nmol/L (P < 0.0001 for both) but is not different at 300 nmol/L.
Results

T cells express increased CT-L proteasome activity and decreased sensitivity to CFZ compared to B cells

A common disadvantage of current therapies for CLL is the negative impact on normal lymphocytes, especially T cells, which leaves patients at increased risk of opportunistic infection. To address the impact of CT-L inhibition on CLL and normal cell types, we first measured the basal levels of CT-L activity in CLL cells compared to T and B cells from healthy volunteers. Interestingly, normal T cells showed significantly higher CT-L activity compared to normal B cells and CLL cells (P < 0.0001 for both). CT-L activity in normal B cells was also significantly higher than in CLL cells (P = 0.0003; Fig. 1A). We next tested the relative cytotoxicity of CFZ in these cell types. Because CFZ exhibits a short in vivo half-life (approximately 30 minutes; refs. 26 and 27), cells in all experiments were incubated with CFZ for 1 hour, washed, and further cultured in fresh media for the indicated times to more closely mimic in vivo exposure. Concentrations used were based on pharmacokinetics data from a phase I clinical trial in CLL now underway at OSU. In this trial, the maximum concentration achieved was 0.876 ± 0.099 μg/mL (1.22 ± 0.138 μmol/L) for the 45 mg/m (2) dose (unpublished results). This is similar to concentrations previously reported to be clinically achievable in patients with hematologic malignancies (27). As shown in Fig. 1B, normal B cells were significantly more sensitive to CFZ than normal T cells at 48 hours using 100 and 300 nmol/L concentrations (P < 0.0001 for both). This finding is consistent with the elevated CT-L activity observed in T cells relative to B cells.

CFZ irreversibly inhibits the CT-L subunit and promotes apoptosis in CLL cells

Because of the lower CT-L activity in CLL cells, we hypothesized that the CT-L–specific inhibitor CFZ would show particular efficacy in these cells. We therefore tested the cytotoxicity of CFZ in CLL patient samples. As BTZ was

Figure 2. Carfilzomib irreversibly inhibits the CT-L subunit and promotes selective apoptosis in CLL cells cultured in human serum. A, CLL cells (n = 8) were treated with various concentrations of CFZ or BTZ for 1 hour in media as indicated. Viability was determined by annexin V/propidium iodide flow cytometry at 24 hours. Horizontal lines represent the mean. B, CLL cells or normal T cells (n = 6 each) were incubated with CFZ in RPMI with 10% HS for 1 hour and cells were harvested at several time points to determine CT-L activity. C, viability of cells from B was determined by annexin/propidium iodide flow cytometry. Because of cell number limitations, T cells (striped bars) were investigated for viability at 24 hours only.
previously shown to be inactivated by human plasma components (17), we also included this agent and tested in the presence of 3 different media: serum-free AIM-V medium, RPMI 1640 with 10% HS, and RPMI 1640 with 10% FBS. In serum-free or FBS-containing media, CFZ was more cytotoxic to CLL cells than BTZ (approximate LC50 in AIM-V and FBS: CFZ = 40 and 80 nmol/L; BTZ = 200 and 170 nmol/L, respectively). In media containing HS, however, CFZ maintained efficacy but BTZ was largely inactive (approximate LC50 of CFZ in HS = 80 nmol/L; Fig. 2A). To more closely approximate in vivo conditions, media with HS was used for the remaining work. Next, CT-L activity was assessed in CLL patient cells and in normal T cells after total incubations of 1, 4, or 12 hours (Fig. 2B). Viability was also determined by propidium iodide flow cytometry (Fig. 2C). CFZ rapidly (within 1 hour) suppressed the CT-L proteasome activity both in CLL samples and in T cells. This was followed by a time-dependent increase in apoptosis (as determined by annexin positivity) and cytotoxicity (as determined by propidium iodide uptake) that was greater in CLL versus T cells. Despite drug washout, CT-L inhibition was not reversible within the 12-hour period following CFZ treatment. Proteasome activity was not determined at later time points because of the high degree of CFZ-induced cell death.

**Stromal protection of CFZ-treated CLL cells is diminished by a pan-PI3K inhibitor**

CLL cells have dysregulated apoptosis in vivo, but in vitro they rapidly undergo spontaneous apoptosis because of the lack of external signals from the microenvironment provided by soluble factors and contact with a variety of cells in the bone marrow and lymph nodes (28). We therefore sought to determine the impact of microenvironment factors on CFZ-mediated cytotoxicity using a previously established stromal cell line, HS-5-GFP (29). CFZ did not substantially affect viability of HS-5-GFP stromal cells at 48 hours at concentrations up to 300 nmol/L (data not shown). To test the protective effects of stroma, CLL cells were incubated with CFZ or vehicle for 1 hour and transferred to plates with or without HS-5-GFP cells. Using flow cytometry, GFP-negative CLL cells were distinguished from GFP-positive HS-5 cells and cytotoxicity was assessed by propidium iodide uptake. CFZ caused significantly more cytotoxicity in CLL cells alone as compared to CLL cells on stroma, although CLL cells on stroma remained sensitive to CFZ at the tested concentrations ($P < 0.0001$; Fig. 3A). The importance of the PI3K signaling pathway to the survival of mature B cells, and specifically co-culture of CLL on stromal cells, has been established (29). Therefore, we sought to determine if the stromal cell protection of CLL cells was mediated by PI3K signaling. As shown in Fig. 3B, the addition of the pan-PI3K inhibitor LY294002 significantly diminished the stromal cell–mediated protection of CFZ-treated CLL cells ($P = 0.0083$). These data indicate that CFZ can mediate cytotoxicity in CLL cells despite the protective effect of stromal cells, and that stromal cell protection from CFZ-mediated cytotoxicity can be diminished by PI3K inhibition.

![Figure 3. Stromal protection of CLL cells treated with CFZ is reversed by pan-PI3K inhibitor. A, CLL cells (n = 5) were incubated with various concentrations of CFZ for 1 hour, washed, and transferred to plates with or without HS-5-GFP stromal cells. Dinaciclib (indicated by +) was included as a positive control (52). Viability of GFP negative (CLL) cells was determined by propidium iodide uptake at 48 hours and the differences were significant ($P < 0.0001$). B, addition of 25 μmol/L pan-PI3K inhibitor LY294002 to 100 nmol/L CFZ treated CLL cells (n = 5) decreased stromal protection as determined by propidium iodide uptake at 48 hours ($*P = 0.0083$). Horizontal lines represent the mean.](#)
Cytotoxicity induced by CFZ in CLL cells is caspase-dependent

CFZ has been reported to mediate caspase-dependent apoptosis in mature B-cell malignancies such as Waldenstrom’s macroglobulinemia (30) and multiple myeloma (31). We sought to determine if similar death pathways were used in less-differentiated malignant B cells. CFZ caused a time-dependent cleavage (Fig. 4A) of the caspase substrate PARP and cytotoxicity (Fig. 4B) that was rescued by the pan-caspase inhibitor Boc-D-fmk (BocD) for 18 hours. PARP cleavage was assessed by immunoblot. A representative immunoblot is shown. B, viability of cells from A was determined by annexin V/propidium iodide at 4, 8, and 18 hours. Differences in cytotoxicity of CFZ with the addition of BocD were significant (P = 0.0003). F-ara A (active metabolite of fludarabine) was included as a positive control for PARP cleavage and caspase-dependent cytotoxicity.

CFZ is cytotoxic to cells independent of p53

Mutations or deletions of p53 are far more common in advanced cases of CLL and are associated with resistance to alkylating agents, disease progression in 1 or 2 years, and overall significantly poorer outcome (32). The proteasome regulates the turnover of p53 as well as several key p53 targets. We therefore sought to determine the effects of proteasome inhibition on p53 and its targets. CLL cells incubated with CFZ were analyzed by immunoblot or real-time RT-PCR. Gamma irradiation was included as a positive control representing a classical p53-inducing treatment. As shown in Fig. 5A, CFZ treatment caused only modest accumulation of p53 compared to irradiated cells, and p27 was unchanged. Puma and Bax proteins were similarly unchanged (data not shown). Significant increases in p21 transcription were noted in all patient samples following CFZ treatment (average fold change of 5.78; P < 0.0001), and Puma transcription was slightly increased (average fold change of 1.97; P = 0.0323; Fig. 5B). However, p53 was found to be downregulated by an average of 0.75 fold (P = 0.0193), and no significant changes were observed in the transcription of Noxa (P = 0.2232) or Bax (P = 0.1531). These findings show that CFZ does not induce a
typical p53 response in CLL cells, and suggests that CFZ-mediated p21 induction may be because of an indirect mechanism rather than direct p53 activity. As was shown previously with BTZ (33), we observed an increase in Noxa protein (Fig. 5A), but not mRNA, with CFZ treatment. Next, we analyzed CLL samples from patients with or without del(17p13.1), the chromosomal site of the p53 gene (Fig. 5C). CFZ was similarly cytotoxic to patient cells irrespective of del(17p13.1) status ($P = 0.3924$). In contrast, significant differences in cytotoxicity were observed between patient samples with and without this deletion for both γ-irradiation and fludarabine treatments ($P < 0.0001$ and $P = 0.0002$, respectively). As p53 is only one of the genes deleted on del(17p13.1), we next sought to test the cytotoxicity of CFZ in cells with wild-type versus defective p53 activity. In CLL, a transition mutation in the p53 DNA binding domain at codon 273 that converts Arg to His has been described as a gain-of-function dominant negative mutation (34). In addition, mutations at codons 281 (Asp → Glu) and 273 (Arg → His) are found in certain leukemias (35). Doxycycline-inducible p53DN constructs representing these mutations were transfected into 697 cells, an acute B-lymphoblastic leukemia cell line that has wild-type p53 and responds to CFZ treatment similarly to CLL cells (Supplementary Fig. S1B). As shown in Fig. 6A, p53 protein was induced in cell lines carrying the p53DN constructs, but not the empty vector, as early as 18 hours after doxycycline addition. Induction of p53DN activity...
increased p53 transcription by more than 5-fold in both p53DN818 and p53DN843 cells but not in empty-vector transfected cells (P < 0.0001; Fig. 6B). Addition of doxycycline also caused a significant reduction in cytotoxicity (Fig. 6C) with γ-irradiation, but not with CFZ treatment, in the cells with p53DN activity (P = 0.0378 and 0.0243 for p53DN818 and p53DN843, respectively). Furthermore, in γ-irradiated cells, although doxycycline caused a 1.5-fold upregulation of p21 transcription in empty-vector control cells, p21 was reduced (0.7-fold) in doxycycline-treated p53DN cells (Fig. 6B). Although these changes did not reach statistical significance, they further support the functional activity of the p53DN constructs in the 697 cells. Cumulatively, these experiments indicate that cytotoxicity of CFZ is not dependent on functional p53 activity in leukemic cells.

**CFZ induces an atypical NF-κB response in CLL cells**

CLL cells are characterized by constitutive activation of NF-κB as indicated by high levels of nuclear NF-κB, p50, and p65 (RelA) proteins. In cutaneous T-cell lymphoma, BTZ was reported to inhibit the NF-κB pathway by preventing the degradation of the phosphorylated form of IκBα, thus preventing the nuclear translocation of p50 and p65 and further activation of NF-κB targets (36). More recently it was shown in multiple myeloma cell lines that IκBα can be degraded by proteasome-independent mechanisms following BTZ treatment to allow for translocation and activation of NF-κB subunits (37). Thus, NF-κB response to proteasome inhibition may vary by cell type or other factors not yet identified. To determine the effects of CFZ on NF-κB activity in CLL, CLL cells were incubated with CFZ for 1 hour and examined by immunoblot. As shown in Fig. 7A–C, IκBα protein decreases in both whole cell extracts as well as nuclear and cytoplasmic fractions. However, phosphorylation of IκBα (p-IκBα) increased in all fractions. To determine if this decrease in IκBα was because of caspase activity as was previously reported (38), CLL cells were incubated with CFZ in the presence or absence of Boc-D-fmk (Fig. 7B). Although Boc-D-fmk rescued cells from CFZ-mediated PARP cleavage and apoptosis, it did not prevent degradation of IκBα. Antibodies recognizing epitopes in both the N- and C-terminal region of IκBα produced identical results (data not shown). It also was reported that IκBα is a substrate of calcains (37) but similar to caspase inhibitors, calpain inhibitors did not prevent degradation of IκBα (data not shown). As proteasome inhibitors were previously shown to induce or inhibit both the canonical and noncanonical NF-κB pathways (36, 37), nuclear and cytoplasmic fractions of CFZ-treated CLL cells were probed for canonical (Fig. 7C) and noncanonical (Fig. 7D) proteins. No significant changes were observed in the nuclear or cytoplasmic levels of NF-κB subunits p50, p65, p52, or p100 in the CFZ treated samples. As controls, Cpg and CD40L increased expression of both canonical and noncanonical proteins in the nuclear fraction.

To determine whether nuclear translocation of pIκBα affects NF-κB activity, NF-κB DNA binding was measured in nuclear extracts prepared from CLL cells treated with CFZ or CD40L. The constitutive NF-κB (p50/p65) DNA binding activity in CLL cells was increased with CFZ treatment in approximately 50% of the samples, whereas there was no change in the remainder (Fig. 7E and F, respectively). Importantly, CFZ was similarly cytotoxic to all CLL samples tested (Fig. 7G). These results indicate that the impact of CFZ on NF-κB is not relevant to its cytotoxic activity in CLL. Finally, to determine whether the increased NF-κB activity observed by EMSA resulted in changes in gene expression, we analyzed several known NF-κB targets by real-time RT-PCR (Fig. 7H). CFZ induced a subset of these targets, including IkBα, IL-6, c-FLIP, and CXCL13, but with substantial interpatient variability (average fold changes for target genes are indicated in Supplementary Table S1). Notably, CFZ did not induce the transcription of several classical NF-κB targets such as Bcl2A1, XIAP, Mcl-1, and p53. Thus, our results show that CFZ induces rather than inhibits NF-κB in CLL patient cells, but that this response is atypical and does not contribute to the CFZ induced cytotoxicity or resistance to this therapy.

**Discussion**

CFZ represents a new class of irreversible proteasome inhibitors that specifically target the CT-L subunit. It is currently in clinical trials for B-cell malignancies, but its mechanism of cell death is poorly understood. Here, we show that CLL cells have a low level of CT-L proteasome activity relative to normal B- and T-lymphocytes. We hypothesized that an irreversible inhibitor of the CT-L subunit would be most effective in cells having the least baseline activity, and used this to justify the examination of CFZ in CLL. In fact, we show that a short (1 hour) exposure of 100 nmol/L CFZ is more cytotoxic to CLL cells compared to normal lymphocytes, even though this concentration similarly inhibits the CT-L subunit in CLL and normal T cells. This finding indicates that the differential sensitivity of CLL versus T cells to CFZ is not directly related to the extent of CT-L inhibition. Unlike BTZ, the cytotoxicity of CFZ is not diminished in media with HS. Importantly, our studies indicate that CFZ induces cytotoxicity irrespective of del (17p13.1) status or p53 function. Stromal cells partially protected CLL cells from CFZ-mediated cytotoxicity, although this protective effect was decreased by the addition of a pan-P13K inhibitor. Finally, we show that CFZ mediates an atypical NF-κB activation that does not associate with transcriptional induction of classical NF-κB targets or with CLL cell death. As previously reported with other proteasome inhibitors, (33), Noxa induction is observed with CFZ treatment concurrent with the onset of apoptosis, suggesting that Noxa-mediated caspase induction is the mechanism of action of CFZ in CLL.

To our knowledge, this is the first study showing the reduced CT-L activity in primary CLL cells versus normal B and T cells. Although activity of proteasome inhibitors is well documented, the mechanism by which these agents preferentially target malignant cells is yet to be elucidated. We speculate that differences in CT-L activity explain the observed therapeutic window between CLL.
tumor cells and normal lymphocytes. This hypothesis could be further tested by comparing the levels of inhibition of the CT-L activities in the constitutive (c20S) and immunoproteasome (i20S). We observed that levels of CT-L activity inhibition did not necessarily correlate with CFZ cytotoxicity. One potential explanation is differing levels of inhibition of the CT-L activity in c20S and i20S by CFZ, although this remains to be tested.

The tumor suppressor protein p53 induces apoptosis under conditions of cellular stress or DNA damage. Although p53 is functional in most CLL patients at the time of diagnosis, the gene becomes mutated or deleted in at least one allele in approximately 40% of patients with advanced CLL (24, 39), corresponding with significantly poorer outcome (32). Thus, agents that work independently of p53 are of significant interest in the development of therapies for drug-resistant CLL. Proteasome inhibitors have been shown to induce apoptosis in both p53-independent (40) and p53-dependent manners (41). Here, we show that CFZ induces cytotoxicity irrespective of del(17p13.1) status or the expression of a dominant negative p53 protein. Given the poor outcome and lack of effective therapies for del (17p13.1) CLL patients, these findings suggest CFZ therapy may be effective in this disease subtype.

Recent advances in CLL biology show the importance of both internal and external (microenvironment) factors in the survival and drug resistance of CLL tumor cells. The PI3K and NF-κB pathways are key regulators of differentiation and survival in B cells and are induced by microenvironmental factors (42, 43). CLL cells cultured with stromal cells were less sensitive to CFZ compared to CLL cells in suspension. This protective effect has been linked to the PI3K pathway (29, 44), and our data show that PI3K inhibition indeed reverses this protection. Further work is necessary to determine the specific PI3K isomorph responsible for this effect, but our data suggest that combination therapy of CFZ with PI3K inhibitors may provide added benefit.

Although the mechanism of proteasome inhibitor–mediated cell death is unclear, these agents have commonly been reported to be inhibitors of the NF-κB pathway (36, 37). This notion is attributed to the observation that the NF-κB inhibitory molecule IκBα is a proteasome substrate, and that proteasome inhibitors should prevent its degradation. More recent studies indicate that proteasome inhibitors can either inhibit or induce NF-κB, again through interaction with IκBα (36, 37). However, these reports are conflicting and effects may vary by cell type. Our data in CLL show that the NF-κB pathway is in fact activated by CFZ as evidenced by degradation of total IκBα, accumulation of phosphorylated IκBα in nuclear and cytoplasmic fractions, and enhanced binding of NF-κB subunits to NF-κB consensus sites. However, NF-κB activation does not correlate with CLL cell death. This result is similar to what was reported in multiple myeloma cell lines using BTZ (37), although our studies in CLL primary cells extend these observations to show that a classical NF-κB gene induction pattern is not observed. Therefore, although the inhibition of proteasome activity is not inhibitory to the NF-κB pathway per se, it induces an atypical response that does not result in the ultimate transcription of canonical NF-κB targets and thus is unlikely to provide a prosurvival effect.

To characterize the mechanism of CFZ-mediated cell death, we assessed the effects of CFZ on the Bcl-2 family of proteins and subsequent caspase activation. We found that inhibition of caspase activity caused a significant reduction in cytotoxicity of CFZ. In addition, of the Bcl-2 family of proteins, only Noxa was consistently upregulated in all CFZ-treated samples. This effect was recently shown to induce caspase activity in CLL cells, further supporting a caspase-dependent pattern of cytotoxicity (33). Proteasome inhibitors can also cause cytotoxicity because of endoplasmic reticulum stress by blocking the degradation of regulatory and misfolded proteins (33, 45). However in our studies CFZ did not cause changes in conventional endoplasmic reticulum stress markers, including splicing of XBP1 or mRNA or protein levels of CHOP, GRP78, or pEIF2α (data not shown). Thus, we concluded that in primary CLL cells, CFZ-mediated cytotoxicity was caused by a caspase-dependent pathway and not via endoplasmic reticulum stress.

To date, BTZ is the only proteasome inhibitor approved for treatment of malignant blood disorders and is now being used in front-line therapy for multiple myeloma (46). In spite of a high degree of cytotoxicity in vitro, BTZ failed to produce any objective responses in CLL patients in a phase II clinical trial (16). Its failure in vivo was hypothesized to be because of the inactivation of the boronate moiety in BTZ by flavonoids in HS (17), which is consistent with our observation that BTZ lacks activity against CLL cells in media containing HS. Despite the initial clinical success of BTZ in hematological disorders, significant populations of patients remain refractory to treatment. Furthermore, toxicities with BTZ treatment such as peripheral neuropathy and thrombocytopenia have increased the intervals between dosing, allowing recovery of the proteasome function (47, 48). This has spurred development of a new class of proteasome inhibitors that lack these toxicities. CFZ is being investigated through clinical trials in newly diagnosed, relapsed, or
Figure 7. CFZ induces a defective NF-κB response. A, CLL cells \((n = 6)\) were incubated 1 hour without or with 300 nmol/L CFZ, and lysates were collected at 8 hours. IκBα and phosphorylated IκBα (pIκBα) were assessed by immunoblot. Untreated and TNF-treated HeLa cells were included as negative and positive controls for pIκBα protein expression, respectively. B, CLL cells \((n = 6)\) were treated as in A, with or without 100 µmol/L Boc-D-fmk. Lysates were isolated at 18 hours and analyzed by immunoblot. CLL cells \((n = 6)\) were incubated with 0, 70, and 350 nmol/L CFZ for 1 hour or 500 ng/mL CD40L for 4 hours. Nuclear (NE) and cytosolic (CE) extracts were collected at 4 hours and analyzed by immunoblot for NF-κB (C) canonical pathway and (D) noncanonical pathway proteins. EMSAs were run using NE from cells treated with 350 nmol/L CFZ (1 hour treatment, 4 hours incubation) or 500 ng/mL CD40L (4 hours; \(n = 14\)), using a probe containing a consensus NF-κB binding site. Representative EMSAs from CFZ-treated NE that displayed (E) increased NF-κB binding and (F) no changes are shown. CD40L and BMS-345541 (IKK inhibitor) treated NE were included as positive and negative controls for p50/p65 binding, respectively. G, viability of all samples analyzed by EMSA was determined by MTS at 48 hours.
refractory multiple myeloma, and has seen promising activity both as a single agent and in combination with immunomodulators (49–51).

In summary, our study indicates that CFZ represents a promising, p53-independent therapeutic for CLL and provides the rationale for development of CFZ in this disease. Based on these data, we have initiated a phase I dose escalation study of this agent in relapsed and refractory CLL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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