Effects of the Proteasome Inhibitor, Bortezomib, on Apoptosis in Isolated Lymphocytes Obtained from Patients with Chronic Lymphocytic Leukemia

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ABSTRACT

Purpose: Bortezomib is a peptide boronic acid inhibitor of the proteasome developed for cancer therapy. The compound is being evaluated currently in Phase II and III clinical trials. Here we characterized the effects and mechanisms of action of bortezomib in cells obtained from patients with chronic lymphocytic leukemia (CLL).

Experimental Design: We exposed isolated CLL lymphocytes from >100 patients to various concentrations of bortezomib or other proteasome inhibitors, and measured DNA fragmentation by propidium iodide staining and flow cytometry. We characterized the effects of bortezomib on release of apoptosis-associated mitochondrial factors and measured downstream effects on caspase activation using a fluorogenic substrate cleavage assay. We assessed potential effects of the drug on inhibitor of apoptosis protein family apoptosis inhibitors by immunoblotting. Finally, we quantified the effects of bortezomib on apoptosis in 5 patients on a Phase II clinical trial.

Results: Bortezomib stimulated apoptosis more rapidly than positive controls (glucocorticoid and fludarabine), although substantial heterogeneity was noted with respect to the concentration of drug required to induce cell death. Bortezomib-induced apoptosis was associated with release of SMAC, apoptosis-inducing factor, and cytochrome c from mitochondria, but the drug did not affect levels of inhibitor of apoptosis protein family cell death inhibitors. Levels of apoptosis were marginally elevated in CLL cells obtained from 2 of 5 fludarabine-refractory patients treated with bortezomib in vivo.

Conclusion: Our data confirm that bortezomib, like other proteasome inhibitors, has proapoptotic activity in CLL cells.

INTRODUCTION

CLL2 is the most common adult leukemia in the Western world (1). Although nucleoside analogues such as fludarabine and 2-chlorodeoxyadenosine (cladribine) have excellent activity in patients who have not received prior therapy, their impact on long-term survival is unclear (2) and few agents display any activity in refractory disease (1–3). Unlike other hematological malignancies, CLL cells do not display a consistent molecular etiology (i.e., chromosomal translocation) that would suggest an obvious therapeutic target (4, 5). Nonetheless, the available evidence suggests that CLL emerges primarily as the result of dysregulated apoptosis rather than unchecked proliferation (6, 7). Thus, agents that selectively target the survival pathway(s) active in CLL cells could reverse drug resistance (7).

Data published previously by our laboratory and others have demonstrated that inhibitors of a multicatalytic protease known as the proteasome act as potent inducers of apoptosis in cancer cells (8–11). Bortezomib is a dipeptide, boronic acid inhibitor of the proteasome that was developed specifically for use in cancer chemotherapy (12). The compound was well tolerated in Phase I studies, and displayed promising antitumoral effects in multiple myeloma and some solid tumors (13–15). Here we examined the effects of bortezomib on apoptosis in isolated CLL lymphocytes and characterized the biochemical mechanisms associated with the response.

MATERIALS AND METHODS

Antibodies and Chemical Reagents. A monoclonal anticytochrome c antibody, monoclonal antibodies to c-IAP-1, c-IAP-2, and X-linked inhibitor of apoptosis protein, and a rabbit polyclonal antibody specific for AIF2 were purchased from BD-PharMingen (San Diego, CA). Phorbol-12,13-dibutyrate was obtained from Sigma (St. Louis, MO). A rabbit polyclonal antibody specific for second mitochondrial activator of caspases/DIABLO antibody was generously provided by Dr. X. Wang (University of Texas Southwestern Medical Center, Dallas, TX) (16). Bortezomib was provided by Millenium (Cambridge, MA), and Dr. D. C. Altieri (University of Massachusetts Medical Center, Worcester, MA) provided the c-IAP-1, c-IAP-2, and X-linked inhibitor of apoptosis protein antibodies. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: CLL, chronic lymphocytic leukemia; IAP, inhibitor of apoptosis protein; AIF, apoptosis-inducing factor; AMC, aminomethyl coumarin; PI, propidium iodide; FACS, fluorescence-activated cell sorting; MPS, methylprednisolone.
MA) provided the rabbit antisurvivin antibody. The peptide caspase substrate DEVD-AMC was purchased from Enzyme Systems Products (Dublin, CA).

**Isolation and Culture of B-CLL Cells.** All of the patients fulfilled the criteria of the National Cancer Institute for the diagnosis of B-CLL (17). Immunophenotyping by dual-parameter flow cytometry showed coexpression of CD5 with B-cell antigen and isotypic light chain expression. Freshly isolated peripheral blood was fractionated by Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) sedimentation. Nonadherent mononuclear cells were immediately suspended in RPMI 1640 supplemented with 2 mM glutamine and 10 mM HEPES (pH 7.5).

**Immunoblotting Analysis.** Cells were lysed for 20 min at 4°C by resuspension in lysis buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM sodium orthovanadate, and a Complete Mini protease inhibitor tablet (Roche Molecular Biochemicals, Indianapolis, IN). Debris was sedimented by centrifugation for 12,000 g, and protein content in the supernatant was determined using the Bradford reagent (18). Supernatants were mixed with equal volumes of 2X Laemmli’s SDS-PAGE sample buffer (19) containing 0.7 M 2-mercaptoethanol and incubated for 5 min at 100°C. Polypeptides were resolved at 150 V on 10–15% polyacrylamide gels and electrophoretically transferred to a 0.2 μm nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH) for 1 h at 100 V. Membranes were blocked for 1 h in Tris-buffered saline-Tween buffer [25 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] containing 3% (w/v) nonfat dried milk. Blots were probed overnight with primary antibody, incubated with species-specific secondary antibodies conjugated to horseradish peroxidase, and immunoreactive material was visualized by enhanced chemiluminescence (Renaissance, NEN, Boston, MA). Relative protein levels were measured by scanning exposed films and quantifying band intensities using Un-Scan-It software (SILK Scientific Corporation). Levels of IAP proteins were standardized to a loading control (actin).

**Quantification of DNA Fragmentation.** Apoptosis was measured by PI staining and FACS analysis (20) as described previously (9). After incubation with various agents in vitro, cells were pelleted by centrifugation and resuspended in PBS containing 50 μg/ml PI, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4°C for 16 h and vortexed before FACS analysis (FL-3 channel).

**Isolation of CD19+ Lymphocytes.** Isolated peripheral blood mononuclear cells obtained from normal donors were resuspended in 80 μl of PBS containing 5 mM EDTA and 20 μl of CD19 microbeads (Miltenyi) per 10^7 cells. Cells were incubated at 4°C for 15 min. Cells were washed in 10–20 volumes of PBS. Labeled cells were layered on a prewashed magnetic column (midiMACS; Miltenyi). The column was washed three times with PBS and unlabeled cells collected. The column was removed from the magnetic field and the CD19+ fraction collected. Cell fractions were labeled with a fluorescein-conjugated CD19 antibody (Becton-Dickinson, San Diego, CA) and analyzed by flow cytometry to confirm purification.

**Evaluation of Mitochondrial Factor Release.** Cells were resuspended in an ice-cold buffer [25 mM Tris and 5 mM MgCl₂ (pH 7.4)], Dounce homogenized (five times), and centrifuged for 5 min at 16,000 × g to isolate cytosolic extracts. These were mixed with 2× Laemmli’s reducing SDS-PAGE sample buffer, and extracts from equal numbers of cells were resolved by 15% SDS-PAGE. Immunoblotting was performed as described above.

**Spectrofluorometric Analysis of Caspase-3 Activation.** Cells were lysed in caspase-3 buffer [100 mM HEPES (pH 7.5)], 10% sucrose, 0.1% 3-[(cholamidopropyldimethylammonio)1-propanesulfonic acid, 1 mM EDTA with freshly added 10 mM DTT, 2 mM phenylmethylsulfonyl fluoride, and a complete mini tablet (Roche Molecular Biochemicals) for 1 h at 4°C. Lysates were incubated at 37°C for 1 h in a total of 2 ml of caspase-3 buffer containing 25 μM DEVD-AMC (Bachem, King of Prussia, PA). Samples were excited at 380 nm and read at 460 nm in a RF-1501 spectrofluorimeter (Shimadzu Scientific Instruments, Columbia, MD). Fold caspase activity was determined by dividing the sample fluorescence by the fluorescence of substrate alone.

**Statistical Analyses.** All of the statistics are described as the average ± SE. Significance was determined using a two-tailed t test not assuming equal variance between the populations.

**RESULTS**

Bortezomib induces high levels of apoptosis in CLL cells. We collected peripheral blood mononuclear cells from 166 patients with CLL. We exposed these cells to 10 μM bortezomib, 10 μM MPS, or 50 μM fludarabine for 24 h and measured apoptosis-associated DNA fragmentation by PI staining and FACS analysis. Bortezomib stimulated significantly higher levels of DNA fragmentation (44.3 ± 1.6%) than MPS (11.6 ± 1.5%) or fludarabine (16.9 ± 2.2%; P < 0.001), both of which served as positive controls (Fig. 1A). We also compared the effects of bortezomib in isolates arbitrarily characterized as “glucocorticoid-sensitive” or “glucocorticoid-resistant” based on the activity of MPS, where 20% DNA fragmentation at 24 h served as the cutpoint between the groups. Bortezomib induced indistinguishable levels of apoptosis in both groups (P = 0.314), indicating that cells resistant to glucocorticoid were not resistant to proteasome inhibitor. In contrast, the levels of fludarabine-induced apoptosis were significantly lower in glucocorticoid-resistant as compared with glucocorticoid-sensitive cells (P < 0.001; Fig. 1B).

**Concentration-dependent Effects of Bortezomib on Apoptosis.** In a subsequent series of experiments we exposed a new panel of CLL isolates (n = 20) for 24 h to increasing concentrations of bortezomib ranging from 1 nM to 10 μM and measured DNA fragmentation as described above. The results revealed significant heterogeneity in drug-induced apoptosis with the isolates segregating into three general categories. One group of isolates (n = 6) displayed maximal levels of apoptosis at low concentrations of bortezomib (≤10 nM; Table 1; Fig. 2A). The second group (n = 7) displayed moderate sensitivity characterized by induction of apoptosis at a 1000-fold higher concentration of drug (10 μM; Table 2; Fig. 2A). The third group (n = 7) displayed little to no increase in apoptosis relative to controls after 24-h exposure to 10 μM bortezomib (Table 3; Fig. 2B). When experiments were carried out to 48 h, some of these isolates did display increased apoptosis, whereas others did not (Table 3). There was no obvious correlation between bort-
Apoptosis (bortezomib versus 3). The average difference was a 15.1 observed in response to treatment with either agent alone (Fig. with bortezomib plus fludarabine compared with the levels isolates we observed higher levels of apoptosis in cells treated /H9252 ezomib-induced apoptosis and Rai stage, WBC count, and Mitochondrial Factor Release.

any of the samples (Fig. 3).

bortezomib did not increase glucocorticoid-induced apoptosis in any of the samples (Fig. 3).

Effects of Bortezomib on Fludarabine-induced Apoptosis. Biological agents can often promote the proapoptotic effects of conventional chemotherapeutic agents. Therefore, we conducted a small scale experiment to characterize the effects of bortezomib (10 nM) on apoptosis induced by MPS or fludarabine in three CLL isolates, all of which displayed modest sensitivity to either agent alone (<20% apoptosis). In two of the three isolates we observed higher levels of apoptosis in cells treated with bortezomib plus fludarabine compared with the levels observed in response to treatment with either agent alone (Fig. 3). The average difference was a 15.1 ± 1.9% increase in apoptosis (bortezomib versus bortezomib+F-ara). In contrast, bortezomib did not increase glucocorticoid-induced apoptosis in any of the samples (Fig. 3).

Kinetics of Bortezomib-induced Caspase Activation and Mitochondrial Factor Release. We exposed 5 additional CLL isolates to 10 μM bortezomib and characterized its time-dependent effects on activation of caspase-3-like protease activity, measured in cytosolic extracts using the fluorogenic peptide substrate DEVD-AMC. Caspase activation was readily detectable in drug-treated isolates by 8 h and reached a peak at 12 h (Fig. 4A). Caspase activation was associated with increases in DNA fragmentation, measured by PI-FACS (Fig. 4B). Minimal caspase activation was detected in untreated cells, and significant levels of DNA fragmentation were not observed until 48 h (data not shown).

Because caspase activation is often initiated by the release of polypeptide factors from mitochondria, we also characterized the time-dependent effects of bortezomib on the release of cytochrome c, SMAC, and AIF by cell fractionation and immunoblotting (Fig. 5A). Release of all three of the factors was readily detectable by 24 h (Fig. 5B). The apparent discrepancy between the kinetics of caspase activation/DNA fragmentation and mitochondrial factor release was probably attributable to the higher sensitivity of the fluorogenic peptide hydrolysis and PI-FACS assays compared with the cell fractionation experiments.

Bortezomib Does Not Promote IAP Stabilization in CLL Cells. A previous study demonstrated that proteasome inhibitors block thymocyte apoptosis by promoting accumulation of IAPs (21). This effect could, in principle, limit the therapeutic efficacy of PS-341 in CLL cells. Therefore, we characterized the effects of bortezomib on the levels of X-IAP, c-IAP-1, and c-IAP-2, in five independent CLL isolates (Fig. 6A). (We were unable to detect significant levels of the IAP family member, survivin, in any of the isolates; data not shown.)

Table 1 Characteristics, in vitro drug sensitivity, and PS-341 dose response of CLL patients in group 1

<table>
<thead>
<tr>
<th>ID#</th>
<th>Rai</th>
<th>WBC</th>
<th>B2M</th>
<th>MPS</th>
<th>F-ara</th>
<th>Prev. treat.</th>
<th>PS-341 (nM)</th>
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<tr>
<td>459</td>
<td>0</td>
<td>17.8</td>
<td>3.8</td>
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<td>3.2</td>
<td>9.6</td>
<td>N</td>
<td>11.1</td>
</tr>
</tbody>
</table>

WBC, white blood cell count; MPS, % apoptosis induced by MPS; F-ara, % apoptosis induced by F-ara; Prev. treat., previous treatment; PS-341, % apoptosis induced by PS-341; Y, Yes; N, No.
Effects of Bortezomib on Apoptosis in Vivo. Previous work suggests that the drug-induced apoptosis observed in CLL cells in vitro does not correlate well with sensitivity to the corresponding drug in vivo (22). Therefore, we conducted a preliminary study to determine the effects of bortezomib on apoptosis in CLL cells isolated from patients enrolled in a Phase II clinical trial. We prepared peripheral blood mononuclear cells from patients just before drug dosing and after they had been exposed to the drug for 1 h or 24 h. Baseline levels of apoptosis were measured, as well as bortezomib- and fludarabine-induced apoptosis in vitro. Although all 5 of the patients were clinically refractory to fludarabine, 2 of 5 displayed significant (>20%) DNA fragmentation after exposure to 50 μM fludarabine for 48 h in vitro, and 4 of 5 isolates displayed at least moderate sensitivity to bortezomib (1 μM for 24 h; Fig. 7B). Interestingly, 2 of 5 patients (#3, 5) displayed a time-dependent increase in baseline levels of DNA fragmentation that probably reflected drug-induced apoptosis in vivo (Fig. 7A). Thus, it appears that bortezomib has detectable biological activity in fludarabine-refractory CLL patients.

DISCUSSION

Previous work from our laboratory and others has demonstrated that proteasome inhibitors induce apoptosis in CLL cells (8–11). Here we examined the effects of the proteasome inhibitor, bortezomib, in ~200 peripheral blood mononuclear cell isolates from patients with CLL. Cells incubated for 24 h with a concentration of 10 μM bortezomib displayed an average of 44.3 ± 1.8% DNA fragmentation characteristic of apoptosis, a level that was significantly higher than was observed with optimal concentrations of MPS or fludarabine (11.6 ± 1.0 and 16.9 ± 1.4, respectively). More importantly, bortezomib was equally effective in glucocorticoid-sensitive and -resistant isolates. These results contrast with the effects of fludarabine, which exhibited less potency in the glucocorticoid-resistant isolates. Preliminary experiments also demonstrated that combined treatment with bortezomib and fludarabine induced more DNA fragmentation than treatment with either bortezomib or fludarabine alone. Together, our data demonstrate that bortezomib effectively interrupts endogenous survival signal(s) in CLL isolates. These effects might be exploited to improve the effects of fludarabine in patients who are otherwise refractory to the drug.

Previous work demonstrated that bortezomib inhibits the activity of the 20S proteasome at low nanomolar concentrations, consistent with its mean IC_{50} (7 nM) in the National Cancer Institute panel of 60 cell lines (12). Although the rapid clearance of bortezomib from the serum prohibits accurate measurement of serum bortezomib levels in patients treated with maximally tolerated doses of the drug (23), bioassays of 20S activity in preclinical studies in rodents and primates have demonstrated that levels of bortezomib that produce up to 80% inhibition of the proteasome are very well tolerated, but higher doses are toxic and can lead to rapid mortality (12). We performed pilot measurements of 20S proteasome activity in patient isolates that were not included in this study and found that 10 μM bortezomib produced ~100% inhibition in all of them (n = 6). Therefore, we were surprised that the CLL isolates included in this study displayed such heterogeneous responses to low concentrations of bortezomib.

We examined the effects of bortezomib at a time (8 h) when cells first displayed significant caspase activation. In all of the cases IAP levels were standardized to levels of an endogenous protein control (actin). Exposure to MPS induced a decrease in c-IAP-1 (Fig. 6B). However, neither bortezomib alone nor the combination of bortezomib and either fludarabine or MPS resulted in detectable stabilization of c-IAP-1, c-IAP-2, or xIAP (Fig. 6B). Thus, bortezomib does not appear to have functionally significant effects on IAP levels in CLL cells.
of bortezomib. Roughly one third of the 20 isolates examined displayed significant DNA fragmentation after 24-h exposure to 10 nM drug, another third responded to a 1000-fold higher concentration, and one third was refractory to the drug, even at the highest concentration. Clinical factors such as Rai stage or WBC count showed no obvious correlation with DNA fragmentation within these groups. Irrespective of the absolute incidence of resistance, the data strongly suggest that bortezomib will be biologically active in a significant fraction of CLL cells at concentrations that can be achieved in vivo. The cause of bortezomib resistance was not identified in our study. However, given the relatively homogenous effects of the drug on 20S proteasome inhibition, it appears that some cells tolerate sustained proteasome inhibition better than others.

Our dose-response studies also demonstrated that bortezomib stimulated apoptosis in normal peripheral blood mononuclear cells, including normal CD19+ B cells. The drug sensitivity of normal peripheral blood mononuclear cells fell between the hypersensitive and moderately sensitive groups of CLL isolates, suggesting that bortezomib demonstrates some selectivity for the transformed cells. A previous report suggested that the naturally occurring proteasome inhibitor, lactacystin, displayed more selectivity in CLL cells than the synthetic inhibitor, MG-132 (10), suggesting that the therapeutic index in CLL could be increased with a different proteasome inhibitor. As discussed above, it is unlikely that bortezomib concentrations ever exceed 10 nM in vivo, so we would not expect to observe much apoptosis in normal cells at clinically achievable concentrations of the drug. Consistent with this idea, bortezomib has caused little to no myelosuppression or other hematological toxicity in Phase I trials (14). Whether or not the drug will exacerbate the effects of fludarabine on normal T cells (24) remains to be determined.
In a preliminary attempt to characterize the biochemical mechanisms involved in bortezomib-induced apoptosis, we measured its effects on mitochondrial factor release (cytochrome c, SMAC, and AIF), caspase activation, and DNA fragmentation. Overall, the data strongly suggest that proteasome inhibitors induce cell death via direct or indirect effects on mitochondria. Our data are consistent with results obtained recently by another group, who implicated mitochondrial events in apoptosis induced by another proteasome inhibitor in CLL cells (11). Whereas our data do not formally exclude a role for death receptor(s) such as Fas, CLL cells are generally very refractory to Fas-mediated cell death in the absence of costimulation with inflammatory cytokines (25–27). (Fas resistance has been linked to CD40 engagement; Refs. 28, 29.) However, bortezomib may engage death receptors in other hematological malignancies, because recent work demonstrated that the drug activates caspase-8 in multiple myeloma cells (30).

Recent work demonstrated that proteasome inhibitors promote the accumulation of IAP family proteins in thymocytes (21), an effect that might explain why proteasome inhibitors block multiple apoptotic pathways in thymocytes and neuronal cells (32, 33). Some of the IAP family proteins contain RING domains that can act as ubiquitin-conjugating enzymes (34). IAP-mediated ubiquitin conjugation may regulate the stability of procaspase-9 and the IAPs themselves (21, 35). Therefore, we were concerned that bortezomib might also cause IAP proteins to accumulate in CLL. Our concern was heightened by a previous report demonstrating that increased xIAP expression promoted resistance of CLL cells to bryostatin (29). Immunoblotting analyses confirmed that all of the CLL isolates we analyzed expressed xIAP, cIAP-1, and c-IAP-2. However, the levels of these proteins were not significantly altered by exposure to a high concentration (10 μM) of bortezomib.

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does not cause additional stabilization of the proteins. Regardless of the explanation, the data demonstrate that IAP stabilization does not play a major role in determining bortezomib sensitivity or resistance.

Combined with previous observations with other proteasome inhibitors, the results of this study prompted the design and initiation of a Phase II trial of bortezomib in patients with fludarabine-refractory CLL. This multicenter trial is being conducted by the CLL consortium and involves acquisition of peripheral blood samples just before and 1–24 h after therapy with bortezomib, and DNA fragmentation was measured by PI staining and FACS analysis. Results from 5 evaluable patients are shown. B, evaluation of in vitro sensitivity to bortezomib or fludarabine. The "pretreatment" isolates described in A were exposed to 1 μM bortezomib for 24 h or 50 μM fludarabine for 48 h, and DNA fragmentation was measured by PI staining and FACS analysis.

Fig. 6 Effects of bortezomib on IAP expression. CLL cells were treated for 8 h with 10 μM MPS, 50 μM F-ara, 10 μM bortezomib, or a combination of these drugs. cIAP-1, xIAP, and cIAP-2 expression was determined in Triton X-100 extracts by immunoblot analysis. A, representative immunoblots. Top panels, IAPs; bottom panels, actin loading controls. B, quantification of protein expression. Levels in five different isolates were measured by densitometric scanning. Results were standardized to actin loading controls.

Fig. 7 Effects of bortezomib on DNA fragmentation in vivo. A, peripheral blood mononuclear cells were isolated from patients enrolled on a Phase II clinical trial just before (pre) or 1 h/24 h after (post) therapy with bortezomib, and DNA fragmentation was measured by PI staining and FACS analysis. Results from 5 evaluable patients are shown. B, evaluation of in vitro sensitivity to bortezomib or fludarabine. The "pretreatment" isolates described in A were exposed to 1 μM bortezomib for 24 h or 50 μM fludarabine for 48 h, and DNA fragmentation was measured by PI staining and FACS analysis.
inary results presented in Fig. 7 support the idea that bortezomib will have some biological activity in fludarabine-refractory pa-
tients, although the extent of this activity and whether or not it translates into clinical benefit (i.e., responses) will only be known after the trial closes and the samples from all of the patients enrolled on the trial have been evaluated.

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Clinical Cancer Research

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