A Phase 1 Study of the Proteasome Inhibitor Bortezomib in Pediatric Patients with Refractory Leukemia: a Children's Oncology Group Study

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Abstract

Purpose: A phase 1 study to determine the maximum-tolerated dose, dose-limiting toxicity, pharmacokinetics, and biological effects of bortezomib in children with recurrent/refractory leukemia.

Experimental Design: Bortezomib was administered twice weekly for 2 consecutive weeks at either 1.3 or 1.7 mg/m^2 dose followed by a 1-week rest. Bortezomib pharmacokinetics and nuclear factor κB (NF-κB) binding activity were evaluated during the first treatment cycle.

Results: Twelve patients (nine with acute lymphoblastic leukemia, three with acute myelogenous leukemia), median age 11 years (range, 1-18 years), were enrolled between May 2004 and November 2005, of whom seven were not fully evaluable for toxicity due to rapidly progressive disease or uncontrolled infection. Dose-limiting toxicities occurred in two patients at the 1.7 mg/m^2 dose level. One patient experienced grade 3 confusion and the other patient had grade 4 febrile neutropenia associated with grade 4 hypotension and grade 3 creatinine. Pharmacokinetic analysis at 1.3 mg/m^2 revealed a clearance of 11 mL/h/m^2, a central volume of distribution of 6.7 L/m^2, and a terminal half-life of 12.6 h. NF-κB activity was examined in five patients and was noted to transiently increase and then decrease 4- to 6-fold by 24 h following bortezomib in two patients. There were no objective clinical responses.

Conclusions: For children with leukemia, the recommended phase 2 dose of bortezomib, administered twice weekly for 2 weeks followed by a 1-week rest, is 1.3 mg/m^2/dose. Although bortezomib treatment inhibited NF-κB activity, bortezomib had little activity as a single agent in this population.

Although cure rates for children with newly diagnosed leukemia approach 85% to 90%, the prognosis for children with relapsed leukemia remains poor. Novel treatment approaches are needed for these patients. Bortezomib, a dipeptidyl boronic acid, is a selective inhibitor of the ubiquitin-proteasome pathway, which is essential for the degradation of most short-lived and many long-lived regulatory intracellular proteins in eukaryotic cells (1). Bortezomib specifically inhibits the 26S proteasome, an ATP-dependent multi-subunit protein that degrades proteins involved in cell cycle regulation, transcription factor activation, peptide processing, apoptosis, and cell trafficking (2). Proteins affected by inhibition of the ubiquitin-proteasome pathway system include nuclear factor κB (NF-κB), p53, and cell cycle regulatory proteins such as the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (3). Proteasome inhibition sensitizes malignant cells, but not normal hematopoietic progenitors, to apoptosis (4–6). Bortezomib has potent preclinical antitumor activity in a variety of malignancies and is approved for use in multiple myeloma. Bortezomib has been administered to adults using a variety of dosing schedules. The recommended adult dose of bortezomib given on days 1, 4, 8, and 11 every 21 days is 1.3 mg/m^2; however, bortezomib has also been well-tolerated at doses of 1.5 mg/m^2 (7, 8).

The Children's Oncology Group completed a phase 1 study of bortezomib in pediatric patients with relapsed/refractory solid tumors (9). Bortezomib was administered twice weekly for 2 consecutive weeks at either 1.2 or 1.6 mg/m^2 followed by a 10-day rest period. The recommended phase 2 dose for children with nonhematologic malignancies was 1.2 mg/m^2 and the dose-limiting toxicity was thrombocytopenia.

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This report presents the results of a phase 1 trial and pharmacokinetic study of bortezomib given twice weekly for 2 consecutive weeks every 21 days in pediatric patients with refractory leukemia. The objectives of this study were to identify the optimal bortezomib dose for phase 2 pediatric trials in leukemia, to determine the incidence and severity of toxicities associated with bortezomib, and to examine the pharmacokinetics and biological activity of bortezomib.

Patients and Methods

Patient eligibility. Patients between the ages of 1 and 21 years (inclusive) with histologically confirmed disease (>25% blasts in bone marrow aspirate or biopsy) of refractory acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), or chronic myelogenous leukemia in blast crisis were eligible for this trial. Other eligibility criteria included: (*a*) a Karnofsky or Lanksy performance score of ≥50, (*b*) recovery from the acute toxic effects of prior chemotherapy, radiotherapy, or immunotherapy with a minimum elapsed period of at least 7 days since the last dose of corticosteroids or hematopoietic growth factors, (*c*) at least 3 months since the last stem cell transplant, and (*d*) at least 3 months since any prior craniospinal radiation, pelvic radiation, or total body irradiation; at least 2 weeks since local palliative radiation; and at least 6 weeks since other substantial radiation. Patients could have no evidence of active graft versus host disease. Patients were required to have adequate renal function (serum creatinine below the upper limits of normal for age or a creatinine clearance or radioisotope glomerular filtration rate ≥70 mL/min/1.73 m^2); adequate liver function (serum bilirubin ≤1.5 mg/dL, alanine aminotransferase ≤5 times the institutional upper limit of normal for age, and albumin ≥2 g/dL); a platelet count ≥20,000/μL and a hemoglobin count of ≥8 g/dL. Transfusion support was allowed if necessary to meet hematologic criteria. Study exclusion criteria included: central nervous system leukemia, pregnancy or lactation in women of child-bearing age, uncontrolled infection, hyperleukocytosis (WBC > 100,000 cells/μL), receipt of concomitant enzyme-inducing anticonvulsants or concomitant use of other experimental agents. Hydroxyurea was permitted up to 24 h before the start of therapy if needed for cytoreduction. Informed consent from the patient or their parent(s) and assent, as appropriate, were obtained in accordance with the U.S. National Cancer Institute, Children's Oncology Group, and individual institutional review board policies prior to study entry.

Dosage and drug administration. Bortezomib was supplied by the Cancer Therapy Evaluation Program (National Cancer Institute, Bethesda, MD) as a lyophilized formulation containing 35 mg of mannitol. A 3.5 mg vial of the drug product was reconstituted with 3.5 mL normal saline (U.S. Pharmacopeia), such that the reconstituted solution contained 1 mg/mL of bortezomib. The appropriate dose of drug was administered as an intravenous push over 3 to 5 s.

Trial design. The starting dose of bortezomib in this pediatric phase 1 trial was 1.3 mg/m^2/dose, which was the maximum tolerated dose of bortezomib in adults with hematologic diseases using the same schedule (10), and was within 10% of the maximum tolerated dose for children with solid tumors. Subsequent planned dose escalations were in increments of 30%. Drug was administered twice weekly for 2 consecutive weeks (days 1, 4, 8, and 11) followed by a 1-week rest. Cycles were repeated every 21 days if there was no evidence of progressive disease (i.e., <25% increase in percentage of peripheral blood or bone marrow aspirate tumor cells), the platelet count was ≥20,000/μL, the hemoglobin count was ≥8 g/dL (with transfusion support), and other treatment-related adverse events were grade 1 or lower. Doses were not held or omitted for hematologic toxicity during a cycle of therapy.

A minimum of three patients were entered at each dose level and the dose level was expanded to up to six patients if one patient experienced dose-limiting toxicity during the first cycle of therapy. When dose-limiting toxicity was observed in two patients of a cohort of three to six patients receiving the same dose of drug, the maximum tolerated dose was exceeded. The maximum tolerated dose of bortezomib was defined as the dose level immediately below the level at which at least two patients experienced dose-limiting toxicities in the first cycle of therapy.

Toxicities were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0). Dose-limiting nonhematologic toxicity was defined as any grade 3 or 4 adverse event attributable to the study drug with the specific exclusion of grade 3 nausea or vomiting, grade 3 hepatic transaminase (aspartate aminotransferase or/or alanine aminotransferase) elevation returning to grade 1 or lower before the next treatment cycle, and grade 3 fever or infection. Dose-limiting hematologic toxicity was defined as bone marrow aplasia in the absence of malignant cell infiltration for ≥6 weeks of duration from the first treatment day; specifically, failure to recover a peripheral absolute neutrophil count of ≥500/μL and platelets ≥20,000/μL.

Criteria for assessment of response. Complete response was defined as the attainment of an M1 bone marrow (<5% blasts and adequate marrow cellularity) with no evidence of circulating blasts or extramedullary disease. Partial response was defined as the complete disappearance of circulating blasts and achievement of M2 marrow status (≥5% or <25% blast cells and adequate cellularity). Progressive disease was defined as an increase of at least 25%, or an absolute increase of at least 5,000 cells/mm^3 (whichever was greater) in the number of circulating leukemia cells, development of extramedullary disease, or other laboratory or clinical evidence of progressive disease. Stable disease was defined as failing to fulfill the criteria for a complete response, partial response, or progressive disease. Two cycles of therapy and two objective status determinations of complete response or partial response before progression were required.

Pharmacokinetic studies. The concentration of bortezomib in plasma was determined using a high-pressure liquid chromatography/mass spectroscopy assay. Two hundred microliters of plasma were diluted with 50 μL of 0.1% formic acid in water followed by 50 μL of a stable isotope-labeled internal standard (^{13}C-bortezomib) prepared in 50:50 acetonitrile/water with 0.1% formic acid. The samples were then extracted with methyl tert-butyl ether, evaporated, reconstituted, and injected onto the liquid chromatography/mass spectroscopy system for analysis. A reverse-phase gradient method provided sample stacking and separation. Bortezomib and the internal standard were ionized under a positive ion spray mode and detected through multiple reaction monitoring. The lower limit of quantitation for bortezomib in plasma was 0.1 ng/mL, and the assay was linear between 0.1 and 20 ng/mL. The coefficient of variation was 6.9% at the lower limit of detection and was 1.5% at the upper limit of quantitation. The correlation coefficient was ≥0.997. The interassay coefficient of variation of bortezomib (e.g., triplicate) in quality control standards was ≤8% for all control samples.

Bortezomib concentration time data were modeled with ADAPT II using maximum likelihood estimation (11). Two- and three-compartment models were fit to the data and compared using Akaike's information criterion (12). Peak plasma concentration (C_{max}) was the maximum measured plasma concentration determined 5 min after drug administration unless otherwise noted. Clearance (Cl) was calculated from the central compartment volume of distribution and the central compartment elimination rate constant (Cl = K_{10} ×Vc). The half-lives for each phase were calculated from the model fit. Area under the curve (AUC_{inf}) was calculated directly from the concentration time data using the linear trapezoidal method (AUC_{0-t}) and was extrapolated to infinity using the equation AUC_{inf} = AUC $_{(0-t)}$ + C_{last}/K_t, where C_{last} was the final quantifiable concentration and K_t was the terminal elimination rate constant fit to the final three time points.

Flow cytometry. Bone marrow aspirate cells were analyzed before treatment, 3 to 6 h after bortezomib treatment on day 8, and prior to cycle 2 (day 18) of cycle 1. Tumor cells were gated using $CD45_{dim}$, CD19, CD10 (ALL), or CD33 (AML) using established techniques.

Apoptotic cells were identified with either FITC- or PE-coupled Annexin V and 7-amino-actinomycin D (Medical Biology Laboratories, Watertown, MA).

Determination of NF-κB activity by ELISA. Peripheral blood mononuclear cells (PBMC) or bone marrow aspirate cells were isolated from diluted patient blood or marrow samples using Lymphoprep solution (Axis-Shield, Oslo, Norway). PBMC were obtained before bortezomib treatment and at 6, 12, and 24 h following the first bortezomib treatment; bone marrow aspirate cells were similarly isolated from diluted bone marrow aspirate samples prior to bortezomib treatment, 3 to 6 h following bortezomib administration on day 8 (unless otherwise noted), and prior to the next cycle of therapy (day 18). All samples contained >90% tumor cells unless otherwise noted as determined by flow cytometry (see above). Mononuclear cells were washed twice with cold PBS, counted, and frozen as cell pellets at −80°C. PBMC were either isolated at our institution or isolated at the treating facility, frozen, and shipped to our institution as a cell pellet as noted below. Samples from each patient were processed in an identical manner. The viability of each patient sample was confirmed to be >90% in each sample, as determined by trypan blue exclusion. NF-κB activity tested in normal lymphocytes was similar before and after sample shipment. Nuclear lysates were prepared and active p65 NF-κB determined by ELISA (TransAM NF-κB p65 transcription factor assay kit; ActiveMotif, Carlsbad, CA) according to the manufacturer's directions. The absorbance of each sample was determined using a microplate reader (SpectraFluor Plus, Tecan, NC) set at 450 nm. The assay was done on each sample as follows: patient 1 is a weighted average of three independent ELISA determinations done in triplicate (pretreatment and posttreatment, blood, and bone marrow), patients 2 (pretreatment and posttreatment) and 3 (pretreatment only) are an average of two independent determinations, and patients 4 and 5 (pretreatment only) are weighted averages from two independent determinations done in triplicate. Three patients with either no peripheral lymphoblasts ($n = 2$) or a mixed population of lymphoblasts and lymphocytes ($n = 1$) were excluded from the analysis. Protein integrity and protein quantification was confirmed by immunoblot using a β-actin antibody (see below).

Immunoblot analysis. Patient bone marrow aspirate cells and peripheral PBMC were isolated and the percentage of tumor cells after isolation was determined by flow cytometry as described above. Cells were washed twice in cold PBS, pelleted by low-speed centrifugation (800 × *g* for 5 min) and frozen at −80°C. Cell pellets were slowly thawed on ice and lysed in PBSTDS buffer (PBS, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) containing protease and phosphatase inhibitors [1 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride, one tablet of complete miniTab protease inhibitor cocktail (Roche, Basel, Switzerland), 100 mmol/L sodium orthovanadate, and 100 mmol/L sodium fluoride (Sigma, St. Louis, MO)] for 10 to 15 min on ice. Lysates were then centrifuged at 1,000 × *g* for 20 min and the supernatants aliquoted and frozen at −80°C. After protein quantification [using detergent-compatible protein dye and bovine serum albumin; Bio-Rad standards (Hercules, CA)], 10 to 40 μg of protein extracts was electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). The filters were blocked for nonspecific binding in Odyssey blocking buffer (LiCor Biotech, Lincoln, NE) for 1 h at room temperature. The filters were then incubated overnight (4°C) with anti-ubiquitin (1:1,000; EMD Biosciences, Darmstadt, Germany), IκB (1:500; Cell Signaling Technology), caspase 8 (1:1,000; Cell Signaling Technology), caspase 9 (1:1,000; Cell Signaling Technology), or β-actin (1:10,000; Sigma) antibodies diluted in Odyssey blocking buffer. Bound primary antibodies were detected with IR-800 or IR-700 dye-labeled, appropriate species-specific secondary antisera and the signal was visualized on a Li-Cor Odyssey IR scanner. The intensity of the gel bands was quantified using ImageQuant software (GE Healthcare, Uppsala, Sweden).

Statistical analysis. Descriptive statistics and bar graphs are used to summarize NF-κB ELISA assays from blood and bone marrow aspirate at each time point of measurement. Pairwise comparisons from pretreatment to each posttreatment level were made using paired *t* test on the log-transformed data. Normality assumptions were assessed on the log-transformed data.

Results

Twelve patients, nine with ALL and three with AML, were enrolled in this study between April 2004 and November 2005. Seven patients were not fully assessable for toxicity because they did not complete the first course of bortezomib due to disease progression ($n = 6$) or did not receive drug due to infection ($n = 1$). The inevaluable patients did not experience unusual or severe bortezomib-related toxicities. In general, patients were heavily pretreated prior to receiving bortezomib. The patient cohort had received an average of four prior chemotherapy regimens; five patients had undergone prior stem cell transplantation and eight patients had received prior radiation (three prior total body irradiation, four prior craniospinal radiation, one prior testicular radiation, and one prior total body irradiation/testicular radiation). Patient characteristics for the eligible patients are listed in Table 1.

Dose-limiting toxicities. Dose-limiting toxicities that were possibly, probably, or definitely related to bortezomib for all patients who received at least one dose of bortezomib are reported in Table 2. One patient, an 18-year-old white male with B-precursor (pre-B) ALL treated at the 1.7 mg/m^2 dose level, experienced grade 3 confusion and a cranial nerve XII palsy that was initially considered to be possibly related to bortezomib. Although a lumbar puncture was not done at this time due to parent refusal, the patient subsequently developed symptoms consistent with central nervous system leukemia, including dysarthria that improved with radiation therapy. A second patient, a 10-year-old white male with pre-B ALL treated at the 1.7 mg/m^2/dose level, was hospitalized on day 6 of cycle

Table 1. Characteristics for eligible patients ($n = 12$)

Characteristic	Number
Age (y)	
Median (range)	12.5 (1-18)
Sex	
Male	8
Female	4
Race	
White	9
Black or African-American	2
Unknown	1
Ethnicity	
Non-Hispanic	9
Hispanic	3
Diagnosis	
ALL (pre-B)	9
AML	3
Prior treatment regimens	
Median (range)	4 (1-7)
Prior bone marrow transplant	5
Prior radiation therapy	8
On-study hematologic variables	
Median absolute neutrophil count, ×10^3 (range)	2.8 (0.1-20.9)
Evaluable patents ($n = 5$)	1.5 (0.1-20.9)
Median platelet count, ×10^3 (range)	55 (23-136)
Evaluable patients ($n = 5$)	67 (34-94)

Table 2. Dose-limiting toxicities during the first course of each dose level

Dose level (mg/m^2)	Number enrolled	Number evaluable	Number of patients with dose-limiting toxicities during cycle 1	Dose-limiting toxicities (no. of patients)
1.3	6	3	0	—
1.7	6	2	2	Confusion/CN XII palsy (n = 1), Grade 4 hypotension, grade 4 febrile neutropenia and grade 3 creatinine (n = 1)

1 with febrile neutropenia (absolute neutrophil count, 560), hypotension, and dehydration. His fever and hypotension resolved by day 8 and the patient received a third dose of bortezomib. Eleven hours following bortezomib administration, the patient developed severe hypotension unresponsive to dopamine, epinephrine, and milrinone. The patient subsequently developed anuria, acute respiratory distress, and multisystem organ failure resulting in death on day 10. Because no bacterial or fungal organisms were isolated from multiple cultures obtained during his hospital course, it is possible that the adverse events in this patient were related to bortezomib administration.

Non–dose-limiting toxicities attributable to bortezomib in cycle 1 at the1.3 mg^2 level included one patient each with grade 1 rigors/chills, pruritus, hypocalcemia; grade 2 fatigue, hypocalcemia, bone pain and hypoalbuminemia; and grade 3 nausea, vomiting, bacteremia, and pneumonia. Additional non–dose-limiting toxicities at the 1.7 mg/m^2 dose level included one patient each with grade 1 hypercalcemia, hyperphosphatemia, hypokalemia, and hyperuricemia; and grade 2 hypocalcemia, hyperkalemia, aspartate aminotransferase elevation, anorexia, and vomiting. One patient who received two cycles of drug (1.3 mg/m^2) experienced transient grade 2 and 3 elevations in aspartate aminotransferase and alanine aminotransferase, respectively, following cycle 2 that returned to baseline within 16 days. This patient also experienced grade 2 weight loss and fatigue; grade 3 nausea, vomiting, and anorexia; and grade 3 gastrointestinal bleeding (coffee-ground emesis and stomach erosions) with thrombocytopenia.

Response. There were no objective antitumor responses using bortezomib as a single agent. One patient with pre-B ALL treated with 1.3 mg/m^2 bortezomib had stable disease for two cycles and a decrease in peripheral WBC from 26,000 cells/μL to 400 cells/μL, but the bone marrow aspirate remained infiltrated (88% marrow lymphoblasts after cycle 2). An additional patient (pre-B ALL, 1.3 mg/m^2 bortezomib) had stable disease at the end of cycle 1 (decrease in marrow lymphoblasts from 76% to 35%), but had evidence of progressive disease following the second cycle of therapy. Although there were no objective responses to treatment using bortezomib as a single agent, one patient had a significant response to chemotherapy given immediately after bortezomib. This patient, an 18-year-old with relapsed pre-B ALL refractory to three prior treatment regimens (including two induction regimens), achieved a complete response (2% blasts in bone marrow) when treated with dexamethasone, Adriamycin, and vincristine immediately following bortezomib. This patient subsequently underwent a bone marrow transplant and lived for an additional year before developing progressive disease.

Pharmacokinetics. Five of the 11 patients that received bortezomib consented to pharmacokinetic sampling. Pharmacokinetic variables are presented in Table 3. In four of five patients, the three-compartment model provided the best model fit according to Akaike's criteria (12). There was little apparent relationship between peak plasma concentration (C_{max}) and dose, which was likely due to the small sample size and variability in the timing of initial sample acquisition. All patients had a rapid initial distribution half-life ($t_{1/2}\alpha$, 1.4-6.6 min), followed by a longer redistribution half-life ($t_{1/2}\beta$, 32-79 min), and a sustained terminal elimination half-life ($t_{1/2}\gamma$, 72-1,534 min). Terminal half-life and clearance for one patient with progressive disease (1.7 mg/m^2) were outliers that resulted in a large $t_{1/2}\gamma$ and low clearance estimates.

Accumulation of ubiquitinated proteins. Ubiquitinated proteins accumulated in lymphoblasts obtained 3 h after the day 8 dose of bortezomib in the one patient with stable disease

Table 3. Pharmacokinetic variables from noncompartmental analysis (C_{max} and AUC) and a three-compartment model analysis ($t_{1/2}\alpha$, $t_{1/2}\beta$ and $t_{1/2}\gamma$, Vc and Cl) of bortezomib in plasma

Dose (mg/m^2)	C_{max} (ng/mL)	AUC_{inf} (h ng/mL)	$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (min)	$t_{1/2}\gamma$ (min)	V_c (L/m^2)	Cl ($mL/h/m^2$)
1.3	44	30	3.5	32	600	13	41
1.3	73	28	1.4	34	816	2.1	31
1.3	70	23	3.0	41	758	5.6	42
Mean	**63**	**27**	**2.7**	**36**	**725**	**6.7**	**38**
SD	**16**	**3.0**	**0.9**	**4.1**	**91**	**4.5**	**4.8**
1.7	54	42	6.0	45	719	16	37
1.7	41*	47	6.6	79	1,534	13	22

Abbreviations: C_{max}, maximum serum concentration at 5 min; AUC_{inf}, area under the concentration-time curve extrapolated to infinity; $t_{1/2}\alpha$, distribution half-life; $t_{1/2}\gamma$, elimination half-life; Vc, volume of distribution in the central compartment; Cl, clearance.
*C_{max} determined at 13 min.

(Fig. 1). However, ubiquitinated conjugates did not seem to accumulate following the day 1 dose of bortezomib in a second patient with progressive disease. Of note, although this patient with AML had little or no accumulation of ubiquitin conjugates prior to bortezomib treatment (Fig. 1B, lane 2), eight of nine patients on this study (five of five with ALL and two of three with AML) had significantly elevated levels of ubiquitinated conjugates prior to bortezomib treatment (data for seven patients shown in Fig. 1A, lane 4, lane 7, and Fig. 1B, lanes 1, 3-6). The presence of elevated ubiquitin conjugates was also noted in a limited independent set of leukemia bone marrow samples (four of seven ALL lymphoblast samples, four of six AML myeloblast samples; data not shown). Elevated pretreatment ubiquitin conjugates may indicate a dysfunctional ubiquitin-proteasome pathway system in some patients with relapsed leukemia.

NF-κB activity and IκB expression following bortezomib treatment. Because one mechanism for bortezomib's antitumor activity may be IκB stabilization and subsequent inhibition of NF-κB activity, we analyzed IκB protein expression and NF-κB activity before and after bortezomib treatment (Fig. 2; Table 4). One patient with stable disease following bortezomib treatment had evidence of limited bone marrow leukemia cell apoptosis on day 8, as shown by an increase in tumor cell Annexin V expression (11% Annexin positivity; data not shown). This patient also had evidence of caspase 9 cleavage 3 h after bortezomib treatment on day 8 (Fig. 2A). Unexpectedly, this patient had decreased IκB protein expression 3 h after bortezomib treatment on day 8 (Fig. 2A, left). A similar pattern of IκB expression was seen in patient 2 following day 1 bortezomib administration; in this patient, IκB expression decreased 6 h after bortezomib treatment (2-fold decrease), then normalized after 24 h. In contrast to IκB protein expression, there seemed to be little change in protein expression of the γ-subunit of IκB kinase (IKKγ, NEMO), bcl-2, JNK kinase (Fig. 2A, left), or caspase 8 (data not shown) in patient 1 following bortezomib treatment.

Consistent with short-term decreases in IκB expression, there was a short-term increase in peripheral lymphoblast NF-κB activity after bortezomib treatment followed by a significant decrease in NF-κB activity after 24 h. The average pretreatment NF-κB binding activity in peripheral blood lymphoblasts for

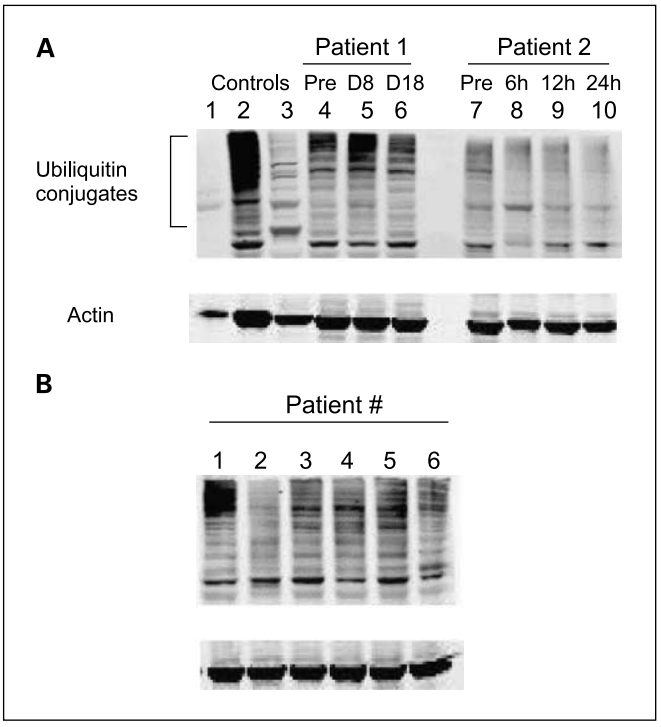

Fig. 1. The pattern of ubiquitinated proteins present before and after bortezomib treatment. *A,* immunoblot of ubiquitinated proteins in bone marrow aspirate cells from patient 1 with pre-B ALL (98% tumor) before treatment (*lane 4*), 3 h following bortezomib treatment on day 8 (*lane 5*), and in a recovery bone marrow obtained 1 wk after the last dose of bortezomib in cycle 1 (day 18, *lane 6*). Ubiquitinated protein expression is also shown in cells isolated from peripheral blood (94% blasts) in a second patient with AML who did not respond to bortezomib treatment (*lanes 7-10*). Samples were obtained before bortezomib (*lane 7*) and at 6, 12, and 24 h following the first bortezomib dose (*lanes 8-10*). Controls: normal bone marrow mononuclear cells (*lane 1*), Jurkat T cells 4 h after treatment with the proteasome inhibitor MG132 (*lane 2*), and normal PBMCs (*lane 3*). *B,* the pattern of ubiquitinated protein conjugates in tumor cells obtained from six patient samples before bortezomib treatment. Percentage of tumor cells and subtype of leukemia for each patient: lane 1, pre-B ALL (95% blasts); lane 2, AML (same sample as patient 2 in *A*); lane 3, pre-B ALL (92% blasts); lane 4, pre-B ALL (85% blasts); lane 5, AML (80% blasts); and lane 6, AML (23% blasts).

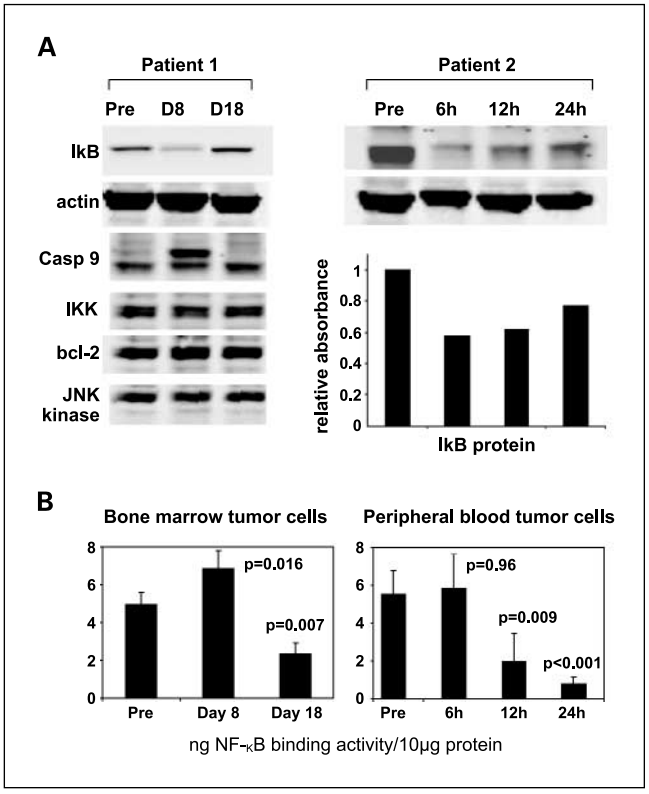

Fig. 2. Characterization of apoptosis and the NF-κB pathway proteins in two patients following bortezomib treatment. *A,* IκB expression in two patients treated with bortezomib. Both patients were treated with 1.3 mg/m^2 bortezomib on days 1, 4, 8, and 11. Marrow and blood samples for patient 1 were shipped prior to processing; blood samples obtained from patient 2 were processed locally. Left, bone marrow cells (98% blasts) were obtained from patient 1 prior to treatment (*lane 1*), 3 h following bortezomib treatment on day 8 (*lane 2*), and 1 wk after last bortezomib dose on day 18 (*lane 3*). PBMCs (94% blasts) were obtained from a second patient (*right*) before bortezomib treatment (*lane 2*) and at 6, 12, and 24 h following the first dose of bortezomib (*lanes 2-4*). IκB expression in the second patient is normalized to actin and quantitated (*right*). Expression of the γ-subunit of IκB kinase (IKKγ, NEMO), bcl-2, and JNK kinase are also shown for patient 1 (*left*). *B,* NF-κB quantitation in bone marrow aspirate (*left*) and tumor PBMCs (*right*) from patient 1 using an active NF-κB p65 ELISA. Significant differences are noted as determined by a Student's *t* test. Posttreatment samples were compared with pretreatment samples from the same patient as noted.

Table 4. NF-κB activity in peripheral leukemia cells before and after bortezomib treatment

Patient no.	NF-κB activity (ng/10 μg protein)			
	Pre	6 h	12 h	24 h
1*	5.5 ± 1.2	5.8 ± 1.8	2.0 ± 1.5	0.8 ± 0.3
2†	7.6 ± 0.5	—	10.3 ± 2.4	1.8 ± 0.4

*Samples shipped to our institution prior to NF-κB determination.
†Tumor cells isolated locally prior to NF-κB determination.

the five patients with >90% lymphoblasts was 7.1 ± 0.7 ng NF-κB binding activity/10 μg protein. Two of these patients had matched tumor peripheral blood samples collected before and after bortezomib treatment, and the NF-κB binding activity decreased 4- to 6-fold 24 h after bortezomib treatment (Fig. 2B, right; $P < 0.001$; Table 4). Similarly, there was an increase in NF-κB activity 3 h after the bortezomib dose on day 8 ($P = 0.016$), followed by a significant decrease in NF-κB activity in malignant bone marrow lymphoblasts in one patient by the end of the first cycle ($P = 0.007$; Fig. 2B, left).

Discussion

In this study, we examined the toxicity and tolerable dose of bortezomib given as a single agent to pediatric patients with relapsed or refractory leukemia. Drug disposition of bortezomib in children (Table 3) was similar to that observed in adults (13). Due to the high inevaluability rate secondary to disease progression, and because one patient experienced severe hypotension at the second dose level (1.7 mg/m^2), the recommended phase 2 dose is 1.3 mg/m^2. Severe hypotension following bortezomib has been a rare, but previously reported, adverse event in adults (14, 15).

As noted above, one refractory patient entered complete response to a steroid-containing regimen following bortezomib. Four other patients were treated with steroid-containing regimens after bortezomib; two of these patients had transient decreases in tumor burden (i.e., decreases in peripheral blood counts with no improvement in bone marrow involvement), and two patients had only a minimal response. There has been a prior case report of a complete response following treatment of pediatric relapsed ALL with the combination of dexamethasone and bortezomib (16). Prior *in vitro* studies have also suggested that bortezomib and dexamethasone may be synergistic in leukemia (17). Together, these data suggest that although bortezomib is ineffective as a single agent, it may act as a chemosensitizer.

Because bortezomib is expected to stabilize ubiquitinated proteins, we examined the effect of bortezomib treatment on ubiquitinated protein conjugates (Fig. 1). There were an unusual number of ubiquitin conjugates present prior to bortezomib treatment in patient samples (Fig. 1) and in banked lymphoblasts (data not shown). Although the accumulation of ubiquitin protein conjugates has not been previously reported in acute leukemia, Masdehors et al. (18) has previously reported an accumulation of ubiquitin conjugates in adults with chronic lymphocytic leukemia. This may indicate that ubiquitin-mediated degradation is at least partially dysfunctional in pediatric acute leukemia and it is possible that preexisting proteasome dysfunction can attenuate the cytotoxic effects of proteasome inhibition. Other researchers, however, have suggested that cells with deregulated ubiquitin conjugates may be more susceptible to proteasome-induced apoptosis because the cells are already stressed with an abnormal increase in ubiquitinated proteins (5). Investigators have postulated that proteasome-induced stress in the endoplasmic reticulum can induce apoptosis independent of the NF-κB or other signaling pathways (19).

Unexpectedly, we observed a short-term decrease in IκB expression 3 to 6 h following bortezomib treatment in both bone marrow and peripheral blood (Fig. 2A). Consistent with this finding, there was an initial increase in NF-κB binding activity at 3 to 6 h following bortezomib treatment, followed by a 4- to 6-fold decrease in NF-κB activity at later time points (24 h). It is possible that the initial increase in NF-κB was a stress response induced by bortezomib treatment. This would suggest that both the limited apoptosis (data not shown) and the increase in caspase 9 cleavage (Fig. 2A) induced by bortezomib after 3 to 6 h may be NF-κB–independent. One explanation of this finding is that other proteasome pathway proteins are responsible for bortezomib-induced apoptosis in this patient population. Several other cell regulatory proteins are controlled by proteasome-dependent degradation, including cyclins, cyclin kinase inhibitors, and bcl-2 family members; and destabilization of these pathways may have contributed to the observed apoptosis.

In summary, 12 pediatric patients with relapsed leukemia were enrolled in this phase 1 trial of bortezomib and we recommend a phase 2 dose of 1.3 mg/m^2 in children with relapsed leukemia. We observed limited tumor cell apoptosis and caspase 9 cleavage early after treatment with delayed inhibition of tumor cell NF-κB DNA binding activity in two patients. Although bortezomib was ineffective as a single agent in this population, its role as a potential chemosensitizing agent when combined with cytotoxic chemotherapy merits further study.

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