Clinical and pharmacodynamic activity of bortezomib and decitabine in acute myeloid leukemia

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We recently reported promising clinical activity for a 10-day regimen of decitabine in older AML patients; high *miR-29b* expression associated with clinical response. Subsequent preclinical studies with bortezomib in AML cells have shown drug-induced *miR-29b* up-regulation, resulting in loss of transcriptional activation for several genes relevant to myeloid leukemogenesis, including DNA methyltransferases and receptor tyrosine kinases. Thus, a phase 1 trial of bortezomib and decitabine was developed. Nineteen poor-risk AML patients (median age 70 years; range, 32-84 years) enrolled. Induction with decitabine (20 mg/m^2 intravenously on days 1-10) plus bortezomib (escalated up to the target 1.3 mg/m^2 on days 5, 8, 12, and 15) was tolerable, but bortezomib-related neuropathy developed after repetitive cycles. Of previously untreated patients (age ≥ 65 years), 5 of 10 had CR (complete remission, n = 4) or incomplete CR (CRi, n = 1); 7 of 19 overall had CR/CRi. Pharmacodynamic analysis showed *FLT3* down-regulation on day 26 of cycle 1 (*P* = .02). Additional mechanistic studies showed that *FLT3* down-regulation was due to bortezomib-induced *miR-29b* up-regulation; this led to *SP1* down-regulation and destruction of the SP1/NF-κB complex that transactivated *FLT3*. This study demonstrates the feasibility and preliminary clinical activity of decitabine plus bortezomib in AML and identifies *FLT3* as a novel pharmacodynamic end point for future trials. This study is registered at http://www.clinicaltrials.gov as NCT00703300. (*Blood*. 2012;119(25): 6025-6031)

Introduction

Despite progress made in understanding the mechanisms of leukemogenesis and the identification of cytogenetic and molecular markers for risk stratification, most adult patients with acute myeloid leukemia (AML) are not cured when treated with conventional chemotherapy, especially elderly patients.[1,2] Thus, novel approaches to improve outcomes for patients with AML are needed.

Bortezomib is a proteasome inhibitor approved for the treatment of multiple myeloma and mantle cell lymphoma, but only transient hematologic improvements were noted in a single-agent phase 1 study of bortezomib in AML.[3] Despite lack of single-agent activity, bortezomib has shown promise when used in combination regimens for AML.[4] We recently demonstrated a unique mechanism of activity of bortezomib: the drug is an indirect transcriptional inhibitor for several target genes that are relevant to AML.[5,6] We showed an important role for bortezomib in disrupting a network that operates on the basis of interactions of *miR-29b*, the transcription factor SP1, and NF-κB(p65). This network affects the expression of several genes in myeloid leukemia cells, including DNA methyltransferase enzymes (DNMT) and the receptor tyrosine kinase (RTK) *KIT*.[5,6] We showed that activating *KIT* mutations, frequently found in core binding factor AML, led to MYC-dependent *miR-29b* repression, resulting in increased levels of SP1 (a *miR-29b* target).[5] Up-regulated SP1 bound NF-κB(p65) and transactivated *KIT*. Therefore, activated KIT ultimately induced its own transcription via *miR-29b*.[5] We demonstrated that bortezomib-induced disruption of the SP1/NF-κB(p65) complex inhibited the growth of leukemic cells via up-regulation of *miR-29b*.[5] The results supported the notion that *miR-29b*/SP1/NF-κB(p65) complex–dependent *KIT* overexpression contributed to the growth of leukemia and could be targeted by bortezomib.[5] Because most AML cells express FLT3 (another member of the RTK family) and because of the relevance of both wild-type and mutated *FLT3* expression for AML cell growth and survival, FLT3 is an important target in AML[7-11]; we hypothesized that the aforementioned mechanisms also extend to *FLT3* expression.

In the current study, we sought to deepen our understanding of the transcriptional inhibitory activity bortezomib and its potential use in patients with AML by combining clinical, pharmacodynamic, and additional in vitro mechanistic experiments. Recently, we reported that the DNA hypomethylating agent decitabine is active in AML. In a phase 2 study conducted at our institution, the complete remission (CR) rate was 47%, the overall response

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rate 64%, and the median overall survival duration approximately 1 year with a 10-day induction regimen of low-dose decitabine in untreated older patients with AML (≥ 60 years, not candidates/refused intensive therapy).[12] Although these results were promising, we viewed the regimen as a framework on which future investigations might build and improve. Given that (1) greater *miR-29b* levels were associated with response to decitabine in that trial[12] and (2) preclinical work showed bortezomib to be an inducer of *miR-29b* expression, bortezomib was a very appealing agent for combination studies with decitabine. Therefore, we performed a phase 1 clinical trial of bortezomib with decitabine in poor-risk AML patients to test feasibility and provide preliminary clinical response data for this combination; we further developed our understanding of the role bortezomib in AML via pharmacodynamic and additional in vitro studies.

Methods

Eligibility criteria and study design

Eligible patients were adults with either (1) relapsed or refractory AML or (2) previously untreated AML who were ≥ 65 years of age. Patients were required to have total bilirubin ≤ 2× the upper limit normal, creatinine ≤ 2.0 mg/dL, alanine aminotransferase/aspartate aminotransferase ≤ 5× upper limit normal, left ventricular ejection fraction at least 40%, and Eastern Cooperative Oncology Group performance status ≤ 2. Exclusion criteria included chemotherapy or radiotherapy within 2 weeks, active other malignancies (within 3 years), active CNS disease or granulocytic sarcoma as sole site of disease, uncontrolled intercurrent illness, and pre-existing neuropathy grade 2 or greater. Informed written consent approved by The Ohio State University (OSU) Human Studies Committee was obtained on all patients before they were entered into the study, in accordance with the Declaration of Helsinki.

Patients were given induction cycles of decitabine 20 mg/m^2 intravenously over 1 hour on days 1-10 with cycles repeated every 28 days until bone marrow (BM) blasts were < 5%, at which time decitabine dosing was cut to 3-5 days/cycle as previously described.[12] Bortezomib was administered immediately after the decitabine dose by intravenous push (IVP). Bortezomib was dose escalated according to the following dose-escalation plan: dose level 1, 0.7 mg/m^2 IVP on days 5 and 8; dose level 2, 0.7 mg/m^2 IVP on days 5, 8, 12, and 15; dose level 3, 1.0 mg/m^2 IVP on days 5, 8, 12, and 15; and dose level 4, 1.3 mg/m^2 IVP on days 5, 8, 12, and 15. Treatment delays of ≥ 10 days were permitted for patients with a BM cellularity of ≤ 10% and no evidence of disease in the marrow, until at least partial restoration of hematopoiesis occurred (defined as BM cellularity > 10% or absolute neutrophil count [ANC] > 1000/μL). Hydroxyurea was permitted to control white blood count to < 40 000/μL, if necessary, before and during cycle 1, but no other antileukemic therapies were permitted. In the absence of a hypoplastic marrow (≤ 10% cellularity), clearly progressive increase in BM blasts (after at least 2 cycles of administration, if possible), ongoing/uncontrolled infection, or serious hemorrhagic complications, dosing was to be continued every 4 weeks without delay. Treatment continued indefinitely until disease progression or unacceptable toxicity occurred, except that bortezomib was discontinued in patients who did not have an objective response after 3 cycles of treatment. Reponses were defined according to the International Working Group criteria for AML, including CR and CR with incomplete count recovery (CRi).[13]

Definition of dosing-limiting toxicity

Adverse events were graded according to the National Cancer Institute (NCI) Common Toxicity Criteria for Adverse Events Version 3.0. Dose-limiting toxicity (DLT) was defined with cycle 1 of therapy. Drug-related nonhematologic toxicity of grade 4 was considered DLT with the exception of alopecia, nausea and vomiting controllable with antiemetic therapy, infection, and fatigue. Given the frequency of infectious complications with conventional chemotherapy in this population and prevalence of disease-related cytopenias, infectious complications were not mandated as DLT unless the severity or duration was longer than that expected with conventional treatment. For DLT, if the toxicity occurred in 2 or more patients at a single-dose level, that dose was deemed intolerable and the next lower dose level was expanded to increase confidence in toxicity assessment at the maximum tolerable dose. Hematologic DLT was defined as follows: failure to recover neutrophil and/or platelet counts by day 42 in patients with < 5% blasts in the BM, absence of myelodysplastic changes, and/or absence of evidence of disease by flow cytometry in the BM. Six additional patients were treated at the maximum tolerable dose.

Cytogenetics, molecular markers, and correlative studies

Standard cytogenetic analyses were performed on BM samples. For patients who consented to and had additional material available for molecular studies, the presence or absence of *FLT3*-ITD and *FLT3*-TKD was determined as previously described.[7,11] Correlative studies included the measurement of *miR-29b*, *FLT3*, *DNMT1*, *DNMT3A*, *DNMT3B*, and estrogen receptor (*ESR*) mRNA expression in BM at pretreatment and at approximately day 26 of cycle 1 (± 2 days) with the use of RT-PCR as previously described.[6,12] In brief, total RNA was extracted with Trizol (Invitrogen) reagent, and cDNA was synthesized from total RNA. Gene expression of *FLT3*, *DNMT1*, *DNMT3A*, *DNMT3B*, and *ESR* were normalized to *ABL1*. For *miR-29b* expression, quantitative RT-PCR was performed by TaqMan MicroRNA Assays (Applied Biosystems) according to the manufacturer's protocol and normalized by *U44* as previously described.[12] Expression of the target genes were measured by use of the ΔCT approach. All Taqman Assays for gene and microRNA expression were purchased from Applied Biosystems

Cell culture and treatment

MV4-11, KG1, and HEK293T cell cultures were in standard fashion. Cells were treated with bortezomib (Millennium Pharmaceuticals) used at concentrations, times, and schedules indicated in the Results section. For additional in vitro mechanistic studies, mononuclear cells from BM samples with > 70% blasts from an AML patient were obtained from OSU Leukemia Tissue Bank. Primary patient blasts were cultured in StemSpan SFEM media (StemCell Technologies), supplemented with StemSpan CC100 (StemCell Technologies) containing FLT3-ligand, stem cell factor, IL-1, and IL-6. Patients signed an informed consent to store and use their tissue for discovery studies according to OSU institutional guidelines.

Transient transfections

Construction of the human SP1 and NF-κB(p65) expression vectors were performed as previously described.[5] On-target plus Smart pool siRNA for *SP1*, *NFKBP65*, and controls were purchased from Thermo Fisher Scientific. Precursor *miR-29b* was obtained from Applied Biosystems. siRNA, miRNA, or plasmid constructs were introduced into the leukemia cell lines MV4-11 and KG1 by Nucleofector Kit (Lonza Walkersville) according to the manufacturer's instructions and as previously reported.[5] Transient transfections of HEK293T cells for the luciferase experiments were performed with the use of Lipofectamine reagent (Invitrogen) according to the manufacturer's description.

ChIP assays

ChIP assays were performed with the EZ ChIP Assay Kit (Millipore) per manufacturer recommendations and as previously described.[5] DNA was quantified by the use of quantitative RT-PCR with SYBR green incorporation (Applied Biosystems). The antibodies used were as follows: SP1 (Cell Signaling) and NFkB p65 (Millipore). Quantitative PCR was used to measure the fraction of *FLT3* promoter DNA enrichment in the immunoprecipitated with SP1 and NFkB p65 antibody.

Table 1. Patient characteristics

Previously untreated patients, age, y/sex	Secondary or de novo	Diagnostic karyotype	Presenting WBC × $10^3/\mu L$	% BM blasts	Response
81/F	De novo	46,XX,del(5)(q13q33)(2)/46,sl,del(11)(p12)(cp2)/46,sl,t(X;6)(p22.1;q13),del(12)(p11.2), del(16)(q12.1)(15)/ nonclonal with clonal abnormalities(1)	4.8	71	CRi
66/F	Secondary	Insufficient Metaphase (46,XX(6)/nonclonal(2))	1.5	48	CR
84/M	De novo	85-88 < 4n > ,XXYY,i(1)(q10),-2,-3,-7,-9,-17,i(17)(q10),+18(cp15)/46,XY(4)/8n(1)	1	76	
65/F	De novo	Normal	5.2	20	CR
68/M	De novo	44,X-Y,-3,del(5)(q22q35),del(6)(q13), del(17)(p11.2)(5)/43,sl,-11,der(19)t(1 1;19) (q12;p13.3)(3)/44,sl,+mar1(cp3)/ 44,sl,add(19)(p13.3)(cp2)/44,sl,+mar2(2)/46,XY(5)	0.5	14	
70/M	De novo	Normal	11.3	65	
73/M	Secondary	46,XY,del(7)(q21)(13)/46,XY(6)/nonclonal(1)	39.8	39	
67/M	De novo	43,XY,-3,del(5)(q13),-7,del(8)(p12), dic(11;12)(p11.2;p11.2), add(15)(p11.1), 16,add(17)(p11.2),+mar(18)/46,XY(1)/nonclonal(1)	0.2	15	
70/M	De novo	46,XY,inv(3)(q21q26)(10)/46,XY(10)	0.5	23	CR
83/M	Secondary	46,XY,del(12)(p11.2p13)(3)/45,idem,-7(22).ish del(12)(ETV6-)	0.8	34	CRu

Cru indicates unconfirmed complete remission for the elderly patient who had peripheral blood count recovery but who refused BM evaluation after treatment, as noted in the text; and WBC, white blood cell.

Luciferase assays

Luciferase assays were conducted with the use of HEK293T cells. A 700-bp upstream region of *FLT3* promoter spanning SP1 and NFkB p65 binding sites was cloned into a pGL2-luciferase-reporter (Promega) vector using the *Hin*dIII restriction enzyme site with primers as indicated in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Firefly luciferase and *Renilla* luciferase activities were assessed according to the recommendations detailed in the Dual-Luciferase Reporter Assay System (Promega).

Electromobility shift assay

Nuclear proteins were extracted from MV4-11 cells using the Nuclear Extract Kit (Active Motif) according to the manufacturer's instructions. 5′-biotinylated DNA containing the predicted binding sites for SP1 (SP1-site1 and SP1-site2) and NF-κB (NF-κB-site 1 and NF-κB-site 2) were obtained from Integrated DNA Technologies. For annealing, concentrated complementary oligonucleotides were mixed at a 1:1 molar ratio and incubated at 95°C for 5 minutes. The heat was then gradually reduced over hours until the oligonucleotides reached room temperature 24 hours before start of the experiment. Annealed oligos were diluted to a final concentration of 10 fmol. The Thermo Scientific LightShift Chemiluminescent EMSA Kit (Pierce/Thermo Fisher Scientific) was used according to the manufacturer's instructions. For supershift experiments, SP1 antibody (Santa Cruz Biotechnology) or NFkB p65 antibody (Cell Signaling Technology) were added.

Western blotting

The western blots were preformed as previously described.[5] The antibodies used were as follows: SP1 and actin (Santa Cruz Biotechnology), NFkB p65 (Millipore), and FLT3 (Cell Signaling Technology).

Statistical analysis

Data were compared using the Student 2-tailed t test. $P \leq .05$ was considered statistically significant. All analyses were performed using the R 2.14.1 software package (available at http://www.r-project.org).

Results

Patient characteristics

The enrollment of 19 poor-risk AML patients occurred during the course of 11 months. Clinical and the cytogenetic characteristics of the patients enrolled on the clinical trial are summarized in Tables 1 and 2. The median age was 70 years (range, 32-84 years). Median white blood cell count was 3.9 × $10^3/\mu L$ (range, 1.3-69.9). Previously untreated patients (n = 10) were of median age 70 years (range, 65-84 years) and presented with intermediate or adverse cytogenetic risk according to Cancer and Leukemia Group B criteria[2]; 7 had de novo AML, and 3 had secondary/therapy-related

Table 2. Patient response

Relapsed/ refractory patients, age, y/sex	No. previous inductions	Pretreatment karyotype	Presenting WBC × $10^3/\mu L$	% BM blasts	Response
75/M	3	46,XY,dup(1)(q21q41)(2)/46,XY,del(3)(p21.1p21.3)(2)/46,XY(16)	1.8	4	
66//F	2	46,XX,del(5)(q22q33)(1)/55–56,sl,+1,+2,+8, del(8)(p11.2p23)x2,+9,+10,+11, del(12)(q13q15),+13,+14,+14,add(14)(q32),-17,add(18)(p11.2), add(19)(p13.2), +21,+22,del(22)(q13), +mar1, +mar2,+mar3,+mar4(cp8)/55–56,sdl1,add(4)(q32), -add(14)(q32)(cp5)/46,XX(5)/nonclonal(1)	0.9	42	
67/M	1	Normal	0.6	42	CRi
50/F	2	46X,t(x;10;11),del(12p)	22	86	
77/F	2	Unobtainable (dry tap)	2.1	63	
32/M	3	45,X,-Y,t(8;21)(q22;q22)cp7)/46,XY(13)	1.6	18	
70/M	3	94 < 4n > ,XXYY,+13,+13(9)/46,XY(11)	1.2	31	
73/M	1	Normal	0.4	19	CRi
57/M	4	47,XY,+8[16]/47,idem,t(2;12)(p16;q21)[1]/non-clonal abnormalities[3]	6.8	81	

WBC indicates white blood cell.

AML. Relapsed/refractory patients (n = 9) had a median age of 67 years (range, 32-75 years). Eight had did not respond to previous anthracycline/high-dose cytarabine therapy. All 4 patients who enrolled with AML in untreated first relapse had CR1 duration of < 1 year. Overall, 8 patients consented to a diagnostic assessment of *FLT3* mutational status; none harbored a *FLT3*-TKD, and 1 patient had a *FLT3*-ITD. All 8 patients expressed robust *FLT3* levels as measured by real-time RT-PCR (not shown). Five patients had serial material available for pharmacodynamic studies.

Dose escalation and treatment

Three patients were treated at dose level 1. Four patients were treated at dose level 2 because 1 patient was removed from study as the result of disease-related thrombosis. This event was not judged to be related to the drug because the patient had presented with AML originally with extensive deep-venous thrombosis thought to be related to malignancy and had an inferior vena cava filter placed 11 months earlier. Dose level 3 was expanded to 6 patients because of safety concerns after an infection-related death during induction in 1 patient. Six additional patients were treated at the highest planned dose of bortezomib (1.3 mg/m^2 on days 5, 8, 12, and 15); 1 respiratory death before cycle 2 occurred at this dose. Patients received a median of 2 cycles of treatment (range, 1-14). Only 2 patients received treatment with both drugs beyond 3 cycles. By design, bortezomib treatment was discontinued after 3 cycles in patients without response; neuropathy occurring after cycle 2 required the discontinuation of bortezomib in 3 patients. One patient received the combination of both drugs for 8 cycles, then decitabine alone for 6 additional cycles; the other received the combination for 4 cycles, then decitabine alone for 6 additional cycles.

Toxicities

Typically, induction death in AML is described at 30 days, but given the less-intensive nature of decitabine-based treatments with delayed response and the need for repetitive cycles of administration, 8-week mortality may be a better measure with this agent. Four patients (2 with refractory/relapsed disease, 2 with previously untreated disease) died within 8 weeks of treatment because of infection (n = 2), disease progression (n = 1), and respiratory failure from pulmonary fibrosis (n = 1). Only 1 of these deaths (from disease progression) occurred within 30 days of study entry. Infections or febrile neutropenia were commonly encountered, occurring in 11 patients during cycles 1 and 2 of treatment. Grade 3 or greater toxicities regardless of attribution during the first 2 cycles are listed in Table 3. Through the entire duration of the study, grade 3 or greater neuropathy occurred in 3 patients (none during cycle 1). For 1 of these patients, the neuropathy was autonomic and disabling. No hematologic DLT was observed.

Clinical responses

In previously untreated patients (all ages 65 years and older), CR/CRi occurred in 4 patients; another CR not confirmed by marrow evaluation occurred in a patient who refused BM evaluation after treatment (described in this paragraph), for an overall remission rate of 50% (5/10) in this subset. For the 4 previously untreated patients with documented CR/CRi, the best response and duration of response were as follows: CR, 12 months; CR, 9 months; CR, 10 months (died of myocardial infarction in remission); and CRi, 3 months (incomplete response was ANC < 1000/μL). The fifth responder was an 84-year-old patient who refused BM reevaluation after 2 cycles of treatment, but the patient had complete count recovery with no blood blasts and lived for 17 months before dying of unknown cause (the patient refused further follow-up after discontinuing trial participation). Although we could not classify this patient as a CR by International Working Group criteria because of lack of morphologic documentation by BM aspirate and biopsy, it is likely that this patient achieved CR. Among patients with relapsed or refractory AML, 2 of 9 achieved CRi (CR was incomplete in both cases because of ANC < 1000/μL). Response duration in one was only 2 months, but the other proceeded to allogeneic transplant quickly with no relapse more than 18 months after transplantation. On the basis of the pattern of the response, the patient likely would have met ANC recovery for CR had transplant not immediately occurred. Including the 1 patient with unconfirmed BM CR, the remission rate was 50% (5/10) for those with previously untreated disease and 37% (7/19) for the whole cohort. The median number of cycles to best response was 2 (range, 2-4).

Table 3. Toxicities: grade 3 or greater nonhematologic toxicities regardless of attribution during cycles 1-2

Infections/febrile neutropenia	11 patients
Pneumonia	4
Cellulitis	1
Bloodstream	2
Neutropenic fever	4
Organ toxicities*	23 events
Neurotoxicity	3
Gastrointestinal	3
Pulmonary	3
DVT/PE	2
Hyperglycemia	6
Confusion	1
Rash	1
Acute renal failure	1
Atrial fibrillation	1
Syncope	2
Death	4 patients
Infection	2
Pulmonary fibrosis	1
Disease	1

DVT indicates deep-vein thrombosis; and PE, pulmonary embolism.
*Toxicities that recurred in individual patients in both cycles 1 and 2 are listed as 2 events.

Pharmacodynamic validation studies

The expression levels of *miR-29b* and *FLT3* mRNA were measured in pretreatment and on day 26 of the first cycle in patients who consented to correlative studies and had suitable serial BM samples available (n = 5). At the day 26 posttreatment time point, none of these patients had achieved CR. We observed a trend toward a greater expression of *miR-29b* ($P = .19$) and noted statistically significant lower expression of *FLT3* mRNA ($P = .02$) on day 26 with respect to pretreatment expression levels (Figure 1). The patient with *FLT3*-ITD had 50% lower expression of *FLT3* after 1 cycle (BM blasts % was the same as pretreatment). The median fold-change increase for *miR-29b* expression at day 26 with respect to baseline levels was 2.9 ($P = .2$), whereas the median fold-change decrease for *FLT3* mRNA at day 26 with respect to baseline levels was 0.4 ($P < .01$).

Because we previously showed that *miR-29b* directly or indirectly targets *DNMT1*, *DNMT3A*, and *DNMT3B*,[14] we also assessed the mRNA expression of these genes (supplemental Figure 1A). Posttreatment day 26 down-regulation of all 3 *DNMT* isoforms

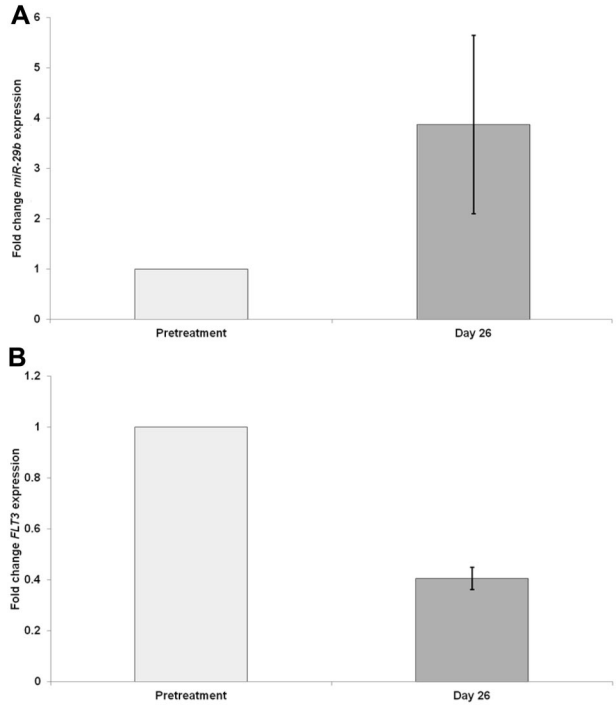

Figure 1. Posttreatment down-regulation of *FLT3* in AML patients. Pretreatment and day 26 expression levels of *miR-29b* (A) and *FLT3* mRNA (B) in patients with serial bone marrow from the decitabine-bortezomib clinical trial (n = 5).

compared with baseline was observed, ie, *DNMT1* (median fold-change: 0.4), *DNMT3A* (median fold-change: 0.2), and *DNMT3B* (median fold-change: 0.4). Furthermore, posttreatment day 26 expression of the *ESR* gene, often hypermethylated and silenced in AML, was increased (supplemental Figure 1B; median fold-change: 2.5). These changes did not achieve statistical significance.

Bortezomib-dependent mechanisms of FLT3 down-regulation

Because down-regulation of *FLT3* expression was observed in all patients at day 26 after treatment, we further investigated in vitro the mechanisms of this pharmacologic effect. We recently showed that a NF-κB(p65)/SP1 complex drives the expression of *KIT*, a member of the RTK family. Thus, we postulated that the SP1/NF-κB(p65) complex could also transactivate the *FLT3* gene. First, we identified 2 putative SP1 and NF-κB(p65) binding sites (sites 1 and 2) within the promoter region of *FLT3* promoter in MV4-11 cells that harbor a *FLT3*-ITD and express *FLT3* at high levels (Figure 2A). Then, using electromobility shift assays, we validated the binding of the SP1/NF-κB(p65) complex to the promoter binding sites for SP1 (only Site 1; Figure 2B) and NF-κB(p65) (both Sites 1 and 2; Figure 2B). The binding affinity of SP1 and NF-κB(p65) on these putative binding sites was confirmed by ChIP assays. Using primers spanning the region of the first SP1 and NF-κB(p65) binding sites, we showed enrichment of both SP1 and NF-κB(p65) on the *FLT3* promoter in MV4-11 cells (Figure 2C).

To assess transactivating activity of SP1/NFκB(p65) on *FLT3* promoter regulatory sequences, we cloned a 700-bp spanning region of the *FLT3* promoter containing the SP1/NF-κB(p65) binding sites into a luciferase-reporter vector. When the *FLT3* luciferase-reporter vector was cotransfected with SP1 or NF-κB(p65) overexpression vector in HEK293T cells, promoter activity was enhanced by SP1 or NF-κB(p65) overexpression compared with negative controls (Figure 2D). Conversely, SP1 or NF-κB(p65) knockdown using siRNAs resulted in the down-regulation

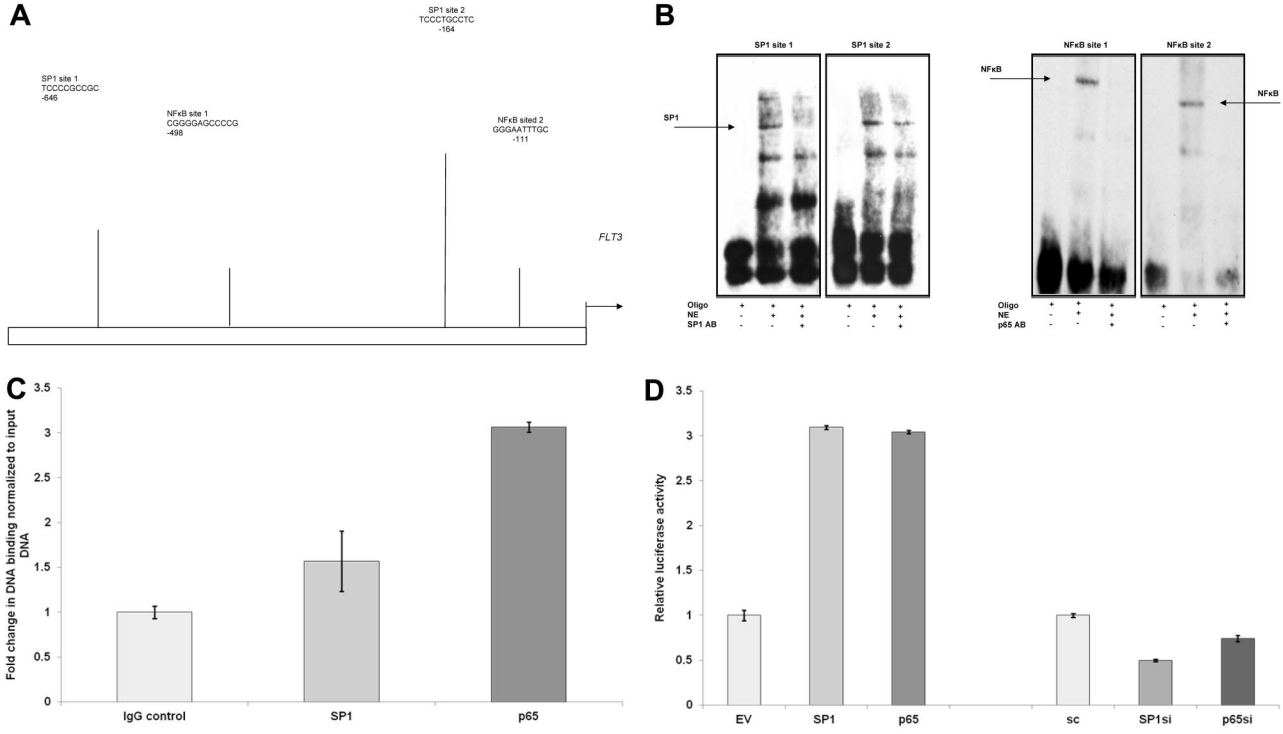

Figure 2. Regulation of *FLT3* expression via the SP1/NF-κB(p65) complex. (A) SP1 and NF-κB(p65) binding sites in the promoter region of the *FLT3* gene. (B) EMSA assays of the 2 identified binding sites for SP1 and NF-κB(p65), demonstrating specific binding of SP1 to the first site and binding of NF-κB(p65) to both sites using specific antibody to supershift the DNA-protein complexes. (C) Chromatin (ChIP) for the region containing binding sites of SP1 and of NF-κB(p65). (D) Luciferase promoter activity reporter assay including 700 bp of the promoter region of the *FLT3* gene containing all identified SP1 and NF-κB(p65) binding sites, demonstrating increased activity after cotransfection with constructs to overexpress SP1 or NF-κB(p65) and decreased activity after siRNA mediated knockdown of SP1 and NFκB(p65). EV indicates empty vector; and sc, scramble oligo.

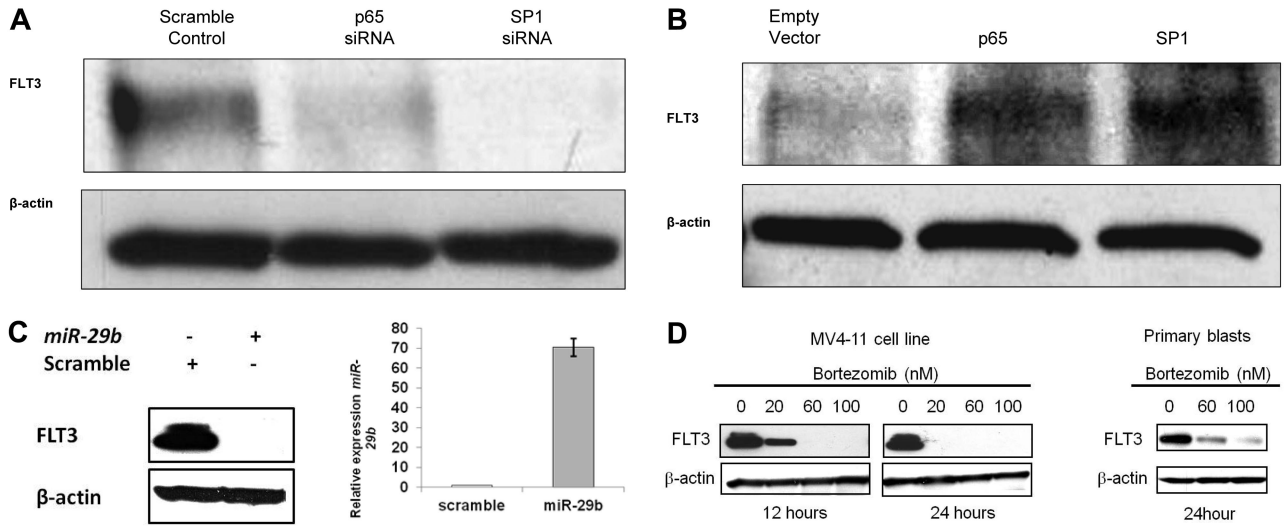

Figure 3. Bortezomib-induced FLT3 down-regulation via interaction with the SP1/NF-κB(p65) complex. (A) SiRNA mediated knock-down of SP1 or NFκ-B(p65) down-regulates FLT3 expression in MV4-11 cells that harbor a *FLT3*-ITD and express *FLT3* at high levels. (B) Overexpression of SP1 or NF-κB(p65) increases the expression of FLT3 in the KG1 cell line that usually has low expression of FLT3. (C) Increasing *miR-29b* in the MV4-11 cell line decreases FLT3 expression in MV4-11 cells. (D) Bortezomib treatment decreases FLT3 expression in a time- and dose-dependent manner in the MV4-11 cell line and in primary patient blasts (obtained from patients not enrolled on the current clinical trial; samples were procured in the OSU Leukemia Tissue Bank).

of the luciferase activity (Figure 2D). Gain and loss of function experiments further supported the regulatory role of SP1 and NF-κB(p65) on *FLT3* expression. SiRNA-mediated knock-down of SP1 or NF-κB(p65) led to decreased *FLT3* expression in *FLT3*-ITD–positive and *FLT3*-high expressing MV4-11 cells (Figure 3A). In contrast, overexpression of SP1 or NF-κB(p65) led to increased *FLT3* expression in *FLT3* wild-type and *FLT3*-low expressing wild-type KG1 cells (Figure 3B). BecauseSP1 is a bona fide target of *miR-29b*, we also reasoned that *miR-29b* is likely to participate in *FLT3* transcriptional regulation through modulating SP1 expression. Forced *miR-29b* expression indeed resulted in *FLT3* down-regulation in MV4-11 (Figure 3C).

Having validated our hypothesis that the NFκB/SP1 complex up-regulates *FLT3* expression and that *miR-29b* causes down-regulation of this RTK, we reasoned that a pharmacologic intervention that would increase *miR-29b* expression would also down-regulate *FLT3* by interfering with the SP1/NF-κB(p65) complex. We have already reported that bortezomib induced miR-29b and disrupts the SP1/NF-κB(p65) complex.[6] Consistent with these observations, we showed dose- and time-dependent *FLT3* down-regulation in bortezomib treated *FLT3*-ITD–positive MV4-11 cell lines and primary AML blasts (Figure 3D), confirming the observation from patients treated on the clinical trial.

Discussion

We report here the results of a phase 1 trial of bortezomib and decitabine in patients with poor-risk AML. In addition to the clinical results, we provide pharmacodynamic evidence that *FLT3* expression is a novel target for this combination and describe the mechanisms through which bortezomib contributes to *FLT3* down-regulation.

With regard to the clinical trial, the combination of bortezomib and decitabine was tolerable and active in this cohort of AML patients. We observed a 50% CR/CRi rate in previously untreated older AML patients, whereas for patients with refractory or relapsed disease, the CR/CRi rate was 22%. The maximal planned dose of bortezomib in combination with decitabine was reached, but 3 patients experienced serious neuropathy after multiple cycles of therapy. Although the incidence of neuropathy in this trial was similar to that observed with bortezomib in other malignancies, strategies to prolong the duration of exposure to bortezomib and decitabine without increasing the frequency of neurotoxicity must be considered with further development of this regimen. Emerging data suggest that, at least in multiagent regimens, modification of the traditional bortezomib schedule of administration on days 1, 4, 8, and 11 to a once-weekly approach substantially reduces neurotoxicity without a detrimental effect on clinical response end points or survival.

In several studies, authors have noted reduced toxicity with preserved efficacy for once-weekly bortezomib compared with the traditional schedule.[15,16] Most notably, in a phase 3 study in myeloma, clinical outcomes including 3-year progression-free and overall survival were similar between different bortezomib dosing groups (nonrandomized) but with a markedly lower incidence of neuropathy with for weekly dosing versus the traditional schedule.[17,18] Subcutaneous administration of bortezomib appears to be another alternative with reduced neurotoxicity.[18] This consideration is an important one for future phase 2/3 studies of bortezomib and decitabine in AML because hypomethylating agent therapy requires prolonged and repetitive exposure to maximize benefit.

The results of the pharmacodynamic analyses showing a trend for *miR-29b* up-regulation and significant *FLT3* down-regulation, albeit limited by small sample size, led us to further dissect the mechanisms through which bortezomib could target the activity of the *FLT3* gene. We have previously shown that bortezomib interferes with the transcription complex SP1/NF-κB(p65) by increasing *miR-29b* that targets *SP1*.[6] Here, we showed that the SP1/NF-κB(p65) complex transactivated *FLT3* and that the activity of the complex was coregulated by *miR-29b*. Down-regulation of *miR-29b* in AML resulted in greater activity of the SP1/NF-κB(p65) complex (because of elevated levels of SP1),[6] and this in turn caused *FLT3* up-regulation. Reversing this constituted the basis for bortezomib-induced *FLT3* down-regulation in vitro and in vivo. Because overexpression of wild-type or mutated *FLT3* is frequent in AML blasts and because FLT3 activation promotes leukemia cell growth and survival, the finding of pharmacologic

FLT3 transcriptional inhibition in AML patients may represent a novel therapeutic strategy.

Our understanding of the role for bortezomib as a modulator of the SP1/NF-κB(p65) complex[5] has developed over time. Given current understanding of bortezomib's ability to up-regulate *miR-29b*, in turn disrupting expression of genes dependent on this complex and potentially sensitizing patients to decitabine, future clinical studies of decitabine and bortezomib in AML should alter the sequence of administration and test bortezomib given before decitabine, rather than after. We have previously reported that greater expression of *miR-29b* associated with response to decitabine.[12] It is tempting to hypothesize that this occurs because of correspondingly low expression of *miR-29b* targets, including FLT3, that play a relevant role in supporting myeloid leukemia growth and treatment resistance. With the heterogeneity of patients and the small sample size, it is difficult to compare remission rates in untreated older AML patients from this trial to our previously reported study with decitabine alone.[12] However, given that the remission rates appear similar, to determine whether bortezomib increases the clinical efficacy of decitabine by increasing *miR-29b* and down-regulating *miR-29b* targets (ie, FLT3) will require randomization and a larger trial, likely via the use of an alternative route (subcutaneous) or schedule (weekly) of bortezomib administration to ameliorate neurotoxicity concerns. A recently activated Alliance phase 2 trial in previously untreated older AML patients randomized to decitabine versus decitabine plus bortezomib (subcutaneous) will investigate this question (Alliance 11002).

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Authorship

Contribution: W.B. was the principal investigator of the clinical study and takes primary responsibility for the paper; S.S. and G.M. designed the experimental studies; G.M. served as the mentor on this project and provided input into the initial study design, implementation, manuscript preparation, and editing; W.B., A.W., R.K., J.C.B., S.M.D., and G.M. recruited and/or treated the patients; S.S. and A.-K.E. performed the laboratory experiments; S.G. performed statistical analyses; C.G. and C.K. coordinated the research; W.B., S.S., R.G., and G.M. wrote the manuscript; the remaining authors provided materials and/or scientific support for the experiments performed; and all authors agreed on the final version.

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