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

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**ABSTRACT**

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* upregulation, resulting in loss of transcriptional activation for several genes relevant to myeloid leukemogenesis including DNA methyltransferases (DNMTs) and receptor tyrosine kinases (RTKs). Thus, a phase I trial of bortezomib and decitabine was developed. Nineteen poor-risk AML patients (median age 70 years; range, 32-84) enrolled. Induction with decitabine (20mg/m$^2$ IV 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), 5/10 had CR (complete remission, N=4) or incomplete CR (CRi, N=1); 7/19 overall had CR/CRi. Pharmacodynamic analysis showed *FLT3* downregulation on day 26 of cycle 1 (P=0.02). Additional mechanistic studies showed that *FLT3* downregulation was due to bortezomib-induced *miR-29b* upregulation; this led to *SP1* downregulation and destruction of the SP1/NFκB complex that transactivated *FLT3*. In conclusion, this study demonstrated feasibility and preliminary clinical activity of decitabine plus bortezomib in AML and identified *FLT3* as a novel pharmacodynamic endpoint for future trials. This study is registered at the NCI Clinical Trials Network as NCT00703300.

## INTRODUCTION

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

Bortezomib is a proteasome inhibitor approved for treatment of multiple myeloma and mantle cell lymphoma, but only transient hematologic improvements were noted in a single-agent phase I study of bortezomib in AML.[3] Despite lack of single agent activity, bortezomib has shown promise when used in combination regimens for AML.[17] 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.[4,5] We showed an important role for bortezomib in disrupting a network which operates based on interactions of *miR-29b*, the transcription factor SP1, and NFκB(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.*[4,5] 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).[4] Upregulated SP1 bound NFκB(p65) and transactivated *KIT*. Therefore, activated KIT ultimately induced its own transcription via *miR-29b*.[4] We demonstrated that bortezomib-induced disruption of the SP1/NFκB(p65) complex inhibited the growth of leukemic cells via upregulation of *miR-29b*.[4] The results supported the notion that *miR-29b*/SP1/NFκB(p65) complex-dependent *KIT* overexpression contributed to leukemia growth and could be targeted by bortezomib.[4] As most AML cells express FLT3 (another member of the RTK family) and due to the relevance of both wild-type and mutated *FLT3* expression for AML cell growth and survival, FLT3 is an

important target in AML;[6-10] we hypothesized that the mechanisms described above 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 AML patients 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 II study conducted at our institution, the complete remission (CR) rate was 47%, the overall response rate 64%, and the median overall survival duration approximately 1 year with a 10-day induction regimen of low dose decitabine in untreated older AML patients (≥60 years, not candidates/refused intensive therapy).[11] Although these results were promising, we viewed the regimen as a framework upon which future investigations might build and improve. Given that 1) higher *miR-29b* levels were associated with response to decitabine in that trial[11] 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 I 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.

## PATIENTS AND METHODS

### *Eligibility Criteria and Study Design*

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

Patients were given induction cycles of decitabine 20mg/m$^2$ intravenously (IV) over 1 hour on days 1-10 with cycles repeated every 28 days, until blood and marrow (BM) blasts were less than 5% at which time decitabine dosing was cut to 3-5 days/cycle as previously described.[11] Bortezomib was administered immediately following the decitabine dose by intravenous push (IVP). Bortezomib was dose escalated according to the following dose escalation plan: dose level 1, 0.7mg/m$^2$ IVP on days 5 and 8; dose level 2, 0.7mg/m$^2$ IVP on days 5, 8, 12, 15; dose level 3, 1.0 mg/m$^2$ IVP on days 5, 8, 12, 15; and dose level 4, 1.3 mg/m$^2$ IVP on days 5, 8, 12, 15. Treatment delays of ≥10 days were permitted for patients with 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/uL]. Hydroxyurea was permitted to control white blood count (WBC) to <40,000/uL, if necessary, before and during cycle 1, but no other anti-leukemic 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 objective response after 3 cycles of treatment. Reponses were defined according to the International Working Group (IWG) criteria for AML, including CR and CR with incomplete count recovery (CRi).[12]

***Definition of Dosing Limiting Toxicity***

Adverse events were graded according to the National Cancer Institute (NCI) Common Toxicity Criteria for Adverse Events (CTCAE), version 3.0. Dose limiting toxicity (DLT) was defined with cycle 1 of therapy. Drug related non-hematologic toxicity of grade 4 was considered DLT with the exception of alopecia, nausea and vomiting controllable with anti-emetic 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 two 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 (MTD). Hematologic DLT was defined as: 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 MTD.

***Cytogenetics, molecular markers, and correlative studies***

Standard cytogenetic analyses were performed on BM samples. For patients who consented for and had additional material available for molecular studies, the presence or absence of *FLT3*-

ITD and *FLT3*-TKD was determined as previously described.[6,10] 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) using RT-PCR as previously described.[5,11] Briefly, total RNA was extracted using Trizol (Invitrogen) reagent, and complementary DNA was synthesized from total RNA. Gene expression of *FLT3, DNMT1, DNMT3A, DNMT3B,* and *ESR* were normalized to *ABL1*. For *miR-29b* expression, qRT-PCR was carried out by TaqMan MicroRNA Assays (Applied Biosystems) according to the manufacturer's protocol and normalized by *U44* as previously described.[11] Expression of the target genes were measured using 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 Inc) 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 The Ohio State University (OSU) Leukemia Tissue Bank. Primary patient blasts were cultured in StemSpan SFEM media (Stem Cell Technologies), supplemented with StemSpan CC100 (Stem Cell 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 NFκB(p65) expression vectors were done as previously described.[4] 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 Inc) according to the manufacturer's instructions and as previously reported.[4] Transient transfections of HEK293T cells for the luciferase experiments were performed using Lipofectamine reagent (Invitrogene) according to the manufacturer's description.

**Chromatin immunoprecipitation (ChIP)**

ChIP assays were performed using the EZ ChIP Assay Kit (Millipore) per manufacturer recommendations and as previously discribed.[4] DNA was quantified using qRT-PCR with SYBR green incorporation (Applied Biosystems). The antibodies used were: SP1 (Cell Signaling) and NFkBp65 (Millipore). Quantitative PCR was utilized to measure the fraction of *FLT3* promoter DNA enrichment in the immunoprecipitated with SP1 and NFkBp65 antibody.

**Luciferase Assays**

Luciferase assays were conducted utilizing 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 utilizing the HindIII restriction enzyme site using primers as indicated in the supplemental material. Firefly luciferase and *Renilla* luciferase activities were assessed according to the recommendations detailed in the Dual-Luciferase® Reporter Assay System (Promega).

**Electrophoretic Mobility Shift Assay (EMSA)**

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 NFκB (NFκB-site 1 and NFκB-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 min. The heat was then gradually reduced over hours until the oligonucleotides reached room temperature 24 h 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 NFkBp65 antibody (Cell Signaling Technology) were added.

**Western Blotting**

The western blots were preformed as previously described.[4] The antibodies used were: SP1 and Actin (Santa Cruz Biotechnology), NFkBp65 (Millipore), and FLT3 (Cell Signaling Technology).

***Statistical Analysis***

Data were compared using the Student 2-tailed t test. A $P \leq 0.05$ was considered statistically significant. All analyses were performed by using the R 2.14.1 software package (available at www.r-project.org).

## RESULTS

### *Patient characteristics*

Enrollment of 19 poor risk AML patients occurred over 11 months. Clinical and cytogenetic characteristics of the patients enrolled on the clinical trial are summarized in Table 1. The median age was 70 years (range, 32-84). Median WBC count was 3.9 x $10^3$/uL (range, 1.3-69.9). Previously untreated patients (N=10) were of median age 70 years (range, 65-84) and presented with intermediate or adverse cytogenetic risk according to CALGB criteria;[2] seven had *de novo* AML, and three had secondary/therapy-related AML. Relapsed/refractory patients (N=9) had median age of 67 years (range, 32-75). Eight had failed prior anthracycline/high-dose cytarabine therapy. All four patients who enrolled with AML in untreated first relapse had CR1 duration of <1 year. Overall, eight patients consented for diagnostic assessment of *FLT3* mutational status; none harbored a *FLT3*-TKD, one patient had a *FLT3*-ITD. All eight 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, as one patient was removed from study due to disease-related thrombosis. This event was not judged to be related to the drug, as the patient had presented with AML originally with extensive deep venous thrombosis thought to be malignancy-related and had an inferior vena cava filter placed 11 months earlier. Dose level 3 was expanded to six patients due to safety concerns following an infection-related death during induction in one patient. Six additional patients were treated at the highest planned dose of bortezomib (1.3mg/$m^2$ on days 5, 8, 12, 15), one respiratory death before cycle 2 occurred at this dose. Patients received a median of 2 cycles of treatment (range, 1-14). Only two 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 bortezomib discontinuation 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 (two with refractory/relapsed disease, two with previously untreated disease) expired within 8 weeks of treatment due to infection (n=2), disease progression (n=1), and respiratory failure from pulmonary fibrosis (n=1). Only one 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 higher toxicities regardless of attribution during the first two cycles are listed in Table 2. Through the entire duration of the study, grade 3 or higher neuropathy occurred in 3 patients (none during cycle 1). For one of these patients, the neuropathy was autonomic and disabling. No hematologic DLT was observed.

***Clinical Responses***

In previously untreated patients (all aged 65 and above), CR/CRi occurred in four patients; another CR not confirmed by marrow evaluation occurred in a patient who refused BM evaluation after treatment (described more below), for an overall remission rate of 50% (5/10) in this subset. For the four 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); CRi, 3 months (incomplete response was

ANC<1000/uL). The fifth responder was an 84 year old patient who refused BM re-evaluation 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 IWG 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 due to ANC<1000/uL). 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. Based on the pattern of the response, the patient likely would have met ANC recovery for CR had transplant not immediately occurred. Including the one 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).

***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 post-treatment timepoint, none of these patients had achieved CR. We observed a trend towards a higher expression of *miR-29b* (P=0.19) and noted statistically significant lower expression of *FLT3* mRNA (P=0.02) on day 26 with respect to pretreatment expression levels (Figure 1). The patient with *FLT3*-ITD had 50% lower expression of *FLT3* after one 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=0.2), while the median fold change decrease for *FLT3* mRNA at day 26 with respect to baseline levels was 0.4 (P<0.01).

Since we previously showed that *miR-29b* directly or indirectly targets *DNMT1*, *DNMT3A* and *DNMT3B,*[18] we also assessed the mRNA expression of these genes (Figure S1A). Post-treatment day 26 downregulation of all three *DNMT* isoforms compared to baseline was observed i.e. *DNMT1* (median fold-change: 0.4), *DNMT3A* (median fold-change: 0.2), and *DNMT3B* (median fold-change: 0.4). Furthermore, post-treatment day 26 expression of the *ESR* gene, often hypermethylated and silenced in AML, was increased (Figure S1B; median fold-change: 2.5). These changes did not achieve statistical significance.

***Bortezomib-dependent mechanisms of FLT3* downregulation**

Since downregulation of *FLT3* expression was observed in all patients at day 26 post-treatment, we further investigated *in vitro* the mechanisms of this pharmacologic effect. We recently showed that a NFκB(p65)/SP1 complex drives the expression of *KIT*, a member of the RTK family. Thus, we postulated that the SP1/NFκB(p65) complex could also transactivate the *FLT3* gene. First, we identified two putative SP1 and NFκB(p65) binding sites (Site 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, utilizing electromobility shift assays, we validated the binding of the SP1/NFκB(p65) complex to the promoter binding sites for SP1 (only Site 1; Figure 2B) and NFκB(p65) (both Site 1 and 2; Figure 2B). The binding affinity of SP1 and NFκB(p65) on these putative binding sites was confirmed by ChIP assays. Utilizing primers spanning the region of the first SP1 and NFκB(p65) binding sites, we showed enrichment of both SP1 and NFκB(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 700bp-spanning region of the *FLT3* promoter containing the SP1/NFκB(p65) binding sites into a luciferase-reporter vector. When the *FLT3* luciferase-reporter vector was co-

transfected with SP1 or NFκB(p65) overexpression vector in HEK293T cells, promoter activity was enhanced by SP1 or NFκB(p65) overexpression compared with negative controls (Figure 2D). Conversely, SP1 or NFκB(p65) knock-down using siRNAs resulted in the downregulation of the luciferase activity (Figure 2D). Gain and loss of function experiments further supported the regulatory role of SP1 and NFκB(p65) on *FLT3* expression. SiRNA-mediated knock-down of SP1 or NFκB(p65) led to decreased *FLT3* expression in *FLT3*-ITD-positive and *FLT3*-high expressing MV4-11 cells (Figure 3A). In contrast, over expression of SP1 or NFκB(p65) led to increased *FLT3* expression in *FLT3* wild-type and *FLT3*-low expressing wild-type KG1 cells (Figure 3B). Since SP1 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* downregulation in MV4-11 (Figure 3C).

Having validated our hypothesis that the NFκB/SP1 complex upregulates *FLT3* expression and that *miR-29b* causes downregulation of this RTK, we reasoned that a pharmacologic intervention that would increase *miR-29b* expression would also downregulate *FLT3* by interfering with the SP1/NFκB(p65) complex. We have already reported that bortezomib induced miR-29b and disrupts the SP1/NFκB(p65) complex.[5] Consistent with these observations, we showed dose- and time-dependent *FLT3* downregulation 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 I trial of bortezomib and decitabine in 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* downregulation.

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, while 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 three patients experienced serious neuropathy after multiple cycles of therapy. Though the incidence of neuropathy in this trial was similar to that seen 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, 11 to a once weekly approach substantially reduces neurotoxicity without a detrimental effect on clinical response endpoints or survival. Several studies have noted reduced toxicity with preserved efficacy for once weekly bortezomib compared to the traditional schedule.[13,14] Most notably, in a phase III study in myeloma, clinical outcomes including 3-year progression-free and overall survival were similar between different bortezomib dosing groups (non-randomized) but with a markedly lower incidence of neuropathy with for weekly dosing vs. the traditional schedule.[15,16] Subcutaneous administration of bortezomib appears to be another alternative with reduced neurotoxicity.[16] This is an important consideration for future phase II/III studies of bortezomib and decitabine in AML, since hypomethylating agent therapy requires prolonged and repetitive exposure to maximize benefit.

The results of the pharmacodynamic analyses showing a trend for *miR-29b* upregulation and significant *FLT3* downregulation, 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/NFκB(p65) by increasing *miR-29b* that targets *SP1.*[5] Here, we showed that the SP1/NFκB(p65) complex transactivated *FLT3* and that the activity of the complex was co-regulated by *miR-29b*. Downregulation of *miR-29b* in AML resulted in higher activity of the SP1/NFκB(p65) complex (due to elevated levels of SP1),[5] and this in turn caused *FLT3* upregulation. Reversing this constituted the basis for bortezomib-induced *FLT3* downregulation *in vitro* and *in vivo*. As overexpression of wild type or mutated *FLT3* is frequent in AML blasts and since 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/NFκB(p65) complex[4] has developed over time. Given current understanding of bortezomib's ability to upregulate *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 prior to decitabine, rather than after. We have previously reported that higher expression of *miR-29b* associated with response to decitabine.[11] It is tempting to hypothesize that this occurs due to 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.[11] However, given that the remission rates appear similar, to determine whether bortezomib increases the clinical efficacy

of decitabine by increasing *miR-29b* and downregulating *miR-29b* targets (i.e., FLT3) will require randomization and a larger trial, likely using an alternative route (subcutaneous) or schedule (weekly) of bortezomib administration to ameliorate neurotoxicity concerns. A recently activated Alliance phase II trial in previously untreated older AML patients randomized to decitabine vs. decitabine plus bortezomib (subcutaneous) will investigate this question (Alliance 11002).

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## Authorship Contributions

WB was the principal investigator of the clinical study and takes primary responsibility for the paper. SS and GM designed the experimental studies. GM served as the mentor on this project and provided input into the initial study design, implementation, manuscript preparation, and editing. WB, GM, RBK, AW, SMD, JCB recruited and/or treated the patients. KH, SL, LJS coordinated data and the trial operation. SS, TS, AKE and S.L. performed the laboratory experiments. SG performed statistical analyses. WB, SS, RG, and GM wrote the manuscript and all authors agreed on the final version. This investigator initiated trial was sponsored by the Cancer Therapy Evaluation Program of the NCI.

## Disclosure of Conflicts of Interest

The authors declare no conflicts of interest.

## REFERENCES


1. Breems DA, Van Putten WL, Huijgens PC, et al. Prognostic index for adult patients with acute myeloid leukemia in first relapse. *J Clin Oncol.* 2005; 23(9): 1969-78.
2. Farag SS, Archer KJ, Mrozek K, et al. Pretreatment cytogenetics add to other prognostic factors predicting complete remission and long-term outcome in patients 60 years of age or older with acute myeloid leukemia: results from Cancer and Leukemia Group B 8461. *Blood.* 2006; 108(1): 63-73.
3. Cortes J, Thomas D, Koller C, et al. Phase I study of bortezomib in refractory or relapsed acute leukemias. *Clin Cancer Res.* 2004; 10(10): 3371-6.
4. Liu S, Liu Z, Xie Z, et al. Bortezomib induces DNA hypomethylation and silenced gene transcription by interfering with Sp1/NF-kappaB-dependent DNA methyltransferase activity in acute myeloid leukemia. *Blood.* 2008; 111(4): 2364-73.
5. Liu S, Wu LC, Pang J, et al. Sp1/NFkappaB/HDAC/miR-29b regulatory network in KIT-driven myeloid leukemia. *Cancer Cell.* 2010; 17(4): 333-47.
6. Whitman SP, Maharry K, Radmacher MD, et al. FLT3 internal tandem duplication associates with adverse outcome and gene- and microRNA-expression signatures in patients 60 years of age or older with primary cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *Blood.* 2010; 116(18): 3622-26.
7. Ozeki K, Kiyoi H, Hirose Y, et al. Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. *Blood.* 2004; 103: 1901-8.
8. Masson K, Rönnstrand L. Oncogenic signaling from the hematopoietic growth factor receptors c-Kit and Flt3. *Cell Signal.* 2009; 12: 1717-26.
9. Sanz M, Burnett A, Lo-Coco F, Löwenberg B. FLT3 inhibition as a targeted therapy for acute myeloid leukemia. *Curr Opin Oncol.* 2009; 21: 594-600.
10. Whitman SP, Ruppert AS, Radmacher MD, et al. FLT3 D835/I836 mutations are associated with poor disease-free survival and a distinct gene-expression signature

among younger adults with de novo cytogenetically normal acute myeloid leukemia lacking FLT3 internal tandem duplications. *Blood.* 2008; 111: 1552-9.

11. Blum W, Garzon R, Klisovic RB, et al. Clinical response and miR-29b predictive significance in older AML patients treated with a 10-day schedule of decitabine. *Proc Natl Acad Sci U S A.* 2010; 107(16): 7473-8.
12. Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. *J Clin Oncol.* 2003; 21(24): 4642-9.
13. de Vos S, Goy A, Dakhil SR, et al. Multicenter Randomized Phase II Study of Weekly or Twice-Weekly Bortezomib Plus Rituximab in Patients With Relapsed or Refractory Follicular or Marginal-Zone B-Cell Lymphoma. *J Clin Oncol.* 2009; 27: 5023-30.
14. Gerecitano J, Portlock C, Moskowitz C, et al. Phase 2 study of weekly bortezomib in mantle cell and follicular lymphoma. *Br J Haematol.* 2009; 146(6): 652-5.
15. Hainsworth JD, Spigel DR, Barton J, et al. Weekly treatment with bortezomib for patients with recurrent or refractory multiple myeloma: a phase 2 trial of the Minnie Pearl Cancer Research Network. *Cancer.* 2008; 113(4): 765-71.
16. Moreau P, Pylypenko H, Grosicki S, et al. Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, phase 3, non-inferiority study. *Lancet Oncol.* 2011; 12: 431-40.
17. Attar EC, De Angelo DJ, Supko JG, et al. Phase I and pharmacokinetic study of bortezomib in combination with idarubicin and cytarabine in patients with acute myelogenous leukemia. *Clin Cancer Res*. 2008; 14(5): 1446-54.

18. Garzon R, Liu S, Fabbri M, et al. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood*. 2009; 113(25): 6411-8.

## Table 1: Patient characteristics and response

| **Previously untreated patients** | | | | | |
|---|---|---|---|---|---|
| **Age in years/Sex** | **Secondary or *de novo*** | **Diagnostic Karyotype** | **Presenting WBC x $10^3$/uL** | **% 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(11;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 |
| **Relapsed/Refractory patients** | | | | | |
| **Age in years/Sex** | **No. of Prior Inductions** | **Pretreatment Karyotype** | **Presenting WBC x $10^3$/uL** | **% 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 | |

**CR; complete remission**
**CRi; CR with incomplete count recovery**
**CRu; unconfirmed CR for the elderly patient who had peripheral blood count recovery but who refused BM evaluation post treatment, as noted in the text**

**Table 2: Toxicities**

Grade 3 or higher non-hematologic 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 |

*Toxicities that recurred in individual patients in both cycles 1 and 2 are listed as two events

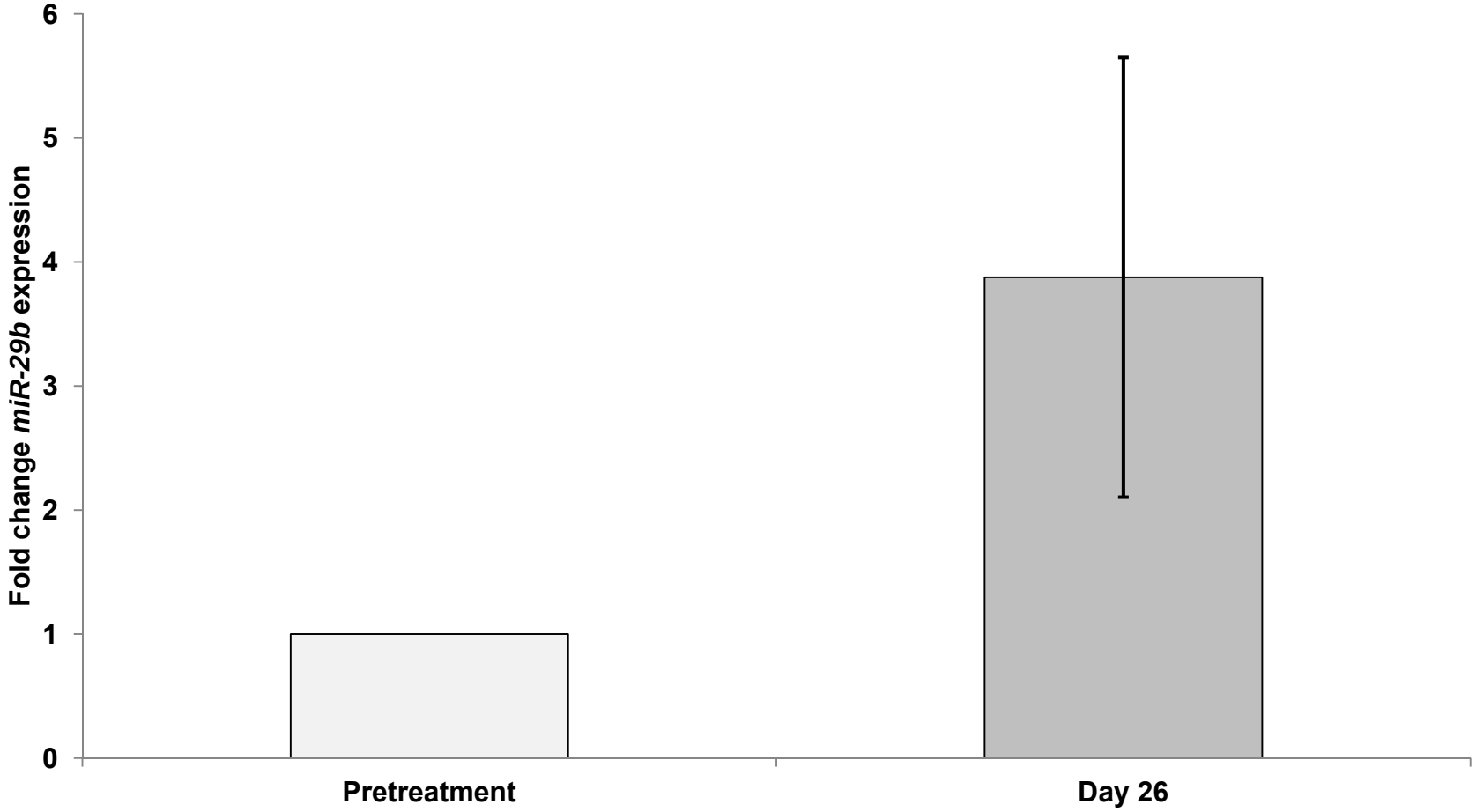
## FIGURE LEGENDS

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

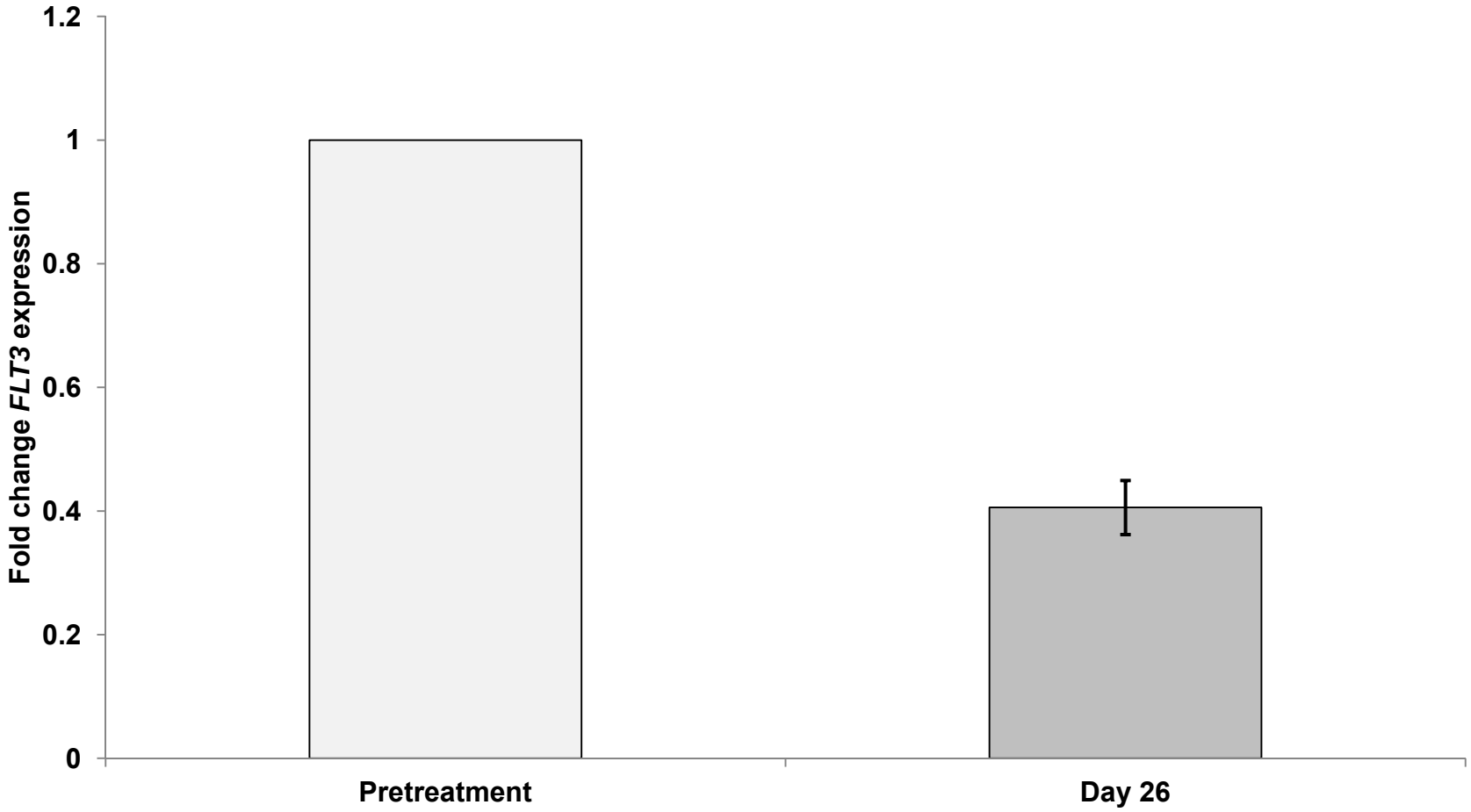
**Figure 2. Regulation of *FLT3* expression via the SP1/NFκB(p65) complex (A)** SP1 and NFκB(p65) binding sites in the promoter region of the *FLT3* gene. **(B)** Electromobility shift (EMSA) assays of the two identified binding sites for SP1 and NFκB(p65), demonstrating specific binding of SP1 to the first site and binding of NFκB(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 NFκB(p65). **(D)** Luciferase promoter activity reporter assay including 700bp of the promoter region of the *FLT3* gene containing all identified SP1 and NFκB(p65) binding sites, demonstrating increased activity after cotransfection with constructs to overexpress SP1 or NFκB(p65) and decreased activity after siRNA mediated knock-down of SP1 and NFκB(p65).

**Figure 3. Bortezomib-induced FLT3 downregulation via interaction with the SP1/NFκB(p65) complex (A)** SiRNA mediated knock-down of SP1 or NFκB(p65) downregulates FLT3 expression in MV4-11 cells that harbor a *FLT3*-ITD and express *FLT3* at high levels. **(B)** Overexpression of SP1 or NFκB(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).

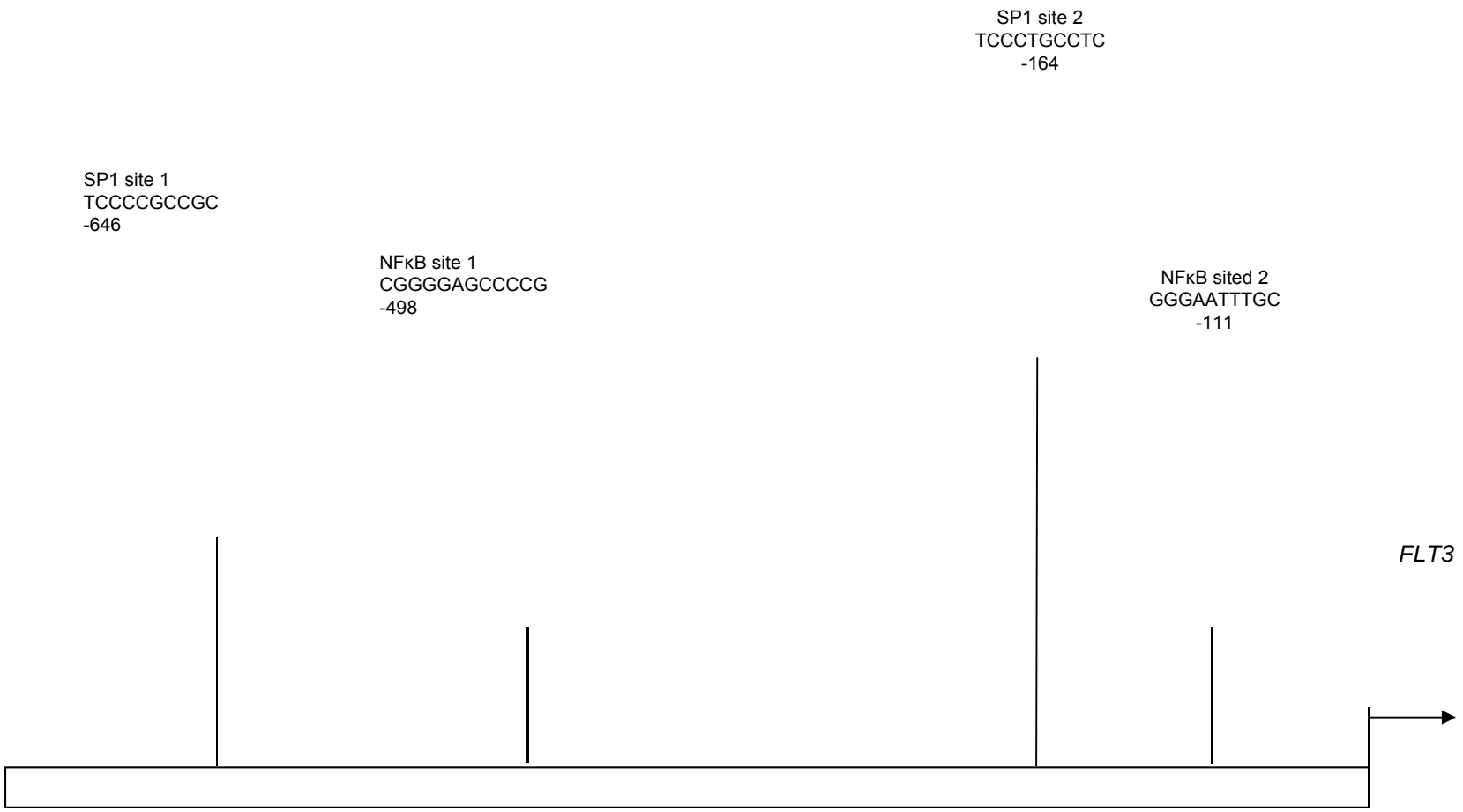
# Figure 1 A


Fold change miR-29b expression
6
5
4
3
2
1
0
Pretreatment
Day 26

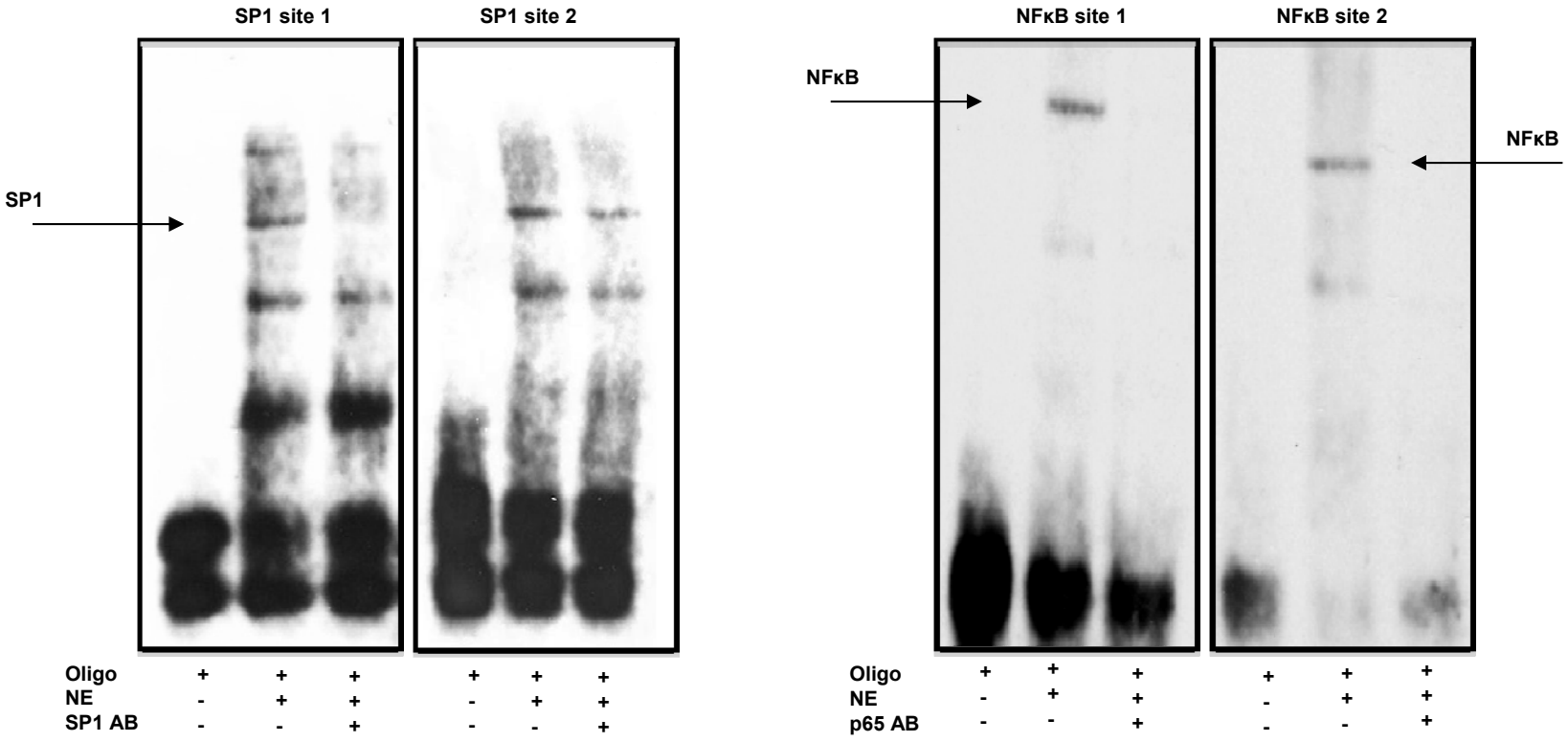
# Figure 1 B


1.2
1
0.8
0.6
0.4
0.2
0
Fold change FLT3 expression
Pretreatment
Day 26

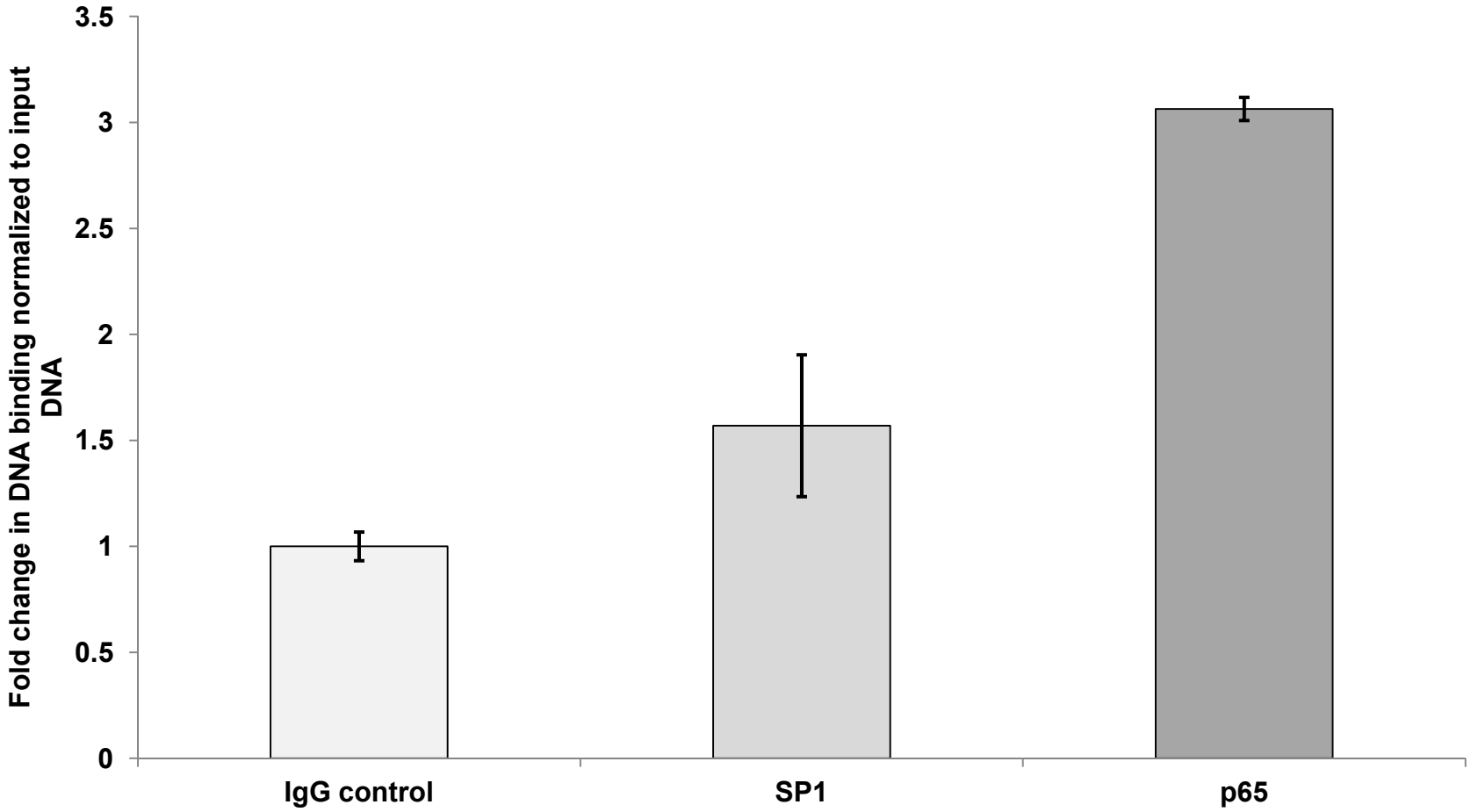
# Figure 2 A


SP1 site 2
TCCCTGCCTC
-164
SP1 site 1
TCCCCGCCGC
-646
NFκB site 1
CGGGGAGCCCCG
-498
NFκB sited 2
GGGAATTTGC
-111
FLT3

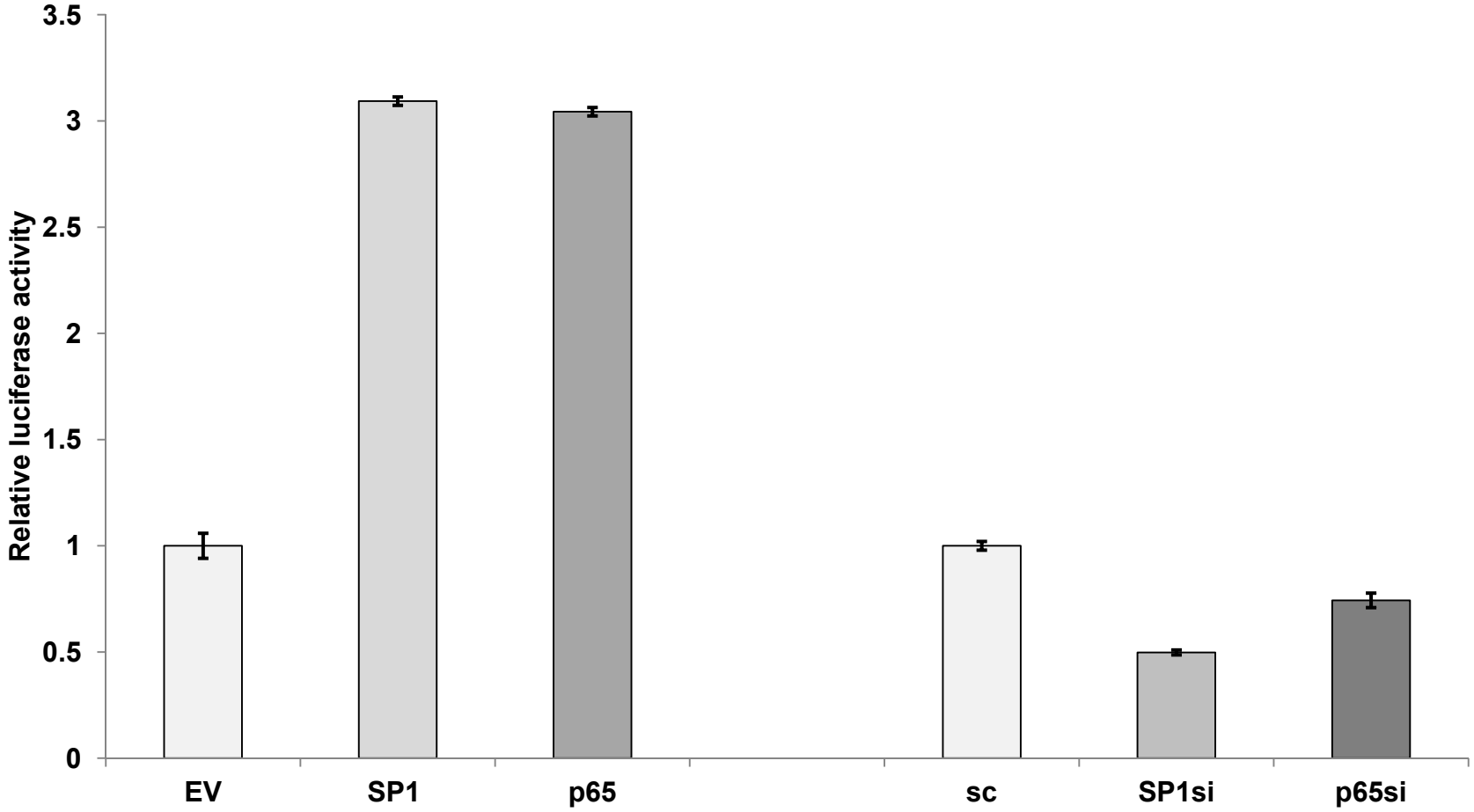
# Figure 2 B


SP1 site 1
SP1 site 2
SP1
Oligo + + + + + +
NE - + + - + +
SP1 AB - - + - - +
NFκB site 1
NFκB site 2
NFκB
NFκB
Oligo + + + + + +
NE - + + - + +
p65 AB - - + - - +

# Figure 2 C


Fold change in DNA binding normalized to input DNA
3.5
3
2.5
2
1.5
1
0.5
0
IgG control
SP1
p65

# Figure 2 D


Relative luciferase activity
3.5
3
2.5
2
1.5
1
0.5
0
EV
SP1
p65
sc
SP1si
p65si

# Figure 3 A

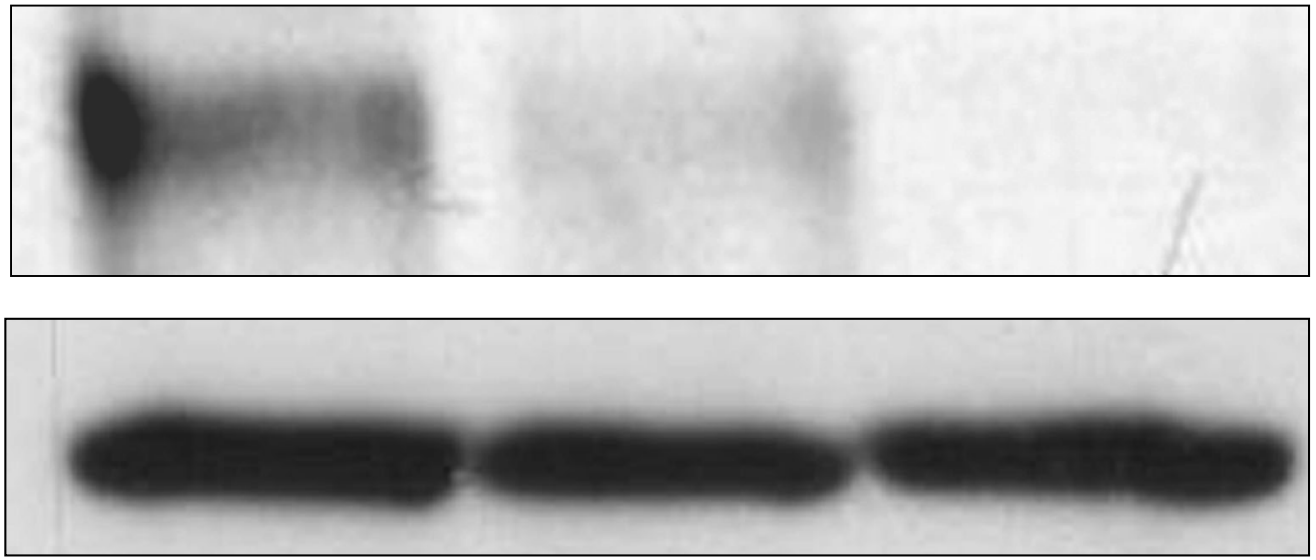
Scramble Control

p65 siRNA

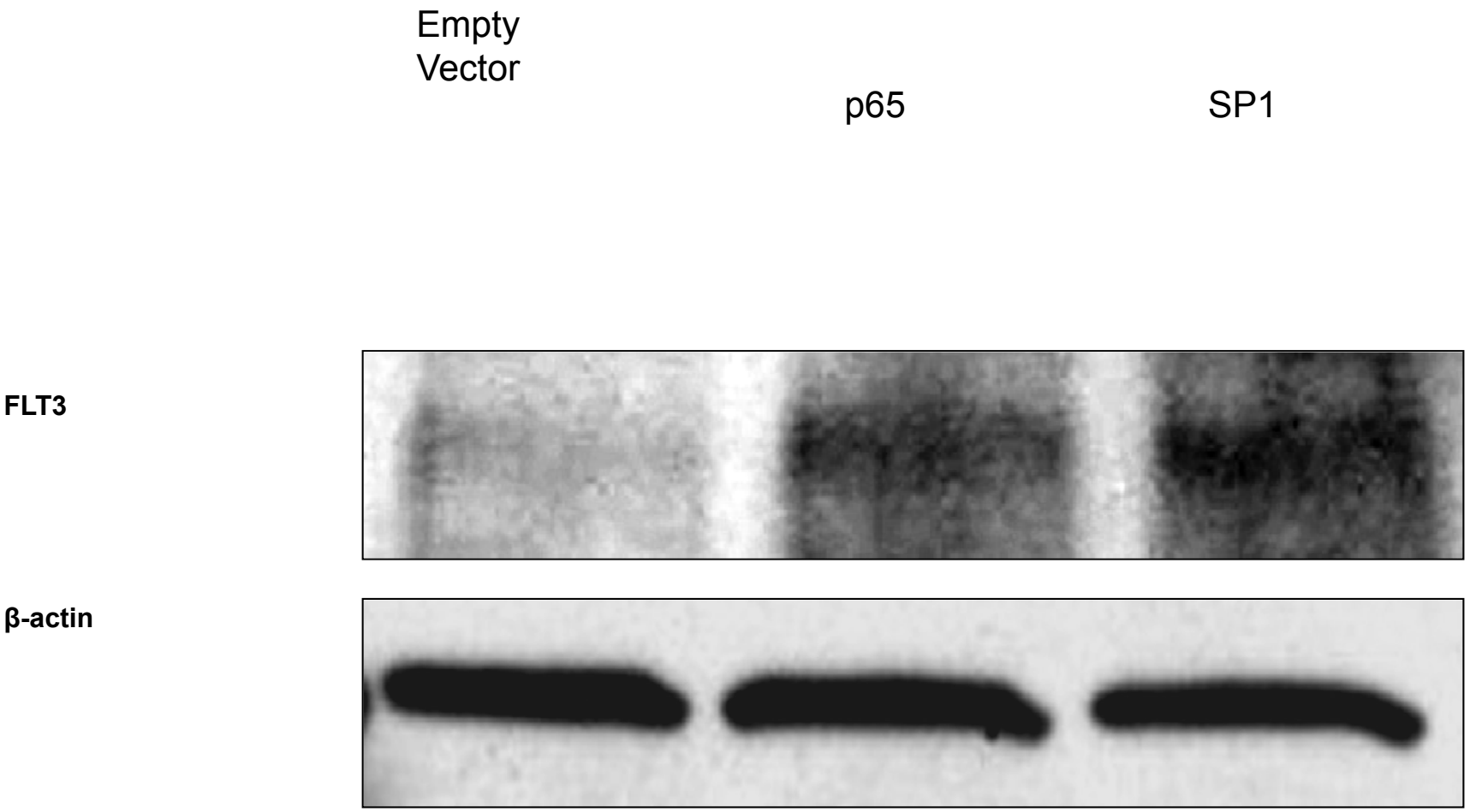
SP1 siRNA

**FLT3**

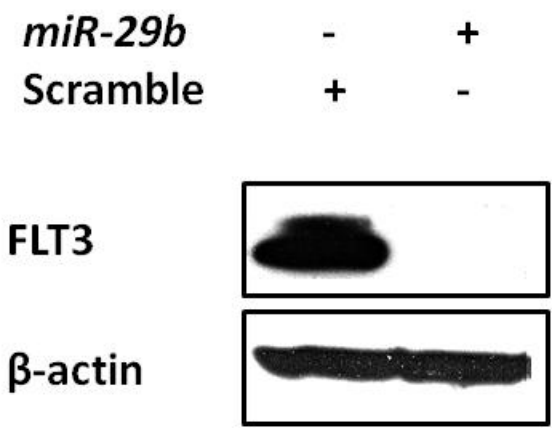
**β-actin**

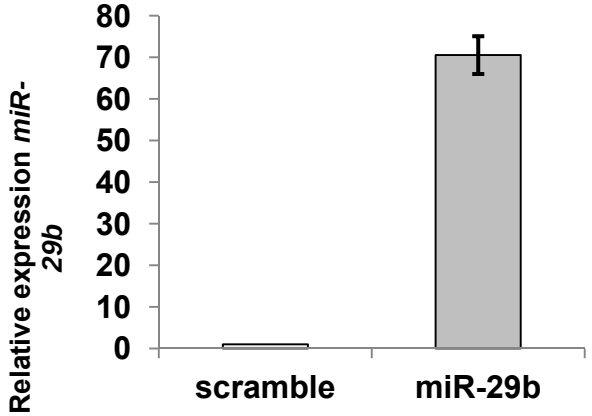


# Figure 3 B

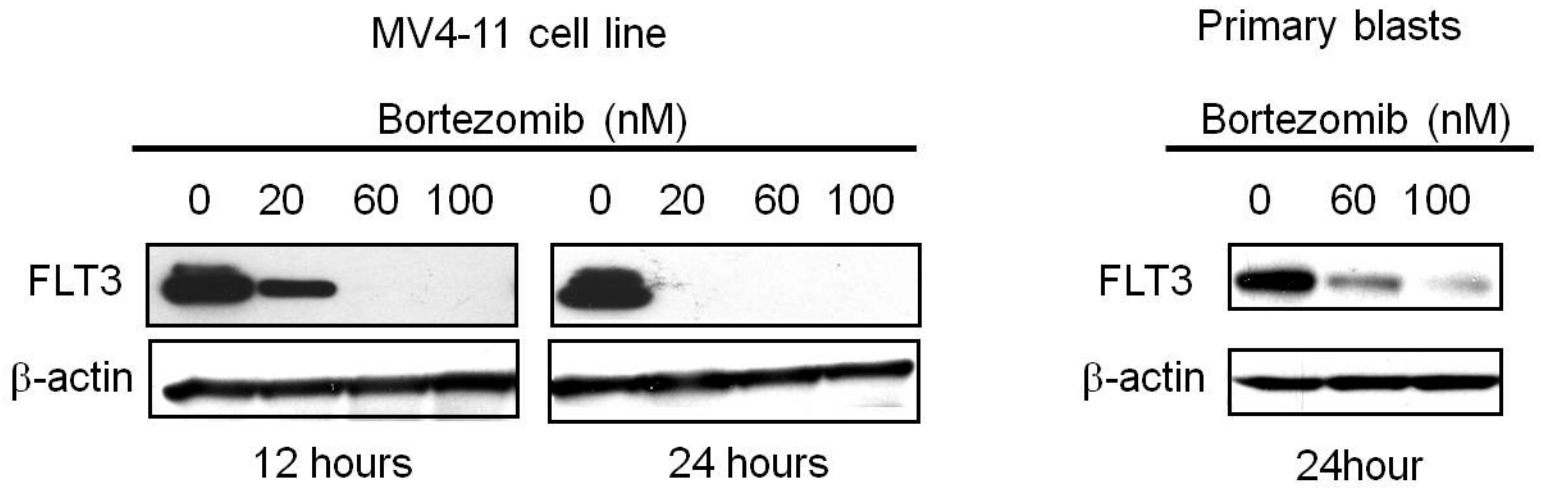


# Figure 3 C


miR-29b - +
Scramble + -
FLT3
β-actin


Relative expression miR-29b
80
70
60
50
40
30
20
10
0
scramble
miR-29b

# Figure 3 D


MV4-11 cell line
Bortezomib (nM)
0 20 60 100 0 20 60 100
FLT3
β-actin
12 hours
24 hours
Primary blasts
Bortezomib (nM)
0 60 100
FLT3
β-actin
24hour

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

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