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# Phase I/II study of single-agent bortezomib for the treatment of patients with myelofibrosis. Clinical and biological effects of proteasome inhibition

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**A phase I/II trial was undertaken to determine maximum tolerated dose (MTD), toxicity, clinical efficacy, and biological activity of bortezomib in patients with advanced stage primary or postpolycythemia vera/postessential thrombocythemia myelofibrosis (MF). Bortezomib (0.8, 1.0, or 1.3 mg/m<sup>2</sup>) was administered on days 1, 4, 8, and 11 by intravenous push to patients previously resistant to at least one line of therapy, or with an intermediate/high-risk score of International Working Group (IWG) [1]. Therapy was repeated every 28 days for six cycles. At 1.3 mg/m<sup>2</sup> dose, one of six patients experienced a dose limiting toxicity, and this was determined to be the MTD. Neither remissions nor clinical improvements were recorded in 16 patients treated at this dose level, fulfilling the early stopping rule in the Simon two-stage study design. Major toxicity was on thrombocytopenia. In 9 of 15 patients bortezomib proved that it is able to reduce bone marrow vessel density. However, the agent was associated with worsening of markers of disease activity, such as enhancement of hematopoietic CD34-positive progenitor cell mobilization, WT-1 gene expression in mononuclear cells, and downregulation of CXCR4 expression on CD34-positive cells. Occurrence of both beneficial and detrimental biological effects claims further investigation on the mechanisms of the drug in MF.**

The proteasome inhibitor bortezomib (Velcade<sup>®</sup>, Millennium Pharmaceuticals, and Johnson & Johnson Pharmaceutical Research and Development, LLC, Cambridge, MA) induces tumor cell death by inhibiting the degradation of several intracellular proteins involved in cell cycle regulation, and inhibits degradation of I $\kappa$ B blocking the multifunctional transcription factor nuclear factor- $\kappa$ B (NF $\kappa$ B) leading to reduced levels of transforming growth factor  $\beta$ -1 (TGF- $\beta$ 1). In addition, bortezomib indirectly inhibits angiogenesis and prevents tumor adaptation to hypoxia by functional inhibition of hypoxia inducible factor 1- $\alpha$  (HIF-1 $\alpha$ ). In MF, several lines of evidence are in favor of a crucial role of the TGF- $\beta$ 1, which is released by clonal proliferation of megakaryocytes or monocytes via activation of NF- $\kappa$ B [2,3]. Moreover, MF shows enhanced bone marrow and spleen angiogenesis that has been documented to be associated with worse prognosis [4,5]. Thus, NF- $\kappa$ B signaling pathway and angiogenesis are candidate targets for bortezomib in MF. Based on these assumptions, in 2007 we initiated a phase I/II trial with the aim to evaluate the safety and efficacy of bortezomib in patients with MF, to evaluate its effect on bone marrow angiogenesis and fibrosis, and on biomarkers of severity and progression of the disease.

Twelve patients were enrolled onto phase I of the study. The baseline characteristics of these patients are listed in Table I. Three patients treated at the 0.8 mg/m<sup>2</sup> dose level, and three treated at the 1 mg/m<sup>2</sup> dose level had no dose limiting toxicity (DLT). One of six patients treated at the 1.3 mg/m<sup>2</sup> dose level experienced acute severe pulmonary distress syndrome during the first cycle of treatment and this dose level was defined as MTD. Sixteen patients were enrolled onto the phase II portion of the study. One patient did not complete the first cycle of treatment, 13 patients (81%) completed four cycles of treatment, and nine (56%) patients completed the six cycles of treatment. The primary reason for early withdrawal from the study was unacceptable adverse events (AEs) (three patients), patient's refusal (two patients), and physician's decision (two patients).

At intention to treat analysis in which all patients who received at least one dose of the drug in the phase II study were evaluable (16 patients), no responses were recorded according the IWG response criteria [6]. At the per protocol analysis in which patients who received at least four cycles of treatment were evaluable, 13 of the 16 patients in the phase II study were evaluable. No patient had clinical improvement. As a matter of fact, no patient had Hb increasing >2 g/dL by the end of the study, and none of the transfusion-dependent patients (n = 4) had decrease in blood transfusion

need. One patient with absolute neutrophil count below  $1 \times 10^9/L$  at baseline did not increase neutrophil count by at least 100%. None of the patients had >50% spleen reduction. A patient with  $4,448 \times 10^9/L$  platelet count at baseline decreased the platelet count by 67% by the end of the study, but this response is not included in the criteria for clinical improvement. As depicted in Table II, the most frequent Grade 3 or 4 toxicity was thrombocytopenia.

At analysis of individual changes in cellularity, CD34+ cell content, and fibrosis in the 14 patients who completed the six cycles of treatment at any dose and had serial bone marrow specimens available for review, no statistically significant changes in none of the parameters resulted after therapy. At baseline, the patients had a significantly higher level of TGF- $\beta$ 1 than our control normal population (4738 pg/mL vs. 2404 pg/mL;  $P = 0.015$ ). No correlation was evidenced between baseline bone marrow fibrosis grade and plasma TGF- $\beta$ 1 level. Bortezomib treatment did not significantly decrease total TGF- $\beta$ 1 plasma levels (TGF- $\beta$ 1 final, 4959.5 pg/mL) from baseline (Wilcoxon test,  $P = NS$ ).

In the whole population of patients, bortezomib treatment did not significantly reduce the median vessels density, vessels area, and vessels perimeter. However, a decrease in vessels density was evidenced in 9 of the 15 (60%) patients studied (Table III). The percent decrease in vessels density ranged from 1.4% to 51%. Vessels area and vessels perimeter were reduced in 40% and 66% of cases, respectively. At baseline, the median value of plasma vascular endothelial growth factor (VEGF) in MF patients was 78.9 pg/mL (range, from 15.6 to 236.4 pg/mL), significantly higher than in normal controls (median, 30.16 pg/mL; range, from 15.6 to 130.6 pg/mL;  $P = 0.001$ ). Bortezomib treatment did not significantly decrease VEGF levels from baseline (Wilcoxon test,  $P = NS$ ).

At analysis of the 17 patients who completed at least four cycles of treatment at any dose, and had serial measurements available, the median baseline hematopoietic CD34+ cell number was  $114.1 \times 10^6/L$  (range, 15.5 to  $3026 \times 10^6/L$ ), whereas it was  $143.1 \times 10^6/L$  (range, 17.2 to  $3688.3 \times 10^6/L$ ) at the end of the study (Wilcoxon test,  $P = 0.05$ ). Increase in CD34+ cells in peripheral blood at the end of the study was detected in 11 of 17 (64.7%) patients, and the increase at the end of the therapy ranged from 4% to 1125% of basal value.

At analysis of the 14 patients who completed at least four cycles of treatment at any dose, and had serial measurements available, median WT1 expression at baseline was 6,870.68 copies/ $10^4$  ABL copies (range, 221.31 to 67,842.21 copies). After bortezomib, median WT1 expression did not significantly change (Wilcoxon test, NS). However, WT-1 expression increased in 8 of 14 patients (57.1%) with an increase ranging from 10% to 820% of basal value.

At analysis of the 18 patients who completed at least four cycles of treatment at any dose, CXCR4 expression on circulating CD34-positive cells was downmodulated at baseline in patients involved in this study (median, 22%; range, 6.2–92%), as compared with our historical normal controls (median, 76.7%; range 37–97%;  $P < 0.001$ ). By the end of the study, the value of CXCR4 expression was significantly lower than at baseline (median, 15.2%; range, 5.9–90%; Wilcoxon test,  $P = 0.05$ ). Reduction was documented in 10 of the 18 patients analyzed (55.7%). Granulocyte DNA-derived JAK2 617F allele burden was measured in 13 patients at baseline and after completion of at least four cycles of treatment. Twelve patients were JAK2V617F mutated with a median allele burden of 42.5% (range 4–100%). In none of the patients, the V617F burden variation was >10%. No significant changes in plasma SDF-1, IL-8, IL-6, and TNF were revealed at the end of the study.

In summary, with this phase I/II study, we found that none of the 22 patients either treated with the MTD of 1.3 mg/m<sup>2</sup>, or with lower doses in the phase I of the study, achieved a clinical response. Our results are in agreement with the lack of any clinical efficacy described by Mesa et al.

**TABLE I. Baseline Characteristics of the Study Populations Entering the Phase I and Phase II of the Study**

Characteristic	Phase I (N = 12)		Phase II (N = 16)	
	No of patients (%)	Median (range)	No of patients (%)	Median (range)
Age, years		57 (22–69)		58 (46–72)
Sex				
Female	5 (41.7)		6 (37.5)	
Male	7 (58.3)		10 (62.5)	
Type of Myelofibrosis				
Primary	11 (91.6)		10 (62.5)	
Post-PV	1 (8.4)		4 (25)	
Post-ET	0		2 (12.5)	
Prior treatment for myelofibrosis				
Hydroxyurea	8 (66.6)		13 (81.2)	
Splenectomy	1 (8.4)		2 (12.5)	
Danazol	1 (8.4)		2 (12.5)	
Thalidomide	1 (8.4)		2 (12.5)	
Duration of the disease (months)		44.5 (1–228)		35 (1–156)
Transfusion dependent patients	2 (16.6)		3 (18.7)	
Transfusion-independent patients with initial hemoglobin <10 g/dL	5 (41.7)		5 (31.2)	
White blood cell count ( $\times 10^9/L$ )		7.9 (3.7–71.3)		13.2 (1.7–71.3)
Myeloblasts in peripheral blood (%)		2 (0–7)		1 (0–7)
Immature myeloid cells (nonblasts) in peripheral blood (%)		1 (0–12)		3 (0–15)
Erythroblasts (% leukocytes) in peripheral blood		3 (0–45)		4 (0–45)
Platelet count ( $\times 10^9/L$ )		302 (106–1066)		285 (70–3405)
Spleen size below the costal margin, cm		15 (2–20)		15 (2–25)
Dupriez prognostic score				
Score 0	4 (33.3)		7 (43.8)	
Score 1	6 (50)		6 (37.5)	
Score 2	2 (16.7)		3 (18.7)	
Serum lactate dehydrogenase (mU/mL)		1486 (358–3024)		1408 (489–2658)
Chromosomal abnormalities <sup>a</sup>				
Not available	5 (41.7)		7 (43.8)	
No	5 (41.7)		4 (25)	
chromosomal abnormalities				
Chromosomal abnormalities <sup>b</sup>	2 (16.7) <sup>b</sup>		5 (31.2) <sup>c</sup>	

<sup>a</sup>In all patients, analysis of chromosomal abnormalities was performed on peripheral blood; <sup>b</sup>del5, del7; <sup>c</sup>del20, t(x;20), del6/del14, del5, del7.

who reported the results of a pilot phase II study with bortezomib in nine patients with MF and two with systemic mastocytosis or chronic myelomonocytic leukemia, showing lack of any clinical efficacy of the drug [7].

The results of this trial contrast with the 31–80% response rate in multiple myeloma [8], mantle-cell lymphoma [9], amyloidosis [10], cutaneous T-cell lymphoma [11], Waldstrom macroglobulinemia [12], or mucosa-associated lymphoid tissue (MALT) lymphoma [13] when bortezomib was used as single agent. In an attempt to clarify how bortezomib affects the pathogenetic mechanisms that sustain MF, in this trial we evaluated bone marrow and blood biomarkers variations as secondary endpoints of the study. In a great proportion of patients, the density of bone marrow microvessels was less

**TABLE II. Toxicity Summary during Treatment with Bortezomib**

Event	All adverse events	Grade 3 events
Thrombocytopenia	8	3
Fatigue	4	0
Rash	2	0
Pyrexia	3	0
Dyspnoea with pulmonary distress syndrome	1	1
Dyspnoea with pulmonary hypertension	1	1
Cutaneous vasculitis	1	1
Peripheral neuropathy	1	0
Cutaneous infectious ulcer	1	1

**TABLE III. Bone Marrow Vessels Density during Bortezomib Trial in 15 Patients Who Had Serial Bone Marrow Specimens Available for Review**

Case	Bortezomib dose mg/m <sup>2</sup>	Number of vessels ( $\times 10^{-3} \mu^2$ )		Change from baseline (%)
		Baseline	Final	
1	0.8	1.41	2.54	80.1
2	0.8	2.33	2.78	19.3
3	0.8	2.66	2.17	−18.4
4	1	3.32	3.02	−9.0
5	1	2.17	2.14	−1.4
6	1.3	1.30	2.49	91.5
7	1.3	2.66	1.40	−47.4
8	1.3	3.65	1.79	−50.9
9	1.3	1.92	2.43	26.5
10	1.3	4.09	3.20	−21.8
11	1.3	2.76	2.77	0.4
12	1.3	4.56	3.15	−30.9
13	1.3	5.93	3.41	−42.5
14	1.3	2.06	2.30	11.6
15	1.3	3.96	2.54	−36.1
Median		2.66	2.53	−9.03

after treatment than at baseline, reaching up to 51% reduction. The role of proteasome inhibition in angiogenesis has been documented in several pre-clinical studies [14–17] and one in vivo study in humans [18]. We were not able to document that the effect on angiogenesis could be associated with a decrease of plasma VEGF. This was in accordance with the results in multiple myeloma [19].

In contrast with the potentially beneficial effect on angiogenesis, we documented that the therapy had the potential to exert detrimental effects on biomarkers that mirror disease activity and progression. CXCR4 downregulation seems to represent the most relevant biological consequence of bortezomib therapy in patients with MF. Downregulation of cell surface proteins is a general mechanism of bortezomib [20–22]. However, the decrease of CXCR4 on CD34+ cells of patients with MF seems to be an unique example of chemokine receptor downregulation, because bortezomib has no effect on CXCR4 expression in multiple myeloma cells [23]. Furthermore, because the downregulation of the above-mentioned receptors on the cell surface is potentially beneficial, such as overcoming cell adhesion-mediated drug resistance for VLA-4 downregulation [21], in MF CXCR4 downregulation exacerbates a detrimental disease characteristic that specifically is responsible for hematopoietic cell mobilization and myelopoiesis derangement. We hypothesize that the strong influence of bortezomib on the bone marrow microenvironment may interact with the migration and adhesion mechanisms of hematopoietic stem cells operating in MF, and disrupt a homeostatic equilibrium that is unique and specific for the disease. A better understanding of these mechanisms is necessary for planning a better targeted use of bortezomib in MF.

## Methods

### Study design

For the Phase I portion of the study, DLT was defined as any Grade 3 or 4 treatment-related nonhematologic toxicity (National Cancer Institute Common Terminology Criteria of Adverse Events, version 3.0); any Grade 4 treatment-related hematologic toxicity; or any Grade 3 treatment-related hematologic toxicity requiring treatment delay of more than 2 weeks. Three patients were to be enrolled at each dose level starting at dose level 1. If no DLT was observed in cycle one, three patients were enrolled at the next

dose level. If one DLT was observed, the dose level was expanded to six patients. If two DLTs were observed, the MTD was exceeded and the previous dose level was expanded to six patients. The recommended phase II dose was the highest dose level at which one or less of six patients experienced a DLT. Three dose levels were planned (0.8, 1, and 1.3 mg/m<sup>2</sup>). No intra patient dose escalation was allowed.

For the Phase II efficacy analysis, we used an optimum Simon 2 stage design to test the null hypothesis that the complete or major response rate was  $\leq 0.05$  versus the alternative that this response rate was  $\geq 0.20$  at an alpha level of 0.05 with 80% power. At the evaluation of response at 18 weeks, if there were no or one responses (complete or major) of first 16 patients, the trial would be terminated for lack of efficacy. If the trial continued to a second stage, a total of 30 patients would be studied.

Bortezomib was administered intravenously on days 1, 4, 8, and 11 of a 21-day cycle. A total of six cycles were planned while on study. Dose reduction was allowed for Grade 3 or 4 thrombocytopenia or any Grade 3 or 4 nonhematologic toxicity.

All patients provided written informed consent. The study protocol was approved by the ethics committee of the IRCCS Policlinico S. Matteo Foundation, Pavia, and of the Florence University Hospital, Florence. The study was conducted in accordance with the policies of the MPD Research Consortium.

### Bone marrow histology and microvascular proliferation

Bone marrow samples were obtained before treatment and at the patient's final evaluation. Cellularity and fibrosis were assessed using the EUMNET score [24]. The rate of CD34+ progenitor cells and degree of microvascular proliferation were evaluated on sections stained with antiCD34 (mouse monoclonal Thermo Scientific, Fremont, CA). For microvascular proliferation, sections were evaluated on five randomly selected fields and images digitally acquired using an Olympus BX-60 microscope equipped with the DP-70 camera (Olympus Optical Corporation, Japan). From the total area, the area occupied by bone or eventual art factual spaces was subtracted, and the absolute number, the perimeter, and the area of CD34 positive vascular structures, including small vessels but not arterioles or sinusoids, were measured using CELL<sup>^</sup>F 2.5 software (Olympus Soft Imaging Solution, Olympus). All the data were parameterized to 10,000  $\mu^2$ .

### Biomarkers

Blood samples for the measure of biomarkers were obtained on day 0 of treatment cycle one and at the patient's final evaluation. The percentage of circulating CD34-positive hematopoietic progenitor cells was calculated according to the guidelines from the International Society of Hematotherapy and Graft Engineering [25]. For plasma TGF- $\beta$ 1 measurement, human TGF- $\beta$ 1 immunoassay was used (Quantikine kit, R&D Systems). Plasma levels of SDF-1, VEGF, IL-8, IL-6, and TNF were determined with the appropriate human Quantikine kits from R&D Systems according to the instructions of the manufacturer. Samples were assessed in duplicate. Seventeen normal individuals were used as controls for the cytokine level assays. They were 10 men and 7 women, with a median age of 49 years (range 32–65 years). Levels of WT1 mRNA were measured on mononuclear cells according to the previously reported method [26]. For CXCR4 expression measurement, cells were stained with specific monoclonal antibodies and analyzed using flow cytometry (Becton Dickinson, Oxford, UK) as described earlier [27]. Analysis of JAK2V617F mutational status and mutated allele burden was performed as described [28].

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