Proteasome inhibitor bortezomib impairs both myelofibrosis and osteosclerosis induced by high thrombopoietin levels in mice

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Primary myelofibrosis (PMF) is the most serious myeloproliferative disorder, characterized by clonal myeloproliferation associated with cytokine-mediated bone marrow stromal reaction including fibrosis and osteosclerosis. Current drug therapy remains mainly palliative. Because the NF-κB pathway is implicated in the abnormal release of cytokines in PMF, the proteasome inhibitor bortezomib might be a potential therapy. To test its effect, we used the lethal murine model of myelofibrosis induced by thrombopoietin (TPO) overexpression. In this TPO-high model, the development of the disease is related to a deregulated MPL signaling, as recently described in PMF patients. We first demonstrated that bortezomib was able to inhibit TPO-induced NF-κB activation in vitro in murine megakaryocytes. It also inhibited NF-κB activation in vivo in TPO-high mice leading to decreased IL-1α plasma levels. After 4 weeks of treatment, bortezomib decreased TGF-β1 levels in narrow fluids and impaired narrow and spleen fibrosis development. After 12 weeks of treatment, bortezomib also impaired osteosclerosis development through osteoprotegerin inhibition. Moreover, this drug reduced myeloproliferation induced by high TPO level. Finally, bortezomib dramatically improved TPO-high mouse survival (89% vs 8% at week 52). We conclude that bortezomib appears as a promising therapy for future treatment of PMF patients.

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Introduction

Primary myelofibrosis (PMF) is a myeloproliferative disorder known as a clonal stem-cell disorder, whereas the associated stromal reaction in the bone marrow environment, leading to fibrosis (excessive deposits of extracellular matrix proteins) and osteosclerosis (new bone formation), is considered to be reactive and cytokine mediated. Because the description of mice overexpressing thrombopoietin (TPO), known as TPO-high mice, featuring numerous aspects of the human disease including dysmegakaryopoiesis, the implication of the TPO/MPL pathway in PMF has been demonstrated. Recently, two activating mutations of the TPO receptor MPL, MPLV617F and MPLV615K, have been detected in 5% of PMF patients and have been shown to induce fibrosis in mice. Activated MPL is known to stimulate the members of the Janus family of protein tyrosine kinases, JAKs. Indeed, the activating JAK2V617F mutation, directly linked to deregulated MPL signaling, is present in 50% of PMF patients and also induces fibrosis in mice. However, how these unique JAK2 or MPL mutations may lead to bone marrow fibrosis development is not yet understood. Notably, both mutations can be found in essential thrombocythemia, a myeloproliferative disorder without fibrosis. Thus, PMF is related to TPO/MPL pathway alterations and the TPO-high model, mimicking deregulated MPL signaling, appears to be the most relevant to test drugs in vivo.

Several lines of evidence obtained from both studies of patients with PMF and of murine models ending with myelofibrosis are in favor of a crucial role (i) of the pleiotropic cytokine transforming growth factor β1 (TGF-β1), released by clonal proliferation of megakaryocytes or monocytes or both, in reticulin fiber deposition; (ii) and of stroma-derived osteoprotegerin (OPG) in osteosclerosis development. The NF-κB pathway, shown to contribute to hematopoietic differentiation, may be involved in the abnormal release of these cytokines. Rameshwar et al first reported a spontaneous activation of NF-κB in monocytes from PMF patients leading to IL-1 production, which induces TGF-β1 production through autocrine means. In addition, the NF-κB pathway is also activated in megakaryocytes and in circulating CD34+ cells from PMF patients. We have recently described that TPO-high mice exhibit high plasma levels of IL-1α, suggesting a role of the NF-κB pathway in this model of myelofibrosis development.

We therefore hypothesized that the NF-κB signaling pathway might be a candidate target for therapy, to limit reticulin fiber deposition. We assessed the effects of NF-κB inhibition on myelofibrosis development induced by high levels of TPO and chose to affect this pathway with the proteasome inhibitor bortezomib. Among proteasome inhibitors, bortezomib is currently the most effective drug used in patients with multiple myeloma. TPO-high mice were treated twice a week with either bortezomib or vehicle. Bortezomib was able to inhibit NF-κB activation in vitro and in vivo in TPO-high mice and to decrease IL-1α plasma levels. We demonstrated that bortezomib impaired fibrosis development through...
marrow cells were collected and cocultured with 10^6 MPZenTPO virus–(1 injection of 150 mg/kg administered intraperitoneally), total bone colony-forming cells (CFCs) as previously described.4,19 CFCs were picked in clonogenic progenitor assays to determine the percentage of infected 4 days, nonadherent cells were harvested. An aliquot was immediately used (100 U/mL), murine IL-6 (20 ng/mL), and murine SCF (20 ng/mL). After E-86 cells in Dulbecco modified Eagle medium (DMEM) maintained at the IGR animal facility under specific pathogen-free conditions. ethidesge committee. C57BL/6J mice (Janvier, Le Genest, France) were Mice and bone marrow transduction (San Diego, CA) and used for lineage-positive depletion: anti–Ly-6/GR-1 methyl rhodamine isothiocyanate (TRITC)–labeled secondary Abs were anti–rabbit horseradish peroxidase–labeled and donkey anti–rabbit tetra methyl rhodamine isothiocyanate (TRITC)–labeled secondary Abs were purchased from Jackson Immunoresearch (West Grove, PA). The following rat monoclonal Abs were purchased from Pharmingen (San Diego, CA) and used for lineage-positive depletions: anti–Ly-6/GR-1 (RB6–8C5), anti–CD11b/MAC-1 (M1/70), anti-B220 (RA3–6B2), anti–CD4 (GK1.5), anti–CD8 (Lyt-1), and anticytometry TEI-119. Mice and bone marrow transduction All procedures were approved by the local Institut Gustave Roussy (IGR) ethics committee. C57BL/6 mice (Janvier, Le Genest, France) were maintained at the IGR animal facility under specific pathogen-free conditions. Six to 8-week-old male mice were used as bone marrow donors. Seven to 10 week-old female mice were recipients. The infection was performed as previously described.4,19,21 Briefly, 4 days after 5-fluorouracil treatment (1 injection of 150 mg/kg administered intraperitoneally), total bone marrow cells were collected and cocultured with 10^6 MPZenTPO virus–producing GP+E-E-86 cells in Dulbecco modified Eagle medium (DMEM) supplemented with antibiotics, 20% fetal calf serum (FCS), murine IL-3 (100 U/mL), murine IL-6 (20 ng/mL), and murine SCF (20 ng/mL). After 4 days, nonadherent cells were harvested. An aliquot was immediately used in clonogenic progenitor assays to determine the percentage of infected colony-forming cells (CFCs) as previously described.4,19 CFCs were picked from methylcellulose and analyzed by polymerase chain reaction (PCR) with specific primers for the viral TPO gene and actin primers to ascertain the presence of material. The remaining cells were inoculated intravenously via the retro-orbital sinus into irradiated hosts (9.5 Gy, X-ray apparatus, single dose) in a ratio of one donor per one recipient. Three independent infection experiments were performed with a total of 80 engrafted hosts. The percentage of CFCs demonstrating the integrated TPO cDNA was comparable in the 3 experiments (95% ± 2%). Bortezomib administration One month after engraftment with TPO-overexpressing hematopoietic cells, 4 groups of 20 immunocompetent C57BL/6 mice displaying similar myeloproliferation (evaluated by similar platelet number) were constituted. Engrafted mice were treated intravenously twice a week, with either bortezomib in NaCl or vehicle (NaCl), for 4 to 12 weeks. Mice were weighed before each injection. Hematologic evaluation Blood from the orbital plexus was collected in citrated tubes at indicated times. Nucleated blood cells, hematocrit level, and platelet counts were determined using an automated blood counter calibrated for mouse blood (MS9; Melet Schloessing, Cergy-Pontoise, France). Differential cell counts were performed after May–Grünewald-Giemsas staining. Platelet-poor plasma was used for determination of TPO, IL-1α, TGF–β1, and OPG levels. Fresh spleen and blood cells (10^6) were grown in semisolid medium CFCs analyzed as previously described.4,19 Extracellular fluids of bone marrow were prepared by flushing 1 femur and 1 tibia freshly excised with 700 µL DMEM supplemented with 10% heat-inactivated FCS and antibiotics. Histopathology For histologic analysis, bones were excised and cleaned of soft tissue. One femur and 1 tibia were fixed in formaldehyde, decalcified, and paraffin embedded. Spleen, liver, and pulmonary samples were fixed and embedded in the same manner. Sections (4.5 µm) were stained with hematoxylin eosin, periodic acid Schiff, and Giemsa for overall cytology analysis. Reticulin fibers were revealed by silver staining according to Gordon Sweet method. Images were obtained using a Leica DMRB microscope (Leica, Solms, Germany) with 25×0.85 NA (magnification ×250), with 10×0.3 NA (magnification ×100) and with 2.5×0.075 NA (magnification ×25) objectives, and acquired with a Video 3 charge–coupled device (CCD) Sony Leica Power hole accumulated diode (HAD) camera (Sony, Tokyo, Japan). Determination of chimerism Fluorescent in situ hybridization (FISH) analysis of Y chromosome was performed on bone marrow cells from mice killed 8 weeks after engraftment as previously described.25 Enzyme-linked immunosorbent assay (ELISA) TPO, IL-1α, and OPG levels in plasma or supernatants were determined with the appropriate murine Quantikine Kits from R&D Systems according to the manufacturer’s instructions. Sensitivity limits of the assays were 62.5 pg/mL, 31.2 pg/mL, and 4.69 pg/mL for TPO, IL-1α, and OPG, respectively. Human TGF–β1 immunoassay, which cross-reacts with murine TGF–β1, was used to determine TGF–β1 levels in plasma and supernatants (Quantikine Kit; R&D Systems). Because this assay detects only the active form of TGF–β1, samples were assayed before (active forms) and after acidification (active + latent forms) according to the manufacturer’s instructions. The sensitivity of the assay was 31.2 pg/mL active TGF–β1. Cell culture Human factor-dependent cell line UT7/c-MPL (clone 110C1) was maintained in DMEM supplemented with 10% heat-inactivated FCS, antibiotics, and 5 ng/mL of rhGM-CSF. Murine megakaryocytes were derived from fetal livers of 14-day-old embryos. Single-cell suspensions were enriched for progenitor cells (Lin– fraction) by immunomagnetic selection (Dynabeads M-450; Dynal AS, Oslo, Norway) using lineage-specific Abs. The Lin– fraction was grown for 3 to 5 days as previously described.25 Adherent murine stromal cells were cultured as initially described by Dexter et al.27 Briefly, total bone marrow cells from one femur and one tibia were cultured in αMEM supplemented with 20% horse serum and 10^-7 M hydrocortisone sodium hemisuccinate. Half-growth medium was replaced weekly. At week 4, the entire medium was switched from horse serum to FCS. One week later, murine IL–1α (5 ng/mL) was added in culture medium. After a 24-hour incubation period, culture supernatants were harvested and used for determination of the OPG level. Image of adherent stromal cell layer was obtained using a Nikon microscope (Nikon, Tokyo, Japan) with 20×0.45 NA objective, a Zeiss AxiosCam MrC camera, and the Axio Vision Rel.4.3 acquisition software (Zeiss). Immunofluorescence and Western-blot analysis For in vitro analysis, UT7/c-mpl cells and murine megakaryocytes (4-day-old) were cytokine deprived for 16 hours in DMEM supplemented with 1%
FCS or IMDM supplemented with 3% FCS, respectively. For stimulation, TNF-α (5 ng/mL), rhGM-CSF (20 ng/mL), and rhTPO (100 ng/mL) in the absence or presence of bortezomib (2.5 ng/mL) were added to the culture medium. At the indicated time points, cells were centrifuged on coverslips, fixed and permeabilized with ice-cold methanol for 5 minutes, washed in PBS, stained with anti–NF-κBp65, and revealed with a donkey antirabbit TRITC-labeled Ab. Nuclei were counterstained with DAPI (Vectashield with DAPI; Vector Laboratories, Burlingame, CA). Localization of NF-κBp65 in the different UT7/c-mpl cells or murine megakaryocytes was analyzed using a fluorescence microscope (Nikon Eclipse 600; Tokyo, Japan) with Zeiss Plan-apochromat 63×/1.4 numerical aperture (NA) oil objective, a Zeiss AxioCam Mrc camera, and the AxioVision Rel.4.3 acquisition software (all from Zeiss, Oberkochen, Germany). Images were processed with the Adobe Photoshop CS software.

For in vivo analysis, fresh spleen cells from mice were dissociated and nuclear extracts were prepared and analyzed by Western blotting with the adequate Abs as previously described.†

Statistical analysis

Differences between data groups were evaluated for significance using the Wilcoxon test. A P value of less than .05 was considered significant. The data are presented as the mean plus or minus the standard error of the mean (±SEM). Dose effect was evaluated using an analysis of variance of the

Results

Bortezomib inhibits TPO-induced NF-κB activation in vitro in UT7/c-MPL cells and in murine megakaryocytes

Bortezomib has been demonstrated, both in vitro and in vivo, to block degradation of IκB through proteasome inhibition, leading to impaired NF-κB activation.24 A spontaneous activation of NF-κB was detected in monocytes,21 megakaryocytes, and circulating CD34+ cells22 from MMM patients. Moreover, we recently described that TPOhigh mice exhibit high plasma levels of IL-1α,23 suggesting a role of the NF-κB pathway activation in the TPO-induced myelofibrosis development. Therefore, we assessed the effect of bortezomib on myelofibrosis induced by high TPO levels.

We first confirmed that TPO was able to activate the NF-κB pathway in vitro in the MPL-expressing cell line UT7 and assessed the effect of bortezomib treatment on these cells. Stimulation with TPO, as well as TNF-α and GM-CSF, chosen as controls, induced NF-κB activation. Indeed, immunofluorescence staining showed a rapid accumulation of the NF-κB subunit p65 in the nucleus, observed as early as 10 minutes of TPO treatment (data not shown). P65 nuclear translocation was significantly inhibited by bortezomib treatment (Figure 1A). Similar results were obtained in normal murine megakaryocytes (Figure 1B).

Bortezomib inhibits NF-κB activation in vivo in TPOhigh mice

We assessed whether NF-κB was also activated in our in vivo model, the TPOhigh mice, and whether bortezomib was able to inhibit this activation. Eighty lethally irradiated female mice were hematologically repopulated with 10⁶ male bone marrow cells transduced with the murine Tpo gene using a retroviral gene transfer protocol, as previously described elsewhere.4,19,23

Four weeks after engraftment with the transduced hematopoietic cells, 4 groups of 20 mice each displaying similar TPO-induced myeloproliferation (evaluated by similar platelet number) were constituted. Engrafted mice were treated intravenously twice a week, with either bortezomib in NaCl (1 mg/kg, 0.5 mg/kg, and 0.25 mg/kg) or vehicle (NaCl), for 4 to 12 weeks. Plasma levels of TPO were monitored over time using an ELISA. Four weeks after engraftment, the TPO concentration in plasma was more than 1000-fold higher in engrafted mice than in the controls. No difference between treated and untreated TPOhigh mice was observed at week 8 (Figure 1E) and at week 16 (data not shown). Chimerism was analyzed by FISH on Y chromosome (bone marrow donors were male, recipients were female) on whole nucleated bone marrow cells. Chimerism levels were more than 90% and were similar in both treated and untreated mice (data not shown).

We studied what bortezomib would effect on NF-κB activation in vivo. Western blots were performed on nuclear spleen extracts prepared from control, treated TPOhigh (bortezomib 1 mg/kg), and untreated TPOhigh mice (vehicle) killed 8 weeks after engraftment. As expected, nuclear levels of the two NF-κB subunits p65 and p50 were increased in TPOhigh spleen cells and bortezomib largely reduced this translocation (Figure 1C-D). It has been demonstrated that NF-κB activation leads to IL-1α production,21 explaining that TPOhigh mice exhibit high plasma levels of IL-1α.23 Bortezomib treatment was able to significantly decrease the plasma concentration of IL-1α in a statistically significant dose-dependent manner (Figure 1F).

The most common side effect of bortezomib treatment in mice is weight loss; therefore, treatment was adapted to each mouse weight, and their weights were closely monitored throughout the follow-up. A bortezomib dose of 1 mg/kg led to the death of 50% of the animals within 4 weeks accompanied with statistically significant weight loss. Surviving mice were nevertheless analyzed. This toxicity of bortezomib at 1 mg/kg was higher than previously reported and may be related to the total body irradiation regimen that preceded the bortezomib administration. We then chose to repeat experiments with lower doses of bortezomib. Mice treated with 0.5 mg/kg and 0.25 mg/kg doses lost weight rapidly after the first bortezomib injections but progressively recovered their initial weights over time.

Bortezomib reduces myeloproliferation induced by high TPO levels

We then assessed the effects of bortezomib treatment on the TPOhigh myeloproliferative syndrome. We studied blood and spleen parameters (Figure 2). Leukocytosis (Figure 2A) and thrombocyto- sis (Figure 2B) displayed by TPOhigh mice were decreased in treated mice in a statistically significant dose-dependent fashion. Conversely, bortezomib did not significantly improve anemia of TPOhigh mice (Figure 2C).

The number of progenitor cells in the spleen and the blood was studied 8 weeks after engraftment. The increase of blood-circulating CFCs displayed by the TPOhigh mice was dramatically reduced by bortezomib treatment in a statistically significant dose-dependent manner (Figure 2D). Moreover, the high number of spleen CFCs was also impaired by bortezomib treatment (Figure 2E), in parallel to a dramatic decrease in the splenomegaly displayed by the TPOhigh mice (Figure 2F). Surprisingly, our data provide evidence that bortezomib is able to reduce the TPOhigh myeloproliferative syndrome in mice.

Bortezomib impairs marrow and spleen fibrosis development induced by high TPO levels through TGF-β1 inhibition

The development of fibrosis is characterized by the excessive deposits of extracellular matrix proteins. Bone marrow fibrosis
has been reported to be a direct consequence of high TGF-$
\beta_1$ levels in blood and bone marrow fluids in the TPOhigh model.\textsuperscript{16} Furthermore, to exert its biologic effects, TGF-$
\beta_1$ has to be activated at secretion sites within the hematopoietic environment. The mechanisms responsible for TGF-$
\beta_1$ activation remain unclear. Total and active forms of TGF-$
\beta_1$ were measured in treated and untreated mice (Figure 3A-C). As previously reported,\textsuperscript{16} the level of TGF-$
\beta_1$ in the plasma increased as early as 4 weeks after engraftment and reached a level 4 times higher than in control mice by week 8 (Figure 3A). Moreover, 8 weeks after engraftment, we observed an augmentation in total TGF-$
\beta_1$ levels in extracellular fluids of marrow in TPO high compared with control mice (Figure 3B). As expected, the active form of TGF-$
\beta_1$ was detected only in engrafted mice and was absent in control mice (Figure 3C). Bortezomib treatment significantly decreased total TGF-$\beta_1$ plasma levels (Figure 3A) and both the total (Figure 3B) and active (Figure 3C) forms of TGF-$\beta_1$ in extracellular fluids of marrow in a statistically significant dose-dependent manner.

Three mice of each group were killed after 4 weeks of treatment (8 weeks after engraftment). Macroscopic examination of the femora of control mice, excised and cleaned of soft tissue, appeared dark red, full of marrow cells (Figure 3D left). In contrast, fibrotic bones from untreated TPOhigh mice appeared white (Figure 3D right), suggesting fewer deposits of extracellular matrix proteins. As expected, histologic sections of femora and spleens of TPOhigh mice revealed a massive hyperplasia of dysmorphic megakaryocytes and granulocytic cells in both treated and untreated mice (Figure 3E,G,I,K). Silver impregnation, used to examine the degree of fibrosis, showed densification of the reticulin network with deposition surrounding megakaryocytes in untreated mice (Figure 3F bone marrow; Figure 3J spleen). In contrast, mice treated with bortezomib (1 mg/kg) displayed impaired reticulin fibers in both the bone marrow (Figure 3H) and the spleen (Figure 3L), confirming the macroscopic observation of their femora. Lower doses of bortezomib (0.5 and 0.25 mg/kg) have much fewer effects (data not shown).

**Bortezomib impairs osteosclerosis development induced by high TPO levels through OPG inhibition**

Osteosclerosis is another stromal change displayed by TPOhigh mice. It has been demonstrated that OPG secreted by the bone marrow microenvironment, more precisely by the stromal cells, is required for this abnormal bone growth.\textsuperscript{19} The mechanism leading to stromal OPG up-regulation remains unknown but it is not due to
we therefore made the in vitro to stimulate OPG production by human osteoblast lineage ezomib impaired OPG plasma levels (Figure 4C) and especially extracellular fluids (Figure 4D) 8 weeks after engraftment. Bortezomib dramatically increased OPG levels in fluids of marrow, regardless of dosage (Figure 4D).

Osteosclerosis development, which occurs later than bone marrow fibrosis, was assessed 16 weeks after engraftment. Therefore, 3 mice of each group were killed after 12 weeks of treatment. As expected, histologic sections of femora from untreated TPOhigh mice revealed a dense new bone formation, almost filling the medullar cavity (Figure 4E-F). Since OPG levels were decreased in bone marrow extracellular fluids of 0.5 mg/kg bortezomib–treated TPOhigh mice, a rare bone growth was observed in femora collected from these mice (Figure 4G-H). A minimal dose of 0.25 mg/kg also dramatically reduced osteosclerosis development (data not shown).

**Bortezomib dramatically improves survival in TPOhigh mice**

The TPOhigh model is known to induce a severe myeloproliferative disorder that mimics the evolution of the MMM disease in humans, leading to the death of all animals within 10 months after TPO-infected bone marrow cell engraftment.4 We assessed the effect of bortezomib treatment on TPOhigh mice survival after 12 weeks of treatment (from 16 to 52 weeks after engraftment). As TPOhigh mice invariably die with severe anemia,6 we decided not to bleed them anymore to avoid artificial death. Therefore, we observed a delayed lethality (up to 12 months) in untreated (vehicle) TPOhigh mice (Figure 5) compared with the survival curve initially described.4 Bortezomib (0.5 mg/kg) significantly improved survival in TPOhigh mice (89% vs 8% at week 52; \(P < .001\)).

**Discussion**

We show here that bortezomib reduces the myeloproliferative disorder, impairs both bone marrow fibrosis and osteosclerosis development, and dramatically improves survival in a murine model mimicking PMF. This TPOhigh model, induced by systemic TPO overexpression, is one of the two extensively studied experimental models of myelofibrosis in mice, together with the GATA-1low model.52 In the latter model, the knockout mice specifically express a low amount of the transcriptional factor GATA-1 in the megakaryocyte lineage. They display a megakaryocyte hyperplasia in the bone marrow and the spleen, like the TPOhigh mice. Myeloproliferation observed in both models does not originate from a clonal malignant event, as is the case in the human disease,2,3 but leads to similar stromal changes including bone marrow fibrosis and osteosclerosis. No alteration in either the structure or the expression of GATA-133 or its cofactor FOG-134 genes has yet been described in PMF. Conversely, three activating mutations implicating the TPO/MPL pathway (JAK2V617F, MPLW515L, and MPLW515K) have recently been identified.5,10 JAK2V617F and MPLW515L induce fibrosis in mice.5,11,12 However, JAK2V617F mice display a disease mimicking more polycythemia vera progressing to bone marrow fibrosis than PMF.11,12 MPLW515L mice develop a myeloproliferative disorder, fatal within 30 days after engraftment.5 In fact, this model is not really relevant, since it does not behave like a slowly evolving disorder such as PMF. Indeed, the activating MPL is transduced in total bone marrow cells, regardless of the hematopoietic lineage. On the contrary, in the TPOhigh model, only hematopoietic cells physiologically expressing MPL respond to TPO overexpression, mimicking deregulated MPL signaling in PMF cells. Therefore, the TPOhigh model appears more relevant to human PMF than either the MPLW515L or the...
In this study, we assessed the effects of the proteasome inhibitor bortezomib in the stromal changes displayed by the TPOhigh model. Moreover, TPOhigh mice develop a myeloproliferative disorder with associated myelofibrosis and osteosclerosis, and all the engrafted animals display a delayed death, like the natural evolution of human PMF. Therefore the TPOhigh model is an interesting in vivo model for testing candidate drugs.

In this study, we assessed the effects of the proteasome inhibitor bortezomib in the stromal changes displayed by the TPOhigh mice. Because the minimal dose of 1 mg/kg recommended by Millenium Pharmaceuticals for in vivo studies led to the death of all our irradiated and engrafted mice, we successfully decreased the dose to 0.5 and 0.25 mg/kg. Bortezomib impaired bone marrow fibrosis development through inhibition of TGF-β1 in a dose-dependent fashion (the minimal dose 0.25 mg/kg had few effects), as well as the myeloid proliferation. Conversely, bortezomib considerably reduced osteosclerosis development through OPG inhibition, regardless of dosage. It also dramatically improved TPOhigh mice survival but not at the minimal dose of 0.25 mg/kg. Thus, bortezomib seems to have dissociated effects on fibrosis and osteosclerosis development induced by TPO overexpression. Even a lower dose of 0.25 mg/kg dramatically reduced OPG production and osteosclerosis without significantly improving mice survival. Hence, osteosclerosis does not seem to be a major determinant in TPOhigh mouse lethality. In addition, our results shed light on osteosclerosis pathogenesis. Indeed, we have demonstrated that IL-1α, which originates in part from platelets in TPOhigh mice, is able to stimulate OPG secretion by stromal cells. Decreased IL-1α secretion in TPOhigh mice treated with bortezomib may be directly linked to NF-κB pathway inhibition and may lead to the inhibition of osteosclerosis onset.

Furthermore, reduction of reticulin fiber deposition appears to be dose dependent and paralleled the decreased in TGF-β1 secretion. TGF-β1 production seems to be directly related to the megakaryocyte/platelet compartment. In TPOhigh mice, a 2- to 4-fold increase in the TGF-β1 plasma level has been described with a similar augmentation in circulating platelet number. Therefore, only a slight increase in TGF-β1 transcript was found in TPOhigh platelets, unlike the IL-1α transcript as previously described. To date, the mechanism responsible for the abnormal release of TGF-β1 by the megakaryocytes remains unknown. It could be a consequence of either dysmegakaryopoiesis with abnormal emperipolesis or only of the megakaryocyte hyperplasia. According to
Bortezomib treatment (0.5 mg/kg) impairs considerably new bone formation (G-H). Development with accumulation of bone trabeculae in the medullar cavity (arrow).

The latter hypothesis, genetically modified mice\textsuperscript{5,11,12,32,36-42} displaying megakaryocyte hyperplasia lead invariably to bone marrow or spleen fibrosis development. The decreased TGF-\(\beta\)1 secretion that we have reported here could be linked either indirectly to myeloproliferation reduction (and thus to megakaryocyte/platelet compartment reduction) by bortezomib or directly to NF-\(\kappa\)B inhibition by bortezomib. In favor of the latter hypothesis, it has been demonstrated that NF-\(\kappa\)B inhibition leads to decreased TGF-\(\beta\)1 in both megakaryocytes\textsuperscript{22} and monocytes.\textsuperscript{21} A spectacular increase in survival was observed when TPO\textsuperscript{high} mice were treated with 0.5 mg/kg bortezomib. The role of the myelofibrosis in the poor prognosis of myeloproliferative disorders is still subject to debate. Recently, it has also been hypothesized that in TPO\textsuperscript{high} mice it is mostly the severity of the myeloproliferative disorder rather than bone marrow fibrosis that leads to death.\textsuperscript{43} As bortezomib decreases bone marrow fibrosis as well as myeloproliferative disorder, it is difficult to determine the origin of TPO\textsuperscript{high} lethality.

Altogether, our results allow us to propose a physiopathologic model for the stromal reaction in TPO\textsuperscript{high} mice, probably recapitulating bone marrow changes observed in the human disease (Figure 6).

Here, we used bortezomib to target the NF-\(\kappa\)B signaling pathway.\textsuperscript{24} However, this molecule has much broader effects and can interfere with synthesis of numerous cytokines, especially at the level of bone marrow environment. Anderson’s group (Roccaro et al\textsuperscript{44}) has shown that bortezomib has antiangiogenic effects in multiple myeloma. In TPO\textsuperscript{high} mice, there are some abnormalities of angiogenesis.\textsuperscript{45} PMF patients display a similar increase in bone marrow microvessel density, and neo-angiogenesis appears to be a component of the bone marrow stromal reaction in PMF.\textsuperscript{46} Thus we cannot exclude that part of bortezomib effects may also be related to an inhibition of neo-angiogenesis.

Moreover, the sole inhibition of the NF-\(\kappa\)B pathway cannot fully explain the antimyeloproliferative effect we observed in this mouse model, since we have previously reported that NF-\(\kappa\)B inhibition has no impact on megakaryocytic proliferation.\textsuperscript{22} However, in both human and mouse, bortezomib is known to induce thrombocytopenia through an unknown mechanism.\textsuperscript{47} However, it is unlikely to be related to marrow injury or decreased TPO production.\textsuperscript{46} Thus we cannot exclude that the NF-\(\kappa\)B pathway may play a role in megakaryopoiesis.

Figure 5. Bortezomib dramatically improves TPO\textsuperscript{high} mice survival. Kaplan-Meier plots of untreated (vehicle) or treated (bortezomib 0.5 mg/kg and 0.25 mg/kg) TPO\textsuperscript{high} mice. Bortezomib (0.5 mg/kg) significantly improves survival (\(P < 0.001\)). Pooled data from 9 to 13 animals per experimental group studied from 16 (after 12 weeks of treatment) to 52 weeks after engraftment. Bzb indicates bortezomib.

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cytokine responsible of this OPG up-regulation. /H9251 IL-1 release in hematopoietic cells, monocytes, and platelets, may be the /H9251 osteoclastogenesis mediated by an increased secretion of stromal cell–derived OPG. /H9252 proliferation stimulated by TGF- /H9252 and, on the other hand, to the inhibition of /H9252 required within hematopoietic microenvironment for the fibroblast-mediated fibrosis /H9252 activation of TGF- /H9252 and, on the other hand, to the inhibition of osteoclastogenesis mediated by an increased secretion of stromal cell–derived OPG. IL-1α, released by hematopoietic cells, monocytes, and platelets, may be the cytokine responsible of this OPG up-regulation.

**Acknowledgments**

This work was supported by grants from Institut National de la Santé et la Recherche Médicale (INSERM) and La Ligue Nationale contre le Cancer (équipe labellisée 2004 and 2007). O.W.B. was supported by a fellowship from INSERM. T.G. was supported by La Ligue Nationale contre le Cancer. D.F.P. was supported by Institut Gustave Roussy.

We are grateful to Annie Rouchès and Patrice Ardouin for managing the animals and to Caroline Lefebvre and Caroline Marty for improving the English manuscript. We thank Anna-Lila Kaushik and Sébastien Giroux for providing embryos.

**Authorship**

Contribution: O.W.B. performed cell-culture studies, animal studies, ELISA, FISH, and immunofluorescence; generated figures; helped design the study; and wrote the manuscript. D.F.P. performed Western blot analysis and helped generate figures and write the manuscript. T.G. performed animal studies and ELISA. M.T. performed histologic analysis. R.C. performed cell-culture and Western blot analysis. C.L. performed bortezomib injections. F.A. performed murine megakaryocytes cultures and helped generate figures. J.-L.V. provided MPZenTPO virus–producing GP+E86 cells. P.G. performed statistical analysis. W.V. and S.G. designed studies, analyzed data, and drafted and edited the manuscript.

D.F.P. and T.G. contributed equally to this work.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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**References**


Proteasome inhibitor bortezomib impairs both myelofibrosis and osteosclerosis induced by high thrombopoietin levels in mice

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