Bortezomib Alone or in Combination with the Histone Deacetylase Inhibitor JNJ-26481585: Effect on Myeloma Bone Disease in the 5T2MM Murine Model of Myeloma

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Abstract

The proteasome inhibitor bortezomib (Velcade) is currently approved as second-line treatment of multiple myeloma (MM). MM-related bone disease is one of the most debilitating complications of MM. Besides supportive care with bisphosphonates, which have proven efficacy in reducing and delaying skeletal-related events, there is no specific treatment of lytic bone lesions. The present study investigated the effect of bortezomib alone or in combination with a hydroxamate-based histone deacetylase inhibitor, JNJ-26481585 on tumor burden, and MM bone disease in the 5T2MM model. Injection of 5T2MM cells into C57BL/KaLwRij mice resulted in MM bone disease, characterized by an increase in the percentage osteoclasts, a decrease in osteoblasts, trabecular bone volume, trabecular number, and the development of bone lesions. Treatment of 5T2MM-bearing mice with bortezomib significantly reduced tumor burden, angiogenesis, and MM bone disease. More importantly, the combination of bortezomib with JNJ-26481585 resulted in a more pronounced reduction of osteoclasts and increase of osteoblasts, trabecular bone volume, and trabecular number compared with bortezomib as single agent. These data suggest that bortezomib has bone remodeling properties that can be improved in combination with low dose JNJ-26481585. The study indicates that this combination therapy could be a useful strategy for the treatment of MM patients, especially in those patients with skeletal complications.  [Cancer Res 2009;69(13):5307–11]

Introduction

Multiple myeloma (MM) is an incurable B-cell malignancy characterized by an accumulation of plasma cells in the bone marrow (BM) secreting monoclonal immunoglobulins. Reciprocal interactions between the tumor cells and the BM microenvironment result in increased cell survival and growth, release of cytokines, development of drug resistance, angiogenesis, and induction of bone disease. The latter is caused by a MM-induced imbalance between bone resorption and bone formation, resulting in osteolytic bone destruction and subsequent clinical complications such as pathologic bone fractures, hypercalcaemia, and bone pain (1).

The introduction of novel targeting agents in the treatment of MM such as bortezomib, thalidomide, and lenalidomide, which not only affect the MM cell directly, but also its interactions with the BM microenvironment, has been a major step forward in the therapeutic options for patients. Despite these improvements, osteolytic bone disease remains an important cause of morbidity in these patients. Besides supportive care with bisphosphonates, there are currently limited agents available that can prevent development of MM-related bone disease (2).

Bortezomib, a first-in-class, potent, and reversible proteasome inhibitor, acts on different targets in MM. Bortezomib is an effective therapy for patients with relapsed or refractory MM, with recent proven superiority even in newly diagnosed patients requiring therapy (3).

Preclinical and clinical data suggest that bortezomib also has a positive effect on bone remodeling by inhibiting osteoclast formation and stimulating osteoblast differentiation as reviewed by Terpos and colleagues (4). The effect of bortezomib on bone disease is thought to be direct and not only a consequence of the anti-MM effect. However, the mechanism by which bortezomib regulates MM bone disease in vivo is unclear and therefore further studies are required.

The aim of the present work was to investigate the effect of bortezomib in vivo in the 5T2MM murine model of MM with emphasis on the associated key processes in MM microenvironment such as BM osteolysis and angiogenesis. In addition, we investigated whether JNJ-26481585, a novel “second generation” pyrimidyl-hydroxamate–based histone deacetylase (HDAC) inhibitor, had a more pronounced effect when combined with bortezomib. Compared with J&J first generation HDAC inhibitor (HDACi) R306465, JNJ-26481585 has significantly improved pharmacodynamic properties with, e.g., a longer half-life and a sustained pharmacodynamic response in vivo.5

Materials and Methods

Mice

C57BL/KaLwRij mice were purchased from Harlan. They were housed and treated following conditions approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (license no. LA1230281).

5TMM Model
The in vivo growing 5T2MM cells originated spontaneously in elderly C57BL/KaLwRij mice and have since been propagated by i.v. transfer of diseased marrow in young syngeneic immunocompetent mice (5, 6).

We used the 5T2MM model that mimics the human disease closely having a selective growth in the BM, inducing angiogenesis and MM-associated bone disease. Because freshly isolated 5T2MM cells only survive for short periods, the 5T33MMvt cell line was used for incubation periods of 72 h.

Drugs
Bortezomib (Janssen Pharmaceutica N.V., J&J PRD), mixed with mannitol in a ratio of 1:10, was solubilized in 0.9% sodium chloride at the appropriate concentration before each s.c. injection.
The HDACi, JNJ-26481585 (Johnson & Johnson), was prepared in a solvent containing 10% HP-ßCD, 0.8% HCl 0.1 N, 0.9% NaOH 0.1N, 3.4% mannitol, and pyrogen-free water.

For in vitro studies, bortezomib and JNJ-26481585 stock solutions were prepared at 1 and 5 mmol/L, respectively, in tissue-culture grade DMSO.

Assessment of Viability in 5T33MMvt Cells
The MTS colorimetric assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega] was used as previously described to assess the viability of 5T33MMvt cells (7).

Therapeutic Treatment of 5T2MM-Bearing Mice
Treatment with bortezomib as single therapy. Two groups of 10 mice were injected i.v. with 2.10^6 5T2MM cells, one group of 10 mice was included as negative control (naive, nontumor-bearing mice). Progression of MM in 5T2MM-bearing mice was assessed by measuring serum paraprotein concentrations, using protein electrophoresis. From the time paraprotein was detectable by serum electrophoresis (at week 8), treatment started with either bortezomib (0.8 mg/kg, twice weekly, s.c.; n = 10) or vehicle (NaCl 0.9%; n = 10).

Treatment with bortezomib + JNJ-26481585. Four groups of 10 mice each were injected i.v. with 2.10^6 5T2MM cells and one group was included as negative control (n = 10). Treatment was administered s.c. in tumor-bearing mice and started when paraprotein was detectable, at week 8. Four different treatment groups were defined: group 1, vehicle bortezomib (NaCl 0.9%); group 2, bortezomib (0.6 mg/kg, twice weekly); group 3, JNJ-26481585 (1.25 mg/kg, every other day); group 4, bortezomib (0.6 mg/kg, twice weekly) + JNJ-26481585 (1.25 mg/kg, every other day).

All mice were sacrificed at week 12, the time point when the vehicle group showed signs of morbidity (paralysis of the hind legs). The tibia was dissected free of soft tissue and processed for bone histomorphometric analysis as described below. BM was isolated from hind legs and tumor burden was assessed as described below. One femur was fixed in zinc fixative and further processed to assess BM angiogenesis. Blood samples were obtained to determine serum paraprotein concentrations (5).

Assessment of Tumor Burden
Serum paraprotein was measured by electrophoresis and plasmacytosis was determined on May-Grüenwald-Giemsa stained cytosmears of mononuclear BM cells.

Assessment of Angiogenesis
The microvessel density was determined by CD31 staining as previously described (8). The number of blood vessels was counted in an area of 0.22 mm², in the area with the highest blood vessel density (hotspot).
Analysis of Bone Disease

Tibiae were scanned using a microCT scanner (model 1172; SkyScan) at 50 kV and 200 μA with a pixel size of 4.3 μm, an image being captured every 0.7° through 180° rotation of each bone. Scanned images were reconstructed and analyzed using the SkyScan Recon and SkyScan CT analysis software, respectively. Trabecular volume, thickness, and number were evaluated in a standardized region of interest. The region of interest was defined as a volume starting 0.2 mm from the growth plate and extending 1 mm in length, the cross-sectional area being limited by the border with the cortical bone. All trabecular bone within this region was measured; the bone being differentiated from soft tissue by thresholding the grayscale images. Subsequently, the right tibiae were decalcified, embedded in paraffin, and 3-μm sections cut and stained with H&E or enzymatically stained for tartrate resistant acid phosphatase. Osteoblasts were identified on the basic of characteristic morphology and the osteoclasts were identified after tartrate resistant acid phosphatase staining. The number of osteoclasts (/mm) and osteoblasts (/mm) was measured using the Osteomeasure bone histomorphometry software (Osteometrics).

Statistics

Values represent the means ± SE. The significance between variables was determined using Student’s t test. The results were considered significant if P value is <0.05.

Results and Discussion

In the present study, we observed that bortezomib has potent antimyeloma effects on 5T33MMvt cells in vitro at low nmol/L concentrations. After 72 hours of incubation, bortezomib inhibited the growth in a dose-dependent manner with an IC50 of 4.33 ± 0.56 nmol/L (data not shown), consistent with previous reports using human MM cell lines and freshly isolated patient MM cells (9) and thus indicated similar sensitivity of the 5TMM cells to bortezomib as human MM cells.

We subsequently analyzed the effect of bortezomib in vivo on MM cell growth, angiogenesis, and osteolytic bone lesions in the 5T2MM model (5, 6). In the first series of experiments, treatment of the 5T2MM mice with bortezomib started when serum paraprotein could be detected in tumor-bearing mice, thereby simulating a therapeutic setting. In the 5T2MM tumor-bearing mice, treatment with bortezomib resulted in a near-normalization of microvessel density in the BM (Fig. 1C). This reduction in the microvessel density could not only be a consequence of the reduction of tumor burden but also
by direct effects of bortezomib on endothelial cells. Roccaro and colleagues (10) showed in vitro and in vivo direct effects of bortezomib by reducing the proliferation, chemotaxis, and capillary formation of endothelial cells.

The development of the MM-related bone disease in the vehicle-treated mice was characterized by an induction in osteolytic bone lesions as illustrated by microCT images (Fig. 2A), a decrease in trabecular bone volume (P < 0.05; Fig. 2B), a decrease in trabecular number (P < 0.05; Fig. 2C), an increase in the number of osteoclasts (P < 0.05; Fig. 3A), and a decrease in the number of osteoblasts (P < 0.05; Fig. 3B). Treatment of the tumor-bearing mice with bortezomib (0.8 mg/kg) resulted in a decrease of osteolytic lesions and a 3.5-fold increase in trabecular number and trabecular bone volume compared with the vehicle group (P < 0.05; Fig. 2). Decrease of MM-related bone disease may reflect a direct effect on the MM cells as a result of a reduction of tumor burden or due to a direct effect on osteoclasts and/or osteoblasts. In tumor-bearing mice, treated with bortezomib, we observed that the number of osteoclasts was reduced by 50% (P < 0.05) and returned to levels seen in naive mice (Fig. 3A). The number of osteoblasts was increased for 400% compared with the vehicle group and was also ~50% greater than that seen in naive tumor-free mice (P < 0.05; Fig. 3). This suggests that, apart from the indirect effect of bortezomib, it may also directly affect osteoblasts in a more pronounced way than osteoclasts, confirming previous results (11, 12). Preclinical studies have shown that bortezomib stimulates the Wnt pathway in osteoblasts by reducing DKK-1 levels in the osteoblasts (11, 12). Preclinical studies have shown that bortezomib stimulates the Wnt pathway in osteoblasts by reducing DKK-1 levels in the osteoblasts. In support of this, Feng and colleagues (17) showed in vitro that drug combination of bortezomib and the HDACi, PXD101, resulted in a synergistic inhibition of MM cell survival and osteoclast formation. Therefore, in the present study, we evaluated in vivo the effect of HDAC inhibition in MM and its effects on MM-related bone disease.

We combined bortezomib (0.6 mg/kg, twice weekly, s.c.) at a concentration where 99% of the tumor load was reduced in the 5T2MM model (P < 0.05; Fig. 1A) and bone disease was reduced partially (P < 0.05; Figs. 2B and C and 3), with a suboptimal dose of JNJ-26481585 (1.25 mg/kg, every other day, s.c.). 5T2MM mice treated with JNJ-26481585 at this low dose showed no significant reduction in the tumor burden in the BM (P > 0.05; Fig. 1A) and did not reduce the bone disease significantly (Fig. 2). JNJ-26481585 treatment at a dose of 20 mg/kg every other day in the 5T2MM model resulted in a dramatic reduction in tumor burden, angiogenesis, and MM bone disease, demonstrating the potent antmyeloma activity of this compound at higher concentrations (18). As we observed a reduction of almost 100% in the tumor load (Fig. 2A) when bortezomib was used as single agent, we could not observe any additive reduction on this parameter with the combination of both drugs. Despite this lack of additional effect on tumor burden, we did observe a increase in bone volume (Fig. 2B), trabecular number (Fig. 2C), and the bone surface covered by osteoblasts (Fig. 3B), when we combined both drugs compared with their effect as single agents. In addition, a further reduction in the proportion of bone surface covered by osteoblasts was observed (Fig. 3A). The effect in the combination treatment is more significant on osteoclast than osteoblast because bortezomib as single agent already had an effect on the number of osteoclasts, and it is unlikely that it is possible to

Figure 3. Effect of bortezomib (0.8 mg/kg, twice weekly, s.c.), bortezomib (0.6 mg/kg, twice weekly, s.c.), and JNJ-26481585 (1.25 mg/kg, every other day, s.c.) as single agents and the combination bortezomib (0.6 mg/kg, twice weekly, s.c.) + JNJ-26481585 (1.25 mg/kg, every other day, s.c.) on the number of osteoclasts and osteoblasts per mm. A, Nr osteoclasts. B, Nr osteoblasts. Columns, mean of six groups of 10 mice [Naive, 5T2MM+Vehicle, 5T2MM+Bz (0.6 mg/kg), 5T2MM+JNJ-85, and 5T2MM+Bz (0.6 mg/kg) + JNJ-85; *, P < 0.05]; bars, SE.
References


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