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Stimulation of new bone formation by the proteasome inhibitor, bortezomib: implications for myeloma bone disease

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Summary

Impaired bone formation contributes to the lack of bone healing in multiple myeloma and there is a need for agents with bone anabolic properties to reverse the bone deficit in patients. Bortezomib, a proteasome inhibitor with antitumour efficacy in myeloma patients, enhanced new bone formation in mouse calvarial cultures; this effect was blocked by dickkopf 1(Dkk1), an antagonist of Wnt signalling implicated in myeloma bone disease. Bortezomib inhibited Dkk1 expression in calvariae and bone marrow-derived stromal cells, suggesting a novel mechanism by which bortezomib exerts its effects in bone. Clinical trials in patients with myeloma bone disease are needed to validate these results.

Keywords

bortezomib; osteoblast; myeloma; Dkk1; bone

Proteasome inhibition is increasingly being employed as an antitumour strategy in several cancers including multiple myeloma. Bortezomib (Velcade™; PS-341), the first-in-class proteasome inhibitor approved for use in myeloma, has been evaluated in several completed and on-going clinical trials. Results have documented a striking antitumour effect of bortezomib in myeloma patients at first presentation or after relapse, alone or in combination with corticosteroids and/or chemotherapeutic agents (Richardson *et al*, 2006).

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Myeloma bone disease is commonly associated with tumour-induced osteolysis and, until recently, most attention has focused on the increased number and activity of osteoclasts. However, in myeloma patients, there is also an impaired bone formation response characterized by a failure of osteoblasts to respond appropriately to replace resorbed bone. The impaired bone formation contributes to the lack of bone healing in myeloma patients even when the tumour is in remission. Osteoclast-mediated bone resorption becomes progressively uncoupled from bone formation resulting in osteopenia that is in some cases generalized, manifesting as markedly reduced serum bone alkaline phosphatase activity (Bataille *et al*, 1990; Taube *et al*, 1992; Withold *et al*, 1996; Dhodapkar *et al*, 1998; Woitge *et al*, 2001). Although osteoblast dysfunction in myeloma bone disease is well recognized and described, no treatment is available to target this dysfunction. Bisphosphonates, potent inhibitors of bone resorption and the current standard of care for cancer-induced bone diseases, stop further bone loss but do not stimulate new bone formation. There is, therefore, a need to understand the mechanisms mediating the impaired bone formation response in myeloma and to develop new and effective therapies that target both the tumour and the associated bone deficit.

Herein, we report that bortezomib stimulated osteoblast differentiation and bone formation in bone organ cultures in a bone morphogenetic protein (BMP)-dependent manner. Dickkopf 1 (Dkk1), an antagonist of Wnt signalling recently implicated in myeloma bone disease (Tian *et al*, 2003), was also found to block bortezomib-induced new bone formation. Finally, bortezomib was shown to inhibit Dkk1 expression in bone and bone-derived cells, suggesting a novel mechanism by which the proteasome inhibitor may modulate bone formation in the bone microenvironment.

Materials and methods

Calvaria organ cultures

Calvarial bone organ cultures established as described from 4-d-old ICR Swiss mice (Harlan, Indianapolis, IN, USA; Garrett, 2003) were cultured in the presence or absence of bortezomib. After fixation for 24 h in buffered formalin, calvarie were decalcified in EDTA, embedded in paraffin, and 4- μ m-thick sections stained with Hematoxylin and Eosin. Images of sections visualized using $\times 20$ objective magnification were captured with a Pro-Series 128 Capture Kit frame-grabber board and area of new bone formed measured as previously detailed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA; Garrett *et al*, 2003).

Reverse transcription polymerase chain reaction (RT-PCR)

Polymerase chain reaction was performed on first-strand cDNA reverse-transcribed from total RNA using oligo-dT and Super-Script II kit (Invitrogen, Carlsbad, CA, USA). After preheating to 94°C for 4 min, amplification was performed for 35 cycles (58°C for 45 s, 72°C for 45 s and 94°C for 30 s followed by a final extension at 72°C for 2 min) using published murine Dkk1 primers (Stump *et al*, 2003). Amplicons were purified and sequenced in the University of Texas Health Science Center's Advanced Nucleic Acids Core Facility to verify identity with published murine Dkk1 sequences in GenBank.

Transfections and luciferase assay

Promoter firefly luciferase fusion DNA reporter in pGL3-basic constructs were co-transfected with a pSV- β -galactosidase construct (Promega, Madison, WI, USA; for transfection efficiency control) using Lipofectamine (Invitrogen) and standard protocols. Cell lysate luciferase and β -galactosidase activities were determined using Bright-Glo™ luciferase assay and β -Glo® assay kits (Promega), respectively.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Cells treated with or without bortezomib in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) for 48 h and then in serum-free DMEM for another 24 h were harvested and whole-cell lysates, prepared according to standard protocol in radioimmunoprecipitation assay (RIPA) buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany), were resolved electrophoretically on a 10% SDS polyacrylamide gel. 50 ng recombinant murine (rm) Dkk1 (R&D Systems, Minneapolis, MN, USA) was included in one lane as a positive control. Separated proteins were transblotted onto a polyvinylidene difluoride membrane, probed with an affinity-purified polyclonal goat antibody raised against a peptide mapping to an internal region of human Dkk1 (Y-17) (sc-14949; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:100 dilution. Following several washes, the membrane was then incubated with rabbit anti-goat antibody conjugated to horseradish peroxidase (HRP; Sigma Chemicals, St Louis, MO, USA) and signals visualized by chemi-luminescence (New England Nuclear, NEN; Boston, MA, USA). To control for equal loading, the membrane was then completely stripped and re-probed with a mouse monoclonal antibody to rabbit GAPDH (6C5; Santa Cruz) that cross reacts with mouse GAPDH followed by an appropriate HRP-conjugated secondary antibody.

Statistical analysis

Except otherwise stated, data were analysed using one-way analysis of variance with *post-hoc* testing using Fisher's Protected Least Significant Difference and accepted as significantly different given $P \leq 0.05$.

Results and discussion

It remains unknown whether bortezomib stimulates bone formation or can reverse the bone deficit in myeloma. To test this concept, we examined the effects of bortezomib on bone formation in neonatal mouse calvarial organ cultures, a model system that we and others have shown is predictive of bone anabolic compounds that are active *in vivo* when administered systemically (Garrett, 2003; Garrett *et al*, 2003). In two separate experiments, bortezomib, when added to the cultures on day 0, dose-dependently enhanced bone formation by day 5 (Fig 1A). This bone anabolic effect of bortezomib was clearly evident even at low nanomolar concentrations shown to be effective in killing myeloma cells in culture. We next determined if the effect of bortezomib in this context was similarly BMP-2-dependent. Murine 2T3 pre-osteoblasts were transfected with a BMP-2 promoter (-2712/+165)-luciferase expression construct (Garrett *et al*, 2003) in the presence or

absence of bortezomib or another structurally-unrelated peptidyl aldehyde proteasome inhibitor, Proteasome Inhibitor 1 (PSI), for 24 h. Bortezomib was found to be an extremely potent inducer of BMP-2 transcription, again with effects seen at low nanomolar concentrations (Fig 1B), and it was even more potent in this regard than reported previously with PSI (Garrett *et al*, 2003). These data support our notion that all proteasome inhibitors, including bortezomib, enhance bone formation by increasing BMP-2 production.

Dkk1 is a known antagonist of Wnt signalling, which has been increasingly associated with postnatal mammalian bone formation (Holmen *et al*, 2005). As Dkk1 has recently been implicated in myeloma bone disease (Tian *et al*, 2003), we next examined the effects of Dkk1 on bortezomib-induced new bone formation. Calvariae were incubated either with noggin-Fc (a BMP antagonist), murine Dkk1, or a combination of both for an initial period of 24 h, and then together with bortezomib for the subsequent 96 h. Dkk1 and noggin-Fc incubated together completely prevented bortezomib-stimulated osteoblast accumulation and bone formation, although bortezomib-induced bone formation was only partially blocked by either reagent individually (Fig 1C).

To gain further mechanistic insight into how bortezomib may modulate bone formation *in vivo*, we next investigated whether the proteasome inhibitor regulates Dkk1 expression. To this end, mouse calvariae and bone marrow stromal 14M1 cells, derived from a murine 5T Radl myeloma-bearing mouse, were treated with bortezomib for 24 h, and murine Dkk1 expression was determined by RT-PCR. Bortezomib was found to be a powerful inhibitor of Dkk1 gene (*Dkk1*) expression in both calvariae and 14M1 cells (Fig 2A & B). Consistent with this effect, the same concentrations of bortezomib that inhibited *Dkk1* expression also inhibited expression of a human *DKK1* promoter (−2338/+112)-luciferase reporter construct (Gonzalez-Sancho *et al*, 2005) when transfected into 14M1 cells (data not shown). Immunoblotting with extracts of 14M1 stromal cells treated with bortezomib for 72 h confirmed the inhibitory effect of bortezomib on Dkk1 protein expression (Fig 2C). Consistent with these present observations, it has recently been reported that circulating Dkk1 levels were reduced in myeloma patients receiving bortezomib (Terpos *et al*, 2006).

Together, the above data demonstrated that bortezomib is a powerful stimulator of new bone formation. Consistent with these findings, there are preliminary reports indicating elevation of surrogate indices of bone formation, such as serum bone-specific alkaline phosphatase in bortezomib-treated myeloma patients (Zangari *et al*, 2005). Bortezomib may potentially reverse the associated bone deficit in these patients, thereby reducing risk of complications such as pathological fractures. These results provide additional rationale to investigate the potential bone anabolic efficacy of this drug directly in preclinical models of myeloma bone disease and in clinical trials in patients with myeloma bone disease.

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Fig 1.

(A) Bortezomib stimulates bone formation dose-dependently in neonatal mouse calvariae. Hemi-calvariae were treated with or without bortezomib ($n = 4/\text{group}$) at indicated concentrations for 5 d. Photomicrograph of treated calvariae showing dose-dependent induction of robust osteoblast proliferation and new bone formation by bortezomib (left panel); new bone area in calvariae assessed morphometrically. Data presented as mean $\pm$ SEM for each concentration tested (right panel) (veh, vehicle); (B) Dose-dependent effect of bortezomib on BMP-2 promoter activity. Murine 2T3 preosteoblasts were co-transfected with a murine BMP-2 promoter ($-2712/+165$)-luciferase and a RSV- β -galactosidase reporter vectors and then treated with or without bortezomib or PSI at the indicated concentrations for 24 h. Luciferase (*Luc*) and galactosidase (β -gal) assays were performed using standard procedures. Data represent ratios of *Luc*/ β -gal activities in cells treated with bortezomib (in two experiments) or PSI expressed as fold induction compared with results in vehicle-treated (unstimulated) cells transfected with the same reporter. Note, bortezomib was even more potent than PSI [closed circles, bortezomib – (two separate experiments); open circles, PSI]; (C) Noggin and Dkk1 oppose the effect of bortezomib to enhance bone formation. Mouse hemi-calvariae were incubated with 2 $\mu\text{g}/\text{ml}$ recombinant noggin-Fc (a BMP antagonist), 200 ng/ml recombinant murine (rm) Dkk1 or a combination of both for the first 24 h $\pm$ bortezomib for the remaining 4 d. Data represent mean $\pm$ SEM ($n = 4$) for each concentration tested (right panel; open bar, media alone; filled bars, 200 ng/ml rmDkk1). *versus new bone area in vehicle-treated cultures, #versus new bone area in bortezomib-treated cultures without Dkk1. (Recombinant proteins were from R&D Systems, Minneapolis, MN, USA).

Fig 2.

(A,B) Bortezomib inhibits Dkk1 gene expression. RT-PCR in 14M1 marrow stromal cells (A) and neonatal mouse calvariae (B) treated with bortezomib showing amplicon of predicted size in vehicle-treated cells; (C) Inhibition of Dkk1 protein expression by Bortezomib. Western blotting of extracts of 14M1 cells treated with bortezomib for 72 h using a goat-anti mouse Dkk1 antibody (Santa Cruz) as primary antibody. Positive control was rmDkk1 (R&D Systems) migrating, as predicted, as a doublet of 35–40 kDa. Note the presence of multiple glycosylated forms of Dkk1 as previously reported (Bhat *et al*, 2004). Blot was completely stripped and reprobed with an antibody recognizing mouse GAPDH, used as an internal loading control.