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PARATHYROID HORMONE RECEPTOR MEDIATES THE ANTI-MYELOMA EFFECT OF PROTEASOME INHIBITORS

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Abstract

Clinically significant serum parathyroid hormone (PTH) variations have been reported in multiple myeloma (MM) patients treated with proteasome inhibitors. To elucidate the association between serum PTH variations and proteasome inhibition in MM, the effect of PTH and PTHR1 ligands on the proteasome inhibitors bortezomib and carfilzomib *in vitro* and *in vivo* was determined. The MM cell lines ARP1, OC1 and 5TGM1 expressed mRNA and protein encoding PTH receptor 1 (PTHR1). Treatment of 5TGM1 cells with either PTH(1–34), bortezomib or carfilzomib alone dose-dependently inhibited 5TGM1 cell proliferation. However, treatment with the potent PTHR1 antagonist [TYR34]PTH(7–34) (PTH(7–34)) had no significant effect on myeloma cell proliferation and cell viability. In contrast, when used in combination with bortezomib or carfilzomib, PTH(7–34) treatment significantly reduced the bortezomib or carfilzomib-associated decrease in cell proliferation. Treatment of the C57BL/KaLwRij mouse myeloma model with either bortezomib or carfilzomib provided a significantly prolonged survival benefit compared to controls ($p=0.04$; $p=0.01$ respectively). This potent anti-myeloma effect was completely abrogated by concomitant treatment with PTH(7–34). These results suggest an important role of the PTHR1 in the anti-myeloma effect of proteasome inhibition.

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Keywords

multiple myeloma; proteasome inhibition; PTH receptor

INTRODUCTION

The pathogenesis of disease progression in multiple myeloma (MM) is a complex phenomenon and numerous processes and pathways have been implicated in the regulation of osteoblast-induced bone formation and bone resorption by osteoclasts.[1, 2] In fact, bone disease is one of the most debilitating manifestations of the disease. The ubiquitin proteasome pathway is an essential cellular degradative system has been shown to play an important regulatory role in multiple myeloma.[3] A number of clinical studies have demonstrated that proteasome inhibition increases osteoblast precursor differentiation through interference with canonical Wnt signaling and increased DKK1.[4–6] The effect of proteasome inhibition on MM bone disease is believed to be direct and not merely a consequence of the anti-myeloma properties. [7] In addition, retrospective and prospective clinical studies examining variations in serum bone alkaline phosphatase in response to proteasome inhibition with bortezomib in myeloma patients have demonstrated a close correlation between drug activity and increased bone anabolic activity.[8, 9] As such, proteasome inhibitor treatment is an attractive therapeutic option that may combine potent anti-myeloma activity with perceived beneficial effects on the skeleton.

Parathyroid hormone (PTH) is a 84 amino acid peptide synthesized by the parathyroid glands that is stored in secretory vesicles and dense core granules.[10] Extracellular calcium levels sensed by the calcium receptor on parathyroid chief cells regulate PTH release, thereby controlling serum calcium levels systemically.[10] The clinical use of intermittent PTH(1–34) therapy in the postmenopausal osteoporosis setting elicits a rapid increase in bone-formation markers, followed by increases in bone-resorption markers.[11] PTH(1–34) is able to reverse the structural damage seen in postmenopausal osteoporosis and restores the structure and strength of trabecular bone.[12] In myeloma patients, during the first Bortezomib treatment cycle, serial serum PTH measurements demonstrated a significant difference in the first treatment cycle in responders versus non-responders patients.[13] The cyclic PTH variations associated with the potent anti-myeloma effect of proteasome inhibition suggested an unknown mechanism of action, therefore, we tested the role of the PTH axis in MM cell lines and in a myeloma mouse model during proteasome inhibitor treatment.

MATERIALS AND METHODS

Tissue culture, peptides and proteasome inhibitors

All tissue culture and laboratory plasticware was purchased from (Fisher Scientific Inc.), and medium was purchased from (Invitrogen NY, USA). Human PTH(1–34) and the antagonist [TYR³⁴]PTH(7–34) (PTH(7–34)) were purchased (Bachem Pharmaceutical Company, Torrance, CA). The proteasome inhibitors bortezomib and carfilzomib were provided by the manufacturers (Millennium The TAKEDA Oncology Company, Cambridge MA; Onyx Pharmaceutical, San Francisco, CA). 5TGM1, ARP1 and OC1 myeloma cells were grown in Hyclone classical liquid media RPMI 1640 (Thermo Scientific Hyclone, Logan, UT) supplemented with heat-inactivated fetal bovine serum (Thermo Scientific Hyclone, Logan, UT) and 1× penicillin-streptomycin solution (GIBCO, Grand Island, NY) at 37°C in 5% CO₂ atmosphere.

Evaluation of PTHR1 expression

Briefly ARP1, OC1 and 5TGM1 cell lysates were prepared as previously described.[14] Samples (30 ug/lane) were then subjected to 8% SDS-PAGE and immunoblotted onto nitrocellulose. Blots were incubated with 0.1 mg/ml anti-PTHr1 antibody (5G1.3) specific for PTHR1.[14] Bound primary antibody was detected by incubation with fluorescently-labeled secondary antibody (goat-anti mouse) in Odyssey Blocking Buffer (1:40000 dilution in 1/10 in 5% Evaporated milk (stock is 20%) and PBS followed by Odyssey infrared detection (Li-Cor Biosciences, Lincoln, NE USA).

5TGM1 Cell-based experiments

5TGM1 myeloma cells were plated in 24 well plates at 10^5 cells per milliliter in RPMI-1640 then supplemented with 2% FBS in the presence or absence of Bortezomib, PTH(1–34) or PTH(7–34). Cell number and viability were measured by Trypan Blue exclusion at various time intervals every day (from day 0 to 5) and the total number of cells and the number of Trypan blue-positive cells counted using a Neubauer chamber under bright field illumination.

The 5TGM1 cell line was exposed to bortezomib, carfilzomib, PTH(1–34) and PTH(7–34) at various concentrations and combinations. Bortezomib was added as single agent at 5nM and 10nM to the medium of 5TGM1 cells and replenished every other day when the medium was changed. Carfilzomib was tested at 2.5nM and 5nM concentration following the same treatment regimen. PTH(1–34) was added at 10nM, 50nM and 100nM concentrations daily from day 0. The PTHR1 antagonist PTH(7–34) was added at 100nM, 500nM and 1µM on days –3 to –1 before the addition of each proteasome inhibitor. Bortezomib and carfilzomib were tested (at the above concentrations) in combination with PTH(1–34) and with PTH(7–34).

Mouse Model of Myeloma

All procedures involving these mice were approved by the local ethics committee at the University of Utah. The 5TGM1 murine model of myeloma was provided by Dr. Greg Mundy, Vanderbilt Center for Bone Biology, Department of Cancer Biology, Vanderbilt University, Nashville, Tennessee, USA,

All animals, including the respective un-injected, non-tumor bearing, control groups, were sacrificed at 12 weeks for the 5TGM1 bearing mice (n=15, naïve control n=10). Inoculation of 5TGM1 cells in the inbred C57BL/KaLwRij induced myeloma disease (evident 3 weeks after inoculation) characterized by severe osteolysis and IgG2b production along with terminal involvement of extramedullary sites such as liver and kidney. After tail vein inoculation of 0.5×10^6 5TGM1 cells (in 100 µL PBS) at day 0, tumors were allowed to grow for 1 week. One week after injection of the 5TGM1 cells, the test compounds were infused. A total of 10 untreated mice were used as controls (5 mice were used for each drug tested), 5 mice were treated with either bortezomib or carfilzomib, 10 mice received PTH(7–34), and 5 in combination with each drug. One week after cells were inoculated, bortezomib was infused at 1 mg/kg twice a week IV (via tail vein) and carfilzomib was infused IV (via tail vein) at 1.5 mg/kg twice a week. PTH(1–34) was injected subcutaneously at 80 ug/kg daily as described.[15] PTH(7–34) was injected subcutaneously at 50mg/kg on day –3, –2, –1 prior to proteasome inhibitor delivery.

Serum IgG2b levels were measured as described,[16] using a Mouse IgG2b ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's instructions. All steps were performed at room temperature. The reaction product was quantified spectrophotometrically at 450 nm using a microplate reader..

Statistical analysis

Data were expressed as the mean $\pm$ SEM. Comparison between groups was performed by Mann-Whitney U test, Chi-square analysis or Student's unpaired *t* test, as appropriate. Overall mouse survival was assessed using the log-rank test. In all cases, $p < 0.05$ was considered statistically significant and indicated as such.

RESULTS

The effect of PTH(1–34) and PTH(7–34) on proteasome inhibition of MM cells in vitro

A number of well-accepted myeloma cell lines (5TGM1, ARP1 and OC1) [17–19] were first examined for expression of the human PTH receptor (PTHr1). Western blot analysis demonstrated the robust expression of PTHr1 at similar levels in all three myeloma cell lines (Figure 1A) as well as in primary human myeloma cells by gene expression analysis (data not shown). As a result of this analysis, we chose 5TGM1 cells for further detailed experimental evaluation since transplanting these cells develops MM in C57BL6/KaLwRij mouse.

Next, the effect of bortezomib treatment on 5TGM1 myeloma cell proliferation was determined. After 4 days of bortezomib treatment (50nM and 100nM) the proliferation of the 5TGM1 cells was significantly ($p < 0.05$) inhibited (Figure 1B). Similarly, treatment of 5TGM1 cells with PTH(1–34) also induced a significant decrease in cell proliferation by day 4 at both 50nM and 100nM PTH(1–34) (Figure 1C) ($p < 0.05$). Importantly, the potent PTHr1 antagonist PTH(7–34) alone had no significant effect on the proliferation of 5TGM1 myeloma cells at any dose tested (Figure 1D).

Next, the effect of combined bortezomib (50nM) and PTH(1–34) (50nM) treatment on the proliferation of 5TGM1 myeloma cells was examined. The two drug combination exerted a significant reduction in 5TGM1 cell proliferation compared to the control group by day 4 ($p = 0.001$) (Figure 2A). Interestingly, the inhibitory effect of bortezomib treatment on 5TGM1 cell proliferation was significantly inhibited ($p = 0.00025$) by the addition of 500nM PTH(7–34) (Figure 2B), suggesting the involvement of the PTH signal in the anti-myeloma effect of bortezomib treatment.

These effects were not proteasome inhibitor-specific since similar inhibitory effects ($p < 0.05$) on 5TGM1 cell proliferation were also observed after treatment of 5TGM1 cells with carfilzomib at 2.5 and 5nM (Figure 2C). The inhibition of 5TGM1 cell proliferation of 5TGM1 cell by carfilzomib (2.5nM) was also significantly ($p < 0.05$) reduced by concomitant treatment with PTH(7–34) at either 500nM or 1 μ M (Figures 2D and 2E).

The effect of PTH(1–34) and PTH(7–34) on MM growth and survival in the presence of proteasome inhibition *in vivo*

We next determined the effect of bortezomib, PTH(1–34) and PTH(7–34) on the overall survival of C57BL/KaLwRij mice after transplantation of 5TGM1 myeloma cells. As expected, MM-bearing mice treated with bortezomib (1 mg/kg) had a significantly longer survival compared to untreated control animals ($p = 0.04$) (Figure 3A). No untreated control mouse lived longer than 5 weeks, whereas bortezomib only treated animals survived for up to 7 weeks (a 40% increase). The survival of PTH(1–34) treated mice was also improved compared with the untreated control mice, but was significantly less than the bortezomib-treated animals (Figure 3A). The combination of PTH(1–34) and bortezomib was not different from bortezomib alone (Figure 3A). Strikingly, the combination of bortezomib and the PTHr1 antagonist PTH(7–34) completely abrogated the beneficial effect of bortezomib treatment ($p = 0.04$ on day +30) (Figure 3A). The PTH(7–34) and bortezomib- treated

animals had poor survival, identical to the untreated MM-bearing control group (Figure 3A). Both the PTH(1–34) treated and bortezomib treated animals had a significant survival advantage over the bortezomib plus PTH(7–34) combination group ($p=0.04$; Figure 3A).

The significant survival benefits were associated with treatment-induced decreases in serum IgG2b (M-protein) levels (Figure 3B), which is a direct reflection of changes in tumor burden *in vivo*. [13] Treatment with bortezomib or PTH(1–34) as single agents or in combination produced significantly lower serum IgG2b levels compared to untreated controls ($p=0.02$; $p=0.03$; $p=0.02$, respectively; Figure 3B). Similar to the overall survival data, the beneficial effect of bortezomib on tumor burden (serum IgG2b) was lost with PTH(7–34) treatment (Figure 3B). Serum IgG2b levels in the bortezomib plus PTH(7–34) group were significantly higher than levels in the bortezomib treated animals ($p=0.03$) and identical to untreated 5TGM1 myeloma-bearing animals (Figure 3B). As expected, a positive effect of carfilzomib on survival compared to MM-bearing controls was observed (Figure 3) ($p=0.01$). However, when mice were treated with the combination of carfilzomib and PTH(7–34), the survival benefit was lost and the animals had a significantly shorter lifespan compared to carfilzomib only treated animals ($p=0.02$). In sum, these data implicate the PTHR1 in the anti-myeloma effects of bortezomib and carfilzomib therapy.

DISCUSSION

The proteasome inhibitor bortezomib is widely incorporated into multiple myeloma treatment strategies and has demonstrated substantial antitumor activity. [20]. Interestingly, accumulated data suggest that the activity of proteasome inhibitors extends beyond the tumor cell, to include significant effects the microenvironment and bone metabolism. [7] Indeed, we have previously shown that the treatment of myeloma patients with bortezomib induced significant increases in bone architectural parameters, such as bone volume/total volume (BV/TV) and trabecular thickness. [13] The significant bone anabolism in 70–80% of the responding patients was preceded by a significant and reproducible pulsatile increase in serum PTH levels, with no significant concomitant changes in Ca, Mg, or phosphorus levels. [21] These changes are similar to the rapid increases in serum PTH associated with bone anabolism in PTH-treated post-menopausal osteoporosis patients. [11, 22] In addition other clinical data has suggested that bortezomib directly stimulates osteoblast growth and differentiation in combination with inhibition of osteoclast development/activity; an activity that may be associated with PTH secretion. [23, 24]

Myeloma cell lines express the specific PTHR1 and the proliferation of myeloma cell lines are exquisitely sensitive to treatment with bortezomib and carfilzomib. [23, 25] Bortezomib and PTH(1–34) dose-dependently inhibited myeloma cell proliferation and the anti-myeloma effect of bortezomib was not affected by concomitant PTH(1–34) administration. In contrast, the inhibition of PTHR1 function by the specific receptor antagonist PTH(7–34) significantly decreased the anti-myeloma effect associated with either bortezomib or carfilzomib treatment, leading to the conclusion that the PTHR1 plays a role in the anti-myeloma action of proteasome inhibitors.

The 5TGM1 cell line was initially derived from 5T33 myeloma cells that originated spontaneously in C57BL/KaLwRij mice. [26, 27] In fact, the 5TGM1-derived MM model is considered a model that closely resembles the human disease, displaying rampant osteolysis and elevated IgG2B levels. [28] Using this immunocompetent model, a significant positive effect on survival after treatment with either proteasome inhibitor was observed. The myeloma survival advantage afforded by proteasome inhibition was maintained in the presence of concomitant PTH(1–34) administration. Surprisingly, the survival benefit was completely lost when bortezomib or carfilzomib treatment was combined with the co-

administration of PTH(7–34), confirming the idea developed *in vitro* using MM cell lines that the anti-tumor effect is related to PTHR1. In sum, the data provide a compelling argument aligning PTHR1 with the anti-myeloma activity of proteasome inhibitors and provide a mechanistic basis for the positive effect of these molecules on bone metabolism that requires PTHR1.

The findings presented here offer a promising new understanding of the mechanism of action of proteasome inhibition in MM. The data demonstrate for the first time that the inhibitory effect of two chemically distinct proteasome inhibitors on myeloma growth is dependent on the presence of functional PTHR1, suggesting that PTH/PTHR1 pathway may be associated with the antitumor activity of this class of drug.

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Highlights

- Parathyroid hormone receptor involved in antimyeloma effect of proteasome inhibition
- PTHR1 antagonism inhibits antimyeloma effects in mice
- Treatment options may involve identifying PTHR1 expression in myeloma

Figure 1. PTHR1 expression and effect of PTH and proteasome inhibitors on myeloma cell proliferation

(A) Western Blot Analysis for PTHR1 expression in ARP1, OC1 and 5TGM1 myeloma cell lines. Arrow shows the position of PTHR1 at ~80kDa. (B) Concentration dependent inhibitory effect of bortezomib (50 nM and 100 nM) on the 5TGM1 cell proliferation. (C) Concentration dependent inhibitory effect of PTH (10, 50, 100nM) on 5TGM1 cell. (D) No significant effect of PTH(7-34) (100nM, 500nM, 1μM) on 5TGM1 cell proliferation. (* denotes $p < 0.05$)

Figure 2. Effect of PTH analogs on proteasome inhibition of 5TGM1 cell proliferation
(A) PTH(1–34) (50 nM) does not attenuate the inhibitory effect of 5nM bortezomib on 5TGM1 cell proliferation. **(B)** PTH(7–34) (500 nM) significantly attenuates the inhibitory effect of 5nM bortezomib on 5TGM1 cell proliferation. **(C)** Inhibition of 5TGM1 cell proliferation by carfilzomib. **(D)** PTH(7–34) (500nM) treatment has no effect of cell proliferation. PTH(7–34) (500nM) treatment in combination with concomitant exposure of carfilzomib (2.5nM) significantly attenuated carfilzomib-mediated inhibition of cell proliferation. **(E)** PTH(7–34) (1uM) treatment has no effect of cell proliferation. PTH(7–34) (1uM) treatment in combination with concomitant exposure of carfilzomib (2.5nM) significantly attenuated carfilzomib-mediated inhibition of cell proliferation. (* denotes $p < 0.05$; ** denotes $p < 0.01$)

Figure 3. Effect of PTH analogs on the improved 5TGM1 myeloma cell survival with proteasome inhibition

(A) Overall survival of 5TGM1 transplanted C57BL/KaLwRij mice treated with Bortezomib, PTH(1–34), PTH(7–34) and their combination. (Con vs Bortezomib $p=0.044$, Con vs Bortezomib+PTH(7–34) $p=0.5$, Bortezomib vs Bortezomib+PTH(7–34) $P=0.044$, PTH(1–34) vs PTH(7–34)+Bortezomib $p=0.047$). (B) Serum IgG2b (M-protein) changes of the different cohorts of mice treated with Bortezomib, PTH(1–34), PTH(7–34) and their combinations. (Con vs Bortezomib $p=0.017$, Con vs Bortezomib+PTH(1–34) $p=0.017$, Bortezomib vs Bortezomib+PTH(7–34) $p=0.034$, PTH(1–34) vs PTH(7–34)+Bortezomib $p=0.029$). (C) Overall survival of 5TGM1 C57BL/KaLwRij mice after treatment with

carfilzomib and PTH(7–34) as single agent and in combination (Carfilzomib vs. Control $p=0.01$, Carfilzomib Vs Carfilzomib + PTH(7–34) $p=0.026$)