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Proteasome inhibitors abrogate osteoclast differentiation and osteoclast function

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Abstract

Cancer-induced bone disease results in bone destruction, pathological fractures, and pain. We hypothesized that the inhibition of the proteasome–ubiquitin system in osteoclasts could abolish the receptor activator of NF-κB ligand (RANKL) mediated osteoclast differentiation and function, since RANKL-mediated downstream signaling plays a crucial role in osteoclast life cycle. In this study, we examined the effects of the proteasome inhibitors MG-132 and MG-262 on RANKL-induced osteoclast differentiation and function. Osteoclast precursors from peripheral blood mononuclear cells were cultured in the presence of RANKL and M-CSF. Osteoclasts were identified as multi-nucleated TRAP-positive cells. Osteoclast function was quantified with the extent of dentine resorption and TRAP activity in culture supernatants. For the evaluation of the effects of proteasome inhibitors towards osteoclastogenesis, sub-apoptotic concentrations of MG-132 and MG-262 were used. Effects on NF-κB were obtained in treated and untreated osteoclasts. MG-132 and MG-262 inhibit both osteoclast differentiation and osteoclast function. 0.01 μM MG-132 induced a 3.2-fold ($P = 0.004$) and 0.001 μM MG-262 a 3.3-fold ($P = 0.004$) reduction of osteoclast differentiation, respectively. The resorption capacity was decreased 2.6- and 11.1-fold ($P = 0.003$) by treatment with 0.01 and 0.1 μM MG-132, and 14.2- and 16.6-fold ($P = 0.003$) by 0.001 and 0.01 μM MG-262, respectively. This decrease correlated with the extent of NF-κB binding capacity. In conclusion, this study shows for the first time that proteasome inhibitors act on osteoclast development and function at low concentrations and should be considered as potential drugs for the treatment of cancer-induced osteolytic bone disease.

Keywords: Cancer-induced bone disease; NF-κB; Osteoclast; Proteasome; Proteasome inhibitors

Osteolytic bone disease is a common manifestation in cancer, e.g., in multiple myeloma and breast carcinoma [1]. Cancer-induced bone disease can lead to severe bone pain, pathological fractures, symptoms of hypercalcemia, and reduced quality of life. The excessive differentiation and activation of bone-resorbing osteoclasts may result from a direct contact between tumor cells and osteoclast precursors in the bone marrow, stimulation by various cytokines called osteoclast activating factors (OAFs) produced by tumor and stroma, or both [2–4]. The bi-directional interactions between tumor and microenvironment may result in a vicious circle with enhanced tumor growth and spread.

In multiple myeloma, which causes exclusively osteolytic bone lesions and thus acts as a model disease for cancer-induced bone destruction, the highest level of bone remodeling occurs notably in regions close to myeloma cell conglomerates [5]. Osteoclast activating factors are either produced locally by myeloma cells or from stromal cells in response to myeloma cells, and particularly include receptor activator of NF-κB ligand (RANKL) [6] and macrophage inflammatory

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protein-1α (MIP-1α) [7]. The newly identified tumor necrosis factor-ligand family member RANKL [8,9], which exists in a membrane-bound and in a soluble form, was shown to be expressed on some tumor cells [5,10–12] and in osteoblastic lineage cells [13]. On the other side, both osteoclast precursors and osteoclasts express RANK, a transmembrane receptor of RANKL. The binding of RANKL to RANK activates TNF-receptor-associated cytoplasmatic factors (TRAFs), predominantly TRAF-6, and in consequence NF-κB, AP-1, and p38 pathways, which are crucial for osteoclast differentiation, survival, and function [14,15]. Furthermore, the levels of RANKL expression on bone marrow myeloma cells from patients correlated with the presence or absence of osteolytic bone lesions [16], which highlights the impact of RANKL on osteoclast activation. The binding of RANKL to its transmembrane receptor RANK activates several signaling cascades, including the NF-κB-pathway. Blocking the NF-κB activation by dominant negative mutant of I-κB kinase IKK 2 resulted in reduced osteoclast differentiation, which highlights its decisive role [17]. A cell permeable I-κB kinase inhibitor showed similar effects [18].

NF-κB is a transcription factor which is composed of homo- and heterodimeric complexes of members of the Rel family. There are five subunits of the family in mammals: p50, p65 (RelA), c-Rel, p52, and RelB3 [19]. In the majority of cells, NF-κB exists in an inactive form in the cytoplasm, bound to the inhibitory protein I-κB. When liberated from I-κB, NF-κB translocates into the nucleus, where it promotes gene transcription [20]. NF-κB activation can be prevented by prolonged stabilization of its antagonist I-κB, which is normally degraded by the 26S proteasome. Newly designed proteasome inhibitors (bortezomib, MG-262, and MG-132) were shown to induce apoptosis in malignant cells [21–24] and to prevent the NF-κB activation by inducing I-κB stabilization [25].

We hypothesized that proteasome inhibitors might abolish osteoclast differentiation and function by inhibiting RANKL-induced NF-κB activation. This study shows for the first time that the inhibition of the proteasome–ubiquitin pathway significantly reduces both osteoclast differentiation and resorptional activity.

Materials and methods

Cultivation of osteoclasts. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of healthy volunteers, using a Ficoll–Hypaque density gradient ($\varphi = 1.007$). After centrifugation at 400*g* for 30 min at 21 °C, the buffy layer was removed with a Pasteur pipette and washed twice in PBS. Isolated mononuclear cells were cultured at a density of 2×10^6 cells/cm^2 in 10 cm petri dishes in α-MEM (Sigma–Aldrich Chemie, Taufkirchen, Germany) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml M-CSF, and 25 ng/ml RANKL (osteoclastogenic medium) at 37 °C in a humidified 5% CO_2 atmosphere. After 24 h of culture, the non-adherent cell fraction was discarded and the adherent population was washed with PBS (PAA Laboratories, Pasching, Austria). Thereafter, trypsin was added, and the cells and trypsin were incubated at 37 °C for approximately 6 min. The cells were scraped off and reseeded as indicated. Readherent cells were subjected to further immunophenotypic characterization and were identified as committed preosteoclasts (pOCs) [26].

The experiments have been approved by the Ethics Committee of the University Hospital Charité Berlin, in accordance with the Declaration of Helsinki. An informed consent was obtained from all volunteers.

Effects of proteasome inhibitors on osteoclast precursors. Preosteoclasts were generated as mentioned above. Cells were reseeded at a cell density of 2×10^5/cm^2 in 96-well plates and incubated with indicated doses of MG-132 and MG-262 for 3 days. Cell viability was measured using an MTT-assay. For the last 4 h of culture, cells were pulsed with 10 μl of the MTT labeling reagent (Sigma–Aldrich) at a final concentration of 0.5 mg/ml. This assay is based on the cleavage of the yellow tetrazolium salt MTT to purpure formazan crystals by metabolically active cells. To solubilize the crystals, 100 μl of a solubilization solution, containing 10% SDS in 0.01 M HCl, was added into each well, and the plate was allowed to stand overnight in the incubator in a humidified 37 °C/5% CO_2 atmosphere. Finally, the absorbance was measured spectrophotometrically, using a 550 nm wavelength ELISA-reader and Anthos-software.

Osteoclast differentiation assay. Preosteoclasts were seeded at a density of 2×10^5 cells/cm^2 in 24-well plates. Half of the media was replaced every 2 days. For studies concerning the differentiation inhibition, media were supplemented with MG-132 (0.1 or 0.01 μM) or MG-262 (0.01 or 0.001 μM). After a culture period of 28 days, cells were stained for TRAP activity, using a commercially available Leukocyte Acid Phosphatase Kit (Sigma–Aldrich). Mature osteoclasts were identified as large cells, containing more than two nuclei and positive for TRAP staining.

Osteoclast activity assay. The ability of osteoclasts, generated from PBMC, to resorb bone was assessed using ivory dentine pits (received from German customs in accordance with international laws for the protection of species). Briefly, dentine slides were sterilized in 96% ethanol overnight, washed three times with sterile PBS, and incubated in FCS containing medium for 2 days to ensure the sterility. Preosteoclasts were cultured in osteoclastogenic medium as described above in the presence or absence of MG-132 and MG-262. After 28 days of culture, cells were removed using natriumhypochlorite. Resorption lacunae were visualized by using 1% toluidine-blue solution and quantified by light microscopy.

Tartrate resistant acid phosphatase activity assay. TRAP activity was measured in supernatants at the end of the culture period by adding a colorimetric substrate consisting of 7.6 mM *p*-nitrophenylphosphate, 100 nM sodium acetate in the presence of 50 mM sodium tartrate at pH 5.5. The reaction products were quantified by measuring the optical absorbance at 405 nm wavelength.

Flow cytometry. The expression of vitronectin receptor on osteoclast precursors was quantified using a mouse monoclonal IgG1 antibody against vitronectin-R (Serotec, Oxford, UK). Briefly, adherent cells at the day +1 of culture were washed three times with PBS and trypsinated for 5 min. Trypsin activity was stopped with FCS. Cells (2×10^5) were stained with either 10 μl of isotypic antibody (100 μg/ml, Immunotech, Marseille, France), diluted 1:4, or 10 μl of anti-vitronectin-R antibody (1 mg/ml), diluted 1:40. Fluorescence intensities were measured using a secondary PE-conjugated F(ab′)2 fragment goat anti-mouse IgG antibody (Immunotech) by a FACSCalibur and Cell Quest Pro software (Becton–Dickinson, Mountain View, CA).

Transcription factor ELISA. In order to quantify the RANKL-induced NF-κB activation using different treatment schedules with proteasome inhibitors, we used an ELISA-based kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. Briefly, osteoclasts were lysed with the provided complete lysis buffer

and total protein amounts were quantified by BCA-assay (Pierce, Rockford, IL). Using a provided 96-well plate, containing an immobilized oligonucleotide, which is specific for NF-κB consensus site (5′-GGGACTTTCC-3′), 20 μg of cell extract per well was allowed to react. The NF-κB complex bound to the oligonucleotide was detected by an antibody against NF-κB p65 subunit. An HRP-conjugated secondary antibody allowed a sensitive colorimetric readout.

Chemicals. Proteasome inhibitors MG-132 and MG-262 (Biomol, Hamburg, Germany) were dissolved in an appropriate solvent (DMSO or distilled water, according to manufacturer's instructions) to 1 mM stock solutions. Aliquots were stored at −80 °C. Further dilution of required concentrations followed in medium before use. The solvent did not exceed 0.0001% in the final solution. RANKL and M-CSF (Sigma–Aldrich) were dissolved in PBS, adjusted to working concentrations of 10 μg/ml, and stored at −20 °C until use.

Statistical analysis. Descriptive statistics and significance levels were determined using the SPSS Version 11.0 and Sigma Plot. All experiments were conducted in triplicate and reproduced at least twice with similar results. The data demonstrate mean values of at least six measured values. Error bars represent standard deviations.

Results

Expression of TRAP and Vitronectin-R in adherent osteoclast precursors

To characterize the early adherent cell population derived from peripheral blood mononuclear cells, cells were subjected to immunocytochemistry and flow cytometry. On day +1 of culture, we found that cells expressed vitronectin-R, an αVβ 3 family member of the integrin superfamily, which mediates the adhesion to extracellular matrix proteins (Fig. 1A). On day +7, all cells stained positive for TRAP (Fig. 1B), implicating the feasibility of monocytic precursors from the day +1 to differentiate into preosteoclasts.

Indicated dosages of proteasome inhibitors do not reduce preosteoclast survival

For indicated experiments, we used sub-apoptotic dosages of proteasome inhibitors, evaluated in multiple myeloma cells in a previous work [22]. To clearly exclude the toxic effects of proteasome inhibitors on preosteoclasts, we performed an MTT-assay. Adherent day +1 pOCL were treated with 0.1 and 0.01 μM MG-132 or 0.01 and 0.001 μM MG-262 for three days, respectively. Cell viability was similar in all experimental settings, indicating that used dosages of proteasome inhibitors did not have any cytotoxic effects on preosteoclasts (data not shown).

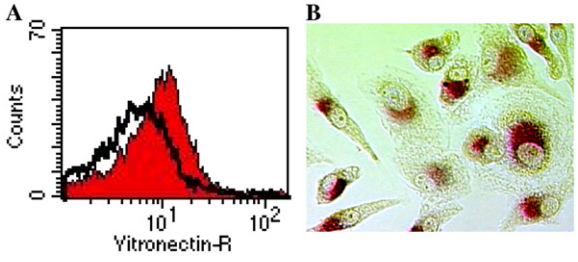

Fig. 1. Identification of preosteoclasts by vitronectin-R expression and TRAP activity. On day +1, cells were stained with a monoclonal antibody against vitronectin-R. As shown by the FACS analyses, cells on day +1 express vitronectin-R, a member of integrin superfamily, which mediates the adhesion to the extracellular matrix (A). After osteoclast precursors had been cultured with osteoclastogenic media containing 50 ng/ml M-CSF and 25 ng/ml RANKL for one week, all cells became positive for TRAP activity (B).

Proteasome inhibitors abrogate osteoclast differentiation from osteoclast precursors

In the next group of experiments, we investigated the impact of proteasome inhibitors on osteoclast differentiation. Preosteoclasts were cultured as described in Materials and methods in the presence or absence of RANKL and M-CSF. On day +1, treatment with 0.01 or 0.1 μM MG-132 and 0.001 or 0.01 μM MG-262 was started. After 28 days of culture, TRAP positive and multi-nucleated osteoclasts were quantified by light microscopy. The confluence was equal in treated and untreated samples. In this experiment, we could demonstrate that the used proteasome inhibitors were sufficient for significant inhibition of osteoclast formation, while the effects occurred in a dose-dependent manner (Fig. 2). For MG-132 at 0.1 μM, a reduction in the number of osteoclasts to 24% (range: 12–46, $P < 0.005$), and at 0.01 μM to 31% (range: 16–41, $P < 0.005$) was observed. When MG-262 was used, the differentiation was reduced to 27% (range: 12–45, $P < 0.005$) for 0.01 μM and to 30% (range: 17–42, $P < 0.005$) for 0.001 μM.

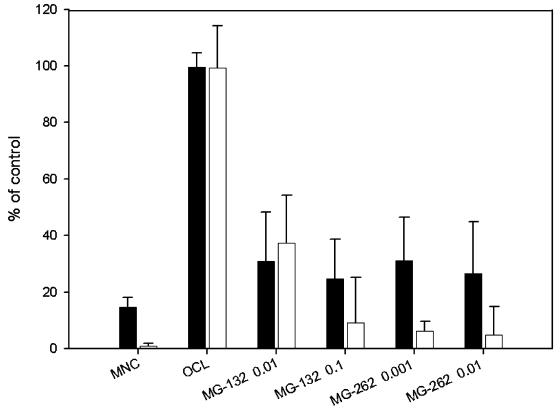

Fig. 2. Proteasome inhibitors induce inhibition of osteoclast differentiation and function at subapoptotic concentrations. Concentrations of proteasome inhibitors are given in micromolar. Unstimulated mononuclear cells (MNC) served as negative control, while RANKL-stimulated osteoclasts (OCL) as positive control. The cell density was equal in all samples. Black bars represent the number of TRAP positive multinucleated osteoclasts after treatment with indicated dosages of proteasome inhibitors for 4 weeks. White bars represent the number of lacunae resorbed by osteoclasts. For MG-132 at 0.1 μM and for MG-262 at 0.01 and 0.001 μM, a greater inhibition of osteoclast function was found in comparison to inhibition of osteoclast differentiation.

Proteasome inhibitors inhibit bone resorption

When osteoclasts were cultivated on dentine pits, bone resorption was considered as a marker of osteoclast activity. Resorption lacunae were envisualized by toluidine-blue and counted microscopically. In the first experimental setting, the treatment was started at the day +1 of culture. After 28 days, the bone resorption was inhibited similarly to the osteoclast differentiation. The number of resorption lacunae decreased to 9% of control value when cells were treated with 0.1 μM MG-132 ($P = 0.003$), and to 6% ($P = 0.003$) and 7% ($P = 0.003$) of control with 0.01, and 0.001 μM MG-262, respectively. Interestingly, the extent of inhibition of bone resorption was even higher than the inhibition of osteoclast differentiation, indicating that not only the differentiation, but also the osteoclast function was abrogated by proteasome inhibitors.

Proteasome inhibitors reduce TRAP activity

Tartrate resistant acid phosphatase activity is a hallmark of osteoclast function. To determine the extent of the inhibition of activity culture supernatants at the end of the experimental settings were collected. According to the data collected from the dentine pits, a reduction in TRAP activity was observed in a dose-dependent manner (Fig. 3).

Proteasome inhibitors prevent the NF-κB activation in osteoclasts

Since NF-κB activation is one of the central consequences of the RANKL binding to RANK, and the physiologic NF-κB inhibitor I-κB is a well-documented

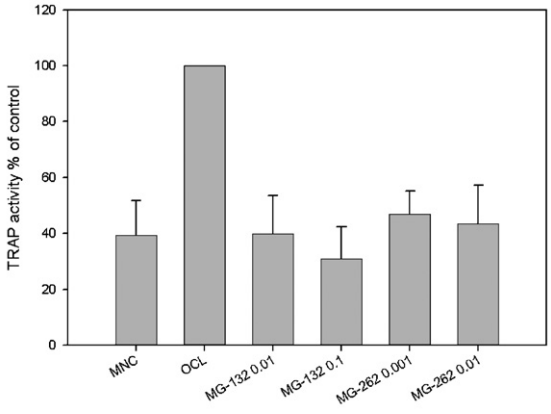

Fig. 3. Inhibition of osteoclast activity reflected in the reduction of TRAP activity. TRAP activity was measured from culture supernatants after 4 weeks of treatment. Untreated osteoclasts (OCL, with RANKL stimulation) served as a positive control, while untreated mononuclear cells (MNC, without RANKL stimulation) as negative control. When cells were treated with indicated doses of MG-132 and MG-262 (concentrations are given in micromolar), a significant reduction of TRAP activity was observed.

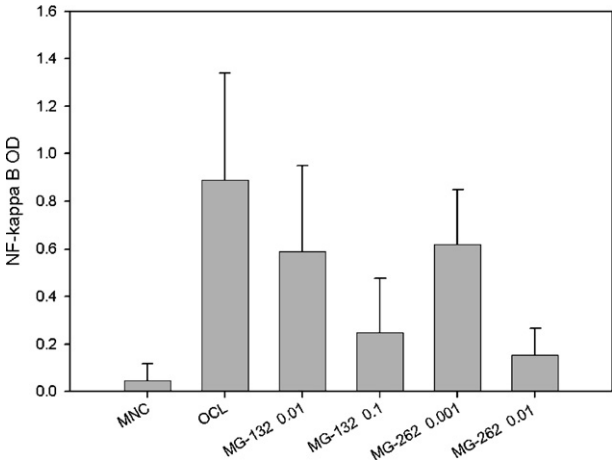

Fig. 4. Proteasome inhibitors act through NF-κB inhibition. Osteoclasts on day +28 were pretreated with indicated doses (concentrations are given in micromolar) of proteasome inhibitors for 48 h and then stimulated with 25 ng/ml RANKL for 30 min. Untreated osteoclasts served as a positive control (OCL, stimulated with RANKL), and unstimulated mononuclear cells (MNC, without RANKL stimulation) as negative control. In this experimental setting, a dose-dependent reduction of NF-κB activity was observed.

substrate of the 26S proteasome, we assumed that the inhibition of 26S proteasome by proteasome inhibitors might result in a reduction of RANKL-induced NF-κB activation. In order to quantify the NF-κB activation in untreated and treated osteoclasts reliably, we performed an ELISA-based assay as described above. After 30 min of stimulation with or without RANKL, M-CSF, and proteasome inhibitors, cells were lysed and subjected to examination. Mature osteoclasts, solely stimulated with RANKL and M-CSF, exhibited a strong NF-κB activation. In contrast, osteoclasts treated with proteasome inhibitors exhibited a dose-dependent reduction of NF-κB activation (Fig. 4), which correlated with the diminished osteoclast differentiation ($P = 0.037$).

Discussion

Bone destruction is a common phenomenon in a variety of malignancies. In cancer-induced bone disease due to, e.g., multiple myeloma or breast cancer, increased bone resorption results from enhanced osteoclast differentiation and activity. The consequences are skeletal complications in terms of pathologic fractures, neurological symptoms, and hypercalcaemia, which are associated with reduced quality of life and overall survival.

The standard therapy of osteolytic bone disease is bisphosphonates. They inhibit the osteoclast function by interaction with intracellular signaling [27] and by accumulation in the mineralized bone matrix making it more resistant to dissolution by osteoclasts [28]. Compounds such as pamidronate and zoledronic acid

have been shown to reduce skeletal events in patients with cancer-induced lytic bone disease [29–31]. However, the progression of cancer-induced bone disease is only partially inhibited by bisphosphonates, thus alternative or additional treatment strategies are urgently needed.

Physiologically, osteoclasts are terminally differentiated cells that evolve from monocytic precursors. In cancer-induced lytic bone disease, osteoclast differentiation is enhanced either directly by cell–cell contact with tumor cells, or by paracrine stimulation by circulating factors, summarized as OAFs [5]. The most common OAFs include cytokines IL-1, IL-6, PTHrP, MIP-1α, and RANKL, which are produced by tumor and stromal cells. Enhanced osteoclast activation leads to growth stimulation and protection of tumor cells from apoptosis and consequently to tumor growth and spread [11].

The latest cytokine system, essential for the osteoclast activation, was identified as a member of the TNF superfamily [8]. It consists of an effector, RANKL, and two receptors, of which one was identified as a soluble decoy receptor OPG [32] and the other as a transmembrane receptor RANK [9], which is located on osteoclast precursors and mature osteoclasts [14]. The RANKL/RANK binding activates a downstream signaling cascade, which results in NF-κB activation and enhanced transcription of NF-κB related genes, and in consequence in enhanced osteoclast differentiation and function [15]. In a previous study, we found that multiple myeloma cells expressed RANKL [10,33], and furthermore, the levels of RANKL expression correlated with the presence or absence of osteolytic bone lesions, evident on conventional radiography examinations of multiple myeloma patients [16], which indicates the crucial role of cell–cell contact in RANKL-induced osteoclast activation. Besides, NF-κB is constitutionally upregulated in myeloma cells. The newly designed and for the treatment of relapsed multiple myeloma approved proteasome inhibitor bortezomib acts through NF-κB inhibition. The purpose of this study was to elucidate, if proteasome inhibitors may inhibit osteoclast differentiation and function by affecting the NF-κB pathway. The use of a drug inhibiting both tumor cell growth and osteoclast activity may effectively interrupt the vicious cycle and reduce complications that result from osteolytic bone disease.

First, we demonstrated that the applicated doses of proteasome inhibitors did not have any effect on cell viability. As shown in a previous study, the used dosages have not had any growth inhibitory effects on myeloma cell growth [22]. After 4 weeks of culture, we showed that even low concentrations of MG-132 and MG-262, 0.1 and 0.01 or 0.01 and 0.001 μM, respectively, could significantly reduce both osteoclast formation and osteoclast function, as reflected by the decreased number of multi-nucleated osteoclasts and resorption lacunae. The confluence was equal in treated and untreated samples, confirming that the observed effects were not induced by apoptosis and reduction in cell survival. The extent of inhibition of the bone resorption was even greater than the inhibition of osteoclast formation. This finding implicated that proteasome inhibitors act on osteoclast precursors to inhibit differentiation and may furthermore inhibit the activity of mature osteoclasts. Since TRAP was considered to be a reliable marker of osteoclast function, we evaluated if proteasome inhibitors could abrogate the TRAP activity in osteoclasts. In fact, the measurement from the culture supernatants revealed a significant reduction of TRAP activity. The residual TRAP activity was higher than the capability of osteoclasts to resorb bone, which indicates that not only TRAP, but also other lytic enzymes may be modulated by proteasome inhibitors. In addition, we examined the effects of proteasome inhibitors on NF-κB activation induced by RANKL. In all treatment schedules, a marked inhibition of NF-κB was found, whereas the extent of NF-κB inhibition correlated with the number of osteoclasts and resorption lacunae. Our observations indicate that low concentrations of proteasome inhibitors are capable of inhibiting the NF-κB activation in osteoclasts, reflected in diminished osteoclastic differentiation and bone resorption.

In conclusion, these findings are of emerging importance, since they demonstrate for the first time that proteasome inhibitors abrogate osteoclast differentiation and osteoclast function. Thus, proteasome inhibitors may not only be effective in the treatment of some types of cancer like multiple myeloma, but may particularly improve the outcome of cancer-induced bone disease.

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