The Proteasome Inhibitor Carfilzomib Suppresses Parathyroid Hormone-induced Osteoclastogenesis through a RANKL-mediated Signaling Pathway*

Received for publication, May 6, 2015, and in revised form, May 15, 2015. Published, JBC Papers in Press, May 15, 2015, DOI 10.1074/jbc.M115.663963

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Parathyroid hormone (PTH) induces osteoclast formation and activity by increasing the ratio of RANKL/OPG in osteoblasts. The proteasome inhibitor carfilzomib (CFZ) has been used as an effective therapy for multiple myeloma via the inhibition of pathologic bone destruction. However, the effect of combination of PTH and CFZ on osteoclastogenesis is unknown. We now report that CFZ inhibits PTH-induced RANKL expression and secretion without affecting PTH inhibition of OPG expression, and it does so by blocking HDAC4 proteasomal degradation in osteoblasts. Furthermore, we used different types of culture systems, including co-culture, indirect co-culture, and transactivation, to assess the effect of CFZ on PTH action to induce osteoclastogenesis. Our results demonstrated that CFZ blocks PTH-induced osteoclast formation and bone resorption by its additional effect to inhibit RANKL-mediated NF-κB degradation and NF-κB activation in osteoclasts. This study showed for the first time that CFZ targets both osteoblasts and osteoclasts to suppress PTH-induced osteoclast differentiation and bone resorption. These findings warrant further investigation of this novel combination in animal models of osteoporosis and in patients.

Background: PTH induces RANKL expression in osteoblasts, and proteasome inhibitors suppress bone resorption.

Results: Carfilzomib inhibited PTH-induced RANKL expression in osteoblasts to inhibit osteoclast activity.

Conclusion: Carfilzomib inhibits PTH-induced osteoclastogenesis.

Significance: Carfilzomib can improve the therapeutic efficacy of PTH by mitigating the catabolic effects of PTH.

Osteoporosis results from a disruption of the balance between osteoblastic bone formation and osteoclastic bone resorption. Intermittent administration of parathyroid hormone (PTH) increases bone formation, whereas continuous infusion of PTH causes bone resorption (1, 2). However, the molecular and cellular mechanisms underlying these effects are poorly understood. The type 1 parathyroid hormone receptor (PTHr), a member of the G protein-coupled receptor superfamily, mediates PTH actions to maintain calcium homeostasis and bone remodeling (3). One established mechanism by which PTH exerts its effects on bone involves the induction of RANKL. PTH binds to PTHR on stromal/osteoblastic cells to effect signaling that induces the expression and secretion of RANKL. Osteoclasts do not express PTHR, yet PTH can regulate osteoclast formation and activity via the induction of RANKL in osteoblasts. RANKL binds to its receptor RANK at the surface of the osteoclast precursor cells to mediate RANK downstream signaling and cause osteoclast formation and bone resorption. Although recombinant PTH1–34 (teriparatide) is currently used as an anabolic agent for the treatment of osteoporosis, the administration of dosing and daily subcutaneous injection is problematic. PTH treatment can cause an increase in serum calcium and hypercalcemia in some osteoporosis patients, which can prompt cessation of PTH treatment (4, 5).

A better understanding of those mechanisms mediating the anabolic and catabolic effects of PTH could overcome the current limitations of PTH-based treatment of osteoporosis. Recent studies suggest that the ubiquitin-proteasome pathway plays an important role in regulating and controlling bone metabolism (6, 7). The first generation proteasome inhibitor bortezomib has been used as an effective therapy for the treatment of multiple myeloma, a disease characterized by an increase in the numbers and activity of osteoclasts and a decrease in the number and function of osteoblasts adjacent to tumor cells in the bone marrow (8). Carfilzomib (CFZ), a next generation selective proteasome inhibitor, exhibits potent anti-myeloma efficacy and decreased toxicity when compared with bortezomib and has been recently approved in the United States for the treatment of relapsed and refractory multiple myeloma (9). Both bortezomib and CFZ have been shown to directly inhibit osteoclast formation and bone resorption *in vitro* (10, 11), and bortezomib was reported to inhibit PTH-
induced Rankl mRNA expression in osteoblasts (12). However, how PTH and proteosomal inhibitors collectively regulate the complex interplay between osteoblasts and osteoclasts to in turn regulate bone resorption is poorly understood.

In the present study, we demonstrate that CFZ blocks PTH-induced proteosomal degradation of HDAC4 (histone deacetylase 4) and reduces RANKL expression and production in osteoblasts. In addition, we employed osteoblast/osteoclast co-culture and other cell models to elucidate the mechanisms by which CFZ reduces both PTH-induced osteoclast differentiation and resorptinal activity. These findings suggest that CFZ can be employed as a means to improve the therapeutic efficacy of PTH by mitigating the catabolic effects of PTH.

Experimental Procedures

Materials—CFZ was purchased from LA Laboratories (Woburn, MA), prepared in a 10 mM stock solution in DMSO, and diluted in media just prior to use. Human PTH (1–34) was purchased from Bachem (Torrance, CA). Protease inhibitor mixture set I and H89 were from Calbiochem. HDAC4 polyclonal antibody, 1eB-α polyclonal antibody, ubiquitonal monoclonal antibody, actin polyclonal antibody, HDAC4 siRNA, and scrambled nontargeting siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TRizol, DNase, Lipofectamine 2000, and α-minimum essential medium (α-MEM) were from Invitrogen. AccuScript high fidelity first strand cDNA synthesis kit was from Stratagene (La Jolla, CA). iTagTM SYBR Green Supermix with ROX was from Bio-Rad. Bovine cDNA synthesis kit was from Stratagene (La Jolla, CA). Opg/16919 and Opg-1 luc, and different concentrations of CFZ were added to UAMS-32P cell culture. One-half of the medium in each cavity was replaced with fresh medium including PTH and CFZ every 2 days. After 6 days, the viability of cells in both upper and bottom layer and the osteoclast formation in the bottom layer were assessed.

Separated Co-cultures—Nonadherent bone marrow cells were seeded on the bottom layer of a 24-well plate at a density of 2 × 10^4 cells/cm² and UAMS-32P cells were placed into a Transwell insert at a density of 5 × 10^3 cells/cm² (18). Vehicle, PTH (10 nM), and different concentrations of CFZ were added to UAMS-32P cell culture. One-half of the medium in each cavity was replaced with fresh medium including PTH and CFZ every 2 days. After 6 days, the viability of cells in both upper and bottom layer and the osteoclast formation in the bottom layer were assessed.

Cell Viability Assay—3-(4,5-Dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) was added to each well at a final concentration of 500 μg/ml. The cells were further incubated for 1 h at 37 °C in 5% CO₂ atmosphere, and the liquid in the wells was removed thereafter. DMSO was then added to each well, and the absorbance was measured at 570 nm (17).

Rankl and Osteoprotegerin (Opg) Promoter Activity Assay—UAMS-32P cells in 6-well plates were transiently transfected with 2 μg of Rankl or Opg promoter construct linked to luciferase cDNA (pGL3-basic-Rankl-luc and pGL3-basic-Opg-luc, kindly provided by Dr. Gerard Karsenty (Columbia University, New York, NY) and by Dr. Malayannan Subramaniam (Mayo Clinic, Rochester, MN), respectively) using Lipofectamine 2000 as described previously (19, 20). The cells were then incubated for 36 h at 37 °C in 5% CO₂. After the media were changed to 0.1% FBS, the transfected cells were cultured with vehicle, PTH (10 nM), and different concentrations of CFZ for another 16 h. Luciferase activity from the cell extracts was assayed by the chemiluminescence according to the instructions of the manufacturer (Promega, Madison, WI).

Preparation of Conditioned Media—UAMS-32P cells were passaged onto 10-cm dishes and grown to confluence. The cells were treated with vehicle, PTH (10 nM), and the indicated CFZ...
concentrations for 72 h at 37 °C in 5% CO₂ (21). Ten ml of supernatants were collected and concentrated using a 10,000-Da molecular mass cutoff Amicon centrifugal filter (Millipore, Bedford, MA) by centrifugation at 4,000 rpm for 8 min. The concentrated conditioned media (1 ml) were applied to nonadherent bone marrow cells on 6-well plates with 1 ml of fresh medium. After 3 days, both IκB-α expression and NF-κB activity were detected as described below.

Determination of Soluble RANKL in Conditioned Media—Soluble RANKL was quantified in conditioned media using the R&D System ELISA kits MTR00 according to the protocols of the manufacturers (R&D System, Minneapolis, MN). Briefly, 50 μl of supernatant or RANKL standards was added to the provided 96-well plate coated with a specific RANKL polyclonal antibody. A HRP-conjugated secondary antibody allowed a sensitive colorimetric readout. The RANKL content in each sample was analyzed using the RANKL standard curve.

NF-κB Activity Assay—Nuclear extracts from the nonadherent bone marrow cells treated with conditioned media were prepared using the nuclear extract kit (Active Motif). Total protein amounts were measured by BCA assay (Pierce). NF-κB activity was measured using the TransAM NF-κB p65 assay kit according to the manufacturer’s recommendations. Briefly, 5 μg of nuclear extract per sample was added to the 96-well plate, in which oligonucleotide containing the NF-κB consensus site (5’-GGGACCTTTCC-3’) was immobilized. The active form of NF-κB bound to the oligonucleotide was detected using an antibody against NF-κB p65 subunit. An HRP-conjugated secondary antibody provided a sensitive colorimetric readout that was quantified by spectrophotometry.

siRNA-mediated Knockdown of Hdac4—To knockdown Hdac4 in UAMS-32P cells, siRNA duplexes targeting the mouse Hdac4 were used for transient transfection, and scrambled nontargeting siRNA was used as a control (22). The UAMS-32P cells were seeded in 6-well plates and grown to 80% confluence, and Hdac4 siRNA and scrambled siRNA were transfected using Lipofectamine 2000 according to the manufacturer’s instructions. 36 h after transfection, these cells were treated with vehicle, PTH, and CFZ as before, and the Rankl promoter activity and HDAC4 protein level were then detected.

mRNA Abundance Quantification Using Quantitative Real Time PCR—RNA from primary calvarial osteoblasts or UAMS-32P cells was extracted with phenol and guanidine isothiocyanate (TRIzol), treated with DNase, converted to cDNA using the Accuscript high fidelity first strand cDNA synthesis kit, and subjected to quantitative real time PCR. The primers utilized for assessing expression of Rankl and Opg mRNA abundance are listed in Table 1. Aliquots of first strand cDNA were amplified using iTaqTM SYBR Green Supermix with ROX under the following conditions: initial denaturation for 10 min at 94 °C, followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C, followed by melting curve analysis. The mRNA expression levels of the target gene were normalized to β-actin mRNA. The data are presented as fold induction.

Immunoprecipitation and Immunoblot Analysis—UAMS-32P cells were lysed with radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl) supplemented with protease inhibitor mixture I. To detect HDAC4 ubiquitination, 10 mM N-ethylmaleimide, which inhibits deubiquitinase activity, was added to the lysis buffer and incubated with lyses for 30 min on ice. Solubilized materials were incubated with HDAC4 polyclonal antibody for 1 h at 4 °C, and then protein A-Sepharose 4B conjugate was added to each sample and incubated overnight at 4 °C. Total lyses and immunoprecipitated protein, eluted by the addition of SDS sample buffer, were analyzed by SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) using the semi-dry method (Bio-Rad). Membranes were blocked with 3% bovine serum albumin in TBST buffer at room temperature for 1 h and incubated with different antibodies (polyclonal anti-HDAC4 (1:1000), monoclonal anti-RANKL (1:1000), or polyclonal anti-actin (1:2000)) overnight at 4 °C. The membranes were then washed and incubated with IRDye 800CW goat anti-rabbit IgG or IRDye 680RD goat anti-mouse IgG at room temperature for 1 h. Band intensity was quantified using the Licor Odyssey system.

Statistical Analysis—The data are presented as the means ± S.E., where n indicates the number of independent experiments. Multiple comparisons were evaluated by analysis of variance with post-test repeated measures analyzed by the Bonferroni procedure (Prism; GraphPad). p values < 0.05 were considered sufficient to reject the null hypothesis.

Results

CFZ Inhibits PTH-induced Rankl Expression—PTH binds to PTHR on osteoblasts and enhances Rankl gene expression (23, 24). Because osteoclasts do not express PTHR, the effect of PTH on osteoclast formation and bone resorption is mediated by RANKL. To characterize the effects of CFZ on PTH-induced osteoclast differentiation and activity, we first investigated whether CFZ affects PTH-induced Rankl mRNA expression in primary osteoblasts. Mouse primary osteoblastic cells were treated with ascorbic acid (50 μg/ml) for 7 days to induce their differentiation (18). The cells were cultured in the medium with 0.1% FBS for 15 h and then treated with vehicle, PTH (10 nM), and CFZ (0–50 nM) for 4 h. PTH significantly increased Rankl mRNA expression (Fig. 1A). CFZ did not affect Rankl mRNA expression but inhibited the ability of PTH to up-regulate Rankl expression in a dose-dependent manner (Fig. 1A).

Osteoclast development depends strictly on the support provided by stromal/osteoblastic cells (23, 24). Previous studies demonstrated that PTH increases RANKL expression in the stromal/osteoblast cell line UAMS-32P (25, 26).

### Table 1

| Gene     | Forward            | Reverse           | Accession number |
|----------|--------------------|-------------------|------------------|
| Rankl    | 5'-CCCTCGAAGCTGTTGAA | 5'-CTTCACTCTGCACCTTCT | NM_016133.3     |
| Opg      | 5'-TGGTCTGGACCCCTGCAAA | 5'-AACAGCCGATGACCATCC | NM_008764.3     |
| β-Actin  | 5'-AGCCACGGTACCTGACCATCC | 5'-GTCACCTGCTGCTGGTGA | NM_007393      |

*Primer sequences for real time PCR*
Consistent with these findings and the observed effect of CFZ on primary osteoblasts, CFZ inhibited PTH-induced Rankl expression in UAMS-32P cells in a dose-dependent manner (Fig. 1A). OPG that is recognized as a decoy receptor for RANKL negatively regulates the effect of RANKL on osteoclastogenesis (25, 27). Consistent with these reports, PTH inhibited Opg mRNA expression in primary osteoblasts and UAMS-32P cells (Fig. 1B). However, CFZ failed to reverse the inhibition of Opg promoter activity by PTH. The data are summarized as the means ± S.E. of four independent experiments. a, p < 0.05, compared with vehicle control; b, p < 0.05, compared with PTH group.

Effects of CFZ on Rankl and Opg Promoter Activity—To assess whether CFZ inhibits PTH-induced transcription of the Rankl gene, UAMS-32P cells were transfected with a promoter construct in which luciferase is driven by 3 kb of the Rankl promoter (19). PTH (10 nM) markedly increased Rankl promoter activity as shown in Fig. 2A. CFZ suppressed PTH-induced Rankl promoter activity in a concentration-dependent manner. Consistent with its effect on PTH-induced Opg mRNA expression, CFZ had no effect on PTH inhibition of Opg promoter activity (Fig. 2B). For the rest of this research, we there-
fore sought to determine how CFZ affects PTH-induced Rankl expression and its downstream signaling on osteoclast formation and activity.

**CFZ Inhibits PTH-induced Rankl Promoter Activity by Blocking HDAC4 Degradation**—PTH is known to increase Rankl expression on osteoblasts in a cAMP-dependent manner (23, 24). In UAMS-32P cells, PTH, as well as forskolin, which stimulates adenyl cyclase activity to generate intracellular cAMP, dramatically increased Rankl promoter activity (Fig. 3A). Addition of the PKA inhibitor H89 or CFZ blocked the effects of both PTH and forskolin on Rankl promoter activity, indicating that CFZ inhibits PTH-induced Rankl promoter activity through cAMP/PKA signaling pathway.

Obri et al. (12) recently reported that HDAC4 inhibits Rankl expression in osteoblasts and cAMP/PKA signaling activates Smurf2 (Smad ubiquitin regulatory factor 2), an E3 ubiquitin ligase, which mediates HDAC4 degradation via the ubiquitin-proteasome pathway. In addition, bortezomib blocked PTH-induced HDAC4 degradation in osteoblasts. Similarly, we demonstrated that CFZ, in a concentration-dependent manner, increased accumulation of ubiquitinated HDAC4 protein in the presence of PTH (Fig. 3B). PTH-induced HDAC4 degradation...
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CFZ Blocks PTH-induced Osteoclast Formation and Resorptive Activity—Osteoclast differentiation requires the support provided by the contact with osteoblasts/bone marrow stromal cells or secretion of their products, such as RANKL (23, 24). To determine the effects of CFZ on PTH-induced osteoclast formation and bone resorption by activation of osteoblasts, we used co-culture of UAMS-32P cells and osteoclast precursor cells. The effects of CFZ on PTH-induced osteoclast formation were investigated first. Vehicle, PTH (10 nM), and different concentrations of CFZ were added to the 24-well plates as indicated. One-half volume of media was changed every other day. Osteoclast formation was detected by TRAP staining on day 7. PTH treatment significantly augmented osteoclast formation with mature osteoclasts identified as large cells, containing more than two nuclei and positive for TRAP staining (Fig. 5, A–C). In addition, PTH failed to increase osteoclast formation in cell cultures that only had UAMS-32P cells (Fig. 5D), suggesting that no osteoclast precursors existed in UAMS-32P cell line. Whereas a low concentration (3.2 nM) of CFZ only modestly reduced osteoclast formation, a higher concentration (12.5 nM) of CFZ significantly inhibited osteoclast formation by 80%. We then examined the effects of CFZ on PTH-induced osteoclastic bone resorption. UAMS-32P cells and nonadherent bone marrow cells were seeded on the bone slice into a 96-well plate. Vehicle, PTH (10 nm), and different concentrations of CFZ were added to the wells as before. Bone resorption pit formation was measured on day 7. Consistent with osteoclast formation, PTH treatment significantly increased bone resorption (Fig. 5, E and F). CFZ concentration-dependently suppressed osteoclast activity.

CFZ Suppresses Osteoclastogenesis without Inducing Cytotoxicity—To exclude the possibility that CFZ induces cytotoxicity in UAMS-32P cells or osteoclast precursor cells, we performed indirect co-culture of UAMS-32P cells and nonadherent bone marrow cells. We placed nonadherent bone marrow cells into the bottom layer and seeded UAMS-32P cells on the cell culture insert. This indirect co-culture system spatially separates the nonadherent marrow cells and UAMS-32P cells but allows exchange of soluble factors. Vehicle, PTH, and CFZ were added to the UAMS-32P cell culture. One-half of the medium was changed every 2 days. The cell viability on both bottom and insert layer was assessed by MTT assay on day 7. Results in Fig. 6A demonstrated that CFZ (3.2 and 12.5 nM) used in the osteoclastogenesis studies did not cause cytotoxicity in either UAMS-32P cells or nonadherent bone marrow cells. Previous reports suggest that the separation of osteoblasts from osteoclast precursors in co-cultures can induce osteoclast differentiation (18). Similarly, we observed that PTH treatment increased the formation of mature osteoclasts in the separated co-culture (Fig. 6, B and C). CFZ (12.5 nM) decreased PTH-induced osteoclastogenesis. Collectively, these data clearly show that the inhibitory effects on PTH-induced osteoclastogenesis by CFZ are not caused by cytotoxicity.

Effects of CFZ on PTH-Induced RANKL/Wb/NF-κB Signaling in Osteoclasts—RANKL binds RANK on osteoclast precursor cells to activate its downstream signaling cascade. The ubiquitination of several proteins including tumor necrosis factor receptor-associated factor 6 (TRAF6) and IκB in osteoclasts was blocked by CFZ in UAMS-32P cells (Fig. 3C). We further demonstrated that knockdown of Hdac4 with siRNA (~88% reduction) eliminated the effect of CFZ inhibition of PTH-induced Rankl promoter activity (Fig. 3, D–F).

**CFZ Inhibits PTH-induced Soluble RANKL Production**—RANKL exists as either a membrane-bound cytokine or a soluble factor secreted by stromal/osteoblastic cells. We then hypothesized that CFZ regulates PTH-induced soluble RANKL production. The data in Fig. 4A showed that PTH significantly induced soluble RANKL protein levels assayed in supernatants of UAMS-32P cells. Indeed, CFZ inhibited this induction in a concentration-dependent manner, with the maximal inhibition reaching 46%. Knockdown of Hdac4 with siRNA abolished the effect of CFZ inhibition of PTH-induced RANKL production (Fig. 4B). Collectively, these data strongly indicate CFZ inhibits PTH-induced RANKL expression and production by blocking HDAC4 degradation through the ubiquitin-proteasome pathway.

**FIGURE 4.** CFZ suppresses PTH-induced soluble RANKL production in UAMS32P cells. A, CFZ inhibits PTH-induced RANKL production in a concentration-dependent manner. UAMS-32P cells were treated with vehicle, PTH (10 nM), and the indicated concentrations of CFZ for 3 days. The cell supernatants were collected, and the RANKL protein level was detected by ELISA. The data are summarized as the means ± S.E. of four independent experiments. a, p < 0.05, compared with vehicle control; b, p < 0.05, compared with PTH group. B, knockdown of Hdac4 abolishes CFZ inhibition of PTH-induced RANKL production. The data are summarized as the means ± S.E. of four independent experiments. a, p < 0.01, compared with scrambled siRNA control; b, p < 0.05, compared with PTH plus scrambled siRNA; c, p < 0.05, compared with Hdac4 siRNA group.
leads to NF-κB activation, which is required for the induction of osteoclastogenesis (7, 28). The outcome results in enhancing osteoclast differentiation and function. Whereas CFZ inhibited PTH-induced soluble RANKL production in UAMS-32P cells by ~46% (Fig. 4), a much greater inhibition (up to 80%) of PTH-induced osteoclast formation and bone resorption was observed (Figs. 5 and 6). These findings suggest an additional mechanism whereby CFZ inhibits osteoclast formation and bone resorption. To test this idea, we collected conditioned media from the supernatants in UAMS-32P cells treated with vehicle, PTH, and CFZ. The 10 ml of conditioned media were concentrated to 1 ml using a 10,000-Da molecular mass cutoff Amicon centrifugal filter device by centrifugation. This procedure increased soluble RANKL (>20 kDa) levels and reduced CFZ and PTH(1–34) (both < 5 kDa) amounts in the concentrated conditioned media (Fig. 7A). Stimulation of nonadherent bone marrow cells with the concentrated medium from UAMS-32P cells treated with both PTH and CFZ markedly reduced 1κB-α degradation and decreased NF-κB activation (Fig. 7B–E, group 3).
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Discussion

Bone formation by osteoblasts and bone resorption by osteoclasts are tightly coupled processes, and their balance controls bone mass. Previous studies have shown that unlike osteoblasts, osteoclasts do not express PTHR, and the effects of PTH on osteoclasts are secondary to PTH effects on stromal/osteoblastic cells (23, 24). Recent findings have indicated that the magnitude of osteoclast formation is directly proportional to the level of RANKL secretion by osteoblasts (25, 26). Thus, the modulation of RANKL expression or activity represents a means of reducing the catabolic effect of PTH on bone.

In the present study, we explored the effects of CFZ on PTH-induced RANKL expression and its downstream signaling to activate osteoclast formation and bone resorption. We first tested whether CFZ affects PTH-induced Rankl mRNA expression in primary osteoblasts and UAMS-32P cells, a stromal/osteoblastic cell line. We demonstrated that CFZ similarly inhibits PTH-induced Rankl gene expression, without affecting PTH inhibition of Opg expression and its promoter activity, in both cell cultures (Figs. 1 and 2). Several proteins that are involved in osteoblast differentiation and formation are degraded through the ubiquitin-proteasome pathway (29–31).

In particular, PTH promotes proteasomal degradation of Runx2 (runt-related transcription factor 2) to limit the anti-apoptotic effect of Runx2 in osteoblasts (29). Runx2 is not required for PTH regulation of RANKL, although it is capable of binding to the Rankl gene promoter (32–34). The Wnt/β-catenin pathway contributes to PTH action on bone formation (35–37), and the ubiquitin-dependent degradation of β-catenin is blocked by CFZ (6). E3 ubiquitin ligase Smurf1 negatively regulates osteoblast differentiation and proliferation by controlling MEKK2 and JunB protein stability through the ubiquitin-proteasome pathway (31, 38).

The discovery of proteasomal degradation of HDAC4 provided a novel mechanism to explain how PTHR activation stimulates RANKL expression (39, 40). Rankl promoter has three binding sites of transcription factor MEF2c. HDAC4 interacts with MEF2c and inhibits Rankl expression (12, 41). Obri et al. (12) reported that PTH induces HDAC4 ubiquitination by E3 ubiquitin ligase Smurf2, which can release MEF2c from Rankl promoter and activate Rankl expression. We demonstrated that CFZ concentration-dependently increases accumulation of ubiquitinated HDAC4 protein in the presence of PTH (Fig. 3). Knockdown of Hdac4 eliminates the CFZ inhibition of PTH-induced Rankl expression. Therefore, CFZ inhibits PTH-induced Rankl expression by blocking ubiquitin-dependent degradation of HDAC4 in stromal/osteoblastic cells.

Osteoblasts stimulate osteoclast differentiation and activation through their contacts with osteoclast precursor cells. We performed different cell culture systems to characterize how CFZ affects PTH-induced osteoclast formation and resorptive activity. First, to determine the effects of CFZ on PTH-induced osteoclast formation and bone resorption by activation of osteoblasts, we used co-culture of UAMS-32P cells and osteoclast precursors and seeded these cells on the bone slice. Our data indicated that CFZ at a concentration of 12.5 nM significantly inhibits osteoclast formation and activity by up to 80%.
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(Fig. 5). However, CFZ inhibited PTH-stimulated RANKL production at concentrations of 12.5 nM by up to 50% (Fig. 4). These findings raise two possibilities: that CFZ has cytotoxicity after culture with cells for 6 days or that an additional mechanism inhibits PTH-induced RANKL downstream signaling in osteoclasts. To exclude the possibility that CFZ caused cytotoxicity on UAMS-32P cells or nonadherent bone marrow cells, we performed the indirect co-culture to separate the contact of these two types of cells, allowing the exchange of soluble factors secreted from cells. Our results showed that the concentrations of CFZ used for osteoclastogenesis studies did not induce cytotoxicity in both cell types (Fig. 6). We also displayed that the separated co-culture of these cells can induce osteoclast differentiation in the presence of PTH (18) and that this osteoclast formation was inhibited by CFZ. These findings suggest there is an additional CFZ effect to inhibit PTH-induced RANKL downstream signaling in osteoclasts.

The ubiquitination of TRAF6, the crucial adaptor molecule of RANK is required for the induction of NF-κB activation and osteoclastogenesis (28) (Fig. 8). Activation of the IκB kinase complex results in the phosphorylation and subsequent proteasomal degradation of IκB-α. NF-κB proteins are then released and translocated from the cytoplasm to the nucleus, thereby promoting gene transcription to induce osteoclast differentiation (7, 28) (Fig. 8). To elucidate whether CFZ directly inhibits PTH-induced RANKL downstream signaling in osteoclasts, we utilized concentrated, conditioned media from the supernatants in UAMS-32P cells treated with vehicle, PTH, and CFZ, which generates media with increased soluble RANKL and reduced CFZ and PTH(1–34) levels. The IκB-α expression and nuclear NF-κB activity were then measured in each group. Our data demonstrated that the concentrated conditioned media from UAMS-32P cells treated with PTH promotes IκB-α degradation and increases NF-κB activity in nonadherent bone marrow cells (Fig. 7, group 2). The conditioned medium from UAMS-32P cells treated with both PTH and CFZ markedly attenuated IκB-α degradation and decreased NF-κB activity.

Because nonadherent bone marrow cells do not express PTHR, as expected, direct addition of PTH to nonadherent bone marrow cells did not affect IκB-α degradation or stimulate NF-κB activity. Because nonadherent bone marrow cells do not express PTHR, as expected, direct addition of PTH to nonadherent bone marrow cells did not affect IκB-α degradation or stimulate NF-κB activity. Because nonadherent bone marrow cells do not express PTHR, as expected, direct addition of PTH to nonadherent bone marrow cells did not affect IκB-α degradation or stimulate NF-κB activity. Because nonadherent bone marrow cells do not express PTHR, as expected, direct addition of PTH to nonadherent bone marrow cells did not affect IκB-α degradation or stimulate NF-κB activity.

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media, and in turn, stimulates osteoclastogenesis in nonadherent bone marrow cells. Therefore, we propose that CFZ directly inhibits RANKL-induced NF-κB activity by blocking the proteasomal degradation of the proteins, which are upstream effectors of NF-κB in osteoclasts (Fig. 8). Although other proteasome inhibitors block the degradation of TRAF6 (6, 7), further studies will be necessary to confirm the view that CFZ suppresses proteasomal degradation of TRAF6 proteins in osteoclasts caused by PTH-induced RANKL downstream signaling.

In conclusion, we demonstrate that CFZ inhibits PTH-induced RANKL expression and its indirect effect on osteoclastogenesis by blocking proteasomal degradation of HDAC4 in osteoblasts and NF-κB activity in osteoclasts, respectively (Fig. 8). These findings indicate that CFZ may reduce the catabolic effect of PTH on bone. Whereas previous studies have emphasized the therapeutic benefit of bortezomib and CFZ in treating both the tumor burden and bone loss associated with multiple myeloma, the present study asserts a clear utility for CFZ as an adjunct in the treatment of osteoporosis and other bone resorptive diseases with PTH.

Author Contributions—B. W. designed the study and wrote the paper. Y. Y. and B. W. performed and analyzed the experiments. H. C. B. provided technical assistance and contributed to the preparation of the figures. H. C. B. and I. M. S. reviewed the results and discussed the manuscript. All authors approved the final version of the manuscript.

Acknowledgment—We thank Dr. Raymond B. Penn for advice and help in the completion of this work.

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