

Suppression by HSP90 inhibitors of BMP-4-stimulated osteoprotegerin synthesis in osteoblasts: Attenuation of p70 S6 kinase

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Abstract. Heat shock protein 90 (HSP90) is an ATP-dependent ubiquitous molecular chaperon which is important in cell homeostasis. The authors previously demonstrated that bone morphogenetic protein (BMP)-4 stimulates osteoprotegerin (OPG) production in osteoblast-like MC3T3-E1 cells, and that p70 S6 kinase positively regulates the OPG synthesis by BMP-4. The present study investigated the involvement of HSP90 in the BMP-4-stimulated OPG synthesis and the mechanism in MC3T3-E1 cells. HSP90 inhibitors, 17-allylamino-17-demethoxy-geldanamycin (17-AAG), 17-dimethylamino-ethylamino-17-demethoxy-geldanamycin (17-DMAG) and geldanamycin significantly suppressed the BMP-4-stimulated OPG release. Geldanamycin markedly reduced the BMP-4-induced mRNA expression of OPG. 17-AAG and 17-DMAG significantly attenuated the phosphorylation of p70 S6 kinase induced by BMP-4 without affecting the BMP-4-induced phosphorylation of mothers against decapentaplegic homolog 1/5. The results suggest that HSP90 inhibitors suppress the BMP-4-stimulated OPG synthesis in osteoblasts, and that their suppressive effects are exerted through downregulating p70 S6 kinase.

Introduction

Bone metabolism is strictly coordinated by two functional cells, osteoclasts and osteoblasts (1). The former cells are

responsible for bone resorption and the latter cells are for bone formation (1). Bone tissue in the skeleton is continuously regenerated and renewed to maintain the quality and quantity through the bone remodeling process (2). The remodeling process begins with osteoclastic bone resorption, followed by osteoblastic bone formation (3). The imbalance of bone remodeling causes metabolic bone diseases such as osteoporosis and the increased risk of age-related bone fracture.

Currently, it is well recognized that osteoblasts play a crucial role in regulating bone resorption via the expression of receptor activator of nuclear factor- κ B (RANK) ligand (RANKL), which responds to a variety of bone resorptive agents (1-3). Osteoprotegerin (OPG), which is synthesized in osteoblasts and secreted, belongs to the tumor necrosis factor receptor family as well as RANK on osteoclasts (4). OPG binds to RANKL as a decoy receptor, and prevents RANKL from binding to RANK, resulting in the suppression of bone resorption via inhibiting osteoclastogenesis (4). OPG-knock out mice reportedly suffer from severe osteoporosis (5). Therefore, it is currently recognized that the RANK/RANKL/OPG axis plays a central regulatory system in osteoclast functions (6). It has been shown that bone morphogenetic protein (BMP)-2 stimulates OPG production in human osteoblastic cell line (7). BMPs, multifunctional cytokines, belong to the transforming growth factor- β (TGF- β) superfamily (8). Regarding the intracellular signaling of BMPs, it is firmly established that the effects of BMPs are exerted mainly through the Smad-dependent pathway (8). In addition, accumulating evidence indicates that the Smad-independent pathway mediates numerous effects of BMP (9). We have recently shown that BMP-4 stimulates the synthesis of OPG at least in part through the activation of p70 S6 kinase in osteoblast-like MC3T3-E1 cells (10). However, the exact mechanism behind the BMP-induced OPG synthesis in osteoblasts has not yet been clarified.

Heat shock proteins (HSPs) are induced in response to various environmental stress such as heat (11). HSPs play an essential role as molecular chaperones in protein folding and the prevention of aggregation. Among them, HSP90 (also

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known as HSPC) is abundantly expressed in a variety type of unstressed cells and represents 1-2% of total cellular proteins, which increases to 4-6% under the stress conditions (12). Since client proteins of HSP90 are involved in a variety of oncogenic signaling pathways, HSP90 inhibition has emerged as one of the strategies for anticancer chemotherapeutics, and HSP90 inhibitors including 17-allylamino-17-demethoxy-geldanamycin (17-AAG), 17-dimethylamino-ethylamino-17-demethoxy-geldanamycin (17-DMAG) and geldanamycin, are developed (13-18). With regard to HSP90 inhibitor-effects on bone metabolism, 17-AAG reportedly amplifies osteoclast formation and potentiates osteolytic bone metastasis in bone metastasis of breast cancer cells (19). In addition, it has been shown that geldanamycin induces autophagy and apoptosis of osteosarcoma cells (20). However, the exact roles of HSP90 in osteoblast functions remains to be elucidated.

In the present study, we investigated whether HSP90 is involved in the BMP-4-induced OPG synthesis in osteoblast-like MC3T3-E1 cells using HSP90 inhibitors. We herein demonstrate that HSP90 inhibitors suppress the BMP-4-stimulated OPG synthesis through downregulating p70 S6 kinase in osteoblasts.

Materials and methods

Materials. 17-AAG and 17-DMAG were purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). Geldanamycin was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). BMP-4 and mouse OPG enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Phospho-specific Smad1/5 antibodies, phospho-specific p70 S6 kinase antibodies and p70 S6 kinase antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An ECL Western blotting detection system was obtained from GE Healthcare Life Sciences (Chalfont, UK). Other materials and chemicals were obtained from commercial sources. 17-AAG, 17-DMAG and geldanamycin were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for OPG, real-time RT-PCR or western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells that have been derived from newborn mouse calvaria (21) were maintained as previously described (22). Briefly, the cells were cultured in α -minimum essential medium (α -MEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂/95% air. The cells were seeded into 35-mm diameter dishes (5x10⁴ cells/dish) or 90-mm diameter dishes (2x10⁵ cells/dish) in α -MEM containing 10% FBS. After 5 days, the medium was exchanged for α -MEM containing 0.3% FBS. The cells were used for experiments after 48 h.

Measurement of OPG. The cultured cells were stimulated by 30 ng/ml of BMP-4 or vehicle in 1 ml of α -MEM containing 0.3% FBS for 48 h. When indicated, the cells were pretreated with various doses of 17-AAG, 17-DMAG or geldanamycin for 60 min. The conditioned medium was collected at the end of

incubation, and the OPG concentration was then measured using the OPG ELISA kit according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cultured cells were pretreated with 0.3 μ M of geldanamycin or vehicle for 60 min, and then stimulated by 30 ng/ml of BMP-4 or vehicle in 1 ml of α -MEM containing 0.3% FBS for 6 h. Total RNA was isolated and reverse transcribed into complementary DNA using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Heysham, Lancashire, UK) and Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA, USA), respectively. RT-qPCR was performed in capillaries using a Light Cycler system with the Light Cycler Fast Start DNA Master SYBR-Green I (Roche Diagnostics, Basel, Switzerland). Sense and antisense primers for mouse OPG mRNA or GAPDH mRNA were purchased from Takara Bio, Inc. (Tokyo, Japan; primer set ID, MA026526). The amplified products were determined by melting curve analysis and agarose electrophoresis. The OPG mRNA levels were normalized to those of GAPDH mRNA.

Western blot analysis. The cultured cells were pretreated with various doses of 17-AAG or 17-DMAG for 60 min, and then stimulated by 30 ng/ml of BMP-4 or vehicle in 1 ml α -MEM containing 0.3% FBS for the indicated periods. The cells were washed twice with phosphate-buffered saline, and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (23) in 10% polyacrylamide gels. The protein was fractionated and transferred onto an Immun-Blot PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween (TBS-T; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 h before incubation with primary antibodies. A western blot analysis was performed as described previously (24) using antibodies against phospho-specific Smad1/5 antibodies, GAPDH, phospho-specific p70 S6 kinase antibodies or p70 S6 kinase as primary antibodies at a dilution of 1:1,000 in 5% milk in TBS-T overnight at 4°C. Peroxidase-labeled antibodies raised in goat against rabbit IgG (KPL, Inc., Gaithersburg, MD, USA) were used as secondary antibodies at a dilution of 1:1,000 in 5% milk in TBS-T for 1 h at room temperature. The peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

Densitometric analysis. A densitometric analysis of the western blots was performed using a scanner and image analysis software program (Image J, version 1.48; National Institutes of Health, Bethesda, MD, USA). The phosphorylated protein levels were calculated as follows: The background-subtracted signal intensity of each phosphorylation signal was respectively normalized to the total protein signal and plotted as the fold increase in comparison to that of the control cells treated without stimulation.

Statistical analysis. The data were analyzed by ANOVA followed by Bonferroni method for multiple comparisons

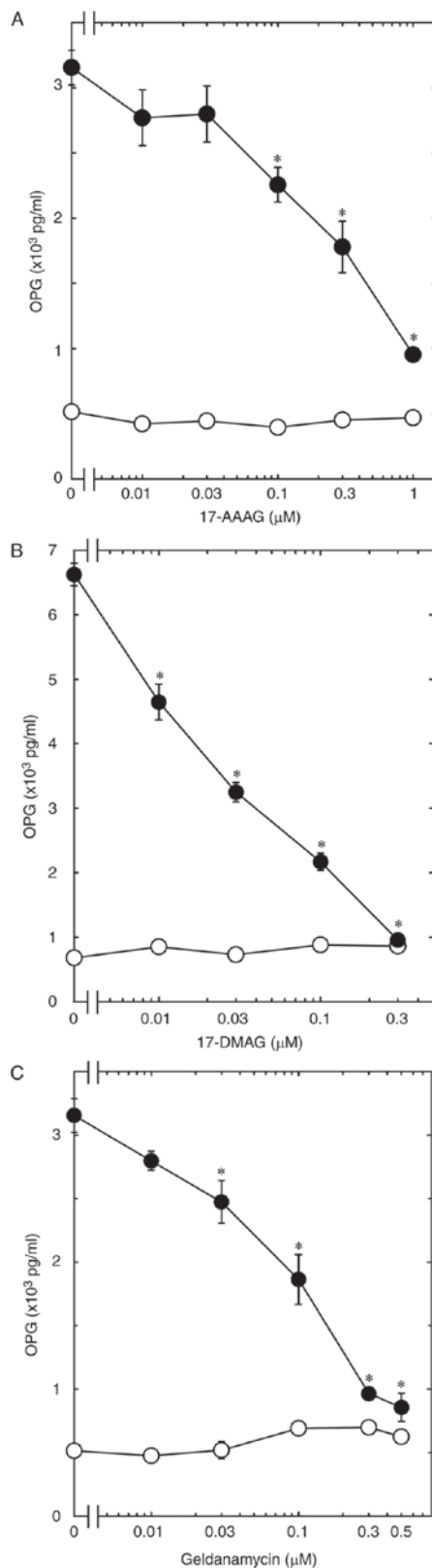


Figure 1. Effects of 17-AAG, 17-DMAG or geldanamycin on the BMP-4-stimulated release of OPG in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) 17-AAG, (B) 17-DMAG or (C) geldanamycin for 60 min, and then stimulated by 30 ng/ml of BMP-4 (●) or vehicle (○) for 48 h. The OPG concentrations in the conditioned medium were determined by ELISA. Each value represents the mean \pm SEM of triplicate determinations from three independent cell preparations. * $P < 0.05$, compared to the value of BMP-4 alone. 17-AAG, 17-allylamino-17-demethoxy-geldanamycin; 17-DMAG, 17-dimethylamino-ethylamino-17-demethoxy-geldanamycin; BMP, bone morphogenetic protein; OPG, osteoprotegerin; ELISA, enzyme-linked immunosorbent assay.

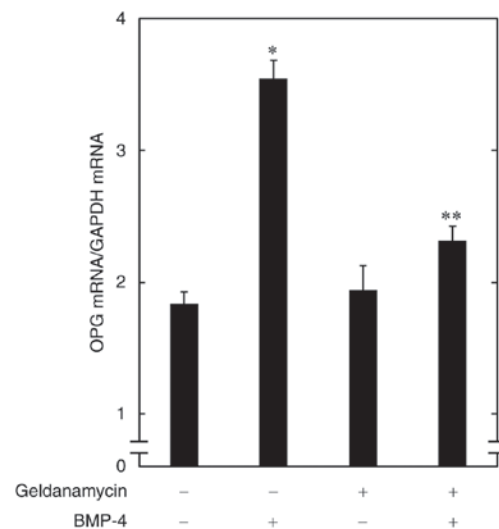


Figure 2. Effect of geldanamycin on the BMP-4-induced expression of OPG mRNA in MC3T3-E1 cells. The cultured cells were pretreated with 0.3 μ M of geldanamycin or vehicle for 60 min, and then stimulated by 30 ng/ml of BMP-4 or vehicle for 6 h. The respective total RNA was then isolated and transcribed into cDNA. The expressions of OPG mRNA and GAPDH mRNA were quantified by real-time RT-PCR. The OPG mRNA levels were normalized to those of GAPDH mRNA. Each value represents the mean \pm SEM of triplicate determinations from three independent cell preparations. * $P < 0.05$, compared to the value of control. ** $P < 0.05$, compared to the value of BMP-4 alone. BMP, bone morphogenetic protein; OPG, osteoprotegerin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

between pairs, and $P < 0.05$ was considered to be statistically significant. All data are presented as the mean \pm SEM of triplicate determinations from three independent cell preparations.

Results

Effects of 17-AAG, 17-DMAG or geldanamycin on the BMP-4-stimulated OPG release in MC3T3-E1 cells. In order to investigate the involvement of HSP90 in the BMP-4-induced synthesis of OPG in osteoblast-like MC3T3-E1 cells, we first examined the effects of 17-AAG (13), 17-DMAG (15) and geldanamycin (14), as HSP90 inhibitors, on the BMP-4-stimulated release of OPG. 17-AAG, which alone had little effect on the release, significantly reduced the BMP-4-stimulated OPG release in a dose-dependent manner over the range 0.01 and 1 μ M (Fig. 1A). The maximum effect of 17-AAG was observed at 1 μ M, which caused an approximately 80% decrease in the BMP-4-effect. In addition, 17-DMAG and geldanamycin as well as 17-AAG markedly suppressed the OPG release (Fig. 1B and C). The maximum effects of 17-DMAG and geldanamycin were observed at 0.3 and 0.5 μ M, respectively, which caused almost complete suppression in the BMP-4-effect.

Effect of geldanamycin on the BMP-4-induced expression levels of OPG mRNA in MC3T3-E1 cells. To clarify whether the inhibition by HSP90 inhibitors of the BMP-4-induced OPG release is mediated through transcriptional events, we examined the effect of geldanamycin on the OPG mRNA expression induced by BMP-4 in osteoblast-like MC3T3-E1 cells. Geldanamycin, which by itself had little effect on the basal levels, significantly suppressed the BMP-4-induced expression levels of OPG mRNA (Fig. 2).

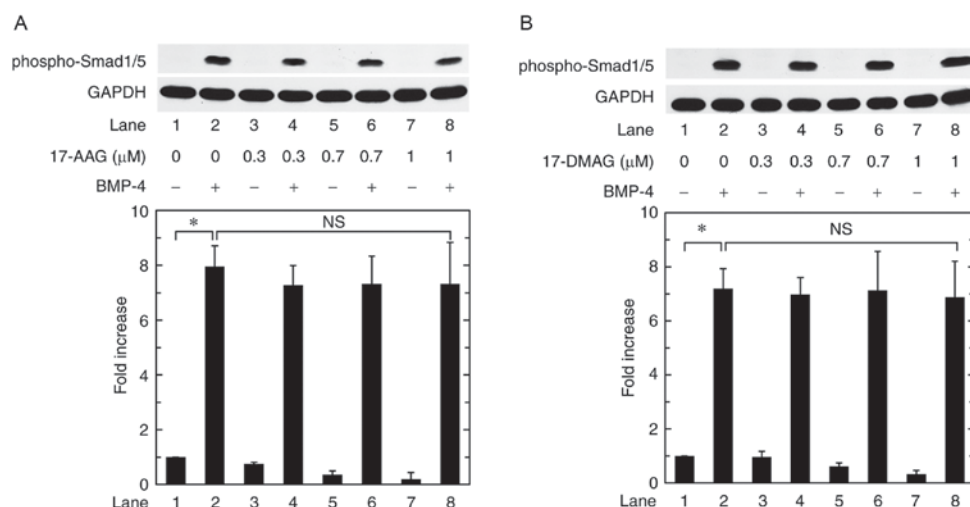


Figure 3. Effects of 17-AAG or 17-DMAG on the BMP-4-induced phosphorylation of Smad1/5 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) 17-AAG or (B) 17-DMAG for 60 min, and then stimulated by 30 ng/ml of BMP-4 or vehicle for 45 min. The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific Smad1/5 or GAPDH. The histogram shows the quantitative representations of the BMP-4-induced levels from laser densitometric analysis of three independent experiments. The density levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean \pm SEM of triplicate determinations from three independent cell preparations. * P <0.05, compared to the value of control. N.S. designates no significant difference between the indicated pairs. 17-AAG, 17-allylamino-17-demethoxy-geldanamycin; 17-DMAG, 17-dimethylamino-ethylamino-17-demethoxy-geldanamycin; BMP, bone morphogenetic protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

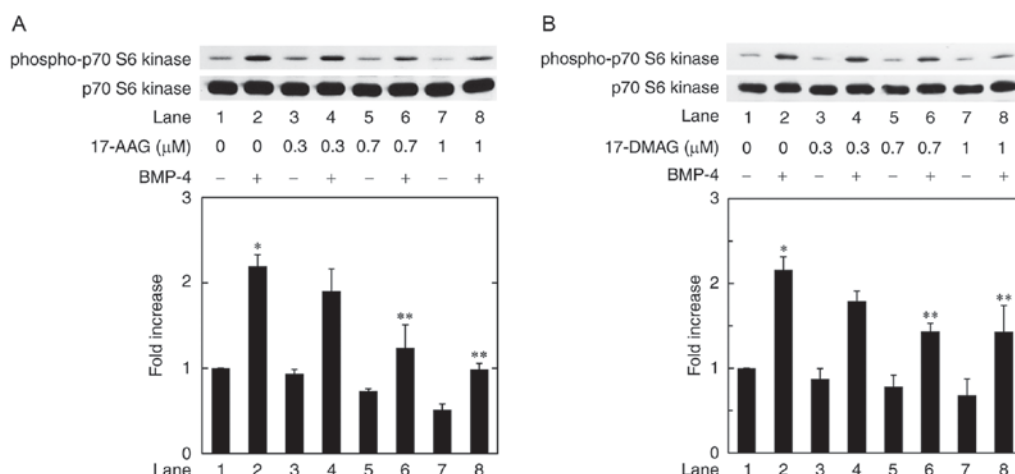


Figure 4. Effects of 17-AAG or 17-DMAG on the BMP-4-induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) 17-AAG or (B) 17-DMAG for 60 min, and then stimulated by 30 ng/ml of BMP-4 or vehicle for 120 min. The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p70 S6 kinase or p70 S6 kinase. The histogram shows the quantitative representations of the BMP-4-induced levels from laser densitometric analysis of three independent experiments. The density levels were expressed as the fold increase to the basal levels presented as lane 1. Each value represents the mean \pm SEM of triplicate determinations from three independent cell preparations. * P <0.05, compared to the value of control. ** P <0.05, compared to the value of BMP-4 alone. 17-AAG, 17-allylamino-17-demethoxy-geldanamycin; 17-DMAG, 17-dimethylamino-ethylamino-17-demethoxy-geldanamycin; BMP, bone morphogenetic protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Effects of 17-AAG or 17-DMAG on the BMP-4-induced phosphorylation of Smad1/5 in MC3T3-E1 cells. Regarding the intracellular signaling of BMPs, the Smad protein family such as Smad1, Smad5 and Smad8 plays an important role (8). Therefore, we examined the effect of 17-AAG or 17-DMAG on the BMP-4-induced phosphorylation of Smad1/5 in osteoblast-like MC3T3-E1 cells. However, neither 17-AAG nor 17-DMAG affected the BMP-4-induced phosphorylation of Smad1/5 up to 1 μ M (Fig. 3A and B).

Effects of 17-AAG or 17-DMAG on the BMP-4-induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. It is currently

recognized that not only the Smad-dependent pathway but also the Smad-independent pathways mediate the effects of BMPs (9). We have recently demonstrated that p70 S6 kinase functions as a positive regulator in the BMP-4-stimulated synthesis of OPG in osteoblast-like MC3T3-E1 cells (10). In order to investigate whether the activation of p70 S6 kinase is implicated in the HSP90 inhibitor-effect on the BMP-4-induced OPG synthesis in MC3T3-E1 cells, we examined the effect of 17-AAG on the BMP-4-induced phosphorylation of p70 S6 kinase. 17-AAG at 0.7 and 1.0 μ M significantly attenuated the BMP-4-induced phosphorylation of p70 S6 kinase dose dependently over the range 0.3 and 1 μ M (Fig. 4A). In

addition, the phosphorylation of p70 S6 kinase was remarkably inhibited by 17-DMAG (Fig. 4B).

Discussion

In the present study, we demonstrated that HSP90 inhibitors including 17-AAG (13), 17-DMAG (15) and geldanamycin (14) significantly attenuated the BMP-4-stimulated release of OPG in osteoblast-like MC3T3-E1 cells. In addition, the expression levels of OPG mRNA induced by BMP-4 were markedly suppressed by geldanamycin. Therefore, our findings suggest that the suppression by HSP90 inhibitors of the BMP-4-stimulated synthesis of OPG is exerted at a point upstream of transcriptional levels in MC3T3-E1 cells. This is probably the first report showing the attenuation by HSP90 inhibitors of BMP-stimulated OPG synthesis in osteoblasts as far as we know. Thus, we next investigated the exact mechanism behind the suppression by HSP90 inhibitors of the BMP-4-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells.

Regarding the intracellular signaling in the TGF- β superfamily including BMPs, it is firmly established that Smad proteins act as central mediators (8). Among the Smad proteins, BMPs employ the activation of 1, 5 and Smad8 as receptor-activated Smads (8). Thus, in order to investigate whether the activation of these Smads is implicated in the inhibitory effects of HSP90 inhibitors on the BMP-4-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells, we examined the effects of 17-AAG or 17-DMAG on the BMP-4-induced phosphorylation of Smad1/5. However, we found that 17-AAG and 17-DMAG failed to affect the BMP-4-induced phosphorylation of Smad1/5. Based on these findings, it seems unlikely that the suppression by HSP90 inhibitors of the OPG synthesis stimulated by BMP-4 is mediated through the Smad-dependent signaling pathway. On the other hand, accumulating evidence indicates that the TGF- β superfamily exerts their effects on a variety of biological functions via the Smad-independent signaling pathways in addition to the Smad-dependent pathway (9). In our recent study (10), we have shown that BMP-4 stimulates OPG synthesis at least in part via p70 S6 kinase activation in osteoblast-like MC3T3-E1 cells. Thus, to clarify whether HSP90 inhibitors affect the BMP-4-induced activation of p70 S6 kinase in MC3T3-E1 cells, we examined the effects of 17-AAG or 17-DMAG on the BMP-4-induced phosphorylation of p70 S6 kinase. We showed here that the phosphorylation levels of p70 S6 kinase induced by BMP-4 were remarkably reduced by both 17-AAG and 17-DMAG. Taking our findings into account, it is most likely that HSP90 inhibitors suppress the BMP-4-stimulated OPG synthesis via attenuating p70 S6 kinase in osteoblast-like MC3T3-E1 cells.

HSP90 is a ubiquitous molecular chaperone which is involved in the folding and stabilization of a variety of proteins (25,26). It is currently recognized that HSP90 plays important roles in cell homeostasis including the regulation of glucocorticoid receptors (25,26). We have found that the expression levels of HSP90 protein are quite high in osteoblast-like MC3T3-E1 cells (27). HSP90 inhibitors, including 17-AAG, 17-DMAG and geldanamycin, are developed as anticancer agents since numerous client proteins of HSP90 are involved in the progression of cancer (26). On the other hand, OPG,

which has been identified as an osteoclastogenesis inhibitory factor, functions as a negative regulator of RANKL-mediated osteoclastic bone resorption (1). In physiological bone remodeling, bone resorption is the primary step, and bone formation is subsequently developed (1,3). To maintain the quality and quantity of bone, proper remodeling cooperated by osteoclasts and osteoblasts is required to remove old fragile skeleton and regenerate new bone. Our present findings, demonstrating that HSP90 inhibitors reduced the BMP-4-stimulated OPG synthesis in osteoblast-like MC3T3-E1 cells, make us to speculate that HSP90 could act as a positive regulator in the OPG synthesis in osteoblasts. Taking our present results into account as a whole, it is possible that the upregulation of HSP90 activity in BMP-4-stimulated OPG synthesis in osteoblasts leads bone metabolism toward the increase of bone formation due to the attenuation of osteoclastic bone resorption. Therefore, our present findings might provide a novel insight for HSP90 as a pivotal modulator of bone remodeling, which possesses a potentiality of therapeutic strategy for the remedy of metabolic bone diseases including osteoporosis. HSP90 inhibitors are generally recognized as anticancer agents (13-18), however, 17-AAG reportedly potentiates osteolytic bone metastasis of breast cancer cells (19). On the other hand, BMP is a potent osteoinductive cytokine (8). Based on our present findings, it is possible that HSP90 inhibitors upregulate RANKL-RANK-mediating bone resorption through the reduction of OPG synthesis by BMP-4, leading to the potentiation of osteolysis consistent with the previous report. Thus, it seems necessary to pay attention to the possibility of bone resorption enhanced by HSP90 inhibitors. In addition, we used only one cell line, osteoblast-like MC3T3-E1 cells in the present study. Therefore, our findings about HSP90 inhibitor-effects on MC3T3-E1 cells should be confirmed in other types of osteoblasts including primary cultured cells. Further investigations would be required to clarify the details underlying the roles of HSP90 in bone metabolism.

In conclusion, our results strongly suggest that HSP90 inhibitors suppress the BMP-4-stimulated OPG synthesis in osteoblasts, and that their inhibitory effects are exerted through downregulating p70 S6 kinase.

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