

# HSP90 limits thrombin-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells: Regulation of p38 MAPK

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**Abstract.** Heat shock protein 90 (HSP90), expressed abundantly in a variety of cell types, is a molecular chaperone, and has a central role in protein homeostasis under stress conditions. In our previous study, it was shown that thrombin stimulates interleukin-6 (IL-6) synthesis via p44/p42 mitogen-activated protein kinase (MAPK) and p38 MAPK in osteoblast-like MC3T3-E1 cells, and that Rho-kinase acts as a positive regulator at a point upstream of p38 MAPK, but not p44/p42 MAPK. The present study investigated whether or not HSP90 is involved in the thrombin-stimulated synthesis of IL-6 and examined the mechanism by which HSP90 is involved in MC3T3-E1 cells. Cultured cells were stimulated by treatment with thrombin. IL-6 concentrations in MC3T3-E1 cells were determined using an ELISA assay, and levels of phosphorylated p38 MAPK, p44/p42 MAPK and myosin phosphatase targeting subunit, a substrate of Rho-kinase; were analyzed by western blotting. The 17-allylamino-17-demethoxy-geldanamycin (17-AAG) and 17-dimethylamino-ethylamino-17-demethoxy-geldanamycin (17-DMAG) HSP90 inhibitors significantly enhanced the thrombin-stimulated release of IL-6. Geldanamycin, another inhibitor of HSP90, also upregulated the release and mRNA expression of IL-6. 17-AAG and geldanamycin markedly potentiated the thrombin-induced phosphorylation of p38 MAPK without affecting the phosphorylation of p44/p42 MAPK or myosin phosphatase targeting subunit, a substrate of Rho-kinase. Additionally, the enhancement by 17-AAG of the thrombin-stimulated release of IL-6 was significantly reduced by SB203580, an inhibitor of p38 MAPK. These results suggested that the thrombin-stimulated synthesis of IL-6 was

limited by HSP90 in osteoblasts, and that the effects of HSP90 were exerted at the point between Rho-kinase and p38 MAPK.

## Introduction

Bone metabolism is coordinated mainly by two types of functional cells, osteoclasts and osteoblasts (1). Osteoclasts are responsible for bone resorption, whereas osteoblasts are responsible for bone formation (1). Skeletal tissue is constantly regenerated through a sequential process of resorption and formation, known as 'bone remodeling', to preserve sufficient bone quality and quantity, which is strictly regulated by numerous hormones and cytokines (2,3). Metabolic bone diseases, including osteoporosis, are caused by impairment in the bone remodeling process.

Interleukin-6 (IL-6), a potent and multifunctional cytokine, is known to perform pivotal physiological actions, including the promotion of B-cell differentiation and the induction of acute-phase proteins (4,5). Regarding bone metabolism, it has been considered that IL-6 stimulates bone resorption and induces osteoclast formation (5). However, IL-6 is also reportedly essential in the process of bone fracture repair (6). Therefore, IL-6 may osteotropically modulate bone formation under conditions of increased bone turnover (7).

Heat shock proteins (HSPs), are molecular chaperones, which function as central regulators of proteostasis under various stresses, including heat and chemicals (8). HSPs that are responsible for the quality control of protein folding, facilitate the refolding of misfolded proteins or assist in their deletion (8). Accumulating evidence indicates that HSPs are implicated in various physiological cellular functions, including the immune response and regulation of the cytoskeleton, in addition to protein folding (8). Among the HSP family, HSP90, a homodimeric ATPase, is constitutively expressed in various types of unstressed mammalian cell (9,10). It is well known that HSP90 is essential in the regulation of steroid hormone receptor under physiological conditions (8). Furthermore, it has been shown that the expression of HSP90 is elevated in several types of cancer, and that client proteins of HSP90 contribute to several oncogenic signaling pathways (11,12). As such, HSP90 inhibitors, including 17-allylamino-17-demethoxy-geldanamycin

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(17-AAG) (13), 17-dimethylamino-ethylamino-17-demethoxygeldanamycin (17-DMAG) (14) and geldanamycin (15), have received attention for their potential utility as anticancer chemotherapeutics (11,12).

In terms of the effects of HSP90 inhibitors on bone metabolism, it has been reported that 17-AAG induces osteoclastogenesis and potentiates osteolytic bone metastasis in the bone metastasis of breast cancer cells (16). Additionally, geldanamycin reportedly induces autophagy and apoptosis in osteosarcoma cells (17). In our previous study (18), HSP90 was shown to negatively regulate the prostaglandin  $F_{2\alpha}$ -stimulated synthesis of IL-6 in osteoblast-like MC3T3-E1 cells, and the effect of HSP90 on the synthesis of IL-6 was exerted by regulating p38 mitogen-activated protein kinase (MAPK) of the MAPK superfamily (19). However, the details regarding the role of HSP90 in osteoblasts remain to be fully elucidated.

It is firmly established that thrombin, a serine protease, is key in the blood coagulation cascade, promoting the cleavage of fibrinogen to fibrin (20). Accumulating evidence suggests that thrombin also affects the cell functions of various types of cells, including osteoblasts, through specific receptors on the cell surface termed protease-activated receptors (21). It has been reported that thrombin stimulates the proliferation of osteoblasts and secretion of IL-6, and suppresses alkaline phosphatase activity, a phenotype of osteoblastic differentiation (21).

In terms of IL-6 synthesis in osteoblasts, our previous investigations demonstrated that thrombin induces IL-6 synthesis, at least in part, via p38 MAPK and p44/p42 MAPK of the MAPK superfamily in osteoblast-like MC3T3-E1 cells (22,23). Additionally, it was revealed that Rho-kinase regulates the thrombin-stimulated synthesis of IL-6 at a point upstream of p38 MAPK in these cells (23). However, the mechanism underlying the thrombin-stimulated synthesis of IL-6 in osteoblasts has not been clarified.

In the present study, the involvement of HSP90 in thrombin-induced IL-6 synthesis was investigated in osteoblast-like MC3T3-E1 cells using HSP90 inhibitors. It was shown that the thrombin-stimulated synthesis of IL-6 was amplified by HSP90 inhibitors in osteoblasts, and that the effect of HSP90 inhibitors was exerted at the point between Rho-kinase and p38 MAPK.

## Materials and methods

**Materials.** 17-AAG, 17-DMAG and SB203580 were purchased from Calbiochem; EMD Millipore (Billerica, MA, USA). Thrombin and geldanamycin were obtained from Sigma-Aldrich; EMD Millipore. A mouse IL-6 enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Phospho-specific p44/p42 MAPK antibodies (cat. no. 9101), p44/p42 MAPK antibodies (cat. no. 9102), phospho-specific p38 MAPK antibodies (cat. no. 4511), p38 MAPK antibodies (cat. no. 9212) and phospho-specific myosin phosphatase targeting subunit (MYPT-1) antibodies (cat. no. 4563) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (cat. no. sc-25778) were purchased from Santa Cruz

Biotechnology, Inc. (Santa Cruz, CA, USA). An ECL western blot detection system was purchased from GE Healthcare Life Sciences (Chalfont, UK). Other materials and chemicals were obtained from commercial sources. 17-AAG, 17-DMAG, geldanamycin and SB203580 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect assays for IL-6, reverse transcription-polymerase chain reaction (RT-PCR) analysis or western blot analysis.

**Cell culture.** Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvariae (24) were provided by Dr. M. Kumegawa (Meikai University, Sakado, Japan) and maintained as described previously (25). In brief, the cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The cells were seeded into 35-mm diameter dishes (5x10<sup>4</sup> cells/dish) for the IL-6 assay and RT-PCR analysis, or in 90-mm diameter dishes (2x10<sup>5</sup> cells/dish) for western blot analysis in  $\alpha$ -MEM containing 10% FBS. After 5 days, the medium was replenished with  $\alpha$ -MEM containing 0.3% FBS at 37°C for 48 h. The cells were then used in the experiments described below.

**Assay for IL-6.** The cultured MC3T3-E1 cells were stimulated by 1 U/ml thrombin or vehicle in 1 ml of  $\alpha$ -MEM containing 0.3% FBS for 48 h and then pretreated with various doses of 17-AAG or 17-DMAG, or 1  $\mu$ M of geldanamycin at 37°C for 60 min. Preincubation with 10  $\mu$ M of SB203580 or vehicle was performed at 37°C for 60 min prior to pretreatment with 17-AAG. The conditioned medium was collected at the end of the incubation, and the concentration of IL-6 in the medium was then measured using the mouse IL-6 ELISA kit according to the manufacturer's protocol.

**RT-PCR analysis.** The cultured MC3T3-E1 cells were pretreated with 1  $\mu$ M of geldanamycin or vehicle for 60 min, and then stimulated by 1 U/ml of thrombin or vehicle in  $\alpha$ -MEM containing 0.3% FBS for 3 h. Total RNA was isolated and reverse transcribed into complementary DNA at 37°C for 60 min and then 95°C for 5 min using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), an Omniscript Reverse Transcriptase kit (Qiagen, Inc., Valencia, CA, USA) and Oligo (dT) 12-18 Primers (Thermo Fisher Scientific, Inc.), respectively. RT-PCR analysis was performed in capillaries using a Light Cycler system with Fast Start DNA Master SYBR Green I (Roche Diagnostics, Basel, Switzerland). Samples (cDNA 2.5 ng/assay, primers 0.5 mM) were subjected to the following PCR thermocycling conditions: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 1 sec, annealing at 60°C for 5 sec and elongation at 72°C for 7 sec. Primers used for PCR were purchased from Takara Bio, Inc. (Otsu, Japan) and had the following sequences: IL-6 forward, 5'-CCATTCACAAGTCGAGGCTTA-3' and reverse, GCAAGTGCA TCATCGTTGTTTCATAC; and GAPDH forward, 5'-TGTGTCCGTCGTGGATCTGA-3' and reverse, 5'-TTGCTGTTGAAGTCGCAGGAG-3'.

**Western blot analysis.** The cultured MC3T3-E1 cells were pretreated with various doses of 17-AAG or geldanamycin for 60 min, and then stimulated by 1 U/ml of thrombin or vehicle in  $\alpha$ -MEM containing 0.3% FBS for the indicated periods. The cells were then 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 was performed using the method of Laemmli (26) using 10% polyacrylamide gels. Protein quantification was performed using the Bradford method using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The protein (10  $\mu$ g/lane) 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 [20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% Tween-20] for 1 h prior to incubation with primary antibodies. The western blot analysis was performed as described previously (27) using phospho-specific p44/p42 MAPK antibodies, p44/p42 MAPK antibodies, phospho-specific p38 MAPK antibodies, p38 MAPK antibodies, phospho-specific MYPT-1 antibodies and GAPDH antibodies as primary antibodies. Peroxidase-labeled antibodies raised in goat against rabbit IgG (cat. no. 5220-0336; KPL, Inc., Gaithersburg, MD, USA) were used as secondary antibodies. The primary and secondary antibodies were diluted at 1:1,000 with 5% fat-free dry milk in the Tris-buffered saline-Tween. The peroxidase activity on the PVDF sheet was visualized on X-ray film using the ECL western blot detection system.

**Densitometric analysis.** Densitometric analysis of the western blots was performed using a scanner and image analysis software program (Image J version 1.48; NIH, Bethesda, MD, USA). The phosphorylated protein levels were calculated as follows: Background-subtracted signal intensity of each phosphorylation signal was respectively normalized to the total protein signal and plotted as the fold increase compared with that of the control cells without stimulation.

**Statistical analysis.** The data were analyzed using one-way analysis of variance followed by Bonferroni's method for multiple comparisons between pairs using StatView (ver.5.0; SAS Institute, Inc., Cary, NC, USA).  $P < 0.05$  was considered to indicate a statistically significant difference. All data are presented as the mean  $\pm$  standard deviation of triplicate determinations from three independent cell preparations.

## Results

**Effects of 17-AAG, 17-DMAG or geldanamycin on the thrombin-stimulated release of IL-6 in MC3T3-E1 cells.** HSP90 inhibitors, 17-AAG, 17-DMAG and geldanamycin, which are all benzoquinone ansamycin antibiotics, bind to the N-terminal domain ATP binding site of HSP90, inhibiting the ATP-dependent HSP90 chaperone activity (13-15). To clarify whether or not HSP90 is involved in the thrombin-induced synthesis of IL-6 in osteoblast-like MC3T3-E1 cells, the present

Table I. Effect of geldanamycin on the thrombin-stimulated release of IL-6 in MC3T3-E1 cells.

Geldanamycin (1 $\mu$ M)	Thrombin (1 U/ml)	IL-6 (pg/ml)
-	-	10.0 $\pm$ 2.0
-	+	468.7 $\pm$ 65.4 <sup>a</sup>
+	-	102.2 $\pm$ 5.5
+	+	3,757.9 $\pm$ 458.3 <sup>b</sup>

Cultured cells were pretreated with 1 mM of geldanamycin or vehicle for 60 min, and then with 1 U/ml of thrombin or vehicle for 48 h. IL-6 concentrations in the conditioned medium were determined using an enzyme-linked immunosorbent assay. Values are presented as the mean  $\pm$  standard deviation of triplicate determinations from three independent cell preparations. <sup>a</sup> $P < 0.05$ , compared with cells without thrombin or geldanamycin; <sup>b</sup> $P < 0.05$ , compared with cells stimulated with thrombin without geldanamycin pretreatment. IL-6, interleukin-6.

study examined the effects of these HSP90 inhibitors on the release of IL-6. The effect of 17-AAG was dose-dependent over the range of 0.1-1  $\mu$ M, and the maximum effect was observed at 0.7  $\mu$ M with 1.9 $\times 10^3$  pg/ml IL-6, which induced an increase of  $\sim 310\%$  from the effect of thrombin at 0.6 $\times 10^3$  pg/ml IL-6 ( $P = 1.6 \times 10^{-5}$ ; Fig. 1A). Additionally, the effect of 17-DMAG was dose-dependent over the range of 0.01-0.1  $\mu$ M, and the maximum effect was observed at 0.1  $\mu$ M with 3.1 $\times 10^3$  pg/ml IL-6, which induced an increase of  $\sim 560\%$  from the effect of thrombin at 0.5 $\times 10^3$  pg/ml IL-6 ( $P = 8.0 \times 10^{-6}$ ; Fig. 1B).

The release of IL-6 by thrombin was significantly increased by geldanamycin (1  $\mu$ M) with an IL-6 level of 3.8 $\times 10^3$  pg/ml, which was an increase of  $\sim 700\%$  from the effect of thrombin at 0.5 $\times 10^3$  pg/ml IL-6 ( $P = 3.0 \times 10^{-4}$ ; Table I).

**Effect of geldanamycin on the thrombin-induced mRNA expression of IL-6 in MC3T3-E1 cells.** To elucidate whether or not the enhancement by HSP90 inhibitors of the thrombin-stimulated release of IL-6 is mediated through transcriptional events in MC3T3-E1 cells, the present study examined the effect of geldanamycin on the thrombin-induced mRNA expression of IL-6. Geldanamycin (1  $\mu$ M) markedly amplified the expression of mRNA induced by thrombin (Fig. 2).

**Effects of 17-AAG or geldanamycin on the thrombin-induced phosphorylation of p44/p42 MAPK in MC3T3-E1 cells.** Regarding to the intracellular signaling pathway of thrombin in osteoblasts, our previous study showed that thrombin stimulates the synthesis of IL-6, at least in part, via the activation of p44/p42 MAPK in osteoblast-like MC3T3-E1 cells (23). Therefore, the present study examined the effects of 17-AAG or geldanamycin on the phosphorylation of p44/p42 MAPK induced by thrombin in MC3T3-E1 cells. However, 17-AAG had no significant effect on the phosphorylation of p44/p42 MAPK (Fig. 3A). Geldanamycin also had no significant effect on the phosphorylation of p44/p42 MAPK (Fig. 3B).

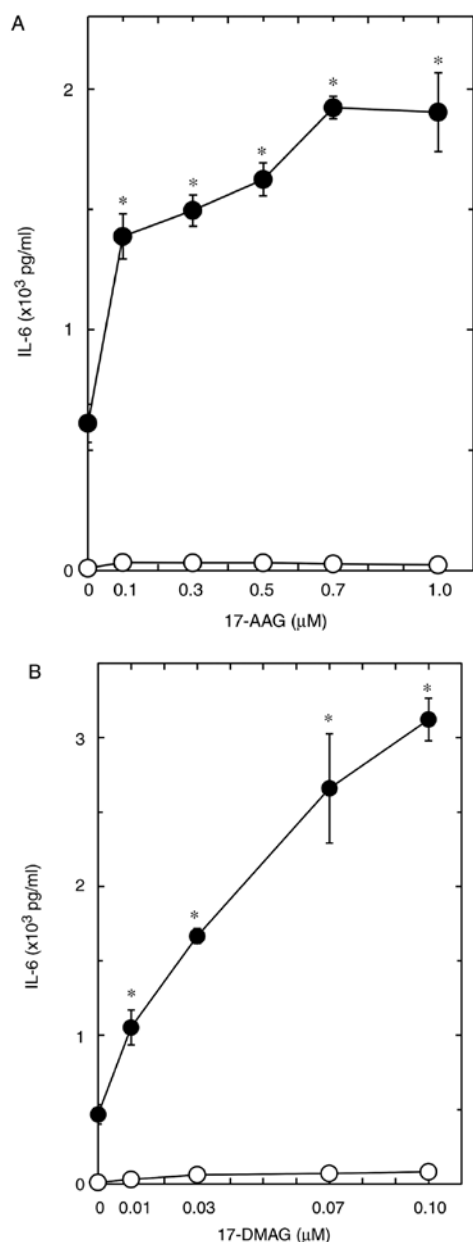


Figure 1. (A) Effect of 17-AAG on the thrombin-stimulated release of IL-6 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of 17-AAG for 60 min, and then stimulated by 1 U/ml of thrombin (●) or vehicle (○) for 48 h. The IL-6 concentrations in the conditioned medium were determined using an ELISA. Each value is presented as the mean  $\pm$  standard deviation of triplicate determinations from three independent cell preparations. \* $P < 0.05$ , compared with cells stimulated with thrombin without 17-AAG pretreatment. (B) Effect of 17-DMAG on the thrombin-stimulated release of IL-6 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of 17-DMAG for 60 min, and then stimulated by 1 U/ml of thrombin (●) or vehicle (○) for 48 h. The IL-6 concentrations in the conditioned medium were determined using an ELISA. Each value is presented as the mean  $\pm$  standard deviation of triplicate determinations from three independent cell preparations. \* $P < 0.05$ , compared with the cells stimulated with thrombin without 17-DMAG pretreatment. IL-6, interleukin-6; 17-AAG, 17-allylamino-17-demethoxy-geldanamycin; 17-DMAG, 17-dimethylamino-ethylamino-17-demethoxy-geldanamycin; ELISA, enzyme-linked immunosorbent assay.

**Effects of 17-AAG or geldanamycin on the thrombin-induced phosphorylation of p38 MAPK in MC3T3-E1 cells.** Our previous study showed that p38 MAPK and p44/p42 MAPK act as positive regulators in the thrombin-stimulated synthesis

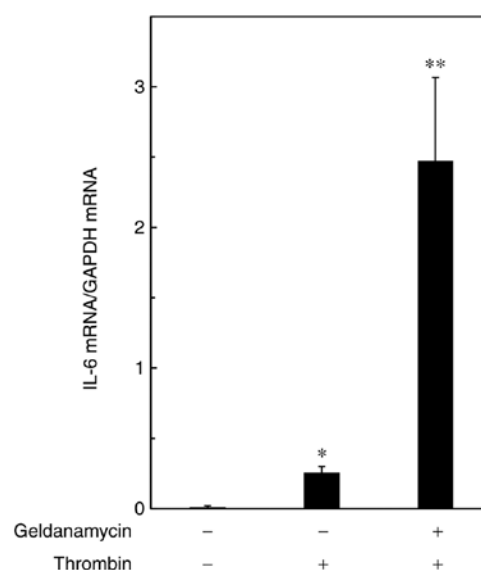
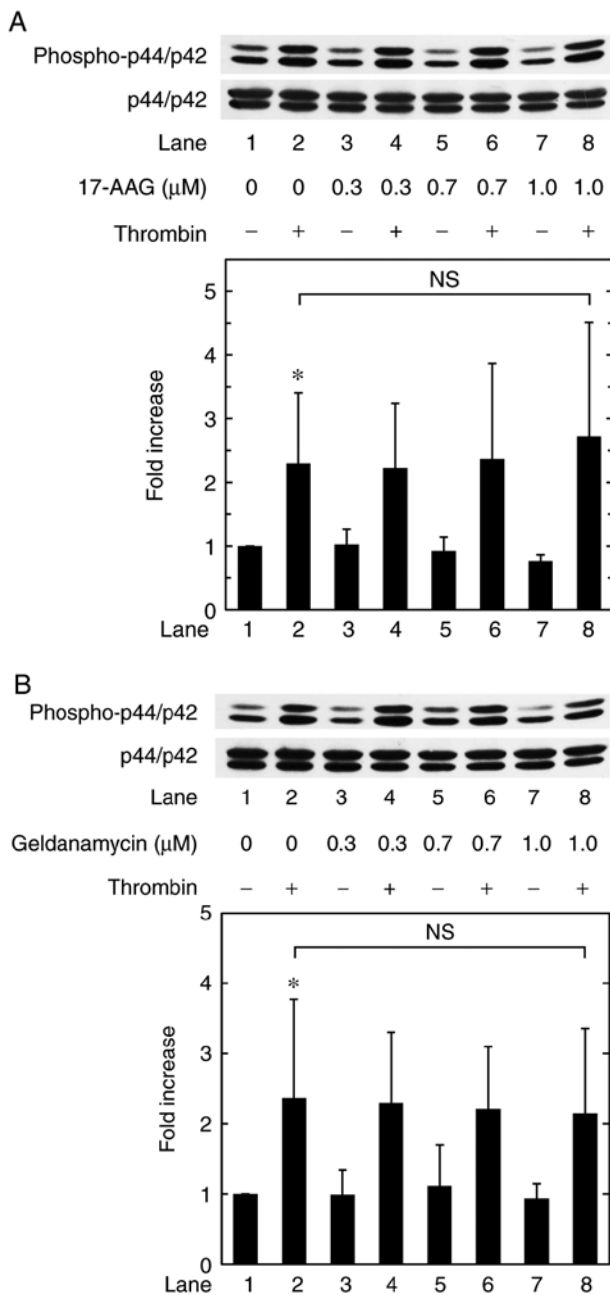


Figure 2. Effect of geldanamycin on the thrombin-induced mRNA expression levels of IL-6 in MC3T3-E1 cells. The cultured cells were pretreated with 1  $\mu$ M of geldanamycin or vehicle for 60 min, and then stimulated by 1 U/ml of thrombin or vehicle for 3 h. The total RNA was then isolated, and the respective mRNA expression levels of IL-6 and GAPDH were quantified using reverse transcription-polymerase chain reaction analysis. The mRNA levels of IL-6 were normalized to those of GAPDH. Each value is presented as the mean  $\pm$  standard deviation of triplicate determinations from three independent cell preparations. \* $P < 0.05$ , compared with cells without thrombin or geldanamycin; \*\* $P < 0.05$ , compared with cells stimulated with thrombin without geldanamycin pretreatment. IL-6, interleukin-6.

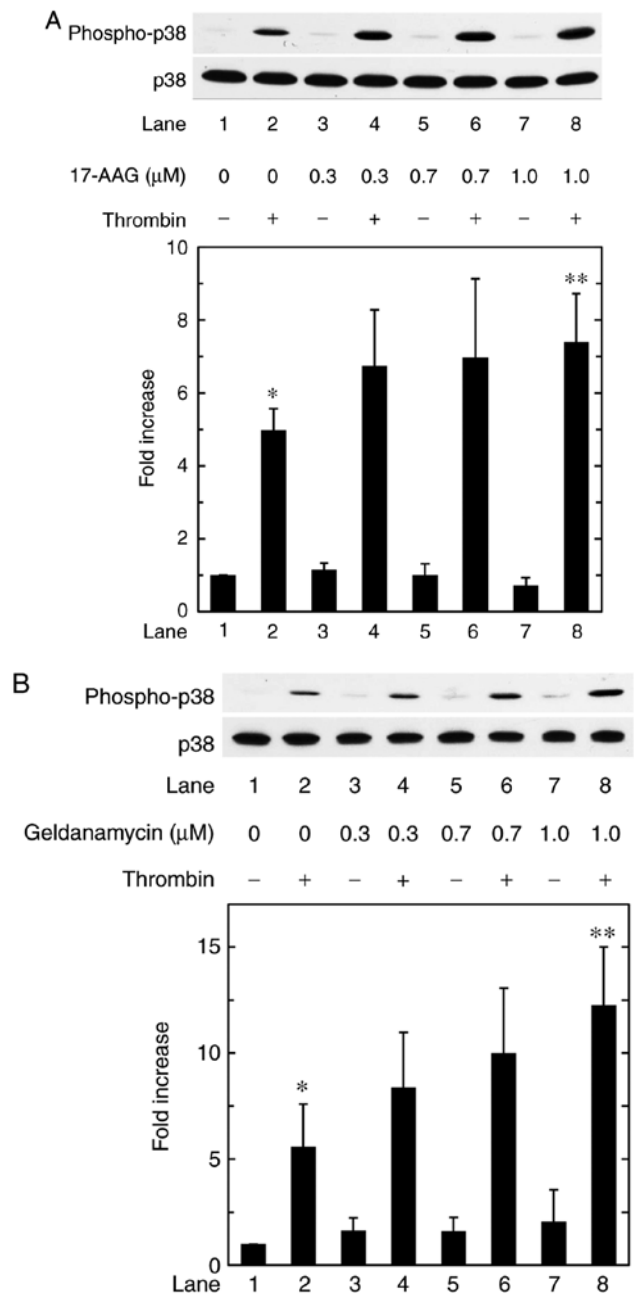
of IL-6 in osteoblast-like MC3T3-E1 cells (23). To investigate whether or not the amplification by HSP90 inhibitors of the thrombin-induced synthesis of IL-6 is exerted through the modulation of p38 MAPK activity in MC3T3-E1 cells, the present study examined the effects of 17-AAG or geldanamycin on the phosphorylation of p38 MAPK induced by thrombin. Only the 17-AAG concentration of 1.0  $\mu$ M and geldanamycin concentration of 1.0  $\mu$ M led to a significant difference compared with thrombin alone ( $P = 0.04$  and  $P = 0.03$ , respectively). The other concentrations had no significant effect (Fig. 4A and B).

**Effects of 17-AAG or geldanamycin on the thrombin-induced phosphorylation of MYPT-1 in MC3T3-E1 cells.** In our previous study (23), it was also shown that Rho-kinase positively regulates the thrombin-stimulated synthesis of IL-6 at a point upstream of p38 MAPK, but not p44/p42 MAPK, in osteoblast-like MC3T3-E1 cells. It is currently recognized that Rho-kinase phosphorylates MYPT-1, a component of myosin phosphatase, as a direct downstream substrate (28,29). Therefore, the present study further examined the effects of 17-AAG or geldanamycin on the phosphorylation of MYPT-1 induced by thrombin in MC3T3-E1 cells. However, 17-AAG failed to affect the thrombin-induced phosphorylation of MYPT-1 up to 1  $\mu$ M (Fig. 5A). Geldanamycin also had minimal effect on the thrombin-induced phosphorylation of MYPT-1 up to 1  $\mu$ M (Fig. 5B).

**Effect of SB203580 on the amplification by 17-AAG of the thrombin-stimulated release of IL-6 in MC3T3-E1 cells.** The



**Figure 3.** Effects of 17-AAG or geldanamycin on the thrombin-induced phosphorylation of p44/p42 MAPK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) 17-AAG or (B) geldanamycin for 60 min, and then stimulated with 1 U/ml of thrombin or vehicle for 15 min. The cell extracts were then subjected to sodium dodecyl sulfate-PAGE with a subsequent western blot analysis with antibodies against phospho-specific p44/p42 MAPK or p44/p42 MAPK. The histogram shows the quantitative representations of the levels of phospho-p44/p42 MAPK following normalization with respect to p44/p42 MAPK obtained from laser densitometric analysis. The levels are expressed as the fold increase compared to the basal levels shown in lane 1. Each value is presented as the mean  $\pm$  standard deviation of triplicate determinations from three independent cell preparations. \* $P < 0.05$ , compared with cells without thrombin or HSP90 inhibitor. NS, no significant difference; 17-AAG, 17-allylamino-17demethoxy-geldanamycin; phospho, phosphorylated.



**Figure 4.** Effects of 17-AAG or geldanamycin on the thrombin-induced phosphorylation of p38 MAPK in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) 17-AAG or (B) geldanamycin for 60 min, and then stimulated with 1 U/ml of thrombin or vehicle for 5 min. The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p38 MAPK or p38 MAPK. The histogram shows the quantitative representations of the levels of phospho-p38 MAPK following normalization with respect to p38 MAPK obtained from laser densitometric analysis. The levels are expressed as the fold increase compared to the basal levels shown in lane 1. Each value is presented as the mean  $\pm$  standard deviation of triplicate determinations from three independent cell preparations. \* $P < 0.05$ , compared with cells without thrombin or HSP90 inhibitor; \*\* $P < 0.05$ , compared with the cells stimulated by thrombin without HSP90 inhibitor pretreatment. MAPK, mitogen-activated protein kinase; 17-AAG, 17-allylamino-17demethoxy-geldanamycin; phospho, phosphorylated.

present study further investigated the role of p38 MAPK in the upregulation by HSP90 inhibitors of the thrombin-stimulated synthesis of IL-6 in osteoblast-like MC3T3-E1 cells. It was confirmed that SB203580, an inhibitor of p38 MAPK (30),

suppressed the thrombin-induced release of IL-6, as previously reported (24) (Fig. 6). SB203580 significantly reduced the enhancement by 17-AAG of the thrombin-stimulated release of IL-6 ( $0.4 \times 10^3$  pg/ml IL-6). SB203580 induced a decrease

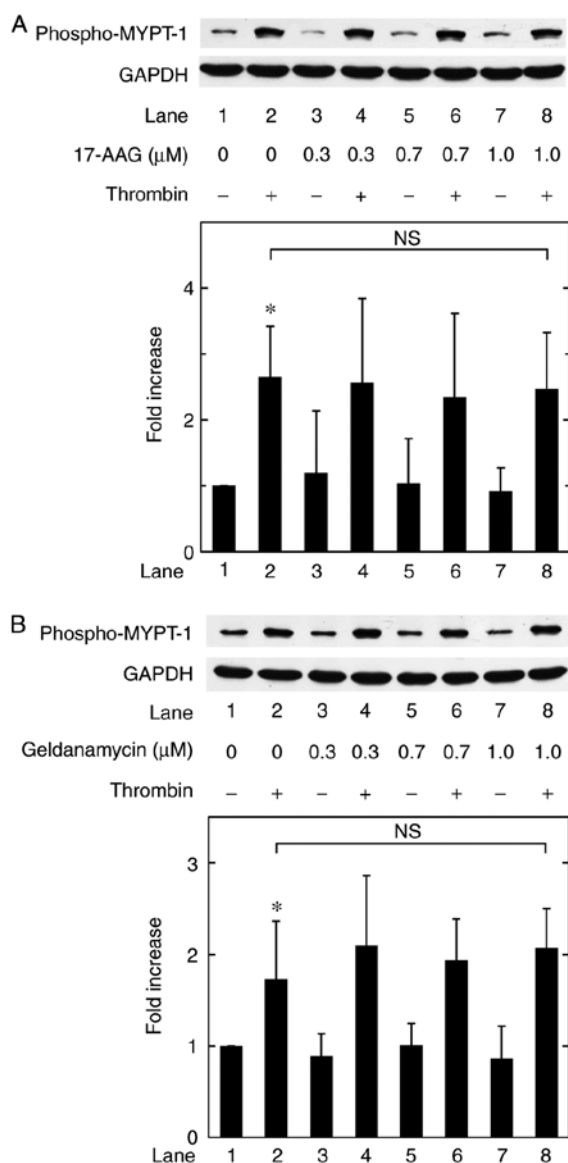


Figure 5. Effects of 17-AAG or geldanamycin on the thrombin-induced phosphorylation of MYPT-1 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of (A) 17-AAG or (B) geldanamycin for 60 min, and then stimulated with 1 U/ml of thrombin or vehicle for 15 min. The cell extracts were then subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific MYPT-1 or GAPDH. The histogram shows the quantitative representations of the levels of phospho-MYPT-1 following normalization with respect to GAPDH obtained from laser densitometric analysis. The levels are expressed as the fold increase compared to the basal levels shown in lane 1. Each value is presented as the mean  $\pm$  standard deviation of triplicate determinations from three independent cell preparations. \* $P < 0.05$ , compared with cells without thrombin or HSP90 inhibitor. NS, no significant difference; 17-AAG, 17-allylamino-17demethoxy-geldanamycin; MYPT, myosin phosphatase targeting subunit; phospho, phosphorylated.

of  $\sim 85\%$  in the effect of thrombin and 17-AAG ( $2.3 \times 10^3$  pg/ml IL-6;  $P = 1.0 \times 10^{-6}$ ; Fig. 6).

## Discussion

In the present study, it was shown that the thrombin-stimulated release of IL-6 was significantly amplified by representative HSP90 inhibitors, including 7-AAG, 17-DMAG

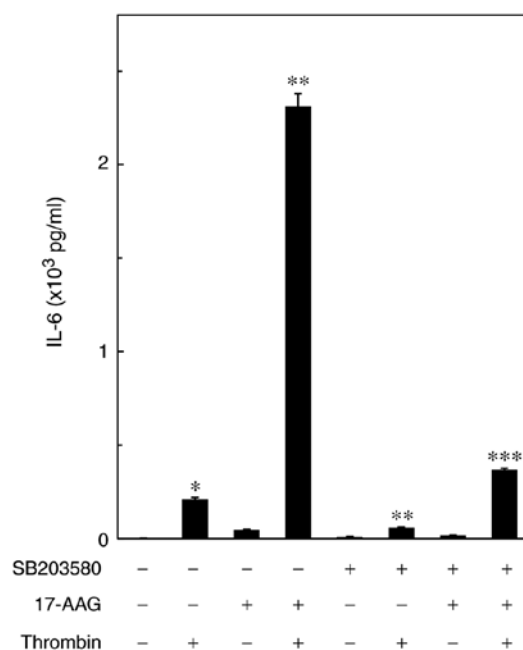


Figure 6. Effect of SB203580 on the enhancement by 17-AAG of the thrombin-induced release of IL-6 in MC3T3-E1 cells. The cultured cells were preincubated with 10  $\mu$ M of SB203580 or vehicle for 60 min, subsequently pretreated with 0.7  $\mu$ M of 17-AAG or vehicle for 60 min, and then stimulated with 1 U/ml of thrombin or vehicle for 48 h. The IL-6 concentrations of the conditioned mediums were determined using an enzyme-linked immunosorbent assay. Each value is presented as the mean  $\pm$  standard deviation of triplicate determinations from three independent cell preparations. \* $P < 0.05$ , compared with cells without thrombin, 17-AAG or SB203580; \*\* $P < 0.05$ , compared with cells stimulated with thrombin without 17-AAG or SB203580; \*\*\* $P < 0.05$ , compared with cells stimulated with thrombin with 17-AAG pretreatment without SB203580. 17-AAG, 17-allylamino-17demethoxy-geldanamycin; IL-6, interleukin-6.

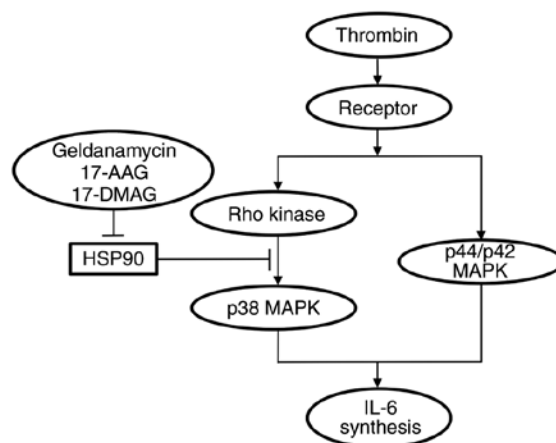


Figure 7. Schematic illustration of the regulatory mechanism underlying the amplifying effect of HSP90 inhibitors on thrombin-induced IL-6 synthesis in osteoblast-like MC3T3-E1 cells. MAPK, mitogen-activated protein kinase; HSP90, heat shock protein 90; IL-6, interleukin-6; 17-AAG, 17-(Allylamino) geldanamycin; 17-DMAG, 17-dimethylamino-ethylamino-17-demethoxy-geldanamycin.

and geldanamycin, in osteoblast-like MC3T3-E1 cells. Additionally, it was revealed that geldanamycin markedly enhanced the thrombin-induced mRNA expression of IL-6. These findings suggested that the HSP90 inhibitors exerted



an amplifying effect on the thrombin-stimulated release of IL-6 through gene transcription in these cells. It is recognized that geldanamycin and its less toxic derivatives, 17-AAG and 17-DMAG, inhibit ATP-dependent HSP90 chaperone activity in a common mechanism (13-15). Therefore, it was considered likely that 17-AAG and 17-DMAG possess a positive effect on the mRNA expression levels of IL-6 induced by thrombin and geldanamycin. Taking the findings of the present study into account, it is possible that HSP90 possesses inhibitory activity on the thrombin-stimulated synthesis of IL-6 in osteoblast-like MC3T3-E1 cells. Therefore, using HSP90 inhibitors, the present study investigated the mechanism underlying the suppressive effect of HSP90 on the synthesis of IL-6 stimulated by thrombin in osteoblast-like MC3T3-E1 cells.

In terms of the intracellular signal transduction of thrombin in osteoblasts, our previous study revealed that thrombin induces the activation of p38 MAPK, p44/p42 MAPK and stress-activated protein kinase/c-Jun N-terminal kinase in osteoblast-like MC3T3-E1 cells, and that among the MAPKs, p38 MAPK and p44/p42 MAPK lead to the stimulation of IL-6 synthesis (23). Based on these previous findings, the present study investigated whether or not the thrombin-induced activation of p38 MAPK and/or p44/p42 MAPK are affected by HSP90 inhibitors in osteoblast-like MC3T3-E1 cells. It was found that the phosphorylation of p44/p42 MAPK by thrombin was not affected by either 17-AAG or geldanamycin. By contrast, the thrombin-induced phosphorylation of p38 MAPK was significantly increased by 17-AAG and geldanamycin. It is likely that similar results can be obtained using 17-DMAG, as geldanamycin and its derivatives suppress the ATP-dependent chaperone activity of HSP90 in common mechanism (13-15). Therefore, these results suggested that the upregulation of p38 MAPK, rather than p44/p42 MAPK, is implicated in the enhancement by HSP90 inhibitors of thrombin-stimulated IL-6 synthesis in osteoblast-like MC3T3-E1 cells.

It was noted that HSP90 inhibitors combined with thrombin treatment resulted in the amplification of IL-6 synthesis to high levels, compared with those induced by thrombin or the inhibitors alone, whereas neither geldanamycin nor 17-AAG caused the enhancement of thrombin-induced phosphorylation of p38 MAPK to similar levels. It is likely that the sustained enhancement of thrombin-induced p38 MAPK activity by HSP90 inhibitors results in high levels of IL-6 synthesis in osteoblasts. However, it has been reported that 17-AAG itself enhances osteoblastic differentiation in C3H10T1/2 cells and primary calvarial osteoblasts (28). Therefore, the amplification of thrombin-induced IL-6 synthesis by HSP90 inhibitors in the present study may have been affected by the enhancement of osteoblastic differentiation induced by HSP90 inhibitors.

In our previous study (23), it was also shown that the thrombin-stimulated synthesis of IL-6 is positively regulated by Rho-kinase at a point upstream of p38 MAPK in these cells. However, it was shown in the present study that, unlike p38 MAPK, the thrombin-induced phosphorylation of MYPT-1 was not affected by either 17-AAG or geldanamycin. As MYPT-1 is a well-established substrate of Rho-kinase (29,30), it is unlikely that the amplifying effect of HSP90 inhibitors on the synthesis of IL-6 stimulated by thrombin is exerted at a point upstream of Rho-kinase. These findings suggested that the thrombin-stimulated IL-6 synthesis was negatively

regulated by HSP90 in osteoblast-like MC3T3-E1 cells, and that HSP90 exerted its inhibitory effect on IL-6 synthesis at the point between Rho-kinase and p38 MAPK. The present study also showed that the amplification of the thrombin-stimulated release of IL-6 by 17-AAG was markedly reduced by SB203580, a p38 MAPK inhibitor (31), in these cells. Taken together, these results suggested that the thrombin-stimulated synthesis of IL-6 was inhibited by HSP90 in the osteoblast-like MC3T3-E1 cells, and that the suppressive effect of HSP90 was exerted at the point between Rho-kinase and p38 MAPK. The potential mechanism underlying amplification of thrombin-stimulated IL-6 synthesis by HSP90 inhibitors in osteoblasts is summarized in Fig. 7.

Bone remodeling is initiated with bone resorption, followed by bone formation (3). In order to maintain the quantity and quality of the adult skeleton, the regulation of bone remodeling handled by osteoblasts and osteoclasts must be well orchestrated. It is generally known that IL-6 is a potent bone resorptive agent promoting osteoclastogenesis (5). In addition, accumulating evidence suggests that IL-6 functions as an osteotropic factor under the conditions of increased bone turnover, inducing bone formation (7). Therefore, IL-6 is currently recognized to act as a bone-remodeling agent in bone metabolism. In our previous study, it was demonstrated that HSP90 is expressed at high levels in osteoblast-like MC3T3-E1 cells, even in their resting state (32). Therefore, the results of the present study, showing that thrombin-stimulated IL-6 synthesis was upregulated by HSP90 inhibitors in MC3T3-E1 cells, support the physiological function of HSP90 in osteoblasts as a fundamental bone remodeling modulator. Several HSP90 inhibitors have been adopted in clinical trials as anticancer agents (33). Taken together, the findings of the present study suggested the role of HSP90 inhibitors as a bone-remodeling agent through the amplification of IL-6 synthesis in osteoblasts.

In the present study, all experiments were performed using murine osteoblast-like MC3T3-E1 cells. Further investigations using another types of osteoblastic cells, for example primary human osteoblasts, are required to clarify the exact mechanism and the clinical relevance of HSP90 inhibitors in bone metabolism. In addition, supporting evidence to clarify whether HSP90 inhibitors affect the synthesis or the activity of HSP90 in osteoblasts is required.

In conclusion, the results of the present study suggested that thrombin-stimulated IL-6 synthesis is negatively regulated by HSP90 in osteoblasts, and that the effect of HSP90 on the synthesis of IL-6 is exerted at the point between Rho-kinase and p38 MAPK.

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### Availability of data and materials

All data generated or analyzed during this study are included within.

### Authors' contribution

KF, OK and HT conceived and designed the experiments. KF, TK, SK and GS performed the experiments. KF, TO, RM-N, OK and HT analyzed the data. KF, OK and HT wrote the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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