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Molecular Stress-inducing Compounds Increase Osteoclast Formation in a Heat Shock Factor 1 Protein-dependent Manner*

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Background: HSP90 inhibitors increase osteoclast formation and bone loss.

Results: Altered Hsf1 activity impacts the ability of stress-inducing compounds to modulate osteoclast formation.

Conclusion: Hsf1 plays an important role in stress-associated osteoclast formation, potentially via MITF.

Significance: We identified a novel pathway whereby agents inducing stress can enhance osteoclast formation.

Many anticancer therapeutic agents cause bone loss, which increases the risk of fractures that severely reduce quality of life. Thus, in drug development, it is critical to identify and understand such effects. Anticancer therapeutic and HSP90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) causes bone loss by increasing osteoclast formation, but the mechanism underlying this is not understood. 17-AAG activates heat shock factor 1 (Hsf1), the master transcriptional regulator of heat shock/cell stress responses, which may be involved in this negative action of 17-AAG upon bone. Using mouse bone marrow and RAW264.7 osteoclast differentiation models we found that HSP90 inhibitors that induced a heat shock response also enhanced osteoclast formation, whereas HSP90 inhibitors that did not (including coumermycin A1 and novobiocin) did not affect osteoclast formation. Pharmacological inhibition or shRNAmir knockdown of Hsf1 in RAW264.7 cells as well as the use of *Hsf1* null mouse bone marrow cells demonstrated that 17-AAG-enhanced osteoclast formation was Hsf1-dependent. Moreover, ectopic overexpression of Hsf1 enhanced 17-AAG effects upon osteoclast formation. Consistent with these findings, protein levels of the essential osteoclast transcription factor micropthalmia-associated transcription factor were increased by 17-AAG in an Hsf1-dependent manner. In addition to HSP90 inhibitors, we also identified that other agents that induced cellular stress, such as ethanol, doxorubicin, and methotrexate, also directly increased osteoclast formation, potentially in an Hsf1-dependent manner. These results, therefore, indicate that cellular stress can enhance osteoclast differentiation via Hsf1-dependent mechanisms

and may significantly contribute to pathological and therapeutic related bone loss.

Maintaining bone mass and quality is critical for sustained health and quality of life by preventing fracture (1). For this reason, bone undergoes continual remodeling throughout adult life to optimize bone quality and structural integrity. This remodeling process involves cycles of bone resorption and formation, mediated by osteoclasts and osteoblasts, respectively (2, 3). Many factors can negatively impact bone health, including a poor diet, gonadal hormonal insufficiency, pathological insult, as well as a range of therapeutic agents (4–7) that often compound the loss of bone mass seen with aging. Factors that are deleterious to bone generally cause a net increase in the formation of the specialized bone-resorbing cell, the osteoclast, causing sustained bone loss that can result in low bone mass, *i.e.* osteopenia or osteoporosis (8, 9), that is not compensated for by increased bone formation. Such bone loss is associated with decreased bone strength and, thus, an increased fracture risk, particularly in the spine, hip, and wrist, with any resulting fractures ultimately leading to a severely diminished quality of life and increased rate of mortality, particularly in elderly patients (10). Localized rapid bone loss may also cause pain and hypercalcemia (4).

It is increasingly recognized that chemotherapeutic agents have a major negative impact upon bone by increasing bone loss and fracture risk more rapidly and severely than seen in normal age-related bone loss (4, 6). Although both hormonal and non-hormonal cancer therapies promote bone loss by inducing hypogonadism, chemotherapeutics can also directly impact osteoclasts (as well as the bone-forming osteoblasts) to cause loss of bone mass and structural integrity, although the mechanisms that underlie this have still to be fully elucidated (4, 11–13). Because of the effectiveness of a number of cancer treatments providing improved survival rates, especially in older patients who may already have low bone mass, it is of

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increasing importance to determine the effect of therapeutics on bone turnover and bone loss. Moreover, it is important to identify the mechanisms by which anticancer agents may result in bone loss so that preventative measures, such as administration of antiosteolytic treatments, may be designed effectively.

The process of osteoclast formation is fundamental to the resorption of bone during both physiological and pathophysiological bone resorption. Osteoclasts are multinucleated, hematopoietically derived cells (3) that are highly active and relatively short-lived. Thus, their formation is a highly regulated point of control for bone resorption and is dependent upon the action of RANKL,³ a TNF-related molecule whose production is locally regulated by many osteotropic hormones. RANKL typically acts in concert with M-CSF, a survival and proliferation factor for osteoclast progenitors and macrophages. RANKL, through interaction with its cognate receptor RANK, activates a cascade of critical transcription factors in osteoclast progenitors, notably involving NF κ B, AP-1 (cFos/cJun dimer), NFATc1, and MITF. These factors, in turn, activate osteoclastic gene expression and induce cell fusion, resulting in mature, functional, multinucleated osteoclasts (14, 15).

Heat shock protein 90 (HSP90) is a molecular chaperone that is required for the stability and functionality of a diverse range of proteins (16). In particular, its action is critical for the stability and activity of mutated and overexpressed oncogenic proteins that enhance the survival, growth, and invasive potential of cancer cells (16, 17). Consistent with this, HSP90 is highly expressed in many tumor types and has been associated with poor patient outcomes (16–18). Thus, HSP90 has emerged as a major cancer therapeutic target and, as such, a number of HSP90 inhibitors have been developed, many of which have undergone or are currently in clinical trials (19).

We have found previously that the geldanamycin-derived HSP90 inhibitor and anticancer agent 17-AAG increases bone loss in mouse models through the direct stimulation of osteoclast formation (20). Furthermore, although 17-AAG proved to be effective in reducing the tumor burden at extraosseous sites, it actually increased the tumor burden within the bone and caused elevated bone loss even in the absence of tumor cells (20). Increased tumor growth in bone probably reflects the well characterized effects of the release of tumor growth factors from the bone matrix and is, therefore, secondary to the bone destruction caused by the pro-osteoclastic effects of 17-AAG. Consistent with our findings, Yano *et al.* (21) demonstrated that 17-AAG treatment enhanced prostate tumor growth in the bones of mice, which could be abrogated by the administration of inhibitors of osteoclast formation and function. In addition to 17-AAG, we have demonstrated that other structurally unrelated HSP90 inhibitors also enhance osteoclast formation (20, 22). To date, the mechanism by which HSP90 inhibitors stimulate osteoclast formation has not been clearly defined,

although Src kinase and the elevated expression of the essential osteoclast transcription factor microphthalmia-associated transcription factor (MITF) may play roles (21, 22). However, HSP90 inhibition itself seems unlikely to be directly critical in 17-AAG actions on osteoclasts because many of the RANKL signaling pathways required for osteoclast formation (*e.g.* NF κ B activation) are at least partly HSP90-dependent.

An alternative possibility is that the ability of 17-AAG to activate the transcription factor heat shock factor 1 (Hsf1) may play a central role in its effects on osteoclasts. Hsf1 is a critical regulator of stress responses in mammalian cells and is essential for the response to a broad range of stress stimuli, including the regulation of heat shock proteins (HSPs) (23–25). Fundamental to this response is the fact that Hsf1 associates with HSP90 under normal conditions, maintaining Hsf1 in an inactive monomeric state (26, 27). However, upon 17-AAG binding to the N-terminal ATPase domain of HSP90 or upon cellular stress, Hsf1 dissociates from the HSP90 complex, forming homotrimeric complexes, undergoes phosphorylation and SUMOylation (28), and binds to heat shock element sites within the promoters of target genes (28, 29). This results in a characteristic pattern of gene expression that is observed during stress (*e.g.* elevated levels of HSP70 and other HSPs), aiding cell survival. Thus, we examined whether the Hsf1-mediated stress response induced by HSP90 inhibition is responsible for enhancing osteoclast formation.

In this study, we report that the effects of 17-AAG upon osteoclast formation are indeed Hsf1-dependent and that, consistent with this, other Hsf1-inducing stressors have similar effects. Moreover, within the context of the stress response, we found that Hsf1 plays a major role in enhancing the levels of the critical osteoclast formation factor MITF. Our results implicate, for the first time, the role of Hsf1 in osteoclast formation and the influence of stress-induced MITF expression, which points to a direct effect of cell stress and MITF in inducing bone loss that may be important in many diseases that affect bone.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The HSP90 inhibitors 17-AAG, 17-DMAG, and radicicol were obtained from LC Labs (Woburn, MA), and coumermycin A1 and novobiocin were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). KNK437 was a gift from Kaneka Corp. (Takasago, Japan), and quercetin, methotrexate, and doxorubicin were purchased from Merck Millipore (Kilsyth, VIC, Australia). Anti-HSP70 (HSPA1A) antibody (catalog no. ADI-SPA-812) was purchased from Enzo Life Sciences (San Diego, CA). Anti-HSP105/110 (HSPH1) antibody (catalog no. SC-6241) was purchased from Santa Cruz Biotechnology (Dallas, TX). The pan anti-actin antibody (catalog no. MS-1295-P) was purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia), and the anti-Hsf1 antibody (catalog no. 4356) was obtained from Cell Signaling Technology (Danvers, MA). IgG HRP-conjugated secondary antibodies for immunoblotting were purchased from Thermo Fisher Scientific. Recombinant murine soluble RANKL (RANKL^{158–316}-GST fusion protein) was obtained from Oriental Yeast Co. (Tokyo, Japan), and human M-CSF and TGF β (TGF β 1 isoform) were from R&D Systems (Minneapolis, MN). L-cell conditioned medium (a source of secreted murine

³ The abbreviations used are: RANKL, receptor activator of nuclear factor κ B ligand; MITF, microphthalmia-associated transcription factor; 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; HSP, heat shock protein; TRAP, tartrate-resistant acid phosphatase; MEM, minimal essential medium; BMM, bone marrow macrophage(s); MNC, mononuclear cell; ANOVA, analysis of variance.

Stress-induced Osteoclast Formation via *Hsf1*

M-CSF) was prepared as described by Yeung *et al.* (30). For tartrate resistant acid phosphatase (TRAP) histochemical staining, fast red violet LB salt (F-1625), naphthol AS-MX phosphate, and dimethylformamide were purchased from Sigma-Aldrich.

Animals—C57Black/6 mice were obtained from Monash Animal Services (Monash University, Clayton, VIC, Australia). The mice were maintained at the Monash Medical Centre Animal Facility (Clayton, VIC, Australia) according to procedures approved by the Monash Medical Centre Animal Ethics Committee B (Clayton, VIC, Australia), authorization no. MMCB-2011/19. The C;129-*Hsf1*^{tm11jb}/J (stock no. 010543) (31) were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained in the Animal Resource Laboratories of Monash University (Clayton, VIC, Australia) according to standard husbandry and breeding procedures approved by the Monash Animal Research Platform (MARP) 2 Animal Ethics Committee (Clayton, VIC Australia), authorization no. SOBSB/B/2010/28BC. Mice were maintained on a BALB/cx 129SvEV background, and intercrossed *Hsf1*^{+/-} mice were used to generate *Hsf1*^{+/+}, *Hsf1*^{+/-}, and *Hsf1*^{-/-} mice. Mouse genotypes were determined by PCR according to the standard protocol for the C;129-*Hsf1*^{tm11jb}/J strain provided by The Jackson Laboratory. All mice used in the experiments were age-matched females.

Cell Lines and Culture—RAW264.7 cells were purchased from the ATCC and were maintained in minimal essential medium- α (MEM) (Invitrogen) containing 10% FBS (Thermo Fisher Scientific), penicillin (10000 units/ml), and streptomycin (10000 units/ml) (Invitrogen), and HEPES (Invitrogen). All osteoclast formation assays utilized this medium (MEM/FBS). Primary bone marrow cells for culture were immediately isolated from humanely killed, 6- to 12-week-old mice by flushing the bone marrow cavity of the long bones with PBS in accordance with the MARP Animal Ethics Committee (Monash University, Clayton, VIC, Australia) authorization MARP/2011/048. Primary bone marrow macrophage (BMM) cultures were maintained in L-cell conditioned medium to induce BMM proliferation, as described previously (32) in RPMI 1640 medium (Invitrogen) supplemented with penicillin and streptomycin and 10% heat-inactivated FBS (MEM/HIFBS). All cells were maintained in a 37 °C incubator in a humidified atmosphere containing 5% CO₂.

***Hsf1* shRNAmir Knockdown and *Hsf1* Ectopic Overexpression**—For *Hsf1* knockdown, GIPZ lentiviral shRNAmir constructs (V2LMM_226824, V2LMM_82329, V2LMM_82328, V3LMM_415511, and V3LMM_415512) targeted toward mouse *Hsf1* and a GIPZ non-silencing control lentiviral shRNAmir construct (RHS4346) were purchased from Thermo Scientific. The non-silencing control and the targeted mouse GIPZ shRNA constructs were transiently cotransfected with psPAX2 and pMD2.G packaging constructs into HEK293T cells using Lipofectamine LTX according to the instructions of the manufacturer (Invitrogen). The medium was replaced 16 h later, and, after a further 24 h, the lentiviral-conditioned medium was collected and filtered using a 0.45- μ m filter. RAW264.7 cells were transduced by the addition of the lentiviral-conditioned medium for a period of 24 h with the addition of 10 mg/ml of Polybrene. Cells were then grown in standard medium, and transduced cells were

selected on the basis of GFP expression using FACS (Flowcore Platform, Monash University) with the selection gates being set to normalize GFP fluorescence intensity between the non-silencing and *Hsf1*-silencing shRNAmir-expressing cells. The most efficient knockdowns were achieved by using the V3LMM_415512 and V2LMM_82329 shRNAmirs, which were used for subsequent experiments and are referred to as mir4 and mir5, respectively.

To ectopically overexpress mouse *Hsf1* in RAW264.7 cells, a retroviral expression system was employed. The pBABE-*Hsf1*-IRES-mCherry retroviral construct was generated by excision of mouse *Hsf1* from the *Hsf1* construct pcDNA3.1(+)-mHsf1 (provided by Richard Voellmy, University of Miami, FL) using HindIII endonuclease refilled by T4 DNA polymerase to generate blunt ends, and this was further digested with EcoRI endonuclease. The resulting product was then ligated with pBABE-puro-IRES-mCherry (33) that had been linearized by BamHI digestion, end-filled with T4 DNA polymerase, and then digested with EcoRI endonuclease. The correct orientation of the mHSF1 insert was confirmed by diagnostic endonuclease digestion. HEK293T cells were cotransfected with pCL-Ampho packaging vector (Imgenex, San Diego, CA) and pBABE-*Hsf1*-IRES-mCherry using Lipofectamine LTX (Invitrogen). Retroviral-conditioned medium generation, RAW264.7 transduction, and selection of mCherry-expressing transduced cells by FACS were performed according to the lentiviral approaches stated previously.

Osteoclast Progenitor Differentiation and Survival Assays—Osteoclasts were generated by culturing RAW264.7 cells for a 6-day period in 96-well plates at a density of 5×10^3 cells/well in MEM/FBS, 20 ng/ml RANKL and in the presence or absence (vehicle control) of HSP90 inhibitors and other stress-inducing agents, as indicated under “Results.” The medium and the agents were replaced at day 3, and on day 6, cells were fixed with 4% formaldehyde and histochemically stained for TRAP as described previously (34). TRAP-positive multinucleated cells (MNCs) containing three or more nuclei per cell, quantified using an inverted light microscope, were counted as osteoclasts. To generate osteoclasts from primary murine cells, bone marrow cells were flushed from bisected long bones of C57black6/J, wild-type (*Hsf1*^{+/+}), heterozygous (*Hsf1*^{+/-}), or knockout (*Hsf1*^{-/-}) C;129-*Hsf1*^{tm11jb}/J mice with PBS. Cells were centrifuged and then resuspended in MEM/FBS. Bone marrow cells (10^5 cells/well) were stimulated by 20 ng/ml RANKL and 25 ng/ml M-CSF in the presence or absence of the HSP90 inhibitor 17-AAG for 6 days. Cells were then fixed and stained histochemically for TRAP and osteoclast numbers counted. For BMM preparation for Western blotting, bone marrow cells (10^6 cells/ml) were suspended in RPMI/HIFBS supplemented with 30% L-cell-conditioned medium (30, 32), incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 3 days, and then, the non-adherent cell fraction was removed. The resulting adherent proliferating cells were then prepared for analysis. These cells were able to form numerous osteoclasts with RANKL/M-CSF treatment, as described previously (32). For cell survival assays RAW264.7 cells were seeded at 5×10^3 cells/well and treated with a range of HSP90 inhibitor concentrations. After a period of 96 h, cells were fixed in 50%

TCA at 4 °C for 1 h, followed by five washes in distilled water. Cells were stained with sulforhodamine B (Sigma-Aldrich), rinsed, and then cell-bound sulforhodamine B was solubilized in 150 μ l of 10 mM Tris-HCl (pH 10.5). The absorbance at 550 nm was measured by spectrophotometry using a Multiskan FC absorbance plate reader (Thermo-Lab Systems, MA).

Immunoblot Analysis—Immunoblot analysis was performed as described previously (20, 22, 35). Briefly, cell lysates were generated using modified radioimmune precipitation assay buffer (50 mmol/liter Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/liter NaCl) containing phosphatase and protease inhibitor mixture (Sigma-Aldrich), sonicated, and then clarified by centrifugation. Protein concentrations were determined using the BCA protein assay according to the instructions of the manufacturer (Thermo Fisher Scientific). Cell lysates were run on 4–12% BisTris gradient SDS-PAGE electrophoresis gels with MES SDS running buffer (Invitrogen) under reducing conditions and transferred to Immobilon-P PVDF membranes (Merck Millipore). Membranes were blocked for 1 h with 3% milk powder (Diploma, Fonterra Food Services, Mount Waverley, Australia) dissolved in PBST (PBS + 0.1% Tween 20). Membranes were then incubated overnight at 4 °C with appropriate primary antibodies. Immunoblot visualization was achieved by incubation with appropriate IgG HRP-conjugated secondary antibodies and an ECL detection system (Supersignal West Pico, Thermo Fisher Scientific) according to the instructions of the manufacturer.

Statistical Analysis—Data were analyzed using Prism 5 software (GraphPad, San Diego, CA), and statistical significance was determined using ANOVA/Dunnett's post hoc test. Quantitative data are presented as mean \pm S.E. of three or more pooled experiments, and significance is represented graphically by *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$.

RESULTS

HSP90 Inhibitors Enhance Osteoclast Formation in Association with Induction of the Heat Shock Response—To investigate the role of the HSR and, more specifically, Hsf1, in 17-AAG actions on osteoclastogenesis, we first examined the effects of different HSP90 inhibitors on the maturation of murine RAW264.7 cells, a bipotential osteoclast/macrophage progenitor cell line that responds strongly to RANKL treatment by forming osteoclasts. RAW264.7 cells were treated with a submaximal concentration of RANKL (20 ng/ml) that is sufficient to cause low levels of osteoclast formation over 6 days of incubation. Osteoclast formation was completely dependent on RANKL treatment; *i.e.* in cultures where RANKL was omitted, osteoclast formation or mononuclear TRAP⁺ cells were never seen, as described previously (22). Unlike primary bone marrow cells or BMM, RAW264.7 cells form osteoclasts without M-CSF treatment; *i.e.* they require only RANKL stimulation.

Treatment of RAW264.7 cells with 17-AAG induced a dose-dependent increase in Hsp70 (Hspa1a) protein expression, consistent with induction of the HSR (Fig. 1A), and, consistent with previous findings (20, 21), 17-AAG also increased RANKL-stimulated osteoclast formation in a dose-dependent manner (Fig. 1, B and C). To confirm that this response was not unique to RAW264.7 cells, examination of the effects of 17-AAG in

RANKL- and M-CSF-stimulated primary bone marrow cells derived from C57Black/6 mice was performed. As with that of the RAW264.7 cell line, 17-AAG significantly increased Hsp70 expression (Fig. 1D), and this was associated with a marked increase in osteoclast formation (Fig. 1, E and F). It was noted that the primary bone marrow cultures were more sensitive to 17-AAG with respect to the induction of the HSR and that this correlated with increased osteoclast formation.

In RAW264.7 cells, 17-DMAG, a HSP90 inhibitor that is structurally related to 17-AAG but more potently inhibits HSP90, also enhanced both Hsp70 protein levels (Fig. 2A) and RANKL-induced osteoclast formation (Fig. 2B) in a dose-dependent manner, although at notably lower concentrations to that of 17-AAG (Fig. 1, A and B).

To determine the scope of the effect, we examined whether the structurally unrelated HSP90 inhibitor radicicol had similar effects to that of 17-AAG and 17-DMAG. Despite the differing structure of radicicol, it was also found to significantly increase Hsp70 levels (Fig. 2C) and significantly increase osteoclast formation (Fig. 1D). However, in contrast to these findings, the HSP90 inhibitors coumermycin A1 (Fig. 2E) and novobiocin (Fig. 2G) did not significantly increase Hsp70 levels, thus failing to induce a robust HSR. Moreover, coumermycin A1 (Fig. 2F) and novobiocin (Fig. 2H) did not significantly increase osteoclast formation. These two HSP90 inhibitors bind the C-terminal region of HSP90 and, thus, have a different mode of action to that of 17-AAG, 17-DMAG, and radicicol. These results are consistent with the notion that activation of Hsf1 and its ability to induce the HSR induced by N-terminal HSP90 inhibitors enhances RANKL-elicited osteoclast formation.

Pharmacological Inhibition of Hsf1 Reduces 17-AAG Enhancement of RANKL-induced Osteoclast Differentiation—To further investigate whether the HSR was mechanistically important for 17-AAG-enhanced osteoclast formation, we examined whether pharmacological inhibition of the HSR would abrogate the 17-AAG-mediated effect. Quercetin and KNK437 are two compounds that have been shown to inhibit the HSR via impacting Hsf1 functionality (36, 37). Consistent with this, treatment of RAW264.7 cells with KNK437 (Fig. 3A) dose-dependently inhibited the induction of Hsp70 protein expression by 17-AAG and significantly abrogated the effects of 17-AAG on RANKL-stimulated osteoclast formation (Fig. 3B), indicating a potential influence of Hsf1 on osteoclast formation. Quercetin was also found to inhibit Hsp70 induction by 17-AAG (Fig. 3C) and also significantly reduced the effects of 17-AAG upon osteoclast formation (Fig. 3D). Of the two compounds, KNK437 was more potent at inhibiting Hsp70 induction and, consistent with this, was more effective at blocking 17-AAG effects upon osteoclast formation (Fig. 3B). Interestingly, the enhancement of osteoclast differentiation by TGF β , a cytokine known to stimulate NFATc1 expression (unlike 17-AAG (22)) and augment RANKL signals (38, 39), was not affected by KNK437-mediated inhibition of Hsf1 (Fig. 3E). Thus, this demonstrated a specificity of action of KNK437 upon stress-mediated osteoclast formation and, more importantly, that of Hsf1 in 17-AAG-enhanced osteoclast differentiation.

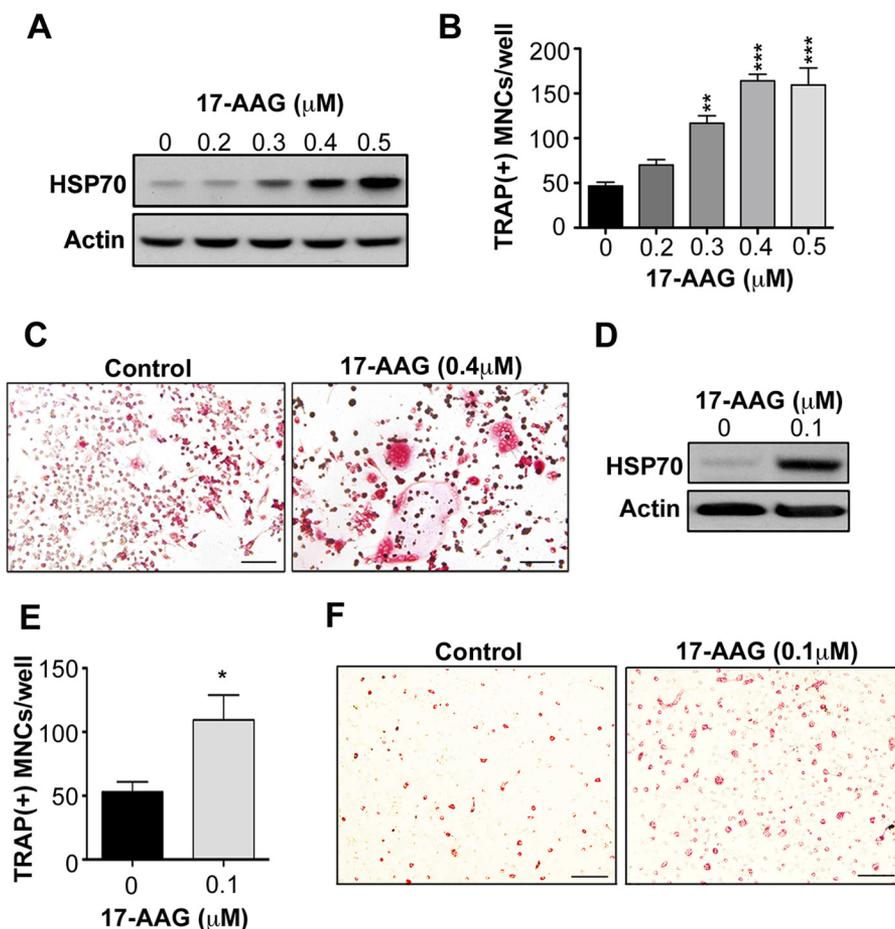


FIGURE 1. 17-AAG enhances RANKL-dependent osteoclastogenesis and induces an HSR. *A*, murine osteoclast progenitor RAW264.7 cells showed an increase in Hsp70 levels (immunoblot analysis) after treatment with indicated concentrations of 17-AAG for 24 h. *B*, RAW264.7 cells were cultured in 20 ng/ml RANKL and the indicated concentrations of 17-AAG for 6 days, and osteoclasts (TRAP-positive MNCs) were counted. 17-AAG treatment dose-dependently enhanced RANKL-dependent osteoclast formation. *C*, photomicrographs of osteoclasts formed in RANKL-treated RAW264.7 cell cultures showing increased osteoclast formation in 17-AAG-treated (0.4 μM) cells compared with vehicle control (*Veh. Ctl.*). *Red*, TRAP staining. *Scale bars* = 100 μm. *D*, immunoblot analysis of BMMs demonstrated elevated Hsp70 protein levels after treatment with 17-AAG (0.1 μM) after a 24-h treatment period. *E*, as with RAW264.7 cells, mouse bone marrow cells cultured in 20 ng/ml RANKL and M-CSF for 6 days demonstrated a significant increase in TRAP-positive MNCs with 0.1 μM 17-AAG treatment. *F*, photomicrographs of osteoclasts formed in mouse bone marrow cells cultured in 20 ng/ml RANKL and M-CSF for 6 days. Cultures with 17-AAG (0.1 μM) showed an increase in TRAP-positive MNCs when compared with vehicle control. *Red*, TRAP staining. *Scale bars* = 100 μm. *Error bars* represent the mean ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to RANKL (20 ng/ml)-treated vehicle control using ANOVA/Dunnett's post hoc test.

Hsf1 Knockdown Impairs the Effect of 17-AAG on Osteoclastogenesis—To demonstrate a specific involvement of Hsf1 upon 17-AAG actions in osteoclast formation, we reduced Hsf1 expression by RNA interference methods. We utilized an shRNAmir approach on the basis of the design of the primary microRNA-30 transcript allowing for processing via the endogenous RNAi pathways and allowing for more specific silencing than conventional shRNAs. RAW264.7 cells were transduced using lentiviral constructs that expressed either a non-silencing shRNAmir that had no homology to any known mammalian genes or shRNAmirs with specificity for mouse *Hsf1*. Immunoblot analysis of Hsf1 levels confirmed the efficient knockdown of Hsf1 in RAW264.7 cells using two independent shRNAmirs (Fig. 4A). Consistent with the knockdown of Hsf1, the 17-AAG-mediated induction of Hsp70 was abrogated significantly (Fig. 4A). In non-silencing shRNAmir control RAW264.7 cells, 17-AAG strongly enhanced osteoclast formation, whereas knockdown of Hsf1 by mir4 or mir5 significantly reduced the effects of 17-AAG upon osteoclast formation (Fig. 4B), consis-

tent with the effects of the pharmacological inhibition of Hsf1. Of interest, 17-AAG did not increase the steady-state levels of Hsf1, but, consistent with it being a HSP90 client protein, Hsf1 levels were reduced. To ensure that abrogation of 17-AAG-mediated effects upon osteoclast formation because of Hsf1 knockdown was not merely a result of an increased cell death, we tested the sensitivity of the RAW264.7 cells to 17-AAG-mediated cell death. In standard cell survival assays examining increasing 17-AAG concentrations, no differences were observed between the non-silencing control and Hsf1 knockdown cells (Fig. 4C), indicating that any effects on osteoclast formation were not due to alterations in cell survival.

Overexpression of Hsf1 Enhances 17-AAG Effects on RANKL-induced Osteoclast Differentiation—To further investigate the influence of Hsf1 in 17-AAG-enhanced osteoclast differentiation, we examined the effect of overexpressing mouse wild-type Hsf1 in the RAW264.7 cell line. To achieve this, we transduced RAW264.7 cells with retroviral vectors expressing mCherry (control) or wild-type Hsf1 (Hsf1^{WT}). Immunoblot analysis

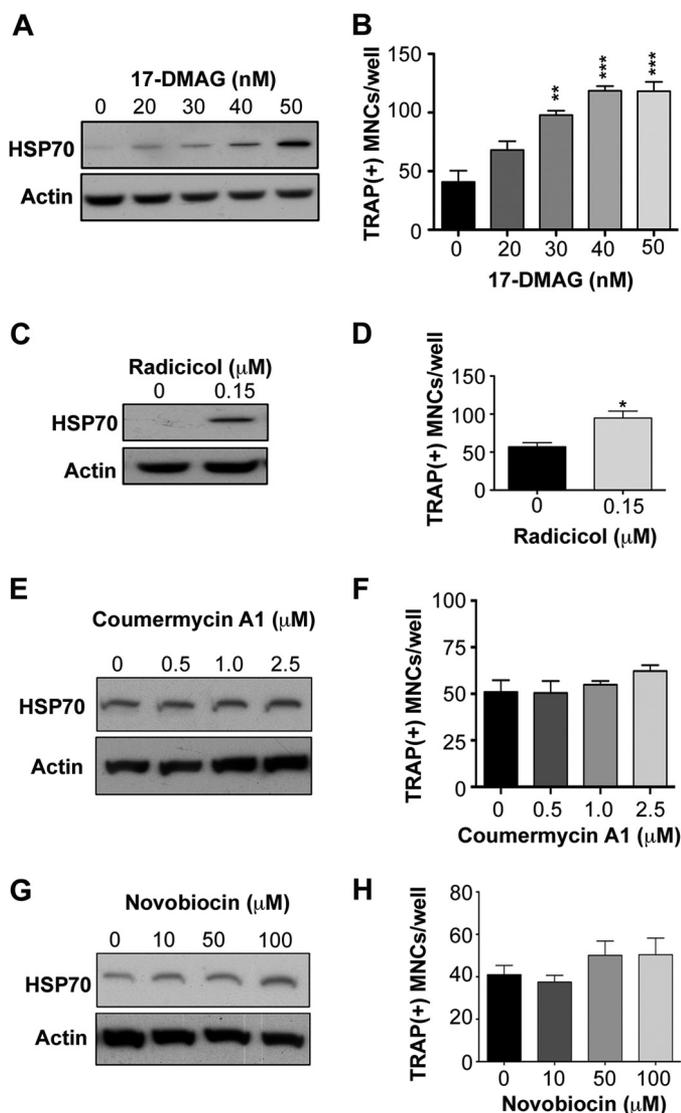


FIGURE 2. Induction of an HSR by HSP90 inhibitors associates with increased osteoclast formation. A, Hsp70 protein levels in RAW264.7 were dose-dependently increased by 17-DMAG treatment after a 24-h period. B, 17-DMAG significantly increased osteoclast formation in RANKL-treated RAW264.7 cells. C, the structurally unrelated HSP90 inhibitor radicicol increased Hsp70 levels in RAW264.7 cells after a 24-h treatment period. D, radicicol significantly increased osteoclast formation in RAW264.7 cells when compared with vehicle control. The coumermycin A1 (E and F) and novobiocin (G and H) HSP90 inhibitors did not induce a heat shock response, indicated by a failure to increase Hsp70 levels in RAW264.7 cells and also failed to increase osteoclast formation. Error bars represent the mean \pm S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to RANKL (20 ng/ml)-treated vehicle control using ANOVA/Dunnett's post hoc test.

demonstrated that, although there was a strong overexpression of Hsf1 in the pBABE-Hsf1WT-transduced RAW264.7 cells, Hsf1 was maintained in an inactive state, as demonstrated by the comparative steady-state levels of Hsp70 and Hsp105 between control cells and Hsf1-overexpressing cells (Fig. 5A). However, upon treatment with increasing concentrations of 17-AAG, Hsf1-overexpressing cells demonstrated an augmented response, denoted by increased Hsp70 and Hsp105 expression (Fig. 5A) in comparison with the vector control (pBABE-mCherry)-transduced cells. Consistent with this finding, RAW264.7 cells overexpressing Hsf1 were more sensitive

to the effects of 17-AAG in enhancing osteoclast differentiation (Fig. 5B). Notably, however, Hsf1 overexpression did not alter the osteoclastogenic potential of RAW264.7 cells in the absence of 17-AAG. Therefore, elevated Hsf1 levels appeared to sensitize cells to the actions of 17-AAG rather than directly enhance osteoclast differentiation.

17-AAG-enhanced Osteoclast Formation Is Impaired in Primary Bone Marrow Cells Derived from Hsf1^{-/-} Mice—To extend our findings, we examined the role of Hsf1 in 17-AAG-enhanced osteoclast formation in primary cells using bone marrow cells derived from mice that were wild-type (Hsf1^{+/+}), heterozygous (Hsf1^{+/-}), and null (Hsf1^{-/-}) for Hsf1. Immunoblot analysis showed that the expression level of Hsf1 in BMM derived from Hsf1^{-/-} mice was undetectable, whereas its level of expression in Hsf1^{+/-} BMM was observed to be significantly lower than those isolated from wild-type (Hsf1^{+/+}) mice (Fig. 6A). Consistent with the steady-state levels of Hsf1 in isolated BMM, the induction of Hsp70 by 17-AAG was absent in the Hsf1^{-/-} BMM and reduced significantly in the Hsf1^{+/-} BMM when compared with Hsf1^{+/+} BMM (Fig. 6A). We then examined osteoclast formation in Hsf1^{+/+}, Hsf1^{+/-}, and Hsf1^{-/-} bone marrow cells (stimulated with 20 ng/ml RANKL and M-CSF) in the presence of 17-AAG. We found that, in Hsf1^{-/-} cell cultures, 17-AAG failed to significantly elevate osteoclast numbers relative to vehicle control cultures, whereas in Hsf1^{+/+} bone marrow cultures, 17-AAG significantly enhanced osteoclast differentiation (Fig. 6, B and C). Bone marrow cell cultures from Hsf1^{+/-} mice also showed a marked impairment of 17-AAG-enhanced osteoclast formation, consistent with the decreased steady-state and activated levels of Hsf1 in these cells (Figs. 6, B and C).

17-AAG Treatment Enhances MITF Levels in an Hsf1-dependent Manner—MITF has been shown to be a critical regulator of osteoclast formation and function (40–42), although the regulation of MITF protein expression in osteoclasts is not well characterized. We have shown previously that, although 17-AAG has no enhancing effect upon major RANKL-elicited intracellular signaling components (e.g. NFκB, c-fos, and NFATc1), we determined that 17-AAG did potently enhance the cellular protein levels of MITF (22). Therefore, we investigated whether 17-AAG enhanced MITF protein levels in a manner that was mediated via Hsf1. We found that increased MITF protein levels caused by 17-AAG treatment of RAW264.7 cells was reduced significantly by pharmacological inhibition of Hsf1 by KNK437 treatment (Fig. 7A). Similarly, knockdown of Hsf1 by shRNAmir also inhibited the effect of 17-AAG upon MITF protein induction (Fig. 7B). Examination of primary BMM cultures isolated from Hsf1^{-/-} mice demonstrated that MITF protein was low or undetectable by immunoblot analysis, either with or without 17-AAG treatment (Fig. 7C). Conversely, overexpression of Hsf1 in RAW264.7 cells resulted in the elevation of MITF levels when compared with control cells and was increased further with 17-AAG treatment (Fig. 7D). These results indicate that 17-AAG increased RANKL-induced osteoclast differentiation with increased MITF protein levels, which was mediated by the action of Hsf1.

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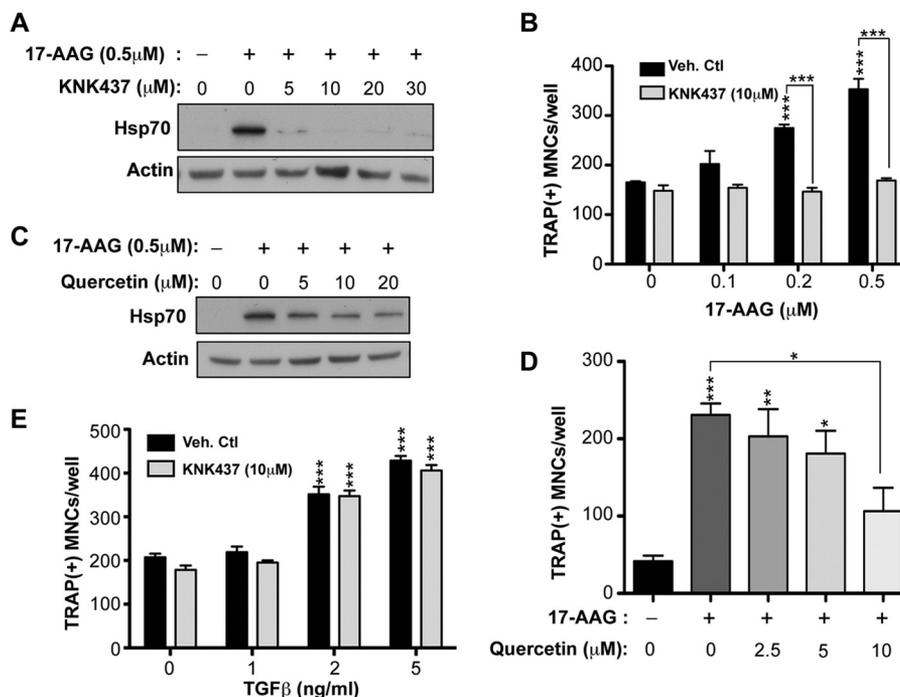


FIGURE 3. Inhibitors of Hsf1 decrease the effects of 17-AAG, but not TGF β , on osteoclastogenesis in RANKL-treated RAW264.7 cells. *A*, immunoblot analysis demonstrated that 24-h cotreatment of RAW264.7 cells with KNK437 ablated 17-AAG-induced Hsp70 protein induction. *B*, KNK437 inhibited 17-AAG-enhanced osteoclast formation in RAW264.7 cells cultured for 6 days in the presence of 20 ng/ml RANKL. *Veh. Ctl.*, vehicle control. Quercetin reduced the effects of 17-AAG treatment on Hsp70 protein expression after 24 h of treatment (*C*) and inhibited the effects of 17-AAG upon enhanced osteoclast numbers in RANKL (20 ng/ml) treated RAW264.7 cells (*D*). *E*, RAW264.7 cells cultured with RANKL showed an increase in osteoclast numbers with TGF β treatment. However, no effects of KNK437 treatment were observed. Error bars represent the mean \pm S.E. of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to RANKL-treated (20 ng/ml) vehicle control using ANOVA/Dunnett's post hoc test.

Ethanol and Chemotherapeutic Agents Enhance Osteoclast Differentiation Potentially Mediated by Hsf1—Hsf1 is activated to counteract cellular damage and death caused by proteotoxicity of a wide variety of chemical agents. In addition to HSP90 inhibitors, many cytotoxic chemotherapeutic agents also potently activate Hsf1 and the HSR (43–46). Interestingly, some cytotoxic chemotherapeutic agents are also known to promote osteoclast formation (13). We hypothesized that at least some stress-inducing agents, including some currently used cytotoxic chemotherapeutics, may directly enhance osteoclastogenesis via activation of Hsf1 and the HSR (in a manner similar to that of 17-AAG) if the agent was not too directly toxic to RAW264.7 cells. We examined both ethanol, an oxidative stressor that has been shown previously to enhance osteoclastogenesis and induce the HSR (47, 48), and two cytotoxic chemotherapeutic agents, doxorubicin and methotrexate. Immunoblot analysis demonstrated that ethanol (Fig. 8A), doxorubicin (Fig. 8B), and methotrexate (Fig. 8C) all increased Hsp70 protein expression in a dose-dependent manner, consistent with their activation of Hsf1 and the HSR. Consistent with our previous findings regarding HSP90 inhibitors and the HSR, we observed that these three stressors, ethanol, doxorubicin, and methotrexate, all enhanced RANKL-dependent osteoclast formation in a dose-dependent manner in RAW264.7 cells (Figs. 7, D–F). As with 17-AAG, pharmacological inhibition of the HSR and Hsf1 by KNK437 in RAW264.7 cultures treated with ethanol (Fig. 7G), doxorubicin (Fig. 7H), or methotrexate (Fig. 7I) inhibited the pro-osteoclastic effects of the agents, although KN437 did not completely ablate the

effects of methotrexate. In sum, these results demonstrate that, in addition to 17-AAG, other compounds that can induce Hsf1 activation and the HSR are also able to enhance RANKL-induced osteoclastogenesis, potentially through a mechanism that is at least partly Hsf1-dependent.

DISCUSSION

The ability of 17-AAG to cause bone loss and to increase breast and prostate tumor growth and invasion in bone in murine models indicates that this compound has potentially serious negative effects on bone mass (20, 21). Although 17-AAG itself is not likely to be used clinically, functionally similar, second-generation HSP90 inhibitors are currently undergoing clinical trials and may enter the clinic in the future. Thus, it is imperative to elucidate their effects on bone. 17-AAG and other HSP90 inhibitors have profound stimulatory effects on osteoclast formation (20–22, 49), although contributing influences of other cells to the observed bone loss cannot be ruled out (50). In addition, increases in osteoclast numbers and consequent increased bone resorption potentially increase the risk of metastatic tumor growth in bone because of the release of tumor growth factors from the bone matrix (51).

In this study, we demonstrated that 17-AAG and other stressors act in an Hsf1-dependent manner to increase osteoclast formation from their progenitors and that this may involve an increase in the levels of the transcription factor MITF. We investigated Hsf1 involvement in 17-AAG using a number of approaches, including the use of pharmacological inhibition of Hsf1 and HSR by KNK437. This compound is a potent inhibitor

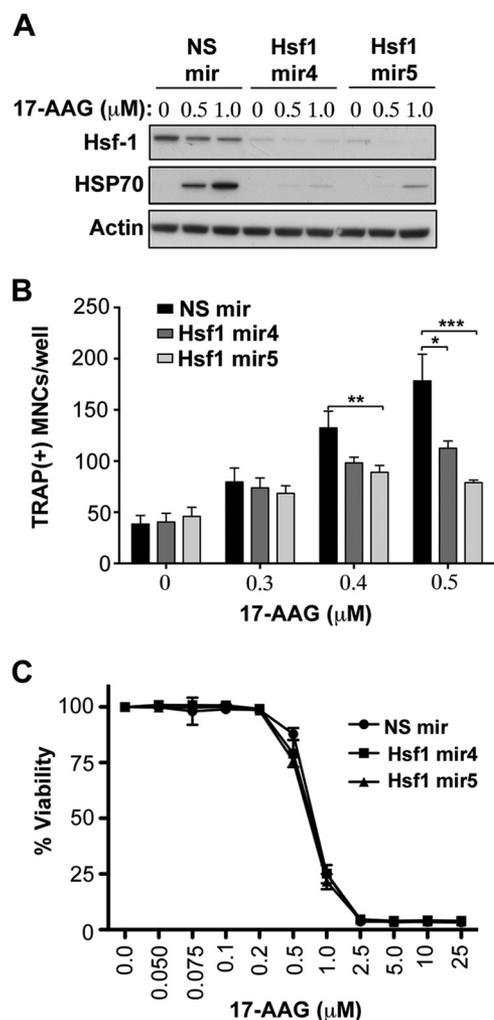


FIGURE 4. Hsf1 knockdown greatly reduces the effect of 17-AAG upon RANKL-induced osteoclastogenesis. A, lentivirus-transduced RAW264.7 cells with non-silencing (*NS mir*) or *Hsf1*-targeting shRNAmir (*mir4* and *mir5*) constructs were treated with the indicated concentrations of 17-AAG for 24 h. Immunoblot analysis demonstrated decreased Hsf1 protein levels, confirming knockdown. 17-AAG induction of Hsp70 protein was also impaired in the *Hsf1* knockdown RAW264.7 cells. B, *Hsf1* knockdown resulted in a significant reduction of the effects of 17-AAG on osteoclast formation in RAW264.7 cells cultured in 20 ng/ml RANKL. C, a dose-response survival assay over 96 h in RAW264.7 cells with indicated concentrations of 17-AAG demonstrated that *Hsf1* knockdown had no significant effect upon RAW264.7 cell survival. Error bars represent the mean \pm S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to RANKL-treated (20 ng/ml) vehicle control using ANOVA/Dunnett's post hoc test.

of Hsf1-induced expression of HSPs, such as HSP70, but does not affect the basal levels of their constitutively expressed isoforms (36). It was notable that KNK437 administration at 10 μ M completely ablated 17-AAG actions on osteoclast formation but did not decrease it below the control baseline levels induced by 20 ng/ml RANKL alone. This concentration of KNK437 also blocked Hsp70 induction. Quercetin, a widely distributed, naturally occurring flavonoid, also reduces HSP induction and has acceptable toxicity in clinical trials (52, 53). However, it should be noted that quercetin also inhibits *c-fos* and NF κ B actions that play a role in osteoclastogenesis, so its effects cannot be assumed to be via Hsf1 alone (54, 55). Quercetin is not used in any currently approved therapies but has been investigated for anticancer and anti-inflammatory actions, so its clinical use to

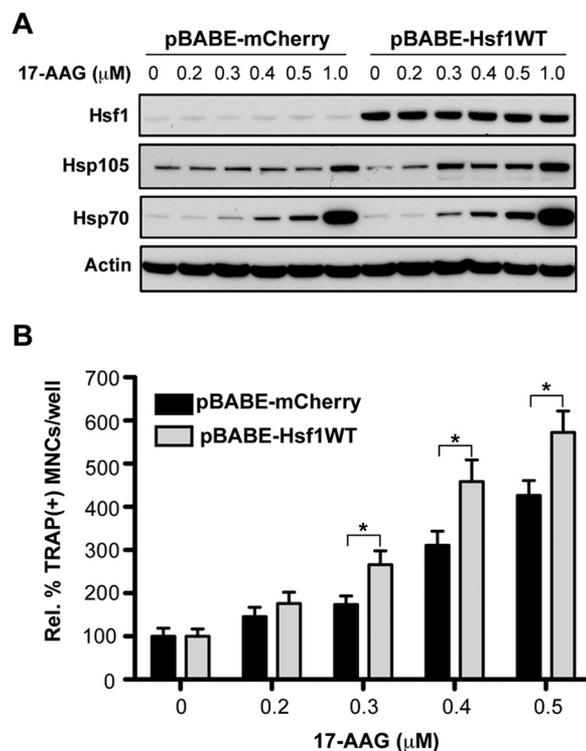


FIGURE 5. Overexpression of Hsf1 enhances 17-AAG effects on RANKL-induced osteoclastogenesis. A, RAW264.7 cells that had been retrovirus-transduced with pBABE-mCherry control or pBABE-Hsf1WT-mCherry constructs were treated with the indicated concentrations of 17-AAG for 24 h. Immunoblot analysis demonstrated that ectopic expression of Hsf1 increased the levels of Hsp70 and Hsp105 induced by 17-AAG in RAW264.7 cells. B, *Hsf1* overexpression resulted in a significant increase in TRAP-positive osteoclasts in RAW264.7 cells (cultured in 20 ng/ml RANKL) at increasing concentrations of 17-AAG for 6 days relative to vehicle control. Data are presented as the proportion relative to control (Rel. %) \pm S.E. from three independent experiments. *, $p < 0.05$ relative to RANKL-treated (20 ng/ml) vehicle control using ANOVA/Dunnett's post hoc test.

ameliorate pathological bone loss is possible but has not yet been investigated properly.

To more specifically address the role of Hsf1 in 17-AAG osteoclast effects, we targeted *Hsf1* expression by shRNAmirs in RAW264.7 cells. Knockdown of Hsf1 had a similar effect to that of KNK437 in decreasing the effect of 17-AAG on RANKL-induced osteoclast formation as well as inhibiting the induction of HSP70 by 17-AAG. Bone marrow cells from *Hsf1*^{-/-} mice were similarly defective in 17-AAG induction of osteoclast formation. However, the ability of the progenitors to form osteoclasts was not impaired because osteoclast formation in response to RANKL in *Hsf1*^{-/-}, *Hsf1*^{+/-}, and *Hsf1*^{+/+} were all comparable. Unfortunately, because of fertility problems in these mice (56), we have not been able to undertake a systematic study of the bones or the influence of stressors on their bone parameters. However, with a role for Hsf1 being established, we also sought to identify the sufficiency of Hsf1 induction in mediating 17-AAG actions on osteoclasts. Ectopic overexpression of Hsf1 (28) did not increase osteoclast formation itself but did significantly increase the osteoclastic responsiveness of RAW264.7 cells to 17-AAG. The overexpressed Hsf1 probably remained in an inactive state because we observed no alteration in the steady-state levels of HSP70, indicating that

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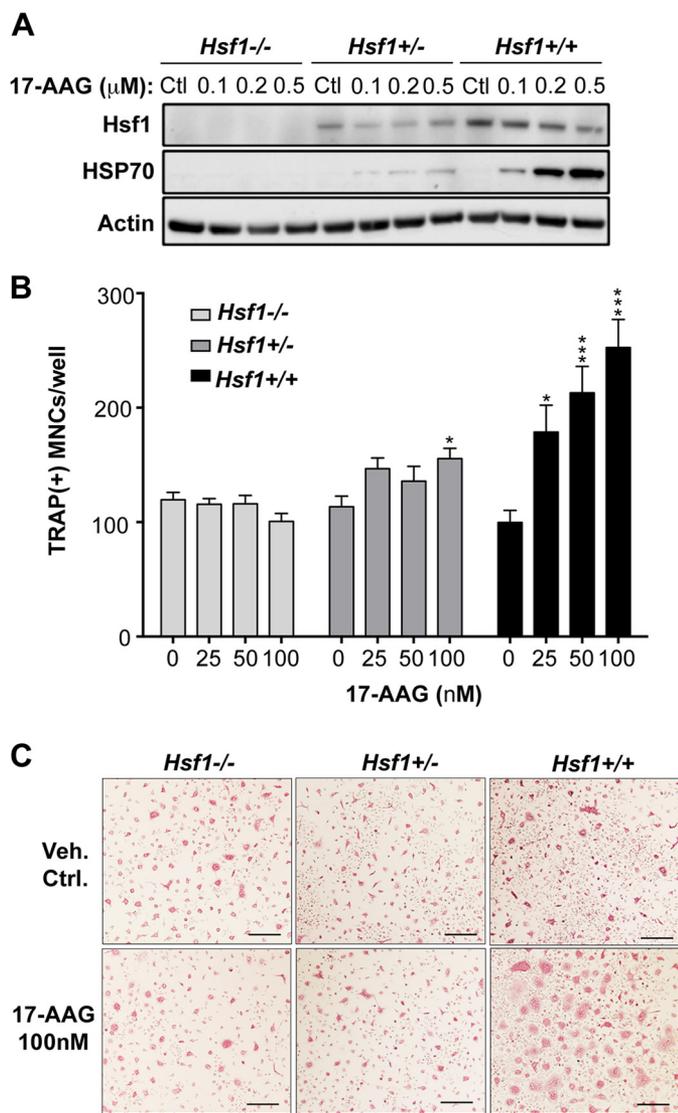


FIGURE 6. The pro-osteoclastic effects of 17-AAG are impaired in bone marrow cells derived from Hsf1 null mice. *A*, BMM derived from *Hsf1*^{-/-}, *Hsf1*^{+/-}, and *Hsf1*^{+/+} mice were treated with the indicated concentrations of 17-AAG for 24 h. Immunoblot analysis showed a complete and partial reduction of Hsf1 protein in *Hsf1*^{-/-} and *Hsf1*^{+/-} cells, respectively. Consistent with this, no induction of Hsp70 was observed in *Hsf1*^{-/-} cells, whereas in *Hsf1*^{+/-} cells, there was a substantial reduction. *Ctl*, control. *B*, primary bone marrow cells derived from *Hsf1*^{-/-}, *Hsf1*^{+/-}, and *Hsf1*^{+/+} mice were cultured in 20 ng/ml RANKL, M-CSF, and the indicated concentrations of 17-AAG for 6 days, fixed, and stained histochemically, and then TRAP-positive MNCs were counted. *Hsf1*^{-/-} cells showed a lack of response to 17-AAG, whereas *Hsf1*^{+/-} cells demonstrated a marked diminished response to 17-AAG treatment in terms of increased osteoclast formation. *C*, photomicrographs of TRAP-positive (red) osteoclast formation in *Hsf1*^{-/-}, *Hsf1*^{+/-}, and *Hsf1*^{+/+} bone marrow cultures. *Veh. Ctrl.*, vehicle control. Scale bars = 200 μm. Error bars represent the mean ± S.E. of three independent experiments. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001 relative to RANKL-treated (20 ng/ml) *Hsf1*^{+/+} control using ANOVA/Dunnett's post hoc test.

Hsf1 expression in itself is insufficient to increase RANKL-induced osteoclast formation but requires activation.

In addition to 17-AAG, we have found other benzoquinone ansamycins, such as herbimycin (20) and 17-DMAG, to also significantly increase RANKL-induced osteoclast formation. However, this action was not limited to this class of compounds because other structurally distinct HSP90 inhibitors, such as radicicol, NVP-AUY922, and CCT018159, also increased oste-

oclast formation (20, 22). Because these compounds all interact with the N-terminal ATPase site of HSP90, causing inhibition, it could be argued that ATPase site binding may be required for their common actions, and although they greatly stimulate the HSR, it may actually be the inhibition of HSP90 that is mechanistically important for enhanced osteoclast formation (28, 57). However, the HSP90 inhibitor novobiocin and its derivative coumermycin A1, known to inhibit HSP90 by binding the C-terminal of HSP90 and inhibiting its autophosphorylation (thus altering both its chaperone activity and client protein interactions), did not enhance RANKL-induced osteoclast formation (58–60). Moreover, we have found no clear correlation between the potency of the HSP90 inhibitors and their ability to induce osteoclast formation. It should also be noted that these compounds had a minimal effect on the induction of HSR, consistent with previous observations that novobiocin causes a dose-dependent decrease in Hsf1 DNA-binding and transcriptional activities (61). Combined, these results suggest that HSP90 inhibition *per se* may not enhance osteoclast formation and is consistent with a role for the involvement of Hsf1 downstream target involvement.

Although Hsf1 itself has not been suggested previously to play a role in osteoclast formation, several types of Hsf1-dependent cellular stressors have been implicated in pathological bone loss. These include chemotherapeutic agents, such as doxorubicin and methotrexate, that have been shown to cause a decrease in trabecular bone volume in a rat model (13, 62). Similarly, ethanol has been associated with the induction of cellular stress and enhances bone loss *in vivo* through the increase of osteoclast numbers (5). These observations provide circumstantial evidence that Hsf1-dependent cell stress induced by stimuli other than HSP90 inhibitors might indeed enhance osteoclastogenesis, although there is no reason to expect their actions to depend on a single mechanism. However, we confirmed here that ethanol, doxorubicin, and methotrexate cause both enhanced osteoclast formation and a cellular stress response that could be ablated by Hsf1 inhibition by KNK437. Thus, our results demonstrate, for the first time, that compounds capable of activating Hsf1-dependent stress pathways can enhance osteoclastogenesis in a manner similar to that of HSP90 inhibitors. It is important to note, however, that compounds that are simply very toxic to cells or that inhibit signaling essential to RANKL responses may not necessarily drive increased osteoclast formation.

Our findings that MITF levels may be involved in the actions of 17-AAG are particularly interesting. MITF is a transcription factor that is critical for osteoclast formation, as evidenced by the *mi/mi* strain of mice that lack MITF and are devoid of osteoclasts (63). In osteoclast progenitors, MITF levels are also enhanced by RANKL, which triggers a signaling cascade by its interaction with RANK, involving rapid induction of NFκB, p38, AP-1 and NFATc1 activity, and leading to increased MITF levels, typically after 24–48 h. The mechanism linking the elevation of MITF levels to the induction by RANKL treatment is currently controversial, but MITF is essential for many (but not all) gene expression that is required by mature osteoclasts, including TRAP (*acp5*), cathepsin K (*ctsk*), and H⁺ ion pump components (40, 64). This requires cooperation

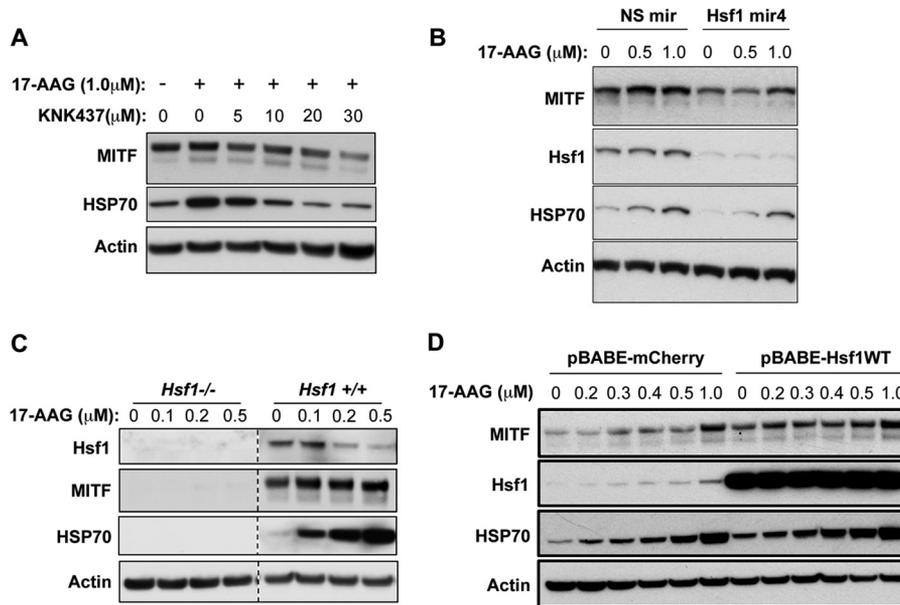


FIGURE 7. **The induction of MITF by 17-AAG osteoclast progenitor cells is dependent upon Hsf1.** A–D, protein expression was assessed by immunoblotting of the indicated cell lysates. A, Hsf1 inhibition by KNK437 ablated 17-AAG-induced MITF and Hsp70 protein levels after 24 h in RAW264.7 cells. B, RAW264.7 cells stably transduced with a lentiviral construct expressing Hsf1 shRNA mir showed a decrease in 17-AAG-induced MITF protein expression after 24 h. NS; nonsilencing. C, BMM derived from *Hsf1*^{-/-} and *Hsf1*^{+/+} mice were treated with M-CSF and the indicated concentrations of 17-AAG for 24 h. *Hsf1*^{-/-} BMM showed lower MITF protein expression both with and without 17-AAG. D, RAW264.7 stably transduced with a retroviral construct expressing Hsf1WT showed an increase in MITF protein expression after 24 h of 17-AAG treatment.

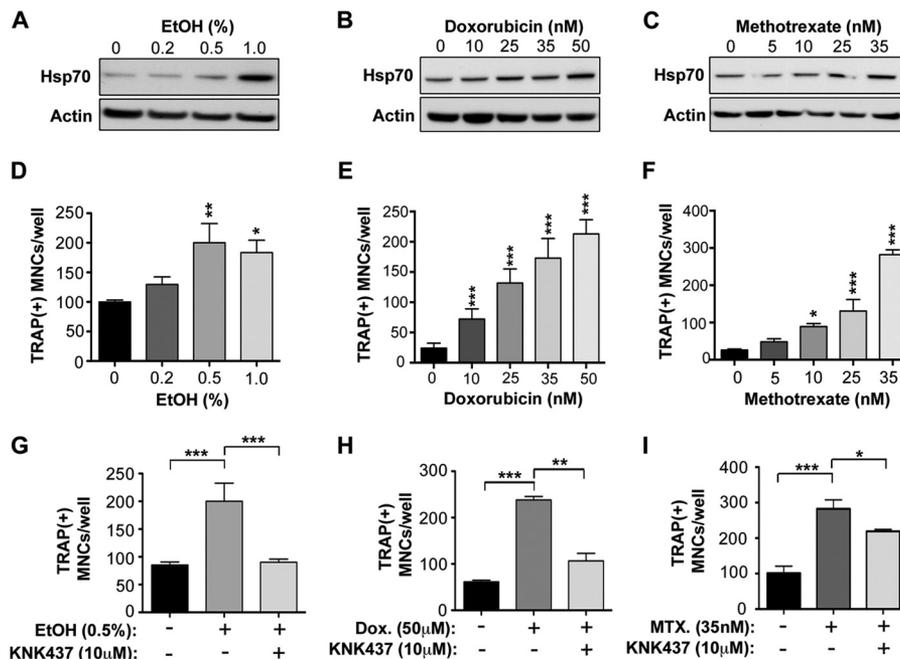


FIGURE 8. **Chemotherapeutic agents and ethanol induce a heat shock response and enhance RANKL-dependent osteoclastogenesis.** Hsp70 protein levels were induced in RAW264.7 cells by EtOH (A), doxorubicin (B), and methotrexate (C) over 24 h, as demonstrated by immunoblot analyses. D, RAW264.7 cells treated with RANKL showed a significant increase in osteoclast numbers after 6 days incubation with the indicated concentrations of ethanol. This was also observed when cultures were treated with doxorubicin (E) and methotrexate (F). G, KNK437 treatment inhibited the action of ethanol as well as that of doxorubicin (Dox., H) and methotrexate (MTX, I) on osteoclast formation in RANKL-treated RAW264.7 cells. Error bars represent the mean \pm S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to untreated control using ANOVA/Dunnett's post hoc test.

between MITF and the transcription factor PU.1 (the latter is not RANKL-dependent but binds MITF directly) together with AP-1, NF κ B, and NFATc1. MITF is a relatively late-activated factor in osteoclast commitment, and it is possible that its induction by 17-AAG results in an increased pool of MITF that may be otherwise rate-limiting. Consistent with

the latter, overexpression of MITF or the MITF-E isoform (the latter isoform is a particular target of RANKL) enhances osteoclast formation and action (65, 66). It should be noted that because MITF ablation abolishes osteoclast formation, its inhibition is not informative in addressing MITF mediation of 17-AAG effects.

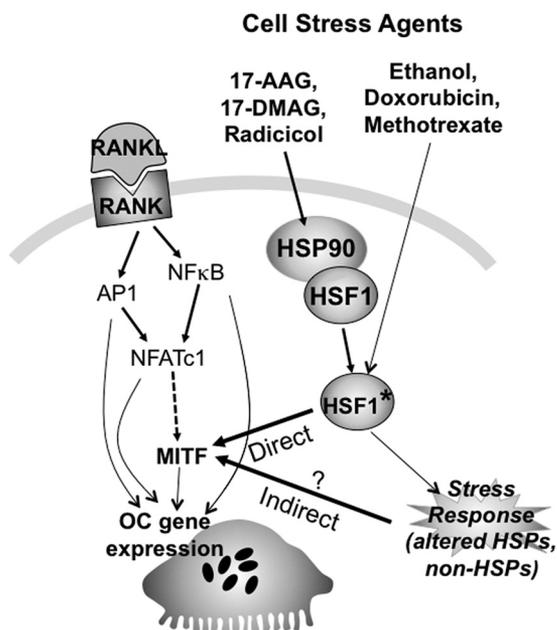


FIGURE 9. A proposed model of 17-AAG and other cell stress agents upon osteoclast formation. RANKL binding of RANK induces activation of the NF κ B, AP1, and NFATc1 transcription factors. Subsequently, expression of MIFF is raised. All four transcription factors are required for osteoclast gene expression. 17-AAG and other HSP90 inhibitors bind to HSP90, inducing a cellular stress response via Hsf1 activation that, in turn, enhances MIFF levels either by directly stimulating the *MITF* promoter or indirectly by altering the expression of HSP and/or non-HSP target genes. Other stressor agents (e.g. chemotherapeutics) induce a stress response and activate Hsf1, leading to a similar series of events. Elevated MIFF levels would, thus, result in an enhanced differentiation response of the osteoclast progenitors to RANKL and greater osteoclast formation. *Hsf1**, activated Hsf1; OC, osteoclast.

On the basis of our findings that 17-AAG increases MIFF levels and osteoclast formation in a manner sensitive to Hsf1 inhibition, we propose that 17-AAG-induced Hsf1 enhances MIFF protein levels and, thereby, amplifies the osteoclastogenic actions of RANKL. This proposed mechanism is summarized in Fig. 9 and incorporates our earlier finding that transcription factors activated early in the RANKL-dependent signaling cascade are not induced by 17-AAG, including NF κ B, c-fos (the regulated subunit of AP-1), and NFATc1 (22). The latter findings suggested to us the possibility that a late-acting factor such as MIFF would be a more obvious candidate for mediating 17-AAG actions. There is some evidence that MIFF can be induced by heat shock elicited by Hsf1 via a direct action on the *MITF* promoter (67). However, for stress-stimulated osteoclast formation, it still has to be determined whether Hsf1 acts directly via *MITF* promoter interaction, by an indirect mechanism such as increased HSP expression that may increase MIFF protein stability, or by a combination of both direct and indirect mechanisms. Nevertheless, our findings raise the possibility that any type of cell stress might enhance the levels of this transcription factor, contingent upon other effects the stressor exerts on cells. For example, some recently developed HSP90 inhibitors, such as SNX-2112 and PF-04928473, do not increase osteoclast formation at therapeutically relevant concentrations (49, 68) because, probably, these agents are more potent than 17-AAG at causing degradation of a number of HSP90 clients, such as NF κ B, c-fos, NFATc1, and PU.1, which are critical for osteoclast differentiation.

In summary, we have identified a new role for Hsf1 and cell stress in the enhanced formation of osteoclasts that may be highly significant in bone physiology and pathophysiology beyond our focus here on the HSP90 inhibitor 17-AAG. This may result from enhancement of MIFF levels, potentially through a direct action of Hsf1 on the *MITF* promoter. 17-AAG actions on osteoclasts may not be solely due to stress or Hsf1 induction, but, nevertheless, inhibition of Hsf1 seems to be a potentially useful approach to reducing osteoclast formation and osteolysis that may be induced by stressor compounds. If stress responses do directly increase the formation of osteoclasts by increasing the responsiveness of osteoclast progenitors to RANKL, we would speculate that other pathological osteolytic stimuli might act, at least in part, by increasing stress via Hsf1 activation and, thus, MIFF levels, rather than increasing local net RANKL levels. This raises the possibility that blocking cell stress might reduce excessive pathological osteolysis without necessarily abolishing the bone resorption required for normal bone repair and remodeling.

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