The Heat Shock Protein 90 Inhibitor, 17-Allylamino-17-demethoxygeldanamycin, Enhances Osteoclast Formation and Potentiates Bone Metastasis of a Human Breast Cancer Cell Line

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Abstract

Breast cancer metastasis to the bone occurs frequently, causing numerous complications including severe pain, fracture, hypercalcemia, and paralysis. Despite its prevalence and severity, few effective therapies exist. To address this, we examined whether the heat shock protein 90 (Hsp90) inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), would be efficacious in inhibiting breast cancer metastasis to bone. Utilizing the human breast cancer subline, MDA-MB-231SA, previously in vivo selected for its enhanced ability to generate osteolytic bone lesions, we determined that 17-AAG potently inhibited its in vitro proliferation and migration. Moreover, 17-AAG significantly reduced MDA-MB-231SA tumor growth in the mammary-fat pad of nude mice. Despite these findings, 17-AAG enhanced the incidence of bone metastasis and osteolytic lesions following intracardiac inoculation in the nude mouse. Consistent with these findings, 17-AAG enhanced osteoclast formation 2- to 4-fold in mouse bone marrow/osteoblast cocultures, receptor activator of nuclear factor κB ligand (RANKL)-stimulated bone marrow, and RAW264.7 cell models of in vitro osteoclastogenesis. Moreover, the drug enhanced osteoclastogenesis in human cord blood progenitor cells, demonstrating that its effects were not limited to mouse models. In addition to 17-AAG, other Hsp90 inhibitors, such as radicicol and herbimycin A, also enhanced osteoclastogenesis. A pro-osteolytic action of 17-AAG independent of tumor presence was also determined in vivo, in which 17-AAG–treated tumor-naive mice had reduced trabecular bone volume with an associated increase in osteoclast number. Thus, HSP90 inhibitors can stimulate osteoclast formation, which may underlie the increased incidence of osteolysis and skeletal tumor incidence caused by 17-AAG in vivo. These data suggest an important contraindication to the Hsp90 targeted cancer therapy currently undergoing clinical trial. (Cancer Res 2005; 65(11): 4929-38)

Introduction

The dissemination of tumor cells from their primary site of growth to distant organs is the major cause of morbidity and death among cancer patients (1, 2). It is well recognized that rather than being a random process, different cancer types display a predestination for metastasis to particular organs (3). In the case of breast cancer, the great majority of patients with advanced disease develop osteolytic metastases (4). This aspect of breast cancer causes a number of major complications for patients, including severe pain, pathologic fractures, hypercalcemia, and paralysis due to nerve compression (4–6). Moreover, once tumors have spread to the bone, they frequently do not respond to therapy, with only 20% of breast cancer patients surviving for 5 years after the discovery of bone metastasis (7).

The growth of metastases at distant sites is known to depend upon interactions between tumor cells and the host microenvironment (8). It is unknown why breast cancer cells preferentially metastasize to bone; however, it is known that the complex interplay between tumor cells and the bone microenvironment plays an important role (5, 9). Although breast cancer metastases can grow in the marrow cavity, the tumors normally promote the destruction and invasion of the bone tissue itself, which compromises the structural integrity of the bone. To achieve this, the invading tumors recruit osteoclasts, highly specialized bone resorbing cells that form rapidly from myelomonocytic progenitors present locally in large numbers in bone marrow and the circulation. This bone destruction itself can release factors that encourage tumor growth (4, 10). The role of the osteoclast in bone metastasis has led to the use of agents that target bone resorption, such as bisphosphonates, in the treatment of patients with bone metastasis (11). However, although the bisphosphonates have provided promising results (4, 12), these treatments are often palliative and do not provide substantial life-prolonging benefits to patients suffering from bone metastasis (11, 12). Therefore, there is a need to identify new approaches that would provide useful adjunct therapies for the treatment of cancers that metastasize to bone.

Heat shock protein 90 (Hsp90) is a molecular chaperone that is ubiquitously and abundantly expressed. Numerous proteins involved in the control of physiologic and pathophysiologic processes require Hsp90 for their biogenesis, regulation, and functionality (13, 14). In cancer, the expression of Hsp90 is increased when compared with that of normal tissues. Moreover, with many of its “client” proteins, such as Akt, Her2/Neu, and Raf-1,
important participants in pathways driving tumor cell survival, proliferation, and progression, Hsp90 is believed to be an excellent molecular target in cancer therapy (15–17). Moreover, a number of Hsp90 clients, such as c-Src, epidermal growth factor receptor, and matrix metalloproteinase-2 (14, 18), have been shown experimentally to play a role in bone metastasis, suggesting that Hsp90 inhibition may not only prove effective in primary tumor growth but could also be an effective inhibitor of bone metastasis (19–22). In addition, we have recently identified that Hsp90 expression correlates with bone metastasis in an in vivo mouse model (23).

Naturally occurring compounds that inhibit Hsp90 action, such as geldanamycin and radicicol, have provided evidence of the importance of Hsp90 in cancer cell growth and progression (24–26). These compounds bind to the ATP/ADP binding pocket in the NH2-terminal domain with high affinity, inhibiting Hsp90, and thus are potent inhibitors of cancer cell growth and the malignant phenotype in a number of cancer cell types, including breast (27–29). In addition, analogue compounds, such as the geldanamycin derivative 17 allylamin-17-demethoxygeldanamycin (17-AAG), have been shown to possess antitumor activity in several human xenograft models, including colon, breast, and prostate cancer (30–32) and have been used in patient clinical trials (15, 25, 33). 17-AAG has completed multi-institutional phase I clinical trials in which different schedules of drug administration have been examined and phase II trials have commenced (17, 34).

The aim of this study was to investigate whether pharmacologic inhibition of Hsp90 by the geldanamycin analogue, 17-AAG, would be effective in inhibiting the incidence and growth of osteolytic bone metastasis in an animal model of experimental bone metastasis using the human breast cancer subline, MDA-MB-231SA.

Materials and Methods

Drugs and reagents. 17-AAG was kindly provided by Dr. E. Sausville (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD); geldanamycin, radicicol, herbimycin A, human collagen IV, and prostaglandin E2 (PGE2) were obtained from Sigma Chemical, Co. (Castle Hill, NSW, Australia); Collagen I (Vitrogen 100) was obtained from Cohesion (Palo Alto, CA) and the growth factors insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF) were obtained from Becton Dickinson (Bedford, MA). Antibodies toward Hsp90α and Hsp90β were purchased from Stressgen (San Diego, CA) and antibodies to actin were purchased from Neomarkers (Freemont, CA). Recombinant murine GST-RANKL158–316 (receptor activator of nuclear factor-kB ligand, RANKL) was produced in Escherichia coli BL21 cells using a protein expression construct kindly provided by Prof. F. Patrick Ross (Washington University School of Medicine, St. Louis, MO). Human macrophage colony stimulating factor (M-CSF) was obtained from R&D Systems (Minneapolis, MN), whereas 1,25(OH)2 vitamin D3 [1,25(OH)2D3] was purchased from Wako Pure Chemical, Co. (Osaka, Japan). The C57BL/6 and BALB/c-nu/nu (nude) mice strains were obtained from Animal Resource Centre (Perth, WA, Australia).

Cell lines. The estrogen-independent human breast cancer cell subline, MDA-MB-231SA, was kindly provided by T. Yoneda (University of Texas Southwestern Medical Center, Dallas, TX). These cells had been previously generated from MDA-MB-231 cells by intracardiac inoculation and in vivo selection of cells displaying the ability to spread and grow in bone. The MDA-MB-231SA cells were isolated after being in vivo selected by passing and reselecting seven times through nude mice as described previously (35) and displayed an enhanced onset and severity of bone metastasis. MDA-MB-231SA cells were cultured routinely in DME with 10% fetal bovine serum (FBS, JHR Biosciences, Lenexa, KS). MDA-MB-231SArfp (red fluorescent protein, RFP) cells were generated through stable transfection of the pDsRed2-N1 construct (Clontech). Briefly, pDsRed2-N1 was transfected into MDA-MB-231SA using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Twenty-four hours after transfection, RFP-positive cells were isolated by fluorescence-activated cell sorting (FACS, DIVA, Becton Dickinson) and grown in culture with G-418 selection (0.675 mg/mL). After 2 weeks of selection, RFP-positive cells were once again sorted by FACS analysis and cells expressing the highest levels of RFP (10% of the total population) were retained. Cells were selected for a further 2 weeks in G-418 (Invitrogen) after which time a final round of FACS-based cell sorting was done in which 10% of the highest RFP-expressing cells were selected. The resulting cells were termed MDA-MB-231SArfp.

RAW264.7 cells and mouse primary osteoblasts were cultured in α minimal essential medium (Life Technologies, Gaithersburg, MD) containing 10% FBS (CSL Biosciences, Parkville, Australia; MEM/FBS). All osteoclast formation assays utilized this medium. RPMI 1640 (Life Technologies) containing 10% heat-inactivated FBS (RPMI/HIFBS) was used for the preparation of bone marrow macrophages (BMM) as indicated.

Western blot analysis. Cell lysates were prepared in modified radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% Na deoxycholate, 150 mmol/L NaCl] with EDTA free Complete Protease Inhibitors (Roche, Kew, Victoria, Australia). Resulting cell lysates were sonicated and protein concentrations determined by BCA protein assay following the manufacturer’s protocol (Pierce, Rockford, IL). Cell lysates were electrophoresed on a 10% SDS-PAGE gel under reducing conditions. Proteins were transferred to polyvinylidene difluoride membranes and probed with appropriate antibodies and incubated with horseradish peroxidase–conjugated secondary antibodies. Western blots were visualized by ECL detection system according to manufacturer’s instructions.

Tumor cell proliferation analysis. Cell growth was determined by the sulforhodamine B assay in a 96-well format as previously described by Skehan et al. (36).

Tumor cell migration analysis. Cell migration was determined using a 48-well microchemotaxis chamber assay using collagen type I–coated 8 μm polycarbonate membranes (Neuroprobe, Gaithersburg, MD) as previously described (37). Briefly, IGF-I and EGF were used as chemoattractants at the concentrations indicated and added to the lower wells. Cells (1 × 105/mL) were resuspended in serum-free DME containing 0.1% bovine serum albumin (DME/BSA) and added to the upper chambers (56 μL/well). Chambers were incubated at 37°C in a humidified incubator in an atmosphere of 5% CO2/95% air for 4 hours, after which the filters were removed, fixed, and stained with Diff-Quik (Baxter Scientific, McGaw Park, IL) and mounted on glass slides. Nonmigrated cells were removed by wiping with a cotton swab. At least four random fields per well (∼20 objective) were counted for quantitation of cell migration. Triplicate wells were done in each assay and the assay was repeated at least thrice.

Intracardiac inoculation model. Intracardiac injection of the MDA-MB-231SArfp cells was done as previously described (22, 38). Briefly, subconfluent cells were fed with fresh media 24 hours before intracardiac injections. Cells were removed nonenzymatically (PBS-EDTA), resuspended in DME/10% FBS medium, and then were washed thrice with PBS and resuspended in PBS at 106 cells/mL. Mice (45-week-old female BALB/c-nu/nu; Animal Resource Centre) were anesthetized with ketamine (40 mg/kg/mouse) and mounted on glass slides. Nonmigrated cells were removed by wiping with a cotton swab. At least four random fields per well (∼20 objective) were counted for quantitation of cell migration. Triplicate wells were done in each assay and the assay was repeated at least thrice.

Treatement schedules. 17-AAG was reconstituted in 10% DMSO/0.05% Tween 80 in PBS. Mice were treated on a 4-day treatment/3-day rest schedule with the daily dose of 70 mg/kg/mouse being given as a split dose.
of 35 mg/kg/mouse separated by a period of 8 hours. Administration of the drug was by i.p. injection and drug treatments were commenced 1 day after intracardiac or mammary-fat pad inoculation of tumor cells.

Analysis of bone metastases. Radiographic analysis and fluorescent imaging were used to determine tumor incidence in the bones of mice. For radiographic analysis, animals were anesthetized, laid in the prone position against film (22 × 27 cm; X-Omat AR; Eastman Kodak, Co., Rochester, NY), and exposed to an X-ray at 35 kV for 10 seconds using a Faxitron instrument (Model MX-20-20, local source; Faxitron, Corp., Buffalo, IL). Films were developed and inspected for visible bone lesions. For fluorescent imaging, at the time of harvest, culled mice were skinned and soft organs removed to enhance fluorescent sensitivity. The mouse was then imaged under fluorescence using the LAS1000Plus instrument (Fujifilm Scientific, Tokyo, Japan) using excitation-dominant wavelength of 530 nm and green emission filter of 585LP. Images were captured using Science Lab 99 software (Fujifilm Scientific).

Mammary-fat pad inoculation. Groups of 8 to 10 mice (4-5-week-old female BALB/c-nu/nu; Animal Resource Centre) received mammary-fat pad inoculation of MDA-MB-231SA cells in a mixture (1:1) of PBS and Matrigel (1.5 × 10^6 cells/15 μL) as previously described (36). Tumor growth was assessed by measuring the length and width of tumors with electronic calipers every 3 to 4 days continuously after the tumor became palpable. Volumes were calculated using the formula (length × width)^2 / 2, where the length was determined as the larger measurement. Mice were sacrificed when tumors approached 1,500 mm^3.

Murine osteoclast formation assays. Bone marrow cells were flushed from bisected long bones of C57BL/6 mice with PBS using a 10 mL syringe. Cells were centrifuged and resuspended (10^6 cells/mL) in MEM/FBS. For BMM preparation, bone marrow cells (10^6 cells/mL) were suspended in RPMI/HIFBS supplemented with 30% L-cell–conditioned medium (an impure source of secreted murine M-CSF) prepared in described in ref. 39) and incubated at 37°C for 3 days after which time the nonadherent cell fraction was removed, centrifuged, and resuspended in MEM/FBS. Primary osteoclasts were prepared by sequential collagenase digestion of neonatal mouse calvaria as previously described (40).

All osteoclast assays were done in 10-mm-diameter tissue culture wells. Primary osteoclasts (4 × 10^5 cells/well) were cocultured with bone marrow cells (10^5 cells/well) in the presence of 10 nmol/L 1,25(OH)2D3 and 100 nmol/L PGE2 in the presence or absence of 17-AAG or vehicle for 7 days. Cells were fixed and histochemically stained for tartrate-resistant acid phosphatase (TRAP) as described previously (41). TRAP-positive cells with three or more nuclei were counted as osteoclasts. Osteoclasts were also generated from bone marrow cells (10^6 cells/well) in the absence of osteoblasts using stimulation by 100 ng/mL RANKL and 25 ng/mL M-CSF (with or without 17-AAG or vehicle) for 7 days. Similarly, osteoclasts were generated from RANKL- and M-CSF–stimulated BMMs (10^5 cells/well). Experiments were done four times, each with five replicate cultures per experiment. Final representations of results were pooled data from the four individual experiments.

Human osteoclast formation assays. Human cord blood–derived progenitors were stimulated to form osteoclasts as previously described (42). Briefly, cord blood mononuclear cells were cultured in semisolid medium containing interleukin (IL)-3, granulocyte M-CSF, and stem cell factor (42) for 7 days. The resulting expanded progenitors were cultured in 6-mm-diameter wells (4 × 10^5 cells/well) in MEM/FBS with RANKL and M-CSF (with or without 17-AAG or vehicle) and TRAP-positive multinucleated (>3 nuclei) osteoclasts were counted at day 7.

Human cord blood was obtained in accordance with full ethical approval of the human ethics committee of the University of Melbourne. Experiments were done thrice, each with five replicate cultures per experiment. Final representations of results were pooled data from the three individual experiments.

Histologic analysis. Hind limbs were fixed in 10% neutral buffered formalin for 48 hours, decalcified in EDTA (pH 7.2) for 2 weeks, and processed for conventional paraffin-embedded H&E staining.

Histomorphometric analysis of long bones. Tissue specimens were fixed in 4% paraformaldehyde in PBS and embedded in methylmethacrylate as described previously (43). Undecalcified 5 μm sections of the proximal tibial were stained with toluidine blue and the secondary spongiosa was analyzed by histomorphometry according to standard procedures using the Osteomeasure system (OsteoMetrics, Inc, Decatur, GA; ref. 43).

Statistical analyses. Results are represented as mean ± SD unless otherwise stated. χ² analysis and Fisher’s exact probability test were used for tumor incidence, whereas the significance of MFP growth was determined by two-way repeated measures ANOVA (GraphPad Prism, San Diego, CA). Cell migration and osteoclast generation assays were analyzed by Student’s t test.

Results

Selection of MDA-MB-231 cells enhances incidence of bone lesions in the intracardiac inoculation nude mouse model. To study bone metastasis (i.e., the seeding and growth of tumor cells in the bone microenvironment), the intracardiac inoculation model using BALB/c-nu/nu (nude) mice was utilized (22). This methodology directly seeds cells into the arterial circulation of mice through inoculation into the left ventricle, thus allowing the stages of metastasis postintransavasation to be examined. Although this model results in bone lesions, seeding to visceral organs is also achieved (21). However, to examine bone metastasis more precisely, we obtained the human breast cancer cell subline, MDA-MB-231SA (T. Yoneda). This line, previously generated by serial in vivo passage of MDA-MB-231 cells through the bones of nude mice, displays a higher incidence, severity, and specificity for bone metastasis. In our hands, when MDA-MB-231SA cells were compared with a standard MDA MB 231 cell line in the intracardiac inoculation model, the MDA-MB-231SA displayed a bone lesion incidence of between 90% and 100% as determined by hind limb X-ray analysis after 4 weeks (Fig. 1A). In contrast, parental MDA-MB-231 cells only displayed a 20% to 30% incidence of bone metastasis after a similar period of time (Fig. 1A). Moreover, bone lesions generated by the MDA-MB-231SA cells were much larger in size when compared with those produced by MDA-MB-231 cells (Fig. 1B). Previously, we have identified two subclones of the MDA-MB-231 cell line, namely, MDA-MB-231#16 and MDA-MB-231#17, which have a high and low bone metastatic potential in the intracardiac inoculation model, respectively. Gene array profiling of these two subclones and subsequent postarray validation by Western blot analysis (Fig. 1C) showed that Hsp90β was expressed at higher levels in the highly bone metastatic MDA-MB-231#16 subclone (23). Therefore, we examined whether Hsp90 protein levels would be higher in the highly bone metastatic MDA-MB-231SA cells when compared with the parental cell line. Western blot analysis showed that Hsp90α and Hsp90β isoforms were both found to be expressed at higher levels in the MDA-MB-231SA cells (Fig. 1C), recapitulating our findings within the MDA-MB-231#16 and MDA-MB-231#17 model.

17-Allylamino-17-demethoxygeldanamycin treatment inhibits MDA-MB-231SA proliferation and chemotactic migration. To determine whether the MDA-MB-231SA cells were sensitive to Hsp90 inhibition by 17-AAG with respect to their growth, we examined the effects of varying concentrations of the drug in an adhesion-dependent proliferation assay (36). At lower concentrations of 17-AAG (0.01 and 0.1 μmol/L), there was no detectable effect upon the proliferation of the MDA-MB-231SA cells (Fig. 2A). However, when the concentration of the drug was increased to 1.0 μmol/L, a significant reduction in the rate of cellular growth was observed (Fig. 2A). In addition to 17-AAG, the Hsp90 inhibitors, radicicol and herbimycin A, were also...
inhibiting EGF-induced or the random background migration of the cells (Fig. 2B). MDA-MB-231 cells have been shown to be sensitive to effects of Hsp90 inhibition upon the IGF-I signaling pathways with reduction of IRS-1 and IRS-2 phosphorylation and the degradation of the IGF-I type 1 receptor (46). Therefore, 17-AAG is effective in inhibiting MDA-MB-231SA cell proliferation and IGF-I- and EGF-induced migration in vitro.

17-Allylamino-17-demethoxygeldanamycin treatment enhances bone metastasis to the long bones as determined by X-ray analysis. Although it is well accepted that 17-AAG is a potent inhibitor of xenograft tumor growth in a number of models, including those of breast, prostate, and colon cancers (30–32), the efficacy of the drug has yet to be assessed for its ability to reduce tumor spread to and growth in bone. To address this, we used the intracardiac inoculation model, administering 17-AAG (70 mg/kg/mouse) to the mice as a split dose on a 4-day treatment, 3-day rest schedule commencing the day after the inoculation of tumor cells. Similar dose schedules as well as higher dose schedules of 17-AAG have been previously used to show 17-AAG efficacy in breast, prostate, melanoma, and colon tumor models (30–32, 47). Moreover, in our hands, this schedule is well tolerated in nontumor and mammary-fat pad tumor-bearing mice, being below the maximum tolerated dose of 80 mg/kg/d (48).

Contrary to our expectations, mice treated with 17-AAG displayed a 2-fold increase in bone lesion incidence after 21 days when compared with the vehicle control group (Fig. 2C). Due to the general poor health and weight loss of the mice in the 17-AAG treatment group, the experiment was terminated at this point, 10 to 14 days earlier than typical. No gross histomorphologic differences were observed in the tumors of treated and nontreated groups (Fig. 2D).

17-Allylamino-17-demethoxygeldanamycin enhances spread to skeletal sites other than the long bones of nude mice. To enhance our ability to observe tumor cell spread to other sites of the skeleton that are generally more difficult to detect by X-ray analysis alone, we generated a stably transfected cell line from the MDA-MB-231SA cells that constitutively expressed RFP allowing the in vivo fluorescent imaging of tumors. The MDA-MB-231SArfp cells were generated in a nonclonal manner and displayed similar morphologic and growth characteristics as the parental MDA-MB-231SA cells (Fig. 3A and B). These cells were also sensitive to 17-AAG with respect to their in vitro proliferation (data not shown). Intracardiac inoculation of these cells into nude mice, which were subsequently treated with 17-AAG as previously described, resulted in enhanced bone metastasis as observed in the MDA-MB-231SA cells (Fig. 3C). Once again, the experiment had to be terminated after 21 days because of the poor health of the animals.

The use of full body fluorescent imaging showed the presence of the tumor cells not only in the long bones of the mice but also in the jaw, spine, and skull (Fig. 4). In addition, examination of tumor incidence in the skeleton by fluorescent imaging revealed a significant increase in the incidence of metastasis in the long bones and in the jaws of the mice (Fig. 4), the latter providing a possible explanation for the weight loss in the 17-AAG–treated mice. Although there was only a trend to an increased incidence of bone metastases in the spine, the spinal lesions detected were much larger in size (Fig. 4).

17-Allylamino-17-demethoxygeldanamycin reduces mammary-fat pad MDA-MB-231SArfp tumor growth in nude mice. To determine if 17-AAG inhibited MDA-MB-231SA tumor cell growth at the orthotopic site, MDA-MB-231SArfp cells were effective in inhibiting growth of the MDA-MB-231SA cells (data not shown). In addition to the MDA-MB-231SA cell line, we have also found that other human breast cancer lines, namely MDA-MB-231, MCF-7, MDA-MB-435, and BT-474 cells, are also growth inhibited by 17-AAG (data not shown). In addition to growth, we also examined the effect of Hsp90 inhibition upon another important parameter of tumor metastasis, that of cellular migration (44). MDA-MB-231 cells have been previously shown to migrate toward a number of growth factors and cytokines (37, 45). Using a standard microchemotaxis assay, we examined the effect of 17-AAG on MDA-MB-231SA cell migration toward EGF and IGF-I. IGF-I–induced chemomigration of MDA-MB-231SA was significantly decreased at all concentrations of 17-AAG, while only the highest concentration of 17-AAG was effective at inhibiting EGF-induced or the random background migration of
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inoculated into the mammary-fat pads of nude mice and treated with 17-AAG. As has been previously shown in breast and prostate xenograft models (31, 32), 17-AAG significantly inhibited the growth of tumors at the mammary-fat pad (Fig. 4H). Therefore, depending upon the microenvironment, 17-AAG had differential effects.

17-Allylamino-17-demethoxygeldanamycin enhances in vitro osteoclastogenesis.

To colonize bone, tumors recruit osteoclasts, which are formed from local hemopoietic progenitors. In addition, it has previously been shown that resorption of bone by osteoclasts is important in the process of bone metastasis in the intracardiac inoculation model (49). Therefore, we initially determined whether 17-AAG would affect osteoclastogenesis in vitro. Treatment of osteoblasts with osteolytic factors, such as 1,25(OH)2D3, show enhanced expression of RANKL and decreased expression of osteoprotegerin, permitting osteoclast formation (50). We thus examined the effect of 17-AAG upon osteoclastogenesis in a coculture assay in which a primary calvarial osteoblast feeder layer and the addition of PGE2 and 1,25(OH)2D3 provided the stimulus for osteoclastogenesis from bone marrow cultures. Treatment of these cocultures with 17-AAG significantly enhanced the number of osteoclasts after 7 days, although at concentrations higher than 100 nmol/L, 17-AAG resulted in no osteoclast formation due to its cellular toxicity (Fig. 5A). Whereas this data indicates that 17-AAG can enhance osteoclastogenesis, it does not define whether this is via a direct action upon the osteoclast progenitors or by an indirect mechanism through the osteoblasts. Thus, bone marrow cultures were stimulated with recombinant RANKL and M-CSF to generate osteoclasts over a 7-day period. 17-AAG treatment of these cultures resulted in a significant increase in osteoclast numbers over the period of the assay (Fig. 5B). Once again, concentrations of 17-AAG >100 nmol/L resulted in cellular toxicity and decreased the number of osteoclasts generated. 17-AAG also increased osteoclast formation in RANKL- and M-CSF–stimulated BMM, which are a highly enriched population of osteoclast/macrophage progenitors (Fig. 5C). Moreover, the osteoclast formation of the RAW264.7 cell line when these were utilized as the osteoclast progenitor cells was also increased by 17-AAG (data not shown). We have determined that 17-AAG has maximal activity upon osteoclast formation when present over the entire culture period of 7 days. However, when cultures were treated with 17-AAG for 0 to 3 or 4 to 7 days, increases in osteoclast numbers were noted although a submaximal effect was observed (data not shown).

Therefore, although this does not rule out an indirect action of 17-AAG acting through the osteoblast, it does confirm that 17-AAG can act directly upon osteoclast progenitors to enhance osteoclastogenesis. To rule out a nonspecific mechanism by which osteoclastogenesis is enhanced before cell death, cultures were treated with a number of metabolic inhibitors, proteosomal inhibitors, and rapamycin, each of which resulted in inhibition of osteoclastogenesis and, at higher concentrations, cell death. None of these agents were observed to increase osteoclastogenesis before cellular toxicity (data not shown). Moreover, use of other Hsp90 inhibitors, herbimycin A (Fig. 5D), or radicicol (data not shown), also resulted in enhanced osteoclastogenesis in RANKL/M-CSF BMM cultures, demonstrating that this effect was not specific to 17-AAG but a more general phenomenon of Hsp90 inhibitors. It was also observed that these inhibitors not only enhanced osteoclast numbers but also enhanced osteoclast fusion, increasing the nuclear number and size of the osteoclasts (Fig. 5E). Thus, the effect of the drugs on osteoclast numbers may be greater than suggested by the osteoclast numbers alone.

We next evaluated whether the actions of 17-AAG were limited to mouse models of osteoclastogenesis or whether the drug had the...
was determined by increase in hind limb metastasis as determined by X-ray analysis. Significance of mice inoculated with the MDA-MB-231SArfp cells showed a significant done at 21 days postinoculation of MDA-MB-231SArfp cells. 17-AAG treatment dependent cell growth assay.

The identification of several naturally occurring anticancer antibiotics, including geldanamycin, radicicol, and herbimycin A, which selectively inhibit the function of the molecular chaperone Hsp90, has led to the candidacy of this molecule as a novel target for cancer drug therapy. These agents and derivatives have proven highly effective at inhibiting the growth of xenograft models of colon, breast, and prostate cancer (30–32), as well as enhancing conventional therapies, such as Taxol, when used in combination (33).

However, it is the ability of a tumor cell to metastasize, rather than the growth of the primary tumor, that is the major cause of treatment failure, morbidity, and death in breast cancer patients. Despite this, the majority of preclinical investigations have examined the effects of 17-AAG upon tumor growth in subcutaneous or orthotopic models, with little being done to investigate its efficacy in the inhibition of tumor cell metastasis. To address this, we utilized an intracardiac inoculation model of tumor spread, focusing on metastasis and invasion of the bone, the most common and often the earliest site of breast cancer spread. Moreover, the bone is a tissue that represents a very different microenvironment to that of the primary tumor site, often allowing tumors to be refractory to conventional therapies (7, 51).

Our initial in vitro investigations of the effects of 17-AAG upon the proliferation and chemotactic migration of MDA-MB-231SA cells are entirely consistent with previous findings in which 17-AAG inhibited proliferation and reduced IGF-I–mediated signaling in MDA-MB-231 cells (46, 53). Additionally, our observation that 17-AAG inhibits the growth of MDA-MB-231SArfp tumors at the orthotopic site are in accordance with findings in other breast cancer models of tumor growth (27, 28). However, in contrast to these very positive indications, we have made the surprising observation that 17-AAG increases the incidence of osteolytic bone metastases and, consistent with this, had the ability to enhance the formation of osteoclasts, the specialized bone-resorbing cells essential for the destruction of bone necessary for tumor colonization. Furthermore, and consistent with this pattern of 17-AAG osteolytic action we observed, 17-AAG caused an increase treated with 17-AAG with no prior inoculation of tumor cells, using the same treatment regime of 17-AAG as in tumor-challenged mice. Despite the already low bone mass of these mice (Fig. 6A), histomorphometric analysis of the long bones after 21 days of treatment showed a significant reduction in the trabecular bone volume (Fig 6A). However, the very low bone mass in these mice meant that osteoclast and osteoblast numbers could not be properly analyzed. For this reason, and because of the immunocompromised nature of the nude mice, we examined the effects of 17-AAG upon the C57BL/6 mouse strain. As observed in the nude mice, the trabecular bone volume was significantly decreased in the C57BL/6 mice (Fig. 6A). Moreover, due to the higher bone mass in the untreated C57BL/6 mice relative to the nude mice, more meaningful data could be obtained with respect to osteoblast and osteoclast numbers. No difference was observed in osteoblast numbers between the 17-AAG– and vehicle-treated groups (Fig. 6C); however, as observed in vitro, 17-AAG significantly enhanced the number of osteoclasts (Fig. 6B). Therefore, in the absence of tumor cells, 17-AAG treatment resulted in loss of bone volume with a concomitant increase in osteoclast numbers.

Discussion

The identification of several naturally occurring anticancer antibiotics, including geldanamycin, radicicol, and herbimycin A, which selectively inhibit the function of the molecular chaperone Hsp90, has led to the candidacy of this molecule as a novel target for cancer drug therapy. These agents and derivatives have proven highly effective at inhibiting the growth of xenograft models of colon, breast, and prostate cancer (30–32), as well as enhancing conventional therapies, such as Taxol, when used in combination (33).

However, it is the ability of a tumor cell to metastasize, rather than the growth of the primary tumor, that is the major cause of treatment failure, morbidity, and death in breast cancer patients. Despite this, the majority of preclinical investigations have examined the effects of 17-AAG upon tumor growth in subcutaneous or orthotopic models, with little being done to investigate its efficacy in the inhibition of tumor cell metastasis. To address this, we utilized an intracardiac inoculation model of tumor spread, focusing on metastasis and invasion of the bone, the most common and often the earliest site of breast cancer spread. Moreover, the bone is a tissue that represents a very different microenvironment to that of the primary tumor site, often allowing tumors to be refractory to conventional therapies (7, 51).

Our initial in vitro investigations of the effects of 17-AAG upon the proliferation and chemotactic migration of MDA-MB-231SA cells are entirely consistent with previous findings in which 17-AAG inhibited proliferation and reduced IGF-I–mediated signaling in MDA-MB-231 cells (46, 53). Additionally, our observation that 17-AAG inhibits the growth of MDA-MB-231SArfp tumors at the orthotopic site are in accordance with findings in other breast cancer models of tumor growth (27, 28). However, in contrast to these very positive indications, we have made the surprising observation that 17-AAG increases the incidence of osteolytic bone metastases and, consistent with this, had the ability to enhance the formation of osteoclasts, the specialized bone-resorbing cells essential for the destruction of bone necessary for tumor colonization. Furthermore, and consistent with this pattern of 17-AAG osteolytic action we observed, 17-AAG caused an increase.

Figure 3. MDA-MB-231SA cells were engineered to constitutively express RFP (rfp). A, phase contrast microscopy showed no morphologic changes in the MDA-MB-231SArfp when compared with parental MDA-MB-231SA cells. B, in addition, no significant alteration in the in vitro proliferation of the MDA-MB-231SArfp cells was identified as determined by an adhesion-dependent cell growth assay. C, X-ray analysis of the hind limbs of mice was done at 21 days postinoculation of MDA-MB-231SArfp cells. 17-AAG treatment of mice inoculated with the MDA-MB-231SArfp cells showed a significant increase in hind limb metastasis as determined by X-ray analysis. Significance was determined by χ² analysis.
in osteoclast numbers in both nude and C57BL/6 mice in the absence of any tumor challenge. As a result, 17-AAG reduced the bone mass in these mice, an osteopenic effect that in itself could pose a significant problem for patients undergoing therapy with 17-AAG, regardless of the presence or progression of their cancer. This risk may not be limited to 17-AAG, as enhanced osteoclast formation, albeit in vitro, was observed with herbimycin A and the structurally unrelated Hsp90 inhibitor, radicicol, suggesting that the osteopenic effect may be a more general phenomenon of Hsp90 inhibitors.

In the osteolytic metastasis that are typically associated with breast cancer, it is believed that complex cellular interactions occur within the bone. Tumor cells release factors, such as parathyroid hormone–related protein, that stimulate the local mesenchymal cells of bone (osteoblasts) to produce RANKL, a tumor necrosis factor-related molecule required for osteoclast formation and activation (4). The resulting bone resorption by the osteoclast release factors, such as transforming growth factor β (TGFβ) and IGF-1, which can further stimulate tumor growth, forming a positive feedback loop or “vicious cycle” of local pro-osteolytic and tumor proliferation stimuli (4). It is not clear whether this mechanism is important in the establishment of tumor metastases in all models or that it is critical in the human disease, and indeed there is emerging evidence of the importance of other tumor-derived factors such as IL-8, IL-11, and connective tissue growth factor (52, 53). However, this and other evidence suggest that not only are the bone and bone marrow a permissive microenvironment or “soil” for metastatic breast cancer growth, but that bone undergoing high turnover or breakdown may be particularly permissive to tumor establishment and growth (48). Therefore, our finding that 17-AAG reduces bone mass in mice in the absence of tumor invasion may indicate that, in addition to any effects on the behavior of the tumor cells themselves, 17-AAG can act as a pro-osteolytic factor, enhancing the capability of tumor cells to establish and grow in the bone.

Although the precise mechanism of the reduction in bone mass after 17-AAG administration (even in the absence of tumor challenge) is difficult to determine, the in vitro data suggests that it may be the result of enhanced osteoclast differentiation and/or survival. The increased numbers of osteoclasts in the absence of an accompanying increase in osteoblast numbers in vivo indicate that this is indeed likely to be the major cause of the lower bone mass in these mice. Moreover, because this was evident in nude mice, which already have low bone mass, it suggests that 17-AAG has potent effects upon the osteoclast lineage and that this is not dependent on the presence or absence of lymphocytes.

To date, the in vivo effects of 17-AAG in the tumor models and the tumor naïve mice have only been examined at a single dose using a single treatment schedule. Designed to observe maximal benefit of the drug, it was, therefore, surprising to see a strong enhancement of bone metastasis at a drug concentration close to the maximal tolerated dose. Further dosing and treatment schedules will be required to further define the potential severity of the effects of 17-AAG upon the bone microenvironment and its impact upon tumor growth.

Our studies in vitro are in strong accord with our in vivo findings. Bone marrow cells (containing the hematopoietic progenitors from which osteoclasts derive) were cocultured with osteoblasts in the presence of a hormonal osteolytic stimulus provided by high concentrations of 1,25(OH)2D3 and PGE2, a type of in vitro model

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**Figure 4.** The presence of tumors at sites of the skeleton other than that of the hind limbs was determined by fluorescent imaging. A, X-ray analysis of the hind limb showed the presence of osteolytic lesions (arrows) after 21 days in a 17-AAG–treated mouse. B, corresponding fluorescent image of the hind limb osteolytic tumor shown by fluorescent imaging. Fluorescent imaging also revealed metastases to the jaws (C), skull (D), and spine (E) of 17-AGG–treated nude mice. F, spine metastases were also identified in the vehicle control group as previously reported for this model; however, these were less numerous and smaller in size. G, graphical representation of the incidence of bone metastasis to various sites determined by fluorescent imaging. A significant increase in metastasis to the hind limbs and the jaws was observed as determined by Fischer exact probability test. *P < 0.05. H, 17-AAG treatment of mice that had been orthotopically inoculated with MDA-MB-231SA/rho cells showed a significant decrease in tumor growth when compared with the vehicle control mice (8 mice/group). Columns, mean; bars, SD.
thought to recapitulate the cell-to-cell interactions at the bone surface and used to elucidate the mechanisms controlling osteoclast formation. 17-AAG greatly increased the osteoclast formation in this model; however, this action of 17-AAG did not require the presence of osteoblasts: RANKL-stimulated bone marrow cells also formed more osteoclasts in the presence of 17-AAG. Furthermore, BMM, a highly enriched source of osteoclast/macrophage progenitors, and the RAW 264.7 pre-osteoclast cell line (data not shown) also formed more osteoclasts with 17-AAG treatment. This suggests that 17-AAG pro-osteolytic effects require only the presence of hematopoietic-derived cells and may indeed act directly on osteoclast progenitors to increase their response to RANKL. The only other exogenous factor known to do this is TGFβ (and, less potently, the related bone morphogenetic protein-2), and whereas we cannot exclude an induction of TGFβ secretion by 17-AAG, TGFβ strongly inhibits osteoclast formation in the coculture model as it suppresses RANKL stimulation by the osteoblasts (55); this is inconsistent with our results with 17-AAG. A number of endogenous factors are known to increase osteoclast formation, e.g., thioredoxin expression (56), and it is possible that 17-AAG might induce such factors in osteoclasts. This is supported by previous findings that heat shock and oxidative stress in the premonocytic line U937 increases thioredoxin expression via activation of HSF (57). It is also known that 17-AAG and other Hsp90 inhibitors, such as herbimycin A and radicicol, induce a potent heat shock response in cells through the release and subsequent activation of HSF-1 from the Hsp90 molecular chaperone complex (58). Therefore, Hsp90 inhibitors may have an indirect effect on osteoclast formation via HSF-1–mediated up-regulation of gene transcription. It is also conceivable that 17-AAG may have a direct effect upon osteoclast formation in that endogenous repressors of the process may exist and that these...
molecules are Hsp90 “client” proteins. Upon Hsp90 inhibition by 17-AAG or other Hsp90 inhibitors, these proteins may lose functionality and/or are degraded resulting in the release of the repression leading to enhanced osteoclast formation.

Although our results clearly define an action of 17-AAG upon the host cells of the bone, it is not known at this time whether 17-AAG can act directly upon the tumor cells, modifying their phenotype and enabling them to survive and grow more efficiently in bone. 17-AAG is known to have profound effects upon cancer cell gene expression (59), increasing the expression of genes that may aid in tumor cell survival and/or growth in bone. Thus, in different microenvironments with differing growth pressures, tumor cells may escape 17-AAG–mediated toxicity, allowing the drug to modify the gene expression profile of the tumor cell and ultimately aid in the generation of bone metastases.

These findings come at a critical time in the clinical development of 17-AAG as well as the preclinical development of other Hsp90 inhibitors. Although phase I clinical trials have served as a proof of principle that Hsp90 pharmacologic inhibition can be achieved with tolerable toxicity in humans, its longer-term application will determine whether our experimental findings will be translated to the clinical situation. If so, the identification of a pro-osteolytic action of 17-AAG in the bone microenvironment needs to be addressed, especially in patients with a high likelihood of bone metastasis, such as in breast and prostate cancer and multiple myeloma. However, the potential deleterious effects of 17-AAG upon the bone may be overcome by the administration of existing effective anti-osteolytic therapies, such as the bisphosphonates, which are currently clinically approved and being used routinely (2). The use of such approaches deserves careful consideration for those investigating HSP90 inhibitors, and suggests that its evaluation using in vivo murine models is desirable.

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References


The Heat Shock Protein 90 Inhibitor, 17-Allylamino-17-demethoxygeldanamycin, Enhances Osteoclast Formation and Potentiates Bone Metastasis of a Human Breast Cancer Cell Line


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