Antiangiogenic Properties of 17-(Dimethylaminoethylamino)-17-Demethoxygeldanamycin: An Orally Bioavailable Heat Shock Protein 90 Modulator

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ABSTRACT

Purpose: **The purpose of this study was to investigate the antiangiogenic properties of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG; NSC707545), a water-soluble benzoquinone ansamycin.**

Experimental Design: **The activity of 17-DMAG, *in vivo*, was evaluated for inhibition of fibroblast growth factor (FGF)-2-induced angiogenesis in s.c. implanted Matrigel in mice. *In vitro*, the activity of 17-DMAG on endothelial cells (human umbilical vein endothelial cells; HUVEC) was tested in FGF-2; and vascular endothelial growth factor (VEGF)-induced proliferation and apoptosis, motility, and extracellular matrix invasion; and on the alignment of capillary like structures in Matrigel. The protein level of heat shock protein (Hsp)90 and client proteins was examined by Western blot in FGF-2 and VEGF-stimulated HUVEC.**

Results: **Daily oral administration of 17-DMAG affected the angiogenic response in Matrigel in a dose-dependent manner. The hemoglobin content in the Matrigel implants was significantly inhibited, and the histological analysis confirmed a decrease of $CD31^+$ endothelial cells and of structures organized in cord and erythrocyte-containing vessels. *In vitro*, the compound inhibited dose-dependently the migration and the extracellular matrix-invasiveness of HUVEC and their capacity to form capillary like structures in Matrigel. 17-DMAG treatment also inhibited FGF-2 and VEGF-induced HUVEC proliferation and resulted in apoptosis. Accordingly, the expression of Hsp90 direct client proteins (pAkt and c-Raf-1) or their downstream substrates including pERK was also affected. 17-DMAG consistently increased the expression of Hsp70. Throughout the study similar results were obtained with 17-allylamino-17-demethoxygeldanamycin (17-AAG; NSC330507), the analog compound currently undergoing clinical trials.**

Conclusions: **We show that the Hsp90 targeting agents 17-DMAG and 17-AAG inhibit angiogenesis. The strong effects on endothelial cell functions, *in vitro*, indicate that the antiangiogenic activity of 17-DMAG/17-AAG could also be due to a direct effect on endothelial cells. The oral bioavailability of 17-DMAG might be of advantage in investigating the potential of this compound in clinical trials with antiangiogenic as well as antiproliferative endpoints.**

INTRODUCTION

The molecular chaperone heat shock protein (Hsp) 90 is a highly conserved, constitutively expressed protein. Hsp90 mediates cellular response to stress by acting as a molecular chaperone to assist the refolding of proteins, which may be damaged by various environmental stresses. It can account for 2–5% of total cellular proteins even in the absence of stress (1). Recent interest in Hsp90 as a target for cancer drug development has arisen with the elucidation of Hsp90 as the binding partner for the benzoquinone ansamycin class of antineoplastic agents (2, 3). Drugs targeting Hsp90 might directly address important targets in the pathogenesis of cancer, because Hsp90 protein is required for the proper function of signal transduction kinases and transcription factors, including v-*src*, c-Raf-1, c-*erb*B2, *met*, CDK4, and steroid receptors (4–6). Mutated or chimeric signaling proteins (*e.g.*, p53 and Bcr-Abl) are also Hsp90 clients (7). Very recently, tumor cells were shown to possess Hsp90-containing complexes with intrinsically higher affinity for geldanamycins than those derived from normal cells and tissues (8).

Evidence for a role for Hsp90 in regulating angiogenic responses is accumulating. For example, Hsp90 is a major regulator of the stability, function (9), and activation (10) of the transcription factor hypoxia-inducible factor (HIF)-1α during the angiogenic response induced by hypoxia. Hsp90 mediates the antiapoptotic effects of vascular endothelial growth factor (VEGF) in endothelial and leukemic cells (11) and plays an important role in regulating VEGF-induced endothelial cell migration *in vitro* (12). Furthermore, Hsp90 facilitates signaling mediated by growth factors, G-protein, and mechanotransduction pathways that lead to the activation of iNOS and consequently to the proangiogenic effects mediated by nitric oxide (13, 14). HIF-1α drives the transcription of many genes in-

Received 12/29/03; revised 3/23/04; accepted 4/15/04.
Grant support: National Cancer Institute, NIH, under contract N01-CO-12400 (to R. Giavazzi).
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volved in tumor cell hypoxia adaptation, including VEGF, which is essential for angiogenesis (15). The existence of Hsp90-dependent pathways for degradation of HIF-1α predicts that Hsp90 antagonists will possess antiangiogenic activity (9, 16). However, the potential capacity for Hsp90-directed agents to affect pathways involved in angiogenesis has not been directly addressed. This might occur indirectly through the effects of the agent on tumor cell-mediated promotion of angiogenesis or through direct effects on endothelial cell functions.

17-Allylamino-17-demethoxygeldanamycin (17-AAG), a semisynthetic geldanamycin currently in clinical trials (17), is unsuitable as a candidate regulator of angiogenesis, because it is poorly soluble and not orally bioavailable. Because an antiangiogenic agent will likely require persistent dosing through ideally an oral route of administration, a compound possessing those attributes would be desirable (18). 17- (Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) is a water-soluble benzoquinone ansamycin and, like 17-AAG, also destabilizes Hsp90 client proteins (19). Moreover, in contrast to 17-AAG or other analogs studied to this point, 17-DMAG has several potential advantages. 17-DMAG is water soluble and displays an oral bioavailability twice that of orally delivered 17-AAG. 17-DMAG does not give rise to potentially toxic metabolites (20). Preclinical evaluation of 17-DMAG has shown that in the National Cancer Institute 60-cell line panel *in vitro* activity screen and in animal models, 17-DMAG is more potent than 17-AAG (21).

In this study we show that *in vivo* 17-AAG and 17-DMAG equally reduced the angiogenic response to growth factor-impregnated Matrigel plugs. However, 17-DMAG was active when administered by the oral route. 17-DMAG inhibited fibroblast growth factor (FGF)-2 and VEGF induced endothelial cell proliferation causing their apoptosis. Other endothelial cell functions related to the angiogenic process, such as migration, extracellular matrix invasion, and the formation of capillary-like structures were also affected by 17-DMAG treatment. Endothelial cells exposed to 17-DMAG underwent degradation of AKT, c-Raf-1, and extracellular signal-regulated kinase (ERK) protein kinases at concentrations that also caused cell growth inhibition and apoptosis. Our studies indicate that 17-DMAG has antiangiogenic properties through direct effects on endothelial cell functions. These appear to be mediated by Hsp90 as a molecular chaperone for client proteins involved in endothelial cell functions related to angiogenesis. The oral bioavailability of 17-DMAG over 17-AAG might be of advantage in additionally investigating the potential of 17-DMAG in clinical trials with antiangiogenic as well as antiproliferative endpoints.

MATERIALS AND METHODS

Materials and Reagents. 17-AAG (NSC 330507) and 17-DMAG (NSC 707545) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). For *in vitro* studies 17-AAG and 17- DMAG were dissolved in DMSO to yield 20 mM stock, stored at −20°C in aliquots, and additionally diluted in working medium at the concentrations indicated in the results. For *in vivo* studies, 17-AAG was prepared fresh daily in DMSO (22) and 17-DMAG in water (pH 4.0) and administered at the doses indicated in the "Results." The antibodies used were anti-pAKT, anti-AKT, anti-pERK, and anti-ERK (Cell Signaling, Beverly, MA); anti-c-Raf-1 (BD Biosciences, Franklin Lakes, NJ); anti-actin (Sigma-Aldrich Corp., St. Louis, MO) and anti-Hsp70 and anti-Hsp90 (Stressgen, San Diego, CA). Human umbilical vein endothelial cells (HUVEC) were purchased from VEC Technology (New York, NY) and human dermal micro vascular endothelial cells (HDMEC) were from Cambrex Bio-Science (Walkersville, MD). Endothelial cells were cultured in growth factor supplemented EBM-2 (Clonetic #CC3162 Bullet kit) medium with 2% fetal bovine serum. HUVEC and HDMEC were used between passages two and five.

Angiogenesis Assay in Matrigel. The method described by Passaniti *et al.* (23) was used with some modifications. Briefly, FGF-2 (300 ng/pellet) was embedded in a chilled pellet of Matrigel (12.5 mg/ml; 0.5 ml); Becton Dickinson, Bedford, MA) and injected s.c. in pathogen-free, 4-week-old female C57BL/6N mice (Charles River, Calco, Italy) following institutional guidelines and national and international laws and policies. Mice received 17-AAG or 17-DMAG daily six times as indicated in the "Results." Control mice received the same volume of vehicle. At the end of the treatment period the pellet was removed, and the hemoglobin content was measured with Drabkin's procedure (Drabkin reagent kit; Sigma, St. Louis, MO).

For histological analysis, the Matrigel plug with the surrounding skin and tissues were fixed in a solution of zinc acetate and zinc chloride in a Tris-Ca^{+2} acetate buffer for 24 h and embedded in paraffin. Five-μm sections were stained with H&E, and observers blinded to the treatment conditions analyzed the slides. The angiogenic response was subjectively graded, based on the amount of infiltrating cells and the presence of cords and erythrocyte-containing vessels as described previously (24). For immunohistochemical analysis of CD31-positive endothelial cells, sections were immunostained with a rat anti-mouse CD31 monoclonal antibody (PharMingen, Becton Dickinson, San Diego, CA) accordingly to the described procedure (25, 26). Microvessel count was carried out on six fields (×400) chosen within the highest vascularized areas. Any endothelial cell or cluster of endothelial cells positive for CD31 was counted. Statistical significance was determined by the two-tailed Mann-Whitney *U* test. The limit of statistical significance was $P \leq 0.005$.

Growth Inhibition Assay. HUVEC (2.5×10^3) or HDMEC (5×10^3) were plated in a 96-well plate (Falcon, Bedford, MA) in 100 μl of basal medium (EBM-2 containing 2% FCS). After 24 h (day 0), the test compound (100 μl) was added to each well at twice the desired concentration prepared in basal medium or in VEGF (10 ng/ml final concentration) or FGF-2 (10 ng/ml final concentration) containing medium. On day 0, one plate was stained with 0.5% crystal violet in 20% methanol, rinsed with water, and air-dried. The remaining plates were incubated at 37°C. After 24, 48, or 72 h, plates were stained with crystal violet as described above. The stain was eluted with a solution of 0.1 M sodium citrate and ethanol (1:1). Absorbance was measured at 540 nm with an ELISA reader (Dynex Technology, Chantilly, VA). Day 0 absorbance was subtracted from the test plates, and data were plotted as percentage of control proliferation (vehicle-treated cells). IC_{50} (drug concentration

causing 50% inhibition) was calculated from the plotted data using regression analysis.

Quantification of Apoptotic Cells. To determine cell survival, 1×10^5 HUVEC were plated in basal medium in a six-well plate. After 24 h medium was replaced with EBM-2 supplemented with VEGF (10 ng/ml) or FGF-2 (10 ng/ml), and the test compound was added at various concentrations. At indicated times media and cells were collected, washed, centrifuged, and analyzed for the presence of apoptotic cells using the Annexin V staining kit (Guava Nexin kit) from Guava Technologies (Hayward, CA). Samples were analyzed on Guava Personal Cell Analyses System (Guava Technologies). Triplicates were used in all of the experiments, and each experiment was repeated at least three times.

Cell Migration Assay. Migration assay was performed in a 96-well disposable chamber (Chemotx 101–8; Neuroprobe, Gaithersburg, MD). Both sides of framed filter of the 96-well chamber were coated with 25 μl/well of rat tail collagen type I (0.1 mg/ml) for 30 min and left dried in laminar flow hood. Basal medium containing 0.1% BSA for negative control or containing VEGF or FGF-2 (10 ng/ml) was added as chemoattractant to the wells of the bottom plate. HUVEC were pretreated for 24 h with various concentrations of 17-DMAG or vehicle. Cells were then harvested, washed, and resuspended in assay medium.

17-DMAG and vehicle-treated cells (3×10^4 cells in 30 μl) were placed on top of each well of the filter and incubated at 37°C for 4 h. At the end of incubation period, the filter was fixed and stained in Diff Quick (Marz-Dade, Dudingen, Switzerland). Migrated cells were counted from five high-power fields. Data are expressed as the percentage of control migration (vehicle-treated cells), and IC_{50} was calculated.

Chemoinvasion Assay. Endothelial cell invasiveness was assayed using modified Boyden chambers, with 8-μm pore size, polycarbonate PVP-free Nucleopore filters. NIH-3T3 supernatant was used as the chemoattractant and was added to the lower compartment of the chamber. Filters were coated with a layer of the reconstituted basement membrane Matrigel (Becton Dickinson, 0.5 mg/ml). HUVEC were treated with vehicle or 17-DMAG for 24 h at the concentrations indicated in "Results." The cells were harvested, washed in basal medium with 0.1% BSA, and resuspended at the concentration of 1×10^6/ml. Cell suspension was added to the upper compartment of the chamber. After 6 h the filters were stained with Diff-Quik and the migrated cells in 10 high-power fields were counted. Data are expressed as the percentage of control migration (vehicle-treated cells), and IC_{50} was calculated.

Cord Formation on Matrigel. The formation of capillary-like structures was assessed by plating HUVEC on Matrigel (Becton Dickinson; 10 mg/ml). Briefly, cells were treated with vehicle or various concentrations of 17-DMAG for 24 h harvested, washed, and resuspended in growth factor-supplemented EBM-2 medium before distributing in 96-well plates (2×10^5/100ul). After 16 h, cord formation was observed using an inverted phase contrast microscope (DM-IRB; Leica Inc.), and images were captured with a CCD camera. The cords were quantitated by measuring the length of tubes and counting junctions in three random fields from each well (two wells per point) using Bioquant Image analysis system. Data were plotted, and IC_{50} was calculated.

Protein Extraction and Immunoblotting. HUVEC (1.5×10^5) were plated on 100-mm dishes in basal media. After 24 h, FGF-2 or VEGF (50 ng/ml) were added. Unstimulated or stimulated cells were treated with various concentration of 17-DMAG for 4 or 24 h, scraped from dishes, and washed with PBS. Cell pellets were lysed in cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1%v/v Triton X-100, 1 mM $EGTA^2$ mM EDTA, 1 mM sodium orthovanadate, 2.5 mM sodium PP_i, 1 mM B-glycerophosphate, 10 μg/ml leupeptin, and 1 mM phenylmethane-sulfonyl fluoride]. Cell lysates were incubated on ice for 15 min, sonicated for 5 s, and cleared by centrifugation at $14{,}000 \times g$ for 15 min. Protein concentrations were determined by Bradford protein assay (Pierce, Rockford, IL). Equal amounts of proteins were resolved by SDS-PAGE on 4–20% Tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membrane (Millipore, Boston, MA).

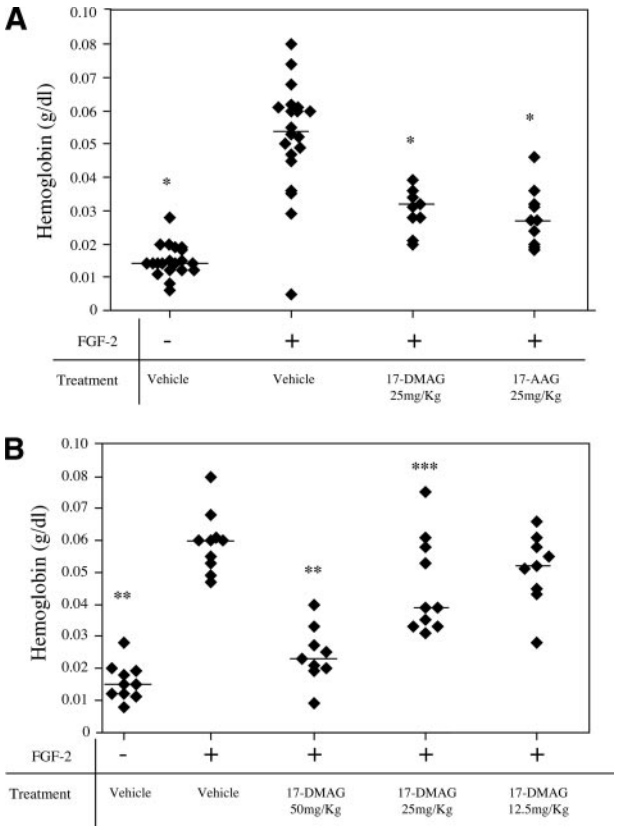

Fig. 1 Effect of 17-demethoxygeldanamycin (*17-AAG*) and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (*17-DMAG*) on angiogenesis in Matrigel (Becton Dickinson). Matrigel containing fibroblast growth factor-2 (FGF-2; 300 ng/pellet) was injected s.c. in mice. *A*, mice were treated. i.p. with vehicle or 17-AAG and 17-DMAG (25 mg/kg, daily ×6). *B*, mice were treated p.o. daily with vehicle or 17-DMAG (50, 25, 12 mg/kg, daily ×6). At day 7, pellets were collected, and the angiogenic response was evaluated by measuring the hemoglobin content of the pellets. Data are expressed as hemoglobin content (g/dl) for each pellet. *Horizontal bars*, median. ∗, $P < 0.0001$, ∗∗, $P < 0.0002$, ∗∗∗, $P < 0.04$ compared with vehicle-treated, FGF-2-containing pellets (Mann-Whitney *U* test).

After blocking, blots were probed with the antibody of interest overnight at 4°C. After incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were visualized by chemiluminescence (Amersham Corp., Piscataway, NJ).

RESULTS

17-DMAG Inhibits Angiogenesis *in vivo*. The effect of 17-DMAG on angiogenesis, *in vivo*, was evaluated in the Matrigel plug assay, where angiogenesis is induced by FGF-2 embedded in a pellet of Matrigel, which is, in turn, implanted s.c. in mice. Seven days after implantation, FGF-2 induced a strong angiogenic response, with significant increase in the hemoglobin content of the pellets, compared with Matrigel pellets without FGF-2 (Fig. 1, *A* and *B*). 17-DMAG reduced the angiogenic response to FGF-2 at the same extent as 17-AAG administered by i.p. route at the same schedule and dose (Fig. 1*A*). The median hemoglobin content of FGF-2 containing pellets was significantly lower in 17-DMAG and 17-AAG treated mice (median, 0.031; range, 0.020–0.039 g/dl and median, 0.027; range, 0.018–0.046 g/dl, respectively) than in vehicle-treated mice (median, 0.054; range, 0.029–0.080 g/dl). The effect was confirmed by histological analysis of the angiogenic response where no difference between the two compounds was observed (data not shown). In another experiment (Fig. 1*B*), 17-DMAG administered by the oral route significantly affected the angiogenic response in a dose-dependent manner with the maximum effect observed at the maximum tolerated dose of 50 mg/kg (median hemoglobin, 0.023; range, 0.009–0.040 g/dl) compared with vehicle-treated mice bearing FGF-2-containing pellets (median hemoglobin, 0.060; range, 0.047–0.080 g/dl). No significant toxicity was observed in mice treated with 17-DMAG at 50 mg/kg with a maximum of 3% of body weight loss at the end of treatment. Due to the limited solubility and reduced bioavailability, it was not possible to give 17-AAG orally (27).

Histological analysis of the Matrigel pellets confirmed the antiangiogenic activity of 17-DMAG (Fig. 2, *A*, *C*, and *E*) given orally. FGF-2 induced an obvious angiogenic response, with endothelial cells infiltrating the Matrigel organized in thin cords,

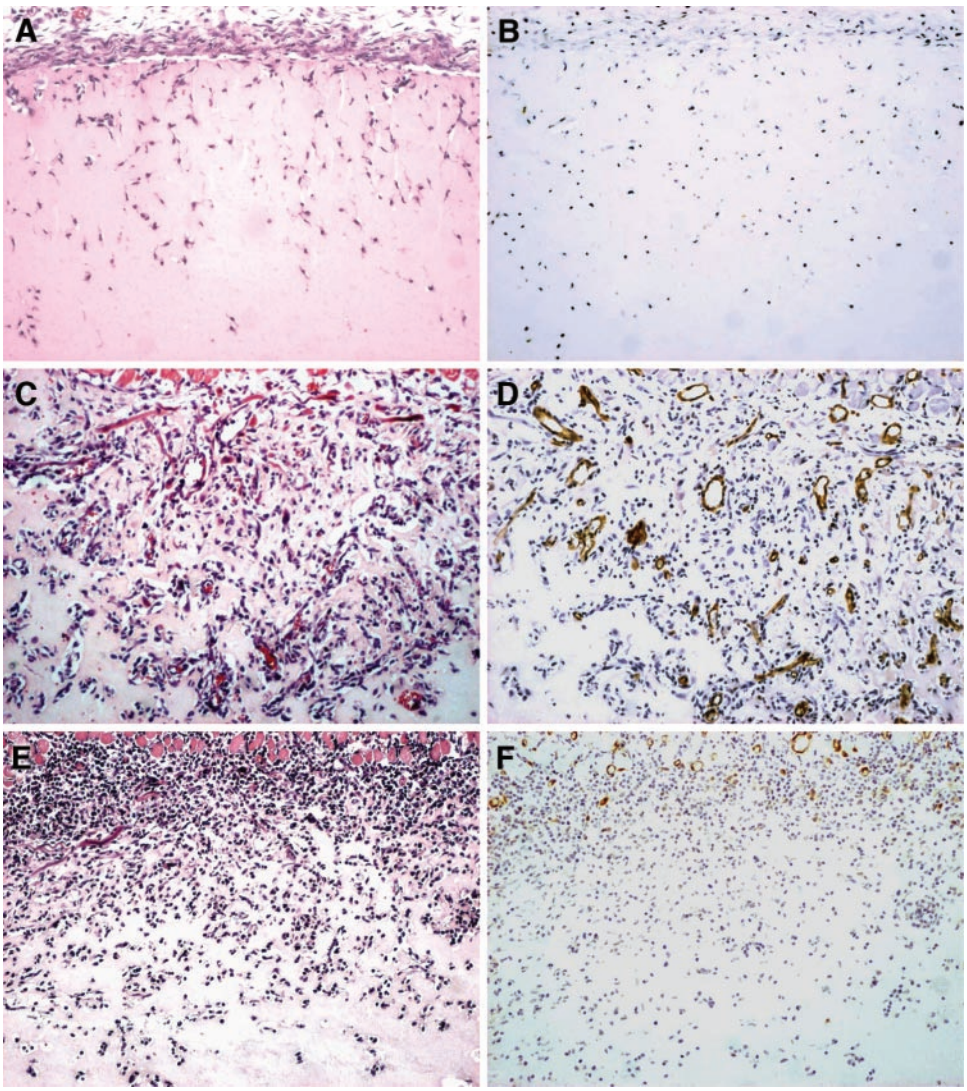

Fig. 2 Histological analysis of Matrigel treated with 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG). Experiment was performed as in Fig. 1. Negative control (w/o fibroblast growth factor-2; FGF-2): Matrigel containing no angiogenic stimulus showed few infiltrating cells (*A*) and a weak CD31 immunostaining. *B*, positive control (FGF-2): Matrigel containing FGF-2 in vehicle-treated mice presented a high degree of cellularity, and the presence of blood-containing vessels (*C*), confirmed by a high number of CD31 positive vessels (*D*); with 17-DMAG-treated mice (50 mg/kg, p.o.): the FGF-2-containing Matrigel pellets presented a reduced number of blood-containing vessels (*E*) and no positivity for CD31 (*F*). Results show a representative example of histological analysis and CD31 immunostaining. $n = 5$ mice per group.

tubules, and blood-containing vessels (Fig. 2*C*). Pellet sections of 17-DMAG-treated mice showed fewer infiltrating cells, cords, and erythrocyte-containing vessels than vehicle-treated pellets (Fig. 2*E*). The antiangiogenic effect of 17-DMAG was confirmed by CD31 immunostaining of endothelial cells (Fig. 2, *B, D,* and *F*). CD31 immunostaining was weak or absent in FGF-2- unstimulated controls (median CD31-positive vessels, 0; range, 0–3.3; Fig. 2*B*), whereas a high number of CD31-positive vessels was observed in FGF-2-stimulated pellets (median, 14; range, 9–15.8; Fig. 2*D*). The treatment with 17-DMAG completely abolished the staining for CD31 (Fig. 2*F*).

17-DMAG Inhibits Endothelial Cell Proliferation. HUVEC were plated in medium devoid of growth factors for 24 h and then stimulated with 10 ng/ml of FGF-2 (Fig. 3*A*) or VEGF (Fig. 3*B*) in presence of various concentrations of 17-DMAG. A 4-h exposure did not affect the growth of HUVEC, whereas 24-, 48-, and 72-h treatment with 17-DMAG inhibited the proliferation of HUVEC in a time- and concentration-dependent manner. As shown in Fig. 3, *A* and B, 17-DMAG equally blocked endothelial cell proliferation induced by FGF-2 or VEGF (IC_{50} 20 nM and 22 nM, respectively, in 72-h exposure). The response of HDMEC, stimulated by FGF-2 or VEGF and treated with 17-DMAG, was not significantly different from HUVEC (IC_{50} 35 nM and 30 nM, respectively, in 72-h exposure). Similar results were obtained with 17-AAG (data not shown).

17-DMAG Induces Apoptosis in Endothelial Cells. The survival of HUVEC after exposure to 17-DMAG in the presence of FGF-2 (Fig. 4*A*) or VEGF (Fig. 4*B*) was studied in time-dose response experiments. As determined by annexin V staining, a significant increase of apoptotic cells was observed in the presence of different concentrations of 17-DMAG. Forty-eight h exposure at a concentration >25 nM induced ≥40% increase of apoptotic cells. As observed for the inhibition of proliferation, the apoptotic index induced by 17-DMAG treatments was similar for FGF-2- or VEGF-stimulated HUVEC.

17-DMAG Inhibits Endothelial Cell Migration and Invasion. 17-DMAG inhibited the motility response of HUVEC to VEGF and FGF-2 in a dose-dependent manner. The IC_{50} was 50 nM for FGF-2 and 23 nM for VEGF-induced migration. (Fig. 5*A*). HUVEC invasiveness through a layer of reconstituted basement membrane (Matrigel) in response to NIH-3T3-conditioned medium, a condition rich in stimuli for cellular motility,

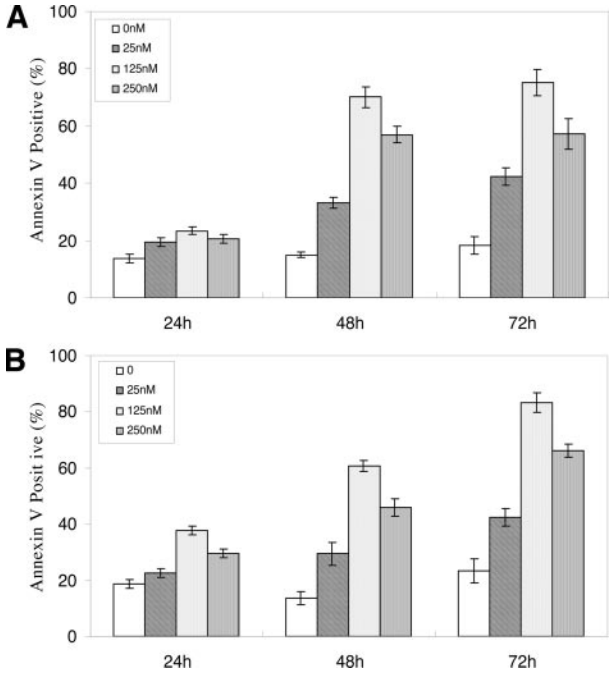

Fig. 4 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DAMG) induces apoptosis in human umbilical vascular endothelial cells. To determine the apoptotic population, human umbilical vascular endothelial cells were incubated with 17-DMAG in the presence of fibroblast growth factor-2 10 ng/ml (*A*) or vascular endothelial growth factor 10 ng/ml (*B*). At the indicated times, apoptotic cells were determined by Annexin V staining as described in "Materials and Methods." Data are plotted as percentage of Annexin V-positive population. Results are the mean of triplicate and representative of three independent experiments; *bars,* ±SD.

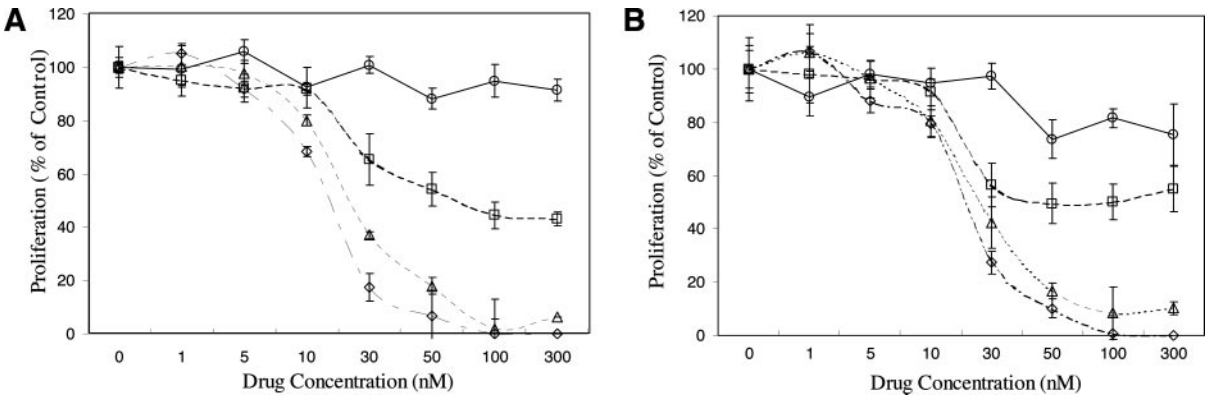

Fig. 3 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) induce growth inhibition of endothelial cells. Human umbilical vascular endothelial cells were plated in media devoid of growth factors for 24 h and then stimulated with 10 ng/ml of fibroblast growth factor-2 (*A*) or 10 ng/ml of vascular endothelial growth factor (*B*) in the presence of various concentrations of 17-DMAG. Cells were incubated for 4 h (○), 24 h (□), 48 h (△), and 72 h (◇). Results are expressed as percentage of control (vehicle-treated cells) and are the mean of triplicate; *bars,* ±SD. Data are from one experiment representative of four.

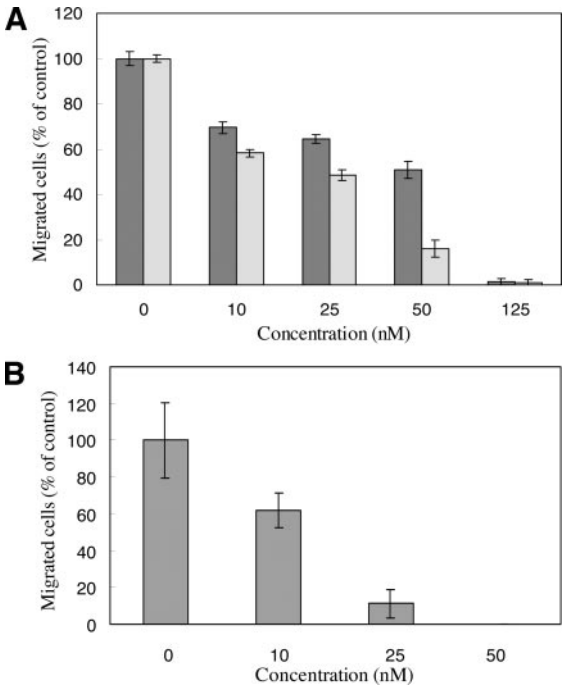

Fig. 5 Effect of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) on endothelial cell migration and invasion. *A*, endothelial cells were exposed to a different concentration of 17-DMAG for 24 h and then tested in migration assay as described in "Materials and Methods." Fibroblast growth factor-2 (10 ng/ml; ■) and vascular endothelial growth factor (10 ng/ml; □) were used as chemoattractants. Results are expressed as percentage of control (vehicle-treated cells) and are the mean of triplicate; *bars*, ±SD. Data are from one experiment representative of three. *B*, endothelial cells were exposed to different concentration of 17-DMAG for 24 h and then tested in invasion assay as described. Results are expressed as percentage of control (vehicle-treated cells) and are the mean of triplicate. Data are from one experiment representative of two; *bars*, ±SD.

was also inhibited by 17-DMAG. Treatment with 17-DMAG resulted in a dose-dependent inhibition of invasion, with an IC_{50} at only 14 nM (Fig. 5*B*). Similar results were obtained with 17-AAG (data not shown).

17-DMAG Affects Endothelial Cell Cord Formation. In addition to its effects on endothelial invasiveness, 17-DMAG also prevented another endothelial cell function crucial to angiogenesis, the alignment of endothelial cells in capillary-like structures. HUVEC were plated on a three-dimensional layer of Matrigel where they align, forming cords, which already were evident a few hours after plating. Treatment of endothelial cells with 17-DMAG significantly inhibited cord formation. The inhibition was dose-dependent (Fig. 6, *A–F*) with IC_{50} = 73 nM for cord junctions and 86 nM for cord length (Fig. 6*G*). These findings additionally confirm that 17-DMAG is able to modify endothelial cell functions at concentrations and exposure times at which cell proliferation at 24 h is only marginally affected, thus underscoring that 17-DMAG might contribute to inhibition of angiogenesis.

17-DMAG Causes Down-Regulation of Signaling Molecules. Modulation of Hsp90 function by ansamycins causes proteasomal degradation of several client proteins. We quantitated the protein levels of Hsp90 and several other client proteins in FGF-2 (Fig. 7*A*) or VEGF (Fig. 7*B*) stimulated HUVEC after 4-h and 24-h exposure to 17- DMAG. Phospho-AKT and total AKT were not affected at early time points but protein was degraded at 250 nM after 24-h treatment with 17-DMAG. C-Raf-1 levels also decreased after 17-DMAG treatments. Interestingly, it was the most sensitive and rapidly degraded target of 17-DMAG in FGF-2-stimulated HUVEC (4 h treatment at 25 nM). 17-DMAG consistently increased the expression of Hsp70, and to a lesser extent Hsp90, in a time- and dose-dependent manner (Fig. 7, *A* and *B*). The effect was already evident at

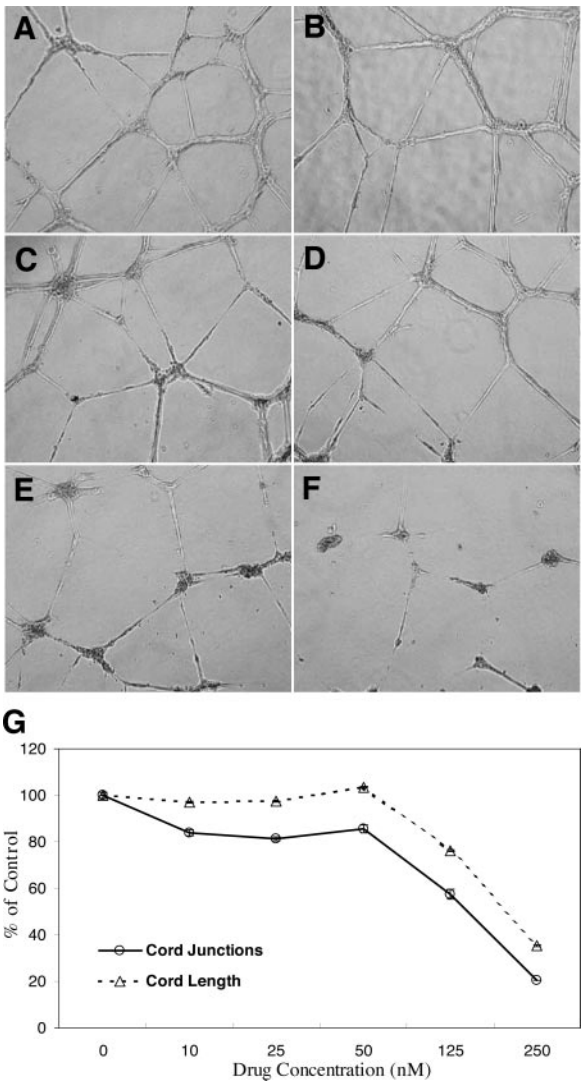

Fig. 6 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) pretreatment inhibits cord formation. Endothelial cells were plated on a three-dimensional layer of Matrigel where they aligned, forming cord-like structures. Treatment of human umbilical vascular endothelial cells with vehicle (*A*) or 17-DMAG 10 nM (*B*), 25 nM (*C*), 50 nM (*D*), 125 nM (*E*), and 250 nM (*F*) caused a concentration-dependent inhibition of cord formation as it can be seen by the inhibition of cord junctions (○) and cord length (△). *G*, data are expressed as percentage of control (vehicle-treated cells). Data are from one experiment representative of three.

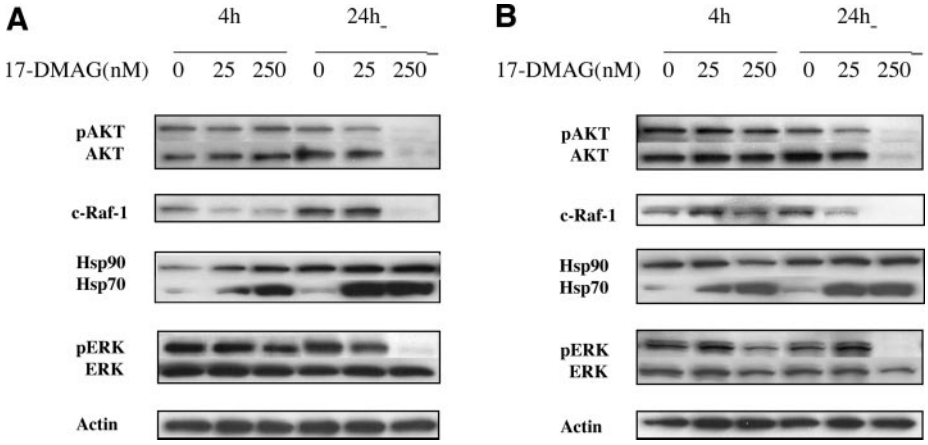

Fig. 7 Effect of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (*17-DMAG*) on signaling proteins in endothelial cells. Fibroblast growth factor-2 (50 ng/ml; *A*) or vascular endothelial growth factor (50 ng/ml; *B*) -stimulated human umbilical vascular endothelial cells were treated for 4 h and 24 h with 17-DMAG at the indicated doses or vehicle as control. Expression levels of pAKT, AKT, c-Raf-1 heat shock protein (*Hsp*)70, Hsp90, pERK, and extracellular signal-regulated kinase (*ERK*) proteins were evaluated by Western blot analysis. Actin was used to demonstrate protein loading (40 μg).

concentrations of drug (25 nM), which just begin to inhibit cell growth (IC_{50} for cell growth ~30 nM). 17-DMAG treatments inhibited the phosphorylation of pERK, whereas total ERK was only marginally affected.

DISCUSSION

We have shown here that 17-DMAG, the water-soluble analog of the benzoquinone ansamycin compound 17-AAG, has antiangiogenic activity *in vivo* in a Matrigel plug assay when administrated to mice. This was associated with the inhibition of a number of endothelial cell functions related to new vessel formation *in vitro*. Whereas previous reports have extensively documented the antitumor effect of geldanamycin derivatives, including 17- AAG and 17-DMAG (28), a direct effect of these compounds on endothelial cell functions has never been reported. The results shown in this study provide evidence that antiangiogenic activity of 17-DMAG/17-AAG may represent an additional mechanism of their antineoplastic activity. Moreover, we define *in vivo* antiangiogenic activity by 17-DMAG when administered by the oral route. The antiangiogenic activities of Hsp90 inhibitors could derive from a direct effect on endothelial cells, as demonstrated here. Our findings that 17-DMAG and 17-AAG blocked endothelial cell proliferation, migration, invasion, and cord formation and induced endothelial cell apoptosis speak in favor of such a direct effect on the angiogenic process. This conclusion is in accordance with previous results showing geldanamycin-induced apoptosis of leukemic and endothelial cells throughout a VEGF-activated Hsp90 pathway (11). Inhibition of hepatocytes growth factor/scatter factor-mediated cell motility and invasion, associated with a reduction in Met expression, has also been reported (29). We recognize, however, that the parent compound of 17-DMAG, geldanamycin, did induce HIF-1α protein degradation, accompanied by inhibition in HIF-1α-induced transcriptional activity. Because VEGF elaboration is "downstream" of an hypoxia-induced angiogenesis (9, 16), this aspect of benzoquinone ansamycin action provides an additional basis for inhibiting an angiogenic response, through effects of the drug on the tumor cell compartment. Moreover, 17-AAG was very effective in inhibiting breast tumors growing in a hypoxic environment (30), where Hsp90 plays an enhanced role by regulating HIF-1α activation (10). The dual action of Hsp90 inhibitors on tumor cells and on endothelial cells might potentiate the antiangiogenic outcome of these compounds.

As shown by Western blot analysis, 17-DMAG inhibited the VEGF- and FGF-2- induced expression of protein kinases involved in the angiogenic pathways of endothelial cells, known to be Hsp90 client proteins (pAkt and c-Raf-1) or their downstream substrates (pERK). These findings on one hand are expected from an Hsp90-directed inhibitor in any cell type but confirm in a mechanistic way the antiangiogenic potential of 17-DMAG. Kamal *et al.* (8) have demonstrated recently that Hsp90 derived from tumor cells has 100-fold higher binding affinity for 17-AAG than does Hsp90 from normal unstimulated cells. This has been hypothesized to be the result of engagement of Hsp90 in chaperoning client oncoproteins or other "stress response" elements up-regulated in tumor as opposed to normal cells. It is plausible that a similar selectivity for proliferating endothelial cells could arise because of growth factor or microenvironment-induced "stress." Endothelial cell activation in these circumstances might lead to the formation of multichaperone protein complexes with high affinity for Hsp90 inhibitors. The fact that 17-DMAG and 17-AAG inhibited potently both *in vivo* and *in vitro* endothelial cell responses induced by FGF-2 or VEGF is in favor of this hypothesis. This might imply a selective activity of 17-DMAG/17-AAG against "angiogenic" endothelial cells exposed to an environment rich in angiogenic stimuli. Additional studies are in process to address this hypothesis.

The many potential consequences of 17-DMAG modulations of Hsp90 protein interactions require careful consideration of the clinical scenarios and endpoints that will be the subject of initial clinical investigations. Pharmacodynamic evaluation of drug-target effects has emerged appropriately as a focus of great interest in early clinical trials with "targeted" therapeutics (31). Our data support the possibility that 17-DMAG might possess antiangiogenic activity by effects on the proliferating vascular compartment of tumors. Therefore, inclusion of angiogenesis-related endpoints in future clinical trials with 17-DMAG is reasonable and warranted by the data presented here. Schirner *et al.* (32) had documented by use of tumor cells growing in agarose plugs implanted into animals that conventional chemotherapy agents could be segregated into groups of agents that had direct antiangiogenic effects (*e.g.*, vincristine, bleomycin,

and TNP470) and those that acted to varying degrees indirectly, through effects on tumor cell-directed functions (*e.g.*, cyclophosphamide, 5-fluorouracil, doxorubicin, and etoposide). Our experiments reported here would place 17-DMAG into the former group of agents in that it had direct antiendothelial cell-directed effects *in vivo* in a similar growth-factor impregnated Matrigel plug system.

Previous detailed pharmacokinetic evaluation of 17-DMAG had emphasized its excellent bioavailability to well-vascularized tissues, with the exception of brain (19), and related this in part to its reduced plasma protein binding compared with 17-AAG. Other distinct pharmacological advantages on the part of 17-DMAG emerged in that study, including ~50% oral bioavailability and a lack of noteworthy metabolism to circulating active metabolites, which could cloud interpretation of toxicity emerging during protracted dosing. This is in contrast to the prominent generation of 17-aminogeldanamycin by 17-AAG. This latter feature is of particular relevance to the use of 17-DMAG in antiangiogenic strategies, as prolonged exposure to doses below the maximal tolerated dose in a "metronomic" schedules of drug administration has emerged as a key desired feature of strategies that would target the vascular compartment, even with agents that also have obvious antitumor cell directed activities (33, 34). 17-DMAG would appear to be a suitable candidate Hsp90 modulator for such strategies, based on the capacity of orally administered doses to convey antiendothelial cell-directed effects reported here and on the clear demonstration of antitumor effects of 17-DMAG when administered by the oral route to animals bearing hepatic metastasis models (21).

In conclusion, our data highlight a new aspect of geldanamycin analog action, specifically, that 17-DMAG exerts antiangiogenic activity when administrated by the oral route. This appears to be relatable to degradation of Hsp90 client proteins in endothelial cells. These studies encourage the addition of angiogenic endpoints to clinical trials involving benzoquinone ansamycins to analyze and verify the contribution of the antiangiogenic effect to the antineoplastic actions of these agents. The oral bioavailability and solubility of 17-DMAG could be advantageous in easily allowing a chronic drug administration schedule to maintain the inhibition of Hsp90 for prolonged periods, hence ensuring sustained depletion of client proteins. Future pharmacological and toxicological evaluations must query the plasma concentrations and doses tolerated over longer periods of observation than addressed here to gain a more thorough appreciation of the potential of the drug for chronic dosing. Consideration of distinct oral formulation strategies to investigate the utility of more immediate as well as more delayed release dose forms is also warranted. As our results suggest here, this could be of value in modulating Hsp90-related targets in both the endothelial as well as the tumor cell compartments.

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Clin Cancer Res 2004;10:4813-4821.