Inhibition of heat shock protein 90 impairs epidermal growth factor–mediated signaling in gastric cancer cells and reduces tumor growth and vascularization in vivo

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Abstract
Oncogenic signaling through activation of epidermal growth factor receptor (EGFR), HER-2, and hypoxia-inducible-factor-1α (HIF-1α) has been implicated in gastric cancer growth and angiogenesis through up-regulation of vascular endothelial growth factor (VEGF). Recently, heat shock protein 90 (Hsp90) has been identified as a critical regulator of oncogenic protein stability, including EGFR, HER-2, and HIF-1α. We hypothesized that inhibition of Hsp90 impairs EGFR- and hypoxia-mediated angiogenic signaling in gastric cancer cells and consequently inhibits angiogenesis and tumor growth. In vitro, the geldanamycin derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) led to marked reduction in constitutive and inducible activation of extracellular signal-regulated kinase 1/2, Akt, and signal transducer and activator of transcription 3 and decreased nuclear HIF-1α protein. In addition, EGFR and HER-2 were downregulated after Hsp90 inhibition. With respect to regulation of angiogenic molecules, 17-AAG significantly reduced EGFR-mediated VEGF secretion. Phosphorylation of focal adhesion kinase and paxillin were both abrogated by 17-AAG, which resulted in significant impairment of cancer cell motility. Interestingly, cytotoxic effects of 17-AAG in vitro were higher on cancer cells and gastric fibroblasts than on pericytes. In vivo, the water-soluble compound 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; 25 mg/kg, thrice per week) significantly reduced s.c. xenografted tumor growth. By immunohistochemistry, 17-DMAG significantly reduced vessel area and numbers of proliferating tumor cells in sections. Furthermore, similar significant growth-inhibitory effects of 17-DMAG were achieved when administered as low-dose therapy (5 mg/kg, thrice per week). In conclusion, blocking Hsp90 disrupts multiple proangiogenic signaling pathways in gastric cancer cells and inhibits xenografted tumor growth in vivo. Hence, gastric cancer harbors attractive molecular targets for therapy with Hsp90 inhibitors, which could lead to improved efficacy of antineoplastic therapy regimens. [Mol Cancer Ther 2007;6(3):1123–32]

Introduction
Angiogenesis is an essential process for growth and metastasis of solid malignancies, including gastrointestinal cancers. One of the most potent endothelial mitogens and mediators of angiogenesis is vascular endothelial growth factor (VEGF-A; ref. 1). In gastric cancer, expression of VEGF-A has been correlated with tumor progression and poor survival (2, 3). The epidermal growth factor receptor (EGFR) and HER-2/new have been identified as important regulators of gastric cancer growth and angiogenesis, which in part involves up-regulation of VEGF-A (4, 5). This induction of VEGF-A in cancer cells can be mediated through activation of various signaling pathways such as phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (Erk). Interestingly, PI3K/Akt has been reported to be highly activated in tissues of patients with gastric adenocarcinomas (6). In addition, both hypoxic and oncogenic activation of the transcription factor hypoxia-inducible factor-1α (HIF-1α) induce VEGF-A and promote tumor growth and metastasis (7–9). Using an experimental model of gastric cancer, we have previously shown that HIF-1α is an important regulator of gastric cancer growth and angiogenesis, suggesting that inhibition of HIF-1α function might be therapeutically efficacious (10). However, specific inhibitors of HIF-1α are still under development, and, to date, it is still not clear whether targeting solely HIF-1α is effective for cancer therapy because cancer cells may upregulate compensatory pathways to promote angiogenesis (11). Hence, targeting multiple receptor systems and...
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signaling substrates, including HIF-1α, may prove to be more effective.

Recently, the chaperone heat shock protein (Hsp90) has emerged as a promising target for cancer therapy because the function of multiple oncogenic proteins seems to be highly dependent on Hsp90, which therefore is of great importance for cancer cells survival (12). With respect to gastric cancer, Hsp90 client proteins comprise mutated EGFR (13), HER-2 (14), p53, mitogen-activated protein kinase/Erk, Akt (reviewed in ref. 15), and the transcription factor HIF-1α (16). Efficacy of geldanamycin and its derivatives to inhibit tumor growth has been shown in various preclinical xenografted tumor models (17–19), and some inhibitors (17-AAG) are currently being investigated in phase I/II trials (20–22). Importantly, Hsp90 is constitutively expressed at 2- to 10-fold higher levels in tumor cells compared with their normal counterparts, suggesting that tumor cells should be a selective target of Hsp90 inhibitors (23). In addition, recent reports also showed some antiangiogenic effects of Hsp90 inhibitors (24) and, when administered at maximal tolerated doses, even direct effects of these inhibitors on normal vasculature in terms of down-regulation of various VEGF systems (25).

In the present study, we hypothesized that targeting Hsp90 in gastric cancer cells would be highly efficacious through inhibition of multiple oncogenic pathways; hence, therapy with Hsp90 inhibitors would not require administration of maximal tolerated doses to achieve potent antiangiogenic and growth-inhibitory effects in vivo. Our results show that gastric cancer can be targeted at multiple levels, making low-dose Hsp90 inhibitor therapy an attractive new anticancer concept.

Materials and Methods

Cells and Culture Conditions

The human gastric cancer cell line TMK-1 was obtained from Dr. Eiichi Tahara (University of Hiroshima, Hiroshima, Japan), and KKLS cells were obtained from Yutaka Takahashi (Cancer Research Institute, Kanazawa University, Kanazawa, Japan). Human gastric fibroblasts and vascular smooth muscle cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM (Life Technologies, Karlsruhe, Germany) supplemented with 10% or 15% FCS and maintained in 5% CO2 at 37°C, as previously described (10, 26). All in vitro experiments were done at 60% to 70% cell density to avoid effects of cell confluence on HIF-1α and VEGF expression. For in vivo experiments, trypsinized cells were resuspended in HBSS.

Reagents and Antibodies

The Hsp90 inhibitors 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) were obtained from Invivogen (Toulouse, France). For in vitro experiments, 17-AAG was dissolved in DMSO (0.1%) and added to culture media, and equal concentrations of DMSO alone served as control a in all experiments. Water-soluble 17-DMAG was used for in vivo studies. Antibodies against signal transducer and activator of transcription 3 (STAT3), phosphorylated STAT3 Tyr705, Erk1/2, phosphorylated Erk1/2 Thr202/Tyr204, Akt, phosphorylated Akt Thr473, cyclic AMP–responsive element binding protein (CREB), focal adhesion kinase (FAK), phosphorylated FAK Tyr925, paxillin, phosphorylated paxillin Tyr118, EGFR, phosphorylated EGFR Tyr1068, phosphorylated EGFR Tyr845, phosphorylated p70S6K Thr389/Ser405, phosphorylated p85/PI3K Tyr377, and HER-2/neu were purchased from Cell Signaling Technologies (Beverly, MA). Probing with an anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) served as a loading control in Western blot analyses. Antibodies to HIF-1α (NB100-105) were purchased from Novus Biologicals (Littleton, CO), and anti-VEGF-A was purchased from R&D Systems (Minneapolis, MN). For stimulation assays, recombinant human EGF was used (R&D Systems).

Immunoblot Analysis of Constitutive and Inducible Signaling Intermediates

To determine the effects of Hsp90 inhibition on constitutive signaling pathway activation, cancer cells were incubated for 4 and 24 h in 10% FCS-MEM containing 17-AAG (100 nmol/L). Dose-dependent effects of 17-AAG on inducible signaling and EGFR activation were investigated by preincubating cells with 17-AAG (100 nmol/L, 16 h) followed by a stimulation with recombinant human EGF (40 ng/mL, 15 min) under serum-reduced conditions (1% FCS-MEM). Cells were lysed for Western blotting, as described previously (27). Protein samples (50 μg) were subjected to Western blotting on a denaturating 10% SDS-PAGE. Analyses for HIF-1α and CREB were done under nuclear protein extracts, as described (10). Desferroxamine for HIF-1α induction was obtained from Sigma-Aldrich (Deisenhofen, Germany) Nuclear protein was extracted using a commercially available kit (NucBuster; Novagen, Merck Biosciences, Darmstadt, Germany).

Immunoprecipitation of HER-2/neu

For detecting changes in HER-2/neu expression in gastric cancer cells, whole-cell lysates (200 μg) were immunoprecipitated overnight at 4°C using an antibody to HER-2/neu (1 μg) and A/G Plus agarose (Santa Cruz Biotechnology). Western blotting was subsequently done on a denaturating 8% SDS-PAGE, as described (27).

ELISA for VEGF-A Secretion

VEGF-A secretion by cancer cells was determined with an ELISA kit specific for human VEGF-A (BioSource Europe, Nivelles, Belgium). Gastric cancer cells were plated at 40% to 50% density and incubated with or without 17-AAG (100 nmol/L) and/or EGF (40 ng/mL) for 48 h. Analysis of culture supernatant was done according to the manufacturer’s protocol. Cells were harvested by trypsinization and counted. Detected VEGF levels were calculated as pg/mL (10).

Real-time PCR for VEGF-A Expression

Total RNA was isolated using Trizol Reagent (Invitrogen, Karlsruhe, Germany) and subsequently purified with ethanol precipitation. For each RNA sample, a 1 μg aliquot was reversed transcribed into cDNA (Superscript II kit,
vascular smooth muscle cells, cells (10³ per well) were tumor cells (TMK-1 and KKLS), gastric fibroblasts, and average numbers were calculated. Newark, NJ). Cells were counted in four random fields, cells were stained (Diff-Quick reagent, Dade Behring, Germany) using Roche Fast-Start LightCycler-Master Hybridization Probes master mix.

Migration and Invasion Assays
To determine the effect of 17-AAG treatment (100 nmol/L) on cancer cell motility in vitro, migration and invasion assays were done using modified Boyden chambers, as described (28). Briefly, 1 x 10⁵ cells were resuspended in 1% FCS-DMEM and seeded into inserts with 8-μm filter pores that were either uncoated (migration assay), or coated with growth factor–reduced Matrigel (invasion; Becton Dickinson Bioscience, Heidelberg, Germany), and 10% FCS-DMEM with or without EGF (40 ng/mL) served as a chemoattractant. After 48 h, cells were fixed, and migrated cells were stained (Diff-Quick reagent, Dade Behring, Newark, NJ). Cells were counted in four random fields, and average numbers were calculated.

Methythiazole Tetrazolium Analyses
To evaluate effects of 17-AAG on the proliferation of tumor cells (TMK-1 and KKLS), gastric fibroblasts, and vascular smooth muscle cells, cells (10⁵ per well) were seeded into 96-well plates and exposed to various concentrations of 17-AAG for an indicated time at 37°C. We used the methythiazole tetrazolium assay to assess cell numbers, as previously described (27). In addition, cytotoxicity was determined using assays where cells (10⁴ per well) were seeded into six-well plates and treated with 17-AAG (100 nmol/L), as described above. Viable cancer cells were counted thereafter using trypan blue staining.

Animal Model
Eight-week-old male athymic nude mice (BALB/c nu/nu, Charles River, Sulzfeld, Germany) were used for experiments, as approved by the Institutional Animal Care and Use Committee of the University of Regensburg and the regional authorities. In addition, experiments were conducted according to Guidelines for the Welfare of Animals in Experimental Neoplasia published by the United Kingdom Coordinating Committee on Cancer Research. The effects of Hsp90 inhibition on the growth of human gastric cancer cells were investigated in a s.c. xenograft tumor model. TMK-1 (or KKLS) cells (1 x 10⁶) were injected into the subcutis (right flank) of nude mice. Mice were randomized (n = 5–9 per group) and assigned to treatment groups. i.p. injection of 17-DMAg was started when mean tumor volumes had reached 100 mm³. Tumor diameters of tumor-bearing mice were measured every other day, and tumor volumes were calculated (width² x length x 0.5). When the experiment was terminated, s.c. tumors were excised, weighed, and prepared for immunohistochemical analyses. Western blot analyses on tumor tissues were done by using Lysis buffer for protein extraction and subsequent SDS-PAGE, as described above.

Immunohistochemical Analysis of Vessel Area and Tumor Cell Proliferation
Multiple cryosections were obtained from tumors for immunohistochemical analyses. For assessment of vessel area, rat anti-mouse CD31/platelet/endothelial cell adhesion molecule 1 antibody (PharMingen, San Diego, CA) and peroxidase-conjugated goat anti-rat IgG (Jackson Research Laboratories, West Grove, PA) were used (10). Antibody binding was visualized using diaminobenzidine. Images were obtained in four different quadrants of each tumor section (2 mm inside the tumor-normal tissue interface) at ×40 magnification. Measurement of vessel area of CD31-stained vessels was done by converting images to grayscale and setting a consistent threshold for all slides using ImageJ software (version 1.33; NIH, Bethesda, MD). Vessel areas were expressed as pixels per high-power field (27).

To determine proliferating tumor cells, mice received i.p. injections of bromodeoxyuridine (BrdUrd; Sigma-Aldrich; 1.0 mg per mouse) 2 h before termination of animal studies. A BrdUrd detection kit (Becton Dickinson Bioscience) was used to visualize BrdUrd uptake of cells in sections of tumors. Briefly, sections were incubated with anti-BrdUrd antibody solution followed by streptavidin-conjugated horseradish peroxidase–linked goat anti-mouse IgG2. Antibody binding was visualized by incubating slides in diaminobenzidine with hematoxylin counterstaining. BrdUrd-positive tumor cells were counted in four fields per tumor section at ×20 magnification, and averages were calculated (10).

Statistical Analyses and Densitometry
Statistical analyses were done using SigmaStat (version 3.0). Results of in vivo experiments were analyzed for outliers using the Grubb’s test. Tumor-associated variables in in vitro experiments were tested for statistical significance using the Mann-Whitney U test. The two-sided Student’s t test was applied for analysis of in vitro data. All results are expressed as the mean ± SE. Densitometry was done on Western blots to quantify changes in expression and phosphorylation of EGFR, HER-2, and FAK.

Results
Effect of Hsp90 Inhibition on EGF-Mediated Signaling Cascades in Gastric Cancer Cells
Because the EGF/EGFR system mediates its proliferative and proangiogenic effects in part through activating signaling cascades, which involve PI3K/Akt and mitogen-activated protein kinase/Erk, we first investigated whether inhibition of Hsp90 with the geldanamycin derivate 17-AAG would affect activity of these pathways under constitutive conditions. Experiments were done and confirmed in both cell lines; however, results are being illustrated representatively from TMK-1 cells. After 4 h,
marked changes in phosphorylation of Erk1/2 and Akt were not detectable upon treatment with 17-AAG. However, treating gastric cancer cells for 24 h with 17-AAG substantially reduced constitutive phosphorylation of both Erk1/2 and Akt (273) (Fig. 1A). In addition, constitutive activation of STAT3, which is another important transcription factor for regulating VEGF-A in cancer cells, was diminished upon Hsp90 blockade (Fig. 1A). We concluded that blocking Hsp90 could potentially interfere with important EGF-mediated and angiogenesis-related signaling cascades in gastric cancer cells. To determine optimal inhibitory doses of 17-AAG in vitro, TMK-1 cells were subsequently incubated under serum-reduced conditions with various concentrations of 17-AAG followed by stimulation with EGF. 17-AAG inhibited EGF-mediated activation of Erk1/2 and Akt in a dose-dependent manner and diminished constitutive STAT3 phosphorylation (Fig. 1B). The lowest inhibitory dose of 17-AAG (100 nmol/L) was used for all subsequent in vitro experiments.

Next, we investigated the effect of Hsp90 blockade on functionality and expression of EGFR in cancer cells because 17-AAG could directly interfere with receptor phosphorylation. Pretreatment of TMK-1 cells with 17-AAG markedly diminished EGFR phosphorylation upon stimulation with EGF (Fig. 1C). However, this reduction in receptor activation was paralleled by a dose-dependent down-regulation of EGFR itself (Fig. 1D), which was already detectable after 4 h of treatment with 17-AAG (data not shown). Similar to other reports (29–31), blocking Hsp90 markedly down-regulated HER-2/neu expression as well as phosphorylation in cancer cells, which also is an important mediator of gastric cancer growth (Fig. 1E). We concluded from these results that interference with Hsp90 could effectively impair EGFR signaling in gastric cancer through inhibition of downstream signaling and down-regulation of EGF receptor systems.

**Effect of Hsp90 Inhibition on Nuclear HIF-α and CREB Expression**

The ability of geldanamycin and its derivates to inhibit HIF-1α has been previously described (16). However, this effect seems to be dependent on the cell type. HIF-1α plays an important role in gastric cancer growth and angiogenesis (10). Hence, we investigated whether 17-AAG could interfere with hypoxia-mediated signaling in gastric cancer cells. Western blotting of nuclear protein extracts showed that blocking Hsp90 with 17-AAG completely abrogated desferroxamine-induced HIF-1α activation in cancer cells (Fig. 2A). Because hypoxia-induced stress signaling may be also mediated through HIF-1α–independent mechanisms, we next investigated a potential Hsp90-regulated expression of the transcription factor CREB, which has been implicated in cancer growth and angiogenesis (32).

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**Figure 1.** Effect of Hsp90 inhibition on activation of signaling cascades in gastric cancer cells. **A,** Western blot for activated signaling intermediates. Hsp90 inhibition led to a marked decrease in constitutive phosphorylation of Erk1/2, Akt, and STAT3 after 24 h. **B,** Western blot analysis for constitutive and EGF-mediated signaling pathway activation upon Hsp90 blockade. Cells were pretreated with increasing doses of 17-AAG and subsequently stimulated with EGF (15 min). 17-AAG led to a dose-dependent inhibition of constitutive and inducible Erk1/2 and Akt activation. In addition, constitutive STAT3 phosphorylation was dose-dependently reduced. Phosphorylation of PI3K/p85 subunit was not affected by 17-AAG. In contrast, activation of the Akt downstream substrate pS6K1 appeared diminished. β-Actin served as loading control. **C,** to determine the effects of Hsp90 inhibition on EGFR function, TMK-1 cells were treated for 16 h with 17-AAG and subsequently stimulated with EGF. Inhibition of Hsp90 led to a 60% reduction in receptor activation. **D,** this effect was paralleled by a similar decrease in receptor expression that therefore accounted for observed changes in receptor phosphorylation. **E,** immunoprecipitation analysis for HER-2 activation in cancer cells upon Hsp90 inhibition. Similar to effects on EGFR, blockade of Hsp90 decreased HER-2 activation via down-regulation of receptor expression. Results were additionally confirmed in KKLS cells (data not shown). IP, immunoprecipitation; WB, Western blot.
For angiogenesis, VEGF-A is a critical target gene, which is regulated in part by PI3K, mitogen-activated protein kinase, and HIF-1α signaling cascades. We therefore determined effects of Hsp90 inhibition on hypoxic induction of HIF-1α in cells was investigated by Western blotting of nuclear protein. Cells were incubated for 16 h under the presence or absence of 17-AAG and subsequently exposed to desferroxamine (DFX) for mimicking hypoxia. 17-AAG effectively blunted desferroxamine-mediated induction of HIF-1α protein.

To determine the effects of Hsp90 inhibition on EGF-mediated VEGF-A secretion, ELISA was done on culture supernatants. After 48 h, 17-AAG significantly reduced constitutive VEGF secretion and abrogated EGF-mediated (40 ng/mL) induction of VEGF-A. * P < 0.01.

Figure 2. Effects of Hsp90 blockade on HIF-1α, CREB, and VEGF-A. A, effect of Hsp90 inhibition on hypoxic induction of HIF-1α in cells was investigated by Western blotting of nuclear protein. Cells were incubated for 16 h under the presence or absence of 17-AAG and subsequently exposed to desferroxamine (DFX) for mimicking hypoxia. 17-AAG effectively blunted desferroxamine-mediated induction of HIF-1α protein. B, Western blot for nuclear CREB protein upon treatment with 17-AAG. Cells were incubated with 17-AAG and subsequently exposed to desferroxamine for various time points because CREB activity may be regulated through growth factor– and HIF-1α–independent hypoxic induction. Desferroxamine only slightly increased (1.5-fold) nuclear CREB; however, treatment with 17-AAG (100 nmol/L) markedly diminished CREB protein. C, to determine effects of Hsp90 inhibition on EGF-mediated VEGF secretion, ELISA was done on culture supernatants. After 48 h, 17-AAG significantly reduced constitutive VEGF secretion and abrogated EGF-mediated (40 ng/mL) induction of VEGF-A. * P < 0.01. D, Western blot for cellular VEGF-A protein upon hypoxic stimulation with desferroxamine (24 h). Treatment with 17-AAG markedly reduced cytoplasmatic VEGF protein content in cancer cells (in the presence of desferroxamine to mimic hypoxia), as determined by Western blotting of whole-cell lysates from viable cells (Fig. 2D). As determined by reverse transcription-PCR, 17-AAG also markedly reduced constitutive (46%) and inducible VEGF-A mRNA (86% for EGF) expression in cancer cells (data not shown), thus validating its functional interference with transcriptional regulators of VEGF. These results suggest that Hsp90 inhibitors may elicit antangiogenic activity in vivo through potent down-regulation of VEGF-A and inhibition of stress signaling.

Effect of Hsp90 Inhibition on Cancer Cell Migration and Invasion In vitro

Next, we addressed the effect of Hsp90 block on functional aspects of tumor cell activity, including cell migration and invasion. The basis for this examination comes from studies that identified FAK as client protein of Hsp90 (15, 33), suggesting that cancer cell motility might be impaired by 17-AAG. In Western blot analysis, we did observe down-regulation of FAK in TMK-1 cells, within 6 h of 17-AAG treatment. In contrast to other studies, we also detected a direct inhibitory effect of 17-AAG on EGF-mediated FAK phosphorylation, which occurred as early as 2 h after exposure to 17-AAG. In these experiments, 17-AAG reduced EGF-mediated FAK phosphorylation by 50% in addition to a subsequent down-regulation of FAK (with >2 h of exposure to 17-AAG; Fig. 3A). Furthermore, 17-AAG also diminished EGF-induced phosphorylation of paxillin, an important downstream effector of FAK (Fig. 3B). However, inhibition of paxillin activation by 17-AAG occurred only through down-regulation of this substrate. Therefore, Hsp90 inhibition potentially inhibits molecules important for tumor cell motility.

To determine the effects of Hsp90 inhibition on EGF-mediated cell motility, cancer cells (TMK-1) were subjected to migration and invasion assays. EGF significantly induced cancer cell migration and invasiveness (Fig. 3C and D). The presence of 17-AAG in the bottom of the chamber significantly blocked cancer cell migration and invasion both in the absence or presence of EGF (P < 0.01 for both). These results suggest that inhibitors of Hsp90 have the potential to reduce invasiveness and metastatic spread of gastric cancer cells.

Cytotoxic Effects of Hsp90 Inhibition on Tumor Cells, Gastric Fibroblasts, and Vascular Smooth Muscle Cells In vitro

To determine direct cytotoxic effects of Hsp90 inhibition on tumor cells in vitro, we did cell death assays using 17-AAG on gastric cancer cells (TMK-1 and KKLs). Results show that 17-AAG (100 nmol/L) significantly reduces tumor cell numbers (TMK-1) after 24 h [control: 32 ± 4 cells (10⁶ per well) versus 17-AAG treated: 18 ± 5 cells (10⁶ per well)] and 48 h [control: 75 ± 7 cells (10⁶ per well) versus 17-AAG treated: 14 ± 4 cells (10⁶ per well); P < 0.01 for both]. Dose escalation of 17-AAG up to 10 μmol/L in media resulted only in a modest increase of cytotoxic effects compared with the lowest effective dose of 17-AAG (100 nmol/L; data not available).

Treatment of gastric cancer cells with 17-AAG substantially reduced nuclear CREB protein content under both constitutive and desferroxamine-induced hypoxic conditions (Fig. 2B). Hence, we propose that blocking Hsp90 effectively interferes with hypoxic stress signaling in gastric cancer cells mediated through HIF-1α and CREB.

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We therefore hypothesize that Hsp90 inhibitors could be effective at low doses \textit{in vivo} to achieve potent growth-inhibitory effects in cancer therapy.

Because the tumor microenvironment also consists of nonmalignant cell compartments such as fibroblasts and pericytes, we next investigated whether 17-AAG would also affect proliferation and survival of gastric fibroblasts and vascular smooth muscle cells (representative for pericytes). To address this issue, methylthiazole tetrazolium analyses were done using an identical dose escalation scheme as mentioned above. Similar to gastric cancer cells, numbers of gastric fibroblasts were significantly reduced after 48 h (33% reduction) and 72 h (63% reduction) by 17-AAG treatment at 100 nmol/L compared with controls ($P < 0.01$ for both). Interestingly, in terms of reduction of cell numbers after 48 h, vascular smooth muscle cells did not respond to doses up to 100 nmol/L 17-AAG. To achieve similar cytotoxic effects, 17-AAG had to be used at 1 pmol/L, suggesting that targeting pericytes (with a direct cytotoxic effect) for antiangiogenic therapy would require higher doses of Hsp90 inhibitors \textit{in vivo} (data not shown).

**Effect of Hsp90 Blockade on Growth of Gastric Cancer Tumors \textit{In vivo}**

To estimate potential growth inhibitory and antiangiogenic effects of the Hsp90 inhibitor 17-DMAG \textit{in vivo}, we subsequently implanted TMK-1 cells s.c. into mice. Inhibition of Hsp90 with 17-DMAG (25 mg/kg, thrice per week) significantly reduced the growth of TMK-1 tumors compared with controls (Fig. 4A). This was also reflected by final tumor weights on day 28, which were significantly lower in the 17-DMAG treatment group (Fig. 4B). Similar growth inhibitory effects of 17-DMAG therapy were achieved in KKLS tumors, which resulted in a 60% reduction in final tumor volumes ($P < 0.05$; data not shown). In addition, tumor vascularization in TMK-1 tumors terms of CD31-positive vessel area was significantly reduced in tumor sections of the 17-DMAG group (Fig. 5A). Furthermore, 17-DMAG reduced the number of
proliferating (BrdUrd positive) cells within the s.c. tumors (Fig. 5B). Interestingly, we noticed that necrotic areas in 17-DMAG–treated tumors were detectable within a shorter distance to the closest microvessel compared with tissue sections from tumors of the control group. This would suggest an antiangiogenic effect contributing to growth inhibition with Hsp90 inhibitor treatment, in addition to impairing cancer cell survival (Fig. 5B).

Because blocking Hsp90 inhibited multiple signaling pathways involved in angiogenesis and cancer progression, we hypothesized that 17-DMAG would elicit substantial growth-inhibitory effects at much lower doses than reported previously. Therefore, we next investigated whether Hsp90 inhibition could also effectively reduce tumor growth in vivo using low-dose 17-DMAG therapy (5 mg/kg, thrice per week i.p.). Indeed, low-dose 17-DMAG therapy exhibited potent growth-inhibitory effects in vivo, which led to a significant reduction in final tumor volumes and tumor weights, compared with controls (Fig. 6A and B). The in vivo effect of low-dose 17-DMAG treatment was nearly identical to the “high-dose” regimen. Notably, the “high-dose” regimen already represents a dosing of 17-DMAG, which is half of the dose reported by other groups. Moreover, we investigated whether EGFR could be down-regulated by this therapy regimen. Surprisingly, results from Western blot analyses of tumor tissues revealed that only high-dose therapy indeed down-regulated EGFR in tissues (Fig. 6C). However, a substantial decrease in phosphorylation of Akt (as a downstream mediator of EGFR) was also effectively achieved by low-dose 17-DMAG therapy (Fig. 6C). Changes in Erk1/2 or STAT3 phosphorylation were only minor in both treatment groups, which may be due to the ability of a “fast recovery”

**Figure 5.** Immunohistochemical analysis of tumor vascularization and tumor cell proliferation in tissues. **A,** densitometric analysis of images from CD31-stained tissue section showed that the vessel area was significantly reduced in 17-DMAG–treated tumors. *, **P** < 0.05. **B,** 17-DMAG significantly decreased the number of proliferating (BrdUrd positive) tumor cells in tissue sections. *, **P** < 0.01. Representative images for both vessel area and cell proliferation data. **Columns,** mean; **bars,** SE.

**Figure 6.** Effect of low-dose 17-DMAG therapy on s.c. tumor growth in vivo. TMK-1 cells were implanted s.c., and mice received the water-soluble Hsp90 inhibitor 17-DMAG at either “high-dose” (25 mg/kg, thrice per week) or low-dose (5 mg/kg, thrice per week) concentration, whereas controls received diluent. **A,** treatment with low-dose 17-DMAG led to a significant reduction in final tumor volumes compared with the high-dose regimen. *, **P** < 0.05. **B,** this result was also reflected by final weights of excised tumors, where 17-DMAG (n = 5–6) significantly lowered tumor burden compared with tumors of the control group (n = 5). *, **P** < 0.05. **C,** Western blot analyses for EGFR and Akt in tumor tissues. High-dose therapy markedly diminished EGFR expression, whereas low-dose therapy was sufficient to inhibit Akt activation in tumors. **Columns,** mean; **bars,** SE.
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Discussion

Our study shows efficacy of Hsp90 inhibitors for therapy of gastric cancer based on three important findings: (a) EGFR and HER-2 are targeted by Hsp90 inhibitors in gastric cancer cell lines despite the lack of receptor mutations; (b) inhibition of Hsp90 impairs multiple angiogenic signaling pathways in vitro and reduces the growth and vascularization of gastric cancer xenografts in vivo; and (c) low-dose therapy with 17-DMAG proved to be efficacious in vivo.

Previous studies have described growth-inhibitory effects of Hsp90 inhibitors and their interference with oncogenic signaling cascades (12, 15, 25, 35, 36). Here, we focused on the efficacy of Hsp90 inhibitors to specifically disrupt EGFR and hypoxia-mediated angiogenic signaling pathways in gastric cancer cells because activation of EGFR and/or HIF-1α have been associated with growth and angiogenesis of human gastric cancer (10, 37, 38). In our experiments, blocking Hsp90 substantially disrupted EGFR-mediated signaling in gastric cancer cells. This interference occurred in part through inhibition of constitutive and inducible mitogen-activated protein kinase/Erk1/2 and Akt phosphorylation as well as through reduction in constitutive activation of STAT3, which is another important pathway for mediating VEGF induction (39, 40). In addition, we detected a significant down-regulation of EGFR upon Hsp90 blockade. In contrast to other reports, down-regulation of EGFR was already detectable in gastric cancer cells after 4 h, which was a similar timeframe for reduction observed in HER-2 expression. However, Hsp90 inhibition did not have a direct functional inhibition on EGFR in terms of EGFR-mediated receptor phosphorylation. Interestingly, EGFR seemed to be a direct target of Hsp90 inhibition, although this receptor system was not mutated, as determined by sequencing of our gastric cancer cell lines (data not shown). In contrast, Shimamura et al. recently showed that in non–small cell lung cancer, efficacy of geldanamycin in terms of EGFR degradation highly depends on the presence of somatic mutations in the kinase domain of the EGFR in cancer cells, which is frequently detectable in non–small cell lung cancer (13). Hence, our data suggest that in gastric cancer, disruption of EGFR function by Hsp90 inhibitors, such as 17-DMAG, does not critically depend on the presence of such somatic EGFR mutations. Moreover, HER-2 seems to be another growth factor receptor system, which is of relevance for gastric cancer growth and angiogenesis, that is being efficiently targeted by Hsp90 inhibitors (34, 37).

Another important observation from our study is that inhibition of Hsp90 reduced activation of multiple signaling pathways in gastric cancer cells associated with VEGF-A expression and angiogenesis, such as Erk1/2, Akt, STAT3, and HIF-1α. Furthermore, 17-AAG treatment diminished EGF- and hypoxia-mediated VEGF-A secretion in our in vitro analyses. Interestingly, blocking Hsp90 also reduced nuclear protein content of another transcription factor involved in hypoxia- and growth factor–mediated VEGF regulation (independently of HIF-1α), which is CREB (41, 42). This potential antiangiogenic effect of Hsp90 inhibition was also reflected by results from our in vivo tumor models. 17-DMA treatment significantly reduced vascularization of TMK-1 tumors and altered vessel morphology. Tumor vessels in the treatment group seemed to be fractionated and less functional because necrotic areas seemed to occur within a shorter distance from the nearest microvessel compared with controls. However, we speculate that these effects on neoplastic vasculature are indirect (e.g., reduction of VEGF-A) rather than direct (e.g., effect on endothelial cells), as Hsp90 inhibitors bind with a much higher affinity to Hsp90 located in tumor cells than to Hsp90 in normal nonneoplastic cells (23). Nevertheless, some direct antiangiogenic effect of Hsp90 inhibitors, such as 17-DMAG, have also been reported (25, 43). In one study, 17-DMA treatment decreased vascularization and hemoglobin content in a Matrigel angiogenesis assay in vivo (43). Furthermore, Sanderson et al. recently showed that 17-AAG significantly reduces expression of three VEGF receptors (VEGF-R1, VEGF-R2, and VEGF-R3) on mouse vasculature in vivo (25). However, both studies used maximal tolerated doses of respective Hsp90 inhibitors to achieve these direct antiangiogenic effects on endothelial cells or vasculature in vivo. In our hands, vascular smooth muscle cells (pericytes) had to be treated with 10-fold higher doses of Hsp90 inhibitor 17-AAG to achieve similar inhibition of cell proliferation than gastric cancer cells. In addition, as the microenvironment is also of great importance for angiogenesis and tumor growth, we found gastric fibroblasts to respond to 17-AAG treatment as sensitive as gastric cancer cells in vitro, suggesting that these cell types would potentially be targeted in vivo. We therefore hypothesized that targeting gastric cancer (tumor and stroma) with Hsp90 inhibitors does not require high-dose therapy to achieve antiangiogenic and growth-inhibitory effects in vivo. This seems of particular importance for reducing side effects with long-term antiangiogenic treatment regimens for cancer patients.

Based on the results from our in vitro experiments and combined with the pleiotropism of Hsp90 inhibition on intracellular signaling pathways, we further investigated the effect of different dosing regimens in vivo. To test the concept of low-dose therapy, we used 17-DMAG either at 75 mg/kg/wk, which represents a medium-dose regimen compared with other reports (25, 35, 43), or 15 mg/kg/wk (low dose) for therapy of s.c. tumors. As predicted, low-dose 17-DMAG therapy was as effective as the high-dose therapy at inhibiting tumor growth, suggesting that targeting multiple oncogenic pathways by Hsp90 inhibition is very efficacious for cancer therapy. Surprisingly, we...
found that only high-dose therapy reduced EGFR expression in tumors. However, low-dose therapy already effectively inhibited Akt activation, suggesting that signaling pathway inhibition indeed was sufficient. In contrast, Erk1/2 and STAT3 were diminished to a lesser extent (data not shown), which could be due to a suboptimal therapy regimen (three per week), allowing a recovery (within 24 h) of these signaling intermediates, as reported by others (34). Furthermore, it has to be realized that tumor volumes differ substantially among groups (treatment versus control), thus demanding caution interpretation. Together, these results show that gastric cancer uses molecular pathways suitable for targeted therapy concepts with Hsp90 inhibitors.

In addition to the antineoplastic/antiangiogenic potential of Hsp90 inhibitors, this study also emphasizes the potential of Hsp90 inhibitors to reduce cancer cell invasiveness. Blockade of Hsp90 reduced cancer cell migration and led to a direct functional inhibition of EGF-mediated FAK phosphorylation, before its down-regulation. Moreover, phosphorylation of Paxillin, as an important mediator of FAK, seemed to be down-regulated by 17-AAG, suggesting that Hsp90 inhibitors may inhibit gastric cancer metastasis. This is of particular relevance because Price et al. recently described that Hsp90 inhibitors promoted formation of bone metastases through activating osteoclasts, which was shown in an experimental model of breast cancer (44). However, formation of bone metastases is not a predominant phenomenon of gastric cancer. Therefore, we suggest that effect of Hsp90 blockade on tumor cell motility is highly dependent on tumor entity and tumor cell line. The role of Hsp90 inhibitors for reducing metastasis of gastrointestinal cancers warrants further investigation.

In conclusion, the present study shows that gastric cancer cells harbor multiple attractive molecular targets that can be effectively neutralized by Hsp90 inhibitors. Furthermore, therapy with Hsp90 inhibitors does not seem to require treatment at maximum tolerated dose levels to achieve significant growth-inhibitory and antiangiogenic effects \emph{in vivo}. This finding should have important implications for future gastric cancer clinical trials, particularly in light of new data showing that drug efficacy of Hsp90 inhibitors can be monitored in patients (31). Moreover, synthetically inhibitors to Hsp90 that can be administered orally are under development (34).

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References


Inhibition of heat shock protein 90 impairs epidermal growth factor –mediated signaling in gastric cancer cells and reduces tumor growth and vascularization \textit{in vivo}

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