Up-regulation of Heat Shock Protein 27 Induces Resistance to 17-Allylamino-Demethoxygeldanamycin through a Glutathione-Mediated Mechanism

Andrea K. McCollum, Cynthia J. TenEyck, Brian M. Sauer, David O. Toft, and Charles Erlichman

Abstract
17-Allylamino-demethoxygeldanamycin (17-AAG), currently in phase I and II clinical trials as an anticancer agent, binds to the ATP pocket of heat shock protein (Hsp90). This binding induces a cellular stress response that up-regulates many proteins including Hsp27, a member of the small heat shock protein family that has cytoprotective roles, including chaperoning of cellular proteins, regulation of apoptotic signaling, and modulation of oxidative stress. Therefore, we hypothesized that Hsp27 expression may affect cancer cell sensitivity to 17-AAG. In colony-forming assays, overexpression of Hsp27 increased cell resistance to 17-AAG whereas down-regulation of Hsp27 by siRNA increased sensitivity. Because Hsp27 is known to modulate levels of glutathione (GSH), we examined cellular levels of GSH and found that it was decreased in cells transfected with Hsp27 siRNA when compared with control siRNA. Treatment with buthionine sulfoximine, an inhibitor of GSH synthesis, also sensitized cells to 17-AAG. Conversely, treatment of Hsp27 siRNA-transfected cells with N-acetylcysteine, an antioxidant and GSH precursor, reversed their sensitivity to 17-AAG. A cell line selected for stable resistance to geldanamycin relative to parent cells showed increased Hsp27 expression. When these geldanamycin- and 17-AAG-resistant cells were transfected with Hsp27 siRNA, 17-AAG resistance was dramatically diminished. Our results suggest that Hsp27 up-regulation has a significant role in 17-AAG resistance, which may be mediated in part through GSH regulation. Clinical modulation of GSH may therefore enhance the efficacy of Hsp90-directed therapy. (Cancer Res 2006; 66(22): 10967-75)

Introduction
Heat shock protein (Hsp90) is an abundant cytosolic molecular chaperone found within multimeric chaperone complexes known to participate in regulating protein homeostasis within cells. Structural and biochemical studies have shown that ansamycin antibiotics such as geldanamycin (GA) and 17-allylamino-demethoxygeldanamycin (17-AAG) bind to the conserved ATP pocket of Hsp90 (1–3). Because ansamycins have shown tumoricidal effects in vitro and in vivo, Hsp90 inhibitors are being developed as anticancer agents (4). GA derivatives such as 17-AAG and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), which have similar activity to GA, are now in phase I and phase II clinical trials (5–8). Treatment with these compounds produces a variety of responses in cells, including inhibition of Hsp90 chaperone complex activity (1), degradation of client proteins (9), and up-regulation of proteins such as Hsp70 and Hsp27 (10–12).

The cellular stress response is characterized by up-regulation of many proteins by the transcription factor heat shock factor-1 (HSF-1), particularly members of the Hsp70 family (13), as well as small heat shock proteins, including Hsp27 (14, 15). Because these proteins are dramatically up-regulated in response to treatment with Hsp90 inhibitors such as GA or 17-AAG (10–12), their potential involvement in resistance to Hsp90 inhibitors in the clinic has recently gained interest (16). Other studies have shown the protection of cancer cells from 17-AAG-induced death by Hsp70, which is known to be up-regulated in response to Hsp90-directed agents (17). However, other proteins that are up-regulated in an HSF-1-mediated stress response may also contribute to differences in 17-AAG sensitivity.

One such protein is Hsp27, a 205-amino-acid member of the small heat shock protein family that is known to confer thermotolerance in cells with expression (18). Hsp27 has also been identified as a participant in cellular chemoresistance in response to treatment with anticancer drugs such as cisplatin (19) and has been shown to be up-regulated in response to GA and 17-AAG (10–12). The conserved α-crystallin domain that characterizes the small heat shock protein family is found in the COOH terminus of Hsp27 and serves to mediate protein-protein interactions that govern the formation of high molecular weight oligomers (14, 20). These oligomers are thought to be the active form of Hsp27 that perform chaperoning functions, as well as regulation of oxidative stress through modulation of intracellular glutathione (GSH) levels (20).

Hsp27-mediated regulation of GSH, or γ-glutamylcysteinylglycine, was first identified through studies to determine mechanisms of resistance to tumor necrosis factor α (TNF-α). Those studies have shown that Hsp27 expression increases survival after TNF-α treatment while lowering intracellular oxidative species (21). Depletion of GSH by buthionine sulfoximine (BSO), a compound that binds γ-glutamylcysteinyl synthase and causes GSH production inhibition, showed that Hsp27 does not block TNF-α-induced cell death through another mechanism (22). More recent studies have shown that Hsp27 participates in regulation of several detoxifying enzymes. In particular, Hsp27 overexpression increases expression and activity of glucose-6-phosphate dehydrogenase, an enzyme that functions to reduce NADP⁺ during cellular recycling and reduction of GSH (22). Other detoxifying enzymes also have increased activity due to overexpression of Hsp27, such as...
glutathione-S-transferase and glutathione reductase, and Hsp27 expression serves to protect the activity of these enzymes after exposure to hydrogen peroxide (22).

In this study, we have identified Hsp27 as a modulator of 17-AAG sensitivity in tumor cells. Our data show that Hsp27 expression affects sensitivity to 17-AAG without affecting client protein down-regulation or stress response induction after treatment. To determine a potential mechanism for the Hsp27-modulated sensitivity to 17-AAG, we examined Hsp27 regulation of GSH in cells treated with 17-AAG. Our results suggest that Hsp27 contributes to 17-AAG resistance, at least in part, through regulation of GSH and that modulation of GSH clinically may enhance 17-AAG efficacy in patients.

Materials and Methods

Materials. Reagents were obtained from the following sources: GA from Dr. V.L. Narayanan (Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD); 17-AAG from Dr. R. Johnson (Kosan Biosciences, Hayward, CA); BSO and N-acetylcysteine (NAC) from Sigma (St. Louis, MO); and enhanced chemiluminesence reagents from Amersham Pharmacia Biotechnology (Piscataway, NJ).

Antibodies. H9010 mouse monoclonal antibody recognizing Hsp90 was previously described (23). The remaining antibodies were purchased from the following suppliers: peroxidase-coupled affinity-purified goat anti-mouse and goat anti-rabbit secondary antibodies from Kirkegaard & Perry Laboratories (Gaithersburg, MD); mouse monoclonal anti–poly(ADP-ribose) polymerase from BD Transduction Labs (Lexington, KY); mouse monoclonal anti-Hsp70, mouse monoclonal anti-Hsp27, and mouse monoclonal anti-Raf-1 from Stressgen (San Diego, CA); rabbit polyclonal anti-Akt from Cell Signaling Technologies (Beverly, MA); and mouse monoclonal anti-actin from Sigma.

Cell culture. HeLa and PC3 cell lines were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 units/ml penicillin. A549 and A549GARS were cultured in RPMI 1640 with 5% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin.

Creation of A549GARS. A549 cells were incubated with 30 nmol/L GA in RPMI 1640 with 5% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin. Surviving cells were allowed to grow to confluence, then GA was added. Cells were trypsinized and replated for clonogenic assays. For overexpression of Hsp27, PC3 cells were transfected with either DMSO or 1 μmol/L 17-AAG (lanes 2 and 4) for 24 hours. Cell lysates were probed by Western blotting for the presence of Hsp90, Hsp70, and Hsp27, and actin expression. Overexpression of Hsp27 increases resistance to 17-AAG. PC3 cells were transfected and sorted as in (B), then used in clonogenic assays. Cells were treated with DMSO or 17-AAG in concentrations indicated. After 24-hour treatment, cells were washed in serum-free medium, then incubated in fresh medium for 12 days to allow colonies to form. Final DMSO concentration was 1% in all experiments. Bars, SE (n = 3).

Transfections. For siRNA, HeLa cells were plated in six-well plates at a density of 5 × 10⁵ per well and allowed to adhere for 20 to 24 hours. Four hundred nanomoles of control siRNA #1 (Dharmacon, Lafayette, CO), Hsp70-specific siRNA (accession no. NM_005346, tcatcagggactgtcaca), or Hsp27-specific siRNA (accession no. BC073768, ttagactgcccagtaa) were complexed with 10 μL of Lipofectamine 2000 in 0.5 mL of Opti-MEM for 10 minutes. Cells were incubated for 4 hours with complexed lipid-siRNA, after which 1 mL of Opti-MEM containing 35% FBS was added. The next day, cultures were washed once with serum-free medium, and fresh medium was added. Cells were trypsinized and replated for clonogenic assays or immunoblotting the next day. For overexpression of Hsp27, PC3 cells were plated in six-well plates at a density of 5 × 10⁵ per well and allowed to adhere for 20 to 24 hours. Cells were transfected as above with 1 μg of pEGFP-N1, containing enhanced green fluorescent protein (GFP), from Clontech (Mountain View, CA) or 5 μg of pSV2711 (kindly provided by Drs. Eileen Hickey and Lee Weber, University of Nevada, Reno, NV; ref. 18), containing wild-type Hsp27 and 1 μg pEGFP-N1. Cells were trypsinized and sorted by a Becton Dickinson FACS Vantage Sorter (San Jose, CA) for wide-range fluorescence-activated cell sorting.

\[ \text{GFP} \]
\[ \text{IC}_{50} = 9 \text{nM} \]
\[ \text{pSV2711} \]
\[ \text{IC}_{50} = 28 \text{nM} \]

Figure 1. Hsp27 expression induces 17-AAG resistance. A, Hsp27 is induced by 17-AAG. HeLa and PC3 cells were treated for 24 hours with diluent alone (lanes C) or 17-AAG at 1 nmol/L (lanes 2 and 7), 10 nmol/L (lanes 3 and 8), 100 nmol/L (lanes 4 and 9), or 1000 nmol/L (lanes 5 and 10). Fifty micrograms of cell lysates were resolved by SDS-PAGE and detected by Western blotting. Actin was used as a loading control in all experiments. B, overexpression of Hsp27. PC3 cells were transfected with GFP or GFP + pSV2711, then sorted by fluorescence-activated cell sorting. Cells were treated with either DMSO (lanes 1 and 3) or 1 μmol/L 17-AAG (lanes 2 and 4) for 24 hours. Cell lysates were probed by Western blotting for the presence of Hsp90, Hsp70, Hsp27, and actin expression. C, overexpression of Hsp27 increases resistance to 17-AAG. PC3 cells were transfected and sorted as in (B), then used in clonogenic assays. Cells were treated with DMSO or 17-AAG in concentrations indicated. After 24-hour treatment, cells were washed in serum-free medium, then incubated in fresh medium for 12 days to allow colonies to form. Final DMSO concentration was 1% in all experiments. Bars, SE (n = 3).
GFP expression, then plated for either clonogenic assays or Western blot analysis, as described below.

**Hoechst 33258 staining.** Transfected cells were grown in 100-mm dishes and harvested by scraping. Adherent and nonadherent cells were combined and pelleted at 250 × g for 5 minutes at 4°C, washed once with ice-cold PBS, and fixed in 3:1 methanol/acetic acid overnight at room temperature. Fixed cells were applied to coverslips, then stained with 1 μg/mL Hoechst 33258 in 50 mmol/L Tris-HCl (pH 7.4 at 21°C) containing 50% (v/v) glycerol. The presence of apoptosis was determined by examining slides by fluorescence microscopy and recording the number of cells that showed apoptotic nuclear morphology. At least 500 cells were counted per slide.

**Clonogenic assays.** A549, A549 GARS, transfected or untransfected HeLa, or transfected PC3 cells were trypsinized and plated in 60-mm tissue culture plates to a density of 1,500, 1,500, 500, 500 or 3,000 per plate, respectively. Cells were allowed to adhere for 22 to 24 hours, then drugs were added as indicated to final concentrations from 100-fold concentrated stocks. After 24-hour incubation, plates were washed twice with serum-free medium, then fresh medium was added, and cells were incubated until colonies were visible. The plates were washed once with PBS and stained with Coomassie brilliant blue. Visible colonies were counted, with typical plating efficiencies of 250 to 400, 250 to 300, 250 to 300, 250 to 400, or 100 to 150 colonies for A549, A549 GARS, transfected or untransfected HeLa, or transfected PC3 cells, respectively.

**Immunoblotting.** Cells were plated on 100-mm dishes, allowed to adhere for 22 to 24 hours, then treated as described. Adherent cells were lifted from plates by scraping, combined with nonadherent cells, pelleted at 100 × g for 5 minutes at 4°C, rinsed once with ice-cold PBS, and then lysed in lysis buffer containing 10 mmol/L HEPES (pH 7.4), 20 mmol/L sodium molybdate, 150 mmol/L KCl, 10 mmol/L MgCl₂, 0.1% NP40, 1 mmol/L Na₃VO₄, and protease inhibitors (Complete, mini, EDTA-free tablets; Roche, Indianapolis, IN). After a 10-minute incubation on ice, the detergent insoluble fractions were pelleted at 18,000 × g for 2 minutes at 4°C. Total protein concentrations of supernatants were estimated by the bicinchoninic acid (BCA) method (24). Samples containing 50 μg of total protein were separated by one-dimensional SDS-PAGE on a 10% acrylamide gel. The separated polypeptides were transferred to nitrocellulose, probed with antibodies, and visualized by enhanced chemiluminescence as previously described (25).

**GSH assay.** Cells were plated on 100-mm dishes, allowed to adhere for 22 to 24 hours, then left untreated or treated with 100 μmol/L BSO for 1 hour, 100 μmol/L BSO for 25 hours, 1 μmol/L 17-AAG for 24 hours, or 100 μmol/L BSO for 1 hour followed by addition of 17-AAG for 24 hours as indicated. Adherent cells were lifted from plates by scraping, combined with nonadherent cells, pelleted at 100 × g for 5 minutes at 4°C, and rinsed once with ice-cold PBS. Cells were resuspended in 0.5 mL of ice-cold MES buffer [containing 0.4 mol/L 2-(N-morpholino) ethanesulfonic acid, 0.1 mol/L phosphate, and 2 mmol/L EDTA, pH 6.0], then sonicated for 15 bursts. Cell lysates were then sedimented at 18,000 × g for 15 minutes at 4°C. One volume of fresh 1.25 mol/L metaphosphoric acid was added to the supernatants after a small sample was removed for protein concentration determination by BCA method (24). After incubation with metaphosphoric acid for 5 minutes at room temperature, insoluble material was sedimented at 18,000 × g for 3 minutes. Supernatants were then stored frozen at −20°C until use (all samples were used within 2 weeks of collection). Total GSH concentration was determined with the Glutathione Assay Kit from Cayman Chemical (Ann Arbor, MI) according to the instructions of the manufacturer.

Figure 2. Knockdown of Hsp27 increases sensitivity to 17-AAG. A, HeLa cells were transfected with siRNA as described in Materials and Methods, then plated for clonogenic assays. Cells were treated with 17-AAG for 24 hours in concentrations indicated, then washed in serum-free medium, and incubated in drug-free medium for 7 days to allow colonies to form. Bars, SE (n = 3). B, HeLa cells were transfected with control or Hsp27 siRNA as indicated, then treated with DMSO (lanes 1 and 2) or 1 μmol/L 17-AAG (lane 3) for 24 hours. Western blotting was done with 50 μg of cell lysates harvested after treatment and separated by SDS-PAGE. C, HeLa cells were transfected with control, Hsp27, Hsp70, or both Hsp27 and Hsp70 siRNA as indicated, then plated for clonogenic assays. Cells were treated with 17-AAG for 24 hours in concentrations indicated, then washed in serum-free medium, and incubated in drug-free medium for 7 days to allow colonies to form. Bars, SE (n = 3). D, HeLa cells were transfected as indicated, then treated with DMSO (lanes 1, 3, 5 and 7) or 1 μmol/L 17-AAG (lanes 2, 4, 6 and 8) for 24 hours. Western blotting was done with 50 μg of cell lysates harvested after treatment and separated by SDS-PAGE.
Statistical analysis. Statistical analysis consisted of the Student’s t test. *P < 0.05 indicated statistical significance.

Results

Overexpression of Hsp27 decreases sensitivity to 17-AAG. Previous studies have shown that Hsp27 is highly up-regulated after treatment of cells with Hsp90-directed agents such as 17-AAG (10, 12). This prompted us to hypothesize that up-regulation of Hsp27 would decrease 17-AAG cell sensitivity and contribute to 17-AAG resistance.

We first defined the basal and 17-AAG-induced expression levels of Hsp27 in two cancer cell lines and found that HeLa cells expressed high levels of Hsp27, whereas PC3 cells expressed low levels. Both cell lines showed dramatic up-regulation of Hsp27 and Hsp70 after 24 hours of treatment with 17-AAG (Fig. 1A). As HeLa cells have a high basal level of Hsp27, overexpressing Hsp27 in these cells may not lead to a significant up-regulation of Hsp27, and thus may not produce a dramatic difference in sensitivity to 17-AAG. Therefore, to assess the effect of constitutive high-level Hsp27 expression on tumor cell survival after 17-AAG treatment, we exogenously expressed wild-type Hsp27 in PC3 cells and were able to achieve high expression of Hsp27 as compared with GFP-only transfected cells. Although these cells overexpress Hsp27, a robust increase in Hsp70 is still observed after 17-AAG treatment (Fig. 1B).

Western blotting shows that cells transfected with Hsp27 siRNA had dramatically decreased expression of Hsp27 as compared with control, and it remained suppressed after 17-AAG treatment. Concomitantly, 17-AAG caused an increase in Hsp70 and a decrease in Hsp90 client proteins Akt and Raf-1 kinases (Fig. 2B). This indicates that the decreased IC₅₀ is caused by the down-regulation of Hsp27, and not by interference with the general up-regulation of cell stress proteins or with degradation of Hsp90 client proteins.

Because Hsp70 is up-regulated after 17-AAG treatment, and previous studies have shown that Hsp70 offers protection to tumor cells against 17-AAG (17), we chose to compare the protection offered by Hsp27 and Hsp70 against 17-AAG-induced cytotoxicity. As predicted, knockdown of either Hsp27 or Hsp70 (Fig. 2D) decreased the 17-AAG IC₅₀ as compared with cells transfected with control siRNA (39 ± 10.7, 18 ± 6.5, and 82 ± 20.1 nmol/L, respectively; Fig. 2C). Simultaneous knockdown of Hsp27 and Hsp70 with siRNA resulted in an additional 66% or 28% decrease in IC₅₀ as compared with Hsp27 or Hsp70 siRNA alone (IC₅₀ of 13 ± 4.5, 39 ± 10.7, and 18 ± 6.5 nmol/L, respectively; Fig. 2C). Western blotting was done to confirm knockdown by siRNA (Fig. 2D). These results suggest that inhibition of both Hsp27 and Hsp70 could decrease resistance to 17-AAG.

Hsp27 regulation of GSH affects 17-AAG sensitivity. Previous studies indicate that Hsp27 contributes to the regulation of GSH.
through several possible mechanisms (22). This information led us to hypothesize that down-regulation of Hsp27 may lead to decreased GSH levels and that this decrease may affect 17-AAG sensitivity. Transfection of Hsp27 siRNA caused an ∼50\% depletion of GSH as compared with control (P < 0.05; Fig. 3A). The resulting decrease in GSH is similar to that observed after treatment of cells with 100 μmol/L BSO (P < 0.05), an inhibitor known to cause GSH depletion through binding to γ-glutamylcysteinyl synthase; this concentration is not toxic to HeLa cells in clonogenic assays (data not shown).

To confirm the role that GSH plays in 17-AAG sensitivity, HeLa cells were first treated with 100 μmol/L BSO for 1 hour to block GSH synthesis during the cellular stress response, then 17-AAG was added for an additional 24 hours. This 25-hour BSO treatment resulted in a decreased IC50 for 17-AAG (68 ± 11.5 and 20 ± 3.1 nmol/L for 17-AAG alone and BSO + 17-AAG, respectively; Fig. 3B). Although 17-AAG treatment caused a slight increase in GSH (P < 0.02; Fig. 3C), likely due to up-regulation of Hsp27, BSO treatment for 25 hours or BSO + 17-AAG treatment for 25 hours (treatment for 1 hour with BSO alone, followed by 24 hours with 17-AAG) resulted in a dramatic decrease in GSH (P < 0.005; Fig. 3C). Because depletion of GSH by BSO causes increased sensitivity to 17-AAG, we conclude that GSH is acting to protect the cells from 17-AAG-induced cell death. This is consistent with the 17-AAG-induced stress response, manifested in part by increased Hsp27 modulating tumor cell GSH levels, contributing to 17-AAG resistance.

NAC protects cells from 17-AAG-induced cell death. Because depleting cells of GSH sensitized them to 17-AAG treatment, we hypothesized that treatment with NAC would protect cells from 17-AAG-induced cell death by raising intracellular GSH levels. HeLa cells were transfected with either control siRNA or Hsp27 siRNA, and 17-AAG cytotoxicity with and without NAC treatment was determined. Cells were treated for 1 hour with diluent (sterile water) or 5 mmol/L NAC, then diluted (DMSO) or 17-AAG was added for an additional 24 hours. Our data indicate that whereas NAC does offer a small protective effect for control siRNA–transfected cells (69 ± 8.5 and 97 ± 16.1 nmol/L, without and with NAC, respectively; Fig. 4A), Hsp27 siRNA–transfected cells were ~3-fold less sensitive to 17-AAG treatment (37 ± 4.8 and 120 ± 17.1 nmol/L, respectively; Fig. 4A). In fact, NAC stimulated survival in Hsp27 siRNA–transfected cells to a similar level as control siRNA–transfected cells that were treated with NAC. This protection likely results from the 2-fold increase in GSH observed after 1-hour NAC treatment (P < 0.02; Fig. 4B).

To confirm that this protection is a result of inhibition of cell death, we examined nuclear morphology and poly(ADP-ribose) polymerase cleavage for evidence of apoptosis after treatment. Cells transfected with either control siRNA or Hsp27 siRNA were treated with diluent (sterile water) or 5 mmol/L NAC for 1 hour,

Figure 4. NAC protects Hsp27 siRNA–transfected cells from apoptosis. A, HeLa cells were transfected with either control siRNA or Hsp27 siRNA, then plated for clonogenic assays. Cells were treated with either diluent (sterile water) or 5 mmol/L NAC for 1 hour, followed by addition of either diluent (DMSO) or varying concentrations of 17-AAG for 24 hours. Bars, SE (n = 3). B, HeLa cells were treated with diluent (sterile water), 5 mmol/L NAC for 1 hour, or 5 mmol/L NAC for 25 hours, then harvested for GSH detection as described in Materials and Methods. Total GSH (μmol/L) was determined with the GSH assay kit from Cayman Chemical. GSH was normalized according to protein concentration by dividing total GSH by mg/mL protein determined by BCA assay. Average GSH for diluent-treated cells was used to find percent control. Bars, SE; *, P < 0.02, compared with diluent-treated cells. C, transfected HeLa cells were treated with either diluent (sterile water) or 5 mmol/L NAC for 1 hour, followed by 24-hour treatment with diluent (DMSO) or 1 μmol/L 17-AAG. Cells were examined for apoptotic morphology with Hoechst staining; at least 500 cells were counted per slide. *, P < 0.05, compared with control siRNA–transfected cells treated with 17-AAG. **, P < 0.05, compared with cells transfected with Hsp27 siRNA treated with 17-AAG alone. Bars, SE (n = 3). D, cell lysates were taken from one experiment in (C) and Western blotting was used to confirm presence of poly(ADP-ribose) polymerase (PARP) cleavage, a hallmark of apoptosis.
followed by 24-hour treatment with diluent (DMSO) or 1 μmol/L 17-AAG. Hoechst 33258 staining revealed that transfection with Hsp27 siRNA significantly increased apoptosis after 17-AAG treatment as compared with control siRNA (P < 0.01; Fig. 4C). Treatment with NAC reduced apoptosis as compared with 17-AAG alone in Hsp27 siRNA–transfected cells (P < 0.05; Fig. 4C), whereas there was not a significant change in apoptosis with NAC treatment in control siRNA–transfected cells. Poly(ADP-ribose) polymerase cleavage (PARP) confirmed these results in Western blots, with cleavage of this caspase substrate only occurring in Hsp27 siRNA–transfected cells after 17-AAG treatment (Fig. 4D). Cell death in the control-transfected cells treated with NAC was slightly increased as compared with 17-AAG only, with some poly(ADP-ribose) polymerase cleavage also visible, consistent with a small amount of NAC-induced apoptosis as previously reported (26).

Hsp27 is overexpressed in GA-resistant cells. To confirm the role of Hsp27 in 17-AAG resistance, we created a cell line derived from A549 cells that is highly resistant to GA and 17-AAG (A549GARS). Although the cells have been removed from drug-containing medium, they maintain resistance to GA, with a 10-fold increase in GA IC50, as determined by comparison with A549 parent cells in a clonogenic assay (1,217 ± 145.5 and 116 ± 19.3 nmol/L, respectively; Fig. 5A).

To examine the difference in proteins expressed in A549GARS versus A549 parent cells, we did Western blot analysis on cells that were left untreated or treated with DMSO or 1 μmol/L 17-AAG. As expected with ansamycin treatment, A549 cells have an up-regulation of stress-inducible proteins Hsp70 and Hsp27 after treatment with 1 μmol/L 17-AAG, whereas Hsp90-dependent client proteins such as Akt and Raf-1 are decreased (Fig. 5B). Surprisingly, A549GARS cells have a similar basal level of Hsp70 as compared with A549 cells, and they also have up-regulated Hsp70 and decreased Hsp90 clients Akt and Raf-1 after 17-AAG treatment (Fig. 5B). However, A549GARS cells contain an increased basal level of Hsp27 as compared with A549 cells. These data indicate that Hsp70 up-regulation does not play a significant role in A549GARS resistance to GA or 17-AAG, whereas Hsp27 may contribute to the ability of A549GARS to maintain resistance to ansamycins.

GSH is increased in A549GARS cells. Because Hsp27 is up-regulated in A549GARS cells, and Hsp27 has been implicated in cellular GSH regulation, we hypothesized that A549GARS cells would have increased total GSH as compared with A549 cells. As predicted, A549GARS had significantly increased GSH as compared with A549 cells (Fig. 5C; P < 0.02). This increase of GSH in A549GARS cells suggests that Hsp27 can contribute to increased...
in intracellular levels of GSH, which may be a possible mechanism of GA and 17-AAG resistance in these cells.

**Knockdown of Hsp27 sensitizes A549GARS to 17-AAG.** To directly assess the contribution of Hsp27 in cellular resistance to 17-AAG, clonogenic assays were done on A549GARS transfected with either control siRNA or Hsp27 siRNA. Knockdown of Hsp27 with siRNA rendered the cells dramatically more sensitive to 17-AAG than with control siRNA (IC_{50} of 48 ± 27.2 and 836 ± 153.7 nmol/L, respectively; Fig. 6A). Whereas A549GARS cells have a higher level of Hsp27 expression than A549 cells, transfection with Hsp27-directed siRNA resulted in a dramatic decrease of Hsp27 protein as compared with control siRNA (Fig. 6B). Knockdown of Hsp27 was also maintained in the presence of 17-AAG as compared with control, whereas Hsp70 was still up-regulated (Fig. 6C), indicating that expression of other stress-responsive proteins is unaffected by Hsp27 siRNA. We conclude that Hsp27 contributes to A549GARS resistance to ansamycins GA and 17-AAG.

**Discussion**

Hsp90 inhibitors are currently in phase II clinical trials and phase I trials testing combinations of 17-AAG with chemotherapy agents (27). Despite evidence that multiple oncogenic kinases are degraded with 17-AAG treatment (28), these trials have shown little clinical activity for 17-AAG, although in vitro and in vivo testing predicted significant potential for 17-AAG as an anticancer agent (29–31). A number of explanations can be proposed. First, it is possible that the levels of drug exposure in vitro cannot be achieved in tumors in vivo. This is unlikely, as tumor cells seem to take up and retain significant amounts of 17-AAG (32). Second, the tumor models that have shown exquisite sensitivity to Hsp90-targeted therapy are highly dependent on specific Hsp90 client proteins for survival and proliferation, such as the breast cancer models SKBr3 and BT-474 that rely on the HER-2 receptor tyrosine kinase (33). Tumors in humans are more heterogeneous and not necessarily dependent on one kinase, but likely have a network of redundant signaling pathways to ensure survival, and thereby may be more resistant to 17-AAG treatment. Alternatively, the ability of tumors to rapidly resynthesize client proteins may limit the effect of 17-AAG in patients because of intermittent dosing. However, Banerji et al. (34) have shown that, in some cases, the degradation of a client protein may be maintained for prolonged periods.

Other studies point to transport proteins or metabolizing enzymes as contributors to 17-AAG resistance. Cells treated with 17-AAG and examined by microarray had increased mRNA levels of certain multidrug transporters, such as multidrug resistance 1 (MDR1), or MDR-associated protein 1 (35). These transporters, which render cells resistant to a variety of structurally and functionally unrelated antitumor drugs, can bind and transport these drugs out of the cell in an ATP-dependent manner. Benchekroun et al. (36) showed that members of the ansamycin family of antibiotics could inhibit MDR activity and that MDR1-overexpressing cells were resistant to GA. MDR1 was also increased 2-fold in GA-resistant cells, and verapamil, an inhibitor of MDR, was shown to increase intracellular GA concentration in cells. However, Radjukovic et al. (37) found that 17-AAG enhances activity of imatinib in imatinib-resistance cells that overexpress MDR1. This suggests that 17-AAG transport may be a variable factor contributing to resistance. Our data show that 17-AAG is able to down-regulate Hsp90 client proteins and up-regulate Hsp70 in the A549GARS resistant cell line, suggesting that even if MDR1 was present in these cells, it is not able to completely prevent 17-AAG accumulation in these cells. This suggests that MDR expression may influence resistance in some tumors, but cannot account for all possible mechanisms.

17-AAG sensitivity may also be influenced by expression of NAD(P)H:quinone oxidoreductase 1 (NQO1). Kelland et al. (38) showed that 17-AAG is an NQO1 substrate and that NQO1 expression increased cell resistance to 17-AAG. Subsequently, Guo et al. (39) identified an NQO1-dependent metabolite of 17-AAG that would potentially retain Hsp90-binding ability. However, Brunton et al. (40) suggest that NQO1 expression in cell lines does not correlate with their sensitivity to 17-AAG. Moreover,
in human studies we have previously shown that NQO1 polymorphisms do not affect 17-AAG clearance or toxicity (5).

An alternate explanation is that targeting Hsp90 induces a stress response mediated through HSF-1, which mitigates the effect of 17-AAG inhibition of Hsp90 chaperone activity. Bagatell et al. (11) showed that HSF-1−/− cells were significantly more sensitive to Hsp90-targeted agents, indicating the potential role of the HSF-1-mediated stress response in 17-AAG resistance. Guo et al. (17) showed the importance of Hsp70 in mediating sensitivity to 17-AAG by inhibiting apoptotic signaling in leukemia. This is in agreement with a previous study by Nishimura et al. (41) that suggested that Hsp70 is a major factor in determining cell death after heat stress. However, other stress-responsive proteins may also play a role in protecting cells from 17-AAG-induced death.

Hsp27 is dramatically up-regulated in the cellular response to 17-AAG (10–12), which prompted us to explore the relationship between Hsp27 and 17-AAG sensitivity. We have shown that exogenous expression of Hsp27 decreases sensitivity to 17-AAG, whereas knockdown of Hsp27 had the opposite effect, indicating that Hsp27 is involved in protection of cells from 17-AAG-induced death. Our findings with Hsp27 further support the hypothesis that a stress-induced cellular response protects cells from 17-AAG cytotoxicity. In this case, the resistance caused by Hsp27 up-regulation seems to be mediated, at least in part, by modulation of GSH levels. However, we cannot rule out the possibility that other pleotropic cytoprotective effects of Hsp27 may be contributing.

Hsp27 regulation of GSH provides many potential intracellular mechanisms to regulate 17-AAG sensitivity. First, GA and 17-AAG were recently shown to bind GSH in a nonenzymatic reaction in vitro (42). Direct binding of 17-AAG to GSH may reduce binding to Hsp90 in vivo and could therefore explain protection of tumor cells by increased GSH levels. Second, ansamycins were previously shown to produce reactive oxygen species in cells. Particularly, Benchehroun et al. (43) showed that GA and herbimycin A were activated by cytochrome P450 enzymes, and this led to the formation of •OH radicals. Subsequently, Dikalov et al. (44) described semiquinone and superoxide formation by cells treated with GA and confirmed that cytochrome P450 contributes to this radical production. Dey and Cederbaum (45) have shown that GA caused depletion of GSH in cells that overexpress CYP2E1, indicating that the oxidation of GA by this enzyme may contribute to GA-induced reactive oxygen species. Alternatively, because 17-AAG-induced apoptosis is known to proceed through the intrinsic mitochondrial pathway (46), the loss of mitochondrial membrane integrity after 17-AAG treatment may cause reactive oxygen species to leak into the cytoplasm from the mitochondria. In each case, GSH may eliminate the ability of 17-AAG to form free radicals, by either direct binding to 17-AAG, binding to oxygen species formed during 17-AAG treatment, such as superoxide, or binding oxidative species from mitochondrial leakage observed during 17-AAG-mediated apoptosis. Third, GSH may play an important role in oxidative protein folding that is disrupted as a result of 17-AAG blockade of Hsp90 chaperoning functions. Recent studies have shown that GSH is required to regulate formation of certain native disulfide bonds, thereby contributing to correct folding of substrate proteins (47). When this process is disrupted due to endoplasmic reticulum stress, which may occur as a result of ansamycin treatment, reactive oxygen species may be released from the endoplasmic reticulum. Increased levels of GSH in tumor cells could prevent cell damage and possibly death resulting from these oxidative species. In any of these events, GSH is needed to regulate cellular homeostasis and prevent cell death as a result of 17-AAG treatment.

Our studies raise the possibility of further pharmacologic intervention to enhance 17-AAG activity. High levels of GSH in tumors have been correlated with resistance to agents such as cisplatin and melphalan (48). BSO, an inhibitor of the rate-limiting step of GSH synthesis, has been used clinically in combination with melphalan and was found to have minimal toxicity alone, with more frequent toxicity occurring in combination therapy (49, 50). In these trials, GSH was dramatically reduced in patients, indicating that BSO had biological activity. Our studies suggest that modulation of GSH may increase 17-AAG efficacy. Although Hsp27 regulation of GSH modulates tumor sensitivity to 17-AAG, Hsp27 is currently not a target for any known therapeutic agents. If the role of Hsp27 in 17-AAG sensitivity is predominantly due to modulation of GSH, it may be possible that combining GSH depletion by BSO with 17-AAG could result in greater clinical response to 17-AAG. However, if Hsp27 is contributing to 17-AAG resistance through other mechanisms, targeting Hsp27 directly may be an alternate strategy to enhance Hsp90-directed therapy.

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References


Up-regulation of Heat Shock Protein 27 Induces Resistance to 17-Allylamino-Demethoxygeldanamycin through a Glutathione-Mediated Mechanism
