P-Glycoprotein–Mediated Resistance to Hsp90-Directed Therapy Is Eclipsed by the Heat Shock Response

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Abstract

Despite studies that show the antitumor activity of Hsp90 inhibitors, such as geldanamycin (GA) and its derivative 17-allylamino-demethoxygeldanamycin (17-AAG), recent reports indicate that these inhibitors lack significant single-agent clinical activity. Resistance to Hsp90 inhibitors has been previously linked to expression of P-glycoprotein (Pgp) and the multidrug resistant (MDR) phenotype. However, the stress response induced by GA treatment can also cause resistance to Hsp90-targeted therapy. Therefore, we chose to further investigate the relative importance of Pgp and the stress response in 17-AAG resistance. Colony-forming assays revealed that high expression of Pgp could increase the 17-AAG IC50 6-fold in cells transfected with Pgp compared with parent cells. A549 cells selected for resistance to GA overexpressed Pgp, but verapamil did not reverse the resistance. These cells also overexpressed Hsp27, and Hsp70 was induced with 17-AAG treatment. When the GA and 17-AAG resistant cells were transfected with Hsp27 and/or Hsp70 small interfering RNA (siRNA), the 17-AAG IC50 decreased 10-fold compared with control transfected cells. Transfection with siRNA directed against Hsp27, Hsp70, or Hsp27 and Hsp70 also increased sensitivity to EC78, a purine scaffold-based Hsp90 inhibitor that is not a Pgp substrate. We conclude that Pgp may contribute, in part, to resistance to 17-AAG, but induction of stress response proteins, such as Hsp27 and Hsp70, by Hsp90-targeted therapy plays a larger role. Taken together, our results indicate that targeting of Hsp27 and Hsp70 should be exploited to increase the clinical efficacy of Hsp90-directed therapy. [Cancer Res 2008;68(18):7419–27]

Introduction

Heat shock proteins (Hsp) are a family of molecular chaperone proteins important in folding newly synthesized polypeptides, stabilization and refolding of proteins during stress, and protein trafficking. Hsp90 is a core component of a multimolecular chaperone complex that includes many other chaperone and cochaperone proteins (1). Client proteins of this chaperone complex include transcription factors, tyrosine kinases, serine/threonine kinases, and other oncoproteins (2). By controlled binding and release of these client proteins, the Hsp90 chaperone complex plays a regulatory role in cellular protein fates.

The Hsp90 chaperone machinery is dependent on ATP binding to fulfill its chaperone function (3, 4). Geldanamycin (GA) has been shown by crystallographic (5) and biochemical analyses (6, 7) to bind the Hsp90 ATP/ADP-binding domain, constraining the chaperone in its ADP-bound conformation and inhibiting its folding, trafficking, and stabilization functions (8). In this ADP-bound conformation, multiple kinases and other proteins in critical signal transduction pathways are degraded by the proteasome in response to GA treatment (9). Concomitantly, binding of Hsp90 inhibitors also induces a stress response through the release and activation of heat shock factor-1 (HSF-1; refs. 10, 11), a transcription factor that binds heat shock elements (HSE) within promoters of stress-responsive genes, such as Hsp70 and Hsp27, resulting in increased transcription and translation of these genes (12). Whereas GA has shown effective preclinical activity (13–15), observed hepatotoxicity limited its clinical development (16). However, an analogue of GA, 17-allylamino-demethoxygeldanamycin (17-AAG), has shown a favorable toxicologic profile, and several formulations of 17-AAG have completed phase 1 testing phase 2 trials for 17-AAG are currently under way (17, 18). Because 17-AAG is not water soluble, unstable in solution, and not orally bioavailable, other Hsp90 inhibitors are being developed for clinical use. Currently, the orally bioavailable GA analogue 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; ref. 19), as well as purine and pyrazole scaffold-based Hsp90-targeted agents, which also possess favorable characteristics, are also under development for future clinical application (20–22).

Unfortunately, data from many 17-AAG phase 1 studies indicate that, as a single agent, 17-AAG has not resulted in the predicted significant clinical activity. These data have raised the question of targeting mechanisms of intrinsic 17-AAG resistance to increase clinical response. P-glycoprotein (Pgp), a member of the ATP-binding cassette (ABC) transporter family encoded by the gene MDR1 and previously implicated in the multidrug resistance (MDR) phenotype (23), acts as an efflux pump for many common anticancer agents and ansamycin benzoquinones, such as GA and 17-AAG (24). MDR1 gene amplification in cells selected for resistance to Pgp substrates has been frequently described (25). However, it is notable that increased transcription of MDR1 after cell stress has been related to transcriptional activation at the identified HSE in the promoter region (26, 27). The potential contribution of an HSF-1–mediated stress response to 17-AAG clinical resistance has been posited by several groups (12, 28). In agreement with this premise, Hsp70 down-regulation was recently shown to sensitize cells to 17-AAG–induced apoptosis (29, 30). Subsequently, we identified the role of Hsp27 and glutathione in Hsp90 inhibitor resistance (31). This connection between the transcriptional activation of Pgp and stress-induced proteins, such as Hsp27 and Hsp70, through HSF-1 binding of HSEs prompted us

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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to assess the relative contribution of these proteins to Hsp90-targeted therapy resistance.

In this study, we have confirmed that P-gp expression affects sensitivity to 17-AAG, although it does not prevent cells from up-regulating stress response proteins. We also show that GA analogues cause acute up-regulation of P-gp. In cells with acquired resistance to 17-AAG, we found that P-gp was possibly overexpressed due to a chromosomal duplication event. Whereas P-gp may contribute, at least in part, to 17-AAG resistance, our data indicate that Hsp27 and Hsp70 induction plays a much larger role. Knockdown of either Hsp27 or Hsp70 was sufficient to increase sensitivity to 17-AAG by as much as 10-fold and renders 17-AAG–resistant cells more sensitive to EC78, a purine-scaffold Hsp90 inhibitor that is not a P-gp substrate. Collectively, these results indicate that targeting stress-responsive proteins, such as Hsp70 and Hsp27, will be important for preventing resistance to current and future generations of Hsp90 inhibitors.

Materials and Methods

Materials. Reagents were obtained from the following sources: GA and 17-DMAG from Dr. V.L. Narayanan, Drug Synthesis and Chemistry Branch, National Cancer Institute; 17-AAG and EC78 from Dr. Francis Burrows, Conforma Therapeutics; enhanced chemiluminescent reagents from Amersham Pharmacia Biotechnology; daunorubicin and verapamil (VP) from Sigma.

Antibodies. H9010 and J3 mouse monoclonal antibodies recognizing Hsp90 and p23, respectively, were previously described (32, 33). The remaining antibodies were purchased from the following suppliers: peroxidase-coupled affinity-purified goat anti-mouse, goat anti-rat, and goat anti-rabbit secondary antibodies from Kirkgaard & Perry; mouse monoclonal anti-Hsp70, mouse monoclonal anti-Hsp27, and rat monoclonal anti-Hsp90 from Stressgen; rabbit polyclonal anti-Akt from Cell Signaling Technologies; mouse monoclonal anti–P-gp (C219) from Calbiochem; mouse monoclonal antiactin from Sigma; mouse monoclonal anti-BCRP from Calbiochem.

Clonogenic assays. A549, A549GARS, KB3-1, KB-T10, or MCF-7/ADR cells were trypsinized and plated in 60-mm tissue culture plates to a density of 5 × 10^4 per well and allowed to adhere for 22 to 24 h. Control siRNA #1 (400 nmol), Hsp70-specific siRNA, and/or Hsp27-specific siRNA (Dharmacon; ref. 31) were complexed with 10 μL of Lipofectamine 2000 (Invitrogen) in 0.5 mL Opti-MEM (Invitrogen) for 10 min. Cells were then incubated for 4 h with complexed lipid-siRNA, after which 1 mL of Opti-MEM containing 35% FBS was added. On 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, as described below. For MCF-7/ADR cells, wells were coated with 5 μL of Lipofectamine RNAIMAX, 500 μL Opti-MEM, and 100 pmol siRNA. 1 × 10^5 cells in 2.5-mL medium was added to the well and incubated for 48 h before trypsinization.

Immunoblotting. Cells were plated on 100-mm dishes, allowed to adhere for 22 to 24 h, and then treated as described. Cells were lifted from plates by scraping, pelleted at 250 × g for 5 min 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% Nonidet P-40, 1 mmol/L Na3VO4, and protease inhibitors (complete, mini, EDTA-free, tablets; Roche). After 10-min incubation on ice, the detergent insoluble fractions were pelleted at 18,000 x g for 2 min at 4°C. Total protein concentration of supernatants was estimated by the bicinchoninic acid (BCA) method (31). For P-gp samples, cells were plated in 75-mm flasks, allowed to adhere for 22 to 24 h, and then treated as described. Adherent cells were washed twice with ice-cold PBS, harvested by scraping after adding 500 µL of 4X SDS sample buffer to flask, and then sonicated. Total protein concentration was estimated by the BCA method (31). To prepare samples for SDS-PAGE, 1/10 volume DTT was added to be final concentration of 100 µmol/L and then samples were boiled for 15 min. For all Western blots, samples containing 50 to 150 µg of total protein were separated polypeptides were transferred to nitrocellulose, probed with antibodies, and visualized by enhanced chemiluminescence as previously described (31).

**Immunoprecipitation.** p23 immunoprecipitations were performed as follows: J3 antibody was rotated with Protein A Sepharose beads for 1 h at room temperature, washed thrice with 0.1 mol/L potassium phosphate (pH 8.0), washed once in cold lysis buffer, and then resuspended in one volume of lysis buffer to form antibody-bead slurry. One milligram of bead slurry-precleared protein was added to 50 µL of antibody-bead slurry and rotated overnight at 4°C. Antibody complexes were washed three times with lysis buffer, and then resuspended in one volume of 2X SDS sample buffer. Protein binding to p23 was determined by separating immunoprecipitations by SDS-PAGE, and then Western blotting for proteins was indicated.

**Microarray analysis.** RNA was prepared from parent and resistant A549 cells using the RNeasy Total RNA Isolation kit (QIAGEN). RNA quality assessment, probe labeling, and hybridization to the Affymetrix U133 plus 2.0 Human Genome Array Chip were performed by the Mayo Clinic Cancer Center Microarray Shared Resource. In the preliminary analysis, one array was hybridized to a sample of parent A549 and another array was hybridized to a sample of resistant A549 cells. The samples were analyzed in duplicate using chips from the same lot. These arrays were normalized using fastlo normalization (36). Gene expression values were generated using the perfect match probes of the corresponding probeset using a mixed linear model similar to that described by Chu and colleagues (37). An MVA plot was used to determine the number of genes that were statistically, significantly, and substantially, in fold-change magnitude, altered between the parent and resistant cells.

17-AAG retention. Exponentially growing parent or A549GARS cells were treated with 217 nmol/L [3H]17-AAG for 1 h at 37°C. After washing with PBS, drug-free medium was added to the cells, which were harvested immediately (0 h), or incubated for an additional 4 h at 37°C. To harvest, cells were washed with thrieve with room temperature 1X PBS, twice with ice-cold 1X PBS, and then resuspended in about four volumes of buffer {10 mmol/L Tris, 0.1 mmol/L EDTA (pH 7.5) plus protease inhibitors: 0.1 mmol/L leupeptin, 0.1 mg/mL bacitracin, 77 µg/mL aprotinin, 1.5 µmol/L pepstatin, and 1 mmol/L AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride]} and lysed by brief sonication. Radioactivity from the cell lysates was measured using a liquid scintillation counter. Excess unlabelled GA was used to saturate specific binding before treatment in background control flasks.

Fig. 2. P-gp is overexpressed in GA-resistant cells. A, A549 cells were left untreated (lane 1), treated with DMSO (lane 2) or 1 µmol/L 17-DMAG, 1 µmol/L 17-AAG, or 1µmol/L GA for 24 or 48 h as indicated. Protein (150 µg) was resolved by SDS-PAGE and probed by Western blotting. B, A549 and A549GARS expression of mRNA for P-gp, BCRP, v-actin, Hsp27, Hsp70, and Hsp90 were determined by microarray analysis. Results for signal were graphed as percentage of A549 signal, with 100 indicating no change between A549 and A549GARS expression. Below 100 represents less expression in A549GARS versus A549, whereas above 100 represents more expression in A549GARS. *, P < 0.001 compared with A549. C, to examine P-gp protein expression, 100 µg of A549 and A549GARS (GARS) cells were resolved by SDS-PAGE and probed by Western blotting. D, FISH analysis was performed on A549 and A549GARS cells with representative metaphase cells shown. A centromere 7 probe (green) together with a locus-specific probe at 7q21.1 for MDR1 gene (red) were used to examine potential gene amplification in A549GARS cells. White arrows indicate the existence of iso(7q) formation in A549GARS.
Statistical analysis. Identification of genes with statistically significant \((P < 0.05)\) different expression between the groups was done with a mixed linear model; the independent variables in the model were the probe values and a group status (e.g., parental versus resistant cells). Genes were ranked by smallest to largest \(P\) value. Because this was an exploratory analysis (versus a confirmatory analysis), no correction was made for multiple comparisons.

Results

P-gp expression can affect sensitivity to 17-AAG. Previous studies have shown that P-gp in tumor cells may participate in the efflux of Hsp90-directed agents, such as 17-AAG (39). To test P-gp influence on 17-AAG sensitivity, we performed clonogenic assays on KB3-1 cells, a human epidermoid carcinoma, and KB-T10 cells, a colchicine-resistant KB3-1 variant that overexpresses P-gp (23), but not Hsp90, Hsp70, and Hsp27 (Fig. 1A). As predicted, expression of P-gp increased resistance to 17-AAG; the IC\(_{50}\) for KB3-1 parent cells was 36 ± 16 nmol/L, whereas the IC\(_{50}\) in the P-gp–expressing KB-T10 line was 218 ± 43 nmol/L (Fig. 1B). These data indicate that high basal P-gp expression can contribute to 17-AAG resistance.

To examine possible mechanisms for the observed increase in 17-AAG IC\(_{50}\) in cells expressing P-gp, we chose to assess the function of Hsp90 in these cells. Hsp90 activity was monitored by determining Hsp90 binding by Western blotting, we assessed in the presence of ATP (40). By immunoprecipitating p23, then examining its binding to p23, a cochaperone that binds Hsp90 only if Hsp90 is in an ATP-bound conformation. Because Johnson and colleagues previously showed that p23 binding to Hsp90 is increased by 17-AAG treatment (33), we hypothesized that Hsp90–p23 binding would be less affected in cells overexpressing P-gp than in nontransfected cells due to efflux of 17-AAG. To isolate the contribution of P-gp, we included cells treated with VP, a known inhibitor of P-gp. KB3-1 and KB-T10 cells were treated with vehicle (DMSO), 100 nmol/L 17-AAG, 5 μmol/L VP, or both 17-AAG and VP simultaneously for 24 h. Immunoprecipitation of p23 showed that 17-AAG was able to completely abolish Hsp90 binding to p23 in the KB3-1 cells compared with DMSO-treated cells (Fig. 1C, lanes 5 and 3, respectively), indicating that Hsp90 function was disrupted. However, in KB-T10 cells that overexpress P-gp, Hsp90–p23 binding was not disrupted to the same extent as in KB3-1 cells with 17-AAG treatment (lane 9). The lack of Hsp90 inhibition likely results from the KB-T10 cells effluxing 17-AAG, thereby resulting in lower intracellular concentrations than are found in the KB3-1 cell line. Addition of VP restored 17-AAG–mediated disruption of Hsp90 in KB-T10 cells (lane 10), indicating that P-gp expression alone can cause resistance to 17-AAG–mediated disruption of Hsp90.

Because stress response proteins, such as Hsp70 are dramatically up-regulated in response to Hsp90-directed agents (12), we examined immunoblots of the lysates used for the p23 immunoprecipitation to assess Hsp70 expression after treatment with 17-AAG. Our data revealed that Hsp70 was up-regulated in response to 17-AAG in both KB3-1 cells and KB-T10 cells, both with and without VP treatment, indicating that 17-AAG stimulates the stress response in both cell types independently of P-gp function (Fig. 1D).

From these data, we conclude that, whereas P-gp expression may increase the 17-AAG IC\(_{50}\) in cells, up-regulation of heat shock proteins such as Hsp70 contributes to resistance to 17-AAG.

Figure 3. P-gp–mediated drug efflux does not contribute to GA resistance in A549GARS. A, A549 and A549GARS cells were treated with 217 nmol/L [\(^{3}H\)]17-AAG and then medium was replaced with fresh medium. Medium and cell lysate was collected after 2 h loading treatment (0 hr, dark columns), and again 4 h after medium change (4 hr, light columns). To control for nonspecific binding, 10 μmol/L GA was added for 30 min before [\(^{3}H\)]17-AAG and shown in middle bars as +GA (cold). Bars, SE \((n = 2)\). B, A549GARS cells were treated with 17-AAG for 24 h with doses indicated, without (○) or with (■) 5 μmol/L VP. After 24-h treatment, cells were washed in serum-free medium, then incubated in fresh medium for 7 d to allow colonies to form. Final DMSO concentration was 1% in all experiments. Bars, SE \((n = 3)\). C, for immunoprecipitation (IP), 1 mg of protein from cells treated with DMSO, 1 μmol/L 17-AAG, 5 μmol/L VP, or both 17-AAG and VP was precleared for 1 h at 4 °C, then incubated with 50 μL JJS-2 bead slurry. Immunoprecipitated proteins were resolved by SDS-PAGE and detected by Western blotting. A blot of p23 was used as a loading control, lane 1 contains unconjugated beads with lysate only (no antibody), and lane 2 contains antibody only. D, 50 μg of protein from cell lysates used in C were separated by SDS-PAGE and probed by Western blotting.
proteins indicates that some 17-AAG is still able to enter these cells and disrupt Hsp90 activity.

**P-gp is up-regulated in GA-resistant cells.** Because P-gp is known to contain an HSE and its production is stimulated by heat shock (26), we hypothesized that Hsp90 inhibitor treatment would cause up-regulation of P-gp. To examine this question, we treated A549 cells with 1 μmol/L GA, 1 μmol/L 17-AAG, or 1 μmol/L 17-DMAG for 24 or 48 hours. Western analysis revealed that all three Hsp90 inhibitors caused up-regulation of P-gp at both 24 and 48 hours (Fig. 2A, lanes 3–5 and 6–8, respectively), indicating that treatment with Hsp90 inhibitors could result in P-gp-mediated resistance to those and other drugs.

To further assess the role of P-gp in Hsp90 inhibitor-induced resistance, we created a cell line derived from parent A549 cells that is highly resistant to GA and 17-AAG. Resistant cells (A549GARS) were selected by growing A549 cells in the presence of increasing concentrations of GA, until the cells were determined to be stably resistant in drug-free medium with a 10-fold increase in GA IC50 in comparison with A549 parent cells in a clonogenic assay (31). Using microarray technology, we compared differences in gene expression between A549 and A549GARS cells and found that increased gene expression for MDR1 in A549GARS (Fig. 2B) was correlated with increased protein expression by Western blotting (Fig. 2C). Interestingly, we did not observe significant differences between A549 and A549GARS cells in either mRNA or protein expression for Hsp90, BCRP, or the stress-inducible isoform of Hsp70, although Hsp27 protein expression was slightly increased. Supplementary Table S1 includes the 20 genes with the greatest mean difference (either increased or decreased) identified by differential expression analysis and ranked according to P value. However, the P values are not corrected for multiple tests and used as ranking and hypothesis generation tools only.

To further investigate possible mechanisms of MDR1 gene up-regulation in A549GARS, we chose to perform FISH analysis to compare A549 and A549GARS chromosomal differences because MDR1 is often duplicated or amplified in cells that develop resistance to various chemotherapeutics (25). In Fig. 2D, A549 cells are shown to have three full copies of chromosome 7 (green) but no evidence of duplication or amplification of MDR1 (red). However, A549GARS metaphase nuclei revealed that these cells have an extra iso(7q) chromosome resulting in an increased copy number for MDR1, although there was no evidence of amplification of MDR1 on 7q21.1 (Fig. 2D). This chromosomal abnormality may explain the increased expression of P-gp in these GA-resistant cells observed at the levels of both mRNA and protein expression (Fig. 2B and C).

In an attempt to determine if P-gp expression was causing decreased retention of 17-AAG in A549GARS cells, we loaded cells with [3H]17-AAG and then washed with drug-free medium and allowed 17-AAG to efflux over 4 hours into drug-free medium. The amount of [3H]17-AAG remaining in the cells was used as an indirect measure of drug efflux. Our data show that A549GARS had less initial drug uptake than A549 cells (Fig. 3A, black columns) and retained proportionally less 17-AAG after 4 hours than A549 cells (white columns), consistent with A549GARS cells effluxing more drug than A549 cells. We used a 100-fold excess of unlabeled GA as a control for nonspecific binding (middle columns). These data show that A549 cells take up and retain more 17-AAG at 4 hours than A549GARS. Experiments using flow cytometry to measure the retention of daunorubicin provide additional evidence of P-gp efflux in A549GARS (Supplementary Fig. S1).

**P-gp inhibition does not affect 17-AAG IC50 in GA-resistant cells.** 17-AAG has been identified as both a substrate and inhibitor of P-gp activity (41, 42). However, it remains unclear whether P-gp activity directly affects cellular sensitivity in cells that have acquired resistance to 17-AAG, such as A549GARS. Clonogenic assays revealed that the IC50 of A549GARS cells was not significantly different for 17-AAG combined with VP (1048 ± 121 nmol/L) versus 17-AAG alone (957 ± 123 nmol/L; Fig. 3B). This suggests that P-gp does not play a major role in the resistance to 17-AAG observed in A549GARS cells. Additionally, overexpression of BCRP in MDA-MB-231 cells, another drug efflux pump, failed to increase 17-AAG resistance (Supplementary Fig. S2).

To confirm that P-gp is not inhibiting 17-AAG disruption of Hsp90 activity, we performed a p23 immunoprecipitation in A549 and A549GARS cells after treatment. Cells were treated with vehicle (DMSO), 100 nmol/L 17-AAG, 5 μmol/L VP, or both 17-AAG and VP simultaneously, then Hsp90 binding to p23 was determined. Western analysis revealed that, whereas VP alone had no effect on Hsp90 binding to p23 (Fig. 3C, lanes 4 and 8), this binding was disrupted in both cell lines with 17-AAG treatment alone or in combination with VP (lanes 5 and 9 and 6 and 10, respectively; Fig. 3C). This is unlike KB-T10 cells, in which VP enhanced 17-AAG disruption of Hsp90-p23 binding (Fig. 1C, lane 10). Importantly,
we observed no difference in p23 binding between the A549 parent cell line and the GA-resistant A549GARS. We conclude that P-gp is unlikely to contribute significantly to the 17-AAG resistance observed in A549GARS cells. Interestingly, A549 and A549GARS cells both up-regulated Hsp70 in response to 17-AAG treatment independently of VP treatment, consistent with induction of a stress response in these cells, despite P-gp expression (Fig. 3D). This raises the possibility that proteins induced during the cellular stress response mediate resistance to 17-AAG.

**Knockdown of stress proteins Hsp27 or Hsp70 in GA-resistant cells increases sensitivity to 17-AAG.** Previously, we showed that Hsp27 contributes to 17-AAG resistance through a glutathione-mediated mechanism (31). We also found that the effect of knockdown of Hsp27 alone or combined knockdown of Hsp27 and Hsp70 does not offer any advantage over knockdown of Hsp70 alone in cells that have not been selected for GA resistance. However, A549GARS cells have higher expression of Hsp27 than parent A549 cells (Fig. 2C) and may be more dependent on Hsp70 expression for survival after 17-AAG treatment. Consequently, we chose to examine the effect of Hsp27 and Hsp70 expression on 17-AAG cytotoxicity in A549GARS cells. Simultaneous knockdown of Hsp27 and Hsp70 with siRNA resulted in a similar IC₅₀ as knockdown of Hsp27 or Hsp70 alone (Fig. 4A; 228 ± 22, 235 ± 42, and 179 ± 67, respectively; control siRNA had an IC₅₀ of 816 ± 89 nmol/L). Western blotting was performed to confirm siRNA efficacy (Fig. 4B). The similar effect of knocking down Hsp27 or Hsp70 is in striking contrast to our previous finding that Hsp27 seemed less critical than Hsp70 for survival in cells not selected for GA resistance. These data are consistent with cells chronically exposed to 17-AAG, developing resistance through up-regulation of stress-responsive proteins, particularly Hsp27.

**Inhibition of P-gp function does not increase sensitivity to 17-AAG when added to knockdown of stress response proteins.** To test the possibility that P-gp may cause additive resistance that is masked by Hsp27 overexpression in A549GARS cells, we performed clonogenic assays with cells that were transfected with Hsp27 siRNA and then treated with 17-AAG or 17-AAG and VP simultaneously. Treatment with VP did not cause a significant change in the IC₅₀ for 17-AAG in cells with decreased levels of Hsp27 compared with 17-AAG alone (IC₅₀ of 283 ± 66 and 276 ± 48 nmol/L, respectively; Fig. 5A), further indicating that P-gp activity is not necessary for A549GARS resistance to Hsp90 inhibitors. Western blotting confirmed that cells transfected with Hsp27-directed siRNA have dramatically decreased Hsp27 compared with cells transfected with control siRNA (Fig. 5B). However, knockdown of Hsp27 did not affect down-regulation of Hsp90 client protein Akt after 17-AAG treatment.

To further assess the role of Hsp27 in resistance to 17-AAG in cells which overexpress P-gp, Hsp27 was knocked down in MCF-7 cells that have a high expression of P-gp due to acquired resistance to Adriamycin (MCF-7/ADRR; ref. 34), which are also resistant to GA (39, 43). The IC₅₀ for 17-AAG was 1,080 ± 134 nmol/L in cells that were transfected with Hsp27 siRNA compared with 2,760 ± 155 nmol/L for control siRNA (Fig. 5C). Whereas VP treatment was more sensitizing to 17-AAG than Hsp27 siRNA, combining Hsp27 knockdown and VP treatment in these cells resulted in a small additive effect (IC₅₀ of 429 ± 121, 1,080 ± 134, and 255 ± 112 nmol/L, respectively; Fig. 5C). Western blotting confirmed that

![Figure 5. Hsp27 expression does not mask P-gp contribution to 17-AAG resistance. A, A549GARS cells were transfected with Hsp27 siRNA and then plated for clonogenic assays. Cells were treated with 17-AAG for 24 h with doses indicated, without (●) or with (○) 5 μmol/L VP, washed in serum-free medium, and then incubated in drug-free medium for 7 d to allow colonies to form. Bars, SE (n = 3). B, A549GARS cells were transfected with control or Hsp27 siRNA as indicated and then treated with DMSO (lanes 1 and 5) or 1 μmol/L 17-AAG (lanes 2, 4, 6, and 8) and/or 5 μmol/L VP (lanes 3, 4, 7, and 8) for 24 h as indicated. Western blotting was performed using 50 μg of cell lysates harvested after treatment and separated by SDS-PAGE. C, MCF-7/ADRR cells were transfected with control (●) or Hsp27 siRNA (○) treated with 17-AAG or 17-AAG + 5 μmol/L VP (● for control siRNA + VP, and ○ for Hsp27 siRNA + VP), then plated for clonogenic assays. Cells were treated with 17-AAG with doses indicated for 24 h, washed in serum-free medium, then incubated in drug-free medium for 7 d to allow colonies to form. Bars, SE (n = 3). D, MCF-7/ADRR cells were transfected with control or Hsp27 siRNA as indicated and then Western blotting was performed using 50 μg of cell lysates harvested after treatment and separated by SDS-PAGE.](image-url)
Hsp27 was knocked down after transfection with Hsp27-targeted siRNA (Fig. 5D).

**Knockdown of Hsp27 or Hsp70 increases sensitivity to EC78.**

We elected to assess whether Hsp27 and Hsp70 would affect sensitivity to non-ansamycin-based Hsp90 inhibitors. EC78 is an orally bioavailable, purine-based inhibitor of Hsp90 with both in vitro and in vivo tumoricidal activity. Importantly, EC78 sensitivity was not significantly affected by P-gp expression in cells (44). Immunoprecipitation of p23 from A549GARS cells treated with DMSO, 1 μmol/L 17-AAG, or 2 μmol/L EC78 revealed that EC78 inhibits Hsp90 binding to p23 (Fig. 6A, top). This indicates that EC78 is an effective Hsp90 inhibitor in vitro. Notably, EC78 also caused an up-regulation of Hsp70 in a manner similar to 17-AAG (Fig. 6A, bottom). Because sensitivity to EC78 is independent of P-gp activity (44), we assessed whether knockdown of Hsp27 and Hsp70 influenced resistance to EC78. Western blotting confirmed the siRNA-induced knockdown even in the presence of EC78 (Fig. 6B). Clonogenic assays revealed that Hsp27, Hsp70, or combined Hsp27 and Hsp70 knockdown resulted in similar decreases in EC78 resistance in A549GARS cells [IC50 of 178 ± 126 nmol/L (P < 0.04), 301 ± 138 nmol/L (P < 0.007), and 299 ± 121 nmol/L (P < 0.007), respectively, compared with control siRNA IC50 of 970 ± 238 nmol/L; Fig. 6C]. These results suggest that inhibition of Hsp27 and/or Hsp70 may decrease resistance to 17-AAG, as well as new classes of Hsp90 inhibitors.

**Discussion**

Hsp90 inhibitors, specifically the GA analogue 17-AAG, have generated interest as cancer therapeutics. Unfortunately, despite in vitro and in vivo tumoricidal activity (13–15), 17-AAG has had limited single-agent clinical efficacy to date (45). Previous studies have shown that this inactivity is not likely due to lack of client protein degradation (9), decreased drug uptake by tumor cells (46), or metabolism by NAD(P)H/quinone oxidoreductase I (47).

Studies to identify resistance mechanisms to GA indicate that P-gp expression may contribute to decreased 17-AAG sensitivity. Benckhoun and colleagues first identified a potential role for P-gp in GA resistance by showing that MCF7/ADR was 100-fold resistant to GA and treatment with the P-gp inhibitor VP increased sensitivity to GA (39). A second study by this group revealed that GA interacts with P-gp and that cells resistant to GA or ADR had decreased uptake of GA (43). Huang and colleagues reported that cyclosporine A, an inhibitor of ABC transporters, sensitized resistant cells to GA and indicated that GA may be both an inhibitor and substrate for P-gp activity (42). Studies by Radujkovic and colleagues also support the hypothesis that GA analogues can act as both substrates and inhibitors of P-gp (41). More recently, other studies have indicated that other drug transporters, such as multidrug resistance protein 1, may also be implicated in 17-AAG resistance (48).

Treatment with Hsp90-directed agents, such as 17-AAG, induces a dramatic up-regulation of stress-responsive proteins, including Hsp70 and Hsp27. Interestingly, P-gp is also induced by stress, such as heat shock, which is consistent with the discovery of an HSE promoter located in the MDRI gene (26). We have found that treatment with GA or its analogues induces P-gp expression, likely as a result of the HSF-1-mediated cellular stress response. There are two important implications of this finding. First, when 17-AAG is combined with chemotherapeutic agents that are P-gp substrates, it would be advisable to treat in a sequence with the

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Knockdown of Hsp27 sensitizes cells to EC78. A, for immunoprecipitation (IP), 1 mg of protein from either A549 or A549GARS cells treated with DMSO, 1 μmol/L 17-AAG, or 2 μmol/L EC78 was precleared for 1 h at 4°C, then incubated with 50 μL JJS-bead slurry. Immunoprecipitated proteins were resolved by SDS-PAGE and detected by Western blotting. B, A549GARS cells were transfected with siRNA indicated and then treated with DMSO (lanes 1, 3, 5, and 7) or 1 μmol/L 17-AAG (lanes 2, 4, 6, and 8) for 24 h. Western blotting was performed using 50 μg protein from cell lysates harvested before and after treatment and separated by SDS-PAGE. C, A549GARS cells were transfected with control, Hsp27, Hsp70, or both Hsp27 and Hsp70 siRNA, as indicated, then plated for clonogenic assays. Cells were treated with EC78 for 24 h in concentrations indicated, washed in serum-free medium, then incubated in drug-free medium for 7 d to allow colonies to form. Bars, SE (n = 3).
chemotherapeutic first followed by 17-AAG. It is of note that Munster and colleagues (49) found that Taxol followed by 17-AAG was synergistic, attributing this result to an Rb-dependent cell cycle effect. However, when 17-AAG was followed by Taxol, less apoptotic cell death occurred. Based on our results, P-gp induction by 17-AAG may contribute to this effect because Taxol is an identified P-gp substrate. The second implication of P-gp induction by 17-AAG is that chronic treatment with Hsp90-targeted agents could cause resistance to future cycles of therapy because GA can cause an MDR1 duplication event that would lead to a more permanent MDR phenotype.

Whereas P-gp did confer GA resistance in cells selected for P-gp–mediated resistance (MCF-7/ADR), it seems to play a minor role in cells selected for GA resistance. In contrast, stress-inducible proteins Hsp27 and Hsp70 had dramatic effects on GA resistance. Our data showed that knockdown of Hsp27 or Hsp70 alone was sufficient to reverse resistance to 17-AAG and EC78 in A549GARS, whereas inhibition of P-gp by VP was not. Furthermore, VP treatment did not contribute to reversal of resistance by Hsp27 knockdown in A549GARS, which is in contrast to MCF-7/ADR1. Although we conclude that P-gp may contribute in part to 17-AAG resistance, our data are consistent with induction of Hsp70 and Hsp27 playing a much larger role. The mechanism whereby these two proteins may contribute to resistance has been elucidated in part. Hsp70 may contribute to 17-AAG resistance by inhibiting 17-AAG–induced Bax conformational and localization change and mitochondria-initiated apoptosis (29). Schmitt and colleagues showed that inhibiting Hsp70 function by treatment with a peptide that mimics the Hsp70 binding partner apoptosis-inducing factor resulted in additive cell killing when combined with Hsp90 inhibitors (30). Subsequently, studies in our laboratory have shown that Hsp27 can contribute to 17-AAG resistance through increased glutathione synthesis (31). It is notable that Hsp27, which we previously reported to contribute to 17-AAG resistance, is found within 1 million bp of P-gp on the long arm of chromosome 7, which is duplicated in the A549GARS. This may explain the pivotal role of Hsp27 in the resistance phenotype of A547GARS. However, our data do not exclude participation of Hsp70 and Hsp27 in 17-AAG resistance through other prosurvival functions, including management of misfolded and aggregated proteins. It is important to remember that other studies using cells with acquired GA resistance suggest that more than one resistance mechanism can occur in cells chronically treated with ansamycin-based Hsp90-targeted agents (39). In particular, it remains to be determined if other stress-inducible proteins add to Hsp90 inhibitor resistance.

From our data, we conclude that induction of a stress response increases resistance to Hsp90-directed agents. This is important because this induction will likely result in limited effectiveness of single-agent Hsp90-targeted therapy, which is consistent with the results of a number of clinical trials reported to date (50). Taken together, our results would support the development of agents that target proteins involved in the stress response, such as HSF-1, Hsp27, and/or Hsp70. These new agents could be combined with Hsp90-directed therapy to inhibit the stress response induced by 17-AAG and other Hsp90-targeted therapies. Whether such a strategy will have an effective therapeutic index will necessitate the evaluation of such combinations in vivo.

Disclosure of Potential Conflicts of Interest

C. Eichelman: Clinical trial funding, Conforma. The other authors disclosed no potential conflicts of interest.

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