Silencing of HSP90 Cochaperone AHA1 Expression Decreases Client Protein Activation and Increases Cellular Sensitivity to the HSP90 Inhibitor 17-Allylamino-17-Demethoxygeldanamycin

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Abstract

AHA1 (activator of HSP90 ATPase) is a cochaperone of the ATP-dependent molecular chaperone, HSP90, which is involved in the maturation, stabilization/degradation, and function of oncoproteins. HSP90 operates in a multi-meric complex driven by the binding and hydrolysis of ATP. Treatment of cells with the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) results in the degradation of client proteins via the ubiquitin-proteasome pathway. As AHA1 increases the ATPase activity of HSP90, we hypothesized that modulation of AHA1 expression could influence the activity of client proteins and/or the cellular response to 17-AAG. We show that the basal expression of AHA1 is different across a panel of human cancer cell lines, and that treatment with 17-AAG resulted in sustained AHA1 up-regulation. Increasing the expression of AHA1 did not affect the sensitivity to 17-AAG, but did increase C-RAF activity and the levels of phosphorylated MEK1/2 and ERK1/2 without affecting total levels of these proteins or of client proteins C-RAF, ERBB2, or CDK4. Conversely, small interfering RNA–selective knockdown of >80% of AHA1 expression decreased C-RAF activity and the levels of phosphorylated MEK1/2 and ERK1/2 phosphorylation. Moreover, the AHA1 knockdown resulted in a significant (P < 0.05) increase in sensitivity to 17-AAG, due in part to a 2- to 3-fold increase in apoptosis. These results show that the reduction of AHA1 levels could decrease the phosphorylation of key signal transduction proteins, and for the first time, separate the activation and stabilization functions of HSP90. Furthermore, AHA1 knockdown could sensitize cancer cells to 17-AAG. We conclude that modulation of AHA1 might be a potential therapeutic strategy to increase sensitivity to HSP90 inhibitors. [Cancer Res 2008;68(4):1187-96]

Introduction

HSP90 is a unique and essential molecular chaperone in all eukaryotic cells (1). It forms the core component of a multimeric chaperone complex that regulates the balance of stabilization, activation, and degradation of client proteins including numerous transcription factors and kinases. Client protein binding to HSP90 promotes conformational states that permit their phosphorylation or interactions with specific cognate ligands such as steroid hormones (2). Of note, HSP90 plays an especially important role when cells are under stress (3). HSP90 is required to maintain the conformational stability and function of a number of key oncopogenic client proteins, e.g., C-RAF, CDK4, and ERBB2 which are involved in signaling pathways crucial for the development and maintenance of the malignant phenotype (2, 4). By chaperoning otherwise relatively unstable proteins (5, 6), HSP90 can enable these signaling proteins to remain poised for activation (7). Furthermore, in cancer cells, HSP90 allows proteins with activating but potentially destabilizing mutations to continue to function rather than being degraded, and hence supports malignant transformation and progression (8–13).

AHA1 (activator of HSP90 ATPase) is a cochaperone of HSP90 which has been identified in our institution (14). It interacts with HSP90 along with other cochaperones including HOP/Sti1 (15), P50/Cdc37 (16), P23/Sba1 (17, 18), Cyp40/Cpr6 (19), and Cpr7 (20). These various accessory proteins, and others, interact in a chaperone cycle driven by ATP binding and hydrolysis (21, 22). The ATP binding state of HSP90 determines which combination of cochaperones is bound at particular stages of the cycle, and the ATPase activity of HSP90 controls the rate of the chaperone cycle (22, 23). The intrinsic ATPase activity of human HSP90 is relatively low (24) compared with HSP90 counterparts from other organisms such as yeast (21, 25). However, the cochaperone AHA1 increases the ATPase activity of human HSP90 in biochemical assays, suggesting that it may play a significant role in the ATP-driven HSP90 chaperone cycle in the cell (14).

HSP90 is an important target for cancer drugs because its inhibition causes the depletion of client proteins involved in tumor cell proliferation, survival, and indeed, all the hallmark traits of malignancy (26, 27). Therapeutic selectivity of HSP90 inhibitors can result from the exploitation of oncogene addiction and dependence on the stress response (28). HSP90 inhibitors block the NH2-terminal ATP pocket of HSP90 and thereby inhibit the essential hydrolysis step in the chaperone cycle (29), leading to the degradation of multiple oncogenic proteins by the ubiquitin-proteasome pathway (30, 31). Phase 1 trials with the first-in-class HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) provided proof of principle for HSP90 inhibition in human tumors and showed, in our own study, prolonged stable disease in two patients with advanced malignant melanoma (32). Activity was also seen in combination studies in breast cancer and multiple myeloma (33). 17-AAG causes the induction of HSP72 and the depletion of client protein levels (e.g., C-RAF, CDK4, ERBB2), which represents the molecular signature of HSP90 inhibition (34). As AHA1 increases the ATPase activity of HSP90, understanding the effects of AHA1 on HSP90 and its client proteins in cancer cells and determining whether AHA1 is involved in cellular sensitivity to 17-AAG is therefore of great interest.

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

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We hypothesized that modulation of AHA1 expression could affect the activity of HSP90 and influence the response of cancer cells to 17-AAG. We have used both overexpression and small interfering RNA (siRNA) knockdown models to alter AHA1 protein expression in cancer cells. We show that modulation of AHA1 levels could alter the activity of representative HSP90 client proteins and the phosphorylation status of key signaling proteins MEK1/2, ERK1/2, and AKT without affecting the total levels of these proteins or of the client proteins C-RAF, ERBB2, or CDK4. We also show that the depletion of AHA1 increases cellular sensitivity to 17-AAG. The results suggest that AHA1 is involved in regulating the activation status, but not the stability, of several signal transduction proteins and also indicate that AHA1 levels may play a role in cellular response to HSP90 inhibitors. In addition, our findings support the consideration of targeting AHA1 as a potential therapeutic strategy.

Materials and Methods

Cell lines. Human cancer cell lines were obtained from American Type Culture Collection (ATCC). All lines were grown in DMEM (Sigma-Aldrich) supplemented with 10% (FCS; Invitrogen), 2 mmol/L of l-glutamine, and 1× nonessential amino acids in a humidified atmosphere of 5% CO2 at 37°C. All lines were free of Mycoplasma ( VenorGeM mycoplasma PCR detection kit; Minerva Biolabs).

Transfection with siRNA oligos. The siRNA oligos were synthesized by Dharmaco Research, Inc. The siRNA oligos corresponded to nucleotides 390 to 409 and 428 to 446 of the human AHA1 coding region (GenBank accession no: XM_0121111). Controls were designed with the central 4 bp of the active oligo sequences inverted. To minimize off-target effects, all sequences were BLAST-searched against the human genome sequence to ensure 75% homology matches to other genes. HSF-1 oligos used were the siGENOME smartpool M-012109-00 (Dharmacon Research). Oligos were transfected into HCT116 human colon cancer cells using oligofectamine reagent (Invitrogen) according to the manufacturer’s instructions.

Cell growth inhibition studies. The sulforhodamine B assay (SRB) was used as described (35). The IC50 was calculated as the drug concentration that inhibited cell growth by 50% compared with vehicle controls (<0.1% DMSO).

Western blotting. Protein extraction, Western blotting, and antibodies used are described in the Supplementary Data.

Kinase assays. C-RAF (Upstate) and AKT (Cell Signaling Technology, Inc.) immunoprecipitation-kinase assay kits were used as described in the Supplementary Data. The resultant immunoprecipitated client-chaperone complexes were analyzed by Western blotting.

Morphologic analysis. Cells transfected with siRNA oligos were incubated with 1 mg/mL of RNase A and 1 mg/mL of propidium iodide for 30 min at 37°C. Samples were mounted on microscope slides and images collected using a Leica SP confocal microscope (Leica).

Vector construction and transfection. A derivative of the bicistronic plasmid vector pEFires-P (36) known as F1005 was used to express the open reading frame of AHA1 (14), kindly provided by Dr. Chris Prodromou, The Institute of Cancer Research, London, United Kingdom (Fig. S1). The resultant AHA1-containing F1007 vector or the empty vector F1005 were transfected into the HCT116 and HT29 colon cancer cell lines using the cationic liposome-mediated transfection (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate) method (Roche Diagnostics) as described in the Supplementary Data.

Statistical analysis. The single-factor ANOVA test was used to compare the IC50 values determined by SRB assay. Paired t tests were used for the statistical analysis of data from cell-counting experiments combining siRNA against AHA1 and 17-AAG treatment. In all analyses, P < 0.05 was considered statistically significant. Pearson’s correlation was used to compare basal AHA1 expression and IC50 values.

Results

Differential expression of AHA1 and sensitivity to 17-AAG in human cancer cell lines. A panel of human cancer cell lines including five different tumor types was selected for initial analysis. Constitutive protein expression of AHA1, HSP90, and HSP72 was determined by Western blotting (Fig. 1A). There were reproducible differences (three independent repeats) in the expression of all three proteins across the various cancer cell lines. However, there was no obvious relationship between the levels of AHA1 and those of HSP90 and HSP72. For example, the HT29 and HCT116 colon cancer cell lines express high levels of HSP72, but the former exhibits low expression of AHA1, whereas the latter has high levels. Similar differences can be seen in the melanoma and ovarian cancer lines (Fig. 1A).

The IC50 values for 17-AAG in these cancer cell lines, as determined in the NCI60 cell line panel,1 were compared with the relative basal levels of AHA1 as measured by densitometry. There was no correlation between sensitivity to 17-AAG and basal AHA1 protein expression (r2 = 0.00002448, P = 0.9878). These results suggest that AHA1 expression alone does not explain the sensitivity to 17-AAG. This is not surprising as many factors are involved in the response to HSP90 inhibitors (37), including the expression of other heat shock proteins and cochaperones such as HSP72 and HSP27 (37). To control for the effects of other factors, we therefore investigated the role of AHA1 in isogenic overexpression and knockdown models (see below).

Induction of AHA1 by 17-AAG via HSF-1. A smaller group of human cancer cell lines, i.e., HCT116 (colon), HT29 (colon), and A2780 (ovarian) cells, were selected for further studies based on their different levels of constitutive AHA1 and HSP90 expression (Fig. 1A). When HCT116 cells were treated with 5× IC50 of 17-AAG (108 nmol/L) over 96 h, AHA1 was induced as early as 8 h and remained up-regulated at 96 h when compared with control cells (Fig. 1B). A rapid induction of HSP72 and a depletion of C-RAF were also observed, consistent with the molecular signature of HSP90 inhibition (34). Recovery of C-RAF was seen, and in some experiments (e.g., Fig. 1B), expression at 72 and 96 h was higher than at baseline. Treatment of HT29 and A2780 cells with 17-AAG resulted in similar AHA1 induction to that seen in HCT116 cells (data not shown). Interestingly, however, nontumorigenic PNT2 human prostate epithelial cells did not show the induction of AHA1 expression when treated with an equimolar concentration of 17-AAG over 96 h, although HSP72 was induced and C-RAF depleted followed by recovery (Fig. 1C).

The AHA1 gene contains heat shock element (HSE) sites in its promoter (14), suggesting that the induction observed in cancer cells could be regulated by HSF-1. Consistent with this hypothesis, siRNA knockdown of HSF-1 in both A2780 (Fig. 1D) and HCT116 cells (Fig. S2) resulted in decreased AHA1, HSP72, and HSP27 induction following 17-AAG treatment, with no effect on the levels of these proteins in the vehicle-treated controls.

Increased expression of AHA1 enhances the phosphorylation of signal transduction proteins. A stable AHA1 overexpression model was successfully developed in the HT29 cell line, which has low constitutive levels of AHA1 (Fig. 1A). The resultant clones had significantly increased AHA1 expression compared

1 http://dftp.nci.nih.gov/docs/dftp_search.html
with the parental HT29 cell line, as detected by Western blotting (Fig. 2A). The empty vector control clones (E and H) had levels of AHA1 expression similar to those of the parental cell line (Fig. 2A).

Clones 2 and 3 with ~10-fold and 3.5-fold more AHA1 than parental HT29 cells, respectively, were selected for further investigation. There was no significant difference in growth rates between parental, empty vector, and overexpressing cells, with doubling times of ~24 h in all cases (data not shown). Overexpression of AHA1 did not affect the levels of HSP90 or the commonly studied client proteins C-RAF or CDK4 (Fig. 2B). Interestingly, however, there was an increase in the phosphorylation of MEK1/2 and ERK1/2 in the AHA1-overexpressing HT29 clones 2 and 3 compared with the parental and empty vector controls, but no change in the total levels of these proteins (Fig. 2B). The extent of phosphorylation of MEK1/2 and ERK1/2 was in proportion with the degree of overexpression of AHA1 in the two clones.

Next, we investigated the possible cause of the increased MEK1/2 and ERK1/2 activation seen with overexpression of AHA1. Following immunoprecipitation of C-RAF from the AHA1-overexpressing HT29 cells, we showed increased kinase activity of C-RAF, using recombinant MEK1 as a substrate, compared with the empty vector controls (Fig. 2C), providing an explanation for the enhanced phosphorylation of the downstream kinases. The immunoprecipitated complex in which the activated C-RAF was present was associated with increased levels of AHA1 but similar levels of HSP90 compared with the control. Thus, the results suggest that the enhanced C-RAF activation is due to the greater level of AHA1 present in the immunoprecipitated HSP90/C-RAF complex, resulting in increased MEK1/2 and ERK1/2 phosphorylation in the cell.

The activation of another HSP90 client protein was investigated. Phosphorylation of AKT was relatively difficult to detect in HT29 cells. However, Fig. 2D shows that in HCT116 cells overexpressing AHA1, there was an increase in phosphorylation of AKT at Ser473 with no change in total AKT or of the 3-phosphoinositide-dependent protein kinase 1 (PDK1) that phosphorylates AKT. The AKT immunoprecipitated from the AHA1-overexpressing HCT116 cells was shown to have increased kinase activity using recombinant GSK3β as a substrate (Fig. 2C). The immunoprecipitated activated AKT complex contained higher levels of AHA1 but similar levels of HSP90 compared with the control in an analogous way to the result noted above for activated C-RAF. Because there was no change in PDK1 expression, it could be hypothesized that PDK1 activity may also be increased with AHA1 overexpression, leading to greater AKT phosphorylation and activation.

Taken together, the results indicate that overexpression of AHA1 increases the phosphorylation of the signal transduction proteins MEK1/2, ERK1/2, and AKT in the cell, without affecting the respective total protein levels. This was explained by the increased
association of AHA1 with the HSP90/client protein complex, resulting in enhanced kinase activity of C-RAF and AKT.

**Increased expression of AHA1 does not affect cellular sensitivity to 17-AAG.** Next, the effects of AHA1 overexpression on the cellular sensitivity of HT29 cells to 17-AAG were evaluated by SRB assay. Expression of AHA1, HSP72, and representative client proteins C-RAF and CDK4 was also determined by Western blotting. The higher levels of AHA1 in the overexpressing clones compared with the controls were maintained but not obviously further induced following treatment with 17-AAG (Fig. 3). Depletion of C-RAF and CDK4 together with up-regulation of HSP72 confirmed the same molecular signature of HSP90 inhibition by 17-AAG in both AHA1-overexpressing and control cells. Table 1 shows that increased expression of AHA1 had no significant effect on the sensitivity of HT29 cells to 17-AAG (ANOVA, \( P > 0.05 \)). In addition, no difference was seen in the sensitivity to 17-AAG of HCT116 cells overexpressing AHA1 when compared with the empty vector controls (\( P > 0.05 \); Table 1).

**siRNA knockdown of AHA1 decreases the phosphorylation of signal transduction proteins.** To complement the overexpression studies, an siRNA knockdown approach was also used. The effects of two different 21-mer oligonucleotides that were designed to target distinct regions of the AHA1 sequence were determined in HCT116 cells, which have relatively high constitutive AHA1 levels (Fig. 1A). The active siRNA oligonucleotides [oligo 1 (O1) and oligo 2 (O2)] successfully knocked down >80% of AHA1 protein expression, as measured by Western blotting and densitometry, at 48 and 72 h posttransfection (Fig. 4A). Controls were designed for each of the active oligos with a 4 bp central invert (IC1 and IC2). Mock-transfected (control 1) and lipid-free transfected (control 2) controls were also included. The control oligos and transfection controls had no effect on AHA1 expression.

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**Figure 2.** Overexpression of AHA1 protein and effects on the expression and activation status of selected proteins in human cancer cells. AHA1 protein expression was detected using Western blotting. A, HT29 human colon cancer clones expressing the F1007 vector containing the AHA1 open reading frame (clones 1–10) and HT29 clones expressing the empty vector F1005 (E and H). Parental HT29 cell line (WT) was also included. His-tagged recombinant AHA1 protein produced in bacteria (rAHA1) was included as a positive control; the different mobilities were due to the His-tag. B, wild-type HT29 cells and selected HT29 clones transfected with either the overexpression vector F1007 (2 and 3) or empty vector control clones (E and H) were harvested and the constitutive levels of C-RAF, CDK4, phosphorylated MEK1/2 and total MEK2, and phosphorylated ERK1/2 and total ERK2 were assayed by Western blotting. C, selected HT29 and HCT116 overexpressing clones and empty vector controls were used to assay C-RAF and AKT kinase activity, respectively, using immunoprecipitation kinase assays. Immunoprecipitated C-RAF kinase activity was measured by the extent of phosphorylation of an inactive MEK1 protein; C-RAF acted as a loading control. Recombinant C-RAF protein (rRAF) was included as a positive control. Immunoprecipitated AKT kinase activity was measured by the extent of phosphorylation of inactive GSK3β protein; AKT acted as a loading control. AHA1 and HSP90 were also determined using the corresponding antibodies. D, wild-type HCT116 human colon cancer cells and selected HCT116 clones transfected with either the overexpression vector F1007 (C, G, and N) or empty vector control clones (2 and 3) were harvested and the constitutive levels of HSP90, PDK1, phosphorylated AKT, and total AKT were assayed by Western blotting. GAPDH was included as loading control.
at 48 or 72 h (Fig. 4A), indicating that the silencing of AHA1 was a specific effect of the targeted siRNA oligos. The selectivity of the siRNA oligonucleotides was also shown by the fact there was no effect on the expression of HSP90, HSP72 (Fig. 4A), or cochaperones CDC37 or P23 (data not shown).

In addition to having no effect on other chaperones and cochaperones (see previous paragraph), knocking down AHA1 protein expression over a 72-h time course also had no effect on the levels of client proteins C-RAF, CDK4, or ERBB2 (Fig. 4A). However, a decrease in the phosphorylation of MEK1/2 and ERK1/2 proteins was observed when AHA1 was knocked down, with no change in the levels of total MEK2 or ERK2 (Fig. 4B). In addition, a reduction in the kinase activity of immunoprecipitated C-RAF was also observed in the cells with decreased AHA1 (Fig. 4C). Furthermore, the immunoprecipitated complex with the less active C-RAF contained a decreased level of AHA1 but comparable levels of C-RAF and HSP90 compared with the controls (Fig. 4C). Taken together with the overexpression data, which gave the entirely complementary result (Fig. 2C), these data suggest that AHA1 is involved in regulating the activation status, but not the stability of signal transduction proteins such as C-RAF and AKT.

**siRNA knockdown of AHA1 increases the cellular sensitivity to 17-AAG.** siRNA knockdown of AHA1 in HCT116 cells treated with 5× IC₅₀ 17-AAG for 48 h resulted in significantly greater sensitivity to 17-AAG, as shown by a 2- to 3-fold increase in detached cells when compared with both inactive inverted controls and mock-transfected controls (P < 0.05; Fig. 5A). Similar results were obtained in the A2780 ovarian cell line in which the increase in detached cells was 3- to 6-fold (P < 0.02; Fig. S3). The cellular sensitivity following AHA1 depletion evaluated by SRB assay showed a significant decrease in IC₅₀ value with the active oligos compared with all the controls (ANOVA, P < 0.05; Fig. 5B). The results suggest that when AHA1 levels become limiting, cellular sensitivity to HSP90 inhibition by 17-AAG is increased.

Western blotting was used to assess the expression of relevant proteins under the same experimental conditions as the sensitivity studies. Knockdown of AHA1 was confirmed in samples transfected with the active siRNA oligos, with no detectable change in any of the control samples (Fig. 5C). Cells treated with 17-AAG also showed an induction of HSP72 and depletion of C-RAF regardless of AHA1 silencing. Of note, the siRNA against AHA1 completely blocked the induction of AHA1, as well as reducing the endogenous expression (Fig. 5C).

Figure 5D shows that nuclear fragmentation, which is indicative of apoptosis, was observed in all the detached cell populations but not in the attached cells. Western blotting also showed PARP

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**Table 1. Effect of increased AHA1 protein expression on the sensitivity of human colon cancer cells to the HSP90 inhibitor 17-AAG**

<table>
<thead>
<tr>
<th></th>
<th>Overexpressing clones</th>
<th>Empty vector clones</th>
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<tr>
<td></td>
<td>Parental HT29</td>
<td>F1007-2</td>
</tr>
<tr>
<td>17-AAG (nmol/L)</td>
<td>5.9 (±0.81)</td>
<td>3.2 (±0.68)</td>
</tr>
<tr>
<td>Parental HCT116</td>
<td>F1007-C</td>
<td>F1007-G</td>
</tr>
<tr>
<td>17-AAG (nmol/L)</td>
<td>22 (±3.6)</td>
<td>17 (±3.1)</td>
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**NOTE:** Ninety-six hour SRB growth inhibition assays were performed with parental HT29 cells, HCT116 cells, or cells transfected with either the overexpression vector F1007 or empty vector control, following treatment with 17-AAG. IC₅₀ values were measured as the drug concentration required to inhibit cell growth by 50% compared with control cells (n = 3, ±SD). Increased expression of AHA1 had no significant effect on cellular sensitivity to 17-AAG (ANOVA, P > 0.05 in all cases).
cleavage in the detached but not in the attached cells (Fig. 5C). Cleaved PARP was present in a small number of detached cells in the control population, indicating a low endogenous rate of apoptosis (~2.5% detached cells). Figure 5A shows a statistically significant 2- to 3-fold increase to 7% detached apoptotic cells after siRNA knockdown of AHA1 (P < 0.05). Thus, the increase in cell sensitivity to 17-AAG caused by a reduction in AHA1 expression, as measured by SRB assay, was accompanied by an increase in apoptosis.

Discussion

In conjunction with its cochaperones, which operate together in a chaperone cycle, HSP90 is involved in chaperoning a large array of client proteins. As well as controlling the stabilization/ degradation balance for client proteins, HSP90 maintains its clients in conformational states that facilitate activation events, such as ligand binding and phosphorylation, and thereby regulates signal transduction. ATP hydrolysis by HSP90 is important to drive the chaperone cycle and to enable controlled chaperone function. Changes in the conformation of HSP90 and in the binding of cochaperones have been reported to affect the rate of hydrolysis, depending on the stage of the cycle. For example, CDC37 binds to the NH2-terminal domain of HSP90 in the early complex state, causing steric hindrance of the NH2-terminal ATP binding site to facilitate client protein loading. AHA1, however, binds to the middle domain of HSP90. Structural studies of yeast Aha1 indicate that upon binding to Hsp90, Aha1 facilitates a conformational switch in the binding loop of Hsp90 (41). The switch from the retracted and inactive state to the extended conformation of this loop enables the catalytic Arg380 residue in the yeast Aha1 to access the ATP bound to the NH2-terminal of yeast Hsp90, resulting in increased ATPase activity.

Although regulation of the ATPase cycle of HSP90 by its cochaperones is well defined, the current understanding of how the ATPase cycle of HSP90 is coupled to activation and/or stabilization of its client proteins in the cell is very limited. To gain greater insight into the role of AHA1 in human cancer cells and to investigate its potential therapeutic effect, the present studies have focused on two aspects of AHA1 biology and pharmacology. First, we looked at the effect of manipulating the levels of AHA1 in cancer cells on the activity of HSP90, as determined by client protein levels and the phosphorylation status of key signaling proteins that lie on oncogenic pathways downstream of representative oncogenic client proteins. Second, we determined the effects of altering AHA1 levels on the cellular sensitivity to the first-in-class HSP90 inhibitor 17-AAG, which is currently in phase 2 clinical trials.

Initial investigation of the relationship between the basal expression of AHA1 in several human cancer cell lines showed that a range of levels were present. However, there was no obvious relationship between the levels of AHA1 and the expression of HSP90 and the cochaperone HSP72. Furthermore, there was no correlation between basal expression of AHA1 and sensitivity to 17-AAG. This is not surprising as many genes and proteins affect sensitivity to HSP90 inhibitors such as 17-AAG (37). For example, the levels of the antiapoptotic heat shock protein and cochaperone HSP72 affect 17-AAG sensitivity (42), and we show here that AHA1 and HSP72 levels are not related (Fig. 1A). Based on constitutive AHA1 expression levels, HT29 and HCT116 human colon cancer cell lines, together with A2780 human ovarian cancer cells, were selected for further investigation. HT29 cells had the lowest expression of AHA1 in the panel, whereas HCT116 and A2780 represent lines with relatively high levels (Fig. 1A).

When the function of HSP90 is impaired by pharmacologic inhibition of the chaperone’s intrinsic ATPase activity, the role of HSP90 shifts from stabilization to degradation, resulting in a depletion of the client protein via the ubiquitin-proteasome pathway. Interestingly, despite its role in the activation of...
Figure 5. Effects of siRNA knockdown of AHA1 on cellular sensitivity to 17-AAG and 17-AAG–induced expression of selected proteins in HCT116 human colon cancer cells. A, effect of 5 × IC50 17-AAG (108 nmol/L; blue columns) or vehicle (red columns) on cell detachment. The detached and attached cells were counted by hemocytometer and detached cells were expressed as a percentage of the total cell count. Bars, SE (n = 3). Statistical significance of the difference was analyzed by paired t test (*, P < 0.05). B, effects, determined by SRB growth inhibition assay, of 48 h of 17-AAG treatment performed on HCT116 human colon cancer cells transfected with active [O1 (•) and O2 (▼)], inverted control oligos [IC1 (▲) and IC2 (△)], mock-transfected control (★), or lipid-free control (□). The concentration-response curve shows the number of cells remaining posttreatment as a percentage of control untreated cells (n = 3, ± SE). C, expression of AHA1, HSP72, and C-RAF in attached cells treated in A with or without 17-AAG determined by Western blotting. Cleavage of PARP in detached cell populations treated in A with 17-AAG at 108 nmol/L (5 × IC50) or vehicle for 48 h. Note that, as shown by the presence of cleaved PARP, all the detached cells were apoptotic, including the small number of detached cells in control populations; however, A shows a much greater number of these detached apoptotic cells after siRNA knockdown of AHA1. GAPDH was included as loading control. D, morphology was determined by propidium iodide staining: a, typical morphology of mock- and siRNA-transfected (oligo 1 and oligo 2) HCT116-attached cells after 48 h of 17-AAG treatment; b, morphology of nuclear fragmentation in the detached cells indicative of apoptosis of both mock- and siRNA-transfected cells (oligo 1 and oligo 2).
HSP90 ATPase activity (14), our results show that siRNA knockdown of AHA1 by ~80% had no effect on the expression of the representative HSP90 client proteins C-RAF, ERBB2, or CDK4 in HCT116 cancer cells (Fig. 4). In addition, overexpression of AHA1 by 3.5- to 10-fold had no effect on the expression of these HSP90 client proteins (Fig. 2B). The results suggest that AHA1 is not essential for the stabilization of the kinase clients investigated.

intriguingly, however, our results show that modulation of AHA1 does affect the phosphorylation and activation of important oncogenic signal transduction proteins in cancer cells. Thus, we have shown that siRNA knockdown of AHA1 in HCT116 cancer cells results in a decrease in phosphorylated, activated forms of MEK1/2 and ERK1/2 which could be explained by a reduction in the kinase activity of immunoprecipitated C-RAF, but no change in the total amounts of any of these proteins (Fig. 4). In complementary studies in HT29 cancer cells, overexpression of AHA1 resulted in an increase in the phosphorylated forms of MEK1/2 and ERK1/2 associated with an increase in the kinase activity of immunoprecipitated C-RAF, again with no change in the total amounts of the corresponding proteins (Fig. 2B and C). Taken together, the siRNA knockdown and overexpression data are consistent with a model in which AHA1 plays a role in regulating the ERK1/2 pathway by modulating C-RAF activity, but without affecting the amount of HSP90 client proteins. Further evidence that AHA1 may be involved in client protein activation rather than stability was provided by the observed increase in AKT phosphorylation and immunoprecipitated AKT catalytic activity when AHA1 was overexpressed in HCT116 cells, once more with no change in the corresponding total protein level (Fig. 2C). Because the total levels of PDK1 were also unchanged (Fig. 2D), the results suggest that the activity of this AKT-activating kinase may also be increased by AHA1 overexpression.

To investigate the potential mechanism by which AHA1 modulation affected the activation of the client proteins C-RAF and AKT, we carried out immunoprecipitation experiments to determine the amount of AHA1 that was associated with the representative HSP90 client proteins C-RAF and AKT in the siRNA knockdown model whereas more AHA1 was associated with C-RAF and AKT in the overexpression model (Figs. 2C and 4C). In contrast, the amount of HSP90 associated with client proteins was constant. These results therefore support a model in which AHA1 regulates the activation status of C-RAF and AKT, but is not required, or is needed to a lesser extent, for the stability of these client proteins.

Previous studies have suggested that AHA1 may increase the activity of the glucocorticoid receptor, which is a non–kinase client protein, as measured by a reporter gene assay in both W303 yeast cells and HEK-293 human embryonic kidney cells (44). Furthermore, using cellular phosphotyrosine profiling in yeast, a decrease in tyrosine phosphorylation of proteins by V-SRC was observed when the Aha1 gene was deleted in yeast (14). Taken together, the results suggest that AHA1 may operate in the cell not to regulate the balance of stabilization versus degradation of client proteins, but rather, to control the activity of certain client proteins.

It is likely, although not definitively proven, that the activation of client proteins occurs via the stimulation of HSP90/ATPase activity in the HSP90/AHA1/client protein complex. On the other hand, we cannot rule out alternative or additional effects of AHA1. However, because activation of HSP90 ATPase activity is the only known biochemical function of AHA1, we propose that this is the most likely hypothesis to explain the findings.

The results we have seen here with the modulation of AHA1 expression are different from those seen in equivalent studies with other HSP90 cochaperones in cancer cells. Thus, knockdown of HSP72 and HSC70 in the same HCT116 cell model resulted in decreased expression of client proteins C-RAF, CDK4, and ERBB2, an effect not seen with AHA1 silencing. In addition, knockdown of CDC37 in these models also resulted in decreased expression of kinase client proteins C-RAF and CDK4. Further studies are required to investigate the effects of the large number of additional HSP90 cochaperones.

The first-in-class HSP90 inhibitor 17-AAG binds to the NH2-terminal ATP pocket of HSP90, thus inhibiting its activity (29). Treatment with this drug has been shown to cause cell cycle arrest and cytostasis in adherent cell populations, with a concomitant increase in detached apoptotic cells (45). In the present study, we showed that when HCT116 colon cancer cells were treated with 17-AAG, the levels of AHA1 protein were elevated (Fig. 1B). This was also observed with A2780 ovarian cancer cells (46) and HT29 colon cancer cells (data not shown). Interestingly, AHA1 induction was not observed in the non-tumorigenic human prostate cell line PNT2 at equivalent drug concentrations, although HSP72 was induced and client proteins depleted (Fig. 1C). Malignant cells are known to be more dependent on HSP90 due to the oncogenic stress and microenvironmental conditions found in tumors (47). As a result, HSP90 is present with its cochaperones in superchaperone complexes in cancer but not “normal” cells (48). HSP90 inhibitors can bind with higher affinity to these superchaperone complexes, providing an explanation for the therapeutic window observed between normal and tumor cells (48), and the decreased induction of stress-regulated proteins, as observed here for AHA1.

It is well established that induction of HSP72 occurs at the mRNA and protein level in response to HSP90 inhibitors (49). In stressed cells, heat shock proteins are up-regulated to protect the cell from unfavorable conditions by preventing protein aggregation, aiding protein folding, and supporting protein trafficking (47). For stress-induced transcription to occur, activation of HSF-1 is required, which once phosphorylated, can bind to HSEs in the promoters of heat shock protein genes (47). HSEs are characterized as multiple tandem inverted repeats of the short consensus sequence 5′-nGAAn-3′ (50). The yeast Aha1 promoter contains three HSEs (14, 51) and both yeast and human genes are induced when cells are exposed to heat shock (14). Further confirmation that HSF-1 regulates the induction of AHA1 was provided by our demonstration of decreased induction of AHA1 after treatment of HCT116 human colon cancer cells with 17-AAG when HSF-1 levels were depleted by siRNA (Fig. 1D). The present studies show that induction of AHA1 by 17-AAG is less extensive than is seen with HSP72, which contains four HSEs in its promoter (52). Nevertheless, despite the more limited response to 17-AAG compared with HSP72, the induction of the HSP90-activating protein AHA1 could potentially represent a protective mechanism for the cancer cell through the activation of HSP90 and potentially other unknown mechanisms. We show here that siRNA to AHA1 decreased basal

3 M. Powers, P. Clarke and P. Workman, unpublished data.
4 J. Smith, P. Clarke, and P. Workman, unpublished data.
expression and completely blocked its induction by 17-AAG, without affecting client protein expression levels. Furthermore, we also developed overexpression models for AHA1 in both low and high basal expression cancer cell lines. We therefore investigated the effect of modulating AHA1 on the sensitivity of cancer cells to 17-AAG.

Overexpression of AHA1 did not significantly ($P > 0.05$) affect the sensitivity to the HSP90 inhibitor 17-AAG in either of the two human colon cancer cell lines tested (HT29 and HCT116). Importantly, however, depletion of AHA1 by siRNA increased the sensitivity of cancer cells to 17-AAG (Fig. 5A). HCT116 colon cancer cells showed a significant ($P < 0.05$) increase in the number of detached apoptotic cells (2- to 3-fold) after 17-AAG treatment when AHA1 was depleted (Fig. 5A). These detached cells were shown to be apoptotic by morphology (nuclear fragmentation) and the appearance of cleaved PARP (Fig. 5C).

Cells were shown to be apoptotic by morphology (nuclear fragmentation) and the appearance of cleaved PARP (Fig. 5C). A corresponding increase in sensitivity to 17-AAG was observed in the SRB growth inhibition assay in HCT116 cells after AHA1 levels had been depleted ($P < 0.05$; Fig. 5). The SRB assay is sensitive to drug effects on cell number via apoptosis and other mechanisms, including cytostasis. Our previous detailed studies have shown that 17-AAG exhibits both apoptotic and cytostatic (cell cycle arrest) effects in HCT116 cells (45). Further studies are required to dissect out the precise contributions of changes in apoptosis and cytostasis to the increased sensitivity of cancer cells to 17-AAG following AHA1 knockdown. These studies could include the analysis of additional apoptotic markers in the adherent population of treated cells. It is, however, clear that AHA1 does increase the sensitivity of cancer cells to 17-AAG, as measured either by the SRB assay or by apoptosis biomarkers. The fact that depletion of AHA1 by ~80% increases the sensitivity of cancer cells to 17-AAG, whereas overexpression does not affect the sensitivity, indicates that expression above a certain threshold has no further effect on cellular responsiveness to HSP90 inhibition.

The precise molecular mechanism by which depletion of AHA1 sensitizes cells to HSP90 inhibition remains to be established. The effect is not mediated by a direct effect on HSP90-dependent stabilization of kinase clients because levels of C-RAF, ERBB2, and CDK4 were not depleted further by AHA1 knockdown as compared with the effect of 17-AAG alone (Fig. 4). The protective effect of AHA1 may relate to the ability we have identified for this cochaperone to activate the ERK1/2 mitogen-activated protein kinase and AKT signal transduction pathways that regulate cell survival. Alternatively, or in addition, the protective effects of AHA1 could occur via similar effects on other kinases or ligand-activated clients. Published studies investigating the Hsp90 complex in yeast have shown that mutations which disrupt the binding of cochaperones to the complex or depletion of a cochaperone can increase sensitivity to the HSP90 inhibitor geldanamycin (53, 54). In particular, loss of the yeast cochaperone Sti1 (the functional equivalent of the mammalian HOP) resulted in a significant increase in sensitivity to geldanamycin. HOP has been reported to displace geldanamycin from the HSP90-inhibitor complex (55). Depletion of Sti1/HOP would prevent this from occurring, which could explain the increased drug sensitivity. Mutational analysis of Hsp90 in yeast has also shown that certain key mutations could increase the sensitivity to geldanamycin and radicicol. Of note, a mutation in Hsp90 which dramatically reduced the in vitro ATPase activity of the purified protein was shown to cause significantly increased sensitivity to geldanamycin (54). Furthermore, the cochaperone cyclophilin 40 (CyP40), another component of the Hsp90 chaperone complex which can (to a more limited extent than Aha1) increase the ATPase activity of Hsp90 (14), has also been shown to affect the sensitivity of yeast to geldanamycin. CyP40 has two functional homologues in yeast, Cpr6 and Cpr7. Yeast strains lacking Cpr7 are reported to be hypersensitive to geldanamycin (53). Together with the results presented here, these data suggest that the levels of cochaperones may affect the cellular sensitivity to HSP90 inhibitors and that modulation of HSP90 ATPase activity via interactions with various cochaperones that affect this activity may in turn affect therapeutic responses to HSP90 inhibitors.

A model can be suggested to explain our observations that depletion of AHA1 increases sensitivity to 17-AAG whereas overexpression has no effect. We propose that the induction of AHA1, upon treatment with 17-AAG, represents a protective mechanism within the cell which increases HSP90 activity and limits the effects of the inhibitor. According to this model, the maximum protective effect that AHA1 can have in the cell has already been achieved by the extent of AHA1 induction seen upon treatment with 17-AAG. Therefore, no additional protective benefit to the cells is obtained by artificially overexpressing AHA1. However, when AHA1 levels are reduced by siRNA, the protective response is attenuated, resulting in the observed increase in sensitivity to 17-AAG. Our results support the consideration of potential therapeutic approaches to enhance the activity of HSP90 inhibitors either by depleting AHA1, for example, by RNA interference, or by identifying small molecule inhibitors of the HSP90-AHA1 interaction (56). It is important to note, however, that care will have to be taken to determine the effect of AHA1 depletion in malignant versus normal cells. We believe that this will best be determined in the context of the whole animal. However, there is potential for therapeutic selectivity towards malignant cells based on their greater dependence on HSP90, the much higher proportion of HSP90 that is present with cochaperones in a superchaperone complex, and the increased sensitivity to 17-AAG of this chaperone complex that is present in cancer cells (48).

In summary, our results show that modulation of the levels of the HSP90 ATPase–activating protein AHA1 affects the activation status of client proteins and that depletion of AHA1 could increase cellular sensitivity to the HSP90 inhibitor 17-AAG. Because HSP90 inhibitors continue to show promise in early clinical trials, this work should stimulate further research into AHA1.

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Silencing of HSP90 Co-chaperone AHA1 Expression Decreases Client Protein Activation and Increases Cellular Sensitivity to the HSP90 Inhibitor 17-Allylamino-17-Demethoxygeldanamycin


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