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Transcription Inhibition of Heat Shock Proteins: A Strategy for Combination of 17-allylamino-17-demethoxygeldanamycin and Actinomycin D

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Abstract

The heat shock protein (HSP) 90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) is currently in clinical trials because of its unique mechanism of action and antitumor activity. However, 17-AAG triggers the transcription and elevation of antiapoptotic HSP90, HSP70, and HSP27, which lead to chemoresistance in tumor cells. We hypothesized that inhibiting HSP90, HSP70, and HSP27 transcription may enhance 17-AAG-induced cell death in multiple myeloma (MM) cell lines. Actinomycin D (Act D), a clinically used agent and transcription inhibitor, was combined with 17-AAG. The concentrations for 17-AAG and Act D were selected based on the target actions and plasma levels during therapy. Inducible and constitutive HSP27, HSP70, and HSP90 mRNA and protein levels were measured by real-time RT-PCR and immunoblot assays. Compared to no treatment, Act D alone decreased HSP mRNA levels in the MM.1S and RPMI-8226 cell lines. Combining of Act D with 17-AAG did not attenuate 17-AAG-mediated increases in transcript levels of inducible HSP70; however, constitutive HSP mRNA levels were decreased. In contrast to its effect on mRNA levels, Act D was able to abrogate 17-AAG-mediated increases in all HSP protein levels. The cytotoxicity of combined Act D and 17-AAG was assessed. Treatment with Act D alone caused less than 40% cell death, while the combination of 17-AAG and Act D resulted in an increase of cell death in both MM cell lines. In conclusion, these results indicate that 17-AAG-mediated induction of HSP70 and HSP27 expression can be attenuated by Act D and therefore can potentially improve the clinical treatment of MM.

Keywords

multiple myeloma; 17-AAG; actinomycin D; heat shock proteins; transcription

Introduction

In 1962, Ritossa described a chromosomal puffing in *Drosophila* salivary glands after being exposed to heat, thus discovering the heat shock response (1). The heat shock protein (HSP) family is a group of related proteins that act as molecular chaperones to aid and stabilize the correct folding of proteins (2). Heat shock protein 90 (HSP90) aids in the stabilization of the functional conformation of stress-denatured client oncoproteins (3). To date, more than 200 proteins are known to be regulated by HSP90 (list of HSP90 client proteins by Dr. Picard, <http://www.picard.ch/downloads/Hsp90interactors.pdf>). Most of these client proteins are protein kinases and transcription factors known to be important players in the signaling pathways that drive survival and proliferation of malignant tumor cells (4). The HSP90-chaperone cycle is an ATP-dependent process (5,6). The denatured client protein is recognized by a set of cochaperones that allow it to form a complex with HSP90. Upon ATP binding to the ATPase pocket of HSP90, another set of cochaperones interacts with HSP90 in order to catalyze the conformational maturation of the client protein in the complex. In this mature conformation, the client protein is able to interact with its ligand or be activated through phosphorylation (7,8).

Because of the importance of this chaperone functionality in the stability of oncoproteins, several small molecules have been synthesized to down-regulate numerous signaling cascades simultaneously (9). One target of this effort has been HSP90. HSP90 is an abundant cytosolic molecular chaperone that is induced when the cell is subjected to physiological stress (including heat, heavy metals, hypoxia, and low pH) or when geldanamycin, a benzoquinone ansamycin antibiotic, binds to its ATP pocket (10). The geldanamycin derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) reduces hepatotoxicity encountered by geldanamycin and inhibits the chaperone function of HSP90 (11). 17-AAG mimics ATP and binds to the ATP pocket on the N-terminus of HSP90, blocking the binding of the natural substrate ATP (5). Since ATP binding to HSP90 is necessary for this chaperone to stabilize client proteins, binding of 17-AAG hinders the chaperone activities of HSP90. As a result, the HSP90 chaperone is unable to aid and allow the stabilization of the client to a functional protein. In turn, the unfolded client oncoproteins are labeled with ubiquitin and targeted for degradation by the proteasome (12). Because of this mode of action, 17-AAG was the first benzoquinone derivative to enter clinical trials (13). Phase I studies of this agent in patients with diverse malignancies showed that this drug was well-tolerated, down-regulated client protein expression, and resulted in stable disease in patients. However with this therapy as a response to HSP90 inhibition, an increase in HSP70 was observed in tumor and surrogate tissues (13–15).

Under normal physiological conditions, HSP90, is bound to the transcription factor heat shock factor 1 (HSF-1) (16–18). Stress to the cell causes the release of HSF-1 from HSP90 (18). Once released, HSF-1 undergoes trimerization and phosphorylation to achieve active conformation (16,19). The HSF-1 trimer translocates to the nucleus, binds to heat shock elements present in the promoter of HSP genes, and triggers transcription of HSP. Consequently, there is an increase in HSP90, HSP70, and HSP27 proteins (20). HSP90, HSP70, and HSP27 play a role in hindering the apoptotic process, interfering not only with the function of several proapoptotic proteins, such as cytochrome C and apoptosis-inducing factor, but also with the proper assembly of the apoptosome complex (21–24). Furthermore, expression of HSP90, HSP70, and HSP27 has been associated with apoptotic resistance to several chemotherapeutic agents (25–27). Conversely, silencing of HSP90, HSP70, and HSP27 expression results in apoptosis and sensitization to chemotherapeutic agents (28,29,30). Similarly, dual targeting of the constitutive HSP70 homologue (HSC70) and inducible HSP70 genes induced apoptosis in tumor cells (31).

Actinomycin D (Act D) was the first antibiotic reported to have anti-cancer activity. Aside from being an established transcription inhibitor, Act D is also used in the clinic against various pediatric malignancies (32). Act D induces cytotoxicity by intercalating its phenoxazone ring into the minor groove of DNA, thus blocking the binding of RNA polymerase II (Pol II) and subsequently inhibiting RNA synthesis (33). We hypothesized that transcription induction of HSP in response to 17-AAG could be inhibited by a transcription inhibitor such as Act D. To determine whether transcription of antiapoptotic HSP could be targeted, the transcription inhibitor Act D was evaluated in combination with 17-AAG in multiple myeloma (MM) cell lines.

Materials and Methods

Cell lines

MM.1S cell line was obtained from Drs. Nancy Krett and Steve Rosen (Laurie Comprehensive Cancer Center, Northwestern University, Chicago, IL) (34). RPMI-8226 cell line was obtained from Dr. William Dalton (Moffitt Cancer Center and Research Institute, Tampa, FL) (35). Cells were maintained in RPMI-1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum in the presence of 5% CO₂ at 37°C. Cells were routinely tested for *Mycoplasma* using Gen-Probe's kit (San Diego, CA).

Materials

Act D and 17-AAG were purchased from Sigma-Aldrich Co. (St Louis, MO). Drugs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1000 µg/mL for Act D and 1 mM for 17-AAG and stored at -20°C.

RNA synthesis

Global RNA synthesis was measured using [³H]uridine incorporation (37.2 Ci/mmol; Moraveck Biochemical, Inc., Brea, CA). Cells were treated with 0.05 µg/mL Act D, then labeled with [³H]uridine (1 µCi) at 37°C for 1 h, harvested, washed, and transferred to glass fiber filters (Whatman Inc., Clifton, NJ) using a Millipore vacuum manifold (Fisher Scientific, Pittsburgh, PA). The radioactivity on the filters was quantified by a liquid scintillation counter (Packard, Ramsey, MN).

Isolation of RNA and quantitative real-time RT-PCR

A combination experiment was designed where cells were either incubated without any drug for 12 h, with 0.5 µM 17-AAG for 8 h, with 0.05 µg/mL Act D for 12 h, or with the combination of 0.05 µg/mL Act D for 4 h followed by 0.5 µM 17-AAG for 8 h. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA). Relative transcript levels were assessed using TaqMan One Step RT-PCR master mix reagents (Applied Biosystems, Foster City, CA) on an ABI prism 7900HT sequence detection system. Predesigned primers and TaqMan probes for thioredoxin 2 (TXN2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HSP90α (HS00743767_sH), HSP90β (HS00607336_gH), HSP70 (HS00359147_s1), HSC70 (HS01683591_g1), HSP27 (HS00356629_g1) and HSF-1 (HS01027619_M1) were purchased from Applied Biosystems. Contaminating DNA was removed from RNA preparations by using a DNA-free kit (Ambion, Austin, TX). To verify the absence of DNA in each RNA sample, control reactions without reverse transcriptase were performed. Relative levels of HSP gene expression were determined by using standard curves and normalized with the endogenous GAPDH and TXN2. The results of the triplicate experiments were plotted relative to those for untreated cells.

Protein expression analysis

Cells were treated as described above, harvested, washed, and lysed. Protein samples were electrophoresed and transferred to nitrocellulose membranes. HSP90 α/β , HSP70, HSC70, HSP27, HSF-1, (Stressgen, Ann Harbor, MI), and β -actin (Sigma, St. Louis, MO) bands were imaged and quantitated using Odyssey imaging system (LI-COR Biosciences, Lincoln, NE).

Cell death assay

Cells were treated as previously described, and cytotoxicity of Act D and 17-AAG was assessed by Annexin V/7-AAD binding assay (BD Biosciences Pharmingen). The endogenous cell death was subtracted from all the conditions. The fractional two-drug analysis was used to compare the expected and observed (Annexin V/7-AAD staining) levels of cell death for the combination. The expected cell survival for the combination was calculated by multiplying the percentage of cells surviving 17-AAG treatment (100% X% Annexin V/7-AAD staining) by the percentage of cells surviving Act D treatment (100% X% Annexin V/7-AAD staining) divided by 100.

MTS cell viability assay

Cell proliferation was assessed using the CellTiter 96 (Promega, Madison, WI) following the instructions of the manufacturer. The results were calculated as a percentage of untreated control.

Statistical analysis

One-tailed paired Student's *t*-test analyses were done using GraphPad Prism (GraphPad Software, San Diego, CA).

Results

Dose-response and time-course experiments with Act D and 17-AAG

A dose-response experiment was conducted in order to select a concentration of 17-AAG that would result in induction of HSP in MM.1S and RPMI-8226 (Fig. 1A) cell lines. Cells were incubated with escalating concentrations of 17-AAG, for 8 h. Additionally, a time-course experiment was performed where the cells were incubated with 0.5 μ M 17-AAG for several time periods (Fig. 1B). Protein levels were measured for HSP90 α/β , HSP70, and HSP27. All HSP were present in both cell lines, and these levels were induced after treatment with 17-AAG in a dose- and time-dependent manner. In both cell lines, 0.5 μ M 17-AAG incubated for 8 h resulted in an increase in the levels of all three HSPs; based on these data, we selected 8 h treatment with 0.5 μ M 17-AAG for all experiments. In a similar manner, a dose-response (Fig. 1C) and a time-course experiment (Fig. 1D) were also performed by incubating cells with Act D. A 12 h incubation with 0.05 μ g/mL Act D resulted in a decline in all HSPs in both cell lines; based on these data, 0.05 μ g/mL Act D for 12 h was selected for future experiments. These concentrations of 17-AAG and Act D are achievable in the clinic (13,36).

Inhibition of global RNA synthesis by Act D

To determine the extent of RNA synthesis inhibition by 0.05 μ g/mL Act D, a uridine incorporation assay was performed. Cells were treated with Act D for 4, 8, and 12 h (Fig. 1E), and 1 h prior to the end of treatment, [3 H]uridine was added. A higher inhibition of total RNA synthesis was observed for the cell line RPMI-8226 than for MM.1S. At 4, 8, and 12 h, global RNA synthesis for MM.1S was inhibited by 55, 70, and 80%, respectively. For RPMI-8226, at 4, 8, and 12 h, RNA synthesis was inhibited by 70, 80, and 90%, respectively.

Effect of Act D and 17-AAG on HSP and HSF-1 mRNA levels

In order to test if RNA synthesis inhibition by Act D also abrogated the 17-AAG-induced antiapoptotic HSP and their transcription factor, HSF-1, the mRNA levels for these genes were determined using real-time RT-PCR. Inducible and constitutive HSP genes and HSF-1 were probed using specific primers. Transcript levels were plotted as fold change relative to the untreated control using the endogenous genes TXN2 (Fig. 2A–F) and GAPDH (data not shown), for normalization in order to verify that the obtained data were reproducible. Cells were either untreated or treated with 0.05 $\mu\text{g/mL}$ Act D for 12 h, 0.5 μM 17-AAG for 8 h, or Act D for 4 h followed by 0.5 μM 17-AAG for 8 h. Since there are two HSP90 isoforms in humans, we probed for both the stress-inducible alpha gene (Fig. 2A) and the constitutive beta gene (Fig. 2B). As expected, transcript levels for HSP90 α in both cell lines were 3–4 times higher upon 17-AAG treatment. Act D alone had a minor effect on endogenous levels of HSP90 α and was able to diminish the 17-AAG-induced increase by 30% in the combination condition. In contrast to HSP90 α , HSP90 β mRNA was induced to only a 1.5-fold higher level for MM.1S and only a 2.5-fold higher level for RPMI-8226 cells by 17-AAG, and Act D reduced induction to nearly endogenous levels in the combination culture (Fig. 2B).

Inducible HSP70 (Fig. 2C) and constitutive HSC70 (Fig. 2D) mRNA levels were also measured. Incubation of cells with 17-AAG resulted in a 4- and 6-fold induction of inducible HSP70 transcripts in MM.1S and RPMI-8226 cells, respectively. Treatment with Act D alone also induced a stress response, resulting in 1.5- and 2-fold increase in HSP70 transcript levels in these cell lines. Interestingly, combination of Act D and 17-AAG resulted in 6- and 8-fold inductions of HSP70 mRNA levels in MM.1S and RPMI-8226 cells, respectively. In contrast to mRNA levels of HSP70, mRNA levels of the constitutive homologue of HSP70 (HSC70, Fig. 2D) were only slightly induced (2- and 3.5-fold) by 17-AAG; the increase was abrogated by Act D in the combination treatment. Furthermore, HSC70 mRNA levels were reduced by 80% when cells were treated with Act D alone.

HSP27 mRNA levels were also measured in both cell lines (Fig. 2E). Upon 17-AAG treatment, there were 21- and 10-fold increases in the transcript levels of HSP27 in the MM.1S and RPMI-8226 cells, respectively. Act D reduced HSP27 mRNA by 50% and by more than 70% in the combination treatment versus 17-AAG alone in the MM.1S and RPMI-8226 cells, respectively. Compared to the untreated control, Act D alone had minor effects on HSP27 mRNA levels.

The transcription factor HSF-1 mRNA levels were also determined (Fig. 2F). Treatment with 17-AAG did not enhance the mRNA levels of HSF-1 on MM.1S or RPMI-8226. However the combination treatment diminished HSF-1 transcript levels on both MM cell lines below 50% compared to the untreated control and similar levels of attenuation of this transcript were also observed for Act D as single treatment.

Parallel effects on HSP and HSF-1 transcript levels were obtained with the second endogenous gene used, GAPDH (data not shown), in comparison to TXN2 (Fig. 2A–E). The results were qualitative similar to these obtained with TXN2 (data not shown).

Effect of Act D and 17-AAG on HSP and HSF-1 expression

To investigate if the changes in the mRNA levels of HSPs and HSF-1 also resulted in an effect on protein expression, immunoblot assays were performed on the MM cell lines treated with Act D and 17-AAG in combination or as single agents following the previously described drug sequence. As expected, 17-AAG treatment resulted in induction of all HSP in both MM.1S (Fig. 3A) and RPMI-8226 (Fig. 3B) cell lines. Conversely, in the combination treatment, Act D reduced 17-AAG-mediated induction of all HSPs to endogenous or lower levels. Similar

immunoblot assays of inducible and constitutive HSP proteins and transcription factor HSF-1 were also completed. Following this, data were quantitated, normalized with β -actin, and plotted for MM.1S and RPMI-8226 cells (Fig. 4A–E). As shown in these graphs and associated statistical analysis, 17-AAG treatment induced expression of all HSPs. The combination of Act D and 17-AAG caused a significant decrease in the expression of HSC-70 in both cell lines. HSP27 was also decreased with the combination treatment; however, this decrease was only statistically significant in the RPMI-8226 cell line. This decrease in HSPs is mechanism-based and sequence specific as treatment of cells first with 17-AAG followed by Act D maintained increased levels of all HSPs (data not shown). As expected, HSF-1 protein levels were not induced with treatment with 17-AAG as a single agent in neither MM cell line (Fig. 4E). Overall, compared to HSPs, there was only a modest change of HSF-1 expression levels.

Cytotoxicity of Act D in combination with 17-AAG in MM cells

To determine if the combination of Act D and 17-AAG resulted in increased cytotoxicity, Annexin V/7-AAD assay was performed. There was 18% endogenous cell death in both MM.1S and RPMI-8226 cell lines and DMSO (0.05%) did not increase this value. This was subtracted from each condition treatment (Fig. 5). When both cell lines were treated with 0.5 μ M 17-AAG alone, there was less than 6% annexin positivity. With 0.05 μ g/mL Act D alone, there was 38% cell death in MM.1S and 34% in RPMI-8226 cells. The combination of these two drugs resulted in 49 and 41% cell death for MM.1S and RPMI-8226 cells, respectively, which is more than the expected additive in the fractional two-drug combination analysis. Similar cytotoxic effects were obtained when an MTS assay was performed for the same combination (data not shown). Since the combination of Act D and 17-AAG at these concentrations resulted in elevated cytotoxicity, additional concentrations of Act D were tested.

MM.1S and RPMI-8226 cells were treated with varying concentrations (0.01, 0.025, or 0.05 μ g/mL) of Act D for 12 h, alone or in combination with 17-AAG (Table 1). In both MM cell lines, at this low concentration of 17-AAG, the measured cell death was 5% or less. Both MM cell lines, when treated with the transcription inhibitor, showed a dose-dependent increase in cytotoxicity. MM.1S cells treated with 0.05, 0.025, and 0.01 μ g/mL of Act D resulted in 38, 30, and 7% cell death, respectively. RPMI-8226 cells treated with 0.05, 0.025, and 0.01 μ g/mL of Act D resulted in 34, 30, and 11% cell death, respectively. As described in material and methods a fractional two-drug combination assay was performed, and the expected cell death for each concentration of Act D in combination with 17-AAG was calculated; the expected cell death calculated for the combination was compared to the actual cell death observed for each cell line. Using the Student's *t*-test analysis, it was found that there was a significant difference in cell death between each combination treatment (MM.1S, $P = 0.027$; RPMI-8226, $P = 0.0006$; both cell lines together, $P = 0.0001$).

Discussion

Some tumor cells rely on the chaperone activity of HSP90 for the proper folding and function of client proteins, such as protein kinases and transcription factors (37). Therefore, targeting HSP90 activity with an inhibitor will disrupt several signaling molecules, leading to antitumor activity. Conversely, treatment with 17-AAG triggers a stress response in cells that leads to activation of the transcription factor HSF-1, resulting in elevation of antiapoptotic HSP90, HSP70, and HSP27. These chaperones aid in the conformational maturation of unfolded proteins; additionally, HSPs hinder the apoptotic process by binding and inhibiting the assembly or activation of important pro-apoptotic proteins (21,25,38).

Overexpression of cytoprotective HSP90, HSP70, and HSP27 has been shown to confer to cells resistance to such chemotherapeutic agents as imatinib, doxorubicin, and dexamethasone in hematological malignancies and solid tumors (26,27,39). Additionally, clinical trials

administering 17-AAG twice on a weekly schedule resulted in a marked decrease of client proteins coupled with a sustained increase in HSP70 levels (40). This increase could potentially contribute to resistance to 17-AAG. Furthermore, silencing the antiapoptotic HSP90, HSP70, and HSP27 proteins with small interfering RNA (siRNA) results in cell death and sensitization to chemotherapeutic agents (28,29,30). Inhibition of both inducible HSP70 and constitutive HSC70 by siRNA techniques clearly demonstrated that suppression of these HSPs augments 17-AAG-mediated cell death (31). While this siRNA approach provides a proof-of-principle for the role of HSP90, HSP70, and HSP27, different therapeutic approaches are needed because of the lack of practical utility of siRNA-based therapeutics in the clinic.

The use of a transcription inhibitor is an alternative approach to block the stress response elicited by 17-AAG that leads to the transcriptional induction of cytoprotective HSP. We postulated that Act D would be a suitable transcription inhibitor to be tested in combination with 17-AAG for four reasons. First, Act D is a global transcription inhibitor that blocks the binding of RNA Pol II to the DNA, hence hindering transcript elongation (33). Second, Act D has been used in the clinic for more than 40 years in the treatment of rare cancers in children and young adults (36). Third, Act D is effective in the clinic without untoward toxicity (41). Fourth, adults treated with 10–15 $\mu\text{g/kg}$ Act D achieved peak plasma levels of 0.01–0.1 $\mu\text{g/mL}$, and children treated with 0.75–1.50 mg/m^2 Act D obtained peak plasma levels ranging from 0.02–0.05 $\mu\text{g/mL}$ (36,42). These plasma concentrations of Act D result in inhibition of global RNA synthesis (32). Therefore, we selected Act D to determine whether transcription of cytoprotective HSP may be targeted in combination with 17-AAG in two different MM cell lines.

The concentrations of Act D (0.05 $\mu\text{g/mL}$) and 17-AAG (0.5 μM) used in this study are achievable in the clinic. A clinical trial with the HSP90 inhibitor 17-AAG reported a dose of 450 $\text{mg/m}^2/\text{week}$, which was the highest administered dose, achieved a peak plasma concentration of 16 μM with a half-life of 6 h (13). A pediatric clinical trial with Act D reported a median peak plasma concentration of 0.025 $\mu\text{g/mL}$ (range, 0.003–0.1 $\mu\text{g/mL}$) with a half-life ranging from 14 to 43 h (36).

At the selected concentration of Act D, more than 80 and 90% inhibition of global RNA synthesis was observed at 12 h for MM.1S and RPMI-8226 cells, respectively (Fig. 1E). Treatment with Act D as single agent resulted in a decrease in endogenous HSF-1 mRNA which was also observed in the combination treatment. This treatment condition also decreased HSP levels (Fig. 1C–D). Yet, as shown by real-time RT-PCR, Act D was not able to abrogate 17-AAG mediated induction of HSP70 (Fig. 2C). The combination treatment showed a further increase in HSP70 transcript levels from the treatment with 17-AAG alone. HSP70 is an inducible gene that is transcribed because of a stress response. While this is the first report for HSP70, similar results of transcription inhibitors not being able to block transcription of inducible genes have also been reported by others (43,44). Transcription inhibitors targeting RNA Pol II, such as Act D, DRB and α -amanitin, have been shown to trigger a stress response resulting in stabilization of p53 protein and induction of p21, a gene that is transcriptionally activated by p53 (45). In addition, the cyclin-dependent kinase (CDK) inhibitor roscovitine—which inhibits CDK7 and CDK9, resulting in a decrease in phosphorylation of RNA Pol II and, hence, global transcription inhibition—has also been reported to enhance induction of p21 levels via p53 activation (46). Mechanistic studies using chromatin immunoprecipitation assays demonstrated that a pharmacological inhibitor, such as DRB, leads to global inhibition of mRNA synthesis and a decrease in HPRT and SDHA, both of which are housekeeping and non-inducible genes, but a 5- to 10-fold increase in PUMA and p21, which are p53-inducible genes (43).

Even though, there was an increase in HSP70 mRNA, induction at the protein level was either null or minor with the combination treatment (Fig. 4A–D). These data suggest that the combination-induced HSP70 transcripts are either non-functional or short-lived. The 3'-noncoding region of HSP70 mRNA contains the sequence AUUUA (47). This motif is associated with faster degradation of this mRNA. Additional studies are needed to determine the fate of HSP70 transcripts when both drugs are used in combination. Compared to 17-AAG alone, the combination treatment reduced the HSP protein levels in both MM.1S (Fig. 3A) and RPMI-8226 cells (Fig. 3B); hence, Act D in combination with 17-AAG would be expected to result in a greater increase in cytotoxicity than the single agents.

The abrogation of 17-AAG-induced antiapoptotic HSP protein levels by the transcription inhibitor Act D resulted in an increase in cytotoxicity as determined by Annexin V/7-AAD assay (Fig. 5). Treatment of MM cell lines with 0.5 μ M 17-AAG resulted in less than 6% cell death. Incubation of cell lines with 0.05 μ g/mL Act D resulted in 38 and 34% cell death in MM.1S and RPMI-8226 cells, respectively. However, the combination of these two agents caused an induction in cytotoxicity resulting in 49 and 41% cell death for the MM1.S and RPMI-8226 lines, respectively. Analysis of these data shows a more than additive effect in cytotoxicity. Since the clinical plasma levels achieved in patients treated with Act D ranges from 0.003 to 0.1 μ g/mL, lower concentrations of Act D (0.01 and 0.025 μ g/mL) were also tested in combination with 0.5 μ M 17-AAG for 8 h (Table 1). Statistical analysis also demonstrated that cytotoxicity in MM.1S and RPMI-8226 cells with these lower concentrations of Act D in combination with 17-AAG was also more than additive or synergistic (MM.1S, $P = 0.027$; RPMI-8226, $P = 0.0006$; both cell lines together, $P = 0.0001$).

The geldanamycin analogue 17-AAG inhibits the chaperone activity of HSP90 in stabilizing client oncoproteins. However, 17-AAG elicits a stress response in the cell resulting in the induction of antiapoptotic HSP that may lead to cell death resistance. Act D, because of its transcription inhibitory actions, can be used to block transcription of antiapoptotic HSP. Thus, this combination strategy represents a potential novel approach to the treatment of hematological malignancies.

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Abbreviations

MM	multiple myeloma
17-AAG	17-allylamino-17-demethoxygeldanamycin
Act D	actinomycin D
HSP	heat shock proteins
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

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Figure 1. Effect of 17-AAG or Act D on HSPs and effect of Act D on global RNA synthesis
 Cells were treated for 8 h with 17-AAG for the indicated doses (A) or with 0.5 μM for the indicated time points (B). In parallel, cells were treated for 12 h with Act D for the indicated doses (C) or with 0.05 μg/mL for the indicated time points (D). Protein extracts were analyzed for inducible HSP90α and constitutive HSP90β, inducible HSP70, HSP27, and β-actin. Inhibition of global RNA synthesis by Act D (E). Cells were treated with 0.05 μg/mL Act D for the indicated time points and labeled with [³H]uridine for 1 h before being harvest. Each column is plotted as the percentage of untreated control and represents the mean and SD of three independent experiments.

Figure 2. Effect of Act D and 17-AAG alone and in combination on HSP and HSF-1 transcript levels in MM.1S and RPMI-8226

Cells were not treated or treated with 0.5 μ M 17-AAG for 8 h, 0.05 μ g/mL Act D for 12 h, or the combination of Act D for 4 h and then 17-AAG for 8 h. RNA was isolated and HSP or HSF-1 mRNA levels were measured by using the real-time RT-PCR technique. TXN2 was used as the endogenous gene for normalization. mRNA levels are plotted as fold change in comparison with the untreated control for inducible HSP90 α (A), constitutive HSP90 β (B), inducible HSP70 (C), constitutive HSC70 (D), HSP27 (E) and HSF-1 (F). Each column represents the mean and SD of triplicate experiments. Values in parentheses are *P* values obtained by comparing 17-AAG alone with the combination condition.

Figure 3A

Figure 3B

Figure 3. Effect of Act D and 17-AAG alone and in combination on HSP and HSF-1 protein levels in MM.1S and RPMI-8226 cells

Cells were not treated (U) or treated with 0.5 μ M 17-AAG for 8 h, 0.05 μ g/mL Act D for 12 h, or the combination of Act D for 4 h and then 17-AAG for 8 h. HSP and HSF-1 protein levels for these conditions were visualized on immunoblots using antibodies for inducible HSP90 α and constitutive HSP90 β , inducible HSP70, constitutive HSC70, HSP27, HSF-1, and the loading control β -actin for MM.1S (A) and for RPMI-8226 (B) cells.

Figure 4. Effect of Act D and 17-AAG alone and in combination on HSP and HSF-1 protein levels in MM.1S and RPMI-8226 cells

Immunoblot assays were performed in triplicate and quantitated using Odyssey infrared imaging system application software version 1.2. Protein levels for HSP90α/β (A), inducible HSP70 (B), constitutive HSC70 (C), HSP27 (D), and HSF-1 (E) were normalized to β-actin. The plots represent data from three independent immunoblots plotted as means plus SD. Values in parentheses are *P* values obtained by comparing 17-AAG alone with the combination treatment.

Figure 5. Effect of Act D and 17-AAG on cell death

Percentages of cell death in untreated cells or cells treated with 0.5 μ M 17-AAG for 8 h, 0.05 μ g/mL Act D for 12 h, or the combination of Act D for 4 h and then 17-AAG for 8 h. Flow cytometry was performed to determine the amount of cell death, which was determined as the percentage of cells staining positive for Annexin V/7-AAD after each treatment minus endogenous cell death. The data are mean and SD from triplicate experiments.

Table 1
Cytotoxicity of Act D and 17-AAG alone and in combination

Cells were treated with 0.5 μ M 17-AAG for 8 h, different concentrations of Act D for 12 h, or the combination of Act D for 4 h and then 17-AAG for 8 h. Cytotoxicity was determined by Annexin V/7-AAD staining as described in Materials and Methods.

	17-AAG	Act D	Combination	
MM.1S			<i>Expected</i>	<i>Observed</i>
	0.5 μM	0.05 μg/mL		
% Cell Death	5	38	41	49
	0.5 μM	0.025 μg/mL		
% Cell Death	2	30	31	37
	0.5 μM	0.01 μg/mL		
% Cell Death	2	7	9	11
RPMI-8226			<i>Expected</i>	<i>Observed</i>
	0.5 μM	0.05 μg/mL		
% Cell Death	4	34	37	41
	0.5 μM	0.025 μg/mL		
% Cell Death	4	30	32	44
	0.5 μM	0.01 μg/mL		
% Cell Death	4	11	14	35