Inhibition of Hsp90 Leads to Cell Cycle Arrest and Apoptosis in Human Malignant Pleural Mesothelioma

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Introduction: Heat shock protein 90 (Hsp90) is an abundant molecular chaperone that mediates the maturation and stability of a variety of proteins associated with the promotion of cell growth and survival. Inhibition of Hsp90 function leads to proteasomal degradation of its mis-folded client proteins. Recently, Hsp90 has emerged as being of prime importance to the growth and survival of cancer cells and its inhibitors have already been used in phase I and II clinical trials.

Methods: We investigated how 17-allylamino-17-demethoxygeldanamycin (17-AAG), a small molecule inhibitor of Hsp90, is implicated in human malignant pleural mesothelioma (MM).

Results: We found that 17-AAG led to significant G1 or G2/M cell cycle arrest, inhibition of cell proliferation, and decrease of AKT, AKT1, and survivin expression in all human malignant pleural mesothelioma cell lines examined. We also observed significant apoptosis induction in all MM cell lines treated with 17-AAG. Furthermore, 17-AAG induced apoptosis in freshly cultured primary MM cells and caused signaling changes identical to those in 17-AAG treated MM cell lines.

Conclusion: These results suggest that Hsp90 is strongly associated with the growth and survival of MM and that inhibition of Hsp90 may have therapeutic potential in the treatment of MM.

Key Words: Hsp90, 17-AAG, Cell cycle arrest, Apoptosis, Mesothelioma.

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Malignant pleural mesothelioma[1] (MM) is an asbestos-related malignancy characterized by rapidly progressing, diffuse, local growths, late metastases, and poor prognoses.[2] Approximately 3000 patients are diagnosed with MM each year in the United States, and the incidences are expected to steadily rise over the next two decades.[3] Unfortunately, the prognosis even after standard therapies (surgery, chemotherapy, and radiation) remains dismal, and most patients die within 12 to 15 months of their first symptoms if left untreated.[4] New therapies based on improved understanding of the molecular mechanisms behind MM are, therefore, imperative.

Heat shock protein 90 (Hsp90) is a molecular chaperone that participates in the folding, assembly, maturation, and stabilization of a variety of proteins.[5] Hsp90 is ubiquitously expressed at 2- to 10-fold higher levels in tumor cells than in their normal counterparts. Hsp90 is present entirely in multichaperone complexes with high adenosine triphosphatase activity in tumor tissues, whereas in normal tissues it is in a latent, uncomplexed state.[6,7] Thus, Hsp90 may be critically important for tumor cell growth and survival. Inhibition of Hsp90 function disrupts the complex and leads to degradation of client proteins in a proteasome-dependent manner.[8]

A small molecule inhibitor of Hsp90, 17-allylamino-17-demethoxygeldanamycin (17-AAG) is a less-toxic derivative of the ansamycin antibiotic geldanamycin.[8] It directly binds to the adenosine triphosphate/adenosine diphosphate-binding pocket, thereby replacing the nucleotide and inhibiting Hsp90 function as a molecular chaperone for its client proteins.[9] It has been shown that 17-AAG has a 100-fold higher binding affinity in tumor cells than that in normal cells, and it has antitumor activity in several human xenograft models.[7,10,11] This promising drug is currently completing multi-institution phase I clinical trials, and phase II trials are being planned and performed.[1,6,12–18]

AKT is a serine/threonine kinase that lies downstream of phosphatidylinositol 3-kinase and mediates a wide variety of biological responses to epidermal growth factor receptor and other growth factor receptors[19,20]. The AKT signaling pathway is involved in the regulation of a large variety of cellular processes including growth, cell cycle, death, and sur-

vival. It has been demonstrated that AKT is constitutively active in many types of human cancers and that aberrant activation of the phosphatidylinositol 3-kinase/AKT survival pathway leads to an increase in antiapoptotic signals[19] that inhibit the effectiveness of conventional chemotherapy.[21] Recent results show that AKT is active in MM and relies on Hsp90 for its stability and activity.[5,11,22–24]

Survivin is a structurally unique member of the inhibitor of apoptosis proteins family, and is involved in the control of mitotic progression and inhibition of apoptosis.[25] Several retrospective studies have found that survivin is a reliable marker of aggressive and unfavorable disease progression in various malignancies and is associated with abbreviated overall survival.[25] Survivin is found highly overexpressed in MM[26] and is also a client protein of Hsp90.[27]

Taken together, these reports suggest that inhibition of Hsp90 function may have implications in the treatment of refractory MM. In the present study, we demonstrate a possible therapeutic role of 17-AAG in the treatment of MM cells.

MATERIALS AND METHODS

Cell Lines and Tissue Samples

Human mesothelioma cell lines were obtained from the following sources: H2052, H28, and 211H from American Type Culture Collection (Manassas, VA), H290 from National Institutes of Health (Frederick, MD), and REN through a generous gift from Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA). All cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2.

Fresh MM tissue samples obtained directly from surgical resection were cut into pieces (1–2 mm in diameter) and digested with Triple Enzyme Solution containing collagenase, deoxyribonuclease, and hyaluronidase (Sigma, St. Louis, MO) at room temperature for 16 hours according to National Cancer Institute laboratory protocol. Single cells from the digestion were spun down and the cell pellets were washed twice using Hanks Balanced Salt Solution (Invitrogen, Carlsbad, CA). The cells were resuspended in RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml), and cultured in 6-well plates at 37°C in a humidified incubator with 5% CO_2 until ready for treatment. Other fresh MM tissue samples were immediately snap-frozen in liquid nitrogen. They were preserved at −170°C in a liquid nitrogen freezer before use.

Drug

A 17-AAG was purchased from Sigma (St. Louis, MO). Stock solution was prepared in 100% DMSO and stored at −20°C. The drug was diluted in fresh media before each experiment.

Cell Cycle Analysis

Cells were plated on six-well plates, incubated for 24 hours, and then treated with DMSO, 1 μm 17-AAG, or 2 μm 17-AAG. For flow cytometry, cells were trypsinized and fixed in 70% ethanol at −20°C, washed and stained with 30 μg/ml propidium iodide (PI) (Sigma, St. Louis, MO), and then incubated with 10 μg/ml RNase (Roche, Indianapolis, IN) for 1 hour at room temperature. Cells were evaluated on a FACScan machine (Becton Dickinson, Franklin Lake, NJ) and the data analyzed with the ModFit LT 3.1 Mac software for modeling cell cycle distribution. Experiments were performed in triplicate, and data were expressed as mean ± SD.

Cell Proliferation Assay

Cell proliferation assays were performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's protocol. Cells were plated at 1000 cells/well in 100 μl of media in 96-well plates. After 24 hours, the cells were treated with DMSO or 17-AAG (2 μm) and incubated for 48 hours. Methanethoiosulfonate/phenazine methosulfate solution (20 μl/well) was added and incubated for 2 hours at 37°C in a humid incubator with 5% CO_2. Absorbance was read at 490 nm using a microplate reader. Cell viability was calculated according to the following formula. Cell viability = OD490 (DMSO treated cells or 17-AAG treated cells)/OD490 (nontreated cells).

Western Blotting

Whole cell lysates of MM cell lines and primary tissue cultures were obtained using CytoBuster Protein Extraction Reagent (Novagen, Madison, WI). Protein samples were separated on 4 to 15% gradient sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilion-P (Millipore, Bedford, MA) membranes. Antigen-antibody complexes were detected by the enhanced chemiluminescence blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ). The following primary antibodies were used: Hsp90 and Hsp70 (Stressgen, Victoria, BC, Canada); AKT, cleaved PARP (Cell Signaling Technology, Beverly, MA), AKT1, survivin (Santa Cruz Biotechnology, Santa Cruz, CA), p53 and β-Actin (Sigma Chemical Co., St Louis, MO).

Apoptosis Analysis

Following drug treatment, cells were harvested by trypsinization and stained using an Annexin V-FITC Apoptosis kit (BioSource, Camarillo, CA) according to the manufacturer's protocol. Stained cells were immediately analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin Lake, NJ). Early apoptotic cells were characterized by exposed phosphatidylserine bound to Annexin V-FITC but not to propidium iodide. Cells in necrotic or late apoptotic stages were labeled both with Annexin V-FITC and with PI. Experiments were performed in triplicate and a total of 20,000 cells were analyzed in each individual experiment.

Statistical Analysis

The data presented represent mean values (±SD). Statistical comparisons were made with a two-sided Student t test. A p value of less than 0.05 was considered to be statistically significant. Asterisks (*) represent statistical significance (*$p < 0.05$; **$p < 0.01$).

RESULTS

Expression of Hsp90 in MM Cell Lines and Primary MM Tissue Samples

It has been reported that Hsp90 is one of the most abundant cellular proteins, accounting for about 1 to 2% of total protein under nonstressed conditions.[5] To confirm the expression of Hsp90 in MM, we performed Western blotting analysis using 13 MM tissue samples and 5 MM cell lines and found that all MM tissue samples and cell lines that we examined expressed Hsp90 protein (Figures 1*A, B*).

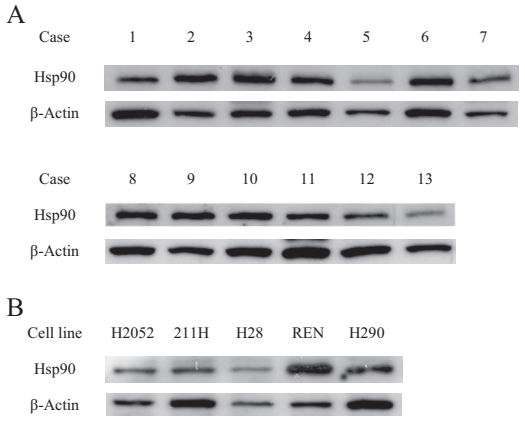

FIGURE 1. Western analysis of the Hsp90 expression in 13 MM tissue samples (*A*) and in 5 MM cell lines (*B*). β-Actin served as loading control.

A 17-AAG Leads to G1 or G2/M Cell Cycle Arrest and Inhibits Cell Proliferation in MM Cells

Hsp90 has been observed to play an important role in human cancer cells. For example, inhibition of Hsp90 by 17-AAG leads to G1 or G2/M cell cycle arrest in gynecologic cancer cells and breast cancer cells.[5,28,29] To examine the role of Hsp90 function in MM, we first treated five MM cell lines for 24 hours using either DMSO, 1 μm 17-AAG, or 2 μm 17-AAG, and then performed cell cycle analysis. We found significant G0/G1 arrest in H2052 and 211H cells after treatment with 17-AAG (Figure 2*A*). Specifically, in H2052 cells significant accumulation of cells in G1 phase was observed in both the 1 μm and the 2 μm 17-AAG treated cells as opposed to in the DMSO treated ones (1 μm: $p = 0.03$, 2 μm: $p < 0.01$). In 211H cells, significant accumulation of cells in G1 phase was observed in 2 μm 17-AAG treated cells compared with the DMSO treated ones (1 μm: $p = 0.06$, 2 μm: $p < 0.01$). No obvious changes in accumulation at G2/M phases after 17-AAG treatment were observed in either H2052 or 211H cell lines (data not shown).

On the other hand, we observed significant accumulation of cells in G2/M phases in both the 1 μm and the 2 μm 17-AAG treated cells as opposed to in the DMSO treated cells in several other MM cell lines; H28 (1 μm: $p < 0.01$, 2 μm: $p < 0.01$), REN (1 μm: $p < 0.01$, 2 μm: $p = 0.04$), and H290 (1 μm: $p < 0.01$, 2 μm: $p < 0.01$) (Figure 2*B*). In these latter cell lines, no obvious changes in accumulation at G1/G0 phases were observed following 17-AAG treatment (data not shown).

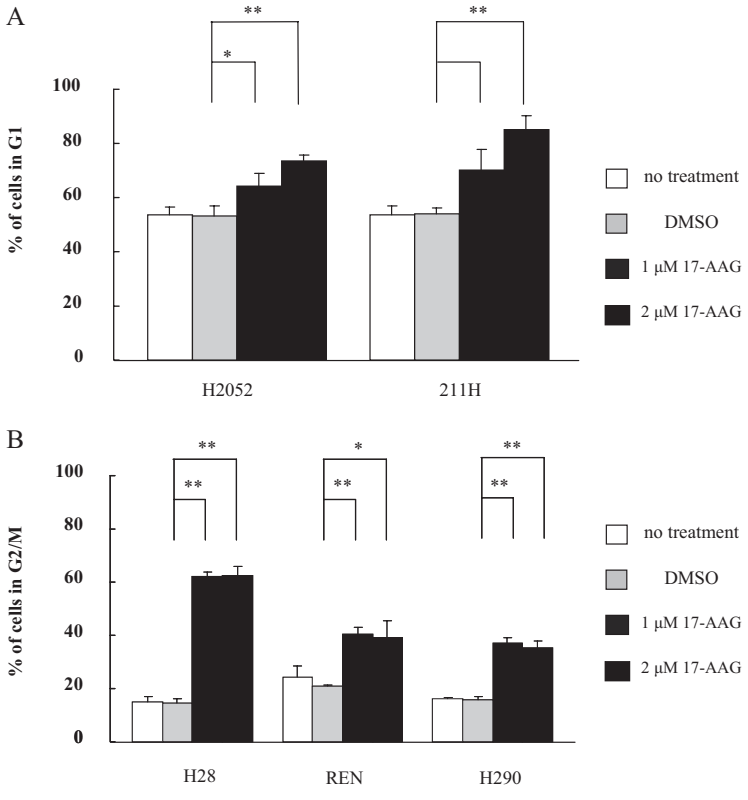

FIGURE 2. Cell cycle analysis of MM cells after 17-AAG treatments. (*A*) 17-AAG induces G1 arrest in MM cell lines. (*B*) 17-AAG induces G2/M cell cycle arrest in MM cell lines. All cell lines were treated with DMSO, 1 μm and 2 μm 17-AAG for 24 hours.

To confirm cell cycle arrest after 17-AAG treatment in MM cell lines, we performed cell proliferation assays. Across all cell lines, after 48 hours of treatment we observed significant inhibition of cell proliferation in 17-AAG (2 μm) treated cells compared with DMSO treated ones (H2052: $p = 0.02$, other cell lines: $p < 0.01$) (Figure 3*A*). Our results indicate that cell cycle arrest correlates with the growth suppression of these MM cells.

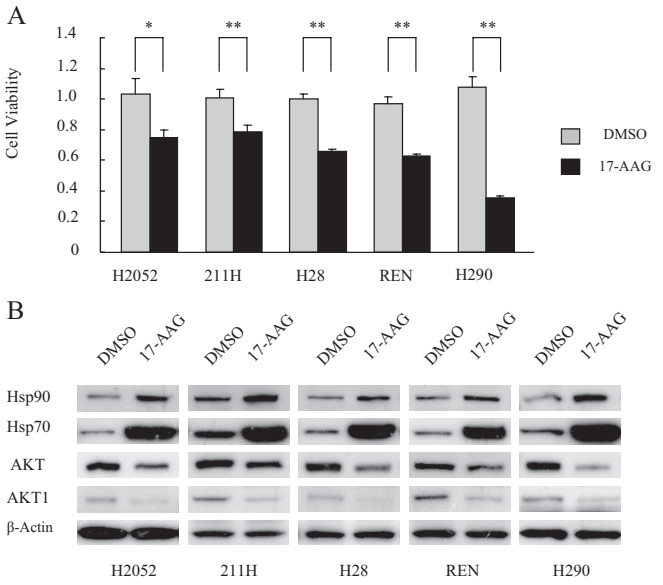

FIGURE 3. Proliferation assay of MM cells after 17-AAG treatments. (*A*) MTT assay after 48 hours treatment with 17-AAG. A 17-AAG inhibits growth of MM cells. Gray: OD490 (DMSO treated cells)/OD490 (nontreated cells); black: OD490 (17-AAG treated cells)/OD490 (nontreated cells). (*B*) Western analysis of Hsp90, Hsp70, AKT, AKT1, and Survivin expression level in MM cell lines. β-Actin served as loading control.

The Hsp70 and Hsp90 chaperone systems are linked by the adaptor protein HOP/p60, which interacts with the C-terminals of both Hsp70 and Hsp90 via its tetra-copeptide repeat domain.[30] Increased levels of Hsp90 and Hsp70 are an indication of cellular stress response, and inhibition of Hsp90 function has been previously reported to increase Hsp90 and Hsp70 levels in various cancer cell types.[14,16,18,31] Consistently, we found that 17-AAG treatment (2 μm for 24 hours) also increased expression levels of Hsp90 and Hsp70 in all MM cell lines (Figure 3*B*). The AKT pathway plays a crucial role in cell growth and survival.[20] To analyze whether 17-AAG suppresses cell growth by interfering with this pathway, we also analyzed AKT and AKT1 expression levels after 17-AAG treatment (Figure 3*B*) and found that expression of both AKT and AKT1 decreased after 17-AAG treatment in all MM cell lines. Taken together our results revealed that in MM, decreased cell viability was associated with up-regulated expression of Hsp90 and Hsp70, and that inhibition of Hsp90 function by 17-AAG suppressed cell proliferation through AKT-dependent cell cycle arrest.

A 17-AAG-Induced Apoptosis in MM Cell Lines

Reduced levels of PI3-AKT pathway-associated proteins increase apoptosis.[20] To determine whether 17-AAG leads to apoptosis in addition to cell growth suppression in MM cells, the AnnexinV apoptosis assay was used to examine MM cells after treatment with 1 and 2 μm 17-AAG. After 2 days of treatment, significant apoptosis induction was observed in three MM cell lines (REN, 1 μm: $p < 0.01$, 2 μm: $p < 0.01$; H290, 1 μm: $p = 0.02$, 2 μm: $p < 0.01$; H28, 2 μm, $p = 0.03$) (Figure 4*A*). In H2052 and 211H cells, significant apoptosis induction was observed after 3 days of treatment with 2 μm 17-AAG (H2052: $p < 0.01$; 211H: $p = 0.02$). Consistently, we found that after 2 μm 17-AAG treatment for 2 days, the level of cleaved poly (ADP-ribose) polymerase protein (active form) was increased in four MM cell lines (H28, REN, and H290) (Figure 4*B*). In H2052 and 211H, the expres-

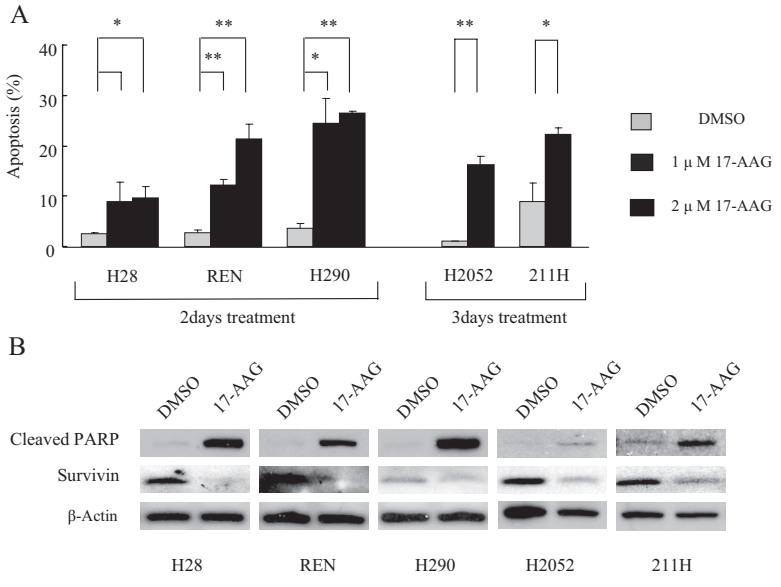

FIGURE 4. A 17-AAG induces apoptosis in MM cell lines. (*A*) FACS analysis of apoptosis after17-AAG treatment in MM cell lines. Two days treatment: H28, REN, and H290. Three days treatment: H2052 and 211H. (*B*) Western analysis of expression levels of cleaved PARP and Survivin in MM cell lines. β-Actin served as loading control.

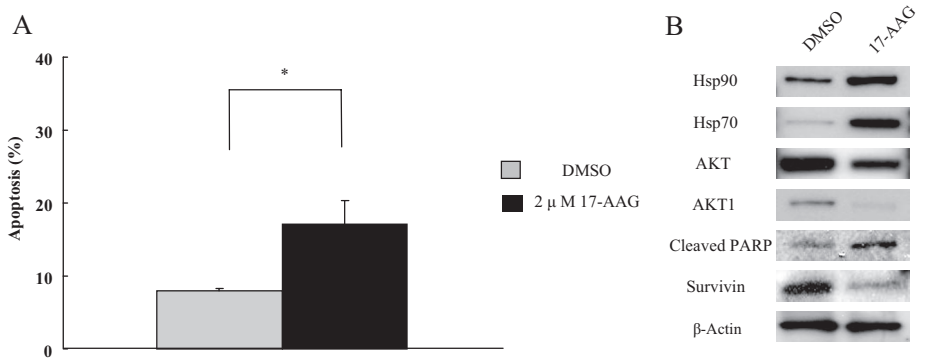

FIGURE 5. A 17-AAG induces apoptosis in MM primary tissue culture. (*A*) FACS analysis of apoptosis after 17-AAG treatment in MM primary culture. (*B*) Western analysis after 17-AAG treatment in MM primary culture. β-Actin served as loading control.

sion of cleaved PARP protein increased after 3 days of treatment with 2 μm 17-AAG. Furthermore, the expression level of survivin, an apoptosis inhibitor, decreased in all MM cell lines after 2 μm 17-AAG treatment for 2 days (Figure 4*B*).

A 17-AAG-Induced Apoptosis in Primary Cultured MM Tissues

We next investigated the efficacy of 17-AAG in the treatment of primary MM tissue cultures. After 3 days, significant apoptosis was observed in 17-AAG (2 μm) treated cells compared with those treated with DMSO alone ($p = 0.04$) (Figure 5*A*). Consistently, we observed increased expression of cleaved PARP and decreased expression of survivin (Figure 5*B*). Moreover, in line with our observations in 17-AAG treated MM cell lines, we observed increased levels of Hsp90 and Hsp70 as well as decreased levels of AKT, suggesting that the 17-AAG inhibitory mechanism is similar in both MM cell lines and primary MM cultures (Figure 5*B*). Taken together, these results suggest that 17-AAG may have a therapeutic role in the treatment of MM.

DISCUSSION

Chemotherapy, radiotherapy, and surgery have long been standard treatments for MM. Several MM chemotherapy regimens such as Pemetrexed plus Cisplatin or Gemcitabine plus Cisplatin are valuable for palliation. These treatments not only decrease tumor burden but also relieve symptoms such as pain and breathlessness. However, no chemotherapy regimen for mesothelioma has yet proven wholly curative. Clinical trials show objective response rates of 41 to 48%, median survivals are less than 12 months, and the therapies themselves are still controversial.[32–35] Radiotherapy has been used in MM for about 30 years but the results have been largely disappointing. Although the operative mortality rate is now around 6% for the procedure,[32,36,37] extrapleural pneumonectomies are performed in select patients at specialized centers to completely remove all MM; however, adjuvant therapy after surgery still remains necessary.

Recently, molecular target medicines, such as imanitib (2-phenylaminopyrimidine tyrosine kinase inhibitor) and gefinitib (EGFR inhibitor) have also been investigated in MM patients. Nevertheless, these drugs do not appear to be effective against MM according to early studies.[38,39] Therefore, it is vital that more effective therapies against MM are developed.

Heat shock proteins are a group of chaperones important in maintaining the stability and function of their client proteins. Hsp90 is one of the most abundant heat shock proteins found in human cells. Nevertheless, Hsp90 is distinct from other heat shock proteins in that it does not participate in general protein folding. Instead, it acts by regulating the stability and function of several signal transduction proteins, and plays an important role in biological processes that include hormone signaling, cell cycle control, and development.[5–7]

A 17-AAG is currently in clinical trials as a drug against a variety of solid tumors[1,13–18,40,41] and shows a time- and dose-dependent growth inhibition of Hodgkin lymphoma cell lines[31] as well. There are extensive preclinical data both in vitro and in vivo suggesting that inhibition of Hsp90 is a rational therapeutic approach to cancer, either alone or in combination with standard chemotherapeutic drugs. Several investigators have also found that 17-AAG can be used to sensitize cancer cells to radiation therapy.[42,43] Nevertheless, studies of Hsp90 in MM have been limited.

In our study, we investigated cell cycle arrest and suppression of cell growth after 17-AAG treatment in MM cell lines. Our results revealed that Hsp90 function is strongly associated with cell growth in MM. Previous studies showed that 17-AAG led to G1 arrest in retinoblastoma (Rb)-positive breast cancer cell lines and to G2/M arrest in Rb-negative ones.[29] In cervical carcinoma cells, another Hsp90 inhibitor17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) induced G2/M arrest through AKT regulation.[44] All MM cell lines examined in our study were found to be Rb-positive[45] (data not shown). Nevertheless, 17-AAG was observed to induce both G1 arrest (in H2052 and 211H) and G2/M arrest (in H28, REN, and H290) (Figure 2). These results suggest that 17-AAG can induce either G1 arrest or G2/M arrest through suppression of the AKT pathway in Rb-positive MM cells. Our interpretation is that Hsp90 chaperones a number of different cell cycle regulatory proteins, including those involved in both G_0-G_1 and G_2-M entries in MM cells. The final predominant effect on the cell cycle may depend on the net effect on all these proteins.[44,46] Notably, about 50% of the untreated H2052 and 211H cells were also in G1 phase (Figure 2*A*). This is possibly due to cell-cell contact inhibition[47] resulting from their fast-growing nature.

A 17-AAG not only suppresses cell growth but also induces apoptosis in a variety of cancer cells.[8] Our results

reveal that 17-AAG leads to apoptosis as well as to decreased expression levels of AKT and survivin in MM. AKT and survivin have antiapoptotic functions and are active or overexpressed in many cancer cells.[25,48] Georgakis et al.[31] showed that 17-AAG causes AKT down-regulation and Hsp70 up-regulation in Hodgkin lymphoma cell lines. In addition, Sain et al.[48] suggested that 17-AAG causes p-AKT and AKT down-regulation and Hsp70 up-regulation in ovarian carcinoma cell lines. It has also been reported that inhibition of AKT leads to suppression of cell growth and apoptosis in MM.[22,23] In addition, down-regulation of survivin, which predicts poor prognosis,[49,50] induces apoptosis in MM.[26] These reports are consistent with our results and support the observation that 17-AAG leads to apoptosis in MM.

In summary, we demonstrated that 17-AAG, an Hsp90 inhibitor, leads to G1 or G2/M cell cycle arrest, to suppression of cell growth, and to apoptosis resulting from decreased levels of AKT and survivin in human MM cell lines. We also demonstrated that this small molecule induces apoptosis in MM primary cultures (Figure 5). Our findings suggest that inhibition of Hsp90 function is a promising therapeutic target for a highly aggressive and inexorably fatal cancer.

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