Abrogation of Heat Shock Protein 70 Induction as a Strategy to Increase Antileukemia Activity of Heat Shock Protein 90 Inhibitor 17-Allylamo-Demethoxy Geldanamycin

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Abstract

17-Allylamo-demethoxy geldanamycin (17-AAG) inhibits the chaperone association of heat shock protein 90 (hsp90) with the heat shock factor-1 (HSF-1), which induces the mRNA and protein levels of hsp70. Increased hsp70 levels inhibit death receptor and mitochondria-initiated signaling for apoptosis. Here, we show that ectopic overexpression of hsp70 in human acute myelogenous leukemia HL-60 cells (HL-60/hsp70) and high endogenous hsp70 levels in Bcr-Abl-expressing cultured CML-BC K562 cells confers resistance to 17-AAG-induced apoptosis. In HL-60/hsp70 cells, hsp70 was bound to Bax, inhibited 17-AAG-mediated Bax conformation change and mitochondrial localization, thereby inhibiting the mitochondria-initiated events of apoptosis. Treatment with 17-AAG attenuated the levels of phospho-AKT, AKT, and c-Raf but increased hsp70 levels to a similar extent in the control HL-60/Neo and HL-60/hsp70 cells. Pretreatment with 17-AAG, which induced hsp70, inhibited 1-β-D-arabinofuranosylcytosine or etoposide-induced apoptosis in HL-60 cells. Stable transfection of a small interfering RNA (siRNA) to hsp70 completely abrogated the endogenous levels of hsp70 and blocked 17-AAG-mediated hsp70 induction, resulting in sensitizing K562/siRNA-hsp70 cells to 17-AAG-induced apoptosis. This was associated with decreased binding of Bax to hsp70 and increased 17-AAG-induced Bax conformation change. 17-AAG-mediated decline in the levels of AKT, c-Raf, and Bcr-Abl was similar in K562 and K562/siRNA-hsp70 cells. Cotreatment with KNK437, a benzylidine lactam inhibitor of hsp70 induction and thermotolerance, attenuated 17-AAG-mediated hsp70 induction and increased 17-AAG-induced apoptosis and loss of clonogenic survival of HL-60 cells. Collectively, these data indicate that induction of hsp70 attenuates the apoptotic effects of 17-AAG, and abrogation of hsp70 induction significantly enhances the antileukemia activity of 17-AAG.

Introduction

A number of leukemia associated, newly synthesized, or stress-denatured client proteins, including Bcr-Abl, c-Raf, AKT, c-KIT, and FLT-3, require interaction with heat shock protein 90 (hsp90) to maintain a mature, stable, and functional conformation (1, 2). Hsp90 is an ATP-dependent molecular chaperone, which binds and releases client proteins, driven by ATP binding and hydrolysis (3). ATP/ADP binding to the hydrophobic NH2 terminus pocket alters the conformation of hsp90, resulting in its interaction with the cochaperone complex that protects or stabilizes the client proteins, or with an alternative subset of cochaperones that directs the misfolded proteins to a covalent linkage with polyubiquitin and subsequent degradation by the 26S proteasome (1–4). Benzoxai-none ansamycin antibiotic geldanamycin and its less toxic analogue 17-allylamo-demethoxy geldanamycin (17-AAG) directly bind to the ATP/ADP binding pocket, thereby replacing the nucleotide and inhibiting hsp90 function as a molecular chaperone for the client proteins (5). By blocking ATP binding to hsp90, 17-AAG stabilizes the hsp90 conformation that recruits hsp70-based cochaperone complex associated with the misfolded client proteins (1, 2, 6). This results in the ubiquitin-dependent proteasomal degradation of the client proteins (1, 2).

Recent studies from our laboratory have shown that the antipoptotic effects of Bcr-Abl in acute leukemia cells are partially mediated by increased expression of hsp70 (7). Hsp70 is also an ATP-dependent molecular chaperone, which is induced by cellular stress due to misfolded and denatured proteins (8, 9). In normal nontransformed cells, the expression of hsp70 is low and largely stress inducible (8, 9). However, hsp70 is abundantly expressed in most cancer cells (8, 9). Hsp70 has been shown to play an active role in oncogenic transformation, and turning off the hsp70 expression was shown to reverse the transformed phenotype of Rat-1 fibroblasts (10–12). Ectopic overexpression or induced endogenous levels of hsp70 potently inhibits apoptosis (9, 13, 14). Several reports have documented that hsp70 inhibits the mitochondrial pathway of apoptosis by blocking Apaf-1-mediated activation of caspase-9 and caspase-3, as well as by repressing the activity of caspase-3 (15–17). Additionally, hsp70 can also inhibit caspase-independent apoptosis by directly interacting with AIF, thereby preventing nuclear import and DNA fragmentation by AIF (18, 19). Conversely, hsp70 depletion by antisense oligonucleotides or ectopic transfection and expression of a fragment of hsp70 DNA in the antisense orientation has been shown to induce apoptosis (7, 20, 21).

Heat-damaged proteins, reactive oxygen species, or oncogenic stress is known to induce hsp70 levels (8, 9). This is mediated through the transcriptional activity of the heat shock factor-1 (HSF-1; refs. 8, 9). Stress-induced activation of HSF-1 involves phosphorylation, trimerization, nuclear localization, and binding of HSF-1 to the heat shock elements (HSE) in the promoter of the hsp70 gene, resulting in induction of hsp70 levels (9, 22, 23). Hsp90 binds and blocks the activation of HSF-1 (22). During stress...
response, denatured proteins bind hsp90 and displace HSF-1 from hsp90, allowing nuclear localization and activity of HSF-1, resulting in up-regulation of hsp70 levels (9, 22, 23). KNK437 is a novel benzylidine lactam compound, which is known to inhibit the development of thermotolerance by inhibiting the induction of heat shock proteins, including hsp70 (24, 25). Treatment with 17-AAG disrupts the association between hsp90 and HSF-1, thereby promoting nuclear localization, HSE binding, and activity of HSF-1, resulting in induction of hsp70 levels (22, 23, 26). 17-AAG was shown to induce hsp70 in the transformed fibroblasts derived from mice with wild-type HSF-1 but not from the HSF-1 knockout mice (26). Indeed, the hsf-1−/− fibroblasts were significantly more sensitive than hsf-1+/+ fibroblasts to 17-AAG-mediated cytotoxic effect. Therefore, the question arises whether 17-AAG-induced hsp70 attenuates the apoptotic effect of 17-AAG in human leukemia cells, and whether pretreatment with 17-AAG, through induction of hsp70, would inhibit apoptosis induced by conventional antileukemia agents. In the present studies, we examined these issues, as well as determined whether abrogation of hsp70 induction by small interfering RNA (siRNA) to hsp70 or by cotreatment with KNK437 would sensitize human leukemia cells to 17-AAG and other antileukemia agents.

Materials and Methods

Reagents and antibodies. 17-AAG was a gift from Kanso Bioscience (South San Francisco, CA; ref. 27). KNK437 was a gift from Kaneka Corp. (Takasago, Japan). Etoposide and 1-(β- arabino-furanosylcotosine (ara-C) were purchased from Sigma Chemical Co. (St. Louis, MO) and prepared as a 10 mmol/L stock solution in sterile PBS and diluted in RPMI 1640 before use. Anti-hsp70, HSF-1, and anti-hsp90 antibodies were purchased from Stressgen Biotechnologies Corp. (Victoria, British Columbia, Canada). Anti-β-galactosidase (β-gal) reporter construct-containing vector pλ730R was also purchased from Stressgen Biotechnologies. The monoclonal anti-Ab and polyclonal anti-Bax antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-poly(ADP-ribose) polymerase (PARP) was purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-AKT (p-AKT), c-Raf, and anti-AKT antibodies were procured as previously described (28).

Cultured cells. Human chronic myelogenous leukemia blast crisis K562 and acute myelogenous leukemia HL-60 cells were maintained in culture as previously described (29). Cells were passaged as previously reported (29). Logarithmically growing cells were used for the studies described below.

Creation and culture of HL-60/hsp70 and K562/hsp70AS cells. pcDNA3-hsp70 plasmid was kindly provided by Dr. Richard Morimoto (Northwestern University, Urbana, IL; ref. 30). This construct was stably transfected into HL-60 cells, and the stable clones were selected, subcloned by limiting dilution, and maintained in 500 μg/mL of G418 (7). To create the pcDNA3-hsp70AS construct, pcDNA3-hsp70 was used as a template for PCR amplification using forward primer containing XhoI restriction site (5′-CCCTCTGAGAGGATGCGGGTGTGATCG-3′) and a reverse primer containing HindIII restriction site (5′-CCCAAAGGTTCGGTGCTGCGAGCAGAACGAG-3′; ref. 20). The PCR product was digested with XhoI and HindIII and subcloned into pcDNA3.1 vector. K562 cells were stably transfected with the pcDNA3-hsp70AS construct and maintained in 500 μg/mL of G418.

Creation and culture of K562/siRNA-hsp70 cells. K562 cells were stably transfected by Amaxa nucleofector, using T-16 protocol of the manufacturer (Gathiersburg, MD), with the pRNAIn-H1.2/Neo vector containing hsp70 siRNA GAAAGCCAGGGTTGCGACMAA or the control sequence (K562 control cells; GenScript, Piscataway, NJ; refs. 7, 31). Stable clones were selected and maintained in 500 μg/mL G418 selection medium.

Western analyses of proteins. Western analyses of Bcr-Abl, pAKT, AKT, c-Raf, Caspase-3, PARP, hsp70, hsp90 and HSF-1, and β-actin were done using specific antisera or monoclonal antibodies according to previously reported protocols (29, 32).

Bax conformation change analysis. Cells were lysed in 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid lysis buffer, containing 150 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), and 1% [3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid] containing protease inhibitors. Immunoprecipitation is done in lysis buffer by using 500 μg of total cell lysate and 2.5 μg of anti-Bax 6A7 monoclonal antibody (Sigma Chemical). The resulting immune complexes were subjected to immunoblotting analysis with anti-Bax polyclonal antibody, as described previously (32, 33).

Apoptosis assessment by Annexin V staining. After drug treatments, percentage apoptotic cells (either stained only with Annexin V or with both Annexin V and propidium iodide) were estimated by flow cytometry, as previously described (28, 29).

Colony growth inhibition. Following treatment with the designated concentrations of 17-AAG and/or KNK437, untreated and drug-treated cells were washed in RPMI 1640. Approximately 200 cells treated under each condition were resuspended in 100 μL of RPMI 1640 containing 10% fetal bovine serum and then plated in duplicate wells in a 12- well plate containing 1.0 mL of Methocult medium (Stem Cell Technologies, Inc., Vancouver, Canada) per well, according to the manufacturer’s protocol. The plates were placed in an incubator at 37°C with 5% CO2 for 10 days. Following this incubation, colonies consisting of ≥50 cells, in each well, were counted by an inverted microscope and % colony growth inhibition compared with the untreated control cells were calculated.

Morphology of apoptotic cells. After drug treatment, 50 × 103 cells were washed and resuspended in 1× PBS (pH 7.4). Cytosin preparations of the cell suspensions were fixed and stained with Wright stain. Cell morphology was determined and the percentage of apoptotic cells was calculated for each experiment, as described previously (29).

Nuclear and S100 fraction isolation. Cells were lysed in hypotonic buffer containing 20 mmol/L HEPES (pH 7.9), 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L NaF, 1 mmol/L Na3P2O7, 1 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF). 0.5 μg/mL leupeptin, 50 μg/mL antipain, and 2 μg/mL aprotinin. Cell lysates were left on ice for 10 minutes and then centrifuged at 2,000 × g for 30 seconds. Pellets were resuspended in hypotonic buffer containing 0.05% NP40. The nuclei were released and pelleted by centrifugation at 2,000 × g for 2 minutes. Nuclei were resuspended in hypotonic buffer C, containing 20 mmol/L HEPES (pH 7.9), 420 NaCl, 20% glycerol, 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L NaF, 1 mmol/L Na3P2O7, 1 mmol/L DTT, and 0.5 mmol/L PMSF, 0.5 μg/mL leupeptin, 50 μg/mL antipain, and 2 μg/mL aprotinin, mixed well, and rotating at 4°C for 30 minutes. Finally, nuclear extracts were collected as supernatant after centrifugation at 12,000 × g at 4°C for 30 minutes, as previously described (7, 32). S100 fractions were collected as previously described (7, 32).

Heat shock factor-1 immunofluorescence. Approximately 50,000 cells were cytospun, immediately fixed with 4% paraformaldehyde, and kept overnight at 4°C. The cells were then permeabilized in 0.5% Triton X-100/PBS for 15 minutes. After preblocking with 3% bovine serum albumin/PBS, cells were incubated with anti-HSF-1 primary antibody (Stressgen, British Columbia, Canada; 1:100) at room temperature for 1.5 hours followed by three washes in PBS and incubated with FITC-conjugated secondary antibody (1:200) at room temperature for 30 minutes. Cells were washed in PBS before nuclear staining with 0.1 μg/mL of 4,6-diamino-2-phenylindole and analysis by fluorescence microscopy, as described previously (34).

β-Galactosidase reporter assay. Cells were transfected with hsp70 promoter/-gal reporter vector pλ730R by nucleoection (Amaxa) and incubated at 37°C for 36 hours. Cells were then pretreated with KNK437 (400 μmol/L) for 1 hour followed by KNK437 plus 17 AAG (5 μmol/L) for 6 hours. Alternatively, cells were heat shocked (42°C) for 1 hour followed by recovery at 37°C for 5 hours. Subsequently, cells were harvested and total cell lysates obtained. Ten micrograms of the protein were incubated with chloroformoloid-β-galactopyranoside (Roche, Indianapolis, IN) for 4 hours and analyzed using a microplate reader at 570 nm.

Statistical analysis. Significant differences between values obtained in a population of leukemia cells treated with different experimental conditions were determined using the Student’s t test.
Results

Overexpression of heat shock protein 70 confers resistance against 17-allylamino-demethoxy geldanamycin–induced apoptosis of acute leukemia cells. First, we compared the apoptotic effects of 17-AAG in the control (HL-60/Neo) versus the HL-60/hsp70 cells with ectopic overexpression of hsp70. As shown in Fig. 1A, treatment with 0.5, 2.0, or 5.0 μmol/L of 17-AAG induced significantly less apoptosis in HL-60/hsp70 compared with HL-60/Neo cells. This was associated with reduced processing of PARP in HL-60/hsp70 cells (Fig. 1C). However, exposure to 17-AAG depleted p-AKT, AKT, and c-Raf levels in both cell types, with slightly greater inhibition of p-AKT and AKT levels in HL-60/hsp70 cells (Fig. 1B). A more pronounced effect noted on p-AKT than AKT levels may be due to increased activity of the protein phosphatase 1 (PP1) induced by treatment with 17-AAG (35). 17-AAG also induced the expression of hsp70, with only a slight increase in hsp90 levels, in both HL-60/Neo and HL-60/hsp70 cells (Fig. 1C).

Hsp70 inhibits 17-allylamino-demethoxy geldanamycin–induced Bax conformation change and localization to the mitochondria. In unperturbed cells the multi-Bcl-2 homology domain-containing prosapoptotic molecule Bax is predominantly localized in the cytosol (36). Following exposure to an apoptotic stimulus, Bax undergoes a conformational change, detected by the 6A7 antibody, leading to exposure of its NH₂ and COOH termini and localization to the mitochondria (33, 37). These results in mitochondrial permeabilization and release of the pro-death molecules cytochrome c, Smac, Omi, and AIF (27, 33, 38). Compared with HL-60/Neo, in HL-60/hsp70 cells, more hsp70 could be coimmunoprecipitated with intracellular Bax, with or without treatment with 17-AAG (Fig. 2A). These results are consistent with a recent report, where hsp70 was shown to inhibit endoplasmic reticulum stress-induced apoptosis by binding to Bax and preventing its translocation to the mitochondria (39). A dose-dependent increase in 17-AAG-mediated Bax conformation change in HL-60/Neo versus the control K562 cells was observed, which was markedly inhibited in HL-60/hsp70 cells (Fig. 2B). Consistent with the reduced 17-AAG-induced Bax conformation change, 17-AAG also did not attenuate Bax levels in the cytosolic S100 fraction or increased Bax levels in the mitochondria that are present in the heavy membrane fraction of HL-60/hsp70 cells, as was seen in HL-60/Neo cells (Fig. 2C).

Abrogation of heat shock protein 70 induction sensitizes 17-allylamino-demethoxy geldanamycin–induced apoptosis. We had previously reported that Bcr-Abl-expressing chronic myelogenous leukemia cells (e.g., K562 cells) possess markedly higher expression of hsp70 but not hsp90 (7). The direct role of hsp70 in mediating resistance to apoptosis was determined in K562 cells stably transfected with the siRNA to hsp70 (K562/siRNA–hsp70), or in K562 cells with stable transfection of the cDNA of hsp70 in the reversed orientation (K562/hsp70AS cells). Figure 3A shows that, compared with the control K562, untreated K562/siRNA–hsp70 cells do not express hsp70. Notably, treatment with 17-AAG induced marked induction of hsp70 levels in the control K562 but not in K562/siRNA–hsp70 cells. Concomitantly, exposure to 17-AAG induced significantly more Bax conformation change in K562/siRNA–hsp70 versus the control K562 cells (Fig. 3A). Consistent with this, compared with K562/control, K562/siRNA–hsp70 cells were more sensitive to 17-AAG-induced apoptosis (Fig. 3B). Despite this, treatment with 17-AAG caused similar decline in the levels of Bcr-Abl, AKT, and c-Raf levels in both cell types (Fig. 3C). These data indicate that induction of hsp70 by 17-AAG inhibits Bax-mediated signaling for apoptosis induced by 17-AAG, without abrogating 17-AAG-mediated depletion of hsp90 client proteins c-Raf, AKT, and Bcr-Abl in K562/control cells. Treatment with 17-AAG also induced hsp90 but failed to induce hsp70 in K562/hsp70AS cells (Fig. 3A). Consequently, as in K562/siRNA–hsp70 cells, the sensitivity to 17-AAG-induced apoptosis was markedly restored in K562/hsp70AS cells (Fig. 3B). There was no significant difference in Bcr-Abl, c-Raf, p-AKT, or AKT levels in K562/hsp70AS versus K562/control cells (data not shown).
Pretreatment with 17-allylamino-demethoxy geldanamycin induces heat shock protein 70 and confers resistance to apoptosis due to antileukemia agents. We next determined the sequence-dependent effects of cotreatment with 17-AAG on the cytotoxic effects of etoposide (1.0 μmol/L) or ara-C (1.0 μmol/L; data not shown) on human acute myelogenous leukemia HL-60 cells. Whereas exposure to etoposide had no effect, treatment with 0.5 μmol/L of 17-AAG for 24 hours induced hsp70 levels in HL-60 cells, which were unaffected by exposure to etoposide before or after 17-AAG treatment (Fig. 5A). Similar effects of ara-C on hsp70 induction were observed (data not shown). Concomitantly, apoptosis following sequential treatment with etoposide followed by 17-AAG (each for 24 hours) was significantly more than the sequential treatment with 17-AAG followed by etoposide, or following treatment with either drug alone for 24 hours followed by culture of the cells in the drug-free medium for 24 hours (P < 0.05; Fig. 5B). A similar sequence-dependent effect of 17-AAG was also observed with ara-C (data not shown). These data suggest that induction of hsp70 levels by 17-AAG pretreatment may confer

Figure 2. Hsp70 binds to Bax and inhibits the conformational change and translocation of Bax to the mitochondria. A, HL-60/Neo and HL-60/hsp70 cells were treated with 17-AAG for 24 hours. Following this treatment, the cell lysates were immunoprecipitated with anti-Bax antibody and immunoblotted with either anti-hsp70 or anti-Bax antibody. B, HL-60/Neo and HL-60/hsp70 cells were treated with 17-AAG at indicated concentrations for 24 hours. Following this treatment, the cytosolic (S100) and heavy membrane fractions (HMF) were separated and immunoblotted with anti-Bax antibody. β-Actin levels were used as a loading control.

Figure 3. Ectopic expression of siRNA-hsp70 depletes hsp70 levels and sensitizes cells to 17-AAG-induced apoptosis. A, control K562 and K562/siRNA-hsp70 cells were treated with 17-AAG for 24 hours. Following this treatment, the cell lysates were immunoprecipitated with anti-Bax antibody and immunoblotted with either hsp70 or Bax antibodies. Alternatively, the cell lysates were immunoprecipitated with 6A7 antibody that detects the conformationally changed Bax and immunoblotted with polyclonal anti-Bax antibody. C, HL-60/Neo and HL-60/hsp70 cells were treated with 17-AAG at indicated concentrations for 24 hours. Following this treatment, the cytosolic (S100) and heavy membrane fractions (HMF) were separated and immunoblotted with anti-Bax antibody. β-Actin levels were used as a loading control.
resistance to apoptosis secondary to a subsequent treatment with etoposide or ara-C.

**Cotreatment with KNK437 inhibits 17-allylamino-demethoxy geldanamycin-mediated heat shock protein 70 induction but enhances 17-allylamino-demethoxy geldanamycin-induced apoptosis.** Previous reports have indicated that the induction of hsp70 expression is dependent on the phosphorylation, oligomerization, nuclear localization, and binding of HSF-1 to the HSEs present upstream of the hsp70 promoter (8, 9, 22, 23, 26). Because KNK437 pretreatment has been shown to inhibit the induction of the mRNA and protein levels of hsp70 following heat shock (24, 25), we determined the mechanism by which KNK437 inhibits 17-AAG-mediated hsp70 induction, as well as evaluated the effect of cotreatment with KNK437 on 17-AAG-induced apoptosis. Figure 6A shows that treatment with KNK437 did not inhibit the phosphorylation of HSF-1 in K562 cells, based on the absence of any inhibitory effect on the slower migrating band on the immunoblot of HSF-1, which is abolished by cotreatment with PP1 (Calbiochem, San Diego, CA; data not shown). Pretreatment for 1 hour followed by cotreatment with KNK437 did not inhibit 17-AAG-mediated increase in the nuclear HSF-1 immunofluorescence (Fig. 6C). In contrast, in cells transfected with the hsp70 promoter/β-gal reporter plasmid, pretreatment and cotreatment with KNK437 significantly inhibited heat shock– or 17-AAG-induced β-gal expression in K562 cells (P < 0.05; Fig. 6D). We next determined the effect of KNK437 on 17-AAG-induced hsp70 and apoptosis. HL-60 cells were pretreated with KNK37 (100 or 400 μmol/L) for 1 hour and then cotreated with 17-AAG and KNK437 for 24 hours. Figure 7A shows that pretreatment and cotreatment with KNK437 inhibited 17-AAG-mediated hsp70 induction in HL-60 cells. Compared with treatment with KNK437 (100 or 400 μmol/L) or 17-AAG (0.5 or 2.0 μmol/L) alone, each of the combinations of treatments with KNK437 and 17-AAG, as indicated, induced significantly more apoptosis of HL-60 cells (P < 0.05; Fig. 7B). Notably, cotreatment with KNK437 and 17-AAG also significantly increased the loss of clonogenic survival of HL-60 cells compared with the treatment with either agent alone (P < 0.01; Fig. 7C).

**Discussion**

Previous reports have clearly established that stress-induced ectopic or endogenous expression of hsp70 exerts an antiapoptotic effect upstream and downstream of the mitochondria and inhibits apoptosis due to a variety of antileukemia agents (13–17, 20, 21).

![Figure 4](image4.png)  
**Figure 4.** Ectopic expression of cDNA in the reverse orientation depletes hsp70 levels and sensitizes CML-BC cells to 17-AAG-induced apoptosis. A, K562 and K562/hsp70 AS cells were treated with 17-AAG at indicated concentrations for 24 hours. Following these treatments, total cell lysates were immunoblotted with hsp70 and hsp90 antibodies. β-Actin levels were used as a loading control. B, control K562 and K562/hsp70 AS cells were treated with the indicated concentrations of 17-AAG for 48 hours. Following these treatments, the percentages of apoptotic cells were determined by Annexin V staining and flow cytometry. Points, mean of three experiments; bars, ±SE.

![Figure 5](image5.png)  
**Figure 5.** Pretreatment with 17-AAG inhibits etoposide-induced apoptosis in HL-60 cells. A, HL-60 cells were treated with 17-AAG (0.5 μmol/L) for 24 hours followed by incubation in drug-free medium for 24 hours or washed and treated with etoposide (1.0 μmol/L) for 24 hours. Drugs were also administered in the reverse sequence. Following these treatments, the levels of hsp70 were observed by Western blot analysis. β-Actin levels were used as a loading control. B, HL-60 cells were treated with 17-AAG (0.5 μmol/L) for 24 hours followed by either incubation in drug-free medium for 24 hours or washed and treated with etoposide (1.0 μmol/L) for 24 hours. Drugs were also administered in the reverse sequence. Following these treatments, the percentage of apoptotic cells was determined at 48 hours by Annexin V staining and flow cytometry. Columns, mean of three experiments; bars, ±SE.
Hsp70 has also been reported to bind AIF and abrogate the execution of the non–caspase-dependent nuclear DNA fragmentation triggered by the release of AIF from the mitochondria (18, 19). Additionally, hsp70 has been shown to promote cell survival by inhibiting lysosomal membrane permeabilization (40). Conversely, attenuation of hsp70 levels has been shown to sensitize human leukemia cells to conventional and novel antileukemia agents (e.g., etoposide, ara-C, and tumor necrosis factor [TNF]–related apoptosis-inducing ligand; ref. 7). In addition, a lack of hsp70 induction in human leukemia cells by a variety of maneuvers (e.g., siRNA to hsp70), ectopic expression of the cDNA of hsp70 in the reverse orientation or treatment with KNK437, sensitizes human leukemia cells to 17-AAG-induced Bax conformation change, mitochondrial localization, and apoptosis.

Figure 6. KNK437 inhibits transactivation of hsp70 by HSF-1 without affecting phosphorylation and nuclear localization of HSF-1. A, K562 cells were treated with KNK437 at the indicated concentrations for 24 hours. Following this treatment, total cell lysates were immunoblotted with the anti-HSF-1 antibody. β-Actin levels were used as a loading control. B, K562 cells were treated with KNK437 (400 μmol/L) for 1 hour followed by 17-AAG (5 μmol/L) plus KNK437 for 4 hours. Following this treatment, the S100 and nuclear fractions were separated and immunoblotted with anti-HSF-1 antibody. Histone H1 and β-actin levels were used as loading controls. C, K562 cells were treated with KNK437 (400 μmol/L) for 1 hour followed by 17-AAG (5 μmol/L) plus KNK437 for 4 hours. Following this treatment, cells were immunostained with anti-HSF-1 antibody then incubated in FITC-conjugated secondary antibody. Nuclear material was stained with 4',6-diamino-2-phenylindole and visualized with immunofluorescent microscopy. D, K562 cells were transfected with the hsp70 promoter/β-gal reporter construct-containing vector p173OR by nucleofection (Amaxa) and incubated at 37°C for 36 hours. Following this, cells were treated with the indicated concentrations of either KNK437 for 7 hours, 17-AAG for 6 hours, heat shock of 42°C for 1 hour, or KNK437 for 1 hour followed by KNK437 plus 17-AAG or heat shock. Columns, mean levels (of three experiments) of β-gal determined by a colorimetric assay; bars, ±SE.
without affecting 17-AAG-mediated hsp90 inhibition and attenuation of the levels of the progrowth and prosurvival proteins AKT and c-Raf.

Recent reports have shown that TNF-α-mediated activation of c-Jun NH2-terminus kinase (JNK) induces caspase-8-independent cleavage of Bid into jBid at a distinct site, which results in the translocation of jBid to the mitochondria with the selective release of Smac from the mitochondria into the cytosol (44). Although overexpression of hsp70 inhibits JNK activation and Bid cleavage, JNK inhibition was shown to be insufficient for the antia apoptotic function of hsp70 (45–47). Apoptosis signal-regulating kinase 1 (ASK1) is a serine/threonine kinase, which functions in apoptosis induced by TNF-α and agonistic antibody to Fas (48). Hsp70 was also shown to inhibit the homo-oligomerization of ASK1 and ASK1-dependent apoptosis (49). Conversely, antisense oligonucleotides to hsp70 were shown to derepress JNK- and ASK1-induced apoptosis (46, 49). However, the role of JNK and ASK1 in regulating 17-AAG-induced apoptosis is not known. Ectopic overexpression or high endogenous levels of hsp70 were also recently shown to up-regulate signal transducers and activators of transcription 5 (STAT5) and the antiapoptosis genes transactivated by it (e.g., Bcl-xL and Pim-2), thereby conferring resistance to apoptosis induced by antileukemia agents (7). Conversely, the dominant-negative STAT5 sensitized these cells, albeit not completely, to apoptosis induced by the antileukemia agents (7). These reports further highlight how induction of hsp70 could attenuate 17-AAG-mediated apoptosis in human leukemia cells through multiple mechanisms. They also show that attenuating hsp70 induction and targeting these mechanisms could potentially amplify the antileukemia activity of 17-AAG.

Presumably because in tumor cells hsp90 is more ATP bound and complexed with its cochaperones and client proteins on which the tumor cells are dependent for their growth and survival, 17-AAG has been recently shown to have a greater affinity for hsp90 from tumor versus normal cells (50). Because of a greater dependency on the progrowth and prosurvival signaling mediated by the hsp90 client proteins Bcr-Abl, FLT-3, c-Kit, AKT, and c-Raf, the leukemia progenitors may also be more vulnerable to the effects mediated by hsp90 inhibitors through the attenuation of the hsp90 client proteins, which could be another basis for the relatively selective preclinical antitumor activity of 17-AAG inhibitors. 17-AAG is currently being evaluated in phase I and II clinical trials administered alone or in combination with other anticancer agents (51, 52). It is clearly important to design combinations in which the sequence of administration of 17-AAG with other agents (e.g., etoposide and ara-C) is optimal and the combination exerts potent antileukemia effects. Indeed, our findings show that pretreatment with 17-AAG induces hsp70, which inhibits apoptosis due to etoposide and ara-C. The reversed sequence of administration of etoposide or ara-C followed by 17-AAG induces more apoptosis than either agent alone or 17-AAG followed by etoposide. It is possible that, when administered after etoposide or ara-C,
HSF-1 (58). However, clearly, cotreatment with KNK437 partially attenuates heat shock– or 17-AAG-induced hsp70 through HSEs, comprehensively reported. Our present studies show that KNK437 attenuates heat shock– or 17-AAG-induced hsp70 through HSEs, without affecting the phosphorylation and nuclear localization of HSF-1. In our studies, why heat shock is more potent than 17-AAG in inducing hsp70 may possibly be due to the distinct stimulus-specific histone modifications at the hsp70 chromatin targeted by HSF-1 (58). However, clearly, cotreatment with KNK437 partially sensitizes leukemia cells to apoptosis and loss of clonogenic survival due to treatment with 17-AAG. It is obviously important to develop more specific and potent inhibitors of hsp70 induction, which could potentially increase the sensitivity of tumor cells to 17-AAG and the other hsp90 inhibitors. One avenue would be to inhibit the serine phosphorylation of monomeric HSF-1, which may affect its nuclear localization, oligomerization, and/or transactivation of the heat shock proteins (8, 9, 22, 23). Under physiologic and stress conditions, multiple protein kinases are likely to be involved in phosphorylating HSF-1 (8, 9, 22, 23). Among these, JNK and Plk1 activities may be relevant (59, 60). Therefore, inhibitors of these kinases may undermine HSF-1-mediated transactivation of hsp70. Parenthetically, we recently reported that the endogenous or ectopic expression of Bcr-Abl in human leukemia cells leads to increased mRNA and protein levels of hsp70, and inhibition of Bcr-Abl tyrosine kinase activity with imatinib resulted in attenuation of p-HSF and hsp70 levels. However, a direct mechanistic link between Bcr-Abl and the downstream phosphatidylinositol 3-kinase/AKT to the phosphorylation of HSF-1 and induction of hsp70 in Bcr-Abl-expressing acute leukemia cells remains to be established. Collectively, whether it is through inhibition of HSF-1 phosphorylation or through attenuation of the transcriptional activation of hsp70, these hsp70-targeted strategies are likely to enhance the antileukemia effects of hsp90 inhibitors, especially where the mutant versions of the client proteins (e.g., Bcr-Abl, FLT-3, and c-Kit) are responsible for the phenotype of the leukemia (2, 61, 62).

Acknowledgments

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Abrogation of Heat Shock Protein 70 Induction as a Strategy to Increase Antileukemia Activity of Heat Shock Protein 90 Inhibitor 17-Allylamino-Demethoxy Geldanamycin

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