Induction of a Heat Shock Factor 1-dependent Stress Response Alters the Cytotoxic Activity of Hsp90-binding Agents[1]

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ABSTRACT

In addition to its classic role in the cellular stress response, heat shock protein 90 (Hsp90) plays a critical but less well appreciated role in regulating signal transduction pathways that control cell growth and survival under basal, nonstress conditions. Over the past 5 years, the antitumor antibiotics geldanamycin and radicicol have become recognized as selective Hsp90-binding agents (HBA) with a novel ability to alter the activity of many of the receptors, kinases, and transcription factors involved in these cancer-associated pathways. As a consequence of their interaction with Hsp90, however, these agents also induce a marked cellular heat shock response. To study the mechanism of this response and assess its relevance to the anticancer action of the HBA, we verified that the compounds could activate a reporter construct containing consensus binding sites for heat shock factor 1 (HSF1), the major transcriptional regulator of the vertebrate heat shock response. We then used transformed fibroblasts derived from *HSF1* knock-out mice to show that unlike conventional chemotherapeutics, HBA increased the synthesis and cellular levels of heat shock proteins in an HSF1-dependent manner. Compared with transformed fibroblasts derived from wild-type mice, *HSF1* knock-out cells were significantly more sensitive to the cytotoxic effects of HBA but not to doxorubicin or cisplatin. Consistent with these *in vitro* data, we found that systemic administration of an HBA led to marked increases in the level of Hsp72 in both normal mouse tissues and human tumor xenografts. We conclude that HBA are useful probes for studying molecular mechanisms regulating the heat shock response both in cells and in whole animals. Moreover, induction of the heat shock response by HBA will be an important consideration in the clinical application of these drugs, both in terms of modulating their cytotoxic activity as well as monitoring their biological activity in individual patients.

INTRODUCTION

The molecular chaperone Hsp[3]90 plays an essential role in stress tolerance, protein folding, and posttranslational control of the stability and function of many key regulators of cell growth, differentiation, and apoptosis (reviewed in Refs. 1 and 2). Recently, small molecule natural products have been identified that bind Hsp90 with high affinity and selectively disrupt many of its chaperone functions (3). The chemically distinct compounds RD and GA have now been shown by crystallographic (4, 5) and biochemical (6, 7) analyses to bind as nucleotide mimetics to the NH_2-terminal ATP/ADP-binding domain within Hsp90, locking the chaperone in its ADP-bound conformation and compromising its function. Clinical trials have now begun in an effort to develop HBAs as anticancer drugs based on their unique ability to inhibit the wide range of cancer-associated "client" proteins with which Hsp90 is known to associate including steroid hormone receptors (8–10), nitric oxide synthase (11), transforming tyrosine kinases (12, 13), serine/threonine kinases such as c-raf (14–16), and mutant transcription factors such as p53 (17–19).

Distinct from their inhibition of proliferation-associated signaling pathways, however, HBAs have also been shown to act as potent inducers of the cellular heat shock or stress response (20–22). Recent mechanistic work *in vitro* has demonstrated that Hsp90 forms complexes with the major transcriptional regulator of the vertebrate heat shock response, HSF1 (23, 24). The Hsp90 association with HSF1 appears to maintain HSF1 in a repressed, transcriptionally inactive form, and HBAs are thought to initiate the stress response in a novel, nonproteotoxic manner by disrupting Hsp90-HSF1 interaction (25). Whether HBA-mediated induction of a stress response in this novel way contributes to the potent cytotoxic activity of these compounds or acts in a cytoprotective fashion to limit cell damage after drug exposure is unknown. To address this issue, we used transformed fibroblasts derived from either wild-type or homozygous *HSF1* knock-out mice and performed quantitative dose-response analyses of cell proliferation/survival after exposure to HBA and conventional chemotherapeutic

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[3] The abbreviations used are: Hsp, heat shock protein; RD, radicicol; GA, geldanamycin; HBA, Hsp90-binding agent; HSF1, heat shock factor 1; 17AAG, 17-allylaminogeldanamycin; CDDP, cisplatin; EGFP, enhanced green fluorescent protein; IP, immunoprecipitation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

agents. To assess the relevance of these *in vitro* findings to the therapeutic application of HBAs, we then examined Hsp induction in tumor-bearing mice after systemic administration of an HBA. Our results indicate that induction of the heat shock response by HBAs will be an important consideration as the toxicity and activity of these drugs are explored in current and upcoming clinical trials.

MATERIALS AND METHODS

Cells and Reagents. Embryonic fibroblasts from wild-type and *HSF1* knock-out BALB/c mice were transformed with E6/E7 as described previously (26). NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C under 10% CO_2 in air using DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA), 10 mM HEPES (Life Technologies, Inc.), and 2 mM L-glutamine (Life Technologies, Inc.). The culture medium for embryonic fibroblasts was also supplemented with 0.75 mM 2-mercaptoethanol (Sigma Chemical Co, St. Louis, MO) and 10 μg/ml ciprofloxacin (Bayer Pharmaceutical, West Haven, CT). Cells were confirmed negative for *Mycoplasma* contamination by ELISA, and all experiments were performed within 10 serial passages. RD and its derivative KF58333 were supplied by the Pharmaceutical Research Institute (Kyowa Hakko Kogyo Co. Ltd., Shizuoka, Japan). GA and 17AAG were provided by the Developmental Therapeutics Program of the National Cancer Institute. All other drugs were obtained from Sigma, except as indicated, and formulated in DMSO except CDDP, which was dissolved in water. Stock solutions were maintained at −20°C in the dark until use. Mouse monoclonal antibodies to Hsp90 (AC88) and Hsp72 (C-92) were obtained from Stressgen (Victoria, British Columbia, Canada). Antibody to Hsp70 (BB70) was provided by D. Smith (Mayo Clinic, Scottsdale, AZ).

Analysis of HSF1-regulated Transcription. NIH 3T3 cells were transfected with a reporter construct encoding EGFP (Clontech, Palo Alto, CA) under the transcriptional control of a 400-bp promoter fragment derived from the *HSP70B* gene (kindly provided by T. Tsang, University of Arizona). Stable transformants were selected in G418 (geneticin, 500 μg/ml; Life Technologies, Inc.) for 3 weeks. Cells were heat shocked (42°C for 30 min) to induce EGFP expression and fluorescence activated cell sorting was performed 24 h later to isolate a population of cells displaying a robust reporter response to heat stress. To assess reporter activation by drugs, these stably transfected, sorted 3T3 cells were exposed to GA or cadmium, followed by wash-out and refeeding with drug-free medium. Cells were fixed 24 h later with 4% paraformaldehyde and viewed using a Zeiss Axiovert epifluorescence microscope and FITC filter set. Images were acquired electronically on a SenSys cooled HCCD camera (Photometrics, Tucson, AZ) and processed using Adobe Photoshop software (San Jose, CA).

Drug-mediated Hsp Induction. To examine the effects of drug treatment on cellular protein synthesis, replicate dishes of wild-type and *HSF1* knock-out fibroblasts were rinsed with methionine/cysteine-free DMEM supplemented with 10% dialyzed fetal bovine serum and incubated at 37°C for 2 h in drug-containing medium, followed by addition of [^{35}S]methionine/[^{35}S]cysteine (Translabel, 10.5 mCi/ml; ICN, Costa Mesa, CA) to yield 100 μCi/ml. Incubation was continued for an additional hour. Cell lysates were then prepared and analyzed by SDS-PAGE, as described previously (12). To examine drug effects on the synthesis of specific Hsps, IP was performed from metabolically labeled cell lysates using antibodies to Hsp90 and Hsp70, as described previously (17). To evaluate total cellular levels of specific Hsps in drug-treated cells, replicate dishes of wild-type and *HSF1* knock-out fibroblasts were lysed in nonionic detergent buffer, and immunoblotting was performed using 50 μg of total protein/sample, as described previously (17). Chemiluminescent substrate and exposure to Kodak XAR-5 film were used for detection. Multiple exposure times were evaluated for each blot to ensure that the band intensities observed were within the dynamic response range of the film.

Quantitation of Cell Survival/Proliferation. To assess the role of HSF1 in modulating drug sensitivity, a semiautomated assay of relative viable cell number based on the mitochondrial reduction of MTT (Sigma) was used as described previously (27). Briefly, *HSF1* knock-out and wild-type fibroblasts were plated in 96-well plates (2.5×10^3 cells/well) and treated 24 h after plating with varying concentrations of 17AAG, KF 58333, doxorubicin, or CDDP. After 24 h incubation at 37°C, the medium was removed, and cells washed twice with fresh medium and then incubated for an additional 48 h in drug-free medium. MTT (500 μg/ml) was then added to each well, and plates were incubated for 2 h at 37°C. Medium was removed, and DMSO (150 μl/well) was added, followed by gentle agitation for 10 min in the dark. Absorbance was determined at 540 nm, and values for drug-treated wells were compared with those for vehicle-treated control wells assayed on the same plate. All determinations were performed in triplicate, and each experiment was repeated three times. Results were calculated as a percentage of control absorbance, and dose-response curves were compared using a two-way ANOVA with $P < 0.05$ considered significant.

Heat Shock Induction *in Vivo*. SCID mice (University of Arizona Breeding Colony) were treated with 75 mg/kg 17AAG formulated in DMSO and injected i.p. daily for 2 days. Animals were sacrificed 24 h after the last drug dose, and organs were harvested. Snap-frozen brain, liver, and lung tissues were pulverized, and cytosolic extracts were prepared in hypotonic lysis buffer (pH 8.2) containing 10 mM HEPES, 1 mM EDTA, and 10 mM sodium molybdate. Lysates (50 μg/lane) were fractionated by 7.5% SDS-PAGE, and immunoblotting was performed using anti-Hsp72 primary antibody. Additional SCID mice were inoculated s.c. with MCF7 human breast cancer cells, as described previously (28). Tumor-bearing mice received i.p. injections of 17AAG at dose levels of either 50 or 100 mg/kg daily for 4 consecutive days. Control mice received i.p. injections of an equal volume of DMSO. Mice were sacrificed, tumors were resected 18 h after final drug injection, and tumors were analyzed for Hsp72 levels as above. All experiments involving mice were carried out under protocols approved by the University of Arizona Institutional Animal Care and Use Committee.

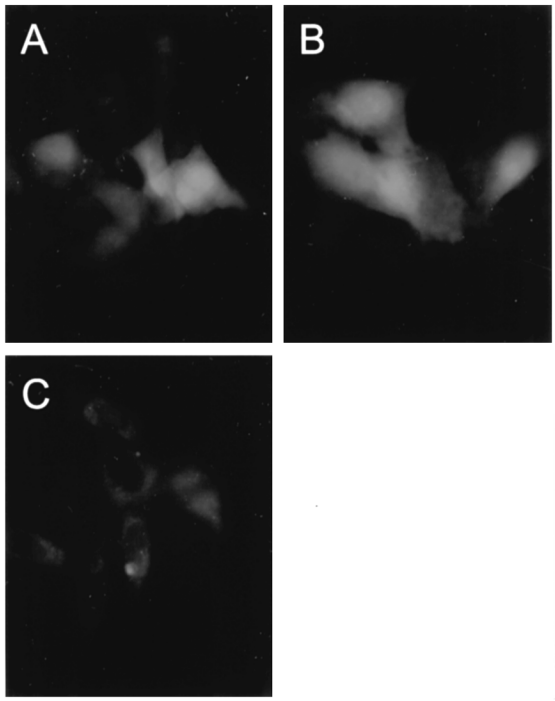

Fig. 1 GA induces transcriptional activation of a heat shock element. NIH3T3 cells stably transfected with a reporter construct encoding EGFP under the transcriptional control of promoter elements from the *Hsp70B* gene were exposed to GA (1.8 μM for 30 min; *A*), cadmium chloride (100 μM for 60 min; *B*), or drug diluent only (*C*). Cells were fixed and examined by fluorescence microscopy 24 h later. All images were acquired using the same magnification and exposure conditions.

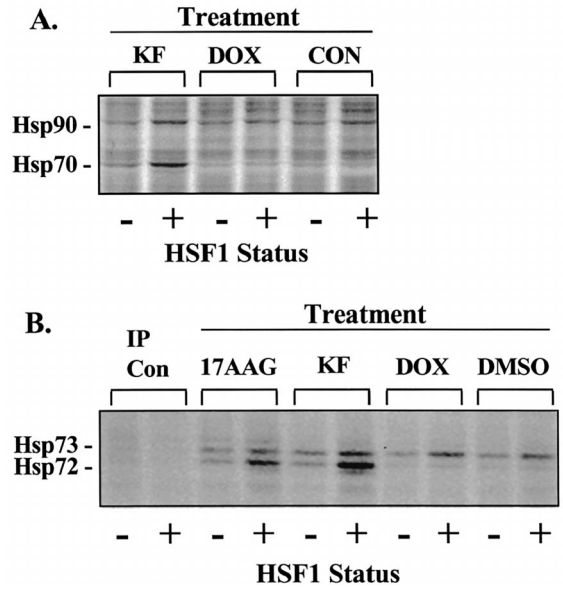

Fig. 2 HBA treatment increases the rate of synthesis of inducible Hsps. Wild-type and *HSF1* knock-out fibroblasts were treated with 17AAG, KF 58333 (*KF*), doxorubicin (*DOX*) or an equal volume of DMSO (*CON*) for 2 h, followed by metabolic labeling with [^{35}S]methionine/[^{35}S]cysteine. After extensive washing, lysates were prepared in nonionic detergent buffer. The HSF1 status of the cells from which each lysate was prepared is indicated below the respective lane. *A*, equal amounts of total radioactivity were fractionated by SDS-PAGE, and proteins were visualized by autoradiography. *B*, IP was performed from metabolically labeled lysates using equal amounts of total radioactivity and anti-Hsp70 antibody (BB70) or mouse IgG (*IP Con*). Precipitates were fractionated by SDS-PAGE, and proteins visualized by autoradiography.

RESULTS

HBAs Transcriptionally Activate a Heat Shock Element. We used NIH3T3 cells stably transfected with an expression vector encoding a heat-inducible EGFP reporter to examine the effect of GA on HSE-controlled gene expression in mammalian cells. Stimulation of a stress response using the conventional proteotoxic agent cadmium chloride led to detection of very strong green signal by fluorescence microscopy (Fig. 1*B*). A strong but somewhat less intense signal was observed when cells were exposed briefly to GA (Fig. 1*A*), indicating that HBA treatment transcriptionally activated the reporter construct. In comparison, cells treated only with drug diluent demonstrate minimal signal (Fig. 1*C*), consistent with autofluorescence.

HSF1 Mediates the HBA-induced Heat Shock Response in Cells. To examine the role of HSF1 in regulating the heat shock response associated with drug treatment of tumor cells, mouse fibroblasts were stably transformed with the E6 and E7 proteins of human papillomavirus. Cell lines were derived from either wild-type BALB/c embryos (*HSF1*+/+) or homozygous *HSF1* knock-out embryos (*HSF1*−/−). They displayed equivalent plating efficiencies and growth kinetics (doubling time of 20 h) under basal conditions. Metabolic labeling was used to assess the effects of HBAs on the rate of Hsp synthesis in these wild-type and knock-out fibroblasts. For greater clinical relevance, data are presented using derivatives of RD (KF58333) and GA (17AAG), which have been developed for clinical application. Similar results were obtained with the parent compounds (not shown). In the experiment depicted in Fig. 2*A*, cells were exposed to KF58333 or the conventional DNA-damaging agent doxorubicin. Autoradiography of total lysate prepared from wild-type cells treated with KF58333 demonstrated a clear increase in the levels of newly synthesized proteins with apparent molecular sizes of 90 and 70 M_r, presumably representing Hsp90 and Hsp70 isoforms. No such increases were seen with doxorubicin, and no increases were seen in *HSF*−/− cells treated with either agent. Immunoprecipitations from radiolabeled lysates with anti-Hsp70 antibody were performed to verify the identity of the 70 kDa bands seen in Fig. 2*A* as Hsp70 isoforms. Two bands are evident in these precipitations, one representing Hsp73 (also known as Hsc70), the major constitutive Hsp70 isoform and the other Hsp72, a highly inducible isoform that is expressed at very low levels under nonstress conditions in most tissues (Fig. 2*B*). Little variation was seen in Hsp73 signal between samples, but both KF58333 and 17AAG elicited a marked increase in the level of Hsp72 in cells with normal HSF1 function. Isogenic cells without HSF1 displayed minimal increases. Consistent with total lysate data presented in Fig. 2*A*, increased Hsp72 synthesis was not evident in precipitations using lysate from either wild-type or knock-out cells treated with doxorubicin or DMSO vehicle. The absence of signal in the control IP lanes

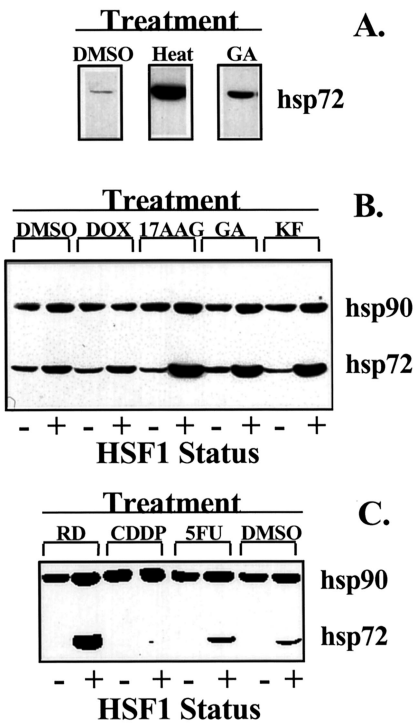

Fig. 3 HBAs increase total cellular levels of inducible Hsps. *A*, wild-type embryonic fibroblasts were exposed to either heat shock (42°C × 30 min), GA (1 μM), or drug diluent (DMSO). Drug exposure was 24 h in duration. Cells were then lysed, and Hsp72 levels were evaluated by immunoblotting. *B* and *C*, HSF1(+) and HSF1(−) fibroblasts were treated with either doxorubicin (*DOX*, 2 μM), 17AAG (1 μM), GA (1 μM), KF58333 (*KF*, 1 μM), RD (1 μM), CDDP (1 μM), 5-fluorouracil (*5FU*, 5 μg/ml), or an equal volume of DMSO vehicle. Twenty-four h later, lysates were made, and the levels of Hsp72 and Hsp90 were evaluated by immunoblotting.

confirms the specificity of the immunoprecipitation conditions used.

To determine whether increased synthesis of Hsp72 and Hsp90 after exposure to HBAs led to increased cellular levels of these proteins, we used SDS-PAGE followed by Western blotting. In Fig. 3*A*, wild-type cells were exposed to GA or incubated at 42°C for 30 min, and lysates were prepared 24 h later to compare the Hsp levels induced by these two stimuli. *HSF1* knock-out cells were not examined because, as reported previously (26), these cells fail to induce Hsp synthesis, even after incubation at 43°C for 30 min. Although exposure of wild-type cells to GA resulted in substantial Hsp72 induction compared with control, we found that the level of detectable Hsp72 was considerably greater after moderate heat shock. We next prepared lysates from wild-type and knock-out cells 18 h after treatment with several HBAs and conventional chemotherapeutic agents. Small but consistent differences in the levels of Hsp72 and Hsp90 were observed between the two cell types under control conditions (DMSO vehicle alone), perhaps reflecting a role for HSF1 in regulating basal Hsp expression or a response of cells with intact HSF1 function to the mild stresses inherent in cell culture (Fig. 3, *B* and *C*). After exposure to 17AAG, RD, and KF58333, a marked increase in Hsp72 level and a less apparent increase in Hsp90 level were seen in wild-type cells but not in *HSF1* knock-out cells. No increase in Hsp72 levels over their respective control levels were seen in either cell type, however, after exposure to conventional cytotoxic agents. Neither an intercalator/topoisomerase inhibitor (doxorubicin), nor an alkylator (CDDP), nor an antimetabolite (5-fluorouracil) caused detectable increases in Hsp levels in these cells under conditions demonstrated by quantitative dose-response analysis to reduce proliferation/survival by at least 50% (see below; Fig. 4, *C* and *D*). These data demonstrate that the ability of HBAs to induce a heat shock response requires HSF1 function, and that induction of this response is a biological property of these agents distinct from that of DNA-targeted chemotherapeutics.

Heat Shock Induction by HBAs Is Cytoprotective. Having shown a clear difference in the ability of wild-type and *HSF1* knock-out cells to mount a stress response after exposure to HBAs, we examined whether wild-type cells were more or less sensitive to the cytotoxic activity of these drugs. Cell proliferation and survival were quantitated by MTT assay for two clinically relevant HBAs as well as two mechanistically distinct conventional chemotherapeutics. Absorbance as an indicator of viable cell number was measured for drug-treated cells and compared with that of control cells grown in the same plate but treated with vehicle alone. For the experiments presented in Fig. 4, dose-response data were obtained two days after a 24-h exposure to various drug concentrations. Knock-out cells were significantly more sensitive to 17AAG (Fig. 4*A*) and KF58333 (*B*) than their wild-type counterparts, as determined by two-way ANOVA ($P < 0.0001$). In contrast, no statistically significant difference was observed between the two cell types when treated with CDDP (Fig. 4*C*; $P > 0.6$) and doxorubicin (Fig. 4*D*; $P > 0.37$).

HBA Treatment Induces a Heat Shock Response *in Vivo*. To examine the clinical implications of our *in vitro* findings, we assessed the effects of systemic 17AAG exposure on normal tissues and tumor xenografts in SCID mice. After IP administration of 17AAG or an equal volume of DMSO to non-tumor-bearing mice, organs were harvested. Elevated levels of Hsp72 were found in liver and lung from animals treated with 17AAG, as shown in Fig. 5*A*. Surprisingly, brain tissue from animals treated with the HBAs showed no increase in Hsp72 level. We also assessed heat shock induction by 17AAG in established human breast tumor xenografts. Systemic drug treatment of tumor-bearing mice at a well-tolerated dose induced a readily detected increase in tumor hsp72 level compared with that seen in tumors from DMSO-treated control mice (Fig. 5*B*).

DISCUSSION

Recently, microbial fermentation products have been identified that bind Hsp90 with high affinity and selectively alter its function. These HBAs have proven useful in defining a long controversial role for ATP in Hsp90 chaperone activity (4, 7). They have also established a role for Hsp90 in regulating the function, stability, and degradation of multiple signal transduction molecules relevant to oncogenic transformation (29). We now report the use of HBAs and *HSF-1* knock-out cells to clarify the mechanism by which Hsp90 regulates both its own expression and that of other stress-inducible chaperones. Control of chaperone protein expression in vertebrate animals is

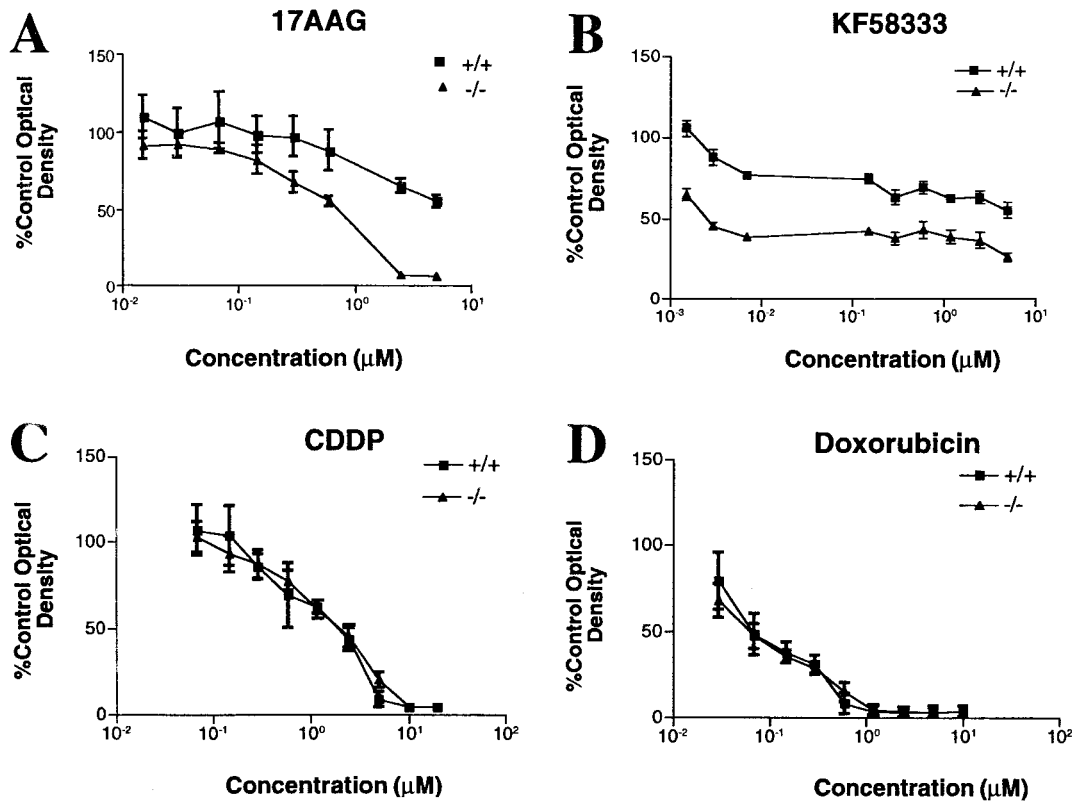

Fig. 4 Cytotoxic activity of HBA is greater in *HSF1*(−/−) cells than in *HSF1*(+/+) cells. Wild-type and *HSF1* knock-out fibroblasts were exposed to various concentrations of 17AAG (*A*), KF58333 (*B*), CDDP (*C*), or doxorubicin (*D*) for 24 h. Cells were washed and reincubated for an additional 48 h with drug-free medium. Cell survival/proliferation was assessed by MTT assay. Absorbance values are expressed as a percentage of diluent-treated cells. All determinations were performed in triplicate, and experiments were repeated three times. The means are depicted; *bars*, SE.

complex. At least four distinct HSFs have been identified that bind consensus sequences within the promoter elements of major heat shock genes, but HSF1 appears to be the most important in terms of regulating initial responses to heat and other stressors (reviewed in Ref. 30). Although much remains to be learned about how HSF1 functions, a picture is emerging in which the protein resides as an inactive monomer in the cytoplasm of unstressed cells. These monomers are held inactive in complexes with Hsp90 and possibly additional molecular chaperones (25). Nonnative proteins resulting from heat stress or other proteotoxic insult are thought to compete with HSF1 for binding to Hsp90, thus leading to the appearance of unbound HSF1 monomer that is free to trimerize, translocate to the nucleus, undergo phosphorylation, and activate gene expression (31). Experiments in reticulocyte lysate using HBAs have lent support to this model by demonstrating that HBA interaction with Hsp90 stimulates HSF1 trimerization and sequence-specific DNA binding (21, 25). Although DNA binding is clearly necessary for HSF1 to activate gene expression, it is not sufficient. Experiments with nonsteroidal anti-inflammatory drugs, such as indomethacin and salicylate, have shown that these drugs stimulate trimer formation and DNA binding but fail to activate gene expression (32). As a result, they augment heat shock responses, but in the absence of other stimuli, they fail to induce expression on their own. To address this issue in regard to the HBAs, we examined the ability of the drugs to induce heat shock-regulated gene expression at the transcriptional level using a reporter construct (Fig. 2) and at the translational level using [S^{35}]methionine labeling (Fig. 3) and Western blotting (Fig. 4). Consistent with previous reports using herbimycin A (21, 22) but in contrast to a recent report by Ali *et al.* (33), we found that HBA exposure resulted in robust induction of Hsp expression. Ali *et al.* (33) made use of *Xenopus* oocytes into which a heat-inducible reporter construct was microinjected. Whether the discrepancy between their results and ours reflects a fundamental difference in heat shock regulation between frogs and mammals or a technical difference in the reporter constructs and transfection techniques used is not clear.

Our findings indicate that exposure of cells to concentrations of HBAs that cause changes in survival and proliferation do not induce Hsp expression to the same extent as that seen in cells exposed to heat or heavy metals. Compared with HBAs, these relatively nonspecific stressors may stimulate more robust responses because they activate not only HSF-1 but additional cofactors such as HSF-2 (30). In addition, these physical agents have been shown to affect attenuators of the heat shock response such as Hsp70-family chaperones, which may allow them to generate a more prolonged, exaggerated response than that seen with the HBAs, which act only on Hsp90 (34). Alternatively, HBAs are known to inhibit the activity of multiple kinases involved in signal

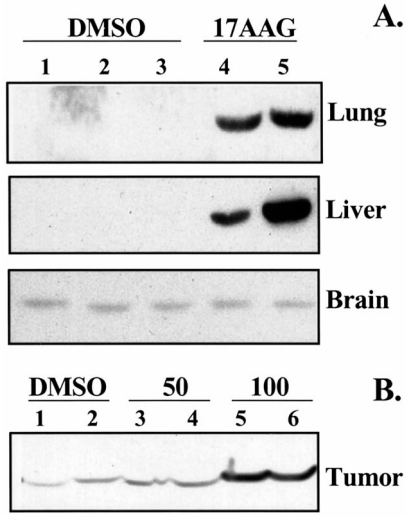

Fig. 5 *A*, severe combined immunodeficient mice were treated i.p. with DMSO drug diluent (*Lanes 1–3*) or with 75 mg/kg 17AAG (*Lanes 4* and *5*) daily for two doses. Brain, liver, and lung extracts were prepared from independent animals in hypotonic lysis buffer. Hsp72 levels in equal amounts of total protein were evaluated by immunoblotting. *B*, SCID mice bearing MCF-7 xenografts were treated i.p. with 17AAG (50 or 100 mg/kg, as indicated *above* the lanes) or an equal volume of DMSO daily for 4 days. Tumors from two independent mice per treatment group were resected, and extracts were analyzed by Western blotting for Hsp72 level as in (*A*).

transduction. Perhaps exposure to HBAs stimulates HSF1 trimer formation but also impairs the activity of the as yet unidentified kinase(s) that are required to inducibly phosphorylate HSF1 and render it active. Additional work comparing the effects of heat, nonsteroidal anti-inflammatory drugs, and HBAs on HSF1 phosphorylation may prove useful in identifying the kinase(s) involved in regulating HSF1 function.

Mechanistically, it is perhaps not surprising that *HSF1* knock-out cells were more sensitive to the cytotoxic action of HBA than wild-type cells. Hsp90 function is known to be essential for survival in eukaryotes (35). The ability of wild-type cells to increase Hsp90 levels probably allowed them to restore normal Hsp90 function more effectively than knock-out cells after drug exposure, thus leading to their enhanced survival. The ability of normal cells and tissues to up-regulate Hsp90 levels in the face of HBA exposure may explain why the compounds are less toxic than might be expected (given the essential nature of Hsp90 function), and this ability suggests that at noncytotoxic doses, the compounds may prove useful as "biological response modifiers" for therapeutic manipulation of the stress response in diseases involving processes such as inflammation or ischemia. It has been suggested that the benzoquinone ansamycins may not act primarily through modulating Hsp90 function but rather by inducing more general oxidative damage (36) or alkylating target proteins such as src kinase (37, 38). Our data argue strongly against these possibilities: (*a*) we found that RD and its derivative KF58333 induce a heat shock response, and yet these compounds lack the quinone ring responsible for proposed free radical formation (see Ref. 4 for structures); (*b*) HSF1-deficient cells were more sensitive to HBAs than wild-type cells, but no such difference was seen with another redox active, quinone-containing agent, doxorubicin, which does not interact with Hsp90.

Increased levels of certain Hsps have been reported to confer drug resistance to cancer cells (39, 40). Whether the heat shock response, *per se*, is cytoprotective in the face of exposure to conventional genotoxic chemotherapeutics has remained unclear. Our data with transformed cells in which the heat shock response has been disabled indicate that this response does not play a major role in modulating the cytotoxicity of several distinct classes of chemotherapeutic agents. Although there are obvious limitations to an *in vitro* model involving rodent cells, the cell lines used in the experiments reported here were transformed with E6 and E7, rendering them functionally p53 and Rb deficient, as is the case with many human cancers. Our findings have several other important implications for the clinical application of HBA as anticancer drugs: (*a*) given the cytoprotective effect of the heat shock response in cells exposed to HBA (Fig. 4), it may be important to administer these agents in a pulsed fashion, with a sufficient interval between exposures to allow drug-induced heat shock responses to extinguish; (*b*) we did not detect an increase in Hsp72 levels in brain tissue after administration of 17AAG (Fig. 5) or KF58333 (not shown). Either these drugs do not penetrate the blood brain barrier or as suggested by others, induction of the heat shock response is regulated differently in neurons compared with other cells (41). If the latter is the case, neurotoxicity may be an important potential complication when administering these agents to patients; and (*c*) the finding that HBAs induce a measurable change in cellular Hsp levels suggests that this biological response will prove a useful pharmacodynamic end point for therapeutic monitoring *in vivo*. In fact, measurement of Hsp72 levels in tumor tissue and peripheral blood lymphocytes has been incorporated into the design of the Phase I clinical trials of 17AAG that have now begun in patients with refractory malignancies.

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