HSP27 Knockdown Produces Synergistic Induction of Apoptosis by HSP90 and Kinase Inhibitors in Glioblastoma Multiforme

LOUIZA BELKACEMI and MATTHEW O. HEBB

Department of Clinical Neurological Sciences, University of Western Ontario, London, ON, Canada

Abstract. *Background/Aim: The heat-shock proteins HSP27 and HSP90 perpetuate the malignant nature of glioblastoma multiforme (GBM) and offer promise as targets for novel cancer therapeutics. The present study sought to define synergistic antitumor benefits of concurrent HSP27-knockdown and the HSP90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) or, comparatively, the non-selective kinase inhibitor, staurosporine, in GBM cells. Materials and Methods: Dose–response relations were determined for 17-AAG and staurosporine in three GBM cell lines. HSP27-targeted siRNA was administered alone or in combination with subtherapeutic concentrations of each drug and cells were evaluated for viability, proliferation and apoptosis. Results: Adjuvant HSP27 knockdown with 17-AAG or staurosporine produced marked and synergistic decrease in GBM cell viability and proliferation, with robust elevation of apoptotic fractions and caspase-3 activation. Conclusion: HSP27 knockdown confers potent chemosensitization of GBM cells. These novel data support the development of HSP-targeting strategies and, specifically, anti-HSP27 agents for the treatment of GBM.*

Glioblastoma multiforme (GBM) accounts for up to 15% of all intracranial neoplasms and is the most common and malignant primary central nervous system (CNS) tumor in adults. The lesion incites robust neovascularization however, the blood supply becomes overwhelmed by growth and metabolic demand, producing hypoxia and acidosis within and surrounding the lesion. Despite these hostile conditions, GBM cells are highly proliferative and invasive, with frequent extension through multiple cerebral lobes and across midline commissures. The current standards-of-care entail maximal safe resection followed by adjuvant chemoradiation and afford a median survival of only 12-18 months (1, 2).

Heat-shock proteins (HSPs) are highly-conserved stress proteins that are categorized by molecular weight (*e.g.*, HSP110, HSP90, HSP70, HSP60, HSP40, small HSPs) and play potent cytoprotective roles in normal and neoplastic cells (3, 4). HSPs are a major contributor to the recalcitrant nature of various types of cancer and pharmacological inhibitors that impede expression or function offer tremendous potential as novel cancer therapeutics (5-7). The genetic and metabolic aberrations in GBM incite high levels of HSPs, including HSP27 and HSP90, that support rampant growth and treatment resistance (8-10). HSP90 inhibitors such as the ansamycin derivatives, 17-N-allylamino-17-demethoxy-geldanamycin (17-AAG) and others, as well as new small-molecule inhibitors, are some of the best characterized and most promising anti-HSP agents available. These drugs interfere with tumor growth by disrupting the HSP90 chaperoning cycle and promoting degradation of client oncoproteins involved in cancer cell dysregulation (11-15). Several HSP90 inhibitors have robust anticancer effects in pre-clinical models and have been, or are currently being, investigated in clinical trials of various somatic cancers (*e.g.* breast, lung, ovarian, prostate, and renal). Unfortunately, these agents have shown only modest clinical efficacy in humans, with the suboptimal results attributed to dose and bioavailability limitations, off-target effects and variable tumor dependence on HSP90 client proteins (16, 17). The main driver of human HSP expression is the transcription factor, heat-shock factor-1 (HSF1), which is also a client protein and stabilized in inactive form by HSP90. Because of this relationship, many HSP90 inhibitors produce an elevation in active HSF1 and induction of multiple HSPs that support cancer cell viability and detract from the therapeutic benefit (18). The challenges associated with HSP90 inhibition have led to the search for alternative or complementary HSP targets to elicit more effective tumor control.

We propose that HSP27 offers an effective substrate for novel molecular or gene-targeting strategies in GBM. The *HSP27* gene is regulated by various promoter motifs,

Correspondence to: Matthew O. Hebb, MD, Ph.D., FRCSC, Department of Clinical Neurological Sciences, University of Western Ontario, 339 Windermere Road, C7-134, London, ON N6A 5A5, Canada. Tel: +1 5196632981, Fax: +1 5196633130, e-mail: mhebb@uwo.ca

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including two heat-shock elements that bind HSF1 (19, 20). Its protein product is distinguished by a low molecular weight and an alpha-crystallin domain that facilitates oligomerization. HSP27 confers cytoprotection against physiological stressors, including hypoxia, hyperthermia, chemical toxins, among others, and may also alter the production of survival and apoptosis proteins at transcriptional or translational levels. HSP27 binds other stress proteins to form oligomeric complexes that inhibit aggregation of denatured peptides (21-24). Stress-activated kinases, such as mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKT, are overactive in GBM and phosphorylate HSP27 to produce changes in isoform and function. Serine phosphorylation in the amino terminus alters the quaternary structure of HSP27 and stimulates oligomer disassembly and the generation of phosphorylated monomers and dimers that inhibit cell death processes (25). The protective action of HSP27 is conferred, in part, by a filament-capping function that stabilizes cell structure and attenuates release of pro-apoptotic factors such as cytochrome *c* and procaspases. This influence is integral to lamellopodia repair and motility of normal cells but also drives migration and the invasive properties of cancer cells, including those in GBM (26-30).

HSP27 is overexpressed in and contributes to the malignant character of numerous types of cancers (31-36). Pre-clinical and patient studies demonstrated that its targeted knockdown reduces proliferation and increases the susceptibility of somatic tumor cells to chemotherapy and radiation (37-45). These data have led to development of anti-HSP27 strategies in clinical trials for prostate, ovarian, lung, breast and bladder cancer (46-49). There has been notably less progress for cancer of the brain and, although the level of HSP27 expression has been shown to correlate with the histological grade of gliomas, the potential for HSP27 as a therapeutic target in GBM has not been defined (50, 51). Drugs that interfere with HSP27 activity are not expected to directly stimulate HSP expression and may provide efficacious stand-alone therapy or potentiate the benefits of HSP90 inhibitors or other chemoradiation options. The objectives of this study were to evaluate the potential for synergistic tumoristatic and tumoricidal actions of HSP27 and HSP90 targeting and to define the mechanism of cell death produced by HSP inhibition in GBM.

Materials and Methods

Cell maintenance and transfection. Human LN229, U87 (American Type Culture Collection, Manassas, VA, USA), and U251 (a kind gift from Dr. Voon Wee Yong, University of Calgary, AB, Canada) GBM cell lines were maintained in Dulbecco's modified Eagle's medium (Life Technologies Inc., Burlington, ON, Canada) supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere of 5% CO_2. The cells were passaged every 2-3 days with 0.25% trypsin-EDTA (Wisent Inc., St-Bruno, QC, Canada). At the exponential phase of growth, cells were seeded in either 6-well or 96-well plates (Sarstadt, Montreal, QC, Canada) in maintenance medium for 24 h prior to treatment.

Cells were transfected with siRNA targeting human *HSP27* mRNA (HSP27i; 50 nM; Sigma-Aldrich Canada Co., Oakville, ON, Canada) or an equivalent concentration of a non-specific Control Oligomer (siRNA Universal Negative Control; Sigma-Aldrich Canada Co., Oakville, ON, Canada) using jetPRIME™ transfection reagent (Polyplus Transfection, Illkirch, France, EU), according to the manufacturer's instructions. Briefly, LN229, U87 and U251 cells (1.5×10^5 cells/ml) were plated into 6-well plates in 2 ml of maintenance medium. The following day, the culture medium was replaced with 210 μl of jetPRIME-siRNA complex with 2 ml maintenance medium. The transfected cells were incubated at 37˚C in humidified air with 5% CO_2 for 24 hours, after which the cells were returned to maintenance medium. Forty-eight hours following transfection, the extent of HSP27 knockdown was assessed by immunocytochemistry and western blot analysis.

Evaluation of HSP knockdown. HSP27 and HSP90 immunocytochemistry and western blot analysis were performed two days following transfection. HSP90 analysis was included to ensure neither off-target suppression nor compensatory increase in HSP90 expression due to HSP27i administration. Cells (1.5×10^5 cells/ml) were washed with ice-cold phosphate buffered saline (PBS) and sequentially incubated in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 4 minutes on ice. Cells were then washed four times for 5 minutes each in PBS containing 0.3% Triton X-100 (PBS-T). Non-specific binding was blocked in PBS-T containing 5% albumin (BioShop Canada Inc. Burlington, ON, Canada) for 1 h at room temperature. Subsequently, cells were incubated with primary rabbit polyclonal antibodies to human HSP27 (1:200) or HSP90 (1:100; Millipore, Etobicoke , ON, Canada) overnight at 4˚C, followed by three washes of 10 min in PBS-T. An Alexa Fluor® 546-conjugated goat anti-rabbit secondary antibody (1:200; Life Technologies Inc., Burlington, ON, Canada) was subsequently used to visualize the antigen. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies Inc., Burlington, ON, Canada).

Chemotherapeutic agents and cell viability assay. The experimental chemotoxins included the HSP90 inhibitor, 17-AAG, and protein kinase inhibitor, staurosporine (STS; Sigma-Aldrich Canada Co., Oakville, ON, Canada). 17-AAG is representative of the benzoquinone ansamycin class of antibiotics currently being widely explored as cancer therapeutics (12, 13, 52). STS was evaluated in parallel with the HSP90 studies to determine whether pharmacological synergy by adjuvant HSP27 inhibitor could be conferred to agents that do not directly impact HSP activity. STS has a broad selectivity and toxicity profile that restrict its use to research applications, however, numerous STS analogs have been developed and are in clinical trials to treat various types of cancer, including GBM (53, 54). All drugs were dissolved in dimethyl sulfoxide (DMSO) and each experimental series included control cultures exposed to DMSO concentrations equivalent to those used in the corresponding drug treatment groups. LN229, U87 and U251 GBM cells (2.5×10^3 cells/well) were seeded in 96-well plates for 48 hours prior to being exposed to a panel of escalating doses of 17-AAG or STS for 24 h, after which cell viability was evaluated using the 3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) spectral analysis. This colorimetric assay measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase to an insoluble, dark purple formosan product. Twenty microliters of MTT dye (5 mg/ml) were added to each well of a 96-well plate and incubated for 3 hours at 37˚C in a humidified atmosphere with 5% CO_2. The cells were then lysed to release the purple formosan product by the addition of 150 µl DMSO for 15 minutes at room temperature. Absorbance was measured using an enzyme-linked immunosorbent assay plate reader (Fisher Scientific, Nepean, ON, Canada). Cell viability was estimated using optical density values at 570 nm with references at 655 nm detected in each well.

Dose–response studies were conducted with both 17-AAG and STS in all GBM lines by defining the relative cell viability following treatment with escalating concentrations of each agent. To evaluate the cytotoxic properties of HSP27i alone and in combination with the chemotherapeutic agents, LN229, U87, and U251 cells (2.5×10^3 cells/well) were transfected with HSP27i or the Control Oligomer in 96-well plates for 24 h, after which they were exposed to 17-AAG or STS for a further 24 h. The dose used for each agent and cell line was determined from the dose–response studies as the highest concentration that did not significantly reduce cell viability. MTT spectrophotometric values of the treated cultures were normalized to the mean spectrophotometric value of the untreated cultures to generate relative viability fractions for each treatment group.

Ki-67 immunochemical staining. The Ki-67 index was used to compare proliferation between GBM control and treatment groups. U251 cells (2.5×10^3), transfected with either HSP27i or an equal concentration of Control Oligomer, were grown in 96-well plates for 24 hours before being subjected to either 17-AAG or STS treatment for a further 24 hours. The cells were then fixed in 4% paraformaldehyde in 0.01M PBS (pH 7.4) for 20 min. Following three 10-minute washes in PBS, endogenous peroxidases were quenched using 0.3% hydrogen peroxide in methanol for 10 minutes. Cells were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate buffer (pH 6.0) on ice, then incubated overnight with a mouse monoclonal antibody to Ki-67 (1:200; Millipore, Etobicoke , ON, Canada). The next day, the cells were washed three times for 10 minutes then incubated for 1 hour with a horseradish peroxidase-conjugated anti-mouse secondary antibody (1:200; Life Technologies Inc., Burlington, ON, Canada). Colorimetric detection was achieved using a mixture of 50 µl 3-3'-diaminobenzidine tetrahydrochloride (DAB;Sigma-Aldrich Canada Co., Oakville, ON, Canada). Ki-67 experiments were run in triplicates.

Apoptosis evaluation. Apoptosis was detected and quantified using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), and immunocytochemical and western blot analysis of activated caspase-3 in U251 GBM cells. Cells were grown on coverslips in 6-well plates (1.5×10^5 cells per well) or directly in 96-well plates (2.5×10^3 cells per well). Naive, transfected (*i.e.* with HSP27i or Control Oligomer) and combined transfected plus drug (*i.e.* 17-AAG or STS) treatment groups, along with appropriate control cell groups were processed, as described above. TUNEL analysis was performed with a standard cell death detection kit according to the manufacturer's guidelines (Roche Diagnostics, Laval, QC, Canada). After fixation and permeabilization, cells were incubated in a TUNEL reaction mixture for 1 hour at 37˚C, washed with PBS, and reacted with 50 µl Converter-POD solution prior to incubation with a rabbit polyclonal antibody to detect the 17 kDa/19 kDa cleaved (activated) caspase-3 (Cell Signaling Technologies, Danvers, MA, USA) at 4˚C overnight followed by the addition of Alexa Fluor® 633-conjugated anti-rabbit secondary antibody (1:200; Life Technologies Inc., Burlington, ON, Canada). Cell nuclei were counterstained with DAPI (Life Technologies Inc., Burlington, ON, Canada). TUNEL and activated caspase-3 labeling of cells on coverslips were visualized by confocal microscopy. Cells in 96-well plates incubated with the TUNEL mixture were reacted with a combination of 50 µl DAB as a chromogen and hydrogen peroxide as substrate for horseradish peroxidase (Sigma-Aldrich Canada Co., Oakville, ON, Canada) for 5 min and observed with brightfield microscopy. Cells exposed to TUNEL reaction without terminal transferase enzyme, or with PBS instead of activated caspase-3 antibody served as negative controls. All experiments were run in triplicate.

Microscopic observations and cell counts. Digital images of Ki-67 and TUNEL DAB labeling were captured using a Motic AE31 inverted microscope fitted with an Infinity1-3 scientific complementary metal-oxide semiconductor camera (Lumenera Corp., Ottawa, ON, Canada) at a magnification of ×20. Labeling was quantified in five randomly selected fields, providing a minimum of 1000 cells per treatment group, using the National Institute of Health open-source ImageJ software (http://rsb.info.nih.gov/ij/). Results were expressed as the percentage of Ki-67- or TUNEL-positive cells per total number of cells. High definition images of other immunocytochemical labeling were obtained using a Zeiss LSM-510 META laser-scanning microscope with a Zeiss ×63 NA 1.4 oil immersion lens, suitable filters and AIM software (Carl Zeiss GmbH, Jena, Germany, EU).

Western blot analysis. Forty-eight hours after transfection, LN229, U87 and U251 cells were washed twice with PBS, collected in lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P40, pH 7.4) supplemented with SIGMA FAST™ protease inhibitor cocktail (1:10; Sigma-Aldrich Canada Co., Oakville, ON, Canada), and incubated on ice for 15 min, after which cells were sonicated. The protein lysates were then centrifuged at 12,000 × *g* for 15 min and supernatant collected. Protein concentrations were determined using a DC™ Protein Assay kit (BioRad, Mississauga, ON, Canada) and all fractions were frozen at −80˚C until use. Ten to 20 micrograms of each protein extract were separated on a 12% sodium dodecyl sulphate polyacrylamide gel and transferred electrophoretically to Immobilon-P membranes (BioRad, Mississauga, ON, Canada). Non-specific binding was blocked in 5% powdered non-fat milk in Tris-buffered saline with 0.01% Tween-20 (TBS-T) at room temperature for 1 hour. The membranes were then incubated overnight at 4˚C in the same solution containing a primary antibody to either HSP27 (1:2,000), HSP90 (1:1,000; Millipore, Etobicoke , ON, Canada) or activated caspase-3 (1:2,000; Cell Signaling Technologies, Danvers, MA, USA). After washing in TBS-T, membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:3,000; BioRad, Mississauga, ON, Canada) for 1 h at room temperature. To ensure equal loading, protein blots were stripped and re-probed for β-actin (1:10,000; Abcam, Toronto, ON, Canada). Peroxidase activity was visualized using enhanced chemiluminescence and detection system imager (Alpha Innotech Corporation, San Leandro, CA, USA). The scanned membranes were then subject to densitometric analysis using the manufacturer's software. Treatment and control conditions were independently evaluated in quadruplicate for these studies.

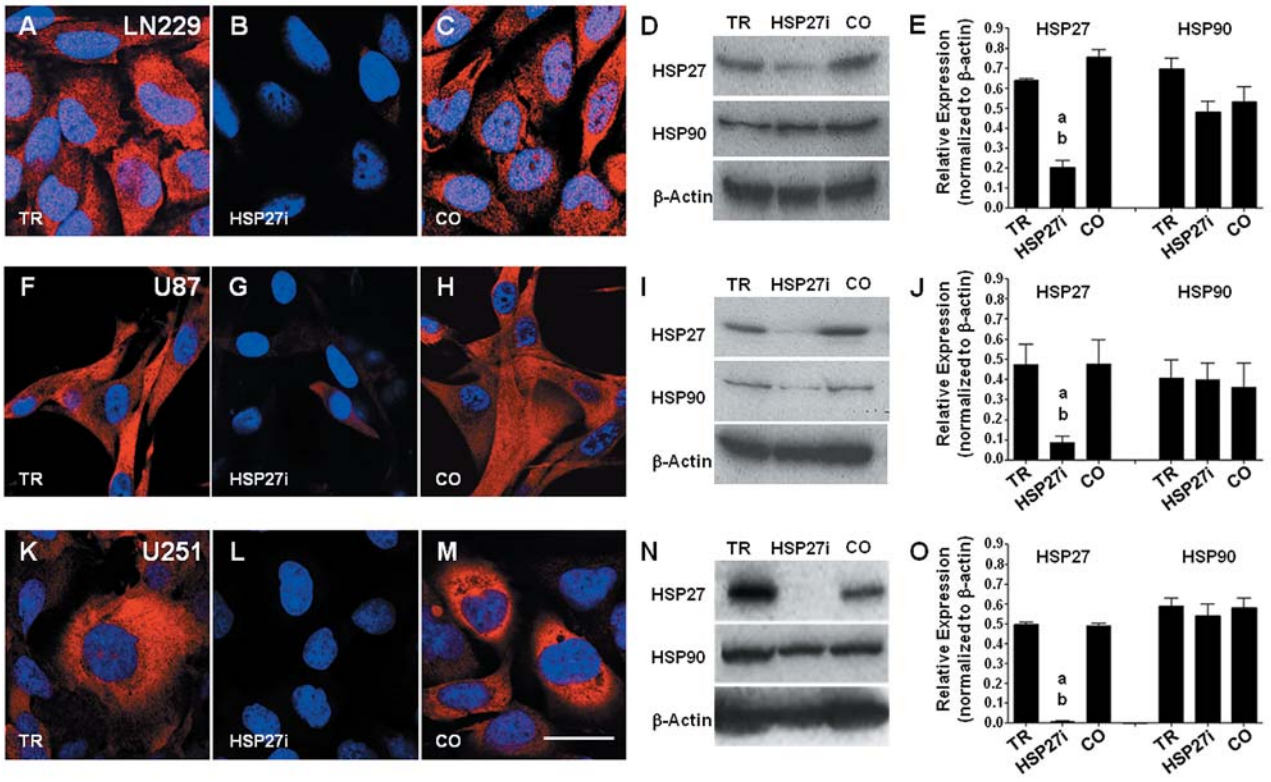

Figure 1. *Heat shock protein-27 (HSP27) knockdown in glioblastoma multiforme (GBM) cells. Immunocytochemistry and western blot analysis was used to evaluate HSP27 expression in LN229 (A-E), U87 (F-J) and U251 (K-O) cells following treatment with the transfection reagent (TR) alone, HSP27 siRNA (HSP27i), or Control Oligomer (CO). HSP27i, but not control conditions, produced a marked reduction in HSP27 expression in all GBM lines. Western blot and corresponding densitometric analysis (N=3-7 per group) demonstrated significant knockdown of the target protein by HSP27i, with no significant effect on HSP27 or HSP90 expression by either the transfection reagent alone or Control Oligomer in the three GBM lines. The statistical qualifiers labeled 'a' and 'b' refer to a significant difference at $p<0.05$ compared to the transfection agent or Control Oligomer treatment group, respectively. The scale bar in M represents 10 μm for all photomicrographs.*

Statistical analysis. The relative viability fractions, Ki-67 labeling indices and western blot densitometry values were analyzed using PRISM software version 4 (GraphPad Inc., La Jolla, CA). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Bonferroni *post-hoc* for multiple comparisons. All data were presented as mean±standard deviation and were considered significant at $p<0.05$.

Results

HSP27i robustly inhibits target protein expression in GBM cells. Gene silencing using HSP27i was assessed in LN229, U87 and U251 GBM cells with confocal microscopy and western blot analysis. Under baseline conditions, or in the presence of the Control Oligomer, HSP27 was robustly expressed in the cytosol of all three GBM lines. Forty-eight hours following HSP27i transfection, HSP27 expression levels were reduced by ~79% in LN229, 92% in U87, and 98% in U251 cells. In contrast, HSP90 expression was neither elevated nor suppressed after treatment with HSP27i, consistent with a lack of compensatory induction or off-target effect. There were no alterations in either HSP27 or HSP90 levels following exposure to the transfection reagent alone or the Control Oligomer (Figure 1).

HSP27 knockdown potentiates the tumoricidal effect of 17-AAG and STS. Dose–response and the estimated minimum effective dose (MED) were defined for 17-AAG (Figure 2A-C) and STS (Figure 3A-C) in all GBM lines. The working concentration of each agent was subsequently chosen as that directly below the MED in the dose-response studies (Table I). The intent of using such biologically-ineffective concentrations of 17-AAG and STS was to determine whether adjuvant HSP27i treatment could produce a leftward shift in the MED (*i.e.*, potentiate the therapeutic effect). The MTT viability assay was used to evaluate GBM cell responsiveness to single treatment HSP27i, 17-AAG and STS, or combination therapy using HSP27i with 17-AAG or STS. The viability studies showed

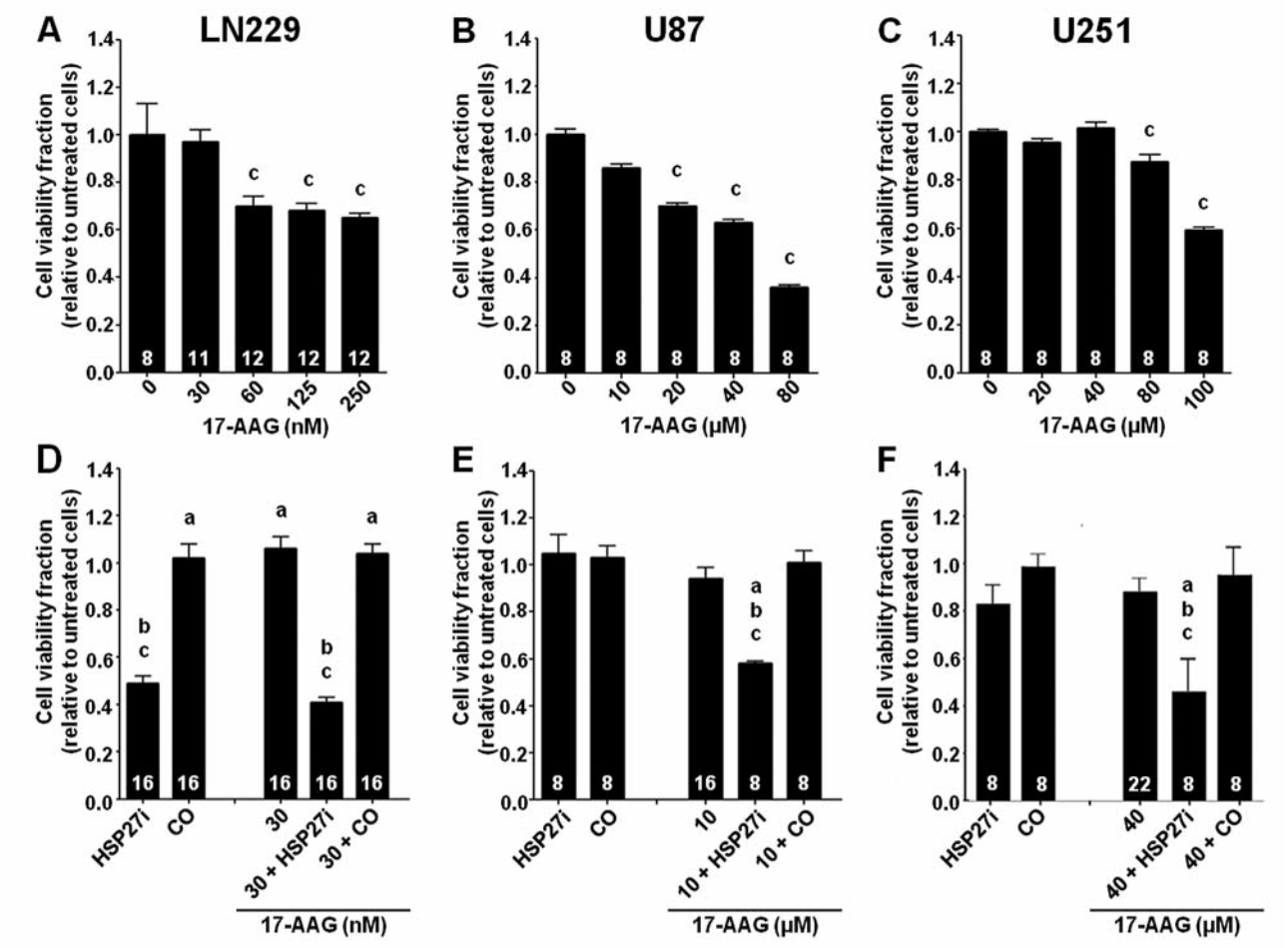

Figure 2. *Heat shock protein-27 (HSP27) knockdown sensitizes glioblastoma multiforme (GBM) cells to cytotoxic effects of 17-N-allylamino-17-demethoxygeldanamycin (17-AAG). MTT viability studies for LN229 (A, D), U87 (B, E) and U251 (C, F) GBM cell lines. Dose–response relations (A-C) were assessed for 17-AAG and the minimum effective dose (MED) to produce significant cytotoxicity was estimated for each cell line. The drug concentration immediately below the MED was used as a therapeutically ineffective dose to determine whether HSP27 knockdown sensitized GBM cells to 17-AAG, as measured by a leftward shift in the MED (D-F). All GBM lines exhibited a progressive decrease in viability with escalating 17-AAG concentrations. LN229 cells were exquisitely sensitive to 17-AAG, with a MED in the nanomolar range and more than 300- and 1300-fold less than that of U87 and U251 lines, respectively (MED: LN229=60 nM, U87=20 μM, U251=80 μM). The working concentrations of 17-AAG used to assess the sensitization effect of HSP27 knockdown were: LN229 30 nM, U87 10 μM, and U251 40 μM. There was a profound reduction in viability following HSP27 knockdown in LN229, but not U87 or U251 cells. The addition of low-dose 17-AAG alone or in the presence of Control Oligomer (CO) produced no significant effect on the viability fraction in any GBM line. In contrast, there was a marked and potentiated cytotoxic effect of low-dose 17-AAG in all cell lines following treatment with HSP27i. Data are presented as mean viability fraction±standard deviation. The statistical qualifiers refer to a significant difference at $p<0.05$ compared to: a, cells treated with HSP27i alone; b, cells treated with the respective concentration of 17-AAG alone (i.e., 30 nM, 10 μM or 40 μM); and c, untreated cells. The values in the histogram bars indicate the number of replicates per group.*

that U87 and U251 cells had similar sensitivities to 17-AAG, whereas LN229 cells exhibited marked toxicity at concentrations several hundred-to over one thousand-fold lower (Figure 2A-C). In contrast, there were no such disparities in responsiveness to STS between the GBM cells (Figure 3A-C). Similar sensitivity profiles were revealed with HSP27i treatment that produced a robust tumoricidal effect in LN229 cells but had no significant impact on the viability of U87 and U251 cultures, despite marked target protein knockdown. The use of adjuvant HSP27i dramatically potentiated the tumoricidal effects of low-dose 17-AAG and STS. In contrast, the Control Oligomer and the transfection reagent without HSP27i did not produce cytotoxic effects when administered alone, nor did they sensitize any of the GBM lines to the chemotherapeutic agents (Figures 2D-F, 3D-F).

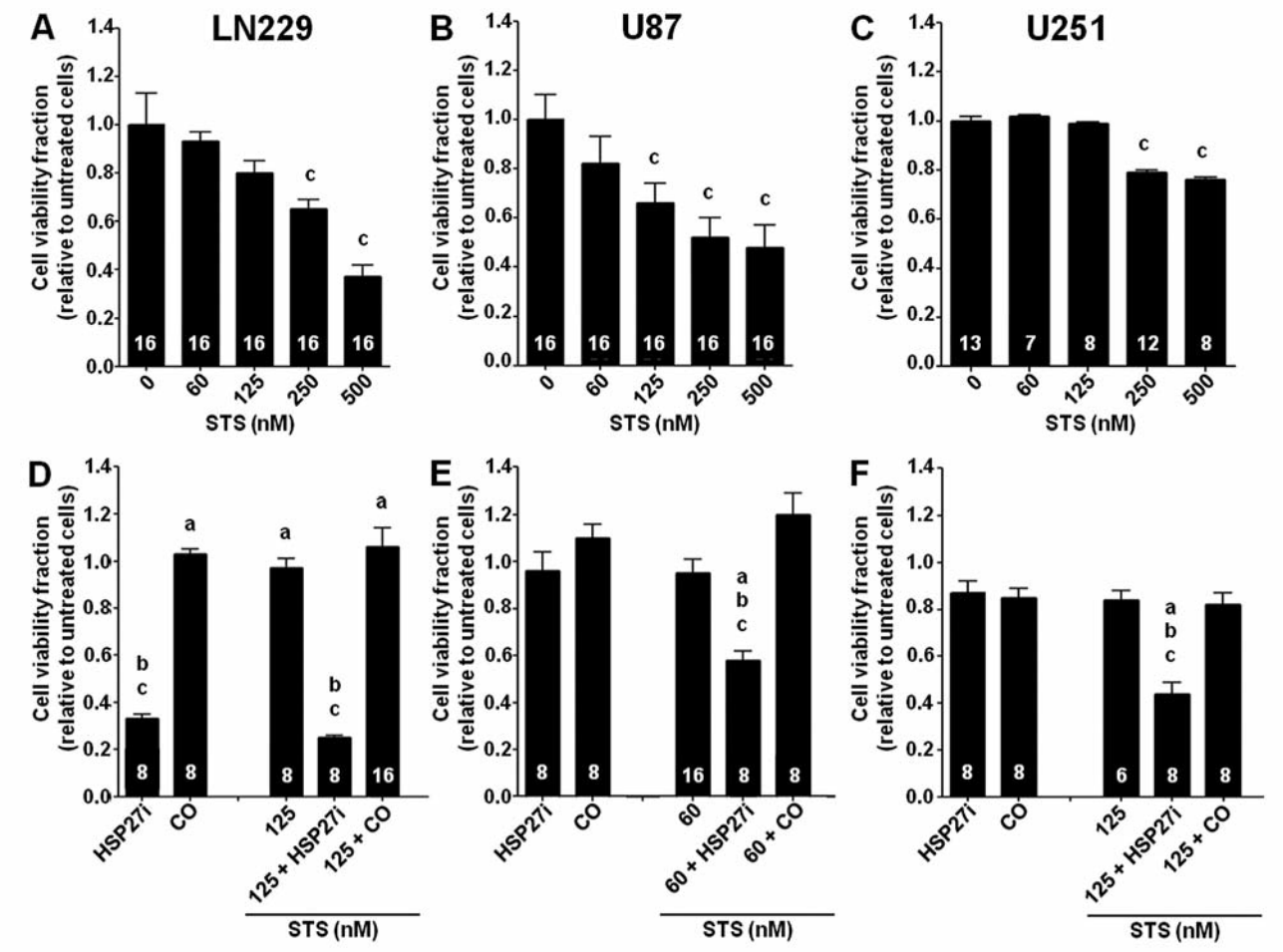

Figure 3. *Heat shock protein-27 (HSP27) knockdown sensitizes glioblastoma multiforme (GBM) cells to cytotoxic effects of staurosporine (STS). MTT viability studies for LN229 (A, D), U87 (B, E) and U251 (C, F) GBM cell lines. Dose–response relations (A-C) were assessed for STS and the minimum effective dose (MED) to produce significant cytotoxicity was estimated for each cell line. The drug concentration immediately below the MED was used as a therapeutically ineffective dose to determine whether HSP27 knockdown sensitized GBM cells to STS, as measured by a leftward shift in the MED (D-F). Unlike the variable sensitivity to 17-AAG, all GBM lines exhibited a comparable response and progressive decrease in viability with escalating concentrations of STS. The working concentrations of STS used to assess the sensitization effect of HSP27 knockdown were: LN229 125 nM, U87 60 nM, U251 125 nM. There was a profound reduction in viability following HSP27 knockdown in LN229, but not U87 or U251 cells. The addition of low-dose STS alone or in the presence of Control Oligomer (CO) produced no significant effect on the viability fraction in any GBM line. In contrast, there was a marked and potentiated cytotoxic effect of low-dose STS in all cell lines following treatment with HSP27i. Data are presented as mean viability fraction±standard deviation. The statistical qualifiers refer to a significant difference at $p<0.05$ compared to: a, cells treated with HSP27i alone; b, cells treated with the respective concentration of STS alone (i.e., 60 or 125 nM); and c, untreated cells. The values in the histogram bars indicate the number of replicates per group.*

Combined HSP targeting synergistically reduces proliferation and enhances apoptosis with caspase-3 activation in GBM cells. U251 cells exhibited the greatest *HSP27* knockdown amongst the three GBM cell lines tested. The altered protein expression did not produce direct toxicity yet conferred potent sensitization to chemotherapeutic challenge. These cells were selected on this basis to explore the impact of stand-alone and adjuvant HSP27 targeting on GBM cell turnover and the biological mechanism associated with decreased tumor cell viability secondary to HSP inhibition. Cell proliferation was assessed using the Ki-67 labeling index which, as expected, was consistently high (~75-85%) in GBM cultures under control conditions. There was no significant reduction with the low-dose 17-AAG (40 μM) with or without adjuvant Control Oligomer. In contrast, concurrent inhibition of HSP27 expression and HSP90 function using adjuvant HSP27i with the otherwise innocuous, low-dose 17-AAG resulted in markedly reduced proliferation indices. This sensitization of

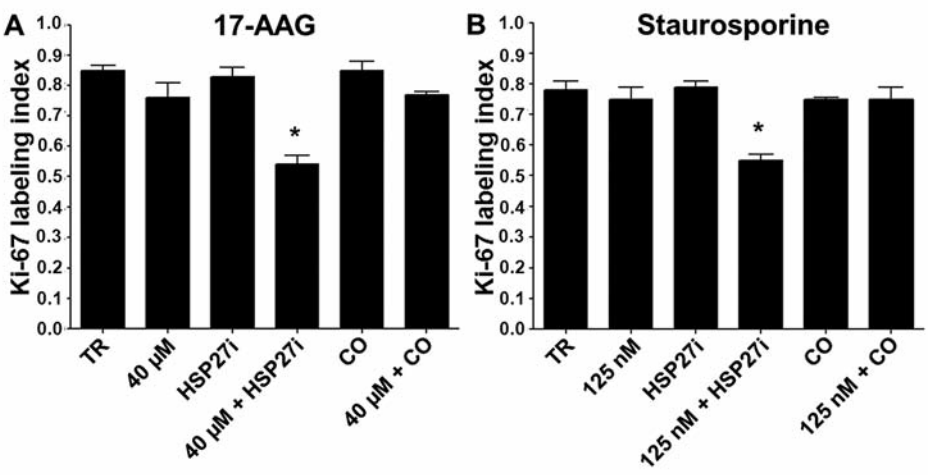

Figure 4. *Adjuvant heat shock protein-27 (HSP27) targeting synergistically enhances the antiproliferative effect of chemotoxins in glioblastoma multiforme (GBM) cells. The Ki-67 labeling index was used to measure proliferation in U251 cells exposed to control and experimental conditions. Single-agent therapy with the transfection reagent (TR), low-dose drug (either 17-AAG or STS) or HSP27 siRNA (HSP27i) did not notably impact proliferation. In contrast, cells treated with HSP27i prior to drug challenge with 40 μM 17-AAG (A) or 125 nM STS (B) exhibited a significant reduction in Ki-67 labeling (N=3 per group). Data are presented as mean±standard deviation. *Significant difference at p<0.05 relative to the control (i.e., TR only). CO, Control Oligomer.*

Table I. *Drug concentrations used to assess synergy with heat shock protein-27 (HSP27) knockdown in glioblastoma multiforme (GBM) lines. The selected concentrations were lower than the estimated minimum effective dose for each agent, as determined using the MTT viability studies in all cell lines. Note the exquisite sensitivity of LN229 cells to the HSP90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), that was paralleled by a comparable reduction in viability following HSP27 knockdown (see Figure 2). In contrast, there were no notable differences in response to staurosporine among the different GBM lines.*

GBM cell line	17-AAG (μM)	Staurosporine (nM)
LN229	0.030	125
U87	10	60
U251	40	125

tumoristatic effect was also produced by *HSP27* knockdown in the presence of low-dose STS (Figure 4).

The relative fraction of apoptotic cells in GBM cultures was compared among the various treatment and control conditions using TUNEL analysis and immunodetection of cleaved (activated) caspase-3. Apoptotic cells were infrequent (approximately 5% of the total cell number) in cultures treated with the transfection reagent only, low-dose drug (either 17-AAG or STS), HSP27i alone, or Control Oligomer with or without drug. Consistent with the MTT metabolic findings, adjuvant HSP27i therapy resulted in significantly elevated TUNEL immunostaining (Figures 5 and 6) and overall fraction of TUNEL-positive nuclei when combined with 17-AAG (Figure 7A) or STS (Figure 8A). Activated caspase-3 protein expression was negligible in cells treated with the transfection reagent only, HSP27i, or Control Oligomer. There were scant levels induced by low-dose drug (either 17-AAG or STS) alone, and when combined with the Control Oligomer. In contrast, cells treated with adjuvant HSP27i in the presence of 17-AAG or STS exhibited a marked rise in activated caspase-3 expression that co-localized and correlated with the extent of TUNEL signal (Figures 5 and 6). Cells incubated without primary antibody showed negligible fluorochrome signal (data not shown). The immunocytochemistry results were confirmed and quantified using western blot analysis (n=4 per group). The large fragments (17/19 kDa) of activated caspase-3 were present in lysates from cells exposed to either 17-AAG or STS with and without HSP27i or Control oligomer, whereas cells treated with transfection reagent, HSP27i or Control Oligomer alone showed negligible expression. The treatment with adjuvant HSP27i, but not the Control Oligomer, produced a robust and synergistic increase in 17-AAG- or STS-mediated caspase-3 activation ($p<0.05$; Figures 7 and 8).

Discussion

The function of HSPs in perpetuating the growth, dissemination and treatment resistance of somatic and CNS neoplasms, supports the development of HSP-targeting agents as novel cancer therapeutics (55, 56). A major focus has been put on HSP90, a highly-conserved stress chaperone with a wide range of client proteins, including HSF1 and oncogene

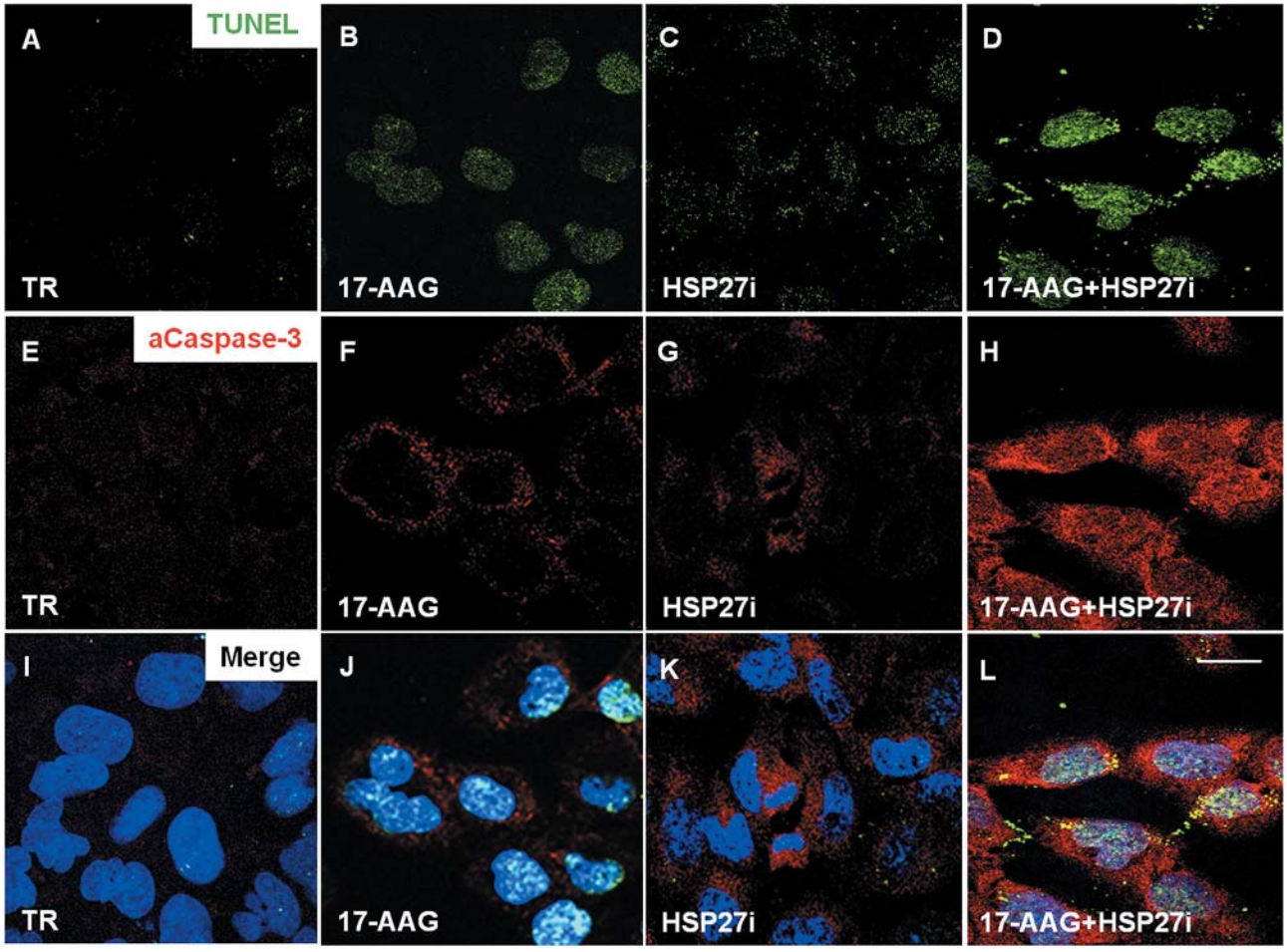

Figure 5. *Heat shock protein-27 (HSP27) inhibition sensitizes glioblastoma multiforme (GBM) cells to 17-N-allylamino-17-demethoxygeldanamycin (17-AAG)-mediated apoptosis. Representative confocal micrographs of the apoptosis markers, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL; A-D) and activated caspase-3 (E-H), as well as merged (I-L) imaging of U251 cells exposed to control and experimental treatment conditions. The cells exhibited negligible evidence of apoptosis under control conditions [transfection reagent (TR) only], with a small but notable increase in marker labeling following single-agent therapy with low-dose 17-AAG (40 μM) or HSP27 siRNA (HSP27i). In contrast, there was a marked increase in co-localized TUNEL and activated caspase-3 (aCaspase-3) labeling following concurrent HSP27i and 17-AAG treatment. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). The scale bar in L applies to all panels and represents 10 μm. CO, Control Oligomer.*

products (*e.g.*, p53, AKT, RAF, EGFR, MEK, among others) involved in growth, survival and cell-cycle regulatory pathways (16, 52). Three operational regions of HSP90 facilitate protein folding, localization and/or proteosomal degradation: an N-terminal ATPase domain, a central domain which participates in client protein binding, and a C-terminal dimerization domain that binds ATP and co-chaperone proteins. The chaperoning cycle is initiated by client protein complexes with HSP40 and HSP70 that recruit co-chaperones and HSP90 molecules. ATP-binding promotes dissociation from HSP40 and HSP70, leaving the client bound with HSP90 to recruit effector proteins that propagate the associated molecular cascade. The stabilizing effects of HSP90 on a broad host of oncogenic molecules has peaked interest in its potential as a novel target for cancer therapies (12, 16, 17, 52). The benzoquinone ansamycin, geldanamycin, was originally identified from *Streptomyces hygrocopicus* in 1970 (11, 13). Newer, less toxic and more water-soluble derivatives are now available, out of which 17-AAG is one of the most extensively characterized. These agents inhibit HSP90 function by competing with ATP to disrupt the chaperoning cycle and recruit ubiquitin ligase which tags client proteins for proteosomal degradation. HSP90 inhibitors show robust efficacy in pre-clinical models and have been extensively studied in human cancer trials. Unfortunately, the clinical benefit of these agents has been mitigated by various factors, including a compensatory up-regulation of HSF1-regulated

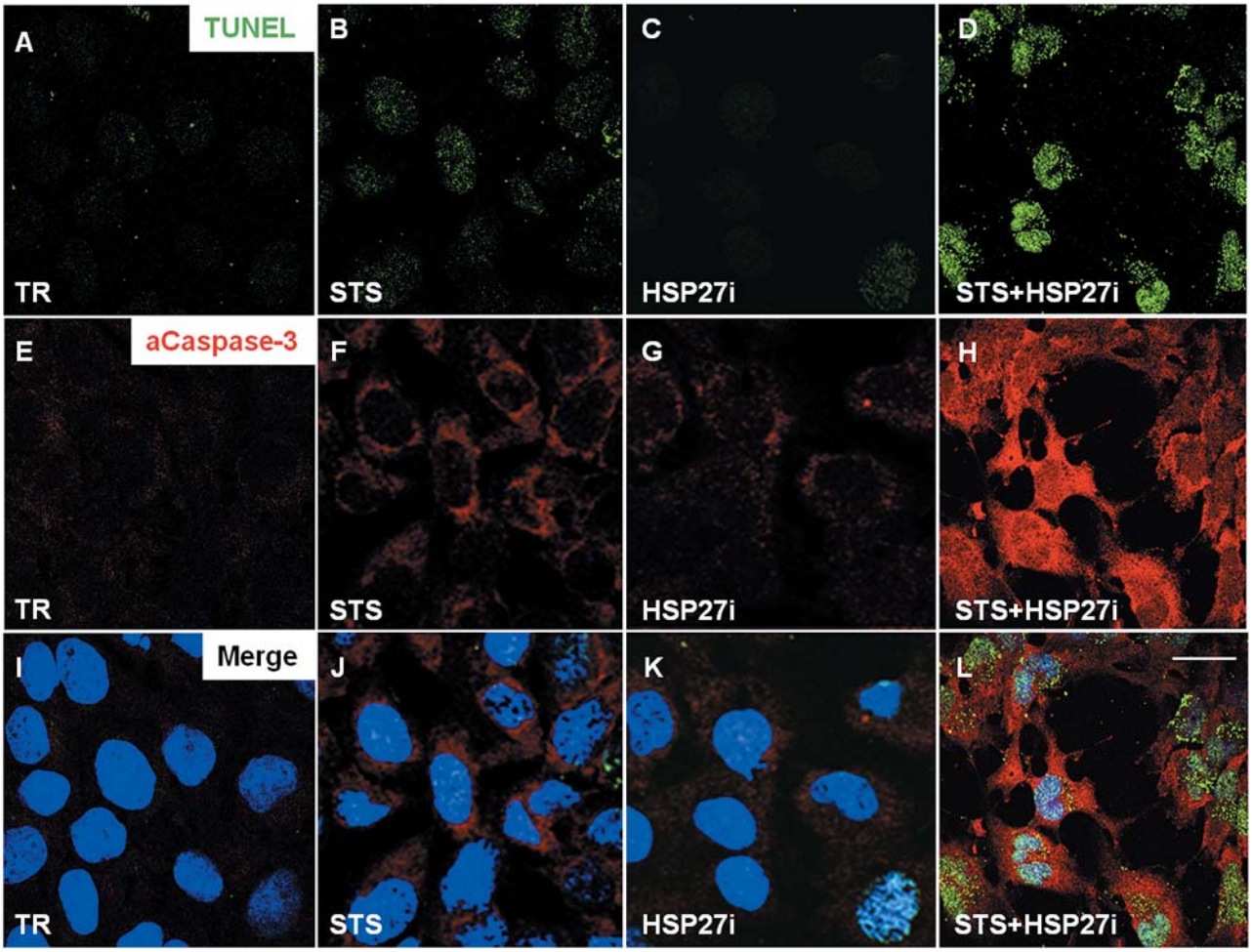

Figure 6. *Heat shock protein-27 (HSP27) inhibition sensitizes glioblastoma multiforme (GBM) cells to staurosporine (STS)-induced apoptosis. The combination of HSP27 siRNA (HSP27i) and STS was assessed to determine if the chemosensitization produced by HSP27 knockdown could be achieved when used as adjuvant to agents with biological mechanisms other than direct HSP inhibition. Representative confocal micrographs of the apoptosis markers, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL; A-D) and activated caspase-3 (E-H), as well as merged (I-L) imaging of U251 cells exposed to control and experimental treatment conditions are shown. The cells exhibited negligible evidence of apoptosis under control conditions [transfection reagent (TR) only], with a small but notable increase in marker labeling following single-agent therapy with low dose STS (125 nM) or HSP27i. In contrast, there was a marked increase in co-localized TUNEL and activated caspase-3 (aCaspase-3) labeling following concurrent HSP27i and STS treatment. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). The scale bar in L applies to all panels and represents 10 μm. CO, Control Oligomer.*

stress proteins, such as HSP27, HSP70 and HSP90 (12, 16, 17, 52, 57, 58). This effect is mediated by reduced HSF1 binding to HSP90 and a resultant rise in free (active) HSF1 available for nuclear translocation. Increased levels of cytosolic peptides from degraded protein may also compete for HSP90 and contribute to HSF1 availability. The resultant broad induction of HSPs counteracts the therapeutic effects of HSP90 inhibitors by bolstering the recalcitrant attributes of neoplastic cells.

There is now considerable evidence to support the development of HSP27-targeting strategies to treat various forms of cancer. The heightened expression and aberrant control by oncogenic signaling pathways relegate the functions of HSP27 as a molecular chaperone, anti-apoptotic and cytoskeletal modulator in favor of cancer growth (46). It has been shown that high HSP27 levels are a predictive marker of aggressive tumor behavior and poor clinical outcome in prostate, pancreatic and colorectal cancer, among others (34-36). The known role in fundamental processes associated with cell proliferation, migration and survival, as well as the development of promising anti-HSP27 trials in genitourinary cancer, raises the possibility that HSP27 may be an effective target for GBM control. The current study sought to explore

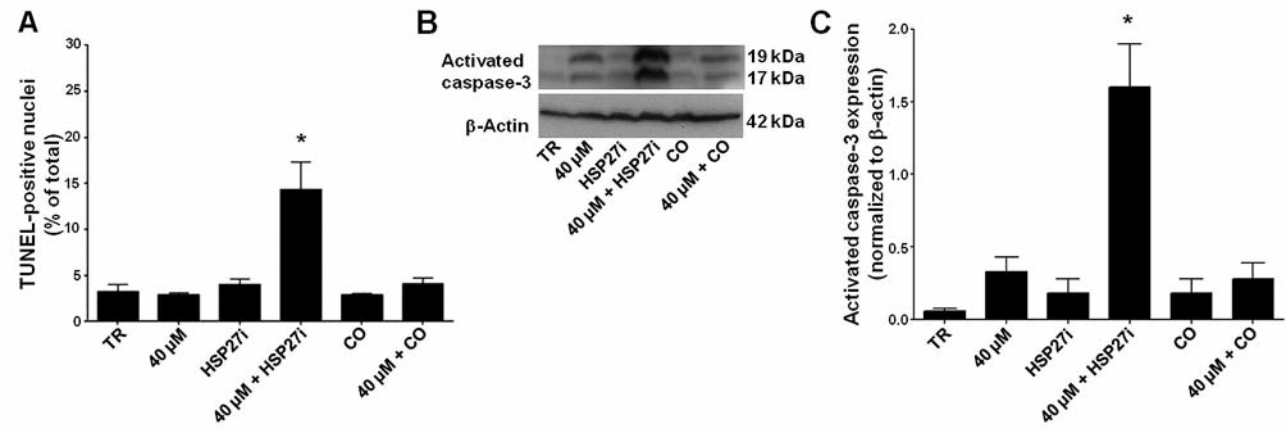

Figure 7. *Concurrent heat shock protein-27 (HSP27) and heat shock protein-90 (HSP90) targeting produces synergistic induction of apoptosis and caspase-3 activation in glioblastoma multiforme (GBM) cells. A: Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) quantification in U251 cells. There was no significant impact on the relative extent of apoptosis of GBM cells following exposure to control conditions [transfection reagent (TR) only] or single-agent treatment with either HSP27 siRNA (HSP27i) or low-dose 17-N-allylamino-17-demethoxygeldanamycin (17-AAG; 40 μM). The Control Oligomer (CO) alone and as adjuvant to low-dose 17-AAG also produced no significant change in the fraction of TUNEL-positive cells. In contrast, HSP27i markedly sensitized the GBM cells to low-dose 17-AAG, producing a dramatic rise in the number of apoptotic cells. B: Activated caspase-3 expression was assessed by western blot analysis. C: Corresponding densitometric measures in GBM cells exposed to control or experimental conditions. There was a minor increase in caspase-3 activation with single-agent therapy (i.e., HSP27i or low-dose 17-AAG) but a marked and synergistic rise (>5-fold) when GBM cells were treated concurrently with both HSP27 and HSP90 inhibitors. Data are presented as the mean±standard deviation.*Significant difference relative to control (TR only) at $p<0.05$; N=3 per group.*

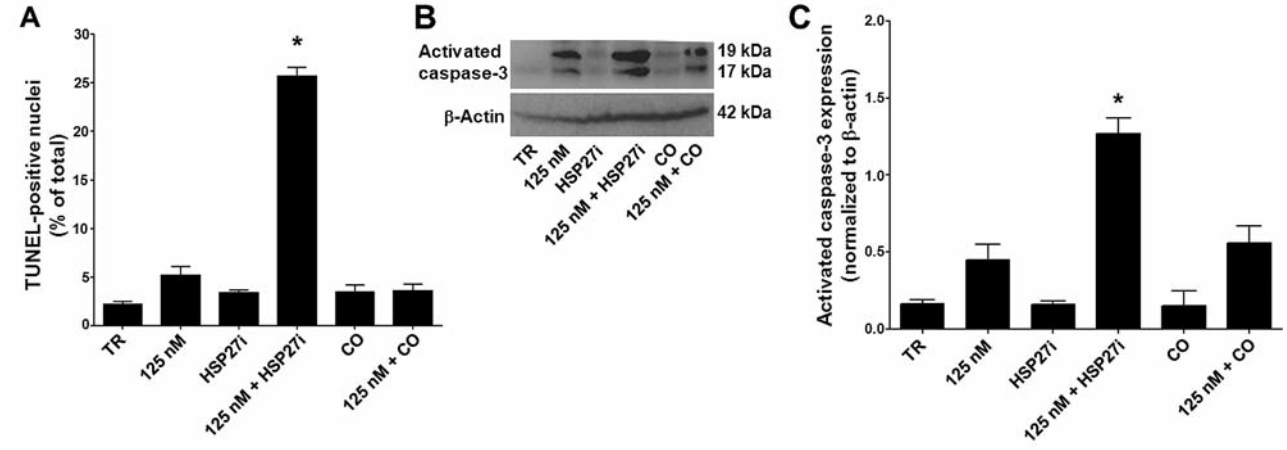

Figure 8. *HSP27 knockdown synergistically enhances apoptosis and caspase-3 activation by staurosporine (STS) in glioblastoma multiforme (GBM) cells. STS is a non-specific kinase inhibitor and potent cytotoxin that was used to determine if an HSP27-targeting strategy could also produce GBM sensitization to agents that do not function primarily through HSP pathways. A: Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) quantification in U251 cells. Low concentration of STS (125 nM) produced a discernible but non-significant increase in the number of TUNEL-positive cells. As previously noted, there was no significant impact on the relative extent of GBM apoptosis following exposure to control conditions [transfection reagent (TR) only] or single-agent treatment with HSP27 siRNA (HSP27i). The Control Oligomer (CO) alone or as adjuvant to low-dose STS also produced no significant change in the number of TUNEL-positive cells. In contrast, there was a dramatic sensitization to STS by HSP27i with a significant rise in the observed fraction of apoptotic cells. B: Activated caspase-3 expression was assessed by western blot analysis. C: Corresponding densitometric measures in GBM cells exposed to control or experimental conditions. There was a non-significant increase in caspase-3 activation with single-agent therapy (i.e., HSP27i or low-dose STS) but a marked and synergistic rise when GBM cells were treated concurrently with both HSP27i and STS. Data are presented as the mean±standard deviation. *Significant difference relative to control (TR only) at $p<0.05$; N=3 per group.*

this notion by evaluating the impact of an HSP27 siRNA (HSP27i) on the proliferation and viability of multiple GBM cell lines. Given the developing advances in HSP90 inhibitors, the potential for synergy using dual HSP27 and HSP90-targeting strategies was also evaluated. There was variation in the susceptibility to both HSP27i and 17-AAG among the GBM lines tested. Cell viability was consistently reduced to less than 50% by HSP27i in LN229 cells, with no significant cell loss observed in either U251 or U87 lines, despite robust target knockdown. The dose–response studies for 17-AAG also demonstrated markedly greater sensitivity of the LN229 cells compared to the other GBM lines. In contrast, there were comparable STS dose responses among the different GBM lines and, together, these data support a critical dependence on HSP function rather than broad chemosensitivity in LN229 cells. Such variability in treatment response is well recognized in GBM and likely reflects the oncogene addiction status and the role of tumor-related HSP client proteins in specific lesions (12, 59-62). Treatment with HSP27i robustly potentiated the antiproliferative and cytotoxic actions of an otherwise innocuous concentration of 17-AAG, demonstrating a clear advantage of dual HSP targeting to reduce dose requirements and maintain tumor control. TUNEL and activated caspase-3 studies further provided impressive evidence of synergistic tumoricidal action (apoptosis) imparted by concurrent HSP27i and 17-AAG treatment. A similar effect observed with low-dose STS suggests that HSP27i mediates GBM sensitization independent of HSP90 status and may have potential to broadly enhance the benefits of current and developing chemoradiation options.

The genetic heterogeneity among patients and within any single GBM tumor poses significant challenges in defining reliable targets for disease-modifying therapies. Highly deregulated and redundant trophic signaling involves diverse cellular elements controlled by post-translational modifications (*e.g.* phosphorylation, glycosylation, methylation) and counter-regulatory molecules (63, 64). The complexity of these processes limits the efficacy of any single modality or cellular targeting therapy and there remains a critical need for novel strategies that disrupt parallel survival mechanisms in GBM. Inhibition of HSP expression or function is highly effective at reducing cancer cell proliferation and viability, as well as acting as a sensitizing method for other chemoradiation treatments in preclinical models (16, 17, 46, 65). The challenges met in clinical trials likely reflect specific pharmacological limitations and off-target actions of these agents. Novel strategies that block the activity of multiple HSPs are being investigated to reduce dose requirements and more effectively restrict common and complementary tumor-promoting pathways (66, 67). HSP27 and HSP90 are stress-induced chaperones that overlap in function but carry out distinct cellular roles *via* ATP-independent and dependent mechanisms, respectively (3, 4). A recent study has demonstrated marked potentiation of cytotoxic benefit achieved in breast cancer stem-like cells using a combination of anti-HSP27 oligomers and ansamycin HSP90 inhibitors (68). These findings align with the present data in GBM cells and offer corroborating evidence for the broad efficacy of this targeting approach.

There remains little data available on the utility of HSP27 inhibitors or combined anti-HSP strategies for the treatment of brain tumors. The current study provides new information regarding the *in vitro* efficacy of HSP27 knockdown, and the marked synergy in tumoristatic and tumoricidal effects achieved when combined with HSP90 inhibition in GBM cells. These findings are consistent with a growing literature that supports the development of anti-HSP strategies for somatic and CNS tumors. The full potential of HSP27 inhibitors alone, as well as an adjuvant therapy in GBM has yet to be realized.

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