Geldanamycin and 17-Allylamino-17-demethoxygeldanamycin Potentiate the in Vitro and in Vivo Radiation Response of Cervical Tumor Cells via the Heat Shock Protein 90-Mediated Intracellular Signaling and Cytotoxicity

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ABSTRACT

Ansamycin antibiotics inhibit function of the heat shock protein (HSP) 90, causing selective degradation of several intracellular proteins regulating such processes as proliferation, cell cycle regulation, and pro survival signaling cascades. HSP90 has been identified previously as a molecular target for anticancer agents, including ionizing radiation (IR). Therefore, we hypothesized that the ansamycin geldanamycin and its 17-allylamo-17-demethoxy analog (17-AAG), which inhibit HSP90, would enhance tumor cell susceptibility to the cytotoxicity of IR. Treatment of two human cervical carcinoma cell lines (HeLa and SiHa) with geldanamycin and 17-AAG resulted in cytotoxicity and, when combined with IR, enhanced the radiation response, each effect with a temporal range from 6 to 48 h after drug exposure. In addition, mouse in vitro models using 17-AAG at clinically achievable concentrations yielded results that paralleled the in vitro radiosensitization studies of both single and fractioned courses of irradiation. The increase in IR-induced cell death appears to be attributable to a combination of both programmed and nonprogrammed cell death. We also measured total levels of several prosurvival and apoptotic signaling proteins. Akt1, extracellular signal-regulated kinase-1, Glut-1, HER-2/neu, Lyn, cAMP-dependent protein kinase, Raf-1, and vascular endothelial growth factor expression were down-regulated in 17-AAG-treated cells, identifying these factors as molecular markers and potential therapeutic targets. Finally, a series of immortalized and human papillomavirus-transformed cell lines were used to demonstrate that the radiosensitizing effects of 17-AAG were limited to transformed cells, suggesting a possible differential cytotoxic effect. This work shows that altered HSP90 function induces significant tumor cytotoxicity and radiosensitization, suggesting a potential therapeutic utility.

INTRODUCTION

The development of new anticancer agents involves identifying novel molecular targets to improve therapeutic outcome, ideally with efficacy both alone and when combined with existing treatment modalities. Among others, the heat shock protein (HSP) class of chaperones has emerged as a molecular target candidate in that members of this group are key intracellular mediators of the eukaryotic cellular response to damage induced by environment stressors, including anticancer agents (1, 2). Particularly, the importance of HSP90 to numerous oncogenic processes makes it a promising target molecule. HSP90 has been implicated in maintaining the conformation, stability, and function of key client proteins involved in signal transduction pathways related to proliferation, cell cycle progression, and apoptosis, and it is also involved in processes characteristic of the malignant phenotype, including invasion, angiogenesis, and metastasis (3, 4). In addition, HSP90 expression is up-regulated in tumors as compared with normal tissues, which implies that tumor cells might be preferentially affected by HSP90-targeted therapies (5). Therefore, HSP90 may represent a potentially attractive target for specific molecular anticancer agents.

Geldanamycin (GA) is a naturally occurring ansamycin antibiotic that, along with its clinically used analog 17-allylamo-17-demethoxygeldanamycin (17-AAG), has significant anticancer properties (6, 7). These agents disrupt HSP90 association with client proteins by occupying the nucleotide-binding site of HSP90 (8–10), thereby preventing binding of HSP90 with ATP and profoundly affecting the composition of HSP90-containing multimolecular chaperone complexes (11–13). Interestingly, tumor cells have increased levels of HSP90, and many cytoprotective and prosurvival pathways are functionally stabilized by HSP90 (14–16). GA and 17-AAG induce the proteasomal degradation of a subset of HSP90 client proteins involved in signal transduction (5, 17, 18) and oncogenes known to be involved in transformation (19–21), but the compounds demonstrate little effect in the untransformed counterparts of malignant tissues (22, 23). Taken together, these findings suggest that HSP90 may be a unique target to preferentially destroy abnormal cells while sparing normal tissue.

Greater than half of all cancer patients receive radiation therapy, thereby emphasizing the need to understand the cellular and molecular events after exposure to ionizing radiation (IR; Refs. 24–27). Translational research with hyperthermia and nonsteroidal anti-inflammatory agents, both of which induce some aspects of the heat shock response, suggests that at least one target of these anticancer modalities may be the functional activity of HSP90 (28–42). GA and 17-AAG inhibit HSP90, resulting in the inhibition of several intracellular prosurvival signaling factors (9–18). Therefore, we hypothesized that HSP90 inhibition by these agents might increase IR-induced tumor cell death. This work was initiated with intent to validate the idea that GA and 17-AAG would sensitize malignant cells both in vitro and in vivo to the cytotoxic effects of IR and identify potential molecular markers that could be used in future clinical studies.

Using two cervical tumor cell lines, we demonstrated that both GA and 17-AAG function as cytotoxic and powerful radiosensitizing agents in time- and dose-dependent manners. GA and 17-AAG exhibit toxic effects in tumor cells beginning at 6 h and lasting for 48 h after initial exposure through a mechanism involving increases in both programmed and nonprogrammed cell death pathways. Biochemical analysis determined that several prosurviving signaling factors or molecular markers are decreased after exposure, including Akt1, extracellular signal-regulated kinase (ERK) 1, Glut-1, Her-2/neu, Lyn, cAMP-dependent protein kinase, Raf-1, and vascular endothelial growth factor (VEGF) expression. A HeLa xenograft mouse model
system was used to test the consistency of these results in vivo, and results from these studies demonstrate a greater effect on tumor control in combined 17-AAG and IR treatment groups relative to either 17-AAG or IR alone. These results suggest that GA and 17-AAG induce a significant increase in IR-induced cytotoxicity in vitro and in vivo as well as identify several molecular markers that can potentially be used in vivo.

MATERIALS AND METHODS

Cell Culture, GA and 17-AAG Treatment, and IR Exposure. HeLa (human cervical carcinoma) cells were grown in MEM, α modification, supplemented with 10% heat-inactivated (56°C, 30 min) calf serum. SiHa cells were grown in MEM containing Earle’s balanced salt solution and 10% heat-inactivated fetal bovine serum. The HFF3/LXSN (normal fibroblasts), HFF3/LXSN/16E6 [fibroblasts transduced with human papillomavirus (HPV) 16 E6], HFF3/LXSN/16E7 (fibroblasts transduced with HPV16 E7), and HFF3/LXSN/16E6/E7 (fibroblasts transduced with both HPV16 E6 and E7) were obtained from Dr. Denise Galloway (University of Washington) and grown in DMEM with high glucose containing 10% heat-inactivated fetal bovine serum. Medium was supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml). All cells were plated at densities of 2 × 10^5 cells/100-mm dish, grown in a humidified 5% CO_2 incubator at 37°C, and passaged when they reached 75% confluence. GA and 17-AAG (NSC 330507) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD), dissolved in DMSO to yield 10 mM stock solutions, and stored at −80°C. Agents were added to the growth medium of designated cells to achieve specific molar concentrations, after which they were returned to 37°C. Corresponding volumes of DMSO vehicle were added to designated sham controls in each experiment. Cells were exposed to IR in a Therapax high-frequency X-ray generator (Agfa NDT, Lewistown, PA), operated at 300 V and 10 mA with 2-mm Al filtration to achieve a dose rate of 2.1 Gy/min. After exposure, cells were returned to 37°C.

Clonogenic Cell Survival Assays. Cells were assayed for the cytotoxic effects of GA or 17-AAG, and/or IR after cell survival according to the established methods of performing the clonogenic assay (30, 41). Briefly, HeLa or SiHa cells were seeded at densities of 2 × 10^3 cells/100-mm tissue culture dish and allowed to grow in a 37°C incubator until they reached 75% confluence. After attaining sufficient growth, GA, 17-AAG, or DMSO was added to the growth media for the designated times, the plates were exposed to IR at doses of 2–8 Gy, and cells were immediately trypsinized, diluted, counted, and seeded into 6-well tissue culture plates. Colonies were allowed to form from surviving cells in a humidified, 5% CO_2, 37°C environment for 10 days, after which they were fixed, stained, and counted. Individual assays were performed at multiple dilutions with a total of six observations per data point and repeated twice for a total of three identical experiments. The results of these trials are shown in a linear versus logarithmic plot relating IR exposure to survival. Statistical significance of survival differences between vehicle and treated cells was determined by two-sample, two-sided t tests performed at the 0.05 level of significance.

Kinetics Protein Kinase and Apoptosis Immunoblot Analysis. Whole cell lysates were analyzed using the Kinetworks Protein Kinase Screen (KPKS) 1.2 Protein Kinase and Kinetworks Apoptosis Protein Screen 1.0 (Kinexus Bioinformatics Corp., Vancouver, British Columbia, Canada) after their preparation in accordance with the company’s instructions. Detailed protocols for the Kinetworks analyses can be found online. Briefly, protein extracts were applied into separate lanes of a 20-lane multiblotter (Immunetics, Cambridge, MA), separated by electrophoresis, transferred to a membrane, and probed with mixes of primary antibodies from commercial sources. The immunoblots were developed with enhanced chemiluminescence plus reagent (Amersham Biosciences, Piscataway, NJ), and signals were captured by Fluor-S MultiImager and quantified using Quantity One software (Bio-Rad, Hercules, CA). Antibodies and techniques were validated for reliable immunoblotted performance (43).

**SDS-PAGE and Western Blot Analysis.** Extracts were prepared from whole cells by a method modified from Dignam (44). Nuclear and cytoplasmic extracts were obtained via methods overviewed in Curry et al. (33). Total protein concentrations were determined via a deoxycholate/urea protein assay (Bio-Rad) on a Wallac Victor (Perkin-Elmer, Boston, MA) microplate reader. After preparation and quantification, all samples were stored at −80°C and thawed on ice. Equal amounts of protein (10–30 µg) were mixed with 4X Laemmli lysis buffer, boiled for 5 min, separated on a denaturing SDS-polyacrylamide gel, and transferred to a nitrocellulose filter using a semi-dry transfer apparatus (Owl, Inc., Portsmouth, NH). Nonspecific antibody-antigen binding on the nitrocellulose filter was blocked by incubating in 7.0% milk, PBS, and 0.05% Tween 20 solution for 1 h. This was followed by hybridizing the membranes overnight with antibodies against HSP70 (Stressgen, Vancouver, British Columbia, Canada), Glut-1 (Lab Vision, Fremont, CA), actin (Chemicon, Temecula, CA), Akt1–1, E6AP, ERK1, phosphorylated (p)-ERK (pY204), HSP90, Her-2/neu, p-Her-2/neu (pTyr1248), Lyn, p53, CAM-dependent protein kinase, Raf-1, p-Raf-1 (Tyr204), or VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), diluted in 3.5% milk, PBS, and 0.05% Tween 20 solution. The nitrocellulose filter was washed with PBS containing 0.05% Tween 20 and then incubated with the appropriate secondary antibody (Santa Cruz Biotechnology) conjugated with horseradish peroxidase at room temperature for 1 h. Protein banding was analyzed by an enhanced chemiluminescence method (Amersham Biosciences, Piscataway, NJ) and resolved on X-ray film (Eastman-Kodak, Rochester, NY) according to the manufacturer’s specifications.

Detection of Apoptosis. Assays for apoptosis detection and measurement were performed according to the manufacturer’s instructions (Annexin V-FITC Apoptosis Detection Kit; Oncogene Research, San Diego, CA). Briefly, phosphatidylserine is located on the cytoplasmic surface of the cell membrane in viable cells, but apoptosis-induced alterations in phospholipid organization result in cell surface externalization of phosphatidylserine, where it can be detected with the antigen-binding Annexin V. Annexin V-FITC conjugate and propidium iodide were used in a flow cytometric method to distinguish between viable early apoptotic and necrotic or late apoptotic cells. HeLa cells were plated at a density of 5 × 10^5 cells/dish, returned to the incubator for 12 h, treated with 17-AAG for 12 h, and exposed to IR. Cells were washed with PBS to remove the drug from the dishes, fresh medium was added, and cells were allowed to recover in the incubator for designated times after the treatment. Spent media were collected on ice, and attached cells were trypsinized and resuspended in the spent media. One-half million cells were transferred to a microcentrifuge tube, incubated with media-binding reagent and Annexin V-FITC, and centrifuged at 900 rpm for 5 min at 4°C. Media were removed and resuspended in binding buffer and propidium iodide. Samples were kept on ice in the dark until processing by flow cytometry. Data from 10,000 events were collected on a FACSCalibur cytometer (BD Immunocytometry, Fullerton, CA) and analyzed using CellQuest/ModFit software. Results for early and late apoptosis were added together to calculate the total amount of apoptosis as the sum of the total amount of apoptosis from two time points. Samples treated with camptothecin (4 µM, 42 h, Sigma, St. Louis, MO) were used as positive controls.

**In Vivo Mouse Tumor Models.** Mouse experimentation protocols were approved by the Animal Care and Use Committee (National Cancer Institute, NIH, Bethesda, MD). Female nude mice were supplied by the Frederick Cancer Research and Development Center Animal Production Area (Frederick, MD). The animals were received at 6 weeks of age, housed five per cage, and allowed autoclaved food and water ad libitum. Experiments were performed at 60–80 days of age (body weight, 22–27 g) and in accordance with principles outlined in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). Mice were housed in the Animal Research Facility (ARF) of the Laboratory of Cellular and Molecular Biology, National Institutes of Health, Bethesda, MD. Tumors were introduced by s.c. injection of HeLa single cell suspensions (1 × 10^6) in right hind legs and allowed to grow in volume for approximately 13 days to a size of 1 cm^3. Where designated, 17-AAG (125 mg/kg) was injected i.p. 16 h before IR treatment. SCC VII/ISF (SCC) was derived from spontaneous abdominal wall squamous cell cancer (obtained from Dr. T. Phillips, UCSF, San Francisco, CA) and propagated in C3H/Hen mice. For growth delay studies, 2 × 10^5 viable SCC VII cells suspended in 100 µL PBS were injected into the subcutaneous space of the right hind leg of ten week old female C3H/Hen. Tumor growth was followed until the diameter of tumor reached 1+− 0.1 cm as measured by caliper. Irradiation of the tumor was accomplished by securing
each animal in a specially designed Lucite jig that allowed treatment of the tumor bearing leg, yet lead-shielded the body from radiation. A Therapax DXT300 X-ray irradiator (Pantak, Inc., East Haven, CT) using 2.0 mm A1 filtration (300 KVp) at a dose rate of 1.9 Gy/min was used as the X-ray source. For these experiments 17-AAG was administered the night before irradiation on days 1 and 4 followed by 2 Gy of IR on days 2–6. Designated animals were given IR to the tumor using customized Lucite jigs that allow for immobilization and selective irradiation of the tumor-bearing leg; special care was taken to avoid irradiation of other body parts using lead shields designed specifically as a part of the jigs. After irradiation, the mice were returned to their cages, and tumors were measured three times each week thereafter to assess tumor response and regrowth. Tumors were not allowed to grow beyond 6 cm³.

RESULTS

GA and 17-AAG Are Cytotoxic and Radiosensitizing Agents in Vitro. It has long been hypothesized that HSP90 is a molecular target for anticancer agents, including IR; because of this, we hypothesized that GA and 17-AAG would enhance tumor cell death. Therefore, to address the IR component of this hypothesis, we used two agents (GA and 17-AAG) that inhibit HSP90 function to determine the relationship of HSP90 with increased IR-induced cytotoxicity. Where possible, clonogenic cell survival assays are considered the most sensitive measure of in vitro cell killing, and therefore, we used this technique in two human cervical model systems (HeLa and SiHa) to determine cell death induced by GA and 17-AAG either alone or in combination with IR.

Using clonogenic survival experiments, we first assayed the cytotoxic effects induced by GA and 17-AAG. After treatment with GA (100 nM) or 17-AAG (150 nM) for 24 h, HeLa and SiHa cells were trypsinized, plated into cloning dishes at measured cell densities, and left undisturbed at 37°C for colony formation from surviving cells. These experiments clearly demonstrate that both GA and 17-AAG are

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Fig. 1. Geldanamycin (GA) and 17-allylamino-17-demethoxygeldanamycin (17-AAG) are cytotoxic as well as radiosensitizing agents in vitro. A, clonogenic cell survival assays depicting HeLa and SiHa cells treated with either GA (100 nM) or 17-AAG (150 nM) for 24 h. After drug exposure, cells were trypsinized and plated at various densities into compartments of a 6-well plate. After 10 days, colonies were stained and scored, and the surviving fraction was graphed as a function of GA or 17-AAG doses. The results presented represent the average of at least three separate experiments. For these experiments 17-AAG was administered the night before irradiation on days 1 and 4 followed by 2 Gy of IR on days 2–6. Designated animals were given IR to the tumor using customized Lucite jigs that allow for immobilization and selective irradiation of the tumor-bearing leg; special care was taken to avoid irradiation of other body parts using lead shields designed specifically as a part of the jigs. After irradiation, the mice were returned to their cages, and tumors were measured three times each week thereafter to assess tumor response and regrowth. Tumors were not allowed to grow beyond 6 cm³.

Table 1. Effect of GA or 17-AAG on radiosensitization

Dose modifying factor (DMF) at 10% isosurvival and plating efficiency (PE) for cell lines treated with geldanamycin (GA) or 17-allylamino-17-demethoxygeldanamycin (17-AAG) followed by ionizing radiation at 100 and 150 nM, respectively.
considerably cytotoxic to cervical tumor cell lines and that the effect of each drug is slightly different between cell lines (Fig. 1A). Particularly, 17-AAG is significantly more cytotoxic to HeLa than to SiHa cells, whereas SiHa cells are more sensitive to GA. However, this difference in the relative effect of each agent does not diminish the overall cytotoxic response. To address the effect of HSP90 functional inhibition on IR-induced cytotoxicity, HeLa cells were pretreated with 17-AAG for 24 h before exposure to IR at 2, 4, and 6 Gy (Fig. 1B). The dose-modifying factor and plating efficacy are shown (Table 1). These results demonstrate approximately 60% cell killing (or 40% cell survival) with 17-AAG (150 nM) alone and 25% (75% cell survival) cell killing with IR (2 Gy). Therefore, the expected additive cell survival after exposure to these two modalities would be roughly 30% (i.e., $0.40 \times 0.75 = 0.30$). However, cell survival after exposure to both 2 Gy of IR and 17-AAG was only 5% (or 95% cell killing); this value surpasses 99% killing at 4 Gy of IR with 17-AAG and 99.9% killing at 6 Gy of IR with 17-AAG (Fig. 1B). These results are indicative of a synergistic or superadditive radiosensitizing cytotoxic effect when 17-AAG is coupled with IR.

**GA and 17-AAG Are Dose- and Time-Dependent Radiosensitizing Agents.** To address the possibility of dose-dependent effects on cytotoxicity, HeLa and SiHa cells were treated with increasing concentrations of GA and 17-AAG and analyzed for cytotoxicity using clonogenic assays. Cell killing patterns reflect an increasing dose response in HeLa (Fig. 2A) and SiHa (Fig. 2B) cells exposed to 50 and 100 nM GA for 24 h. At concentrations of GA below 50 nM,
only mild radiosensitization was observed (data not shown). It should be noted that data in these curves have been normalized for the cytotoxic effect of drug alone to reflect only the radiosensitizing effect. Identical experiments were performed using 75 and 150 nM 17-AAG (Fig. 3, A and B) and show similar results, with no effect observed below 75 nM 17-AAG (data not shown). The dose-modifying factor and plating efficacy results for Figs. 2 and 3 are shown (Table 1).

Finally, HeLa cells were exposed to 150 nM 17-AAG for various time points before exposure to IR. The results of these experiments demonstrate that 4–6 h of drug exposure is the minimum necessary to induce radiosensitization, and the maximum radiosensitizing effect occurs at 12 h of exposure (Fig. 4). Similar results were observed with GA and in SiHa cells (data not shown). Because the effect of 17-AAG in HeLa cells at 12 and 24 h is nearly identical (compare Fig. 3A, ▲, with Fig. 4, ▲), experiments were designed for exposure times of 12 h and concentrations of 17-AAG in the range of 75–150 nM. These experiments clearly demonstrate that GA and 17-AAG are significant dose- and time-dependent cytotoxic and radiosensitizing agents.

17-AAG Inhibits Multiple Prosurvival Signaling Pathways in HeLa Cells. GA and 17-AAG are unique agents in that, although they are directed toward a specific molecular target (HSP90), they simultaneously inhibit multiple signaling pathways on which cancer cells depend for growth and survival (13–16). Many of these targets are involved in signaling pathways used by tumor cells to protect against both endogenous and exogenous signals that induce several different modes of cell death (4, 7–9). Therefore, we examined a series of signaling pathways using a combination of antibodies against factors identified previously. Specifically, using KPKS and conventional Western blot assay, we observed expression of proteins from pathways that should be susceptible to GA and 17-AAG. To initially determine any signaling factors altered by exposure to 17-AAG in cervical tumor cells, we used the KPKS, thereby identifying several signaling factors that decrease after exposure to 17-AAG. These
protein kinases, as well as those with unchanged expression levels after 17-AAG exposure, are listed (see supplemental data available via the Cancer Research web site).

Validations of the KPKS results and the additional targets are shown in Fig. 5A. We observed decreases in expression of several signaling factors after 17-AAG (50–150 nM for 12 h) exposure, including Raf-1, ERK1/2, Akt1, and HER-2/neu. The timing, concentrations, and kinetics of the decreased protein levels of these signaling proteins are consistent with the results presented for 17-AAG-induced radiosensitization (Fig. 4) and are significant because Raf-1, ERK1/2, Lyn, and Akt1 have been shown previously to protect against IR-induced cytotoxicity in other cell types (25, 45, 46). In addition, we observed decreases in several signaling and/or prosurvival factors that have not been shown previously to be sensitive to 17-AAG, including cAMP-dependent protein kinase, Glut-1, and VEGF. Interestingly, Lyn, Glut-1, and VEGF have been shown to play a role in how tumor cells respond to the cytotoxicity of IR (45, 46). In addition, two kinases, Janus-activated kinase 1 and p38, which were not altered by 17-AAG exposure, were added as controls (Fig. 4). These results validate what has been shown previously for Raf-1, ERK1/2, Akt1, and HER-2/neu (3, 4, 9–16) and identify several proteins not shown previously to be sensitive to degradation after GA exposure. Because these proteins do not appear to require HSP90 for stabilization and or trafficking, it is possible that the decreased protein levels are attributable to the indirect effects via the inhibition of upstream factors regulating their expression. For example, transcription of both Glut-1 and VEGF is activated by hypoxia-inducible factor-1, a factor shown to be inhibited by GA (47), suggesting a possible mechanism for the decrease in protein levels observed after exposure.

After establishing a dose response for these factors, several were also validated for a time-dependent relationship of expression with 17-AAG exposure. Similar to the results for radiosensitization, a significant decrease in protein levels was observed between 4 and 8 h (Fig. 5B), which is consistent with that observed in Fig. 4. Finally, several of these factors have phospho-specific antibodies that give insight into protein function. Consistent with the decrease in protein levels, a decrease in p-Raf, p-ERK1/2, and p-HER-2/neu were observed (Fig. 5C).

17-AAG Cytotoxicity Is Programmed Cell Death Dependent and Independent. To address the mode of cell death induced by GA and 17-AAG, HeLa cells were treated as described above and assayed for cell death attributable to apoptosis. Specifically, HeLa cells were treated with 17-AAG alone (150 nM for 12 h), IR (2 and 4 Gy), or 17-AAG for 12 h before exposure to IR. After exposure, programmed cell death was measured at two time points using an Annexin V-FITC apoptosis detection protocol to distinguish between viable early and late apoptotic and necrotic cells. Results for early and late apoptosis were added together to calculate the total amount of apoptosis. These results demonstrate that exposure to 17-AAG alone increased rates of apoptosis to roughly 20%; IR exposure alone caused increases to 8% and 16% at 2 and 4 Gy of IR, respectively (Fig. 6A). When cells were pretreated with 17-AAG for 12 h prior to IR, an increase was observed to 44% and 52% at 2 and 4 Gy of IR, respectively. When these results are compared with those observed in Fig. 1, it is evident that a programmed/apoptotic mode of cell death cannot entirely account for the cytotoxicity induced by 17-AAG, either alone or in combination with IR. This observation suggests that other mechanisms of 17-AAG-induced cytotoxicity and radiosensitization are also induced.

Given that multiple prodeath and prosurvival intracellular factors regulate programmed cell death, we next determined the specific

Fig. 4. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is a time-dependent radiosensitizing agent in HeLa cells in vitro. Clonogenic cell survival assays depict the effects of 17-AAG pretreatment for 4, 6, or 12 h on radiation response of HeLa cells. After 17-AAG treatment (150 nm), cells were exposed to ionizing radiation (IR) doses as indicated (2, 4, 6, or 8 Gy), trypsinized, and plated at various densities into compartments of a 6-well plate. After 10 days, colonies were stained and scored, and the surviving fraction was plotted versus IR dose. Curves are normalized to account for drug-induced cytotoxicity. The results presented represent the average of three separate experiments. Error bars around data points represent 1 SD about the arithmetic mean, and statistical significance was established by Student’s t test (P < 0.05).
17-AAG Is a Cytotoxic and Radiosensitizing Agent In Vivo.

Although in vitro data provide an important preliminary background to advance the potential use of new anticancer agents, these results must be confirmed and validated in an in vivo tumor model system before clinical trial. Therefore, we established a HeLa xenograft protocol to use as an in vivo model system and introduce tumors in the flanks of mice. Once the tumors reached 1 cm³ in size, the mice were separated into the following four groups: (a) control, untreated mice; (b) mice treated i.p. with 150 mg/kg 17-AAG; (c) mice exposed to 12 Gy of IR; and (d) mice treated i.p. with 125 mg/kg 17-AAG for 1 h followed by 12 Gy of IR (Fig. 7A). Local irradiation was delivered to the tumor site only, and tumor sizes were monitored three times each week after treatment. The experiments demonstrate that IR and 17-AAG alone induce a tumor growth delay that is significantly increased when both anticancer agents are added. The results of these experiments confirm the in vitro results that 17-AAG is moderately cytotoxic as well as a strong radiosensitizing agent.

The clinical application of therapeutic IR relies on fractionated doses of radiation that, for definitive therapy, are given daily for several weeks. Therefore, we determined the anticancer effects of fractionated radiation therapy combined with twice weekly 17-AAG. For these experiments, 17-AAG was administered during the evenings of days 1 and 4, followed by fractionated irradiation (2 Gy/fraction) on days 2–6. The time required to determine the appropriate dose and timing to establish a fractionated HeLa cell xenograft tumor model system is extensive; because of this, we used an existing model system with SCCVII squamous carcinoma cells that have properties similar to cervical squamous carcinoma cells. As above, xenograft tumors were allowed to grow to 1 cm³, and mice were separated into four treatment groups. These results clearly show a significant growth delay when two doses of 17-AAG are combined with a 5-day fractionated course of radiation (Fig. 7B). The results of these experiments confirm and extend our in vitro results to a fractionated weekly scheme consistent with clinical treatment protocols.

17-AAG Is Preferentially Cytotoxic in Transformed Cells Versus Immortalized Cells. One of the major shortcomings of anticancer and sensitizing agents is increased cell killing of normal cells in addition to their malignant counterparts. Therefore, uncovering a way to preferentially treat cancer cells while leaving adjacent normal tissue unharmed would be a significant advancement in therapy. In this regard, cervical tumors and the genetics of papilloma viruses provide ideal model systems. The HPV16 E6 and E7 genes, which are expressed in cervical malignancies, play different roles in the initiation process leading to cellular transformation. For example, most tumors derived from the E7-transgenic mice are benign, whereas the majority of the tumors from the E6-transgenic mice are malignant (48–50); mice expressing both E6 and E7 are frankly malignant. In tissue culture systems, similar results are also observed, i.e., E7-expressing...
fibroblasts demonstrate increased proliferation but not other morphology consistent with malignant transformation (51, 52). However, cell lines expressing E6 and, to a greater extent, E6 and E7 display a frankly malignant phenotype as measured by contact inhibition, proliferation in soft agar, and growth in nude mice (51–53). As such, cell lines expressing HPV16 E7, E6, and both E6 and E7 provide a model system, albeit imperfect, that is similar to normal (immortalized) cells, benign transformed (HPV16 E6-expressing) cells, and malignant transformed (HPV16 E6-expressing and HPV E6/E7-expressing) cells, respectively.

Therefore, a series of fibroblast cell lines (HFF3) that were transfected with and selected for either empty vector (LXSN), viral expression vectors encoding HPV16 E6 (E6), E7 (E7), or both E6 and E7 (E6/E7) were treated with 17-AAG, IR, or both. Although not a perfect model system to address our research question, these four cell lines resemble nontransformed or benign (LXSN and E6), malignant (E6), and frankly malignant cells (E6/E7) and provide a cervical tumor model system to determine the in vitro therapeutic index of new anticancer agents such as 17-AAG. The protein levels of E6 and E7 in these cells have been shown previously (54, 55).

17-AAG treatment alone had little effect on clonogenicity in the LXSN- or E7-overexpressing fibroblasts, but the E6- and E6/E7-overexpressing cells demonstrated significant cytotoxicity (data not shown), consistent with that observed in HeLa and SiHa cells (Fig. 1A). When these cells were treated with 17-AAG before IR, the LXSN- or E7-overexpressing fibroblasts exhibited very little drug-induced radiosensitization, but the E6-overexpressing cells and, to a great extent, the E6/E7-overexpressing cells demonstrated significant 17-AAG-induced radiosensitization (Fig. 8). The dose-modifying factor and plating efficacy results for Fig. 8 are shown (Table 1). These experiments demonstrate that the E6/E7-overexpressing fibroblasts exhibit a significant degree of radiosensitization (Fig. 7B), consistent with HeLa and SiHa cells (Figs. 2 and 3), whereas little effect is observed in LXSN- or E7-overexpressing fibroblasts. Together, the results of these experiments suggest that the cytotoxic and radiosensitizing effect of 17-AAG is more significant in transformed than nontransformed cells in vitro and imply a potentially favorable therapeutic index for the use of these agents.

DISCUSSION

Novel therapeutic agents, such as Iressa and Gleevec, inhibit specific intracellular factors in tumor cells and therefore offer considerable promise in the arena of anticancer therapy (56–59). However, tumors exhibit considerable genetic plasticity, and because of this, they may quickly adapt to the cytotoxicity of such endogenous anticancer agents with finite molecular targets (4, 60). The resulting potential for resistance to single treatment modalities emphasizes the
need for combining anticancer agents or using those with multiple targets of action (3, 59). GA and 17-AAG represent two such agents in that both alter the intracellular function of multiple signaling factors shown to up-regulate prosurvival pathways (6–10), including those that protect tumor cells from the cytotoxicity of IR (45, 46). Additional lines of evidence show that altering the functionality of intracellular HSPs either via thermal stress or exposure to nonsteroidal anti-inflammatory agents sensitizes tumor cells to the cytotoxicity of therapeutic IR (41, 42, 61). GA and 17-AAG, through their binding to the catalytic subdomain of HSP90, similarly alter the chaperone functionality of key HSPs (3–5). Therefore, it is plausible that GA and 17-AAG, through abrogation of prosurvival pathways and alteration of HSP90 conformation, may increase tumor cell death when combined with IR.

In in vitro cervical tumor models using HeLa or SiHa cells, both GA and 17-AAG were shown to be cytotoxic and radiosensitizing in a manner and degree similar to that observed for hyperthermic radiosensitization (Figs. 2 and 3). In addition, the effects of GA and 17-AAG were concentration- and time-dependent, with a minimum of 6 h and 75 nM concentrations of 17-AAG necessary to induce significant radiosensitization. Although the mechanism of cell death appears to be complex, both programmed cell death-dependent and -independent pathways are likely involved. To begin to address this idea, over 100 programmed cell death and intracellular signaling factors were examined through several techniques. Similar results were obtained with GA (data not shown; see supplemental data available via the Cancer Research web site). These results provide several possible molecular targets to be further examined and even

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**Fig. 7.** 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is a cytotoxic and a radiosensitizing agent in vivo using both single and fractionated exposures. A, HeLa cell tumors were separated into four treatment groups: (a) control, untreated mice; (b) mice treated i.p. with 150 mg/kg 17-AAG (c) mice exposed to 12 Gy of ionizing radiation (IR); and (d) mice treated i.p. with 150 mg/kg 17-AAG followed by 12 Gy of IR 16 h later. Tumors sizes were measured three times each week, and the data presented represent the average of at least 10 mice/treatment group. B, SCCVII squamous carcinoma cells were separated into four groups as described above, except 125 mg/kg 17-AAG was given on the evening of days 1 and 4 (groups 2 and 4), and the mice were irradiated with 2 Gy of IR on days 2–6 (group 4). Experiments were repeated at least twice. Error bars around data points represent 1 SD about the arithmetic mean.
more potential molecular markers that are currently being validated in vitro and in vivo.

The mechanism of 17-AAG cytotoxicity and radiosensitization is likely complex and inclusive of multiple pathways, including those related to programmed cell death, cell cycle regulation, mitotic catastrophe, and others. This hypothesis is consistent with the alterations in prosurvival pathways (Fig. 5, A and B) and cell death (Fig. 6B) factors. Experiments involving 17-AAG treatment concentration and duration confirm that the decreases in immunoreactive protein levels correspond to the radiosensitizing effects reflected in Fig. 4A. Several of the prosurvival factors degraded in response to 17-AAG have been identified previously (i.e., Akt1, ERK, HER-2/neu, Raf-1, VEGF, and Lyn) to protect against IR-induced cell death (45, 46). Furthermore, we show evidence that two additional signaling factors, Lyn and Glut-1, which have also been shown to be protective against IR or oxidative stress (46), are also decreased in cells treated with 17-AAG. Several of these factors (Akt1, ERK, HER-2/neu, Raf-1, and Lyn) have been shown previously to be sensitive to 17-AAG- or GA-induced degradation (3, 4), whereas several others (VEGF and Glut-1) have not been shown to be client proteins of HSP90, suggesting an alternative process. Both these genes are regulated by a series of intercommunicating signaling pathways, several of which are disrupted by GA and 17-AAG, including hypoxia-inducible factor-dependent transcriptional control of VEGF (47).

An intriguing aspect of using 17-AAG as a radiosensitizing agent, and one that perhaps offers the most potential for therapeutic utility, is the differential effect in normal and transformed cells. This idea was addressed using a series of permanently transfected fibroblast cell lines expressing the control vector, HPV16 E6, E7, or both E6 and E7 genes. In general terms, these cell lines provide a reasonable in vitro model for normal, transformed, benign, and frankly malignant tissues or cells, respectively. Survival assays with 17-AAG and/or IR demonstrated that 17-AAG enhancement of IR-induced cell death occurred only in the HPV16 E6-expressing and HPV16 E6/E7-expressing cells. These preliminary results suggest a differential cytotoxic effect depending on transformation status, conferring a possible favorable therapeutic index in patients.

Although the mechanism for the differential effect of 17-AAG on transformed versus immortalized cells is unknown, we would suggest two possibilities. First, it has been shown recently that HSP90 derived from tumor cells has a 100-fold higher binding affinity for 17-AAG attributable to the presence of HSP90 in multichaperone complexes.
with high ATPase activity. In contrast, HSP90 from normal cells is in active protein levels in the E6- and E6/E7-expressing cell lines as compared to geldanamycin. In contrast, HSP90 from normal cells is in active protein levels in the E6- and E6/E7-expressing cell lines as compared to geldanamycin. In contrast, HSP90 from normal cells is in active protein levels in the E6- and E6/E7-expressing cell lines as compared to geldanamycin.


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Geldanamycin and 17-Allylamino-17-demethoxygeldanamycin Potentiate the *in Vitro* and *in Vivo* Radiation Response of Cervical Tumor Cells via the Heat Shock Protein 90-Mediated Intracellular Signaling and Cytotoxicity

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