Efficacy of the HSP90 inhibitor 17-AAG in human glioma cell lines and tumorigenic glioma stem cells

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Glioblastoma multiforme (GBM) arises from genetic and signaling abnormalities in components of signal transduction pathways involved in proliferation, survival, and the cell cycle axis. Studies to date with single-agent targeted molecular therapy have revealed only modest effects in attenuating the growth of these tumors, suggesting that targeting multiple aberrant pathways may be more beneficial. Heat-shock protein 90 (HSP90) is a molecular chaperone that is involved in the conformational maturation of a defined group of client proteins, many of which are deregulated in GBM. 17-allylamino-17-demethoxygeldanamycin (17-AAG) is a well-characterized HSP90 inhibitor that should be able to target many of the aberrant signal transduction pathways in GBM. We assessed the ability of 17-AAG to inhibit the growth of glioma cell lines and glioma stem cells both in vitro and in vivo and assessed its ability to synergize with radiation and/or temozolomide, the standard therapies for GBM. Our results reveal that 17-AAG is able to inhibit the growth of both human glioma cell lines and glioma stem cells in vitro and is able to target the appropriate proteins within these cells. In addition, 17-AAG can inhibit the growth of intracranial tumors and can synergize with radiation both in tissue culture and in intracranial tumors. This compound was not found to synergize with temozolomide in any of our models of gliomas. Our results suggest that HSP90 inhibitors like 17-AAG may have therapeutic potential in GBM, either as a single agent or in combination with radiation.
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Malignant gliomas are the most common type of brain tumors, occurring in 5–7 out of 100,000 individuals per year.[1] The most common and malignant form of gliomas is known as glioblastoma multiforme (GBM), which accounts for approximately 60%–70% of the cases. GBM is characterized by highly aggressive growth, invasiveness, and poor prognosis. Current standard-of-care therapy for GBM consists of surgical resection, followed by treatment with radiation therapy (RT) and the chemotherapeutic alkylating agent temozolomide (TMZ), the combination of which confers a median survival period of only 14.6 months.[2]

Although the cellular origins of GBMs still have not been definitively identified, there is mounting evidence

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that the malignant phenotype is propelled by a subset of cells with stemlike qualities. Indeed, recent work has revealed the existence of a small population of cells within GBMs that exhibit characteristics of stem cells, enabling them to self-renew and give rise to the heterogeneous mass of cells that make up these tumors.[3–6] The origin of these glioma stem cells is still debated; they arise either from endogenous stem cells, which have acquired transforming events, or from more differentiated glioma cells, which have accrued a panel of mutations that confer stem cell characteristics upon them.[7]

On a molecular level, GBMs exhibit a number of genetic and signaling abnormalities that ultimately lead to the uncontrollable growth, invasiveness, and angiogenesis that are characteristic of these tumors. Included among these is an abnormal activation of the Akt survival and growth signal transduction pathway. Approximately 30%–40% of GBMs exhibit mutations within the phosphatase and tensin homologue (PTEN) deleted on chromosome 10 tumor suppressor gene.[8] PTEN is a lipid phosphatase whose activity restricts growth and survival signals mediated by the phosphoinositide-3 kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway.[9] Loss of functional PTEN in tumor cells leads to constitutive activation of Akt and hence uncontrollable growth. In addition to PTEN mutations, 5%–27% of GBMs have gain-of-function mutations of PI3K.[10,11] As with PTEN mutations, these activating PI3K mutations result in constitutive activation of Akt signaling.

In addition to mutations that activate the Akt survival pathway, about 40% of GBMs have amplified epidermal growth factor receptor (EGFR).[12–15] Overactivity of this receptor results in cellular proliferation, tumor invasiveness, increased angiogenesis and tumor cell motility, and inhibition of apoptosis.[16] In GBM tumors exhibiting this gene amplification, 40% also express a constitutively autophosphorylated variant of the EGFR that lacks the extracellular ligand-binding domain known as EGFR variant III (EGFRvIII),[12,17–19] which has been shown to enhance tumorigenicity by increasing proliferation and decreasing apoptosis in brain tumors.[18,20] Constitutive activation of this receptor tyrosine kinase results in overactivation of both the Ras/Raf/MAPK (mitogen-activated protein kinase) and the PI3K Akt signal transduction pathways and contributes to progression of the disease.[21–24]

GBMs also exhibit alterations in the platelet-derived growth factor (PDGF) signaling pathway. Coexpression of the PDGF-A and -B ligands and the PDGF-α receptor are common in most glioma cell lines, primary cells, and fresh surgical isolates, whereas the PDGF-β receptor is frequently expressed in glioma and endothelial cells.[25–27] Such coexpression of receptors and ligands within the same cells leads to autocrine loops, which can drive cell proliferation. These autocrine loops are thought to both initiate the transformation process and contribute to the transformed phenotype within these cells.[28–30] PDGF receptor (PDGFR) mRNA expression, which is normally not detectable in the vessels of the normal brain, is also upregulated in proliferating endothelial cells of the tumor vasculature,[31] suggesting that the PDGF produced by glioma cells would also be able to bind neighboring endothelial cells to promote angiogenesis.

Heat-shock protein 90 (HSP90) is a molecular chaperone that is involved in regulating cellular processes such as protein folding, protein degradation, and signal transduction. Within this context, HSP90 can bind an array of well-characterized client proteins to regulate their conformation, stability, phosphorylation states, and fates. These client proteins include many oncogenic signaling proteins such as ErbB2, EGFR, Raf, MEK, FAK, Flt3, PDGFR, Cdk-4, -6, and -9, Akt, and mutated p53 and Kit.[32] Significantly, many of these proteins are the ones that are mutated or overexpressed in GBM.

17-allylamino-17-demethoxygeldanamycin (17-AAG), a benzoquinone antibiotic derived from geldanamycin, is an HSP90 inhibitor that has been shown to promote growth inhibition in a number of tumor cell lines, as well as antitumor activity in vivo and in preclinical models.[33–43] HSP90 inhibitors act by binding to HSP90 and inducing proteasomal degradation of HSP90 client proteins.[44–49] The ability of HSP90 inhibitors to simultaneously target multiple signal transduction pathways involved in proliferation and survival makes these compounds ideal therapeutic candidates in cancers such as GBM, whose etiology is so multifaceted.

Although HSP90 is expressed in most cells, the HSP90 exhibits a 100-fold higher binding affinity for 17-AAG in tumor cells than in normal cells,[50] enabling selective drug targeting of tumor cells. Indeed, selective accumulation of HSP90 inhibitors has been noted in a number of in vivo human tumor xenografts.[51,52] Moreover, the highly lipophilic nature of this compound, which would enable it to penetrate the blood–brain barrier, makes it a particularly promising therapy for targeting GBMs.

Clinical studies to date with molecular agents directed at single targets have produced only modest activity, due to coactivation of multiple tyrosine kinases and the presence of redundant signaling pathways.[53] Given the ability of 17-AAG to target multiple signal transduction pathways that are aberrant in GBM, we were interested in examining its effects on glioma cell growth and survival both as a single agent and in combination with RT and TMZ, which are the standard therapies for GBM. Toward this end, three models of gliomas were tested, consisting of genetically diverse human glioma cell lines, genetically defined tumorigenic murine neural stem cells, and human glioma stem cells that have been isolated from primary GBM tumor specimens. Within these models, we show that 17-AAG is able to inhibit the growth of a large panel of human gliomas, irrespective of PTEN, p53, and EGFR status, as well as both the murine and human tumorigenic glioma stem cells. In all cases, growth inhibition with 17-AAG is accompanied by the degradation of HSP90 client proteins that are involved in the pathology of GBM. We additionally show that 17-AAG is able to inhibit the in vivo growth of orthotopic U87 and human glioma stem cell tumors. Finally, we show that 17-AAG acts as a radiosensitizer both in vitro and in vivo but does not synergize with TMZ. Our results identify 17-AAG as a potentially promising agent for the treatment of GBM.

Materials and Methods

Cell Culture

U87 (American Type Culture Collection [ATCC], Manassas, VA, USA), U343 (provided by the laboratory of Ab Guha, Hospital for Sick Children, University of Toronto, ON, Canada), U373 (provided by the laboratory of Charles Stiles, Dana-Farber Cancer Institute, Boston, MA, USA), U118 (ATCC), U87-LucNeo glioma lines, and Balb/c3T3 fibroblasts (provided by the laboratory of Charles Scherr, St Jude Children's Research Hospital, Memphis, TN, USA) were incubated in 10% bovine calf serum in Dulbecco's modified Eagle's medium supplemented with 0.1 units/ml penicillin, 0.001 µg/ml streptomycin, and 4 mM glutamine. A172 (ATCC), T98G (ATCC), U138 (ATCC), LN428 (provided by the laboratory of Rosalind Segal, Dana-Farber Cancer Institute), LN827 (provided by the laboratory of Andrew Kung, Dana-Farber Cancer Institute), and U87-EGFRvIII glioma cells were incubated in a similar medium consisting of 10% fetal bovine serum rather than bovine calf serum. U87-LucNeo and U87-EGFRvIII cells were generated by retroviral infection of the parent U87 cells. The LucNeo construct (provided by the laboratory of Andrew Kung, Dana-Farber Cancer Institute) is described in Rubin et al.[54] The EGFRvIII construct (originating from the laboratory of Ronald DePinho, Dana-Farber Cancer Institute) is described in Bachoo et al.[55] U87-LucNeo and U87-EGFRvIII cell cultures were supplemented with 400 µg/ml G418 to maintain the expression of luciferase and EGFRvIII. Cells were maintained in a humidified incubator at 37°C with 10% CO_2. Murine neural stem cells (NSCs) were obtained by dissecting mouse E14.5 Ink4a/ARF$^{-/-}$ or wild-type ganglionic eminences, triturating the cells, resuspending them in NSC medium as described elsewhere,[56] plating them on nonadherent poly(2-hydroxyethylmethacrylate)–coated plates, and allowing them to grow into neurospheres. Ink4a/ARF$^{-/-}$ neurospheres were further infected with an EGFRvIII retrovirus, followed by selection with puromycin. Cells were maintained in a humidified incubator at 37°C with 5% CO_2. Cells were always maintained in log phase growth and used only at low passage number. Human glioma stem cells were isolated from glioma surgical specimens and passaged subcutaneously in mice in order to maintain the genetic integrity of the cells. BT37 glioma stem cells were obtained from Brigham and Women's Hospital (Boston, MA, USA) following approval from its institutional review board for collection and use of fresh and discarded human tumor tissue. BT70 glioma stem cells were obtained from the University of California, San Francisco (San Francisco, CA, USA), courtesy of C. David James. For each of these lines, after frozen section diagnosis of malignant glioma by the attending neuropathologist, tumor material was grossly dissected from the tissue sample. Portions of the tumors were collected in chilled media with antibiotics for the studies described here, and other portions were allocated for paraffin embedding for histological diagnosis and for genotyping. Tissue was washed in artificial cerebrospinal fluid (CSF) and acutely dissociated as described previously[5] using a modified tissue dissociation medium containing collagenase IV (1 mg/ml; Worthington, St. Louis, MO, USA), hyaluronidase (0.67 mg/ml; Sigma, Lakewood, NJ, USA), DNase I (0.4 mg/ml; Worthington), kynurenic acid, and *N*-acetylcysteine (60 µg/ml; Sigma). For all experiments reported, animal husbandry was performed according to protocols approved by the Dana Farber Cancer Institute's Animal Care and Use Committee. Immunocompromised spontaneous mutant T and B cell–deficient (SCID) homozygous mice were obtained from Taconic Inc. (Hudson, NY, USA; model ICRSC-M). Dissociated tumor cells were injected subcutaneously in SCID mice and followed for tumor growth. When tumors reached approximately 1 cm, tumors were disaggregated and cells were counted and then grown in serum-free media with EGF, fibroblast growth factor, and leukemia inhibitory factor as described previously[4,57] to form tumor neurospheres.

Characterization of Glioma Cell Lines

Characterization of p53 and PTEN mutations within our cell lines and human glioma stem cells was done by PCR amplification of genomic DNA extracted from cells at low passage number. Genomic DNA was extracted using Qiagen's Genomic DNA Extraction Kit. Primer pairs were generated to span each exon of both genes, as well as flanking intronic sequence, and PCR products were purified using Qiagen's (Valencia, CA, USA) QIAquick PCR purification kit. PCR products were subsequently sequenced and analyzed for mutations. EGFR protein levels were assessed by Western blot analysis (see below) on protein lysates harvested from cell lines at low passage.

Compounds

17-AAG was generously provided by Kosan Bioscience, Inc. (Hayward, CA, USA) in the form of a lyophilized powder. 17-AAG in this state was stored in the dark at room temperature and reconstituted in dimethyl sulfoxide (DMSO) before use. Temodar (TMZ) was obtained from a pharmacy and resuspended immediately prior to use in DMSO for in vitro assays and in 0.5% carboxymethyl cellulose and 0.1% Tween 80 for in vivo experiments.

Cell Viability Assays

U138, A172, U118, U343, and U373 cells and glioma stem cells were plated in 96-well plates at a density of 2,000 cells/well, while U87, U87-EGFRvIII, T98G, LN428, LN827, and Balb/c3T3 cells were plated at a density of 1,000 cells/well. The following day, triplicate wells were treated with increasing concentrations of 17-AAG and redosed 48 h later. Four days after the first treatment, cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell

proliferation assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA), and plotted as surviving fraction of cells relative to the no-treatment condition. For combinatorial assays with 17-AAG and TMZ, cells were either dosed once simultaneously for concurrent assays or dosed alone with TMZ for 24 h followed by 17-AAG treatment for sequential assays.

Clonogenic Assays

U87 cells were plated at densities of 500–7,500 cells per 10-cm plate. Cells were allowed to adhere for 8 h and were then treated with either 0, 50, or 100 nM 17-AAG for 16 h. Cells were then irradiated with graded doses of γ-radiation, and media were changed 1 h following irradiation. Six days later, colonies were fixed and stained with crystal violet, and the number of colonies containing at least 50 cells were scored. The surviving fraction of cells was calculated at each dose of 17-AAG and normalized to the radiation-naive condition. Sensitizer enhancement ratios were calculated as ratios of observed growth inhibition at each dose of radiation alone, relative to growth inhibition observed at the equivalent dose of radiation combined with 17-AAG treatment.

Western Blot Analyses

Cells were rinsed once with cold phosphate-buffered saline (PBS), lysed with radioimmunoprecipitation assay (RIPA) buffer (20 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES] [pH 8], 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 2.5 mM ethylene glycol tetraacetic acid (EGTA), 2.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM sodium orthovanadate) at 4°C for 15 min on a rocker, followed by centrifugation at 12,000*g* for 15 min at 4°C. Protein supernatants were collected and quantified using Bio-Rad (Hercules, CA, USA) Protein Assay reagent. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P PMVF nitrocellulose membrane (Millipore, Billerica, MA, USA). Blots were rinsed once in Tris-buffered saline Tween (TBST), blocked 1 h at room temperature in 5% Blotto, then incubated with primary antibodies in 5% bovine serum albumin overnight at 4°C. Anti-EGFR, anti-Akt, anti-pAkt, and anti-pRb (Cell Signaling, Danvers, MA, USA) were used at a 1:1,000 dilution; anti-PDGFR-α (Santa Cruz, Santa Cruz, CA, USA) and anti-Ras (Ab-3) (Oncogene Research Products, San Diego, CA, USA) were used at a dilution of 1:500; anti-ERK 1/2 and anti-ACTIVE MAPK (Promega) were used at a 1:5,000 dilution; and anti-β-actin (Abcam, Cambridge, MA, USA) was used at a dilution of 1:10,000. The blots were then washed three times with TBST, then probed with horseradish peroxidase-conjugated secondary (Pierce, Rockford, IL, USA) at a dilution of 1:5,000 at room temperature in 5% Blotto, washed three more times in TBST, and then developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

U87 Intracranial Implants

All protocols involving animals were performed in accordance with an approved Institutional Animal Care and Use Committee protocol. U87 cells stably infected with a luciferase expression vector were resuspended in sterile PBS at a density of 3×10^6 cells/ml, and 30,000 cells were stereotactically implanted in the right cerebral cortices of nude athymic (nu/nu) or SCID mice (male; Charles River Laboratories, Shrewsbury, MA, USA). One week following implantation, animals received 58 mg/kg D-luciferin, and tumor implantation was verified by luciferase bioluminescence using the Xenogen (Hopkinton, MA, USA) In Vivo Imaging System. Animals with established tumors were assigned to their respective treatment groups, and treatment began 10 days after tumor implantation. For efficacy as a single agent, animals received either vehicle (90% saline, 0.05% Tween 20, and 9.95% DMSO) or 80-mg/kg 17-AAG resuspended in vehicle intraperitoneally for 5 days on, 2 days off, and then 5 days on. Tumor growth was assessed weekly by luciferase bioluminescence. For efficacy in conjunction with RT, 17-AAG and vehicle were administered as described above. Animals in the RT groups received 2.5 Gy radiation for 2 days. Animals receiving both 17-AAG and RT received radiation on days 2 and 4 of 17-AAG treatment, 4 h after the 17-AAG treatment. For efficacy in conjunction with RT and TMZ treatments, animals received 17-AAG, vehicle, and radiation at doses described above. Animals in the TMZ group received 5 mg/kg/day TMZ for 2 days by oral gavage. The 17-AAG and TMZ were dosed either 4 or 1 h prior to radiation, respectively.

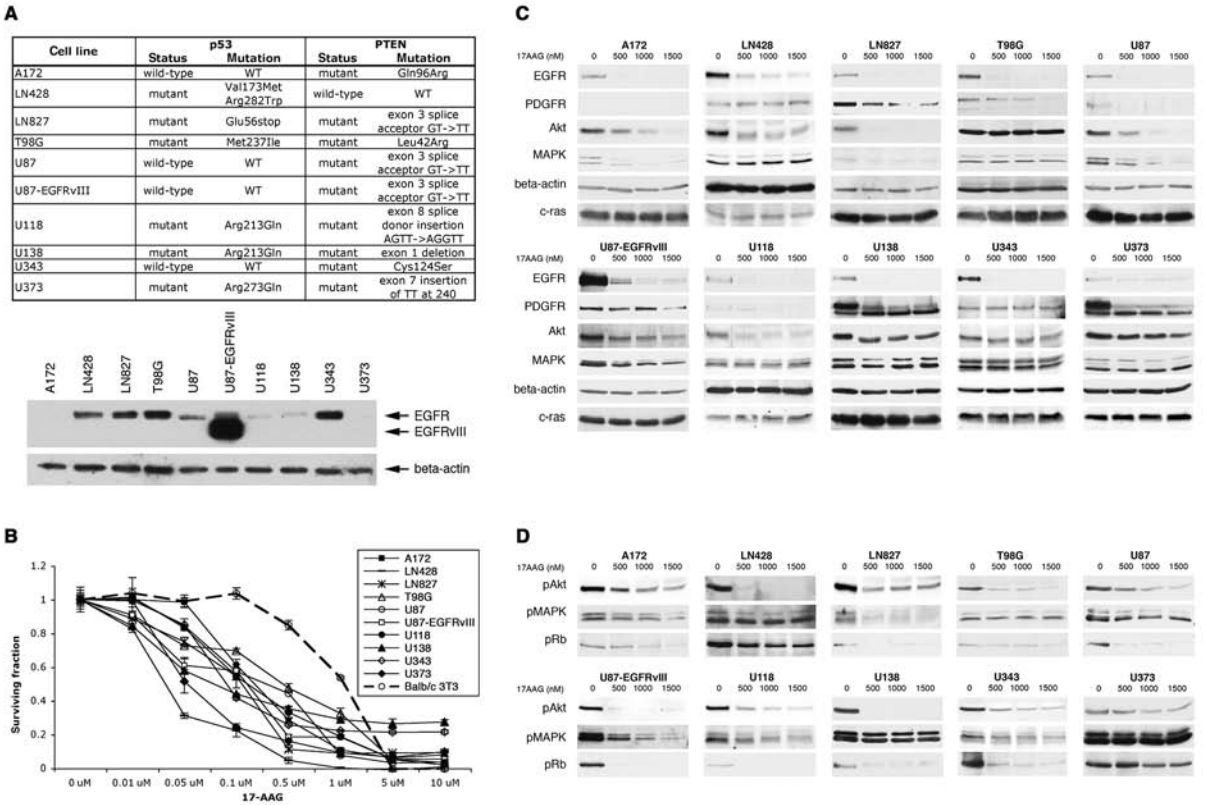
Human NSC Intracranial Implants

Primary human glioma stem cells grown in the flanks of SCID mice were dissected out, dissociated, plated, and treated immediately in vitro with either 10 μM 17-AAG or DMSO for 24 h. Cells were then manually dissociated and the number of viable cells was assessed by trypan blue exclusion. Cells for injection were resuspended in PBS at a concentration of 20,000 viable cells/5 μl, and 5 μl was injected into the right striatum as previously described.[57] Animals were placed into survival or time point cohorts and were sacrificed at the onset of neurological symptoms or once moribund.

Results

17-AAG Inhibits the Growth of Glioma Cell Lines Irrespective of p53, PTEN, or EGFR Status

Previous reports have shown that 17-AAG is able to inhibit the growth of numerous tumor cell lines, including some human glioma cell lines.[38,58–60] We were interested in determining the effectiveness of 17-AAG in inhibiting the growth of a larger panel of genetically diverse human glioma cell lines, as well as in assessing whether the genetic backgrounds of these cells correlate



Cell line	p53 Status	p53 Mutation	PTEN Status	PTEN Mutation
A172	wild-type	WT	mutant	Gln96Arg
LN428	mutant	Val173Met Arg282Trp	wild-type	WT
LN827	mutant	Glu56stop	mutant	exon 3 splice acceptor GT->TT
T98G	mutant	Met237Ile	mutant	Leu42Arg
U87	wild-type	WT	mutant	exon 3 splice acceptor GT->TT
U87-EGFRvIII	wild-type	WT	mutant	exon 3 splice acceptor GT->TT
U118	mutant	Arg213Gln	mutant	exon 8 splice donor insertion AGTT->AGGTT
U138	mutant	Arg213Gln	mutant	exon 1 deletion
U343	wild-type	WT	mutant	Cys124Ser
U373	mutant	Arg273Gln	mutant	exon 7 insertion of TT at 240

Fig. 1. 17-allylamino-17-demethoxygeldanamycin (17-AAG) inhibits the growth of genetically diverse glioma cell lines via degradation of heat shock protein 90 (HSP90) client proteins. (A) Assessment of phosphatase and tensin homolog (PTEN), p53, and epidermal growth factor receptor (EGFR) status of human glioma cell lines. Genomic DNA from human glioma cell lines was extracted from cells at low passage, and mutations in p53 and PTEN were determined by sequence analysis. Levels of EGFR and EGFR variant III (EGFRvIII) were assessed by Western blot analysis. (B) 17-AAG inhibits the growth of human glioma cell lines. Human glioma cell lines and a nontumorigenic fibroblast cell line were treated with increasing concentrations of 17-AAG for 4 days, and the effect on cell growth was assessed by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. The 50% inhibitory concentration (IC_{50}) of all glioma cell lines ranged from 0.05 to 0.5 μM 17-AAG, as compared to 1.5 μM for the nontumorigenic cells. (C) 17-AAG promotes the degradation of HSP90 client proteins that are involved in the pathology of glioblastoma multiforme (GBM). Glioma cell lines were treated with increasing concentrations of 17-AAG for 48 h, and protein lysates were run on Western blots. HSP90 client proteins known to be involved in gliomagenesis showed degradation while nonclient proteins remained unaffected by 17-AAG treatment. (D) The phosphorylated states of signaling proteins involved in glioma growth are also affected by 17-AAG treatment.

with their responsiveness to this compound. Toward this end, DNA from the following ten human glioma cell lines was extracted for genetic characterization: A172, U87, LN827, LN428, U118, U373, U343, U138, T98G, and U87-EGFRvIII. Mutations in p53 and PTEN were determined by sequence analysis, whereas differences in EGFR proteins levels were examined by Western blot analysis. These results are summarized in Fig. 1A and are consistent with mutations described by Ishii et al.[61] These cells' responsiveness to 17-AAG was then evaluated with an MTS cell viability assay. Cells were treated twice over 4 days with increasing concentrations of 17-AAG, and the ratio of viable cells remaining in treatment groups relative to no treatment is plotted in Fig. 1B. The 50% inhibitory concentration (IC_{50}) range of all ten glioma cell lines ranged from 50–500 nM. In addition, consistent with reports showing that 17-AAG preferentially targets tumorigenic cells, nontumorigenic Balb/c3T3 cells treated with 17-AAG exhibited an IC_{50} of 1.5 μM. There was no correlation between p53, PTEN, and EGFR status and responsiveness, suggesting that this compound does not target a defined subpopulation of gliomas and could be used generically on gliomas with diverse genetic backgrounds.

17-AAG Promotes Degradation of HSP90 Client Proteins in Glioma Cell Lines

GBMs exhibit a number of signaling abnormalities involved in the progression and pathology of this tumor type, including EGFR amplifications, inactivating PTEN mutations, and PDGF autocrine loops, with subsequent overactivation of their associated downstream signal generators. Significantly, many of these proteins are HSP90 client proteins.[32] To assess the mechanism by which 17-AAG is able to inhibit the growth of glioma cells, we stimulated all our glioma cell lines with increasing concentrations of 17-AAG and then assessed levels

of known client proteins involved in GBM pathology. As shown in Fig. 1C, examination of EGFR, PDGFR, Akt, and MAPK reveals a dose-dependent decrease in these proteins in a number of our glioma cell lines. Proteins known not to be HSP90 client proteins, including Ras and β-actin, did not show a decrease in expression, which is consistent with the idea that the responsiveness of these cells to 17AAG is a result of the degradation of HSP90 client proteins. Changes in phosphorylation states of the signaling proteins pAkt, pMAPK, and pRb are shown in Fig. 1D. These decreases in phosphoproteins could result both from a decrease in baseline quantities of the nonphosphorylated versions of these proteins, as well as from the degradation of upstream proteins that normally phosphorylate Akt, MAPK, and Rb. Indeed, MEK, which is upstream of MAPK, and Cdk 4 and 6, which are upstream of Rb, have been documented as HSP90 client proteins in other systems.[32]

17-AAG Inhibits Intracranial Tumor Growth as a Single Agent

Previous work has shown the efficacy of 17-AAG in inhibiting the growth of rat glioma C6 cells in a subcutaneous model.[38] To assess if 17-AAG is able to inhibit the growth of human glioma cells in an orthotopic model, we implanted U87 cells expressing the firefly luciferase gene in the cortices of either nude or SCID mice and noninvasively assessed the effects of treatment with 17-AAG on tumor growth by use of luciferase imaging with the Xenogen In Vivo Imaging System. Tumors were implanted and allowed to grow, and then animals were treated with either vehicle or 80 mg/kg/day of 17-AAG for 5 days on, 2 days off, and 5 days on. Tumor bioluminescence was monitored weekly. As shown in Fig. 2A, tumor growth was significantly delayed in the animals receiving 17-AAG relative to animals receiving

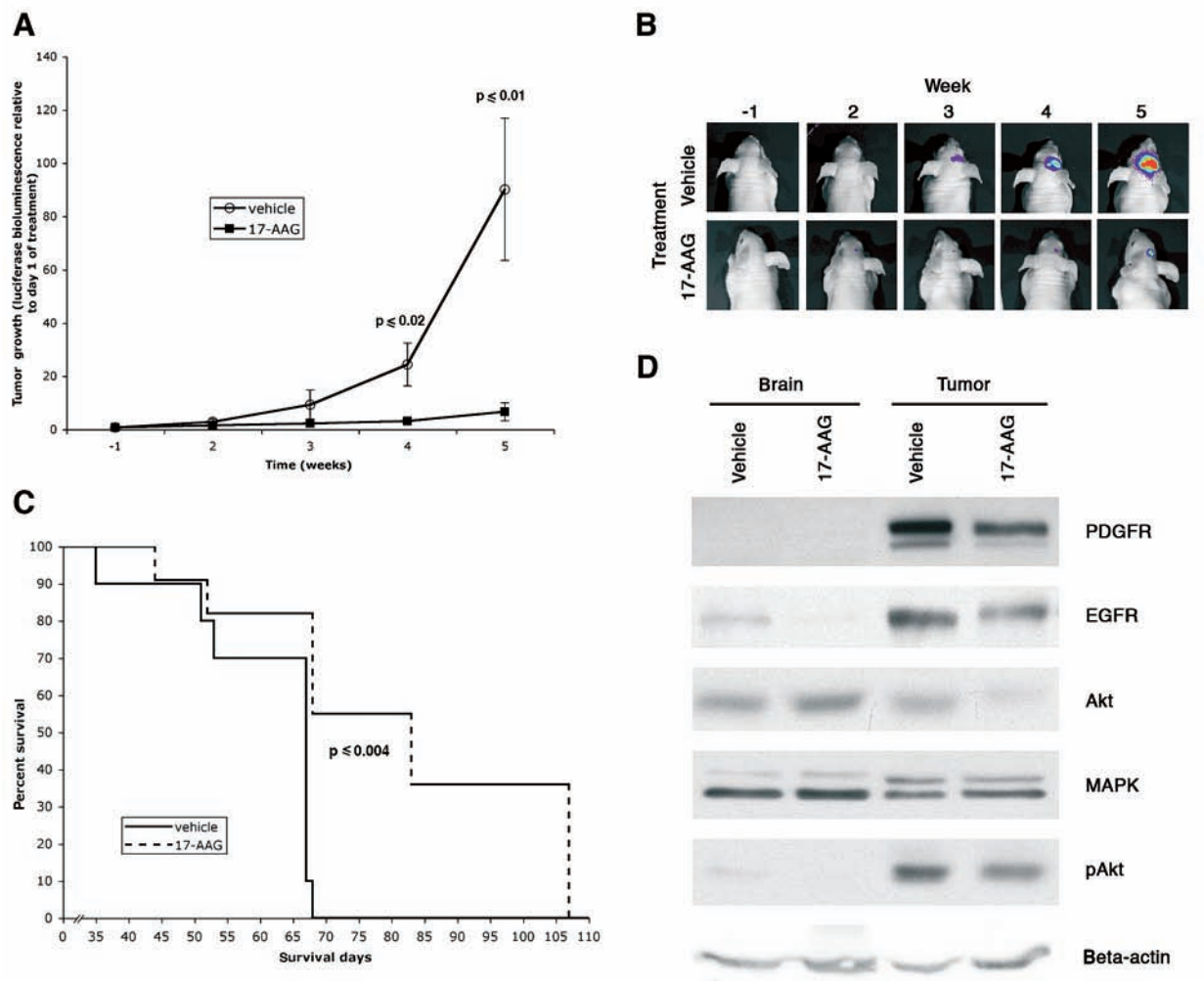


Fig. 2. 17-allylamino-17-demethoxygeldanamycin (17-AAG) inhibits the growth of orthotopic U87 glioma cells. U87-LucNeo cells were stereotaxically implanted in the right cerebral cortices of immunodeficient mice, which were subsequently treated with either vehicle or 80 mg/kg 17-AAG for 5 days on, 2 days off, and 5 days on. (A) Tumor size was assessed weekly by luciferase bioluminescence imaging and plotted relative to bioluminescence on the first day of treatment. (B) Representative examples of luciferase bioluminescence in either vehicle-treated or 17-AAG–treated mice. (C) 17-AAG–treated animals survived significantly longer than vehicle-treated animals. (D) Proteins harvested from either vehicle- or 17-AAG–treated tumors or contralateral cortices reveal a decrease in client protein expression in the 17-AAG–treated animals.

vehicle (data pooled from two independent repeats of the experiment; Student's *t*-test: $p \leq 0.02$ at week 4, and $p \leq 0.01$ at week 5). Fig. 2B shows examples of luciferase images of either vehicle- or 17-AAG–treated animals as a function of time. During the period of drug treatment, there were neither significant changes in weight nor any deaths. 17-AAG–treated animals survived significantly longer than vehicle-treated animals (log-rank test, $p \leq 0.004$) (Fig. 2C), with greater than 50% of the 17-AAG animals still surviving when all of the vehicle-treated animals had died. In addition, Western blot analyses of HSP90 client proteins in intracranial tumors revealed a decrease in the quantity of HSP90 client proteins in treated tumors compared to vehicle-treated tumors (Fig. 2D).

17-AAG Acts as a Glioma Radiosensitizer in Glioma Cell Lines Both In Vitro and In Vivo

Radiation has traditionally been the primary therapeutic intervention given to patients following surgical resection and at the time of tumor recurrence. As previous work has revealed synergistic activity of 17-AAG with RT in two glioma cell lines in vitro,[60] we were interested in assessing if radiosensitization can also occur in vivo. We began by verifying that human glioma U87 cells exhibit radiosensitization in vitro by a clonogenic cell survival assay of cells treated with two doses of 17-AAG and clinically relevant doses of γ-irradiation. Cells pretreated for 16 h with 17-AAG, then exposed to RT, showed a decrease in colony-forming ability relative to cells exposed to RT alone (Fig. 3A). Radiosensitization was verified by sensitizer enhancement ratios (SERs) and calculated by generating a ratio of observed versus predicted colony number, which revealed SERs of 2.5 and 2.9 with 50 nM or 100 nM 17-AAG, respectively, at 800-cGy doses of RT. To assess if 17-AAG is also able to radiosensitize in vivo, U87 cells expressing the firefly luciferase gene were intracranially implanted in nude mice. Mice were then treated with suboptimal doses of 17-AAG and/or γ-irradiation, or a combination of both of these agents, in order to assess if the combination has greater efficacy in inhibiting tumor growth than either of the single agents alone. Suboptimal doses were used to ensure that we were not achieving maximal growth inhibition with either agent alone. As such, mice received either vehicle, two fractions of 250 cGy γ-radiation, 80 mg/kg/day 17-AAG for only 5 days, or a combination of the RT and 17-AAG, and tumor growth was monitored noninvasively over time by luciferase bioluminescence. As shown in Fig. 3B, the combination of 17-AAG and RT yielded a statistically significant effect on growth inhibition relative to efficacy with either treatment modality alone (data pooled from three biological repeats; 17-AAG/RT relative to RT, Student's *t*-test, $p = 0.01$; 17-AAG/RT relative to 17-AAG, $p = 0.01$; 17-AAG/RT relative to vehicle, $p = 0.006$).

17-AAG Does Not Synergize with TMZ Treatment in Glioma Cell Lines

TMZ has recently been added to the standard-of-care regimen for GBM by virtue of its ability to increase survival from an average of 12 months to 14.6 months relative to radiation alone.[2] We were interested in examining the ability of 17-AAG to synergize with TMZ alone and to assess its efficacy in combination with both TMZ and RT. The effect on growth and cell viability of concurrent or sequential treatment of 17-AAG and TMZ was examined in all our glioma cell lines in vitro by MTS assays. Cells were treated either concurrently, being exposed to both compounds for 6 days, or sequentially, being exposed to TMZ for 24 h prior to the addition of 17-AAG for an additional 5 days, and the ability of these compounds to synergize was assessed by interaction analyses. For this analysis, a ratio of the observed growth inhibition seen following treatment with both compounds, relative to the amount of inhibition expected if either compound was working independently, was calculated. Within this analysis, a ratio of 1 demonstrates additive growth inhibition, a ratio less than 1 reveals subadditive or antagonistic effects on growth inhibition, and a ratio greater than 1 demonstrates synergistic growth inhibition. Our results on U87 cells, shown in Fig. 3C, indicate that 17-AAG does not synergize with TMZ. This analysis was performed on all of our ten glioma cell lines, with comparable results seen in all of the cell lines (data not shown). Given that the current standard-of-care therapy for gliomas combines RT with TMZ, we were interested in seeing if this treatment modality supplemented with 17-AAG would be able to provide greater efficacy in growth inhibition in vivo than treatment with RT and TMZ alone. Nu/nu mice were implanted with U87 cells stably expressing luciferase and treated with combinations of suboptimal doses of RT (2.5 Gy radiation × 2 days), TMZ (5 mg/kg TMZ × 2 days), and 17-AAG (80 mg/kg 17-AAG on days 1–5), and tumor growth over time was monitored noninvasively by luciferase bioluminescence. Our results reveal that the addition of 17-AAG to TMZ and RT does not provide additional efficacy on growth inhibition of glioma tumors (Fig. 3D; data pooled from two biological repeats; vehicle relative to TMZ/RT or TMZ/RT/17-AAG; Student's *t*-test, $p \leq 0.01$). When considered with results from Fig. 3B, these data suggest that rather than administering 17-AAG in conjunction with TMZ and RT it should be administered solely with RT, perhaps as a treatment option for recurrent GBM.

17-AAG Inhibits the Growth of Tumorigenic Glioma Stem Cells

Recent evidence has suggested that the cell of origin of gliomas are glioma stem cells (GSCs).[3–6] We used two models to address if 17-AAG can also inhibit the growth of GSCs. The first consisted of genetically defined murine NSCs that were driven by loss of Ink4a/ARF in conjunction with expression of EGFRvIII. This model was

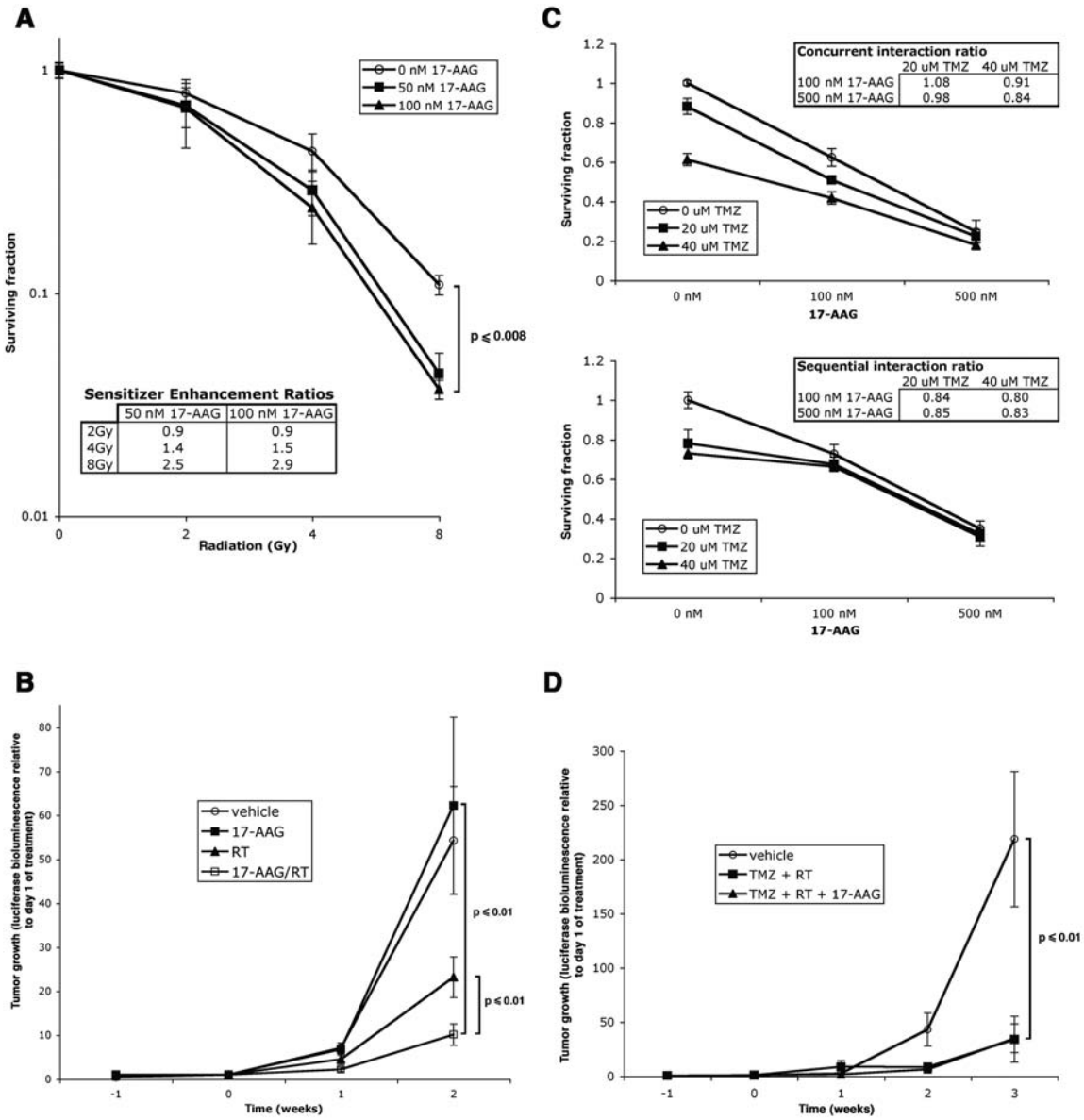


Fig. 3. 17-allylamino-17-demethoxygeldanamycin (17-AAG) radiosensitizes but does not chemosensitize glioma cell lines in vitro and in vivo. (A) The ability of 17-AAG to radiosensitize U87 glioma cells in vitro was assessed by clonogenic cell survival assays, in which the cells plated at clonal density were pretreated for 16 h with two doses of 17-AAG and then exposed to increasing doses of γ-irradiation. Colonies containing greater than 50 cells were counted after 6 days of growth, and sensitizer enhancement ratios were determined by calculating a ratio of observed versus predicted colony number (inset). (B) The ability of 17-AAG to radiosensitize in vivo was assessed by orthotopic implantation of U87 cells expressing the firefly luciferase gene, followed by treatment of tumor-bearing animals with suboptimal doses of either 17-AAG, radiation, or a combination of both treatment agents. Tumor growth was assessed over time with luciferase bioluminescence. The combination of 17-AAG and radiation therapy (RT) yielded a statistically significant effect on tumor growth inhibition relative to treatment with single agents. (C) Efficacy of either concurrent or sequential treatment of temozolomide (TMZ) and 17-AAG in U87 glioma cells in vitro was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays. Interaction ratios were calculated to determine synergy between these compounds. This ratio is calculated as the observed growth inhibition seen with both compounds relative to the amount of predicted growth inhibition if either compound is acting independently. A ratio of 1 reveals additive growth inhibition, a ratio greater than 1 demonstrates synergistic growth inhibition, while a ratio less than 1 suggests subadditive effects on growth inhibition. (D) The effect of 17-AAG on the combination of TMZ and RT in vivo was assessed using orthotopically implanted U87-LucNeo cells in immunodeficient mice. Mice were treated with either vehicle, RT, and TMZ (2.5 Gy radiation × 2 days and 5mg/kg TMZ × 2 days), or radiation, TMZ, and 17-AAG (2.5 Gy radiation × 2 days, 5 mg/kg TMZ × 2 days, and 80 mg/kg 17-AAG on days 1–5), and tumor growth was monitored by luciferase bioluminescence. Addition of 17-AAG to TMZ and RT does not provide additional efficacy on growth inhibition of orthotopic glioma tumors.

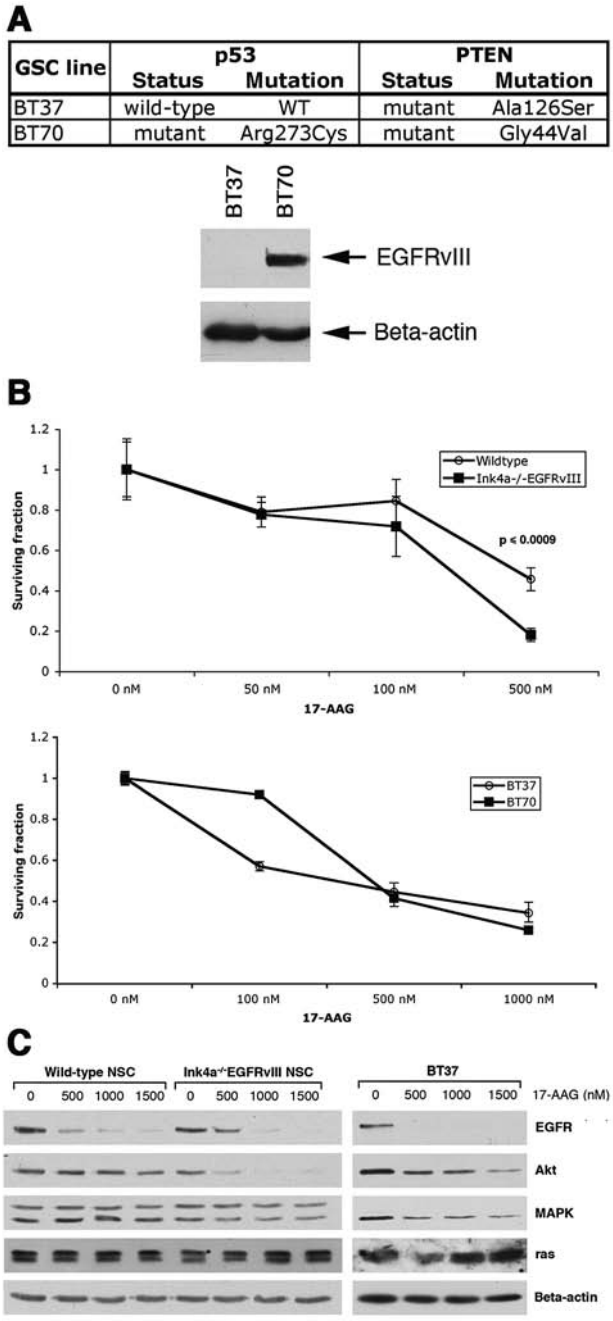


Fig. 4. 17-allylamino-17-demethoxygeldanamycin (17-AAG) inhibits the growth of genetically diverse tumorigenic glioma stem cells via degradation of heat shock protein (HSP90) client proteins. (A) Phosphatase and tensin homolog (PTEN), p53, and epidermal growth factor receptor variant III (EGFRvIII) status of human glioma stem cell lines. Genomic DNA was extracted from neurosphere cultures plated from dissociated subcutaneous tumors, and mutations in p53 and PTEN were determined by sequence analysis. Levels of EGFRvIII were assessed by Western blot analysis. (B) Stem cells were treated with increasing concentrations of 17-AAG, and the effect on cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell viability assays. In the murine model, cell viability in the tumorigenic Ink4a$^{-/-}$EGFRvIII was significantly lower than in their wild-type counterparts, consistent with the ability of 17-AAG to preferentially target tumorigenic cells over nontumorigenic cells. Dose-dependent growth inhibition was also seen in all human glioma stem cells tested. (C) 17-AAG results in degradation of HSP90 client proteins in glioma stem cells. Murine neural stem cells (NSCs) and human BT37 glioma stem cells were treated with increasing concentrations of 17-AAG, and protein lysates were harvested after 48 h. In both models, proteins involved in glioma growth show a dose-dependent decrease in expression levels, with no change in expression levels of proteins that are not HSP90 client proteins.

established and described by Bachoo et al. in 2002[55] and has been shown to be capable of giving rise to tumors that recapitulate the biological features of high-grade gliomas.[55] Wild-type NSCs were used as a control for these cells. The second model consisted of human GSCs that have been isolated from fresh GBM surgical isolates. These tumors were serially passaged in the flanks of mice so that they were not genetically altered by the in vitro environment and were used immediately for experimental purposes following removal from animals.

The responsiveness of GSCs from both models to 17-AAG was assessed by MTS cell viability assays. Cell viability results in murine NSCs reveal that the tumorigenic Ink4a/ARF$^{-/-}$ EGFRvIII cells are more responsive to 17-AAG than wild-type NSCs (IC_{50} = 200 nM vs. 400 nM, respectively; Student's *t*-test, $p \leq 0.0009$) (Fig. 4B), which is consistent with the ability of 17-AAG to preferentially target tumorigenic cells over nontumorigenic cells.

Human GSCs were successfully isolated from two primary GBM surgical specimens. Mutations within the PTEN and p53 loci were identified by sequence analysis, while expression of EGFRvIII was assessed by Western blot analysis. These results are summarized in Fig. 4A. Treatment of these cells with increasing concentrations of 17-AAG revealed dose-dependent growth inhibition, with an IC_{50} range of 200–500 nM in growth inhibition assays (Fig. 4B). As noted in the glioma cell lines, responsiveness of these cells does not appear to be dictated by genetic differences between the GSC lines.

17-AAG Causes Degradation of HSP90 Client Proteins in Glioma Stem Cells

A number of HSP90 client proteins are known to be deregulated in gliomas, including Akt and EGFR.[32] To address the mechanism by which 17-AAG inhibits the growth of GSCs we stimulated both tumorigenic and wild-type murine NSCs, as well as one human GSC line, with increasing concentrations of 17-AAG, and its effect on HSP90 client proteins was assessed by Western blot analysis. Our results reveal a dose-dependent decrease in the HSP90 client proteins EGFR, Akt, and MAPK in all our GSC models, with no changes in the expression of Ras and β-actin, which are not HSP90 client proteins (Fig. 4C), suggesting that the growth inhibition seen in these cells following exposure to 17-AAG is mediated

by HSP90 client protein degradation. Differential degradation of client proteins such as Akt in Ink4a/ARF$^{-/-}$ EGFRvIII versus wild-type cells may explain the higher efficacy of 17-AAG in tumorigenic stem cells than in their wild-type counterparts.

17-AAG Inhibits the Growth of Intracranial Human Glioma Stem Cells

There is increasing evidence that gliomas can arise from a small population of cells with stemlike qualities, capable of giving rise to the heterogeneous mass of glial tumors. We were therefore interested in assessing if 17-AAG is able to reduce the number of glioma stem cells capable of giving rise to tumors in vivo. Toward this end, human glioma stem cells were transiently treated in vitro with either 10 μM 17-AAG or DMSO for 24 h, and then 20,000 viable cells from each condition were implanted in the right striatum of immunodeficient mice. Mice harboring 17-AAG-treated glioma stem cells survived significantly longer than control animals (log-rank test, $p \leq 0.04$) (Fig. 5), with all 17-AAG–treated mice surviving at a time when more than 50% of the control mice had died. The gross morphology of tumors from either of these treatment groups was not different, as would be expected given the long-term nature of this experiment following transient exposure to 17-AAG. These data suggest that 17-AAG significantly reduces the tumor-initiating ability of human glioma stem cells in an orthotopic model of gliomas.

Discussion

GBMs are characterized by dysregulation of numerous signal transduction pathways involved in proliferation and survival. These include overactivation of the PI3K/Akt and the Ras/MAPK signaling pathways, as well as mutations, deletions, and amplification of proteins in the cell cycle axis, including Rb, p14ARF, p16Ink4a, MDM2, and p53. Standard therapy consisting of surgery and radiation therapy with concomitant TMZ has increased survival from less than 1 year to around 15 months, but there is a need for more effective therapies.

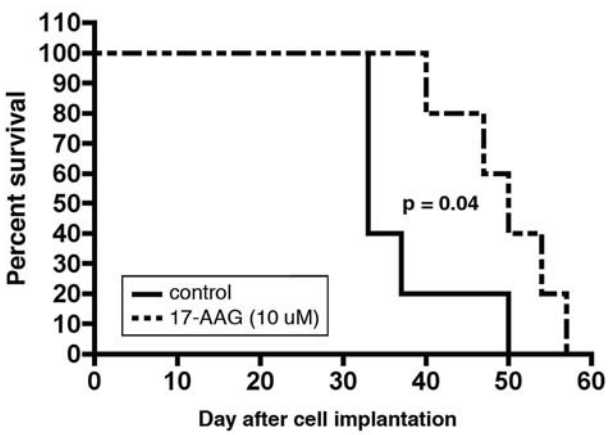


Fig. 5. Transient exposure to 17-allylamino-17-demethoxygeldanamycin (17-AAG) in vitro significantly reduces the growth of human glioma stem cells in vivo. Human glioma stem cells were treated for 24 h in vitro with either 10 μM 17-AAG or dimethyl sulfoxide, then 20,000 viable cells were orthotopically implanted in immunodeficient mice, and survival of mice was determined over time. Mice implanted with the 17-AAG–treated glioma stem cells survived significantly longer than control animals.

Much research within the last few years has focused on small-molecule inhibitors that target specific signal transduction proteins. Such targeted inhibitors were generated to focus on the underlying signaling mechanisms driving tumorigenesis, with the hope that they could extend survival and standard-of-life of cancer patients. Unfortunately, clinical studies thus far with such compounds have revealed only modest effects in gliomas,[62] suggesting that a multitargeted strategy may be more efficacious for this disease. The HSP90 inhibitor 17-AAG is able to bind HSP90 and promote the degradation of HSP90 client proteins. Significantly, many of the HSP90 client proteins are proteins known to be deregulated in GBM, including EGFR, PDGFR, Akt, and mutated p53. Hence, this compound is an excellent candidate as a multitargeted therapy for GBM, which could offer a better prognosis than single targeted agents.

We have shown that 17-AAG as a single agent is able to inhibit the growth of a panel of genetically diverse human glioma cell lines at a lower concentration than is needed to inhibit the growth of a nontumorigenic cell line. The ability of this compound to target tumor cells over normal cells is critical in the clinical setting, where a window of dosing can be established such that the glioma cells are primarily targeted. In addition, the lipophilic nature of this compound, which can facilitate its passage through the blood–brain barrier, further increases the ability of this compound to target the affected tissue.

Glial cells were traditionally thought to be the only proliferating cells within the adult brain and, as such, were assumed to be the only cells capable of acquiring transforming mutations that could result in gliomas. The discovery of multipotent neural stem cells within the adult brain[56] revealed another population of cells that could be the cell of origin of gliomas. Indeed, recent work has identified the existence of stem cells within primary gliomas capable of giving rise to high-grade gliomas indistinguishable from their parent gliomas.[3–6] A truly therapeutic compound for GBM should therefore be able to target both the mass of the tumor as well as these cells of origin. Our results reveal that 17-AAG is effective in inhibiting the growth not only of glioma cell lines, which are representative of the cells within the tumor mass, but also of glioma stem cells, which are tumor-initiating cells capable of giving rise to high-grade gliomas. These data suggest that 17-AAG would be effective both in targeting the bulk of the tumor as well as eventually minimizing recurrence that may occur through the proliferation and differentiation of cancer stem cells. In addition to its ability to target both the tumor mass and the stem cells, the fact that 17-AAG does not discriminate against different genetic abnor-

malities within different tumors would make this compound globally useful in the clinical setting.

Current therapy for GBM following surgical resection combines radiation therapy with temozolomide treatment. We were interested in assessing if addition of 17-AAG to this treatment regimen would provide additional therapeutic efficacy against the growth of these gliomas. Our results indicate that while 17-AAG is able to radiosensitize, it has a slight antagonistic effect on growth inhibition with temozolomide. The ability of 17-AAG to synergize with radiation but not with temozolomide may result from the selective nature of HSP90 client protein. Specifically, proteins that are known to be involved in radioresistance in gliomas, including Raf-1, Akt, and ErbB1,[60,63–65] are known HSP90 client proteins, while proteins implicated in temozolomide chemoresistance, including O^6-alkylguanine-DNA alkyltransferase and mismatch repair proteins,[66,67] are not HSP90 clients. Such selective degradation of client proteins may mediate the differential synergistic responses to either of these two treatment modalities.

In summary, our results suggest that 17-AAG has antitumor activity in GBM. This compound is currently in phase II trials for a number of cancers and could potentially be evaluated either as a single agent in the treatment of recurrent GBM or in combination with RT in newly diagnosed GBM. Although RT with concomitant TMZ is the standard of care for patients with GBM, most of the patients who benefit from TMZ have epigenetic silencing of the O^6-methylguanine-DNA methyl-transferase (MGMT) DNA-repair gene by promoter methylation.[68] Tumors from approximately 55% of GBM patients do not have methylation of the MGMT promoter and are relatively insensitive to TMZ.[68] This group of patients would potentially be suitable for treatment with RT and 17-AAG alone. In addition to 17-AAG, several novel potent HSP90 inhibitors are currently being developed that are easier to administer and may have less toxicity. The preclinical work described herein suggests that HSP90 inhibitors may have therapeutic potential in GBM.

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