Cotreatment With a Novel Phosphoinositide Analogue Inhibitor and Carmustine Enhances Chemotherapeutic Efficacy by Attenuating AKT Activity in Gliomas

Timothy E. Van Meter, PhD
William C. Broaddus, MD, PhD
Dana Cash, BS
Helen Fillmore, PhD

Department of Neurosurgery, Virginia Commonwealth University, Medical College of Virginia Campus, Richmond, Virginia.

Supported generously by the Medical College of Virginia Foundation Brain Tumor Research Funds and by the Hord, Crone, and Cullather families and supported in part by pediatric neurooncology research fellowship funding from the family of Michael Bergen, Jr.

We thank Dr. Geoffrey Krystal for receipt of the AKT1 mutant DNA variants.

Address for reprints: Timothy E. Van Meter, PhD, Department of Neurosurgery, P. O. Box 980631, West Hospital, 8th Floor, Virginia Commonwealth University, Medical College of Virginia Campus, Richmond, VA 23230; Fax: (804) 827-1487; E-mail: tevanmet@hsc.vcu.edu

Received February 21, 2006; revision received August 8, 2006; accepted August 14, 2006.

BACKGROUND. Heightened activity of the AKT signaling pathway is prominent in malignant gliomas and has been suggested to play a role in treatment resistance. Selective targeting of AKT, therefore, may increase chemosensitivity. Recently, a novel class of AKT-selective inhibitors has been described, including SH-6, a phosphatidylinositol analogue.

METHODS. The effects of SH-6 on AKT signaling were tested in glioma cells, and the putative role of AKT signaling in chemoresistance was tested by attenuating AKT signaling pharmacologically and genetically. The initial characterization of SH-6 included treatment of glioma cells with increasing doses of SH-6 (0.30–30 μM) and examining the effects on AKT signaling proteins by Western blot analyses and in kinase assays with immunoprecipitated AKT1. Dose-response studies with SH-6 administered to glioma cell lines were performed using a luminescent cell-viability assay (0.1–30 μM). Studies examining the effect of carmustine, either alone or in combination with either the phosphatidylinositol 3-kinase inhibitor LY294002 or SH-6, were performed by cell viability assays and clonogenic survival assays. The effect of carmustine on AKT activity as a response to treatment also was examined. Caspase assays were used to examine the potential role of apoptosis in SH-6/ carmustine -elicited cell death. Finally, the induction of a dominant-negative AKT1 transgene was used in combination with carmustine to demonstrate the role of AKT1 in carmustine chemoresistance.

RESULTS. Serum-stimulated phosphorylation of AKT1 was inhibited by SH-6 at doses $\geq$10 μM (>70% decrease in Threonine 308 and Serine 473 phosphorylation of AKT1). In adenosine triphosphate assays, 72 hours of treatment with SH-6 led to 50% lethal doses near 10 μM for 2 cell lines tested. SH-6 enhancement of carmustine-mediated cell death led to synergistic increases in Caspase 3/Capsase 7 activity, implicating apoptosis as the cell death mechanism. In clonogenic assays, SH-6 cotreatment with carmustine significantly decreased the number of colonies at 10 μM ($P < .05$) compared with carmustine alone. No decrease was observed in cells that were treated with SH-6 alone (10 μM). LY294002 (10 μM) was also able to enhance the effects of carmustine significantly in both cell lines.

CONCLUSIONS. In the current study, the authors characterized the efficacy of a new class of adjuvant chemotherapeutics that show promise in enhancing the efficacy of standard chemotherapy regimens in gliomas. ***Cancer*** **2006;107:2446–54.**

KEYWORDS: AKT, phosphatidylinositide analogue, survival, carmustine, brain tumor.

The serine-threonine kinase AKT/protein kinase B is an oncogenic serine/threonine kinase with 3 known isotypes, each encoded by a separate gene, AKT1, AKT2, AKT3 (protein kinase Bα, protein kinase Bβ, and protein kinase Bγ.[1] Recent studies in cancer research have

DOI 10.1002/cncr.22248
Published online 13 October 2006 in Wiley InterScience (www.interscience.wiley.com).

implicated AKT isotypes in critical aspects of malignant behavior, including the suppression of apoptosis, the expression of genes critical to invasiveness, and cell cycle progression.[2–4] In gliomas, heightened AKT activity has been demonstrated both in model cell lines and in clinical tissue samples and has been correlated with loss of function of the phosphatase and tensin homolog (PTEN) tumor suppressor on chromosome 10q23.[5] Furthermore, in human glioblastoma tissues, AKT activity has been correlated with poor overall survival.[6]

One consequence of deregulated AKT activity that may underlie treatment failure derives from the role of AKT in directly regulating target signaling proteins through phosphorylation, including apoptotic machinery like Caspase 9 and Bad.[7,8] Furthermore, because it has been found that AKT activity is deregulated in tumors without PTEN deficiency, direct targeting of AKT kinase with selective inhibitors may be a more effective means of diminishing antiapoptotic signaling in chemoresistant glioma cells. Recent studies have indicated that inhibition of AKT signaling through PTEN restoration in PTEN mutant cell lines can sensitize glioma cells to radiotherapy. The results presented in the current study support a role for AKT signaling in resistance to chemotherapy-induced cell death in glioma by demonstrating an enhanced chemosensitivity of glioma cells to carmustine when they are cotreated with SH-6, a novel phosphatidylinositide analogue inhibitor of AKT kinases.[7] Genetic evidence using dominant-negative AKT1 is provided to support further the notion that AKT signaling plays a significant role in the chemoresistant phenotype in glioma.

MATERIALS AND METHODS

Cell Culture

U251MG cells were obtained from Dr. Geoffrey Pilkington (Department of Neuropathology, University of London). LN-z308 glioma cells were obtained from Dr. Erwin van Meir (Department of Neurosurgery, Winship Cancer Institute, Emory University). Cells were cultured under identical growth conditions in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37°C in a humidified incubator with 5% carbon dioxide (all materials from Invitrogen, Gaithersburg, MD). Subculturing prior to cell counting and seeding of cell for experiments was performed by briefly washing cultures, incubation for 5 minutes using 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Invitrogen), centrifuging cell suspensions at $\times 330$ *g* for 5 minutes to collect cell pellets, and resuspending cells in fresh culture medium prior to plating. Cell density counts were performed using the Trypan Blue dye-exclusion method and a Brightline Hemocytometer (Sigma Chemical Company, St. Louis, MO).

Pharmacologic Inhibitors and Chemotherapeutic Agents

Chemical inhibitors that were selective for phosphatidylinositol 3 (PI3) kinase (LY294002) and AKT (SH-6) were obtained from EMD Biosciences-Calbiochem (San Diego, CA) and were reconstituted in sterile dimethyl sulfoxide (DMSO) (Sigma Chemical Company) prior to freezing in aliquots at -20°C. Each aliquot was thawed only once, immediately prior to use for experiments in vitro. Optimal concentrations for achieving inhibition of PI3 kinases and AKT were determined empirically in dose-response time-course studies by analyzing phosphorylation states of downstream phosphorylation targets by Western blot (data not shown). LY294002 was used at a concentration of 1 μM or 10 μM for the inhibition of PI3 kinase activity, and SH-6 was used at a concentration of 10 μM for the inhibition of AKT kinase activity. Growth media with inhibitors at final concentrations were prepared immediately prior to addition on cells.

Carmustine (Bristol-Myers Squibb or Sigma Chemical Company) was prepared as a 50-mM stock solution using sterile 100% ethanol according to the manufacturer's suggested protocol and was used only once because of the known instability of the compound. Concentrations from 0.5 μM to 50 μM were used to treat cells in vitro. Single and multiple administrations were used, depending on the experimental design. During cell treatments, carmustine was added to the growth medium and remained until fresh medium was added or for the duration of the experiment. Experimental conditions were performed in quadruplicate wells, and each independent experiment was conducted 3 times.

Cell Lysate Preparation

Cell lysates were prepared in a modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride, and 1 mM activated sodium orthovanadate [all from Sigma Chemical Company] supplemented with $1\times$ Protease Inhibitor Cocktail I, and $1\times$ Phosphate Inhibitor Cocktail I [EMD Biosciences–Calbiochem, San Diego, CA]). Monolayers were rinsed briefly in phosphate-buffered saline [pH 7.4] and from 0.4 mL to 1 mL chilled, modified RIPA was added per 10^7 cells. Cells were lysed for 30 minutes on ice with agitation, scraped with sterile cell scrapers, and supernatant fluids were procured after centrifugation for 10 minutes at 14,000 revolutions per minute (rpm) in an Eppendorf 1513 C

microcentrifuge at 4°C. Lysates were assayed for protein concentration using the DC Protein Assay (Pierce Chemical Company, Rockville, IL). For enzyme assays, the same procedure was used, except that mammalian protein extraction reagent was used as cell lysis buffer (Pierce Chemical Company).

Immunoblotting

Total protein was investigated in 10-μg samples by using Western blot analysis. Briefly, samples were diluted in nanopure water in 4× NuPAGE sample buffer with dithiothreitol as a reducing agent. Samples were heated for 10 minutes at 70°C and were loaded directly into prepoured 4% to 20% Bis-Tris NuPAGE gels along with prestained molecular weight standards (SeeBlue2; all reagents from Invitrogen). Electrophoresis was performed in 2-(N-morpholino)ethanesulfonic acid (MES) running buffer at a constant 200 volts for 50 minutes. Gels were rinsed briefly in ultrapure water, equilibrated for an additional 5 minutes with the transfer buffer (40% methanol, NuPAGE transfer buffer), and transferred to 0.2-μm pore nitrocellulose paper (Invitrogen) on ice at 260 mA constant current for 2 to 3 hours. After that transfer, blots were transferred briefly to Tris-buffered saline rinsing buffer (TBST) (10 mM Tris, 150 mM NaCl, and 0.5% Tween-20 [pH 8.0]), blocked for 1 hour at room temperature in TBST supplemented with 5% nonfat dry milk, and the primary antibody was applied overnight at 4°C on a rocking platform in fresh blocking buffer. After 4 10-minute washes with TBST, species-specific, horseradish peroxidase-conjugated secondary antiserum (1:5000 dilution; Rockland, Gilbertsville, PA) was applied for 1.5 hour at room temperature with agitation, the samples were washed for 1.5 hours with frequent buffer changes, and they were treated with enhanced chemiluminescence reagents (Amersham Lifesciences, Piscataway, NJ) for 1 minute at room temperature before they were subjected to autoradiographic film (Marsh BioProducts, Inc., Rochester, NY). Film was developed on a Kodak X-Omat automatic film processor; then, densitometry was performed and analyzed with Imagequant software. Antisera were used to detect AKT1 protein or phosphorylation state-specific AKT1 protein, and the ratio of active AKT was computed after normalization to a Cyclophilin-A loading control.

Immunopreciptation Kinase Assays

Total cell lysates were prepared as described above after serum starvation for 16 hours and treatment for 15 minutes with 5% serum preincubated with SH-6, LY294002, or control solvent solution. Equivalent amounts of lysate per sample (200 μg per condition) were incubated overnight with 2 μg agarose bead-conjugated monoclonal anti-AKT1 antibody (clone 2H10; Cell Signaling Technologies, Beverly, MA) at 4°C. After 3 10-minute washes with TBST, beads were collected by centrifugation, and the remaining buffer was discarded. AKT1 immunoprecipitates were resuspended in kinase assay buffer (Cell Signaling Technologies), according to the manufacturer's suggestion, along with 3000 mCi ^{32}P-γ adenosine triphosphate (ATP) and GSK3β substrate peptide and were incubated for 30 minutes at room temperature. Scintillation counting was performed on triplicate samples applied to phosphocellulose paper after washing 3 times in 0.75% phosphoric acid. Alternately, reaction immunoprecipitates were boiled in 2 × Laemmli sample buffer for 5 minutes and subjected to Western blot analysis to assess AKT1 pull down and for the detection of phosphorylated GSK3β peptide with rabbit polyclonal antisera specific to the Serine-9 site (Cell Signaling Technologies).

Cell Growth and Viability Assays

Cell growth and viability studies were performed using the Cell Titer-Glo luminescent ATP assay (Promega Inc., Madison, WI). Glioma cells were added to white, opaque-walled, sterile, 96-well plates at a concentration of 500 cells per 100 μL medium per well in 6 wells per condition and were allowed to settle overnight (for 16 hours) in 10% serum-containing DMEM. The next day, medium was replaced with 5% serum-supplemented DMEM with the following additives: Preincubation for 1 hour was performed with the AKT inhibitor SH-6 (10 μM) or the PI3 kinase inhibitor LY294002 (10 μM) versus equimolar, sterile, cell-culture tested DMSO (Sigma-Aldrich, St. Louis, MO) as a solvent control. Next, media were replaced with fresh inhibitor in the presence or absence of carmustine (10 μM or 50 μM) Viable cell numbers were then determined by adding 100 μL Cell-Glo lysis reagent, incubating at room temperature for 2 minutes with manual agitation, incubating for an additional 8 minutes on a rotating platform at 4°C, equilibrating at room temperature for 15 minutes, and luminescence detected using a BMG Lumistar luminescent plate reader (Cell-Titer Glo Reagents [Promega Inc.]; Lumistar Luminescent Plate Reader [BMG LabTechnologies GMBH, Durham, NC]). Mean relative light units were calculated for replicates within each condition and were compared using Student t tests with the significance threshold set at 95% confidence ($P < .05$).

Clonogenic Survival Assays in Vitro

Clonogenic assays were performed in 5% medium in 6-well plates by plating 200 cells per well (U251MG

cells) or 300 cells per well (LN-z308 cells) based on the determined plating efficiency of each cell line, such that equivalent numbers of clones were achieved in the untreated control wells. Glioma cells were plated in triplicate wells in the presence or absence of inhibitors and/or carmustine. After cell plating, cell plates were left for 8 days in the incubator under standard conditions. Cells were washed briefly with PBS, fixed for 30 minutes in 100% methanol, stained with Geimsa stain, and destained in distilled water. Quantification of clones was performed in replicate wells for each test condition using a counting pen, and cell numbers of each clone were verified by visual inspection using phase-contrast microscopy. Individual clones were defined as islands of at least 25 contiguous cells. Assays were repeated for 3 independent experiments.

Caspase 3 Activity Assay

Caspase 3 activity was measured by using a luminescent peptide cleavage assay (Lumi-Glo Caspase-Glo 3/7 Assay; Promega Inc.) under the following parameters. Glioma cells were plated at 500 cells per 100 μL medium per well in clear, white, opaque-walled, 96-well plates and were allowed to settle for 24 hours. Cells were pretreated for 1 hour with vehicle alone (DMSO) or with varying concentrations of AKT inhibitor (0–10 μM SH-6; EMD Biosciences). After preincubation with inhibitor, cells were treated with carmustine as indicated above and were incubated for 3 days. Medium was removed, and cells were assayed for caspase activity by using the manufacturer's protocol. Assay conditions were performed in triplicate, and 3 independent trials were performed for each assay.

Transfection of AKT Combinational DNAs

LN-z308 glioma cells were a gift from Dr. Erwin Van Meir (Winship Cancer Institute, Emory University and were transfected stably with the pTET-On vector incorporating the puromycin-resistance gene (also a gift from Dr. Erwin Van Meir). LN-Z-Tet-on cells were maintained in 1 μg/mL puromycin, prior to transfection in 6-well plates with 1.2 μg pTRE-AC-AKT (myristoylated, constitutively active, mutant AKT1), pTRE-DN-AKT (dominant-negative AKT1), or empty vector control per 4×10^5 cells by using 3 μL Lipofectamine 2000 (Invitrogen). Transfections were performed in 0.5 mL 2% serum per well in 6-well plates for 4 hours followed by the addition of antibiotic-free 10% DMEM for an additional 20 hours before cells were replated in selection medium with 1 μg/mL puromycin and 300 μg/mL G418 (Invitrogen) or for use in transient expression experiments. Expression of AKT transgene variants was achieved by the addition of 2 μg/mL doxycycline to the growth medium. AKT expression and consequential protein activation were monitored by analyzing protein extracts that were prepared 24 hours after the addition of doxycycline (72 hours posttransfection). Expression levels of AKT1 protein and Myc-tagged transgene expression were measured by analyzing Western blots with specific antisera at a dilution of 1:2000, as described (mouse monoclonal anti-AKT1, clone 2H10; and Mouse monoclonal anti Myc-Tag clone; Cell Signaling Technologies).

RESULTS

Pharmacologic Inhibition of AKT

Glioma cells were examined for phosphorylation levels of AKT under normal growth conditions. Selected cell lines were chosen to demonstrate the efficacy of the phosphatidylinositide analogue SH-6, which has been shown in preliminary studies to prevent AKT phosphorylation.[7] Western blot analyses using antisera specific for total AKT1 or phosphorylation state-specific AKT demonstrated a dose-dependent reduction in detectable phosphorylated AKT relative the detectable amounts of total AKT protein in repeated assays of extracts supplemented with phosphatase inhibitors (Fig. 1A-C). First, to demonstrate the induction of AKT1 phosphorylation, 5% fetal calf serum was used to stimulate serum-starved glioma cells for 15 minutes to generate an increase in AKT phosphorylation. Figure 1A shows that both U251MG and LN-z308 glioma cells showed a pronounced AKT activation in cell lysates, as detected by Western blot analysis using phosphorylation-specific antisera. A >2-fold increase in the active AKT1/total AKT1 ratio was detected in U251MG cells after stimulation (Fig. 1A). Under the same conditions, incubation of glioma cells with 1–30 μM SH-6 caused a significant 60% to 90% abrogation of AKT phosphorylation at doses of 10 μM or greater, as detected at the Threonine 308-AKT and Serine 473 positions with phosphorylation site-specific antisera. To assay the effects of SH-6 on AKT1 activity directly, cell lysates were pretreated for 1 hour with increasing concentrations of SH-6 or the PI3 kinase inhibitor LY294002. Immunoprecipitated AKT1 stimulated with serum for 15 minutes showed a 5-fold increase in phosphotransferase activity toward GSK3β peptide compared with serum-starved controls. Preincubation with SH-6 or LY294002 effectively suppressed the level of phosphotransferase activity in a dose-dependent manner, as shown in Figure 1C. The peptide substrate also was detected by Western blot analysis with phosphorylation-specific antisera, and the levels normalized to detectable amounts of AKT1 in each immu-

noprecipitate (Fig. 1B). In either format, SH-6 doses $\geq$10 μM were necessary to achieve 70% suppression of phosphotransferase activity as detected in immunoprecipitates. Therefore, the dose of 10 μM SH-6 was used in subsequent experiments to suppress AKT activity.

Effects of SH-6 on Glioma Cell Survival in Vitro

To examine the effect of AKT inhibitor treatment on the viability of glioma cells, luminescence-based ATP assays were used. U251MG and LN-z308 cells were treated for 72 hours with increasing doses of SH-6 or equimolar DMSO (solvent control), and viability was determined after 3 days of treatment. Mean relative light units from replicate detected values were converted to percent control and plotted with standard deviation of the means included (Fig. 2). The lethal doses to 50% (LD_{50}s) for the 2 cell lines tested were approximately 10 μM, as indicated in Figure 2 (dotted horizontal line),. Significant decreases in cell survival were observed as low as 1 μM in LN-z308 cells and 3 μM in U251MG cells.

Inhibition of AKT by SH-6 Decreases Clonogenic Survival of Glioma Cells

It is believed that the clonogenic survival of cancer cells reflects tumor aggressiveness. Because AKT signaling plays an important role in cell survival, we tested the response of glioma cells to carmustine, which is a commonly administered chemotherapy agent in gliomas. Figure 3 shows that U251MG gliomas cells that were treated with carmustine responded with an increase in detectable phosphorylated AKT, indicating that the cellular response to carmustine toxicity involves enhanced AKT activity. Next, U251MG and LN-z308 glioma cells were plated in clonogenic assays, left overnight for 16 hours, then pretreated with SH-6 or LY294002 at 10 μM, DMSO solvent control and at 0 μM, 10 μM, or 50 μM carmustine for

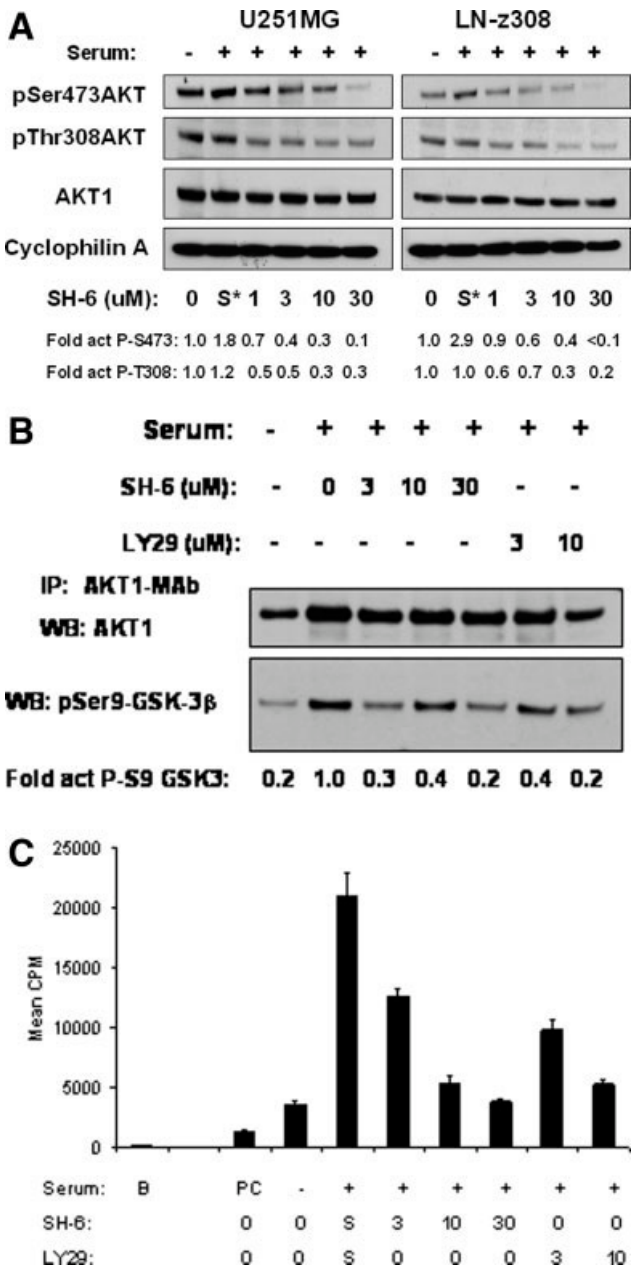

FIGURE 1. The attenuation of AKT signaling by the AKT inhibitor SH-6 (a phosphatidylinositol analogue) is illustrated. (A) Serum stimulation induces activation of AKT in 2 glioma cell lines (U251MG and LN-z308), as detected by Western blot (WB) analysis of 10 μg total protein. Rabbit polyclonal antisera raised against phosphorylated Thr308-AKT (pThr308AKT), phosphorylated Ser473-AKT (pSer473AKT), and anti-AKT1 (AKT1) (all at 1:2000 dilution; Cell Signaling Technologies) were used to detect protein levels of active and total AKT1. S* indicates dimethyl sulfoxide (DMSO) control; −, without serum stimulation; +, stimulated with 5% serum. (B) Serum treatment stimulated the activation of AKT activity toward GSK3β peptide-bearing the Ser9 AKT phosphorylation site after 15 minutes of stimulation (5% serum vs. serum-free medium, SH-6, or the phosphatidylinositol 3-kinase [PI3] inhibitor LY294002). Phosphorylated peptide was detected by immunoblot analysis after 30 minutes of incubation with AKT1 immunoprecipitates from serum stimulated cells that were subjected to SH-6 or LY294002 at the indicated dose for 60 minutes. IP indicates immunoprecipitation; MAb, monoclonal antibody; CPM, ^{32}P counts per minute. (C) Preadministration of SH-6 to serum-starved glioma cells for 30 minutes diminished AKT1 kinase activity against GSK3 in a concentration-dependent fashion. The PI3 inhibitor LY294002 also attenuated this activity in a dose-dependent manner at both Thr308 and Ser473 sites in the regulatory domain of AKT1. Representative results for U251MG are shown (B,C). S indicates DMSO solvent control (0 μM in B and C); B, background CPM signal (in C); PC, peptide input control (without AKT).

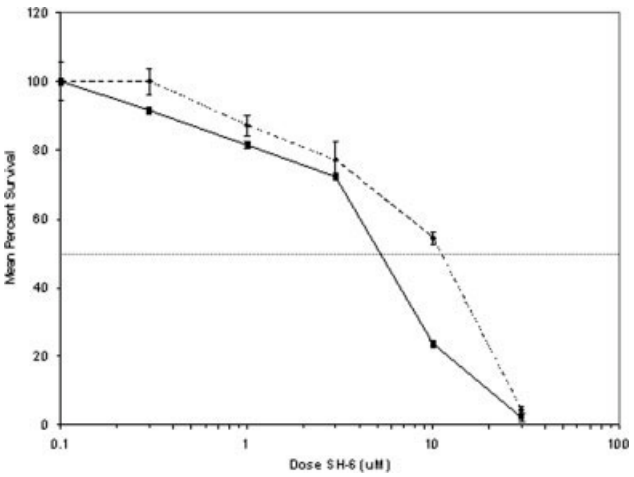

FIGURE 2. This chart illustrates dose-dependent survival response in glioma cell lines that were treated with the phosphatidylinositol analogue AKT inhibitor SH-6. Cell lines were treated for 72 hours with SH-6, and differences in cell survival were measured by using a luminescence based assay for ATP content. (Diamonds, U251MG cells; Squares, LN-z308 cells. Bars indicate standard deviation of the mean. Data represent the mean of 3 independent experiments. The dotted line that intercepts with each curve indicates the lethal dose to 50%, as determined in this assay.

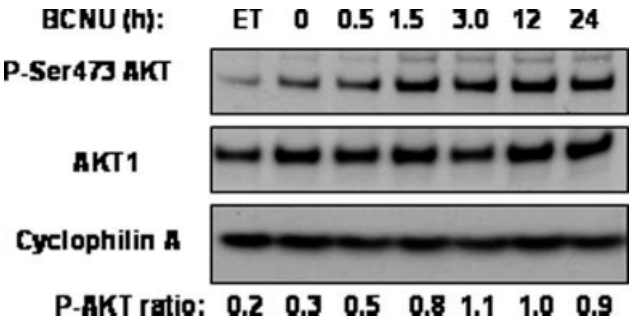

FIGURE 3. These blots illustrate AKT activity in glioma cells that were treated with carmustine (BCNU). Cells from the glioma cell line U251MG were treated for increasing periods over 24 hours (h) with 10 μM BCNU, and phosphorylated AKT (p-AKT) was assessed by Western blot analysis. P-Ser473AKT indicates phosphorylated Ser473-AKT; P-AKT ratio, ratio between p-AKT and AKT; ET, ethanol solvent control.

1 week. Each condition was performed in triplicate for 3 separate independent trials. Cells were fixed in methanol, stained, and counted as described above. Figure 4 shows that, in LN-z308 cells, a significant decrease (69%) in cell death was achieved with 10 μM SH-6 coadministration compared with carmustine alone ($P = .0013$). Repeated assays of U251MG cells showed an additional 31.4% mean decrease in cell viability compared with carmustine alone ($P = .021$; data not shown). It is noteworthy that little effect was

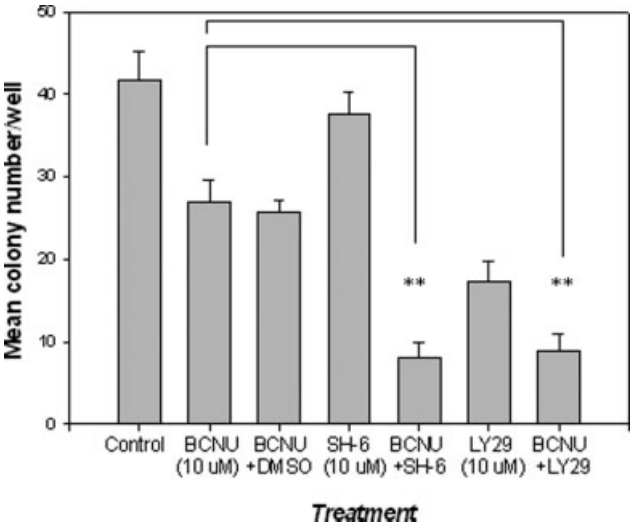

FIGURE 4. Cotreatment of glioma cells with carmustine (BCNU) and AKT signaling inhibitors enhanced cell kill. Glioma cells were treated for 1 week in clonogenic growth assays with BCNU (10 μM), the selective AKT inhibitor SH-6 (10 μM), or the selective phosphatidylinositol 3-kinase inhibitor LY294002 (10 μM) both alone and in combination. A comparison of the mean colony number was performed by using a Student *t* test for unpaired data. Significance is indicated by *P* values $<.05$ (single asterisk) and $<.01$ (double asterisks).

observed on colony numbers with SH-6 treatment alone in either cell line with this assay.

Induction of Caspase Activity after Coadministration of carmustine and SH-6

To begin an examination of the mechanism by which AKT inhibitor augments carmustine-mediated cell death, direct activity of the effector Caspases 3 and 7 was assayed by using a luminescent peptide substrate-cleavage assay. Lysis of cells in assay buffer supplemented with Caspase 3/Caspase 7-specific peptide substrate was performed using a carmustine dose of 10 μM and the dose of SH-6 that was used in the carmustine experiments (10 μM). In LNz-308 cells, which were chosen because they exhibited the greatest sensitivity to both carmustine and SH-6, we observed that treatment with 10 μM SH-6 alone was able to induce a significant decrease in viability, which we assayed by detecting the ATP content per well at 36 hours, proportional to the number of viable cells present (Fig. 2). In the caspase activity assay (Fig. 5), carmustine alone at 10 μM was able to induce a modest but detectable increase in caspase activity (2.7-fold) compared with solvent control at 36 hours. Treatment with SH-6 alone similarly was able to induce caspase activity at 10 μM (3.8-fold). When carmu-

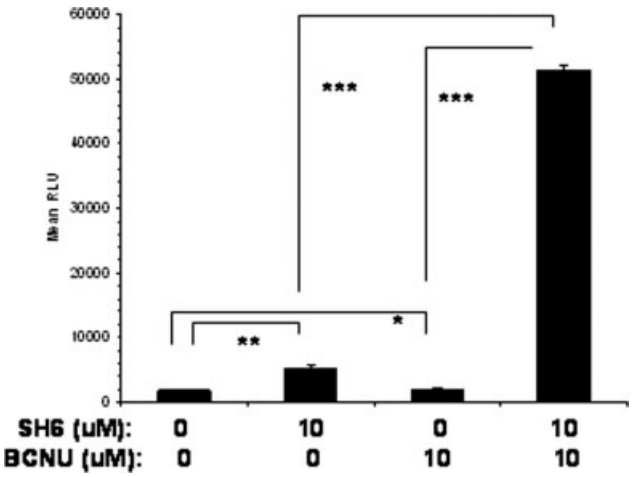

FIGURE 5. AKT inhibition by the selective AKT inhibitor SH-6 enhanced carmustine (BCNU)-mediated Caspase 3 activity. An intermediate dose of 10 μM BCNU was used to determine whether AKT inhibition with SH-6 treatment (10 μM) increased caspase activity after alkylating chemotherapy. Luminescent adenosine triphosphate-based Caspase 3/Caspase 7 assay (Promega Inc.). RLU indicates relative light units. $*P < .05$, $**P < .01$, $***P < .001$.

stine was coadministered with SH-6, a greater than additive increase in caspase activity was detected at doses $\geq$10 μM.

Decreased Clonogenic Growth of Glioma Cells Treated with Dominant-Negative Mutant AKT1

To examine further the impact of AKT signaling on chemoresistant phenotype in gliomas, a tetracycline-inducible dominant-negative mutant transgene for human AKT1 was used, because this isotype of AKT has been implicated the greatest in glioma malignancy. In LN-z308 glioma cells stably cotransfected with the pTet-On vector and either a modified empty pTRE response vector or bearing the dominant-negative Myc-tagged AKT1 transgene, the induction of transgene expression was monitored over the course of 72 hours. Figure 6 shows that robust transgene expression was detected at 48 hours and 72 house after the addition of doxicycline (2 μg/mL) to the culture medium by using an Myc-tag antibody in protein lysates and Western blot analysis. No expression was observed in the parental cell line or in the empty vector controls. These stable transfectants were used in clonogenic assays under the influence of doxicycline.

Dominant-negative AKT1 transgene expression led to a significant decrease in mean colony numbers ($P = .02$; t test), as shown in Figure 7. The administration of increasing dose of carmustine led to decreases in the mean number of colonies that were characteristic of previous assays (see Fig. 4). SH-6 was used as an additional treatment in this assay to determine whether additional cell kill could be achieved by using both genetic and pharmacologic methods of perturbing AKT function simultaneously. In both parental LN-z308 cells and empty vector controls, a small but significant difference in cell viability was observed with the inclusion of SH-6 in the media compared with no SH-6, indicating an enhancement in cell kill

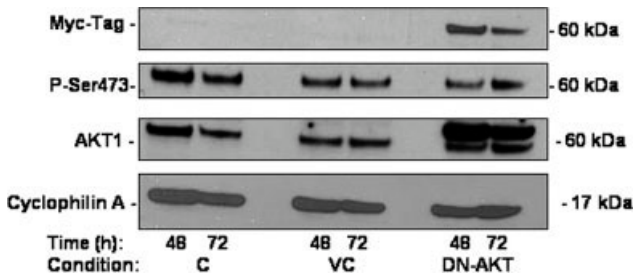

FIGURE 6. These blots illustrate the time course of induction of Myc-tagged (Myc-Tag) DN-AKT1 transgene after doxycycline treatment. Cell lysates were prepared at 48 hours (h) and 72 hours after doxycycline treatment (2 μg/mL) and assayed by Western blot analysis (10 μg total protein). Anti-Myc-Tag monoclonal antibody was used to detect DN-AKT1 (1:1000 dilution; Cell Signaling Technologies). Cyclophilin A protein detection was included as a loading control (1:3000 dilution; rabbit polyclonal antisera; Upstate Biotechnology, Inc.). P-Ser473 indicates phosphorylated Ser473-AKT; C, control; VC, vector control; KDa, kilodaltons.

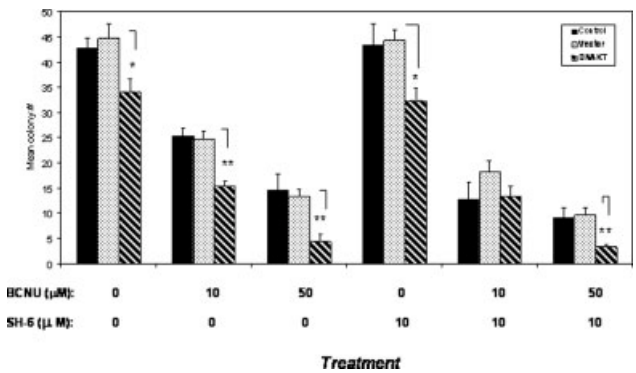

FIGURE 7. Induction of DN-AKT1 in stably Tet-On transfected LN-z308 glioma cells enhanced sensitivity to carmustine (BCNU) in the clonogenic assay. Clonogenic survival assays were conducted with LN-z308 cells that were cotransfected stably with the empty pTRE/pTetOn vector or with the DN-AKT1-pTRE/PTet-On vector, incorporating the dominant-negative AKT1 mutant transgene, and were compared with parental LN-z308 cells in the presence of doxycycline (2 μg/mL) to maintain transgene expression. Sensitivity to BCNU treatment was examined over 8 days in vitro. Colonies were counted after methanol fixation and Geimsa staining. Statistical comparisons of mean colony numbers between all conditions were performed using a Student t test for unpaired data, with resulting P values of $<.05$ (single asterisk) and $<.01$ (double asterisks).

($P < .05$ in all experiments). However, no significant difference was observed under conditions in which DN-AKT1 and SH-6 were present simultaneously, suggesting that 1 method of AKT perturbation may prevent perturbation by a separate mechanism with a similar mode of action.

DISCUSSION

The role of heightened AKT signaling in cancer has been well documented. In malignant gliomas, this increase has been attributed primarily to loss of function of the PTEN tumor suppressor and constitutive activation of growth factor-receptor tyrosine kinase signaling pathways. In the current study, we attempted to target AKT signaling directly, by both pharmacologic and genetic means, to assess whether AKT activity plays a significant role in suppression of apoptotic cell death during the administration of standard chemotherapy in gliomas.[9,10]

First, we tested the ability of a novel phosphatidylinositol analogue inhibitor, SH-6, to diminish AKT activation. SH-6 was characterized previously, and we observed that it was selective for AKT isotypes, particularly AKT1 isoenzyme, which has been identified as the most commonly overactive isozyme in astrocytic gliomas (our own data).[6] Using Western blot assays, we demonstrated that pretreatment of 2 glioma cell lines for 30 minutes with SH-6 at doses of 10 μM led to reductions >60% in active AKT1 under the influence of serum stimulation, as evidenced by detection with phosphorylation-specific antisera. At both the Serine473 and Threonine308 phosphorylation sites, which are the 2 major phosphorylation sites located within the regulatory domain, significant reduction was detected after treatment. To assess the effects of pharmacologic inhibition of AKT on tumor cell viability, glioma cell lines were treated with increasing doses of SH-6 compared with solvent control (DMSO). The LD_{50} values for SH-6 in 2 glioma cell lines were near 10 μM for a 3 day treatment period.

Cotreatment of gliomas with carmustine and SH-6 or with the PI3 kinase inhibitor LY294002 enhanced carmustine -mediated cell death in clonogenic survival assays (Figs. 3 and 4). The significant decreases in glioma cell survival after carmustine treatment and the augmentation observed in AKT phosphorylation after carmustine suggest that part of the response of glioma cells to carmustine treatment involves an AKT-dependent mechanism. It is noteworthy that, with this assay, repeated assay of SH-6 alone at 10 μM did not decrease clonogenicity significantly in these cell lines, which was unexpected. LY294002 (10 μM) was potent in its ability to diminish clonogenicity under the same conditions. This finding suggests that either another putative AKT-independent mechanism or a separate target of SH-6 may be involved with the suppression of cell death under the influence of carmustine treatment. Therefore, additional studies will focus on refining our understanding of the molecular events that underlie the response to carmustine in these cell lines.

By using the expression of a dominant-negative mutant transgene (DN-AKT1) (Fig. 6) to perturb AKT1 function, a decrease in glioma cell survival was achieved, which also enhanced carmustine -mediated cell death compared with controls (Fig. 7). In our hands, additional SH-6 treatment did not enhance cell death further beyond that achieved with DN-AKT1 expression and carmustine, despite the ability of SH-6 to enhance cell death in parental and vector control cells that were treated with carmustine. The differing effects of AKT interference by SH-6 and DN-AKT1 in clonogenic survival may provide important clues about the specific modes of action of these 2 targeting mechanisms during carmustine -mediated cell death and, when they are understood better, may lead to more efficient methods of enhancing chemotherapeutic efficacy by AKT targeting.

To investigate further the precise mechanism of cell death observed in glioma cells treated with 10 μM SH-6 in the presence of 10 μM carmustine, we used caspase activity assays. Treatment with either 10 μM carmustine or 10 μM SH-6 alone significantly increased the activity level of effector Caspase 3 and Caspase 7, as detected in this assay ($P = .035$, and $P = .021$, respectively, compared with solvent controls; Student t test for unpaired data). Concomitant treatment of glioma cells with carmustine and SH-6 led to a greater than additive increase in caspase activity at 10 μM. Comparison of mean values with carmustine treatment alone demonstrated that the elevated response was significant (Student t test for unpaired data). The enhancement of caspase activity by SH-6 and the greater than additive enhancement of carmustine -mediated caspase activity suggest that at least part of the impact of SH-6 on glioma cells derives from an increase in apoptotic sensitivity in response to carmustine. This effect may derive from a release of the suppression of proapoptotic factors upstream of Caspase 3/Caspase 7.

The results of this study suggest that AKT inhibition can lead to enhanced sensitivity to alkylating chemotherapy agents, and our findings suggest that this occurs by relieving the inhibition of apoptotic cellular response in gliomas, a tumor that is notorious for its clinical intractability. In other cancers, the role of AKT1 and AKT2 in antiapoptotic signaling also is

apparent.[8,10] The combined effect of using pharmacologic inhibitors of AKT and standard chemotherapies appears to be in line with an improved clinical outcome both in vitro and in reported studies of animal models.[11,12] This is encouraging for further development of AKT inhibitors for clinical use in patients with cancer.

It has been demonstrated that compounds like SH-6 that target the PH domain of AKT prevent AKT recruitment to signaling complexes in the plasma membrane.[7,13] However, no data have been published to date that address the effect of direct AKT inhibition on carmustine sensitivity, nor have there been detailed studies published on AKT isotype-specific efficacy in vivo. Such studies will be required to justify the further development of these promising novel adjuvant therapies. The significance of AKT signaling in glioma biology has been reinforced by several recent studies in which transgenic mice bearing conditionally overexpressed AKT1 and mutant Ras gave rise to more malignant tumors than Ras over-expressing mice, and have histologic features more akin to glioblastoma versus lower grade glioma.[14–16] Such reports underline the importance of AKT as a factor in the malignancy of gliomas and support the rationale for further testing of AKT inhibitors in vivo and, eventually, in human patients.

REFERENCES

1. Bayascas JR, Alessi DR. Regulation of Akt/PKB Ser473phosphorylation. *Mol Cell*. 2005;18:143–145.
2. Lawlor MA, Alessi DR. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci*. 2001; 114(Pt 16):2903–2910.
3. Liang J, Zubovitz J, Petrocelli T, et al. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nat Med*. 2002;8:1153–1160.
4. Arboleda MJ, Lyons JF, Kabbinavar FF, et al. Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. *Cancer Res*. 2003;63: 196–206.
5. Fan X, Aalto Y, Sanko SG, Knuutila S, Klatzmann D, Castresana JS. Genetic profile, PTEN mutation and therapeutic role of PTEN in glioblastomas. *Int J Oncol*. 2002;21:1141–1150.
6. Ermoian RP, Furniss CS, Lamborn KR, et al. Dysregulation of PTEN and protein kinase B is associated with glioma histology and patient survival. *Clin Cancer Res*. 2002;8:1100–1106.
7. Kozikowski AP, Sun H, Brognard J, Dennis PA. Novel PI analogues selectively block activation of the pro-survival serine/threonine kinase Akt. *J Am Chem Soc*. 2003;125:1144–1145.
8. Gagnon V, Mathieu I, Sexton E, Leblanc K, Asselin E. AKT involvement in cisplatin chemoresistance of human uterine cancer cells. *Gynecol Oncol*. 2004;94:785–795.
9. Cheng JQ, Lindsley CW, Cheng GZ, Yang H, Nicosia SV. The Akt/PKB pathway: molecular target for cancer drug discovery. *Oncogene*. 2005;24:7482–492.
10. Westfall SD, Skinner MK. Inhibition of phosphatidylinositol 3-kinase sensitizes ovarian cancer cells to carboplatin and allows adjunct chemotherapy treatment. *Mol Cancer Ther*. 2005;4:1764–1771.
11. Thomas S, Shah G. Calcitonin induces apoptosis resistance in prostate cancer cell lines against cytotoxic drugs via the Akt/survivin pathway. *Cancer Biol Ther*. 2005;4:1226–1233.
12. Delord JP, Allal C, Canal M, et al. Selective inhibition of HER2 inhibits AKT signal transduction and prolongs disease-free survival in a micrometastasis model of ovarian carcinoma. *Ann Oncol*. 2005;16:1889–1897.
13. Caron RW, Yacoub A, Li M, et al. Activated forms of H-RAS and K-RAS differentially regulate membrane association of PI3K, PDK-1, and AKT and the effect of therapeutic kinase inhibitors on cell survival. *Mol Cancer Ther*. 2005;4:257–270.
14. Hu X, Pandolfi PP, Li Y, Koutcher JA, Rosenblum M, Holland EC. mTOR promotes survival and astrocytic characteristics induced by PTEN/AKT signaling in glioblastoma. *Neoplasia*. 2005;7:356–368.
15. Uhrbom L, Dai C, Celestino JC, Rosenblum MK, Fuller GN, Holland EC. Ink4a-Arf loss cooperates with KRas activation in astrocytes and neural progenitors to generate glioblastomas of various morphologies depending on activated Akt. *Cancer Res*. 2002;62:5551–5558.
16. Holland EC, Celestino J, Dai C, Schaefer L, Sawaya RE, Fuller GN. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. *Nat Genet*. 2000;25:55–57.