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Inhibition of Akt inhibits growth of glioblastoma and glioblastoma stem-like cells

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Abstract

A commonly activated signaling cascade in many human malignancies, including glioblastoma multiforme, is the Akt pathway. This pathway can be activated via numerous upstream alterations including genomic amplification of epidermal growth factor receptor, PTEN deletion, or PIK3CA mutations. In this study, we screened phosphatidylinositol 3-kinase/Akt small-molecule inhibitors in an isogenic cell culture system with an activated Akt pathway secondary to a PIK3CA mutation. One small molecule, A-443654, showed the greatest selective inhibition of cells with the mutant phenotype. Based on these findings, this inhibitor was screened *in vitro* against a panel of glioblastoma multiforme cell lines. All cell lines tested were sensitive to A-443654 with a mean IC_{50} of ~150 nmol/L. An analogue of A-443654, methylated at a region that blocks Akt binding, was on average 36-fold less active. Caspase assays and dual flow cytometric analysis showed an apoptotic mechanism of cell death. A-443654 was further tested in a rat intracranial model of glioblastoma multiforme. Animals treated intracranially with polymers containing A-443654 had significantly extended survival compared with control animals; animals survived 79% and 43% longer than controls when A-443654-containing polymers were implanted simultaneously or in a delayed fashion, respectively. This small molecule also inhibited glioblastoma multiforme stem-like cells with similar efficacy compared with traditionally cultured glioblastoma multiforme cell lines. These results suggest that local delivery of an Akt small-molecule inhibitor is effective against experimental intracranial glioma, with no observed resistance to glioblastoma multiforme cells grown in stem cell conditions. [Mol Cancer Ther 2009;8(2):386–93]

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Introduction

Advances over the past few decades have improved the understanding of glioma tumorigenesis, proliferation, and invasion. The serine/threonine kinase Akt/protein kinase B pathway is a nodal point regulating numerous tumor-associated processes, including cell growth, cell cycle progression, survival, migration, and angiogenesis, and has been shown to be important in many malignancies including glioblastoma. More specifically, the Akt pathway has been shown to be activated in the majority of glioblastoma multiforme (1, 2). In other studies, activation of the Akt pathway in a human astrocytic model of glioma resulted in conversion of anaplastic astrocytoma to glioblastoma multiforme (3), and the combined activation of Akt and Ras in neural progenitors induced glioblastoma multiforme formation in a murine model (1). Recently, activation of PIK3CA [a phosphatidylinositol 3-kinase (PI3K) gene] and Akt pathway members has been shown to be associated with reduced patient survival times (4, 5). In addition to PTEN deletion or genomic amplification of growth factor receptors such as epidermal growth factor receptor, activating mutations in PIK3CA have been identified in many cancers, including adult and pediatric glioblastoma multiforme, and these mutations also activate the Akt pathway (6–8).

In this study, we screened inhibitors of the PI3K/Akt pathway in a genetically controlled cell culture system in which the Akt pathway was activated. Based on the data obtained from our initial screen, we further tested a small-molecule Akt inhibitor *in vitro* against traditionally cultured glioblastoma multiforme cell lines and glioblastoma multiforme stem-like cell (GSLC) lines and *in vivo* in a rat intracranial gliosarcoma model.

Materials and Methods

Cell Lines and Culture Conditions

D-PIK3CA 127 (wild-type D-PIK-Ex1-2) and D-PIK3CA 129 (mutant D-PIK-Ex-1-7) (7) were grown in McCoy's 5A medium supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The human glioblastoma multiforme cell lines D54-MG, H80, H247, H263, H392, H397, H502, H542, H566, H1477, U87-MG, and 1028S and the rat 9L gliosarcoma cell line were grown in

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DMEM supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The human glioblastoma multiforme cell lines SK-15-MG, SK-17-MG, SK-21-MG, and SK-26-MG were grown in RPMI 1640 supplemented with 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 100 μmol/L MEM nonessential amino acids. The human GSLC lines 020913, 040622, 050509, and 060919 were grown in NeuroCult NS-A basal medium (Stem Cell Technologies) containing NeuroCult NS-A proliferation supplements (Stem Cell Technologies), 20 ng/mL human epidermal growth factor (PeproTech), 10 ng/mL human fibroblast growth factor 2 (PeproTech), and 2 μg/mL heparin (Stem Cell Technologies). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Drugs

Akt inhibitor III (SH-6), Akt inhibitor IV, Akt inhibitor V (Triciribine, API-2, NSC 154020, TCN), Akt inhibitor VIII (Akti-1/2), LY294002, naltrindole hydrochloride (NTI), and wortmannin were purchased from Calbiochem. A-443654 and its methylated analogue 2-methyl A-443654 (A-739985) were obtained from Abbott Laboratories. All compounds were dissolved in DMSO, except for NTI, which was dissolved in water.

Protein Extract Preparation and Immunoblotting

Cytoplasmic protein lysates were made from cells during exponential growth using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biochemicals) containing Halt protease and phosphatase inhibitor cocktails (Pierce) according to the recommendations of the manufacturer. Lysates (40 μg) were heated to 95°C in Laemmli sample buffer for 10 min and separated on SDS polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad) in Western transfer buffer [25 mmol/L Tris (pH 8.3), 192 mmol/L glycine, and 20% methanol]. For Western blot analysis, membranes were blocked for 1 h at room temperature in 5% nonfat dry milk in TBST (1× TBS, 0.1% Tween 20) and incubated overnight at 4°C with antibodies against Akt, phospho-Akt (Thr^{308}), phospho-Akt (Ser^{473}; Cell Signaling Technology), or glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology). After washing, membranes were incubated with a horseradish peroxidase-linked goat anti-rabbit antibody for 1 h at room temperature. Antibody detection was achieved by chemiluminescence according to the manufacturer's recommendations (Pierce).

Cytotoxicity Assays

For the initial PI3K/Akt small-molecule inhibitor screen, 2,000 to 3,000 cells containing either a wild-type (127) or a mutant (129) PIK3CA gene were plated in 200 μL in 96-well plates. The following day, cells were treated with vehicle (DMSO or water) or 1 and 10 μmol/L Akt inhibitors III, IV, V, and VIII, A-443654, NTI, and wortmannin for 48 h. LY294002 was used at 50 μmol/L as a positive control (7). For determination of IC_{50} values, 1,500 to 4,000 glioblastoma multiforme cells grown in serum or 5,000 to 8,000 GSLC lines grown in NeuroCult medium were plated in 96-well plates in 200 μL of respective medium and treated the following day with vehicle (DMSO) or various concentrations of A-443654 (1, 5, 10, 50, 100, and 500 nmol/L and 1, 5, and 10 μmol/L) and A-739985 (1, 5, 10, 50, 100, and 500 nmol/L and 1, 5, 10, and 12.5 μmol/L) for 48 h. CCK-8 assays were done according the recommendation of the manufacturer (Alexis Biochemicals). Briefly, 20 μL CCK-8 was added to each well and incubated at 37°C. Absorbance at 450 nm was measured using the Victor^3 microplate reader (Perkin-Elmer). IC_{50} values were calculated by GraphPad Prism (GraphPad Software).

Caspase Activity Assays

Caspase-3 and -7 activities were detected in cell lysates after various treatments using the Caspase-Glo 3/7 Assay according to the recommendations of the manufacturer (Promega). Luminescence was measured on a Victor^3 microplate reader (Perkin-Elmer). Experiments were done in triplicate, with each experiment containing five or six replicates.

Flow Cytometry

Apoptosis was assayed by staining cells with the annexin V-allophycocyanin (APC) apoptosis kit (BD Bioscience) according to the instructions of the manufacturer. 7-aminoactinomycin D was used for dual staining to distinguish cells in the earlier stages of apoptosis (annexin V-APC positive, 7-AAD negative) from those in later stages of apoptosis or that were already dead (annexin V-APC positive, 7-AAD positive). Cells stained with annexin V-APC and 7-AAD were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson).

Polymer Preparation

Polymers were synthesized by incorporating A-443654 into the polyanhydride polymer poly[1,3-bis(carboxyphenoxy)propane-co-sebacic acid] (CPP:SA). Both substances were mixed, dissolved in *N,N*-dimethylformamide (Sigma), briefly sonicated, and dried in a vacuum dessicator overnight. The polymers were molded into disks weighing 10 mg by a stainless-steel molding press. Control CPP:SA polymers containing no drug were made in a similar fashion.

Animals

Female Fischer 344 rats weighing 150 to 200 g were used for this study. The animals were housed in standard animal facilities with up to four rats per cage and allowed free access to Baltimore city water and rodent chow. All animal protocols were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Surgical Technique

Intracranial tumors and polymers were implanted in an analogous fashion. Female Fischer 344 rats were anesthetized and their heads were shaved and prepared with povidone-iodine. A midline scalp incision was made and the coronal and sagittal sutures were identified. Using an electric drill, a 3 mm burr hole was made 3 mm lateral to the sagittal suture and 5 mm posterior to the coronal suture. The dura was incised sharply and a small amount of cortex and white matter was removed with gentle suction. To determine the maximally tolerated A-443654

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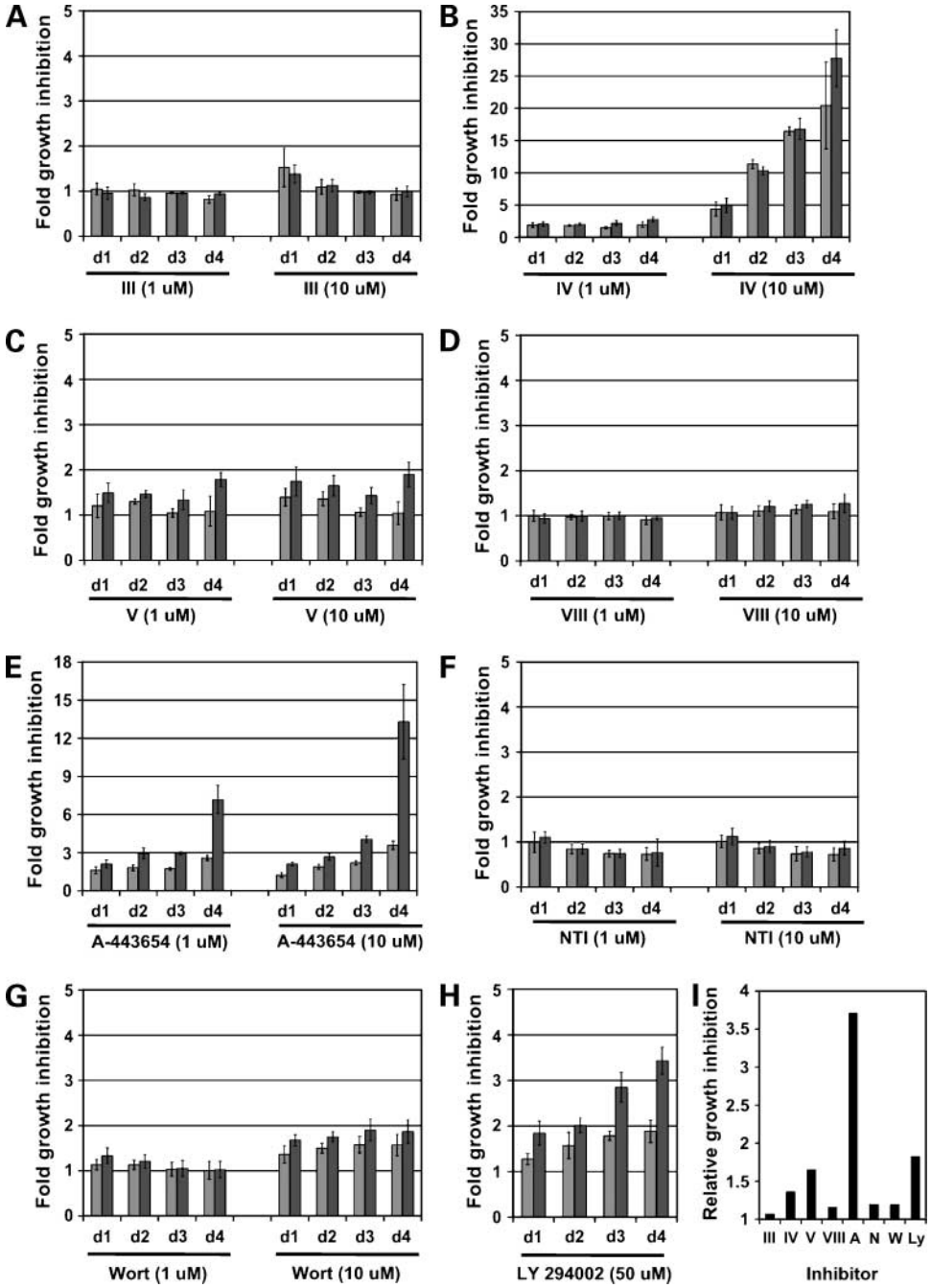

Figure 1. Among various known Akt inhibitors, A-443654 shows the greatest relative activity against PIK3CA mutant cells compared with isogenic cells without the mutation. **A** to **G,** cells containing either a wild-type (*light gray columns*) or a mutant (*dark gray columns*) PIK3CA gene were treated with 1 or 10 μmol/L of Akt inhibitors III, IV, V, and VIII, A-443654, NTI, and wortmannin for 24 h (*d1*), 48 h (*d2*), 72 h (*d3*), or 72 h (*d4*). **H,** cells containing either a wild-type (*light gray columns*) or a mutant (*dark gray columns*) PIK3CA gene were treated with 50 μmol/L LY294002 for 24 h (*d1*), 48 h (*d2*), 72 h (*d3*), or 96 h (*d4*). **A** to **H,** CCK-8 proliferation assays were done and the results were normalized to vehicle-treated cells. Average of 5 or 6 replicates. **I,** relative growth inhibition of cells containing a mutant versus a wild-type PIK3CA treated with 10 μmol/L of Akt inhibitors III, IV, V, and VIII, A-443654, NTI, and wortmannin and 50 μmol/L LY294002 at 96 h.

dose, rats underwent intracerebral implantation of polymers containing 0 (n = 8), 5% (n = 3), 20% (n = 3), 30% (n = 8), 40% (n = 7), and 50% (n = 8) A-443654 CPP:SA wafers. For efficacy studies, 9L gliosarcoma grown in the flank of female carrier Fischer rats was excised, cut into 1 mm^3 pieces, and placed intracranially. For animals receiving simultaneous implantation of tumor and polymers, the tumor piece was implanted first and the polymer was placed next to it. After implantation of tumor and/or polymers, the skin was closed with surgical staples. The delayed treatment group received tumor alone on day 0 and then underwent a second operation on day 4. On day 4, the animals were anesthetized and prepped as above. The midline scalp incision was reopened and the burr hole was identified. Control or A-443654 CPP:SA polymers were placed into the original defect and the scalp incision was closed with surgical staples. Animals were closely monitored for signs of toxicity, including failure to thrive, weight loss, and neurologic deficits.

Statistical Analysis

For the intracranial efficacy studies, death was the primary endpoint. Statistical analysis was done using the

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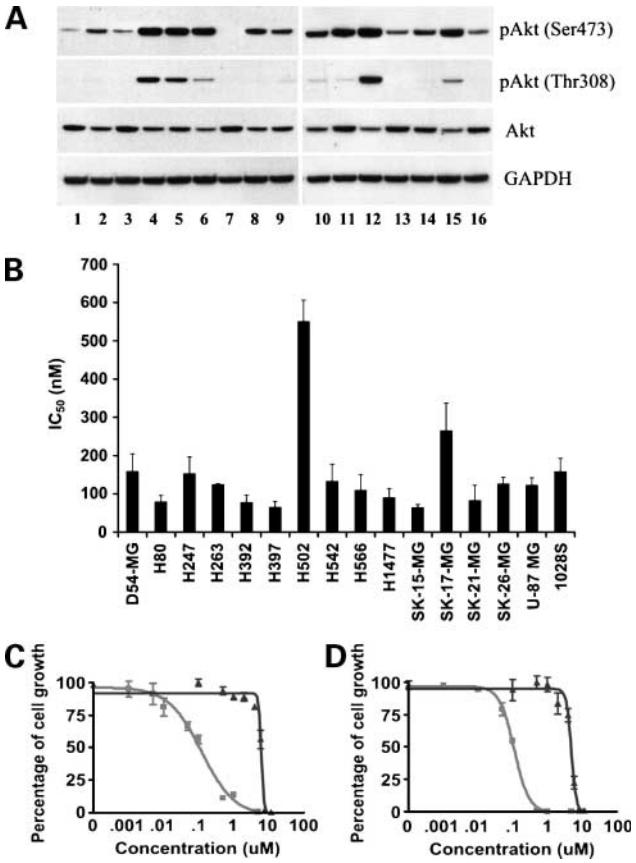

Figure 2. **A,** Akt activity is elevated in human glioblastoma multiforme cell lines. Whole-cell extracts from human glioblastoma multiforme cell lines cultured in 10% serum were analyzed by Western blot analysis using an anti-Akt, anti-phospho-Akt (Thr^{308}), and anti-phospho-Akt (Ser^{473}) antibodies. Blots were probed with an anti-glyceraldehyde 3-phosphate dehydrogenase antibody as a loading control. The order of the cell lines is the same as listed in panel **B**. **B,** A-443654 inhibits growth of glioblastoma multiforme cell lines. Glioblastoma multiforme cell lines cultured in 10% serum were treated with increasing concentrations of A-443654 or an equal volume of DMSO as a control and 48 h IC_{50} values were calculated. At least three independent experiments with each containing five or six replicates. Cell proliferation assays of D54-MG (**C**) and H566 (**D**) treated for 48 h with A-443654 (*light gray lines*) and A-739985, a methylated control that does not bind Akt (*dark gray lines*).

Kaplan-Meier method and the significance of survival differences was evaluated with the log-rank test using the software GraphPad Prism 4. P values < 0.01 were considered statistically significant.

Results

In vitro Isogenic Cell Line Screen of PIK3CA/Akt Inhibitors

The effects of several PI3K/Akt inhibitors were initially investigated in an isogenic cell line culture system. In this isogenic system, cells contained either a wild-type (127) or a mutant (129) PIK3CA with more Akt pathway activation in the mutant PIK3CA-containing cells (7). We tested a panel of known PI3K/Akt inhibitors in this system, seeking selective inhibition of the mutant lines relative to the wild-type control. Cells were plated in 96-well plates and treated with vehicle, 1 or 10 μmol/L of Akt inhibitors III, IV, V, and VIII, A-443654, NTI, and wortmannin and assayed for cell proliferation over the course of 4 days (Fig. 1). LY294002 was used at 50 μmol/L as this concentration has been shown previously to result in reduced cell proliferation to a greater extent in the mutant cells than in the wild-type cells (7). Our results with this concentration (Fig. 1H) were similar to those published previously (7).

At the concentrations tested, Akt inhibitors III, VIII, NTI, and wortmannin did not show any significant selective effect between the mutant and the wild-type cells (Fig. 1A, D, F, and G). Akt inhibitor IV resulted in reduced cell proliferation in both mutant and wild-type cells at 10 μmol/L (Fig. 1B). Akt inhibitor V showed some selectivity for the mutant cells at both concentrations especially at day 4 (Fig. 1C). A-443654 showed the greatest selective

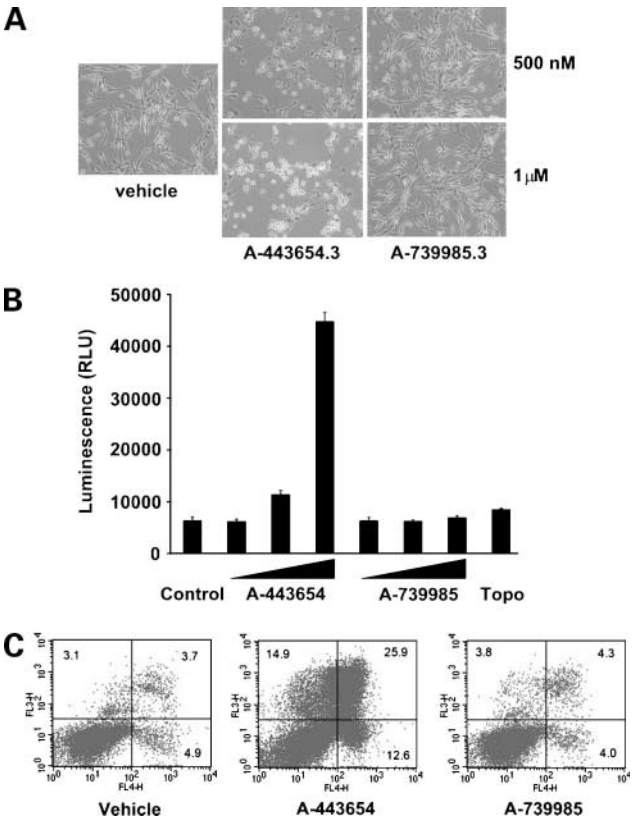

Figure 3. A-443654 induces apoptosis *in vitro*. **A,** U87-MG cells were treated for 24 h with vehicle (DMSO) or 500 nmol/L or 1 μmol/L of either A-443543 or 739985 and photographed. **B,** U87-MG cells were treated with vehicle (DMSO), A-443654, or A-739985 at 500 nmol/L, 1 μmol/L, or 5 μmol/L or topotecan at 20 μmol/L. After 24 h of treatment, caspase activity was determined. **C,** U87-MG cells were treated with vehicle (DMSO) or 5 μmol/L of either A-443654 or A-739985 for 24 h. Two-color flow cytometric analysis was done using APC-Annexin V and 7-amino-actinomycin D. *Bottom left,* viable cells; *bottom right,* percent of early apoptotic cells; *top right,* percent of both late apoptotic and necrotic cells. Representative of at least three independent experiments.

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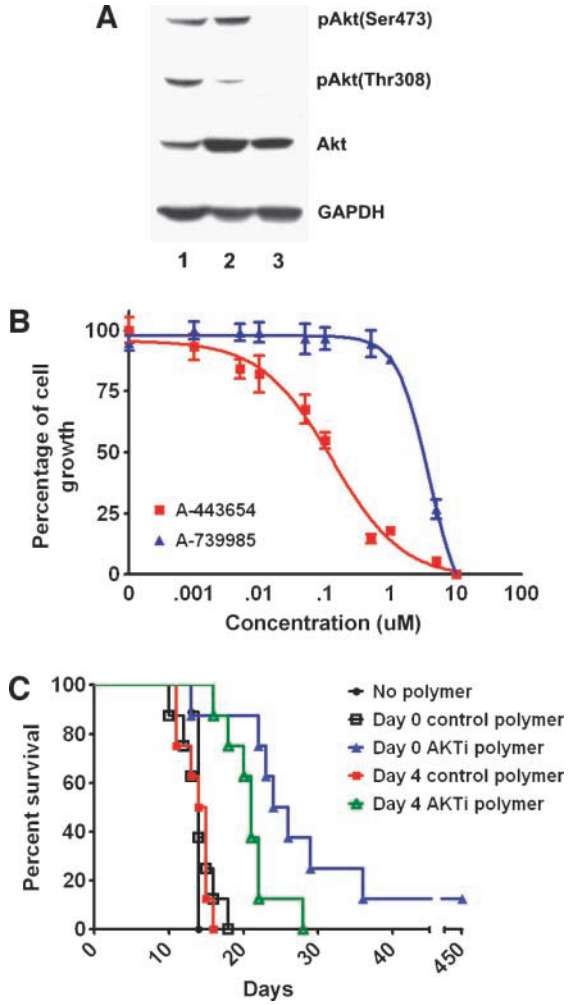

Figure 4. A-443654 increases survival in a syngeneic rat glioma model. **A,** Akt activity is elevated in the 9L rat gliosarcoma cell line. Whole-cell extracts from 9L cells (*lane 1*) were analyzed by Western blot analysis using anti-Akt, anti-phospho-Akt (Thr^{308}), and anti-phospho-Akt (Ser^{473}) antibodies. U87-MG (*lane 2*) and normal Fischer rat brain (*lane 3*) were included as positive and negative controls, respectively. Blots were probed with an anti-glyceraldehyde 3-phosphate dehydrogenase antibody as a loading control. **B,** cell proliferation assays of 9L cells treated for 48 h with A-443654 (*red line*) and A-739985 (*blue line*) showing sensitivity to the A-443654. **C,** Kaplan-Meier survival curves for control F344 rats (no polymer), animals that received simultaneous tumor implantation (day 0) of either empty or A-443654 (AKTi) containing polymers, and animals treated 4 days after tumor implantation (day 4) with either empty or A-443654 containing polymers. Animals treated with polymers containing A-443654 on day 0 or 4 had significantly extended survival compared with control animals. The median survival for the control animals (no polymer), control animals treated with empty polymers on day 0, and control treatment animals treated with empty polymers on day 4 were each 14 versus 20 days for the A-443654-treated animals on day 4 ($P < 0.0001$) and 25 days for the A-443654-treated animals on day 0 ($P = 0.0006$).

effect on the mutant cells compared with the wild-type cells with >3.5-fold relative growth inhibition of the mutant cells (Fig. 1E and I). Based on these findings, this small molecule was selected for further analysis.

Akt Is Activated in the Majority of Glioblastoma Multiforme Cell Lines

To determine if Akt signaling was active in the glioblastoma multiforme cell lines used to test growth inhibition, a series of 16 adherent glioblastoma multiforme cell lines grown in serum (traditionally cultured glioblastoma multiforme cell lines) was evaluated using Western blotting with antibodies specific for Akt and phospho-Akt. Moderate to very strong phosphorylation at one or both known phosphorylation sites of Akt was observed in 15 of 16 glioblastoma multiforme lines (Fig. 2A). One cell line, H502 (lane 7), had, in comparison with the other lines, a very small amount of phospho-Akt, which was observed only with longer exposure.

A-443654 Inhibits Glioblastoma Multiforme Growth *In vitro*

The growth-inhibitory effect of A-443654 was evaluated in the 16 glioblastoma multiforme cell lines using a cell proliferation assay. Cells were plated in 96-well plates and treated for 48 h with increasing concentrations of A-443654. All cell lines tested were sensitive to A-443654 with a mean IC_{50} of 147 nmol/L (range, 64-550 nmol/L). Interestingly, the cell line with the lowest amount of phospho-Akt, H502, had the highest IC_{50} (Fig. 2A and B), suggesting that A-443654 efficacy is related to interfering with activated Akt signaling. A-443654 is a pan-Akt inhibitor that binds to the ATP site of Akt and inhibits Akt-dependent signal transduction (9). To provide further evidence that the mechanism of action of A-443654 involves binding and inhibition of Akt, all 16 glioblastoma multiforme cell lines tested above were plated in 96-well plates and treated for 48 h with increasing concentrations of either A-443654 or its methylated analogue 2-methyl A-443654 (A-739985), which has a significantly reduced binding affinity for Akt. Cells were less sensitive to the methylated analogue by an average of 36-fold. Cell line H502 had the least difference with 4-fold reduced potency; cell line SK-15-MG had the greatest difference between A-443654 and the methylated analogue (76-fold). Two examples are shown in Fig. 2C (cell line D54-MG) and Fig. 2D (cell line H566). This reduced potency of the methylated analogue supports the Akt-mediated activity of A-443654.

A-443654 Induces Apoptosis *In vitro*

Akt inhibitors including A-443654 have been shown to induce apoptosis (9–11). To investigate the mechanism of cell death in glioblastoma multiforme, U87-MG cells were treated with A-443654 and A-739985. After 24 h, cells treated with A-443654 lost their astrocytic appearance and were rounder in comparison with control and A-739985-treated cells (Fig. 3A). To further determine if the inhibition of glioblastoma multiforme cell lines was secondary to an apoptotic mechanism, caspase and dual Annexin V/7-amino-actinomycin D flow cytometric assays were done. U87-MG cells were treated for 24 h with increasing concentrations of A-443654 and A-739985. As shown in Fig. 3B, caspase activity was induced after exposure to A-443654 but not after exposure to A-739985. In the next series of experiments, U87-MG cells were treated

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with vehicle, A-443654, or A-739985 for 24 h and then stained with Annexin V to measure phosphatidylserine translocation to the extracellular membrane (early apoptosis) and 7-amino-actinomycin D to measure the loss of phospholipid membrane integrity (late apoptosis/necrosis). Treatment of U87-MG cells with A-443654 resulted in an increase in both early apoptotic and late apoptotic/necrotic cells compared with the vehicle-treated cells and cells treated with A-739985 (Fig. 3C). Together, these data suggest that the inhibition of glioblastoma multiforme cell lines by A-443654 may, at least in part, occur via an apoptotic mechanism.

Rat Glioma Cell Line 9L Has Activated Akt

Our next major goal was to test the ability to inhibit tumor growth *in vivo* using a well-defined system. The rat gliosarcoma line 9L is commonly used as a high-grade glioma model and forms aggressive, invasive intracranial tumors. To first determine whether the cell line in this model has the presumed target, cells were examined by Western blot analysis for phospho-Akt. As shown in Fig. 4A, 9L cells express phospho-Akt. Cell proliferation assays with 9L cells showed similar IC_{50} values for A-443654 (IC_{50}, 100 nmol/L) and A-739985 (IC_{50}, 2.8 μmol/L; Fig. 4B) to the panel of human traditionally cultured glioblastoma multiforme cell lines tested earlier. Together, these data suggest that this cell line represents an appropriate model for *in vivo* testing of A-443654.

A-443654 Polymer Central Nervous System Toxicity Studies

The next question addressed was how best to deliver A-443654 for animal testing. Because systemic administration of A-443654 in animals has resulted in toxicities such as abnormal glucose metabolism and weight loss (9), we evaluated local delivery of A-443654 to the rat central nervous system. The polymer employed was CPP:SA, the same polymer used for the manufacture of the clinically approved Gliadel wafer used to deliver carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea] to high-grade gliomas. A-443654-loaded CPP:SA polymers containing 0%, 20%, 30%, 40%, and 50% A-443654 were implanted intracranially in non-tumor-bearing animals. A dose-dependent toxicity was observed (data not shown). Animals receiving the 50% and 40% A-443654 CPP:SA polymers began dying at days 7 and 9, respectively, and ≥50% of the animals were dead 21 days after implantation in these two treatment groups. Concentrations below 30% were well tolerated. There was one late death in the 30% A-443654 polymer group at day 65. This animal had a cystic cavity under the implantation site. Based on these findings, an efficacy study was done using 30% A-443654 CPP:SA polymers.

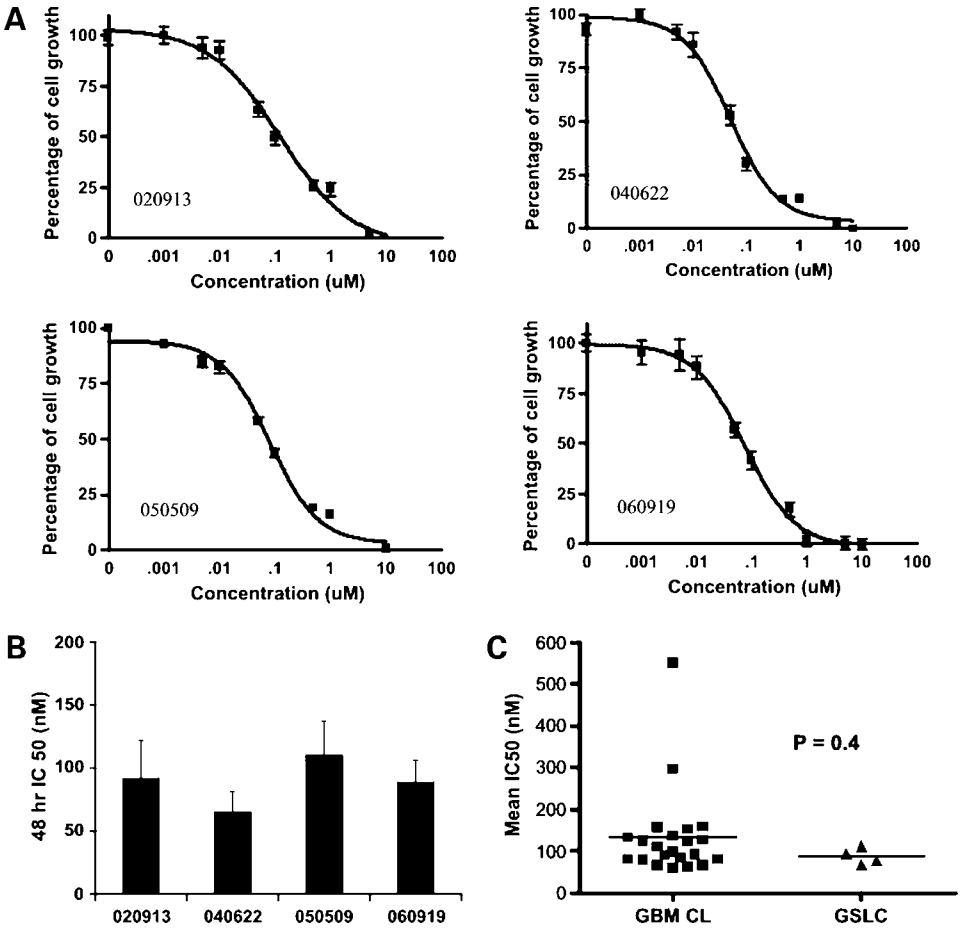

Figure 5. A-443654 inhibits GSLC lines. **A,** cell proliferation assays of four GSLC lines treated with increasing concentrations of A-443654 for 48 h. **B,** IC_{50} values of GSLC lines at 48 h treated with A-443654. At least three independent experiments with each containing five or six replicates. **C,** IC_{50} values at 48 h of four GSLC lines cultured in serum-free medium containing epidermal growth factor and fibroblast growth factor 2. There is no significant difference in sensitivity compared with glioblastoma multiforme lines grown in serum-containing medium ($P = 0.4$).

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A-443654 Extends Survival in an Intracranial Glioma Animal Model

To determine the effectiveness of local delivery of A-443654, tumor-bearing animals underwent implantation of empty or 30% A-443654 polymers either at day 0 or at day 4. Rats that received empty polymers either on day 0 or on day 4 had a median survival of 14 days, similar to untreated control rats (Fig. 4C). The median survival of rats (n = 8) that received 30% polymers implanted on the same day as the tumor increased to 25 days (P = 0.0006), with one animal living >400 days (long-term survivor). This represents a 79% increase in survival compared with controls. This long-term survivor was euthanized on day 450 and had no histopathologic evidence of tumor (data not shown). The median survival of rats (n = 8) that received tumor followed by 30% A-443654 polymers 4 days later increased to 20 days (P < 0.0001). Although there were no long-term survivors in this group, survival was increased by 43%. There was no statistically significant difference between the median survival of animals treated with A-443654 containing polymers at day 0 and day 4.

A-443654 Inhibits Growth of GSLC Lines

Accumulating evidence has suggested that stem-like precursors exist in glioblastoma multiforme. These cells display extensive self-renewal *in vitro* and *in vivo* and are multipotent (12, 13). We next examined the effect of A-443654 on GSLC lines, which are neurosphere cultures of glioblastoma multiforme that are thought to maintain a stem-like cell component. Glioblastoma multiforme neurosphere-forming cells were isolated from human glioblastoma multiforme samples as described previously (14). Flow cytometric analysis was done on four of these GSLC lines and four traditionally cultured adherent glioblastoma multiforme cell lines for the cell surface marker CD133, the best characterized marker to date of the glioblastoma multiforme stem-like population. The majority of cells in the neurosphere cultures were positive for CD133 compared with the traditionally cultured glioblastoma multiforme cell lines, which had no or little CD133 expression (<5%; data not shown). Proliferation assays were then done in the 4 GSLC lines with increasing concentrations of A-443654 (Fig. 5A). The 48 h IC_{50} values ranged from 66 to 110 nmol/L (Fig. 5B). There was no statistical difference between the 48 h IC_{50} values of the 4 GSLC lines compared with the 16 traditionally cultured cell lines (Fig. 5C), suggesting that inhibiting this pathway can kill both cells grown in serum and cells grown serum-free medium containing epidermal growth factor and fibroblast growth factor 2.

Discussion

Targeting kinases in human malignancies including glioblastoma multiforme represents an active area of research and there are numerous kinase inhibitors currently under investigation in human clinical trials. In this study, we screened a panel of PI3K and Akt inhibitors using a PIK3CA isogenic cell culture knockout system in which one cell line possessed a wild-type PIK3CA and the other possessed a mutant PIK3CA and an activated Akt pathway. One small molecule, A-443654, possessed superior activity against the cells containing the mutant PIK3CA compared with the cells with the wild-type PIK3CA. This molecule was further tested against a panel of traditionally cultured glioblastoma multiforme cell lines and displayed activity against all lines. Consistent with previous studies (10), the activity of this compound against this panel of glioblastoma multiforme cell lines was, at least in part, due to induction of apoptosis. Our data also show that local delivery of this inhibitor is efficacious against an intracranial model of glioblastoma multiforme. Furthermore, we were able to show that neurosphere cultures with a large CD133 population were equally sensitive to this Akt inhibitor compared with glioblastoma multiforme cell lines cultured in serum.

The Akt signaling pathway is very important in glioblastoma multiforme. Previous studies have shown that this pathway is activated in the majority of primary glioblastoma multiforme samples (1, 2) as well as in xenografts derived from glioblastoma multiforme tumor samples (15). We observed a similar rate of Akt pathway activation in the panel of 16 glioblastoma multiforme cell lines examined in this study (Fig. 2A) as well as in the GSLC lines (data not shown). Additional experimental evidence for the importance of this pathway includes the formation of glioblastoma multiforme in several animal models (1, 3) as well as the poorer survival of patients in whom this pathway is activated (4, 5). Akt represents a nodal point in cell signaling and can be activated by several upstream events including epidermal growth factor receptor amplification or mutation, loss of PTEN, and PIK3CA mutation. As such, targeting this pathway may be able to block glioblastoma multiforme proliferation secondary to a variety of upstream etiologies.

There are several Akt inhibitors that have been described in glioblastoma multiforme model systems (16, 17). Koul et al (16). reported two Akt-targeting small molecules, KP-372-1 and KP-372-2, which inhibited the *in vitro* growth of six glioblastoma multiforme cell lines and found that this inhibition was due to the induction of apoptosis. Another study by Momota et al (17). showed that perifosine, an oral Akt inhibitor, inhibited Akt and Ras-extracellular signal-regulated kinase 1/2 pathways in murine glial progenitors engineered to have activated Akt and Ras pathways and induced a G_1 and G_2 cell cycle arrest. Additionally, this group showed that perifosine reduced proliferation of a murine platelet-derived growth factor-driven glioma model *in vivo*. Taken together, these observations along with our current study suggest that pharmacologic targeting of Akt represents a potential therapeutic strategy for glioblastoma multiforme. Moreover, the synergy observed between A-443654 and chemotherapeutic agents in nonglial models (9, 10) and the increased radiosensitivity observed with targeting the PI3K/Akt pathway in glioblastoma multiforme (18, 19)

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raise interesting possibilities regarding treatment regimens that combine A-443654 with other therapeutic modalities such as temozolomide and cranial radiotherapy.

In this study, we chose to investigate the *in vivo* efficacy of A-443654 in an intracranial glioblastoma model system. Because of the reported metabolic toxicities of A-443654 and other inhibitors of the Akt/protein kinase B pathway, we chose to use local delivery. Additionally, numerous studies demonstrate that chemotherapeutics used in the treatment of glioblastoma such as BCNU and temozolomide (20, 21) are more efficacious when administered locally. Results from our *in vivo* study show that A-443654-containing polymers delivered locally either at the time of tumor implantation or in a delayed fashion prolonged survival in an experimental intracranial rodent glioblastoma multiforme model. The animals treated in a delayed fashion probably better represent clinical situations.

There is emerging evidence that GSLC lines may better represent primary human tumors (22, 23). Additionally, there are numerous studies suggesting that these cells may be chemoresistant and radioresistant (24–26). Interestingly, in our study, we did not find any difference in the IC_{50} values of traditionally cultured glioma lines compared with GSLC lines. Although further investigations will be necessary to understand this effect, it is of note that activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells (27), suggesting that targeting the Akt pathway may be efficacious in both stem and nonstem cell populations of glioblastoma multiforme.

Disclosure of Potential Conflicts of Interest

V.L. Giranda: employee, Abbott Laboratories. The authors disclosed no other potential conflicts of interest.

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