Inhibition of Akt survival pathway by a small-molecule inhibitor in human glioblastoma

Dimpy Koul,1 Ruijun Shen,1 Sherry Bergh,1 Xiaoyang Sheng,1 Shishir Shishodia,1 Tiffany A. Lafortune,1 Yiling Lu,2 John F. de Groot,1 Gordon B. Mills,2 and W.K. Alfred Yung1

1Brain Tumor Center, Department of Neuro-Oncology and 2Department of Molecular Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract
Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and Akt are important regulators of the phosphatidylinositol 3-kinase (PI3K) pathway and thus are important to the regulation of a wide spectrum of tumor-related biological processes. Akt regulates several critical cellular functions, including cell cycle progression; cell migration, invasion, and survival; and angiogenesis. Decreased expression of PTEN and overexpression of the Akt proto-oncogene, which is located downstream of PI3K, have been shown in a variety of cancers, including glioblastoma. Novel small-molecule inhibitors of receptors and signaling pathways, including inhibitors of the PI3K pathway, have shown antitumor activity, but inhibitors of Akt have not been examined. In this study, we tested our hypothesis that the pharmacologic inhibition of Akt has an antiproliferative effect on gliomas. We showed that two newly developed Akt inhibitors, KP-372-1 and KP-372-2 (herein called KP-1 and KP-2), effectively inhibited the PI3K/Akt signaling cascade. KP-1 and KP-2 blocked both the basal and epidermal growth factor–induced phosphorylation of Akt Ser473 at 125 and 250 nmol/L, which, in turn, reduced the activation of intracellular downstream targets of Akt, including GSK-3β and p70s6k. Furthermore, the treatment of U87 and U251 glioma cells with 125 to 250 nmol/L KP-1 and KP-2 for 48 hours inhibited cell growth by ∼50%. This decrease in cell growth stemmed from the induction of apoptosis. Collectively, these results provide a strong rationale for the pharmacologic targeting of Akt for the treatment of gliomas. [Mol Cancer Ther 2006;5(3):637–44]

Introduction
Malignant gliomas are the most common primary brain tumor in adults, but the prognosis for patients with these tumors remains poor despite advances in diagnosis and standard therapies, such as surgery, radiation therapy, and chemotherapy. Progress in the treatment of gliomas now depends to a great extent on an increased understanding of the biology of these tumors. Recent insights into the biology of gliomas include the finding that tyrosine kinase receptors and signal transduction pathways play a role in tumor initiation and maintenance. The epidermal growth factor receptor, platelet-derived growth factor receptor, vascular endothelial growth factor receptor, Ras/Raf/mitogen-activated protein kinase, and phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathways have all been found to be important for cell survival. The Akt pathway is of particular interest because it regulates several critical cellular functions, including cell cycle progression, migration, invasion, and survival as well as angiogenesis. In addition, the activated PI3K/Akt pathway provides major survival signals to glioblastoma multiforme cells and many other cancer cells (1–4). Furthermore, the ectopic expression of Akt induces cell survival and malignant transformation, whereas the inhibition of Akt activity stimulates apoptosis in a range of mammalian cells (5–8). Highlighting its potential as a therapeutic target is the fact that Akt is activated in 70% of gliomas (9–12). Recent studies have identified the substrates of Akt that are involved in the pro-cell survival effects, which thus far include glycogen synthase kinase-3, mTOR, FKHR, MDM2, p21, HIF-1α, IKK, Bad, and caspase-9 (13, 14). Akt has also been implicated as an important target of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), as the reintroduction of PTEN into PTEN-deficient glial, breast, and prostate cancer cells resulted in a decrease in activated Akt (15–17). Thus, the dysregulation of Akt seems to be an important consequence of the loss of PTEN function.

PTEN is a phospholipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-triphosphate (18, 19) and inhibits PI3K-dependent activation of Akt. The mutation or loss of PTEN leads to constitutively activated Akt. Consistent with this, Akt activity is elevated in about 70% of glioblastoma multiforme cells expressing mutant forms of PTEN (10). Activated Akt phosphorylates and inactivates Bad, caspase-9, and Forkhead transcription factors,
which not only suppresses apoptosis but also promotes cell survival (13, 14, 20). Furthermore, Akt must be activated for glioblastoma multiformes to form from genetically modified neural progenitors and normal human astrocytes, suggesting that the activation of Akt plays an important role in glioma formation and progression (11, 21). These observations establish Akt as an attractive target for cancer therapy, both alone and in conjunction with standard cancer chemotherapies, as a means of reducing the apoptotic threshold and preferentially killing cancer cells. Attention is thus focused on the development of potent small-molecule Akt inhibitors, but the potential of this strategy to inhibit glioma cell growth has not been studied. In the study we report on here, we tested our hypothesis that the pharmacologic inhibition of Akt has an antiproliferative effect on gliomas. We found that the small-molecule inhibitors of Akt, KP-1 and KP-2, inhibited Akt signaling in glioma cells, which inhibited cell growth and induced apoptosis.

Materials and Methods

Cell Lines

U87, U251, LN229, SNB19, U373, and D54 human glioblastoma cells were maintained at 37°C in culture medium (DMEM/F12, 10% fetal bovine serum) in a humidified atmosphere containing 5% CO2. The normal human lung fibroblast line CCD32-Lu was obtained from the American Type Culture Collection (Manassas, VA). Normal human astrocytes were purchased from Clonetics/Bio Whittaker (Walkersville, MD). Normal human astrocyte cultures were maintained in astrocyte growth medium from an AGM-Astroctye Medium Bullet kit obtained from Clonetics/Bio Whittaker.

Drugs

QLT, Inc. (formerly Kinetek, Vancouver, British Columbia, Canada) has provided us with a series of KP compounds [KP-372-1 (KP-1) to KP-372-5 (KP-5)] that target the Akt component of the PI3K pathway. KP compounds used in this study have at least 10-fold selectivity against a limited number of kinase targets, including CDK, CK2, CSK, DNAPK, ERK1, GSK3b, LCK, MEK1, PAK3, PIM, PKA, PKC, and S6K.3 A stock solution of 5 mmol/L was prepared in DMSO and stored at −20°C. Dilutions for all assays were made before use. DMSO has been used to dissolve these drugs and as the vehicle control for all the experiments on these agents.

Cell Proliferation Assays

Five thousand cells were plated into 38-mm2 wells of 96-well tissue culture plates. Cells were incubated with KP-1 and KP-2 (0.0625–2 mmol/L); control cells were incubated with either medium or DMSO alone. After a 2-day incubation, the number of metabolically viable cells was estimated with either medium or DMSO alone. After a 24-hour incubation, cells were trypsinized and plated at a density of either 100 or 500 per well in a six-well plate containing 1 mL of DMEM/F12/10% fetal bovine serum. Cells in culture were then incubated at 37°C in a humidified atmosphere containing 5% CO2. Colony growth was assessed by the size and number of colonies after 28 days. Colonies exceeding the minimum diameter of 80 μm were counted in triplicate wells, and the fraction of treated survival cells was calculated relative to the fraction of control surviving cells, which was taken as 1. Experiments were done in triplicate and repeated thrice.

Antibodies and Western Blotting

Subconfluent monolayers of cells were treated with KP-1 and KP-2 at the indicated doses in serum-free medium. Six hours later, cells were harvested, either with or without stimulation with epidermal growth factor (50 ng/mL) for 10 minutes. Cells were harvested in lysis solution containing 50 mmol/L HEPES (pH 7), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mmol/L Na3VO4, 10 μmol/L pepstatin, 5 mmol/L iodoacetic acid, and 2 μg/mL leupeptin. Proteins equal to 50 μg from U87 cells, either untreated or treated with KP-1 and KP-2 for 48 hours, were resolved by SDS-PAGE, electroblotted to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and then probed with the following primary antibodies: phospho-specific Akt (Ser473), phospho-specific GSK-3β (Ser9), phospho-specific mTOR (S2448), phospho-specific p70S6 kinase, and phospho-specific S6 kinase (Cell Signaling, Boston, MA), as well as poly(ADP-ribose) polymerase (PARP), Bcl-2, and Bax (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

LIVE/DEAD Assay

To measure the cytotoxic effect of KP-1 and KP-2 on glioma cell lines, we did the LIVE/DEAD assay (Molecular Probes, Invitrogen, Carlsbad, CA), which determines intracellular esterase activity and plasma membrane integrity. This assay employs calcein, a polyanionic dye that is retained within live cells and produces green fluorescence. It also employs the ethidium bromide homodimer dye (red fluorescence), which can enter the cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membrane of live cells. Briefly, 1 × 105 cells were treated with KP-1 and KP-2 at the indicated doses for 48 hours at 37°C. Cells were stained with the LIVE/DEAD reagent (5 μmol/L ethidium...
were fixed in 1% paraformaldehyde, stored in 70% ethanol, and analyzed for apoptosis by flow cytometry using the Apo-Bromodeoxyuridine kit (Phoenix Flow Systems, San Diego, CA) according to the manufacturer’s instructions.

We also investigated the effect of the KP-1 and KP-2 on the expression of apoptotic proteins. In particular, we determined the effect of inhibitor treatment on PARP, a well-known substrate for caspase-3, caspase-6, and caspase-7, to determine whether inhibitor-induced apoptosis was mediated through caspase activation. In this experiment, U251 and U87 cells were treated with KP-1 or KP-2 for 48 hours, after which whole-cell extracts were prepared and analyzed for cleavage of PARP. Immunoblot analysis was then done to determine the activation status of the downstream caspases.

**Terminal Deoxynucleotidyl Transferase–Mediated Nick End-Labeling Assay**

We also assayed cytotoxicity using the terminal deoxynucleotidyl transferase–mediated nick end-labeling method, which examines DNA strand breaks during apoptosis. We used the Roche in situ cell death detection reagent (Roche Applied Science, Indianapolis, IN). Briefly, 1 × 10⁵ cells were treated with KP-1 and KP-2 at the indicated doses for 48 hours at 37°C. Thereafter, cells were washed with PBS, air-dried, fixed with 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed, cells were incubated with reaction mixture for 60 minutes at 37°C. Stained cells were mounted and analyzed under a fluorescence microscope (Labophot-2). Pictures were captured using a Photometrics CoolSnap CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).

**Results**

**Akt Inhibitor Induces Cell Growth Inhibition**

We first tested the growth-inhibitory effect of KP-1 and KP-2 on a set of glioma cell lines using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Treatment of cells with KP-1 and KP-2 for 2 days resulted in dose-dependent growth inhibition (Fig. 1A and B). However, the magnitude of the growth inhibition varied among the different cell lines, with the IC₅₀ ranging between 125 and 250 nmol/L. We also tested these drugs on normal human astrocytes and normal human fibroblasts to check the toxicity on these cells. Treatment of these cells with KP-1 and KP-2 had no growth inhibitory effect on normal astrocytes and fibroblasts (Fig. 1C), showing the cancer specific effect of the drugs. We then did a colony-forming assay using two well-characterized and drug-sensitive glioma cell lines, U251 and U87, to confirm the growth-inhibitory effects of these Akt inhibitors. Treatment of the U251 and U87 cells with different concentrations of KP-1 and KP-2 abrogated the ability of the cells to form colonies, confirming the growth-inhibitory and antitumorigenic effects of KP-1 and KP-2 (Fig. 1D). In contrast, untreated U251 and U87 cells efficiently formed colonies reflecting their transformed phenotype.

**Akt Is Activated in Glioma Cell Lines**

In an attempt to delineate the role of Akt signaling in glioma cells, we first profiled the Akt phosphorylation status and the activation (i.e., phosphorylation) status of signaling targets downstream of Akt in a panel of glioma cell lines. As shown in Fig. 2A, glioma cells expressed high levels of activated Akt, as determined by the phosphorylation of Akt at Ser⁴⁷³. In addition, the downstream targets of Akt, including the S6 ribosomal protein, mTOR, and tuberin, were all activated in the cell lines tested. All of the components of the PI3K pathway that were analyzed were maximally activated in the U251 and U87 cells and were consequently used as a model system to delineate the effects of KP-1 and KP-2 on Akt signaling in glioma cells.

**KP Compound Inhibits Akt Phosphorylation and Downstream Targets of Akt**

We next examined the effect of the KP compounds on Akt phosphorylation and on downstream targets of Akt. KP-1 and KP-2 at 125 and 250 nmol/L inhibited both the basal and epidermal growth factor–induced phosphorylation of Akt at Ser⁴⁷³ (Fig. 2B and C). KP-1 and KP-2 also reduced the activation of Akt downstream targets, including the phosphorylation levels of GSK-3β (Ser²¹) and p70s6k (Thr³⁸⁹). At the same time, the expression of total Akt did not change significantly in response to the KP compound, indicating that the Akt inhibitor suppresses Akt activity, thereby decreasing the activation of its downstream targets GSK-3β and p70s6k. However, the KP compounds did not inhibit basal and epidermal growth factor–induced mitogen-activated protein kinase activation (data not shown). These data suggest that the KP compounds induce cell growth arrest specifically by inhibiting the phosphorylation of PI3K downstream targets.

**KP Compounds Induce Cytotoxicity in Glioma Cells**

This ability of KP compounds to selectively inhibit the Akt pathway suggested that they should inhibit cell proliferation and/or induce apoptosis. To investigate this possibility, we did a LIVE/DEAD assay and terminal deoxynucleotidyl transferase–mediated nick end-labeling and propidium iodide staining to determine the effects of KP-1 and KP-2 on both the cell cycle profile and apoptosis. In keeping with this, the LIVE/DEAD assay showed that 250 nmol/L KP-1 and KP-2 were cytotoxic.
Figure 1. Effect of Akt inhibitors on proliferation of human glioma cell lines. Six glioma cell lines were plated in 96-well plates at a density of 5,000 per well. Cells were treated with increasing concentrations of KP-1 (A) or KP-2 (B) in triplicate wells for 48 h under serum-fed conditions, and cell viability was assessed in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay described in Materials and Methods. The value for DMSO-treated cells was set to 100% (Y axis), and the number of cells for all drug doses were normalized as percentage of the number of cells in DMSO. There was a dose-dependent decrease in cell viability in all cell lines tested. C, effect of Akt inhibitor on proliferation of normal human astrocytes and normal human fibroblasts treated similar way as indicated above. D, U251 and U87 cells, either untreated or treated with different concentrations of the Akt inhibitors, were cultured for 28 d. The efficiency of colony formation was determined as described in Materials and Methods. Anchorage-dependent cell growth was abrogated by Akt inhibition.
to 81% and 72% of U87 cells and 89% and 75% of U251 cells, respectively (Fig. 3A). Furthermore, flow cytometry using the Apo-Bromodeoxyuridine kit showed that the Akt inhibitors induced apoptosis in both U87 and U251 glioma cells. Specifically, 22% and 52% of U87 cells treated with 125 and 250 nmol/L KP-1 and 19% and 49% of U87 cells treated with 125 and 250 nmol/L KP-2 showed apoptosis, respectively. Similarly, 38% and 70% of U251 cells treated with 125 and 250 nmol/L KP-1 and 25% and 55% of U251 cells treated with 125 and 250 nmol/L showed apoptosis, respectively. These data indicate that KP-1 is more potent than KP-2 in its ability to induce apoptosis (Fig. 3B). Terminal deoxynucleotidyl transferase–mediated nick end-labeling staining confirmed that the KP compounds induced apoptosis in U87 and U251 cells (Fig. 3C).

Effect of KP Compounds on Apoptotic Proteins

Immunoblot analysis of the extracts from cells treated with KP-1 or KP-2 clearly showed activation of the downstream caspases leading to cleavage of the 118-kDa PARP protein into an 87-kDa fragment, another hallmark of cells undergoing apoptosis (Fig. 3D). Untreated cells did not show any PARP cleavage. Furthermore, the antiapoptotic protein Bcl-2 was down-regulated, and the proapoptotic protein Bax was up-regulated in response to inhibitor treatment. These results indicate that the KP compounds induced apoptosis in glioma cells.

Discussion

In this study, we showed that small-molecule inhibitors of Akt can inhibit the growth of glioma cell lines. In particular, the Akt inhibitors KP-1 and KP-2 blocked both the basal and epidermal growth factor–induced phosphorylation of Akt. They also reduced the activation of downstream targets of Akt, including p70s6k and GSK-3β, consistent with the selective inhibition of Akt, and this was accompanied by inhibition of glioma proliferation. Indeed, KP-1 and KP-2 induced apoptosis enough to significantly inhibit cellular proliferation, although other cellular effects, such as cell cycle arrest, and the possibility of different findings in other cell systems cannot be ruled out.

PI3K is a lipid kinase (22) that promotes diverse biological functions, including cell proliferation, survival, and motility (23). Not surprisingly, therefore, the PI3K signaling pathway is frequently aberrantly activated in glioblastomas as a result of a mutation or loss of the tumor suppressor PTEN. Such PTEN abnormalities result in inappropriate signaling to downstream molecules, including Akt and mTOR. This, plus the fact that Akt is activated in 70% gliomas, makes the deregulation of the PI3K signaling pathway an attractive target for therapy (24, 25). Therefore, elucidating the molecular events associated with activation of this pathway represents an important goal because of the important use this knowledge can be put to in the development and clinical testing of PI3K pathway inhibitors.

The principle that kinase inhibitors can be effective treatments for some types of cancer has already been shown (24, 26), and PI3K pathway inhibitors are already in early clinical trials (27–30). The loss of PTEN expression is correlated with high levels of phosphorylated Akt in glioblastoma. Akt is considered an attractive target for...
chemotherapy, and it has been postulated that the inhibition of Akt alone or in combination with standard cancer chemotherapy will reduce the apoptotic threshold and preferentially kill cancer cells.

Several agents interfering with intracellular targets that are components of key oncogenic signaling pathways, such as the RAF kinase, PI3K/Akt, and Src kinases, are in preclinical and early clinical development. Two commonly used PI3K inhibitors, wortmannin and LY294002, may have limited clinical use, however, because they lack specificity and have potential adverse side effects, poor pharmacologic properties, low stability, and poor solubility (31). Indeed, most small-molecule inhibitors in this nascent field are classic ATP-competitive inhibitors that have little specificity. Nonetheless, there are several advantages to small-molecule drugs that make them worth pursuing; these include good delivery properties, good in vivo stability, a low probability of inducing an immune response, and low cost. Recently, novel allosteric inhibitors have been reported that are pleckstrin homology domain dependent and exhibit selectivity for the Akt isozyme.

Figure 3. Analysis of apoptosis induced by Akt inhibitors in U87 and U251 glioma cells. **A**, U87 and U251 cells were pretreated with Akt inhibitors at a concentration of 125 and 250 nmol/L for 48 h. Cells were stained with LIVE/DEAD assay reagent for 30 min and then analyzed under a fluorescence microscope, as described in Materials and Methods. The KP compounds were cytotoxic in both cell lines. **B**, U87 and U251 cells were treated with KP-1 (left) or KP-2 (right) for 48 h at concentrations of 125 and 250 nmol/L. The percentages of apoptotic cells were determined by terminal deoxynucleotidyl transferase–mediated nick end-labeling followed by flow cytometric analysis. Columns, mean of triplicate determinations; bars, SE. Apoptosis was induced by the KP compounds in both cell lines. **C**, U87 and U251 cells were pretreated with Akt inhibitors for 48 h. Cells were fixed, stained with terminal deoxynucleotidyl transferase–mediated nick end-labeling assay reagent and analyzed under a fluorescence microscope as described in Materials and Methods. Original magnification, ×200. **D**, effect of Akt inhibitors on apoptotic proteins, as shown by immunoblotting.
Similarly, in this report, we describe potential small-molecule inhibitors of the Akt pathway. Specifically, our data have shown that KP compounds can inhibit the proliferation of glioma cells by down-regulating Akt, p70S6k, and GSK-3β phosphorylation. They also induced apoptosis in glioma cell lines expressing high levels of activated Akt. Because we observed more extensive apoptosis in U87 cells (with wild-type p53) than in U251 cells (with mutant p53), we used a dominant-negative p53 variant of the U87 (32) cell line to show the role of p53 in inhibitor-induced apoptosis. Treatment of this cell line with an Akt inhibitor still induced apoptosis irrespective of the p53 status (data not shown). This suggests that KP compounds do not induce apoptosis through a p53-dependent pathway.

In addition to glioblastoma, PTEN is frequently mutated in endometrial and prostate cancer (33, 34), whereas Akt activity is also elevated in many ovarian and breast cancers. In these tumors, however, Akt activation is mainly due to amplification of the Akt oncogene or to activation by upstream regulators (35–37). It will therefore be of interest to determine whether KP compounds can inhibit other types of cancer that display high Akt kinase activity. In this regard, KP compounds have already been shown to inhibit Akt kinase activity and induce apoptosis in thyroid cancer cells with high Akt activity (38). These studies plus our study represent the first critical steps towards developing a small-molecule therapy that targets the Akt oncogenic pathway. This approach may prove effective not only against glioblastomas but also against prostate cancer, endometrial cancer, and other tumors in which PTEN and Akt disruption is common.

Acknowledgments

We thank QLT for providing Akt inhibitors and Betty P. Notzon (Department of Scientific publications, The University of Texas M.D. Anderson Cancer Center) for reading and editing of the article.

References


Mol Cancer Ther 2006;5(3). March 2006
Molecular Cancer Therapeutics

Inhibition of Akt survival pathway by a small-molecule inhibitor in human glioblastoma

Dimpy Koul, Ruijun Shen, Sherry Bergh, et al.

Mol Cancer Ther 2006;5:637-644.

Updated version  Access the most recent version of this article at: http://mct.aacrjournals.org/content/5/3/637

Cited articles  This article cites 35 articles, 18 of which you can access for free at: http://mct.aacrjournals.org/content/5/3/637.full#ref-list-1

Citing articles  This article has been cited by 6 HighWire-hosted articles. Access the articles at: http://mct.aacrjournals.org/content/5/3/637.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.