Targeting integrin-linked kinase inhibits Akt signaling pathways and decreases tumor progression of human glioblastoma

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Abstract
The phosphatidylinositol 3-kinase pathway is an important regulator of a wide spectrum of tumor-related biological processes, including cell proliferation, survival, and motility, as well as neovascularization. Protein kinase B/Akt is activated in a complex manner through the phosphorylation of protein kinase B/Akt on Thr308 and Ser473. Although protein-dependent kinase-1 has been shown to phosphorylate Akt at Thr308, it is not clear whether there is a distinct kinase that exclusively phosphorylates Akt at Ser473. A possible candidate is integrin-linked kinase (ILK), which has been shown to phosphorylate Akt at Ser473 in vitro. ILK is a multidomain focal adhesion protein that is believed to be involved in signal transmission from integrin and growth factor receptors. Further, ILK is implicated in the regulation of anchorage-dependent cell growth/survival, cell cycle progression, invasion and migration, and tumor angiogenesis. In this study, we tested the hypothesis that ILK inhibition would inhibit these processes in gliomas in which it is constitutively expressed. We found that a newly developed small-molecule compound (QLT0267) effectively inhibited signaling through the ILK/Akt cascade in glioma cells by blocking the phosphorylation of Akt and downstream targets, including mammalian target of rapamycin and glycogen synthase kinase-3β. Treatment of glioma cells with 12.5 μmol/L QLT0267 inhibited cell growth by 50% at 48 hours. An anchorage-dependent cell growth assay confirmed the cell growth-inhibitory effect of QLT0267. Further, the decrease in cell growth was associated with a dramatic accumulation of cells in the G2-M phase of the cell cycle. Although the cell growth-inhibitory effects of the ILK inhibitor were achieved only at a high concentration, the QLT0267 was able to reduce cellular invasion and angiogenesis at much lower concentrations as shown by in vitro invasion assays and vascular endothelial growth factor secretion. Thus, blocking the ILK/Akt pathway is a potential strategy for molecular targeted therapy for gliomas. [Mol Cancer Ther 2005;4(11):1681–8]

Introduction
The main pathologic characteristics of glioblastoma multiforme are unregulated cell proliferation, necrosis, and neovascularization, which have been shown to be influenced by the phosphatidylinositol 3-kinase (PI3K) pathway and more specifically by the activity of its downstream target Akt (1–4). Integrin-linked kinase (ILK) is a protein-dependent kinase that has been shown to regulate Akt activity (5) in a PI3K-dependent manner (6).

ILK, an ankyrin repeat–containing serine/threonine protein kinase (7) that interacts with the cytoplasmic domain of β1 and β3 integrins, is required for the localization of ILK in focal adhesion plaques (8) and regulates integrin-dependent functions. A region of amino acids in the primary ILK sequence fits a consensus sequence for phosphoinositide-binding pleckstrin homology domains (9), indicating a mechanism for the PI3K-dependent activation of ILK through its direct interaction with the lipid products of PI3K. It has further been shown in vitro that phosphatidylinositol 3,4,5-trisphosphate stimulates the kinase activity of purified recombinant ILK, suggesting that ILK is activated by a pleckstrin homology domain–mediated interaction with 3′-phosphorylated inositol lipids (10–12). Conversely, the phosphatase and tensin homologue (PTEN) tumor suppressor is a 3′ inositol lipid phosphatase (13) that acts as the key cellular antagonist of PI3K signaling (14). Recently, ILK regulation by PTEN has been shown to occur in glioblastomas in vitro (15).

ILK regulates many diverse processes. For example, it regulates cell cycle progression, survival, and division and changes in cell migration and invasion through its coupling of integrins and growth factors to downstream signaling pathways. The promotion of cell survival by ILK (16–19) is likely to be regulated primarily by the ability of ILK to promote the phosphorylation of Akt on Ser473 (5) and its consequent activation of downstream antiapoptotic pathways accomplished through the activation of nuclear factor-κB, the inhibition of forkhead transcription factors, and the inactivation of proapoptotic proteins, such as BAD,
among other events (20). In particular, ILK can activate nuclear factor-κB in an Akt-dependent manner (21); conversely, the inhibition of ILK with a dominant-negative ILK mutant inhibits Akt and nuclear factor-κB activation (22). Of particular note, ILK activity is constitutively activated in PTEN-negative human cancers, such as prostate cancer, breast cancer, and glioblastomas, and the inhibition of ILK activity in PTEN-negative cells inhibits Akt phosphorylation and downstream Akt targets.

ILK is also an important regulator of invasion and angiogenesis. Inhibition of ILK activity in highly invasive human glioblastoma cells and in ILK-overexpressing SCp2 mammary epithelial cells has been shown to substantially inhibit invasion across Matrigel, showing that ILK is involved in invasion (17). Recent studies have also suggested that ILK plays a significant role in vascular development, vascular morphogenesis, and tumor angiogenesis. Not surprisingly, therefore, because ILK activity is high in PTEN-negative cells, which also express high levels of vascular endothelial growth factor (VEGF), the inhibition of ILK activity decreased VEGF expression in a prostate cancer model (23).

The modulation of ILK in cancer cells is therefore a potential strategy for the efficacious treatment of cancers with increased ILK expression, and such treatment could be achieved through a range of approaches. In the present study, we studied the effect of modulating the activity of ILK in glioblastoma cells using the small-molecule inhibitor QLT0267. In particular, we studied whether QLT0267 inhibited ILK activity in glioma cells, as shown by the inhibition of its downstream targets Akt and glycogen synthase kinase-3β (GSK-3β), and whether this led to cell growth inhibition resulting from G2-M cell cycle arrest and the inhibition of invasion and VEGF secretion.

Materials and Methods

Cell Lines

U87, U251, LN229, SNB-19, U373, and D54 human glioblastoma cell lines were maintained at 37°C in culture medium (DMEM/F-12/10% fetal bovine serum) in a humidified atmosphere containing 5% CO₂.

ILK Activation

The stimulation of ILK activity in the extracellular matrix was assessed in U87 cells. First, the cells were washed and then incubated for various times (30 minutes to 4 hours) in serum-free medium on fibronectin (10 μg/mL) or vitronectin (5 μg/mL). Briefly, six-well tissue culture microplates were coated with 500 μL (5 μg/mL) vitronectin from human plasma (Sigma Chemical Co., St. Louis, MO) and 10 μg/mL fibronectin (Sigma) diluted in PBS. After this, the plates were stored at 4°C overnight, washed with PBS, air-dried, and used immediately. To assess the effect of insulin or serum on ILK activity, U87 cells were serum starved for 24 hours, incubated with insulin (100 nmol/L) or serum for 30 minutes, and then plated on either fibronectin- or vitronectin-coated plates. Cells were harvested at the indicated times and then lysed, after which the proteins were isolated and analyzed by Western blotting. Because Akt phosphorylation at Ser²⁷³ reflects the measure of ILK activity, the membrane was probed with phosphorylated Akt (Ser²⁷³) antibodies.

Drugs

The ILK inhibitor QLT0267 (QLT, Inc., Vancouver, British Columbia, Canada) was shown in a cell-free assay to inhibit the kinase activity of ILK at a concentration of 26 nmol/L. Preliminary experiments suggest that it has ~1,000-fold selectivity for ILK over other kinases tested under similar conditions, including CK2, CSK, DNA protein kinase, PIM1, protein kinase B/Akt, and protein kinase C, and an ~100-fold selectivity for ILK over extracellular signal-regulated kinase 1, GSK-3β, LCK, protein kinase A, p70S6K, and RSK1 (QLT). Of the kinases tested, cyclin-dependent kinases 1, 2, and 5 showed the greatest inhibition by QLT0267, but the ILK inhibitor still showed at least 10-fold selectivity for ILK over these kinases. Drug was dissolved in DMSO, which was used as the vehicle control for all the experiments.

Cell Proliferation Assays

Five thousand cells were plated into 38-mm² wells of 96-well tissue culture plates. Cells were incubated with QLT0267 (1.5–50 μmol/L) and control cells were incubated with either medium or DMSO alone. After a 2-day incubation, the number of metabolically viable cells was determined in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan by metabolically active cells was measured by a MR-5000 96-well microtiter plate reader (Dynatech, Inc., Chantilly, VA) at an absorbance of 570 nm. Growth inhibition was calculated by the following formula: cytostasis (%) = [1 – (A / B)] × 100, where A is the absorbance of treated cells and B is the absorbance of control cells.

Colony-Forming Assays

Cells (1 × 10⁶) were incubated with QLT0267 and control cells were incubated with medium or DMSO alone. After a 24-hour incubation, cells were trypsinized and plated at a density of either 100 or 500 cells per well in a six-well plate containing 1 mL DMEM/F-12/10% 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 surviving 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 QLT0267 at the indicated doses in serum-free medium. Twenty-four 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 7.0), 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, 10 μg/mL aprotinin, 5 mmol/L iodoacetic acid, and 2 μg/mL leupeptin. Proteins were resolved by SDS-PAGE, electroblotted to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and then probed with the following primary antibodies: phosphospecific Akt (Ser473), GSK-3β (Ser9), and mammalian target of rapamycin (mTOR; S2448; Cell Signaling, Boston, MA) and cyclin B1 and CDC2 (pY15; Santa Cruz Biotechnology, Santa Cruz, CA). Anti-β-actin antibody was purchased from Sigma. Anti-hypoxia-inducible factor-1α (HIF-1α) antibody was obtained from Affinity BioReagents (Golden, CO).

**Cell Cycle Analysis**

Cells (2 × 106) were seeded onto a 10-cm tissue culture dish and incubated overnight with serum-free medium followed by treatment with QLT0267 at the indicated doses. Cells were then maintained in medium supplemented with 10% FCS. Cells were harvested after 48 hours of drug treatment. Cells for DNA analysis were fixed in 1% paraformaldehyde, stored in 70% ethanol, and stained with propidium iodide. Analysis was done on a FACScan calculations were done using BD software (Becton Dickinson, Franklin Lakes, NJ).

**Cell Invasion Assay**

Cells were treated with QLT0267 for 24 hours, and the invasion of glioma cells in vitro was assessed by the invasion of the cells through Matrigel-coated Transwell inserts (24). Briefly, Transwell inserts with a 12-μm pore size were coated with a final concentration of 0.78 mg/mL Matrigel in cold serum-free medium. Cells were trypsinized, and cell suspension (500 μL; 1 × 107 cells/mL) was added in triplicate wells. After a 24-hour incubation, the cells that passed through the filter into the lower wells were scored using a phase microscope. The membranes were dissolved in 2% deoxycholic acid and read colorimetrically at 595 nm.

**Gelatin Substrate Gel Zymography**

Zymography was done using a minor modification of the procedure described by Nakajima et al. (25) using 7.5% SDS-polyacrylamide gels impregnated with 0.1% gelatin (w/v) and 10% polyacrylamide (w/v). Supernatants as conditioned medium were collected after 24 hours of QLT0267 treatment, as described above, and equal amounts of protein were mixed with the SDS sample buffer without reducing agent, after which proteins were subjected to SDS-PAGE. After electrophoresis, the gels were washed several times in 2.5% Triton X-100 for 1 hour at room temperature to remove the SDS and then incubated for 24 hours at 37°C in buffer containing 5 mmol/L CaCl2 and 1 μmol/L ZnCl2. The gels were stained with Coomassie blue (0.25%) for 30 minutes and then destained for 1 hour in a solution of acetic acid and methanol. The proteolytic activity was evidenced by clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

**ELISA for VEGF**

Subconfluent cells were treated with QLT0267. After a 24-hour incubation, cell cultures were rinsed twice with serum-free medium and incubated for a further 6 hours. Cells were then incubated with serum-free medium. After 24 hours, conditioned medium was collected and clarified by centrifugation at 4,000 × g for 5 minutes at 4°C and the supernatants were frozen and stored at −80°C until use. The levels of VEGF were measured in conditioned medium samples with the Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Results**

**ILK Activity Is Stimulated by Fibronectin and Vitronectin**

Analysis of ILK activity in cells plated on fibronectin and vitronectin showed that ILK is rapidly activated at 30 minutes, and its activity declines rapidly, such that at 2 hours it is markedly diminished. Because ILK phosphorylates and thereby activates Akt, we used the phosphorylation of Akt at Ser473 as a measure of ILK activity. Our analysis of the effect of growth factors on ILK activity revealed that ILK activity is stimulated by serum and insulin in serum-starved cells (Fig. 1), with activity peaking within 30 minutes of exposure.

**ILK Inhibitor Inhibits Cell Growth**

We next did a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to test whether the ILK inhibitor QLT0267 inhibited the growth of a set of glioma cell lines. Treatment of cells with QLT0267 for 2 days resulted in a dose-dependent growth inhibition (Fig. 2A), with the magnitude of growth inhibition varying among the different cell lines. The IC50 for various cell lines ranged between 5 and 10 μmol/L. We then did a colony formation assay in two well-characterized and drug-sensitive glioma cell lines, U251 and U87, to confirm the growth-inhibitory effects of QLT0267. As expected, treatment of the U251 and U87 cells with different concentrations of QLT0267 abrogated the ability of the cells to form colonies, confirming the growth-inhibitory and antitumorigenic effects of this

**Figure 1.** ILK activity is stimulated by extracellular matrix components: stimulation of ILK in the extracellular matrix was assessed in U87 cells by washing the cells and incubating them for various times (30 min to 4 h) in serum-free medium on either regular tissue culture plates or fibronectin (FN) – coated or vitronectin (VN) – coated plates. The phosphorylated Akt (pAkt) levels were assessed as a measure of ILK activity. U87 cells were also serum starved for 24 h and then incubated with insulin (100 nmol/L) and 10% serum for 30 min to assess the effects of insulin and serum on ILK activity.
inhibitor (Fig. 2B). In contrast, untreated U251 and U87 cells efficiently formed colonies that reflected their transformed phenotype.

**Inhibition of ILK Activation Suppresses Akt and GSK-3β Phosphorylation**

In certain human glioblastoma cell lines (U87 and U251), the PI3K pathway is constitutively activated due to the loss of expression of the tumor suppressor PTEN (26). We have also seen that ILK activity is constitutively activated in these cell lines (Fig. 1). In this experiment, we evaluated the effect of QLT0267 on ILK activity in terms of its effects on the downstream effectors of ILK, Akt and GSK-3β. In both U251 and U87 cells, increasing doses of QLT0267 resulted in significantly reduced levels of phosphorylated Akt concomitant with the inhibition of phosphorylation of GSK-3β (Fig. 3). At the same time, the expression of total Akt did not change significantly in response to QLT0267 treatment, indicating that the ILK inhibitor inhibits ILK activity, thereby decreasing the activation of its downstream targets Akt and GSK-3β.

**Inhibition of ILK Induces G2-M Cell Cycle Arrest**

To examine the effect of ILK inhibition on the cell cycle, we stained the cells with propidium iodide and then did flow cytometry. This showed a marked increase in the fraction of U87 cells in the G2 phase after treatment with QLT0267 for 48 hours (Fig. 4A). Next, we explored the effect of QLT0267 on cell cycle checkpoint signaling. Because CDC2 and cyclin B1 are key mediators of the G2-M transition, high levels of CDC2 phosphorylation at Tyr15 [CDC2 (pY15)] and cyclin B1 were considered indicative of

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**Figure 2.** A, ILK inhibitor inhibits proliferation of human glioma cell lines. Six glioma cell lines were plated in 96-well plates at a density of 5,000 cells per well. Cells were treated with increasing concentrations of ILK inhibitor 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 as described in Materials and Methods. There was a dose-dependent decrease in cell viability in all cell lines tested. B, abrogation of anchorage-dependent cell growth by ILK inhibition. U251 and U87 cells, either untreated or treated with different concentrations of the ILK inhibitor, were cultured for 28 d. The efficiency of colony formation was determined as described in Materials and Methods.
G2-M checkpoint arrest. As shown in Fig. 4B, QLT0267 induced increased levels of CDC2 (pY15) and cyclin B1, and this correlated with an increased 4C population in U87 cells, as shown by flow cytometry, thereby confirming that ILK inhibition induced a sustained G2-M checkpoint arrest.

ILK Inhibition Decreases Cell Invasion and Down-Regulates Matrix Metalloproteinase-2

An in vitro invasion assay was done next to determine if ILK inhibition decreases the invasive capability of glioma cells (24). The cell lines U251 and SNB-19 were treated in vitro with increasing concentrations of QLT0267 for 24 hours (Fig. 5A, B, D, and E). Invasion of both cell lines was inhibited in response to QLT0267 at concentrations below the IC50. Because several matrix metalloproteinases (MMP), including MMP-2, which are involved in tumor invasion, have been shown to be elevated in human gliomas, we next investigated whether there is any correlation between MMP-2 activity and tumor cell invasion under the influence of ILK. Each glioma cell line was treated with QLT0267 in serum-free medium for 24 hours, the supernatant was harvested, protein was quantified, and gelatin zymography was done. In both cell lines, the MMP-2 was down-regulated by QLT0267 (Fig. 5C and F).

Inhibition of ILK Suppresses mTOR/FKBP12-Rapamycin-Associated Protein and Inhibits VEGF Expression in Glioma Cells

To elucidate in more detail the ILK-mediated signaling pathway leading to the stimulation of VEGF and to assess the effect of ILK on VEGF expression in cancer cells, we first treated U87 glioma cells, which express VEGF, with QLT0267 to inactivate ILK expression and activity. As shown by ELISA of the conditioned medium, the inhibition of ILK decreased VEGF secretion (Fig. 6A). Furthermore, the inhibition of ILK significantly inhibited expression of HIF-1α (Fig. 6B). It has been shown recently that Akt can regulate the expression of HIF-1α protein at the translational level by stimulating the phosphorylation of mTOR/FKBP12-rapamycin-associated protein (FRAP), which regulates protein synthesis. We therefore determined whether the ILK-mediated expression of HIF-1α and VEGF also involved mTOR/FRAP. As shown in Fig. 6B, the inhibition of ILK activity led to a substantial decrease in mTOR/FRAP phosphorylation concomitant with the inhibition of HIF-1α and VEGF secretion, suggesting that the ILK-mediated expression of HIF-1α and VEGF also involve mTOR/FRAP.

Discussion

Our results showed that the treatment of glioma cells with QLT0267 in vitro suppressed the phosphorylated protein levels of Akt, GSK-3β, and mTOR without down-regulating...
the total Akt, GSK-3β, and mTOR levels, consistent with selective inhibition of ILK. The inhibition of Akt activation and its downstream targets was accompanied by inhibition of the proliferation of glioma cells. Indeed, the effect of the small-molecule inhibitor on progression through the G2 phase was great enough to significantly inhibit cellular proliferation, although other cellular effects, such as the induction of apoptosis, cannot be ruled out and may depend on the cell system used.

Several approaches have been used to inhibit ILK activity in glioma cells. For example, the inhibition of ILK by antisense ILK suppressed the constitutive phosphorylation of Akt on Ser473, resulting in apoptosis in a U87 glioma cell model (27). Similarly, ILK suppression by antisense ILK caused a tumor growth delay in Rag-2 M mice bearing established human U87 glioblastoma tumors (27), suggesting that ILK is an important therapeutic target.

Because of the oncogenic properties of ILK, we also wanted to determine the effects of ILK inhibition on invasion and angiogenesis. We found that ILK inhibition decreased the in vitro invasive capability of glioma cells, concomitant with a decrease in MMP-2 secretion. VEGF, an important mediator of angiogenesis, and its major transcriptional activator HIF-1α were also reduced in response to ILK inhibition. Because ILK is PI3K dependent and an upstream target of Akt, it is likely to be involved in the regulation of VEGF and HIF-1α expression through its regulation of Akt activity. In addition, because HIF-1α translation is regulated through the regulation of mTOR/FRAP, a downstream target of Akt (28), we also examined whether ILK is essential for the regulation of

Figure 5. ILK inhibition decreases glioma cell invasion. A and D, U251 and SNB-19 glioma cell lines were treated with increasing doses of the ILK inhibitor for 24 h, harvested, and replated onto Matrigel-coated inserts. Cells were allowed to incubate for an additional 24 h, and cells that migrated to the opposite side of the insert were stained with crystal violet. B and E, the membranes with invaded cells were dissolved in 2% deoxycholic acid and read colorimetrically at 595 nm for quantification of invasion. C and F, effect of QLT0267 on MMP-2 secretion as measured by gelatin zymography of conditioned medium.
HIF-1α expression and the consequent production of VEGF in human glioma cells, and our findings bore this out. Conversely, the functional inactivation of ILK by QLT0267 resulted in decreased HIF-1α protein levels and VEGF expression followed by decreases in Akt and mTOR/FRAP phosphorylation. These data suggest that, in glioma cells, ILK plays a crucial role in HIF-1α and VEGF expression through its activation of Akt and the phosphorylation of mTOR/FRAP.

The inactivation of tumor suppressors results in the deregulated activation of immediate downstream effectors; thus, the inhibition of these effectors may represent a selective and alternative means of treating tumors with mutated tumor suppressor genes. The role of ILK in stimulating various signaling pathways has been understood through its constitutive activation and/or overexpression in epithelial cells. This has been achieved with growth factors and loss-of-function strategies. In terms of the latter, transfection with dominant-negative ILK and ILK small interfering RNA has revealed a critical role for ILK in the stimulation of downstream components of many key signaling pathways. ILK and Akt are constitutively activated in human glioblastomas lacking expression of the PTEN tumor suppressor (15), which means that the expression and activity of ILK is increased in gliomas. Targeting ILK is therefore a rational approach to the treatment of glioblastoma, and various approaches have been used to inhibit the ILK activity in various cancers.

In summary, we showed that the inhibition of ILK in glioblastoma cells decreased cell proliferation, invasion, and angiogenesis through the down-regulation of Akt, mTOR/FRAP, and GSK-3β phosphorylation. Our results therefore suggest that ILK inhibition specifically affects those properties responsible for malignant transformation. Thus, targeting ILK in the glioblastoma signal transduction pathway may represent a novel strategy in the treatment of these tumors.

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