Glycogen Synthase Kinase 3β Sustains Invasion of Glioblastoma via the Focal Adhesion Kinase, Rac1, and c-Jun N-Terminal Kinase-Mediated Pathway

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Abstract

The failure of current treatment options for glioblastoma stems from their inability to control tumor cell proliferation and invasion. Biologically targeted therapies offer great hope and one promising target is glycogen synthase kinase-3β (GSK3β), implicated in various diseases, including cancer. We previously reported that inhibition of GSK3β compromises the survival and proliferation of glioblastoma cells, induces their apoptosis, and sensitizes them to temozolomide and radiation. Here, we explore whether GSK3β also contributes to the highly invasive nature of glioblastoma. The effects of GSK3β inhibition on migration and invasion of glioblastoma cells were examined by wound-healing and Transwell assays, as well as in a mouse model of glioblastoma. We also investigated changes in cellular microarchitectures, cytoskeletal components, and proteins responsible for cell motility and invasion. Inhibition of GSK3β attenuated the migration and invasion of glioblastoma cells in vitro and that of tumor cells in a mouse model of glioblastoma. These effects were associated with suppression of the molecular axis involving focal adhesion kinase, guanine nucleotide exchange factors/Rac1 and c-Jun N-terminal kinase. Changes in cellular phenotypes responsible for cell motility and invasion were also observed, including decreased formation of lamellipodia and invadopodium-like microstructures and alterations in the subcellular localization, and activity of Rac1 and F-actin. These changes coincided with decreased expression of matrix metalloproteinases. Our results confirm the potential of GSK3β as an attractive therapeutic target against glioblastoma invasion, thus highlighting a second role in this tumor type in addition to its involvement in chemo- and radioresistance. Mol Cancer Ther; 1–11. ©2014 AACR.

Introduction

Glioblastoma is the most frequent and lethal type of malignant primary brain tumor (1). The mainstay of current treatment for patients with glioblastoma is maximal tumor-reduction surgery where this is safe, followed by chemoradiation therapy (1, 2). The pathologic triad of this disease includes the highly proliferative capacity of the tumor cells, their borderless invasive ability, and tumor neoangiogenesis (1). The invasive nature of glioblastomas prevents complete surgical removal and renders them resistant to temozolomide-based chemotherapy, radiation, and a combination of both (2). Consequently, the median overall survival of patients following diagnosis is just 15 months and has not improved significantly over the past 30 years (1, 3). Recent clinical trials using pharmacologic inhibitors of epidermal growth factor receptor (EGFR) and HER2 and therapeutic antibodies against EGFR failed to show significant clinical benefit (4). This is probably due to extensive heterogeneity at the cellular and molecular levels and the complex interplay between different oncogenic signaling pathways in tumor cells (5). Clinical trials using the antiangiogenic agent bevacizumab, a monoclonal antibody to vascular endothelial growth factor, demonstrated some improvement in progression-free survival in newly diagnosed and recurrent glioblastomas, but failed to show an overall survival benefit (4). An undesired consequence of this therapy is the enhancement of tumor cell invasion in the resulting hypoxic tumor environment due to a metabolic shift toward glycolysis and upregulation of other proangiogenic factors (6, 7). The effective targeting of biologic mechanisms that facilitate tumor cell invasion will be crucial for the development of more successful treatment strategies (8).

Glycogen synthase kinase-3β (GSK3β) is a serine/threonine protein kinase that has emerged as a key enzyme in a number of chronic progressive diseases, including diabetes mellitus, neurodegenerative disorders, and cancer (9, 10). We and others have demonstrated that inhibition of CSK3β attenuates the survival and proliferation of glioblastoma cells by modulating specific

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
molecular pathways (11, 12), thus sensitizing them to chemotherapeutic agents and radiation (12, 13). It has also been reported that GSK3β inhibition with lithium and indirubins reduces the invasive potential of glioblastoma cells (14, 15). However, a recent study indicated both pro- and anti-invasive roles for GSK3β depending on its subcellular localization in glioblastoma (16). Despite the increasing number of studies showing its participation in cell polarity and motility (17), relatively little is known about a putative role for GSK3β in the migration, invasion, and metastasis of tumor cells. Here, we demonstrate that inhibition of GSK3β attenuates the invasion of glioblastoma cells via effects on the proinvasive cellular microarchitectures and on a pivotal pathway involving focal adhesion kinase (FAK). Rac1, and c-Jun N-terminal kinase (JNK).

**Materials and Methods**

**Cell lines**

The human glioblastoma cell lines U87 and U251 were obtained from the American Type Culture Collection, whereas A172 and T98G were obtained from the Human Science Research Resource Bank (Osaka, Japan) and Biomedical Cell Resource Center, Tohoku University Geriatric Medicine Research Institute (Sendai, Japan) in 2009. These cell lines were characterized by the respective resource bank/institute by short tandem repeat profile analysis, and passaged in our laboratory for fewer than 6 months after resuscitation. They were maintained at 37°C with 5% CO₂ in high-glucose Dulbecco's modified Eagle medium (U87, U251, A172) and RPMI-1640 (T98G; Sigma-Aldrich). All medium was supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin-G and 100 µg/mL streptomycin; Gibco).

**Western blotting**

Protein was extracted from cultured cells and mouse brain tissues using lysis buffer (Cellytic-MT; Sigma-Aldrich) containing a mixture of protease and phosphatase inhibitors (Sigma-Aldrich). A 30-µg aliquot of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western immunoblotting for the proteins of interest. Electroblotted membranes (Amersham) were blocked with 5% bovine serum albumin before detection of phosphorylated protein fractions. The source and working dilutions of the primary antibodies are listed in Supplementary Table S1. Signals were developed using enhanced chemiluminescence (ECL; Amersham). The amount of protein extract in each sample was monitored by the expression of β-actin. Immunoblot signals were measured using the CS analyzer (version 2.0; AITTO).

**Assays for cell migration and invasion**

Glioblastoma cell migration and invasion were examined by wound-healing assay and Transwell assays, respectively. Confluent tumor cells grown in the presence of dimethyl sulfoxide (DMSO; Sigma-Aldrich) or the GSK3β inhibitor AR-A014418 (Calbiochem) dissolved in DMSO at the indicated concentrations were scratched with a 20-µL micropipette tip to create a cell-free zone (wound). For each condition studied, the gap distance between the wound edges was measured at three fixed reference points for 12 to 48 hours using a phase-contrast microscope (Axiovert 40 CFI; Zeiss). Cell migration at each time point was calculated as the mean distance of the gap measured at the three reference points. This was compared between cells treated with DMSO and AR-A014418. AR-A014418 does not inhibit the activity of 26 closely related kinases and is therefore considered highly specific for GSK3β (18). Because no subsequent information is available for the effect of AR-A014418 on activity of kinases other than those reported previously (18), the effects of GSK3β RNA interference (RNAi) on cell migration and invasion were examined for cells transfected with either nonspecific small interfering RNA (siRNA; Stealth RNAi Negative Control Low GC Duplex; Invitrogen) or GSK3β-specific siRNA (GSK3β Validated Stealth RNAi; Invitrogen). The effect of RNAi on GSK3β expression was determined by Western blotting using an antibody against both GSK3α and GSK3β (Supplementary Table S1). The specificity of GSK3β-specific siRNA was confirmed in our previous studies (12, 19).

The Transwell assays used a 24-well double-chamber system (BD BioCoat Matrigel Incubation Chamber; BD Bioscience) to examine cell migration and invasion by applying cells to uncoated and Matrigel-coated upper chambers, respectively. Cells were suspended in serum-free medium containing DMSO or AR-A014418 at the indicated concentrations and applied to the upper chamber. The paired lower chamber was filled with medium containing 10% FBS (as a chemoattracting agent) and DMSO or AR-A014418 at the indicated concentrations. The cells transfected with nonspecific or GSK3β-specific siRNA were also subjected for the Transwell assay. Cells were allowed to migrate or invade the Matrigel toward the lower side of the upper chamber. After 22 hours of incubation, cells on the upper side of the chamber were removed with a cotton-swab. Cells on the lower side of the chamber were fixed and stained with the Diff-Quick Kit (Systex). In each assay, the total number of cells per high-power microscopic field on the lower side of the uncoated or Matrigel-coated chamber was counted and scored for migrating or invading cells, respectively. The mean number of cells in five high-power microscopic fields was calculated.

**Cell morphology and immunofluorescence cytochemistry**

Glioblastoma cells grown to 50%-60% confluence on a cover slip were treated with either DMSO or AR-A014418 at the indicated concentrations for 24 hours and then observed under a phase-contrast microscope. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X (Sigma-Aldrich) for immunofluorescence and fluorescence staining. They were incubated with mouse monoclonal antibody to Rac1 (BD Bioscience; diluted 1:200) at 4°C overnight and then with Alexa Flour 488-labeled anti-mouse IgG (Invitrogen; diluted 1:1,000) at room temperature for 40 minutes in the dark. After washing off excess antibody, cells were stained for filamentous (F-)-actin with Alexa Flour 546-labeled phalloidin (Invitrogen; diluted 1:40) for 20 minutes at room temperature. Following Rac1 and F-actin staining, cell nuclei were counterstained with Hoechst 33342 (Molecular Probes) for 20 minutes at room temperature. The cells were observed by fluorescence microscopy (Keyence) for expression and subcellular localization of Rac1 and F-actin.

To quantify effects of GSK3β inhibition on lamellipodia formation, lamellipodia-positive cells were scored for the same cells treated with DMSO and 25 µmol/L AR-A014418 and for those transfected with nonspecific and GSK3β-specific siRNA. The cells were stained for F-actin and observed under phase-contrast and fluorescence microscopy, respectively. In each assay, the mean percentage of lamellipodia-positive cells in five microscopic fields was calculated with standard deviations.
Rac1 activity

Protein was extracted from cells treated with DMSO or AR-A014418 for 24 hours with 25 mmol/L Tris–HCl buffer (pH 7.5) containing 150 mmol/L NaCl, 5 mmol/L MgCl2, 1% NP-40, 1 mmol/L dithiothreitol, and 5% glycerol. Active Rac1 was isolated from the protein sample by the pull-down method using GST-human Pak1-PBD (Thermo) and resins (Glutathione Sepharose 4 Fast Flow; GE Healthcare) according to the manufacturers’ instructions. The fraction of Rac1 bound to guanosine triphosphate (GTP; Rac1-GTP, an active form) was eluted from the resins and detected by Western blot analysis using rabbit polyclonal antibody to Rac1 (diluted 1:1,000; Thermo). Separately, whole cellular protein was probed for total Rac1 using the same antibody. The relative level of Rac1-GTP was quantified by densitometry and normalized to that of total Rac1 in the same cells.

Expression and secretion of matrix metalloproteinases

Expression of matrix metalloproteinase 2 (MMP-2) and membrane type 1 (MT1)-MMP mRNA in the cells was examined by quantitative reverse transcription-PCR (qRT-PCR) as described in our previous report (12) using SYBR Premix Ex Taq II (TakaRa-Bios) and specific primers for the amplification of MMP-2, MT1-MMP, and β-actin (Supplementary Table S2). MMP-2 expression was analyzed by gelatin zymography (20). Glioblastoma cells were seeded on 12-well plates for 48 hours and then treated with DMSO or AR-A014418 (10.25 μmol/L) for 24 hours in serum-free medium. Conditioned medium or treated cells were incubated with SDS sample buffer for 30 minutes at 37°C. Samples were separated on 10% SDS-PAGE containing 0.005% Alexa Fluor 680–labeled gelatin. After electrophoresis, gels were washed in 2.5% Triton X-100 for 2 hours at room temperature to remove SDS and incubated in substrate buffer overnight at 37°C. The gel was scanned using the LI-COR Odyssey IR imaging system.

Expression of guanine nucleotide exchange factors and integrins

Guanine nucleotide exchange factors (GEF) shown to activate Rac1 and promote migration and invasion of glioblastoma include ELMO1, Dock180, Trio, Vav3, and SWAP-70 (21). Among integrin family, integrins α2, α3, α5, αV, β1, β3, and β8 are associated with glioma invasion (22). Changes in the mRNA expression of these GEFs and integrins in glioblastoma cells were examined after treatment with AR-A014418 by qRTPCR (12) using specific primers for these GEFs, integrins, and β-actin (Supplementary Table S2). The expression of GEFs proteins was analyzed by Western blotting with the respective specific antibodies (Supplementary Table S1) and compared between cells treated with DMSO and AR-A014418.

Invadopodia formation

The ability of glioblastoma cells to invade three-dimensional extracellular matrix (ECM) was assessed by observing the formation of invasive “feet” (invadopodia; ref. 23). Glioblastoma cells were cultured on cover slips coated with either poly-1-lysine (Sigma-Aldrich) or type I collagen gel (Nitta Gelatin). The cells were fixed, serially stained for F-actin, cortactin, and nuclei, and observed by fluorescence microscopy as described above to evaluate invadopodia. To examine the effect of GSK3β inhibition on the formation of invadopodia, cells seeded on glass-bottom dishes coated with Oregon Green (OG; Invitrogen)–labeled gelatin were treated with DMSO or 25 μmol/L AR-A014418 for 12 hours. After staining for F-actin, cortactin, and nuclei, the cells were observed by confocal laser-scanning microscopy (LSM5 EXCITER; Carl Zeiss). Area of degradation of OG-labeled gelatin mediated by invadopodia was measured by using the Image J Software.

Animal study, immunohistochemical and biochemical analysis

We generated a mouse brain tumor model of human glioblastoma by retrovirus-mediated introduction of mutant K-ras gene (K-rasG12V) into neurospheres derived from the brain of a p16Ink4A-/-/p19Arf-/- mouse. These were then transplanted into the brains of wild-type mice, as described in our previous study (24). Brain tumors developed in this mouse model as early as 20 days after transplantation, with most mice dying within 40 days. Histologically, the tumors showed dense palli-sades of tumor cells around areas of necrosis, the presence of bizarre giant cells and the proliferation of microvasculature, all of which are characteristic of human glioblastoma (1). The 12 mice were treated by intraperitoneal injection of either DMSO (n = 6) or AR-A014418 (2 mg/kg body weight; n = 6) three times a week, as described earlier (19, 25). All mice were euthanized at the end of 2 weeks of treatment. At autopsy, the brain (with tumor) and vital organs (lungs, liver, pancreas, and kidneys) were assessed histologically and biochemically. All animal experiments followed the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University that covers the national guideline (26).

For histologic and immunohistochemical examinations, mice brains were fixed in 4% paraformaldehyde. Before fixation, the parts of fresh specimens were immediately frozen and stored at −80°C until use. Tissue sections were stained with hematoxylin and eosin and immunostained with the following primary antibodies: anti-nestin (BD Biosciences), anti-glycogen synthase (GS) and the fraction phosphorylated at the serine (S) 641 residue (pGS641; Cell Signaling). The staining signals were visualized with peroxidase-conjugated secondary antibody (Dako) followed by the avidin–biotin complex method (12). The sections were counterstained with hematoxylin. Images were acquired with a BZ-X700 microscope (Keyence) and digitally processed with the Keyence Analysis Software. To evaluate the irregular-shaped tumor size, we calculated the surface included by the contour of each tumor, we calculated the surface included by the contour of each tumor

Statistical analysis

Statistical significance was determined using the Student t test for comparison of two groups and analysis of variance (ANOVA) followed by Bonferroni/Dunn post hoc test for comparison of three groups in wound-healing and Transwell assays. A P value of <0.05 was considered as statistically significant.

Results

Effect of GSK3β inhibition on tumor cell migration and invasion

In the wound-healing assay, treatment with 5 to 25 μmol/L AR-A014418 reduced the migration of all glioblastoma cells in
response to mechanical stimulation (Fig. 1A). The selective effect of AR-A014418 on cell migration is supported by our previous observations that the same concentrations of inhibitor did not affect cell survival after 24 hours of treatment, although 25 µmol/L AR-A014418 slightly attenuated cell survival at 48 hours (12). The use of RNAi for GSK3β produced the same result in this assay as AR-A014418. The Transwell assay showed that AR-A014418 and GSK3β-RNAi inhibited the migration of glioblastoma cells and their invasion of ECM constituents (Fig. 1B and C; Supplementary Fig. S1).

Changes in cell migration phenotypes following GSK3β inhibition

Among the different cellular microarchitectures involved in cell motility and invasion, we focused here on lamellipodia (28) because these structures were frequently observed in glioblastoma cells growing in nonconfluent conditions (Fig. 2, Supplementary Fig. S2). Rac1, a member of the Rho-GTPase family, is involved in the formation of lamellipodia and membrane ruffling, thus promoting cell migration (21, 29). Immunofluorescence staining of glioblastoma cells showed preferential colocalization of Rac1

![Figure 1](image-url)

**Figure 1.**
Effect of GSK3β inhibition on the migration and invasion of glioblastoma cells under mechanical and chemoattractant stimuli. A, top, representative time course of glioblastoma cell migration in the wound-healing assay in the presence of DMSO or AR-A014418 (AR). The assay was performed by scratching confluent cells followed by serial observation of the same reference points at 24 and 48 hours under a phase-contrast microscope. Bottom, the relative widths of wounds at the indicated time after treatment with DMSO or AR-A014418 were measured and expressed as a percentage of the initial gap width at time zero. Values shown are the means ± SD of three separate reference points of observation. B and C, effects of GSK3β inhibition on three-dimensional migration and invasion of glioblastoma cells. Migrating cells through a Transwell chamber (B) and invading cells through a Matrigel-coated Transwell chamber (C) were scored for cells treated with DMSO or AR-A014418 for 22 hours. In each assay, the mean number of cells in five high-power microscopic fields was calculated with SDs. Representative photomicroscopic findings from the assay are shown below each column. *, P < 0.05, statistically significant difference between cells treated with DMSO and AR-A014418.
with actin filaments at the site of lamellipodia. Both proteins are involved in the organization of lamella structure. Treatment of glioblastoma cells with AR-A014418 inhibited lamellipodia formation and the colocalization of Rac1 and F-actin, and the effect was reversible (Fig. 2A and B; Supplementary Fig. S2A and S2B). Treatment with AR-A014418 and GSK3β-specific siRNA significantly decreased the incidence of lamellipodia-positive glioblastoma cells (Fig. 2C, Supplementary Fig. S2C). Associated with these changes, the pull-down assay showed decreased Rac1-GTP (active fraction) in cells treated with AR-A014418 (Fig. 2D).

Rac1 cycles between the active GTP-bound and inactive guanosine diphosphate (GDP)–bound forms (29). Of the three classes of protein that regulate Rho-GTPase activity, GEFs activate GTPase and participate in tumor cell migration and invasion (30). Among the GEFs previously shown to promote glioblastoma invasion via the activation of Rac1 (21), four (Dock180, SWAP-70, ELMO1, Trio) were constitutively expressed but this could be reduced following treatment with AR-A014418. Inhibition of GSK3β also decreased Vav3 expression in T98G and U87 cells (Fig. 3, Supplementary Fig. S3A). Thus, decreased Rac1 activity following...
inhibition of GSK3β is partly due to the downregulation of specific GEFs.

Changes in the invasive phenotype following GSK3β inhibition
The ECM degrading enzymes MMP-2, MMP-9, and MT1-MMP play a major role in glioblastoma invasion (31) and Rac1 has been shown to enhance their expression and/or activity (29). Treatment of glioblastoma cells with AR-A014418 decreased the expression of mRNAs for MMP-2 and MT1-MMP and inhibited the secretion of MMP-2 in the glioblastoma (Fig. 4A and B) other than A172 cells (data not shown). Together with the changes in Rac1 activity (Fig. 2D), these findings indicate a mechanistic link between GSK3β and Rac1 in the dysregulation of MMP expression and secretion in glioblastoma cells.

It has been reported that FAK is regulated by GSK3β and interacts with Rac1 to promote cell motility and tumor cell invasion (32). Phosphorylation of the tyrosine (Y) 397 and Y861 residues of FAK is crucial for its kinase activity and its signal transduction to Rac1, respectively (33). In addition, the enhancement of cell motility and invasion mediated by the FAK–Rac1 axis is associated with JNK activation through phosphorylation of its threonine (T) 183 and/or Y185 residues (34). The phosphorylated fractions of FAK (p-FAKTY397, p-FAKY861) and JNK (p-JNKT183/Y185) in all glioblastoma cells tested were reduced following treatment with 25 μmol/L AR-A014418 (Fig. 4C and D). Together, these results suggest a pivotal role for the GSK3β-induced molecular axis mediated by FAK, Rac1, and JNK in sustaining glioblastoma cell invasion (Supplementary Figure S4). It is known that integrin-mediated signaling plays a pivotal role in activating FAK phosphorylation and invasion of tumor cells (33). Of integrin subunits associated with glioblastoma invasion (22), GSK3β inhibition decreased expression of integrin β8 in three of four cell lines tested (Supplementary Fig. S3B), suggesting an involvement of integrin β8-induced...
Figure 4. Effect of GSK3β inhibition on the expression of MMP-2 and MT1-MMP (A), the secretion of MMP-2 (B), and the expression and phosphorylation of FAK (C) and JNK (D) in glioblastoma cells. A, the expression of mRNA for MMP-2 and MT1-MMP was measured by qRT-PCR in glioblastoma cells after 24 hours of treatment with DMSO or AR-A014418 (AR). Relative levels of mRNA expression shown are the means ± SDs from four separate experiments. *, P < 0.05, statistically significant difference between cells treated with DMSO and AR-A014418. B, glioblastoma cells were grown in regular medium for 24 hours and then in serum-free medium for 2 hours. Both media contained either DMSO (DM) or AR-A014418. The conditioned media were collected from the respective cell cultures and analyzed for MMP-2 by gelatin zymography. The value shown below each lane is the relative amount of MMP-2 quantified by densitometry and normalized to the same cells treated with DMSO. C and D, Western blotting analyses comparing the levels of phosphorylation for FAK (p-FAK(397), p-FAK(861)) and JNK (p-JNK(183/185)) between glioblastoma cells treated with DMSO (DM) or with 25 μmol/L AR-A014418 (AR) for 24 hours. The value below each lane shows the relative level of p-FAK(397), p-FAK(861) or p-JNK(183/185) quantified by densitometry and normalized to total FAK or JNK in the same cells.

GSK3β Sustains Glioblastoma Invasion

Although lamellipodia are important for cell motility, the cell surface microstructure known as invadopodium is critical for stromal degradation and invasion in several tumor types, including glioblastoma. MT1-MMP is one of the key molecules in the formation of invadopodia and is highly active in invasive tumor cells (23, 31, 35). Consistent with previous studies (reviewed in 35), glioblastoma cells in the type I collagen gel formed innumerable invadopodium-like microstructures where F-actin and cortactin colocalized (Fig. 5A). Treatment of cells with 25 μmol/L AR-A014418 decreased the number of invadopodium-like microstructures and inhibited the degradation of OG-labeled gelatin mediated by invadopodia (Fig. 5B, Supplementary Fig. S5). This is in line with the negative effects of GSK3β inhibition on FAK, Rac1, and MMPs described above.

Effect of GSK3β inhibition on glioblastoma cell invasion in an animal model

As described in Materials and Methods, the histology of tumors from our animal model showed several features that are characteristic of human glioblastoma (1). Inhibition of GSK3β by
treatment with AR-A014418 was confirmed by the decreased level of phosphorylated GS observed in the tumor cells (Supplementary Fig. S6). Tumors were smaller in mice treated with AR-A014418 compared with those treated with DMSO but no significant difference was found between the two groups (Fig. 6). The number of diffusely infiltrating tumor cells that stained positive for nestin was significantly decreased (Fig. 6B). This resulted in a clearly demarcated border between the tumor and adjacent normal brain tissues in mice treated with AR-A014418 (Fig. 6A).

Similar to our previous studies (19, 25), no detrimental effects were observed in mice treated with GSK3β inhibitor. There was no significant difference in body weight between groups treated with DMSO and AR-A014418. At necropsy, gross observation and histologic examination revealed no pathologic findings, primary cancers, or metastatic tumors in the major vital organs, including the lungs, liver, gastrointestinal tract, pancreas, and kidneys (data not shown).

Discussion

The highly invasive nature of tumor cells is one of the most challenging hallmarks that prevents cure of refractory cancers, including glioblastoma. In the present study, we have shown that inhibition of GSK3β attenuates the migration and invasion of glioblastoma cells in vitro, as well as the invasion of malignant glioma cells in an animal model that recapitulates human glioblastoma (24). These effects were associated with alterations in molecular pathways mediated by FAK, GEFs/Rac1, and JNK. In addition, changes were observed in the cellular microstructures of lamellipodia and invadopodia, which coordinate to influence the motility and invasion of glioblastoma cells, respectively. We have previously reported that deregulated GSK3β is important for the survival and proliferation of glioblastoma cells and confers them with resistance to chemotherapeutic agents and radiation (12, 13). Taken together, these results establish GSK3β as a therapeutic target with multiple functional roles in glioblastoma.

An earlier study of glioblastoma cells showed an inverse association between cell migration and transcriptional...
coactivation of β-catenin following treatment with indirubin or lithium chloride (15). However, no data were presented to suggest how activated β-catenin might inhibit cell migration and the results are inconsistent with other studies demonstrating a role for the oncogenic Wnt/β-catenin pathway in promoting tumor cell stemness, proliferation, and invasion of glioblastoma cells (36–38). In our previous study, the fraction of β-catenin phosphorylated at its GSK3β phospho-acceptor sites (S33, 37 and/or T41 residues) was frequently detected in clinical samples of glioblastoma at elevated levels compared with adjacent, normal brain tissue (12). Furthermore, we did not find constitutive activation of β-catenin in glioblastoma cells following GSK3β inhibition. The role of β-catenin–mediated signaling in glioblastoma cell motility and invasion therefore requires further investigation.

The proinvasive phenotype of cancer cells includes an epithelial–mesenchymal transition (EMT) state (39) and distinct cellular microstructures such as lamellipodia and invadopodia (23, 28, 35) responsible for cell migration and invasion. In contrast with its role in cell migration (17), GSK3β was shown to inhibit EMT in normal cells by phosphorylating and stabilizing snail, a repressor of E-cadherin transcription (40). This suggests that EMT may not be involved in the mechanism by which GSK3β inhibition suppresses the migration and invasion of glioblastoma cells. As previously reported (28, 35), we observed the formation of lamellipodia and invadopodia in glioblastoma cells grown in nonconfluent conditions and on gelatin-coated slides, respectively. Treatment of cells with GSK3β inhibitor decreased the formation of these cellular microstructures and altered the subcellular colocalization of the cytoskeletal molecules F-actin and Rac1, resulting in their redistribution throughout the cytoplasm. Coinciding with these structural and functional changes in the cell, the inhibition of GSK3β reduced the phosphorylation of FAK and JNK. This in turn reduced their activity as well as that of active Rac1, the expression of GEFs responsible for Rac1 activation (22) and the expression and secretion of MMP-2 and MT1-MMP. Previous studies have shown these molecules (FAK, JNK, GEFs/Rac-1, MMPs other than GSK3β) interact to facilitate cell motility (32–34) and are involved in glioblastoma cell migration and invasion (22, 31, 41, 42). With this in consideration, our findings indicate that a GSK3β-mediated pathway involving FAK, JNK, GEFs/Rac-1 and MT1-MMP MMP-2 plays a major role in promoting glioblastoma invasion (Supplementary Fig. S4) and suggest a novel therapeutic approach to control the invasive nature of this lethal disease.

It is becoming increasingly recognized that the invasive behavior of tumor cells and their resistance to therapy may not be separate properties and could in fact be interconnected processes (43). This notion is particularly relevant for glioblastoma in which a strong association is seen between invasive and therapy-resistant phenotypes (44, 45), leading to dismal survival outcomes (1–4). These two major cancer hallmarks share distinctive molecular pathways with several signaling hubs that include Rac1, FAK, and JNK (reviewed in 43). Rac1 has been reported to sustain stemness and the invasive ability of glioma stem-like cells, thus rendering them resistant to radiotherapy (46). It is also implicated in the radiation-induced enhancement of the invasive potential of primary glioblastoma cells (47). Similar to observations made with gene-manipulated fibroblasts (34), Rac1 has been shown to mediate phorbol 12-myristate 13-acetate–induced migration of glioblastoma cells via phosphorylation and activation of JNK and its translocation to paxillin-containing focal complexes (42). A previous study also showed that Rac1-mediated enhancement of glioma cell invasion in response to radiation was associated with activation of JNK and p38 MAP kinases (48). Moreover, an inhibitor of FAK autophosphorylation (Y15) reduces the viability of glioblastoma cells, induces their apoptosis, inhibits their invasion, and synergizes with temozolomide to increase survival in a murine glioma model (41). We previously reported that glioblastoma cells depend on deregulated GSK3β to survive, proliferate, and resist chemotherapy and radiation via the inactivation of p53- and/or RB-mediated pathways (12).

We have also demonstrated that a specific GSK3β inhibitor (AR-A014418; ref. 18) synergizes with temozolomide against glioblastoma cells by silencing O6-methylguanine DNA methyltransferase expression via c-Myc–mediated promoter methylation (13). GSK3β is, therefore, heavily implicated in the two major pathologic characteristics of glioblastoma cells, invasive activity and therapy resistance, through pivotal oncogenic pathways.

We are currently undertaking a phase I/II clinical trial of recurrent glioblastoma patients treated with temozolomide in combination with drugs known to inhibit GSK3β activity and which are already in clinical use (UMIN:00005111; ref. 49). Our preliminary results show that the repurposed drugs inhibit GSK3β activity in the tumor cells, enhance the therapeutic effect of temozolomide and reduce invasion by the residual tumors, resulting in significantly longer patient survival times compared with patients treated with temozolomide alone (Nakada and colleagues; unpublished data). There is increasing evidence to show that GSK3β has neurodegenerative effects and that its inhibition has neuroprotective consequences (9). This has been observed for example with cranial irradiation-induced neurocognitive dysfunction (50). Therefore, the inhibition of GSK3β can provide dual benefits for the treatment of patients with glioblastoma by first reducing tumor invasion and therapy resistance, and second by protecting the host brain tissue from injury.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Molecular Cancer Therapeutics

Glycogen Synthase Kinase 3β Sustains Invasion of Glioblastoma via the Focal Adhesion Kinase, Rac1, and c-Jun N-Terminal Kinase-Mediated Pathway

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