Glycogen Synthase Kinase-3 Inhibition Induces Glioma Cell Death through c-MYC, Nuclear Factor-κB, and Glucose Regulation

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Abstract
Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase, is involved in diverse cellular processes ranging from nutrient and energy homeostasis to proliferation and apoptosis. Its role in glioblastoma multiforme has yet to be elucidated. We identified GSK3 as a regulator of glioblastoma multiforme cell survival using microarray analysis and small-molecule and genetic inhibitors of GSK3 activity. Various molecular and genetic approaches were then used to dissect out the molecular mechanisms responsible for GSK3 inhibition–induced cytotoxicity. We show that multiple small-molecule inhibitors of GSK3 activity and genetic down-regulation of GSK3 significantly inhibit glioblastoma cell survival and clonogenicity. The potency of the cytotoxic effects is directly correlated with decreased enzyme activity–activating phosphorylation of GSK3α/β Y276/Y216 and with increased enzyme activity inhibitory phosphorylation of GSK3α γ21. Inhibition of GSK3 activity results in c-MYC activation, leading to the induction of Bax, Bim, DR4/DR5, and tumor necrosis factor-related apoptosis-inducing ligand expression and subsequent cytotoxicity. Additionally, down-regulation of GSK3 activity results in alteration of intracellular glucose metabolism resulting in dissociation of hexokinase II from the outer mitochondrial membrane with subsequent mitochondrial destabilization. Finally, inhibition of GSK3 activity causes a dramatic decrease in intracellular nuclear factor-κB activity. Inhibition of GSK3 activity results in c-MYC–dependent glioma cell death through multiple mechanisms, all of which converge on the apoptotic pathways. GSK3 may therefore be an important therapeutic target for gliomas. Future studies will further define the optimal combinations of GSK3 inhibitors and cytotoxic agents for use in gliomas and other cancers. [Cancer Res 2008;68(16):6643–51]

Introduction
Glioblastomas are among the most lethal tumors. New therapeutic approaches using novel molecular targets are clearly needed. In the course of conducting a clinical trial of enzastaurin (LY317615.HCl), a new small-molecule inhibitor of protein kinase Cβ (PKCβ; ref. 1), we noted a number of impressive radiographic responses in patients with recurrent glioblastomas, consistent with a primary cytotoxic mechanism of action rather than, or in addition to, a pure antiangiogenic mechanism of the drug (2). While attempting to elucidate the apparent cytotoxic activity of enzastaurin against gliomas, we discovered that the α and β forms of glycogen synthase kinase 3 (GSK3) were targets of the drug in addition to its primary target, PKCβ (Supplementary Fig. S1). In this study, we therefore evaluated whether GSK3 may be a potentially new therapeutic target in gliomas.

GSK3 was initially identified more than 25 years ago as a protein kinase that phosphorylated and inactivated glycogen synthase (3), the final enzyme in glycogen biosynthesis. Recently, it has been recognized as a key component of a diverse range of cellular functions essential for survival (for reviews, see refs. 4, 5). The role of GSK3 in the regulation of apoptosis is controversial. GSK3 inhibition–induced apoptosis in mouse embryonic fibroblasts derived from these embryos are sensitized to apoptosis (6). However, this observation seems to contradict the finding that overexpression of GSK3 is sufficient to induce apoptosis (7). A recent report has shown that GSK3 is a prosurvival factor in pancreatic tumor cells, partly through its ability to regulate the NF-κB pathway (8). These data are consistent with the recent demonstration that GSK3β can regulate NF-κB stability and activity (6, 9). Finally, new experimental evidence suggests that GSK3 inhibition can promote Bax-mediated apoptosis in colorectal cancer cells (10). These data confirm the complex role of GSK3 in apoptosis and show that the biological outcome of GSK3 signaling is cell type and tissue context dependent.

Little is currently known about the significance of GSK3 to glioma cell survival. We therefore sought to evaluate the role of GSK3 in glioma cell survival, proliferation, and tumorigenicity, both in vitro and in vivo, and the mechanistic consequences of inhibiting GSK3 activity. Our data point to GSK3 as a potentially new target for human glioma therapy.

Materials and Methods

Cell culture and drug treatment. The glioma cell lines, U251, T98, U87, U373, U118, and A172, were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC protocols.

Enzastaurin was kindly provided by Eli-Lilly. Enzastaurin, lithium chloride, and kenpaullone (Sigma) were dissolved in 100% DMSO and used at the concentrations specified in the article. The GSK3 inhibitor LY2064827 was kindly provided by Eli-Lilly, and the GSK3 inhibitors 705701, 708244, and 709125 were kindly provided by The Developmental Therapeutics Program, The National Cancer Institute (Bethesda, MD). Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was from R&D Systems.

Proliferation assays. Cell proliferation assays were done by cell counting using trypan blue staining for dead cell visualization. Alternatively, a fluorescent assay based on Alamar Blue (BioSource) reduction by viable cells was done according to the manufacturer's manual.

Gene silencing by small interfering RNA treatment and generation of stable hairpin RNA cell lines. Small interfering
RNA (siRNA) duplexes were synthesized by Dharmacon: GSK3α, GSK3β, NFκB p65, and c-MYC. Oligofectamine (Invitrogen) was used for transfection of siRNA into cells per manufacturer's instructions. Short hairpin RNA (shRNA) clone for c-MYC V2HS_152060 was from Open Biosystems. Cells stably overexpressing pcDNA6 (Invitrogen) and TMP-MYC (Invitrogen) were selected with 5 μg/mL blasticidin and 1 μg/mL puromycin.

Cell cycle analysis. U251 cells were plated at a density of 750,000 in 15-cm tissue culture dishes 24 h before treatment with enzastaurin started. After a 2-h pulse with 10 mM bromodeoxyuridine (BrdUrd) (BD Biosciences Pharmingen), BrdUrd-labeled cells were detected as indicated in the user's manual. Fluorescence-activated cell sorting analysis was done using FACSVantage SE flow cytometer and Cell Quest Acquisition and Analysis software (BD Biosciences).

RNA expression arrays and analysis. U251 cells were treated in triplicates with 10 μM/L enzastaurin for 4, 10, and 18 h. Total RNA was isolated using TRIzol (Invitrogen) and further purified using RNeasy Mini Kit (Qiagen). Samples were hybridized to Affymetrix Human Genome U133 Plus 2 GeneChip Arrays (Affymetrix) according to the Affymetrix GeneChip Expression Analysis Technical Manual. The initial gene expression analysis data files (CEL files) were generated by using Affymetrix GeneChip Operating Software (GCOS) version 1.2. CEL files were processed as described earlier (11). False discovery rates of 0.05- and 1.5-fold change were applied as thresholds to select up-regulated or down-regulated probes. Data were analyzed by Ingenuity pathway software analysis. The accession number of the files submitted to National Center for Biotechnology Information Gene Expression Omnibus is GSE11283. The sample sets used in Fig. 8 were previously published (11, 12).

Real-time reverse transcription-PCR. mRNA expression levels were quantified in triplicates by real-time reverse transcription-PCR (RT-PCR) on an ABI Prism 7900 sequence detection system (Applied Biosystems). The relative amount of target transcripts quantified by standard curve method was normalized to the amount of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin transcripts found in the same sample. Primers and probes were purchased from Applied Biosystems.

Protein analysis by Western blot, ELISA, and immunohistochemistry. Whole-cell lysates were prepared in Cell Lysis Buffer (Cell Signaling Technology, Inc.). Cell lysate samples were run on a precast 4% to 12% Bis-Tris gel (Invitrogen), transferred to a polyvinylidene difluoride membrane (Invitrogen), and probed with the following antibodies: anti–phospho S21/ S9 GSK3α/β (1:2,000; Cell Signaling); anti-ph-p65 (1:500; Santa Cruz Biotechnology); anti-β-catenin, anti–phospho Ser33/Thr37 and Ser43 of GSK3α/β (1:1,000; BD Biosciences); and anti–phospho S217/221 of c-MYC. Oligofectamine (Invitrogen) was used for transfection with the luciferase assay system (Promega Corp.) or β-galactosidase assay system (Promega) on a 20/20 Luminometer (Turner Biosystems). c-MYC activity assays were done with ELISA-based c-MYC activity assay kit (Active Motif) according to the manufacturer's instructions.

GSK3 and hexokinase in vitro kinase assays. GSK3α and GSK3β kinase activities were measured with the KinaseProfiler Assay Protocol by Upstate. Hexokinase activity assays were done as described elsewhere (15).

In vitro tumorigenicity assays. Colony formation assays were done using the In vitro Tumorigenicity Assay kit from Clontech according to the manufacturer’s instructions. One thousand cells were plated in the upper agar layer per well of 12-well plates. Three or four weeks later, colonies were stained and counted.

Tumor xenograft models. U87 cells were injected intracranially in neonatal severe combined immunodeficient mice or 6- to 8-wk-old athymic nude mice (National Cancer Institute-Frederick Animal Production Area). Stereotoxic coordinates, for injection in adult animals, were 2.8 mm distal to the midline, 1 mm anterior to the coronal suture, and 2.5 mm deep from the dura. All procedures were in accordance with NIH Animal Care and Use Committee protocols. Fifteen days after tumor cells injection, enzastaurin (75 mg/kg) and/or carboplatin (40 mg/kg) was administered. Control animals were treated with vehicle alone. For evaluation of tumorigenicity, animals were observed daily for death or signs of distress. Kaplan-Meier analysis was done using Prism 4 (GraphPad Software, Inc.) or JMP 5.1 (SAS Institute).

S.c. tumor models were generated by injection of 500,000 siRNA-treated U251 cells s.c. on the right thigh of 6- to 8-wk-old athymic male nude mice. Tumors were measured twice a week.

Results

Inhibition of GSK3 activity results in glioma cell death and reduced tumorigenicity. After we found that enzastaurin exerts direct antiproliferative and apoptotic effects in vitro on multiple glioma cell lines (U87, T98, U251, and 373) as well as on primary glioma tumor stem cell–like lines (TSC0308 and TSC1228) at therapeutically achievable concentrations (Figs. 1A and 2D, Supplementary Fig. S11, and data not shown), we addressed the mechanism of enzastaurin-induced cytotoxicity through analysis of global gene expression changes over time. Unexpectedly, we found that enzastaurin abnormally regulated the expression of a number of WNT pathway genes, with axin 2 showing the most significant change with ~100-fold induction (Supplementary Table S1, Supplementary Fig. S1E; other affected genes and pathways, as detected by ANOVA and Ingenuity Pathway Analysis software, are shown in Supplementary Tables S2 and S3, respectively). We confirmed the activation of the WNT pathway through the demonstration of enzastaurin-mediated loss of β-catenin S37/41 phosphorylation with resultant β-catenin accumulation in the cytoplasm and subsequent translocation to the nucleus (Supplementary Fig. S1A–C). This resulted in a dose-dependent increase in the transcriptional activity of β-catenin (Supplementary Fig. S1D and E).

Because GSK3 is known to be a major regulator of β-catenin S37/41 phosphorylation, we asked whether enzastaurin modulates GSK3 activity. We found that the activation-related phosphorylation of Y216/Y276 in GSK3α/β was dramatically decreased following exposure to enzastaurin (Fig. 1B), whereas the inhibitory phosphorylation of GSK3α S21 was significantly up-regulated. Furthermore, the enzymatic activity of both forms of GSK3 was directly down-regulated by 0.5 μmol/L enzastaurin (lowest concentration tested) to <3% of the activity of the wild-type control enzyme as shown by direct in vitro activity assays (Supplementary Fig. S2).

We next evaluated whether inhibition of GSK3 by other GSK3 inhibitors resulted in glioma cell cytotoxicity. Along with the relatively nonselective GSK3 inhibitors, lithium chloride (LiCl; refs. 16, 17) and kenpaullone (ref. 18; Supplementary Fig. S3A and B), we also evaluated several highly selective small-molecule GSK3 inhibitors (705701, 708244, 709125, and LY2064827). Serum-cultured U251, U87, and T98 cell lines and two glioma tumor...
stem cell–like lines (11) were analyzed following treatment with each GSK3 inhibitor (Supplementary Fig. S3). Each drug exerted cytotoxic effects on glioma cell lines in a dose- and time-dependent manner; the most potent of which, LY2064827, showed significant cytotoxicity at concentrations as low as 0.1 μmol/L (Supplementary Fig. S3). Moreover, the potency of the cytotoxic effects of each drug correlated directly with the level of decreased phosphorylation of GSK3α/γ Y276/Y216 and with increased phosphorylation of GSK3α S21 (Fig. 1B). These results suggest that the cytotoxic effects of these drugs are directly related to their ability to inhibit GSK3 activity.

**Direct GSK3 down-regulation inhibits glioma cell proliferation both in vitro and in vivo.** To further address the “on-target” specificity of the cytotoxic effects of the GSK3 inhibitors, we examined whether direct down-regulation of GSK3 protein expression inhibits glioma cell growth. GSK3 siRNA–mediated down-regulation of GSK3 resulted in decreased U251 and T98 glioma cell growth (Fig. 1C and data not shown). Furthermore, we observed a 70% reduction in U251 glioma cell clonogenicity as revealed by colony formation assay in soft agar (Fig. 1C). Moreover, there was significant growth delay from GSK3 siRNA–treated gliomas in vivo compared with siRNA control–treated cells (Fig. 1D). Down-regulation of GSK3 activity by shRNA also resulted in decreased tumor growth in another mouse xenograft model (Supplementary Fig. S12).

GSK3 down-regulation inhibits antiapoptotic mechanisms in mitochondria. Because GSK3 is involved in cellular energy metabolism, we investigated the effects of GSK3 inhibition on several parameters of glioma cell glucose metabolism. GSK3 siRNA and enzastaurin significantly inhibited the phosphorylation of the S640 residue of glycogen synthase, thereby releasing the normal inhibitory effects of GSK3 on glycogen synthase (Fig. 2A and...
Supplementary Fig. S4) and resulting in increased intracytoplasmic glycogen storage and decreased cytoplasmic glucose concentrations (Fig. 2B).

Majewski and colleagues (15) have shown that a drop in intracellular glucose levels negatively affects the association of hexokinase activity with mitochondria, resulting in cellular apoptosis. We therefore evaluated the effects of GSK3 inhibition on mitochondrial hexokinase activity. Mitochondrial hexokinase activity decreased by 2-fold following GSK3 siRNA or a 4-hour exposure to 5 μmol/L enzastaurin (Supplementary Fig. S4B and C). By contrast, total and mitochondrial-associated Bax expression significantly increased in the cells treated with GSK3 siRNA or enzastaurin (Fig. 2C and Supplementary Fig. S4D), resulting in an increase in the mitochondrial-associated Bax/hexokinase II (HKII) ratio (Fig. 2D). Displacement of HKII activity from mitochondria along with increased mitochondrial-associated Bax protein has been shown to lead to a loss of mitochondrial membrane integrity with resultant apoptosis (19, 20). Indeed, mitochondrial cytochrome c release was detected and MitoTracker staining revealed a significant loss of functional mitochondria in enzastaurin-, 705701-, LY2064827-, and GSK3 siRNA–treated glioma cells consistent with mitochondrial-mediated cell death (Fig. 2D and data not shown). The importance of mitochondrial HKII activity in glioma cell survival is supported by the observation that HKII siRNA–mediated down-regulation of HKII induced glioma cell death (Supplementary Fig. S4E).

**GSK3 down-regulation inhibits NF-κB activity essential for glioma cell survival.** We and others have previously shown the importance of prosurvival activities of NF-κB in glioma (21–25). Recent studies have linked inhibition of GSK3β to negative regulation of NF-κB activity (6). We found that NF-κB was inhibited by LiCl, kenpaullone, enzastaurin, and GSK3 siRNA treatments in U251, T98, and U87 (Fig. 3A and B and data not shown). GSK3 inhibition was accompanied by down-regulation of a number of NF-κB–regulated prosurvival genes including IL8, IER3, and BIRC2, as assessed by microarray gene expression analysis and TaqMan RT-PCR (Supplementary Fig. S5A–C). Moreover, NF-κB inhibition resulted in decreased glioma cell survival in vitro and in
inhibition of tumor growth in vivo (Figs. 1C and 3C). Finally, we also show that up-regulation of NF-κB activity by stable over-expression of NF-κB (p65) results in a partial protection of U251 against GSK3 inhibition–induced cell death (Supplementary Fig. S9). Thus, GSK3 inhibition contributes to glioma cell death through negative regulation of a number of NF-κB–associated prosurvival genes.

GSK3 inhibition increases c-MYC activity and DR4/5 expression in glioma cells. Western blot analysis showed that DR4 (TNFRSF10A) and DR5 (TNFRSF10B) proteins and TRAIL were significantly induced by GSK3 inhibition in a time- and dose-dependent manner (Fig. 4A–C and data not shown). Because c-MYC activity is partially regulated by GSK3, and because c-MYC is known to regulate the transcription of DR5 (26, 27), we evaluated c-MYC activity following GSK3 inhibition. An ELISA-based DNA binding assay showed that c-MYC activity was up-regulated after 4 hours of enzastaurin treatment and by GSK3 siRNA (Fig. 5A).

The stability and, thus, activity of c-MYC are specifically regulated by differential phosphorylation of its NH2 terminus through GSK3-mediated inhibitory phosphorylation of c-MYC T58 and through extracellular signal–regulated kinase (ERK)-1/2 activating phosphorylation of c-MYC S62 (28–30). We found that both enzastaurin and GSK3 siRNA induced loss of the inhibitory phosphorylation of c-MYC at T58 and increased phosphorylation of the activating phosphorylation site S62 (Fig. 5B, Supplementary Fig. S10, and data not shown). Consistent with increased c-MYC S62 phosphorylation, treatment with different GSK3 inhibitors induced ERK1/2 phosphorylation (Supplementary Fig. S6). ERK1/2 activation was sustained (Supplementary Fig. S6A) and dose dependent (Supplementary Fig. S6B), suggesting that ERK1/2 might be responsible for the phosphorylation of c-MYC S62 in glioma cells, as has previously been reported for other cell types (28).

We next examined whether c-MYC regulates transcription of Bax- and TRAIL-associated death receptors in glioma cell lines, as has been shown in other cells (for a review see, ref. 31). Treatment of glioma cells with c-MYC siRNA resulted in significant down-regulation of NF-κB activity (Fig. 3A). NF-κB silencing by siRNA resulted in cytotoxicity of U251. Bottom graph shows functional efficiency of NF-κB activity silencing by siRNA in U251 as measured by luciferase reporter assay. In all luciferase assays, cells were cotransfected with luciferase reporters for NF-κB and h-gal. Luciferase activity levels from NF-κB reporter were divided by those from the h-gal reporter. To control for transfection efficiency and cell viability, a CMV-h-gal plasmid was cotransfected. NF-κB and h-galactosidase reporter activities were detected 24 h after transfection and NF-κB values were normalized compared with h-galactosidase levels. Western blot shows the efficiency of NF-κB silencing by siRNA.

Figure 3. Cytotoxicity caused by GSK3 inhibition is accompanied by down-regulation of NF-κB activity. A, NF-κB luciferase reporter assay shows that GSK3 siRNA down-regulates NF-κB activity in U251. B, kenpaullone, LiCl, and enzastaurin down-regulate NF-κB activity in a dose-dependent manner (24-h treatment). C, NF-κB silencing by siRNA resulted in cytotoxicity of U251. Bottom graph shows functional efficiency of NF-κB activity silencing by siRNA in U251 as measured by luciferase reporter assay. In all luciferase assays, cells were cotransfected with luciferase reporters for NF-κB and h-gal. Luciferase activity levels from NF-κB reporter were divided by those from the h-gal reporter. To control for transfection efficiency and cell viability, a CMV-h-gal plasmid was cotransfected. NF-κB and h-galactosidase reporter activities were detected 24 h after transfection and NF-κB values were normalized compared with h-galactosidase levels. Western blot shows the efficiency of NF-κB silencing by siRNA.
transfected glioma cells exposed to 705701 (Supplementary Fig. S7H). By contrast, c-MYC overexpression was cytotoxic to glioma cells (Fig. 6B) and positively regulated the transcription of Bax, DR4, and DR5 (Supplementary Fig. S7D–F).

Finally, we show that GSK3 inhibition resulted in significantly increased RNA and protein levels of Bim, a known Bcl-2 antagonist and c-MYC target gene (Supplementary Fig. S7I and J and data not shown; ref. 32). These observations are consistent with the hypothesis that c-MYC plays a significant, if not predominant, role as a mediator of GSK3 inhibition–induced glioma cell apoptosis. GSK3 inhibitors and TRAIL act synergistically in glioma cell cytotoxicity. We found that in addition to DR4/5, TRAIL was up-regulated by enzastaurin treatment and GSK3 siRNA (Fig. 4C) in several glioma cell lines. Given that glioma cells are relatively resistant to TRAIL-induced apoptosis and that GSK3 inhibition up-regulates the death receptors Bax and TRAIL and inhibits the antiapoptotic HKII/mitochondrial and NF-κB mechanisms, we examined whether GSK3 inhibition would result in increased sensitivity to apoptotic stimuli such as TRAIL itself. We found that TRAIL-mediated cell death was dramatically enhanced when otherwise subcytotoxic doses of TRAIL (25–50 nmol/L) and enzastaurin (1–2.5 μmol/L) were combined in U251 (Fig. 6A) and U87 (data not shown) cell lines. Similar data were obtained with other GSK3 inhibitors, 705701 (0.05–0.1 μmol/L) and LY2064827(0.05 μmol/L), as well as with GSK3 siRNA (data not shown). Moreover, overexpression of c-MYC rendered cells more sensitive to TRAIL-mediated cytotoxicity (Fig. 6B), again supporting the hypothesis of the important role of c-MYC in GSK3-induced cytotoxicity. Finally, enzastaurin combined with carboplatin, a standard DNA-damaging chemotherapeutic agent often used in patients with malignant gliomas, causes synergistic tumor killing both in vitro and in vivo (Fig. 6C and D), confirming our hypothesis that GSK3 inhibition would have additive/synergistic cytotoxic activity in combination with other, unrelated DNA-damaging agents.

Discussion

Although initially viewed as a specific regulator of glycogen metabolism, GSK3 has recently been shown to be a crucial enzymatic regulator of a diverse number of cellular functions including cell structure, metabolism, and survival (reviewed in refs. 4, 5). Our work shows a role for GSK3 inhibition in mediating glioma cell proliferation arrest, decreased clonogenicity, and induction of apoptotic cell death through both the extrinsic and intrinsic apoptotic pathways both in vitro and in vivo, much of which is mediated through c-MYC.

c-MYC is known to play important roles in the regulation of cell proliferation, differentiation, and apoptosis and is deregulated in many human tumors (33, 34), including glioma (Supplementary Fig. S8; refs. 35–37). c-MYC can be activated by phosphorylation at S62 by mitogen-activated protein kinase ERK1/2 whereas it is inhibited by GSK3-mediated phosphorylation at T58 (28). We show that the increase in c-MYC activity in glioma cells after GSK3 inhibition correlates with a loss of T58 phosphorylation and with an increase in S62 phosphorylation. The decrease in c-MYC phosphorylation at T58 observed following enzastaurin or GSK3 siRNA treatment correlates with the inhibition of Y216/Y276 phosphorylation of GSK3α/GSK3β and is therefore likely to be a direct result of decreased GSK3 activity. By contrast, the increase in c-MYC S62 phosphorylation coincides with an increase in activating ERK1/2 phosphorylation consistent with the recent demonstration that GSK3 negatively regulates ERK1/2 activity (38). Thus, inhibition of GSK3 activity results in increased...
phosphorylation and activity of ERK1/2, as we showed in Supplementary Fig. S6. In addition to a likely direct effect of ERK1/2 on activation of c-MYC, sustained phosphorylation of ERK1/2 and its target p90rSk has been shown to induce cell cycle arrest (39), as we observed in our enzastaurin- and GSK3 siRNA–treated cells (Supplementary Fig. S6 and data not shown). Seth and colleagues (40) have suggested that the elevated cyclin-dependent kinase (Cdk)-1/cyclin B levels seen in G2-M may mediate the increased S62 phosphorylation of c-MYC observed in G2-M. This is consistent with our data showing up-regulation of cyclin B following enzastaurin- and GSK3 siRNA–induced G2-M arrest before induction of apoptosis (data not shown). Whether GSK3 inhibition–induced phosphorylation of c-MYC S62 is mediated directly by ERK1/2 and/or G2-M arrested cellular up-regulation of Cdk1/cyclin B remains a subject of future studies.

GSK3 inhibition–mediated up-regulation of c-MYC activity results in increased expression of apoptosis-related molecules DR5, Bim, Bax, and TRAIL and down-regulation of antiapoptotic protein FLIP (data not shown), all known targets of c-MYC (27, 31). DR4 may also be a target of c-MYC transcriptional activity in glioma cells because siRNA-mediated down-regulation of c-MYC resulted in decreased mRNA and protein levels of DR4 whereas overexpression of c-MYC up-regulated DR4 (Supplementary Fig. S7). Our demonstration that reduction of c-MYC activity by shRNA protects glioma cells from GSK3-mediated cytotoxicity, together with data reported by Rottman and colleagues (41), suggests that c-MYC plays a major role as a mediator of GSK3 inhibition–related induction of TRAIL-mediated apoptosis.

In addition to induction of apoptosis through a TRAIL-mediated mechanism, our data also show that GSK3 inhibition may increase the propensity for glioma cell death through direct destabilization of the mitochondrial membrane. Hexokinase can inhibit apoptosis-related mitochondrial cytochrome C release by interfering with the ability of Bax to bind to the outer mitochondrial membrane (19). We show that treatment of glioma cells with GSK3 inhibitors results in increased mitochondria-associated Bax protein, accompanied by a decrease in mitochondria-associated HKII activity (Fig. 2 and Supplementary Fig. S4). Increases in the Bax/HKII ratio on the outer mitochondrial membrane have previously been shown to result in loss of mitochondrial membrane potential with subsequent mitochondrial cytochrome C release and apoptosis, as we observed following GSK3 inhibition in glioma cells.

Figure 5. c-MYC is a mediator of GSK3 inhibition–mediated cytotoxicity. A, GSK3 inhibition by siRNA and enzastaurin (Enza) up-regulates c-MYC activity as revealed by ELISA-based DNA-binding assay. B, Western blot analysis of nuclear extracts prepared from cells treated with 5 μmol/L enzastaurin or DMSO. c-MYC phosphorylation at T58 is down-regulated in U251. This correlates with reduced Y phosphorylation of GSK3, both at 4 and 24 h (shown) of treatment with enzastaurin. Conversely, S62 phosphorylation of c-MYC was up-regulated at 24 h. C, Western blot of total cell lysates: c-MYC siRNA negatively regulates Bax, DR4, and DR5 proteins in U251. D, silencing of c-MYC in U251 by shRNA in two individual clones, c-MYC-shRNA_1 and c-MYC-shRNA_2, effectively protects cells from 0.5 μmol/L 705701 or 0.5 μmol/L LY2064827 (24-h treatment) compared with control cells. Cells were plated at a density of 50,000 per well in a six-well plate.
Although the exact mechanism for hexokinase dissociation from mitochondria following GSK3 inhibition remains the subject of future experiments, it has previously been shown that reduction in intracellular glucose content induces dissociation of hexokinase from mitochondria (15). Thus, it is likely that the nearly 2-fold reduction in intracellular glucose concentration we observed in glioma cells following GSK3 inhibition is at least partially responsible for the hexokinase dissociation from mitochondria. The reduction in intracellular glucose content following GSK3 inhibition is likely a result of decreased GSK3-dependent inhibitory glycogen synthase S640 phosphorylation, resulting in overstimulation of glycogen synthase activity and leading to the increased intracellular glycogen content we observed.

Finally, we showed that down-regulation of GSK3 activity led to a decrease in NF-κB activity within glioma cells, an important finding because down-regulation of NF-κB by siRNA or by pharmacologic means (salicylates, Velcade) results in increased propensity for glioma cell death (23). These observations are in agreement with those from other groups who have shown regulation of mouse embryonic fibroblast and pancreatic cancer cell survival by GSK3β through a NF-κB–dependent pathway (6). Whether the effect of GSK3 inhibition on NF-κB activity in glioma cells is mediated by direct phosphorylation of NF-κB components, as previously described (9, 42), or through the upstream regulators, IκB kinase and IκB (43), and/or through inhibitory binding by increased nuclear β-catenin (44, 45) remains a matter of further study.

It is interesting to note that a significant number of glioblastomas (as well as many other cancer types) have mutations/deletions of the PTEN gene locus leading to constitutive AKT activation. This likely results in constitutive down-regulation of GSK3 activity through S9 phosphorylation by AKT (46). This constitutive inhibition of GSK3 activity may, in part, be responsible for the up-regulation c-MYC activity in gliomas (Supplementary Fig. S8) and a number of other tumors that do not have amplification of the c-MYC gene (35–37). The higher basal level of c-MYC activity found in glioma and tumor cells may subsequently make them significantly more sensitive to c-MYC–induced apoptosis following application of GSK3 inhibitors compared with normal cells that start out with low levels of c-MYC activity. Thus, it is plausible that deregulation of the phosphatidylinositol 3-kinase pathway and activation of AKT may be a synthetic lethal event for further GSK3 inhibition. If so, patients suffering from tumors with constitutively active AKT may ultimately prove to be preferentially sensitive to therapeutic GSK3 inhibition. Additional in vitro and in vivo experimental data will clearly be needed to test this hypothesis.

In conclusion, we have shown that GSK3 inhibition in glioma cells lead to (i) induction of proapoptotic effects through priming of the TRAIL death receptor pathway (GSK3/c-MYC/DR4/DR5/...
TRAIL/Bim); (ii) inhibition of prosurvival signals through inhibition of NF-κB activity; and (iii) induction of mitochondrial permeability (Bax/HKII ratio) through alteration in intracellular glucose regulation (GSK3/glycogen synthase). These data suggest that GSK3 may be an important molecular target for human glioma therapy. Future studies will need to define the optimal combinations of GSK3 inhibitors and cytotoxic agents in additional models of glioma and other cancers to better understand and optimize the most promising therapeutic strategies for evaluation in the clinic.

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

References

GSK3: A Novel Therapeutic Target in Glioma

Acknowledgments
Received 3/5/2008; revised 5/8/2008; accepted 6/3/2008.
Grant support: Intramural Research Program of the NIH, National Cancer Institute, Center for Research.

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We thank The Developmental Therapeutics Program, The National Cancer Institute (Bethesda, MD) and Dr. Zaharevitz for kindly providing GSK3 inhibitors 705701, 708234, and 709125; Dr. Sears for providing us with an aliquot of anti-phospho S62 c-MYC antibody; Dr. Baba for the generous gift of the anti-glycogen-specific antibody; Dr. Nogueira for sharing her detailed protocol with us on Hexokinase activity assays (NF-κB plasmid was kindly provided by Drs. Brien and Pena from Mayo Clinic College of Medicine); and James W. Nagle and Deborah Kauffmann from National Institute of Neurological Disorders and Stroke sequencing facility and Dr. Carolyn L. Smith from National Institute of Neurological Disorders and Stroke light image facility for their generous contributions to this work.

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