Potential Therapeutic Effect of Glycogen Synthase Kinase 3β Inhibition against Human Glioblastoma

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Abstract

Purpose: Glioblastoma represents the malignant brain tumor that is most refractory to treatment and in which the identification of molecular target(s) is urgently required. We investigated the expression, activity, and putative pathologic role of glycogen synthase kinase 3β (GSK3β), an emerging therapeutic target for neurodegenerative diseases, in human glioblastoma.

Experimental Design: The active fraction of GSK3β that is phosphorylated at the tyrosine 216 residue (pGSK3β^{Y216}) was identified in glioblastoma cell lines. GSK3β activity for phosphorylating its substrate was detected in these cells by nonradioisotopic *in vitro* kinase assay.

Results: Higher expression levels of GSK3β and pGSK3β^{Y216} were frequently detected in glioblastomas compared with nonneoplastic brain tissues. Inhibition of GSK3β activity by escalating doses of a small-molecule inhibitor (AR-A014418) or inhibition of its expression by RNA interference induced the apoptosis and attenuated the survival and proliferation of glioblastoma cells *in vitro*. Inhibition of GSK3β was associated with increased expression of p53 and p21 in glioblastoma cells with wild-type *p53* and with decreased Rb phosphorylation and expression of cyclin-dependent kinase 6 in all glioblastoma cell lines. Administration of AR-A014418 at a low dose significantly sensitized glioblastoma cells to temozolomide and 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea, chemotherapeutic agents used in the clinical setting, as well as to ionizing radiation.

Conclusion: These results indicate that GSK3β exerts a pathologic role by promoting the survival and proliferation of glioblastoma cells and by protecting them from apoptosis via the inactivation of p53- and/or Rb-mediated pathways. Consequently, we propose that GSK3β provides a potential therapeutic target in glioblastoma.

Glioblastoma is the most frequent malignant tumor of the brain and represents a subset of cancers that is mostly nonresponsive to currently available anticancer treatments. Biologically, this tumor is characterized by highly proliferative and invasive activity and widespread infiltration of tumor cells into the host brain tissue (1). The aggressive nature of glioblastoma hampers curable surgical intervention and renders it highly resistant to radiation and chemotherapy (2). The median patient survival period is ~12 months and has shown little improvement. Despite several clinical trials conducted over the past 30 years, only a minority of patients (5-10%) survives for 2 years (3). There is clearly an urgent need to develop new classes of treatment modalities, represented by molecular target-directed therapies.

One target with potential clinical relevance is a set of protein kinases that are mostly encoded by proto-oncogenes and that mediate signaling pathways for cell survival and/or proliferation in response to various stimuli, but which are frequently deregulated in cancer (4). The majority of small-molecule inhibitors currently under development for cancer treatment target a family of protein tyrosine kinases that include epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (4, 5). Based on the demonstration of a frequent deregulation of EGFR and/or platelet-derived growth factor receptor in glioblastomas (6, 7), several clinical trials have tested the efficacy of small-molecule inhibitors of these tyrosine kinases on glioblastoma patients. These include the use of gefitinib and erlotinib targeting EGFR and imatinib mesylate targeting platelet-derived growth factor receptor, bcr-abl, and c-kit (8, 9). However, these trials have proven largely unsuccessful and there is insufficient knowledge of the

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Translational Relevance

We showed that glioblastoma cells depend on deregulated glycogen synthase kinase 3β (GSK3β) to survive, proliferate, and resist chemotherapy and radiation. For applying a therapeutic strategy targeting GSK3β to treatment of glioblastoma patients, it is necessary to ascertain the therapeutic effect of GSK3β inhibition against glioblastoma in animal models with no or minimal adverse effects and to determine pharmacokinetic and pharmacodynamic properties of synthetic GSK3β inhibitors and preexisting drugs acting against GSK3β. There is accumulating evidence for a neurodegenerative effect of GSK3β and neuroprotective consequences resulting from its inhibition. Because of the highly invasive properties of the tumor, we expect that GSK3β inhibition provides dual benefits for the treatment of glioblastoma by attenuating tumor proliferation and by protecting host brain tissue from degradation and allowing its repair.

molecular determinants or surrogate markers of response of glioblastomas to tyrosine kinase inhibitors (8, 10).

Among the family of serine/threonine protein kinases, glycogen synthase kinase 3β (GSK3β) has recently emerged as a central player in the underlying molecular mechanism of several chronic, progressive diseases and therefore as a potential treatment target (11). GSK3β is a multifunctional protein kinase that regulates various cellular pathways depending on its substrates for phosphorylation (12). Based on its known biological properties and functions (12) as well as its primary pathologic causalities (13, 14), GSK3β has been a target for drug development in the treatment of non–insulin-dependent (type 2) diabetes mellitus and Alzheimer's disease (15–17). Because GSK3β triggers the degradation and inactivation of several oncogenic transcription factors (e.g., c-Jun and c-Myc) and proto-oncoproteins (e.g., β-catenin) by phosphorylating them, it would be expected to suppress neoplastic transformation and tumor development (18). A different line of observation has implicated GSK3β in the pathway for nuclear factor-κB (NF-κB)–mediated cell survival (19). Recently, we showed that deregulated GSK3β expression and activity are characteristic features of colorectal cancers and that GSK3β maintains the survival and proliferation of colon cancer cells (20). This novel pathologic role for GSK3β is supported by the observation that inhibition of this kinase sustains survival and proliferation and induces apoptosis of human colon cancer cells grown *in vitro* and in rodents (20, 21). We have subsequently shown similar properties for GSK3β in gastrointestinal, pancreas, and liver cancer cells (22).

In the present study, we have investigated the expression, activity, and putative pathologic properties of GSK3β in glioblastoma. We addressed whether this kinase might be a potential target that could lead to a more effective therapeutic strategy for this disease. Of particular interest is the involvement of GSK3β in major diseases of the central nervous system (11, 14). Lithium, a classic GSK3β inhibitor, is widely used in the treatment of bipolar disorder (17). GSK3β phosphorylates tau protein, leading to the abnormal assembly of this protein in nerve cells and increases production and extracellular deposition of amyloid Aβ, both of which are hallmarks of Alzheimer's disease (14). Increased phosphorylation of GSK3β at the tyrosine 216 residue (representing the active kinase form) was reported in degenerating cortical neurons induced by ischemia in a rodent model (23). Cells were rescued from death in this model by lithium treatment. A similar report has shown that GSK3β inhibition reduces neuronal death and acts as a neuroprotector (24). The accumulating evidence for neurodegenerative ability of GSK3β and a neuroprotective effect associated with the kinase inhibition led us to investigate for possible effects of GSK3β on the survival and proliferation of glioblastoma cells that invade and destroy host brain tissue.

Materials and Methods

Cell cultures. Human glioblastoma cell lines U87 and U251 were obtained from the American Type Culture Collection; A172 and T98G were obtained from the Health Science Research Resources Bank (Osaka, Japan) and the Cell Resource Center for Biochemical Research Institute of Development, Aging and Cancer in Tohoku University (Sendai, Japan), respectively. Cells from A172, U87, and U251 were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum. T98G cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum.

Clinical samples and immunohistochemistry. This study included 23 patients with primary glioblastoma who underwent surgical resection in the Department of Neurosurgery, Kanazawa University Hospital, in the period 1997 to 2005. An institutional review board approved the protocol. Most glioblastoma patients died of the disease within 2 years of diagnosis. Fresh tumor tissues were collected from 19 patients with glioblastoma (Supplementary Table S1). During surgery, nonneoplastic brain tissue was identified and obtained from margins of the tumors whenever possible. Surgical specimens of glioblastoma were fixed in 4% paraformaldehyde and embedded in paraffin for routine histopathologic examination and immunohistochemical analysis. Histologic diagnosis of the tumors was made by light microscopic examination of the sections stained with H&E.

Expression and localization of GSK3β in tumors and nonneoplastic brain tissues were examined immunohistochemically by the avidin-biotin-peroxidase complex method following microwave antigen retrieval of paraffin sections as described previously (25) and using rabbit monoclonal antibody to human GSK3β (diluted 1:100; Epitomics) and biotinylated goat anti-rabbit IgG (diluted 1:200; Vector). The positive control specimen for immunostaining was colon cancer that overexpressed active GSK3β as shown by Western immunoblot analysis (data not shown; ref. 20). For the negative control, the primary antibody was replaced by nonimmune rabbit IgG1 (DAKO).

Western immunoblot analysis. Cellular protein was extracted from cultured cells following the treatments indicated below and from fresh surgical specimens using lysis buffer (CelLytic-MT, Sigma-Aldrich) containing a mixture of protease and phosphatase inhibitors (Sigma-Aldrich). A 30-μg aliquot of whole protein extract was analyzed by Western immunoblot for the proteins of interest, as described previously (20). Blots were blocked with 5% bovine serum albumin before detection of phosphorylated protein fractions. The amount of protein extract in each sample was monitored by the expression of β-actin. We used the following primary antibodies at the dilutions shown against total GSK3 (GSK3α and GSK3β; 1:1,000; Upstate Biotechnology); GSK3β (1:1,000; BD Biosciences) and fractions that were phosphorylated in serine 9 (pGSK3β^{S9}; 1:1,000; Cell Signaling Technology) and

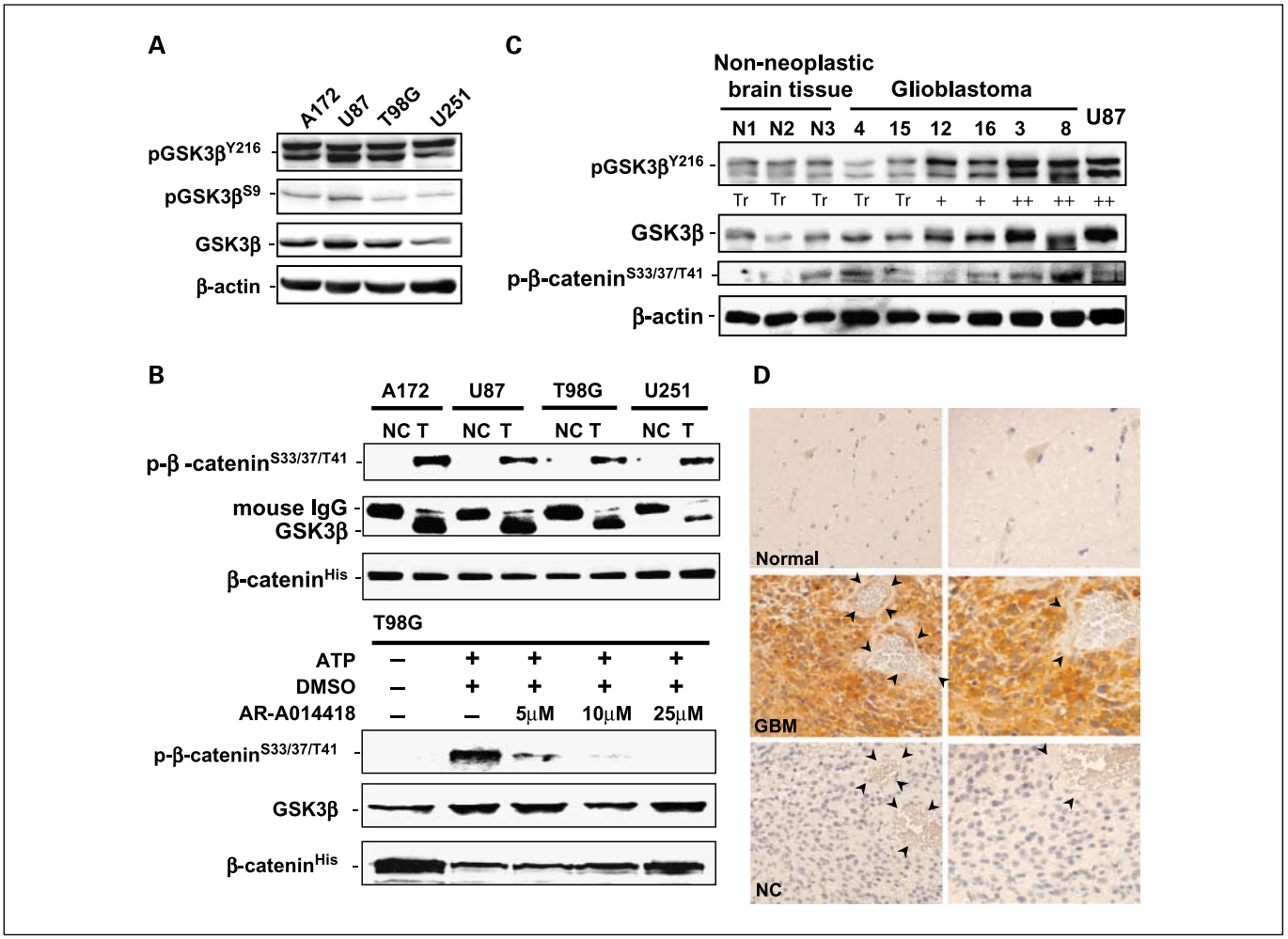

Fig. 1. Expression, phosphorylation, and activity of GSK3β in glioblastoma cells and tumor tissues. *A,* GSK3β and its fractions pGSK3β^{Y216} (active form) and pGSK3β^{S9} (inactive form) were detected in protein extracts from the indicated glioblastoma cell lines by Western immunoblotting analysis. *B,* GSK3β activity, an ability to phosphorylate its substrate (β-catenin), in the glioblastoma cells was detected by NRIKA (22). GSK3β was isolated from 1-mg aliquots of each sample cell lysate by immunoprecipitation. *In vitro* kinase reaction was then carried out in the presence of immunoprecipitated cellular GSK3β, its substrate recombinant human β-catenin protein (β-catenin^{His}), and nonradioisotopic ATP. The resultant products were analyzed by Western immunoblotting for phosphorylation of β-catenin^{His} at the residues serine 33 and 37 and/or threonine 41 (p-β-catenin$^{S33/37/T41}$). As a negative control reaction (*NC*) for each cell line, the mouse monoclonal antibody to GSK3β was replaced by an equal amount of nonimmune mouse IgG in the immunoprecipitation step. GSK3β activity is shown in all glioblastoma cells by expression of p-β-catenin$^{S33/37/T41}$ in the test lanes (*T*) and by the observation of little or no expression of p-β-catenin$^{S33/37/T41}$ in the negative control reaction. The amount of GSK3β and the presence of β-catenin^{His} in the kinase reaction were monitored by immunoblotting with mouse monoclonal antibodies to GSK3β and β-catenin, respectively. The bottom panels show the effect of a small-molecule GSK3β inhibitor (AR-A014418) on cellular GSK3β activity in T98G cells. In the NRIKA, kinase reaction was carried out in the presence of DMSO or different concentration of AR-A014418 at 37°C for 30 min. The presence of AR-A014418 inhibited the ability of T98G cell – derived GSK3β to phosphorylate β-catenin^{His}. *C,* expression of GSK3β, pGSK3β^{Y216}, and p-β-catenin$^{S33/37/T41}$ in surgical specimens removed from patients. A higher expression of GSK3β and pGSK3β^{Y216} was frequently found in the tumors (indicated by ++ and +) of glioblastoma patients compared with nonneoplastic brain tissues (*N1-N3*) showing trace expression (*Tr*). Protein extract from U87 cells was analyzed as a positive control (++). A fraction of p-β-catenin$^{S33/37/T41}$ was detected in the tumor tissues and U87 cells. β-Actin expression was monitored as a loading control in each sample. Full-length blots/gels for *A, B*, and *C* are presented in Supplementary Fig. S3. *D,* paraffin sections of glioblastoma and normal brain tissues were examined for expression and localization of GSK3β by immunohistochemistry using rabbit monoclonal antibody. Weak cytoplasmic expression of GSK3β was observed in neurons in normal brain tissue, whereas higher levels were found in the cytoplasm of glioblastoma cells (*GBM*). The absence of immunoreactivity in blood vessels or RBC (*arrowheads*) indicated the specificity of this immunostain. As a negative control (*NC*), a serial section of the same tumor tissue was immunostained with nonspecific rabbit IgG. Magnification, ×200. Panels on the right show higher magnification views corresponding to those on the left.

tyrosine 216 residues (pGSK3β^{Y216}; 1:1,000; BD Biosciences); β-catenin (1:1,000; BD Biosciences) and its fractions phosphorylated at serine 33, serine 37, and/or threonine 41 residues (p-β-catenin$^{S33/37/T41}$; 1:1,000; Cell Signaling Technology); p53 (1:1,000; DAKO); p21$^{Waf1/Cip1}$ (1:2,000; Cell Signaling Technology); Mdm2 (1:1,000; Cell Signaling Technology); Rb (1:2,000; Cell Signaling Technology) and its fractions phosphorylated in serine 780 (pRb^{S780}), serine 795 (pRb^{S795}), and serine 805/811 (pRb$^{S805/811}$; 1:1,000 each; Cell Signaling Technology); cyclin-dependent kinase 6 (CDK6; 1:2,000; Cell Signaling Technology); cyclin D1 (1:2,000; Cell Signaling Technology); EGFR (1:1,000; Cell Signaling Technology); and β-actin (1:2,000; Ambion).

In vitro *kinase activity assay.* A nonradioisotopic *in vitro* kinase assay (NRIKA) developed in our laboratory (22) was used to detect GSK3β activity in glioblastoma cells in the absence or presence of AR-A014418 (Calbiochem), a small-molecule GSK3β inhibitor.

Effects of GSK3β inhibitors on tumor cells. Glioblastoma cell lines seeded in 96-well plates were treated with DMSO or with AR-A014418 dissolved in DMSO at the indicated final concentrations in the medium. A previous study showed that AR-A014418 does not significantly inhibit 26 closely related protein kinases and is considered highly specific against GSK3β (26). At designated time points (24, 48, 72, and 96 h), the relative numbers of viable cells were

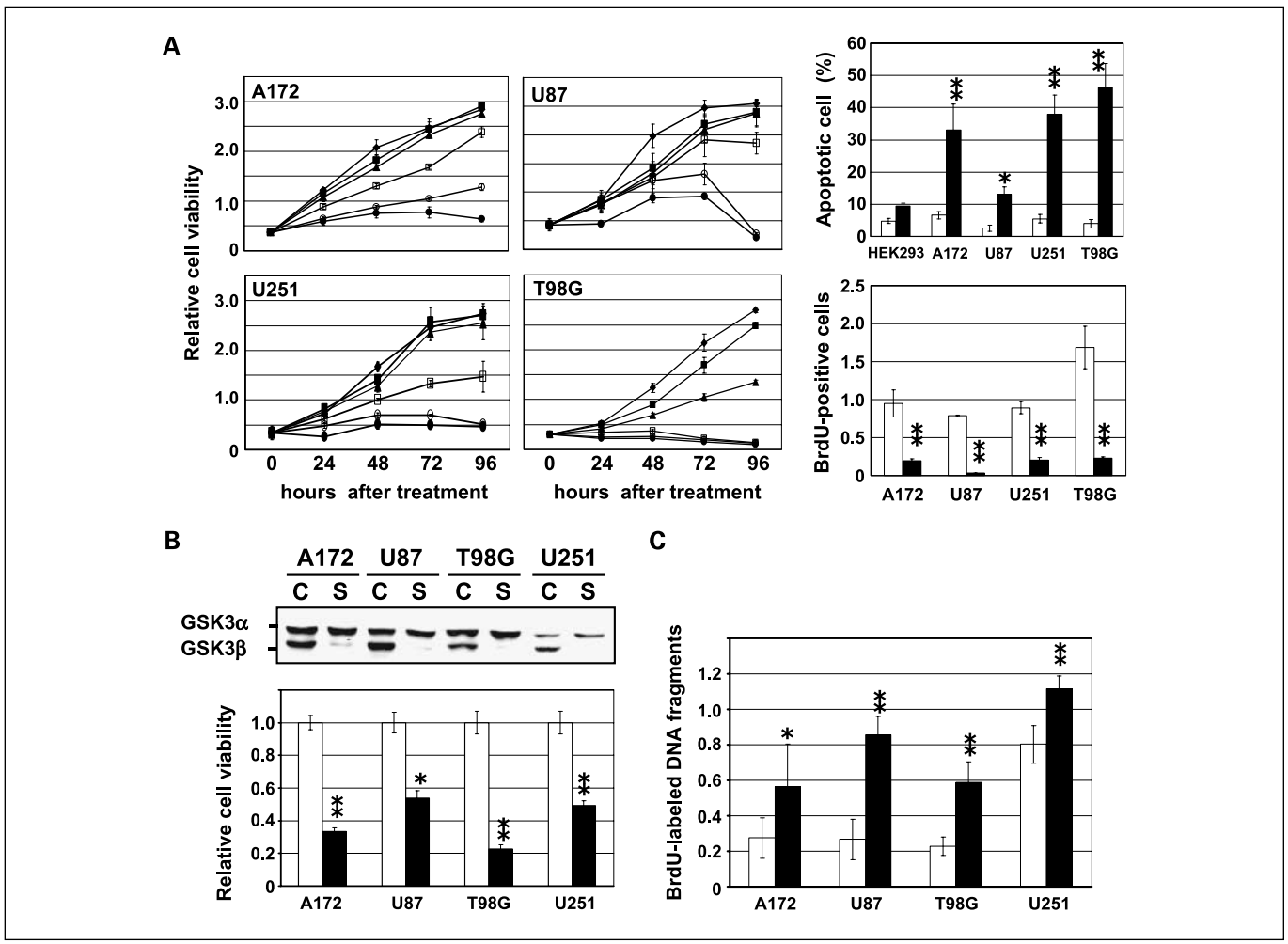

Fig. 2. Effect of GSK3β inhibition on cell survival and proliferation of glioblastoma cells. *A,* the glioblastoma cell lines indicated were grown in 96-well culture plates and treated with either DMSO or a small-molecule GSK3β inhibitor (AR-A014418) at escalating concentrations (5-50 μmol/L) for the designated times. The four panels on the left showed relative viable cell numbers measured by WST-8 assay at the indicated times as described in Materials and Methods. Treatment with DMSO and concentrations of AR-A014418 in the culture medium are indicated by the following symbols: ♦, DMSO; ■, 5 μmol/L; ▲, 10 μmol/L; □, 25 μmol/L; ○, 40 μmol/L; and ●, 50 μmol/L. IC_{50} values of AR-A014418 for A172, U87, T98G, and U251 cells are 16.9, 23.9, 10.6, and 13.5 μmol/L, respectively. Relative numbers of apoptotic cells (*right top*) and proliferating cells (*right bottom*) were determined by scoring apoptotic changes in cells stained with Hoechst 33342 and by measuring the amount of BrdUrd incorporation in cells, respectively, in the presence of DMSO (*open columns*) or 25 μmol/L AR-A014418 (*closed columns*) in culture medium for 72 h. In each case, the result is expressed as mean value ± SD. The effects of AR-A014418 on cell survival, proliferation, and apoptosis were compared with DMSO using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$. *B,* Western immunoblotting analysis using antibody to total GSK3 (GSK3α and GSK3β) showed the specific knockdown of GSK3β, but not its isozyme GSK3α, in all cell lines (*top*). *C,* nonspecific siRNA; S, GSK3β-specific siRNA. A full-length blot/gel is presented in Supplementary Fig. S3. Relative numbers of viable cells were measured and compared by WST-8 assay between the same glioblastoma cells transfected with GSK3β-specific siRNA (10 nmol/L each; *closed column*) and nonspecific siRNA (10 nmol/L each; *open column*) for 72 h (*bottom*). *, $P < 0.05$; **, $P < 0.001$; Student's *t* test. *C,* apoptosis in glioblastoma cells was measured by detecting BrdUrd-labeled DNA fragments using the Cellular DNA Fragmentation ELISA, in the presence of DMSO (*open columns*) or 25 μmol/L AR-A014418 (*closed columns*) in culture medium for 72 h. Bar in each column indicates SD. *, $P < 0.05$; **, $P < 0.001$; Student's *t* test.

determined by measuring absorbance using a WST-8 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) assay kit (Cell counting kit-8, Wako) and spectrophotometry using a microtiter-plate reader (Bio-Rad; refs. 20, 22).

The effects of GSK3β inhibitors on cells were analyzed in terms of apoptosis and cell proliferation. After treatment with DMSO or AR-A014418, cells were stained with Hoechst 33342 (Sigma-Aldrich) to detect apoptotic cells characterized by fragmentation of nuclei or nuclear condensation. A total of 200 nuclei per field was counted and scored for apoptotic changes. The mean scores for apoptosis in all five fields were then calculated. The presence of apoptosis was confirmed biologically using the Cellular DNA Fragmentation ELISA kit (Roche Diagnostics). The relative number of proliferating cells was determined by measuring the amount of bromodeoxyuridine (BrdUrd) incorporation in cells treated with DMSO or AR-A014418, using the Cell Proliferation ELISA BrdUrd Kit (Roche Diagnostics). Changes in NF-κB transcription activity following GSK3β inhibition were detected by luciferase reporter assay in the presence of DMSO or 25 μmol/L AR-A014418. The assay used the NF-κB Luciferase Reporter Vector (Panomics), the phRL-SV40 vector (Promega), and the Dual-Luciferase Reporter Assay System (Promega).

RNA interference. Small interfering RNA (siRNA) specific to human GSK3β (GSK3β Validated Stealth RNAi) and negative control siRNA (Stealth RNAi Negative Control Low GC duplex) were purchased from Invitrogen. The target sequence was 5′-GCUCCAGAUCAUGAGAAAGCUAGAU-3′. Cells were transfected with either the GSK3β-specific siRNA or negative control siRNA by using Lipofectamine RNAiMAX (Invitrogen). The effect of siRNA on GSK3β expression was determined by Western immunoblotting with antibody that binds to both GSK3α and GSK3β (Upstate Biotechnology). Glioblastoma cells seeded into 96-well plates were transfected with the respective siRNA and the relative cell viability was determined

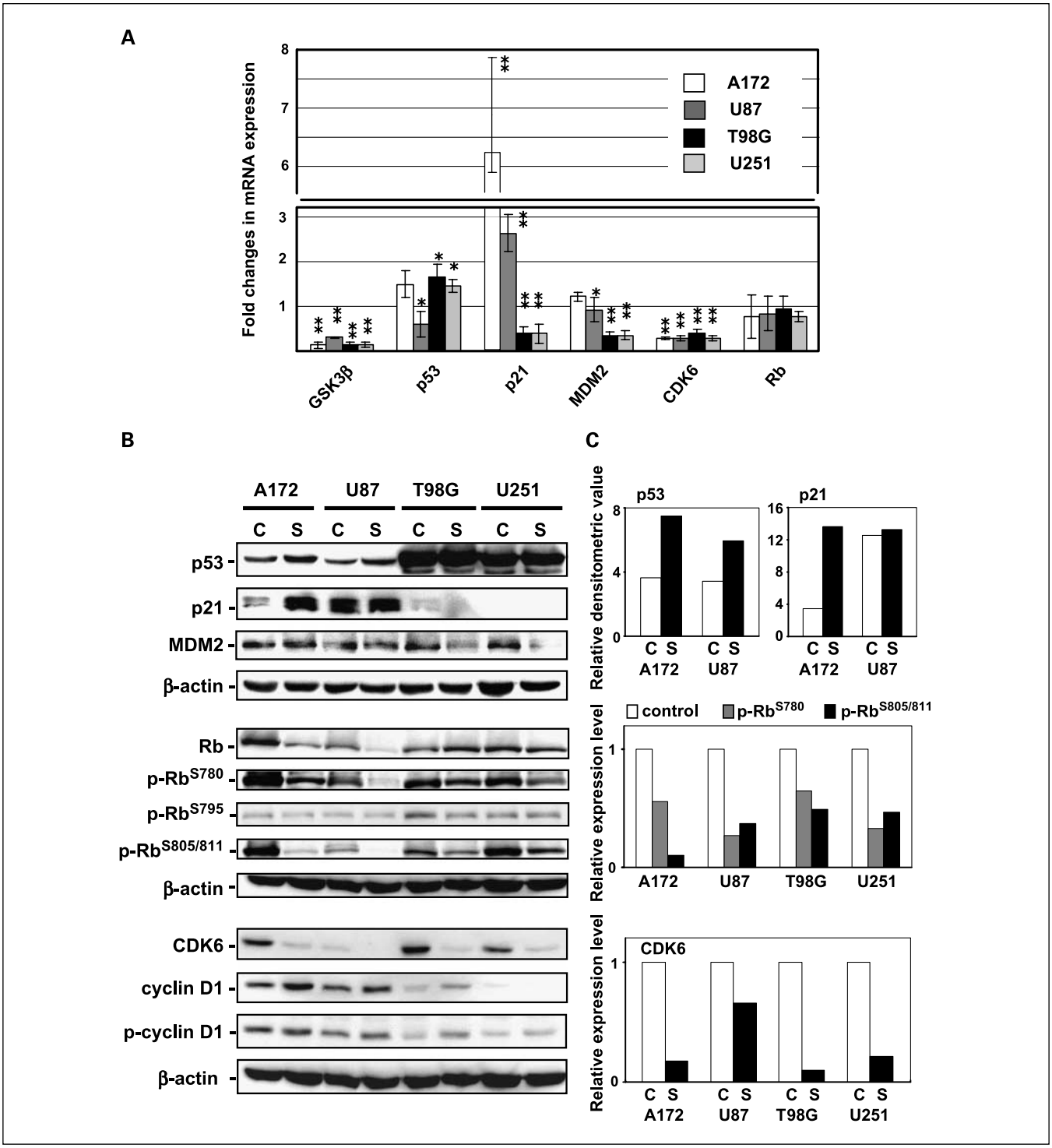

Fig. 3. Changes in expression of transcripts and proteins for genes involved in cell cycle regulation and proliferation following GSK3β depletion in glioblastoma cells. Cells from each glioblastoma cell line were transfected with 10 nmol/L of siRNA specific to GSK3β or negative control siRNA. Total RNA and cellular protein were extracted from the cells 72 h after transfection and subjected to qRT-PCR amplification and Western immunoblotting analysis, respectively. *A,* levels of mRNA expression (*indicated at the bottom of panel*) were measured by qRT-PCR amplification with the respective sets of primers (Supplementary Table S2) and normalized to the level of β-actin mRNA that co-amplified as the endogenous control for monitoring the amount of cDNA in each sample. Relative values of mRNA expression of the respective gene in each cell line transfected with GSK3β-specific siRNA were determined by comparing with the level of the corresponding gene expression in the same cell line transfected with negative control siRNA. Each result is expressed as the mean value ± SD. SD was obtained from three data points. Bar in each column indicates SD. *, $P < 0.05$; **, $P < 0.01$, Student's *t* test. *B,* immunoblotting analysis was carried out to compare expression of p53, p21, Mdm2, Rb, CDK6, cyclin D1, and phosphorylation of Rb and cyclin D1 between the cells transfected with GSK3β-specific siRNA (*S*) and nonspecific siRNA (*C*), respectively. Before this analysis, the efficiency of knockdown of GSK3β was confirmed by qRT-PCR as shown in *A*. β-Actin expression was monitored as a loading control in each sample. Full-length blots/gels are presented in Supplementary Fig. S3. *C,* effect of GSK3β depletion on the expression of each molecule of interest in the same cells was examined by densitometric analysis of signal corresponding to the protein detected in Western immunoblotting. Levels of expression of the respective proteins in each cell line transfected with GSK3β-specific siRNA (*S*) were compared with those of the corresponding protein in the same cell line transfected with negative control siRNA (*C*).

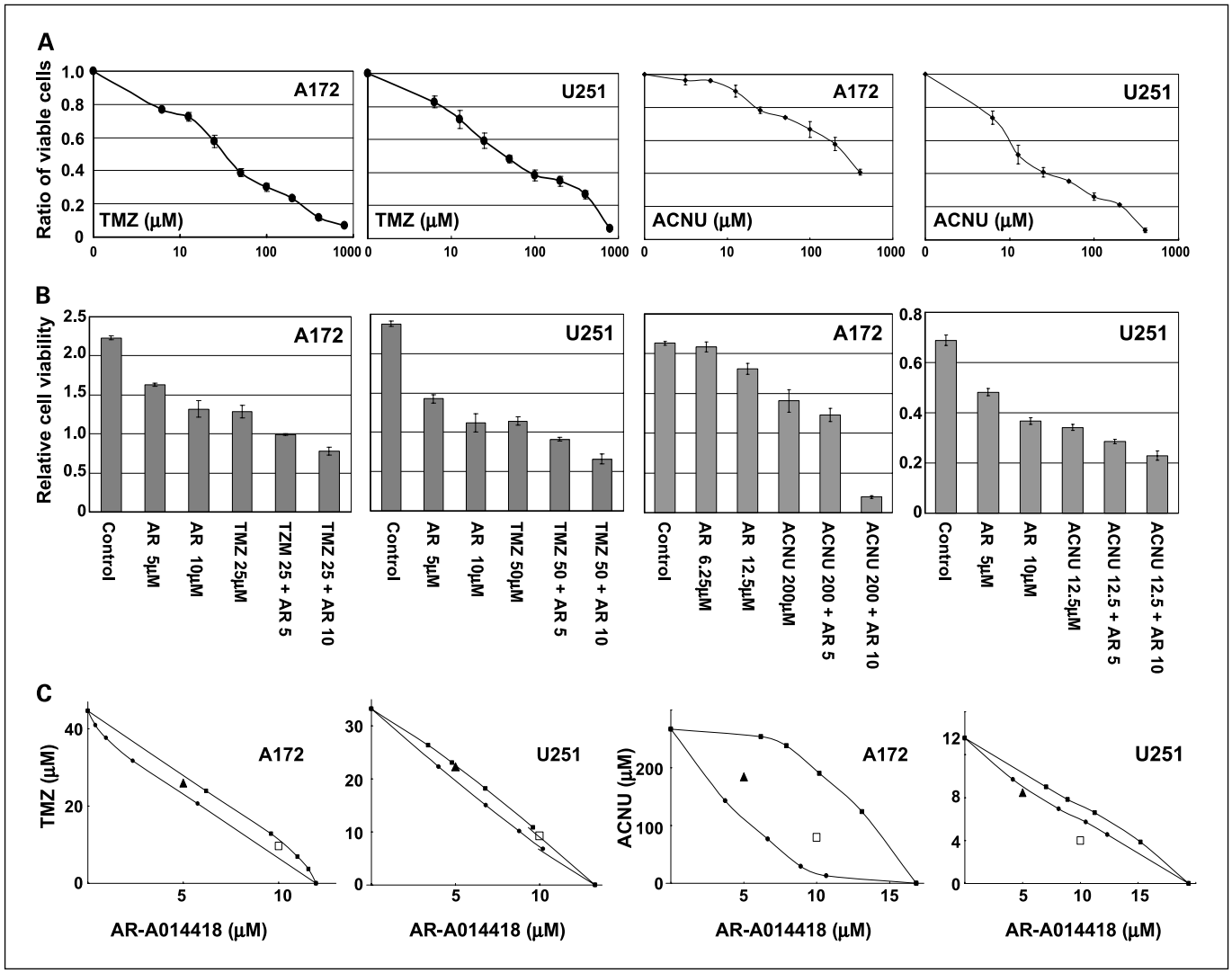

Fig. 4. Combined effect of a chemotherapeutic agent, temozolomide or ACNU, and low dose of a GSK3β inhibitor (AR-A014418) on glioblastoma cells (A172, U251). *A,* glioblastoma cells were treated with escalating concentrations of either temozolomide (*TMZ*) or ACNU as indicated in the respective panels. After treatment for 72 h, relative numbers of viable cells were measured by WST-8 assay to determine the dose-dependent effect and IC_{50} of temozolomide and ACNU against the respective cells. *B,* glioblastoma cells were treated with DMSO (control) or the indicated concentrations and combinations of *TMZ* or ACNU and AR-A014418 (*AR*). After treatment for 72 h, the relative numbers of viable cells were measured by WST-8 assay to observe the influence of low-dose (5 or 10 μmol/L) GSK3β inhibitor on the effect of *TMZ* and ACNU against glioblastoma cells. *C,* the influence of low-dose GSK3β inhibitor on the effect of *TMZ* and ACNU against glioblastoma cells was analyzed using isobologram methods (28) where the IC_{50} of the combination therapy was plotted (*delta sign*). The analysis showed that combined administration of low dose AR (▲, 5 μmol/L; □, 10 μmol/L) enhanced the expected effect of *TMZ* additively against A172 cells and U251 cells. Similarly, combined administration of low-dose AR enhanced the expected effect of ACNU additively against A172 cells and synergistically against U251 cells.

72 h later by the WST assay. The effects of RNAi on expression and phosphorylation of various proteins of interest was investigated by Western immunoblotting.

Quantitative real-time reverse transcription-PCR. Total RNA was isolated from cell pellets using RNA-Bee (Tel-Test) and serial treatment with phenol and chloroform. Complementary DNA was generated from total RNA using a Reverse Transcription Kit (Promega). Quantitative reverse transcription-PCR (qRT-PCR) was done using SYBR Premix Ex Taq (Takara Bio) with the respective sets of sense and antisense primers for amplification of *GSK3β*, *p53*, *p21*, *Mdm2*, *CDK4*, *CDK6*, *Rb*, *EGF*, *EGFR*, and *β-actin* (Supplementary Table S2; all from Takara Bio). The qRT-PCR reaction protocol consisted of an initial primary denaturation step at 95°C for 10 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Threshold cycle values (C_T) were determined with Sequence Detector Software (version 2.0; Applied Biosystems) and relative mRNA expression was calculated using the ΔC_T method described by the manufacturer and using *β-actin* as the calibrator gene. Changes in mRNA expression of multidrug resistance-associated protein 1 (MRP1) following treatment with GSK3β inhibitor were examined by qRT-PCR according to a previously reported method (27).

Effect of GSK3β inhibition on the sensitivity of glioblastoma cells to chemotherapeutic agent and to ionizing radiation. Glioblastoma cells seeded into 96-well plates were treated with escalating concentrations of either of the chemotherapeutic agents temozolomide (LKT Laboratories), 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU; generously provided by Daiichi-Sankyo Co., Ltd.), or AR-A014418. Following treatment for 72 h, the relative number of viable cells was measured by WST-8 assay to determine IC_{50} values for temozolomide, ACNU, and AR-A014418 and to generate isobolograms. Cells were then treated with a dose of temozolomide or ACNU close to IC_{50} in the presence of DMSO or low dose (5 or 10 μmol/L) of

AR-A014418. The isobologram method described previously (28) was used to determine whether the effect of GSK3β inhibitor on glioblastoma cell sensitivity to temozolomide or ACNU was additive, synergistic, or antagonistic.

Cells seeded in 96-well plates were pretreated with DMSO or with 5 or 10 μmol/L of AR-A014418 for 24 h and then irradiated (0, 4, or 8 Gy) using RX-650 (Faxitron X-ray Corporation). The relative numbers of viable cells at 120 h after treatment were measured by WST-8 assay to determine the combined effect of ionizing radiation and GSK3β inhibition on the survival of glioblastoma cells. The combined effect of the GSK3β inhibitor and ionizing radiation was further assessed by a colony-forming survival assay for cells seeded in six-well culture plates.

Statistical analysis. Between-group statistical significance was determined using the Student's *t* test. A *P* value of <0.05 was considered as statistically significant.

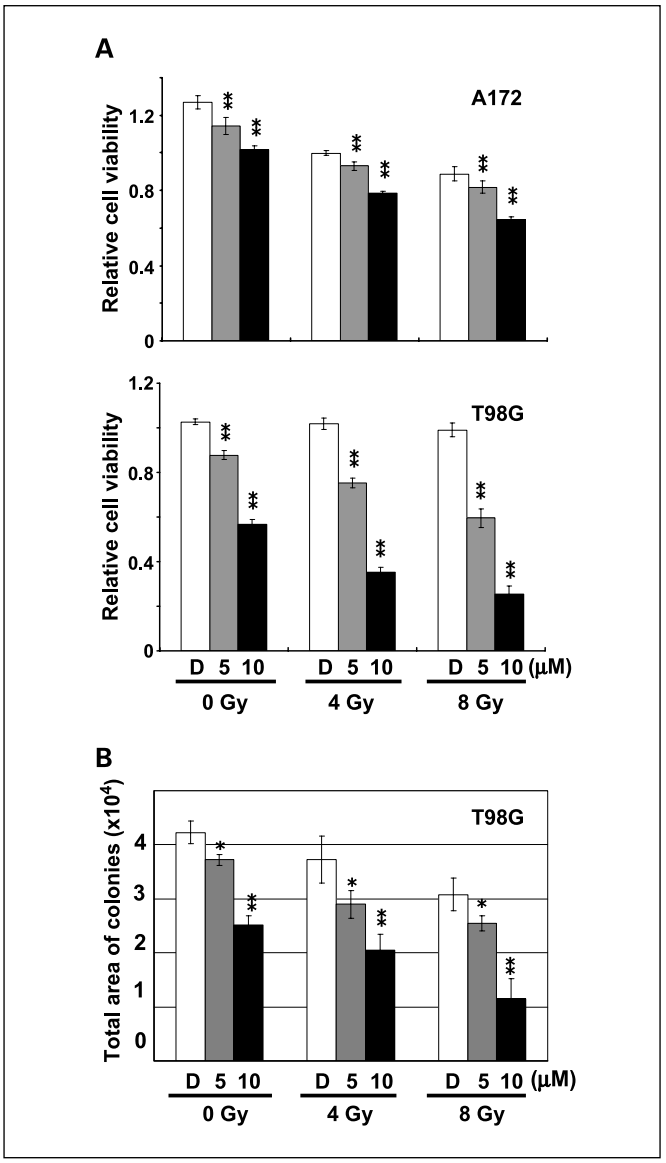

Fig. 5. The combined effect of ionizing radiation and low dose of GSK3β inhibitor (AR-A014418) against glioblastoma cells (A172 and T98G). *A,* the glioblastoma cells were seeded in 96-well culture plates and treated with either DMSO or a low dose (5 or 10 μmol/L) of AR-A014418 for 24 h before irradiation at doses of 0, 4, or 8 Gy. The relative number of viable cells was measured by WST-8 assay at 120 h after irradiation. When compared with cells treated with DMSO (*D*), pretreatment with either 5 μmol/L (*gray column*) or 10 μmol/L (*closed column*) AR-A014418 significantly enhanced the effect of irradiation against glioblastoma cells and particularly the T98G cells that harbor mutant p53 and are normally resistant to radiation (34). **, $P < 0.01$; Student's *t* test. *B,* the combined effect of the GSK3β inhibitor was tested for T98G by colony formation assay in triplicates. In each well of six-well plates, 2,000 of T98G cells were seeded and treated sequentially with DMSO or AR-A014418 and with ionizing radiation as described above. A total area of colonies stained with 0.1% crystal violet in PBS in each well was recorded and measured as arbitrary pixel number (*Y axis*) by the Analytical Digital Photomicrography method provided by Adobe Photoshop software (Adobe Systems, Inc.). *, $P < 0.05$; **, $P < 0.01$; Student's *t* test.

Results

Expression and activity of GSK3β in glioblastoma. We first investigated the expression of GSK3β and its phosphorylated fractions pGSK3β^{S9} (inactive form) and pGSK3β^{Y216} (active form) in glioblastoma cell lines. Western blot analysis showed prominent pGSK3β^{Y216} and faint pGSK3β^{S9} expression in all cell lines (Fig. 1A). NRIKA (22) showed that GSK3β derived from these cells was able to phosphorylate its substrate, β-catenin (Fig. 1B, *top panels*). These results indicate that glioblastoma cells contain constitutively active GSK3β and that its activity is thought to be decontrolled by phosphorylation-dependent regulation. Activity of GSK3β derived from the glioblastoma cells was inhibited by a small-molecule GSK3β inhibitor, AR-A014418 (Fig. 1B, *bottom panels*), that was used for investigating effects of GSK3β inhibition on survival and proliferation of glioblastoma cells, as shown below.

Serial immunoblotting analysis of tissue specimens from patients showed that a higher expression of GSK3β and pGSK3β^{Y216} was detected frequently in glioblastomas compared with nonneoplastic brain tissues (Fig. 1C; Supplementary Table S1). A fraction of p-β-catenin$^{S33/37/T41}$ was detected in the tumor tissues and U87 cells and its levels in the tumor tissues were higher than those in nonneoplastic brain tissues (Fig. 1C), indicating that GSK3β is functional in the tumors. Using immunohistochemistry, overexpression of GSK3β was observed in the cytoplasm of tumor cells in 6 of 11 (55%) glioblastomas, whereas weak expression only was observed in the cytoplasm of neurons from nonneoplastic tissue (Fig. 1D). Thus, it is likely that overexpression of active GSK3β is a pathologic characteristic of glioblastoma cell lines and clinical glioblastomas. In these patients, there was no correlation between levels of GSK3β expression or its Y216 phosphorylation and clinical characteristics of the patients, including response to the treatment and outcome (Supplementary Table S1).

Inhibition of GSK3β suppresses glioblastoma cell viability and proliferation and induces apoptosis. The above results indicate that GSK3β has a putative pathologic role in glioblastoma. Therefore, we investigated the consequences of inhibition of GSK3β activity and down-regulation of its expression on the viability and proliferation of glioblastoma cells *in vitro*. Treatment with escalating concentrations (5-50 μmol/L) of AR-A014418 suppressed the viability of all glioblastoma cell lines examined in a dose-dependent manner (Fig. 2A). NRIKA (22) was used to confirm inhibition of GSK3β activity by AR-A014418 (Fig. 1B, *bottom panels*). This effect was associated with a significant decrease in cell proliferation and increase in apoptosis (Fig. 2A). On the basis of the result in NRIKA (Fig. 1B), we expect that therapeutic effect of 25 μmol/L

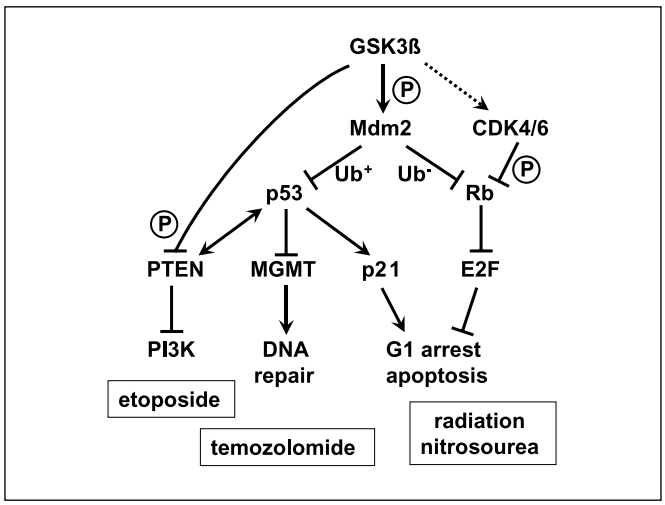

Fig. 6. Putative molecular pathways showing how GSK3β regulates the fate of tumor cells (survival, proliferation, or apoptosis) and influences their sensitivity to different chemotherapeutic agents (etoposide, temozolomide, ACNU) and radiation. The pathways are generated according to the results of the present study and to previous reports, as described in the text. Positive feedback regulation is suggested to exist between the expression of p53 and PTEN (47). The molecular mechanism leading to the pathway indicated by the dotted arrow remains to be determined. Circled P, phosphorylation; Ub^+, ubiquitin system dependent; Ub^-, ubiquitin system independent.

AR-A014418 against glioblastoma cells is attributed to inhibition of GSK3β activity.

RNA interference (RNAi) was then used to down-regulate GSK3β expression. Western blotting showed that transfection of GSK3β-specific siRNA reduced the expression of GSK3β but not GSK3α, indicating specificity of RNAi for GSK3β (Fig. 2B, *top*). Depletion of GSK3β by siRNA led to a significant decrease in the relative number of viable cells in all glioblastoma cell lines (Fig. 2B, *bottom*). This was associated with attenuated cell proliferation and induction of apoptosis; the latter was confirmed by cellular DNA fragmentation analysis (Fig. 2C). These results indicate a pathologic role for GSK3β in promoting tumor cell survival and proliferation in glioblastoma.

Molecular alterations associated with GSK3β inhibition in glioblastoma cells. The molecular mechanisms that underlie a putative pathologic role for GSK3β in glioblastoma are of particular relevance because of the resistance to chemotherapy and radiation shown by this tumor (2, 3). Therefore, we investigated whether inhibition of GSK3β modifies the expression of EGFR and MRP1 and the transcriptional activity of NF-κB. These factors are involved in the progression of glioblastoma and in its resistance to apoptosis-based treatments (2, 6, 27). Although the glioblastoma cells expressed variable levels of EGFR and MRP1 proteins, no concordant changes in their expression was observed using GSK3β knockdown (Supplementary Fig. S1A and B). In contrast to a previous report in pancreatic cancer cells suggesting a putative role for GSK3β in NF-κB-mediated gene transcription (29), the inhibition of GSK3β in glioblastoma cells caused little effect or even increased NF-κB transcriptional activity (Supplementary Fig. S1C).

Based on preliminary microarray-based studies of GSK3β-regulated transcripts in colon and pancreatic cancer cells,[6] glioblastoma cells were examined by qRT-PCR for GSK3β knockdown-induced changes in the expression of genes involved in cell cycle and apoptosis pathways. As shown in Fig. 3A, the relative expression of *p21* was significantly increased in A172 and U87 cells, both with wild-type *p53* (30), but significantly decreased in T98G and U251 cells, both with mutant *p53* (30). Increased expression of *p53* and decreased expression of *CDK6* were also found in these cells.

Previous reports showed that Mdm2 and cyclin D1 are substrates for GSK3β-dependent phosphorylation (18, 31). Mdm2 regulates p53 abundance and cyclin D1 controls the activity of CDK6. The results of qRT-PCR analyses and the known roles of GSK3β in regulating Mdm2 function and cyclin D1 stability suggest that Mdm2-p53-p21 and cyclin D1-CDK6-Rb pathways are involved in the molecular mechanism(s) downstream of GSK3β inhibition. Therefore, we further analyzed protein expression levels of the components of these pathways following GSK3β-specific siRNA treatment. In addition, phosphorylated fractions of Rb, including p-Rb^{S780}, p-Rb^{S795}, and p-$Rb^{S805/811}$, were compared between cells treated with GSK3β-specific and nonspecific siRNAs. Upon GSK3β knockdown, the level of p53 protein was elevated in A172 and U87 cells, and the expression of p21 was increased markedly in the former cells and slightly in the latter cells. No changes in the level of mutant p53 protein and only trace expression of p21 protein were observed in T98G and U251 cells. No concordant changes in basal levels of Mdm2 expression were seen in these cells (Fig. 3B and C, *top panels*). As shown in Fig. 3B and C (*middle panels*), GSK3β knockdown did not always induce coincident changes in Rb protein levels, but decreased the levels of p-Rb^{S780} and p-$Rb^{S805/811}$ in all glioblastoma cell lines. Decreased Rb phosphorylation was associated with down-regulation of CDK6 (Fig. 3B and C, *bottom panels*), a known CDK that phosphorylates Rb and thus renders it unable to bind and repress E2F-mediated gene transcription (32). Contrary to a previous study showing that overexpression of a constitutively active form of GSK3β increased cyclin D1 expression in ovarian cancer cells (33), depletion of GSK3β in the present study caused up-regulation of cyclin D1 in glioblastoma cells (Fig. 3B, *bottom panels*). This is consistent with the known primary role of GSK3β in phosphorylating cyclin D1 and targeting it for ubiquitin-dependent degradation (18). Consequently, the current study indicates that restoration of p53- and Rb-mediated pathways could explain mechanisms by which GSK3β inhibition attenuates the survival and proliferation of glioblastoma cells.

Low-dose GSK3β inhibitor sensitizes glioblastoma cells to a chemotherapeutic agent and to ionizing radiation. Recognition of a pathologic role for GSK3β in glioblastoma encouraged further experiments to address whether its inhibition could enhance the benefits of chemotherapy and irradiation, the standard treatment modalities for glioblastoma (1). The restoration of p53- and Rb-mediated tumor suppressor pathways induced by GSK3β inhibition was a mechanistic base of our hypothesis.

GSK3β inhibitor alone at a high dose (25-50 μmol/L in cell culture) had a therapeutic effect against tumor cells (Fig. 2A). Thus, we tested for possible effects of relatively low doses (5 or 10 μmol/L in cell culture) of GSK3β inhibitor in combination with a chemotherapeutic agent, temozolomide or ACNU, or

[6] Unpublished results.

ionizing radiation. In preliminary experiments, we determined the dose-dependent effects of temozolomide and ACNU on glioblastoma cells (Fig. 4A). When cells were treated with temozolomide or ACNU at a dose close to its IC_{50}, the combination with 10 μmol/L AR-A014418 significantly reduced cell viability than treatment with temozolomide, ACNU, or 10 to 12.5 μmol/L AR-A014418 alone (Fig. 4B). Isobologram analysis was used to evaluate whether low-dose AR-A014418 potentiates the effect of temozolomide or ACNU against glioblastoma cell lines. The analysis determined that low-dose AR-A014418 in combination with temozolomide was additive in A172 and U251 cells. Similarly, its effect in combination with ACNU was additive in A172 and U87 cells and synergistic in U251 and T98G cells (Fig. 4C; Supplementary Fig. S2). Combined administration with low-dose GSK3β inhibitor therefore sensitizes glioblastoma cells to the anticancer drugs.

The effect of low-dose GSK3β inhibitor combined with ionizing radiation was tested in A172 and T98G cells, known to be radiosensitive and radioresistant, respectively (34). In both WST-8 and colony-forming survival assays, combination with 5 or 10 μmol/L AR-A014418 reduced cell viability, particularly in T98G cells (Fig. 5). The results indicate that concurrent administration of low-dose GSK3β inhibitor sensitizes glioblastoma cells to ionizing radiation.

Discussion

GSK3β is a multifunctional kinase implicated in predisposition to neurodegenerative diseases (14, 17). The present study showed a pathologic role for deregulated GSK3β in promoting the survival and proliferation of glioblastoma cells and a therapeutic effect of GSK3β inhibition in this tumor. Deregulated expression and activity of GSK3β was observed in both the established glioblastoma cell lines and the majority of clinical tumors. Because of the highly destructive and invasive properties of this tumor, we expect that GSK3β inhibition provides dual benefits for the treatment of glioblastoma by attenuating tumor proliferation and by protecting host brain tissue from degradation and allowing regeneration. The small-molecule GSK3β inhibitor acted synergistically or additively to enhance the effect of chemotherapeutic agents (temozolomide, ACNU) and of ionizing radiation against glioblastoma cells. Therefore, inhibitory drugs against GSK3β activity could be a promising therapeutic option for glioblastoma, in line with recent reports on other refractory cancers, including pancreatic cancer (22, 29), malignant melanoma (35), and medullary thyroid cancer (36). Nonneoplastic brain tissue showed considerably less expression of the active fraction of GSK3β ($pGSK3\beta^{Y216}$) compared with glioblastomas. The primary function of GSK3β in inducing neuronal apoptosis (14, 17) suggests that few adverse effects should result from its inhibition in central nervous system if used for the treatment of glioblastoma. This is supported by our earlier studies followed by other investigators' reports showing that neither GSK3β inhibitors influenced cell survival or growth in culture of human embryonic kidney (HEK293) cells, mammary epithelial cells, embryonic lung fibroblasts, or mouse embryonic fibroblasts (NIH-3T3; refs. 20, 22, 29, 35) and that no detrimental effects were observed in rodents treated with i.p. injection of AR-A014418 for 5 weeks (21) or 10 weeks.[7]

As discussed below and hypothesized in Fig. 6, there is considerable interest in the molecular mechanism that underlies the putative pathologic role of GSK3β in glioblastoma. Of particular interest is the effect of GSK3β inhibition on tumor cell survival and proliferation and on susceptibility to chemotherapeutic agents and ionizing radiation. Inhibition of GSK3β was found to induce the expression of p53 and p21 proteins in the glioblastoma cell lines A172 and U87 harboring wild-type *p53*, but not in T98G and U251 harboring mutant *p53*. The present results are consistent with recent findings in colon cancer (HCT116; ref. 37) and melanoma (35) cell lines with wild-type *p53*. Overall, they account for suppression of cell proliferation and induction of apoptosis in glioblastoma cells via the inhibition of GSK3β in a p53-dependent manner. Whereas the depletion of GSK3β by siRNA did not always induce concordant changes in the expression of Rb protein, it decreased the levels of Rb phosphorylation at serine 780 and 805/811 residues in all cell lines irrespective of *p53* mutation status. This effect could be attributed to the down-regulation of CDK6 mediated by GSK3β inhibition and resulting in the decreased phosphorylation and activation of Rb (32). A previous study showed that CDK6 expression is increased in glioblastoma but not in normal brain tissue and is undetectable in antecedent low-grade tumor in the same patients (38). A pharmacologic inhibitor of CDK4 and CDK6 is reported to induce G_1 arrest and suppress proliferation of tumor cells via the reduction of $p\text{-}Rb^{S780/795}$ and subsequent down-regulation of its target genes under the transcriptional control of E2F (39). Therefore, our results indicate that inhibition of GSK3β reduces cell survival and proliferation and induces apoptosis in glioblastoma cells with wild-type or mutant *p53* via activating Rb-mediated pathway. CDK6 seems to exert a critical regulatory role for cell proliferation in these tumors. Further work is necessary to understand how GSK3β regulates the expression and/or activity of CDK6.

The current standard therapy for newly diagnosed glioblastoma consists of surgical resection of the tumor to the extent that is safe and feasible, followed by chemotherapy and irradiation. A few years ago, ACNU was one of the commonly prescribed anticancer drugs for adjuvant chemotherapy of glioblastoma in Japan (3). Meta-analysis of 12 randomized clinical trials using nitrosourea in high-grade glioma has shown only a moderate survival benefit of 6% for 1-year survival rate and 2 months for median survival time (3). Recently, temozolomide has been substituted for ACNU and used clinically as standard chemotherapy for glioblastoma, but its efficiency is not sufficient even by combination with radiation (40). This is thought to be largely due to tumor resistance to both therapies (2). There has been an emerging paradigm for the combination of chemotherapy and molecular targeted therapy to improve therapeutic efficiency (10). Importantly, we have shown in the present study that the GSK3β inhibitor AR-A014418, even at low concentrations (5 or 10 μmol/L) having little therapeutic effect, could significantly sensitize glioblastoma cells to temozolomide and ACNU. As discussed above and below, understanding the molecular basis by which GSK3β inhibition alters the chemosensitivity of glioblastoma

[7] W. Mai, et al. Unpublished results.

is critical for possible future clinical applications and for predicting possible adverse effect(s).

Several genes have been reported to influence the susceptibility and resistance of glioblastoma to chemotherapy (2). Well documented among the molecular determinants are EGFR, MRP1, and NF-κB (2, 6, 27). In the present study, GSK3β inhibition by RNAi did not show any consistent correlation with the expression of EGFR protein or MRP1 transcript. Activation of NF-κB has been implicated in the acquisition of resistance to apoptosis-based chemotherapy in many cancer types (41), including glioblastoma (2). It has been reported that NF-κB inhibition sensitizes human glioblastoma cells to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; ref. 42) and that GSK3β suppresses TRAIL-induced apoptosis in prostate cancer cells (43). These studies suggest a possible involvement of NF-κB in the molecular mechanism by which GSK3β inhibition enhances chemotherapeutic efficiency. In line with observations made in GSK3β knockout mice (19), another report showed that GSK3β inhibition decreased the activity of NF-κB for transactivation of its target genes in pancreatic cancer cells (29). However, in the present study, GSK3β inhibition had little influence or may have even increased the transcriptional activity of NF-κB in glioblastoma cells. We have made similar observations in colon cancer cells[8] and the data seem consistent with the proposal that NF-κB exerts bipartite functions in cell survival and apoptosis depending on the cell type and the context of tissue kinetics (41). Overall, the data suggest that NF-κB is unlikely to be involved in the mechanism by which inhibition of GSK3β sensitizes glioblastoma cells to ACNU-induced apoptosis.

Other molecules potentially involved in the chemosensitization associated with GSK3β inhibition in glioblastoma cells include O^6-methylguanine-DNA methyltransferase (MGMT) and PTEN. The expression level of MGMT, a DNA repair protein, has been shown to correlate inversely with chemosensitivity and prognosis in glioblastoma patients (44). Induction of p53 also attenuates MGMT expression in glioblastoma cells and renders them sensitive to temozolomide (45). Thus, the present results showing that GSK3β inhibition induced the expression of p53 in cells with wild-type *p53* suggests that it may also attenuate MGMT expression in a p53-dependent manner and sensitize glioblastoma cells with wild-type *p53* to anticancer drugs. Another molecule of interest is the PTEN tumor suppressor protein, a phospholipid phosphatase that dephosphorylates phosphatydylinositol 3,4,5,-triphosphate and inhibits phosphoinositide 3-kinase–dependent signaling. *PTEN* is mutated or deleted in ~40% of primary glioblastomas and these tend to be particularly refractory to chemotherapy (3). A recent study showed that GSK3β phosphorylates the threonine 366 residue of PTEN, resulting in its destabilization, and that GSK3β inhibition rescues the stability of PTEN in glioblastoma cells (T98G and U87 with exogenous PTEN; ref. 46). In *PTEN*-null U87 cells, retroviral transduction of PTEN protects p53 from Mdm2, augments the expression of p53 target genes, and sensitizes the cells to the chemotherapeutic agent etoposide (47). Together, these studies suggest that PTEN stabilization via inhibition of GSK3β can lead to increased p53 activity and induction of cell cycle arrest and chemosensitivity in glioblastoma cells. Further studies are required to address whether alterations in MGMT and PTEN are responsible for the chemosensitization of glioblastoma cells following GSK3β inhibition.

Although radiotherapy remains a primary treatment modality for glioblastoma, this tumor is poorly responsive to current treatment regimens (1–3) that include combination with temozolomide (40). The ability to enhance radiosensitivity would likely be of clinical benefit in the treatment of this disease. Importantly, the present study found that pretreatment with low-dose GSK3β inhibitor enhanced the cytocidal effect of ionizing radiation to glioblastoma cells. This could be due mainly to the reactivation of pathways mediated by p53 (48) and Rb (32). The radiosensitizing effect in the current study was more prominent in T98G cells containing mutant *p53*, reported to be highly radioresistant (34), than A172 cells containing wild-type *p53*. Glioblastomas could acquire enhanced radiosensitivity following GSK3β inhibition through decreases in Rb phosphorylation and CDK6 expression. This is consistent with a report that CDK inhibitor enhances radiosensitivity of esophageal adenocarcinoma by up-regulating Rb (49). It was recently reported that inhibition of protein kinase B (PKB/Akt) does not enhance the radiosensitivity of glioblastoma cells (50). This indirectly supports the present finding that active GSK3β confers radioresistance to glioblastoma cells, based on the known relationship between PKB/Akt and GSK3β (12). In a preliminary study including >40 glioblastoma patients, we have found no correlation in expression or phosphorylation between PKB/Akt and GSK3β in the tumors,[9] which is consistent with our previous study for colorectal cancer showing frequent activation of PKB/Akt by phosphorylation in the tumors (20). Taken together, it would be possible that GSK3β is constitutively active in glioblastoma cells and that an undetermined pathway other than that mediated by PKB/Akt prevents GSK3β S9 phosphorylation, thus allowing it to be constitutively active in tumor cells.

In summary, we report here a previously unrecognized pathologic role for deregulated GSK3β in human glioblastoma. This kinase is involved in tumor resistance to the chemotherapeutic agents and to ionizing radiation, both commonly used in the clinical setting against glioblastoma. Changes in expression were found in several molecules that regulate cell cycle, proliferation, and apoptosis and that are considered to underlie the observed effect of GSK3β inhibition against glioblastoma. Investigation of the broader mechanisms responsible for deregulation of GSK3β activity and its downstream pathologic effects should provide useful insights into the aberrant molecular pathways leading to tumor development and progression and should also suggest novel therapeutic strategies for glioblastoma, the most fatal human cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[8] Unpublished results.

[9] M. Nakada, unpublished observation.

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