Akt Activation Suppresses Chk2-Mediated, Methylating Agent–Induced G_2 Arrest and Protects from Temozolomide-Induced Mitotic Catastrophe and Cellular Senescence

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Abstract

Pharmacologic inhibition of the DNA signal transducers Chk1 and p38 blocks G_2 arrest and sensitizes glioblastoma cells to chemotherapeutic methylating agent–induced cytotoxicity. Because Akt pathway activation has been suggested to also block G_2 arrest induced by DNA-damaging agents and because glioma cells frequently have high levels of Akt activation, we examined the contribution of the Akt pathway to methylating agent–induced G_2 arrest and toxicity. U87MG human glioma cells containing an inducible Akt expression construct were incubated with inducing agent or vehicle, after which the cells were exposed to temozolomide and assayed for activation of the components of the G_2 arrest pathway and survival. Temozolomide-treated control cells activated the DNA damage signal transducers Chk1, Chk2, and p38, leading to Cdc25C and Cdc2 inactivation, prolonged G_2 arrest, and loss of clonagenicity by a combination of senescence and mitotic catastrophe. Temozolomide-treated cells induced to overexpress Akt, however, exhibited significantly less drug-induced Cdc25C/Cdc2 inactivation and less G_2 arrest. Akt-mediated suppression of G_2 arrest was associated not with alterations in Chk1 or p38 activation but rather with suppression of Chk2 activation and reduced recruitment of Chk2 to sites of damage in chromatin. Unlike bypass of the G_2 checkpoint induced by pharmacologic inhibitors of Chk1 or p38, however, Akt-induced bypass of G_2 arrest suppressed, rather than enhanced, temozolomide-induced senescence and mitotic catastrophe. These results show that whereas Akt activation suppresses temozolomide-induced Chk2 activation and G_2 arrest, the overriding effect is protection from temozolomide-induced cytotoxicity. The Akt pathway therefore represents a new target for the sensitization of gliomas to chemotherapeutic methylating agents such as temozolomide. (Cancer Res 2005; 65(11): 4861-9)

Introduction

Cytotoxic DNA methylating agents are compounds that share the ability to directly or indirectly form methyl adducts in DNA. Whereas these agents all methylate a variety of positions on the bases of DNA, the most relevant adduct to the cytotoxic action of these compounds is O^6-methylguanine. O^6-methylguanine mispairs with thymine during DNA replication creating a DNA mismatch (1). This mismatch is recognized by the DNA mismatch repair system that subsequently removes the mismatched base rather than O^6-methylguanine itself (2). Reinsertion of thymine opposite the O^6-methylguanine during repair DNA resynthesis reinitiates the cycle of futile repair and leads to the creation of DNA single- and double-strand breaks (3). These strand breaks ultimately lead to cytotoxicity via activation of cell death pathways. The unique action of cytotoxic methylating agents makes them a key component of therapeutic regimens targeted at gliomas and lymphoid malignancies (4, 5).

Although the pathway leading from O^6-methylguanine in DNA to cytotoxicity has been defined, cell death is not an inevitable consequence of methylating agent–induced DNA damage. In response to cytotoxic methylating agents, glioma cells and to a lesser extent lymphoid cells activate the G_2 checkpoint (6). Activation of the G_2 checkpoint is believed to allow cells time to repair DNA damage and to avoid the cytotoxic consequences of such damage. Consistent with this idea, glioma cells better able to activate and sustain G_2 arrest in response to the cytotoxic methylating agent temozolomide also exhibit increased survival (6). Similarly, preincubation of glioma cells with the G_2 checkpoint inhibitor UCN-01 blocks temozolomide-induced G_2 arrest and prematurely forces cells into mitosis, leading to enhanced death by mitotic catastrophe (7). These studies suggest that control of the G_2 checkpoint plays a key role in the response of tumor cells to cytotoxic methylating agents.

The G_2 checkpoint is in turn controlled by a variety of interlinked kinases and phosphatases, each of which plays a critical role in the DNA damage response. The ultimate activation of the G_2 checkpoint is controlled by the cyclin-dependent kinase Cdc2, also known as Cdk1. When phosphorylated at Thr^{14} and Tyr^{15} residues, Cdc2 cannot stimulate entry of cells into mitosis (8). The phosphorylation state of Cdc2, however, is controlled by proteins of the Cdc25 family of phosphatases. Cdc25 A, B, and/or C, in their unphosphorylated, active forms dephosphorylate and activate Cdc2 (9–11). The phosphorylation/activation state of Cdc25 family of proteins is in turn controlled by the DNA damage signal transducers Chk1 and/or Chk2 that phosphorylate and inactivate the various Cdc25 proteins in response to DNA damage (12–14). As an example, DNA damage induced by methylating agents leads to Chk1 activation (Ser^{345} phosphorylation), Chk2 activation (Thr^{68} phosphorylation), Cdc25C phosphorylation (Ser^{216})/inactivation, retention of Cdc2 in an inactive, phosphorylated (Tyr^{15}) form, and arrest of the cell cycle at the G_2-M boundary (7). This description, however, is an oversimplification as at least one other pathway involving the stress response protein p38 α is also activated by DNA damage and similarly leads to Cdc2 inactivation (15). Additionally, the exact response pathway

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activated is highly dependent on the type of DNA damage incurred, with ionizing radiation, UV irradiation, and methylating agents each activating different components of the G_2 arrest pathway (15–17). The pathways that control activation of the G_2 checkpoint in response to DNA damage are therefore complex and variable depending on the DNA damage induced.

Whereas an extensive network of proteins are required to work together to initiate G_2 arrest in response to DNA damage, a number of additional proteins have been reported to alter activation and maintenance of the G_2 checkpoint. One of the most interesting of these is Akt, a member of the phosphatidylinositol-3 kinase family that is recruited to the cell membrane and activated in response to the generation of phospholipids by a variety of signaling pathways. Activated Akt in turn signals to a variety of key downstream molecules including mammalian target of rapamycin, glycogen synthetase kinase 3, and S6 kinase, the sum of which is to suppress cell death and to promote cell survival (reviewed in ref. 18). In addition to effects on apoptosis and cell metabolism, Akt activation has recently been reported to suppress activation of the G_2 checkpoint in human colon carcinoma cells exposed to radiation (19). Whereas the mechanism by which Akt suppresses G_2 arrest has not been defined, the observation that Akt influences the G_2 checkpoint is of particular importance to the therapeutic application of cytotoxic methylating agents such as temozolomide whose cytotoxicity is influenced by the G_2 checkpoint and which are frequently used in the treatment of PTEN-deficient, Akt overexpressing gliomas (20, 21). Furthermore, because exposure of glioma cells to methylating agents induces a senescence-like phenomenon and mitotic catastrophe (6), and because bypass of methylating agent–induced G_2 arrest by Chk1 inhibitors enhances, rather than suppresses, glioma cell death (7), the consequences of potential Akt-mediated bypass of methylating agent–induced G_2 arrest on cellular outcome remain uncertain.

In the present study, we addressed the role of Akt in G_2 arrest and cytotoxicity in response to the chemotherapeutic methylating agent temozolomide. Results of the study suggest that Akt activation suppresses temozolomide-induced activation of the G_2 checkpoint by inhibiting the activation of the DNA damage signal transducer Chk2 and the downstream effectors of the G_2 checkpoint, whereas at the same time suppressing temozolomide-induced senescence and mitotic catastrophe in cells that avoid G_2 arrest. The Akt pathway may therefore represent a new target for the sensitization of Akt overexpressing gliomas to chemotherapeutic methylating agents such as temozolomide.

Experimental Procedures

Cell culture, retroviral infection, and drug treatment. Human U87MG glioblastoma cells and a pooled population of U87MG cells expressing human papillomavirus E6 (HPV E6, U87MG-E6) have been described elsewhere (6). Human astrocytes transformed by serial introduction of retroviral constructs encoding E6, E7, hTERT, V12 mutant H-Ras, and constitutively activated Akt (myrAkt δ4-129) have been described elsewhere (22). U87MG and U87MG-E6 human glioma cell lines were grown in DMEM plus 10% FCS, except before retroviral infection when they were shifted to phenol red–free DMEM containing 20 mmol/L HEPES and 10% charcoal dextran–treated, estrogen-free fetal bovine serum (Omega Scientific, Inc., Tarzana, CA).

The retroviral construct pWzl-hygro-M(+)AktER* was a generous gift from Dr. Martin McMahon (UCSF Comprehensive Cancer Center, San Francisco, CA). The construction of this vector has been described previously (23). Briefly, to generate a conditionally active form of Akt, the myristoylated Akt (M+)-encoding construct was fused to a mutant form of the murine estrogen receptor that is responsive to the synthetic steroid 4-hydroxytamoxifen. The fusion protein–encoding regions were subcloned into a retroviral vector pWzl-Hygro that carried a hygromycin resistance gene to create pWzl-hygro-M(+)Akt:ER*. To obtain retrovirus stocks, Phoenix A cells were transfected with pWZL-hygro-M(+)Akt:ER* by lipofection. Retroviral constructs were introduced into U87MG or U87MG-E6 cells with selection by hygromycin B. In all cases, cultures arose from polyclonal expansion of infected cells.

Temozolomide was supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute and was dissolved in dimethyl sufoxide (Sigma, St. Louis, MO). O^6-benzyguanine was obtained from Sigma. 4-Hydroxytamoxifen (Research Biochemicals International, Natick, MA) was prepared as a 1 mmol/L stock in ethanol and was stored at −20°C. Except where noted, unsynchronized U87 cells were preincubated with 4-hydroxytamoxifen (10 nmol/L, 24 hours) or vehicle, after which cells were additionally exposed to temozolomide (0 or 100 μmol/L, 3 hours). After temozolomide treatment, cells were gently washed and incubated in fresh medium containing 10 nmol/L 4-hydroxytamoxifen or vehicle at 37°C. 4-Hydroxytamoxifen was replenished every 4 days [the final dimethyl sulfoxide concentration did not exceed 0.1% (v/v)]. The cells were harvested at subconfluence at various time points. For studies with E7/E7/hTERT/Ras and E7/E7/hTERT/Ras + Akt cells, cells were incubated with O^6-benzylguanine (20 μmol/L) for 24 hours before exposure to temozolomide (0 or 100 μmol/L, 3 hours) and 5 μmol/L O^6-benzylguanine following temozolomide removal.

Protein extracts. For experiments using whole cell lysate samples, cells were washed with ice-cold PBS, scraped from the culture dish, and incubated in tissue lysis buffer containing 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.5% Igepal CA-630, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 10 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 100 mg/mL phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin (all reagents was purchased from Sigma) for 30 minutes on ice. For experiments of subcellular fractionation, cells were scraped and washed as above, incubated in hypotonic protein extract buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 10 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 100 mg/mL phenylmethylsulfonyl fluoride, and 10 mg/mL aprotinin] for 10 minutes on ice and lysed by addition of Igepal CA-630 (final concentration 0.4%) and vigorous vortexing for 10 seconds. The cell lysate was centrifuged at 12,000 × *g* for 5 minutes, and the supernatant was used as cytoplasmic protein extract. The pellet was sonicated and incubated in hypertonic protein extract puffer [10 mmol/L Tris (pH 7.4), 400 mmol/L NaCl, 0.5% Igepal CA-630, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 10 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 100 mg/mL phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin] for 30 minutes on ice, centrifuged at 12,000 × *g* for 5 minutes, and the supernatant was used as nuclear protein extract. Protein samples were stored at −80°C until use. The protein concentration of extracts was measured using Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA).

Immunoblot analyses. Forty micrograms of protein were subjected to SDS-PAGE and electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked in 5% nonfat skim milk (Bio-Rad Laboratories)/TBST [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% Tween 20] at 4°C overnight and probed with antibodies against Akt (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Akt (Ser^{473}, Cell Signaling Technology, Beverly, MA), phosphorylated FKHR (Ser^{256}, Cell Signaling Technology), phosphorylated GSK3β (Ser^9, Cell Signaling Technology), cyclin D1 (Cell Signaling Technology), phosphorylated Cdc2 (Tyr^{15}, Cell Signaling Technology), phosphorylated Cdc25C (Ser^{216}, Cell Signaling Technology), phosphorylated Chk1 (Ser^{345}, Cell Signaling Technology), phosphorylated Chk2 (Thr^{68}, Cell Signaling Technology), phosphorylated p38 (Thr^{180}/Tyr^{182}, Cell Signaling), and α-tubulin (Santa Cruz Biotechnology) for 16 hours at 4°C. Bound antibody was detected with horse radish peroxidase–conjugated secondary IgG (Santa Cruz Biotechnology) using

enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Immunofluorescence. U87MG cells containing a conditional Akt expression construct (5×10^3) were seeded onto 18-mm glass coverslips. One day later, the cells were pretreated with 4-hydroxytamoxifen for 24 hours and incubated with temozolomide (100 μmol/L, 3 hours) as described above. Three days after temozolomide treatment the cells were fixed and processed for immunofluorescent staining as described previously (24). Cells were incubated overnight at 4°C with phospho-Chk2 antibody (Thr^{68}, Cell Signaling Technology) at 1:200 dilution followed by Alexa 488–conjugated secondary antibody (Molecular Probes, Eugene, OR) for 30 minutes at room temperature. Cells were washed, counterstained with 4′,6′-diamidino-2-phenylindole (DAPI), and mounted with Prolong anti-fade reagent (Molecular Probes). Cells were viewed at Zeiss Axioplan fluorescent microscope using Axiovision Software. At least 200 nuclei were scored for phospho-Chk2 foci and percentages of cells displaying foci were calculated for each treatment.

Cell cycle studies. At various time points following temozolomide exposure, cells attached to culture dishes were trypsinized and collected together with the cells floating in the media. Cells were then washed in PBS, fixed in 70% (v/v) ethanol, and stored for up to 2 weeks at -20°C. The cells were washed once with PBS followed by incubation in PBS containing 40 μg/mL propidium iodine (Sigma) and 200 μg/mL RNase A (Sigma) for 1 hour at room temperature in the dark. Stained nuclei were then analyzed using a Becton Dickinson FACScan (San Jose, CA) with 20,000 events per determination. ModFit LT software (Verity Software House, Inc., Topsham, ME) was used to assess cell cycle distribution (6).

Chk2 inhibitory RNA (RNAi) studies. U87 MG cells (5×10^5 cells) were plated on 6-well culture plates 24 hours before transfection. The cells were transfected with an optimized amount (160 nmol/L) of inhibitory RNA (RNAi) targeting Chk2 (SMARTPool Chk2) or Cyclophylin B (siCONTROL Cyclophylin B), both purchased from Dharmacon (Lafayette, CO), using LipofectAMINE (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were washed, treated with temozolomide for 3 hours in complete medium free of RNAi, washed again, and placed in temozolomide-free medium until harvested for analysis of protein expression and cell cycle distribution at the times indicated.

Colony formation efficiency. Cells were plated in triplicate at a concentration of 50 cells per well into wells of 6-well culture plates and allowed to attach the plate by 24-hour incubation with or without 4-hydroxytamoxifen at 37°C. These cultures were then incubated with temozolomide as described above and allowed to form colonies. Seventeen days after temozolomide exposure, cells were stained with methylene blue (Sigma) and colonies of over 50 cells were counted.

Senescence-associated β-galactosidase staining. Senescence-associated β-galactosidase (SA-β-gal) staining was done as described by Dimiri et al. (25). Briefly, at various times after temozolomide exposure, control or temozolomide-treated cells were washed in PBS, fixed with 2% formamide/0.2% glutaraldehyde in PBS for 5 minutes at room temperature, and incubated overnight at 37°C in fresh 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution [1 mg/mL X-Gal, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L NaCl, and 2 mmol/L $MgCl_2$ in 40 mmol/L citric acid/sodium phosphate buffer (pH 6.0)]. The next day, cells were rinsed with PBS, and the percentages of SA-β-gal positive (blue) cells were determined after scoring 500 cells for each sample using a bright-field microscope.

4′,6-Diamidino-2-phenylindole staining. At various times after temozolomide exposure, control or temozolomide-treated cells were fixed with 4% paraformaldehyde/PBS for 1 hour at room temperature, rinsed with PBS, incubated with 0.5% Igepal CA-630/PBS for 1 hour at room temperature, and stained with DAPI. Nuclei were visualized by fluorescence microscopy, and >100 nuclei were examined for each experiment.

Results

Akt suppresses temozolomide-induced Chk2 activation and G_2 arrest. To begin to address the consequences of Akt activation on methylating agent–induced G_2 arrest, U87MG human glioma cells were infected with a retroviral construct encoding a modified Akt (AktERM+) protein which, by virtue of deletion of the parathyroid hormone domain, fusion to a c-Src myristoylation signal, and fusion to a modified form of the mouse ER hormone binding domain, has been shown rapidly activated in response to 4-hydroxytamoxifen (23, 26). Following selection of a pooled cell population with hygromycin, cells were incubated for 24 hours with various concentrations of 4-hydroxytamoxifen or vehicle, after which levels of endogenous Akt and exogenous AktER were examined by Western blot. As shown in Fig. 1, the U87MG cells containing the AktERM+ construct expressed both endogenous Akt (Fig. 1*A*) and phosphorylated endogenous Akt (Fig. 1*B*), levels of which were not altered by addition of 4-hydroxytamoxifen. Although AktER fusion protein could be detected in the absence of 4-hydroxytamoxifen (Fig. 1*A*), levels of AktER rose in response to exposures of 4-hydroxytamoxifen of >5 nmol/L. Whereas phosphorylated, activated AktER could not be detected in the U87MG cells containing the AktERM+ construct in the absence of 4-hydroxytamoxifen (Fig. 1*B*), levels were detectable following exposure of cells to 1 nmol/L 4-hydroxytamoxifen, and were 3- to 4-fold higher than levels of endogenous phospho-Akt following exposure to 10 nmol/L 4-hydroxytamoxifen.

Having created cells with inducible levels of activated Akt, we addressed the consequences of Akt pathway activation on temozolomide-induced G_2 arrest. U87MG cells containing the AktERM+ construct were preincubated with 0 or 10 nmol/L of 4-hydroxytamoxifen for 24 hours. Cells were then exposed to 100 μmol/L temozolomide for 3 hours, washed free of drug, and replated in drug-free or 4-hydroxytamoxifen–containing medium. In the absence of 4-hydroxytamoxifen, U87MG cells containing the AktERM+ construct had undetectable levels of exogenous, phosphorylated AktER both before and after exposure to temozolomide, whereas levels of phosphorylated endogenous Akt were detectable but decreased 2 and 3 days after temozolomide exposure (Fig 2*A*, *left*). U87MG cells not induced to overexpress AktER also exhibited modest phosphorylation of the Akt downstream targets FKHR and GSK3β, and detectable levels of cyclin D1, although consistent with temozolomide-induced reduction of phospho-Akt levels, significant decreases in the levels of phosphorylated FKHR and cyclin D1 and were noted 2 to 3 days

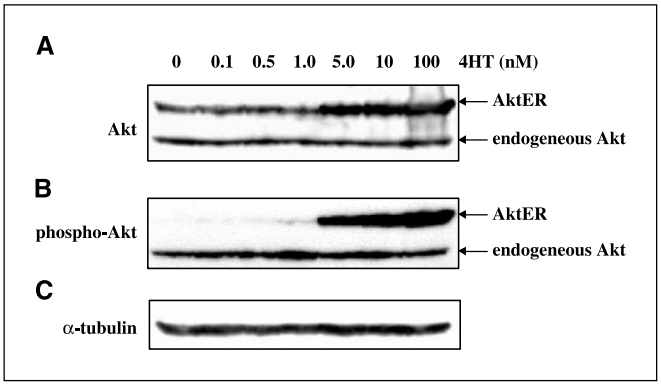

Figure 1. 4-Hydroxytamoxifen (*4HT*)–mediated induction of AktER in U87MG cells containing an AktERM+ encoding construct. U87MG cells containing an AktERM+ encoding construct were exposed to 0 to 100 nmol/L 4-hydroxytamoxifen for 24 hours, after which cellular proteins were subjected to Western blot analysis using antibodies selective for total Akt (*A*), phospho-Akt (Ser^{473}, *B*), or α-tubulin (panel *C*). Representative of three analyses.

following temozolomide exposure (Fig. 2*B* and *D*). The U87MG cells not exposed to 4-hydroxytamoxifen exhibited Chk1 phosphorylation (Ser^{345}) and Chk 2 phosphorylation (Thr^{68}) within 1 to 2 days of temozolomide exposure and also showed biphasic p38 phosphorylation (Thr^{180}/Tyr^{182}) in response to temozolomide, as previously reported (Fig. 2*E-G*; refs. 7, 17). These events occurred in the absence of changes in total Chk2 (Fig. 2*H*), Chk1, and p38 protein levels (data not shown) and were associated with Cdc25C (Ser^{216}) phosphorylation/inactivation (Fig. 2*I*), Cdc2 (Tyr^{15}) phosphorylation/inactivation (Fig. 2*J*), and G_2 arrest (here defined as a greater percentage of cells with 4n DNA content than 2n DNA content) 3 to 5 days following temozolomide exposure (Fig. 3*A*, 4-hydroxytamoxifen, 0 nmol/L). Whereas paired U87MG cells in which AktERM+ was activated by preincubation and post-incubation with 4-hydroxytamoxifen also exhibited temozolomide-induced suppression of endogenous phospho-Akt, these cells showed high levels of phosphorylated exogenous AktER and consequently also had persistently elevated levels of phosphorylated

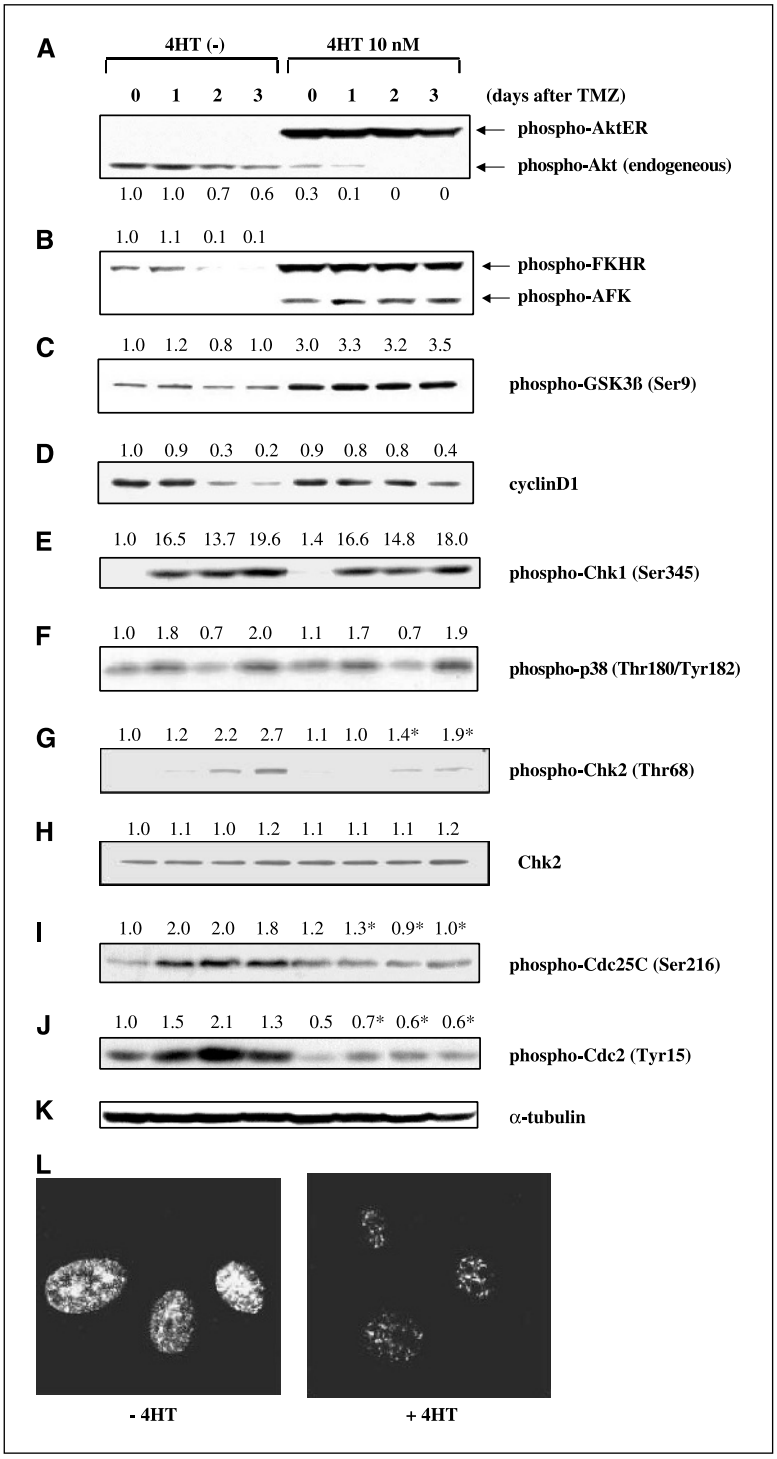

Figure 2. Effect of Akt overexpression on the temozolomide (*TMZ*)–induced phosphorylation/activation of key proteins in the G_2 checkpoint pathway. U87MG cells containing an AktERM+ encoding construct were exposed to 0 nmol/L (*left*) or 10 nmol/L (*right*) 4-hydroxytamoxifen (*4HT*) for 24 hours, after which the cells were exposed to 100 μmol/L temozolomide for 3 hours, washed free of drug, replated in drug-free or 4-hydroxytamoxifen–containing medium, and subjected to Western blot analysis using antibodies selective for phospho-Akt (Ser^{473}, *A*), phospho-FKHR (Ser^{256}), or phospho-AFK (*B*), phospho-GSK3β (Ser^{9}, *C*), cyclin D1 (*D*), phospho-Chk1 (Ser^{345}, *E*), *E*), phospho-p38 (Thr^{180}/Tyr^{182}, *F*), phospho-Chk2 (Thr^{68}, *G*), Chk2 (*H*), phospho-Cdc25C (Ser^{216}, *I*), phospho-Cdc2 (Tyr^{15}, *J*), or or α-tubulin (*K*). Fold induction was based on densitometric measurements and is below relevant immunoreactive bands. α-Tubulin levels were used as a standard for each sample and protein levels in untreated cells were defined as 1.0. *, $P < 0.05$, values differing statistically from controls (Student's *t* test). Similarly, treated cells were also subjected to immunofluorescence analysis of nuclear phospho-Chk2 foci (*L*). Representative of three analyses.

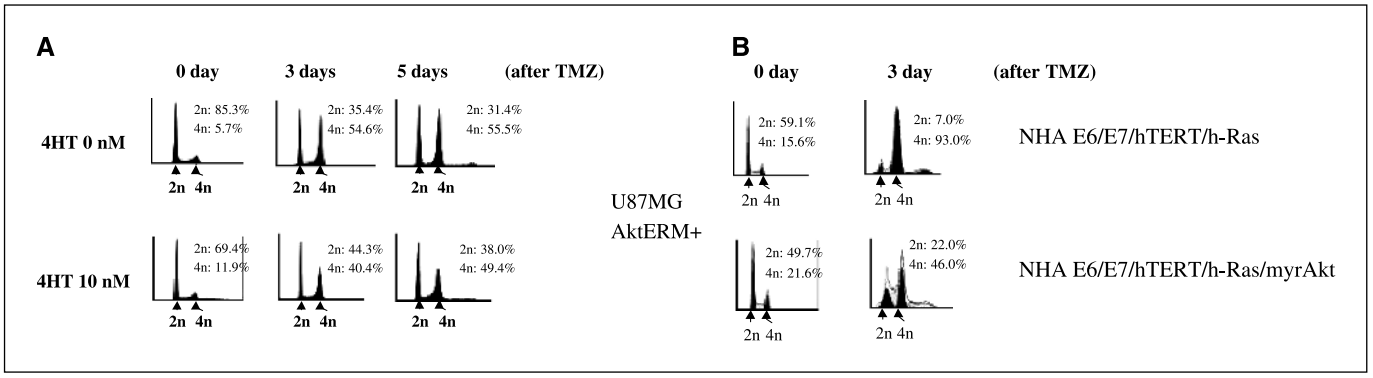

Figure 3. Flourescence-activated cell sorting–based cell cycle analysis of the effects of Akt overexpression on temozolomide (*TMZ*)–induced G2 arrest. *A,* U87MG cells containing an AktERM+ encoding construct were preincubated with 0 or 10 nmol/L 4-hydroxytamoxifen (*4HT*) for 24 hours, after which the cells were exposed to 100 μmol/L temozolomide for 3 hours, washed free of drug, replated in 4-hydroxytamoxifen–containing medium, and subjected to flourescence-activated cell sorting analysis at 0, 3, or 5 days after temozolomide exposure. Representative of three experiments. *B,* E6/E7/hTERT/mutant H-Ras–transformed human astrocytes and paired cells additionally expressing constitutively activated Akt were depleted of MGMT by preincubation with O^6-benzylguanine (20 μmol/L, 24 hours), after which the cells were exposed to 100 μmol/L temozolomide for 3 hours, washed free of drug, replated in O^6-benzylguanine–containing medium (5 μmol/L), and subjected to flourescence-activated cell sorting analysis at 0 or 3 days after temozolomide exposure. Representative of three experiments.

FKHR, AFK, and GSK3β (but not elevated levels of cyclin D1) relative to cells not overexpressing Akt (Fig. 2*A-D*, *right*). The U87MG cells induced to express the AktERM+ construct exhibited temozolomide-induced changes in cyclin D1 levels and phosphorylation/activation of Chk1 and p38, but these changes were no different in extent or timing from those noted in cells that did not overexpress Akt (Fig. 2*D-F*). U87MG cells expressing the AktERM+ construct, however, exhibited a statistically significant reduction in temozolomide-induced Chk2 phosphorylation (but not in total Chk2 levels) relative to that noted in cells not overexpressing Akt (Fig. 2*G* and *H*). Consistent with this observation, the nuclei of U87MG cells containing but not expressing the AktERM+ construct showed extensive clustering of phosphorylated Chk2 at sites of damaged DNA (40% of nuclei strongly positive for foci formation; Fig. 2*L*, *left*) 3 days after temozolomide exposure. In contrast, only 15% ($P < 0.05$) of the nuclei of temozolomide-treated cells induced to express the AktERM+ construct exhibited phospho-Chk2 foci formation, and in these cells the fluorescence intensity of the phospho-Chk2 foci was also weaker (Fig. 2*L*, *right*). Akt overexpression was also associated with statistically significant inhibition ($P < 0.05$) of temozolomide-induced phosphorylation/inactivation of Cdc25C, Cdc2 (Fig. 2*I* and *J*), and temozolomide-induced G_2 arrest (Fig. 3*A*, 4-hydroxytamoxifen 10 nmol/L). The suppression of G_2 arrest noted in temozolomide-treated cells overexpressing Akt, therefore was associated not with enhanced expression of cyclin D1 expression or with suppression of activation of Chk1 and p38, but rather with inhibition of Chk2 activation and recruitment to damaged DNA, and with loss of inactivation of the molecules critical for movement through the G_2 checkpoint and into mitosis.

To verify the results noted in control and Akt overexpressing U87 cells, a second paired cell line was examined for the effects of Akt overexpression on temozolomide-induced G_2 arrest. As shown in Fig. 3*B*, exposure of MGMT-depleted, PTEN-proficient human astrocytes transformed by expression of HPV E6, HPV E7, hTERT, and mutant H-Ras (22) to temozolomide (100 μmol/L, 3 hours) led to G_2 arrest 3 days after drug exposure. Temozolomide-treated MGMT-depleted paired transformed astrocytes expression a constitutively activated Akt, however, exhibited significantly less G_2 arrest at 2, 3, and 4 days following temozolomide exposure (3-day data shown in Fig. 3*B*). These results suggest that Akt-induced suppression of temozolomide-induced G_2 arrest occurs regardless of PTEN status and is not unique to U87 GBM cells.

Chk2 is critical for temozolomide-induced G_2 arrest. To directly assess whether Chk2 activation was critical for temozolomide-induced G_2 arrest, U87MG glioma cells were sham transfected or transfected with RNAi-targeting Chk2, after which the effects of reduced levels of Chk2 on temozolomide-induced G_2 arrest were assessed. As shown in the Western blot in Fig. 4*A*, transfection of cells with RNAi targeting Chk2 significantly ($P < 0.05$) reduced Chk2 protein levels without effecting levels of Chk1 or p38 (the data shown are derived from cells harvested 2 days after RNAi transfection). Exposure of cells to temozolomide and transfection reagent alone (LF only) or to temozolomide and RNAi directed at a non-Chk2 target (here Cyclophylin B) resulted in an accumulation of cells at the G_2-M boundary (and an increase in the ratio of cells with 4N G_2-M DNA content versus 2N G_1-S DNA content; Fig. 4*B*) no different than that noted in sham-transfected, temozolomide-treated cells (no LF, Fig. 4*B*). Transfection of temozolomide-treated cells with RNAi targeting Chk2, however, significantly suppressed the accumulation of temozolomide-treated cells at the G_2-M boundary and suppressed the temozolomide-induced increase in the ratio of cells with 4N DNA content versus 2N DNA content (Fig. 4*B*). These results that are comparable to those derived by pharmacologic inhibition of Chk1 or by RNAi-mediated inhibition of p38 α (7, 17) suggest that Chk2, along with Chk1 and p38 α, is a key initiator of temozolomide-induced G_2 arrest, and that Akt-mediated inhibition of Chk2 activation has the potential to block temozolomide-induced G_2 arrest.

Consequences of Akt-mediated suppression of temozolomide-induced G_2 arrest. Unlike cells with a high apoptotic potential, temozolomide-treated glioma cells lose clonagenicity in association with either the onset of a senescence-like phenomenon, or by death via mitotic catastrophe (6). p53-proficient glioma cells undergo a prolonged temozolomide-induced G_2 arrest and lose clonagenicity primarily by senescence (>60% of cells), whereas p53-deficient cells undergo a shorter temozolomide-induced G_2 arrest and lose clonagenicity in association with mitotic catastrophe (6). We previously showed that inhibition of temozolomide-induced G_2 arrest by Chk1/2 inhibitors forces cells into mitosis and enhances cell death by mitotic catastrophe in both p53-proficient

and p53-deficient glioma cells (7). We therefore questioned whether bypass of temozolomide-induced G_2 arrest by Akt, a protein known to suppress apoptosis in response to radiation-induced DNA damage, would sensitize cells to temozolomide or instead protect cells by suppressing pathways linked to senescence and/or mitotic catastrophe. As shown in Fig 5*A*, exposure of U87MG cells containing a 4-hydroxytamoxifen–inducible AktERM+ construct to suboptimal concentrations of 4-hydroxytamoxifen (0.1 and 1 nmol/L) did not affect temozolomide cytotoxicity. 4-Hydroxytamoxifen exposures that effectively induced AktERM+ activation (10 and 100 nmol/L), however, protected rather than sensitized the p53-proficient U87MG cells to temozolomide-induced cytotoxicity. The cytoprotective effects of Akt overexpression were associated with a reduction in the percentage of cells expressing senescence-associated β-galactosidase activity at 10 days following temozolomide exposure (Fig. 5*B*), suggesting that Akt overexpression not only reduced the percentage of cells undergoing temozolomide-induced G_2 arrest but also reduced the ability of p53-positive glioma cells to undergo a senescence-like phenomenon in response to temozolomide.

To examine the effects of Akt expression on temozolomide-induced mitotic catastrophe, U87MG glioma cells retrovirally infected with constructs encoding HPV E6 and 4-hydroxytamoxifen–inducible AktERM+ were used. Previous studies showed that fewer than 10% of the U87-E6 cells exhibit senescence-associated β-galactosidase expression 10 days following temozolomide exposure, whereas ~30% of the cells displayed characteristics (fragmented or lobulated nuclei, micronuclei) of mitotic catastrophe (7). 4-Hydroxytamoxifen–mediated AktERM+ overexpression in U87-E6 cells suppressed not only temozolomide-induced G_2 arrest (Fig. 6) and loss of clonagenicity (Fig. 5*A*) but also the percentage of cells undergoing death by mitotic catastrophe at both 6 and 10 days following temozolomide exposure (Table 1). These results suggest that Akt overexpression, whereas suppressing temozolomide-induced G_2 arrest, also protects cells from loss of clonagenicity caused by induction of senescence and mitotic catastrophe.

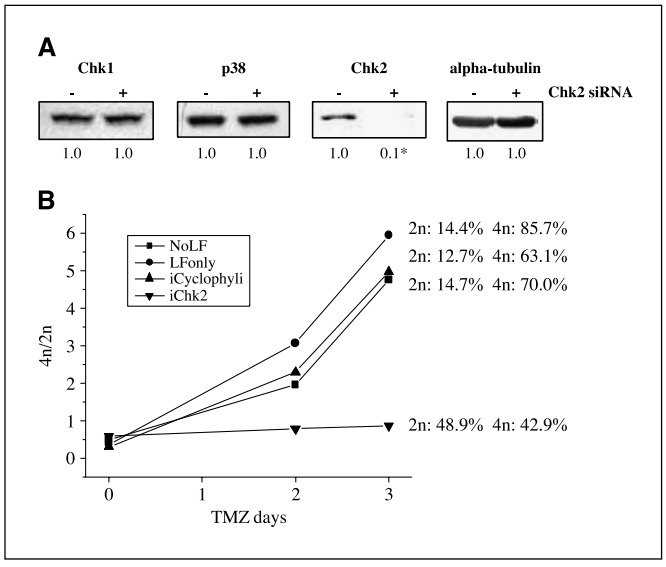

Figure 4. Analysis of the requirement for Chk2 in activation of the G_2 checkpoint in response to temozolomide (*TMZ*). U87MG cells were incubated with vehicle (no LF), transfection reagent alone (LF only), or with transfection reagent plus siRNA targeting Chk2 or the unrelated target Cyclophylin B. Twenty-four hours after transfection, the cells were washed, exposed to 100 μmol/L temozolomide for 3 hours, washed free of drug, incubated in medium free of siRNA and temozolomide, and harvested for analysis of Chk1, p38, Chk2, and α-tubulin expression (*A*, 2 days after temozolomide exposure), and for extent of G_2 arrest, expressed in (*B*) as the ratio of percentage of cells at the G_2-M boundary versus the percentage of cells at the G_1-S boundary 0 to 3 days after temozolomide exposure. Representative of three experiments. *, $P < 0.05$, values differing statistically from controls (Student's *t* test). The values next to each curve in (*B*) are the percentages of cells with 2N or 4N DNA content at 3 days following temozolomide exposure for each group.

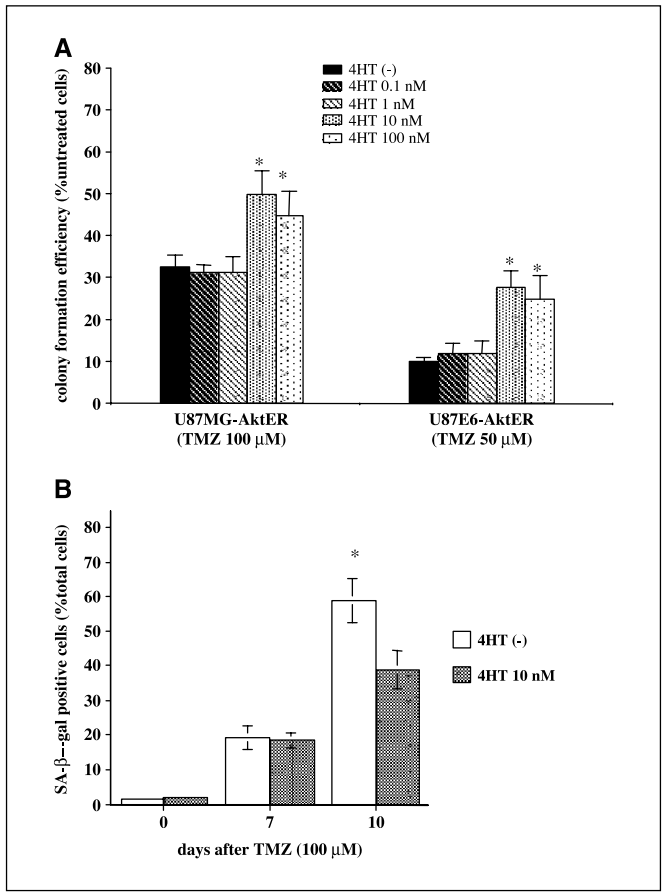

Figure 5. Reduction of temozolomide (*TMZ*)–induced cytotoxicity and senescence-associated β-galactosidase activity by Akt overexpression in p53-proficient and p53-deficient glioma cells. *A,* U87MG p53-proficient cells (*left*) and U87E6 p53-deficient cells (*right*) were exposed to 0 to 100 nmol/L 4-hydroxytamoxifen (*4HT*) for 24 hours, after which the cells were exposed to 100 μmol/L temozolomide for 3 hours, washed free of drug, and replated in 4-hydroxytamoxifen–containing medium. Colonies of >50 cells were counted 18 days after the initial temozolomide exposure. *B,* U87MG p53-proficient cells were exposed to 0 or 10 nmol/L 4-hydroxytamoxifen for 24 hours, after which the cells were exposed to 100 μmol/L temozolomide for 3 hours, washed free of drug, and replated in 4-hydroxytamoxifen–containing medium, and stained for β-galactosidase activity 7 and 10 days after temozolomide exposure. *Columns,* mean from four experiments; *bars,* ±SD. *, $P < 0.05$, values differing statistically from controls (Student's *t* test).

Discussion

Cytotoxic DNA methylating agents such as temozolomide play a key role in the therapeutic management of a variety of malignancies including lymphoma and glioma. Whereas the cytotoxic action of temozolomide and other methylating agents is dependent on mismatch repair–induced DNA damage, the response of cells to this damage can be modulated by the G_2 checkpoint, activation of which can protect cells from methylating agent–induced mitotic

catastrophe and cell death (7, 17). Because Akt overexpression influences cell cycle progression and because gliomas frequently have high levels of Akt activation, we examined the role Akt may play in the G_2 arrest and toxicity induced by the clinically used chemotherapeutic methylating agent temozolomide. The results presented here show that Akt activation suppresses the G_2 checkpoint by selectively altering activation of the DNA damage signal transducer Chk2 and the downstream effectors of the G_2 checkpoint. The overriding effect of Akt activation, however, is suppression of temozolomide-induced senescence and mitotic catastrophe in cells that avoid G_2 arrest. The Akt pathway may therefore contribute to temozolomide resistance in the clinical setting.

The present data clearly suggest that the Akt pathway plays a role in suppressing the G_2 checkpoint in glioma cells following temozolomide exposure. In U87MG cells expressing inactive Akt, temozolomide treatment induced G_2 arrest, whereas at the same time, suppressing levels of endogenous phospho-Akt. These observations suggest that activation of the G_2 checkpoint may be linked to inactivation of Akt, a suggestion supported by the observation that 4-hydroxytamoxifen–induced elevation of phospho-Akt levels suppressed temozolomide-induced G_2 arrest. These observations are consistent with the finding that Akt overexpression also blocks G_2 arrest induced by ionizing radiation (19), an agent that activates G_2 arrest by a pathway considerably different from that used by temozolomide. The ability of Akt to suppress G_2 arrest induced by these disparate agents, as well as by phosphatidylinositol-3-kinase inhibition (27), suggests that Akt may have a broad ability to suppress G_2 arrest regardless of the pathway by which the process is initiated.

Whereas Akt seems to have a broad ability to suppress activation of the G_2 checkpoint, only partial information exists in the literature as to how this might occur. Cyclin D1 plays a key role in cell cycle progression and it is conceivable that Akt, by blocking GSK3β activation and subsequent cyclin D1 proteolysis, could increase cyclin D1 levels and suppress temozolomide-induced G_2 arrest. Whereas inhibitory phosphorylation of GSK3β was noted in the Akt-overexpressing cells examined in the present study, corresponding changes in cyclin D1 levels were not seen, suggesting that effects of Akt on temozolomide-induced G_2 arrest did not involve GSK3β/cyclin D1. Alternatively, because overexpression of constitutively activated Akt in rodent cells reverses 6-thioguanine–mediated suppression of Cdc2 activity, Akt seems to influence the G_2 checkpoint pathway at, or upstream of, the Cdc2-cyclin B complex (19). Overexpression of constitutively activated Akt, however, has also been shown to suppress hydroxyurea-mediated activation of Chk1, suggesting that the effects of Akt on the G_2 arrest pathway occur not only upstream of Cdc2 but also upstream of Chk1 (27). The data derived from the present study of methylating agent–induced G_2 arrest clearly show that Akt overexpression blocks temozolomide-induced Tyr^{15} phosphorylation of Cdc2 as well as the upstream Ser^{216} phosphorylation of Cdc25C. Cdc25C is phosphorylated by Chk1, Chk2, and p38, all of which are activated by phosphorylation in response to temozolomide-induced DNA damage (7, 17). Our previous studies showed that Chk1 and p38 were required for temozolomide-induced activation of the G_2 checkpoint and that RNAi-mediated suppression of either blocked activation of the G_2 checkpoint in response to temozolomide. Data from the present study show that in addition to Chk1 and p38, Chk2 also plays a key role in activating G_2 arrest in response to temozolomide. If Chk1, Chk2, and p38 act coordinately or synergistically to bring about Cdc25C phosphorylation as has been suggested (28), Akt-mediated selective inhibition of any of the sensors could block Cdc25C phosphorylation and temozolomide-induced G_2 arrest to the extent noted in the present study. Akt-mediated phosphorylation of Ser^{280} has been suggested to suppress Chk1 activity (27) although in the present study, Akt did not influence temozolomide-induced Ser^{280} Chk1 phosphorylation (data not shown). It also remains possible that Akt-mediated modification of Chk1 or p38 at sites not examined in the present study suppresses activity to the extent that even in their Ser^{345}/Thr^{180}/Tyr^{182} phosphorylated forms, the kinases are inactive. The data from the present study, however, suggest that Chk2 may to be of particular importance in the ability of Akt to bypass temozolomide-induced G_2 arrest because only Chk2 phosphorylation and function were inhibited by the Akt overexpression that also led to suppression of temozolomide-induced G_2 arrest. The exact connection between Akt activation and inhibition of Chk2 phosphorylation remains unclear. Histone γ-H2AX, 53BP1, and Chk2, however, all assemble in nuclear foci within minutes of creation of double strand breaks by ionizing radiation, and it has been recently shown that nuclear factor with BRCT domains protein 1 (NFBD1) and 53BP1 both regulate ionizing radiation-induced Chk2 phosphorylation/activation via ataxia telangiectasia mutated (29). In this model, NFBD1 and 53BP1 serve as adaptor molecules, which in nuclear foci bring upstream signal molecules such as γ-H2AX in close physical proximity with Chk2. Although the assembly of nuclear foci in response to methylating

Table 1. Effect of Akt activation on mitotic catastrophe induced by temozolomide

		6 d	10 d
U87MG-E6-AktER	4HT (−)	17.1 ± 2.9%	30.1 ± 4.0%
	4HT 10 nmol/L	9.3 ± 3.2%	16.8 ± 3.8%

Abbreviation: 4HT, 4-hydroxytamoxifen.

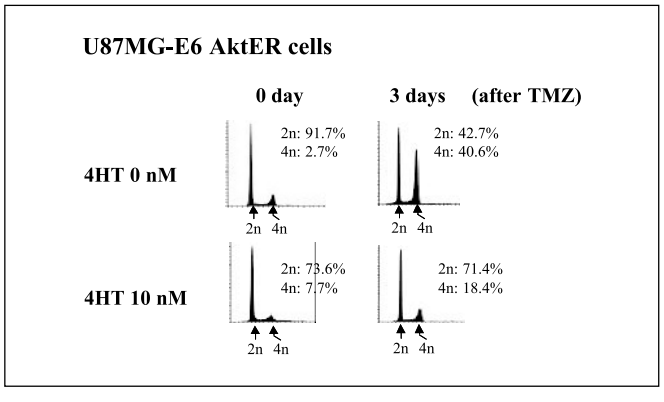

Figure 6. Flourescence-activated cell sorting–based cell cycle analysis of the effects of Akt overexpression on temozolomide (*TMZ*)–induced G_2 arrest. p53-deficient U87MG-E6 cells containing an AktERM+ encoding construct were preincubated with 0 or 10 nmol/L 4-hydroxytamoxifen (*4HT*) for 24 hours, after which the cells were exposed to 100 μmol/L temozolomide for 3 hours, washed free of drug, replated in 4-hydroxytamoxifen–containing medium, and subjected to flourescence-activated cell sorting analysis at 0 or 3 days after temozolomide exposure. Representative of three experiments.

agent–induced DNA damage has not been well examined, it may be possible that Akt overexpression alters the formation of such damage responsive foci and thus blocks Chk2 phosphorylation and ultimately initiation of temozolomide-induced G_2 arrest.

If Akt served solely as an inhibitor of Chk2 activation, it would be expected that Akt activation would lead to bypass of temozolomide-induced G_2 arrest and increased cytotoxicity as noted following exposure of temozolomide-treated GBM cells to the Chk1/2 inhibitor UCN-01. The results of the present study, however, show that Akt overexpression not only bypasses temozolomide-induced G_2 arrest, but also protects cells from temozolomide-induced senescence and mitotic catastrophe. Although the ability of Akt to inactivate Bad and caspase-9 and to suppress apoptosis induced by ionizing radiation and other DNA damaging agents, the means by which Akt protect GBM cells from temozolomide-induced senescence and mitotic catastrophe are likely very different from those which protect from apoptosis. With regard to senescence, elimination of tuberous sclerosis complex-1, a downstream target of Akt, has been shown to induce premature senescence (30). Similarly, several studies have shown that senescent cells exhibit suppressed Akt activity (31, 32). Akt activation has also been shown to increase the proliferation of aged fibroblasts to platelet-derived growth factor (31), suggesting that in the simplest sense Akt may suppress senescence by increasing the sensitivity of cells to proliferative signals. With regard to suppression of mitotic catastrophe, a variety of proteins contribute to stabilization of DNA during mitosis as well as to orderly mitotic progression. These proteins include forkhead transcription factors and polo-like kinases, the activities of which are intimately linked to Akt (33, 34). Akt may therefore contribute to the activity of these proteins in a way that favors the integrity of the mitotic process. The ability of Akt overexpression to suppress temozolomide-induced mitotic catastrophe as well as temozolomide-induced senescence suggests that Akt has broader cytoprotective functions than previously reported.

In addition to identifying the mechanism by which Akt alters G_2 checkpoint activation and subsequent connections to cell death pathways, the present studies also may have clinical significance. Methylating agents such as temozolomide are important compounds in the treatment of human gliomas, although only a small percentage of gliomas respond to temozolomide-based therapies. It is interesting to note that most high-grade human gliomas have high levels of Akt activation, which are believed to be a consequence of PTEN deletion. Given the role Akt plays in moving cells through the G_2 checkpoint and in suppressing temozolomide-induced cytotoxicity, it seems likely that an analysis of Akt pathway activation in gliomas before therapy may help identify those individuals for whom temozolomide-based therapies are most likely to succeed. Similarly, strategies combining temozolomide with inhibitors of the Akt pathway may enhance the likelihood of success. Because Akt overexpression has also been reported to increase the mutagenicity of agents that induce G_2 arrest (19), presumably by promoting cell survival in the absence of genuine DNA repair, strategies designed to suppress Akt may contribute not only to improved tumor cell kill but also to suppression of unwanted mutagenic effects which might otherwise contribute to secondary malignancies. It is worth noting, however, that the cells used in this study contained constitutively or conditionally activated Akt which is not subjected to the same regulation (including perhaps temozolomide-induced regulation) as noted with endogenous Akt. It will therefore be important to test the ideas derived from the present study in clinical material. Such studies may allow an even better understanding of he connections between Akt activation, G_2 arrest, and cell survival and may allow for the development of better therapeutic strategies for treating what are currently nearly incurable malignancies.

Acknowledgments

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