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AKT Is Activated in an Ataxia-Telangiectasia and Rad3-Related-Dependent Manner in Response to Temozolomide and Confers Protection against Drug-Induced Cell Growth Inhibition

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ABSTRACT

The phosphatidylinositol 3-kinase/AKT pathway is activated frequently in human cancer, and it has been implicated in tumor cell proliferation, survival, and chemoresistance. In this study, we addressed the role of AKT in cellular responses to the therapeutic methylating agent temozolomide (TMZ), and we investigated the possible link between TMZ-induced modulation of AKT function and activation of ataxia-telangiectasia and Rad3-related (ATR)- and ataxia telangiectasia mutated (ATM)-dependent signaling pathways. We found that clinically relevant concentrations of TMZ caused activation of endogenous AKT in lymphoblastoid cells, and in colon and breast cancer cells, and that this molecular event required a functional mismatch repair system. Transfection of a dominant-negative kinase-dead form of AKT1 into breast cancer cells abrogated TMZ-induced activation of endogenous AKT, and it markedly enhanced cell sensitivity to the drug. Likewise, exposure of the MMR-proficient cell lines to the AKT inhibitor D-3-deoxy-2-*O*-methyl-*myo* inositol 1-[(*R*)-2-methoxy-3-(octadecyloxy)-propyl hydrogen phosphate] (SH-5) impaired AKT phosphorylation in response to TMZ, and it significantly increased cell chemosensitivity. Furthermore, small interfering RNA (siRNA)-mediated reduction of AKT1 expression in colon cancer cells potentiated the growth inhibitory effects of TMZ. Inhibition of ATM expression in colon cancer cells by siRNA did not impair TMZ-induced activation of AKT, whereas siRNA-mediated inhibition of ATR prevented AKT activation in response to the drug and increased cell chemosensitivity. These results strongly support the hypothesis that clinical benefit could be obtained by combining TMZ with inhibitors of the AKT pathway. Moreover, they provide the first evidence of a novel function of ATR as an upstream activator of AKT in response to DNA damage induced by O^6-guanine-methylating agents.

The serine/threonine protein kinase AKT is a critical regulator of major cellular processes, including gene expression, glycogen metabolism, migration, proliferation, and survival (for review, see Bellacosa et al., 2005). Three isoforms of AKT, encoded by three different genes, are present in mammalian cells. Each isoform comprises an NH_2-terminal pleckstrin homology domain, a catalytic protein kinase domain, and a short COOH-terminal region. All AKT isoforms contain two main regulatory phosphorylation sites, one within the kinase domain (Thr308/AKT1, Thr309/AKT2, Thr305/AKT3), and one in the COOH-terminal region (Ser473/AKT1, Ser474/AKT2, Ser472/AKT3).

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ABBREVIATIONS: PI3K, phosphatidylinositol 3-kinase; PDK, 3-phosphoinositide-dependent protein kinase; IKK, IκB kinase; GSK, glycogen synthase kinase; TMZ, 8-carbamoyl-3-methyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (temozolomide); O^6-G, O^6-guanine; MGMT, O^6-methylguanine-DNA methyltransferase; BG, O^6-benzylguanine; MMR, mismatch repair; ATR, ataxia-telangiectasia and Rad3-related; ATM, ataxia telangiectasia mutated; SH-5, D-3-deoxy-2-*O*-methyl-*myo* inositol 1-[(*R*)-2-methoxy-3-(octadecyloxy) propyl hydrogen phosphate]; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence; SA-β-gal, senescence-associated β-galactosidase; siRNA, small interfering RNA; siATR, small-interfering RNA directed against ATR; siCONTROL, nonsilencing control small-interfering RNA; siATM, small-interfering RNA directed against ATM; siAKT1, small-interfering RNA directed against AKT1; scrATM, scramble small-interfering RNA negative control for ATM; scrAKT1, scramble small-interfering RNA negative control for AKT1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; DSB, double-strand break; mAb, monoclonal antibody; p-, phospho-.

AKT is a major downstream target of growth factor receptor tyrosine kinases that signal through phosphatidylinositol 3-kinase (PI3K). Upon PI3K activation, the generation of phosphatidylinositol 3,4,5-triphosphate recruits AKT at the plasma membrane, where the kinase is activated through phosphorylation on the regulatory threonine residue by phosphoinositide-dependent protein kinase (PDK)-1 and on the regulatory serine residue by a kinase termed PDK-2. So far, at least 10 kinases have been shown to function as a PDK-2, including integrin-linked kinase, double-stranded DNA-dependent protein kinase, protein kinase Cα, and the mammalian target of rapamycin (for review, see Dong and Liu, 2005).

Once active, AKT controls cellular functions through phosphorylation of a variety of substrates that include, among others, BAD, caspase-9, the Forkhead family of transcription factors, IκB kinase (IKK), glycogen synthase kinase (GSK)-3β, $p21^{Waf1}$, and $p27^{Kip1}$(Bellacosa et al., 2005). Inappropriate activation of the PI3K/AKT pathway has been associated with the development of diseases such as diabetes, autoimmunity, and cancer (Bellacosa et al., 2005).

In addition to activation by receptor tyrosine kinases, AKT can also be activated by many forms of cellular stress, including exposure to a number of chemotherapeutic agents (for review, see West et al., 2002). Activation of AKT in response to cellular stress may be considered a compensatory protective mechanism activated by the cell to escape death. The activity of PI3K/AKT pathway has consistently been linked to tumor cell resistance to antineoplastic agents (West et al., 2002; Bellacosa et al., 2005).

Temozolomide (TMZ) is a methylating agent (Newlands et al., 1997) recently approved for the treatment of recurrent high-grade gliomas and in phase II/III clinical trials for the treatment of melanoma and other solid neoplasias (Payne et al., 2005).

The cytotoxicity of TMZ is due primarily to the methylation of the O^6 position of guanine (O^6-G) in DNA. This is supported by the inverse correlation existing between sensitivity of tumor cells to TMZ and the activity of O^6-methylguanine-DNA methyltransferase (MGMT; EC 2.1.1.63), which removes small alkyl groups from O^6-G in DNA (for reviews, see Gerson, 2002; Margison et al., 2003; Rabik et al., 2006; Sabharwal and Middleton, 2006). Depletion of MGMT activity by the competitive inhibitors O^6-benzylguanine (BG) and O^6-(4-bromothenyl)guanine consistently increases tumor cell sensitivity to TMZ both in vitro and in vivo (Gerson, 2002; Margison et al., 2003; Rabik et al., 2006; Sabharwal and Middleton, 2006).

The cytotoxic effects of unrepaired O^6-methylguanine (O^6-MeG) rely on the formation of O^6-MeG:T mispairs in the course of DNA duplication and the subsequent engagement of the mismatch repair (MMR) system (for review, see Jiricny, 2006). According to the "futile repair" model (Bignami et al., 2000), DNA damage produced by the unsuccessful processing of O^6-MeG:T mispairs by the MMR system activates a signaling cascade, resulting in cell cycle arrest at the G_2 phase of the second cell doubling event, which is followed by either apoptosis, mitotic catastrophe, or a senescence-like state (Kaina et al., 1997; D'Atri et al., 1998; Hirose et al., 2003; Stojic et al., 2004b). According to the "signaling" model (Fishel, 1999), after the recognition of O^6-MeG:T mispairs, the MMR system transmits the damage signal directly to the checkpoint machinery, without the need for DNA processing. Cells with a defective MMR are highly resistant to TMZ and other O^6-G-methylating agents regardless of their MGMT activity, and MGMT inhibitors fail to increase drug sensitivity in these cells.

In the past few years, the early molecular events occurring in cells exposed to O^6-G-methylating agents and their dependence on an intact MMR system have begun to be clarified (for reviews, see Stojic et al., 2004a; O'Brien and Brown, 2006; Roos and Kaina, 2006; Wang and Edelmann, 2006). In particular, it has been shown that in MMR-proficient cells exposed to clinically achievable concentrations of TMZ, cell cycle arrest is linked to the activation of signaling pathways controlled by p38 mitogen-activated protein kinase and ataxia-telengiectasia mutated and Rad3-related (ATR) kinase (Hirose et al., 2003; Caporali et al., 2004). Ataxia-telengiectasia mutated (ATM) is also activated in TMZ-treated cells, and it confers protection against drug-induced cytotoxicity (Caporali et al., 2004). However, ATM was found to be dispensable for cell cycle arrest and for Chk1 and Chk2 phosphorylation that have been observed upon exposure to the drug (Caporali et al., 2004). In addition, Hirose et al. (2005) showed that glioblastoma cells induced to overexpress activated AKT exhibit reduced Chk2 phosphorylation and G_2 arrest upon treatment with TMZ, and they are protected against drug-induced cytotoxicity. Therefore, the AKT pathway seems to be a promising target to increase the efficacy of TMZ.

In the present study, we investigated whether endogenous AKT is activated in TMZ-treated cells and whether inhibition of AKT function is associated with increased cell sensitivity to the drug. Moreover, we investigated whether ATM and ATR can have a role in TMZ-induced activation of AKT.

Materials and Methods

Cell Lines. The MMR-proficient human breast carcinoma cell line MCF-7, the MCF-7/B1 clone derived from MCF-7 cells by limiting dilution, the MMR-proficient human B lymphoblastoid cell line TK6 and its MMR-deficient subline MT1 were maintained in RPMI 1640 medium (HyClone Europe, Cramlington, UK) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and 50 μg/ml gentamicin (Invitrogen, Paisley, UK). The MMR-deficient human colorectal cancer cell line HCT116 and its MMR-proficient subline HCT116/3-6 were maintained in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum, L-glutamine, gentamicin, and, in the case of HCT116/3-6 cells, 400 μg/ml G418 (Sigma-Aldrich). The MT1 cell line harbors a different missense mutation in both alleles of the *hMSH6* locus (Papadopoulos et al., 1995). HCT116 cells have a hemizygous nonsense mutation in the *hMLH1* gene located on chromosome 3 (Papadopoulos et al., 1994). The HCT116/3-6 clone was created by microcell chromosome transfer of a single normal human chromosome 3 into HCT116 cells (Koi et al., 1994). TK6 and MT1 cells are MGMT-deficient; thus, they fail to remove methyl adducts from O^6-G (Goldmacher et al., 1986). MCF-7, HCT116, and HCT116/3-6 cells are MGMT-proficient (Vernole et al., 2003; Clemons et al., 2005).

Drugs and Reagents. TMZ was kindly provided by Schering Plough (Kenilworth, NJ), BG and insulin were purchased from Sigma-Aldrich, and the AKT inhibitor SH-5 (Kozikowski et al., 2003) was obtained from Alexis Biochemicals (San Diego, CA). TMZ was always prepared fresh in RPMI 1640 medium, because the drug readily decomposes in aqueous solution. BG was dissolved in ethanol (2.4 mg/ml), stored as stock solution at −80°C, and diluted in culture medium just before use. The final concentrations of ethanol did not

affect cell growth (data not shown). SH-5 was prepared in RPMI 1640 medium, and it was stored as stock solution (5 μM) at 4°C.

Stable Transfection. The pUSEamp(+) expression vector encoding for a dominant-negative kinase-dead form of AKT1 (mutation K179M) (Franke et al., 1995) and the empty pUSEamp(+) vector were purchased from Upstate (Lake Placid, NY). The expression vector contains a Myc-His tag at the 3′ end of the AKT1 open reading frame.

The MCF-7/B1 clone was transfected with the expression construct, or the empty vector, by using the FuGENE6 transfection reagent (Roche Diagnostic, Mannheim, Germany), according to the manufacturer's protocol. Stably transfected subclones were selected in the presence of 400 μg/ml G418 (Sigma-Aldrich). Isolated subclones were expanded, and the expression of AKT1(K179M) and endogenous AKT was confirmed by Western blot analysis using 2 μg/ml anti-Myc tag (Upstate) and anti-AKT (1:1000 dilution; Cell Signaling Technology Inc., Danvers, MA)-specific antibodies. The pUSE2 subclone, transfected with the empty vector, and the KD12 subclone, expressing AKT1(K179M), were selected for the subsequent studies. The subclones were maintained in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 400 μg/ml G418.

Cell Treatment for Western Blot Analysis or in Vitro AKT Kinase Assay. TK6 and MT1 cells were suspended (1×10^5 cells/ml) in culture medium or culture medium containing 12.5 μM TMZ, and cultured in flasks (Falcon; BD Discovery Labware, Bedford, MA) at 37°C in a 5% CO_2 atmosphere. At the desired time points, the cells were collected, washed in phosphate-buffered saline (PBS), and processed for Western blot analysis or in vitro AKT kinase assay.

The MGMT-proficient cell lines HCT116, HC116/3-6, pUSE2, and KD12 were suspended in culture medium, seeded on 15-cm dishes (Falcon; BD Discovery Labware), and allowed to adhere at 37°C for 18 h. BG (10 μM) and TMZ (50 μM) were then added to the dishes, and the cultures were maintained at 37°C in a 5% CO_2 atmosphere. The MGMT inhibitor was added to the dishes 2 h before the addition of TMZ, and control groups were treated with BG alone. At the desired time points, the cells were harvested and processed for Western blot analysis or in vitro AKT kinase assay.

For treatment with insulin, pUSE2 and KD12 cells were cultured in RPMI 1640 medium, supplemented with 0.5% fetal calf serum, 2 mM L-glutamine, and 400 μg/ml G418 for 24 h. Insulin (10 μg/ml) was then added to the cultures, and after a 30-min incubation, the cells were recovered, washed in PBS, and processed for Western blot analysis.

For treatment with SH-5, TK6, pUSE2, and HCT116/3-6 cells were seeded in flasks or dishes as described above. The AKT inhibitor (1 μM for TK6 cells and 5 μM for pUSE2 and HCT116/3-6 cells) was added to the cultures, followed by TMZ 6 h later. Cells of pUSE2 and HCT116/3-6 lines were also treated with 10 μM BG that was added to the cultures 4 h after SH-5. Seventy-two hours after TMZ exposure, the cells were recovered, washed in PBS, and processed for Western blot analysis.

Evaluation of Cell Sensitivity to TMZ. Adherent cells (pUSE2, KD12, HCT116, and HCT116/3-6 cell lines) were seeded (3.3×10^3 cells/well for pUSE2 and KD12 cell lines and 2×10^3cells/well for HCT116 and HCT116/3-6 cell lines) in 24-well plates (Falcon; BD Discovery Labware), and then they were allowed to adhere at 37°C in a 5% CO_2 atmosphere for 18 h. BG (10 μM) and TMZ (at concentrations ranging from 6.25 to 50 μM) were then added to the cultures. Six days after the addition of TMZ, the cells were harvested by trypsinization and resuspended in culture medium to perform viable cell count. Nonadherent TK6 and MT1 cells were suspended in culture medium (1×10^5 cells/ml), seeded in 24-well plates (Falcon; BD Discovery Labware), and incubated with TMZ (at concentrations ranging from 1.56 to 12.5 μM) at 37°C in a 5% CO_2 atmosphere. Viable cell count was performed after 72 h of culture. In all cases, three replica wells were used for controls and drug-treated groups. Cells were manually counted using a hemocytometer, and cell viability was determined by trypan blue dye exclusion test.

To evaluate the effect of the AKT inhibitor SH-5 on cell sensitivity to TMZ, the cells were exposed to SH-5 (1 μM for TK6 and MT1 cells and 5 μM for pUSE2, HCT116 and HCT116/3-6 cells) for 6 h, before TMZ addition to the cultures. The adherent cells were also treated with 10 μM BG that was added to the cultures 4 h after SH-5.

Senescence-Associated β-Galactosidase Staining. pUSE2, KD12, and HCT116/3-6 cells were plated as describe for cell growth evaluation. SH-5 (5 μM), BG (10 μM, 4 h after SH-5), and TMZ (50 μM, 2 h after BG) were then added to the cultures, and the plates were incubated at 37°C in a 5% CO_2 atmosphere for 6 days. At the end of the incubation period, the cells were fixed and stained using the Senescence β-Galactosidase Staining kit from Cell Signaling Technology Inc., according to the manufacturer's protocol. Senescence-associated β-galactosidase (SA-β-gal)-positive cells were scored by counting three different fields per samples under a bright-field microscopy

RNA Interference. The pool of small-interfering RNAs (siRNAs) directed against ATR (ATR Smart Pool siRNA) (siATR) and the pool of nonsilencing control siRNAs (siCONTROL Nontargeting siRNA Pool) (siCONTROL) were obtained from Dharmacon RNA Technologies (Lafayette, CO). The siRNA directed against ATM (AAUGUCUUUGAGUAGUAUG) (siATM), the siRNA directed against AKT1 (CUCACAGCCCUGAAGUACU) (siAKT1), and the corresponding scramble siRNAs GUUGCUUUACUUCGUAUAU (scrATM) and GAUCCUAUAUUCGGUUAGU (scrAKT1) used as controls were purchased from Sigma-Proligo (The Woodlands, TX).

HCT116/3-6 cells were seeded on 6-cm dishes (Falcon; BD Discovery Labware), and then they were allowed to adhere at 37°C for 18 h. The cells were then transfected with siATR, siCONTROL, siATM, siAKT1, or the scramble siRNAs at a final concentration of 100 nM using Oligofectamine (Invitrogen). After 72 h of culture at 37°C, the cells were recovered, seeded on 15-cm dishes (Falcon; BD Discovery Labware), allowed to adhere for 18 h, and then subjected to a second transfection as described above. After 18 h, 10 μM BG and 50 μM TMZ were added to the cells transfected with siCONTROL, siATR, scrATM, or siATM, whereas cells transfected with scrAKT1 or siAKT1 were left untreated. All dishes were incubated at 37°C for additional 48 h, and the cells were harvested and processed for Western blot analysis.

For treatment with insulin, 18 h after the second transfection with either siATR or siCONTROL the cells were subjected to a 24 h-starvation in McCoy's 5A medium, supplemented with 0.5% fetal calf serum and 2 mM L-glutamine. The cells were then exposed to 10 μg/ml insulin for 30 min, recovered, washed in PBS, and processed for Western blot analysis.

To evaluate the effects of inhibition of AKT1 or ATR expression on cell proliferation, HCT116/3-6 cells recovered after the first transfection were seeded (1.3×10^3 cells/well; four replica wells) into flat-bottomed 96-well plates (Falcon; BD Discovery Labware), allowed to adhere for 18 h, and then subjected to the second transfection. After 18 h, BG (10 μM) and graded concentrations of TMZ were added to the cultures, and the plates were incubated at 37°C for 6 or 8 days. Cell growth was then evaluated by the MTT assay, as described previously (Vernole et al., 2003).

Western Blot Analysis. Rabbit polyclonal antibodies against AKT, phospho-AKT (Ser473 or Thr308), and phospho-GSK3β (Ser9) were purchased from Cell Signaling Technology Inc. Rabbit polyclonal antibody against phospho-IKKα/β (Thr23) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody against ATR (Ab2) was purchased from Oncogene Science (Cambridge, MA), rabbit polyclonal antibody against ATM (ab91) was obtained from Abcam plc (Cambridge, UK), and mouse monoclonal antibody against actin (clone AC-40) was obtained from Sigma-Aldrich.

Total cellular extracts were prepared as described previously (Caporali et al., 2004). Twenty-five micrograms of protein per sample

were run on a 10 or 6% SDS-polyacrylamide gel, transferred to nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), and blocked with 5% nonfat milk in Tris-buffered saline supplemented with 0.1% Tween 20 for 1 h at room temperature. The membranes were then incubated in the same solution overnight at 4°C with primary antibodies at the following dilutions: anti-AKT, 1:1000; anti-phospho-AKT (Ser473 or Thr308), 1:500; anti-phospho-GSK3β, 1:1000; anti-phospho-IKKα/β, 1 μg/ml; anti-ATR, 1:500; anti-ATM, 1:1500; and anti-actin, 1:1000. The latter antibody was used as an internal standard for loading. Immunodetection was carried out using appropriate horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (ECL) detection reagents. Where indicated, films were scanned on a GS-710 calibrated imaging densitometer and analyzed by means of Quantity One, version 4.1.1 software (Bio-Rad, Hercules, CA).

In Vitro AKT Kinase Assay. AKT kinase assays were performed using a nonradioactive kit from Cell Signaling Technology Inc. Only minor modifications were introduced into the manufacturer's protocol. In brief, AKT was immunoprecipitated from cell extracts using an immobilized anti-AKT antibody from Upstate. The immunoprecipitates were then washed two times with lysis buffer (Cell Signaling Technology Inc.) and two times with kinase buffer (Cell Signaling Technology Inc). The in vitro kinase reaction was carried out in 50 μl of kinase buffer containing immunoprecipitated AKT, 200 μM ATP, and 1 μg of a GSK3 fusion protein that served as the substrate. After an incubation of 30 min at 30°C, the reaction was stopped by the addition of SDS sample buffer. Samples were boiled for 5 min, separated on a 12% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. The level of phosphorylated GSK3 fusion protein was detected using an anti-phospho-GSK3α/β (Ser9/21) polyclonal antibody (1:1000 dilution; Cell Signaling Technology Inc.). The immunocomplexes were also subjected to immunoblotting with the anti-AKT polyclonal antibody to ensure equivalent kinase abundance.

Results

TMZ Treatment Induces MMR-Dependent AKT Activation. To determine whether the AKT pathway is activated in response to TMZ treatment, and the role played by the MMR system in this molecular event, the MMR-proficient/MGMT-deficient lymphoblastoid cell line TK6 and its MMR-deficient/MGMT-deficient subline MT1 were cultured in the presence of 12.5 μM TMZ (i.e., a drug concentration shown previously to induce G_2 arrest and apoptosis in TK6 but not in MT1 cells) (D'Atri et al., 1998; Caporali et al., 2004) for 72 h, and then they were analyzed for the phosphorylation status of AKT and its downstream targets GSK3β and IKKα/β every 24 h.

In TMZ-treated TK6 cells, increased phosphorylation of AKT on Ser473, GSK3β on Ser9 and IKKα/β on Thr23 was detected at all time points analyzed, whereas no changes were observed in the phosphorylation status of AKT at Thr308, and in the total amount of the kinase (Fig. 1A). Neither phosphorylation of AKT nor phosphorylation of its

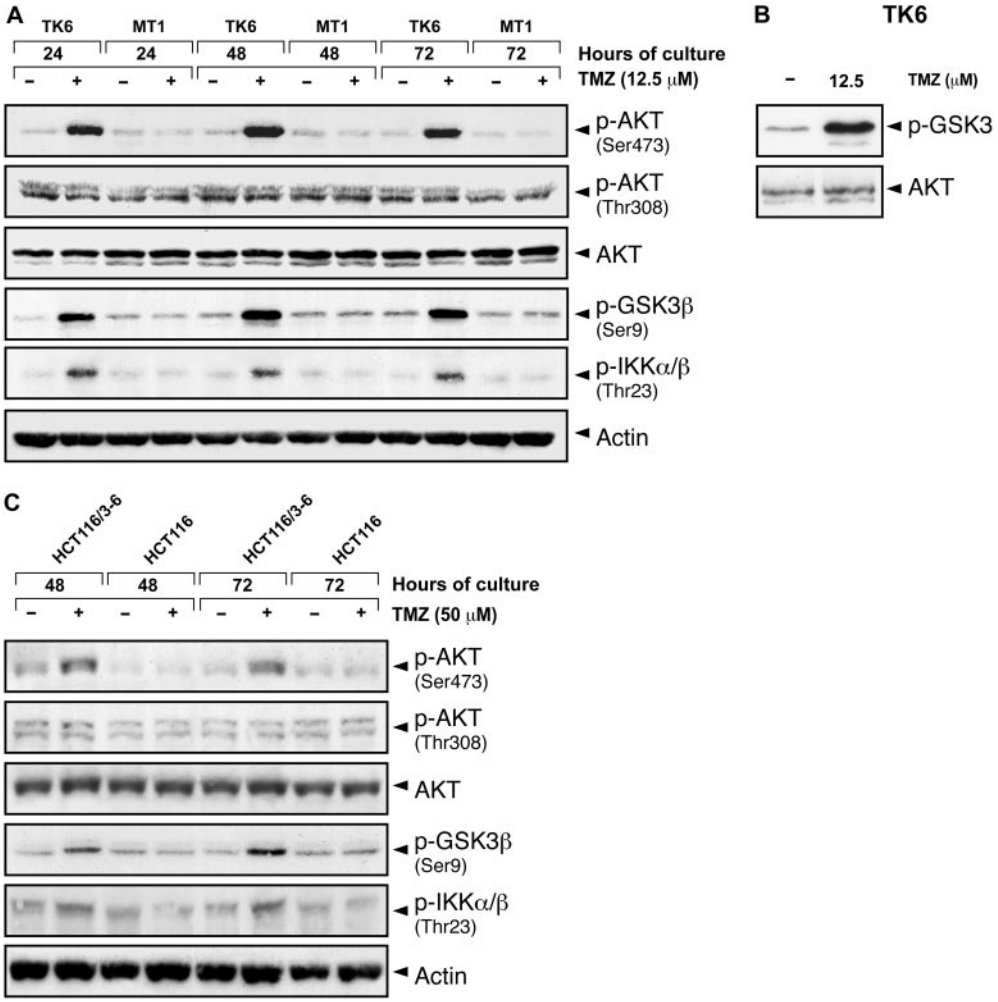

Fig. 1. Endogenous AKT is activated in TMZ-treated cells in a MMR-dependent manner. A, TK6 and MT1 cells were cultured in the absence or in the presence of 12.5 μM TMZ for the indicated times. Total cell extracts were resolved on 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against phospho-AKT (Ser473 or Thr308), AKT, phospho-GSK3β (Ser9), and phospho-IKKα/β (Thr23). Incubation with the anti-actin mAb was performed as a loading control. The immune complexes were visualized using ECL. B, TK6 cells were cultured in the absence or in the presence of 12.5 μM TMZ for 72 h. Cell lysates were prepared, and AKT was immunoprecipitated with an immobilized anti-AKT antibody. The in vitro AKT kinase assay was then performed as described under *Materials and Methods*, using a GSK3 fusion protein as the substrate. At the end of the assay, samples were resolved on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and probed with antibodies against phospho-GSK3α/β (Ser9/Ser21), and AKT to ensure equivalent kinase abundance. C, HCT116/3-6 and HCT116 cells were maintained in culture medium containing 10 μM BG or BG plus 50 μM TMZ for 72 h. At the indicated time points, cell total cell extracts were prepared and subjected to Western blot analysis as described in A. The results are representative of three independent experiments.

downstream targets occurred in TMZ-treated MT1 cells (Fig. 1A).

To test whether AKT phosphorylation at Ser473 was associated with a functional activation of the kinase, TK6 cells were cultured in the presence of 12.5 μM TMZ for 72 h, and then cell extracts were assayed for AKT activity using the in vitro kinase assay described under *Materials and Methods*. The results illustrated in Fig. 1B show that the level of phosphorylated GSK3 fusion protein was markedly increased in TMZ-treated cells with respect to untreated control, indicating that AKT phosphorylation on Ser473 was sufficient to increase the kinase activity.

To further confirm that AKT was activated in response to TMZ in an MMR-dependent manner, we performed additional experiments using the MMR-deficient cell line HCT116 and its MMR-proficient counterpart HCT116/3-6. The cells were incubated with 50 μM TMZ, in the presence of 10 μM BG to abrogate MGMT activity, and analyzed for phosphorylation of AKT, GSK3β, and IKKα/β after 24, 48, and 72 h of drug exposure. In both cell lines, no changes in the phosphorylation status of the proteins were observed after 24 h of drug treatment (data not shown). After 48 and 72 h of TMZ exposure, increased phosphorylation of AKT (Ser473), GSK3β (Ser9), and IKKα/β (Thr23) was detected in HCT116/3-6 but not in HCT116 cells (Fig. 1C). In both cell lines, the phosphorylation status of AKT at Thr308 and the total amount of the kinase were not affected by TMZ treatment (Fig. 1C). AKT kinase assay performed on cell extracts obtained from control and TMZ-treated HCT116/3-6 cells after 48 h of culture confirmed drug-induced activation of AKT (data not shown).

Inhibition of AKT Function Increases Tumor Cell Sensitivity to TMZ. To assess whether activation of endogenous AKT protects against TMZ cytotoxicity, a cell clone derived from the MMR-proficient/MGMT-proficient cell line MCF-7 (i.e., the MCF-7/B1 clone) was stably transfected with the pUSEamp(+) plasmid encoding a Myc-tagged dominant-negative kinase-dead mutant of AKT1 [i.e., AKT1(K179M)] (Franke et al., 1995) or with the empty vector. Transfected subclones were selected in the presence of G418, expanded, and subsequently examined for AKT and Myc-tagged AKT expression (data not shown). Two clones, i.e., the KD12 subclone transfected with mutant AKT1 and the pUSE2 subclone transfected with the empty vector (Fig. 2A), were chosen for the subsequent studies.

We first tested the dominant-negative effect of mutant AKT1. To this end, KD12 and pUSE2 subclones were treated with 10 μg/ml insulin for 30 min, and then they were analyzed for phosphorylation of AKT on Ser473 and GSK3β on Ser9. As illustrated in Fig. 2B, phosphorylation of endogenous AKT and GSK3β was increased by insulin treatment in pUSE2 cells. In contrast, insulin-induced up-regulation of endogenous AKT and GSK3β phosphorylation did not occur in KD12 cells transfected with the dominant-negative AKT1(K179M) mutant. In these cells, however, phosphorylation of the exogenous AKT1(K179M) protein was found to be constitutively up-regulated.

We next performed a time course evaluation of TMZ-induced activation of AKT in KD12 and pUSE2 subclones. The cells were cultured in the presence of 50 μM TMZ plus 10 μM BG for 72 h, and then they were analyzed for the phosphorylation status of AKT, GSK3β, and IKKα/β every 24 h. In TMZ-treated pUSE2 cells, no changes in the phopshorylation status of the proteins were observed after 24 h of drug exposure (data not shown), whereas increased phosphorylation of endogenous AKT on Ser473, GSK3β, and IKKα/β was detectable after 48 and 72 h of culture (Fig. 2C). Similarly to TMZ-treated TK6 and HCT116/3-6 cells, pUSE2 cells exposed to the drug did not show increased phosphorylation of the Thr308 residue of AKT. No changes in the levels of phosphorylated AKT (endogenous and mutant), GSK3β, and IKKα/β were observed in TMZ-treated KD12 cells (Fig. 2C). AKT kinase assay performed on cell extracts obtained from control and TMZ-treated pUSE2 cells after 48 h of culture confirmed drug-induced activation of AKT (data not shown).

Having demonstrated that the expression of AKT1/K179M mutant prevents TMZ-induced activation of endogenous AKT in KD12 cells, we evaluated sensitivity to TMZ of pUSE2 and

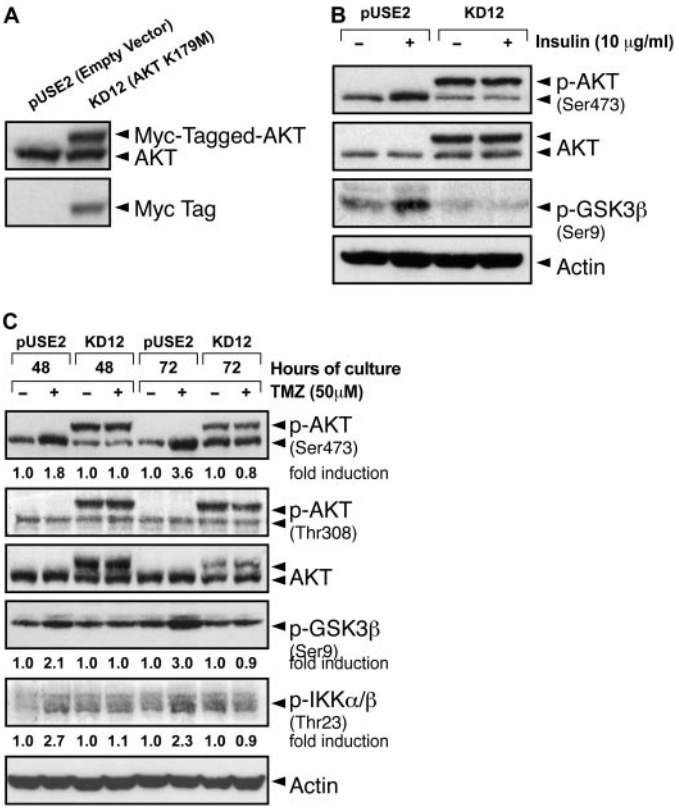

Fig. 2. Analysis of AKT and GSK3β phosphorylation in pUSE2 and KD12 cells treated with insulin or TMZ. A, whole cell extracts were resolved on 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and probed with anti-Myc tag and anti-AKT antibodies. The immune complexes were visualized using ECL. B, pUSE2 and KD12 cells were serum starved for 24 h, and then they were exposed to 10 μg/ml insulin for 30 min. Total cell lysates were prepared and analyzed as described in A using antibodies against phospho-AKT (Ser473), AKT, and phospho-GSK3β (Ser9). Incubation with the anti-actin mAb was performed as a loading control. C, pUSE2 and KD12 cells were maintained in culture medium containing 10 μM BG or BG plus 50 μM TMZ for 72 h. At the indicated time points, total cell extracts were prepared and subjected to Western blot analysis as described in A, using antibodies against phospho-AKT (Ser473 or Thr308), AKT, phospho-GSK3β (Ser9), and phospho-IKKα/β (Thr23). Incubation with the anti-actin mAb was performed as a loading control. For each sample, the densitometric levels of endogenous p-AKT (Ser473), p-GSK3β, and p-IKKα/β were normalized to the respective levels of actin. The -fold induction of p-AKT, p-GSK3β, and p-IKKα/β in TMZ-treated samples was then calculated with respect to the protein levels in the corresponding controls, to which the arbitrary value of 1.0 was assigned. The results are representative of two independent experiments.

KD12 subclones. The cells were cultured in the presence of graded concentrations of the drug (plus 10 μM BG) for 6 days, and then they were analyzed for cell growth. The results illustrated in Fig. 3 show that KD12 cells were significantly more sensitive to TMZ than pUSE2 cells at all drug concentrations tested.

To further confirm that inhibition of AKT function was able to increase cell sensitivity to TMZ, we evaluated the combined effect of the drug and the AKT inhibitor SH-5 on TK6, pUSE2, and HCT116/3-6 cell proliferation. The same drug combination was used to perform control experiments using MT1 and HCT116 cells, which do not activate AKT upon exposure to TMZ concentrations that are effective in their MMR-proficient counterparts. The cells were incubated with graded concentrations of TMZ in the absence or in the presence of SH-5, and they were monitored for proliferation after 72 h (TK6 and MT1 cells) or 6 days (pUSE2, HCT116/3-6, and HCT116 cell) of culture. In the breast and colon cancer cell lines, TMZ treatment was performed in the presence of 10 μM BG. TMZ sensitivity of the MMR-proficient cell lines was significantly increased by SH-5 (Fig. 4, A–C). Moreover, at the concentrations used, TMZ, either alone or combined with SH-5, did not inhibit proliferation of the MMR-deficient MT1 and HCT116 cells (data not shown).

To confirm the ability of SH-5 to impair AKT activation upon cell exposure to TMZ, TK6, pUSE2, and HCT116/3-6 cells were cultured with the drug in the absence or in the presence of SH-5 for 72 h, and then they were analyzed for phosphorylation of AKT (on Ser473), and GSK3β. A reduction in the levels of phosphorylated AKT and GSK3β was observed in all the three cell lines treated with TMZ plus SH-5 with respect to the cell lines treated with the methylating agent alone (Fig. 4D; data not shown).

Additional experiments were performed to investigate whether inhibition of AKT1 expression by RNA interference technology was also associated with increased cell sensitivity to TMZ. HCT116/3-6 cells were subjected to two sequential transfections with 100 nM siAKT1 or scrAKT1, and then they were tested for AKT1 expression and sensitivity to TMZ. The amount of AKT1 was markedly reduced in the cells transfected with siAKT1 with respect to the cells transfected with scrAKT1 (Fig. 5A). Moreover, inhibition of AKT expression was accompanied by a significant increase in cell sensitivity to TMZ (Fig. 5B)

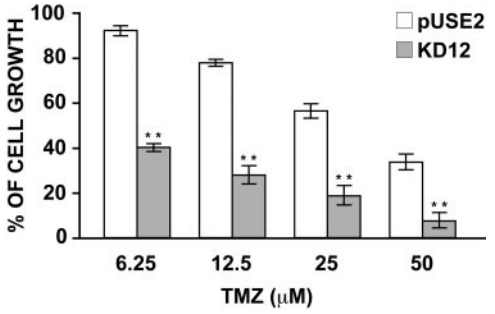

Fig. 3. Overexpression of the kinase-dead dominant-negative AKT1(K179M) mutant increases cell sensitivity to TMZ. pUSE2 and KD12 cells were cultured in the presence of 10 μM BG or BG plus the indicated concentrations of TMZ for 6 days. Cell growth was then evaluated in terms of viable cell count. Data are expressed in terms of percentage of cell growth of target cells treated with BG + TMZ with respect to cells exposed to BG alone. Each value represents the mean of three independent experiments performed with triplicate samples, with bars indicating standard error of the mean. **, $p < 0.01$, according to Student's t test, comparing the percentages of cell growth of KD12 cells with those of pUSE2 cells. Percentages were subjected to angular transformation to obtain normally distributed data. Thereafter, conventional standard error calculation and Student's t test statistics were performed on converted data. However, the data in the figure are expressed in nontransformed percentages, after conversion of the transformed data into the original values.

Inhibition of AKT Function Increases TMZ-Induced Senescence. Previous studies showed that glioma cells induced to overexpress activated AKT are protected against TMZ-induced senescence (Hirose et al., 2005). We therefore investigated whether inhibition of endogenous AKT function as a consequence of either overexpression of the AKT1(K179M) mutant or cell treatment with SH-5 was accompanied by an increase in the percentage of cells undergoing a senescence-like phenomenon in response to TMZ. To this end, pUSE2, KD12, and HCT116/3-6 cells were incubated with 10 μM BG and 50 μM TMZ in the absence or in the presence of 5 μM SH-5, and the percentages of SA-β-gal-positive cells were determined after 6 days of culture. In the absence of SH-5, TMZ induced a significantly higher percentage of SA-β-gal-positive cells in the KD12 cell line than in the syngeneic pUSE2 cell line (Fig. 6). SH-5 had no effect on the percentage of TMZ-treated KD12 cells that were SA-β-gal-positive, whereas it significantly increased the proportion of TMZ-treated pUSE2 cells undergoing senescence (Fig. 6). HCT116/3-6 cells were less susceptible than breast cancer cells to TMZ-induced senescence (Fig. 6). Nonetheless, as in the case of pUSE2 cells, pharmacological inhibition of AKT by SH-5 caused a significant increase in the percentage of TMZ-induced SA-β-gal-positive cells (Fig. 6).

Inhibition of ATR but Not ATM Impairs Activation of AKT in Response to TMZ. We previously showed that cell cycle arrest and apoptosis triggered by TMZ in MMR-proficient cells is accompanied by activation of the ATM and ATR kinases (Caporali et al., 2004). Moreover, ATM has been recently implicated in the activation of AKT in response to insulin and ionizing radiation (Viniegra et al., 2005). We therefore investigated whether TMZ-induced activation of AKT was dependent on ATM and/or ATR function. To this end, HCT116/3-6 cells were subjected to two sequential transfections with 1) a pool of siRNAs targeting ATR (siATR) or a pool of nontargeting siRNAs (siCONTROL) as a control; and 2) a siRNA against ATM (siATM) or a scramble siRNA (scrATM) as a control. Eighteen hours after the second transfection, 10 μM BG and 50 μM TMZ were added to the cultures, and the levels of phosphorylated AKT (Thr308 and/or Ser473) and GSK3β were determined 48 h later. To establish whether inhibition of ATR expression could impair, as described previously for ATM, cell response to insulin, the same parameters were also evaluated in HCT116/3-6 cells transfected with siATR or siCONTROL and treated with 10 μg/ml agent for 30 min.

Transfection of siATM efficiently inhibited ATM expression in HCT116/3-6 cells (Fig. 7A). However, upon TMZ treatment a comparable increase in the level of phosphorylated AKT (Ser473) and GSK3β was detected in the cells transfected with siATM or scrATM (Fig. 7A).

ATR expression was markedly reduced in siATR-transfected cells (Fig. 7B). TMZ treatment was able to enhance the level of phosphorylated AKT (Ser473) and GSK3β in HCT116/3-6 cells transfected with siCONTROL but not in

the cells transfected with siATR (Fig. 7B). In agreement with previous findings (Bellacosa et al., 2005), insulin was able to induce Ser473 and Thr308 phosphorylation of AKT, as well as phosphorylation of GSK3β, in HCT116/3-6 cells transfected with siCONTROL (Fig. 7B). A reduction of insulin-induced phosphorylation of AKT and GSK3β was observed in the cells transfected with siATR.

We demonstrated previously that U2OS cells overexpressing a dominant-negative kinase-dead mutant form of ATR are more sensitive to TMZ than their wild-type counterparts (Caporali et al., 2004). We therefore investigated whether inhibition of ATR expression using RNA interference technology was also accompanied by increased cell sensitivity to TMZ. To this end, HCT116/3-6 cells transfected with either siATR or siCONTROL were cultured in the presence of graded concentrations of TMZ plus 10 μM BG for 8 days, and

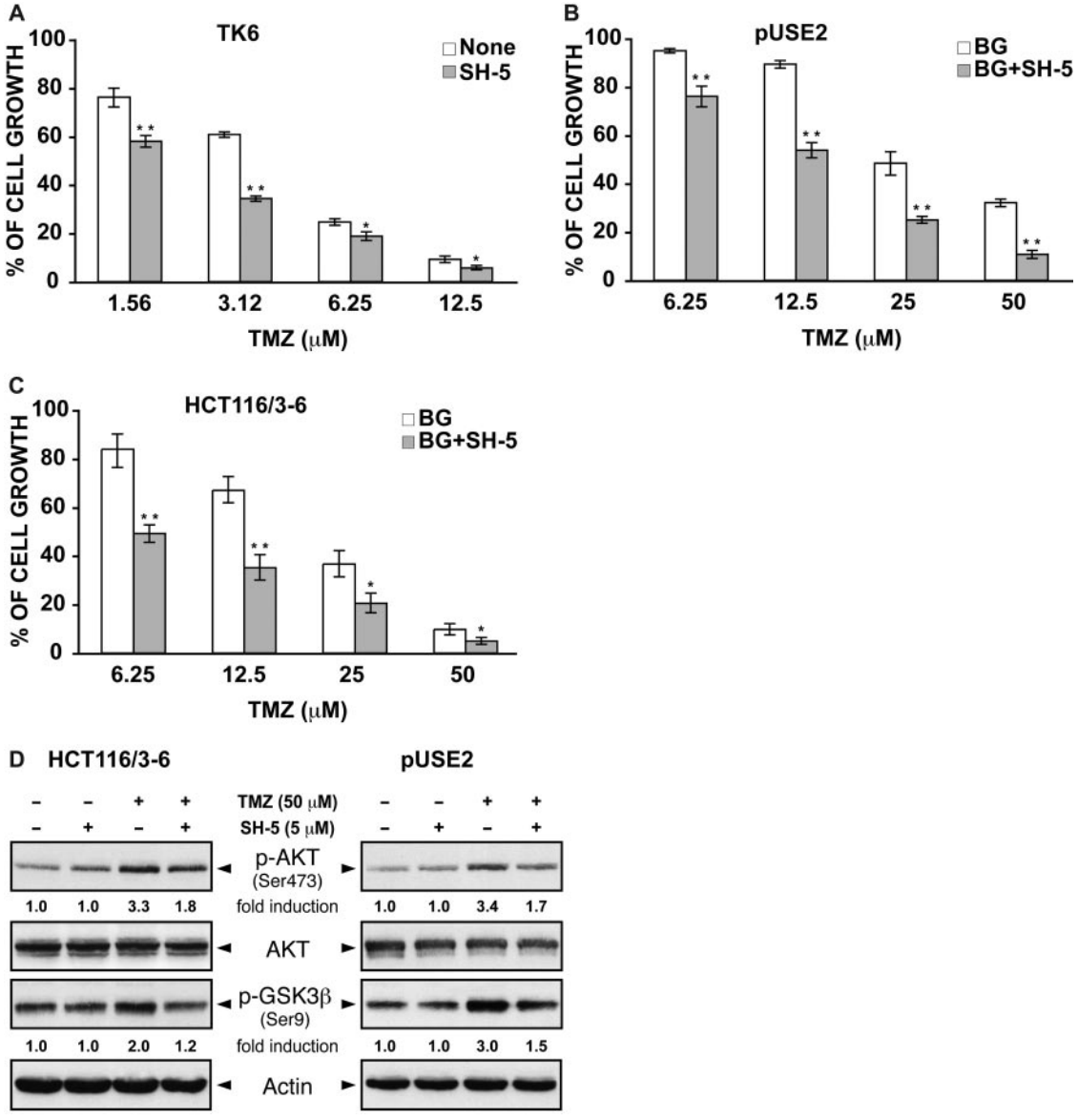

Fig. 4. The AKT inhibitor SH-5 increases sensitivity to TMZ of MMR-proficient cells and inhibits drug-induced activation of AKT. Cells of TK6 (A), pUSE2 (B), and HCT116/3-6 (C) lines were cultured in the presence of TMZ or TMZ plus SH-5 (1 μM for TK6 cells and 5 μM for pUSE2 and HCT116/3-6 cells) for 72 h (TK6) or 6 days (pUSE2 and HCT116/3-6). BG (10 μM) was also added to the cultures of pUSE2 and HCT116/3-6 cells as described under *Materials and Methods*. Control groups were exposed to culture medium alone or culture medium containing SH-5 (TK6), or to culture medium containing BG alone or BG plus SH-5 (pUSE2 and HCT116/3-6). At the end of the incubation period, cell growth was evaluated in terms of viable cell count. Data are expressed in terms of percentage of cell growth of target cells treated with TMZ with respect to the appropriate control group not exposed to the drug. Each value represents the mean of three independent experiments performed with triplicate samples, with bars indicating standard error of the mean. $**$, $p < 0.01$; $*$, $p < 0.05$, according to Student's t test, comparing the percentages of cell growth obtained in the presence of SH-5 with those obtained in the absence of the AKT inhibitor. Percentages were subjected to angular transformation and statistical analysis as described in the legend of Fig. 2. D, 5 μM SH-5, 10 μM BG, and 50 μM TMZ were sequentially added to pUSE2 and HCT116/3-6 cell cultures as described under *Materials and Methods*. Seventy-two hours after the addition of TMZ, total cell extracts were resolved on 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against phospho-AKT (Ser473), AKT, and phospho-GSK3β (Ser9). Incubation with the anti-actin mAb was performed as a loading control. The immune complexes were visualized using ECL. For each sample, the densitometric levels of p-AKT, and p-GSK3β were normalized to the respective levels of actin. The -fold induction of p-AKT and p-GSK3β in TMZ-treated samples was then calculated with respect to the protein levels in the corresponding controls, to which the arbitrary value of 1.0 was assigned. The results are representative of two independent experiments.

then they were assayed for proliferation using the MTT assay. The results illustrated in Fig. 8 show that inhibition of ATR expression significantly enhanced cell sensitivity to TMZ.

Discussion

A large body of experimental evidence indicates that constitutive activation of the PI3K/AKT pathway is associated with tumor cell resistance to conventional chemotherapy (West et al., 2002; Bellacosa et al., 2005). Moreover, it has been shown that several cytotoxic antitumor agents can modulate, either positively or negatively, the PI3K/AKT pathway (West et al., 2002; Bellacosa et al., 2005). A better understanding of the connection between drug-induced modulation of the PI3K/AKT pathway and response to chemotherapy may provide rational bases for the development of combination regimens in which selected cytotoxic drugs are associated with agents targeting components of the pathway.

A recent study by Hirose et al. (2005) suggests that the AKT pathway may represent a new target for the sensitization of gliomas to TMZ. The authors overexpressed in human glioblastoma cells a modified AKT protein, which is rapidly activated by 4-hydroxytamoxifen, and they found that the cells exposed to this agent exhibited reduced Chk2 phosphorylation and G_2 arrest upon treatment with TMZ. Moreover, the 4-hydroxytamoxifen-treated cells were significantly more resistant than control cells to senescence and mitotic catastrophe induced by TMZ. In the present study, using B lymphoblastoid, colon cancer, and breast cancer cell lines, we further addressed the role of AKT pathway in cellular response to TMZ.

Our results demonstrate that endogenous AKT was phosphorylated on Ser473 in target cells exposed to TMZ and that this molecular event was accompanied by an increase in the levels of the phosphorylated form of GSK3β and IKK, two direct downstream targets of AKT. No detectable phosphorylation of Thr308 was induced by TMZ, although this site was readily phosphorylated in the cells exposed to insulin, a standard AKT stimulus. Moreover, no changes in the level of total AKT were observed in TMZ-treated cells. Under the

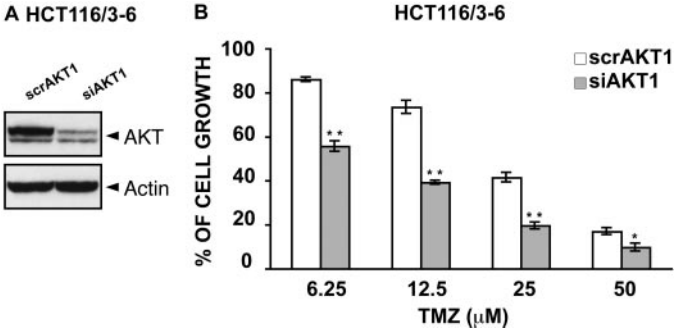

Fig. 5. Inhibition of AKT expression by siRNAs increases cell sensitivity to TMZ. A. HCT116/3-6 cells were transfected with a siRNA targeting AKT1 (siAKT1) or with a scramble siRNA (scrAKT1) as described under *Materials and Methods*. Whole cell extracts were prepared and resolved on 10% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with antibodies against AKT. Incubation with the anti-actin mAb was performed as a loading control. The immune complexes were visualized using ECL. B, siAKT1- and scrAKT1-transfected cells were cultured in the presence of 10 μM BG or BG plus the indicated concentrations of TMZ for 6 days. Cell proliferation was then evaluated by the MTT assay. Data are expressed in terms of percentage of cell growth of target cells treated with BG + TMZ with respect to cells exposed to BG alone. Each value represents the mean of three independent experiments performed with quadruplicate samples, with bars indicating standard error of the mean. **, $p < 0.01$; *, $p < 0.05$, according to Student's t test, comparing the percentages of cell growth of siAKT1-transfected/TMZ-treated cells with those of scrAKT1-transfected/TMZ-treated cells. Percentages were subjected to angular transformation and statistical analysis as described in the legend of Fig. 2.

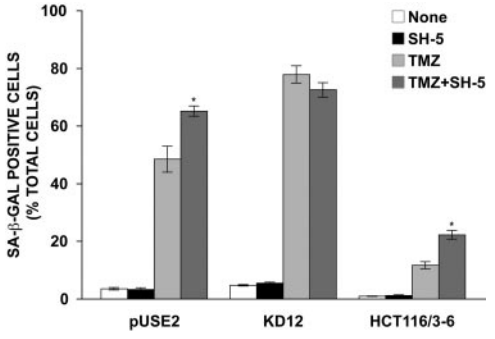

Fig. 6. Inhibition of AKT function increases TMZ-induced senescence. pUSE2, KD12, and HCT116/3-6 cells were incubated with 10 μM BG (CTRL) or with BG plus 5 μM SH-5 (SH-5), 50 μM TMZ (TMZ), or TMZ and SH-5 (TMZ + SH-5), and then they were monitored 6 day later for the percentage of SA-β-gal-positive cells. Each value represents the mean of three independent experiments, with bars indicating standard error of the mean. *, $p < 0.05$, according to paired Student's t test, comparing the percentages of positive cells of TMZ + SH5-treated cultures with those of TMZ-treated cultures. None = control.

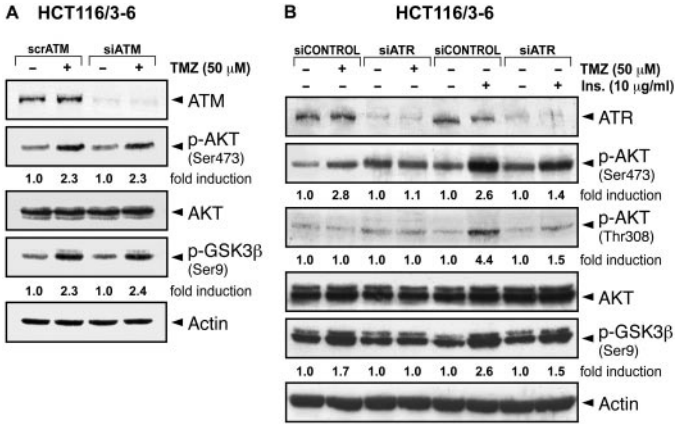

Fig. 7. ATR but not ATM is required for AKT activation in response to TMZ. A, HCT116/3-6 cells were transfected with a siRNA targeting ATM (siATM) or with a scramble siRNA (scrATM) as described under *Materials and Methods*, and then they were exposed to 10 μM BG or BG plus 50 μM TMZ for 48 h. Whole cell extracts were prepared and resolved on 6% (ATM) or 10% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with antibodies against ATM, phospho-AKT (Ser473), AKT, and phospho-GSK3β (Ser9). Incubation with the anti-actin mAb was performed as a loading control. For each sample, the densitometric levels of p-AKT and p-GSK3β were normalized to the respective levels of actin. The -fold induction of p-AKT and p-GSK3β in TMZ-treated samples was then calculated with respect to the protein levels in the corresponding controls, to which the arbitrary value of 1.0 was assigned. The results are representative of three independent experiments. B, HCT116/3-6 cells were transfected with a pool of siRNAs targeting ATR (siATR) or with a pool of nontargeting siRNAs (siCONTROL) as described under *Materials and Methods*, and then they were exposed to 10 μM BG or BG plus 50 μM TMZ for 48 h. Transfected cells were also serum starved for 24 h, and then they were treated with 10 μg/ml insulin for 30 min. Whole cell extracts were prepared and subjected to Western blot analysis as described in A, using antibodies against ATR, phospho-AKT (Ser473 or Thr308), AKT, and phospho-GSK3β (Ser9). Incubation with the anti-actin mAb was performed as a loading control. The -fold induction of p-AKT and p-GSK3β was determined as indicated in A. The results are representative of three independent experiments.

experimental conditions used in the study, namely, cell exposure to low concentrations of TMZ, phosphorylation of AKT, GSK3β, and IKK in response to TMZ was absolutely dependent on a functional MMR system, because it occurred in the MMR-proficient cell lines TK6 and HCT116/3-6 but not in their corresponding MMR-deficient MT1 and HCT116 counterparts.

Despite the lack of detectable Thr308 phosphorylation, AKT kinase activity was substantially increased by TMZ, as demonstrated not only by the kinetics of phosphorylation of GSK3β and IKK, which parallels that of AKT, but also by the results of the in vitro AKT kinase assays performed on control and TMZ-treated cells. It is noteworthy that in the study by Hirose et al. (2005), glioblastoma cells overexpressing the 4-hydroxytamoxifen-inducible form of AKT, either exposed or not to the inducing agent, showed a decrease in the level of phosphorylated (Ser473) endogenous AKT upon treatment with TMZ. It has been previously shown that several chemotherapeutic agents can either stimulate or inhibit the AKT pathway (West et al., 2002), suggesting that the genetic asset of individual cell lines might have a role in determining the cellular response to treatment in terms of AKT modulation. It is therefore reasonable to hypothesize that the discrepancy between our data and those of Hirose et al. (2005) simply stems from the use of different cell lines.

Previous studies have demonstrated that maximal AKT activation in response to insulin or growth factor stimulation is achieved through phosphorylation of both Thr308 and Ser473 (Bellacosa et al., 2005). However, there are some conflicting data about the interdependence and the specific role of these phosphorylation events in AKT activation. For example, Alessi et al. (1996) demonstrated that phosphorylation of Thr308 is not dependent on phosphorylation of Ser473 or vice versa in cells exposed to insulin or insulin-like growth factor-1 and that phosphorylation of either Ser473 or Thr308 leads to partial AKT activation in vitro. Conversely, Toker and Newton (2000) reported that phosphorylation at Thr308 is a prerequisite for Ser473 phosphorylation in response to IGF-1. Likewise, Yamada et al. (2001) suggested that phosphorylation of Thr308 in response to insulin may serve as a trigger for AKT activation, even though they observed that Thr308 phosphorylation occurs transiently and that AKT activation correlated with Ser473 phosphorylation. Finally, it has been recently shown that AKT phosphorylation at Thr308 was primary dependent on prior phosphorylation at Ser473 (Scheid et al., 2002; Sarbassov et al., 2005). In the present study, we analyzed the AKT phosphorylation status after 24 to 72 h of cell exposure to TMZ. Therefore, we cannot exclude that Thr308 was phosphorylated at earlier time points after drug treatment and then rapidly dephosphorylated after triggering Ser473 phosphorylation. Nevertheless, our data clearly demonstrate that, in the cell lines under investigation, the AKT pathway is activated in response to TMZ, and they confirm previous findings (Alessi et al., 1996; Yamada et al., 2001) showing that AKT phosphorylated only at Ser473 has increased kinase activity.

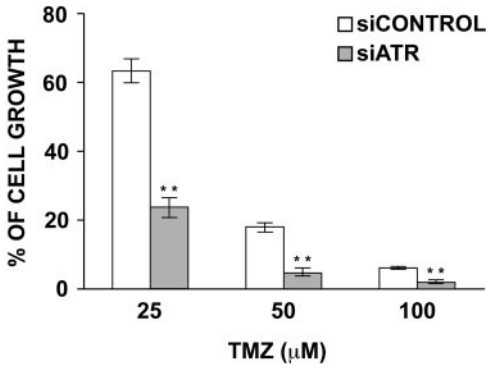

Fig. 8. Inhibition of ATR expression by siRNAs increases cell sensitivity to TMZ. HCT116/3-6 cells were transfected with a pool of siRNAs targeting ATR (siATR) or with a pool of nontargeting siRNAs (siCONTROL) as described under *Materials and Methods*. The cells were then cultured in the presence of 10 μM BG or BG plus the indicated concentrations of TMZ for 8 days. Cell proliferation was evaluated by the MTT assay. Data are expressed in terms of percentage of cell growth of target cells treated with BG + TMZ with respect to cells exposed to BG alone. Each value represents the mean of three independent experiments performed with quadruplicate samples, with bars indicating standard error of the mean. **, $p < 0.01$, according to Student's t test, comparing the percentages of cell growth of siATR-transfected/TMZ-treated cells with those of siCONTROL-transfected/TMZ-treated cells. Percentages were subjected to angular transformation and statistical analysis as described in the legend of Fig. 2.

As mentioned, it has been shown that ectopic overexpression of an active form of AKT increases glioma cell resistance to TMZ by attenuating drug-induced G_2 arrest, senescence, and mitotic catastrophe (Hirose et al., 2005). In the present investigation, we demonstrate that inhibition of endogenous AKT function sensitizes cells to TMZ, thus providing additional experimental bases for a possible clinical use of AKT inhibitors in combination with the drug. Indeed, overexpression of the kinase-dead dominant-negative AKT1(K179M) mutant in KD12 cells prevented TMZ-induced activation of endogenous AKT, and it markedly increased the growth inhibitory effect of the drug. Likewise, the AKT inhibitor SH-5 reduced substantially TMZ-induced activation of the kinase in the MMR-proficient cell lines pUSE2, TK6, and HCT116/3-6 cells and significantly enhanced their sensitivity to the drug. The inhibitor consistently did not affect TMZ sensitivity of the MMR-deficient MT1 and HCT116 cells, which do not activate AKT in response to the drug. Finally, the selective inhibition of AKT1 expression by siRNA significantly increased HCT116/3-6 cell sensitivity to TMZ. In agreement with previous findings (Hirose et al., 2005), we also found that TMZ-mediated activation of endogenous AKT is able to antagonize drug-induced target cell senescence. Therefore, this could be considered one of the possible mechanisms underlying malignant cell resistance to the triazene compound.

In response to genotoxic stress, cells activate checkpoint signaling pathways that coordinate cell cycle progression with DNA repair and apoptosis to minimize the probability of replicating and segregating damaged DNA (for review, see Bakkenist and Kastan, 2004). The serine/threonine protein kinases ATM and ATR, which belong to the PI3K-related kinase family, are essential transducers of these checkpoints. In response to DNA damage, they phosphorylate numerous target proteins involved in cell cycle arrest, DNA repair, and apoptosis (Bakkenist and Kastan, 2004). ATM is rapidly activated after cell exposure to double-strand break (DSB)-inducing agents, such as ionizing radiation or radiomimetic drugs. ATR is the principal signal transducer when cells are challenged with UV light or with agents that interfere with DNA replication, such as aphidicoline or hydroxyurea, and it

cooperates with ATM to enforce and sustain the checkpoints induced by DSBs (Bakkenist and Kastan, 2004). In a recent study by Viniegra et al. (2005), it was demonstrated that in cells exposed to insulin or ionizing radiation, phosphorylation of AKT at Ser473 is strictly dependent on functional ATM. Moreover, a previous report by Feng et al. (2004) identified the PI3K-related DNA-dependent protein kinase, which plays a critical role in DNA DSB repair by nonhomologous end-joining (Bakkenist and Kastan, 2004), as a prominent Ser473 kinase in cells treated with insulin or pervanadate. These findings suggest a potential intersection of transmembrane signal transduction pathways and DNA damage response pathways. We showed previously that cell cycle arrest and apoptosis triggered by clinically relevant TMZ concentrations in MMR-proficient cells is accompanied by activation of the ATM and ATR kinases and that ATR is the major kinase responsible for triggering the G_2 cell cycle checkpoint (Caporali et al., 2004). Here, we show that ATR, but not ATM is required for AKT phosphorylation at Ser473 in response to TMZ. Indeed, siRNA-mediated knockdown of ATR expression in HCT116/3-6 cells was associated with abrogation of TMZ-induced phosphorylation of AKT and GSK3β, whereas siRNA-induced inhibition of ATM expression in the same cells did not affect phosphorylation of both proteins in response to TMZ.

The mechanism of AKT activation mediated by ATR is presently unknown. In the study by Viniegra et al. (2005), it was suggested that ATM promotes AKT phosphorylation at Ser473 through an indirect mechanism. It is possible to speculate that also ATR does not directly phosphorylate AKT in response to TMZ but that it mediates the activation of a third kinase able to phosphorylate Ser473 of AKT. It is noteworthy that inhibition of ATR expression in HCT116/3-6 cells significantly increased TMZ-induced inhibition of cell growth. This finding suggests that the protective role of ATR activation in cells treated with O^6-G-methylating agents (Caporali et al., 2004, Stojic et al., 2004b) can be, at least in part, mediated through activation of AKT.

In conclusion, our data demonstrate that endogenous AKT is activated in response to clinically relevant concentrations of TMZ. This molecular event is strictly dependent on a functional MMR system, requires the expression of ATR, and confers protection against drug-induced cell growth inhibition.

Previous investigations have shown that the engagement of the MMR system is necessary to trigger signaling transduction pathways leading to cell cycle arrest and cell death in response to unrepaired O^6-MeG. Our study shows, for the first time, that activation of the MMR system by O^6-MeG:T mispairs also induces a survival signal through the activation of AKT. Several inhibitors of individual components of PI3K/AKT pathway are currently being developed for clinical use, either as single agents or in combination with conventional chemotherapy (for review, see Granville et al., 2006). Our findings strongly suggest that a clinical benefit could be obtained by combining TMZ with this kind of therapeutic molecules.

Furthermore, our data constitute the first evidence of a novel function of ATR as the main AKT activator downstream the triggering of the MMR system. While the manuscript for this article was in preparation, Matsuoka et al. (2007) reported that more than 700 proteins undergo phosphorylation on ATM and ATR consensus sites in response to ionizing radiation, including proteins in the PI3K/AKT pathway. Our findings are consistent with this report, because they clearly demonstrate a link between the DNA damage response to unrepaired O^6-MeG and activation of the AKT pathway. Future investigations are required to identify the proteins that might interact with and regulate the ATR-AKT signaling pathway.

Acknowledgments

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