Research Article

Molecular Cancer Therapeutics

Caffeine Confers Radiosensitization of *PTEN*-Deficient Malignant Glioma Cells by Enhancing Ionizing Radiation–Induced G_1 Arrest and Negatively Regulating Akt Phosphorylation

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Abstract

PTEN mutations are frequently found in malignant glioma and can result in activated phosphatidylinositol-3-kinase/Akt survival signaling associated with resistance to radiotherapy. Strategies to interfere with aberrant PI3K/Akt activity are therefore being developed to improve the therapeutic efficacy of radiotherapy in patients with malignant glioma. The methylxanthine caffeine has been described as a PI3K inhibitor and is also known to sensitize cells to ionizing radiation. However, a direct association between these two caffeine-mediated effects has not been reported yet. Therefore, we asked whether caffeine or its derivative pentoxifylline differentially affect the radiosensitivity of malignant gliomas with different *PTEN* status. As models, we used the radiosensitive EA14 malignant glioma cell line containing wild-type *PTEN* and the radioresistant U87MG malignant glioma cell line harboring mutant *PTEN*. Our study revealed that caffeine and pentoxifylline radiosensitized *PTEN*-deficient but not *PTEN*-proficient glioma cells. Radiosensitization of *PTEN*-deficient U87MG cells by caffeine was significantly correlated with the activation of the G_1 DNA damage checkpoint that occurred independently of *de novo* synthesis of p53 and p21. The p53 independency was also confirmed by a significant caffeine-mediated radiosensitization of the glioma cell lines T98G and U373MG that are deficient for both *PTEN* and *p53*. Furthermore, caffeine-mediated radiosensitization was associated with the inhibition of Akt hyperphosphorylation in PTEN-deficient cells to a level comparable with PTEN-proficient cells. Our data suggest that the methylxanthine caffeine or its derivative pentoxifylline are promising candidate drugs for the radiosensitization of glioma cells particularly with *PTEN* mutations. *Mol Cancer Ther; 9(2); 480–8. ©2010 AACR.*

Introduction

Glioblastoma multiforme (GBM) is the most frequent primary malignancy of the adult central nervous system and the most malignant form of glioma (1). Current treatment strategies involve neurosurgery, polychemotherapy, and ionizing radiation (IR). Long-term survival for patients with GBM is still limited, especially due to the emergence of resistance to DNA-damaging procedures such as IR.

The mechanisms of glioma cell resistance to IR are not completely understood. However, despite the expression of wild-type p53 and p21 in primary GBM cells, they often fail to arrest in cell cycle following IR-induced DNA damage (2). In general, cells arrest in cell cycle to enable DNA repair and, in case of irreparable damage, undergo apoptosis; both mechanisms are governed by p53 (3). However, cell fate is not only dependent on the amount and type of damage but also on the functional interaction of various damage-inducible signal transduction cascades. The ability of p53 to induce cell cycle arrest or apoptosis on IR can be antagonized by survival signals, such as by the phosphatidylinositol-3-kinase (PI3K)–dependent activation of Akt/protein kinase B, which indirectly leads to the inhibition of p53 functions by activating its negative regulator MDM2 (4). Because Akt is constitutively activated in cells with mutations of the *phosphatase and tension homologue gene* (*PTEN*; refs. 5, 6) and because *PTEN* mutations are frequently found in GBM (7–9), a pivotal role of Akt in functional p53 deficiency (4, 10) and radioresistance of GBM can be expected.

Caffeine, a methylxanthine, is well known to radiosensitize cancer cells including malignant gliomas through the inhibition of ATM and ATR kinase activity and the abrogation of the G_2-M checkpoint (11). However, it has also been reported that caffeine has inhibitory effects on

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the phosphoinositide metabolism, including inhibition of the enzymatic activity of PI3K (12). Furthermore, caffeine and PTEN have been found to act synergistically regarding growth inhibition and the induction of apoptosis in cancer cells by the downregulation of the PI3K/Akt pathway (13), suggesting the antiproliferative effects of caffeine that are comparable with those of PTEN. Based on these reports and because only limited data exist on the effects of caffeine on PI3K/Akt signaling and the cell cycle checkpoints in malignant glioma, we asked if caffeine differentially affects the radiosensitivity of malignant glioma cells displaying different *PTEN* status. We further aimed at determining by which underlying signaling pathways a potential radiosensitization might be mediated.

Materials and Methods

Cell Lines

The U87MG GBM cell line, which is deficient for *PTEN* but contains wild-type *p53* (14), was obtained from the American Type Culture Collection. The EA14 malignant glioma cell line containing wild-type *PTEN* and p53 has originally been established by Stuschke et al. (15) from biopsy samples of an anaplastic astrocytoma WHO grade III. The wild-type p53 status of both cell lines (15, 16) was confirmed by our group by temperature gradient gel electrophoresis followed by sequence analysis of the exons 5, 6, 7, and 8 of the *p53* gene (data not shown). The T98G and U373MG cell lines, which harbor mutations both in *PTEN* and *p53* (16), were obtained from American Type Culture Collection.

Cells were cultured as previously described (15). In brief, cells were grown in monolayers in MEM (Life Technologies Ltd.) supplemented with 15% heat-inactivated FCS, 1% nonessential amino acids, penicillin (1×10^5 U/L), and streptomycin (100 mg/L). Cultures were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

Treatment

Before treatment, cells were arrested in the G_0-G_1 phase of the cell cycle by contact inhibition. Synchronization of cultures was confirmed by flow cytometric analysis. Cells were then harvested by trypsinization and were plated in cell culture flasks (25 cm^2) at a defined cell density. Because caffeine has been shown to interfere both with G_1 and G_2-M checkpoints of cell cycle, we irradiated cells either 5 h after plating when >90% of cells were still in G_0-G_1 (stationary phase; Fig. 1A and B) or 24 h (U87MG) or 28 h (EA14) after plating when at least 20% of cells were in the G_2-M phase of the cell cycle (exponential growth phase; Fig. 1C and D), respectively. IR with graded doses up to 8 Gy was performed by using a ^{60}Co source at 1 to 2 Gy/min. Caffeine (Sigma) was used at a final concentration of 2 mmol/L because this dosage has been previously reported to effectively inhibit ATR and ATM (11). Because caffeine plasma concentrations of 2 mmol/L cannot be clinically achieved because of the dose-limiting toxicity of this drug, we also tested pentoxifylline (Sigma), a methylxanthine derivative with reduced toxicity, for its radiosensitizing effect in PTEN-deficient and PTEN-proficient glioma cells. Furthermore, the selective Akt inhibitor triciribine (Calbiochem) was used to determine whether Akt or ATM/ATR are central targets of caffeine-modulated radiosensitivity of *PTEN*-deficient glioma cells. Caffeine (2 mmol/L), pentoxifylline (2 mmol/L), or triciribine (5 μmol/L) were added to the medium 30 min before IR and were removed by the exchange of culture medium 72 h later.

Cell Cycle Analysis

Flow cytometric analysis was used to quantify the relative number of cells in different phases of the first (G_0-G_1, S, and G_2-M) as well as the consecutive cell cycle (G_0'/G_1', S', and G_2'/M'; ref. 17). Briefly, cells were labeled continuously during the whole culture period with 5-bromodeoxyuridine (BrdUrd; Serva) and, at the end of the experiment, they were stained with the DNA dyes Hoechst 33258 and ethidium bromide (EB; Sigma-Aldrich). Because incorporated BrdUrd quenches the fluorescence of DNA-bound Hoechst dye and because the amount of incorporated BrdUrd positively correlates with the number of replications, cells can be distinguished according to how many cell cycles they have passed. In contrast, labeling with EB is unaffected by BrdUrd and allows one to differentiate between cells in the cell cycle phases G_0-G_1, S, and G_2-M.

In detail, cells were continuously labeled with 60 μmol/L BrdUrd immediately after IR. Cells were then harvested at different time points between 0 and 60 h after IR and stored at −80°C in cell culture medium containing 10% DMSO until analyzed. After thawing, samples were pelleted by centrifugation and resuspended in 1 mL ice-cold Hoechst staining buffer (1.2 μg/mL Hoechst 33258, 10 mmol/L Tris-HCl, 154 mmol/L NaCl, 0.5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 0.1% Nonidet P40, and 0.2% bovine serum albumin). Cells were incubated at 4°C for 15 min. EB was added to a final concentration of 0.2 μg/mL, and samples were incubated again at 4°C for 15 min. Flow cytometric analysis was done by means of a PAS III three-channel flow cytometer (PARTEC GmbH) equipped with an HBO 100 mercury arc lamp source. Excitation at 365 nm and detection of red fluorescence for EB at 570 nm versus blue fluorescence for Hoechst 33258 at 435 nm were used. A minimum of 2×10^4 cells was analyzed per sample. Analysis of the DNA content distribution in each cell cycle phase was done using WINMDI (supplied by Dr. Joe Trotter, Salk Institute, La Jolla, CA).

Clonogenic Cell Survival

Cell survival after IR alone or in the presence of caffeine or pentoxifylline was determined using a clonogenic survival assay. Cells were arrested in G_0-G_1 by contact inhibition and subsequently seeded into 25-cm^2 culture flasks at defined densities. In their stationary

or exponential growth phase, cells were treated with caffeine or pentoxifylline, IR, or a combination of both. Colonies were grown for 14 d, then fixed with 70% ethanol and stained with Giemsa (Sigma). Colonies with >50 cells were counted. Plating efficiency and survival fractions for given treatments were calculated on the basis of survival of nonirradiated cells treated with the solvent control, or caffeine or pentoxifylline alone. Each sample was done in triplicate and at least three independent experiments were carried out.

p53, p21^{Waf1}, and Phosphorylated Akt Expression

The quantitation of expression levels of p53, p21^{WAF1}, pan-Akt, or Akt phosphorylated at serine 473 (p-Akt^{Ser473}) in cell lysates from EA14 and U87MG cells was done by standard Western blot analysis. Briefly, standard SDS-PAGE was done using 90 μg of total protein per cell lysate, followed by transfer to nitrocellulose membranes (Amersham GE Healthcare). The following antibodies were used for detection: mouse anti-p53 (clone DO-1, Santa Cruz Biotechnology), mouse anti-p21^{WAF1} (clone

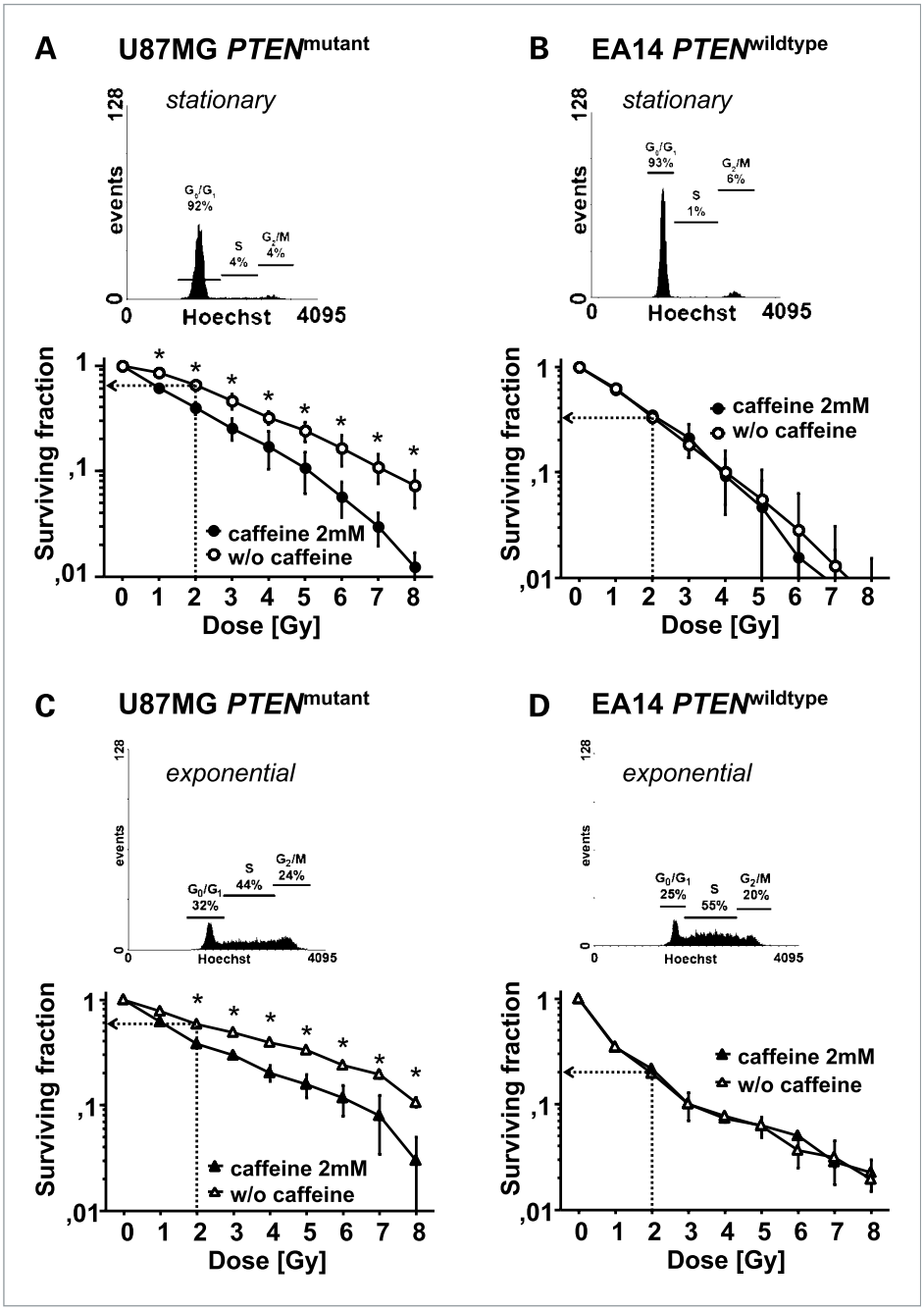

Figure 1. Caffeine increases the radiosensitivity of *PTEN*-deficient but not *PTEN*-proficient glioma cells. Cell survival of U87MG (A and C) and EA14 cells (B and D) after IR in the absence or presence of caffeine was determined using a clonogenic survival assay. Cells were arrested in G_0-G_1 by contact inhibition and subsequently seeded into 25-cm^2 culture flasks. In stationary (A and B) or exponential growth phase (C and D), cells were treated with 2 mmol/L caffeine, IR, or a combination of both. Cells were then left to grow undisturbed for 14 d and the established colonies (>50 cells) were stained and counted. Points, mean surviving fractions from three independent experiments done in triplicates; bars, SD. *, significant differences ($P < 0.01$) in surviving fractions of cells irradiated in the absence or presence of caffeine. Top, the representative cell cycle distribution of U87MG and EA14 cells in their stationary or exponential growth phase as determined by flow cytometric analysis of Hoechst 33258 staining.

EA10, Calbiochem), mouse pan-Akt (clone 40D4) and mouse anti–p-Akt^{Ser473} (clone 587F11, both from Cell Signaling Technology), mouse anti–β-actin (clone AC-74, Sigma), and peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories).

Statistical Analysis

All statistical analyses were done using StatView 5.1 (Abacus Concepts, Inc.). The significance of differences in surviving fractions and cell cycle distribution of cell lines treated with increasing doses in the absence or presence of caffeine was determined by paired *t* test. The significance of differences in the effect of caffeine, pentoxifylline, or triciribine on surviving fractions at 2 Gy (SF2) in U87MG, EA14, T98G, and U373MG cells (Table 1A and B; Fig. 5) was determined using paired *t* test for normally distributed and Wilcoxon-Signed rank test for non-normally distributed data, respectively.

Results

Caffeine Increases Radiosensitivity of *PTEN*-Deficient but not *PTEN*-Proficient Glioma Cells

We first assessed the basal radiosensitivity of synchronized cell cultures from the *PTEN*-deficient U87MG and the *PTEN*-proficient EA14 cells by irradiating them with increasing doses (0–8 Gy) either in their stationary growth phase (>90% of cells were in G_0-G_1; Fig. 1A and B) or exponential growth phase (≥20% of cells were in G_2-M; Fig. 1C and D). Following a culture period of 14 days, survival fractions were determined. As reported earlier (15), U87MG cells were relatively resistant to IR (Fig. 1A and C). In contrast, the EA14 cell line was significantly more sensitive (Table 1; Fig. 1B and D). Because caffeine has been shown to inhibit PI3K activity and should therefore counteract constitutive PI3K activity in the *PTEN*-deficient U87MG cells, we treated EA14 and U87MG cells with 2 mmol/L caffeine 30 minutes before irradiation and again assessed their clonogenic survival. As shown in Fig. 1A and C and Table 1A, pretreatment with caffeine significantly sensitized U87MG to IR. In contrast, caffeine pretreatment had no radiosensitizing effects on the *PTEN*-proficient E414 cells (Table 1A; Fig. 1B and D).

Because the concentration of caffeine that we used for our *in vitro* studies cannot be achieved in a clinical setting because of the dose-limiting toxicity of caffeine, we also tested pentoxifylline, a less toxic methylxanthine derivative for its radiosensitizing effects. Of clinical importance, we also observed a selective radiosensitizing activity of pentoxifylline in PTEN-deficient glioma cells (Table 1B).

Caffeine Increases IR-Induced G_1 Arrest in *PTEN*-Deficient but not *PTEN*-Proficient Glioma Cells

We next addressed whether the selective interaction of caffeine with radiosensitivity of *PTEN*-deficient cells occurred at distinct cell cycle checkpoints. We first focused on the G_1 DNA damage checkpoint. We therefore irradiated U87MG and EA14 cells in their stationary growth phase with 6 Gy in the absence or presence of caffeine and analyzed whether the IR-induced G_1 checkpoint of

Table 1. Surviving fractions of glioma cells after IR in their stationary growth phase

A: Surviving fractions of glioma cells after IR in their stationary growth phase with 2 Gy (SF2) in the absence or presence of caffeine

Cell line	Gene status		SF2 (% ± SD)*		*P*†
	PTEN	*p53*	– caffeine	+ caffeine	
EA14	Wt	Wt	34 ± 2.9	35 ± 2.2	0.18
U87MG	Mut	Wt	64 ± 2.9	40 ± 5.5	0.0003
T98G	Mut	Mut	73 ± 11.4	64 ± 11.7	0.07
U373MG	Mut	Mut	56 ± 4.4	43 ± 5.0	0.03

B: Surviving fractions of glioma cells after IR in their stationary growth phase with 2 Gy (SF2) in the absence or presence of pentoxifylline

Cell line	Gene status		SF2 (% ± SD)*		*P*†
	PTEN	*p53*	– pentoxifylline	+ pentoxifylline	
EA14	Wt	Wt	33 ± 1.5	34 ± 1.2	0.52
U87MG	Mut	Wt	64 ± 1.7	35 ± 1.2	0.0017

Abbreviations: Wt, wild-type; mut, mutant.

*The results for each cell line representing the mean surviving fractions after IR with 2 Gy ± SD from at least four independent experiments are presented.

†Significance levels were determined using paired *t* test for normally distributed and Wilcoxon signed-rank test for not-normally distributed data, respectively.

the ongoing cell cycle was modulated by caffeine in a *PTEN*-specific manner. As shown in Fig. 2A (top), in the *PTEN*-deficient and radioresistant U87MG cells, only a minor percentage of cells remained arrested in G_1 overtime following IR of cells with 6 Gy. Pretreatment with 2 mmol/L caffeine significantly increased the number of U87MG cells that remained arrested in G_1. This temporal extension of the G_1 checkpoint by caffeine in U87MG cells was sustained because 30% ± 1.5% of cells remained in G_1 for up to 60 h after IR (Fig. 2A, top). Caffeine-mediated prolongation of G_1 arrest of irradiated U87MG cells was also reflected by a decreased number of cells progressing to G_2-M (Fig. 2A, bottom). In contrast, IR with 6 Gy alone induced an arrest in G_1 in the radiosensitive *PTEN*-proficient EA14 cells, and caffeine did neither affect this IR-induced G_1 arrest (Fig. 2B, top) nor the IR-induced inhibition of cell cycle progression to G_2-M (Fig. 2B, bottom).

Caffeine Abrogates IR-Induced G_2 Arrest in a *PTEN*-Independent Manner

Besides the modulation of the G_1 checkpoint, caffeine has been shown to cause an override of the IR-induced G_2-M checkpoint (11); however, the role of PTEN at this checkpoint and the effect of caffeine therein are rather unclear. We therefore irradiated U87MG and EA14 cells with 6 Gy in their exponential growth phase in the absence or presence of caffeine and analyzed the relative numbers of U87MG and EA14 cells at G_2-M of the ongoing cell cycle at 0 to 60 h postirradiation. In contrast to the observed significant differences in the extent and duration of the G_1 checkpoint, both U87MG and EA14 cells became initially arrested in G_2 for up to 20 h on IR (Fig. 3A and B, top). Pretreatment of cells with 2 mmol/L caffeine completely abrogated this IR-induced G_2 arrest both in the *PTEN*-deficient and *PTEN*-proficient cells: twenty hours after IR in the presence of caffeine, only 15% + 2% of U87MG cells and 9% ± 0.6% of EA14 were still in G_2 (Fig. 3A and B, top). This abrogation of the IR-induced G_2 arrest by caffeine was again persistent because even 60 hours after IR, both cell lines still revealed a significantly lower proportion of cells in G_2 after caffeine pretreatment when compared with IR alone (Fig. 3A and B, top).

Caffeine Mediates an Increase in IR-Induced G_1' Arrest Both in *PTEN*-Proficient and *PTEN*-Deficient Exponentially Growing Glioma Cells

We next investigated whether caffeine would also modulate the G_1 checkpoint of cells in which DNA damage had occurred in a cell cycle phase other than G_1. We irradiated U87MG and EA14 cells with 6 Gy in their exponential growth phase in the absence or presence of caffeine and again used the BrdUrd/Hoechst quenching technique for the discrimination between the ongoing and consecutive cell cycles. We found that both U87MG and EA14 cells irradiated during the exponential growth phase reached the G_1 phase of the consecutive cell cycle (G_1') to a degree

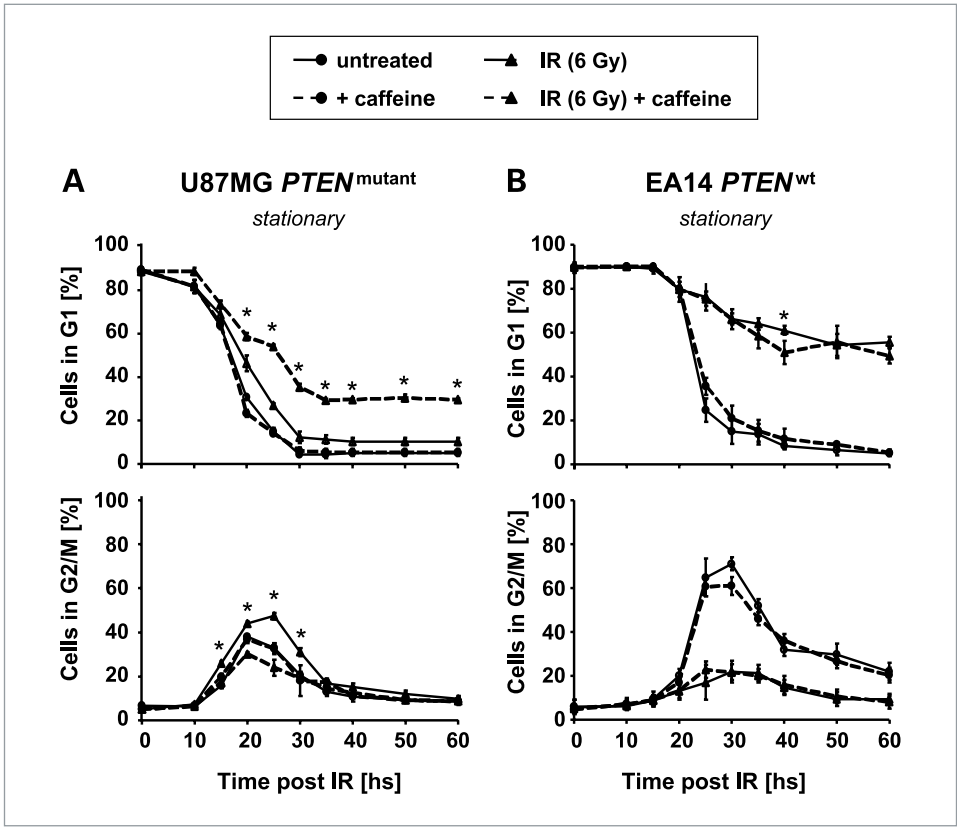

Figure 2. Caffeine increases IR-induced G_1 arrest in *PTEN*-deficient but not *PTEN*-proficient glioma cells. U87MG (A) and EA14 cells (B) were synchronized by contact inhibition and seeded into 25-cm^2 culture flasks. In their stationary growth phase, cells were irradiated in the absence or presence of 2 mmol/L caffeine. The kinetics of cell cycle distribution from 0 to 60 h after IR was determined by flow cytometry using the BrdUrd-Hoechst quenching technique. The results are expressed as percentage of cells in G_0-G_1 (top) or G_2-M phase (bottom) of the ongoing cell cycle. Points, mean percentages from at least three independent experiments are presented; bars, SD. *, significant differences ($P < 0.01$) in cell cycle distribution of cells irradiated in the absence or presence of caffeine.

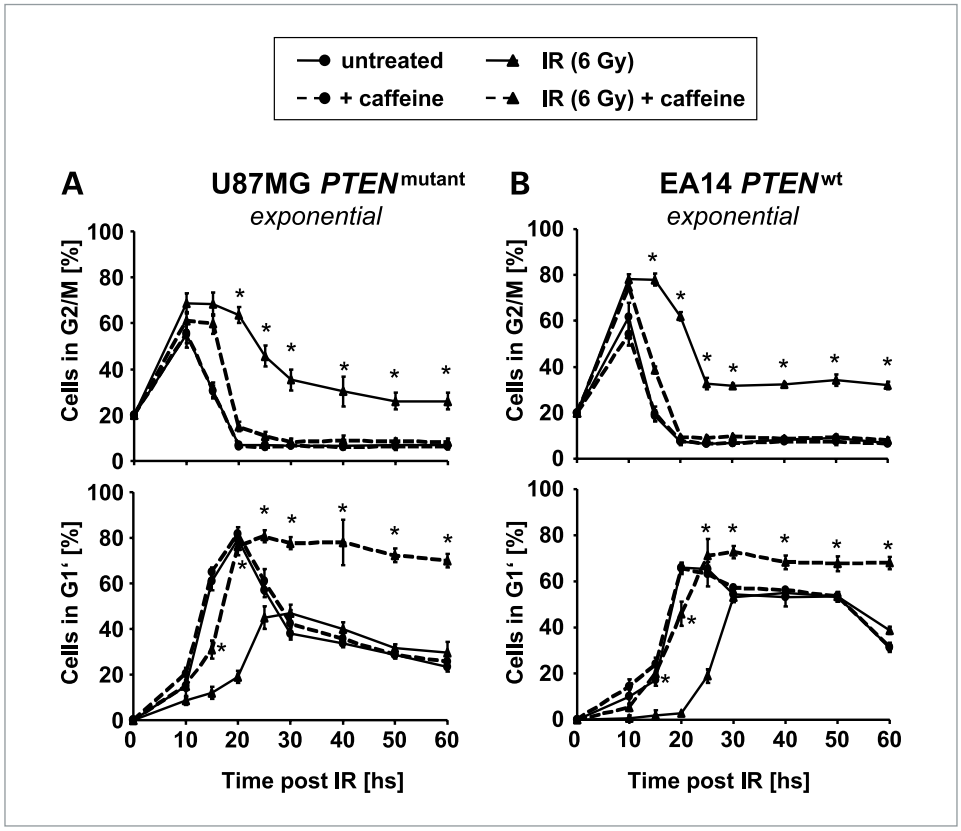

Figure 3. Caffeine mediates an increase in IR-induced G_1' arrest both in *PTEN*-proficient and *PTEN*-deficient exponentially growing glioma cells. U87MG (A) and EA14 cells (B) were synchronized by contact inhibition and irradiated with 6 Gy in their exponential growth phase in the absence or presence of 2 mmol/L caffeine. Using the BrdUrd-Hoechst quenching technique, the relative numbers of cells in the G_2-M phase of the ongoing cell cycle (top) or the G_0-G_1 phase of the consecutive cell cycle (G_0'/G_1') were determined by flow cytometry. Points, percentages from at least three independent experiments are presented; bars, SD.

that, 60 hours after IR, was similar to that of untreated controls or to cells treated with caffeine alone (Fig. 3A and B, bottom). Pretreatment of both cell lines with 2 mmol/L caffeine resulted in a significant IR-induced arrest in G_1' (Fig. 3A and B, bottom). In EA14 cells, the inhibitory effect of caffeine on the G_2 block was equalized by its augmenting effect at the G_1' checkpoint activation, which might explain the overall absent effect of caffeine on clonogenic survival after IR. In contrast, in U87MG cells, caffeine more efficiently increased the G_1' checkpoint activation than it decreased the IR-induced G_2 arrest, resulting in an overall inhibition of cell cycle progression.

Increase of Irradiated Malignant Glioma Cells in G_1 Arrest after Pretreatment with Caffeine Is Independent of p53 Function

In our model, caffeine did not affect the radiosensitivity of *PTEN*-proficient cells (Fig. 1B) but significantly increased the radiosensitivity of *PTEN*-deficient cells (Fig. 1A), which was preceded by an increase of IR-induced G_1 arrest by caffeine in cells with impaired PTEN function. DNA damage–induced G_1 checkpoint is particularly regulated through the induction of $p21^{Waf1}$ expression by p53 (18), the function of which is downregulated in *PTEN*-deficient glioma cells (10). We therefore asked whether the interaction of caffeine with the G_1 checkpoint in *PTEN*-deficient cells would involve p53 reactivation. First, we analyzed p53 and $p21^{Waf1}$ protein expression in U87MG and EA14 cells after IR in the absence or presence of caffeine. In both U87MG (Fig. 4A) and EA14 cells (Fig. 4B), p53 was induced 2 hours after IR with 6 Gy. Accordingly, $p21^{Waf1}$ expression levels were also significantly increased in both cell lines 6 hours after IR (Fig. 4A and B), suggesting that the observed impaired IR-induced G_1 checkpoint in U87MG cells (Fig. 2A, top) was not a result from low to absent $p21^{Waf1}$ induction. Pretreatment of U87MG (Fig. 4A) or EA14 cells (Fig. 4B) with caffeine resulted only in a slight inhibition of IR-induced p53 or $p21^{Waf1}$ expression. This moderate effect of caffeine on IR-induced p53 and p21 expression is in line with the absent interaction of caffeine with the extent or duration of the IR-induced G_1 checkpoint in EA14 cells (Fig. 2B, top). Our data further suggest that the significant effect of caffeine on the G_1 checkpoint activation (Fig. 2A) in *PTEN*-deficient cells is independent of an enhancement of IR-induced p53 or $p21^{Waf1}$ upregulation. To confirm the p53 independency of the caffeine-mediated radiosensitization of *PTEN*-deficient glioma cells, we used T98G and U373MG cells, which carry mutations in *PTEN* and *p53*. Indeed, in both cell lines, caffeine pretreatment significantly reduced surviving fractions after IR with 2 Gy (Table 1A).

Caffeine Negatively Regulates Phosphorylation of Akt after DNA Damage

Caffeine has been shown to inhibit PI3K (12), the upstream activator kinase of Akt. Because increased Akt

activation in *PTEN*-deficient cells has been directly linked with their resistance to IR (19), caffeine might exert its radiosensitizing effects by reducing Akt activation in our model. Therefore, we investigated the levels of p-Akt^{Ser473} in *PTEN*-proficient and *PTEN*-deficient glioma cells at the time points 30, 60, or 120 minutes after the cells had been irradiated in the absence or presence of caffeine. As shown in Fig. 4A, untreated U87MG cells contained significantly higher levels of p-Akt^{Ser473} compared with untreated EA14 cells (Fig. 4B). No significant changes of p-Akt^{Ser473} expression levels were induced by IR in either cell line. Independently of the *PTEN* status, caffeine rapidly, i.e., already 30 minutes after IR, and significantly decreased the expression of p-Akt^{Ser473} in U87MG (Fig. 4A) and

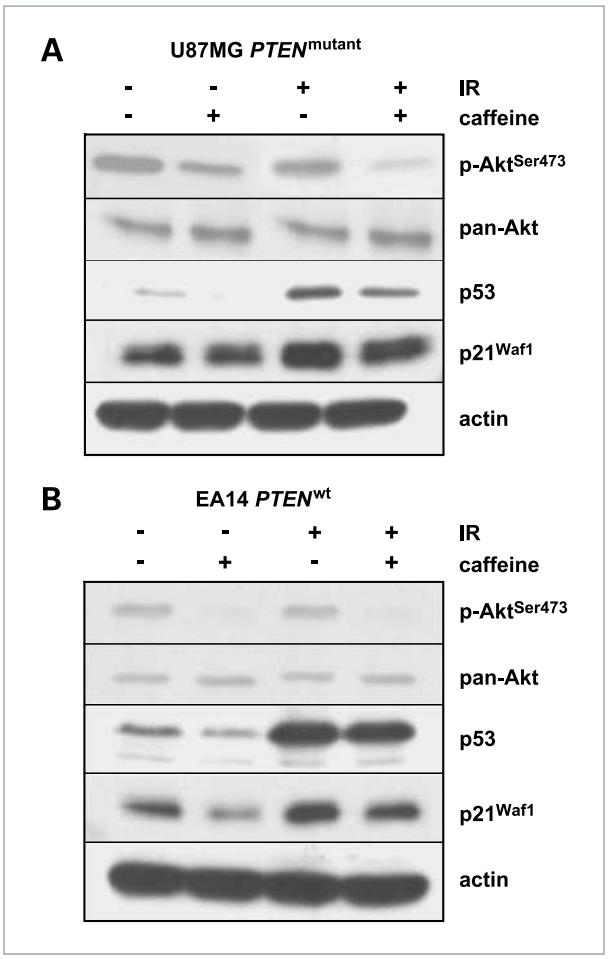

Figure 4. The interaction of caffeine with p53/p21^{Waf1} and p-Akt^{Ser437} expression levels. U87MG (A) and EA14 cells (B) were irradiated with 6 Gy in their stationary growth phase in the presence or absence of 2 mmol/L caffeine. Thirty minutes after IR, cell lysates from U87MG and EA14 cells were prepared and analyzed by Western blot analysis for expression levels of p-Akt^{Ser473} or pan-Akt. In addition, 2 or 6 h after IR, nuclear protein extracts from cell lysates were prepared and analyzed by Western blot for expression levels of p53 or its transcriptional target p21^{Waf1}, respectively. The expression levels of actin were determined as internal loading control. Representative blots from three independent experiments are shown.

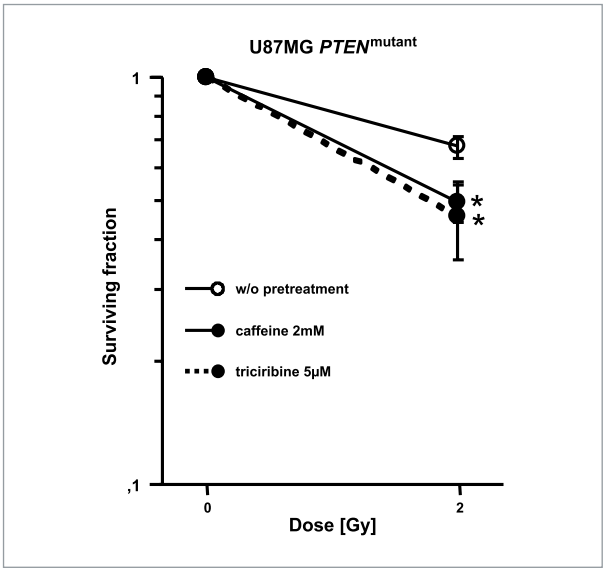

Figure 5. Radiosensitization of PTEN-deficient glioma cells by the specific Akt inhibitor triciribine. Cell survival of U87MG cells after IR in the absence or presence of caffeine or the specific Akt inhibitor triciribine was determined using a clonogenic survival assay. Cells in stationary growth phase were left untreated or were pretreated with 2 mmol/L caffeine or 5 µmol/L triciribine. Subsequently, cells were irradiated with 2 Gy. After a culture period of 14 d, the established colonies (>50 cells) were stained and counted. Points, mean surviving fractions from five independent experiments done in triplicates; bars, SD. Both caffeine and triciribine significantly reduced the survival of U87MG cells following IR with 2 Gy (caffeine versus without pretreatment: $P = 0.001$; triciribine versus without pretreatment: $P = 0.003$) with comparable efficacy (caffeine versus triciribine: $P = 0.25$).

EA14 cells (Fig. 4B). The extent of inhibition of p-Akt levels was comparable for each time point analyzed (data not shown for 60 and 120 minutes following IR). Of note, caffeine treatment before IR decreased the p-Akt^{Ser473} levels of U87MG cells to a level comparable with untreated E414 cells (Fig. 4A and B), which well correlated with its radiosensitizing efficacy in U87MG cells (Table 1A).

Because caffeine also negatively interferes with ATM/ATR (11) aside from inhibiting the PI3K/Akt pathway, we next asked whether the radiosensitizing effect of caffeine in PTEN-deficient glioma cells is mainly due to the inhibition of the PI3K/AKT pathway or whether inhibition of ATM/ATR is also involved. We therefore compared the radiosensitizing effect of caffeine with that of the specific Akt inhibitor triciribine. As shown in Fig. 5, the pretreatment of U87MG cells with triciribine (5 µmol/L) before IR with 2 Gy significantly reduced the surviving fractions of U87MG cells with an efficacy comparable with caffeine (Fig. 5).

Discussion

It has been shown by numerous studies that the activation of the PI3K/Akt signaling pathway in tumor cells, either by increased growth factor receptor signaling (20, 21) or decreased activity of specific pathway inhibitors such as

PTEN (14), is significantly associated with resistance to radiotherapy. We show in this study that constitutive activation of the PI3K/Akt signaling pathway in *PTEN*-deficient glioma cells can be targeted by caffeine, resulting in radiosensitization. Interestingly, this caffeine-mediated radiosensitization was absent in *PTEN*-proficient glioma cells (Fig. 1). Our results corroborate findings from a recent study that revealed the higher efficacy of several small-molecule inhibitors of PI3K in radiosensitizing glioma cells lacking wild-type PTEN (22). Of importance, not only caffeine but also the less toxic pentoxifylline selectively radiosensitized *PTEN*-deficient glioma cells (Table 1B), supporting the clinical relevance of our finding.

The identification of agents that preferentially radiosensitize tumor cells deficient in genes required for treatment efficacy is clinically highly relevant. This would not only increase the desired effects of therapy in tumor tissue but would also decrease the unwanted side effects because normal tissue lacking such genetic abnormalities would not be affected. It has already been shown in the past that caffeine might belong to such an interesting class of agents because it proved to effectively radiosensitize *p53*-deficient cells but was significantly less effective in *p53*-proficient cells (23, 24). Our results confirmed the p53 independency of caffeine-mediated radiosensitization (23, 24): caffeine radiosensitized U87MG cells (Fig. 1) despite its concurrent slight inhibition of p53 expression and transcriptional activity (Fig. 4) and it was able to radiosensitize the *p53*-deficient cell lines T98G and U373MG (Table 1A). However, our study also provides new evidence for a preferential activity of caffeine in *PTEN*-deficient tumors (Table 1A; Fig. 1). Our study further supports the important role of Akt rather than ATM/ATR in caffeine-mediated radiosensitization of PTEN-deficient glioma cells, and thereby corroborate conclusions from previous reports (19, 22) and a very recent study (25) that Akt represents an important therapeutic target in combination with IR in the treatment of PTEN-deficient brain tumors.

Given the ability of PTEN to indirectly stabilize p53 protein through antagonizing the Akt-MDM2 pathway (4, 10, 26) or by directly increasing p53 acetylation (27), decreased p53 activity in *PTEN*-deficient tumor cells should be expected and would explain the comparably preferential activity of caffeine in radiosensitizing *p53*-deficient (Table 1A; refs. 23, 24) and *PTEN*-deficient tumor cells (Table 1A; Fig. 1). However, the results from the mechanistical studies performed by us (Figs. 2 and 3) by and Powell et al. (1) suggest a distinct mode of interaction of caffeine in cells with *p53*- or *PTEN*-deficiencies: the study of Powell et al. (1) revealed a quantitative difference at the IR-induced G_2-M checkpoint and a different threshold for the caffeine-induced override of G_2-M arrest in *p53*-null compared with *p53* to wild-type cells. In contrast, we observed a significant difference in IR-induced G_1 checkpoint activation and in the potential of caffeine to interfere at this specific DNA damage checkpoint particularly in *PTEN*-deficient compared with *PTEN*-proficient cells (Fig. 2), whereas the degree of G_2-M checkpoint activation and its well-known modulation by caffeine was the same for both cell lines in our model (Fig. 3). The results from our cell cycle studies are in accordance with the recent study of Sturgeon and Roberge (28) who showed in a very comprehensive analysis that caffeine was only able to radiosensitize breast cancer cell lines if given at the time when most cells progress from G_1 into S phase rather than at the time of maximal G_2 arrest. Furthermore, our results are in line with the study of Deplanque et al. (29) who showed in the model of normal human skin fibroblasts that the radiosensitizing effect of caffeine was associated with modulation of G_1 rather than G_2 checkpoint.

In our study, the caffeine-mediated increase of IR-induced G_1 checkpoint activation in U87MG cells did not depend on an increase in IR-induced $p21^{Waf1}$ expression. This result was somewhat surprising because constitutive activation of Akt in *PTEN*-deficient cells should downregulate p53 transcriptional activity and block p53-induced $p21^{Waf1}$ induction (4, 10), both of which should be rescued by PI3K/Akt inhibition by caffeine. This suggests that cell cycle regulators other than $p21^{Waf1}$ might mediate the positive effect of caffeine on G_1 DNA damage checkpoint activation in our model. Alternatively, the interaction of Akt with posttranslational modification rather than transcriptional regulation of $p21^{Waf1}$ might be targeted by caffeine: besides the indirect suppression of $p21^{Waf1}$ expression by triggering MDM2-mediated p53 degradation, Akt has been shown to directly phosphorylate $p21^{Waf1}$ within its nuclear localization sequence, thereby causing its cytoplasmic accumulation and preventing its access to nuclear cyclin-Cdk targets (30). The role of p21 mislocalization rather than reduced transcription in G_1 checkpoint defects and radioresistance of *PTEN*-deficient cells remains to be determined in future studies.

The negative cross-talk between Akt and p53 signaling pathways is not one sided but mutual in its nature. It is well know that the activation of p53 and its target genes such as *PTEN* by IR can negatively interfere with Akt activation (31). Thus, the increased reduction of $p\text{-}Akt^{437}$ levels observed in U87MG cells irradiated in the presence of caffeine (Fig. 4A) might result from IR-induced p53 activation. However, a role of PTEN as mediator of such p53-dependent inhibition of Akt phosphorylation can be excluded in our model of PTEN-deficient glioma cells. Whether or not PHLDA3, a further p53-regulated repressor of Akt identified very recently (32), contributes to the suppression of Akt phosphorylation in PTEN-deficient glioma cells irradiated in the presence of caffeine remains to be determined in future studies.

In conclusion, we have confirmed previous studies that the PI3K/AKT signaling pathway is an attractive target for radiosensitization of glioma cells (19). We show for the first time that the methylxanthine caffeine or the less toxic derivative pentoxifylline are candidate drugs for targeting PI3K/Akt signaling particularly in *PTEN*-deficient glioma cells. Future direct comparison of the therapeutic ratio of caffeine or the related methylxanthine derivative pentoxifyllline with improved pharmacologic

features (24) in tumor compared with normal tissue will reveal the clinical potential of this substance class in the treatment of *PTEN*-deficient glioma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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