Telomere Targeting with a New G4 Ligand Enhances Radiation-Induced Killing of Human Glioblastoma Cells

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Abstract

The aim of this study was to test in vitro the efficacy of TAC, an original G-quadruplex ligand, as a potential radiosensitizing agent for glioblastoma multiforme (GBM). Two human radioresistant telomerase-positive GBM cell lines (SF763 and SF767) were analyzed, with and without TAC treatment, for telomere length, cell proliferation, apoptosis, cell-cycle distribution, gene expression, cytogenetic aberrations, clonogenic survival assay, 53BP1 immunofluorescence staining, and γH2AX phosphorylation. We found that low concentrations of TAC (0.5 and 1 μmol/L) inhibited the proliferation of GBM cells in a concentration-dependent manner after only 1 week of treatment, with minimal effects on cell cycle and apoptosis. TAC treatment had no visible effect on average telomere length but modified expression levels of telomere-related genes (hTERT, TRF1, and TRF2) and induced concentration-dependent DNA damage response and dicentric chromosomes. Survival curves analysis showed that exposure to nontoxic, subapoptotic concentrations of TAC enhanced radiation-induced killing of GBM cells. Analysis of DNA repair after irradiation revealed delayed repair kinetics in GBM cells treated with TAC. Furthermore, the combined treatment (TAC and radiation) significantly increased the frequency of chromosomal aberrations as compared with radiation alone. These findings provide the first evidence that exposure to a G4 ligand radiosensitizes human glioblastoma cells and suggest the prospect of future therapeutic applications. Mol Cancer Ther; 10(10); 1784–95. ©2011 AACR.

Introduction

Glioblastoma multiforme (GBM) is the most common human primary brain tumor with a very poor prognosis. GBM patients are treated by neurosurgery followed by radiotherapy with concomitant administration of temozolomide (1). This treatment is not curative, and the tumor almost always reappears inside the irradiated tumor volume. Increasing the sensitivity of tumor cells to radiotherapy would improve outcome in patients with GBM.

Telomeres are nucleoprotein structures at the end of chromosomes that stabilize and protect them from nucleotide degradation and end-to-end fusion. Telomerase is a ribonucleoprotein complex, with reverse transcriptase activity maintaining telomere length by adding hexameric GGTTAG repeats to the chromosomal ends (2). Telomerase activity is regulated at the expression level of the human telomerase reverse transcriptase (hTERT) gene. In humans, telomerase is expressed in germlinal and stem cells but is repressed in most somatic cells. In contrast, telomerase is active in more than 85% of cancers and allows unlimited proliferation of tumor cells (3). hTERT expression is strongly associated with GBM (4), and patients with high hTERT levels have very short survival (5). Suppression of hTERT in GBM cells induces senescence and inhibits proliferation (6). The telomerase antagonist, imetelstat, can inhibit GBM growth, showing an increased efficacy in combination with radiation and temozolomide (7). In addition, telomeric dysfunction due to genetic deficiency of telomerase activity increases radiosensitivity and decreases the capacity of DNA repair (8). Thus, there is a pathogenetic link between telomere, telomerase, and radiosensitivity.
Targeting telomeres and/or telomerase using G-quadruplex (G4) ligands is a promising strategy for cancer treatment (9). G4 ligands bind to a family of DNA secondary structures called G-quadruplexes, which result from the cation-stabilized stacking of several G-quartets (typically 2–5), each quartet being composed of 4 coplanar guanines. Quadruplexes can be very stable under physiologic conditions. Any genomic sequence containing a repetitive motif with several consecutive guanines can in principle form a quadruplex (10). This is the case for the telomeric TTAGGG motif, which is single stranded at chromosomal ends. Thus, G4 ligands interact with telomeres (11), induce telomere uncapping, and, indirectly, affect telomerase function by preventing telomerase binding on its target (12). Some of these ligands induce proliferation arrest and apoptosis in GBM cell lines (13).

Here, we assessed the ability of an original G4 ligand to inhibit tumor cell proliferation and to enhance the sensitivity to ionizing radiation of 2 human telomerase-positive GBM cell lines.

Materials and Methods

Cell lines

Human GBM cell lines, SF763 and SF767, were obtained from the Brain Tumor Research Center Tissue Bank of the University of California. Cell lines were Mycoplasma free (Mycoplasma Detection Kit; InvivoGen). No authentication of cell lines was done by the authors. Normal skin fibroblasts were obtained from a healthy 30-year-old female donor. Cultures were carried out in Dulbecco’s Modified Eagle’s Media supplemented with 10% of FBS, 1% of nonessential amino acids, and 1% of pyruvate of sodium and gentamicin (Gibco Invitrogen).

Chemical compounds

TAC consists of N-methylated trivalent derivatives of 4,6-bis-(6-(acrid-9-yl)-pyridin-2-yl)-pyrimidine (U.S. patent 20080119492; see Supplementary Methods for synthesis details). Mass spectrometric analyses (liquid chromatography/mass spectrometry and electrospray-mass spectrometry; Supplementary Fig. S1–3) indicated that TAC is a 2:1 mixture of bis-methylated and tris-methylated products. Because of the small amount of material available, the 2 compounds were not separated and the 2:1 mixture was used as such for biological experiments. TAC was dissolved in dimethyl sulfoxide at a concentration of 2 mM/L to produce aliquots of stock solution. The aliquots were stored at −20°C and diluted in culture medium immediately before use to obtain a working solution at 100 µmol/L.

Equilibrium dialysis and fluorescence resonance energy transfer melting assays

Competitive dialysis is an effective method to compare the binding of a compound to different nucleic acid structures. Experiments were carried out as previously described on a panel of single strands, duplexes, i-motif, and quadruplexes (14). Binding was quantified by the absorbance and fluorescence signals of TAC.

Fluorescence resonance energy transfer (FRET) melting assays allow a semiquantitative assessment of quadruplex stabilization. The F21T oligonucleotide was double labeled with fluorescein or carboxytetramethylrhodamine (TAMRA), and apparent melting temperatures were determined in sodium and potassium conditions, with or without added ligand, at various concentrations (micromolar range) as previously described (15).

Proliferation assessment

Cells were grown for 14 days with an intermediate passage after 7 days of culture. After harvesting, trypan blue-negative viable cells were counted. The number of population doublings (PD) after 7 and 14 days was calculated as follows: PD = \log_2(N_f/N_0), where \(N_f\) is the final cell number and \(N_0\) is the initial number of seeded cells.

Cell-cycle analysis

Cell membranes were lysed by immersion in liquid nitrogen. The cell pellet was then resuspended in 500 µL of ribonuclease A (Sigma) and 500 µL of propidium iodide (PI; Sigma) for 20 minutes at room temperature. Cell-cycle distribution and sub-G0–G1 fraction were determined by flow cytometry (EPICS XL; Beckman Coulter) using CellQuest software.

Apoptosis assessment

The Annexin V kit of Caltag Laboratories, Inc., was used to assess apoptosis. Cells (1 × 10^5) were double labeled with PI and anti-human Annexin V antibody and then analyzed by flow cytometry (EPICS XL). Early apoptotic cells were scored as the percentage of Annexin V-positive/PI-negative cells, and late apoptotic and necrotic cells were scored as the percentage of cells staining positive for both Annexin V and PI.

Quantitative real-time reverse transcriptase PCR

Total RNA was extracted from tumor cells using TRIzol reagent (Invitrogen). One microgram of RNA was reverse transcribed with SuperScript II enzyme (Invitrogen). Quantitative real-time reverse transcriptase PCR (qRT-PCR) was carried out with the Light-Cycler system (Roche Diagnostics). The primers used for amplification of hTERT, TRF1, and TRF2 (telomeric repeat binding factors 1 and 2) have been described elsewhere (16, 17). In addition, the amount of ABL transcripts was quantified in all samples as an internal control to normalize the results (18).

Cytogenetics

Cells were harvested after colcemide exposure (0.01 µg/mL) for 20 hours. Chromosomal slides were prepared and stained according to standard methods.
The frequency of chromosomal aberrations was determined by the analysis of 100 metaphases.

Cell irradiation
Photon irradiations were conducted at room temperature with a linear accelerator PHILIPS SL 75-5 (in X-ray production mode) at a dose rate of 3 Gy/min.

Clonogenic survival assay
Clonogenic cell survival was determined by a standard colony-forming assay. Cells (1,500) were plated in culture flasks and were allowed to attach for 24 hours. The following day, flasks were irradiated with 0 to 8 Gy. Cells were reincubated for 10 days, then fixed with methanol, and stained with Giemsa. Colonies containing more than 50 cells were scored. Surviving fractions at each dose were calculated relative to the plating efficiency of nonirradiated control cells.

53BP1 immunofluorescence staining
Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. For immunostaining, cells were incubated with a mouse monoclonal anti-53BP1 (p53-binding protein 1) antibody (Santa Cruz Biotechnology) overnight at 4°C. Cells were then exposed to the secondary fluorescein isothiocyanate–labeled rabbit anti-mouse antibody (Santa Cruz Biotechnology) for 1 hour. The nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI). Fluorescence signals were analyzed with a Zeiss confocal laser scanning microscope.

Determination of γH2AX phosphorylation by flow cytometry
The level of γH2AX phosphorylation was determined by flow cytometry using H2AX Phosphorylation Assay Kit (Upstate) according to the manufacturer’s instructions. Fluorescence intensity, in arbitrary units, was expressed relative to the nonirradiated control. For each experiment, all the compared samples were analyzed the same day.

Statistical analysis
Standard errors were calculated for each mean, and statistical differences between groups were determined by Student’s t test, Kruskal–Wallis H test, or ANOVA using SEM software (19). Survival data were fitted according to the linear quadratic model using KaleidaGraph software (4.0).

Results
Chemical properties of TAC and its interaction with quadruplex (telomeric) DNA
The major component (70%) of TAC (TAC-Me2) comprises 2 acridinium moieties joined by a heterotriaryl spacer containing 2 pyridine rings and 1 pyrimidine ring in alternation (abbreviated as py-pym-py; Fig. 1A). The minor component (TAC-Me3, 30%; Supplementary Fig. S4) has a third methylated site that cannot be precisely attributed (methylated pyridine or pyrimidine). Hence, throughout the present study, all discussion of the data refers to the bis-methylated compound. Owing to the propensity of the py-pym-py spacer to fold in a U-shaped
conformation, TAC-Me2 may possibly adopt a tweezer-type conformation in water (Fig. 1B), similar to that observed for the uncharged and monomethylated analogues in the solid state and in organic solvents (20). Interestingly, tweezer-type molecules holding 2 potentially intercalating units 7 Å apart have a moderate affinity for duplex DNA based on the neighbor exclusion principle (21, 22), which excludes bis-intercalation between contiguous base pairs. In contrast, such compounds can display strong binding affinity for non-B DNA architectures that can provide less sterically constrained binding sites (23). In particular, G4 DNA can accommodate a large diversity of small molecules due to the presence of 3 loops and 4 grooves in the vicinity of the guanines quartets. Although the first generations of G4 ligands were mainly restricted to planar aromatic molecules (24–26), several recent studies reported the design of second-generation quadruplex binders that exhibit a more complex 3-dimensional topology (27–31). For instance, molecular modeling studies have suggested that ligands possessing 2 intercalative aromatic units can establish a dual quartet–loop interaction (32). It was thus of great interest to assess the ability of TAC to bind the quadruplex form of human telomeric DNA, as it is now suggested that this unusual DNA structure affects cancer cell viability. A monomeric acridinium salt CH12-4 was prepared and used as control (Supplementary Fig. S5). This molecule has a planar conformation and most likely behaves in the same manner as a classical duplex DNA intercalator.

Two complementary methods were used to show that TAC is a quadruplex ligand. Competitive dialysis experiments (Fig. 1D) indicate that this compound has a preference for quadruplex structures. These experiments compare the amount of dye bound to a variety of structures (typically 12–20), each trapped in a small dialysis chamber immersed in a large bath containing 1 μmol/L TAC. Binding to the 2 quadruplex samples [22AG and 24G20, corresponding to the human telomeric motif [A(GGGTTA)2GGG] and T2G2T2 sequence, respectively] was stronger than that for duplexes and DNA and RNA single strands [poly(rA), poly(rU), poly(dT), and poly(dU)] or i-motifs (formed with C-rich sequences). Binding to quadruplexes was not exclusive, as lower but proportional topological units (7 Å) were bound to quadruplexes in the solid state and in organic solvents (20). Interestingly, the TRAP assay should be considered as a useful technique to confirm quadruplex recognition rather than one to test its activity against telomerase.

Short-term treatment with G4 ligand TAC inhibits GBM cell growth

SF763 and SF767 cell lines were maintained in culture for 14 days in the presence of 0.5, 1.0, and 2.0 μmol/L of TAC. This treatment inhibited the growth of GBM cells in a concentration-dependent way (ANOVA; \( P < 10^{-7} \); Fig. 2A), with a more marked effect on SF763 cells. Analysis of cellular viability indicated that the treatments were not toxic (<10% Trypan blue–positive cells).

In parallel, we tested the same concentrations of TAC on control normal human fibroblasts. No changes in proliferation were observed (Supplementary Fig. S6), in line with previous studies with other G4 ligands showing that they did not affect the proliferation and viability of normal human telomerase-negative cells like fibroblasts (34) or astrocytes (13). However, the toxicity of TAC to telomerase-dependent normal human cells as hematopoietic cells or other rapidly proliferating cells remains to be explored. In addition, the control compound CH12-4, a classical duplex DNA intercalator, did not inhibit GBM cell proliferation (Supplementary Fig. S7). Taken together, these results indicate the specificity of TAC effects on tumor cell proliferation.

We next analyzed the distribution of cells in different phases of the cell cycle by flow cytometry (Fig. 2B). As compared with untreated controls, the distribution was not significantly modified in SF763 and SF767 cells treated with TAC for 14 days, with only a moderate cell accumulation in the G2–M phase.

To determine whether inhibition of tumor cell growth was related to apoptosis induction, the fraction of early apoptotic cells was evaluated by flow cytometry after 14 days of treatment (0.5–2 μmol/L). The apoptotic fractions were lower (<10%) and closely similar in controls and in cells treated with 0.5 and 1 μmol/L TAC. In contrast, the early apoptotic fraction increased to 32% and 23% (Student’s \( t \) test; \( P < 10^{-3} \)) in SF763 and SF767 cells treated with 2 μmol/L of TAC, respectively (Fig. 2C). There was no significant increase in the fraction of late apoptotic and necrotic cells, which is in agreement with the low percentage of cells stained with Trypan blue. In addition, sub-G0/G1 analysis also detected a significant increase in apoptosis in both cell lines treated with 2 μmol/L TAC (Supplementary Fig. S8).

In the absence of significant changes in cell-cycle distribution and apoptosis rate after treatment with low concentration of TAC, the observed proliferation inhibition may be related to an increase in the cell-cycle duration. To address this point, we estimated the length of cell doubling time in the exponential phase of cell growth, which roughly equals the cell-cycle duration. This estimation was done after 10 days of culturing, with an intermediate passage of cultures after 5 days. The treatment with
1 μmol/L TAC significantly increased the duration of the cell cycle from 23.2 ± 1.3 to 45.4 ± 2.4 hours in SF763 cells (Student’s t test; \( P = 9 \times 10^{-4} \)) and from 22.1 ± 0.9 to 34.0 ± 0.9 hours in SF767 cells \( (P = 7 \times 10^{-4}) \).

**TAC triggers hTERT overexpression and DNA damage but does not induce telomere shortening**

SF763 and SF767 cell lines have relatively short telomeres, with an average length for the telomeric restriction fragments of 4.4 and 3.3 kb, respectively. The treatment with 1 μmol/L TAC did not induce any significant reduction of total or 3'-overhang telomeric DNA, as radioactive signal corresponding to 3'-telomeric single-stranded DNA was roughly the same before and after 14 days of treatment for similar DNA quantities (Supplementary Fig. S9).

The positive telomerase status of the GBM cell lines was confirmed with qRT-PCR, which revealed hTERT expression. We then evaluated expression levels of the hTERT gene as well as TRF1 and TRF2 genes, which encode the core members of the shelterin telomere protection complex, in cells treated with TAC for 14 days (Fig. 3A). In comparison with untreated controls, the expression of hTERT was greatly increased (3-fold for SF763 and 10-fold for SF767 cells) after TAC treatment (ANOVA, \( P = 2.5 \times 10^{-7} \) and \( P = 1.7 \times 10^{-7} \), respectively). The treatment also led to a 2-fold increase in TRF2 expression \( (SF763, P = 6.6 \times 10^{-5}; SF767, P = 4.4 \times 10^{-5}) \) whereas the expression of TRF1 gene was decreased \( (SF763, P = 1.6 \times 10^{-5}; SF767, P = 6.0 \times 10^{-6}) \).

To study the induction of DNA double-strand breaks (DSB), we counted the number of phosphorylated 53BP1 foci by *in situ* immunofluorescence staining. G4 ligands have been reported to induce DNA damage after only a few hours of treatment (34). Indeed, we found that 24-hour treatment with TAC significantly increased the number of 53BP1 foci both in SF763 cells exposed to 0.5 μmol/L TAC \( (P = 4 \times 10^{-4}; H\text{ test}) \),...
The intrinsic radiosensitivity of SF763 and SF767 cells (0–8 Gy). Both GBM lines were found to be radioresistant, as previously observed (35). The survival fraction at 2 Gy was higher for SF763 cells (86%) than for SF767 cells (65%), indicating an even stronger resistance of SF763 cells to radiation.

Subapoptotic, nontoxic concentrations of TAC (0.5 and 1 μmol/L) were selected for combination experiments. Of note, these concentrations did not reduce plating efficiency (data not shown). SF763 and SF767 cells were treated with TAC for 14 days, the time point where TAC had shown an appreciable antiproliferative effect in both cell lines. These cells were then irradiated and clonogenic survival assay was conducted. During incubation, TAC was present in the culture medium. The comparison between survival curves obtained after irradiation alone and after the combined treatment showed a strong concentration-dependent enhancement of radiation sensitivity after TAC treatment (Fig. 4; ANOVA, P < 10−7), showing a synergistic effect in both cell lines. Survival (S) data after irradiation dose (D) were fitted according to the linear quadratic model as follows: S(D)/S(0) = exp[−αD − βD²]. The α and β parameters were used to calculate the dose enhancement factor at 10% survival (DEF10). The DEF10 values for 0.5 and 1 μmol/L TAC were 1.5 and 2.0, respectively.

TAC enhances radiation-induced killing of GBM cells

The intrinsic radiosensitivity of SF763 and SF767 cells was first studied in triplicate experiments by clonogenic survival assay after increasing doses of radiation (0–8 Gy). Both GBM lines were found to be radioresistant, as previously observed (35). The survival fraction at 2 Gy was higher for SF763 cells (86%) than for SF767 cells (65%), indicating an even stronger resistance of SF763 cells to radiation.

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Figure 3. TAC effects at RNA and DNA levels. A, qRT-PCR results for the expression of hTERT and telomere-related genes TRF1 and TRF2. In both cell lines, the treatment with TAC led to an increase in the expression of hTERT and TRF2 genes whereas the expression of TRF1 was decreased after TAC treatment. B and C, DNA breaks in GBM cells, nontreated (NT) or treated with TAC, detected with 53BP1 immunofluorescence. Immunofluorescence staining of SF763 cells was carried out after treatment for 24 hours with TAC. Nuclei were stained with DAPI (blue) and 53BP1 (green). TAC increased the number of 53BP1 foci per cell in a concentration-dependent manner (B). This increase in the number of 53BP1 foci per cell was significant in SF763 cells exposed to 0.5, 1, and 2 μmol/L TAC and for SF767 cells exposed to 1 and 2 μmol/L TAC (C). *, statistically significant difference (P values are given in the text).
TAC were 1.68 and 2.32 in SF763 cells and 1.96 and 2.92 in SF767 cells, respectively.

TAC modifies the repair of DNA DSBs induced by radiation

To investigate possible radiosensitization mechanisms of TAC, we assessed the induction and repair of DSB after irradiation through the study of phosphorylated 53BP1 and γH2AX foci that colocalize with DNA DSB.

Despite extensive analysis (at least 100 cells), we did not detect any consistent increase in DNA break induction after combined TAC (0.5 μmol/L) and radiation (2 Gy) treatment by in situ immunofluorescence analysis for 53BP1. Initial levels of foci at 0.5 hours after irradiation were around 30 per cell. The repair was apparently efficient after this dose, as the numbers of residual foci 24 hours following irradiation were very low (<3 foci per cell).

Because the radiosensitizing effect of TAC was stronger at higher radiation doses, we applied a different approach with flow cytometric analysis of phosphorylated γH2AX (36), which enabled us to use the radiation dose of 6 Gy. We measured the initial peak at 0.5 hours and signal reduction at 3 and 24 hours after irradiation. No significant difference was noted at 0.5 and 3 hours between treated (0.5 μmol/L) and nontreated cells. In contrast, a higher residual level in the presence of TAC was observed at 24 hours (P = 0.016 for SF763 and P = 0.04 for SF767; Fig. 5A), indicating a significant deceleration of DNA repair.

These results suggest that TAC affected DNA repair after irradiation. To further investigate this hypothesis, we analyzed the frequency of radiation-induced chromosomal aberrations after irradiation with 4 Gy. The treatment with 0.5 μmol/L TAC for 14 days significantly increased the frequency of both chromosome-type and chromatid-type aberrations (Table 1). Of note, the highest increase was observed in the frequency of dicentric chromosomes (Fig. 5B) after subtraction of the background incidence of dicentrics induced by TAC alone.

In addition, we conducted cell-cycle analysis at 48 hours after irradiation with 6 Gy in untreated cultures and in cultures treated with 1 μmol/L of TAC for 14 days. Both GBM cell lines treated with TAC exhibited a significant increase in percentage of cells in the G2–M phase (P < 0.01), reflecting a more important radiation-induced G2-phase block (Fig. 5C).

To address the issue of apoptosis induction after TAC and radiation, we conducted Annexin V/PI staining and flow cytometric analysis of cells harvested 3, 6, 24, 48, and 72 hours after treatments with either radiation (6 Gy) alone or radiation (6 Gy) in combination with 1 μmol/L TAC. During this time course, both early and late apoptosis/necrosis was slightly but not significantly induced by the treatments (data not shown).

Discussion

TAC is a quadruplex ligand active on telomeric DNA in vitro. Telomere damage and DNA damage response induced by G4 ligands such as RHP54, BRACO19, and
telomestatin (37–39) lead to a short-term inhibition of proliferation and rapid apoptosis of tumor cells. In line with these reports, we found that low concentrations of TAC (0.5 and 1 μmol/L) inhibited the proliferation of GBM cells in a concentration-dependent manner after only 1 week of treatment. The effects of these low concentrations on cell-cycle distribution and apoptosis were minimal, but the treatment apparently increased the cell-cycle length, which can explain the decreased cell proliferation rate. However, TAC could also induce rapid and substantial apoptosis (up to 32% of cells) at a higher concentration (2 μmol/L). Antitumor properties of several G4 ligands (pyridine dicarboxamide derivatives) have already been tested on GBM cell lines by Pennarun and colleagues (13). They showed an early antiproliferative effect and a massive apoptosis (35% of cells) in cultures treated with a G4 ligand at 5 μmol/L concentration. Tumor cell apoptosis could be the linking factor between telomere instability, DNA damage response, and decreased proliferation.

G4 ligands were initially evaluated as telomerase inhibitors, but their antiproliferative effect may occur quickly, before any telomere shortening takes place (40). This short-term effect cannot be explained by telomerase inhibition, which would lead to a gradual shortening of telomeres after a certain number of cell divisions. The observations that G4 ligands delocalize telomeric proteins from telomeres (34, 41), activate DNA damage...
response at telomeres, and induce chromosomal end-to-end fusions (38) strongly suggest that their direct target is the telomere and not the telomerase enzyme.

In agreement with these reports, we found that TAC caused DNA damage without significant telomere shortening. This damage was, in particular, revealed by an increase in chromosomal fusions in both GBM cell lines. Pennarun and colleagues (13) also found in GBM cells treated with G4 ligands a very clear increase in telomeric fusions in metaphase and anaphasic bridges, evidence of telomeric instability not related to telomere attrition.

The chromosome end fusions are formed when telomeres are dysfunctional and are recognized as DSBs by repair machinery, which joins chromosomal ends. Dysfunctional (uncapped) telomeres, resulting from quadruplex DNA stabilization by G4 ligand, associate directly with many DNA damage response proteins. This DNA damage response may be evidenced by the appearance of phosphorylated 53BP1 or H2AX foci in cells exposed to G4 ligands, of which about 70% are located at telomeres (34). In the present study, we found a rapid and significant increase in the number of 53BP1 foci after TAC treatment.

The structure and function of telomeres are controlled both by telomerase and by the shelterin complex. Previous studies showed that tumor cells overexpressing hTERT or TRF2, a shelterin complex component, become resistant to treatments with G4 ligands (34, 42). These changes were considered to represent compensatory defense mechanisms activated in response to G4 ligand treatment, which is consistent with the view that telomere is a direct target of G4 ligands (34, 42). In our study, the effect of TAC on telomeres was also evidenced by changes in the expression of telomere-related genes in treated cells. In particular, we observed a strong increase in the expression of hTERT, which might counteract the deleterious effects of the stabilization of G4 DNA by a ligand through the extension of the single-stranded G-rich telomeric portion, which is termed 3’ overhang (42). We also found increased TRF2 expression, which could facilitate the formation of a loop structure (t-loop), masking the 3’ overhang against G4 ligand binding, and/or the recruitment and stimulation of RecQ helicases, which resolve G4 DNA structures (32). In addition, we observed a concomitant decrease in the expression of TRF1, which might contribute to hTERT-dependent telomere elongation. TRF1 controls the access of telomerase to telomeric DNA, and its downregulation was shown to result in the elongation of telomeres (43).

Thus, our results indicate that TAC targets telomeres, thereby increasing telomeric instability in tumor cells, and this inhibits tumor growth and, at higher doses, leads to tumor cell apoptosis. As telomeric dysfunction was reported to increase radiosensitivity and to decrease DNA repair capacity (8), we then investigated the impact of telomeric instability induced by TAC on the radiosensitization of GBM cells.

Applying low concentrations of TAC, which itself did not induce apoptosis or cell-cycle changes, we found a strong concentration-dependent radiosensitizing effect on GBM cell lines within a wide dose range, including the dose corresponding to the daily therapeutic radiation dose (2 Gy) administered to GBM patients in clinical practice. This result is significant, because the administration of temozolomide, the new standard chemotherapy (1) given concomitantly with radiotherapy, is based on

### Table 1. Chromosome aberration frequencies in GBM cell lines after irradiation and after combined treatment with radiation and G4 ligand TAC

<table>
<thead>
<tr>
<th>Cells analyzed</th>
<th>Acentries per cell (95% CI)</th>
<th>Dicentrics per cell (95% CI)</th>
<th>Chromatid fragments per cell (95% CI)</th>
<th>Chromatid exchanges per cell (95% CI)</th>
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<tbody>
<tr>
<td>SF763 cells</td>
<td></td>
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<tr>
<td>Irradiated (4 Gy)</td>
<td>0.34 (0.21–0.47)</td>
<td>0.32 (0.19–0.44)</td>
<td>1.12 (0.89–1.34)</td>
<td>0.31 (0.19–0.43)</td>
</tr>
<tr>
<td>Irradiated (4 Gy) and treated with 0.5 μmol/L TAC</td>
<td>0.85 (0.62–1.08)</td>
<td>1.02 (0.83–1.21)*</td>
<td>1.72 (1.45–1.99)</td>
<td>0.81 (0.58–1.04)</td>
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<tr>
<td>P (H test)</td>
<td>0.011</td>
<td>&lt;10^-6</td>
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<tr>
<td>SF767 cells</td>
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<tr>
<td>Irradiated (4 Gy)</td>
<td>0.46 (0.32–0.59)</td>
<td>0.38 (0.24–0.52)</td>
<td>0.86 (0.63–1.09)</td>
<td>0.24 (0.11–0.37)</td>
</tr>
<tr>
<td>Irradiated (4 Gy) and treated with 0.5 μmol/L TAC</td>
<td>0.86 (0.64–1.07)</td>
<td>0.80 (0.62–0.98)*</td>
<td>1.42 (1.12–1.72)</td>
<td>0.58 (0.39–0.77)</td>
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<tr>
<td>P (H test)</td>
<td>0.043</td>
<td>0.00076</td>
<td>0.0093</td>
<td>0.0068</td>
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</tbody>
</table>

*The background frequencies of dicentric chromosomes induced by TAC alone in nonirradiated cells (0.09 and 0.06 for SF763 and SF767 cells, respectively) were subtracted.
preclinical data (44, 45), which suggested that a combination of temozolomide with radiation was essentially additive.

Radiosensitivity in an animal model with dysfunctional telomeres correlated with a deceleration of DNA repair kinetics and persistence of chromosomal aberrations (8). We investigated whether TAC induced these effects in GBM cell lines. There is a correspondence between the formation and subsequent loss of phosphorylated γH2AX or 53BP1 foci and the recognition and subsequent repair of DNA damage. On the basis of this, MacPhail and colleagues (36) and Olive and Banath (46) proposed that the half-loss time of phosphorylated γH2AX and the residual rate could be significant factors in the evaluation of cell response to ionizing radiation, with more rapid loss and less retention associated with more radioreistant cell lines. In our study, analysis of DNA repair after irradiation by a similar approach revealed delayed repair kinetics in GBM cells treated with TAC. The persistence of significant amounts of residual phosphorylated γH2AX was observed in both cell lines, indicating that the GBM cells had become more sensitive to radiation.

An increase in radiosensitivity was also correlated with a higher frequency of radiation-induced chromosomal aberrations after combined treatment with radiation and TAC as compared with radiation alone. Dysfunctional telomeres, which act as DNA breaks, might join radiation-induced DNA DSB, thus increasing radiation sensitivity (47). As TAC induces telomere dysfunction, we suggest that this mechanism was involved in the radiosensitization of GBM cells observed after TAC treatment. Notably, the highest increase was seen in the frequency of dicentric chromosomes, which could be due to DSB–telomere joinings.

Cells exposed to ionizing radiation show delay or arrest in the cell-cycle progression. In particular, G2-phase block prevents G2 cells with damaged DNA entering mitosis. The DNA damage can be reduced by means of DNA repair, but heavily damaged cells are unable to reach mitosis and are arrested in the G2 phase. G2–M accumulation after irradiation in GBM cells treated with TAC compared with untreated cells may reflect the presence of cell population with increased DNA damage. This cell-cycle arrest can explain, at least in part, the radiosensitizing effect of TAC at the cellular level.

We did not observe any significant apoptosis induction up to 72 hours after treatment with radiation and TAC. This result may be explained by the fact that the cells were blocked in the G2–M phase after radiation, which was particularly marked after the combination of radiation and TAC. This blockage delays cell death, which could occur much later, after 1 or more cell divisions (48).

Here, we showed efficacy of TAC combined with radiation on GBM cells in vitro. Next step will be to test the radiosensitizing effect of intratumoral injections of TAC in vivo, using subcutaneous human GBM xenografts in animals. Because one of the major problems in brain tumor therapy remains the difficulty of drug delivery across the blood–brain barrier, it will also be essential to evaluate in an orthotopic model whether TAC is capable of crossing the blood–brain barrier to reach and radiosensitize GBM tumors. Another possibility can be the direct injection of G4 ligand into the tumor-infiltrated brain parenchyma under positive pressure using convection-enhanced delivery. Conversely to conventional diffusion, convection-enhanced delivery creates a homogenous drug concentration extending over several centimeters in diameter, allowing to target brain tumor tissue as well as the area surrounding the tumor, which may contain tumor cells. This approach is successfully used to test novel antitumor agents in animal glioma models and in clinical trials with acceptable safety (49).

In conclusion, we showed that TAC, an original G4 ligand, could enhance the sensitivity to ionizing radiation of 2 human hTERT-positive GBM cell lines. A possible mechanism of radiosensitization is related to delayed DNA break repair after irradiation, which increases the number of chromosomal abnormalities such as dicentrics, leading to cell death. Our work thus provides the first evidence that exposure to a G4 ligand radiosensitizes radiosensitive human GBM cells. Radiosensitizing effects will be investigated with other G4 ligands. Many elements concerning molecular mechanisms of altering tumor growth with TAC have to be elucidated. In particular, it will be interesting to study the effects of TAC on G4 ligands located outside telomeric regions, in promoters of human genes, which are involved in cell proliferation (50). Further studies in animal models using human GBM tumor xenografts are necessary to explore the therapeutic potential of this radiosensitizing approach.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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