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PRIORITY REPORT

Protection of normal brain cells from γ-irradiation-induced apoptosis by a mitochondria-targeted triphenyl-phosphonium-nitroxide: a possible utility in glioblastoma therapy

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Abstract Glioblastoma multiforme is the most frequent and aggressive primary brain tumor. A strong rationale to identify innovative approaches to treat these tumors is required since treatment failures result in local recurrences and median survivals range from 9 to 12 months. Glioma cells are reported to have less mitochondrial content compared to adjacent normal brain cells. Based on this difference, we suggest a new strategy, utilizing protection of normal brain cells by mitochondria-targeted electron scavengers and antioxidants—nitroxides—thus allowing for the escalation of the radiation doses. In this paper, we report that a conjugate of nitroxide with a hydrophobic cation, triphenyl-phosphonium (TPEY-Tempo), significantly protected brain endothelial cells from γ-irradiation-induced apoptosis while radiosensitizing brain tumor cells. Thus, TPEY-Tempo may be a promising adjunct in the treatment of glioblastoma with the potential to not only prolong survival but also to maintain quality of life and reduce treatment toxicity.

Keywords Glioblastoma · Mitochondria · Triphenyl-phosphonium conjugated nitroxide · γ-irradiation · Apoptosis

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Abbreviations

CL	Cardiolipin
COX IV	Cytochrome *c* oxidase subunit IV
Cyt	Cytochrome
PS	Phosphatidylserine
ROS	Reactive oxygen species
TPEY-Tempo	[2-(1-Oxyl-2,2,6,6-tetramethyl-piperidin-4-ylimino)-ethyl]-triphenyl-phosphonium

Introduction

Glioblastoma multiforme is a locally aggressive and invasive glial tumor of the central nervous system. Median survivals range from 9 to 12 months despite decades of progress in surgical technique, advances in the delivery of radiation therapy and the development of new chemotherapeutics. These dour statistics provide a strong rationale to identify innovative approaches to treat these tumors. Current therapy for malignant gliomas includes post-operative radiation therapy combined with temozolomide chemotherapy [1]. Radiation therapy has been shown to be the single most active treatment for these tumors after surgical resection. Standardization of the dose to 60 Gy in 30 fractions at 2 Gy per fraction delivered over a 6-week period has yielded only modest increases in survival [2]. The initial radiation induced tumor cell death is eventually overwhelmed by local recurrence leading to patient's death. Effective radiation and chemotherapy dose escalation is limited by treatment-induced neurological damage of surrounding normal brain cells.

Apoptosis is one of the major mechanisms of radiation induced cell death [3], which is mainly through a mitochondria-dependent intrinsic apoptotic pathway. The early

essential event of this pathway is mitochondria-specific phospholipid-cardiolipin oxidation by cytochrome (cyt) *c* following formation of reactive intermediates of peroxidase complexes of cytochrome *c* with cardiolipin (CL) and enhanced production of reactive oxygen species [4]. Redistribution of CL from the inner into the outer mitochondrial membrane, formation of the peroxidase cyt *c*/CL complex, peroxidation of CL, mitochondrial permeabilization and release of cyt *c*, along with other pro-apoptotic factors, from mitochondria into the cytosol designate a point-of-no-return in the execution of apoptotic program [5]. Superoxide radicals and their dismutation product, H_2O_2, produced spontaneously or via superoxide dismutase catalyzed reactions, are essential for feeding the peroxidase cycle of CL oxidation [5]. For this reason, elimination of intracellular reactive oxygen species (ROS), particularly its major source, mitochondrial ROS, by antioxidants may be effective in protecting cells against irradiation-induced apoptosis, thus offering a promising therapeutic opportunity for radioprotection/mitigation. Many nitroxides have demonstrated radioprotection in a standard radiation dose–response clonogenic assay [6]. TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl)—a generic nitroxide has been extensively studied for its radioprotection effect in vitro and in vivo [7–11]. Though it is found radioprotective, the required high millimolar concentrations, partially due to its poor partitioning into cells and mitochondria, limits for broader applications [12]. Recent developments in targeted delivery of antioxidants, from nitroxides to mitochondria, offer new opportunities in the radioprotection of cells and tissues [13, 14]. Triphenylphosphonium derivatives of vitamin E, ubiquinone, *N-tert*-butyl-α-phenylnitrone (and lately nitroxides) were the first antioxidant molecules found to predominantly compartmentalized into mitochondria [15, 16].

Previously, we have shown that a conjugate of 4-amino-2,2,6,6-tetramethyl-piperidine-*N*-oxyl with a hydrophobic cation, triphenyl-phosphonium moiety, TPEY-Tempo (Fig. 2a)—accumulated preferentially in the mitochondria of mouse embryonic cells resulting in decreased ROS generation and protection of the cells from γ-irradiation-induced apoptosis [16]. We reasoned that the decreased mitochondrial content in tumor versus normal tissue can be utilized for the therapeutically beneficial prevention of normal cell death by mitochondria-targeted antioxidants. Our target in this model is the radiation-sensitive endothelial cells, which has been shown to play an important role in the late central nervous system toxicity after radiation [17]. Endothelial cells may therefore represent an important target for protective strategies aimed at dose escalation to effectively eradicate tumor.

In the current work, we compared the mitochondria level in human brain microvascular endothelial cells (BB19) and glioma cells (T98G) and showed that the former had significantly higher content of mitochondria than the latter. Accordingly, TPEY-Tempo dramatically increased its integration efficiency in intracellular and mitochondrial compartments of BB19 cells and protected them against γ-irradiation-induced apoptosis, while significantly sensitized glioma cells to irradiation-induced death.

Materials and methods

Materials

Glioblastoma clinical specimens were from tumor bank of University of Pittsburgh. TPEY-Tempo was prepared as described previously [16]. The Annexin V kit was purchased from Biovision (Mountain View, CA). Antibody against cytochrome *c* oxidase subunit IV (COX IV) was purchased from Abcam (Cambridge, MA). Anti-cyt *c* antibody was purchased from BD Pharmingen (San Diego, CA) and anti-actin antibody was from Novus (Littleton, CO). Unless indicated, all other reagents were from Sigma (St. Louis, MO).

Cell culture

Immortalized human brain endothelial cells (BB19, generous gift from Dr. Moses, Vaccine and Gene Therapy Institute, Oregon Health Sciences University) maintained in human Endo-SFM (Invitrogen, Carlsbad, CA) containing 10% human AB-serum (Sigma), endothelial cell growth supplement (ECGS, Fisher, Pittsburgh, PA), 100 U/l penicillin, and 100 μg/l streptomycin. T98G human glioblastoma cells (ATCC) were grown in minimal essential medium (MEM) supplemented with Earle's, 10% fetal bovine serum (FBS), 1× nonessential amino acids, 110 mg/l pyruvate, 100 U/l penicillin, and 100 μg/l streptomycin.

Irradiation

Cells were cultured in fresh medium, prior to irradiation and were γ-irradiated with a Shepherd model 143–45A irradiator (J.L. Shepherd & Associates, CA) at indicated dose. Cells were incubated with nitroxides in complete culture medium during pre- (30 min) or post-irradiation (30 min) treatment. Cells were then incubated at 37°C in 5% CO_2 incubator until harvest.

Western blot analysis

Samples were separated on 15% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with antibodies against COX IV (Abcam), cyt *c* (clone 7H8.2C12,

BD Pharmingen) or actin (Novus) (loading control) followed by horseradish peroxidase-coupled detection. The protein band profile was analyzed by densitometry using Labworks Image Acquisition and Analysis Software (UVP, Upland, CA).

Mitochondria staining by MitoTracker Red

Cells were incubated with 100 nM MitoTracker Red (Invitrogen) for 30 min in 5% CO_2 incubator and then applied to slides. The slides were mounted in Fluoromount-G (Southern Biotech) and examined using an Olympus Fluoview 1000 confocal microscope (Malvern, NY, USA).

EPR-based analysis of nitroxides partitioning into mitochondria

To examine the partitioning efficiency, BB19 and T98G cells (2×10^6/mL) were incubated with 10 μM nitroxide for 30 min in medium at room temperature. At the end of incubation, cells were washed twice with medium. ESR spectra of nitroxide radicals in cells or the mitochondrial fraction were recorded after mixing with acetonitrile (1:1 v/v) after a 5-min incubation with 2 mM $K_3Fe(CN)_6$ using JEOL-RE1X EPR spectrometer under the following conditions: 3350G center field; 25G scan range; 0.79G field modulation, 20 mW microwave power; 0.1 s time constant; 4 min scan time. Mitochondria-enriched fraction was obtained by differential centrifugation. Briefly, cells were suspended in mitochondria isolation buffer (210 mM mannitol, 70 mM sucrose, 10 mM Hepes KOH, pH 7.4, 1 mM EDTA, 0.1% BSA and cocktail protease inhibitor) and disrupted by Dounce homogenization. Unbroken cells, nuclei, and debris were removed by 10 min centrifugation at $700 \times g$ at 4°C. Mitochondria-rich fraction was obtained by 10 min centrifugation at $5{,}000 \times g$, and washed twice with mitochondria isolation buffer. Partitioning efficiency was calculated as percentage of initial signal. Amounts of nitroxide radicals integrated into mitochondria were normalized to the content of COX IV.

Measurements of phosphatidylserine externalization and caspase 3/7 activity

Externalization of phosphatidylserine was analyzed by flow cytometry using Annexin V kit (Biovision). In brief, harvested cells were stained with Annexin V-fluorescein isothiocyanate and propidium iodide 5 min in the dark before flow cytometry analysis. Cell debris as represented by distinct low forward and side scatter were gated out for analysis. Ten thousand events were collected on a FACScan flow cytometry (BD Biosciences, Rutherford, NJ) supplied with CellQuest software. Caspase 3/7 activity was measured using a luminescence Caspase–GloTM 3/7 assay kit (Promega, Madison, WI) according to the manufacturer's instruction.

Assessments of cyt *c* release from mitochondria

Cells were harvested and re-suspended in lysis buffer A (250 mM sucrose, 20 mM HEPES KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) containing 0.05% digitonin for 4 min on ice, and then centrifuged at $10{,}000 \times g$ for 10 min. The resulting supernatants were collected as cytosolic fractions.

Statistics

Data are expressed as mean ± S.D. as indicated in figure legends. Changes in variables for different assays were analyzed by a two-tailed Student's *t*-test for comparisons. Differences were considered significant at $P < 0.05$.

Results

Evaluation of mitochondria levels in normal brain tissues and glioma tissues

To determine the content of mitochondria in human glioma operative specimens and normal brain specimens obtained at the time of resection, we assessed the level of a mitochondria-specific protein—cytochrome *c* oxidase subunit IV (COX IV)—by Western blotting. We measured 35 clinical specimens including nine normal brain samples collected during the surgical approach to deep tumors and 26 glioma samples from the tumor bank at the University of Pittsburgh. The combined results from all the clinical specimens showed that relative amounts of COX IV in glioma tissues were significantly lower when compared to normal brain tissues ($\sim$15.3-fold, $P < 0.01$) (Fig. 1b). In addition, we also demonstrated in matched pairs of normal and tumors specimens form the same patients that the glioma samples had dramatically decreased COX IV amounts compared to paired adjacent normal brain specimens as demonstrated in Fig. 1a.

Analysis of the mitochondrial content in BB19 cells and T98G cells

To quantify the mitochondrial content in BB19 and T98G cells, we initially used a mitochondria-specific staining with Mitotracker Red. Figure 1c shows that BB19 cells had a much stronger fluorescence than that detected from T98G cells, indicating higher mitochondrial mass in BB19 cells.

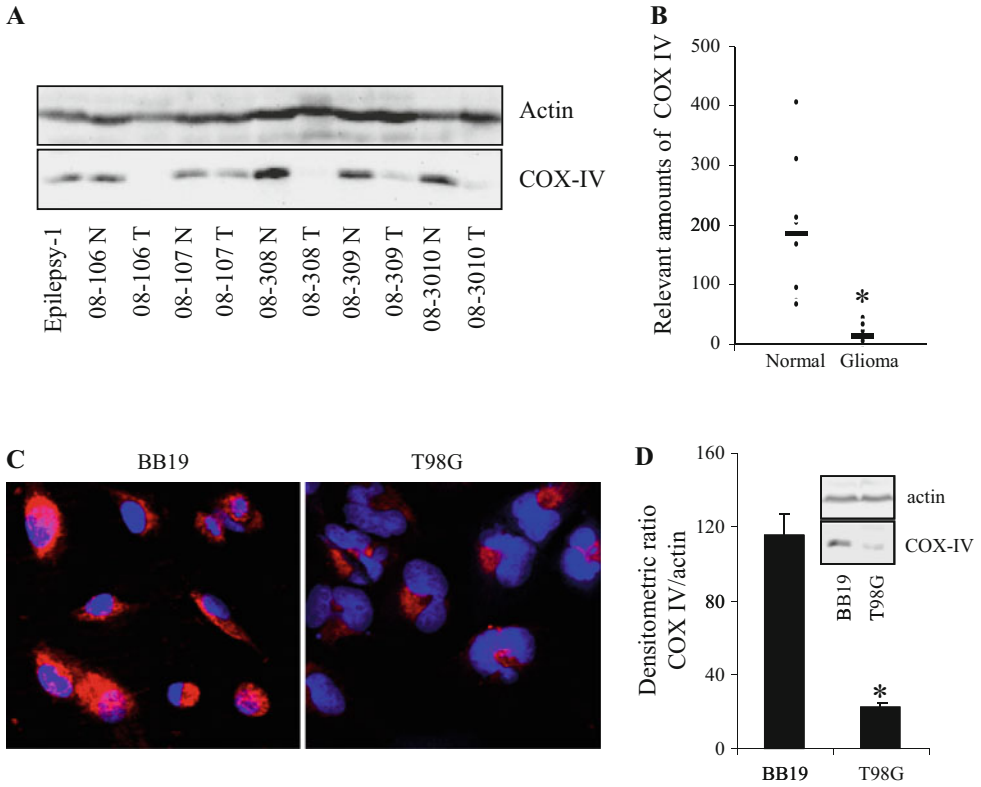

Fig. 1 Evaluation mitochondrial content in normal brain tissues, glioma tissues, BB19 and T98G cells. **a** Representative Western blotting analysis of COX IV level in paired normal brain tissues (N) and glioblastoma specimens (T). **b** Statistical results of relevant amounts of COX IV in 9 normal brain tissues and 26 glioblastoma specimens. * $P < 0.01$ vs. normal brain tissues. **c** Evaluation of mitochondria level by MitoTracker Red staining. Cells were incubated with 100 nM MitoTracker Red for 30 min and then applied to slides, which were mounted in Fluoromount-G and examined using an Olympus Fluoview 1000 confocal microscope (20× objective len). Representative confocal image was presented. **d** Analysis of COX IV level in BB19 and T98G cells. The amount of COX IV was determined by Western blotting and densitometry. The relative level of COX IV was expressed as the mean densitometric ratio of COX IV over actin (loading control). *Insert* was a representative Western blotting of three independent experiments. Data presented are means ± S.D. ($n = 3$). * $P < 0.01$ vs. BB19 cells

Since the accumulation of MitoTracker Red in mitochondria is membrane-potential dependent, the estimate of mitochondria mass might be cell type specific. Further support for the decreased mitochondrial content in T98G cells was obtained by the examination of mitochondria-specific COX IV by Western blotting. As shown in Fig. 1d, relative amounts of COX IV in T98G cells were significantly lower than those in BB19 cells (~5.2-fold, $P < 0.01$). Taken together, we suggested that T98G cells have decreased the mitochondrial content compared to BB19 cells.

Integration of TPEY-Tempo into cells and mitochondria

Integration of triphenylphosphonium-conjugated compounds into cells and mitochondria have been reported to be selectively targeted into mitochondria [15, 16]. We measured the cellular and mitochondrial integration of TPEY-Tempo in BB19 and T98G cells by EPR spectroscopy. After incubation of cells with 10 μM TPEY-Tempo at room temperature for 30 min, no EPR signal from the nitroxide moiety was detected in cell pellet (Fig. 2b). However, a characteristic triplet signal was recovered from cells, upon addition of an one-electron oxidant, ferricyanide (Fig. 2b). Similarly, in mitochondrial fractions, detectable EPR signals were only recorded in the presence of ferricyanide. This indicates that the integrated nitroxide was reduced to corresponding hydroxylamine, likely by mitochondrial electron transport, resulting in the loss of its radical nature and EPR signal. The mitochondrial integration of TPEY-Tempo versus its total amount integrated in BB19 and T98G cells, were 38.5 ± 7.7 and 16.4 ± 2.5%, respectively (Fig. 2d).

TPEY-Tempo protects BB19 from γ-irradiation-induced apoptosis and enhances radiosensitivity of T98G cells

Time-dependent response of T98G to γ-irradiation was checked using PS externalization. As shown in Fig. 3a,

only marginal amount of apoptotic cells was observed 24 h post irradiation, while at 96 h post irradiation about 41.1% percent of cells externalized PS on their surface. In the subsequent experiments, we chose 96 h as the end time-point for measuring apoptotic events (PS externalization, cyt *c* release and caspase activation) to evaluate the radioprotective effect of TPEY-Tempo. Cells were incubated with nitroxides (25, 50 and 100 μM) for 30 min prior to γ-irradiation. PS externalization revealed that TPEY-Tempo significantly protected BB19 cells from γ-irradiation-induced PS exposure on cell surface. On the contrary TPEY-Tempo enhanced PS externalization in T98G cells. As demonstrated in Fig. 3b and c, after 96 h, percentage of PS positive cells was decreased from 31.6 to 15.8% in BB19 cells treated with 25 μM TPEY-Tempo ($P < 0.01$ vs. irradiated cells without TPEY-Tempo treatment), while increased from 42.3 to 59.3% ($P < 0.05$ vs. irradiated cells without TPEY-Tempo treatment) in T98G cells. At higher concentrations of TPEY-Tempo (50 and 100 μM), BB19 cells were also protected from γ-irradiation-induced PS externalization. Notably, more than 98% of T98G cells treated with these higher doses of TPEY-Tempo were PS positive thus demonstrating a significant radiosensitization effect of TPEY-Tempo on tumor cells.

We further assessed effect of TPEY-Tempo on cyt *c* release induced by irradiation in both types of cells. Cyt *c* release from mitochondria into the cytosol was significantly suppressed by TPEY-Tempo added to BB19 cells either pre- or post- irradiation ($P < 0.01$ vs. irradiated cells). In contrast, T98G cells loaded with TPEY-Tempo responded by a markedly increased cyt c accumulation in the cytosol (Fig. 3d and e). Furthermore, TPEY-Tempo (25 μM) attenuated irradiation-induced caspase 3/7 activation in BB19 cells (a 3.8-fold decrease vs. irradiated cells without TPEY-Tempo). The caspase activity was significantly potentiated by TPEY-Tempo in irradiated T98G cells under the same conditions (a 1.3-fold increase vs. irradiated cells without TPEY-Tempo treatment) as shown in Fig. 3f.

This phenomenon of elevated radiosensitivity was also observed in another glioma cell line, U87MG. As shown in

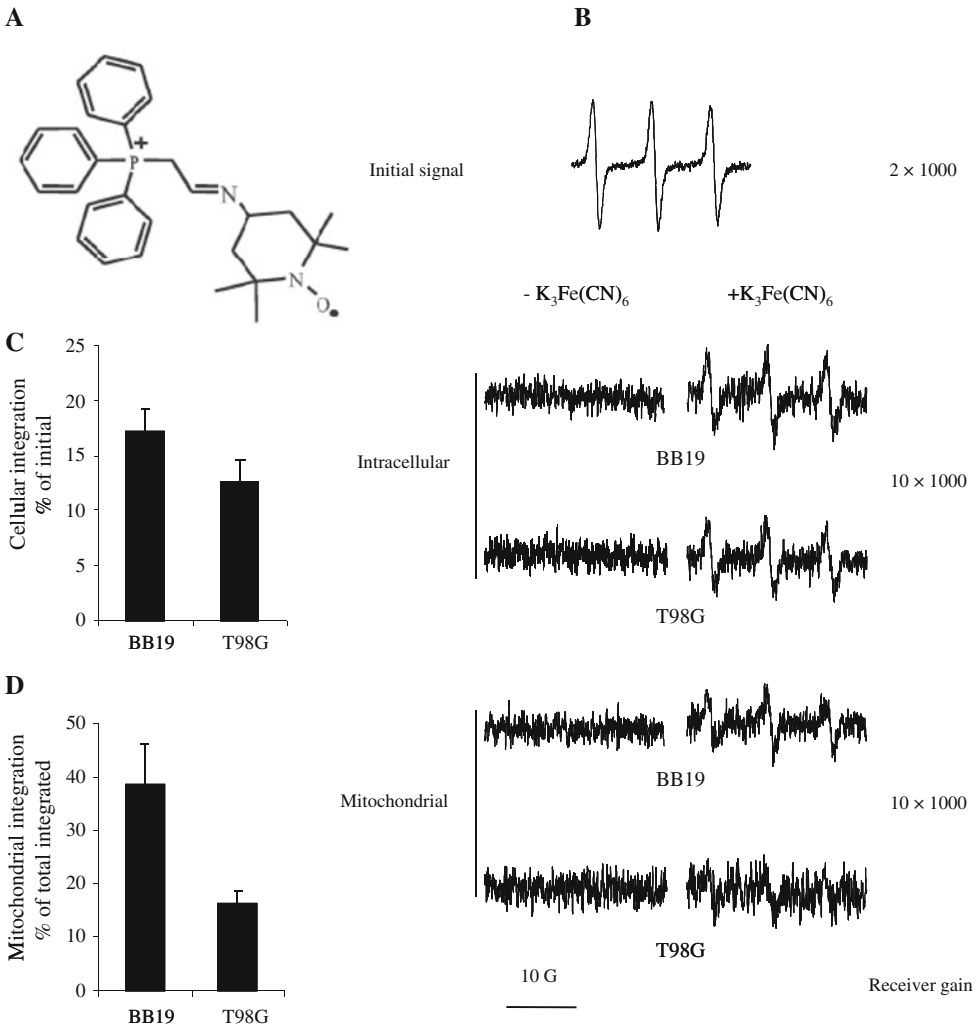

Fig. 2 **a** Structure of TPEY-Tempo. **b** Intracellular and mitochondrial integration of TPEY-Tempo in BB19 and T98G cells by EPR spectrometry. Cells were incubated with 10 μM nitroxide for 30 min at room temperature. Mitochondria were obtained by differential centrifugation. EPR spectra of nitroxide radicals in cells or mitochondrial fraction were recorded after 5-min incubation with 2 mM $K_3Fe(CN)_6$ using JEOL-RE1X EPR spectrometer under the following conditions: 3350G center field; 25G scan range; 0.79G field modulation, 20 mW microwave power; 0.1 s time constant; 4 min scan time. Spectra presented are representative of the three independent measurements. **c**, **d** Quantitative analysis of cellular and mitochondrial integration of TPEY-Tempo. Partitioning efficiency was calculated as the percentage of initial signal. Amounts of nitroxide radicals integrated into mitochondria were normalized to the content of COX IV

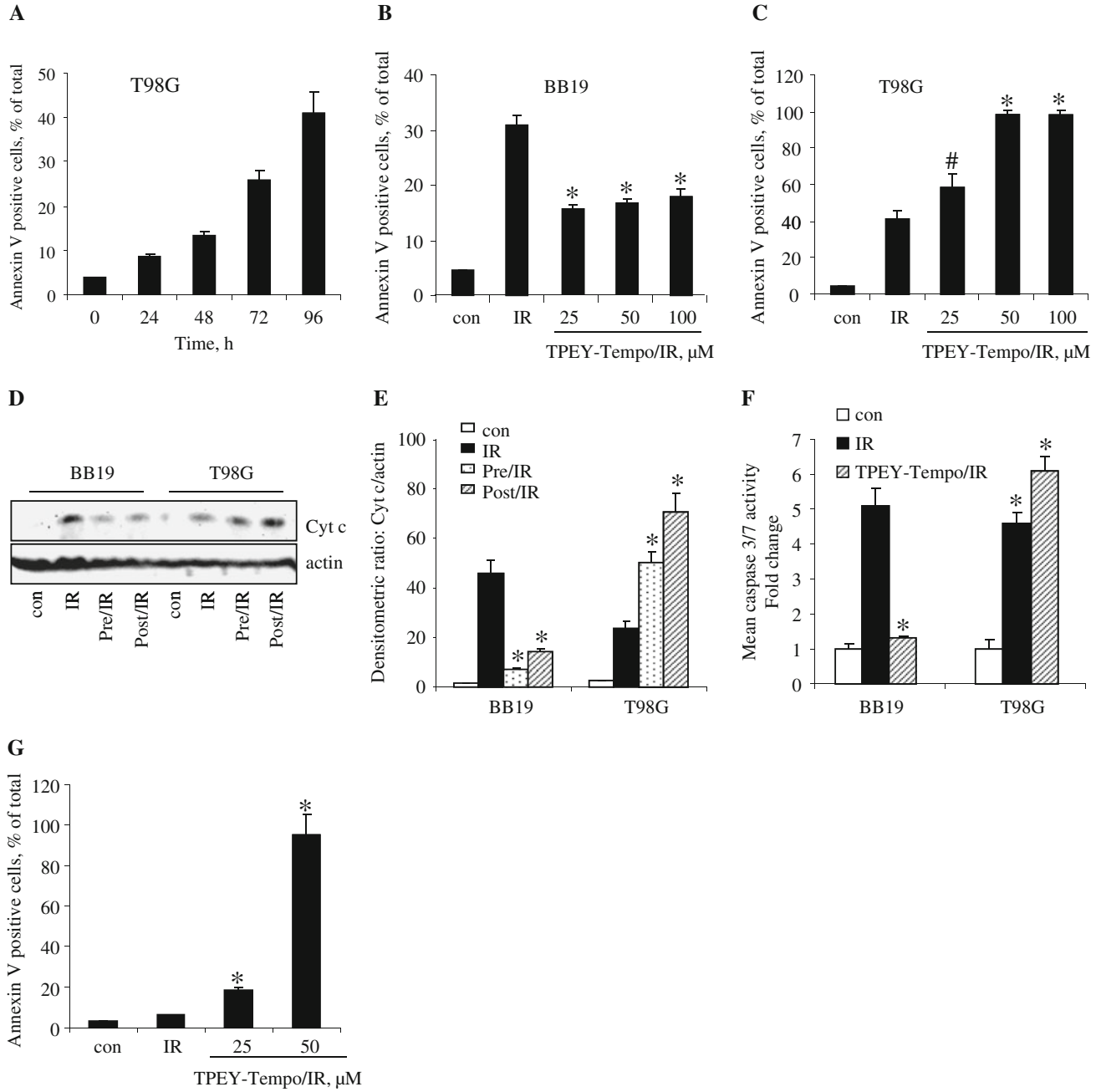

Fig. 3 Effect of TEPY-Tempo on γ-irradiation-induced apoptosis in BB19 and T98G cells. **a** Evaluation time course of phosphatidylserine externalization induced by γ-irradiation (25 Gy) in T98G cells. **b**, **c** Effect of TPEY-Tempo on γ-irradiation-induced phosphatidylserine externalization in BB19 and T98G cells. Cells were treated with TPEY-Tempo (25, 50, and 100 μM) 30 min prior to γ-irradiation (25 Gy). Externalization of phosphatidylserine was assessed by flow cytometry using an Annexin V/PI kit after 96 h post-irradiation incubation. **d**, **e** Effect of TPEY-Tempo on γ-irradiation-induced cyt *c* release in BB19 and T98G cells by Western blotting and densitometry. Cells were treated with TPEY-Tempo (25 μM) 30 min prior to γ-irradiation (25 Gy, Pre/IR) or 10 min after radiation (Post/IR). Samples were harvested after 96 h post-irradiation incubation and re-suspended in lysis buffer containing 0.05% digitonin for 4 min on ice. Supernatants were collected after centrifugation for 10 min at 10,000×*g*. Equal amounts of protein (40 μg) were subject to 15% SDS-PAGE and detected by Western blotting using antibodies against cyt *c* and actin (loading control). **f** Effect of TPEY-Tempo on γ-irradiation-induced caspase 3/7 activation in BB19 and T98G cells. Caspase 3/7 activity was measured 96 h post-irradiation using a caspase 3/7 assay kit. **g** Effect of TPEY-Tempo on γ-irradiation-induced phosphatidylserine externalization in U87MG cells. Cells were treated with TPEY-Tempo (25 and 50 μM) 30 min prior to γ-irradiation (25 Gy). Externalization of phosphatidylserine was assessed after 72 h post-irradiation incubation by flow cytometry using Annexin V/PI kit. Data presented are the means ± S.D. ($n = 3$). $^{\#}$ $P < 0.05$ vs. irradiated cells, * $P < 0.01$ vs. irradiated cells

Fig. 3g, percentage of PS positive cells was increased from 6.2 to 18.3% with 25 μM TPEY-Tempo treatment ($P < 0.01$ vs. irradiated cells without TPEY-Tempo) after 72 h post-irradiation incubation. At a higher concentration (50 μM) of TPEY-Tempo treatment, more than 95% of cells were Annexin V positive.

Discussion

Malignant gliomas are locally aggressive and invasive tumors of the central nervous system. The prognosis is very poor for the patients with these cancers despite of a decade's progress. The focal point of current research is centered on the development of improved anti-tumor therapies since our current therapies, while effective initially, are plagued by recurrences. For example, radiation therapy has been shown to be the single most active treatment for malignant gliomas after surgical resection. Despite the therapeutic benefit, almost all patients suffer local recurrence completely inside the planning target volume receiving the maximal dose of radiation. These patients eventually succumb to this recurrence within a median of 12 months. Attempts to escalate the total radiation doses above 60 Gy have not yielded survival advantages, and there is concern for increased toxicity to the normal brain [18]. In fact, dose-related toxicity to normal brain remains a major dose-limiting factor in the clinical radiotherapy of brain tumor volumes.

The pathogenesis of radiation induced central nervous system toxicity includes white matter demyelination (loss of oligodendrocytes and their precursors) and vascular endothelial damage. The endothelial damage causes alterations in permeability and blood–brain barrier breakdown in the acute and subacute phases [17]. Vascular changes also include telangiectasia, hyalinosis and fibrinoid deposits in the vessels wall in the late phase of radiation induced injury. Endothelial cells have been implicated in the development of demyelination and white matter necrosis [17]. Our preliminary data shows that the endothelial cell has exquisite sensitivity to irradiation-induced apoptosis (data not presented). Our novel approach aims at protecting normal brain and escalating doses of currently effective therapies in an attempt to further reduce the tumor population and perhaps effect toxic doses to radiation-resistant cancerous stem cells.

Irradiation-induced cell death occurs mainly through a mitochondria-dependent apoptotic pathway. In this pathway, the early essential event is ROS generation and CL oxidation by cyt *c* followed by cyt *c*, apoptosis-inducing factor and other apoptotic factors release into cytosol from mitochondria, which designate a point of no return in the process of apoptosis [4, 5]. Malignant gliomas cells have been shown in this report to have decreased mitochondrial content with relative amounts of COX IV 5.2-fold lower in T98G tumors cells compared to immortalized human brain endothelial cells. Dmitrenko et al. [19] have shown a reduction of the transcription level of the mitochondrial genome in human glioblastoma. Oudard et al. [20] also reported that three of four glioma tissues had reduced mitochondria number compared to normal tissues as evidenced by electron microscopy. This mitochondrial content difference provides a therapeutic target between normal and tumor cells. We hope to capitalize on this mitochondrial content differential to reduce central nervous system toxicity and attempt radiation dose escalation to better control glioblastoma.

Nitroxides, as shown in Fig. 2b and in our previous studies [16], once integrated into cells, could undergo one-electron reduction to their corresponding hydroxylamine forms. Importantly, nitroxides, but not hydroxylamines, are effective radioprotectants [21]. Furthermore, nitroxides have been found to exert differential cytotoxicity to tumor cells compared with normal cells [12] and confer greater radioprotection to normal tissue than to tumor tissue [22]. The selective radioprotection was attributed to the different bio-reduction rate of nitroxides in tumor cells versus normal cells [23]. In addition to its direct radical scavenging properties, it is also possible that other non-electron scavenging and non-antioxidant mechanisms may participate appreciably in the radioprotective effect of TPEY-Tempo. For instance, Suy et al. [24] reported that Tempol stimulated the ERK signaling pathway that primarily promotes cell growth and proliferation/survival.

In our previous study, we demonstrated that TPEY-Tempo significantly decreased irradiation-induced ROS generation in mouse embryonic cells using mitoSOX assay. Importantly, attenuated ROS generation was associated with decreased CL oxidation and increased cell survival [16]. In this report, we chose one of the most irradiation-sensitive brain cells, endothelial cells, as our targeting cells, and showed that mitochondria-targeted nitroxide, TPEY-Tempo, protected endothelial cells from the irradiation-induced apoptosis, while enhancing the radiosensitivity of T98G tumor cells. While it has been known that tumor cells display markedly higher requirements than normal cells in oxidants such as H_2O_2, necessary for maintenance, of their high proliferative potential [25], the specific radiosensitization mechanisms of the TPEY-Tempo compounds on tumor cells needs to be further investigated. Our novel strategy of protecting normal brain cells from the apoptotic effect of ionizing radiation with simultaneous sensitization of tumor cells provides a promising adjunct in the treatment of glioblastoma with the potential to not only prolong survival but also maintain quality of life and reduce treatment toxicity.

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