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ORIGINAL ARTICLE

Combination treatment for glioblastoma with temozolomide, DFMO and radiation

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Summary

Purpose: *Glioblastoma multiforme (GBM) is the most malignant primary brain tumor with dismal prognosis. This tumor is characterized by extensive heterogeneity, thus is difficult to treat and every established or new treatment faces significant hazard of resistance. Temozolomide (TMZ), an oral alkylating agent, is the first-line treatment for GBM, but resistance to TMZ is a major problem. Herewith, we investigated the combined effect of TMZ, difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, and radiation in GBM cell lines.*

Methods: *We used the U87G, U251MG and T98G GBM cell lines. A linac 6MV accelerator (Varian Medical Systems) was used for cell irradiation. Viability and proliferation of the cells were examined with trypan blue exclusion assay, crystal violet and xCELLigence system. Cell cycle and activation of caspase-8 were evaluated with flow cytometry.*

Results: *The combination treatment resulted in a consistent higher suppression of proliferation in all cell lines treated and induced a significant higher cell cycle arrest in G2/M phase in U251MG and T98G cell lines. In U251MG cells caspase-8 was increased with each treatment alone, however the combination treatment had lower level of caspase-8 induction, suggesting a co-existence of another mechanism of cell death apart from apoptosis. In T98G cells the combination treatment increased the activation of caspase-8.*

Conclusion: *Combination treatment with DFMO, TMZ and radiation significantly reduced cell viability in all cell lines tested. Given that both TMZ and DFMO can be administered orally and are related to minimal toxicities, this combination treatment may be a novel treatment strategy for GBM that deserves further investigation.*

Key words: *DFMO, glioblastoma, radiation, temozolomide*

Introduction

GBM is the most malignant primary brain tumor. Despite intensive clinical investigation and several novel therapeutic approaches, the median survival remains in the range of about 15 months and the 5-year survival rate does not exceed 2% [1]. Current treatment involves surgical resection followed by radiotherapy and chemotherapy with the alkylating agent temozolomide [1]. Nevertheless, recurrence is nearly universal, since the tumor displays significant resistance to therapy. GBM is a heterogeneous brain tumor with evident pathological and genomic variants [2,3]. This heterogeneity makes GBM difficult to treat as every established or new treatment faces significant hazard of resistance [4].

The polyamines spermidine and spermine and their precursor putrescine are required for cell growth and proliferation and high levels have

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been found in neoplastic cells [5]. DFMO, an inhibitor of ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine synthesis, as a chemotherapeutic agent has the advantage of low toxicity and oral bioavailability, which makes the drug particularly suitable for therapy [5]. DFMO showed promise for glioma treatment [6,7]. Given that both temozolomide and DFMO can be administered orally we set out to investigate the combination of temozolomide, DFMO and radiation in GBM cell lines.

Methods

Cell lines and treatment conditions

The human GBM cell lines U251MG and U87G were obtained from Dr W.K. Alfred Yung (Department of Neuro-Oncology, M.D. Anderson Cancer Center, Houston, TX) and T98G was obtained from ATCC (Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco BRL), at 37°C in a 5% CO_2 atmosphere. Temozolomide was purchased from Schering Plough Ltd (Welwyn Garden City, UK) and DFMO was obtained from Tocris Bioscience (Ellisville, MO, U.S.A.). Both drugs were dissolved in distilled water to form a stock solution of 1 M, sterilized by filtration and stored at 20°C. Prior to each experiment, the stock solution was diluted to the final concentration with growth medium. Cultures of malignant glioma cells were treated with TMZ (0.5 mM) alone, DFMO (5 mM) alone or TMZ (0.5 mM) and DFMO (5 mM), with and without radiation. The concentration of DFMO used has been previously established [7]. Cells were plated and 24 hrs later TMZ and/or DFMO was administered, followed by radiotherapy on the next day.

Cell adhesion assay

We used the xCELLigence Real-Time Cell Analyzer (Roche Diagnostics) to monitor the biological condition of cells. This analyzer contains wells similar to that of 96-well plate and calculates cell adhesion and number by measuring electrical impedance on the bottom of tissue culture plates (E-Plates). The greater the number of cells attached on E-Plates, the higher the impedance value. The analyzer automatically calculates the electrical impedance as a cell index (CI). Plated cells were treated after 24 hrs with TMZ alone (0.5 mM) DFMO alone (5 mM), or both, with and without radiation and monitored for 8 to 10 days. Cell medium was changed every 4 days. Values of normalized CI are presented as mean of four different measurements.

Viability assay

Cultures of human glioma cells were treated with TMZ alone at concentration of 0.5mM, DFMO at concentration of 5mM, radiation and their combination. Cell viability was calculated by both crystal violet and trypan blue exclusion test. Each assay was performed at least 3 times and is represented as cell viability relative to untreated cells (equal to 100%). The assays were performed on day 5. Cell proliferation was additionally monitored for 10 days every 30 min using the xCELLigence system.

Flow cytometric analysis of DNA cell cycle

Cells were treated with TMZ 0.5mM, DFMO 5mM, radiation and their combination with and without radiation. Untreated cells were used as a negative control. Each experiment was performed in triplicate. Flow cytometric analysis was performed on day 5. In order to perform DNA cell cycle, cells were first trypsinized, centrifuged and washed with PBS. Then cells were incubated with PI-working solution (50 µg/mL PI and 20 mg/mL RNase A and 0.1% Triton X-100) for 20 min at 37°C in the dark [8,9]. For each sample the PI fluorescence of 10,000 individual nuclei was measured using a flow cytometer (FACScalibur, Becton Dickinson San Jose, Cali-

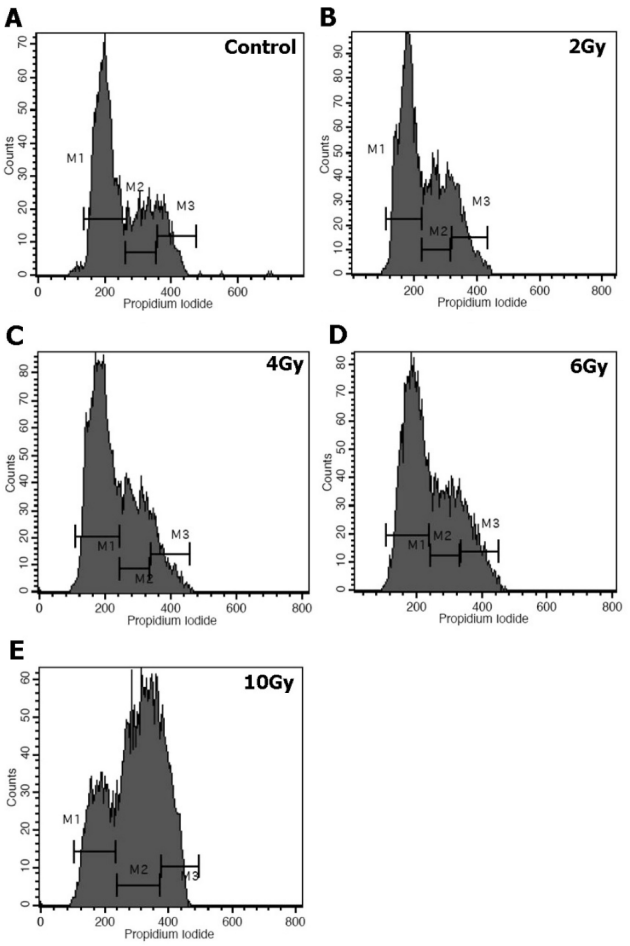

Figure 1. Effect of radiation treatment of U251MG cells. To determine the most appropriate radiation dose in U251MG cells, escalating single doses of ionizing radiation were used (0, 2, 4, 6 and 10 Gy). 10^4 U251MG cells were seeded in 24-well plates and 24 hrs later were exposed to escalating doses of ionizing radiation. The radiation effect on cell cycle was examined 24 hrs after irradiation using flow cytometry. In U251MG, cell cycle arrest was induced in S phase in a dose-dependent manner. The radiation effect was more pronounced at 10 Gy **(E)**.

fornia, USA). The cell fractions in G0/G1, S, and G2/M phases were analyzed using Cell Quest software program (BD Biosciences).

Caspase-8 activity

The activity of caspase-8 was quantified with the Fluorescein Active Caspase-8 Staining Kit (Abnova, Taiwan). Plated cells at 10,000/well were treated after 24 hrs as described in the viability assay. The cells were then trypsinized and 1 μl of FITC-IETD-FMK was added into each tube and incubated at 37°C incubator with 5% CO_2 for 0.5-1 hr. Cells were then centrifuged at 3,000 rpm for 5 min and resuspended in 0.5 ml of Wash Buffer and centrifuged again. Flow cytometry was used for quantification of fluorescent cells.

Radiation treatment

A linac 6MV accelerator (Varian Medical Systems) was used for cell irradiation. Cells (10^5) were plated in 24-well plates, and irradiated with the chosen Gy of X-rays with a dose rate of 300MU/min. Radiation was delivered via a single irradiation field of 13x17cm at 180°. A 1.5cm of added filtration (plexiglass) was used for the build-up effect to be compensated. Plates of Perspex were positioned below a receptacle that contained the well plate, filled with water at a height of about 5cm to guarantee a homogeneous dose distribution at the periphery wells. Perspex plate was positioned above the well plates.

The Philips Pinnacle 3 radiation therapy planning system was used to calculate the delivered dose customized with the geometric and dosimetric characteristics of

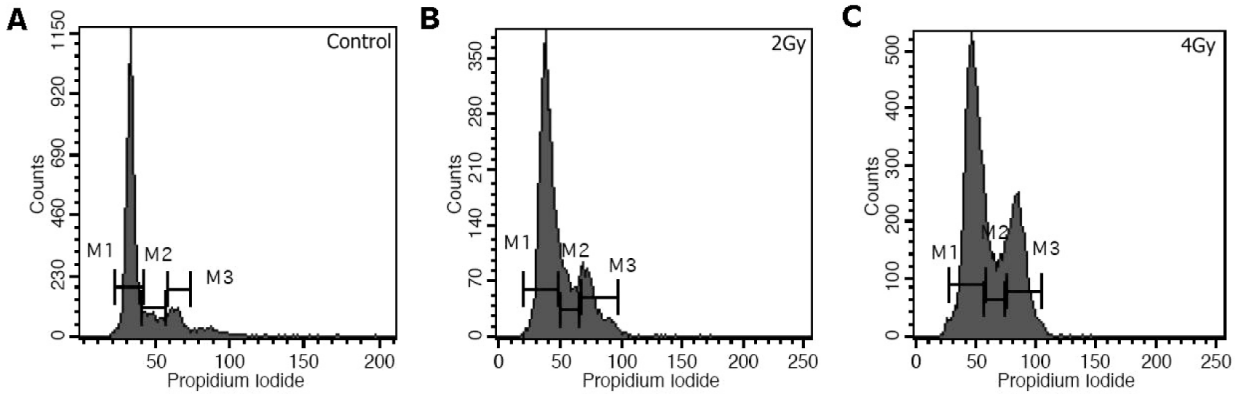

Figure 2. Effect of radiation treatment of T98G cells. To determine the most appropriate radiation dose in T98G cells, the following escalating single doses of ionizing radiation were used (0 **(A)**, 2 **(B)**, 4 Gy **(C)**). 10^4 T98G cells were seeded in 24-well plates and 24 hrs later were exposed to escalating doses of ionizing radiation. The radiation effect on cell cycle was examined 24 hrs after irradiation. Cell cycle arrest was induced in G2/M phase and was more pronounced at 4 Gy.

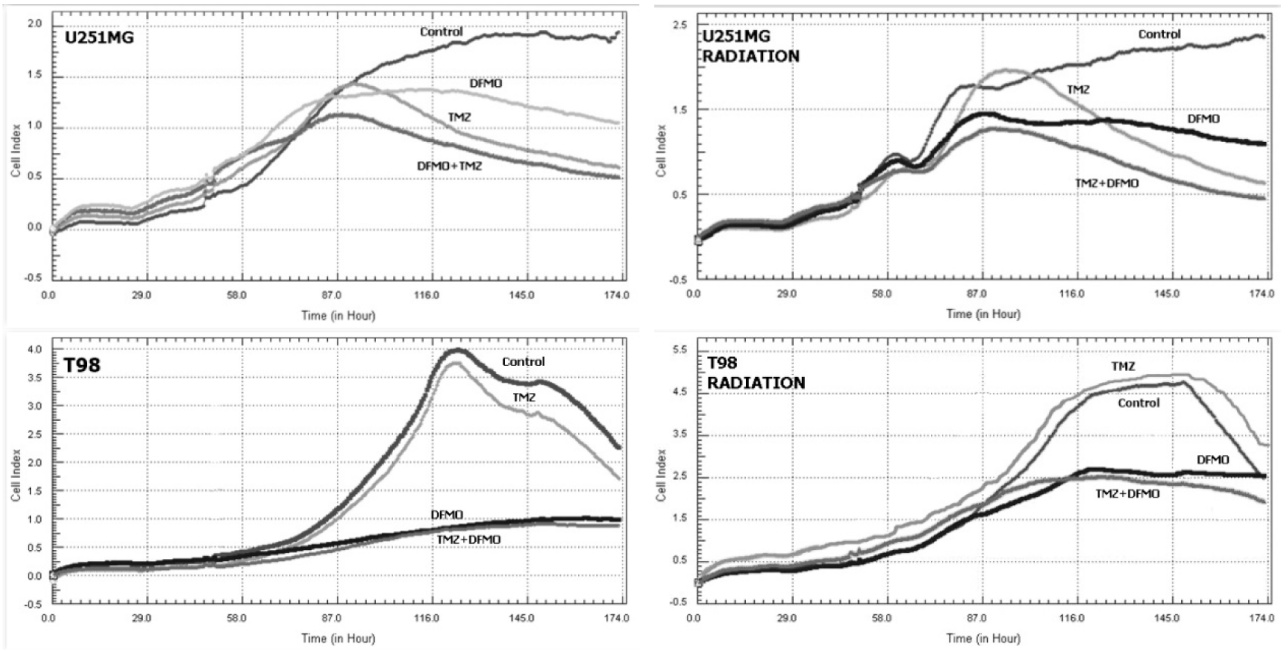

Figure 3. Cell index curves of U251MG and T98G cell lines as produced by xCELLigence RTCA. Cells were seeded and 24 hrs later were treated with either TMZ (0.5 mM), DFMO (5mM), radiation, or their combination. Combination treatment resulted in a significant higher suppression of proliferation in both cell lines ($p<0.05$).

the accelerator. A CT scan of the whole set-up was used in order to calculate the dose distribution. The resulting treatment plan showed satisfying calculated dose uniformity. Dosimetry was based on an absolute dose calibration of the linac output according to IAEA TRS398 protocol. Increasing radiation doses were used in U251 and T98 cells. A radiation dose of 4Gy was chosen as the most appropriate dose, approximating the daily dose of 2Gy used in humans [Figures 1 and 2].

Statistics

Data were expressed as mean ± SD. A 2-tailed Student's *t*-test was used to investigate the significance of differences between experimental conditions. Differences were considered significant at P values less than 0.05.

Results

Combination of TMZ, DFMO and radiation effectively increases cell death

In the xCELLigence system, RTCA measurements showed significant decreased CI values for cells treated with the combination of TMZ, DFMO and radiation compared to non-treated cells and cells treated with only TMZ, DFMO or radiation, for

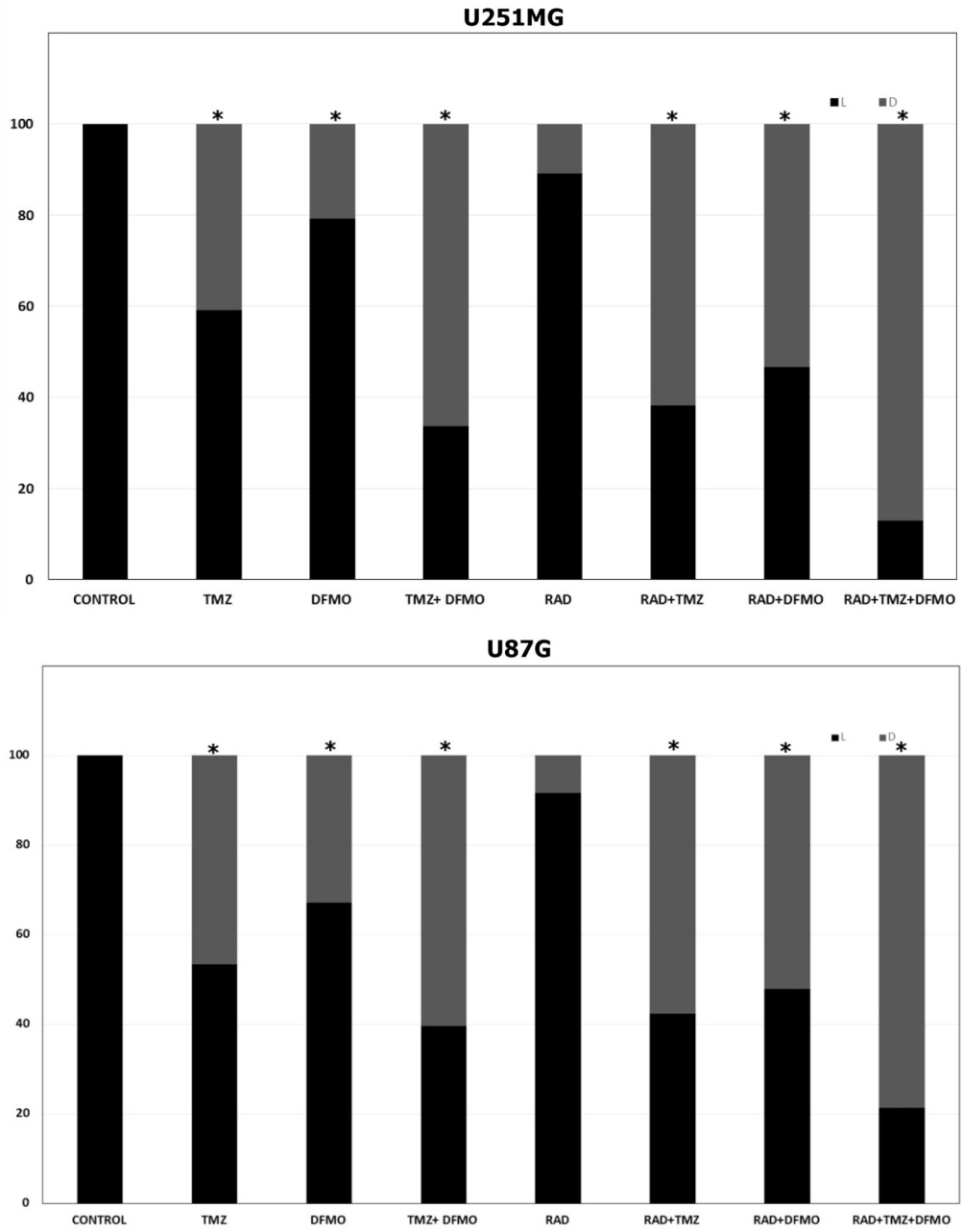

Figure 4. Viability of U251MG and U87G cells following treatments. Cell viability was assessed by the trypan blue exclusion test. 10^4 U251MG and U87G cells were seeded in a 24-well plate and 24 hrs later cells were treated with DFMO (5mM), TMZ (0.5 mM), DFMO plus TMZ, with or without radiation. Viability tests were performed on day 5. Values are normalized to nontreated cells. *p<0.05 vs control.

both T98G and U251MG cells ($p<0.05$). As expected, T98G cells displayed resistance to TMZ, whereas U251MG were sensitive to TMZ treatment (Figure 3).

For the cell viability, experiments with crystal violet and trypan blue exclusion test cell lines (U251MG and U87G) were exposed to TMZ (0.5mM), DFMO (5mM), radiation and their combination. Cell lines displayed differential sensitivity to each treatment. The cytotoxic effect was significant more pronounced when the combination of the three treatments was used. U251MG and U87G demonstrated a strong cytopathic effect at day 5 (Figure 4).

Effect of TMZ, DFMO and radiation on cell cycle of GBM cell lines

The effect of TMZ, DFMO and radiation was tested on the 3rd day after treatment in U251MG

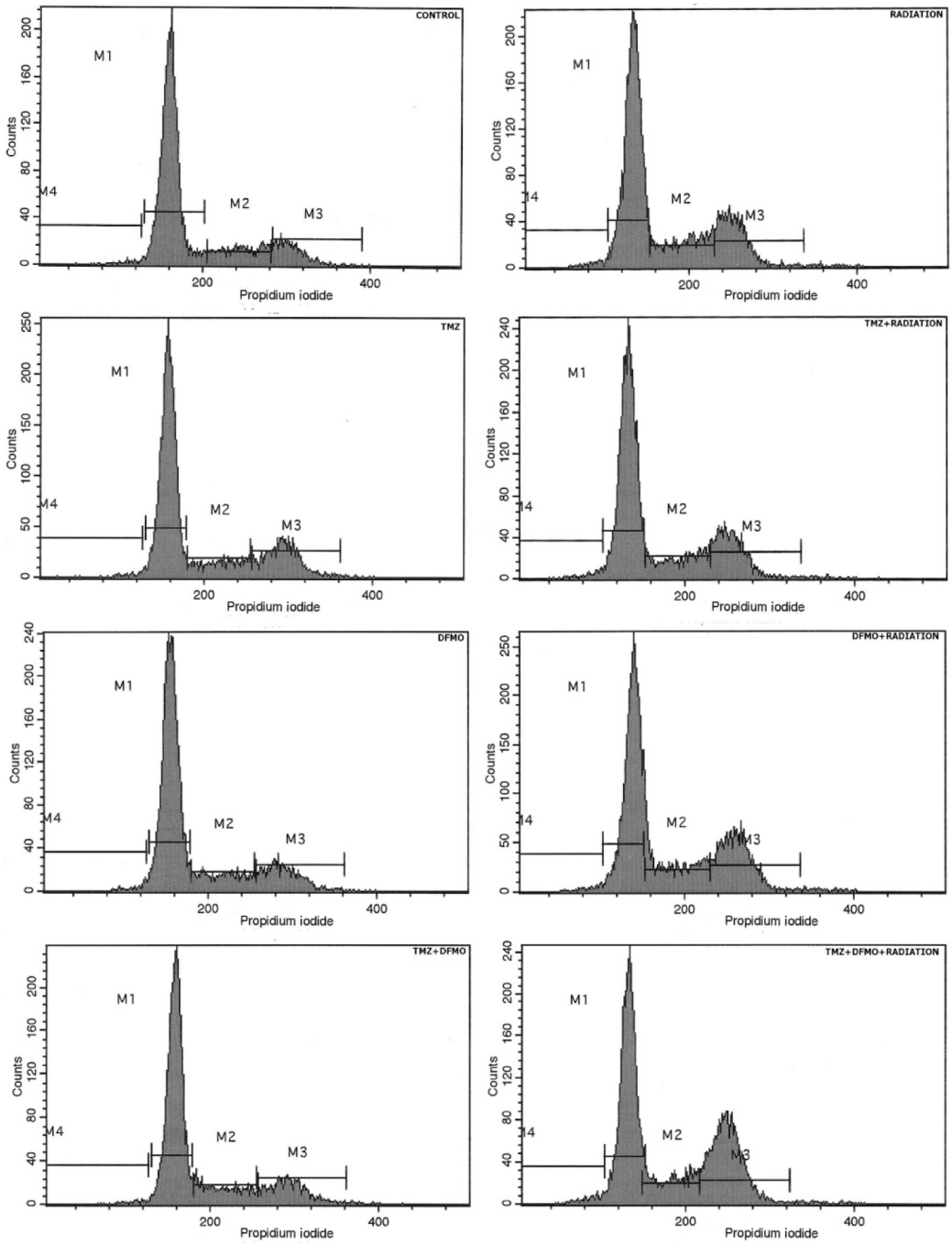

Figure 5. Effects of TMZ (0.05 mM), DFMO (5 mM), radiation and combination of all three on cell cycle. T98G (10^4) cells were seeded in 24-well plate and after 24 hrs were exposed to the various treatments indicated. At 72 hrs, cells were stained by propidium iodide and the DNA content was evaluated by flow cytometry. Radiation alone induced G2/M arrest. DFMO alone induced a G0/G1 cell cycle arrest and TMZ alone had a modest effect in the TMZ resistant T98G cells. Combination treatment produced a significant higher G2/M cell cycle arrest.

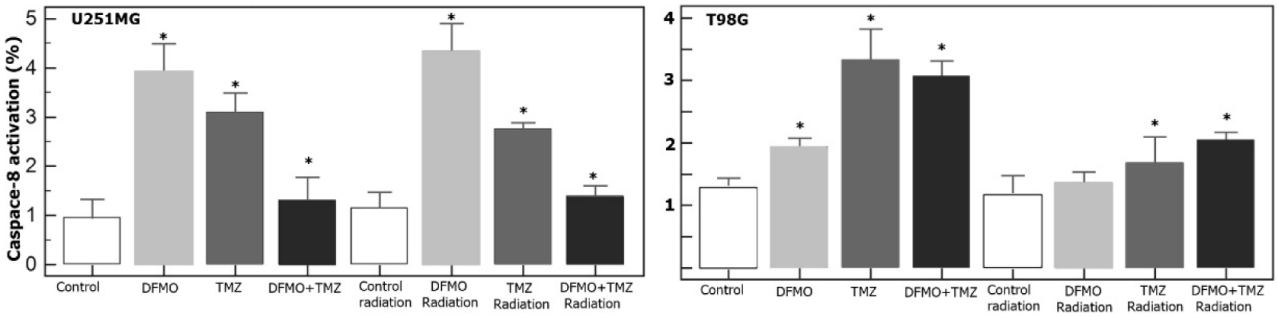

Figure 6. Caspace-8 activation as assessed by flow cytometry. U251MG and T98G (10^4) cells were seeded in 24-well plate and after 24 hrs were exposed to treatment with either TMZ (0.5 mM), DFMO (5mM), TMZ (0.5 mM) plus DFMO (5mM), with or without radiation. Measurements were performed on the 5th day after treatment. Combined treatment had the most pronounced effect in T98G cells. In U251MG cells combination treatment resulted in lower levels of caspace-8 activation than each treatment alone, possibly suggesting the co-existence of another mechanism for cell death apart from apoptosis. *p<0.05 vs control.

and T98G cells. Analysis of DNA content and cell cycle was carried out with flow cytometry and PI single staining of both cell lines. DFMO alone produced cell cycle arrest in G1 phase in both cell lines. As expected, TMZ caused G2/M cell cycle arrest more pronounced in U251MG cells. Radiation alone produced cell cycle arrest in G2/M phase in both cell lines. The combination treatment induced a significant higher cell cycle arrest in G2/M phase in both cell lines (Figure 5).

TMZ, DFMO and radiation augment activation of Caspase-8 in T98G cells

In order to investigate the mechanism by which inhibition of cell growth was produced we evaluated the levels of caspase-8 activation. The data from caspase-8 activity assay showed that in U251MG cells caspase-8 was increased with each treatment alone, however the combination treatment produced lower level of caspase-8 induction, suggesting a co-existance of another mechanism of cell death apart from apoptosis. In T98G cells the combination treatment increased the activation of caspase-8 (Figure 6).

Discussion

This study is the first to determine the activity of DFMO in combination with TMZ and radiotherapy against GBM *in vitro*. Exposure of glioma cells to the combination was superior to each treatment alone and decreased the proliferation and survival of glioma cell lines. GBM is a highly heterogeneous malignant tumor that effects tumor resistance and prognosis. Radioresistance is another feature of GBM due to the presence of cancer stem cells [10]. Since both TMZ and DFMO can be administered orally the results of the present study provide a strong rational for further investigation in *in vivo* models.

The alkylating agent TMZ is the first-line therapy drug used for glioma treatment and the combination with radiotherapy resulted in significant increased survival [11]. The methylation status of the methyl-guanine methyl transferase gene, *MGMT*, is a predictor of outcome and benefit of temozolomide. *MGMT* gene encodes a DNA repair protein. Epigenetic silencing of the MGMT gene by promoter methylation is associated with diminished DNA-repair activity [12]. In T98G cell line there is MGMT protein expression and is TMZ-resistant, whereas in U251 cell line there is no MGMT protein expression and is TMZ-sensitive [12,13]. This was verified in the present study using xCELLigence system, since T98G exhibited significant resistance to TMZ treatment alone. Several studies investigated agents that sensitize temozolomide-resistant glioma cells, such as valproic acid, methoxyamine and deferiprone [13-15].

DFMO is an irreversible inhibitor of ornithine decarboxylase and has been approved by the US Food and Drug Administration as a treatment for forms of African sleeping sickness [16]. DFMO is a cytostatic agent causing suppression of cell proliferation and has been previously tested for cancer treatment [5]. A phase III study in anaplastic glioma patients demonstrated a survival advantage for patients who were treated with the nitrosourea combination of PCV and DFMO [15], but not for glioblastoma patients [18]. DFMO proved to have a low toxicity profile [17,18]. In glioma cell lines DFMO inhibited spheroid growth and cell migration and the combination with radiation had a greater effect on the survival rate of a rat glioma model than single therapies alone, the effect being additive [19,20]. Ueda et al. studied the DFMO effects on Y79 retinoblastoma cells and found cells

accumulated in the G1 and S phase. DFMO induced p27/Kip1 protein expression, p107 dephosphorylation and accumulation of p107/E2F-4 complex in Y79 cells [21]. Treatment of glioblastoma cell lines with combination of DFMO,

TRAIL and radiation showed an enhanced effect [7]. In the present study we also found DFMO to cause G1/S cell cycle arrest. An induction of apoptosis via overexpression of BAX, BAD and underexpression of bcl-2 has been reported [7,22]. Increased apoptosis has been reported in other studies in several cell lines [23,24]. However, DFMO alone did not increase apoptosis in a mouse model of squamous cell carcinoma [25].

Radiotherapy constitutes the most effective treatment of glioblastoma [10]. One of the major strengths of the present study is the use of radiation, which is scarcely used for the investigation of combination treatments for GBM. Irradiation results in cell cycle arrest, but also induces apoptosis, which proceeds principally via the mitochondrial pathway [26]. In the present study in U251MG cell line, cell cycle arrest was induced in S phase and started at a dose of 2Gy. The radiation effect was more pronounced in 10 Gy. In T98G cell line cell cycle arrest was induced in G2/M phase and was more pronounced at 4Gy. One limitation of the present study is lack of an *in vivo* model to test the effect of this therapeutic approach.

In conclusion, we believe that the combination of TMZ, DFMO and radiotherapy constitutes a promising therapy for GBM, mainly because both agents can be administered orally and have a low toxicity profile. However, the results of the present study need to be validated in a glioma xenograft model.

Authors' contribution

George A. Alexiou, Evrysthenis Vartholomatos, Konstantinos I. Tsamis, Evangelia Peponi, George Markopoulos, Vasiliki A. Papathanasopoulou and Ifigeneia Tasiou performed the experimental procedures. George A. Alexiou and Vasiliki Galani designed the research, analyzed the experimental data and wrote the paper. Athanasios P. Kyritsis and Vasiliki Galani supervised the experimental procedures and contributed to the analysis of the experimental data. Periklis Tsekeris and Vassilios Ragos contributed to the analysis of the bibliography.

Conflict of interests

The authors declare no conflict of interests.

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