Manuscript thesis
for obtaining the scientific degree Master of Science

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Effect of photodynamic therapy on glioblastoma cell lines.

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2022

Abstract: Photodynamic therapy (PDT) utilises light-activated activation of 5-aminolevulinic acid (5-ALA) to reduce the viability of glioblastoma (GBM) cells. Primary patient-derived cell lines Ox5 core and G7 were exposed to light excitation at 410 nm, 528 nm & 810 nm wavelengths +/- 5-ALA (protoporphyrin IX). The primary objective is to evaluate whether PDT +/- 5-ALA is effective in reducing cell viability and clonogenic expansion, relative to the standard of care in (Temozolomide +/- Radiotherapy).

Results demonstrated significant cell death >70% in Ox5 after PDT/ 410nm + 5-ALA 50uM, and >23% in G7 cells utilising the MTT viability assay (n=4), $p< 0.05, 0.001$.

Clonogenic assays using G7 cells showed a severe inhibition of clonogenic potential upon PTD (PDT/410nm + 5-ALA 50uM) therapy. Apoptosis was detected in both, Ox5 core and G7 using immunohistochemistry for cleaved caspase-3. Additionally, the standard of care (TMZ monotherapy) was tested, and 4 biological repeats demonstrated no statistical significance, $p>0.05$, on the relative viability of Ox5 or G7 cells. Clonogenic assays treated with the standard of care regime (TMZ+/-Radiotherapy) demonstrated remaining colonies at the higher doses of radiation (4 & 6 Gy).

Therefore, PDT/410nm + 5-ALA 50uM causes cell death in Ox5 and G7 cells offering a potential novel therapy in the treatment of GBM.

Introduction.

Glioblastoma (GBM) is a very aggressive primary brain tumour with a lethal prognosis. Despite successful surgical resections, chemotherapy and radiotherapy (CRT), the survival range fluctuates from around one year up to 15 months (42). Newly discovered reasons responsible for the high recurrence and chemoresistance in GBM are TGFb and microtubes formation, however, this phenomenon is rather one of the many others described in the last decade (20).

Surgical resection utilising 5-aminolevulinic acid (5-ALA) and fluorescein-guided surgery of the brain has gained in popularity, allowing the visualisation of the tumour, and demarking the tumour boundaries; however, neoplasms which are growing in the deepest brain structures and/or functionally significant brain zones (e.g. Broca's area, basal ganglia) cannot be removed, and aggressively resected because of their function, and localisation (33). However, there is evidence emerging that the ability of 5-ALA to accumulate into glioma cells combined with its excitation at specific

wavelengths of light results in photodynamic therapy (PDT) (7,24,41) capable of killing cells.

The principles of PDT for surgical resection and treatment

Neurosurgery: PDT works by the photosensitizer absorbing light and then emitting it at a different wavelength. The most common photosensitizers in clinical use are porfimer sodium, talaporfin sodium, and 5-ALA (40). As malignant gliomas take up to 300-fold higher concentrations (31), of the photosensitizer, they glow when illuminated allowing their surgical resection.

Longer wavelengths of photo-illumination can penetrate deeper structures in the brain illuminating hard or impossible regions to resect. The average wavelength of these photosensitizers is about 660 nm (2). The mostly described photosensitizers are porfimer sodium and 5-ALA (16).

Treatment: In addition to their ability to demark the tumour this family of photoreactive chemicals produce reactive O_2 species (ROS) during their excitation /emission chemistry. The fundamentals of PDT involve photosensitizer photo-activation that is accumulated into the neoplastic cells. Consequently, photo-illumination activity activated by the photosensitizer transmits the

energy to the sensitiser in the PDT machine, and causes molecular O_2 release in neoplasms, leading to singlet/triplet phases. Oxygenated tumour tissues, as the consequence of the photosensitizer's energy transformation into molecular O_2 (the ground state), leads to singlet/triplet energy production (17). Induction of cell death by ROS occurs, as a result, damages to intracellular organelles, where O_2 in PDT acts as a pivotal key element in cell death (14). In terms of significance, singlet O_2 can only induce tumour cell death in situ, because of brief lifetime bioactivity. Thus, triplet bioactivity production is the essential phase which must be reached while performing PDT.

From the biochemistry perspective, 5-ALA is a precursor of protoporphyrin IX (PpIX), one of the proteins involved in the heme biosynthesis pathway, that is biosynthesised in the mitochondria. When PpIX is photoexcited at 625–635 nm, reactive O_2 species (ROS) are generated. Most cancer therapies rely on the rapidly dividing properties of the tumour to deliver their chemotoxic effects, however, GBM dormant cells can concentrate high peaks of PpIX levels, enhancing sensitiveness to 5-ALA PTD (17).

While both radiation therapy (RT) and PDT require molecular O_2, their course of action is distinct. Ionising radiation activates DNA damage and apoptosis (35,36). PDT, instead, damages cell organelles (e.g.mitochondria, lysosomes) and can synergise with the agents responsible for DNA damage afterwards.

Clinical trials involving PTD therapy

Historical review backs to 1972, when Diamond and colleagues, firstly described PDT in GBM experiments (38). The first clinical trial was conducted by Perria, and coworkers in 1980 (12). Since those times PDT has been rooted in clinical practice and research as an adjuvant treatment for malignant gliomas till modern times (30, 32).

Intriguingly, O_2 in glioblastoma cell lines tend to be at the minimum levels, thus, it might induce poor clinical effects while PDT utilisation (15). However, the experiments with the use of hyperbaric oxygenation didn't demonstrate clinical approval (5).

The beneficial impact of PDT application has been reported by a trial in 350 patients conducted at Royal Melbourne Hospital (14).

Moreover, a Japanese trial of 145 malignant glioma patients with Hematiporphyrin Derivative (HpD) and PDT application, followed by fractioned radiotherapy and chemotherapy (CRT), demonstrated the average survival from 76.5 to 14.3 months (27). Despite the high indexes of survival which varied approximately 3 years (Stepp et al., 2014; Muragaci et al., 2013; Kaneko et al, 2012; Eljamel et al., 2010; Muller and Wilson 2006; Kostron et al., 2006; Stilli et al., 2005; Rosenthal et al., 2003; Popovic et al., 1995), unfortunately, these trials failed to distinguish the clear effect of PDT because all the patients were treated with CRT following PDT.

An additional study conducted at the Kashiwaba neurosurgical hospital used PTD after resection surgery, as well as intraoperatively (26). Although the favourable impact on the patient's survival was detected up to 3 years, it is difficult to determine the clear evidence of PDT impact on GBM patients, as patients were subsequently placed on CRT treatment regimes. These studies fail to demonstrate a clear effect of PDT therapy, as all patients enrolled in these trials had access to CRT treatment following surgery, and PDT whilst proven to be safe requires the establishment of in vitro preclinical models to show efficacy in patients without access to CRT, be it for financial or medical

reasons. Such models would then form the basis to conduct a clinical trial to compare PDT against CRT.

The two Japanese trials utilised wavelengths ranging from 400 to 660 nm, although this is insufficient to target deep GBM tumours(6). The optimal therapeutic wavelength is up to 800 nm (15).

To date, there are no reports in the literature about the action of PDT/5-ALA with the commonly used chemotherapy agents Cycloheximide and Methotrexate, which may boost the 4-fold-killing cancer cells (21). The experiments weren't performed in vivo/ in vitro, and clinically with GBM patients.

Chemotherapy resistance and mechanisms of PDT.

Chemotherapy works by eliminating cancer cells, either by generically targeting rapidly dividing cells or by targeting a specific pathway that the tumour is utilising. However, even highly targeted therapies can result in a multitude of adverse effects (e.g. excessive bleeding due to thrombocytopenia, numbness, excessive fatigue, etc.) (24). Conversely, PDT which comprises a light and a photosensitizing drug induce an apoptosis cascade in glioma cell lines via ROS production. 5-ALA is absorbed by the body's organs and concentrates in cancerous cells. The photosensitizer is well

tolerated by patients and has no unfavourable effects on bodily organs. Focused light treatment of only the tumour and its immediate surrounding area, results in the highly localised chemical activation of the photosensitizer, causing oxidation and producing ROS, and cell death. PDT is capable of generating several types of ROS (e.g. superoxide anion, hydrogen peroxide, simple oxygen, hydroxyl radical, peroxide) (10,44).

GBM has 4 subtypes (e.g. proneural, classical, neural and mesenchymal) (3). Each of these subtypes is classified by its signalling pathway dysregulation signature. Proneural GBM has PDGFR, IDH, and PIK3CD alterations, while classical GBM displays EGFR alterations; NF1 and NFkB relate to the mesenchymal subtype. The IDH1 and IDH2 mutations which convey a favourable clinical outcome to GBM patients has only a 5 % incidence in the worldwide population (45). These subtypes theoretically present targets for subtype-specific targeted chemotherapy, however, all subtypes should be susceptible to PDT.

(BBB) blood-brain barrier penetration remains a major hurdle in the effective delivery of chemotherapy to brain tumours, despite the many promising druggable pathways presented by the GBM subtypes (1,9,21).

All this mentioned above highlights the phenomenon of PDT discovery, however, its mechanisms of resistance may be more immunogenic in GBM than the other treatments.

The possible role of PDT to overcome chemoresistance.

Apoptosis in PDT results from the activation of cell death pathways, as well as an expression by specific proteins (4). The exploitation of molecular-targeted PDT via protein extraction may indicate possible resistance mechanisms in GBM. For example, autophagy has a defensive mechanism for malignant glioma cell endurance, after PDT illumination, and anti-tumour immunity, as the firm evidence of successful PDT illumination. Furthermore, cell membrane damage in GBM leads to necrosis, apoptosis as well as autophagy (37). Anti-tumour immunity is another important aspect that can be regulated by PDT. Straightaway after PDT illumination on cancer cells, anti-tumour antigens production increases when dendritic cells are activated. Therefore, antigens induce natural killer cells (NK) and CD_{8+} cytotoxic T-cells. NK and CD_{8+} T-cells can suppress further tumour growth via their immune memory defensive mechanisms (28). Therefore, it is crucial to highlight the beneficial PDT effect on cancerous cell lines in GBM.

Chemoresistance mechanisms of PDT with mitochondria structure involvement were mentioned in several studies (19,27,39). Luna and Gomer, 1991 described several cell lines' chemoresistance after PDT, as well as in vivo models. The research by Casas and colleagues discussed 5-ALA-induced protoporphyrin IX-PDT resistant cell lines (16). The evidence suggests increasing mitochondria and decreasing PpIX protein merits further research. Even though PDT can potentiate chemotherapy and radiotherapy (CRT), in particular, after surgical resections, the effect and role of PDT on a variety of GBM cell lines weren't described recently.

Hypothesis: to investigate whether PDT +/- 5- ALA causes cell death in primary GBM cell lines (Ox5 & G7).

Objective of the research:

1. Determine the efficacy of PDT on MGMT methylated and unmethylated GBM cell lines (G7 and Ox5).
2. Test whether the addition of 5-ALA enhances the cytotoxic effects of PDT.
3. Compare effects with the standard of care (radiotherapy +/- Temozolomide).

4. Investigate the timing & mode of cell death in GBM cells treated with PDT +/- 5-ALA.

Results dissemination:

Results of this research will be disseminated between Wolfson Wohl Cancer Research Centre, Institute of Cancer Research, Glasgow University and the Centre of Clinical Brain Sciences, University of Edinburgh.

Materials & Methods

Ox5 (Unmethylated), and G7 (Methylated) cell lines were anonymously derived from GBM patients, and obtained from Chalmers Lab. Cell lines were repeatedly cultured on Matrigel-coated plates (0.234 mg in ADVANCED/DMEM) in cancer stem serum-free medium with Advanced/DMEM/F12 (Gibco) enriched by supplements - 0.5% Invitrogen, 10 ng/mL fibroblast growth factor 2 (Sigma), 1 % Invitrogen, 4 uM/mL heparin, 1% L-glutamine, and 20 ng/mL EGF (Sigma). All the cell lines in the experiments were grown in 75 cm_2 tissue cultured flasks (Greiner, BioOne, Frichenhausen, Germany). Cell lines were kept in the incubator at 37_oC and 5 % CO_2 and harvested in acutase, utilisation of cell lines

was between passages 2 and 10. Cell lines were between 70- 80 % confluent and checked under microscope view control (Leica Microsystems, Germany).

Ethical approval

In Vitro research was in compliance with all regulatory guidelines according to the Human Tissue Act 2006, Scotland, and regulated by Home Office in the United Kingdom.

Photodynamic therapy

PDT was performed on both cell lines with different passages (4 complete biological repeats with at least 4 technical replicates for each tested group including controls). Cells were seeded in 96 Matrigel-coated plates (Catalog No-167008, Corning$_{TM}$, Costar$_{TM}$, flat-bottom) with seeding density (0.01×10^6) and incubated 24 hours before PDT illumination. 5-ALA- (A3785, Sigma-Aldrich, USA) were added straight away in the dark hood with no light 24 hours after incubation time and incubated at least 4 hours before PDT illumination. Different concentrations of 5-ALA (50 and 100 uM) were added per well and mixed with fresh stem media. In some 96-well plates, PDT was performed without 5-ALA with the controls.

LEDs (RS Components Ltd, UK) of various wavelengths (410 nm, 528 nm, and 810 nm) were performed inside a dark UV box. The distance from the LED to the sample was adjusted and measured using an intensity sensor (Thorlabs Optical Power Meter PM100D and Thorlabs sensor S/N: 16100711) to give the intensity of 10 mW for all LEDs. The distance from the LED to the sample for 410 nm was 3.5 cm, 528 nm- 3 cm and 810 nm- 7.5 cm. The time of illumination for all the cell lines was 4 minutes in 4 biological repeats.

MTT assay

MTT (Thyazolyl blue tetrazolium bromide) viability assay (Sigma M2128-10g) was performed according to the manufacturer's protocol (Merck KGaA, Darmstadt, Germany). MTT working stock solution was 5 mg/ml. 1 g MTT was dissolved in 200 PBS and protected from light (bottles were wrapped in foil) and stored at 4 C0. Glycine buffer was prepared beforehand (0.1 M NaCl, 0.1 M glycine with 1 M NaOH, were adjusted to ph 10.5) and stored in the cold room. 24 hours after PDT illumination, the medium in 96 well-plates was carefully aspirated, and 50 uL MTT solution was added without light in the hood (including wells with no cells) and mixed

with fresh stem media (200 uL total per each well). Plates were wrapped in silver foil and placed in the incubator for 4 hours. Aspiration from each well was performed straight away after the incubation time, quickly 200 uL of DMSO was added (purple dye was realised), additionally, 25 uL glycine buffer was added per each well. Results were obtained on the TECAN counter, measured at 570 wavelengths, and the plates were shaken for 180 sec.

Additional 96 Matrigel-coated plates were seeded as described above. Treatment with TMZ (Temozolomide)- (T 2577, Sigma-Aldrich, US) without PDT with 10 and 50 uM concentrations, including controls and was performed 24 hours after incubation time, and MTT assay was performed 4 hours after incubation afterwards.

Clonogenic assay

G7 was seeded in Matrigel-coated Petri dishes and stayed in the incubator. Seeding density was 250 cells per one Petri-dish, fourteen hours afterwards 5-ALA- (A3785, Sigma-Aldrich, USA), (50 uM) were added in the hood without light, samples stayed in the incubator for at least 4 hours, PDT illumination was performed at 410 nm with the intensity of 10mW, and the distance 3.5 cm from

the LED to the sample. Cells were incubated 2.5 weeks before the fixation with methanol and crystal violet for 2D staining conditions. Visible colonies were counted manually and represented in the bar graph.

Additionally, Ox5 and G7 were seeded in Matrigel-coated 6 well plates with seeding density 0 and 2 Gy- 250 cells per well; 4 and 6 Gy 400 cells per well. Fourteen hours afterwards half of the 6 well plates were treated with Temozolomide (T 2577, Sigma-Aldrich, US)- 10 uM concentration. Cells stayed in the incubator for 3 hours and were irradiated at the indicated doses of radiation above. Fixation with methanol and crystal violet, as well as manual counting was performed. Visible colonies were represented in the line graphs.

IHC cleaved with caspase-3

Cell lines Ox5 and G7, obtained from patients were seeded in small 6 chambers for IHC (Immunohistochemistry slides) with a seeding density of 10^3 cells per chamber. Cell lines were incubated for fourteen hours afterwards, 5-ALA (A3785, Sigma-Aldrich, USA) - 50

uM concentration were added mixed with fresh stem media in the hood without light and incubated for at least 4 hours. PDT Illumination has performed straight away at 410 nm wavelength, with an intensity of 10mW, and a distance of 3.5 cm from the LED to the sample. 24 hours after incubation time immunohistochemical analysis was performed using cleaved caspase-3 (Cell Signalling Technology Inc, Massachusetts, USA), (Asp 175) (5A1E) Rabbit mAb in dilution 1:2000 paraffin-embedded fixed cells. Images of apoptosis in both cell lines were taken under the microscope view control (Leica Microsystems, Germany).

Statistical analysis

Statistical analyses were performed using single factor variance (ANOVA) and T-test followed by Bonferroni multiple comparison (Post-Hoc) analysis. Statistical tests were considered statistically significant at probability levels $P < 0.05$, and $P< 0.001$.

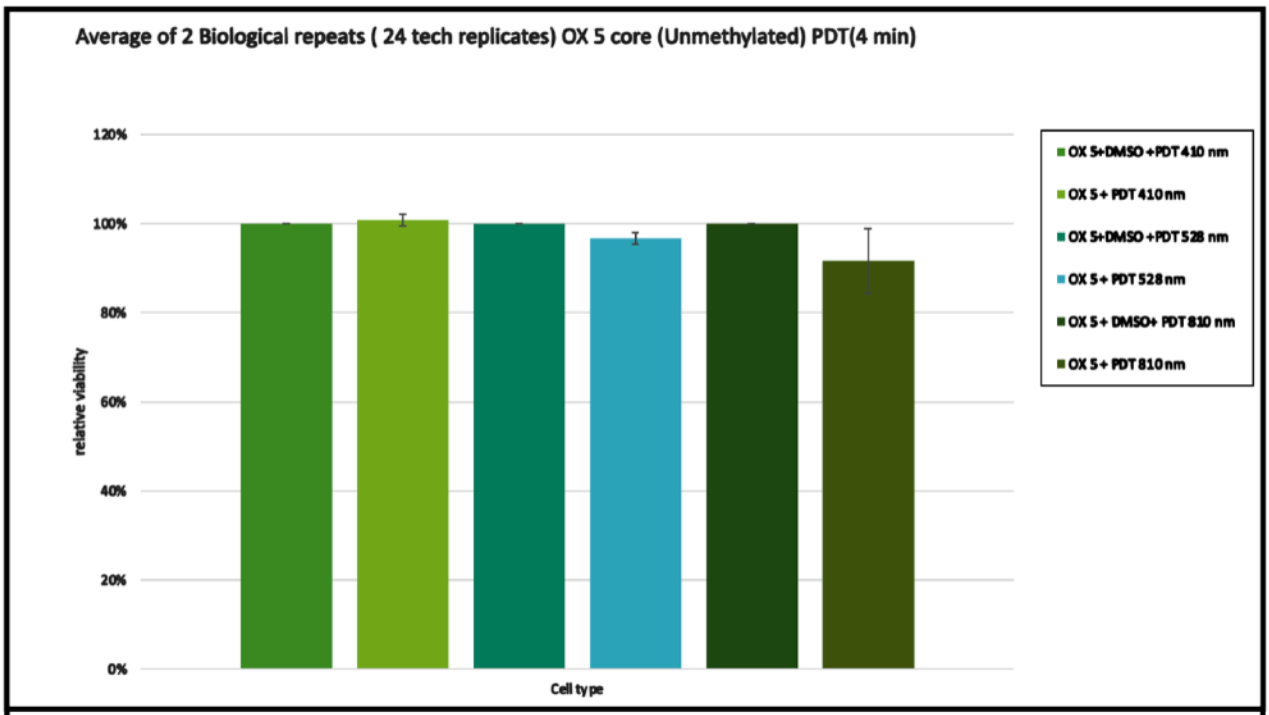
Results

In the experiments, there was no difference during PDT illumination for 4 minutes between different wavelengths without adding 5-ALA in Ox5 and G7. The average of 2 biological repeats is represented

in **(Fig. 1)**. According to the data analysis comparison, there was no statistical significance in Ox5 PDT without 5-ALA - between different wavelengths, ANOVA single factor analysis demonstrated P- value - 0.14, P>0.05.

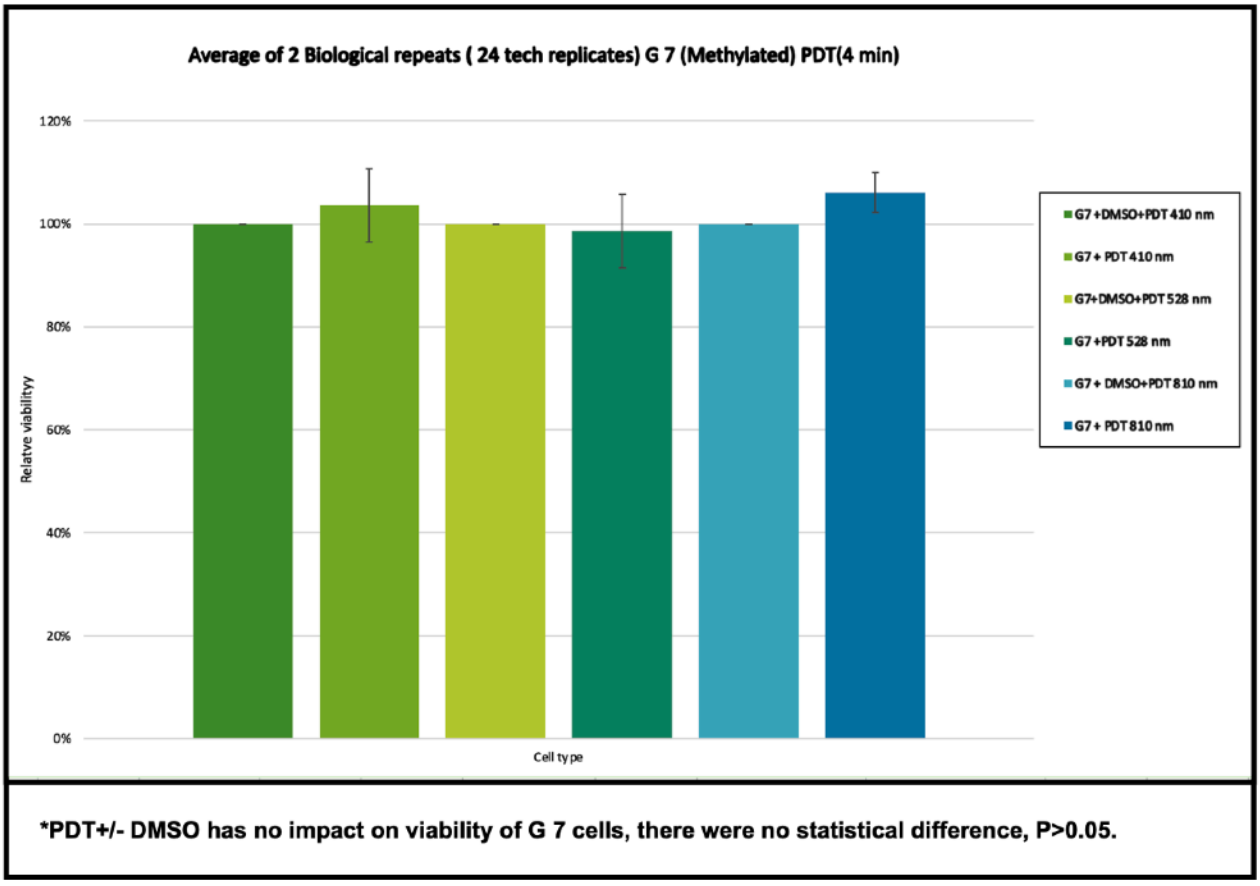
Consequently, the averages of 2 biological repeats in G7

Figure 1.



demonstrated no statistical difference between different wavelengths and controls performance PDT without 5-ALA **(Fig. 2)**, ANOVA single factor analysis showed P- value 0.74, P>0.05.

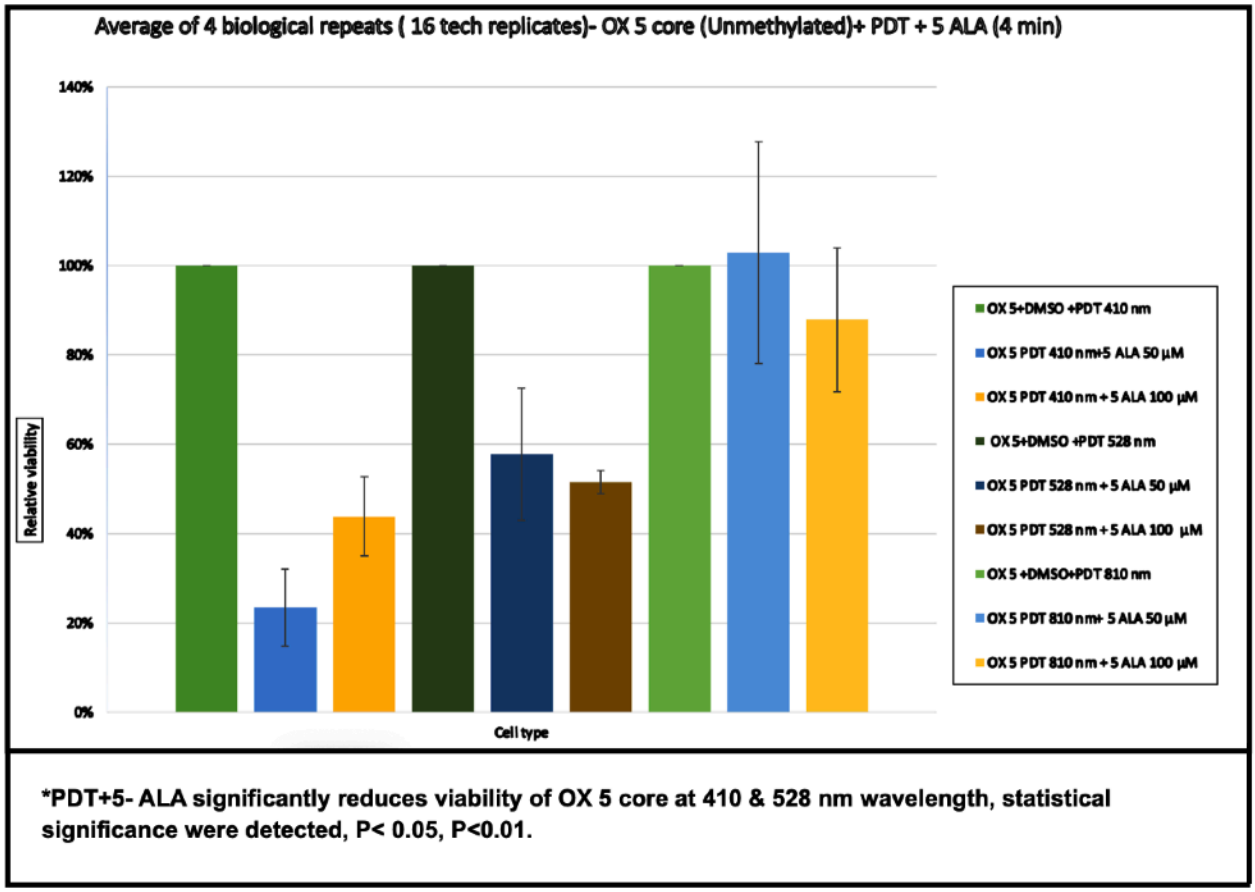
Figure 2.



*PDT+/- DMSO has no impact on viability of G 7 cells, there were no statistical difference, P>0.05.

Significant cells death after PDT with 5-ALA (50 and 100 uM concentrations) was detected in the Unmethylated Ox5 cell line **(Fig.3)** at wavelengths 410 and 528 nm with the intensity of 10mW, 3.5 cm from the Led to the target. Statistical significance showed the difference between the averages of 410 nm & 810 nm wavelengths, P-value 0.0136, P< 0.05. Statistical confirmation was followed by Bonferroni Post-Hoc Analysis, where Alpha 0.0166> 0.0136.

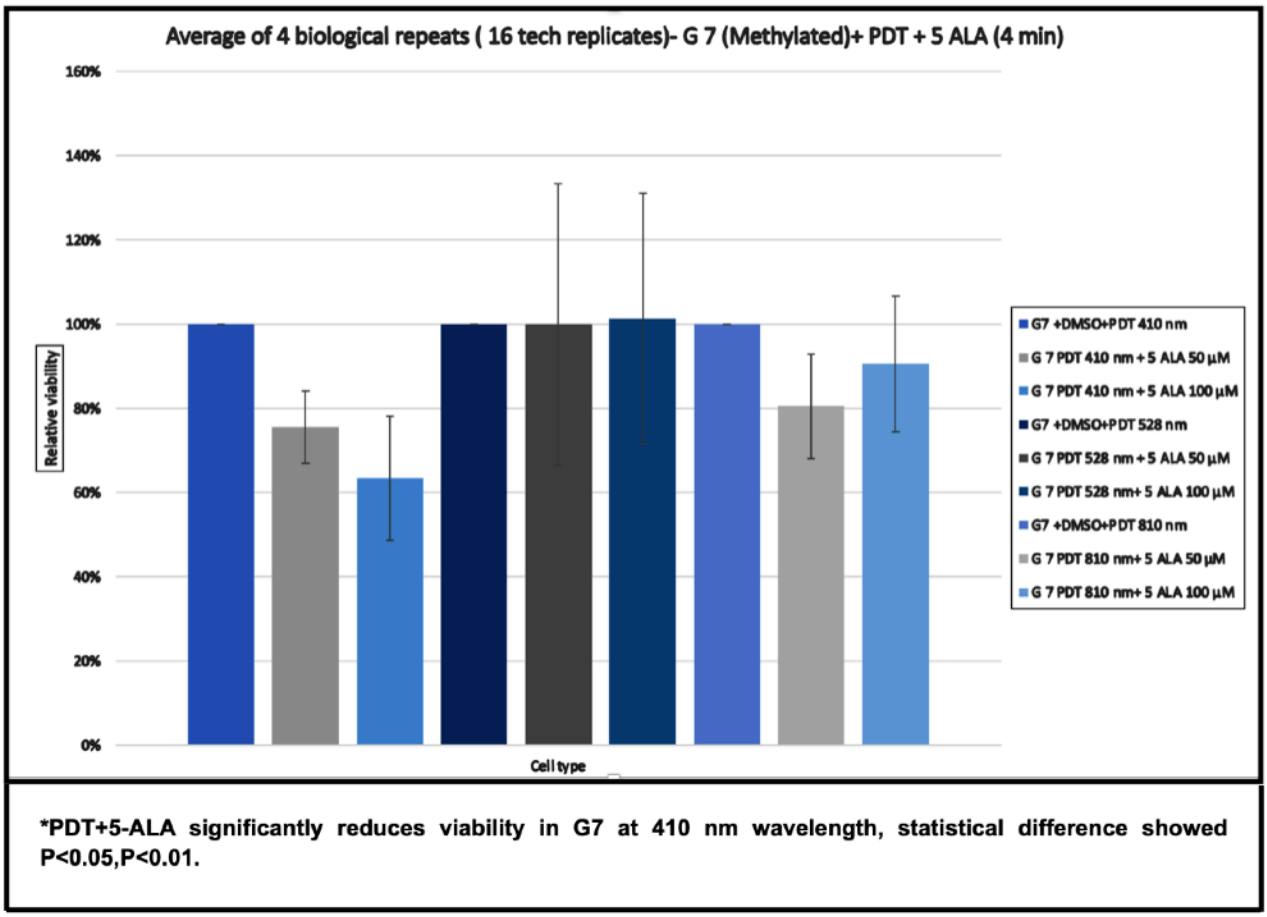
Figure 3.



*PDT+5- ALA significantly reduces viability of OX 5 core at 410 & 528 nm wavelength, statistical significance were detected, P< 0.05, P<0.01.

Following (Unmethylated) Ox5, in G7 (Methylated) cell line **(Fig. 4)** different spectrums of PDT light were tested with 5-ALA with the same concentrations. Therefore, significant cell death was detected at 410 nm wavelength and statistical differences were noticed between averages of 410 nm & 528 nm wavelengths, P- value 0.0125, P<0.05. Bonferroni Post-Hoc Analysis demonstrated the confirmation of the significance, where Alpha 0.0166>0.0125.

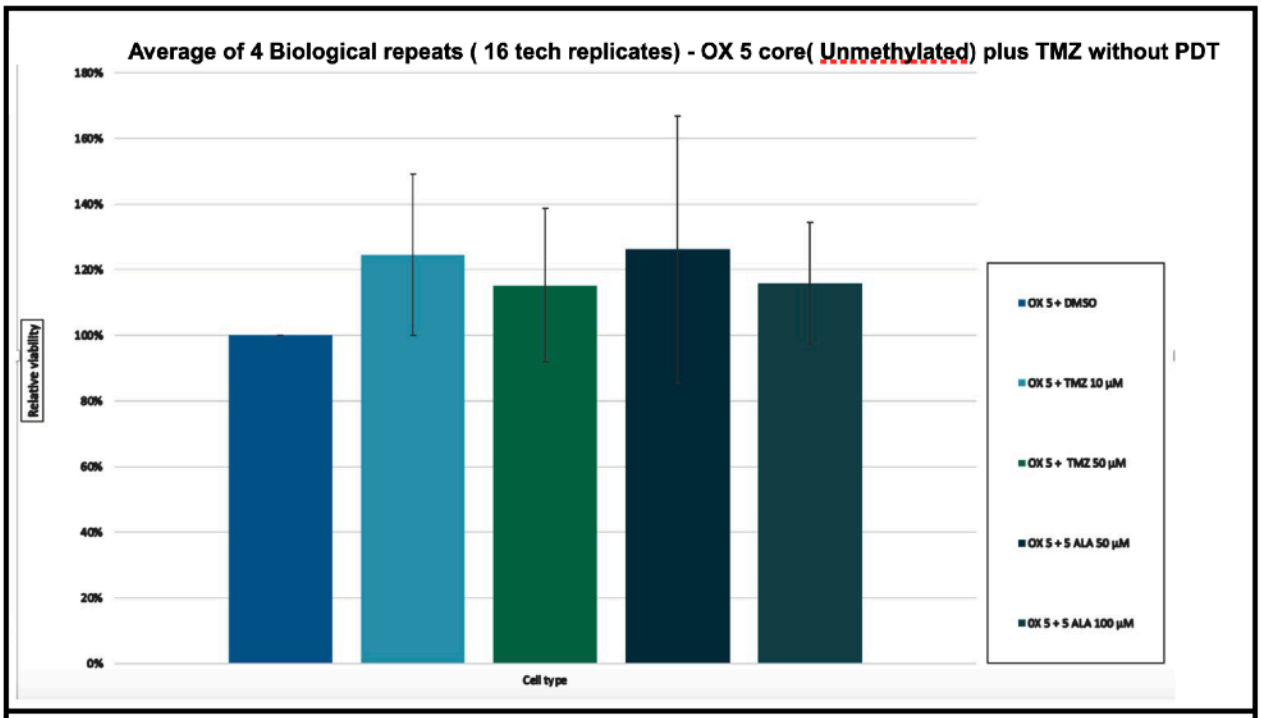
Figure 4.



*PDT+5-ALA significantly reduces viability in G7 at 410 nm wavelength, statistical difference showed P<0.05,P<0.01.

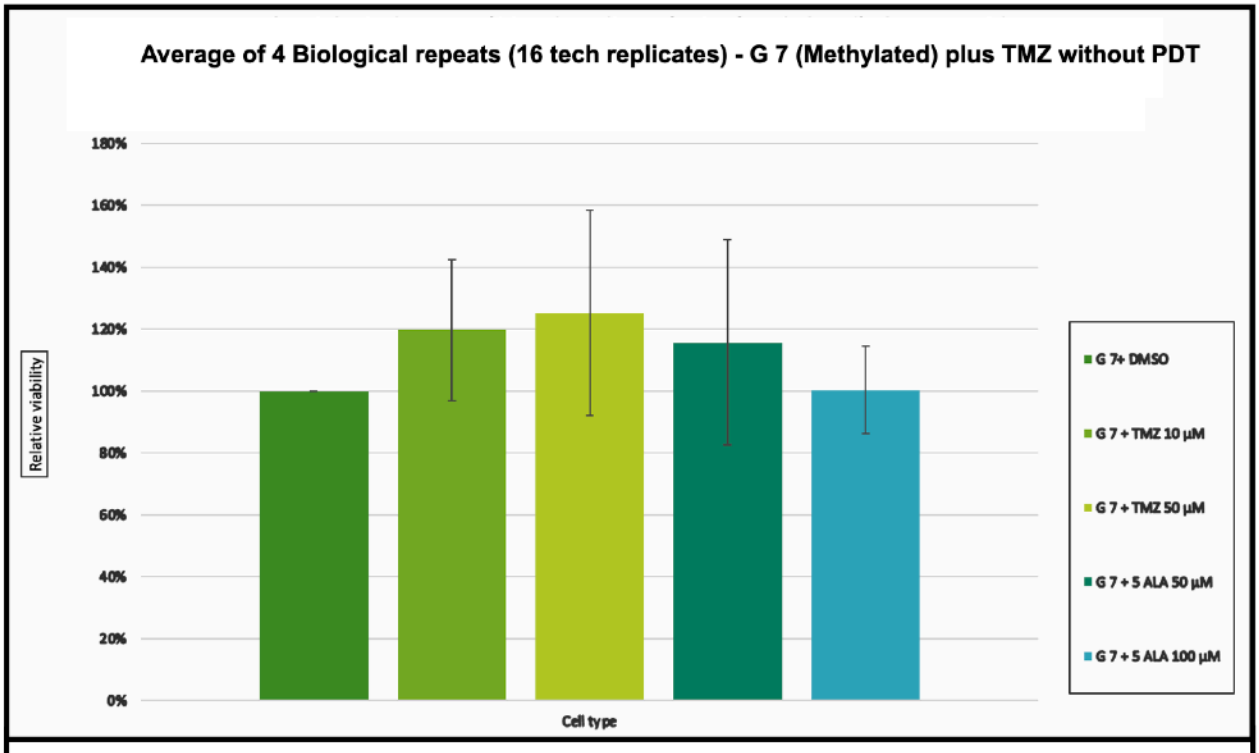
Clinically relevant doses of TMZ (Temozolomide - 10 and 50 uM concentrations) in Ox5 and G7 were tested. The average of 4 biological repeats in Ox5 primary GBM cell line **(Fig.5)**, single factor ANOVA had p-value 0.6> 0.05, as well as in G7 primary GBM cell line **(Fig.6)** p-value 0.7 > 0.05. Therefore, there was no difference in both cell lines with untreated controls. Additionally, 5-ALA (50 and 100 uM) were tested in both cell lines and didn't show any effect on the relative viability of Ox5 or G7, there were no statistical differences in both cell lines, P >0.05.

Figure 5.



* No statistical difference in averages of TMZ doses (10 & 50 uM) in OX 5 core, P>0.05.

Figure 6.



* No statistical difference in averages of TMZ doses (10 & 50 uM) in G 7, P>0.05.

Clonogenic assay G7 with PDT plus 5-ALA

Image 1.

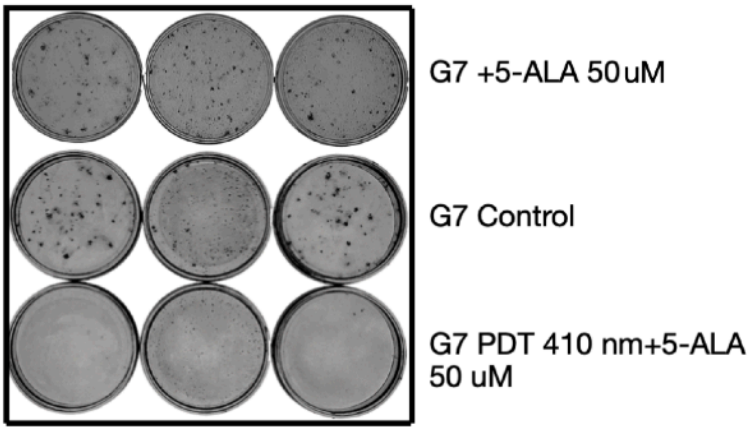


Figure 7.

In the clonogenic experiment, there were no colonies detected in G7 cell line after PDT illumination of 410 nm plus 5-ALA 50 uM with the intensity of 10mW, and the distance of 3.5 cm from the LED to the sample **(Fig.7; Supplementary img.1)**. However, colonies were formed in the additional control group 5-ALA 50 uM concentration without PDT.

In the clonogenic assay experiment after 2.5 weeks, there were no colonies observed in Ox5 with PDT 410 nm wavelength with the intensity of 10mW, and the distance of 3.5 cm from the LED to the sample applied, however, the experiment should be repeated for biological confirmation.

For comparative reasons radiotherapy with different doses of radiation combined with chemotherapy -(TMZ) were performed in the next experiment set-up.

Image 2.

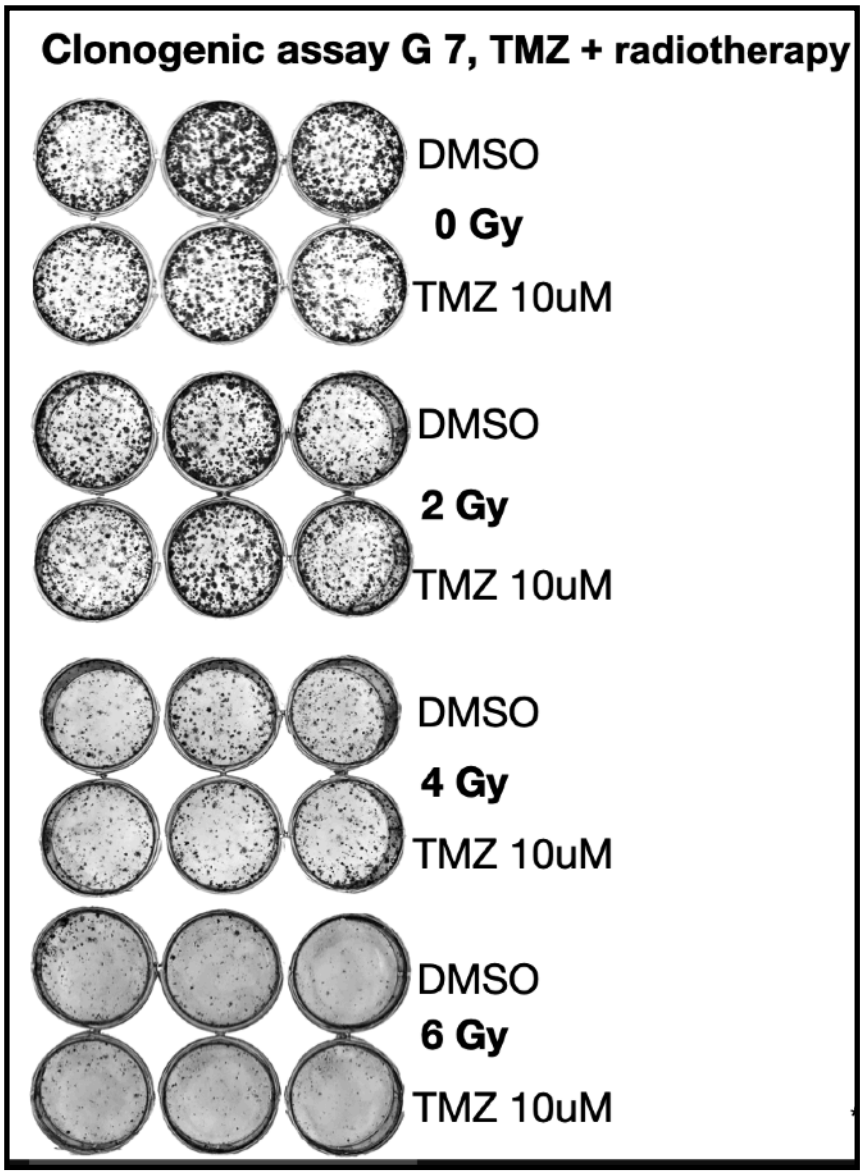


Figure 8.

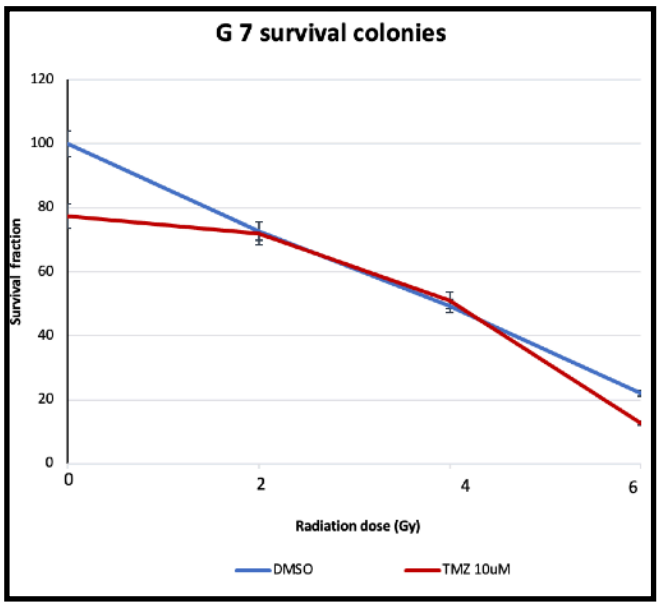


Image 3.

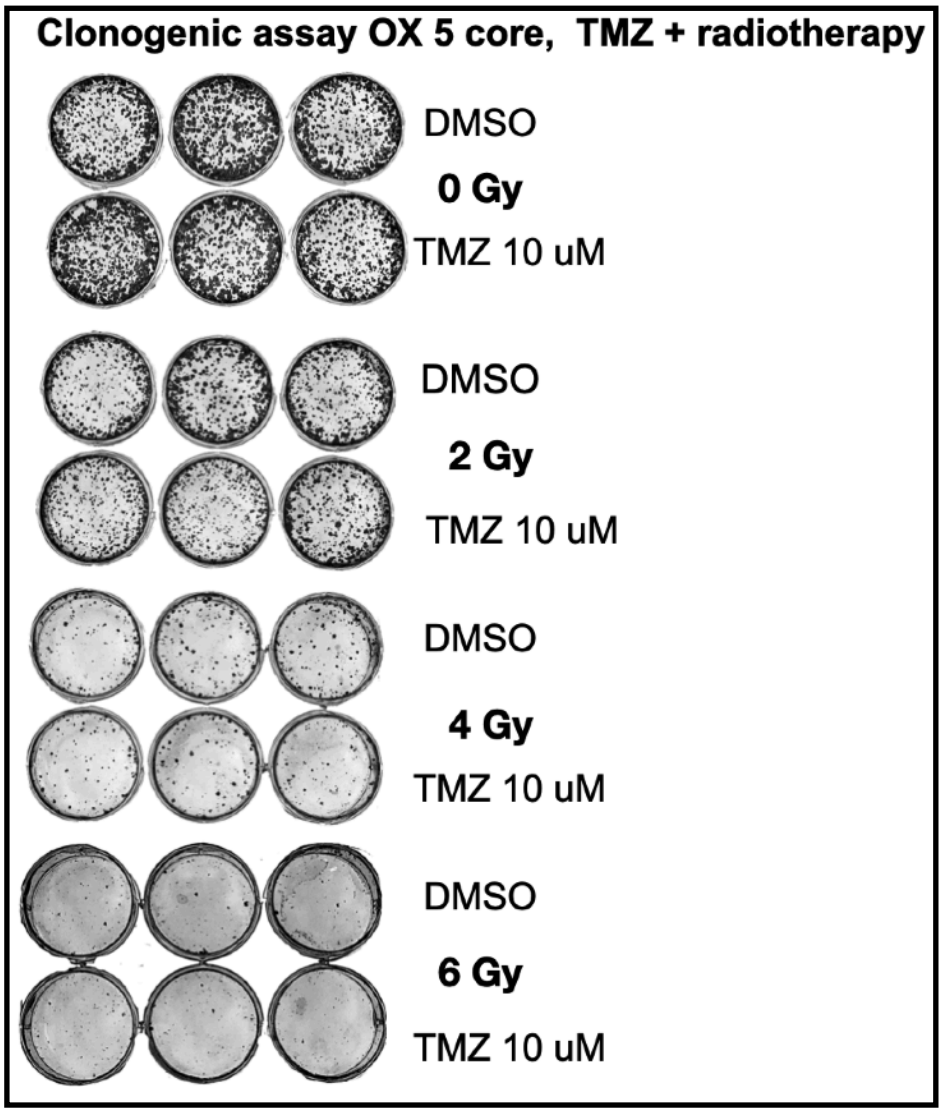
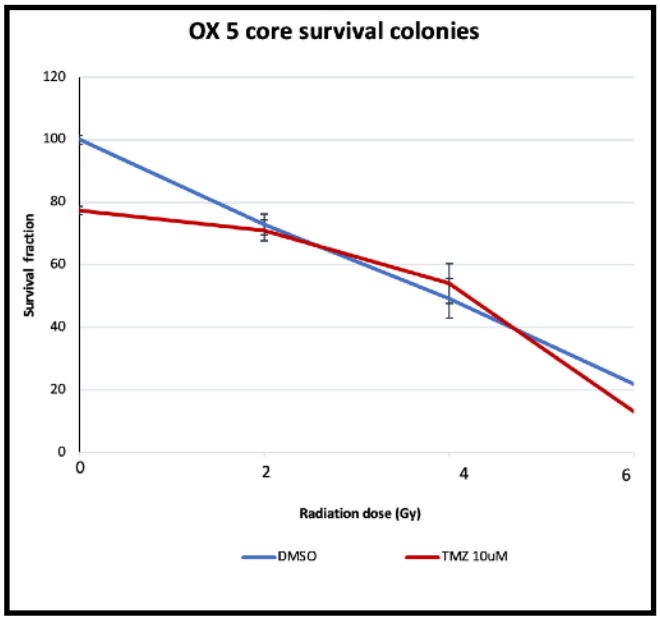


Figure 9.

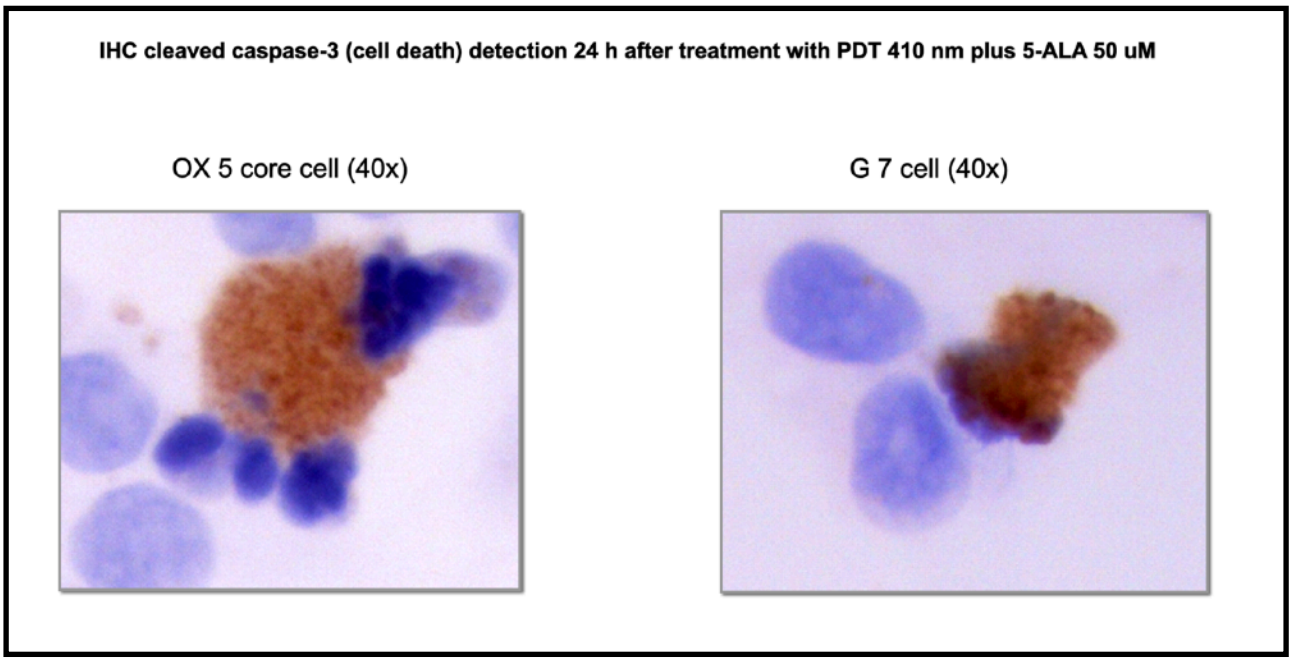


In the experiment with the clonogenic assay, G7 cell line demonstrated a significant fall in the number of the survived colonies at 4 and 6 Gy, although there was an insignificant difference between the dose of TMZ 10 uM in all plates and controls,(P>0.05). The average number of colonies irradiated from 0, 2, 4 and 6 Gy in combination with TMZ 10 uM is presented in **(Fig.8; Supplementary Img.2)**.

Clonogenic assay with Ox5 demonstrated a significant fall in the relation to the dose of radiation at 4 and 6 Gy in combination with TMZ 10 uM, however, a number of colonies remained almost the same within one plate comparatively with the controls (P>0.05), irrespectively of the dose of TMZ in all plates **(Fig.9; Supplementary Img.3)**.

IHC cleaved caspase-3 detected apoptosis in both cell lines (Ox5 & G7) 24 hours after illumination demonstrated on **(Img.4)**. PDT was performed at 410 nm/ 5-ALA 50 uM with the intensity of 10mW, and the distance of 3.5 cm from the LEDs to the samples. The time of photodynamic illumination was 4 minutes.

Image 4.



Discussion

This research demonstrated the efficacy of PDT with 5-ALA in Unmethylated Ox5 and Methylated G7 glioblastoma cell lines. Significant cell death was detected at 410 nm wavelength (PDT) photodynamic therapy with the intensity of 10 mW at 4 minutes. The relative viability and the confirmation of the cell death were performed in a number of ways via different assays with 4 complete biological repeats. The clonogenic assay demonstrated no colonies after PDT was performed in both cell lines. Therefore, after careful examination of the cells in the clonogenic assay, in particular, those treated with PDT with 5-ALA, under the microscope, the hypothesis

was made about the type of senescence that happened after PDT/ 5-ALA illumination. IHC cleaved caspase-3 illustrated the images of cell death in both cell lines. Interestingly, the difference in relative viability between Ox5 (Unmethylated) and G7 (Methylated) cell lines was noticed. Significant cell death was noticed in Ox5 at 77 % at 410 nm wavelength and 50 uM 5-ALA, while G7 showed 25.3 % of cell death under the same condition. However, more GMB cell lines are required to investigate MGMT status in relation to PDT with 5-ALA.

The Standard of care for GMB was mimicked in this research to demonstrate the efficacy of monochemotherapy (Temozolomide)- and radiotherapy with different doses of radiation ranging from 2 to 6 Gy in combination with chemotherapy treatment. The relative viability in both cell lines after 4 biological repeats didn't demonstrate any effect of TMZ monotherapy. Clinical doses of 10 and 50 uM were taken to investigate the efficacy of this treatment. Although radiotherapy combination with TMZ showed the decrease of the colonies at 4 and 6 Gy, survived colonies treated with TMZ still remained. The effect of TMZ didn't show any significance with irradiated and non-irradiated controls, within one plate, in the

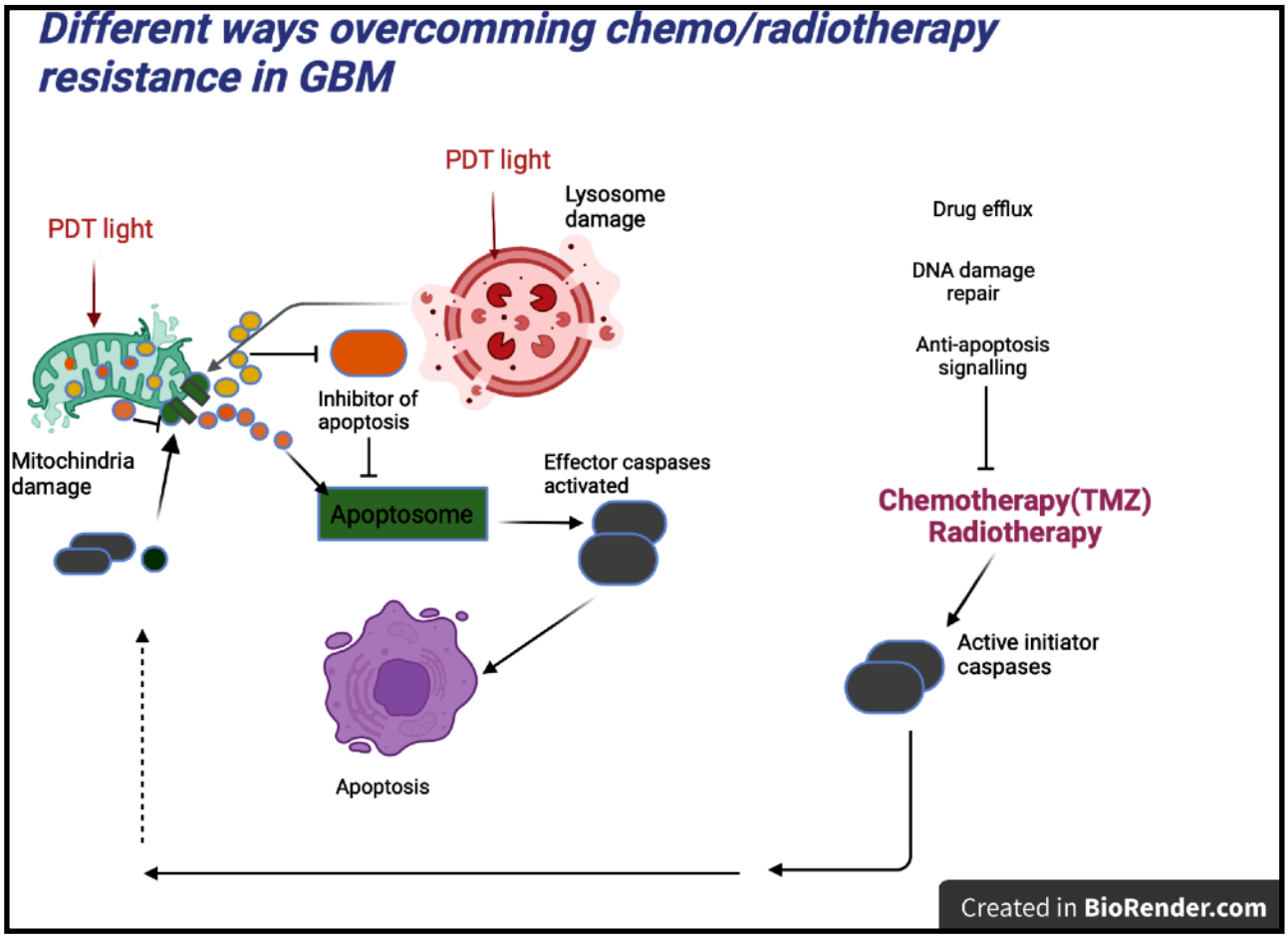
clonogenic assay. The problem of TMZ (O^6-methylguanine-DNA methyltransferase) and radiotherapy resistance is the biggest dilemma these days (18,43,50). Moreover, complications after chemotherapy in combination with radiotherapy, in particular, for GBM patients are the main issues in the countries with no insurance where patients have risks of thrombocytopenia and lack of affordability and governmental support. Despite this TMZ remains the priority for the vast majority of patients with high-grade glioma treatment. According to statistics, more than half of GBM patients treated with TMZ do not have the effect of chemotherapy (8). The knowledge about predictive markers for the TMZ is far beyond the MGMT status of glioma cell lines. In particular, glioma stem cells (GSCs) have heterogeneous nature in GBM which makes an opportunity for selecting specific clonal cell populations with growth advantages and forces mutation. GSCs as recurrent tumour-initiating cells are responsible for the regrowth of the tumour. MGMT is the main gene promotor enzyme and TMZ treatment response, in particular, in methylated glioma cell lines with better prognosis (18). However, under conditions given of chemotherapy treatment, the MGMT enzyme removes the methyl group in O_6-methylguanine,

therefore, DNA damage is neutralised leading to a dramatic decrease of TMZ effect on GBM. Moreover, base excision repair (BER), which repairs single nucleotides, involves proteins in the BER pathway and makes TMZ resistant to treatment. In particular, high-mobility group A_2 - stem cell factor, DNA glycosylase MPG, etc. Mismatch repair (MMR) demonstrates TMZ resistance via the progression of MMR mutation in response and de novo (51). Another crucial factor is the expression level of BCL-2, BCL-X1 and pro-apoptotic proteins, known as BOX, BAK, and BAX overexpression of those increases BCL-2 and BAK and leads to the resistance of TMZ and escape the drug from the GBM cell. The function of the Wnt and B-catenin pathways is the main in glioma progression, therefore Wnt pathway induces stemness in GBM leading to the induction of TMZ resistance. Additionally, B-catenin is another factor of TMZ resistance in glioma cells (52). Autophagy has a specific and cytoprotective role in tumour cells, although during apoptosis it is considered a chemoresistance mechanism development (23,35).

Thus, the ways of overcoming the chemoresistance may be demonstrated in Photodynamic therapy (PDT) **(Img. 5)**. However,

these mechanisms should be investigated in more detail via protein extraction in multiple ways (e.g. Western Blot, etc.). PDT light activates the intrinsic apoptosis pathway by damaging mitochondria outer membrane, which is regulated by BCL-2 family proteins and associated with BAX (homologous antagonist killer) (21).

Image 5.



Consequently, it forms pores in the mitochondria membrane, where 5-ALA is converted to protoporphyrin IX due to PDT light, and releases cytochrome-c, leading to the formation of the APAF-

apoptosome. This, in turn, activates caspase-9, caspase-3 and caspase-7 resulting in apoptosis. It is important to note that, caspase-8 serves as a crosstalk in intrinsic and extrinsic apoptosis pathways. This cleaves pro-apoptotic BH3-interacting domain death (BID), therefore BAK and BAX can be activated. Interestingly, during PDT, damage-associated molecular patterns (DAMPs), which are released from dying GBM cell lines, activate Toll-like death receptors (TLR) which form inflammasomes and activate caspase-1. Following this, caspase-1 activates gasdermin D (GSDMD), which forms pores in GBM cells, cleaves IL 1-B and IL-18, and finally causes pyroptosis (44).

Necroptosis is another form that may be activated via PDT with 5-ALA. PDT light affects lysosomes by damaging its membrane, therefore it activates tumour necrosis factor (TMF), binding to (TNFR) tumour necrosis factor receptor leads to pleiotropic GBM cell signalling, receptor-interacting serine/threonine protein kinase 1 (RIPK1) and (RIPK3) activation. As a result, necrosome phosphorylates mixed-lineage kinase-like (MLKL), leading to GBM membrane destabilisation and necroptosis (21).

Limitations and future directions:

Nevertheless, all the experiments were performed on primary GBM cells derived from patients under normal conditions and were time-consuming, therefore, various GBM cell lines are required to investigate the efficacy of PDT/5-ALA. Therefore, an investigation of the variability of GBM cell lines (2D & 3D models) will potentially demonstrate the relation between MGMT status and PDT/5-ALA. Characterisation of cell death pathways activated by PDT/5-ALA via protein extraction (e.g. Western Blot). Investigation of mechanisms underlying resistance to PDT/5-ALA. Investigation of small molecule modulators and their ability of cell death pathways to enhance sensitivity is required in the long-term study (e.g. BH3 mimetics). Further in vivo investigation is required to understand the molecular mechanisms of GBM cell death after PDT/5-ALA. In the mice models, stereotactic implantation of methylated and unmethylated GBM cell lines can be conducted and tumour growth can be checked under MRI control. Surgical resection of GBM tissue in vivo, treatment with PDT/5-ALA +/- cell modulators (e.g. BH3 mimetics). Ex vivo GBM culture models to check immune cells behaviour and/or immune cells purified from blood/tissue after PDT/

5-ALA. Panels of orthotropic xenograft and allograft GBM models with PDT set-up.

Conclusion

Current in vitro research demonstrates the efficacy of PDT with 5-ALA in malignant glioma. There is significant cell death in MGMT promotor unmethylated Ox5 and methylated G7 at PDT 410 nm wavelength with 50uM 5-ALA. Adding 5-ALA at 410 nm wavelength has a cytotoxic effect in both cell lines in the clonogenic assay. Standard of care (TMZ) didn't demonstrate any effect in Ox5 and G7 GBM cell lines. Radiotherapy combination with TMZ showed remaining colonies at a higher radiation dose (6 Gy). Cell death was detected via apoptotic pathway (cleaved caspase-3) after 24 h for both cell lines (PDT 4 minutes plus 50 uM 5-ALA illumination). The results of this manuscript have future potential tools to explore the effect of photodynamic therapy in malignant brain tumours, in particular, GBM.

Acknowledgements:

I would like to express my appreciation to Anthony Chalmers, Karin Williams and Paul Brennan for supervision, reviewing and accepting the manuscript thesis. Rebeca Ginesi for helping with the PDT set-up.

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