Photodiagnostics and photodynamic treatment of stem cells cultivated from human glioblastoma tumors

L Zaharieva¹ ² *, E Borisova¹ ³, D Kyurkchiev⁴, K Tumangelova-Yuzeir⁴, E Ivanova-Todorova⁴, I Angelov¹ ⁵, Ts Genova¹, B Kolev¹, A Gisbrecht¹, L Avramov¹, O Semyachkina-Glushkovskaya³, P Karazapryanov⁶ and K Minkin⁶

¹ Institute of Electronics, Bulgarian Academy of Sciences, 72 Tsarigradsko Chaussee blvd., 1784 Sofia, Bulgaria
² Faculty of Physics, St. Kliment Ohridski University of Sofia, 5 James Bourchier blvd., 1164 Sofia, Bulgaria
³ Biology Department, Saratov State University, 83, Astrakhanskaya str., 410012 Saratov, Russia
⁴ Laboratory of Clinical Immunology, St. Ivan Rilski University Hospital, Department of Clinical Laboratory and Clinical Immunology, Medical University of Sofia, 15 Acad. Ivan Evstratiev Geshov blvd., 1431 Sofia, Bulgaria
⁵ Institute of Organic Chemistry with Center on Phytochemistry, Bulgarian Academy of Sciences, 9 Acad. G. Bonchev str., 1113 Sofia, Bulgaria
⁶ Department of Neurosurgery, St. Ivan Rilski University Hospital, 15 Acad. Ivan Evstratiev Geshov blvd., 1431 Sofia, Bulgaria

*E-mail: zaharievalidia@gmail.com

Abstract. Porphyrins used as photosensitizers are accumulated selectively in glioblastoma cells due to the latter heme metabolism disorders and could be used effectively for intraoperative fluorescence staining of the tumor formation and differentiation from normal brain parenchyma. The aim of this work was to investigate the photodynamic properties of 5-ALA/PpIX on stem cell cultures isolated from glioblastoma. Supernatant samples of photosensitizer-treated cell lines were used for evaluation of photosensitizers’ accumulation in the cell lines investigated using excitation in the spectral ranges of Soret’ band (360 – 410 nm) and Q-band (600 – 650 nm). The emission detected was used to evaluate the efficacy of the photodynamic treatment during PDT irradiation inducing apoptosis and necrosis processes in cell lines treated with photosensitizers. Several variables were studied, such as histochemical and genetic variants of glioblastoma, and various photosensitizers’ concentrations and light-emitting parameters. The results of this in vitro work will be used as the basis for a further in vivo application on animal models of glioblastoma with applying irradiation using intracranial light sources and for a subsequent transfer of the obtained protocols of photodynamic treatment of glioblastoma lesions in such model systems for the needs of human medicine.

1. Introduction
This work focuses on photodiagnostics (PD) and photodynamic therapy (PDT) of brain cancer and in particular on glioblastoma – a malignancy of the central nervous system (CNS). This form of brain cancer is considered as the most aggressive and is the last stage of the most common form of brain tumor...
– astrocytoma [1]. Glioblastoma (GBM) is the most common and most severe type of brain tumor. The average survival rate in patients with newly diagnosed GBM is only 12 – 14 months, notwithstanding the use of aggressive surgery, radiation therapy and chemotherapy. Glioblastoma, unlike other malignant tumors, rarely spreads outside of the brain and the main cause of death is the local intracranial progression. Surgery and subsequent radiotherapy and chemotherapy do not lead to effective results in the treatment of this type of malignancy [1-3]. Therefore, scientists are trying to find alternative methods that would lead to more effective and long-term treatment of glioblastoma cells after conventional clinical therapy, such as photodynamic treatment [4].

Photodynamic therapy (PDT) and photodiagnostics (PD) are unconventional methods for treating and diagnosing malignant tumors. The therapeutic effect of PDT is based on the photodynamic effect associated with the properties of free radicals (singlet oxygen) to oxidize lipids and proteins. Radicals are activated after light exposure of photosensitive molecules called photosensitizers (PS) that are accumulated selectively in cancer cells, which after light irradiation transfer the excitation energy to produce singlet oxygen. Photodiagnostics is based on the fluorescence properties of such accumulated photosensitizers, whose emission could be observed after light irradiation and used as an indicator of tumor cells presence [4, 5].

PDT and PD made their first significant steps in the treatment and diagnosis of cancer cells nearly 50 years ago. Initially, the experiments were performed on rodents; it was found that the fluorescent signal of porphyrins in the red end of the visible spectrum carries with it diagnostic information for the presence of tumors. In the last few years, this information has been used to determine the boundaries of lesions. The researchers then found that hematoporphyrin, a type of porphyrin, could be used to selectively destroy malignant cells, and later managed to completely cure the tumor through using its derivative, the so-called hematoporphyrin derivative (HpD) [6]. Nowadays, many different compounds are in use for PD and PDT applications, but brain tumors photodynamic treatment is still under investigation and optimization due to the limited number of photosensitive drugs that could penetrate the blood-brain barrier and effectively treat the tumor cells.

The absorption peak of meta-tetra (hydroxyphenyl) chlorin – mTHPC (trade name Foscan®) is located at longer wavelengths than that of porphyrins, namely, near the end of the red spectrum (652 – 662 nm), which allows for a better treatment at a greater depth due to the higher penetration length of this light into the tissue. Other chlorins, with absorption bands in the region 660 – 670 nm, such as Talaporfin®, LS11, MACE, Npe6, Fotolon® (PVP – Ce6), Radachlorin®, Photodithazine® are also being investigated as possible photosensitive drugs applicable to brain tumor treatment [4, 7, 8].

Styli et al. conducted one of the largest studies for PDT of glioblastoma multiforme at Royal Melbourne Hospital in Australia. A group of 375 people were treated with HpD and laser irradiation. The development of 138 patients was monitored for over 3 years – 78 of them with GBM, 58 with AA. The procedure was as follows – 5 mg/kg HpD were injected intravenously 24 hours before surgery and then irradiated at 532 nm at a dose between 70 – 260 J/cm². The median survival of newly diagnosed patients with glioblastoma was 14.3 months; that of relapsed patients was 13.5 months. The two-year life expectancy for GBM was 28% and for AA patients, 37% [9].

Delta-aminolevulinic acid (5-ALA) in combination with HpD is also being used as an option for the diagnosis and treatment of CNS tumors. It is applied in fluorescence-guided resection and sensitization of cancer cells, and with HpD for sensitization of interstitial tumor tissue and blood vessels that supply the tumor [10]. The molecular weight of 5-ALA is low, so that it could cross the blood-brain barrier and accumulate in tumor cells. It is not photosensitive by itself, but is a precursor of protoporphyrin IX (PpIX), which is a photosensitizer. As a result of the 5-ALA metabolism in the cells, the concentration of PpIX in glioblastoma is much higher than in the surrounding healthy cells (over 19 times). Subsequently, tumor fluorescence can be induced and used for the so-called fluorescent-guided resection, when the outline of the lesion is clearly visible during surgery due to the fluorescence emission of the PpIX. PDT with the same photosensitizer (PpIX) could be applied after the surgical removal of the main tumor mass to kill residual tumor cells in the tumor bed. The first studies on PDT using 5-
ALA/PpIX in advanced gliomas showed an overall improvement in patients' life expectancy and an increase from 18% to 28% in their two-year survival [4, 11].

Photodynamic therapy (PDT) using 5-ALA/PpIX from the family of porphyrins has proven its effectiveness in the treatment of different neoplasia, including brain ones. There is still room for optimizing the photodynamic therapy procedures for effective treatment of GBM. Furthermore, the fact that it leads to positive results stimulates scientists and doctors to continue working in this direction.

2. Methods and materials

After surgical removal of glioblastoma tumors from patients at the St. Ivan Rilski University Hospital, Sofia, cells with adherent morphology were grown in six-well plates and prepared for photodynamic treatment in the Laboratory of Clinical Immunology of the hospital. Glioblastoma cells were cultivated with 5-ALA, in order to achieve a proper concentration of PpIX in the cells. The next step included delivering a selected light dose to the prepared adherent cell layers and preliminary assessment of the apoptosis and necrosis levels using flow cytometry detection with markers for early and late apoptosis. The experiments were carried out using a light dose of 60 J/cm², in accordance with the results of the optimization procedure in our previous study [12].

Depending on the incubation with photosensitizer and irradiation with a certain dose of light, the control cells were divided into three main groups. The first was the so-called "zero control group" – no incubation with a photosensitizer and no light irradiation. In the second "light control group", again there was no incubation with a photosensitizer, but the cells were irradiated with light at the same dose as the treated cells to which a sensitizer was added. Finally, in the so-called "drug control group" the cells were incubated with 5-ALA, but were not irradiated.

The three control groups were studied to distinguish the effects of light irradiation and the so-called dark toxicity of photosensitizing substances on apoptotic changes observed in cell samples derived from real glioblastoma tumors extracted during surgical resection of the lesion.

In the experimental group, 5-ALA was administered at a fixed dose of 25 μg/ml for each of the six wells. The well contained cells from the corresponding group planned for treatment with a photosensitizer. The procedure was performed four hours before irradiation with light at the wavelength of 635 nm. Upon completion of PDT, the treated cell wells were divided into two groups. In the first one, the cells were studied one hour after light irradiation in view of detecting early apoptotic changes; in the second group the cells were studied 24 hours after the photodynamic treatment seeking late apoptotic changes.

The appearance of PpIX in the cultivated GBM tumor cells was evaluated by way of spectroscopic measurements of the fluorescent signal from the supernatant by means of a FluoroLog 3 spectrofluorimeter (HORIBA JY, France) with excitation at 405 nm, where absorption in the Soret band of the PpIX takes place, and at 615 – 630 nm, where the Q-absorption lines of the photosensitizer are located.

3. Results and discussion

Figures 1 (a) and 1 (b) present the fluorescence spectra of supernatants of the control and experimental groups of glioblastoma cells treated with 5-ALA and PDT procedures performed on two different samples obtained from two different patients with GBM. They represent high- and medium-effective PDT of the cells.

In the control group, no statistically significant fluorescence emission peak was observed in the range 630 – 670 nm, where the fluorescence of PpIX was detected. In the case of the 5-ALA-treated cells, a maximum was observed due to the accumulated photosensitizer PpIX.

The ratio of the fluorescent signal in the red spectral region between the emission of the control and experimental groups of cells provides a semi-quantitative indicator of the degree of accumulation of the photosensitizing agent, which correlates with the degree of development of the tumor formation from which the glioblastoma cells were taken.
Figure 1. (a) Fluorescence spectra of supernatants of control and experimental groups of glioblastoma cell samples treated with 5-ALA/PpIX on 05.05.2020 (a) 15.05.2020 (b).

The following graphs (figures 2 (a), 2 (b) and 2 (c)) represent the differences between the control groups and the PDT-treated cell samples, respectively, with respect to the observed early apoptosis, late apoptosis (after 24 hours) and necrosis in the treated cells, using 5-ALA/PpIX as a photosensitizer.

Figure 2. Recorded levels of differences between treated and control glioblastoma cell lines for cells with induced early apoptosis changes (a), late apoptosis changes (b) and necrosis changes (c).

The mean value of the late apoptosis (after 24 hours) is the highest, followed by that of early apoptosis and finally necrosis. It is seen that the process of apoptosis is better manifested than the induction of necrotic changes in PDT-treated cells. The fact that necrotic cell death is less pronounced after PDT treatment allows us to conclude that when administered in vivo, the side effects associated with the necrosis toxic response of the body will be minimal. Further analyzes of these results need to be made as they could contribute to improving the effectiveness of the PD treatment.

4. Conclusions

Glioblastoma is one of the deadliest types of cancer. There is still no efficient therapy to completely deal with this disease. That is why many researchers worldwide try to improve the effectiveness of PDT and PD in this area of oncologic applications.

In our experimental studies, the best results for treatment of GBM adherent cells were obtained when cells from surgically removed GBM tumors were irradiated with a light dose of 60 J/cm² after exogenous 5-ALA application at a dose of 25 μg/ml.

A positive PDT reaction was observed in the whole set of cell samples taken from different patients, but a wide distribution of the response to treatment was also found, with the selected dose being optimal in terms of maximum response. This means that at values equal to or higher than the applied light dose, all cell samples showed apoptotic changes. Also, the apoptotic reaction was more pronounced than the necrotic one, which allows us to assume that the in vivo treatment will lead to fewer adverse side effects related to systematic necrotic changes as the body reacts to the PDT procedure applied.
Further analysis of the results obtained is forthcoming; we believe that these will contribute to the better understanding of the mechanism of apoptotic death of glioblastoma cells resulting from PDT.

5. Acknowledgements

This experimental work was supported by the National Science Fund of Bulgaria under grant #KP06-N23/8/2018 “Innovative photodynamic methods for treatment of stem cells cultivated from glioblastoma tumors”. The work of I. Angelov, B. Kolev, and Al. Gisbrecht was partially supported under grant #KP06-N38/13/2019 “Development of methods of biophotonics as a basis of oncological theranostics – 2”. The spectrofluorimetric equipment used is purchased under project DO-02-112/2008 "National center of biomedical photonics“.

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