PDT and PD of Human Glioblastoma with 5-ALA/PpIX and n-GalLuPc

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Abstract. Glioblastoma (GBM) is the most common and severe type of brain tumor. Surgery and subsequent radiotherapy and chemotherapy do not lead to sufficient results in the treatment of this type of malignancy, mostly due to its specific morphology. Only about 6% of the patients of advanced age survive 5 years after being diagnosed with GBM. Therefore, scientists are working on alternative therapies that would lead to more effective and long-term treatment of glioblastoma. Photodynamic therapy (PDT) and photodiagnostics (PD) are such unconventional methods for treating and diagnosing malignant tumors. During our work, more than 20 experiments were carried out with stem cells cultivated from human glioblastoma tumors. We used two types of photosensitizers – delta-aminolevulinic acid (5-ALA) as a precursor of protoporphyrin IX (PpIX) and non-peripherally galactosylated lutetium phthalocyanine (n-GalLuPc). After the irradiation supernatant samples of photosensitizer-treated cell lines were used for evaluation of photosensitizers' accumulation in the cell lines investigated. The emitting spectra is correlated with the total induced cell death in the treated cells. After considering the overall effectiveness of the two photosensitizers, n-GalLuPc showed higher efficiency. These *in vitro* results prompted the future investigation of the phthalocyanine for *in vivo* application in GBM treatments.

1. Introduction

In this work we have focused our attention on photodetection (PD) and photodynamic therapy of brain cancer. In details, we have investigated PD and PDT of glioblastoma cells, using porphyrins and phthalocyanines as photosensitizers (PS) and fluorescence markers.

Glioblastoma is a malignancy of the central nervous system. It is considered as one of the most aggressive types of tumors and the most common and severe type of brain tumors. Patients diagnosed with GBM have approximately 12–14 months survival rate, even after they have been treated with all

types of conventional clinical therapy – surgery, radiation therapy and chemotherapy [1,2]. This is due to the specific morphology of the tumor and the presence of small subpopulation of cells with a self-renewal function (glioma stem cells) and also to the so-called blood-brain barrier, which has selective permeability [3].

Therefore, physicians and scientists are diligently working on the implementation of some new methods for diagnostics and therapy, such as PD and PDT, which could lead to more effective and permanent treatment of glioblastoma cells. Although these unconventional methods have been used for treatment and diagnosis of cancer for nearly 50 years, their clinical application is still a great subject of improvement and development of new, more effective photosensitizers [1].

The utilization of wavelengths with deeper penetration and PS with appropriate absorption helps to minimize the amount of PS (drug dose), which is necessary for successful therapy [4]. Using a single pure compound reduces operating costs and leads to subsequent quality control and the possibility of long-term storage. Ideally, the PS should be water-soluble or at least soluble in another solvent. In addition, it must be aggregated evenly in the biological environment, because otherwise its photochemical activity will be reduced [5,6].

Photosensitizers with tetrapyrrole structure, are the ones to be commonly used in PDT. This type of bond is found in several important biomolecules, such as heme and chlorophyll. Their absorption peaks are in the red end of the spectrum. In addition to these molecules, phthalocyanines also have a wide peak in the 670-nanometer range.

Phthalocyanines were one of the first compounds to be studied in the 1980s and 1990s. The unmodified zinc phthalocyanine in liposomal form has been used experimentally as a PS with both animals and humans. Cationic phthalocyanines such as RLP068 have been studied for antimicrobial applications *in vivo* and clinically for diabetic infected foot ulcers. Under the clinical name Photosens – a photosensitizer of phthalocyanine type, is approved for clinical use in Russia for cancer of the head and neck, stomach, bladder, and others [6].

We have already explored and described the PD and PDT with porphyrins using 5-ALA as a precursor to PpIX in a previous work [7]. Therefore, now we are focusing on applying phthalocyanines as PS and continuing our research with 5-ALA for better statistics of the results.

2. Materials and Methods

GBM stem cells were excised from human glioblastoma tumors, which were surgically removed from patients at the University Hospital "St. Ivan Rilski" – Sofia. This study is conducted in accordance with ethical principles and all investigated samples were obtained with the consent of the patients. Then, the GBM cells were grown in 6-well plates and prepared for PDT. Before the irradiation (4-6 hours), 5-ALA or n-GalLuPc was exogenously administered into the GBM cells environment at a fixed dose of 25 μg/ml for the 5-ALA and 20 μM for the n-GalLuPc, in order for the cells to accumulate a proper concentration of the photosensitizing agent – PpIX or n-GalLuPc, respectively.

In figure 1 is shown the experimental set-up arrangement for PDT treatment of cells samples. Light at 635 nm, with intensity of 30 mW/cm^2, measured at solvent surface, was applied for about 30 min to achieve irradiation dose of 60 J/cm^2. The wide area light emitter is based on 25 pcs high power red LED's. This light source gives us the possibility to achieve a light intensity of the order of 100 mW/cm^2 per area of 25 cm^2, with an unfocused beam, in order to irradiate all samples on the 6-well plate. The irradiation intensity was chosen so that the temperature of the samples would not increase with more than 1°C during the irradiation. The light intensity and the light dose were chosen, according to the results for optimization of the PDT procedure from our previous studies [8-9]. The second light channel realized through a diode laser with low power, optical system and a spectra analyzer Ocean Optics, was used to observe the bleaching process of the photosensitizers, during the irradiation. At the used conditions, the level of the bleaching was negligible for both PSs.

After the irradiation, apoptosis and necrosis levels were assessed, using flow cytometry detection with markers for early apoptosis and late apoptosis. For the experimental group the incubation procedure was performed 4 hours before the irradiation at 635 nm. After the PDT, the treated cell wells were

separated into two groups. One was used one hour after the PDT to detect early apoptotic changes, and the other – 24 hours after the irradiation, to detect late apoptotic alterations.

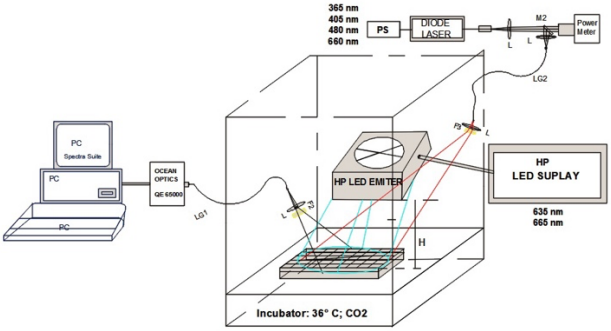

Figure 1. Experimental set-up arrangement for PDT treatment of GBM cells.

The next step was fluorescence spectra measurement of the supernatants from the control (without irradiation) and PS' treated cell lines with FluoroLog 3 spectrofluorometer (HORIBA JY, France). To all supernatant samples was applied an excitation at 630 nm.

The aim of the last measurement is to determine the trace concentrations of protoporphyrin IX and n-GalLuPc in the respective supernatants. With this experimental approach we evaluate the efficiency of inducing apoptosis and/or necrosis, in the studied cell samples, and the possible correlation between these induced cell death processes, expressed in percentages, and the fluorescence signal from the supernatants.

3. Results and Discussion

In figure 2 is presented the obtained data for the levels of induced (late) apoptosis, early apoptosis and necrosis, after the cells were treated with the corresponding photosensitizing agent. For comparison of the distribution of the obtained results for the different processes of cell death, we have chosen the box and whisker plot, representing the mean value, medium value and the dispersion of the data set.

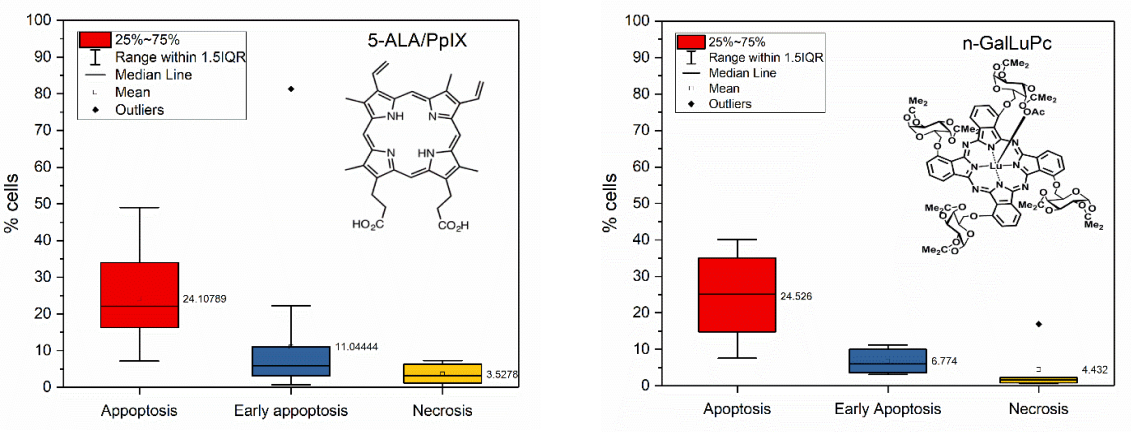

Figure 2. Evaluated levels of induced cell death after PDT of the experimental groups of glioblastoma cell samples treated with 5-ALA/PpIX (a) and n-GalLuPc (b).

With both PSs we observe the same trend – apoptosis is with the highest mean value of inducement in the cells, followed by early apoptosis and necrosis. This corresponds well with our expectations and leads to the conclusion that for both PSs the cell death is caused primary by apoptosis, which reduces

the toxic effect from the necrosis. At the same time the average percentage of the induced cell death in the control groups is much lower, which confirms the efficacy of the PDT.

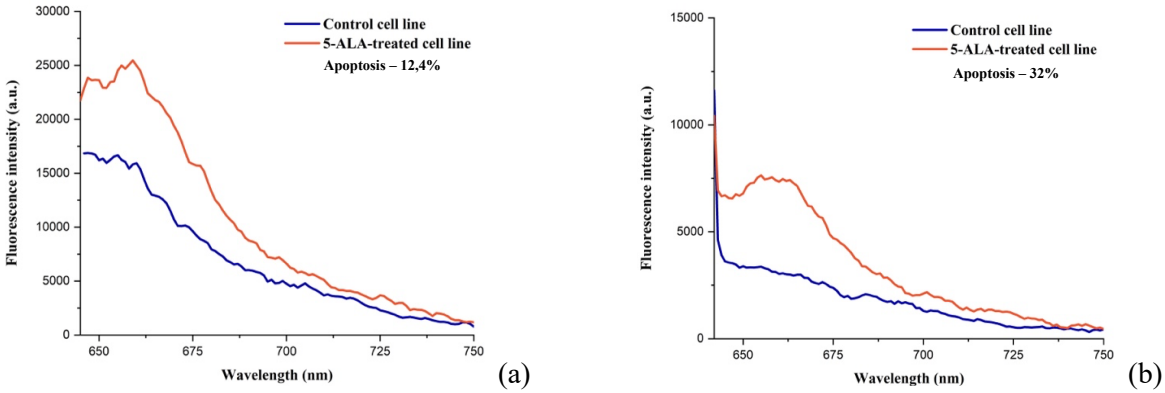

Figure 3. (a) Fluorescence spectra of supernatants of control and experimental groups of glioblastoma cell samples treated with 5-ALA/PpIX at 12.03.2020 (a) 20.07.2021 (b).

In the following figures are shown the fluorescence spectra of the supernatants of the control and experimental groups of GMB cell samples treated with 5-ALA (figure 3) and n-GalLuPc (figure 4). Figure 2(a) represents the fluorescence spectra for a sample treated with 5-ALA, demonstrating medium PDT efficacy and figure 2(b) represents the fluorescence spectra from a sample with high PDT efficacy. Similarly, results are presented on figure 3(a) and (b), for samples treated with n-GalLuPc.

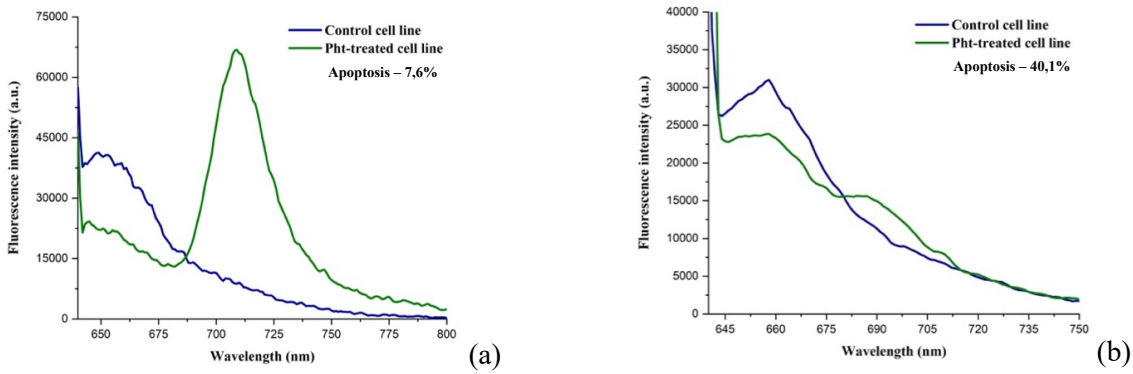

Figure 4. (a) Fluorescence spectra of supernatants of control and experimental groups of glioblastoma cell samples treated with n-GalLuPc at 04.11.2021 (a) 20.07.2021 (b).

5-ALA is a precursor of PpIX. We inject the acid exogenously into the cellular environment, so the endogenous induction of the photosensitizer PpIX is additionally stimulated to achieve effective concentrations for PDT. We observe a fluorescence maximum at around 660 nm, which coincides with the characteristic fluorescence of PpIX. From the spectra we see that the increase in the fluorescence ratio between the control and the treated cell lines, coincides with higher rate of induced cell death after the PDT.

When we analyze the spectra of the supernatants of the cells treated with phthalocyanine, we observe a peak around 690-730 nm, which confirms the presence of n-GalLuPc in them. Here, reduction of the fluorescence ratio corresponds with higher percentage of induced cell deaths. The reason behind this inversion is that the phthalocyanine is an exogenous photosensitizer that is introduced into the cell environment and subsequently interacts to various degrees with the glioblastoma cells.

In the experimental studies, the best results were obtained after cells' irradiation with a light dose of 60 J/cm^2 at a 36°C when in the cell environment was injected a drug dose of 25 μg/ml for the 5-ALA and 20 μM for the n-GalLuPc.

As it can be seen, PDT treatment is successful in most cases, but there is a high standard deviation (SD) in the results for the different types of induced cell death. The results are ambiguous and one of the reasons is that the glioblastoma cells were taken from 9 women and 11 men. Hence the large SDs when examining the parameters of early apoptosis, late apoptosis and necrosis, could be caused by high variety of mutations in the cells. Considering that they originate from 20 different patients and mutations could be observed even in samples from the same patient. The average age of the patients is 60 years, but again there is large age gap between the youngest being 34 and the oldest being 88. Nevertheless, we do not observe any specific dependence on the patient's gender and age in the effectiveness of the method's impact on cell cultures.

When comparing the efficacy of the photodynamic effect of the two photosensitizers, almost the same PDT response was observed. Nevertheless, after analyzing the average percentage of the total induced cell death, n-GalLuPc showed higher efficacy, which leads to the assumption that the galactose in the compound has increased the selectivity of the photosensitizer and the affinity of the tumor cells to accumulate it.

4. Conclusions and discussion

Glioblastoma is one of the most severe malignancies, which leads to high mortality among the patients. The reason is that there is still not efficient enough therapy, which can completely and permanently treat this disease. Therefore, researchers all over the world, work very hard to improve the efficacy of PDT and PD in this oncologic field.

Despite the mentioned facts, we observe a wide distribution of treatment response, with the selected dose being optimal. This means that at values equal to or higher than the applied light dose, all cell samples showed different response from PDT treatment. This can be explained with the presence of a various number of glioma stem cells in each tumor, which according to the literature, is responsible for the different resistance to the used procedure [10].

We foresee more investigations to verify our observations and to further improve the structure and thus the selectivity of the phthalocyanine. We are hoping that after additional improvement this compound will be applicable for *in vivo* clinical stages.

Acknowledgements

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