Effect of Photosensitizers Photosens, Photodithazine and Hypericin on Glioma Cells and Primary Neuronal Cultures: a Comparative Analysis

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The aim of the study was to compare the effect of photosensitizers photosens, photodithazine, and hypericin on primary brain cell cultures, and assess their toxic effect on tumor and normal nervous cells in order to choose the optimal photodynamic agent for glioma therapy.

Materials and Methods. The cytotoxicity of photosens (NIOPIK, Russia), photodithazine (Veta-grand, Russia) and hypericin (Merck KGaA; Sigma-Aldrich, Germany) was assessed on primary brain cell cultures obtained from C57BL/6 mice (gestation day 18). On day 14 of cultivation, the tested photosensitizers were added to a culture medium at concentrations of 0.1, 1, 10, 50, and 100 μM. Then the cultures were placed in a CO_2-incubator in the dark. The viability of primary neuronal cultures was estimated on days 3 and 7 after photosensitizer application. Using confocal microscopy, we analyzed the rate of entry and subcellular localization of the tested agents in the primary neuronal cells. Statistical analysis was performed in SigmaPlot 11.0 (Systat Software Inc., USA) using ANOVA.

Results. We analyzed the absorption and fluorescence spectra of the tested photosensitizers. Photosens and photodithazine showed the presence of absorption maximum in short- and long-wave spectral ranges. Hypericin was characterized by a complex spectrum with many peaks in both blue-violet and orange-red spectral ranges. Cell viability analysis revealed that high concentrations of photosensitizers caused a pronounced toxic effect on nervous cells. The most marked effect was shown for photodithazine. Photosens exhibited the lowest accumulation rate in primary neuronal cells. Photosens and hypericin were found to have a high phototoxic effect on glioma, and demonstrated low dark toxicity for normal brain cells.

Conclusion. The photosensitizers hypericin and photosens are the least toxic for nervous tissue, though effectively penetrating in tumor cells. These properties enable to consider them as prospective photodynamic agents for clinic.

Key words: photosensitizers; photosens; photodithazine; hypericin; photodynamic therapy; neurooncology; cytotoxicity; primary neuronal cultures.

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Introduction

According to Central Brain Tumor Registry of the United States (CBTRUS) the frequency of diagnosed malignant and benign brain tumors is 10.71 cases per 100,000 people under 39 years old, and 40.10 cases per 100,000 of population over 40 years of age [1, 2]. Glioma is the most common brain tumor (75–80% of primary malignancies) characterized by marked histological heterogeneity. Among these are astrocytomas (astrocytoma, anaplastic astrocytoma, glioblastoma multiforme), oligodendrogliomas and mixed gliomas. A distinctive feature of gliomas is rapid invasive growth, high proliferative activity, and intensive angiogenesis, which significantly reduce the effectiveness of therapy [3].

Contemporary anti-cancer therapy of brain tumors consists of a complex of procedures, including microsurgical tumor resection with distant postoperative radio- and chemotherapy [4]. Although gliomas have pronounced infiltrative growth and ability to involve functionally important parts of the brain to malignant process, conduction of radical surgical tumor eradication is not possible in most of the cases. Moreover, 96% of patients with maximum tumor resection have a relapse in a perifocal area within a short period of time [5, 6]. Blood-brain barrier (BBB) is also a significant challenge for anti-cancer therapy of brain tumors. Despite the fact that tumor growth is accompanied by BBB damage and an increase in its permeability, the use of drugs and their entry into tumor is impeded [7]. Screening of currently known cytostatic agents has revealed their low efficiency in brain tumor therapy, resulting in a limited list of officially approved drugs for malignant gliomas treatment [8, 9]. Thus, prognoses for patients with malignant glioma are still extremely unfavorable, even in case of using advanced treatment techniques. A lifetime after surgical tumor resection in population is about 10 months [10]. Low efficiency of traditional therapy of brain tumors and poor patient prognoses require improvement of the applied techniques, as well as a search for alternative approaches to treat this pathology.

Photodynamic therapy (PDT) is regarded as the most attractive strategy for effective adjuvant therapy of gliomas [11]. The method suggests the use of photosensitizers able to cross BBB and selectively accumulate in tumor [9, 12]. When exposed to visible light irradiation, an activated photosensitizer generates singlet oxygen and radical active oxygen species that have a direct cytotoxic effect on tumor cells causing their death, including an immunogenic pathway [13].

PDT also damages microvascular tumor environment due to vascular stagnation, thrombosis, and hemorrhage resulting in hypoxia and subsequent cell death [14, 15]. The efficiency of photodynamic damage effect depends on intracellular concentration of a photosensitizer, its localization in a cell, photochemical activity, and an irradiation dose.

Currently, the derivatives of hematoporphyrin (HpD), chlorin, phthalocyanine, as well as 5-aminolevulinic acid, the precursor of endogenous hematoporphyrin IX, are commonly used as photosensitizers in photodynamic therapy of brain tumors [16]. In European Union, hypericin (Sigma-Aldrich, Germany) is actively used photosensitizer [17], which has been shown to induce immunogenic cell death [18]. Photosens — sulfonated aluminium phthalocyanine (NIOPIK) [19] and a natural chlorin-based product — photodithazine (Veta-grand) are used for these purposes in Russia [20].

However, photodynamic agents may penetrate both a tumor and healthy nervous tissue, and there is still a question whether the concentrations of compounds, effective in photodynamic therapy, are toxic for non-tumor nervous cells. The study of dark toxicity of photodynamic agents will enable to assess possible side effects on brain neuronal networks and choose the safest compounds.

The aim of the study was to compare the effect of photosensitizers photosens, photodithazine, and hypericin on primary brain cell cultures, and assess their toxic effect on tumor and normal nervous cells in order to choose the optimal agent for glioma therapy.

Materials and Methods

Photodynamic agents. The following photosensitizers widely used currently in clinical practice have been analyzed (Figure 1):

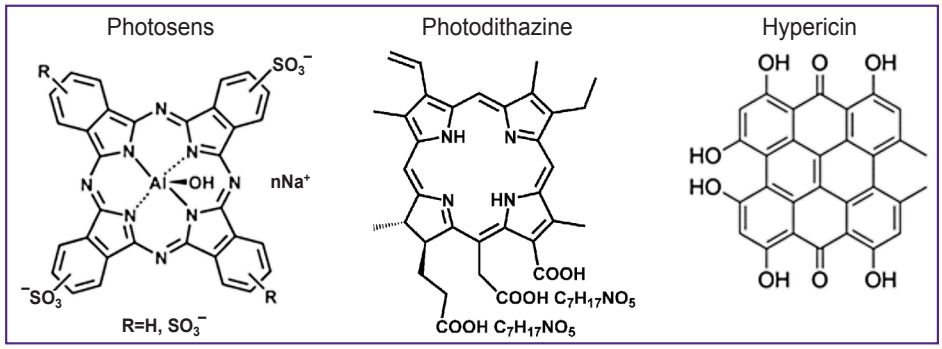

Figure 1. Structures of the photosensitizers

Photosens is a mixture of di-, tri-, and tetra-substituted fractions of sulfonated aluminium phthalocyanine with the number of sulfo group 3,4 (NIOPIK, Russia);

Photodithazine is bis-N-methylglucamine salt of chlorin e6 (Veta-grand, Russia);

Hypericin (4,5,7,4′,5′,7′-hexahydroxy-2,2′-dimethyl-naphthodiatrone) (Merck KGaA; Sigma-Aldrich, Germany).

Primary brain cell cultures. Primary brain cell cultures were obtained from C57BL/6 murine embryos (gestation day 18). All procedures with animals were performed according to Rules for the Work using Experimental Animals (Russia, 2010), International Guiding Principles for Biomedical Research Involving Animals (CIOMS and ICLAS, 2012), as well as the ethic principles established by European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 2006). The study was also approved by the Bioethics Committee of National Research Lobachevsky State University of Nizhni Novgorod.

Isolation and long-term cultivation of dissociated brain cells were performed on coverslips (18×18 mm) according to the previously developed protocol [21]. Pregnant female mice were sacrificed by cervical vertebra dislocation. Then the embryos were removed from the uterus, and brain tissue was isolated followed by mechanical and 20-minute enzymatic treatment with 0.25% trypsin solution (Life Technologies, USA). Initial cell density was 4500 cells/cm^2. Cell viability was supported in a CO_2-incubator (Sheldon Manufacturing, USA). During cultivation, dissociated neuronal cells formed neuron-glial networks with specific spatial topology and functional activity. The key stages of neuron-glial networks formation were assessed using an inverted DMIL HC fluorescent microscope (Leica Biosystems, Germany).

Determination of dark toxicity of photosensitizers. In order to access the dark toxicity of photosensitizers for normal brain cells, the studied agents were added to a culture medium on day 14 of cultures development *in vitro* (DIV) at concentrations of 0.1, 1, 10, 50, and 100 μM. Then, the cultures were placed in a CO_2 incubator and protected from light.

The viability of primary neuronal cultures were estimated on day 3 and 7 after compounds application using an inverted fluorescent microscope DMIL HC (Leica Biosystems, Germany) and specific fluorescent dyes — propidium iodide (Sigma-Aldrich, USA) and bis-benzimide (Sigma-Aldrich) in order to calculate the number of nuclei of dead cells relative to the total number of cells in a culture [21].

Accumulation dynamics of photosensitizers in normal brain cells. The features of penetration of photodynamic agents into the primary neuronal cells were assessed by confocal microscopy and a LSM-510 NLO system (Carl Zeiss, Germany). In order to visualize the cytoplasm of metabolically active cells a fluorescent calcium-sensitive dye Oregon Green 488 BAPTA-1 AM — OGB1 (Thermo Fisher Scientific, USA) was used.

Fluorescence of OGB1 was excited at 488 nm by argon laser radiation, and emission was recorded in the range of 500 to 530 nm. The fluorescence of tested photosensitizers was excited at 633 nm by He-Ne-laser radiation, and recorded in the range of 650 to 715 nm. The concentration of the photosensitizers was 10 μM. The peculiarities of the agents accumulated in neuronal cells were assessed in 2 and 4 h after the start of incubation.

Analysis of subcellular distribution of photosensitizer in primary brain cell culture. To determine features of intracellular distribution of tested photosensitizers we performed a co-localization analysis using confocal microscopy and specific fluorescent dyes for different cellular organelles (Life Technologies, USA). The following parameters for fluorescence detection were used:

LysoTracker Green DND-26 is a lysosome marker; excitation wavelength: 488 nm; signal detection range is 500–560 nm; fluorescence maximum is at 511 nm;

MitoTracker Green FM is a mitochondrial marker; excitation wavelength: 488 nm; signal detection range is 500–560 nm; fluorescence maximum is at 516 nm;

ER-Tracker Green is an endoplasmic reticulum (ER) marker; excitation wavelength: 488 nm; signal detection range is 500–560 nm; fluorescence maximum is at 511 nm.

On DIV 14, the culture medium was replaced by a serum-free medium containing the tested photosensitizer at concentration of 10 μM, and was incubated for 4 h. The fluorescence marker of lysosomes, mitochondria or ER at concentration of 0.5 μM was added to the culture medium 30 min before the end of incubation. Then the medium containing photosensitizer and organelle marker was replaced by a fresh serum-free medium.

On the obtained confocal images, the co-localization of a respective photodynamic agent and an organelle-specific marker was confirmed by comparing signal distribution profiles in corresponding fluorescent channels.

Statistical data analysis. The obtained data are presented as a mean ± a standard error of the mean (M±SEM). The statistical analysis was performed using ANOVA implemented in Sigma Plot 11.0 software (Systat Software Inc., USA). Differences between groups were considered significant if the corresponding p-value was less than 0.05.

Results

The main characteristics of the photosensitizers. First, we studied the absorption and fluorescent bands of the studied photodynamic agents (Table 1). Tetrapyrrol compounds — photosens and photodithazine — showed in spectra the presence of absorption maximum in short-

Table 1

Spectral characteristics of the photosensitizers

Photosensitizer	λ_{abs} (nm)	ε ($l \cdot mol^{-1} \cdot cm^{-1}$)	λ_{em} (nm)
Photosens	678*	$30 \cdot 10^{-4}$	690*
Photodithazine	404* 643*	$30 \cdot 10^{-4}$ $4.6 \cdot 10^{-4}$	652*
Hypericin	600**	$30 \cdot 10^{-4}$	604**

Note: the used solvents: * water; ** DMSO (dimethyl sulfoxide). Here: λ_{abs} — absorption maximum; λ_{em} — fluorescence maximum.

wave (Soret band) and long-wave (Q-band) spectral regions. Photodithazine is characterized by a short-wave band dominating that is typical of chlorin dye group. A complex absorption spectrum with many maxima both in blue-violet and orange-red bands was found for hypericin.

Analysis of the dark toxicity of photosensitizers for normal neuronal cells. Next, we analyzed the dark toxicity effects of studied photosensitizers for primary neuronal cultures. It should be noted that nervous cells have certain patterns of long neuronal network degradation under stress; therefore, it is not enough to assess short-term toxic effects of the photodynamic agents. Thus, two main periods for toxicity assessment were chosen. Day 3 after a photosensitizer addition is a period when most cells exposed to a toxic effect die; and day 7 after exposure when a process of cell death ends, the cells have lost a great number of intercellular connections and received an internal signal for programmed death [22]. Moreover, it is necessary to assess the overall toxicity of the compounds in order to reveal the period of their adequate effect on the nervous system.

High concentrations of tested photosensitizers were found to have a marked toxic effect on nervous cells (Figure 2). The percentage of dead cells on day 7 after photosensitizer addition at concentration of 100 μM was exceeded 40% (photosens: 44.98±4.93%; hypericin: 63.8±4.12%; photodithazine: 45.62±5.67%). Low concentrations of photosens (0.1, 1, and 10 μM) had no marked toxic effect: the number of dead cells in the cultures was 8.04±0.72, 13.50±1.27, and 8.56±0.75%, respectively. It should be noted that the viability of cultures on day 7 after addition of 0.1 and 10 μM of photosens did not differ from the intact group (7.20±1.28%).

Hypericin at concentration of 10 μM exhibited a light cytotoxic effect. The percentage of nonviable cells exceeded the intact values and amounted to 15.76±1.40%. Lower concentrations of hypericin had no marked cytoxic effect on primary neuronal cultures. The number of dead cells on day 7 after photosensitizer

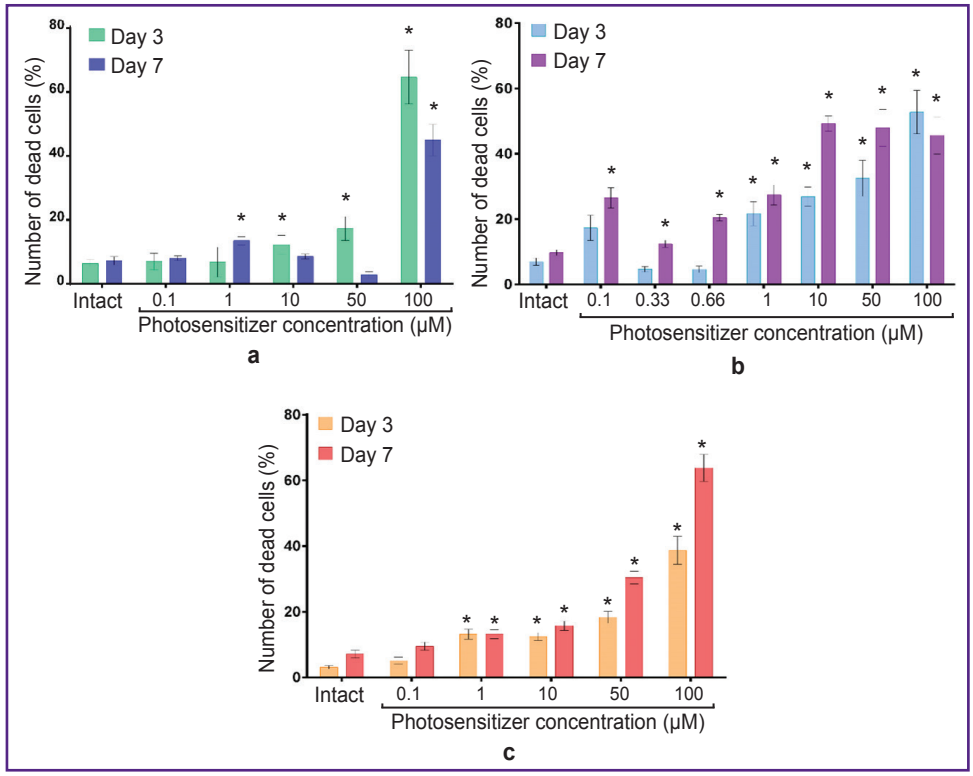

Figure 2. Dark toxicity assessment of commercial photosensitizers for primary brain cell cultures on day 3 and 7 after addition:
(a) photosens; (b) photodithazine; (c) hypericin; Y-axis — percentage of propidium iodide positive (dead) cells related to the total number of cells; * versus "Intact", p<0.05, ANOVA

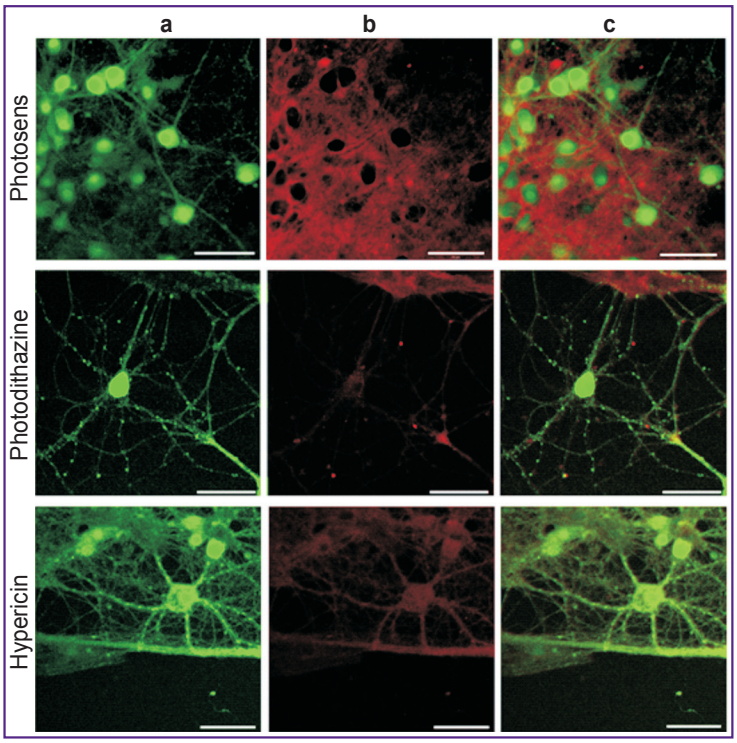

Figure 3. Representative confocal images of primary brain cell cultures 2 h after incubation with commercial photosensitizers: (a) a fluorescent channel of calcium-sensitive dye Oregon Green 488 BAPTA-1 AM; (b) a fluorescent channel of a photosensitizer; (c) merge: overlay of the fluorescence channels; scale bars — 50 μm

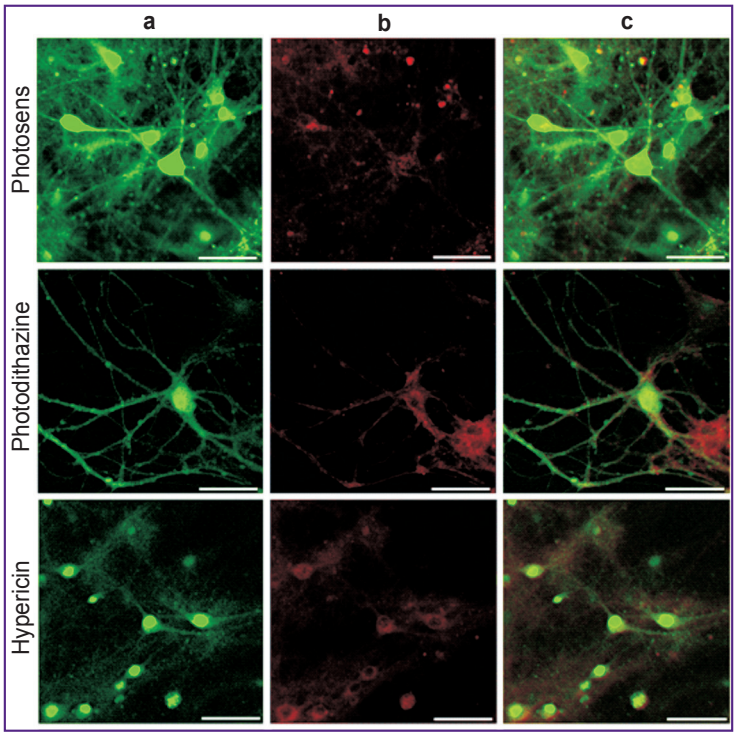

Figure 4. Representative confocal images of primary brain cell cultures 4 h after incubation with commercial photosensitizers: (a) a fluorescent channel of calcium-sensitive dye Oregon Green 488 BAPTA-1 AM; (b) a fluorescent channel of a photosensitizer; (c) merge: overlay of the fluorescence channels; scale bars — 50 μm

application was 9.50±1.23% (0.1 μM) and 13.21±1.36% (1 μM).

Among the tested commercial photosensitizers, photoditazine has the most pronounced cytoxic effect. Even in low concentrations, a dose-dependent increase in the percentage of dead cells in cultures was observed by day 7 after its addition. The number of nonviable cells at application of 1 and 10 μM of photoditazine was amounted to 30.08±4.40 and 48.11±4.13%, respectively. Due to the identified high toxicity of photodithazine, additional concentrations of the photosensitizer were analyzed. It was shown that the use of 0.66 μM was found to have average toxicity for primary neuronal cultures. The percentage of dead cells in the experimental group was 19.63±1.62%.

Analysis of cellular uptake rate of the photosensitizers on primary brain cell cultures. In contrast to other tissues, the nervous cells have specific metabolic peculiarities. Neuroglia specializes in performing auxiliary functions for neurons, including trophic function, neurosecretion, and regulatory function, while neurons are characterized by decreased ability to uptake various compounds. The feature of tumor cells as a high metabolic rate, and often a glial nature, enables to select the compounds that fast accumulate in malignant cells and therefore exhibit minimal damaging effect on neurons.

Using confocal microscopy, we analyzed the uptake rate of the tested photodynamic agents for primary neuronal cultures. Hypericin and photodithazine were found to actively accumulate in neuron bodies and outgrowths as well as in glial cells 2 h after the start of incubation (Figure 3). The photosensitizers were observed in all cell types of primary neuronal cultures 4 h after their addition (Figure 4).

By contrast, the uptake rate of photosens by neurons was decreased. Photosens was detected only in glial elements of neuron-glial networks 2 h after application. The presence of the photosensitizer in neuron bodies and outgrowths was revealed after 4-hour incubation period.

Thus, among the tested photosensitizers, photosens showed the lowest accumulation rate in the nervous cells.

Intracellular distribution of photosensitizers in primary brain cell cultures. Apart from an uptake rate, intracellular localization of photodynamic agents is equally important aspect. This determines primary targets of light irradiation, molecular

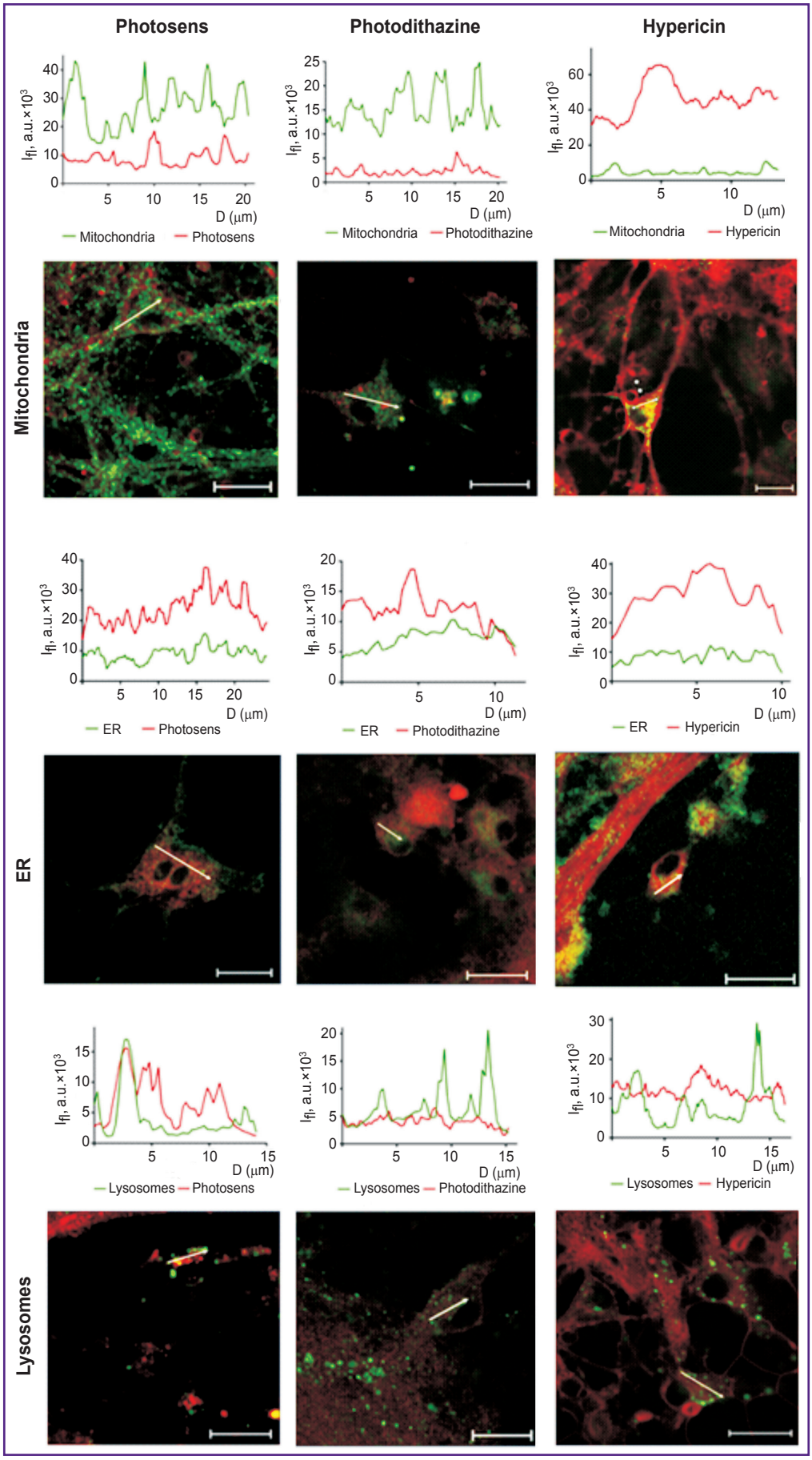

Figure 5. Analysis of intracellular localization of commercial photosensitizers in primary brain cell cultures

Primary brain cell cultures were preincubated with a photodynamic agent at concentration of 10 μM in 2 h. Then the primary neuronal cultures were stained with the following organelle dyes: LysoTracker Green DND-26 for lysosomes; MitoTracker Green FM for mitochondria; ER-Tracker Green for endoplasmatic reticulum (ER). Scale bars — 50 μm. In graphs: fluorescent signal profiles in the area of overlay of the fluorescence channels (white arrows on confocal images). I_{fl} — fluorescence intensity; D — the distance along the arrow

mechanisms of a response and an immunogenic nature of cell death [23, 24].

In this regard, we studied a series of confocal images of primary brain cell cultures registered 2 h after the incubation with tested compounds. Co-localization of a photosensitizer fluorescent signal and a signal of dyes specifically staining such cellular organelles as lysosomes, ER, and mitochondria (Figure 5) was analyzed.

It was shown that photosens is mostly accumulated in the ER, and partially in lysosomes. Other two photosensitizers were found to locate in ER only.

A comparative analysis of glioma cells and primary neuronal cultures death induced by photodynamic treatment. In order to choose optimal photodynamic agents and select their concentrations exhibited both minimal dark toxicity for normal brain cells and a pronounced therapeutic effect in PDT, we performed a comparative analysis of the effect of photosensitizers at pre-established concentrations of IC_{50} for murine glioma GL261 cells (light dose of 20 J/cm^2) on non-PDT treated primary brain cell cultures. Glioma GL261 cells were exposed to irradiation using a LED light source at a power of density 20 mW/cm^2 (λ_{ex} 630 nm) during 16 min 40 s. For analysis, we used a cytotoxicity score according to ISO 10993-5-2009 (Table 2).

Study on photosensitizer concentrations enables to reveal the compounds, that may have potentially high phototoxic effect on glioma cells, but are low-toxic for nervous system cells, even under their long-term circulation in cerebrospinal fluid and intercellular space. Based on our results, hypericin and photosens can be definitely referred to this group (Table 3).

Table 2

Cytotoxicity score according to the ISO 10993-5-2009

Cytotoxicity (points)	Number of dead cells in culture (%)	Interpretation of cytotoxicity
0	0–10	Non-toxic
1	10–20	Light
2	20–30	Average
3	Over 30	Significant

Table 3

Comparative assessment of cytotoxicity of the photosensitizers for glioma GL261 cells and primary neuronal cultures

Compounds	IC_{50} for glioma GL261 cells, μM (a light dose of 20 J/cm^2)	Dark cytotoxicity for the primary neuronal cultures in the area of IC_{50} of glioma GL261 cells
Photodithazin	0.8 [0.67; 0.92]	3 — severe cytotoxicity
Photosens	0.96 [0.79; 1.18]	0 — non-toxic
Hypericin	0.015 [0.013; 0.017]	0 — non-toxic

Discussion

Glioma therapy is currently an unresolved issue for global health care. Rapid course of the disease, high malignancy grade, and the shortest life expectancy make this pathology extremely difficult to treat and achieve persistent remission. A number of morphological characteristics of glioma, primarily, active migration of tumor cells to normal brain tissue increase risks of metastasis [3, 25, 26].

In case of adjuvant photodynamic therapy, it should take into consideration the effect of any pharmacological agents, including photosensitizers, on normal nervous cells. Brain neurons have certain metabolic peculiarities making them extremely sensitive to a toxic load. Differentiated neurons have a limited set of antioxidant enzymes incapable for division and regeneration, therefore, the loss of most functionally active connections lead to their death [27, 28].

In the present study, we have analyzed a toxic effect of some commercial photosensitizers on normal brain cells. The dark toxicity assessment of the tested compounds suggests that photodithazine has a marked toxic effect even in the absence of photo-induced cell death.

The data on uptake rate of the photosensitizers in brain cells and the agent distribution between neurons and astrocytes are of special interest. Photoinduction time calculation and the development of active detoxication measures can be a key to a novel therapeutic strategy based on photodynamic agents. Glioma has a higher metabolic rate compared to non-tumor cells [29, 30] and can accumulate photosensitizers faster than nervous system cells. Therefore, it is reasonable to suggest that a photodynamic agent can have minimal side effects for normal cells if its internalization rate in neurons is minimal, the rate being rather high for tumor cells. Among the studied compounds it is photosens.

Moreover, we analyzed the features of subcellular localization of the photosensitizers in primary brain cell cultures. The peculiarity of photosens that we have revealed is its localization not only in ER, but partially in lysosomes. In addition, photosens is characterized by vesicular localization in tumor cells. Photosens being a weak base due to nitrogen atoms of tetrapyrrol ring easily penetrates a lysosomal membrane, and remains in these organelles after dissociation under low pH [31]. It should be assumed that photosens redistribution between organelles will reduce the risk of normal cells death under photodynamic treatment [31–33].

In recent years, there have been intensive studies of molecular mechanisms of action of the compounds capable of providing cytotoxic effect, and also activate an anti-tumor immune response. If tumor cells die by an immunogenic pathway, then there occurs the secretion of damage-associated molecular patterns (DAMPs) [13, 34]. DAMPs emission, in turn, leads to the induction of a strong T-cell anti-tumor immune response, which

targets tumor cells remained after surgical resection and resistant to chemotherapy [34–36]. Previous studies have demonstrated an ability to induce immunogenic cell death through hypericin-based photodynamic therapy [33]. Since all the photosensitizers analyzed in this study accumulate in endoplasmic reticulum, they potentially have an ability to induce an ER stress by immunogenic pathway [13]. Therefore, further studies on capabilities of photodynamic agents to induce immunogenic cell death will enable to determine the most prospective agents for glioma therapy.

Conclusion

This work allowed us to specify the photosensitizers which have low toxic effects on normal brain cells, and therefore, can be used in practice for photodynamic glioma therapy. The established property of both normal and also tumor glial cells to quickly accumulate all tested photosensitizers can be used in the development of new therapeutic strategy. Hypericin and photosens showed the lowest toxicity for normal nervous tissue, and effectively penetrate tumor cells.

Study funding. We have determined spectral characteristics of photosensitizers, analysed dark toxicity and penetration rate of photodynamic agents, and a comparative assessment of normal and tumor cells death under photosensitizers' treatment was carried out with the support of Russian Science Foundation (project 18-15-00279). The study on the peculiarities of intracellular localization of photosensitizers in primary neuronal cultures was supported by a grant of the President of the Russian Federation (МК-1485.2019.4).

Conflicts of interest. The authors have no conflicts of interest to declare regarding the publication of this research.

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