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THE EFFECT OF HYPOXIA ON PHOTOCYTOTOXICITY OF TICS TRICARBOCYANINE DYE IN VITRO

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Aim: **To evaluate the effect of cell oxygenation on photocytotoxicity of a novel tricarbocyanine indolenine dye covalently bound to glucose (TICS).** ***Methods:*** **HeLa cells were incubated with 5 μM TICS, 2 h later irradiated by laser at 740 nm with a light dose of 10 J/cm², delivered at a power density of 10, 20, 25 or 30 mW/cm², in air or in argon atmosphere, and then scored for viability.** ***Results:*** **The photocytotoxicity of TICS increased dramatically as the power density was reduced. Under hypoxia TICS-photosensitized cell death was determined but its value was lowered, compared to photoirradiation in the air.** ***Conclusion:*** **Photosensitizing effect of TICS is only partially dependent on the oxygenation of tumor cells.**

Key words: **photosensitizers, cyanine dyes, HeLa cells, photodynamic therapy, hypoxia.**

In most human malignancies, oxygen delivery to the neoplastic and stromal cells is reduced as a result of structural and functional abnormalities of tumor microcirculation and limited diffusion. Hypoxia in solid tumors may restrict tumor curability, as hypoxic cells have been shown to be resistant to standard radiotherapy and chemotherapy [1].

Photodynamic therapy (PDT) of malignant tumors is based on the activation of tumor-localizing photosensitizing agents by specific light. With most photosensitizers (PS) under investigation, the PDT effect is mediated by singlet oxygen and so is also oxygen-dependent. Indeed, direct cell phototoxicity of Photofrin is reduced at oxygen concentration of 0.5% [2]. Photocytotoxicity of Foscan under anoxic conditions was almost completely inhibited [3]. Cytotoxic effects induced by photoactivated hypericin [4] or phthalocyanines [5] are also completely oxygen-dependent. The tumor cell response to aminolaevulinic acid-based PDT was abolished by hypoxia, as a result of both reduced protoporphyrin IX synthesis and reduced PDT toxicity [6]. However, in the case of bacteriochlorophyll-serine, a phototoxic effect was also seen under hypoxic conditions [7]. Some other PS (psoralens, kryptocyanines) can act in the absence of oxygen [8].

PDT treatment efficacy may be reduced not only by pre-existing tumor hypoxia but also by oxygen depletion during the therapy itself as a result of oxygen consumption by photooxidative reactions, especially with high rate of light delivery (power density) [9]. Therefore, it is important to know if the applied PS is oxygen-dependent and to use an oxygen-conserving regimen of light treatment that determines the outcome of malignant tumor PDT [10].

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Abbreviations used: Chl – chlorin e6; PDT – photodynamic therapy; PS – photosensitizer; TICS – tricarbocyanine indolenine dye covalently bound to glucose.

Tricarbocyanine indolenine dye covalently bound to glucose (TICS) is a novel promising PS for PDT. TICS has strong light absorption in the long-wave region, low toxicity in the dark, accumulates in tumor cells and tissues, and was proved to be an effective PS in HeLa tumor cells culture and in rats with transplanted tumors [11, 12]. In the present study we investigated the impact of cell oxygenation and rate of light delivery on TICS photocytotoxicity, in comparison with chlorin e6 which acts as PS due to a predominantly singlet oxygen mechanism [13].

Chemicals. Symmetrical tricarbocyanine indolenine dye with 4-chlorosubstituted conjugate heptamethine chain and with glucose connected to nitrogen (TICS) has been developed and synthesized at Spectroscopy Laboratory of A. N. Sevchenko Research Institute of Applied Physical Problems (Minsk, Belarus) [12]. The stock solution (10 mM) was prepared by dissolving the dye in ethanol and stored at 4°C at dark until use.

Chlorin e6 (Chl) was obtained from Photochemistry Laboratory of Byelorussian Academy of Sciences (Minsk, Belarus) [14] and dissolved in saline solution. Further PS dilutions were made in 199 culture medium. 199 medium, fetal calf serum and Hanks' balanced salt solution were obtained from Research Institute of Epidemiology and Microbiology (Minsk, Belarus).

Cell culture. HeLa cells (human cervical epithelioid carcinoma) were obtained from the cell culture collection of Research Institute of Epidemiology and Microbiology (Minsk, Belarus). The cells were cultured as a monolayer in 199 medium supplemented with 10% fetal calf serum and 100 μg/ml kanamycin.

Photocytotoxicity study. For the experiments, HeLa cells were plated at 1×10^5 cells in 2 ml of culture medium glass flasks (with diameter of 3 cm²), allowed to get attached to the bottom and grow for 72 h before being tested. All the experiments were carried out on exponentially growing cells. On the day of the experiment, 100 μl of TICS at a concentration of 100 μM or Chl at a concentration of 40 μM were added into the flasks with cell monolayer in order to obtain a final concentration of 5 μM TICS or 2 μM Chl. After incubation

at 37 °C at a dark for 2 h with TICS or for 3 h with Chl for maximal accumulation of PS in cells, the medium containing PS was removed, cell monolayers were washed three times with ice-cold Hanks' balanced salt solution, and the flasks were refilled with fresh medium without PS. To perform experiments in argon atmosphere with exclusion of oxygen, the monolayer of cells was covered with deoxygenated (by bubbling 99.99% argon gas with 50 ml/min for 2 h) medium, and then further saturated with argon by bubbling argon gas with 6 ml/min through the medium for 30 min. In such conditions the oxygen content in the medium was below 0.1–0.2 μg/ml [4]. Phototreatment of the cell monolayer was carried out at 0–4 °C with semiconductor laser (740 nm, Lotis, Belarus, in the case of TICS; or 668 nm, BioSpec, Russia, in the case of Chl). The size of a light spot adjusted by diaphragm was 2 cm in diameter. At the surface of the flask bottom the power density was of 10, 20, 25 or 30 mW/cm^2, as measured by LM-2 power meter (Carl Zeiss, Jena, Germany). Immediately after irradiation the hypoxic medium was removed and replaced with fresh normoxic culture medium. Treated and control cells were cultured for additional 20 h and scored for viability by determining the number of viable cells in the flasks. This involves dispersal of cells with 0.02% versen (DIALEK, Belarus) for 10 min at 37 °C, pipetting and counting in Goryaev's chamber under phase-contrast microscope. All manipulations were carried out in reduced light. The results are presented as the percentage of cell survival relative to the controls. The mean ± standard deviation was calculated from three experiments.

PS accumulation in cells. Cell content of TICS or Chl was measured by a fluorimetric assay. HeLa cell monolayers cultured in glass flasks over 72 h, as for cytotoxicity evaluation, were incubated with 5 μM TICS or 2 μM Chl at 37 °C at dark as described above. After incubation with PS, the medium containing PS was removed and cell monolayers were washed three times with ice-cold Hanks' balanced salt solution without phenol red. The cells were removed from the flask bottoms with 0.02% versen (10 min at 37 °C), counted in Goryaev's chamber and suspended in butanol for dye extraction [14] in the case of TICS or were dissolved in 2% Triton X-100 detergent solution in phosphate buffer at pH 7.4 in the case of Chl [15]. Fluorescence intensity of the samples was measured by Fluorolog Spex fluorescence spectrophotometer (USA) at 750 nm (excitation wavelength, 715 nm) in the case of TICS or at 668 nm (excitation wavelength, 500 nm) in the case of Chl. Just before measurements of the samples spectrofluorimeter was calibrated using standards of known PS concentration. All manipulations were carried out in reduced light.

RESULTS AND DISCUSSION

To eliminate oxygen from the cell monolayer, the culture medium was saturated with argon by bubbling argon gas through the medium with continuous stirring. To compare the photodynamic activity of TICS and Chl in hypoxic condition, we used isoeffective amounts of each PS and light (near 10% survival) under normal oxygenation. Thus, under normoxia, the survival of HeLa cells after incubation with 5 μM TICS followed by light irradiation at 740 nm with 10 J/cm^2 (25 mW/cm^2) was 12 ± 3%, and after incubation with 2 μM Chl with subsequent light irradiation at 668 nm with 3 J/cm^2 (25 mW/cm^2) was 7 ± 2%. In hypoxic condition, the same phototreatment resulted in 24 ± 4% cell survival with TICS and 97±4% cell survival with Chl (Fig.1). No differences in viability were noticed when cells were incubated with 5 μM TICS or 2 μM Chl (see Fig.1) without phototreatment or when cells were irradiated with light in the absence of PS, both under normoxic and hypoxic conditions.

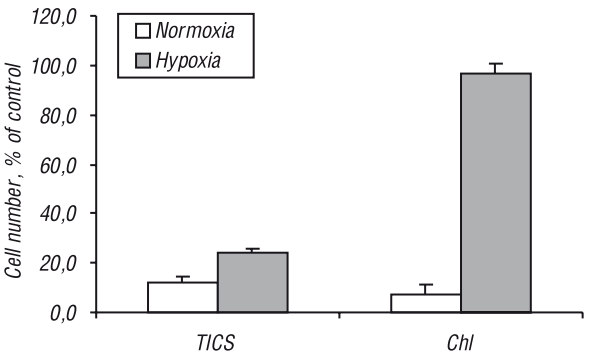

Fig.1. Survival of HeLa cells after photodynamic treatment with TICS or Chl in normoxic and hypoxic conditions. The cell monolayers were incubated with 5 μM TICS or 2 μM Chl, exposed to light under normoxia or hypoxia and scored for viable cell number 20 h later. Each data point represents the mean ± standard deviation of three experiments

Thus, at the attained level of hypoxia most of the Chl photocytotoxicity was lost, while phototoxic effect of TICS was partly retained. PS content in cells just before phototreatment was 4.6×10^7 moleculus/cell for TICS and 5.7×10^7 moleculus/cell for Chl, and it did not depend on the oxygenation level. Hence the differences between normoxic and hypoxic conditions could not be ascribed to intracellular PS concentration.

The photocytotoxicity of TICS under normal oxygenation was greatly dependent on the rate of light delivery (Fig. 2). As the power density was reduced from 25 mW/cm^2 to 20 mW/cm^2 or 10 mW/cm^2, the cell survival was markedly diminished (from 12.4 ± 2.2% to 2.6 ± 0.9% or 1.7 ± 0.3%, respectively) with an applied fixed light dose of 10 J/cm^2. Cell survival following the same light dose of 10 J/cm^2 administered at a power density of 30 mW/cm^2 was 28.0 ± 4.3% under normoxic conditions. Thus, with increasing the rate of light delivery, the photocytotoxicity of TICS was dramatically decreased under normoxic conditions, while under hypoxic conditions similar phenomenon was less marked. It should be noted that survival of cells treated with the highest light delivery rate (30 mW/cm^2) under hypoxic conditions, was determined as 35.9±4.7%. This value did not significantly differ from those in normoxic conditions 28.0±4.3%, and most likely indicated that the decrease of TICS photocytotoxicity under normal oxy-

genation with increase of light delivery rate was caused by oxygen consumption within phototreatment. Similar results with light-delivery rate effect on cell survival were obtained in Colo 26 multicell spheroids photosensitized by meta-tetra (hydroxyphenyl) chlorin and irradiated at 5, 30 or 90 mW/cm^2 [16].

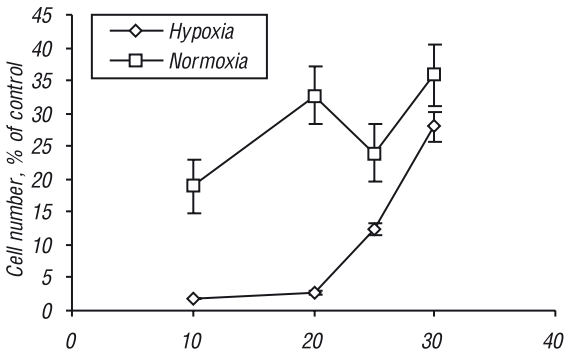

Fig.2. Survival of HeLa cells after TICS-PDT as a function of light delivery rate and oxygenation conditions. The cell monolayers were incubated with 5 μM TICS, exposed to light irradiation at 10 J/cm^2, delivered at 10, 20, 25 or 30 mW/cm^2 under normoxia or hypoxia and scored for viable cell number 20 h later. Points: means ± standard deviation of three experiments

Obtained results allow to suggest that molecular oxygen is involved in TICS-sensitized cell photo inactivation under normal oxygenation. The photocytotoxicity of TICS under the hypoxia condition was retained but lowered, in contrast to the loss of the phototoxic effect of Chl. Thus, we can assume that the mechanism of the photosensitizing effect exhibited by TICS is only partly dependent on oxygen. TICS ability for photodynamic activity in hypoxic conditions makes it possible to overcome the resistance of hypoxic cells in malignant tumors to PDT.

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ВЛИЯНИЕ ГИПОКСИИ НА ФОТОЦИТОТОКСИЧНОСТЬ ТРИКАРБОЦИАНИНОВОГО КРАСИТЕЛЯ ТИКС IN VITRO

Цель: **исследование влияния оксигенации клеток на фотоцитотоксичность нового трикарбоцианинового индоленинового красителя, ковалентно связанного с глюкозой (ТИКС).** ***Методы:*** **клетки HeLa инкубировали в среде, содержащей 5 мкМ ТИКС, а через 2 ч на воздухе или в атмосфере аргона, облучили светом лазера 740 нм в дозе 10 Дж/см^2 при плотности мощности 10, 20, 25 или 30 мВт/см^2. Затем была определена их жизнеспособность.** ***Результаты:*** **фотоцитотоксичность ТИКС значительно возрастала при уменьшении плотности мощности облучения. В условиях гипоксии гибель клеток фотосенсибилизированная ТИКС сохранялась, но несколько уменьшалась в сравнении с результатами фотооблучения на воздухе.** ***Выводы:*** **фотосенсибилизирующий эффект ТИКС только частично зависит от оксигенации опухолевых клеток.**
Ключевые слова: **фотосенсибилизаторы, цианиновые красители, клетки HeLa, фотодинамическая терапия, гипоксия.**