Fluorescent and photodynamic properties of near-infrared photosensitizer bacteriochlorophyllide-serine

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ABSTRACT

Study is devoted to investigation of fluorescent and photodynamic properties of near-infrared photosensitizer bacteriochlorophyllide-serine. The peculiarities of its fluorescence excited by different lasers, dynamics and selectivity of accumulation were investigated. Photodynamic therapy of mice and investigation of tissue de-oxygenation during tumor irradiation were performed. It was shown that bacteriochlorophyllide-serine is an effective photosensitizer for PDT and fluorescent diagnostics of tumors.

Keywords: bacteriochlorophyllide-serine, Bchl-Ser, photosensitizer, laser, PDT, fluorescence, tumor, vessel alterations.

1. INTRODUCTION

High photodynamic efficiency and quantum yield of fluorescence, suitable spectral range of absorption and photostability play the important role for the choice of photosensitizer (PS) for photodynamic therapy (PDT) and fluorescent diagnostics (FD).

First of all, the choice of a spectral range is connected to the depth of photodynamic treatment, which is limited to the depth of penetration of light with wavelength corresponding to a spectral maximum of PS absorption in tissue. PS which absorb in spectral range exceeding 770 nm¹ provide the greatest depth of photodynamic effect due to the minimal intrinsic absorbance of non-sensitized tissue in this range. The same spectral range also gives advantage for PDT of melanoma and pigmented malignancies. Another factor, determining a choice of suitable spectral range for PDT, is an availability of suitable and accessible sources of laser radiation in this range (in particular, laser diodes).

High fluorescent properties of PS play an important role at estimation of their bio-distribution and PDT results, development of tactics of treatment.

In present work the fluorescent and photodynamic properties of bacteriochlorophyllide-serine were studied. This PS of a near infrared range was synthesized and investigated earlier²⁻⁶, however the peculiarities of processes of sensitization as well as photodynamic action of bacteriochlorophyllide-serine need additional investigations.

2. MATERIALS AND METHODS

Bacteriochlorophyllide-serine (Bchl-Ser) was synthesized in the Weizmann Institute of Science (Rehovot, Israel).

The investigations of absorption and fluorescence spectra of Bchl-Ser, as well as the level of blood oxygenation in tumor vessels, were carried out using spectra-analyzer "LESA-01-Biospec" ("Biospec", Russia)^{7,8}. The semiconductor laser (775 nm) and He-Ne laser (633 nm) were used as a light source for investigations of fluorescence.

For estimation of concentration of PS in biological tissue, it is important to compare the intensity of fluorescence and the scattered laser radiation used for excitation. As a rule, the ratio of these values is around 10⁻⁴. We established on the

input of spectra-analyzer the cut-off filter made of the KS19 glass (for the excitation with He-Ne laser), or polymethylmethacrylate solid solution of the copper tetra-3-dimethylamino-tetra-5-*tret*-buthyl-phthalocyanine (for the excitation with semiconductor laser). The transparency spectra of these filters are shown on the Fig.1. Use of these filters allowed to increase the dynamic range of spectra-analyzer to estimate the level of accumulation of PS in tissue in wide range of concentrations.

High light transparency of the polymethylmethacrylate filter in the infrared (>830 nm) and green-to-orange (500-600 nm) spectral ranges allowed to monitor the fluorescence of PS as well as the blood oxygenation during the therapeutic irradiation. However, use of this filter also caused suppression of the short-wavelength component of the fluorescence spectra of PS. Thus, in all following experiments for investigations of the dynamics and selectivity of the photosensitizer accumulation using the semiconductor laser with polymethylmethacrylate filter, we took into account only the long-wavelength component of the fluorescence.

Additionally, on the output of the semiconductor laser, we have established an interference filter, with sharp decrease of transparency above 780 nm (Fig. 3), in order to suppress luminescence "noise" in the near-IR range from the output of the semiconductor laser. It increased the sensitivity of the method for the work with low concentration of PS.

Fig. 2. Transparency spectra of filters on the input of spectraanalyzer: 1 - KS19, 2 - polymethylmethacrylate solid solution of the copper tetra-3-dimethylamino-tetra-5-*tret*-buthyl-phthalocyanine

Fig. 3. Transparency spectrum of the interference filter

We have accepted that under conditions we used, the intensity of fluorescence of PS is proportional to its concentration at the zone of measurement. We also assumed that skin, muscle and malignant tissue are characterized by approximately the same parameters of scattering of the exciting laser radiation. That's why we estimated the dynamics and distribution of photosensitizer concentration in tissue from intensity of fluorescence at the zone of measurement, normalized to the intensity of the exciting laser radiation in the same point, thus avoiding the effect of instability of exciting radiation. Accordingly, the selectivity of accumulation of PS in tumor in comparison to normal tissue was determined as a ratio of normalized fluorescence intensities in tumor and respective zone of normal tissue.

The powerful light source based of xenon arc lamp equipped with tunable interference filter was developed for PDT irradiation. It provided an irradiation of a tumor site by narrow-band quasi-monochromatic radiation with varied wavelength of a spectral maximum and spectral half-width 30-40 nm (Fig.4), power of a therapeutic radiation up to 800 mW at a diameter of a light beam about 16 mm.

Fig. 3. Emission spectra of the arc lamp light source with tunable interference filter, with different wavelengths of spectral maximum: 1 - 730 nm, 2 - 753 nm, 3 - 779 nm, 4 - 827 nm. 5 - emission spectrum of the semiconductor laser.

Investigations *in vivo* were done using F₁ mice bearing solid form of Erlich tumor (ELD), 7 days after inoculation (when the average volume of tumor achieves 0.8-1.0 cm³). Bchl-Ser was administrated intravenously at a dose of 20 mg/kg. The studies of biodistribution of Bchl-Ser were carried out by study of fluorescence intensity in tumor, muscle, skin, liver, kidney and spleen of groups of animals, sacrificed at 15, 30 min, 1, 2, 4 and 24 hours after administration of PS.

For PDT research, two groups of mice were used. The tumors of one group of mice were illuminated with narrow-band radiation from the lamp light source with spectral maximum at 775 nm and power density of 250 mW/cm² for 20 minutes (delivered dose of irradiation 300 J/cm²). Other group of mice was used as control. The spectra of tumor fluorescence were investigated before and after irradiation in order to estimate the photo-bleaching of Bchl-Ser *in vivo*. The level of blood oxygenation in tumor vessels was measured during all time of irradiation and continuing for approximately 10 minutes after it was finished using method described by J. Zilberstein *et al*⁶.

During 10 days period after PDT the tumor's dimensions were measured and the tumor growth inhibition index (TGI) was calculated, using from the following expression:

$$\text{TGI (\%)} = \frac{(V_c - V_T)}{V_c} \times 100 ,$$

where V_c – average tumor volume in control group (mm³);
 V_T – average tumor volume in group with treatment (mm³).

Then the animals were sacrificed by overexposition to ether and the morphological study of tumors was performed, which included macro- and microscopical examination of paraffin embedded sections, stained by hematoxylin and eosin.

3. RESULTS AND DISCUSSION

Investigation of biodistribution and selectivity of accumulation of Bchl-Ser in tissue of experimental animals.

The spectra of fluorescence of Bchl-Ser in tissue of experimental animals, which were registered by spectra-analyzer "LESA-01-Biospec" at excitation with semiconductor laser with wavelength 775 nm, have one peak with a maximum on wavelength 840 nm (Fig.4A) and only amplitude differences in malignant and normal tissue.

The spectra of fluorescence of Bchl-Ser in sensitized malignant and normal tissue at excitation with the He-Ne laser with wavelength 633 nm essentially differ each from other (Fig.4B). The spectra of fluorescence in malignant tissue have two peaks with maximums at 695 and 775 nm, with the clearly expressed downfall between them in a spectral

range of 730-745 nm. At the same time the spectrum of fluorescence in normal tissue consists of peak with a spectral maximum about 690 nm and flat plateau in long-wavelength range (approximately 730-790 nm) or poorly expressed peak at 760-770 nm (the downfall between maximums in this case does not exceed 8 %).

The comparison of changes of Bchl-Ser spectra of fluorescence in tissue *in vivo* and in different biological solutions with earlier data^{3,4} allows us to speculate that these spectral changes of fluorescence are caused by processes of enzymatic metabolism and oxidation of Bchl-Ser in a biological tissue with formation as products of oxidation of PS with a spectral maximum of fluorescence in the range of 690-700 nm, supposedly chlorines, as well as product of demetallization of Bchl-Ser (bacteriopheophytin) with a spectral maximum near 760 nm. The peculiarities of these processes of biodegradation of Bchl-Ser in malignant and normal tissue require additional study.

Fig. 5. Fluorescence spectra of Bchl-Ser in mice tissue, 17 min after administration: 1 – tumor, 2 – normal tissue. A – excitation at 775 nm, B – excitation at 633 nm.

The results of research of changes of concentration of Bchl-Ser in tumor, skin, muscle, liver, kidney, spleen of mice (Fig.8) were similar to results reported by V. Rosenbach-Belkin *et al.*³, however it was shown that the concentration of Bchl-Ser achieves the maximal value in 15-20 minutes after its intravenous administration, and then is reduced more than twice within 4 hours because of its clearance and metabolism; in internal organs this decrease occurs essentially faster.

Fig. 8. Dynamics of the fluorescence intensity of Bchl-Ser in mice different tissue and organs.
A - with excitation at 775 nm; B - with excitation at 633 nm.

By estimation from the fluorescence intensity at 840 nm (excited by the semiconductor laser with wavelength 775 nm), the selectivity index of accumulation of PS in tumor in comparison to normal tissue achieved appreciable level

(approximately 4) in 15-20 minutes after PS administration. In 240 min after administration, the selectivity of accumulation achieved its maximum value of $8 \div 10$. The selectivity of accumulation estimated on intensity of fluorescence in a spectral range 770-780 nm (excited by He-Ne laser with wavelength 633 nm), gives the same value (4 in the initial period, 8-10 in 240 min), as with excitation on 775 nm (Fig. 5).

Fig. 6. Selectivity of accumulation of Bchl-Ser in tumor in comparison to normal tissue estimated from fluorescence excited on: ■ - 775 nm, △ - 633 nm.

Possible mechanisms of accumulation of bacteriochlorophyllide-serine in tumor

Selective accumulation of bacteriochlorophyllide-serine (Bchl-Ser) in tumor is caused (provided) both by differences in rate of clearance of photosensitizer and different behavior of processes of its biodegradation in tumor and normal tissue.

One of the main mechanisms of selective accumulation of Bchl-Ser, as well as other derivatives of bacteriochlorophylls, is accomplished due to their affinity to low density lipoproteins (LDL)^{9,10}. It is well known that actively proliferating tumor cells produce increased amounts of LDL-receptors, which participate in receptor-mediated endocytosis of LDL complexes (molecules) as a source of building material of cell¹⁰, thus providing a transportation of LDL-associated photosensitizers into the cell.

On the other hand, comparison of the fluorescence spectra *in vivo* of animal tissue sensitized by Bchl-Ser (see Fig. 5B) with earlier data^{3,4} allows to assume that the observed differences in shape and dynamics of fluorescence spectra are related to processes of oxidation and enzymatic metabolism of Bchl-Ser in biological tissue resulting in formation of products of demetalation (bacteriopheophytine, including Bphe-Ser conjugate) characterized by spectral maximum of fluorescence at 760-770 nm, as well as products of oxidation (chlorines, including Chln-Ser) with maximum of fluorescence at 680-700 nm (Fig. 7).

Analysis of obtained fluorescence spectra of tumors and normal tissue of mice sensitized by bacteriochlorophyllide-serine indicates (shows) that the rate of accumulation (formation, production) of bacteriopheophytine due to biodegradation of Bchl-Ser is much higher in normal tissue than in tumors (Fig. 8). Similar results can be produced by processing of experimental data obtained by V. Rosenbach-Belkin *et al.*³. The difference between the rates of accumulation of chlorines (products of oxidative degradation of Bchl-Ser) in tumor and normal tissue is even

All considered mechanisms result in significant decrease of concentration of bacteriochlorophyllide-serine in normal tissue in comparison to tumor, what causes additional increase of selectivity of its accumulation in tumor.

Fig. 9. Time dependence of the chlorine / bacteriochlorophyllide-serine ratio in tumor (1) and normal tissue (2) of mice after i.v. administration of Bchl-Ser.

Our measurements of fluorescence of Bchl-Ser in both malignant and normal tissue for more than two hours have shown that, despite of essential decrease of fluorescence intensity, the ratio of intensity values at 770-780 nm and 730-745 nm remains close to constant for a long time, but essentially different for malignant and normal tissue. We have proposed the parameter I of the normalized fluorescence for an estimation of this ratio, which can characterize this feature of fluorescence of Bchl-Ser in each point of an investigated zone:

$$I = \frac{I_{fl} - I_{fall}}{I_{fl} + I_{fall}},$$

where I_{fl} – intensity of fluorescence in a spectral range 770-780 nm,
 I_{fall} – intensity of fluorescence in a spectral range 730-745 nm.

The value of this parameter in normal tissue did not exceed 0.04 while in a pathological tissue it was more than 0.8 (Fig. 10). Its numerical value does not depend on intensity of exciting radiation as well as on non-homogeneity of distribution of optical properties on a surface of a tissue, and is correlated only to properties of tissue in the point of investigation. Thus, as normalization is based on values of intensity of fluorescence in close spectral range, the error caused by non-homogeneity of distribution of intensity of exciting radiation or optical properties on a surface of a tissue is minimized. The value of this parameter in malignant and normal tissue can be connected with enough reliability to differences in their biochemical and biophysical properties.

Fig. 10. Dynamics of the parameter of normalized fluorescence of Bchl-Ser in mice tissue: 1 – tumor, 2 – normal tissue.

Investigation of a photodynamic efficiency and photobleaching of Bchl-Ser *in vivo*

The received dynamic data of Bchl-Ser concentration in different tissue have allowed us to choose the optimal time interval for a photodynamic irradiation in 15-35 minutes after administration. It is in a good agreement with the results of the authors [5], who have done the experimental PDT of melanoma using Bchl-Ser. They found that PDT is most effective if the irradiation is carried out within the first 30 minutes after administration.

For PDT investigations the tumors of mice were illuminated for 20 minutes with radiation of a lamp light source with wavelength 775 nm and power 500 mW into a spot with diameter of 16 mm (power density about 250 mW/cm²). Illumination was started 15 minutes after administration of Bchl-Ser, when the concentration of PS in the tumor tissue was maximal. Fig. 11 shows that illumination of a tumor by therapeutic light irradiation with a dose of 200 J/cm² results in complete irreversible blood de-oxygenation.

Fig. 11. Decrease of the blood oxygenation level monitored through PDT. End of illumination is indicated with an arrow.

That allows to assume the vascular mechanism of a photodynamic damage of tumor by PDT with Bchl-Ser. It was confirmed results of morphological study (Fig.12) of tumor after PDT, which have shown a strong vascular damages (hemorrhages (A) and thrombosis (B)) as well as expressed necrosis (A,B) and apoptosis (C) of malignant tissue. These results are in a good agreement with the data of J. Zilberstein *et al*^{5,6}.

Size of tumors was measured before and for 23 days after PDT, and the inhibition of tumors growth (in comparison with tumors of nontreated mice) was estimated. Results of monitoring are presented on Fig. 13. They show that PDT with Bchl-Ser causes the significant inhibition of a tumor growth exceeding 75 %.

After irradiation at a dose of 300 J/cm² the intensity of fluorescence of Bchl-Ser falls practically down to zero due to photobleaching (Fig. 14) simultaneously with the increase of a fluorescent band with a spectral maximum in a range of 760-770 nm. This band can be associated to bacteriopheophytin, which is a product of photo-degradation of bacteriochlorophyllide⁴.

A
Hemorrhages and necrosis
(hematoxylin-eosin, ×400)

B
Thrombosis and necrosis
(hematoxylin-eosin, ×100)

C
Apoptosis (hematoxylin-eosin, ×400)

D
Control (hematoxylin-eosin, ×100)

Fig. 12. Results of the morphological study of tumor tissue after PDT.

Fig. 13. Growth of tumor volume (A) and tumor growth inhibition (B) after PDT with Bchl-Ser administered 15 minutes before irradiation at a dose of 20 mg/kg: 1 – nontreated (control) group; 2 – group after photodynamic treatment ($p < 0.05$).

Fig. 14. Fluorescence spectra of the P-388 tumor tissue before (1,2) and after PDT (3,4).

4. CONCLUSION

Bacteriochlorophyllide-serine seem to be promising for fluorescent diagnostics of malignancies due to fast achievement of high value of selectivity of accumulation and presence of spectral features of fluorescence of Bchl-Ser in malignant tissue in comparison with normal tissue. The irradiation of a tumor by therapeutic light irradiation with a dose 150-200 J/cm² provides essential inhibition of a tumor growth (up to 76%) with the complete irreversible blood deoxygenation in a tumor vessels, thus allows to speculate the vascular mechanism of a photodynamic alteration of a tumor. The results of morphological study of tumor alteration have shown strong vascular damages (hemorrhages and thrombosis), expressed necrosis and apoptosis of malignant tissue.

Thus, the work shows that bacteriochlorophyllide-serine is effective infrared photosensitizer for fluorescent diagnostics and photodynamic therapy.

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