Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Photochemistry and Photobiology B: Biology 80 (2005) 57–64
Journal of Photochemistry and Photobiology B:Biology
www.elsevier.com/locate/jphotobiol

Photodynamic activity of dibiotinylated aluminum sulfophthalocyanine in vitro and in vivo

Irina G. Meerovich [a], VictoriaV. Jerdeva [a], Valentina M. Derkacheva [b], Gennady A. Meerovich [c], Eugeny A. Lukyanets [b], Eugenia A. Kogan [d], Alexander P. Savitsky [a,*]

[a] *Laboratory of Physical Biochemistry, A.N.Bach Institute of Biochemistry of Russian Academy of Sciences, 33, Leninsky prospect, 119071 Moscow, Russian Federation*
[b] *Organic Intermediates and Dyes Institute, 1/4, B. Sadovaya, 103787 Moscow, Russian Federation*
[c] *Natural Sciences Center of A.M. Prokhorov General Physics Institute, 38, Vavilov str., 119991 Moscow, Russian Federation*
[d] *I.M. Sechenov, Moscow Medical Academy, 6, B. Pyrogovskaya str., 119021 Moscow, Russian Federation*

Received 28 February 2004; received in revised form 26 December 2004; accepted 26 December 2004
Available online 18 April 2005

Abstract

The photodynamic activity of dibiotinylated aluminum sulfophthalocyanine was studied in vitro and in vivo. Dibiotinylated aluminum sulfophthalocyanine provided enhanced phototoxic action on OAT-75 cell monolayers as compared with the parent drug. Photodynamic therapy of mice with Ehrlich carcinoma using dibiotinylated aluminum sulfophthalocyanine (0.25 mg/kg) resulted in enhanced inhibition of tumor growth, pronounced vascular damage (thrombosis and destruction of vascular walls) and eventual tumor necrosis.

Keywords: Photodynamic therapy; Biotin; Aluminum sulfophthalocyanine; Necrosis; Apoptosis; Proliferative activity

1. Introduction

Phthalocyanine derivatives belong to a promising class of synthetic photosensitizers [1–3]. They are extensively studied because of favorable photophysical and physicochemical properties such as solubility and hydrophobicity which can be further optimized through derivatization of the macrocycle periphery.

Tumor uptake of a photosensitizer depends on hydrophobicity/hydrophilicity of a sensitizer. Margaron et al. [4] reported that the photodynamic activity of amphiphilic photosensitizers was enhanced as compared with the symmetric hydrophobic or hydrophilic molecules. It was assumed that amphiphilic photosensitizers are localized on the hydrophobic–hydrophilic interfaces of cell membranes and protein surfaces. According to Margaron et al. [4], introduction of (*tert*-butyl)-benzo- and (*tert*-butyl)-naphtha groups to zinc trisulfophthalocyanine molecule increased the phototoxic effect in the experiments with EMT-6 murine tumor cell line, while the introduction of the fourth sulfobenzo group decreased the phototoxic activity as compared to the parent molecule.

A number of naturally occurring compounds have been used as substituents to achieve optimal amphiphilicity of photosensitizers. For example, water-soluble chlorophyll (Chl) and bacteriochlorophyll (Bchl) derivatives were synthesized by trans-esterification with serine (Ser) at the propionyl residue and tested as potential agents for photodynamic therapy (PDT) [5]. High

* Corresponding author. Tel.: +7 095 9548725; fax: +7 095 9542732.
E-mail address: apsavitsky@inbi.ras.ru (A.P. Savitsky).

1011-1344/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotobiol.2004.12.008

phototoxicity and fast tissue clearance rates reported in [5] proved that the polar conjugates of Chl and Bchl could be highly effective PDT agents.

One of the goals of our investigations was to study new phthalocyanine derivatives that can provide high photodynamic effect at relatively low concentrations.

Well-known photosensitizers based on aluminum sulfophthalocyanine [3], such as "Photosens", as a rule, have several hydrophilic sulfo groups. It is important to study phototoxic properties of aluminum sulfophthalocyanine photosensitizers with natural hydrophobic substituents which were introduced to change their amphiphilicity. In this paper, we describe the results of the studies of the photodynamic activity of dibiotinylated aluminum sulfophthalocyanine in vitro and in vivo.

2. Materials and methods

2.1. Reagents and materials

Biotin–cadaverine and 5(6)-carboxyfluorescein diacetate (mixed isomers) were from Molecular Probes Europe BV (Holland); neutravidin was from Pierce (USA). Buffer components (sodium chloride, magnesium chloride, calcium chloride, sodium monophosphate, sodium diphosphate, potassium diphosphate, potassium chloride, hydrochloric acid, and sodium hydroxide), dimethyl sulfoxide (DMSO), and triethylamine were from Dia-M (Russia). DMEM and antibiotics were from Paneco (Russia). Fetal bovine serum was from Flow (Germany). All chemical reagents were of analytical grade.

Sterile culture flasks and test-tubes were from Costar (USA), culture microplates were from Dynatech (USA), and sterile 0.22 μm filters were from Millipore (USA).

Aluminum sulfophthalocyanine derivatives: photosensitizer "Photosens" (mixture of aluminum di- (30%), tri- (50%) and tetra- (20%) sulfophthalocyanines [3]) and aluminum tetra-4-sulfophthalocyanine were synthesized in "NIOPIK" (Russia). The composition of "Photosens" is strictly controlled by the manufacturer using HPLC and other methods.

All solutions were prepared using Milli Q water.

2.2. Spectra

Absorption spectra of aluminum sulfophthalocyanines were recorded on a Jenway 6405 spectrophotometer (UK). Fluorescence spectra were obtained on a Hitachi MPF-4 spectrofluorimeter (Japan). Fluorescence intensity of solutions of carboxyfluorescein in microplate wells was measured on a Fluoroscan II instrument (Labsystems, Finland); λ_{em} 538 nm, λ_{ex} 485 nm.

2.3. Synthesis, purification, and characterization of dibiotinylated aluminum sulfophthalocyanine

Biotinylation of aluminum sulfochloride phthalocyanine (reactive derivative of aluminum sulfophthalocyanine) was performed by the procedure described in [6,7] (Fig. 1). The initial molar ratio of biotin–cadaverine:phthalocyanine was (2.1–2.3):1. The conjugate was purified by equilibrium dialysis against phosphate-buffered saline (PBS) buffer (pH 7.4) using a 3500 D cut-off membrane (Pierce, USA). Concentration of "Photosens" in the conjugate solution was measured by the absorption at 678 nm ($\varepsilon = 1.35 \times 10^5\ M^{-1}\ cm^{-1}$ [3]). Concentration of biotin in the conjugate solution was determined by fluorometric titration of NeutrAvidin as described in [8]. The degree of biotinylation was calculated as a ratio of concentrations of biotin and phthalocyanine in the conjugate. The biotinylation degree of the conjugates was 1.9–2.1.

2.4. Cell culture

Cells (human small cell lung carcinoma OAT-75 [9]) were grown in sixty inner wells of 96-well culture microplates in DMEM supplemented with 5% fetal bovine serum and antibiotics at 37 °C in CO_2-incubator for two days. Cell monolayers were used for the evaluation of the phototoxic properties of photosensitizers.

2.5. Light irradiation device

Cell irradiation was accomplished using a special lamp source of light LS-3PDT (Biospec, Russia) with a multi-fiber optical bundle (Fig. 2) that allowed independent irradiation of each well of a microplate [7]. Light source consisted of a high-pressure 300 W arc xenon lamp and elliptical reflector that could focus light in a 10-mm spot. Light passes sequentially through a water filter (cutting off the infrared radiation of a lamp), a special ceramic filter (cutting off radiation in the ultraviolet and short-wave visible range), and then a cut-off glass filter providing light transmission in the appropriate wavelength range. Light output in the red spectral range is above 5 W. The entrance end of a fiber bundle is fixed in the focus of an elliptical reflector. At the distal end of a bundle, 54 fibers are fixed in such a way that each of them is placed strictly against one well in a culture microplate (with the exception of last rows), while six fibers remain non-fixed and are used for the control of stability of radiation parameters. The distribution of light intensity on the entrance end of the optical bundle is characterized by a non-uniform (cupola-shaped) form. Hence, values of light output delivered by different fibers are also different comprising relatively uniform series, which allows simultaneous evaluation of the phototoxic effect in a wide range of irradiation doses. Distribution of irradiation power in the range

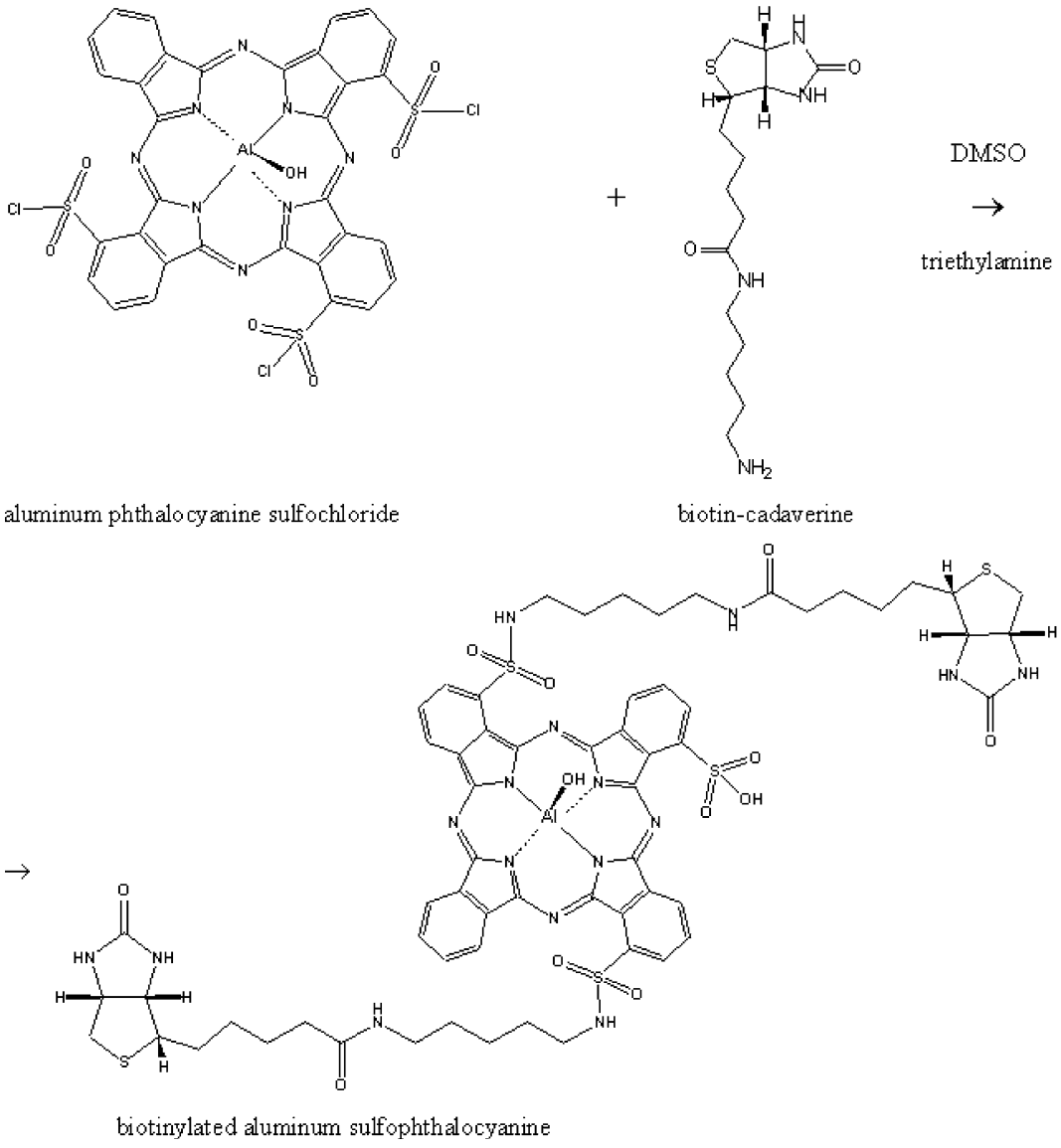

Fig. 1. Synthesis of biotinylated aluminum sulfophthalocyanine.

of 620–700 nm (at the absorption maxima of "Photosens" and some other photosensitizers) along the inner wells of the microplate is shown in Fig. 3.

2.6. Evaluation of phototoxic properties of aluminum dibiotinylated sulfophthalocyanine in vitro

Monolayers of OAT-75 cells were incubated with the photosensitizers ("Photosens", dibiotinylated "Photosens", or dibiotinylated aluminum tetra-4-sulfophthalocyanine) and dissolved in DMEM containing 5% fetal bovine serum. Untreated cells were used as the control. Then the medium was discarded and the cells were washed once with DMEM. After that the fresh medium was added, and the cells were irradiated using LS-3PDT lamp light delivery system (Biospec, Russia). Irradiation was performed through the bottom of the microplate.

Cytotoxicity of photosensitizers was estimated using proliferation test with carboxyfluorescein diacetate (CFDA). Upon hydrolysis by intracellular non-specific esterases, CFDA forms carboxyfluorescein. Carboxyfluorescein contains extra negative charges and is, therefore, better retained in cells as compared with fluorescein [10]. Upon excitation with blue light, carboxyfluorescein exhibits green fluorescence. According to Riordan et al. [11], the results on tumor cells viability obtained with FDA test are in good correlation with the clonogenic assay data.

The cells were washed three times with Dulbecco's phosphate-buffered saline (PBD) and then the solution of CFDA in PBD (concentration 1 μg/ml) was added. After 45 min of incubation, the intensity of carboxyfluorescein fluorescence was measured using Fluoroscan II. The calibration curve, i.e., the dependence of the

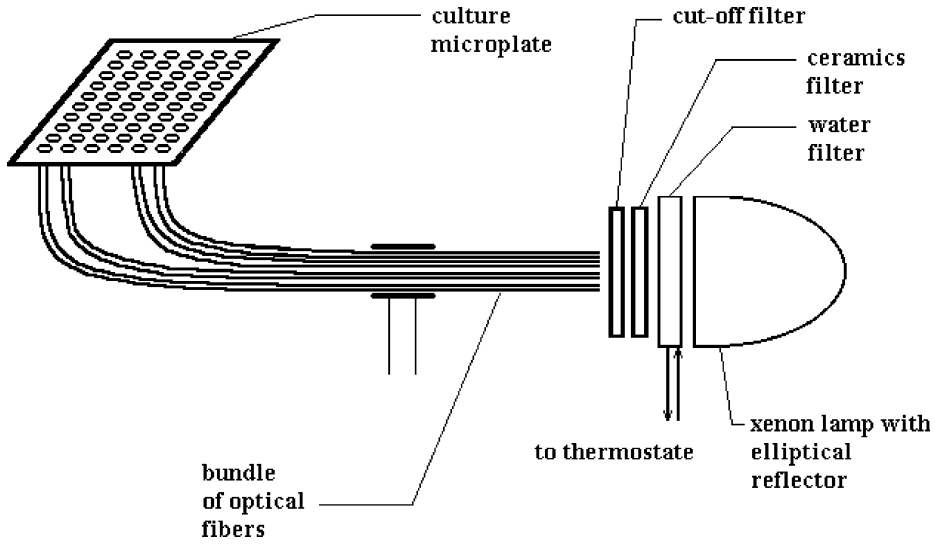

Fig. 2. Light delivery device for the evaluation of photodynamic action on cell monolayers in 96-well culture microplate.

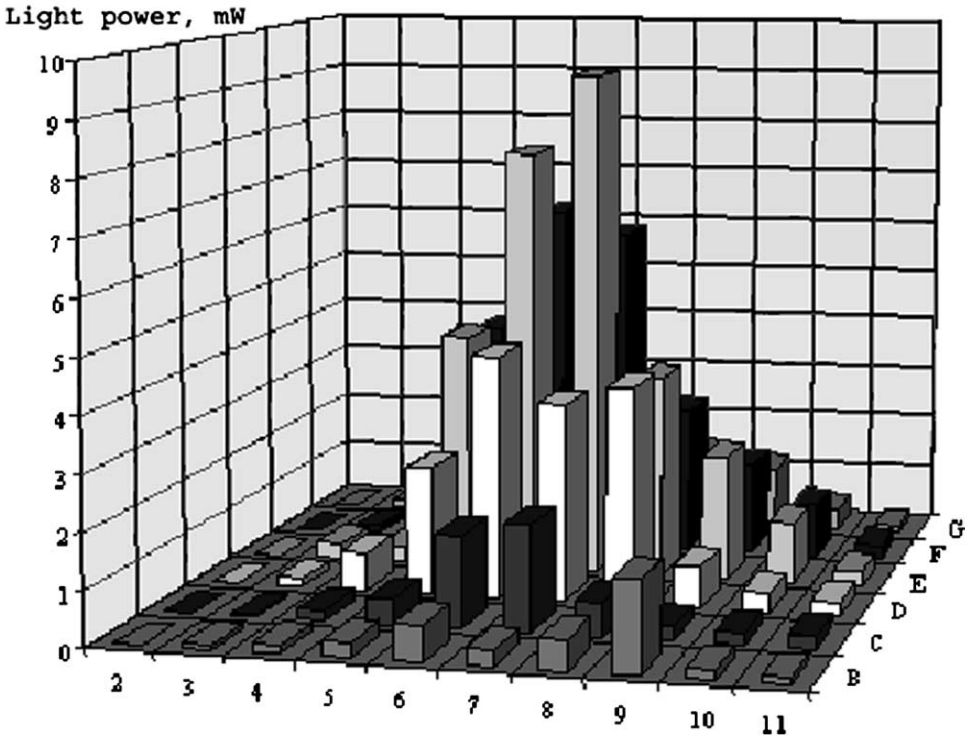

Fig. 3. Light distribution between the wells of culture microplate. Numbers 2–11 refer to the vertical row number, letters B–G refer to the horizontal row number of a culture microplate.

fluorescence intensity on the number of cultured cells was plotted; each value was the average of 6 measurements. The number of surviving cells was determined by extrapolation of the calibration curve to zero time. Phototoxic activity of photosensitizers was expressed as ED_{90} and ED_{50} (light dose causing death of 90% and 50% of cells, respectively).

2.7. Photodynamic therapy of tumor-bearing mice using aluminum dibiotinylated sulfophthalocyanine in vivo

The experiment was performed on 35 mice (F_1, male, 22–24 g) bearing Ehrlich adenocarcinoma.

The suspension of tumor cells was inoculated intramuscularly into a mice right leg. Photosensitizers were

injected intravenously on day 6 in the doses of 0.25 and 0.5 mg/kg. Twenty-four hours after the photosensitizer injection, the tumor sites were irradiated using a semiconductor laser (wavelength 670 nm, power density 200 mW/cm^2, light dose density 250 J/cm^2). Mice treated with equal doses of "Photosens" and intact mice were used as controls.

During the first few days after PDT, the "Photosens"-based photosensitizers caused significant hypostasis, therefore, the tumor volume was not measured immediately after the PDT. The first measurements were carried out after 3–4 days, after the decrease in the hypostasis.

PDT efficacy was estimated by tumor growth inhibition, morphological changes of tumor tissue, such as necrosis and vascular damages, proliferative activity of surviving cells, and cell apoptosis. Desmoplastic and inflammatory reactions characterizing body anti-tumor defense were also studied [12].

The tumor size was measured during 9 days after irradiation. On 9th day the animals were sacrificed and the morphological study was carried out, including micro- and macroscopic studies of paraffin-embedded sections stained with haematoxylin-eosin.

The results of morphological study were estimated semi-quantitatively by the following parameters:

1. area of intact tumor tissue (the percentage of the total tumor area in the section);
2. level of apoptosis estimated according to the pictures of karyorrhexis, karyopycnosis and cytoplasm condensation of tumor cells [13]. Maximum level corresponding to >20% apoptotic cells was estimated as 6 scores, 4 scores corresponded to 10–20% apoptotic cells, 2 scores corresponded to <10% apoptotic cells;
3. proliferative activity – the number of mitotic figures in a field of view at 400× magnification; 6 scores corresponded to maximum activity (5 mitotic figures), moderate activity (4 scores) – 2–5 mitoses, weak activity (2 scores) – 1–2 mitoses;
4. desmoplastic reaction was estimated as follows: expressed (6 scores) – formation of a capsule, moderate expressed (4 scores) – detection of capsule's fragments around tumor (pseudocapsule), weak (2 scores) – proliferation of myelofibroblasts on tumor's border;
5. inflammatory reaction – expression of inflammatory infiltrate around the tumor (from 6 to 0 scores).

Animal experiments were performed in according with "Guidelines for experimental (pre-clinical) studying of new pharmacological substances" of Ministry of Health of Russian Federation.

3. Results and discussion

3.1. Phototoxic effect of dibiotinylated aluminum sulfophthalocyanine in vitro

"Photosens" is a "controlled mixture" of aluminum sulphophthalocyanines with different degree of sulfonation: di-, tri- and tetra-sulfophthalocyanines. The mixture is an efficient sensitizer, whereas all the individual components are inactive. The reason of this is that the components in the mixture, unlike components themselves, do not aggregate in aqueous solutions [1].

From the practical point of view it is important to improve the activity of the sensitizer. We have chosen "Photosens", the active sensitizer, which was to-date used for the treatment of more than 2000 patients.

We compared the photodynamic efficiency of dibiotinylated "Photosens" with non-modified "Photosens" from the same preparation. For comparison, we have studied the conjugate of biotin with the individual tetra-4-substituted compound.

The influence of amphiphilicity of the phthalocyanine on the phototoxic effect was demonstrated in the experiment where the effect of "Photosens" possessing three hydrophilic sulfo-groups was compared with the effect of dibiotinylated "Photosens" and dibiotinylated aluminum tetra-4-sulfophthalocyanine with increased lipophilicity due to the introduction of the two biotin residues.

The results of this experiment demonstrated that dibiotinylated "Photosens" provided enhancement of phototoxic action on monolayers of OAT-75 cells in

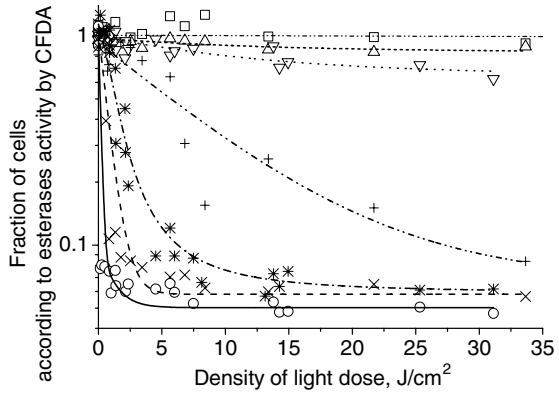

Fig. 4. Dependence of phototoxic action of dibiotinylated "Photosens" on monolayers of cells OAT-75 on the power of irradiation. Concentration of the photosensitizer: △, 0.375 μM; ▽, 1.12 μM; +, 3.75 μM; ✻, 11.2 μM; ×, 37.5 μM; ○, 112.4 μM; □, control without photosensitizer. Incubation with the photosensitizer for 6 h, irradiation time 20 min. (The dependences of single experiment are presented, for one cell culture seeding. For each concentration of the photosensitizer from the range 1.12–112.4 μM from 2 to 6 experiments were conducted with similar trends and with similar ratios between dependences at the different concentrations.)

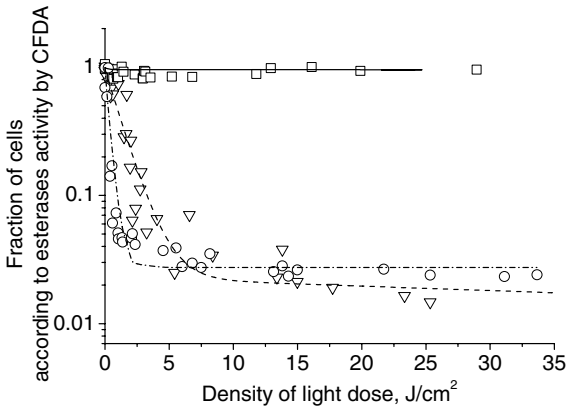

Fig. 5. Dependence of phototoxic action of dibiotinylated "Photosens" and "Photosens" on monolayers of cells OAT-75 on the power of irradiation. Incubation with the photosensitizers for 6 h, irradiation time 20 min. ▽, "Photosens"; ○, dibiotinylated "Photosens"; □, control. Concentration of the photosensitizers: 112.4 μM. (The dependences of single experiment are presented, for one cell culture seeding; 4 experiments were conducted with similar trends and with similar ratios between dependences for these photosensitizers.)

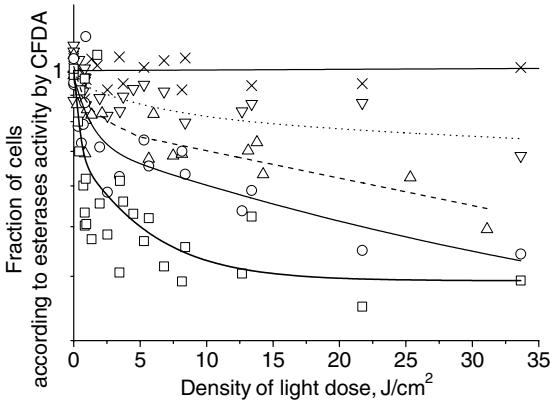

Fig. 6. Influence of irradiation power on phototoxic action of dibiotinylated tetra-4-sulfo aluminum phthalocyanine on monolayers of OAT-75 cells. Concentration of the photosensitizer: ▽, 3.75 μM; △, 11.2 μM; ○, 37.5 μM; □, 112.4 μM; ×, control without photosensitizer. Incubation with the photosensitizer for 6 h, irradiation time 20 min.

comparison with both non-modified "Photosens" and dibiotinylated aluminum tetra-4-sulfophthalocyanine (Figs. 4–6, Table 1). ED_{50} for dibiotinylated "Photosens" was 1.6–2.8 times lower as compared with "Photosens", and more than 20 times lower as compared with dibiotinylated aluminum tetra-4-sulfophthalocyanine. In our opinion, these results can be explained by the increased amphiphilicity of the photosensitizers due to the addition of the hydrophobic substituents (biotin–cadaverine residues) that is in good correlation with the literature data [4]. On the other hand, higher hydrophilicity due to the increased number of sulfo-groups led to considerably impaired phototoxic action of dibiotinylated aluminum tetra-4-sulfophthalocyanine in comparison with dibiotinylated "Photosens". Even at maximum concentrations (112.4 μM) and maximum irradiation power, the amount of dead cells for dibiotinylated aluminum tetra-4-sulfophthalocyanine did not exceed 60%. It could be presumed that a certain fraction of cells was resistant to dibiotinylated aluminum tetra-4-sulfophthalocyanine since the OAT-75 cell culture is asynchronous. Hence, the resistance can be associated with different vulnerability of cells to photo-damage at different phases of growth.

No dark toxicity was detected for any of the photosensitizers.

3.2. Phototoxic action of dibiotinylated aluminum sulfophthalocyanine in vivo

The results of PDT of mice bearing Ehrlich carcinoma using dibiotinylated "Photosens" are presented in Fig. 7. The results for the photosensitizers without irradiation and for the irradiation without photosensitizers did not differ from the intact control results. It can be seen that dibiotinylated "Photosens" provided

Table 1
Phototoxicity of dibiotinylated derivatives of aluminum phthalocyanine[a]

Parameters	ED_{50} (J/cm^2)				ED_{90} (J/cm^2)
Photosensitizers	Dibiotinylated "Photosens"		"Photosens"	Dibiotinylated tetra-4-sulfo aluminum phthalocyanine	Dibiotinylated "Photosens"
Time of incubation (h)	6	2	2	6	6
Concentration of photosensitizer (μM)					
112.4	–[b]	0.36	1.01	5.06[c]	0.36
37.5	0.62[c]	–	–	21.7	1.99[c]
11.2	1.44	5.42	8.70	39.8	6.24
3.75	6.15[c]	57.8	93.0[c]	148.3[c]	28.3[c]
1.12	39.8	–	–	–	73.4
0.375	112.5[c]	–	–	–	202.9[c]

Irradiation time 20 min.

[a] The ED data were obtained by inter- or extrapolation of the dependences of surviving fraction of cells on light dose; for the same light dose SD of "surviving fraction" value did not exceed 10%.

[b] $ED_{50} < 0.14$ J/cm^2.

[c] $R^2 < 0.9$.

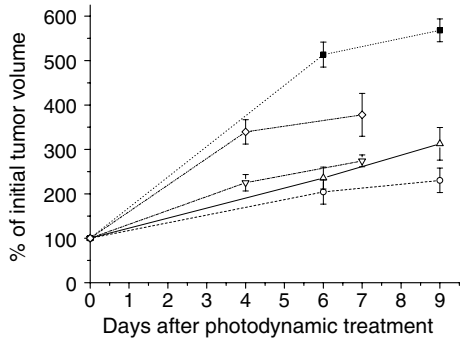

Fig. 7. Tumor growth inhibition after PDT. △, dibiotinylated "Photosens", 0.5 mg/kg; ○, dibiotinylated "Photosens", 0.25 mg/kg; ▽, "Photosens", 0.5 mg/kg; ◇, "Photosens", 0.25 mg/kg; ■, control without photosensitizer. The tumor volume before PDT was $0.71 \pm 0.10\ cm^3$.

more efficient tumor growth inhibition as compared with "Photosens". The morphological study of tumor sections has revealed high level of cell death (up to total), pronounced vascular damage (thrombosis and destruction of vascular walls), and inhibition of tumor cell proliferation activity even at the dose as low as 0.25 mg/kg (Fig. 8(a)–(c)). Some results of the morphological studies of tumors after PDT are presented in Table 2.

The total tumor cure was not observed in our experiments that can be explained by the comparatively low doses of the photosensitizers.

The results obtained demonstrate that the application of the dibiotinylated "Photosens" at the dose of 0.25 mg/kg sufficiently decreased the area of surviving tumor cells (6 times as compared with "Photosens", from 30% down to 5%), increased the extent of necrotic area from 70% up to almost total (95%), and the level of apoptosis exceeded 50%. Additionally, the proliferative activity of the tumor cells decreased from the moderate down to the weak, the desmoplastic reaction extent changed from the moderately expressed (detection of capsule's fragments around tumor, so called pseudo-capsule) down to the weakly expressed (proliferation of myelofibroblasts on the tumor border).

"Photosens" and dibiotinylated "Photosens" differ from each other by their amphiphilicity. We propose, that dibiotinylated "Photosens" may penetrate the cell membranes more easily, accumulate inside tumor cells in higher concentrations, and induce additional tumor injury – necrosis and apoptosis. We can also suppose that the lipophilic moiety of dibiotinylated "Photosens" molecules may enhance membrane's destruction and activate the mechanisms of cell death.

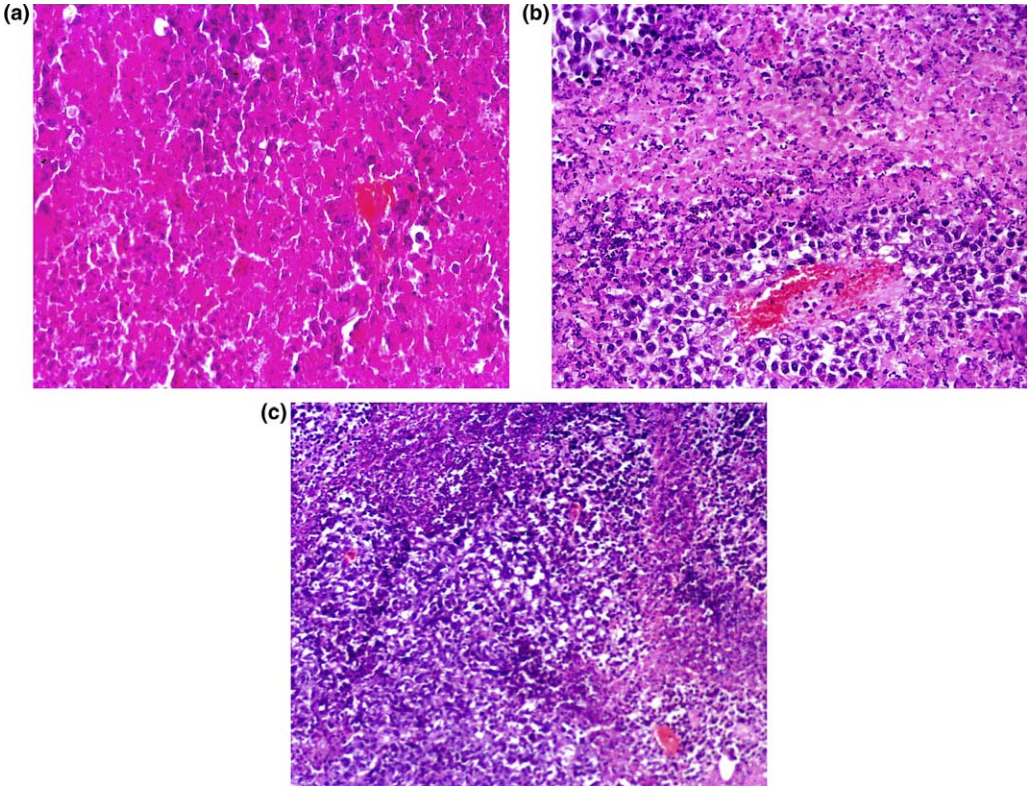

Fig. 8. Microphotographs of murine tumor sections stained with haematoxylin and eosine, ×200. (a) PDT with dibiotinylated "Photosens", 0.5 mg/kg; (b) PDT with "Photosens", 0.5 mg/kg; (c) control without the photosensitizer.

Table 2
Morphological study of tumor sections after PDT of mice bearing Ehrlich carcinoma

Photosensitizer	Dose (mg/kg)	Area of surviving tumor cells (%)	Damage of tumor cells		Proliferative activity of tumor cells, scores	Desmoplastic reaction, scores	Borderline inflammatory reaction, scores
			Necrotic area (%)	Apoptosis, scores			
"Photosens"	0.25	30*,**	70*,**	4	4*,**	4*,**	1
"Photosens"	0.5	25*,**	75*,**	4	3*,**	4*,**	1
"Photosens"-biotin	0.25	5*,**	95*,**	6	1*,**	1**	1
"Photosens"-biotin	0.5	2*,**	98*,**	6	1*,**	1**	1
Control	0	60	40*,**	4	6	0	0

* $p < 0.05$ for PDT as compared with control.
** $p < 0.05$ for PDT with different photosensitizers at the same dose; statistical analysis was performed using White's criterium for small groups [14].

4. Conclusions

Our results demonstrate that the photosensitizing effect of "Photosens" can be improved by the increase of its amphiphilicity through the introduction of biotin residues. PDT of the experimental tumor using dibiotinylated "Photosens" provided more effective tumor destruction and inhibition of tumor cell proliferation in comparison with the parent drug.

5. Abbreviation

BChl	bacteriochlorophyll
CFDA	carboxyfluorescein diacetate
Chl	chlorophyll
DMEM	Dulbecco's modified Eagle's medium
FDA	fluorescein diacetate
ED_{90}	(ED_{50}) effective dose of light that caused the decreasing by 90% and 50%, respectively, of cell fraction determined according to esterases activity by CFDA
PBD	Dulbecco's phosphate-buffered saline
PBS	phosphate-buffered saline
PDT	photodynamic therapy

Acknowledgments

This research was partly supported by INTAS, Grant No. 0554. The authors E. Luckyanets, V. Derkacheva and G. Meerovich were supported by the Russian Ministry of Science and Moscow municipal government.

References

[1] H. Ali, J.E. van Lier, Metal complexes as photo- and radiosensitizers, Chem. Rev. 99 (1999) 2379–2450.
[2] I.J. McDonald, T.G. Dougherty, Basic principles of photodynamic therapy, J. Porphyr. Phthalocya. 5 (2001) 105–129.
[3] E.A. Lukyanets, New photosensitizers for photodynamic therapy, Rossiiskii khimicheskii zhurnal XLII (1998) 9–16 (in Russian).
[4] P. Margaron, R. Langlois, J.E. van Lier, S. Gaspard, Photodynamic properties of naphthosulfobenzoporphyrazines, novel asymmetric, amphiphilic phthalocyanine derivatives, J. Photochem. Photobiol. B: Biol. 14 (3) (1992) 187–199.
[5] V. Rosenbach-Belkin, L. Chen, L. Feodor, I. Tregub, F. Pavlotsky, V. Brumfeld, Y. Salomon, A. Scherz, Serine conjugates of chlorophyll and bacteriochlorophyll: photocytotoxicity in vitro and tissue distributuon in mice bearing melanoma tumors, Photochem. Photobiol. 64 (1996) 174–181.
[6] S. Kudrevich, Basseur, C. La Madeleine, S. Gilbert, J.E. van Lier, Synthesis and photodynamic activities of novel trisulfonated zinc phthalocyanine derivatives, J. Med. Chem. 40 (1997) 3897–3904.
[7] A.P. Savitsky, I.G. Meerovich, G.A. Meerovich, Device for studying of photodynamic activity of photosensitizers in vitro, Patent of Russian Federation No. 2160897, published on 20.12.2000.
[8] G.P. Der-Balian, B. Gomes, R.S. Masino, J.W. Parce, A fluorometric method for determining the degree of biotinylation of proteins, J. Immunol. Meth. 172 (1994) 255–264.
[9] H. Ohara, T. Okamoto, A new in vitro cell line established from human OAT carcinoma of the scores lung, Cancer Res. 37 (1977) 3088–3095.
[10] J.R. Sellers, S. Cook, V.S. Goldmacher, A cytotoxicity assay utilizing a fluorescent dye that determines accurate surviving fractions of cells, J. Immunol. Meth. 172 (1994) 255–264.
[11] H.D. Riordan, N.H. Riordan, X. Meng, J. Zhong, J.A. Jackson, Improved microplate fluorometer counting of viable tumor and normal cells, Anticancer Res. 14 (1994) 927–931.
[12] H.C. Pitot, Fundamentals of Oncology, third ed., Marcel Dekker, New York, 1986.
[13] J.F.R. Kerr, A.H. Wylie, A.H. Currie, Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics, Br. J. Cancer 26 (1972) 239–257.
[14] G.F. Lakin, Biometry, Moscow, Vysshaya shkola, 1990 (in Russian).