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Photodynamic activity of the boronated chlorin e6 amide in artificial and cellular membranes

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ABSTRACT

Photodynamic tumor-destroying activity of the boronated chlorin e6 derivative BACE (chlorin e6 13(1)-N-{2-[N-(1-carba-*closo*-dodecaboran-1-yl)methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester), previously described in Moisenovich et al. (2010) PLoS ONE 5(9) e12717, was shown here to be enormously higher than that of unsubstituted chlorin e6, being supported by the data on much higher photocytotoxicity of BACE in M-1 sarcoma cell culture. To validate membrane damaging effect as the basis of the enhanced tumoricidal activity, BACE was compared with unsubstituted chlorin e6 in the potency to photosensitize dye leakage from liposomes, transbilayer lipid flip-flop, inactivation of gramicidin A ionic channels in planar lipid membranes and erythrocyte hemolysis. In all the models comprising artificial and cellular membranes, the photodynamic effect of BACE exceeded that of chlorin e6. BACE substantially differed from chlorin e6 in the affinity to liposomes and erythrocytes, as monitored by fluorescence spectroscopy, flow cytometry and centrifugation. The results support the key role of membrane binding in the photodynamic effect of the boronated chlorin e6 amide.

1. Introduction

Photodynamic therapy (PDT), a non-surgical cancer treatment modality, uses the ability of dyes termed photosensitizers to generate cell-killing singlet oxygen and other reactive oxygen species. Because the basis of PDT lies in photosensitized oxidation of lipids, proteins and/or DNA [1], its efficacy essentially depends on the photosensitizer ability to absorb light in the tissue transparency window and the potency to generate singlet oxygen. Chlorin derivatives having an intense absorption band in the far-red region and a high quantum yield of singlet oxygen generation generally meet the above requirements and, therefore, are widely used for design and testing of new photodynamic drugs [2]. One of the most intriguing and so far unresolved issues is associated with causes of selective accumulation/retention of the PDT agents in malignant tumors and approaches to its improvement. Based on photophysical, photochemical and pharmacokinetic studies [3–6], the photodynamic activity of chlorin and bacteriochlorin derivatives, among which several are clinically used [7–12], can be modulated by varying substituents at the periphery of the tetrapyrrole ring [13,14]. In particular, chlorin e6 ethylenediamide and pheophorbide *a* ethanolamide exhibited a higher light-dependent tumor-destroying potency than unsubstituted chlorin e6 [15] and pheophorbide *a* [16], respectively. Significantly pronounced photodynamic suppression of tumor growth was found with boronated chlorin e6 derivatives [17,18]. It is worth noting that synthesis and examination of carboranyl-containing porphyrins [19,20], phthalocyanines [21,22] and chlorins [17,23–25] were prompted by a promising opportunity of using these compounds as boron-delivery agents for anticancer boron neutron capture therapy (BNCT), because of the selective uptake and persistence in tumor over many normal tissues. In particular, for some boronated chlorin e6 and phthalocyanine

Abbreviations: PDT, photodynamic therapy; BACE, chlorin e_6 13(1)-N-{2-[N-(1-carba-*closo*-dodecaboran-1-yl)methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester sodium; DPhPC, diphytanoylphosphatidylcholine; EggPC, egg yolk phosphatidylcholine; Chol, cholesterol; pyPC, 1-lauroyl-2-(1′pyrenebutyroyl)-sn-glycero-3-phosphocholine; gA, gramicidin A; CF, 5(6)-carboxyfluorescein; BLM, bilayer lipid membrane; RBC, red blood cell; MTT, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer solution

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conjugates, high propensity to accumulation in tumor cells was recently reported [26,27].

Having revealed high photocytotoxicity of chlorin e6 13(1)-N-{2-[N-(1-carba-*closo*-dodecaboran-1-yl) methyl] aminoethyl} amide-15(2), 17(3)-dimethyl ester (boronated chlorin e6 amide, BACE), our previous study [18] left its basis unclear. According to our data, the quantum yield of singlet oxygen generation of BACE was close to that of chlorin e6. The high photodynamic activity of BACE in vivo was tentatively ascribed to the increased membrane-penetrating ability of this compound [18]. Here we compared the activity of BACE with that of unsubstituted chlorin e6 in a variety of membrane systems, ranging from lipid and protein perturbation in model membranes to erythrocyte photohemolysis and antitumor activity. Chlorin e6 has long been thoroughly studied as a photosensitizer, therefore it would be reasonable to use it as a reference agent while estimating the photodynamic potency of BACE.

2. Materials and methods

2.1. Synthesis of the BACE photosensitizer

Synthesis of the BACE photosensitizer was described previously [18]. Briefly, we used methylpheophorbide *a* (**1**; Fig. S1, Supplementary material) as the starting compound for preparation of free base boronated chlorin e_6 derivatives. The synthesis proceeded via the formation of the amide derivative **2** obtained after the nucleophilic opening of the exocyclic ring in **1** with ethylenediamine. Alkylation of amino group in **2** with 1-trifluoromethanesulfonylmethyl-1-carba-*closo*-dodecaborate cesium **3** [28,23] in tetrahydrofuran in the presence of N,O-bis(trimethylsilyl)acetamide led to chlorin e_6 13(1)-N-{2-[N-(1-carba-*closo*-dodecaboran-1-yl) methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester cesium **4**. For biological studies the sodium salt, chlorin e_6 13(1)-N-{2-[N-(1-carba-*closo*-dodecaboran-1-yl)methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester sodium (BACE) was obtained from cesium salt **4** (Fig. S1).

Egg yolk phosphatidylcholine (EggPC), diphytanoylphosphatidylcholine (DPhPC), and cholesterol (Chol) were from Avanti polar lipids (Alabaster, AL). 5,6-Carboxyfluorescein (CF) was from Sigma-Aldrich (St. Louis, MO). Chlorin e6 was obtained from Porphyrin Products (Logan, UT). 1-Lauroyl-2-(1′pyrenebutyroyl)-sn-glycero-3-phosphocholine (pyPC) was prepared by Dr. Sergei Kovalchuk from Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences as described in [29].

Liposomes were prepared by evaporation under a stream of nitrogen of 2% solution of EggPC in chloroform followed by hydration with a buffer solution containing 150 mM NaCl, 10 mM KH_2PO_4, pH 7.4. The mixture was vortexed, passed through a cycle of freezing and thawing, and extruded through Nucleopore polycarbonate membranes (0.1-μm pore size) using an Avanti Mini-Extruder.

2.2. Fluorescence spectra and polarization degree

Fluorescence spectra and polarization degree were measured with a Horiba Jobin Yvon FluoroMax-3 spectrofluorometer.

2.3. Flip-flop of pyPC on liposomes

The measurement of lipid flip-flop was performed as described in [30]. To label EggPC/Chol vesicles with synthetic lipid probe pyPC (1-lauroyl-2-(1′pyrenebutyroyl)-sn-glycero-3-phosphocholine) exclusively on the outer leaflet, 5 μM pyPC dissolved in ethanol was added to the buffer solution containing liposomes (final lipid concentration 50 μg/ml). Incorporation of pyPC into the outer membrane leaflet was followed by measuring the ratio of fluorescence intensities of monomers and excimers at 395 and 480 nm, respectively. The fluorescence was excited at 344 nm.

2.4. Sensitized photoinactivation of gramicidin-mediated current in planar bilayers

Bilayer lipid membranes (BLMs) were formed on a 0.55-mm diameter hole in a Teflon partition separating two compartments of a cell containing aqueous solutions of 100 mM KCl, 10 mM MES, and 10 mM Tris at pH 7.0. The membrane-forming solutions contained 20 mg of diphytanoylphosphatidylcholine (DPhPC) in 1 ml of n-decane. Gramicidin A (gA) was added from stock solutions in ethanol (1 μg/ml) to the bathing solutions at both sides of the BLM and routinely incubated for 15 min with constant stirring. Experiments were carried out at room temperature (24–26 °C). The dyes were added to the bathing solution at the trans side (the cis side is the front side with respect to the illumination lamp). The electric current (I) was recorded under voltage-clamp conditions with a Keithley 428 amplifier, digitized by a LabPC 1200 (National Instruments, Austin, TX) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, U.K.). Ag–AgCl electrodes were placed directly into the cell, and a voltage of 50 mV was applied to BLM. The initial level of the gramicidin-mediated current amounted to 0.41 ± 0.12 μA in all the experiments. The xenon lamp was placed at the *cis*-side of the membrane. Illumination of the bilayer was performed by single flashes (flash energy of 400 mJ/cm^2 and flash duration of 2 ms). The light was passed through a cutoff filter (for wavelengths ≤ 500 nm). In the presence of the photosensitizer, a light flash is known to decrease the gramicidin-mediated transmembrane current, I(t) [31]. The latter is a monoexponential function of time: $I(t) = (I_0 - I_\infty)e^{-t/\tau} + I_\infty$, where I_0, I_∞, and τ are the initial current before illumination, the steady-state current measured after light exposure, and the characteristic time of photoinactivation, respectively. It has been shown that τ is not related to the rate of photoinactivation, but rather to the dissociation kinetics of gramicidin dimers [31]. Another important parameter is the relative amplitude of photoinactivation α: $\alpha = (I_0 - I_\infty) / I_0$. Because the decrease in the gramicidin-mediated current is due to the reduction of the number of open channels, while the single-channel conductance remains unaltered [32], α is equal to the portion of photoinactivated gramicidin channels. This parameter allows us to compare the efficiency of different photosensitizers.

2.5. Blood collection, preparation of red blood cells and kinetics of hemolysis

Blood was collected from healthy volunteers into tubes containing EDTA as anticoagulant. Red blood cells (RBCs) were harvested by centrifugation (1500 ×*g* for 2 min), the plasma was discarded, and the erythrocyte pellet was washed thrice with sterile buffered saline. A 1% (v/v) RBC suspension was made in PBS (l0 mM Na phosphate, 150 mM NaCl, pH 7.4). Several experiments (described in Figs. 4 and 5) were run with mouse erythrocytes. Blood from mice was collected from the saphenous vein as described [33]. The time course of RBC lysis induced by photodynamic treatment was followed spectrophotometrically at 650 nm with an Amersham Ultrospec 1100 spectrophotometer in a 1-cm cuvette. Human RBCs were suspended at a concentration of 0.1% in PBS buffer. Initially the A650 value was approximately 0.5.

2.6. Flow cytometry

RBCs were incubated with 1 μM of the dyes for 15 min, and then washed with PBS. Initial gating on RBCs was performed according to light scatter characteristics. The fluorescence of BACE and chlorin e6 was measured with excitation at 641 nm, Em: LP 690 nm on a FACS Aria SORP (Becton Dickinson, San Jose, CA). The collected data contained at least 20,000 events per gate.

2.7. Microscopy

Mouse RBCs were isolated in the Hanks medium HBSS containing 1/10 volume of 0.5 mM EDTA. RBCs were placed on round 24 mm glass cover slips and were incubated with 1 μM of dyes for 5 min. Digital images were acquired using an *Nikon Eclipse Ti-E A1* laser-scanning confocal system. The 512 × 512 or 1024 × 1024 pixel confocal images were recorded with an Apo TIRF 60×/1.49 oil objective. Fluorescence of photosensitizers was excited with a 642 nm laser, and emission was recorded with a 663–738 nm band pass filter. The pinholes for high-resolution images were set up according to the manufacturer's instructions. Images were analyzed with the NIS-Element imaging software.

2.8. Cell culture and cytotoxicity assays

M1 sarcoma cells were isolated by incubating the tumor tissue in the solution of 0.25% Trypsin–EDTA for 40 min. The resulting cell suspension was evenly distributed into wells of a 96-well plate placing 1.5–10^4 cells in 100 μl per well and incubated for 24 h at 37 °C in the dark in an atmosphere containing 6.1% CO_2. Then, wells of the same plate were supplemented with the compounds under study (100 μl per well) at various concentrations in a medium containing 10% fetal calf serum. Cells were incubated with the compounds for 1 h at 37 °C in an atmosphere containing 6.1% CO_2. After the incubation, cells were irradiated for 30 min by using a source of monochromatic light with a wavelength of 633 nm. Then the cells were incubated in the dark for 48 h at 37 °C in an atmosphere containing 6.1% CO_2. Then the medium with the compounds was removed and 100 μl of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at a concentration of 0.5 mg/ml and incubated for 24 h at 37 °C. The pellet was dissolved in 200 μl dimethyl sulfoxide (DMSO). Colorimetric measurements were performed with Labsystem Multiscan plus plate reader at 540 nm. The optical density was directly proportional to the number of living cells.

Chlorin e6 and BACE were dissolved to obtain 5 mM stock solutions in dimethyl sulfoxide, and serial dilutions were made immediately before the experiments. The cells were incubated with the dyes in a 96-well plate for 1 h in the dark. Then cells were illuminated for 10 min using a monochromatic light source (Lebedev Institute of Physics, Moscow; λ = 650 nm, light energy density = 200 J/cm^2) followed by incubation in fresh medium for 48 h. Then 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added for additional 2 h, and the formation of formazan (MTT test [34]) was detected by the absorbance at 540 nm using a Multiscan Plus reader (LabSystems).

2.9. Animals and in vivo PDT

Female rats weighing 180–200 g were used in the experiments. Sarcoma M1 (a 0.2-g piece of tumor tissue) was inoculated subcutaneously into the left thigh. After the tumor nodules were reached, 5 mm in diameter, the animals were divided into 3 cohorts (10 animals per group). Rats in group 1 were injected with PBS, followed by light illumination in the absence of photosensitizers. Rats in groups 2–3 were injected with 2.5 mg/kg of either chlorin e6 or BACE, respectively, followed by light illumination. PBS or photosensitizers were administered i.p. In the preliminary experiments we found that the maximal accumulation of BACE in the tumors was detectable 1.5 h after i.p. injection. Therefore, we used a 1.5-h drug-light interval (i.e., the time between drug injection and tumor illumination). Hair around the tumor was epilated prior to illumination. PDT was performed with the laser beam source Atcus-2 (Semi-Conductor Devices, St. Petersburg, Russian Federation, λ = 661 nm, light energy density = 300 mJ/cm^2). The tumor size was measured immediately before illumination and at 3, 7, 14 and 21 days post illumination. The volume V (cm^3) of the tumor was calculated using the equation:

$$V_T = \frac{1}{6}\pi \times d_1 \times d_2 \times d_3$$

where d_1, d_2 and d_3 are perpendicular diameters (cm) of the tumor mass. PDT effectiveness was evaluated using a parameter of tumor volume divided by initial volume (V_0), i.e. $V_T - V_0/V_0$. Tumor-bearing animals in groups 1–4 were sacrificed by day 14 after PDT. Animals in groups 5–6 were monitored for 60 days post PDT.

For histological examination, tumors of the control and experimental animals were isolated by using thiopental anesthesia (sodium thiopental, 0.1% solution in a dose of 0.25 ml/100 g body weight) together with the surrounding soft tissues. The tumors were cut in the proximal–distal direction, fixed in 10% neutralized formalin solution, dehydrated with increasing concentrations of ethanol and acetone, and then embedded in paraffin (Histomix, BioVitrum, Russia). The 5-μm histological sections were stained with hematoxylin and eosin and examined with microscope "Olympus CX41".

2.10. Ethics

The verbal informed consent was obtained from all participants involved in this study. The written consent was not necessary since all participants worked as a multidisciplinary group and openly shared their results on a regular basis, from the study planning to the finalized manuscript. The research involving laboratory mice and rats has been approved by the Institutional Review Board at Medical Radiological Research Center, Obninsk where the animals were hosted and all animal experiments were performed. No specific approval was required for this particular study since in this institution the experiments with small laboratory animals were performed under the guidance of the Ministry of Health and Social Development of Russian Federation, document no 755 issued on 12.08.1977, and the Declaration of Helsinki of World Medical Association (2000).

3. Results

3.1. Comparison of the antitumor effect of BACE and chlorin e6 on rat M-1 sarcoma

It was shown in our previous work that BACE exhibited pronounced antitumor activity upon photodynamic treatment [18]. Here we compared the activity of BACE with that of chlorin e6. After the tumor nodules reached ~6–8 mm in diameter, the animals were divided into 3 cohorts (10 animals per group): rats were injected with 10 μg/kg BACE, chlorin e6, and equivalent volume of buffer saline as a control. After illumination with red light the tumor volume was measured at time intervals indicated in Fig. 1. Tumor volumes were normalized to their initial levels as described in Materials and methods. Fig. 1 shows that chlorin e6 slowed down the tumor growth while BACE blocked the growth at the same dosage. Actually, animals treated with BACE exhibited gradual shrinkage of the tumors after PDT [18].

To analyze the increased activity of BACE in vivo, histological sections were prepared from tumors subjected or not to PDT. Figs. S2–S4 display typical sections of a 5-day control (unilluminated) tumor (Fig. S2), a 5-day photodynamically treated tumor (Fig. S3) and a tumor remaining 21 days after PDT (Fig. S4). According to Fig. S2, a solid part of the control tumor was composed of polymorphic cells having weakly basophilic cytoplasm and a large nucleus with dispersed chromatin and a large nucleolus, mitotic figures and apoptotic bodies were present in the tumor cells. A section of the tumor subjected to PDT (Fig. S3) shows that a superficial part of the tumor parenchyma was composed of cellular elements having densely eosinophilic cytoplasm and a small nucleus with a poorly discernible nucleolus, and mitotic figures were observed

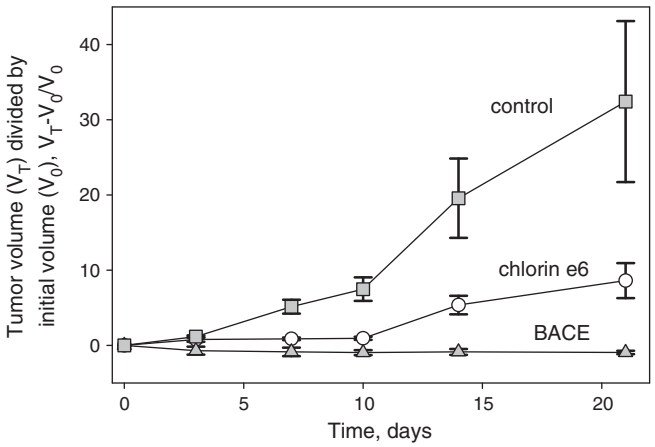

Fig. 1. Comparison of BACE and chlorin e6 as antitumor photosensitizers. Rats bearing s.c. transplant of M-1 sarcoma were injected i.p. with PBS or 2.5 mg/kg of BACE or chlorin e6. Ninety minutes after injection the tumors were illuminated with red light (see Materials and methods). The tumor volume was measured at indicated time intervals.

very rarely. Three weeks after PDT a dark coarse scab was found at the site of the tumor. The remainder of the tumor was located directly beneath the scab and partially inside the skin microfistula. According to Fig. S4, the parenchyma consisted of disintegrating tumor cells having densely eosinophilic cytoplasm and a nucleus with condensed chromatin. A substantial fraction of cells showed hallmarks of necrosis and apoptosis, and numerous apoptotic bodies were seen. The parenchyma was infiltrated by lymphocytes and polymorphonuclear leukocytes (Fig. S4). Thus, total necrosis along with partial or complete regression of the tumor was observed 21 days after PDT. Concomitantly, there was focal necrosis of surrounding soft tissue with immune cell infiltration and replacement of necrotic tissue with fibrous growths. Besides, there was a partial recovery of nerve fibers and blood vessels with mild symptoms of edema and hemodynamic instability in the irradiated region.

3.2. Cytotoxicity of BACE and chlorin e6 upon light exposure

Fig. 2 shows survival of sarcoma M-1 cell culture after light exposure in the presence of BACE and chlorin e6 as determined by the MTT test. The photocytotoxicity of BACE exceeded many-fold that of chlorin e6 at doses of 0.03–0.3 μM. No growth retardation or cell death was recorded upon the same treatment in the dark.

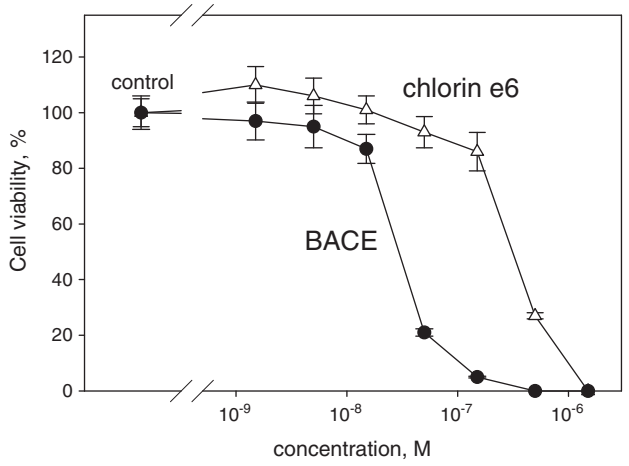

Fig. 2. Viability of cultured M-1 sarcoma cells according to the MTT test after photodynamic treatment with appropriate concentration of BACE or chlorin e6. Experiments were performed 3 times and 3 wells per each concentration were analyzed.

3.3. Photohemolysis of erythrocytes

To support the idea of cellular membrane being one of the major targets for photodynamic action sensitized by BACE and chlorin e6, we performed a comparative study of erythrocyte photohemolysis. Illumination of human erythrocyte suspension at pH 7.2 (Fig. 3A) in the presence of the BACE at a concentration of 2 nM brought about erythrocyte hemolysis, as evidenced by a decrease in light scattering with $t_{1/2}$ about 2 min. By contrast, no photohemolysis was observed with chlorin e6 at the same concentration (black curve), and the comparable photodynamic effect was achieved only at concentrations higher than 40 nM (green curve). The concentration dependence of $t_{1/2}$ for chlorin e6 was shifted substantially towards higher concentrations, compared to that for BACE (Fig. 3B). Therefore, BACE appeared to be a much more potent photosensitizer for erythrocyte hemolysis than chlorin e6. Earlier Gurinovich et al. [35] have shown that chlorin e6 ethylenediamide is more effective in sensitizing erythrocyte photohemolysis than chlorin e6.

3.4. Photohemolysis and photosensitizer binding

A large difference in BACE and chlorin e6 binding to mouse erythrocytes was demonstrated by flow cytometry (Fig. 4). The maximum in brightness histogram was shifted to the right with BACE compared to chlorin e6. In these experiments erythrocytes were incubated with the dyes for 15 min before centrifugation, then resuspended in the buffer solution and detected after 45 min. The results showed that BACE binds more effectively to erythrocyte membranes, than chlorin e6, and thus there is a correlation between photohemolytic activity and membrane binding. Similar correlation was earlier obtained for chlorin derivatives in [15] and [36], and for bacteriochlorin derivatives in [37].

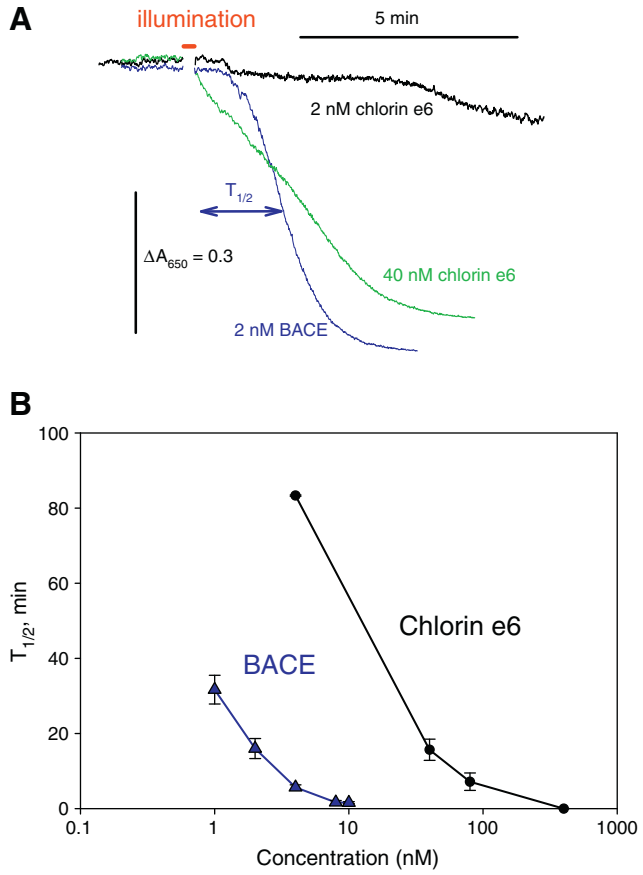

Fig. 3. Photohemolysis of human erythrocytes in the presence of BACE and chlorin e6. A. Time course of a change in absorbance at 650 nm after irradiation with red light. B. The dependence of photohemolysis half-time on the concentration of BACE and chlorin e6.

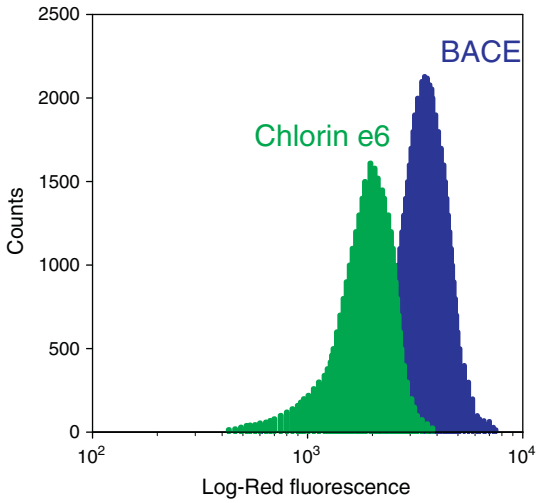

Fig. 4. Flow cytometry analysis of fluorescence emission of mouse red blood cells stained with BACE or chlorin e6. RBCs were incubated with 1 μM of the dyes for 15 min, washed with PBS and counted after 45 min.

3.5. Intra-erythrocyte distribution of BACE and chlorin e6

Fig. 5 displays a typical micrograph of a mouse erythrocyte showing distribution of BACE (A) and chlorin e6 (B) fluorescence. It is seen that BACE was associated predominantly with plasma membrane, whereas a major portion of chlorin e6 fluorescence originated from cytoplasm. Transmission light images show that in the case of chlorin e6, some erythrocytes changed their shape in a way resembling echinocyte transformation.

Images of the time series of successive scans obtained with the confocal microscope revealed a substantial difference in photocytotoxicity between BACE and chlorin e6. With BACE (1 μM), destruction of erythrocytes was observed in a 3–4-fold shorter time interval, than with chlorin e6 at the same concentration under similar conditions of scanning. Remarkably, the destruction manifested itself as abrupt disappearance of cellular structure.

3.6. Lipid flip-flop

As shown earlier in our lab, photodynamic treatment of bilayer membranes containing unsaturated lipids facilitated flip-flop of lipid molecules from one monolayer to another [38]. To follow lipid flip-flop, we tracked the evolution of the ratio of excimer/monomer fluorescence intensities (I_E/I_M) of pyrene-labeled phosphatidylcholine (pyPC), similar to our previous study [38]. If added to water solution, pyPC inserts solely into an outer monolayer of a liposome membrane, and induction of flip-flop leads to redistribution of the labeled lipid between the two monolayers, manifesting itself in a reduction of the I_E/I_M ratio due to a decrease in a local pyPC concentration [30]. Fig. 6 shows time courses of pyPC flip-flop initiated by photodynamic treatment of liposomes with BACE or chlorin e6. A rather fast (on a minute scale) decrease in fluorescence of pyPC excimers paralleled by an increase in fluorescence of pyPC monomers (Fig. 6A), resulting in a drop of the I_E/I_M ratio (Fig. 6B), was recorded after exposure of EggPC/Chol liposomes to red light in the presence of 0.5 μM of the photosensitizers. In the case of BACE, the flip-flop kinetics was substantially faster, than with chlorin e6: estimation of the flip-flop half-time from the monoexponential curve fitting of 3–4 experiments gave the value (Mean ± S.E.) of 165 ± 15 s for BACE and 330 ± 25 s for chlorin e6. In control experiments, practically no change of the I_E/I_M ratio was observed after similar

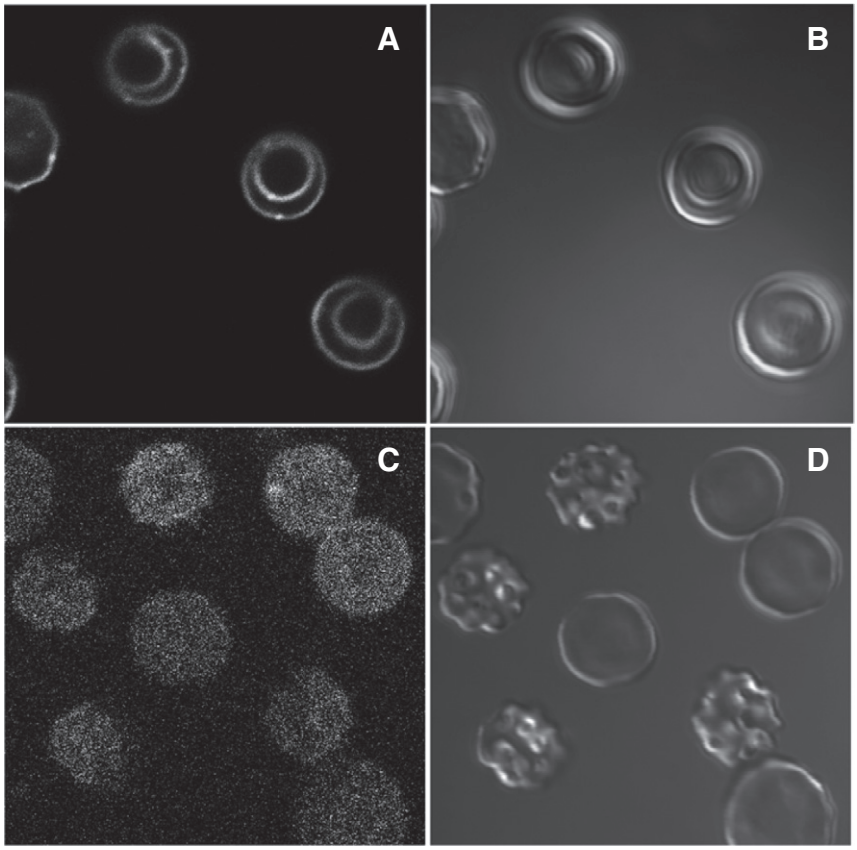

Fig. 5. Intracellular distribution of BACE (A) and chlorin e6 (B) in mouse red blood cells (left two panels) and corresponding transmission light images (C, D). RBCs were incubated with 1 μM of the dyes for 5 min, washed with PBS and imaged.

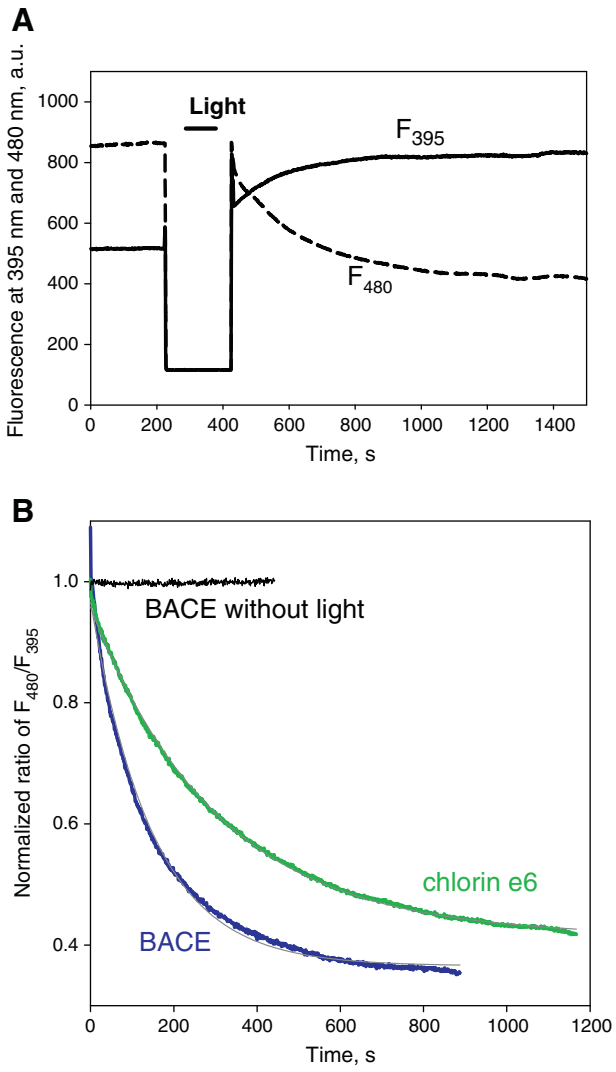

Fig. 6. Lipid flip-flop sensitized by BACE and chlorin e6 as evidenced by the transbilayer diffusion of pyPC in liposomes induced by photodynamic treatment. Panel A shows the recordings of fluorescence of pyPC at 395 nm (solid line) and 480 nm (dashed line). Panel B shows the recordings of the ratio of pyPC fluorescence at 480 nm and 395 nm induced by 1-min exposure to visible light in the presence of 0.5 μM BACE (blue curve), and 0.5 μM chlorin e6 (green curve). Gray curves are monoexponential fits with characteristic times 147 s (BACE) and 305 s (chlorin e6). Solution was 100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4. pyPC concentration was 5 μM. Lipid concentration was 50 μg/ml.

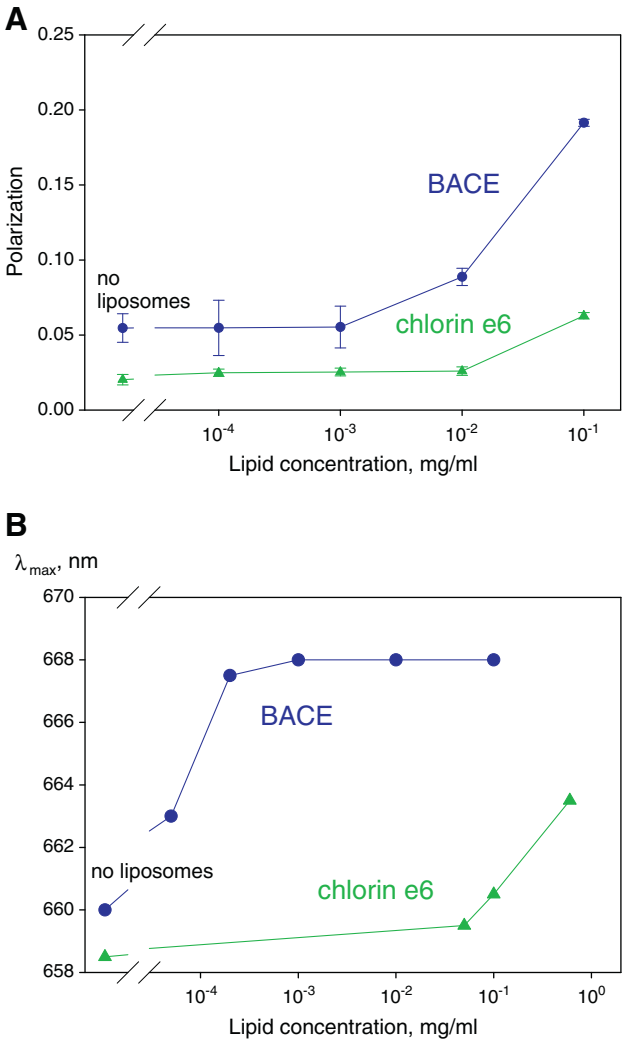

Fig. 7. Binding of BACE and chlorin e6 to liposomes made from EggPC. A. Dependence of the degree of fluorescence polarization of BACE and chlorin e6 on lipid concentration. Emission at 670 nm, and excitation at 650 nm. B. Dependence of the bathochromic shift of the fluorescence maxima of BACE and chlorin e6 on lipid concentration. Excitation at 407 nm.

treatment of liposomes formed from the saturated lipid DPhPC (data not shown).

3.7. Binding of photosensitizers to liposomes

To examine the relationship between photodynamic activity and photosensitizer binding to liposomes, we performed fluorescence polarization and spectral measurements. As seen from Fig. 7A, interaction of BACE with EggPC liposomes led to a larger increase in the degree of fluorescence polarization than that of chlorin e6, favoring a higher membrane affinity of BACE compared to chlorin e6. This conclusion was also supported by a significant difference in the magnitudes of the bathochromic shift of the fluorescence maxima observed upon interaction of BACE and chlorin e6 (cf. [39]) with EggPC liposomes (Fig. 7B). It is worth noting that BACE fluorescence intensity depended non-monotonously on liposome concentration, namely: the addition of liposomes at a low concentration led to quenching of BACE fluorescence, which was followed by a fluorescence increase at higher liposome concentrations (Fig. 8). A similar pattern of the concentration dependence was observed previously for the interaction of cationic aluminum phthalocyanine with liposomes [40].

3.8. Photosensitized inactivation of gramicidin channels in planar bilayers

The photodynamic activity of photosensitizers in BLM can be also evaluated by a very convenient method developed in our laboratory, which consists in measuring damage to the membrane-active peptide gramicidin A [31,41,42] that is known to form ionic channels selective to alkaline metal cations. Illumination of planar BLM with visible light in the presence of a photosensitizer is known to suppress the gramicidin-mediated transmembrane current [32,31,41,43]. The photoinactivation of gramicidin A results from the damage to its tryptophan residues caused by reactive oxygen species that are generated upon interaction of excited photosensitizer molecules with oxygen [32,31,44,45]. It has been shown that the light-induced decrease in the gramicidin-mediated current is due to the reduction of the number of open channels, while the single channel conductance remains unaltered [32]. Therefore, the relative decrease in the current, $\alpha = (I_0 - I_\infty) / I_0$, induced by illumination is equal to the damaged portion of gramicidin channels. This parameter enables one to compare the efficacy of different photosensitizers. As seen

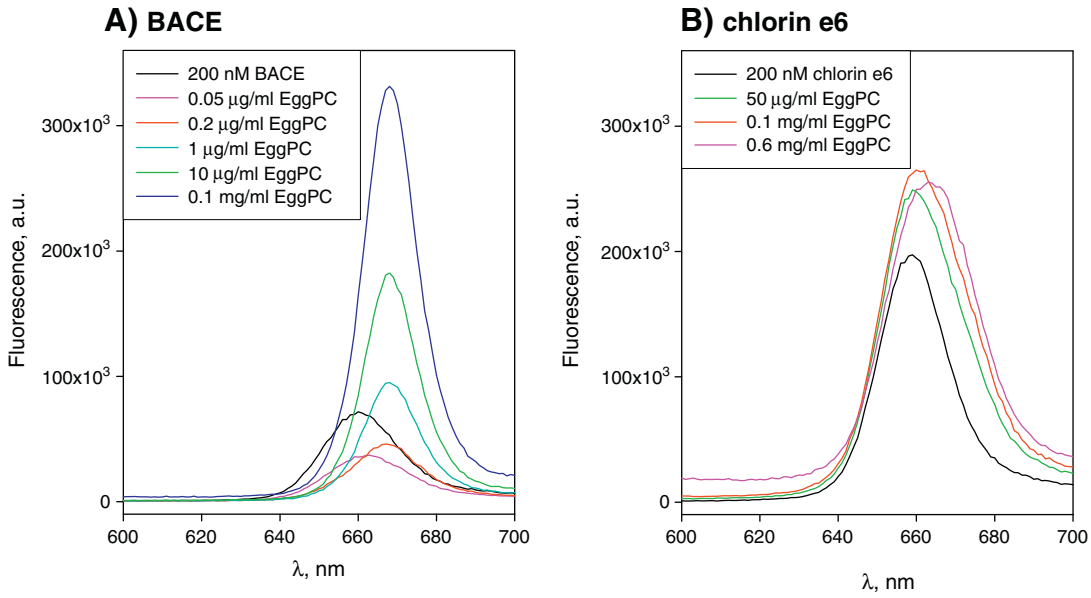

Fig. 8. Fluorescence spectra of BACE (A) and chlorin e6 (B) in the presence of different concentrations of liposomes made from EggPC. Excitation at 407 nm.

from Fig. 9A, exposure of planar BLM containing gramicidin channels to a flash of visible light in the presence of BACE led to a decrease in the gramicidin-mediated transmembrane current of potassium ions, in agreement with the data obtained previously with different substituted aluminum and zinc phthalocyanines [32,31,41,40,46–48]. Fig. 9B shows the dependence of gramicidin photodamage on the concentration of BACE and chlorin e6 in BLM formed from DPhPC, a saturated lipid not susceptible itself to photodynamic action. In agreement with the results of the liposome experiments (Fig. 6), BACE was also more effective than chlorin e6 in the gramicidin assay. Noteworthy, in this system the difference between BACE and chlorin e6 activity was rather modest and became indiscernible at low concentrations of the photosensitizers, in contrast to a marked difference between efficacies of these agents in promoting damage to unsaturated lipids as measured by photosensitized leakage of fluorescent dyes from liposomes [18]. This result could be associated with the dependence of the photosensitizing efficiency on the porphyrin depth in a lipid bilayer [49–51] and the relative location of target and photosensitizer molecules [52–54]. It could be suggested that BACE, being more hydrophobic than chlorin e6, resides deeper in a membrane and thus closer to the location of lipid double bonds, but further from gramicidin tryptophan residues tending to localize at water–hydrocarbon core interface [55]. The position of chlorin e6 in close proximity to tryptophans might explain its rather high efficacy in sensitizing gramicidin photoinactivation.

4. Discussion

In our previous study we demonstrated a remarkable potency of the boronated chlorin e_6 derivative BACE as an antitumor photosensitizer [18]. It was suggested that photoactivation of BACE triggered intracellular ROS generation followed by rapid (within the initial minutes after illumination) damage of multiple membrane organelles, leading to cell necrosis. The membrane photodamage and necrosis required nanomolar-to-submicromolar concentrations of the novel photosensitizer. The mechanism of the high photodynamic activity of BACE remained unclear. According to our data, the quantum yield of singlet oxygen generation of BACE was close to that of chlorin e6, whereas the electric current relaxation experiments revealed the remarkably high ability of BACE to permeate through the BLM. Therefore, membrane penetrativity was suggested to account for the enhanced photodynamic activity of BACE [18], although

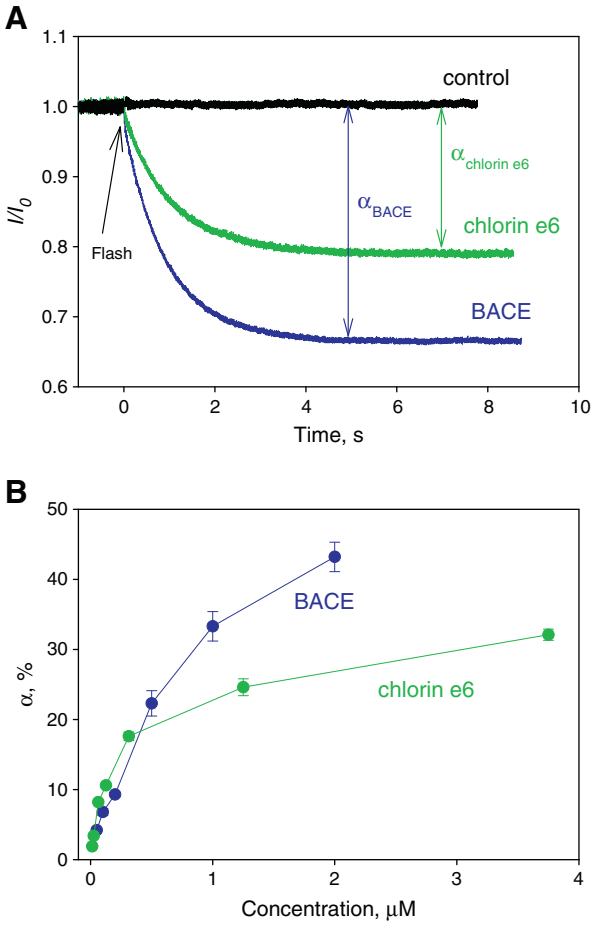

Fig. 9. Sensitized photoinactivation of gramicidin A channels in BLM formed from DPhPC in the presence of BACE and chlorin e6. A. The time course of the decrease in the gramicidin-mediated current (I) with respect to initial current (I_0) across a planar lipid membrane as a result of illumination with a flash of visible light (marked by an arrow). Black curve was a control without a photosensitizer, blue curve was obtained with 0.5 μM BACE, and green curve with 0.5 μM chlorin e6. B. Concentration dependence of the decrease in the gramicidin-mediated current across a planar lipid membrane as a result of photodynamic action. The solution was 100 mM KCl, 10 mM Tris, pH 7.4.

the relaxation technique did not allow us to monitor membrane translocation of unsubstituted chlorin e6. Noteworthy, rather diverse data on the ability of chlorins to cross lipid membranes were obtained in different laboratories [39,56–59]. According to earlier studies, methyl esters of chlorin e6 and bacteriochlorin exhibit higher affinity to liposomes [35] and erythrocytes [36,37], as compared to the parent compounds, which may be associated with an increase in the membrane/water partition coefficient upon blocking carboxylic groups by methylation. However, an increase in hydrophobicity must not obligatorily lead to enhancement of photodynamic efficacy, because it can reduce water solubility, and thereby decrease accessibility for cells. In our experiments, the increased accumulation in cells (Fig. 4) and higher colocalization with the plasma membrane (Fig. 5) were observed for BACE, as compared to chlorin e6, in erythrocytes. These results are consistent with the idea that necrosis being characterized by the loss of plasma membrane integrity represents the predominant mechanism of BACE-mediated photodynamic cell killing. Membrane permeabilization that underlies this mechanism most probably results from the light-induced peroxidation of unsaturated lipids.

In the present study we scanned the photodynamic efficacy of BACE with respect to that of unsubstituted chlorin in a series of in vivo and in vitro models, making the emphasis on the importance of photosensitizer–membrane interaction for the photodynamic potency. In all the experiments described, BACE appeared to cause a larger photoeffect than unsubstituted chlorin e6. It can be supposed that in vivo, photosensitized perturbation of different membrane (lipid and protein) components might synergistically result in a profoundly enhanced photodynamic potency of BACE.

The photodynamic efficacy of chlorin e6 in cell culture can be significantly increased by conjugating the photosensitizer with protein vectors [60,61], thereby providing its targeted delivery, e.g., to nuclei [61]. In particular, a modular transporter containing both a receptor-binding ligand and a nuclear localization sequence represents an elegant, though laborious, example of such a construct [62]. The present study demonstrates an alternative way of multiple enhancement of chlorin e6 efficacy based upon linking the photosensitizer to membranotropic boron clusters which switches the primary target of the photodynamic damage from DNA to cell membranes. In this case selectivity of tumor damage could be achieved via directed illumination and preferential accumulation/retention of photosensitizers in tumor tissues due to impaired lymphatic drainage.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.11.012.

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