Confocal microscopy and spectral imaging technique: contribution to the development of neutron sensitizers for anticancer BNCT

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Conjugation of chlorin e6 with boron nanoparticles is an attractive approach to the development of advanced neutronsensitizers for anticancer boron neutron capture therapy (BNCT). Here we report on the properties of chlorin e6 conjugated in different ways with a closo-dodecaborate nanocluster (6, 7), one (1, 2) or two (3, 4, 5) cobalt bis(dicarbollide) nanoparticles. Chlorin e6 fluoresces that allows one to use confocal laser scanning microscopy (CLSM) and confocal spectral imaging technique (CSIT) to study interactions of the conjugates with cancer cells, namely: ability of conjugates to penetrate in cells, intracellular distribution and localization, quantitative characteristics of intracellular accumulation. It is revealed that conjugates 1-7 penetrate in cancer cells and have similar cytoplasmic localization with dominant accumulation in lysosomes. The most drastic and important difference between studied conjugates is a level of their intracellular accumulation. A structure of linker connecting boron nanoparticles with chlorin e6 moiety is the critical structural factor affecting ability of conjugates to deliver boron nanoparticles in cancer cells. It is demonstrated that without activation with neutrons the conjugates are not toxic for cells in a wide range of concentrations. Due to enhanced intracellular accumulation conjugates 4 and 5 are promising for BNCT and fluorescence diagnostics of cancer.

Keywords confocal; fluorescence; chlorin e6; closo-dodecaborate; cobalt bis(dicarbollide); boron neutron capture therapy

1. Introduction

Boron neutron capture therapy (BNCT) is one of the actual approaches to the cancer treatment. BNCT is considered to be most promising for the treatment of tumors localized in the neck and head area (e.g. glioblastoma), as well as for the treatment of melanoma A basis of BNCT is selective accumulation of nonradioactive 10B isotopes in a tumor followed by their activation with thermal or epi-thermal neutrons. Thermal neutrons are captured by 10B and the excited nucleus breaks into two particles,  α  and 7Li, with high linear energy transfer (Fig. 1) and high capability to damage cells [1]. Carbon, hydrogen, nitrogen, oxygen and other elements of biological molecules have very low cross sections of thermal neutron capture and do not interact directly with the neutrons.

![Fig. 1 Scheme of 10B interaction with a neutron](image)

The emitted particles have a short free path in tissue (< 14 μm), that is comparable with cellular diameter, and therefore destroy first of all those cells, where 10B isotopes are located. Evidently, BNCT efficiency depends critically on ability of 10B-bearing compounds (neutronsensitizers) to penetrate and accumulate in cancer cells. There are different biologically active molecules that are considered as probable transporters for 10B isotopes, including amino acids and peptides, carbohydrates, precursors of nucleic acids, acridine derivatives, different polyamines, phthalocyanines and porphyrins [2]. In particular, an idea to use porphyrins is based on the data obtained during development of photosensitizers for photodynamic therapy. It was found that many porphyrin derivatives accumulate in malignant cells and provide efficiently their photoinduced death. As a rule, porphyrins fluoresce in a red region that facilitates considerably in vitro and in vivo analysis of their distribution in cells and tissues.

As some other research groups we are trying to develop porphyrin derivatives highly loaded with 10B and targeted to cancer cells. We use a chlorin e6 derivative as a transporter and conjugate it by means of different linkers with boron nanoclusters and nanoparticles as a cargo (Fig. 2). Confocal spectral imaging technique (CSIT) together with confocal laser scanning microscopy (CLSM) is applied by us to compare cellular properties of conjugates and find the most advanced design. In the present paper we summarize the results obtained for conjugates 1-7 (Fig. 2).
2. Experimental

2.1 Reagents

Cremophor EL (CrEL), Hoechst 33342, propidium iodide (PI), human α- and β-globulins were supplied by Sigma-Aldrich (St. Louis, MO, USA). The essentially fatty acid–free human and bovine serum albumins (HSA, BSA), deoxyribonucleic acid (DNA) from calf thymus and ribonucleic acid (RNA) from yeast were received from MP Biomedicals (USA). Triton X-100 and acridine orange (AO) were purchased from DiaM (Moscow, Russia). Other chemicals were reagent grade and used as received from commercial vendors. Conjugates 1-7 were synthesized and characterized as described elsewhere [3-5]. The purity of the compounds was verified by analytical TLC on Merck Kieselgel 60 F245 plates (chloroform : methanol 25 : 1) and 1H NMR spectroscopy. Concentrated solutions of 1-7 were prepared by chafing the powders in a small quantity of 100% CrEL (polyoxyethylene derivative of hydrogenated castor oil) followed by 20-fold dilution with a sodium phosphate buffer (50 mM, pH 7.0). Concentration of conjugates 1-7 in 5% CrEL was 0.5 mM.

![Fig. 2 Structures of conjugates 1-7](image)

<table>
<thead>
<tr>
<th>R</th>
<th>X^2</th>
<th>n</th>
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<tbody>
<tr>
<td>1</td>
<td>i-Pr2EtNH^+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Me,NH^+</td>
<td></td>
</tr>
<tr>
<td>6, 7</td>
<td>2(Bu4N^+)</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>i-Pr2EtNH^+</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>i-Pr2EtNH^+</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>i-Pr2EtNH^+</td>
<td>6</td>
</tr>
</tbody>
</table>

2.2 Cells and their treatment

A549 human lung adenocarcinoma cells were grown in MEM-Eagle medium supplemented with 2 mM L-glutamine and 8% fetal calf serum (37 °C, 5% CO₂), abbreviated below as a complete medium. To study cellular accumulation and distribution of 1-7 cells were incubated with 4 μM of 1, 2 or 6 for 3 h, 0.5 μM of 3-5 or 7 for 2 h in a complete medium at 37 °C. For the survival assays cells were seeded into 96-well plates. Twenty-four hours later, 1-7 were added gradually into the wells to achieve concentrations ranging from 0.05 to 16 μM with a two-fold increment. The cytotoxicity was determined after a 5 h incubation of cells with 1-7 in the dark. Control cells were incubated with CrEl emulsion at the equivalent concentrations. The phototoxicity was determined on cells incubated with 1-7 in a complete medium for 2 h and irradiated with a 500 W halogen lamp through a 5 cm water filter and a band-pass filter (transmission 640–1000 nm, 10–12 mW cm⁻², 10 J cm⁻²). After irradiation the cells were incubated at standard conditions during 3 h and subjected to examination for viability. For evaluation of cell viability the fluorescent dyes Hoechst 33342 (4 μM) and PI (6 μM) were added to cells for 15 min, and the cells were examined under the inverted fluorescence microscope with a long-working distance 20× objective (Axio Observer, Carl Zeiss AG, Germany). A PI/Hoechst 33342 viability assay is based on: (i) penetration of Hoechst 33342 through plasma membrane and bright staining of DNA in the nuclei of both living and dead cells; (ii) impermeability of plasma membrane for PI in living
cells and bright staining of DNA with PI in the nuclei of dead cells. A filter unit with the 530–585 nm excitation filter, the 600 nm dichroic mirror and the 615 nm barrier emission filter was used to register fluorescent images of dead cells having red PI fluorescence in the nucleus. A filter unit with the 359–371 nm excitation filter, the 390 nm dichroic mirror and the 410 nm barrier emission filter was used to register fluorescent images of all cells due to blue nuclear fluorescence of Hoechst33342. Both types of images were captured from each examined field of view and treated with the Image J software (National Institutes of Health, USA) to count total number of cells and dead cells.

2.3 Confocal microscopic measurements

CSIT [6-10] was used to study microenvironment of 1-7 in cells and to estimate quantitatively intracellular accumulation of 1-7. CSIT was realized with an installation (Fig. 3) on the basis of the OMARS-89 spectrograph (Dilor, France), Olympus BH-2 microscope (Japan), and a motorized scanning stage (Marzhauser- Wetzlar, Germany). An air-Peltier cooled CCD camera (1024×256 pixels, Wright Instruments Ltd, UK) was used as a detection system. Fluorescence excitation was carried out with a continuous Nd$^{3+}$-YAG laser (532 nm, 12 μW). Lateral, axial and spectral resolutions were 0.6 μm, 3 μm and 1 μm, respectively. CSIT is based on measurement of the spectrum at each point of a cell. The 20×20 or 30×30 voxel spectral images were recorded and treated as described previously [6-10]. The concentration of the monomeric fluorescent form of 1-7 within cells was deduced from the integrated intensities of intracellular fluorescence spectra. The quantitative map of 1-7 distribution was calculated for each measured cell and treated with the Image J software to estimate an average cytoplasmic concentration of 1-7. The results were averaged over 30–40 cells for each extracellular concentration of 1-7 used in the experiments. The experiments repeated at least three times. Detailed intracellular distribution and localization of 1-7 were studied with CLSM using a LSM-710 confocal laser scanning microscope (Carl Zeiss AG, Germany). The CLSM images were obtained with a 40× C-Apochromat water-immersion objective (NA = 1.2) at 0.3 μm lateral and 1.5 μm axial resolution. Studying the intracellular distribution of 1-7, the fluorescence was excited with an Ar$^+$-laser (514.5 nm, 20 μW) and emission was registered with the 650-nm long-wavelength barrier filter. Identification of cellular structures accumulating the conjugates was performed with CLSM as described elsewhere [6].

![Fig. 3 A scheme of the experimental installation for CSIT.](image)

3. Results

3.1 Spectral properties and molecular interactions of 1-7 in solution

Conjugates 1-7 dissolve in chloroform, alcohols and dimethylsulfoxide, but are not soluble in water. To use them in cellular studies the conjugates were solubilized with CrEL. CrEL is non-toxic for cells at moderate concentrations and according to our experience is one of the best solubilizers for different chlorin derivatives [6-10]. Absorption and
fluorescence spectra of 1-7 in 1% CrEL are very similar, except for increased molar extinctions of conjugates 2 and 6 (Table 1).

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>λQ/nm*</th>
<th>ε(λQ)×10^{-3}/M^{-1}cm^{-1}</th>
<th>λf/nm</th>
<th>K</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>665</td>
<td>-</td>
<td>670</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2</td>
<td>665</td>
<td>45± 2</td>
<td>670</td>
<td>0.10± 0.05</td>
</tr>
<tr>
<td>3</td>
<td>665</td>
<td>35± 2</td>
<td>670</td>
<td>0.20± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>665</td>
<td>34± 2</td>
<td>670</td>
<td>5± 1</td>
</tr>
<tr>
<td>5</td>
<td>665</td>
<td>32± 2</td>
<td>670</td>
<td>9± 1</td>
</tr>
<tr>
<td>6</td>
<td>665</td>
<td>45± 2</td>
<td>670</td>
<td>0.15± 0.05</td>
</tr>
<tr>
<td>7</td>
<td>665</td>
<td>33± 2</td>
<td>670</td>
<td>1.5± 0.2</td>
</tr>
</tbody>
</table>

*λQ is a Q-band absorption maximum in 1% aqueous emulsion of CrEL. ε(λQ) is a molar extinction at the λQ wavelength. λf is a maximum of fluorescence emission in 1% aqueous emulsion of CrEL. K is a distribution factor, i.e. a ratio of the average cytoplasmic concentration of conjugate 1-7 to its extracellular concentration.

To understand deeper the properties of 1-7 that can affect transport of the conjugates in biological systems, their penetration into cells and intracellular localization we have studied interactions of the conjugates with particular proteins (BSA, HSA, α-β- and γ-globulins), nucleic acids (DNA, RNA) and membrane mimetic systems (emulsion of CrEL, micelles of Triton X-100). These studies were performed using absorption and fluorescence spectroscopy. The main results are summarized briefly below, whereas extended set of data concerning molecular interactions of 4 and 7 can be found elsewhere [6]. The conjugates 1-7 do not bind to DNA and RNA. They interact with proteins weakly, and these interactions are traceable (if any) at very high concentration of proteins (4-10 g l^{-1}). The conjugates incorporate readily in Triton X-100 micelles and CrEL emulsion, and their fluorescence spectra do not change, when pH varies from 4 to 8. One can conclude that the binding to lipid structures and membranes is a main type of interaction of the conjugates in biological environment.

3.2 Intracellular accumulation and distribution of 1-7

Using CLSM it was revealed that 1-7 penetrated in A549 human lung adenocarcinoma cells and accumulated in cytoplasm, but intracellular fluorescence of 1-7 was very different in intensity. The features of cytoplasmic distribution of 1-7 were similar, namely, diffuse staining of cytoplasm complemented with enhanced accumulation in submicron vesicles (Fig. 4). Conjugates 1-7 did not penetrate in a cell nucleus. Analyzing co-localization of the conjugates and fluorescent markers of different cellular organelles the vesicles accumulating 1-7 were identified as lysosomes. Typical CLSM images proving co-localization of AO, a fluorescent marker of lysosomes, and conjugate 5 accumulated in vesicles are shown in Fig. 5.

As analyzed with CSIT the intracellular fluorescence spectra of conjugates 1-7 have the same shape and maximum (670 nm) in every point of cytoplasm including lysosomes. The intracellular spectra coincide exactly in the shape and maximum with the fluorescence spectra of the conjugates in CrEL emulsion (Fig. 6). Therefore, we can conclude that conjugates 1-7 are situated in cells in a lipid microenvironment, apparently in lysosomal membranes and cytoplasmic membrane structures.

Combining data for molecular interactions of the conjugates with cellular CSIT studies we get a possibility to estimate quantitatively intracellular accumulation of 1-7. Having a model system (CrEL emulsion) that reproduces an influence of cellular microenvironment on fluorescence spectra of conjugates 1-7, fixing all the essential experimental parameters and measuring the fluorescence spectrum in a confocal mode from equal microvolumes in both cells and model solution, we can calibrate dependence of fluorescence intensity on the conjugate concentration. Using such calibration the confocal images describing intracellular distribution of conjugate fluorescence intensity can be recalculated into concentration maps (Fig. 4). Analysis of these maps shows that the concentration of conjugates in lysosomes is usually 4-5 fold higher than their concentration in surrounding cytoplasm. Besides, it is clearly observed that intracellular concentration of the conjugates is very different (Fig. 43).

To compare quantitatively ability of conjugates to penetrate in cells, the distribution factor K was calculated. K is a ratio of the average cytoplasmic concentration of a conjugate to its extracellular concentration within a linear range of the concentration dependence of intracellular accumulation of the conjugate. Intracellular accumulation of 1-3 and 6 is weak (K = 0.1÷0.2). Conjugate 7 is characterized by moderate K value. Conjugates 4 and 5 are the most potent in intracellular accumulation (Table 1).
Fig. 4 Intracellular distributions of 1 (a, b), 2 (c, d), 3 (e, f), 4 (g, h), 5 (i, j), 6 (k, l) and 7 (m, n) in A549 cells. (a, c, e, g, i, k, m)-confocal fluorescence images describing the subcellular distribution of 1-7. (b, d, f, h, j, l, n)-conventional light-microscope images of the cells. N marks the nucleus; bar represents 5 μm. Brightness scales show the concentration of conjugates 1-7 in μM. Cells were incubated with 1, 2 and 6 (4 μM) for 3 h or with 3-5, 7 (0.5 μM) for 2 h.

Cellular experiments were performed both in the presence of 8% fetal calf serum and without it. It was revealed that the serum does not affect intracellular localization and accumulation of the conjugates including their distribution factors. Therefore, serum components are not involved in the interactions of 1-7 with cells.

Fig. 5 Staining of lysosomes with AO in living A549 cells loaded with 5. A typical cell is shown. (a) A conventional light microscope image of the cell. N indicates the nucleus. Bar represents 5 μm. (c, d) CLSM images describing intracellular distribution of 5 (c) and AO (d). (b) Overlap of images showing intracellular distribution of 5 (red) and AO (green). Yellow color indicates co-localization of 5 and AO.
Without irradiation conjugates 1-7 were found to be not toxic for A549 cells. Cytotoxicity was evaluated when the conjugate concentration in a culture medium was below 8 μM (1) or 16 μM (2-7), and incubation time was 5 h.

Chlorin e₆ is a well known photosensitizer. Accordingly, photoinduced cytotoxicity of the conjugates 1-7 was studied to characterize their photosensitizing properties. The irradiation of A549 cells with light after incubation with conjugate 1 (0.5-8 μM) or conjugates 2-6 (0.5–16 μM) for 3 h does not result in cell death. Concentration-dependent photodynamic effect was observed for conjugate 7 only. As reported by us earlier [6], conjugate 7 caused photoinduced cell death at micromolar concentrations (LD₅₀=2.1 μM).

### 4 Discussion

In the present study we have compared seven conjugates of the chlorin e₆ derivative with either a closo-dodecaborate nanocluster ((B₃H₁₂)₃⁺; conjugates 6, 7) or cobalt bis(dicarbollide) nanoparticle(s) ([3,3′-Co(1,2-C₂B₉H₁₁)₂]; conjugates 1-5). An additional variable was a structure of linker connecting both parts of a conjugate (Fig. 2). Our aim was to understand how structural features affected properties of these conjugates, especially their ability to accumulate in cancer cells, and to find the best design.

We found that linkers and boron cargo induced no changes in spectral properties of the conjugates such as shape and maxima of absorption and fluorescence spectra. The spectral properties were defined by chlorin e₆ chromophore solely. Differences in a conjugate structure did not become apparent in interactions with DNA, RNA, studied proteins and lipids. All the conjugates have dominating affinity to lipid structures due to their hydrophobicity. Charged boron nanoparticles and linkers changes in the conjugate structure. Our experience in the study of lipophilic chlorin derivatives testifies that a structure, polarity and a charge of substituent groups determine intracellular accumulation. Boron nanoparticles and linkers connecting them with the chlorin moiety play a role of such substituent groups in a case of conjugates 1-7. Considering a factor of charge one can conclude that mono- vs. di-anionic character of the conjugates do not explain the differences in K-values (compare 2 and 6, 4 and 7). Here a structure of linker is most critical. Flexible linkers containing a poly-alkyl fragment are more profitable than rigid linkers containing aromatic cycles. Moreover, as demonstrated with 3-5 an increase in a length of the poly-alkyl fragment improves considerably intracellular penetration of the conjugates (Fig. 4, Table 1).

The obtained results indicate that cobalt bis(dicarbollide) suits for boron intracellular delivery much better than closo-dodecaborate. First, conjugate 4 with cobalt bis(dicarbollide) has higher intracellular accumulation than the most similar in structure conjugate 7 with closo-dodecaborate. Second, a conjugate bears two nanoparticles of cobalt bis(dicarbollide) but only one nanocluster of closo-dodecaborate. Third, cobalt bis(dicarbollide) contains 18 boron atoms, whereas closo-dodecaborate contains just 12 boron atoms. Accordingly, when extracellular conjugate concentration is 1.5 μM the average cytoplasmic concentration of boron atoms achieves 270, 486 and 27 μM for conjugates 4, 5 and 7, respectively. Assuming that a cell volume is 1.7×10⁻⁷ μl and a nucleus volume is 35% of the cell volume, one can estimate that conjugates 4, 5 and 7 deliver ca. 2×10⁵, 3.6×10⁵ and 2×10⁴ boron atoms per cell, respectively. Theoretically, ca. 10⁵ boron atoms per cell are required for efficient BNCT, and conjugates 4 and 5 meet such requirement. Importantly, considerable accumulation of 4 and 5 in cells is not accompanied with cytotoxicity, and only local irradiation of a tumor with neutrons will produce selective antitumor effect.

Fig. 6 Typical intracellular fluorescence spectra of 5 (dotted line; (a)- in lysosomes, (b) – in cytoplasm) and their description with a model spectrum of 5 in 1% CrEL (solid line).
Usually, chlorin containing conjugates possess photoinduced cytotoxicity as, for example, conjugate 7 does [6]. But it is not a case for chlorin conjugates bearing two cobalt bis(dicarbollide) nanoparticles. Conjugates 4, 5 and 7 have high quantum yields of singlet oxygen generation (0.55 and 0.85 for 4 and 7 in solution, respectively [6]), identical intracellular localization and similar lipid-like microenvironment. Intracellular accumulation of conjugates 4 and 5 is higher than that of conjugate 7. Nevertheless, photoinduced cytotoxicity is not a property of 4 and 5. A presence of cobalt complexes in conjugates 4 and 5 is supposed to be a reason of the observed antioxidative effect in cellular environment [6]. A mechanism of the antioxidative effect is not clear and needs further study. It is probably associated with quenching of free radicals or reactive oxygen species other than singlet oxygen [6].

On the one hand, absence of photoinduced cytotoxicity excludes application of conjugates 4 and 5 to photodynamic therapy. On the other hand, biological properties of conjugates 4 and 5 such as their accumulation, distribution and retention in cells and tissues can be followed with fluorescence techniques without concomitant photoinduced toxicity. Enhanced accumulation in cancer cells and intrinsic fluorescence make conjugates 4 and 5 suitable for fluorescent diagnostics of cancer and fluorescent navigation over an operative field in anticancer surgery.

In conclusion, among studied conjugates (Fig. 2) conjugate 5 is the most promising neutron sensitizer for BNCT. Moreover, it can be also considered as a safe contrast agent for fluorescence navigation and diagnostics of cancer.

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References


