



Photocytotoxicity of a cyanine dye with two chromophores toward melanoma and normal cells

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ABSTRACT

Background: Due to high optical absorption, triplet quantum yield and affinity to biological structures bichromophoric cyanine dyes (BCDs) can be considered promising sensitizers for application in photodynamic therapy (PDT). In this work, we report on the study of the BCD photocytotoxicity toward melanoma and normal cells in comparison with that of commercial photosensitizer Photogem®.

Methods: The cytotoxic and phototoxic effects were measured by standard tests of cell viability. The drug uptake was obtained by the flow cytometry and optical absorption techniques. The BCD intracellular distribution was obtained by the fluorescence image microscopy using specific organelle markers.

Results: Both drugs demonstrated increased cytotoxicity under irradiation, while in darkness their cytotoxic effect at concentrations lower than 20 μM after 24 h of incubation did not exceed 20%. For 5 h of incubation, BCD photocytotoxicity in relation to melanoma cells reached 100% already at concentrations below 5 μM , while for normal cells the effect did not exceed 70% even for the 20 μM concentration. It is shown that BCD penetrates into the cells and is located predominantly in perinuclear cytoplasmic structures.

Conclusions: The BCD photosensitizing characteristics appear more adequate for application in PDT than that of the actually applied commercial photosensitizer Photogem®. Higher light absorption by BCD in the near IR region and its preferential localization in mitochondria can explain its high photocytotoxicity.

General significance: BCD can be considered as a new promising photosensitizer class for cancer PDT.

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1. Introduction

Cyanine dyes (CDs) are widely applied in biology as fluorescence probes due to their high optical absorption, intensive fluorescence in the visible region of the spectrum and high affinity to cell structures [1, 2]. CD absorption spectrum can easily be shifted to red and near infrared spectral region by increasing π -conjugated chromophore chain length. This stimulates the interest on the use of CD as photosensitizers (PS) in photodynamic therapy of cancer (PDT) and as fluorescence probes in cancer fluorescence diagnostics (PDD) [3–6]. Unfortunately, a significant increase of the π -conjugated chromophore chain length necessary to shift the absorption to desirable spectrum region increases the flexibility of the chain, thus reducing the fluorescence and triplet state quantum yields and reducing consequently the photochemical activity of the

molecule. Moreover, photoisomerization, characteristic for CD [7–10] opens a channel for the excitation energy dissipation, reducing excited state lifetimes and quantum yields even more and thus reducing the CD efficacy in both PDT and PDD.

The class of cyanine dyes with two chromophores linked by a central heterocycle (biscyanine dyes, BCDs) is free from these disadvantages because the red shift of their absorption spectra is achieved due to dipole–dipole chromophore interaction and electron tunneling through the central heterocycle [11,12] rather than due to the increase of chromophore chain lengths. BCD possesses high molar absorption coefficients ($\epsilon > 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ in the spectral range of 600–700 nm), high affinity to cell membranes and biomolecules [13] and high triplet quantum yields [14,15]. This stimulates our interest to study BCD photocytotoxicity toward malignant cell cultures, taking into account their possible application as PS in PDT.

In this work, we report on the study of the BCD photocytotoxicity effect on B16F10 (mouse) and C8161 (human) melanoma cell lines, compared to a normal cell line (PBMC) as a function of the dye concentration,

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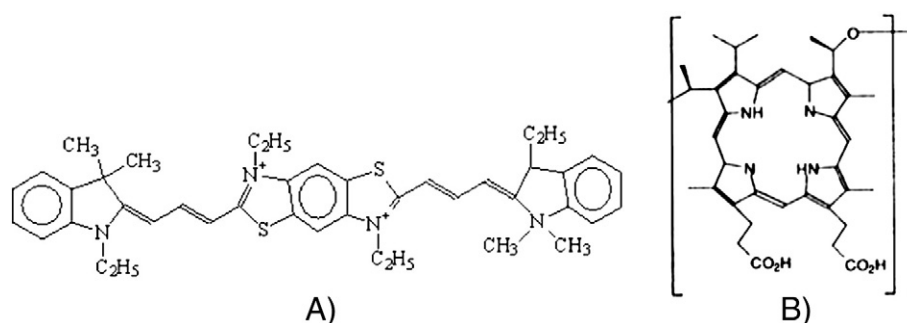


Fig. 1. (A) BCD and (B) Photogem® molecular structures.

incubation time and irradiation dose. The BCD photocytotoxicity was compared to Photogem®, a Russian Photofrin® analogue PS used in clinics for cancer treatment by PDT.

2. Materials and methods

2.1. Photosensitizers

Benzo[1,2-d:4,5-d']bisthiazolium,2,6-bis[3-(1,3-dihydro-1-ethyl,3,3-dimethyl-2H-indol-2-ylidene)-1-propenyl]-3,7-diethyl (Fig. 1A), the bichromophoric cyanine dye, is one of a series of BCD synthesized by Dr. Felix Mikhailenko in the Institute of Organic Chemistry of the Academy of Science of Ukraine. BCD was purified by Dr. Mikhailenko, and the purity

degree was controlled by High Pressure Liquid Chromatography, optical absorption and fluorescence spectroscopy methods. The specialities of BCD synthesis and purification are presented in [16]. Photogem® (Fig. 1B) was obtained from PHOTOGEN LLC (Moscow, Russia) and used without additional purification.

BCD and Photogem® stock solutions were prepared in ethanol and water, respectively, concentrations being controlled by optical absorption using the absorption molar coefficients $\epsilon = 1.33 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 632 \text{ nm}$ for BCD [13] and $\epsilon = 1.17 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 630 \text{ nm}$ for Photogem® [17].

Sodium dodecyl sulfate (SDS) was used to measure the BCD absorption spectra at its interaction with micelles as the simplified membrane model. SDS concentration used was 20 mM, which is twice higher than

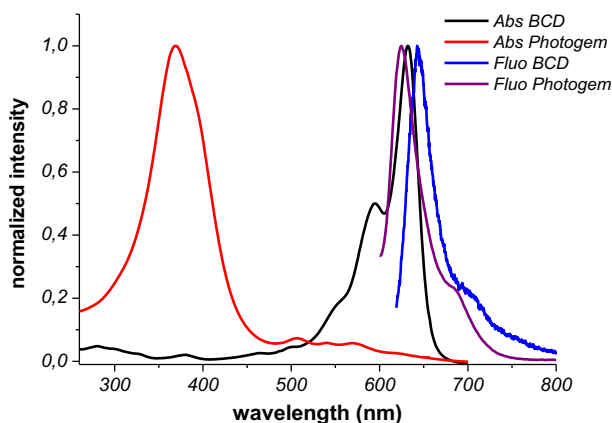


Fig. 2. Normalized absorption and fluorescence spectra of BCD and Photogem® in PBS.

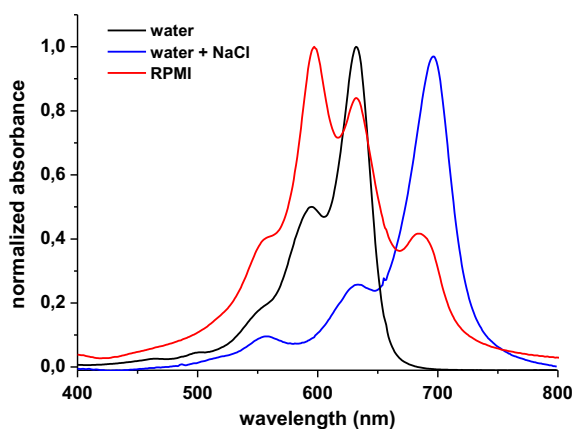


Fig. 3. Normalized absorption spectra of [BCD] = 3.5 μM in an aqueous solution; in the presence of [NaCl] = 2 M and in the RPMI medium.

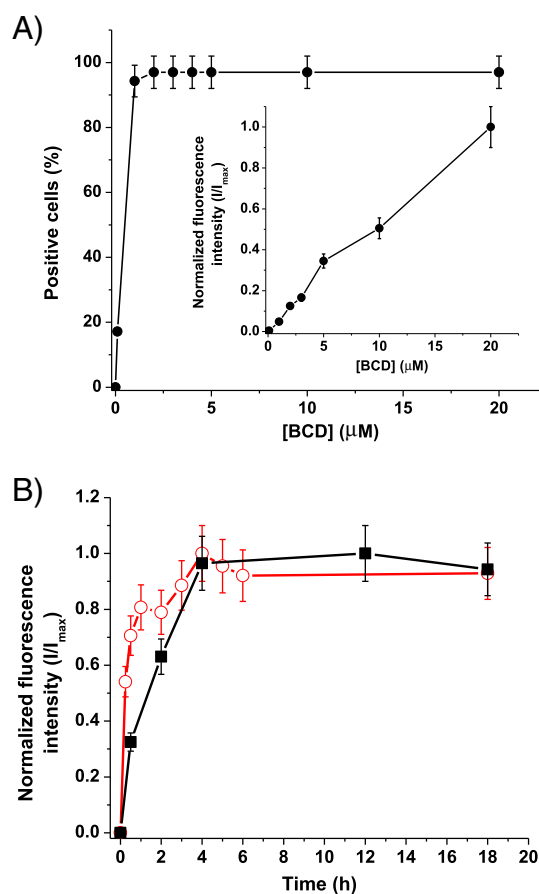


Fig. 4. Cellular uptake of BCD in B16F10 cells. (A) Concentration dependence on the BCD internalization after 6 h of incubation obtained by flow cytometry. Inset: Dependence of integral fluorescence intensity in positive cells on BCD concentration. (B) Comparison between BCD uptake determined by (○) flow cytometry, [BCD] = 2 μM and (■) optical absorption, as a function of time.

its critical micelle concentration (c.m.c). SDS was obtained from Sigma Co. and used without additional purification.

2.2. Cell culture conditions

B16F10 murine melanoma and human melanoma (C8161) cell lines were originally obtained from American Type Culture Collection (Rockville, MD). Peripheral blood mononuclear cells (PBMC) were extracted from healthy human volunteers by centrifugation in density gradient using the Ficoll–Hypaque technique (Sigma Diagnostics, Inc., Missouri, USA).

All cell lines were allowed to grow to confluence in RPMI 1640 medium with phenol red and supplemented with 10% FCS, glutamine and antibiotics, at 37 °C in 5% CO₂, 95% air, and a humidified atmosphere. All reagents were obtained from Gibco–Invitrogen, Carlsbad, CA.

The adherent cells were washed 3 times with PBS and subcultured by dispersal with trypsin–EDTA (2.5 g/L) (Sigma–Aldrich, St. Louis, Mo, USA), centrifuged at 1000 rpm for 15 min at 10 °C in complete medium and seeded. Cells were placed in 96-well plates with 0.2 mL of culture medium per well at a density of 5×10^4 cells per well. After 24 h of culture, the medium was removed and fresh RPMI with different concentrations of BCD formulations (1–20 μ M) was added to the wells (stock solutions of sensitizers (5 mg/mL) were prepared in ethanol (BCD) and PBS (Photogem®) and dilutions were performed in RPMI).

Cells were incubated and then the sensitizer solutions were removed before a fresh complete medium was added. Since the absorption spectrum of phenol red typically presented in RPMI 1640 medium coincides with BCD spectrum, in these experiments, we used culture medium free of phenol red.

2.3. Cellular uptake

- Flow cytometry:** the cellular uptake of BCD in B16F10 cells was performed using a fluorescence-activated cell scanner (BD FACSCanto, Becton Dickinson) equipped with an argon ion laser providing excitation at 488 nm. Cells were incubated with BCD in the dark from 15 min to 18 h and then washed and resuspended in PBS. Cell suspensions were excited and fluorescence signal of drug uptake was detected using a 670 nm long-pass filter. The analysis was performed using FACSDiva software. Each analyzed sample contained a minimum of 10^4 cells.
- Optical absorption:** 1×10^6 cells were seeded onto 50 cm³ flask and allowed to grow for 24 h prior to treatment with BCD at the concentration of 50 μ M in complete culture medium. Cells were incubated with BCD in the dark from 30 min to 18 h and then washed 5 times with PBS and resuspended in 5 mL of NaOH 0.1 M solution containing SDS 1%. After each interval of incubation, the absorbance of the sample was recorded and the resulting concentration was obtained with a calibration curve of the PS in a cell lysate SDS solution (procedure modified from [18]).

2.4. Cellular uptake and intracellular localization by fluorescence imaging microscopy

Experiments were carried out with a Leica Microsystem DM6000B microscope. Specific organelles staining probes were used. Mitochondria were stained with both Mitotracker Green™ (Molecular Probe) and Rhodamine 123 (Sigma Chemical Co.). 4',6-Diamino-2-phenylindole (DAPI)

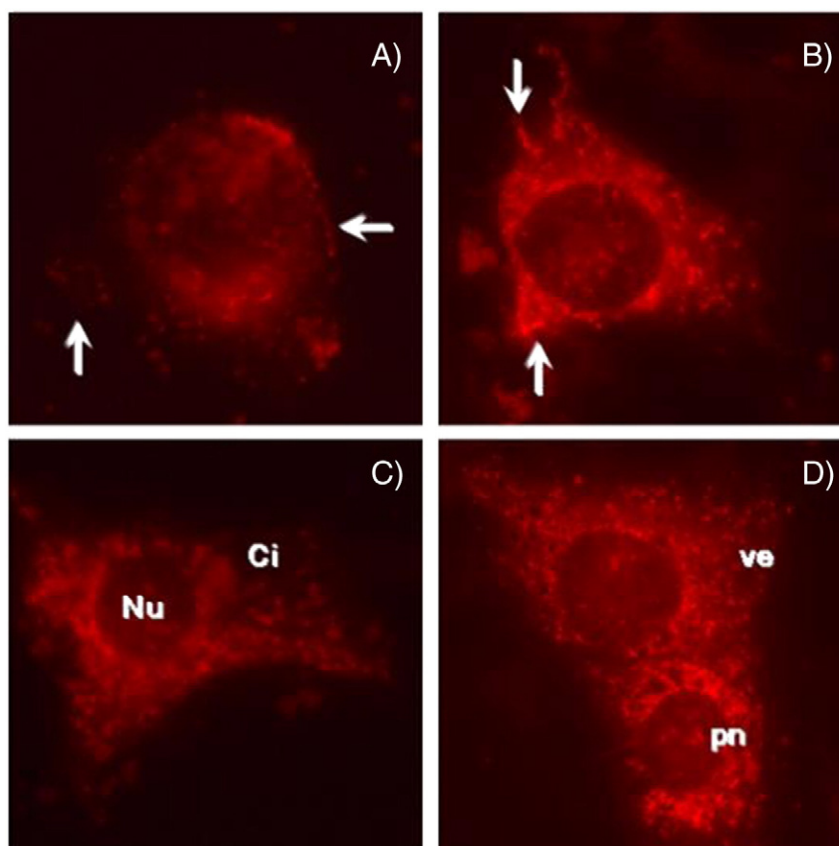


Fig. 5. BCD cell uptake and intracellular localization in B16F10 cells obtained with a Leica Microsystem DM6000B Fluorescence Microscope with an N2.1 filter. Cells were treated with BCD and incubated during (A) 5 min, (B) 15 min, (C) 30 min and (D) 120 min.

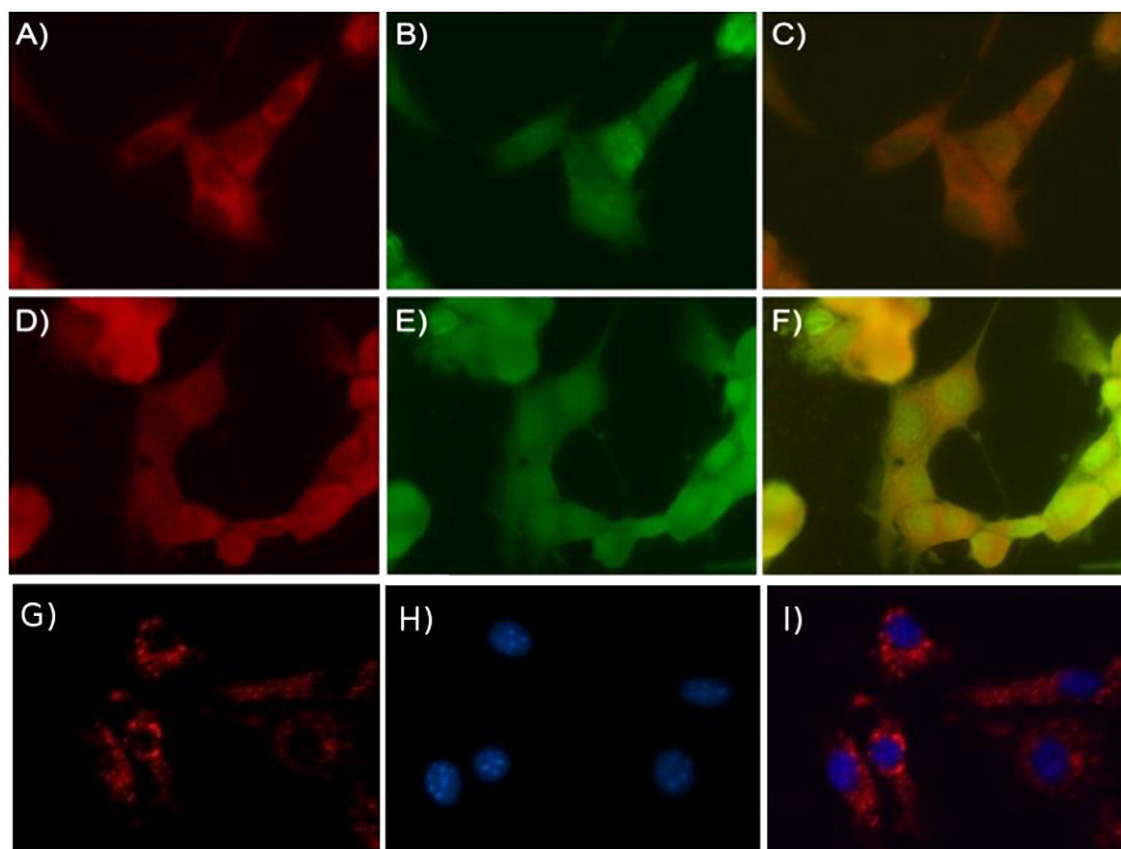


Fig. 6. B16F10 cells after incubation with: (A), (D) and (G) BCD (120 min); (B) Rhodamine 123 (30 min); (C) superposition of A and B; (E) Mitotracker Green™ (30 min); (F) superposition of D and E; (H) DAPI (30 min) and (I) superposition of G and H.

(Molecular Probe) was used as a nuclei probe. A filter set N2.1 (BP 515–560 nm, FT 580 nm, LP 590 nm) was used for BCD; L5 (BP 480 nm, FT 505 nm, LP 527 nm) for Mitotracker Green™ and Rhodamine 123, and filter set A for DAPI (BP 340–380 nm, FT 400 nm, LP 425 nm). In all fluorescence imaging experiments, cells were grown in 96-well culture plates and were washed after incubation and prior to measurements.

2.5. Photocytotoxicity assay

Irradiations were performed using the standard light source with a halogen lamp (250 W) equipped with a color 3-69 KOPP glass filter with the light transmittance range $\lambda > 500$ nm (Fig. S1 in Supplementary data).

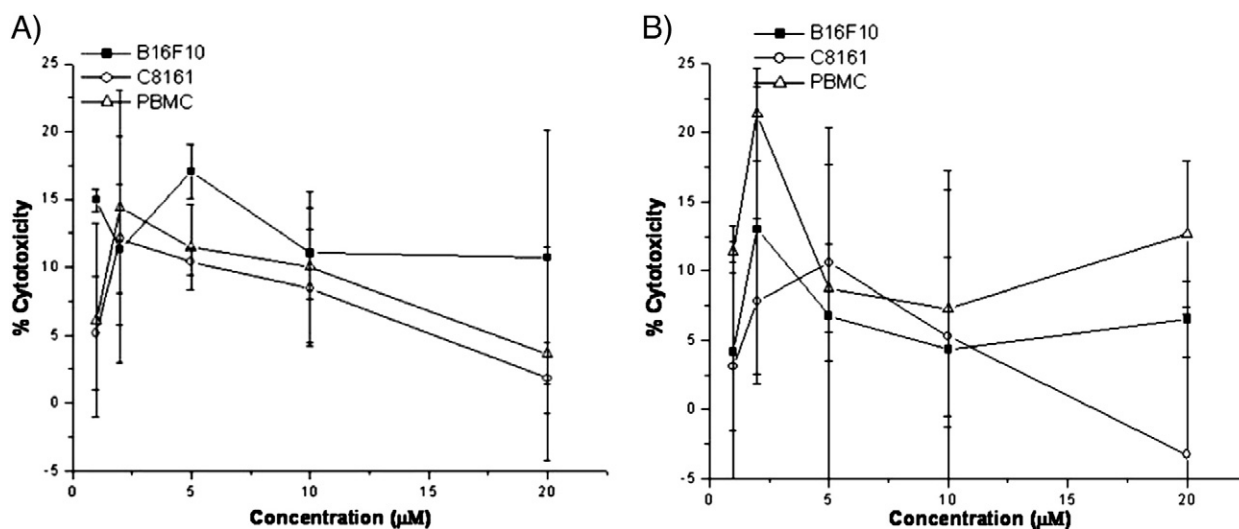


Fig. 7. Dark cytotoxic effect (CTE) of BCD (A) and Photogem® (B) after 24 h of incubation as a function of drug concentration in B16F10-murine melanoma cells; C8161-human melanoma cells and PBMC-human peripheral blood mononuclear cells.

Irradiation intensity at the plate position was 11 mW/cm² measured by a Spectra-Physics 407A radiometer and irradiation times were adjusted in order to obtain 10, 20, 30, 40, 50, 60 and 70 J/cm² fluences. In all experiments controls were as follows: wells containing cells treated with photosensitizer but not exposed to light, wells containing cells without photosensitizer and without light, and wells containing cells without photosensitizer and exposed to light. The cells with and without PS (control) were irradiated directly in the culture plates, under sterile conditions. After irradiation, all cells were incubated for 24 h at 37 °C in 5% CO₂, 95% air and a humidified atmosphere.

Cell viability was measured by determination of mitochondrial activity using the 3-(4,5-dimethylthiazol-2-yl) diphenyltetrazolium bromide (MTT) assay, according to the method described by Mosmann [19]. As such, the culture medium was replaced and 100 µL of MTT (0.5 mg/mL) was added to each well, and then the cells were incubated for a further 3 h at 37 °C. Subsequently, the formed formazan crystals were dissolved in DMSO (200 µL/well) for 2 h. The absorbance of each well was measured at 570 nm using a µQuant ELISA microplate reader (BioTek Instruments, Winooski, VT). The cytotoxic effect (CTE) was calculated as follows:

$$CTE(\%) = \left(1 - \frac{\text{absorbance treated cells}}{\text{absorbance untreated cells}}\right) \times 100.$$

BCD and Photogem® 50% lethal concentrations (LC₅₀) were determined from CTE dependence on PS concentration at a fixed irradiation dose of 40 J/cm² for B16-F10, C8161 and PBMC cell lines.

3. Statistical analysis

The results are given as an arithmetic mean value ± standard deviation ($\bar{X} \pm S.D.$) of 6 independent experiments. Statistical comparisons were made using Student's t-test with $P < 0.05$ as the minimal level of significance using Prism 4.0 Version Software.

3.1. Optical absorption measurements

Absorbance of the samples at $\lambda = 570$ nm, as well as BCD absorption spectra in the spectral range of 500–700 nm in the RPMI1640 medium with and without cell cultures before the irradiation, were monitored by a µ-Quant BioTek Instruments spectrophotometer directly in the culture plates. BCD absorption spectra in the range of 500–700 nm were also monitored in aqueous and SDS micelle solutions in 1 cm quartz cuvette using a Beckmann Coulter DU640 spectrophotometer.

4. Results and discussion

4.1. BCD spectral characteristics

To give a brief introduction on the spectral characteristics of biscyanine (BCD) in comparison to that of the Photogem®, the absorption and emission spectra of both photosensitizers are presented in Fig. 2. As a porphyrin derivative, Photogem® is characterized by a high molar absorption coefficient in the Soret region ($10^5 \text{ M}^{-1} \text{ cm}^{-1}$) and relatively weak absorption in the Q region ($\epsilon_{630 \text{ nm}} = 1.17 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) [17]. On the other hand, in dilute solutions ($<10^{-6} \text{ M}$) BCD has high light absorption in the region between 500 and 700 nm ($\epsilon_{632 \text{ nm}} = 1.33 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) [13]. The BCD fluorescence emission spectrum shows the maximum at 644 nm, with a shoulder around 706 nm. These characteristics are associated with BCD in monomeric form. The Photogem® fluorescence maximum is localized at 626 nm. The fluorescence quantum yields in PBS are 0.16 and 0.018 for Photogem® and BCD, respectively.

Fig. 3 shows the changes in the absorption spectra of BCD in different situations. In the RPMI medium a new absorption band in BCD spectrum with the maximum localized at $\lambda \approx 690$ nm appears. The same band was also observed in BCD solution in the presence of NaCl. The relative intensity of this band increases when BCD and/or salt concentrations increase. This absorption band was associated with the formation of BCD J-aggregates [13]. This fact can be essential for BCD application, since in general the aggregation reduces lifetimes and quantum yields of photosensitizer excited states [13,20–22], thus reducing its efficacy in PDT.

However, at the BCD interaction with nanoorganized structures, such as SDS micelles (Fig. S2, Supplementary data) or DNA [13], the absorption band at 690 nm disappears, indicating BCD disaggregation. Based on this fact we may expect that the BCD interaction with cell culture will induce its disaggregation. Really, the monitoring of absorption spectrum of BCD incubated with B16F10 cells as a function of time showed that for incubation times between 30 min and 24 h BCD in relatively high concentration (50 µM) remains in its monomeric form (Fig. S2, Supplementary data).

4.2. Cell internalization

Cellular uptake of BCD was determined on the basis of the cellular fluorescence intensity using flow cytometry and fluorescence image microscopy.

Fig. 4 shows the concentration and time dependences of BCD internalization obtained by flow cytometry. After 6 h of incubation in the cells, the BCD saturation concentration was found to be around 2 µM (Fig. 4A). The sensitizer uptake increased during the first 3 h reaching

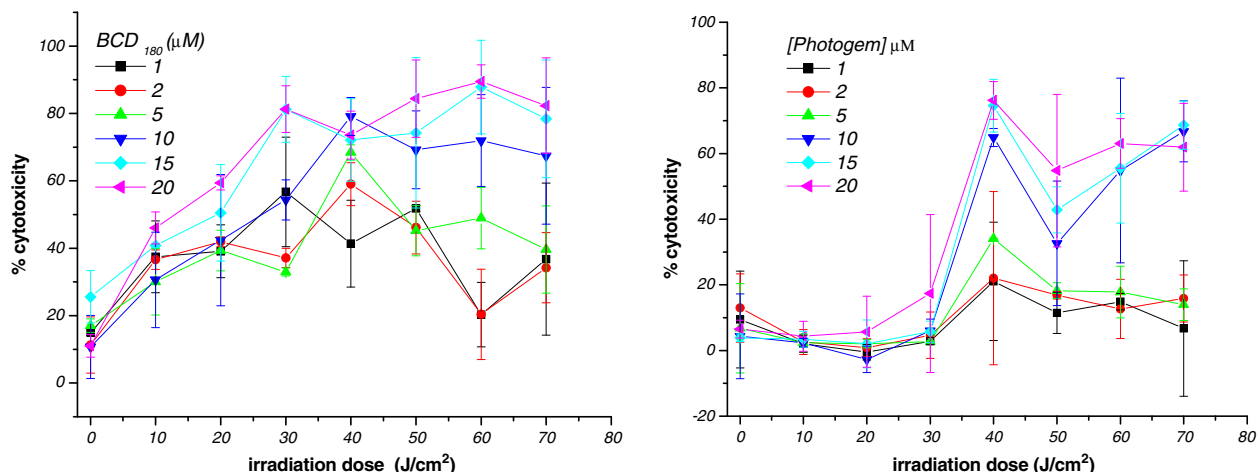


Fig. 8. Dependence of the photocytotoxicity of BCD and Photogem® on the irradiation dose for different PS concentrations after 2 h of incubation in B16F10 cells.

the saturation for incubation times longer than 4 h (Fig. 4B). We should note that similar levels of fluorescence intensity were found after 18 h of incubation with 2 μM of BCD, confirming the negligible drug release during this time interval (Fig. 4B). The high BCD fluorescence intensity

(Fig. 4A, inset) shows that inside the cells this compound is found in the monomeric form.

Similar results were obtained by fluorescence microscopy. Cells were incubated with 5 μM of BCD and the images of the sensitizer

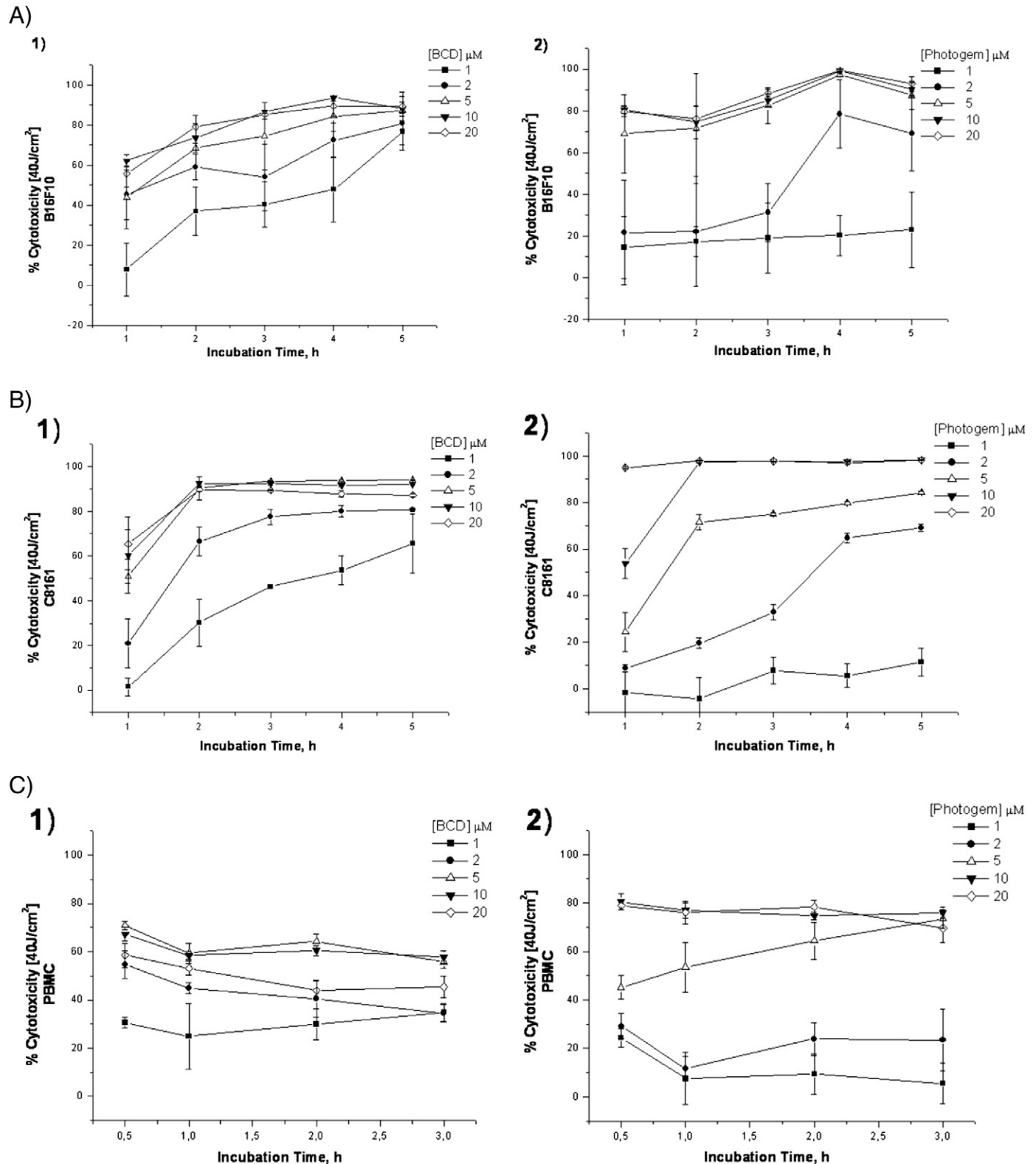


Fig. 9. Photocytotoxic effect (CTE) of (1) BCD and (2) Photogem® against: A) B16F10–murine melanoma cells; B) C8161–human melanoma cells; C) PBMC–human peripheral blood mononuclear cells at different concentrations and incubation times at and irradiation dose of 40 J/cm².

fluorescence emission in cellular medium as a function of incubation time were viewed with the help of a fluorescence microscope using an appropriate filter set. Fig. 5 shows the time-course of BCD internalization.

After 5 min of incubation with BCD, multiple fluorescent points at the membrane surface were observed, suggesting the binding of BCD with the cell membrane (Fig. 5A). With 15 min of incubation, BCD fluorescence at the membrane surface becomes more intense (Fig. 5B). This increase of the BCD fluorescence intensity may be associated with BCD monomer binding with cell structures. It confirms the optical absorption data shown earlier, indicating that BCD is in its monomeric form in the presence of cells, since BCD aggregates have no fluorescence. Formerly [13] it was demonstrated that the fluorescence quantum yield of BCD monomers, being just 0.02 in homogeneous aqueous solutions, increases up to 0.45 at its binding with micelles or with DNA. The observed images demonstrated that the association of BCD with cell structures also increases its fluorescence intensity confirming that it is in the monomeric form.

After 30 min of incubation, BCD fluorescence was diffusively distributed throughout the cytoplasm and detected inside endosome vesicles in the cell cytosolic compartments (Fig. 5C). Finally, after 120 min we observed that an intensive BCD fluorescence was still at perinuclear cytoplasm structures (Fig. 5D).

To evaluate more precisely the site of intracellular localization of BCD, comparative analysis of sensitizer fluorescence emission pattern to those characteristics of standard probes was performed (Fig. 6).

The similarity between the fluorescence pattern of BCD and mitochondria probes (Fig. 6A–F) shows that BCD localizes preferentially in a region near to the mitochondria. This was not observed with the nuclei probe DAPI (Fig. 6G–I), suggesting an unlikely nuclear BCD localization.

Mitochondria have been found to be a very important subcellular target for many PS used in PDT, including the first generation ones, such as Photogem® and its analogue Photofrin®. The mitochondrial damage after these PS illuminations leads to cell death by apoptosis [23] emphasizing the importance of subcellular location/target of the PS in PDT.

4.3. Photocytotoxicity

After 24 h of incubation, BCD and Photogem® cytotoxic effect (*CTE*) in darkness for drug concentrations varying from 1 to 20 μM did not exceed 20% (Fig. 7). *CTE* values were calculated as described in Section 2.5. Cells incubated without drug were used as the control.

In the view to choose the adequate irradiation dose for the photocytotoxicity tests, B16F10 cells were incubated for 2 h with BCD and Photogem® and then exposed to fluences from 10 to 70 J/cm^2 (Fig. 8).

It was observed that BCD had a significant *CTE* due to light exposure at all fluences, while for Photogem® the *CTE* was expressive only when the irradiation doses were $\geq 40 \text{ J}/\text{cm}^2$. Besides, BCD presented a higher *CTE* when compared to Photogem® in all concentrations used. Similar results were obtained using C8161 cells (data not shown). Since the saturation at 40 J/cm^2 was observed for both compounds, this dose was chosen to perform the photocytotoxic assays with all cell lines. *CTE* of both BCD and Photogem® in relation to B16F10, C8161 and PBMC as a function of the incubation time and drug concentration at 40 J/cm^2 irradiation dose are shown in Fig. 9A–C.

When illuminated, both drugs demonstrated cytotoxicity much higher than in darkness, the effect depending on the incubation time. BCD photocytotoxicity reached saturation after 4 h of incubation for B16F10 cells and after 2 h for C8161 cells, whereas Photogem® needed just 1 h of incubation for both cell lines (Fig. 9A and B). The photocytotoxicity of both BCD and Photogem® in relation to B16F10 cells reached the maximum at concentrations approaching 5 μM , whereas in relation to C8161 cells concentrations were 5 μM for BCD

Table 1

BCD and Photogem® LC_{50} under illumination (irradiation dose of 40 J/cm^2).

Cell line	Incubation time (h)	BCD LC_{50} (μM)	Photogem® LC_{50} (μM)
B16F10	5.0	<1	1.46
C8161	2.0	1.61	3.80
PBMC	0.5	1.80	5.63

and 20 μM for Photogem®. For PBMC cells, the incubation time necessary to achieve the maximum photocytotoxicity was 0.5 h for both drugs (Fig. 9C).

The LC_{50} values (Table 1) were determined from *CTE* dependence on BCD concentration at a fixed irradiation dose of 40 J/cm^2 , according to the data from Fig. 9, and the incubation times of 5 h for B16F10, 2 h for C8161 and 0.5 h for PBMC cells were chosen, respectively. For B16F10 and C8161 cells, Photogem® LC_{50} was, approximately, twice higher than BCD LC_{50} .

These results are in good agreement with those obtained by flow cytometry experiments, since the maximum uptake for BCD in B16F10 cells was obtained after 4 h of incubation and at the concentration of 1 μM it was higher than 97%.

We have also observed that under illumination BCD LC_{50} for PBMC cells was 3 times lower than for Photogem®. On the other hand, maximal BCD photocytotoxic effect for PBMC cells did not exceed 70%, whereas for Photogem® it reached 80%. Thus, it may be concluded that in similar conditions Photogem® photocytotoxicity against PBMC cells is higher, as compared with BCD.

So, this study demonstrated that BCD photocytotoxicity for melanoma cells is similar or higher than Photogem® one, whereas for normal cells it is lower than the latter. The incubation time necessary for BCD photocytotoxicity reaching maximum was about 4 h. Then at concentrations $< 5 \mu\text{M}$ it demonstrated 100% *CTE* in relation to melanoma cells. At the same time, its cytotoxicity in darkness toward all used cell lines did not exceed 20%. The mitochondrial localization of BCD can explain its high photocytotoxicity since the main mechanisms of cellular death, both necrosis and apoptosis, are associated with mitochondria [23].

Based on these results, we conclude that BCD can be considered as a promising compound for photodynamic therapy of cancer.

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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.bbagen.2014.12.005>.

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