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Photosensitizing properties of a boronated phthalocyanine: studies at the molecular and cellular level

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Abstract

A synthetic procedure has been developed for the preparation of a Zn-phthalocyanine peripherally substituted with a dodecaborane. The absorption spectrum of the derivative is typical of the phthalocyanine chromophore. Moreover, the boronated phthalocyanine exhibits a high photosensitizing efficiency against a model biological substrate, such as *N*-acetyl-L-tryptophanamide, and a singlet oxygen quantum yield of 0.53 in dimethylformamide. Even though the presence of the dodecaborane moiety appears to decrease the affinity of the phthalocyanine for HT-1080 transformed human fibroblasts, the boronated phthalocyanine causes an essentially complete loss of cell viability upon irradiation with 600–700 nm light under mild conditions (1 μM concentration, 5-min irradiation at 10 mW/cm^2).

Keywords: Boronated phthalocyanines; Photosensitization; Radiosensitization; BNCT; PDT

1. Introduction

The approval of photodynamic therapy (PDT) for the treatment of selected solid tumors [1] has prompted several investigators to perform studies aimed at enhancing its efficacy and broadening its field of application. In particular, some approaches have been proposed for combining PDT with other therapeutic modalities, such as surgery, chemotherapy or radiotherapy [2].

Recent papers [3,4] have mentioned the possibility to use some boronated porphyrins and phthalocyanines as tumor radiosensitizing agents in the so-called boron neutron capture therapy (BNCT). This technique is based [5] on the interaction of non-radioactive ^{10}B in the tumor with low energy (thermal) neutrons to generate ^{11}B which decomposes into high linear energy transfer particles, $^{4}He^{2+}$ (α particle) and $^{7}Li^{3+}$: those fission fragments have mean free pathways which are approximately equivalent to the average diameter of mammalian cells, hence they can induce confined biological damage through ionization. Thus, the success of BNCT is dependent on: (a) the selectivity of the ionization of boronated-sensitizers in the tumor tissue, and (b) the possibility to achieve a sufficiently large endocellular concentration of boron in the neoplastic lesion. It has been calculated [6] that the minimum effective ^{10}B concentration in the tumor is around 20 μg per g of tissue. At present, phenylalanine is most frequently used [5] for delivering boron to tumour tissues. However, the selectivity of tumour targeting by boronated phenylalanine is generally low [4,5].

Since many porphyrins and phthalocyanines are accumulated by a variety of malignant lesions in amounts which are of this order of magnitude and exhibit some selectivity for tumors over most normal tissues [7], it appears logical to use such compounds as boron carriers to the tumor. In this way, two interesting perspectives are opened, namely: (a) to develop a novel radiosensitizer for BNCT; and (b) to test the possibility of using one radio/photosensitizing agent for the treatment of tumors by a

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combination of BNCT and PDT. The latter application obviously requires that the boronated porphyrin or phthalocyanine derivatives retain a significant photodynamic activity, as well as a high affinity for neoplastic cells. These requirements are favorably confirmed by the results obtained in the present investigation.

2. Material and methods

2.1. Chemicals

Borocaptate (mercapto-undecahydro-*closo*-dodecaborate, BSH) disodium salt was a generous gift from Dr Otomar Kritz (Katchem s.r.o, Czech Republic) and was used as received without any further purification. All other chemicals and solvents used were commercially available products of at least analytical grade.

N-Acetyl-L-tryptophanamide (NATA) and 9,10-dimethyl-anthracene (DMA) were obtained from Sigma (St Louis, MO). Fetal calf serum (FCS) was a product of Life Technologies. The Dulbecco's modified Eagle medium (DMEM) used for cell growth was supplied by Sigma. Trypan blue was also a Sigma product while sodium dodecylsulfate (SDS) was obtained from Merck.

2.2. Synthesis of 2-[(4-undecahydro-closo-dodecaboromercaptocarbonyl)benzoxy]zn(ii)-phthalocyanine (compound I) [8]

To 30 mg (0.04 mmol) of 2-(4-carboxyphenoxy) phthalocyaninato Zn(II) (compound II) [9] in 6 ml of anhydrous tetrahydrofuran (THF) at 0°C under an inert atmosphere was sequentially added *N*-ethyl morpholine (NEM) (11.5 μl, 1.2 mmol) and ethyl cloroformiate (4 μl, 0.04 mmol). The reaction mixture was kept at 0°C until the presence of residual compound (II) was no longer revealed by thin layer chromatography, then *N*-ethyl-morpholine (11.5 μl, 1.2 mmol) and 10 min later disodium borocaptate (18.4 mg, 0.08 mmol) solubilized in 2 ml of anhydrous THF were added. The reaction mixture was first left at room temperature for 8 h, refluxed for 12 h and finally cooled to room temperature again. By adding diethyl ether to the reaction mixture a blue precipitate was obtained which was collected by centrifugation.

The desired product (I) was isolated from the crude reaction mixture through several washes with $CHCl_3$ and precipitation from acetone with ethyl ether (26 mg, 71% overall yield).

^{1}H NMR: (200 MHz, d_6DMSO) δ 9.48–9.40 (m, 7H), 9.07 (bs, 1H), 8.37 (d, J=8.7 Hz, 2H), 8.27–8.19 (m, 7H), 8.04 (d, J=8.6 Hz, 1H), 7.60 (d, J=8.7 Hz, 2H), 2.20–0.10 (bm, 9H, borocaptate cluster) UV–Vis: (DMF) maxima at 672 nm (107 800 M^{-1} cm^{-1}), 606 nm, 343 nm.

2.3. Photokinetic studies

All the irradiation studies were performed using a Waldmann halogen light source, which was equipped with a set of bandpass filters to isolate the 600–700 nm spectral interval.

The rate of photobleaching of compound I was followed spectrophotometrically by measuring the phthalocyanine absorption spectrum in the 550–750 nm range upon exposure of a 2 μM solution of compound I (3 ml) in dimethylformamide (DMF) to the Waldmann lamp operating at a fluence rate of 100 mW/cm^2. The solution of compound I was placed in a quartz cuvette of 1 cm optical path and was gently stirred during irradiation.

The photosensitizing efficiency of compound I was measured by irradiation (100 mW/cm^2) of a DMF solution containing 10 μM NATA and 2 μM compound I.

The decrease in NATA concentration as a function of the irradiation time was followed by measuring the tryptophan fluorescence emission in the 300–400 nm wavelength range ($\lambda_{exc.}$=290 nm). The solution (3 ml) was placed in a 1-cm quartz cuvette with gentle magnetic stirring. Parallel photokinetic studies were performed under identical experimental conditions using the corresponding non-boronated phthalocyanine (compound II).

2.4. Cell accumulation and photosensitization studies

HT-1080 transformed human fibroblasts (ATCC CCL121) were harvested in DMEM with 10% FCS added at 37°C in a humidified atmosphere containing 5% CO_2. At 24 h prior to the incubation experiments, the cells were seeded in a Petri dish at a concentration of 10^4 cell/cm^2.

The cell growth medium was then removed and replaced by the DMEM containing 3% FCS and 1% dimethylsulfoxide (DMSO) where the desired concentration of compound I or its non-boronated analogue compound II was dissolved. The phthalocyanine-cell incubation was prolonged for 24 h in the dark at 37°C. The medium was then removed, the cells were washed twice with phosphate-buffered saline (PBS, 2 ml) containing Ca^{2+} and Mg^{2+} ions and were then solubilized in 2% aqueous SDS (1 ml). After 1-h incubation, the suspension thus obtained was diluted 1:5 (v/v) with 2% SDS and the phthalocyanine concentration was determined using a spectrophotofluorimetric procedure [10] ($\lambda_{exc.}$ 610 nm, $\lambda_{em.}$ 640–800 nm).

In cell photosensitization studies, the phthalocyanine-loaded cells were washed twice with PBS, added again with PBS containing Ca^{2+} and Mg^{2+} ions (1 ml) and exposed to the 600–700 nm light from the Waldmann lamp operating at a fluence rate of 10 mW/cm^2.

The cell survival was measured by means of the trypan blue exclusion test [11]: (a) after irradiation for 0.5–10 min in the presence of 0.5 μM phthalocyanine, or (b) after 3 or

5 min irradiation in the presence of 0.5–5 μM phthalocyanine concentrations.

2.5. Determination of the quantum yield for singlet oxygen generation

The quantum yield of singlet oxygen generation by both compound I and its non-boronated analogue compound II was measured using 9,10-dimethyl-anthracene as a substrate [12]. Typically, 2 ml of a DMF solution containing 10 μM DMA and 4 μM phthalocyanine were placed in a quartz cuvette of 1 cm optical path and gently stirred during exposure to the Waldmann lamp (100 mW/cm^2). Since compounds I and II have essentially identical molar extinction coefficients, identical numbers of photons were absorbed under our irradiation conditions. The spectrophotofluorimetric analysis to determine the DMA content was performed at 5-s intervals up to a total irradiation time of 30 s; the unirradiated and irradiated DMA solutions were excited at 360 nm and the fluorescence emission was observed in the 380–550 nm wavelength range [12]. The DMA concentration was obtained by measuring the fluorescence intensity of the longer wavelength emission peak; the DMA photooxidation followed first-order kinetics: thus, the rate constant of the photoprocess was obtained from the slope of the semilog plot (decrease in DMA concentration vs. irradiation time) and was converted into quantum yield of singlet oxygen generation by comparison with the rate constant for DMA photooxidation sensitized by unsubstituted Zn(II)-phthalocyanine for which the quantum yield $\Phi_\Delta = 0.53$ [13]. The absorbance of Zn(II)-phthalocyanine ($\epsilon = 168\,000$ M^{-1} cm^{-1} at 673 nm) was adjusted in order to match the absorbance of compounds I and II.

3. Results

3.1. Spectroscopic and photosensitizing properties

The straightforward one pot synthetic procedure adopted by us to prepare compound I relies on the reaction between the non isolated asymmetric anhydride of compound II previously described [8,9], which was prepared through the reaction with ethyl chloroformiate and commercially available borocaptate disodium salt.

The reaction is carried out in anhydrous tetrahydrofuran as a solvent at 50°C and allows one to prepare the desired product in an overall 71% yield. Characterization of compound I is based on the assignment of NMR signals. UV–visible spectra for both compound I and its non-boronated analogue compound II in DMF solution are shown in Fig. 2a and b, respectively. No appreciable differences in the molar extinction coefficient and position of the λ_{max} at 671 nm are noticed, as expected from the chemical structure of the two compounds (Fig. 1), i.e. from the linkage of the borocaptate moiety far from the phthalocyanine nucleus, so that no significant perturbation of its electronic structure takes place.

Both compounds II and I underwent a readily detectable photobleaching when irradiated by 600–700 nm light. As one can see in Fig. 2, the presence of the peripheral borocaptate substituent caused a modest increase in the rate of the photoprocess. In any case, the decrease in absorbance involved all the visible absorption bands of the phthalocyanine with no concomitant appearance of new bands in the 300–800 nm wavelength range (data not shown).

This suggests that the photobleaching reflects an irreversible and extensive degradation of the tetraazaisoindole macrocycle, similar to what had been observed by previous authors for tetrasulphonated phthalocyanine [14].

Both compounds I and II appeared to photosensitize the oxidation of the indole moiety in NATA, according to first-order kinetics (Fig. 3a,b); this behavior is typical of most photodynamically active photosensitizers [15]. For each compound, two photokinetic experiments were performed: as one can see the overlap between the two sets of data is very high.

The slopes of the semilogarithmic plots for both compounds I and II were essentially identical, thus indicating that the two phthalocyanine derivatives are characterized by a closely similar photosensitizing activity.

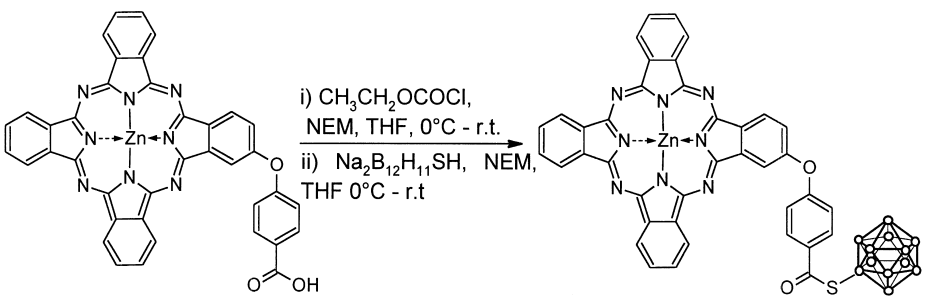

Fig. 1. Scheme for the synthetic preparation of phthalocyanine–borocaptate conjugate.

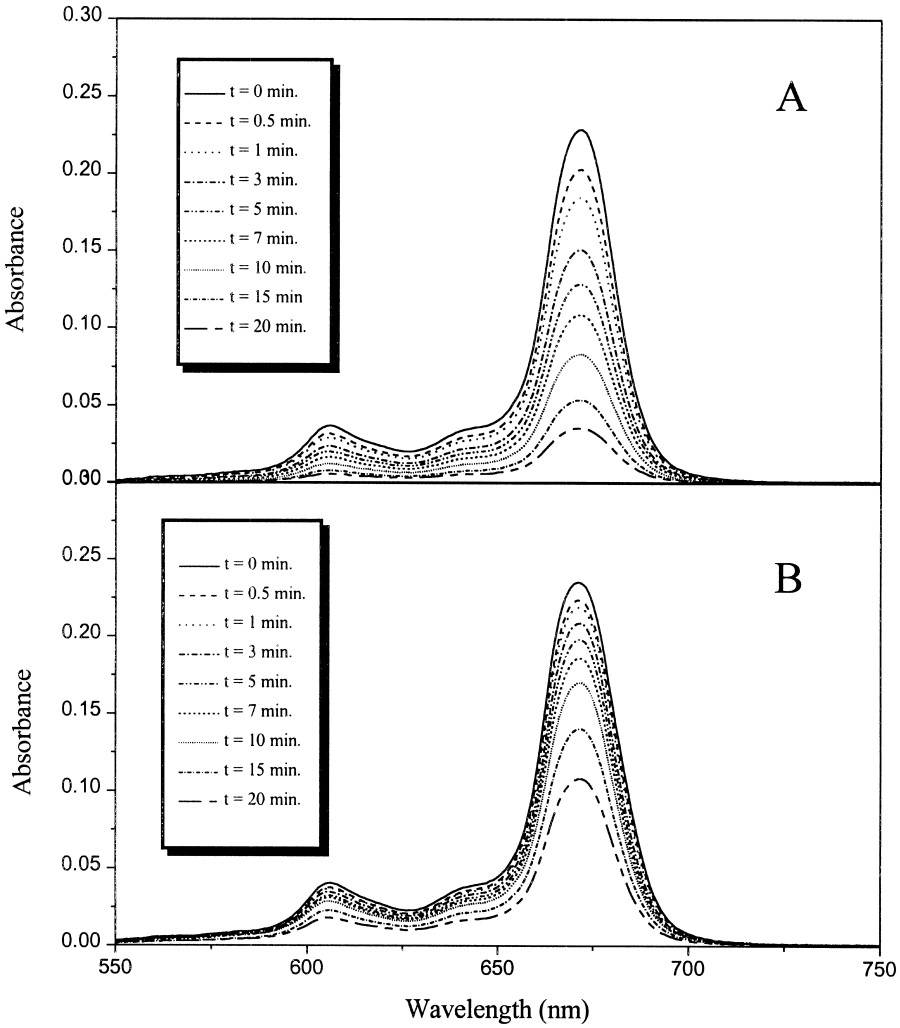

Fig. 2. Photobleaching of 2 μM DMF solution of compound I (A) and compound II (B) upon irradiation with a Waldmann lamp (600–700 nm) at 100 mW/cm^2.

Even for the photooxidation of DMA, compound I and the non-boronated analogue exhibited a very similar efficiency with a first-order rate constant of 7.14×10^{-2} and 7.49×10^{-2} s^{-1}, respectively. These values are to be compared with a rate constant of 7.23×10^{-2} s^{-1} measured for Zn(II)-phthalocyanine indicating that the three phthalocyanine samples have essentially identical quantum yields for singlet oxygen generation, namely around 0.5.

3.2. Cell accumulation and photosensitization studies

The extent of accumulation of compounds I and II by HT1080 transformed human fibroblasts is shown in Fig. 4.

For both phthalocyanine derivatives, the amount of cell-bound drugs steadily increased with increasing photosensitizer concentration in the incubation medium: no apparent saturation of the binding process was detected for 5 μM phthalocyanine, i.e. the highest concentration investigated by us. In any case, the phthalocyanine recoveries (expressed as nmol of phthalocyanine/mg of cell protein) were found to be appreciably lower for compound I compared with compound II, except at the lowest photosensitizer concentration tested by us, i.e. 0.5 μM (Fig. 4). Thus, it is reasonable to conclude that the presence of the peripheral boronated cluster caused some inhibition of the phthalocyanine accumulation by HT1080 fibroblasts to an extent which was particularly evident at higher phthalocyanine doses. Therefore, the subsequent photosensitization experiments were performed in the presence of 0.5 μM phthalocyanine, since the substantially similar endocellular accumulation of compounds I and II would make a comparison of their cell-photosensitizing properties more meaningful.

As one can observe in Fig. 5, when the fibroblasts were irradiated after incubation with 0.5 μM phthalocyanine, a markedly faster drop in cell survival was observed for compound II: thus, after 1 min irradiation with compound II as the photosensitizer, the residual cell survival was

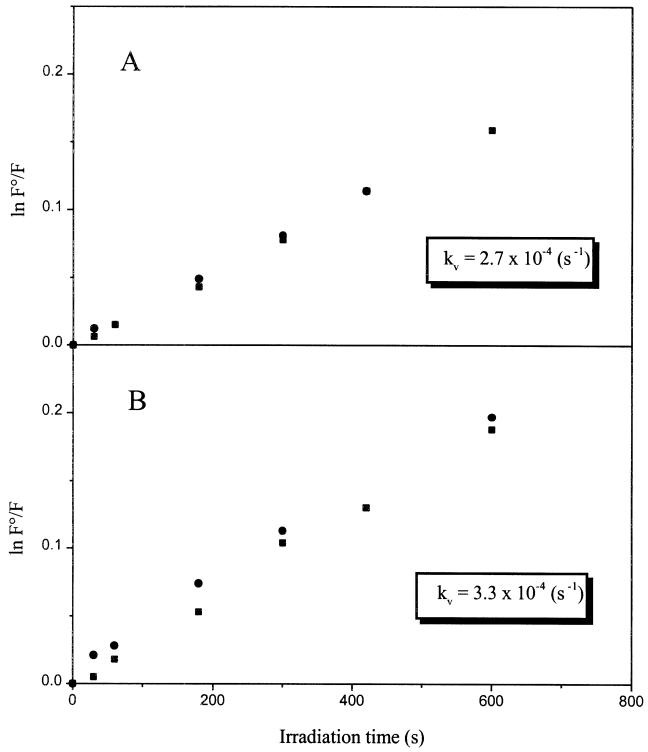

Fig. 3. Photooxidation of 10 μM NATA in DMF solution sensitized by 2 μM compound I (A) and compound II (B). k_v is the first-order rate constant for the photoprocess as deduced from the slope of the semilog plot.

below the detection limit of our assay system. However, the photosensitizing activity of compound I was still significant since increasing the concentration of this phthalocyanine to 1 and 2 μM again caused an essentially

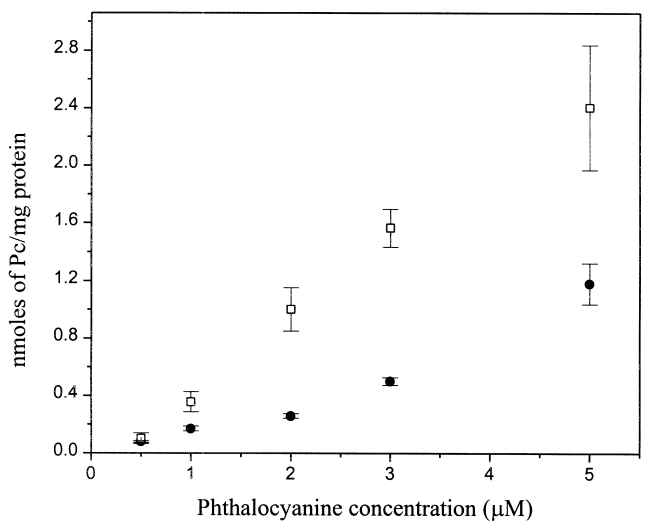

Fig. 4. Effect of phthalocyanine concentration on the uptake of compound I (●) and compound II (□) by HT-1080 cells after 24-h incubation.

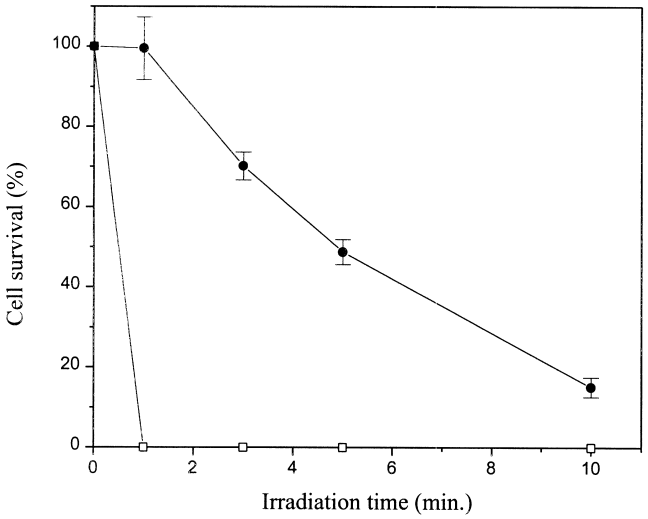

Fig. 5. Effect of irradiation time (600–700 nm, 10 mW/cm^2) on the survival of HT-1080 cells incubated for 24 h with 0.5 μM compound I (●) and compound II (□).

complete cell death after 5 min and, respectively, 3 min of exposure to light (Fig. 6).

4. Discussion

The synthetic straightforward procedure used in the present investigations demonstrates the possibility to prepare phthalocyanine derivatives which retain efficient photosensitizing properties and have a boron cluster for application in BNCT. The synthesis is just one example out of various possible pathways and the boronated

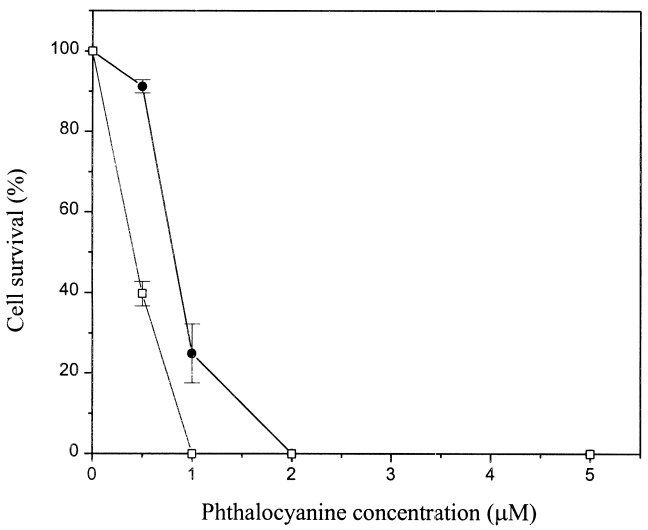

Fig. 6. Effect of phthalocyanine concentration on the survival of HT-1080 cells irradiated (600–700 nm, 10 mW/cm^2) for 3 min (●) or 5 min (□) after 24-h incubation with compound I.

phthalocyanine product represents a model of what can be obtained by the application of several synthetic strategies previously described by us [9]. Actually, our data clearly show that the introduction of a borocaptate substituent in the peripheral position of the phthalocyanine macrocycle does not induce major changes in its photochemical and photosensitizing properties. In particular, no significant variations can be detected in the quantum yields for the generation of singlet oxygen, a main reactive intermediate in many photosensitizing processes [15], as well as in the efficiency of photo-oxidative modification of tryptophan, that is a substrate which can be attacked by both type I and type II photosensitization pathways [16]. Thus, boronated phthalocyanines appear to be photodynamic sensitizers of the same level of efficiency as their parent non-boronated compounds. These conclusions can represent a basis for the utilization of boronated phthalocyanines as photo-/radio-therapeutic agents in a combined PDT+BNCT approach to tumor treatment.

Further support for this proposal is lent by the observation that the boronated compound I displays an appreciable affinity for transformed human fibroblasts, even though the cell-bound amount is generally somewhat smaller than that typical of non-boronated phthalocyanines; the latter compounds are well known to be among the most promising photosensitizers for therapeutic applications [17]: this receives further support by the very efficient photosensitization of fibroblasts killing by compound II (Fig. 5). As a consequence, the red light irradiation of compound I-loaded fibroblasts leads to a fast and extensive cell inactivation in the presence of phthalocyanine concentrations (1–2 μM) which are again typical values for efficient sensitizers [15]. Our results are also in agreement with previous reports [18] showing that boronated porphyrins are good tumor localizers and photosensitizers in vivo. The relatively lower cell photoinactivating efficacy of compound I compared with compound II, even under conditions yielding similar endocellular concentrations of the two phthalocyanines, could reflect a faster photobleaching as suggested by the experiments reported in Fig. 2, as well as an at least partially different pattern of subcellular distribution caused by steric factors and/or differences in the degree of hydrophilicity or hydrophobicity. This point is currently being addressed in our laboratory. In any case, from the data on phthalocyanine accumulation by cells (Fig. 4), one can estimate that the amount of boron delivered to the tumour cells is around 10–20 μg per g of protein. This is close to the boron concentration which is expected to induce an efficient response to BNCT (see Introduction).

We are also defining the most effective sequence for the application of the two sensitization modalities such as BNCT and PDT. In this connection, the observed tendency of compound I to undergo a relatively large photodegradation upon visible light irradiation would suggest that PDT is used after having performed the BNCT treatment, provided the phthalocyanine molecule is stable to the action of thermal neutrons. We have not yet addressed this issue specifically, however literature reports [4,5] suggest that boronated porphyrins are not significantly destroyed upon irradiation with neutrons.

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