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Novel boronated chlorin e_6-based photosensitizers: Synthesis, binding to albumin and antitumour efficacy

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ABSTRACT

Chlorins, a class of plant porphyrins, are perspective as photosensitizing agents due to light absorption in the long wavelength spectral region and deeper photodamage of tissues. Aiming at optimization of antitumour properties of chlorins, we synthesized a series of boronated derivatives of chlorin e_6 and their complexes containing Zn(II), Pd(II) or Sn(IV). The compounds were synthesized by alkylation of amino or hydroxy derivatives of chlorin e_6 with 1-trifluoromethanesulfonylmethyl-*o*-carborane. Chlorin e_6 13(1)-*N*-{2-[*N*-(*o*-carboran-1-yl)methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester (compound **5**) formed complexes with serum albumin, a major porphyrin carrier. The binding constant of these complexes was ~4 times bigger than the respective value for the complexes of albumin with boron-free aminochlorin e_6. Compound **5** potently sensitized rat fibroblasts to illumination with monochromatic red light: >98% of cells were necrotic by 24 h post-illumination with 1 μM of **5**. This compound demonstrated high efficacy in photodynamic therapy of rat M-1 sarcoma. After PDT with 25 mg/kg of **5** the residual tumours were significantly smaller than in animals subjected to PDT with equal concentration of boron-free aminochlorin e_6. No signs of general toxicity were detectable after PDT with **5**. Thus, boronation can enhance the potency of chlorins in PDT, in particular, due to an increased binding to albumin. Our data expand the therapeutic applicability of boronated chlorins beyond boron neutron capture therapy; these agents emerge as dual efficacy photoradiosensitizers.

1. Introduction

Tetrapyrrole containing compounds, that is, porphyrins and structurally close chlorins, are extensively investigated as photosensitizers for photodynamic therapy (PDT) of a variety of human diseases.[1–3] These compounds are accumulated predominantly in metabolically active cells, in particular, malignant tumours; activation of porphyrins and chlorins by light triggers oxygen burst and cell photodamage.[4] The porphyrin-containing pharmaceuticals are used in the clinical PDT.[5] Furthermore, second- and third-generations of photosensitizers with improved photochemical characteristics are under development.[6,7] Chlorins are promising photosensitizers because they strongly absorb light in the long-wavelength spectrum region ($\lambda_{excitation} > 650$ nm) along with increased molar extinction coefficients.[5,8] These properties of chlorins allow for deeper photodamage of tissues and better therapeutic effect compared with porphyrins.

Chemical modifications of porphyrins, in particular, the conjugation of boron containing moieties to the porphyrin macrocycle, have been initially aimed at obtaining the radiosensitizers for boron neutron capture therapy (BNCT).[9–16] However, besides their applicability for this therapeutic modality, the boronated derivatives proved to be preferential in PDT over the boron-free porphyrins.[17,18] We have demonstrated a significantly higher efficacy of water-soluble boronated derivative of natural protohaemin IX (compared with boron-free protohaemin IX) in PDT of sarcoma-bearing rats.[18] Importantly, the complete cure of animals was accomplished with the concentrations of boronated protohaemin IX that caused no dark toxicity and were well tolerated. Thus, boronation not merely retains the photosensitizing properties of initial porphyrins. Rather, the porphyrin–boron conjugates might possess an enhanced therapeutic potency. Tentatively, boronation of porphyrins can confer optimal amphiphilicity, an increased

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binding of the conjugate to its target, and an improved biodistribution in the body. Keeping in mind these factors (or their combinations) and the aforementioned photochemical advantages of chlorins, one can expect that boronation would potentiate the efficacy of chlorins in PDT.

In this work we report the synthesis, in vitro drug–target interactions and in vivo antitumour efficacy of novel conjugates of chlorin e_6 with neutral carboranes. The water-soluble chlorin e_6 13(1)-*N*-{2-[*N*-(*o*-carboran-1-yl) methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester (**5**) caused no dark cytotoxicity but potent light toxicity against cultured mammalian cells. At tolerable concentrations **5** demonstrated higher sensitization of transplanted rat sarcoma to PDT than the initial boron-free aminochlorin e_6 (**2**). This effect was associated with higher ability of the boronated chlorin e_6 to form complexes with albumin, a major vehicle of many endogenous and exogenous compounds including porphyrins and chlorins. These data provide evidence that boronated chlorins are perspective candidates for further preclinical development.

2. Results

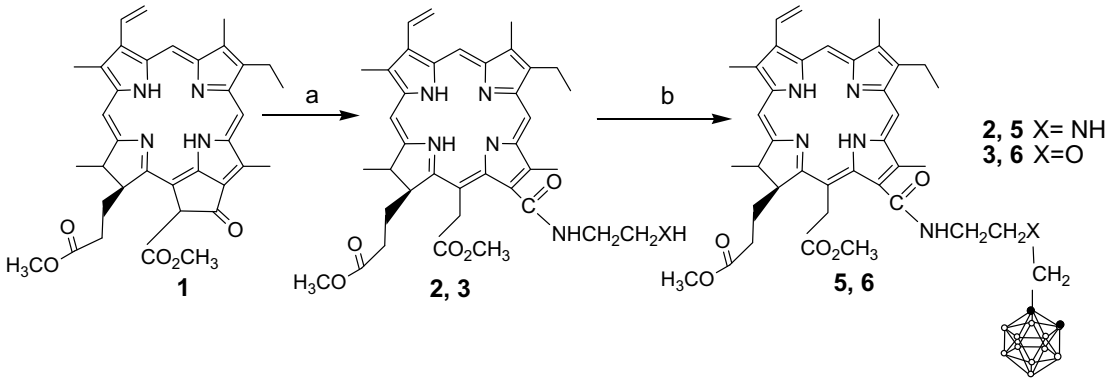
2.1. Chemistry

We developed the synthesis of novel carborane derivatives of chlorin e_6, starting from methylpheophorbide *a* (**1**). Compound **1** is an excellent source for preparing clinical photosensitizers due to strong absorption at λ = 670 nm (ε = 50,000 in dichloromethane) and high quantum yield of generated singlet $^1O_2$. However, **1** is of somewhat limited practical use because of the possibility of C13(1)–C13(2) bond cleavage by bases, resulting in the opening of the exo-ring V in **1**.[3,19] We took advantage of high reactivity of the exocyclic fragment for introducing the boron-containing polyhedra into the macrocycle of **1**. The synthesis proceeded via the formation of the amino (compound **2**) and hydroxy (compound **3**) derivatives of chlorin e_6. These compounds were obtained by the nucleophilic opening of the exocyclic fragment in **1** with ethylenediamine and ethanolamine, respectively.[20] Alkylation of amino and hydroxy groups in **2** and **3** with carboranylmethyl triflate **4** (boiling in THF in the presence of NaOAc) led to carboranylchlorins **5** and **6** isolated in 20% and 12% yield, respectively (Scheme 1). As expected, the reactivity of aminochlorin **2** in these reactions was higher than that of hydroxychlorin **3** owing to higher nucleophilicity of the amino group. Higher reactivity of **2** was manifested in an accelerated reaction and in an enhanced yield of the target carboranylchlorin **5**.

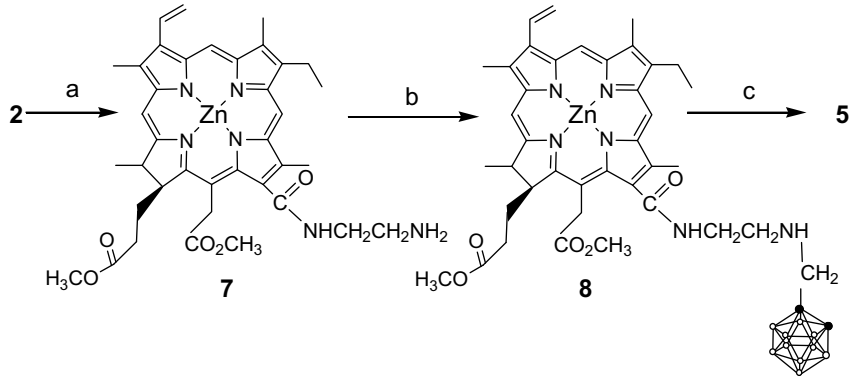
Still, the yields of carboranylchlorins **5** and **6** in the above reactions were unsatisfactory, considering that these compounds are being developed for clinical purposes. We hypothesized that low yields of **5** and **6** could be due to the side reactions of triflate **4** with NH groups of the chlorin macrocycle. To increase the yield of carboranylchlorin **5**, a candidate antitumour photosensitizer (see below), we used Zn(II) complex of **2**. Reflux of **2** with the excess of zinc acetate in chloroform–methanol mixture yielded compound **7** (75% yield).[21] Treatment of **7** with triflate **4** in refluxing THF smoothly produced carboranylchlorin **8** in 53% yield. Removal of Zn from the coordination sphere of **8** with trifluoroacetic acid in methylene chloride afforded metal-free carboranylchlorin **5** (overall 50% yield). This approach of preparing carboranylchlorin **5** via the formation of intermediate zinc complex **8** followed by its demetallation may serve as a common method of synthesis of metal-free chlorin derivatives with reasonable yields (Scheme 2).

Insertion of metal cations into the coordination sphere of porphyrins and chlorins can substantially change physico-chemical properties of the compounds, thereby modulating their therapeutic effects.[22] The metal chlorins have been studied as potential photosensitizers.[23–28] In particular, excellent efficacy of Tookad, the Pd(II) derivative of bacteriochlorophyll *a*, has been shown in vascular targeted PDT of experimental and clinical tumours.[29–31] However, the synthetic studies of boronated metal chlorins are scarce. We modified **5** by introducing Pd(II) and Sn(IV) into the coordination sphere of the chlorin macrocycle (compounds **11** and **14**; Scheme 3). The procedure for the preparation of **11** included the formation of Pd(II) complex of methylpheophorbide *a* (compound **9**) upon refluxing of **1** with $PdCl_2$ in CH_3CN and opening of the exo-cycle in **9** with ethylenediamine in $CHCl_3$ to produce Pd(II) complex of chlorin e_6 (compound **10**). The subsequent carboranylmethylation of **10** with triflate **4** yielded Pd(II)–carboranylchlorin **11.** The synthetic route to boronated Sn(IV) complex **14** was similar and included the preparation of Sn(IV) complex of methylpheophorbide (compound **12**) upon reflux of **1** with the excess of hydrated $SnCl_2$ in methanol.[23–28] Hydrolysis of the chloride complex with water yielded the hydroxo complex **12** (Scheme 3). Further transformations of the exo-cycle in **12** with ethylenediamine and alkylation with triflate **4** yielded Sn(IV) complexes **13** and **14**. It is noteworthy that opening of the exo-cycle in **9** and **12** occurred readily with ethylenediamine, whereas the exo-cycle in the Zn complex of methylpheophorbide remained unchanged by this treatment.

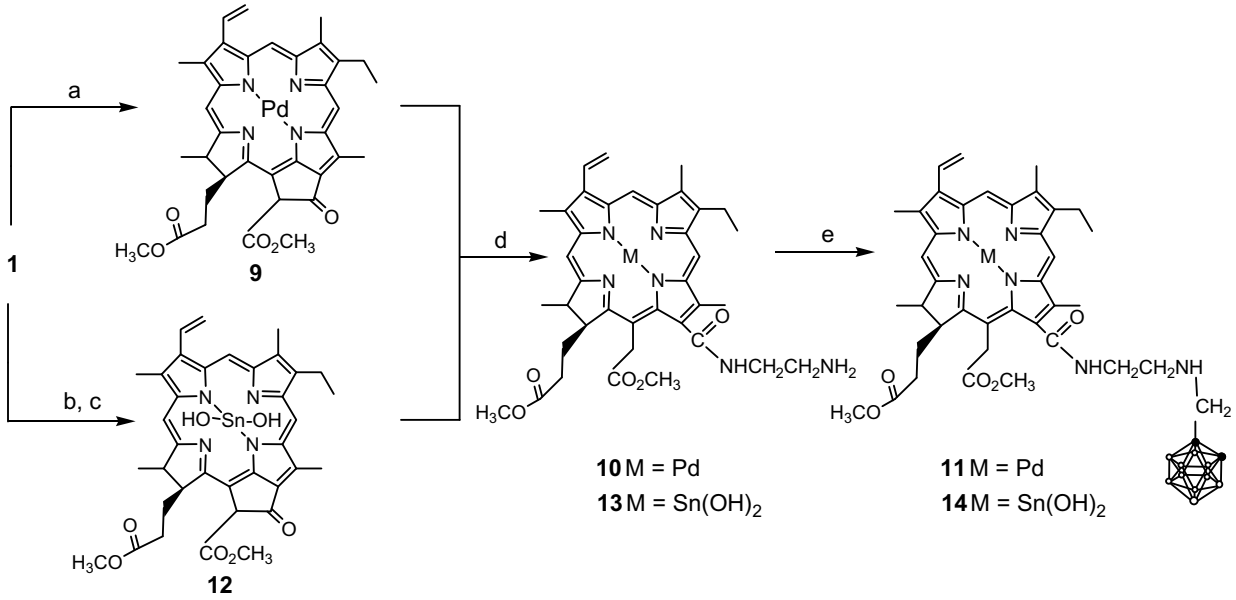
The structure of compounds **2, 3, 5**–**14** was confirmed by NMR, IR and UV–vis and mass spectroscopy. The ^1H NMR spectra of **5–8**, **10**, **11**, **13** and **14** lacked the singlet signal of the proton in the 13(2)-position of the exo-ring revealed in the spectrum of the initial compound **1** (6.27 ppm). Instead, we observed the doublet signals of nonequivalent protons of the methylene group in the 15(1)-position at 5.0–5.5 ppm that resulted from the opening of the exo-ring. The broadened signals appeared that corresponded



Scheme 1. Synthesis of neutral carboranylchlorins **5**, **6**. Reagents and conditions: (a) $H_2NCH_2CH_2XH$, $CHCl_3$, room temperature, 12 h; (b) *o*-$HCB_{10}H_{10}CCH_2OTf$ (**4**), THF, NaOAc, under Ar, 66 °C, 24 h.



Scheme 2. Synthesis of compound **5** through Zn(II)–carboranylchlorin complex **8**. Reagents and conditions: (a) $Zn(OAc)_2$, $CHCl_3$–MeOH, under Ar, room temperature, 1 h; (b) **4**, THF, NaOAc, under Ar, 66 °C, 24 h; (c) CH_2Cl_2, CF_3COOH, under Ar, room temperature, 2 h.



Scheme 3. Synthesis of Pd(II)– and Sn(IV)–carboranylchlorin complexes **11**, **14**. Reagents and conditions: (a) $PdCl_2$, CH_3CN, 85 °C, 3 h; (b) $SnCl_2{\cdot}2H_2O$, CH_3OH, 65 °C, 48 h; (c) H_2O; (d) $H_2NCH_2CH_2NH_2$, $CHCl_3$, room temperature, 1 h; (e) **4**, THF, NaOAc, under Ar, 66 °C, 24 h.

to NH protons of the amide groups. The protons of pheophorbide macrocycle exhibited the signals in the downfield region at 9.6–8.6 ppm. The protons of the BH group of the carborane moiety in **5, 6, 8**, **11** and **14** were observed only in the high-field region, and the carborane CH protons were detected at $\delta = 3.52$ ppm. The protons of the methylene group in the carborane were magnetically nonequivalent and manifested themselves as doublets at ~2.8 ppm. The ^{11}B NMR data confirmed that the carborane fragments in **5**, **6, 8, 11** and **14** were unaltered: the signals typical for *closo*-carborane were observed whereas no *nido*-derivatives were detectable. The IR spectra of **2** and **3**, in comparison with that of **1**, showed no band corresponding to the stretching vibrations of the keto group in the 13(1)-position at 1706 cm^{-1}. In contrast, we detected the amide I and amide II absorption bands at 1636 and 1524 cm^{-1}, respectively. The IR spectra of **5**, **6, 8, 11** and **14** showed the stretching vibrations of CH groups (3069 cm^{-1}) and BH groups (2600 cm^{-1}) of the carborane fragment. A broad band at 3437 cm^{-1} confirmed the presence of OH group in **12**–**14**.

Finally, the chlorin moiety was clearly detectable in the electronic spectra of carboranylchlorins **5, 6, 8, 11** and **14**. All spectra exhibited an intense Soret band and some bands in Q region resulted from $\pi \rightarrow \pi^*$ transition. It is worth noting that the position and intensity of Soret band as well as Q bands of **1**, **2** and **5** changed after the introduction of Zn, Sn and Pd. We observed the red-shift of Soret band (from ~405 nm in **1**, **2** and **5** to ~415–420 in the metal complexes), the drop of its intensity, the blue-shift of Q(0.0) band (from 670 nm in **1, 2** and **5** to 640–660 nm in the metal compounds) and an increase of its intensity. These effects may be due to the influence of metals on the conjugated π-electron system of the chlorin moiety. In all our chlorin–metal complexes the number and the intensities of other Q bands within 510–615 nm decreased compared to **1** and **2**. The reason for this phenomenon is that some electron transitions became forbidden, as the chlorin fragment acquired symmetry due to the removal of protons in metal complexes.

We selected **5** for studies of antitumour photosensitization because this compound was obtained in reasonable yield and was readily soluble in water. Compound **2**, the boron-free synthetic precursor of **5**, was used as a reference agent.

2.2. Interaction of 2 and 5 with serum albumin

We investigated the in vitro binding of **2** and **5** to serum albumin, a major carrier of porphyrins and chlorins in the

body.[32] Figure 1 shows the absorption spectra of **2** and **5** in phosphate buffered saline, pH 7.0 (PBS) without albumin and in PBS supplemented with high amount of albumin. The spectra of **2** and **5** in PBS were represented by narrow bands identical to those observed in dimethyl sulfoxide (DMSO) (in which **2** and **5** are monomeric). This fact, as well as the absence of absorption bands of aggregates or dimers indicated that in PBS compounds **2** and **5** were present exclusively as monomers. In PBS containing high concentration of albumin at which all **2** and **5** are complexed with the protein, the maximum peaks of absorption were blue shifted compared with the respective peaks in albumin-free PBS. Furthermore, the formation of complexes of **2** and **5** with albumin led to the hyperchromic effect: the intensities of absorption by the complexes increased by ~20% compared with the absorption intensities of free **2** and **5** (Fig. 1). These spectral changes differed from the findings of Mishra et al.[33] who reported a batochromic shift of absorption bands of chlorin p_6 and purpurin 18 complexed with albumin. Also, chlorin p_6 and purpurin 18 formed aggregates in PBS whereas **2** and **5** did not, further substantiating the therapeutic advantage of **2** and **5**.

The spectra of fluorescence of **2** and **5** in PBS supplemented with albumin are shown in Figure 2, insets. Even low concentrations of albumin significantly changed the spectra, demonstrating that **2** and **5** formed complexes with this protein. We observed the batochromic shifts of fluorescence of complexed **2** and **5**, compared to unbound drugs, by 6 nm and 8 nm, respectively. These shifts were much smaller than a 30 nm shift of fluorescence spectra registered for the complexes of chlorin p_6 and albumin in phosphate buffer, pH 5.[33] The intensity of fluorescence increased with the elevation of [HSA], and the plateau of the curves (Fig. 2A and B) indicated the saturation of drug–albumin complex formation. These curves were used for Scatchard plots from which we calculated the constants of complex formation of **2** and **5** with albumin. Figure 3 shows linear function θ from $\frac{\theta}{[HSA]_{free}}$ for **2** and **5**. From this plot we calculated the albumin binding constants: $K_b = 2 \times 10^5\ M^{-1}$ for **2** and $K_b = 8 \times 10^5\ M^{-1}$ for **5**.

To further characterize the interaction of carboranylchlorins with their carrier, we measured the lifetime of singlet excitation states τ of unbound and albumin-bound **2** and **5**. The kinetics of fluorescence decay of free **2** and **5** and in complexes with albumin was mono-exponential, indicating that only one state of fluorescence was detectable in excited singlets of **2** and **5**. The fluorescence lifetime of each compound bound to albumin increased in comparison with lifetime of the unbound drug. The values τ for **2** in the albumin-free buffer and in complex with albumin were 4.5 ns and 4.9 ns, respectively, whereas these time intervals for **5** were 4.1 ns and 5.0 ns, respectively.

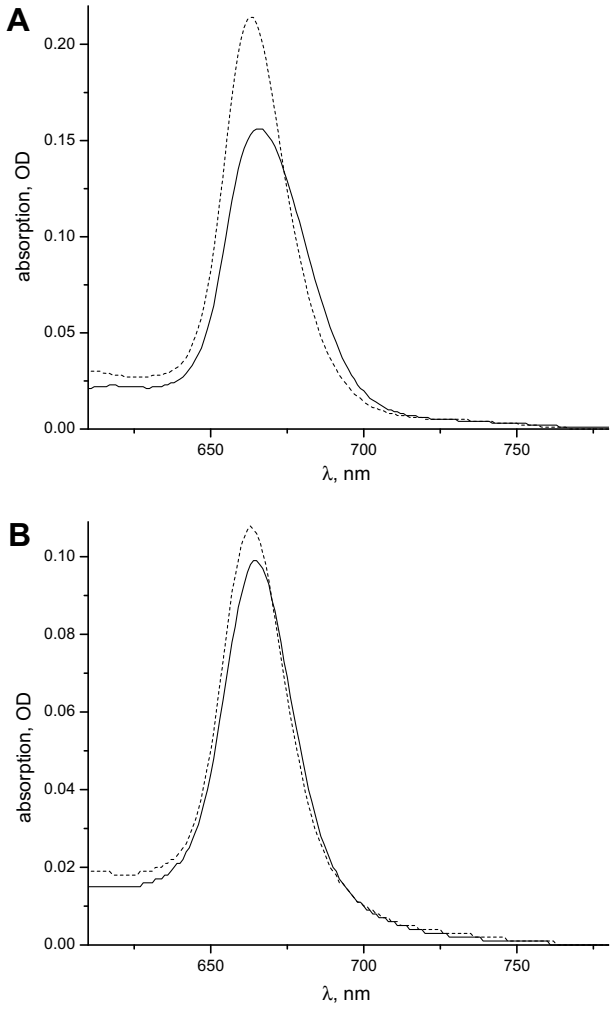


Figure 1. Absorption spectra of compounds **2** and **5** in PBS at pH 7.0. (A) compound **2**, (B) compound **5**. Solid lines, compounds in PBS (max. 665.5 nm), dotted lines, compounds in PBS with 50 μM HSA (max. 663 nm).

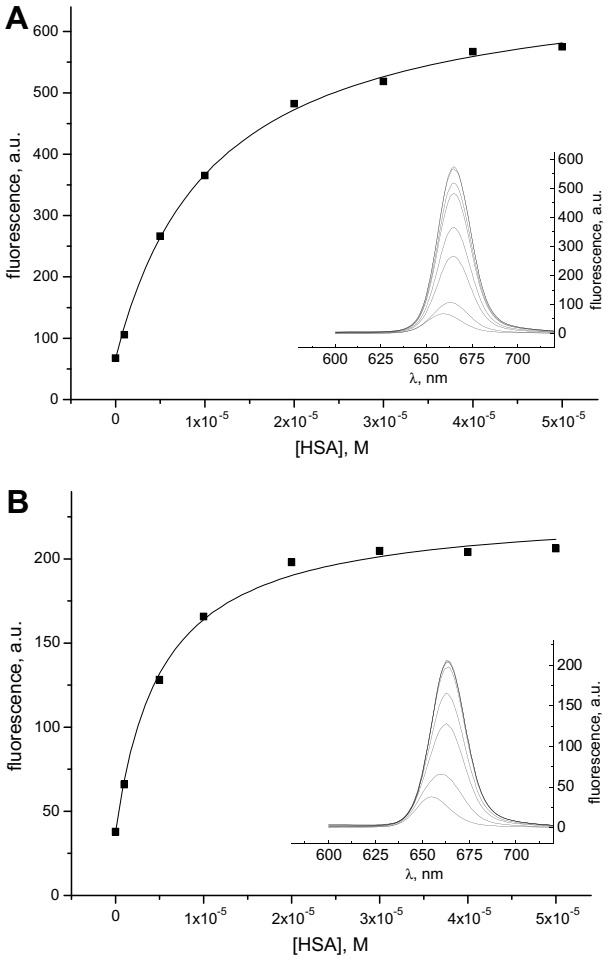


Figure 2. Dependence of fluorescence intensity of compounds **2** and **5** on [HSA], (A) compound **2**. λ_{ex} = 400 nm, λ_{em}: 659 nm (free drug), 665 nm (bound drug), (B) compound **5**. λ_{ex} = 400 nm, λ_{em}: 655 nm (free drug), 663 nm (bound drug). Insets, spectra of fluorescence. A.u., arbitrary units.

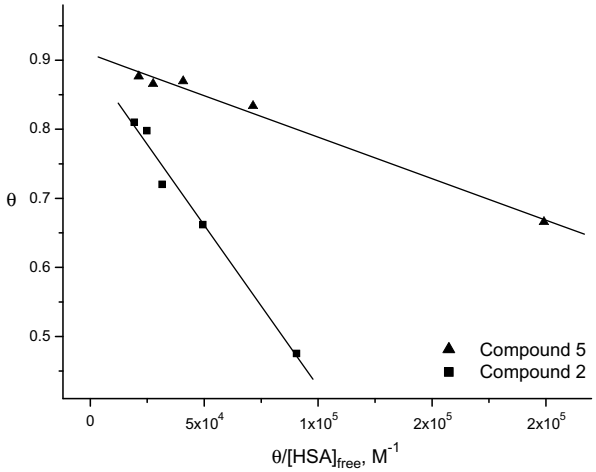


Figure 3. Scatchard analysis of complexes with HSA. The concentrations of **2** and **5** were 10 μM. Binding was measured in PBS at pH 7.0 Squares, compound **2**; triangles, compound **5**.

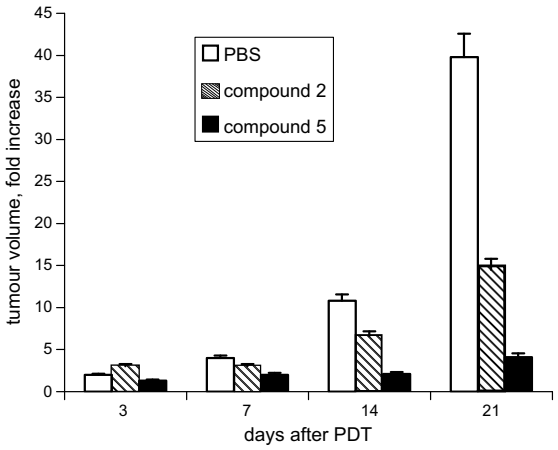


Figure 4. Antitumour potencies of boron-free aminochlorin e_6 and its boronated derivative. Rats bearing s.c. M-1 sarcoma node were injected i.p. with PBS or 25 mg/kg **2** or 25 mg/kg **5**. Ninety minutes after injection the tumours were illuminated with red light (see 'Section 5'). The tumour volume was measured at indicated time intervals and plotted as mean fold increase of the tumour volume before illumination (regarded as 1). One representative experiment out of a total of three is shown. * indicates $p < 0.05$ between 'compound **2**' group and 'compound **5**' group.

2.3. Biological testing

An important prerequisite for the clinical use of photosensitizers is their minimal dark toxicity. We studied the cytotoxicity of **5** for McA 7777 rat liver epithelial cell line, Rat-1 and REF fibroblasts, MCF-10A human breast epithelial cell line and HCT116 human colon epithelial cell line, and human peripheral blood lymphocytes. Cells were continuously exposed to various concentrations (up to 25 μM) of **5** for 72 h, and cell viability was assessed in MTT-test.[34] In this range of concentrations **5** was readily soluble in water. No growth retardation or cell death was registered in any of tested cell lines after the above treatments. Thus, **5** was nontoxic at concentrations that did not affect its solubility in culture medium.

To verify the potency of **5** in cellular PDT, we first studied its intracellular accumulation and distribution. Treatment of Rat-1 fibroblast cell line with 1 μM of **5** for 0.5–24 h resulted in clearly detectable diffuse cytoplasmic staining, as determined by fluorescent microscopy (not shown). The patterns of intracellular distribution and the mean brightness of cells did not change significantly after 30 min with the drug; we therefore used this time interval for cell loading with 1 μM of **5** followed by drug withdrawal and light illumination (LI) of cells (see 'Section 5' for details). Already after the initial minutes post LI the phototoxic effect was clearly detectable. Cells lost their polygonal shape and became rounded. Flow cytometry assisted analysis revealed that 30 ± 8% of cells (mean ± standard deviation, results of 3 experiments) were propidium iodide (PI)-positive as soon as 20 min after illumination, and >70% of cells were PI-positive by 2 h post LI. Twenty four hours later >99% of cells were positively stained with PI whereas this parameter was <5% in cells exposed to **5** alone or in cells treated with LI in the absence of **5**. Thus, high photodamaging potency of **5** at the concentration that caused virtually no dark toxicity suggested that this carboranylchlorin might be perspective in animal studies.

We compared the antitumour efficacy of **5** and its boron-free precursor **2** in the model of rat M-1 sarcoma.[18] After the subcutaneously implanted tumour noduli became palpable, animals were divided into eight cohorts and were mock-treated or injected with **2** (25 mg/kg) or **5** (5–25 mg/kg) followed by LI (see 'Section 5' for details). As shown in Figure 4, tumours in the group 1 (PBS) grew fast: by days 14 and 21 the mean fold increase of tumour volume were 10.8 and 39.8, respectively. Similar rates of tumour growth were registered in groups 2–4 (not shown). In striking contrast, LI in the presence of **5** (groups 6–8) caused a dose-dependent retardation of tumour growth. The most pronounced therapeutic effect was achieved with 25 mg/kg of **5**: by days 14 and 21 the mean fold increase of tumour volume were only 2.2 and 4.1, respectively. This effect was accomplished with the dose of **5** that caused no general toxicity as judged by unaltered behaviour, nutritional habits and hair cover of animals, as well as by unchanged blood cell count. Importantly, compound **2** at equal concentration was less efficient than **5**: the mean coefficients of tumour volume increase after PDT with 25 mg/kg of **2** were 6.6 and 14.5 (Fig. 4). These data proved that boronation can improve the photodamaging potency of chlorins in antitumour PDT.

3. Discussion

Chlorin-based photosensitizers are widely investigated as the agents for PDT due to strong absorption of light in the red and near-infrared spectrum regions and deep photodamage of pathological tissues. Chemical modifications of chlorins are aimed at increased water solubility,[35,36] better recognition of and interaction with cell surface molecules,[37–39] and enhanced specificity of the conjugate to the tumour cells.[40] Various substituents cause no significant perturbation to the structure of the chlorin macrocycle, therefore, the advantageous photochemical properties of this moiety are retained in the conjugates. Furthermore, the combinations of individual components (e.g., metals or functional groups) render the chlorin-based conjugates suitable for optimization as photosensitizers by modulating the solar spectrum, long-lived excited states, red-region fluorescence and biological characteristics.[41] Indeed, the conjugation of chlorins with *closo*- and *nido*-carboranes yielded the compounds with very low dark toxicity, efficient photosensitizing properties and significantly improved uptake by glioma cells.[42] In the present study we synthesized a new series of carboranylchlorins by alkylation of amino or hydroxy derivatives of chlorin e_6 with 1-trifluoromethanesulfonylmethyl-*o*-carborane. Also, we obtained novel metal containing carboranylchlorins and proved that the yield of carboranylchlorins can be substantially improved via the formation of an intermediate metal complex followed by demetallation. The selected water-soluble chlorin e_6 13(1)-*N*-{2-[*N*-(*o*-carboran-1-yl) methyl]amino-

ethyl}amide-15(2), 17(3)-dimethyl ester (compound **5**) demonstrated a negligible dark cytotoxicity and high potency as a photosensitizer in PDT of cultured mammalian cells and in in vivo photodamage of rat M-1 sarcoma. Most importantly, the boronated derivative caused a longer antitumour effect than the parental boron-free aminochlorin. Together with a significantly higher efficacy of boronated derivative of protohaemin IX than that of boron-free protohaemin IX,[18] the present results further substantiate the role of boronation in improving the potency of tetrapyrrole containing compounds as antitumour photosensitizers.

In this study we report new series of boronated chlorins with metals in the coordination sphere. We found that Zn(II) carboranylchlorin **8** exerted dark cytotoxicity for HCT116 colon carcinoma cell line (IC_{50} = 5.4 μM) whereas Pd(II) or Sn(IV) complexes **11** and **14** caused no cell death even at 12 μM within a 72 h continuous exposure. The binding constants of **8**, **11** and **14** to albumin (calculated by the same method as for **5** and **2**) were $2.4 \times 10^5\ M^{-1}$, $1 \times 10^6\ M^{-1}$ and $5.0 \times 10^4\ M^{-1}$, respectively. Based on these results we excluded **8** from the list of promising photosensitizers (regardless of its relatively high albumin binding constant) since this compound was cytotoxic in the dark. Compounds **11** and **14** were tested as photosensitizers in cell culture. Similarly to compound **5** (this study) and **2** (not shown), **11** and **14** at 1 μM each sensitized rat fibroblasts to red light, triggering necrosis within 20 min post-illumination. Thus, Pd(II) and Sn(IV) containing carboranylchlorins are potent photosensitizers, and their potency is comparable with that of compound **5**.

To get insight into the mechanisms of higher efficacy of boronated versus boron-free chlorins, we investigated the interactions of **2** and **5** with serum albumin. The electrostatic and hydrophobic interactions may stabilize the complexes of chlorins and purpurins with albumin.[43,44] To evaluate relative impact of electric charge, authors modulated pH of solutions of chlorins with albumin.[33,45] Acidification led to neutralization of chlorin molecules, thereby increasing the binding to albumin. Therefore, the electrostatic interactions are unlikely to be important for complex formation. In the present study we addressed the role for hydrophobicity in the stability of the complexes of chlorin and boronated chlorin with albumin. Measurements of drug–albumin complex formation were performed at the same pH of the buffer, so the electric charges of **2** and **5** were equal. Our results support the key role of hydrophobic interactions in the formation of complexes of **2** and **5** with albumin.

The difference in the interaction of **2** and **5** with albumin is also reflected by lifetime of singlet excited states τ. This parameter strongly depends on pH, viscosity, dielectric permeability constant, electrostatic and hydrophobic interactions. The fluorescence lifetime depends on radiation and radiationless processes. Because the absorption spectra of **2** and **5** were similar, the rates of radiation processes for both compounds (unbound as well as bound to albumin) were virtually the same. Therefore, the above differences between lifetimes of fluorescence should reflect the dependence of radiationless transitions on the structure of **2** and **5** and on the close surrounding of these compounds bound to albumin. A shorter lifetime of fluorescence of free **5** compared to that of free **2** is likely to be associated with the bulky carborane moiety in **5** that might accelerate the internal conversion of singlet excited state into the main state. A somewhat bigger increase of fluorescence lifetime upon binding of **5** to albumin compared with the respective increase for **2**, can be associated with sterically hindered intramolecular vibrations of the carborane fragment upon binding of **5** with albumin. Similarity of fluorescence lifetimes of complexes of **2** and **5** with albumin (4.9 ns vs 5.0 ns, respectively) is explained by 'freezing' of the carborane vibrations which decreases the rate of radiationless processes in the complex of **5** with albumin. Since **2** lacks the bulky substituents, its binding to albumin changed the fluorescence lifetime only slightly. These considerations corroborate an important role of carborane in the interactions of boronated chlorins with albumin.

At least two important factors should be considered regarding the complexes of chlorins with their carriers. First, although we found a tighter binding to albumin of carboranylchlorin **5** compared with boron-free **2**, the boron polyhedra can differentially influence on the binding constants of chlorin–albumin complexes. We observed a decreased binding to albumin of carboranylpyropheophorbide compared with pyropheophorbide (our unpublished work). Currently our group generates an in silico model of carboranylchlorin–albumin interactions for rational design of the conjugates with desired properties. Second, photosensitizers have been reported to bind plasma proteins other than albumin. Chlorin e_6 interacts with low density lipoproteins (LDL; ~10 high affinity binding sites), although albumin was the major carrier (binding constants $5.7 \times 10^7\ M^{-1}$ vs $1.8 \times 10^8\ M^{-1}$, respectively).[45] Importantly, interactions of chlorin e_6 with LDL and albumin depended on pH: acidification led to stronger binding to LDL. The LDL receptors are overexpressed in many tumour types,[46] and the acidic pH is frequently detectable in solid tumours. Thus, binding to LDL emerges as an important prerequisite for the efficacy of chlorin-based photosensitizers. Our preliminary results suggest that compounds **2** and **5** are high affinity LDL ligands; studies are in progress to analyze these interactions in detail.

It remains to be elucidated whether boronation can influence the intracellular localization of the photosensitizer, a key factor of its therapeutic efficacy. *Meta*-tetra (hydroxyphenyl) chlorin has been shown to be localized in mitochondria and Golgi apparatus of HT29 human colon carcinoma cells[47] or in the nuclear envelope of EMT6 murine mammary sarcoma cells.[48] 13,15-*N*-Cycloimide derivatives of chlorin p_6 were observed in the lysosomes.[49] Also, chlorins can be targeted to the nucleus.[50–52] The boronated protoporphyrin and *nido*-carboranylchlorin were localized in the lysosomes.[42,53] We observed a diffuse staining of the cytoplasm (but not the nuclei) of Rat-1 fibroblasts and HCT116 colon carcinoma cells exposed to compounds **2** and **5** (data not shown). Clearly, the modifications of carboranylchlorins by varying the electric charge of the carborane and therefore modulating the amphiphilicity of the whole conjugate, as well as by conjugating the organelle-specific nucleic acid or amino acid sequences with the chlorin macrocycle, open the road to novel classes of chlorin-based drugs with preferential selectivity to the nuclei, mitochondria or the plasma membrane, the most vulnerable compartments for cell photodamage.

4. Conclusion

In summary, work of other authors[42,54,55] and our data (Ref. 8 and this study) expand the applicability of boronated derivatives of tetrapyrrole containing compounds beyond BNCT. These agents emerge as the drugs potentially efficient in binary anticancer strategies, that is, both PDT and BNCT. Considering the unique possibilities for chemical modifications of pyrrole rings, including those that confer cytotoxic properties to porphyrins and chlorins independently of cell illumination,[56,57] the therapeutic value of this class becomes all the more high.

5. Experimental

5.1. Chemistry

Methylpheophorbide *a* **1** was synthesized from chlorophyll *a*, which was extracted from the alga *Spirulina platensis* and then converted to compound **1**.[58] Compounds **2**–**4** were prepared as de-

scribed earlier.[20,59] IR spectra were recorded on a Specord M-82 of Carl Zeiss spectrometer in KBr tablets. The UV–vis spectra were measured on a spectrophotometer Jasco UV 7800 in $CHCl_3$. ^1H and ^{11}B NMR spectra were recorded on a Bruker Avance-400 spectrometer in $CDCl_3$. Chemical shifts (δ) are given in ppm relative to internal chloroform. Mass spectra were obtained using Vision 2000 (MALDI) mass spectrometer. Merck silica gel L 0.040–0.08 mesh was used for column chromatography. The identities of new compounds were verified on TLC 60 F_{254} plates (Merck) in $CHCl_3$–CH_3OH (9:1) solvent system. The solvents were purified according to standard procedures.

5.1.1. General procedures of synthesis of carboranylchlorins 5, 6, 8, 11 and 14

A solution of compounds **2, 3, 7, 10** or **13** (0.15 mmol) and triflate **4** (92 mg, 0.3 mmol) in THF (8 ml) was stirred with NaOAc (49 mg, 0.6 mmol) at 66 °C for 24 h. Then the reaction mixture was diluted with $CHCl_3$ (50 ml), washed with water (3 × 100 ml), dried over Na_2SO_4 and evaporated to dryness in vacuo. The crude product was purified by column chromatography on SiO_2 using $CHCl_3$–CH_3OH (9:1) as an eluent to give pure **5**, **6, 8, 11** or **14**, respectively.

5.1.2. Chlorin e_6 13(1)-*N*-{2-[*N*-(*o*-carboran-1-yl) methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester (5)

Method A. According to the general procedure from 100 mg (0.15 mmol) chlorin **2**, 25 mg (20%) of carboranylchlorin **5** was obtained. IR (KBr) ν_{max} 3309 (NH), 3069 (carborane CH), 2591 (BH), 1732 (CO), 1605 (chlorin band), 1636 (amide I), and 1522 cm^{-1} (amide II); UV ($CHCl_3$) λ_{max} 404 (ε 68,200), 501 (8890), 535 (2870), 611 (3760) and 663 nm (29,800); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.62 (1H, s, 10H), 9.60 (1H, s, 5H), 8.80 (1H, s, 20H), 8.39 (1H, dd, J = 17.6 and 11.2 Hz, 3^1H), 6.60 (1H, s, 13^1NH), 6.32 (1H, d, J = 18.0 Hz, 3^2H(trans)), 6.11 (1H, d, J = 11.6 Hz, 3^2H(cis)), 15^1CH_2: 5.40 (1H, d, J = 19.2 Hz) and 5.19 (1H, d, J = 18.6 Hz), 4.47 (1H, q, J = 6.8 Hz, 18H), 4.34 (1H, d, J = 9.2 Hz, 17H), 3.75 (2H, q, J = 7.6 Hz, 8^1CH_2), 3.64 (3H, s, 15^3CH_3), 3.61 (4H, m, 13^2CH_2, 13^3CH_2), 3.52 (1H, br s, carborane CH), 3.59 (3H, s, 17^4CH_3), 3.46 (3H, s, 12^1CH_3), 3.38 (3H, s, 2^1CH_3), 3.27 (3H, s, 7^1CH_3), 3.14 (1H, s, 13^3NH), NH–CH_2–C(carborane): 2.78 (1H, d, J = 14.0 Hz) and 2.74 (1H, d, J = 13.6 Hz), 2.57 (2H, m, 17^2CH_2), 2.17 (2H, m, 17^1CH_2), 3.0–1.4 (10H, br m, BH), 1.71 (3H, t, J = 7.6 Hz, 8^2CH_3), 1.71 (3H, d, J = 6.8 Hz, 18^1CH_3), −1.60 (1H, br s, I-NH), and −1.82 (1H, br s, III-NH); ^{11}B NMR ($CDCl_3$) (128.38 MHz) δ −1.70 (1B, d, J = 154 Hz), −4.21 (1B, d, J = 152 Hz), −8.04 (2B, d, J = 150 Hz), −10.44 (2B, d, J = 167 Hz), and −12.00 (4B, d, J = 175 Hz); MS (MALDI) m/z 825 ($M+H^+$).

Method B. To a solution of chlorin **8** (50 mg, 0.06 mmol) in CH_2Cl_2 (5 ml) CF_3COOH (0.50 ml, 0.8 mmol) was added. The mixture was stirred at room temperature for 2 h. Then the reaction mixture was diluted with CH_2Cl_2 (50 ml), washed with water (3 × 100 ml), dried over Na_2SO_4 and evaporated to dryness under vacuo to give pure **5** (46 mg, 99%). The structure of **5** obtaned by this method is identical to the resulting compound prepared by method A.

5.1.3. Chlorin e_6 13(1)-*N*-{2-[*O*-(*o*-carboran-1-yl)methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester (6)

From 100 mg (0.15 mmol) of chlorin **3** and 92 mg (0.3 mmol) of triflate **4**, 15 mg (12%) of carboranylchlorin **6** was obtained. IR (KBr) ν_{max} 3069 (carborane CH), 2591 (BH), 1732 (CO), 1605 (chlorin band), 1636 (amide I), and 1522 cm^{-1} (amide II); UV ($CHCl_3$) λ_{max} 404 (ε 141,100), 505 (13,200), 534 (5100), 608 (7800), and 665 nm (38,900); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.63 (1H, s, 10H), 9.59 (1H, s, 5H), 8.79 (1H, s, 20H), 8.04 (1H, dd, J = 18.0 and 11.6 Hz, 3^1H), 6.68 (1H, br s, 13^1NH), 6.32 (1H, d, J = 18.4 Hz, 3^2H(trans)), 6.11 (1H, d, J = 11.0 Hz, 3^2H(cis)), 15^1CH_2: 5.42 (1H, d, J = 18.4 Hz) and 5.21 (1H, d, J = 18.4 Hz), 4.46 (1H, q, J = 6.4 Hz, 18H), 4.34 (1H, d, J = 9.2 Hz, 17H), 3.76 (4H, m, 13^2CH_2, 13^3CH_2), 3.55 (2H, q, J = 7.6 Hz, 8^1CH_2), 3.70 (3H, s, 15^3CH_3), 3.59 (3H, s, 17^4CH_3), 3.46 (3H, s, 12^1CH_3), 3.43 (3H, s, 2^1CH_3), 3.27 (3H, s, 7^1CH_3), 2.56 (2H, s, O–CH_2–C (carborane)), 2.03 (1H, br s, carborane CH), 3.0–1.4 (10H, br m, BH), 1.61 (6H, m, 8^2CH_3, 18^1CH_3), 1.27 (4H, m, 17^2CH_2, 17^1CH_2), −1.57 (1H, br s, I-NH), and −1.80 (1H, br s, III-NH); ^{11}B NMR ($CDCl_3$) (128.38 MHz) δ −1.65 (1B, d, J = 149 Hz), −3.56 (1B, d, J = 151 Hz), −7.90 (2B, d, J = 155 Hz), −10.55 (2B, d, J = 170 Hz), and −11.77 (4B, d, J = 177 Hz); MS (MALDI) m/z 825 (M^+).

5.1.4. Zinc(II) complex of chlorin e_6 13(1)-*N*-{2-[*N*-(*o*-carboran-1-yl)methyl]aminoethyl} amide-15(2), 17(3)-dimethyl ester (8)

From 109 mg (0.15 mmol) of chlorin **7** and 92 mg (0.3 mmol) of triflate **4**, 70 mg (53%) of carboranylchlorin **8** was obtained. IR (KBr) ν_{max} 3312 (NH), 3069 (carborane CH), 2583 (BH), 1722 (CO), 1605 (chlorin band), 1647 (amide I), and 1522 cm^{-1} (amide II); UV ($CHCl_3$) λ_{max} 414 (ε 118,000), 515 (3,210), 598 (4870), and 642 nm (49,100); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.57 (1H, s, 10H), 9.51 (1H, s, 5H), 8.57 (1H, s, 20H), 8.03 (1H, dd, J = 17.6 and 11.7 Hz, 3^1H), 5.79 (1H, br s, 13^1NH), 6.18 (1H, d, J = 17.6 Hz, 3^2H(trans)), 6.03 (1H, d, J = 11.7 Hz, 3^2H(cis)), 15^1CH_2: 5.28 (1H, d, J = 19.2 Hz) and 5.07 (1H, d, J = 19.2 Hz), 4.35 (1H, q, J = 7.2 Hz, 18H), 4.15 (1H, d, J = 9.6 Hz, 17H), 4.14 (4H, m, 13^2CH_2, 13^3CH_2), 3.78 (3H, s, 15^3CH_3), 3.73 (2H, q, J = 7.2 Hz, 8^1CH_2), 3.57 (3H, s, 17^4CH_3), 3.51 (1H, br s, carborane CH), 3.35 (3H, s, 12^1CH_3), 3.31 (3H, s, 2^1CH_3), 3.29 (3H, s, 7^1CH_3), NH–CH_2–C (carborane): 3.27 (1H, d, J = 13.6 Hz) and 3.16 (1H, d, J = 13.8 Hz), 3.03 (1H, br s, 13^3NH), 2.52 (2H, m, 17^2CH_2), 2.23 (2H, m, 17^1CH_2), 3.0–1.4 (10H, br m, BH), 1.68 (3H, d, J = 7.2 Hz, 18^1CH_3), and 1.64 (3H, t, J = 7.2 Hz, 8^2CH_3); ^{11}B NMR ($CDCl_3$) (128.38 MHz) δ −2.89 (1B, d, J = 150 Hz), −5.09 (1B, d, J = 152 Hz), −9.24 (3B, d, J = 149 Hz), −11.79 (2B, d, J = 175 Hz), and −13.18 (3B, d, J = 175 Hz); MS (MALDI) m/z 822 ($M-Zn^+$).

5.1.5. Pd(II) complex of chlorin e_6 13(1)-*N*-{2-[*N*-(*o*-carboran-1-yl)methyl]aminoethyl} amide-15(2), 17(3)-dimethyl ester (11)

From 116 mg (0.15 mmol) of chlorin **10** and 92 mg (0.3 mmol) of triflate **4**, 88 mg (63%) of carboranylchlorin **11** was obtained. IR (KBr) ν_{max} 3300 (NH), 3057 (carborane CH), 2579 (BH), 1735 (CO), 1608 (chlorin band), 1637 (amide I), and 1520 cm^{-1} (amide II); UV ($CHCl_3$) λ_{max} 404 (ε 61,225), 492 (4500), 575 (9300), and 622 nm (37,100); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.58 (1H, s, 10H), 9.49 (1H, s, 5H), 8.64 (1H, s, 20H), 7.95 (1H, dd, J = 17.8 and 11.4 Hz, 3^1H), 6.54 (1H, s, 13^1NH), 6.14 (1H, d, J = 18.0 Hz, 3^2H(trans)), 6.01 (1H, d, J = 11.3 Hz, 3^2H(cis)), 15^1CH_2: 5.27 (1H, d, J = 19.2 Hz) and 4.93 (1H, d, J = 19.4 Hz), 4.44 (1H, q, J = 7.2 Hz, 18H), 4.31 (1H, d, J = 10.6 Hz, 17H), 4.08 (4H, m, 13^2CH_2, 13^3CH_2), 3.78 (3H, s, 15^3CH_3), 3.75 (2H, q, J = 7.9 Hz, 8^1CH_2), 3.60 (3H, s, 17^4CH_3), 3.52 (1H, br s, carborane CH), 3.32 (3H, s, 12^1CH_3), 3.31 (3H, s, 2^1CH_3), 3.27 (3H, s, 7^1CH_3), NH–CH_2–C (carborane): 3.26 (1H, d, J = 13.6 Hz) and 3.18 (1H, d, J = 13.6 Hz), 2.98 (1H, br s, 13^3NH), 2.49 (2H, m, 17^2CH_2), 2.34 (2H, m, 17^1CH_2), 3.0–1.2 (10H, br m, BH), 1.66 (3H, d, J = 7.3 Hz, 18^1CH_3), and 1.69 (3H, t, J = 7.9 Hz, 8^2CH_3); ^{11}B NMR ($CDCl_3$) (128.38 MHz) δ −2.80 (1B, d, J = 145 Hz), −5.08 (1B, d, J =147 Hz), −9.18 (3B, d, J = 150 Hz), and −13.22 (5B, d, J = 175 Hz); MS (MALDI) m/z 927 (M^+).

5.1.6. Sn(IV) dihydroxide complex of chlorin e_6 13(1)-*N*-{2-[*N*-(*o*-carboran-1-yl)methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester (14)

From 123 mg (0.15 mmol) of chlorin **13** and 92 mg (0.3 mmol) of triflate **4**, 71 mg (48%) of carboranylchlorin **14** was obtained. IR (KBr) ν_{max} 3437 (OH, NH), 3065 (carborane CH), 2580 (BH),

1726 (CO), 1610 (chlorin band), 1631 (amide I), and 1519 cm^{-1} (amide II); UV ($CHCl_3$) λ_{max} 410 (ε 34,300), 502 (3300), and 634 nm (25,900); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.67 (1H, s, 10H), 9.61 (1H, s, 5H), 8.79 (1H, s, 20H), 8.04 (1H, dd, J = 17.6 and 11.2 Hz, 3^1H), 6.32 (1H, s, 13^1NH), 6.21 (1H, d, J = 17.8 Hz, 3^2H(trans)), 6.13 (1H, d, J = 11.3 Hz, 3^2H(cis)), 15^1CH_2: 5.29 (1H, d, J = 19.2 Hz) and 5.11 (1H, d, J = 19.2 Hz), 4.45 (1H, q, J = 7.2 Hz, 18H), 4.32 (1H, d, J = 10.6 Hz, 17H), 4.10 (4H, m, 13^2CH_2, 13^3CH_2), 3.79 (3H, s, 15^3CH_3), 3.73 (2H, q, J = 7.9 Hz, 8^1CH_2), 3.61 (3H, s, 17^4CH_3), 3.53 (1H, br s, carborane CH), 3.36 (3H, s, 12^1CH_3), 3.32 (3H, s, 2^1CH_3), 3.29 (3H, s, 7^1CH_3), NH–CH_2–C (carborane): 3.24 (1H, d, J = 13.6 Hz) and 3.20 (1H, d, J = 13.8 Hz), 2.97 (1H, br s, 13^3NH), 2.48 (2H, m, 17^2CH_2), 2.32 (2H, m, 17^1CH_2), 3.0–1.3 (10H, br m, BH), 1.69 (3H, t, J = 7.9 Hz, 8^2CH_3), and 1.66 (3H, d, J = 7.2 Hz, 18^1CH_3); ^{11}B NMR ($CDCl_3$) (128.38 MHz) δ −2.70 (1B, d, J = 145 Hz), −5.50 (1B, d, J = 153 Hz), −9.22 (3B, d, J = 146 Hz), −11.82 (2B, d, J = 175 Hz), and −13.20 (3B, d, J = 154 Hz); MS (MALDI) m/z 976 (M^+).

5.1.7. Zinc(II) complex of chlorin e_6 13(1)-*N*-(2-aminoethyl)amide-15(2), 17(3)-dimethyl ester (7)

To a solution of chlorin **2** (50 mg, 0.08 mmol) in $CHCl_3$ (5 ml) and MeOH (1 ml) zinc acetate (60 mg, 0.3 mmol) was added. The resulting mixture was stirred at room temperature for 1 h. Then the reaction mixture was diluted with $CHCl_3$ (50 ml), washed with water (3 × 100 ml), dried over Na_2SO_4 and evaporated to dryness under vacuo. The residue was isolated by column chromatography on SiO_2 (eluent $CHCl_3$–CH_3OH 9:1) to give pure chlorin **7** (41 mg, 75%). IR (KBr) ν_{max} 3311 (NH), 1722 (CO), 1610 (chlorin band), 1647 (amide I), and 1523 cm^{-1} (amide II); UV ($CHCl_3$) λ_{max} 418 (ε 54,480), 522 (3520), 597 (5280), and 669 nm (28,120); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.76 (1H, s, 10H), 9.74 (1H, s, 5H), 9.52 (1H, s, 20H), 7.71 (1H, dd, J = 18.0 and 12.9 Hz, 3^1H), 6.31 (1H, br s, 13^1NH), 6.21 (1H, d, J = 17.8 Hz, 3^2H(trans), 6.00 (1H, d, J = 12.9 Hz, 3^2H(cis)), 15^1CH_2: 5.54 (1H, d, J = 19.2 Hz) and 5.27 (1H, d, J = 19.2 Hz), 4.35 (1H, q, J = 9.2 Hz, 18H), 4.34 (1H, d, J = 7.2 Hz, 17H), 4.14 (4H, m, 13^2CH_2, 13^3CH_2), 3.92 (2H, m, 8^1CH_2), 3.75 (3H, s, 15^3CH_3), 3.72 (3H, s, 17^4CH_3), 3.59 (3H, s, 12^1CH_3), 3.34 (3H, s, 2^1CH_3), 3.31 (3H, s, 7^1CH_3), 3.29 (2H, s, 13^3NH_2), 2.22 (2H, m, 17^2CH_2), 1.85 (2H, m, 17^1CH_2), and 1.34 (6H, m, 8^2CH_3, 18^1CH_3); MS (MALDI) m/z 664 ($M-Zn^+$).

5.1.8. Pd(II) complex of methylpheophorbide *a* (9)

To a solution of **1** (100 mg, 0.165 mmol) in CH_3CN (10 ml) a solution of palladium dichloride (35 mg, 0.2 mmol) in CH_3CN (50 ml) was added. The resulting mixture was stirred at 85 °C for 3 h and then was diluted with $CHCl_3$ (100 ml), washed with water (3 × 100 ml), dried over Na_2SO_4 and evaporated to dryness under vacuo. The residue was isolated by column chromatography on SiO_2 (eluent $CHCl_3$–CH_3OH 9:1) to give pure chlorin **9** (97 mg, 83%). IR (KBr) ν_{max} 1736 (CO ester), 1696 (CO 13(1)), and 1629 cm^{-1} (chlorin band); UV ($CHCl_3$) λ_{max} 407 (ε 69,500), 501 (5890), 534 (6450), 569 (3300), and 625 nm (39,100); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.54 (1H, s, 10H), 9.34 (1H, s, 5H), 8.55 (1H, s, 20H), 7.97 (1H, dd, J = 17.9 and 11.6 Hz, 3^1H), 6.34 (1H, s, 13^2H), 6.15 (1H, dd, J = 17.8 and 1.4 Hz, 3^2H(trans)), 6.03 (1H, dd, J = 11.6 and 1.4 Hz, 3^2H(cis)), 4.55 (1H, m, 18H), 4.26 (1H, m, 17H), 3.91 (3H, s, 13^3CH_3), 3.63 (2H, q, J = 7.6 Hz, 8^1CH_2), 3.62 (3H, s, 12^1CH_3), 3.55 (3H, s, 17^4CH_3), 3.41 (3H, s, 2^1CH_3), 3.17 (3H, s, 7^1CH_3), 2.48 (4H, m, 17^1CH_2, 17^2CH_2), 1.84 (3H, d, J = 7.4 Hz, 18^1CH_3), and 1.65 (3H, t, J = 7.6 Hz, 8^2CH_3); MS (MALDI) m/z 711 ($M+H^+$).

5.1.9. Pd(II) complex of chlorin e_6 13(1)-*N*-(2-aminoethyl)amide-15(2), 17(3)-dimethyl ester (10)

A solution of Pd(II) complex **9** (100 mg, 0.14 mmol) in $CHCl_3$ (10 ml) was stirred with ethylenediamine (0.8 ml, 1.2 mmol) at room temperature for 1 h. Then the reaction mixture was diluted with $CHCl_3$ (50 ml), washed with water (3 × 100 ml), dried over Na_2SO_4 and evaporated to dryness under vacuo. The residue was isolated by column chromatography on SiO_2 (eluent $CHCl_3$–CH_3OH 10:1) to give pure chlorin **10** (58 mg, 53.5%). IR (KBr) ν_{max} 3350 (NH), 1725 (CO), 1606 (chlorin band), 1643 (amide I), and 1522 cm^{-1} (amide II); UV ($CHCl_3$) λ_{max} 407 (ε 53,870), 501 (3470), 534 (5410) and 625 nm (17,100); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.56 (1H, s, 10H), 9.44 (1H, s, 5H), 8.63 (1H, s, 20H), 7.96 (1H, dd, J = 17.6 and 11.3 Hz, 3^1H), 6.85 (1H, br s, 13^1NH), 6.13 (1H, d, J = 17.8 Hz, 3^2H(trans)), 5.99 (1H, d, J = 11.8 Hz, 3^2H(cis)), 15^1CH_2: 5.37 (1H, d, J = 19.0 Hz) and 4.98 (1H, d, J = 18.7 Hz), 4.44 (1H, q, J = 6.8 Hz, 18H), 4.36 (1H, br d, J = 8.1 Hz, 17H), 4.12 (4H, m, 13^2CH_2, 13^3CH_2), 3.81 (3H, s, 15^3CH_3), 3.69 (2H, q, J = 7.0 Hz, 8^1CH_2), 3.59 (3H, s, 17^4CH_3), 3.35 (3H, s, 12^1CH_3), 3.31 (3H, s, 2^1CH_3), 3.25 (3H, s, 7^1CH_3), 3.18 (2H, s, 13^3NH_2), 2.49 (2H, m, 17^1CH_2), 2.04 (2H, m, 17^2CH_2), and 1.68 (6H, m, 8^2CH_3, 18^1CH_3); MS (MALDI) m/z 771 ($M+H^+$).

5.1.10. Sn(IV) dihydroxide complex of methylpheophorbide *a* (12)

To a solution of **1** (100 mg, 0.165 mmol) in CH_3OH (10 ml) hydrated tin dichloride (76 mg, 0.336 mmol) was added. The resulting mixture was stirred at 65 °C for 48 h and then was diluted with $CHCl_3$ (100 ml), washed with water (3 × 100 ml), dried over Na_2SO_4 and evaporated to dryness under vacuo. The residue was isolated by column chromatography on SiO_2 (eluent $CHCl_3$–CH_3OH 9:1) to give pure chlorin **12** (89 mg, 71%). IR (KBr) ν_{max} 3437 (OH), 1739 (CO ester), 1708 (CO 13(1)), and 1632 cm^{-1} (chlorin band); UV ($CHCl_3$) λ_{max} 420 (ε 79,750), 524 (2900), 569 (10,300), 610 (22,300), and 658 nm (56,700); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.94 (1H, s, 10H), 9.75 (1H, s, 5H), 8.79 (1H, s, 20H), 7.93 (1H, dd, J = 17.8 and 11.3 Hz, 3^1H), 6.28 (1H, dd, J = 17.8 and 1.5 Hz, 3^2H(trans)), 6.24 (1H, s, 13^2H), 6.21 (1H, dd, J = 17.5 and 1.5 Hz, 3^2H(cis)), 4.56 (1H, m, 18H), 4.45 (1H, m, 17H), 3.93 (3H, s, 13^3CH_3), 3.73 (3H, s, 12^1CH_3), 3.85 (2H, q, J = 7.4 Hz, 8^1CH_2), 3.60 (3H, s, 17^4CH_3), 3.39 (6H, s, 2^1CH_3, 7^1CH_3), 2.51 (2H, m, 17^1CH_2), 2.17 (2H, m, 17^2CH_2), 1.92 (3H, d, J = 7.2 Hz, 18^1CH_3), and 1.79 (3H, t, J = 7.4 Hz, 8^2CH_3); MS (MALDI) m/z 757 ($M+H^+$).

5.1.11. Sn(IV) dihydroxide complex of chlorin e_6 13(1)-*N*-(2-aminoethyl)amide-15(2), 17(3)-dimethyl ester (13)

A solution of Sn(IV) complex **12** (100 mg, 0.13 mmol) in $CHCl_3$ (10 ml) was stirred with ethylenediamine (0.8 ml, 1.2 mmol) at room temperature for 1 h. Then the reaction mixture was diluted with $CHCl_3$ (50 ml), washed with water (3 × 100 ml), dried over Na_2SO_4 and evaporated to dryness under vacuo. The residue was isolated by column chromatography on SiO_2 (eluent $CHCl_3$–CH_3OH 9:1) to give pure chlorin **13** (68 mg, 63%). IR (KBr) ν_{max} 3438 (OH, NH), 1727 (CO), 1733 (chlorin band), 1654 (amide I), and 1524 cm^{-1} (amide II); UV ($CHCl_3$) λ_{max} 420 (ε 59,700), 502 (3470), 610 (6230), and 658 nm (31,700); ^1H NMR ($CDCl_3$) (400 MHz) δ 9.67 (1H, s, 10H), 9.60 (1H, s, 5H), 8.87 (1H, s, 20H), 7.98 (1H, dd, J = 17.7 and 11.3 Hz, 3^1H), 6.96 (1H, br s, 13^1NH), 6.20 (1H, d, J = 17.9 Hz, 3^2H(trans)), 6.11 (1H, d, J = 11.2 Hz, 3^2H(cis)), 15^1CH_2: 5.53 (1H, d, J = 19.0 Hz) and 5.26 (1H, d, J = 19.0 Hz), 4.45 (1H, d, J = 6.9 Hz, 17H), 4.65 (1H, q, J = 8.2 Hz, 18H), 4.10 (4H, m, 13^2CH_2, 13^3CH_2), 3.88 (2H, q, J = 7.6 Hz, 8^1CH_2), 3.79 (3H, s, 15^3CH_3), 3.75 (3H, s, 17^4CH_3), 3.66 (3H, s, 12^1CH_3), 3.58 (3H, s, 2^1CH_3), 3.53 (3H, s, 7^1CH_3), 3.10 (2H, br s, 13^3NH_2), 2.56 (4H, m, 17^1CH_2, 17^2CH_2), and 1.23 (6H, m, 8^2CH_3, 18^1CH_3); MS (MALDI) m/z 817 ($M+H^+$).

5.2. Measurement of drug–albumin binding constants

The binding constants $K_b = 1/K_d$ were calculated on the basis of fluorescence spectra. For plotting of data we hypothesized that one molecule of **2** or **5** interacted with one binding site in HSA:

$$[\mathrm{Ch}l] + [\mathrm{HSA}] \leftrightarrow [\mathrm{Complex}], \quad (1)$$

where [Chl] is the concentration of **2** or **5**, [HSA] is the concentration of albumin, [Complex] is the concentration of complexes of **2** or **5** with HSA.

From this equation

$$K_d = \frac{[\mathrm{Chl}][\mathrm{HSA}]}{[\mathrm{Complex}]}, \quad (2)$$

$$\theta = \frac{[\mathrm{Ch}l]_b}{[\mathrm{Ch}l]_t} = \frac{[\mathrm{Complex}]}{[\mathrm{Ch}l] + [\mathrm{Complex}]}, \quad (3)$$

where θ is the fraction of **2** or **5** bound to HSA.

Eqs. (2) and (3) can be transformed into the following form of Scatchard's equation

$$\theta = 1 - K_d \times \frac{\theta}{[\mathrm{HSA}]_f}, \quad (4)$$

where $\theta = [\mathrm{Chl}]_b/[\mathrm{Chl}]_t = [\mathrm{HSA}]_b/[\mathrm{Chl}]_t$, $[\mathrm{HSA}]_f = [\mathrm{HSA}]_t - [\mathrm{HSA}]_b = [\mathrm{HSA}]_t - [\mathrm{Chl}]_t \times (F - F_0)/(F_{max} - F_0)$, $[\mathrm{Chl}]_f = [\mathrm{Chl}]_t \times (F_{max} - F)/(F_{max} - F_0)$, $[\mathrm{Chl}]_b = [\mathrm{Chl}]_t \times (F - F_0)/(F_{max} - F_0)$, $[\mathrm{HSA}]_f$, $[\mathrm{HSA}]_b$ and $[\mathrm{HSA}]_t$ are HSA concentrations: free, bound and initial (total), respectively, $[\mathrm{Chl}]_f$, $[\mathrm{Chl}]_b$ and $[\mathrm{Chl}]_t$ are the concentrations of **2** or **5**: free, bound and total, respectively, F_0 is fluorescence intensity of **2** or **5** at [HSA] = 0, F_{max} is fluorescence intensity in the excess of HSA (at $[\mathrm{HSA}] > 4 \times 10^{-5}$ M; Fig. 3) when only the complexes of **2** or **5** with HSA were present in the solution, F is fluorescence intensity of **2** or **5** at $[\mathrm{HSA}] < 4 \times 10^{-5}$ M.

The excited-state lifetime in the picosecond scale was measured with time-correlated single photon counting (TCSPC) technique (Fluorescence Lifetime Spectrometer FluoTime200; PicoQuant). Excitation was performed at 437 nm with a pulse laser diode (PicoQuant model LDH-P-C-400B; pulse duration ~60 ps, average power 0.5 mW at 20 MHz). Fluorescence was collected at 90°, and passed through monochromator (Sciencetech Model 9030) located in front of a single photon sensitive detector (Hamamatsu photomultiplier tube, H5783). The detector output was connected to the input of TCSPC computer board module (TimeHarp 200). The full width on the half maximum of the instrument response was ~100 ps. Measurements were performed in 1 cm quartz cell. The accuracy of measurement was ~0.1 ns.

5.3. Cell lines, dark cytotoxicity studies and PDT in culture

The McA 7777 rat liver epithelial cell line, Rat-1 and REF fibroblasts and MCF-10A human breast epithelial cell lines (all from American Type Culture Collection, Manassas, VA) were propagated in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, 5% CO_2 in humidified atmosphere. Human peripheral blood mononuclear cells were freshly isolated from donor blood (pool of 4 adult donors) using standard Ficoll gradient technique. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO unless specified otherwise. Newly synthesized compounds were dissolved as 10 mM stock solutions in DMSO, and serial aqueous dilutions were made immediately before experiments. The compounds were kept away from light. The experiments were performed in the dark. Cytotoxicity was tested in a 96-well format by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (MTT-test).[34] Each concentration of **5** was tested in three independent experiments. For PDT in culture, Rat-1 fibroblasts were seeded overnight into a 6-well plate (Costar; 5×10^4 cells per well) followed by the addition of **5** (final concentration 1 μM) for 30 min at 37 °C, 5% CO_2. After the completion of exposure the culture medium was replaced with 0.5 ml PBS, and cell monolayers were illuminated with the monochromatic red light (λ = 670 nm, density of light emission energy 200 J/cm^2) for 10 min. An aqueous solution of $NaNO_2$ was placed between the light source and the cells to protect the monolayer from warming. After illumination cells were replenished with fresh culture medium and incubated at 37 °C, 5% CO_2 for an additional 24 h. Control cells were left untreated (no drug, no light) or treated with 1 μM of **5** alone or illuminated in the absence of **5**. For quantitation of dead cells, PI (10 μg/ml) was added, and cells were analyzed by flow cytometry on FL2 (Becton Dickinson FACSCalibur System).

5.4. Animals and in vivo PDT

Rats were hosted in the animal facility of Medical Radiological Research Center, Obninsk, Russia. Animals were given food and water ad libitum. For transplantation of M-1 sarcoma, tumour cells were freshly isolated from the sarcoma-bearing animal. Three million tumour cells in 0.5 ml PBS were injected under the skin of rear extremities (one inoculum per animal). After the subcutaneously implanted tumour noduli reached ~0.3 cm^3 in volume, animals were divided into eight cohorts (10 animals per group). Rats in the group 1 were injected with PBS, rats in the group 2 were given LI in the absence of photosensitizers. Animals in the groups 3–4 were injected i.p. with 25 mg/kg of either **2** or **5**, respectively; no LI was administered in these groups. Rats in the group 5 were injected with 25 mg/kg of **2** followed by LI, and animals in the groups 6–8 were injected with either 5 mg/kg, 10 mg/kg or 25 mg/kg of **5**, respectively, followed by LI. In preliminary experiments we found that maximal accumulation of **5** in the tumours was achieved ~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 tumour illumination). Hair around the tumour was epilated prior to LI. The laser beam ('Atcus', λ = 670 nm, density of light emission energy 300 J/cm^2) was used for LI. The duration of exposure was calculated by the formula:

$$T = (D^2 \times E \times 13.09)/P, \quad (5)$$

where T is time (min) of LI, D is the biggest diameter (cm) of the tumour, E is the density of absorbed light energy (J/cm^2) and P is the power (mW) of emitted light.

The tumour size was measured immediately before LI and at days 3, 7, 14 and 21 post LI. The volume V (cm^3) of the tumour was calculated using the equation:

$$V = \frac{1}{6}\pi \times d_1 \times d_2 \times d_3, \quad (6)$$

where d_1, d_2 and d_3 are perpendicular diameters (cm) of the tumour mass.

Statistical analysis was performed using Student's t-test.

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References and notes

1. Pandey, R. K.; Bellnier, D. A.; Smith, K. M.; Dougherty, T. J. *Photochem. Photobiol.* **1991**, *53*, 65.

2. Nyman, E. S.; Hynninen, P. H. *J. Photochem. Photobiol. B: Biol.* **2004**, *73*, 1.
3. Pavlov, V. Yu.; Ponomarev, G. V. *Chem. Heterocycl. Comp.* **2004**, *40*, 393.
4. Sternberg, E. D.; Dolphin, D.; Brückner, C. *Tetrahedron* **1998**, *54*, 4151.
5. Wainwright, M. *Anti-Cancer Agents Med. Chem.* **2008**, *8*, 280.
6. Mody, T. D. *J. Porphyrins Phthalocyanines* **2000**, *4*, 362.
7. Josefsen, L. B.; Boyle, R. W. *Br. J. Pharmacol.* **2008**, *154*, 1.
8. Spikes, J. D. *J. Photochem. Photobiol. B: Biol.* **1990**, *6*, 259.
9. Kostron, H.; Obwegeser, A.; Jakober, R. J. *Photochem. Photobiol.* **1996**, *36*, 157.
10. Soloway, A. H.; Tjarks, W.; Barnum, B. A.; Rong, F.-G.; Barth, R. F.; Codogni, I. M.; Wilson, J. G. *Chem. Rev.* **1998**, *98*, 1515.
11. Evstigneeva, R. P.; Zaitsev, A. V.; Luzgina, V. N.; Ol'shevskaya, V. A.; Shtil, A. A. *Curr. Med. Chem. Anti-Cancer Agents* **2003**, *2*, 383.
12. Dozzo, P.; Koo, M.-S.; Berger, S.; Forte, T. M.; Kahl, S. B. *J. Med. Chem.* **2005**, *48*, 357.
13. Ratajski, M.; Osterloh, J.; Gabel, D. *Anti-Cancer Agents Med. Chem.* **2006**, *6*, 159.
14. Renner, M. W.; Miura, M. M.; Easson, W.; Vicente, M. G. H. *Anti-Cancer Agents Med. Chem.* **2006**, *6*, 145.
15. Gottumukkala, V.; Ongayi, O.; Baker, D. G.; Lomax, L. G.; Vicente, M. G. H. *Bioorg. Med. Chem.* **2006**, *14*, 1871.
16. Ol'shevskaya, V. A.; Zaitsev, A. V.; Savchenko, A. N.; Shtil, A. A.; Chan, Seong Cheong; Kalinin, V. N. *Bull. Korean Chem. Soc.* **2007**, *28*, 1910.
17. Spiuirril, P. G.; Hill, J. S.; Kahl, S. B.; Ghiggino, K. P. *Photochem. Photobiol.* **1996**, *64*, 975.
18. Ol'shevskaya, V. A.; Nikitina, R. G.; Zaitsev, A. V.; Luzgina, V. N.; Kononova, E. G.; Morozova, T. G.; Drozhzhina, V. V.; Ivanov, O. G.; Kaplan, M. A.; Kalinin, V. N.; Shtil, A. A. *Org. Biomol. Chem.* **2006**, *4*, 3815.
19. Zheng, G.; Aoudia, M.; Lee, D.; Rodgers, M. A.; Smith, K. M.; Dougherty, T. J.; Pandey, R. K. *J. Chem. Soc., Perkin Trans. 1* **2000**, *18*, 3113.
20. Belykh, D. V.; Karmanova, L. V.; Spirikhin, L. V.; Kutchin, A. V. *Mendeleev Commun.* **2002**, *12*, 77.
21. Tamiaki, H.; Amakawal, M.; Shimonol, Y.; Tanikaga, R.; Hotzwarth, A. R.; Schaffner, K. *Photochem. Photobiol.* **1996**, *63*, 92.
22. Szaciłowski, K.; Macyk, W.; Drzewiecka-Matuszek, A.; Brindell, M.; Stochel, G. *Chem. Rev.* **2005**, *105*, 2647.
23. Morgan, A. R.; Garbo, G. M.; Keck, R. W.; Selman, S. H. *Cancer Res.* **1988**, *48*, 194.
24. Kessel, D.; Morgan, A.; Garbo, G. M. *Photochem. Photobiol.* **1991**, *54*, 193.
25. Pogue, B. W.; Redmond, R. W.; Trivedi, N.; Hasan, T. *Photochem. Photobiol.* **1998**, *68*, 809.
26. Arnold, D. P.; Blok, J. *Coord. Chem. Rev.* **2004**, *248*, 299.
27. Weersink, R. A.; Bogaards, A.; Gertner, M.; Davidson, S. R. H.; Zhang, K.; Netchev, G.; Trachtenberg, J.; Wilson, B. C. *J. Photochem. Photobiol. B: Biol.* **2005**, *79*, 211.
28. Zhu, T. C.; Finlay, J. C. *Photodiagn. Photodyn. Ther.* **2006**, *3*, 234.
29. Schreiber, S.; Gross, S.; Brandis, A.; Harmelin, A.; Rosenbach-Belkin, V.; Scherz, A.; Salomon, Y. *Int. J. Cancer* **2002**, *99*, 279.
30. Woodhams, J. H.; MacRobert, A. J.; Novelli, M.; Bown, S. G. *Int. J. Cancer* **2006**, *118*, 477.
31. Trachtenberg, J.; Bogaards, A.; Weersink, R. A.; Haider, M. A.; Evans, A.; McCluskey, S. A.; Scherz, A.; Gertner, M. R.; Yue, C.; Appu, S.; Aprikian, A.; Savard, J.; Wilson, B. C.; Elhilali, M. *J. Urol.* **2007**, *178*, 1974.
32. Kessel, D.; Thompson, P.; Saatio, K.; Nantwi, K. D. *Photochem. Photobiol.* **1987**, *45*, 787.
33. Mishra, P.; Patel, S.; Datta, A. *J. Phys. Chem. B* **2006**, *110*, 21238.
34. Sidorova, T. A.; Nigmatov, A. G.; Kakpakova, E. S.; Stavrovskaya, A. A.; Gerassimova, G. K.; Shtil, A. A.; Serebryakov, E. P. *J. Med. Chem.* **2002**, *45*, 5330.
35. Borbas, K. E.; Mroz, P.; Hamblin, M. R.; Lindsey, J. S. *Bioconjug. Chem.* **2006**, *17*, 638.
36. Borbas, K. E.; Chandrashaker, V.; Muthiah, C.; Kee, H. L.; Holten, D.; Lindsey, J. S. *J. Org. Chem.* **2008**, *73*, 3145.
37. Muthiah, C.; Taniguchi, M.; Kim, H. J.; Schmidt, I.; Kee, H. L.; Holten, D.; Bocian, D. F.; Lindsey, J. S. *Photochem Photobiol.* **2007**, *83*, 1513.
38. Al-Omari, S. *Biomed. Mater.* **2007**, *2*, 107.
39. Thomas, N.; Tirand, L.; Chatelut, E.; Plénat, F.; Frochot, C.; Dodeller, M.; Guillemin, F.; Barberi-Heyob, M. *Photochem. Photobiol. Sci.* **2008**, *7*, 433.
40. Zheng, X.; Pandey, R. K. *Anti-Cancer Agents Med. Chem.* **2008**, *8*, 241.
41. Kee, H. L.; Kirmaier, C.; Tang, Q.; Diers, J. R.; Muthiah, C.; Taniguchi, M.; Laha, J. K.; Ptaszek, M.; Lindsey, J. S.; Bocian, D. F.; Holten, D. *Photochem. Photobiol.* **2007**, *83*, 1110.
42. Luguya, R.; Jensen, T. J.; Smith, K. M.; Vicente, M. G. *Bioorg. Med. Chem.* **2006**, *14*, 5890.
43. Bardos-Nagy, I.; Galantay, R.; Fidy, J. *Biochim. Biophys. Acta* **2001**, *1512*, 125.
44. Chowdhary, R. K.; Shariff, I.; Dolphin, D. *J. Pharm. Pharm. Sci.* **2003**, *6*, 13.
45. Mojzisova, H.; Bonneau, S.; Vever-Bizet, C.; Brault, D. *Biochim. Biophys. Acta* **2007**, *1768*, 366.
46. Novick, S.; Laster, B.; Quastel, M. R. *Int. J. Biochem. Cell Biol.* **2006**, *38*, 1374.
47. Melnikova, V. O.; Bezdetnaya, L. N.; Bour, C.; Festor, E.; Gramain, M. P.; Merlin, J. L.; Potapenko, A. Ya.; Guillemin, F. *J. Photochem. Photobiol. B* **1999**, *49*, 96.
48. Foster, T. H.; Pearson, B. D.; Mitra, S.; Bigelow, C. E. *Photochem. Photobiol.* **2005**, *81*, 1544.
49. Nazarova, A.; Ignatova, A.; Feofanov, A.; Karmakova, T.; Pljutinskaya, A.; Mass, O.; Grin, M.; Yakubovskaya, R.; Mironov, A.; Maurizot, J. C. *Photochem. Photobiol. Sci.* **2007**, *6*, 1184.
50. Akhlynina, T. V.; Jans, D. A.; Statsyuk, N. V.; Balashova, I. Y.; Toth, G.; Pavo, I.; Rosenkranz, A. A.; Naroditsky, B. S.; Sobolev, A. S. *Int. J. Cancer* **1999**, *81*, 734.
51. Ogura, S.; Yazaki, K.; Yamaguchi, K.; Kamachi, T.; Okura, I. *J. Control Release* **2005**, *103*, 1.
52. Swamy, N.; Purohit, A.; Fernandez-Gacio, A.; Jones, G. B.; Ray, R. *J. Cell Biochem.* **2006**, *99*, 966.
53. Callahan, D. E.; Forte, T. M.; Afzal, S. M.; Deen, D. F.; Kahl, S. B.; Bjornstad, K. A.; Bauer, W. F.; Blakely, E. A. *Int. J. Radiat. Oncol. Biol. Phys.* **1999**, *45*, 761.
54. Ozawa, T.; Santos, R. A.; Lamborn, K. R.; Bauer, W. F.; Koo, M. S.; Kahl, S. B.; Deen, D. F. *Mol. Pharm.* **2004**, *1*, 368.
55. Koo, M. S.; Ozawa, T.; Santos, R. A.; Lamborn, K. R.; Bollen, A. W.; Deen, D. F.; Kahl, S. B. *J. Med. Chem.* **2007**, *50*, 820.
56. Wongsinkongman, P.; Brossi, A.; Wang, H. K.; Bastow, K. F.; Lee, K. H. *Bioorg. Med. Chem.* **2002**, *10*, 583.
57. Ol'shevskaya, V. A.; Zaitsev, A. V.; Luzgina, V. N.; Kondratieva, T. T.; Kononova, E. G.; Petrovskii, P. V.; Mironov, A. F.; Kalinin, V. N.; Hofmann, J.; Shtil, A. A. *Bioorg. Med. Chem.* **2006**, *14*, 109.
58. Smith, K. M.; Goff, D. F.; Simpson, D. J. *J. Am. Chem. Soc.* **1985**, *107*, 4946.
59. Kalinin, V. N.; Rys, E. G.; Tyutyunov, A. A.; Starikova, Z. A.; Korlyukov, A. A.; Ol'shevskaya, V. A.; Sung, D. D.; Ponomaryov, A. B.; Petrovskii, P. V.; Hey-Hawkins, E. *Dalton Trans.* **2005**, *5*, 903.