Boronated protohaemins: synthesis and *in vivo* antitumour efficacy

Valentina A. Ol'shevskaya,*[a] Roza G. Nikitina,[b] Andrei V. Zaitsev,[a] Valentina N. Luzgina,[c] Elena G. Kononova,[a] Tamila G. Morozova,[b] Valentina V. Drozhzhina,[b] Oleg G. Ivanov,[d] Mikhail A. Kaplan,[b] Valery N. Kalinin[a] and Alexander A. Shtil[d]

Received 31st May 2006, Accepted 18th August 2006
First published as an Advance Article on the web 1st September 2006
DOI: 10.1039/b607766h

The conjugates of porphyrin macrocycles with boron-containing polyhedra are under investigation as agents for binary treatment strategies of cancer. Aiming at the design of photoactive compounds with low-to-zero dark toxicity, we synthesized a series of carboranyl and monocarbon-carboranyl derivatives of protohaemin IX using the activation of porphyrin carboxylic groups with di-*tert*-butyl pyrocarbonate or pivaloyl chloride. The water-soluble 1,3,5,8-tetramethyl-2,4-divinyl-6(7)–[2′-(*closo*-monocarbon-carborane-1″-yl)methoxycarbonylethyl]-7(6)-(2′-carboxyethyl)porphyrin Fe(III) (compound **9**) exerted no discernible cytotoxicity for cultured mammalian cells, nor did it cause general toxicity in rats. Importantly, **9** demonstrated dose-dependent activity as a phototoxin in photodynamic therapy of M-1 sarcoma-bearing rats. In animals injected with 20 mg kg^{-1} of **9**, the tumours shrank by day 3 after one single irradiation of the tumour with red laser light. Between 7 and 14 days post-irradiation, 88.9% of rats were tumour-free; no recurrence of the disease was detectable within at least 90 days. Protohaemin IX alone was without effect, indicating that boronation is important for the phototoxic activity of **9**. This is the first study that presents the synthesis and preclinical *in vivo* efficacy of boronated derivatives of protohaemin as phototoxins. The applicability in photodynamic treatment broadens the therapeutic potential of boronated porphyrins beyond their conventional role as radiosensitizers in boron neutron capture therapy.

Introduction

Photodynamic therapy (PDT) and boron neutron capture therapy (BNCT) emerge as important treatment modalities in patients with locally advanced cancer.[1,2] The efficacy of these binary strategies is based on two critical events: accumulation of the photo- or radiosensitizing drug in the tumour site and triggering cytotoxicity *via* drug activation by external irradiation.[3] Both these goals can be accomplished by design of agents containing porphyrins, the macrocyclic compounds abundantly present in the cells being the components of prosthetic groups of many enzymes.[4] The following features of porphyrins favour their use as anticancer agents: 1) highly reactive free-oxygen species are generated upon tumour irradiation; 2) porphyrins are entrapped predominantly by actively proliferating cells; therefore, the conjugates of porphyrins with other potential killers would be targeted to the tumour while sparing normal tissues; 3) due to functional groups at the periphery of the macrocycle, porphyrins are suitable for conjugating other potentially cytotoxic moieties including boron-containing polyhedra (carboranes).[5,6] Indeed, boronated derivatives of porphyrins have been the subjects of an extensive investigation as tentative agents for binary anticancer strategies.[7]

A major problem that limits clinical use of porphyrin-based compounds is general (dark) toxicity. Although boronated porphyrins demonstrate therapeutic efficacy due to high ratio of tumour-to-tissue content and the ability to generate intratumoural ionization processes,[2,6,8] these compounds may cause toxicity prior to irradiation. Our carborane-substituted derivatives of 5,10,15,20-tetraphenylporphyrin and their metal complexes were toxic to cultured human cells, largely due to the cytotoxicity of the porphyrin moiety.[9] Although the anticancer activity of carbonylporphyrins without irradiation is important,[9] their dark toxicity might be unfavourable in clinical situations. Thus, use of non-toxic porphyrins for design of carboranylporphyrins should attenuate general toxicity while their antitumour activity in binary treatments is expected to be retained. Photofrin®, a mixture of porphyrin oligomers derived from natural products, recently entered clinical trials as a photosensitizer for PDT of bladder, stomach, lung, esophageal and cervical tumours. However, skin photosensitivity emerged as an unfavourable effect, and a series of novel porphyrins and chlorins have been synthesized to obtain active antitumour compounds with attenuated general toxicity.[10] Studies of BOPP [the tetrakiscarborane carboxylate ester of 2,4-bis(α,β-dihydroxyethyl)deuteroporphyrin IX disodium salt], a water-soluble boronated porphyrin, demonstrated its excellent characteristics such as selective tumour uptake, mitochondrial localization and an anticancer effect in PDT of experimental intracranial tumours and in phase I clinical trials.[11,12] However, thrombocytopenia was a dose-limiting factor, and skin photosensitivity needed to be taken into consideration.[11]

[a]*A. N. Nesmeyanov Institute of Organoelement Compounds, 28 Vavilov Street, 119991, Moscow, Russia. E-mail: olshevsk@ineos.ac.ru; Fax: +7 (495) 135-5085; Tel: +7 (495) 135-7933*
[b]*Medical Radiological Research Center, 4 Korolev Street, 249020, Obninsk, Russia*
[c]*M. V. Lomonosov Moscow State Academy of Fine Chemical Technology, 86 Vernadsky Avenue, 117571, Moscow, Russia*
[d]*N. N. Blokhin Cancer Center, 24 Kashirskoye shosse, 115478, Moscow, Russia*

We have developed the synthesis of carboranyl derivatives of deuteroporphyrin IX and protoporphyrin IX using the activation of porphyrin carboxylic groups with di-*tert*-butyl pyrocarbonate (Boc_2O) or pivaloyl chloride.[13] This method allowed us to obtain the neutral and anionic congeners in which the boron polyhedra are linked to the porphyrin ring by ester or amide bonds. We report here the synthesis of a series of carboranyl and monocarbon-carboranyl derivatives of protohaemin IX (**1**), a component of haem-containing proteins. Importantly, 1,3,5,8-tetramethyl-2,4-divinyl-6(7)-[2′-(*closo*-monocarbon-carborane-1″-yl)methoxycarbonylethyl]-7(6)-(2′-carboxyethyl)porphyrin Fe(III) (compound **9**), being non-toxic and well tolerated by tumour-bearing animals, demonstrated high antitumour activity in PDT of experimental sarcoma.

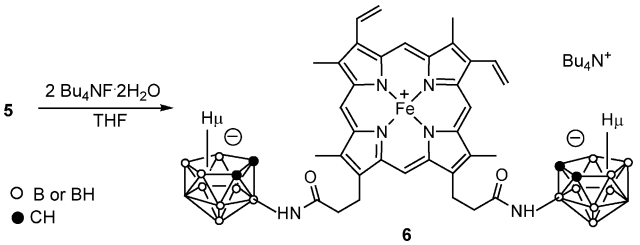
Results and discussion

Chemistry

The reaction of porphyrin **1** with neutral 9-hydroxymethyl-*m*-carborane **2** in a Py–CH_2Cl_2 system in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP) resulted in the cationic dicarboranyl-substituted porphyrin **3** (Scheme 1). The carboxylic groups of **1** were activated by Boc_2O.

The carborane fragment in **3** is linked to the porphyrin macrocycle by an ester bond through the boron atom of the polyhedron. Under the same reaction conditions compound **1** reacted with neutral 3-amino-*o*-carborane **4** to yield compound **5**, in which the carborane moiety is bound to porphyrin *via* the amide bond through the boron atom of the polyhedron (Scheme 2).

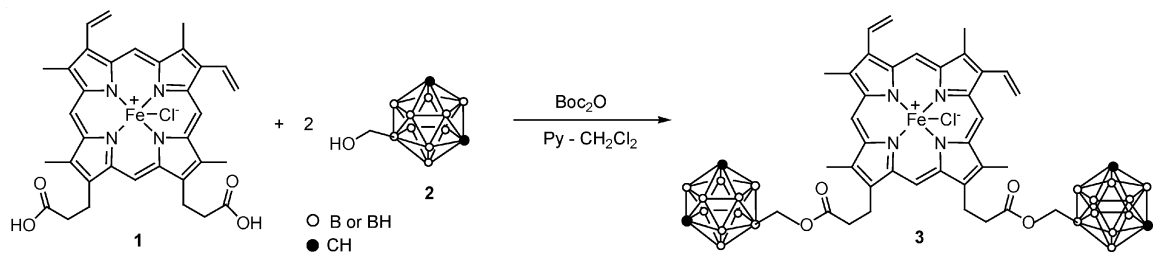
Regardless of the fact that **3** and **5** are cations, these compounds were hydrophobic and poorly soluble in hydroxyl-containing solvents. Given that amphiphilicity is a prerequisite for biological use of carboranylporphyrins, we improved the hydrophilicity by 1) modifying the *closo*-polyhedra into their water soluble *nido*-analogues, and 2) direct introduction of anionic *closo*-monocarbon-carborane polyhedra into the porphyrin system. Deboronation of *closo*-polyhedra in porphyrin **5** into their corresponding *nido*-analogues was performed by the reaction of porphyrin **5** with $Bu_4NF{\cdot}2H_2O$ in THF to yield the anionic porphyrin **6** with two amphiphilic *nido*-7,8-dicarbaundecaborate substituents (Scheme 3). The porphyrin **6** was isolated as its tetrabutylammonium salt with 85% yield.



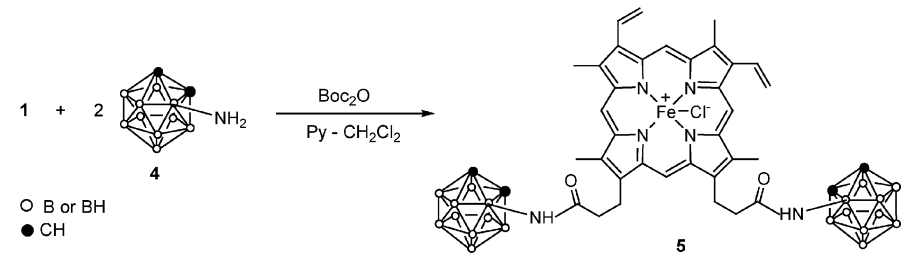
Scheme 3

To introduce the *closo*-monocarbon-carborane anion into the porphyrin system, we used porphyrin **1** and (1-hydroxymethyl-*closo*-monocarbon-carborane)caesium **7** or {1-[1′-hydroxy(phenyl)methyl]-*closo*-monocarbon-carborane}caesium **8** to yield porphyrins **9** and **10**. Although alcohols **7** and **8** were used in a 4-fold excess relative to compound **1**, we isolated only mono-substituted zwitterionic monocarbon-carboranylporphyrins **9** and **10**. This fact is likely to be due to specific features of the electronic structure of the monocarbon-carborane polyhedron, which make the formation of the zwitterionic product preferable. In contrast to the anionic monocarbon-carboranes, neutral carboranes **2** and **4** readily formed disubstituted protohaemins **3** and **5**. The monocarbon-carboranes in **9** and **10** were linked to the porphyrin by an ester bond through the carbon atom of the polyhedron (Scheme 4).

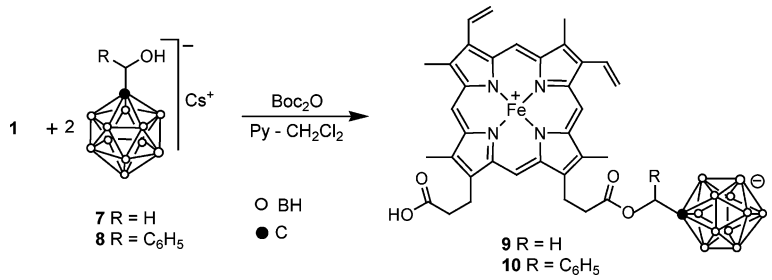
The free carboxylic group in compounds **9** and **10** allows for the introduction of various cations, which might improve water solubility of the whole conjugate. In our experiments the free carboxylic group in **9** was used for conjugating the neutral *closo*-carborane substituent to further increase the boron content



Scheme 1

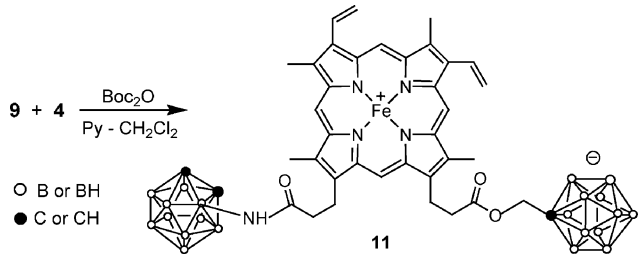


Scheme 2



Scheme 4

in the complex. The reaction of **9** with carborane **4** resulted in disubstituted protohaemin **11** in 63% yield. Compound **11** contains the anionic *closo*-monocarbon-carborane and neutral *closo*-carborane polyhedra linked to the porphyrin macrocycle by ester and amide bonds, respectively (Scheme 5). This approach demonstrates a way of synthesizing derivatives of haemin with higher boron content.



Scheme 5

Thus, we obtained a series of novel derivatives of protohaemin IX (**1**). As we have demonstrated for other carboranylporphyrins,[13] the mixed anhydride formed upon interaction of the carboxylic groups of **1** and Boc_2O is an effective acylating agent for introducing both anionic and neutral carboranes into protohaemin IX. The water-soluble derivatives of boronated protohaemin IX can be good candidates for binary anticancer treatments. Most importantly, the hydrolytically stable *closo*-monocarbon-carborane makes compounds **9** and **10** clinically promising.

All synthesized compounds were isolated by column chromatography as dark red crystals soluble in chloroform, methylene chloride, THF, pyridine, acetone, and acetonitrile. The identity of all compounds was confirmed by mass spectra, and electronic and infrared spectroscopy.

Biological testing

We considered two major criteria, *i.e.*, the solubility in water and the boron content, for selecting novel boronated derivatives of protohaemin IX as phototoxins in experimental therapy for sarcoma. In this study we selected compound **9**, notwithstanding the fact that the boron content in **9** is somewhat lower than in **11** (~16% and ~25%, respectively). Although both **9** and **10** are zwitterionic, at therapeutic concentrations (see below) **10** was less hydrophilic than **9**, probably due to the phenyl group in **10**. The higher water solubility of **9** can be attributed to the anionic boron polyhedron and free carboxylic group. In the initial experiments we tested the toxicity of **9** on cultured cells. Treatment with **9** (up to 100 μM for 72 h) caused no death or growth retardation of McA 7777 liver epithelium, Rat-1 and REF fibroblasts, freshly isolated peripheral blood lymphocytes or human breast epithelium (MCF-10A cell line) (data not shown). At concentrations >100 μM the solubility of **9** in culture medium was limited. Therefore, at least at the doses not exceeding the solubility limit, compound **9** had no effect upon the tested mammalian cells.

Next, we tested the activity of **9** as a photosensitizer in PDT of cultured Rat-1 cells. Compound **9** (10 μM or 20 μM) was added to the cells over a period of 3 h, followed by withdrawal of the drug and irradiation of cells with the laser beam (see the Experimental for details). Twenty-four hours post-irradiation, the phototoxic effect was clearly detectable. At 10 μM of **9** almost all cells lost their polygonal shape and became rounded; ~30–50% of cells detached from the plastic and floated in the medium. At 20 μM of **9** we observed severe damage to cells, leakage of the cytoplasm and disruption of the nuclei. No signs of cytotoxicity were detected in untreated cells, in cells exposed to **9** alone or in cells irradiated in the absence of **9**. The phototoxic effect was reproduced in three experiments, proving that **9** is promising for further investigation as a photosensitizer *in vivo*.

We set out to study the *in vivo* efficacy of **9** in PDT of M-1 rat sarcoma.[14] After the subcutaneously transplanted tumour noduli reached ~0.1 cm^3 in volume, animals were divided into four cohorts (9 animals per group). Rats in group 1 were left untreated (no drug, no irradiation). Animals in group 2 were injected i.p. with saline followed by PDT (see below). Animals in groups 3 and 4 were injected i.p. with compound **9**; group 3 was subjected to PDT, whereas group 4 received no laser treatment. Thus, groups 1, 2 and 4 were controls for group 3 (experimental). In preliminary experiments we found that the doses of **9** up to 20 mg kg^{-1} i.p. did not affect animals' behavior, their nutritional habits, nor did we notice hair loss or altered blood cell count. These data suggested no general toxicity of **9** within this range of concentrations. Tumour irradiation performed 3 h or 24 h after injection of **9** resulted in a similar therapeutic outcome; however, the complete responses (see the Experimental) were more frequent with the latter regimen (data not shown). Therefore, in the subsequent experiments we injected **9** (2.5–20 mg kg^{-1}) i.p. followed by tumour irradiation 24 h later.

In all control cohorts the tumours grew exponentially with similar rates. Tumour irradiation in group 2 or injection of **9** without subsequent irradiation had no effect on the growth of sarcoma compared to untreated animals (data not shown). In striking contrast, PDT in rats injected with 2.5 mg kg^{-1} of compound **9** led to a significant reduction of the tumour size (Fig. 1).

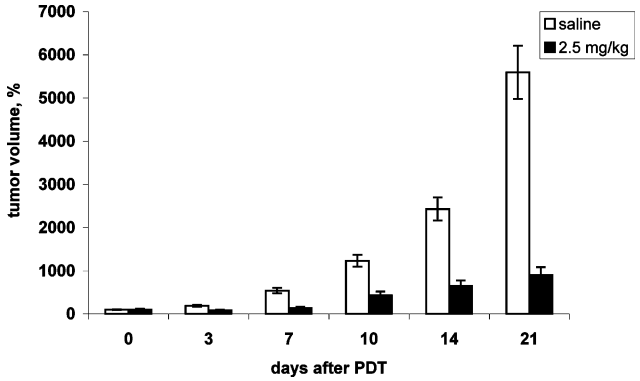


Fig. 1 Phototoxic activity of compound **9**. Rats bearing s.c. transplanted sarcoma M-1 node were injected i.p. with saline or 2.5 mg kg^{-1} of compound **9**. Twenty-four hours later the tumours were irradiated with red laser (see the Experimental). Tumour size was measured at the indicated time intervals and plotted as the percentage of the size of the tumour before irradiation (100%). One representative experiment out of a total of three is presented. $P < 0.05$ between the 'saline' group and the 'compound **9**' group at days 7, 10, 14 and 21.

Retardation of tumour growth was observed early after irradiation (by days 3–6) and remained sustained during the period of follow-up (until day 21). With this schedule a complete response resulted in one animal out of a total of 9 in the group. These data proved the principle that our boronated derivative of protohaemin IX can be a candidate agent for anticancer PDT.

We next tested higher doses of compound **9**, given that this agent caused no general toxicity. At 5 mg kg^{-1} the tumour shrinkage (up to 50% of the initial volume) was observed in 8 out of 9 rats by day 3 post-irradiation. However, all tumours eventually resumed growth; overall the therapeutic effect was <50% (Fig. 2). A complete response was registered only in 2 out of 9 rats (22.2%). With 10 mg kg^{-1} of compound **9** the therapeutic effect was statistically higher during the whole follow-up period (~60–80%; Fig. 2); however, only 3 out of 9 (33.3%) rats were cured by day 21 post-irradiation. The most robust effect of PDT was achieved in rats injected with 20 mg kg^{-1} of compound **9**. A marked decrease of the tumour size (down to 10–20% of its initial volume) was detected in all animals as early as by day 3 post-irradiation. Remarkably, 7 out of 9 animals were cured by days 7–9; in one rat the palpable tumour disappeared by day 14. Therefore, 88.9% of animals were cured within the initial 1–2 weeks of PDT and remained tumour-free for at least 90 days. Re-growth of sarcoma after partial response (~50% of the initial volume) was registered only in one rat. No signs of general toxicity (such as anaemia and loss of weight or hair) were observed during the entire time of monitoring of the animals. In the experiments with matched concentrations of protohaemin IX and **9**, no phototoxic activity of the former compound was observed, *i.e.* no decrease of tumour size was detected in rats injected with 2.5–20 mg kg^{-1} of protohaemin IX followed by laser treatment (data not shown).

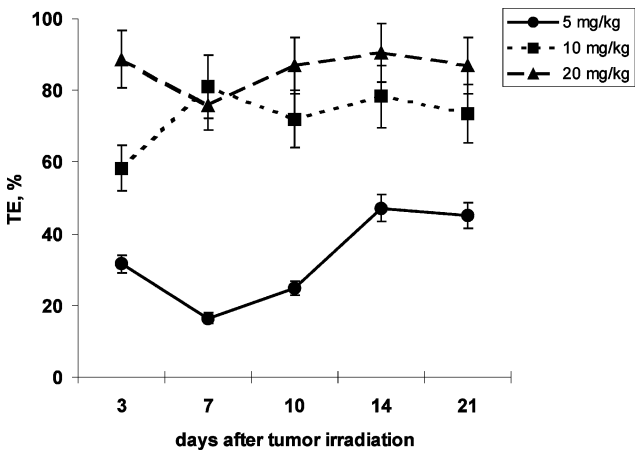


Fig. 2 The antitumour effect of PDT is dependent on the concentration of compound **9**. Tumour-bearing animals were injected i.p. with compound **9** at the indicated concentrations and subjected to PDT. Tumour size was monitored until 21 days post-irradiation. Therapeutic effect (TE) was calculated as described in the Experimental section. Values are mean ± S.D. of three independent experiments. $P < 0.05$ between the '5 mg kg^{-1}' group and the two other groups.

General cytotoxicity of synthetic porphyrin-based compounds (dark toxicity) frequently hampers their clinical use. Therefore, non-toxic porphyrins are likely to be more suitable for the design of practically applicable drugs. Indeed, the efficacy of protohaemin IX as a photosensitizer has been demonstrated. In these protocols protohaemin IX was generated in the body from its precursor, 5-aminolevulinic acid (5-ALA), in the haem cycle.[15,16] The effect of PDT largely depended on the delivery of a sufficient amount of 5-ALA to the tumour site and metabolic conversion of the precursor into protohaemin IX in cells across the tumour.[17] However, intratumoural accumulation of the precursor can be limited by impaired blood supply and by the fact that at physiological pH 5-ALA is hydrophilic and zwitterionic.[18] Thus, design of more lipophilic 5-ALA derivatives and their activation in tumour cells remain as yet unresolved issues.[16,19] Furthermore, the biosynthesis of protohaemin IX is regulated by cell size, cell density, differentiation status and cell cycle phase.[20,21] Therefore, tumour heterogeneity is likely to be the reason for unequal production of protohaemin IX in different areas within the tumour. Therefore, the metabolic conversion of the precursor as an approach for achieving a sufficient amount of the photoactive drug in the tumour has its limitations; we believe that exogenous protohaemin IX-based compounds could be advantageous, as they bypass the requirement for being metabolized to yield a therapeutically active phototoxin.

Aiming at the design of efficient antitumour agents with minimal general toxicity, we used protohaemin IX for conjugation with boron-containing polyhedra. Our data demonstrate that the water-soluble compound **9** has virtually no effect upon human and rat cells. In contrast, **9** markedly potentiated the cytotoxic effect of laser treatment in cultured fibroblasts. Most importantly, PDT with non-toxic concentrations of **9** completely cured 88.9% of animals. Thus, this boronated derivative of protohaemin IX is a prospective anticancer agent.

The exact mechanism of phototoxicity of boronated protohaemin IX remains to be elucidated; however, other studies have attributed it to the biodistribution of boronated protohaemins. Mitochondrial localization has been shown to be an important prerequisite for high phototoxicity of boronated porphyrins,[22] and the mitochondrial anti-apoptotic Bcl-2 protein was found to be a major target of PDT.[23] However, species-specific patterns of intracellular distribution of boronated porphyrins should be taken

into consideration, given that BOPP was entrapped in lysosomes in human glioma cells and in mitochondria in rat glioma cells.[24] Investigating the intracellular distribution of compound **9**, we found that it had cytoplasmic but not nuclear localization; furthermore, **9** did not bind double-stranded DNA in aqueous solution (manuscript in preparation). If **9** is entrapped in mitochondria, this compound should not be genotoxic and therefore should lack a mutagenic effect. Although the intratumoural accumulations of **9** and protohaemin IX have yet to be compared accurately, an increased uptake of **9** and its 'favourable' intracellular localization could explain, at least in part, the higher phototoxic activity of the boronated derivative of protohaemin IX.

Because the conjugation of carboranes is critical for the tumouricidal efficacy of the congjugate, boronated protohaemins with more boron atoms should be therapeutically advantageous. In particular, further work is needed to increase water solubility of disubstituted boronated protohaemins (compound **11** as a prototype) and to obtain practically applicable derivatives with higher boron content and different carborane structure.[7] Moreover, boronated protohaemins can be used in both PDT and boron neutron capture therapy, and the latter modality is important if the tumour response to PDT is incomplete. Escalation of the dose of the photosensitizer has been proven efficient in experimental models and patient protocols.[21] Kostenich and co-workers have shown that the effect of PDT on M-1 sarcoma was dependent on the dose of chlorin e_6;[14] our present data demonstrate the direct correlation of tumour response to PDT with the concentration of compound **9**. These results and the fact that the animals were cured with tolerable concentrations of **9** imply that, if necessary, the amount of this agent in the body can be elevated and the laser irradiation repeated.

Concluding remarks

We synthesized novel carboranyl and monocarbon-carboranyl derivatives of protohaemin IX using the activation of porphyrin carboxylic groups with di-*tert*-butyl pyrocarbonate or pivaloyl chloride. In tolerable concentrations the water-soluble compound 1,3,5,8-tetramethyl-2,4-divinyl-6(7)-[2′-(*closo*-monocarbon-carborane-1″-yl)methoxycarbonylethyl]-7(6)-(2′-carboxyethyl)porphyrin Fe(III) demonstrated dose-dependent therapeutic efficacy against transplanted M-1 sarcoma in rats. Protohaemin IX alone was without effect, indicating that boronation accounts for the phototoxic activity of the conjugate. Thus, boronated derivatives of protohaemin IX have potential for further development as agents for PDT. Given that boronated porphyrins are used in boron neutron-capture therapy, these compounds might be of dual advantage as agents for both types of binary anticancer strategies.

Experimental

The carboranes **2**, **4**, **7** and **8** were obtained as reported by us previously.[25] The IR-spectra were obtained on a UR-20 instrument in KBr pellets. The electronic spectra were recorded on a Hitachi UV-557 instrument. The mass spectra were measured on a VISION-2000 (MALDI) spectrophotometer. The purity of the compounds was assessed by TLC on Silufol plates with a 9 : 1 $CHCl_3$–MeOH solvent system. All carboranylporphyrins were isolated by column chromatography carried out on L silica gel (40–100 μm) using the same solvent system. The solvents were purified according to standard protocols.

General procedure for the synthesis of carboranyl-substituted porphyrins 3, 5, 9 and 10

To the solution of 100 mg (0.15 mmol) of porphyrin **1** in a mixture of 8 mL CH_2Cl_2 and 8 mL Py, 100 mg (0.46 mmol) Boc_2O was added at 0 °C followed by mixing for 10 min to form the mixed anhydride (TLC in 9 : 1 $CHCl_3$–MeOH, R_f = 0.6). Then the respective carborane (**2**, **4**, **7** or **8**) and 10 mg of DMAP were added, and the mixture was kept at 20 °C for 4 h. After removal of the solvents *in vacuo*, the residue was isolated by column chromatography on SiO_2 as dark red crystals soluble in $CHCl_3$, CH_2Cl_2, THF and pyridine. The structures of all newly synthesized compounds were confirmed by elemental analysis, their mass spectra, and electronic and IR spectra.

1,3,5,8-Tetramethyl-2,4-divinyl-6,7-di[2′-(*m*-carborane-9″-yl)-methoxycarbonylethyl]porphyrin Fe(III) chloride 3. From 100 mg (0.15 mmol) of porphyrin **1** and 40 mg (0.23 mmol) of carborane **2**, 102.7 mg (71%) of carboranylporphyrin **3** was obtained. (Found: C, 49.76; N, 5.69; H,5.93. Calc. for $C_{40}H_{56}N_4O_4B_{20}FeCl$: C, 49.82; N, 5.81; H, 5.85%); $\lambda_{max}(CHCl_3)$/nm 389.2 (ε/dm^3 mol^{-1} cm^{-1} 99 000), 511.4 (11 190); 542 (10 780) and 643 (5220); ν_{max}(KBr)/cm^{-1} 2590 (BH), 1713 (CO) and 1602 (–CH=CH_2); MS (MALDI) m/z 964 (M^+).

1,3,5,8-Tetramethyl-2,4-divinyl-6,7-di[2′-*N*-(*o*-carborane-3″-yl)carbamoylethyl]porphyrin Fe(III) chloride 5. From 100 mg (0.15 mmol) of porphyrin **1** and 48.6 mg (0.30 mmol) of carborane **4**, 86.7 mg (62%) of carboranylporphyrin **5** was obtained. (Found: C, 48.96; N, 8.75; H, 5.94. Calc. for $C_{38}H_{54}N_6O_2B_{20}FeCl$: C, 48.85; N, 8.99; H, 5.83%); $\lambda_{max}(CHCl_3)$/nm 386.8 (ε/dm^3 mol^{-1} cm^{-1} 61 100) 511.2 (6650), 545.2 (6120) and 642.2 (2960); ν_{max}(KBr)/cm^{-1} 2585 (BH), 1737 (CO) and 1656 (–CH=CH_2); MS (MALDI) m/z 934 (M^+).

1,3,5,8-Tetramethyl-2,4-divinyl-6,7-di[2′-(*nido*-7,8-dicarbaundecaborate-3″-yl)carbamoylethyl]porphyrin Fe(III) tetrabutylammonium salt 6. To the solution of 50 mg (0.05 mmol) of carboranylporphyrin **5** in 15 mL THF, 39.2 mg (0.15 mmol) Bu_4NF was added, and the mixture was boiled for 4 h. The THF was evaporated and the residue dissolved in 15 mL water, filtered and dried to give 47.5 mg (85%) of carboranylporphyrin **6**. (Found: C,58.36; N,8.85; H, 7.48. Calc. for $C_{54}H_{84}N_7O_2B_{18}Fe$: C, 58.24; N, 8.80; H, 7.60%); $\lambda_{max}(CHCl_3)$/nm 415 (ε/dm^3 mol^{-1} cm^{-1} 17 070), 512 (1180) and 588 (380); ν_{max}(KBr)/cm^{-1} 2522 (BH), 1725 (CO) and 1656 (–CH=CH_2); MS (MALDI) m/z 1113 (M^+).

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2′-(*closo*-monocarbon-carborane-1″-yl)methoxy-carbonylethyl]-7(6)-(2′-carboxyethyl)porphyrin Fe(III) 9. From 100 mg (0.15 mmol) of porphyrin **1** and 55 mg (0.18 mmol) of carborane **7**, 65.8 mg (57%) of monocarboranylporphyrin **9** were obtained. (Found: C, 55.86; N, 7.13; H, 5.92. Calc. for $C_{36}H_{44}N_4O_4B_{11}Fe$: C, 56.04; N, 7.26; H, 5.75%); $\lambda_{max}(CHCl_3)$/nm 389.4 (ε/dm^3 mol^{-1} cm^{-1} 85 790), 510.6 (7370), 539.6 (7050) and 642.8 (3820); ν_{max}(KBr)/cm^{-1} 2560 (BH), 1737 (CO) and 1649 (–CH=CH_2).); MS (MALDI) m/z 771 (M^+).

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2′-*closo*-monocarbon-carborane-1″-yl-2″-phenyl)methoxycarbonylethyl]-7(6)-(2′-carboxyethyl)porphyrin Fe(III) (10). From 100 mg (0.15 mmol) of porphyrin **1** and 48.6 mg (0.30 mmol) of monocarborane **8**, 74.9 mg (59%) of monocarboranylporphyrin **10** were obtained. (Found: C, 59.75; N, 6.48; H, 5.79. Calc. for $C_{42}H_{48}N_4O_4B_{11}Fe$: C, 59.51; N, 6.61; H, 5.71%); $\lambda_{max}(CHCl_3)$/nm 387.4 (ε/dm^3 mol^{-1} cm^{-1} 37 700), 511.2 (4190), 543.6 (3860) and 642.2 (1810); ν_{max}(KBr)/cm^{-1} 2531 (BH), 1726 (CO) and 1624 (–CH=CH$_2$, Ph); MS (MALDI) m/z 847 (M$^+$).

1,3,5,8-Tetramethyl-2,4-divinyl-6(7)-[2′-(*closo*-monocarbon-carborane-1″-yl)methoxycarbonylethyl-7(6)-[2′-*N*-(*o*-carborane-3″-yl)-carbamoylethyl]porphyrin Fe(III) 11. From 50 mg (0.065 mmol) of monocarboranylporphyrin **9** and 10.3 mg (0.065 mmol) of carborane **4**, 37.4 mg (63%) of disubstituted carboranylporphyrin **11** were obtained. (Found: C, 49.83; N, 7.55; H, 6.10. Calc. for $C_{38}H_{55}N_5O_3B_{21}Fe$: C, 50.01; N, 7.67; H, 6.07%); $\lambda_{max}(CHCl_3)$/nm 361.8 (ε/dm^3 mol^{-1} cm^{-1} 3890), 411 (2450), 492 (4150), 553 (870) and 592 (910); ν_{max}(KBr)/cm^{-1} 2587 (BH) and 1682 (CO). MS (MALDI) m/z 912 (M$^+$).

Cell culture and cytotoxicity assays and cell irradiation

The McA 7777 rat liver epithelial cell line, Rat-1 and REF fibroblasts and MCF-10A human breast epithelial cell lines were propagated in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37 °C, 5% CO_2 in a humidified atmosphere. Novel carboranylporphyrins were dissolved in dimethyl sulfoxide (DMSO) to give 10 mM stock solutions, and serial aqueous dilutions were made immediately before experiments. All porphyrin-containing compounds were kept away from light, and the experiments were performed in the dark. For testing the cytotoxicity, cells (3×10^3 in 100 μL of culture medium) were plated into a 96-well plate (Becton Dickinson, Franklin Lakes, NJ) and treated with vehicle control (DMSO) or compound **9** (each concentration in triplicate) for 72 h. After the completion of drug exposure, 100 μg MTT in 20 μL of aqueous solution were added into each well for an additional 2 h. Formazan was dissolved in acidified DMSO, and the absorbance at $\lambda = 540$ nm was measured on a Flow Multiscan plate reader (LKB, Sweden). Cell survival was calculated as ratio of OD_{540} in wells with the respective drug concentrations to the OD_{540} of wells containing vehicle control (100%). DMSO at ≤1% caused no discernible toxicity, growth arrest or morphological changes in cells within the time of experiments. For PDT, Rat-1 fibroblasts were seeded into a 24-well plate (5×10^4 cells per well) overnight followed by the addition of compound **9** at final concentrations of 10 μM or 20 μM for 3 h at 37 °C, 5% CO_2. After the completion of exposure, the culture medium was replaced with 0.5 mL saline, and cell monolayers were irradiated with the laser beam ($\lambda =$ 630 nm, density of light emission energy 200 J cm^{-2}). The saline was removed, fresh culture medium was added to the wells, and cells were incubated at 37 °C, 5% CO_2 for an additional 24 h. Control cells were left untreated (no drug, no irradiation) or exposed to **9** alone or irradiated in the absence of **9**. The cytotoxicity was determined by morphological examination. To confirm the effects observed in unstained cells, glass coverslips were placed into the wells, cells were grown on this support, treated as described above, washed with saline, fixed in 10% formalin and stained with hematoxylin and eosin. Each concentration of **9** was tested in three independent experiments.

Animals and *in vivo* PDT

Rats were hosted in the animal facility of the Medical Radiological Research Center, Obninsk, Russia. Animals were given food and water *ad libitum*. Transplantation of M-1 sarcoma was performed as described.[14] Briefly, tumour cells were freshly isolated from the sarcoma-bearing animal. Three million tumour cells in 0.5 mL saline were injected under the skin of rear extremities (one inoculum per animal). After the implanted tumours reached ~0.1 cm^3 in volume (normally 8–10 days post-implantation) rats were divided into four groups (9 animals per group). Group 1 received no treatment, group 2 was injected i.p. with saline, and groups 3 and 4 were injected i.p. with compound **9**. Twenty-four hours later, tumours in groups 1–3 were irradiated with the laser beam (lamp ATO-150, filter 630 nm, density of light emission energy 300 J cm^{-2}). Hair in the area of the tumour was epilated prior to tumour irradiation. The duration of exposure was calculated by the formula:

$$T = (D^2 \times E \times 13.09)/P \quad (1)$$

where T is time (min) of laser light exposure, 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 evaluated before irradiation (day 0) and at days 3, 7, 10, 14 and 21 post-irradiation. The volume V (cm^3) of the tumour was calculated using the equation:

$$\tfrac{1}{6}\pi \times d_1 \times d_2 \times d_2 \quad (2)$$

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

The therapeutic effect TE (determined as the change of tumour size in the course of PDT) was calculated by the formula:

$$TE = (V_c - V_i)/V_c \times 100\% \quad (3)$$

where V_c is the tumour volume in untreated rats, and V_i is the tumour volume at the respective day after irradiation. Group 4 animals received no laser treatment. The complete response to PDT was estimated as disappearance of the tumour and cure of animals, *i.e.*, rats were tumour-free for at least 90 days post-irradiation. The tumour-bearing animals were sacrificed by day 22 post-irradiation.

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

Acknowledgements

This work was supported by grants from the Russian Foundation for Basic Research (05-03-08155-ofi-a) and the Protec company.

References

1 (*a*) D. Nowis, M. Legat, T. Grzela, J. Niderla, E. Wilczek, G. M. Wilczynski, E. Glodkowska, P. Mrowka, T. Issat, J. Dulak, A. Jozkowicz, H. Was, M. Adamek, A. Wrosek, S. Nazarewski, M. Makovski, T. Stoklova, M. Jakobisiak and J. Golab, *Oncogene*, 2006, **25**, 3365–3374; (*b*) H. C. Wolfsen, *J. Clin. Gastroenterol.*, 2005, **39**, 189–202; (*c*) H. B. Ris, *Lung Cancer*, 2005, **49**, S65–S68.

2 R. F. Barth, J. A. Coderre, M. G. H. Vicente and T. E. Blue, *Clin. Cancer Res.*, 2005, **11**, 3987–4002.
3 K. Plaetzer, T. Kiesslich, C. B. Oberdanner and B. Krammer, *Curr. Pharm. Des.*, 2005, **11**, 1151–1165.
4 T. J. Dougherty, C. J. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan and Q. Peng, *J. Natl. Cancer Inst.*, 1998, **90**, 889–905.
5 (*a*) M. A. Dagrosa, M. Viaggi, R. J. Rebagliati, D. Batistoni, S. B. Kahl, G. J. Juvenal and M. A. Pisarev, *Mol. Pharmacol.*, 2005, 2151–2156; (*b*) V. Gottumukkala, R. Luguya, F. R. Fronczek and M. G. H. Vicente, *Bioorg. Med. Chem.*, 2005, **13**, 1633–1640; (*c*) M. G. H. Vicente, A. Weickramasinghe, D. J. Nurco, H. J. Wang, M. M. Nawrocky, M. S. Makar and M. Miura, *Bioorg. Med. Chem.*, 2003, **11**, 3101–3108.
6 R. P. Evstigneeva, A. V. Zaitsev, V. N. Luzgina, V. A. Ol'shevskaya and A. A. Shtil, *Curr. Med. Chem.: Anti-Cancer Agents*, 2003, **3**, 383–392.
7 (*a*) T. D. Mody, *J. Porphyrins Phthalocyanines*, 2000, **4**, 362–367; (*b*) M. Miura, D. Gabel, G. Oenbrink and R. G. Fairchild, *Tetrahedron Lett.*, 1990, **31**, 2247–2250; (*c*) M. Muira, P. L. Micca, C. D. Fisher, C. R. Gordon, J. C. Heinrichs and D. N. Slatkin, *Br. J. Radiol.*, 1998, **71**, 773–781.
8 (*a*) M. W. Renner, M. Miura, M. W. Easson and M. G. H. Vicente, *Anti-Cancer Agents Med. Chem.*, 2006, **6**, 145–157; (*b*) M. G. H. Vicente, *Curr. Med. Chem.: Anti-Cancer Agents*, 2001, **1**, 175–194.
9 V. A. Ol'shevskaya, A. V. Zaitsev, V. N. Luzgina, T. T. Kondratieva, E. G. Kononova, P. V. Petrovskii, A. F. Mironov, V. N. Kalinin, I. Hofmann and A. A. Shtil, *Bioorg. Med. Chem.*, 2006, **14**, 109–120.
10 S. Banfi, E. Caruso, S. Caprioli, L. Mazzagatti, G. Canti, R. Ravizza, M. Gariboldi and E. Monti, *Bioorg. Med. Chem.*, 2004, **12**, 4853–4860.
11 M. A. Rosental, B. Kavar, J. S. Hill, D. J. Morgan, R. L. Nation, S. S. Stylli, R. L. Basser, S. Uren, H. Geldard, M. D. Green, S. B. Kahl and A. H. Kaye, *J. Clin. Oncol.*, 2001, **19**, 519–524.
12 (*a*) J. Tibbitts, N. C. Sambol, J. R. Fike, W. F. Bauer and S. B. Kahl, *J. Pharm. Sci.*, 2000, **89**, 469–477; (*b*) J. Tibbitts, J. R. Fike, K. R. Lamborn, A. W. Bollen and S. B. Kahl, *Photochem. Photobiol.*, 1999, **69**, 587–594; (*c*) P. G. Spizzirri, J. S. Hill, S. B. Kahl and K. P. Ghiggino, *Photochem. Photobiol.*, 1996, **64**, 975–983.
13 (*a*) L. I. Zakharkin, V. A. Ol'shevskaya, S. Yu. Panfilova, P. V. Petrovskii, V. N. Luzgina and R. P. Evstigneeva, *Russ. Chem. Bull.*, 1999, **48**, 2237–2239; (*b*) V. A. Ol'shevskaya, R. P. Evstigneeva, V. N. Luzgina, M. A. Gyul'malieva, P. V. Petrovskii, J. H. Morris and L. I. Zakharkin, *Mendeleev Commun.*, 2001, **11**, 14–15.
14 (*a*) G. A. Kostenich, I. N. Zhuravkin, A. V. Furmanchuk and E. A. Zhavrid, *J. Photochem. Photobiol.*, 1993, **17**, 187–194; (*b*) G. A. Kostenich, I. N. Zhuravkin and E. A. Zhavrid, *J. Photochem. Photobiol.*, 1994, **22**, 211–217.
15 (*a*) Z. Ades, *Int. J. Biochem.*, 1990, **22**, 565–578; (*b*) S. Collaud, A. Juzeniene, J. Moan and N. Lange, *Curr. Med. Chem.: Anti-Cancer Agents*, 2004, **4**, 301–316.
16 R. F. Lopez, N. Lange, R. Guy and M. V. Bentley, *Adv. Drug Delivery Rev.*, 2004, **56**, 77–94.
17 J. Kloek and J. M. J. Beijersbergen van Henegouwen, *Photochem. Photobiol.*, 1996, **64**, 994–1000.
18 F. S. De, Rosa, A. C. Tedesco, R. F. Lopez, M. B. Pierre, N. Lange, J. M. Marchetti, J. C. Rotta and M. V. Bentley, *J. Controlled Release*, 2003, **89**, 261–269.
19 (*a*) C. Perotti, H. Fukuda, G. DiVenosa, A. J. MacRobert, A. Batlle and A. Casas, *Br. J. Cancer*, 2004, **90**, 1660–1665; (*b*) Y. Berger, L. Ingrassia, R. Neier and L. Juillerat-Jeanneret, *Bioorg. Med. Chem.*, 2003, **11**, 1343–1351; (*c*) H. Brunner, F. Hausmann and R. Knuechel, *Photochem. Photobiol.*, 2003, **78**, 481–486.
20 J. Moan, O. Bech, J. M. Gaullier, T. Stokke, H. B. Steen, L. W. Ma and K. Berg, *Int. J. Cancer*, 1998, **75**, 134–139.
21 D. Ickowicz Schwartz, Y. Gozlan, L. Greenbaum, T. Babushkina, D. J. Katcoff and Z. Malik, *Br. J. Cancer*, 2004, **90**, 1833–1841.
22 (*a*) D. Kessel, R. Luguya and M. G. Vicente, *Photochem. Photobiol.*, 2003, **78**, 431–435; (*b*) L. Y. Xue, S. M. Chiu and N. L. Oleinick, *Oncogene*, 2005, **24**, 6987–6992; (*c*) G. Li, A. Graham, Y. Chen, M. P. Dobhal, J. Morgan, G. Zheng, A. Kozyrev, A. Ozeroff, T. J. Dougberty and R. K. Padney, *J. Med. Chem.*, 2003, **46**, 5349–5359.
23 J. Usuda, S. M. Chiu, E. S. Murphy, M. Lam, A. L. Nieminen and N. L. Oleinick, *J. Biol. Chem.*, 2003, **278**, 2021–2029.
24 D. E. Callahan, T. M. Forte, S. M. Afzal, D. F. Deen, S. B. Kahl, K. A. Bjornstad, W. F. Bauer and E. A. Blakely, *Int. J. Radiat. Oncol., Biol., Phys.*, 1999, **45**, 761–771.
25 (*a*) L. I. Zakharkin, V. A. Ol'shevskaya and N. B. Boiko, *Russ. Chem. Bull.*, 1996, 719–722; (*b*) L. I. Zakharkin, V. N. Kalinin and V. V. Gedymin, *J. Organomet. Chem.*, 1969, **16**, 371–374; (*c*) L. I. Zakharkin, V. A. Ol'shevskaya, P. V. Petrovskii and J. H. Morris, *Mendeleev Commun.*, 2000, **10**, 71–72.