Synthesis and biological investigations of tetrakis(*p*-carboranylthio-tetrafluorophenyl)chlorin (TPFC)†

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We describe the total synthesis and biological properties of a new carboranyl-containing chlorin (TPFC) that might find application as a dual sensitizer in the PDT and BNCT treatment of cancer. TPFC was found to be non-toxic in the dark but showed extensive photosensitizing ability both *in vitro* and *in vivo* despite its relatively low singlet oxygen quantum yield. In particular, TPFC exhibited significant photosensitizing activity against highly pigmented melanotic melanoma tumors in mice.

Introduction

Boron neutron capture therapy (BNCT)[1] and photodynamic therapy (PDT),[2] are two binary modalities for cancer treatment that rely on the selective accumulation of a sensitizer within tumor tissue, followed by its activation upon irradiation with either low-energy neutrons (in BNCT) or red light (in PDT). The combination of BNCT and PDT using a single drug has several advantages, including increased therapeutic effect due to the targeting of different cellular components and/or mechanisms of tumor cell destruction. Boronated porphyrins and their derivatives, in particular dihydroporphyrins or chlorins, are promising dual BNCT and PDT sensitizers because they usually have low dark toxicity, preferentially accumulate within tumor tissues and persist there for a considerable amount of time.[3–5] Moreover, their significant absorption of light wavelengths in the far-red region (>650 nm), which is characterized by the power of deep penetration into most human tissues,[6] makes them ideal candidates for *in vivo* phototherapeutic applications;[2] on the other hand, their large boron content enhances the efficiency of their interaction with thermal neutrons. We have previously reported a boronated chlorin that was synthesized in 4 steps from a boronated porphyrin.[7] This compound showed very low intrinsic cytotoxicity towards human glioma T98G cells, moderate phototoxicity and high cellular uptake. In our continuing effort to develop expeditious synthetic routes to boronated porphyrin derivatives for therapeutic applications,[8] we choose 5,10,15,20-tetrakis(2,3,4,5,6-pentafluorophenyl) porphyrin (TPPF) as the core platform for the preparation of a new carboranyl-substituted chlorin (TPFC). Our methodology allows the introduction of the carborane clusters on a pre-formed porphyrin, under exceptionally mild conditions and good (48%) overall yield.

TPPF is commercially available and can be routinely made in multi-gram amounts using either the procedures of Adler *et al.*[9] or Lindsey *et al*.[10] TPPF was selected as the starting material for functionalization for the following reasons: (1) it is readily available in multi-gram amounts; (2) the *p*-phenyl fluoride is known to be reactive towards nucleophilic substitution reactions;[11–13] (3) it can be easily functionalized at the β-positions to form chlorins and other reduced porphyrins;[14] (4) TPPF derivatives can be studied by *in vivo* spectroscopy and imaging; and (5) fluorinated porphyrins have been shown in previous investigations to have higher photodynamic activities than the corresponding non-fluorinated analogs.[15,16] Furthermore, ^{19}F NMR is a highly sensitive technique with low endogenous background signals and has been used to study metabolism, tumor growth, and blood flow.[17] In addition, fluorinated porphyrins have been shown to have greater triplet state quantum yields and increased biological efficacy compared with their non-fluorinated analogs.

The substitution of the *p*-phenyl fluoride of TPPF using nucleophilic reagents has been previously exploited,[11–13] and the application of this method to the attachment of biomolecules, such as sugars, to porphyrins has been reported.[18,19] Recently, Drain's group has also reported an efficient method using microwave heating.[20] The use of TPPF derivatives as PDT agents has been previously reported,[17–20] but surprisingly their corresponding chlorins have not been investigated, although chlorins have significantly stronger absorptions within the so-called PDT therapeutic window (600–800 nm).[2] Herein we report the expeditious synthesis of a new boronated chlorin from TPPF, and its characterization. Sulfur, rather than oxygen, was used as the nucleophilic species in the *p*-fluoride substitution reactions, since sulfur is known to induce greater stability towards acid hydrolysis than oxygen, as a result of its weaker basicity and lower affinity for protons.

Results and discussion

Synthesis

TPFC was synthesized in 48% overall yield as shown in Scheme 1, from commercially available TPPF. The reaction of TPPF with CH_3NHCH_2COOH and paraformaldehyde in toluene gave the corresponding chlorin, which reacted with excess 1-mercapto-*o*-carborane and K_2CO_3 in DMF at room temperature to give a mixture of partially deboronated tetra(*o*-carboranylthio) chlorins,

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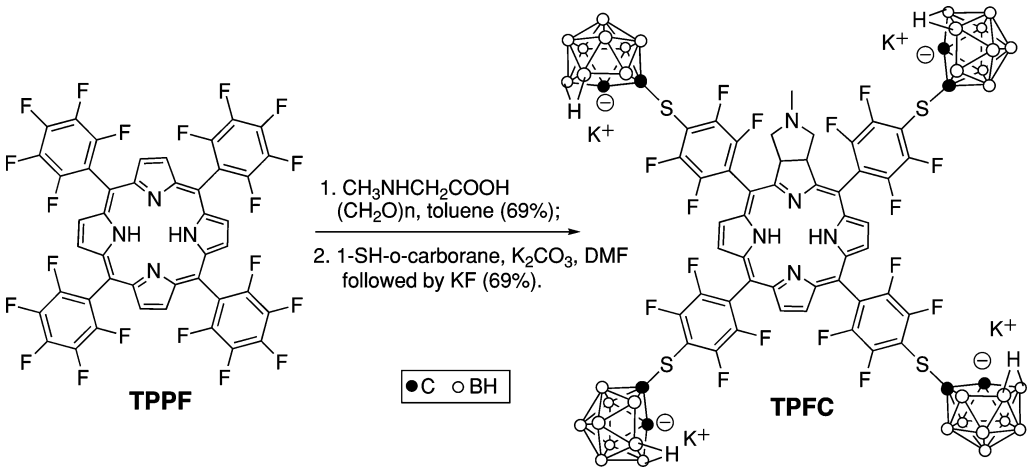

Scheme 1 Synthesis of TPFC (48% overall yield).

after two days. The partial degradation of the icosahedral *o*-caborane cages to the corresponding open-face *nido*-carboranes under the above reaction conditions is not surprising. It has been previously observed that the *o*-carborane cages of a sugar derivative[21] and of a porphyrin macrocycle[22] were degraded upon heating in a polar solvent. To complete the deboronation reaction fluoride ion was used, as previously reported.[23,24] TPFC was obtained after purification by silica gel column chromatography using ethyl acetate–acetone 1 : 1 for elution, and characterized by UV–vis and ^{1}H-NMR spectroscopy, HRMS and HPLC.

TPFC shows a characteristic long wavelength absorption at 650 nm in its optical spectrum, characteristic of dihydroporphyrins, that is 14 times stronger than that of the corresponding porphyrin.[25] Therefore TPFC is potentially more suitable for application as a PDT sensitizer than the boronated porphyrin. In addition TPFC showed an intense fluorescence band at 658 nm upon excitation at 410 nm, and a fluorescence quantum yield of 0.22 in DMSO. The fluorescence properties of TPFC will not only allow the detection of tumors *via* minimally invasive fluorescence-based techniques, but also the determination and quantification of tumor-localized boron, which facilitates radiation microdosimetry in BNCT. The HRMS (ESI) of TPFC showed isotope patterns matched perfectly with those calculated (see ESI†). TPFC is very soluble in polar organic solvents such as methanol, ethyl acetate, THF, DMSO and DMF, but has limited solubility in water.

Photochemical and photophysical investigations

The irradiation of TPFC in a DMSO solution by red light wavelengths, using experimental conditions identical with those adopted for cell photosensitization, induced a very modest decrease in the intensity of the visible absorption bands of the chlorin (less than 10% decrease after 30 min irradiation). The formation of new absorption bands was not detected even after prolonged illumination. The high degree of photostability displayed by TPFC is fairly unusual since most derivatives having a tetrapyrrolic macrocycle are known to undergo extensive photobleaching upon exposure to visible light.[26] These observations imply that the concentration of TPFC in the irradiated tissue remains at reasonably large levels throughout the light exposure time, thereby ensuring an efficient absorption of the incident light during the phototherapeutic treatment. On the other hand, the singlet oxygen quantum yield (Φ_Δ) for TPFC was found to be 0.1, a significantly lower value compared with *ca.* 0.5, the typical singlet oxygen quantum yield value for most PDT photosensitizers.[27] The high photochemical stability and comparatively low oxygen quantum yield observed for TPFC are probably a consequence of the presence of the four carborane cages, as previously observed for other carborane-containing molecules.[28]

Cellular investigations

Since drug toxicity is a very important property that determines its therapeutic use, the dark cytotoxicity of TPFC was investigated in human carcinoma HEp2 cells at concentrations up to 100 μM for 24 h. The HEp2 cell line was used because a substantial amount of data on the dark cytotoxicity of porphyrin derivatives already exists using this cell line. TPFC was found to be non-toxic in the dark under these conditions, as shown in Fig. 1.

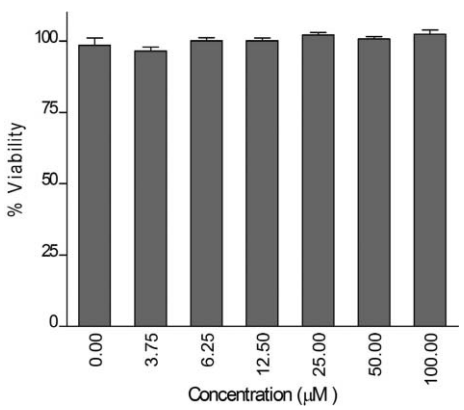

Fig. 1 Dark toxicity of TPFC toward human HEp2 cells using the Cell Titer Blue assay.

In spite of the low Φ_Δ, TPFC appears to be a very efficient photosensitizing agent toward tumor cells. As shown in Fig. 2A TPFC accumulated in significant amounts within melanoma B16F1 cells. The endocellular concentration of TPFC gradually increased by more than 8 times upon increasing the chlorin concentration in the incubation medium over the 1–20 μM interval. No appreciable

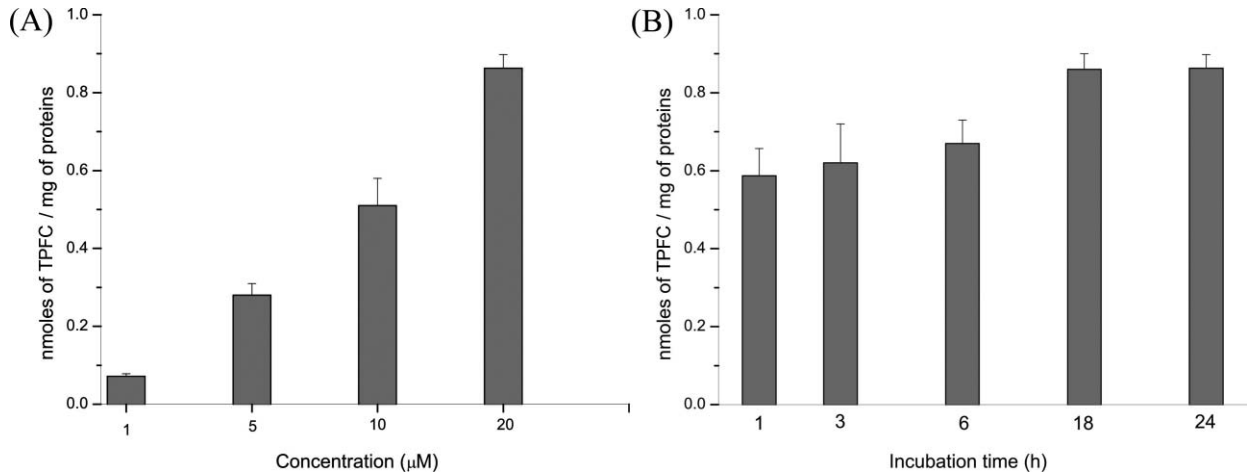

Fig. 2 (A) Effect of TPFC concentration on the uptake by melanotic melanoma B16F1 cells after 24 h incubation in DMEM containing 0.3% DMSO. (B) Effect of incubation time in DMEM containing 0.3% DMSO on the uptake of 20 μM TPFC by B16F1 cells.

toxic effect on the melanotic melanoma cells was noticed even at the largest photosensitizer dose investigated. For the 20 μM TPFC concentration the uptake process appeared to be fairly rapid, since a TPFC recovery from the melanotic melanoma cells of about 0.6 nmoles mg^{-1} of cell protein was obtained after incubation times as short as 1 h, with a further increase to about 0.85 nmoles mg^{-1} of cell protein when the incubation time was prolonged to 18 h (see Fig. 2B). The latter value underwent no appreciable increase when the incubation was extended to 24 h, suggesting that the 20 μM TPFC binding with this cell type reaches a plateau value.

Under these experimental conditions (24 h incubation with 20 μM TPFC) irradiation of B16F1 cells with 600–700 nm light caused an essentially complete mortality after 5 min irradiation, as shown in Fig. 3. Interestingly, when the cells were irradiated after 3 h incubation with 20 μM TPFC, the degree of photosensitivity markedly dropped (about 25% decrease in survival after 5 min exposure to light) in spite of the fact that the amount of cell-bound chlorin was decreased by less than 30% over this incubation time interval (Fig. 2B). Moreover, under these experimental conditions, the cell photosensitivity was significantly lower than that observed upon irradiation after 24 h incubation in the presence of 10 μM TPFC, *i.e.* under conditions yielding endocellular chlorin concentrations that are very similar to those obtained after 3 h incubation with 20 μM TPFC. A similar dependence of the photosensitivity on the incubation time was recently observed for melanotic melanoma cells photosensitized by naphthalocyanine derivatives and was ascribed to a different pattern of subcellular distribution of the photosensitizing agent.[29] In our case, fluorescence microscopy observations of the B16F1 cells after 24 h incubation with 20 μM TPFC clearly indicate that the chlorin is mostly localized at the level of the cytoplasmic membrane (Fig. 4), which should consequently represent the preferential target of the photosensitized process. Thus, the integrity of the plasma membrane appears to be critical for cell survival.

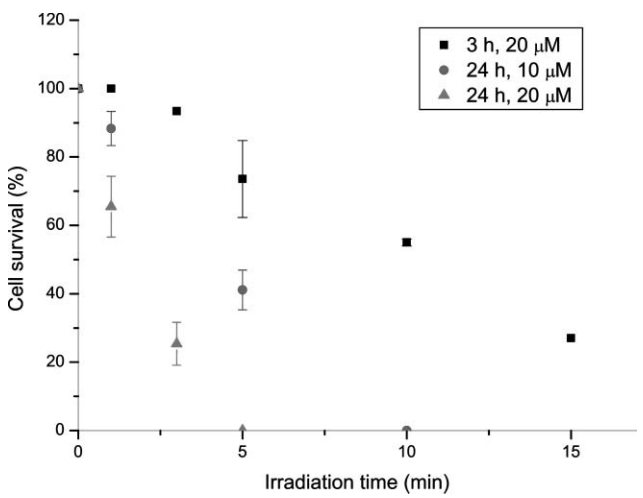

Fig. 3 Survival of B16F1 melanotic melanoma cells irradiated with red light (600–700 nm) at 20 mW cm^{-2} for different time periods after incubation for 3 h or 24 h with 10 or 20 μM TPFC.

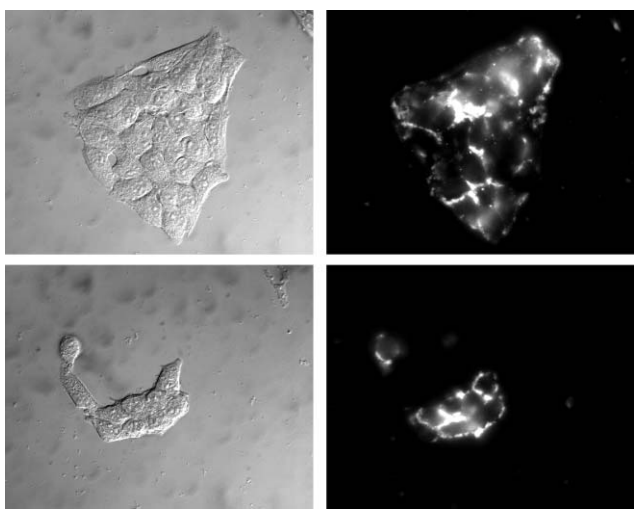

Fig. 4 Fluorescence micrographs of B16F1 cells after 24 h incubation with TPFC 20 μM in DMEM with 10% FBS and 0.5% DMSO and their corresponding bright field images. The cells were grown for 48 h before incubation with TPFC.

The caspase-3 assays, performed as described in the experimental section, showed no significant difference between the control and extensively photosensitized cells, suggesting that apoptotic processes give a minor, if any, contribution to the photoinduced cell death. Actually, caspase-3 is known to be a typical marker of PDT-induced cell apoptosis.[30] This observation was further supported by fluorescence microscopy after incubation of control and photosensitized cells with the chromatin probe Hoechst (Fig. 5). Such analysis monitors the possible occurrence of caspase-independent apoptotic pathways. Clearly, the micrographs obtained for B16F1 cells that had been extensively photoinactivated show no increase in the nuclear fluorescence as compared with control cells, suggesting that no significant increase in chromatin condensation (a process which is again typical of apoptosis) had occurred as a consequence of TPFC photosensitization. Thus, it is likely that TPFC predominantly performs its photosensitizing activity through random necrotic pathways, at least in the case of B16F1 cells.

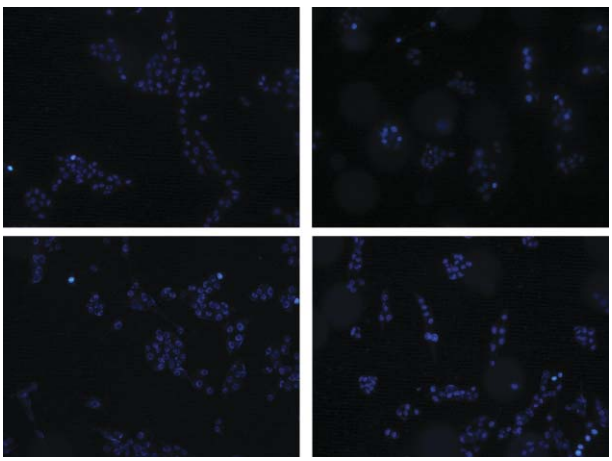

Fig. 5 Fluorescence micrographs of B16F1 melanotic melanoma cells after 10 min incubation with Hoechst dye HO33342. The pictures on the left side refer to control untreated cells; the pictures on the right side refer to cells that have been incubated for 24 h with TPFC 20 μM, irradiated with 600–700 nm light for 3 min and observed 2 h after the end of the phototreatment.

It is likely that such conclusions do not completely apply to other cell types, where different modes of TPFC interaction with the cell constituents can take place. In actual fact, the preferential sites of intracellular localization of TPFC in human carcinoma HEp2 cells at 10 μM for 24 h were also investigated (Fig. 6). The green fluorescing organelle tracers BODIPY Ceramide (Golgi network), LysoSensor Green (lysosomes) and MitoTracker Green (mitochondria) were used in colocalization experiments. A punctuate fluorescence pattern was observed (Fig. 6b) that correlated to some extent with the cell lysosomes, as seen by the characteristic orange–yellow regions in Fig. 6f. No additional areas of colocalization were observed in the overlaid images with the organelle tracers BODIPY Ceramide and MitoTracker Green (Fig. 6d,h).

In vivo studies

As shown in Fig. 7, intravenously injected TPFC exhibited some selectivity for tumor targeting at relatively short times after administration. The maximum ratio of photosensitizer concentration

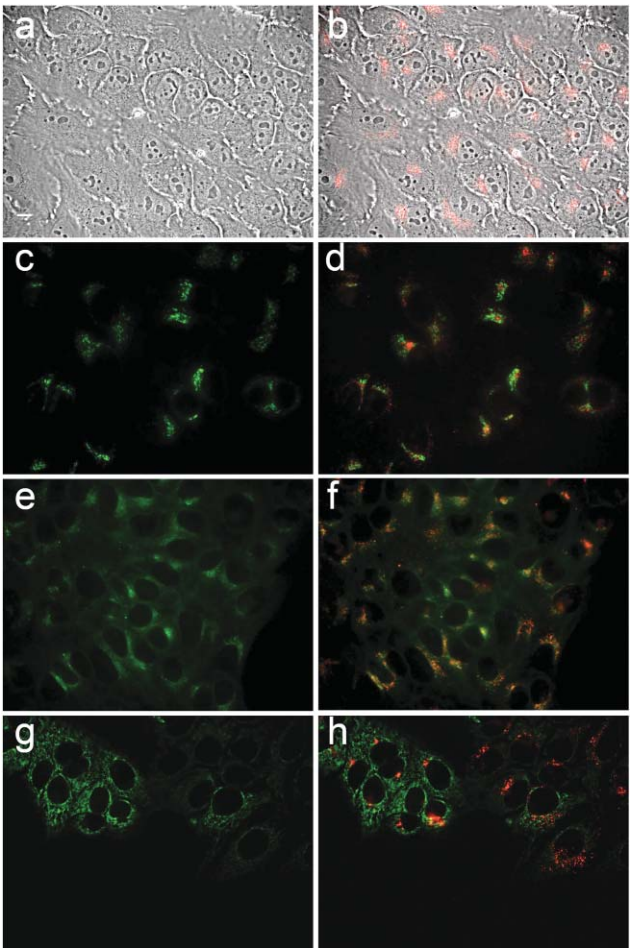

Fig. 6 Subcellular localization of TPFC in HEp2 cells at 10 μM for 24 h. (a) Phase contrast, (b) overlay of TPFC fluorescence with phase contrast, (c) BODIPY Ceramide fluorescence, (d) overlay of BODIPY Ceramide with TPFC fluorescence, (e) LysoSensor Green fluorescence, (f) overlay of Lysosensor Green with TPFC fluorescence, (g) MitoTracker Green fluorescence, (h) overlay of MitoTracker Green with TPFC fluorescence.

in the melanotic melanoma to the peritumoral cutaneous tissue, namely 2.5, was measured at 3 h post-injection. Clearly, the chlorin underwent a faster clearance from the tumor as compared with the adjacent skin compartments, so that no residual selectivity was noticed after 18 h. This behaviour was observed for other photodynamic agents[2,17] and can be related to some degree of radial infiltration of the melanotic melanoma into the surrounding tissue.[29] On the other hand, only minor amounts of TPFC were recovered from tumor-distal skin at all post-injection times, which should reduce the risk of a persistent or generalized cutaneous photosensitivity; the latter represents an undesired side effect of PDT, especially when first generation PDT agents, such as Photofrin, are used.[2,27]

As is typical of hydrophobic porphyrin-type derivatives, the largest accumulation of TPFC takes place (Fig. 8a) at the level of the liver and, to a lesser extent, of the spleen, which reflects both the affinity of such compounds for the components of the reticuloendothelial system and their predominant clearance from the organism *via* the liver–gut pathway.[6,31] In this case, however, significant amounts of TPFC are also found in the kidneys, which could indicate a partial elimination of the chlorin in the

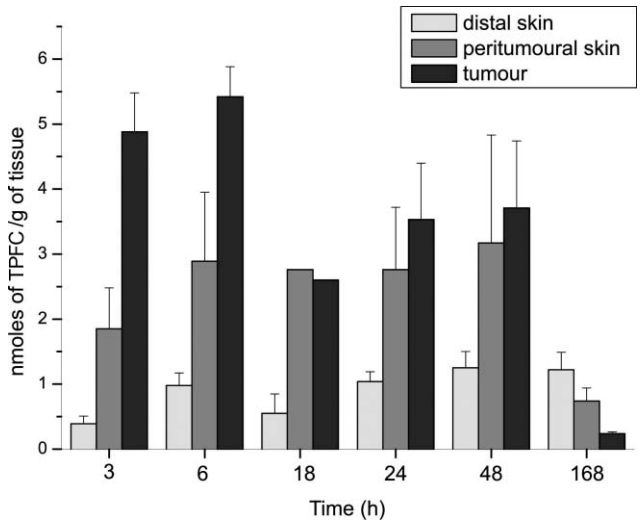

Fig. 7 Recovery of TPFC from tumor and skin of C57BL/6 mice bearing a s.c.-transplanted B16 pigmented melanoma at various times after i.v. injection of 10 mg kg^{-1} of photosensitizer dissolved in a ternary mixture composed of 20% DMSO, 30% PEG 400 and 50% water.

urine. Metabolic investigations to clarify this issue are currently in progress. In any case, a decrease in the TPFC concentration from all tissues analysed by us is evident at one week after injection, hence the onset of toxic effects as a consequence of prolonged retention of the chlorin in such tissues is unlikely.

The lack of appreciable TPFC accumulation in the brain is in agreement with the known inability of such compounds to cross the blood–brain barrier.[32] Lastly, the clearance of TPFC from serum is essentially complete after 18 h (Fig. 8b), which is again suggesting that the systemic administration of this chlorin should not be accompanied by the appearance of generalized photosensivity.

In actual fact, when skin areas distal to the melanotic melanoma were irradiated at 3 h or 24 h after the injection of TPFC by using the same protocol as adopted for the PDT of the neoplastic lesion, no indication of cutaneous phototoxicity was obtained, in full agreement with the indications provided by the pharmacokinetic studies. On the other hand, at both post-injection times, the melanotic melanoma showed a significant response to the PDT treatment (Fig. 9). Apparently, all the mice irradiated after injection of the chlorin remained tumor-free for about 5 days, while the untreated mice or the mice irradiated in the absence of the photosensitizing agent displayed a fairly rapid tumor growth during this time interval. This result is certainly appreciable since this kind of melanoma is known to be poorly responsive to PDT owing to the filtering action toward the incident light caused by the large concentration of melanin-type pigments.[27] The regrowth of the tumor starting on the 6th day after the end of PDT can actually reflect the insufficient penetration of the red light used by us to the deeper tumor layers. In any case, as one can see in Fig. 9, the growth of the tumor for both controls and PDT-treated mice eventually proceeds at a very aggressive rate, hence the animals need to be sacrificed when the tumor volume becomes larger than 1.5 cm^3 in order to avoid undue suffering caused by too large a size of the neoplasia and/or the spread of metastases to other organs (*e.g.*, liver or lungs).

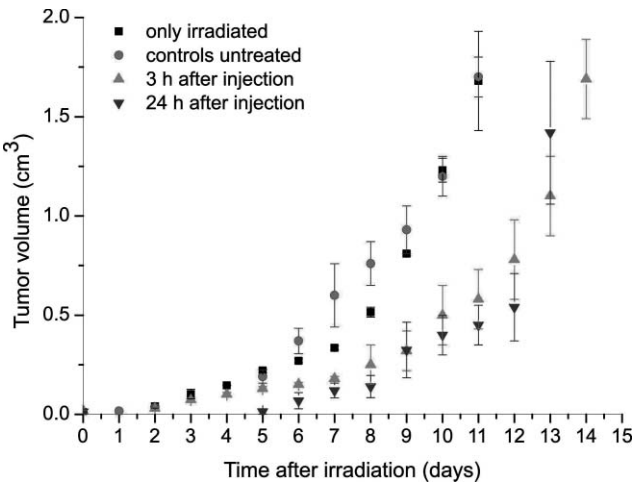

Fig. 9 Growth rate of pigmented melanoma B16F1 in mice C57BL/6 injected with 10 mg kg^{-1} of TPFC and irradiated after 3 h (▲) and 24 h (▼) with 135 mW cm^{-2} (81 J cm^{-2}) of 600–700 nm light. Comparison with the tumor growth rate in untreated (▨) and only irradiated (■) mice.

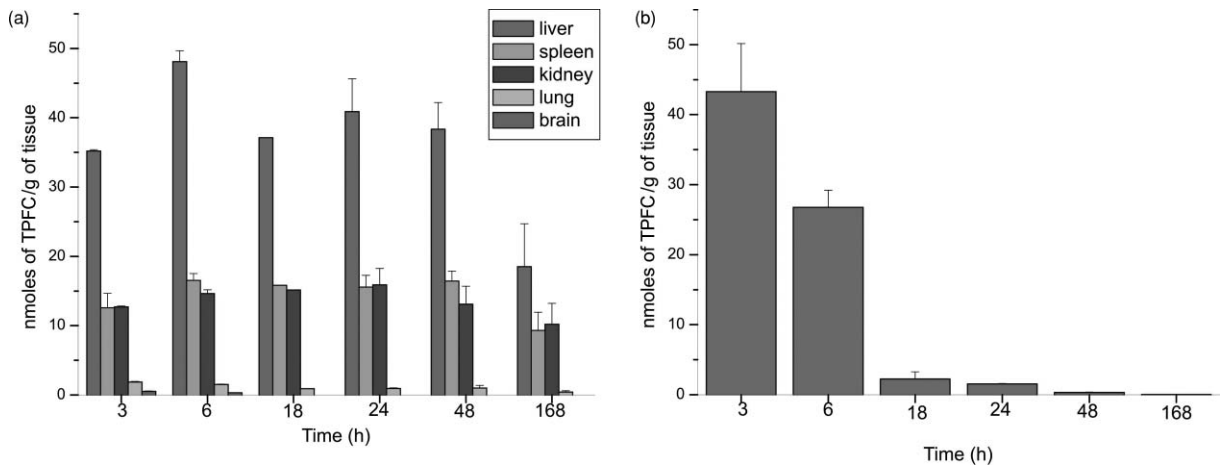

Fig. 8 Recovery of TPFC from different tissues (a) and serum (b) of C57BL/6 mice bearing a s.c.-transplanted B16 pigmented melanoma at various times after i.v. injection of 10 mg kg^{-1} of photosensitizer dissolved in a ternary mixture composed of 20% DMSO, 30% PEG 400 and 50% water.

Conclusions

In summary, a new carborane-containing chlorin TPFC was synthesized in 48% overall yield from commercially available materials. The presence of the four carborane cages does not impair the affinity of this chlorin for tumor cells, as shown by our findings with two different cell lines. Moreover, the boronated chlorin exhibits an efficient photosensitizing activity against melanotic melanoma cells, namely a tumor type which is usually poorly responsive to PDT. Interestingly, TPFC appears to induce an extensive cell photoinactivation under relatively mild irradiation conditions even though its singlet oxygen quantum yield is rather low, at least in a homogeneous solution. This observation can be interpreted by assuming that reaction pathways not involving singlet oxygen occur in the subcellular microenvironment of the boronated chlorin, *e.g.* type I mechanisms promoted by radical species, which are generated *via* electron transfer from the photoexcited triplet chlorin.[27] Alternatively, the efficiency of the photoprocess can be enhanced by the close proximity between the cell-bound TPFC and specific constituents whose integrity is critical for cell survival.[33] Our fluorescence microscopy images indicate that TPFC is largely partitioned into the cytoplasmic membrane, where several potential targets of photosensitized reactions, especially proteins and unsaturated lipids, are present. A multi-target nature of the photoprocess would also be in agreement with the observed predominance of random necrosis rather than apoptosis as the main mechanism of melanotic melanoma cell death. On the other hand, TPFC was found to be non-toxic in the dark toward human carcinoma cells up to the 100 μM concentration investigated, and to localize subcellularly preferentially within the cell lysosomes.

The *in vivo* data appear to confirm the indications obtained through cellular studies in regard to both the lack of detectable toxic effects of TPFC at photodynamically active doses, and the efficiency of this chlorin in inducing a significant response of the melanotic melanoma to PDT. This observation is very encouraging since a variety of photodynamic sensitizers have been previously shown to be inefficient toward highly pigmented tumors.[2,27] Only photosensitizers absorbing in the infrared spectral region have the possibility of favourably competing with melanin for the incident light; however, such photosensitizers cannot act photodynamically due to insufficient energy in their electronically excited states for the generation of singlet oxygen.[29] Since BNCT acts on tumors *via* completely different mechanisms, it appears reasonable to hypothesize that activation of the porphyrin-bound boron atoms by irradiation with thermal neutrons could promote a synergistic effect with PDT, enhancing the overall damage to pigmented tumors and providing novel perspectives for their therapeutic treatment. The use of boronated compounds for therapeutic applications must also address the problem of generalized toxicity, which may arise as a consequence of slow boron release from the carborane clusters, although carboranes are among the most stable boron clusters known.[34] Preliminary studies performed with healthy mice that had been intravenously injected with TPFC at doses up to 4 μmol kg^{-1} body weight showed that no apparent toxic effect occurred up to one month after administration. In any case, we plan to perform more focused toxicological studies especially for those organs, such as liver and spleen, that accumulate larger amounts of the boronated compound and release it at a slow rate, as we have previously reported for related boronated porphyrins.[34]

Experimental section

Synthesis of TPFC

All reactions were monitored by TLC using 0.25 mm silica gel plates (60F-254). The carborane group was detected by TLC upon coating with an aqueous HCl solution of $PdCl_2$(1 g $PdCl_2$ in 80 mL water and 20 mL concentrated HCl solution) followed by heating. Silica gel Sorbent Technologies 32–63 μm was used for flash column chromatography. ^{1}H NMR were obtained on a ARX-250 Bruker spectrometer. Chemical shifts (δ) are given in ppm relative to acetone-d_6 (2.05 ppm, ^{1}H). Electronic absorption spectra were measured on a Perkin Elmer Lambda 35 UV–vis spectrophotometer. Fluorescence spectra were measured on a Perkin Elmer LS55 spectrometer in the 500–800 nm wavelength regions with 1 nm accuracy, excitation at 410 nm. The fluorescence quantum yield was measured using the standard method and 5,10,15,20-tetraphenylporphyrin as the standard (quantum yield is 0.11), according to the literature.[31] Exact mass was obtained using ESI-TOF and the isotope peaks were perfectly matched with the calculated patterns (only the most abundant peaks are listed below). All materials obtained from commercial suppliers were used without further purification.

TPFC. TPPF was converted into the corresponding chlorin in 69% yield, as previously reported in the literature.[14] The resulting chlorin (20.6 mg, 20 μmol), 1-HS-*o*-carborane[35] (35 mg, 200 μmol) and K_2CO_3 (28 mg, 200 μmol) were added to a reaction tube containing 5 mL of DMF. The mixture was stirred at room temperature for two days. KF (12 mg, 200 μmol) was added and the reaction mixture was heated to reflux for 2 h. The mixture was poured into 50 mL of aqueous KCl and extracted with EtOAc (3 × 50 mL). The organic layers were washed three times with aqueous KCl, the solvent evaporated under vacuum and the resulting residue was purified by column chromatography on silica gel using 1 : 1 acetone–ethyl acetate. The major fraction was collected and recrystallized from ethyl acetate and hexane to give the target chlorin TPFC (24.4 mg) in 69% yield. ^{1}H NMR (250 MHz, acetone-d_6) δ 8.89–8.92 (6H, m), 6.14 (2H, br), 4.03–4.06 (4H, m), 3.21–3.29 (2H, m), 2.75–2.76 (2H, m), 2.38 (3H, s), 1.5–3.5 (36H, br), −1.83 (2H, s), −2.51–2.56 (4H, br). HRMS (ESI) m/z 538.2360, calcd. for $C_{55}H_{63}B_{36}F_{16}N_5S_4$: 538.2857. UV–vis (DMSO) 414 nm (137 000), 506 (12 200), 599 (4220), 618 (3200), 652 (28 960).

Photochemical and photophysical studies

The stability of TPFC to red light irradiation was determined by exposure of a chlorin solution in DMSO (absorbance 0.25 at 410 nm) to 600–700 nm light (100 mW cm^{-2}) isolated from the emission of a quartz-halogen lamp (Teclas, Lugano, Switzerland) by optical filtering. The solution was placed in a quartz cuvette with a 1 cm optical path and kept at 25 °C under gentle magnetic stirring. The light beam was driven to the target by a bundle of optical fibers (external diameter = 8 mm). After predetermined irradiation times, the absorbance spectrum was recorded by a Cary Eclipse 50 scan spectrophotometer and the value of the absorbance

at the maximum peak at time 0 was compared with those recorded after various irradiation times.

The quantum yield (Φ_Δ) of singlet oxygen generation by TPFC was measured by following the decrease in the fluorescence emission of 9,10-dimethyl-anthracene (DMA) upon its photosensitized conversion into the corresponding non-fluorescent 9,10-endoperoxide.[32] A 20 μM DMA and TPFC solution in DMSO was irradiated at 20 ± 2 °C under gentle magnetic stirring (600–700 nm, 100 mW cm^{-2}) for different periods of time. The DMA fluorescence emission was recorded in the 380–550 nm wavelength range with excitation at 360 nm. The first-order rate constant of the photoprocess was obtained by plotting ln F_0/F as a function of the irradiation time, where F_0 and F represent the fluorescence intensity at time 0 and at time t, respectively. The rate constant was converted into the $^1O_2$ quantum yield by comparison with the rate constant for DMA photooxidation sensitized by unsubstituted Zn(II)-phthalocyanine (ZnPc) for which Φ_Δ was shown to be 0.55.[36]

Cell accumulation and photosensitization studies

Melanotic murine melanoma cells (B16F1) were seeded in 25 cm^2 flasks and approximately 24 h later the cells were washed twice with PBS. The cells were incubated with TPFC in DMEM containing 0.3% DMSO in a humid atmosphere containing 5% CO_2 at a constant temperature of 37 °C. Control studies showed that incubation of the cells with the solvent both in the presence and the absence of the boronated chlorin had no effect on cell survival. The cells were incubated in DMEM with TPFC in a humid atmosphere containing 5% CO_2 at a constant temperature of 37 °C. At the end, the cells were washed twice with PBS without Ca^{2+} and Mg^{2+} and removed from the flasks by exposure to trypsin. The cells were centrifuged at 1100 rpm for 7 min; then 2 mL of a 2% sodium dodecyl sulphate (SDS) solution per flask were added and the cells incubated for 60 min under gentle magnetic stirring. An aliquot was diluted with 2% SDS and the fluorescence emission was measured. A second aliquot was used to measure the protein concentration by the bicinchoninic acid test.[37] The chlorin recovery was expressed as nanomoles of TPFC per mg of cell protein.

For the dark toxicity experiments, human carcinoma HEp2 cells were plated at 10 000 per well in a Costar 96 well plate and allowed 36–48 h to attach. The cells were exposed to increasing concentrations of TPFC up to 100 μM and incubated overnight. The loading medium was then removed and the cells fed medium containing Cell Titer Blue (Promega) as per the manufacturer's instructions. Cell viability was measured by reading the fluorescence at 520/584nm using a BMG FLUOstar plate reader. The signal was normalized to 100% viable (untreated) cells and 0% viable (treated with 0.2% saponin from Sigma) cells.

For the phototoxicity experiments, the B16F1 cells were seeded in Petri dishes (35 mm diameter) and after 24 h the growth medium was removed, replaced with DMEM enriched with 10% FCS at the desired concentration of TPFC. The cells incubated at 37 °C for 24 h or 3 h were then washed twice with 2 ml of PBS containing Ca^{2+} and Mg^{2+}, and irradiated in the same buffer for various periods of time with 600–700 nm light from a high pressure sodium lamp (Waldmann, Schwenningen, Germany). The fluence rate at the level of the cell monolayer was 20 mW cm^{-2}. At 24 h after irradiation the cells were washed twice and trypsinized. Then the surviving cells were counted in a Burker chamber with a standard trypan blue staining. The survival percentage was calculated by comparing the counts obtained for TPFC-incubated and irradiated cells to those obtained for cells irradiated in the absence of TPFC. Control studies showed that irradiation alone or chlorin in the dark had no detectable effect on cell survival.

TPFC intracellular localization

The B16F1 cells were seeded on a round cover glass in Petri dishes (5×10^4 cells per dish). After 48 h the cells were incubated with 20 μM TPFC for 24 h and then observed with an Olympus IMT-2 fluorescence microscope equipped with a refrigerated CCD camera (Micromax; Princeton Instruments). A 75 W xenon lamp was used as the excitation source. Fluorescence images obtained with a 40 × 1.4 NA oil immersion objective (Olympus) were acquired with the imaging software Methamorph (Universal Imaging). The TPFC fluorescence was detected by means of a set of filters with 400 nm excitation and 620 nm emission.

The HEp2 cells were plated on LabTek 2 chamber coverslips and incubated overnight, before being exposed to 10 μM of TPFC for 24 h. For the co-localization experiments the cells were incubated for 24 h concurrently with TPFC and one of the following organelle tracers, for 30 min: MitoTracker Green (Molecular Probes) 250 nM, LysoSensor Green (Molecular Probes), and BODIPY FL C_5-ceramide at 50 nM (Golgi network). The slides were washed three times with growth medium and new medium containing 50 mM HEPES pH 7.4 was added. Fluorescence microscopy was performed using a Zeiss Axiovert 200M inverted fluorescence microscope fitted with standard FITC and Texas Red filter sets (Chroma). The images were aquired with a Zeiss Axiocam MRM CCD camera fitted to the microscope.

Mechanisms of photosensitized cell death

Specific aspects of the mechanisms involved in the TPFC-photosensitized death of melanotic B16F1 cells were investigated by measuring the activity of caspase-3a. Typically, cells were incubated with 20 μM TPFC for 24 h and then irradiated for 1 min and 3 min under the same experimental conditions as specified above. The cells were collected at 2 h and 6 h after the end of the irradiation. According to the manufacturer-recommended procedure of the ApoAlert CPP32 kit (Clontech, Palo Alto, CA), 10^6 cells were counted, centrifuged, resuspended in 50 μl of lysis buffer, and held for 10 min on ice. Then, 50 μL of reaction buffer containing DTT (dithiothreitol) and 5 μl of DEVD (Asp-Glu-Val-Asp-)-7-amino-4-trifluoromethyl-coumarin were added to the cell lysate, and the fluorescence emitted at 505 nm ($\lambda_{ex} = 400$ nm) was measured with a Perkin Elmer LS50 spectrometer. The caspase-3 activity in the treated cells was expressed as an x-fold increase in the emitted fluorescence, taking the fluorescence from untreated cells as a reference.

In a parallel experimental session, cells irradiated as previously described were collected at 2 h and 6 h post-irradiation, washed twice with PBS and incubated in the dark for 10 min with PBS containing Hoechst dye HO33342 (HO342) at a concentration of 5 μg ml^{-1}. Nuclear fragmentation was assessed by fluorescence microscopy (Zeiss, Germany). The excitation wavelength was 353–377 nm with emission monitored at 420–450 nm.

Pharmacokinetic studies

Female C57BL/6 mice, 20–22 g body weight, were obtained from Charles River (Como, Italy) and housed in specifically designed cages (up to 10 mice per cage) with free access to standard dietary chow and tap water. All the *in vivo* investigations were performed by following the regulations established by the Italian Ministry of Health ethical committee for the humane treatment of experimental animals. The animal experiments were also approved by the Italian Ministry of Health.

To study the pharmacokinetic properties of TPFC, the mice (5 mice for each time point) were subcutaneously injected in the upper flank with 10^6 B16F1 cells suspended in 20 μl of DMEM. At about 7 days after transplantation, when the malignant lesion had reached a volume of about 0.02 cm^3, TPFC (10 mg kg^{-1} body weight) was injected into the tail vein after solubilization in a ternary mixture containing 20% DMSO, 30% PEG 400 and 50% water. Control studies had previously shown that this solvent mixture, as well as the TPFC at the administered dose, had no detectable toxic effects on the mice. At predetermined time intervals after i.v. injection of the PDT agent, the mice were sacrificed by euthanasia and the blood and selected organs were quickly removed. The serum was isolated by centrifugation at 3000 rpm for 15 min at room temperature. The tissues were washed once with PBS, homogenized with 2% aqueous SDS and kept under gentle magnetic stirring for 2 h. At the end, the samples were diluted with a suitable amount of 2% aqueous SDS in order to obtain an absorbance lower than 0.1 at 410 nm, namely the wavelength to be used for spectrophotofluorimetric analysis, in order to avoid any artifact due to inner filter effects. The 410 nm-excited fluorescence emission typical of TPFC was collected in the 600–800 nm wavelength interval and its intensity was converted into the TPFC concentration (nmoles of chlorin per g of tissue) by interpolation with a calibration plot built with solutions of known TPFC concentration in 2% SDS. Previous investigations showed that this procedure allows the recovery of 90–95% tissue-bound photosensitizer.[4]

Photodynamic therapy studies

In a typical experiment, groups of 6 to 8 mice bearing the s.c.-transplanted tumor were injected in the tail vein with TPFC (10 mg kg^{-1}) by following the same procedure as described for the pharmacokinetic experiments. At 3 h or 24 h after injection of the chlorin, the neoplastic lesion was irradiated with 600–700 nm light isolated from the emission of a quartz-halogen lamp (Teclas, Lugano, Switzerland) by means of a band-pass optical filter. The light source was equipped with UV- and heat-reflecting filters, while the light beam was piloted to the irradiated area by a bundle of optical fibers (total external diameter: 0.5 cm). The irradiation was carried out for 10 min at a fluence rate of 135 mW cm^{-2} (total delivered light dose: 81 J cm^{-2}). The response of the melanotic melanoma to the photodynamic treatment was determined by measuring the volume of the tumor with a calliper at daily intervals.[29] No effect on the rate of tumor growth was caused by irradiation of the melanoma under identical conditions but in the absence of the photosensitizer, as well as by injection of TPFC without exposure to light.

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