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Synthesis and cellular studies of a carboranylchlorin for the PDT and BNCT of tumors

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Abstract—The syntheses of *closo*- and *nido*-carboranylchlorins **4** and **5** from a known carboranylporphyrin are described. Water-soluble *nido*-carboranylporphyrin **5** was found to have very low dark cytotoxicity ($IC_{50} > 500$ μM using a MTT-based assay) but to be toxic in the presence of red light ($IC_{50} = 80$ μM at 0.55 J/cm^2 light dose). Under the same experimental conditions, carboranylchlorin **5** was taken up by human glioma T98G cells to a significantly higher extent than chlorin e_6, a chlorophyll degradation product. The preferred sites of subcellular localization of carboranylchlorin **5** were found to be the cell lysosomes. Our results suggest that carboranylchlorin **5** is a promising new dual sensitizer for the PDT and BNCT treatment of tumors.

1. Introduction

Boronated porphyrins and related macrocycles are currently being investigated as dual sensitizers for the boron neutron capture therapy (BNCT) and the photodynamic therapy (PDT) of cancer.[1–8] Both these therapies involve the selective accumulation of a sensitizer within tumor tissue, followed by irradiation of the tumor with low energy neutrons (in BNCT) or with red light (in PDT). The two cytotoxic particles produced from the boron-10 neutron capture reaction in BNCT are $^4He^{2+}$ and $^7Li^{3+}$, which cause irreversible damage to tissue via ionization processes.[9,10] In PDT the main cytotoxic species generated is highly reactive singlet oxygen, upon energy transfer from electronically excited triplet sensitizer and molecular oxygen.[11,12] All the above cytotoxic species have limited ranges in tissue (approximately 0.1 μm for $^1O_2$, 9 μm for $^4He^{2+}$, and 5 μm for $^7Li^{3+}$) and therefore the toxic effect is highly localized to sensitizer-containing tissues. The combination of BNCT and PDT using a single drug has added advantages in that it can be easy to deliver with minimal invasiveness, while leading to increased therapeutic effect due to the targeting of different mechanisms of tumor cell destruction. This combined technique could be particularly attractive for the treatment of high-grade gliomas and metastatic brain tumors, which cause about 13,000 deaths/year in the US.[13,14] The currently available treatments for these tumors involve surgical resection, whole-brain irradiations, and adjuvant chemotherapy, but these modalities lack local control of the disease which is especially critical in the brain. Phase I/II BNCT brain tumor clinical trials conducted in the US, Japan, and Europe using either disodium mercapto-*closo*-dodecaborane (BSH) or L-4-dihydroxyborylphenylalanine (BPA) have led to increased patient survival time.[15–19] On the other hand, the adjuvant treatment of malignant brain tumors using Photofrin®—PDT provided moderate palliation of recurrent brain tumors and a significant increase in the patient survival time.[20,21] The development of more tumor-selective sensitizers for both the BNCT and PDT treatment of brain tumors can potentially increase the effectiveness of these two localized modalities and their combination.[22] Ideal dual sensitizers are amphiphilic long wavelength-absorbing chromophores of high boron content, able to accumulate in glioma cells and to persist there for a considerable amount of time, with low dark toxicity, but toxic upon activation by low energy neutrons and by red light. Dihydroporphyrins (e.g., chlorins) of high boron content are particularly promising for dual application in BNCT and PDT because of their stronger absorptions in the red region of the optical spectrum, where light penetration through tissue is considerably increased, compared

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with porphyrins.[12,23] Other reported promising dual sensitizers are boron-containing tetrabenzoporphyrins[24,25] and phthalocyanines.[3,7,8,26]

Herein, we report the synthesis of *closo*- and *nido*-carboranylchlorins **4** and **5** from a known carboranylporphyrin,[5] in just four steps. We also report cellular studies of water-soluble *nido*-carboranylchlorin **5**, including dark and light cytotoxicity, cellular uptake in comparison with chlorin e_6 (Fig. 1), and preferential sites of intracellular localization in human glioma T98G cells.

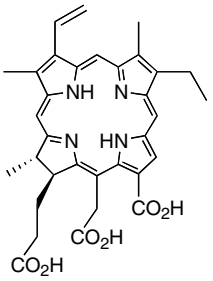
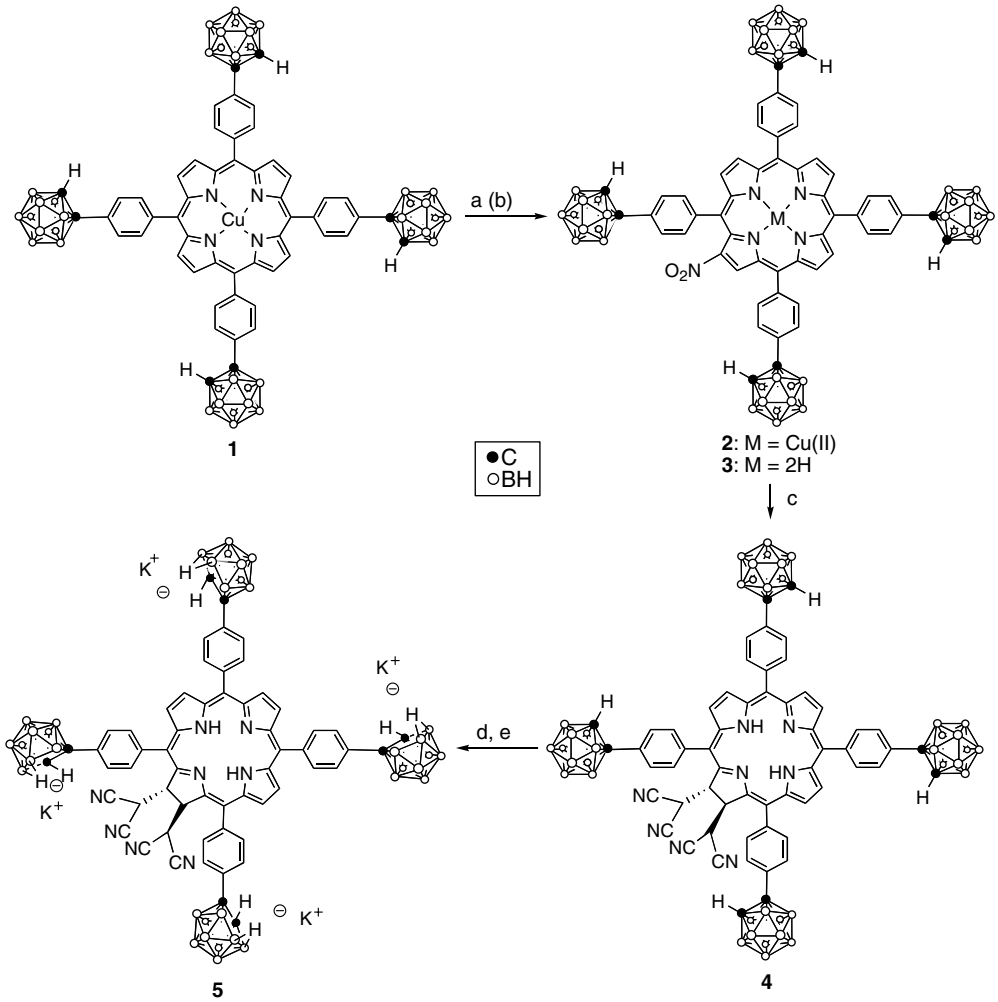


Figure 1. Structure of chlorin e_6.

2. Results

2.1. Chlorin synthesis

Carboranylchlorins **4** and **5**, containing 30 and 28% boron by weight, respectively, were prepared from Cu(II) carboranylporphyrin **1**, as shown in Scheme 1.[23] The starting porphyrin **1** was prepared in 31% overall yield from condensation of 4-(*o*-carboranyl)benzaldehyde with pyrrole under mild conditions, as we have previously described,[5] followed by the insertion of copper(II) upon refluxing a solution of the porphyrin in chloroform in the presence of copper(II) acetate. Complex **1** was nitrated at a β-pyrrolic position using copper(II) nitrate in acetic acid/acetic anhydride to afford the copper(II) complex **2** in 86% yield.[27,28] Lithium nitrate was also investigated as the nitrating agent, but only 14% of complex **2** was obtained under these conditions even after a 6 h reaction time. Demetalation of complex **2** proceeded in quantitative yield using 2% H_2SO_4 in trifluoroacetic acid (TFA) at room temperature. The reaction of nitroporphyrin **3** with excess of malononitrile in the presence of potassium carbonate gave *trans*-chlorin **4** in 41% yield.[29] In order to achieve water-solubility,



Scheme 1. Reagents and conditions: (a) $Cu(NO_3)_2$, CH_2Cl_2, CH_3COOH, $(CH_3CO)_2O$, reflux, 35 min (86%); (b) 2% H_2SO_4 in TFA, rt, 1 h (99%); (c) $CNCH_2CN$, K_2CO_3, THF, 65 °C, 7 h (41%); (d) pyridine/piperidine 3:1, room temperature, 36 h; (e) Dowex resin in K^+ form (99% from **4**).

trans-chlorin **4** was converted into the *nido* derivative **5** by basic degradation of the *closo*-carborane cages, using pyridine/piperidine (3:1), followed by ion exchange using a Dowex resin in the potassium form.[5] As the tetra-potassium salt, chlorin **5** is highly soluble in water and in polar aprotic organic solvents such as acetone and DMSO.

2.2. Cytotoxicity

The dark cytotoxicity of chlorin **5** was investigated in human glioma T98G cells at chlorin concentrations up to 500 μM for 24 h. As a comparison, the cytotoxicity of chlorin e_6 (Fig. 1) was also determined under the same conditions, and the results are shown in Figure 2. Both chlorins were non-toxic in the dark, even at the highest concentration studied, and $IC_{50} > 500$ μM was found for both chlorins. Chlorin **5** was significantly more toxic in the presence of light ($IC_{50} = 50$ μM) toward T98G cells than in the absence of light (Fig. 3). The phototoxicity was evaluated at chlorin **5** concentrations up to 100 μM, upon exposure to 0.55 J/cm^2 light from a halogen lamp equipped with a 610 nm long pass filter.

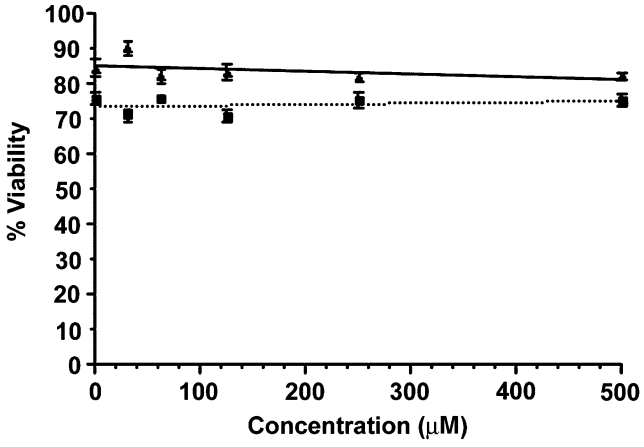


Figure 2. Dark cytotoxicity of chlorin **5** (dotted line) and chlorin e_6 (full line) toward T98G cells using a MTT assay.

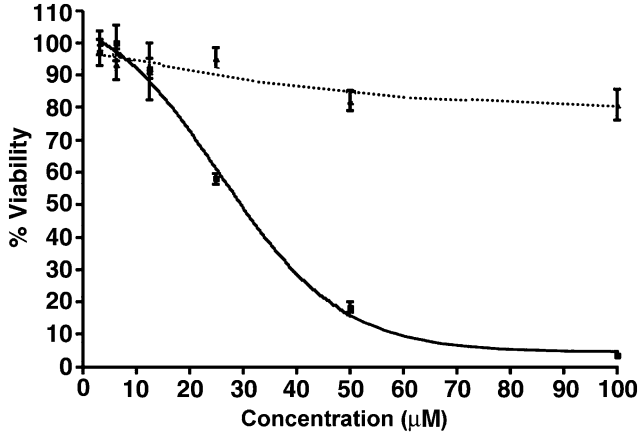


Figure 3. Phototoxicity of chlorin **5** toward T98G cells in the absence (dotted line) and presence (full line) of 0.55 J/cm^2 dose light using a MTT assay.

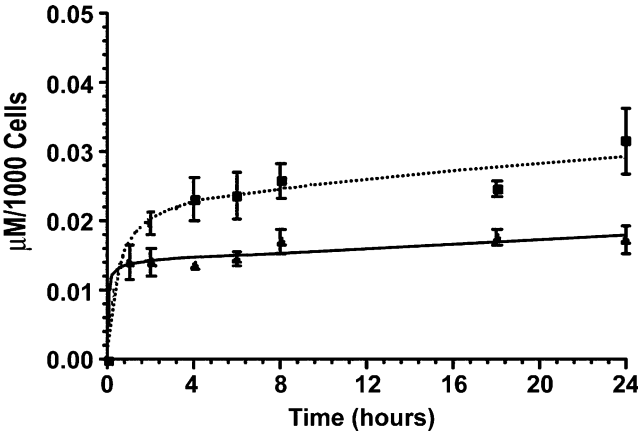


Figure 4. Time-dependent uptake of chlorin **5** (dotted line) and chlorin e_6 (full line) at 10 μM by T98G cells.

2.3. Time-dependent cellular uptake

The time-dependent uptake of chlorin **5** and chlorin e_6 was investigated using human T98G cells for time periods up to 24 h and chlorin concentrations of 50 μM (Fig. 4). Both chlorins were taken up rapidly in the first 1–2 h, after which a plateau was reached. Chlorin **5** was taken up by T98G cells to a significantly higher extent than chlorin e_6 at all time points studied.

2.4. Intracellular localization

Fluorescence microscopy was used to examine the preferential sites of intracellular localization of chlorin **5** in T98G cells, as shown in Figure 5. The observed fluorescence had a punctate pattern characteristic of the cell lysosomes (Fig. 5b), similar to that found with the lysosome-specific probe LysoSensor Green (Fig. 5e). The overlay of the two fluorescences, as shown in Figure 5f, clearly suggests that the two fluorophores are localized in the same organelle. We observed no co-localization of chlorin **5** with other organelle-specific probes, such as BODIPY FL C5-ceramide (5c,d) and MitoTracker Green (5g,h).

3. Discussion

Boron-containing porphyrins and derivatives are highly promising BNCT agents because of their high selectivity and persistence in tumor cells.[1,2,30] Among these, chlorins, tetrabenzoporphyrins, and phthalocyanines are of greater potential use in dual BNCT/PDT than porphyrins, due to their longer-wavelength absorptions along with increased molar extinction coefficients, enabling lower energy light to be used with concomitant increased penetration through tissue. Indeed, most second-generation PDT sensitizers, for example, benzoporphyrin derivative monoacid, mono-aspartyl chlorin e_6, tetra(3-hydroxyphenyl)chlorin, and pyropheophoride a hexyl ether, are chlorins.[31] Other than simple reduction, few methods have been developed for conversion of tetraarylporphyrins into chlorins. During attempts to prepare pyrroloporphyrins from zinc(II) 2-nitro-tetra-

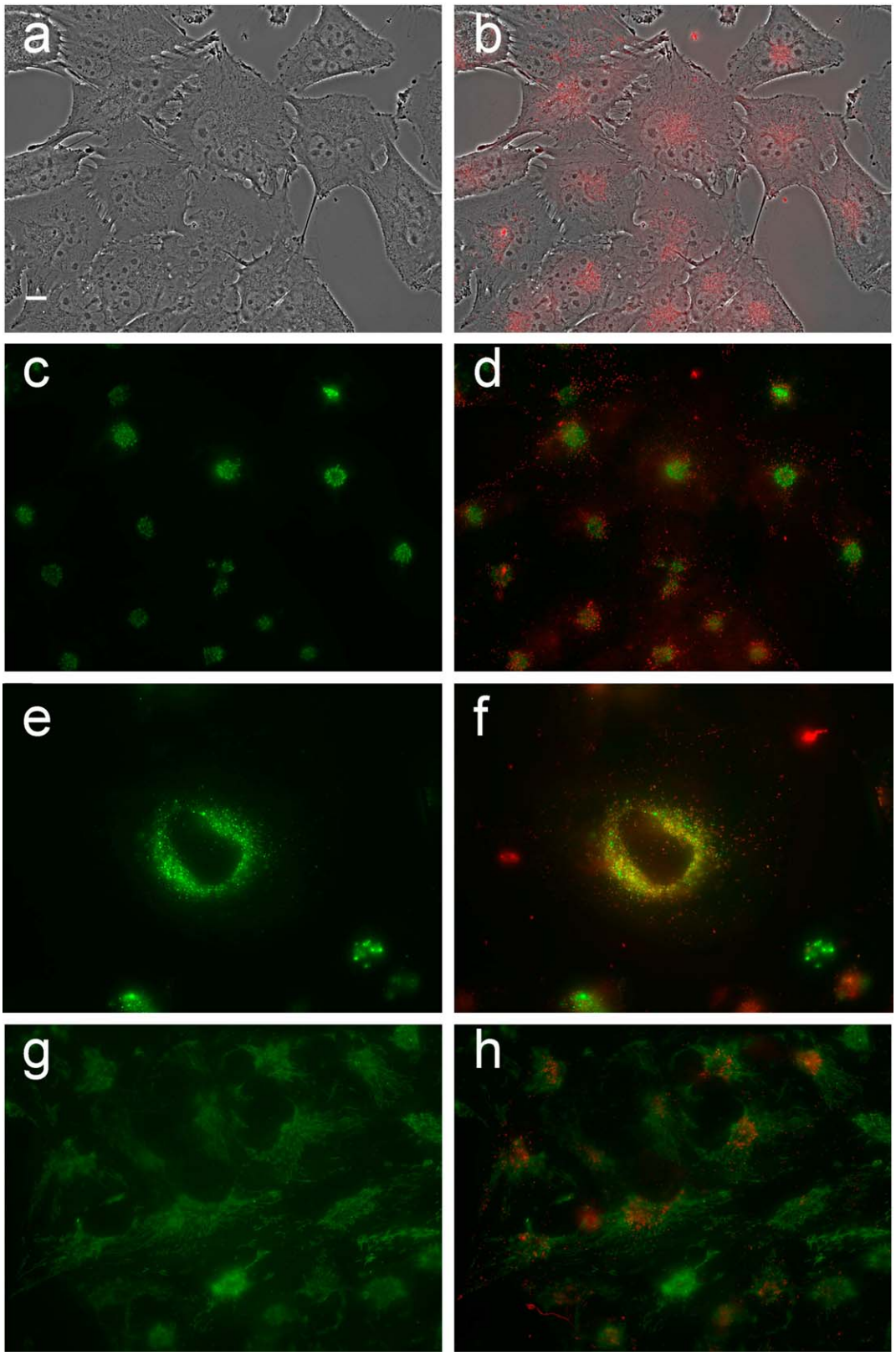


Figure 5. Intracellular localization of chlorin **5** in T98G cells. (a) Phase contrast; (b) overlay of chlorin **5** fluorescence and phase contrast; (c) BODIPY FL C5-ceramide fluorescence; (e) LysoSensor green fluorescence; (g) MitoTracker green fluorescence; (d, f and h) overlays. Scale bar: 10 μm.

arylporphyrins it was noted that under certain conditions large amounts of β,β′-methanochlorins were formed. These methanochlorins could be further ring-opened to afford *trans*-chlorins bearing large substituents that prevented reoxidation back to porphyrin.[32] Conditions were subsequently developed to provide a

reproducible method for preparation of methanochlorins and *trans*-chlorins using active methylene compounds (e.g., diethyl malonate, malononitrile, etc.) in the presence of a base.[29] Michael addition of the anion from the active methylene compound to the nitropyrrole subunit gives a nitronate species that, in the presence of excess anion, is transformed into a *trans*-chlorin, almost certainly by ring-opening of a β,β′-methanochlorin. Using this procedure chlorin **4** was prepared from nitroporphyrin **3** in high overall yield and the carborane cages were stable under these conditions (Scheme 1). Due to the bulkiness of the dicyanomethyl groups on the reduced pyrrole ring, *trans*-chlorin **4** is highly resistant to dehydrogenation. The water-soluble *nido*-chlorin **5** was obtained in quantitative yield from **4** by base-induced deboronation of the *closo*-carborane cages, as previously reported.[5] We have recently reported the X-ray structures of porphyrins **1** and **3**.[23]

Human glioblastoma T98G cells were used in the cellular studies and chlorin e_6 (Fig. 1), a commercially available chlorophyll *a* derivative, was used in the dark toxicity and uptake experiments for comparison purposes. Similarly to chlorin e_6, chlorin **5** was found to be non-toxic in the dark ($IC_{50} > 500$ μM) using a MTT-based assay (Fig. 2), indicating that the presence of *nido*-carborane cages has no effect on the dark toxicity of chlorin macrocycles. Similar results have been found for the related *nido*-carboranylporphyrins[5,6,33,34] and -tetrabenzoporphyrins.[24,25] However in the presence of light, chlorin **5** is significantly more toxic toward T98G cells, even at a low light dose of 0.55 J/cm^2, as seen in Figure 3. The observed phototoxicity is probably due to the generation of singlet oxygen, as has been determined for *nido*-carboranylporphyrins.[5,6] Boronated phthalocyanines have also been shown to have high quantum yields for singlet oxygen generation and to be efficient dual PDT/BNCT sensitizers.[3,7,8]

Chlorin **5** was taken up by human glioma T98G cells to a significantly higher extent than chlorin e_6, as shown in Figure 4, probably due to the higher hydrophobic character of **5** compared with chlorin e_6. The kinetics of uptake were similar for both chlorins; in the first hour both compounds rapidly accumulated within cells, but between 2 and 24 h the uptake was much slower, nearly defining a plateau. These results are in agreement with literature reports on the uptake kinetics of *nido*-carboranylporphyrins,[33,34] suggesting similar mechanisms of uptake for these macrocycles. Since chlorin **5** is comprised of 28% boron by weight and is readily accumulated within human glioblastoma cells, it can potentially deliver large amounts of boron to brain tumors. Concentrations of ^{10}B in the range of 15–30 μm ^{10}B/g tumor are necessary for effective BNCT.[22]

The main sites of subcellular localization of chlorin **5** in T98G cells are the lysosomes (Fig. 5). Similar results have been reported for carboranyl-containing porphyrins.[33,34] The preferential lysosome localization of these macrocycles might be due to their endocytic mechanism of uptake and their negative charge.

4. Conclusions

Long-wavelength absorbing carboranylchlorins can be synthesized from carboranylporphyrins in just a few steps and in 35% overall yield. Under the mild conditions employed, the carborane cages are highly stable to the necessary metalation, nitration, and addition reactions on the porphyrin macrocycle. The water-soluble *nido*-carboranylchlorin **5** is non-toxic in the dark toward human glioma T98G cells ($IC_{50} > 500$ μM, using a MTT-based assay), but it is toxic under low light doses ($IC_{50} = 80$ μM at 0.55 J/cm^2). Chlorin **5** is taken up by T98G cells in culture to a significantly higher extent than is chlorin e_6, a chlorophyll degradation product, probably due to the higher hydrophobic character of **5**. Both chlorins display similar uptake kinetics, accumulating rapidly within tumor cells in the first 1–2 h and are subsequently taken up at a much slower rate, eventually reaching a plateau. The preferential sites of subcellular accumulation of chlorin **5** were found to be the cell lysosomes, as determined by fluorescence microscopy. Since carboranylchlorin **5** was found to be phototoxic, efficiently taken up by T98G cells localizing mainly within the lysosomes, and to be non-toxic in the dark, it is a promising sensitizer for dual application in both the BNCT and the PDT of treatment of malignant brain tumors.

5. Experimental

5.1. Chemistry

All reactions were carried out under an argon atmosphere in dried solvents. Commercially available starting compounds were purchased from Sigma–Aldrich and used directly without further purification. Silica gel 60 (70–230 mesh, Merck) was used for column chromatography. Analytical thin-layer chromatography (TLC) was performed using Merck 60 F254 silica gel (precoated sheets, 0.2 mm thick). ^{1}H NMR spectra were obtained using a Bruker DPX 250 MHz spectrometer; chemical shifts are expressed in ppm relative to TMS (0 ppm). Electronic absorption spectra were measured on a Perkin Elmer Lambda 35 UV–vis spectrophotometer and fluorescence spectra were measured on a Perkin Elmer LS55 spectrometer. Mass spectra were obtained on a Bruker Prolix III MALDI-TOF mass spectrometer. Melting points were measured on a MELT-TEMP apparatus.

5.2. Cu(II) 5,10,15,20-tetra[4-(*o*-carboranyl)phenyl]porphyrin (1)

To a solution of *meso*-tetra[4-(*o*-carboranyl)phenyl]porphyrin[5] (80 mg, 0.068 mmol) in dry chloroform (6 mL) was added 8 mL of a saturated solution of $Cu(CH_3COO)_2{\cdot}2H_2O$ in methanol. The reaction mixture was refluxed for 48 h and monitored by UV–vis spectroscopy. The solvent was removed under vacuum, the residue dissolved in dichloromethane (50 mL), washed with water (2× 50 mL), and then dried over anhydrous Na_2SO_4. The volume of the solvent was reduced under

vacuum, and the residue was purified by filtration through a silica gel plug using dichloromethane for elution. Recrystallization from methanol gave 79 mg (94%) of the title compound. Mp: >300 °C; HRMS (MALDI-QTOF) for $C_{52}H_{68}B_{40}CuN_4$+H: calcd *m*/*z* 1245.8840, found 1245.8818; UV–vis ($CHCl_3$) λ_{max}: 415 nm (ε 398,000) and 538 (20,000).

5.3. Cu(II) 2-nitro-5,10,15,20-tetra[(4-*o*-carboranyl)phenyl]porphyrin (2)

Cu(II) porphyrin (**1**) (30 mg, 0.024 mmol) was added to dry dichloromethane (20 mL) and was warmed to 32 °C until dissolved. A solution of $Cu(NO_3)_2{\cdot}2.5H_2O$ (24 mg, 0.103 mmol) in acetic acid (5.0 mL) and acetic anhydride (4.4 mL) was added slowly while stirring vigorously. The reaction was followed by TLC and was completed in 45 min. The reaction mixture was washed with water (2× 50 mL), the solvent was removed under vacuum, and the resulting residue was recrystallized from methanol to give 27 mg (86%) of the title product. Mp: >300 °C; HRMS (MALDI-QTOF) for $C_{52}H_{67}B_{40}CuN_5O_2$+H: calcd *m*/*z* 1312.8624, found 1312.8730; UV–vis ($CHCl_3$) λ_{max}: 427 nm (ε 184,000), 546 (10,400), 591 (6400) and 662 (2800).

5.4. 2-Nitro-5,10,15,20-tetra[(4-*o*-carboranyl)phenyl]porphyrin (3)

To a solution of Cu(II) nitroporphyrin (**2**) (51 mg, 0.039 mmol) in dry dichloromethane (0.5 mL) was carefully added 2% H_2SO_4 in TFA (10 mL). The mixture was stirred under argon for 1 h, while monitoring by spectrophotometry. The reaction mixture was added to water, neutralized with $NaHCO_3$, and extracted with dichloromethane (3× 20 mL). The solvent was removed under vacuum and the residue was recrystallized from methanol to give 48 mg (99%) of the title porphyrin. Mp: >300 °C; HRMS (MALDI-QTOF) for $C_{52}H_{69}B_{40}N_5O_2$+H: calcd *m*/z 1228.9572, found 1228.9475; ^{1}H NMR ($CDCl_3$) δ ppm: −2.71 (br, 2H), 1.5–3.3 (br, 40 H), 4.29 (br, 4H), 7.90 (m, 8H), 8.18 (m, 8H), 8.66 (s, 2H), 8.88 (m, 4H), 8.98 (s, 1H). UV–vis ($CHCl_3$) λ_{max}: 429 nm (ε 211,200), 526 (15,400), 602 (4400) and 663 (8300).

5.5. *trans*-2,3-Dihydro-2,3-bis(dicyanomethyl)-5,10,15,20-tetra[(4-*o*-carboranyl)phenyl]chlorin (4)

A mixture of K_2CO_3 (54 mg, 0.39 mmol) and malononitrile (36 mg, 0.55 mmol) in dry THF (6.0 mL) was refluxed for 1 h under argon. The reaction mixture was cooled to room temperature and nitroporphyrin (**3**) (48.3 mg, 0.039 mmol) dissolved in dry THF (5 mL) was added to the mixture. The temperature was slowly increased to 65 °C and the mixture was allowed to stir for another 6 h. The reaction mixture was cooled to room temperature, diluted with dichloromethane (20 mL), washed with water (2× 50 mL), and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum and the crude residue was purified by column chromatography using dichloromethane/hexane 3:2 for elution. The main band was collected and recrystallized from hexane to afford 21 mg (41%) of the title carboranylchlorin. Mp: >300 °C; HRMS (MALDI-QTOF) for $C_{58}H_{72}B_{40}N_8$+H: calcd *m*/*z* 1314.9972, found 1314.9916; ^{1}H NMR ($CDCl_3$) δ ppm: −1.96 (br, 2H), 1.6–3.4 (br, 40 H), 4.26 (br, 6H), 5.30 (br, 2H), 7.89 (m, 8H), 8.04 (m, 8H), 8.22 (br, 2H), 8.43 (s, 2H), 8.66 (br, 2H). UV–vis ($CHCl_3$) λ_{max}: 410 nm (ε 227,000), 514 (15,200), 543 (17,000), 591 (8000) and 642 (29,300).

5.6. *trans*-2,3-Dihydro-2,3-bis(dicyanomethyl)-5,10,15,20-tetra[(4-*nido*-carboranyl)phenyl] chlorin (5)

Chlorin **4** (15 mg, 0.011 mmol) was dissolved in pyridine/piperidine 3:1 (3 mL) and allowed to stir at room temperature for 48 h under argon. After removing the pyridine and piperidine under vacuum, the residue was dissolved in 40% aqueous acetone and passed slowly through a Dowex 50WX2-100 resin in the sodium form. The chlorin fraction was collected, dried under vacuum, redissolved in 70% aqueous acetone, and again passed through the ion-exchange resin. After removal of the solvent under vacuum, the title *nido*-carboranylporphyrin (15 mg) was obtained in quantitative yield. Mp: >300 °C; MS (MALDI-QTOF) for $C_{58}H_{72}B_{36}N_8Na_4$: calcd *m*/*z* 317.11, found 318.23; ^{1}H NMR (acetone-d_6) δ ppm: −2.40 (br, 2 H), 1.2–3.5 (br, 40 H), 4.26 (br, 6H), 5.30 (br, 2H), 7.40 (br, 8 H), 8.02 (br, 8H), 8.40 (br, 2H), 8.78 (br, 2H), 8.95 (br, 2H): UV–vis (ethanol) λ_{max}: 422 nm (ε 84,500), 514 (6900), 550 (7400), 595 (3800) and 646 (8000).

5.7. Cell culture

Human glioblastoma T98G cell lines were purchased from ATCC, phosphate-buffered saline (PBS), fetal bovine serum (FBS), and trypsin were purchased from Gibco. The T98G cells were maintained in a 1:1 mixture of DMEM and advanced MEM supplemented with 10% FBS. The CyQUANT cell proliferation assay kit was purchased from Molecular Probes. The MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide] CellTiter 96 non-radioactive cell viability kit was purchased from Promega. Microscopy was performed using a Zeiss Axiovert 200M inverted fluorescent microscope fitted with standard Texas Red (λ_{max} 625 nm at exc. 560 nm) and FITC filter sets (λ_{max} 535 nm at exc. 460 nm) (Chroma technology Corp.).

5.8. Cytotoxicity

The dark toxicity experiments for *nido*-carboranylchlorin **5** and chlorin e_6 were evaluated in human glioma T98G cells, which were seeded onto 96-well tissue culture plates at 10,000 cells per well. The cells were allowed to settle and attached for 48 h. A 2X stock solution of each chlorin dissolved in DMSO was prepared to give 1 mM solutions in 1% DMSO-containing medium and then filter sterilized. A 2-fold dilution series was prepared on the plate to give final chlorin concentration of 500, 250, 125, 62.5, 32.25, and 0 μM, while maintaining 1% DMSO throughout. The cells were incubated for 24 h in the dark. After incubation, the cells were washed with fresh medium and fed with 100 μL

fresh growth medium. Cell viability was then measured using the MTT-based CellTiter 96 non-radioactive cell proliferation assay kit.

The phototoxicity experiments were performed using T98G cells plated at 10,000 cells per well in a Costar 96-well tissue culture dish and incubated overnight. The cells were exposed to chlorin **5** at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, and 0 μM for 18 h. After incubation, the loading medium was removed and replaced with growth medium containing 50 mM HEPES buffer at pH 7.4. The dark controls were left in the incubator, while a duplicate plate was exposed to light from a halogen lamp equipped with a 610 nm long pass filter. The exposure time was 10 min with a measured flux of 3200 lux for a total light dose of 0.55 J/cm^2. During exposure, the cells were kept on ice and the IR radiation was filtered using 5 mm of water contained in an inverted culture plate lid placed over the plate containing the cells. After exposure, the cells were incubated overnight and the cell viability was measured after 18 h using the CellTiter Blue viability assay.

5.9. Time-dependent cellular uptake

The uptake experiments for chlorin **5** and chlorin e_6 were performed on T98G cells which were seeded as described above. The cells were exposed to 50 μM of each chlorin in medium for specific periods of time, between 30 min and 24 h. At the end of the loading period, the compound was removed, the cells were washed with PBS and solubilized with 0.25% Triton X-100 in PBS. The intracellular accumulation of the chlorins was determined by measuring the compound's fluorescence emission using a BMG FluoStar Optima plate reader, while the cell numbers were measured using the CyQUANT cell proliferation assay.

5.10. Intracellular localization

T98G cells were seeded in a Lab-Tek II chamber coverglass system with standard medium and allowed to attach overnight. *nido*-Carboranylchlorin **5** was added to the cells at a concentration of 10 μM and the cells were incubated in the dark for 18 h. The cells were washed with drug-free medium and fed medium containing 50 mM HEPES, pH 7.2. Coverslips were examined using a Zeiss Axiovert 200M inverted fluorescent microscope. Organelle co-localization experiments utilized three different organelle tracers from Molecular Probes; lysosomes were labeled using LysoSensor Green DND-189 at 50 nM, mitochondria were labeled using Mito Tracker green FM at 250 nm, and the Golgi complex was labeled using BODIPY FL C_5-ceramide at 50 nM. All organelle tracers were diluted in medium and incubated concurrently with compound for 30 min prior to washing and microscopy.

Acknowledgment

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