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Introduction

In the past few decades, photodynamic therapy (PDT) has emerged as a minimally invasive regimen for the treatment of cancers.^{1–5} Today, PDT offers an attractive new direction or addition to conventional therapies for diseases including skin lesions, age-related macular degeneration, and cancers (bladder, breast, brain, esophagus, head/neck, lung, and skin). Its therapeutic action is based on the generation of singlet oxygen ($^{1}O_{2}$) and other reactive oxygen species (ROS) that are formed upon light activation of the photosensitizer (PS) which is taken up with higher affinity in malignant than in nonmalignant cells. After photoexcitation, the excited PS transfers its energy to molecular tissue oxygen to generate ROS, of which singlet oxygen is assumed to be the key cytotoxic species causing localized oxidative cell damage and death. In

Synthesis and characterization of novel zinc phthalocyanines as potential photosensitizers for photodynamic therapy of cancers

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Two novel zinc phthalocyanines (Pcs): tetramethyl tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(II) (**4**) and (the negatively charged form) tetrakis-2,(3)-[(3-carboxylicacid-6-sulfanylpyridine)phthalocyaninato] zinc(II) (**5**), water soluble by virtue of their ionic substituent groups were synthesized. The spectroscopic properties of both compounds were determined and their photodynamic activities were investigated in a human tumor cell model. In aqueous media the two peripherally substituted water soluble Pcs are highly aggregated. The phototoxic activity of the two novel Pcs (Pc **4** and Pc **5**; 0–20 μ M) was shown to be time- and dose-dependent in human pancreatic carcinoid BON cells, leading to a reduction of tumor cells of >80% compared to the controls. The effectiveness of the treatment appeared to be attenuated by the aggregation of Pcs under aqueous conditions. Interestingly, even those cells that were not immediately killed by the photoactivated photosensitizer seemed to be affected by the Pc photodynamic activity, as a single PDT induced long-lasting effects on cell survival. Even 4 days after PDT, the number of surviving cells did not re-increase or still dropped, as compared to control cells. The underlying mechanism of this observation has to be deciphered in future investigations.

> spite of great advances, PDT is not yet an integral part of clinical practice due to serious drawbacks of the few regulatory approved PSs, and these include poor solubility in water.

> Notably, tetrapyrroles such as porphyrins,^{6–8} chlorins^{9–12} and phthalocyanines (Pcs)^{13–15} are being used as photoactive agents in PDT. It should be mentioned that Pcs have been shown to be some of the most efficient PSs to date¹⁶ since they have strong absorption in the far red spectral region, demonstrate high intersystem crossing (ISC) to the first excited triplet state and a high efficiency of singlet oxygen generation. The photophysical properties of Pcs can be tuned by rational modification of their structure.¹⁷ This tuning may be achieved through the addition of substituent groups on the periphery of the Pc macrocycle as well as complexing the ligand with different metal ions giving metallated Pcs (MPcs).^{17,18} The photophysical properties of the latter are highly dependent on the central metal ion employed.¹⁷

The suitability of Pcs for PDT is highly dependent on their water-solubility. Thus, some of the highest efficiencies of tumor targeting in photodynamic activity are achieved with water soluble Pcs.^{19,20} In order to accomplish water solubility, water solubilizing groups may be introduced onto the Pc on the periphery and non-periphery of the macrocycle or even as axial ligands for Pcs that have been complexed with multivalent central metal ions such as Si⁴⁺, Al³⁺ Ga³⁺ and In^{3+,21-23} The most common groups used for rendering Pcs water

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Scheme 1 Synthetic route for 4-(4-methyl-2-pyridyloxy)phthalonitrile (1), 4-(3-carboxylicacid-6-sulfanylpyridine)phthalonitrile (2) and phthalocyanines 3, 4 and 5 (where (i) pentanol, zinc acetate, DBU, heating at 160 °C and (ii) dimethyl sulfate and heating at 120 °C under an inert gas).

soluble include carboxy, sulfonate and phosphono groups.^{24–26} Quaternization may also be employed for Pcs bearing pyridino substituents.²⁷

This work reports on the synthesis (Scheme 1) and characterization of new tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(π) (3) and its water soluble quaternized (positively charged) form tetramethyl tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(π) (4) and (the water soluble negatively charged form) tetrakis-2,(3)-[(3-carboxylicacid-6-sulfanylpyridine)phthalocyaninato] zinc(π) (5) phthalocyanines and their photodynamic activity on human pancreatic carcinoid BON cells.²⁸

Experimental methods

Materials

N,*N*-Dimethyl formamide (DMF), 4-nitrophthalonitrile, 2hydroxy-4-methylpyridine, 6-mercaptopyridine-3-carboxylic acid, ethanol, chloroform (CHCl₃), dichloromethane (DCM), tetrahydrofuran (THF) and acetone were obtained from Sigma Aldrich. Bio-beads S-X1 were obtained from Bio-Rad. Ultrapure water was obtained from a Milli-Q Water System (Millipore Corp, Bedford, MA, USA).

Phthalocyanine solutions

Stock solutions of the phthalocyanine complexes were prepared in water and stored at 4 °C. The drugs were diluted in

fresh media before each experiment. In order to evaluate the effect of Pcs on the cells, the cells were incubated with either control medium or medium containing increasing concentrations of the respective Pcs.

Cell line

The human pancreatic carcinoid cell line BON was cultured in a 1:1 mixture of DMEM and HAM's F-12 medium containing 10% of FCS (Biochrom, Berlin, Germany) and 1% L-glutamine. Cells were maintained at 37 °C under a 5% CO₂ humidified atmosphere.^{28–30} Under these conditions, the doubling time of BON cells was 34 ± 4 h. Incubation and subsequent experiments were performed while the cells were in the log phase of their growth.

Methods and instruments

IR data were obtained by using the Perkin-Elmer spectrum 2000 FTIR spectrometer. ¹H NMR spectra were recorded using a Bruker AMX 600 MHz spectrometer. Elemental analyses were carried out on a Vario EL III MicroCube CHNS Analyzer. Mass spectral data were collected using a Bruker AutoFLEX III Smart beam TOF/TOF mass spectrometer. The instrument was operated in positive ion mode using an m/z range of 400–3000. The voltages of the ion sources were set at 19 and 16.7 kV for ion sources 1 and 2 respectively, while the lens was set at 8.50 kV. The reflector 1 and 2 voltages were set at 21 and 9.7 kV respectively. The spectra were acquired using dithranol as the MALDI matrix, and a 354 nm Nd:YAG laser.

Steady-state absorption and fluorescence spectroscopies

The ground-state absorption spectra were recorded using a commercial spectrophotometer Shimadzu UV-2501PC. Steadystate fluorescence spectra were measured in 1 cm \times 1 cm quartz cells using a combination of a cw-xenon lamp (XBO 150) and a monochromator (Lot-Oriel, bandwidth 10 nm) for excitation and a polychromator with a cooled CCD matrix as the detector system (Lot-Oriel, Instaspec IV).

Picosecond transient absorption spectroscopy

The setup for pump-probe experiments with picosecond time resolution was described previously.³¹ Briefly, to measure the transient absorption spectra, a white light continuum was generated as a test beam in a cell with a D₂O-H₂O mixture using intense 25 ps pulses from a Nd³⁺:YAG laser (PL 2143A, Ekspla) at 1064 nm. Before passing through the sample, the continuum radiation was split to obtain a reference spectrum. The transmitted as well as the reference beams were focused into two optical fibers and were recorded simultaneously at different traces on a cooled CCD-matrix (Lot-Oriel, Instaspec IV). Tunable radiation from an OPG/OPA (Ekspla PG 401/SH, tuning range 200-2300 nm) pumped by the third harmonic of the same laser was used as an excitation beam. The mechanical delay line allowed the measurement of light-induced changes of the absorption spectrum at different delays up to 15 ns after excitation. Analysis of experimental data was performed using the compensation method.³²

Light source and irradiation

The radiation unit used a 100 W halogen lamp (HLX 64627, Osram, Germany) as the light source. The spectral output of the lamp ranged from 400 to 800 nm. To prevent infrared irradiation, a heat-reflecting filter (Präzisions Glas & Optik, Iserlohn, Germany) with a cutting-off band for $\lambda \geq 700$ nm was inserted into the optical path. The illuminated area (11 × 14 cm) had an average power density of incident light of 15 mW cm⁻². The light energy dose was measured using a P-9710 radiometer controlled by a silicon photocell (RW-3703-2 radiometer) from Gigahertz-Optik (Munich, Germany). The total light energy dose was calculated by integrating the energy signal over the entire period of irradiation. Cells were illuminated for 33 or 66 min to achieve a total light dose of 30 or 60 J cm⁻², respectively.⁵

Measurement of growth inhibition

PS induced phototoxicity as well as dark toxic effects of the Pcs were determined by crystal violet staining of phthalocyanine (0-20 µM) treated BON cells after incubation and subsequent light irradiation (phototoxicity) or without illumination (dark toxicity). BON cells were seeded at a density of 5000 cells per well into 96-well microtiter plates and incubated with the zinc phthalocyanine photosensitizers for 6 and 12 h periods respectively. After the incubation period had lapsed the phthalocyanine medium solution was replaced with new medium solution and the plates were irradiated for 33 or 66 min by using an incoherent white light source and they were then incubated for 2 day (48 h) and 4 day (96 h) periods respectively. Crystal violet staining was carried out as previously described.33,34 Cells were washed with phosphate buffer solution (PBS) and fixed with 1% gluteraldehyde. Following this step, cells were washed once more with PBS and then stained with 0.1% crystal violet. The unbound crystal violet dye was removed by washing. The crystal violet that had been absorbed onto the cells was solubilized with 0.2% Triton X-100. After this, light extinction was analyzed at 570 nm using an ELISA reader.

Synthetic procedures

Procedure for the synthesis of 4-(4-methyl-2-pyridyloxy)phthalonitrile (1). The reagents 2-hydroxy-4-methylpyridine (1.08 g, 9.9 mmol) and 4-nitrophthalonitrile (1.7 g, 9.8 mmol) were dissolved in DMF (30 ml) under argon. After stirring for 30 min at room temperature, finely ground anhydrous potassium carbonate (2.8 g, 20 mmol) was added in portions for 4 h with efficient stirring. The reaction mixture was stirred under an argon atmosphere at room temperature for 3 days. Then the mixture was poured into 200 ml iced water, and the precipitate was filtered off, washed with water and then dried. The crude product was recrystallized from ethanol. Finally the pure product was dried in a vacuum to give a light purple powder. Yield: 1.6 g (68.67%). IR [(KBr) $ν_{max}/cm^{-1}$]: 3185 (C–H), 2229 (C=N), 1656 (C–C), 1441, 1406 (C=C), 1360, 1279, 1206 (C–O– C), 1494(C=N). ¹H NMR (DMSO-d₆); δ ppm: 8.36 (s, 1H, Ar–H), 8.29 (d, 1H, Ar–H), 8.16 (d, 1H, Ar–H), 7.63–7.68 (m, 1H, pyridyl-H), 7.13 (d, 1H, pyridyl-H), 7.07 (d, 1H, pyridyl-H), 2.20 (s, 3H, pyridyl-H). MS m/z: Calc.: 235.14; found: 236.08 $[M + H]^+$.

Procedure for the synthesis of 4-(3-carboxylicacid-6-sulfanylpyridine)phthalonitrile (2). The synthesis of 2 was similar to that of 1, except that 6-mercaptopyridine-3-carboxylic acid (2 g, 12 mmol) was employed instead of 2-hydroxy-4-methylpyridine. The amount of 4-nitrophthalonitrile used was 2 g (12 mmol). The quantity of anhydrous potassium carbonate used was 3 g (21.0 mmol). The phthalonitrile was precipitated out of the solution by addition of the DMF slurry to acetone. Finally the pure product was dried in a vacuum to give a brownish yellow solid. Yield: 1.3 g (38.46%). IR [(KBr) ν_{max} / cm⁻¹]: 3301 (COOH), 3150 (C−H), 2240 (C≡N), 1362, 1208 (C-S-C), 1656 (C-C), 1439, 1409 (C=C), 1497 (C=N). ¹H NMR $(DMSO-d_6)$; δ ppm: 8.87 (s, 1H, pyridyl carboxyl-H), 8.82 (s, 1H, Ar-H), 7.94 (s, 1H, pyridyl-H), 7.89 (d, 1H, Ar-H), 7.78 (d, 1H, Ar-H), 7.39 (d, 1H, pyridyl-H), 7.17 (d, 1H, pyridyl-H). MS m/z: Calc.: 281.28; found: 282.04 [M + H]⁺.

Procedure for the synthesis of tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(II) (3). A mixture of anhydrous zinc(II) acetate (0.6 g, 2.8 mmol), 4-(4-methyl-2-pyridyloxy) phthalonitrile (1) (0.22 g, 0.94 mmol), DBU (0.27 mL, 2 mmol) and pentanol (25 mL) was stirred at 160 °C for 5 h under a nitrogen atmosphere. After cooling, the solution was mixed with *n*-hexane. The blue solid product was precipitated and collected by filtration and washed with *n*-hexane. The crude product was purified by passing through a silica gel column using chloroform to elute the product. The eluted product was then recrystallized from DMF using methanol. The product was dried in the oven at 100 °C overnight. Yield: 100 mg (42%). UV/vis (DMSO): λ_{max} nm (log ε); 676 (5.02), 610 (4.30), 360 (4.50); IR (KBr): $\nu_{\text{max}}/\text{cm}^{-1}$; 3098, 2606 (C-H), 1659 (C-C), 1495 (C=N), 1439, 1410 (C=C), 1360, 1213 (C-O-C). ¹H NMR (400 MHz, DMSO-d₆) δ ppm: 9.06-8.96 (m, 4H, Ar-H), 8.35-8.32 (m, 4H, pyridyl-H), 8.16-8.12 (m, 4H, pyridyl-H), 7.97-7.90 (m, 4H, pyridyl-H), 7.65 (d, 4H, Ar-H), 7.20-7.17 (m, 4H, Ar-H), 1.22 (s, 12H, pyridyl methyl-H). Calc. for C₅₆H₃₆N₁₂O₄Zn·2CH₃OH: C: 65.08, H: 4.14, N: 15.70. Found: C: 64.17, H: 5.09, N: 12.5. MALDI-TOF-MS m/z: Calc.: 1006.35.41; found: 1007.38 $[M + H]^+$.

(Quaternized) tetramethyl tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(II) (4). Yield: 28 mg (56%). UV/ vis (DMSO): λ_{max} nm (log ε); 675 (4.91), 612 (4.17), 343 (4.51); IR (KBr): ν_{max} /cm⁻¹; 3050, 2618, 2380 (C–H), 1658 (C–C), 1498 (C=N), 1440, 1409 (C=C), 1360, 1213 (C–O–C). ¹H NMR (400 MHz, DMSO-d₆) δ ppm: 8.09 (bs, 4H, Ar–H), 7.96 (bs, 4H, pyridyl-H), 7.86–7.81 (m, 4H, Ar–H), 7.52 (d, 4H, pyridyl-H), 7.29 (d, 4H, Ar–H), 7.22 (d, 4H, pyridyl-H), 1.62 (s, 12H, pyridyl methyl-H), 1.28 (s, 12H, CH₃). Calc. for C₆₀H₄₈N₁₂O₁₂S₂Zn: C: 57.26, H: 3.84, N: 13.35, S: 5.09. Found: C: 58.11, H: 4.85, N: 19.62, S: 6.31. MALDI-TOF-MS *m*/*z*: Calc.: 1258.65; found: 1261.86 [M + 3H]³⁺.

Procedure for the synthesis of tetrakis-2,(3)-(3-carboxylicacid-6-sulfanylpyridine)phthalocyaninato $zinc(\pi)$ (5). The syn-

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thesis of 5 was as outlined for 3, except that compound 2 (0.22 g, 0.77 mmol) instead of compound 1 was employed. The amounts of the other reagents were as follows: anhydrous zinc acetate (0.23 g, 1.13 mmol), dry pentanol (25 ml) and DBU (0.25 ml, 0.16 mmol). A dark green product was obtained after washing with ethanol. It was purified by multiple dissolutions into 0.1 M sodium hydroxide solution and repeatedly precipitating out using 20% HCl. The final precipitated product was washed with methanol, acetone and chloroform. The product was finally dried in the oven overnight at 90 °C. Yield: 30 mg (30%). UV/vis (DMSO): λ_{max} nm (log ε) 683 (5.08), 616 (4.32), 368 (4.53). IR spectrum (cm⁻¹): 3350 (COOH), 3082, 2622 (Ar-CH), 1440, 1400 (C=C), 1657 (C-C), 1498 (C=N), 1361, 1250 (C–S–C). ¹H NMR (DMSO-d₆) δ : 8.35 (bs, 4H, pyridyl-H), 8.25-8.23 (m, 4H, Ar-H), 8.16-8.14 (m, 8H, pyridyl-H), 7.97 (s, 4H, pyridyl-H), 7.84 (s, 4H, Ar-H), 7.33-7.26 (m, 4H, Ar-H). Calc. for C₅₆H₂₈N₁₂S₄O₈Zn·2CHCl₃: C: 48.74, H: 2.13, N: 11.76, S: 8.90%. Found: C: 47.59, H: 3.16, N: 11.65, S: 8.09%. MS (ES-MS) m/z: Calc.: 1190.58; Found: 1192.64 $[M + 2H]^{2+}$.

Results and discussion

Characterization of complexes 3, 4 and 5

The new complexes **3**, **4** and **5** were characterized using UV-Vis, IR, NMR spectroscopies, MALDI-TOF mass spectrometry and elemental analysis. The analyses are all consistent with that expected as shown in the Experimental section with the exception of an elevated nitrogen content for the nitrogen for the elemental of the quaternized complex **4**. Complex **3** exhibited solubility in organic solvents such as CHCl₃ and DCM, DMF and DMSO while complexes **4** and **5** were soluble in the latter two solvents and aqueous media.

The mass spectra of the Pcs were obtained by the relatively soft ionization MALDI-TOF technique with the molecular ion peaks observed at 1007.38 for **3**, 1261.86 for **4** and 1192.64 for **5**.

The ¹H NMR spectra of the complexes **3**, **4** and **5** show complex patterns due to the mixed isomer character of the tetra substituted MPc derivatives and perhaps also due to aggregation. Complexes **3**, **4** and **5** were found to be pure by ¹H NMR with all the substituent protons and the ring protons observed in their respective regions.

UV/Vis absorption and fluorescence spectroscopy

The newly synthesized complexes **3**, **4** and **5** showed no signs of aggregation in the respective absorption spectra in DMF (shown in Fig. 1). However strong aggregation was observed when the compounds were dissolved in water as evidenced by the intense broadening of absorption bands compared to the spectra of these compounds in DMF. Moreover, there is a hypsochromic shift of corresponding Q-band maxima when the samples are dissolved in water (from 676 to 632 nm for **3**, from 675 to 641 nm for **4** and from 683 to 643 nm for **5** respectively, see Table 1). It seems that aggregation for the quaternized Pc **4** is not as intense compared to the two other



Fig. 1 Normalized UV/Vis absorption spectra of **3** (*a*), **4** (*b*) and **5** (*c*) dissolved in DMF and in water. Comparison of the absorption spectra of these Pcs in aqueous solution is given in (*d*).

 Table 1
 Absorption, emission, photophysical and fluorescence lifetime data

Complex	Solvent	$\log \varepsilon$	Q band ^{<i>a</i>} λ_{\max} (nm)	Emission λ_{\max} (nm)	$\Phi_{ m ISC}$	τ (ns)
3	DMF	5.02	676	687	0.50	$\begin{array}{c} 2.71 \pm 0.15 \\ 2.93 \pm 0.18 \\ 2.49 \pm 0.17 \end{array}$
4	DMF	4.91	675	686	0.51	
5	DMF	5.08	683	695	0.62	

^{*a*} When dissolved in water, the Q-band maxima of the complexes showed a hypsochromic shift in the following order: from 676 to 632 nm for 3, from 675 to 641 nm for 4 and from 683 to 643 nm for 5 respectively.

Pcs, since the observed broadening of its absorption Q-band is lower than that for 3 and 5 when dissolved in water (see Fig. 1).

It was not possible to detect any fluorescence signal for the Pcs **3**, **4** and **5** in water since their fluorescence intensities lie below the signal-to-noise ratio of the steady-state fluorescence setup employed for this study. This observation is due to aggregation of the Pc molecules in aqueous solution (due to the likely formation of non-fluorescent aggregates, where fast and very efficient radiationless deactivation of the first excited singlet state follows photoexcitation of a Pc chromophore).^{35–37}

The situation is completely different for the same Pcs when dissolved in DMF (fluorescence spectra are shown in Fig. 2). The fluorescence maximum is located at 687 nm for 3, at 686 nm for 4 and at 695 nm for 5, respectively (Table 1).

Picosecond transient absorption spectroscopy (ps-TAS)

In order to bring to light the dynamics of the excited state population–depopulation, transient absorption experiments with picosecond time resolution (ps-TAS) were carried out. As readily seen from Fig. 3, the transient absorption (TA) spectra



Fig. 2 Normalized fluorescence spectra of the Pcs 3, 4 and 5 dissolved in DMF.



Fig. 3 Transient absorption spectra at different delay times after excitation (a, c, e) and ground state bleaching (mono-exponential fit is shown as a red line) (b, d, f) of the phthalocyanines 3 (a, b), 4 (c, d) and 5 (e, f) dissolved in DMF.

of the Pcs dissolved in DMF have a strong negative signal with a maximum around 680 nm which corresponds to the bleaching of the ground state absorption. The recovery of the ground state population after photoexcitation occurs mono-exponentially with a characteristic time (τ) of 2.71 ± 0.15 ns (3), 2.93 ± 0.18 ns (4) and 2.49 ± 0.17 ns (5) respectively (results shown in Table 1).

Furthermore, it was observed that even 15 ns after excitation, a ground state bleaching signal still remained. This means that excitation energy is partly stored in a long-lived excited state, namely the first excited triplet state. It is wellknown that the lifetime of a triplet state is much longer compared to the first excited singlet state (normally it lies in the microsecond or even millisecond range) due to the fact that the T₁-S₀ transition is spin-forbidden.^{17,38} In DMF the ISC $S_1 \rightarrow T_1$ quantum yield was calculated to be 0.50, 0.51 and 0.62 for phthalocyanines 3, 4 and 5 respectively (see Table 1). It should be emphasized that the ISC quantum yield correlates with that of the singlet oxygen generation (as both normally have very close values). The quantum yield for singlet oxygen production is a very important parameter for the use of Pcs as PSs in PDT of cancers.^{17,38,39} Since all Pcs under investigation show appreciable ISC quantum yield, they could be considered promising candidates for use as photosensitizers in PDT.

Unfortunately, the situation changed dramatically when the samples were dissolved in water. It was not even possible to measure the TA spectra of Pc 5, due to high scattering of the excitation light on the aggregates. It was possible, however, to carry out pump-probe experiments for the Pcs 3 and 4, and the results are presented in Fig. 4.

The TA spectra for the Pc **3** in aqueous solution were very noisy and also the intensity of its ground bleaching was quite low. It seems that the depopulation of the first excited singlet state occurs extremely fast and practically follows an excitation pulse (25 ps, FWHM). The ISC quantum yield value was estimated to be practically 0.

The change in optical density (Δ OD) signal of Pc 4 dissolved in water is much more pronounced compared to that of compound 3, and this is expected as 4 is in the water soluble quaternized form. Nevertheless, the ground state bleaching decays very fast with a characteristic time of 25 ± 10 ps, and the quantum efficiency of population of the first excited triplet state *via* ISC was estimated to be only 2%. This 2% efficiency



Fig. 4 Transient absorption spectra (a, c) and ground state bleaching (mono-exponential fit is shown as a red line) (b, d) of Pcs 3 (a, b) and 4 (c, d) dissolved in water.

of triplet state population is rather low and thus is less likely to result in significant PDT efficiency.

In summary, it was found that Pcs **3**, **4** and **5** have a very high tendency to aggregate when dissolved in water. This aggregation tendency in water results in a quenching of their fluorescence as well as ISC quantum yields compared to the corresponding results of the phthalocyanines when dissolved in DMF.

In vitro determination of Pc-induced phototoxicity

The photodestructive potency of water soluble complexes 4 and 5 (high aggregation tendency in water) was investigated in BON cells, which were used as a model cell line of human pancreatic carcinoid tumors.³⁰ Water soluble complexes were targeted for synthesis and investigation in this work because favorable and high efficiencies of tumor targeting in photodynamic activity are achieved with the use of such Pcs. For both compounds dark- and phototoxicity measurements were carried out at concentrations ranging from 0 to 20 µM (Fig. 5). BON cells were incubated with complexes 4 and 5 for 6 h and 12 h prior to illumination, which was performed with a light dose of 30 or 60 J cm⁻², respectively. Irradiation dose-dependently decreased the cell numbers of BON cells pretreated with either complex 4 or 5 compared to untreated controls (Fig. 5). Non-photoactivated Pcs (0-20 µM) did not affect cell proliferation of BON cells (figure not shown).

The IC₅₀ value of Pc 5 phototoxicity (12 h Pc incubation) calculated two days after PDT was found to be 5.7 \pm 1.6 μ M. The Pc 5-induced cell number reduction on day 4 was not adequate to allow for the calculation of its IC₅₀ value. BON cells showed maximal effects with a decrease in cell number of approximately 80% at complex 5 concentration of 10 μ M and a light dose of 60 J cm⁻² (Fig. 5d). The fact that the number of cells in complex 5 PDT treated samples did not markedly



Fig. 5 Effects of photoactivated Pcs on human cancer cells. BON cells incubated for 6 h or 12 h with complex 4 (*a*, *b*) and complex 5 (*c*, *d*) were irradiated with 30 or 60 J cm⁻² light dose. Changes in the cell number were determined by the crystal violet staining-method 48 and 96 h after PDT treatment. Cell number changes are shown as a percentage of irradiated but Pc-untreated control cells, which were set at 100%. Data are given as mean values \pm SEM of n = 4 independent experiments.

re-increase even after 4 days indicated that there was no appreciable re-proliferation, which is of particular interest for a compound that is well-suited for PDT. It shows that even those cells that were not immediately killed by the photoactivated photosensitizer were nevertheless affected by the PDT. Whether the underlying mode of action involved is apoptosis, necrosis, a stop or at least delay in cancer cell mitosis, or a combination of these, this has to be deciphered in future investigations; as the applied crystal violet-method does not measure these events, but solely determines changes in the cell number as compared to control cells.

Pc 4 showed lower phototoxicity compared to Pc 5 with the result that the cell number reduction induced by Pc 4 was not sufficient to allow for the calculation of its IC₅₀ value. BON cells showed reasonable effects with a decrease in cell number of approximately 50% at complex 4 concentration of 20 µM and a light dose of 60 J cm^{-2} (Fig. 5b). It was also noted that the number of cells in complex 4 PDT treated samples did not show a considerable re-increase even after 4 days. The IC_{50} values obtained in this work were higher than those obtained in work by other researchers using ZnPc and AlPc derivatives.^{21,22} However, PDT conditions and especially illumination (laser, wavelength, and light dose) are different. Therefore, a direct comparison of IC50 values measured here with those found in the literature is not appropriate. In order to estimate the effectiveness of our Pcs, additional experiments were conducted with the clinically approved photosensitizer Photofrin (0-10 µM) using the same PDT conditions (6 h of incubation and cell number detection at 48 h after PDT with 30 or 60 J cm⁻² irradiation doses). Photofrin-PDT dosedependently reduced the BON cell number by up to 85% (data not shown) which is in the range of the cell number reduction induced by PDT from the Pcs used in this work (>80% with Pc 5).

The differences in photodynamic activity of the two complexes 4 and 5 may be a result of differences in charge borne by each complex. Complex 4 is tetra positively charged by virtue of its being a quaternized nitrogen compound, while complex 5 is negatively charged because of the ionizable carboxyl groups. These differences in charge borne by complexes 4 and 5 might possibly influence and favor the uptake of 5 over 4 thereby resulting in enhanced photodynamic activity of complex 5. It seems that the cellular environment reduces the probability of aggregation possibly leading to moderate generation of cytotoxic singlet oxygen upon photoexcitation of the phthalocyanine chromophores. However, the cytotoxic capacities of complexes in the cells might possibly be associated with the subcellular localization of the Pcs once taken into the cell.^{40,41}

Conclusions

Two water soluble novel Zn(II)Pc complexes 4 and 5 were synthesized and *in vitro* studies were carried out to determine their photodynamic activity. The two complexes did not exhibit dark toxicity but led to phototoxic cell killing after light activation. The Pcs **4** and **5** have shown moderate photodynamic activity in *in vitro* experiments. Compound **5** demonstrated even more pronounced phototoxic and growth inhibitory effects as compared to compound **4**. This may be due to a more favored cellular uptake of the tetra negatively charged complex **5**. Further investigations will have to clarify the exact uptake mechanism and kinetic behavior of complex **5** in human tumor cells. As the photodestructive potency of compound **5** is promising for novel approaches in PDT it merits further elucidation.

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Notes and references

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