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Cationic versus Anionic Phthalocyanines for Photodynamic Therapy: What a Difference the Charge Makes.

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ABSTRACT

The literature reports on cationic and anionic phthalocyanines (Pcs) for photodynamic therapy suggest systematically significant differences in activity. In this work, ten different zinc(II) Pcs with carboxylate functions or guaternary nitrogens (hydrophilic anionic, hydrophilic cationic, amphiphilic anionic and amphiphilic cationic) were investigated, with the aim of revealing reasons for such differences. In vitro assays on HeLa, MCF-7 and HCT-116 cells confirmed higher photoactivity for cationic Pcs (EC₅₀ \sim 3-50 nM) than for anionic Pcs (EC₅₀ ~ 0.3-10 μ M), the latter being additionally significantly more active in serum-free medium. The environmental pH, binding to serum proteins, interaction with biomembranes, differences in subcellular localization and relocalization after irradiation were found to be the main factors contributing to the generally lower photoactivity of anionic Pcs than of the cationic derivatives. This result is not limited only to the presented derivatives and should be considered in the design of novel photosensitizers.

INTRODUCTION

Photodynamic therapy (PDT) is a clinically approved therapy of primarily cancerous conditions that combines three essentially nontoxic components - light, oxygen and a photosensitizer (PS).¹ The PS absorbs light energy and is able to convert it into the production of highly reactive singlet oxygen, which is the main destructive species in PDT. Cancer cells die as a consequence of singlet oxygen and other reactive oxygen species damaging various biomolecules and subsequently destroying subcellular organelles.^{2,3} A key component in PDT is always a PS that should have high activity and low toxicity and ideally absorb the light strongly in the optical window of biological tissues, *i.e.*, in the range of 650-800 nm. A number of various macrocycles have been tested and even clinically approved for PDT: porphyrins, chlorins, bacteriochlorins and phthalocyanines (Pcs).^{4,5} The last group is of particular interest due to optimal photophysical properties (strong absorption at approximately 700 nm, good singlet oxygen production) and variability in macrocycle substitution, which may also tune physicochemical properties.^{6,7} The typical problem for Pcs is, however, their

hydrophobic planar macrocycle, which tends to strongly aggregate, particularly in water.

Aggregation is considered undesirable since it lowers or even eliminates the production of singlet oxygen and makes Pcs inactive. For this reason, the clinically approved Pc (sulfonated hydroxyaluminum Pc, S₃AlOHPc) and the majority of newly tested Pcs are functionalized with water-solubilizing substituents, typically cationic or anionic, which may substantially reduce aggregation on the basis of electrostatic repulsive forces.⁷ During our long-term observations of the photodynamic properties of various Pcs published by our group or by other researchers, we noticed substantial differences between anionic (containing carboxy or sulfo groups) and cationic (containing guaternary ammonium groups) Pcs. Analysis of almost sixty sets of data published for the *in vitro* photodynamic activity of anionic and cationic Pcs in the literature (Figure 1; for source data, see Table S2, Supporting Information) supported this hypothesis, with two sets being significantly different (p < 0.01). Despite the heterogeneity of the source data (different cell lines, different irradiation conditions, different structural features), it appears that the anionic Pcs are generally much less active than the cationic Pcs. The reason behind this difference remains unclear, although it may substantially affect the

selection of substituents in the rational design of newly synthesized PSs with improved

photodynamic properties.



Figure 1. Comparison of the *in vitro* photodynamic activity of anionic (red) and cationic (blue) Pcs reported in the literature. For the purpose of this comparison, the activity is expressed as the IC_{50} value (μ M) multiplied by the fluence (J cm⁻²) to correct the heterogeneous data for different irradiation conditions. The mean and standard deviation are shown in black. Statistical analysis was performed by Welch's t-test. For the source data, see Table S2.

In this work, we have focused on the series of Pcs (Chart 1) and investigated their photophysical, physicochemical, binding and biological properties with the aim of finding

the parameters and/or factors that may contribute to the substantial difference between Pcs bearing opposite charges on peripheral substituents. Four different sets of compounds were introduced into the study, namely anionic hydrophilic (1-3), cationic hydrophilic (4-6), anionic amphiphilic (7, 8) and cationic amphiphilic (9, 10), to compare both the influence of the charge type and its distribution on the macrocycle core. The color code (red = anionic, blue = cationic) is used in all illustrations in the whole article.

Chart 1. Structures of compounds investigated in this work.



RESULTS

Design and synthesis. The design of the studied compounds was primarily driven by

attempts to keep the hydrophilic molecules fully monomeric in water solutions. For this reason, we selected hexadeca-substitution by carboxylates or by quaternary ammonium groups that have been reported in the literature to fully monomerize such Pcs.^{8,9} In the case of 6, twelve charges in the molecule were reported to keep this molecule also fully monomeric in water.¹⁰ Both positive and negative charges are located on the bulky substituents that are forced to be oriented almost perpendicularly to the Pc plane due to steric hindrance. Such spatial orientation places the charges rigidly over and below the Pc plane and protects the hydrophobic core from aggregation based on electrostatic repulsive forces. The selected hydrophilic groups were also included in the amphiphilic compounds, where the presence of a lipophilic unsubstituted Pc core was expected to promote more interactions with biomembranes.

While syntheses of compounds **1**, **4-6** and **9** have been described in the literature, synthetic routes to compounds **2**, **3**, **7**, **8**, and **10** were developed *de novo* or modified published procedure were used (Scheme 1).⁹⁻¹³ The phthalonitrile precursor **12** was obtained by the Suzuki-Miyaura cross-coupling reaction between 4,5-

dichlorophthalonitrile and 11. Compound 11 was prepared as reported in the literature.¹⁴

The coupling reaction was optimized with respect to different solvents (dioxane, water, toluene, THF), catalysts (Pd(AcO)₂, Pd(PPh₃)₄, PdCl₂(PPh₃)₂) and cocatalyst (SPhos, XPhos), yielding finally the best result with Pd(AcO)₂, XPhos, and K₃PO₄ in a mixture of THF and water. Subsequent cyclotetramerization of **12** using magnesium butoxide as the initiator was employed to obtain the corresponding magnesium Pc. In agreement with the literature, the methyl esters were transesterified to butyl esters during cyclotetramerization.¹⁵⁻¹⁷ The magnesium complex was directly converted to a metalfree ligand by treatment with p-toluenesulfonic acid in THF and then converted to zinc complex 2Bu with zinc acetate in pyridine. Direct synthesis of zinc(II) complex 2Bu from 12 with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and zinc(II) acetate in butanol gave a very low yield of approximately 3%. Hydrolysis of the ester groups of 2Bu using NaOH in a mixed solvent system (water/MeOH/THF) gave the highly water-soluble phthalocyanine 2. The synthesis of symmetrical Pc 3 was modified from the original report.¹⁵ Precursor 14 was prepared by nucleophilic substitution of 13 with 4,5 dichlorophthalonitrile with K₂CO₃ in DMF. Heating of phthalonitrile **14** in butanol with

zinc(II) acetate and a catalytic amount of DBU led to zinc phthalocyanine **3Bu** with butylesters on the periphery that were subsequently hydrolyzed to **3**.

Unsymmetrical Pcs **7** and **8** were obtained by statistical condensation of phthalonitrile (precursor A) with **12** or **14**, respectively, (precursor B) using magnesium butoxide as the initiator (Scheme 1). Similarly to above, the methyl esters were changed to butyl esters. The reaction led to the mixture of six different congeners, of which the required congener of AAAB type was isolated by column chromatography. The magnesium complexes were then converted to corresponding metal-free ligands and then to zinc(II) complexes, with an overall yield of 9% for **7Bu** and 15% for **8Bu** (based on the starting phthalonitriles). Such yields are typical for the synthesis of unsymmetrical AAAB-type Pcs using a statistical condensation approach.^{18,19} Finally, the ester bonds were hydrolyzed to yield **7** and **8**.

Low symmetrical cationic Pc **10** was prepared by statistical condensation of phthalonitrile (precursor A) with **15** (precursor B) initiated by magnesium butoxide. The corresponding magnesium AAAB congener was isolated by column chromatography, converted to a metal-free ligand by *p*-toluenesulfonic acid in THF and then converted to

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zinc(II) complex **10Zn** by zinc(II) acetate in pyridine, with a yield of 8%. Finally, the imidazole and pyridine nitrogen atoms were alkylated by iodomethane in dry DMF under an argon atmosphere (83% yield).

Scheme 1. Synthesis of studied Pcs.^a



^aReagents and conditions: i) K_3PO_4 , $Pd(AcO)_2$, XPhos, THF/water, rt; ii) Mg, BuOH, reflux; then ρ -TsOH, THF rt; then $Zn(AcO)_2$, pyridine, reflux; iii) NaOH, water/MeOH/THF, rt; then HCl, rt; iv) equimolar NaOH, water, rt; v) K_2CO_3 , DMF, 60 °C; vi) $Zn(AcO)_2$, DBU, butanol, reflux; vii) Mg, butanol, reflux; then ρ -TsOH, THF/CHCl₃/MeOH rt; then $Zn(AcO)_2$, pyridine, reflux; viii) CH₃I, DMF, 80 °C.

Solubility and absorption spectra. All hydrophilic compounds (1-6) and anionic amphiphilic Pcs (7, 8) were perfectly soluble in water and phosphate-buffered saline (PBS) even at high concentrations (>> 1 mM). High solubility (> 20 mM) of some of the studied derivatives has already been noted in the literature.⁹ On the other hand, much lower solubility (~ 100 µM) was observed for amphiphilic cationic 9 and 10, which precipitated in water above this concentration. These data were further supported by the determination of the partition coefficient between octanol and water (PBS, pH 7.4), where **1-8** were distributed almost exclusively in the water phase (log P < -2, Table 1), while 9 and 10 were more lipophilic, with log P -0.07 and 0.72, respectively. Direct comparison of the log *P* values of **7** or **8** with **9** indicated that even when the number of charges was the same and the molecule had a very similar structure, the carboxylate functions made the Pcs much more water soluble than the quaternary ammonium groups.

Table 1. Log *P*, photophysical data of compounds 1-10 in DMF and association constants with BSA and DOPC liposomes in PBS.^a

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| Compound | Log P | λ_{\max} / nm (log ϵ) | <i>I</i> _F / nm | ${\cal P}_{\sf F}{}^{\sf b}$ | $arPhi_{\!\Delta}$ | <i>K</i> _b × 10⁻ ⁶ (M⁻¹) | <i>K</i> _L × 10 ⁻ ³ (M ⁻¹) |
|----------|--------------------|---|-------------------------------|---------------------------------------|--------------------|---|--|
| 1 | -2.15 | 647 ^c | 657° | -, (0.36) ^c | 0.23 ^d | 0.712 | - |
| 2 | -2.45 | 693 | 702 | 0.12 (0.27) | 0.45 | 1.24 | - |
| 3 | -2.68 | 674 | 681 | 0.19 (0.25) ^e | 0.42 ^e | 2.80 | - |
| 4 | -2.42 | 681 | 683 | 0.15 ^f (0.15) | 0.61 ^f | - | - |
| 5 | -3.0 ^g | 680 | 687 | 0.15 ^g (0.18) | 0.49 ^g | - | - |
| 6 | -1.99 ^h | 710 | 721 | 0.13 ^h (0.10) | 0.69 ^h | - | - |
| 7 | -2.45 | 676 | 686 | 0.17 (0.23) ⁱ | 0.33 | 0.0514 | 4.76 |
| 8 | -2.44 | 672, 681 | 682 | 0.09 (0.33) ⁱ | 0.41 | 0.527 | 13.2 |
| 9 | -0.07 | 673 | 681 | 0.25 ^j (0.25) ⁱ | 0.57 ^j | 0.0435 | 7.07 |
| 10 | 0.72 | 671, 689 | 694 | 0.20 (0.22) ⁱ | 0.66 | - | 11.6 |

^aPartition coefficient between 1-octanol and PBS in logarithmic scale (Log *P*), absorption maximum (λ_{max}), emission maximum (λ_F), fluorescence quantum yield (Φ_F), singlet oxygen quantum yield (Φ_Δ), association constant with BSA (K_b), association constant with DOPC liposomes (K_L). Due to solubility issues, the data for anionic compounds 1-3, 7, 8 in DMF were determined for their nonionized analogs containing free carboxylic acid. ^bValues in brackets were determined in PBS. ^cIn H₂O, ref. ¹¹. ^dIn D₂O, ref. ¹¹. ^eSimilar values (Φ_F = 0.26 (water, pH >7), Φ_Δ = 0.40 (DMF)) have been reported for **3COOH** in literature⁸. ^{fref. 9}. ^g ref. ¹². ^href. ¹⁰. ⁱin PBS with liposomes (c_(dye) = 1 uM, dye-to-lipid ratio 1:1200). ^jref. ¹³.

The absorption spectra of all studied derivatives collected in DMF were typical for monomeric species of the Pcs, with an intense Q-band between 670 and 690 nm and B-As band approximately 350-370 (Figure 2). exceptions, at nm two the tetrapyrazinoporphyrazine (TPyzPz) core in 1 caused the blueshift of the absorption Qband to 647 nm, and the nonperipheral substitution in 6 shifted the Q-band to 710 nm. The unsymmetrical character of the amphiphilic compounds 7-10 may potentially lead to splitting of Q-band, but this was only for **10**. The increased polarity and ionic strength of the environment in PBS induced aggregation for all amphiphilic compounds (7-10), while fully monomeric character was maintained for all hydrophilic Pcs 1-6 in PBS (Figure 2).



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Figure 2. Absorption spectra of **1-10** in DMF (full lines) and in PBS (dashed lines) at a concentration of 1 μ M. The samples were diluted from 100 μ M stock solutions prepared in PBS (**1-8**) or in DMF (**9**, **10**). Unlike the stable positive charge on quaternary ammonium groups, the ionization states

of carboxylates may be affected by changes in pH.^{8,11,15} The protonation of carboxylate groups in compounds 1-3 at lower pH induced strong aggregation, as deduced from the decreased intensity, broadening and blueshift of the Q-band (Figure 3, Figure S3). This effect can be easily attributed to the loss of electrostatic repulsive forces once the carboxyl function is nonionized. The highly electron-deficient character of the TPyzPz core^{20,21} in **1** substantially increased the acidity of the carboxyl functions, and the changes in aggregation were observed at much lower pH values than for Pc derivatives 2 and 3. All anionic derivatives irrespective of the macrocycle core were strongly aggregated at pH 5, which is typical for the intralysosomal compartment of cells,^{11,22} and these derivatives were photophysically inactivated since the fluorescence quantum yield $(\Phi_{\rm F})$ was substantially decreased (Figure 3, Figure S3). Amphiphilic anionic derivatives

7 and **8** were aggregated irrespective of pH but the aggregation increased at lower pH values (Figure S3). On the other hand, the effect of pH on cationic derivatives **4-6**, **9** and **10** was rather limited, with only a small increase in both the Q-band intensity and fluorescence quantum yield at lower pH values (Figure 3, Figure S4).



Figure 3. a, c, e, g) Changes in the absorption spectra of compounds 3 (a), 7 (c), 5 (e) and 10 (g) (c = 1 μ M) in buffers of different pH values. b, f) Changes in emission spectra of 3 (b) and 5 (f) (c = 1 μ M, λ_{ex} = 610 nm) in buffers of different pH values. d, h) Dependence of the φ_{F} values of anionic (d) and cationic (h) Pcs on the pH of the buffer.

Dotted vertical lines indicate physiological pH 7.4 and intralysosomal pH 5.

Photophysical characterization. All studied compounds emitted red fluorescence a typical shape of emission spectra for Pcs and only a small Stokes shift. The values of $\phi_{\rm F}$ in DMF were typically above 0.10 and in a number of cases even increased when the data were collected in PBS. For measurements in PBS, the amphiphilic compounds 7-10 had to be formulated in dioleoylphosphatidylcholine (DOPC) liposomes (dye-tolipid ratio 1:1200 was sufficient to fully monomerize the samples, see below) to monomerize them (only 9 had some weak fluorescence in pure PBS, $\phi_{\rm F} \sim 0.01$). The production of singlet oxygen, the most important reactive species in PDT, was also determined in DMF using an established scavenger of singlet oxygen, 1,3diphenylisobenzofuran. The obtained values of singlet oxygen quantum yields (ϕ_{Λ}) ranged between 0.4 and 0.7, falling within the generally published values for zinc(II) Pcs.²³ The data suggested a small difference between anionic and cationic Pcs at the photophysical level. While the ϕ_{Λ} values for anionic Pcs were lower than 0.45, those for cationic species typically exceeded a value of 0.5 (Table 1).

Interaction with bovine serum albumin. The biological environment is a very complex system consisting of a number of biomolecules. Serum proteins and lipidic bilayers of cell membranes are the most important biomolecules and may substantially affect the aggregation state, photophysics and even pharmacokinetics of the PSs and consequently the ultimate photodynamic effect.²⁴⁻²⁷ For this reason, the effect of bovine serum albumin (BSA), the most abundant serum protein, and the interaction of the studied Pcs with the lipidic bilayer of liposomes was investigated.

Albumins, despite relatively low isoelectric point of approximately 4.7, are well-known to strongly bind the acidic drugs mainly into the positively charged binding sites formed prevalently by basic amino acid residues (Lys, Arg, His).²⁸ The binding of anionic Pcs to albumins is also well known in the literature,^{24,25,29} but reports on the interaction of cationic Pcs with this protein have also been reported.^{25,26} However, in line with our previous observations,¹² no qualitative changes in the absorption and emission spectra or $\varphi_{\rm F}$ values of Pcs were observed upon the addition of BSA into a PBS solution of hydrophilic cationic Pcs **4-6**, indicating no detectable interaction with this protein (Figure

S6, S7). Stepwise addition of BSA to anionic hydrophilic Pcs 1-3 (monomeric in PBS) induced a small redshift of the Q-band and emission maxima (approximately 7 nm) with clear isosbestic points in the absorption spectra (Figure 4a, Figure S5), indicating binding of the Pcs to albumin.³⁰ The absorption changes plotted against the BSA concentration were used to calculate the binding constant ($K_{\rm b}$) values, which were found to be approximately 10^6 M^{-1} ($K_{\text{b}} = 7.12 \times 10^5 \text{ M}^{-1}$, $1.24 \times 10^6 \text{ M}^{-1}$, and $2.80 \times 10^6 \text{ M}^{-1}$ for 1, 2, and 3, respectively, Figure 4b, Table 1). These values were roughly on the same level as that for other anionic hydrophilic zinc(II) Pcs or anionic porphyrins reported in the literature and indicated a strong interaction with BSA.³¹⁻³³ The fluorescence intensity of 1 and 3 (Figure 4c) decreased upon the addition of BSA as a consequence of guenching of excited states, and the emission maximum shifted bathochromically in line with a small redshift of the absorption maximum. Interestingly, BSA had no effect on the fluorescence intensity of 2; it resulted only in a bathochromic shift of the emission maximum by 4 nm. The quenching of excited states was only moderate for Pc 3 (~ 80% residual fluorescence at full binding), but it was very strong for TPzyPz 1 (< 10% of residual fluorescence at full binding, Figure 4d). Together with the very strong

reported for octasulfonated TPyzPz recently (< 5% of residual quenching fluorescence³⁴), it appears that the interaction of BSA with this type of aza-analog of Pcs causes strong quenching of the excited state but has a rather limited effect on the excited states of the Pc macrocycle. Amphiphilic Pcs 7-10 were found to strongly aggregate in PBS, and in this case, the addition of BSA induced monomerization (Figure 4e, Figure S5, S6). This effect was very well observed particularly for anionic Pcs 7 and 8 exhibiting $K_{\rm b}$ = 5.14 × 10⁴ M⁻¹ and 5.27 × 10⁵ M⁻¹, respectively, with significant restoration of the photoactivity (Figure 4g); however, the photoactivity was not fully restored to the values typical for monomers in PBS with liposomes (~ 40% and \sim 75% activity at 35 µM BSA for 7 and 8, respectively, Figure 4h), suggesting quenching of the excited states upon binding to albumin. In contrast, the interaction of cationic 9 and 10 with BSA was much weaker ($K_b = 4.35 \times 10^4$ M⁻¹ for 9, K_b could not be properly determined for 10), and even at high concentrations of BSA (> 100 µM), they remained partially (9) or predominantly (10) aggregated, with very low fluorescence (Figure 4h, Figure S6).



Figure 4. a, e) Changes in the absorption spectra of **3** (a) and **8** (e) (c = 1 μ M) upon the addition of BSA at concentrations ranging from 0 μ M (green line) to the final concentration for each compound (magenta line) (inset: detail of the Q-band). b, f) Binding isotherms of 1-3 (b) and 7-10 (f) to BSA. c, g) Changes in the fluorescence of **3** (c) and **8** (g) (c = 1 μ M) upon the addition of BSA (λ_{ex} = 618 nm and 612 nm, respectively). Inset: normalized fluorescence spectra. d, h) Changes in fluorescence intensity upon addition of BSA expressed as $\Phi_F/\Phi_{F(max)}$ (value of Φ_F in PBS with DOPC liposomes (dye-to-lipid ratio 1:1200) was considered $\Phi_{F(max)}$ for 7-10). The typical concentration of BSA in serum-containing medium (SCM) (35 μ M) is indicated by a vertical dotted line.

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Further, considering that the target organelles were lysosomes (see also the biological data below) with acidic environments (pH \sim 5), an experiment focusing on the spectral and photophysical changes induced by lower pH in the presence of BSA (20 µM) was performed (Figure S3, S4). For hydrophilic compounds 2-6, the effect of changing the pH in the presence of BSA mirrored the behavior observed without BSA (see above Figure 3). In the case of 1 bearing the TPyzPz core, the presence of BSA lowered the activity at pH 7.4 to approximately one tenth, but it remained on the same level irrespective of pH, as discussed in a previous article.¹¹ This result further emphasized the difference between the Pc and TPyzPz cores in the interaction with BSA. The addition of BSA (20 µM) to the fully aggregated amphiphilic anionic derivatives 7 and 8 induced (partial or almost full, respectively) monomerization at pH 7.4, but a lower pH led to more pronounced aggregation, most likely due to protonation of carboxylates and lowered interaction with basic groups in BSA. In the case of cationic amphiphilic compounds 9 and 10, their interaction with BSA was very weak, the compounds remained mostly aggregated at pH 7.4, and the aggregation intensified at a lower pH. In

this case it might be due to protonation of carboxylate functions of amino acid residues in BSA, whose negative charges may support weak interactions with amphiphilic cationic Pcs at pH 7.4 (Figure S4).

LC-MS analysis of bovine serum albumin interaction with singlet oxygen. Proteins are common targets of singlet oxygen. For this reason, the susceptibility of BSA to oxidative damage initiated by photodynamic treatment ($\lambda > 570$ nm, 12.4 mW cm⁻², 15 min, 11.2 J cm⁻²) was investigated with compounds 3 and 4 as representatives of anionic and cationic derivatives, respectively, with the use of LC-MS and subsequent quantitative analysis of identified peptides. The data were compared with the results of control experiments with nonirradiated BSA samples with PSs, irradiated BSA samples without PSs, and finally with untreated BSA. The screening was focused on the amino acids that were previously described to be attacked by singlet oxygen, *i.e.* Cys, His, Met, Tyr, and Trp.³⁵⁻³⁸ A number of tryptic peptides with oxidative modifications (His + 14, His + 16, His + 32, Met + 16, Met + 32, Cys + 32, Cys + 48, Trp + 16, and Trp + 32) was detected by LC-MS in the irradiated samples that contained PSs. The level of these modifications was significantly higher than that in the control samples without irradiation,

irradiated without PS or in untreated BSA (for representative examples, see Figure 5; for all significantly modified amino acids, see Figure S2). His was the most susceptible amino acid since almost all residues were oxidatively modified. From all modified His residues, it appears that ¹⁴⁵His, ²⁴¹His, ³⁷⁸His and ⁵⁰⁹His were the amino acids most accessible and susceptible to singlet oxygen in BSA (Figure S2), with a modification abundance sometimes even over 30% under particular irradiation conditions. Typical oxidation of sulfide to sulfoxide of three Met residues (87Met, 445Met, 547Met) was observed, in particular for ⁵⁴⁷Met with abundance about 35% (Figure 5). Further oxidation of Met to sulfone was negligible, not exceeding 0.5% abundance in any Met residue. Oxidation of Cys residues to sulfinic or sulfonic acid was significant only at ³⁴Cys, which is the only free Cys in BSA. Other Cys residues stabilize the folded form of BSA by disulfide bonds, and their oxidation was either not observed at all or the abundance was below 0.5%, with no significant difference between treated and untreated samples (Figure S2). Hydroxylation (Trp + 16) and subsequent formation of formylkynurenin (Trp + 32) was detected only for ²¹³Trp; the second Trp residue remained unaffected. Contrary to the literature data,^{35,37} no oxidative modifications to

Tyr residues were observed. The LC-MS analysis data suggested that BSA is an important target of singlet oxygen produced by both bound (anionic 3) and unbound (cationic 4) PSs.



Figure 5. Representative examples of the oxidative modification of selected His (a),

Met (b) and Trp (c) amino acid residues in BSA upon photodynamic treatment with Pc 3

or 4. Four independent experiments were performed and analyzed by Welch's t-test.*, p

< 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

Interaction with liposomes. Liposomes are often considered simple models of

biomembranes that may be targets of a number of lipophilic or amphiphilic PSs or other drugs.³⁹ In this work, no absorption or fluorescence changes were observed after the addition of DOPC liposomes to hydrophilic compounds 1-6 in PBS, suggesting no interaction, as recently shown for cationic 6 in the literature (Figure S8).¹³ On the other hand, the addition of liposomes to a PBS solution of aggregated amphiphilic Pcs 7-10 (1 µM) clearly induced monomerization, as observed in the absorption spectra, with full restoration of the fluorescence properties (Figure 6a-c, Figure S8). The amphiphilic derivatives are inserted by their lipophilic core into the liposomal bilayer leading thus to separation of individual molecules. Unlike for binding to BSA, the monomerization was complete even for cationic 9 and 10. The fluorescence data were then used to calculate of association constants of these Pcs with DOPC lipids (K_L), which were determined to be $K_1 = 4.76 \times 10^3 \text{ M}^{-1}$, $1.32 \times 10^4 \text{ M}^{-1}$, $7.07 \times 10^3 \text{ M}^{-1}$ and $1.16 \times 10^4 \text{ M}^{-1}$ for **7**, **8**, **9**, and 10, respectively (Figure 6d, Table 1). In comparison with published data, these values are typical for the association of zinc(II) Pcs with liposomes.^{40,41} The order of K_L values

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did not fully correlate with the log P values, suggesting that other factors (*e.g.*, the strength of aggregation forces in the aggregates) may also contribute to this order.^{40,42}



Figure 6. a, b) Changes in the absorption spectra of 7 (a) and 9 (b) (c = 1 μ M) upon the addition of DOPC liposomes at concentrations ranging from 0 mM (green line) to 1.2 mM (magenta line). c) Changes in the emission spectra of 7 (c = 1 μ M, λ_{exc} = 350 nm) upon the addition of DOPC liposomes. d) Binding isotherms of 7-10 (c = 1 μ M) interacting with DOPC liposomes. The fraction of bound Pcs is expressed as the fluorescence intensity of the monomeric form.

Since the amphiphilic Pcs 7-10 appear to interact with both BSA and lipids, it is also interesting to evaluate their preference for a particular type of biomolecule. The

positions of emission maxima of 7, 9 and 10 were slightly different when the compounds were associated with BSA or liposomes (in case of 8, the emission spectrum was much broader in liposomes than in BSA), which allowed the identification of which biomolecules they were associated with. Thus, the emission spectra of Pcs in PBS were collected first after the addition of liposomes ($c_{(DOPC)} = 1.2$ mM, concentration at which all Pcs 7-10 were fully associated with DOPC lipids in the above experiment) and then after the addition of BSA (35 µM, corresponding to the amount of BSA in serumcontaining medium (SCM) used in *in vitro* assays) and compared with the spectra obtained in the presence of only BSA (Figure 7, Figure S9). In another experiment, the order of addition of biomolecules was reversed (*i.e.*, first BSA then liposomes) but yielded the same results. The anionic Pcs 7 and 8 preferred BSA – they associated with BSA upon its addition to liposomal suspension and they were not removed from BSA upon addition of liposomes in the reverse experiment. The opposite behavior was observed for cationic Pcs 9 and 10, which preferred liposomes over BSA; *i.e.*, they remained associated with lipids irrespective of the presence or absence of BSA which is in line with their rather lower affinity to BSA, as shown above.



Figure 7. Normalized emission spectra (λ_{exc} = 608 nm, c = 1 µM in PBS) of **10** (a) and **7** (b) in BSA (35 µM, green), in DOPC liposomes (1.2 mM, red), and in the presence of DOPC liposomes after the addition of BSA (black dashed).

Cytotoxicity studies. *In vitro* assessment of photodynamic activity and cytotoxicity was primarily performed on a human cervical carcinoma cell line (HeLa). The uptake of the tested Pcs by cells had a typical profile reaching a steady state after approximately 12 h (Figure 8a), which was later used as the incubation time during the assessment of photodynamic activity. The total amount of the dyes typically varied between 0.1 and 0.2 nmol *per* mg of protein (Table 2) and had no significant relationship with the Pc structure. The uptake of representative of each structural type (compounds **2**, **5**, **8** and **10**) was also performed in the presence of dynasore (three different concentrations), a well-characterized chemical inhibitor of endocytosis which affects mainly clathrin-mediated

pathway of endocytosis.⁴³⁻⁴⁵ It is a noncompetitive inhibitor of dynamin GTPase activity which blocks dynamin-dependent endocytosis in cells. Although the differences between the uptake in the presence and absence of the inhibitor were on the limit of significance, slightly reduced uptake after 4 h of incubation was observed for all tested compounds (Figure S10). Together with exclusive (hydrophilic Pcs) or predominant (amphiphilic Pcs) localization of all derivatives in endolysosomal compartment (see below), we may conclude that the compounds were preferentially taken up by cells by endocytosis.

Subsequently, the photodynamic activity of all Pcs was evaluated after activation by light ($\lambda > 570$ nm, 11.2 J cm⁻², expressed as the half-maximal effective concentration, EC₅₀) on HeLa (Figure 8b), human colorectal carcinoma (HCT 116, Figure S11) and human breast carcinoma (MCF 7, Figure S12) and nonmalignant mouse fibroblasts (3T3, Figure S15) cell lines. The anionic hydrophilic Pcs **1-3** were the least active in this series, with EC₅₀ values (HeLa) approximately 5-10 μ M, while the hydrophilic cationic **4**-**6** exerted almost three orders of magnitude lower EC₅₀ values, ranging between 3.8 nm and 38 nM (Table 2). Similarly, the anionic amphiphilic compounds **7** and **8** (EC₅₀ ~ 350 nM) were approximately an order of magnitude less active than the corresponding

cationic derivatives 9 and 10 (EC₅₀ ~ 38 nM), confirming the general trend of higher activity for cationic Pcs (Figure 8b). The data obtained on HeLa cells were also confirmed by identical tendencies on other cell lines (MCF 7 and HCT 116). Previously published data for some of the above studied derivatives indicated also that there is no or only a small difference in photodynamic activity between cancerous and nonmalignant cell lines.^{9,10,12,15} In line with these observations, the EC₅₀ values found for 4-10 on 3T3 cells were comparable with the malignant cell lines (Table 2). Surprisingly, much higher phototoxicity was observed for anionic hydrophilic derivatives 1-3. Although we have no clear explanation for this behavior at this moment, these compounds should not be excluded from further investigation as PSs since the selectivity of PDT is based primarily on irradiation of target area only. The inherent toxicity of Pcs without light activation (*i.e.*, dark toxicity) was determined in HeLa cells (expressed as the half-maximal toxic concentration, TC_{50} , Figure S13). The dark toxicity of all compounds was very low, and a general trend of lower toxicity for anionic Pcs $(TC_{50} > 1000 \ \mu M)$ than for cationic Pcs was observed. Nevertheless, despite the cationic species being slightly more toxic without irradiation, their extraordinarily high

photodynamic activity resulted in a much better therapeutic ratio TC_{50}/EC_{50} , which reached a value over 100 000 for the best compound **6** (Table 2). For comparison, sulfonated hydroxyaluminum Pc (S₃AlOHPc) that has been approved in Russia for clinical use in PDT⁴⁶ was tested under the same conditions. Both the photodynamic activity and dark toxicity of S₃AlOHPc were comparable with those of our hydrophilic anionic Pcs **1-3**, which correlated well with its hydrophilic anionic nature.

Keeping in mind the strong binding of some investigated Pcs to BSA as shown above, the photodynamic activity of all Pcs on HeLa cells was also determined in serum-free medium (SFM) that is protein-free (Table 2, Figure S14). Statistically significant differences in the EC₅₀ values between results in SCM and SFM were obtained for anionic species: approximately ten times better activity for **1-3** and almost two orders of magnitude better activity for **7** and **8** were obtained in SFM than in SCM (Figure 8c). On the other hand, no significant difference was observed for cationic species in experiments performed in SCM and SFM (Figure 8c). In line with the results for anionic Pcs, an increase in the photodynamic activity against HeLa cells by two orders of

magnitude when going from SCM to SFM was also reported recently for zinc(II) octa(4-

sulfonatophenyl)TPyzPz.³⁴



Figure 8. a) Example of the cellular uptake of 2, 8 and 9 by HeLa cells incubated with 4 μ M dye. b) Photodynamic activity of compounds 1-10 on HeLa cells in SCM ($\lambda > 570$ nm, 12.4 mW cm⁻², 15 min, 11.2 J cm⁻²). c) EC₅₀ values of 1-10 and S₃AlOHPc on HeLa cells in SCM (blank columns) and SFM (full columns). At least four independent experiments each in quadruplicate were performed. n.s. (nonsignificant), p > 0.05; ***, p < 0.001; ****, p < 0.0001. Cationic derivatives are drawn in blue, and anionic derivatives are drawn in red in all graphs.

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Table 2. Comparison of uptake, dark toxicity (TC₅₀ values) and photodynamic activity (EC₅₀ values) of studied Pcs in

different cell lines.^a

| Compoun d Uptake (nmol <i>per</i> mg of protein), HeLa | Uptake (nmol <i>per</i> | EC ₅₀ (μM) | | | | | TC ₅₀ (μΜ) | TC ₅₀ / EC ₅₀ |
|--|----------------------------|--------------------------------------|---------|---------------|------------------------------|------|-----------------------|--|
| | HeLa ^b | Ratio SCM / SFM | HCT 116 | MCF-7 3T3 | 3 | HeLa | HeLa | |
| 1 | 0.07 | 5.7 ± 1.1 (0.55 ± 0.09) ^c | 10.36 | 4.04 ± 0.59 | 3.01±0.2050.740.038 | ± | > 1500 | > 263 |
| 2 | 0.15 | 10.31 ± 1.02 (0.98 ± 0.56) | 10.52 | 16.7 ± 2.65 | 5.09 ± 0.594 1.24 0.108 | ± | 1043 ± 169 | 101 |
| 3 ^d | 0.027 | 5.16 ± 1.03 (0.31 ± 0.18) | 16.49 | 6.35 ± 0.45 | 3.47±0.4030.670.024 | ± | > 1500 | > 291 |
| 4 | 0.29 | 0.037 ^e (0.038 ± 0.013) | 0.97 | 0.014 ± 0.001 | 0.011 ± 0.022 0.003 0.011 | ± | 628 ^e | 16 972 |
| 5 | 0.20 | $0.012 \pm 0.004^{f} (0.013 \pm$ | 0.92 | 0.0087 = | 0.0053 0.014 ± | ± | 369 ± 50 ^f | 29 916 |
| | | | | | | | | |
| | | 0.005) | | 0.0002 ^f | 0.0008 ^f | 0.014 | | |
|--------------|------|---|-------|---------------------|-----------------------------|--------------------------------|-----------------------|---------|
| 6 | 0.20 | 0.0038 ± 0.0002 ^g (0.0049 ± 0.0019) | 0.76 | 0.0068 ± 0.0035 | 0.0028 ± 0.00008 g | 0.015 ± 0.0053 ⁹ | 435 ± 26 ⁹ | 114 473 |
| 7 | 0.09 | 0.41 ± 0.156 (0.0077 ± 0.0036) | 51.5 | 0.615 ± 0.078 | 0.584 ± 0.088 | 0.287 ± 0.015 | > 1000 | > 2 427 |
| 8 | 0.10 | 0.29 ± 0.078 (0.0032 ± 0.0009) | 95.67 | 0.453 ± 0.019 | 0.453 ± 0.026 | 0.330 ± 0.015 | > 1000 | > 3 484 |
| 9 | 0.18 | 0.048 ± 0.019 (0.12 ± 0.029) | 0.27 | 0.082 ± 0.005 | 0.021 ± 0.008 | 0.105 ± 0.067 | > 150 ^h | > 3125 |
| 10 | 0.48 | 0.027 ± 0.009 (0.044 ± 0.004) | 0.61 | 0.042 ± 0.002 | 0.035 ± 0.002 | 0.082 ± 0.039 | > 100 ^h | > 3 703 |
| S₃AIOHP c | 0.11 | 2.07 ± 0.29 ^g (0.49 ± 0.07) | 4.24 | 1.61 ± 0.12 | 2.04 ± 0.31 | 2.37 ± 0.63 | > 1500 | > 725 |

15 min, 11.2 J cm⁻². All data were determined in SCM. ^bValues in brackets are EC₅₀ values obtained in SFM. ^cData from

ACS Paragon Plus Environment

ref. ¹¹. ^dThe following data were obtained for this compound in literature: EC₅₀ ~ 1 μ M (J774 cells, λ > 600 nm, 48 J cm⁻²,

ref.⁸), EC₅₀ = 4.5 μ M (Hep2 cells, λ > 610 nm, 1 J cm⁻²⁰, ref. ¹⁵), TC₅₀ > 400 μ M (V79, Hep2 cells, ref. ¹⁵). ^eData from ref. ⁹.

^fData from ref. ¹². ^gData from ref. ¹⁰. ^hLimit of solubility. The compounds precipitated above this concentration.

Subcellular localization and relocalization upon irradiation. Localization of PSs within the cell is crucial for triggering cell death. All studied compounds were found localized to endolysosomal vesicles after 12 h incubation, which is in accordance with previously published studies aimed at in vitro evaluation of Pc-based photosensitizers.11,12,34,47 While hydrophilic compounds – both cationic and anionic – were exclusively localized to lysosomes, all amphiphilic compounds were also detected in the cellular membrane by means of fluorescence microcopy (Figure 9, Figure S16-S19). The subcellular localization of PS is the primary target of oxidative damage upon irradiation. Relocalization of compounds during the irradiation period is equivalently important as PSs are further producing ${}^{1}O_{2}$ within these additional loci in the cell. Similar to what has already been reported for Pc 6,10 hydrophilic cationic Pcs 4-6 relocalized to the cytoplasm upon irradiation and damaged the nuclear membrane in a quite short time after the start of irradiation (observable as an influx of propidium iodide (PI) into the cells and subsequently into the nucleus during the irradiation period; Figure 10). The fluorescence signal of 1-3 in the cells was noticeably weaker (than the signal of hydrophilic cationic compounds) due to binding to BSA and guenching of the excited

state (for 1) and lower pH in these acidic organelles, leading to protonation of carboxylate functions and aggregation (for 1-3). The effect of the acidic environment is most notable with 2, where its signal in the cells is barely detectable but increased within the first minute of irradiation due to rupture of lysosomes and an increased pH in the local environment of these Pcs (see also attached video, Supporting Information). Afterwards, the red signal in the lysosomes and cytoplasm decreased, probably due to extensive photobleaching, and was faintly detectable as a punctuate fluorescence in some cells as well as diffuse fluorescence in the cytoplasm. The destructive effect of hydrophilic dyes 2 and 6 on endolysosomal vesicles and redistribution to the cytoplasm can also be compared directly in video (attached Supporting information). It is clearly shown that under the same irradiation conditions, both hydrophilic Pcs redistributed to the cytoplasm, but in the case of the cationic compounds this process is faster and also the bleaching of PS was to a lower extent, indicating remained unaffected ability to inflict damage to other organelles in the cells.



Figure 9. Subcellular localization of **2**, **5**, **8** and **10**. Cells were stained for (A) mitochondria and lysosomes or (B) membrane and nuclei by organelle-specific fluorescent probes. Line C is a control experiment without any PS. The bars in the photomicrographs indicate measured parts of respective images for intensity profiles.

Owing to their amphiphilic nature, Pcs 7-10 were detected not only in the endolysosomal compartment but also in cell membranes (Figure 9). During and after irradiation, subcellular localization of amphiphilic cationic PSs did not change significantly – owing to morphological alterations, even after irradiation, the signals of 9 and 10 were easily detectable in the cell membrane as a thin layer due to membrane detachment from the cytoskeleton and formation of balloon-like membrane protrusions. also called blisters (Figure S19). Even after release from lysosomes, these PSs primarily re-localized to membrane (or were already located in the lysosomal membrane and not in lysosomal lumen). The fluorescence signals of 7 and 8 were found diffused in the cytoplasm (in addition to localization in the membrane) shortly after irradiation as a consequence of their release from lysosomal lumen. In lysosomes before irradiation, they were expected to be localized both in lysosomal membrane and on BSA in lumen due to strong interactions with this protein. The diffused cytoplasmic signal of 7 and 8 after irradiation indicated that the compounds are still bound to BSA and do not redistribute further to membranous structures. Later, the signal was barely detectable in

the cytoplasm and even weaker in the cell membrane due to rapid photobleaching

(Figure S18).



Figure 10. Time-lapse PI staining of HeLa cells due to damage to membranes induced by the photodynamic action of **2**, **5**, **8** or **10**. All nuclei are stained blue by Hoechst 33342. The cytoplasm and subsequently also nuclei in cells with compromised

membranes are stained red by PI. Line C is a control experiment without any PS (cells were irradiated). Bar represents 100 µm.

PI, a cell-membrane-impermeable fluorescent intercalating agent, was used to evaluate the speed and extent of damage to the irradiated cells during irradiation and within the first minutes after irradiation (Figure 10). For irradiation we employed a microscope fluorescence light source in combination with a Cy5 filter set (λ = 604-644 nm, 100.5 mW cm⁻², 15 min, 90.4 J cm⁻²). Cellular damage inflicted by the action of anionic hydrophilic 2 was not sufficient to induce ample damage to the membranes, as PI was unable to stain the cells and nuclei in most of the cells even 45 min after irradiation. A markedly higher influx of PI was induced by anionic amphiphilic 8; PI staining was detectable after 15 min post-irradiation. Due to the delay of the signal appearance, this effect is most likely caused by induced cel death processes and not by the direct effect of the produced singlet oxygen. The damage induced by cationic compounds was, however, more prompt and severe; both hydrophilic 5 and amphiphilic

10 induced oxidative damage that allowed PI to leak into cytoplasm as early as within the first 5 min of irradiation and to begin stain nuclei within the next 5 min of irradiation,

which seems to be a consequence of a direct significant photodynamic effect.

Morphological changes. Treating cells with concentrations of Pcs corresponding to their IC₁₅ values did not induce any noticeable morphological changes after irradiation (λ > 570 nm, 11.2 J cm⁻²). In contrast, a high dose (corresponding to IC_{85}) induced severe morphological changes and a decrease in the number of living cells (Figure 11, Figure S21-S24). Even though these changes cannot *per se* serve as confirmation of a particular cell death pathway, they could hint at the likeliest possibility. Changes in the nuclear shape and volume (shrinkage), mitochondria and F-actin cytoskeleton (microfilaments) were detected. Cationic compounds induced chromatin condensation and pyknosis in dead cells, which were also characterized by weak fluorescence signals of microfilaments and diffuse and barely detectable signals of MitoTracker; indicating rapid cell death such as necrosis. Anionic compounds, on the other hand, also presented different phenotypes of dead cells containing karyorrhectic or pyknotic nuclei,

depolymerized or reorganized F-actin and changes in the MitoTracker signal (diminishing diffuse signal or strong signal of shortened and rounded mitochondria) – suggesting slower cell death with ongoing cellular processes involved in disintegration of cellular components, possibly apoptosis (Figure 11). These findings are in accordance with those obtained by PI staining.



Figure 11. Morphological changes manifested 24 h after irradiation ($\lambda > 570$ nm, 11.2 J cm⁻²) of the cells incubated with amphiphilic anionic **8** and amphiphilic cationic **10**. While low concentration (corresponding to EC₁₅) did not induce any significant changes, high dose (corresponding to EC₈₅) induced pyknosis, karyorrhexis, diminishing signal of both mitochondria and cytoskeleton. Blue – nuclei, red – mitochondria, green – F-actin. Bar represents 50 µm.

DISCUSSION

The *in vitro* photodynamic activity experiments on cells unequivocally confirmed a substantial difference in the activity between anionic and cationic Pcs. In this work, we revealed several factors that may contribute to such results:

1) Singlet oxygen was produced slightly less by anionic Pcs than by cationic Pcs.

2) The charge in anionic species is not stable, and the carboxylate functions can be

protonated at lower pH values, leading to elimination of the charge that is responsible

for monomerization of the molecules. At pH 5, which is typical for the lysosomal environment (the primary localization site for all tested Pcs), all the anionic species were found to be aggregated, with substantial reduction of the photoactivity irrespective of whether they were bound to BSA or not. This result was further confirmed by the weak fluorescence signal in lysosomes, which that increased substantially after their rupture and change of the pH due to neutralization by cytoplasm influx (see video). The cationic derivatives with stable charge were not significantly affected by the change in pH.

3) Binding of the tested Pcs to BSA had very important consequences for the activity. Strong binding with high K_b values was observed primarily for anionic hydrophilic derivatives 1-3, bit a slightly weaker binding for anionic amphiphilic Pcs 7 and 8. The interaction of BSA with cationic derivatives was not observed (4-6) or was very weak (for amphiphilic cationic 9 and 10). Moreover, the last two compounds had a much stronger preference for biomembranes than for BSA (Figure 7); *i.e.*, they are expected to be localized preferentially in biomembranes rather than BSA under

biological conditions. This expectation was confirmed by observation of their signal in cells where the cationic amphiphilic 9 and 10 relocalized to membranes after rupture of lysosomes. On the other hand, the signals of anionic amphiphilic 7 and 8 diffused in the cytoplasm after their release from lysosomes, suggesting that they were still bound to BSA. Binding to BSA led to a substantially lower photodynamic activity of all anionic derivatives 1-3, 7 and 8 on cells; significantly higher EC_{50} values were observed on HeLa cells in SCM (containing 35 µM BSA) than in SFM. This result might be a consequence of several factors. First, the binding to BSA guenched the excited states of anionic Pcs. Particularly in the case of the TPyzPz aza-analogs, this process is a very important deactivation pathway (Figure 4). Second, binding to BSA sterically limits efficient diffusion of the ground state oxygen to the excited macrocycle leading to lower quenching of the triplet states by oxygen, as demonstrated recently for octasulfonated zinc(II) TPyzPz and 5,10,15,20-tetrakis(4sulfophenyl)porphyrin³⁴ and on other Pcs and porphyrins.³⁰ Finally, the spatial arrangement upon binding of Pc to BSA leads to the situation in which the produced singlet oxygen is chemically guenched by reaction with BSA as the closest available

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molecule.³⁰ Physical guenching of singlet oxygen by BSA has been well described in the literature as well.^{37,48} For this reason, singlet oxygen is not released into solution in sufficient amounts and its damage to other biomolecules and organelles in the cells is significantly reduced. This fact is well demonstrated by the limited oxidative damage of the anionic Pcs to the cell membrane, as indicated by the slow influx of PI (Figure 10). Once BSA is eliminated from the incubation medium, *e.g.*, using SFM for in vitro assays on cells, the produced amount and diffusion of singlet oxygen increased, and thus, the effect on subcellular organelles was more destructive. This effect is further supported by the good binding properties of anionic amphiphilic derivatives 7 and 8 to biomembranes in the absence of BSA, where singlet oxygen may destroy the biomolecules in closest proximity with increased efficiency and for longer time (lifetime of singlet oxygen in water is τ_{Δ} = 3.5 µs,⁴⁹ while in dimyristoylphosphatidyl choline and in lecithin, components of biomembranes, it is reported approximately an order of magnitude longer τ_{Δ} = 36.4 µs and 12.2 µs, respectively⁵⁰). This consideration may also help to explain the extremely better photodynamic activity of 7 and 8 on cells in SFM than is SCM (Table 2).

4) The cationic derivatives may additionally profit from a "double punch" including damage to nuclei and other organelles. After quick release from lysosomes, these Pcs relocate to cytoplasm and other organelles and damage the nuclear membrane as well documented by staining the nuclei by PI (Figure 10). This process happens in a short time after the start of irradiation, and the cells may undergo additional damage taking into account that the cationic compounds photobleached much slower than anionic derivatives. The effect seems to be stronger (based on EC₅₀ values) for the hydrophilic compounds 4-6 that are located diffused in cytoplasm after release from lysosomes (*i.e.* singlet oxygen can easily harm all other organelles) than for the amphiphilic ones 9 and 10 that are located primarily in residues of membranous structures and thus the singlet oxygen is generated only locally.

CONCLUSION

In conclusion, on the series of ten compounds bearing positive or negative charges variously distributed on the macrocycle periphery, we confirmed the anticipated difference between anionic and cationic Pcs in terms of their photodynamic activity on

cells. A lower pH in the lysosomal compartment and binding to albumin were shown to be responsible for large differences, leading to a substantially lower photodynamic activity for anionic derivatives, particularly those that were fully hydrophilic. Based on the collected published photodynamic data for various anionic and cationic Pcs (Figure 1), it seems that this observation may be generalized and should be considered during the design of novel PSs.

EXPERIMENTAL SECTION

General. All of the organic solvents used in the synthesis were of analytical grade. Anhydrous butanol for the cyclotetramerization was freshly distilled from magnesium. Unsubstituted zinc phthalocyanine (ZnPc) was purchased from Sigma-Aldrich. All the other chemicals for the syntheses were purchased from certified suppliers (i.e., Sigma-Aldrich, TCI Europe, Acros, and Merck) and used as received. Thin layer chromatography was performed on Merck aluminum sheets coated with silica gel 60 F254. Merck Kieselgel 60 (0.040–0.063 mm) was used for column chromatography. The melting points were measured on an Electrothermal IA9200-series digital melting-point

apparatus (Electrothermal Engineering, Southend-on-Sea, Essex, Great Britain). The infrared spectra were measured on a Nicolet 6700 spectrometer in ATR mode. The ¹H and ¹³C NMR spectra were recorded on a VNMR S500 NMR spectrometer. The chemical shifts are reported as δ values in ppm and are indirectly referenced to Si(CH₃)₄ via the signal from the solvent. J values are given in Hz. The UV-Vis spectra were recorded using a Shimadzu UV-2600 spectrophotometer. HRMS spectra were measured at UHPLC system Acquity UPLC I-class (Waters, Millford, USA) coupled to high resolution mass spectrometer (HRMS) Synapt G2Si (Waters, Manchester, UK) based on Q-TOF were used for HRMS spectra measurement. Chromatography was carried out using Acquity UPLC Protein BEH C4 (2.1 × 50 mm, 1.7 µm, 300 Å) column using gradient elution with acetonitrile (ACN) and 0.1% formic acid at flow-rate 0.4 ml/min. Electrospray ionization (ESI) was operated in positive ion mode. The ESI spectra were recorded in the range 50 - 5000 m/z using leucine-enkefaline as a lock mass reference and sodium iodide for external calibration or in the range 50 - 1200 m/z using leucine-enkefaline as a lock mass reference and sodium formate for external calibration. The MALDI-TOF mass spectra were recorded in positive reflectron mode on

a 4800 MALDI TOF/TOF mass spectrometer (AB Sciex, Framingham, MA, USA) in trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile, which was used as a matrix. The instrument was calibrated externally with a five-point calibration using a Peptide Calibration Mix1 kit (LaserBio Laboratories, Sophia- Antipolis, France). 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-Starting materials dimethyl vl)isophthalate¹⁴ (**11**, vield 86 %), 4.5-[(3,5-bismethoxycarbonyl)phenoxy]phthalonitrile¹⁵ (14, %), and 2-(2,6-bis[(1*H*-imidazol-1-yl)methyl]-4-methylphenoxy)5,6yield dimethylpyridine-3,4-dicarbonitrile¹⁰ (15, yield 73 %), were prepared according to literature. Compounds 1, 4-6 and 9 were available in the laboratory from previously published projects.^{11,12} Purity of novel compounds **2**, **3**, **7**, **8**, and **10** was assessed by HPLC and was found in all cases >96% (Supporting Information). Preparation of 2,3,9,10,16,17,23,24-octakis[3,5-bis(butoxycarbonyl)phenyl]

phthalocyaninato zinc(II) (2Bu). Magnesium turnings (65 mg, 2.7 mmol) were refluxed in anhydrous butanol (20 mL) with a small crystal of iodine for 5 h until all magnesium was converted to magnesium butoxide. Phthalonitrile **12** (185 mg, 0.36 mmol) was added, and the reflux continued for the next 20 h. The mixture was cooled, and approximately

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half of the solvent was removed under reduced pressure. Then, the dark mixture was poured into water/methanol/acetic acid (25:25:1, 100 mL), and the suspension was stirred at rt for 60 min to remove unreacted magnesium butoxide. The green precipitate was collected by filtration and washed with water and methanol. Magnesium complex was characterized by MALDI-TOF (m/z calculated for C₁₆₀H₁₇₆MgN₈O₃₂ [M]⁺: 2745.2. Found: 2745.0) and directly converted to metal-free ligand.

The crude magnesium complex (240 mg) and *p*-toluenesulfonic acid monohydrate (166 mg, 0.87 mmol) were dissolved in tetrahydrofuran (THF, 20 ml). The solution was stirred at rt for 1 h and then the volatiles were removed under reduced pressure. The solid was washed with water and methanol. The crude product was purified *via* column chromatography on silica with toluene/THF (20:1, $R_f = 0.47$) as the eluent. The purified product was crystallized by addition of highly concentrated chloroform solution of the dye into methanol. Green crystals of metal-free ligand were filtered, dried and characterized by MALDI-TOF (*m/z* calculated for C₁₆₀H₁₇₈N₈O₃₂ [M]⁺: 2723.3. Found: 2723.0).

Metal-free ligand (142 mg, 0.052 mmol) was dissolved in pyridine (30 ml) and zinc acetate (26 mg, 0.142 mmol) was added. The mixture was refluxed for 6 h and then pyridine was removed under reduced pressure. The solid was washed with water and methanol. The crude product was purified by column chromatography on silica with chloroform/ethyl acetate (50:1, $R_f = 0.20$). The purified product was crystallized by slow addition of 2Bu chloroform solution into methanol. The solid was collected and dried yielding dark green solid. Yield 112 mg (45 % based on 12). MS MALDI-TOF: m/z calculated for C₁₆₀H₁₇₆N₈O₃₂Zn [M]⁺:2785.2. Found: 2784.9. ¹H NMR (500 MHz, CDCl3/pyridine-d5) δ 9.88 (s, 8H), 8.85 (t, J = 1.6 Hz, 8H), 8.61 (d, J = 1.6 Hz, 16H), 4.41 (t, J = 6.6 Hz, 32H), 1.83 – 1.75 (m, 32H), 1.55 – 1.46 (m, 32H), 1.04 (t, J = 7.4 Hz, 48H). ¹³C NMR (126 MHz, CDCl3/pyridine-d5) δ 165.61, 154.47, 142.19, 140.72, 138.74, 131.41, 129.57, 125.52, 65.32, 30.76, 19.31, 13.80. One aromatic signal was overlapped by signal of solvent. λ_{max} (DMF, 1 μ M)/nm 694 (ϵ /dm³ mol⁻¹ cm⁻¹ 325 240), 625 (49 170), 368 (105 550). *λ*_{max} (THF, 1 μM)/nm 687 (*ε*/dm³ mol⁻¹ cm⁻¹ 368 200), 619 (55 780) 363 (134 500). IR (ATR) $\nu = 2960$ (C-H_{aliph}), 2873 (C-H_{aliph}), 1723 cm⁻¹ (CO).

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Preparation hexadecasodium 2,3,9,10,16,17,23,24-octakis(3,5of salt of dicarboxylatophenyl)phthalocyaninato zinc(II) (2). Zinc phthalocyanine 2Bu (112 mg; 40 µmol) was dissolved in THF (7 ml) and added slowly to a saturated NaOH solution in water/methanol (1:5) (50 mL). The mixture was stirred at 40 °C for 4 h, and the resulting precipitate was filtered and washed repeatedly with MeOH and chloroform. The crude product was dissolved in water and acidified using 1 M HCl until pH 1. The precipitate was collected by filtration, resuspended in water with drop of 1% HCl, centrifuged and carefully decanted. This has been repeated twice. After the removal of the liquid phase, the green solid was suspended in a mixture of MeOH, acetone and pyridine, sonicated and transferred to flask and evaporated. The solid was then suspended in diethyl ether, crystals were filtrated and carefully dried to yield 79 mg (89 %) of green solid. Even after careful washing with diethylether and drying, the sample (according to NMR including 2D NMR) contained approximately four molecules of pyridine per one Pc. The free acid was then quantitatively converted to its sodium salt 2. The solid 2COOH (65 mg, 34 µmol) was dissolved in 1.0 M NaOH solution (544 µL, 544 µmol) and water was evaporated to dryness. Data for 2COOH: Yield : 79 mg (89%). MS MALDI-TOF: m/z

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| calculated for $C_{96}H_{48}N_8O_{32}Zn$ [M] ⁺ : 1888.2. Found: 1888.0. ¹ H NMR (500 MHz, DMSO- |
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| d ₆) δ 13.31 (s, 16H), 9.66 (s, 8H), 8.47 (t, J = 1.6 Hz, 8H), 8.32 (d, J = 1.6 Hz, 16H) + |
| signals of pyridine 8.68-8.64 (m, 8H), 8.03-7.97 (m, 4H), 7.59-7.54 (m, 8H). The ratio of |
| Pc:pyridine signals indicated presence of four pyridine molecules per one Pc. ¹³ C NMR |
| (126 MHz, DMSO-d_6) δ 166.82, 154.04, 148.00, 142.15, 140.65, 138.55, 135.61, |
| 131.81, 125.28 + signals of pyridine 139.42, 129.12, 124.73. $\textit{\lambda}_{max}$ (DMF, 1 μM)/nm 693 |
| (ϵ /dm ³ mol ⁻¹ cm ⁻¹ 213 010), 626 (39 240), 368 (79 100). IR (ATR) ν = 3068 (C-H _{aliph}), |
| 1704 cm ⁻¹ (C=O). Data for 2 : ¹ H NMR (500 MHz, D ₂ O) δ 9.60 (d, <i>J</i> = 2.5 Hz, 8H), 8.23 – |
| 8.19 (m, 8H), 8.14 – 8.10 (m, 16H). ¹³ C NMR (126 MHz, D ₂ O) δ 174.63, 154.53, 142.34, |
| 141.09, 137.23, 136.62, 133.36, 128.02, 124.72. λ_{max} (PBS, 1 μ M)/nm 698 (ϵ /dm ³ mol ⁻¹ |
| cm ⁻¹ 22 1910), 628 (35 300), 362 (92 440). λ_{max} (DMF, 1 μ M)/nm 696 (ϵ /dm ³ mol ⁻¹ cm ⁻¹ |
| 203 740), 627 (36 340), 367 (80 280). IR (ATR) v = 1611, 1563(C=O). HPLC analysis: |
| <i>t</i> _r = 18.41 min; purity 96.4 %. |

Preparationof2,3,9,10,16,17,23,24-octakis[3,5-bis(butoxycarbonyl)phenoxy]phthalocyaninatozinc(II)(3Bu).A mixture of dinitrile14(915 mg, 1.68 mmol), zincacetate(150 mg, 0.68 mmol)and few drops of DBU in *n*-butanol (20 mL) was heated at

| 140 °C for 18 h. The volatiles were removed under reduced pressure to give a greenish |
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| blue solid, which was purified by column chromatography on silica using |
| chloroform/ethyl acetate (100:1, R_f = 0.62) for elution. The purified product was |
| crystallized by slow addition of 3Bu chloroform solution into methanol. The solid was |
| collected and dried yielding dark green solid (502 mg, 41%). MS MALDI-TOF: m/z |
| calculated for $C_{160}H_{176}N_8O_{40}Zn$ [M] ⁺ : 2913.1. Found: 2913.1. ¹ H NMR (500 MHz, |
| CDCl ₃ /pyridine-d ₅ 3:1) δ 8.89 (s, 8H), 8.37 (t, <i>J</i> = 1.5 Hz, 8H), 8.17 (d, <i>J</i> = 1.5 Hz, 16H), |
| 4.31-4.23 (m, 32H), 1.75 – 1.68 (m, 32H), 1.45 – 1.36 (m, 32H), 0.83 (t, J = 7.4 Hz, |
| 48H). $^{13}\mathrm{C}$ NMR (126 MHz, CDCl_3) δ 164.85, 158.02, 150.97, 148.78, 132.66, 125.43, |
| 122.52, 115.87, 65.42, 30.71, 19.27, 13.76. One aromatic signal was overlapped by |
| signal of solvent. λ_{max} (THF, 1 µM)/nm 671 (ϵ /dm ³ mol ⁻¹ cm ⁻¹ 120 900), 640 (91 630), |
| 350 (107 730). $\lambda_{\rm max}$ (DMF, 1 μ M)/nm 640 (ϵ /dm ³ mol ⁻¹ cm ⁻¹ 92 660), 350 (98 910). IR |
| (ATR) ν = 3088 (C-H _{arom}), 2960 (C-H _{aliph}), 2874 (C-H _{aliph}), 1723 cm ⁻¹ (C=O). |

Preparation of hexadecasodium salt of 2,3,9,10,16,17,23,24-octakis(3,5-dicarboxylatophenoxy)phthalocyaninato zinc (II) (3). Compound **3Bu** (290 mg, 100 μmol) was dissolved in THF (7 mL) and added slowly to a saturated NaOH solution in

water/methanol (1:5) (90 mL). The mixture was stirred at 40 °C for 4 h, and the resulting precipitate was filtered and washed repeatedly with MeOH and chloroform. The crude product was dissolved in water and acidified using 1 M HCl until pH 1. The blue precipitate was collected by filtration, resuspended in water with drop of 1% HCl, centrifuged and carefully decanted. This has been repeated twice more. After the removal of the liquid phase, the green solid was suspended in acetone, sonicated (a small amount of the product dissolved) and transferred to flask and evaporated. This has been repeated twice more until the acetone remained colorless. The solid was then washed three times with acetone and diethyl ether and dried to yield 103 mg (51 %) of blue solid. The free acid was then quantitatively converted to sodium salt. The solid **3COOH** (103 mg, 51 µmol) was dissolved in 1.0 M NaOH solution (816 µL, 816 µmol) and water was evaporated to dryness. ¹H NMR measurements in pure D₂O indicated equilibrium between ionized and nonionized form similarly as reported earlier.¹⁵ Data for **3COOH**: MS MALDI-TOF: *m/z* calculated for C₉₆H₄₈N₈O₄₀Zn [M]⁺: 2016.1 Found: 2016.0 ¹H NMR (500 MHz, DMSO-d₆) δ 12.94 (s, ~13H), 8.91 – 8.61 (m, 8H), 8.00 – 7.90 (m, 8H), 7.85 – 7.66 (m, 16H). ¹³C NMR (126 MHz, DMSO-d6) δ 166.02, 157.51, 150.56,

| 148.37, 135.28, 133.16, 125.65, 121.70. Due to poor signal, the spectrum could not be |
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| properly analyzed and one carbon is missing. A_{max} (DMF, 1 µM)/nm 674 (ϵ /dm ³ mol ⁻¹ |
| cm ⁻¹ 216 950), 610 (35 070), 361 (76 970). IR (ATR) ν =: 3090 (C-H _{aliph}), 1701 cm ⁻¹ |
| (C=O). Data for 3 : ¹ H NMR (500 MHz, D ₂ O) δ 9.22 (s, 8H), 7.98 (s, 8H), 7.62 (d, J = 1.5 |
| Hz, 16H). ¹³ C NMR (126 MHz, D ₂ O) δ 174.61, 157.74, 154.21, 149.65, 139.09, 136.07, |
| 124.96, 120.56, 117.24. <i>λ</i> _{max} (PBS, 1 μM)/nm 680 (<i>ε</i> /dm ³ mol ⁻¹ cm ⁻¹ 193 260), 613 (32 |
| 060), 355 (81 780). λ _{max} (DMF, 1 μM)/nm 674 (<i>ε</i> /dm ³ mol ⁻¹ cm ⁻¹ 205 950), 610 (33 240 |
|), 362 (72 930). IR (ATR) $v = 1614 \text{ cm}^{-1}$ (C=O). HPLC analysis: $t_r = 20.34 \text{ min}$; purity |
| 99.2 %. |

Preparation of 2,3-bis[3,5-bis(butoxycarbonyl)phenyl]phthalocyaninato zinc(II) (7Bu). Magnesium turnings (394 mg, 16.2 mmol) were refluxed in anhydrous butanol (20 mL) with a small crystal of iodine for 5 h until all magnesium was converted to magnesium butoxide. Phthalonitrile **12** (300 mg, 0.59 mmol) and phthalonitrile (225 mg, 1.76 mmol) were added, and the reflux continued for the next 20 h. The mixture was cooled, concentrated and poured into water/methanol/acetic acid (25:25:1, 100 mL). The suspension was stirred at rt for 60 min to remove unreacted magnesium butoxide. The

green precipitate was collected by filtration and washed with water and methanol. Crude magnesium complex was purified by column chromatography using chloroform/THF (20:1, $R_f = 0.30$) for elution to isolate the desired AAAB type isomer as the second most intense blue fraction. The crude product was recrystallized from chloroform/MeOH to give a blue solid (123 mg, 19%), which was characterized by MALDI-TOF (*m/z* calculated for $C_{64}H_{56}MgN_8O_8$ [M]⁺: 1088.4. Found: 1088.3) and directly converted to metal-free ligand.

Magnesium complex (123 mg, 113 µmol) and *p*-toluenesulfonic acid hydrate (214 mg, 1.13 mmol) were dissolved in THF (10 mL). The solution was stirred at rt for 2 h and then the volatiles were removed under reduced pressure. The solid was washed with water and methanol. Crude product was purified by column chromatography on silica using chloroform/toluene/THF (25:25:1, $R_f = 0.3$) for elution. The crude product was recrystallized from chloroform/MeOH to give blue solid (62 mg, 51%). Metal-free ligand was characterized by MALDI-TOF (*m*/*z* calculated for C₆₄H₅₈N₈O₈ [M]⁺: 1066.4. Found: 1066.4) and directly converted to zinc(II) complex **7Bu**.

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| Metal-free ligand (62 mg, 58 μ mol) was dissolved in pyridine (7 ml) and zinc(II) |
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| acetate (76 mg, 0.41 mmol) was added. The mixture was refluxed for 6 h and then |
| pyridine was removed under reduced pressure. The solid was washed with water and |
| methanol. The crude product was purified by column chromatography on silica with |
| chloroform/THF/ethylacetate (30:1:1, R_f = 0.52) as the eluent. The purified product was |
| crystallized by slow addition of methanol to solution of 7Bu in chloroform yielding dark |
| blue solid (58 mg, 88 %). MS MALDI-TOF: m/z calculated for C ₆₄ H ₅₆ N ₈ O ₈ Zn [M] ⁺ : |
| 1128.4. Found: 1128.3. HRMS (ESI): m/z calculated for $C_{64}H_{56}N_8O_8Zn + H^+ [M + H^+]$: |
| 1129.3590. Found: 1129.3547. ¹ H NMR (500 MHz, CDCl ₃ /pyridine-d ₅ 3:1) δ 9.35 – 9.29 |
| (m, 4H), 9.25 – 9.17 (m, 4H), 8.95 (s, 2H), 8.72 (d, <i>J</i> = 1.6 Hz, 4H), 8.18 (dd, <i>J</i> = 5.6, 2.8 |
| Hz, 2H), 8.16 – 8.11 (m, 4H), 4.53 (t, J = 6.6 Hz, 8H), 1.91 (q, J = 8.0, 7.4 Hz, 8H), 1.65 |
| – 1.56 (m, 8H), 1.12 (t, J = 7.4 Hz, 12H). ¹³ C NMR (126 MHz, CDCl ₃ /pyridine-d ₅ 3:1) δ |
| 165.88, 153.80, 153.53, 152.73, 151.38, 142.67, 139.14, 138.64, 138.31, 138.29, |
| 137.86, 135.97, 131.42, 129.47, 129.30, 129.15, 129.09, 124.85, 122.89, 122.77, |
| 122.68, 65.45, 30.94, 19.46, 13.94. $\lambda_{\rm max}$ (DMF, 1 μ M)/nm 681 (ϵ /dm ³ mol ⁻¹ cm ⁻¹ 176 |
| 200), 609 (31 570), 352 (64 150). λ_{max} (THF, 1 μ M)/nm 676 (ϵ /dm ³ mol ⁻¹ cm ⁻¹ 223 680), |

Preparation of tetrasodium salt of 2,3-bis(3,5-dicarboxylatophenyl)phthalocyaninato zinc(II) (7). Compound 7Bu (58 mg, 51 µmol) was dissolved in THF (5 mL) and added slowly to a saturated NaOH solution in water/methanol (1:5) (30 mL). The mixture was stirred at 40 °C for 4 h, and the resulting precipitate was collected. The crude product was dissolved in water and acidified using 1 M HCl until pH 1. The precipitate was collected by filtration, resuspended in water with drop of 1% HCI, centrifuged and carefully decanted. This has been repeated twice more. After drying, the solid phthalocyanine was dissolved in a small amount of pyridine and was dropped to diethylether. The blue crystals were filtered and dried yielding blue solid (37 mg, 80%) of **7COOH**. The free acid was then converted to sodium salt. The solid **7COOH** (37 mg, 1 µmol) was dissolved in 0.1 M NaOH (1.63 mL, 163 µmol) and the water was evaporated to dryness. Blue solid was suspended in EtOH, centrifuged and washed with pure EtOH. Blue crystals of salt 7 were collected and dried. Data for 7COOH: MS MALDI-TOF: *m/z* calculated for C₄₈H₂₄N₈O₈Zn [M]⁺: 904.1. Found: 904.0. HRMS (ESI):

 $\ensuremath{\textit{m/z}}$ calculated for $C_{48}H_{24}N_8O_8Zn$ +[M]+: 904.1003. Found: 904.0987. 1H NMR (500

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| MHz, pyridine-d ₅) δ 9.95 (s, 2H), 9.79 (dd, <i>J</i> = 5.5, 3.0 Hz, 2H), 9.73 (d, <i>J</i> = 6.3 Hz, 2H), |
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| 9.67 (d, J = 6.4 Hz, 2H), 9.57 (d, J = 1.7 Hz, 2H), 9.20 (d, J = 1.6 Hz, 4H), 8.30 (dd, J = |
| 5.6, 2.9 Hz, 2H), 8.25 (tt, J = 7.2, 3.6 Hz, 4H). $^{13}\mathrm{C}$ NMR (126 MHz, pyridine-d_5) δ |
| 168.48, 154.76, 154.66, 154.30, 153.52, 142.88, 141.20, 139.30, 139.20, 139.16, |
| 138.68, 133.52, 130.50, 130.01, 129.95, 129.89, 125.47. Some aromatic signals were |
| overlapped by signal of solvent. λ_{max} (DMF, 1 μ M)/nm 676 (ϵ /dm ³ mol ⁻¹ cm ⁻¹ 158 130), |
| 610 (29 680), 345 (49 820). IR (ATR) v 3063(C-H _{arom}), 1711 cm ⁻¹ (C=O) cm ⁻¹ . Data for |
| 7 : ¹ H NMR (500 MHz, DMSO-d ₆ /D ₂ O 1:1) δ 8.97 (dd, J = 5.4, 2.9 Hz, 2H), 8.78 (d, J = |
| 7.3 Hz, 2H), 8.69 (d, J = 7.3 Hz, 2H), 8.63 (s, 2H), 8.39 (s, 2H), 8.35 (s, 4H), 8.06 (t, |
| 4H), 7.96 (t, J = 7.1 Hz, 2H). ¹³ C NMR (125 MHz, DMSO-d ₆ /D ₂ O 1:1) δ 172.74, 152.26, |
| 152.02, 151.76, 151.00, 141.57, 141.41, 137.80, 137.24, 136.90, 136.82, 136.08, |
| 133.25, 129.71, 129.52, 128.56, 124.49, 122.82, 122.62, 122.57. One signal was not |
| detected or overlapped with other signals. ${\it A}_{max}$ (PBS, 1 $\mu M)/nm$ 640 (ϵ/dm^3 mol^1 cm^{-1} |
| 69 530), 339 (53 980). <i>λ</i> _{max} (DMF, 1 μM)/nm 677 (<i>ε</i> /dm ³ mol ⁻¹ cm ⁻¹ 167 840), 610 (29 |

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010), 354 (60 050). IR (ATR) ν = 1558 (C=O) 1614 cm⁻¹ (C=O). HPLC analysis: t_r = 16.83 min; purity 99.3 %.

Preparation of 2,3-bis[3,5-bis(butoxycarbonyl)phenoxy]phthalocyaninato zinc(II) (8Bu). Magnesium turnings (1.95 g, 81.25 mmol) and a small crystal of iodine were refluxed in freshly distilled anhydrous butanol (50 mL) for 4 h until all magnesium was converted to magnesium butoxide. Phthalonitrile (1.12 g, 8.73 mmol) and compound 14 (1.58 g, 2.91 mmol) were then added to the reaction mixture and the reflux was continued for next 20 h. The reaction mixture was then cooled to room temperature and concentrated under reduced pressure. Then, the dark mixture was poured into water/methanol/acetic acid (10:10:1, 300 mL), and the suspension was stirred at rt overnight to remove unreacted magnesium butoxide. The green precipitate was collected by filtration and washed with water and small of amount of methanol. Crude magnesium complex was purified by column chromatography on silica using chloroform/THF (20:1, R_f =0.55) for elution to isolate the desired AAAB type isomer as the second most intense blue fraction. Pure fractions were collected and dried yielding 857 mg (26%) of dark solid of corresponding Mg(II) Pc that was directly converted to metal-free ligand.

Thus, magnesium complex (857 mg, 0.764 mmol) and p-toluenesulfonic acid hydrate

(1451 mg, 7.64 mmol) were dissolved in THF (100 mL). The solution was stirred at rt for 3 h and then the volatiles were removed under reduced pressure. The solid was washed with water and methanol. The crude product (approximately 600 mg) was dissolved in pyridine (50 ml) and zinc(II) acetate (700 mg, 3.81 mmol) was added. The mixture was refluxed overnight and then pyridine was removed under reduced pressure. The solid was washed with water and methanol. The crude product was purified via column chromatography on silica with chloroform/THF (100:1, $R_f = 0.71$) as the eluent. The purified product was crystallized by slow addition of concentrated chloroform solution of 8Bu into methanol. The solid was collected and dried yielding dark solid (514 mg, 15 % based on starting phthalonitriles). HRMS (ESI): m/z calculated for C₆₄H₅₆N₈O₁₀Zn + H⁺ [M + H⁺]: 1161.3484. Found: 1161.3455. ¹H NMR (500 MHz, CDCl₃-d) δ 9.21 (dd, J = 5.5, 3.0 Hz, 2H), 9.18 – 9.16 (m, 2H), 9.13 – 9.10 (m, 2H), 8.99 (s, 2H), 8.67 (t, J = 1.5 Hz, 2H), 8.28 (d, J = 1.5 Hz, 4H), 8.19 – 8.14 (m, 2H), 8.14 – 8.06 (m, 4H), 4.41 (t, J = 6.7 Hz, 8H), 1.83 – 1.76 (m, 8H), 1.52 – 1.42 (m, 8H), 0.91 (t, J = 7.4 Hz, 12H). ¹³C NMR (126 MHz, CDCl₃) δ 165.37, 158.32, 153.69, 153.50, 152.81,

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| 151.05, 146.52, 156.50, 156.51, 156.50, 155.00, 129.25, 129.14, 129.09, 125.54, |
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| 122.93, 122.80, 122.69, 122.65, 115.77, 65.56, 30.80, 19.35, 13.80. One aromatic |
| signal was overlapped by signal of solvent. ${\it A}_{max}$ (DMF, 1 μM)/nm 673 (ϵ/dm^3 mol^1 cm^- |
| 1 227 220), 605 (37 830), 350 (72 940). $\textit{\lambda}_{max}$ (THF, 1 μM)/nm 669 (\textit{e}/dm^3 mol 1 cm 1 213 |
| 900), 602 (35 320), 346 (76 290). IR (ATR) v=: 3063(C-H _{arom}), 2959 (C-H _{aliph}), 2873 (C- |
| H _{aliph}), 1725 cm ⁻¹ (C=O). |

Preparation of 2,3-bis(3,5-dicarboxylatophenoxy)phthalocyaninato zinc(II) (8COOH). Compound 8Bu (392 mg, 0.337 mmol) was dissolved in THF (12 mL) and added slowly to a saturated NaOH solution in water/methanol (1:5) (100 mL). The mixture was stirred at 40 °C for 4 h, and the resulting precipitate was concentrated under reduced pressure. The crude product was dissolved in water and acidified using 1 M HCl until pH 1. The precipitate was collected by filtration, resuspended in water with drop of 1% HCl, centrifuged and carefully decanted. This has been repeated twice more. After drying, the solid phthalocyanine was dissolved in a small amount of pyridine and was dropped to diethylether. The blue crystals were filtered and dried yielding blue solid (220 mg, 70 %) of 8COOH. The free acid was then converted to sodium salt. The solid 8COOH (220

| mg, 234 $\mu\text{mol})$ was dissolved in 0.1 M NaOH (9.36 mL, 936 $\mu\text{mol})$ and the water was |
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| evaporated to dryness. Blue solid was suspended in EtOH and centrifuged and washed |
| with pure EtOH. Blue crystals of salt 8 were collected and dried. Data for 8COOH: Yield |
| 220 mg (70 %). HRMS (ESI): m/z calculated for $C_{48}H_{24}N_8O_{10}Zn + H^+$ [M + H ⁺]: |
| 937.0980. Found: 937.0981. ¹ H NMR (500 MHz, pyridine-d ₅) δ 9.63 – 9.57 (m, 6H), 9.56 |
| (d, J = 7.3 Hz, 2H), 9.37 – 9.34 (m, 2H), 8.84 – 8.79 (m, 4H), 8.26 – 8.14 (m, 6H). ¹³ C |
| NMR (126 MHz, pyridine-d ₅) δ 168.04, 158.70, 154.47, 154.45, 154.03, 152.69, 150.28, |
| 139.10, 139.08, 139.01, 136.31, 136.00, 129.85, 129.74, 126.72, 124.04, 123.08, |
| 116.35. Some signals were overlapped by signal of solvent. λ_{max} (DMF, 1 μ M)/nm 681 |
| (ɛ/dm³ mol-1 cm-1 174 120), 673 (185 470), 608 (28 880), 353 (46 380). IR (ATR) v = |
| 3072 (C-H _{arom}), 1719 cm ⁻¹ (C=O). Data for 8: ¹ H NMR (500 MHz, DMSO-d ₆ /D ₂ O 1:1) δ |
| 9.01 (dd, J = 5.5, 2.9 Hz, 2H), 8.73 (d, J = 5.2 Hz, 2H), 8.45 (d, J = 5.0 Hz, 2H), 8.28 (s, |
| 2H), 8.25 – 8.19 (m, 2H), 8.06 – 7.99 (m, 8H), 7.93 (s, 2H). ¹³ C NMR (126 MHz, DMSO- |
| d ₆ /D ₂ O 1:1) δ 171.80, 156.53, 152.24, 151.89, 151.39, 149.89, 149.31, 140.23, 137.37, |
| 136.80, 132.81, 129.77, 129.48, 129.44, 125.68, 122.89, 122.51, 121.74, 112.53. Two |
| signals were not detected. λ_{max} (PBS, 1 μ M)/nm 635 (ϵ /dm ³ mol ⁻¹ cm ⁻¹ 68 410), 337 (51 |

 000). *λ*_{max} (DMF, 1 μM)/nm 672 (*ε*/dm³ mol⁻¹ cm⁻¹ 156 920), 608 (30 150), 345 (48 130). IR (ATR) *ν* = 1614 (C=O), 1567 cm⁻¹ (C=O). HPLC analysis: *t*_r = 17.24 min; purity 98.6 %.

Preparation of 1-(2,6-bis[(3-methyl-1H-imidazol-3-ium-1-yl)methyl]-4-methylphenoxy)-3,4-dimethyl-tribenzo[*g,l,q*]-*N*-methylpyridino[3,4-*b*]porphyrazinato zinc(II) triiodide (10). Magnesium turnings (680 mg, 29.0 mmol) were refluxed with few crystals of iodine in freshly distilled butanol (30 mL) for 3 hours to be converted at magnesium butoxide. Compound 15 (423 mg, 1.00 mmol) and phthalonitrile (384 mg, 3.0 mmol) were added to reaction mixture and refluxed for 20 hours. Butanol was evaporated from reaction mixture under reduced pressure. Mixture of chloroform and THF (2:1) was added to residue in flask. Flask was treated by ultrasound, undissolved material was filtered off and washed carefully with chloroform, THF and pyridine. Collected organic solution was evaporated to dryness under reduced pressure. Crude product was purified by column chromatography on silica (chloroform/pyridine/methanol – 10:1:1, R_f = 0.40, the second most intense green fraction) yielding 240 mg of magnesium complex that was

characterized by MALDI-TOF (m/z calculated for C₄₈H₃₃MgN₁₃O [M]⁺: 831.3. Found: 831.2).

Magnesium complex (240 mg, 0.29 mmol) and *p*-toluenesulfonic acid monohydrate (544 mg, 2.86 mmol) were dissolved in a mixture of methanol, THF, chloroform (15 mL, 1:1:1) and stirred for 2 hours at room temperature. Solvents were evaporated and the dry residue was suspended in 5% solution of NaHCO₃ (40 mL). Suspension was filtrated, precipitate was collected, washed with water and dried. Obtained metal free ligand (231 mg) was characterized by MALDI-TOF (*m/z* calculated for $C_{48}H_{35}N_{13}O$ [M]⁺: 809.3. Found: 809.3).

Metal-free ligand (231 mg, 0.29 mmol) was dissolved in pyridine (10 mL) and zinc acetate (524 mg, 2.86 mmol) was added. The reaction mixture was refluxed for 2 hours. Pyridine was evaporated from reaction under reduced pressure. A crude product was suspended in water, filtered, precipitate was washed several times with water and dried. The zinc compound was extracted from precipitate by mixture of solvents (methanol, chloroform, pyridine). The solution was filtered, evaporated to dryness and washed with

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hexane yielding 70 mg of dark blue zinc complex. Zinc complex was characterized by MALDI-TOF (m/z calculated for C₄₈H₃₃N₁₃OZn [M]⁺: 871.2. Found: 871.2).

The zinc complex (57 mg, 65 µmol) was dissolved in anhydrous DMF (4 mL) under argon atmosphere. Methyliodide (205 µL, 3.29) mmol) was added into reaction which was heated at 80 °C for 20 hours. Volatile compounds were evaporated, and the product was washed with diethylether. Yield: 45 mg (4 % based on starting phthalonitriles) of dark solid. ¹H NMR (500 MHz, DMSO-d₆) δ 9.48 (d, J = 7.5 Hz, 1H), 9.42 (d, J = 6.8 Hz, 1H), 9.34 (d, J = 6.7 Hz, 1H), 9.31 – 9.26 (m, 1H), 9.22 – 9.17 (m, 1H), 9.07 (d, J = 7.7 Hz, 1H), 8.98 (d, J = 6.0 Hz, 1H), 8.94 (s, 2H), 8.39 – 8.33 (m, 1H), 8.33 – 8.25 (m, 2H), 8.22 – 8.16 (m, 2H), 8.16 – 8.09 (m, 1H), 7.87 (s, 2H), 7.77 (s, 2H), 7.36 (s, 2H), 5.63 (d, J = 14.7 Hz, 2H), 5.50 (d, J = 14.6 Hz, 2H), 3.79 (s, 3H), 3.31 (s, 3H), 3.10 (s, 6H), 2.72 (s, 3H), 2.57 (s, 3H). ¹³C NMR (126 MHz, DMSO-d₆) δ 155.63, 155.12, 154.94, 154.67, 154.55, 153.97, 153.47, 151.98, 151.18, 150.03, 149.28, 145.70, 145.39, 144.96, 138.49, 138.29, 138.26, 138.17, 138.09, 137.71, 136.85, 136.54, 133.46, 130.48, 130.41, 130.12, 129.98, 128.68, 127.86, 123.88, 123.80, 123.17, 122.96, 122.72, 122.48, 122.34, 116.49, 54.57, 48.40, 35.62, 22.53, 20.81,
14.42. One aromatic signal was not detected. λ_{max} (water, 1 µM)/nm 636 (ϵ /dm³ mol⁻¹ cm⁻¹ 37 560), 334 (34 630). λ_{max} (PBS, 1 µM)/nm 630 (ϵ /dm3 mol-1 cm-1 25 050), 341 (27 730). λ_{max} (DMF, 1 µM)/nm 689 (ϵ /dm3 mol-1 cm-1 96 190), 671 (71 310), 612 (16 680), 354 (27 110). IR (ATR) ν =3014 cm⁻¹ (C-H_{aliph}). HPLC analysis: t_r = 20.69 min; purity 98.6 %.

Preparation of 4,5-bis[3,5-bis(methoxycarbonyl)phenyl]phthalonitrile (12). Compound **11** (5.02 g, 15.7 mmol), 4,5-dichlorophthalonitrile (1.05 g; 5.3 mmol), K_3PO_4 (3.27 g; 15.4 mmol), palladium(II) acetate (56 mg; 0.25 mmol), XPhos (362 mg; 0.76 mmol) were dissolved in anhydrous THF (110 ml) and stirred at rt for 24 hod under argon. After that water (5 ml) was added and the reaction was stirred at rt for next 24 h. Organic solvent was evaporated and the product was extracted three times with ethyl acetate. Organic fractions were collected, dried over sodium sulfate, filtered and evaporated to dryness. Crude compound 12 was purified via column chromatography on silica with hexane/ethyl acetate (2:1, R_f = 0.28). The purified product was recrystallized from chloroform/MeOH to give the title compound **12** as white crystals. Yield: 2.25 g (82 %). Mp = 207.8-208.4 °C (chloroform/MeOH). ¹H NMR (500 MHz, CDCl₃) δ 8.60 (s, 2H),

(ATR) v = 2957 (C-H_{aliph}), 2235 (CN), 1716 (C=O). AUTHOR INFORMATION **Corresponding Author** Notes ACKNOWLEDGMENT

7.96 (d, J = 1.6 Hz, 4H), 7.93 (s, 2H), 3.89 (s, 12H). ¹³C NMR (125 MHz, CDCl₃) δ 165.06, 143.79, 137.51, 135.43, 134.22, 131.33, 130.80, 115.66, 114.69, 52.60. IR

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ABBREVIATIONS

BSA, bovine serum albumin; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DOPC, 1,2-

dioleoylphosphatidylcholine; EC₅₀, half-maximal effective concentration; PBS,

phosphate-buffered saline; Pc, phthalocyanine; PDT, photodynamic therapy; PS,

photosensitizer; SCM, serum-containing medium; SFM, serum-free medium, TC₅₀, half-

maximal toxic concentration; TPyzPz, tetrapyrazinoporphyrazine.

ASSOCIATED CONTENT

Supporting Information

The following files are available free of charge.

NMR spectra (PDF)

Video of intracellular relocalization of Pcs 2 and 6 in HeLa cells upon irradiation (video

file .mp4)

Additional experimental details (PDF).

Molecular formula strings (CSV file)

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Figure 4

177x71mm (300 x 300 DPI)





Figure 5 84x98mm (300 x 300 DPI)





56

57 58



Figure 6

84x59mm (300 x 300 DPI)



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Figure 10



Figure 11 84x123mm (300 x 300 DPI)