Effect of zinc insertion and hydrophobicity on the membrane interactions and PDT activity of porphyrin photosensitizers

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A series of photosensitizers (PS), which are *meso*-substituted tetra-cationic porphyrins, was synthesized in order to study the role of amphiphilicity and zinc insertion in photodynamic therapy (PDT) efficacy. Several properties of the PS were evaluated and compared within the series including photophysical properties (absorption spectra, fluorescence quantum yield Φ_f, and singlet oxygen quantum yield Φ_Δ), uptake by vesicles, mitochondria and HeLa cells, dark and phototoxicity in HeLa cells. The photophysical properties of all compounds are quite similar ($\Phi_f \leq 0.02$; $\Phi_\Delta \sim 0.8$). An increase in lipophilicity and the presence of zinc in the porphyrin ring result in higher vesicle and cell uptake. Binding in mitochondria is dependent on the PS lipophilicity and on the electrochemical membrane potential, *i.e.*, in uncoupled mitochondria PS binding decreases by up to 53%. The porphyrin substituted with octyl groups (TC8PyP) is the compound that is most enriched in mitochondria, and its zinc derivative (ZnTC8PyP) has the highest global uptake. The stronger membrane interaction of the zinc-substituted porphyrins is attributed to a complexing effect with phosphate groups of the phospholipids. Zinc insertion was also shown to decrease the interaction with isolated mitochondria and with the mitochondria of HeLa cells, an effect that has been explained by the particular characteristics of the mitochondrial internal membrane. Phototoxicity was shown to increase proportionally with membrane binding efficiency, which is attributed to favorable membrane interactions which allow more efficient membrane photooxidation. For this series of compounds, photodynamic efficiency is directly proportional to the membrane binding and cell uptake, but it is not totally related to mitochondrial targeting.

1. Introduction

Photodynamic therapy (PDT) is a promising modality for the treatment of some tumors and nonmalignant diseases. It is based on the administration of photosensitizers (PS), which are partially retained by the diseased tissue, followed by exposure of the diseased area to light of appropriate wavelengths. This process produces reactive species such as singlet oxygen and radicals that lead to tissue destruction. PDT selectivity is based on differences between the photosensitizer concentrations in normal and diseased tissues and on the selective illumination of the site being treated.[1–3]

Different kinds of PS have been synthesized and studied as PDT agents: pro-drugs and derivatives of porphyrins,[2–5] chlorins,[6,7] phthalocyanines,[8] and phenothiazines.[9,10] A good photosensitizer is preferably a pure compound that has strong light absorption, large triplet quantum yield and efficient formation of singlet oxygen ($^1O_2$).[11] It should be non-toxic in the absence of light, enriched in the target tissue and be eliminated from the body efficiently and quickly to avoid generalized skin photosensitization.[1–3]

Singlet oxygen is a powerful oxidant that can react with many kinds of biomolecules. It is considered the main oxidizing species that leads cells to an apoptotic response[12] and the main agent of tumor damage. As singlet oxygen is short lived (10^{-6}–10^{-9} s), the subcellular structures damaged in PDT are those that are close to the site of $^1O_2$ formation.[1–3,12–15] As a result, targeting important subcellular structures seems to be a good way to improve PDT efficacy.[12–16]

The site of photodamage depends on the photosensitizer structure, since different PS accumulate in different cell compartments.[13–16] Consequently the cellular photosensitization efficiency is strongly dependent on the photosensitizer structure. The distribution of polar and hydrophobic substituents around the macrocycle as well as the charge of the side chains play significant roles.[17,18] Cationic photosensitizers may be especially effective clinical agents because they can accumulate in mitochondria, an effect that is driven by the transmembrane potential of the inner mitochondrial membrane.[9,13,14,18] In fact, mitochondria targeting is considered particularly relevant for several anticancer therapies.[19] However, it is puzzling that to date there is no positively charged PS that is in widespread use in clinics, although phenothiazines have shown interesting results.[20] Knowing the structure–activity relationship of positively charged PS may help to bring them to clinical PDT.

Photosensitizers that localize in mitochondria have been reported to be more efficient at killing cells than those that localize at other cellular sites.[21] Several researchers have also shown that PS which bind to mitochondria induce apoptosis upon irradiation, whereas those which localize in other cell compartments also kill cells, but by non-apoptotic mechanisms.[16,21–23] However, there are also reports showing apoptotic cell death caused by PS that

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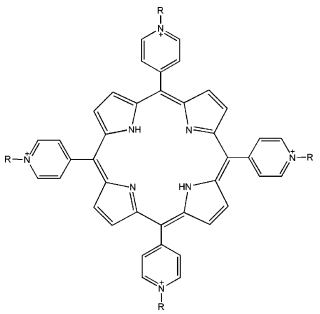
accumulate in lysosomes.[24] Although several results indicate the possible enhanced activity of mitochondrial PS, the quantification of this effect awaits better definition.

Photophysical properties of *meso*-substituted cationic porphyrins have been widely studied.[2,12–14,18–28] The quantum yield of singlet oxygen production (Φ_Δ) and membrane binding were evaluated and it is known that increasing lipophilicity is important for PDT efficiency.[16–18] The site of subcellular localization is also considered to be important, although the relationship between molecular structure, subcellular localization and PDT efficiency is still a matter of debate. For example, cationic porphyrins containing just one alkyl chain studied by Richelli *et al.*[16] showed lysosomal targeting. On the other hand, Zimmermann and Cernay[14] showed that 5,10,15,20-tetrakis(1-decylpyridinium-4-yl)porphyrin tetrabromide promotes selective mitochondria photosensitization.

Another modification in the molecular structure of porphyrin PS that seems to improve PDT efficiency is the addition of zinc into the porphyrin ring.[2,26] This substitution has been frequently used to stabilize the porphyrin ring, maintaining the photophysical properties necessary for PDT. Pashkovskaya and coworkers have recently referred to the role of metal–phosphate coordination to explain binding of phthalocyanines in membranes.[29] However, the effect of zinc on membrane binding properties, cell subcellular localization, and cell phototoxicity has never been carefully investigated.

In this study, we performed the synthesis of a series of amphiphilic *meso*-substituted porphyrins varying the balance between hydro- and lipophilicity through replacement of the four methyl groups by alkyl chains and by inserting zinc (see molecular structure of the free-base PS in Scheme 1). By studying the photophysical and photobiological properties relevant to PDT within this series of compounds we were able to show how hydrophobicity and zinc substitution affect membrane interactions and the photodynamic efficacy of porphyrins.



TMePyP R = CH_3 I^-
5,10,15,20-tetrakis-(N-methylpyridinium-4-yl)porphyrin tetraiodide

TC8PyP R = C_8H_{17} Br^-
5,10,15,20-tetrakis-(N-1-octylpyridinium-4-yl)porphyrin tetrabromide

TC14PyP R = $C_{14}H_{29}$ Br^-
5,10,15,20-tetrakis-(N-1-tetradecylpyridinium-4-yl)porphyrin tetrabromide

Scheme 1 Porphyrin structures.

2. Results and discussion

2.1 Spectroscopic and photophysical studies

The absorption spectra of the synthesized compounds are typical of porphyrins (Fig. 1). Absorption maxima and the molar absorptivity values were determined (Table 1). The free-base compounds present Soret bands with high extinction coefficients

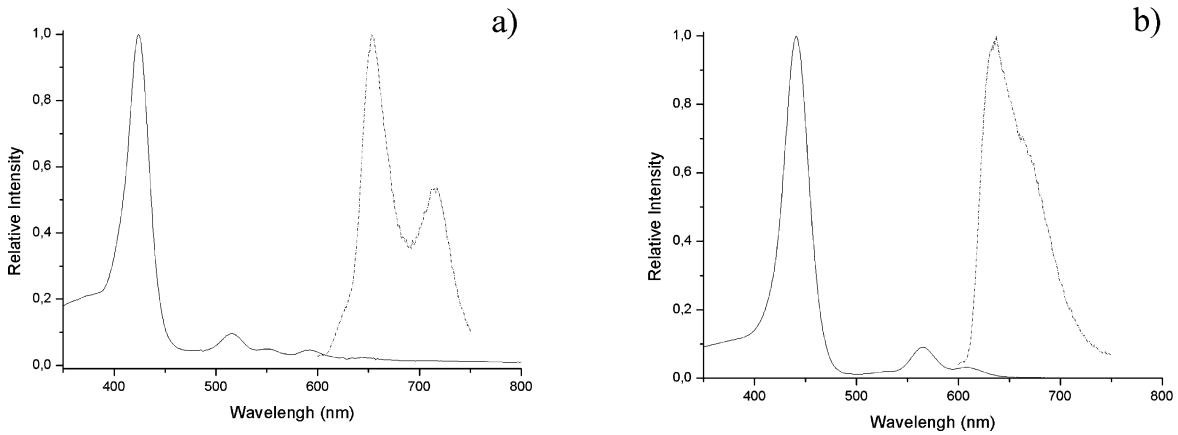


Fig. 1 (a) Normalized absorption (solid line) and emission (dotted line) spectra of TMePyP at 5.3 μmol L^{-1} and 1.0 μmol L^{-1}, respectively, in methanol; (b) normalized absorption (solid line) and emission (dotted line) spectra of ZnTC8PyP at 3.5 μmol L^{-1} and 1.0 μmol L^{-1}, respectively, in methanol.

Table 1 Molar absorptivity values in the band maxima, emission bands, fluorescence and singlet oxygen quantum yields (methanol)

Porphyrin	Soret band	Absorption λ/nm (log ε)				Emission λ/nm		Φ_f[a]	Φ_Δ[b]
		Q bands				Q (0,0)	Q (1,0)		
TMePyP	424 (5.28)	516 (4.15)	554 (3.66)	592 (3.69)	648 (3.08)	654	715	0.014	0.78 ± 0.07
ZnTMePyP	440 (5.08)		565 (4.00)		607 (3.45)	635	700	0.012	0.78 ± 0.05
TC8PyP	426 (5.25)	516 (4.11)	555 (3.73)	592 (3.66)	648 (3.15)	656	716	0.015	0.74 ± 0.06
ZnTC8PyP	442 (5.46)		566 (4.35)		610 (3.67)	635	663	0.020	0.76 ± 0.07
TC14PyP	426 (5.32)	516 (4.18)	552 (3.79)	592 (3.74)	648 (3.08)	655	717	0.011	0.77 ± 0.06

[a] Excitation 515 nm, emission filter > 606, slits: 10 nm on excitation and 1 nm on emission. [b] Excitation 532 nm, laser Nd:YAG, 5 ns, 10 Hz.

and four less intense Q bands.[30,31] The zinc porphyrins show a red-shifted Soret band and two less intense Q bands.[25] Because of the interaction between the central zinc atom and the π-conjugate system, the Q bands of the zinc porphyrins have stronger molar absorptivity coefficients compared with the free bases. Absorption spectra of the TC8PyP, ZnTC8PyP and TC14PyP porphyrins are strikingly similar to that of *meso*-tetrakis(4-*N*-methylpyridyl)porphyrin (TMePyP) (Fig. 1, Table 1), indicating that the increase in the length of the alkyl chain does not affect the electronic configuration of the porphyrins.[26,27] The fluorescence spectra of the free-base compounds have two maxima (650 nm and 715 nm), which are very close to those of other similar compounds.[26,27] The fluorescence spectra of zinc porphyrins have two maxima, which are blue shifted when compared with the emission of their respective free-base analogues, as previously observed for similar porphyrins.[25]

Table 1 also shows the fluorescence quantum yield (Φ_f) obtained for this series of porphyrins. All porphyrin derivatives exhibit low fluorescence quantum yields. The values of Φ_f are predominantly below 0.02, indicating that the radiative decay from the first excited singlet state is of minor importance among the competitive relaxation processes. Consequently, intersystem crossing and/or internal conversion play important roles in the decay of their excited singlet states. These results are in agreement with those obtained for similar porphyrins[25–27] including TMePyP.[32] Zinc insertion causes a small increase in the fluorescence quantum yield for TC8PyP while there is a decrease for TMePyP. Literature reports show that the effects caused by zinc insertion vary according to the porphyrin structure. On the one hand, Quimby and Longo[33] and Durantini *et al.*[34] showed that zinc insertion into halogenated tetraarylporphyrins and *meso*-tetrakis-(4-methoxyphenyl)porphyrin results in reduced fluorescence quantum yield. On the other hand, Mosinger and Kubat[35] described that inserting metals of electronic configuration d^0 and d^{10} into *meso*-tetrakis(4-sulfonatophenyl)porphyrin leads to high fluorescence quantum yields.

The quantum yields of singlet oxygen production Φ_Δ were also evaluated and Φ_Δ values varied from 0.74 to 0.78, indicating that all compounds are efficient singlet oxygen generators. Therefore, the increase in the length of the alkyl chain does not affect the photophysical properties significantly.[36] By studying this series of porphyrins (with the exception of TC14PyP) we were able to carefully evaluate other parameters related to the chemical structure that are relevant to PDT efficiency. The C14 derivative was not further studied because of its low aqueous solubility.

2.2 Porphyrin binding to vesicles and uptake in HeLa cells

Negative vesicles made of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and phosphatidylglycerol (PG), PG : DOPC (30 : 70 w/w) were chosen to mimic binding to the biological membranes, which are usually negatively charged. Note that the binding efficiency in vesicles and incorporation in HeLa cells is enhanced with the increase in the length of the N-alkyl substituent from one to eight carbon atoms (Fig. 2). There is a 10-fold increase in binding in vesicles and a ~3-fold increase in binding in HeLa cells. As has been shown previously,[16,18] the uptake process is strongly influenced by the length of the side chain for porphyrins with analogous structures. More importantly, stronger interaction

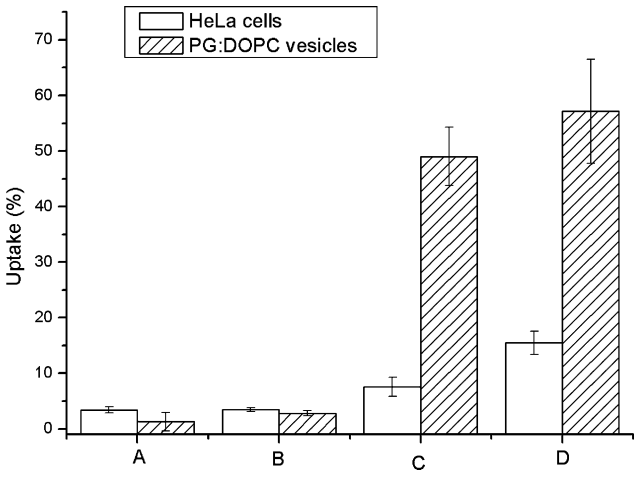


Fig. 2 Uptake of (A) TMePyP, (B) ZnTMePyP, (C) TC8PyP, (D) ZnTC8PyP by vesicles and HeLa cells at pH = 7.2. Vesicles: porphyrins ~3 μmol L^{-1} in phosphate buffer solution 0.1 mol L^{-1}; HeLa cells: porphyrins ~15 μmol L^{-1} in culture medium DMEM.

with membranes is usually related to a larger inclusion of the PS in the membrane and consequently to a stronger photodynamic efficiency in model systems.[17,18]

The interactions of photosensitizers with membranes may occur through several types of forces including electrostatic, hydrophobic, hydration and coordination.[9,10,16–18,29] Cell membranes and the synthetic vesicles used in this study are composed of a certain fraction of negatively charged lipids. Cell membranes also have charged proteins and carbohydrates. Therefore, the initial interaction between the positively-charged porphyrins and a specific type of membrane will take place *via* electrostatic attraction, which is supposedly the same for all compounds (all porphyrins have four positive charges). Binding of TMePyP and ZnTMePyP in cells and in synthetic vesicles (Fig. 2, A and B) is considerably smaller than binding of the hydrophobic porphyrins TC8PyP in both systems. Interestingly, TMePyP and ZnTMePyP bind more strongly to cells than to synthetic vesicles. The hydrophobic effect, which is dependent on the available hydrophobic surfaces of the porphyrins, starts to take place in the vicinity of the membrane. The increase in binding efficiency with increasing alkyl chain length can be explained by this effect. However, in the case of the more hydrophobic porphyrins there is stronger binding to the vesicles than to the cells (compare C and D in Fig. 2). This is probably due to the difficulty of these hydrophobic compounds in interacting with the hydrophobic moieties of the lipids in the cell membranes. Therefore, it is clear that the forces that govern binding to vesicles play only a small role in whole HeLa cell binding, probably because cell membranes have differences in lipid packing and the presence of proteins and carbohydrates.[9,10,16–18]

Zinc porphyrins show stronger binding to both synthetic and biological membranes than their respective free-base analogues (Fig. 2). This is surprising because zinc insertion is known to decrease hydrophobicity.[38] However, one should consider that binding of zinc porphyrins to phospholipid membranes may also be determined by metal–phosphate coordination.[29] This is in agreement with the fact that the formation of coordination complexes with phosphate groups is essential for binding of cations to lipid membranes.[39,40]

2.3 Binding to mitochondria

Mitochondria have two membranes: the external membrane which allows the passage of large molecules such as small proteins, and the internal membrane, which has a large percentage of proteins (more than 50%) and is highly impermeable to charged species.[37] The internal mitochondrial membrane is responsible for maintaining an electrochemical potential difference ($\Delta\Psi$) of around –180 mV in energized mitochondria.[37] Consequently, binding to mitochondria has a stronger electrostatic component compared with binding to other membranes.[9] Note the stronger binding of the polar porphyrins (A and B in Fig. 3) compared with binding to the other investigated membrane systems (A and B in Fig. 2). As expected, porphyrin binding to mitochondria is also affected by the length of the alkyl chain (Fig. 3), since changing the alkyl chain from one to eight carbon atoms increases porphyrin binding of both free-bases and zinc porphyrins. This increase can be explained by the increase in the hydrophobic effect. Interestingly, zinc insertion seems to disfavor binding to mitochondria (compare A with B and C with D in Fig. 3), which indicates that zinc–phosphate coordination does not play an important role in porphyrin binding to mitochondria. Binding seems to be affected mainly by the hydrophobic effect and the electrostatic component. Therefore, addition of zinc increases the hydrophilic character of these porphyrins and decreases the hydrophobic interaction with the membranes.[38] Another possibility to explain the stronger binding of free-base porphyrins compared to zinc porphyrins would be that free-base porphyrins can get chelated in biological media by free or partially chelated metals, *i.e.*, they could get chelated in the mitochondria increasing its binding efficiency. However, this hypothesis still needs experimental proof.

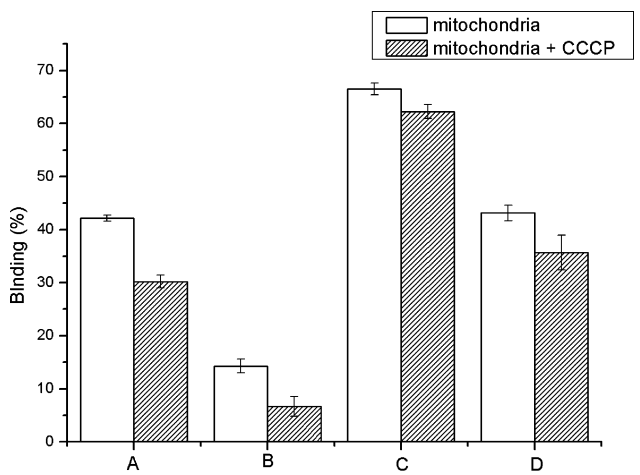


Fig. 3 Mitochondrial binding of (A) TMePyP, (B) ZnTMePyP, (C) TC8PyP and (D) ZnTC8PyP, pH = 7.4; mitochondria 2.0 mg mL^{-1} and porphyrins ~ 5 μmol L^{-1}.

In order to confirm that the mechanism of porphyrin accumulation is modulated by charge effects, binding experiments were carried out after collapsing $\Delta\Psi$ using the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).[9] When CCCP was added, porphyrin binding to mitochondria was reduced in all cases, confirming that porphyrin binding is affected by $\Delta\Psi$. The influence of $\Delta\Psi$ on porphyrin binding is stronger in hydrophilic (26% and 54% decrease for TMePyP and ZnTMePyP, respectively) than in hydrophobic (6% and 17% decrease for TC8PyP and ZnTC8PyP, respectively) porphyrins. It is also more pronounced in zinc (54% and 17%) than in free-base porphyrins (24% and 6%).

2.4 Cytotoxicity and phototoxicity studies

Cytotoxicity tests showed that these porphyrins do not have any dark toxicity with concentrations ranging from 2.5 to 30 μmol L^{-1} (data not shown). Note that the decrease in cell survival and consequent increase in phototoxicity parallels the increase in membrane binding and uptake by the cell (Fig. 2), *i.e.*, ZnTC8PyP > TC8PyP > ZnTMePyP > TMePyP (Fig. 4). This direct relationship between porphyrin uptake and phototoxicity is expected considering that all porphyrins show almost the same Φ_Δ and increase in membrane affinity is usually related to better PDT efficiency.[17,18]

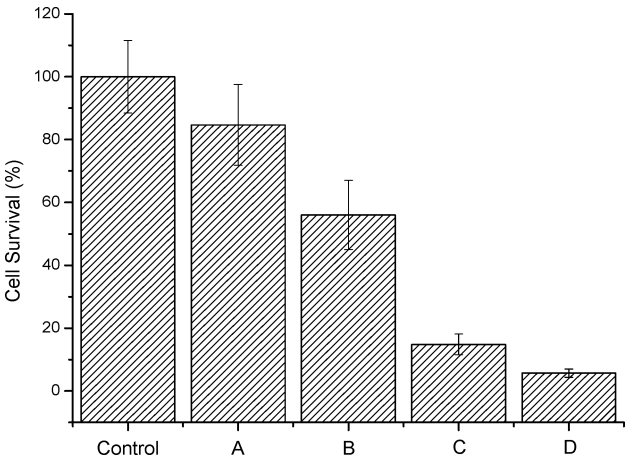


Fig. 4 Phototoxicity in HeLa cells. (A) TMePyP 13 μmol L^{-1}, (B) ZnTMePyP 24 μmol L^{-1}, (C) TC8PyP 10 μmol L^{-1}, (D) ZnTC8PyP 9 μmol L^{-1} in culture medium DMEM, pH = 7.2. Light dose 175 mJ cm^{-2}, A and C irradiated with a 650 nm laser and B and D with a 532 nm laser. Control: cells without PS and light treatments.

Literature data indicate that zinc porphyrins have better efficiency compared with metal-free porphyrins.[28] We were able to reproduce this effect and to show that it is due to the favorable membrane binding that causes the highest uptake in cells. Therefore, membrane binding seems to be the major factor affecting phototoxicity. Although the presence of zinc facilitates membrane binding, the results in Fig. 3 indicate that it decreases interactions with mitochondria, suggesting that mitochondria accumulation is less important for the final photodynamic efficiency than the general efficiency of the membrane interaction. However, before reaching such a conclusion, it is important to evaluate whether zinc affects mitochondria accumulation of PS in cells.

2.5 Subcellular localization

We evaluated the fluorescence distribution of the porphyrins by comparing it with that of rhodamine 123, which is a well-known mitochondrial probe.[16,41] Fig. 5 shows the confocal micrograph patterns of HeLa cells stained with the cationic porphyrins and rhodamine 123 (columns I and II, respectively, Fig. 5). The red fluorescence of these photosensitizers is distributed through the entire cytoplasm and remains outside the nucleus (column I).

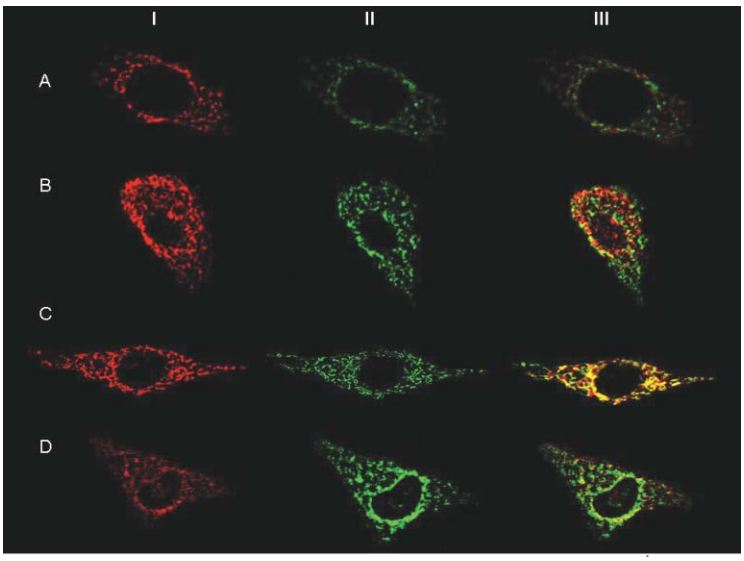


Fig. 5 Confocal images. Left: Intracellular localization of porphyrins 10 μmol L^{-1} in culture medium DMEM (red images, (A) TMePyP, (B) ZnTMePyP, (C) TC8PyP, (D) ZnTC8PyP) after 3 h of incubation at 37 °C. Middle: Rhodamine 123 fluorescence (green images). Right: Overlay of porphyrin and rhodamine 123 fluorescence images.

Table 2 Overlay percentages of porphyrins and probes

Porphyrin/Probe	Rhodamine 123
TMePyP	26.7
ZnTMePyP	17.5
TC8PyP	42.8
ZnTC8PyP	11.4

Mitochondrial localization is shown by the green spots due to the fluorescence of rhodamine 123 (column II). The colocalization between green and red fluorescence can be visualized due to the presence of the yellow color arising from the overlay of the two fluorescence images (column III). The presence of yellow spots is more evident in the cells treated with metal-free porphyrins than in those treated with zinc porphyrins. The area of colocalization was calculated and is compatible with the higher mitochondrial accumulation for the metal-free porphyrins (Table 2), which is in agreement with the results obtained with isolated mitochondria (Fig. 3). Therefore, changing the carbon chain from one to eight carbon atoms results in increased porphyrin accumulation in mitochondria for free-base porphyrins. On the other hand, zinc insertion leads to decreased accumulation. ZnTC8PyP has the lowest mitochondrial uptake and the highest cell uptake, indicating that this molecule tends to accumulate unspecifically in phospholipid bilayers instead of concentrating in mitochondria. We hypothesize that this behavior is mainly due to zinc–phosphate coordination. The fact that the most efficient photosensitizer is the one that least accumulates in mitochondria suggests that mitochondrial binding is a factor that is less important for the total photodynamic efficiency compared with the efficiency of membrane binding and consequently the efficiency of the destruction of the membranes through photooxidation.[17,18,42] Chelation of free-base porphyrins in the mitochondria may also contribute to these results and we are undertaking further studies to test this hypothesis.

3. Conclusions

By studying photosensitizers that have similar photophysical properties but different molecular structures we were able to investigate the effect of hydrophobicity and zinc insertion on the final photodynamic efficiency of amphiphilic porphyrins. Photodynamic efficiency is directly proportional to membrane binding and is not totally related to mitochondria accumulation, indicating that favorable membrane interaction is a key factor in achieving improved PDT efficiency. The presence of zinc decreases mitochondrial binding and increases membrane interactions, leading to improved PDT efficiency. These conclusions were based solely on *in vitro* tests and therefore they should be confirmed in whole-animal studies and/or in clinical protocols.

4. Methods

4.1 Synthesis

5,10,15,20-Tetrakis(4-pyridyl)porphyrin (TPyP) was purchased from Aldrich and used as received. In order to obtain the 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (TMePyP) the quaternization of TPyP was carried out in *N*,*N*-dimethylformamide using methyl iodide, as described previously by Menieur *et al.*[43] Purification was accomplished by crystallization in acetone. TMePyP was obtained with 65% yield (135 mg). The other amphiphilic compounds 5,10,15,20-tetrakis(*N*-1-tetradecylpyridinium-4-yl)porphyrin tetrabromide (TC14PyP) and 5,10,15,20-tetrakis(*N*-1-octylpyridinium-4-yl)porphyrin tetrabromide (TC8PyP) were obtained by coupling the TPyP with the appropriate bromide, as described by Calvin *et al.*[44] We obtained the pure products by column chromatography using aluminum oxide as the stationary phase and a mixture of dichloromethane and methanol (1 : 9 v/v) as the mobile phase. TC14PyP was obtained in a 49% yield (68 mg) and TC8PyP was obtained in 67% yield (78 mg). All samples were

characterized by electrospray ionization mass spectrometry (TMePyP *m/z* 169.7; TC8PyP *m/z* 267.8, TC14PyP *m/z* 352.9).[44] The zinc complexes 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrinate zinc(II) (ZnTMePyP) and 5,10,15,20-tetrakis(*N*-1-octylpyridinium-4-yl)porphyrinate zinc(II) (ZnTC8PyP) were obtained by magnetic stirring of the free-base porphyrin with zinc(II) acetate in methanol for 2 h. ZnTC8PyP was purified by column adsorption chromatography using aluminum oxide as the stationary phase and a mixture of water, dichloromethane and acetone (1 : 10 : 29 v/v/v) as the mobile phase. TMePyP and ZnTMePyP are commercially available and commonly found in the scientific literature. TC8PyP, ZnTC8PyP and TC14PyP are not so common but they are not new either.[25,44–46] However, no one has published their elemental analysis. We have obtained: TC8PyP·2C_2H_6O, Calc. (%) C, 61.54; H, 7.20; N, 7.55; Found. (%) C, 61,73; H, 7,60; N, 8,01; ZnTC8PyP·2H_2O, Calc.(%) C, 58,02; H, 6,49; N, 7,52 Found (%) C, 58,15; H, 6,69; N, 7,35; TC14PyP, Calc. (%) C, 66,73; H, 8,28; N, 6,49; Found. (%) C, 67,01; H 8,89; N 6,49. Elemental analysis of these compounds is complicated because charged porphyrins often retain significant amounts of solvent when they precipitate.[44–46] Considering this fact, the microanalysis data described above give us confidence that the compounds are pure.

4.2 Spectroscopic and photophysical studies

Ground-state absorption spectra were obtained using an HP 8452A Diode Array Spectrophotometer and the molar absorptivity values in the band maxima were determined by measuring absorption from 350 to 800 nm as a function of porphyrin concentration. Fluorescence spectra were recorded with a Spex Fluorolog 1934D spectrofluorimeter. Fluorescence quantum yields of porphyrins (Φ_f) were determined by measuring the area under the emission spectra (600–800 nm range, excitation 515 nm), using methylene blue in methanol ($\Phi_f = 0.03$) as the reference.[10,47,48] In all cases, the absorbance values of the sample and reference solutions were kept below 0.1 at the excitation wavelength to minimize inner filter effects.

The quantum yield of singlet oxygen production (Φ_Δ) was determined by using a phosphorescence detection method. A Continuum Surelite III Nd:YAG laser was used as the excitation source operating at 532 nm (5 ns, 10 Hz). The radiation emitted at 1270 nm was detected at right angles by a liquid nitrogen cooled photomultiplier from Hamamatsu R5509.[9,18,45–48] Five different concentrations of the porphyrins in methanol were tested. The absorbance of the samples and of the standard (5,10,15,20-tetrakis(sulfonatophenyl)porphyrin, $\Phi_\Delta = 0.57$) were matched at the values of 0.05; 0.10; 0.15; 0.20; and 0.30 in 532 nm (1 cm path length cuvette).[18,49] Since there was not an evident variation in Φ_Δ as a function of concentration, average and standard deviations were calculated using the five different concentrations and kept over ice until the experiments were conducted. The value of Φ_Δ was calculated by measuring and comparing the emissions of samples and standards.

4.3 Binding of porphyrins in vesicles

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) : phosphatidylglycerol (PG) (70 : 30 w/w) vesicles were prepared by mechanical stirring. First, a stock solution of the lipids in chloroform was dried to form a film. Lipids were hydrated with 0.1 mol L^{-1} phosphate buffer pH = 7.2 and stirred. A fixed volume of this vesicle suspension (50 μL) was then added to a porphyrin solution (0.1 mol L^{-1} phosphate buffer pH = 7.2; absorbance approximately 0.5). The mixtures were submitted to mechanical agitation and after 1 h they were centrifuged for 3 min at 13 000 rpm and 25 °C. The absorbance on the Soret band was measured before and after incorporation.[18,50]

4.4 Mitochondrial uptake of photosensitizers

Mitochondria were isolated from the liver of female rats, as described previously.[9,51] Protein concentration in the final mitochondrial suspension was determined by the biuret method. The incubation was carried out using a buffer containing 250 mmol L^{-1} sucrose, 10 mmol L^{-1} HEPES, 1 mmol L^{-1} EGTA, 2 mmol L^{-1} succinate, 1 mmol L^{-1} sodium phosphate, pH 7.4, oligomycin (1 μmol L^{-1}), rotenone (1 μmol L^{-1}) and CCCP (2.5 μmol L^{-1}).

Porphyrin uptake was determined after incubation of the porphyrin solutions with a suspension of the isolated mitochondria (2.0 mg ml^{-1}) for 30 s. The suspensions were centrifuged at 10 000 rpm for 2 min in an Eppendorf centrifuge (Mini-spin plus). The porphyrin concentration was determined by UV-Vis spectra (absorption on the Soret band) of the supernatant and of the initial incubation solution.

4.5 Cells and culture conditions

The human cervical adenocarcinoma cell line (HeLa) was routinely grown in 75 cm^2 plastic tissue culture in Dulbecco's Minimum Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin, and maintained at 37 °C in a humid incubator with 5% CO_2.

4.5.1 Photosensitizer uptake in cells (HeLa). Cells (10^5 cells per well) were seeded in a flask and incubated for 18 h for attachment to the flask. The HeLa cells were then exposed to photosensitizers (10^{-6} mol L^{-1}) in DMEM supplemented with 10% FCS and 1% penicilin/streptomycin for 3 h in the dark. The cellular uptake was obtained by dissolving cells in 50 mmol L^{-1} sodium dodecylsulfate (SDS) and determining the absorbance in the Soret band (Shimadzu UV-2401PC spectrophotometer) of the cell lysate and the incubation solution (added in the same amount of SDS). Each porphyrin was tested in triplicate.

4.5.2 Cytotoxicity studies. Cells were seeded at the initial density of 10^5 cells per well. Eighteen hours after plating, the cells were exposed for 3 h to porphyrin solutions (2.5; 5.0; 7.5; 10.0; 15.0; and 30.0 μmol L^{-1}) in DMEM without phenol red and supplemented with 10% FCS and 1% penicillin/streptomycin. The medium was then removed, cells were washed with PBS, and fresh medium was added. Cell survival was measured 48 h later, using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay.[52]

4.5.3 Phototoxicity studies. The initial steps were carried out in the same way as those of the cytotoxicity studies, exposing cells to photosensitizer solutions for 3 h followed by washing with PBS. The cells were then irradiated using the Laser line INOVA 300 mW emitting at 650 nm (0.175 J cm^{-2}) or the Morgotron Laser 20 mW emitting at 532 nm (0.175 J cm^{-2}). Fresh medium

was added and cell survival was also measured 48 h later using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay.

4.5.4 Subcellular localization. We examined the subcellular localization of a series of new porphyrin photosensitizers by staining the HeLa cells (10^4 cells per well) with the porphyrin at the appropriate concentration (taking into consideration different photosensitizer uptakes) and rhodamine 123 was used as a mitochondrial marker. Cells were exposed to porphyrins (10.0 μmol L^{-1}) for 3 h, and rhodamine 123 (250 nmol L^{-1}) for 1 h, at 37 °C. The cells were then washed with PBS and the fluorescence images were obtained using a confocal microscope (Zeiss LSM510). For the porphyrin fluorescence, a set of filters with excitation at 514 nm and emission from 600 to 800 nm was used. For the rhodamine 123, excitation at 488 nm and emission from 500 to 530 nm were employed. The images were treated using the software Metamorph 6.3r2 (Molecular Devices, 2005) by 2D deconvolution techniques.

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