Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 13 (2005) 39–46
Bioorganic & Medicinal Chemistry

Synthesis and photodynamic activity of zinc(II) phthalocyanine derivatives bearing methoxy and trifluoromethylbenzyloxy substituents in homogeneous and biological media

E. Inés Yslas,[b] Viviana Rivarola[b] and Edgardo N. Durantini[a,*]

[a]*Departamento de Química, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, X580BYA Río Cuarto, Argentina*
[b]*Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, X580BYA Río Cuarto, Argentina*

Received 10 September 2004; revised 1 October 2004; accepted 4 October 2004
Available online 28 October 2004

Abstract—Two zinc(II) phthalocyanines bearing either four methoxy (ZnPc **3**) or trifluoromethylbenzyloxy (ZnPc **4**) substituents have been synthesized by a two-step procedure starting from 4-nitrophthalonitrile. Absorption and fluorescence spectroscopic studies were analyzed in different media. These compounds are essentially non-aggregated in the organic solvent. Fluorescence quantum yields (ϕ_F) of 0.26 for ZnPc **3** and 0.25 for ZnPc **4** were calculated in tetrahydrofuran (THF). The photodynamic activity of these compounds was compared in both THF containing photooxidizable substrates and in vitro on Hep-2 human larynx-carcinoma cell line. The production of singlet molecular oxygen, $O_2(^1\Delta_g)$, was determined using 9,10-dimethylanthracene yielding values of ~0.56 for both sensitizers. Under these conditions, the addition of β-carotene (Car) suppresses the $O_2(^1\Delta_g)$-mediated photooxidation. In biological medium, no dark cytotoxicity was found for cells incubated with 0.1 μM of phthalocyanines **3** and **4** for 24 h. However, under similar conditions 0.5 μM of ZnPc **4** was toxic (70% cell survival). The uptake into Hep-2 cells was evaluated using 0.1 μM of sensitizer, reaching values of ~0.05 nmol/10^6 cells after 3 h of incubation at 37 °C. The cell survival after irradiation of the cultures with visible light was dependent upon both light exposure level and intracellular sensitizer concentration. A higher photocytotoxic effect was found for ZnPc **3** with respect to **4** (32%/70% cell survival after 15 min of irradiation). Also, these studies were performed treating the cells with 0.5 μM of ZnPc **3**. In this case, an increase in the uptake (~0.28 nmol/10^6 cells) was observed, which is accompanied by a higher photocytotoxic activity (20% cell survival). These results show that even though both sensitizer present similar photophysical properties in homogeneous medium, the photodynamic behavior in cellular media can significantly be changed.

1. Introduction

One of more recent and promising applications of phthalocyanine in medicine is in the detection and cure of tumors.[1,2] Photodynamic therapy (PDT) is an early new technique for treating cancer.[3,4] After administration of a photosensitizer, which is selectively retained by tumor cells, the subsequent irradiation with visible light in the presence of oxygen specifically inactivates neoplastic cells.[5,6] Basically two types of reactions can occur after photoactivation of the photosensitizer. One involves the generation of free radicals (type I photochemical reaction) and in the other, the production of singlet molecular oxygen, $O_2(^1\Delta_g)$, (type II) which is the main species responsible for cell inactivation.[1] Both reactions can occur simultaneously and the ratio between two processes depends on the sensitizer, substrate and the nature of the medium.[1]

Phthalocyanines derivatives exhibit a high absorption coefficient in the visible region of the spectrum, mainly in the phototherapeutic window (600–800 nm) and a long lifetime of triplet excited state to produce efficiently $O_2(^1\Delta_g)$.[1,2] Liposomal zinc(II) phthalocyanine (ZnPc) has proved very useful for photokilling of HeLa cells and tumor localization in vivo and PDT of murine tumors.[7,8] Also, the photobiological properties of various phthalocyanine derivatives indicate that they can be very promising photosensitizers for clinical application of PDT.[2,6]

In previous studies, we have investigated the photodynamic activity of porphyrin derivatives substituted by

Keywords: Phthalocyanines; Photosensitizer; Singlet oxygen; Photoinactivation; Photodynamic therapy.
* Corresponding author. Tel.: +54 0358 4676157; fax: +54 0358 4676233; e-mail: edurantini@exa.unrc.edu.ar

0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.10.003

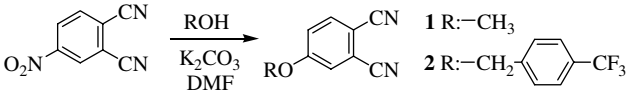
methoxy groups in different biomimetic and biological media.[9–12] The presence of methoxy groups appears to be beneficial from the standpoint of tumor localization.[13,14] Also, a novel *meso*-2,4,6-trimethoxyphenyl porphyrin covalently linked to a trifluoromethylphenyl derivative was evaluated as interesting photosensitizer.[15,16] An attractive photobiological feature is its ability to inactivate cultured tumor cells with high efficiency by apoptotic or necrotic modes depending on the light dose.[16,17] The influence of the trifluoromethyl group in biologically active molecules is often associated with the increased lipophilicity that this substituent imparts.[18] Fluorinated porphyrin derivatives have been synthesized for application in diagnosis and therapy of cancer.[19,20] Likewise, fluorinated zinc phthalocyanines offer some advantages over non-fluorinated derivatives as photosensitizer.[21] Also, studies using zinc hexadecafluorophthalocyanine in mice indicate a favorable tumor/muscle ratio.[22]

Taking into account these considerations, in this paper we report the synthesis and the photodynamic activity of two zinc(II) phthalocyanine derivatives substituted by either four methoxy (ZnPc **3**) or four trifluoromethylbenzyloxy (ZnPc **4**) groups. In particular, the presence of four –CF3 groups was used to obtain a highly lipophilic sensitizer.[18] The photosensitizing properties of these compounds were compared in homogeneous medium bearing photooxidizable substrates and in Hep-2 human larynx-carcinoma cell line. The results contribute to understand the photodynamic process induced by these agents in PDT treatments.

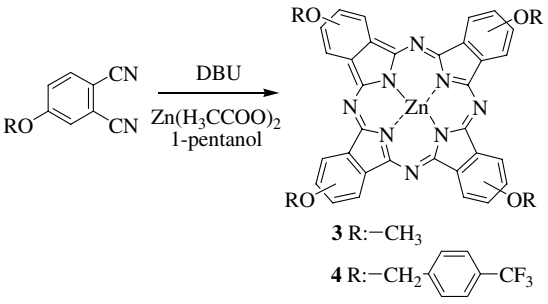
2. Results and discussion

2.1. Synthesis

Zinc(II) phthalocyanines (ZnPcs) **3** and **4** were synthesized by a two-step procedure.[23,24] First, dinitriles **1** and **2** were prepared by a nucleophilic *ipso*-nitro substitution reaction of 4-nitrophthalonitrile with the correspondent alcohol in the presence of K_2CO_3 (Scheme 1). Dinitriles **1** and **2** were isolated by flash chromatography with 68% and 60% yield, respectively. The cyclotetramerization of dinitriles **1** and **2** with zinc(II) acetate in the presence of organic base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was performed in *n*-pentanol. After reflux for 10 h, the reaction results in the formation of the corresponding ZnPcs **3** and **4** as mixtures of constitutional isomers, which were purified by re-crystallization to yield 38% and 41%, respectively Scheme 2.



Scheme 1. Synthesis of nitrile derivatives.



Scheme 2. Synthesis of phthalocyanine derivatives.

2.2. Spectroscopic studies

The absorption spectra of ZnPcs **3** and **4** show the typical Soret and *Q*-bands bands characteristic of zinc(II) phthalocyanine (ZnPc).[25] Figure 1A compares the

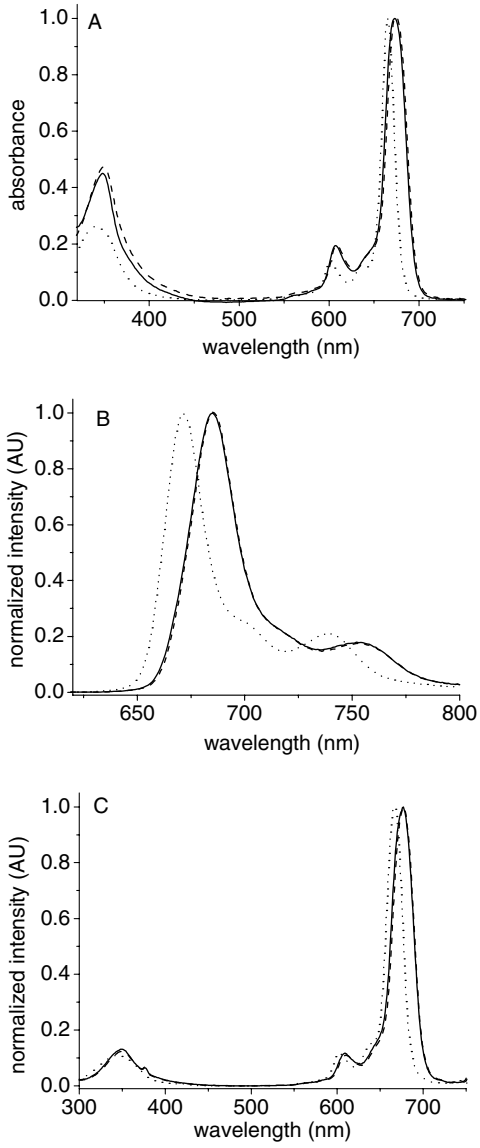


Figure 1. (A) Absorption spectra; (B) fluorescence emission spectra (λ_{exc} = 608 nm) and (C) fluorescence excitation spectra (λ_{em} = 740 nm) of ZnPc **3** (solid line), ZnPc **4** (dashed line) and ZnPc (dotted line) in THF.

Table 1. Absorption and fluorescence emission data for phthalocyanines **3** and **4** in different solvents

Solvent	Absorption λ_{max} (nm)			Emission λ_{max} (nm)[b]	
ZnPc **3**					
Toluene	352	611	678	687	758
THF	347	608	675	685	753
Pyridine	358	614	682	693	766
FCS[a]	339	627	682	690	764
ZnPc **4**					
Toluene	358	612	680	688	759
THF	349	607	673	685	755
Pyridine	357	615	684	693	764
FCS[a]	345	628	683	687	758

[a] 10% vol/vol in PBS.
[b] λ_{exc} = 608 nm.

ZnPcs spectra recorded in THF. Values of $\varepsilon = 2.15 \times 10^5\,M^{-1}s^{-1}$ at 675 and $2.20 \times 10^5\,M^{-1}s^{-1}$ at 673 were calculated for **3** and **4**, respectively. The *Q*-band presents a bathochromic shift by ~10 nm when compared with that of ZnPc. In addition, the wavelength absorption maxima of these ZnPcs were studied in different media (Table 1). A sharp absorption band was obtained indicating that there is no aggregation of these ZnPcs in the organic solvents. A small solvatochromic effect in pure solvents is observed on the location of the absorption band showing a slight red-shift upon solubilization in pyridine.[25] These ZnPcs are not soluble in water as monomers, the absorption spectrum shows a unique broad band at ~630 nm (not shown). Many phthalocyanines have tendency to aggregate in aqueous solutions especially of high ionic strength.[1] The addition of an excess of protein into such solutions usually breaks aggregates to monomers followed by the binding of the monomer.[26] In our cases, when fetal calf serum (FCS) is added to a fixed amount of PcZns (1 nmol) in saline PBS solution produces a narrowing and enhancement in intensity of *Q*-bands (Table 1). Under these conditions, lipophilic phthalocyanines are preferentially bound to the hydrophobic environment of the lipoproteins in the serum.[27]

The steady-state fluorescence emission spectra of these ZnPcs present two maxima, as shown in Figure 1B and Table 1. As can be observed, the λ_{em} values are not very different in these media and no changes in the fluorescence profile were observed, showing that the same species were responsible for the fluorescence emission under these conditions. The fluorescence quantum yields (ϕ_F) of the ZnPcs were calculated by steady state comparative method using ZnPc as a reference ($\phi_F = 0.28$ in THF). Values of $\phi_F = 0.26 \pm 0.01$ and 0.25 ± 0.01 were obtained for **3** and **4**, respectively. These results are consistent with those reported for similar ZnPcs.[1] The fluorescence of these sensitizers are appropriate for detection and quantification of the agent located in biological media.[12]

Also, fluorescence excitation spectra of ZnPcs **3** and **4** were recorded in the different media. In Figure 1C they are compared in THF. In all cases, a close relationship is found between the absorption and excitation spectra, indicating that sensitizers **3** and **4** are essentially nonaggregated in these solutions.

2.3. Photosensitized decomposition of substrates and singlet oxygen production

The aerobic irradiation with monochromatic light of photosensitizer **3** and **4** in THF was performed in the presence of 9,10-dimethylanthracene (DMA). Figure 2A shows typical semilogarithmic plots describing the progress of the reaction for DMA. From these plots the values of the observed rate constant (k_{obs}^{DMA}) were obtained, irradiating the system with light of 675 nm (Table 2). From the slopes of Figure 2A the quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) were calculated comparing the slope for the phthalocyanines with the corresponding slope obtained for the reference, ZnPc.[28] The results show a similar value of $\Phi_\Delta \sim 0.56$ for both phthalocyanines and it is a quite reasonable value for ZnPc derivatives in this solvent.[25,28]

To evaluate the mediation of $O_2(^1\Delta_g)$ in the photooxidation of DMA sensitized by ZnPcs, the reaction was studied in the presence of β-carotene (Car, 12 μM). Under this condition, Car can quench $O_2(^1\Delta_g)$ through energy transfer or chemical reaction.[29–32] From the semilogarithmic plots (Fig. 2B) the values of the observed rate constant in the presence ($k_{obs}^{DMA + Car}$) of Car were calculated. The results are gathered in Table 2.

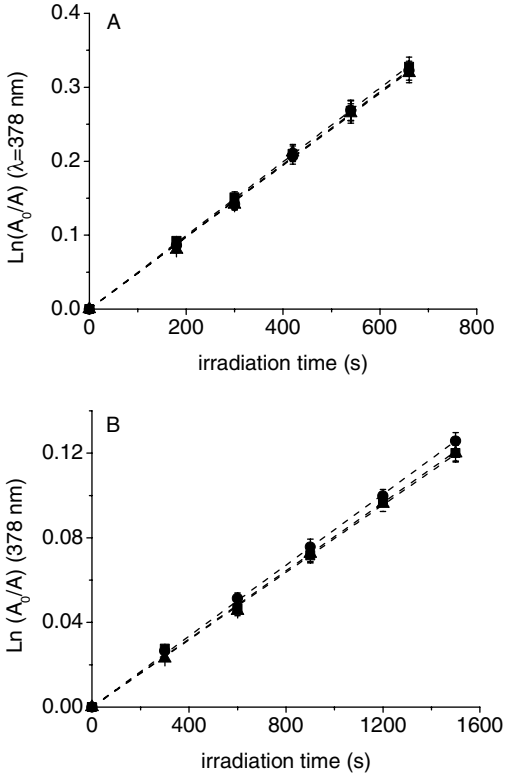


Figure 2. First-order plots for the photooxidation of (A) DMA (30 μM) and (B) DMA (30 μM) in the presence of Car (12 μM) by ZnPc **3** (■), ZnPc **4** (●) and ZnPc (▲), in THF, λ_{irr} = 675 nm. Values represent mean ± standard deviation.

Table 2. Kinetic parameters for the photooxidation reaction of DMA without β-carotene and in the presence of β-carotene (12 μM) in THF

Phthalocyanine	k_{obs}^{DMA} (s^{-1})	$k_{obs}^{DMA+Car}$ (s^{-1})[b]	η_q[a]
ZnPc	$(4.92 \pm 0.05) \times 10^{-4}$	$(8.1 \pm 0.1) \times 10^{-5}$	0.84
3	$(4.95 \pm 0.07) \times 10^{-4}$	$(7.9 \pm 0.1) \times 10^{-5}$	0.84
4	$(4.94 \pm 0.06) \times 10^{-4}$	$(8.3 \pm 0.1) \times 10^{-5}$	0.83

[a] $\eta_q = (1 - k_{obs}^{DMA+Car}/k_{obs}^{DMA})$.

As can be observed the reaction of DMA was quenched with an efficiency ($\eta_q = 1 - k_{obs}^{DMA+Car}/k_{obs}^{DMA}$) of ~0.84 for both phthalocyanines as sensitizers.

On the other hand, the Stern–Volmer equation ($k_{obs}^{DMA}/k_{obs}^{DMA+Car} = 1 + K_{SV}$ [Car], $K_{SV} = \tau^0 k_q$ where k_q represents the quenching constant of Car and τ^0 the excited state lifetime of $O_2(^1\Delta_g)$ in the absence of Car) was used to analyze the $O_2(^1\Delta_g)$ mediation in this precess.[12] Taking into account the value of $K_{SV} = 3.9 \pm 0.2 \times 10^5\ M^{-1}$ previously reported in THF, a ratio of $k_{obs}^{DMA+Car}/k_{obs}^{DMA} = 0.17$ was calculated for [Car] = 12 μM.[33] This result is in agreement with that found for η_q sensitized by ZnPcs **3** and **4**, indicating that $O_2(^1\Delta_g)$ is the main species responsible for DMA photooxidation in THF.

2.4. Studies in vitro on Hep-2 cells

The uptake and photodynamic activity of ZnPcs **3** and **4** were compared in vitro using Hep-2 human larynx-carcinoma cell line. Both sensitizers are not soluble in PBS, therefore they were added to the cell cultures from a liposomal solution of L,D-α-dipalmitoyl phosphatidylethanolamine containing 20% moles of cholesterol.

Cell toxicity induced by the photosensitizers was first analyzed in the dark. The Hep-2 cellular cultures were treated with 0.1 μM of ZnPcs **3** and **4** at 37 °C in the dark. Under these experimental conditions, sensitizers **3** and **4** were not significant toxic after 24 h of incubation. Also, no toxicity was found using 0.5 μM of ZnPc **3** but **4** produced a decrease in cell survival of ~30% after 24 h of dark incubation.

Therefore, the uptake of ZnPcs **3** and **4** into Hep-2 cells was evaluated at different incubation times using 0.1 μM. In each case, the sensitizer intracellular concentration was determined by fluorescence analysis (see Experimental section) and the results are summarized in Figure 3A. As can be seen, both phthalocyanines are rapidly incorporated in the Hep-2 cells in the initial time (<3 h) and the uptake tends to a saturation value at incubation times ⩾3 h. These values were estimated as ~0.05 nmol/10^6 cells for ZnPc **3** and ~0.06 nmol/10^6 cells for ZnPc **4**. Also, ZnPc **3** was studied using 0.5 μM. Under this condition, the profile is similar to that found at lower concentration (0.1 μM), reaching a saturation value of ~0.28 nmol/10^6 cells. As can be noted, the amount of ZnPc **3** incorporated is approximately proportional to the concentration of sensitizer used to incubate the cells at least in the range of concentrations studied. This

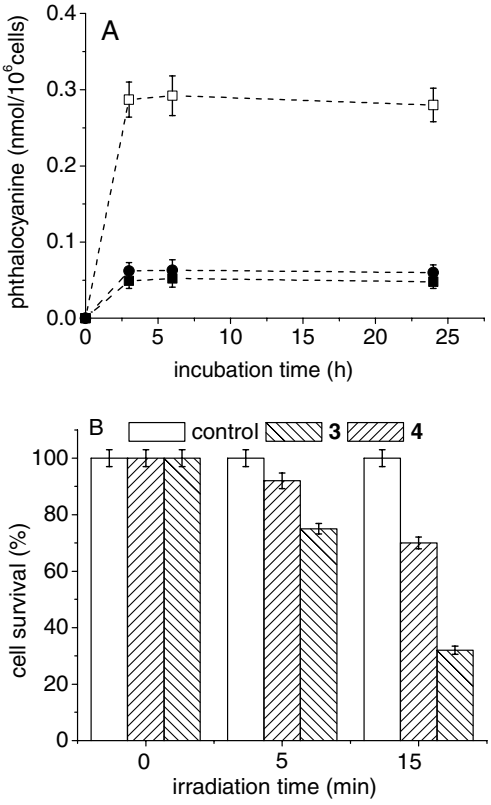


Figure 3. (A) Uptake of ZnPc **3** (■) 0.1 μM, ZnPc **3** (□) 0.5 μM and ZnPc **4** (●) 0.1 μM into Hep-2 cells as a function of incubation time; (B) inactivation of Hep-2 cells treated with phthalocyanine and exposed to different irradiation times with visible light; [phthalocyanine] = 0.1 μM; incubation time 24 h. Values represent mean ± standard deviation of three separate experiments.

value of uptake is lower than those previously found for porphyrin substituted by methoxy groups (~2 nmol/10^6 cells) in Hep-2 cells.[11] However, in the last case the cells were incubated with 1 μM of porphyrin.

After the treatment of Hep-2 cells with 0.1 μM of the ZnPcs **3** or **4** for 24 h, the cultures were irradiated with visible light. The irradiation system used in these studies is described in Experimental section. The corresponding cell survival values for each photosensitizer are shown in Figure 3B. No significant lethality was found for cell cultures not treated with the sensitizer and irradiated; indicating that the cell mortality obtained after irradiation of the cultures treated with the phthalocyanine is due to the photosensitization effect of the agent produced by visible light.

The cell survival after irradiation of the cells with visible light was dependent upon light exposure level. As shown in Figure 3B, the cell survival fraction was 0.32 for cells treated with ZnPc **3** and 0.70 for ZnPc **4** after 15 min of irradiation. Taking into account the light intensity at the treatment site, the light exposure levels required to inactivate 50% of cell population (D_{50}) were estimated. Value of $D_{50} = 28$ and 67 J/cm^2 were found for sensitizer **3** and **4**, respectively. Thus, even though these phthalocyanines present similar uptake by Hep-2 cells, a higher

photodynamic efficiency is obtained using ZnPc **3** than **4** under the same conditions. Also, these experiments were performed using 0.5 μM of ZnPc **3**. Under these conditions, an increase in the cell photoinactivation was obtained. The cell survivals were 62% and 20% after 5 and 15 min of irradiation, respectively. From these results, a value of D_{50} = 19 J/cm^2 was estimated for ZnPc **3**. A similar photokilling behavior was previously observed for HeLa cells treated with different concentration of ZnPc.[7] On the other hand, a small photocytotoxicity was found for HeLa cells treated with 0.1 μM of zinc hexadecafluorophthalocyanine for 2 h and irradiated with 31 J/cm^2.[21] The low efficiency of this hydrophobic compound was apparently influenced by the dissociation degree of the sensitizer and the phthalocyanine localization site in the cells. Also, sensitizer **3** is more effective than 5,10,15,20-tetrakis(4-methoxyphenyl)porphyrin (D_{50} = 45 J/cm^2) and its metal complex with Zn(II) (D_{50} = 30 J/cm^2) in Hep-2 cells.[11]

In conclusion, two zinc(II) phthalocyanine derivatives have been synthesized bearing either four methoxy (ZnPc **3**) or trifluoromethylbenzyloxy (ZnPc **4**) groups by a two-step procedure starting from 4-nitrophthalonitrile. Both phthalocyanines show similar spectroscopic properties and production of $O_2(^1\Delta_g)$ in organic solvent. In biological medium, the uptake of these ZnPcs into Hep-2 cells reaches similar values (~0.05 nmol/10^6 cells) after 3 h of incubation with 0.1 μM of sensitizer at 37 °C. However, the photocytotoxic effect was considerably higher for ZnPc **3** than ZnPc **4**. The low photokilling efficiency of highly lipophilic sensitizer **4** could be due to the formation of aggregate in the cellular microenvironment where the agent is localized. Also, a different intracellular distribution could be affecting the photodynamic activity of ZnPc **4**. This comparative study indicates that the behavior observed in homogeneous solution is not always the case in cellular systems, where the biological microenvironment of the phthalocyanines can induce significant changes in the photophysics of the sensitizers.[34,35] Further in vitro studies concerning the mechanism of cell death produced by the photoactivation of ZnPcs **3** and **4** are presently in progress in our laboratory.

3. Experimental

3.1. General

UV–vis and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker ARX 300 multinuclear spectrometer at 300 MHz. Mass spectra were taken with a ZAB-SEQ Micromass equipment. Uniplate Silica gel GHLF 250 microns thin layer chromatography plates from Analtech (Newark, DE, USA) was used. All the chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Sodium dodecyl sulfate (SDS) from Merck (Darmstadt, Germany) and β-carotene (Car) from Sigma (St. Louis, MO, USA) were used as received. Solvents (GR grade) from Merck were distilled. Ultrapure water was obtained from Labonco equipment model 90901-01.

3.2. Synthesis

4-Methoxyphthalonitrile **1**. To a mixture of 4-nitriphthalonitrile (400 mg, 2.31 mmol) and methanol (1 mL, 38.25 mmol) in DMF (15 mL) was added anhydrous potassium carbonate (1.2 g, 8.68 mmol). The mixture was heated for 3 h at 70 °C. Then, the mixture was partitioned between dichloromethane (50 mL) and water (50 mL). The aqueous layer was separated and extracted with dichloromethane (3 × 50 mL). The solvents of combined organic layers were evaporated under reduced pressure. The residue was subjected to flash column chromatography (silica gel) using dichloromethane as eluent to give 244 mg (68%) of pure dinitrile **1**. R_f (dichloromethane) = 0.51. MS [*m*/*z*] 158 (M^+) (158.0481 calculated for $C_9H_6N_2O_1$). Anal. Calcd C 68.35, H 3.82, N 17.71; found C 68.28, H 3.84, N 17.75.

4-(4′-Trifluoromethylbezyloxy)phthalonitrile **2**. The reaction was performed as described above for compound **1**, using 4-nitrophthalonitrile (433 mg, 2.50 mmol), and 4-(trifluoromethyl)benzyl alcohol (500 mg, 2.84 mmol) to give 450 mg (60%) of pure dinitrile **2**. R_f (dichloromethane) = 0.60. MS [*m*/*z*] 302 (M^+) (302.0668 calculated for $C_{16}H_9F_3N_2O_1$). Anal. Calcd C 63.58, H 3.00, N 9.27; found C 63.62, H 3.05, N 9.23.

Zinc(II) 2,9,16,23-tetrakis(methoxy)phthalocyanine **3**. A mixture of dinitrile **1** (90 mg, 0.57 mmol) and zinc acetate dihydrate (47 mg, 0.21 mmol) in *n*-pentanol (5 mL) was heated at 90 °C. Then, DBU, (86 μL, 0.57 mmol) was added and the mixture was stirred for 10 h at reflux. The volatiles were removed under reduced pressure and the solid obtained was dissolved in a minimum amount of THF. Then methanol and water were added to induce precipitation. The solid was filtered and washed sequentially with water (15 mL), methanol (15 mL), and *n*-hexane (25 mL). The solid was redissolved in THF and the precipitation process repeated one more time. The product was dried under vacuum to yield 38 mg (38%) of pure ZnPc **3**. R_f (ethyl acetate/*n*-hexane 1:1) = 0.20. ^{1}H NMR (300.08 MHz, $CDCl_3$, TMS) δ [ppm] 3.7 (br, 12H, –OCH_3); 7.3–8.0 (m, 12H). MS [*m*/*z*] 696 (M^+) (696.1212 calculated for $C_{36}H_{24}N_8O_4Zn$). Anal. Calcd C 61.95, H 3.47, N 16.05; found C 61.87, H 3.54, N 16.14.

Zinc(II) 2,9,16,23-tetrakis(4-trifluoromethylbenzyloxy)phthalocyanine **4**. According to the above procedure described for ZnPc **3**, using dinitrile **2** (100 mg, 0.33 mmol) and zinc acetate dihydrate (30 mg, 0.14 mmol) to yield 43 mg (41%) of pure ZnPc **4**. R_f (ethyl acetate/*n*-hexane 1:1) = 0.53. ^{1}H NMR (300.08 MHz, $CDCl_3$, TMS) δ [ppm] 5.2 (br, 8 H, –O–CH_2–Ph) 7.3–8.0 (m, 28H). MS [*m*/*z*] 1272 (M^+) (1272.1959 calculated for $C_{64}H_{36}F_{12}N_8O_4Zn$). Anal. Calcd C 60.32, H 2.85, N 8.79; found C 60.25, H 2.78, N 8.83.

3.3. Spectroscopic studies

Absorption spectra were recorded at 25.0 ± 0.5 °C using 1 cm path length cells. The fluorescence quantum yield (ϕ_F) of phthalocyanines were calculated by comparison of the area below the corrected emission spectrum in tetrahydrofuran (THF) with that of zinc phthalocyanine (ZnPc) as a fluorescence standard, exciting at λ_{ex} = 608 nm.[36] A value of ϕ_F = 0.28 for ZnPc in THF was calculated by the comparison with the fluorescence spectrum in pyridine using ϕ_F = 0.30 and taking into account the refractive index of the solvents.[36,37]

3.4. Steady state photolysis

Solutions of 9,10-dimethylanthracene (DMA, 30 μM) and photosensitizer (λ = 675 nm, absorbance 0.3) in THF (2 mL) were irradiated in quartz cuvettes with monochromatic light at λ = 675 nm from a 75 W high-pressure Xe lamp through a high intensity grating monochromator (Photon Technology Instrument). The light intensity was determined as 1.5 mW/cm^2 (Radiometer Laser Mate-Q, Coherent). The kinetics of DMA photooxidation were studied by following the decrease of the absorbance (A) at λ_{max} = 378 nm. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of the semilogarithmic plot of Ln A_0/A. Photooxidation of DMA was used to determine singlet molecular oxygen, $O_2(^1\Delta_g)$, production by the photosensitizers.[15,28] ZnPc was used as the standard (Φ_Δ = 0.56).[38] Measurements of the sample and reference under the same conditions afforded Φ_Δ for ZnPcs **3** and **4** by direct comparison of the slopes in the linear region of the plots. The studies in the presence of β-carotene (Car, 12 μM) were performed using the same condition described above. The pooled standard deviation of the kinetic data, using different prepared samples, was less than 5%.

3.5. Cell photosensitization studies and quantification

The incorporation of the phthalocyanines into the phospholipid bilayer of the D,L-α-dipalmitoyl phosphatidylethanolamine was carried out according to the previously described procedure.[12] Phthalocyanine (1 μMol), phospholipid (33 μMol), and cholesterol (7 μMol) were dissolved in ethanol–THF binary mixture (400 μL, 1:1 vol/vol). The solution was injected into 5 mL of phosphate-buffered saline (PBS) solution at 80 °C. The injection was performed at a speed of 50 μL/min with magnetic stirring and the final volume was reduced to 5 mL by evaporation of organic solvent. The phthalocyanine concentration (~0.2 mM) in the liposomal solution was determined by absorption spectroscopy diluting 10 μL of the sample in 2 mL of THF.

The Hep-2 human larynx-carcinoma cell line (Asociación Banco Argentino de Células, ABAC, Instituto Nacinal de Enfermedades Virales Humanas, Pergamino, Argentina) was maintained and grown as previously described.[11,12] The cells (~1×10^6 cells) were inoculated in 35 mm culture dishes and incubated to obtain nearly confluent cell layers. Then, an appropriate amount of the phthalocyanine incorporated into liposomes was added to a stock of medium bearing 4% fetal calf serum (FCS) to yield the desired final concentration of treatment. An aliquot (2 mL) of this medium was added to the culture dishes. The cells were treated with the sensitizer for 24 h in the dark. Afterwards, the medium containing the photosensitizer was discarded. Cells were washed three times with medium and kept in 2 mL of it containing 7% of FCS. The dishes were exposed for different time intervals to visible light. The light source used was a Novamat 130 AF slide projector equipped with a 150 W lamp. The light was filtered through a 3 cm glass cuvette filled with water to absorb heat. A wavelength range between 350 and 800 nm was selected by optical filters. The light intensity at the treatment site was 45 mW/cm^2 (λ = 670 nm) (Radiometer Laser Mate-Q, Coherent).[39] After each irradiation time, the viability of the cells was estimated 24 h after treatments by 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) spectrophotometric method.[40] Also, the viability was established by microscopy with trypan blue exclusion test using a Neubauer chamber counter.[12] In both cases, similar results were obtained. The same procedure was carried out without irradiation for determining dark toxicity. The uptake was determined adding 1.0 mL of 6% SDS in PBS solution to 1 mL of cellular suspension. The mixture was incubated further for 24 h in the dark and room temperature, sonicated for 15 min and centrifuged at 9000 rpm for 30 min. Then, 1 mL of THF was added to the supernatant. The concentration of sensitizer in the supernatant was measured by spectrofluorimetry (λ_{exc} = 672 nm, λ_{em} = 682 nm). The fluorescence values obtained from each sample were referred to the total number of cells contained in the suspension. The concentration of the phthalocyanine in this sample was estimated by comparison with a calibration curve obtained with standard solutions of the sensitizer in the same medium (2% SDS in water/THF 2:1, [sensitizer] ~ 0.05–0.5 μM). Five culture dishes were used for each incubation time. Every experiment was compared with a culture control without photosensitizer.

Acknowledgements

Authors are grateful to Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina and Fundación Antorchas for financial support. E.N.D. is Scientific Members of CONICET.

References and notes

1. Bonnett, R. *Chemical Aspects of Photodynamic Therapy*; Gordon and Breach Science: Amsterdam, 2000.
2. Chan, W.-S.; Zuk, M.; Ben-Hur, E. In *Photodynamic Tumor Therapy, 2nd and 3rd Genaration Photosensitizers. Phthalocyanines*; Moser, J. G., Ed.; Harwood Academic: Amsterdam, 1998; pp 63–73, Chapter 2.4.
3. Ochsner, M. Photophysical and photobiological processes in the photodynamic therapy of tumours. *J. Photochem. Photobiol. B: Biol.* **1997**, *39*, 1–18.

4. Penning, L. C.; Dubbelman, T. M. Fundamentals of photodynamic therapy: cellular and biochemical aspects. *Anti-Cancer Drugs* **1994**, *5*, 139–146.
5. Dougherty, T. J. Photodynamic therapy. *Photochem. Photobiol.* **1993**, *58*, 895–900.
6. Villanueva, A.; Vidania, R.; Stockert, J. C.; Cañete, M.; Juarrans, A. Photodynamic effects on cultured tumor cells. Cytoskeleton alterations and cell death mechanisms. In *Handbook of Photochemistry and Photobiology*; Nalwa, H. S., Ed.; Photobiology; American Scientific, 2002; Vol. 4, pp 79–117, Chapter 3.
7. Villanueva, A.; Domínguez, V.; Polo, S.; Vendrell, V. D.; Sanz, C.; Cañete, M.; Juarranz, A.; Stockert, J. C. Photokilling mechanisms induced by zinc (II) 9-phthalocyanine on cultured tumor cells. *Oncol. Res.* **1999**, *11*, 447–453.
8. Reddi, E.; Zhou, C.; Biolo, R.; Menegaldo, E.; Jori, G. Liposome or LDL-administered Zn(II)-phthalocyanine as a photodynamic agent for tumour. I. Pharmacokinetic properties and phototherapeutic efficiency. *Br. J. Cancer* **1994**, *69*, 40–45.
9. La Penna, M.; Alvarez, M. G.; Yslas, E. I.; Rivarola, V.; Durantini, E. N. Characterization of photodynamic effects of meso-tetrakis(4-methoxyphenyl) porphyrin: biological consequences in a human carcinoma cell line. *Dyad Pigmants* **2001**, *49*, 75–82.
10. Yslas, I.; Alvarez, M. G.; Marty, C.; Mori, G.; Durantini, E. N.; Rivarola, V. Expression of Fas antigen and apoptosis caused by 5,10,15,20-tetra(4-methoxyphenyl)porphyrin (TMP) on carcinoma cells: implication for photodynamic therapy. *Toxicology* **2000**, *149*, 69–74.
11. Milanesio, M. E.; Alvarez, M. G.; Yslas, E. I.; Borsarelli, C. D.; Silber, J. J.; Rivarola, V.; Durantini, E. N. Photodynamic studies of metallo 5,10,15,20-tetrakis(4-methoxyphenyl) porphyrin: photochemical characterization and biological consequences in a human carcinoma cell line. *Photochem. Photobiol.* **2001**, *74*, 14–21.
12. Milanesio, M. E.; Alvarez, M. G.; Silber, J. J.; Rivarola, V.; Durantini, E. N. Photodynamic activity of monocationic and non-charged methoxyphenyl porphyrin derivatives in homogeneous and biological medium. *Photochem. Photobiol. Sci.* **2003**, *2*, 926–933.
13. Reddi, E.; Segalla, A.; Jori, G.; Kerrigan, P. K.; Liddell, P. A.; Moore, A. L.; Moore, T. A.; Gust, D. Carotenoporphyrins as selective photodiagnostic agents for tumours. *Br. J. Cancer* **1994**, *69*, 40–45.
14. Faustino, M. A. F.; Neves, M. G. P. M. S.; Cavaleiro, J. A. S.; Neumann, M.; Brauer, H.-D.; Jori, G. meso-Tetraphenylporphyrin dimer derivatives as potential photosensitizer in photodynamic therapy. Part 2. *Photochem. Photobiol.* **2000**, *72*, 217–225.
15. Milanesio, M. E.; Moran, M. S.; Yslas, E. I.; Alvarez, M. G.; Rivarola, V.; Durantini, E. N. Synthesis and biological evaluation of methoxyphenyl porphyrin derivatives as potential photodynamic agents. *Bioorg. Med. Chem.* **2001**, *9*, 1943–1949.
16. Villanueva, A.; Durantini, E. N.; Stockert, J. C.; Rello, S.; Vidania, R.; Cañete, M.; Juarranz, A.; Arranz, R.; Rivarola, V. Photokilling of cultured tumour cells by the porphyrin derivative CF3. *Anti-Cancer Drug Design* **2002**, *16*, 279–290.
17. Alvarez, M. G.; Morán, F.; Yslas, E. I.; Rumie Vittar, N. B.; Bertuzzi, M.; Durantini, E. N.; Rivarola, V. Pharmacokinetic and tumour-photosensitizing properties of methoxyphenyl porphyrin derivative. *Biomed. Pharmacother.* **2003**, *57*, 163–168.
18. McClinton, M. A.; McClinton, D. A. Trifluoromethylations and related reactions in organic chemistry. *Tetrahedron* **1992**, *48*, 6555–6666.
19. Ando, A.; Kumadaki, I. Progress on the syntheses of fluorine analogs of natural porphyrins potentially useful for the diagnosis and therapy of certain cancers. *J. Fluor. Chem.* **1999**, *100*, 135–146.
20. Grancho, J. C. P.; Pereira, M. M.; Miguel, M. da. G.; Rocha Gosnsalves, A. M.; Burrows, H. D. Synthesis spectra and photophysics of some free base tetrafluoroalkyl and tetrafluoroaryl porphyrins with potential applications in imaging. *Photochem. Photobiol.* **2002**, *75*, 249–256.
21. Oda, K.; Ogura, S.; Okura, I. Preparation of a water-soluble fluorinated zinc phthalocyanine and its effect for photodynamic therapy. *J. Photochem. Photobiol. B: Biol.* **2000**, *59*, 20–25.
22. Alleman, E.; Brasser, N.; Benrezzak, O.; Rousseau, J.; Kudrevich, S.; Boyle, V.; Leroux, R. W.; Gurny, J.-L.; van Lier, R. PEG-coated poly(lactic acid) nanoparticles for the delivery of hexadecafluore zinc phthalocyanine to EMT-6 mouse mammary tumors. *J. Pharm. Pharmacol.* **1995**, *47*, 382–387.
23. Wöhrle, D.; Iskander, N.; Graschew, G.; Sinn, H.; Friedrich, E. A.; Maier-Borst, W.; Stern, J.; Schlag, P. Synthesis of positively charged phthalocyanines and their activity in the photodynamic therapy of cancer cells. *Photochem. Photobiol.* **1990**, *51*, 351–356.
24. Ng, A. C. H.; Ng, D. K. P. Synthesis and photophysical properties of nonaggregated phthalocyanines bearing dendritic substituents. *Macromolecules* **1999**, *32*, 5292–5298.
25. Ogunsipe, A.; Maree, D.; Nyokong, T. Solvent effect on the photochemical and fluorescence properties of zinc phthalocyanine derivatives. *J. Mol. Struct.* **2003**, *650*, 131–140.
26. Lang, K.; Mosinger, J.; Wagnerová, D. M. Photophysical properties of porphyrinoid sensitizers non-covalently bound to host molecules; models for photodynamic therapy. *Coordination Chem. Rev.* **2004**, *248*, 321–350.
27. Jori, G.; Reddi, E. The role of lipoproteins in the delivery of tumour-targeting photosensitizers. *Int. J. Biochem.* **1993**, *25*, 1369–1375.
28. Redmond, R. W.; Gamlin, J. N. A compilation of singlet yields from biologically relevant molecules. *Photochem. Photobiol.* **1999**, *70*, 391–475.
29. Gust, D.; Moore, T. A.; Moore, A. L.; Jori, G.; Reddi, E. The photochemistry of carotenoids some photosynthetic and photomedical aspects. *Ann. New York Academy Sci.* **1993**, *691*, 32–47.
30. Baltschun, D.; Beutner, S.; Briviba, K.; Martin, H.-D.; Paust, J.; Peters, M.; Röver, S.; Stahl, H.; Steigel, A.; Stenhorst, F. Singlet oxygen quenching abilities of carotenoid. *Liebigs Ann.* **1997**, 1887–1893.
31. Martin, H. D.; Ruck, C.; Schumidt, M.; Sell, S.; Beutner, S.; Mayer, B.; Walsh, R. Chemistry of carotenoid oxidation and free radical reactions. *Pure Appl. Chem.* **1999**, *71*, 2253–2262.
32. Montenegro, M. A.; Nazareno, M. A.; Durantini, E. N.; Borsarelli, C. D. Singlet molecular oxygen quenching ability of carotenoids in reverse-micelle membrane mimetic system. *Photochem. Photobiol.* **2002**, *75*, 353–361.
33. Scalise, I.; Durantini, E. N. Photodynamic effect of metallo 5-(4-carboxyphenyl)-10,15,20-tris(4-methylphenyl) porphyrins in biomimetic media. *J. Photochem. Photobiol. A: Chem.* **2004**, *162*, 105–113.
34. Aveline, B. M.; Redmond, R. W. Exclusive free radical mechanisms of cellular photosensitization. *Photochem. Photobiol.* **1998**, *68*, 266–275.
35. Aveline, B. M.; Redmond, R. W. Can cellular phototoxicity be accurately predicted on the basis of sensitizer photophysics? *Photochem. Photobiol.* **1999**, *69*, 306–316.

36. Demas, J. N.; Crosby, G. A. The measurement of photoluminescence quantum yields. *J. Phys. Chem.* **1971**, *75*, 991–1024.
37. Seybold, P. G.; Gouterman, M. Porphyrins XIII: fluorescence spectra and quantum yields. *J. Mol. Spectrosc.* **1969**, *31*, 1–13.
38. Michelsen, U.; Kliesch, H.; Schnurpfeil, G.; Sobbi, A. K.; Wöhrle, D. Unsymmetrically substituted benzonaphthoporphyrazines: a new class of cationic photosensitizers for the photodynamic therapy of cancer. *Photochem. Photobiol.* **1996**, *64*, 694–701.
39. Alvarez, M. G.; La Penna, M.; Yslas, E. I.; Rivarola, V.; Durantini, E. N. Photodamaging effects of porphyrin in a human carcinoma cell line. *Chem. Educator* **2000**, *5*, 24.
40. Merlin, J.; Azzi, S.; Lignon, D.; Ramacci, C.; Zeghari, N.; Guillemin, F. MTT assays allow quick and reliable measurement of the response of human tumor cells to photodynamic therapy. *Eur. J. Cancer* **1992**, *28*, 1452–1458.