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Photodynamic Studies of Metallo 5,10,15,20-Tetrakis(4-methoxyphenyl) porphyrin: Photochemical Characterization and Biological Consequences in a Human Carcinoma Cell Line¶

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ABSTRACT

The photodynamic activities of the free-base 5,10,15,20-tetrakis(4-methoxyphenyl)porphyrin (TMP) and their metal complexes with zinc(II) (ZnTMP), copper(II) (CuTMP) and cadmium(II) (CdTMP) have been compared in two systems: reverse micelle of *n*-heptane/sodium bis(2-ethylhexyl)sulfosuccinate/water bearing photooxidizable substrates and Hep-2 human larynx carcinoma cell line. The quantum yields of singlet molecular oxygen, $O_2(^1\Delta_g)$, production (Φ_Δ) of TMP, ZnTMP and CdTMP in tetrahydrofuran, were determined yielding values of 0.65, 0.73 and 0.73, respectively, while $O_2(^1\Delta_g)$ formation was not detected for CuTMP. In the reverse micellar system, the amino acid L-tryptophan (Trp) was used as biological substrate to analyze the $O_2(^1\Delta_g)$-mediated photooxidation. The observed rate constants for Trp photooxidation (k_{obs}^{Trp}) were proportional to the sensitizer quantum yield of $O_2(^1\Delta_g)$. A value of $\sim 2 \times 10^7$ s^{-1} M^{-1} was found for the second-order rate constant of Trp (k_r^{Try}) in this system. The response of Hep-2 cells to cytotoxicity photoinduced by these agents in a biological medium was studied. The Hep-2 cultures were treated with 1 μ*M* of porphyrin for 24 h at 37°C and the cells exposed to visible light. The cell survival at different light exposure levels was dependent on Φ_Δ. Under these conditions, the cytotoxic effect increases in the order: CuTMP ≪ TMP < ZnTMP ~ CdTMP, correlating with the production of $O_2(^1\Delta_g)$. A similar behavior was observed in both the chemical and biological media indicating that the $O_2(^1\Delta_g)$ mediation appears to be mainly responsible for the cell inactivation.

INTRODUCTION

The tetrapyrrolic macrocycles play highly diverse roles in biological systems (1). One of the more recent and promising applications of porphyrins in medicine is in the detection and cure of tumors (2,3). Photodynamic therapy (PDT)† is an early new technique for treating cancer. 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 (4,5). Recently, several porphyrin derivatives with potential use in the treatment of tumors have been synthesized (6–10). Basically two types of reactions can occur after photoactivation of the photosensitizer. One involves the generation of free radicals (type-I photochemical reaction) and the other the production of singlet molecular oxygen, $O_2(^1\Delta_g)$ (type II) as the main species responsible for cell inactivation (5). Evidence favors the role of the type-II process in cells, although the photodynamic process of the sensitizers on neoplastic tissues is still not well understood (11). The mechanism of PDT effects may involve a direct tumor cell injury and also an indirect cell killing *via* microcirculatory changes resulting in reduced blood flow in the tumor (5,12,13).

Photosensitizers are deemed to have specific chemical and biological properties (2). Two of the photochemical requisites are a high absorption coefficient in the visible region of the spectrum, mainly in the phototherapeutic window (600–1000 nm) and a long lifetime of the triplet excited state to efficiently produce $O_2(^1\Delta_g)$ (3). Studies on the triplet state carried out with a series of 5,10,15,20-tetrakis (methoxyphenyl) porphyrins have shown that these synthetic porphyrins are effective photosensitizers which can be used as model compounds to investigate the theoretical and instrumental aspects of PDT (14,15). The spectroscopic properties and quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) of porphyrins can

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†*Abbreviations:* AOT, sodium bis(2-ethylhexyl)sulfosuccinate; CdTMP, cadmium 5,10,15,20-tetra(4-methoxyphenyl)porphyrin; CuTMP, copper 5,10,15,20-tetra(4-methoxyphenyl)porphyrin; DMA, 9,10-dimethylanthracene; ϵ, extinction coefficient; FCS, fetal calf serum; ^{1}HNMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; PDT, photodynamic therapy; SDS, sodium dodecyl sulfate; TB, tryptan blue; TLC, thin-layer chromatography; TMP, 5,10,15,20-tetra(4-methoxyphenyl)porphyrin; TPP, tetraphenylporphyrin; Trp, L-tryptophan; ZnTMP, zinc 5,10,15,20-tetra(4-methoxyphenyl)porphyrin.

be significantly changed by forming complexes with metals (16–19).

The aim of this study was to compare the photodynamic effect of metal-free 5,10,15,20-tetrakis(4-methoxyphenyl) porphyrin (TMP), zinc (ZnTMP), copper (CuTMP) and cadmium (CdTMP), first in a simple biological model formed by reverse micelles bearing a photooxidizable amino acid and then in a more complex system of a human carcinoma cell line. Tetrapyrrolic macrocycle–bearing methoxy substituents in the *meso*-phenyl groups appear to present adequate photophysical properties to be used as a sensitizer model (14) and the increase in polarity with respect to tetraphenylporphyrin (TPP), keeping its lipophilic character, could favor the interaction with biological media (20). In most cases, the formation of metalloporphyrin complexes cause changes in the free-base photophysical properties (21). Usually the presence of Zn(II)- and Cd(II)-forming complexes with porphyrins produces an increase in the $O_2(^1\Delta_g)$ generation, while the complex with Cu(II) presents a lack of photodynamic activity. Thus, the Cu(II) complex can be used to analyze the influence of type-I photoreaction in the biological system studied (16,17,21).

The absorption and emission spectroscopic properties of the porphyrins were first studied in a set of pure solvents and in various heterogeneous media. The $O_2(^1\Delta_g)$ quantum yields (Φ_Δ) of the porphyrins were evaluated in a solution of tetrahydrofuran.

Reverse micelles of *n*-heptane/sodium bis(2-ethylhexyl)sulfosuccinate (AOT)/water bearing photooxidizable biological substrate L-tryptophan (Trp) were used to study the mediation of $O_2(^1\Delta_g)$ in the photooxidation mechanism. This amino acid is subject to $O_2(^1\Delta_g)$ damage and it has been used to analyze the sensitizer activity toward biological substrates in different systems (22–24). On the other hand, microheterogeneous systems such as micelles and reverse micelles are frequently used as simplified models for biological membranes and enzyme pockets where various photodynamic effects can take place (25–27).

The response of Hep-2 human larynx carcinoma cell line to cytotoxicity photoinduced by the porphyrins was evaluated in the biological system. The survival of the irradiated cells, previously treated with the porphyrin, was dependent on both light exposure levels and sensitizer photochemical properties. The effect in both the media shows a correlation between the $O_2(^1\Delta_g)$ production and the photodynamic activity upon photooxidizable substrates and Hep-2 cells. Therefore, the intermediacy of $O_2(^1\Delta_g)$ appears to be mainly responsible for the photocytotoxic activities of these porphyrins. The results contribute to the understanding of the photodynamic process induced by these agents and the sensitivity of Hep-2 cells to this photodamage.

MATERIALS AND METHODS

General. UV–visible and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. Proton nuclear magnetic resonance (^{1}HNMR) spectra was recorded on a Varian Gemini spectrometer at 300 MHz. Mass spectra were taken with a Varian Matt 312 operating in EI mode at 70 eV. The high-performance liquid chromatography (HPLC) chromatograms were performed in a Varian 5000 liquid chromatograph equipped with a Varian 2550 UV–visible variable-wavelength detector and Varian MicroPak SI-5 column (3% 2-propanol/*n*-hexane, flow 0.5). Uniplate Silica gel GHLF 250 μm thin-layer chromatography (TLC) plates from Analtech (Newark, DE) and silica gel 200–400 mesh for column chromatography from Aldrich (Milwaukee, WI) were used.

All the chemicals from Aldrich were used without further purification. Sodium dodecyl sulfate (SDS) from Merck (Deutschland, Germany) was used as received. D,L-α-Dipalmitoyl phosphatidylethanolamine from Sigma (St. Louis, MO) was used in liposome preparation. Solvents from Merck (GR grade) were distilled and stored over 4 Å molecule sieves. Tetrahydrofuran was distilled from lithium aluminium hydride under an argon atmosphere. AOT from Sigma was dried under vacuum and used without further purification. Ultrapure water was obtained from Labonco equipment model 90901-01.

Porphyrins. TMP was synthesized by the condensation of 4-methoxybenzaldehyde and pyrrole, using the Lindsey method (28). A solution of 4-methoxybenzaldehyde (0.152 mL, 1.5 mmol) and pyrrole (0.10 mL, 1.5 mmol) in 100 mL of dichloromethane was treated with trifluoroacetic acid (35 μL, 0.45 mmol) for 60 min at room temperature in argon atmosphere. Then 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (0.34 g, 1.5 mmol) was added and the mixture was stirred for an additional 60 min, open to the atmosphere. The solvent was removed under reduced pressure and flash column chromatography (silica gel, dichloromethane) afforded 98 mg (35%) of pure TMP. TLC (silica gel, dichloromethane) R_f 0.46. HPLC t_R 7.4 min. ^{1}HNMR ($CDCl_3$, tetramethylsilane) δ −2.76 (brs, 2H, pyrrole N–H), 4.08 (s, 12H, Ar-OCH_3), 7.26 (d, 8H, J = 8.5 Hz, 5,10,15,20-Ar 3,5-H), 8.10 (d, 8H, J = 8.5 Hz, 10,15,20-Ar 2,6-H), 8.8–8.90 (s, 8H, pyrrole). MS [m/z] 734.3 (M^+) (734.2895 calculated for $C_{48}H_{38}N_4O_4$). Absorption spectrum λ_{max} (dichloromethane, nm) (extinction coefficient [ε], $dm^3\ mol^{-1}\ cm^{-1}$) 422 (463 200), 518 (14 130), 556 (9750), 593 (4340), 650 (4820).

For the preparation of ZnTMP a solution of TMP (20 mg, 0.027 mmol) in 7 mL of dichloromethane was treated with 3 mL of a saturated solution of zinc(II) acetate in methanol. The mixture was stirred for 30 min at room temperature. Solvents were evaporated under reduced pressure and flash chromatography (silica gel, dichloromethane) afforded 20 mg (94%) of pure ZnTMP. TLC (silica gel, dichloromethane) R_f 0.41. HPLC t_R 7.9 min. MS [m/z] 797.7 (M^+) (797.666 calculated for $C_{48}H_{36}N_4O_4Zn$). Absorption spectrum λ_{max} (dichloromethane, nm) (ε, $dm^3\ mol^{-1}\ cm^{-1}$) 425 (406 800), 558 (13 510), 598 (6940).

CuTMP was prepared by using a solution of TMP (20 mg, 0.027 mmol), copper(II) acetate monohydrate (156 mg, 0.78 mmol) and glacial acetic acid (5 mL) in 3 mL of chloroform which was refluxed for 2 h in argon atmosphere. The solvents were removed under vacuum. Flash column chromatography (silica gel, dichloromethane/methanol 0.7%) afforded 16 mg (75%) of pure CuTMP. TLC (silica gel, dichloromethane) R_f 0.17; HPLC t_R 9.2 min. MS [m/z] 795.8 (M^+) (795.8202 calculated for $C_{48}H_{36}N_4O_4Cu$). Absorption spectrum λ_{max} (dichloromethane, nm) (ε, $dm^3\ mol^{-1}\ cm^{-1}$) 418 (325 000), 541 (17 540), 578 (3480).

For the preparation of CdTMP TMP (20 mg, 0.027 mmol) in pyridine (8 mL) and saturated potassium hydroxide in methanol (4 mL) were treated with cadmium acetate dihydrate (18 mg, 0.067 mmol). After a 2 h reflux the solution was cooled and treated with water (30 mL). The organic phase was extracted with three portions of dichloromethane (15 mL each) and the solvents removed under vacuum to give 14 mg (61%) of pure CdTMP. MS [m/z] 844.7 (M^+) (844.6857 calculated for $C_{48}H_{36}N_4O_4Cd$). Absorption spectrum λ_{max} (dichloromethane, nm) (ε, $dm^3\ mol^{-1}\ cm^{-1}$) 436 (367 700), 572 (15 020), 616 (15 870).

Irradiation. The light source used was a Kodak slide projector equipped with a 150 W lamp. The light was filtered through a glass filter and a 3 cm water layer to absorb heat. In biological studies, a wavelength range between 350 and 800 nm was selected by optical filters (29), while a range of 455–800 nm (GG455 cut-off filter) was used in the micellar system. The light intensity at the treatment site was 18.0 and 15.5 mW cm^{-2} (Radiometer Laser Mate-Q, Coherent, Hilton, Australia).

Spectroscopic studies. Absorption spectra were recorded at 25.0 ± 0.5°C using 1 cm path length cells. Wavelength maxima (λ_{max}) were measured by taking the midpoint between the two positions of the spectrum where the absorbance of the band is equal to 0.9 A_{max}

(30). The fluorescence quantum yield (ϕ_F) of porphyrins was calculated by a comparison of the area below the corrected emission spectrum in tetrahydrofuran with that of TPP as a fluorescence standard, exciting at λ_{ex} = 550 nm (31). A value of ϕ_F = 0.10 for TPP in tetrahydrofuran was calculated by comparison with the fluorescence spectrum in toluene using ϕ_F = 0.11 (32) and taking into account the refractive index of the solvents (31). Singlet molecular oxygen $O_2(^1\Delta_g)$ sensitization was measured by using time-resolved phosphorescence detection method. A Q-switched Nd:YAG laser (Spectron SL400, Warwickshire, England) was used as the excitation source operating at 532 nm (20 ns halfwidth) in order to excite the porphyrins. The emitted radiation (mainly 1270 nm) was detected at right angles with an amplified Judson J16/8Sp germanium detector after passing it through appropriate filters. The output of the detector was coupled to a digital oscilloscope (Hewlett–Packard HP-54504A). About 20 shots are usually needed for averaging decay times in order to get a good signal to noise ratio. No change, indicating significant photodegradation of the sensitizer, was observed in the porphyrin absorption spectrum after these experiments. The averaged signals were analyzed as single exponential decays by using the calculation software (Microcal Origin 4.1, Northampton, MA). TPP in tetrahydrofuran was used as the standard (Φ_Δ = 0.62) (33). The absorbances of the sample and reference were matched at the irradiation wavelength. Measurements of these solutions under the same conditions afforded Φ_Δ for the porphyrins studied. This was obtained by a direct comparison of the slopes in the linear region of the plots of phosphorescence amplitude extrapolate to zero *vs* the laser total energy (34).

Studies in reverse micelles. A stock solution of AOT 0.1 *M* was prepared by weighing and dilution in *n*-heptane. The addition of water to the corresponding solution was performed using a calibrated microsyringe. The amount of water present in the system was expressed as the molar ratio between water and the AOT present in the reverse micelle (W = $[H_2O]/[AOT]$). In all experiments, W = 10 was used. The appropriate amount of stock solution (2 mL) was transferred into a quartz cuvette of 1 cm optical path and appropriate amounts of TMP, 9,10-dimethylanthracene (DMA) and Trp in stock solution of AOT (0.1 *M*, W = 10) were added. The mixtures were sonicated to obtain a perfectly clear micellar system. The photooxidation of Trp was evaluated using DMA as the actinometer under the same experimental conditions. The kinetics of DMA and Trp photooxidation in *n*-heptane/AOT/water reverse micelles were studied by following the decrease of the absorbance (A) at λ_{max} = 378 nm and the fluorescence intensity (F) at λ = 350 nm. The Trp fluorescence was excited by 290 nm light (24). A control experiment showed that under these experimental conditions the fluorescence intensity is linearly correlated with Trp concentration. The observed rate constants (k_{obs}) were obtained by a linear least squares fit of the semilogarithmic plot of ln F_0/F or ln A_0/A *vs* time. All the experiments were performed at 25.0 ± 0.5°C. The pooled standard deviation of the kinetic data, using different prepared samples, was less than 5%.

Liposome preparation. The incorporation of the porphyrins into the phospholipid bilayer of the D,L-α-dipalmitoyl phosphatidylethanolamine was achieved by a modification of the ethanol injection procedure of Kremer *et al.* (35). Typically, 2 mL of a solution bearing 9.60 m*M* of phospholipid, 1.91 m*M* of cholesterol and 0.27 m*M* of porphyrin in ethanol–tetrahydrofuran binary mixture (1:1, vol/vol) was injected into 10 mL of phosphate-buffered saline (PBS) at 80°C. The injection was performed at a speed of 50 μL min^{-1} with magnetic stirring (36). The final volume was reduced to 10 mL by evaporation of organic solvents.

Cell culture. The Hep-2 human larynx carcinoma cell line (Asociación Banco Argentino de Células, ABAC, Instituto Nacinal de Enfermedades Virales Humanas, Pergamino, Argentina) was maintained frozen in liquid nitrogen. The cells were grown as a monolayer employing Dulbecco modified Eagle medium containing 10% fetal calf serum (FCS) and 50 μg gentamycin as antibiotic. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere and the medium was changed daily. The cell line was routinely checked for the absence of mycoplasma contamination.

Cell photosensitization studies and quantification. An appropriate number of cells (~1 × 10^6 cells) was inoculated in 25 cm^2 culture flasks and incubated to obtain nearly confluent cell layers. Then 100 μL of porphyrin, incorporated into liposomes, was added to the culture flask bearing 5 mL of the medium. The cells were treated with 1 μ*M* of porphyrin for 24 h in dark condition, the medium containing the photosensitizer was later discarded. Cells were washed three times with the medium and kept in 5 mL of the same. The dishes were exposed to visible light for different time intervals. After each irradiation time the viability of the cells was estimated by microscopy with trypan blue (TB) exclusion test using a Neubauer chamber counter (29). The same procedure without irradiation was carried out to determine dark toxicity. The uptake of porphyrin by Hep-2 cells was determined by treating the cells with 2.0 mL of tetrahydrofuran. The mixture was sonicated for 15 min and centrifuged at 9000 rpm for 30 min. The concentration of the porphyrin in the supernatant was measured by HPLC using the condition described above. The pick area values obtained from each sample refer to the total number of cells contained in the suspension. The concentration of the porphyrin in this sample was estimated by comparison with a calibration curve obtained with standard solutions of the porphyrin in tetrahydrofuran ([porphyrin] ~ 0.1–5 μ*M*). For CdTMP, which decomposes on silica gel column, the uptake was determined by adding 1.0 mL of 4% SDS to 1 mL of the cellular suspension. The mixture was incubated for a further 15 min (in the dark and at room temperature) and centrifuged at 9000 rpm for 30 min. The concentration of CdTMP in the supernatant was measured by spectrofluorimetry in a solution of 2% SDS in PBS. The fluorescence values obtained from each sample referred to the total number of cells contained in the suspension. The concentration of the porphyrin in this sample was estimated by comparison with a calibration curve obtained with standard solutions of CdTMP in 2% SDS ([CdTMP] ~ 0.1–5 μ*M*). Four culture flasks were used for each incubation time. Every experiment was compared with a culture control without porphyrin.

Table 1. The Soret band absorbance peak of porphyrins in different media

Medium	Soret band λ_{max} (nm)			
	TMP	ZnTMP	CuTMP	CdTMP
n-Heptane	417.8	419.7	415.2	432.0
Tetrahydrofuran	419.9	425.4	417.7	434.5
Dichloromethane	421.2	422.3	418.0	437.8
N,*N*-Dimethylformamide	421.9	428.0	418.9	439.7
AOT reverse micelles*	417.7	420.7	415.3	432.4
FCS suspension†	407.0	408.8	408.5	408.2

**n*-heptane/AOT (0.1 *M*)/water (*W* = 10).
†10% vol/vol in PBS.

RESULTS AND DISCUSSION

Spectroscopic studies

The absorption spectrum of TMP, ZnTMP, CuTMP and CdTMP in dichloromethane shows the typical Soret and *Q*-bands, characteristic of a free base and the corresponding matalloporphyrins (see ''Materials and Methods'') (18). In addition the wavelength of the Soret band absorption maximum of these porphyrins was studied in different media. The results are presented in Table 1. A sharp absorption band was obtained in every case indicating that there is no aggregation of the porphyrin in the environment in which the photosensitizer is localized (19,37,38). The solvatochromic effect on the location of the Soret absorption band in pure solvents shows a slight blueshift upon solubilization in a low-polarity medium.

In AOT reverse micelles an insignificant shift with respect to *n*-heptane was observed, indicating no strong association

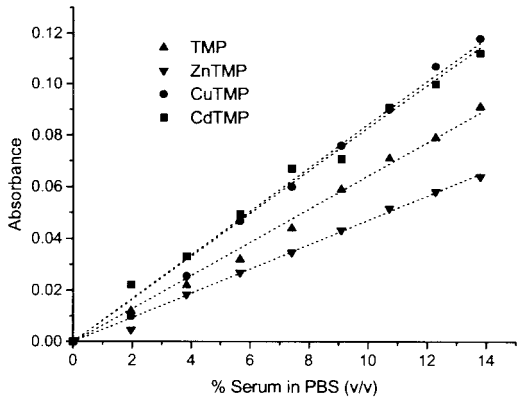

Figure 1. Effect on the Soret band maximum absorption of TMP (▲), ZnTMP (▼), CuTMP (●) and CdTMP (■) produced by the addition of FCS in PBS.

with the polar micellar interface (27,30). On the other hand, these porphyrins are not soluble in saline solution. However, when a fixed amount of precipitated porphyrin (1 nmol) in PBS was treated with FCS, which is used in the biological medium to incubate the cell line (see ''Materials and Methods''), a Soret band appears at ~408 nm. An increase in the amount of FCS added produces a narrowing and enhancement in the intensity of the Soret band. Linear plots of the Soret band absorption with the amount of FCS in PBS were obtained under these conditions for all the porphyrins studied (Fig. 1). In a similar experiment using serum albumin no significant disaggregating was observed by absorption or fluorescence. These results indicate that FCS produces a monomerization of the porphyrin which is preferentially bound to the hydrophobic environment of the lipoproteins in the serum (39). The selective transport by lipoproteins can contribute to enhance the specificity of tumor targeting by the photosensitizer, since mainly the low-density lipoprotein (LDL) develops a very active interaction with neoplastic cells (36,40).

The steady-state fluorescence emission spectra of these porphyrins were analyzed in different media. No detectable fluorescence bands were observed for CuTMP, while TMP, ZnTMP and CdTMP present two maxima, as shown in Table 2. The same values of λ_{em} were obtained on exciting the sample at the wavelength of maximum absorption of the Soret and the *Q*-bands. The two bands are characteristic of similar porphyrins and they have been assigned to *Q*(0–0) and *Q*(0–1) transitions (16). A small Stokes shift is expected for TPP derivatives indicating that the spectroscopic energy is nearly identical to the relaxed energy of the singlet state (16). 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 (monomer) was responsible for the fluorescence emission under these conditions. The fluorescence quantum yield (ϕ_F) of the porphyrins were calculated by the steady-state comparative method using TPP as a reference (31,32). The values of ϕ_F = 0.14 ± 0.01, 0.049 ± 0.004 and 0.018 ± 0.003 were obtained in tetrahydrofuran for TMP, ZnTMP and CdTMP, respectively. These results are consistent with that obtained for similar metalloporphyrins (16).

Table 2. Fluorescence emission maxima of porphyrins in different media

Solvent	λ_{em} (nm)					
	TMP		ZnTMP		CdTMP	
n-Heptane	657	721	593	643	621	665
Dichloromethane	657	722	605	655	622	667
Tetrahydrofuran	658	722	608	659	624	668
AOT*	657	721	593	643	621	665
FCS†	655	720	616	662	620	664

**n*-heptane/AOT (0.1 *M*)/water (*W* = 10).
†10% vol/vol in PBS.

Singlet oxygen production and photooxidation in AOT reverse micelles

In the presence of air, the emission signal of the $O_2(^1\Delta_g)$ formed after laser excitation of the porphyrin solution in tetrahydrofuran showed a first-order exponential decay. Typical results for ZnTMP are shown in Fig. 2. The initial $O_2(^1\Delta_g)$ emission intensities (I_0) were calculated from extrapolation to zero time (determined by the laser shot). These initial signal intensities were plotted *vs* laser total energy (between 20 and 1 mJ; Fig. 2, inset). The quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) was calculated from the slope of the plots for the porphyrins compared with the corresponding slope obtained for the reference (TPP). The values of Φ_Δ in tetrahydrofuran are shown in Table 3. The results for Φ_Δ follow the order CdTMP ~ ZnTMP > TMP, while singlet molecular oxygen ($^1\Delta_g$) was not detected when CuTMP was used as the photosensitizer. The values of Φ_Δ are comparable to those reported before for similar porphyrin derivatives (14–16,17).

The aerobic irradiation of *n*-heptane/AOT (0.1 *M*)/water (W = 10) reverse micelles with visible light containing the photosensitizer was carried out in the presence of Trp. A time-dependent decrease in the amino acid concentration was observed following a decrease in its fluorescence emission at 350 nm. The photoprocess follows first-order kinetics with respect to Trp concentration as shown below using different [Trp] (Fig. 3A). The photoexcitation of the porphyrins in the AOT micellar system was also performed in the pres-

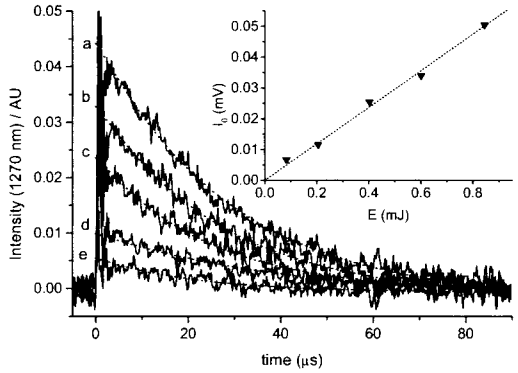

Figure 2. Decay traces of $O_2(^1\Delta_g)$ emission sensitized by ZnTMP in tetrahydrofuran at different laser energies: (A) 0.84; (B) 0.60; (C) 0.40; (D) 0.20; and (E) 0.08 mJ. Inset shows a linear plot between the initial $O_2(^1\Delta_g)$ emission intensities (I_0) *vs* laser total energies (E).

Table 3. Kinetic parameters for the photooxidation reaction of DMA and Trp in *n*-heptane/AOT (0.1 *M*)/water ($W = 10$) reverse micelles

Porphyrin	Φ_Δ*	k_{obs}^{DMA} (s^{-1})†	k_{obs}^{Trp} (s^{-1})†	k_r^{Trp} ($s^{-1}\ M^{-1}$)‡
TMP	0.65	$(3.2 \pm 0.2) \times 10^{-4}$	$(8.5 \pm 0.3) \times 10^{-5}$	$(1.9 \pm 0.1) \times 10^{7}$
ZnTMP	0.73	$(3.8 \pm 0.3) \times 10^{-4}$	$(10.1 \pm 0.4) \times 10^{-5}$	$(1.9 \pm 0.1) \times 10^{7}$
CuTMP	—	—	$(0.6 \pm 0.1) \times 10^{-5}$§	—
CdTMP	0.73	$(3.9 \pm 0.2) \times 10^{-4}$	$(9.8 \pm 0.4) \times 10^{-5}$	$(1.8 \pm 0.1) \times 10^{7}$

*Φ_Δ in tetrahydrofuran.
†Spectral irradiated area 0.20 kK (kK = kilo-Kaiser = 10^3 cm^{-1}; wavelength range 455–800 nm).
‡Calculated considering the mechanism described by Rodgers and Lee (25) and Borsarelli et al. (27) (Scheme 1).
§[Trp] = 15 μM.

ence of DMA. It is known that this substrate quenches $O_2(^1\Delta_g)$ exclusively by chemical reaction (27). Similar behavior to Trp was observed for DMA by following a decrease in its absorbance (Fig. 3B). From the plots in Fig. 3, the values of the observed rate constant (k_{obs}) were calculated for both substrates. The results are shown in Table 3. As can be observed, the increase in k_{obs} is proportional to the sensitizer quantum yield of $O_2(^1\Delta_g)$. Moreover, for TMP, ZnTMP and CdTMP, the observed rate constant for Trp (k_{obs}^{Trp}) does not change when the Trp concentration is varied within the 5–15 μM interval, while for CuTMP the reaction was only detected using highest Trp concentration. The independence of k_{obs}^{Trp} photooxidation over a wide range of Trp concentrations shows that TMP, ZnTMP and CdTMP appear to perform their photosensitizing actions *via* the intermediacy of $O_2(^1\Delta_g)$. On the other hand, alternative type-I photoreaction pathways involving a direct interaction between the excited photosensitizer and the substrate are concentration dependent. This can occur when CuTMP is used as the sensitizer (19).

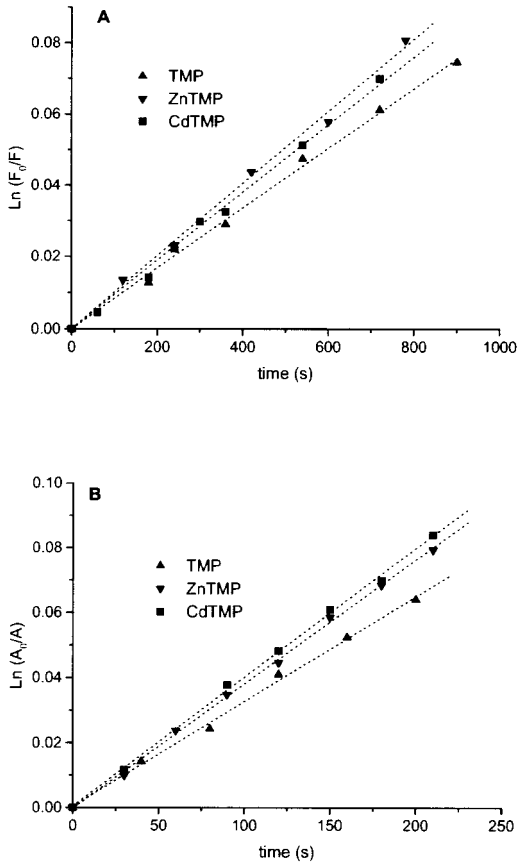

Figure 3. First-order plots for the photooxidation of: (A) Trp (5 μM); and (B) DMA (35 μM) photosensitized by TMP (▲), ZnTMP (■) and CdTMP (●) in AOT reverse micelles.

In this type of microheterogeneous system, Trp is exclusively solubilized in the polar side of the interface and it is oxidized by $O_2(^1\Delta_g)$ (25,41). Taking into account the mechanism described before for the photooxidation reactions in AOT reverse micelles (25,27), the kinetic sequences shown in Scheme 1 can be proposed, where subscripts f and b indicate the organic phase and the micellar pseudophase, respectively. S, ^{1}S and ^{3}S represent the ground, excited singlet and triplet state of the sensitizer, respectively, Δ and Σ represent $O_2(^1\Delta_g)$ and the ground state of molecular oxygen, $O_2(\Sigma_g)^-$, respectively.

$$S_f \xrightarrow{h\upsilon} {}^1S_f \xrightarrow{k_{isc}} {}^3S_f \quad (1)$$

$${}^3S_f + \Sigma_f \xrightarrow{k_{ET}} \Delta_f + S_f \quad (2)$$

$$\Delta_f \underset{}{\overset{K}{\rightleftharpoons}} \Delta_b \quad (3)$$

$$\Delta_f \xrightarrow{k_{d,f}} \Delta_f \quad (4)$$

$$\Delta_b \xrightarrow{k_{d,b}} \Delta_b \quad (5)$$

$$\Delta_b + Trp_b \xrightarrow{k_{r,b}} \text{Products} \quad (6)$$

$$\Delta_b + Trp_b \xrightarrow{k_{q,b}} \Sigma_b + Trp_b \quad (7)$$

To evaluate the reaction rate constant of Trp photooxidation (k_r^{Trp}) in this system, DMA was used as the actinometer under the same experimental conditions. From the ratio of the first-order slopes between the Trp and the actinometer (Eq. 8), the values of k_r^{Trp} were calculated in the micellar system using TMP, ZnTMP and CdTMP as sensitizers.

$$k_{obs}^{Trp}/k_{obs}^{DMA} = k_r^{Trp}[\Delta_b]/k_r^{DMA}[\Delta_f] = k_r^{Trp}K/k_r^{DMA} \quad (8)$$

The values of $K = 0.11$ (25) and $k_r^{DMA} = 0.8 \times 10^7$ s^{-1} M^{-1} (27) reported earlier were used in these calculations. The results for k_r^{Trp} are shown in Table 3. As can be observed very close values were obtained on photoexciting any of these porphyrins. The mean value ($k_r^{Trp} = 1.9 \times 10^7$ s^{-1} M^{-1}) is close to the value reported before ($k_r^{Trp} = 2.3 \times 10^7$ s^{-1} M^{-1}) in a similar AOT reverse micellar system using rose bengal as the sensitizer at $W = 22.2$ (25). However, both results for

Table 4. Porphyrin uptake and cell inactivation parameters

Porphyrin	Uptake (μmol/10^6 cells)	t_{50} (min)*	D_{50} (J cm^{-2})†
TMP	$(2.5 \pm 0.1) \times 10^{-3}$‡	42 ± 3	45 ± 3
ZnTMP	$(2.1 \pm 0.1) \times 10^{-3}$‡	28 ± 2	30 ± 2
CuTMP	$(2.1 \pm 0.1) \times 10^{-3}$‡	—	—
CdTMP	$(1.7 \pm 0.1) \times 10^{-3}$§	29 ± 2	31 ± 2

*Irradiation time at which 50% of cells were inactivated under this experimental condition.
†Light exposure level required to inactivate 50% of cell population.
‡Determined by HPLC.
§Determined by fluorescence.

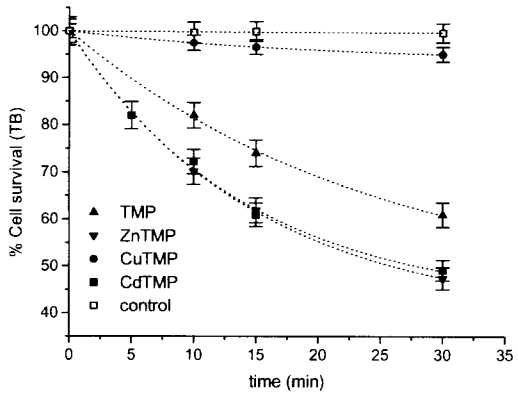

Figure 4. Inactivation of the cells incubated with TMP (▲), ZnTMP (■) CuTMP and CdTMP (●) and exposed to different irradiation times with visible light; (□) corresponding controls in dark conditions; [sensitizer] 1 μM, time 24 h. Values represent mean ± standard deviation of three separate experiments.

k_r^{Trp} are lower than the value reported in water ($k_r^{Trp} = 6.1 \times 10^7$ s^{-1} M^{-1}) (25) indicating that Trp at these W is not able to quench $O_2(^1\Delta_g)$ in the water pool of AOT reverse micelles with the same effectiveness as in bulk water.

Studies *in vitro* on Hep-2 cells

Since these porphyrins are water-insoluble, they were added to the cell cultures from a liposomal solution of L,D-α-dipalmitoyl phosphatidylethanolamine bearing 20 mol% of cholesterol. The use of phospholipid liposomes as vehicles to transport hydrophobic photosensitizers could constitute an advantage for its use in PDT, since liposomes optimizes the release of lipophilic sensitizer to LDL (36). The Hep-2 cells were treated with 1 μM of the sensitizer for 24 h at 37°C. Similar values of porphyrin uptake were obtained for ZnTMP and CuTMP (see Table 4), while a slightly higher value was found for TMP and lower for CdTMP. It was reported before that cellular uptake of porphyrins increase with lipophilicity, which could be explained by a reduction in the membrane permeability barrier toward lipophilic compounds (42).

Cell toxicity induced by the porphyrins was first analyzed in dark condition and no toxicity in terms of cell survival (measured by microscopy in the presence of TB) was detected at any evaluated time (Fig. 4, control). Similar results were found for cell cultures not treated with the sensitizer and irradiated with visible light for 30 min. Therefore, the cell mortality obtained after irradiation of the cultures treated with the porphyrin is due to the photosensitization effect of the agent produced by visible light.

The survival curves obtained after the combined treatment of cells with the sensitizer and visible light are shown in Fig. 4. The irradiation system used in these studies is described in ''Materials and Methods.'' As expected, Hep-2 cell inactivation depends on the light exposure level employed. After an irradiation of 32.4 J cm^{-2}, the survival fractions were 0.61, 0.48 and 0.49 for cell line treated with TMP, ZnTMP and CdTMP, respectively, while a very small effect (~0.94) was found for CuTMP. The results show higher cell mortality with an increase of the $O_2(^1\Delta_g)$ production by the porphyrins. The cell inactivation curves show approximately exponential decay (Fig. 4) for TMP, ZnTMP and CdTMP. From these, the irradiation time required to inactivate 50% of cell population (t_{50}) was obtained (Table 4). Taking into account the light intensity at the treatment site (see ''Materials and Methods'') the corresponding light exposure levels (D_{50}) were calculated. The results are shown in Table 4. Under these conditions, the cytotoxic effect increases in the order: CuTMP $\ll$ TMP $<$ ZnTMP $\sim$ CdTMP, correlating with the sensitizer production of $O_2(^1\Delta_g)$. This behavior is not always the case in cellular systems, where the biological microenvironment of the porphyrins can induce significant changes in the photophysics of the sensitizer established in the homogeneous solution (43,44).

In conclusion, these studies provide information on the photodynamic activity of lipophilic metalloporphyrin derivatives. Although many other factors can contribute to photodamage in cellular systems, a higher damage of Hep-2 cells could be obtained by forming complexes of porphyrin with metal which increase the $O_2(^1\Delta_g)$ generation. TMP and their metallic complexes with Zn(II) and Cd(II) produce $O_2(^1\Delta_g)$ with considerable quantum yield, while the generation of $O_2(^1\Delta_g)$ by Cu(II) complex was negligible. When TMP, ZnTMP and CdTMP were used as photosensitizers in the reverse micellar system, the photooxidation rates of DMA were proportional to the corresponding Φ_Δ. Photooxidation of DMA was not observed with CuTMP. When Trp was used as a model of the biological substrate, it was rapidly photodegraded by the photodynamic activity of TMP, ZnTMP or CdTMP. Depending on the amino acid concentration it was also slowly decomposed in the presence of CuTMP. In biological medium, the relative photosensitizing effectiveness of the porphyrins expressed as the photoinactivation rate ($1/D_{50}$) were 0.022, 0.033 and 0.032 J^{-1} cm^2 for TMP, ZnTMP and CdTMP, respectively, while under these conditions CuTMP produced comparatively small photodamage on Hep-2 cells. These observations are consistent with the reported lack of type-II activity for CuTMP, which is presumed to undergo a type-I photodynamic response (19,21). A similar behavior in both the media indicated that the photodynamic effect of these porphyrins correlate with the $O_2(^1\Delta_g)$ production. Therefore, porphyrins with considerable Φ_Δ appear to act on Hep-2 cells through a mechanism which involves mainly intermediacy of $O_2(^1\Delta_g)$, although, in minor extension a type-I photoreaction process could be occurring in this biological medium.

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