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Photodynamic Activity of 5,10,15,20-Tetrakis(4-methoxyphenyl)porphyrin on the Hep-2 Human Carcinoma Cell Line: Effect of Light Dose and Wavelength Range

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The photodynamic activity of 5,10,15,20-tetrakis(4-methoxyphenyl)porphyrin (TMP) has been investigated in two systems: reverse micelles of *n*-heptane/sodium bis(2-ethylhexyl)sulfosuccinate (AOT)/water-bearing photooxidizable substrates and on a Hep-2 human carcinoma cell line. The effect of variation in the light dose and wavelength range (360–800, 455–800, and 590–800 nm) was compared in both media. The aerobic singlet oxygen-mediated photooxidation of L-tryptophan (Trp) was used as a model of biological substrate in a micellar system. A considerable increase of the observed rate constants of Trp (k_{obs}^{Trp}) was noted, increasing the irradiated area of the TMP spectrum. *In vitro*, the survival curves of Hep-2 cells, treated with TMP, were markedly dependent on the light wavelength ranges used for irradiation. A linear behavior between k_{obs}^{Trp} and the photoinactivation rate of Hep-2 cells was found, indicating that the singlet oxygen ($^1O_2$) is the main species responsible for cell inactivation. These results contributed to an understanding of the photodynamic process yielded by this porphyrin *in vitro* and the sensitivity of Hep-2 cells to photodamage.

Key Words: porphyrin; photodynamic therapy; photosensitizer; singlet oxygen; reverse micelles; carcinoma cell line.

INTRODUCTION

The tetrapyrrolic macrocycles play essential roles in biological systems (*1*). One of the more recent and promising applications of porphyrins in medicine is the detection and cure of tumors by means of photodynamic therapy (PDT) (*1–3*). This method involves the administration of a photosensitizer that becomes concentrated in tumor cells and, upon subsequent irradiation with visible light in the presence of oxygen, specifically destroys the cells (*4,5*). Basically, two types of reactions can

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occur after photoactivation of the sensitizer. In one mechanism the excited photosensitizer reacts directly with substrate molecules in the tissue by electron or hydrogen-transfer reactions (Type I process). In the other, it transfers energy to the ground state of molecular oxygen, generating singlet oxygen ($^1O_2$), which is the tissue-damaging species (Type II process). The photodynamic process of the sensitizers on neoplastic tissues is still not well understood, although it is generally accepted that $^1O_2$, produced after the exposure of the sensitizer to light, is the main species responsible for cell inactivation (*6*).

The first reports of clinical trials of hematoporphyrin derivatives (HPD) in PDT were followed by systematic research for new efficient photosensitizers (*2,3*). Recently, several porphyrin derivatives were synthesized as potential therapeutic agents (*7–12*). Spectroscopic studies of the triplet state carried out with a series of 5,10,15,20-tetrakis(methoxyphenyl)porphyrins have shown that these more structurally simple synthetic porphyrins are effective photosensitizers which can be used as model compounds for investigating the theoretical and instrumental aspects of PDT (*13,14*). These compounds show typical electronic spectra, with a Soret band near 420 nm and four less intense visible Q bands in the range of 510–650 nm. The vast majority of PDT applications utilize light wavelengths in the visible region (350–800 nm) of the light spectrum. However, a better penetration of light in tissue is obtained using long wavelengths. In this way, excitation of photosensitizer at wavelengths in the range of 600–800 nm allows the treatment of deeper neoplastic tissues inside the body by an external source (*1,2*).

This paper reports the photodynamic effect of 5,10,15,20-tetrakis(4-methoxyphenyl)porphyrin (TMP) in two media: reverse micelles bearing photo-oxidizable substrates and a Hep-2 human carcinoma cell line. 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 (*15–17*). In these studies, reverse micelles of *n*-heptane/sodium bis(2-ethylhexyl)sulfosuccinate (AOT)/water were employed as a simple organized model for analyzing the singlet molecular oxygen-mediated photooxidation of L-tryptophan (Trp). This amino acid is subject to $^1O_2$ damage and it has been used to analyze sensitizer activity toward biological substrates in different systems (*18,19*). In biological medium, TMP incorporated in Hep-2 cells was irradiated under the same conditions and cell survival was studied at different light doses and wavelength ranges. Similar behavior was observed in both media, confirming that $^1O_2$ mediation is the main factor responsible for cell inactivation, when TMP is used as sensitizer. These results contributed to an understanding of the photodynamic process yielded by TMP in biological medium and the sensitivity of Hep-2 cells to photodamage.

MATERIALS AND METHODS

Chemicals. 5,10,15,20-Tetrakis(4-methoxyphenyl)porphyrin was synthesized by the condensation of 4-methoxybenzaldehyde and pyrrole, using the Lindsey method (*20*) (TMP Beilstein Reference 26(4),2056). Flash column chromatography (silica gel 200–400 mesh from Aldrich, dichloromethane) afforded pure TMP as confirmed by TLC (Uniplate Silica Gel GHLF 250 μm thin-layer chromatography plates from

Analtech, dichloromethane), R_f 0.45; HPLC (Varian 5000 liquid chromatograph, Varian 2550 UV-visible variable-wavelength detector, Varian MicroPak SI-5 column, 3% 2-propanol/*n*-hexane, flow 0.5), t_R 7.45 min; ^{1}H NMR (Varian Gemini 300 MHz spectrometer, $CDCl_3$, TMS), 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), and MS (Varian Matt 312 operating in EI mode at 70 eV) [*m*/*z*], 734.3 (M^+) (734,2895 calculated for $C_{48}H_{38}N_4O_4$). 9,10-dimethylanthracene (DMA) and L-tryptophan from Aldrich were used without further purification. Sodium dodecyl sulfate (SDS) from Merck was used as received. D,L-α-Dipalmitoyl phosphatidylethanolamine from Sigma was used in liposome preparation. Solvents (GR grade) from Merck was distilled and stored over 4 Å molecule sieves. Sodium bis(2-ethylhexyl)sulfosuccinate from Sigma was dried under vacuum and used without further purification. Water was triply distilled.

Irradiation system. The light source used was a Kodak slide projector equipped with a 150 W lamp. The light was filtered through a 3 cm water layer to absorb heat, a glass filter to remove IR, and a selected wavelength filter (*21*). The light intensity at the treatment site was 18.0 mW/cm^2 for a wavelength range between 360 and 800 nm (GW360 cutoff filter), 15.5 mW/cm^2 for 455–800 nm (GG455 cutoff filter), and 14.0 mW/cm^2 for 590–800 nm (OG590 cutoff filter, Radiometer Laser Mate-Q, Coherent).

Spectroscopic 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, 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 actinometer under the same experimental conditions (*17*). The quenching process of $^1O_2$ by this compound goes exclusively through a chemical reaction. A value of 0.8×10^7 s^{-1} M^{-1} was calculated for the second-order rate constant of DMA in this system (*17*). The kinetics of DMA and Trp photooxidation in *n*-heptane/AOT/water reverse micelles were studied by following the decrease of the absorbance at λ_{max} = 378 nm and the fluorescence intensity at λ = 350 nm, respectively. The Trp fluorescence was excited by 290 nm light (*22*). 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 experiment were performed at 25.0 ± 0.5°C. The pooled standard deviation of the kinetic data, using different prepared samples, was less than 5%. Absorption spectra were recorded on a Shimadzu UV-2401PC spectrophotometer. The fluorescence measurements were performed on a Spex FluoroMax spectrofluorimeter.

Liposome preparation. The incorporation of TMP into the phospholipid bilayer of the dipalmitoylphosphatidylethanolamine was achieved by a modification of the ethanol injection procedure of Kremer *et al.* (*23*). Typically, 2 mL of a solution bearing

9.60 mM of phospholipid, 1.91 mM of cholesterol, and 0.27 mM of TMP in ethanol-tetrahydrofuran binary mixture (1:1, vol/vol) was injected into 10 mL of phosphate-buffered saline solution (PBS) at 80°C. The injection was performed at a speed of 50 μL/min with magnetic stirring (*24*).

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's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 50 μg/mL 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. An appropriate number of cells ($\sim 1 \times 10^6$ cells) were inoculated in 25 cm^2 culture flasks and incubated to obtain nearly confluent cell layers. Then, 100 μL of the TMP, incorporated into liposomes, was added to the culture flask bearing 5 mL of medium. Thus, cells were treated with 1 μM TMP concentration for 24 h under dark conditions. Afterward, the medium containing the photosensitizer was discharged. Cells were washed three times with medium and kept in 5 mL of it. The culture flasks were exposed for different time intervals to visible light of different wavelength ranges. After each irradiation time, the viability of the cells was estimated by microscopy using trypan blue (TB) exclusion method (*21*,*25*), a probe for plasma membrane damage, considered as indicator of cytotoxicity (*26*). The TB assay was performed 1 h after irradiation, in each case. The same procedure without irradiation was carried out for determining dark toxicity. The uptake of TMP by Hep-2 cells was determined by fluorescence spectroscopy. The cells were trypsinized and resuspended in 1 mL of PBS. The number of cells in each suspension was estimated by TB test using a Neubauer chamber counter. Next, 1.0 mL of 4% sodium dodecyl sulfate (Merck) was added to the cellular suspension (*21*). The mixture was incubated further for 15 min (in the dark and room temperature) and centrifuged at 9000 rpm for 30 min. The concentration of the sensitizer in the supernatant was measured by spectrofluorimetry (λ_{exc} = 420 nm, λ_{em} = 658 nm). The fluorescence emission spectrum of TMP presents two maximum at 658 and 725 nm in solution of 2% SDS in PBS. The concentration of TMP in this sample was estimated by comparison with a calibration curve obtained with standard solutions of TMP in 2% SDS ([TMP]$\sim$0.1–10 μM). The fluorescence values obtained from each sample were referred to the total number of cells contained in the suspension.

In order to determine the values of light dose required to inactivate 50% of the cell population (D_{50}), a first-order exponential decay of cell inactivation was considered (*27*). Semilogarithmic plots of Ln (100/% cell survival) vs light dose show a linear correlation in the three cases, whose slopes were used to estimate the values of D_{50}.

Four culture flasks were used for each incubation time and three independent experiments were performed in each case. Any experiments were compared with a control culture without TMP.

RESULTS AND DISCUSSION

The photodynamic effect of TMP was analyzed in two media, reverse micelles of AOT and the Hep-2 cell line, using different light doses and wavelength ranges. The TMP spectrum and the light wavelength ranges employed are shows in Fig. 1. The absorption spectrum for TMP in dichloromethane shows the typical Soret band at 422 nm (ε_{422} = 463270 $dm^3\ mol^{-1}\ cm^{-1}$) and four less intense Q bands at 518 (ε_{518} = 14130 $dm^3\ mol^{-1}\ cm^{-1}$), 556 (ε_{556} = 9750 $dm^3\ mol^{-1}\ cm^{-1}$), 593 (ε_{518} = 4347 $dm^3\ mol^{-1}\ cm^{-1}$), and 650 nm (ε_{518} = 4827 $dm^3\ mol^{-1}\ cm^{-1}$). The photosensitizer was excited with visible light of 360–800, 455–800, and 590–800 nm, which irradiated the Soret and Q bands, Q bands, and approximately Q_I and Q_{II} bands of TMP, respectively (Fig. 1).

Photooxidation of L-Tryptophan in n-Heptane/AOT/Water System

The aerobic irradiation with visible light of *n*-heptane/AOT (0.1 M)/water (W = 10) reverse micelles containing TMP was carried out in the presence of either L-tryptophan or 9,10-dimethylanthracene. The photooxidation reactions for both substrates were studied using different light wavelength ranges, as described above. The stability of TMP was monitored by absorption spectroscopy and no consumption of the photosensitizer was observed during 1 h of irradiation. When the system was irradiated in the presence of Trp, a time-dependent decrease in the amino acid concentration was observed by following a decrease in its fluorescence emission at 350 nm. The photo-process follows first-order kinetics with respect to Trp concentration. Figure 2 shows the semilogarithmic plots describing the progress of the reaction for Trp, using different light wavelength ranges. On the other hand, when photo-excitation of TMP in an AOT system is performed in the presence of DMA, similar plots were obtained by following a decrease in its absorbance (Fig. 3). From these plots the values of the observed rate constant (k_{obs}) were calculated for both substrates. The

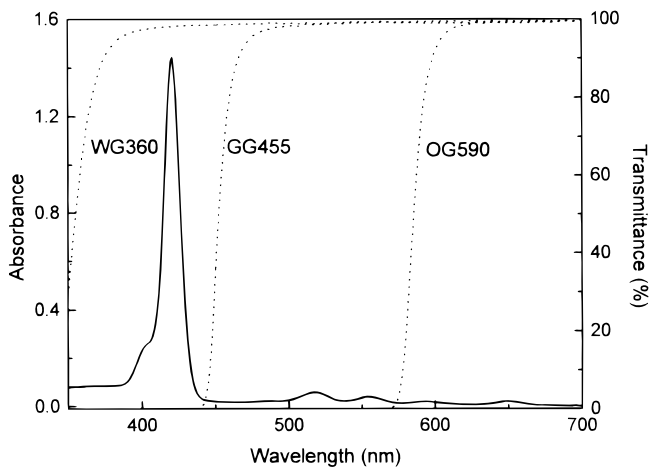

FIG. 1. Absorption spectrum of TMP in dichloromethane [TMP] = 3.1 μM and optic filter transmittances.