Photodamaging Effects of Porphyrin in a Human Carcinoma Cell Line

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Abstract: A convenient procedure to visualize the photodynamic effect of porphyrins on cell lines is shown. 5,10,15,20-Tetra(4-methoxyphenyl)porphyrin (TMP) was used as photosensitizer. The culture flasks bearing a Hep-2 cell line were incubated for 24 h with 1 μM of TMP. Under these conditions saturation of the TMP intracellular concentration is obtained. The irradiation of cell cultures for 30 min produces 90% cell mortality, while no toxicity was observed under dark conditions or under irradiation without TMP. This methodology can be used to demonstrate the photodynamic therapy (PDT) process in a laboratory experiment.

Introduction

Porphyrins and their analogs have attracted much attention as phototherapeutic agents. Photosensitizing porphyrins have a potential use in the treatment of tumors [1]. Photodynamic therapy (PDT) is based on the principle that these porphyrins become concentrated in tumor cells and, upon subsequent irradiation with visible light in the presence of oxygen, specifically destroy the cells [2, 3]. The photodynamic process of the sensitizers on neoplastic tissues is still not well understood although it is generally accepted that singlet oxygen ($^1O_2$), produced after the exposure of the sensitizer to light is the main species responsible for cell inactivation [4]. Basically, two types of reactions can occur after the photoactivation of porphyrin. One reaction involves the generation of free radicals (type I photochemical reaction) and, in the other, singlet oxygen ($^1O_2$) is generated (type II). In both reactions the ground-state sensitizer (^0S) is excited by the absorption of light to a higher-energy excited singlet state (^1S). This is followed by intersystem crossing (ISC) to a triplet state (^3S). This triplet state can react with a suitable substrate forming free radicals (type I), or the energy can be transferred to ground state triplet oxygen ($^3O_2$), and consequently singlet oxygen ($^1O_2$) is produced (type II). A $^1O_2$ molecule can diffuse about 0.1 μm during its lifetime in tissue. This limits the primary reactions to the initial localization sites [1]. Mainly, excited porphyrins act by a type II reaction, which is shown in Scheme 1 [2].

Scheme 1

$$^0S \xrightarrow{h\upsilon} {}^1S \xrightarrow{ISC} {}^3S$$

$$^3S + {}^3O_2 \longrightarrow {}^0S + {}^1O_2$$

Recently, several porphyrin derivatives, such as galactopyranosyl-substituted porphyrins [5], *meso*-glycosylarylporphyrins [6], glycosylated cationic porphyrins [7] and *meso*-substituted porphyrins bound to a polyphenylene chain [8], have been synthesized as potential tumor-photosensitizing agents with interesting applications in PDT.

The purpose of this laboratory class experiment is to study the photodynamic effect of porphyrins on cell lines. Figure 1 shows the structure of 5,10,15,20-tetra(4-methoxyphenyl)-porphyrin (TMP), which was used as photosensitizer. A Hep-2 human carcinoma cell line was used in the experiment. The Hep-2 cell line was incubated for 24 h with 1 μM of TMP. Under these experimental conditions, an intracellular concentration of TMP near the point of saturation is reached. The irradiation of cell cultures with visible light for 30 min produces 90% cell lethality, while no toxicity was observed in the dark condition. This methodology could be easily used as either an undergraduate project for natural science advanced students or in a postgraduate practical training course to show the photodynamic therapy (PDT) process.

Experimental

Spectroscopy. UV–vis and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrophotometer and a Spex FluoroMax fluorimeter, respectively.

TMP solution preparation. A stock solution of porphyrin (1×10^{-4} M) was prepared by dissolving 3 mg of TMP (Aldrich) in 40 mL of ethanol/tetrahydrofuran (1:1). An aliquot of TMP stock solution (50 μL) was added to the culture flasks bearing 5 mL of medium.

Cell culture. The Hep-2 human carcinoma cell line (Asociación Banco Argentino de Células, ABAC, Instituto Nacional de Enfermedades Virales Humanas, Pergamino, Argentina) was maintained frozen in liquid nitrogen. The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal-calf serum (FCS) and gentamicyn as antibiotic. The cells were incubated at 37 °C and 5% humidity. The cell line was routinely checked for the absence of micoplasma contamination [9]. After the experiment the cells were destroyed by autoclave.

Irradiation. Figure 2 shows the irradiation system. The light source used was a slide projector equipped with a 150-W lamp. The light was filtered through a 3-cm water layer to absorb heat. The length between the slide projector and the culture dish is about 30 cm. The light intensity at the treatment site was 25 mW cm^{-2}, which was measured with a radiometer (Laser Mate-Q, Coherent).

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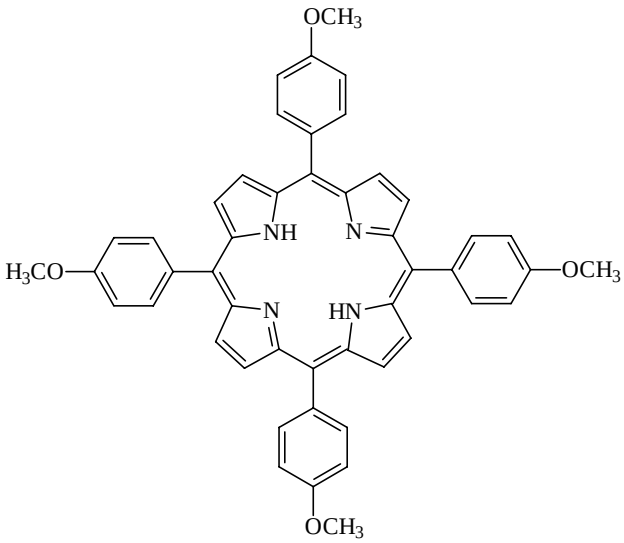

Figure 1. 5,10,15,20-Tetra(4-methoxyphenyl) porphyrin (TMP).

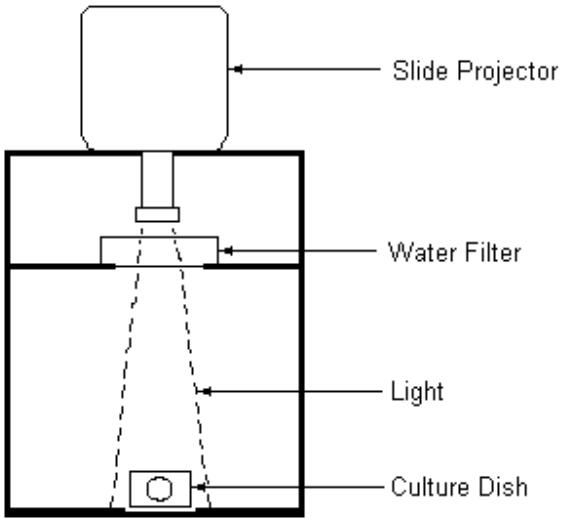

Figure 2. The Irradiation System.

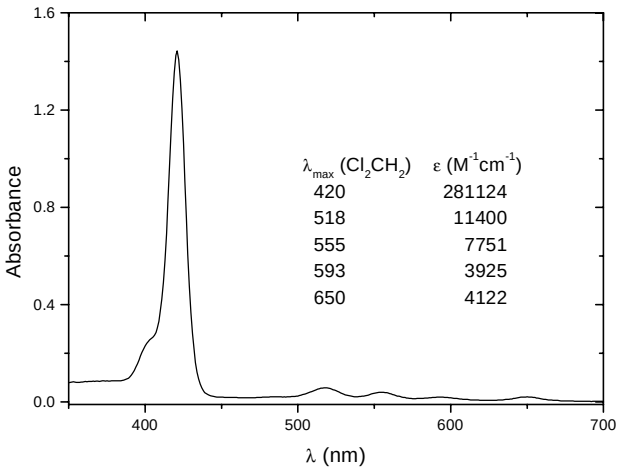

Figure 3. UV-vis absorption spectrum of TMP in dichloromethane, [TMP] = 5.07 μM.

Cell photosensitization studies. About 1×10^6 cells were inoculated in 25 cm^2 culture flasks and incubated to obtain nearly-confluent cell layers. Then, 50 μL of the TMP solution was added to the culture flask bearing 5 mL of medium. The cells were treated with 1 μM of TMP for 24 h in the dark. Following this treatment, the intracellular concentration of TMP is about the saturation value. (The intracellular concentration of TMP was determined at different incubation times. Uptake is rapid at incubation times below 5 h, but slows and eventually reaches a saturation value at ~24 h.) Afterwards, the medium containing the photosensitizer was removed and the cells were washed three times with PBS and kept in 5 mL of medium. The dishes were exposed to visible light (as shown in Figure 2) for different time intervals. After each irradiation time, the viability of the cells was estimated by microscopy using trypan blue (TB) [10]. The number of stained cells and nonstained cells in a given area on the hemocytometer are counted. The percentage of viable cells is:

$$\% \text{ viable cells} = \frac{\text{the number of nonstained cells}}{\text{the number of stained + the number of nonstained}} \times 100$$

The same procedure without irradiation was used for determining the toxicity in the dark. Four culture flasks were used for each incubation time. Any experiments were compared with a culture control without TMP.

TMP quantification. The medium containing the photosensitizer was removed; the cells were washed three times with PBS and suspended in 1 mL of PBS. The number of cells in the suspension was estimated by TB exclusion test using a Neubauer chamber counter. Next, 1.0 mL of 4% sodium dodecyl sulphate (SDS, Merck) was added to the cellular suspension; this breaks the cellular membrane dissolving TMP into a micellar solution. The mixture was incubated for an additional 15 min (in the dark and at 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 value obtained from the sample is proportional to the total number of cells contained in the suspension. 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]~0.5–5 μM).

Results and Discussion

The experiment can be completed in three laboratory periods of about 4 h each. This requires that the instructor or stock-room prepare the flasks with the confluent cell line before the first laboratory period. During the first laboratory period, the spectroscopic characterization of porphyrin (Section I) is carried out and the porphyrin solution is added to the culture flasks. The cells can be incubated for 24 h with TMP during the following day to be ready for the cell survival experiments (Section II), which are conducted during the second laboratory. Section III, in which the intracellular concentration of TMP is determined, is optional. It can be performed in a third laboratory period.

Section I: Spectroscopy. The visible absorption spectrum data for TMP are summarized in Figure 3. The porphyrin Soret band is clearly evident at about 420 nm, and the *Q*-band can be discerned at 518, 555, 593 and 650 nm. This indicates that TMP can be excited by absorption of light in the visible region; therefore, cell cultures were irradiated with visible light filtered through 3 cm of water to eliminate IR irradiation.

The fluorescence emission spectrum of TMP in a solution of 2% SDS in PBS (Figure 4) presents two maxima at 658 and 725 nm. These bands, particularly the more sensitive band at

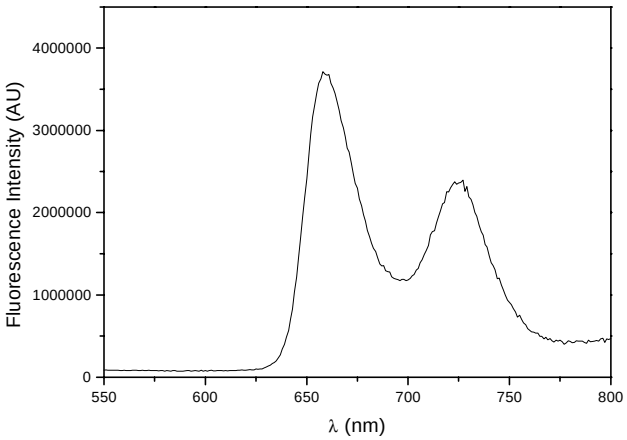

Figure 4. Fluorescence spectrum of TMP in a solution of 2% SDS in PBS, [TMP] = 2.54 μM.

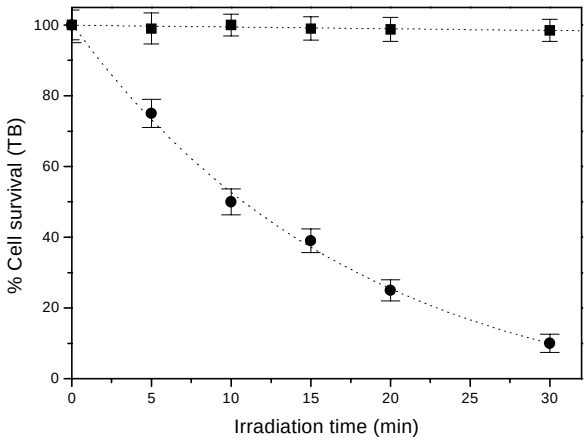

Figure 5. Percent survival of cells exposed to visible light (●) for the indicated irradiation time or kept in dark conditions (■). Both sets were first incubated for 24 h with 1 μM of TMP.

λ = 658 nm, can be satisfactorily used for the quantification of the intracellular concentration of TMP after suspension of a cell culture in a solution of SDS.

Section II: Cell Survival under Irradiation Conditions. Cell survival was determined by microscopy in the presence of TB. The survival curve obtained after the combined treatment of the cells for 24 h with 1 μM of TMP and light irradiation, is shown in Figure 5. Under these conditions, the cell mortality is about 90% after 30 min of irradiation with visible light at an intensity of 25 mW cm^{-2}. Cell toxicity induced by TMP without irradiation was also considered. In this case no toxicity was observed. Those results are also shown in Figure 5. Furthermore, when cells were irradiated with visible light, but were not treated with TMP, no cell mortality was observed; thus, the cell mortality is the result of the exposure of the cell cultures to visible light and the photosensitization effect of TMP.

Section III: Quantification of Intracellular TMP. The uptake of TMP by Hep-2 cells was determined by fluorescence spectroscopy. The cell line was suspended in a 2% solution of SDS and centrifuged. The fluorescence intensity was obtained using the more sensitive band at λ_{em} = 658 nm (λ_{exc} = 420 nm). This value was compared with those obtained for standard solutions of TMP in 2% SDS suspension to obtain the concentration of TMP in the cellular solution. The total number of cells contained in the suspension was estimated by TB exclusion test. The concentration of TMP in the cellular solution was divided by the total number of cells contained in the suspension to obtain a value of ~5×10^9 TMP molecules per cell for the experimental conditions described above.

Conclusion

This laboratory experiment, in which the photodynamic effect of porphyrin on cell lines is demonstrated, is proposed as either an undergraduate project for natural science advanced students or as a practical experiment for the training of postgraduates. Both types of students successfully performed this experiment.

The use of porphyrin to cause photodamage in a human carcinoma cell line is a practical laboratory that provides an interesting method for students to learn about the PDT process.

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