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ENDOGENOUS AND EXOGENOUS PORPHYRINS AS PHOTOSENSITIZERS IN THE HEP-2 HUMAN CARCINOMA CELL LINE

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Abstract – The photodynamic activity of three photosensitizers (PS): ALA-induced PPIX, the porphyrin derivative 5-(4-trimethylammoniumphenyl)-10, 5, 20-tris (2,4,6- trimethoxyphenyl) porphyrin (CP) and the molecular dyad porphyrin-C_{60} (P-C_{60}), the last two incorporated into liposomal vesicles, was evaluated on Hep-2 human larynx carcinoma cell line. ALA-induced accumulation of the endogenous PS PPIX, reached saturation values between 5 and 24 h incubation time; the maximal PPIX content was 5.7 nmol/10^6 cells. The same intracellular level was accumulated when the cationic porphyrin CP was used, while the amount of P-C_{60} attained was 1.5 nmol/10^6 cells. Under violet-blue exciting light, the fluorescence of PPIX and P-C_{60} was found in the cytoplasm showing a granular appearance indicating lysosomal localization. CP was mainly detected as a filamentous pattern characteristic of mitochondrial localization. No dark cytotoxicity was observed using 1mM ALA, 5 µM CP and 1 µM P-C_{60} after 24 h incubation. Cell morphology was analyzed using Hoechst-33258, toluidine blue staining, TUNEL assay and DNA fragmentation, 24 h after irradiation with 54 J/cm2. When photosensitized with ALA and P-C_{60}, chromatine condensation characteristic of apoptotic cell death was found; instead, 58 % of necrotic cells were observed with CP. The results show that in the Hep-2 cells, of the three PS analyzed, the molecular dyad P-C_{60} was more efficient than CP and PPIX, and confirm that PDT can induce different mechanisms of cell death depending on the PS and the irradiation dose.

Key words: photodynamic therapy, cancer, photosensitization, phototherapeutic agents, 5 aminolevulinic acid; PPIX, apoptosis, necrosis

INTRODUCTION

Photodynamic properties of porphyrins are a well known characteristic of these compounds. A clear demonstration that porphyrins are unique and powerful endogenous photosensitizer are the human disorders known as porphyrias, in which porphyrin intermediates are accumulated because of the deficiency of some enzymes of the heme biosynthetic pathway. In the cutaneous porphyrias, abnormal quantities of circulating porphyrins result in skin photosensitivity.

On the other way, biotransformation of ALA to photoactive PpIX has been used in the so called Photodynamic Therapy (PDT), a cancer modality gaining worldwide popularity, based on the preferential accumulation of a given photosensitizing agent (PS) in malignant tissue. It involves the administration of the PS and its subsequent activation by light, to generate singlet oxygen ($^1O_2$) and other reactive oxygen species (ROS), selectively destroying the target cells (2).

Two oxidative mechanisms are considered to be principally implicated in the photodamage of cells. In the type I photochemical reaction, the PS interacts with a biomolecule to produce free radicals, while in the type II mechanism, cell inactivation takes place mainly through the generation of $^1O_2$. Both mechanisms can occur simultaneously and the ratio between the two processes depends mainly of the PS, substrate

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and the nature of the medium (24) However, the mechanism of action of PDT is still not completely understood, since the effects may involve direct tumor cell injury and also indirect cell death as a result of vascular damage (1).

Many PS are being studied in ongoing trials; among them, porphyrins and porphyrin derivatives are the most widely used. In recent years, 5-aminolevulinic acid (ALA)-mediated PDT has become one of the most promising fields in cancer research. After ALA administration, the PS Protoporphyrin IX (PPIX) is generated through the haem biosynthetic pathway, and selectively accumulated in malignant cells (5, 28). Recently, several porphyrin derivatives covalently linked to active molecules have been tested for a potential use in the treatment of tumors (16, 19).

Another interesting approach is the use of compounds such as molecular dyads designed in order to stabilize the photoinduced charge transfer state to inactivate cells. Water-soluble fullerene C60 derivatives have been proposed as efficient PS, as they can be accumulated in the tumor and induce cell death upon illumination (30).

In the present work we compare the photokilling activity of three photosensitizers: ALA-induced PpIX, the porphyrin derivative 5-(4-trimethylammoniumphenyl)-10,5,20-tris (2,4,6-trimethoxyphenyl) porphyrin (CP) and the molecular dyad porphyrin-C60 (P-C60) on the Hep-2 human larynx-carcinoma cell line. Squamous cell carcinoma comprises more than 95% of laryngeal carcinomas and links to tobacco and alcohol consumption. The incidence of these tumors is increasing in developed countries; treatment involves total removal of the larynx and mortality rate is high (31), so it is relevant to investigate strategies to treat this malignancy.

MATERIALS AND METHODS

Chemicals

ALA was purchased from Sigma Chem Co and dissolved in Dulbecco's phosphate buffered saline (PBS) adjusting the pH with sodium hydroxide. CP was synthesized by the reaction of 5-(4-aminophenyl)-10,15,20-tris (2,4,6-trimethoxy phenyl) porphyrin with methyl iodide (19), and P-C60 was synthesized according to Alvarez et al (4). As CP and PC60 are not completely soluble in water, they were incorporated into liposomes of D,L-α-dipalmitoyl phosphatidylethanolamine by a modification of the ethanol injection procedure of Kremer et al (15). Absorption spectra and fluorescence emission spectra of P-C_{60} and CP in *N,N*-dimethylformamide are shown in Fig 1.

Cell culture

The Hep-2 human larynx-carcinoma cell line (Asociación Banco Argentino de Células, ABAC, Instituto Nacional de Enfermedades Virales Humanas, Pergamino, Argentina) was kept frozen in liquid nitrogen. The cells were grown as a monolayer in 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% CO2 atmosphere and the medium was changed daily. Cell line was routinely checked for the absence of mycoplasma contamination.

Cell number

The number of cells seeded per dish were determined by counting viable cells with the trypan blue (TB) exclusion method using a Neubauer chamber counter.

Assays

An appropriate number of cells (~$5x10^5$ cells/ml) were seeded in triplicate in 30 mm diameter dishes (for ALA testing) or in 25 cm2 culture flasks (for CP and PC60), and incubated 24 h at 37° C. Afterwards, the cells were incubated in the dark with specific concentrations of ALA during different time periods (in serum free medium) or with CP and P-C60 (in medium with 1% of serum) incorporated into the liposomes for 24 h.

Measurement of porphyrin synthesis and porphyrin quantification

At the end of incubations, medium containing PS was removed. Total PPIX accumulated within the cells was extracted twice with 5% HCl leaving the cells for half an hour in the presence of the acid at 37° C. Measurements were made in a Shimadzu RF-510 spectrofluorometer, using 406 nm excitation and 604 nm emission wavelengths, and PPIX (Porphyrin Products, Logan, St Louis) as standard reference.

Uptake of CP and P-C60 were determined adding 1.0 ml of 4 % SDS to 1ml of the cellular suspension in PBS. The mixture was further incubated for 15 min in the dark and at room temperature, and centrifuged 30 min at 9000 rpm. The concentration of the PS in the supernatant was measured in a spectrofluorometer (λ exc=422 nm, λ em=650 nm), and quantified in comparison with a calibration curve obtained with standard solutions of the PSs in 2 % SDS ([PS]~0.01-0.5 μM).

Cell photosensitization

At the end of incubations, medium containing PS was removed, cells were washed three times with PBS and fresh medium was added. Cultures were exposed to visible light for different time intervals. After each irradiation time, cells were incubated for another 24 h in the dark to let photodamage occur and then tested for viability by the TB exclusion test, or the MTT assay reading the absorbance of the resulting formazan crystals at 540 nm (18). The same procedure without irradiation was carried out to determine dark toxicity. Cell viability was expressed as a percentage of non-treated control cells.

Irradiation

The light source used was a Kodak slide projector equipped with a 150 W lamp and a wavelength range between 350-800 nm was selected using optical filters. The light was filtered through a 3 cm water layer to absorb heat. The light intensity at the treatment site was 60 mW/cm2.

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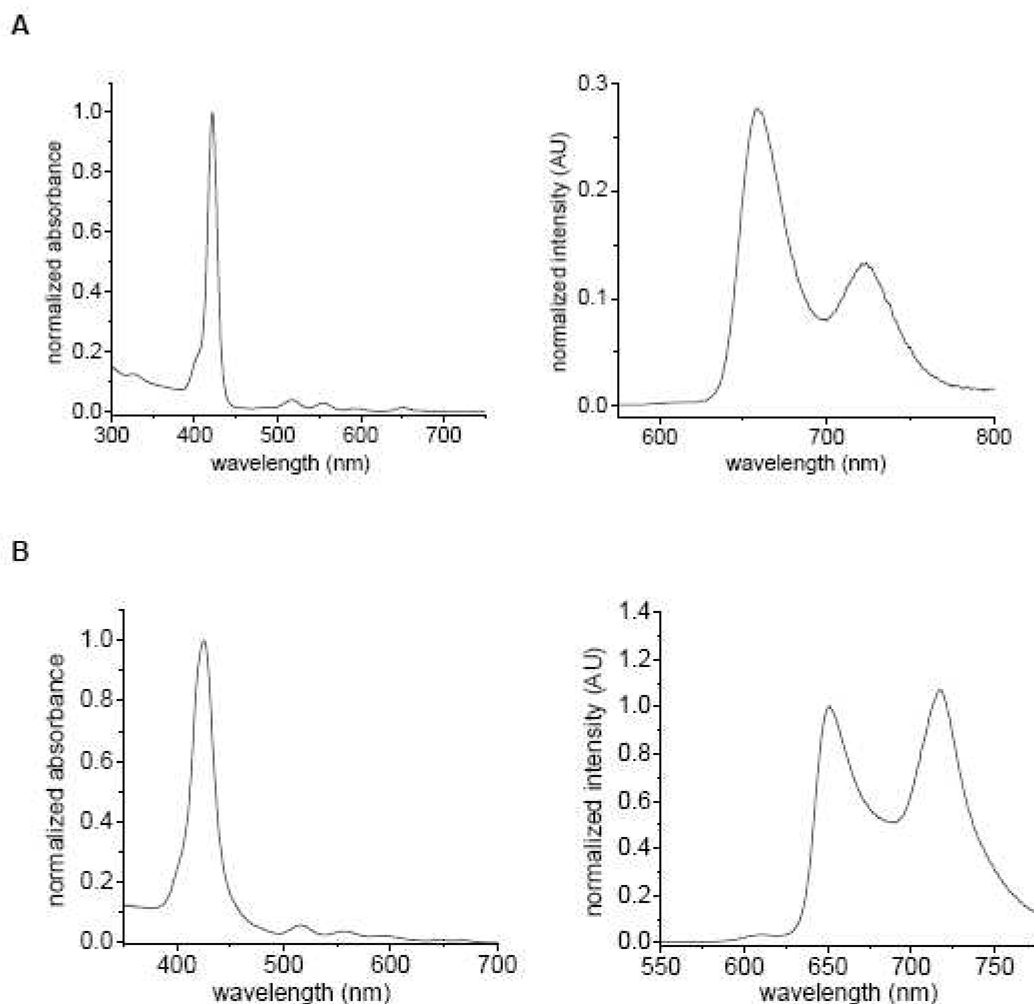

Figure 1. Absorption spectra (left) and fluorescence emission spectra (right) of P-C_{60} (A) and CP (B) in *N,N*-dimethylformamide; λ_{exc} = 554 nm (A); 515 nm.(B).

Morphology and Cell Counting

Changes in cell morphology were analyzed using fluorescence microscopy. After fixation with methanol at -20°C for 10 min, cells were stained with Hoechst-33258 (H-33258, 10 µg/ml in distilled water) for 5 min to visualize the DNA chromatin. Preparations were washed and air-dryed, then mounted in a mixture of distyrene, plasticizer and xylene (DPX, Serva, Heidelberg, Germany), and observed under UV excitation.

Intracellular localization

Typically $3x10^5$ cells were incubated 5 h with 1mM ALA, 3 h with 5 µM CP or 1 µM PC60. Then, medium was removed, cells were washed three times and resuspended in PBS. Fluorescence of PPIX, CP and P-C60 was analyzed under fluorescence microscopy, using violet-blue exciting light (exc=406 nm, em=604 nm for PPIX; exc=422 nm, em=650 nm for CP and P-C60). On the other hand, cells were incubated with 10 µg/ml of mitochondria localizing dye rhodamine 123 (R123) for 15 min, then medium was removed and cells were resuspended in PBS. Fluorescence of R123 was analyzed on account of its red emission, using 450-490 nm excitation and 520-600 nm emission wavelengths. Parallel experiments were run with the fluorescent probes MitoTracker Green and LysoTracker Green (Invitrogen) using a fluorescence microscope (Axiovert S100, Zeiss, Germany) with a color camera (SONY MC-3254) and Axiovision 3.06 software.

Statistical treatment

The values in figures are expressed as means ± standard error of the mean, and they are the average of three independent experiments run in triplicate. A paired two-tailed Student t-test was used to determine statistical significance between means. p values <0.05 were considered significant.

RESULTS

PPIX synthesis and porphyrin accumulation

The endogenous PS PpIX is synthesized from ALA and is rapidly accumulated in the cells; after 5 h incubation, saturation is attained reaching 5.7 nmol PPIX/10^6 cells, with no changes up to 24 h. Similar results were observed when the cell line was treated with 1 µM P-C60;

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in this case, the plateau value reached was 1.5 nmol P-C60 /10^6 cells (Fig 2). At 5 µM, CP uptake was about 5.7±0.3 nmol/10^6 cells. Incorporation of CP into the cells must not be an energy dependent process, as no difference was observed when incubation medium was supplemented with sodium azide and dinitrophenol (3). No saturation uptake occurred up to 10 µM CP and higher concentrations were toxic (Fig 3).

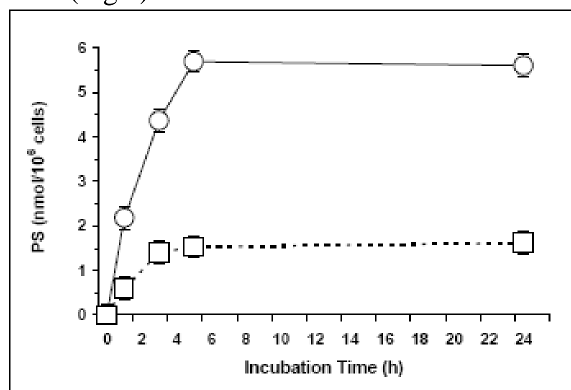

Figure 2. Time course of intracellular accumulation of ALA-induced PPIX (O) and uptake of P-C_{60} (□). Cells were incubated with 1 mM ALA or 1µM P-C_{60}; at the indicated times, medium was removed, and PPIX or P-C_{60} content was determined as described in Materials and Methods.

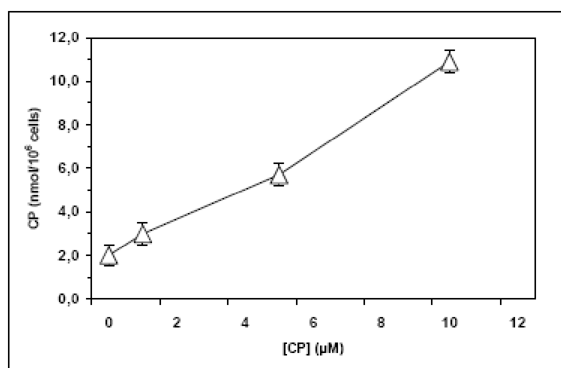

Figure 3. Uptake of CP. Cells were incubated 24 h at 37° C with the CP concentrations indicated, and the amount of intracellular CP content was determined as described in Materials and Methods.

Microscopic localization

Intracellular localization of ALA-induced PPIX, CP and P-C60, was analyzed by means of fluorescence microscopy and confirmed with the fluorescent molecular probes MitoTracker Green and LysoTracker Green. Fluorescence of PPIX and P-C_{60} was mainly observed as granular pattern in the cytoplasm indicating lysosomal localization, while CP showed the bright red fluorescence of porphyrin as filamentous pattern characteristic of mitochondria (Fig 4). The granular fluorescence signal of P-C 60 and PPIX was entirely similar to that observed after lysosome labeling with LysoTracker, and very different from the filamentous pattern of mitochondria labeled by R123 or MitoTracker Green (6,7).

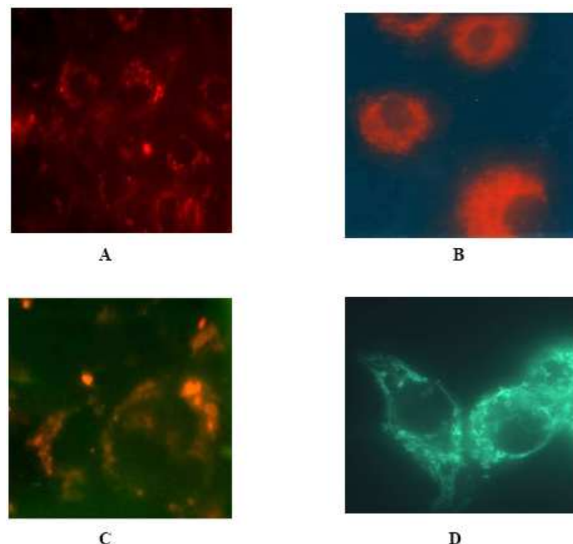

Figure 4. Fluorescence micrographs of Hep-2 cells incubated 5 h with 1 mM ALA (A), 3 h with 5 µM CP (B), 3 h with 1 µM P-C_{60} (C), 30 min with MitoTracker Green (D).

Dark toxicity

Cytotoxic effects in the absence of light were studied using 1 mM ALA, 1-10 µM CP and 0.1-10 µM P-C60 after 24 h incubation. The three compounds did not show any dark toxicity at the concentrations tested, as evaluated by the MTT method 24 h after treatments. Also, the viability was established by microscopy with TB exclusion test using a Neubauer chamber counter. In both cases, similar results were obtained

Photosensitization and photodynamic effect on cell morphology

As expected, light irradiation of cells treated with ALA-induced PPIX, CP and P-C60, diminished the viability of cells in a manner dependent on the PS, the irradiation dose (Fig 5) and the PS concentration (data not shown). Treatment of cells with 1 mM ALA for 5 h induced the accumulation of 5.7 nmol PPIX/10^6 cells, and by illumination with 54 J/cm2 fluence rate, produced 50 % of cell death (fig 4), mainly through apoptotic pathway (Fig 5). After 24 h incubation, 5 µM CP produced the accumulation of 5.7 nmol CP /10^6 cells; this amount induced an important cell death: by illumination with 54 J/cm2 fluence rate, only 20 % of cells survived

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(Fig 5) and 58 % of died cells were necrotic (Fig 6). So, in terms of the intracellular PS level, CP was more effective compared to PPIX. The molecular dyad P-C60 showed a high photodynamic effect, as a concentration of 1.5 nmol P-C60 /10^6 cells was able to inactivate 80% of cells, mainly by apoptosis (Figs 5 and 6).

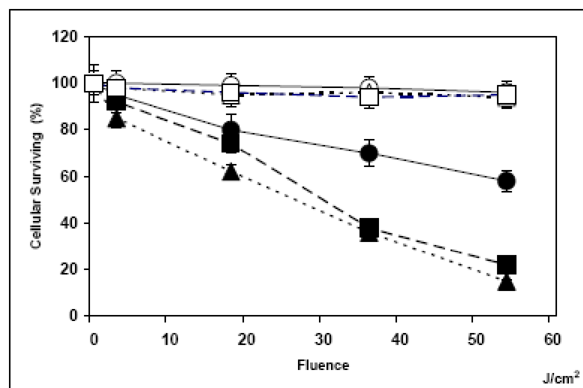

Figure 5. Photoinactivation of Hep-2 cells treated with ALA-induced PPIX, CP or P-C_{60} at different fluence rates. Cells were incubated with 1 mM ALA for 5 h (●), 5 μM CP (▲) or 1 μM P-C_{60} (■) for 24 h at 37 C. At the end of incubations, spent medium was removed, and illuminations were performed as described in Materials and Methods. Surviving cells are expressed as % of the non-PS treated cells (open symbols).

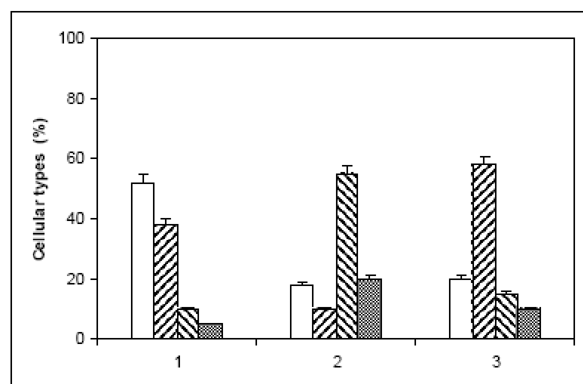

Figure 6. Evaluation of cell death mechanisms after PDT of Hep-2 cells with ALA-PPIX, CP and P-C_{60}. Cells were incubated 5 h with 1mM ALA (1), 24 h with 5 μM CP (2) or 1 μM P-C_{60} (3), and then irradiated with 54 J/cm^2 fluence rate. Values represent mean ± standard deviation of three separated experiments.
( ): normal cells; ( ): apoptotic cells; ( ): necrotic cells; ( ): ghost.

DISCUSSION

In the field of PDT, numerous authors have focused on the study of new porphyrin and non-porphyrin photosensitizers (11, 25). In the present work we compare the photokilling activity of three porphyrin-related photosensitizers: ALA-induced PpIX, the porphyrin derivative 5-(4-trimethylammoniumphenyl)-10,5,20-tris(2,4,6-trimethoxyphenyl)porphyrin (CP) and the molecular dyad porphyrin-C60 (P-C60) on the Hep-2 human larynx-carcinoma cell line.

When adding exogenous ALA, PPIX is accumulated because the step catalized by the rate-limiting enzyme, ALA synthetase, is by-passed (5). Endogenously synthesized PPIX is rapidly eliminated, avoiding the long lasting cutaneous photosensitivity observed with other PS, a great advantage over them (10). ALA-induced PDT has been successfully applied in dermatology, urology and gastroenterology, including the photodiagnosis of tumors (29).

It was observed that higher amounts of PPIX are accumulated in malignant cells compared to normal cells, attributed to differences in the metabolizing ability of the heme pathway (9). In our case, the Hep-2 human larynx-carcinoma cell line accumulates 5.7 nmol PPIX /10^6 cells, which corresponds to 3.2 μg/10^6 cells, a value lower than that obtained in MCF-7c3 cells (2). This amount, however, is similar to the PS level attained with the porphyrin derivative CP, although the photodynamic efficiency was different: while CP was able to produce more than 80 % of cell death, PPIX at the same concentration induced 50% cell inactivation.

The mechanism of cell death was mainly apoptotic in the case of PPIX, and necrotic with CP. It is known that PS, light and molecular oxygen are the three crucial agents in PDT, and depending on the treatment modality, either cellular repair-survival or cell death by an apoptotic or necrotic fashion, is produced (26). There are many evidences that responses to PDT depend primarily on the subcellular localization pattern of a PS, a fact that can influence the mechanism of cell death induced by the PDT treatment (14,17,22), but other factors such as cell line (21) and PDT doses (17) play important roles as well.

It has been reported that mitochondria localized PS are able to induce apoptosis very rapidly, and lysosomal localized PS can elicit either a necrotic or apoptotic response (20). In the Hep-2 cells, microscopic fluorescence studies comparing CP and R123, present a similar filamentous pattern indicating that CP accumulates mainly in mitochondria. The presence of a cationic charge on the macrocycle could be influencing this subcellular localization.

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Although PS accumulated in mitochondria often produce rapid apoptotic responses (27), in our case we found cell death occurred mainly by necrosis. However it was reported that at lower light dose apoptotic cells were predominant (3), indicating that cell inactivation depends not only on the PS intracellular localization but also on the irradiation dose.

ALA-induced PPIX is synthesized in the mitochondria and therefore, it is expected to be primarily confined to this organelle; however, diffusion through the cytoplasm can take place (13). We found the PPIX fluorescence in the cytoplasm in a granular appearance suggesting lysosome localization, confirmed in a parallel experiment with the lysosomal probe LysoTracker Green.

Porphyrin-fullerene C60 dyad presents high capacity to form a photo-induced charge separated state (4). This compound was incorporated into the Hep-2 cells reaching 1.5 nmol P-C60 $/10^6$ cells, and this intracellular concentration causes 80 % cell death upon irradiation with a light dose of 54 J/cm^2. This inactivation was of the same level to that obtained with the cationic porphyrin CP, however comparing the intracellular concentrations, the dyad was more efficient. Alvarez et al (4) reported that under low oxygen concentration, in the case of P-C60, necrosis predominated over the apoptotic pathway. The shift from apoptotic to necrotic death may arise from an excess of oxidative damage and/or the abrogation of cellular energy metabolism.(8,23)

In conclusion, the present paper shows that, in the Hep-2 cells, in terms of intracellular PS concentration the molecular dyad P-C_{60} was the most efficient of the three PSs evaluated. On the other hand, it is confirmed that the mode of cell death by means of PDT is to be dependent on the PS utilized, the different intracellular localization, the drug concentration and incubation time, as re-localization of the PS can occur, and the light dose, inducing cell inactivation by an apoptotic or necrotic pathway.

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