IOP Publishing | Astro Ltd
Laser Physics
Laser Phys. **24** (2014) 045603 (8pp)
doi:10.1088/1054-660X/24/4/045603

Cationic porphyrin derivatives for application in photodynamic therapy of cancer

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Received 16 May 2013
Accepted for publication 11 January 2014
Published 7 March 2014

Abstract

Current studies in photodynamic therapy (PDT) against cancer are focused on the development of new photosensitizers (PSs), with higher phototoxic action. The aim of this study was to compare the therapeutic efficiency of tri-cationic *meso*-substituted porphyrin derivatives (Tri-Py$^+$–Me–PF, Tri-Py$^+$–Me–Ph, Tri-Py$^+$–Me–CO_2Me and Tri-Py$^+$–Me–CO_2H) with the well-known tetra-cationic T_4PM. The phototoxic action of these derivatives was assessed in human colon adenocarcinoma cells by cell viability, intracellular localization and nuclear morphology analysis. In the experimental conditions used we determined that after light activation –PF, –Ph and –CO_2Me cause a more significant decline of cell viability compared to –CO_2H and T_4PM. These results suggest that the nature of the peripheral substituent influences the extent of cell photodamage. Moreover, we have demonstrated that PS concentration, physicochemical properties and further light activation determine the PDT response. All porphyrins were clearly localized as a punctuated pattern in the cytoplasm of the cells, and the PDT scheme resulted in apoptotic cell death after 3 h post-PDT. The tri-cationic porphyrin derivatives Tri-Py$^+$–Me–PF, Tri-Py$^+$–Me–Ph and Tri-Py$^+$–Me–CO_2Me showed a promising ability, making them good photosensitizer candidates for oncological PDT.

Keywords: PDT, cancer, cationic porphyrin, apoptosis

(Some figures may appear in colour only in the online journal)

1. Introduction

Photodynamic therapy (PDT) or photochemotherapy offers a new potential therapy in the field of cancer treatment, based on the interaction of three non-toxic elements: a light-sensitive molecule called a photosensitizer (PS), light and oxygen, which are combined in order to destroy neoplastic tissues [1, 2]. The photodynamic process occurs when the PS cause tumor cell death as the result of oxidative stress due to singlet oxygen ($^1O_2$) as well as other reactive oxygen species (ROS) produced by the photochemical reaction in the PS, induced by a light source at a specific wavelength [3]. New advances in light dosimetry [4, 5], as well as the search for new photosensitizers,

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have allowed the inclusion of PDT protocols in hospitals to treat patients with different types of tumor [6].

Currently, scientists are looking at the development of novel PSs to improve the therapeutic outcome and reduce the nonspecific side-effects of these anticancer agents.

Tetrapyrrolic compounds have attracted considerable attention as phototherapeutic agents, and several porphyrin derivatives have been synthesized and tested for potential use in PDT [7]. The compound 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (T_4PM) is a tetra-cationic porphyrin that is commercially available and is being widely studied for the photoinactivation of environmental microorganisms as well as tumor growth inhibition *in vivo* [8, 9].

Some authors suggest that the molecular structure, in particular the number and position of the positively charged groups and the hydrophobic character of the molecule, appears to play an important role in interactions with biological targets [10]. Positively charged porphyrins are believed to interact electrostatically with the negative charges present on tumor cell membranes and bacterial surfaces, facilitating their penetration through membranes and their consequent PDT efficacy [11].

Previously we have reported that tri-cationic porphyrin derivatives from T_4PM were highly efficient PSs against bacterial strains [12–15]. In the present study, we evaluated the therapeutic efficiency on human cancer cells of tri-cationic porphyrin derivatives that differ in one of the *meso*-substituent groups to explore their possibility as potential PS for oncological PDT.

2. Experimental details

2.1. Photosensitizers

The 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (T_4PM), 5-(pentafluorophenyl)-10,15,20-Tris (1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py$^+$–Me–PF or –PF), 5-(4-methoxicarbonylphenyl)-10,15,20-Tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py$^+$–Me–CO_2Me or –CO_2Me), 5-(4-carboxyphenyl)-10,15,20-Tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py$^+$–Me–CO_2H or –CO_2H) and the 5-phenyl-10,15,20-Tris (1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py$^+$–Me–Ph or –Ph) (figure 1) used in this work were synthesized in accordance to the literature [12, 16]. First, the neutral compounds were obtained from the reaction of pyrrole and adequate aldehydes (pyridine-4-carbaldehyde and 4-formylbenzoic acid or benzaldehyde or pentafluorobenzaldehyde) in refluxing acetic acid and nitrobenzene. These reagents were purchased from Sigma-Aldrich. The resulting porphyrins, after purification by column chromatography (silica), were quaternized by reaction with methyl iodide. Porphyrin Tri-Py$^+$–Me–CO_2Me was obtained by esterification of the corresponding acid derivative with methanol/sulfuric acid, followed by quaternization with methyl iodide. All porphyrins were purified by crystallization from chloroform/methanol/petroleum ether and their purities were confirmed by thin-layer chromatography and ^{1}H NMR spectroscopy [12]. Stock solutions (10 mM) of each PS in dimethyl sulfoxide (DMSO) were prepared by dissolving an adequate amount of the desired cationic porphyrin in a known volume.

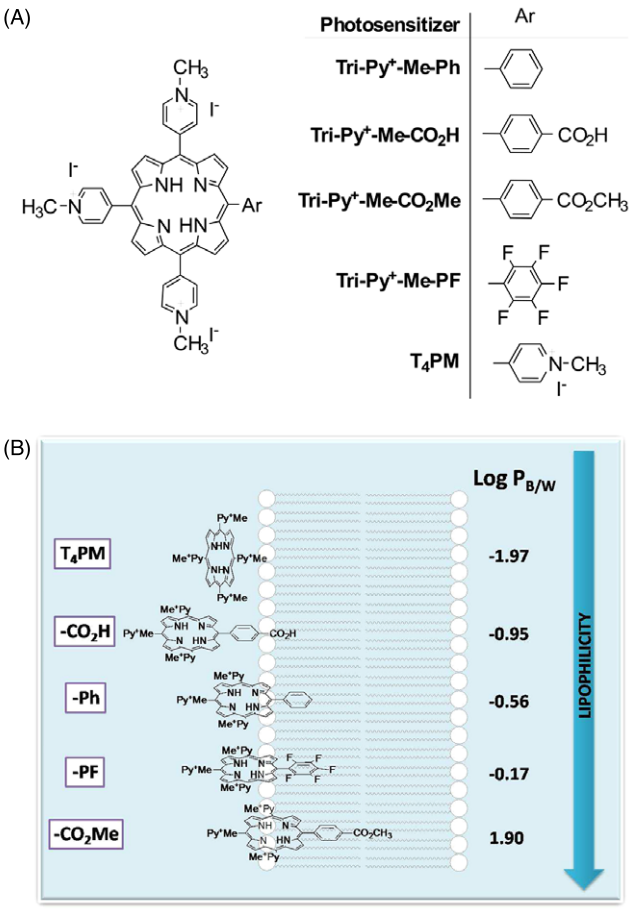

Figure 1. Cationic porphyrin derivatives used in the photodynamic therapy of human colon cancer cells. (A) Chemical structure of 5-phenyl-10,15,20-Tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py$^+$–Me–Ph or –Ph); 5-(4-carboxyphenyl)-10,15,20-Tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py$^+$–Me–CO_2H or –CO_2H); 5-(4-methoxicarbonylphenyl)-10,15,20-Tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py$^+$–Me–CO_2Me or –CO_2Me); 5-(pentafluorophenyl)-10,15,20-Tris(1-methylpyridinium-4-yl)porphyrin tri-iodide(Tri-Py$^+$–Me–PF or –PF); and 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (T_4PM). (B) Scheme of the cationic porphyrin disposition in biological membranes according to the Log *P* value.

2.2. Cell culture

The human colon adenocarcinoma cell line $CaCO_2$ was cultured in complete medium DMEM (Dulbecco's modified Eagle medium high glucose 1X Gibco) supplemented with 20% fetal bovine serum (FBS) (PAA Laboratories), 1% glutamine (GlutaMAX^{TM} 100X Gibco), 1% antibiotic (Penicillin 10 000 units ml^{-1}–streptomycin 10 000 μg ml^{-1} Gibco) and 1% of sodium pyruvate 100 mM (Gibco). Cells were incubated in 5% CO_2 and 95% air at 37 °C in a humidified incubator.

2.3. Photodynamic treatment

The treatment involved incubating the cells with different concentrations of T_4PM, –PF, –Ph, –CO_2Me or –CO_2H (1,

10, 50, 100 μM in DMEM supplemented with 1% FBS, 1% glutamine, 1% sodium pyruvate and 1% antibiotic–antimicotic) for 6,18 or 24 h at 37 °C under dark conditions. The red light source used to perform the PDT treatment was a multi-LED system (Coherent Light) delivering light with the following parameters: max 635 nm; 2.90 mW cm^{-2}; up to 1 J cm^{-2}. After irradiation the medium containing the PS was changed for complete medium and the cultures were left in the incubator for various periods of time. Controls were cells treated with PS only and sham-irradiated.

2.4. Analysis of cell metabolism (MTT assay)

Cell metabolism was assessed by the cytochemical demonstration of mitochondrial dehydrogenase activity, which is a measure of the mitochondrial respiration of cells, employing the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide assay (MTT) [17]. Briefly, cells were seeded in a 96-well microtiter plate at a density of 1.0×10^4 cells per well and left at 37 °C overnight. To evaluate the effect of PDT treatment the mitochondrial activity was assessed 24 h after PDT. For dark toxicity, the protocol followed was the same, but without the irradiation step. The MTT (1 mg ml^{-1}) solution was added to the cells in each well for 3 h at 37 °C and the plate read at 540 nm on a spectrophotometer (Thermo Scientific, Multiskan FC). Absorbance results were represented as a percentage of the untreated control values. Results are reported as the mean ± SD and were realized two times to confirm results. We have considered this assay as an indirect cell viability indicator.

2.5. Cell morphology

To observe the morphological changes in response to photodamage, $CaCO_2$ cells were cultured in 35 mm culture plates and incubated with 1 μM of Tri-Py$^+$–Me–PF or Tri-Py$^+$–Me–Ph for 18 h and 1 μM of Tri-Py$^+$–Me–CO_2Me for 6 h, then irradiated with a total light dose of 3 J cm^{-2}. $CaCO_2$ cells were observed under microscope (Axiophot, Carl Zeiss, Germany) and photographed (AxioCam HRc, Carl Zeiss, Germany) at 24 h post-PDT. For the images the AxioVision Rel. 4.3 software was used.

2.6. Nuclear morphology analysis

To assess the changes in nuclear morphology, $CaCO_2$ cells were cultured in 35 mm culture plates and incubated with 1 μM of –PF or –Ph for 18 h and 1 μM of –CO_2Me for 6 h, then irradiated at a total light dose of 3 J cm^{-2}. At 1 and 3 h after PDT treatment, cells grown on coverslips were fixed in PBS containing 3.7% formaldehyde. The times post-PDT and the irradiation doses were selected by the cell morphology analysis in bright field. After rinsing twice with PBS, cells were stained with 1–5 μg ml^{-1} Hoechst 33342 (H-33342, Molecular Probes) for 15 min at room temperature. Cells were then washed twice with PBS and subsequently immersed in a mounting medium containing anti-fade reagent (Molecular Probes) and visualized under an Axiophot microscope (Carl Zeiss). Nuclei were classified by the characteristic of the chromatin being condensed, fragmented and non-modified. For the quantification of the percentages of the nuclear morphology a total of 200 cells were counted.

2.7. Subcellular localization of photosensitizers

Cells were seeded in 35 mm culture dishes and incubated with DMEM medium containing 1 μM –PF or –Ph for 18 h, or 1 μM –CO_2Me for 6 h, followed by adding a fluorescent probe (H-33342) for nuclear morphology visualization for 10 min. After being washed twice with cold PBS, the subcellular localization pattern of each PS, together with the H-33342, was studied by fluorescence microscopy (Zeiss Axiophot, Carl Zeiss) with a 100 W halogen lamp. Fluorescence images were captured using an AxioCam HRc (Carl Zeiss, Germany) camera and subsequently processed using AxioVision Rel. 4.3 software.

3. Results and discussion

3.1. Photocytotoxicity

Figure 1(A) shows the chemical structure of the cationic porphyrin derivatives tested with respect to their potential use as photosensitizers for PDT using the human colon adenocarcinoma cell line. The porphyrins were derived from the parent compound T_4PM, in which one *meso*-substituted group was replaced, thus inducing a different charge number and butan-l-ol/water partition coefficient (log $P_{B/W}$). Regarding the chemical structure and the log $P_{B/W}$ values, a schematic diagram shows the probable disposition of the cationic porphyrin molecules in biological membranes (figure 1(B)). For the selection of the optimum PDT dose and based on the essential requirement for photosensitizers' minimal dark toxicity, various concentration of porphyrins ranging from 0 to 100 μM were tested on $CaCO_2$ cells in the absence of light. As shown in figure 2, all cationic porphyrin (tri- and tetra-) did not affect the cell metabolism at lower concentrations (1–10 μM), whereas the decrease was slightly evident from 50 μM, dismissing its therapeutic application.

The tri-cationic entities –PF, –CO_2Me and –CO_2H, originated through the insertion of non-charged substituents at the peripheral position of the porphyrin, were more efficient than the tetra-cationic one in ROS production (such as singlet oxygen) [12], and compared to the parental increased their lipophilic/hydrophilic ratio [10, 12]. In order to test the photocytotoxic activity of these modified compounds compared to the parental, 1 and 10 μM PS with 5 J cm^{-2} as the irradiation energy was set for PDT. Figure 3 shows cell metabolism 24 h after PDT treatment as a function of PS concentration and the activating light dose. For this series of tri-cationic porphyrins, the photodynamic efficiency was found to be the highest, according to reduction of cellular metabolism, when 10 μM PS was combined with light. However, the major cell metabolism injury with a lower concentration of PS (1 μM) was done with some tri-cationic compounds. This observed result may indicate that the reduction on viability obtained could be correlated with the acquired molecular properties. If we focused our attention on the porphyrin derivatives with similar generation of cytotoxic species, –CO_2H and –CO_2Me, the basis of the photokilling is given by the log $P_{B/W}$ parameter [12]. The most lipophilic porphyrin, –CO_2Me, showed to be more biologically effective, leading to a lower 15% cell

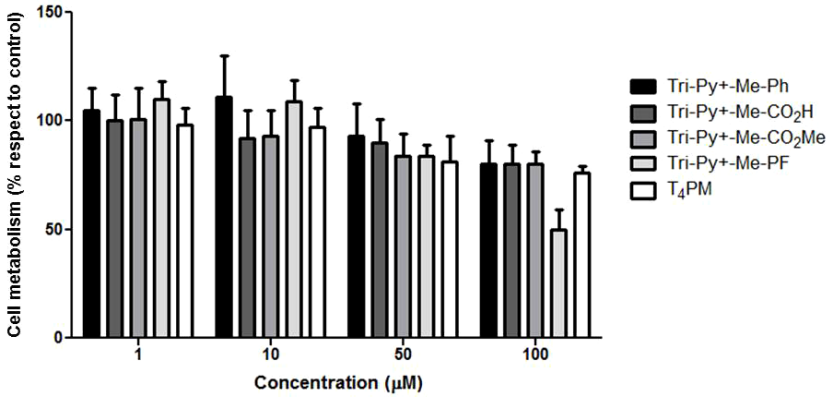

Figure 2. Cell viability of $CaCO_2$ cells after incubation with cationic porphyrin derivatives. The cells were incubated for 24 h in the dark with the cationic compounds at 1, 10, 50 and 100 μM in DMEM 1% FBS. The cell viability was measured by MTT assay. Data are expressed as the percentage of control $\pm$ SD ($n = 8$).

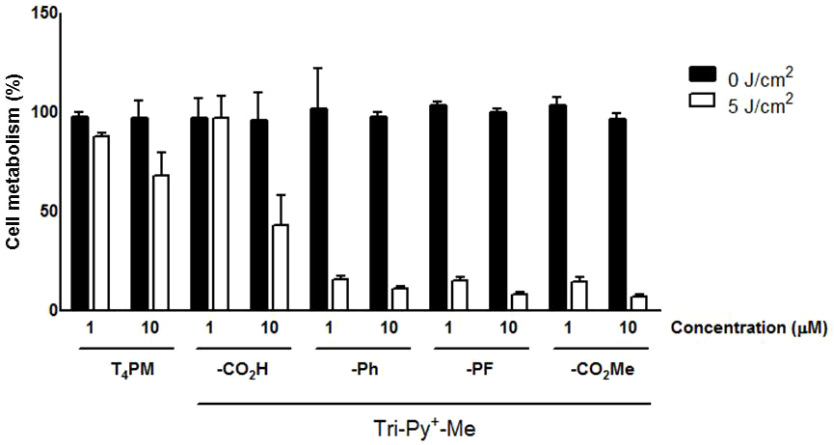

Figure 3. Effect of PS activation on $CaCO_2$ cell viability. The cells were incubated for 24 h in the dark with the cationic porphyrin derivatives T_4PM, $–CO_2H$, –Ph, –PF and $–CO_2Me$ and then exposed to irradiation energies of 0 and 5 J cm^{-2}. After 24 h to the end of treatment the cell viability was measured by MTT assay. Controls consisted of cells exposed to light only, PS only or no treatment. Cell survival was not affected by light or PS alone; therefore, all control values were averaged, and the experimental values were referred to them. Data represent the mean $\pm$ SD of duplicate plates from three independent experiments.

metabolism decline (figure 3). This finding suggests that the introduction of the carboxylic group could be the molecular reason for this enhanced activity. Given the relevance of the observed effect we have dismissed $–CO_2H$ for further studies.

Surprisingly, –Ph and –PF were highly similar in their ability to promote cell metabolism reduction, but only –PF was more effective than T_4PM as a PS singlet oxygen generator. Therefore, the photodynamic efficiency of –Ph and –PF can be ascribed solely to their lipophilic behavior (figure 3) [12, 18]. As a first selection regarding cellular PS sensitization, these results highlight that compounds –Ph, –PF and $–CO_2Me$ were more photochemically active than T_4PM and $–CO_2H$. Among all the proposed sensitizers, –Ph, –PF and $–CO_2Me$ have received special attention since they caused 80–90% of cell destruction upon irradiation. The enhanced photokilling of these tri-cationic porphyrins would be attributed by the hydrophobic degree given with the introduction of a different *meso*-substituent group at one position of the porphyrin macrocycle.

Reviewing the literature, it can be said that there are some factors which increase the photodynamic action, such as strong absorption at wavelengths longer than 650 nm, high quantum efficiency of singlet oxygen generation, and the membrane partition coefficient [19–24]. The results obtained in this study are in agreement with authors suggesting that the interaction/incorporation of PSs in biological membranes predicted by log P value [10] define the success of the PDT treatment [10, 24, 25]. Furthermore, it is important to mention that cellular membranes and organelles are considered the main target of photodynamic action [26, 27]. Thereby, after PS accumulation, if the porphyrin is anchored within the membrane the reactive species formed after light excitation will probably react with membrane components.

Moreover, the PDT efficacy is linked to the total amount of light delivered. ‘Photodynamic dose’ is referred to homogeneous and sufficient light delivery to the target tissue in the presence of an optimal concentration of PS.

It has been proposed that is possible to modulate the magnitude of the PDT damage towards the tumor by manipulating the light fluence, timing of illumination or a combination of these factors. The selection of light dose, one of the major controllable factors in the PDT treatment, is also paramount to the success. Our purpose was to minimize the PS dose needed to achieve the desired effect. For this reason, we modified other variables to generate appropriate treatment parameters. The benefit in this approach could be better efficacy, with a lower side effect profile. In this context, we have decided to assess the lower PS concentration (1 μM) in order to examine different PS light intervals (PS-LI, time interval between PS administration and irradiation) under activation at different irradiation energy densities (1, 3, 5 J cm^{-2}) and study how changes of these variables will affect the final PDT outcome. The results obtained post-PDT are summarized in figure 4. In our experimental data we have observed for –CO_2Me, that the reduction of cell metabolism became evident (40%) with an applied treatment schedule: PS-LI 6 h + 3 J cm^{-2}, whereas for –PF the regimen to achieve the same extent of death was PS-LI 6 h + 5 J cm^{-2} or PS-LI 18 h + 3 J cm^{-2}; and –Ph PDT scheme: PS-LI 18 h + 3 J cm^{-2}. Our results demonstrated that –CO_2Me turned out to be the more photoactive tri-cationic porphyrin derivative because the cytotoxicity is obtained when lower parameters are applied (PS-LI + J cm^{-2}). We assumed that the reason for this photodamage could be attributed to both a significant rate of $^1O_2$ production and its lipophilic behavior compared to the pattern [12]. In the present study we have demonstrated that PS concentration, physicochemical properties and further light activation influence the extent of cell death, but that the mode of cell death triggered would not always be the same.

3.2. Cell death photoinduced by porphyrins

It is known that PDT can evoke at least three main cell death pathways: apoptosis, necrosis and autophagy-associated cell death [28, 29]. Apoptosis is generally the major cell death modality in cells responding to PDT [2, 30], and numerous photosensitizers, including porphyrins, had been characterized to induced apoptosis after their photoactivation [31]. Regarding the cell death mechanism, an explosion of investigation and explorations in the field of cell biology have elucidated many of the pathways that mammalian cells undergo when PS are illuminated. The lethal mechanisms initiated by the photosensitization process appear to encompass the three major morphologies of programmed cell death, i.e., apoptotic, necrotic and autophagic cell death [32, 33]. The most widely studied of these mechanisms is apoptosis. In this context, we and others have observed the occurrence of apoptosis in many tumor cell lines after PDT with a variety of porphyrin [34–36], phthalocyanine [37, 38], and other photosensitizers [39].

In our PDT studies a prominent cytotoxic effect was observed in response to a sub-lethal dose (60% cell survival). The morphological changes in response to treatment consist in shrinkage and the arising of cells debris (figure 5). To characterize the mode of cell death, nuclear morphology analysis allowed us to quantifiably discriminate between

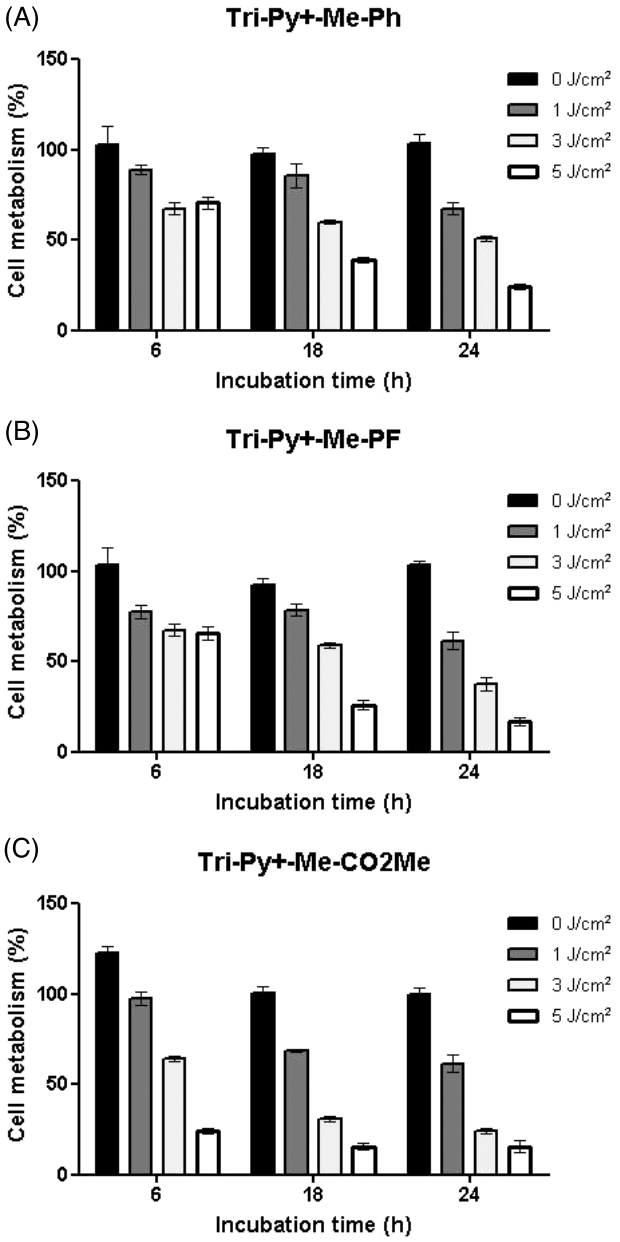

Figure 4. Effect of porphyrin derivatives at different photosensitizer-light interval. Cells were incubated with 1 μM PS in DMEM 1% FBS for 6, 18 and 24 h in the dark. (A) Tri-Py$^+$–Me–Ph, (B) Py$^+$–Me–PF and (C) Tri-Py$^+$–Me–CO_2Me. After incubation, $CaCO_2$ cells were exposed to 0, 1, 3 and 5 J cm^{-2} of irradiation. MTT cytotoxicity assay was carried out 24 h after PDT. Cell survival was not affected by light or PS alone. Data represent media ±SD ($n = 8$).

condensed and fragmented nuclei, the latter considered a hallmark of the end apoptotic cell death stage.

The results indicate that the nuclear morphology changed along with the time after PDT, increasing for all tri-cationic porphyrins towards an apoptotic cell death. Representative images are shown in figure 6 and the calculated percentages of nuclei with condensed or fragmented chromatin in table 1. The treatment of $CaCO_2$ cells with –PF or –Ph and light was found to induce a noticeable apoptotic response in a large fraction of cells. The appearance of chromatin condensation as early as

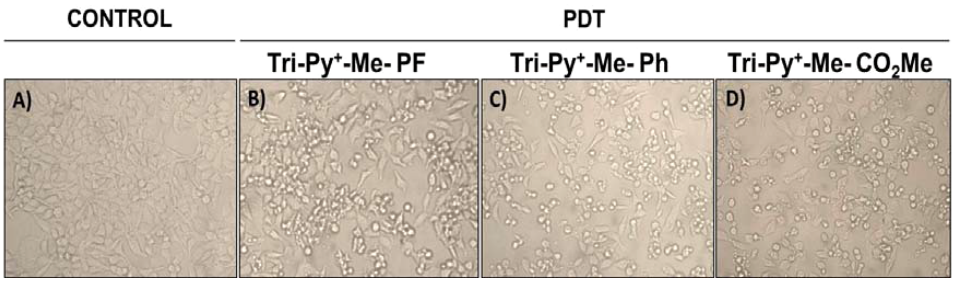

Figure 5. Cell morphology after PDT. $CaCO_2$ cells were incubated for 18 h with 1 μM of Tri-Py^+–Me–PF or Tri-Py^+–Me–Ph and for 6 h with 1 μM of Tri-Py^+–Me–CO_2Me. Cells were irradiated at 3 J cm^{-2} and 24 h after PDT observed under microscopy. (A) Control cells,(B) at 24 h post-PDT with Tri-Py^+–Me–PF, (C) at 24 h post-PDT with Tri-Py^+–Me–Ph and (D) at 24 h post-PDT with Tri-Py^+–Me–CO_2Me.

Table 1. Percentages of cells containing nuclei with apoptotic features were calculated for different times after PDT. (A) Tri-Py^+–Me–Ph, (B) Tri-Py^+–Me–PF, (C) Tri-Py^+–Me–CO_2Me. $CaCO_2$ cells were incubated with 1 μM PS and then irradiated at 3 J cm^{-2}. At 1 and 3 h after PDT, cells were stained with H-33258 and the nuclei classified as condensed, fragmented or non-modified. NT (non treated cells), LC (light control), PS (photosensitizer). Data represent the mean ± SD of at least 200 cells from each cover slip from three experiments.

	Nuclear morphology		
	Non-modified	Condensed	Fragmented
(A)			
NT	98.00 ± 1.2%	2.00 ± 0.11%	0.00 ± 0.00%
LC	98.90 ± 1.1%	1.00 ± 0.19%	0.10 ± 0.27%
PS	96.00 ± 1.0%	3.50 ± 0.17%	0.50 ± 0.10%
PDT1 h	19.80 ± 3.0%	67.40 ± 2.9%	12.8 ± 2.6%
PDT3 h	00.00 ± 0.00%	30.20 ± 2.0%	69.80 ± 2.6%
(B)			
NT	98.30 ± 1.2%	1.70 ± 0.30%	0.00 ± 0.00%
LC	96.90 ± 1.5%	2.40 ± 0.22%	0.60 ± 0.10%
PS	89.90 ± 2.0%	7.50 ± 0.34%	2.50 ± 0.17%
PDT1 h	20.60 ± 1.0%	67.40 ± 2.3%	12.0 ± 0.6%
PDT3 h	00.00 ± 0.00%	35.40 ± 2.1%	64.60 ± 2.0%
(C)			
NT	98.32 ± 1.9%	0.84 ± 0.21%	0.84 ± 0.28%
LC	100 ± 1.5%	0.00 ± 0.00%	0.00 ± 0.00%
PS	99.09 ± 2.0%	0.00 ± 0.00%	0.00 ± 0.00%
PDT1 h	74.07 ± 1.3%	22.22 ± 2.4%	3.70 ± 0.23%
PDT3 h	45.00 ± 0.40%	10.00 ± 0.1%	45.00 ± 2.1%

1 h was evident after light irradiation (figures 6(B) and (E)). Fragmentation of DNA, a fundamental characteristic of cells undergoing apoptosis, was evident within 3 h following PDT, reaching a maximum of about 65–70% (table 1(A) and (B)). However, studies with –CO_2Me have shown that this PS produced few cells with condensed nuclear morphology within 1 h after PDT, indicating that the photodamaged cells are in apoptosis later with respect to –PF and –Ph PDT (figure 6 and table 1(C)).

3.3. Intracellular localization

One of the crucial factors in determining the type of cell death is the subcellular localization of the PS. Due to the photochemically generated ROS having a short half-life and acting close to their site of generation, the degree and type of photodamage that occurs in cells loaded with PS and illuminated depends on the precise subcellular localization [16]. With the aim of determining the subcellular localization of the PSs, $CaCO_2$ cells were incubated with –PF, –Ph or –CO_2Me at a concentration of 1 μM. As shown in figure 7, all PS were clearly localized as a punctuated pattern in the cytoplasm of the cells.

Cellular sub-localization is affected by the lipophilicity of the PS and the nature of its electronic charge (positive or negative). In particular, it has been described that cationic porphyrins have affinity to plasma membrane [40], mitochondria [41], lysosome [24] and endoplasmic reticle [42]. Studies of the tetra-cationic porphyrin T_4PM have shown nuclear localization [43] and induction of apoptosis by DNA damage after irradiation [44]. Also, studies involving several tri-cationic porphyrin derivatives (including the –CO_2Me) have demonstrated localization in endocytotic and pinocytotic vesicles, except nucleus and mitochondria [45]. These antecedents and our results seem to indicate that a modification in one substituent within the porphyrinic macrocycle modifies the subcellular localization. In addition, previous studies show that tri-cationic sensitizers target membranous compartments preferentially in mitochondria. Probably, regarding the log *P* value and the tended intracellular distribution would allow us to assume that the derivatives respond in the same way as was described above. To confirm, more precise determination of their localization involving specific organelle markers is required for further studies.

Conclusions

The aim of the current research was a comprehensive study of the chemical and photochemical properties of the tri-cationic porphyrin derivatives as PS candidate molecules. It has been studied that the proper combination of porphyrin and light doses, and perhaps the proper timing of irradiation, may cause maximal damage to cancerous cells while carrying out PDT. In the experimental conditions used we determined that after light activation –PF, –Ph and –CO_2Me cause a more significant decline of cell viability compared to –CO_2H and the well-known T_4PM. These results suggest that the nature of the peripheral substituent influences

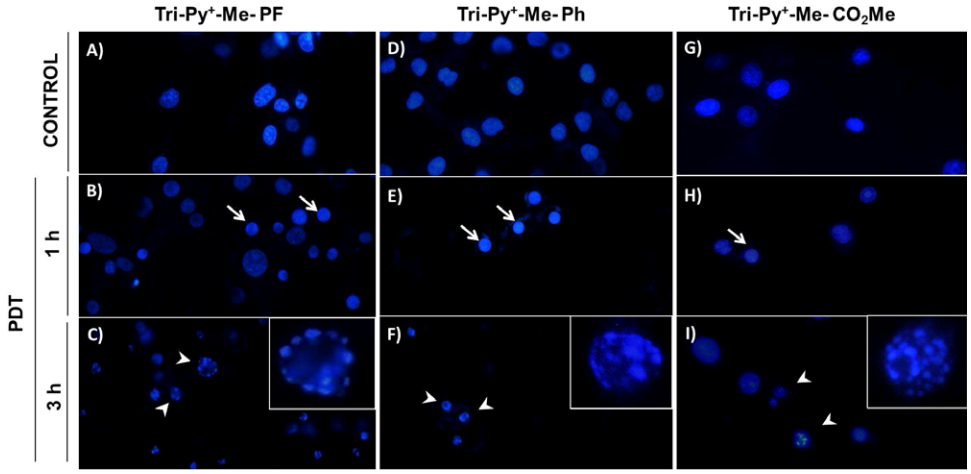

Figure 6. Nuclear morphology in $CaCO_2$ cells after PDT. Cells growing on cover slips were treated with PDT or not (control); at 1 and 3 h thereafter, the cells were fixed, stained with Hoechst 33342, and visualized by fluorescence microscopy. Representative micrographs from PDT-treated ((B), (C), (E), (F), (H), (I)) and untreated cells ((A), (D), and (G)). Arrows and head arrows indicate condensed and fragmented nuclei, respectively. The inserts in (C), (F) and (I) show a magnification of the fragmented nucleus.

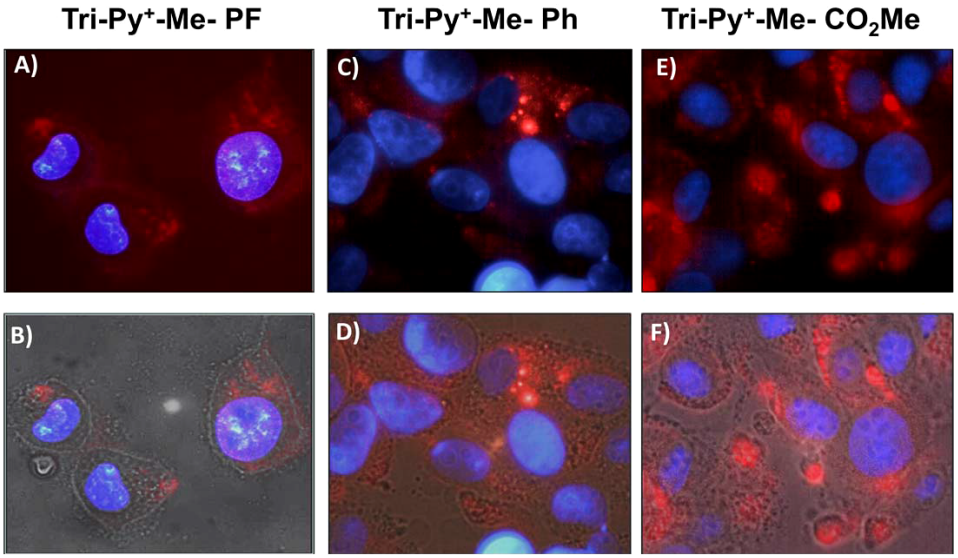

Figure 7. Intracellular localization of tri-cationic porphyrins in $CaCO_2$ cells. Cells were incubated with 1 μM of Tri-Py^+–Me–PF, Tri-Py^+–Me–Ph or Tri-Py^+–Me–CO_2Me. The intracellular distribution of Tri-Py^+–Me–PF and Tri-Py^+–Me–Ph was assessed after 18 h incubation and 6 h for Tri-Py^+–Me–CO_2Me. Immediately after, cells were stained with the nuclear probe H-33342. Red fluorescence corresponds to PS while blue fluorescence represents the signal from H-33342. Images are representative of those obtained in three independent experiments. (A) Tri-Py^+–Me–PF and H-33342 signal merged picture, (B) Tri-Py^+–Me–PF, H-33342 signals and phase contrast merged picture, (C) Tri-Py^+–Me–Ph and H-33342 signal merged picture, (D) Tri-Py^+–Me–Ph, H-33342 signals and phase contrast merged picture, (E) Tri-Py^+–Me–CO_2Me and H-33342 signal merged picture, and (F) Tri-Py^+–Me–CO_2Me, H-33342 signals and phase contrast merged picture.

the extent of cell photodamage. The ultimate therapeutic efficacy could be inflicted upon the PS light interval parameters.

Another important question to be addressed was whether cancer cells were susceptible to PDT-induced apoptotic cell death. PDT with –PF and –Ph showed an early PDT time-dependent increase in apoptosis compared to –CO_2Me. Although further studies are needed, our preliminary results have demonstrated that tri-cationic porphyrin derivatives –PF, –Ph and –CO_2Me are promising PSs candidates for PDT.

Acknowledgments

This research was supported by Consejo Nacional de Investigación Científica y Tecnológica (CONICET), Agencia FONCyT (PICT) and Secretaría de Ciencia y Técnica (SeCyT) of Universidad Nacional de Río Cuarto (UNRC). Also, we thank the University of Aveiro, Fundaçãopara a Ciência e a Tecnologia (FCT) and FEDER for funding the QOPNA unit (project PEst-C/QUI/UI0062/2011). Carla M B Carvalho (SFRH/BD/38611/2007) is also grateful to FCT for a grant.

References

[1] Dougherty T J, Gomer C J, Henderson B W, Jori G, Kessel D, Korbelik M, Moan J and Peng Q 1998 *J. Natl Cancer Inst.* **90** 889

[2] Agostinis P, Berg K, Cengel K A, Foster T H, Girotti A W, Gollnick S O, Hahn S M, Hamblin M R, Juzeniene A and Kessel D 2011 *CA Cancer J. Clin.* **61** 250

[3] Castano A P, Demidova T N and Hamblin M R 2005 *Photodiagn. Photodyn. Ther.* **2** 91

[4] Gill K R S, Wolfsen H C, Preyer N W, Scott M V, Gross S A, Wallace M B and Jones L R 2009 *Clin. Cancer Res.* **15** 1830

[5] Swartling J, Axelsson J, Ahlgren G, Kälkner K, Nilsson S, Svanberg S, Svanberg K and Andersson-Engels S 2010 *J. Biomed. Opt.* **15** 058003

[6] O'Connor A E, Gallagher W M and Byrne A T 2009 *Photochem. Photobiol.* **85** 1053

[7] Sharma S K, Mroz P, Dai T, Huang Y-Y, St Denis T G and Hamblin M R 2012 *Isr. J. Chem.* **52** 691

[8] Grand C L, Han H, Muñoz R M, Weitman S, Von Hoff D D, Hurley L H and Bearss D J 2002 *Mol. Cancer Ther.* **1** 565

[9] Almeida A, Cunha A, Faustino M A F, Tomé A C and Neves M G P M S 2011 *Photodynam* (Cambridge: Royal Society of Chemistry) p 83

[10] Engelmann F M, Mayer I, Gabrielli D S, Toma H E, Kowaltowski A J, Araki K and Baptista M S 2007 *J. Bioenerg. Biomembr.* **39** 175

[11] Jensen T J, Vicente M G H, Luguya R, Norton J, Fronczek F R and Smith K M 2010 *J. Photochem. Photobiol.* B **100** 100

[12] Alves E, Costa L, Carvalho C M B, Tomé J P C, Faustino M A, Neves M G P M S, Tomé A C, Cavaleiro J A S, Cunha A and Almeida A 2009 *BMC Microbiol.* **9** 70

[13] Costa L, Alves E, Carvalho C, Tomé J, Faustino M, Neves M, Tomé A, Cavaleiro J, Cunha A and Almeida A 2008 *Photochem. Photobiol. Sci.* **7** 415

[14] Oliveira A, Almeida A, Carvalho C M B, Tomé J P C, Faustino M A F, Neves M G P M S, Tomé a C, Cavaleiro J A S and Cunha A 2009 *J. Appl. Microbiol.* **106** 1986

[15] Carvalho C M B *et al* 2007 *J. Photochem. Photobiol.* B **88** 112

[16] Carvalho C *et al* 2010 *ACS Nano* **4** 7133

[17] Ribeiro A P D, Pavarina A C, Trindade F Z, Bagnato V S, Kurachi C and Souza Costa C A 2010 *Laser Phys.* **21** 229

[18] Engelmann F M, Rocha S V O, Toma H E, Araki K and Baptista M S 2007 *Int. J. Pharm.* **329** 12

[19] Araki K, Engelmann F M, Mayer I, Toma H E, Baptista M S, Maeda H, Osuka A and Furuta H 2003 *Chem. Lett.* **32** 244

[20] Engelmann F M, Losco P, Winnischofer H, Araki K and Toma H E 2002 *J. Porphyr. Phthalocya.* **2** 33

[21] Engelmann F M, Mayer I, Araki K, Toma H E, Baptista M S, Maeda H, Osuka A and Furuta H 2004 *J. Photochem. Photobiol.* A **163** 403

[22] Hudson R, Savoie H and Boyle R 2005 *Photodiagn. Photodyn. Ther.* **2** 193

[23] Porphyrins C, Reddi E, Ceccon M, Valduga G, Jori G, Bommer J C, Elisei F, Biologia D and Chimica D 2002 *Photochem. Photobiol.* **75** 462

[24] Ricchelli F, Franchi L, Miotto G, Borsetto L, Gobbo S, Nikolov P, Bommer J and Reddi E 2005 *Int. J. Biochem. Cell Biol.* **37** 306

[25] Silva J N *et al* 2010 *Biochem. Pharmacol.* **80** 1373

[26] Villanueva A and Jori G 1993 *Cancer Lett.* **73** 59

[27] Ben Amor T, Bortolotto L and Jori G 2000 *Photochem. Photobiol.* **71** 124

[28] Panzarini E, Inguscio V and Dini L 2011 *Cell Death Dis.* **2** e169

[29] Buytaert E, Dewaele M and Agostinis P 2007 *Biochim. Biophys. Acta* **1776** 86

[30] Oleinick N L, Morris R L and Belichenko I 2002 *Photochem. Photobiol. Sci.* **1** 1

[31] Almeida R D, Manadas B J, Carvalho A P and Duarte C B 2004 *Biochim. Biophys. Acta* **1704** 59

[32] Buytaert E, Callewaert G, Vandenheede J R and Agostinis P 2006 *Autophagy* **2** 238

[33] Buytaert E, Callewaert G, Hendrickx N, Scorrano L, Hartmann D, Missiaen L, Vandenheede J R, Heirman I, Grooten J and Agostinis P 2006 *FASEB J.* **20** 756

[34] Alvarez M, Príncipe F, Milanesio M, Durantini E and Rivarola V 2005 *Int. J. Biochem. Cell Biol.* **37** 2504

[35] Alvarez M, Prucca C, Milanesio M, Durantini E and Rivarola V 2006 *Int. J. Biochem. Cell Biol.* **38** 2092

[36] Kriska T, Korytowski W and Girotti A W 2005 *Arch. Biochem. Biophys.* **433** 435

[37] Usuda J, Azizuddin K, Chiu S and Oleinick N L 2003 *Photochem. Photobiol.* **78** 1

[38] Vittar N, Awruch J, Azizuddin K and Rivarola V 2010 *Int. J. Biochem. Cell Biol.* **42** 1123

[39] Ichinose S, Usuda J, Hirata T, Inoue T, Ohtani K and Maehara S *et al* 2006 *Int. J. Oncol.* **29** 349

[40] Kessel D 2004 *J. Porphyr. Phthalocya.* **8** 1009

[41] Sibrian-Vazquez M, Nesterova I, Jensen T and Vicente M 2008 *Bioconjug. Chem.* **19** 705

[42] Sibrian-Vazquez M, Jensen T, Fronczek F, Hammer R and Vicente M 2005 *Bioconjug. Chem.* **16** 852

[43] Mita H, Ohyama T, Tanaka Y and Yamamoto Y 2006 *Biochemistry* **45** 6765

[44] Tada-Oikawa S, Oikawa S, Hirayama J, Hirakawa K and Kawanishi S 2009 *Photochem. Photobiol.* **85** 1391

[45] Silva J *et al* 2010 *Biochem. Pharmacol.* **80** 1373