Acta Biochimica Polonica

Vol. 50 No. 4/2003

1165–1174

QUARTERLY

Photodynamic effects of two water soluble porphyrins evaluated on human malignant melanoma cells *in vitro*

Agnieszka Szurko[1,2], Gabriela Krämer-Marek[1✉], Maria Wideł[2], Alicja Ratuszna[1], Jan Habdas[3] and Piotr Kuś[3]

[1]*Institute of Physics, University of Silesia, Katowice, Poland;* [2]*Department of Experimental and Clinical Radiobiology, Center of Oncology, Gliwice, Poland;* [3]*Institute of Chemistry, University of Silesia, Katowice, Poland*

Received: 17 May, 2003; revised: 28 September, 2003; accepted: 14 November, 2003

Key words: photodynamic therapy, synthetic porphyrins, malignant melanoma, tumour megacolonies *in vitro*, apoptosis, necrosis

Two water soluble porphyrins: meso-tetra-4-*N*-methylpyridyl-porphyrin iodide (P_1) and 5,10-di-(4-acetamidophenyl)-15,20-di-(4-*N*-methylpyridyl) porphyrin (P_2) were synthesised and evaluated in respect to their photochemical and photophysical properties as well as biological activity. Cytotoxic and phototoxic effects were evaluated in human malignant melanoma Me45 line using clonogenic assay, cytological study of micronuclei, apoptosis and necrosis frequency and inhibition of growth of megacolonies. Both porphyrins were characterised by high UV and low visible light absorptions. Dark toxicity measured on the basis of the clonogenic assay and inhibition of megacolony growth area indicated that P_1 was non-toxic at concentrations up to 50 μg/ml (42.14 μM) and P_2 at concentrations up to 20 μg/ml (16.86 μM). The photodynamic effect induced by red light above 630 nm indicated that both porphyrins were able to inhibit growth of melanoma megacolonies at non-toxic concentrations. Cytologic examination showed that the predominant mode of cell death was necrosis.

Photodynamic therapy (PDT), involving incorporation of photosensitising molecules into tumour cells and their destruction after excitation with visible light (Oleinick & Evans, 1998) offers an alternative treatment to conventional therapies and is increasingly accepted as a therapeutic modality in oncology (Wilson *et al.*, 1992; Nseyo *et al.*, 1998; Lavie

✉Corresponding author: Gabriela Krämer-Marek, Institute of Physics, University of Silesia, Uniwersytecka 4, 40-007 Katowice, Poland; tel. (48 32) 359 1501; fax (48 32) 258 8431; e-mail: rysia1@go2.pl

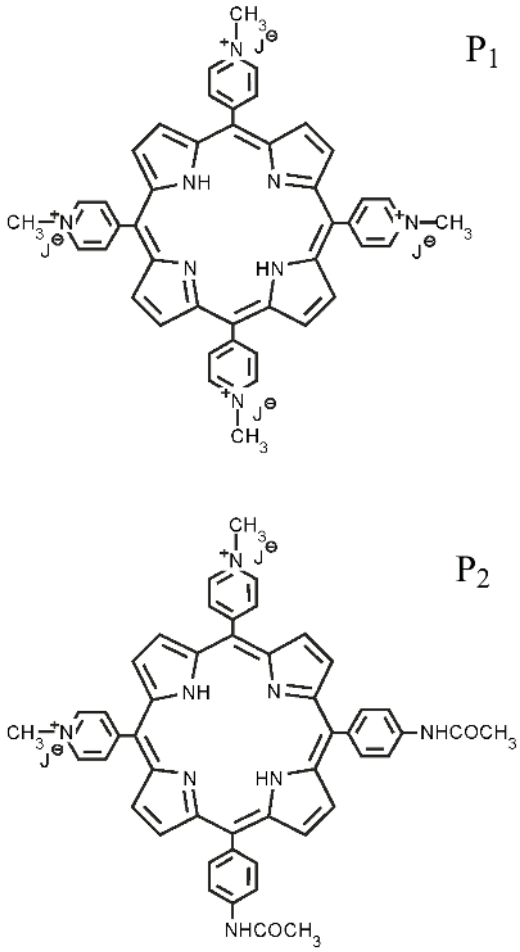
Abbreviations: DAPI, 4',6'-diamidino-2-phenyl · HCl; PDT, photodynamic therapy; P_1, meso-tetra-4-*N*-methylpyridyl-porphyrin iodide; P_2, 5,10 di-(4-acetamidophenyl)-15,20 di-(4-*N*-methylpyridyl) porphyrin.

et al., 1999; Dougherty, 2002). The most widely investigated photosensitisers in PDT are hematoporphyrin derivatives (HPD), among them Photofrin II, which has recently been approved for clinical treatment (Dougherty *et al.*, 1998). However, its use may be limited because of weak absorbance at the shorter range of the red region of the spectrum, and induction of long lasting skin photosensitivity. Furthermore, Photofrin II is not a pure substance but a mixture of hematoporphyrin monomers, dimers, oligomers and their dehydration products. Due to these limitations chemists were challenged to replace Photofrin with other porphyrins or new agents with bioactivity suitable for use in PDT. Currently, various second-generation photosensitisers, such as chlorins, phtalocyanins and others are under investigation (Wesley *et al.*, 2000; Drzewiecka *et al.*, 2001; Lottner *et al.*, 2002).

In the present study we tested the physicochemical and biological properties of two newly synthesised, water soluble porphyrins: meso-tetra-4-*N*-methylpyridyl-porphyrin iodide (P_1), and 5,10-di-(4-acetamidophenyl)-15,20-di-(4-*N*-methylpyridyl)-porphyrin (P_2). Cytotoxic and phototoxic effects were evaluated in human malignant melanoma cells using clonogenic assay, cytologic examination of micronuclei and analysis of apoptosis and necrosis frequency and inhibition of growth of megacolonies. Megacolonies introduced by Kummermehr *et al.* (1996) have been successfully used for investigations of radiotherapy response of recurrent clones of murine carcinoma in comparison with parent cells (Tarnawski *et al.*, 1998). Recently this system was used for the study of tumour cell repopulation during conventional and accelerated radiotherapy (Tarnawski *et al.*, 2003). Megacolonies, multicellular, flat structures growing attached to the bottom of culture flasks resemble in some respect tumours growing *in vivo*. A poorly proliferating multilayer dense core is surrounded by a monolayer rim of actively proliferating cells. The cytotoxic and phototoxic effect on a single megacolony can be measured as inhibition of growth of the megacolony and changes of its area relative to the initial size. This paper presents results of a study of the photodynamic efficiency of two water soluble porphyrins using the megacolony system and monolayer culture.

MATERIALS AND METHODS

The porphyrins tested. The compounds studied were: meso-tetra-4-*N*-methylpyridyl-porphyrin iodide, and 5,10-di-(4-acetamidophenyl)-15,20-di-(4-*N*-methylpyridyl)-porphyrin. Both agents were synthesised by the



Scheme 1. Porphyrin structures.

P_1, meso-tetra-4-*N*-methylpyridyl-porphyrin iodide and P_2, 5,10-di-(4-acetamidophenyl)-15,20-di-(4-*N*-methylpyridyl)-porphyrin.

Adler–Longo method involving refluxing 4-pirydyl aldehyde, or 4-acetamide benzaldehyde and 4-pirydyl aldehyde in propionic acid (Adler, Longo *et al.*, 1967). The porphyrins were made water soluble by converting into methyl iodide salts by treatment with a mixture of methyl iodide and nitromethane (Sersuro & Shoko, 1977; Lindsey *et al.*, 1989).

Cell line, culture and experimental model. The cell line used throughout the study was human malignant melanoma, Me45. This line was derived from a primary culture of lymph node metastasis of skin melanoma originally located on the temple of a 35-year-old male patient and was established at the Department of Experimental and Clinical Radiobiology, Center of Oncology in 1997 (Kumala *et al.*, 2003). Cells were routinely maintained as a monolayer culture in growth medium, DMEM (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich, Germany) supplemented with 12% foetal bovine serum (Gibco BRL, Scotland) and antibiotics: 100 IU/ml penicillin, 100 μg/ml streptomycin and 80 μg/ml gentamycin (Polfa, Poland).

To generate megacolonies, the technique described by Tarnawski *et al.* (2003) was used. Briefly, about 50 μl of cell suspension (about 5×10^4 cells) was carefully placed as single drops at the bottom of flasks (5 drops per 25 cm^2 flask, Nunc) and left in a humidified incubator (with 5% CO_2) for 24 h for attachment. Then the cultures were washed with serum-free medium to remove non-attached cells and 5 ml of fresh growth medium was added. Following further 4 or 5 days of incubation the megacolonies reached diameters of about 5 mm and at that size were used for experiments.

Clonogenic assay of cytotoxicity. In the first step dark toxicity of the porphyrins was estimated in clonogenic cell survival assay, a method often used in PDT studies (West & Moore, 1988). Appropriate aliquots of cells were seeded onto culture dishes (Nunc) of 5-cm diameter and left in a CO_2 incubator for attachment. Eighteen hours later the medium was replaced by 5 ml of fresh medium containing different concentrations of porphyrins (0–100 μg/ml) and the cells were incubated for 72 h. Then the cells were washed, to remove any traces of porphyrins, resuspended in fresh medium and left for another 7 days to obtain macroscopically visible colonies. The colonies were fixed in ethanol and stained with 1% crystal violet. Only colonies containing at least 50 cells were counted. Surviving fractions were calculated as the ratios of plating efficiencies (number of colonies/number of cells seeded) for a given concentration of the agent to the plating efficiency of the control.

Cytotoxicity and phototoxicity assay by the megacolony system. When the megacolonies reached a diameter of about 5 mm, the initial megacolony contour was drawn with a thin marker, copied onto transparent foil and scanned by computer scanner. The growth areas were then measured using the computer software "Image Tool".

For cytotoxicity experiments the growth medium was replaced by fresh one containing different concentrations of porphyrins (0–100 μg/ml). After 48 or 72 h the incubation medium with porphyrins was replaced by fresh complete medium and the cells were further incubated for 7–14 days. The cytotoxic effect was calculated as percent inhibition of megacolony growth at a particular porphyrin concentration in relation to the initial megacolony size.

To evaluate the photosensitising efficiency, megacolonies were incubated for 48–72 h with nontoxic (chosen on the basis of the cytotoxicity experiments) concentrations of porphyrins (20 μg/ml and 15 g/ml for P_1 and P_2, respectively). Prior to irradiation the megacolonies were washed and covered with 2 ml of ice cold phosphate buffered saline, pH 7.4 (PBS). Each colony was exposed to different doses of energy ranging between 13.5–81 J/cm^2 (irradiation time 5–30 min) at room temperature (21°C). Light was generated by a halogen lamp, type LH313K, equipped with a

filter cutting off the wavelengths below 630 nm. Following irradiation the colonies were incubated in growth medium for 7–14 days and growth areas were measured. Experiments were performed in triplicate.

Cytologic estimation of cell damage. To determine the biological effects of porphyrins on cell viability, cells were seeded into 96-well microplates, allowed to attach for 24 h and then the test compounds, prepared in growth medium, were added. After further 48 h of incubation in a humidified CO_2 incubator, the medium was replaced by serum-free medium without the compounds added and individual wells were irradiated for 20 min. Following irradiation serum was added to the wells to reach a final concentration of 12% and the cells were further incubated for 24, 48 and 72 h, or immediately used for analysis. Additional batch of microplates was incubated without being irradiated for the dark toxicity assessment. At a proper time, floating and attached cells (trypsinized) were combined, centrifuged and fixed in 96% ice cold ethanol. After re-fixation in a methanol/acetic acid (3:1, v/v) mixture the cells were dropped onto microscope slides and dried overnight. Then the slides were stained with the DNA binding fluorescent dye DAPI (4′,6′-diamidino-2-phenyl · HCl, Serva Germany) and analyzed with a fluorescent microscope (Axiophot, Zeiss, Germany) simultaneously using phase contrast to visualise the cell membrane. Intact (healthy) cells, those containing micronuclei as well as apoptotic and necrotic cells were scored according to morphologic criteria (Guo *et al.*, 1997; Fenech *et al.*, 1999). Healthy cells were discriminated based on their regular cell and nuclear shape and also nuclear staining weaker than in damaged cells. For examination of photodynamic properties 4000 cells were scored in total for each dose (1000 cells per slide, four slides per point prepared in two independent experiments) and the percentage of apoptotic and necrotic cells as well as cells with micronuclei were calculated.

Measurement of spectral properties. The absorbance was measured in the wavelength range of 380–700 nm using double-beam UV/VIS spectrophotometer Yasco V-530. For absorbance spectra analysis both porphyrins were dissolved in water in a range of concentrations.

RESULTS

Absorption spectra. Absorption spectra of both porphyrins are characterised by a Soret band in the violet region and four wide Q-bands located in the 500–650 nm region (Table1). The absorption coefficients of the bands were obtained from the Beer–Lambert law using 10^{-4}–10^{-6} M solutions. They were comparable to those for similar compounds (Kwaśny & Graczyk, 1990). No tendency to aggregate was observed. However, this does not exclude the possibility of aggregation inside cells, which is often found for such compounds. No significant difference was observed in the shape of the UV-VIS spectra of the compounds studied (not presented). Only a small difference in the peak positions was seen. This similarity may indicate that the photodynamic properties should not vary considerably from one compound to another.

The high absorbance in the Soret band and significantly lower one for the Q bands indicate suitability of the studied porphyrins in photodynamic therapy (PDT) and diagnosis (PDD). The longest-wave absorption bands at 642 and 648 nm observed for P_1 and P_2, respectively, are the most important ones regarding PDT, because only red light has sufficient tissue penetration ability.

Porphyrin cytotoxicity

As shown on the photograms (Fig. 1) apoptotic cells were characterised by cellular shrinkage and nuclear condensation in early stages of apoptosis and nuclear fragmentation in later ones. Necrotic cells were charac-

Table 1. Absorption spectra for meso-tetra-4-*N*-methylpyridyl-porphyrin (P_1) and 5,10-di-(4-acetamidophenyl)-15,20-di-(4-N-methylpyridyl) porphyrin (P_2)

		P_1*	P_2**
Q_x	λ_{max}[nm]	422	433
	$\varepsilon_{0\text{-}0}\left[\frac{dm^3}{mol \cdot cm}\right]$	3.26×10^5	3.80×10^4
Q_x	λ_{max}[nm]	518	523
	$\varepsilon_{1\text{-}0}\left[\frac{dm^3}{mol \cdot cm}\right]$	2.20×10^4	6.9×10^3
Q_y	λ_{max}[nm]	554	566
	$\varepsilon_{0\text{-}0}\left[\frac{dm^3}{mol \cdot cm}\right]$	8.1×10^3	5.1×10^3
Q_y	λ_{max}[nm]	584	588
	$\varepsilon_{1\text{-}0}\left[\frac{dm^3}{mol \cdot cm}\right]$	9.3×10^3	4.3×10^3
B	λ_{max}[nm]	648	642
	$\varepsilon_{0\text{-}0}\left[\frac{dm^3}{mol \cdot cm}\right]$	2.5×10^3	2.8×10^3

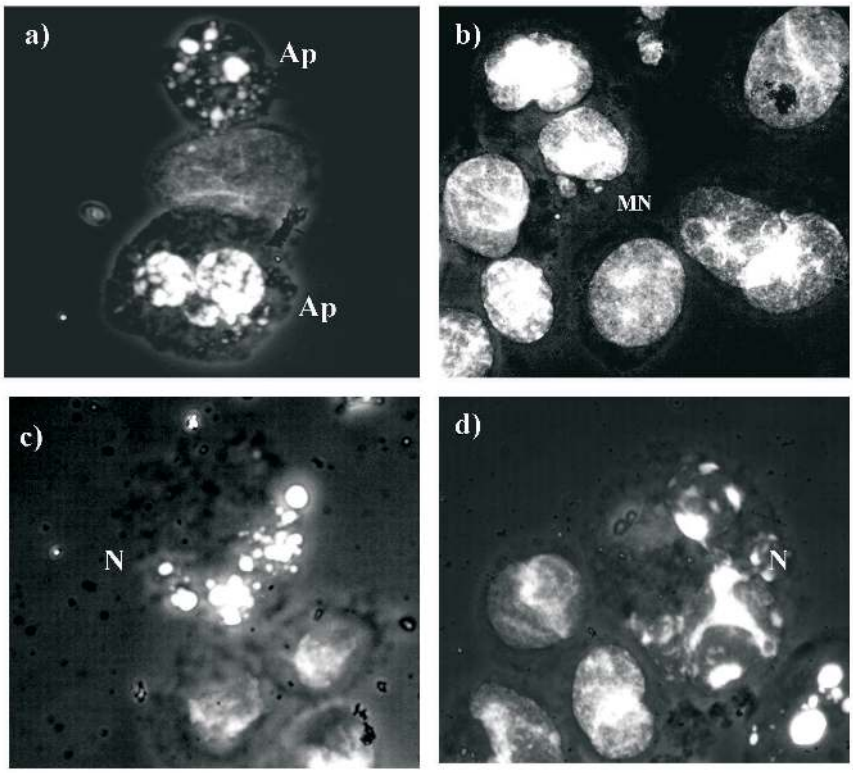


Figure 1. Pictures of apoptotic (a), necrotic (c, d) and cells with micronuclei (b) after treatment of Me45 melanoma with P_2 porphyrin and light exposure.

DAPI-stained cells observed in fluorescence and phase contrast; original magnification 400×.

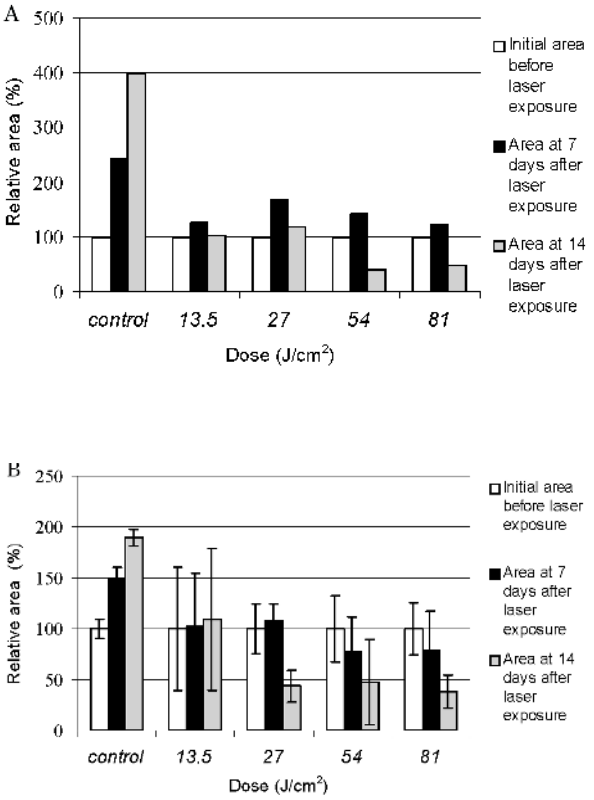


Figure 2. Reduction of areas of Me45 megacolonies after exposition to laser light and 48 h pre-treatment with P_1 porphyrin at 20 μg/ml (A), and with P_2 porphyrin at 15 μg/ml (B).

Significant inhibition of growth of P_1-treated and lighted for 20 min (P = 0.034) and P_2-treated and lighted for 10–30 min ($P < 0.02$) megacolonies was observed after 14 days post-treatment incubation. One way ANOVA was used for statistical analysis.

terised by cellular swelling, plasma membrane disintegration and irregular nuclear fragmentation. On long term incubation with melanoma cells porphyrin P_1 was relatively non-toxic and the surviving fraction of clonogenic cells was close to 1.0 up to the concentration of 20 μg/ml. The surviving fraction of P_2-treated clonogenic cells was unchanged up to 15 μg/ml, but started to drop at higher concentrations, reaching the value of 0.5 at 50 μg/ml (not presented). Treatment of megacolonies with 0–100 μg/ml of the porphyrins for 48 h also had no significant cytotoxic influence on their growth as measured by area increment. The growth area of Me45 megacolonies measured 7 day post treatment with 20 μg/ml P_1 increased to 150 ± 7% of the pre-treatment size, whereas for control it increased to 145 ± 10% (not presented). Under the same conditions P_2 was non-toxic at concentrations up to 15 μg/ml but at higher doses a slight, insignificant inhibition of growth areas was observed (not presented). On the basis of these data we considered the concentration of 20 μg/ml and 15 μg/ml for P_1 and P_2, respectively, as non-toxic and used them for photodynamic study.

Photodynamic effect

The Me45 melanoma was sensitive to photodamage dependent on the energy applied. Growth of megacolonies was initially inhibited and later the megacolonies were partially destroyed, therefore their areas diminished. The most pronounced shrinkage of megacolonies was observed after 14 days for megacolonies treated with P_1 and exposed to light for 20 and 30 min (Fig. 2A). A considerable megacolony shrinkage after P_2 treatment was observed on the 14th day post exposition to light for 10–30 min (Fig. 2B). Control experiment showed that irradiation alone did not inhibit megacolony growth (not presented).

Micronuclei, apoptosis and necrosis

Microscopic analysis of cellular damage indicated that cell death due to necrosis was the predominant biological effect of photodynamic action of both porphyrins under study (Fig. 3A). No necrotic cells were present right after 20 min of irradiation, however after 24 h they amounted 20% and after 48 h they constitute 30% in the case of both compounds. At 72 h post irradiation the level of necrosis reached almost 40 and 45% for P_1 and P_2, respectively. The frequency of necrosis induced by photodynamic treatment with either agent was significantly higher as compared to the effect of irradiation alone, porphyrin alone or

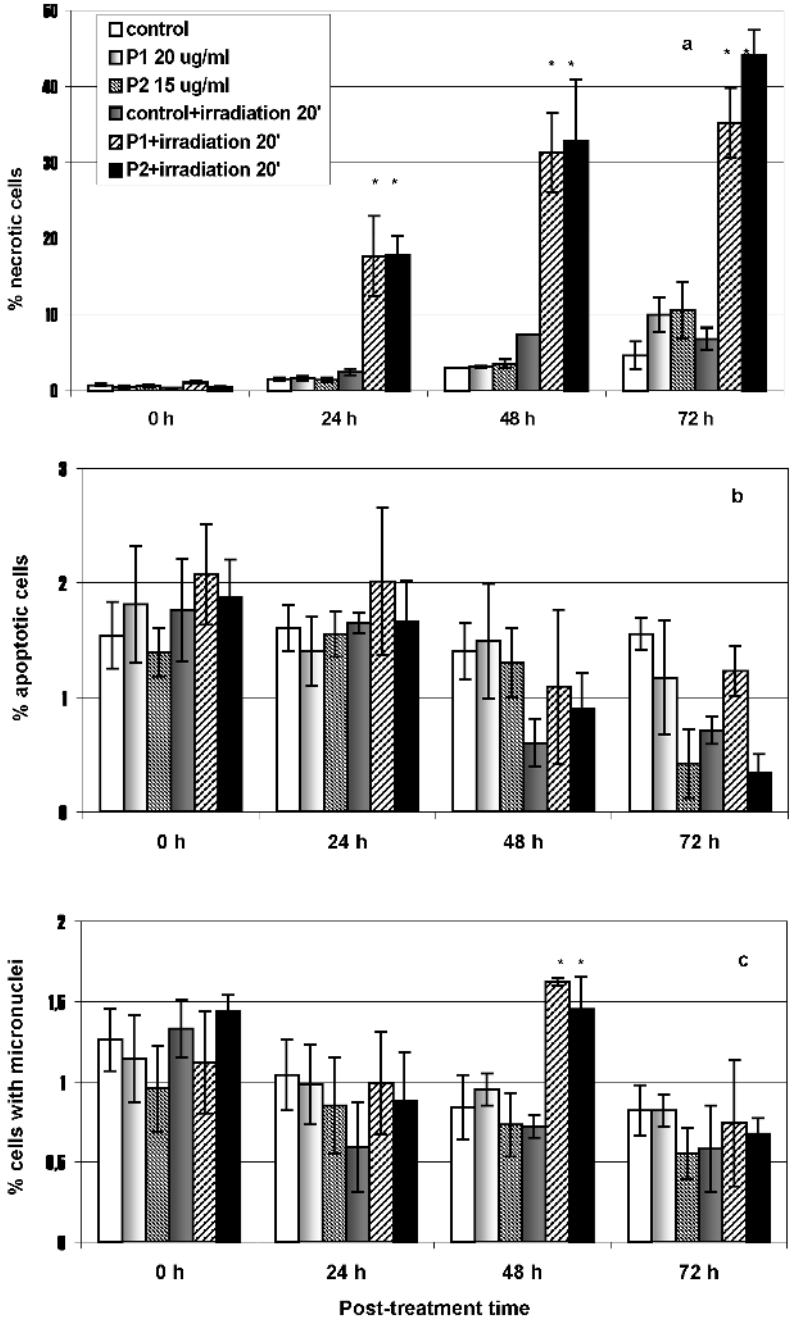


Figure 3. Morphologic study of cellular damage: micronuclei (A), apoptosis (B) and necrosis (C).
Significant difference with appropriate non-irradiated control at $P < 0.05$ level indicated by asterisks.

no treatment at all ($P < 0.05$). At the same time when necrosis increased, apoptosis frequency remained unchanged (24 h), or was diminished in comparison with untreated control (Fig. 3B, 48 h and 72 h). Slight but significant increase of micronuclei frequency was observed only after 48 h post irradiation in both groups treated with porphyrins and light (Fig. 3C).

DISCUSSION

This study is a part of a complex project aimed at synthesis of new porphyrin derivatives, their physico-chemical characterisation and evaluation of biological efficiency. The assays most frequently used in experimental PDT are cell survival assay based on colony formation (West & Moore, 1988) or an assay

based on cell viability, e.g. tetrazolium reduction colorimetric method (MTT assay) (Yow *et al.*, 2000). In our study we used a new model of tumour *in vitro* culture, the megacolonies. In our opinion these multicellular, flat but multilayer structures have some advantage over monolayer or cell suspension cultures since megacolonies can be observed for a long period without the necessity of passaging. The agents used in this study are chemically defined porphyrin derivatives which can be superior to Photofrin. Their red absorption bands are all at longer wavelengths than the bands of Photofrin II, but the differences are not considerable (642 nm for P_1 and 648 P_2 compared with 627 for Photofrin). Thus, they are not expected to produce a large increase in tissue penetration by light. However, both agents exerted their photodynamic effect at non-toxic concentration towards human melanoma cells *in vitro*. Although the concentrations of the porphyrins used in the present work were high, they were within the range of hematoporphyrin concentrations applied by other authors (Yow *et al.*, 2000).

We used tumour growth inhibition as a main criterion of the photodynamic activity of our compounds. In fact we observed prolonged cell death, visible both as megacolony growth inhibition or even shrinkage, and substantial cell loss from the megacolonies. However, inhibition of megacolony growth alone does not explain the nature of cellular death, apoptosis or necrosis, since either can be the way of death after PDT (Ahmad & Mukhtar, 2000). These mechanisms can be different in different cell lines. Therefore, we wanted to investigate the exact nature of the cell death that was induced in M45 melanoma cells by our porphyrin derivatives and to this end we studied cellular damage and viability. Cell viability has been widely studied *in vitro* and *in vivo* and the morphological patterns of apoptosis and necrosis are well known. Microscopic assessment of apoptosis and necrosis is possible due to differential fluorescent staining (Foglieni *et al.*, 2001; Jajte *et al.*, 2002). Combining fluorescent staining with DAPI with simultaneous observation of cells in phase contrast allows successful measurement of three types of cell damage: micronuclei, apoptosis and necrosis (Przybyszewski *et al.*, 2002; Tarnawski *et al.*, 2003). We applied the same technique in the present study in order to gain some insight into the nature of cell death occurring as an effect of the photodynamic action of porphyrin. As can be seen from Fig. 3 the porphyrins studied were not genotoxic in the dark when cytogenic damage, micronucleus induction was measured. However, after 48 h a slight but statistically significant increase of micronucleus frequency was noticed following photodynamic action of P_1 and P_2 (Fig. 3C). There was also no increase of apoptosis after irradiation with either porphyrin in comparison with controls (Fig. 3B). This may be an intrinsic property of the cell line used in this study, as it has been observed that Me45 line is resistant to apoptosis following ionizing irradiation and also after treatment with cytostatic drugs (Kumala *et al.*, 2003). Furthermore, the observed necrosis can result from the relatively high concentration of porphyrins and high doses of light (81 J/cm^2). In experiments performed on HEC1-B endometrial carcinoma cells, Varriale *et al.* (2002) observed that hypericin, another type of photosensitizer, used with light doses ranging between 2 and 5 J/cm^2 induced mainly apoptosis, while above 6 J/cm^2 necrosis prevailed. The low efficiency of induction of DNA damage leading to micronuclei and lack of apoptosis after photodynamic action of both porphyrins and high incidence of necrosis suggest that the main targets for their photobiological activity are cellular membranes. However, elucidation of the mechanism requires further studies.

We are very grateful to Dr. J. Oszajca from the Department of Biophysics, Jagiellonian University for making available the halogen lamp.

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