

# The effect of porphyrins on normal and transformed mouse cell lines in the presence of visible light

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## Abstract

Photodynamic therapy consists of the uptake of a photosensitizing dye, often a porphyrin, by tumor tissue and subsequent irradiation of the tumor with visible light of an appropriate wavelength matched to the absorption spectrum of the photosensitizing dye. This class of molecules produces reactive oxygen species when activated by light, resulting in a direct or indirect cytotoxic effect on the target cells. Photodynamic therapy has been used in the treatment of cancer but the technology has a potential for the treatment of several disease conditions mainly because of its selectivity. However, it is not clear why the porphyrins are retained preferentially by abnormal tissue. This paper describes a study of the effect of the association of porphyrin and visible light on two mouse fibroblast cell lines: A31, normal cells and B61, an EJ-ras transformed variant of A31. Two water-soluble porphyrins were used, a positively charged one, tetra(N-methyl-4-pyridyl)porphyrin chloride, and a negatively charged one, tetra(4-sulfonatophenyl)porphyrin-Na salt (TPPS<sub>4</sub>) in order to assess the effect on cell survival. The results suggest that the B61 cell line is more sensitive to incubation with the anionic porphyrin (TPPS<sub>4</sub>) followed by light irradiation and that the anionic porphyrin is more efficient in killing the cells than the cationic porphyrin.

## Key words

- Water-soluble porphyrins
- Mouse fibroblast lines A31 and B61
- Photodynamic therapy
- Visible light

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## Introduction

Photodynamic therapy (PDT) is a relatively new modality used for cancer treatment, consisting of the combined use of systemically administered photosensitizing porphyrins and local applications of light emitted by lamps or lasers in the presence of oxygen. The technique is based on the preferential accumulation of porphyrin in tumor tissue with subsequent cytotoxicity mediated by singlet oxygen production (1). Its therapeutic usefulness is due to selective and rapid tumor necrosis after illumination of the

porphyrin-containing tumor with visible light (2). Tumor necrosis seems to be preceded by significant alterations in the vascular system of the tumor (1,2). Hematoporphyrin derivative (HpD), first developed by Lipson in 1960, and a purified fraction, dihematoporphyrin ether, known as Photofrin II<sup>®</sup>, are complex mixtures used for tumor localization and treatment (3). Since then, HpD has been applied to endoscopic detection and PDT of various malignant neoplasms including superficial bladder cancer, lung cancer, and cancer of the skin and upper aerodigestive tract (4). HpD has low toxicity and can

be administered at high doses, having no therapeutic value by itself for treating cancer. However, this dye has photodynamic properties, i.e., in the presence of oxygen and visible light it becomes highly destructive to its immediate environment (5). Porphyrin has been the preferred drug used as a photosensitizing substance due to its high affinity for the tumor cell and its strong effect under irradiation with light (6). Different photosensitizers have been tested for localization in human tumor tissues, including fluorescein, cyanine dyes, porphyrin compounds, eosin, tetracycline, acridine orange and Rhodamine-123 (7). *In vivo*, the photosensitizer mainly accumulates in organs of the reticuloendothelial system (liver, spleen, kidney, and lungs), atherosclerotic plaques, areas of inflammation and healing wounds (8).

The cationic, meso-substituted porphyrin tetra(N-methyl-4-pyridyl)porphyrin chloride (TMPyP) has been described as a potent photosensitizer *in vivo* (9) and *in vitro* (10). This porphyrin is a DNA intercalator *in vitro* (11) and can photoinduce genotoxic effects in tumor cells in culture (10). Many water-soluble porphyrins form complexes with albumins, the main protein of blood plasma (12,13). Water-soluble porphyrins compete with hydrophobic species for binding sites in albumin, thus increasing the binding of water-soluble porphyrins to membranes and consequently the effectiveness of PDT (14, 15). Moreover, the high affinity and phototoxicity of water-soluble sulfonated meso-tetraphenylporphyrins (TPPS<sub>4</sub>) themselves for tumor cells allow their consideration as promising compounds for use in PDT (16).

This paper presents the results of a study using two water-soluble porphyrins associated with visible light in two cell lines in order to examine their effect on cell survival. A cationic porphyrin (TMPyP) and an anionic porphyrin (TPPS<sub>4</sub>) were used in their free base form on a normal cell line originating from mouse fibroblasts (A31) and a transformed cell line originating from A31 by

transfection (B61). The choice of this class of porphyrins as models is justifiable for this study since one can compare the effect of the charges on the molecules on their cytotoxic effect on cells.

## Material and Methods

### Chemicals

Tetra(N-methyl-4-pyridyl)porphyrin chloride (TMPyP) and tetra(4-sulfonatophenyl)porphyrin-Na salt (TPPS<sub>4</sub>) were purchased from Mid Century Chemicals (Posen, IL, USA). Stock solutions were prepared in water and diluted in Dulbecco's modified Eagle's medium (DMEM) with 1% FBS just before use.

### Cell lines

The normal cell line used was A31, originating from genetically pure BALBc/3T3 mice (17) and consisting of embryo fibroblasts. The transformed cell line B61, also originating from BALBc mice, was obtained from A31 by transfection of the human EJ-ras oncogene; B61 cells are transformed and tumorigenic (18). The cells were grown in 25-cm<sup>2</sup> flasks containing DMEM supplemented with 10% FBS, streptomycin and ampicillin. The cells were kept at 37°C and 5% CO<sub>2</sub> and harvested with 0.2% trypsin + 0.02% EDTA. Cell viability was assessed by the Trypan blue method.

### Cytotoxic assays

The cells were plated at a density of 10<sup>2</sup> cells/ml with DMEM + 10% FBS onto 35-mm polypropylene Petri dishes, grown at 37°C and 5% CO<sub>2</sub> for 24 h, washed with PBS, and treated with cationic or anionic porphyrin at concentrations ranging from 0 to 30 μM for 1 h at 37°C. After drug removal, the cells were washed three times with PBS and incubated for several days in ordinary

supplemented medium until macroscopic colonies could be observed. The cells were then stained with Giemsa and the colonies counted directly on the plates or on a computer screen after scanning the plates.

### Effect of the combination of porphyrin and light

The cells were plated in triplicate onto 35-mm polypropylene Petri dishes containing DMEM + 10% FBS at a density of  $5 \times 10^4$  cells/ml. Cells were grown at  $37^\circ\text{C}$  for about 48 h in order to reach a density of about  $10^5$  cells/ml. Cells were harvested and counted every day. Immediately before drug addition, three plates were counted. Then the monolayer was washed with PBS and treated with  $9 \mu\text{M}$  porphyrin in DMEM + 1% FBS for 20 min. The cells were washed three times with DMEM + 1% FBS and ordinary supplemented medium was added. The cells were then exposed to visible light going through a 3-cm high layer of water in order to avoid cell heating. The medium temperature was monitored with a thermocouple. The distance between the cells and the 150-W lamp was 13 cm and the illumination time was 45 min. A blue filter was used to select the wavelength in the range of 500 to 650 nm. The cells were then incubated in triplicate and counted after 30 min, 2 h, 4 h and then every day until the time when they were trypsinized, stained with Trypan blue and counted in a hemocytometer. Viability was calculated and viability curves as a function of time were obtained.

### Results

The cytotoxic assays performed with the B61 cell line allowed the determination of the  $\text{IC}_{50}$  values for the porphyrins. The  $\text{IC}_{50}$  for porphyrins in B61 cells were  $2 \pm 1 \mu\text{M}$  ( $N = 3$ ) and  $5 \pm 1 \mu\text{M}$  ( $N = 2$ ) for TMPyP and TPPS<sub>4</sub>, respectively, at pH 7.0 in PBS. The results suggest that the cationic porphyrin is more cytotoxic for this transformed cell line,

since its  $\text{IC}_{50}$  was about 2.5-fold lower than the one obtained for the anionic porphyrin.

In order to assess the effect of the combination of porphyrin and light, the growth curves were monitored in the presence of porphyrin with and without illumination. It should be mentioned that, as a control, in all experiments the cells were exposed to light under the same conditions and for the same period of time in the absence of a photosensitizer. No alteration was observed in any of the parameters of the growth curves for either cell line. This was seen as an indication that light by itself does not affect the cells in the absence of a photosensitizer.

Figure 1 presents the growth curves, i.e., the number of cells as a function of time for B61 (A) and A31 (B) cells alone, in the presence of anionic porphyrin and anionic porphyrin associated with light. It was observed that all the parameters of the curve were changed. From the figure, we may assume that the B61 cells will take more time to become confluent after being treated with the drug, a fact that was still more pro-

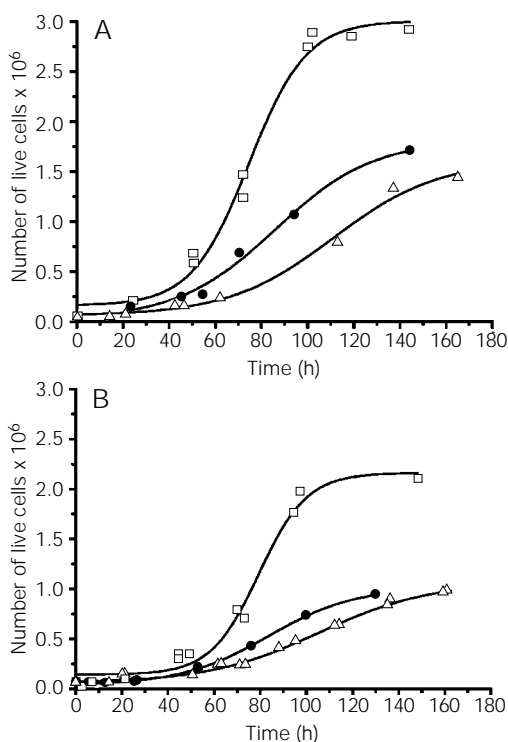


Figure 1. Growth curves for B61 cells (A) and for A31 cells (B) treated with  $9 \mu\text{M}$  of anionic porphyrin (TPPS<sub>4</sub>). Control: cells with no photosensitizer and no light (squares). Cells with photosensitizer and no light (circles); cells with photosensitizer after light irradiation for 45 min (triangles).

nounced in the presence of light. The values for the doubling time were affected after incubation with TPPS<sub>4</sub> alone (a 66% increase for B61 and a 54% increase for A31) and with light (89 and 87% increases for B61 and A31, respectively). Viability determined on the basis of the time the cells reached saturation density decreased from 100 to about 66% in the presence of TPPS<sub>4</sub> alone and to 52% in the presence of TPPS<sub>4</sub> plus light for B61. It can be seen that incubation with porphyrin killed about 34% of B61 cells and incubation with porphyrin plus light killed as much as 48% of the cells. We may suggest that the toxicity of irradiation not only affected the population of living

cells but also that the damage continued during growth, preventing the recovery of normal metabolism. These results support the idea that photochemical treatment of the cells greatly increases the efficiency of porphyrin in the control of cell number in a tumor. For the normal A31 cells, cell viability remained high (around 95%) before and after the treatment and irradiation.

Figure 2 presents cell viability as a function of time for both cell lines after treatment with the anionic porphyrin (TPPS<sub>4</sub>) and light irradiation. From the figure one can see that in fact the cells can recover from treatment to a different extent, but it is quite clear that B61 cells are much more affected and cannot reach more than 50% of viability even after a long time, while A31 can recover quite well.

Figure 3 shows the effects of the positively charged TMPyP on the growth of B61 cells. These curves suggest that these cells are not very much sensitive to the presence of this porphyrin both as a drug itself or as a photosensitizer, i.e., after irradiation. In the presence of this porphyrin, the log phase had not suffered alteration and the doubling time presented an increase of just 21% after incubation with porphyrin and about 36% with light exposure. The viability of B61 cells decreased by 23% after TMPyP treatment and by 32% when treatment was combined with light irradiation. Thus, the results show that cationic porphyrin is less cytotoxic than anionic porphyrin both in the dark and after light irradiation.

Figure 2. Viability as a function of time for A31 (squares) and B61 cells (circles) after treatment with the anionic porphyrin (TPPS<sub>4</sub>) and light irradiation.

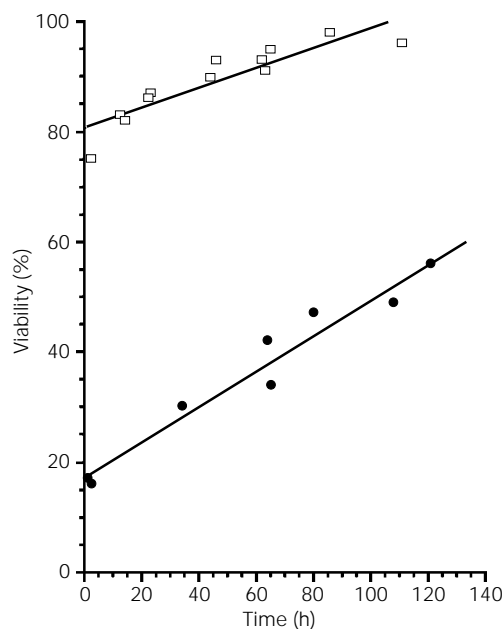
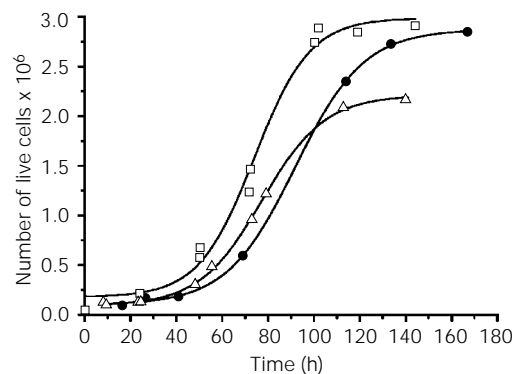


Figure 3. Growth curves for B61 cells treated with 9  $\mu$ M of cationic porphyrin (TMPyP). Control: cells with no photosensitizer and no light (squares). Cells with photosensitizer and no light (circles); cells with photosensitizer after light irradiation for 45 min (triangles).



## Discussion

Both porphyrins used in this study presented a slight toxicity for both cell lines in the dark, i.e., the ability to kill cells in the absence of light when used at therapeutic doses. A relatively low porphyrin concentration was used, 9  $\mu$ M (which corresponds to 7 mg/ml for cationic porphyrin and 11 mg/ml for anionic porphyrin) and the exposure time

to the porphyrins was only 20 min. It is desirable that the photosensitizer have a high toxicity just after illumination, a parameter that defines the therapeutic effectiveness of the compound. Both porphyrins also meet the conventional requirement for photosensitizers to have a homogeneous chemical composition and high solubility in water, facilitating intravenous administration to the patient.

The viability curve as a function of time for both cell lines after treatment with the photosensitizer and light irradiation clearly showed that in fact transformed and normal cells can recover from treatment to a different extent. It is evident that B61 cells are much more affected by treatment with the anionic porphyrin and cannot reach more than 50% viability even after a long period of time, while A31 can recover completely.

We may conclude that in the experiments performed with the anionic porphyrin (TPPS<sub>4</sub>) with no light irradiation the more affected cell line was B61 since the doubling time was more affected but cell viability was the parameter showing more sensitivity to the presence of this anionic porphyrin. In the experiments where light exposure was used after porphyrin incubation, B61 was also the more affected cell line.

In the experiments with the cationic porphyrin, the cell line B61 presented only a slight increase in doubling time (36%) compared to the results obtained for the anionic porphyrin (89%) after visible light irradiation. The viability determined by the time the cells reached saturation density was about 68%, higher than for TPPS<sub>4</sub> in this cell line (52%). Thus, these numbers suggest that the B61 cell line is more sensitive to the effects of the anionic porphyrin (TPPS<sub>4</sub>).

On the basis of the results obtained, we may conclude that, for both cell lines studied, the anionic porphyrin is more efficient in killing the cells as a photosensitizer when incubated for 20 min and submitted to light irradiation for 45 min. The present results showed that, in fact, there is a considerable

decrease in the cell population after porphyrin incubation followed by light irradiation, suggesting that this treatment can be employed as a method to kill tumor cells.

It is well known that PDT leads to apoptosis. However, this event was not investigated in the present study and it is important to say that the approach used here may have a limitation by underestimating the number of affected cells after treatment.

It is interesting to point out that B61 cells (transformed cells) were considerably more affected than A31 cells (normal cells), supporting the hypothesis that porphyrins accumulate rapidly and preferentially in tumor cells. This fact is extremely important for the application of PDT to the treatment of cancer, since the porphyrin is administered intravenously and is supposed to kill only the malignant cells after light irradiation. Thus, the therapeutic usefulness of PDT is based on the preferential accumulation of porphyrin in tumor tissue. To the best of our knowledge, no report in the literature showing this enormous difference in the effect of porphyrin comparing normal and transformed or tumor cell lines has been previously presented.

Our results also suggest that important membrane alterations should take place in the tumor cells causing the selectivity of cell accumulation of the photosensitizing dye that will generate the toxic species leading to cell death.

The results presented here concerning the higher effectiveness of the anionic porphyrin (TPPS<sub>4</sub>) compared with the cationic one (TMPyP) have been confirmed in our laboratory using a human tumor cell line irradiated with laser light.

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## References

1. Karrer S, Szeimies R-M, Abels C & Landthaler M (1998). The use of photodynamic therapy for skin cancer. *Onkologie*, 21: 20-27.
2. Dougherty TJ, Potter WR & Weishaupt KR (1984). The structure of the active components of haematoporphyrin derivative. In: Andreoni A & Cubedda R (Editors), *Porphyryns in Tumour Phototherapy*. Plenum Press, New York, 23-35.
3. Sibata CH, Colussi VC, Oleinick NL & Kinsella TJ (2000). Photodynamic therapy: a new concept in medical treatment. *Brazilian Journal of Medical and Biological Research*, 33: 869-880.
4. Schuitmaker JJ, Baas P, van Leengoed HLLM, van der Meulen FW, Star WM & van Zandwijk N (1996). Photodynamic therapy: a promising new modality for treatment of cancer. *Journal of Photochemistry and Photobiology. B: Biology*, 34: 3-12.
5. Sternberg ED, Dolphin D & Brucker C (1998). Porphyrin-based photosensitizers for use in photodynamic therapy. *Tetrahedron*, 54: 4151-4202.
6. Sekiya S, Kubota K, Kasai T, Iwasaki H, Yamauchi K, Takamizawa H & Tenjin Y (1988). Cytocidal effects of hematoporphyrin derivative and argon dye laser on human gynecologic tumor cells in vitro. *International Journal of Gynecology and Obstetrics*, 26: 151-158.
7. Haghghat S, Castro DJ, Lufkin RB, Fetterman HR, Castro DJ, Soudant J, Ward PH & Saxton RE (1992). Laser dyes for experimental phototherapy of human cancer: comparison of three rhodamines. *Laryngoscope*, 102: 81-87.
8. Hamblin MR & Newman EL (1994). On the mechanism of the tumor localizing effect in photodynamic therapy. *Journal of Photochemistry and Photobiology. B: Biology*, 23: 3-8.
9. Villanueva A & Jori G (1993). Pharmacokinetic and tumour-photosensitizing properties of the cationic porphyrin meso-tetra (4N-methylpyridyl) porphine. *Cancer Letters*, 73: 59-64.
10. Villanueva A, Juarranz A, Diaz V, Gomez J & Cañete M (1992). Photodynamic effects of a cationic mesosubstituted porphyrin in cell cultures. *Anti-Cancer Drug Design*, 7: 297-303.
11. Fiel RJ, Datta-Gupta N, Mark EH & Howard JC (1979). Interaction of DNA with a porphyrin ligand: evidence for intercalation. *Nucleic Acids Research*, 6: 3093-3118.
12. Lamola AA, Asher I, Muller-Eberhard U & Poh-Fitzpatrick M (1981). Fluorimetric study of the binding of protoporphyrin to hemopexin and albumin. *Biochemical Journal*, 63: 693-698.
13. Datta-Gupta N, Malakar D & Dozier J (1989). Binding studies of 4 free base porphyrins and 6 iron (+3) porphyrins with human-serum albumin. *Research Communications in Chemical Pathology and Pharmacology*, 63: 289-292.
14. El-Far MA & Pimstone NR (1986). Selective in vivo tumor-localization of uroporphyrin isomer-I in mouse mammary carcinoma - superiority over other porphyrins in a comparative study. *Cancer Research*, 46: 4390-4394.
15. El-Far MA, El-Hamil NA & Ghoneim M (1988). Selective in vivo localization of heptacarboxylic porphyrin isomer in a bladder tumor model - a novel technique to modulate porphyrin localization. *Biochemistry*, 70: 1379-1384.
16. Berg K, Bommer JC, Winkelman JW & Moan J (1990). Cellular uptake and relative efficiency in cell inactivation by photoactivated sulfonated meso-tetraphenylporphyrins. *Photochemistry and Photobiology*, 52: 775-781.
17. Aaronson SA & Todaro GJ (1968). Development of 3T3-like lines from BALB-c mouse embryo cultures: transformation susceptibility to SV-40. *Journal of Cellular Physiology*, 72: 141-148.
18. Kovary K, Armelin MCS & Armelin HA (1989). HA-RAS-1 oncogene dosage differentially affects BALB/3T3 cells growth-factor requirement and tumorigenicity. *Oncogene Research*, 4: 55-64.