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In vitro evaluation of the genotoxic and clastogenic potential of photodynamic therapy

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Photodynamic therapy (PDT) was recently introduced in clinical practice for the management of cancer. As far as PDT relies on the combined action of a photosensitizer and a laser source, there is a need to evaluate the genotoxic and mutagenic potential of this treatment modality. This paper reports the effects of various photosensitizer and photo-irradiation doses on lethality to the MIA PaCa cell line using $ZnPcS_4$ as the photosensitizer. The sister chromatid exchange (SCE) assay was used to evaluate the genotoxicity of various photosensitizer and photo-irradiation doses. Also, chromosomal aberrations at various time intervals post-irradiation were evaluated. The results showed that a combination of 3 J/cm^2 irradiance with 5 μM $ZnPcS_4$ concentration leads to the LD_{90} 72 h post-irradiation. Eight days post-irradiation the LD_{90} level was achieved using a light dose of 3 J/cm^2, independent of $ZnPcS_4$ concentration. The SCE assay showed that cells treated with various light and drug doses presented no genotoxic potential, as SCE levels were not different from untreated (control) cells. Chromosomal analysis after PDT treatment at various time intervals post-irradiation showed that there was no significant chromosomal damage in cells treated photodynamically compared with untreated controls. The results show that the cell killing mechanism after PDT is not at the chromosome level, but may be at a different cellular level, such as plasma membranes, mitochondria, etc.

Introduction

Pancreatic cancer is the second most common cancer of the gastrointestinal tract. Its incidence is increasing and in addition has one of the poorest prognoses of any cancer. Most patients die 6 months after diagnosis and the 5 year survival fraction doesn't exceed 1–2% (Whittington *et al*., 1981; Kalser and Ellenberg, 1985).

Photodynamic therapy (PDT) is a new therapeutic modality for the management of a variety of solid tumours. During PDT, visible or near infrared light is used to activate a non-toxic drug, the photosensitizer. As the energy transferred from the light to the photosensitizer is dissipated, damage to tumour cells and the tumour vasculature occurs which ultimately leads to widespread tumour destruction (Gomer, 1991; Dougherty and Marcus, 1992; Henderson and Dougherty, 1992; Dougherty, 1993; Pass, 1993).

The effectiveness of this treatment modality has been proven experimentally for a large variety of tumours and recently has been introduced as a conjuctant therapy for the treatment of pancreatic cancer (Tralau *et al*., 1987; Moesta *et al*., 1995).

PDT is highly tumour selective. This is thought to be because tumour tissue retains the photosensitizer at higher concentrations than normal tissue and, secondly, the drug is only cytotoxic when activated by appropriate light (Delaney and Glatstein, 1988; Dougherty *et al*., 1990). In this way PDT can minimize or avoid destruction of normal tissue. Nevertheless, the mechanisms for these effects are not well understood (Tralau *et al*., 1987; Dougherty *et al*., 1990).

The photosensitizer that has received the most extensive evaluation in PDT protocols has been Photofrin, which is a mixture of several different porphyrins and which is approved by the US Food and Drug Administration for palliative therapy for solid tumours. Although effective, Photofrin has a number of undesirable features that make it less than ideal as a photosensitizing agent. These include the fact that it is not a pure compound, it is activated by wavelengths of light (625–630 nm) with relatively limited depths of penetration in tissue and it is associated with severe cutaneous photosensitivity, a problem that may persist for 1–2 months after it is administered (Bellnier and Dougherty, 1989; Tralau *et al*., 1989; Richter *et al*., 1991). Because of these characteristics, a number of second generation photosensitizers have been evaluated that minimize the undesirable characteristics of Photofrin. Phthalocyanines (PC) (Oleinick *et al*., 1993; Paardekooper *et al*., 1994) are second generation photosensitizers and can be produced as highly chemically purified compounds. They absorb light at ~650 nm or longer (which means an increase of penetration depth in tissue) and induce little or no general skin photosensitivity compared with Photofrin. In addition, the increased wavelength at which phthalocyanines can be photoactivated, in contrast to the wavelength of activation of Photofrin, allows the treatment of larger tissue volumes and the possibility of using new light sources, such as diode laser sources.

From a conceptual point of view PDT is complicated, as it relies on the combined action of a photosensitizer and a light source. Although clinical trials have been performed, there have been no studies performed for possible side-effects that PDT might induce, for example whether or not PDT has genotoxic or mutagenic potential.

In this paper we evaluated the effects *in vitro* of various concentrations of $ZnPcS_4$ and irradiation doses using a novel diode laser system as light source, emitting at 655 nm. Experiments were designed to correlate cell death with control-

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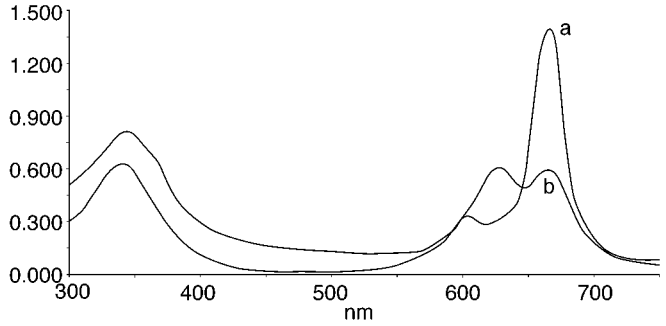

Fig. 1. UV/visible absorption spectra of $ZnPcS_4$ (**a**) in methanol and (**b**) in PBS (concentration 16 μg/ml in both cases) in a 1 cm path cell.

Table I. Technical and performance characteristics of the diode laser source

Maximum output power (mW) (at 18°C)	100
Output power via FD diffuser device (mW) (at 18°C)	>60
Mode of operation	Continuous wave mode (CW)
Current threshold (mA)	134.6
Operational current (mA)	250
Emission wavelength (nm) (at 18°C)	655

ling conditions of illumination and photosensitizer concentration. In addition we have evaluated possible genotoxicity of PDT using a sister chromatid exchange (SCE) assay and chromosomal rearrangement.

Materials and methods

Photosensitizer

Zinc phthalocyanine was purchased from Eastman Kodak (Rochester, NY) and sulfonated according to the method of Ambroz *et al.* (1991) with minor modifications. Products were purified by HPLC, using a preparative Nucleosil ODS 10μ packed column, with a mobile phase of methanol/water. A stock solution of $ZnPcS_4$ in 2 mM phosphate-buffered saline (PBS) was prepared and used within 20 days after its preparation. To avoid the loss of photosensitizing activity, the stock solution was kept in the dark at 4°C. The absorption spectrum of $ZnPcS_4$ was recorded in PBS by a computer controlled spectrometer (model Lambda 16; Perkin Elmer, Norwalk, CT) and is presented in Figure 1.

Cell cultures

MIA Pa-Ca 2 cells (Yunis *et al*., 1977), obtained from an undifferentiated human pancreatic adenocarcinoma, from stock cultures were used. The cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM, high glucose), supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (50 U/ml potassium penicillin G_1, 50 μg/ml streptomycin sulphate). Cells were grown at 37°C in a water-jacketed incubator, in a humidified 5% CO_2 atmosphere. Stock cultures were maintained *in vitro* by routinely subculturing (every fourth day) at an initial density of 2×10^4 cells in 75 cm^2 culture flasks. In all experiments described below, the suspensions of cells placed in the culture dishes/wells were incubated for 6 h to allow cell attachment before adding the photosensitizer or performing any kind of phototreatment.

Irradiation source

A compact diode laser source was used as light source for activating the $ZnPcS_4$ photosensitizer. This laser system (developed by our laboratory) has an emission wavelength at 655 nm (at 18°C), terminating at a special frontal diffuser device which provides uniform illumination, in circular geometry (homogeneity >98%), at the irradiation area (cells). The maximum power produced by the diode laser at the edge of the diffuser device (measured by a Mells Griot power meter, model 13PEM001, at 1 cm distance from the edge of the diffuser device) is >60 mW and for all the experiments the fluence was adjusted to 41 mW/cm^2. Technical and performance characteristics of the diode laser source are given in Table I.

Direct phototoxicity measurement using the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay

The cell killing efficacy was first determined using the MTT microtitre assay (Mosmann, 1983; Griffon *et al*., 1995). Cells growing in almost confluent (80–90%) monolayers were trypsinized and seeded at a density of 5×10^4 MIA cells/well in 96-well round bottom microtitre plates (Corning, UK). Five different wells were plated per sample and were incubated at 37°C in a humidified 5% CO_2 atmosphere. For each experiment two identical 96-well plates were used. Each experiment was repeated at least three times. Plates were incubated at 37°C for 6 h to allow cell attachment. After cell attachment, $ZnPcS_4$ solution in PBS at concentrations which varied from 0 to 5 μM was added to each culture. Treated cultures were incubated for 1 h at 37°C in the dark. The supernatant of each well was removed, cells were washed twice in PBS (Seromed, DE) and finally 20 μl of PBS were added to each well, to protect the cells. Each plate was placed in a heat chamber at 37°C, to avoid cell death during irradiation. A fibre optic frontal diffuser device was lead over each well using an *xyz*-positioner and focused exactly to the cell growth area of each well. The total delivered dose to each well was varied from 0 to 9 J/cm^2. After irradiation culture medium (DMEM) supplemented with 10% fetal calf serum, was added to each well (200 μl DMEM/well) and cells were returned to the incubator for 72 h.

Cell survival was then assessed by means of colourimetric MTT assay

MTT was obtained from Sigma (St Louis, MO). The MTT microtitre assay gives a quantitative estimation of the mitochondrial and cytoplasmic reductase system activity and in particular the succinate dehydrogenase activity of the viable cells (Berridge and Tan, 1993). Aliquots of 20 μl of MTT solution (2.5 g/l in 0.9% NaCl solution) were added to each well 24 and 72 h post-irradiation. After a further incubation of 4 h, 100 μl of acidic isopropanol (0.04 N HCl in absolute isopropanol) were added per well. Plates were gently shaken for a few minutes to dissolve the formazan crystals and the absorbance of converted dye was measured at 570 nm by an ELISA plate reader (Dynatech model MR 5000). Background wells containing MTT but no cells were measured and subtracted from all samples. The mean absorbance of photosensitizer-treated wells was expressed as a percentage of the controls.

Phototoxicity evaluation by the colony-forming assay

Cell survival of MIA cells was determined using a colony-forming assay, as described by Brasseur *et al*. (1988), with minor modifications. Five hundred cells were each plated into 60 mm Petri dishes containing 5 ml growth medium (DMEM) supplemented with 10% fetal calf serum and incubated in a dark humid atmosphere containing 5% CO_2 at 37°C to allow cell attachment.

After 6 h $ZnPcS_4$ solution in PBS was added to each culture at concentrations which varied from 0 to 5 μM. Treated cultures were incubated for 1 h in the dark. After that the dye solution was aspirated, the cells were washed twice in PBS and, finally, a few microlitres of PBS were added to each dish, to protect cells.

Each dish was placed in a heat chamber at 37°C and irradiated as described above and cultured for 8 days to allow colony formation. After 8 days, the cells were fixed in ethanol, stained with a 1% solution of crystal violet and colonies were counted to assess clonogenic survival. Each colony measured consisted of ⩾50 cells. Experiments were repeated three times using four dishes for each treatment. Control plates were treated in the same manner but were not exposed to laser light and/or photosensitizer.

Sister chromatid exchange (SCE) assay

The purpose of this experiment was to evaluate the incidence of SCE after PDT.

For SCE experiments, suspensions of 50×10^4 MIA cells in 1.5 ml culture medium supplemented with 10% fetal calf serum were plated in each well of a 48-well flat bottom culture plate (Corning). Four different wells were plated per sample and for each experiment two identical 48-well plates were used. Each experiment was repeated three times.

Plates were incubated at 37°C for 6 h to allow for cell attachment. Afterwards, $ZnPcS_4$ solution in PBS at concentrations which varied from 0 to 5 μM was added to each culture. Treated cultures were incubated for 1 h at 37°C in the dark. After that the supernatant of each well was removed, cells were washed twice in PBS (Seromed) and finally a few millilitres of PBS were added to each well, to protect cells, and cells were irradiated as described above. For the SCE studies irradiances of ⩾1 J/cm^2 (up to 9 J/cm^2) and $ZnPcS_4$ doses of ⩾1 μM were used, because photodynamic toxicity was very low at these doses.

Determination of SCEs in MIA cell cultures was performed according to the methods described previously (Perry and Wolff, 1974). Twenty four hours after irradiation and 48 h before harvesting, bromodeoxyuridine (BrdU) was added to each culture at a final concentration of 10 μg/ml. At the end of the incubation period, colcemid was added (0.1 μg/ml) for 3 h, to accumulate metaphases, and afterwards the cells were harvested, swollen for 10 min in warm 0.075 M KCl solution, centrifuged and the pellet fixed with methanol/

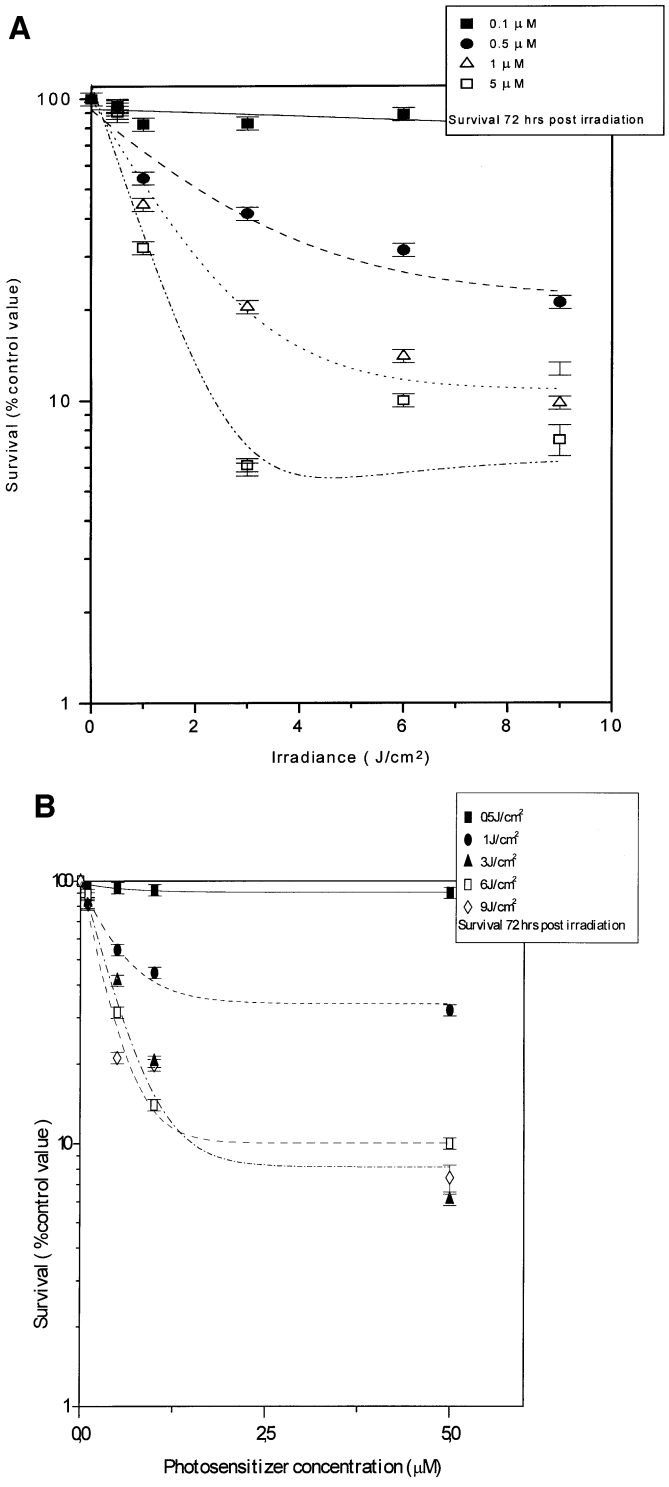

Fig. 2. (**a**) Per cent survival (% control value) of MIA cells as a function of total delivered dose, after treatment with various $ZnPcS_4$ concentrations, 72 h post-irradiation. Laser light (λ = 655 nm) was used as irradiation source and the fluence was adjusted to 41 mW/cm^2. Error bars represent standard deviation of data from three individual experiments. (**b**) Per cent survival (% control value) of MIA cells as a function of $ZnPcS_4$ concentration, after exposure at various irradiances, 72 h post-irradiation. Laser light (λ = 655 nm) was used as irradiation source and the fluence was adjusted to 41 mW/cm^2. Error bars represent standard deviation of data from three individual experiments.

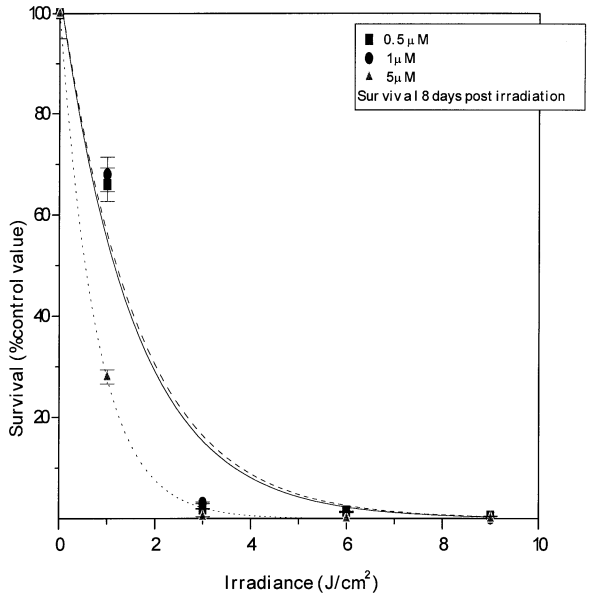

Fig. 3. Per cent survival (% control value) of MIA cells as a function of total delivered dose, after treatment with various $ZnPcS_4$ concentrations, 8 days post-irradiation. Laser light (λ = 655 nm) was used as irradiation source and the fluence was adjusted to 41 mW/cm^2. Error bars represent standard deviation of data from three individual experiments.

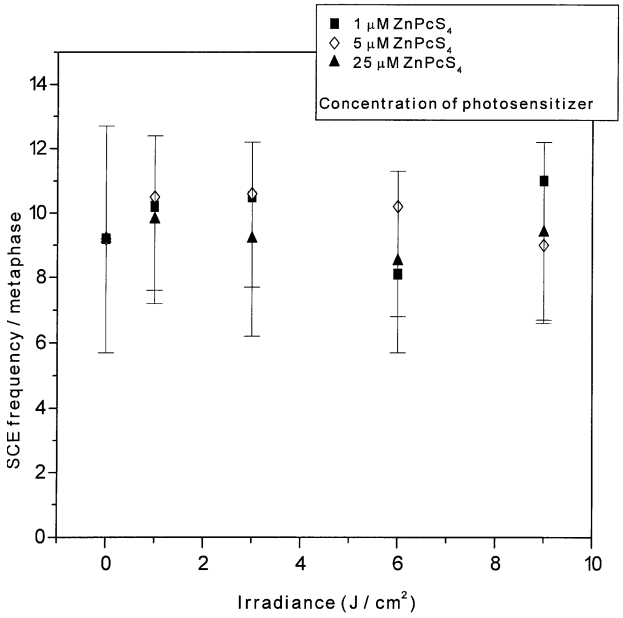

Fig. 4. Frequency of sister chromatid exchanges in MIA cells induced by different irradiances and for three different concentrations of $ZnPcS_4$ (squares, 1 μM; diamonds, 5 μM; triangles, 25 μM). Error bars represent standard deviation of data from three individual experiments, where 70 metaphase spreads were examined for each treatment.

acetic acid (3:1) for 30 min. The fixative was renewed and, after an additional 30 min, the cells were resuspended in fresh fixative and small drops spread on clean wet microscope slides (two drops of 15 μl each were spread on each slide). After drying overnight, the cells were stained with Hoechst 33258 (5 μg/ml in Sorensen phosphate buffer, pH 6.8) for 30 min in the dark, washed and exposed while wet with Sorensen buffer to UV light (360–400 nm) for 1 h. After washing again, the cells were stained for 20 min in 3% Giemsa blood stain in Sorensen buffer, pH 6.8. After washing briefly in buffer followed by distilled water, the slides were dried and mounted in Depex. At least 70 complete metaphase spreads were scored for each treatment. SCE frequency was calculated as SCEs/cell ± SD.

Table 2. Chromosomal aberrations induced on MIA cells

	Cells scored	Chromatid type		Chromosome type		
		Breaks (per cell)	Exchanges (per cell)	Dicentrics (per cell)	Rings (per cell)	Fragments (per cell)
0 h post-irradiation						
Control	1000	0.003	0	0.004	0.002	0.004
MIA + 5 μM $ZnPcS_4$	1000	0	0	0.011	0.002	0.002
MIA + 5 μM $ZnPcS_4$ + 6 J/cm^2 irradiance	250	0	0	0	0	0
3 h post-irradiation						
Control	1000	0	0	0.008	0	0
MIA + 5 μM $ZnPcS_4$	1000	0.004	0	0.004	0	0
MIA + 5 μM $ZnPcS_4$ + 6 J/cm^2 irradiance	40	0	0	0	0	0
6 h post-irradiation						
Control	1000	0	0	0.007	0	0
MIA + 5 μM $ZnPcS_4$	1000	0	0	0.006	0	0
MIA + 5 μM $ZnPcS_4$ + 6 J/cm^2 irradiance	6	0	0	0	0	0
24 h post-irradiation						
Control	1000	0	0	0.008	0	0
MIA + 5 μM $ZnPcS_4$	1000	0	0	0.004	0.004	0
MIA + 5 μM $ZnPcS_4$ + 6 J/cm^2 irradiance	4	0	0	0	0	0

Chromosomal analysis

For chromosomal analysis experiments exponentially growing MIA cells were used. Using the results obtained from cell survival experiments, the protocol of photodynamic treatment was 5 μM $ZnPcS_4$ and a light dose of 6 J/cm^2 for each cell culture. Three hours before irradiation colcemid was added to each well at a final concentration 0.05 μg/ml. After irradiation each culture was shaken and mitotic cells were collected. Then fresh culture medium and colcemid, at the same concentrations as above, were added to each culture, which was maintained for a further 3 h incubation time, to obtain the next wave of mitoses. The same procedure was repeated to obtain mitotic cells 3 and 6 h post-irradiation. The cultures were harvested 24 h post-irradiation. Slides were prepared for all the waves of mitotic cells (0, 3, 6 and 24 h post-irradiation) using the standard procedures.

Scoring was performed for the exclusively first division metaphases for unstable chromosome aberrations including dicentrings, rings and acentric fragments. Experiments for evaluating chromosomal aberrations after PDT were repeated three times.

Statistical evaluation of differences between treated and control samples was performed using Student's *t*-test, where $P < 0.001$ was considered as an indication of statistical significance.

Results

Dark toxicity and determination of laser light cytotoxicity

MIA cells incubated up to 72 h in growth medium containing up to 10^{-4} M $ZnPcS_4$, in the absence of laser light, did not present any loss of cell viability. No significant cell phototoxicity was observed upon exposure of MIA cells to red light alone, at light doses up to 9 J/cm^2.

Photocytotoxicity

The effects of dye concentration and laser light dose on cell survival were investigated. In both cases, similar survival curves were obtained following 72 h post-irradiation incubation (Figure 2a and b). Photocytotoxicity increases rapidly as light dose increases. From Figure 2a it can be seen that a combination of 3 J/cm^2 irradiance with a $ZnPcS_4$ dose of 5 μM leads to 90% of cells killed (LD_{90}). All irradiances above this value in combination with a $ZnPcS_4$ dose of 5 μM gave survival levels almost constant and below the LD_{90} level.

The influence of $ZnPcS_4$ concentration on cell survival 72 h after exposure to laser radiation is presented in Figure 2b. Figure 2b shows that cell survival depends mainly on light irradiance rather than on photosensitizer dose. Survival levels decreased when irradiance was increased and a light dose of 3 J/cm^2, when the $ZnPcS_4$ concentration was 5 μM, was enough to obtain killing levels of 90% or more. From that irradiance and above cell survival remains almost constant and below the LD_{90}.

The effect of post-irradiation time on cell survival

Control MIA cells incubated in growth medium without photosensitizer, but containing 10% fetal calf serum, did not show any lethality when exposed at the highest irradiance (9 J/cm^2). Their plating efficiency was taken as 100% cell survival. Survival curves of MIA cells as a function of laser irradiance 8 days post-irradiation are presented in Figure 3. From this figure it can be seen that a light dose of 3 J/cm^2, independent of $ZnPcS_4$ concentration, leads to the LD_{90}. Comparing Figure 2a with Figure 3 it can be concluded that cell survival decreases with post-irradiation time.

SCE induction by PDT

Control MIA cells show a background SCE frequency. Mean SCE values ± SD for various combinations of light and $ZnPcS_4$ doses are presented in Figure 4. No significant change in the incidence of SCEs, within statistical significance ($P < 0.001$), was observed after treatment with any combination of light and photosensitizer dose, as compared with SCEs obtained from untreated control samples.

Chromosomal analysis after PDT

The yields of unstable chromosomal aberrations in MIA cells induced by PDT immediately and 3, 6 and 24 h post-irradiation are listed in Table II. The aberrations are recorded as breaks, exchanges (chromatid type), rings, dicentrics and acentric fragments (chromosome type). Chromosomal aberrations are presented in control MIA samples (Table II). This observation is not unexpected, because MIA cells are immortalized cells from a cancer patient. However, Table II shows that there was no significant increase in chromosomal damage of samples treated photodynamically compared with the values for control samples. Similar results were obtained from samples that were irradiated with laser light only, but were not treated with $ZnPcS_4$.

Discussion

The results presented in this work show that cell viability decreases rapidly as the light dose increases and that it depends

on the drug concentration. Brasseur *et al*. (1988) presented results which showed a dependence of cell viability on $ZnPcS_4$ concentration up to 1 μM. Our results confirm these results and show that, in addition, for higher concentrations viability remains almost independent of drug concentration, up to 25 μM $ZnPcS_4$. One possible explanation is based on the different levels of photosensitivity according to the type of cell, as Moesta *et al*. (1995) suggested for MIA cells. Another explanation is that the photosensitizer has been partially aggregated, as shown in Figure 1 and as has been confirmed for zinc phthalocyanines in recent literature (Fingar *et al*., 1993; Morgan *et al*., 1994; Cook *et al*., 1995; Jori, 1996). Even though there is some aggregation of the photosensitizer, our results show that a small but constant level of photosensitizer remains photochemically active and this is probably the reason for the observed viability. This active amount of the photosensitizer seems to be enough, according to our results, to achieve lethality levels above the LD_{90} value.

Indeed, by using a protocol of 3 J/cm^2 irradiance (or more) in combination with drug doses >5 μM 72 h post-irradiation, LD_{90} levels of viability were achieved. Light doses required to obtain LD_{90} levels of viability are lower than those determined by Moesta *et al*. (1995) using Photofrin as the photosensitizer for the same type of cell [a protocol of 10 μM Photofrin combined with 50 J/cm^2 irradiance was needed by Moesta *et al*. (1995) to obtain LD_{90} levels of viability]). The light and drug doses used in this study are lower than those usually applied in clinical practice.

The results shows that cell survival depends mainly on light dose rather than on drug dose. Also, cell survival seems to decrease as post-irradiation time increases up to 8 days. These results could lead to indirect evidence about the cellular site of impact of PDT with phthalocyanines. In the past researchers have claimed (Ben-Hur and Rosenthal, 1985; Ramakrishnan *et al*., 1989; Agarwal *et al*., 1991; Rosenthal, 1991) that phthalocyanines could cause damage to plasma membranes, chromosomes and DNA. Our results have shown that SCE and chromosomal damage were not induced by PDT with phthalocyanines. Moreover, over a wide spread of light irradiances and drug concentrations used, the SCE assay indicated that no genotoxic effects on MIA cells were induced. So the results presented here clearly show that PDT using $ZnPcS_4$ and laser light has no genotoxic potential and should provide evidence that no risk of genotoxicity is associated with the application of this treatment modality.

One possible explanation of cell death after PDT treatment is that $ZnPcS_4$ activated by laser light caused damage to plasma membranes, by oxidation after free radical production, or/and damage to other subcellular structures such as mitochondria, lysosomes, etc. In this way cells could lose their integrity and could be killed. Another possible explanation of cell killing after PDT is that cells are killed by apoptosis (K.Halkiotis *et al*., unpublished data). However, it is not quite clear which is the exact mechanism of cell killing after PDT treatment.

The results obtained by applying PDT using $ZnPcS_4$ as photosensitizer and a diode laser source at 655 nm on MIA cells have shown that a high percentage of cancer cell killing is achieved *in vitro*. This may be evidence that *in vivo* application of this specific PDT treatment in pancreatic tumours should achieve acceptable tumor necrosis, without inducing genotoxic effects.

However, further studies are needed to reveal the exact cellular site of damage caused by PDT with phthalocyanines and our future work will be concentrated towards these objectives.

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