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Photosensitization of lymphoblastoid cells with phthalocyanines at different saturating incubation times

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Abstract

Photodynamic therapy of cancer is a promising treatment based on the tumor-specific accumulation of photosensitizers followed by irradiation with visible light which induces tumor cell death. The effect of different preincubation times on the photosensitization efficiency of the phthalocyanines AlPc and $AlPcS_4$ was investigated in lymphoblastoid CCRF-CEM cells under conditions that allow maximal uptake of the sensitizers. First, the time course for the uptake of $AlPcS_4$ and AlPc by CCRF-CEM cells and by the pheochromocytoma PC12 cells was compared. The uptake of $AlPcS_4$ by CCRF-CEM cells was not significantly different after 6 h or 24 h incubation, but the photosensitization efficiency of the phthalocyanine was much higher when a 24 h preincubation period was used, with a fluence rate of 5 mW/cm^2. However, for a fluence rate of 10 mW/cm^2, the photosensitization efficiency of $AlPcS_4$ was almost completely independent of the preincubation time (6 h vs. 24 h) with the phthalocyanine. When the cells were preincubated with 1 μmol/L AlPc for 10 min or 6 h, which allows the same accumulation of sensitizer by the cells, no significant effect of the incubation time on the photodynamic inactivation of CCRF-CEM cells was observed, with fluence rates of 5 mW/cm^2 or 10 mW/cm^2, for different light doses. Confocal fluorescence microscopy studies did not reveal differences in the localization of the phthalocyanines after maximal uptake was reached. The results show that the preincubation time with $AlPcS_4$, after the maximal uptake is reached, affects cell growth to an extent depending on the fluence rate used, and this effect was not due to a major redistribution of the sensitizer during incubation. However, this was not observed when AlPc was used.

Abbreviations: AlPc, aluminum phthalocyanine; AlPcS, sulfonated aluminum phthalocyanine; $AlPcS_2$, bisulfonated aluminum phthalocyanine; $AlPcS_4$, tetrasulfonated aluminum phthalocyanine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDT, photodynamic therapy

Introduction

In photodynamic therapy (PDT) of cancer, a sensitizer, light, and oxygen are used to induce death of tumor cells. When the sensitizer absorbs light, the energy is transferred to oxygen, generating singlet oxygen that causes cell damage (Henderson and Dougherty, 1992;

Moore et al., 1997). Phthalocyanines are a new generation of photosensitizers for PDT (Brasseur et al., 1985; Ben-Hur and Rosenthal, 1985), used successfully in clinical trials (Sokolov et al., 1996). This group of compounds has several advantages over the sensitizers currently used, which are based on hematoporphyrin (Van Lier, 1990; Rosenthal, 1991), including a red-shifted Q-band at 700 nm and the fact that they do not induce skin photosensitivity.

The uptake of phthalocyanines by tumor cells is time dependent (Rosenthal et al., 1987; Gantchev et al., 1994), and the amount of phthalocyanines taken up by cells *in vitro* is correlated with phototoxicity when subsaturating incubation periods are used (Rosenthal et al., 1987; Paquette et al., 1988; Margaron et al., 1996). The intracellular distribution of polar phthalocyanines may also change when longer preillumination incubation periods are used (Bottiroli et al., 1992), and this may be responsible for differences in the photosensitizing efficiency of these phthalocyanines (Gantchev et al., 1994). Another important factor in PDT treatment is the light fluence rate used, but this parameter has shown contradictory effects on cell viability depending on the system used (Gomer et al., 1985; Ben-Hur et al., 1987; Foster et al., 1993; Veenhuizen and Stewart, 1995; Moor et al., 1997). However, the evaluation of the effect of fluence rates in PDT is difficult because different authors use different photosensitizers and illumination techniques, as well as distinct tumor models and normal tissues.

In the present work we first compared the time course for the uptake of $AlPcS_4$ and AlPc in two different cell lines, the lymphoblastoid CCRF-CEM cells and the pheochromocytoma PC12 cells, which have very different phenotypes. We then used CCRF-CEM cells as a model to investigate the effect of different incubation periods (which allowed maximal uptake of the sensitizer, i.e. the same amount of sensitizer inside the cell) on the photosensitization efficiency of $AlPcS_4$ and AlPc. These two phthalocyanines have very different polarities and, therefore, are distributed inside the cells in a distinct manner (Peng et al., 1991). The effect of different fluence rates under these conditions was also studied. Photosensitization was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay, which has been used to measure cellular growth and survival in PDT and other studies (Mosmann, 1983; Merlin et al., 1992; Margaron et al., 1996; Allemann et al., 1997).

Materials and methods

Chemicals

Tetrasulfonated aluminum phthalocyanine ($AlPcS_4$) was kindly provided by Ciba-Geigy (Basel, Switzerland) and aluminum phthalocyanine (AlPc) was purchased from Acros Organics (Geel, Belgium). Fetal calf serum and horse serum were from Biochrom (Berlin, Germany) and Life Technologies (Paisley, UK), respectively. All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany). Cell lines were from ATCC (Rockville, MO, USA).

Stock solutions of $AlPcS_4$ (10 mmol/L) (Gantchev et al., 1994) and AlPc (1 mmol/L) (Ramakrishnan et al., 1989) were prepared in Na^+-medium (140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5.5 mmol/L glucose, 20 mmol/L HEPES, and 1 mmol/L NaH_2PO_4, pH 7.4) and dimethylformamide, respectively, and were stored at –20°C, in the dark. At the final concentration reached in the culture medium, DMF was not toxic to the cells (not shown).

Cell culture

Rat pheochromocytoma PC12 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum (FCS), 23.8 mmol/L $NaHCO_3$, 25 mmol/L HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin, at pH 7.4. The human lymphoblastoid CCRF-CEM cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 23.8 mmol/L $NaHCO_3$, 25 mmol/L HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin, at pH 7.4. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Incubation of cells with phthalocyanines

CCRF-CEM cell density was kept in the exponential phase of growth (0.5×10^6 cells/ml), for 48 h before the experiments. On the day before the experiments, PC12 cells were transferred to a culture medium with a composition identical to that used to culture CCRF-CEM cells, at a density of 0.7×10^6 cells/ml. Cell counting was performed using a Coulter Counter ZF. Phthalocyanines were added to the cultures (0.5×10^6 cells/ml), directly from the stock solutions, and the incubation was performed at 37°C, in the dark, under a humidified atmosphere of 5% CO_2/95% air.

Quantification of AlPc uptake

The uptake of AlPc by PC12 cells and by CCRF-CEM cells was measured as described previously (Chan et al., 1990). After incubation with the phthalocyanine in culture medium, the cells were collected rapidly on Whatman GF/A filters prewashed with 5 ml of Na^+-medium. The filters were then washed with 20 ml ice-cold Na^+-medium and allowed to dry. The filters were placed into vials containing 2 ml of ethanol and incubated overnight at 4°C, in order to extract the intracellular AlPc. The resulting solutions were centrifuged at 15 800*g*, and the absorbance of the supernatants was measured at 674 nm, using a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer. The absorbance due to endogenous intracellular components was determined by filtering the same number of cells not preincubated with AlPc. The binding of AlPc to the filters was measured by filtering culture medium with the phthalocyanine but free of cells. The intracellular accumulation of AlPc was determined by subtracting these values from the absorbance measured in cells incubated with the phthalocyanine. The amount of AlPc taken up by the cells under each experimental condition was determined using a calibration curve prepared with solutions containing different concentrations of AlPc in ethanol.

Quantification of $AlPcS_4$ uptake

After incubation of the cells with $AlPcS_4$, they were washed twice with ice-cold Na^+ medium, and resuspended in the same medium at 37°C. $AlPcS_4$ fluorescence in the cells was determined at 37°C, under stirring, using a Perkin-Elmer LS-5B spectrofluorometer, with excitation at 365 nm and emission at 680 nm (10 nm slits). The fluorescence of the cells not preincubated with the phthalocyanine, at the same excitation and emission wavelengths, was measured as a control, and was subtracted from the fluorescence of cells containing $AlPcS_4$. The uptake of $AlPcS_4$ was calculated using a calibration curve, prepared by adding known quantities of phthalocyanine to control cell suspensions at 37°C.

Irradiation of the cells

The cells (0.5×10^6 cells/ml) were incubated with 1 μmol/L AlPc for 10 min or 6 h, or with 25 μmol/L $AlPcS_4$ for 6 h or 24 h, in culture medium. The uptake of phthalocyanines was

stopped by washing the cells twice with sterile phosphate-buffered saline (PBS) (150 mmol/L NaCl, 2.7 mmol/L KCl, 2 mmol/L NaH_2PO_4, 8 mmol/L Na_2HPO_4, pH 7.4), at 4°C. The cells were then resuspended in culture medium at the initial density, and transferred to 48-well cluster plates. The cells were irradiated at 37°C, using an apparatus consisting of a 2000 W tungsten/halogen lamp (Osram, Germany), with a cut-off filter at 600 nm. Fluence rate was measured with an OPHIR Nova laser power/energy monitor equipped with a 2A-SH head (Ophir Optronics, Israel). After irradiation, cells were transferred to the incubator and maintained in cell culture conditions for the indicated period of time. Cell growth was evaluated using the MTT assay.

MTT assay

CCRF-CEM cells were incubated with 0.5 mg/ml MTT for 45 min at 37°C, in culture medium. MTT, when taken up by live cells, is reduced to a water-insoluble blue-colored product (Mosmann, 1983). After incubation with MTT, the cells were centrifuged for 15 s at 15 800*g*, and the resulting pellets were dissolved in a 1:1 mixture of PBS–acidified isopropanol (0.04 mol/L HCl). Formazan quantification (MTT reduction) was performed at 570 nm, with a reference filter of 620 nm, using a Spectra II automatic plate reader (SLT, Austria). Control experiments revealed that phthalocyanines taken up by the cells did not contribute to the absorbance measured at 570 nm (not shown).

Localization studies with confocal microscopy

CCRF-CEM cells (0.5×10^6 cells/ml) were incubated with 1 μmol/L AlPc for 10 min or 6 h, or with 25 μmol/L $AlPcS_4$ for 6 h or 24 h, in culture medium. The uptake of phthalocyanines was stopped by washing the cells twice with sterile phosphate-buffered saline (PBS) (150 mmol/L NaCl, 2.7 mmol/L KCl; 2 mmol/L NaH_2PO_4, 8 mmol/L Na_2HPO_4, pH 7.4), at 4°C, and the cells were then resuspended in the same buffer. Phthalocyanine fluorescence was then visualized using a ×100 objective, with an MRC600 confocal imaging system (Bio-Rad Laboratories, Milan, Italy) linked to a Nikon Optiphot-2 fluorescence microscope. A krypton/argon mixed laser was used in combination with a 647 nm bandpass filter (excitation) and a 680 nm longpass filter (emission). Confocal Assistant software (Bio-Rad) was used for image processing.

Statistical analysis

Results are presented as means ± SEM for the indicated number of experiments. Statistical significance was determined using two-tailed unpaired Student's *t*-test, using GraphPad Prism 2.01.

Results

Phthalocyanine uptake by CCRF-CEM cells and by PC12 cells

The time course for the uptake of AlPc (1 μmol/L) and $AlPcS_4$ (25 μmol/L) is shown in Figure 1. The uptake of AlPc was higher in CCRF-CEM cells than in PC12 cells for each time investigated, but the maximal uptake of AlPc was reached after 10 min incubation in both cell lines (Figure 1a). The accumulation of $AlPcS_4$ was much slower, the maximal uptake being observed after 6 h incubation in both cell lines (Figure 1b). For periods of incubation longer than 3 h, the uptake of $AlPcS_4$ by PC12 cells was significantly higher than that observed in CCRF-CEM cells.

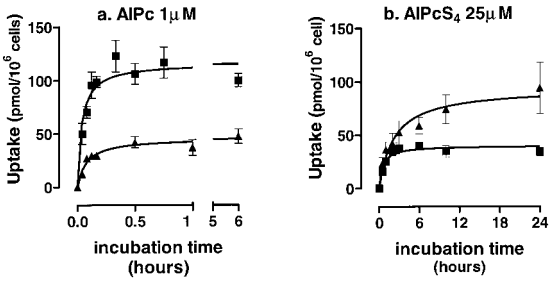

Figure 1. Time course for the uptake of AlPc (1 μmol/L; a) and $AlPcS_4$ (25 μmol/L; b) by CCRF-CEM (■) and PC12 (▲) cells in culture medium. Cells were exposed to the phthalocyanines for the indicated periods of time, and the amount of AlPc or $AlPcS_4$ taken up by the cells was determined as indicated in Material and Methods. Data are presented as means ± SEM of independent experiments ($n = 3$–4). Whenever the error bars are not visible they are contained within the symbols.

Time dependence of $AlPcS_4$-induced photosensitization in CCRF-CEM cells

Since the uptake and intracellular localization of tetrasulfonated phthalocyanines is determined by the incubation period, and this affects photodynamic inactivation of cells (Gantchev et al., 1994; Bottiroli et al., 1992), we investigated the photodynamic effects of $AlPcS_4$ (25 μmol/L) on CCRF-CEM cells after preincubating for 6 h or 24 h, which allows maximal uptake of the phthalocyanine (Figure 1b). The MTT reduction by cells incubated with phthalocyanines in the absence of light, or by cells irradiated without phthalocyanines, was not different from the MTT reduction by control cells maintained in the absence of light and not incubated with phthalocyanines (not shown). There was a time-dependent increase in the absorbance of formazan produced by the reduction of MTT, in the absence of light, due to cell growth (author's unpublished observation, see also Mosmann, 1983). Forty eight hours after seeding CCRF-CEM cells at 0.5×10^6 cells/ml, the slope of the curve decreased (0 J/cm^2, Figures 2 and 3) because the cells reached the plateau phase of the growth curve at this time (not shown).

When the cells were incubated with $AlPcS_4$ for 6 h and were irradiated with 5 mW/cm^2, using light doses ranging from 2 to 6 J/cm^2 (Figure 2a), there was a small decrease on MTT reduction, relative to the control, 15–24 h after irradiation but not after 3 h or 48 h. The same fluence rate (5 mW/cm^2) was much more effective in decreasing MTT reduction by CCRF-CEM cells, as measured 15–24 h after irradiation, when the cells were preincubated with $AlPcS_4$ for 24 h ($p < 0.01$) (Figure 2b).

When the fluence rate was increased to 10 mW/cm^2 (Figure 2c,d), the phototoxicity was almost completely independent of the preincubation time with $AlPcS_4$, in contrast to the results obtained when 5 mW/cm^2 was used. When we compared the photosensitizing efficiency of $AlPcS_4$ on CCRF-CEM cells, using fluence rates of 5 or 10 mW/cm^2, the most striking differences were found between cells preincubated with the phthalocyanines for 6 h (Figure 2a,c), and only small differences were observed between cells preincubated with the phthalocyanine for 24 h (Figure 2b,d).

Time dependence of AlPc-induced photosensitization in CCRF-CEM cells

A similar study was performed with the same cell line, CCRF-CEM cells, but using AlPc. The cells were incubated with 1 μmol/L AlPc for 10 min or 6 h, which allows for the uptake of the same amount of the phthalocyanine (Figure 1a). The effect of using different incubation times with AlPc on cell growth after irradiation with different fluence rates and light doses is shown in Figure 3.

For all light doses tested, the effect of irradiation on cell growth was nearly independent of the preincubation period with the phthalocyanine when the cells were irradiated with 5 mW/cm^2 (Figure 3a,b) or 3 mW/cm^2 (data not shown). When the fluence rate was increased to 10 mW/cm^2 (Figure 3c,d), the preincubation time with the sensitizer was still

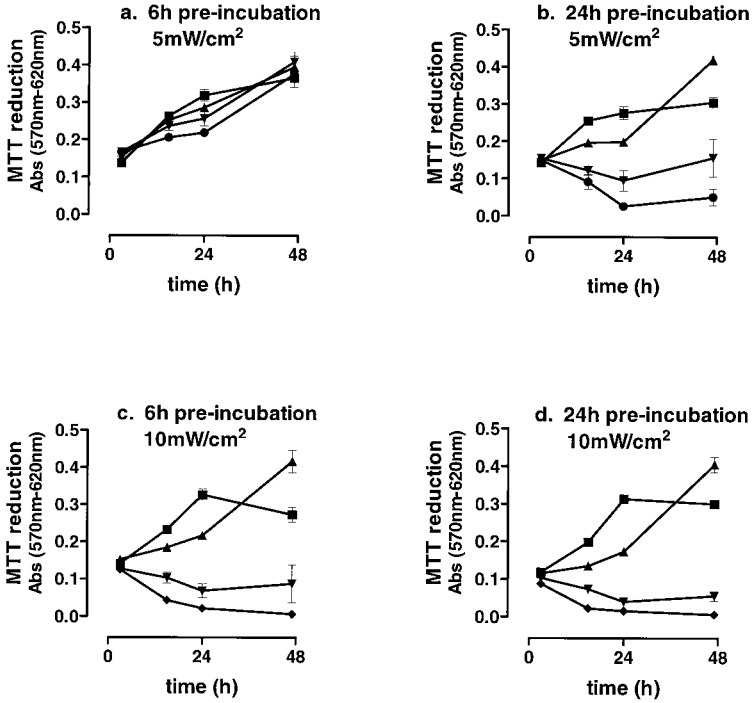

Figure 2. Effect of fluence rate and light dose on the growth of CCRF-CEM cells, preincubated with 25 μmol/L $AlPcS_4$ for 6 h (a, c) or 24 h (b, d). Cells were irradiated with different light doses (■, 0 J/cm^2; ▲, 2 J/cm^2; ▼, 4 J/cm^2; ●, 6 J/cm^2; ◆, 8 J/cm^2), using fluence rates of 5 mW/cm^2 (a, b) or 10 mW/cm^2 (c, d). Cell growth was measured using the MTT assay. Data are presented as means ± SEM from independent experiments ($n = 4$–6). Whenever the error bars are not visible they are contained within the symbols.

without effect on cell growth, when cells were irradiated with 4 J/cm^2. However, when the cells were irradiated with 2 J/cm^2, using the same fluence rate, we observed a significant difference ($p < 0.001$) on MTT reduction 3 h after irradiation between cells preincubated with AlPc for 10 min and for 6 h. This difference decreased 15 h after irradiation ($p < 0.05$) and was not significant 24 h after irradiation.

Comparing the effect of fluence rate on cell growth, using the same preincubation period with AlPc (Figure 3a vs. Figure 3c and Figure 3b vs. Figure 3d), we observed that the phototoxicity was higher when the cells were irradiated with 10 mW/cm^2 than when 5 or 3 mW/cm^2 was used.

Localization of phthalocyanines

In order to evaluate whether the effect of the incubation time on the photosensitizing efficiency of the phthalocyanines was due to redistribution of the sensitizer after the maximal uptake was reached, intracellular localization of both phthalocyanines was determined after 6 h or 24 h incubation with 25 μmol/L $AlPcS_4$ and 10 min or 6 h incubation with 1 μmol/L AlPc (Figure 4).

The distribution of $AlPcS_4$ was confined to the cytoplasm of the cells, exhibiting a granular pattern (Figure 4a,c), and this distribution was the same for both incubation times (6 h or 24 h). AlPc distribution was more diffuse throughout the cell and the pattern was not altered with the incubation period (Figure 4b,d). Neither phthalocyanine was localized in the nucleus.

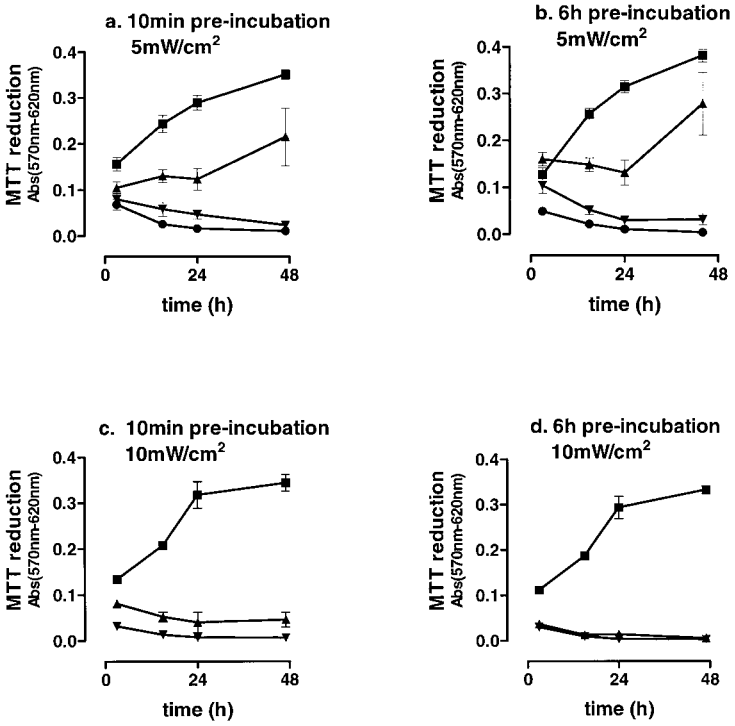

Figure 3. Effect of fluence rate and light dose on the growth of CCRF-CEM cells preincubated with 1 μmol/L AlPc, for 10 min (a, c) or 6 h (b, d). The cells were irradiated with 5 mW/cm^2 (a, b) or 10 mW/cm^2 (c, d), using different light doses (■, 0 J/cm^2; ▲, 2 J/cm^2; ▼, 4 J/cm^2; ●, 6 J/cm^2). Cell growth was measured using the MTT assay. Data are presented as means ± SEM from independent experiments ($n = 4$–8). Whenever the error bars are not visible they are contained within the symbols.

Discussion

In the present work we compared the phototoxicity of two different phthalocyanines, $AlPcS_4$ and AlPc, in the lymphoblastoid cell line CCRF-CEM cells; different preincubation periods with the phthalocyanine were used, which allowed maximal uptake of $AlPcS_4$ and AlPc. The time-dependent uptake of the two phthalocyanines by the lymphoblastoid CCRF-CEM cells was compared with the uptake properties of an excitable cell line, the pheochromocytoma PC12 cells. Our results revealed that under conditions of maximal uptake of $AlPcS_4$, the extent of photosensitization was proportional to the length of preincubation period when a fluence rate of 5 mW/cm^2 was used. This effect was no longer observed when a fluence rate of 10 mW/cm^2 was used, with $AlPcS_4$ as a sensitizer, or when AlPc was used. We also showed that these results apparently were not due to the redistribution of $AlPcS_4$ after the maximal uptake was reached, as determined by confocal microscopy.

AlPc and AlPcS4 are taken up differently by lymphoblastoid and pheochromocytoma cell lines

As expected from the differences in the polarity of AlPc and $AlPcS_4$ molecules, the uptake of AlPc by the two cell lines was faster than that

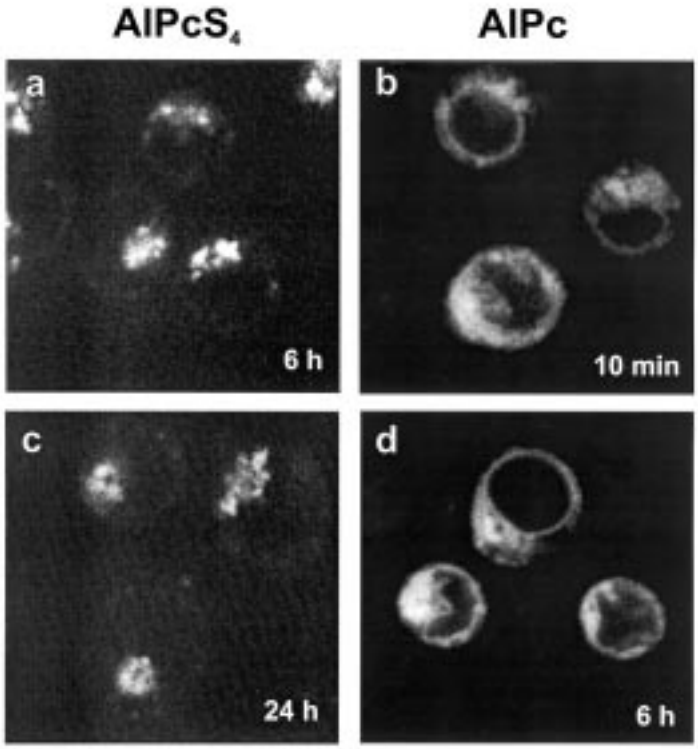

Figure 4. Localization of $AlPcS_4$ and AlPc after incubation for 6 h (a) or 24 h (c) with 25 μmol/L $AlPcS_4$, or 10 min (b) or 6 h (d) incubation with 1 μmol/L AlPc. The images are representative of at least two independent experiments. No measurable autofluorescence was detected in CCRF-CEM cells not incubated with phthalocyanines (not shown).

of $AlPcS_4$. The rapid uptake of AlPc by CCRF-CEM cells and by PC12 cells confirms previous observations in three strains of a mouse lymphoma cell line (L5178Y) (He et al., 1998; Ramakrishnan et al., 1989). However, in our experiments the maximal uptake of AlPc (1 μmol/L) was reached within 1 hour of incubation, whereas in the V79 cell line the uptake of AlPc (0.4 μmol/L) reached a plateau 3 h after incubation with the sensitizer (Ben-Hur and Rosenthal, 1986).

Interestingly, we found that the uptake of this sensitizer by CCRF-CEM cells was higher and faster than the accumulation of the phthalocyanine by PC12 cells (Figure 1a). These results correlate with previous observations showing that L5178Y-5 cells, a mouse leukemia cell line, take up more sulfonated aluminum phthalocyanine (AlPcS), a hydrophobic phthalocyanine, than do EJG-HBP cells (a bovine endothelial cell line), V79 cells (Chinese hamster fibroblasts), and human peripheral blood lymphocytes (Rosenthal and Ben-Hur, 1989).

In contrast to what we observed with AlPc, the uptake of $AlPcS_4$ was higher in PC12 cells than in CCRF-CEM cells. This could be due to the fact that sulfonated phthalocyanines are primarily taken up by endocytosis (Roberts and Berns, 1989) and this mechanism may be more efficient in PC12 cells. The time-dependent uptake of 25 μmol/L $AlPcS_4$ by CCRF-CEM cells was well correlated with a study made with Namalva cells (Burkitt's lymphoma

cells) (Gantchev et al., 1994), where it was observed that the uptake of 20 μmol/L $AlPcS_4$ or 20 μmol/L $ZnPcS_4$ was maximal after 6 h incubation in culture medium. The uptake of $ZnPcS_4$ (25 μmol/L) by V79 cells was linear up to 24 h (Rosenthal et al., 1987), which to some extent resembles the properties of $AlPcS_4$ uptake by PC12 cells.

The total amount of $AlPcS_4$ taken up by the cells may have been underestimated due to aggregation of the phthalocyanine inside the cell, since in the aggregated form $AlPcS_4$ is not fluorescent (MacRobert et al., 1989). However, this is not expected to account for the large differences between the measured accumulation of AlPc and $AlPcS_4$. Accordingly, after incubation of V79 cells with 10 μmol/L $AlPcS_4$ for 18 h, only 16% of the intracellular phthalocyanine was found in the aggregated, non-fluorescent, form (Berg et al., 1989). In less sulfonated phthalocyanines the percentage of the dye in the monomeric form decreases with the decrease in sulfonation (Berg et al., 1989) and, therefore, the uptake of AlPc by PC12 cells and by CCRF-CEM cells was quantified in this study after extraction.

From the uptake studies reported here, we can conclude that the amount of phthalocyanines taken up by the cells is dependent on the type of cell line used; however, it is not clear at the present time why this is so.

Phthalocyanine phototoxicity in CCRF-CEM cells: effect of preincubation time and of fluence rate

Using the MTT assay to measure cell growth, we found that the photosensitizing efficiency of $AlPcS_4$ was dependent on the preincubation time with the phthalocyanine and on the fluence rate used. When CCRF-CEM cells were irradiated with 5 mW/cm^2, only minor damage was detected in cells preincubated with $AlPcS_4$ (25 μmol/L) for 6 h, even when a light dose of 6 J/cm^2 was used, in contrast to the results obtained in cells preincubated with the phthalocyanine for 24 h. Since the amount of $AlPcS_4$ taken up by the cells in the two experimental conditions (6 h vs. 24 h) was similar (Figure 1b), the results suggest that there may be a redistribution of the sensitizer during long incubation periods; however, this was not observed when we looked at the fluorescence of the intracellular phthalocyanines by confocal microscopy (Figure 4). The localization of the phthalocyanines is important for photosensitization since this process is thought to be due to formation of singlet oxygen (Rosenthal and Ben-Hur, 1995), which can diffuse less than 0.1 μm because of its short lifetime (Moan, 1990).

Fluorescence microscopy studies revealed that after incubation of the melanoma cell line LOX with $AlPcS_4$ (1–100 μmol/L), for 18 h, the phthalocyanine is localized in lysosomes (Moan et al., 1989; Peng et al., 1991). However, in that study it was not investigated how the intracellular distribution of the phthalocyanine changed with the incubation time. The fluorescence pattern of human embryonic epithelium cells incubated with bisulfonated aluminum phthalocyanine ($AlPcS_2$), for 30 min, characterized by both bright fluorescence spots and a diffuse fluorescence, was found to change when the cells were incubated with the phthalocyanine for 16 h (Bottiroli et al., 1992). In the latter experimental conditions, brighter and localized fluorescence spots were observed in an endocytic compartment, whereas the diffuse fluorescence was rather low. It is possible that in our experimental conditions, although the images obtained did not reveal any alteration in the subcellular distribution of the sensitizer with the incubation time, short incubation periods with $AlPcS_4$ may not allow the sensitizer to reach its main target; longer incubation periods may allow microenvironmental alterations on $AlPcS_4$ localization and/or interaction with cellular components, increasing the photosensitization efficiency.

When a fluence rate of 10 mW/cm^2 was used, the photodynamic efficiency of $AlPcS_4$ was no longer strictly dependent of the pre-incubation period with the phthalocyanine; only a small effect was detected for the higher light-doses employed. In an ideal situation, where the photophysical properties of the sensitizer are not altered during irradiation, the fluence rate is proportional to the rate of formation of the triplet state of the sensitizer and this formation is proportional to the rate of singlet oxygen production. In this same ideal situation, the light dose is proportional to the total amount of singlet oxygen produced. Therefore, the concentration of singlet oxygen is increased during irradiation using 10 mW/cm^2, as compared with 5 mW/cm^2, although the total amount produced is the same for the same light dose. If the same mechanism applies to intact cells, the increase in the concentration of singlet oxygen, when higher fluence rates are used, could be sufficient to induce irreparable cell damage, even when the phthalocyanine is not located in the more sensitive loci.

When the cells were preincubated for 24 h with $AlPcS_4$, the increase of fluence rate from 5 mW/cm^2 to 10 mW/cm^2 induced a small increase of cell death, as measured 24 h after irradiation. This is probably due to the fact that $AlPcS_4$ was already located in a very sensitive locus and, therefore, the singlet oxygen concentration produced using a fluence rate of 5 mW/cm^2 was sufficient to induce cell death.

The differences obtained regarding the photosensitizing effect of $AlPcS_4$, depending on the preincubation time, could also be due to different states of aggregation of the sensitizer inside the cell, as suggested previously (Margaron et al., 1996). When the cells are incubated with $AlPcS_4$ for 24 h, the amount of sensitizer in the monomeric form may increase, and this may increase singlet oxygen production after irradiation. However, this is not in agreement with the observation that the fluorescence of intracellular monomeric $AlPcS_4$ was not significantly different in cells preincubated with the phthalocyanine for 6 h or for 24 h.

Taken together, our results with $AlPcS_4$ show that CCRF-CEM cells can tolerate a certain level of oxidative stress, such as that caused by irradiation using a fluence rate of 5 mW/cm^2, upon incubation with the phthalocyanine for 6 h. A higher photosensitization efficiency can be achieved by increasing the incubation time with the phthalocyanine and/or by enhancing the fluence rate. The former mechanism may be necessary for the sensitizer to reach the loci where singlet oxygen is efficient in inducing cell death, whereas the latter process increases the rate of production of singlet oxygen.

When we incubated the cells with AlPc no significant effect of the incubation time was observed when cells were irradiated with 3 mW/cm^2 (data not shown), 5 mW/cm^2 or 10 mW/cm^2 (Figure 3), under conditions where maximal uptake of the phthalocyanine was achieved. This is perhaps related to the fact that this phthalocyanine is diffusely distributed in the cytoplasm of the cells when observed with confocal microscopy (Figure 4; see also: Ben Hur et al., 1992; Malham et al., 1996). Indeed, since AlPc is very hydrophobic, it is probably embedded in the lipids of the cell. This sensitizer has a very fast uptake since the maximal concentration inside the cell was achieved after 10 min incubation (Figure 1) and the mechanism of uptake is possibly by simple diffusion through the cell membrane (Ben-Hur and Rosenthal, 1986). Therefore, no difference in its location inside the cell was observed with the different incubation periods used (Figure 4). In the experiments where AlPc was used, photosensitization was influenced mostly by the fluence rate used. Interestingly, our results are in contrast with a recent publication where a decrease in the efficacy of PDT with AlPc was observed for lymphoblastic cell lines as the incubation time was increased (He et al., 1998).

Conclusions

The results show that the preincubation time with $AlPcS_4$ affects photosensitization for the lower fluence rate used under conditions where the same amount of the phthalocyanine was taken up by the cells. However, increasing the fluence rate reduced the effect of the preincubation time on cell damage. Photosensitization with AlPc was affected by the fluence rate but not by the preincubation period with the phthalocyanine. These effects did not appear to be due to redistribution of the sensitizer inside the cells after the maximal uptake was reached, as observed by confocal microscopy.

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References

Allemann E, Brasseur N, Kudrevich SV, Lamadeleine C, Vanlier JE. Photodynamic activities and biodistribution of fluorinated zinc phthalocyanine derivatives in the murine EMT-6 tumour model. Int J Cancer. 1997;72:289–94.

Ben-Hur E, Rosenthal I. Photosensitized inactivation of Chinese hamster cells by phthalocyanines. Photochem Photobiol. 1985;42:129–33

Ben-Hur E, Rosenthal I. Photosensitization of Chinese hamster cells by water-soluble phthalocyanines. Photochem Photobiol. 1986;43:615–9

Ben-Hur E, Kol R, Riklis E, Marko R, Rosenthal I. Effect of light fluence rate on mammalian cells photosensitization by chloroaluminium phthalocyanine tetrasulphonate. Int J Radiat Biol. 1987;51:467–76

Ben Hur E, Nagelkerke JF, Dubbelman TM, Van Steveninck J. The effect of photodynamic-induced fluorescence changes of aluminium phthalocyanine in chinese hamster cells. Int J Radiat Biol. 1992;61:767–72.

Berg K, Bommer JC, Moan J. Evaluation of sulfonated aluminum phthalocyanines for use in photochemotherapy. A study on the relative efficiencies of photoinactivation. Photochem Photobiol. 1989;49:587–94

Bottiroli G, Croce AC, Ramponi R, Vaghi P. Distribution of disulfonated aluminum phthalocyanine and Photofrin II in living cells: a comparative fluorometric study. Photochem Photobiol. 1992;55:575–88

Brasseur N, Ali H, Autenrieth D, Langlois R, van Lier JE. Biological activities of phthalocyanines–III. Photoinactivation of V-79 Chinese hamster cells by tetrasulfophthalocyanines. Photochem Photobiol. 1985;42:515–21

Chan WS, Marshall JF, Svensen R, Bedwell J, Hart IR. Effect of sulfonation on the cell and tissue distribution of the photosensitizer aluminum phthalocyanine. Cancer Res. 1990;50:4533–8

Foster TH, Hartley DF, Nichols MG, Hilf R. Fluence rate effects in photodynamic therapy of multicell tumor spheroids. Cancer Res. 1993;53:1249–54

Gantchev TG, Urumov IJ, Hunting DJ, Van Lier JE. Photocytotoxicity and intracellular generation of free radicals by tetrasulphonated Al- and Zn-phthalocyanines. Int J Radiat Biol. 1994;65:289–98

Gomer CJ, Rucker N, Razum NJ, Murphree AL. In vitro and in vivo light dose rate effects related to hematoporphyrin derivative photodynamic therapy. Cancer Res. 1985;45: 1973–7

He J, Horng M, Deahl JT, Oleinick NL, Evans HH. Variation in photodynamic efficacy during the cellular uptake of two phthalocyanine photosensitizers. Photochem Photobiol. 1998;67:720–8

Henderson BW, Dougherty TJ. How does photodynamic therapy work? Photochem Photobiol. 1992;55:145–57

MacRobert AJ, Bown SG, Phillips D. What are the ideal photoproperties for a sensitizer? In: Harnett S, eds. Photosensitizing compounds: their chemistry, biology and clinical use. Chichester: Wiley & Sons; 1989:4–12.

Malham GM, Thomsen RJ, Finlay GJ, Baguley BC. Subcellular distribution and photocytotoxicity of aluminium phthalocyanines and haematoporphyrin derivative in cultured human meningioma cells. Br J Neurosurg. 1996;10:51–7

Margaron P, Gregoire MJ, Scasnar V, Ali H, van Lier JE. Structure–photodynamic activity relationships of a series of 4-substituted zinc phthalocyanines. Photochem Photobiol. 1996;63:217–23

Merlin JL, Azzi S, Lignon D, Ramacci C, Zeghari N, Guillemin F. MTT assays allow quick and reliable measurement of the response of human tumour cells to photodynamic therapy. Eur J Cancer. 1992;28A:1452–8

Moan J. On the diffusion length of singlet oxygen in cells and tissues. J Photochem Photobiol B: Biol. 1990;6:343–4

Moan J, Berg K, Kvam E et al. Intracellular localization of photosensitizers. In: Harnett S, ed. Photosensitizing compounds: their chemistry, biology and clinical use. Chichester: Wiley; 1989:95–107.

Moor ACE, Lagerberg JWN, Tijssen K et al. In vitro fluence rate effects in photodynamic reactions with $AlPcS_4$ as sensitizer. Photochem Photobiol. 1997a;66:860–5.

Moore JV, West CML, Whitehurst C. The biology of photodynamic therapy. Phys Med Biol. 1997b;42:913–35.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–63.

Paquette B, Ali H, Langlois R, van Lier JE. Biological activities of phthalocyanines–VIII. Cellular distribution in V-79 Chinese hamster cells and phototoxicity of selectively sulfonated aluminum phthalocyanines. Photochem Photobiol. 1988;47:215–20.

Peng Q, Farrants GW, Madslien K et al. Subcellular localization, redistribution and photobleaching of sulfonated aluminum phthalocyanines in a human melanoma cell line. Int J Cancer. 1991;49:290–5.

Ramakrishnan N, Oleinick NL, Clay ME, Horng MF, Antunez AR, Evans HH. DNA lesions and DNA degradation in mouse lymphoma L5178Y cells after photodynamic treatment sensitized by chloroaluminum phthalocyanine. Photochem Photobiol. 1989;50:373–8.

Roberts WG, Berns MW. In vitro photosensitization I. Cellular uptake and subcellular localization of mono-L-aspartyl chlorin e_6, chloro-aluminum sulfonated phthalocyanine, and photofrin II. Lasers Surg Med. 1989;9:90–101.

Rosenthal I. Phthalocyanines as photodynamic sensitizers. Photochem Photobiol. 1991;53:859–70.

Rosenthal I, Ben-Hur E. Phthalocyanines in photobiology. In: Leznoff CC and Lever ABP, eds. Phthalocyanines: properties and applications. New York: VCH; 1989:395–425.

Rosenthal I, Ben-Hur E. Role of oxygen in the phototoxicity of phthalocyanines. Int J Radiat Biol. 1995;67:85–91.

Rosenthal I, Ben Hur E, Greenberg S, Conception-Lam A, Drew DM, Leznoff CC. The effect of substituents on phthalocyanine photocytotoxicity. Photochem Photobiol. 1987;46:959–63.

Sokolov VV, Chissov VI, Yakubivskya RI et al. Photodynamic therapy (PDT) of malignant tumors by photosensitizer Photosens: results of 45 clinical cases. Proc SPIE. 1996; 2625:281–7.

Van Lier JE. Prospects of phthalocyanines as photosensitizers for the photodynamic therapy of cancer. In: Gomer CJ, ed. Future directions and applications in photodynamic therapy; 1990:107–4.

Veenhuizen RB, Stewart FA. The importance of fluence rate in photodynamic therapy: is there a parallel with ionizing radiation dose-rate effects? Radiother Oncol. 1995;37:131–5.

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