Preliminary studies of phthalocyanine sensitizers incorporated into human leukemia cells from two cell-lines✪

Krzysztof Wiktorowicz[1], Justyna Cofta[1], Alina Dudkowiak[2], Aneta Waszkowiak[2] and Danuta Frąckowiak[2✉]

[1]*Department of Biology and Environmental Sciences, K. Marcinkowski University of Medical Sciences, Poznań, Poland,* [2]*Institute of Physics, Poznań University of Technology, Poznań, Poland*

Three phthalocyanine dyes-sensitizers were incorporated into two types of human T leukemia cells from two cell-lines: CCRF and MOLT 4. The efficiency of the dye incorporation into cells and photochemical properties of stained cells were investigated using fluorescence spectroscopy. The dyes exhibited different properties in each of the two cell-lines. Small differences in cell membrane properties have a strong influence on the efficiency of dye incorporation and on the course of photodynamic reaction. It is suggested that, for a given patient, an optimal dye-sensitizer should be established before photodynamic treatment.

Phthalocyanines (Pcs) are promising candidates for medical applications because they show a strong Q band in the absorption spectrum, located in the red region in which the tissue is rather transparent (Rosenthal *et al.*, 1991; Frąckowiak *et al.*, 1998; 2001b; Waszkowiak *et al.*, 2002, Wiktorowicz *et al.*, 2004) and they are fluorescent, which provides an opportunity for the establishment of their localization in the tissue (Pogue *et al.*, 2001; Wood *et al.*, 1997). Therefore, they have already been widely investigated in view of their possible applications in photodynamic therapy (PDT) and photodynamic diagnosis (PDD) (Wood *et al.*, 1997; Cubeddu *et al.*, 2000; Trivedi *et al.*, 2000; Pogue *et al.*, 2001).

Synthetic dyes are still useful for PDT, in addition to the naturally occurring compounds which can be metabolized to photosensitive products (Kelty *et al.,* 2002). Cells activated by mitogens (e.g. phytohemagglutinin), investigated previously (Waszkowiak *et al.,* 2002; Wiktorowicz *et al.,* 2004), are model systems of malignant cells. To be an efficient photosensitizer for PDT, the dye-sensitizer should be (i) efficiently incorporated into malignant cells, (ii) non-toxic for healthy cells, and (iii) exhibit high yield of triplet states generation. The last condition is fulfilled for most of the Pcs investigated by us, which in solutions exhibit different, but always high yield of S→T intersystem crossing transitions (Frąckowiak *et al.,* 2001a). It was shown (Wiktorowicz *et al.,* 2004) that the photodynamic reaction in activated cells is very efficient for dyes exhibiting high efficiency of triplet state generation in solutions.

The spectral and photochemical properties of dyes in cells and in solution can be different. Therefore, in this work we established the spectral properties of three Pcs incorporated into leukemia cells from two cell-lines. These dyes, whose structures are presented in Fig. 1, were previously investigated in various model systems (Frąckowiak *et al.,* 2001a; 2001b; 2002; Waszkowiak *et al.,* 2002; Wiktorowicz *et al.,* 2004).

The absorption spectra of stained and unstained cells were not much different even in the region of maximal absorption of the applied dyes (at about 610 nm) and were additionally perturbed by light scattering. Therefore, in this preliminary study, the fluorescence spectra were used for monitoring the photoreactions occurring in the cells.

MATERIAL AND METHODS

Leukemia T cells from two continuous culture lines, obtained from the Institute of Immunology and Experimental Therapy (Polish Academy of Sciences, Wrocław, Poland),

were incubated in Pcs solutions. The first line (CCRF) was derived from a Caucasian girl with acute lymphoblastic leukemia (ALL). The membrane properties of these cells were: CD2–, CD3+, CD4+, CD5+, CD6+, CD7+, CD8–, CD13–, CD14–, CD15+, CD19–, CD33–, CD34–, CD68–, CDw90–, HLA-DR–, TCRα/ β+, TCRγ/δ– (CD — cluster of differentiation, numbers describe the type of proteins (glycoproteins) in cell membranes; TCR — antigenic receptor of lymphocytes). The morphology of the cells was: round cells growing singly in suspension and as adherent epitheloid cells forming a monolayer. The cells from the second line (MOLT–4) were from a 19 year old man with ALL. These cells exhibit the following characteristics: CD2+, CD3+, CD4+, CD5+, CD6+, CD7+, CD8+, CD13–, CD19–, CD34+, TCRα/β–, TCRγ/δ–. The morphology of these cells was: round cells growing in suspension singly or in clusters.

The cells were grown in stationary suspension cultures, using standard culture conditions (37°C, 5% CO_2) in RPMI 1640 medium with 2 mM L-glutamine and supplemented with 10% FBS (fetal bovine serum, Sigma).

The Pcs were purchased from Sigma-Aldrich and used without further purification. The molecular structures of these Pcs and their abbreviated notation are shown in Fig. 1.

Dye solution (10^{-5} M) in dimethyl sulfoxide (Me_2SO) (with 0.5% H_2O) was added to such a volume of cell suspension that 1 ml of final suspension contained 10^6 cells. Incubation was carried out at (37 ± 1)°C for 1 h in the dark. Then the stained cells were washed three times and resuspended in 0.9% NaCl in H_2O. The cells incubated in pure Me_2SO (without dye) used as a reference samples were investigated cytometrically. The results show that the number of dead cells in such samples was always lower than 10%.

Samples were illuminated with a He-Ne laser light (λ_{las} = 632.8 nm, light intensity 2 mW/cm^2) for 1, 2 or 3 h. During the illumination the temperature was stable to ± 2°C. The
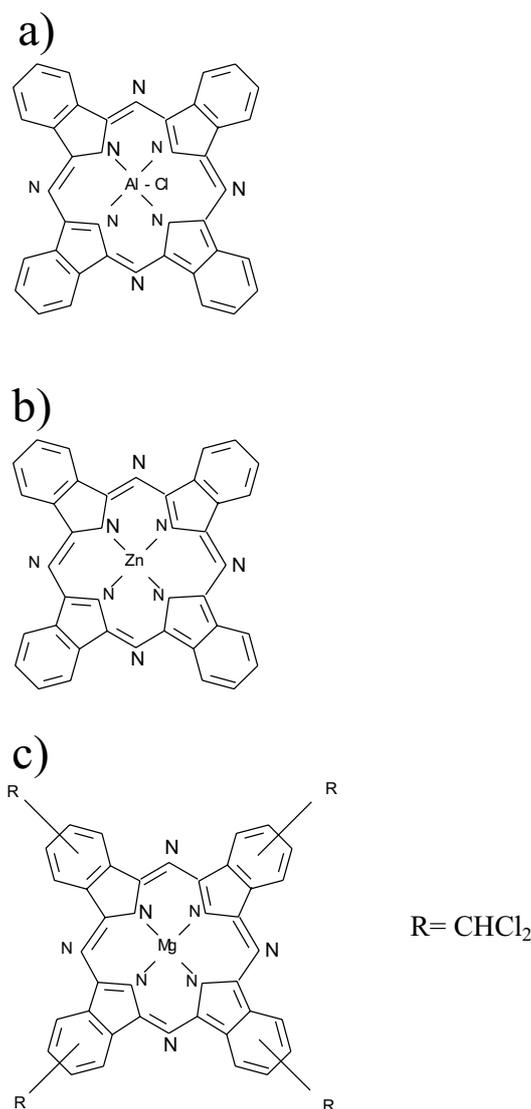
Figure 1. Structures of the investigated phthalocyanines: (a) AlPcCl, (b) ZnPc, (c) MgPc.

dose of light energy delivered during 1 h was about 7.2 J/cm^2. It was almost 1.5 fold higher than that used in (Pogue *et al.,* 2001) for killing 50% of cancer cells. The reference sample (non-stained) cells after the same illuminations were also investigated.

Absorption spectra were recorded using a Specord M40 Carl Zeiss (Jena, Germany) spectrophotometer, fluorescence spectra by means of a Hitachi F 4500 (Japan). The width of the spectral band used in excitation and emission beams was 5 nm. Even unstained cells exhibit some autofluorescence of cell material (Frąckowiak *et al.,* 1998; Pogue *et al.,* 2001; Monici *et al.,* 2003). The changes in this emission caused by illumination, measured for stained and unstained cells, have been recently used for monitoring the course of photodynamic reactions (Monici *et al.,* 2003). The intensity of fluorescence of a dye incorporated into cells depends on the efficiency of the dye incorporation and on the yield of its fluorescence. The yields of dye fluorescence in various cell-lines can be different because of possible differences in their location in different types of cells, resulting in different interactions of the dye molecule with the surroundings.

Fluorescence was excited in two regions: in the region of the incorporated dye absorption (at 610 nm) and in the region of the cell material absorption (at 295 nm). The fluorescence of the incorporated dye (measured in the 640–800 nm range) provided information on the efficiency of the dye incorporation into the cells and on the possible dye bleaching during illumination. The changes in the short wavelength (300–500 nm) endogenous fluorescence of the cell material can be related to changes in cell properties caused by the dye incorporation and by the illumination. From our preliminary results, it is not possible to establish the character of the photochemical or conformational changes occurring in the cell material because in the region of fluorescence excitation contributions from different compounds superimpose.

RESULTS

The absorption spectra of MOLT 4 cells stained by various Pcs and of the same cells incubated in Me$_2$SO without dyes were measured before and after illumination. The illumination caused different changes in the absorption in the 200–250 nm range for non-stained cells as well as for cells stained by the same dye in a case of cells obtained from

two cell-lines (not shown). The absorption of the dyes in this region can be neglected. The long wavelength absorption (about 600–700 nm) in which the Pcs absorption can be expected (Rosenthal, 1991; Waszkowiak *et al.,* 2002) for the Pc dyes incorporated into cells was very low and perturbed by light scattering (not shown). The changes in the cell material absorption and endogenous cell fluorescence due to the cell staining as well as the fluorescence of the Pcs incorporated into cells showed clearly that the dye molecules were present in the stained cells. The emission of non-stained cells (excited at 610 nm) in the longwavelength region was not measurable.

Figure 2 shows the fluorescence excitation spectra of the two types of cells with AlPcCl incorporated. The changes in the fluorescence at 340 nm (i.e. in the region of the cell material emission) observed as a result of illumination are different for the cells stained with the same dye but coming from the two lines. Figure 3 presents the fluorescence spectra of the cells stained by the same dye excited and measured in two wavelength regions. The emission of the cell material (excited at 295 nm, measured from 300 to 500 nm) and the emission of the cell-incorporated dye (excited at 610 nm, observed from 640 to 800 nm) were observed as a function of the time of illumination and shown to be different for the two types of cells. The fluorescence of the dye was less intense than that of the cell material but well measurable. It is known (Frąckowiak *et al.,* 2001b; Wiktorowicz *et al.,* 2004) that the Pcs fluorescence yield in the cells is about tenfold lower than in most solvents. The low fluorescence intensity can be observed in the cells, even at a high level of dye incorporation, as established by photothermal spectral measurements (Frąckowiak *et al.,* 1998). The higher intensity of AlPcCl fluorescence in CCRF cells than in MOLT 4 cells may be due to a more efficient incorporation of the dye into the former and/or to a higher yield of the dye fluorescence due to different intracellular location of the dye molecules.
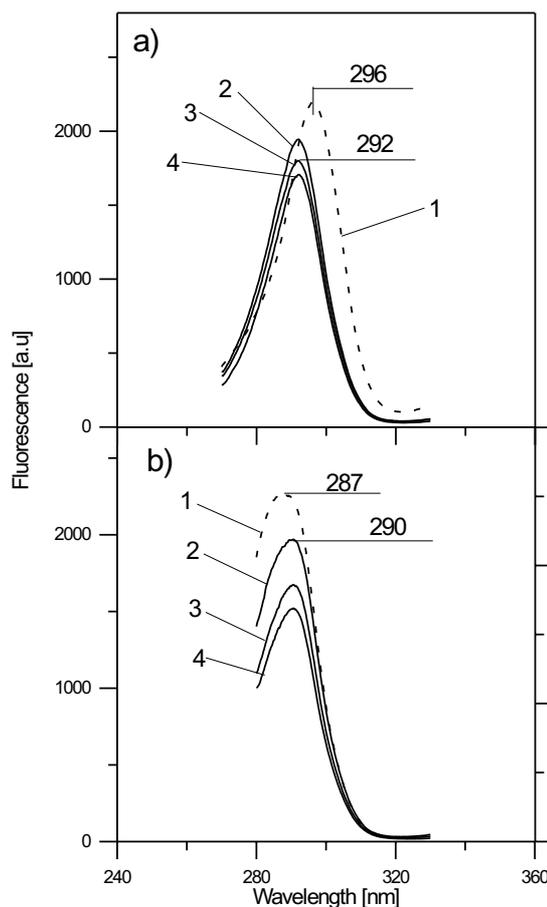
Figure 2. Fluorescence excitation spectra of CCRF (a) and MOLT 4 (b) cells stained by AlPcCl measured, at 340 nm, after illumination for: 0 h (1), 1 h (2), 2 h (3) and 3 h (4).

Figure 4 presents the changes in the intensities of the main fluorescence bands caused by illumination of the samples incubated with AlPcCl, ZnPc and MgPc, in comparison with the changes observed for unstained cells. The endogenous cell fluorescence can be related to several compounds (tryptophan, flavins, nicotinamide adenine dinucleotide) (Anderson-Engels *et al.,* 1991; Pogue *et al.,* 2001; Waszkowiak *et al.,* 2002; Monici *et al.,* 2003). Using only one wavelength of excitation (in fluorescence spectra) and one region of emission observation (in excitation spectra) it is not possible to establish the contributions from all sources.

The fluorescence intensity of stained non-illuminated cells depends on the volume of the cells in the sample, the efficiency of the dye incorporation and the perturbation in the cell
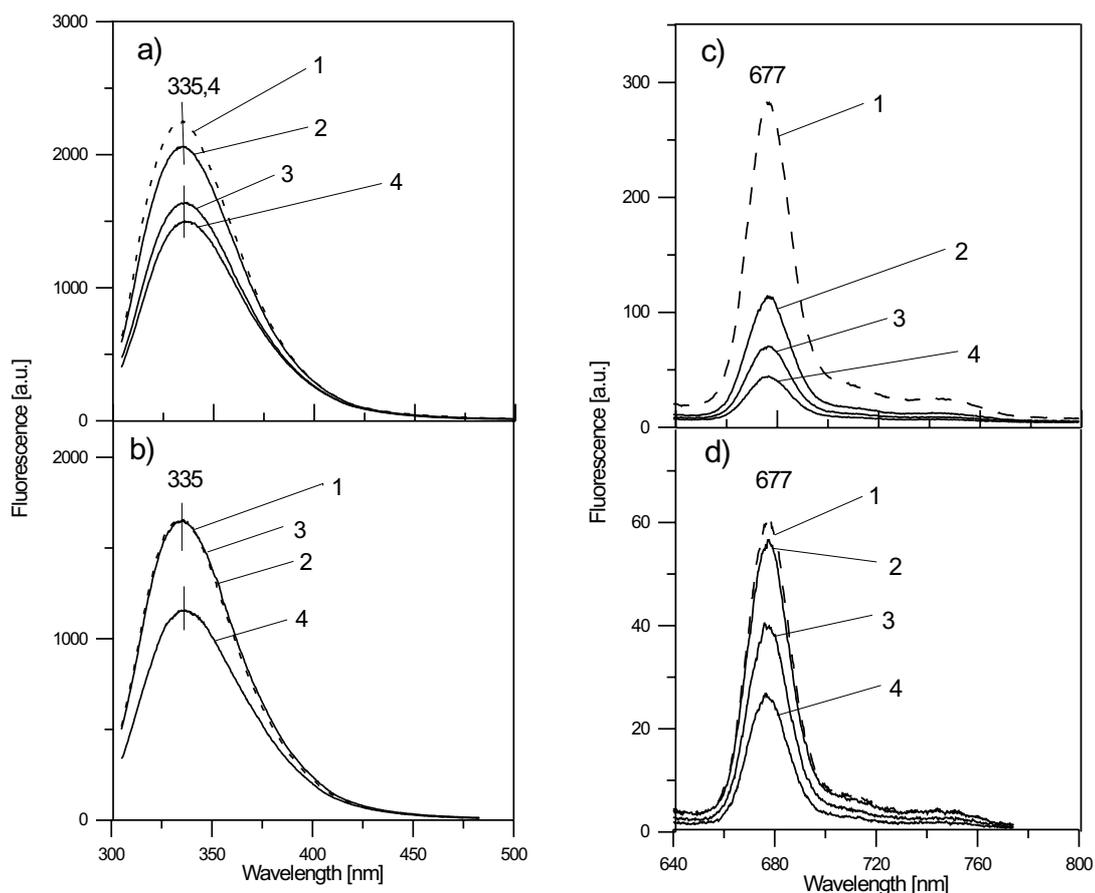
Figure 3. Fluorescence spectra excited at 295 nm (a, b) and at 610 nm (c, d) for the CCRF (a, c) and MOLT 4 (b, d) leukemia cells stained by AlPcCl, illuminated for: 0 h (1), 1 h (2), 2 h (3), 3 h (4).

caused by the incorporation of dye molecules. To eliminate these unknown effects, in Fig. 4 the fluorescence intensities of non-illuminated cells of every type are normalized, and the kinetics of photoreactions are shown as changes in the emission intensity.

As it follows from Fig. 4, the effect of illumination is different in non-stained and stained cells and is different for various Pcs introduced, but always depends strongly on the type of the cells. The photosensitivity of unstained cells from the two lines is different (Fig. 4 a, b). Also the photodestruction of a dye depends on the cell line (Fig. 4 c, d). For cells stained by some dyes the changes in cell material emission are in some cases lower than for unstained cells (Fig. 4 b). The significant changes as a result of illumination observed for the cells from the two lines suggest different modifications of the cell structure or/and different photochemical sensitivity of the samples.

DISCUSSION

The modes of interactions of dye photosensitizers with lipids and proteins are different. It is known (Schafer *et al.,* 1999) that singlet oxygen $^1O_2$ generated by exited Photofrin is quenched by lipids only by a chemical mechanism, while proteins quench it by chemical or physical mechanisms. Dye and illumination affect membrane permeability of leukemia cells. This effect differs for cells from various lines (Schafer *et al.,* 1999). The damage to the membranes depends on the types and amounts of lipid and proteins in the cells. Therefore, it is different for cells from various lines. It seems that proteins can
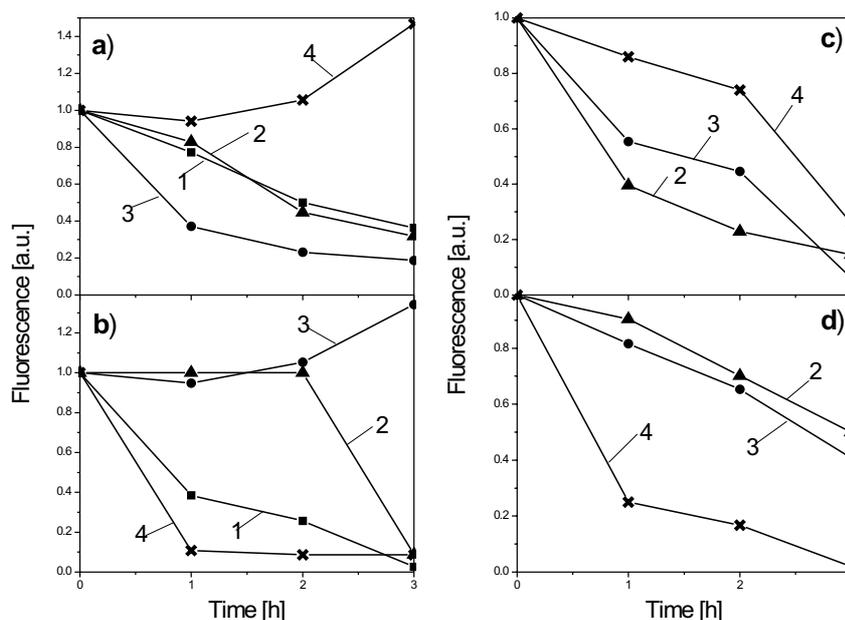
Figure 4. Dependence of intensities at main fluorescence band as a function of illumination time.

Cells obtained from CCRF (a, c) and MOLT 4 (b, d) cell lines. Unstained cells (curves 1); cells stained by AlPcCl (curves 2), ZnPc (curves 3) and MgPc (curves 4). Wavelengths of fluorescence excitation (λ_{exc}) and observation (λ_{obs}): in the case of cell material emission (a, b) λ_{exc} = 295 nm and λ_{obs} = 335 nm; in the case of dye emission (c, d): λ_{exc} = 610 nm and λ_{obs} = 667 nm for AlPcCl, λ_{obs} = 674 nm for ZnPc, λ_{obs} = 675 nm for MgPc.

protect leukemia cells against the damage caused by photosensitizers and illumination by quenching singlet oxygen. Larger cells are better protected than smaller ones (Schafer *et al.,* 1999). The sizes of the cells studied in this work are rather similar. The experiments showed that the kinetics of the photoreactions in stained cells from the two lines are different and that these differences are dependent on the dye used. The cell size can influence the course of photoreactions (Schafer *et al.,* 1999), but this factor cannot explain the different behavior of various Pcs. There are still several open questions and they will be the subject of further investigations. We plan to extend the investigation to several cell-lines and more dyes. The establishment of the precise localization of the dye molecules in cell structures is very important (Cubeddu *et al.,* 2000; Pogue *et al.,* s24 2001; Monici *et al.,* 2003). It is known that Pcs engaged in different interactions with the sur-

roundings have different spectral properties (Frąckowiak *et al.,* 2002). The different photochemical properties of various Pcs in the cells suggest that they interact with different macromolecules, because in solutions all these dyes exhibit similar high yields of triplet states generation (Frąckowiak *et al.,* 2001a).

Triplet states of investigated Pcs in solutions are to a similar degree quenched by oxygen (Frąckowiak *et al.,* 2001a). Therefore, one can predict that these dyes will have a similar photochemical sensitivity. We found out (Frąckowiak *et al.,* 2002) that the Pcs with the same central metal ion and different side groups can be ordered differently with respect to polymer chains. A similar situation can occur in the cell material where these dyes can differently interact with macromolecules because of their various orientation. This corresponds to the data reported by Feofanov *et al.* (2002) that Pcs with the same

central metal and different side groups can exhibit different activity in PDT. It is known (Langlois *et al.,* 1986) that some Pcs are able to oxidize tryptophan and cholesterol. Further studies are necessary in order to establish if after illumination the observed spectral changes in cell material are due to photochemical or to conformational changes.

On the basis of the current knowledge of the mechanisms of interaction between Pcs and cell material, it is not easy to predict the efficiency of anti-tumor activity of a given Pc in the patient cells. Therefore, it seems that it would be practical to work out a simple test providing information about the interaction of patient cells with the photosensitizer.

The presented results show that small differences in the properties of leukemia T cells cause significant differences in the yield of the dye incorporation as well as in the course of the photoreactions occurring in the stained illuminated cells. Different dyes are optimal photosensitizers for various cell-lines. It is not easy to find the dye which can be an optimal sensitizer for all types of the cells. This suggests that before PDT treatment the interaction of a dye with the patient's cells should be investigated. Working out a simple test to the above effect will be our future task.

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