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Preliminary Note

A Transient Reduction of the Fluorescence of Aluminium Phthalocyanine Tetrasulphonate in Tumours During Photodynamic Therapy

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1. Introduction

Phthalocyanines are a group of sensitizers that possess promising properties for future use in photochemotherapy of cancer [1 - 7]. Several of these compounds show some selectivity with respect to tumour uptake or retention. Furthermore, they have a strong absorption in the wavelength region above 600 nm and a significant photosensitizing efficiency, *in vitro* as well as *in vivo*.

In this paper, we report a surprising behaviour of the fluorescence of aluminium phthalocyanine tetrasulphonate ($AlPcS_4$) in tumours during laser exposure under conditions relevant for photodynamic therapy (PDT). This behaviour should be taken into consideration when optimal exposure schemes are sought for PDT with this dye.

2. Materials and methods

$AlPcS_4$ was obtained from Porphyrin Products, Logan, UT. Its purity was tested using high performance liquid chromatography (HPLC) as described elsewhere [8]. Essentially, the dye is free from impurities (less than 5%) but contains several isomers of slightly different polarity [8]. Stock solutions of 2.5 mg ml^{-1} were prepared in Dulbecco's phosphate-buffered saline (PBS) and kept frozen until use.

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Female BALB/c athymic nude mice (G. Bomholt gaard, Ry., Denmark) were used. At the start of the experiments the mice were 5 - 6 weeks old and weighed 18 - 25 g. They were housed 8 - 10 per cage, and were given standard laboratory diet. The mice were challenged with cells of the human tumour line LOX, established as a subcutaneous (s.c.) xenograft from a patient with malignant melanoma [9]. Cells of this line were grown in monolayer cultures in RPMI 1640 medium with L-glutamine, penicillin–streptomycin and 10% foetal calf serum (FCS), detached with ethylenediaminetetraacetic acid (EDTA) and injected s.c. on the dorsal side of the right foot (10^6 cells in 50 μl PBS per injection). When the tumours had reached a volume of 75 - 100 mm^3, the mice were injected intraperitoneally with 20 mg kg^{-1} of the sensitizer. The mice were placed in a holder fitted into the sample compartment of a Perkin–Elmer LS-5 luminescence spectrometer 24 h after injection of the sensitizer. The tumour was gently positioned against a quartz plate for surface excitation at an angle of 50° with the excitation beam.

Using fibre optics the tumour was exposed to laser light during the fluorescence measurements. The diameter of the laser beam at the tumour surface was 5 mm, covering the entire tumour. Since there was a pulsed excitation source and a phase sensitive detection system in the fluorescence spectrometer, it was possible to measure the fluence rate of the pulsed fluorescence during continuous laser irradiation. A filter eliminating light of wavelengths below 680 nm was used to reduce the fluence rate of scattered laser light reaching the detection system of the spectrometer. Above a certain fluence rate of the laser light (approximately 150 mW cm^{-2}) artefacts were introduced into the measurements due to saturation of the photomultiplier of the fluorescence spectrometer. It was thoroughly checked that these artefacts were absent from the results shown below.

The laser system consisted of a 4-dicyanomethylene-2-methyl-6-*p*-dimethylaminostyryl-4H-pyran (DCM) dye laser (Spectra Physics 375 B) pumped with a 5 W argon ion laser (Spectra Physics 164). The laser beam was led by a 600 μm quartz optical fibre to the tumour for surface exposure. At the tumour surface the fluence rate was between 50 and 200 mW cm^{-2}, depending on the chosen wavelength.

3. Results

Figure 1 shows the *in vivo* fluorescence of a LOX tumour containing $AlPcS_4$. To avoid any artefacts due to saturation of the detection system of the fluorescence spectrometer, the slits of the spectrometer were closed during the laser irradiation which was carried out at the clinically relevant fluence rate of 150 mW cm^{-2} (675 nm). The slits were opened approximately 0.1 s after the laser irradiation. At this time the fluence rate of the phthalocyanine fluorescence from the tumour was less than 50% of the value before irradiation. However, within 3 min the fluence rate increased to almost the initial level (Fig. 1). This process could be repeated several times.

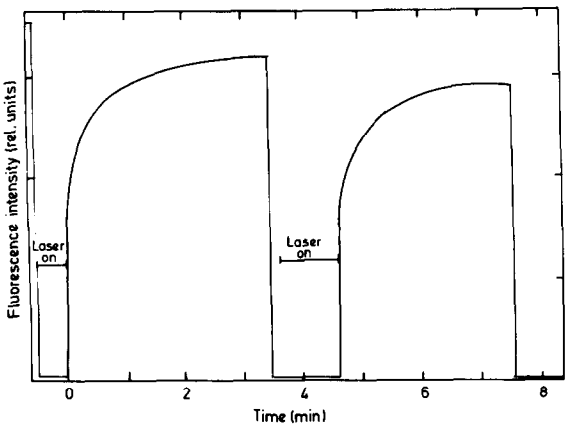

Fig. 1. Phthalocyanine fluorescence registered *in vivo* in a LOX tumour. The tumour-bearing mouse was injected intraperitoneally with 20 mg kg^{-1} $AlPcS_4$ 24 h before the measurement. The excitation and emission wavelengths were 360 nm and 685 nm respectively. Similar results were also found with an excitation wavelength of 610 nm. The wavelength of the laser light was tuned to 675 nm and the fluence rate was about 150 mW cm^{-2} at the surface of the tumour.

At extended laser exposures of 10 min or more, a permanent reduction in the phthalocyanine fluorescence was observed, supposedly due to irreversible photodamage to the phthalocyanine molecules in the tumour.

When the laser was tuned to 620 nm, the fluence rate at the tumour surface adjusted to about 100 mW cm^{-2} and a 680 nm cut-off filter placed in front of the emission slit of the fluorescence spectrometer, it was possible to detect the fluorescence during laser irradiation (data not shown). During laser irradiation the fluence rate of the fluorescence decreased. To a first approximation the decrease was proportional to the fluence rate of the laser light. The emission spectrum of phthalocyanine fluorescence was similar before and during laser exposure; this indicates that fluorescent photoproducts cannot explain the observations.

Furthermore, the observations cannot be due to replacement of the photodegraded dye by fresh dye via the blood stream, since the results were similar in excised tumours and in tumours *in vivo*. Neither can the observations be explained by a diffusion of molecules from unexposed tissue to exposed tissue. The diffusion constant of a lipophilic dye in tissue is of the order of 10^{-8} cm^2 s^{-1} [10]. This is an upper limit for $AlPcS_4$ molecules in tissue since they are probably bound to proteins. For a molecule with a diffusion constant of this magnitude it would take the following time to diffuse a distance of 1 mm: $t = r^2/6D = 0.1^2/6 \times 10^{-8}$ s = 46 h. This is three orders of magnitude larger than the time required for a 90% recovery of the fluorescence in the tumour after laser exposure (Fig. 1).

We tentatively suggest that the decrease in fluorescence during laser irradiation is related to a change in the binding sites or the localization of the fluorescing molecules in the tumour. It remains to be determined whether

the decrease in the fluorescence of $AlPcS_4$ in the tumour during exposure to light at high intensities is accompanied by a similar decrease in its photosensitizing efficiency.

The fluorescence behaviour of $AlPcS_4$ in a tumour during exposure is in contrast with the fluorescence behaviour of the same dye in cells *in vitro*. We have shown that in the latter case the fluorescence increases during exposure to light at high intensities [11]. In cells, the fluorescence, to a large extent, is confined to grains in the cytoplasm, supposedly the lysosomes. During light exposure these grains disrupt, giving rise to a dilution of the dye as well as a change in its intracellular localization. The fluorescence increase is supposedly related to these processes. It should be noted that free phthalocyanines have fluorescence quantum yields which are about an order of magnitude larger than those of phthalocyanines bound to a protein such as human serum albumin [12]. A possible explanation of the striking difference between the fluorescence behaviour of $AlPcS_4$ in cells and in tumours is that the dye is mainly localized in extracellular structures in the tumours and not in the lysosomes of cells.

This is in agreement with our preliminary fluorescence microscopic studies of frozen sections of the LOX tumour (Fig. 2). These micrographs were taken 4 h after injection of 20 mg kg^{-1} $AlPcS_4$ intraperitoneally in the mice. The fluorescence was at a maximum in the tumour 4 h after injection. The fluorescence was too weak to produce good micrographs 24 h after injection, but visually it appeared to be distributed in a similar manner to that shown in Fig. 2. It can be seen that the fluorescence originates, to a large extent, from the extracellular space and not from intracellular structures.

Fig. 2. Micrographs of a LOX tumour in an athymic nude mouse prepared 4 h after intraperitoneal injection of 20 mg kg^{-1} $AlPcS_4$ (bar, 10 μm). Left: transmission optical micrograph; right: fluorescence micrograph obtained using a red-sensitive video camera. Details of the preparation of the samples and of the microscopic procedures are given elsewhere [13].

4. Conclusions

This preliminary report shows that the fluorescence of $AlPcS_4$ in a human LOX tumour transplanted into nude mice decreases during exposure to laser light; the fluorescence recovers to almost the initial value 3 min after the laser light has been switched off. The dye is probably localized extracellularly in the LOX tumour, since the fluorescence intensity of $AlPcS_4$ in cells *in vitro* increases during light exposure in contrast with the decrease seen in the tumour.

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