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Fluorescence Imaging During Photodynamic Therapy of Experimental Tumors in Mice Sensitized with Disulfonated Aluminum Phthalocyanine¶

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ABSTRACT

A fluorescence imaging system was used to monitor the emission of disulfonated aluminum phthalocyanine (AlS_2Pc) during the photodynamic therapy (PDT) of murine tumors. Cells of the MS-2 fibrosarcoma were injected in mice in two compartments in order to cause the development of tumors in different host tissues. Two drug doses and two uptake times were considered. Moreover, the fluorescence of the AlS_2Pc was excited using two wavelengths on the opposite sides of the absorption peak to detect a possible change in the absorption spectrum of the sensitizer induced by the PDT. In the tumors, the treatment induces a variation of the fluorescence intensity: in some mice a mild photobleaching takes place, in others a fluorescence enhancement occurs. Which effect predominates depends on the experimental conditions, even though a large spread of data was found amongst mice of the same group. In all mice, independently of the drug dose, uptake time or tumor compartment, a marked increase in the fluorescence signal takes place at the borders of the irradiated area. To quantify this effect we evaluated the ratio between the fluorescence intensities in the peritumoral area and in the tumor itself. This ratio increases monotonically during the PDT, showing a different behavior with the two excitation wavelengths. This indicates that the AlS_2Pc absorption spectrum shifts toward shorter wavelengths as a result of the irradiation.

INTRODUCTION

The success of the photodynamic therapy (PDT)† of tumors depends on the right combination of photosensitizer and activation light in the tumor. For several sensitizers, optimal protocols in terms of drug dose, uptake time and light dose have been devised for many tumor types, on the basis of extensive trials. Nevertheless, the amount of drug that really accumulates in tumor tissues in any patient is largely dependent on several metabolic factors, usually not well predictable. As a consequence, in some cases the therapy is ineffective, in other cases excess damage takes place in healthy tissues surrounding the neoplasia. Therefore, a real-time monitoring of the PDT process would be appealing. Some clues of the PDT effectiveness can be possibly found in the fluorescence characteristics of the sensitizer during light irradiation. The complex biochemical reactions occurring during the irradiation might alter the binding site of the sensitizer and lead to the photodegradation of the drug with formation of photoproducts. Very often fluorescence can reveal such effects, since they result in intensity or spectral changes of the exogenous emission. In this context, an imaging system is appealing to check the accumulation of the drug in the tumor before the PDT and to investigate the changes in emission occurring during the irradiation. Any insight that were gathered in the photobiological reactions mediated by the PDT might allow a better understanding of its mechanism and constitute the first step to set-up a real-time monitor of its therapeutic effectiveness.

To investigate this subject, we considered the PDT of experimental tumors transplanted in mice. Animals were injected with disulfonated aluminum phthalocyanine (AlS_2Pc), which is a second-generation sensitizer exhibiting a strong absorption band in the far-red, high selectivity in transplanted tumors and good phototoxic effects (1–5). Several researchers have already observed appreciable variation of the AlS_2Pc fluorescence in cells or tissues upon activation by red light either *in vitro* (6–8) or *in vivo* (9–11).

From fluorescence measurements on cells, it has been observed that water-soluble phthalocyanines, like the disulfonated AlS_2Pc or the tetrasulfonated AlS_4Pc, localize mainly in lysosomes (6,8,10). Time-resolved fluorescence studies carried out with a mode-locked laser and a time-correlated single photon counting system suggest that the AlS_2Pc is aggregated when confined to organelles. Therefore, it is weakly fluorescent and exhibits a biexponential decay time (8). The photodamage that occurs upon activation leads to permeabilization of the organelle membranes and redistribution of the sensitizer into the cytoplasm. This causes the fluorescence intensity to increase and the decay curve to change from biexponential to monoexponential behavior (8).

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†*Abbreviations:* AlS_2Pc, disulfonated aluminum phthalocyanine; b.w., body weight; MST, median survival time; PDT, photodynamic therapy.

On increasing the light dose, photobleaching has been reported by several investigators (6,8).

Fluorescence measurements performed *in vivo* during the PDT of mice sensitized with aluminum phthalocyanine also show an initial enhancement of the signal, followed by photobleaching (10,11). Yet, no spectral changes have been reported in fluorescence emission.

To the authors' knowledge, no measurements have been performed *in vivo* using an imaging system. The release of the AlS_2Pc from organelles and eventually from the extracellular matrix might lead to a spatial redistribution of the fluorescence signal and to a possible change in the absorption spectrum. To investigate these aspects, we monitored the fluorescence signal during the PDT using an intensified video camera and two excitation wavelengths on the opposite sides of the absorption peak of AlS_2Pc, which *in vivo* is centered around 685 nm. We performed the measurements at different times after the administration of the drug, during the irradiation and after the end of the treatment to evaluate the accumulation of AlS_2Pc in tissues and the effects of the activation light on this drug.

MATERIALS AND METHODS

Experimental set-up. The fluorescence imaging system used for this study is similar to the one already described in Cubeddu *et al.* (12). The excitation light is provided by a dye laser pumped by a nitrogen laser (LN203C Laser Photonic, Orlando, FL). Two dyes were used alternatively for emission at 660 nm (DCM, Exciton Chemical Co., Dayton, OH) or 690 nm (Nile Blue + Rhodamine 690, Exciton Chemical). The full width half maximum of the laser spectra was ≈15 nm for the 660 nm light and ≈10 nm for the 690 nm light. The excitation beam was coupled to an optical fiber and delivered to the mouse in a circle ≈4 cm in diameter, wide enough to include both tumors. The irradiance was 50 $\mu W/cm^2$ for the 660 nm radiation and 20 $\mu W/cm^2$ for the 690 nm radiation. Care was taken to preserve the irradiance constant throughout the experiment. Further, a fluorescent strip of white paper dumped in a diluted solution of AlS_2Pc was included in all the images to provide a fluorescence reference to be used for normalization. The images were acquired by means of a charge-coupled device video camera provided with a fast-gateable light intensifier (ICCD225, Photek, St. Leonards-on-Sea, England). A 720 nm interference filter allowed us to remove most of the excitation light. An acquisition window 100 ns wide and 2 ns delayed with respect to the excitation pulses was used to eliminate the room light and the residual excitation light. In fact, notwithstanding the interference filter, the gated acquisition proved essential for a reliable detection of the fluorescence signal due to the small separation between the excitation and the detection wavelengths. For each mouse, the gain of the light intensifier was kept constant during all the measurements. Image analysis was performed offline by means of a homemade software written in MATLAB (MathWorks, Natick, MA).

Fluorescence spectra were acquired using a second nitrogen laser (VSL337, Laser Science, Franklin, MA) emitting at 337 nm and an optical multichannel analyzer (OMA EG&G Princeton Applied Research, Princeton, NJ). The excitation light was delivered through the central fiber of a bundle and the fluorescence signal was collected by 20 fibers arranged in two circles. The bundle was positioned in contact with the intact skin above the tumors.

The PDT treatment was performed with a dye laser (DCM, Exciton Chemical Co.), pumped by an Argon laser (CR-18, Coherent, Palo Alto, CA) and tuned at 685 nm. The laser beam was coupled to an optical fiber and the animals were irradiated with 100 mW/cm^2 for 10 min.

Chemicals and tumor models. Aluminum phthalocyanine with an average degree of sulfonation of 2.1 (AlS_2Pc) was kindly provided by Dr. A. McLennan (Paisley College of Technology, Paisley, UK), and diluted in saline to a concentration of 2.5 mg/mL. The degree of sulfonation was ascertained by means of chromatography. The compound consists of a mixture of isomers with sulfonic groups in both adjacent and opposite positions.

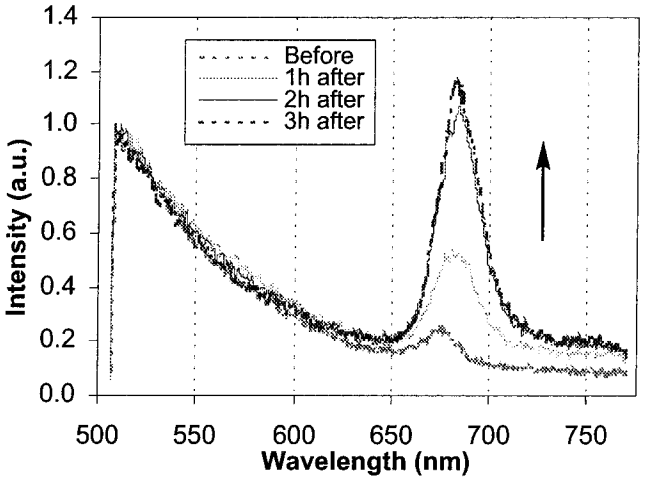

Figure 1. Fluorescence spectra of a tumor in the leg before the injection of 5 mg/kg b.w. of AlS_2Pc and 1, 2 and 3 h after.

For the experiment, two tumors originating from the same cell line were injected in different compartments in each mouse. The tumor line was an MS-2 fibrosarcoma originally induced by the Moloney murine sarcoma virus and maintained by weekly intramuscular passage of tumor cell homogenate into the right hind leg of inbred Balb/C mice (Charles River, Calco, Italy). Five days before the measurements, 10^6 tumor cells were injected intradermally in the lower dorsum and intramuscularly into the right hind leg of hybrid DBA/2 × Balb/C mice (Charles River). At the time of measurements, the tumors on the back had reached a mean diameter of 6 mm, while the tumors in the leg extended for about 1 cm in the muscular tissue. All the tumors were free of evident necrosis.

Experimental procedure. Mice were carefully shaved before the measurements. Two drug doses (*i.e.* 2.5 and 5 mg/kg body weight [b.w.]) and two uptake times (*i.e.* 3 and 12 h) were considered in this experiment. Six mice were injected intraperitoneally for each drug dose and uptake time. For a group of mice (uptake time 3 h, drug dose 5 mg/kg b.w.), fluorescence spectra were recorded 1, 2 and 3 h after the drug injection. For the acquisition of fluorescence images during the PDT, the mice were anesthetized with Ketamine (Inoketam 500, Virbac, Italy) and Xilazine (Rompun, Bayer, Italy) and two treatments were carried out in sequence, first on the leg and then on the back. For both tumors the irradiated area was delimited by an opaque screen having a 12 mm diameter hole in its center. After 5 min the PDT light was stopped and a fluorescence image was taken in few seconds. Then, the light was switched on for 5 more minutes till the completion of the treatment and another fluorescence image was taken. Finally, two fluorescence images were taken 5 and 10 min after the end of the PDT. Four mice not bearing any tumor were also treated according to the protocols described before.

It is worth noting that, in a preliminary test made to optimize the experimental procedure, fluorescence images of six tumor-bearing mice (drug dose 5 mg/kg b.w. and uptake time 3 h) were recorded every 2 min from the beginning of the PDT till 10 min after the end of the irradiation. Since appreciable changes in fluorescence intensity took place only in 4–6 min, this time interval between images was adopted for the final experiment in order to allow an easier control of the procedure.

After the experiments, the mice were followed for more than 90 days to evaluate the effect of PDT.

All the experiments were carried out in accordance with protocols approved by the local experimental animal welfare committee and conformed to national regulations for animal experimentation.

RESULTS

Using the optical multichannel analyzer we checked the progressive accumulation of the sensitizer in mice after the ad-

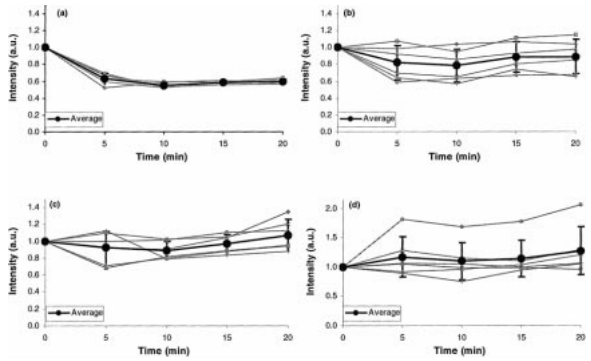

Figure 2. Average fluorescence intensity as a function of time measured from images acquired during and after the irradiation of mice sensitized with 2.5 mg/kg b.w. of AlS_2Pc: (a) tumor on the back after 12 h; (b) tumor in the back after 3 h; (c) tumor on the leg after 12 h; and (d) tumor in the leg after 3 h. The measurements were taken with excitation at 690 nm.

ministration of 5 mg/kg b.w. of AlS_2Pc. Figure 1 shows typical fluorescence spectra taken from the tumor in the leg before the injection of the drug and 1, 2 or 3 h after. Due to the short penetration depth of the UV light, the fluorescence emission comes mainly from the skin. However, this excitation wavelength also allows the detection of the natural fluorescence, which has been used for normalization. The small peak at 655 nm observed before the injection of the AlS_2Pc can be ascribed to the fluorescence of the mouse feed. The well-pronounced peak around 670 nm, which increases with time, is clearly attributed to AlS_2Pc. The amplitude of the AlS_2Pc peak correlates with the intensity in fluorescence images, hence for the other groups of mice only images were considered.

After both the uptake times, a good fluorescence signal was observed in all the animals but three, which presented an unexpected low accumulation of AlS_2Pc in the tumors, possibly due to an improper injection of the drug. These mice were excluded from the fluorescence study and were replaced by other mice. However, they underwent PDT and were followed up to their death, according to our protocol.

During irradiation complex phenomena take place resulting in a change both in the average fluorescence intensity and in its spatial distribution.

Figure 2a–d shows the average intensities as a function of time measured from the images of six mice for the two tumor compartments and the two uptake times. The drug dose was 2.5 mg/kg b.w. and the measurements were taken with excitation at 690 nm. Images taken with excitation at 660 nm were very similar to those at 690 nm. For the four groups the following considerations can be done: (1) tumors on the back of mice injected 12 h before the PDT—a marked photobleaching takes place in all the mice since the beginning of PDT. At the end of the treatment the average intensity is equal to 60% of the initial value. Taking the initial value as a reference, the reduction in the intensity is significant at a confidence level $P < 0.001$ (Student's *t*-test); (2) tumors on the back of mice injected 3 h before the PDT—in three mice an initial increase of the fluorescence intensity is followed by a moderate photobleaching, in the other three animals the photobleaching takes place since the beginning of the treatment; (3) tumors in the leg of mice injected 12 h before the PDT—a marked photobleaching occurs in three mice, while in three mice an initial increase of the fluorescence intensity is followed by a moderate photobleaching; and (4) tumors in the leg of mice injected 3 h before the PDT—an enhancement of the fluorescence takes place in four mice, while in two a moderate bleaching occurs. For the cases (2)–(4), owing to the spread of behaviors, the intensity in the tumor at the end of the treatment does not differ from the initial value at $P < 0.05$. However, as a rule of thumb, we can say that the intensity enhancement predominates for the tumor in the leg, while the photobleaching prevails for the tumor on the back. During the observation time following the PDT (10 min) the fluorescence signal slightly increases in all mice.

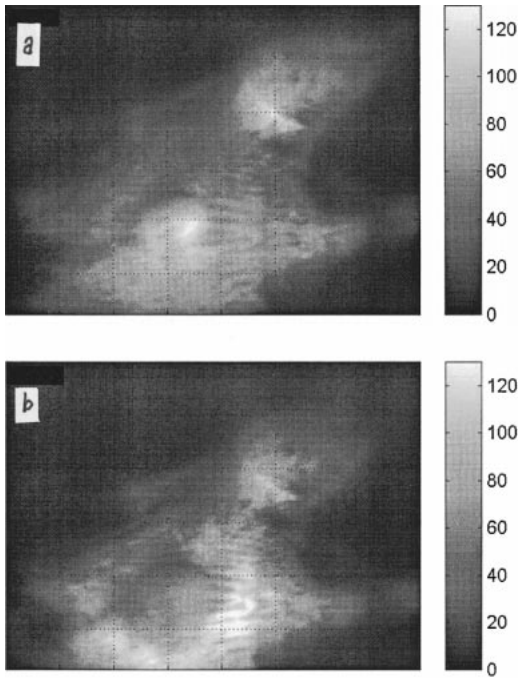

Figure 3. Fluorescence images of a tumor on the back: (a) before; and (b) after the irradiation with the PDT light. Drug dose: 5 mg/kg b.w., uptake time: 3 h, excitation light: 660 nm.

A comparable behavior was observed also in fluorescence images acquired from mice injected with 5 mg/kg b.w of AlS_2Pc. Data analysis basically gave the same results.

In all the conditions considered in this experiment (drug dose, uptake time, tumor compartment, *etc.*) an unexpected effect takes place at the tumor borders, independent of the behavior of the fluorescence emission in the tumor during the irradiation. The signal at the borders rises up to an intensity even double with respect to the initial value. As an example, Fig. 3a,b shows the fluorescence images of the tumor on the back of a mouse before and after the PDT, respectively. The uptake time was 3 h, the drug dose was 5 mg/kg b.w. and the images were taken with excitation at 660 nm. Photobleaching in the tumor and fluorescence enhancement at the borders can be clearly observed. Even though the described phenomena show up with both excitation wavelengths, differences can be appreciated between the measurements performed with 660 or 690 nm. To refer the fluorescence enhancement to the tumor behavior we calculated the ratio between the intensity at the tumor borders and

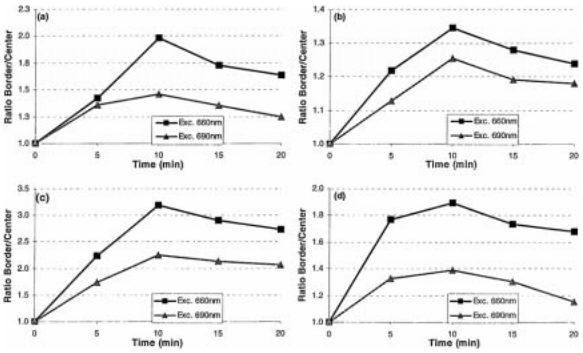

Figure 4. Average ratio of the fluorescence intensity in the tumor border and in the tumor center as a function of time during and after the irradiation of mice sensitized with 2.5 mg/kg b.w. of AlS_2Pc: (a) tumor on the back after 3 h; (b) tumor in the leg after 3 h; (c) tumor on the back after 12 h; (d) tumor in the leg after 12 h. Fluorescence intensities were measured from images.

in the tumor center for the two neoplasias and for the two excitation lights. Figure 4a–d represents these ratios averaged over six mice for the two uptake times. The drug dose was 2.5 mg/kg b.w. It turns out that for both tumors the ratio increases by a factor of 2–3 during the light exposure. Moreover, it increases more for the 660 nm excitation than for that at 690 nm ($P < 0.05$), suggesting a change in the absorption spectrum of the drug. A similar behavior was observed also with the drug dose of 5 mg/kg b.w.

Measurements performed every 2 min during the preliminary experiment basically gave the same results of measurements performed every 5 min during the final experiment on mice of the same group (5 mg/kg b.w.).

Mice not bearing any tumor showed only a mild enhancement of the fluorescence in the irradiated area, followed by photobleaching, while no effects were observed outside.

The PDT treatment with both AlS_2Pc drug doses was able to eradicate the tumors on the back, whereas it induced only a growth delay for the tumors in the leg, with respect to tumor-bearing mice that received neither the drug nor the light (control mice). The tumors on the back have been injected intradermally and remain confined within the skin while they grow. The tumors in the leg infiltrate the muscle and spread out from the original location. Moreover, there are differences in the vascularization of the two compartments. These features might account for the different response of the two tumors to PDT. The median survival time (MST) of mice of the various groups is shown in Table 1 along with the MST of control mice. For both the drug doses and the uptake times the difference in the survival time of treated mice with respect to control mice is significant at $P < 0.05$, according to the Mann–Whitney *U*-test (13). The differences in MST amongst the groups of treated mice are not significant.

For the three mice that showed a low drug uptake in the tumors, the PDT treatment was ineffective.

DISCUSSION

It has been observed *in vitro* that hydrophilic sensitizers like AlS_2Pc, accumulate in extranuclear granules like lysosomes and redistribute in the cytoplasm during irradiation (6,10). The increase in fluorescence intensity that takes place at the beginning of the activation has been ascribed to the release of the dye from the organelles, where it suffers from concentration quenching (8). Thereafter, the fluorescence decreases for a photobleaching effect. Fluorescence enhancement followed by photobleaching was observed also *in vivo* (11) on the skin of nude mice sensitized with low (1 mg/kg b.w.) or high (100 mg/kg b.w.) doses of AlS_2Pc.

Our experiment differs from the one described in Moan *et al.* (11) in two main respects. Irradiation was performed on tumor-bearing mice instead of healthy ones, and the fluorescence was measured using an imaging system, thus leading to a detailed evaluation of the effects that take place in a large area of the animals. From the results of a previous study performed *in vivo* on the absorption spectra of mice sensitized with the same drug (14) we can estimate that, using the excitation light at 660/690 nm, the fluorescence images collect the emission coming from tissue layers as deep as 3–5 mm. For the tumor on the leg this correspond to half the tumor diameter, while for the tumor on the back the emission comes from a region as deep as the tumor. The mouse skin is so thin that it presumably gives a minor contribution to the measurements.

Mice without tumor showed only a mild variation of the fluorescence intensity, in agreement with that observed by Moan *et al.*, at low AlS_2Pc dose (11). In tumor-bearing mice two main effects were observed, referring to the neoplasia and to the nearby area: a large spread of behaviors of the fluorescence intensity in the tumors during the PDT and a regular enhancement of the fluorescence signal outside the tumor. In some cases the latter effect was restricted to the

Table 1. MST and number of dead animals over total (D/T) for the four groups of mice considered in this experiment and for the control mice

Treated mice	MST	D/T	Control mice	MST	D/T
PDT (5 mg/kg b.w.; uptake time 3h)	82* (75–92)	6/6	No treatment	48 (39–60)	6/6
PDT (5 mg/kg b.w.; uptake time 12 h)	80* (78–93)	6/6	No treatment	48 (39–60)	6/6
PDT (2.5 mg/kg b.w.; uptake time 3 h)	78* (70–89)	6/6	No treatment	45 (39–54)	6/6
PDT (2.5 mg/kg b.w.; uptake time 12 h)	74* (69–83)	6/6	No treatment	45 (39–54)	6/6

*$P < 0.05$ by Mann–Whitney *U*-test *vs* control group.

peritumoral region, in other cases the fluorescence enhancement involved a large part of the animal body, looking like a systemic effect.

Before attempting to explain these findings it would be useful to recall the results of the cited experiments on the measurements of the absorption spectrum of the AlS_2Pc *in vivo* (14,15). It was observed that the absorption peak of the AlS_2Pc measured in mice deviates from the one in solution by 10–15 nm toward longer wavelengths due to the binding of the sensitizer to the biological substrate. Yet, at high concentration and after a long uptake time the peak of the absorption spectrum returns around 672 nm, in the position typical of water solution. This indicates the presence of a free form of phthalocyanine in those conditions. A redshift of the absorption peak of aluminum phthalocyanine has been detected *in vivo* also in different experimental situations (16,17).

The first effect observed in the present experiment, *i.e.* the large spread of behaviors of the fluorescence intensity in the tumors during the PDT, is very likely the result of two opposite phenomena. In some cases the signal increases, possibly due to the release of the AlS_2Pc from cellular organelles, in other cases the photochemical reactions that lead to drug degradation prevail. In few mice either the enhancement or the bleaching is very pronounced, while on average the net effect is moderate in all the experimental conditions, except for the case of tumors on the back of mice treated 12 h after the injection either of 2.5 or 5 mg/kg b.w. In this case, the fluorescence signal during the irradiation neatly decreases by a factor of 1.6, showing a similar behavior for all the mice. This result seems to confirm that after a longer uptake time the free form of AlS_2Pc predominates, thus less release of the drug can take place and only the photodegradation occurs. For what concerns the difference between the two tumors, one should take into account that the neoplasia on the back has been implanted intradermally, therefore it grows up in a capsule, well separated from the tissue underneath. Possibly, less blood flow and a lower exchange of metabolites takes place in this tumor than in the one implanted in the muscle of the leg, thus explaining a more pronounced photobleaching. In fact, less-efficient transport mechanisms can supply a lower amount of free AlS_2Pc to replace the one destroyed by the PDT light.

The second effect, *i.e.* the fluorescence enhancement at the border of the lesion, was really unexpected and deserves some remarks. This region did not receive direct light due to the protective screen, but it has been exposed, anyhow, to the diffused radiation. Since the fluence is lower than in the tumor center, one might suppose that only the AlS_2Pc redistribution takes place, while no photobleaching can occur. Nevertheless, this mechanism cannot completely account for such a strong fluorescence enhancement, otherwise a similar increase would also be regularly observed in the tumor at the beginning of the therapy, which is not the case, according to the results of the measurements made every 2 min. These findings are probably related to a macroscopic transport mechanism of the AlS_2Pc from the irradiated area to the surrounding region. The PDT in the tumor might alter the binding status of a significant fraction of AlS_2Pc molecules, which diffuse *via* systemic channels. It is likely that the PDT upsets the equilibrium of the free/bound phthalocyanine forms, leading to an increase in the free one, which might migrate from the irradiated area to the outer region *via* lymphatic vessels or microcirculation. In the tumor, the increase of the free form of AlS_2Pc might be counterbalanced by the photodegradation, which would prevent the fluorescence enhancement. This interpretation is confirmed by the change in absorption spectrum revealed by the higher increase in the fluorescence ratio border/tumor with the 660 nm excitation with respect to the 690 nm excitation. This means that the free phthalocyanine goes from the tumor to the border as a result of the PDT. In fact, the free form of AlS_2Pc is more effectively excited by the 660 nm radiation. It is worth noting that such a strong fluorescence enhancement seems to be related to the presence of a tumor, since it has not been observed in healthy mice.

As a conclusion of this work, we can say that fluorescence imaging proved effective to check the drug accumulation in the tumor before the PDT treatment and to evidence the complex mechanisms that take place during the light irradiation. Changes observed in fluorescence intensity and absorption spectrum of the AlS_2Pc before and after the PDT probably result from a different equilibrium of the free/bound forms of the sensitizer in the tumor and outside. These effects might be considered as real-time indices of therapeutic effectiveness, provided that they correlate with an increase in animal survival time. Further studies at lower doses of drug and light are required to verify this hypothesis.

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