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Uptake and phototoxic effects of aluminum-chlorophthalocyanine (AlSPc) in human bladder carcinoma cells

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Abstract In vitro experiments were performed on human bladder carcinoma cells to evaluate the uptake of aluminum-chlorophthalocyanine (AlSPc) and the subcellular target of phototoxicity. In order to quantify the correlation of intracellular uptake and incubation time and to identify the primary subcellular target of phototoxicity, fluorescence and absorption measurements have been carried out as well as electron microscopic studies. Absorption and fluorescence measurements showed the largest value after 24 h of incubation time. Fluorescence microscopic studies suggested the sensitizer to be located in a brighter patch within cytoplasm. Electron microscopic studies using DAB (3,3′ diaminobenzidine) staining showed that the mitochondria are the primary target of phototoxic activity of AlSPc and that the majority of vacuoles of treated cells were originally mitochondria.

Key words Phototoxicity · Aluminum-chlorophtalocyanine · Electron microscopy · DAB staining

Although photodynamic therapy (PDT) is already used in clinical phase III studies, its biological, photophysical and photochemical mechanisms are only partly understood. In a clinical setting, hematoporphyrine derivatives have been used exclusively so far. However, the properties of these substances with regard to chemical definition, tumor-selective uptake and phototoxicity are far from ideal. As a consequence, a number of alternative photosensitizers have been investigated in an experimental setting. One interesting group of substances are the phthalocyanines. Their basic photodynamic efficiency has been demonstrated in numerous experiments [1, 2, 3, 5, 7], however, it is still unclear where phthalocyanines are stored on a subcellular level and what the primary mechanism of photodynamic toxicity is. Aluminum-chlorophtalocyanine, a water soluble tri- and tetrasulfonated phthalocyanine, is an efficient photosensitizer in vitro and in vivo. The phthalocyanines as well as the hematoporphyrins tend to accumulate in membrane fractions. Functional and morphological changes in membranous organelles have been observed following photodynamic treatment [2]. The following experiments have been conducted in order to localize the sensitizer within bladder tumor cells, to quantify the correlation of intracellular uptake and incubation time, and to identify the subcellular components where the mechanism of phototoxicity takes place.

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Materials and methods

The bladder carcinoma cell line DAH (medium grade urothelial cancer from cystectomy specimen) was used. The cell line was kept for 2 years in the laboratory and was periodically examined with regard to a possible contamination with microorganisms. Cells were grown in monolayer cultures using Dulbecco's modified Eagle's medium (DMEM) + 15% fetal calf serum (FCS) + 2% glutamine + 1% penicillin streptomycine. The pH-value was set at 7.2 and cells were only used until the 6th passage. For transmission electron microscopy cells were grown on Thermanox tissue culture cover slips (NUNC, Naperville, USA). The Thermanox cover slips were placed in the bottom of the multiwells.

For experiments, cells were incubated with culture medium containing only 1% FCS plus photosensitizer in order to provide a high biological availability of the sensitizer. During incubation time cells were kept in the dark.

DAB-staining and electron microscopic investigations

In order to investigate whether the vacuoles in the cytoplasm of treated cells had primarily been mitochondria, we used a specific histochemical staining method for mitochondria: DAB-staining. DAB is a multicyclic aromate which is linked to the cytochrome-oxidase in a Redox-reaction. The mechanism of action of DAB within the cellular oxidation process is shown in Fig. 2. Following oxidation, DAB produces a non-soluble osmiophylic substance, yielding a marked contrast after fixation with osmium tetraoxide solution, as a specific marker for mitochondria (Fig. 1).

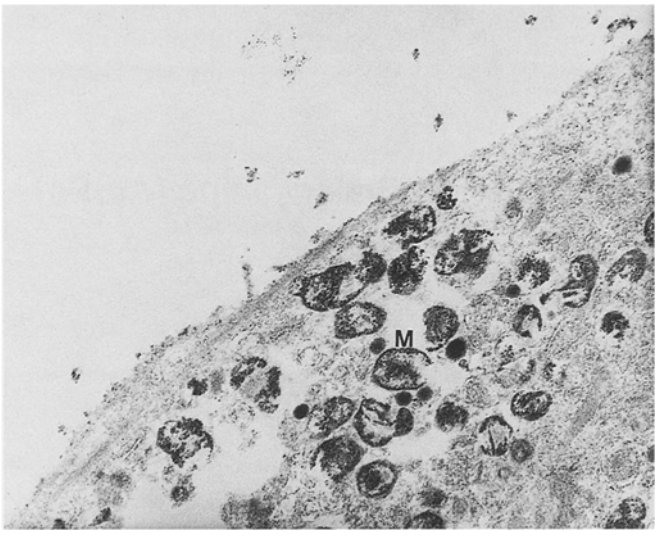

Fig. 1 DAB-staining produces marked contrast of the mitochondria (electron microscope, magnification 8000×). In the course of oxidation DAB produces a non-soluble osmiophylic substance yielding a marked contrast after fixation with osmium tetraoxide. *M*, mitochondria

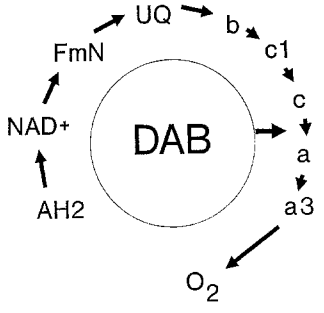

Fig. 2 Mechanism of action of DAB within cellular oxidation. AH_2 → representative substrates for dehydrogenase enzyme; NAD^+ → nicotinamide – adenine – dinucleotide – co-enzyme; Fm N → Flavine – mononucleotide co-enzyme; UQ → ubiquinone; $b/c_1/c/a/a_3$ → individual cytochroms involved in electron transfer; DAB → 3,3′ diaminobenzidine

After 4 h incubation with 40 µg/ml phthalocyanine at 37°C, cells were refed in PBS. In order to remove the rest of sensitizer, the cells were rinsed three times with PBS and irradiated. After irradiation the cells were washed again with PBS and then incubated with DAB solution for 2 h at 37°C (5 mg DAB in tris buffer + 1 ml catalase solution 20 µg/ml, 10 mg cytochrome c and 750 mg sucrose). Following this incubation time cells were rinsed using buffered sucrose (0,22 M sucrose + 0,05 M sodium phosphate) and fixed with 3,5% glutaraldehyde + 2% sacharose for better demonstration of ultrastructural details. After postfixation in 2% osmium tetraoxide for 1–2 h, the cells were dehydrated in a graded ethanol and propylen oxide and then embedded in Epon 812 resin. This procedure was followed by blockcontrasting with 2% uranyl acetat.

The electron microscopic investigations were carried out with a Zeiss EM10 microscope. Besides the irradiated cells, untreated cells and cells treated with light or the sensitizer only were used as controls.

Photosensitizer

A mixture of tri- and tetrasulfonated phthalocyanines (AlSPc) was used, kindly provided by BASF, Ludwigshafen. AlSPc was dissolved in PBS before application, but no further chemical manipulations were done. The cells were incubated with 40 µg/ml phthalocyanine. The absorption spectrum of AlSPc shows that the main absorption is in the UV and red light regions with a peak at 675 nm in Dulbecco's buffer.

Irradiation

Irradiation was carried out using red light of 675 nm. A Krypton-pumped dye-laser was used for that purpose; the laser light was coupled via a quartz fiber into a special microscopic irradiation device. Fluence used was 2 J/cm^2.

Fluorescence and absorption measurements

The ability of tumor cells to accumulate AlSPc was determined in two different ways:

1. Detection of fluorescence after 4 h of incubation with AlSPc 40 µg/ml without irradiation (qualitative proof).
2. Extraction of accumulated photosensitizer from treated cells (method of Ben-Hur and J. Rosenthal) [1] and measurements by spectrophotometry.

For the fluorescence detection the cells were cultured on slides in Quadriperm plates (Heraeus, Hanau Germany). There were 100000 cells per slide in 5 ml medium. For intracellular evidence of fluorescence we used a combination of fluorescence microscopy, video intensified detection and image processing [15]. Fluorescence of the dye in the cells was made visible by excitation with a Hg-lamp at 546 nm. Irradiance was kept below 100 mW/cm^2 in order to prevent photobleaching. The fluorescence before irradiation and its intracellular distribution were presented as proof of intracellular accumulation of dye.

For the extraction, cells were inoculated in culture flasks at 10^5 cells and allowed to grow for 72 h. The cells were incubated with 40 µg AlSPc/ml and kept in the dark. After 2, 4, 6 and 24 h, respectively, the medium was removed and the cells were washed three times with PBS. The accumulated cellular amount of photosensitizer was extracted by incubating the cells with 3 ml 0,1 M NaOH for 1 h. The absorbance of the extract at 675 nm was determined with a spectrophotometer (in accordance with the absorption spectrum of this photosensitizer). The accumulated amount of photosensitizer is expressed in µg per total number of cells (2.5×10^6).

Reference samples contained extraction solution from an equal cell concentration which had not been incubated with AlSPc. A standard curve was obtained by adding known amounts of AlSPc. Simultaneously to the absorption measurements, fluorescence of extract was measured at 675 nm, with an excitation wavelength of 349 nm.

Results

Using a fluorescence microscope uptake of AlSPc could be demonstrated. AlSPc fluorescence after 4 h of incubation was found as a brighter patch within the cytoplasm (Fig. 3). Diffuse fluorescence was evident outside this area, but was much less intense. The nuclear region did not label.

Quantitative levels of AlSPc in bladder tumor cells are shown in Fig. 4 and 5. Phthalocyanine concentration has been expressed in µg per total number of cells (2.5×10^6). Using absorption measurements the uptake of AlSPc shows a proportional correlation with the incubation time, reaching its largest value after 24 h of incubation time. Two hours after incubation the value of uptake of AlSPc

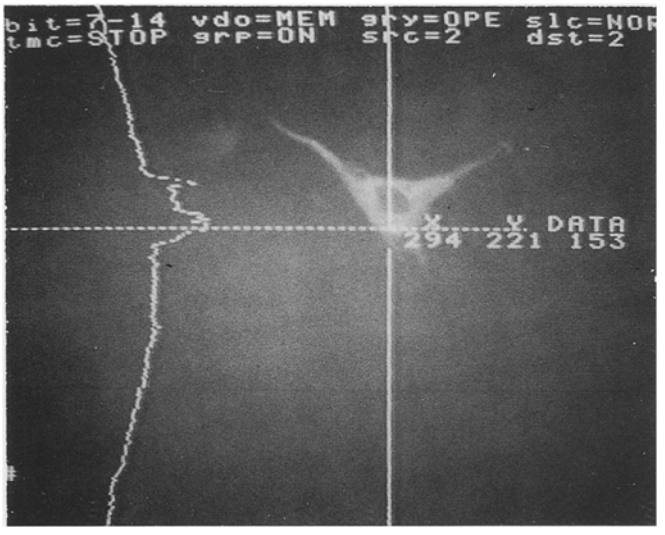

Fig. 3 Intracellular distribution of fluorescence prior to PDT. After 4 h of incubation with 40 µg AlSPc/ml medium, distribution of fluorescence is relatively homogenous in the cytoplasm. The intensity profile curves along the full lines are displayed on the left. Only weak fluorescence was found in the nucleus

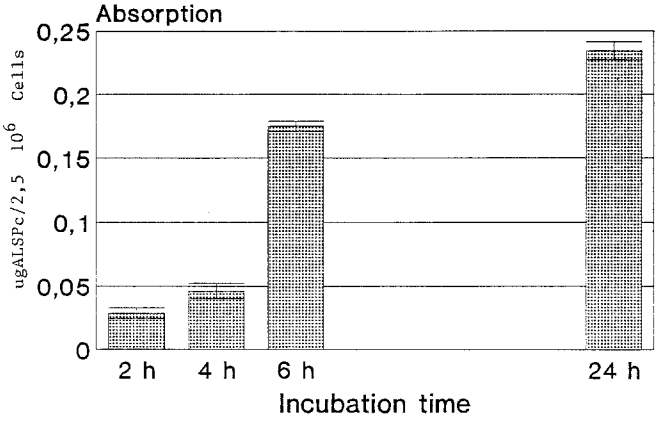

Fig. 4 Cellular uptake of AlSPc (40 µg/ml) in DAH cells (bladder carcinoma cell line) after different incubation times (2, 4, 6, 24 h). Quantitative levels of AlSPc have been expressed in µg per 2.5×10^6 cells

was 0,024 µg per 2.5×10^6 cells. After 4, 6 and 24 h, the concentration of photosensitizer was 0,048 µg, 0,176 µg and 0,242 µg per 2.5×10^6 cells respectively. These results showed a direct correlation between the values of concentration of photosensitizer in the cells and the incubation time. AlSPc was taken up linearly for 24 h of incubation time. Fluorescence measurements showed a slightly different pattern, with similar values for 2, 4 and 6 h of incubation time. There is no correlation between the fluorescence values and incubation time. The relative fluorescence of AlSPc reached its highest level after an incubation time of 24 h (Fig. 5). The fluorescence detection method for the uptake of AlSPc has a limited sensitivity.

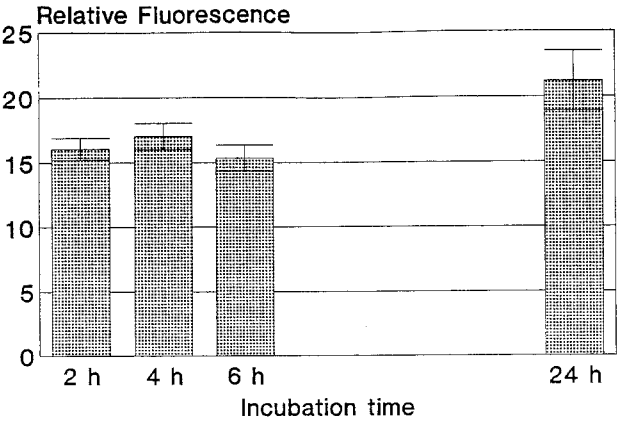

Fig. 5 Relative fluorescence of extract (DAH cells – 2.5×10^6) in correlation to incubation time (2, 4, 6, 24 h)

For transmission electron microscopy untreated cells were compared with treated cells (40 µg AlSPc/ml, 4 h incubation and irradiation with 2 J/cm^2). The tumor cells that received either sensitizer alone or light alone did not show any significant change when compared to control cells without any treatment (Fig. 6). The nucleus with an aggregation of heterochromatine and various organelles (lysosomes, endoplasmic reticulum, mitochondria, ribosomes and lipid bodies) could be identified in the cytoplasm (Fig. 6). The mitochondria appear oval shaped or lengthy (depending on the particular cross section) and show regular cristae with a right angled direction to the longitudinal axes. There is an amorphous matrix between the cristae (Fig. 7).

Treated cells show marked changes of the mitochondria. The size is increased and the structure of the cristae is destroyed with the dissolution beginning in the central part of the mitochondria (Fig. 8). Later a complete dissolution of the inner matrix of the mitochondria, leading to vacuolization, can be seen (Fig. 9). Using this specific staining method the vacuoles appear with a dark contrast indicating that these vacuoles are damaged mitochondria. Without this staining method the vacuoles could not be identified. Through use of this method we can affirm that the numerous vacuoles in the treated tumor cells have primarily been mitochondria. The number of normal mitochondria in treated cells is significantly lower than in the control cells. Moreover, a higher number of secondary lysosomes in the treated cells suggest autophagocytoses of the damaged mitochondria.

Discussion

As we could expect from other experiments with different cell lines [1, 2, 5] bladder tumor cells show a significant uptake of AlSPc into the cytoplasm. With regard to the incubation time, the largest uptake was observed following 24 h of incubation. Our results are consistent with data reported by Chan [5] and Ben-Hur [2]. Their experiments

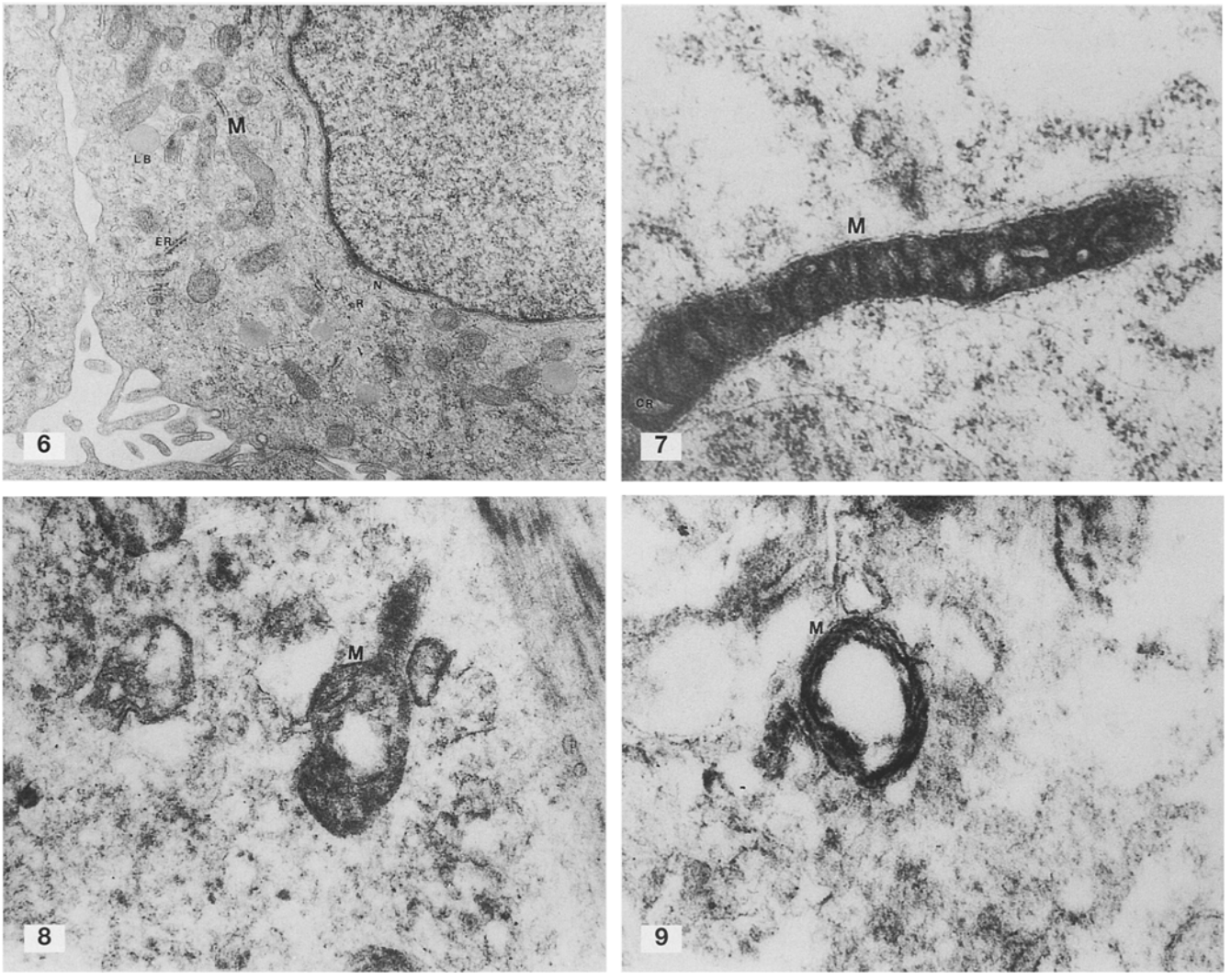

Fig. 6 Untreated control cells with normal structure (EM magnification = 5000×). *N,* nucleus; *M,* mitochondria; *ER,* endoplasmic reticulum; *LB,* lipid body; *R,* ribosomes. **Fig. 7** Mitochondria in an untreated cell (DAB-staining). *M,* mitochondria; *CR,* cristae. **Fig. 8** Central dissolution of mitochondrial structure in a treated cell. **Fig. 9** Complete vacuolization of a destroyed mitochondrion

have shown a similar value of absorption after 24 h of incubation.

We compared the level of concentration of AlSPc at the different time intervals after incubation with the intensity of the relative fluorescence at $\lambda = 675$ nm. Our results did not show a proportional correlation. We believe that due to self-quenching, fluorescence is not proportional to the dye concentration.

Bénoit Paquette [11] has studied water-soluble aluminum phthalocyanines sulfonated to different degrees for cellular distribution and fluorescence. His results revealed that the dye was uniformly distributed in the cytoplasm but the fluorescence intensity is not proportional to the dye concentration. This phenomenon could be correlated with the aggregation state of the dye molecules. It is known that hematoporphyrin fluorescence and corresponding triplet yields are lowered in the presence of the dye aggregation [12]. The formation of aggregates of sulfonated metallophthalocyanine is an unfortunate problem. Aggregation of the dye in water is usually observed spectrophotometrically and disappears upon addition of monomerizing agents [16].

The target of phototoxic action of phthalocyanine in the cell is still a subject of controversy. The plasmatic membrane [2], the microsomes [9] and the mitochondria [11] have been suggested as the main target of the uptake of photosensitizer and phototoxic effect of phthalocyanines. Using HPD-derivatives as photosensitizers, other authors have reported [6, 8, 10, 13, 14] that the mitochondria are the main target of phototoxic action. Using the staining technique with DAB and TEM, our ultrastructural studies on the effect of AlSPc and light on bladder carcinoma cells in vitro demonstrate that mitochondria are the organells being primarily damaged by photodynamic action.

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