Liposome-bound Zn(II)-phthalocyanine. Mechanisms for cellular uptake and photosensitization

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Abstract

In the present study, cellular uptake of a liposomal formulation of ZnPc(CGP 55847) has been studied in human cervix carcinoma cells of the line NHIK 3025. The cellular uptake of ZnPc is found to be completed after 4-8 h of incubation. The maximum level of ZnPc in the cells after incubation with 1 μg/ml ZnPc in E2a medium containing 3% serum is 60 ng/mg protein. The cellular uptake is attenuated by the presence of serum and at low temperature of the incubation medium, but the activation energy (30 kJ/mol) and fluorescence microscopic analysis of cells incubated with ZnPc at 0°C indicate that ZnPc is taken up into cells by a diffusion-mediated pathway. Measurements of subcellular marker enzymes have been performed immediately after light exposure of ZnPc-treated cells. The mitochondrial marker enzyme (cytochrome c oxidase) and the marker enzyme for the Golgi apparatus (UDP galactosyl transferase), but not those for lysosomes (β-N-acetyl-d-glucosaminidase) and endoplasmic reticulum (NADPH cytochrome c reductase), are inactivated upon photodynamic treatment. These results indicate that ZnPc is mainly located in the Golgi apparatus and the mitochondria of NHIK 3025 cells. In contrast, photoactivated Photofrin is found to reduce the activity of UDP galactosyl transferase, but not that of NADPH cytochrome c reductase. The tetraphenylporphine TPPS₄ and light reduce the activity of NADPH cytochrome c reductase, without influencing the activity of UDP galactosyl transferase. TPPS₄ and light do not attenuate the activities of UDP galactosyl transferase and NADPH cytochrome c reductase. © 1998 Elsevier Science S.A. All rights reserved.

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1. Introduction

Photodynamic therapy (PDT) of cancer is promising as a palliative and even a curative treatment for some types of solid tumours [1-3]. The therapy is based on selective retention of a sensitizer in the tumour tissue and its photocytotoxic effect. The most widely used sensitizers for this purpose are haematoporphyrin derivative (HpD) and a somewhat purified version named Photofrin. However, HpD and Photofrin are far from ideal. The ratio of the concentration of photosensitizer in the tumour and the normal surrounding tissue is not significantly larger than one in some cases and the absorption of light is weak in the red area of the spectrum where light transmission through tissue is optimal [4]. Phthalocyanines (Pcs) have absorption peaks above 600 nm with relatively high extinction coefficients [5,6], have been reported to localize selectively in tumour tissue [7] and have been proposed as second-generation sensitizers.

The efficiency of a photosensitizer in sensitizing cells to photoinactivation is dependent upon its ability to be taken up by the cells as well as its photochemical properties. The rate of cellular uptake of a drug is highly dependent upon its lipophilic properties, although other factors also influence the rate of uptake, e.g., the uptake of Pcs is larger than that of porphyrins with similar lipophilicity [8]. This may be related to differences in the intracellular localization of the dyes and the local surroundings of photosensitizers influence their quantum yields of photoinactivation. This topic has not been thoroughly studied so far, although it seems that some photosensitizers are less photocytotoxic when located in the plasma membrane than in other cellular compartments [9]. There is also some evidence for a lower quantum yield of cell inactivation for lysosomally located dyes than that for the non-lysosomally located ones [10]; and mitochondrialy locating dyes have been found to be highly efficient dyes in sensitizing cells to photoinactivation [11]. In order to develop structure-activity-relationship models for photosensitizer targeting of specific subcellular organelles, detailed knowledge of uptake mechanisms and intracellular localiza-
tion of a large variety of photosensitizers is needed. Thus, in
the present study the mechanisms for cellular uptake and the
intracellular targets of a liposome-bound Zn(II)-phthalocya-
nine (ZnPc) have been studied in NHIK 3025 cells.

2. Materials and methods

2.1. Chemicals

A liposome formulation of Zn(II)-phthalocyanine (ZnPc)
(CPG 55847) was kindly provided by Ciba-Geigy (Basel,
Switzerland). Photofrin was supplied by Lederle Parenterals
(Carolina, Puerto Rico), and the tetraphenylporphines
TPPS$_a$, TPPS$_{2a}$, and TPPS$_{1}$, were provided by Porphyrin
Products (Logan, UT). Nocodazol, cytochrome c, β-nicotinamide
adenine dinucleotide phosphate, reduced form (β-NADPH),
and p-nitrophenyl-N-acetyl-β-D-glucosaminide were pur-
chased from Sigma (St. Louis, MO). Uridine diphospho-D-
[6-3H] galactose was purchased from Amersham Life
Science. All chemicals were of the highest purity available.

2.2. Cell culture

The established cell line NHIK 3025, derived from a car-
cinoma in situ of the cervix [12], was used. The cells were
subcultured twice a week (split ratio 1:100) in E2a medium
The cells displayed a doubling time of 18 h.

2.3. Labelling with photosensitizer and irradiation

The cells were inoculated in 10 cm$^2$ dishes (Falcon, UK)
or in 25 cm$^2$ flasks (Nunc, Denmark) containing E2a medium
with 11.5% serum (6% human serum and 5.5% horse serum)
and left at 37°C for 4–5 h for proper attachment to the
substratum. Subsequently, the cells were washed three times
with E2a medium with 3% serum and then exposed to the photo-
sensitizer in E2a medium with 3% serum (2% human serum
and 1% horse serum) or as otherwise described. The follow-
ing concentrations of sensitizers were used: ZnPc (CPG
55847), 1 or 5 µg/ml; Photofrin, 6 µg/ml; TPPS$_a$, 75 µg/
ml; TPPS$_{2a}$, 3.2 µg/ml; TPPS$_{1}$, 3.1 µg/ml.

If not otherwise described, the cells were incubated with
photosensitizer for 18 h, washed three times in E2a medium
containing 11.5% serum and incubated in the same medium
for 1 h before light exposure. The cells were subsequently
exposed to red light (Phillips TL 20 W/09) filtered through a
Cinemoid 35 filter (Fig. 1). In some experiments the cells
were exposed to 1 µg/ml nocodazole for 1 h before light
exposure. Cells incubated with Photofrin, TPPS$_a$, TPPS$_{2a}$ or
TPPS$_{1}$, were exposed to blue light (Appl. Photophysics,
model 3026, London). The fluorescence reaching the cells
were 1.35 and 1.5 mW/cm$^2$ from the red and the blue lamps,
respectively. The temperature of the medium during irradia-

2.4. Cytotoxicity assay

$2 \times 10^3$ cells were seeded out in 25 cm$^2$ flasks (Nunc,
Denmark) and treated as described above. Cytotoxicity was
determined by measuring the colony-forming ability of the
cells as previously described [14]. After the light exposure
the cells were incubated for 7–8 days at 37°C. The colonies
were fixed with ethanol (96%), stained with methylene blue
and scored manually.

2.5. Fluorescence and absorption measurements of
cell-bound ZnPc

$2 \times 10^6$ cells were seeded in 20 cm$^2$ dishes (Falcon, UK)
and treated with 5 µg/ml ZnPc for 18 h as described above.
After incubation the cells were washed three times with ice
cold Dulbecco’s phosphate-buffered saline (PBS) and
scraped off the dishes in 2 ml of PBS. The cell suspensions
were centrifuged and resuspended in 2 ml PBS before ZnPc
was measured spectrofluorometrically (Perkin-Elmer LS-5).
The fluorescence was measured by exciting the samples at
344 nm and by detection of the emission at 672 nm. Cut-off
filters were used both on the excitation (345 nm) and on the
emission (545 nm) sides. Subsequently, absorption was
measured in a Perkin-Elmer Lambda 15 spectrophotometer
equipped with an integrating sphere. The fluorescence exci-
tation spectra were corrected for the low excitation coefficient
of the quantum counter rhodamine above 600 nm. This was
performed by multiplying the spectra with the ratio between
the absorption and the fluorescence excitation spectra of
monomeric AlPcS$_4$ in the wavelength range 580–700 nm.
2.6. Quantitative measurements of cell-bound ZnPc

5 × 10^5 cells were seeded out in 10 cm^2 dishes and labelled with ZnPc. At the end of the incubation time the cells were washed three times with ice-cold PBS. The cells were scraped off the substratum in 1 ml PBS and 20 μl 5 M KOH was added and mixed into the solution. ZnPc was measured spectrofluorometrically (Perkin-Elmer LS-5) using a 1 ml cuvette. The fluorescence was measured by exciting the samples at 344 nm and by detecting the emission at 672 nm. Cut-off filters were used both on the excitation (345 nm) and on the emission (545 nm) sides. For quantification, a standard of known concentration of ZnPc (in PBS) was added to the samples to increase the fluorescence by 50–100%. Dishes without cells but otherwise treated as described above always showed fluorescence intensities less than 4% of those of the corresponding dishes with cells. For quantification of cellular uptake of ZnPc this background was subtracted. The cell layer was approximately 10–20% from being confluent when scraped off the substratum. When the cells were incubated outside an incubator containing CO₂, 25 mM Hepes was added to the E2a medium, or the E2a medium was replaced by 5 mM glucose in PBS. Protein was measured by the Bio-Rad assay according to Bradford [15].

2.7. Fluorescence microscopy

1 × 10^4 cells incubated in 10 cm^2 dishes were treated with ZnPc as described above and then washed once in PBS. A cover glass was gently put on the top of the PBS layer and surplus PBS sucked off. The cells were subsequently studied by a Zeiss Axioplan microscope equipped with epifluorescence. A HBO/100 W mercury lamp was used for excitation. The microscope was equipped with a cooled charge-coupled device (CCD) camera (RE2, Astromed, Cambridge). A computer controlled the camera operation and was used for digital image processing and storage. The microscope was equipped with a 365 nm bandpass excitation filter, a 395 nm emission (545 nm) side. For quantification, a standard of known concentration of ZnPc (in PBS) was added to the samples to increase the fluorescence by 50–100%. Dishes without cells but otherwise treated as described above always showed fluorescence intensities less than 4% of those of the corresponding dishes with cells. For quantification of cellular uptake of ZnPc this background was subtracted. The cell layer was approximately 10–20% from being confluent when scraped off the substratum. When the cells were incubated outside an incubator containing CO₂, 25 mM Hepes was added to the E2a medium, or the E2a medium was replaced by 5 mM glucose in PBS. Protein was measured by the Bio-Rad assay according to Bradford [15].

2.8. Enzyme analysis

2.8.1. β-N-acetyl-D-glucosaminidase (β-AGA)

A confluent 75 cm^2 culture flask (Nunc) was treated with ZnPc as described above. At the end of the incubation period, the cells were trypsinized, pelleted and seeded out on 10 cm^2 dishes (Falcon 1008, UK) with E2a medium containing 11.5% serum. The cells were trypsinized, pelleted and seeded out in 10 cm^3 dishes (Falcon 1008, UK) with E2a medium containing 11.5% serum. The cells were irradiated and pelleted immediately after irradiation. The cells were incubated for 3 h in a solution containing 0.1% Brij 35, 100 mM acetate buffer (pH 4.6), 0.1% Triton X-100 and 2 mM p-nitrophenyl-N-acetyl-β-glucosamine according to Beaufay et al. [16]. The reaction was stopped by adding ice-cold 0.5 M Na₂CO₃. The method is based on the formation of p-nitrophenol (from the substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide) which can be registered spectrophotometrically at 410 nm. The amounts of photosensitizers in the cells did not influence the measurements.

2.8.2. NADPH cytochrome c reductase

50 × 10^5 cells were seeded out in 10 cm^2 dishes and treated with sensitizer as described above. The NADPH cytochrome c reductase activity was measured as described by Beaufay et al. [16]. The method is based on the formation of the reduced form of cytochrome c, cytochrome c<sub>red</sub>, induced by NADPH, which can be recorded spectrophotometrically at 550 nm.

2.8.3. UDP galactosyl transferase

50 × 10^5 cells were seeded out in 10 cm^2 dishes and treated with sensitizer as described above. The UDP galactosyl transferase activity was measured as described by Brandli et al. [17]. The method is based on binding of radioactive-labelled uridine diphospho-D-[6-³H] galactose (Amersham) to ovalbumin, which can be detected with an MR 300 automatic liquid scintillation system.

2.8.4. Cytochrome c oxidase

75 cm² flasks, confluent with cells, were treated with ZnPc as described above. At the end of the incubation period, the cells were trypsinized, pelleted and seeded out on 10 cm² dishes (Falcon 1008) (1 × 10^6 cells in MEM containing 10% serum). Immediately after the irradiation, the mitochondria were isolated from the cells by electropermeabilization. The permeabilized cells were ruptured by repeated pumping through a needle followed by differential centrifugation. The probes were first centrifuged at 2500 rpm for 7 min in a Hettich microtitre centrifuge. Then the supernatant was centrifuged at 1500 rpm for 20 min in the same centrifuge. The cytochrome c oxidase activity was then measured as described by Gibson and Hilf [18]. The method is based on spectrophotometric recording of the disappearance of cytochrome c<sub>red</sub> (reduced form of the substrate cytochrome c (Sigma)) at 550 nm.

3. Results

3.1. Cellular uptake and efflux of ZnPc in NHIK 3025 cells

The cellular uptake of 1 μg/ml ZnPc in NHIK 3025 cells at 37°C was measured for up to 24 h (Fig. 2(a)). The uptake was approximately linearly increasing with time for the first 2–4 h of incubation. A plateau of about 60 ng/mg protein
Fig. 2. Time course of cellular uptake (a) and efflux (b) of ZnPc in NHIK 3025 cells. (a) The cells were incubated with 1 µg/ml ZnPc in E2a medium in the presence of 3% serum and cell-bound ZnPc analysed as described in Section 2.6. (b) NHIK 3025 cells were incubated with 1 µg/ml ZnPc for 18 h. The medium was then replaced by ZnPc-free medium and cell-bound ZnPc analysed as described in Section 2.6. Bars, SD from three dishes.

ZnPc was reached within 8 h. Longer incubation times had no further effect on the cellular level of ZnPc. The absorption and the corrected fluorescence excitation spectra for ZnPc bound to NHIK 3025 cells were almost similar, and showed a main peak at about 672 nm (Fig. 1).

The intracellular concentration of ZnPc could be estimated from the mean diameter of NHIK 3025 cells (15 µm), the number of cells per mg protein (= 1.7 × 10⁶ cells/mg protein) and the data in Fig. 2(a). At a maximum level of 60 ng/mg protein ZnPc reached after 4–8 h of incubation with 1 µg/ml ZnPc, the intracellular concentration of ZnPc could thus be estimated to 150 µg/ml, i.e. about 150 times larger than the concentration in the incubation medium.

After 18 h of incubation with 1 µg/ml ZnPc, the medium was replaced with fresh medium without ZnPc to evaluate the kinetics of ZnPc efflux (Fig. 2(b)). The efflux was clearly biphasic with a small fraction (25%, upon extrapolation of the regression line to the ordinate) lost within 1 h of incubation in sensitizer-free medium. The efflux of the remaining ZnPc seems to follow first-order kinetics, with half the cell-bound ZnPc released in 5 h. 24 h after removing ZnPc from the medium, less than 3% ZnPc remained cell-bound. No changes in the intracellular localization of ZnPc during the efflux could be observed by fluorescence microscopy (data not shown).

3.2. Cell inactivation after photochemical treatment

The clonogenicity of the NHIK 3025 cells was not influenced by treatment with 25 µg/ml ZnPc for 18 h in the presence or absence of 3% serum (data not shown). However, cells exposed to 1 µg/ml ZnPc for 18 h followed by 1 h in sensitizer-free medium were highly sensitive to light exposure (Fig. 3). 90% of the cells were inhibited from forming colonies after 90 s of light exposure, corresponding to 0.16 J/cm².

It has previously been shown that the temperature of the medium surrounding the cells during light exposure can be of great importance for the cytotoxic effect of photodynamic therapy. NHIK 3025 cells treated with haematoporphyrin were about twice as sensitive to PDT when exposed to light at 0°C than at 37°C [19]. Cells were treated overnight with 1 µg/ml ZnPc followed by 1 h in sensitizer-free medium prior to light exposure on ice or at 34–35°C (Fig. 3(a)). In contrast to the results with haematoporphyrin and light, the temperature during light exposure had only a slight and insignificant influence on the photocytotoxic effect of ZnPc.

The temperature of the medium was monitored during light exposure and was found to increase during light exposure in the medium of cells exposed to light on ice (Fig. 3(a), inset).
This might have influenced the outcome of the experiments, although reduction of the temperature of the medium from 37 to 20°C during light exposure induced a clear reduction in cell survival in the case of haematoporphyrin [20].

The unpolymerized form of tubulin has previously been shown to be targeted by several photosensitizers in combination with light [21-25]. The rapid and highly reversible inhibition of tubulin polymerization by nocodazole [26] makes this drug suitable for studying photochemical effects on the same target. Treatment of ZnPc-loaded cells with nocodazole shortly before light exposure increased the photosensitivity of the cells slightly, but not significantly (Fig. 3(b)). Nocodazole given alone before light exposure was non-toxic to the cells (Fig. 3(b)).

3.3. Serum dependency on cellular uptake of ZnPc

It has previously been shown that ZnPc incorporated into small unilamellar liposomes interacts with the lipoproteins in serum and is almost exclusively bound to the three major lipoprotein components VLDL, HDL and LDL [27]. The rate of transfer of ZnPc from liposomes to LDL is highly dependent on the type of liposome, ranging from a few minutes to several hours for completion [28]. The uptake of ZnPc in the NHK 3025 cells was measured after incubation with ZnPc in the absence and presence of serum (Fig. 4). In the absence of serum, the rate of ZnPc uptake was increased more than two-fold. The rate of cellular uptake of ZnPc incubated in 3% serum was independent of a 4 h pre-incubation period of ZnPc in the incubation medium before exposure to cells (Fig. 4).

The photoinactivation efficiency appears to be related to the intracellular ZnPc concentration: for a 2 h incubation in the absence of serum, the dose of light that killed 90% of the cells \( D_{10} \) was 1.6-2.1-fold higher than in the presence of serum (data not shown). In accordance with the uptake studies, cell survival was independent of a 4 h pre-incubation period of ZnPc in the medium before exposure to cells.

3.4. Temperature dependency of cellular uptake of ZnPc

The temperature dependency of the cellular uptake of ZnPc was determined by comparing the rates of uptake at 37 and 0°C. The rate of ZnPc uptake was substantially reduced by lowering the incubation temperature to 0°C (Fig. 5). Linear regression analysis indicates an initial rate of ZnPc uptake of 17 ng/mg protein × h at 37°C (based on a linear uptake for only the first 2 h, \( r^2 = 0.998 \)) and 3.5 ng/mg protein × h at 0°C (\( r^2 = 0.71 \)). The activation energy can be estimated from these results to be about 30 kJ/mol assuming a linear Arrhenius plot within this temperature range.

The temperature dependency of the cellular uptake of ZnPc was also studied by fluorescence microscopy (Fig. 6). In cells exposed to a 1 h pulse at 0°C with the sensitizer the fluorescence could be seen as a rim around the cells, implicating primarily plasma membrane localization of the sensitizer. When this treatment was extended to 4 h, the cells fluoresced more intensely and the fluorescence clearly originated from intracellular areas. A similar pattern was observed in cells treated with ZnPc at 18°C, although the fluorescence intensity was higher than when the cells were incubated at 0°C. In cells treated at 37°C the fluorescence from ZnPc was clearly increasing with time, but the fluorescence pattern seemed to depend less on the time of incubation. The intracellular localization of ZnPc was mainly extranuclear, inhomogeneous and covering most of the cytoplasm. However, after 18 h of incubation the fluorescence pattern was more granular than after 1-4 h of incubation.
3.5. Inactivation of intracellular enzymes

Photosensitized damage to enzymes known as markers for subcellular organelles gives information about the intracellular localization of the sensitizer [29]. The enzymatic activity of UDP galactosyl transferase associated with the Golgi apparatus was reduced by approximately 60% after the treatment with ZnPc and 3 min of light, while the activity of the mitochondrial cytochrome c oxidase was reduced by approximately 40% after 2 min of light (Fig. 7). Lysosomal β-AGA and NADPH cytochrome c reductase located in endoplasmic reticulum showed no significant loss in enzymatic activities after treatment with ZnPc and light (Fig. 7).

For comparison, the inactivation of subcellular marker enzymes as performed with ZnPc and light was also carried out using other photosensitizers with different intracellular localizations, i.e., Photofrin, TPPS4 and TPPS2a (Fig. 8). Neither UDP galactosyl transferase nor NADPH cytochrome c reductase was affected by TPPS4 and light, while photoactivated TPPS2a was able to inactivate NADPH cytochrome c reductase, but not UDP galactosyl transferase (Fig. 8). Light exposure in the presence of TPPS2a has previously been shown to relocate the dye from lysosomes to membranous extralysosomal areas after small light doses [14]. However, UDP galactosyl transferase was not inactivated even when cells treated with TPPS2a were first exposed to a small light dose sufficient to release TPPS2a from lysosomes and then to a second larger dose of light (data not shown). UDP galactosyl transferase was not inactivated by TPPS4 and light (data not shown). In the case of Photofrin and light, NADPH cytochrome c reductase was not significantly affected, in contrast to UDP galactosyl transferase activity which was reduced by 40% (Fig. 8).

4. Discussion

Photosensitizers may enter cells either by penetrating the plasma membrane via passive diffusion [30,31] or by endo-
cytosis [29]. Plasma membrane transporters have not been shown to be involved in the cellular uptake of photosensitizers, although the prodrug 5-aminolaevulinic acid seems to be taken up through β-amino acid and γ-aminobutyric acid transporters in colon adenocarcinomas (Rud and Berg, unpublished observations). The present results indicate that cellular uptake of ZnPc is due to a diffusion-controlled process. The uptake of ZnPc was temperature dependent, but the activation energy for the process was only about 30 kJ/mol, which is within the range of diffusion-controlled uptake mechanisms (Fig. 5, [32]). In contrast, active transport mechanisms are more temperature dependent with activation energies of about 100 kJ/mol [33]. Furthermore, active transport is completely inhibited at 0°C, while ZnPc clearly penetrated the plasma membrane at 0°C (Fig. 6).

Intracellular transport from endosomes to lysosomes and the Golgi apparatus is inhibited at 18°C, while transport from the plasma membrane to endosomes is still active [34]. Photosensitizers that are located in intracellular membranes, like in the endoplasmic reticulum and in the Golgi apparatus (see below), may have been endocytosed and further transported retrogradely to these organelles. ZnPc given at 18°C should therefore accumulate in endosomes if this photosensitizer is transported retrogradely through endosomes to the Golgi apparatus. However, the intracellular localization of ZnPc was not influenced by reducing the incubation temperature from 37 to 18°C (Fig. 6), indicating that endocytosis is not involved in the cellular uptake of ZnPc.

It has previously been shown that ZnPc almost exclusively binds to the three major lipid components, VLDL, HDL and LDL, of serum [27] and that the transfer of ZnPc from liposomes to serum lipoprotein is highly dependent on the constitution of the liposomes [28]. The presence of serum reduced the rate of ZnPc uptake into NHK 3025 cells (Fig. 4) as well as their sensitivity to photoinactivation. These results and the lack of effect of pre-incubating ZnPc in the serum-containing medium strongly indicate that ZnPc is rapidly transferred from the liposomes to the lipoproteins and adsorbed to the cells as a complex with the lipoproteins. It has previously been suggested that several photosensitizers, including ZnPc [35], are taken up both in vitro and in vivo through the LDL pathway. However, although ZnPc may be adsorbed to the plasma membrane complexed with LDL, it does not seem to follow the endocytosis of LDL, but instead diffuses across the plasma membrane (Fig. 6). The most likely explanation is that the LDL–ZnPc complex binds to the LDL receptor where ZnPc leaves LDL and penetrates the plasma membrane [36] (Fig. 9). The preferential uptake of LDL-bound photosensitizers into tumour tissues has been suggested by many authors to be due to uptake via the LDL receptor. However, in contrast many of these photosensitizers are not localized in endosomes or lysosomes [35,37]. Thus, the alternative pathway as indicated in Fig. 9 may link the apparently contradictory results together.

The efflux of ZnPc from the NHK 3025 cells followed apparently first-order kinetics from 1 to 24 h of incubation in sensitizer-free medium (Fig. 2(b)). About 25% of cell-bound ZnPc was lost in the first hour of incubation in sensitizer-free medium. This fraction was lost more rapidly than the remaining cell-bound ZnPc, and is most likely due to ZnPc associated with the plasma membrane. This is in accordance with the previous observation that ZnPc binds to the plasma membrane, as revealed by measurements of plasma membrane function after PDT [38].

In the case of Pcs and porphyrins it is well known that aggregates have a low fluorescence quantum yield compared to monomers [39,40]. Aggregated Pcs have absorption spectra that are different from those of monomeric dyes [5,41]. In the present study the absorption and the fluorescence excitation spectra of ZnPc in NHK 3025 cells were found to be similar in shape. This indicates little or no aggregation of ZnPc in NHK 3025 cells even at the high concentrations of ZnPc used in these studies (Fig. 1). This is to be expected for an efficient photosensitizer, since only monomeric species are appreciably photoactive [41–45].

ZnPc in combination with light induced a cytotoxic effect on NHK 3025 cells (Fig. 3) as has previously been found for other cell lines [38,46]. The sensitivity of NHK 3025 cells to photoactivation did not increase significantly when the temperature was lowered during light exposure (Fig. 3), as has been demonstrated after photoactivation of haematoporphyrin [19,20]. It was suggested in the earlier study that the enhanced sensitivity of the cells to light at reduced temperature was due to reduced rate of repair. Since the temperature was raised to 37°C immediately after the end of the exposure to light, the repair influencing the survival of haematoporphyrin-treated cells must occur during or immediately after exposure to light. The present results indicate that such a repair mechanism is not of great importance in ZnPc-induced photoactivation.

The rapid and highly reversible inhibition of tubulin polymerization by nocodazole [26] makes this drug suitable for studying photochemical effects on the same target. Tubulin is the main structural component of microtubules (MTs).
PDT has been shown to induce accumulation of cells in mitosis subsequently followed by cell death [21,22,24,47,48]. This is due to direct damage of the MT constituents in their un polymerized form [23]. Depolymerization of the MTs with nocodazole prior to light exposure of sensitizer-loaded cells has in many cases been found to enhance the cytotoxic effect of the treatment [22,25]. However, the presence of nocodazole during PDT did not lead to an increased ability of ZnPc to sensitize cells to photo inactivation (Fig. 4). The un polymerized form of tubulin therefore seems not to be a target for the ZnPc-mediated photochemical inactivation of NHIK 3025 cells. This is in accordance with the observed reciprocity between the significance of tubulin damage (and accumulation in mitosis) in photocytotoxicity and lipophilicity of photosensitizer, since ZnPc is a highly lipophilic photosensitizer. It should be noted that cold treatment induces depolymerization of MTs [23]. The enhanced sensitivity of cells to photoinactivation with the more hydrophilic haematoporphyrin at reduced temperature may alternatively be due to its effect on tubulin.

Photo inactivation of cells and tissues containing photosensitizers is strongly dependent on the localization sites of the dye [49,50]. In the present work the localization of ZnPc was investigated by fluorescence microscopy (Fig. 6) as well as by measurements of photochemically induced damage to enzymes known as markers for subcellular organelles (Fig. 7). The lack of inactivation of the lysosomal marker enzyme β-AGA by treatment with ZnPc in combination with light indicates low or no lysosomal localization of ZnPc in NHIK 3025 cells. This is in agreement with the uptake studies, which indicated that endocytosis was not involved in the uptake of ZnPc in this cell line as well as the intracellular localization pattern as seen by fluorescence microscopy (Fig. 6). The marker enzyme for endoplasmic reticulum, NADPH cytochrome c reductase, was not inactivated by ZnPc-PDT, indicating minor localization of ZnPc in endoplasmic reticulum. However, the activities of UDP galactosyl transferase, which localizes in the Golgi apparatus, and cytochrome c oxidase, a marker enzyme for the mitochondria, were found to decrease in the cells treated with ZnPc and light (Fig. 7). This is in accordance with the fluorescence microscopic analysis showing a high concentration of fluorescence in an area close to the nuclear membrane resembling the localization of the Golgi apparatus, and a widespread extranuclear granular fluorescence that might resemble the mitochondria (Fig. 6). Thus, the present results may indicate a Golgi apparatus and mitochondrial localization of ZnPc in NHIK 3025 cells and that these organelles are targets in ZnPc-sensitized PDT. Valduga and coworkers found mitochondria and the plasma membrane to be targeted by ZnPc-PDT [38] in accordance with the present results. In their study the cells were incubated for only 2 h before light exposure and more substantial damage to the plasma membrane should be expected than in the present study. Milanesi and coworkers suggest from ultrastructural analysis that the mitochondria and the cytoplasmic membrane of tumour cells are damaged in vivo by treatment with ZnPc and light [35]. It was shown that ZnPc was distributed throughout the cellular membrane systems in HepG2 cells, but was absent from the cell nucleus [51].

In order to validate the use of the selected marker enzymes for analysing subcellular localization of photosensitizers, several other photosensitizers were used for analysing photochemical inactivation of UDP galactosyl transferase and NADPH cytochrome c reductase (Fig. 8). The present results indicate that Photofrin is located in the Golgi apparatus, which is in accordance with previous electron microscopic analysis of subcellular damage after Photofrin-PDT [52]. Previous works have shown that Photofrin is also located in mitochondria, but usually not in lysosomes [29,53,54]. TPPS₂a was found to inactivate the endoplasmic reticulum-located NADPH cytochrome c reductase after light exposure. TPPS₂a is primarily located in lysosomes, but is relocated to nearly all the extranuclear cytoplasm after light exposure [14]. The present results indicate that TPPS₂a is relocated to endoplasmic reticulum after light exposure.

In conclusion, liposome-delivered ZnPc was found to be taken up into NHIK 3025 cells by a diffusion-mediated pathway and seems to be mainly located in the Golgi apparatus and mitochondria of the cytoplasm.

5. Abbreviations

TPPSₙ tetraphenylporphine with n sulfonate groups
TPPS₂a tetraphenylporphine with two sulfonate groups on adjacent phenyl rings
ZnPc zinc-phthalocyanine
PBS Dulbecco’s phosphate-buffered saline
PDT photodynamic therapy

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