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Photodynamic efficiency of liposome-administered tetramethyl hematoporphyrin in two human bladder cancer cell lines

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Abstract The main problems presented by superficial bladder carcinoma, its high recurrence rate and multifocal appearance, require treatment of the bladder as a whole. Photodynamic therapy (PDT) is one such experimental treatment for superficial bladder carcinoma, involving the administration of a photosensitizer that accumulates in the tumor tissue, and subsequent irradiation of the tumor with light. Since the photosensitizers used in PDT suffer from several drawbacks, new photosensitizers are being sought. Drug delivery systems are also being investigated for the administration of hydrophobic photosensitizers and enhancement of photodynamic efficiency and tumor selectivity. In this study we examined a new photosensitizer, tetramethyl hematoporphyrin (TMHP), in two human bladder cancer cell lines. In the first pair of the experiments, TMHP was bound to unilamellar liposomes. Cellular uptake, dark toxicity and photodynamic efficiency were then studied. Fluorescence microscopy showed TMHP localization in the cytoplasm in a perinuclear region, sparing the nucleus. Dark toxicity occurred after incubation of cells with TMHP above a concentration of 20 μg/ml. Irradiation was carried out using an argon-pumped dye laser emitting a wavelength of 630 nm at a fluence of 3.6 and 7.2 J/cm^2. Before irradiation, cells were incubated with TMHP at concentrations of 2.5 and 5 μg/ml for 1 h. Cell survival rates after incubation with 5 μg/ml TMHP and irradiation at 7.2 J/cm^2 were 15.7% of control cells for Rec and 4.5% for Waf cells. Uptake studies showed a higher intracellular TMHP concentration in Waf than in Rec cells. This correlates with the higher PDT efficiency seen in Waf cells. Our results show that TMHP can be encapsulated into unilamellar liposomes without losing its photodynamic efficiency. TMHP is taken up by human bladder carcinoma cells after an incubation time of only 1 h. This short incubation time seems to be appropriate for an intravesical instillation of the photosensitizer for PDT in bladder cancer patients. Intravesical instillation might demonstrate higher phototoxic efficiency with reduced side effects. TMHP acts as a potent photosensitizer and shows drug- and light-dose-dependent cell destruction. Thus, TMHP has the potential for use in PDT in bladder cancer.

Key words Bladder carcinoma · Tetramethyl hematoporphyrin · Liposomes

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Despite the use of transurethral resection, laser surgery, and intravesical chemo- and immunotherapy, superficial bladder carcinoma still poses the problems of a high recurrence rate, multifocal appearance and progression in tumor grading and stage. Therefore, new treatment options are being sought. In photodynamic therapy (PDT), a photosensitizer that accumulates in tumor tissue is administered either systemically or topically [6, 9, 11]. PDT is also used clinically in different specialties to treat various cancers and benign diseases [15]. In urology it is used to treat superficial bladder cancer, especially carcinoma in situ [7, 12, 16]. Upon irradiation of the tumor with light that is absorbed by the photosensitizer, a photochemical reaction leads to tissue necrosis by the formation of singlet oxygen and/or free radicals. For endoscopic irradiation, laser light is used which can be coupled with quartz fibers

[7, 12, 16]. A fiber is inserted into the urinary bladder via a cystoscope, and, using a balltip fiber end, homogeneous light distribution within the whole bladder wall should be achieved. Therefore, as with intravesical drugs, even invisible carcinoma in situ of the bladder can be treated by this method. The clinically most used photosensitizer is a hematoporphyrin derivative (HPD). Since this suffers from several drawbacks, such as chemical impurity, weak absorption of the therapeutically used red light and skin phototoxicity, new photosensitizers are being sought. HPD is a mixture of different porphyrins and the photodynamic active compound is not known. Due to phototoxicity of normal skin, patients have to avoid direct sunlight for several weeks. The side effect of skin phototoxicity might be circumvented by instillation of the photosensitizer into the urinary bladder, when no systemic absorption occurs. Taari et al., however, studied the PDT effect of Photofrin II and Photosan on rabbit bladder after intravenous and intravesical administration [20]. Intravenous injection of the photosensitizers induced bladder wall edema, hemorrhage and total necrosis of the epithelium, whereas intravesical instillation induced only superficial epithelial necrosis [20]. The authors concluded that the depth of necrosis achieved with PDT after an intravesical dose of 5 mg/kg body wt. of Photofrin II or Photosan for 1 h was not sufficient [20]. Therefore, other photosensitizers are being examined, which might show better photodynamic efficiency after intravesical instillation. Besides new photosensitizers, drug delivery systems, such as monoclonal antibodies, liposomes, microspheres and low-density lipoproteins, are being investigated for the administration of hydrophobic photosensitizers and the enhancement of photodynamic efficiency [2, 5, 10, 13, 14, 17]. Tetramethyl hematoporphyrin (TMHP) is a new hydrophobic agent, which is examined in this study on two human bladder carcinoma cells. In contrast to HPD, TMHP is a chemically pure substance. Figure 1 shows its chemical structure and absorption spectrum. The goal of this investigation was to study the intracellular uptake and localization of liposome-encapsulated TMHP and its dark and photodynamic activity.

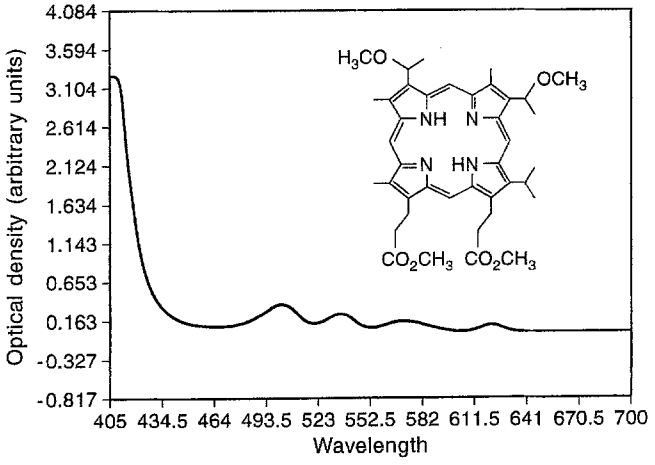


Fig. 1 Chemical structure of TMHP and absorption spectrum of liposome-bound TMHP

Materials and methods

Cell lines

Two human bladder carcinoma cell lines, Rec and Waf, derived from a transitional cell carcinoma, grade 3, were grown as a monolayer culture in DMEM containing 10% fetal calf serum, 2% glutamine and 1% penicillin/streptomycin. Cells were kept in an incubator at 37 °C in a 95% air:5% CO_2 atmosphere. For the experiments, cells were trypsinized, counted and incubated into 35-mm-diameter multiwells (Nunloc, Denmark) at a density of 10^5 cells/well and allowed to grow for 24 h.

Photosensitizer

TMHP was synthesized by Prof. Dr. Müller von der Haegen, Flensburg, FRG (Fig. 1). Phosphatidylcholine was purchased from Sigma and used without further purification. Liposomes were produced using phosphatidylcholine dipalmitoyl as phospholipid (DPPC) as described by Jori [13, 14]. Phospholipid and TMHP were dissolved at a molar ratio of 70:1 in chloroform/methanol 9/1. After evaporation of the solvent, the lipid film was resuspended in aqueous buffer and sonicated (Branson sonifier 250) for 20 min in order to obtain small unilamellar liposomes. The average diameter of the liposomes was determined to be 60 nm by measuring the dynamic light scattering in the Department of Biophysics, University of Ulm. The characteristic absorbance spectra of free and bound TMHP were recorded with a spectrophotometer (DU 64, Beckman).

Biodistribution

Cells were incubated with TMHP for 1 h, washed and the intracellular fluorescence examined using a fluorescence microscope with an excitation wavelength in the range of 450–490 nm and a barrier filter for emission of a wavelength greater than 515 nm. Video microscopy using a high-power field was also used to study fluorescence in single cells.

Extraction of the photosensitizer

After incubation of cells with 5 μg/ml liposomal TMHP for 1 h, cells were washed with PBS and a single-cell suspension was obtained, which was then centrifuged at 2000 rpm for 10 min. The resulting supernatant was removed and 0.1 N NaOH added. The suspension was homogenized and centrifuged again. The fluorescence of the supernatant was measured using a spectrophotometer (Kontron SFM 25) and compared with a standard curve. The autofluorescence of cells was also determined and used for background correction.

Irradiation

Cells were irradiated as monolayer cultures with an argon-pumped dye laser emitting a wavelength of 630 nm, the wavelength of maximum absorbance of TMHP. The fluences were 3.6 and 7.2 J/cm^2 at an irradiance of 30 mW/cm^2.

Dark toxicity

Cells were incubated with increasing concentrations of liposome-bound TMHP between 2.5 and 20 μg/ml for 1 h. Thereafter cells were washed, incubated for 4 days, fixed, stained, counted and compared with control cells.

PDT experiments

Rec or Waf cells were incubated as monolayer with 2.5 or 5 μg/ml liposome-administered TMHP for 1 h, washed, irradiated at 3.6 or 7.2 J/cm^2, incubated again for 4 days, fixed, stained, counted and compared with control cells that were neither incubated with TMHP nor irradiated. Control experiments with cells that were treated with TMHP or light alone were also carried out.

Results

Fluorescence microscopy

After a 1-h incubation of cells with liposome-bound TMHP, the photosensitizer was taken up. Intracellular TMHP fluorescence could be detected using a fluorescence microscope. Fluorescence was found in a punctate pattern in the cytoplasm, sparing the nucleus (Fig. 2). At higher magnification single cells could be studied. No fluorescence was seen in the plasma or nuclear membrane or in the area of the nucleus (Fig. 3).

Extraction of intracellular TMHP

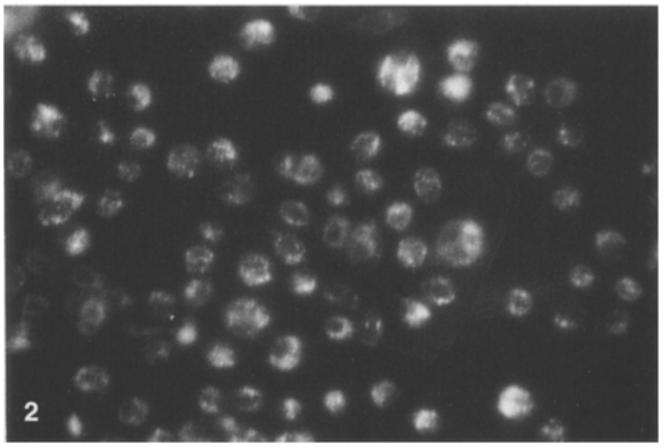
The TMHP concentration was compared with a standard curve and expressed as micrograms TMHP per 1×10^6 cells. After correction for background and autofluorescence, Rec cells were found to contain 0.068 and Waf cells 0.076 μg TMHP/10^6 cells.

Dark toxicity

After incubation of cells with liposome-administered TMHP at a concentration of up to 10 μg/ml, cell survival rate was unaffected compared with control cells. After incubation with 20 μg/ml TMHP, dark toxicity occurred. Therefore, PDT experiments were carried out using a TMHP concentration of only 2.5 and 5 μg/ml, when no dark toxicity was found. Empty liposomes for comparison had no effect on cell survival.

PDT experiments

In all experiments a drug- and light-dose-dependent decrease in cell survival was found. After incubation of Rec cells with 2.5 or 5 μg/ml liposome-bound TMHP and irradiation at 3.6 J/cm^2, cell survival was 47% or 39% that of the control; after irradiation at 7.2 J/cm^2, cell survival was decreased to 22% and 6%, respectively. After incubation of Waf cells at the same TMHP concentration and irradiation at 3.6 J/cm^2, cell survival was 20% and 11% that of control, respectively. After irradiation at 7.2 J/cm^2, cell survival was 16% and 4% of control, respectively (Fig. 4a, b). Figure 5a–d shows the morphology of the two cell lines before and after incubation with TMHP liposomes and irradiation. In control cells, TMHP liposomes or light alone had no effect on cell survival.



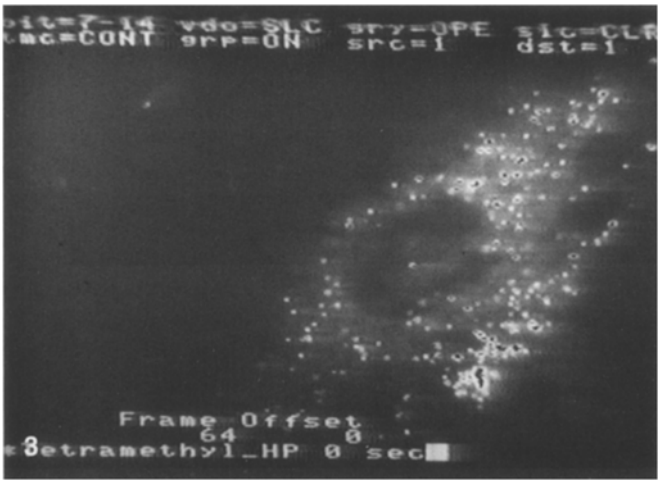


Fig. 2 Waf bladder cancer cells showing fluorescence within the cytoplasm, sparing the nucleus, after a 1-h incubation with TMHP liposomes

Fig. 3 Punctate fluorescence within the cytoplasm of a single cell after incubation with TMHP liposomes

Discussion

New photosensitizers for PDT are being sought, since the photosensitizers used thus far suffer from several drawbacks, such as chemical impurity, weak absorption of the therapeutically used light, skin phototoxicity and poor tumor localization [9, 11]. In order to administer hydrophobic dyes or to enhance photodynamic

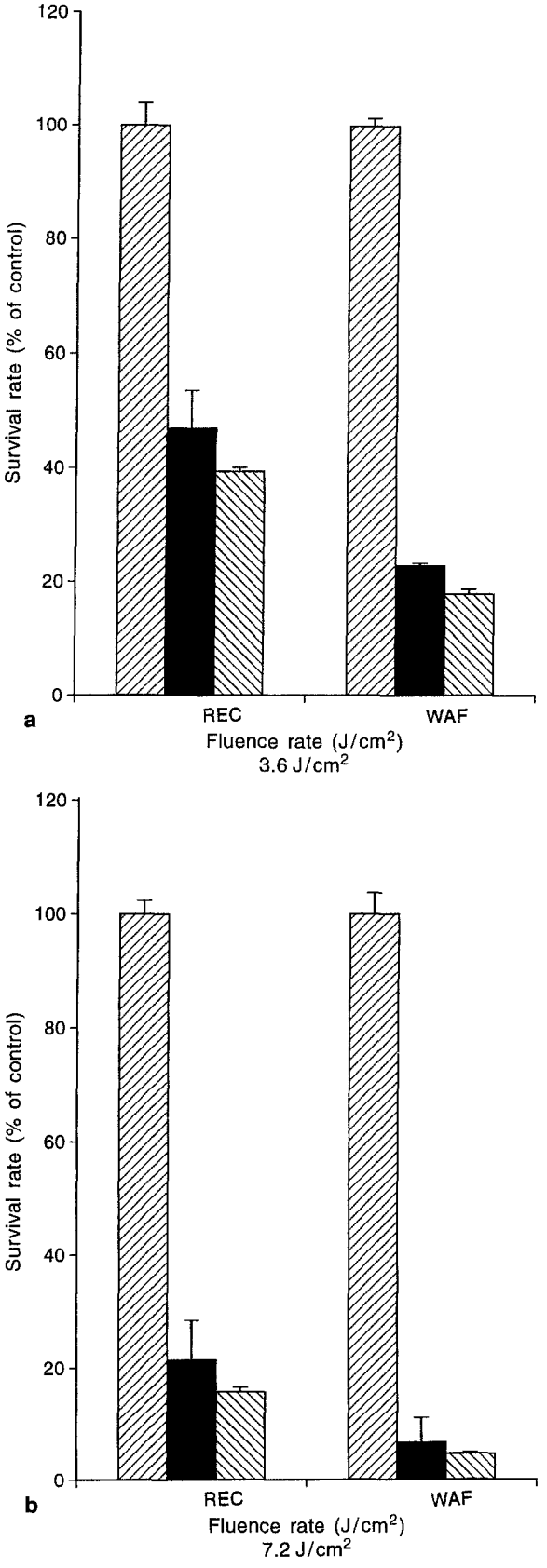


Fig. 4 Effect of PDT on survival rate as a percentage of control in Rec and Waf cells after incubation with 2.5 and 5 μg/ml TMHP liposomes and irradiation at 3.6 (**a**) and 7.2 J/cm² (**b**). ▨ Control; ■ 2.5 μg/ml; ▧ 5.0 μg/ml

efficiency, drug delivery systems are being used [8, 13, 14].

Photosensitizers can be incorporated in the phospholipid bilayer or the internal aqueous compartment of liposomes, depending on the hydro-/liposolubility [14]. It has been demonstrated that binding of photosensitizers to liposomes results in a more efficient delivery with higher intracellular concentration of the photosensitizer and more prolonged retention by tumor cells as compared with free photosensitizer [13, 14]. In in vivo studies on mice bearing an MS-2 sarcoma tumor, localizing properties of porphyrin were improved when it was coupled to liposomes [14]. Liposomes are taken up from the blood by cells via endocytosis and the photosensitizer is released inside the cells [14]. The intracellular distribution of the photosensitizer depends on the hydro-/liposolubility. Thus, different subcellular sites can be targeted by different porphyrins [14]. Polar porphyrins are found in the soluble fraction, whereas hydrophobic porphyrins are localized within the cellular membranes [14]. Taken together, liposomes have two advantages: firstly, polar photosensitizers can pass the cellular membrane barrier, when encapsulated within liposomes; secondly, water-insoluble photosensitizers can be administered intravenously [14]. Therefore, the number of photosensitizers that might be useful for PDT is enlarged by using liposomes as the carrier system [14].

In our study, TMHP, a new hydrophobic photosensitizer, was studied in two human bladder cancer cell lines after binding to liposomes. For PDT of bladder cancer, a topical administration of the photosensitizer – as in intravesical chemotherapy of superficial tumors – seems to be advantageous over intravenous injection [3]. A higher porphyrin concentration might be achieved in the tumor, with increased phototoxicity. On the other hand, side effects both to normal bladder wall and systemically could be reduced. An in vitro study using monolayer cell culture seems to imitate an intravesical photosensitizer instillation, since there is direct contact between the tumor cells and the photosensitizer. Our study shows that TMHP could be incorporated into liposomes without losing its photodynamic efficiency. It was taken up by bladder carcinoma cells within only 1 h of incubation and was found mainly in a perinuclear area. An incubation time of 1 h also seems to be appropriate for clinical application after topical administration.

Chun-Jung also reported a punctate fluorescence pattern in cells that were incubated with liposome-bound hematoporphyrin [4]. This pattern correlates with the intracellular distribution of lysosomes. Liposomes seem to be localized within these organelles after endocytosis [19]. Lysosomal enzymes destroy the lipid bilayer of the liposomes and release the photosensitizer, which acts within the lysosomes or in other cellular compartments [1]. A similar result was obtained with chlorin e6 conjugated to microspheres which were

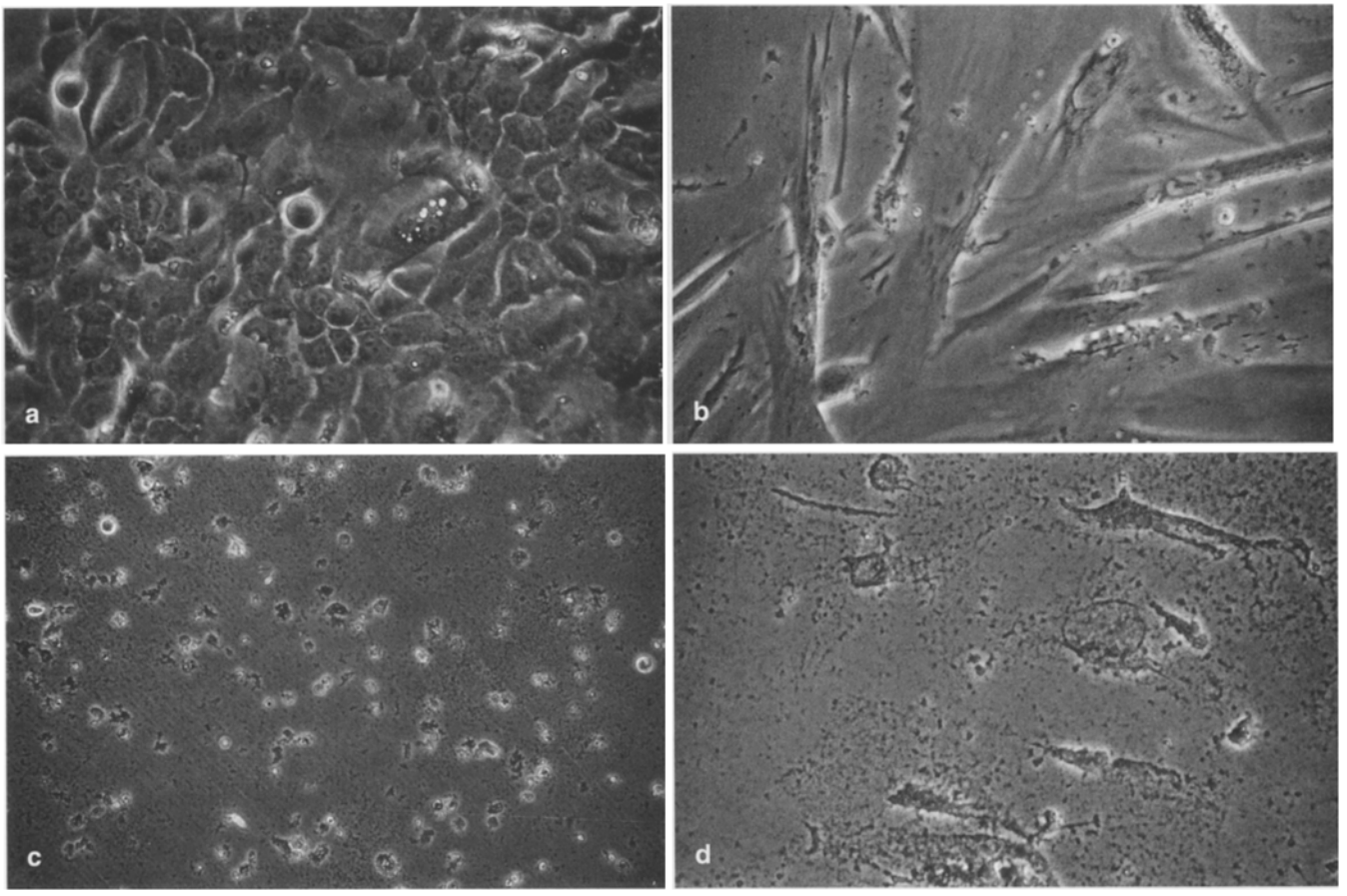


Fig. 5 Bladder cancer cells in monolayer culture: **a** Waf cells, **b** Rec cells: before incubation with TMHP liposomes and irradiation. **c**, **d** after PDT: Waf (**c**) and Rec (**d**) cells showing necrosis, lysis and debris

phagocytosed by bladder carcinoma cells [2]. Counterstaining with acridine orange demonstrated localization within the lysosomes [2]. PDT experiments with liposome-bound TMHP showed a high phototoxic efficiency. A drug- and light-dose-dependent decrease in cell survival was achieved.

After incubation with a low dose of 5 μg/ml TMHP and irradiation at 7.2 J/cm^2, Waf cells demonstrated a survival rate of 4% compared with control cells. In our experiments, Waf cells showed a higher sensitivity to liposome-bound TMHP than Rec cells. This is in accordance with our extraction data, where a higher intracellular TMHP concentration was found in Waf cells.

Figure 4a, b compares the different light and drug doses used. There was a distinct difference in cell survival after irradiation with 3.6 J/cm^2 and 7.2 J/cm^2, respectively. However, an increase in the incubation dose of TMHP from 2.5 μg/ml to 5.0 μg/ml did not show much effect. The reason for this phenomenon might be a saturation of uptake after incubation with a concentration between 2.5 and 5 μg/ml TMHP. The highly phototoxic effect in both cell lines might result from the intracellular localization within lysosomes. After irradiation, lysosomal membranes could be damaged by the photochemical-induced reaction and highly cytotoxic enzymes may be released.

The same phenomenon was found after PDT with chlorin e6 microspheres. The cytotoxic effect of conjugated chlorin e6, which was localized within the lysosomes, was higher than that with the free photosensitizer; free chlorin e6, in contrast, was found within the cellular membranes [2]. Moreover, the retention time of chlorin e6 microspheres was longer than that of free chlorin e6 and a higher intracellular concentration of chlorin e6 was measured after incubation with the microsphere-bound photosensitizer [2]. Spikes studied liposome-incorporated hematoporphyrin dimethylester and found the same degree of phototoxicity over time, suggesting a longer intracellular retention time of liposome-administered photosensitizer than with free dye [18].

In conclusion, TMHP can be incorporated into liposomes without loss of its photodynamic activity. After a 1-h incubation it is taken up by bladder carcinoma cells and leads to cytotoxicity upon irradiation with red light. Therefore, liposome-bound TMHP has the potential to be used in PDT of superficial bladder carcinoma.

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