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Photodynamic therapy of human bladder carcinoma cells in vitro with pH-sensitive liposomes as carriers for 9-acetoxy-tetra-*n*-propylporphycene

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Abstract In vitro experiments were performed on human bladder carcinoma cells to evaluate the efficiency of the recently synthesized photosensitizer 9-acetoxy-tetra-*n*-propylporphycene (ATPPn) for photodynamic therapy. To improve cytoplasmic delivery of this hydrophobic compound, we prepared pH-sensitive liposomes composed of phosphatidylethanolamine (PE) and cholesteryl hemisuccinate (CHEMS) in comparison with pH-insensitive liposomes consisting of phosphatidylcholine (PC) and CHEMS. Dynamic light scattering measurements were used to monitor the acid-induced liposome destabilization. After incubation with liposome-bound ATPPn, bladder carcinoma cells were irradiated by a dye laser with increasing light fluence rates from 1 to 48 J/cm^2. The photodynamic effects were then assessed from cell survival curves. No dark or phospholipid toxicity was measured for 2 μg ATPPn/1.5 ml medium. Qualitative cellular uptake of ATPPn was determined by fluorescence microscopy, while photodamage was elucidated by transmission and scanning electron microscopy. Absorption spectra performed up to 42 days revealed changes in shape for the pH-sensitive liposomes after storage at room temperature. ATPPn was proved to be an encouraging photosensitizer, capable of reducing cell survival to 0.1% after short-term incubation of 60 min with a drug dose of 2 μg ATPPn/1.5 ml medium. Although pH-sensitive PE/CHEMS liposomes showed significantly ($P < 0.05$) more photokilling effects at 24 J/cm^2 and 48 J/cm^2, no further advantages over non-pH-sensitive PC/CHEMS liposomes were found.

Key words Photodynamic therapy · Porphycene · pH-sensitive liposomes · Transmission electron microscopy · Scanning electron microscopy

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The photosensitizer most widely used up to now is hematoporphyrin derivative, especially in its more highly purified form Photofrin II. On account of some important disadvantages, namely the poor absorption in the visible part of the spectrum, the contamination by other porphyrin species and the high degree of skin photosensitization after treatment, the synthesis and evaluation of new photosensitizing agents for the photodynamic therapy (PDT) of cancer continues to be of great interest [15].

Porphycene, a chemically pure synthetic porphin isomer, was described for the first time in 1986 [27]. In the wavelengths above 620 nm, porphycene shows a 10-fold stronger absorption than hematoporphyrin derivatives [17]. Porphycene generates high fluorescence quantum yields that are superior to those of the porphyrins. Molecular singlet oxygen generation by photosensitization with porphycene is comparable to that by other currently used photosensitizers [2]. Because of the strong hydrophobicity of 9-acetoxy-tetra-*n*-propylporphycene (ATPPn), incorporation into lipid matrixes as liposomes is required. Since the early 1980s, reports on the synthesis of pH-sensitive liposomes have been emerging [29]. This special kind of liposomes is stable at neutral pH and becomes fusion competent and leaky at weakly acid pH values. At first, pH-sensitive liposomes were intended to target primary tumors and metastases, where local pH is reduced. However, this theory was soon abandoned and a new one established [4]: after being endocytosed by cells, liposomes encounter the acid environment of the endosomes (prelysosomal compartments) and finally reach the lysosomes, where liposomes and their contents are degraded by enzymes. Conventional liposomes that are not able to escape the endosomal compartment will be inactivated in lysosomes. pH-sensitive liposomes, however, can evade delivery to the lysosome, since the pH is already reduced in the endosome, where pH-sensitive liposomes become destabilized and release their contents into the cytoplasm. This novel type of liposomes is considered by many authors [24, 26] to be an effective cytoplasmic delivery system for different kinds of drugs.

Consequently, we decided to investigate the effectiveness of the new photosensitizer ATPPn mediated by pH-sensitive liposomes.

Materials and methods

Chemicals

ATPPn was synthesized by Professor Vogel at the Institute for Organic Chemistry, University of Cologne, Germany and had a purity >97% as checked by TLC/HPLC and $^{1}H/^{13}C$-NMR-spectroscopy. Egg yolk phosphatidylethanolamine (type III-A; purity >98%), egg yolk phosphatidylcholine (type XI-E; purity >99%) and cholesteryl hemisuccinate (CHEMS) were purchased from Sigma (Deisenhofen, Germany). DMEM (Dulbecco's modified Eagle's medium), FCS (fetal calf serum), PBS (phosphate-buffered saline), *L*-glutamine, trypsin-EDTA (0.05%), streptomycin (10000 µg/ml) and penicillin (10000 IU/ml) were obtained from Gibco (Scotland). Organic solvents and Giemsa were from Merck (Darmstadt, Germany). All lipids were chromatographically pure by high-performance-thin-layer chromatography on silica gel (Merck).

Photosensitizer

A stock solution at a concentration of 1 mg/ml was produced by dissolving ATPPn in chloroform/methanol 9:1 (v/v) and stored at 4°C in the dark. This stock solution was used for all experiments. The characteristic absorbance spectra of liposome-bound ATPPn were recorded by a DU-64 Beckman spectrophotometer.

Liposome preparation

The liposomes were prepared by mixing a chloroform solution of either PE/CHEMS (7:3 mol/mol) for the pH-sensitive liposome composition or PC/CHEMS (7:3 mol/mol) for the pH-insensitive liposome composition with ATPPn at a molar ratio of 70:1 (phospholipid/CHEMS:photosensitizer) [14]. The phospholipid concentrations (PE or PC) were 9 mM. The solvent was removed by vacuum evaporation at 35°C. The dried lipid film was then hydrated with a phosphate-buffered aqueous solution (0.5 M KH_2PO_4-Na_2HPO_4; PBS pH 7.4) by vortexing for 20 min. After probe ultrasonification (Branson sonifier 250) for 20 min, the liposome dispersion was centrifugated at 1000 *g* for 10 min and extruded through a 0.45-µm sterile filter (Millipore).

Dynamic light scattering

Size and polydispersity of the liposomes were measured by dynamic light scattering from an argon-ion laser (Spectra-Physics 2020) employing a Malvern K 7032 multi-8 correlator. The data were transferred to a personal computer obtaining autocorrelation functions based on the method of cumulants [16].

Stability of liposome-bound ATPPn upon storage

For the spectrophotometrical storage assay, ATPPn-containing liposomes were diluted 1:10 with PBS at pH 7.4, resulting in an ATPPn-concentration of 10 µg/ml (0.9 mM phospholipid concentration), and stored under aseptic conditions at room temperature in the dark. Absorbance spectra were periodically recorded up to 42 days.

pH-sensitivity assay

Phospholipid in a concentration of 9 mM was diluted 1:25 in PBS at the desired pH adjusted by adding appropriate amounts of HCl or NaOH. Size and polydispersity of the vesicles were determined after adding the vesicles to the graded pH values. The duration of one measurement was adjusted to 180 s. To exclude possible influences caused by exposure to laser light only liposomes without ATPPn were used for the pH-sensitivity assay.

Cell treatment

The human bladder carcinoma cell line WAF, a medium grade G_2 urothelial cancer from a cystectomy specimen, was grown on DMEM + 15% FCS + 2% L-glutamine + 1% penicillin/streptomycin (complete medium) as monolayer cultures at 37°C, saturated with 5% CO_2. For experiments, cells in passages 14–20 were used. In the log phase, cells were trypsinized and counted. Cells were inoculated into 35-mm-diameter multi-wells (Nunclon, Nunc, Denmark) at 10^5 cells/well and allowed to grow for 24 h. Before incubation with liposome-bound ATPPn, cells were washed twice with PBS (Dulbecco's, Gibco) and then DMEM without FCS was added. After incubation with 2 µg ATPPn/1.5 ml for 60 min at 37°C, cells were refed with complete medium. For dark or phospholipid toxicity evaluation, cells were incubated with liposome-bound ATPPn at concentrations of 2 µg and 20 µg per 1.5 ml and for comparison with the corresponding quantity of empty liposomes (120 µM and 1070 µM phospholipid concentration, respectively) under the same conditions. All experiments concerning the photosensitizer were carried out under subdued light. After treatment, cells were incubated for 3 days at 37°C, then fixed with methanol and stained with Giemsa. To estimate the number of surviving cells, 30 squares were counted under a phase-contrast microscope and compared with controls. Only adherent cells without pyknotic nuclei, damaged cytoplasmic membrane or shrinkage of cytoplasm were counted as vital. For this cell assessment a good correlation with the trypan blue vitality test was observed.

Irradiation procedure

An argon-ion dye laser system (Meditec, Germany) containing DCM dye was adjusted to a wavelength of 642 nm according to the absorption spectrum of ATPPn by means of a monochromator. Light was delivered via a 0.6 nm quartz fiber and the beam was expanded to a spot size of 35 mm covering the monolayer homogeneously. At a power density of 30 mW/cm^2 as measured with a power meter (Gentec, Canada) 1, 3, 6, 12, 24 and 48 J/cm^2 were obtained by varying the irradiation time.

Fluorescence measurements

Fluorescence measurements were made with a Zeiss-IM-35 fluorescence microscope. Cells were cultured on slides in Quadriperm plates (Heraeus, Hanau). Per slide, 10^5 cells were inoculated with 4 µg liposome-bound ATPPn/3 ml medium and incubated for 60 min at 37°C. After excitation of fluorescence in the range of 530–585 nm observation of emission was allowed by a barrier filter at wavelengths greater than 615 nm. Intracellular fluorescence was taken as qualitative measurement of ATPPn uptake.

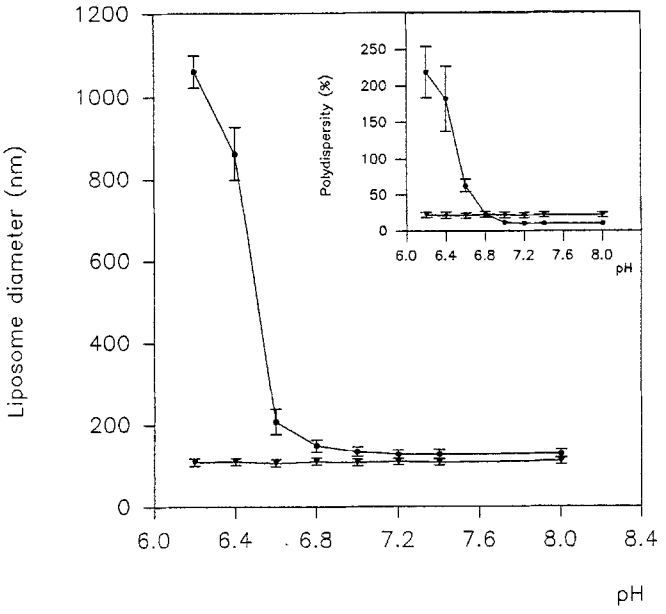


Fig. 1 pH-dependent liposome destabilization. Average diameter of pH-sensitive PE/CHEMS liposomes (●) and pH-insensitive PC/CHEMS liposomes (▼). *Inset:* Polydispersity of PE/CHEMS liposomes (●) and PC/CHEMS liposomes (▼). Each *point* and *bar* represent the mean diameter ± SD or the mean polydispersity ± SD of 5 experiments

Scanning and transmission electron microscopy

For transmission electron microscopy, cells were grown on Thermanox tissue culture coverslips (Nunc, USA). For scanning electron microscopy cells were allowed to adhere on 18-mm glass coverslips. At 1 h and 24 h after irradiation with 48 J/cm^2, specimens for electron microscopic examination were fixed in 2.5% glutaraldehyde, 0.1 M sodium cacodylate and 1% sucrose, pH 7.3. After postfixation in 2% osmium tetroxide for 1–2 h, specimens were dehydrated in a graded ethanol and propylene oxide before being embedded in Epon 812 resin. This procedure was followed by block-contrasting with 2% uranyl acetate. Prior to observation under a Philips EM-301 electron microscope operating at 80 kV, the specimens were counterstained with lead citrate. For scanning electron microscopy, the samples were subjected to critical point drying with liquid CO_2, sputter-coated with gold and observed with a Zeiss-962 digital scanning microscope operating at 5 kV.

Results

Physico-chemical experiments

In order to demonstrate the differences of the two liposome compositions towards acidification, we measured the average size and the polydispersity, a parameter of vesicle homogeneity, by means of dynamic light scattering. As the pH of the buffer decreased, average size and polydispersity of the pH-sensitive liposomes markedly increased as a result of acid-induced vesicle-vesicle aggregation or fusion (Fig. 1). The pH-sensitivity slighty began at pH values lower than pH 7.0. By contrast, pH-insensitive liposomes did not undergo any changes in average size or polydispersity. The pH-sensitive liposomes showed an average diameter of 126.9 nm ± 10.3 (polydispersity: 10.6% ± 1.2), whereas the pH-insensitive liposomes had an average diameter of 108.5 nm ± 8.1 (polydispersity: 22.1% ± 3.4), when kept at pH 7.4. After storage for 24 h at pH 7.4, no change of average size or polydispersity was detectable for both the liposomes.

The effect of storage at room temperature in PBS at pH 7.4 for ATPPn-containing pH-sensitive liposomes (PE/CHEMS) is displayed in Fig. 2. In the visible part of the spectrum the absorbance spectra show peaks at 564 nm, 606 nm, 633 nm and 642 nm in chloroform. For the liposome-bound ATPPn these values are red-shifted for 3–4 nm to 567 nm, 609 nm, 636 nm and 646 nm. After 7 days of storage, the peak at 609 nm showed a loss of intensity, and at 684 nm a shoulder appeared. After 14 days and 21 days of storage, the loss of intensity at 609 nm continued to increase, while the peaks at 636 and 646 nm slowly began to decrease. Furthermore, the difference between the two peaks at 636 nm and 646 nm was fading. Up to 42 days, this tendency was intensified in addition to a beginning loss of intensity at 567 nm. The corresponding absorbance spectra of the pH-insensitive liposomes (PC/CHEMS) revealed no significant changes in general shape or at any peak.

In vitro experiments

The survival curves for WAF cells treated with 2 µg ATPPn/1.5 ml medium for 60 min exhibited an exponential reduction of cell growth rate as a function of increasing light fluence. Data are expressed as survival rates in relation to controls of untreated cells (= 100%) (Fig. 3). Mean survival rates of 58.5%, 36.0%, 18.8%, 6.2%, 1.2% and 0.1% for the pH-sensitive liposomes and 66.5%, 36.7%, 21.9%, 7.3%, 2.0% and 0.3% for the pH-insensitive liposomes were caused by irradiation with 1, 3, 6, 12, 24 and 48 J/cm^2, respectively. There was no significant difference in the effectiveness of ATPPn encapsulated in PE/CHEMS or PC/CHEMS liposomes in the range of 1 to 12 J/cm^2, while significant differences were determined by unpaired Student's t-test ($P < 0.05$) for 24 J/cm^2 and 48 J/cm^2. Neither cells subjected to irradiation without drug exposure nor cells kept in the dark after receiving ATPPn were different in cell growth from untreated control cells. No signs of dark or phospholipid toxicity were manifested after an incubation time of 60 min at 37° C in the dark after administration of 20 µl or 200 µl ATPPn-containing liposomes or empty liposomes (Fig. 4). The liposome dose of 20 µl corresponds to 2 µg ATPPn or 120 µM phospholipid concentration, respectively. The liposome dose of 200 µl corresponds to 20 µg ATPPn or 1070 µM phospholipid concentration, respectively.

Fluorescence microscopy revealed the qualitative uptake of ATPPn. In the cells ATPPn was distributed in a punctate fluorescence pattern at the perinuclear region (Fig. 5a, b). Little or no diffused intracellular fluorescence was detectable. The nucleus was spared, indicating the

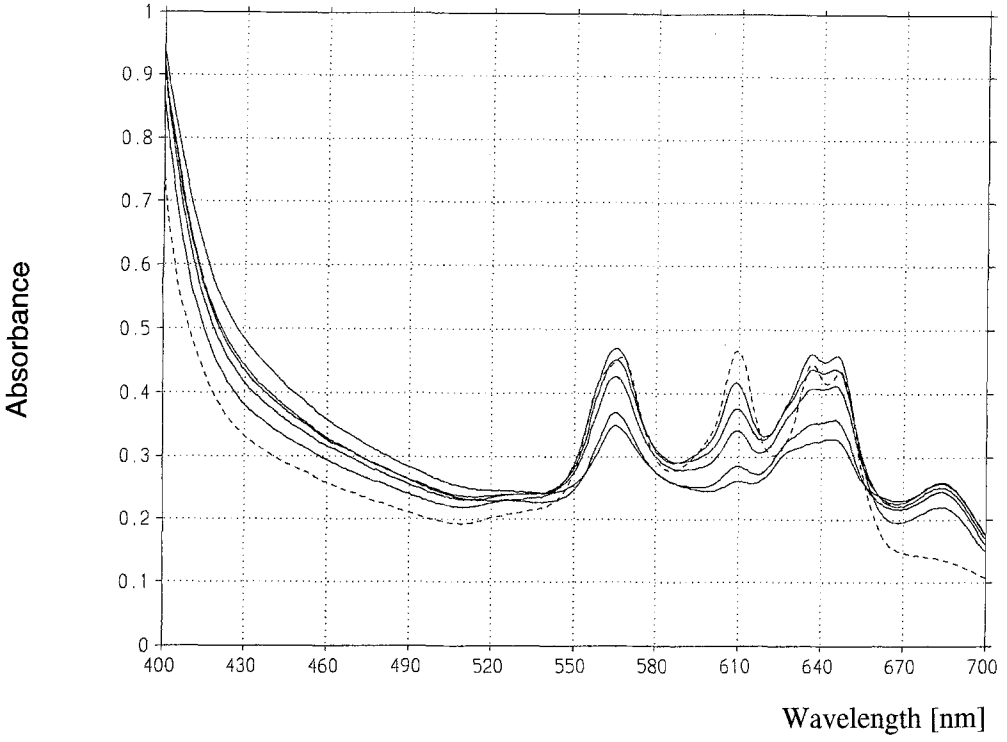


Fig. 2 Absorbance spectra of ATPPn bound to PE/CHEMS liposomes (10 µg ATPPn/ml liposome dispersion) after storage at room temperature in PBS pH 7.4 for 0 days (*dashed line*), 7 days, 14 days, 21 days, 28 days and 42 days, respectively. *Curves* are shown in decreasing order at 609 nm

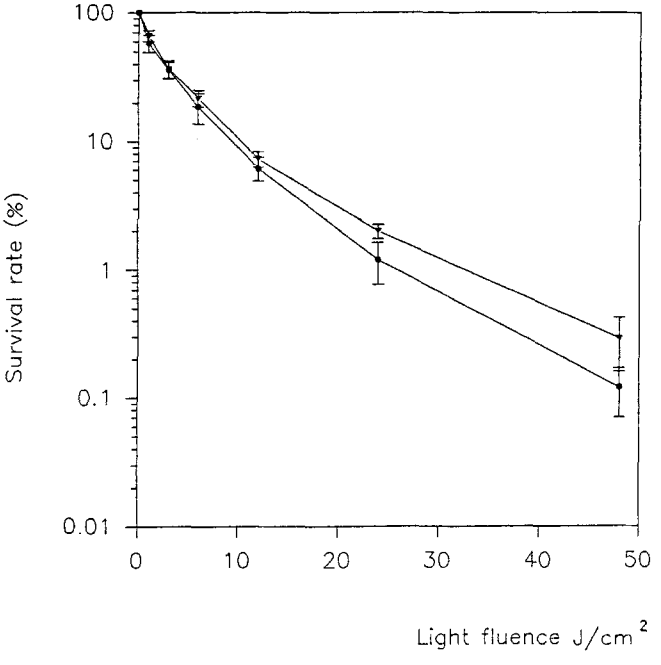


Fig. 3 Survival rate of WAF cells as a function of increasing light fluence after incubation with 2 µg ATPPn/1.5 ml medium for 60 min. ●, pH-sensitive PE/CHEMS liposomes; ▼, pH-insensitive PC/CHEMS liposomes. Each *point* and *bar* represent the mean value ± SD of 7 experiments in triplicate plates

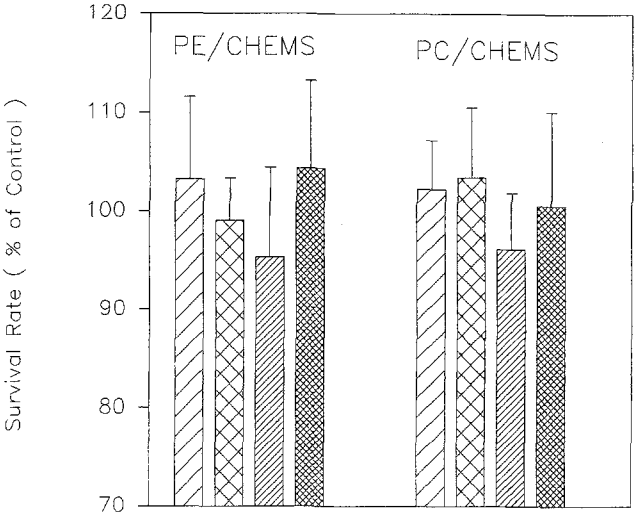
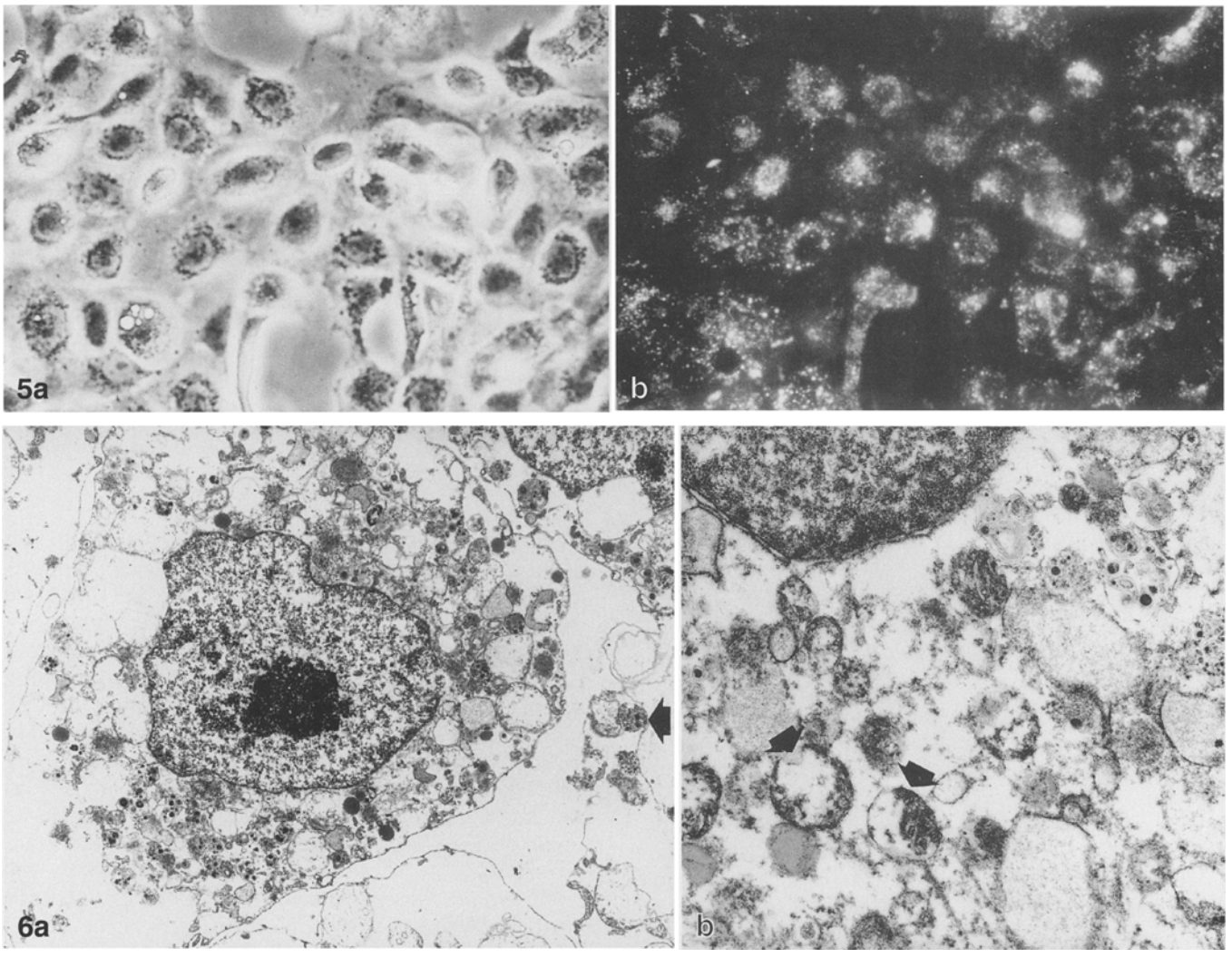


Fig. 4 No dark or phospholipid toxicity can be seen after incubation with 20 µl or 200 µl liposomes per 1.5 ml medium. ▨, 20 µl liposomes corresponding to 120 µM phospholipid concentration; ▩, 200 µl liposomes corresponding to 1070 µM phospholipid concentration; ▨, 20 µl liposomes corresponding to 2 µg ATPPn; ▩, 200 µl liposomes corresponding to 20 µg ATPPn. Each *bar* indicates the mean value ± SD of 3 experiments in triplicate plates



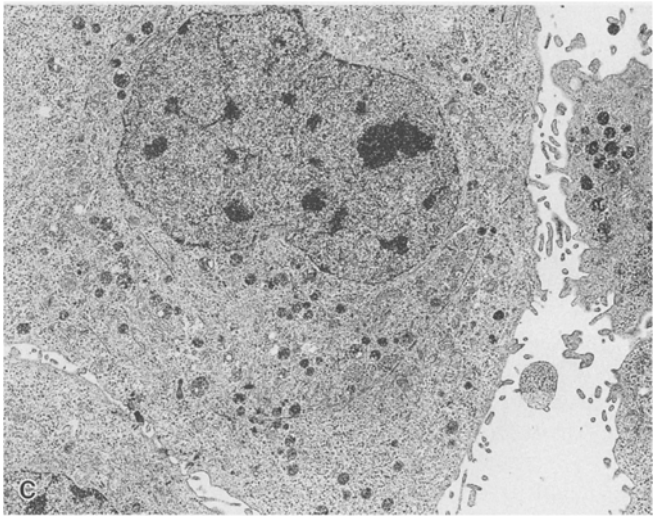


Fig. 5 **a** Phase contrast and **b** fluorescence micrographs of WAF cells incubated with 4 µg liposome-bound ATPPn/3 ml medium for 60 min at 37°C. Cells were transferred to ATPPn-free PBS prior to observation under the microscope, (×320)

Fig. 6a Transmission electron micrographs of cells treated with 2 µg ATPPn/1.5 ml medium after irradiation with 48 J/cm^2, (×2500). Some special lysosomes, multivesicular bodies (*arrows*) are relatively well preserved. **b** At a higher magnification (×9100) mitochondria with disrupted cristae can be seen (*arrows*). **c** Unirradiated control cells (×2500)

incapacity of ATPPn for penetrating the nucleus membrane.

For the transmission and scanning electron microscopy, cells were allowed to grow for 1 h or for 24 h after irradiation with 48 J/cm^2. No signs of recovery or other differences relative to specimens fixed after 1 h were recognizable after 24 h of growth. The photodestruction appeared under the following aspects at the ultrastructural level (Figs. 6a, b, c): the irradiated cells, incubated with liposome-entrapped ATPPn, revealed a cytoplasmic mem-

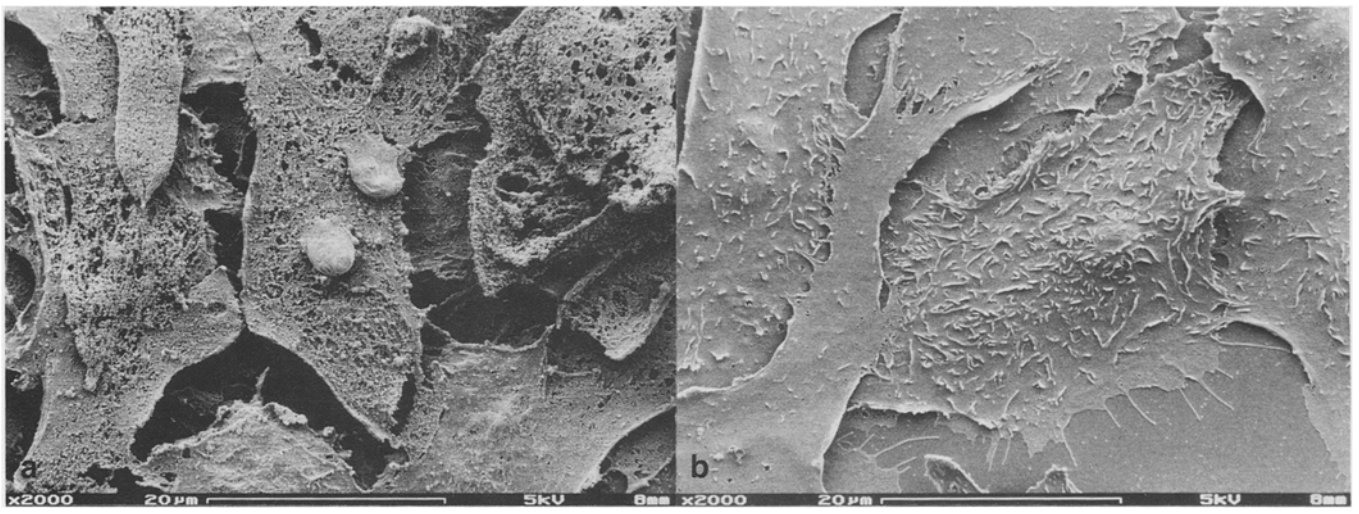


Fig. 7a Scanning electron micrographs of WAF cells treated with 2 µg ATPPn/1.5 ml medium after irradiation with 48 J/cm^2 (×2000). Cytoplasmic membrane is perforated and shows blebs. **b** Unirradiated WAF cells as control

brane that was hard to define and was raised from the residual cytoplasm. The cytoplasm was extensively vacuolated, while the mitochondria showed a disruption of their cristae and a higher electron density than usual. Multivesicular bodies, regarded as a variant of lysosomes and often appearing in WAF cells, seemed to be scarcely affected. The nucleus was rounded and showed an inhomogeneous aspect, corresponding to pyknosis. Scanning electron microscopy demonstrated the extent of cellular injury at the cell's surface (Fig. 7a, b). Irradiated cells were perforated and there were holes in their surface as well as cytoplasmic blebs of different sizes. The microvilli disappeared as soon as the blebs arose. In contrast to the formation of pores by the irradiated cells, unirradiated control cells had a smooth surface with many well-differentiated microvilli.

Discussion

Liposome-mediated drug delivery has been an area of active research in recent years. Vehiculation by liposomes is reported to enhance accumulation in cells and to retard the release of photosensitizing agents in both in vitro and in vivo experiments, so that lower drug doses may be sufficient. In neoplastic cells, these phenomena tend to be more pronounced [7].

pH-sensitive liposomes are generally composed of phosphatidylethanolamine (PE) as the obligatory constituent in combination with certain amphiphiles, such as fatty acids [9] or *N*-acyl-amino acids [6] or CHEMS [10]. We chose CHEMS as amphiphile, because the incorporation of cholesterol reduces the bilayer permeability to non-electrolyte and electrolyte solutes by increasing the packing of phospholipid molecules [8]. This results in a higher rigidity of the bilayer membrane, which is important in the presence of serum. Furthermore, CHEMS is a negatively charged cholesterylester and imparts a negative charge on the liposomes at pH 7.4. Negatively charged liposomes are considered to have a favored binding capacity to cells as well as a better cellular uptake [13, 18]. As soon as the carboxyl group of CHEMS is protonated in an acid environment, the interbilayer repulsions are reduced. The pH-sensitive system collapses into a non-lamellar structure, the inverted hexagonal II (H_{II}) phase, where hexagonally arranged amphiphile cylinders are formed [5].

We monitored the pH-sensitivity of the PE/CHEMS liposomes by means of dynamic light scattering determining the average size and polydispersity of the liposomes. This method requires no further treatment of the vesicles, and size determinations can be made more exactly than by electron microscopy. With dynamic light scattering, however, it is not possible to discriminate between fusion or aggregation of the vesicles.

The narrow polydispersity of the pH-sensitive and pH-insensitive liposomes reflects a high homogeneity of the vesicle population, especially in the case of the PE/CHEMS liposomes, with a polydispersity of about 10%. The size distribution of liposomes and the preparation method by sonication indicate the presence of oligo- or multilamellar vesicles [20]. Liposomes composed of PE/CHEMS destabilized at pH values lower than pH 7, where first a slow and then a sharp increase in average diameter and polydispersity was observed with decreasing pH values. Ellens et al. [10], however, monitored leakage of PE/CHEMS liposomes only at lower pH values than in our experiments, namely between pH 5.5 and pH 5.0.

When PE was replaced by phosphatidylcholine (PC), there were no alterations after an incubation in graded acid buffers at room temperature.

The absorbance spectra of PE/CHEMS and PC/CHEMS liposomes after storage at room temperature in neutral pH were investigated to study the appearance of

contingent changes in shape. While the absorbance spectra of the pH-insensitive liposomes retained their original shape over the entire 42 days, the pH-sensitive liposomes were subjected to several changes, e.g., a loss in peak intensity and a newly arising shoulder. These visible changes in shape suggest that PE/CHEMS liposomes promote the formation of ATPPn metabolites or the aggregation of ATPPn molecules, whereas PC/CHEMS liposomes are a stable vehicle for ATPPn. Dilute aqueous hematoporphyrin solutions are likewise reported to undergo an aging transformation after storage at room temperature [25].

The main purpose of the present study was to test the effectiveness of liposomally delivered ATPPn for photodynamic therapy, both in a pH-sensitive and in a pH-insensitive liposome system. ATPPn, a new second-generation photosensitizer of the group of the porphycenes is recommended as an attractive candidate for photodynamic therapy, since porphycenes, e.g. tetra-*n*-propyl-porphycene and ATPPn, are regarded as efficient in vivo tumor localizers [12, 19].

The survival curves obtained after incubation with 2 μg liposome-bound ATPPn/1.5 ml medium and irradiation in the range of 1–48 J/cm^2 showed an exponential phototoxic effect lacking an initial threshold or shoulder. The initial slope began very steeply and passed into a final slope with reduced steepness above 12 J/cm^2. By increasing the light fluence to 48 J/cm^2, the survival rate was diminished to 0.1% for the pH-sensitive composition and to 0.3% for the pH-insensitive one. Even when the cell surviving rates for pH-sensitive liposomes were significantly lower according to the unpaired Student's *t*-test for 24 J/cm^2 and 48 J/cm^2, the differences at this high light fluence may be assessed as of low clinical relevance.

For better comparison with other photosensitizers, we use the survival rate at the 10% level, where a light fluence of approximately 10 J/cm^2 can be graphically determined. After incubation with 25 μg/ml hematoporphyrin derivative (HPD) for 1 h, an 8-fold lower light fluence was required to reduce cell survival to 10% [11]. In another study a 7-fold higher light fluence was used to achieve this level after application of 50 μg/ml HPD for 2 h [1]. West and Moore [28] incubated 10 μg/ml photosensitizer for 24 h and needed 30-fold and 2-fold higher light fluence rates for $TPPS_4$ [tetrasodium-meso-tetra (4-sulfonatophenyl) porphine] and HPD, respectively. For Photofrin II 1.3 lower light fluence rates were necessary.

Examination of the ultrastructure of irradiated WAF cells incubated with 2 μg liposome-bound ATPPn/1.5 ml medium demonstrated the cellular photodamage. The cytoplasm was highly disintegrated and extensively vacuolated. Only a few residual cell organelles still existed, e.g., more electron-dense mitochondria with disrupted cristae or nuclei with pyknotic structure. A variety of lysosomes, so-called multivesicular bodies [3], appeared relatively well preserved, supporting the theory that hydrophobic compounds such as ATPPn destroy mitochondria and membranes [21, 22]. Scanning electron micrographs revealed the loss of cytoplasmic membrane integrity by demonstrating the formation of vesicles and pores in the cell membrane after irradiation. Since microvilli disappeared as soon as the blebs were formed, blebs are regarded as degenerated former microvilli [23, 30]. When a low light fluence is used, cell vesiculation and swelling are reported to be reversible [23]. At 48 J/cm^2 however, no reversal of the morphological changes was seen when cells were allowed to grow for 24 h after irradiation.

Furthermore, no dark or phospholipid toxicity was ascertained for WAF cells incubated for 60 min with empty or ATPPn-containing liposomes when liposome doses of 20 μl (= 2 μg ATPPn or 120 μM phospholipid concentration) or 200 μl (= 20 μg ATPPn or 1070 μM phospholipid concentration), respectively, were used.

In conclusion, our investigations suggest that ATPPn is a promising photosensitizer. Our results do not, however, confirm the hypothesis of a preferential and improved effectiveness of the pH-sensitive system PE/CHEMS against the pH-insensitive PC/CHEMS liposomes. For the future, we intend to examine different bladder carcinoma cells to exclude a possible cell-line-specific sensitivity or resistance. Moreover, in vivo experiments with local application of liposomes are proposed, in order to assess the clinical relevance of this new photosensitizer.

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References

1. Andreoni A, Cubeddu R, De Silvestri S, Laporta P, Ambesi-Impiombato FS, Esposito M, Mastrocinque M, Tramontano D (1983) Effects of laser irradiation on hematoporphyrin-treated normal and transformed thyroid cells in culture. Cancer Res 43:2076
2. Aramendia PF, Redmond RW, Nonell S, Schuster W, Braslavsky SE, Schaffner K, Vogel E (1986) The photophysical properties of porphycenes: potential photodynamic agents. Photochem Photobiol 44:555
3. Bloom W, Fawcett DW (1975) A textbook of histology, 10th edn. Saunders, Philadelphia London Toronto, p 48
4. Chu CJ, Dijkstra J, Lai MZ, Hong K, Szoka FC (1990) Efficiency of cytoplasmic delivery by pH-sensitive liposomes to cells in culture. Pharm Res 7:824
5. Collins D, Connor J, Ting-Beall HP, Huang L (1990) Proton and divalent cations induce synergistic but mechanistically different destabilizations of pH-sensitive liposomes composed of dioleoyl phosphatidylethanolamine and oleic acid. Chem Phys Lipid 55:339
6. Connor J, Yatvin MB, Huang L (1984) pH-sensitive liposomes: acid-induced liposome fusion. Proc Natl Acad Sci USA 81:1715
7. Cozzani I, Jori G, Reddi E, Tomio L, Zorat PL, Sicuro T, Malvaldi G (1984) Interaction of free and liposome-bound porphyrins with normal and malignant cells: biochemical and photosensitization studies in vitro and in vivo. In: Andreoni A, Cubeddu R (eds) Porphyrins in tumor phototherapy. Plenum Press, New York, p 157
8. Demel RA, Kruyff B de (1976) The function of sterols in membranes. Biochim Biophys Acta 457:109

9. Düzgünes N, Straubinger RM, Baldwin PA, Friend DS, Papahadjopoulos D (1985) Proton-induced fusion of oleic acid-phosphatidylethanolamine liposomes. Biochemistry 24:3091
10. Ellens H, Bentz J, Szoka FC (1984) pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact. Biochemistry 23:1532
11. Gomer CJ, Smith DM (1980) Photoinactivation of chinese hamster cells by hematoporphyrin derivative and red light. Photochem Photobiol 32:341
12. Guardiano M, Biolo R, Jori G, Schaffner K (1989) Tetra-*n*-propylporphycene as a tumour localizer: pharmacokinetic and phototherapeutic studies in mice. Cancer Lett 44:1
13. Johnson JW, Nayar R, Killion JJ, Eschenbach AC von, Fidler IJ (1989) Binding of liposomes to human bladder tumor epithelial cell lines: implications for an intravesical drug delivery system for the treatment of bladder cancer. Select Cancer Therap 5:147
14. Jori G, Garbo GM, Reddi E, Cozzani I (1984) Modulation of the photosensitizing efficiency of porphyrins by their association with liposomes. Med Biol Environ 12:491
15. Kessel D (1984) Hematoporphyrin and HPD: photophysics, photochemistry and phototherapy. Photochem Photobiol 39:851
16. Koppel DE (1972) Analysis of macromolecular polydispersity in intensity correlation spectroscopy: the method of cumulants. J Chem Phys 57:4814
17. Kreimer-Birnbaum M (1989) Modified porphyrins, chlorins, phthalocyanines, and purpurins: second-generation photosensitizers for photodynamic therapy. Semin Hematol 26:157
18. Lee KD, Hong K, Papahadjopoulos D (1992) Recognition of liposomes by cells: in vitro binding and endocytosis mediated by specific headgroups and surface density. Biochim Biophys Acta 1103:185
19. Leunig M, Richert C, Gamarra F, Lumper W, Vogel E, Jocham D, Goetz AE (1993) Tumour localisation kinetics of photofrin and three synthetic porphyrinoids in an amelanotic melanoma of the hamster. Br J Cancer 68:225
20. Lichtenstein D, Barenholz Y (1988) Liposomes: preparation, characterization, and preservation. Methods Anal 33:337
21. Milanesi C, Biolo R, Jori G, Schaffner K (1991) Experimental photodynamic therapy with tetrapropyl-porphycene: ultrastructural studies on the mechanism of tumour photodamage. Lasers Med Sci 6:437
22. Miller K, Reich E, Rück A, Hautmann R (1991) Photodynamic therapy of bladder cancer-uptake and phototoxicity of photosan in vitro. Urol Res 19:353
23. Moan J, Johannessen JV, Christensen T, Espevik T, McGhie JB (1982) Porphyrin-sensitized photoinactivation of human cells in vitro. Am J Pathol 109:184
24. Reddy R, Zhou F, Huang L, Carbone F, Bevan M, Rouse BT (1991) pH-sensitive liposomes provide an efficient means of sensitizing target cells to class I restricted CTL recognition of a soluble protein. J Immunol Methods 141:157
25. Ricchelli F, Grossweiner LI (1984) Properties of a new state of hematoporphyrin in dilute aqueous solution. Photochem Photobiol 40:599
26. Ropert C, Lavignon M, Dubernet C, Couvreur P, Malvy C (1992) Oligonucleotides encapsulated in pH-sensitive liposomes are efficient towards Friend retrovirus. Biochem Biophys Res Commun 183:879
27. Vogel E, Köcher M, Schmickler H, Lex J (1986) Porphycene – a new porphin isomer. Angew Chem 98:262
28. West CML, Moore JV (1989) The photodynamic effects of Photofrin II, hematoporphyrin derivative, hematoporphyrin, and tetrasodium-meso-tetra (4-sulfonatophenyl) porphine in vitro: clonogenic cell survival and drug uptake studies. Photochem Photobiol 49:169
29. Yatvin MB, Kreutz W, Horwitz BA, Shinitzky M (1980) pH-sensitive liposomes: possible clinical implications. Science 210:1253
30. Yu DS, Chang SY, Ma CP (1991) Ultrastructural changes of bladder cancer cells following methylene blue-sensitized photodynamic treatment. Eur Urol 19:322