Urol Res (1991) 19:353–356

Urological Research


# Photodynamic therapy of bladder cancer – uptake and phototoxicity of photosan in vitro*

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Accepted: February 1, 1991

**Summary.** The uptake of photosan and the intracellular sites of photoradiation-induced damage were investigated in vitro in bladder carcinoma cells and in normal bladder cells. Cells were examined by phase contrast, fluorescence and electron microscopy. The concentration of photosan, measured in $\mu g/10^6$ cells, showed a good correlation to the incubation time. At all incubation times, control cells showed a lower uptake when compared with tumor cells. Following photodynamic therapy (PDT), phase-contrast microscopy revealed marked changes in tumor cells, whereas only minor effects could be detected at the cell membrane of the control cells. Following PDT, most of the investigated cells showed changes of the mitochondria and cytoplasma. These changes consisted of dissolution of the cristae, predominantly in the central part of the mitochondria. Twenty-four hours after PDT the shape of the mitochondria had changed markedly and the cristae were found to be completely destroyed. Moreover, the cystoplasma showed numerous vacuoles, and the number of mitochondria was decreased compared to non-treated cells.

**Key words:** Bladder cancer – Electron microscopy – Photodynamic therapy – Photosan – Phototoxicity – Tumor selectivity – Video fluorescence microscopy

The derivatives of hematoporphyrin have been widely used as photosensitizing agents in the past [6, 7]. However, there is still an ongoing discussion about the exact properties of these substances with regard to tumor-selective uptake and phototoxicity. Some authors have not been able to find any significant differences between tumor cells and normal controls [4], whereas other investigators have indeed found tumor-selective uptake in hematoporphyrin derivative (HPD) [8]. Another point of controversy is the nature and location of subcellular damage caused by photodynamic therapy with HPD. The aim of our in vitro study was to measure comparatively the uptake of photosan, a new derivative from the hematoporphyrin group, in normal bladder cells and two bladder cancer cell lines. Moreover, the subcellular morphologic changes after PDT were investigated by electron microscopy.

* Supported by Deutsche Forschungsgemeinschaft Ha 131/1-2

## Materials and methods

### *Cell lines*

Three different cell lines were used. Normal cells of the bladder mucosa, harvested by transurethral cold biopsy, served as controls; two bladder cancer cell lines (WF1 = medium grade urothelial cancer; KH2 = high grade urothelial cancer) obtained from cystectomy specimens were used as tumor cell lines. The cells were grown in monolayer cultures using Dulbecco's modified Engle's medium (DMEM) + 10% fetal calf serum (FCS). In the log phase, the cells were incubated with 10 µg photosan/ml medium + 1% FCS for 4 h.

### *Photosensitizer*

Photosan (code no. 115-5-L/88), a porphyrine mixture with a similar chromatogram to photofrin II (data not shown), was provided by Prof. H. Müller von der Haegen (W-2244 Wesselburenerroog, Seehoflaboratorium, FRG). A stock solution was portioned and stored at -20°C in the dark. Aliquots of this stock solution were used for the experiments. The fluorescence of photosan was measured under a fluorescence microscope (Zeiss/Axiophot) equipped with a highly intensifying silicium intensifying target (SIT) camera and an image-processing system. A fluorescence intensity in the spectral range between 590 and 800 nm was detected after excitation with the 405 or 436 nm bands of a 50 W mercury high-pressure lamp. To avoid photobleaching, the power density was attenuated to less than 100 mW $cm^{-2}$.

Photodynamic therapy was performed using a $Kr^+$-laser-pumped dye laser. The light was connected to the microscope via a 600 µm PCS fiber. The excitation wavelength was 630 nm, the power density of irradiation 3.8 W $cm^{-2}$. Exposure times were 5 × 1 s, followed by 2 × 5 s. After each irradiation, the decrease in fluorescence and the mophological changes in the cells were documented. For each fluorescence measurement an additional radiation dose of 1.0–1.27 J $cm^{-2}$ was applied.

The cells were grown on microscope slides and incubated 4 h with 10 µg $ml^{-1}$ photosan. Immediately before irradiation, the microscope slides were rinsed for about 10 s with PBS. All measurements were carried out within 20 min following incubation and rinsing of the cells.

To assess the uptake of photosan in normal and tumor cells prior to photodynamic therapy (PDT), the fluorescence intensity was measured selectively at the cell site of maximum photosan accumulation. In addition to the video-enhanced microscopy measurements, fluorescence and absorption measurements were carried out. $1 \times 10^6$ cells were incubated with 10 µg/ml photosan and kept in the dark. Culture medium was removed after 2, 4, 6 and 24 h incubation time, respectively. The cells were washed in PBS and then incubated in 3 ml 100% methanol for 1 h at 37°C, to extract photosan from the cells. All phases of the experiment were carried out in the dark. Absorption of the extracted photosan at 396 nm was measured in a spectral photometer. Cells not treated with photosan were used as controls. The concentration of photosan was expressed in µg

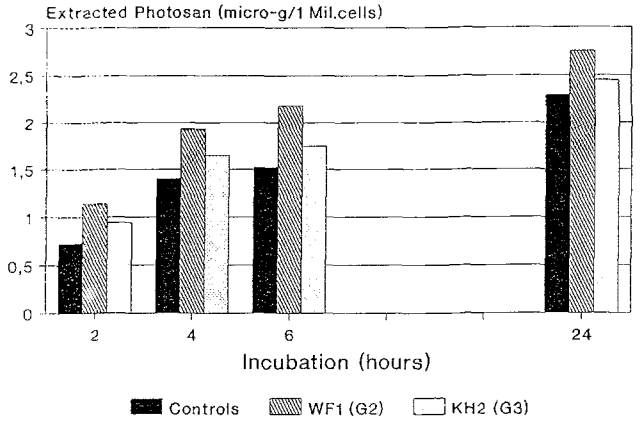


**Fig. 1.** Concentration of photosan in different cell lines following various incubation periods with 10 µg/ml. Results obtained by measurement of absorption (396 nm) of methanol-extractable/ Photosan (for details see text)

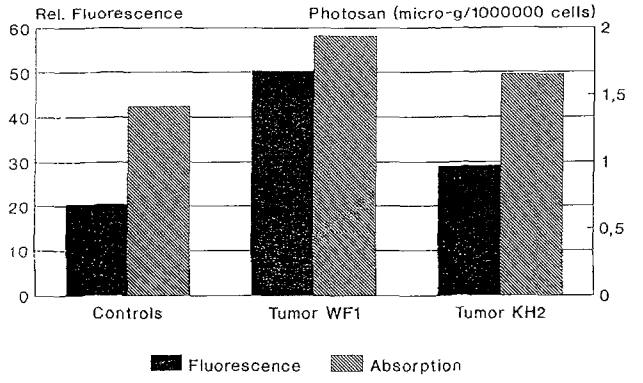


**Fig. 2.** Comparison of relative fluorescence and absorption of photosan following a 4-h incubation period (10 µg/ml)

photosan/$10^6$ cells. Absorption curves of photosan were constructed by measuring known concentrations of photosan in methanol at 396 nm.

### *Morphologic studies*

The morphologic effects after PDT were assessed by two different methods:

1. Changes at the cell membrane were evaluated under light microscopy using a phase-contrast technique. These studies were performed at various intervals following radiation of the cells as described above.
2. The morphologic effects at a subcellular level were assessed under the electron microscope (transmission technique); $1 \times 10^6$ cells, incubated 2, 4, 6 and 24 h with 10 µg/ml photosan, were irradiated homogenously with 12 J $cm^{-2}$ (power density of irradiation 100 mW $cm^{-2}$). Before irradiation, the cells were washed with phosphate-buffered saline (PBS), and after irradiation incubated in fresh medium (10% FCS) and fixed for the electron microscope studies with 3.5% glutaraldehyde, 0.1 mPPP and 2% sucrose (pH 7.3) at various intervals after irradiation (2, 4, 6 and 24 h).

## Results

### *Absorption measurements*

The concentration of photosan, measured in µg/$10^6$ cells showed a good correlation to the incubation time. The effect of the incubation time decreased after 6 h, with only a minimal difference between 6 and 24 h (Fig. 1). At all incubation times, normal cells showed a lower uptake when compared with tumor cells. Of two tumor cell lines, the medium-grade WF1-cell line had a higher uptake than the high-grade KH2 cell line.

### *Fluorescence measurements*

The fluorescence measurements showed a good correlation with the absorption measurements: after 4 h incubation time, the relation of fluorescence between the tumor cell lines and control cell lines paralleled that of the absorption measurement. Again, the control cell lines showed the lowest fluorescence, followed by the KH2 cell line and WF1-cell line with the highest fluorescence values (Fig. 2).

### *Light microscopy studies*

There was a correlation between the fluorescence before irradiation and the effects at the cellular membrane following PDT. Tumor cells showed marked changes (Fig. 3a,–c), whereas only minor effects could be detected at the cell membrane of the control cells (Fig. 4a, b). The changes seen in the KH2 cells were comparable with those in the WF1 cell line.

The distribution of fluorescence within the cells is shown in Fig. 5. These studies under the fluorescence microscope showed a maximum in the vicinity of the nuclear membrane, whereas the cystoplasma and the cell membrane showed little fluorescence.

### *Electron microscope studies*

Untreated control cells showed an almost spherical shape. Heterochromatin is predominently seen in the periphery of the nucleus; numerous organelles are found in the cytoplasma (Fig. 6). The mitochondria have a smooth surface and an oval shape. The cristae are regularly lined up along the longitudinal axes with an amorphic matrix between the cristae (Fig. 7).

Following PDT, most of the investigated cells showed changes of the mitochondria and cytoplasma. These changes depend on the interval following PDT: after 2 h, structural changes of the mitochondria can be seen. These consisted of dissolution of the cristae, predominantly in the central part of the mitochondria (Fig. 8). Twenty-four hours after PDT, the shape of the mitochondria markedly changed and the cristae were completely destroyed. Moreover, the cytoplasma showed numerous vacuoles. Only few organelles still had a normal structure (Fig. 9). The number of mitochondria decreased compared to non-treated cells. Overall, the structural changes following PDT showed a tendency to increase in the period 2 to 24 h following treatment. After 24 h, most of the cells showed signs of cell death.

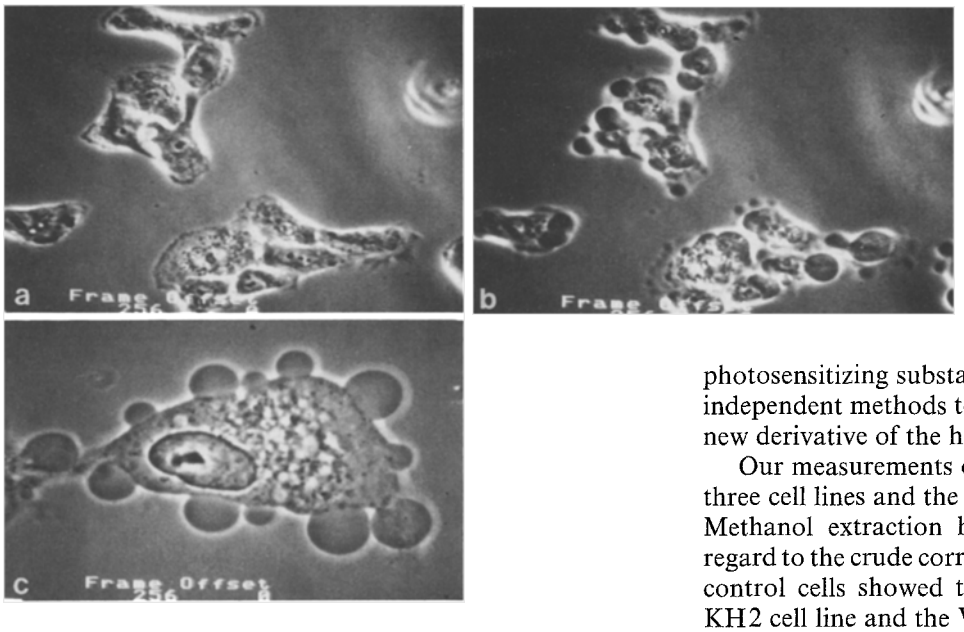

Fig. 3. a Tumor cells (WF1) prior to PDT. b Tumor cells (WF1) following PDT (4 h incubation with photosan). c Single tumor cell following PDT (100× magnification of b)

## Discussion

The primary aim of any oncologic therapy is to combine maximal damage to the tumor with minimal side effects for normal tissue. Many treatment modalities like chemotherapy and radiotherapy are still far from being ideal for many oncologic diseases, particularly with regard to the side effects. Consequently, the tumor-selective uptake of any photosensitizing agent is of utmost interest. There has been ongoing controversy on the relevance of fluorescence measurements to assess the tissue concentration of a photosensitizing substance. Therefore, we have used two independent methods to assess the uptake of photosan, a new derivative of the hematoporphyrin group.

Our measurements of the fluorescence of photosan in three cell lines and the absorption of photosan following Methanol extraction have yielded similar results with regard to the crude correlation between the three cell lines: control cells showed the least uptake, followed by the KH2 cell line and the WF1 cell line. Fluorescence of the WF1 cells was 2.5 times as high as in the control cells, whereas the concentration found by absorption measurements was only 1.35 times higher. Using the same techniques, other groups [5, 9] have found a somewhat higher uptake of HPD in absolute figures, but a similar correlation between normal and tumor cells. Using fluorescence measurements, Shulok et al. [9] found the uptake of HPD in tumor cells to be two times higher than in normal cells 24 h after incubation, which is similar to our results in the fluorescence measurements.

In essence, tumor selectivity could be demonstrated in photosan even though the quantification of this effect is still uncertain. The basis for the relative tumor retention of the sensitizer is still poorly understood. In vivo, the tumor production of bradykinins, with resulting increased sensitizer permeability and greater HPD-protein carrier receptor sites on tumor cells, has been suggested as being responsible for selective sensitizer retention [3]. However,

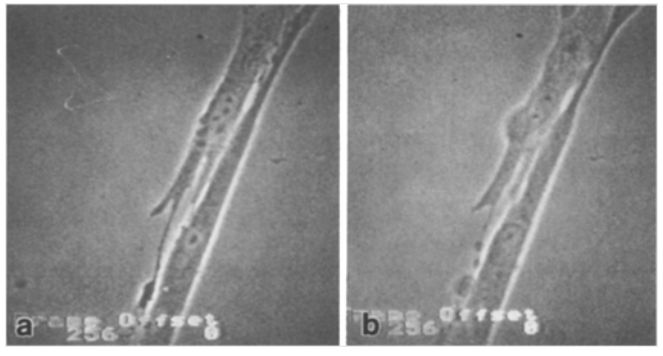

Fig. 4. a Control cells prior to PDT. b Control cells following PDT (4 h incubation with photosan)

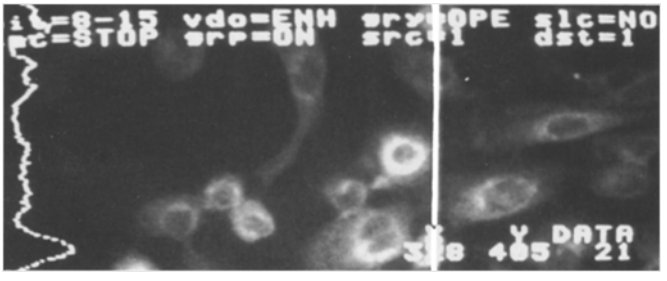

Fig. 5. Pattern of fluorescence within tumor cells (4 h incubation with photosan)

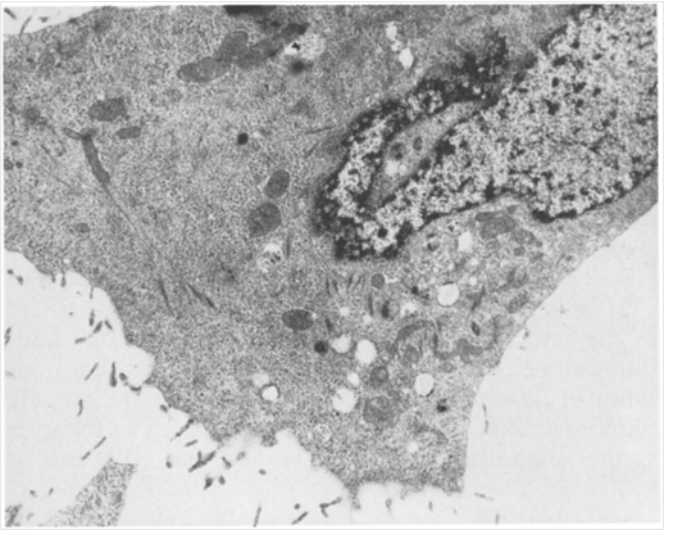

Fig. 6. Transmission electron microscopy of untreated WF1 cell. Peripheral location of nucleaus and numerous mitochondria throughout cytoplasma

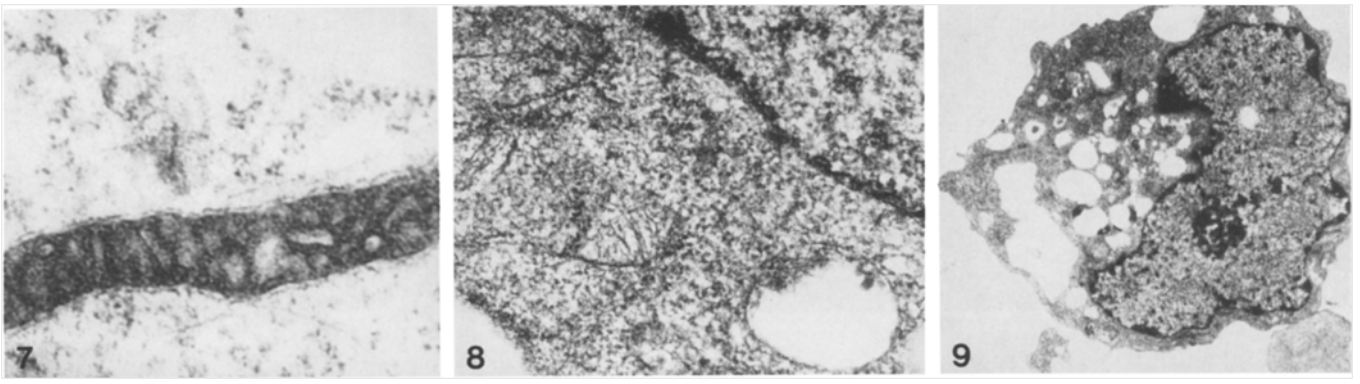

Fig. 7. Mitochondrium in untreated cell at higher magnification: intact cristae traverse the organelle perpendicular to the longitudinal axis. Matrix is uniformly distributed between cristae

Fig. 8. Mitochondrium in a cell 2 h after PDT shows fragmentation of cristae

Fig. 9. Tumor cell (WF 1) 24 h following PDT: marked alterations of the cytoplasma with numerous vacuoles

it must be recognized that the mechanism of uptake in vitro setting may be quite different from the in vivo situation where distribution and concentration of the photosensitizer within the tumor tissue can be influenced by the vasculature.

The phototoxic effects, observed under light microscopy at the cell membrane, correlated with the uptake measurements: after 3 s irradiation, the first changes could be observed, with a progressive tendency dependent on the duration of irradiation. Whereas the normal cells showed only minor effects, dramatic changes were observed at the membrane of the tumor cells. Up to now there have been no data in the literature that tumor cells react differently to the radiation per se; these different effects must therefore be attributed to the higher uptake of the photosensitizer.

Our electron microscopic studies show that treatment of cells with photosan and irradiation resulted in time-dependent alterations in the mitochondria of the majority of cells. These observations are consistent with the work of Shea et al. [8] and Gibson et al. [2]. The effect of photodynamic therapy at the mitochondria may be two-fold: first, there may be direct damaging of the internal membranes and, second, there may be an effect on the energy-producing enzymatic procedures.

Gibson and Hilf [2] have already demonstrated that PDT with hematoporphyrin derivates inhibit the activity of the cytochrome-c-oxidase, which results in the inhibition of cell respiration. The authors suggest that inhibition of cytochrome-c-oxidase may be one of the possible phototoxic effects of PDT with porphyrines. This theory is further supported by the observation that HPD can be detected within the mitochondria by fluorescence microscopy studies [1, 9].

Our data confirm that the mitochondria may be a primary target of photodynamic therapy. Some investigators [9] suggest that the numerous vacuoles observed after PDT may represent damaged mitochondria. Further studies are in progress at our institution to confirm these suggestions.

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