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Original Paper

Photodynamic Effects of 5-Aminolevulinic Acid-induced Porphyrin on Human Bladder Carcinoma Cells *In Vitro*

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The efficiency of 5-aminolevulinic acid (ALA) in photodynamic therapy (PDT) was investigated *in vitro* using urothelial carcinoma cells of various differentiation. HCV29, RT4 and J82 cells were cultured in 96-well plates, incubated with 25–100 μg/ml ALA in serum-containing medium for 4 h, and irradiated at 630, 635 and 640 nm wavelength with light doses of 15–100 J/cm². The degree of reduced tetrazolium bromide corresponding to cell viability was determined with a colorimetric MTT assay 0, 24 and 48 h after PDT. A remarkable reduction of mitochondrial activity occurred in poorly (J82) and well differentiated (RT4) malignant urothelial cells. Twenty-four hours after photodynamic treatment with 100 μg/ml ALA and 50 J/cm², the metabolic activity of malignant cells was nearly extinguished, while HCV29 cells, derived from normal urothelium, behaved similarly to non-irradiated control cells. The photosensitivity of cells depended on presence or absence of fetal bovine serum (FBS) in the ALA-incubation medium. A wavelength of 635 nm was up to 60% more effective compared with 630 nm, which is more frequently applied in PDT. From the results of our *in vitro* studies, we can define a "therapeutic window" for malignant cells without damaging benign cells. The time delayed effects and the strong wavelength dependence are important factors for a clinical application.

Key words: photodynamic therapy, 5-aminolevulinic acid, transitional cell carcinoma, MTT-assay
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INTRODUCTION

PHOTODYNAMIC THERAPY (PDT) appears to be the most effective treatment of superficial cancers such as carcinoma *in situ* of the urinary bladder [1]. PDT is achieved most frequently by intravenous injection of a mixture of oligomeric porphyrins (Photofrin®). Disadvantages of this procedure are low tumour-selective accumulation of the photosensitiser and cutaneous photosensitisation [1, 2].

5-Aminolevulinic acid (ALA), a precursor in the synthetic pathway to haem, induces accumulation of photosensitive protoporphyrin IX (PPIX) after exogenous application in epithelial tissues [3, 4]. Following intravesical application of ALA, the PPIX fluorescence is enhanced by a minimum of 10-fold in human papillary transitional cell carcinoma of the urinary bladder when compared with the surrounding normal mucosa [5]. This ALA-induced tumour-selective, poorly understood biochemical effect of PPIX accumulation in malignant tissues is a successfully used clinical tool in fluorescence detection of hardly visible transitional cell carcinoma of the urinary bladder [5]. PDT on chemically induced tumours of the rat bladder with intravesical ALA instillation followed by integral irradiation of the bladder produced tumour necrosis [6].

We developed an *in vitro* model system that allows investigations of PDT of bladder cancer under reproducible conditions and we investigated drug and phototoxicity, wavelength dependence and different sensitivities of benign and malignant cells using the MTT assay, a method based on the activity of mitochondrial dehydrogenases [7–14], which will be functionally affected by PDT *in vitro* [15]. The clinical use of ALA will depend on a significant increased PPIX accumulation in malignant cells compared with normal cells, and a clear dose dependence of the photodynamic processes.

MATERIALS AND METHODS

Cell lines and culture conditions

Based on extensive characterisations of 22 cell lines derived from normal and neoplastic human urothelium [16], we selected HCV29, RT4 and J82 cells as a representative *in vitro*

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model system for urothelial neoplastic disease. HCV29 cells have an isozyme phenotype characteristic of non-malignant cells [17] similar to normal urothelium. RT4 is derived from a well differentiated [18] and J82 from a poorly differentiated urothelial transitional cell carcinoma [19]. Cell lines were kindly provided by Dr J. Masters, University College London, U.K.

Cells were grown in RPMI 1640 without phenol red (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS); 1 mM sodium pyruvate; 4 mM L-glutamine; 100 μg/ml streptomycin; 100 units/ml penicillin; 0.25 μg/ml amphotericin B (all GIBCO/BRL, Eggenstein, Germany). Cells were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide/95% air as a subconfluent monolayer in 260 ml/83 cm^2 tissue culture flasks (Nunc, Wiesbaden, Germany). Under experimental conditions, cells were incubated at 37°C in FBS-containing medium. Exponentially proliferating cells were harvested with 0.05% trypsin/0.02% EDTA (GIBCO/BRL), resuspended in fresh medium and seeded in 96-well plates (Nunc) with an initial concentration of 10000 cells per well. Twenty-four hours later, drug incubation (4 h) and irradiation for up to 30 min were carried out followed by MTT assays 0, 24 and 48 h after PDT.

ALA application

In order to determine the toxic threshold of ALA, 7.5 mg 5-aminolevulinic acid hydrochloride (Merck, Darmstadt, Germany) were dissolved in 1 ml of a 8.4% sodium hydrogen carbonate/RPMI 1640 (ratio 1:12.5) solution. This pH-neutral stock solution was further diluted in RPMI 1640 and added to the cells in concentrations of 1–5 mg/ml. For irradiation experiments, pH-neutral stock solution consisted of 1 mg/ml 8.4% sodium hydrogen carbonate/RPMI 1640 (ratio 1:100). Concentrations of 25, 50 and 100 μg/ml were achieved by dilution with RPMI 1640.

Twenty-four hours after cell plating, the culture medium was removed and 100 μl of medium containing ALA in different concentrations were added to each well. After 4 h incubation (six wells/dose), a compromise between efficient time interval to achieve sufficient protoporphyrin accumulation and practicable handling of the experiment, cells were washed twice either with RPMI 1640 in drug toxicity experiments or Hank's buffer prior to light exposure. Hank's buffer is a photodynamically inert colourless solution, whereas supplemented RPMI 1640 is partially able to absorb light. After irradiation, Hank's buffer was replaced by fresh medium. Cytotoxicity was evaluated 0, 24 or 48h later, using the MTT assay to demonstrate time delayed physiological cell damage.

Irradiation

In order to irradiate cells cultured in microwell plates, a temperature regulated, computer controlled irradiation box impermeable to light was constructed [41]. An argon pumped dye laser served as a light source (Lambda plus, Coherent, Dieburg, Germany). Irradiation through the lid of a microwell plate would reduce the light dose applied up to 50% by refraction at the fluid meniscus. Therefore, cells were homogeneously illuminated through the transparent bottom with a constant power density of 100 mW/cm^2. The microwell plate was situated on the frame of a table moved by a motorised *X–Y* assembly. The aperture of the diaphragm in the course of rays enabled the simultaneous exposure of six wells. Thus, four different experimental situations with six measurements each could be performed in one plate. Additionally, six wells per plate were used as controls (incubated cells without irradiation), another six wells as blanks (untreated cells in culture medium supplemented with MTT immediately prior to measurement).

MTT assay

We determined the cytotoxic effects of ALA with or without subsequent light exposure on cells of different urothelial cell lines using a modified MTT assay previously described [7]. This assay is based on the reduction of a water soluble tetrazolium salt (MTT) to a purple, unsoluble formazan product by mitochondrial dehydrogenases present only in living, metabolically active cells. MTT (3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Sigma, Deisenhofen, Germany) was dissolved in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) at 5 mg/ml, filtered to sterilise and stored at 4°C. Stock solution was added to each well of an assay (10 μl per 200 μl medium), and plates were incubated at 37°C for 4 h. The resulting formazan crystals were dissolved overnight at 37°C by adding 100 μl per well of a 20% solution of sodium dodecyl sulphate (Sigma) in water. The plates were read on an Emax microplate reader (Molecular Devices Corp., Menlo Park, California, U.S.A.) using a 540 nm bandpass filter. The reader calibrated the air reference for all photodetectors before reading the microplate. Each individual phototoxic experiment was carried out at least twice. The mean optical density of six replicate values as equivalent for mitochondria activity and viability of the cells was expressed in per cent of control values:

$$\text{Optical density (OD) (\%)} = \frac{OD_{experiment} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100 .$$

The linear intracellular reduction of MTT to formazan using increasing numbers of cells between 100 and 1 million per well, and the linearity of reduction accompanying growing cell numbers within the experimental period of 3 days was ascertained prior to phototoxic experiments. The results correspond to previously published observations concerning other cell lines [7, 20].

Statistical analysis

Non-normally distributed values of the optical densities of two differently treated cell groups were statistically compared by the Mann–Whitney *U* test. In the case of three-cell groups, we used the Kruskal–Wallis *H* test. Additionally, we compared pairs of values by the method of Tukey for analysis of variance. All statistical analyses were performed at significance levels of $P < 0.05$ using SPSS for windows.

RESULTS

In order to exclude toxic effects of either light or ALA, MTT metabolism was determined after irradiation with experimental light doses (15–100 J/cm^2 at 635 nm wavelength) as well as after increasing ALA concentrations.

Irradiation without preceding ALA incubation did not affect the viability of cells compared with values of non-irradiated control cells. ALA doses of 2.5 mg/ml (14.9 mM) and above impeded the activity of mitochondria in cells of each cell line (data not shown). Similar results with the three investigated cell lines were seen when the MTT assay was carried out 24 h

after ALA incubation. Thus, the induction of drug toxicity requires a 25- to 100-fold higher drug dose than that which was used in the irradiation experiments (25–100 μg/ml = 0.15–0.6 mM).

Time-dependent phototoxicity

MTT assays performed 0, 24 and 48 h after PDT indicated that the phototoxic damage of irradiated cells increased within the observed time interval of 48 h due to time delayed effects. Immediately after photodynamic treatment of J82 cells with maximal doses (100 μg/ml ALA, 100 J/cm^2 light), a decrease in viability of 70% was observed. Comparable cell damage could be measured at reduced light or drug doses 24 or 48 h after PDT (Figure 1). Additionally, a phototoxic reaction requires a threshold dose of ALA, which induces a sufficient amount of intracellular PPIX, independent of the light energy applied. J82 cells were not impaired by doses below 25 μg/ml.

RT4 cells were more sensitive to PDT. A dose of 50 μg/ml ALA followed by 25 J/cm^2 reduced the activity of mitochondria to 20% of control values 24 h after irradiation (Figure 2). Interestingly, comparable effects in poorly differentiated J82 cells required twice the drug and light doses.

Compared with J82 and RT4, the HCV29 cells were least sensitive to PDT. Maximal drug and light doses (100 μg/ml ALA, 100 J/cm^2 light) were necessary to reduce cell viability to 50% of the control 24 h after PDT (Figure 3). This effect increased with time. However, 48 h after PDT, the activity was still approximately 30% of control values. Thus, HCV29 cells differ significantly from J82 (Figure 1) and from RT4 cells (Figure 2). A 50% reduction of mitochondrial activity

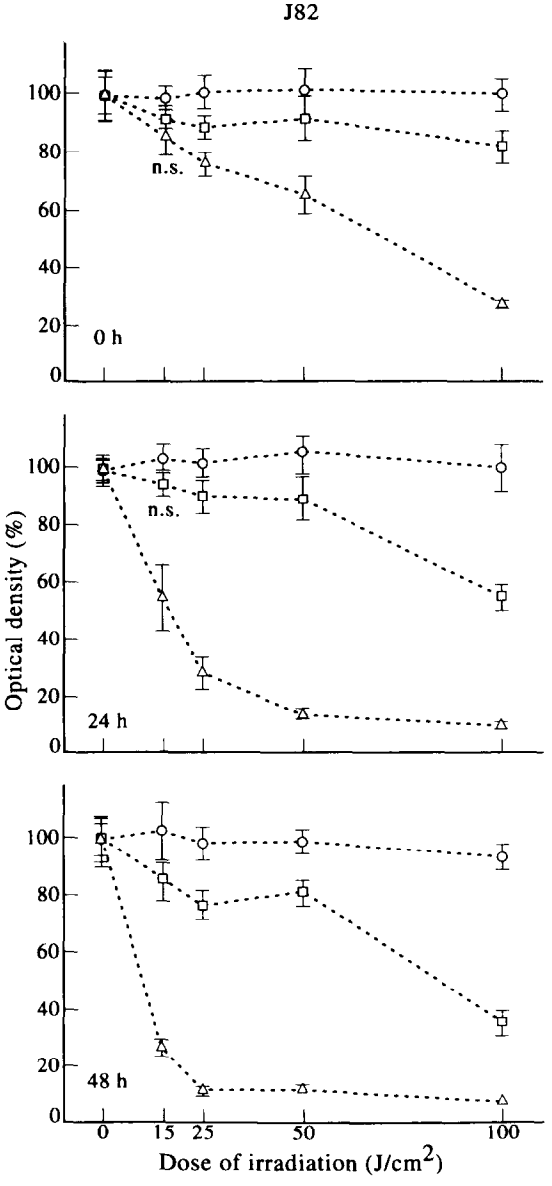

Figure 1. Mitochondria activity of J82 cells in response to ALA application (25–100 μg/ml) and irradiation at 635 nm wavelength (15–100 J/cm^2). ○, 25 μg/ml; □, 50 μg/ml; △, 100 μg/ml. MTT assays were performed immediately, 24 and 48 h after exposure to light. $P < 0.0041$; n.s., not significant. The data along the bottom of the abscissa reflect the limit of the sensitivity of the assay.

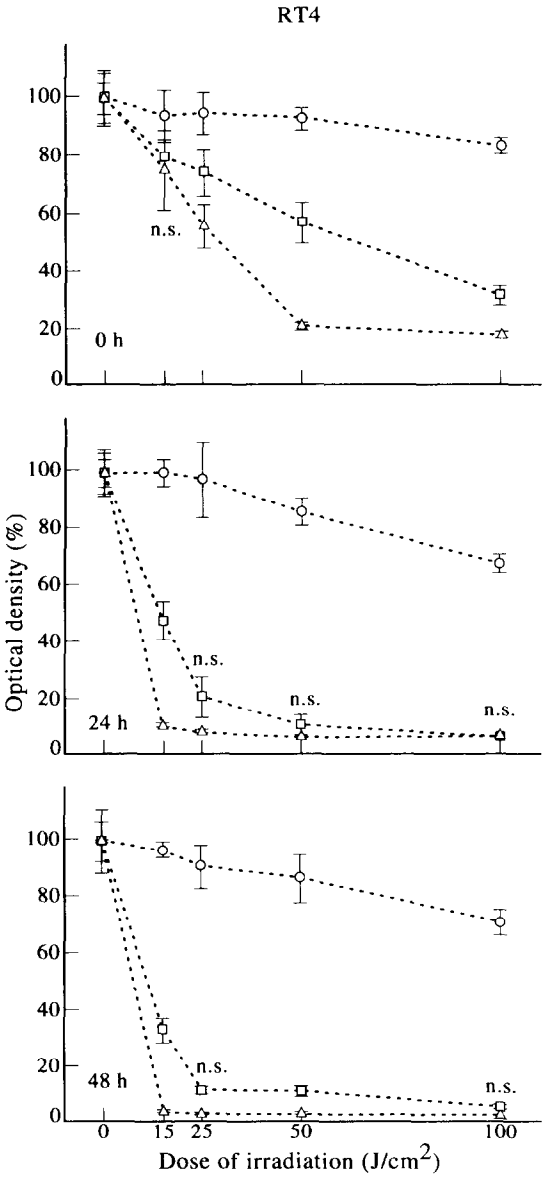

Figure 2. Mitochondria activity of RT4 cells in response to ALA application (25–100 μg/ml) and irradiation at 635 nm wavelength (15–100 J/cm^2). ○, 25 μg/ml; □, 50 μg/ml; △, 100 μg/ml. MTT assays were performed immediately, 24 and 48 h after exposure to light. $P < 0.0022$; n.s., not significant. The data along the bottom of the abscissa reflect the limit of the sensitivity of the assay.

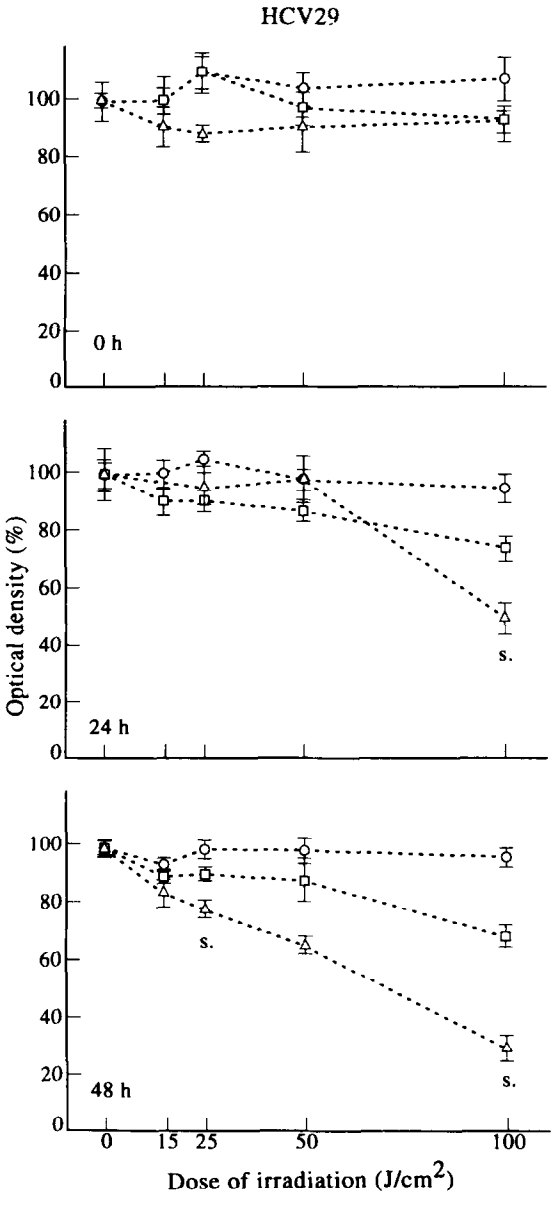

Figure 3. Mitochondria activity of HCV29 cells in response to ALA application (25–100 μg/ml) and irradiation at 635 nm wavelength (15–100 J/cm²). ○, 25 μg/ml; □, 50 μg/ml; △, 100 μg/ml. MTT assays were performed immediately, 24 and 48 h after exposure to light. $P < 0.0005$; s., significant.

24 h after PDT, as described for HCV29 cells using 100 μg/ml ALA and 100 J/cm² light, was obtained at 50 μg/ml ALA and 15 J/cm² light in RT4 cells.

Serum-dependent phototoxicity

The experimental conditions required frequent exchange of media. To maintain stable conditions, the mechanically stressed cells were always cultured in medium supplemented with FBS; with the exception of Hank's buffer during irradiation. Supplementation of medium with 10% FBS promotes cell proliferation and adhesion of monolayer cultures, approximating physiological conditions *in vivo*. Nevertheless, the phototoxic effect was distinctly increased when the 4 h ALA-incubation was carried out in medium without FBS. Twenty-four hours after PDT with 50 μg/ml ALA in FBS-free medium and irradiation at 635 nm wavelength, the activity of J82 cells was reduced to 60% using less than 15 J/cm², whereas 50 μg/ml ALA in FBS-containing medium required more than 100 J/cm² light to achieve the same reduction in cell viability (Figure 4). Iso-effective cell damage required twice as much ALA (100 μg/ml) in medium supplemented with FBS followed by 50 J/cm² compared with incubations in FBS-free medium.

Wavelength and phototoxicity

Synthetic porphyrins in PDT were irradiated most frequently at 630 nm wavelength. However, a red shift of this peak *in vivo* [21] may result in increased phototoxicity after irradiation at 635 or 640 nm. Immediately after PDT on J82 cells with 100 μg/ml ALA, a significant difference in MTT metabolism could be seen using 630 or 640 nm on one hand and 635 nm on the other, applying light doses of 50 and 100 J/cm² (Figure 5). Forty-eight hours after PDT, photoirradiation at 635 nm wavelength was up to 60% more effective than at 630 nm, and 70% more than at 640 nm, respectively, using light doses below 50 J/cm². The differences were significant. Interestingly, irradiation with 630 and 640 nm induced an initial phototoxic effect after 25 J/cm², but, increasing light doses up to 50 J/cm² resulted in less cell damage. This process was intensified within 48 h.

DISCUSSION

Topical ALA-induced PPIX is considered to have clinical value for the treatment of epithelial tumours [22, 23] such as carcinoma *in situ* of the urinary bladder. This emphasises the importance of *in vitro* investigations of PDT using cultured cells to create a basis for the development of new clinical applications, although there are many problems in extrapolating between *in vivo* and *in vitro* experiments. Nevertheless, PDT effects on precancerous lesions or carcinoma *in situ* in the remaining unvascularised epithelium of the bladder after locally applied ALA is perhaps more comparable with isolated cells than with intact vascularised tissues with local differences in oxygenation.

In comparison to the intracellular accumulation of synthetic porphyrins in breast and colon carcinoma cell lines [24], in which maximal values are obtained at approximately 24 h

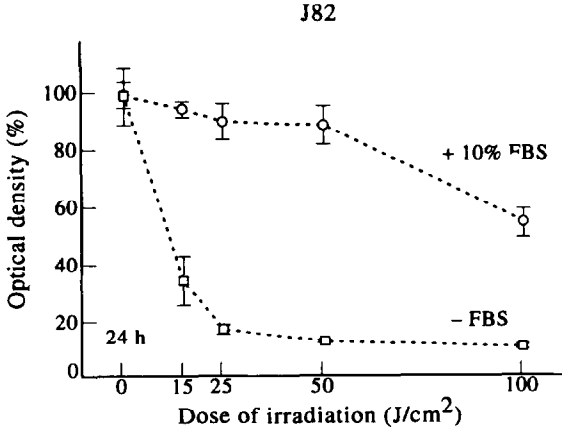

Figure 4. Differences in the inhibition of mitochondria activity of J82 cells after 50 μg/ml ALA incubation in medium with or without fetal bovine serum (FBS) 24 h after irradiation with 15–100 J/cm². $P < 0.004$. The data along the bottom of the abscissa reflect the limit of the sensitivity of the assay.

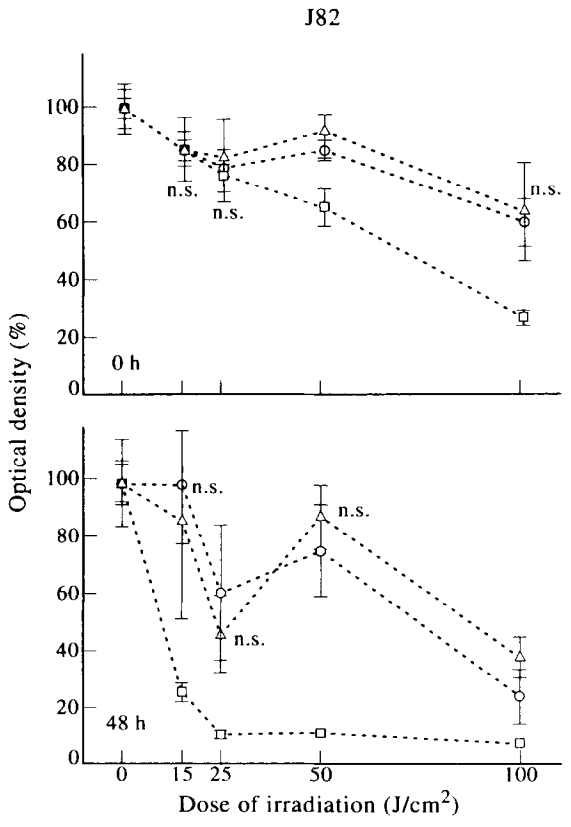

Figure 5. MTT assays of 100 μg/ml ALA incubated J82 cells immediately and 48 h after irradiation at three different wavelengths (630–640 nm) with 15–100 J/cm². ○, 630 nm; □, 635 nm; △, 640 nm. $P < 0.004$; n.s., not significant. The data along the bottom of the abscissa reflect the limit of the sensitivity of the assay.

without a further significant increase in HPD accumulation up to 48 h, the production of PPIX after administration of ALA is maximal up to 4–6 h *in vitro* [25] and *in vivo* [4, 6, 23, 26, 27], in some cell lines by up to 24 h [25]. The fluorescence of ALA-induced intracellular PPIX in J82 and HCV29 cells increased almost linearly up to 4 h without further increase irrespectively of the initial ALA concentrations of 30–300 μg/ml in FBS-containing medium [28]. Therefore, we chose a period of 4 h for the incubation of cells using ALA solution buffered to a pH of 7.0, which is chemically unstable and should be used immediately after it is produced [27]. However, ALA seems to be more stable in low concentrations and a comparison of ALA incubations at different pH-values resulted in maximal intracellular porphyrin fluorescence at physiological pH (data not shown). Additionally, cells can be very sensitive to alterations to their normal milieu.

ALA-induced endogenous photosensitisers are synthesised and accumulated in mitochondria [15, 29, 30]. Hilf and associates [15] demonstrated the functional impairment of mitochondrial dehydrogenases following PDT using haematoporphyrin derivatives. Singlet oxygen, the decisive product of irradiated photosensitisers, must be produced close to the sensitive targets in cells and diffuses over a distance of 100 nm during a lifetime of 1 μs [31]. Electron microscopical studies revealed early irreversible damage of mitochondria even at low power density [25,32]. For this reason, the MTT assay, depending on mitochondrial physiology, seemed to be especially suited for these investigations.

Corresponding results of colorimetric MTT assays and alternative time consuming colony forming assays or dye exclusion assays have been widely described (for review, see ref. [33]). Colony forming assays are restricted to indicate the overall surviving rates of cells exposed to cytotoxic agents. The MTT assay, in contrast, offers the opportunity of establishing time-dependent cytotoxicity by means of mitochondrial enzyme activity [9, 24].

It is of interest to know whether ALA or the induced PPIX is responsible for the observed toxicity threshold. In fluorescence experiments, these three cell lines exhibited a fluorescence saturation of PPIX at about 0.5 mg/ml ALA in FBS-containing medium after an incubation period of 4 h [28]. Therefore, drug toxicity $\geq$ 2.5 mg/ml appears to be attributed to ALA and not to the induced PPIX.

The photoreactive yield is a function of both PPIX absorbance and tissue transmittance. Just below the tissue surface, this yield is about 20% greater for 510 nm light in comparison with 630 nm light; e.g. at a 2.5 mm depth, the value at 630 nm would be greater than that at 510 nm by a factor of 1.5 [34]. Therefore, 630 nm wavelength is conventionally used in PDT with synthetic porphyrins, providing the advantage of a deep tissue penetration due to low competitive absorption of endogenous fluorochromes, such as haem [35]. However, this excitation wavelength of 630 nm is obviously attributed to the fluorescence maxima of PPIX in organic solvents. PPIX dissolved in dimethyl sulphoxide solution has an absorption peak at 629 nm [4], while in methanol it is 632 nm wavelength [21]. The apparent red-shift of approximately 5 nm demonstrated in a rat colonic tumour model by endogenously accumulated ALA-induced PPIX, compared to the spectra in organic solvents, was ascribed to binding with protein substrates, e.g. human serum albumin [4]. Additionally, a comparable effect was observed in the presence of bovine serum albumin [21]. In the present investigation, we experimentally confirmed this observation for the range of 630–640 nm wavelength. Photo-irradiation of 635 nm was up to 60% more effective than at 630 nm (70% at 640 nm) (Figure 5). The unusual shape of the 630 and 640 nm survival curves was similar to that of cells exposed to UVA radiation in the presence of the dihydropyridine calcium antagonist, Nifedipine [36]. This pattern suggests that a toxic photoproduct is formed at 25 J/cm², indicated by an initial toxicity increase with diminished effect after further irradiation.

The different viabilities of HCV29, RT4 and J82 cells, indicated by MTT metabolism following photodynamic treatment, revealed that the malignant cells were more sensitive compared with benign HCV29 cells derived from normal urothelium. The significant differences in cell damage should be attributed to the values of porphyrin fluorescence in ALA-sensitised cells of the respective cell lines since investigations on synthetic porphyrins *in vitro* indicate that intracellular fluorescence intensity may be correlated with prospective phototoxicity [37]. Subconfluent monolayers of J82 and RT4 cells displayed fluorescence intensities 9- and 16-fold higher, respectively, compared with the fluorescence level of benign HCV29 cells [28]. In fact, ratios of the relative optical densities of the individual cell lines 48 h after PDT using 100 μg/ml ALA and 50 J/cm² correspond to the respective ratios of fluorescence intensities.

However, there was no substantial impairment of cell

viability below a threshold of 25 μg/ml ALA, not even at increased light doses. Interestingly, in cells of different cell lines synthesising less than 140 ng PPIX per μg protein following 4 h ALA-incubation, only slight phototoxicity was obtained [25]. Obviously, a minimal amount of intracellular PPIX is necessary to produce phototoxic damage. This is supported by fluorescence experiments using J82, RT4 and HCV29 cells [28].

Clinical application of this technique depends on there being phototoxic damage to neoplastic tissue by means of tumour-selective, ALA-induced PPIX accumulation [4, 38, 39]. Comparison of phototoxic effects on cultured human benign and malignant urothelial cells implies that a "therapeutic window" for malignant cells could be defined without injuring benign cells. However, we found a different dose dependence in both malignant cell lines studied. To produce similar levels of phototoxic cell damage, drug and light doses for J82 cells had to be twice those for RT4 cells. In accordance with other *in vitro* investigations [3, 25], we could not obtain a direct correlation of either the degree of differentiation or the cell doubling time with the phototoxic cell damage.

The reproducibility of the procedure mainly depends on components of the culture medium. As described for haematoporphyrin incubations [30], the presence or absence of 10% FBS in the culture medium resulted in differences in accumulation and retention of PPIX [40] and produced pronounced differences in phototoxic effects. ALA sensitisation in FBS-free medium reduced the viability of J82 cells at low light doses by a factor of five compared with cells in FBS containing medium (Figure 4). This is due to reduced intracellular accumulation of porphyrins and a depletion into the medium [40].

Regarding clinical application of ALA, the described differences in phototoxic effects, dependent on protein binding of PPIX, wavelength, and increasing cell damage within 48 h, may be important factors, encouraging further careful investigations of topical PDT *in vitro* and *in vivo*.

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