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Routine experimental system for defining conditions used in photodynamic therapy and fluorescence photodetection of (non-) neoplastic epithelia

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Abstract. A common method to induce enhanced short-term endogenous porphyrin synthesis and accumulation in cell is the topical, systemic application of 5-aminolevulinic acid or one of its derivatives. This circumvents the intravenous administration of photosensitizers normally used for photodynamic therapy (PDT) of fluorescence photodetection. However, in the majority of potential medical indications, optimal conditions with respect to the porphyrin precursor or its pharmaceutical formulation have not yet been found. Due to ethical restrictions and animal right directives, the number of available test objects is limited. Hence, definition and use of nonanimal test methods are needed. Tissue and organ cultures are a promising approach in replacing cost intensive animal models in early stages of drug development. In this paper, we present a tissue culture, which can among others be used routinely to answer specific questions emerging in the field of photodynamic therapy and fluorescence photodetection. This technique uses mucosae excised from sheep paranasal sinuses or pig bladder, which is cultured under controlled conditions. It allows quasiquantitative testing of different protoporphyrin IX precursors with respect to dose-response curves and pharmacokinetics, as well as the evaluation of different incubation conditions and/or different drug formulations. Furthermore, this approach, when combined with the use of electron microscopy and fluorescence-based methods, can be used to quantitatively determine the therapeutic outcome following protoporphyrin IX-mediated PDT. [DOI: 10.1117/1.1352751]

Keywords: PDT; tissue culture; 5-aminolevulinic acid derivatives; protoporphyrin IX; fluorescence.

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1 Introduction

Following the approval of photodynamic therapy (PDT) as palliative treatment of totally and partially obstructing cancers of esophagus[1] by the US FDA in 1995, research in the field of fluorescence photodetection (PD) and PDT of cancer has been recently given a fresh impetus by several other approvals among which are photodynamic treatment of choroidal neovascularization associated with age-related macular degeneration[2,3] or 5-aminolevulinc acid (ALA)-mediated PDT of actinic keratosis (AK).[4,5] The principle of PD and PDT is based on preferential accumulation of so-called photosensitizers (PSs) in neoplastic tissue and its subsequent irradiation with light of an appropriate wavelength. PS with significant quantum yields for intersystem crossing from the first excited singlet state S_1 to the lowest triplet state T_1, induce cytotoxic effects after interaction with molecular oxygen. Furthermore, the fluorescence of the PS, selectively accumulated in a neoplasm, can be used to visualize otherwise barely visible or invisible lesions. Although most known PS based on a tetrapyrrolic structure that have been tested lack selectivity and thus are nonoptimal in terms of PD, they can still be used for PDT by adapting the manner in which the light is delivered to the lesion and adding the appropriate dosimetry.[6,7]

A more recent strategy involves the exogenous administration of ALA in order to stimulate the intracellular formation of protoporphyrin IX (PpIX). ALA is a naturally occurring intermediate in the heme biosynthetic pathway, which is metabolized to the PD and PDT-active compound PpIX. In most tissues the formation of ALA is the rate limiting step in the

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synthesis of heme and is regulated via a negative feedback control mechanism dependent on the concentration of free heme. Exogenous ALA administration bypasses both the rate-limiting step and the feedback, thereby boosting intracellular accumulation of PpIX. Topical or systemic administration of ALA for PDT or PD has been shown to be a promising approach in this particular field of biomedical optics. Since the pioneering work of Kennedy et al.,[8] this treatment modality has been widely studied clinically in other medical fields such as pulmonology,[9,10] urology,[11–17] gastroenterology,[18] otorhinolaryngology,[19,20] and gynecology[21] for the improved management of cancer. Despite promising results, this methodology often suffers from poor bioavailability of ALA[22–25] caused by its physical–chemical properties.[26] In view of the limited penetration of topically applied ALA and the inhomogeneous distribution of accumulated PpIX, considerable efforts have been undertaken to circumvent these drawbacks by modifying ALA formulations,[27,28] applying physical methods,[29] or chemical derivatization into more lipophilic precursors, i.e., ALA esters.[30–32] However, most formulations of ALA or its derivatives are still far from being adapted to their particular application field. Furthermore, other (e.g., nononcologic) applications,[33,34] will be added to the ''scope of duties'' of ALA-mediated PD and PDT. These will require conditions, to be optimized in realistic preclinical tests. Another factor to be optimized in ALA-based PDT is the choice of the appropriate irradiation wavelength and the optimal irradiation procedure to be used. While for the treatment of AK[4,5] the shallow penetration of blue light seems to be sufficient for effective treatment, other applications require deeper light penetration.

Hence, in order to explore all these parameters, thousands of animal tests would be necessary for a statistically valid analysis of the problem. However, the directive 86/609/EEC on the *Approximation of Laws, Regulations and Administrative Provisions of the Member States Regarding the Protection of Animals Used for Experimental and Other Scientific Purposes*[35] strongly implies the concept of using and developing nonanimal in alternative test methods. In contrast to cell culture models, organ/tissue cultures the original architecture is maintained. These systems are still economical and reduce the use of animals as the samples may be obtained from slaughtered animals.

Recently, we have shown that the use of such tissue cultures can help to considerably accelerate the preclinical development of newly synthesized molecules for the PD and PDT of superficial bladder cancer.[36,32] Here we show how different prerequisites to optimal PD and PDT can be addressed by our experimental *ex vivo* approach. It will also be shown that this method can be used to cultivate tissue from other epithelial origins, such as sheep paranasal mucosa, in which PpIX fluorescence can be generated following exposure to ALA or one of its derivatives. Dose-response curves as well as pharmacokinetics will be established for this type of epithelium. Furthermore, the current approach was used to explore possible experimental setups, defining more quantitatively other relationships between parameters, which are important with respect to PpIX mediated PDT and PD of superficial human bladder cancer, such as *p*H value, irradiation wavelength, and ALA derivative.

1 Materials and Methods

2.1 Chemicals and Solutions

ALA was purchased from Merck (Dietikon, Switzerland). Butyl-(b-ALA) and hexyl-ester (h-ALA) were synthesized as described previously.[32] Appropriate quantities of ALA or one of its derivatives were dissolved in 5 mL of Tyrode solution (see below) to yield final concentration between 1 and 40 mM. These solutions were adjusted to *p*H 5.3 or 6.4 if necessary. Glutaraldeyde and OsO_4 were obtained from Fluka (Buchs, Switzerland). Stock solutions of Sytox-Green (Molecular Probes, Eugene, OR) in DMSO (5 mM) were stored at 4 °C. Prior to incubation they were diluted with Tyrode solution to give a final concentration of 1 μM.

2.2 Preparation of Pig Bladder and Sheep Paranasal Mucosa

Porcine bladders and sheep paranasal mucosae were excised from slaughtered animals. Directly after slaughtering, the tissues were stored at 4 °C in a Tyrode solution containing (in mmol/L): 143.0 Na^+, 2.0 K^+, 0.8 Mg^{2+}, 1.4 Ca^{2+}, 122 Cl^-, 20.0 HCO_3^-, 3 $H_2PO_4^-$, 1.2 SO_4^{2-}, 8 glucose (osmolarity $=$290 mO sm) until use ($\sim$1–6 h postexcision). This solution was saturated with gas mixture containing 95% oxygen and 5% CO_2 (*p*H 7.5). The mucosae were microdissected from the underlying tissue and cut into 7×7 mm fragments, which were mounted in a transparent culture chamber designed for epithelia.[37,38,36] The mucosa divided the chamber into apical (epithelial) and basal (submucosal) compartments. The chamber was fixed onto the plate of an epifluorescence microscope (Leitz Orthoplan) and thermostabilized at 37 ± 0.5 °C. The basal compartment was continuously perfused by oxygenated Tyrode solution. Four mucosae were prepared for each set of conditions and measurements were performed at five spatially separated points on each sample.

2.3 Spectrofluorometry

PpIX accumulation as a function of precursor concentration, time of administration and *p*H value was evaluated by means of the fluorescence intensity at 635 nm. The mucosa was periodically excited by violet light of a filtered 100 W mercury arc lamp [Eppendorf 405 nm (full width half maximum: 10 nm)] at 405 nm. The fluorescence emitted by the cells, taken to be proportional to the cell PpIX concentration, was passed through a low pass filter ($\lambda>610$ nm) and a spectrograph (continuous interference filter Veril, Leitz) and subsequently recorded by an EMI 20 photomultiplier tube. The specificity of the fluorescence signal was systematically checked by analyzing the emission spectra. In order to correct the obtained PpIX fluorescence intensities for day-to-day fluctuations of the excitation light or in the emission pathway, the fluorescence intensity at 635 nm of a solution of Rhodamine 101 ($c=1\times10^{-6}$ mol/L) served as reference.

2.4 Photodynamic Therapy

After incubation with solutions of h-ALA (2 mM) and ALA (180 mM), respectively, to give equal concentrations of PpIX, the mucosa was irradiated at four distinct sites with 405 nm light (20 mW/cm^2) coming from a filtered 100 W mercury arc

lamp or with the white light from an unfiltered Storz D-Light system (Storz, Tuttlingen, Germany) at 20 mW/cm^2 delivered through the ocular of the microscope.

2.5 Fluorescence Microscopy

Two h after irradiation, tissue samples incubated with Sytox-Green solution (1 μM) for 15 min were mounted on a microscope cover slide. Fluorescence imaging was performed with an air-cooled, slow-scan 16 bit-charge coupled device (CCD) camera (EEV P86231, Wright Instr., Endfield, UK) fitted to a fluorescence microscope (Olympus, BH2-RFC) equipped with an Olympus objective (S. Plan Apo 4×, and 10×). Fluorescence excitation controlled by an excitation shutter (Model Uniblitz Driver D122, Vincent Acc., Rochester, NY) was provided by a 100 W mercury arc lamp filtered by an interference filter (λ_{ex}=420–490 nm). Exposure times were controlled by an electronic shutter and set to 1 s. An Olympus microscope excitation ''cube B'' and a long-pass filter (RG 610, Schott, Mainz, Germany) were used to suppress excitation light and record PpIX fluorescence. For Sytox-Green fluorescence recording, a band-pass filter (λ=540–580 nm) was placed in the emission pathway instead of the long-pass filter.

Digital imaging, data display, and storage were performed with an IBM compatible PC connected to the CCD using the AT1 software (Wright Instruments, Endfield, UK).

2.6 Scanning Electron Microscopy

Samples of irradiated bladder mucosa preincubated with h-ALA (2 mM, for 2 h) were fixed overnight with 2% of glutaraldehyde in phosphate buffered saline (PBS). After three washings in PBS, they were dehydrated through 70%, 80%, 90%, and 100% of ethanol and then postfixed with OsO_4 (1%) dissolved in PBS. The samples were then freeze dried with a critical point dryer (CPD 030, Balzers, Lichtenstein) coated with gold (S150 sputter coater, Edwards, Zivy, Basel) and studied by scanning electron microscope (JEOL, Tokyo). Micrographs were recorded on a Kodak Tri-X-Pan (6×7 cm) roll film.

3 Results and Discussion

3.1 PpIX Accumulation in Sheep Paranasal Mucosa

Improved PpIX synthesis induced by the topical application of ALA esters has so far been reported with creams containing ALA methylester (m-ALA) for the therapy of basal cell carcinoma and AK[39] and solutions containing h-ALA for the PD of superficial bladder cancer.[32] Thus, it can be expected that more lipophilic ALA derivatives will also be considered for the use in other medical applications. However, prior to clinical application of such derivatives, extensive testing is necessary to first determine the most promising derivative for the particular application and second to investigate the optimal conditions with respect to the drug formulation. Besides PD and PDT of Barrett's Esophagus, the PpIX mediated PD of early human lung cancer seems to be one of the potential application fields of ALA derivatives. Hence, we exposed freshly excised sheep paranasal mucosae to different ALA esters. Figure 1 shows the PpIX fluorescence intensities relative to the reference obtained in the mucosae as a function of precursor concentration after 3 h. For each ALA derivative the well-known ''bell-shaped'' dose-response curves were obtained. However, the position of fluorescence maxima as well as the total amount of PpIX produced are different as compared to those obtained in porcine urothelia.[36] First, the optimal concentrations for each precursor, where optimal PpIX synthesis occurs, are shifted toward lower concentrations. Second, total fluorescence intensities are smaller. Furthermore, although the fluorescence intensities obtained after incubation with h-ALA are slightly higher as compared to fluorescence intensities with ALA, these differences are not as significant as observed in the case of the urothelia.[36,32] This can probably be attributed to structural differences in the two types of epithelia. While the mucosa of the bladder consists of a lamina propria and a transitional epithelium, which is 5–6 cells in thickness, the respiratory epithelium generally contains only a single layer of cells. Thus in contrast to the bladder epithelium, deeper cell layers cannot contribute to the PpIX fluorescence and the higher tissue penetration capacity of h-ALA with respect to ALA is less important.

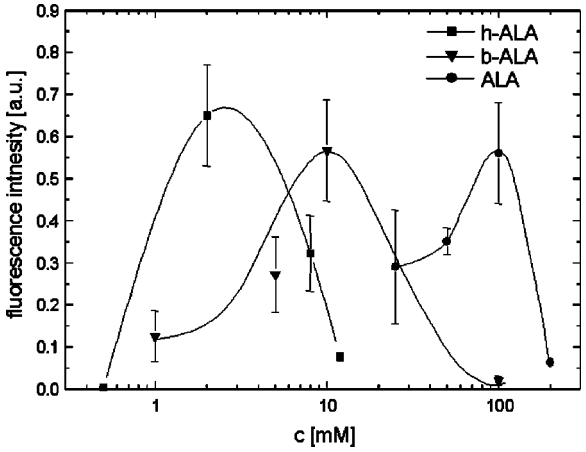

Fig. 1 Concentration dependence of PpIX accumulation in sheep paranasal mucosa after 3 h of incubation with ALA (-●-), b-ALA (-▼-), and h-ALA (-■-).

In Figure 2 the PpIX fluorescence intensity during incubation with ([h-ALA]=2 mM) is plotted against the time. In contrast to the results obtained with the pig bladder mucosa

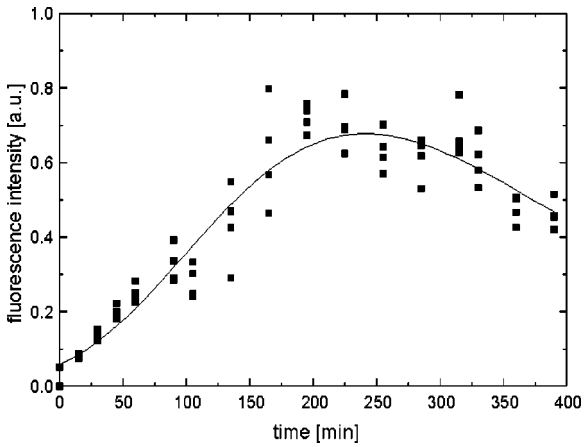

Fig. 2 Pharmacokinetics of PpIX synthesis in sheep paranasal mucosa treated with h-ALA (2 mM).

(see below), continuously increasing PpIX fluorescence was not observed. The highest fluorescence intensity was observed after 3–4 h of continuous incubation. These experiments demonstrate that even very delicate epithelia can be used in our incubation chambers to simulate *in vivo* conditions with respect to PpIX generation. This approach may accelerate the screening procedure for new ALA derivatives in their way to clinical use. Slight modifications of this approach will be used to help to optimize formulations used for the inhalation of ALA or one of its derivatives for PDT and/or photodetection in the bronchi. For these purposes the quartz window of the upper part of the perfusion chamber can be removed, allowing exposure of the respiratory epithelium to a humidified atmosphere. The use of tissue/organ culture also enables us to take into account other environmental factors such as excretion of mucus delivered by goblet cells and glands, which is absent in cell cultures or cocultures.[40] Moreover, the influence of the chemicals under investigation on ciliated cell activity can be investigated in more detail.[37]

In addition, we expect that our model will help in testing different conditions for the improved photodetection of ovarian cancer, thereby reducing the number of animals needed to obtain statistically valid results. Major et al.[41] recently presented a cancer animal model with pathogen free rodents expressing a high number of metastatic ovarian cancer nodules. Apart from nodules observed in the peritoneum, ovarian micrometastases were also observed in the omentum and the bowel mesenterium. However, for each condition at least 4–5 animals were sacrificed. It should be realized that tissue from this origin is rather easy to prepare for tests in our incubation chamber, and many conditions can be tested with one animal under controlled exposure conditions.

3.2 Influence of Total Incubation Time on PpIX Accumulation

Under standard cystoscopic conditions flat multifocal bladder lesions such as carcinoma *in situ* are often barely visible. Hence, complete surgical resection of these lesions is difficult, and may result in higher recurrence rates. The use of ALA-mediated PpIX combined with fluorescence imaging now appears as a reasonable approach to attain essentially complete surgical tumor resection.[11–17] In clinical studies sensitivities between 77% and 97% and specificities ranging from 57% to 67% have been reported. Studies performed by Jichlinski et al.[11] and Koenig et al.[15] suggest that longer time intervals between the instillation of the drug and the start of the fluorescence cystoscopy lead to more detectable neoplastic lesions per patient. Another factor that might influence the sensitivity as well as the specificity of this method is the total time of drug exposure. In clinical trials this time varies between 10 and 420 min but is on average 120 min.

Recently, we have shown that the use of more lipophilic derivatives of ALA, notably the ALA hexylester, can considerably decrease the time of instillation as well as the time between instillation and fluorescence detection.[32] It has been shown that one rate-limiting step in the biosynthetic pathway of heme is mediated by the enzyme porphobilinogen deaminase. Thus, a large pool of the substrate, provided by a rapid passive diffusion through the cellular membrane,[42] is sufficient to maintain a high level PpIX synthesis over a long

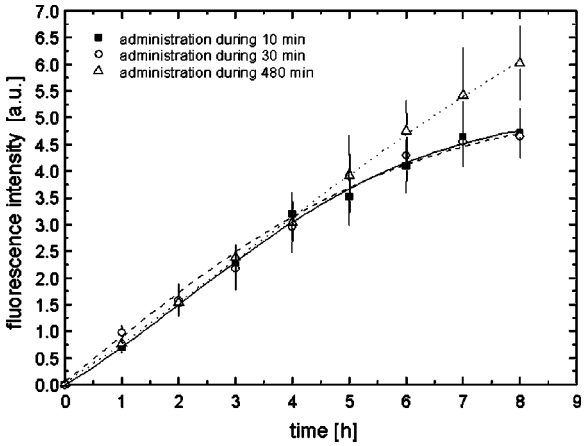

Fig. 3 Pharmacokinetics of PpIX synthesis in pig bladder mucosae after exposure to h-ALA (4 mM) during different time periods: (△) 480 min,; (○) 30 min; (■) 10 min.

period. In order to test the conditions allowing significant reduction of total instillation time and hence an increase of the comfort for patients, we exposed the microdissected pig urothelia for 10 and 30 min, respectively, and followed the PpIX generation by means of fluorescence spectroscopy for 8 h. The resulting fluorescence intensities were compared to those obtained after continuous exposure to h-ALA solutions. The results of these studies are plotted in Figure 3. In all cases the PpIX fluorescence intensity increased in a sigmoidal way as a function of time. No significant differences were observed during the first 3 h of PpIX synthesis, but the PpIX production significantly slowed down after 5 h. Instillation for 30 min has shown a similar picture but with a slight decrease in PpIX formation capacity 5 h after the start of incubation.

Quantitative analysis of the fluorescence spectra after bladder instillation in patients with 50 mL of solutions containing 100 mg h-ALA ([h-ALA]=8 mM) showed that incubation for 2 h with subsequent evacuation of the bladder contents gave higher PpIX fluorescence values as compared to 4 h of continuous exposure to the drug.[32]

The use of slightly higher drug doses than the ''optimal dose,'' i.e., the dose determined by means of our pig bladder model, was necessary in order to compensate for the dilution of the drug due to urine excretion by the patients. However, too high h-ALA doses have been shown to slow down PpIX formation *in vitro*[26,31] and *in vivo*.[32] Thus, evacuation of the product after 2 h avoids exposure of the bladder wall to elevated drug doses and takes into account both the dilution of the compound and its cytotoxic potential.

From Figure 3 it can be seen that even after a short exposure to the prodrug, a continuous PpIX formation can be observed. In contrast to observations *in vivo*, no augmentation of PpIX fluorescence was observed when compared to a continuous application of the drug. One should keep in mind that, in contrast to the clinical practice, the pig bladder was incubated under optimal conditions, determined for minimal toxicity and optimal PpIX synthesis. In order to determine whether or not there will be a different PpIX formation using the clinically established 2+2 concept, the pig bladder should be exposed to slightly higher than optimal drug doses.

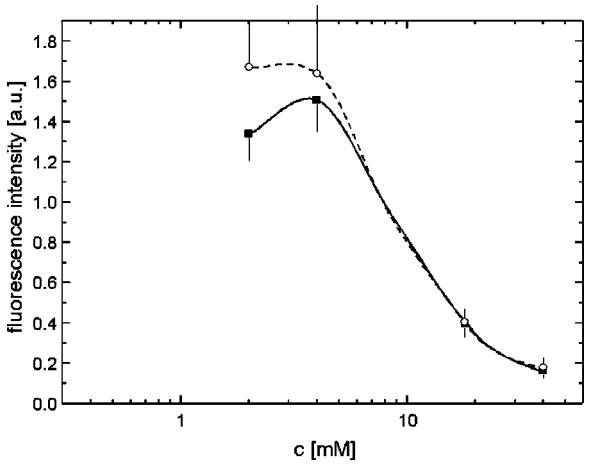

Fig. 4 Concentration dependence of PpIX accumulation in pig bladder mucosae after 2 h of incubation with h-ALA solutions at different *p*H values: (■) *p*H 5.3; (○) *p*H 6.4.

3.3 Influence of *p*H Values on the PpIX Accumulation

Several authors have described the influence of the *p*H value on PpIX synthesis *in vitro* for both ALA and its derivatives.[26,43–45] It was found in different cell lines that PpIX was induced most efficiently when physiological *p*H values were applied. However, due to the instability of ALA in solution under such conditions,[46] applying acidic ALA solutions (*p*H 5.3) for the photodetection of superficial bladder cancer was recommended. Hence, we exposed excised mucosae to different prodrug concentrations using two different *p*H values. This allows us to determine if the *p*H value has an impact on the PpIX production in an intact cellular environment consisting of multiple cell layers, extracellular matrix components, and a submucosa compartment adjusted to *p*H =7.4. From Figure 4 it can be seen that after 2 h of incubation with suboptimal drug concentrations of h-ALA, a significant increase in PpIX generation efficiency with increasing *p*H values can be observed. This is in agreement with previous studies we performed of cell lines derived from a human transitional cell carcinoma of the bladder.[26] The cells were exposed to only half of the optimal concentration in order to avoid a saturation of the biosynthetic pathway. However, compared to cell culture experiments where PpIX synthesis was found to be more than doubled at *p*H=6.4 as compared to *p*H=5.3, this effect was less notable in excised pig bladder mucosae. This can be attributed to the fact that in human bladder even acidic conditions can be considered as physiological *p*H.[46] In this sense the tissue culture approach is closer to the clinical aspects than cell culture experiments. The close relationship between tissue cultures and the clinical photodetection of early human bladder cancer is further demonstrated in Table 1, where optimal concentrations (c_{opt}) of h-ALA solutions at *p*H of 5.3 were assessed in two cell lines of cancerous origin as well as the *ex vivo* pig bladder, and in a clinical pilot study.[32] While the c_{opt} for both cell cultures deviated by more than 1 order of magnitude from clinically determined c_{opt} values, c_{opt}, determined *ex vivo* is only two times lower than optimal conditions determined *in vivo*. Dilution effects as described above can explain this slight difference.

Table 1 Optimal concentration of h-ALA as determined by means of maximal PpIX generation after two hours of exposure to the prodrug at *p*H=5.2.

Test system	J82 cells (*in vitro*)	T24 cells (*in vitro*)	Pig bladder mucosae (*ex vivo*)	Human bladder (*in vivo*)
$[h\text{-}ALA]_{opt}$/mM	0.3[a]	0.2[a]	4[b]	8[c]

[a] Data from Ref. 26.

[b] This study.

[c] Data from Ref. 32.

The long-term impact of *p*H on the PpIX accumulation in intact epithelia was studied by incubation of microdissected pig bladder at c_{opt} and measuring the PpIX fluorescence at 635 nm every hour for 8 h. For both incubation conditions, a sigmoidally increasing PpIX fluorescence was observed and no significant differences between incubation at *p*H 5.3 and *p*H 6.4 were noted (Figure 5). From these experiments it can be concluded that, at least for urological purposes and topical drug application, the *p*H value will be of minor importance if the PpIX precursor remains stable under such conditions.

3.4 PDT on the Pig Bladder Mucosa

A priori, PDT represents a simple treatment modality. It comprises the administration of a photosensitizer and subsequent irradiation by light of an appropriate wavelength. In reality, the therapeutic value of PDT is influenced by complex interactions of multiple parameters such as photosensitizer concentration in tissue and its localization, photosensitizer selectivity, irradiation wavelength, fluence rate, tissue optical properties, tissue metabolic conditions, delay between drug administration and irradiation, and many others. Thus for each new photosensitizer, the best combination of the abovementioned parameters should be determined as closely as possible prior to clinical use. For this purpose, a routine and reproducible model that delivers as quantitative information as possible is essential. In general, this requires screening

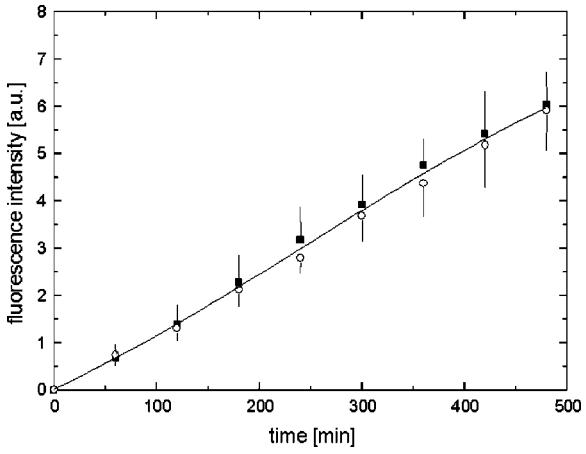

Fig. 5 Pharmacokinetics of PpIX synthesis in pig bladder mucosae after exposure to h-ALA solutions (4 mM) at different *p*H values: (■) *p*H 5.3; (○) *p*H 6.4.

Fig. 6 Reconstituted fluorescence image obtained 3h after PpIX fluorescence and Sytox Green fluorescence imaging (Bar-500 μm); spot A: irradiation with 0.1 J/cm^2 at 405; spot B: irradiation with 0.5 J/cm^2 at 405 nm.

with animal models, which are adapted to the particular clinical problem. As mentioned above, tissue culture models may help in reducing the necessary number of animal tests.

3.4.1 *Determination of Cell Death*

Cell death is the end point of PDT and several methods, allowing the evaluation of this cellular event, have been described in the literature. They are based on morphological evaluations, including light or electron microscopy.[36,47,48] fluorescence-based methods such as the use of propidium iodide[49,50] (which tests for cell permeability) and bioluminescence methods.[51] In this work, we used scanning electron microscopy (SEM) and Sytox-Green-based fluorescence for the quantitative evaluation of h-ALA mediated phototoxicity.

The first evidence of cell membrane rupture was observed about 90 min following PDT as confirmed by Sytox Green fluorescence (data not shown). Figure 6 shows the reconstituted fluorescence image obtained 3 h after PpIX irradiation and Sytox Green fluorescence imaging. The two fluorescent green spots, surrounded by nonbleached areas of characteristic red PpIX fluorescence, were irradiated with different light doses at 405 nm [0.1 J/cm^2 (spot A); 0.7 J/cm^2 (spot B)]. The diameter of the irradiated areas was determined to be 450 $\pm$50 μm depending on the light dose. Less fluorescence intensity was observed in the center of the areas, irradiated with higher light doses. This effect was attributed to the desquamation of dead superficial cell layers following PDT before incubation with Sytox Green. Thus, deeper layers are out of the focal plane of the microscope and the fluorescence resulting from these regions contributes less to the total fluorescence intensity.

Figure 7(A) compares the Sytox-Green fluorescence image of urothelial cell death to a typical SEM micrograph [Figure 7(B)] under the same irradiation conditions (light dose=0.05 J/cm^2 at 405 nm). In the center of Figure 7(B), cells with morphologically changed membranes surrounded by apparently undamaged cells can be seen. On closer inspection [see arrows in Figure 7(B)], areas where the superficial cell layer was removed due to photodynamic action (deeper cells with injured cell membranes) are visible. The diameter of the total affected area was determined to be 350$\pm$50 μm, which was slightly smaller than that determined by fluorescence-based methods. This fact can be ascribed to a higher sensitivity of the fluorescence-based methods since small areas of cell membrane rupture in the outer part of the irradiated area invisible under SEM, and will easily be penetrated by Sytox Green. However, both methods can be used to determine PDT-induced cell death in living tissue.

3.4.2 *Influence of the PpIX Precursor*

In the case of highly selective photosensitizer accumulation into the target tissue, irradiation conditions must be chosen which ensure the destruction of the entire lesion. The high light attenuation by endogenous chromophores, such as flavines or hemoglobin at short wavelengths, restricts the propagation of blue light to only superficial tissue layers. Hence, for the PDT of bulky or nodular lesions more penetrating red/near infrared excitation is required.[52] At present, red light at sufficient fluence rates can only be obtained from relatively high-cost laser sources, which at times may be difficult to handle in clinical practice. A low-cost alternative is the irradiation of target structures with white light, which can be absorbed by the accumulated photosensitizer.

The distribution of PpIX following incubation with both ALA and h-ALA has been measured *ex vivo*[36] and *in vivo* by fluorescence-based methods. In contrast to h-ALA-induced PpIX, a more or less inhomogeneous distribution was observed when ALA was used as substrate. In this case, only small quantities of PpIX were detected in tissue layers near the basement membrane. It was presumed that these small amounts of PpIX, induced by hydrophilic ALA, would not be sufficient for an effective treatment on the basis of papillary bladder tumors. No evidence of cell membrane rupture was observed when incubating the mucosae with h-ALA or ALA alone. Irradiation of microdissected urothelia, preincubated with h-ALA or ALA, always resulted in damage of the tissular network. Using white light and preincubation with h-ALA generally resulted in a complete epithelial destruction yet sparing the basement membrane [Figure 7(C)]. The latter can be explained by very small quantities of the photosensitizer within the basal lamina as determined by Marti et al.[36] While white light was allowed to efficiently destroy the entire urothelium down to the basement membrane when pig bladder mucosae were incubated with h-ALA, similar irradiation

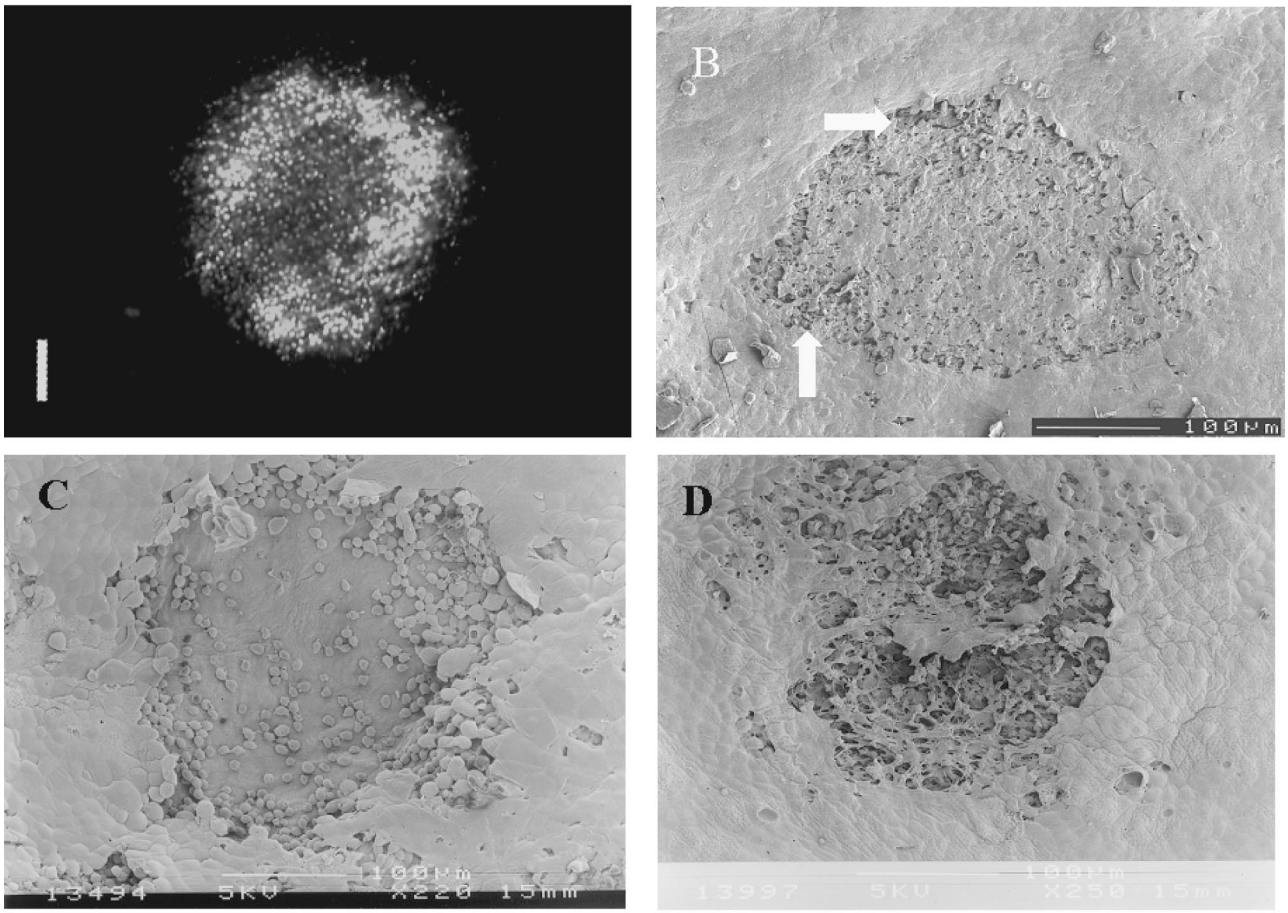

Fig. 7 Sytox Green fluorescence image (A) and SEM micrograph (B) after irradiation with 0.05 J/cm^2 at 405 nm SEM micrographs and Sytox-Green fluorescence images of pig bladder mucosa after incubation with PpIX precursors: (A) Sytox Green fluorescence image after irradiation with 0.05 J/cm^2 at 405 nm; (B) corresponding SEM micrograph after irradiation with 0.05 J/cm^2 at 405 nm (arrows indicate areas where the superficial cell layer was removed due to photodynamic action, deeper cells with evidentially injured cell membranes are visible); (C) 5 h of incubation with h-ALA, irradiation with white light, and a light dose of 2 J/cm^2 after 3 h of incubation; (D) 5 h of incubation with ALA, irradiation with white light, and a light dose of 2 J/cm^2 after 3 h of incubation.

conditions with ALA as PpIX precursor were less efficient. Figures 7(C) and 7(D) clearly demonstrate the differences in the therapeutic outcome when using PpIX precursors of different lipophilicity. The spots in the irradiated areas after incubation with ALA and subsequent irradiation with white light always showed higher heterogeneity as compared to those observed after incubation with h-ALA. Moreover, in all of the conditions tested, the destruction was mostly confined to the superficial urothelial cell layers.

5 Conclusions

The present studies demonstrate the usefulness of the tissue culture approach for screening of fluorescent tumor markers or photosensitizers, in particular of the 5-ALA derivative-induced PpIX. The epithelial cultures are suitable for measuring the relative fluorescence intensities of fluorescence markers using different incubation conditions. The fluorescence intensity as a function of time and concentration of PpIX precursors can be easily followed. The combination with complementary techniques has been shown to be able to respond to major questions in PDT and PD of malignant and nonmalignant superficial lesions.

The use of this tissue culture model can help in reducing the number of animal studies considerably because it can: (a) replace animals in a first stage of a drug developing progress; and (b) test several conditions with the tissue samples obtained from only one animal. It has been shown to deliver highly reproducible results and is easy to handle.

The results shown here mostly document the feasibility of using tissue culture models for studying the pharmacokinetics and PDT effects of new compounds under controlled and well-defined conditions. Additional experiments are in progress to extend the present model to human tissue samples and biopsies also containing pathological cells.

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