Fluorescence microscopy studies on ALA sensitized tissues

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Abstract

Fluorescence microscopy has the potential to study the spatial distribution of photosensitizers in tissue samples with cellular or subcellular resolution. A fluorescence microscope was developed to study the distribution of photosensitizer in tissue samples by acquiring fluorescence images in various spectral ranges and spatially resolved fluorescence spectra both from identical samples. Both methods provide complementary information, since the fluorescence images show the distribution of the sensitizers with a high spatial resolution whereas spatially resolved fluorescence spectra can identify the sensitizers and separate their fluorescence from background light emission by the spectral shape of the fluorescence.

Protoporphyrin IX (PPIX) distribution induced by 5-aminolevulinic acid (ALA) was studied by fluorescence microscopy in basal cell carcinoma (BCC) and in cervical intraepithelial neoplasia (CIN). In an attempt to understand the varying success in treating BCC with topically applied ALA the PPIX distribution was studied in BCC samples of 10 patients. A strong fluorescence was observed in tumor cells as well as in epidermis, sebaceous glands, and hair follicles. The depth of PPIX sensitization of the BCCs ranged from 0.4 to 3 mm and the ratio of tumor versus epidermal fluorescence of uninvolved skin was near one. In the BCCs an uneven sensitization with a lower fluorescence in the center of the tumor was often observed. Samples of the cervical mucosa also showed PPIX fluorescence in the endothelial layer, the malignant tissues and the glands. No increased fluorescence of the dysplastic lesions compared to the epithelium was observed.

1. Introduction

Since photodynamic therapy (PDT) is now approved for certain superficial tumors in several countries (e.g. Canada, USA, Japan, and the Netherlands) [1], there is intensive research in new photosensitizers and in new applications of PDT including non-tumor applications such as the treatment of ocular neovascularization [2] and rheumatoid arthritis (rA) [3, 4]. Beside therapeutic applications, fluorescence diagnosis of early cancer with photosensitizers, especially with 5-aminolevulinic acid (ALA)-induced Protoporphyrin IX (PPIX), is very promising [5]. Both PDT and fluorescence diagnosis rely on a selective accumulation of the photosensitizer in certain tissue structures. Therefore methods for measuring photosensitizer accumulation in tissue with a high spatial resolution are necessary to improve the current procedures and to develop new applications for PDT and fluorescence diagnosis. Radioactive labeling of the sensitizer, extraction with certain solvents, in-situ fluorescence measurements with a fiber spectrometer and fluorescence microscopy can be used to determine the distributions of photosensitizers in tissues. Of these, the only method which can be applied in vivo having a sub-millimeter resolution is fluorescence microscopy of tissue sections.

One has to be careful to interpret the measured fluorescence intensity in terms of photosensitizer concentration because of site-dependent quenching of the fluorescence, but in several experiments

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linear relationships between fluorescence and concentration in certain tissues were shown [6, 7, 8]. In addition the concentration of the photosensitizer might not predict the phototoxicity accurately, since singlet oxygen production and the resulting biological effects depend on the intracellular location of the sensitizer [9]. In contrast to fluorescence measurements with a fiber spectrometer probing large tissue samples the signal measured with fluorescence microscopy is not influenced by scattering and absorption of the tissue.

Fluorescence microscopy allows the acquisition of fluorescence images at different wavelengths and also the measurement of fluorescence spectra with a high spatial resolution. Recording complete emission spectra gives a good discrimination of the fluorescence of different molecules, such as endogenous molecules of the tissue, the photosensitizer, and its photoproducts. Fluorescence images can identify fluorescing structures by their shape and allow a correlation with histology. Ideally a system which records a 2-dimensional array of about 500 x 500 emission spectra with a good spatial resolution would be used. Recently such systems, which use interferometric fourier-transform techniques, have become available, but spectral resolution and sensitivity of these systems are still limited [10].

The experiments reported in this paper were performed with a fluorescence microscope equipped with a slow-scan CCD-camera. With this system fluorescence images and spatially resolved emission spectra can be acquired consecutively from the same sample. The accumulation of ALA-induced PPIX was studied in human skin and the mucosa of the portio uteri in conjunction with the photodynamic therapy of basal cell carcinoma (BCC) and fluorescence diagnosis of cervical intraepithelial neoplasia (CIN).

The photodynamic therapy of basal cell carcinoma (BCC) was introduced by Kennedy and Pottier [11]. Although at first impressive results were reported, the rates of complete responses varied between 50% [12] and 100% [13] in studies published in the last years. Since the recurrence rates of conventional surgery are less than 10% [14], the PDT treatment has to be improved. Therefore fluorescence microscopy was used to study the sensitization of solid and superficial BCC after 4 - 6 hours of ALA application. The study should give information about the depth and selectivity of the BCC sensitization and the PPIX bleaching during irradiation.

Cervical intraepithelial neoplasia (CIN) can be diagnosed by cytological testing (PAP test) and by colposcopy. In a small study the potential of topically applied ALA for the fluorescence diagnosis of CIN was investigated in order to increase the specificity of CIN diagnosis and to determine the exact localization of the dysplasias. Parallel to this study fluorescence microscopy was performed with tissue samples obtained after fluorescence diagnosis in order to investigate the microscopic distribution of the PPIX-fluorescence.

2. Materials and Methods

2.1. Fluorescence Microscope

A fluorescence microscope (Leitz Aristoplan) fitted with a filtered Hg-lamp for excitation and a slow-scan CCD-camera (Spectroscopy Instruments) was used for all experiments [Fig. 1]. The fluorescence images of the microscope can either be directed to the light sensitive area of the CCD-camera or to the entrance slit of a spectrograph (Jobin Yvon), which is corrected to give an astigmatism-free image of the entrance slit. Since the resulting set of spectra - each spectrum is created by one point in the sample - is imaged onto the CCD-camera, a set of spectra belonging to one line in the sample can be recorded simultaneously. By using two moving mirrors the user can switch between images and spectra within seconds. Both spectra and fluorescence images are corrected for background and

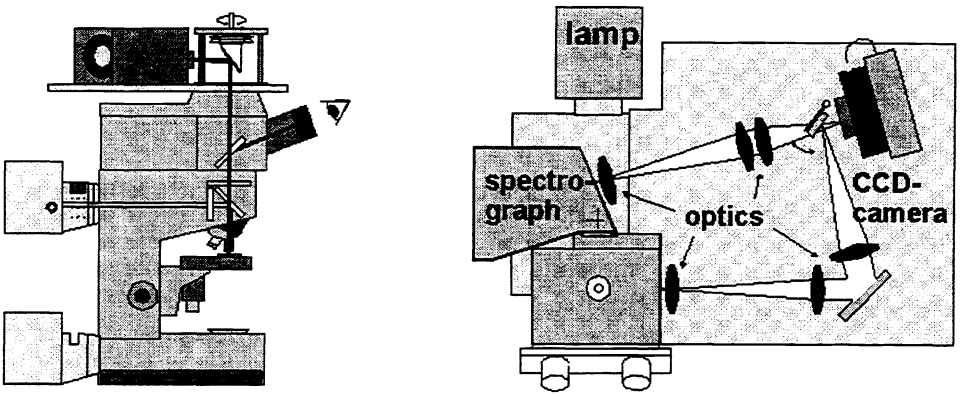

Fig. 1: Setup of the fluorescence microscope, which allows acquisition of fluorescence images and spatially resolved spectra of the same sample.

spatially nonuniform excitation. Additionally the emission spectra are corrected for the spectral sensitivity of spectrograph and CCD camera. The samples were excited in the Soret band of PPIX (400 - 450 nm). Fluorescence images were recorded in two spectral ranges from 500 - 550 nm and above 600 nm in order to image the green autofluorescence of the tissue and the red fluorescence of PPIX. By scaling the green image with an appropriate factor and subtracting it from the red image autofluorescence can be removed from the fluorescence image.

2.2. Preparation of the samples

Tissue samples were removed under dim light in order to prevent photobleaching of PPIX. All further handling, including preparation of the cryo sections and fluorescence microscopy, was performed under the lowest possible light. The absence of the 670 nm band of the photoproduct of PPIX proves that sample preparation is possible with minimal photobleaching (see Fig. 5).

Samples were frozen immediately in liquid nitrogen and stored at this deep temperature until cryo sections of 10 and 20 μm for fluorescence images and 50 μm for fluorescence spectra had been prepared. The samples were afterwards stained with hematoxilin-eosin.

For the fluorescence microscopy of basal cell carcinoma ALA was dissolved in an emulsion of oil in water (Doritin®, 20% w/w). This ointment was applied to solid and superficial BCCs with occlusion, which kept the lesions in the dark. After 4 - 6 hours biopsies were taken. These biopsies were deep frozen in liquid nitrogen.

ALA in a methyl-cellulose gel (5% w/w) was applied to the ectocervix of patients scheduled for a conization after a positive PAP smear test and colposcopy. Under the general anesthesia necessary for the conization fluorescence images of the portio uteri were taken in two spectral ranges. Between 500 and 570 nm the green autofluorescence of the tissue and around 635 nm the red PPIX-fluorescence were recorded. Fluorescence was excited between 380 and 480 nm by a filtered flash lamp. Small tissue samples were obtained from the subsequent excised tissue, which were immediately frozen in liquid nitrogen.

3. Results

3.1. Fluorescence Microscopy of Basal Cell Carcinoma Samples

In superficial BCCs fluorescence over the full thickness of the tumor was observed, whereas in solid BCCs only the upper parts of tumor tissue showed a fluorescence limited to a few millimeters (Fig. 2a). Nevertheless in all superficial lesions the penetration depth of ALA was high enough to sensitize the lesions in full thickness. In a number of samples the fluorescence inside the BCCs varied considerably. In general the margins of the BCCs tended to have a stronger fluorescence than the central parts of tumors (Fig. 2b).

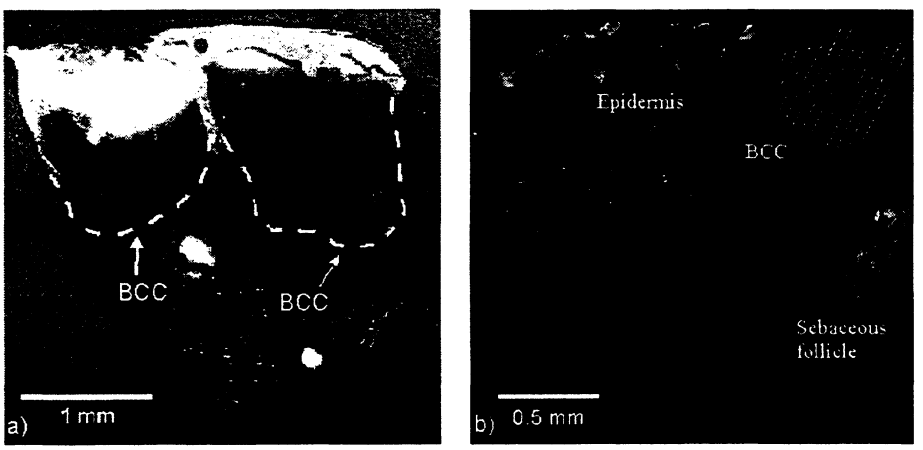

Fig. 2: Fluorescence images of BCC samples after 4h ALA application:
a) Solid BCC with PPIX fluorescence limited to approximately 0.9 mm.
b) Superficial BCC with inhomogeneous sensitization.

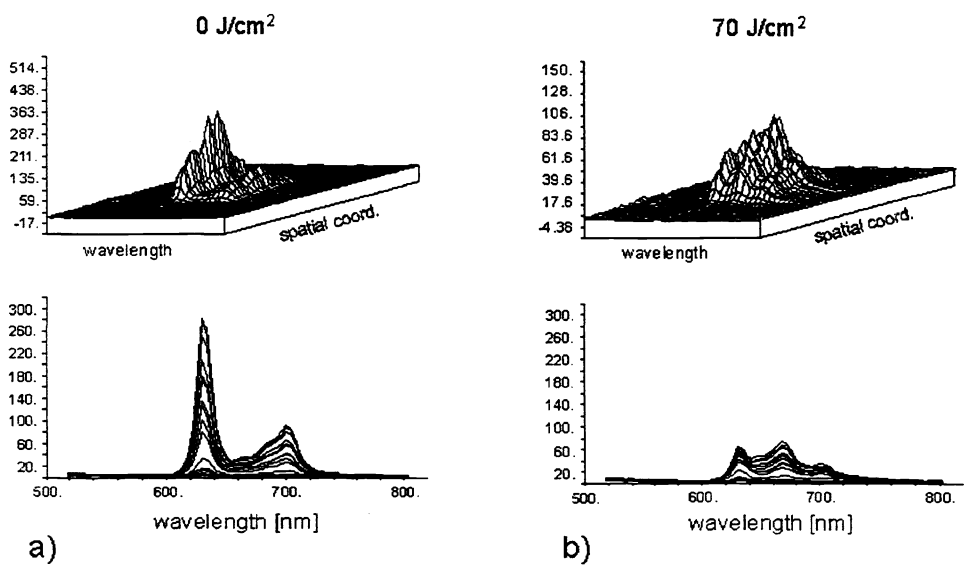

Fig. 3: Spatially resolved microscopic emission spectra of BCC cryo sections a) before irradiation and b) after irradiation with 70 J/cm^2.

All samples showed also fluorescence in the epidermis, sebaceous glands, sweat glands, hair follicles and infiltrating leukocytes. When measured at locations close together, most sections showed similar fluorescence of epidermal cells and BCC cells.

The photobleaching of PPIX during the treatment limits the effect of PDT since an increase in the light dose has no effect if the sensitizer has been destroyed photochemically. By irradiating frozen sections of BCCs under the fluorescence microscope the amount of photobleaching was estimated. After an irradiation of 70 J/cm^2 a strong decrease in the PPIX fluorescence was measured (Fig. 3). Additionally at 670 nm the fluorescence band of a photoproduct of PPIX can be seen.

3.2. Fluorescence microscopy of cervical tissue

All tissue samples showed a good fluorescence in the epithelial layers of squamous and columnar epithelium and the underlying glands [Fig. 4]. Deeper nonepithelial tissue was free of fluorescence. On the average the fluorescence of dysplastic tissue was not higher than the fluorescence of healthy epithelium. Only glandular tissue seems to accumulate PPIX at higher levels. In general a strong variation in the fluorescence was observed in each type of the tissues. Furthermore the thickness of the epithelial tissue varied considerably and no correlation with the malignancies was observed. In Fig. 4 squamous epithelium, which is several times thicker than the dysplastic lesions, is expected to give the strongest macroscopic fluorescence. In a more quantitative analysis the tissue structures were classified in normal tissue (squamous epithelium and glands), light dysplasia (CIN1), medium grade dysplasia (CIN2), severe dysplasia or carcinoma in situ (CIS) and invasive carcinoma. The measured fluorescence intensities of the different types of tissue were normalized in each section by the average fluorescence of the CIN1 lesions in that section. This compensates for a varying time and efficacy of ALA application. A strong variation of fluorescence in healthy and neoplastic tissue was observed [Fig. 5] and differences in healthy and dysplastic tissue fluorescence were not significant.

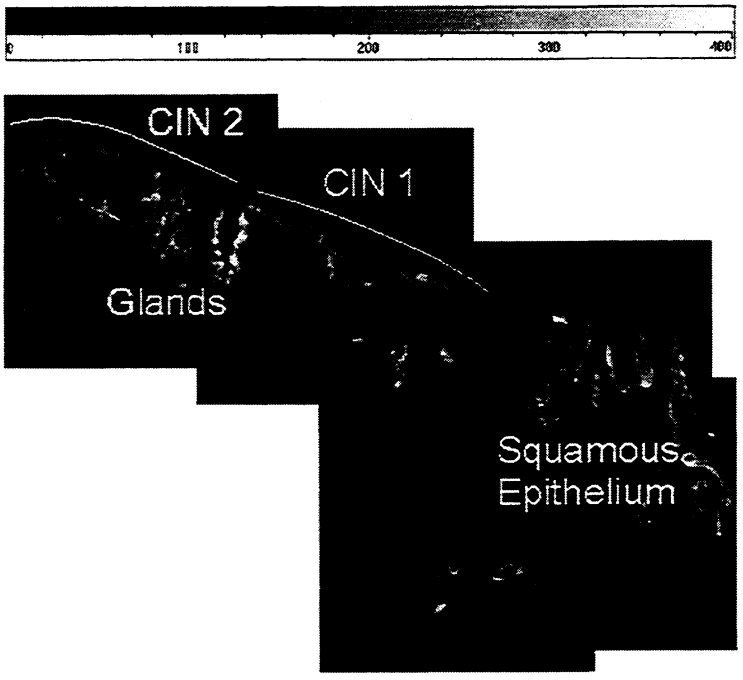

Fig. 4: Fluorescence images of a typical microscopic sample of the cervical mucosa.

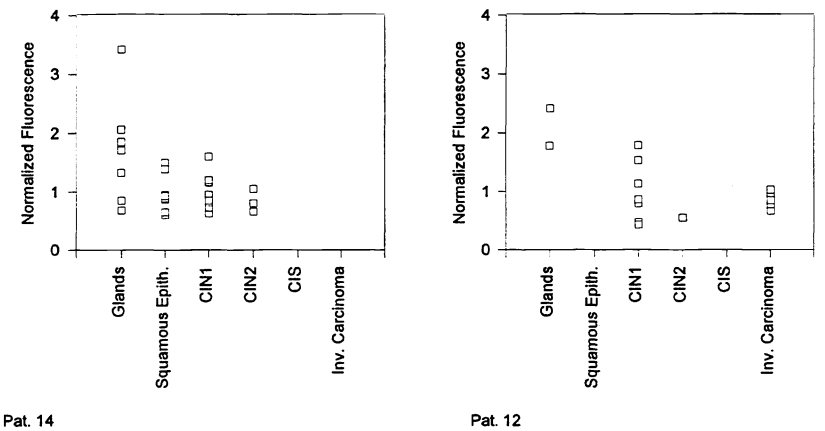

Fig. 5: Fluorescence of different healthy and malignant mucosal tissues measured in the cryo sections of two patients.

4. Discussion

4.1. Fluorescence microscopy of BCC samples

According to our data the treatment of superficial BCC is not limited by the penetration depth of ALA, but rather by an insufficient sensitization of BCC tissue. While the lack of fluorescence in deeper parts of the tumors can be explained by a limited diffusion of ALA, the inhomogeneous fluorescence seems to reflect the individual capacity of the BCC cells to convert ALA to PPIX. Although fluorescence microscopy cannot show whether the accumulation of PPIX in the tumor cells is high enough to cause phototoxicity it can be expected that in the areas with low fluorescence PPIX accumulation is too low to cause phototoxicity. In vitro a strong photobleaching of PPIX was observed in cryo sections. This is in agreement with in vivo measurements of the decrease of PPIX fluorescence during photodynamic treatment [15] and the strong reduction of superficial red fluorescence observed after a treatment of BCC with a radiant exposure of 100 J/cm^2.

The lack of microscopic selectivity seems to be contradictory to the increased macroscopic fluorescence of BCCs after topical application of ALA [13]. In principle three mechanisms might cause an increased tumor fluorescence:

1. A higher accumulation of PPIX in tumor cells at a given ALA concentration.
2. A better penetration of the ALA into the tissue over the tumor due to the destruction of a natural diffusion barrier.
3. A greater thickness of the fluorescing tumor compared to the epidermis.

The last two mechanisms can explain a higher macroscopic fluorescence of the BCCs without microscopic selectivity. The uninvolved stratum corneum is a good diffusion barrier for small hydrophilic molecules like ALA [16]. Since this barrier is often damaged over skin lesions, mechanisms two and three can explain the increased macroscopic fluorescence reasonably.

4.2. Fluorescence diagnosis of cervical neoplasia

Similar to the fluorescence microscopy of BCC samples an increased fluorescence of dysplastic compared to unaltered epithelium was not observed. Hence the intensity in the fluorescence images is dominated by the thickness of the epithelial layers, which varies strongly at the ectocervix, and seems not to be correlated with the malignancies. From these investigations it is expected that only the glands can be diagnosed by a higher fluorescence. These findings can explain the preliminary results of the diagnosis study which shows up to now no correlation between the location of the dysplasias and the macroscopic fluorescence intensity. Because of the varying thickness of the epithelial tissues, a high microscopic selectivity in PPIX accumulation is needed for the fluorescence diagnosis. By changing the concentration of ALA we hope to improve this selectivity.

5. Conclusions

Fluorescence microscopy of tissue samples can give plentiful information on the distribution (homogeneity and depth) of the tumor sensitization, the selectivity, and the photobleaching of the photosensitizer. Two main conclusions can be drawn from these experimental data. On a microscopic level there is not always a selective accumulation of PPIX in epithelial malignancies and premalignancies. Although neither in BCC nor in cervical dysplasias selective fluorescence was observed, BCC lesions exhibit a good demarcation from uninvolved skin by their fluorescence, which might be useful for fluorescence diagnosis. Hence in fluorescence diagnosis with topical application of ALA increased thickness of the lesions and a lack of diffusion barriers have to be considered as a possible mechanism of the fluorescence contrast.

In the PDT with ALA-induced PPIX the accumulation of the sensitizer may not only be limited by the ALA concentration, but also by the metabolic activity of the tumor cells. Therefore parts of the tumors may accumulate PPIX in smaller quantities, as it was observed in BCC. Together with the strong photobleaching of PPIX there might be parts of the tumor which cannot be treated by PDT with topical ALA. In this light the concepts to increase PPIX accumulation by additive substances like EDTA [17, 18] or desferrioxamine [19] are very interesting in order to improve PDT with ALA induced PPIX.

6. References

1. N.A. Buskard, B.C. Wilson, "Introduction to the symposium on photodynamic therapy," *Seminars in Oncology*, **21**, pp. 1-3 (1994).

2. U. Schmidt-Erfurth, T. Hasan, K. Schomacker, T.J. Flotte, R. Birngruber, "In vivo uptake of liposomal benzoporphyrin derivative and photothrombosis in experimental corneal neovascularization," *Lasers Surg. Med.*, **17**, pp. 178-188 (1995).

3. G. Hüttmann, C. Hendrich, R. Birngruber, C. Lehnert, J. Seara, W.E. Siebert, H. Diddens, "Protoporphyrin IX distribution after intra-articular and systemic application of 5-aminolevulinic acid in healthy and arthritic joints," in *Mechanisms and Techniques in Photodynamic Therapy V*, T.J. Dougherty (ed.), **Proc. SPIE 2675**, pp. 238-242 (1996).

4. L.G. Ratkay, R.K. Chowdhary, H.C. Neyndorff, J. Tonzetich, J.D. Waterfield, J.G. Levy, "Photodynamic therapy; a comparison with other immunomodulatory treatments of adjuvant-enhanced arthritis in MRL-lpr mice," *Clin. Exp. Immunol.*, **95**, pp. 373-377 (1994).

5. M. Kriegmair, R. Baumgartner; R. Knuchel; H. Stepp; F. Hofstadter; A. Hofstetter, "Detection of early bladder cancer by 5-aminolevulinic acid induced porphyrin fluorescence," *J. Urol.*, **155,** pp. 105-109 (1996).

6. C.S. Loh, A.J. MacRobert, J. Bedwell, J. Regula, N. Krasner, S.G. Bown, "Oral versus intravenous administration of 5-aminolaevulinic acid for photodynamic therapy," *Br. J. Cancer*, **68**, pp. 41-51 (1993).

7. C.S. Loh, D.I. Vernon, A.J. MacRobert, J. Bedwell, S.G. Bown, S.B. Brown, "Endogenous porphyrin distribution induced by 5-aminolaevulinic acid in the tissue layers of the gastrointestinal tract", *J. Photochem. Photobiol. B: Biol.*, **20**, pp. 47-54 (1993).

8. P.T. Chatlani, J. Bedwell, A.J. MacRobert, H. Barr, P.B. Boulos, N. Krasner, D. Phillips, S.G. Bown, "Comparison of distribution and photodynamic effects of di- and tetra-sulphonated aluminum phthalocyanines in normal rat colon", *Photochem. Photobiol.*, **53**, pp. 745-751 (1991).

9. A. Rück, H. Diddens, "Uptake and subcellular distribution of photosensitizing drugs in malignant cells," The fundamental Bases of Phototherapy, H. Hönigsmann, G. Jori, A.R. Young (ed.), OEMF spa, Milano (1996).

10. Z. Malik, M. Dishi, Y. Garini, "Fourier transform multipixel spectroscopy and spectral imaging of protoporphyrin in single melanoma cells," *Photochem. Photobiol.*, **63**, pp. 608-614 (1996).

11. J.C. Kennedy and R.H. Pottier, " Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy," *J. Photochem. Photobiol. B: Biol.*,**14**, pp. 275-292 (1992).

12. F. Cairnduff, M.R. Stringer, E.J. Hudson, D.V. Ash, S.B. Brown, "Superficial photodynamic therapy with topical 5-aminolaevulinic acid for superficial primary and secondary skin cancer," *Br. J. Cancer*, **69**, pp. 605-608 (1994).

13. K. Svanberg, T. Andersson, D. Killander, I. Wang, U. Stenram, S. Andersson-Engels, R. Berg, J. Johansson and S. Svanberg, "Photodynamic therapy of non-melanoma malignant tumours of the skin using topical δ-amino levulinic acid sensitization and laser irradiation," *Br. J. Dermatol.*, **130**, pp. 743 -751 (1994).

14. D.E. Rowe, R.J. Carroll, C.L. Day, "Long-term recurrence rate in previously untreated (primary) basal cell carcinoma: implications for patient follow-up," *J. Dermatol. Surg. Oncol.*, **15**, pp. 315-328 (1989).

15. R. Sroka, C. Ell, P. Heil, A. Hofstetter, H. Iro, M. Ludwig, E. Unsöld, "Experimentelle und klinische Untersuchungen zum Ausbleichverhalten δ-Aminolävulinsäure induzierter Porphyrine," in Laser in Medicine, W. Waidelich, R. Waidelich, A. Hofstetter (ed.) *Proceedings of the 11th International Congress Laser 93*, pp. 127-130, Springer-Verlag, Berlin (1994).

16. J.S. Nelson, J.L. McCullough, T.C. Glenn, W.H. Wrigth, L.-H.L. Liaw, S.L. Jacques, "Mid-infrared laser ablation of stratum corneum enhances in vitro percutaneous transport of drugs," *J. Invest. Dermatol.*, **97**, pp. 874-879 (1991).

17. A. Orenstein, G. Kostenich, H. Tsur, L. Roitman, B. Ehrenberg, Z. Malik, "Photodynamic therapy of human skin tumors using topical application of 5-aminolevulinic acid, DMSO and EDTA," in *Photodynamic Therapy of Cancer II*, D. Brault, G. Jori, J. Moan, B. Ehrenberg, **Proc. SPIE 2325**, pp. 100-105 (1995).

18. T. Warloe, Q. Peng, J. Moan, H.B. Steen, K.-E. Gierchsky, "Photodynamic therapy with 5-aminolevulinic acid induced porphyrins and DMSO/EDTA for basal cell carcinoma," in 5th Int. Photodynamic Association Biennal Meeting, D.A. Cortese (ed.), **Proc. SPIE 2371**, pp. 226-235 (1995).

19. S. Fijan, H. Hönigsmann, B. Ortel, "Photodynamic therapy of epithelial skin tumours using delta-aminolaevulinic acid and desferrioxamine," *Br. J. Dermatol.*, **133**, pp. 282-288 (1995).