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Aminolevulinic acid for photodynamic therapy of bladder carcinoma cells

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Abstract A new concept in photosensitizing tumor cells is photosensitizer synthesis in situ. Aminolevulinic acid (ALA) is a precursor of protoporphyrin IX (PP IX), a potent photosensitizer. The goal of our study was to examine dark toxicity, phototoxic potential, metabolism of ALA and morphological alterations in Waf bladder cancer cells. Dark toxicity of Waf cells was observed after incubation with ALA, beginning at a concentration of 15 mM. Photodynamic treatment with ALA at concentrations of 1, 5 and 10 mM showed a drug- and light-dose-dependent cell survival rate in comparison to a control group. Two incubation times of 3.5 and 5.5 h were compared for cell survival. After a longer incubation time of 5.5 h, cell survival was decreased in all experiments; this is consistent with our extraction data where higher fluorescence was found after 5.5 than after 3.5 h. The results show that ALA-induced photosensitization has a high potential for photodynamic therapy (PDT) of superficial bladder carcinoma.

Key words Bladder cancer · Photodynamic therapy · Aminolevulinic acid

Introduction

Various cancers in different specialties such as cutaneous, endobronchial, esophageal, gastrointestinal, gynecological, CNS, and head and neck malignancies and also benign diseases are treated with photodynamic therapy (PDT) [6, 19]. In urology it is used for the treatent of superficial bladder carcinoma, especially carcinoma in situ [4, 12, 20]. Thus far, photosensitizers have been administered in their active form. The most frequently administered photosensitizer is a hematoporphyrin derivative (HPD), a mixture of porphyrins. Since it has several drawbacks, such as chemical impurity, weak absorption of the therapeutically used red light, and skin phototoxicity, new photosensitizers are being sought. An alternative approach in PDT is to achieve photosensitizer synthesis in situ with a precursor that is either preferentially localized or preferentially converted to active photosensitizer by target cells. Aminolevulinic acid (ALA) is an endogenous substance in heme synthesis that takes place in mitochondria. Exogenous ALA is not phototoxic and can be used as an indirect photosensitizer [5, 13, 14]. When metabolized it reveals its active form, protoporphyrin IX (PP IX) [5, 13, 14]. PP IX is an effective and well-known photosensitizer which is associated with the extreme skin phototoxicity seen in patients with porphyria. ALA is used clinically after topical application to treat skin lesions, such as basal or squamous cell carcinoma, actinic keratoses, Bowen's disease, xeroderma pigmentosum, cutaneous T-cell lymphoma and skin metastases [3, 13, 22–24]. Complete response in Bowen's disease was found to be 89%, and in more than 100 basal cell carcinomas complete response was found in between 50% and 100% of lesions after a single treatment [3, 13, 22, 23]. It was shown that ALA is converted to PP IX in abnormal epithelium, thus avoiding damage to normal skin after PDT [8, 13]. ALA was also used clinically for PDT of oral and colon cancer after systemic administration [9, 16]. For photodynamic diagnosis, ALA was instilled into the urinary bladder, where a highly selective accumulation of PP IX in urothelial neoplasia was found by fluorescence detection [15]. In the light of these promising results, the potential use of ALA for PDT of

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superficial bladder cancer should be examined. In our study we investigated dark toxicity, phototoxic efficiency, uptake and metabolism of ALA and morphological alterations in a human bladder carcinoma cell line.

Materials and methods

Cell line

Experiments were carried out with Waf human bladder carcinoma cells, grade 3. Cells were grown as monolayer culture in DMEM medium containing 10% fetal calf serum (FCS) plus 2% glutamin and 1% penicillin/streptomycin. Cells were kept in a dark incubator at 37 °C in humidified air with 5% CO_2.

Photosensitizer

ALA and PP IX were purchased from Sigma (Deisenhofen, Germany). For incubation ALA was dissolved in culture medium without FCS. Physiological pH was adjusted with NaOH. PP IX was dissolved in dimethylsulfoxide (DMSO). A stock solution of 1 mg/ml was prepared and stored at 4 °C in the dark. The characteristic absorbance and fluorescence spectra were recorded on a spectrophotometer (DU 64 Beckman, Fullerton, CA, USA).

Light source

An argon-pumped dye laser (Aesculap-Meditec, Model MDS 90, Germany), emitting a wavelength of 630 nm according to the absorbance maximum of PP IX, was used for irradiation. Figure 3 shows an absorbance peak at 630 nm. In vivo the optimum wavelength for PDT with ALA-induced photosensitization is slightly longer. Light was delivered via a 0.6-mm-diameter quartz fiber. The beam was expanded to a spot size of 35 mm in diameter covering the monolayer cell culture homogeneously. The fluences used were 12, 24 and 48 J/cm^2 at an irradiance of 100 mW/cm^2.

Dark toxicity

Cells were incubated with ALA at a concentration between 1 and 40 mM for 3.5 h, kept in a dark incubator for 4 days, fixed with methanol, stained with Giemsa, counted and compared with a control group.

Extraction of PP IX

Waf bladder cancer cells were incubated with ALA at concentrations of 1, 5 and 10 mM, the same concentrations that was used for PDT experiments. After an incubation time of 3.5, 5.5 and 7.5 h, PP IX was extracted with DMSO and the fluorescence of PP IX was measured. For comparison the autofluorescence of cells was also determined.

PDT

In the log phase cells were trypsinized, counted, inoculated into 35 mm-diameter multiwells at 10^5 cells/well and allowed to grow for 24 h. Then cells were incubated with ALA at concentrations of 1, 5 and 10 mM for 3.5 or 5.5 h, washed 3 times with culture medium and irradiated. All experiments concerning the photosensitizer were carried out under subdued light. After irradiation cells were incubated again for 4 days, fixed with methanol, stained with Giemsa, counted and compared with a control group. Cells treated with ALA or light alone served as control group.

Results

Dark toxicity

Cells incubated with ALA at a concentration of up to 10 mM for 3.5 h without light treatment showed no decrease in cell counts compared with a control group. Cells incubated at a concentration of 15 mM showed a cell count of only 15% of control. After incubation with 40 mM ALA, no cell survival was found (Fig. 1). Therefore, PDT experiments were carried out at ALA concentrations of 1, 5 and 10 mM, where no dark toxicity occurred.

Extraction of PP IX

There was a correlation between the ALA concentration for incubation of cells and the PP IX fluorescence. The higher the ALA concentration the higher the PP IX fluorescence. Furthermore, the PP IX fluorescence was dependent on the incubation time. The highest fluorescence was found after an incubation time of 5.5 h. After an incubation time of 7.5 h the fluorescence was decreased and showed similar values to those after an incubation time of 3.5 h. These data are shown in detail in Fig. 2.

PDT

The absorbance spectrum of PP IX shows a maximum at 407 nm and smaller peaks at 505, 540, 577 and

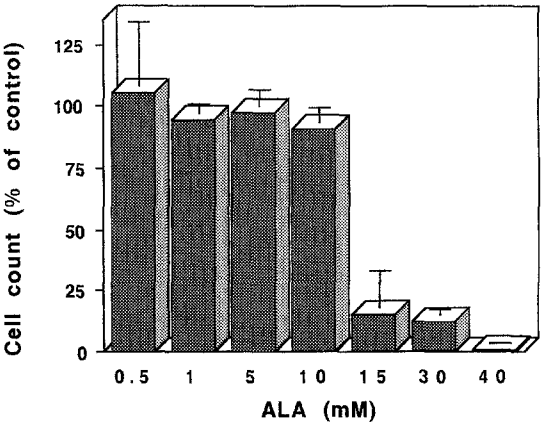

Fig. 1 Dark toxicity of ALA in Waf human bladder carcinoma cells (no irradiation)

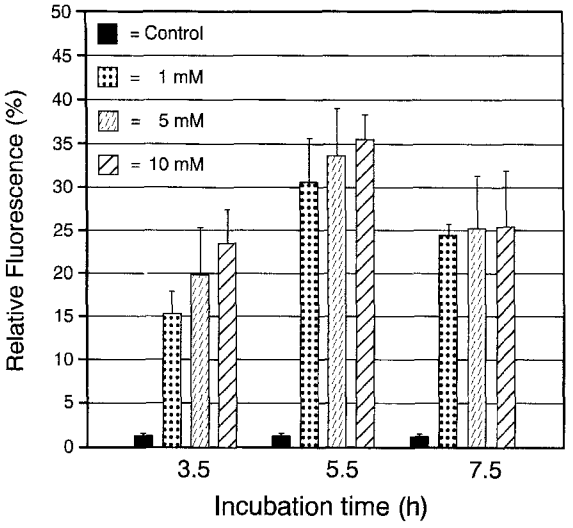

Fig. 2 Relative fluorescence of protoporphyrin IX (PP IX) in control Waf cells and after incubation of Waf cells with ALA at concentrations of 1, 5 and 10 mM for 3.5, 5.5 and 7.5 h and extraction of PP IX

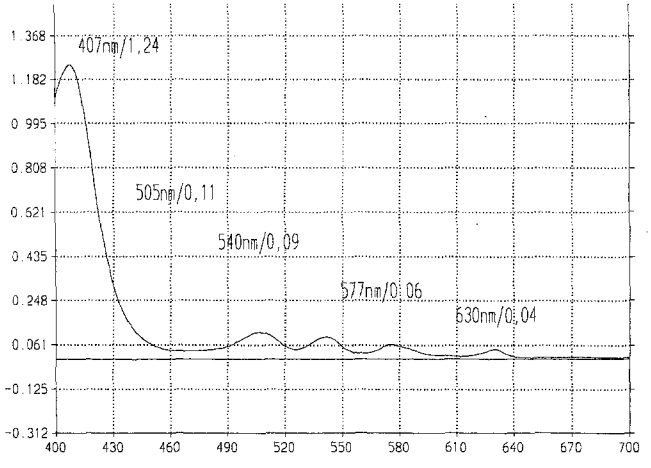

Fig. 3 Absorbance spectrum of protoporphyrin IX (*y-axis* arbitrary units, *x-axis* wavelenghth in nanometers; *numbers following the wavelengths of the maxima* are the absorbance values at these maxima)

630 nm; for PDT experiments the absorbance at 630 nm was used since red light shows the best tissue penetration (Fig. 3). After incubation of Waf cells with ALA at a concentration of 5 mM for 5.5 h, PP IX fluorescence could be detected by fluorescence microscopy. For PDT two different incubation times of 3.5 and 5.5 h were compared in order to see if the time of incubation has an effect on the metabolism of ALA to PP IX and hence on cell survival. Theoretically the best timing for irradiation is when the PP IX concentration reaches its maximum. Both time intervals were chosen on the basis of an experiment reported in the literature and on the basis of our extraction data showing that ALA is converted to PP IX within this period of time.

PDT after an incubation time of 3.5 *h*

After cell incubation with ALA at a concentration of 1 mM, there was only a slight decrease in cell survival after irradiation between 12 and 48 J/cm^2. After incubation with 5 mM ALA, cell survival after irradiation at 12 J/cm^2 was 74.4 ± 9.0%, at 24 J/cm^2 73.3 ± 10.1% and at 48 J/cm^2 10.9 ± 3.2%. After incubation with 10 mM ALA, cell survival was markedly decreased. After irradiation at 12 and 24 J/cm^2, cell survival was approximately 25 ± 7.5%; at 48 J/cm^2 only 6.5 ± 2.7% of cells survived. ALA or light alone had no effect on cell survival in a control group.

PDT after an incubation time of 5.5 *h*

The cell survival rate showed an ALA- and light-dose-dependent decrease. Cell survival after incubation with 1 mM ALA and irradiation between 12 and 48 J/cm^2 was between 50.8 ± 3.8% and 14.6 ± 1.2%. After incubation with 10 mM ALA and irradiation at the above-mentioned fluences, cell survival was markedly decreased to values between 10.4 ± 6.1% and 5.9 ± 1.8%. Figure 4 shows Waf human bladder carcinoma cells in a control group (a) and after incubation with ALA at a concentration of 10 mM for 5.5 h and irradiation where all cells were destroyed (b). ALA or light alone had no effect on cell survival in a control group.

Comparing the two different incubation times, a difference in survival rate was observed. Cell survival was decreased in all experiments after an incubation time of 5.5 h as compared to 3.5 h, independent of the ALA concentration or the light dose. The detailed data for comparison of cell survival after different incubation times are listed in Table 1.

Table 1 Comparison of cell survival (% of control) after incubation with ALA at a concentration of 1, 5, and 10 mM for 3.5 or 5.5 h and irradiation at 12, 24 and 48 J/cm^2 (means ± SE)

ALA (mM)	J/cm^2	Cell survival after an incubation time of:	
		3.5 h	5.5 h
1	12	83.8 ± 7.5	50.8 ± 3.8
5	12	74.4 ± 9.0	27.5 ± 6.7
10	12	21.7 ± 7.1	10.4 ± 6.1
1	24	76.5 ± 6.2	39.9 ± 6.2
5	24	73.3 ± 10.1	18.7 ± 1.5
10	24	30.0 ± 7.9	5.0 ± 3.2
1	48	64.5 ± 7.8	14.6 ± 1.2
5	48	10.9 ± 3.2	10.0 ± 1.2
10	48	6.5 ± 2.7	5.9 ± 1.8

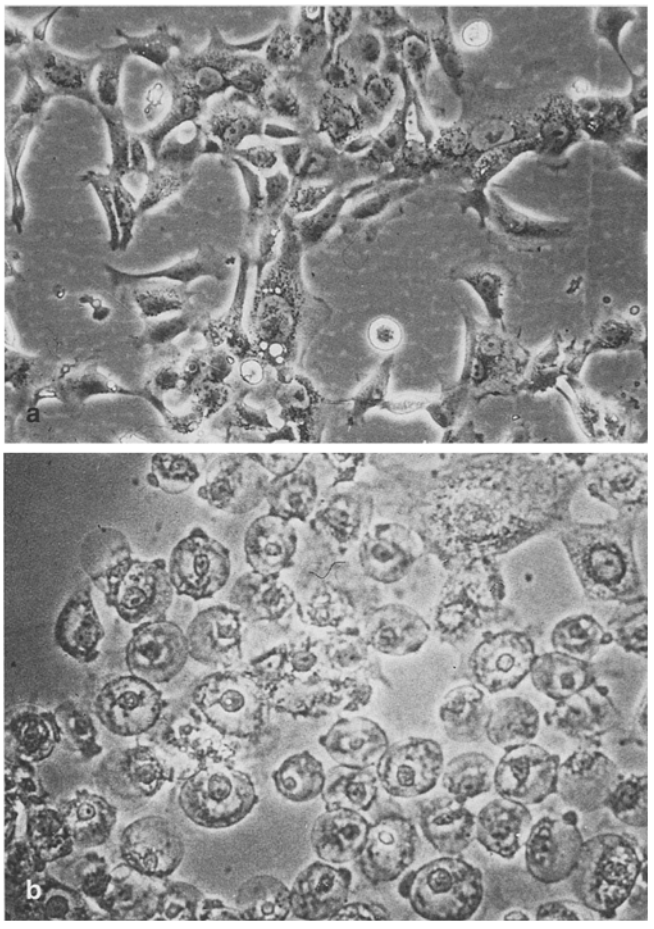

Fig. 4a, b Waf cells **a** in a control group, **b** after incubation with 10 mM ALA for 5.5 h and irradiation showing cell swelling and blebbing as signs of destruction

Discussion

Routine PDT consists of administration of a photosensitizer and subsequent irradiation of the tumor with light that is absorbed by the photosensitizer [6, 19]. A photochemical reaction in which cytotoxic singlet oxygen and/or free radicals are produced leads to tumor necrosis. For endoscopic irradiation, e.g., in the urinary bladder, laser light that is coupled into a flexible quartz fiber is used [4, 12, 20]. Thus far, the photosensitizer most used clinically has been a hematoporphyrin derivative (HPD). A new concept in PDT is the use of endogenous porphyrins as photosensitizer. ALA is a precursor in heme synthesis that is metabolized to PP IX, a potent photosensitizer. It is reported that only certain types of cells become photosensitized when exposed to ALA and others do not [14]. For our purpose, potentially treating superficial bladder cancer with ALA and light, it was of interest to study dark toxicity, photodynamic efficiency and metabolism of ALA in a human bladder carcinoma cell line. Fluorescence microscopy of urinary bladder of mice showed strong PP IX fluorescence in the urothelium but only very little in the underlying muscle of the bladder after systemic administration of ALA [5, 13]. After intravesical instillation of ALA, urothelial neoplasias could be detected by PP IX fluorescence [15]. Therefore, it was expected that exogenous ALA is converted to PP IX by transitional cells and hence intravesical instillation of ALA has the potential to be used for the treatment of superficial bladder carcinoma. In vitro experiments on monolayer cell cultures seem to imitate a topical, intravesical administration where the photosensitizer is in direct contact with the tumor cells. For PDT of bladder cancer it might be advantageous to administer the photosensitizer intravesically in order to achieve higher photosensitizer concentrations in the tumor and to minimize side effects both systemically and to normal bladder wall [1]. The first part of our study was the investigation of dark toxicity in order to find the appropriate concentration of ALA for PDT. It was shown that ALA at a concentration of up to 10 mM exhibited no dark toxicity in our cell line. Therefore, PDT experiments were carried out at ALA concentrations of up to 10 mM. An incubation time of 5.5 h and irradiation showed a lower cell survival rate than an incubation time of 3.5 h. In particular, this was obvious when a lower ALA concentration or light dose was used. There was a good correlation between the PDT results after different incubation times and the extraction data of PP IX. The highest PP IX fluorescence was found after an incubation time of 5.5 h, where better PDT results were obtained compared to a 3.5-h incubation time. Fukuda studied the kinetics of porphyrin accumulation in vitro and also found that the total porphyrin synthesized was a function of ALA concentration and incubation time [7]. The cellular porphyrin content exhibited a saturation pattern reaching a plateau in cells exposed for 6 h [7]. This is consistent with our results showing stronger fluorescence after incubation for 5.5 h in comparison to 3.5 h. Iinuma investigated the efflux of PP IX after incubation of different cell lines with ALA [10], demonstrating that intracellular PP IX accumulation is a dynamic process that is determined by both the efflux of PP IX from the cells and the enzyme activities in the heme biosynthesis pathway [10].

Rebeiz compared the photodestruction of three different cell lines by induction of endogenous accumulation of PP IX [21]. Cells were incubated with ALA alone or in combination with 1,10-phenanthroline (Oph), a porphyrin biosynthesis modulator. To confirm the identity of the accumulated intracellular product after incubation with ALA, its mobility in high-performance liquid chromatography (HPLC) was compared with that of authentic PP IX [21]. Both substances exhibited similar retention times [21]. Also the fluorescence properties were identical to authentic PP IX so that the metabolized product was identified as PP IX

[21]. Slower-growing untransformed cells did not accumulate significant amounts of PP IX following ALA and Oph treatment unless stimulated to proliferate with a mitogenic lectin [21]. This observation is of interest concerning tumor selectivity in vivo. Malik studied porphyrin synthesis after stimulation of different leukemic cell lines and B16 melanoma cells [17, 18]. High-metastatic Esb lymphoma cells accumulated more porphyrin than the low-metastatic Eb cell line [17]. Survival rates after light exposure were dependent on the rate of porphyrin accumulation [17].

A direct comparison between the studies is not possible due to different circumstances. But all authors conclude that different cell lines might show different behavior. Therefore, we studied the behavior of bladder cancer cells. In conclusion, Waf bladder carcinoma cells metabolize exogenous ALA within 3.5 h to PP IX that leads to cell death upon irradiation. This effect is even stronger when an incubation time of 5.5 h is used. Our results warrant further studies using ALA as a photosensitizer for superficial bladder cancer. In vivo, ALA might be superior to conventional photosensitizers as PP IX is synthesized in malignant cells while other sensitizer mainly localize in the vascular stroma of tumors [2]. This could also be shown in a rat bladder cancer model where fluorescence microscopy demonstrated predominantly cellular rather than stromal PP IX fluorescence [11].

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References

1. Bachor R, Flotte TJ, Scholz M, Dretler S, Hasan T(1992) Comparison of intravenous and intravesical administration of chloro-aluminum sulfonated phthalocyanine for photodynamic treatment in a rat bladder cancer model. J Urol 147:1404–1410
2. Bedwell J, MacRobert AJ, Phillips D, Bown SG (1992) Fluorescence distribution and photodynamic effect of ALA-induced PP IX in the DMH rat colonic tumour model. Br J Cancer 65:818–824
3. Cairnduff F, Stringer MR, Hudson EJ, Ash DV, Brown SB (1994) Superficial photodynamic therapy with topical 5-aminolevulinic acid for superficial primary and secondary skin cancer. Br J Cancer 69:605–608
4. D'Hallewin MA, Baert L, Marijnissen JPA, Star WM (1992) Whole bladder wall photodynamic therapy with in situ light dosimetry for carcinoma in situ of the bladder. J Urol 148:1152–1155
5. Divaris DXG, Kennedy JC, Pottier RH (1990) Phototoxic damage to sebaceous glands and hair follicles of mice after systemic administration of 5-aminolevulinic acid correlates with localized protoporphyrin IX fluorescence. Am J Pathol 136:891–897
6. Dougherty TJ (1978) Photosensitizers: therapy and detection of malignant tumors. Photochem Photobiol 45:879–889
7. Fukuda H, Battle AMC, Riley PA (1993) Kinetics of porphyrin accumulation in cultured epithelial cells exposed to ALA. Int J Biochem 25:1407–1410
8. Goff BA, Bachor R, Kollias N, Hasan T (1992) Effects of photodynamic therapy with topical application of 5-aminolevulinic acid on normal skin of hairless guinea pigs. J Photochem Photobiol B: Biol 15:239–251
9. Grant WE, Hopper C, MacRobert AJ, Speight PM, Bown SG (1993) Photodynamic therapy of oral cancer: Photosensitisation with systemic aminolevulinic acid. Lancet 342:147–148
10. Iinuma S, Farshi SS, OPrtel B, Hasan T (1994) A mechanistic study of cellular photodestruction with 5-aminolaevulinic acid-induced porphyrin. Br J Cancer 70:21–28
11. Iinuma S, Bachor R, Flotte T, Hasan T (1995) Biodistribution and phototoxicity of 5-aminolevulinic acid-induced PP IX in an orthotropic rat bladder tumor model. J Urol 153: 802–806
12. Jocham D, Baumgartner R, Stepp H, Unsöld E (1990) Clinical experience with the integral photodynamic therapy of bladder carcinoma. J Photochem Photobiol B: Biol 6:183
13. Kennedy JC, Pottier RH, Pross DC (1990) Photodynamic therapy with endogenous protoporphyrin IX: Basic principles and present clinical experience. J Photochem Photobiol B: Biol 6:143–148
14. Kennedy JC, Pottier RH (1992) Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy. J Photochem Photobiol B: Biol 14:275–292
15. Kriegmair M, Ehsan A, Baumgartner R, Lumper W, Knuechel R, Hofstädter F, Steinbach P, Hofstetter A (1994) Fluorescence photodetection of neoplastic urothelial lesions following intravesical instillation of 5-aminolevulinic acid. Urology 44: 836–841
16. Loh CS, MacRobert AJ, Bedwell J, Regula J, Krasner N, Bown SG (1993) Oral versus intravenous administration of 5-minolaevulinic acid for photodynamic therapy. Br J Cancer 68:41–51
17. Malik Z, Ehrenberg B, Faraggi A (1989) Inactivation of erythrocytic, lymphocytic and myelocytic leukemic cells by photoexcitation of endogenous porphyrins. J Photochem Photobiol B: Biol 4:195–205
18. Malik Z, Babushkina T, Shafran M, Nordenberg Y, Mamet R, Schöenfeld N (1993) Induction of protoporphyrin biosynthesis and photodynamic inactivation of B 16 melanoma cells. SPIE 2078:355–362
19. Manyak MJ, Russo A, Smith PD, Glatstein E (1988) Photodynamic therapy: Review article. J Clin Oncol 6:380–391
20. Prout GR, Lin CW, Benson R, Nseyo UO, Daly JJ, Griffin PP, Kinsey J, Tian ME, Lao YH, Mian YZ, Chen X, Ren FM, Qiao SJ (1987) Photodynamic therapy with hematoporphyrin derivative in the treatment of superficial transitional cell carcinoma of the bladder. N Engl J Med 317:1251
21. Rebeiz N, Rebeiz CC, Arkins S, Kelley KW, Rebeiz CA (1992) Photodestruction of tumor cells by induction of endogenous accumulation of protoporphyrin IX: Enhancement by 1,10-phenanthroline. Photochem Photobiol 55:431–435
22. Svanberg K, Andersson T, Killander D, Wang I, Stenram U, Andersson-Engels S, Berg R, Johansson J, Svanberg S (1994) Photodynamic therapy of non-melanoma malignant tumours of the skin using topical delta-amino-levulinic acid sensitization and laser irradiation. Br J Dermatol 130:743–751
23. Wolf P, Rieger E, Kerl H (1993) Topical photodynamic therapy with endogenous porphyrins after application of 5-aminolevulinic acid. J Am Acad Dermatol 28:17–21
24. Wolf P, Kerl H (1991) Photodynamic therapy in patient with xeroderma pigmentosum. Lancet 337:1613–1614