Optimum porphyrin accumulation in epithelial skin tumours and psoriatic lesions after topical application of \( \text{\textalpha-aminolaevulinic acid} \)

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Summary Photodynamic therapy with topically applied \( \text{\textalpha-aminolaevulinic acid} \) is used to treat skin tumours by employing endogenously formed porphyrins as photosensitizers. This study examines the time course of porphyrin metabolism after topical application of \( \text{\textalpha-aminolaevulinic acid} \). Porphyrin biosynthesis in human skin tumours (basal cell carcinoma, squamous cell carcinoma), in psoriatic lesions, and in normal skin was investigated. Skin areas were treated with \( \text{\textalpha-aminolaevulinic acid} \), and levels of total porphyrins, porphyrin metabolites and proteins were measured in samples excised after 1, 2, 4, 6, 9, 12 and 24 h. There was an increase in porphyrin biosynthesis in all tissues with maximum porphyrin levels in tumours between 2 and 6 h after treatment. The pattern of porphyrin metabolism showed no significant difference between normal and neoplastic skin, protoporphyrin being the predominant metabolite. The results suggest that optimum irradiation time for superficial epithelial skin tumours may be as soon as 2 h after application to \( \text{\textalpha-aminolaevulinic acid} \), whereas for treatment of psoriatic lesions an application time of 6 h is more suitable.

Keywords \( \text{\textalpha-aminolaevulinic acid}; \text{basal cell carcinoma}; \text{squamous cell carcinoma}; \text{psoriasis}; \text{photodynamic therapy}; \text{porphyrin metabolites} \)

Photodynamic therapy (PDT) with \( \text{\textalpha-aminolaevulinic acid} \) (ALA) is based on the administration of ALA, the non-fluorescent first committed compound in the pathway of haem biosynthesis, to the diseased skin (Kennedy et al, 1990). Thus, ALA-synthase is bypassed and porphyrins as photosensitizers are formed, preferentially in neoplastic tissues. Subsequent light irradiation of the sensitized tissue leads to reactive species-induced tumour necrosis by targeting biological membranes (Pass, 1993) and by vascular damage with subsequent tumour cell anoxia (Goetz et al, 1990).

In dermatology, topical ALA-PDT has been used to treat precancerous lesions and neoplasms of the skin without serious adverse effects (Fritsch et al, 1996b). High efficacy of ALA-PDT was achieved for solar keratoses, superficial basal cell carcinomas, and superficial squamous cell carcinomas (Kennedy et al, 1990; Wolf et al, 1993; Calzavara-Pinton, 1995; Fritsch et al, 1996a, 1997d). In addition, there are also reports that psoriatic plaques can be beneficially treated with ALA-PDT (Boehncke et al, 1994; Stringer et al, 1996).

The presence of high intraleisional porphyrin levels is important for effective ALA-PDT. The amount of newly formed porphyrins depends on the application time of ALA. Distribution studies using porphyrin fluorescence revealed maximum fluorescence in tumours up to 24 h after topical or systemic administration of ALA (Kennedy et al, 1990; Abeles et al, 1994; Szeimies et al, 1994; Martin et al, 1995; Peng et al, 1995; Kriegmair et al, 1996). However, biochemical data on the absolute levels of porphyrin metabolites, which have been determined in untreated skin (Goerz et al, 1995), are lacking for ALA-treated lesions. In addition, the time-point of maximum porphyrin accumulation is still not exactly known for specific cutaneous lesions. Therefore, we examined the time-dependent formation of porphyrins after topical application of ALA in epithelial skin tumours, psoriatic lesions and normal skin.

MATERIALS

Tissue samples used were: basal cell carcinomas (BCC; \( n = 32 \)), squamous cell carcinomas (SCC; \( n = 32 \)), psoriatic lesions (PS; \( n = 32 \)), normal skin (NS; \( n = 160 \)). Tumour samples were obtained from patients who underwent surgery. Psoriatic tissue samples were taken by punch biopsy for histopathological examination and analysis of the porphyrin pattern.

Normal skin was examined: (a) from patients with tumours or psoriasis which were treated with ALA and (b) from subjects free of tumour or psoriasis. Tissue samples of normal skin from patients with tumours or psoriasis were either taken from the tumour- or psoriasis-adjacent site (NS-A) or from locations distant from the lesions (NS-D) = individual controls. Additionally, normal skin samples (NS) were obtained from patients free of BCC, SCC or PS = interindividual controls (Table 1). This study design was chosen to investigate the influence of the proximity of normal skin to neoplastic or psoriatic skin on ALA-induced porphyrin accumulation. Each patient received comprehensive information about the scope of the study.

METHODS

ALA-treatment and photodynamic diagnosis (PDD)

Pilot studies on topical ALA application (up to 80 mg cm\(^{-2}\)) to normal skin revealed plateau porphyrin levels (approximately 4 nmol g\(^{-1}\) protein) > 40 mg ALA cm\(^{-2}\) (data not shown). Based on
NS 0.8
BCC, SCC or PS not treated by ALA did not show any fluorescence.

Psoriasis 1.3
Fluorescence intensities of tissues treated by 20% ALA

Table 2

| Disease    | 1 h  | 2 h  | 4 h  | 6 h  | 9 h  | 12 h | 24 h |
|------------|------|------|------|------|------|------|------|
| BCC        | 0.8 ± 0.2 | 0.8 ± 0.2 | 0.8 ± 0.2 | 1.3 ± 0.2 | 1.5 ± 0.3 | 1.5 ± 0.3 | 1.5 ± 0.3 |
| SCC        | 2.8 ± 0.2 | 2.6 ± 0.2 | 2.8 ± 0.2 | 2.9 ± 0.1 | 2.6 ± 0.2 | 2.8 ± 0.2 | 2.5 ± 0.2 |
| Psoriasis  | 1.3 ± 0.2 | 1.8 ± 0.4 | 2.8 ± 0.2 | 2.8 ± 0.2 | 2.5 ± 0.3 | 2.5 ± 0.3 | 1.5 ± 0.3 |

Fluorescence intensities of ALA-treated skin irradiated by Wood’s light (370–405 nm, 10-cm distance, 5 mW cm–2). Values are given according to a fluorescence standard: 0 = no fluorescence, 3 = maximum fluorescence (n = 4; mean ± s.e.m.). Normal skin (NS) was observed in patients free of tumours and psoriasis. NS, BCC, SCC or PS not treated by ALA did not show any fluorescence.

these data and due to clinical experience in ALA-PDT (Calzavara-Pinton, 1995; Wolf et al, 1993) an ALA (hydrochloride) mixture of 20% was prepared in an ointment (Neribas®, Schering, Berlin, Germany). Of the 20% ALA mixture, 0.2 g (40 mg ALA cm–2) were applied to a 1-cm² skin area of BCC, SCC, psoriatic lesions, and all normal skin lesions. Treated skin was covered with an occlusive foil (Tegaderm®, 3M Healthcare, Borken, Germany), gauze, aluminium foil and tape to enhance tissue penetration and avoid photobleaching of the formed porphyrins. After defined incubation times (1, 2, 4, 6, 9, 12 and 24 h) the tape and the ointment were removed and the treated area was illuminated with Wood’s light (Fluotest®, Xenotest, Hanau, Germany; 370–405 nm). Fluorescence intensity was measured and expressed semiquantitatively according to a fluorescence standard as 0 (no), 1 (low), 2 (medium) and 3 (strong fluorescence). The fluorescent area was marked. Basal values were obtained from untreated controls.

According to our clinical experience, BCC and SCC reveal a strong, homogeneous, ALA-induced porphyrin fluorescence under Wood’s light (Figure 1A) (Fritsch et al, 1996a, 1997b), whereas about 20–40% of psoriatic lesions show areas with inhomogeneous or absent fluorescence (Figure 1B). For better comparison of intralesional porphyrin formation in PS and tumours, we selected only uniform fluorescing psoriatic areas for our study. Biochemical analysis was also performed in nonfluorescing psoriatic areas (n = 8) which were treated with ALA for 6 h.

Preparation of skin samples

Only superficial layers of skin samples (< 1 mm) were included in the study due to the limited penetration of topically applied ALA (Széimies et al, 1994; Martin et al, 1995; Peng et al, 1995).

Immediately after excision tissue samples were frozen in liquid nitrogen and stored at –80°C until further examination.

Determination of total porphyrin and protein levels in tissues

Tissue samples were weighed and cut into small pieces. After homogenization with an Ultraturrax and centrifugation at 3000 U min⁻¹ for 10 min, porphyrins were isolated with 1.0 N perchloric acid/methanol (1/1, v/v). In the supernatant, the total porphyrin level was assessed by fluorescence spectroscopy (Perkin Elmer LS-5, Überlingen, Germany); emission was recorded in a range of 520–700 nm at an excitation wavelength of 405 nm (Soret band). For quantification a protoporphyrin standard (Porphyrin Products Inc., Logan, Utah, USA) was used (Fritsch et al, 1997a). Protein levels were determined in the pellet according to Lowry et al (1951).

Determination of porphyrin metabolites in tissues

The supernatant was adjusted with acetic acid to pH 3–4, porphyrins were bound to talcum, esterified and metabolites were identified by HPLC with fluorescence detection (L–7480, Merck Hitachi, Darmstadt, Germany) using a porphyrin standard mixture (Porphyrin Products Inc., Logan, Utah, USA) for quantification (Seubert and Seubert, 1982). The following metabolites were analysed: protoporphyrin, tricarboxylic porphyrin, coproporphyrin, pentacarboxylic porphyrin, hexacarboxylic porphyrin, heptacarboxylic porphyrin and uroporphyrin. The last four porphyrin metabolites are designated as highly carboxylated porphyrins; data are given as the sum of these compounds.
Statistical calculation

Statistical analysis of the data was performed by Student’s t-test. Data are reported as mean ± SEM. Changes were considered statistically significant when P < 0.05.

RESULTS

Fluorescence intensities of treated tissues

The highest macroscopic fluorescence intensities were found in BCC, SCC and PS 4–6 h after ALA application. At all time-points normal skin (NS) revealed only slight fluorescence intensities compared with neoplastic and psoriatic skin (Table 2).

Basal total porphyrin levels in BCC, SCC, PS and skin (all)

In untreated tissues (basal values), the levels of total porphyrins were similarly low in normal skin, tumours and psoriasis (Figure 2).

Total porphyrin levels in BCC, SCC, PS and skin after topical application of ALA

ALA application induced porphyrin synthesis in all tissues. The porphyrin levels in BCC and SCC showed maximum values between 2 h (53.8 ± 19.3 and 46.8 ± 17.3) and 6 h (60.0 ± 13.4 and 49.4 ± 13.1 nmol mg⁻¹ protein). The highest porphyrin accumulation was measured in psoriatic lesions with 91.7 ± 14.4 nmol mg⁻¹ protein at 6 h. Normal skin samples: in NS and NS-D, the accumulation of ALA-induced porphyrins was comparably low with a maximum at 24 h (15.6 ± 6.6 nmol mg⁻¹ protein) (Figure 2); at 6 h, NS-A close to tumours or PS showed higher porphyrin levels (23.8 ± 4.0 and 19.1 ± 4.1 nmol mg⁻¹ protein) than NS or normal skin distant from lesions (NS-D). The ratio of porphyrins measured in BCC vs NS (BCC/NS) showed maximum values between 4.8–5.5 (Figure 3). The maximum ratio of porphyrins SCC/NS (4.8–5.1) was observed 1–4 h after ALA-application. In PS, there was the most distinct maximum of the porphyrin ratio to NS with 7.0 after 6 h. However, using normal skin adjacent to tumours of PS (NS-A) as reference, the ratios of porphyrin levels in lesions vs skin were much lower; maximum values were measured after 2 h for BCC (3.7), after 4 h for SCC (3.3) and after 6 h for PS (4.8).

Basal porphyrin metabolite levels in BCC, SCC, PS and NS (all)

The pattern of porphyrin metabolites was comparable in all untreated tissues with protoporphyrin as the predominant metabolite (82.9–92.3%) followed by uroporphyrin (all tissues) (4.6–6.2%), heptacarboxylic porphyrin (NS: 0.9% and PS: 1.5%), and coproporphyrin (BCC: 2.7% and SCC: 3.9%).

Porphyrin metabolite patterns in BCC, SCC, PS and NS (all) after topical application of ALA

The pattern of metabolites was not changed following ALA-treatment. Protoporphyrin was still the predominant metabolite and
accumulated in all tissues over a period of 24 h (Figure 4A and B). Only at 1 h was there a slight decrease in protoporphyrin and a slight increase in the highly carboxylated porphyrins. At the time-point of the maximum ratio of porphyrins in BCC/NS, SCC/NS or PS/NS (1–12 h, 6 h), protoporphyrin was the prevailing metabolite in the lesions (Table 3, Figure 4A and B).

DISCUSSION

In the present study the time course of porphyrin metabolite formation in human skin after topical application of ALA was investigated. The maximum levels of porphyrins in epithelial tumours and psoriatic lesions had already been detected at 1–6 h after application of ALA. This indicates that ALA is rapidly absorbed by the damaged skin and abnormal cells, and subsequently converted to porphyrins. At the time-point of the maximum ratio of porphyrins in BCC/NS, SCC/NS or PS/NS (1–12 h, 6 h), protoporphyrin was the prevailing metabolite in the lesions (Table 3, Figure 4A and B).

In general, the available data on ALA-induced porphyrin levels in human tissues are based on fluorescence studies (Warloe et al, 1992; Svanberg et al, 1994; Szeimies et al, 1994; Malik et al, 1995; Martin et al, 1995; Peng et al, 1995; Stringer et al, 1996) (Table 4) and only some biochemical studies were performed (Fritsch et al, 1997c, 1998). It was shown by fluorescence microscopy that the major factor limiting the efficacy of topical ALA-PDT is the penetration depth of ALA and ALA-induced

| Total porphyrins (nmol g⁻¹ protein) | Protoporphyrin (%) | Tricarboxylic porphyrin (%) | Coproporphyrin (%) | Pentacarboxylic porphyrin (%) | Hexacarboxylic porphyrin (%) | Heptacarboxylic porphyrin (%) | Uroporphyrin (%) |
|-----------------------------------|-------------------|-----------------------------|-------------------|-----------------------------|-----------------------------|-----------------------------|-----------------|
| NS                                | 4.7 ± 0.8         | 88.3 ± 2.8                  | 0 ± 0             | 0.6 ± 0.5                   | 0.7 ± 0.4                   | 4.0 ± 0.9                    | 7.9 ± 1.3       |
| BCC                               | 15.7 ± 1.1*       | 92.2 ± 1.2                  | 0 ± 0             | 2.1 ± 0.6                   | 0.6 ± 0.3                   | 1.1 ± 0.4                    | 5.3 ± 1.3       |
| SCC                               | 16.7 ± 2.1*       | 92.6 ± 1.7                  | 0 ± 0             | 1.9 ± 0.7                   | 0.4 ± 0.3                   | 3.6 ± 1.1                    | 7.1 ± 1.6       |
| PS                                | 21.9 ± 2.7*       | 83.8 ± 7.6                  | 0 ± 0             | 1.5 ± 1.3                   | 0.5 ± 0.4                   | 2.1 ± 1.3                    | 4.3 ± 2.7       |

Data are given as nmol porphyrin g⁻¹ protein (n = 4; mean ± s.e.m.; *P < 0.005). Porphyrin patterns showed no significant differences between tumours, psoriasis and normal skin.
porphyrins, which is dependent on the incubation time of ALA, the combination with additives and the kind of skin surface treated. In the case of BCC, the penetration was limited to a depth of about 0.75–0.81 mm (Martin et al, 1995; Peng et al, 1995) and could be enhanced by addition of dimethylsulphoxide (DMSO) and ethylenediaminetetraacetic acid disodium salt (EDTA) (up to 1.25 mm). Long-time application of ALA together with 4% EDTA for 20–48 h increased the homogeneity of fluorescence distribution, particularly at the bottom of the lesions (Peng et al, 1995).

Fluorescence studies on the kinetics of the ALA-induced porphyrins showed maximum levels in BCC and PS 4–6 h after ALA application with 3–15-fold higher levels than in normal skin (Svanberg et al, 1994; Stringer et al, 1996). These data are supported by our analysis.

In the psoriatic skin samples which showed no fluorescence 6 h after ALA application, we found no higher porphyrin levels (22.4 ± 8.2 nmol g⁻¹ protein) than in NS-A. The hyperkeratotic lesions may not offer sensitization of normal skin, which lasts up to 48 h with decreasing intensity (data not shown). Psoriatic lesions appear to be the major effect responsible for the intralesional photodynamic diagnosis (PDD), a novel, promising technique in PDT of epithelial skin tumours due to lack of severe side effects (Fritsch et al, 1996). PDT efficacy may be enhanced using ALA esters (Fritsch et al, 1998).

In the case of topical ALA-application, the accumulation of higher levels of total porphyrins in tumours and psoriasis lesions appears to be the major effect responsible for the intralesional photosensitization. Our biochemical data show that the porphyrin metabolite patterns are not significantly changed upon ALA treatment.

Intravenous injection or oral administration of ALA was shown to optimize the homogeneous intralesional accumulation of porphyrins in PS, BCC and oral cavity SCC (Grant et al, 1993; Peng et al, 1995; Stringer et al, 1996). In mice or rats bearing colon or mammary carcinomas and in hamsters bearing an amelanotic melanoma, systemically administered ALA led to earlier (1 h) and higher tumour/skin ratios (6–30) of porphyrins than did topical ALA application (Peng et al, 1992; Hua et al, 1995; Sroka et al, 1996; Fritsch et al, 1997). However, prolonged accumulation of porphyrins in the liver following intravenous injection of ALA was detected (Fritsch et al, 1997) which may be responsible for reported side effects such as nausea, vomiting or transient increases in serum aspartate aminotransferase (Regula et al, 1994; Peng et al, 1995). Thus, topical application of ALA seems to be still the most promising technique in PDT of epithelial skin tumours due to lack of severe side effects (Fritsch et al, 1996). PDT efficacy may be enhanced using ALA esters (Fritsch et al, 1998).

After topical application of ALA (for PDD or PDT), light avoidance for 2 days is recommended due to the prolonged porphyrin sensitization of normal skin, which lasts up to 48 h with decreasing intensity (data not shown). Psoriatic lesions may not offer

### Table 4: Studies on kinetics of ALA-induced porphyrins in various tissues after topical application of ALA

| Human/animals | Tissue | ALA (%) | Additive | Scale (h) | Max. (h) | Ratio Max. (h) | value | Tissues | Pre-treatment levels (h) | Method | Author |
|---------------|--------|---------|----------|-----------|----------|--------------|-------|---------|------------------------|---------|--------|
| Human         | BCC    | 20      | EDTA     | 3–48      | 29–48    | n.d.         | n.d.  | Tumour/skin | n.d.                | FM      | Peng et al, 1995      |
|               | BCC    | 20      | –        | 4–6       | 4–6      | 15           | Tumour/skin | n.d. | PS        | < 180             | LIF     | Svanberg et al, 1994 |
|               | PS     | 20      | –        | 1.5–180   | 4–6      | 4–6          | 3–10 | Psoriasis/skin | 24      | LIF     | Stringer et al, 1996 |
|               | BCC    | 20      | –        | 1–24      | 1–4      | 4.8–5.5      | Tumour/skin | 24   | PS        | > 24              | SP / HPLC | This study          |
|               | SCC    | 20      | –        | 1–24      | 4        | 1–4          | 4.8–5.1 | Tumour/skin | 24     | SP / HPLC | This study          |
|               | PS     | 20      | –        | 1–24      | 6        | 6            | 17   | Psoriasis/skin | 24     | SP / HPLC | This study          |
| Mice          | NS     | 20      | –        | 1–14      | 14       | –            | –    | –       | n.d.                | LIF     | Peng et al, 1996      |
|               | SCC    | 20      | –        | 2–24      | 10       | 10           | 4.5  | Tumour/skin | 24     | F/FM    | Veen et al, 1996     |
|               | Mamma Ca* | 20   | –        | 1–5       | 3        | 3            | 0.2  | Tumour/skin | n.d.       | SP     | Peng et al, 1992      |
|               | Colon Ca* | 20 | EDTA DMSO | 2–6       | 6        | n.d.         | 0.4  | Tumour/skin | n.d. | LiF/SP | Malik et al, 1995     |
| Rat           | Bladder Ca | 50 mg | –        | 4–24      | 4        | 4            | 2    | Tumour / skin or Tumour/Bladder | 24 | FM | linuma et al, 1995 |
| Pig           | NS     | 20      | 0.5–3    | 3         | 3         | –            | –    | –       | n.d.                | F       | Goff et al, 1992      |

*, Subcutaneously growing tumours; Ca, carcinoma; Pre-treatment levels, time-point after ALA-application which was reported to show comparable porphyrin levels prior to ALA treatment; LIF, laser-induced fluorescence analysis; FM, fluorescence microscopy F, fluorescence detection under excitation with UV/visible light; SP, spectrophotometry of extracted porphyrins; HPLC, high performance liquid chromatography; n.d. = no data.
optimum conditions for topical ALA-PDT and should be selected according to the fluorescence pattern under Wood’s light.

**ABBREVIATIONS**

ALA, δ-aminolaevulinic acid; BCC, basal cell carcinoma; DMSO, dimethylsulphoxide; EDTA, ethylenediaminetetraacetic acid; NS, normal skin; NS-A, normal skin adjacent to tumour or psoriasis lesion; NS-D, normal skin distant from tumour or psoriasis lesion; PDD, photodynamic diagnosis; PDT, photodynamic therapy; PS, psoriasis; SCC, squamous cell carcinoma.

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