# Mechanism and Optimization of PDT using ALA induced PpIX

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# Mechanism and Optimization of PDT using ALA induced PpIX

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*Aan mijn Vader, Moeder,*
*Hans en Heleen*

## Preface

Photodynamic therapy (PDT) is an experimental modality for the treatment of benign and malignant lesions. Conventional forms of PDT involve the administration of an exogenous light-sensitive compound (photosensitizer) and subsequent exposure to visible light to induce tissue necrosis.

PDT-research has been conducted in de Dr. Daniel den Hoed Cancer Centre (DDHK) in Rotterdam since 1979. The areas of research are: physical aspects, biological aspects and development of clinical applications. The physical group is mainly interested in the development of instruments for proper light delivery and light dosimetry during the treatment by PDT of, e.g., superficial bladder carcinoma and bronchogenic carcinoma. In the biological group the fluorescence and photodynamic properties of various exogenous photosensitizers are investigated. The exchange of knowledge between both groups is the strength of the PDT group in the DDHK.

In 1989 a new method of tissue photosensitization was introduced by Kennedy *et al.* Instead of administering of a photosensitizer, local photosensitization was induced by the application of 5-aminolevulinic acid (ALA), the precursor of the sensitizer protoporphyrin IX (PpIX). After application, the ALA is transformed into PpIX in the cells through the haem synthesis. In 1993 a project funded by the Dutch Cancer Society started in the DDHK called *'Photodynamic therapy and tissue fluorescence using porphyrin, endogenously generated by application of 5-amino-laevulinic acid: improvement of ALA-bioavailability".* This project was a collabaration between three research groups. The research group in the DDHK was focused on clinical studies on the treatment of superficial skin tumours by ALA-PDT and on pre-clinical studies on the fluorescence kinetics and PDT responses in different animal models after topically and systemically administered ALA. The other two research groups (Division of Medicinal Chemistry and the Division of Pharmaceutical Technology of the center for bio-pharmaceutical sciences of the university of Leiden) were focused on improvement of the bio-availability of ALA by increasing the ALA-penetration using lipophilic ALA-derivatives. The work done in Leiden was promising and three papers were published (Kloek *et al*, 1996; Kloek *et al*, 1998; Roemele *et al*, not yet published). Unfortunately, due to the sudden death of H. Bodde, one of the principal investigaters, and the lack of further funding, research on ALA-PDT in Leiden is minimal at this moment. In the DDHK a follow-up project with clinical and preclinical research using ALA-PDT was started in 1998. This thesis presents the results of the preclinical research performed in the DDHK during the project mentioned above.

Enschede, 1999
Nynke van der Veen

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# Chapter 1

## Introduction

In chapter 1 an outline is given of haem biosynthesis with a short review of the effect of abnormalities in the production of porphyrins, induced by deficiencies of enzymes or by exogenous ALA administration. The general principles of PDT are explained, followed by the basics of systemically and topically administered ALA. The clinical and preclinical studies are discussed separately and the results of some studies are mentioned as an illustration for the obtained effects and problems arising with PDT using ALA. In this chapter no *in vitro* studies concerning ALA have been included. At the end of the chapter an outline of the thesis is given according to the route of ALA administration. The items that will be discussed in this chapter with the corresponding pages are shown below.


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***haem biosynthesis***

Haem synthesis can take place in all mammalian cells but the activity is quantitatively most prominent in bone marrow and liver. A simplified scheme of the haem biosynthetic pathway is shown in Figure 1. The haem biosynthetic pathway comprises eight steps, which are distributed between the mitochondria and the cytosol of the cell. Of all the enzymes in the pathway, ALA synthase (ALAS) has the lowest activity, followed by porphobilinogen deaminase (PBGD), whereas the other enzymes have much higher activities. The rate-limiting step is the conversion of glycine and succinyl CoA to ALA, which is under negative feedback control by haem. In the normal cells the enzymatic activities are strictly regulated to prevent damage due to an accumulation of toxic substrates.

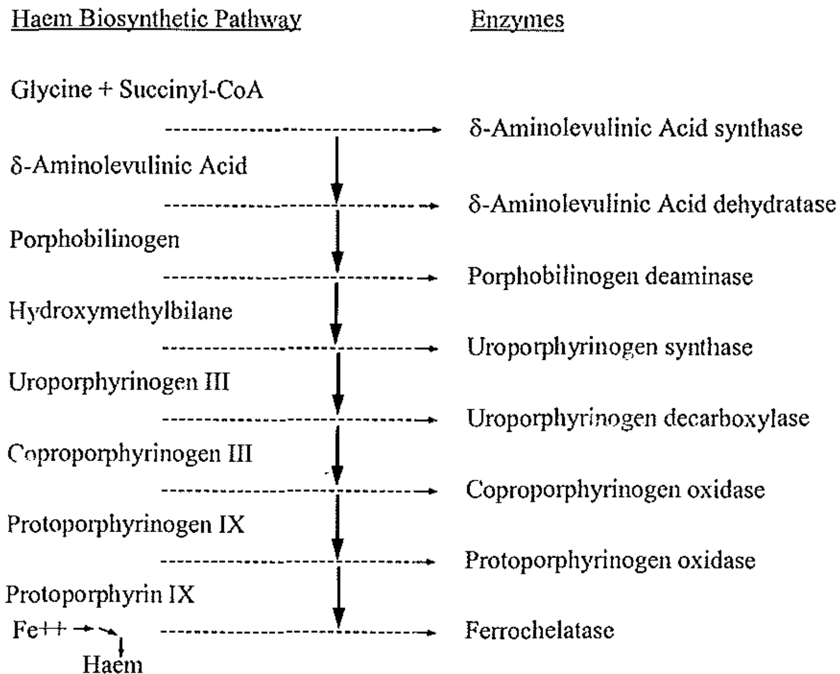


*Figure 1. Simplified biosynthetic pathway for haem.*

***Porphyrin biosynthesis in porphyria***

The effect of metabolic abnormalities in the haem biosynthetic pathway is seen in patients suffering from porphyrias. Human porphyrias are due to a partial deficiency of one of the eight enzymes. The predominant clinical manifestations of the porphyria can be divided into two general areas: neurologic abnormalities (an overproduction of porphyrin precursors) and cutaneous photosensitivity (an overproduction of porphyrins). The neurological manifestations (2 out of 8 porphyrias) are marked by recurrent, acute attacks which may be precipitated by exposure to certain drugs (antibiotics, hormones, sedatives), decreased food intake, infections and by an excess of alcohol intake. During an acute attack the primary clinical manifestations in patients are abdominal pain and psychiatric disturbances and peripheral neuropathy probably caused by the high ALA levels. In 6 out of 8 porphyrias, cutaneous photosensitivity is observed and depending on the deficient enzyme, excess ALA, PBG, copro, uro is observed in urine and protoporphyrin in faeces. Depending on the type of porphyria the clinical manifestation varies from an itching and burning sensation to progressive mutilation and disfiguration of sun exposed areas. The photosensitive porphyrias are mainly non-acute. Severely afflicted patients must live under subdued light levels to prevent skin reactions.

***Porphyrin biosynthesis in tumours***

The first observation of a selective accumulation of porphyrins in malignant tissue was by Policard (1924) who noted typical red fluorescence in tumours of humans and animals when haemolysis was present. Benign and malignant liver tumours were also found to induce the skin photosensitivity as seen in porphyrias. Accordingly, porphyrin and precursor contents were estimated in various malignant tumours including adenocarcinoma (Rasetti *et al.*, 1963) endocrine gland tumours (Zawirska *et al.*, 1979) and metastases of liver and kidney tumours (Zawirska *et al.*, 1979). By the end of the 1970's there was an increasing amount of evidence that the haem biosynthesis pathway could be disturbed in neoplastic disease. In general it was observed that the activity of PBG deaminase was increased and the activities of ALA dehydratase and ferrochelatase were reduced in various malignant and regenerating tissues (Schoenfeld *et al.*, 1987 and 1988).

***Porphyrin biosynthesis after ALA administration***

In search for a good model to investigate porphyrias, the first experimental porphyrin sensitization was performed by Meyer-Betz in 1913. He injected himself with hematoporphyrin which resulted in photosensensitization of the skin lasting for more than two months. In 1956 Berlin *et al.* induced skin photosensitivity by oral ALA administration to animals and humans. The skin of the volunteers was exposed to light when it was cloudy, sunny and sunny in combination with snow. The skin reaction ranged from erythema and tingling sensation on the face, and painful burning followed by desquamation. The skin photosensitivity after ALA administration was limited to several days.

In the 1980's ALA induced photosensitization was investigated as a tool for inducing tumour cell death by photodynamic therapy. Kinetic studies using fluorescence were performed to examine which tissues were sensitive for PpIX accumulation. It was found that normal tissues may also show high PpIX levels after systemic ALA administration. Especially epithelial surfaces like epidermis, oral mucosa, respiratory tract mucosa, vaginal mucosa, rectal mucosa, endometrium and urothelium or glands/organs in continuity with such surfaces like liver, sebaceous glands, salivary glands and seminal vesicles may accumulate PpIX. Tissues of mesodermal origin like muscle, connective tissue, cartilage and blood cells did not show significantly enhanced PpIX after administration of ALA (Kennedy and Pottier, 1992).

In 1990 Kennedy *et al.* introduced topical application of ALA to the skin. Local photosensitization was induced and the PpIX accumulation was found to be sufficient to produce damage to superficial actinic keratoses (AK) and squamous cell carcinoma (SCC) upon illumination. This type of photosensitization drew much attention and a large number of papers have been published on treating superficial non-melanoma skin lesions using topical ALA-PDT.

### *Photochemical reaction*

Protoporphyrin IX can be excited by light of an appropriate wavelength corresponding with the absorption spectrum of the sensitizer. The wavelengths applied with ALA-PDT are commonly 514,5 nm and between 632 and 635 nm. By absorbing photons PpIX can be excited to its excited singlet state and this excited singlet state can then decay back to the singlet ground state and emit light (fluorescence) without altering the PpIX molecule. After excitation to the excited singlet state the sensitizer can also undergo intersystem crossover to its excited triplet state. Then, in the presence of oxygen the triplet excited state photosensitizer exchanges its energy with oxygen through collisions, which will lead to excited (singlet) oxygen (Type II reaction). The triplet photosensitizer can also react with biological substrates and form radicals, which can react with oxygen and form oxidized products (Type I reaction). The triplet photosensitizer thus falls back to the singlet ground state (Dougherty *et al.*, 1984).

### *Fluorescence*

With ALA-PDT, fluorescence is a useful tool to determine the quantity, location and the kinetics of PpIX. HPLC measurements, cryomicroscopy and non-invasive measurements are used to determine the fluorescence. Each method has it own (dis-)advantages which makes a combination of methods necessary. In this thesis non-invasive fluorescence in combination with cryomicroscopy measurements were performed. Three main problems arising with non-invasive fluorescence detection are briefly discussed. One problem may be the relatively high autofluorescence signal of several tissues like the skin. Autofluorescence is the fluorescence of the tissue without the sensitizer. It is due to collagen, NADH and keratin and has an excitation optimum around 400 nm. Depending on the site of the tumour the autofluorescence may strongly contribute to the fluorescence signal which consists of PpIX fluorescence and

autofluorescence. A method to overcome this problem is with dual wavelength excitation. The fluorescence of the target tissue is excited with two wavelengths, one of which is absorbed by both the sensitizer and the autofluorescence and a second wavelength at which only the autofluorescence is excited. By subtracting both signals the sensitizer signal can be estimated. A second problem using fluorescence detection may be the type of tissue surrounding the tumour. As mentioned before, proliferating and metabolically active cells can also produce considerable PpIX levels after ALA administration. The lesion may be difficult to detect when it is located in the liver or on the skin. The third problem is that the specificity of PpIX fluorescence after ALA administration is not high. Necrotic tissue or inflammation reactions may also fluoresce after ALA administration.

***Photodynamic therapy***

Singlet oxygen is the most important agent for inducing tissue damage with PDT. Singlet oxygen is a very short lived product (Foster *et al.*, 1991) and will therefore act locally (Moan, 1990). The site of damage to the cell depends on the site of PpIX production and the localisation of the PpIX molecule within and outside the cell. PpIX is a lipophilic photosensitizer and will mainly cause damage to membranes (e.g. lysosomes, mitochondria, cytoplasm) after exposure to light (Moan *et al.*, 1989; Hilf *et al.*, 1992). Other PDT induced reactions, including apoptosis and the release of vascular mediators, have been described. Besides by this direct effect, an indirect effect may be necessary to achieve complete tumour destruction. Henderson *et al.* (1995) observed in tissues removed immediately after the illumination no complete tumour destruction. Depletion of nutrients by vascular shut down, for a certain amount of time, seems necessary to induce complete tumour destruction.

During PDT the concentration of oxygen in the tissue is reduced by oxygen consumption in the oxidative reactions taking place. The oxygen concentration during illumination is dependent on the rate of oxygen consumption and the rate of reoxygenation of the cell. Three factors influence the oxygen concentration in the cell during treatment.

a Vascularisation of the tissue.

The vascular development can barely keep up with rapidly proliferating tumours. Solid tumour masses have abnormal blood vessel networks unlike vessels in normal tissues. The large intercapillary distances in tumours lead to hypoxic areas (Chapman, 1984) that are less sensitive to PDT (Henderson and Fingar, 1987). During illumination the hypoxic area may expand when the rate of oxygen depletion is higher than the rate of reoxygenation. This effect is more likely to occur in tumour tissues than in normal tissues.

b Therapeutic conditions.
The concentration of PpIX in the cell, the wavelength and the fluence rate of the light applied influence the rate of oxygen consumption during illumination. With a higher PpIX concentration the oxygen consumption is larger. The wavelength used in systemic ALA-PDT is often 514,5 or between 632 and 635 nm. At the lower wavelength the absorption of light is higher which results in a faster oxygen consumption compared to an illumination with 635 nm. Illuminating with a high fluence rate also results in a higher oxygen consumption.

c Vascular constriction.
During illumination vascular constriction may occur. Constriction of arterioles is observed immediately after the start of the illumination, and the venules follow in time during the illumination. When the illumination is stopped the constriction is largely reversible. The explanation for this effect is not yet clear but the consequence is a strongly reduced oxygen concentration in the cell due to a limited reoxygenation. The constriction is believed to occur only after systemically administered ALA. Several studies indicate however that also after topical ALA vascular shutdown occurs. The fluence rate used influences the severity of the vascular constriction. A low fluence rate results in less constriction (this thesis, chapter 6).

***Routes of ALA administration***
ALA can be administered systemically (intravenously and intraperitonially in animals and orally in humans) and topically. Because both routes have their own target tissues and problems they will be discussed separately. For systemic ALA administration more preclinical studies than clinical studies have been published, for topical ALA administration this was reverse. Therefore, preclinical studies on systemic administration will be discussed first whereas for topical ALA-administration clinical studies will be discussed before the preclinical studies.

***Systemic ALA administration***

*Preclinical studies*
In animal studies ALA dissolved in PBS was administered intravenously (i.v.) or intraperitonially (i.p.). The drug doses varied between 100 and 1200 mg/kg but at the high drug dose animals showed toxic reactions and some died. Several studies have been published using orally administered ALA (Van den Boogert *et al.*, 1998; Loh *et al.*, 1993; Van Hillegersberg *et al.*, 1992). Loh *et al.* (1993) examined the difference in PpIX accumulation between orally and i.v. administered ALA (bolus). They observed that i.v. and orally administered ALA gave similar PpIX fluorescence kinetics in tissues, although a higher oral dose was required to achieve the same amount of porphyrins as by i.v. injection. Van Hillegersberg *et al.* (1992) dissolved ALA in the drinking water of rats for 2-11 days which resulted in detectable PpIX levels in a colon tumour transplanted in the liver.

In the initial animal studies PpIX levels in different normal tissues and tumours after ALA administration were determined. In general it was found that in most normal tissues deriving from ecto- and endoderm and in tumours PpIX levels peaked at approximately 1-8 hours after ALA administration and were almost completely eliminated within 24 hours. The interval to maximal fluorescence was dependent on the drug dose used. With a high ALA dose PpIX levels peaked later than with a low ALA dose (Bedwell *et al.*, 1992). Higher PpIX levels could also be obtained by fractionated ALA administration instead of a bolus (Regula *et al.*, 1995). In general it was found that the fluorescence kinetics of PpIX probably depends upon the type of normal tissue and tumour, ALA dose and fractionated ALA administration.

After determining the interval of maximum tissue fluorescence, PDT studies were executed. Although detectable PpIX levels occurred, systemic ALA-PDT often produced insufficient and unreproducible damage.

Present studies are focussed on improving the PDT-efficacy. Besides increasing the bioavailability of ALA, optimization of the oxygen concentration during illumination is being investigated. Messmann *et al.* (1995) observed an increase in depth of necrosis of normal rat colon after a single interruption of about one minute. Hua *et al.* (1995) and Curnow *et al.* (1999) obtained a considerably increased delay in tumour growth when the applied light was fractionated (30 s light, 30 s no light). De Bruijn *et al.* (1999) observed in a solid tumour model an increased tumour growth delay after twofold illumination.

*Clinical studies*

There is a limited number of publications about systemic ALA-PDT in patients. Grant *et al.* (1993) reported the first clinical results for oral ALA. Patients with oral cancer received ALA dissolved in orange juice in doses of 30 and 60 mg/kg ALA. Besides mild, transient nausea or/and transient abnormalities of liver function no other side effects or neurotoxic symptoms were observed. PpIX induced fluorescence peaked 4-6 hours after administration of ALA, depending on the dose, and had disappeared after 24 hours. Several studies were published of limited numbers of patients with rectal adenomas following surgery (Warloe *et al.*, 1995), gastrointestinal tumours and polyps (Regula *et al.*, 1995; Mlkvy *et al.*, 1995), cancer and dysplasia in Barrett's oesophagus (Barr *et al.*, 1996). In all cases only partial necrosis of non-superficial or even superficial tumours could be obtained.

Because of toxicity, the PDT efficacy can not be increased using higher oral ALA doses. A formulation for intravenous ALA administration to patients may resolve this problem. Treatment schemes obtained from animal studies seems not successful in patients. Fan *et al* (1996) treated patients with precancer and cancer of the oral cavity with unsatisfactory results also after fractionated light doses and twofold illuminations. It should be noted that the low ALA doses used in this study are likely to account for the lack of damage after PDT.

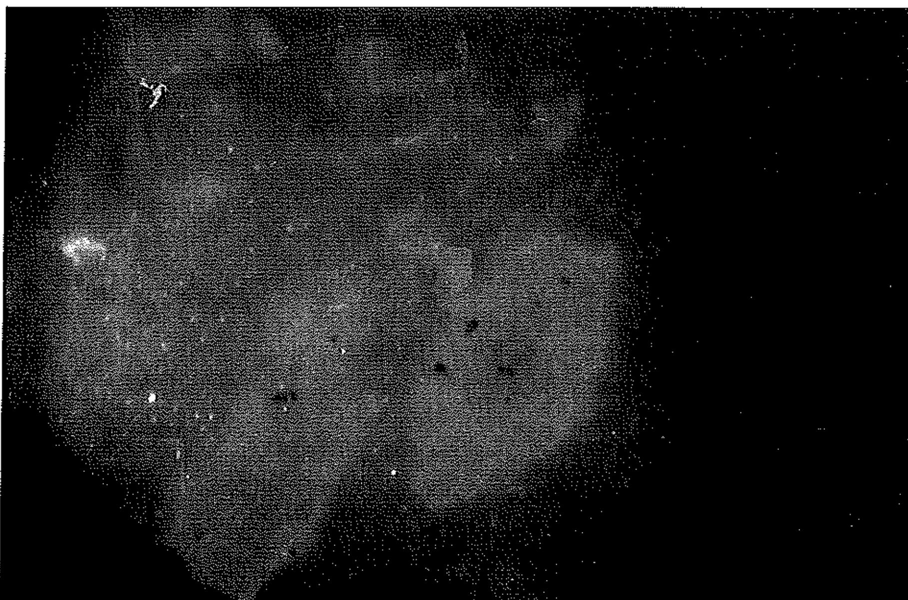

*Figure 2. Fluorescence image of human basal cell carcinoma following a 4 hour ALA-application period. The fluorescence was excited with 514.5 nm light and detected through an OG 570 high pass filter.*

***Topical ALA administration***

*Clinical studies*

The ALA-PDT protocol used by Kennedy *et al.* (1990) still forms the basis for much clinical research to date. The treatment consists of local application of a 20% ALA solution to the target area for 3 to 8 hours followed by illumination. After the application red PpIX fluorescence can be observed in the tumour which is clearly demarcated from the surrounding normal skin (illustration in Figure 2). The photosensitized tumour with a margin of surrounding skin is illuminated with light of a suitable wavelength and dose which results in superficial tumour necrosis. In the past 8 years many investigators studied the efficacy of ALA-PDT for the treatment of superficial actinic keratoses (AK), basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and Bowen's disease. Long term histological complete responses were 92% for AK, 87% for BCC, 81% for SCC and 85% for Bowen's disease (Cairnduff *et al.*, 1994; Lui *et al.*, 1995; Meijnders *et al.*, 1996; Stables *et al.*, 1997; Svanberg *et al.*, 1994; Wolf *et al.*, 1993). Nodular BCC and SCC were also treated with ALA-PDT but the results were disappointing in all studies (between 0 and 50% complete clinical response

rate). Fink-Puches *et al.* (1998) reported a projected disease free rate of 36 months after ALA-PDT of 50% for superficial BCC and only 8% for superficial SCC. The light source, fluence rate and light fluence applied varied markedly between the different studies. Although the results of treating non-melanoma skin lesions with ALA-PDT are promising, large variations within and between patients have been observed. These variations in combination with the large variations in the treatment conditions like wavelength, fluence rate and light fluence, make the results of comparative studies difficult to interpreted. In several studies the fluorescence of the tumour after topically applied ALA was monitored. In normal skin the fluorescence was limited to the epidermal layer and the skin appendages. Also, it was found that the grade of lesions correlated inversely with the level of PpIX fluorescence after topical ALA-application (Jeffes *et al.*, 1997; Szeimies *et al.*, 1996). PpIX fluorescence could only be detected superficially and in nodular lesions no fluorescence was detected in the deeper layers as demonstrated by Martin *et al.* (1995).

The stratum corneum (SC), the protective layer of the skin (Goff *et al.*, 1992) plays a crucial role in the penetration of ALA through the skin. The thickness and the composition of the SC above skin lesions like AK, SCC and BCC is different from normal skin which generally results in an increased penetration of ALA through the abnormal skin. In the SC layer there is hardly any metabolic activity and the SC acts like a passive diffusion medium. The penetration of the drugs in general is correlated with the thickness and composition of the SC (Maibach, 1971). The thickness and composition of the SC vary strongly between different parts of the body. Non-melanoma skin lesions occur most frequently on the head, neck and hands, parts of the body habitually exposed to sunlight. In humans, the skin and AK on the face and scalp show a higher fluorescence intensity than on the extremities or the trunk. (Jeffes *et al.*, 1997; Szeimies *et al.*, 1996). Subsequently, after light irradiation a significantly better response of AKs on the face and scalp (91% complete response) was observed than on the trunk and extremities (45% complete response) (Jeffes *et al.*, 1997).

*Options to improve the therapeutic effectiveness of ALA-PDT.*

Although topical ALA-PDT is promising for superficial lesions, the effectiveness needs to be improved to compete with conventional treatments. Also, the effectiveness of ALA-PDT for nodular lesions must be improved. Several options, mainly focused on improving the ALA penetration through the skin, will be discussed briefly.

- Penetration enhancer. Dimethylsulfoxide (DMSO) is a skin penetration enhancer which is often used in dermatopharmacology for topically applied drugs. DMSO increased the fluorescence after ALA-application of nodular lesions (Peng *et al.*, 1995). However, the PDT effect of treating nodular lesions was not clearly improved (Warloe *et al.*, 1995; Peng *et al.*, 1995).
- Penetration enhancer in combination with an iron chelator. Ethylene-diamine-tetra-acetic acid disodium salt (EDTA) is a chelator and reduces the iron concentration in the tissue and therefore reduces the conversion of PpIX to haem. A combination of ALA with

EDTA/DMSO applied simultaneously or DMSO applied as a prefactor resulted in an increased fluorescence in nodular BCC, although the fluorescence distribution was still inhomogeneous in some cases. Especially the efficacy of PDT for nodular lesions smaller than 2 mm improved after the combination therapy (Orenstein *et al.*, 1995; Warloe *et al.*, 1995). In this group lesions treated with ALA alone showed a complete response rate of 67% whereas lesions treated with a combination of ALA/DMSO/EDTA showed a complete response rate of 91%. The complete response rate of superficial BCCs was not improved (Warloe *et al.* , 1995).

- Prolonged ALA-application. In clinical studies ALA is mostly applied for 3 to 8 hours. Szeimies *et al.* (1993) reported that an application interval of 4 h was too short to detect substantial fluorescence in BCC. Only after 12 h of application, fluorescence was detectable in tumour cells in the deep dermis. Also, application intervals of 16 to 19 hours has been used in clinical studies (Meijnders *et al.*, 1996). After this long interval the fluorescence of the surrounding skin was similar to the fluorescence of the tumour. No comparative studies between a short and a long ALA application interval have been performed to determine the differences on the ALA-PDT induced damage.

- Repeated ALA-PDT. Repeated topical ALA-PDT has been used for lesions that failed to respond to previous treatments. The number of treatments varies. Calzavara-Pinton (1995) treated lesions every other day until complete clinical disappearance. Repeated treatments, which mostly consisted of 2 additional ALA-PDT treatments, did not clearly improve the complete response rate for superficial lesions and for small nodular lesions compared to a single treatment. Only for nodular BCC larger than 2 mm did the complete response rate increase from 43% after a single treatment to 76% after repeated treatments (Svanberg *et al.*, 1994; Warloe *et al.*, 1995; Fijan *et al.*, 1995). Using long application intervals (up to 24 h) and repeated treatments (up to 8 times) cutaneous T-cell lymphoma could be histologically cleared (Shanler *et al.*, 1994).

- Iontophoresis. Iontophoresis is a successful technique to deliver water soluble drugs into the skin. It involves the active delivery of small charged molecules into the skin by the application of an electrical current. Rhodes *et al* (1997) applied this method to normal human skin and depending on the current applied they observed a high increase in fluorescence of the skin compared to skin only with ALA and using only passive diffusion.

*Other clinical applications for topical ALA-based PDT*

Promising topical ALA application methods are instillation of ALA in the bladder to detect and treat carcinoma in situ (Eder *et al.*, 1995; Kriegmair *et al.*, 1994; Stenzl *et al.*, 1996) and inhalation of ALA aerosols to detect and treat early cancer in the bronchi (Baumgartner *et al.*, 1996). Nonneoplastic skin diseases in which the use of topical ALA-PDT is under investigation are, psoriasis (Nelson *et al.*, 1995), condyloma acuminata (Frank *et al.*, 1996) and hirsutism (Grossman *et al.*, 1995).

*Preclinical studies*

Most studies have been performed on tumours transplanted subcutaneously in rats and (nude) mice. Similar experimental conditions were applied as in the clinical studies. With in-vivo and ex-vivo fluorescence studies it was observed that the fluorescence in normal skin was localized in the epidermis, hair follicles and sebaceous glands (Henderson *et al.*, 1995; Peng *et al.*, 1992). The fluorescence of the skin overlying the tumour was the highest followed by normal skin and the least fluorescence was observed in the tumour (Henderson *et al.*, 1995; Peng *et al.*, 1992). The tumour growth delay after topical ALA-PDT was only minor (Cairnduff *et al.*, 1995; Henderson *et al.*, 1995; Peng *et al.*, 1992). Henderson *et al.* (1995) examined the perfusion of the skin after illumination and observed less blood flow compared to the blood flow before the illumination. This indicates that also after topical ALA vascular constriction may occur. These results were supported by our experiments as shown in chapter 6. Malik *et al.* (1995) reported that in a subcutaneously transplanted colon tumour the fluorescence could not significantly be increased after applying ALA in combination with DMSO/EDTA.

***Concluding remarks***

In this chapter the different ALA administration routes have been discussed briefly. With PDT using systemically administered ALA the maximum depth of necrosis in tissues is insufficient, in both preclinical and clinical studies. Topical ALA-PDT for the treatment of superficial non-melanoma skin tumours in humans seems successful. The PDT-efficacy in nodular skin tumours is insufficient.

*Outline of the thesis*
In this thesis the effectiveness of PDT following systemic and topical ALA administration was investigated in different animal models. With both administration routes the fluorescence kinetics were first determined and based on the kinetics, intervals to a therapeutic illumination were chosen. Based on the observed damage effects new treatment protocols were assessed to improve the effectiveness of PDT.

*Studies using systemically administered ALA*

Chapter 2
The rat skinfold observation chamber contains a thin layer of subcutaneous tissue (approx. 0.6 mm thick) with paired small arterioles and venules, into which a syngeneic tumour (mammary tumour) can be transplanted. In this model the fluorescence kinetics after two different doses of systemically administered ALA were monitored. At the time of maximum difference of fluorescence between the tumour and the surrounding tissue, the chambers were illuminated and the differences in effects between a high and a low ALA dose were examined. *During* the illumination vascular constriction of both the arterioles and venules of the subcutaneous tissue was found. The PDT-effect on the bloodflow in arterioles, venules and capillaries of subcutaneous tissue and tumour tissue *after* the illumination was monitored until 7 days post PDT. Based on the observed damage effects treating at maximum selectivity, an illumination earlier after ALA administration and multiple illuminations (early p.i. and at maximum selectivity) were applied after the highest ALA dose to successfully increase the effectiveness of PDT.

Chapter 3
The solid tumour model is an isologous rhabdomyosarcoma which grows subcutaneously on the thigh of rats. When the tumour reached a diameter of 4-5 mm ALA was administered systemically and an illumination was given through the skin. After illumination the tumour growth was measured using callipers and the macroscopical damage effects of the illuminated skin were determined. The solid tumour model was used to rapidly evaluate the ALA-PDT effects of different treatment schemes after systemically administered ALA. The effect of interval p.i. to illumination (PpIX concentration), reduced fluence rate (less constriction and less oxygen consumption), short term light fractionation schemes (reoxygenation) and long term light fractionation scheme (reoxygenation, new PpIX) on the tumour growth delay were determined.

*Study concerning the difference in fluorescence between systemically and topically administered ALA*

Chapter 4
The hairless mouse model develops small skin lesions like AK and SCC on the back as a result of daily UVB illumination. These tumours show a strong resemblance with sunlight induced skin lesions like AK and SCC observed in humans. In this chapter the differences in fluorescence kinetics between intraperitonially and topically administered ALA were determined. At the time of maximum fluorescence tissues were harvested and the localisation of fluorescence was determined using fluorescence cryo-microscopy

*Studies using topically applied ALA*

Chapter 5,6
In chapter 5 and 6 the effectiveness of topically applied ALA-PDT was examined in the hairless mouse model. Based on the fluorescence kinetics presented in chapter 4, two intervals for illumination were chosen: immediately after a 4 hr ALA-application (clinical protocol) and at maximum tumour fluorescence, 6 hours after the end of the 4 hr ALA-application (t=10). The photobleaching during and the fluorescence spectra during PDT were determined to obtain information about the bleaching rate (Chapter 5). In the same chapter re-appearance of fluorescence is shown following PDT. Also, the fluorescence spectra were measured to determine whether the new fluorescence after PDT showed the characteristic PpIX spectrum. In Chapter 6 the results are presented of illumination immediately after the application interval or at maximum fluorescence intensity. The development and recovery of damage to the illuminated area was determined macroscopically up to a maximum of 3 weeks after PDT. Vascular effects during the illumination was examined with two methods: fluence-rate measurements under the skin during the illumination and fluorescein dye exclusion measurements immediately after the illumination. Subsequently, the effect of the fluence-rate and long light fractionation (illuminations at t=4 and t=10) on the effectiveness of PDT was determined.

Chapter 7
In this chapter the PpIX-fluorescence kinetics in normal and tape-stripped human skin was determined during and after ALA application. To examine differences in fluorescence between various locations, the fluorescence was determined in areas on the flexor of the forearm and the lower part of the back. In addition, the effect of a prolonged application interval of 16 hours was compared with an application interval of 4 hours. The fluorescence was excited with 3 different wavelength bands, violet (400 ± 30), green (500 ± 20) and red (625 ± 20) light.

# Chapter 2

## In vivo fluorescence kinetics and photodynamic therapy using ALA-induced porphyrin: increased damage after multiple irradiations.

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## Abstract

The kinetics of fluorescence in tumour (TT) and subcutaneous tissue (ST) and the vascular effects of photodynamic therapy (PDT) were studied using protoporphyrin IX (PpIX), endogenously generated after i.v. administration of 100 and 200 mg/kg 5-aminolevulinic acid (ALA). The experimental model was a rat skinfold observation chamber containing a thin layer of ST in which a small syngeneic mammary tumour grows in a sheetlike fashion. Maximum TT and ST fluorescence following 200 mg/kg ALA was twice the value after 100 mg/kg ALA, but the initial increase with time was the same for the two doses in both TT and ST. The fluorescence increase of ST was slower and the maximum fluorescence was less and appeared later compared to TT. Photodynamic therapy was applied using green argon laser light (514.5 nm, 100 $J/cm^2$). Three groups received a single light treatment at different intervals after administration of 100 or 200 mg/kg ALA. In these groups no correlation was found between the fluorescence intensities and the vascular damage following PDT. A fourth group was treated twice and before the second light treatment some fluorescence had re-appeared after photobleaching due to the first treatment. Only with the twofold light treatment was lasting TT necrosis achieved, and for the first time with any photosensitizer in this model this was accomplished without complete ST necrosis.

## Introduction

Photodynamic therapy (PDT) is an experimental cancer treatment modality using photosensitizers that can produce tissue destruction upon absorbing light of an appropriate wavelength and dose. The photosensitizer Photofrin, a derivative of hematoporphyrin, is currently under clinical investigation. Photofrin consist of a mixture of porphyrins, hydrophillic and hydrophobic, which have different fluorescent and photodynamic properties (Kessel, 1982; Dougherty, 1987). The hydrophillic components fluoresce but are less photodynamically active, whereas the hydrophobic and photodynamically active components generally lack fluorescence. Therefore, the amount of tissue fluorescence after photosensitizer administration is not directly related to the possible PDT effect. Besides this complexity, the major side effect of Photofrin administration is prolonged photosensitivity of the skin (Dougherty *et al.*, 1990). These drawbacks have stimulated a search for better photosensitizers for tumour localization and PDT.

An alternative to administering exogenous photosensitizer is to stimulate cells to generate their own photosensitizer. This can be achieved by 5-aminolevulinic acid (ALA) which can be converted in situ into protoporphyrin IX (PpIX). ALA is present in all mammalian cells and is the first committed intermediate in the haem biosynthesis pathway. Exogenous ALA bypasses the feedback control, which is regulated by haem, and can therefore induce an intracellular accumulation of PpIX. PpIX is an effective photosensitizer which is associated with the skin phototoxicity seen in patients with porphyria (Jarrett *et al.*, 1956; Shanley *et al.*, 1972). Animal experiments have shown that after intravenous (i.v.) ALA administration only certain types of tissues manifest PpIX fluorescence (Bedwell *et al.*, 1992; Loh *et al.*, 1992). This tissue-specific photosensitation provides a basis for using ALA-induced PpIX for tumour localisation and photodynamic therapy. In contrast to Photofrin, the fluorescence intensity after ALA administration is an indication of the PpIX concentration in tissues (Loh *et al.*, 1993). Therefore, fluorescence kinetics can probably be used for determining the optimum interval between ALA administration and light treatment. Another advantage of ALA induced PpIX is the various possibilities for administration of ALA, besides i.v.. Promising results have been obtained in treating basal cell carcinomas using topically applied ALA (Kennedy and Pottier, 1992). With topical application an enhanced selective effect with PDT can be expected because of the restriction of the induced sensitizer to the lesion and the immediately surrounding normal skin. Loh *et al.* (1993) reported about selective fluorescence of inoperable rectal adenocarcinomas after oral administration in two patients. Grant *et al.* (1993) treated patients suffering from oral cavity squamous cell carcinomas with PDT after oral ALA administration. They found no side effects and a rapid clearance of the sensitizer within 24 hours.

In this paper we describe the fluorescence kinetics of PpIX, in rat mammary tumour and subcutaneous tissue, after two i.v. doses of ALA. Based on the fluorescence dynamics different treatment time-points post injection (p.i.) were chosen and a comparison was made between the photodynamic effects with the two drug doses and different intervals between ALA administration and light treatment. Finally, the vascular effects during and after treatment and the role of these effects on the outcome of tumour tissue necrosis are discussed.

## Materials and Methods

### *Animal model*

All studies were performed on 12 weeks old female WAG/Rij rats weighing 110-120 g and supplied by ITRI/TNO, Rijswijk, The Netherlands. The model used was a skinfold observation chamber previously described by Reinhold *et al.* (1979). The transparent chamber (1 cm diameter of visible tissue) contains a thin layer of subcutaneous tissue (approx. 0.6 mm thick), with paired small arterioles (approx. 0.03 mm) and venules (approx. 0.1 mm), in which a transplantable tumour can grow in a sheet-like fashion. Briefly, the chamber was attached to a piece of mica (4.5 × 2.5 cm) which was subcutaneously implanted in the skinfold. A piece of plastic was placed over the skinfold to protect the chamber. After two weeks preparation time a small piece of syngeneic mammary carcinoma (0.5 $mm^3$) was transplanted into the tissue close to a large blood vessel. During all surgical procedures Hypnorm (fluanisol/fentanyl mixture, Janssen Pharmaceutics, Belgium) was used as an anaesthetic and Garamycin (Essex Laboraties) was administered to prevent bacterial infection. To ensure adequate tumour growth the animal was kept in a temperature controlled cabinet at 32 °C. The ambient light level was less than 30 $mW/cm^2$, with a 12/12 h light/dark interval, to prevent unwanted photodynamic damage after sensitization of the animal. Approximately one week after transplantation, when the tumour had grown to about 3 mm diameter and adequate circulation in both tumour and subcutaneous tissue had established, experiments were started.

### *Fluorescence kinetics studies*

The fluorescence set up consisted of a CCD camera with a two stage image intensifier and a 25 mm Leitz Photar macrolens. Fluorescence was excited with 514.5 nm Argon laser light using 0.1 $mW/cm^2$ and fluorescence was detected through a high pass coloured glass filter (RG 665). For each recording the chamber was exposed to the excitation light for a period of 60 seconds. About 10 recordings were made, so that the maximal light dose was 0.06 $J/cm^2$ which proved to be sufficiently low to avoid photodynamic damage. Animals were anaesthetized with Hypnorm and placed on a temperature controlled stage under the CCD

camera. An autofluorescence image was recorded before i.v. ALA administration. ALA was obtained as hydrochloride in 98% pure powder form from Sigma Chemie (Bornem, Belgium). It was dissolved in phosphate buffered saline and immediately administered i.v. via a tail vein. Two groups, each of six animals, received 100 or 200 mg/kg ALA and at various time intervals from 30 up to 360 min p.i. fluorescence images were recorded and stored in the computer. Fluorescence was quantified digitally by calculating the mean grey scale value within selected areas of the recorded fluorescence image. All fluorescence measurements, except those recorded after a light treatment (Fig 3), were corrected for their respective autofluorescence signals in the same area before ALA administration.

*Phototherapy studies*

Four groups, each of six animals receiving 100 or 200 mg/kg ALA, were treated at different starting points p.i.. The treatment starting points were based on the results obtained from the fluorescence experiments (Table I). Animals were anaesthetized and placed on a temperature controlled stage of a microscope. The circulation of arterioles and venules of subcutaneous tissue (ST) and the capillary beds of ST and tumour tissue (TT) was observed and through an optical fibre and a lens system with diaphragm a uniform beam of 514.5 nm light, with a power density of 100 $mW/cm^2$, was projected through the stage onto the back of the entire chamber. Green light was chosen for convenience, since this was also used for fluorescence excitation and is at least as effective for PDT as red light. The tissue layer of the chamber is so thin that green light penetration is sufficient, and we saw no difference in damage between both sides of the chamber. The treatment dose was 100 $J/cm^2$ which required a treatment time of 17 minutes. After 5 minutes (30 $J/cm^2$) the irradiation was briefly interrupted to see whether immediate constriction of the vessels occurred. It should be noted that TT only contains small capillaries and no large venules and arterioles like ST. Up to 7 days after treatment the status of the circulation was determined daily. The total damage to TT and ST was translated into a score on a 0 to 8 scale, i.e. nine levels. Damage scores during and at the end of the treatment were based on other effects than those observed at 1 up to 7 days after treatment. During treatment effects like ischaemia, constriction and stasis were observed. From 1 up to 7 days after treatment vascular stasis was the predominant observation and was therefore also the dominant factor in the damage score (Table II). If the circulation of TT had not completely recovered after 7 days, the content of the chamber was transplanted into the flank of the same animal to see whether regrowth occurred.

**Table 1.** Summary of the phototherapy studies on four groups of animals receiving 100 or 200 mg/kg ALA and treated at different times p.i. (max.= maximal, diff.= difference, TT = tumour tissue, ST = subcutaneous tissue).

| Group | ALA dose (mg/kg) | Light dose ($J/cm^2$) | PDT p.i. (min) | Fluorescence at PDT |
|---|---|---|---|---|
| A | 100 | 100 | 120 | max. diff. TT-ST |
| B | 200 | 100 | 150 | max.diff. TT-ST |
| C | 200 | 100 | 60 | 1/3 of max.diff. TT-ST |
| D | 200 | 100/100 | 60/150 | 1/3 and max.diff. TT-ST |

## Results

*Fluorescence kinetics studies*

The fluorescence data of TT and ST after i.v. administration of 100 mg/kg ALA are shown in Figure 1a and those of 200 mg/kg ALA in Figure 1b. All values in these graphs were corrected for background fluorescence using the autofluorescence image, which had a grey scale level of 25 for both TT and ST. With both drug doses, at all intervals recorded, no fluorescence of the blood vessels could be detected. For TT an almost similar increase of fluorescence was observed during the first 90 min p.i. for both drug doses. After 90 min p.i. the fluorescence intensity in the 100 mg/kg group levelled off reaching a peak at 150 min p.i. after which fluorescence intensity declined rapidly. With 200 mg/kg ALA an increase could be observed up till 240 min p.i. and maximal fluorescence intensity was almost twice the value compared to 100 mg/kg ALA. Fluorescence of ST after both doses increased more slowly compared to TT. With both doses nearly the same increase of fluorescence was observed during the first 150 min. After 150 min p.i. fluorescence maintained the same level during at least 120 min with 100 mg/kg. With 200 mg/kg an increase could be observed up till 330 min p.i. and the maximal intensity was twice compared to 100 mg/kg. With both doses the fluorescence intensity of TT and ST had returned to background level at 24 hours p.i..

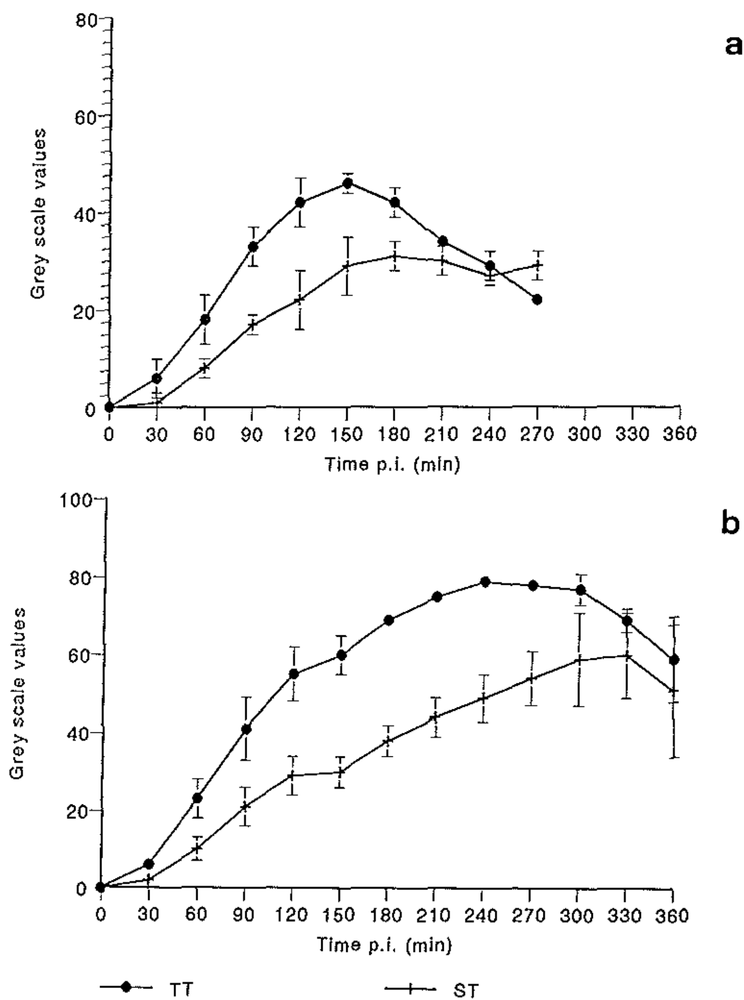



*Figure 1. Fluorescence kinetics, ± standard error of mean (s.e.m.), expressed as grey scale values, of tumour tissue and subcutaneous tissue after administration of 100 (a) and 200 mg/kg (b) ALA. Both doses were studied with six animals each. Fluorescence measurements were corrected for their respective autofluorescence.*

**Table II.** Circulation damage scores used for quantification of vascular damage by PDT to tumor tissue and subcutaneous tissue in the skin fold observation chamber. Intermediate scores were assigned to damage levels between those defined in the table (capil = capillaries; rbcc = red blood cell column).

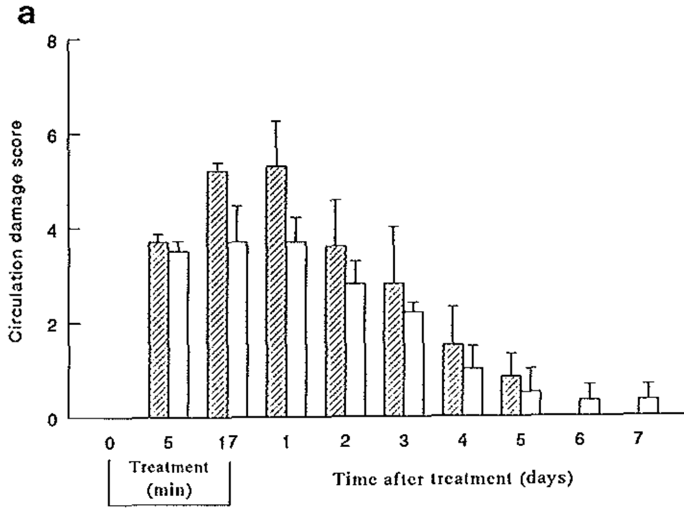
| Circulation | Subcutaneous tissue | | Tumour tissue | |
|---|---|---|---|---|
| Damage Score | During and after PDT | 1-7 days after PDT | During and after PDT | 1-7 days after PDT |
| 0 | no observable damage to capillaries or venules | | | |
| 2 | Arteriolar spasm | ≈ 25% capillary stasis | Ischaemia | ≈25%stasis capil. |
| 4 | ≈25% capillary stasis Mildly reduced RBCC in venules and arteriolar spasm | ≈ 50% capillary stasis Mildly reduced blood flow in vessels | Dilatation and ≈50% capillary stasis | ≈50% capillary stasis |
| 6 | ≈75% capillary stasis Strongly reduced RBCC in venules and arteriolar spasm | ≈70% capillary stasis Strongly reduced blood flow in vessels | Dilatation and ≈75% capillary stasis | ≈75% capillary stasis |
| 8 | no observable circulation to capillaries or venules | | | |

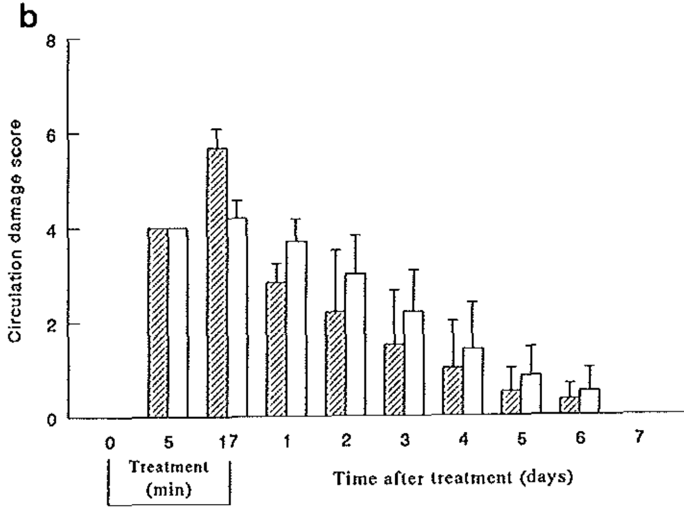
*Phototherapy studies*

Vascular effects of ALA-PDT were examined in four groups of six rats each, differing in ALA drug dose and/or treatment starting-point p.i. (Table I). Treatment starting-points p.i. of group A (120 min p.i.) and B (150 min p.i.) were taken at the maximum difference between TT and ST fluorescence. Observed vascular damage effects during and after treatment, expressed as damage scores using Table II, are shown in Figure 2a for group A and figure 2b for group B. Despite differences in fluorescence intensity between group A and B at the treatment starting point, hardly any differences in circulation damage during and after treatment was observed. After 5 min of light treatment strong constriction of venules, disappearing arterioles in ST and ischaemia in TT were observed. After treatment, constriction was maximal and in TT some vasodilatation was observed. One day after treatment circulation started to recover and 6 days later hardly any damage had remained visible.

Group C was treated at 60 min p.i. using 200 mg/kg ALA. The vascular damage effects during and after light treatment are shown in Figure 2c. There was hardly any constriction of venules during treatment. The arterioles constricted but remained visible and there was a mild ischaemia in TT. Maximum damage was reached 1 day after treatment after which the circulation started to recover. There was a selective TT damage but no complete circulation stop.

Group D (200 mg/kg) received two light treatments, at 60 and 150 min. p.i.. Before and after the treatments fluorescence images were recorded to examine if photobleaching had occurred during the irradiation and if new fluorescence was formed after the first light treatment. This would indicate the presence of new porphyrin that could be used to increase the effectiveness of PDT. The results of these fluorescence recordings are shown in Figure 3. The values in this graph were not corrected for background fluorescence. After the first light treatment photobleaching of fluorescence occurred to a level slightly below the autofluorescence intensity. Just before the second light treatment new fluorescence was observed and after treatment it had returned to the same level as after the first treatment. The vascular damage scores of the two subsequent light treatments are shown in Figure 2d. During the first treatment the same constriction of arterioles (Fig 4b) occurred as in group C. Before the second treatment constriction in ST and the circulation in TT had recovered to some extent. During the second treatment the same vascular constriction of venules and arterioles was seen as in group A and B (fig 4c). At the end of the second treatment complete stasis of the circulation in TT was observed. The damage to ST at day one was maximal (but not complete) and recovered to some extent but even after 7 days some areas remained damaged. Necrosis of TT persisted during 7 days with the exception of one tumour which showed some circulation at its border after 4 days post treatment. In this group the contents of the chambers were re-transplantated after 7 days and only two showed regrowth.




TT ST

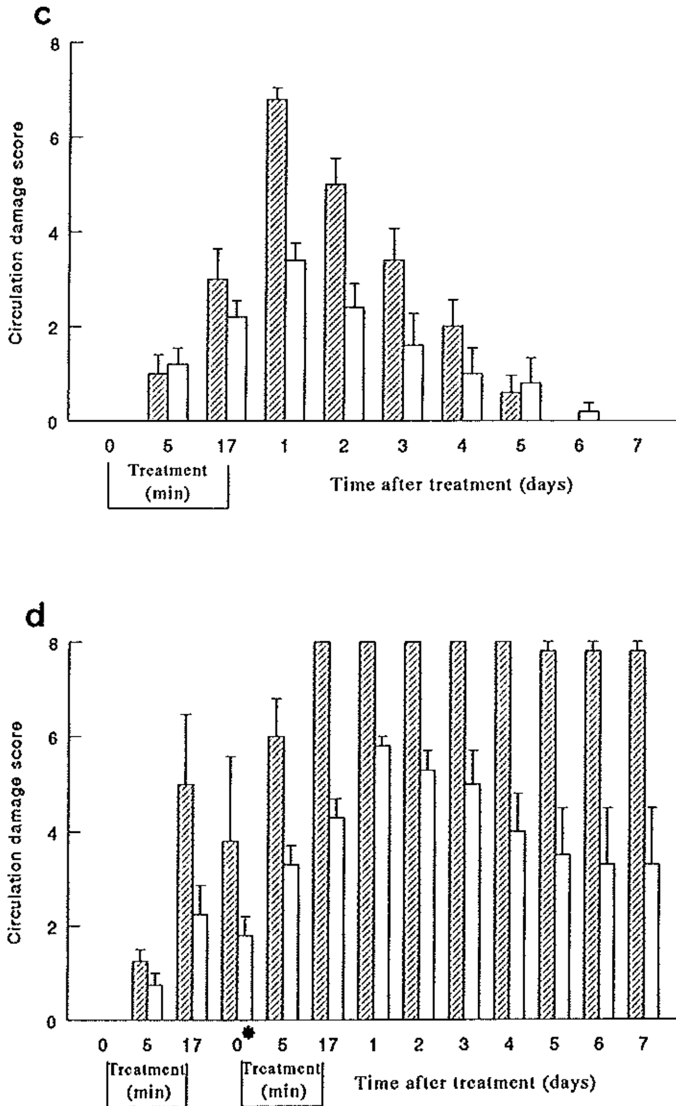



*Figure 2. Circulation damage scores (±s.e.m.) of tumour tissue (TT) and subcutaneous tissue (ST), during (min after start of irradiation) and after light treatment (days). Animals received 100 or 200 mg/kg ALA and were treated with a light dose of 100 J/cm² (100 mW/cm², 514.5 nm), at different time-points p.i. a (100 mg/kg, n=6), 120 min p.i.; b (200 mg/kg, n=6), 150 min p.i.; c (200 mg/kg, n=6), 60 min p.i.; d (200 mg/kg, n=6), 60 (0 = before PDT at 60 min p.i.) and 150 min (0*=before PDT at 150 min p.i.)*

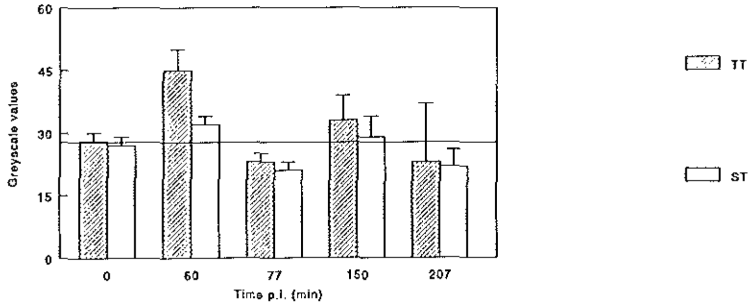



*Figure 3. Photobleaching of fluorescence (± s.e.m.) in tumour (TT) and subcutaneous tissue (ST), recorded in 6 animals treated with 100 J/cm² of 514.5 nm light at 60 as well as 150 min p.i.. The autofluorescence has not been subtracted from the fluorescence measurements after ALA administration.*

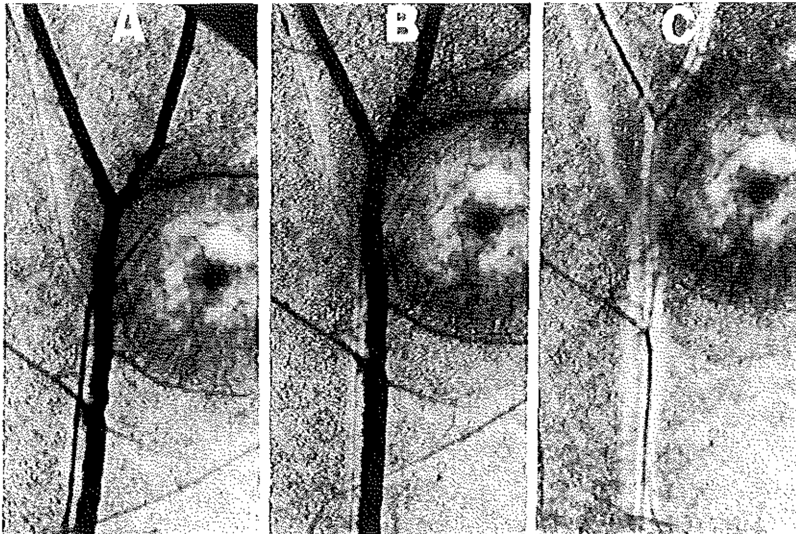



*Figure 4. Status of the vasculature before **a**, and during the first light treatment at 60 min **b**, and the second light treatment at 150 min p.i. **c**. During the early treatment constriction of the arteriole (arrow) occurred, but it remained visible. This arteriole disappeared during the second treatment and a strong constriction of the larger venule was seen. The border of the tumour on the photograph looks dark because of fat cells and there was minor necrosis in the centre of the tumour.*

## Discussion

### *Fluorescence kinetics studies*

In this study we have examined the fluorescence kinetics of TT and ST after 100 and 200 mg/kg of i.v. administered ALA. After both ALA doses the rate of fluorescence increase in TT was higher than in ST. It is known that various malignant tissues have a higher activity of porphobilinogen deaminase (PBGD) and a decreased activity of ferrochelatase (van Hillegersberg *et al.*, 1992). Therefore it is likely that the higher rate of fluorescence increase in TT may represent a higher capacity for conversion of ALA to porphyrin, PpIX to haem or a combination of both.

Another explanation for a higher rate of increase in TT may be a higher ALA uptake. However, if ALA uptake determined the rate of fluorescence increase a steeper rate of increase is expected after a higher ALA dose. This did not occur and although maximal fluorescence intensity was twice that after 200 mg/kg administered ALA, the same maximal rate of increase of fluorescence in both tissues was observed as after 100 mg/kg. As a result, the time to reach maximal fluorescence was longer for the higher ALA dose. This was also found in the normal skin of mice by Pottier *et al.* (1986). It may indicate that the limiting factor in the rate of fluorescence increase is the biosynthesis of haem.

An interesting observation is the difference between TT and ST in time interval of reaching maximal fluorescence. Maximal TT fluorescence was reached earlier than ST and as a result ST fluorescence was still increasing at a point were TT fluorescence already decreased. This is not expected if ALA uptake in TT is higher than ST and if ferrochelatase activity in TT is decreased. It is likely that i.v. administered ALA is rapidly cleared from the circulation. This results in a strong reduction of available ALA for TT in the course of time. It may be possible that maximal ALA accumulation in cells takes place directly after injection and that ALA, or an intermediate of the haem synthesis, is retained there. As a result, ALA or intermediates in the cell will be depleted faster in TT due to the increased PpIX synthesis. This could explain why TT fluorescence started to decrease before ST fluorescence. The difference in maximum fluorescence between TT and ST may then be explained by a difference in ALA-uptake.

We observed no difference in fluorescence kinetics following i.p. or i.v. administered ALA (unpublished data). Loh *et al.* (1993) found that the fluorescence kinetic after oral ALA was similar to i.v. administered ALA although a higher ALA dose was necessary to achieve the same tissue concentrations of PpIX. Therefore, no difference in fluorescence kinetics after oral ALA administration compared to i.v. is expected to occur in our model. Whether fluorescence kinetics after topical ALA is similar to i.v. ALA will be investigated in this model in the future.

*Phototherapy studies*

The vascular effects during and after treatment were examined in four groups of animals differing in ALA dose and interval between ALA administration and light treatment. Treatment time-points were chosen based on the observed fluorescence kinetics after 100 and 200 mg/kg ALA. The chambers of the first two groups (A and B) were treated at maximal difference between TT and ST. These experiments were set up to achieve maximal *selective* TT circulation damage. Therefore, the chambers were treated at maximal difference between TT and ST fluorescence and not at maximal fluorescence.

Despite differences in fluorescence intensities between the two doses of ALA and between TT and ST, no differences in the level of circulation damage could be observed. Furthermore, the overall circulation damage effects were relatively minor, and there was no complete circulation stop in TT. The basis of the apparent discrepancy between the fluorescence intensities and the lack of photodynamic damage may be the strong vascular constriction during treatment observed after both ALA doses. These vascular effects during treatment are comparable with those observed with almost all sensitizers investigated in this model (Star *et al.*, 1986; van Leengoed *et al.*, 1993). An optimal oxygen supply during treatment is necessary to obtain tissue damage with PDT (Henderson and Fingar, 1987; Moan *et al.*, 1985). Therefore, a reduction of tissue oxygenation, as a result of vascular shutdown during treatment, limits the effectiveness of PDT.

No constriction was observed during treatment at 60 min p.i. with group C (200 mg/kg). This lack of constriction during treatment could be caused by a low capacity of endothelial cells to generate PpIX. Although the fluorescence intensity was less, a larger and a more selective level of TT damage was obtained compared to a treatment at maximal difference in fluorescence between TT and ST. This may have been made possible by the blood supply remaining intact during treatment. The increased selective effects on TT treated at 60 min p.i. could also be the result of translocation within the cell of porphyrin from the mitochondrion to less sensitive sites (Malik and Lugaci, 1987; Kessel, 1986). Since PpIX is formed in the mitochondrion and because the mitochondrion is very sensitive for PDT damage (Salet *et al.*, 1986; Hilf *et al.*, 1986) an increased effect may be expected when treating at an interval where the *rate* of fluorescence *increase* is maximal as done in group C.

Summarizing, in this model no correlation was found between the fluorescence intensity and the level of damage after a single light treatment. As discussed, two factors may determine the level of damage after treatment: the quality of blood supply during treatment and the localization of PpIX in the cell in the course of time. Based on the results obtained with our experiments the optimal interval for a single light treatment may be early p.i. where maximal rate of PpIX accumulation is observed and no vascular constriction during treatment seems to occur. The relative importance of the intracellular localization of PpIX and the vascular effects during treatment may be determined by in vitro experiments.

With in vitro studies conditions of oxygen supply can be kept constant and the localization of PpIX in the cell in relation to damage after treatment can be examined.

With two subsequent light treatments, at 60 and 150 min p.i., complete necrosis in TT during the observation period could be achieved while permanently damaging only a relatively small area of ST. With all dyes studied in this model so far (Star *et al.*, 1986: van Leengoed *et al.*, 1993), a large area of permanent ST necrosis was necessary to yield permanent TT necrosis. This could indicate an increased fraction of direct tumour cell kill by ALA-PDT, compared for example with PDT using Photofrin, where this fraction is quite small (Henderson and Fingar, 1987).

During the recovery period of 75 min between the end of the first and the beginning of the second treatment the minor constriction caused by the first treatment recovered. After the first light treatment the fluorescence had bleached to a level slightly below the autofluorescence. This could be caused by bleaching of naturally occurring fluorochromes, for example degradation products of chlorophyll present in animal food (Weagle *et al.*, 1988). However, no photodynamic damage was observed in control animals treated with light alone.

Before the second treatment new fluorescence in TT and ST was observed which was less, in absolute terms, than observed in untreated animals at similar time points. This might indicate that after the first treatment cells were damaged and had therefore lost haem generating capacity. Cells that still have that capacity can form new PpIX. Dan *et al.* (1993) reported about a decreased ferrochelatase activity in cells after a single light treatment. This induced decrease can result in an increase in the *rate* of PpIX accumulation. These cells may be damaged severely by the second treatment which might explain the increased damage effects after two subsequent treatments. Another possible explanation for the severe damage effects with two subsequent treatments may be that cells that are damaged by the first treatment will release PpIX. This PpIX may then cause damage to other cells during the second treatment. However this is not likely to occur because complete bleaching after the first treatment was observed.

In conclusion, no direct correlation was found between fluorescence intensity and the amount of vascular damage to TT and ST in this model after i.v. administration of ALA. With a single light treatment no complete circulation stop in TT was obtained. Only with a double treatment could persistent tumour necrosis be obtained without causing complete necrosis of the surrounding subcutaneous tissue.

*Acknowledgements*

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# Chapter 3

Improvement of Systemic 5-Aminolevulinic acid-based Photodynamic Therapy in vivo using Light Fractionation with a 75 Minute Interval.

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## Abstract

We have studied different single and fractionated illumination schemes after systemic administration of 5-aminolevulinic acid (ALA) to improve the response of nodular tumors to ALA-mediated photodynamic therapy. Tumors transplanted on the thigh of female WAG/Rij rats were transdermally illuminated with red light (633 nm) after systemic ALA administration (200 mg/kg). The effectiveness of each treatment scheme was determined from the tumor volume doubling time. A single illumination (100 J/cm$^2$ at 100 mW/cm$^2$, 2.5 h after ALA administration) yielded a doubling time of 6.6 ± 1.2 days. This was significantly different from the untreated control (doubling time, 1.7 ± 0.1 days). The only treatment scheme that yielded a significant improvement compared to all other schemes studied was illumination at both 1h and 2.5 h after ALA-administration (both 100 J/cm$^2$ at 100 mW/cm$^2$) and resulted in a tumor volume doubling time of 18.9 ± 2.9 days. A possible mechanism to explain this phenomenon is that the protoporphyrin IX formed after administration of ALA is photodegraded by the first illumination. In the 75-min interval, new porphyrin is formed enhancing the effect of the second illumination.

## Introduction

PDT using ALA induced PpIX as a photosensitizer is widely used as an experimental therapy, especially for cutaneous cancer. A complete initial response rate of > 90% has been reported for treatment of human superficial basal cell carcinoma with topically applied ALA-PDT (Kennedy *et al.*, 1992; Calzavara-Pinton *et al.*, 1995; Meijnders *et al.*, 1996). However, for nodular BCC, a much lower complete response of 50% is obtained (Calzavara-Pinton *et al.*, 1995; Peng *et al.*, 1997). An explanation for this lower efficacy might be that topically applied ALA does not penetrate to the deep layers of the tumor (Peng *et al.*, 1995; Martin *et al.*, 1995). Oral or systemic administration of ALA may improve the biodistribution of PpIX (Peng *et al.*, 1995 ; Tope *et al.*, 1998). However, also after systemic ALA-PDT, only superficial necrosis was found in patients treated for dysplasia of the mouth (Fan *et al.*, 1996) or the esophagus (Barr *et al.*, 1996). These clinical reports show the need for improvement of topical and systemic ALA-PDT. A number of animal studies have demonstrated that the response to PDT after systemic ALA administration can be improved by modifying the illumination scheme, for example by reducing the fluence rate, to improve oxygenation (Pogue *et al.*, 1997; Hua *et al.*, 1995; Robinson *et al.*, 1998). Another option is the use of light fractionation with either a short (Pogue *et al.*, 1997; Hua *et al.*, 1995; Messmann *et al.*, 1995) or a long-term interval (Van der Veen *et al.*, 1994). The short-term light fractionation scheme (with one or more interruptions of seconds or min) may allow reoxygenation during the dark period. Theoretically, this will lead to more singlet oxygen formation (Pogue *et al.*, 1997). We define a long-term light fractionation scheme as an illumination scheme with two light fractions separated by an interval of 1 hour or longer. After the first light fraction, PpIX is partially or completely photobleached, and in time, posttreatment new PpIX is formed, which can be used for a second illumination (Van der Veen *et al.*, 1994; Van der Veen *et al.*, 1997). Van der Veen *et al.* (1994) reported complete necrosis of four out of six tumors in a rat skinfold observation chamber model using a long-term light fractionation scheme (with an interval of 75 min) after a single ALA administration. No necrosis was observed after a single illumination. These studies show that improvement of ALA-PDT using different illumination schemes is possible. Our interest here was to improve systemic ALA PDT of nodular tumors. We, therefore, studied the effectiveness of different illumination schemes published by our own group (Robinson *et al.*, 1998; Van der Veen *et al.*, 1994) and others ( Hua *et al.*, 1995; Messmann *et al.*, 1995) by measuring the tumor volume doubling time of a transplantable rat rhabdomyosarcoma after transdermal illumination.

## Materials and Methods

ALA hydrochloride (Finetech , Haifa, Israel) was dissolved in a 0.9% NaCl infusion solution (90 mg $ml^{-1}$). A freshly prepared ALA solution was administered i.v., to a dose of 200 mg $kg^{-1}$ body weight under ether anesthesia. After administration the animals were kept under subdued light conditions.

Rat rhabdomyosarcoma (Rh), originally derived from an isologous undifferentiated rhabdomyosarcoma, was maintained by subcutaneously transplanting small pieces of tumor ($\approx$ 1 $mm^3$) on the thigh of female WAG/Rij rats (12 - 13 weeks old). The tumor growth was monitored daily by measuring the three orthogonal diameters using calipers and the tumor volume was estimated by the formula for an ellipsoid, $V=(\pi/6) * D1 * D2 * D3$. Tumors were randomly assigned to control and treatment groups when their volume reached 50 $mm^3$.

PDT was carried out under general anesthesia using intra muscular, 0.5 ml $kg^{-1}$ Hypnorm (Janssen Pharmaceutica, Tilburg, The Netherlands) and, 2.5 ml $kg^{-1}$ diazepam. Prior to the light treatment the skin overlying the tumor was shaved. The animals were placed on a temperature controlled stage and covered with a black polythene mask. Tumors were transdermally illuminated with a 10 mm diameter plane parallel light beam (633 nm, (Meijnders *et al.*, 1996)). Immediately after PDT, the animals were housed under subdued light conditions at 28 °C for the first 24 h. This was done to minimize the decrease in body temperature caused by the anesthesia. Subsequently, the animals were kept at room temperature.

Ten groups of animals were treated according to various treatment schemes. Groups A, B and C served as controls and were treated either with anesthesia only (n=6), light only (100 J $cm^{-2}$ at 100 mW $cm^{-2}$; n=3) or ALA only (200 mg/kg i.v.; n=3) respectively. Groups D to J, (n=6 for each group), were treated according to different illumination schemes as shown in Fig 1. Each illumination was carried out at either 1 or at 2.5 h after injection of ALA. These time points were based on a pharmacokinetic study performed on this animal model in which we found a maximal PpIX fluorescence of the tumor at 2.5 h postinjection. At 1 h post ALA administration, approximately one third of the maximal PpIX fluorescence was observed.

In group D and E, the tumors were illuminated with a single light fluence of 100 J $cm^{-2}$ at a fluence rate of 100 mW $cm^{-2}$ delivered at either 1 and 2.5 h post-ALA-injection, respectively. In groups F-I the tumors were illuminated at 2.5 h post-injection of ALA. The tumors in group F received 100 J $cm^{-2}$ at 25 mW $cm^{-2}$ so that the treatment time was 4 times longer than in groups D and E. The short-term light fractionation schemes were applied in group G and H. In group G, 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ was delivered with one interruption of 150 seconds after the first 5 J $cm^{-2}$ (Messmann *et al.*, 1995). In group H 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ was delivered with multiple interruptions, being turned off and on every 30 s. (Hua *et al.*, 1995). Groups I and J were both treated with a double light fluence of 200 J $cm^{-2}$ at 100 mW

cm$^{-2}$ given either in one fraction (2.5 h p.i. of ALA) or according to a long-term light fractionation scheme of two equal fractions of 100 J cm$^{-2}$ with an interruption of 75 min (treatment at 1 and 2.5 h p.i. of ALA, (Van der Veen *et al.*, 1994).

The light distribution within the tumors treated in this study was studied in a separate series of experiments using two isotropic probes (500 μm, bulb diameter, Rare Earth Medical, West Yarmouth MA, USA). The isotropic probes were connected to a dosimetry device that enables real-time fluence (rate) measurements to be recorded. One probe was placed on top of the skin at the center of the illuminated area. The second probe was implanted between the base of the tumor and the underlying muscle at the center of the illuminated area. Insertion of the isotropic probe through the skin was performed at a site that was distant from the tumour (>1 cm) to reduce the effect of bleeding on the measurements. The fluence rate was measured continuously during illumination at 100 mW cm$^{-2}$ to a fluence of 100 J cm$^{-2}$ in five tumors 2.5 h after administration of ALA (scheme E). These data were used to estimate the mean optical attenuation coefficient of the combination of tumor and overlying skin.

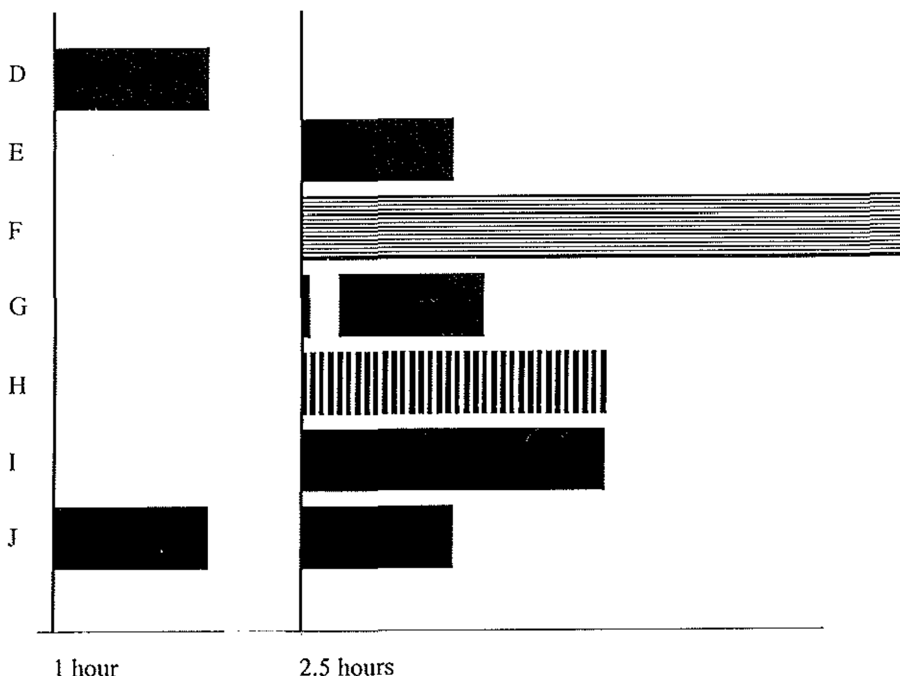


*Figure 1. Schematic diagram of the treatment schemes studied.*

Tumor re-growth and macroscopic changes to the surrounding normal tissue were monitored every 1 or 2 days following therapy until the size of the tumor had reached 5 times its treatment volume. The treatment volume of each tumor (approximately 50 $mm^3$) was defined as 100% and the points in time (in days after treatment) at which the tumor reached certain fixed volumes; 50%, 200%, 500% etc, were linearly interpolated. The effectiveness of each treatment scheme was determined by comparison of the mean tumor volume doubling time of each group, defined as the number of days the tumor required to double its pre-treatment volume. The effect on the tumor growth posttreatment was determined for each group (determined by the number of days the tumor required to grow from 200% to 500%). All results are presented as mean (± SEM). The relative effectiveness of each treatment scheme was statistically compared using the ANOVA followed by a Student-Newman-Keuls test, as necessary. For all tests a $P < 0.05$ was considered to be statistically significant.

## Results

*Normal tissue response to PDT.* Three types of macroscopic normal tissue response were observed: edema of the thigh, discoloration of the skin overlying the tumor and crust formation. None of the total of 12 animals in the three control groups showed any type of normal tissue damage.

The edema was investigated by measuring the thickness of the leg adjacent to the tumor daily. All animals treated with ALA-PDT showed a mild to severe edema of the leg which was found to be maximal on day 1 post-treatment and cleared by day 4. Normally the leg has a thickness of approximately 7 mm, but at day 1 posttreatment the leg could measure 10.7 - 15.8 mm thick (Table I). The edema found for tumors treated at 1 h after administration of ALA was significantly less compared to the other treatment schemes. The edema found for tumors illuminated with 200 J $cm^{-2}$ in one fraction (scheme I) was significantly greater compared to the rest of the treatment schemes.

Almost all treatment schemes induced a bluish/black discoloration of the skin overlying the tumor after treatment which cleared within a few days. The involved area was as large as the illuminated tumor under the skin, that is smaller than the illuminated area. In some treatment schemes severe discolouration was accompanied by crust formation (Table I). To investigate whether the edema, discoloration and crust formation were influenced by the presence of an underlying tumor, a group of 4 animals without a tumor was illuminated according to the treatment scheme used in group J. The edematous response was found to be the same for skin and muscle illuminated in the absence of tumor. The discoloration was found to be less marked being only pale blue for the group with no tumor compared to dark blue/black for the group with a tumor. The crust seemed to be thinner and smaller in size and

appeared only in 50 % of the animals.

To histologically determine the location of the edema and the cause of the discoloration, a separate set of experiments were performed. Four extra animals were illuminated with 200 J $cm^{-2}$ given either in one fraction or according to a long-term light fractionation scheme (groups I and J, respectively). The illuminated area was excised at day 1 post treatment for histology. Sections of the leg, including skin and soft tissues were stained with haemotoxylin and eosine after formalin fixation. The epidermis and the dermal adnexa showed necrosis after both illumination schemes. Severe edema was found in the dermis and the muscle surrounding and underlying the tumor whereas the tumor showed little or no edema. Enlarged blood vessels that were located around and at the border of the tumor were heavily damaged and there was evidence of hemorrhage.

**Table I.** Normal tissue damage caused by the different treatment schemes used.

| Group | Normal tissue damage | |
|---|---|---|
| | Edema (mm) | Crusts (n) |
| D | 10.7 ± 0.3[a] | 2 |
| E | 14.2 ± 0.9 | - |
| F | 13.8 ± 0.3 | 4 |
| G | 12.4 ± 0.2 | 2 |
| H | 13.3 ± 0.7 | 2 |
| I | 15.8 ± 0.3[b] | 3 |
| J | 12.7 ± 0.7 | 6 |

[a)] Significantly less edema than to the other groups
[b)]Significantly more edema than to the other groups

***Tumor volume measurements.*** The error associated with the tumor volume measurements was estimated by comparing the measurements of two independent observers for 14 tumors treated in this study in a range of tumor volumes. The relative error decreased from 5.3 ± 0.9% for tumor volumes below 30 $mm^3$, to 3.7 ± 1.3% for tumor volumes ranging from 30 to 60 $mm^3$, to 3.5 ± 0.7% for tumor volumes ranging from 60 to 120 $mm^3$, to 2.3 ± 0.5% for tumor volumes ranging from 120 to 240 $mm^3$.

***Tumor response to PDT.*** There was no significant difference in treatment volume for the tumors in different groups and the mean treatment volume was measured to be 50.3 ± 1.4 $mm^3$ (n=54). The tumor volume doubling times measured for the three control groups (A-C) were not significantly different. These data were combined and used as a pooled control group for comparison with the remaining treatment schemes. The rhabdomyosarcoma was found to have a mean tumor volume doubling time of 1.7 ± 0.1 days (n=12).

All of the PDT schemes investigated demonstrated a significantly longer tumor volume doubling time compared to control tumors, as shown in Figure 2. Tumors illuminated with a light fluence of 100 J $cm^{-2}$ at 100 mW $cm^{-2}$, 1 and 2.5 h after ALA administration demonstrated a tumor volume doubling time of 5.0 ± 1.5 days and 6.6 ± 1.2 days respectively (group D and E). The use of a short-term light fractionation scheme with a dark interval of 150 s, after the first 5 J $cm^{-2}$ of the total 100 J $cm^{-2}$ was delivered, showed a tumor volume doubling time of 7.5 ± 1.5 days. This was comparable to the tumor volume doubling time found for the other short-term light fractionation scheme (30 s light on/off, 7.5 ± 0.8 days). Although the mean tumor volume doubling time found for both short-term light fractionation schemes is longer compared to illumination with a single fraction (group E), the increase was not found to be statistically significant. Also illumination with a 4 times lower fluence rate (group F) resulted in an increased mean tumor volume doubling time (8.8 ± 1.9 days) compared to group E which was again not statistically significant. Even increasing the fluence to 200 J $cm^{-2}$ (group I) did not increase the mean tumor volume doubling time (8.6 ± 0.8 days) significantly, compared to group E. Only the use of a long-term light fractionation scheme (100 J $cm^{-2}$ at both 1 and 2.5 h p.i. of ALA, group J) showed a significantly increased tumor volume doubling time compared to all the other illumination schemes: 18.9 ± 2.9 days.

None of the investigated protocols resulted in a "cure" of the tumor and only in group J three out of six tumors were not palpable for 10 to 13 days before the tumor was again detectable. No statistically significant difference could be shown in the tumor growth posttreatment defined as the time a tumor required to grow from 200 to 500%. The tumor volume increased by a factor of 2.5 in 2.58 ± 0.06 days.

***Tumor thickness.*** As might be expected since the illumination was superficial, the tumor response to PDT seemed to be correlated to the thickness of the treated tumor. After observation of the growth curves of the individual tumors in the groups there seemed to be a threshold for the thickness. Tumors thinner than 4 mm responded significantly better to treatment with a total fluence dose of 100 J $cm^{-2}$ compared to thick tumors. The mean tumor volume doubling time for thin tumors of groups D - H was 12.0 ± 2.2 days (n=5) compared to a mean tumor volume doubling time of 5.9 ± 2.2 days for thick tumors (n=25). For illuminations with a total light fluence of 200 J $cm^{-2}$ delivered either in one fraction or according to a long-term light fractionation scheme (groups I and J, respectively) this difference in volume doubling time between thin and thick tumors was not found.

***Light distribution.*** No significant variation in the measured fluence rate was observed during irradiation in individual treatments. The fluence rate measured by the probe placed on top of the skin overlying the tumor was 184.4 ± 14 mW $cm^{-2}$ (n=5) in which the incident light fluence rate was 100 mW $cm^{-2}$. The fluence rate measured by the probe placed at depth between the tumor base and the underlying muscle was 42.3 ± 3.2 mW $cm^{-2}$ (n=5). Therefore the fluence rate at the base of the tumor was approximately 23% of the fluence rate measured at the top of the tumor. From these measurements, a mean effective attenuation coefficient, (eff, was calculated to be 3.5 ± 1.2 $cm^{-1}$ ).

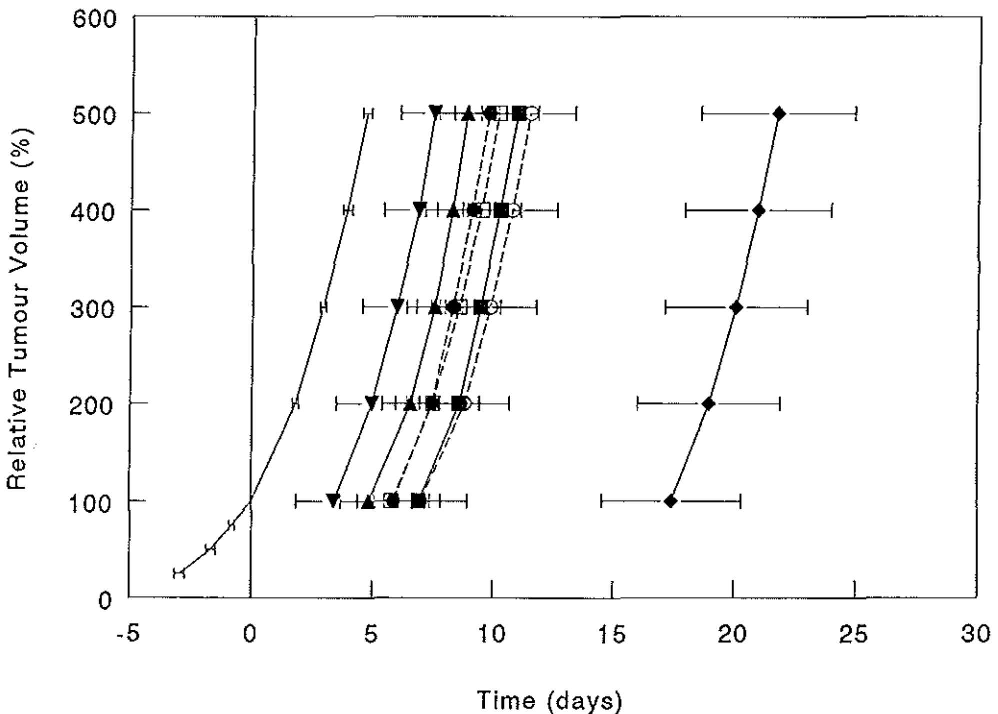


*Figure 2. Relative tumor volume in time after ALA-PDT using different illumination schemes: control (no symbol); scheme D, 1h, 100 J/cm² at 100 mW/cm² (▼); scheme E, 2,5 h, 100 J/cm² at 100 mW/cm² (▲); scheme F, 2,5 h, 100 J/cm² at 25 mW/cm² (○); scheme G, 2,5 h, 100 J/cm² at 100 mW/cm² using a short-term fractionation scheme with one dark interval of 150 s after 5 J/cm² (●); scheme H, 2,5, 100 J/cm² at 100 mW/cm² using a short-term fractionation scheme: 30 sec on/30 sec off (□); scheme I, 2.5 h,, 200 J/cm² at 100 mW/cm² (■) and scheme J, both 1 and 2.5 h, 100 and 100 J/cm² at 100 mW/cm² (◆). Data points, means; bars, ± SEM.*

## Discussion

In this study, we have demonstrated a dramatic increase in tumor volume doubling time following systemic ALA PDT using a long-term light fractionation scheme (two light fractions separated by a dark interval of 75 min). In previous studies, it was shown that new PpIX is formed after complete photobleaching caused by the illumination (Van der Veen *et al.*, 1995; Van der Veen *et al.*, 1997). This newly formed PpIX can be used during a second illumination. Van der Veen *et al.* (1995) showed in a skinfold chamber model that a long-term light fractionation scheme resulted in four out of six tumors with complete necrosis at day 7 posttreatment compared to no necrosis for a single illumination scheme. In this long-term light fractionation scheme a double light fluence (200 J $cm^{-2}$) was delivered (Veen *et al.*, 1995) compared to the single illumination (100 J $cm^{-2}$) which might explain the increased effect. However, when PpIX is completely photobleached, a longer illumination is not expected to be more effective; this is also demonstrated here. Treating the tumor with a double light fluence (200 J $cm^{-2}$) did not significantly increase tumor volume doubling time compared to 100 J $cm^{-2}$ whereas treatment with the same total fluence according to a long-term light fractionation scheme did (Fig. 1). In fact, this scheme increased the tumor volume doubling time by a factor of 2.6. The substantially improved tumor response can only be explained by the use of the dark interval between two light fractions. As we have discussed the long interruption may allow time for the formation of new PpIX which can be used for a second illumination and result in extra cell death. The origin of this new PpIX fluorescence is as yet unknown. One possibility is that ALA is still present in the tissue and can be converted into PpIX by the surviving cells.

The edema formation was not increased using a long-term light fractionation scheme compared to a single illumination of 100 J $cm^{-2}$. The discoloration was more pronounced compared to a single illumination and all the animals formed crust. From the histology it can be concluded that the discoloration was caused by hemorrhage of the blood vessels around and at the border of the tumor. This means that the discoloration and the accompanied crust formation caused by necrosis of epidermal, dermal and tumor tissue was actually a combined normal and tumor tissue response. Thus the fact that we saw more crust after illumination with a long-term light fractionation scheme is not surprising.

In contrast, ALA PDT using a low fluence rate or a short-term light fractionation scheme did not significantly improve the tumor volume doubling time. These illumination schemes were designed to increase the amount of singlet oxygen formation during the treatment by reducing the demand rate for oxygen (Pogue *et al.*, 1997). Several authors have shown that this can enhance the PDT response in a variety of animal models. Robinson *et al.* (1998) reported a higher damage score of normal hairless mouse skin after topical ALA-PDT with a low fluence rate. They observed that the difference in damage score between an illumination with a fluence rate of 150 and 50 mW $cm^{-2}$ was rather small whereas the

difference between these fluence rates and 5 mW cm$^{-2}$ was considerable. Hua *et al.* (1995) showed a 1.5 times longer volume doubling time for tumors illuminated with a 4 times lower fluence rate after systemic ALA administration. The volume doubling time was found to be further enhanced for tumors treated with a 30 seconds light on/off short-term light fractionation scheme. Messmann *et al.* (1995) obtained a greater area of necrosis of normal colon after illumination using several short-term light fractionation schemes. Off course , it is difficult to compare these studies because the animal model used, the ALA doses and the illumination methods are all different. The fact that we could not show an improved tumor response using any of these schemes indicates that little or no extra tumor damage was obtained by the use of a low fluence rate or dark periods of several seconds or min for this tumor model. These results imply that improving the tumor response to ALA PDT is not simply a matter of interrupting the illumination for a few seconds or min and that tumor response may be different both for different sizes of tumor and for different tumor types.

Fan *et al.* (1996) investigated the short and long-term light fractionation schemes in patients treated for mouth dysplasia with orally administered ALA. They were not able to show an improved tumor response using either of these treatment schemes compared to a single fraction illumination. It should be noted that the maximum ALA dose orally administered in patients is 60 mg kg$^{-1}$ whereas experimental animals are given 200 mg kg$^{-1}$ intravenously.

In summary, no significant improved tumor response could be obtained using a low fluence rate or a short-term light fractionation scheme (dark interval of seconds or min) for the illumination of a solid rhabdomyosarcoma transplanted on the thigh of a rat. This could only be achieved by using a long-term light fractionation scheme with an dark period of 75 min between two light treatments.

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# Chapter 4

## Kinetics and localisation of PpIX fluorescence after topical and systemic ALA application, observed in skin and skin tumours of UVB-treated mice

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## Abstract

In this study the kinetics and localisation of protoporphyrin IX (PpIX) fluorescence in skin and skin tumours were examined after topically (20%, for 4 hours) or systemically (200 mg/kg, i.p.) administered 5-aminolevulinic acid (ALA). As a model we used hairless mice with skin lesions (actinic keratoses and squamous cell carcinoma) which were induced by daily UV-B irradiation. The epidermis of the skin surrounding the tumours (T) was altered (AS); due to the UV-B irradiation the epidermis was thicker and less elastic. Therefore, non-UVB irradiated mice were used to assess fluorescence of normal skin (NS). Light from a halogen lamp was used to excite at 500 ± 20 nm and fluorescence was detected through a filter which passes light of 670 ± 50 nm. Maximal fluorescence following i.p. ALA was observed after 2 h p.i. and was 3 times less than after topically applied ALA. Furthermore, after i.p. ALA a lower T selectivity (T/NS) could be obtained than after topically applied ALA. Maximal fluorescence following topically applied ALA was achieved 6 h after the end of the 4 h application time. At that interval fluorescence of T was 2 times higher than directly after the application period. Furthermore, T selectivity (T/NS) after topical ALA at the interval of maximal fluorescence was higher than at the interval directly after application. With fluorescence cryo-microscopy localisation of fluorescence in the skin at the interval of maximal fluorescence was determined after both administration routes. For both cases fluorescence was mainly located in T, epidermis and hair follicles. Fluorescence in subcutis could only be observed at 2 h post i.p. ALA and at 6 h post topical ALA. No fluorescence could be observed in muscle. We conclude that, in this model and with these ALA doses, a higher fluorescence intensity and selectivity (T/NS) was achieved after topically applied ALA than after systemically administered ALA. These results make topically applied ALA more favourable for ALA-PDT of superficial skin tumours in this model. In general these results imply that by optimizing the time after ALA application the efficacy and selectivity of topical ALA-PDT for skin tumours may be improved.

## Introduction

A new approach in photodynamic therapy (PDT) to photosensitize tumour tissue is the use of endogenously produced photosensitizers. This can be achieved by administration of 5-aminolevu-linic acid (ALA), an agent which utilizes the haem biosynthetic pathway, by bypassing the feedback control of this pathway, to create diagnostic and therapeutic levels of the sensitizer protoporphyrin IX (PpIX).

The activity of several enzymes involved in the haem biosynthetic pathway varies in different tissue types. For example, various malignant tissues have an increased porphobilinogen deaminase activity, which converts ALA into porphobilinogen, and a decreased ferrochelatase activity, which converts PpIX into haem (van Hillegersberg *et al*, 1992). This alteration in enzymatic activities may cause an increased fluorescence in tumour tissue compared to normal tissue after ALA administration (van der Veen *et al*, 1994; Bedwell *et al*, 1992). Several normal tissues, especially those originating from ecto- and endoderm may also become photosensitized after exposure to ALA. This in contrast to tissues from mesodermal origin (Divaris *et al*, 1990; Loh *et al*, 1993).

In both human and animal studies ALA has been administered via various routes. Topically applied ALA-PDT has proven to be a successful treatment modality for non-melanoma superficial malignant skin tumours (Kennedy and Pottier, 1990; Svanberg *et al*, 1994). Also, human studies have been performed using orally applied ALA (Grant *et al*, 1993; Regula *et al*, 1995) and topically applied ALA (Kriegmair *et al*, 1994) for endoscopic PDT treatments as well as photodetection of cancer. Sufficient tissue levels of PpIX for diagnostic and treatment purposes can also be achieved by administering ALA intravenously (i.v.) or intraperitonially (i.p.) as observed in animal studies by Iinuma *et al* (1995) and Peng *et al* (1992).

Administering ALA via various routes may reveal a dissimilarity in the bio-availability of ALA which may result in different fluorescence dynamics and localisation of PpIX fluorescence. To examine these differences in more detail the fluorescence kinetics after i.p. and topically administered ALA of small skin tumours were studied. Furthermore, localisation of PpIX after both topical and i.p. ALA was examined using fluorescence cryo-microscopy. As a model we used hairless mice with small skin lesions (actinic keratoses and squamous cell carcinomas) which were induced by UV-B irradiation.

## Materials and Methods

### *Animal model*

The animals used were inbred female hairless albino mice, Skh hr1, 18 weeks old, obtained from the University of Utrecht. These mice were irradiated daily with UV-B light (1.5 KJ $m^{-2}$) using a Westinghouse FS40TL12 lamp according to a method described by de Gruijl *et al* (1983). The lamps were mounted above the cages so that mainly the dorsal skin of the mice were exposed to the UV-B light. The animals developed multiple primary tumours in the exposed area after approximately 80 days. Small tumours (1-2 mm in diameter), which were mainly squamous cell carcinoma, actinic keratoses and seborrhoeic keratoses, were used in the experiments. Non-UVB irradiated animals were used to assess normal skin fluorescence. This was necessary because as a reaction to the daily UV-B irradiation the skin was altered and became thicker and less elastic.

### *ALA*

5-Aminolevulinic acid was obtained as hydrochloride in 98% pure powder form from Sigma Chemie (Bornem, Belgium). For the topical application ALA was dissolved in Carboxymethylcellulose 3% to yield a 20% solution. The solvent was set at pH 5.5 by adding NaOH (2M), to avoid irritation of the skin. Before ALA application the animals received a low dose of diazepam (Centrafarm b.v., Etten-leur, Holland) to avoid anxiety and therefore movement of the solvent. The freshly made solvent was placed on the entire dorsal skin and covered with a gauze. A piece of transparent film dressing (Mölnlycke, Waremme, Belgium) was placed over the gauze to achieve occlusion and to prevent evaporation and movement of the solvent. The solvent was applied to the skin for 4 hours after which it was carefully removed.

For systemic administration ALA was dissolved in PBS after which the solution was set at pH 6 using NaOH (2M) to prevent necrosis at the injection site. The freshly made solution was injected intraperitonially (i.p.) in a dose of 200 mg/kg.

### *Experimental set-up for in vivo experiments*

Fluorescence kinetics after topically and i.p. administered ALA were determined in six UV-B and six non-UVB irradiated mice per administration route. Fluorescence after i.p. administered ALA was recorded every hour for 12 hours. The fluorescence after topical ALA was recorded every two hours for 24 hours. This long observation period after topical ALA made it necessary to compose each 24 hour series from two groups of mice each followed for 12 hours. During the fluorescence recordings the animals were anaesthetized with a combination of ethrane/oxidion/$N_20$ and positioned on a temperature controlled stage under an

intensified CCD-camera. In each UV-B irradiated mouse, after i.p. or topical ALA, three areas each of 1 cm in diameter were recorded; two areas with tumours and one area which was macroscopically free of tumour. In each non-UVB irradiated mouse two randomly chosen areas of 1 cm in diameter on the dorsal skin were recorded. Small pieces of fluorescent plastic positioned in the recorded areas were used for focusing on the skin and for corrections of small variations in output of the excitation light. Light from a halogen lamp and bandpass filter were used to excite fluorescence at a wavelength of 500 ± 20 nm with dose rate of 0.2 mW $cm^{-2}$. Through a dichroic mirror light was projected on the skin and fluorescence was detected through a filter which passes light of 670 ± 50 nm. Fluorescence images were recorded using a CCD camera with a two stage image intensifier and a 50 mm Leitz Photar macrolens. No photodynamic damage, caused by the excitation light, could be observed (total maximum excitation light dose was 0.04 J $cm^{-2}$). During the application period and between the fluorescence recordings the animals were kept in the dark.

The recorded digitized images were analyzed yielding average grey scale values per time interval of selected areas of interest in tumours (T), UV-B irradiated skin (AS) and non-UVB irradiated skin (NS).

***Fluorescence cryo-microscopy studies***

For the fluorescence cryo-microscopy study the same administration routes and ALA doses were applied as used with the fluorescence kinetics studies. Fluorescence in T and in hair-follicles (HF), epidermis (EP), subcutis (SC) and muscle (M) of AS and NS was examined at 2 intervals after topical ALA; directly after 4 h application (t=4) and at maximal fluorescence (t=10; 6 h after the end of the application period). With systemic ALA, fluorescence was only examined at interval of maximum fluorescence which was 2 h p.i. for T and AS and 6 h p.i. for NS. Furthermore, unsensitized animals were used to examine the autofluorescence of T, AS and NS. At each interval 2 animals were sacrificed after which in each animal 2 samples of 0,5 $cm^2$ were excised and immediately frozen in liquid nitrogen. Of each sample 4 transversal sections of 30 μm thick were cut using a cryostat and placed on a slide. The slides were kept in the dark and preserved in the refrigerator.

The fluorescence set-up consisted of a CCD-camera fitted to a Leitz DM fluorescence microscope. Excitation light of 543 ± 28 nm with an irradiance of 1 mW $cm^{-2}$ was used and fluorescence of a freshly made section was detected through a 615 nm high pass filter. No photobleaching was observed after the recordings (total energy was less than 0,01 J $cm^{-2}$). With phase contrast representative areas in 4 sections per sample of T and HF, EP, SC and M of UV-B and non-UVB irradiated skin were identified and recorded. In the resulting digitized images average fluorescence grey scale values of these structures were determined.

## Results

### *Fluorescence kinetics studies*

The fluorescence after topical ALA of T (n=50), AS (n=6) and NS (n=12) observed in 6 animals per interval was assessed every 2 hours during 24 hours (fig. 1a). The results after i.p. administered ALA of T (n=50), AS (n=6) and NS (n=12), measured every hour during 12 hours in 6 animals per interval, are displayed in figure 1b. All values in these graphs were corrected for background fluorescence using the autofluorescence image, which had an average grey scale value of 29 for T, 27 for AS and 24 for NS. The grey scale values in fig. 1a and fig. 1b are proportional to the fluorescence with the same factor.

After topically applied ALA the rate of fluorescence increase of T and AS was higher than NS. Furthermore, maximum fluorescence of T and AS was attained at the same interval, about 10 hours after the start of the application. At that interval the mean fluorescence intensity of tumours was 1,4 times higher than AS and 4 times higher than NS. After 10 hours a rapid decrease in fluorescence was observed but even at 24 hours a certain amount of fluorescence could be detected. Fluorescence of NS increased slower and the fluorescence stayed at the same level between 6 and 24 hours.

After i.p. administered ALA differences in the rate of fluorescence increase between T and NS were observed as shown in figure 1B. However, maximal fluorescence of both T and AS was 3 times less than after topically applied ALA. Maximal fluorescence intensity of NS after i.p. was almost the same as after topically applied ALA. After i.p. ALA maximal fluorescence in T and AS was achieved around 2 hours whereas maximal fluorescence in NS was attained around 6 hours p.i. After interval of maximum fluorescence, a decrease in the same rate as the fluorescence increase could be observed. As a result the fluorescence of T was only higher than AS and NS till 4 hours p.i. and after that time interval the fluorescence of NS exceeded T and AS fluorescence. Twelve hours p.i. fluorescence of T, AS and NS had almost returned to autofluorescence level.

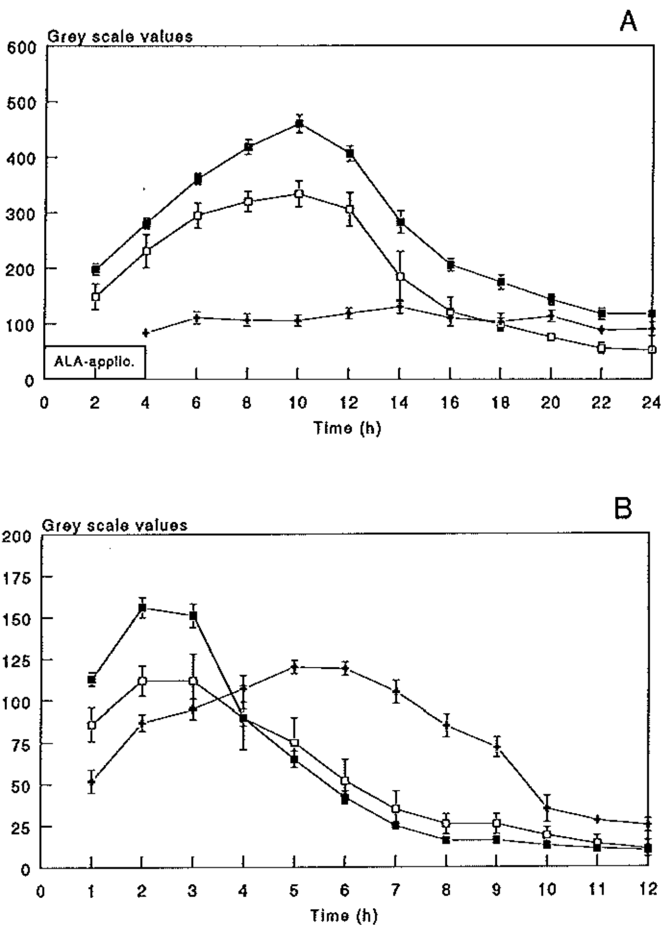



*Figure 1. In vivo fluorescence kinetics, ± standard error of the mean (s.e.m.), expressed as grey scale values, of Tumour (■), Altered skin (□) and Normal skin (+) after topically (A) and systemically (B) administered ALA. At each time interval the mean fluorescence (± s.e.m.) of 6 animals is plotted, each corrected for their respective autofluorescence.*

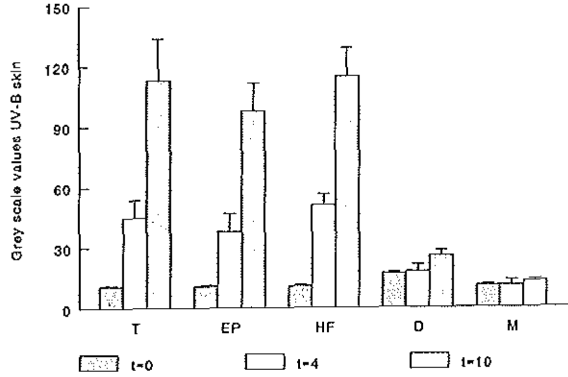
***Fluorescence localisation studies***
Mean fluorescence was determined in 4 sections per sample of tumour (T), epidermal layer (EP), hair follicles (HF), subcutis (SC) and muscle (M) of UV-B and non-UVB irradiated skin without ALA and after topical and systemic ALA. Mean fluorescence per time interval was assessed in 16 sections per 4 samples obtained in 2 animals.

The fluorescence in UV-B and non-UVB irradiated mice examined directly (t=4) and 10 hours after start of topical ALA application is shown in figure 2a and 2b. In unsensitized UV-B and non-UVB irradiated animals only a low autofluorescence signal was observed in all structures. Directly after ALA application fluorescence in UV-B irradiated skin (fig 2a) was mainly located in HF, EP and in T. Between these structures comparable fluorescence intensities were observed. No fluorescence could be detected in SC and M. Fluorescence at 10 h was also located in T, EP and HF and was 2 times higher than at 4 h. No fluorescence could be observed in M but a low fluorescence level in SC was detected at 10 h. In non-UVB irradiated skin (fig 2b) fluorescence could be detected at both 4 and 10 h in EP and HF and not in SC and M. The increase in fluorescence in EP and HF at 10 h was small and not significantly different from 4 h. Differences in fluorescence intensities at 4 h between HF and EP of UV-B and non-UVB irradiated skin were not significantly different. However, at 10 h the fluorescence in EP and HF of UV-B irradiated skin was approximately 2 times higher than in EP and HF of non-UVB irradiated skin.

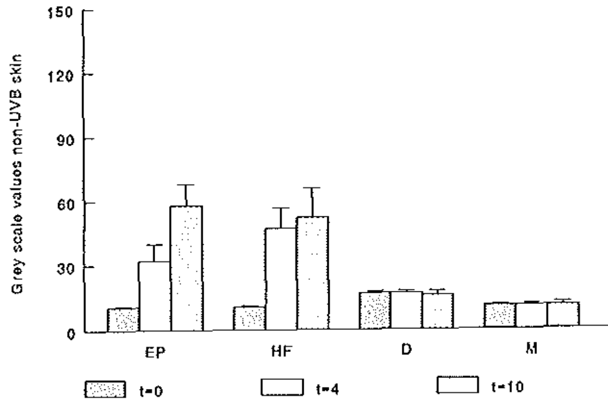
Fluorescence after i.p. administered ALA, shown in fig 2c, was determined at 2 h p.i. for UV-B and 6 h p.i. for non-UVB irradiated mice. In UV-B irradiated skin fluorescence was mainly located in T, HF and EP. A low level of fluorescence could be detected in SC whereas no fluorescence was observed in M. Fluorescence intensity of HF, EP and M at 6 h p.i. in non-UVB irradiated mice was similar to fluorescence of UV-B irradiated mice at 2 h p.i., except in SC of non-UVB irradiated skin where no fluorescence could be detected. The fluorescence intensities of T, EP, HF in UV-B irradiated mice after i.p. ALA were comparable with intensities found in UV-B irradiated mice at interval directly after application (4 h).

***Figure 2.*** *Results of fluorescence cryo-microscopy, expressed as grey scale values (± s.e.m.), in tumour (T), epidermis (EP), hair follicles (HF), subcutis (SC) and muscle (M) are displayed in fig 2a, 2b and 2c. In fig. 2a the fluorescence before (t=0), directly after (t=4) and 6 h after (t=10) topically applied ALA of structures in UV-B irradiated skin are displayed. Fig 2b shows the fluorescence of structures in non-UVB irradiated skin after topical ALA, determined at similar intervals as shown in fig 2a. In fig 2c the fluorescence after i.p. administered ALA, before and at 2 h p.i. in UV-B irradiated skin and at 6 h p.i. in non-UVB irradiated skin are displayed.*

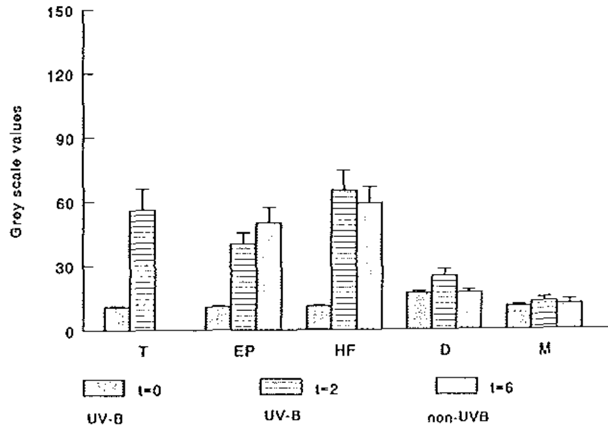
**a**
Topical ALA




**b**
Topical ALA




**c**
i.p. ALA



## Discussion

In this study the differences in kinetics and localisation of PpIX fluorescence between topically and systemically administered ALA of small skin tumours, induced in mice by daily UV-B irradiation, were determined. Human skin cancers, particularly basal cell and squamous cell carcinomas, are also closely associated with chronic, repeated exposure of the skin to solar UV radiation (Fears *et al*, 1976). De Gruijl *et al* (1983) reported that in this model the fraction of actinic keratoses decreases with increasing diameter whereas the fraction of squamous cell carcinoma increases with increasing diameter. This means roughly that tumours smaller than 2 mm mainly consists of actinic keratoses whereas tumours between 2 and 4 mm in diameter mainly consist of squamous cell carcinoma. In this study only small tumours of approximately 2 mm (actinic keratoses and squamous cell carcinoma) were included. Larger tumours revealed considerable variations in appearance and incidence of necrosis or bleeding on top of the tumours.

In human skin a thickening of the epidermis is seen after exposure to sunlight. This reaction is only temporary and the thickness returns to normal values within weeks when the skin is no longer exposed to sunlight. Also in mouse skin this epidermal thickening due to UV-B irradiation was observed. Sterenborg *et al* (1986) found that this reaction is UV-B dose dependent. They also observed that the thickness of the stratum corneum was roughly proportional to the thickness of the whole epidermis. The UV-B doses used in this experiment were at the threshold for producing edema. As a reaction to the high daily doses of UV-B light an acute thickening of epidermis occurred which is no longer UV-B dose dependent (Sterenborg *et al*, 1986). This acute reaction maintains longer and is only partly reversible. This made it necessary to use non-UVB irradiated animals to examine the fluorescence of normal skin.

***Differences in fluorescence kinetics between UV-B and non-UVB irradiated mice.***

The variation in thickness of epidermis between non-UVB and UV-B irradiated mice accounts to some extent for the differences in fluorescence kinetics between T, AS and NS observed after both topically and systemically administered ALA. The thickness of the epidermis in the skin of non-UVB irradiated mice is 23 μm (s.e.m. ± 1) whereas in UV-B irradiated skin the epidermal thickness is 52 μm (s.e.m. ± 5). The thickness in UV-B irradiated skin of tumour with the underlying epidermis is 404 μm (s.e.m. ± 70). We observed with fluorescence cryo-microscopy a significant amount of PpIX fluorescence in the epidermal layer. Therefore a higher fluorescence increase and intensity in UV-B irradiated skin than in non-UVB irradiated skin can be expected with our fluorescence set-up.

The importance of the thickness of the epidermal layer for the fluorescence increase and intensity is confirmed by our fluorescence cryo-microscopy study. With this method, which excludes variations in epidermal thickness and elevated tumours, no significant

differences in fluorescence intensities between T and AS fluorescence were observed. However, with fluorescence cryo-microscopy a significant difference in fluorescence intensity (factor 2) at 6 hr post application between T (and AS) and NS was observed. Therefore, differences in kinetics between a slightly elevated T, a thickened AS and NS can only partly be accounted for variations in thickness of the epidermal layer.

A factor which also may explain the higher rate of fluorescence increase in T and AS compared to NS is an altered activity of enzymes involved in the haem biosynthetic pathway in T and AS. It is known that the activity of two enzymes, porphobilinogen deaminase and ferrochelatase, can be changed in malignant tissues (van Hillegersberg *et al*, 1992). This may result in a steeper rate of fluorescence increase in T and AS compared to NS after both topically and systemically administered ALA. That a disparity in enzymatic activity is an important factor in a different rate of fluorescence increase was supported by a previous study with a different animal model (van der Veen *et al*, 1994). In this model the same differences in rate of fluorescence increase between T and surrounding subcutaneous tissue were observed after i.v. administered ALA. Furthermore, in both T and subcutaneous tissue no enlargement in rate of fluorescence increase occurred after doubling of the administered ALA dose. This observation confirmed that the higher rate of fluorescence increase in T and AS could represent a higher capacity for conversion of ALA to porphyrin or a lower capacity of conversion of PpIX to haem or a combination of both. It also excludes that differences in rate of fluorescence increase after systemic ALA were determined by a disparity in ALA uptake, vascularisation or quality of bloodvessel wall between T and NS. Otherwise, an enhancement in rate of fluorescence increase was expected after a two-fold ALA dose. Variations in ALA uptake and vascularisation may however account for differences in maximal fluorescence intensities after systemically administered ALA.

***Differences in fluorescence kinetics between topically and i.p. administered ALA.***

Between i.p. and topically administered ALA clear differences in fluorescence kinetics of T, AS and NS were observed. There was a large difference in interval of reaching maximal fluorescence. This difference may be the result of a dissimilarity in the bio-availability of ALA between both administration routes.

With systemically administered ALA maximal fluorescence in T and AS occurred 2 h post injection (p.i.) whereas maximal NS fluorescence was reached 6 h p.i. This short interval of fluorescence increase in T and AS may be caused by a combination of a limited retention of ALA in the circulation and an increased PpIX synthesis. After systemic ALA administration a substantial fraction of ALA will be accumulated by the liver. ALA is also rapidly cleared from the circulation resulting in a strong reduction of ALA in the course of time. Therefore, it is likely that maximal ALA accumulation takes place directly p.i. and that ALA, or an intermediate of the haem synthesis, is retained in the cells. As a result, ALA or intermediates will be depleted faster in T and AS than in NS owing to the increased PpIX synthesis in T and AS. This may explain the disparity in time required to reach maximal fluorescence between T

and NS, a phenomenon also observed by Peng *et al* (1992) and van der Veen *et al* (1994).

This difference between tissues in interval to reaching maximal fluorescence may be an important element in determining the time interval p.i. for a PDT treatment. For example if treatment in this model would be performed at 6 h p.i., an interval where fluorescence in NS is 3 times higher than in T, no T damage but severe NS damage can be expected. That relatively small variations in time interval p.i. for PDT treatments may result in large variations in damage effect on T and normal tissues has been illustrated by several studies. Peng *et al* (1992) observed maximal fluorescence in mammary T at 1 h p.i. whereas maximal skin fluorescence was observed 3 h p.i. They observed a delay of T growth treating at 1 h p.i. (maximal fluorescence) but no delay of growth treating at 5 h p.i.. Also, Orth *et al* (1994) examined the fluorescence after systemically administered ALA in mouse skin and in a subcutaneously transplanted colonic tumour. At 3 h p.i. they found a higher fluorescence intensity in skin than in T and no T damage after a single treatment at 3 h p.i. could be obtained. Furthermore, van der Veen *et al* (1995) found no direct correlation between fluorescence intensity and the amount of vascular damage to tumour and normal tissue after systemic ALA administration. These results emphasize the importance of further studies on the relationship between fluorescence kinetics and optimum interval of PDT treatment of tumours and host tissues before systemic ALA-PDT can be a successful and reliable treatment modality for human studies. Furthermore, it also seems necessary to investigate whether the bioavailability of ALA can be improved by for example using liposomes (Fukuda *et al*, 1989) as a carrier system. A successful option for increasing the retention of ALA in the circulation is by administering fractionated ALA doses. Regula *et al* (1995) were able to produce plateau levels of ALA in patients by 6 fractionated ALA doses given orally at hourly intervals.

In contrast to systemically administered ALA, maximal fluorescence in T and AS after topical ALA was reached 6 hours after the end of the application period. Malik *et al* (1995) observed an increase in fluorescence intensity of normal mouse skin till 2-4 hours after the end of 2 hours topically applied ALA. It may be possible that during the 4 hour application, on the skin of hairless mice, a large depot of ALA is formed in the horny layer or in other parts of the skin. This ALA can be metabolized over a long period of time which may explain this long interval of fluorescence increase in T and AS. Fluorescence in NS increased only till 4 hours post application after which the fluorescence stayed at the same level. As a result maximal fluorescence intensity in T was 4 times higher than in NS. This is in contrast with systemically administered ALA where fluorescence of T at 2 h p.i. was only 1.8 times higher than NS fluorescence. The increased selectivity of T and AS over NS after topically applied ALA may be the consequence of an altered skin barrier of T and AS. The abnormal layer of keratin that is produced by some skin tumours like squamous cell carcinoma is rapidly penetrated by ALA. Also skin that shows evidence of chronic sun burn damage and actinic keratoses usually shows an increased penetration of ALA. This was illustrated by Goff *et al* (1992) who induced a disrupted stratum corneum by tape stripping the skin of guinea pigs and found an increased damage effect after topical ALA-PDT compared to ALA-PDT on skin

with an intact stratum corneum. Because of the altered skin barrier of T and AS an increased ALA depot in the skin may be formed during the application period which may result in an increase in fluorescence over a long period of time. In NS less ALA can penetrate the skin during the application period because of an intact skin barrier. This may then result in an increased selectivity of T and AS compared to NS till interval of maximal fluorescence.

At 10 h after the start of application fluorescence intensity in T and AS was at least 1.5 times higher than directly after the application period. Furthermore, the fluorescence ratio of T compared to NS between 4 and 10 h had increased from 3.3 to 4.3. This increase in fluorescence intensity and selectivity implies that treatment directly after the 4-6 h ALA-application, as commonly applied in human studies (Cairnduff *et al*, 1994; Wolf *et al*, 1993; Svanberg *et al*, 1994), may not be optimal. Instead, 4-6 h of ALA-application and postponing illumination for another 4-6 h may result in improved tumour response and improved therapeutic ratio.

Another option for increasing the fluorescence intensities after topical ALA is prolonging the ALA-application period. Szeimies *et al* (1994) observed an increase in fluorescence intensity in human basal cell carcinoma by applying ALA for 12 h instead of a 4 h application period. However, with this prolonged application interval they also observed an increase in fluorescence in surrounding normal tissue. Also in our clinic we have used a prolonged ALA application (16-19 h) for treating skin malignancies with ALA-PDT. With this prolonged application more damage effect after ALA-PDT is observed but in contrast with a 4 hour application period hardly any difference in fluorescence between T and surrounding skin can be observed. Nevertheless, the therapeutic ratio and cosmetic effect are not adversely effected. It may be possible that due to a prolonged ALA application and therefore prolonged occlusion the difference in ALA penetration between T and surrounding skin diminishes. Considering the results obtained with the experiments in hairless mice it may be more favourable to apply ALA for a limited time and illuminate the applied area at a later interval.

In further studies the influence of the interval of ALA application on selectivity and fluorescence intensities will be investigated. Also studies will be performed to examine the correlation between fluorescence intensities and therapeutic effects by treating at different intervals after topically applied ALA.

*Fluorescence localisation study.*
The results obtained with the fluorescence cryo-microscopy study showed no important variations in localisation of PpIX fluorescence in the skin between topically and systemically administered ALA. Fluorescence after both administration routes in NS and AS was mainly localised in the epidermal layer and in the hair follicles. No fluorescence could be detected in the muscle layer. In AS a small increase in fluorescence could be observed in the submucosa at 2 h post i.p. and at 6 h post topical ALA application. For topical ALA this could imply that in the course of time ALA slowly penetrates through the epidermal layer into the dermal layer.

With the fluorescence kinetics study we even observed some fluorescence at 20 h after ALA application. This fluorescence was spotty and inhomogeneous and was mainly observed in the tumours that were macroscopically rough on the top. In a pilot fluorescence cryo-microscopy study it became clear that this fluorescence was located in necrosis of tumours and some areas of the stratum corneum of the tumour. This may be caused by porphyrins excreted from cells and diffused to the necrotic parts in the tumour. Fluorescence in stratum corneum was also observed in humans after a 12 h application period by Szeimies *et al* (1994). They suggested that this fluorescence could also been synthesized by bacteria.

*Summary*
In the hairless mouse model we observed differences in fluorescence kinetics between topically and systemically administered ALA. The most apparent differences between both administration routes were the maximal fluorescence intensity and the interval to reaching maximal fluorescence in T and AS. Because of the disparity in bio-availability maximal fluorescence after systemic ALA in T was reached early (2 h p.i.) but was 3 times lower than maximal fluorescence after topical ALA which occurred at 10 h after start of ALA application. Furthermore, a higher selectivity of T compared to NS could be observed after topically applied ALA. These differences make topically applied ALA probably more favourable for a successful ALA-PDT in this model than systemic ALA.

In general it can be concluded that by optimizing the time of ALA application or interval after ALA application the efficacy and selectivity of topical ALA-PDT for skin tumours may be improved.

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# Chapter 5

## Photobleaching during and re-appearance after photodynamic therapy of topical ALA-induced fluorescence in UVB-treated mouse skin

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## Abstract

Photodynamic therapy (PDT) using protoporphyrin IX (PpIX) induced by topically applied 5-aminolaevulinic acid (ALA) seems a promising alternative for the treatment of superficial non-melanoma skin cancer and actinic keratosis. In this study the kinetics were determined of new PpIX fluorescence arising after a PDT treatment that had photobleached the original fluorescence. The purpose was to examine the feasibility of multiple irradiations, following a single topical ALA-application, to increase the PDT efficacy. In addition the photobleaching during PDT and the fluorescence spectra during and after PDT were studied. As a model we used hairless mice without and with UVB induced skin lesions. ALA was applied to the skin for 4 hours. An illumination was delivered either immediately after application or 6 hours after the end of the application (at interval of maximum fluorescence). *During* PDT, the fluorescence of normal skin decreased at a faster rate than the fluorescence of the skin lesions. In the fluorescence study *after* PDT, the areas treated immediately post application showed a similar fluorescence increase in time as non-treated areas on the same mice. A remarkable result was that the fluorescence of areas treated at maximum fluorescence increased whereas the fluorescence of non-treated areas did not increase in time. With both treatment intervals the new fluorescence showed a characteristic PpIX spectrum. These results demonstrate that a second illumination, when new PpIX-fluorescence has been formed, may increase the PDT efficacy after topical ALA-application. This finding has been demonstrated previously for systemic ALA administration.

## Introduction

Photodynamic therapy (PDT) is an experimental cancer treatment modality using a photosensitizer, which localizes in tumour tissue and can produce tissue destruction upon absorbing light of an appropriate wavelength and dose. 5-Aminolaevulinic acid (ALA) is a prodrug that can be converted in situ into the effective photosensitizer protoporphyrin IX (PpIX), the penultimate step in the haem biosynthetic pathway. A simplified scheme of this biosynthetic pathway for haem is shown in figure 1. Under normal conditions the synthesis of haem is controlled by a feedback mechanism in which the presence of free haem inhibits the synthesis of ALA. An excess of exogenous ALA bypasses this feedback control mechanism to create diagnostic and therapeutic levels of intracellular PpIX. Besides PpIX other photosensitive products like Uroporphyrin (Up) I and III and Coproporphyrin (Cp) I and III are synthesised in situ. Up and Cp are very water soluble and are rapidly removed from the body. Therefore, they are normally not observed in vivo.

Protoporphyrin IX is a photolabile sensitizer absorbing light throughout the near-ultraviolet and visible region. Light absorption brings the PpIX molecule to an excited state from which it may decay back to the groundstate via an intermediate excited state, thereby raising available oxygen from its triplet ground state to the excited singlet state. Singlet oxygen is highly reactive and may cause cellular damage and eventually tissue destruction. Especially the mitochondrion is very sensitive to PDT damage (Hilf *et al.*, 1986). This was demonstrated by Iinuma *et al.* (1994) and Malik *et al.* (1987) who observed early irreversible photodynamic damage of the mitochondrion, the site of PpIX synthesis.

Photo-destruction of PpIX by PDT results in bleaching of fluorescence and the formation of photoproducts. Because photodestruction is dependent on the oxygen concentration the process of photobleaching may be used as an indicator for the effectiveness of ALA-PDT (Robinson *et al.*, 1996). The same may account for the process of photoproduct formation (König *et al.*, 1993; Ahram *et al.*, 1994) but the role of photoproducts in the effectiveness of ALA-PDT is still unclear (Gudgin Dickson *et al.*, 1995).

PDT with topically applied ALA has proven to be effective for a variety of superficial skin malignancies. In clinical studies (Kennedy *et al.*; 1990, Wolf *et al.*; 1993, and Calzavara-Pinton *et al.*, 1995) superficial basal cell carcinoma, actinic keratoses, Bowen's disease and squamous cell carcinoma could be treated successfully (initial complete respons rate of approximately 85%). However, its efficacy for nodular lesions is still insufficient. Multiple irradiations may be a modality for increasing the PDT efficacy. This was illustrated in a previous study by van der Veen *et al.* (1994). In that study two light treatments with an interval of 75 minutes were delivered after one systemically administered ALA dose. After a single treatment, which bleached the fluorescence, no necrosis of tumour tissue was observed. New fluorescence was observed in the course of time after the first treatment and total tumour necrosis could be accomplished after two treatments. This work should be distinguished from

that of Messmann *et al.* (1995) and Hua *et al.* (1995) who studied illumination with brief interuptions to reoxygenate the tissues rather than by using new fluorescence. In the present study the feasibility for multiple irradiations with topically applied ALA was determined by examining the fluorescence kinetics after a single illumination. In addition the bleaching of fluorescence in skin lesions and normal skin during treatment was followed without interrupting the illumination. During and after treatment fluorescence spectra were monitored to examine the formation of photoproducts. As a model we used hairless mice without and with small skin lesions (actinic keratoses and squamous cell carcinomas), which were induced by UVB irradiation.

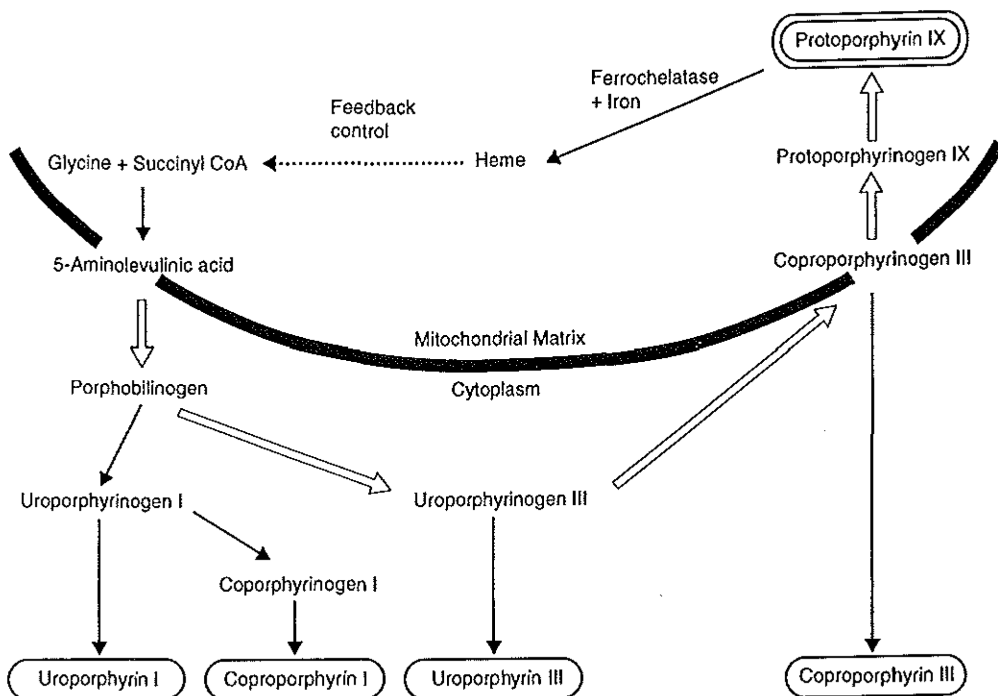



*Figure 1. Simplified biosynthetic pathway for haem. The principal biosynthetic route for ALA-induced protoporphyrin IX is indicated by open arrows. The haem feedback control is indicated with a dashed line. All fluorescent components are encircled; the most potent sensitizer (PpIX) is double encircled.*

## Materials and methods

### *Animal model*

Inbred female albino mice, Skh hr1, of 18 weeks old were obtained from the University of Utrecht. The dorsal skin of the mice had been exposed to a daily UVB dose of 1.5 kJ $m^{-2}$ using a Westinghouse FS40TL12 lamp according to a method previously described by de Gruijl *et al.* (1983). After approximately 80 days multiple primary tumours developed in the exposed area. In the experiments small lesions (1-2 mm diameter) were included, which were mainly squamous cell carcinoma, actinic keratoses and seborrhoeic keratoses. Non-UVB irradiated animals were used to assess the fluorescence of normal skin. This was necessary because as a reaction to the daily UVB dose the exposed dorsal skin showed an acute thickening of the epidermis which was only partly reversible (altered skin). In this article the UVB-irradiated animals will be referred to as UVB animals and the non-UVB irradiated animals will be referred to as n-UVB animals.

### *Preparation and administration of ALA*

5-Aminolevulinic acid hydrochloride (98%) was purchased from Finetech Ltd. (Haifa, Israel). ALA was dissolved in Carboxymethylcellulose 3% after which the solvent was set at pH 5.5 by adding NaOH (2M) to prevent irritation of the skin. Before application the animals received a low dose of Hypnorm (fluanisol/fentanyl mixture, Janssen Pharmaceutics, Belgium) and diazepam (Centrafarm b.v., Etten-Leur, The Netherlands) to avoid anxiety caused by the dressing. A thin gauze was soaked in a solution containing 20% ALA and placed on the entire dorsal skin. A piece of transparent film dressing (Mölnlycke, Waremme, Belgium) was placed over the gauze to achieve occlusion and to prevent evaporation and movement of the solvent. The solvent was applied to the skin for four hours after which it was carefully removed.

## *Experimental apparatus*

### *Fluorescence imaging measurements (FIM)*

Fluorescence images *during* PDT were recorded using a CCD-camera with a two stage image intensifier and a 50 mm Leitz Photar macrolens. Fluorescence was detected through a bandpass filter of 670 ± 50 nm. Excitation light from an Argon laser with an incident power density of 100 mW $cm^{-2}$ and a wavelength of 514 nm was guided by a 400 μm fiber. The fiber was attached to the CCD-camera by a specially developed holder. With a microlens fitted to the end of the fiber a homogeneous light field of 7 mm diameter was projected on the mouse skin at an angle of 25 degrees with the normal. Variations of the beam profile (± 10%) were examined at 9 points in the light field using an isotropic light probe (Van Staveren *et al.*, 1995). The treatment dose was 100 J $cm^{-2}$ which required a treatment time of 16 minutes and

40 seconds. To prevent overload of the CCD-camera by the fluorescence light grey filters were placed in front of the Leitz Photar lens. With this set-up it was possible to record the fluorescence during treatment without interrupting the illumination beam. The recorded digitized images were analyzed yielding average grey scale values of selected areas of interest.

The experimental set-up for the fluorescence recordings *after* PDT was partly similar to the set-up used during PDT. Fluorescence images were also recorded using the CCD-camera with a two stage image intensifier and a 50 mm Leitz Photar macrolens. Light from a halogen lamp and a laser line filter were used to excite fluorescence at a wavelength of 514 nm. Through a dichroic mirror light with a maximal irradiance of 0.2 mW $cm^{-2}$ was projected perpendicularly on the skin. Fluorescence was detected through a bandpass filter of 670 ± 50 nm. The recorded digitized images were analyzed yielding average grey scale values of selected areas of interest.

***Fluorescence spectroscopy measurements (FSM)***

ALA-induced fluorescence emission spectra of UVB and n-UVB animals were determined with an Instaspec IV system (Oriel, Stratford, CT USA). Fluorescence during PDT was excited with 514 nm light from an Argon laser with an incident power density of 100 mW $cm^{-2}$. Fluorescence after PDT was excited with 514 nm from an Argon laser with an incident power density of 75 μW $cm^{-2}$. The fluorescence emission spectrum was recorded above 570 nm using a long pass filter to examine both PpIX emission bands (640 and 710 nm). The animals were placed against a specially developed holder to ascertain a constant distance between skin and fibertip during the recordings.

***Experimental design of FIM and FSM***

The numbers of animals included in the FIM and FSM study are shown in Table I. In the FSM study only a small number of animals were used because the possibility for recording spectra arose at the end of the study. Therefore, the results should be seen as a support and an enhanced understanding of the results obtained with the fluorescing imaging study. The animals were divided in three groups, each group containing UVB and n-UVB animals. The first group (A) served as a control and received a PDT treatment after application of the solvent which contained no ALA. The second group (B) received a PDT treatment at t=4 (directly after the ALA application) and the third group (C) received a PDT treatment at t=10 (6 hours after the end of the application period). These intervals for a PDT treatment were based on the fluorescence kinetics study described by van der Veen *et al.* (1996) which was carried out under similar conditions. Briefly, the interval for PDT in group B was chosen for comparison with clinical treatments and the PDT interval for group C was chosen because maximal fluorescence of UVB skin was observed at that interval. In each animal two areas of 1 cm in diameter were chosen and ALA was applied to both. One area received a PDT-

illumination and the other served as a control.

For the FIM *during* PDT the animals were anaesthetized with a combination of Ethrane/Oxygen/$N_2O$ and placed under the CCD-camera on a temperature controlled stage. To avoid photobleaching and PDT-damage the untreated skin was covered with black plastic. During the treatment of almost 17 minutes, 35 fluorescence recordings were made on-line to examine the photobleaching.

For the FIM *after* PDT the animals were briefly anaesthetized with a combination of Ethrane/Oxygen/$N_2O$ and (re-)positioned under the CCD camera. Before ALA application (autofluorescence) and at several intervals after PDT the fluorescence of the treated and the control area was recorded. The fluorescence after treatment of group A and B was recorded every 15 or 30 minutes for 5 hours. The fluorescence after treatment of group C was recorded every 15 minutes for 120 minutes. Between the fluorescence recordings the animals were housed under subdued light conditions with water and food ad libitum.

In UVB and n-UVB animals of each group spectra of both areas were recorded to examine the fluorescence *after* PDT. The spectra were measured before ALA-application and before PDT, directly after PDT and 1 and 2 hours after PDT. During the recordings the animals were briefly anaesthetized with a combination of Ethrane/Oxygen/N2O and (re-)positioned under the holder. In an additional experiment the spectra during PDT in UVB and n-UVB animals were recorded every 10 seconds to examine the possible formation of photoproducts *during* the treatment. In each animal one area was treated at t=4 and a second area was treated 17 minutes later.

**Table I.** Number of animals (UVB/n-UVB) of 3 groups treated at t=0 (A, before application), t=4 (B) and t=10 (C). The animals were used for fluorescence imaging measurements (FIM) during and after PDT and for fluorescence spectroscopy measurements (FSM) during and after PDT.

| Group | FIM | | FSM | |
|---|---|---|---|---|
| | During PDT | Post PDT | During PDT | Post PDT |
| A | 2/2 | 2/2 | | 2/2 |
| B | 6/6 | 6/6 | 2/2 | 2/2 |
| C | 6/6 | 6/6 | | 2/2 |

## Results

### *FIM during PDT*

As an indication for differences in bleaching rate between tissues and for different PpIX concentrations we examined the light dose at which the fluorescence was reduced to 50% (FBD50). This was determined in UVB and n-UVB skin treated at t=4 (group B) or t=10 (group C). The result for each animal was corrected for the autofluorescence, which was partly photobleached, and for the non-bleached fluorescence. Between UVB and n-UVB skin a clear difference in the FBD50 was observed in both groups. In group B the dose for UVB skin was 9.8 J $cm^{-2}$ (s.e.m. ± 1.1) and for n-UVB skin 4.8 J $cm^{-2}$ (s.e.m. ± 0.8). In group C almost the same values (9.7 ± 1.4 and 5.9 J ± 0.9 $cm^{-2}$ respectively) were obtained.

Treatment with 100 J $cm^{-2}$ at a dose rate of 100 mW $cm^{-2}$ lasted almost 17 minutes. The fast photobleaching, as illustrated by a FBD50 of approximately 10 J $cm^{-2}$, suggest that it may be possible to shorten the treatment time without loss of biological effect. As an indication for the effective treatment time the dose at which 80% (FBD80) and 90% (FBD90) of the fluorescence had bleached was determined. In both UVB and n-UVB in group B and C no difference in FBD80 and FBD90 was observed. Therefore, the results of the two groups were taken together. In the UVB treated skin the FBD80 was already observed after 30 J $cm^{-2}$ (s.e.m. ± 1) and the FBD90 after 49 J $cm^{-2}$ (s.e.m. ± 2). In n-UVB skin this dose was achieved faster, after 20 (s.e.m. ± 2) and 40 J $cm^{-2}$ (s.e.m. ± 2) respectively.

### *FIM post PDT*

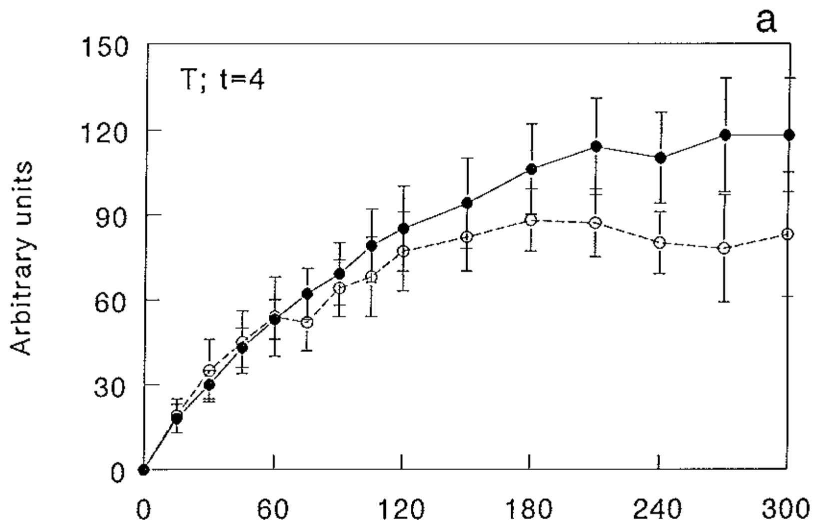
The fluorescence kinetics after PDT of group A, B and C were recorded. Also, the fluorescence kinetics of non-treated areas, which served as controls, in the same animals were recorded. The results are presented in Figure 2 as fluorescence *increase* in time and not as fluorescence intensity. Therefore, the fluorescence values directly post PDT of non-treated or treated areas were subtracted from all further recorded images for each area. Differences in fluorescence intensities before ALA, before treatment and immediately after treatment of the 3 groups are shown in Table II. The fluorescence increase in figure 2 and the fluorescence intensities shown in table II are not corrected for the autofluorescence.

The first group (A) received a PDT treatment without ALA to examine the autofluorescence. Because the autofluorescence of tumour could not be distinguished from the surrounding altered skin the average grey scale value of the entire recorded area was measured as a function of time. The fluorescence levels of both areas before treatment were similar and after treatment the fluorescence levels of the treated areas were approximately 2 times less than the intensities before treatment (see Table II). After PDT no increase of the autofluorescence of both treated and non-treated areas in UVB and n-UVB skin could be observed. This is not shown in figure 2 to limit the number of graphs.

The second group (B) received a treatment at t=4 which was immediately after ALA

application. At t=4 the ALA-induced fluorescence of tumour was approximately 7 times, altered skin 6 times and normal skin 3 times higher than their respective autofluorescence. The fluorescence intensities of treated areas of these tissues directly after PDT decreased almost to the fluorescence intensities observed before ALA-application (see Table II). The fluorescence of treated and non-treated areas increased after PDT which is shown in figure 2a and 2b for tumour and normal skin. During the observation time of 300 minutes the increase of fluorescence in tumour was higher than in altered skin which was higher than in normal skin. In tumour and altered skin a similar increase until 150 minutes was observed in both the treated area and non-treated area. After 150 minutes the increase in fluorescence of the treated area seemed to be slightly higher (not significant) than the non-treated area. In normal skin (Figure 2b) a slight increase in time could be observed which was similar in both areas. This total increase at the end of the observation time was less (2.5 times) than observed in tumour.

The third group (C) received a treatment at t=10 which was 6 hours after the end of the ALA-application period. The fluorescence intensities at this interval in tumour, altered skin and normal skin were approximately 1.4 times higher than at t=4. After PDT the fluorescence in the treated areas of normal skin had decreased to the intensities observed prior to ALA application. In both tumour and altered skin the fluorescence after PDT was higher than the intensities observed prior to ALA application (see Table II). In figure 2c and 2d the fluorescence *increase* of tumour and altered skin is shown. For practical reasons the fluorescence after PDT in group C was monitored for 120 minutes. The results obtained in this group were quite fascinating. In all tissues there was a significant difference in fluorescence *increase* between treated and non-treated areas. In the non-treated areas the fluorescence did not increase whereas in treated areas the fluorescence increased during the observation time. The fluorescence increase in tumour was higher than altered skin which was higher than normal skin. The increase in group C of the treated areas was similar to the increase observed in group B. To illustrate this fluorescence increase 4 images recorded before treatment (I), immediately after treatment (II), 1 (III) and 2 (IV) hours after treatment are displayed in Figure 3. Note that the irradiance of the fluorescence excitation light before treatment (I) was a factor of 3 smaller than after treatment (II, III, IV). In this figure a decrease in fluorescence intensity in both tumour and altered skin after PDT was monitored. In the images recorded 1 and 2 hours after treatment an increase, which was higher in tumour than in altered skin, can be seen.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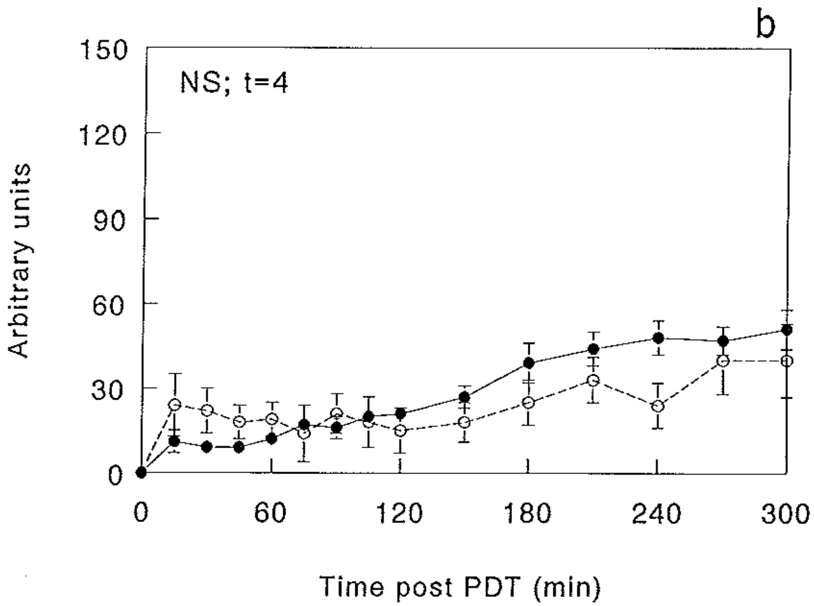
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Time post PDT (min)

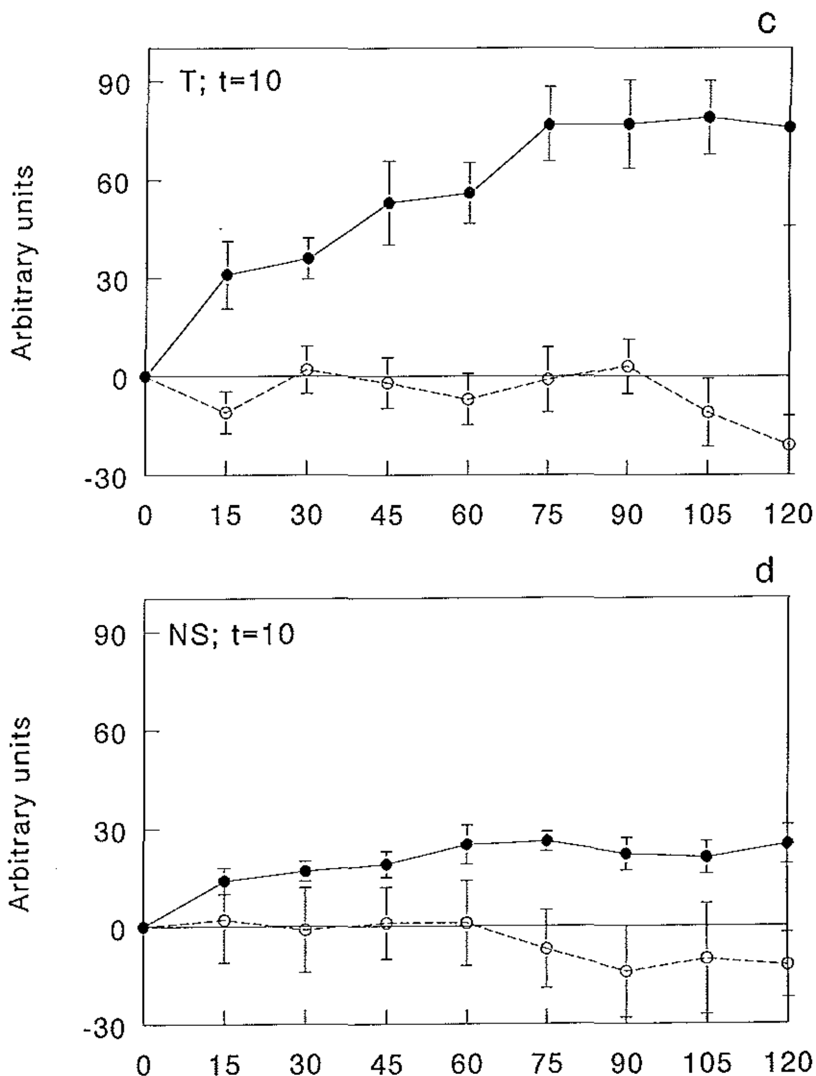



*Figure 2. Fluorescence increase (± s.e.m.) in treated areas (solid lines and closed symbols) after PDT and control areas (dashed lines and open symbols) as a function of time. Animals with skin lesions (T, 2a) and n-UVB animals with normal skin (NS, 2b) of group B received a treatment at t=4. Animals of group C (T; 2c and NS; 2d) received a treatment at t=10. The arbitrary units are the same in 2a, 2b, 2c and 2d. Note the difference in the scale of the horizontal axis between 2a, b and 2c, d, respectively.*

**Table II.** Mean fluorescence intensities expressed in arbitrary units of 3 groups (± s.e.m.) Treated at t=0 (A, before application), t=4 (B) or t=10 (C). In all groups the fluorescence of tumour (T), altered skin (AS) and normal skin (NS) was determined before ALA-application (Autoflu.), prior to PDT (pre-PDT) and immediately after PDT (post PDT).

| | | Autoflu. | Pre-PDT | Post-PDT |
|---|---|---|---|---|
| Group A | T, AS | 42 ± 4 | 45 ± 9 | 26 ± 3 |
| | NS | 45 ± 2 | 44 ± 5 | 28 ± 1 |
| Group B | T | 45 ± 6 | 322 ± 46 | 65 ± 10 |
| | AS | 45 ± 6 | 261 ± 28 | 59 ± 8 |
| | NS | 50 ± 9 | 170 ± 13 | 48 ± 7 |
| Group C | T | 49 ± 7 | 473 ± 49 | 82 ± 4 |
| | AS | 49 ± 7 | 380 ± 37 | 75 ± 4 |
| | NS | 47 ± 10 | 219 ± 22 | 45 ± 4 |

*FSM*

The spectra after PDT of 3 groups consisting each of UVB and n-UVB animals treated at t=0 (no ALA, group A), t=4 (B) and t=10 (C) were recorded. In each animal spectra of the PDT area and the control area were determined before ALA-application and PDT, directly after PDT and subsequently 1 and 2 hours after PDT. All recordings were corrected for background spectra and for small variations in excitation light intensity.

An autofluorescence spectrum was recorded in both areas of all the animals (6 UVB and 6 non-UVB irradiated mice). In 19 out of 24 measurements no clear emission bands between 600 and 720 nm could be distinguished. However, in 5 measurements (3 UVB and 2 n-UVB) a weak emission band around 679 nm could be detected. This band did not always occur in both areas of one mouse and no correlation between the location of the area and the occurrences of this band could be made. Also, within these observations large variations in fluorescence intensities were observed. In group A the spectrum was examined of the bleached autofluorescence of UVB and n-UVB animals. In both groups the areas after PDT showed a lower intensity of the entire spectra (in 2 animals also of the 679 nm band) recorded between 600 and 720 nm whereas the spectra of the control areas did not alter. In both areas no increase of fluorescence in time after PDT could be observed. In group B the spectra before and after PDT at t=4 were determined. A typical example of the spectrum of a treated and a control area of an UVB mouse is shown in figure 4a and 4b. Except for differences in

height no differences in spectra between UVB and n-UVB animals before and after PDT were observed. In both groups typical PpIX spectra with emission bands around 640 nm and 709 nm were detected before PDT. After treatment both emission bands had almost disappeared in the treated areas and the spectra were similar to the autofluorescence spectra. After 1 and 2 hours post PDT a small fluorescence increase of the first emission band (640 nm) was observed indicating a PpIX increase. The fluorescence intensity of the control area increased in time as illustrated in Figure 4b. In group C the spectra before and after PDT at t=10 were determined. In Figure 4c and 4d the spectrum of the treated and the control area of an UVB mouse is shown. Before PDT a similar spectrum but with higher emission bands was observed as at t=4 (group B). Immediately after PDT the spectrum of the treated areas was almost similar to the autofluorescence spectra. At 1 and 2 hours after PDT an increase of the 640 nm band was observed whereas the fluorescence intensity of the control area decreased as shown in Figure 4d. In n-UVB and UVB animals the spectra during PDT were recorded every 10 seconds to examine the formation of photoproducts. A fast decrease with the same rate of the 640 and the 709 nm band was observed. The fluorescence intensity at 680 nm decreased at a lower rate but during PDT no additional bands could be observed (data not shown). After PDT there were no clear emission bands.

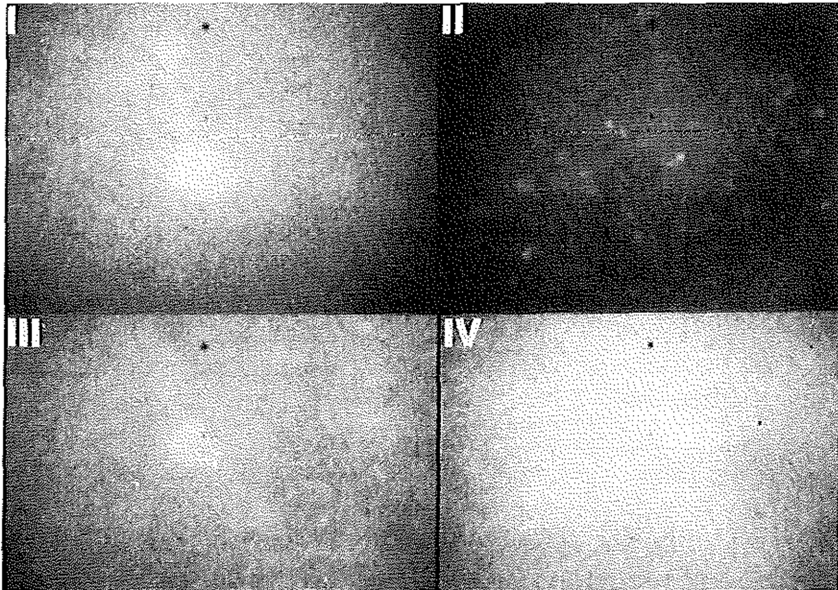
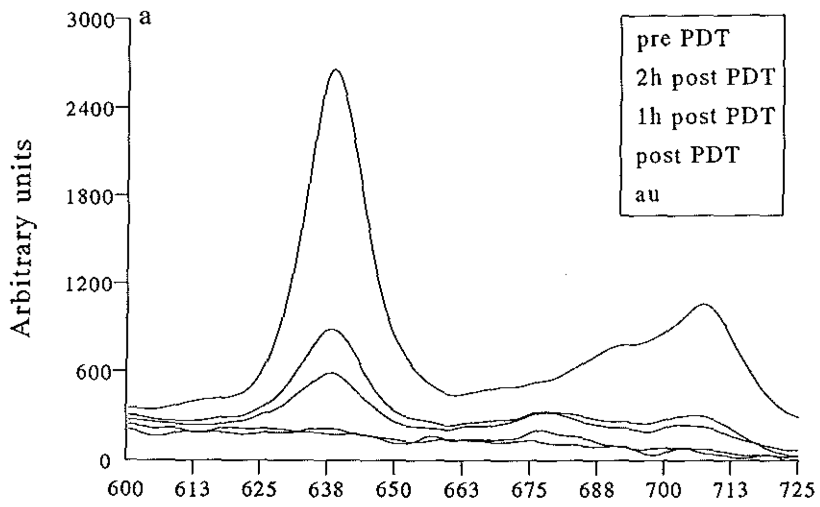



*Figure 3. Fluorescence increase at 4 intervals of UVB skin treated at t=10 (group C): I; before PDT; II, post PDT, III; 1 h post PDT and IV 2 h post PDT. Note that the irradiance of the fluorescence excitation light before treatment (I) was a factor of 3 smaller than after treatment (II, III, IV). T can be recognized in I, III, IV as a bright fluorescent spot in the centre of each image.*


a
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pre PDT
2h post PDT
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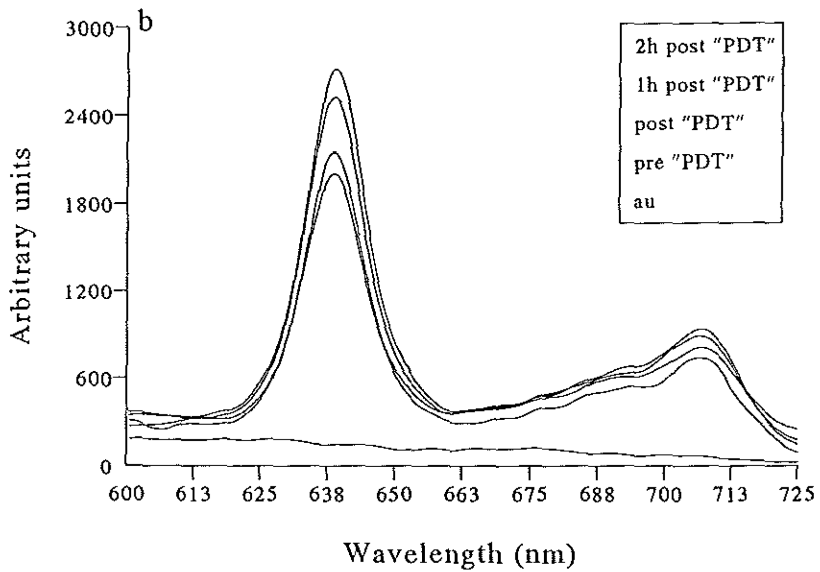
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pre "PDT"
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Wavelength (nm)

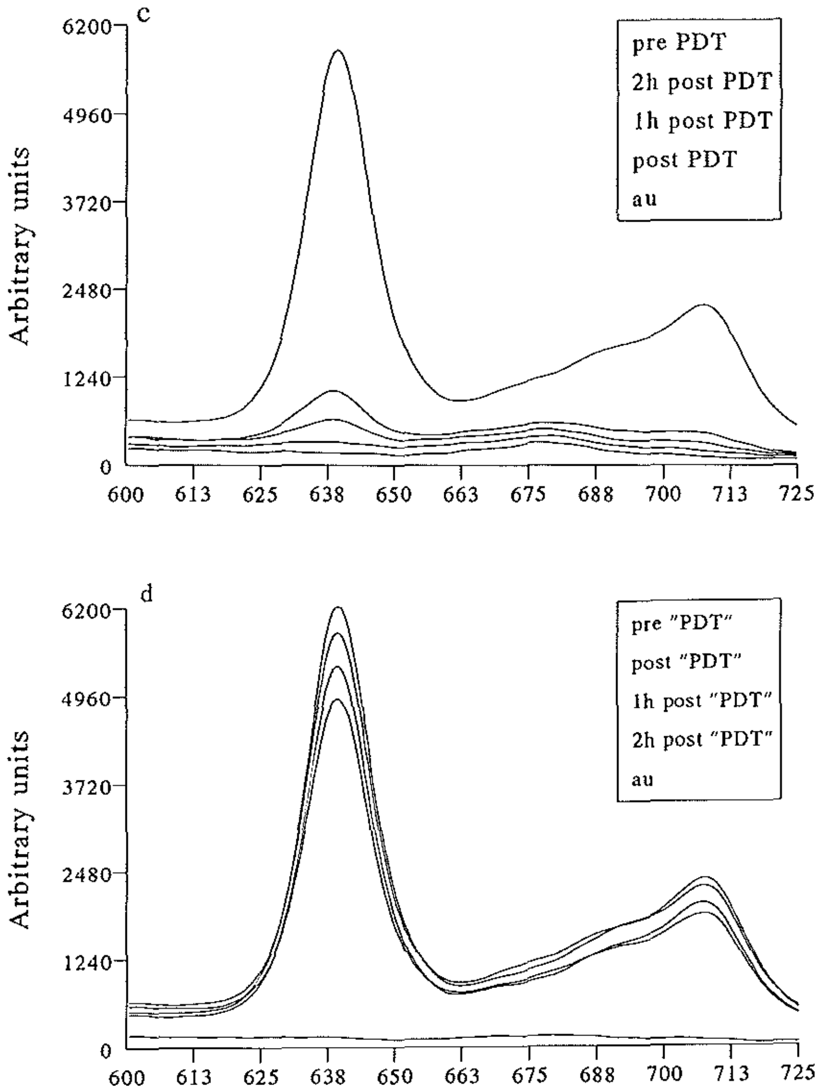


***Figure 4.*** *In vivo fluorescence emission spectra of skin lesions prior to ALA (au); pre PDT; post PDT; 1 h post PDT and 2 h post PDT. In each box the time of measurements of the spectra is indicated in rank order from high to low fluorescence intensity. The data of the control areas, which received no irradiation are indicated by quotation marks. Figure 4a represents the area of one mouse treated at t=4 (group B) and Fig. 4b shows the related spectra of the control area (ALA but no PDT). Figure 4c represents spectra of the area of one mouse treated at t=10 whereas Fig. 4d represents spectra of the control area.*

## Discussion

### *Animal model*

In this study hairless mice were used with small lesions on the dorsal skin which were induced by daily UVB irradiation. De Gruijl *et al.* (1983) observed that the nature of the lesions differed depending on their size. They found that the fraction of actinic keratoses decreases with increasing diameter whereas the fraction of squamous cell carcinoma increases with increasing diameter. This means roughly that lesions smaller than 2 mm mainly consist of actinic keratoses whereas lesions between 2 and 4 mm mainly consist of squamous cell carcinoma. In this study only small lesions of 1 to 2 mm were included to avoid considerable spread in the results. Larger lesions revealed considerable variations in appearance and incidence of necrosis or bleeding on top of these lesions.

The UVB treated hairless mice were chosen for their resemblance with skin lesions like actinic keratoses and squamous cell carcinomas observed in humans. These skin lesions are also closely associated with repeated exposure to UV irradiation (Fears *et al.*, 1976). In both humans and mice a thickening of the epidermis is seen after exposure to UV light. In humans this reaction is only temporary and the thickness returns to normal values within weeks when the skin is no longer exposed to sunlight. The daily UVB dose given to mice was so high that an acute thickening of the epidermis occurred which remained longer and was only partly reversible (altered skin). This made it necessary to use n-UVB mice to examine skin with normal epidermal thickness. Therefore, in this paper we emphasise the comparison between n-UVB skin and skin lesions of UVB skin and not between skin lesions and the surrounding UVB skin.

### *Experimental apparatus*

In this paper two methods (FIM and FSM) were used for fluorescence measurements. These two methods have their own advantages and disadvantages but by combining the methods a good indication about the distribution and nature of ALA-induced fluorescence could be obtained. With FIM the fluorescence distribution could be visualised and after recording of the images the mean fluorescence intensity of several areas of different size could be measured. Additional information about the nature of the fluorescence and especially changes in spectra could be obtained with FSM.

A comparison between these two methods was made with regard to fluorescence kinetics. With FSM the area under the curve of the fluorescence measured between 620 and 720 nm (the band of detection used with FIM) was determined at several intervals and compared with the fluorescence intensities recorded with FIM. With both the photobleaching and the study concerning new fluorescence the same fluorescence kinetics were observed.

For PDT-treatment and for the fluorescence kinetics after treatment excitation light of 514 nm was used. The light penetration in skin was examined by placing an isotropic light

probe (Van Staveren *et al.*, 1995) at the surface or under the skin of a n-UVB and UVB animal on two different areas (no ALA). A homogeneous light field of 7 mm diameter and 100 mW $cm^{-2}$ was projected on the skin. No differences in fluence rate between UVB and n-UVB on the surface of the skin and under the skin could be detected. In both cases the fluence rate at the surface of the skin was approximately 150 mW $cm^{-2}$, due to back scattered light. Under the skin the fluence rate was approximately 100 mW $cm^{-2}$. These measurements indicate that green light has sufficient penetration depth in mouse skin and that differences in results between UVB and n-UVB animals presented in this paper are not the result of differences in light penetration between these tissues.

Light penetration in both UVB and n-UVB skin using 514 nm might alter due to vascular damage effects induced by PDT. This effect may have consequences for the PpIX fluorescence intensities in time. However, the strongest fluorescing layer of the mouse skin is the epidermis whereas hardly any fluorescence can be detected in the dermis (Van der Veen *et al*; 1996). The vessels are located under the epidermis. Therefore, alteration in light penetration due to vascular damage is not likely to be an important factor on the observed PpIX fluorescence intensities in time.

***Fluorescence during PDT***

The 50% fluorescence bleaching light dose (FBD50) of n-UVB skin was about two times smaller than that of UVB skin. The rate of bleaching with a constant fluence rate may depend on the concentration of oxygen and PpIX in the cells. This was investigated in aqueous solution, with either constant oxygen concentration and various PpIX concentrations or the reverse, by Robinson *et al.* (1995). They observed an increase in photobleaching rate after increasing the oxygen or the PpIX concentration in the solution. They also found that the oxygen concentration was rate limiting. Between UVB and n-UVB skin a clear difference could be observed in thickness of the epidermis, which is the strongest fluorescing layer. Due to the UVB irradiation the epidermis and dermis are thickened. The thickness of the epidermis of altered skin is 52 μm (s.e.m. ± 5), of tumour with the underlying epidermis 404 μm (s.e.m. ± 70) whereas the thickness of the epidermis of normal skin (n-UVB) 23 μm (s.e.m. ± 1) is. The non-vascularized epidermis depends on oxygen by diffusion from the dermis. This may result in less oxygen during treatment in the epidermal layer of the thickened UVB skin compared to the epidermal layer of the thin n-UVB skin. The lower oxygen concentration in UVB skin may then result in the lower photobleaching rate compared to n-UVB skin. The faster bleaching rate in n-UVB skin compared to UVB skin may be beneficial for the therapeutic ratio between these tissues. Iinuma *et al.* (1994) suggested that some threshold of cellular PpIX concentration is required for PDT-induced cell killing. The fast bleaching of the sensitiser in n-UVB skin may reduce the concentration of sensitiser to a level below the threshold for producing photodynamic therapy which may enlarge the therapeutic ratio (Moan *et al.*, 1996).

We could not find a correlation between the initial fluorescence intensity and the photobleaching rate. There was a difference (a factor of approximately 1.5) in fluorescence intensities before treatment in both UVB or n-UVB between group B and C (see table II). However, similar values of the FBD50 were observed. A condition for finding a higher rate of photobleaching for higher fluorescence (Robinson *et al.*, 1996) is that optimum oxygen concentration must be available during the treatment. This condition can be realized with in vitro studies. In vivo vascular constriction during PDT is a common phenomenon (van der Veen *et al.*, 1994; Star *et al.*, 1986). Also after topical ALA-PDT a reduced circulation, which was temporary, was observed in this hairless mouse model (results will be presented in a forthcoming paper). Constriction results in less optimal oxygenation of the cells which may explain why different initial fluorescence intensities were not accompanied by different photobleaching rates. Also, in group B the location of PpIX in the cell may be different than in group C. This was indeed found by Malik & Lugaci (1987) who observed a gradual translocation of porphyrins from the mitochondria to less sensitive sites. The influence of this factor on the photobleaching rate has not yet been investigated. However, it seems that the initial fluorescence intensity is less relevant for the photobleaching rate than the oxygen concentration during treatment.

The autofluorescence bleached to approximately 50% of its original values. Depending on the ALA-induced fluorescence intensity this is a factor which should be taken into account. Especially with low ALA-induced fluorescence intensities, which mostly occur in normal tissues, the contribution of the bleached autofluorescence to the total photobleached fluorescence may be significant. The nature of the spectrum before and after PDT is discussed in section 4.4.

By correcting the results for the photobleaching of the autofluorescence the bleaching of ALA-induced fluorescence could be determined. We then found that approximately 18% of the ALA-induced fluorescence intensity before treatment did not bleach. This was independent of the initial fluorescence levels, the different tissues or the different treatment intervals. This phenomenon was also not the result of a low light dose because after 20-30 J $cm^{-2}$ approximately 80% of the ALA-induced fluorescence had already bleached. Spectra of the remaining fluorescence of the treated areas were measured but no clear emission bands could be detected. An overall higher fluorescence intensity of the detected area between 620 and 720 nm was observed. This might indicate that the remaining fluorescence is not from photoproducts, not from PpIX which can not be bleached any further due to lack of oxygen nor from PpIX which is still continuously synthesised in time. We are not aware of studies in which comparable results concerning residual fluorescence were observed. In vivo, especially in humans, it is almost impossible to determine the non-bleached fluorescence by correcting the measured fluorescence for the bleaching of the autofluorescence. Because no clear emission bands were observed and the residual fluorescence could hardly be bleached further the relevance of this phenomenon for the PDT efficacy remains unclear.

After 30 J $cm^{-2}$ in UVB skin and after 20 J $cm^{-2}$ for n-UVB skin already 80% of the

fluorescence was bleached. As mentioned a PpIX threshold level for producing PDT damage was observed by Iinuma *et al.* (1994). It would be interesting to perform a light dose-respons study to examine the PpIX threshold level for producing PDT-damage with topical ALA. Experiments have been started looking at the correlation between photobleaching rate and the damage for different fluorescence levels. If there is a correlation, monitoring the photobleaching during PDT can then be a useful tool to determine the required light dose.

With FSM we examined the formation of photoproducts during PDT. The decrease of fluorescence in the 3 spectral regions (640, 680 and 709 nm) was not proportional. The fluorescence around 680 nm decreased at a lower rate than the other two bands. Several authors have reported (König *et al.*, 1993; Ahram *et al.*, 1994) PpIX photoproduct formation in vitro and in vivo during irradiation. A minor emission peak in the vicinity of 670 nm or a slower decrease of this peak during PDT has been observed which presumably indicates the formation of hydroxyaldehyde chlorin-type of photoproduct. The occurrence of photoproducts and their possible role in the effectiveness of ALA-PDT is unclear. It appeared that the occurrence of photoproducts in vitro depends on the solvent used and on the oxygen concentration (König *et al.,* 1994). König *et al.* (1994) investigated the role of photoproducts on the PDT efficacy in vivo by applying a combined treatment. They used 630 nm for PpIX activation during the first part of the treatment and subsequently 670 nm for activation of the photoproducts during the second part. This combination treatment compared to a single treatment with 630 nm did not enhance the therapeutic efficacy. However, the significance of photoproducts needs to be investigated in more detail before conclusions can be drawn.

***Fluorescence post PDT***

The fluorescence after PDT was followed in animals treated at t=4 (group B) or t=10 (group C) to see whether new fluorescence was formed which might be used for a second illumination, like after systemic ALA (van der Veen *et al.*, 1995).

Because bleaching of the autofluorescence was observed the fluorescence post PDT was also followed to examine a possible recovery of the autofluorescence. In the autofluorescence measurements no clear emission bands were observed before and after treatment. After treatment similar spectra but with lower fluorescence intensities were detected. Also, in the observation time after treatment no increase in fluorescence or changes in spectra could be observed indicating that the increase in fluorescence observed after ALA-PDT is not the result of autofluorescence recovery. In 21% of the autofluorescence spectroscopy recordings a weak emission band in the vicinity of 679 nm could be detected before and/or after treatment. This peak did not always appear on both areas in the same mouse and no clear correlation could be made between the appearance of this band and the location of the area on the mouse. Weagle *et al.* (1988) examined the chromophore responsible for the 679 band in mouse skin. They observed the emission peak at all sites of the mice with the highest intensity at the abdominal side. This fluorescence peak was the result of a degradation product of one of the chlorophyll molecules. Because in our results no

correlation could be established between the sites of measurements and the appearance of the 679 band it is not likely that this band is the result of chow components. It is more likely that the occasional band is the result of stray light from the experimental apparatus.

With FIM, the fluorescence increase in the treated areas after PDT in group B was similar in time as in the control areas. In contrast, in group C an increase in fluorescence in the treated areas was observed whereas the fluorescence of the control areas did not increase in time. These results were similar in both UVB as in n-UVB animals.

The spectra of the new fluorescence of the treated areas after PDT in group B and C were typically PpIX spectra. Other components like Uro and Coproporphyrin (Up and Cp) were expected based on experiments performed by Dietel *et al* (1996). They observed an enhanced formation of Up and Cp under photolysis conditions which was due to mitochondrial damage after PDT. PpIX is synthesized in the mitochondrion (figure 1), a cell organelle initially damaged in PDT (Iinuma *et al.*, 1994; Malik *et al.*, 1987). This damage might lead to an accumulation of various water soluble Up and Cp derivatives. Although water soluble porphyrins are rapidly transported into the blood stream and cleared from the body an increase of these hydrophillic porphyrins in the mice could occur due to a shut down of the circulation caused by the treatment. However, no emission band around 610 nm, indicating Up and Cp accumulation, was observed.

Because ALA was applied to a relative large area of the body a possible explanation for the new fluorescence post PDT was PpIX leakage, from cells surrounding the PDT-treated area or from PpIX synthesised in other parts of the skin and transported through the circulation. However, looking at the images we did not see fluorescence from the edges slowly penetrating into the treated area which then would result in a decreased diameter of the non-fluorescent area in time. In contrast, as illustrated in Figure 3, we did observe a homogenous fluorescence increase in the entire bleached area in time, which makes PpIX leakage from cells surrounding the bleached area unlikely. To examine PpIX leakage from tissues outside the treated area an additional experiment was performed. In this experiment we measured the difference in increase of PpIX fluorescence between animals having ALA applied to a large area (the entire dorsal skin) and animals with ALA applied to an area of the same size as the PDT-treatment field. This experiment was performed at t=10 in UVB mice (n=4); an interval were maximal fluorescence in the skin was observed and where one would expect maximal PpIX leakage. In both the control and in the PDT-treated area no difference in fluorescence increase was observed between a large and a small ALA application area (data not shown). These results suggest that new fluorescence post PDT observed in group B and C probably results from locally formed PpIX and is unlikely to result from PpIX leakage.

The treated areas in both groups revealed a similar increase of fluorescence as the control areas in group B which might indicate that the increase in the treated areas is enzymatically regulated. Dan *et al.* (1993) and Malik *et al.* (1979) observed a decreased ferrochelatase activity in cells after PDT. Also, due to recovery reactions less Iron is available for incorporation in the PpIX molecule. This may result in an increase of PpIX due to a

decreased metabolization of PpIX to haem. As a consequence the fluorescence increase of treated areas in group B should be stronger than the control areas. As illustrated in figure 2a the areas treated in group B showed a slightly stronger increase between 2 and 5 hours after treatment compared to the control areas. In the control areas the fluorescence increase between 2 and 5 hours is not maximal indicating that ALA is no longer in excess available. Information about the importance of this decreased PpIX metabolization to haem may only be gained by applying ALA again to the entire skin (treated and control area) immediately after treatment. In that situation again a large ALA depot is created in the skin and differences between maximal PpIX accumulation of the treated and the control areas can be investigated.

Although no appropriate explanation can be given at this moment it is clear that new PpIX is formed after treatment which can be used for a second treatment. The PDT-induced damage after single treatment delivered at t=4 or at t=10 will be presented in a forthcoming paper. The effect of multiple light treatments, with topically applied ALA, on the therapeutic efficacy will also be presented in a forthcoming paper. The first experiments concerning multiple irradiations are completed and indeed we observed increased damage after multiple irradiations.

**Acknowledgements**

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# Chapter 6

## Photodynamic effectiveness and vasoconstriction in hairless mouse skin after topical ALA and single or twofold illuminations.

Submitted to: Photochemistry and Photobiology

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## Abstract

Several options were investigated to increase the efficacy of Photodynamic Therapy (PDT) using protoporphyrin IX (PpIX) induced by topically applied 5-aminolevulinic acid (ALA). Hairless mice with normal skin or UVB-light-induced skin changes were used as a model. In the first part of the study animals were illuminated immediately (t=4) or 6 hours (t=10, PpIX-fluorescence maximum) after the end of a 4 hour ALA application. A total incident light fluence of 100 J/cm$^2$ (514.5 nm) was delivered at a fluence rate of 100 or 50 mW/cm$^2$. The PDT-induced damage to normal skin was more severe after treatment at t=10 than at t=4. Illumination at 50 mW/cm$^2$ caused more visible damage than when the same total light fluence was given at 100 mW/cm$^2$. For UVB-illuminated skin, different intervals or fluence rates made no significant difference in the severity of damage although some qualitative differences occurred. In situ fluence rate measurements during PDT indicated vasoconstriction almost immediately after the start of the illumination. The second part of the study examined the effect of two illuminations. The first illumination bleaches the PpIX fluorescence. At the start of the second illumination, new PpIX had been formed. Light of 514.5 nm was delivered at 100 mW/cm$^2$ to a total incident light fluence of 200 J/cm$^2$ at t=4 (single illumination) or 100 J/cm$^2$ at t=4 plus 100 J/cm$^2$ at t=10. There was no visual difference in skin damage between 100 and 200 J/cm$^2$ single illumination. Two-fold illumination (100 + 100 J/cm$^2$) caused significantly more skin damage, indicating a potentially successful option for increasing the efficacy of topical ALA-PDT.

## Introduction

Skin lesions associated with sunlight exposure like actinic keratoses (AK), basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the most frequent (pre)-malignant skin lesions in Caucasians. At present, surgical and nonsurgical treatments like excisional surgery, cryosurgery, topical chemotherapy and radiotherapy are used. These methods can be costly, unsuitable for elderly people and the cosmetic outcome is not always satisfactory. Photodynamic therapy (PDT) using topically applied 5-aminolevulinic acid (ALA) appears a promising addition to the treatment modalities of superficial non-melanoma skin lesions such as AK, BCC and SCC. ALA is a prodrug and is converted in tissues in situ through the heam biosynthetic pathway into the photosensitizer protoporphyrin IX (PpIX). An excess of exogenous ALA can lead to diagnostic and therapeutic levels of intracellular PpIX in various malignant tissues. Several normal tissues, especially those originating from ecto- and endoderm may also become sensitized after ALA administration, in contrast to tissues from mesodermal origin (Divaris *et al.*, 1990; Loh *et al.*, 1993).

Kennedy *et al.* introduced topical ALA-PDT for skin lesions and reported 90% (1990) and 80% (1992) complete response rates of superficial BCC. The ALA-PDT protocol used by Kennedy is still the basis for clinical research to date. Briefly, a 20% ALA solution is applied to the target area for 4 to 6 hours. In normal skin, with an intact skin barrier, the penetration is limited. Because SCC, BCC and AK have abnormal keratin layers, ALA may easily penetrate the epidermis to be converted into PpIX in the tumour cells. The difference in skin barrier between tumour and normal skin may result in a selective photosensitization of tumours. This can be observed after the application as red PpIX fluorescence in the tumour which is clearly demarcated from the normal skin. The tumour and a margin of normal skin are then illuminated with light of a suitable wavelength and dose which results in tumour necrosis.

Because of the relative simplicity of the treatment and the promising results published by Kennedy, topical ALA-PDT drew widespread attention. In the past 5 years many investigators studied the efficacy of ALA-PDT for the treatment of superficial AK, BCC, SCC and Bowen's disease. The initial response rates were high. Long term histological complete responses were still 92% for AK, 87% for BCC, 81% for SCC and 85% for Bowen's disease (Cairnduff *et al.*, 1994; Lui *et al.*, 1995; Meijnders *et al.*, 1996; Stables *et al.*, 1997; Svanberg *et al.*, 1994; Wolf *et al.*, 1993). However, Fink-Puches *et al.* (1998) recently reported a projected disease free rate at 36 months after ALA-PDT of 50% for BCC and only 8% for SCC. Nodular BCC and SCC were also treated with ALA-PDT but the results were disappointing in all studies (between 0 and 50% complete clinical response rate). Improvement of the PDT efficacy, especially for nodular lesions is necessary and researchers have examined several options like duration of ALA application (Szeimies *et al.*, 1993), multiple ALA-PDT treatments (Calzavara-Pinton *et al.*, 1995; Warloe *et al.*, 1995; Fijan *et al.*, 1995 and Morton *et al.*, 1995), the use of penetration enhancers and enhancement of PpIX production using iron chelators (Orenstein *et al.*, 1994; Berg *et al.*, 1996). All these options

contribute to some extent to an enlarged PDT effect. In the present study, the effect of interval between ALA-application and illumination, fluence rate and multiple illuminations of normal and altered skin were investigated using the UVB-irradiated mouse skin as a model for (pre-) malignant changes.

## Materials and Methods

### *Animal model*

The protocol of the animal experiments was approved by the local ethical committee. Outbred female albino hairless mice, Skh hr1, 18 weeks old, were obtained from the University of Utrecht. The dorsal skin of the mice had been exposed to UVB light for 80 days, with a daily UVB dose of 1.5 kJ/m$^2$ from a Westinghouse FS40TL12 lamp according to a method previously described by de Gruijl *et al.* (1983). As a result, the entire dorsal skin showed a histological thickening of the epidermis with hyperkeratosis, increased basal mitotic activity and disorganization of the architecture (Fig.1). Also the dermis was thickened, showing an increased amount of dense collagen. Additionally, small intra-epidermal lesions of atypical spinous cells were seen in several animals. No infiltration into the dermis was seen, thus classifying these lesions as actinic keratosis (AK). Technically, it is not possible to shield part of the mouse skin for normal tissue control. Therefore, a comparison was made between UVB-treated skin and the normal skin of non-UVB mice and not between AK and the surrounding skin.

The UVB-treated mice were chosen for the resemblance of their skin lesions to sunlight-induced skin lesions like AK and SCC observed in humans (Fears *et al.*, 1976). In both humans and mice, thickening of the epidermis is seen after exposure to UV irradiation. In humans this reaction is only temporary and the thickness returns to normal values within weeks when the skin is no longer exposed to sunlight. In our model the daily UVB dose given to the mice was so high that an acute thickening of the epidermis and dermis occurred which remained longer and was only partly reversible (Sterenborg *et al.*, 1986).

### *Preparation and administration of ALA*

5-Aminolevulinic acid hydrochloride was purchased from Finetech Ltd (Haifa, Israel). ALA was dissolved in 3% carboxymethylcellulose after which the solution was set at pH 5.5 by adding NaOH (2M) to prevent irritation of the skin. Under anaesthesia using Hypnorm (Janssen, Beerse, Belgium) and diazepam (Centrafarm, Etten-Leur, the Netherlands) a thin gauze soaked in a 20% ALA solution (max. 20*20 mm$^2$) was applied to the dorsal skin for 4 hours. This is the most common topical ALA concentration used clinically. A piece of transparent film dressing (Mölnlycke, Waremme, Belgium) was placed over the gauze.

**Table I**: Summary of the PDT study on 7 groups of animals. One group served as a control. A solution without ALA was applied for 4 hours followed by illumination. On the animals of group A to F, ALA (20%) was applied for 4 hours after which they received an illumination at different time intervals after application with different light fluence rate and fluence. The number of animals in the follow-up study (Visual Skin Damage) and the histological study are shown separately.

| Group | Interval from start ALA-application to illumination (h) | Fluence rate mW/cm$^2$ | Light fluence J/cm$^2$ | Numbers of Animals Normal/UVB | |
|---|---|---|---|---|---|
| | | | | VSD | Histology |
| Control | 4 | 100 | 100 | 4/4 | 4/4 |
| A | 4 | 50 | 100 | 4/4 | 2/2 |
| B | 10 | 50 | 100 | 4/4 | 2/2 |
| C | 4 | 100 | 100 | 6/6 | 3/4 |
| D | 10 | 100 | 100 | 6/6 | 4/4 |
| E | 4 | 100 | 200 | 4/4 | -/- |
| F | 4+10 | 100 | 100+100 | 4/4 | -/- |

***Illumination schemes and visual skin damage score***

In the first part of the study the effect of fluence rate and interval between ALA application and illumination on the damage to the skin was examined. Reducing the fluence rate may improve oxygenation and the interval affects the concentration and distribution of PpIX in the tissues. Four UVB-treated and 4 normal animals were used to examine the effect of light and carboxymethylcellulose (control group). Four groups (A-D) were treated with ALA and light, each group consisting of UVB and normal animals (Table I). Illumination at the end of a 4 h ALA-application is designated "t=4", illumination at maximum fluorescence, 6h later, is designated "t=10". The difference between 50 mW/cm$^2$ (groups A, B) and 100 mW/cm$^2$ (all other groups) is relatively small, but chosen for practical reasons: with 50 mW/cm$^2$ it takes 33 minutes to deliver 100 J/cm$^2$. The effect of ALA-PDT at a very low fluence rate (5 mW/cm$^2$) has been studied before (Robinson *et al.*, 1998). All mice received a light fluence of 100 J/cm$^2$, which causes complete photobleaching of PpIX-fluorescence (and also of the photoproduct (Robinson *et al.*, 1998 and 1999). Intervals for the illumination were based on the PpIX-fluorescence kinetics study of van der Veen *et al* (1996). Briefly, illumination at t=10 was chosen because of maximal PpIX-fluorescence in UVB skin (both in lesions and in surrounding thickened skin). Illumination at t=4 was chosen because clinical ALA-PDT treatment is often applied at this interval. At t=10 the PpIX-fluorescence in UVB skin and in normal skin was 1.3 times higher than at t=4. At both intervals the PpIX-fluorescence in UVB skin was 1.7 times higher than in normal skin, as measured with a CCD-camera (van der Veen *et al*, 1996).

Before illumination, the animals were anaesthetized with a combination of Ethrane/-oxygen/$N_2O$ and placed on a temperature controlled stage. To restrict damage to the target area a black polythene mask was used. A 7 mm diameter homogeneous light field with an incident power density of 50 or 100 mW/cm$^2$ and a wavelength of 514.5 nm (from a Spectra Physics model 2040E Argon ion laser) was projected on the back of the mouse. A black polythene mask was used to facilitate (re)positioning of the beam and to block scattered light from the microlens. The 514.5 nm wavelength was chosen because it allows measurement of the full PpIX-fluorescence emission spectrum during PDT (Robinson *et al*, 1998) and the light penetrates sufficiently deep into mouse skin (see below). Variations of the beam intensity profile (± 10%) were checked in the light field using an isotropic light probe (Van Staveren *et al.*, 1995). The skin response of between 4 and 6 UVB and between 4 and 6 normal animals in each group were determined every day for the first week and every two to three days until 25 days post treatment according to the visual skin damage score (VSD), as shown in table II. Mean damage scores were calculated by summing partial scores weighted with the fraction of the total illuminated area. Accordingly, the mean scores of day 2 to 7 post treatment for each treatment site were used to calculate a mean VSD for each group. In UVB skin each area contained one or two small lesions which were histologically classified as AK. We did not observe any macroscopical or histological differences in damage between the lesion and the surrounding hyperplastic skin. Therefore, no distinction between AK and the

surrounding skin was made in the observations. The hairless mouse model is unsuitable to study long term response to PDT because new skin lesions develop in time in the entire UVB affected skin. Therefore, the VSD was determined until healing of the skin was complete with a maximum of 25 days.

The VSD was based on the prevalence of the visual skin damage with regard to the time of recovery was determined in group A to D. Visual skin reactions like oedema, discolouration and scab formation were observed in UVB and normal skin. Oedema was observed 5-10 hours after the illumination and had disappeared at day two. The degree of oedema did not correlate with the severity of the damage and was therefore not included in the VSD. Discolouration (redness) and scab formation ensued at day 2 after illumination. We could make a distinction between dry desquamation, thin scab and thick scab formation. Dry desquamation had disappeared at day 3 after PDT and healing was complete after an average of 6.1 days (n=21; sem ± 0.1). A thin scab fell off 5 to 7 days after PDT and healing of the skin was complete after an average of 10.3 days (n=7; sem ± 0.9). A thick scab fell of 6 to 8 days after PDT, after which the underlying skin showed strong red discolouration. Complete healing was observed after an average of 17.6 days (n=8; sem ± 2.2). When more than 25% of the area was covered with a thick scab, a thinner and greyer skin was still observed 25 days after PDT.

**Table II**: Visual skin damage (VSD) scoring system for UVB and normal skin

| Grade | Visual Skin Damage |
|---|---|
| 0 | No change in colour |
| 1 | Minimal redness |
| 2 | Redness |
| 3 | Dry desquamation |
| 4 | Thin scab formation |
| 5 | Thick scab formation |

***Histology***

To determine the extent of tissue damage after ALA-PDT with varying treatment parameters, limited numbers of animals were sacrificed for histology (Table I). Forty eight hours after PDT between 2 and 4 UVB and between 2 and 4 normal animals in each group were sacrificed. The illuminated area and an untreated (ALA, no light) area were excised, fixed in buffered formalin, processed for routine tissue sectioning, and then stained with haematoxylin and eosin (HE). Maximum depth and diameter of the lesion in relation to the thickness of epidermis and dermis were measured with an eyepiece graticule in 2 to 3 slides of each

excised area. Because of the rather large variation in these measured values, the difference in thickness of dermis and epidermis between UVB-treated and normal skin was related to the involved structures. The skin was divided into four layers. From the tissue surface they are: layer I the epidermis, layer II the papillary dermis, layer III the reticular dermis not including the dermal cysts (dilated hair follicles) and layer IV the deep dermis including dermal cysts down to muscle (Fig.1). Accordingly, the location (rim or centre) of the deepest extension of damage of the treated area was determined. A study of the histology following twofold illumination (see below) will be presented when this treatment scheme has been optimized.

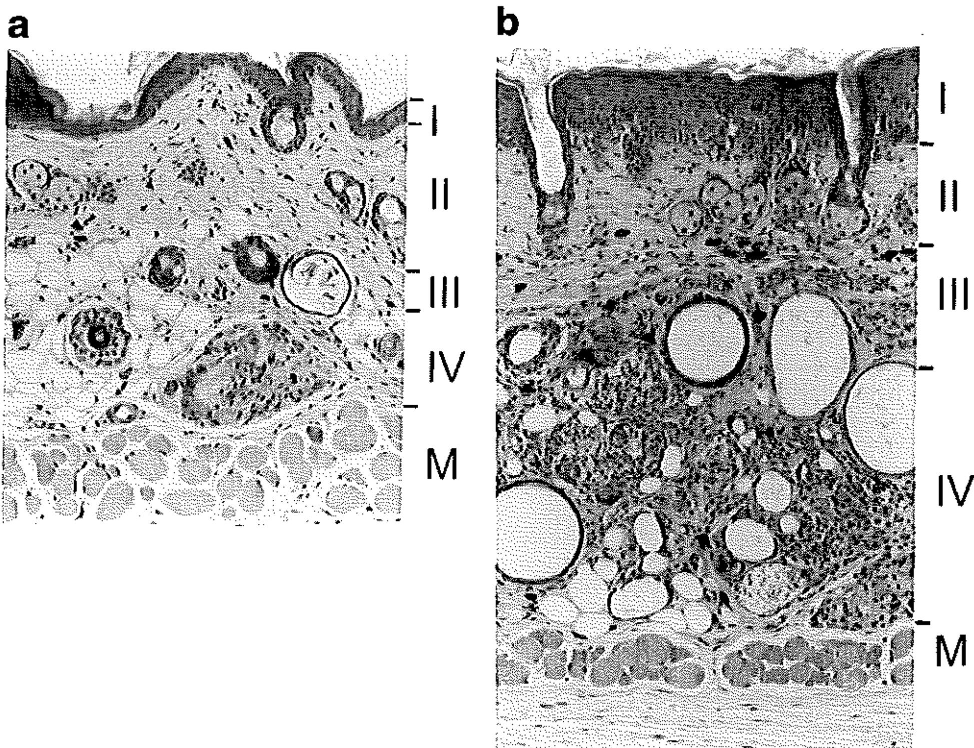



*Figure 1. Histology of normal hairless mouse skin (A) and UVB-treated skin (B). The layers are:I, the epidermis; II, the papillary dermis; III, the reticular dermis not including the dermal cysts (dilated hair follicles); IV, the deep dermis including dermal cysts down to muscle. M=Muscle. Total thickness of layers I-IV as indicated 0.37 mm (A) and 0.69 mm (B), respectively (H&E, objective 10x).*

*Vascular effects*

Two additional experiments were performed to look for possible vascular effects of topical ALA-PDT. In the first experiment we examined the exclusion of fluorescein dye *after* the illumination. This method has been used by several authors (Bellnier *et al.*, 1995; Fingar *et al.*, 1987) and is based on the exclusion of fluorescein dye from tissue vessels with impaired perfusion due to PDT induced vascular damage. Immediately after illumination (performed at t=4 or t=10, 100 mW/cm$^2$ or 50 mW/cm$^2$) 2 UVB and 2 normal animals of each treatment group (a total of 16 mice) were given an intraperitonal injection of 20 mg/kg fluorescein dye (10 mg/ml of sodium fluorescein, ACROS, Geel, Belgium; diluted in phosphate-buffered saline). Fluorescein-fluorescence was excited with 460 $\pm$ 60 nm with a maximum fluence rate of 0.1 mW/cm$^2$ and detected through a long-pass coloured glass filter (OG 530). Before and 5 min after injection the fluorescein fluorescence (FF) of a treated (ALA and light) and an untreated (ALA, no light) area on the same mouse was recorded using a charge-coupled device camera according to a method previously described by van der Veen *et al.* (1996). Mean FF fluorescence intensities of the entire recorded images were calculated and the autofluorescence before fluorescein injection was subtracted. The data are expressed as percentage fluorescein fluorescence of the treated area as compared to the untreated area.

A second experiment was performed to investigate acute vascular constriction *during* the illumination. This was examined using the fact that green light (514 nm) is strongly absorbed by red blood cells. Vascular constriction reduces the tissue blood content and thus reduces absorption of green light. Changes in blood flow can then be detected by measuring the fluence rate under the skin. The fluence rate during illumination (t=4, 100 mW/cm$^2$ or 50 mW/cm$^2$) was measured in 2 normal (4 areas) and 2 UVB (4 areas) animals of each group ( a total of 8 mice) using two isotropic light detectors (van Staveren *et al.*, 1995), which were manufactured in our laboratory. One probe was placed on top of the skin in the centre of the illumination field. A small incision (approximately 1 mm) was made at least 1 cm from the illuminated area and a second probe was placed under the skin beside the first probe. The probes were connected to photodiodes (Photop UDT-455, Graseby Electronics, Orlando FL, USA), whose output was A/D converted and stored in the computer. The fluence rate during illumination of both probes was recorded every 5 seconds. To exclude the effect on light absorption of non-vascular changes in optical properties, as a control 2 areas on each of 2 UVB mice were illuminated at t=4 with 630 nm light (from a Spectra Physics model 375B dye laser pumped by a model 2040E argon ion laser) and a fluence rate of 100 mW/cm$^2$. Because the absorption coefficient ($\mu_a$) of partially oxygenated human blood for red light is 10 times less than for green light (Cheong *et al.*, 1990) no significant increase in fluence rate was expected during red light PDT.

### *Twofold illuminations*

In the second part of the study the effect of twofold illuminations was examined. The basis for this experiment was a previous study by van der Veen *et al* (1997). In that study animals received a light treatment immediately after a 4 hour ALA application time (t=4). After the illumination the PpIX-fluorescence had bleached completely indicating that PpIX was no longer present. However, after the illumination an increase in PpIX-fluorescence was observed, at the same rate as in non-illuminated animals at the same time after AlA-application, indicating new PpIX formation. This phenomenon led to the idea that two illuminations separated by a waiting interval, in which new PpIX was formed, would be more effective than a single illumination.

Two groups (E and F) were assessed, each group containing UVB and normal animals (Table I). Group F received illuminations at t=4 and at t=10 both with a fluence rate of 100 mW/cm$^2$ and a light fluence of 100 J/cm$^2$ (total light fluence 200 J/cm$^2$). Group E served as a control group and received a single illumination at t=4 (immediately after the 4 hr ALA-application) with a fluence rate of 100 mW/cm$^2$ and a total light fluence of 200 J/cm$^2$. The damage scoring protocol (Table II) was the same as that used for the fluence rate and illumination intervals study. The results of group E and F were compared with those of group C.

### *Statistics*

Stastistical tests were performed using the analysis of variance followed by a Student-Newman-Keuls test, as necessary. Whenever the term "significant" is used in this paper we mean that the test yielded a p-value of less than 0.05.

## Results

### *Fluence rate and interval to illumination*

There was no visual damage in the control group of 4 UVB and 4 normal animals, to which solvent without ALA was applied for 4 hours followed by illumination (100 mW/cm$^2$, 100 J/cm$^2$). Figure 2 shows the mean VSD of the first part of the study. In normal skin, illumination with a fluence rate of 100 mW/cm$^2$ caused very mild damage and no significant difference in VSD was observed between both illumination intervals. The damage in both groups (t=4 and t=10) increased when the fluence rate was lowered to 50 mW/cm$^2$. In particular at t=10 the VSD clearly increased (3.5 times higher than with 100 mW/cm$^2$), resulting in a significant difference in VSD between illumination at t=4 and t=10 using 50 mW/cm$^2$. In UVB animals lowering the fluence rate or postponing the illumination had no significant effect on the VSD (Fig 2).

During the observation period a distinct pattern of skin changes was noted that depended on the fluence rate. In both normal skin and UVB-treated skin, maximum skin

change was observed at the rim (high fluence rate) or in the centre (low fluence rate) of the illuminated area. An illustration of rim damage is given in Figure 3. Figure 3A shows an area of UVB mouse skin illuminated with 100 $mW/cm^2$ and Fig. 3B an area illuminated with 50 $mW/cm^2$. Table III presents the number of mice showing rim reactions, including the animals sacrificed at day 2 for histology. Rim reaction occurred most frequently in UVB animals illuminated at 100 $mW/cm^2$ (significantly more than in UVB-treated animals illuminated at 50 $mW/cm^2$) and least frequently in normal animals illuminated at 50 $mW/cm^2$. To examine the occurrences of rim reaction related to the VSD, the UVB-treated animals were pooled and two new groups were formed showing rim or central reaction (mice used for histology were not included here, because no VSD was available). We observed a significantly lower mean VSD in areas showing rim reaction than in areas showing a more central reaction. In eight areas with rim reaction a VSD of 1.76 (± 0.28) was found, while twelve areas with a more central reaction had a VSD of 2.51 (± 0.21).

**Table III**: Number of UVB or normal animals (%, and the numbers in each group), two days post treatment, showing more damage at the rim than in the centre of the illuminated area. The animals used for the histology study are also included.

| Interval from start of ALA-application to illumination | | Fluence rate 50 $mW/cm^2$ | Fluence rate 100 $mW/cm^2$ |
|---|---|---|---|
| t=4 | Normal | 16% (1 out of 6) | 33% (3 out of 9) |
| | UVB | 33% (2 out of 6) | 60% (6 out of 10) |
| t=10 | Normal | 16% (1 out of 6) | 44% (4 out of 9) |
| | UVB | 33% (2 out of 6) | 80% (8 out of 10) |

***Histology***

ALA-PDT caused acute skin changes that were similar in UVB and normal skin (Fig.4). In epidermal lesions they ranged from hyperplasia, coagulation necrosis of the epidermis and crust formation to re-epithelization. In deeper (dermal) lesions necrosis of adnexal structures and blood vessels, and polymorphonuclearinfiltration were seen. No damage was observed to muscles and large blood vessels below layer IV (hair follicles).

The thickness of the epidermis and the diameter and depth of the damage were measured. In all groups we found large variations in diameter and depth. Two days after PDT the thickness of normal epidermis in all groups had increased significantly compared to the untreated areas

(ALA, no light) on the same mouse. No significant increase occurred in UVB-treated skin. Additional significant differences after PDT were in diameter of damage between normal and UVB-treated skin (group C, 1.4 ± 0.4 mm and 7.1 ± 0.9 mm, respectively), as well as between normal skin of group A versus C (3.9 ± 1.4 mm and 1.4 ± 0.4 mm, respectively), and in thickness of normal epidermis (group B versus D, 0.043 ± 0.01 mm versus 0.068 ± 0.008 mm respectively). Generally speaking, the depth and diameter of damage in normal skin increased when the fluence rate reduced from 100 mW/cm$^2$ to 50 mW/cm$^2$. This was not clear in UVB-treated skin. More fluorescence (t=10 versus t=4) did not lead to greater depth or diameter of damage.

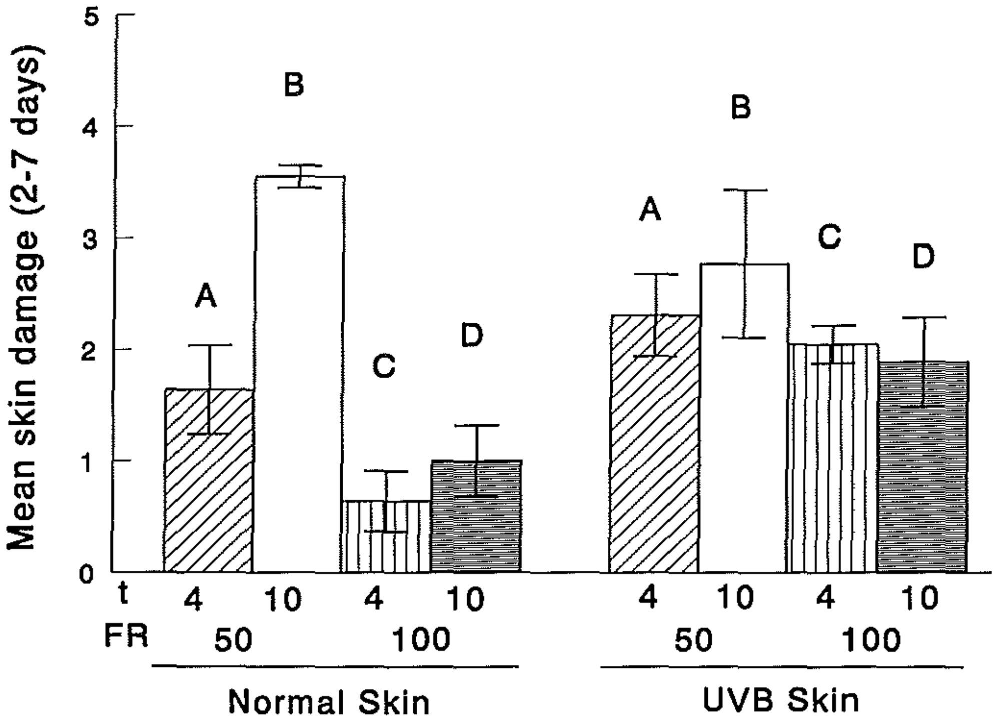
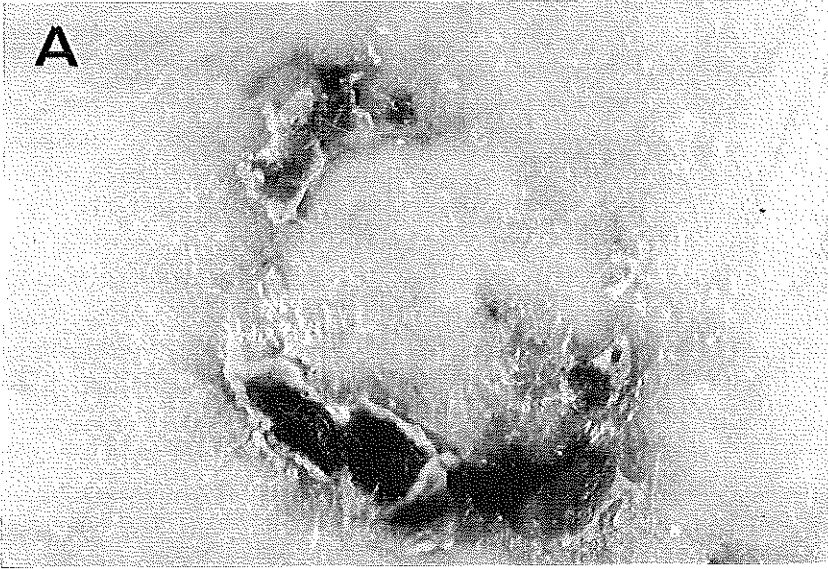



*Figure 2: Mean visual skin damage (2-7 days; ± s.e.m.) of group A, B, C, D (Table I) treated with a fluence rate (FR) of 50 (n=4) or 100 (n=6) mW/cm$^2$ and a light fluence of 100 J/cm$^2$. Areas were illuminated immediately (t=4) or 6 hours after the end of ALA application (t=10).*



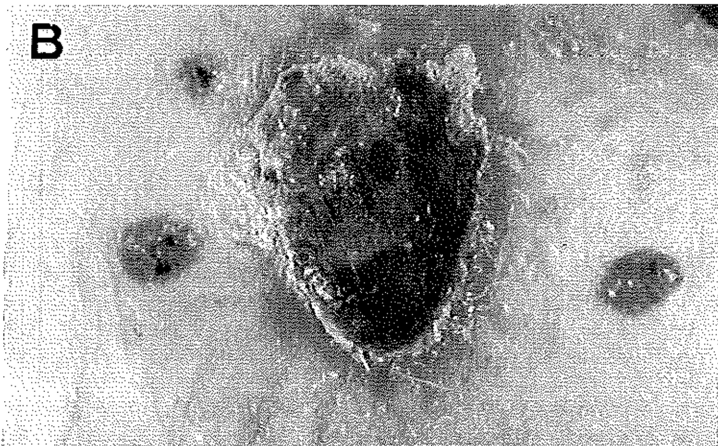
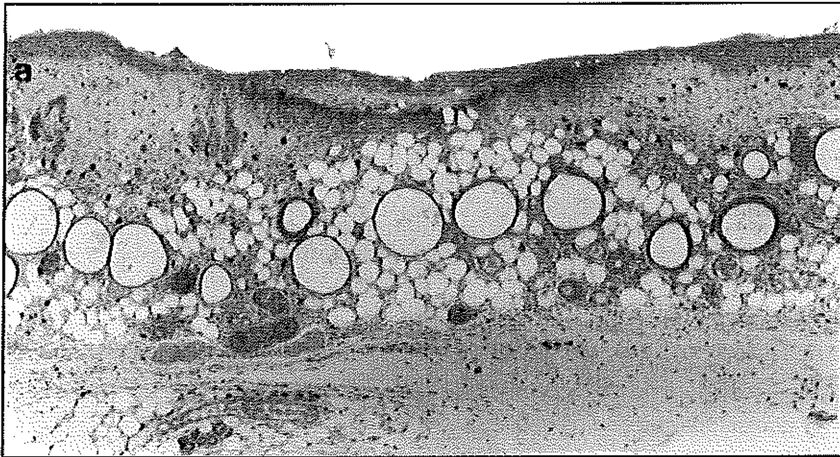




***Figure 3:*** *Illustration of PDT treated areas in UVB mouse skin showing a rim reaction (3A) and a central reaction (2B) at day 4 after illumination (fluence rate was 100 mW/cm² in 3A and 50 mW/cm² in 3B). The diameter of the damage is approximately 7 mm, the diameter of the illuminated area. In Fig. 3B three small lesions are located outside the illuminated area.*



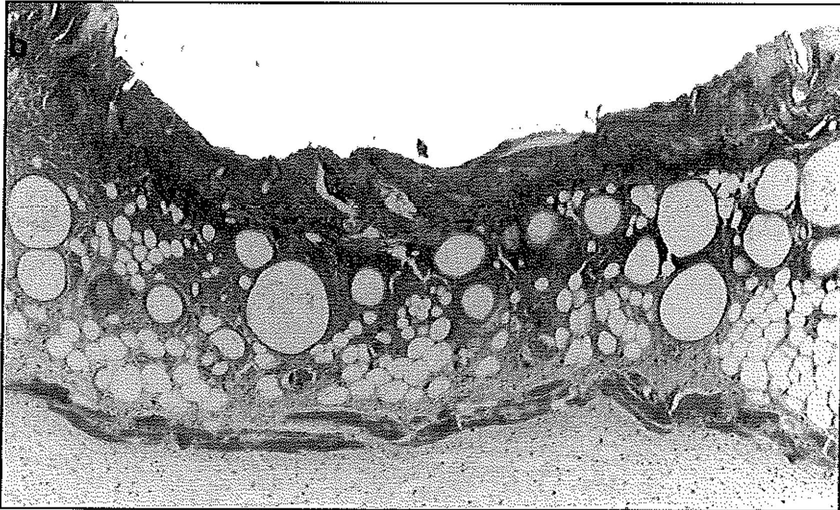




*Figure 4. Histology of hairless mouse skin, 48 h after PDT (H&E, objective 4x). (A) Normal skin treated at t=10 and 100 mW/cm². In the centre a crater is visible, covered with crust. The damage extends down to, but not into, layer IV. Thickness of crust plus layers I-IV at centre 0.6 mm. (B) UVB-treated skin illuminated at t=4 and 100 mW/cm². Damage extends into layer IV and over the full width of the picture. Thickness layers I-IV at centre 0.6 mm.*

The histological examinations (48 h after PDT) showed similar patterns of skin damage as with the daily macroscopical examinations. Therefore, the location of maximum histological damage (rim or centre), the depth of the damage (mm) and the layer into which it extended (I, II, III, IV) were determined and are shown in Table IV. The results of all treated groups were pooled. In both normal and UVB treated skin when damage was located at the centre of the illuminated area, more animals showed extension of damage into the deeper layers III and IV than when damage was located at the rim of the illuminated area. In addition, more UVB animals than normal animals showed extension of damage into skin layers III and IV, irrespectively of the site (centre or rim).

**Table IV:** Histological location of maximum damage (rim or centre) related to the depth of damage and to the skin layer which it extended, in normal and UVB skin (results of group A to D were pooled). The skin was divided into four layers. From outside to inside they consist of: Layer I the epidermis, layer II the papillary dermis, layer III the reticular dermis not including the dermal cysts (dilated hair follicles) and layer IV the deep dermis including dermal cysts down to the muscle. For layers with more than two measurements, mean depths (± sem) are given, otherwise the measured values.

| | Normal skin | | | UVB skin | | |
|---|---|---|---|---|---|---|
| Location of damage | Skin layer | depth of damage (mm) | animals | Skin layer | Depth of Damage (mm) | animals |
| Center | 0 | 0 | 1 | 0 | - | - |
| | II | 0.17 | 1 | II | - | - |
| | III | 0.22<br>0.21 | 2 | III | 0.26 | 1 |
| | IV | 0.39<br>0.50 | 2 | IV | 0.34<br>(± 0.07) | 4 |
| Rim | 0 | 0* | 1 | 0 | - | - |
| | II | 0.09<br>0.07 | 2 | II | 0.18 | 1 |
| | III | 0.15 | 1 | III | 0.15<br>(± 0.04) | 4 |
| | IV | - | - | IV | 0.21<br>0.43 | 2 |

* Only crust remaining at time of histology, 48 h after PDT. Any damage in underlying

tissues had apparently healed at this time.

*Vascular damage effect*

Fluorescein dye exclusion:

In the untreated areas (ALA, no light) we observed no differences in fluorescein fluorescence (FF) intensities between UVB and normal skin. In the areas of both normal and UVB skin after PDT no significant difference was observed FF between illumination at t=4 and t=10 (Fig 5.). Therefore these results were pooled. In UVB-treated skin, after PDT with a fluence rate of 100 mW/cm$^2$, the FF was less than in normal skin. A reduction of the fluence rate to 50 mW/cm$^2$ resulted in an increase in FF in both UVB and normal skin. This was significant (factor 1.6) in normal skin.

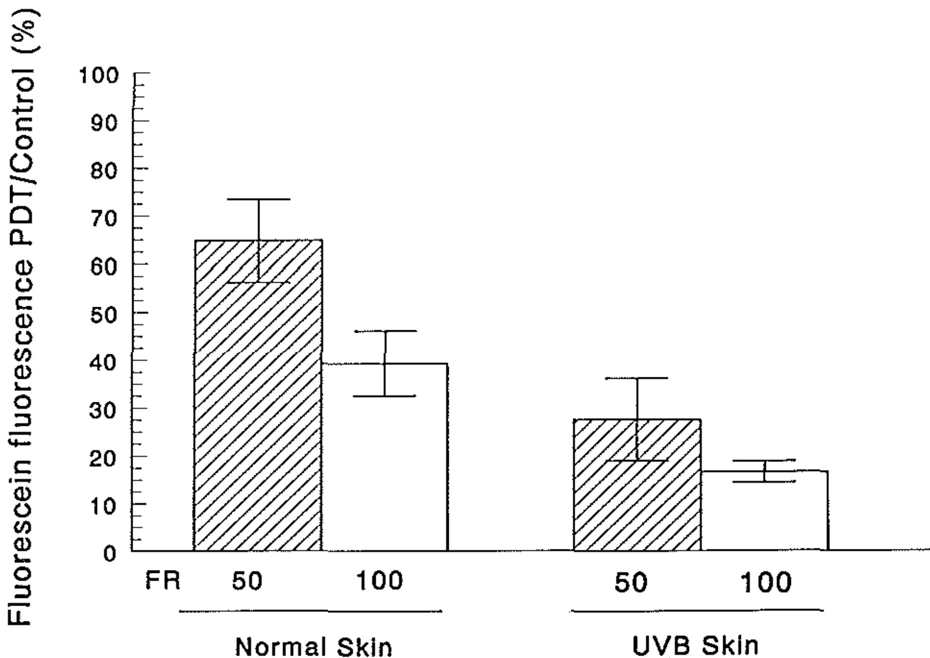



*Figure 5: Fluorescein fluorescence of PDT treated areas (incident fluence rate FR= 50 or 100 mW/cm$^2$) relative to control (ALA, no light) areas. Fluorescence recordings were made 5 minutes after the end of the illumination for PDT. The results of illumination at t=4 and t=10 were pooled for both UVB and normal skin and each group consist of 3 to 4 animals (± s.e.m.).*

Fluence rate measurements:

In the ALA-PDT groups the fluence rate measured with a probe on the skin did not change significantly (± 5 %, interval 2 min) during the illumination. In areas treated by PDT a maximum increase in fluence rate under the skin was measured at approximately 2 minutes after the start of the illumination. Therefore, this interval was chosen to calculate the ratio between the fluence rate under the skin and the fluence rate on the skin. The results of the increase of fluence rate are shown in Figure 6. In normal (n=3) and UVB (n=3) control animals treated with light and solution (no ALA) the fluence rate did not increase during illumination. UVB animals (n=4) illuminated with red light (630 nm) showed a small increase (3,3% ± 1,6). In normal animals treated at 514.5 nm with 100 mW/cm$^2$ (n=4) or 50 mW/cm$^2$ (n=4) there was no significant increase in fluence rate, compared to the control group. In UVB animals illuminated with a fluence rate of 100 mW/cm$^2$ a significant increase in fluence rate (34,3% ± 3,7) was measured 2 min after the start of illumination. With 50 mW/cm$^2$ the increase was less (11% ± 3,4, n=4) but still significantly more than in the control animals. We note that the fluence rate measurements indicate a significant reduction in perfusion *just after the start* of illumination only for UVB-treated skin, whereas the FF indicates the same only for normal skin *after the end* of the illumination.

*Twofold illuminations*

The VSD scores of group C, E and F (normal and UVB animals) are shown in Figure 7. In both normal and UVB skin there was no difference in damage between group C (100 J/cm$^2$) and E (200 J/cm$^2$). However, two illuminations of 100 J/cm$^2$ seperated by a 6 h interval caused a significant increase of damage as shown by group F. The VSD after the multiple illuminations in normal skin was 4.3 times and in UVB skin 2.5 times higher than after a single 200 J/cm$^2$ light dose delivery. In both normal and UVB skin the areas treated with multiple illuminations were still different from normal skin 25 days post treatment. Normal skin showed dermal scars with diameters of 2.1 and 2.3 mm. In the UVB skin scars in the dermis ranged from 1.7 to 3.2 mm diameter. These areas were histologically examined and the long term effects consisted of fibrous scar tissue in the dermis (II, III, IV) with complete local loss of hair follicles.

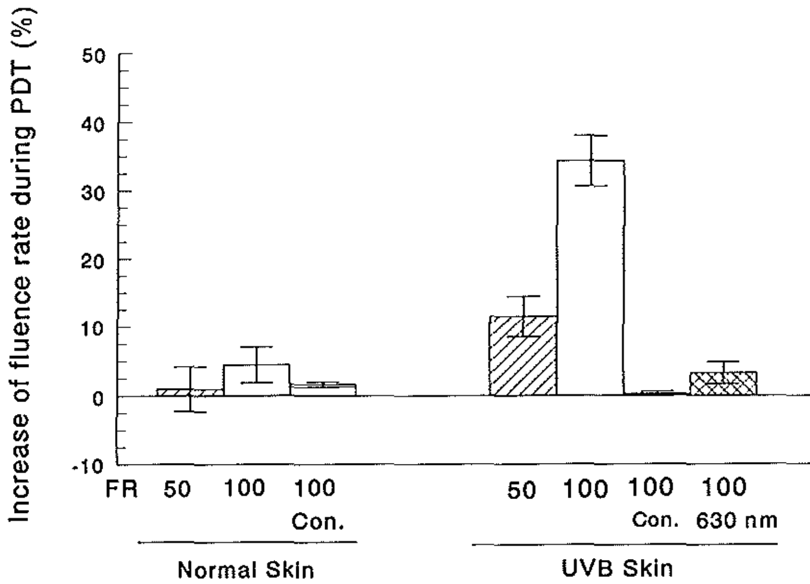



*Figure 6: Increase in fluence rate measured in situ under the skin (± s.e.m.), 2 minutes after the start of the illumination. Illumination of normal and UVB skin occurred at t=4 with a fluence rate (FR) of 50 (n=4) or 100 mW/cm² (n=4) at 514.5 nm wavelength. In both normal (n=3) and UVB (n=3) skin the fluence rates in control (solution without ALA, and light) areas were determined and 4 areas in UVB animals were illuminated with red light (630 nm, 100 mW/cm²).*

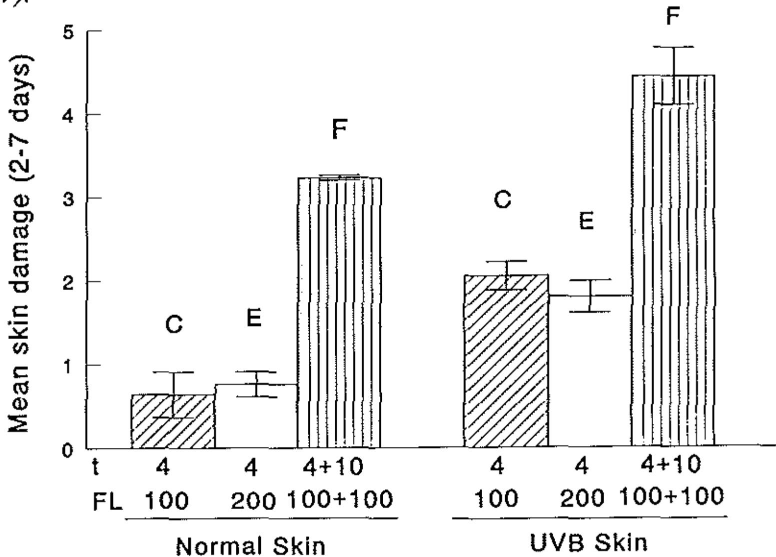



*Figure 7: Mean visual skin damage (2-7 days; ± sem) of group C, E, F (Table I) treated with a fluence rate of 100 mW/cm² and a light fluence (FL) of 100 (n=6), 200 (n=4) continuous and 200 (n=4) J/cm² fractionated. Areas were illuminated immediately after application (t=4).*

## Discussion

### *Fluence rate and interval to illumination*

In the first part of the study the effect of interval between ALA-application and illumination as well as the effect of two different fluence rates on the PDT damage effect were investigated. The basis for these experiments were the fluorescence results described in a previous paper (van der Veen *et al.*, 1996). Based on the fluorescence intensities, an increase in damage to UVB and normal skin was expected for illumination at maximal fluorescence (t=10) compared to an illumination immediately after ALA-application (t=4). At t=10 there is still a clear difference in PpIX-fluorescence between UVB and normal skin in contrast to human BCC and surrounding skin after 16 h ALA-application (Meijnders *et al.*, 1996).

In normal skin a higher PpIX-fluorescence level indeed resulted in increased PDT damage (t=10 versus t=4), which was significant (Fig.2) when treating at 50 $mW/cm^2$. With the lower fluence rate the damage at both treatment intervals was significantly larger than with 100 $mW/cm^2$. Further reduction of the fluence rate (to 5 $mW/cm^2$) leads to further increase of damage (Robinson *et al.*, 1998 and 1999). In UVB skin there was no correlation between the fluorescence intensity and damage. Neither was there an increased effect after lowering the fluence rate from 100 to 50 $mW/cm^2$. This might be due to the larger PpIX levels, causing oxygen depletion even at the lower fluence rate. Further reduction of the fluence rate should then eventually lead to increased PDT-induced damage in UVB-treated skin as well, which has indeed been observed, at 5 $mW/cm^2$ (Robinson *et al.*, 1999). It would therefore seem that lowering the fluence rate would reduce the therapeutic selectivity in our model. This could not be confirmed experimentally in the same animal, because the treated area of transformed skin was always surrounded by transformed skin and not by normal skin. Fluence rate effects were also reported by Hua *et al.* (1995) who observed an increase in tumour growth delay after systemic ALA-PDT treating with 25 $mW/cm^2$ compared to 100 $mW/cm^2$. In a different model De Bruijn *et al.* (1999) could not confirm this effect. The difference may result from differences in tumour oxygenation and perfusion.

### *Histology*

The pattern of damage (rim or centre), observed visually, were studied histologically. In both UVB and normal skin maximum damage which was located at the centre of the illuminated area extended deeper into the skin than maximum damage which was located at the rim of the illuminated field (Table IV). The rim pattern has also been observed in patients (personal communications W.M. Star and A.R. Osseroff) A possible explanation for this phenomenon is oxygen depletion by PDT and reduced oxygen supply to the centre by diffusion, combined with reduced perfusion during treatment, as demonstrated in Figs. 5 and 6. Oxygen is essential for PDT induced damage. During illumination the oxygen concentration in the cells rapidly decreases by consumption in the PDT reaction. Even with an intact circulation the need for oxygen may be greater than the oxygen supply by diffusion. The reduced perfusion

is limited to the illumination field. At the rim of this field the oxygen supply is larger than in the centre of the illumination field as a result of oxygen diffusion from the surrounding untreated tissues. The better availability of oxygen results in more damage at the rim than at the centre of the illuminated field. With a low fluence rate, the demand for oxygen is less so that diffusion might maintain the oxygen concentration at a higher level, resulting in a more central damage in both UVB and normal skin.

*Vascular effects*

So far, vascular effects were believed to be absent or much less for topical ALA-PDT than for PDT with systemically administered ALA (van der Veen *et al.*, 1994). The fluence rate measurements as well as the fluorescein studies show a reduced vascular perfusion during illumination after topically applied ALA. Wang *et al* (1997) reported an increased vascular perfusion after topical ALA-PDT. These assays are complementary. The fluence rate measurements show a significant acute reduction in perfusion for UVB-treated skin, whereas the FF shows a significant reduction in perfusion after illumination for normal skin.Wang *et al.* (1997) reported an increased vascular perfusion after topical ALA-PDT of human BCC. However this occurred predominaly in the surrounding normal skin. The perfusion in the centre of the lesion after PDT appeared almost unchanged.

During and after PDT using i.v. ALA-administration we have directly observed vasoconstriction (1994), which recovered in time after a single illumination. In the present study, we have observed reduced perfusion, which may be the result of vasoconstriction. It is possible that increased interstitial pressure, due to histamine induced reaction, reduces the blood volume. However, edema was only observed after PDT in time, in contrast to the measurements of fluence rate and fluorescein fluorescence.

Concluding the discussion of the first part of the study, we note that in clinical treatments the incident fluence rate is usually made as large as possible, to limit the treatment time. The upper limit is determined by the desire to avoid hyperthermia and by pain felt by the patient. In our experience, reducing the fluence rate may relieve the pain during illumination. Therefore, reducing the fluence rate may serve a dual purpose: less pain and an increased effect. Using mouse data as a guide for clinical protocol requires caution. Nevertheless, it seems safe to conclude that not only the total light fluence , but also the fluence rate should be reported and kept constant in ALA-PDT treatments. The mouse data suggest an association between more central and deeper damage at lower fluence rate and between rim type and less damage at higher fluence rate. Since the rim type damage has also been observed clinically at fluence rates of 100 mW/cm$^2$ or more, it seems wise to keep the fluence rate as low as practically possible. ALA-PDT at very low fluence rates deserves further study. The long treatment times may seem impractical, but with low power, diode lasers and suitable light delivery devices low-fluence rate ALA-PDT may become an attractive option.

*Twofold illuminations*

In the second part of this study the effect of multiple illumination on the PDT damage effects was investigated. Two illuminations separated by a dark period of 6 hour may not be clinically practical. However, this study was designed to achieve maximal damage under the most optimum circumstances concerning PpIX-fluorescence intensities. When the mechanism for the enhanced effect is better understood it may be possible to design a more practical protocol.

Twofold illuminations separated by an almost 6 hours interval resulted in a 2.5-4.3 times higher VSD than after a single illumination. The enhanced damage effect with multiple illuminations was not the result of an increased light fluence, as shown by group E. A 200 J/cm$^2$ single illumination "control" was only performed at t=4 and not at t=10. Since 100 J/cm$^2$ at 100 mW/cm$^2$ completely photobleaches the fluorescence of PpIX as well as the photoproducts (Robinson *et al.*, 1998 and 1999), no increase of damage was expected upon increasing the fluence to 200 J/cm$^2$ and this was confirmed at t=4, for both normal and UVB-treated skin.

After the first illumination the PpIX-fluorescence of the treated areas increased at the same rate as the control (ALA, no light) areas on the same mice (van der Veen *et al.*, 1997). The similar rate of increase may suggest that all cells (damaged or not) still have the capacity to form PpIX. An alternative explanation is that some cells have an increased PpIX accumumlation and that severely damaged cells have lost the capacity to form PpIX. This effect was illustrated by He *et al.* (1993) who observed a decreased ferrochelatase activity in cells after PDT, which may result in an increase of PpIX due to a decreased metabolization of PpIX to haem. Since ALA was applied to a relatively large area of hairless mouse skin, one may wonder if circulating PpIX is not partially responsible for the new fluorescence after photobleaching. We have checkes this by measuring the fluarescence before and after illumination of 7mm diameter spots on UVB-treated mouse skin when ALA was applied to different areas: in one group to a large part of the dorsal skin, in the other group to areas of about 7 mm diameter. There was no significant difference in rate of increase of fluorescence (Van der Veen *et al.*, 1997). New fluorescence was also found to arise in human BCC after ALA-PDT (Orenstein *et al.*, 1997; Af Klinteberg *et al.*, 1999), although relatively less than in hairless mouse skin. Orenstein *et al.* (1997) reported substantial new fluorescence after PDT of nodular BCC. A second illumination was mentioned but not the clinical resultThe PpIX-fluorescence level just before the second illumination in the treated areas given PDT was for UVB-treated skin 2.5 and for normal skin 2.4 times less than the control areas on the same mice at the same time after the ALA-application. This relatively low PpIX-fluorescence level of the treated areas suggests that the increased damage effect after the second illumination is unlikely to be *only* the result of new PpIX. The as yet unknown phenomona responsible for the light fractionation effect reported by Curnow *et al* (1999) may also play a role here. Increased sensitivity for PDT may result from recovery reactions of the

cells initiated by the first illumination. Reoxygenation between the two light fractionations may also play a role, by diffusion and by reperfusion of constricted blood vessels. Nevertheless, even though the mechanism of the observed phenomena remains unclear, ALA-PDT using twofold illumination has been shown to be more effective than using single illumination in different tumour models and with topical (present paper) as well as systemic ALA-administration (de Bruijn et al.,1999; van der Veen et al., 1994).

**Acknowledgements**
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# Chapter 7

## Kinetics of PpIX fluorescence after topical ALA application to normal and tape-stripped human skin.

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## Introduction

Photodynamic therapy using topically applied 5-aminolevulinic acid (ALA) is a relatively new and successful treatment modality for superficial non-melanoma skin tumours. ALA-application to skin leads to an accumulation of the endogenous photosensitizer protoporphyrin IX (PpIX) in epidermal cells. The conversion of ALA to PpIX is accomplished in normal and neoplastic skin cells by enzymes in the haem synthesis pathway. An illumination with light causes a photochemical reaction that results in tissue destruction. In the past 8 years a large number of clinical studies has been performed treating actinic keratoses (AK), basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) (Kennedy *et al.*, 1992; Lui *et al.*, 1995; Meijnders *et al.*, 1996; Stables *et al.*, 1997). Although the results are promising, considerable variation in results was observed within and between different studies.

The stratum corneum (SC) is the limiting barrier to the penetration through the skin of topically applied drugs. The stratum corneum consist of corneocytes embedded in a matrix of lipid bilayers and stacked in overlapping layers. The shape and surface features of the corneocytes are adapted to maintain the integrity of the SC but also to allow desquamation. In the SC layer there is hardly any metabolic activity and the SC acts as a passive diffusion medium. There are important chemical and structural disparities in the SC from one site to another. As a result, the skin barrier function may vary between different sites. As an illustration, the penetration of hydrocortisone in humans at various sites is shown in table 1 (table adapted from Feldmann and Maibach, 1967). Also, with a wide variety of other chemical moieties examined, a general pattern of regional variations was observed (Maibach, 1971). It was found that the penetration of the drug correlated with the thickness of the SC.

The thickness and the structure of the SC above skin lesions like AK, SCC and BCC is different from normal skin. The penetration of drugs into superficial lesions is mostly enhanced due to a decreased skin barrier function. The penetration into non-superficial lesions is mostly insufficient. Jeffes *et al.* (1997) observed an inverse correlation between fluorescence (PpIX production) after topical ALA and the grade of AK. The PDT damage decreased with increasing grade of AK which was also observed for SCC and BCC by Szeimies *et al.* (1996). Besides the grade of the skin lesion, the location of the lesion is also important for the response to PDT. Non-melanoma skin lesions occur most frequently on the head, neck and hands, parts of the body habitually exposed to sunlight. Jeffes *et al.* (1997) examined the differences in damage effect after ALA-PDT between AK's located on the face and scalp and lesions located on the trunk and extremities. They observed a higher fluorescence after ALA-application to lesions located on the scalp and face than to lesions located on the trunk and extremities. Also, in the normal-appearing skin adjacent to the lesions the fluorescence on the face and scalp was higher (but less than the lesions) than on the trunk and extremities. Subsequently, after light irradiation a significantly better response of AK's on the face and scalp (91% complete response) was observed than on the trunk and

extremities (45% complete response). Szeimies *et al.* (1996) reported similar differences in effects after ALA-PDT between AK's located on the head and on the hands. This difference in effect between lesions on the face and scalp and lesions on the trunk and extremities has also been observed after topically applied chemotherapy and chemical peels (Lawrence *et al.*, 1995; Pearlman *et al.*, 1991). An important factor in this difference in damage effect between various sites is the thickness and composition of the SC.

**Table 1.** Penetration of Hydrocortisone in Humans at Various Anatomic Sites.
Table adapted from Feldmann and Maibach (1967).

| Anatomical site | Penetration ratio |
|---|---|
| Forearm (ventral) | 1.0 |
| Forearm (dorsal) | 1.1 |
| Foot (plantar) | 0.14 |
| Ankle (lateral) | 0.42 |
| Palm | 0.83 |
| Back | 1.7 |
| Scalp | 3.5 |
| Axilla | 3.6 |
| Forehead | 6.0 |
| Jaw angle | 13.0 |
| Scrotum | 42.0 |

For a successful ALA-PDT treatment of superficial lesions located on the trunk and extremities and for non-superficial skin lesions better penetration of ALA through the SC is necessary. Several options have been considered to increase the concentration of ALA in the skin. The use of the penetration enhancer dimethylsulfoxide (DMSO) increased the fluorescence after ALA-application to nodular lesions (Peng *et al.*, 1995). However, the response rate after ALA-PDT of nodular lesions was not clearly improved (Warloe *et al.*, 1995; Peng *et al.*, 1995). Iontophoresis was shown to increase the penetration of ALA through the normal SC (Rhodes *et al.*, 1997). This technique involves the active delivery of small charged molecules into the skin by the application of an electrical current. An increased concentration of drug in the skin can also be accomplished by increasing the duration of ALA-

application. With a long application time the increased ALA-availability in combination with a long occlusion time resulted in an increased fluorescence in normal skin (Szeimies *et al.*, 1993; Meijnders *et al.*, 1997). The penetration of the drug can also be enhanced by tape-stripping the skin which reduces the thickness of the SC. Tape-stripping the skin resulted in a fourfold increase in penetration of hydrocortisone (Feldmann and Maibach, 1965). Similarly enhanced factors have been found with other chemical compound (Maibach *et al.*, 1971). The effect of tape-stripping on the penetration of topically applied ALA in human skin is unknown.

In this study we examined the effect of ALA-application time, location and tape-stripping on the fluorescence kinetics. In a first experiment the fluorescence kinetics after 4 and 16 hours ALA-application on normal human skin was examined. ALA was given on two locations: the flexor of the forearm and the lower part of the back. In a second experiment the ALA induced fluorescence kinetics in normal and tape-stripped skin on the flexor arm was examined after a 4 hour ALA-application time. The fluorescence kinetics of both normal and tape-stripped skin was examined by excitation with three wavelengths and therefore three different penetration depths in order to estimate the penetration of ALA induced PpIX in time through the skin.

## Materials and Methods, Experiment I:

*ALA-application*

The first experiment was performed on 7 healthy volunteers, 2 females and 5 males. On each person the fluorescence kinetics was examined on the flexor of both forearms and on the lower part of the back. On each arm 2 areas and on the back 3 to 4 areas of 1.5 $cm^2$ were marked. In some areas the thickness of the SC was reduced by stripping the skin 10 times with tape. The stripping was performed by a trained person according to a method well described by Pinkus *et al.* (1951).

An indication of the skin barrier function before and after tape-stripping was obtained by measuring the transepidermal epidermal water loss (TEWL). Water molecules diffuse across the skin, and this process accounts for approximately 35% of the total water loss from the body. TEWL has been related to the skin barrier function and it was observed that a high TEWL rate has been detected in patients with stratum corneum (SC) disorders, like psoriasis (Frost *et al.*, 1968). In this study the TEWL was measured with an Evaporimeter. The working principle is the measurement of the partial water vapour pressure at 2 points at known distances above the skin surface. The partial pressure gradient is computed according to Fick's law of diffusion and the evaporation rate, i.e. the transdermal water loss rate, is displayed (Nilsson *et al.*, 1977). The actual method and the factors affecting reliable measurements are described by H. Tanojo in his PhD thesis (University of Leiden, 1996):

"Fatty acids as enhancers of drug permeation across human skin". One person who had the flu showed a TEWL 2 to 3 times higher compared to the other persons, due to fever. This person was excluded from the study.

After the TEWL measurements a solution with ALA or a solution without ALA was applied to all marked areas for 4 or 16 hours (Table 2). The solution with ALA (Finetech; Haifa, Israel) was freshly made in a concentration of 20 % (w/w) and 0.2 ml was absorbed into a gauze of 1.5 $cm^2$ which was placed on the skin. A piece of transparent film dressing (Mölnlycke, Waremme, Belgium) was placed over the gauze to prevent evaporation and movement of the solution. After the application period the solution was removed and the skin was carefully cleaned with water and dried with a towel.

**Table 2**. Areas of skin studied in the first experiment (n=6)

| Application Time (h) | ALA | Skin | Number of areas | |
|---|---|---|---|---|
| | | | Arm | Back |
| 4 | + | normal | 15 | 8 |
| 4 | + | stripped | 9 | 8 |
| 4 | - | stripped | 4 | - |
| 16 | + | normal | 4 | 4 |
| 16 | - | normal | - | 4 |

*Fluorescence recordings*

Recordings were made before, immediately after and every 2 hours after ALA-application until 28 hours after the first fluorescence recording. Light from a halogen lamp and a bandpass filter were used to excite fluorescence at a wavelength of 500 ± 40 nm with a fluence rate of 0.2 $mW/cm^2$. The fluorescence was detected through a filter that passes light above 665 nm. Fluorescence images were recorded using a charge-coupled device (CCD) camera with a two-stage image intensifier. The experimental set-up is explained in more detail by van der Veen *et al.* (1994). The fluorescence was quantified digitally by calculating the mean greyscale value within an area as large as possible (approximately 1.5 $cm^2$) of the recorded fluorescence image.

## Results, Experiment I:

*Autofluorescence.*

The intensity of the autofluorescence signal on the flexor part of the arm was nearly the same in 5 out of 6 volunteers. The autofluorescence on the lower part of the back showed a large variation between volunteers. In almost all volunteers the autofluorescence of the back was higher than on the arm by a factor between 1 and 2. Tape-stripping did not alter the autofluorescence signal.

*PpIX-fluorescence of tape-stripped versus normal skin.*

There was a marked variation in the results because of problems with the experimental set-up. Some parts of the experimental set-up were not fixed which resulted in irreproducible measurements. The effect of tape-stripping on the PpIX-fluorescence kinetics was examined again in the second experiment with an improved set-up. Therefore, the results of the first experiment will not be discussed.

*Duration of ALA-application.*

In 4 volunteers ALA was applied for 16 hours on the back and the arm. The results after 16 hours of ALA-application were compared with the results after 4 hours of ALA-application on the same volunteer. The PpIX-fluorescence immediately after 16 hours of ALA application was on the arm 2.3 (s.e.m. ± 0.2 ) and on the back 1.9 (s.e.m. ± 0.2) times higher than on skin with ALA applied for 4 hours. On the arm maximum fluorescence at 16 hours was 2.3 (s.e.m. ± 0.5) times higher than maximum fluorescence after a 4 hour ALA-application time. On the back, maximum PpIX-fluorescence intensity after 16 hours ALA-application was 1.1 (s.e.m. ± 0.5) times higher than maximum fluorescence of skin applied with ALA for 4 hours. After the 16 hours ALA-application maximal PpIX-fluorescence on the arm and on the back was achieved between 0 and 8 hours (t=16 and 24 hours) with an average of approximately 4 hours (t=20) post ALA-application.

*Location.*

In 5 out of 6 volunteers the TEWL value before and after tape-stripping was lower on the arm than on the back. Also, in these volunteers maximal PpIX-fluorescence intensity following 4 hours ALA application on normal skin was higher on the back than on the arm. After 16 hours ALA application there was no difference in maximal PpIX-fluorescence intensity on either back or arm.

## Materials and Methods, Experiment II:

*ALA-application*

The second experiment was performed on 8 healthy volunteers, 1 female and 7 males. In the first experiment we observed that the variations in the results on the arm and back were large. Especially the application of ALA to the back was not optimal due to movement of the gauze with solution resulting from movement of the volunteer. Therefore, in this experiment only the fluorescence kinetics of the flexor of one forearm was examined. On one arm 3 areas were marked and the skin of one area was stripped. In the second experiment the skin was stripped 15 times with tape instead of 10 times as in the first experiment. This was done with the hope of increasing the difference in PpIX-fluorescence intensity between tape-stripped and normal skin. The TEWL before and after tape-stripping and at the end of the experiment was measured. A 20% ALA solution (w/w) was freshly prepared and the solution was set at pH 5.5 by adding sodium hydroxide (2M). On one normal and one tape-stripped area 0.2 ml solution with ALA was applied and covered with a thin gauze of 1.5 cm$^2$. On a third area the solvent without ALA was applied to the skin. A piece of transparent film dressing (Mölnlycke, Waremme, Belgium) was placed over the gauze to prevent evaporation and movement of the solution. The solution was applied to the skin for 4 hours after which it was carefully removed.

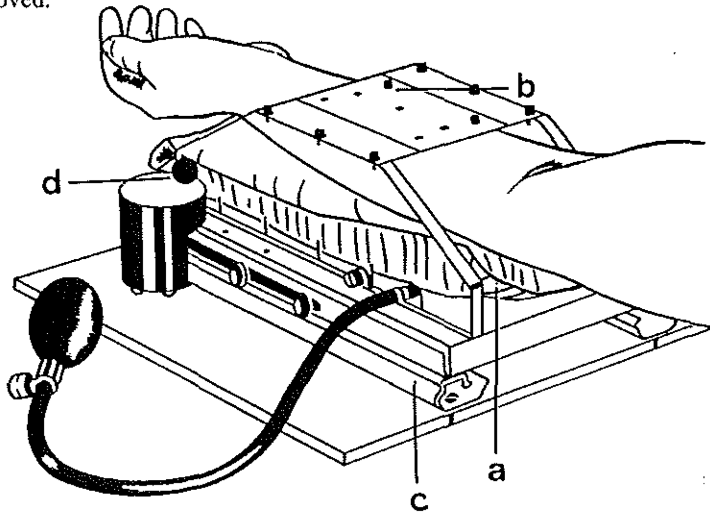



***Figure 1.*** *Template for correct repositioning of the arm. The arm was placed in the template and positioned using marks on the skin. By pumping air in the bag (a) the arm was lifted and fixed. For each wavelength a piece of fluorescent plastic (b) was placed on the template so that small variations in output of the excitation light could be corrected. The areas were repositioned under the CCD-camera using a rail system (c) with stops (d) at certain distances.*

*Fluorescence recordings*

Experience obtained with the first experiment resulted in an improved second experiment. A template (Figure 1) was made to fix the arm during the measurements and for correct repositioning of the arm each time. Also, the CCD camera was fixed to ensure an optimal light field during the entire experiment. Fluorescence recordings were made before and every 2 hours after the start of the ALA-application until 28 hours after the first recording. In the second experiment an extra recording was made during the application, 2 h after the start of the application period. At that time the transparent film dressing above a gauze was carefully removed with scissors without damaging the skin. The gauze containing the ALA was also removed and a fluorescence recording was made. The solution is clear and non-fluorescent so that the recordings could be made without cleaning the skin. After the recording the removed film dressing with the gauze was re-placed on the skin and a new film dressing was placed over the first. Light from a Halogen lamp and bandpass filters located in a filter wheel were used to excite fluorescence at three different wavelengths as shown in table 3. The applied fluence rate and detection filters are also shown in this table. The experimental set-up is explained in more detail by Robinson *et al* (1998).

**Table 3.** Fluorescence excitation and emission wavelengths of the second experiment.

| | Fluence rate ($mW/cm^2$) | Excitation wavelength | Detection wavelength |
|---|---|---|---|
| Violet | 0,03 | 400 ± 30 | 670 ± 50 |
| Green | 0,13 | 500 ± 20 | 670 ± 50 |
| Red | 1,30 | 625 ± 20 | 700 ± 20 |

## Results, experiment II:

*TEWL*

The TEWL before stripping was almost the same in all volunteers (6.9 $g/m^2h$ ± 0.4 s.e.m.). After stripping a large variation in TEWL values between persons was observed. In 3 out of 8 volunteers the TEWL was between 15 and 21 $g/m^2h$. In 4 volunteers the TEWL after stripping was larger, between 40 and 84 $g/m^2h$. One volunteer did not show an increase in TEWL after stripping and this person was excluded from the entire study. The TEWL values were used to compose two groups. Group A consisted of 3 volunteers in whom the TEWL *increased* by 10 to 15 $g/m^2h$ after tape-stripping. Group B consisted of 4 volunteers in whom the TEWL increased by 30 to 75 $g/m^2h$ after tape-stripping. In this group the skin of 2 volunteers with the

highest TEWL increase glistened after tape-stripping indicating moisture and one day after the experiment a superficial crust had been formed on the stripped area.

*Excitation wavelength*

The fluorescence after a 4 hour ALA-application time of both normal and tape-stripped skin was excited with violet (400 ± 30), green (500 ± 20) and red (625 ± 20) light. The effect of the excitation wavelength on the fluorescence kinetics will be discussed only for normal skin (not tape-stripped). In figure 2 the normalised fluorescence kinetics (fluorescence maximum is 100%) of all volunteers excited with violet, green and red light is shown. The rate of fluorescence increase was similar for the 3 excitation wavelengths. There was no difference in the time after ALA-application to maximum PpIX-fluorescence between violet, green and red excitation light. After the PpIX-fluorescence maximum, the fluorescence excited with violet decreased faster than the fluorescence excited with green and red. The decrease of fluorescence excited with green and red was similar.

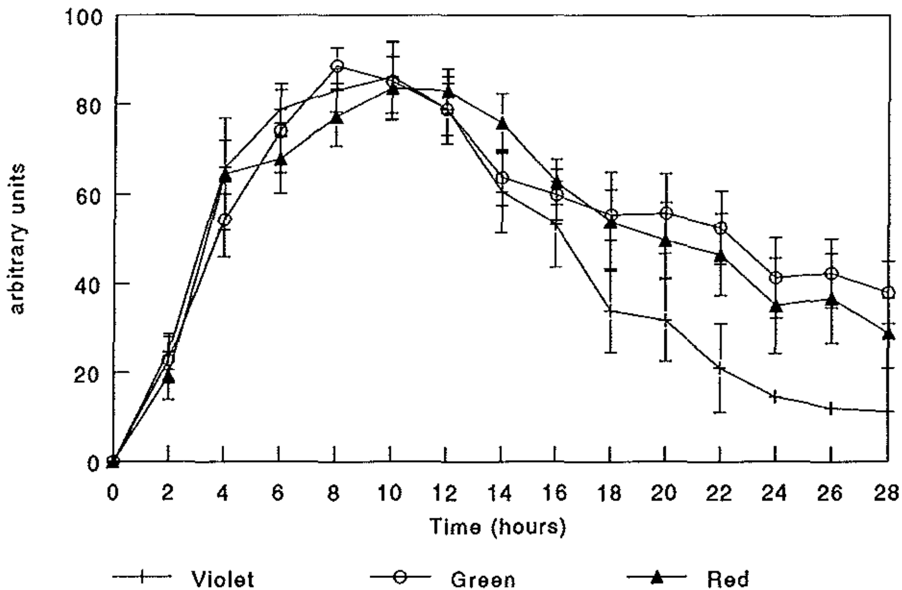


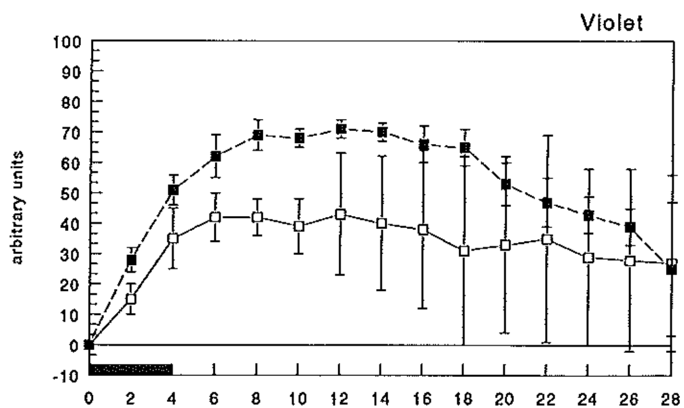

*Figure 2. Mean fluorescence kinetics (normalised at the fluorescence maximum; n=7; ± s.e.m.) of normal skin to which ALA had been applied for 4 hours, excited with violet (400 ± 30), green (500 ± 20) and red (625 ± 20) light. The autofluorescence was subtracted. The timescale indicates time after the start of the 4 hours ALA application.*

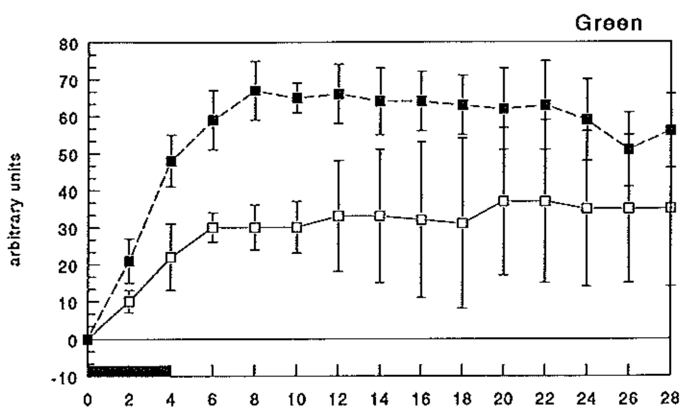
*Tape-stripping*

The PpIX-fluorescence kinetics of tape-stripped and normal skin of group A and B at three different excitation wavelengths are shown in figure 3a to 3f. For this figure the autofluorescence value was subtracted from the PpIX fluorescence data. In group A the fluorescence increase of stripped skin was higher than normal skin with all three excitation wavelengths. In two volunteers the fluorescence of both stripped and normal skin increased until t=10 (6 hours after the 4 h ALA application). After t=10 the fluorescence decreased and at t=18 the fluorescence of normal skin had almost returned to autofluorescence level. The fluorescence of tape-stripped skin decreased only slowly and especially with red excitation light the fluorescence at the end of the experiment was still high. In one volunteer a different fluorescence pattern was observed. The fluorescence of both normal and tape-stripped skin increased until t=18. This resulted in the large s.e.m. after t=10 as observed in figure 3a to 3c.

In group B the fluorescence increased until t=10 for all three excitation wavelengths. With violet light the increase of fluorescence in tape-stripped skin was less than of normal skin. After maximum fluorescence a fast decrease was observed in normal skin whereas the fluorescence of tape-stripped skin hardly decreased. With green and red excitation light the fluorescence of tape-stripped skin at all times was higher than that of normal skin in two out of 4 volunteers. After maximum fluorescence a decrease was observed in normal skin whereas in tape-stripped skin the fluorescence hardly decreased. Even at t=28 the fluorescence intensity was almost similar to the fluorescence intensity observed at time of maximum fluorescence.

The difference in maximum fluorescence intensities and time of maximum PpIX-fluorescence between group A and B is shown in table 4a to 4c. There is no difference in time of maximum fluorescence between the groups and between the different excitation wavelengths. Maximum fluorescence intensity of normal skin was similar in volunteers from group A and B. Maximum fluorescence intensity in group B of tape-stripped skin was similar to normal skin. Only in group A did the tape-stripped skin show a higher maximum fluorescence intensity compared to the other groups.


Violet
arbitrary units
100
90
80
70
60
50
40
30
20
10
0
-10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28


Green
arbitrary units
80
70
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20
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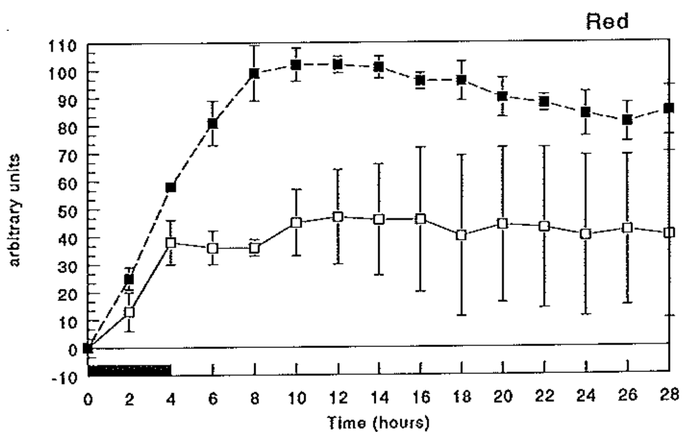
-10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28


Red
arbitrary units
110
100
90
80
70
60
50
40
30
20
10
0
-10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (hours)
normal
stripped

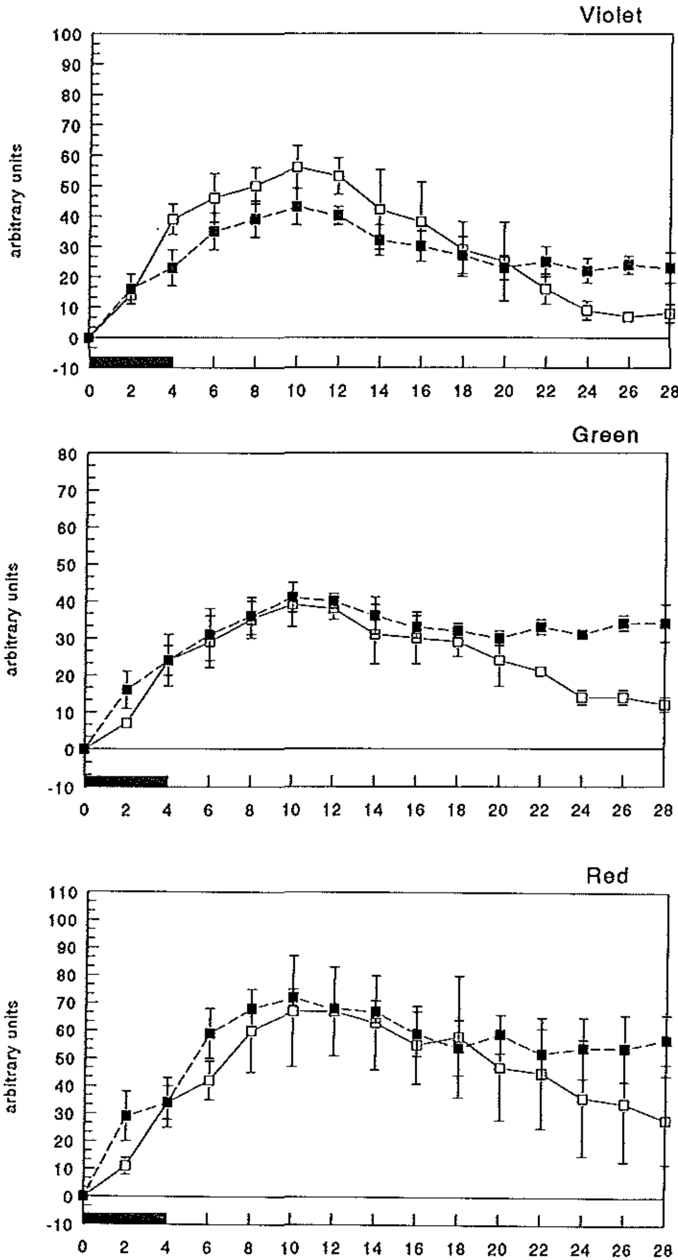



*Figure 3. Fluorescence kinetics ( ± s.e.m.) of group A (figure 3a-c; TEWL increase etween 10 and 15 g/m²h; n=3) and B (figure 3d-f; TEWL increase between 30 and 75 g/m²h; n=4) of normal and tape-stripped skin excited with 3 wavelengths, violet (400 ± 30), green (500 ± 20) and red (625 ± 20) light. The autofluorescence was subtracted in these figures.*

a

| | Violet (400 ± 30 nm) | | | |
|---|---|---|---|---|
| | Group A | | Group B | |
| Skin | normal | stripped | normal | Stripped |
| $Flu_{max}$ (a.u.) | 56 (± 16) | 73 (± 5) | 58 (± 9) | 42 (± 5) |
| $t_{max}$ (hours) | 13 (± 6) | 12 (± 3) | 11 (± 1) | 11 (± 1) |

b

| | Green (500 ± 20 nm) | | | |
|---|---|---|---|---|
| | Group A | | Group B | |
| Skin | normal | stripped | normal | Stripped |
| $Flu_{max}$ (a.u.) | 44 (± 14) | 70 (± 6) | 44 (± 8) | 41 (± 4) |
| $t_{max}$ (hours) | 13 (± 6) | 12 (± 4) | 11 (± 2) | 11 (± 1) |

c

| | Red (625 ± 20 nm) | | | |
|---|---|---|---|---|
| | Group A | | Group B | |
| Skin | normal | stripped | normal | Stripped |
| $Flu_{max}$ (a.u.) | 59 (± 26) | 102 (± 1) | 69 (± 23) | 71 (± 2) |
| $t_{max}$ (hours) | 12 (± 6) | 10 (± 2) | 11 (± 1) | 11 (± 1) |

**Table 4.** Maximum fluorescence and time interval between the start of ALA application and maximum fluorescence of group A (TEWL increase 10-15 $g/m^2h$; ± s.e.m.) and B (TEWL increase 30-75 $g/m^2h$; ± s.e.m.) on normal and tape-stripped skin excited with violet (a), green (b) and red (c) excitation light. The fluorescence units are arbitrary but comparable between groups.

## Discussion

### Experiment I

*Location*

The difference in ALA-induced PpIX-fluorescence between the flexor of the forearm and the lower part of the back was examined. Before ALA-application an autofluorescence recording was made. In 5 out of 6 volunteers the autofluorescence on the back was larger than on the arm. Autofluorescence arises from elastin and collagen, both major compounds of the human dermis. With age and exposure to sunlight these molecules may be altered. This was examined by Leffell *et al.* (1988) who found that the autofluorescence of sun-exposed skin was lower than of sun-protected skin. Furthermore, the degree of pigmentation, or melanin content, which absorbs light, varies between the locations. The effect of pigmentation, and changes in collagen and elastin due to sunlight, is likely to cause the lower autofluorescence intensity on the arm compared to the intensity on the back.

The increase of PpIX-fluorescence, during and after 4 hours ALA-application on normal skin, was higher on the back than on the arm. The interval between the start of ALA application and maximum fluorescence was comparable between the back and the arm. For other topically applied drugs the penetration through the skin was higher on the back than on the skin (table 1). The limiting tissue layer for the penetration of drugs through the skin is the SC. The SC on the back is thinner than on the arm, resulting in a higher penetration of ALA through the SC. This results in a higher PpIX concentration in the skin of the back compared to the skin on the arm.

*Duration of ALA-application.*

With a long application time of 16 hours, the fluorescence was approximately 2 times higher than immediately after a 4 hour application. Also, in clinical studies a longer application time was also successful in increasing the fluorescence intensity in skin lesions (Szeimies *et al.*, 1993; Meijnders *et al.*, 1997). With a long ALA-application time the fluorescence of the skin surrounding the lesions was the same as the fluorescence intensity observed in skin lesions (Meijnders *et al.*, 1997). It may be that with a longer application of ALA the PDT effect can be increased. However, there have been no comparative clinical studies done so far, to examine the difference in damage between a short and a long application time.

*Differences between first and second experiment*

In addition to differences in the experimental set-up and in the number of tape-strippings the applied ALA-solution also differed. In the second experiment the pH of the solution was higher than the solution used in the first experiment. In the first experiment the pH of the ALA-solution was approximately 2.3 (no adjustment of pH). In the second experiment the pH

of the ALA-solution was adjusted with NaOH to pH 5.5. This was done to avoid visible skin reactions like redness, as seen in our laboratory with animal studies. In a pilot study with 3 volunteers the difference in fluorescence kinetics between the solution used in experiment I and II was examined. The ALA solution was applied to the skin for 4 hours and after removal of ALA the PpIX-fluorescence was measured 12, 16 and 20 hours with violet, green and red excitation light using the same experimental set-up as in the second experiment. In all three volunteers we observed a higher PpIX-fluorescence intensity in the areas with the ALA-solution used in the first experiment (pH 2.3). The PpIX-fluorescence intensity of the solution with the low pH (as used in the first experiment) compared to the solution with the higher pH (as used in the second experiment) was the highest 16 hours after removal of ALA (1.30 s.e.m. ± 0.18) and the lowest 12 hours after removal of ALA (1.13 s.e.m. ± 0.01). With all three excitation wavelength the fluorescence intensity following application of the solution with the low pH was higher than the fluorescence kinetics following the solution with the high pH. An enhanced penetration after application of a solution with a low pH, is also seen with other topically applied drugs. A solution with a low pH results in an alteration of the integrity of the SC layer and therefore in alteration of the skin barrier function.

**Experiment II**

*TEWL*

Although the skin of each person was tape-stripped 15 times a large variation in TEWL increase after tape-stripping was observed. Because the SC is not a uniform layer there is no relationship between stripping and the increase of TEWL. The structure, composition and the function of the corneocytes change towards the outer surface of the skin. Cells in the deeper layer of the SC (stratum compactum) are thicker and have more densely packed, organized parallel arrays of keratin filaments, a more fragile cornified cell envelope and a greater variety of modified desmosomes, compared with cells of the outer SC. The deep SC layer has the least water-binding capacity, but the highest water content among other SC regions. Mid-SC has the highest concentration of amino acids and is able to bind water with greater efficiency than cell-layers of either deeper or outer SC regions. The outer SC (stratum disjunction) has the lowest water content. Depending on what part of the SC is stripped away the TEWL increase will be small as seen in group A, or large as seen in group B.

*Excitation wavelength*

The skin is a multilayered organ with one strongly fluorescing layer, the epidermis. The thickness of the different layers varies between different sites but the SC is approximately 8-15 μm thick, the epidermis is 50-100 μm thick and the papillary dermis with the skin appendages is approximately 100-200 μm thick. The approximate depth of light penetration in

fair caucasian skin to a value of 37% of the incident energy density was determined by Anderson and Parrish (The Science of Photomedicine, Chapter 6, Edited by Regan and Parrish, New York, 1982). They found that the approximate penetration depth of 400, 500 and 600 nm light was 90, 230 and 550 μm respectively. This means that in our study with violet (400 ± 30 nm) light, fluorescence could be detected almost in the entire epidermis. With green (500 ± 20) and red (625 ± 20) excitation light PpIX in the epidermis and the papillary dermis could be excited. The fluorescence kinetics between red and green excitation light was similar. With violet excitation light the fluorescence decrease was faster than with green and red excitation light. We had expected to see a shift in time of maximum fluorescence. The maximum of fluorescence excited with 600 nm light was expected to appear at the latest time. However, for all three excitation wavelengths the time of maximum fluorescence was the same. Apparently, the light penetration in normal skin of all three excitation wavelengths was sufficient to excite fluorescence in all PpIX present.

*Tape-stripping versus normal skin*

Tape-stripping is a well known method to increase the penetration of drugs through the skin. For example the penetration ratio of Hydrocortisone after tape-stripping was approximately 4 (Feldmann and Maibach, 1967). Also, tape-stripping in combination with occlusion increased the penetration ratio by a factor of 20 (Feldmann and Maibach, 1967). A similar pattern was observed with other drugs (Feldmann and Maibach, 1967). Therefore, it is realistic to assume that also with ALA-application the penetration in tape-stripped skin is enhanced. Because the skin barrier is the limiting factor in the penetration through the SC, a higher fluorescence in tape-stripped skin was expected. This occurred in group A, volunteers with a relatively small decrease in SC thickness after tape-stripping, but not in group B, volunteers with a strongly decreased SC thickness after tape-stripping. Topically applied ALA penetrates through the SC and is subsequently converted to PpIX in the epidermal cells. In our study the fluorescence is an indication of the concentration of PpIX in the tissue. However, tape-stripping has a strong effect on the epidermal cells This strong epidermal cell reaction to tape-stripping, already visible ½ hour after stripping, might result in alterations in the haem synthesis of these cells. This effect of tape-stripping on the epidermis was examined by Pinkus (1952) who used tape-stripping as a method to study the mechanism of epidermal regeneration. After removing the SC of the flexor forearm he took biopsies ½, 12, 24, 36, 47, and 72 hours after tape-stripping. He examined the changes which take place in the number and sizes of cells, and also, in the number of mitoses in relation to completeness of stripping and to time elapsed after stripping. Already ½ hour after stripping macroscopical changes in the epidermal layer could be observed. The basal cells were larger and more widely spaced than in normal state and the number of prickle cell layers was reduced. Also, there were numerous pyknotic nuclei. At 12 hours the basal cells were more hypertrophic, and their nuclei were situated at varying distances from the base. In the prickle cell layer there were still quite a few pyknotic nuclei

while other nuclei became hypertrophic. The 24 hour stage showed that one third or more of the thickness of the epidermis was made up of the columnar basal cells and that a multiple parakeratotic layer covered the surface. At 47 hours he saw numerous cells in all stages of mitosis. The epidermis had almost doubled its normal thickness. In a second experiment Pinkus varied the number of tape-strippings and biopsies were made 48 hours after stripping. He found that the nuclear diameters and mitotic activity increased with increasing denudation. He concluded that the loss of keratinized cells itself acts as the primary stimulus to induce hypertrophy and mitosis 48 hours after stripping. Furthermore, he concluded that the mitotic activity was delayed in cells that were appreciably damaged.

Two factors are changed in tape-stripped skin. The penetration of ALA through the SC is increased whereas the haem synthesis might be decreased as a result of cellular changes after stripping. Both factors are determined by the decrease of the SC thickness after tape-stripping. This may explain the results observed in this study. Depending on the increase of ALA penetration through the skin in combination with a decrease of haem synthesis in the cells the fluorescence in stripped skin is higher (group A) or lower (group B) than of normal skin.

The slope of the tape-stripped graphs was different from that of normal skin. The fluorescence of tape-stripped skin hardly decreased after maximum fluorescence. This may be the result of the high ALA-concentration in the tape-stripped skin as a result of the decreased skin barrier. Even at t=28 the fluorescence was almost similar to maximum fluorescence. Whether these porphyrins can be used successfully for PDT is unclear. In one volunteer a fluorescence recording was made at t=28. Because some fluorescing dirt was located on the area, the skin was carefully cleaned with water and dried with a towel. After cleaning a second recording was made. With the second recording the fluorescence was clearly less, indicating that the PpIX fluorescence was wiped off.

This study suggests that tape-stripping is not a good and reliable method to increase the PpIX concentration in normal skin tissue. Whether strong cell reactions after tape-stripping also occur in skin lesions is not clear.

**Concluding remarks**

Diffusion of ALA across the SC of the skin is the limiting factor for ALA penetration in normal skin. Important factors that are successful in increasing the fluorescence intensity were, a long application time and a solution with a low pH value. Tape-stripping the skin did not automatically result in an increased fluorescence. In tape-stripped skin the limiting factor is no longer the diffusion across the SC but the rate of the haem synthesis.

# Chapter 8

General Discussion

## General Discussion

*Studies using systemically administered ALA*

There are a limited number of publications about systemic ALA-PDT in patients. Small numbers of patients with rectal adenomas following surgery (Warloe *et al.*, 1995), gastrointestinal tumours and polyps (Regula *et al.*, 1995; Mlkvy *et al.*, 1995), cancer and dysplasia in Barrett's oesophagus (Barr et al., 1996), precancer and cancer of the oral cavity (Fan *et al.*, 1996) have been treated and in all cases only partial necrosis could be obtained. Optimization of systemic ALA-PDT is difficult because to avoid toxic reactions ALA-doses can not be increased above a maximum bolus of 60 mg/kg. Furthermore, the bioavailability after oral ALA is low due to a low uptake from the gut and a poor penetration through cellular membranes. Improvement of the bioavailability is crucial to ensure sufficient PpIX concentration in tissues. The use of fractionated ALA administration and a formulation for intravenous ALA administration are promising options to improve the ALA-bioavailability.

Even when sufficient PpIX concentration in the tissues after oral ALA can be achieved in patients, it will still be difficult to achieve effective tissue damage after PDT. This is illustrated by animal studies. In animal studies high ALA-doses (intravenous, 200-600 mg/kg) have been used and the PDT efficacy using a single illumination was still found to be insufficient. The PDT effect is limited by the available oxygen concentration and oxygen consumption in the tissues and by the rapid photobleaching of PpIX. Reoxygenation of the tissues is limited by vascular constriction during illumination. We found in the observation chamber model that vascular constriction occurred almost immediately after the start of the illumination. The severity of vascular constriction depended on the interval between ALA administration and illumination. A better reoxygenation due to the applied fluence rate or to the use of short light fractionation schemes was found to be successful in increasing the PDT efficacy by Curnow *et al.* (1999), Hua *et al.* (1995) and Messman *et al.* (1995). However, this effect could not be clearly reproduced in our studies. We found that the most promising way to increase PDT efficacy was to use of long term light fractionation schemes (two illuminations separated by 75 minutes). In both the observation chamber model as well as the solid tumour model long term light fractionation induced substantial tumour necrosis. Long term light fractionation will be discussed below in a separate section.

Concluding remarks: Whether PDT using systemically administered ALA can make a significant contribution to the treatment of cancer is not yet clear. Relatively new alternative exogenous photosensitizers are currently under investigation. The drug m-THPC (meta-Tetra-Hydroxy-Phenyl-Chlorin) has found to be very effective in inducing tissue necrosis. However, this drug causes a relatively long period of skin-photosensitivity (several weeks) compared to PpIX (one or two days). The limited skin-photosensitivity makes PDT using systemically administered ALA still attractive. However, problems concerning ALA-

bioavailability and the oxygen concentration during illumination must be solved before PDT using systemically administered ALA can be applied successfully in humans.

*Studies using topically applied ALA*

Since the first publication of Kennedy *et al.* in 1990 many papers have been published about topical ALA-PDT for the treatment of non-melanoma skin tumours in humans. The long term histological response after topical ALA-PDT of superficial AK, BCC, SCC and Bowen's disease was approximately 85%. The efficacy of ALA-PDT for nodular BCC and SCC was disappointing in all studies (between 0 and 50% complete clinical response rate). However, Fink-Puches *et al.* (1998) recently reported a projected disease free rate at 36 months after ALA-PDT of 50% for BCC and only 8% for SCC. In almost all studies the clinical responses were found to vary strongly within and between patients. Jeffes *et al* (1996) found that AK's on the face and scalp responded better to ALA-PDT treatment than AKs on the trunk and extremities. In our study with healthy volunteers (Ch.7) we found that the fluorescence of skin on the trunk after topical ALA application was higher than the fluorescence on the forearm. The difference in thickness and composition of the SC between different sites of the body determines the penetration of ALA into the skin. Present research to increase the PDT efficacy after topical ALA is therefore mainly focused on increasing the ALA penetration into the skin. The use of iontophoresis, penetration enhancers and ALA application time, to increase the ALA penetration into the skin, are options which are currently under investigation.

In the volunteer experiment (Ch.7) the effect of reducing the SC by tape-stripping the skin was investigated. We expected that removing the SC by tape-stripping would substantially increase the ALA-penetration into the skin and therefore increase the PpIX concentration in the cells. However, we observed that removing the SC by tape-stripping of healthy human skin did not automatically result in an increased fluorescence after topical ALA application. Tape-stripping of skin with a relatively thick SC followed by topical ALA application resulted in a higher fluorescence intensity compared with ALA-application to normal skin (no tape-stripping). On the other hand, tape-stripping of skin with a relatively thin SC followed by topical ALA application did not result in a higher fluorescence intensity compared with ALA-application to normal skin (no tape-stripping). Tape-stripping causes epidermal cell reactions which already occur 30 min after tape-stripping (Pinkus, 1952). This reaction is dependent on the thickness of the SC, e.g., a relatively thin SC reacts more strongly to tape-stripping than a relatively thick SC. The epidermal cell reactions may result in alterations in the haem synthesis and therefore in alterations in the PpIX synthesis.

The fluorescence kinetics after topical ALA was examined in normal human skin and in hairless mouse skin (UVB-treated and non-UVB-treated). The most common clinical ALA application time is between 4 and 6 hours. Some institutes use a long ALA application time of 16 to 20 hours. With this long application time the difference in fluorescence between the tumour and the surrounding skin may be lost. In our study on healthy human skin we

observed that the fluorescence after an application time of 16 hours was 2 times higher than immediately after a 4 hour application time. In both the hairless mouse model and in the human skin we observed a continuing increase in fluorescence 6-8 hours after the end of a 4 hour application time. At maximum fluorescence, the intensity was approximately 2 times higher than immediately after application. In the hairless mouse model we observed in time after application an increased difference in fluorescence between UVB-treated and non-UVB-treated skin. It was not possible to examine prolonged ALA-application in the hairless mouse. Considering the results obtained with the hairless mouse and the human skin studies it may be more favourable to apply ALA for a limited time and illuminate the applied area a few hours later.

In the hairless mouse model larger PpIX fluorescence in UVB-treated animals at the maximum (t=10, chapter 6) compared to immediately after ALA-application (t=4) did not result in significantly more damage upon illumination. This may be due to a limited oxygen supply in the tissues during the illumination. We observed vascular constriction in hairless mouse model almost immediately after the start of illumination which was maximal 2 minutes later. The limited oxygen concentration during illumination, partly due to the constriction, resulted in a distinct pattern of skin changes. We often saw more damage at the rim than at the centre of the illuminated area. These reactions were more often observed in UVB-treated mice than in the non-UVB-treated mice. The incidence of rim reactions was less after a lower fluence rate. This damage pattern is also sometimes observed in patients treated with topical ALA-PDT (personal communication W.M. Star). The incidence of this effect is not known because the damage reaction after a clinical treatment is not always followed in detail. The incidence of this effect needs to be investigated further to determine the occurrences of vascular constriction during a clinical ALA-PDT treatment.

In the hairless mouse studies using topically applied ALA, the effect on UVB and non-UVB skin after a single illumination was not severe. The effect after two illuminations (long term light fractionation) separated by a dark period induced a strong damage effect to both UVB- and non-UVB-treated skin. The damage was so severe that after the observation time of 25 days the treated skin showed scar tissue and local loss of hair follicles. Long term light fractionation will be further discussed below.

Concluding remarks: After topical ALA-PDT to hairless mouse skin, vascular constriction occurs which may limit the oxygen supply and therefore limit the PDT efficacy. The extent of these vascular effects in clinical ALA-PDT needs to be investigated.

*Long term light fractionation (Twofold illuminations).*

Long term light fractionation was the most successful way to improve the PDT effectiveness after systemically and topically applied ALA, compared to a single illumination. In all three models, observation chamber model, solid tumour model and hairless mouse model, two illuminations separated by a dark period induced clearly more tissue damage than a continuous illumination with the same light fluence. The damage to normal skin also increased clearly, so that the therapeutic selectivity did not increase. The precise reason for this strongly increased damage effect using long term light fractionation is not clear. Features that may account for this enlarged effect are:

A) **Recovery of vascular constriction during the dark period** and as a result reoxygenation of the tissues.

B) **New PpIX synthetised during the dark period.** In the observation chamber model and in the hairless mouse model the fluorescence after the first illumination was monitored. Immediately after the illumination the PpIX fluorescence was bleached and in time new fluorescence was observed. After topical ALA-PDT the increase of fluorescence in the PDT treated areas was similar or faster (depending on the interval between ALA-application and the first illumination) than in the areas on the same hairless mouse which had received ALA but no illumination. We have shown in the hairless mouse model that the new fluorescence was the result of locally formed PpIX and was unlikely to result from PpIX leakage from the surrounding tissue. After systemic ALA-PDT we also found an increase in fluorescence after illumination in the observation chamber model. The increase was less than the increase in chambers which had received no illumination. The new PpIX synthetised after illumination may arise from cells that still have the capacity to synthetise haem. Furthermore, biochemical changes in the cells as a result of the first treatment may also cause an increased PpIX concentration. Cells can respond to photodynamic treatment by regulation of specific proteins, so called stress proteins (Fisher et al., 1993; Curry and Levy, 1993). One of the three groups of stress proteins is haem oxygenase (Schlesinger, 1990), an enzyme which plays a key role in the regulation of haem turnover. Induction of haem oxygenase due to ALA-PDT may then result in an increased PpIX concentration in the cell.

The new PpIX synthetised in the dark period after the first illumination can be used in the second treatment to increase the PDT efficacy. The new fluorescence is unlikely to be the only determining factor in the increased PDT effect using long term light fractionation. After ALA-application the hairless mice received an illumination 6 hours after the end of the application (t=10) or both immediately after application at t=4 and at t=10 (long term light fractionation). The fluorescence intensity in the skin of the animals illuminated only at t=10 was approximately the same as the sum of the fluorescence in the second group (long term light fractionation) at t=4 (before the first illumination) and at t=10 (the new fluorescence synthetised during the dark period). The PDT damage between both groups however was clearly different.

**C) An increased sensitivity to PDT due to recovery reactions after the first illumination.** ALA-PDT may cause cell damage to membranes of lysosomes, mitochondria and cytoplasm. The cells response to the treatment with a variety of damage protection/repair systems. Cells in this process may become more sensitive to PDT treatment. Also, after illumination the cell cycle phase may change. Cells in the S-phase or G2 are more sensitive to ALA-induced PDT than cells in the G1 phase (Wyld *et al*, 1998). This increased sensitivity in combination with the new PpIX synthetised in the dark period may result in the strong tissue response after the second illumination. This process needs to be investigated to understand the mechanism of the markedly increased tissue damage after long term light fractionation.

Whether ALA-PDT treatment using long term light fractionation will be successful in humans is as yet unclear. There is only one study by Fan *et al.* (1996) who used long term light fractionation after orally administered ALA for the treatment of precancer and cancer of the oral cavity. They observed in all cases only partial necrosis after long and short term light fractionation. However, in this study the systemically administered ALA doses were low compared to the doses used in animal studies and the synthetised PpIX levels were around threshold level for producing tissue damage.

In the DDHK the first patients with superficial skin lesions have been treated with topical ALA-PDT using twofold illuminations. The fluorescence prior to and after the first illumination was monitored and new fluorescence was observed in time after the illumination (Meijnders *et al*, 1996). However, it is too early to tell whether topical ALA-PDT using twofold illumination is more successful than a treatment using a single illumination.

Concluding remarks: Topical and systemical ALA-PDT using long term light fractionation induced the strongest PDT damage effect in tissues in our animal models. In the dark period between the illuminations processes like reoxygenation, the formation of new PpIX and probably an increase of sensitivity to PDT due to recovery reactions, take place that may account for this. Although it is tempting to apply ALA-PDT using long term light fractionation in patients, more information about the mechanism of the strongly induced damage after long term light fractionation is needed.

## Summary of the studies

*Studies using systemically administered ALA*

In chapter 2 PpIX-fluorescence kinetics in the observation chamber model are described after two different doses of intravenously administered ALA. We found a higher fluorescence intensity in tumour tissue (TT) than in the surrounding subcutaneous tissue (ST). The initial maximum rate of fluorescence increase was similar between the two different ALA-doses in both TT and ST. This resulted in a later time of maximum fluorescence after the high ALA-dose. Based on the PpIX-fluorescence kinetics, the TT and ST were illuminated at the time where the difference between TT fluorescence and ST fluorescence was maximal. Immediately after the start of the illumination, vascular constriction was observed and the damage effects on both TT and ST were disappointing. Treatment early after ALA administration resulted in less vascular constriction during the illumination and in a greater tumour tissue damage. After an early illumination new fluorescence, showing new PpIX, was observed which could be used for a second illumination. With two illuminations separated by 75 minutes (long term light fractionation), complete TT necrosis could be obtained while permanently damaging only a relatively small area of ST.

In chapter 3 we show the results of different treatment schemes on the effectiveness of PDT using systemically administered ALA. Solid tumours, subcutaneously transplanted on the thigh of rats, were used as a model. The effects of four parameters on the PDT induced damage were determined by examining the difference in tumour growth delay. These parameters were: interval between ALA administration and illumination, fluence rate, short term light fractionation schemes (30 sec on/30 sec off or 5 J/cm$^2$ then 2.5 minutes dark and 95 J/cm$^2$) and a long term light fractionation scheme (100 J/cm$^2$ then 75 minutes dark and 100 J/cm$^2$). All of the investigated PDT schemes demonstrated a significantly longer tumour volume doubling time compared to control tumours. No significant difference in tumour volume doubling time was observed between the different schemes except the scheme using long term light fractionation. This scheme increased the tumour volume doubling time by a factor of 2.6 over all the other illumination schemes.

*Study of the difference in fluorescence of hairless mouse skin after systemically and topically administered ALA*

The difference in fluorescence kinetics and localisation between topically and systemically administered ALA in the hairless mouse model is presented in chapter 4. The most apparent differences between both administration routes were the maximal fluorescence intensity and the time post administration to reach maximal fluorescence in UVB-treated skin. Maximal fluorescence after systemically administered ALA was reached early (2 h post injection) in UVB treated skin but was 3 times lower than maximal fluorescence after topically applied ALA, which occurred at 6 h after the end of the ALA application. The results obtained with the fluorescence cryo-microscopy study showed no important differences in localisation of PpIX fluorescence in the skin between both ALA administration routes. The fluorescence after both administration routes in both UVB treated and non-UVB skin was mainly located in the epidermal layer and in the hair follicles.

*Studies using topically applied ALA*

In chapters 5 and 6 we present the PpIX-fluorescence *during* and *after* PDT and the damage effects after topically applied ALA-PDT of UVB treated and non-UVB hairless mice are presented. Based on the fluorescence kinetics presented in chapter 4 two times for illumination were chosen, immediately after ALA-application (t=4) and at maximum fluorescence (t=10). The animals received an illumination with a fluence rate of 100 $mW/cm^2$ and a total light fluence of 100 $J/cm^2$. In chapter 5 the results of fluorescence measurements *during* PDT (photobleaching) and *after* PDT are shown. We observed that the fluorescence *during* illumination of normal skin decreased at a faster rate than the fluorescence of UVB treated skin. No difference in bleaching rate was observed between an illumination at t=4 or at t=10. The fluorescence *after* illumination of areas treated immediately after application (t=4) or at maximum fluorescence (t=10) and of non-treated areas (ALA, no light) on the same mouse were examined. The areas treated at t=4 showed a similar increase in time as non-treated areas. Areas treated at t=10 also showed a fluorescence increase after PDT whereas the fluorescence of non-treated areas on the same mouse did not increase (PpIX-fluorescence maximum). With both treatment times the new fluorescence showed a characteristic PpIX spectrum. We demonstrated that the new fluorescence after PDT resulted from locally formed PpIX and was unlikely to result from PpIX leakage out of the surrounding tissue. In chapter 6 the damage effects after PDT at t=4 or t=10 are presented. Although the fluorescence at t=10 of UVB treated skin was higher than at t=4 the macroscopical damage effects were similar. In non-UVB skin the fluorescence at t=4 and t=10 was almost the same but the damage to the skin which was treated at t=10 was higher. In

UVB skin the majority of the illuminated areas showed more damage at the rim than at the centre of the illuminated field. This damage pattern might indicate a limited oxygen supply, especially in the centre of the illumination. With *in situ* fluence rate measurements during illumination we observed vasoconstriction almost immediately after the start of the illumination. Vasoconstriction was also demonstrated using the fluorescein exclusion assay. The vascular perfusion in UVB skin was more sensitive to PDT than the perfusion in non-UVB skin. By lowering the fluence rate (from 100 to 50 $mW/cm^2$) an attempt was made to decrease the oxygen depletion and vasoconstriction during the illumination. This resulted in less constriction and reduced rim reactions but no clearly increased damage effect was observed in UVB skin. In non-UVB skin the damage was clearly greater compared to an illumination with a higher fluence rate.

With long term light fractionation (illumination at t=4 and t=10; 100 $mW/cm^2$), using new PpIX-fluorescence arising after a single illumination, a significantly increased damage in both UVB and non-UVB skin was observed compared to a single illumination to the same total fluence (100 $J/cm^2$). The damage to UVB and non-UVB skin was so severe that after the observation time of 25 days scar tissue and complete local loss of hair follicles was observed.

Chapter 7 describes the effect of location, time of ALA application and tape-stripping on the PpIX-fluorescence kinetics of normal human skin. In this study ALA was applied during 4 hours at two locations, the flexor of the forearm and the lower part of the back. We observed during and after ALA-application a larger PpIX-fluorescence increase on the back than on the arm. The interval between ALA-application and maximum fluorescence on the arm and back was similar. With a prolonged ALA-application time (16 hours compared to 4 hours) the fluorescence at the end of application was approximately 2 times higher than immediately after a 4 hour application time. To examine the ALA penetration into the skin in time the fluorescence was excited with three wavelength bands, each with different penetration depth. However, this method did not yield clear differences in kinetics. A strong variation was observed between volunteers in PpIX-fluorescence kinetics after ALA application on tape-stripped skin. An indication for the permeability of the skin after tape-stripping was given by the transepidermal water loss (TEWL). Based on the TEWL measurements volunteers were divided in two groups. The first group showed a slight increase in TEWL after tape-stripping. In this group, the PpIX-fluorescence increase after ALA-application in tape-stripped skin was higher than of normal skin. The second group showed a strong increase in TEWL after tape-stripping. In this group the increase of fluorescence after ALA-application of tape-stripped skin was similar or less compared to normal skin. The time of PpIX-maximum fluorescence, approximately 8 hours after the end of the application period, was similar in tape-stripped and normal skin.

## Samenvatting van de studies

*Studies met systemisch toegediende ALA*

In hoofdstuk 2 wordt de PpIX-fluorescentie kinetiek na twee verschillende doses systemisch toegediende ALA in het observatie kamertjes model onderzocht. De fluorescentie intensiteit was hoger in tumor weefsel (TT) dan in het omliggende subcutane weefsel (ST). De maximale fluorescentie toename in zowel TT en ST na twee verschillende ALA-doses was hetzelfde. Dit resulteerde bij een hogere ALA-dosis in een later tijdstip van maximale fluorescentie. Na het bepalen van de PpIX-fluorescentie kinetiek werden de weefsels belicht op het tijdstip met maximaal verschil in PpIX-fluorescentie tussen TT en ST. Direct na de start van de belichting werd vasoconstrictie geconstateerd en de door PDT teweeggebrachte schade in zowel TT en ST was teleurstellend. Een belichting kort na ALA toediening resulteerde in minder sterke vasoconstrictie en in een hogere tumor schade. Na een vroege belichting werd in de tijd nieuwe fluorescentie waargenomen; een aanwijzing voor nieuw gevormd PpIX. Deze nieuwe PpIX kan gebruikt worden voor een tweede belichting. Met twee belichtingen (lang licht fractioneringsschema), en daartussen een wachttijd van 75 minuten, werd complete TT necrose verkregen met slechts geringe permanente beschadiging van het ST.

In hoofdstuk 3 werden verschillende behandelingsschema's toegepast om de effectiviteit van PDT na systemisch toegediend ALA te verhogen. De invloed van 4 parameters op de effectiviteit van PDT werd bepaald door verschillen in groeiuitstel te meten van solide tumoren die subcutaan op de dijen van ratten groeien. De onderzochte parameters waren: interval tussen ALA toediening en belichting, fluentie tempo, korte licht fractionerings schema's (30 sec aan/30 sec uit of 5 $J/cm^2$ en 2.5 minuten donker gevolgd door 95 $J/cm^2$) en een lang fractionerings schema (100 $J/cm^2$ en 75 minuten donker gevolgd door 100 $J/cm^2$). In vergelijking met de controle tumoren resulteerden alle onderzochte schema's in een significant langere tumor volume verdubbelingstijd. Met uitzondering van het lange fractionerings schema zagen we geen significant verschil in tumor volume verdubbelingstijd tussen de andere schema's. Met het lange licht fractionerings schema werd een 2.6 keer hogere tumor volume verdubbelingstijd behaald in vergelijking met de andere schema's.

*Studie over het verschil in fluorescentie van de haarloze muizen huid na systemisch en topicaal toegediende ALA.*

In hoofdstuk 4 wordt het verschil in PpIX-fluorescentie kinetiek en lokalisatie tussen topicaal en systemisch toegediende ALA in het haarloze muizen model onderzocht. In huid behandeld met UVB werd het maximale fluorescentie niveau na systemisch ALA vroeg (2 h na toediening) waargenomen. Het fluorescentie niveau was 3 keer lager dan het fluorescentie niveau na topicaal toegediende ALA. Hier werd het fluorescentie-maximum waargenomen 6 uur na het einde van de ALA applicatie. Met fluorescentie cryo-microscopy studies werden tussen beide toedienings manieren geen duidelijke verschillen in lokalisatie van PpIX fluorescentie waargenomen. De fluorescentie was voornamelijk gelokaliseerd in de epidermis en in de haarfollikels van zowel huid behandeld met UVB als onbehandeld huid.

*Studies met topicaal toegediend ALA.*

In de hoofdstukken 5 en 6 worden de schade effecten na topicale ALA-PDT en de PpIX-fluorescentie *tijdens* en *na* de belichting van huid behandeld met UVB en onbehandelde huid gepresenteerd. Aan de hand van de fluorescentie kinetiek (hoofdstuk 4) werden twee tijdstippen voor belichtingen gekozen; direct na ALA applicatie (t=4) en op maximale fluorescentie (t=10). In hoofdstuk 5 worden de resultaten van de PpIX-fluorescentie waarnemingen *tijdens* (bleking) en *na* de PDT behandeling op t=4 en t=10 besproken. We vonden dat de PpIX-fluorescentie *tijdens* de belichting van de onbehandelde huid sneller afnam dan de fluorescentie van huid behandeld met UVB. Het tijdstip van belichten (t=4 of t=10) had geen effect op de blekingssnelheid. Direct na de belichting werd geen PpIX-fluorescentie meer gemeten. De fluorescentie *na* de belichting op t=4 en t=10 werd gevolgd van zowel huid behandeld met UVB als onbehandelde huid. Verder werd van dezelfde dieren de PpIX-fluorescentie gevolgd van een gebied behandeld met ALA maar niet belicht. Op t=4 nam de PpIX-fluorescentie van de belichte gebieden even snel toe als de onbelichte gebieden. Op t=10 nam de fluorescentie van de belichte gebieden sneller toe dan de onbelichte gebieden op dezelfde muis. De nieuwe fluorescentie had het karakteristieke PpIX spectrum. De nieuwe PpIX-fluorescentie in de gebleekte gebieden werd niet veroorzaakt door lekkage uit de nog fluorescerende omliggende gebieden. De schade effecten na belichting op t=4 en t=6 worden gepresenteerd in hoofdstuk 6. In huid behandeld met UVB vonden we ondanks een hogere fluorescentie op t=10 geen verschil in schade tussen beide tijdstippen. In huid onbehandeld met UVB daarentegen was de PpIX-fluorescentie op beide tijdstippen bijna gelijk terwijl de schade van de huid belicht op t=10 hoger was dan op t=4. Tijdens de dagelijkse metingen zagen we dat in de huid behandeld met UVB op zowel t=4 als t=10 het merendeel van de dieren meer schade aan de rand dan in het midden van het belichte gebied hadden. Dit

schadepatroon kan het gevolg zijn van een zuurstofdepletie en een beperkte zuurstofvoorziening in het midden van het belichte gebied. Dit werd bevestigd door de *in situ* fluentie tempo metingen tijdens de belichting. Direct na de start van de belichting werd vasoconstrictie waargenomen welke maximaal was 2 minuten na de start van de belichting. Ook met fluoresceïne fluorescentie metingen na de belichting werd vasoconstrictie aangetoond. We vonden dat de vaten van huid behandeld met UVB gevoeliger voor PDT waren dan de vaten van onbehandelde huid. Een nieuwe groep dieren werd behandeld met een verlaagd fluentietempo ( 50 $mW/cm^2$ versus 100 $mW/cm^2$) met als doel de vasoconstrictie en zuurstofconsumptie te verminderen. In huid behandeld met UVB resulteerde dit in minder vasoconstrictie en minder rand effecten maar niet in een versterkt schade effect. In de onbehandelde huid resulteerde een verlaging van het fluentie tempo in een vermindering van vasoconstrictie en in een grotere schade.

In vergelijking met een continue belichting werd met lange licht fractionering (belichting op t=4 en t=10) in zowel huid behandeld als onbehandeld met UVB een sterk verhoogde schade waargenomen. De schade was zo sterk dat na de observatie tijd van 25 dagen de huid nog niet volledig was hersteld. De haarfollikels waren verdwenen en er was littekenweefsel aanwezig.

Het effect van plaats op het lichaam, ALA applicatie tijd en tape-stripping op de PpIX-fluorescentie kinetiek van normale huid na topicale ALA applicatie wordt gepresenteerd in hoofdstuk 7. ALA werd geappliceerd op twee locaties, de binnenkant van de onderarm en de onderrug. We vonden dat de toename van PpIX-fluorescentie op de rug hoger was dan op de arm terwijl het tijdstip van maximale fluorescentie hetzelfde was. De fluorescentie na een lange ALA-applicatie tijd (16 uur) was 2 keer hoger dan net na een 4 uur durende applicatie. Het exciteren van de fluorescentie met drie golflengtes, om zo inzicht te krijgen in de penetratie diepte van ALA in de tijd, bleek niet het gehoopte resultaat op te leveren. De fluorescentie kinetiek voor alle drie de golflengten was identiek. De fluorescentie kinetiek na het strippen van de huid varieerde sterk tussen alle personen. De permeabiliteit van de huid voor en na strippen was gemeten en aan de hand van deze metingen werden de proef personen in 2 groepen verdeeld. In de eerste groep was er een kleine toename in permeabiliteit na het strippen en na ALA-applicatie vonden we een hogere PpIX-fluorescentie toename in de gestripte huid dan in de niet-gestripte huid. In de tweede groep was er een sterke toename in permeabiliteit na het strippen en na ALA-applicatie vonden we een gelijke of mindere PpIX-fluorescentie toename in de gestripte huid dan in de niet-gestripte huid. In beide groepen was het tijdstip van maximale fluorescentie (8 uur na applicatie) bij zowel gestripte als niet-gestripte huid gelijk.

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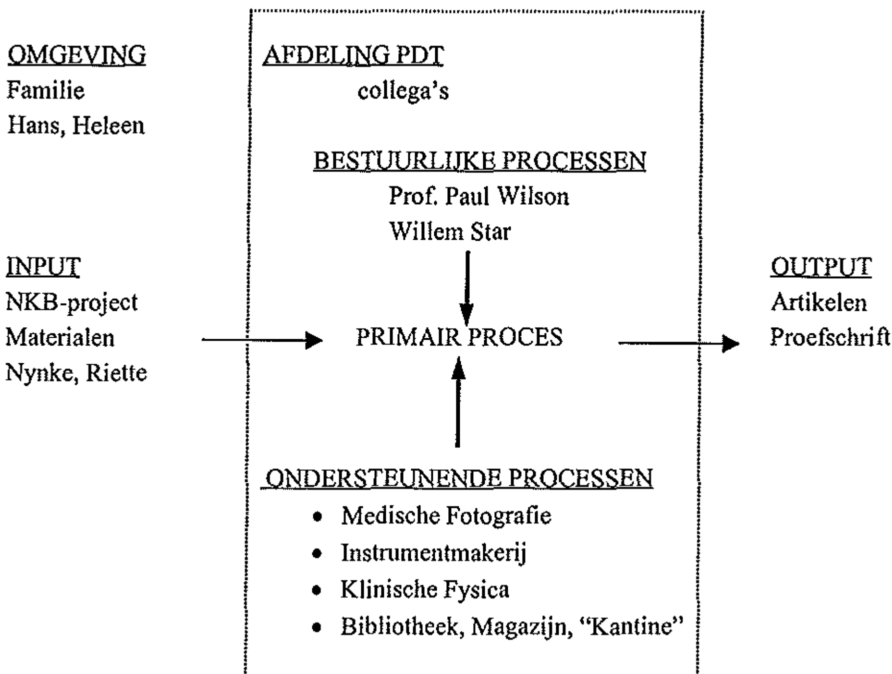
## Abbreviations:

| | |
|---|---|
| AK | Actinic keratosis |
| ALA | 5-Aminolaevulinic acid |
| AS | Altered skin |
| BCC | Basal cell carcinoma |
| Copro | Coproporphyrin |
| FF | Fluorescein fluorescence |
| FIM | Fluorescence imaging measurements |
| FSM | Fluorescence spectroscopy measurements |
| i.p. | Intraperitonial |
| i.v. | Intravenous |
| NS | Normal skin |
| PBG | Porphobilinogen |
| PDT | Photodynamic therapy |
| p.i. | Post injection |
| PpIX | Protoporphyrin IX |
| RH | Rhabdomyosarcoma |
| RMA | Mammary carcinoma |
| SC | Stratum corneum |
| SCC | Squamous cell carcinoma |
| s.e.m. | Standard error of mean |
| T(TT) | Tumour (Tumour Tissue) |
| TEWL | Trans epidermal water loss |
| Uro | Uroporphyrin |
| UVB | Ultra violet B |
| UVB-animals | Animals which received UVB irradiation |
| n-UVB-animals | Animals which received no UVB irradiation |

## Dankwoord

Zo, het proefschrift is eindelijk af. Het dankwoord wil ik schrijven mbv een schematische weergave hoe dit proefschrift tot stand is gekomen en welke mensen daarbij belangrijk waren. Dit schema wordt in bedrijven gebruikt om allerlei organisatorische processen te beschrijven. Het leek mij wel een leuke manier om de invloeden en samenhang tussen de verschillende processen weer te geven.




Uitleg bij bovenstaand schema:

Het primaire proces bestaat uit tal van activiteiten, zoals experimenten, voordrachten, schrijven, vergaderen etc., die nodig waren voor het tot stand komen van de artikelen en het proefschrift (de Output). Voor dit proces was input nodig welke in dit geval het NKB project, materialen en menselijke inspanning waren. Het gehele primaire proces speelde zich af binnen de afdeling photodynamische therapie en stond onder invloed van de omgeving. Voor het goed verlopen van het proces waren instandhoudings processen en bestuurlijke processen nodig.

INPUT/OUTPUT

Het onderzoek was gebaseerd op een project geschreven door Willem Star en gehonoreerd door de Nederlandse Kanker Bestrijding. De duur van het project was totaal 4,5 jaar en in die tijd is er veel veranderd aan de hoeveelheid apparatuur dankzij de inspanningen van Willem. In het begin van het project waren de middelen beperkter dan aan het einde van het project. Dit is ook terug te vinden in de artikelen, vooral in de artikelen gepresenteerd in hoofdstuk 4 en 5 zijn modernere apparaten gebruikt dan in het artikel van hoofdstuk 2. Ondanks moderne middelen bleef het gelukkig noodzakelijk om je creativiteit te gebruiken tijdens de experimenten. De kleurenwaaier als referentiemateriaal is daar een goed voorbeeld van.
Het onderzoek was opgezet en uitgevoerd door mij en Riette. Riette ik wil je hierbij bedanken voor de goede samenwerking gedurende het project. Behalve kwaliteiten die nodig zijn als analiste zoals, nauwkeurigheid, zelfstandigheid, kunnen meedenken, flexibiliteit, heb je ook nog veel andere kwaliteiten die zeker net zo belangrijk waren voor dit project. Ik wil er even enkele noemen:
- Koken. Je zelfgemaakte hartige taarten smaakten altijd prima, zelfs al werden ze gegeten op gesmolten plastic bordjes.
- Kletsen . We hebben er wat af gekletst in die laatste 4 jaar. Onze gesprekken achter het donkere gordijn vond ik erg gezellig en zo waren de wachttijden tussen de fluorescentie opnamen snel voorbij. Ik hoop dat je bij je nieuwe collega's weer een kletspartner hebt gevonden.
- Verder ben je oprecht en spontaan.

Bedankt voor alles en ik hoop dat we nog lang contact met elkaar houden.

AFDELING PDT

In de 8,5 jaar die ik op de afdeling PDT heb rondgelopen zijn er verschillende collega's geweest, Jeanne, Eric, Hans, Otto, Hugo en Lars. Naast deze collega's hebben er ook vele studenten op de afdeling rondgelopen. Ik wil jullie allen bedanken voor de goede en gezellige werksfeer op de afdeling. Jullie waren altijd bereid mij te helpen met woorden en daden. Er zijn legio anekdotes te vertellen maar gelukkig zijn het er teveel om op te noemen. Mede dankzij jullie kijk ik met een goed gevoel terug naar die tijd op de afdeling PDT.

INSTANDHOUDINGSPROCESSN

Deze processen zorgen dat het primaire proces goed verloopt. De medewerkers van de afdelingen Medische Fotografie, Instrumentmakerij, Klinische Fysica, Bibliotheek, Magazijn en "Kantine" waren voor mij belangrijk voor het optimaal verlopen van het proces. Ik wil jullie bedanken voor onder andere de praatjes op de gang, het even kunnen binnenlopen voor advies, het even snel tussendoor kunnen doen, de lekkere koeken en de vriendelijkheid.

BESTUURLIJKE PROCESSEN

Mijn promotor Prof. Paul Wilson wil ik bedanken voor de begeleiding bij het tot stand komen van dit proefschrift. Mijn co-promotor Willem Star wil ik bedanken dat hij mij de mogelijkheid heeft geboden dit promotie onderzoek te doen. Willem, de hulp en ruimte die je mij gaf maakte het mogelijk me optimaal te ontplooien. Ik bewonder je nimmer aflatende optimisme en gedrevenheid die hebben gezorgd dat het PDT-lab in 11 jaar tijd is gegroeid tot een goed uitgerust lab waar mensen met verschillende disciplines succesvol samenwerken.

OMGEVING

De omgeving rond het primaire proces was solide en heeft gezorgd dat het proces zonder veel problemen verliep. Mijn ouders, broers, zus en hun partners hebben mij gestimuleerd en waren de basis waarop ik altijd kon terugvallen. En ondanks dat mijn moeder niet meer leeft weet ik dat ze nu hoog in de wolken trots tegen de andere mensen verteld wat ik heb gepresteerd.

In de loop der jaren is er veel veranderd. Toen ik begon op de Daniel was ik net van school af en nu ben ik weer aan de studie, woon samen, ben moeder, heb een huis, een konijn en een parkiet. Twee personen zijn de afgelopen jaren erg belangrijk voor mij geweest, mijn vriend Hans en mijn dochter Heleen. Hans, bedankt voor het maken van de omslag van dit proefschrift maar vooral bedankt voor je steun in de afgelopen jaren. Heleen, ik geloof niet dat je veel hebt gemerkt van dit proefschrift. Ik hoop dat je later dit proefschrift onder ogen krijgt en weet dan dat the sky the limit is.

Nynke van der Veen

## Curriculum vitae

Nynke van der Veen werd op 3 mei 1966 geboren te Deil. Na het behalen van haar HAVO diploma in 1984 volgde ze de studierichting zöologie aan de hogere laboratorium opleiding te Utrecht. Na het behalen van haar diploma in 1988 ging ze werken als analiste op de afdeling photodynamische therapie van de Dr Daniel den Hoed Kliniek. Van 1993 tot juni 1997 was ze werkzaam als aankomend wetenschappelijk onderzoeker op dezelfde afdeling. Deze werkzaamheden zijn de basis geweest voor dit proefschrift.

## List of Publications

**Van Leengoed HLLM**, Schuitmaker JJ, van der Veen N, Dubbelman, TMAR and Star WM (1993) Fluorescence and photodynamic effects of bacteriochlorin a observed *in vivo* in "sandwich" observation chambers. Br J Cancer, 67, 898-903.

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