# Stellingen

Behorende bij het proefschrift

## *Light Fractionated* ALA-PDT

1. De effectiviteit van ALA-PDT is significant beter wanneer het therapeutische licht niet in een keer maar in twee fracties gegeven wordt en de specifieke belichting parameters zijn hiervoor van cruciaal belang (*dit proefschrift*)

2. Hoewel de re-synthese van PpIX na PDT de aanleiding was om gefractioneerd te belichten na ALA toediening kan de verbeterde effectiviteit hierdoor niet worden verklaard (*dit proefschrift*)

3. Anders dan bij PDT met Photofrin® dragen neutrofielen niet bij aan de effectiviteit van de behandeling van ALA-PDT (*dit proefschrift*)

4. Een gefractioneerde belichting leidt niet tot een verbetering van de effectiviteit bij MAL-PDT (*dit proefschrift*)

5. De locale distributie van PpIX precursor en het cellulaire milieu spelen een rol bij de verhoogde effectiviteit van een gefractioneerde belichting (*dit proefschrift*)

6. Een zwangerschap en geboorte wordt als uniek ervaren wat opmerkelijk is bij een wereldbevolking van 6,6 miljard mensen

7. “Dollartekens in de ogen hebben” lijkt niet te getuigen van koersinzicht

8. Als alles onder controle lijkt ga je niet snel genoeg (Mario Andretti, ex-formule I coureur)

9. De stelling “willen = kunnen” gaat voorbij aan de bijdrage van talent

10. Wie met beide benen op de grond staat komt niet ver (Loesje)

11. Promoveren is het einde van het kleurpotloden tijdperk

H.S. de Bruijn
Zevenbergen 2008

# *Light Fractionated* ALA-PDT

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# *Light Fractionated* ALA-PDT

## ALA-PDT *met gefractioneerde belichting*

**Proefschrift**

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en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

*donderdag 12 juni 2008 om 11:00 uur*

door

Henriëtte Suzanna de Bruijn

geboren te Meppel

***Promotie commissie***

| | |
|---|---|
| Promotoren : | Prof.dr. P.C. Levendag |
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# Contents

# Chapter 1

## General Introduction

# Backgrond

The principle of photodynamic therapy (PDT) is based on the generation of reactive oxygen species, notably singlet oxygen, within cells and tissues. This is achieved by the administration of a photosensitiser, or a photosensitiser precursor, and subsequent illumination with (visible) light of an appropriate wavelength. The photosensitiser absorbs the energy of the photons and transfers it to molecular oxygen in the tissue. This photochemical reaction results in the formation of reactive oxygen species that cause damage to critical cellular and tissue structures. The characteristics of the photosensitiser determine their spatial distribution within cells and tissues which has a strong influence on the response to therapy. For this reason it is important to consider the study of specific photosensitisers and recognise the importance of their specific field of application. PDT has been used to treat various (pre-) malignant and non malignant conditions that range from skin cancer and psoriasis to age-related macular degeneration (AMD) and prostate cancer. In each case the specifics of the disease and photosensitiser are critical parameters for the successful application of the therapy.

***Historical development of Photodynamic Therapy***

PDT is now recognised as the treatment of choice for a small number of important diseases such as non-melanoma skin cancer and AMD. As described briefly below it is under investigation for numerous other conditions. The potential for PDT, based on the use of porphyrin photosensitisers was first recognised at the end of the 19$^{th}$ century [1-4]. Patients suffering from skin photosensitivity were found to excrete Haematoporphyrin (HP) via their urine [1]. Haematoporphyrin (Hp) is a complex mixture of different porphyrins and their aggregates. Lipson showed that photodetection of tumours was enhanced using a derivative of haematoporphyrin; HpD [5]. Clinical treatments using HpD were introduced in 1976 by Dougherty *et al.* [6]. Due to the complex nature of HpD, the results with HpD-PDT were variable and the active fraction was enriched to yield dihaematoporphyrin ether or Photofrin® (PII) [7]. At present PII-PDT is approved by the FDA for the ablation of precancerous lesions (high-grade dysplasia) in Barrett's oesophagus, tumours located in the bronchi and palliative treatment of advanced cancers of the oesophagus. The prolonged skin photosensitisation after administration of HpD and PII, which can last for several weeks, stimulated investigators to design other photosensitisers.

Since then many different photosensitisers have been studied and the most common known and (pre) clinically successful photosensitisers are BPD-MA (benzoporphyrin derivative monoacid ring A), mTHPC (meso-tetra-hydroxyphenyl-chlorin) and ALA (5-aminolevulinic acid) or other precursors of PpIX (protoporphyrin IX). BPD-MA is a vascular photosensitiser that is primarily used to treat abnormal blood vessels. BPD-MA PDT is approved by the FDA for the treatment of subfoveal choroidal neovascularisation (CNV) caused by age-related macular degeneration (AMD) [8], pathological myopia (a form of nearsightedness) and presumed ocular histoplasmosis (a fungal infection of the eye). Light

treatment is typically started shortly after administration of the photosensitiser. This process is repeated every three months for as long is needed to prevent regrowth of the abnormal vessels (usually 6 or 7 times over 2-3 years). The skin photosensitisation associated with this photosensitiser is much shorter compared to PII and patients should stay away from direct sunlight and bright indoor light for 5 days. mTHPC is an other photosensitiser that is highly lipophylic and very potent [9]. mTHPC mediated PDT is approved by the EU and the FDA for the palliative treatment of head and neck cancer and is under investigation for the treatment of various other diseases. In general, pharmacokinetic studies show high plasma levels in the first hours and a retained fraction in (malignant) tissues days after systemic administration. A drug light interval of 4 days is therefore not uncommon and patients are photosensitive for approximately 15 days. Different clinical trials are ongoing investigating the utility of novel photosensitisers like HPPH (2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a) and Npe6 (mono-L-aspartyl chlorine e6). Also the use of photosensitisers bound to carriers like monoclonal antibodies or tumour specific cell surface receptors for selective delivery to tumour is under investigation.

A different approach to administering a photosensitiser is to administer a precursor. The photosensitiser protoporphyrin IX (PpIX) is one of the intermediate products of the haem synthesis pathway that takes place in every cell containing mitochondria. Exogenous administration of 5-aminolevulinic acid (ALA) or methyl aminolevulinate (MAL) leads to the accumulation of PpIX as described in detail below. Kennedy *et al.* [10] were the first to recognise that ALA induced PpIX can be used for PDT to treat basal cell carcinomas (BCC). ALA can also be used for the diagnosis of cancerous lesions. At present ALA-PDT is in clinical trials worldwide to treat a variety of cancers and other disorders. In London a large phase III clinical trial is running investigating ALA-PDT for the treatment of Barrett's oesophagus. Studies are performed investigating the applicability of ALA-PDT for the treatment of bladder, brain and prostate tumours. The use of ALA derivatives or esterified ALA for PDT was first suggested by Kloek *et al.* [11] in 1996. At present methyl aminolevulinate (MAL) is clinically the most successful derivative of ALA and recently also hexyl aminolevulinate (HAL) has gained increasing interest [12]. MAL mediated PDT is indicated, in most European countries, for the treatment of nodular and superficial BCC. ALA-PDT is approved by the FDA to treat actinic keratosis and the treatment of choice for superficial non-melanoma skin cancer [13]. The optimisation of ALA-PDT and its mechanism of action are the subject of this thesis.

## Principles of PDT using porphyrin pre-cursors

***Photochemical reaction***

The cytotoxic effect of photodynamic therapy is the result of the photochemical reaction that is initiated by the absorption of light of the appropriate wavelength by the photosensitiser (Figure 1). As a result the photosensitiser is excited from the ground state to the excited state ($S_1$, $S_2$ etc). This state is unstable and will release the absorbed energy via

one of the two possible routes. It can decay back to its ground state by means of fluorescence emission. Or it undergoes intersystem crossover to its excited triplet state ($T_1$) and becomes photodynamically active. This triplet state is relatively long lived and hence exchanges the energy through collisions with molecules in its environment. Two specific types of reactions are described [14]. The type I reaction involves the collisions with substrate or solvents, forming radicals and radical ions, which after interaction with oxygen can produce oxygenated products. The type II reaction involves the collisions with oxygen, forming the highly reactive singlet oxygen. Although type I reactions can occur under certain conditions it is generally thought that the PDT induced damage is predominantly caused by type II reactions.

Three parameters are critically important for the formation of singlet oxygen; the photosensitiser, light and oxygen. Singlet oxygen is a highly reactive oxygen species (ROS) with a short lifetime (< 200 ns) and a short diffusion range [15]. For these reasons the primary target of PDT is determined by the distribution of the photosensitiser in cells and in tissues.

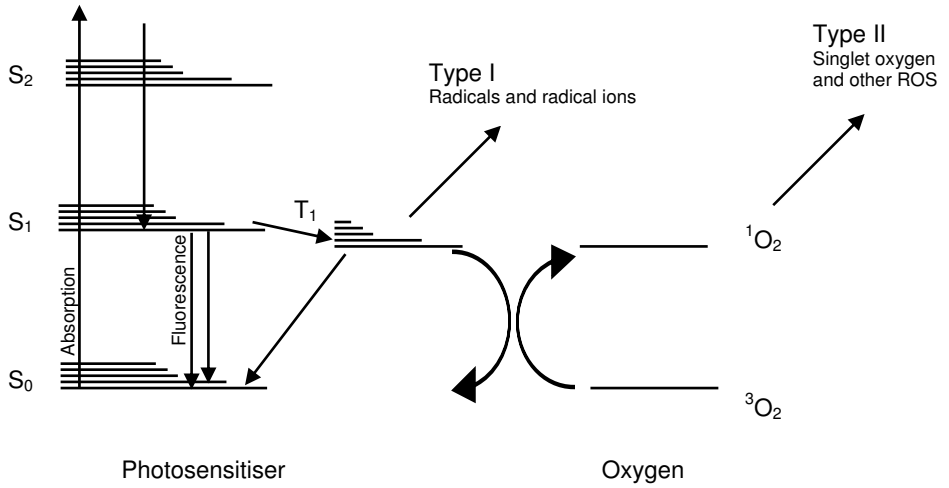


*Figure 1:* Energy level scheme of the photodynamic reaction.

***Haem synthesis and protoporphyrin IX***

As described above exogenous administration of 5-aminolevulinic acid (ALA) leads to the accumulation of the photosensitiser protoporphyrin IX (PpIX) which is the penultimate molecule of the haem synthesis pathway. Haem is synthesised from succinyl CoA and glycine via a series of enzymatic reactions that takes place in and around the mitochondria of almost every living cell (Figure 2). Under normal circumstances the accumulation of photosensitive intermediates like PpIX is avoided by two mechanisms involving the first reaction in this pathway. The condensation of glycine and succinyl CoA to form ALA is rate-limited and feedback controlled by haem.

Exogenous administration of ALA bypasses the feedback inhibition and overloads the cycle. The second step in the pathway is now rate-limiting but also the last step, the

chelation of iron to PpIX to form haem, is relatively slow. As a result of this several photosensitive porphyrins are temporary accumulated of which PpIX is the predominate one. This can also be achieved by the administration of ALA derivatives or esterified ALA like methyl aminolevulinate (MAL) or hexyl aminolevulinate (HAL). A different approach is the administration of iron chelators like desferrioxamine (DFO) and CP94 in combination with ALA [16].

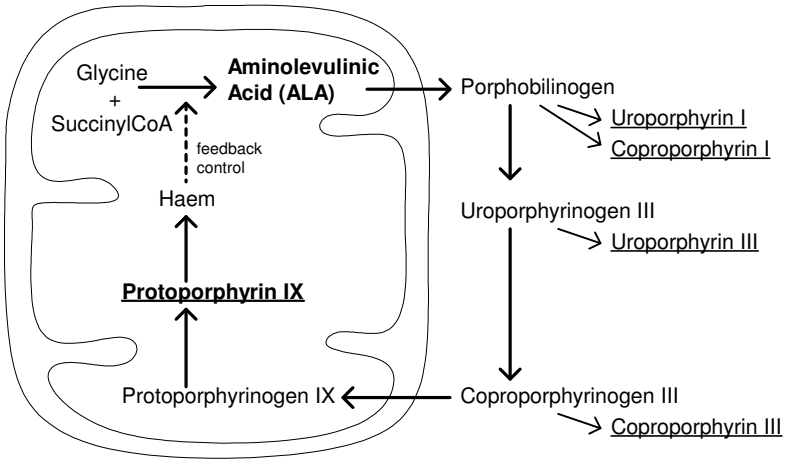


*Figure 2*: Schematic overview of the haem biosynthetic pathway based on Peng *et al.* [17]. Thick arrows indicate the principal biosynthetic route. The dashed arrow indicates the haem feedback control. The porphyrins are fluorescent compounds of which PpIX is the most potent.

***Administration routes and distribution in tissue***

ALA can be administered either systemically or topically depending on the host and target tissue. The most common administration route to treat human skin diseases is the topical application of ALA in a cream using 20% w/w ALA for 3-6 hours. Also in pre-clinical studies topical ALA administration is common using creams containing a dosage of 2 to 40% w/w ALA and application times ranging from 1 to 24 hours. MAL and other ALA-esters are mainly used for topical applications in creams using concentrations between 2 and 16%.

The penetration of a topically applied drug through skin dependents on its biochemical and biophysical characteristics. Also the vehicle in which it is dissolved and the condition of the skin is of influence and should be considered. ALA is highly hydrophylic and has a positive charge. To improve the penetration into the deeper regions of the skin lesions the use of penetration enhancers in the vehicle or prior to application of ALA has been investigated. Tape-stripping the stratum corneum has been shown to increase the ALA penetration through normal skin [18]. Ionthophoresis can be used to shorten the ALA application time. MAL and other ALA-esters are more lipophilic than ALA and this may enhance the cellular uptake. In-vitro studies have shown that cells accumulate more PpIX after ALA-ester compared to ALA administration. In vivo experiments show that MAL and ALA result in similar PpIX fluorescence intensities in the applied areas [19]. That study also showed a significant difference in the distribution of PpIX after topical application of MAL or

ALA. PpIX fluorescence is observed in areas remote from the application site after ALA and not after MAL application suggesting that ALA but not MAL is systemically distributed after topical application.

Systemic administration of ALA is only used when the target tissue can not be reached via topical application or intravesical instillation. As might be expected the toxicity of ALA is more important after systemic than after topical administration. In clinical PDT studies, treating oral cancer, Barrett's oesophagus or gastrointestinal cancer, ALA is dissolved in orange juice and administered orally using doses up to 60 mg $kg^{-1}$ body weight [20,21]. Adverse effects reported [21,22] for these doses include mild nausea, vomiting, transient abnormalities of liver function and decreased blood pressure or non-specific photosensitivity. Lower ALA doses like 5-20 mg $kg^{-1}$ body weight have been used for diagnostic purposes. In pre-clinical models also other systemic administration routes like intravenous and intraperitoneal injections have been investigated. Usually the ALA doses administered in pre-clinical studies are higher compared to clinical studies; a dose of 100-200 mg $kg^{-1}$ body weight is common.

***Distribution of PpIX in tissues and cells***

Although the haem pathway is present in all cells containing mitochondria some tissues accumulate more PpIX than others [23]. In general high accumulation of PpIX is found in tissues deriving from ecto- and endoderm like epidermis, oral mucosa, endometrium, urothelium or glands. In contrast tissues of mesodermal origin like muscle, connective tissue, cartilage and blood cells show low PpIX accumulation.

In vitro studies have shown that cells take up ALA via active transport using ß-amino acid and GABA carriers whereas MAL is taken up by passive transport [24]. Several subcellular localisation studies have shown that PpIX fluorescence is pre-dominantly observed in the mitochondia [25,26].

***Light / Illumination***

Light is one of the three critically important parameters for PDT. The distribution and penetration depth of light in tissue depends on the wavelength used and the optical properties of the tissue (Figure 3). These optical properties depend on the type of scatterers and absorbers and their spatial distribution in tissue. In general photons are scattered due to local change of refractive index or by small particles in tissue. Examples of scatterers in tissue are cell membranes or membrane aggregates, collagen fibres and nuclei. Typical absorbers in tissue are water, lipids and blood (haemoglobine, both oxy-and de-oxygenated).

The optimal wavelength to use for PDT or for monitoring PDT (see below) depends on the absorption characteristics of the photosensitiser, the tissue optical properties and the intended sampling or treatment depth. The absorption spectrum of PpIX shows a high peak in the blue region of the spectrum with a few smaller peaks between 500 and 635 nm. For PDT using PpIX either blue light is used to treat superficial conditions like actinic keratosis or

the deeper penetrating red light (610-640 nm) is used to treat superficial BCC and other skin lesions.

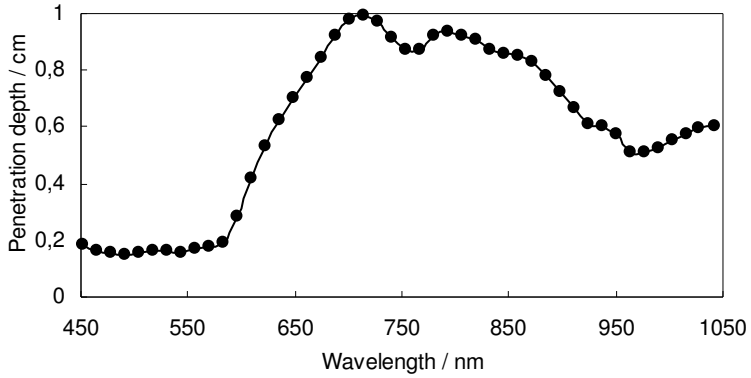


*Figure 3:* An estimate of the penetration depth of light in healthy human skin. Here penetration depth is defined as the depth that is reached by 37% of the light [27].

***PpIX Fluorescence***

The fluorescence emission spectrum of PpIX shows peaks around 635 and 705 nm that can be used for fluorescence detection. To collect the PpIX fluorescence from tissue the contribution of chromophores naturally present should be considered. This autofluorescence typically comes from proteins like collagen, flavins and NADH. Furthermore it has been shown that the derivatives of chlorophyll, pheophorbides, show an emission peak around 670-675 nm. In experimental models the contribution of pheophorbides to the fluorescence signal can be minimised by feeding the animals chlorophyll free food 2 weeks prior to the experiment. The specific contribution of these chromophores to the autofluorescence spectrum varies between tissue types and individuals. Figure 4 shows a typical example of an autofluorescence spectrum and a PpIX fluorescence spectrum collected from mouse skin using 514 nm excitation light.

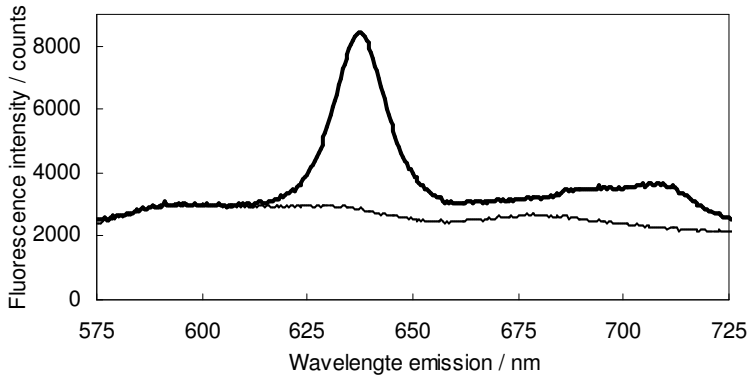


*Figure 4*: An example of a PpIX and autofluorescence spectrum collected from hairless mouse skin using 514 nm excitation light.

***Monitoring PDT induced damage***

In order to optimise therapy it is necessary to measure PDT response. PDT induced damage is usually scored after treatment using different methods depending on the model used. For in-vitro studies it could be the clonogenic assay or a histochemical assay in which a stain is used to determine the vitality of the nucleus (propidium iodide) or the mitochondria (MTT assay). In pre-clinical models there are a variety of different methods ranging from determination of the tumour growth delay or cure to visually observed necrosis of tissue to histologically scored damage in tissues.

In general the response to PDT can be highly variable. This is probably due to the complexity of the photodynamic action, which affects the availability of the three important parameters (light, oxygen and photosensitiser) and vice versa. Oxygen might be depleted locally as the demand for oxygen for the formation of singlet oxygen might be higher than the diffusion rate. The diffusion rate may be hampered by the vascular responses to PDT (see below). Vascular effects in themselves change the tissue optical properties resulting in a modified light distribution within tissue. And last but not least the availability of PpIX decreases as it undergoes self-sensitised photobleaching mediated by the production of the highly reactive singlet oxygen [28,29]. All these different reactions take place during PDT, are difficult to control or predict, and have a significant impact on the response to PDT. Several studies have shown a good correlation between the formation of singlet oxygen, PDT induced damage and PpIX photobleaching during PDT [30,31]. The intrinsic mechanism of photobleaching has been shown to be photosensitiser specific. PpIX undergoes self-sensitised photobleaching resulting in the formation of various photoproducts, in particular the chlorin photoprotoporphyrin. This photoproduct fluoresces around 675 nm and in turn is also photobleached. The influence of the variation in fluorescence intensity of the photoproduct is significant and should be considered while measuring PpIX fluorescence photobleaching rates. Also the changes in tissue optical properties and the possible photobleaching of autofluorescence should be considered.

***Techniques to measure PpIX fluorescence***

PpIX fluorescence can be determined non-invasively in superficial tissues like skin or oesophagus using techniques such as fluorescence imaging or spectral analysis. Fluorescence imaging is generally used to investigate the spatial distribution of PpIX within tissue. The fluorescence emission is collected using a small band pass filter around the emission peak. The contribution of autofluorescence can be accounted for by collecting an autofluorescence image prior to administration of the PpIX precursor and subtracting this from the subsequent fluorescence images.

Fluorescence spectral analysis is a different and more accurate technique to determine the PpIX fluorescence. Changes in tissue optical properties at the emission wavelengths can be corrected for by dividing the fluorescence emission spectrum by the reflectance spectrum [32]. The contribution of photoproducts and possible changes in autofluorescence can be accounted for by the use of single value decomposition (SVD) [33,34]. The basis

spectra of auto-, PpIX- and photoproduct fluorescence are used to fit their contribution to the measured fluorescence emission spectrum to determine the actual PpIX fluorescence intensity.

## Mechanism of action

As described above PDT induced damage is mainly the result of the formation of the highly reactive singlet oxygen that has a short lifetime and diffusion range. The tissue and cellular localisation of the photosensitiser therefore determines the primary target of PDT that is critically important for the mechanism of action. In the literature many different responses to PDT are reported that can be divided into three categories; cellular, vascular and immunological responses. The mechanism of action of tissues to PDT resulting in the overall response is a combination of these responses depending on the tissue oxygen availability, the photosensitiser and the illumination scheme used.

***Cellular response***

Cells either survive or die from the PDT induced damage and many different processes are involved in this. In general three modes of cell death after PDT are described in the literature; necrosis, apoptosis and recently also autophagy. Necrosis is a disorderly cell death that usually results from acute tissue injury. It involves cell swelling, chromatin digestion, disruption of plasma and organelle membranes, and cell lysis. The disruption of the plasma membrane may release harmful proteins and chemicals that damage neighbour cells and provokes an inflammatory response. Damage to the plasma membrane and lysosome membranes generally leads to necrosis. PDT using a photosensitiser that localises in these membranes will therefore result in a necrotic response after PDT.

Apoptosis is a type of programmed cell death that involves a series of biochemical events. It involves blebbing, shrinkage, nuclear fragmentation, chromatin condensation, and DNA fragmentation. In the final stage of apoptosis phagocytes remove the dying cells without eliciting an inflammatory response. Many pathways and signals can lead to apoptosis. Extra-cellular factors like hormones, growth factors or cytokines can initiate apoptosis. It could also be induced intra-cellularly in response to stress. Damage to the mitochondria may lead to the release of intra-cellular apoptotic signals in the cell. Endogenously accumulated PpIX is localised in the mitochondria and subsequent illumination causes mitochondrial damage. The whole apoptotic cell death process requires energy and a functional cell machinery but sometimes the overall damage caused by PDT is so severe that the cell can not complete the chain of reactions involved in apoptosis and it turns into a necrotic cell death.

The role of autophagy in PDT induced cell death or survival is relatively unknown. Autophagy is a process by which cells undergo partial autodigestion through the lysosomes in an attempt to prolong survival. Recently it has been considered as a secondary type of programmed cell death although there is no proof yet that cell death is really caused by

autophagy and not the result of an unsuccessful attempt to prevent it. PDT resulting in ER stress using CPO (a porphycyne photosensitiser) has been shown to induce both apoptosis and autophagy [35].

***Vascular response***

PDT induced damage has been shown to lead to different vascular responses like vasoconstriction or dilatation, adhesion of trombocytes and leucocytes and leakage of tissue fluid and macromolecules [36]. Changes in vessel diameter and platelet aggregation are generally responses that occur (early) during PDT and these responses may be reversible. Leakage of vessels causing the formation of oedema is generally observed immediately after PDT. These reactions could be a direct response to endothelial cell causing them to retract and expose the sub-endothelial matrix. Thrombocytes adhere to the matrix and become activated. They release vasoactive eicosanoids like thromboxane leading to (temporary) constriction of primarily arterioles [37,38]. Neo vessels and especially tumour vessels are usually more sensitive to PDT induced damage than normal vessels. This phenomenon is used in the approach to treat age related macular degeneration utilising a photosensitiser that localises in the vascular endothelium. It has been postulated that the vascular response, causing indirect damage by the deprivation of oxygen and nutrients, is necessary, in addition to the direct cellular damage, to achieve complete tumour destruction [39]. Temporary vascular occlusion and the subsequent re-perfusion also results in ischemia/re-perfusion injury (I/R injury). The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress. Vascular responses could, however, also lead to less effective PDT treatments. As mentioned before oxygen is one of the three crucially important parameters for PDT induced damage. Without the availability of oxygen, as a result of vascular damage, singlet oxygen can not be formed and PDT damage is not induced.

***Immune response***

Besides the cellular and vascular response PDT is also known to activate the immune system via various routes. Cellular necrosis involves the release of the intracellular content including cytokines that usually regulate the inflammatory and immunological responses. It is likely that neutrophils invade the treatment area to remove the necrotic cell debris. Secondary to that, the expression of pro-inflammatory cytokines like IL-6 and 10 was shown to be induced by (Photofrin-mediated) PDT [39]. Furthermore, as a result of the endothelial cell damage, inflammation cells are able to invade the tissue. In Photofrin mediated PDT it is shown that acute inflammatory cells adhere to the vessel walls within 5 minutes after the start of PDT. This results in a rapid and massive accumulation of neutrophils in the treated area [40]. Besides the acute inflammatory reaction also other immune effector cells like lymphocytes and monocytes/macrophages are recruited to the treated area. Recently it has been shown that pro-inflammatory mediators activate antigen presenting cells (APCs) that stimulate cytokine secretion and effector T-cell proliferation [41]. It is suggested that PDT and

the subsequent immunological reaction can be used for in situ vaccination inducing a systemic antitumor response [42]. Studies using pre-clinical animal models that are deprived of neutrophils show a decreased effectiveness of the PDT treatment. This indicates that neutrophils have a more active role than just phagocytosis of cell debris [43].

***Response to ALA-PDT***

ALA mediated PDT induces most of the responses mentioned above. Many in-vitro studies have reported apoptotic cell death after ALA-PDT although there are also studies that report necrotic cell death. The release of cytochrome-c into the cytoplasma in response to mitochondrial damage seems to be the first step, initiating the activation of the different caspase proteins that leads to apoptotic cell death [44]. In-vivo studies show a combination of apoptotic and necrotic cell death in tissue after ALA-PDT. Apoptosis is considered an early event while it occurs within the first hours after PDT preceding the appearance of necrosis. Vascular damage like vasoconstriction and vascular leakage is also observed using either topical or systemic administration of ALA [45,46]. Also I/R injury seems to play a role since the use of known inhibitors of I/R injury diminish the effect of ALA-PDT in the normal rat colon [47]. The role of neutrophils or the immune system in ALA-PDT is unknown.

## Optimisation of ALA-PDT

The initial clinical complete response rate of sBCC to ALA mediated PDT is high, complete response rates (CR) above 90% are reported. However the long term response is concerningly low: CR below 30% have been reported. Also the responses of nodular BCC or other lesions are not optimal. This prompted investigators to search for approaches to improve the response to ALA-PDT. The standard treatment involves the application of ALA for 4 hours and the subsequent light treatment. A number of factors limit the response to ALA-PDT. First of all the availability of ALA to cells in deeper regions of the skin lesions is limited by the penetration depth of topically applied ALA. The PpIX accumulation is further limited by the capacity of the haem synthesis pathway. The actual PDT response is limited by the availability of oxygen and the distribution of light.

Different methods have been used in an attempt to improve the response to PDT. As described above, the uptake of ALA and/or the accumulation of PpIX can be improved by the use of ionthophoresis, ALA derivatives, penetration enhancers or iron chelators. A different approach to improve the PDT response is to change the illumination parameters. The availability of oxygen for PDT can be increased by the use of a lower fluence rate for illumination as this lowers the demand for oxygen for the photodynamic action. Illumination with a lower fluence rate has shown to result in more PDT induced damage in normal hairless mouse skin [31]. Light fractionation using one or more short dark intervals may improve the response due to two mechanisms. The availability of oxygen could be increased since oxygen re-diffuses the treated area during the dark interval [48]. The second mechanism is the inflicted I/R injury caused by (repetitive) light-on/light-off intervals. This type of light

fractionation has shown to increase the PDT induced damage as determined by the size of the necrotic area in normal colon [49].

Light fractionation using a long dark interval between the two light fractions, i.e., a two-fold illumination scheme, is the approach described in this thesis. The design of this type of light fractionation was inspired by a clinical observation of Star [50] who noted the return of PpIX fluorescence in time after treatment in a lesion that showed complete photobleaching during treatment. This increase in PpIX fluorescence in time after PDT has also been reported by other investigators [51,52]. The rationale behind light fractionation using a dark interval of more than one hour was to also utilise this PpIX for PDT. Our first studies using this type of light fractionation were promising [46,53].

## Outline of the thesis:

Light fractionated ALA-PDT is the subject of this thesis. First the influence of the different illumination parameters on the response to PDT is investigated. Second the mechanism behind the increased effectiveness of light fractionated ALA-PDT is studied.

The first three chapters are focussed on the optimisation of light fractionated ALA-PDT. In **Chapter 2** the effect of different illumination schemes is investigated using the growth delay of the rat rhabdomyosarcoma after PDT. The following variables are studied; the influence of drug-light interval, low fluence rate illumination, short term light fractionation using dark intervals of only seconds or minutes and long term light fractionation using a dark interval of 75 minutes. Rhabdomyosarcoma is transplanted on the thigh of the rat and transdermally illuminated after intra-venous injection of ALA. The tumour volume is monitored daily after PDT to determine the delay in growth.

In earlier studies it was shown that light parameters like fluence and fluence rate have a large influence on the effectiveness of PDT. The influence of these parameters for light fractionated ALA-PDT was investigated in **Chapters 3** and **4**. For these studies the hairless mouse model was used and ALA was topically applied, while this is more representative of clinical ALA-PDT than using a solid tumour model and systemic ALA. The fluences of the first and second fraction were varied as well as the fluence rate and the duration of the dark interval. The PpIX fluorescence and photobleaching kinetics were measured before-, during and after PDT and the effectiveness of the treatment was determined by scoring the skin damage visually.

The mechanism behind the increased effectiveness of light fractionated ALA-PDT is the focus of the following chapters. Neutrophils are crucially important for the effectiveness of Photofrin-mediated PDT therefore their role in the response to ALA-PDT is investigated in **Chapter 5**. The rat rhabdomyosarcoma solid tumour model is used again while in this model both the increased effectiveness of light fractionated ALA-PDT and the role of neutrophils in

PII-PDT are shown before. The PpIX fluorescence kinetics pre and post PDT are also investigated in correlation with the delivered fluence.

The source of the increase in PpIX fluorescence observed in time after PDT is investigated in **Chapter 6**. The increase in PpIX fluorescence is either the result of re-distribution or local re-synthesis. In the skin-fold observation chamber the increase in PpIX fluorescence after PDT was determined as a function of the distance from the vasculature. In a separate group the temperature dependence of the increase in PpIX fluorescence after PDT was determined by cooling the tissue for one hour after PDT to 10-12°C, a temperature at which the accumulation of PpIX is inhibited. The increase in PpIX fluorescence after PDT followed by cooling is compared with that measured without cooling.

The effect of light fractionated PDT is studied using MAL in **Chaper 7**. This study is performed on the hairless mouse model using the most effective light fractionation scheme determined for ALA. The visual skin damage observed in time after PDT was compared with the results obtained earlier ALA. The PpIX fluorescence and photobleaching kinetics were monitored and compared after both topical MAL and ALA administration. The difference in response to MAL and ALA-PDT is investigated in **Chapter 8**. In this study the spatial distribution of PpIX fluorescence is investigated in normal mouse skin after 4 hours of topical application of either MAL or ALA using fluorescence microscopy. This is correlated with the PDT response histologically observed at 2.5, 24 and 48 hours after PDT.

The hypothesis that the increased effectiveness of light fractionated ALA-PDT is the result of a cellular mechanism in which the sub-lethally damaged cells are more vulnerable to a second light fraction is investigated in **Chapter 9**. In collaboration with the Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP) of the University of Buenos Aires in Argentina cell survival is investigated after a standard and a light fractionated treatment scheme in-vitro using different cell lines.

In the general discussion, **Chapter 10**, the results of these studies are discussed in the context of the current concepts in the literature and future perspectives are presented.

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# Chapter 2

## 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) in order to improve the response of nodular tumours to ALA-mediated photodynamic therapy (ALA-PDT). Tumours transplanted on the thigh of female WAG/Rij rats were transdermally illuminated with red light (633 nm) after systemic ALA administration (200 mg $kg^{-1}$). The effectiveness of each treatment scheme was determined from the tumour 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 tumour 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 minute interval new porphyrin is formed enhancing the effect of the second illumination.

## Introduction

Photodynamic therapy (PDT) using 5-aminolevulinic-acid (ALA) induced protoporphyrin IX (PpIX) as a photosensitiser is widely used as an experimental therapy, especially for cutaneous cancer. A complete initial response rate (CR) of more than 90% has been reported for treatment of human superficial basal cell carcinoma (BCC) with topically applied ALA-PDT [1-3]. However for nodular BCC a much lower CR, of 50% is obtained [2,4]. An explanation for this lower efficacy might be that topically applied ALA does not penetrate to the deep layers of tumour [5,6]. Oral or systemic administration of ALA may improve the biodistribution of PpIX [5,7]. However, also after systemic ALA-PDT only superficial necrosis was found in patients treated for dysplasia of the mouth [8] or the oesophagus [9]. 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 [10-12]. Another option is the use of light fractionation with either a short [10,11,13] or a long-term interval [14]. The short term light fractionation scheme (with one or more interruptions of seconds or minutes) may allow reoxygenation during the dark period. Theoretically, this will lead to more singlet oxygen formation [10]. 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 post treatment new PpIX is formed which can be used for a second illumination [14,15]. Van der Veen *et al.* [14] reported complete necrosis of 4 out of 6 tumours 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 in the present paper is to improve systemic ALA PDT of nodular tumours. We therefore studied the effectiveness of different illumination schemes published by our own group [12,14] and others [11,13] by measuring the tumour volume doubling time of a transplantable rat rhabdomyosarcoma after transdermal illumination.

## Materials and Methods

5-Aminolevulinic acid hydrochloride (ALA, 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 anaesthesia. 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 tumour (~ 1 $mm^3$) on the thigh of female WAG/Rij rats (12 - 13 weeks old). The tumour

growth was monitored daily by measuring the three orthogonal diameters using callipers and the tumour volume was estimated by the formula for an ellipsoid, $V=(\pi/6)*D1*D2*D3$. Tumours were randomly assigned to control and treatment groups when their volume reached 50 $mm^3$.

PDT was carried out under general anaesthesia using intra muscular Hypnorm, 0.5 ml $kg^{-1}$ (Janssen Pharmaceutica, Tilburg, The Netherlands) and diazepam, 2.5 ml $kg^{-1}$. Prior to the light treatment the skin overlying the tumour was shaved. The animals were placed on a temperature-controlled stage and covered with a black polythene mask. Tumours were transdermally illuminated with a 10 mm diameter plane parallel light beam (633 nm). Immediately after PDT the animals were housed under subdued light conditions at 28 °C for the first 24 hours. This was done to minimise the decrease in body temperature caused by the anaesthesia. 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 anaesthesia only (n=6), light only (100 J $cm^{-2}$ at 100 mW $cm^{-2}$; n=3) or ALA only (200 mg $kg^{-1}$ *i.v.*; n=3) respectively. Groups D to J, (n=6 in each), were treated according to different illumination schemes as shown in Figure 1. Each illumination was carried out at either 1 and/or at 2.5 hours post 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 tumour at 2.5 hours post injection. At one hour post ALA administration approximately one third of the maximal PpIX fluorescence was observed.

In group D and E the tumours 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 or 2.5 hours post ALA injection respectively. In the groups F to I the tumours were illuminated at 2.5 hours post injection of ALA. The tumours in group F received 100 J $cm^{-2}$ at 25 mW $cm^{-2}$ so that the treatment time

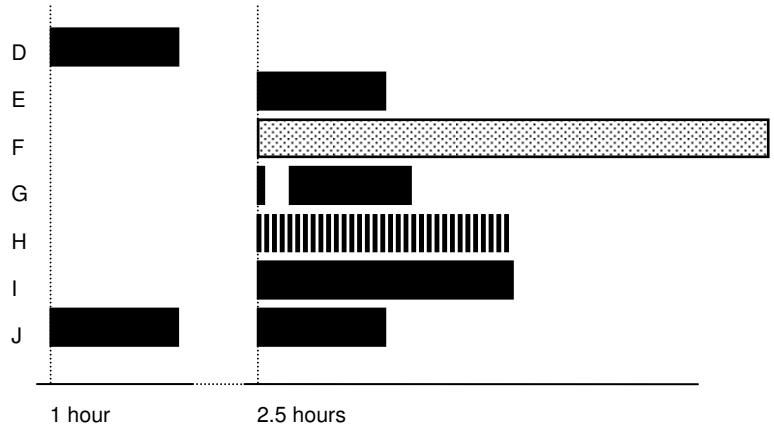


*Figure 1.* Schematic diagram of the treatment schemes studied.

was a factor of 4 longer than that of 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 $cm^{-2}$ [13]. In group H 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ was delivered with multiple interruptions, turning the light off and on every 30 seconds [11]. 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 hours 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 minutes (treatment at 1 and 2.5 hours p.i. of ALA [14]).

The light distribution within the tumours 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 centre of the illuminated area. The second probe was implanted between the base of the tumour and the underlying muscle at the centre of the illuminated area. Insertion of the isotropic probe through the skin was performed at a site 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 5 tumours 2.5 hours after administration of ALA (scheme E). These data were used to estimate the mean optical attenuation coefficient of the combination of tumour and overlying skin.

Tumour re-growth and macroscopic changes to the surrounding normal tissue were monitored every 1 or 2 days following therapy until the size of the tumour had reached 5 times its treatment volume. The treatment volume of each tumour (approximately 50 $mm^3$) was defined as 100% and the points in time (in days after treatment) at which the tumour reached certain fixed volumes; 50%, 200%, 500% etc, were linearly interpolated. The effectiveness of each treatment scheme was determined by comparison of the mean tumour volume doubling time of each group, defined as the number of days the tumour required to double its pre-treatment volume. The effect on the tumour growth post treatment was determined for each group (determined by the number of days the tumour 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 analysis of variance followed by a Student-Newman-Keuls test, as necessary. For all tests a *P* value of less than 0.05 was considered to be statistically significant.

## Results

### *Normal tissue response to PDT*

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

The oedema was investigated by measuring the thickness of the leg adjacent to the tumour daily. All animals treated with ALA-PDT showed a mild to severe oedema 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 post treatment the leg could measure up to be from 10.7 to 15.8 mm thick (Table 1). The oedema found for tumours treated at 1 hour post administration of ALA was significantly less compared to the other treatment schemes. The oedema found for tumours 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 tumour after treatment, which cleared within a few days. The involved area was as large as the illuminated tumour under the skin that is smaller than the illuminated area. In some treatment schemes severe discolouration was accompanied by crust formation (Table 1).

To investigate whether the oedema, discolouration and crust formation were influenced by the presence of an underlying tumour, a group of 4 animals without a tumour was illuminated according to the treatment scheme used in group J. The oedematous response was found to be the same for skin and muscle illuminated in the absence of tumour. The discolouration was found to be less marked being only pale blue for the group with no tumour compared to dark blue/black for the group with a tumour. The crust seemed to be macroscopically thinner and smaller in size and appeared only in 50% of the animals.

*Table 1.* Normal tissue damage caused by the different treatment schemes used.

| Group | Normal tissue damage | |
|---|---|---|
| | Oedema (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 oedema compared to the other groups

[b)] significantly more oedema compared to the other groups

To histologically determine the location of the oedema 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 oedema was found in the dermis and the muscle surrounding and underlying the tumour whereas the tumour showed little or no oedema. Enlarged blood vessels that were located around and at the border of the tumour were heavily damaged and there was evidence of haemorrhage.

***Tumour volume measurements***

The error associated with the tumour volume measurements was estimated by comparing the measurements of two independent observers for 14 tumours treated in this study in a range of tumour volumes. The relative error decreased from 5.3 ± 0.9% for tumour volumes below 30 mm³, to 3.7 ± 1.3% for tumour volumes ranging from 30 to 60 mm³, to 3.5 ± 0.7% for tumour volumes ranging from 60 to 120 mm³, to 2.3 ± 0.5% for tumour volumes ranging from 120 to 240 mm³.

***Tumour response to PDT***

There was no significant difference in treatment volume for the tumours in different groups and the mean treatment volume was measured to be 50.3 ± 1.4 mm³ (n=54). The tumour 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 tumour volume doubling time of 1.7 ± 0.1 days (n=12).

All of the PDT schemes investigated demonstrated a significantly longer tumour volume doubling time compared to control tumours, as shown in Figure 2. Tumours illuminated with a light fluence of 100 J $cm^{-2}$ at 100 mW $cm^{-2}$, 1 or 2.5 hours after ALA administration demonstrated a tumour 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 seconds, after the first 5 J $cm^{-2}$ of the total 100 J $cm^{-2}$ was delivered, showed a tumour volume doubling time of 7.5 ± 1.5 days. This was comparable to the tumour volume doubling time found for the other short term light fractionation scheme (30 seconds

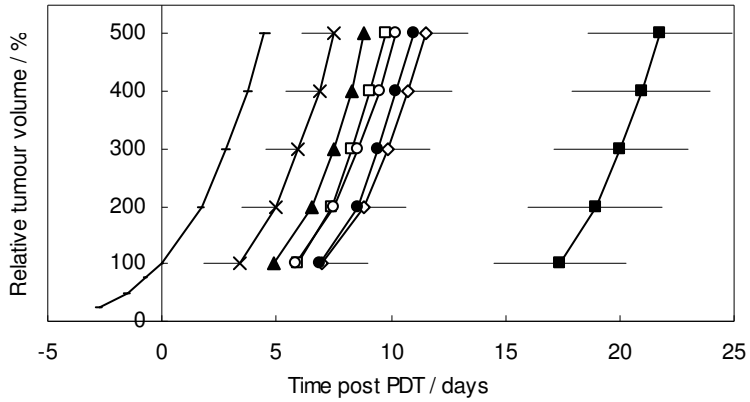


*Figure 2*. Relative tumour volume in time after ALA-PDT using different illumination schemes: control (-); scheme D: 1 hr 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ (×); scheme E: 2.5 hrs 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ (▲); scheme F: 2.5 hrs 100 J $cm^{-2}$ at 25 mW $cm^{-2}$ (◇); scheme G: 2.5 hrs 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ using a short term light fractionation scheme with one dark interval of 150 seconds after 5 J $cm^{-2}$ (□); scheme H: 2.5 hrs 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ using a short term light fractionation scheme: 30 sec on/ 30 sec off (○); scheme I: 2.5 hrs 200 J $cm^{-2}$ at 100 mW $cm^{-2}$ (●) and scheme J: both 1 and 2.5 hrs 100 and 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ (■). Data are shown as mean ± SEM.

light on/off, 7.5 ± 0.8 days). Although the mean tumour 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 tumour 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 tumour 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 hours p.i. of ALA, group J) showed a significantly increased tumour 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 tumour and only in group J three out of six tumours were not palpable for 10 to 13 days before the tumour was again detectable. No statistically significant difference could be shown in the tumour growth post treatment defined as the time a tumour required to grow from 200 to 500%. The tumour volume increased by a factor of 2.5 in 2.58 ± 0.06 days.

***Tumour thickness***

As might be expected since the illumination was superficial, the tumour response to PDT seemed to be correlated to the thickness of the treated tumour. After observation of the growth curves of the individual tumours in the groups there seemed to be a threshold for the thickness. Tumours thinner than 4 mm responded significantly better to treatment with a total fluence dose of 100 J $cm^{-2}$ compared to thick tumours. The mean tumour volume doubling time for thin tumours of groups D to H was 12.0 ± 2.2 days (n=5) compared to a mean tumour volume doubling time of 5.9 ± 2.2 days for thick tumours (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 tumours 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 tumour was 184.4 ± 14 mW $cm^{-2}$ (n=5) where the incident light fluence rate was 100 mW $cm^{-2}$. The fluence rate measured by the probe placed at depth between the tumour base and the underlying muscle was 42.3 ± 3.2 mW $cm^{-2}$ (n=5). Therefore the fluence rate at the base of the tumour was approximately 23% of the fluence rate measured at the top of the tumour. From these measurements a mean effective attenuation coefficient, $\mu_{eff}$, was calculated to be 3.5 ± 1.2 $cm^{-1}$.

## Discussion

In this study we have demonstrated a dramatic increase in tumour volume doubling time following systemic ALA PDT using a long-term light fractionation scheme (two light fractions separated by a dark interval of 75 minutes). In previous studies it has been shown that new PpIX is formed after complete photobleaching caused by the illumination [14,15]. This newly formed PpIX can be utilised during a second illumination. Van der Veen *et al.* [14] showed in a skinfold chamber model that a long term light fractionation scheme resulted in 4 out of 6 tumours with complete necrosis at day 7 post treatment 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 [14] compared to the single illumination (100 J $cm^{-2}$) which might be the explanation for the increased effect. However, when PpIX is completely photobleached, a longer illumination is not expected to be more effective. This is also demonstrated in the present study. Treating the tumour with a double light fluence (200 J $cm^{-2}$) did not significantly increase tumour 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 (Figure 1). In fact, this scheme increased the tumour volume doubling time by a factor of 2.6. The substantially improved tumour 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 oedema formation was not increased using a long-term light fractionation scheme compared to a single illumination of 100 J $cm^{-2}$. The discolouration was more pronounced compared to a single illumination and all the animals formed crust. From the histology it can be concluded that the discolouration was caused by haemorrhage of the blood vessels around and at the border of the tumour. This means that the discolouration and the accompanied crust formation caused by necrosis of epidermal, dermal and tumour tissue was actually a combined normal and tumour tissue response. The fact that we saw more crust after illumination with a long-term light fractionation scheme is then not surprising.

In contrast, ALA PDT using a low fluence rate or a short-term light fractionation scheme did not significantly improve the tumour 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 [10]. Several authors have shown that this can enhance the PDT response in a variety of animal models. Robinson *et al.* [12] 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.* [11] showed a 1.5 times longer volume doubling time for tumours illuminated with a 4 times lower fluence rate after systemic ALA administration. The volume doubling time was found to be further enhanced for tumours treated with a 30 seconds light on/off short term light fractionation scheme. Messmann *et al.*

obtained a greater area of necrosis of normal colon after illumination using several short-term light fractionation schemes [13]. Off course, it is difficult to compare these studies since the animal model used, the ALA doses and the illumination methods are all different. The fact that we could not show an improved tumour response using any of these schemes indicates that little or no extra tumour damage was obtained by the use of a low fluence rate or dark periods of several seconds or minutes for this tumour model. These results imply that improving the tumour response to ALA PDT is not simply a matter of interrupting the illumination for a few seconds or minutes and that tumour response may be different both for different sizes of tumour and for different tumour types.

Fan *et al.* [8] 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 tumour 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 $kg^{-1}$ *intra venously.*

In summary, no significant improved tumour response could be obtained using a low fluence rate or a short-term light fractionation scheme (dark interval of seconds or minutes) 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 a dark period of 75 minutes between two light treatments.

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# Chapter 3

## Fractionated illumination after topical application of 5-aminolevulinic acid on normal skin of hairless mice; the influence of the light parameters

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## Abstract

Light fractionation with dark periods of the order of hours has been shown to considerably increase the efficacy of 5-aminolevulinic acid-photodynamic therapy (ALA-PDT). Recent investigations have suggested that this increase may be due to the resynthesis of protoporphyrin IX (PpIX) during the dark period following the first illumination that is then utilised in the second light fraction. Light parameters are known to influence the response to PDT using single light fractions. In the present study we have investigated the kinetics of PpIX fluorescence and PDT-induced damage during PDT in the normal skin of the SKH1 HR hairless mouse using different light fractionation schemes. ALA was topically applied for 4 hour and PDT was performed using 514 nm light. The results show that the kinetics of PpIX fluorescence after a single light fraction, with light fluences of 5, 10 and 50 J $cm^{-2}$ is dependent on the fluence delivered; the resynthesis of PpIX is progressively inhibited following fluences above 10 J $cm^{-2}$.

All investigated light fractionation schemes show an increased skin response to ALA-PDT compared to a single illumination with the same cumulative fluence delivered 4 or 6 h after the application of ALA. The fluence and fluence rate of the two light fractions are crucially important for the efficacy of the treatment. The most optimal fractionation scheme involves a first fraction of 5 J $cm^{-2}$ delivered at 50 mW $cm^{-2}$ followed by a second light fraction of 95 J $cm^{-2}$ at 50 mW $cm^{-2}$.

The kinetics of PpIX fluorescence do not explain this significant increase in PDT response. Histological sections of the illuminated volume showed a trend toward increasing extent and depth of necrosis for the two-fold illumination scheme in which the first light fraction is 5 J $cm^{-2}$, compared with a single illumination scheme.

## Introduction

Photodynamic therapy (PDT) using topically applied 5-aminolevulinic acid (ALA) is an emerging treatment modality for a number of (pre-) malignant conditions. ALA is converted in situ via the haem biosynthetic pathway into the photosensitiser protoporphyrin IX (PpIX). An excess of exogenous ALA can lead to the accumulation of therapeutic levels of PpIX in various tissues. To date ALA-PDT has been primarily used for the treatment of nonmelanoma skin lesions, such as actinic keratoses (AK), basal cell carcinoma (BCC) and squamous cell carcinoma. It has recently received approval in the United States for the treatment of AK of the face or scalp. Since its introduction by Kennedy *et al.* [1] a number of studies have reported complete initial response rates +/- 85%, for superficial BCC [2,3]. However, for nodular BCC, a much lower complete response rate of 50% is obtained [2-4]. Improvement of PDT efficacy, particularly in nodular tumours, is necessary. Various options have been investigated, which include the use of penetration enhancers, iron chelators, varying the duration of ALA application and modifying the illumination scheme [5-8]. The illumination can be modified in a number of ways: reducing the fluence rate has been shown to improve efficacy by reducing the demand for oxygen during illumination and increasing the total amount of singlet oxygen produced [9-11]. Introducing short-term fractionation (with one or more interruptions of seconds or minutes) allows reoxygenation during the dark intervals and has essentially the same effect as reducing the fluence rate [10].

We have recently reported another type of light fractionation that leads to increased PDT efficacy. Long-term light fractionation, in which two light fractions are delivered, separated by an interval of1 h or longer, has been shown to enhance PDT response in two different model systems [12-14]. We showed that PpIX fluorescence, that had been photobleached during illumination, reappeared in the hours immediately after illumination and postulated that the increase in PDT efficacy was due to the utilisation of this additional PpIX during the second illumination. The optimum two-fold illumination scheme will be determined by the time interval between illuminations and the ‘dose’ delivered during each illumination. With these three parameters it is easy to design numerous complex treatment and control treatment schemes. In the first part of the study we extend our previous findings by (1) investigating, in detail, the kinetics of PpIX fluorescence after illumination; and (2) determining the relationship between the illumination parameters (fluence and fluence rate), the photobleaching of PpIX during illumination, and the PDT effect of two-fold illumination with a 2 h dark interval. The results showed that the dose delivered in the first fraction is an important parameter that determines, at least in part, the response of tissue to a two-fold illumination. We continued this study with a second part in which we investigated the relationship between (3) the fluence delivered during and (4) the timing of the first fraction of a two-fold illumination scheme, the PDT response and the kinetics of PpIX fluorescence during treatment with a cumulative fluence of 100 J $cm^{-2}$ and a 2 h dark interval.

## Materials and Methods

*Animal model*. The experimental protocol was approved by the ‘‘Committee on Animal Research’’ of the Erasmus University Rotterdam. Female inbred albino hairless mice (SKH1 HR, Charles River, Someren, The Netherlands), aged between 8 and 10 weeks, are included in this study. Prior to treatment animals were fed on a diet free of chlorophyll (Hope Farms B.V., Woerden, The Netherlands) for a minimum of 2 weeks in order to remove the autofluorescence emission from mouse skin centered on 675 nm [15] attributed to pheophorbide-*a* a breakdown product of chlorophyll [16]. This fluorescence emission overlaps with those of PpIX and its fluorescent photoproducts, and pheophorbide-*a* is itself a photosensitiser.

*ALA application*. ALA (20%) (Medac, Hamburg, Germany) was dissolved in 3% carboxymethylcellulose in water. To prevent skin irritation each solution was prepared to approximately pH 4 by the addition of NaOH (2 M). ALA was applied topically to a 7 mm diameter area (the same diameter as that illuminated during treatment) on the dorsal skin of each animal and covered with a thin layer of gauze; a polythene dressing (Tegaderm, 3M, The Netherlands) was used to occlude the area for 4 h prior to treatment. Before the application of drug animals received low-dose analgesia (Hypnorm; fluanisol/fentanyl mixture, Janssen Pharmaceutics, BE and 0.05 mL Diazepam, Centrafarm B.V., Etten-Leur, The Netherlands) to alleviate possible anxiety caused by the dressing.

*PDT light delivery and fluorescence/reflectance spectroscopy*. PDT light delivery and fluorescence spectroscopy were performed as described previously [15]. The 514 nm output from an argon ion laser is delivered *via* a 400 μm fibre and imaged to a 7 mm diameter spot of homogeneous profile on the skin of the mouse using a microlens (QLT, Vancouver, BC, Canada). Scattered excitation light and fluorescence emission (550–792 nm) are collected from the whole of the illuminated area using and focused into either a 1 mm core optical fibre or 400 μm optical fibre coupled to a spectrograph (Acton Research, Acton, MA) with a charge-coupled device (CCD) camera (Princeton Instruments Inc., Princeton, NJ) or a 400 μm optical fibre coupled to a fibre optic spectrometer (Ocean Optics, Eerbeek, Netherlands). A long pass filter, OG 570 (Melles Griot, Zevenaar, The Netherlands) is placed in the optical path to block scattered 514 nm excitation light. In addition, immediately prior to treatment and at regular intervals during illumination the 514 nm PDT illumination is interrupted for a short period of time to allow a reflectance spectrum to be acquired using the same spectrograph. The output from a filtered halogen-lamp, (0.15 mW $cm^{-2}$, Stortz, Tutlingen, Germany), delivered by a second 400 μm fibre and microlens is imaged onto the 7 mm diameter treatment spot. A long pass filter (OG 530, Melles Griot) is used to minimise fluorescence excitation of the tissue during reflection measurements. Using this setup both fluorescence and reflectance measurements are acquired using the same source detector geometry. The kinetics of PpIX fluorescence prior to and following PDT were determined using the same excitation and detection system, except that low-intensity excitation (0.1 mW $cm^{-2}$) was used in order to minimise photobleaching. A fluorescence image was also acquired prior to each period of illumination using a CCD camera with double-stage image

intensifier (ADIMEC, Eindhoven, The Netherlands or Lambert Instruments, Leutingwolde, Germany) to locate the area of interrogation and maintain a constant distance between the mouse skin and the head of the spectrometer. During illumination each mouse is placed on a temperature-controlled stage and anesthetised with a combination of 2% Ethrane (Abbott, Amstelveen, The Netherlands) oxygen and $N_2O$. Fluorescence emission spectra (550–792 nm) are acquired at intervals of 5 s during 514 nm illumination using an integration time of 0.5 s or 1.5 s. Reflectance spectra were either acquired before and after illumination or every 30 s using an integration time of 0.5 s. During this procedure the PDT illumination is interrupted for 3 s. In total the PDT illumination is interrupted for 10% of the duration of the treatment.

*Data analysis*. The spectral analysis of data acquired during illumination performed in this study is substantially different from that used in our two previously published studies involving PpIX photobleaching [15,17]. This change is in light of the recent work of Foster and his co-workers [18-21]. During illumination of a highly scattering medium such as tissue, changes in the measured fluorescence may be due to changes in the actual fluorescence intensity of the medium and/or to changes in its optical properties.

To correct for such changes in tissue optical properties during illumination we use a method introduced by Wu *et al.* [22], in which the measured fluorescence emission is divided by the reflectance signal over the same range of wavelengths that the fluorescence is acquired. Since the source-detector geometry described above is identical for both fluorescence and reflectance measurements, the optical properties encountered by fluorescence and reflectance light are the same. Thus dividing the fluorescence emission by the reflectance corrects changes in optical properties of the tissue at the emission wavelengths. Since we have not acquired a reflectance spectrum for each of the fluorescence spectra obtained during illumination we have corrected all the fluorescence spectra acquired during each illumination period with a second-order polynomial interpolation of the reflectance spectra acquired at regular intervals during that illumination. In case the reflectance spectrum was acquired immediately before and after each light fraction, we have corrected all the fluorescence spectra acquired during each illumination period with a linear interpolation of these reflectance spectra.

The fluorescence emission spectra, corrected for tissue optical properties, are analyzed as a linear combination of basis fluorescence spectra [18,19] using single value decomposition (SVD) algorithm. The three basis fluorescence spectra used in this analysis are the autofluorescence of normal mouse skin, PpIX and the hydroxyaldehyde chlorin photoproduct of PpIX. We also investigated the use of other basis fluorescence spectra, in particular that of a blueshifted water-soluble porphyrin with a peak emission in the wavelength range 600–620 nm. In all cases however, this resulted in a reduction in the goodness-of-fit and we were unable to find evidence for any such an emission under the illumination conditions investigated.

An average autofluorescence spectrum was determined from the average of 20 autofluorescence spectra from 20 animals, before the application of ALA. Similarly an average PpIX fluorescence spectrum was determined from the average of 20 fluorescence

spectra acquired from 20 animals, 4 h after the application of ALA. The PpIX basis spectrum was determined by subtracting the average autofluorescence spectrum described above.

In keeping with the analysis of Finlay and Foster [18] we accounted for differences in the autofluorescence between animals by constructing an individual autofluorescence basis spectrum for each animal. The initial spectrum acquired during each illumination was fit using an SVD as a combination of the average autofluorescence, the PpIX basis spectrum and a 61-term Fourier series. The individual autofluorescence basis spectrum is therefore a sum of fitted average autofluorescence and the Fourier series.

The PpIX-induced photoproduct basis spectrum was constructed from the average of 10 spectra acquired from 10 animals that demonstrated sufficient photoproduct fluorescence, *i.e.* had received between 5 and 10 J $cm^{-2}$ of 514 nm illumination at 50 mW $cm^{-2}$. Each spectrum was then fitted using an SVD as a combination of PpIX, average autofluorescence, the individual Fourier series and a single lorentzian. The average of these lorentzian fits, centered on 674 nm with a width of 28 nm full width half maximum was used as the PpIX photoproduct fluorescence spectrum. The three basis spectra, with equal weighting, are now used to fit the contribution of PpIX and its fluorescent photoproducts in all of the spectra acquired in this study.

*PDT illumination schemes*. The normal kinetics of PpIX fluorescence were measured in a control group of five animals for 8 h after the application of ALA. The kinetics of PpIX fluorescence were also measured following illumination with 5, 10 and 50 J $cm^{-2}$, 4 h after the application of ALA for 4 h (n = 5 in each group). The results from these data were used to design the next set of experiments in which we monitored the kinetics of porphyrin fluorescence during illumination and the biological damage induced in the illuminated area following PDT. This was done in a series of six different single and two-fold illumination schemes, with n = 5 animals in each: (1) a single illumination of 100 J $cm^{-2}$ delivered at 50 mW $cm^{-2}$, 4 h after the application of ALA; (2) a single illumination of 100 J $cm^{-2}$ delivered at 50 mW $cm^{-2}$, 6 h after the application of ALA; (3) a two-fold illumination of 50 J $cm^{-2}$ at 50 mW $cm^{-2}$ delivered at 4 and 6 h after the application of ALA (cumulative fluence 100 J $cm^{-2}$: 2h interval between illuminations); (4) a two-fold illumination of 5 J $cm^{-2}$ at 50 mW $cm^{-2}$, 4 h after the application of ALA and 95 J $cm^{-2}$ at 50 mW $cm^{-2}$, 6 h after the application of ALA (cumulative fluence 100 J $cm^{-2}$: 2 h interval between illuminations); (5) a two-fold illumination scheme in which we investigated the effect of reducing the fluence rate of the first light fraction to 5 mW $cm^{-2}$; 5 J $cm^{-2}$ at 5 mW $cm^{-2}$ 4 h after the application of ALA and 95 J $cm^{-2}$ at 50 mW $cm^{-2}$, 6 h after the application of ALA (cumulative fluence 100 J $cm^{-2}$: 2 h interval between illuminations); and (6) a two-fold illumination scheme in which we investigated the effect of reducing the cumulative fluence by reducing the length of the second illumination; 5 J $cm^{-2}$ at 50 mW $cm^{-2}$, 4 h after the application of ALA and 45 J $cm^{-2}$ at 50 mW $cm^{-2}$, 6 h after the application of ALA (cumulative fluence 50 J $cm^{-2}$: 2 h interval between illuminations). Based on these results the study was extended to determine the influence of the illumination parameters of the first light fraction in an extra series of 4 different two-fold illumination schemes with n=12 in each group unless stated differently: (7) a two-fold illumination of 1 J $cm^{-2}$ delivered 4 h after the application of ALA and 99 J $cm^{-2}$ 6 h after the application of ALA;

(8) a two-fold illumination of 2.5 J $cm^{-2}$ delivered 4 h after the application of ALA and 97.5 J $cm^{-2}$ 6 h after the application of ALA; (9) a two-fold illumination of 5 J $cm^{-2}$ delivered 4 h after the application of ALA and 95 J $cm^{-2}$ 6 h after the application of ALA; and (10) a two-fold illumination scheme in which we investigated the effect that shortens the time of ALA application before the first illumination while maintaining the dose delivered during the first illumination; 10 J $cm^{-2}$ delivered 2 h after the application of ALA and 90 J $cm^{-2}$ delivered 4 h after the application of ALA (n=6).

*PDT damage*. Biological damage to the irradiated area was assessed daily using a visual skin scoring system [17] by two independent observers (D.J.R. and H.S.B.) blinded from the treatments. Photographs were also taken daily in order to determine the degree and distribution of damage. Grade 1 represents minimal redness, grades 2, 3, 4 and 5 represent, redness, severe redness, thin and thick scab formation, respectively. Mean damage scores were calculated by scoring areas according to the degree of damage and the contribution to the total illuminated area. The scores from each treatment site were used to calculate a mean skin score for each group. The total PDT damage in a single treatment was quantified by integrating the mean skin score when plotted against time. The formation of scar tissue was not included in the visual skin scoring system. The statistical significance of differences in PDT damage was determined using an analysis of variance followed by a Student–Newman–Keuls test as necessary, use of the word significant corresponds to a $P$ value $<0.05$. The histological damage was also determined 48 h after therapy in each of the illumination schemes investigated. The depth of damage was quantified by dividing the skin into three layers: the epidermis, the upper or papillary dermis and the deep dermis. The extent of necrosis, either partial or complete, was assessed with in each layer.

## Results

***PpIX fluorescence kinetics after a single light fraction.***

The kinetics of PpIX fluorescence in normal hairless mouse skin after topical application of ALA is shown in Figure 1. PpIX fluorescence increases during the first 4 h of application. After the removal of excess ALA at 4 h, the fluorescence intensity reaches a maximum and does not change significantly over the time course investigated in this study. Illumination with 50 mW $cm^{-2}$, 514 nm radiation 4 h after the application of ALA results in significant photobleaching of PpIX. The extent of this photobleaching is dependent on the fluence delivered during illumination, and increases as the fluence is increased from 5 to 10 J $cm^{-2}$ and again when the fluence is increased to 50 J $cm^{-2}$. The increase in PpIX fluorescence in the 4 h after illumination is also dependent on the fluence delivered during illumination. The amount of PpIX fluorescence 2 and 4 h after illumination decreases significantly as the fluence is increased from 5 to 10 and to 50 J $cm^{-2}$. In the 4 h following illumination, PpIX fluorescence does not return to the pre-treatment intensity or to the intensity that would be expected in the absence of illumination. PpIX fluorescence reaches a maximum intensity between 1 and 4 h after illumination, depending on the fluence delivered. The rate of PpIX

resynthesis following each illumination is less than that immediately after the initial application of ALA. It is also less than that at the corresponding PpIX fluorescence intensity in the normal kinetics of PpIX fluorescence before illumination.

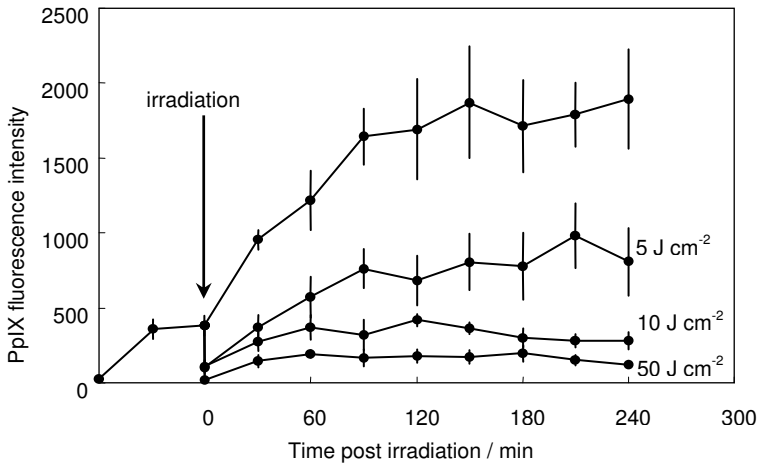


*Figure 1.* PpIX fluorescence kinetics following topical application of ALA, and following illumination 4 h after the application of ALA with 5, 10 and 50 J $cm^{-2}$ at a fluence rate of 50 mW $cm^{-2}$. Note: kinetics do not include time elapsed during illumination.

***PpIX fluorescence photobleaching during illumination***

Figure 2 shows the typical mean normalised PpIX fluorescence intensity during a two-fold illumination. A first illumination of 50 J $cm^{-2}$ is delivered at 50 mW $cm^{-2}$, 4 h after the application of ALA; PpIX is rapidly photobleached during illumination. A dark interval of 2 h results in an increase in PpIX fluorescence (approximately 25% of that present prior to PDT). This PpIX fluorescence is then photobleached during the second illumination, performed 6 h after the start of ALA application.

Figure 3a shows the mean normalised variation in PpIX fluorescence intensity during a single illumination at 50 mW $cm^{-2}$, 4 or 6 h after the start of ALA application (note: the full range of fluence is not shown for clarity). There is no significant difference between the mean rate of photobleaching at 4 or 6 h after ALA application. Figure 3b shows the corresponding normalised variation in PpIX fluorescence during a two-fold illumination at the same fluence rate. The rate of photobleaching of PpIX during the second illumination is significantly less than both that during the first illumination and at the same time point (6 h) in the absence of a first illumination (Figure 3a).

Figure 4 shows the mean normalised PpIX fluorescence intensity during three two-fold illumination schemes that are different with respect to the fluence and/or fluence rate of the first light fraction. In each case the fluorescence intensity during the first and second illumination is plotted in the same panel and normalised to the initial fluorescence intensity before the first illumination. The relative fluorescence intensities immediately before each illumination are therefore on the left edge of each plot. Figure 4a shows the fluorescence

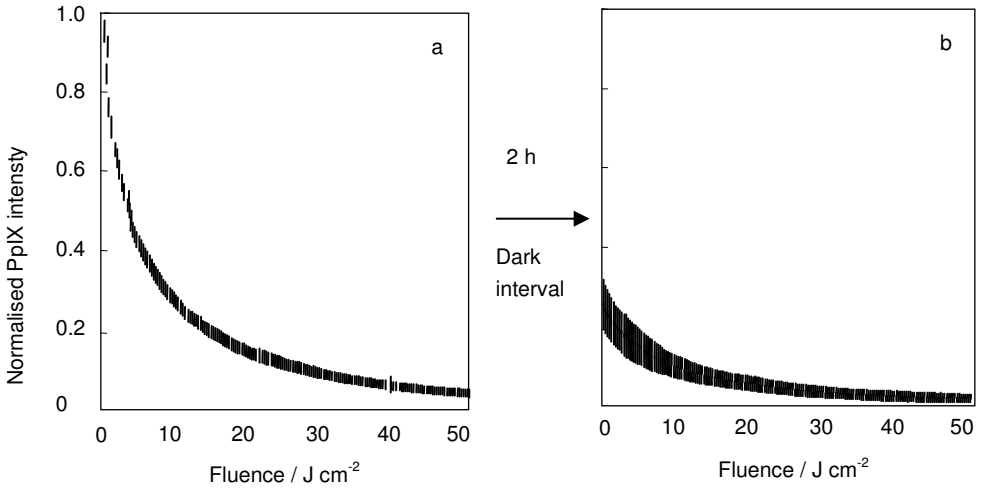


*Figure 2*. Mean normalised PpIX fluorescence intensity during twofold illumination with 514 nm radiation of normal mouse skin (a) 4 h and again at (b) 6 h after the application of ALA with 50 J $cm^{-2}$ delivered in each light fraction. There is a dark interval of 2 h between illuminations. In each case the fluorescence intensity is normalised to that at the start of the first illumination.

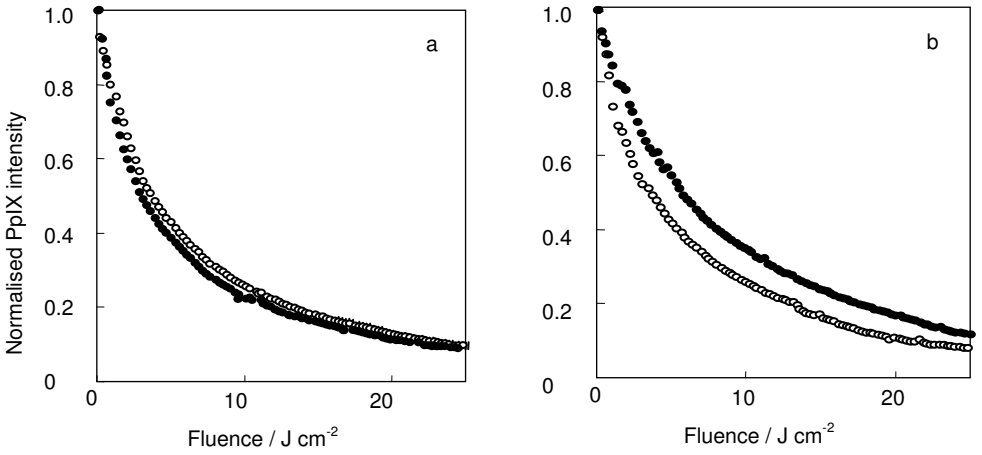


*Figure 3*. (a) Mean normalised PpIX fluorescence intensity during single illumination with 514 nm radiation at 50 mW $cm^{-2}$, (●) 4 h or (○) 6 h after the application of topical ALA; and (b) mean normalised PpIX fluorescence intensity during a two-fold illumination with 514 nm radiation (●) 4 h and again (○) 6 h after the topical application of ALA, with a dark interval of 2 h between illuminations.

intensity during a two-fold illumination where 50+50 J $cm^{-2}$ is delivered with a 2 h dark interval. The mean fluorescence intensity before the second illumination is approximately 25% of that before the first. Figure 4b shows the fluorescence intensity when two illuminations of 5+95 J $cm^{-2}$ are delivered. The mean fluorescence intensity before the second illumination is now significantly greater, approximately 75% of that before the first illumination. Similarly Figure 4c shows the fluorescence intensity during a two-fold illumination; 5+95 J $cm^{-2}$, except that the first illumination is delivered at 5 mW $cm^{-2}$. The rate of photobleaching during the first illumination at 5 mW $cm^{-2}$ is significantly greater than that during illumination at 50 mW $cm^{-2}$ (Figure 4b, open symbols). The mean fluorescence intensity before the second illumination is significantly less than that following 5 J $cm^{-2}$ at 50 mW $cm^{-2}$ (Figure 4b, closed symbols); approximately 35% of that before the first illumination.

Two hours after the application of ALA, the PpIX fluorescence is approximately 50% of this maximum intensity as shown in Figure 5. Illumination with 50 mW $cm^{-2}$ to a fluence of 10 J $cm^{-2}$ results in significant photobleaching of PpIX. Immediately after this illumination, approximately 18% of the PpIX fluorescence remains. In the 2h interval between light fractions, PpIX fluorescence returns. The intensity of PpIX fluorescence immediately before the second light fraction, 4h after the application of ALA, is greater than that before the first light fraction but is significantly less than that would have been present in the absence of the first fraction. During the second light fraction, PpIX fluorescence again decreases rapidly, and after the end of the illumination (90 J $cm^{-2}$) it is not significantly different from the background fluorescence intensity.

***PpIX photoproduct fluorescence during illumination***

Compared to the PpIX fluorescence intensity the mean photoproduct fluorescence intensity is maximal less than 1%. The photoproduct fluorescence kinetics during PDT using the 50+50 J $cm^{-2}$ and the 5+95 J $cm^{-2}$ illumination schemes show distinct differences. Photoproduct fluorescence increases rapidly during the first illumination of the 50+50 J $cm^{-2}$ scheme, reaches a maximum between 10 and 20 J $cm^{-2}$, and is subsequently photobleached during illumination. At the end of the first illumination a significant level of photoproduct fluorescence is present. After the 2 h dark interval, at the start of the second illumination, there is still photoproduct fluorescence that is not significantly different from that present at the end of the first illumination. During the second illumination photoproduct fluorescence increases again and reaches a maximum between 5 and 10 J $cm^{-2}$, and photobleaches during the course of the second illumination. At the end of the second illumination a significant level of photoproduct fluorescence is still present.

In the 5+95 J $cm^{-2}$ scheme the first illumination ceases before the photoproduct fluorescence reaches a maximum intensity and a significant level of photoproduct fluorescence is present at the end of the first illumination. After the 2 h dark interval, at the start of the second illumination, there is still photoproduct fluorescence present that is not significantly different from that at the end of the first illumination. During the second illumination photoproduct fluorescence increases and reaches a maximum after between 10 and 20 J $cm^{-2}$. The increase in photoproduct fluorescence during the second illumination is

greater after a first illumination of 5 J cm$^{-2}$ than that observed following a first illumination of 50 J cm$^{-2}$. The photoproduct again undergoes photobleaching during the second illumination. At the end of the second illumination a significant level of photoproduct fluorescence is again present.

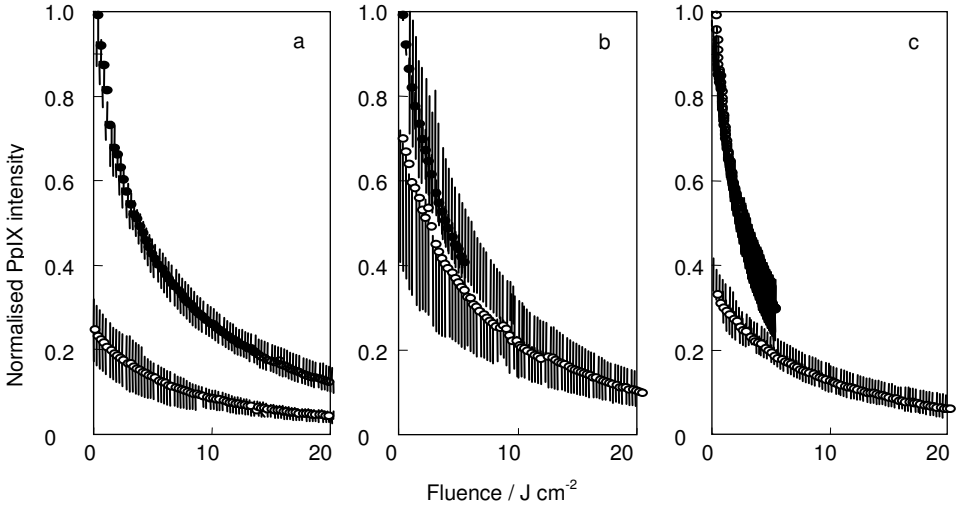


*Figure 4.* Mean normalised PpIX fluorescence intensity during three two-fold illumination schemes with illumination at 4 h (●) and 6 h (○) after the application of ALA; (a) 50 J cm$^{-2}$ + 50 J cm$^{-2}$ at 50 mW cm$^{-2}$ (b) 5 J cm$^{-2}$ + 95 J cm$^{-2}$ at 50 mW cm$^{-2}$ and (c) 5 J cm$^{-2}$ at 5 mW cm$^{-2}$ + 95 J cm$^{-2}$ at 50 mW cm$^{-2}$. In each panel all PpIX fluorescence intensities are normalised to the initial PpIX fluorescence intensity prior to the first illumination.

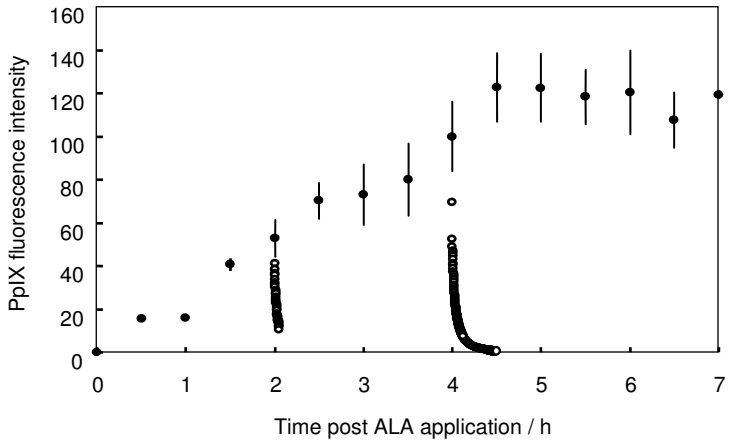


*Figure 5.* PpIX fluorescence kinetics (n = 6) following topical application of ALA to normal hairless mouse skin (●) and PpIX fluorescence kinetics during a two-fold illumination scheme with a 2 h dark interval using 514 nm (○). A first fraction of 10 J cm$^{-2}$ is delivered 2 h after the application of ALA and a second fraction of 90 J cm$^{-2}$ 2 h later (4 h after the application of ALA). A cumulative fluence of 100 J cm$^{-2}$ is delivered at 50 mW cm$^{-2}$.

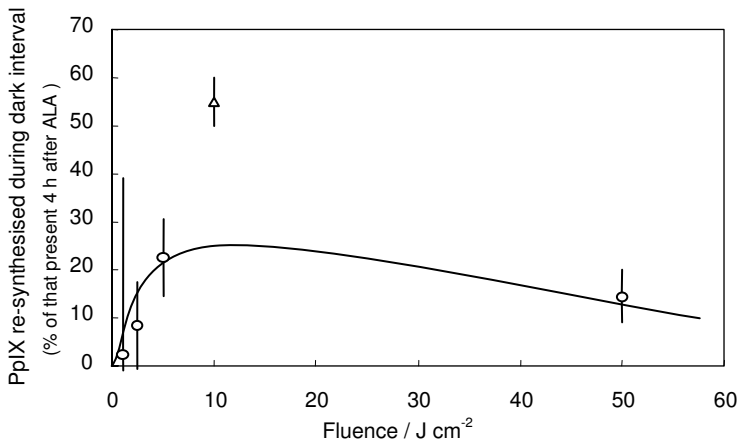


*Figure 6*. Amount of PpIX that is synthesised during the 2 h dark interval between the two light fractions as a function of the fluence of the first light fraction delivered (△) 2 and (○) 4 h after the application of ALA (n 5 6 in each case). In each case the fluorescence intensity is represented as a percentage of that present 4 h after the application of ALA (note that the curve is used as an illustration of the amount of PpIX synthesised after llumination 4 h after ALA application).

***PDT-induced damage***

Control treatments using light only and drug only were performed and did not show any significant PDT induced damage. Figure 7 summarises the PDT damage (quantified by integrating the mean skin score when plotted against time) following each illumination scheme investigated. A single illumination at 4 or 6 h after the start of ALA application results in a maximum visual skin damage score corresponding to severe redness. There is no significant difference between the visual damage score obtained after illumination at either time point. We note that this is also the case for single illuminations of 50 and 200 J $cm^{-2}$ (data not shown).

A two-fold illumination scheme of 50+50 J $cm^{-2}$ separated by a 2 h dark interval results in an increased visual damage score. Reducing the fluence of the first fraction from 50 to 5 J $cm^{-2}$ while keeping the cumulative fluence 100 J $cm^{-2}$ result in a large increase in the visual damage score corresponding to thick crust formation and is the most extensive visual damage we have observed in normal hairless mouse skin to date [12,16]. Reducing the fluence of the second illumination from 95 to 45 J $cm^{-2}$ results in a reduction in the visual damage to approximately that obtained with 50+50 J $cm^{-2}$. Reducing the fluence rate of the first illumination from 50 to 5 mW $cm^{-2}$ results in a similar reduction in the visual damage skin score following treatment. Delivering 2.5+97.5 J $cm^{-2}$ results in the same damage as 5+95 J $cm^{-2}$. Reducing the fluence of the first fraction still further to 1 J $cm^{-2}$ (+99 J $cm^{-2}$) results in significantly less damage such that this scheme is no longer significantly different from 50+50 J $cm^{-2}$. However, this illumination scheme still results in significantly more damage than delivering 100 J $cm^{-2}$ in a single fraction. This Figure also shows that 10+90 J $cm^{-2}$ delivered 2 and 4 h after the application of ALA results in the same damage as at 5+95 J

$cm^{-2}$ delivered 4 and 6 h after ALA.

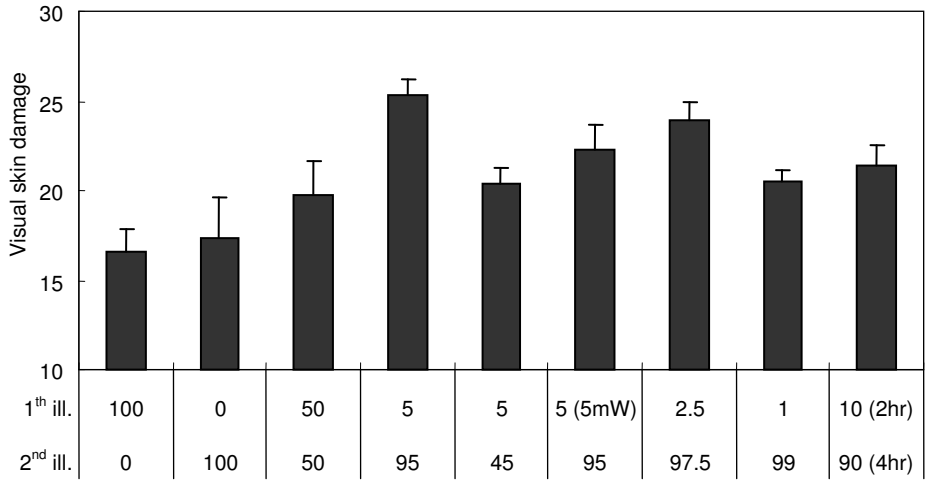


*Figure 7.* Visual skin damage (quantified by integrating the mean skin score for the first 7 days after illumination plotted against time) following each of illumination schemes. Indicated are the fluences delivered during the first and second light fractions at 50 mW $cm^{-2}$, 4 and 6 h (unless indicated in parentheses) after the application of ALA respectively. Results are shown as mean +/- SEM.

***Histological damage***

Figure 8 shows the histological damage 48h after treatment for two of the illumination schemes investigated in this study - a single fraction of 100 J $cm^{-2}$, delivered at 50 mW $cm^{-2}$, 4h after the application of ALA and a two-fold illumination scheme of 5+95 J $cm^{-2}$ delivered 4 and 6h after the application of ALA. Three representative sections from the five animals in each group are shown for each illumination scheme. Normal mouse skin is also shown for comparison. Illumination with a single fraction of 100 J $cm^{-2}$ results in necrosis that is predominately restricted to the epidermis. Complete necrosis of the upper dermis was observed in only one animal, and two animals showed partial necrosis of the upper dermis. After a two-fold illumination (50+50 J $cm^{-2}$), we did not observe a significant increase in the amount or depth of necrosis. However, reducing the fluence of the first light fraction (5+95 J $cm^{-2}$) resulted in more necrosis in both the upper dermis and deep dermis. Four of the five animals showed complete necrosis of the upper dermis and two of the animals showed partial necrosis of the deep dermis. We also observed that the necrosis was accompanied by a large amount of inflammatory infiltrate in the deep dermis after this illumination scheme.

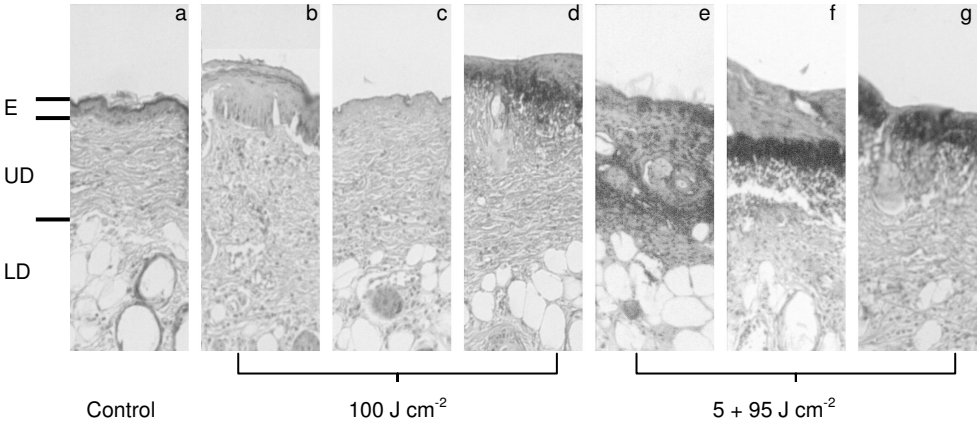


Figure 8. *Histology of hairless mouse skin, (H&E 10x, E; epidermis, U D, upper dermis, L D, lower dermis). (a) normal skin and 48 h after illumination with (b – d) a single fraction of 100 J cm$^{-2}$ delivered at 50 mW cm$^{-2}$, 4 h after the application of ALA, and (e – g) a two-fold illumination scheme in which 5 J cm$^{-2}$ and 95 J cm$^{-2}$ are delivered at 50 mW cm$^{-2}$, 4 and 6 h after the application of ALA respectively.*

## Discussion

***PpIX fluorescence kinetics after illumination***

In the first part of this study we have investigated the kinetics of PpIX fluorescence following illumination and found that the kinetics of PpIX fluorescence after a single light fraction is dependent on the fluence delivered; the resynthesis of PpIX is progressively inhibited following fluences above 10 J cm$^{-2}$. There is a range of factors that may or may not affect the kinetics of PpIX fluorescence within the illuminated volume following ALA-PDT. Before we consider the implications for optimizing therapy it is useful to discuss some of these factors in turn. Between 4 and 6 h after topical application there remains a significant concentration of ALA in normal mouse skin. Illumination in this time period may cause damage to critical cellular components, in particular, the mitochondria, and may decrease or completely inhibit the production of PpIX, despite the presence of ALA. ALA-PDT is also known to reduce the availability of oxygen during illumination [10,11,15,17]. While it is unclear as to the extent of vascular effects following topical ALA-PDT [12], a reduced oxygen supply within the treatment volume, after illumination, may under some circumstances inhibit the ability of the cells to synthesis PpIX [23]. Conversely, PpIX may under some circumstances be present in the circulation, and damage to vessels following PDT, may allow PpIX to return to the volume, from which it has been photobleached during illumination, and thus increase the fluorescence measured within the treatment volume. A similar result may occur if undamaged cells, initially outside the illumination volume, migrate toward it and then synthesise PpIX from ALA already present in the illuminated volume. There is also some in vitro evidence to suggest that the synthesis of PpIX may be modulated by the photoinactivation of the terminal enzyme in haem biosynthesis, ferrochelatase.

Ferrochelatase catalyses the addition of $Fe^{2+}$ to PpIX producing haem. A number of studies have shown that adding ferrochelatase inhibitors such as dimethyl sulfoxide enhances the accumulation of PpIX. The close proximity of PpIX and ferrochelatase during PDT may reduce the activity of ferrochelatase and lead to enhanced PpIX production. It should be noted however that in vivo, heterogeneous cell populations may lead to a complex mixture of these effects.

There are a number of reports in the literature that have investigated the kinetics of PpIX fluorescence after ALA-PDT. To our knowledge there have been two clinical studies where fluorescence of PpIX after illumination has been studied, both during ALA-PDT of human BCC [24,25]. These data are similar to our own unpublished clinical findings that demonstrate a significant increase in PpIX fluorescence in the hours following ALA-PDT, after photobleaching during illumination. There have also been a small number of in vitro studies, the results of which are contradictory. Gibson *et al.* [26] recently reported in an in vitro study a significant reduction in the ability of R3230AC cells to synthesise PpIX immediately after illumination, 3 h after the start of ALA incubation. They showed that this reduction in PpIX synthesis persists for at least 24 h after illumination. As might be expected they found no enhanced efficacy with a second illumination with a dark interval of 24 h. In addition to reduced PpIX synthesis a concomitant reduction in porphobilinogen deaminase (PBGD) activity upon illumination was observed and the authors concluded that PBGD is an important enzyme target when ALA is administered exogenously. In contrast to these findings He *et al.* in two separate studies, reported increased levels of PpIX in two different cell lines between 2 and 48 h after illumination [27,28]. Our data shows that the rate of PpIX resynthesis after illumination is both less than that immediately after the initial application of ALA, and less than that at the corresponding PpIX fluorescence intensity in the normal kinetics of PpIX fluorescence. The absence of an increased rate of production of PpIX after illumination seems to support the hypothesis that ferrochelatase cannot be photoinactivated without inhibiting the production of PpIX.

The continued synthesis of PpIX after illumination has also been demonstrated in UVB-induced tumours in the same animal model used in this study. Van der Veen *et al.* [12] showed an increase in PpIX fluorescence in areas of UVB-irradiated mouse skin after illumination with 100 J $cm^{-2}$ at 100 mW $cm^{-2}$. They reported that the subsequent rate of increase is the same as that in control areas in the same animal (i.e. ALA no illumination). These data seem to indicate a more rapid increase in fluorescence intensity after illumination in UVB-treated skin. However, as we have demonstrated the kinetics of PpIX fluorescence after illumination are dependent on the ‘‘dose’’ delivered. While this is difficult to determine, these treatment parameters, in particular the high fluence rate, may result in the deposition of a small ‘‘dose’’ and explain the rapid increase in PpIX fluorescence intensity after illumination. Van der Veen *et al.* [12] discussed the possibility that circulating porphyrins may play a role in the return of fluorescence in the hairless mouse. They compared two cases (1) where ALA is applied to large area (2 x 2 $cm^2$). This is both large compared to the surface area of the mouse and the illuminated area (7 mm diameter, the same as we have used here); and (2) where ALA is only applied to the illuminated area. They reported no significant difference in

the kinetics of PpIX fluorescence after illumination [29]. We have repeated these measurements (in preparation), and find no significant difference between the two methods of applying topical ALA. This is despite the fact that mouse skin is such that vessels are confined to the most superficial layers and areas are supplied from the periphery and not from beneath as in human skin. These observations seem to support the conclusion that PpIX is not transported back into the treated area after illumination. It is also possible that cells from outside the illuminated volume may enter and themselves synthesise PpIX from the ALA already present.

***PpIX and photoproduct photobleaching during illumination***

The kinetics of PpIX photobleaching are similar to those that we have published previously [15,17]. PpIX is rapidly photobleached during illumination and the rate of photobleaching increases for decreasing fluence rate, supporting an oxygen-dependent mechanism of PpIX photobleaching. Since these data were published there have been two other studies that have reported the absence of fluence rate dependence of PpIX photobleaching [30,31]. While it is difficult to determine the reasons for the difference in results between these studies and our own, there are some specific differences in experimental methods. In each of these studies red light (636 and 630 nm, respectively) was used to both perform PDT illumination and to excite PpIX fluorescence. In addition ALA was administered systemically in each case. Employing long-wavelength excitation and systemic administration of ALA may mean that during illumination the depth from which fluorescence emission is collected increases. Changes in optical properties during illumination may influence the measured kinetics of PpIX fluorescence. The effects of fluorescence originating from progressively deeper layer of tissue are not accounted for. Using shorter 514 nm fluorescence excitation in combination with topical ALA administration and short application times alleviates this problem. Fluorescence spectra were not corrected for changes in tissue optical properties over the emission wavelengths during illumination. In addition Iinuma *et al.* [31] quantified PpIX photobleaching during illumination by integrating the spectral emission between 675 and 720 nm. Within this region PpIX photoproduct fluorescence overlaps with the emission from PpIX and may affect the measured kinetics of PpIX fluorescence.

At constant fluence rate, the rate of photobleaching of PpIX during a single illumination 4 or 6 h after the application of ALA is equivalent. However, this is not the case during a two-fold illumination scheme (Figure 3b), when illumination at 6 h is preceded by illumination at 4 h. In this case the initial rate of photobleaching is significantly less during the second illumination. This not a consequence of the lower fluorescence intensity before the second illumination where the mean fluorescence intensity is not significantly less than the lowest fluorescence intensities prior to the first illumination. Indeed, in the absence of other effects a lower concentration of PpIX would result in faster photobleaching of PpIX due to the reduced demand for oxygen. The reduced rate of photobleaching observed may be due to either, a reduced availability of oxygen at the time of the second illumination or possibly the differential cellular localisation of PpIX.

The kinetics of the fluorescent photoproducts of PpIX, centered on 674 nm, are again similar to those that we have published previously [15]. Using the method of data analysis introduced by Foster and his colleagues we measure less of photoproduct bleaching after large fluences than we have reported previously. However photoproduct photobleaching remains a significant effect. It is interesting to note that we observe similar trends in PpIX and photoproduct photobleaching in individual animals under the same illumination conditions. More rapid PpIX photobleaching corresponds to a faster increase in photoproduct fluorescence and greater photoproduct photobleaching. Also, we do not observe a significant reduction or increase in photoproduct fluorescence during the dark interval between illumination.

***Two-fold illumination***

At constant fluence rate, PDT damage induced by a single illumination in hairless mouse skin is limited by the photobleaching of PpIX. There is no significant difference between the visual damage after illumination with 100 J $cm^{-2}$ at 50 mW $cm^{-2}$ obtained in this study and 50 J $cm^{-2}$ at 50 mW $cm^{-2}$, obtained previously [15]. We have also shown that increasing the fluence of a single illumination beyond 100 to 200 J $cm^{-2}$ does not increase the PDT damage [12].

Two-fold illumination with the same cumulative fluence 50+50 J $cm^{-2}$ results in an increase in PDT damage. Reducing the fluence of the first illumination from 50 to 5 J $cm^{-2}$ results in a large increase in PDT damage which is significantly greater than that of all the other treatment schemes investigated. This is a surprising result. It seems that an illumination scheme with a small first fluence that results in the resynthesis of relatively more PpIX, which is then utilised during the second illumination, is more effective than a scheme in which more PpIX is bleached during the first illumination. It seems that there is a first illumination threshold above which delivering more dose reduces the effectiveness of the second illumination whatever dose is delivered during the second illumination. The difference between the first and second illumination is also demonstrated when we compare delivering 45 and 95 J $cm^{-2}$ in the second illumination after a first illumination of 5 J $cm^{-2}$. There is now significantly less damage when the second illumination is shortened. It seems necessary to deliver a large fluence in this illumination. This does not seem to hold for a single illumination; 100 J $cm^{-2}$ does not cause significantly more damage than 50 J $cm^{-2}$.

The mechanism behind the increase in PDT response demonstrated in this study is unlikely to be solely determined by the amount of PpIX present before each illumination and the extent to which it is photobleached during illumination. Other mechanisms are likely to be complex and beyond the scope of this study. It is possible that a small first dose renders the cells in the illuminated volume sensitive to a second illumination, perhaps by inducing repair mechanisms that are subsequently damaged. It is also possible that the mechanism of cell death, apoptotic or necrotic, may be different after a small initial PDT dose. The dose delivered in the first fraction seems an important parameter that determines, at least in part, the response of tissue to a two-fold illumination.

The optimum dose of the first light fraction is unknown but it is clear that reducing the fluence of the first fraction from 50 to 5 J $cm^{-2}$ is more effective therefore this study was extended to this in more detail. This was done by investigating the further reduction of the fluence of the first light fraction and by reducing the interval between the application of ALA and the first light fraction. Figure 7 shows that reducing the fluence of the first light fraction from 5 to 2.5 J $cm^{-2}$ does not significantly reduce the visual skin damage after illumination. Both illumination schemes remain significantly more effective than a two-fold illumination scheme with equal light fractions and are almost twice as effective as a single illumination of 100 J $cm^{-2}$. Reducing the fluence still further to 1 J $cm^{-2}$ results in a significant reduction in visual skin damage, but this is still significantly greater than for a single illumination scheme. We have shown that the optimum fluence of the first light fraction delivered 4 h after the administration of ALA at a fluence rate of 50 mW $cm^{-2}$ is greater than 1 J $cm^{-2}$. Our results also demonstrate that it is possible to reduce the overall treatment time by a first light fraction of 10 J $cm^{-2}$ delivered 2 h after the application of ALA and the second light fraction delivered 4 h after the application of ALA. Because the results have demonstrated the sensitivity of the response after a twofold illumination to the dose delivered during the first light fraction, we were careful to choose an appropriate fluence for this early first light fraction. The concentration of PpIX available 2 h after the application of ALA is approximately 50% of that at 4 h. We therefore attempted to compensate for the reduced concentration of PpIX by increasing the fluence of the first illumination from 5 to 10 J $cm^{-2}$. As Figure 7 shows, it is possible to reduce the time interval between the application of ALA and the first light fraction from 4 to 2 h with no significant reduction in visual skin damage from that seen with 5 J $cm^{-2}$ delivered 4 h after the application of ALA. The findings described above are important for two reasons. First, they offer an indication of the optimum light dose and timing of a first light fraction that should be delivered to achieve the maximum visual skin damage. Second, they illustrate our lack of understanding of the mechanism behind the increase in PDT induced damage associated with a two-fold illumination scheme. We have shown that in this model the optimum first light fraction in a two-fold illumination scheme with a dark interval of 2 h is between 2.5 and 5 J $cm^{-2}$. It should be noted that this result is only valid for an illumination performed, 4 and 6 h after the application of ALA, at 50 mW $cm^{-2}$. Changing these parameters will obviously affect the dose delivered during the first illumination, which determines the resulting visual skin damage. We have also shown that delivering as little as 1 J $cm^{-2}$ in the first light fraction can significantly increase the visual skin damage above that seen after a single illumination. Again it is clearly demonstrated that the increase in damage is not due to the utilisation of additional PpIX that is synthesised during the 2 h dark interval. Relatively small amounts of PpIX (10% of that present before illumination 4 h after the application of ALA) are resynthesised in the dark interval between the two light fractions, as illustrated in Figure 6. Note that the total amount of additional PpIX photobleached is closely related to that resynthesised in the 2 h dark interval because there is no significant difference in the amount of PpIX that remains after the second light fraction for any of the illumination schemes. The histological damage after illumination shows some interesting results. We observed a trend toward increasing extent and depth of necrosis for

the two-fold illumination scheme in which the first light fraction is 5 J $cm^{-2}$, compared with a single illumination scheme. We did not determine whether these effects were statistically significant, but they represent an area for future study.

The relationship between the increase in PDT damage observed with a two-fold illumination and the length of the dark interval is a different area for future study. The shorter dark interval used in this study (2 not 6 h) does not significantly affect the increase in damage we have observed previously [12]. The time interval between each illumination has a direct effect on the total treatment time, an important factor in the clinic. However a significant dark interval seems necessary (of the order of several tens of minutes). We have shown previously, in the model used in this study, that a single dark interval of 2 min after 3 or 6 J $cm^{-2}$, during illumination to a fluence of 50 J $cm^{-2}$, does not significantly increase the PDT damage [17]. This result is in contrast to the data of Cunrow *et al.* [31], who were able to show an increase in PDT damage with such an illumination scheme. It is not easy to determine the reason for the difference between these two results. However, the bare-fibre illumination geometry used by Cunrow *et al.* makes it difficult to compare their data with those obtained in our model and in clinical ALA-PDT.

In summary, a fractionated illumination scheme in which a cumulative fluence of 100 J $cm^{-2}$ at 50 mW $cm^{-2}$ is delivered in two equal light fractions separated by a dark interval of 2 h has shown to considerably increase the efficacy of ALA-PDT. The efficacy of such a scheme is further increased if the fluence of the first light fraction is reduced to 5 J $cm^{-2}$. The significance of the illumination parameters is shown for both the first and the second light fraction. Reducing the fluence of the first fraction from 5 to 2.5 J $cm^{-2}$ results in the same amount of visual skin damage whereas reducing the fluence to 1 J $cm^{-2}$ or reducing the fluence rate to 5 mW $cm^{-2}$ results in less damage. Reducing the fluence of the second light fraction from 95 to 45 J $cm^{-2}$ also results in less damage but all the two-fold illumination schemes tested remain more effective than a single illumination of 100 J $cm^{-2}$. Also, a first light fraction of 10 J $cm^{-2}$ can be delivered 2 h earlier, 2 h after the application of ALA, with no significant reduction in visual skin damage obtained after a first light fraction of 5 J $cm^{-2}$ delivered 4 h after the application of ALA.

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# Chapter 4

## Fractionated illumination after topical application of 5-aminolevulinic acid on normal skin of hairless mice; the influence of the dark interval

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## Abstract

We have previously shown that light fractionation during topical aminolevulinic acid based photodynamic therapy (ALA-PDT) with a dark interval of 2 hours leads to a significant increase in efficacy in both pre clinical and clinical PDT. However this fractionated illumination scheme required an extended overall treatment time. Therefore we investigated the relationship between the dark interval and PDT response with the aim of reducing the overall treatment time without reducing the efficacy. Five groups of mice were treated with ALA-PDT using a single light fraction or the two fold illumination scheme with a dark interval of 30 minutes, 1, 1.5 and 2 hours. Protoporphyrin IX fluorescence kinetics were monitored during illumination. Visual skin response was monitored in the first 7 days after PDT and assessed as PDT response. The PDT response decreases with decreasing length of the dark interval. Only the dark interval of 2 hours showed significantly more damage compared to all the other dark intervals investigated ($P$<0,05 compared to 1.5 hours and $P$<0.01 compared to 1 hour, 30 minutes and a single illumination). No relationship could be shown between the utilised PpIX fluorescence during the two fold illumination and the PDT response. The rate of photobleaching was comparable for the first and the second light fraction and not dependent of the length of dark interval used. We conclude that in the skin of the hairless mouse the dark interval cannot be reduced below 2 hours without a significant reduction in PDT efficacy.

## Introduction

Illumination with two light fractions separated by a dark interval of two hours significantly improves the therapeutic outcome of 5-aminolevulinc acid based photodynamic therapy (ALA-PDT) for human skin cancer [1,2]. The higher efficacy of a two-fold illumination scheme was initially shown in several pre-clinical studies using either topical or systemic ALA administration [3-7].

In our search for the mechanism behind the increased effectiveness we have shown that the PDT dose of the first as well as the second light fraction are important parameters in both clinically and in pre clinical models [1,2,6,7]. For example, for the hairless mouse model [7] this means that for a total illumination with 100 J $cm^{-2}$ at 50 mW $cm^{-2}$ a first fraction with 5 J $cm^{-2}$ is more effective than 1 J $cm^{-2}$. The details of the relationship between the dark interval and PDT response have not yet been determined. Previously [5,6] we have used intervals of two and six hours after topical ALA application and both were effective in pre-clinical models. Clinically, we routinely use a two-fold illumination scheme with a 2 hours dark interval between illumination at 4 and 6 hours after the start of ALA administration. The overall treatment time of this illumination scheme is relatively long. In the present study we therefore investigated if we could shorten the dark interval between the two light fractions without loss of effectiveness.

Initially we hypothesised that the increased effectiveness could be explained by the utilisation of re-synthesised protoporphyrin IX (PpIX) during the second light fraction after photobleaching during the first light fraction. Although we have subsequently shown that the amount of re-synthesised PpIX is not a critical factor in either topical or systemic ALA-PDT we tested this hypothesis again in the present study [7,8]. In the present study we have determined the amount of re-synthesised PpIX present at the start of the second light fraction and investigated if this is correlated with the response following PDT.

A second hypothesis for increased effectiveness of a fractionated illumination scheme is re-oxygenation of tissue in the dark interval [8-15]. Re-oxygenation of tissue during the dark interval may increase the generation of singlet oxygen and therefore enhance PDT. The rate of photobleaching of PpIX can be used as an indirect marker for the oxygenation of the tissue since a higher oxygenation of the tissue will result in faster photobleaching and more PDT induced damage [16]. To test this hypothesis we have measured the rate of photobleaching during each light fraction to investigate if this is correlated to the PDT induced damage.

## Materials and Methods

*Animal model*. Female inbred albino hairless mice (SKH1 HR, Charles River, Someren, NL), aged between 8 and 10 weeks old, were included in this study. Prior to treatment animals were fed on a diet free of chlorophyll (Hope Farms b.v., Woerden, NL) for a

minimum of two weeks in order to remove the autofluorescence emission from mouse skin centered on 675 nm attributed to pheophorbide-a. The animal experimental committee of the Erasmus University Medical Centre approved the experimental protocol.

*ALA application*. Twenty percent 5-aminolaevulinic acid (ALA, Medac, Hamburg, DE) was dissolved in 3% carboxymethylcellulose in water. To prevent skin irritation, each ALA solution was prepared to approximately pH 4 by the addition NaOH (2 M). One layer of gauze (∅ 7 mm) was soaked in 37.5 mg ALA cream and placed on the dorsal skin. The skin was not pre-treated before ALA application. A polythene dressing (Tegaderm, 3M, NL) was used to occlude the area for 4 hours prior to treatment. Before the application of ALA animals received low dose anaesthesia (Hypnorm; fluanisol/fentanyl mixture, Janssen Pharmaceutics, BE and Diazepam, Centrafarm b.v., Etten-Leur, NL) to alleviate possible anxiety caused by the dressing.

*PDT light delivery and fluorescence detection*. Four groups of mice received a 2-fold illumination scheme: The first light fraction of 5 J $cm^{-2}$ light at 50 mW $cm^{-2}$ was delivered 4 hours after the administration of ALA. The second light fraction of 95 J $cm^{-2}$ at 50 mW $cm^{-2}$ was delivered after a dark interval of 30 minutes, 1, 1.5 or 2 hours. One group of mice received a single illumination (100 J $cm^{-2}$ at 50 mW $cm^{-2}$) 4 hours after the administration of ALA. Other controls (single illumination at 6 hours after ALA, light only and ALA only) have been performed previously and were not repeated [5,6,16]. During the therapeutic illumination animals were anaesthetised with a combination of 2% Ethrane or Isoflurane (Abbott, Amstelveen, NL) oxygen and $N_2O$.

The experimental set-up used for the PDT illumination and PpIX fluorescence detection has been described previously [7]. In short; the illumination was performed using the 514 nm output from an argon ion laser delivered via a 400 µm fibre and imaged to a 7 mm diameter spot of homogeneous profile on the skin of the mouse using a microlens (QLT, Vancouver, BC, Canada). All treated animals were used to assess PDT induced damage as described below. The kinetics of PpIX fluorescence during illumination was determined in a sub-group of 5-6 animals for each illumination group. Scattered excitation light and fluorescence emission (550 - 792 nm) was collected from the whole of the illuminated area and focused into a 400 µm optical fibre coupled to a fibre optic spectrometer (Ocean Optics, Eerbeek, Netherlands). A long pass filter, OG 570 (Melles Griot, Zevenaar, Netherlands) was placed in the optical path to bock scattered 514 nm excitation light. Fluorescence emission spectra were acquired during illumination at intervals of 5 seconds using an integration time of 1.5 seconds. In addition, immediately prior to and after treatment a reflectance spectrum was acquired using the same spectrograph. The output from a filtered halogen-lamp, (0.15 mW $cm^{-2}$, Stortz, Tüttlingen, Germany), delivered by a second 400 µm fibre and microlens was imaged onto the 7 mm diameter treatment spot. A long pass filter (OG 530, Melles Griot, Zevenaar, Netherlands) was used to minimise fluorescence excitation of the tissue during reflection measurements. Using this set-up both fluorescence and reflectance measurements was acquired using the same source-detector geometry.

A fluorescence image was acquired prior to each period of illumination using a CCD camera (Lambert Instruments, Leutingwolde, Germany) to re-locate the area of interrogation

and maintain a constant distance between the mouse skin and the head of the spectrometer.

*Data analysis*. The spectral analysis of fluorescence data acquired in this study was the same as we have described previously [6] and based on that described by Foster and his co-workers [17-19]. We have again corrected for changes in tissue optical properties during illumination using the method introduced by Wu *et al.* [20]. Since we have only acquired a reflectance spectrum immediately before and after each light fraction we have corrected all the fluorescence spectra acquired during each illumination period with a linear interpolation of these reflectance spectra. Fluorescence emission spectra, corrected for tissue optical properties were analyzed as a linear combination of basis fluorescence spectra using single value decomposition (SVD) algorithm. The 3 basis fluorescence spectra used in this analysis were the autofluorescence of normal mouse skin, PpIX and the hydroxyaldehyde chlorin photoproduct of PpIX. In each case basis spectra were determined from the average of 10 spectra from 10 animals. The fitted PpIX values measured during illumination was normalised to the first measurement and in case of a two-fold illumination to the first measurement of each illumination. To determine the initial rate of photobleaching for each light fraction the reciprocal of the normalised PpIX fluorescence was calculated and the slope over the first 5 J $cm^{-2}$ was calculated using linear regression fitting [16].

*PDT damage.* The PDT response within the illuminated area was assessed daily by two independent observers (HSB and APH) blinded from the treatments using a visual skin damage scoring system as described previously [21]. No change in skin colour was scored as 0. Scores 1 to 3 were used for increasing discoloration of the skin, 1 meaning minimal redness and 3 meaning severe redness. Thin crust formation was scored as 4 and thick crust formation was scored as 5. Damage was observed to be inhomogeneous in a number of treatments. Photographs were taken regularly in order to determine the degree and distribution of damage. The damage score of the total illuminated area in one animal at one time point was calculated by scoring areas according to the degree of damage related to the contribution to the total illuminated area. The visual skin damage value of a single mouse was quantified by integrating the damage score over the first 7 days.

*Statistics.* Statistical analysis was performed on the PDT induced damage data using the ANOVA followed by a Student-Newman-Keuls test (SNK) and a *P*-value < 0.05 was considered significant. One way analysis of variance is performed to test for trends.

## Results

### *PDT induced skin damage*

The cumulative visual skin damage score over the first 7 days post ALA-PDT is plotted against the duration of the dark interval between the two fractions in Figure 1. All groups were treated with a cumulative dose of 100 J $cm^{-2}$ delivered at 50 mW $cm^{-2}$. The mean (and SD) of the visual skin damage score for the illumination in a single fraction i.e. no dark

interval was 16.6 (3.5). Increasing the length of the dark interval resulted in an increasing damage score.

Generally PDT using a single light fraction resulted in discoloration of the skin and sometimes in the formation of a thin crust around day 2-3. At day 7 minimal skin damage was seen; the skin was slightly thickened and showed minimal discoloration in most animals. In contrast PDT using a two-fold illumination with a dark interval of two hours resulted in oedema at day one followed by the formation of a thin crust that developed into a thick crust between days 2 to 3. At day 7 after PDT this thick crust was detached but the tissue underneath still showed severe redness that developed, in some animals, in the formation of a second thin crust. The skin damage after a two-fold illumination with shorter time intervals also resulted in the formation of crusts, but these were mostly thin and the area that responded to PDT was smaller and healed quicker.

Statistical analysis showed no significant difference in PDT damage between a single fraction and dark intervals of 30 minutes and 1 hour. PDT with a dark interval of 1.5 hours resulted in significantly more damage compared to PDT with a single light fraction of equal cumulative fluence ($P$<0.05). Treatment with a 2 hours dark interval showed the most severe damage compared to all other groups ($P$<0.05 compared to 1.5 hours and $P$<0.01 compared to 1 hour, 30 minutes and a single illumination). The skin damage shows a trend for increasing skin damage with increasing length of dark interval ($P$< 0.001).

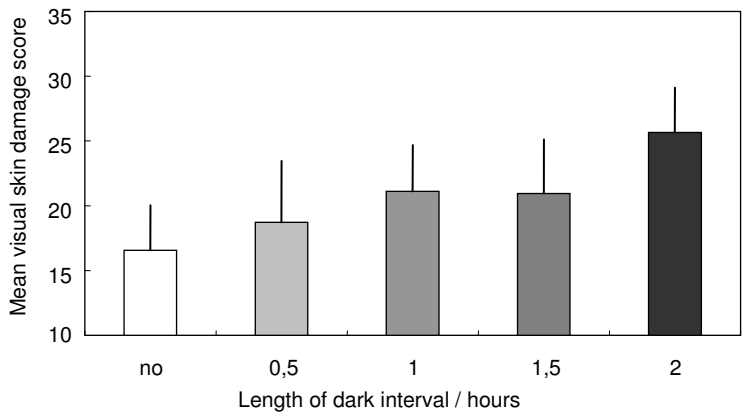


*Figure.1*. The mean visual skin damage score (quantified by integrating the skin damage score for the first 7 days after ALA-PDT) plotted against the length of the dark interval the two light fractions of 5 and 95 J $cm^{-2}$. A control group of mice (n=9) received a single illumination of 100 J $cm^{-2}$ (no dark interval) 4 hours after the administration of ALA. The lengths of dark tested were 0.5 (n=12), 1 (n=11), 1.5 (n=10) and 2 hours (n=15). The maximum skin damage score would be 35. Results are shown as mean ± SD. A 2 hour dark interval resulted in significantly more damage compared to the shorter dark intervals tested.

***PpIX fluorescence kinetics during therapy***

The initial fluorescence was 1744 ± 396 counts. The normalised PpIX fluorescence during the different two fold illumination schemes with variable dark intervals obtained from the recorded fluorescence spectra is shown in Figure 2. PpIX fluorescence was rapidly photobleached during the first illumination of 5 J $cm^{-2}$ at 50 mW $cm^{-2}$ to 41.8 ± 5.5% of the intensity at the start of illumination. In time after the end of this first illumination PpIX fluorescence increased again. At 30 minutes this increase was not statistically significant. At 1, 1.5 and 2 hours the PpIX fluorescence significantly increased ($P$<0.05) although not significantly different from each other ($P$=0.53) as the kinetics of PpIX re-synthesis plateaus. PpIX fluorescence was almost completely photobleached during the second illumination of 95 J $cm^{-2}$ delivered at 50 mW $cm^{-2}$.

The average rate of photobleaching during that first light fraction of 5 J $cm^{-2}$ was determined from each group of animals and found to be 0.29 ± 0.07 $cm^2$ $J^{-1}$. The initial rate of photobleaching during the second light fraction, delivered after a dark interval of 30 minutes, 1, 1.5 and 2 hours, were 0.18 ± 0.03, 0.20 ± 0.03, 0.17 ± 0.03, 0.18 ± 0.04 $cm^2$ $J^{-1}$ respectively, and not significantly different from each other.

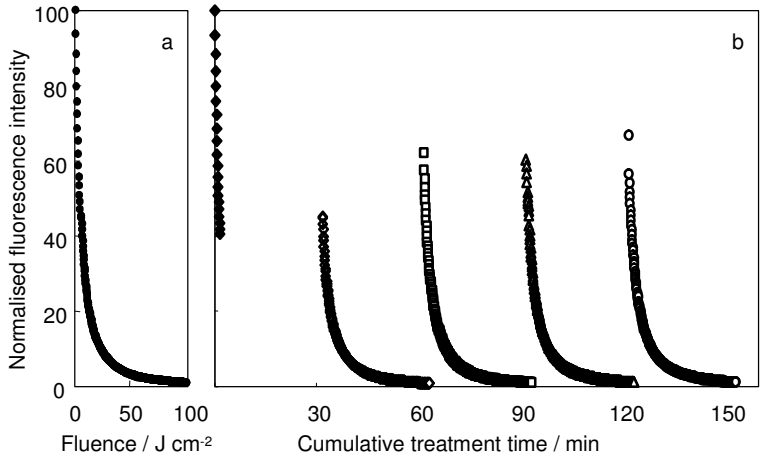


*Figure 2*. Mean normalised PpIX fluorescence intensity during 5 different illumination schemes using 514 nm illumination of normal mouse skin 4 h after the application of ALA. A cumulative fluence of 100 J $cm^{-2}$ was delivered at 50 mW $cm^{-2}$. In panel (a) the results after a single light fraction is presented (●, n=5). In panel (b) the results of the two-fold illuminations of 5 and 95 J $cm^{-2}$ are presented. The results of the first light fraction are combined as this was similar for all treatment schemes (◆, n=21). The second fraction was delivered after a dark interval of 0.5 (◇, n=6), 1 (□, n=5), 1.5 (△, n=5) and 2 hours (○, n=5). The fluorescence intensity is normalised to the PpIX fluorescence at the start of the first light fraction.

## Discussion

In this study we investigated the relationship between the PDT response and the length of the dark interval between the two light fractions of a two-fold illumination scheme. Previously we have investigated the use of a longer (6 h) dark interval in both normal skin and in UVB induced tumours in the mouse [5]. This interval combined with the 4 hours preceding the first light fraction results in an overall treatment time in excess of 10 hours. This is not easily accommodated into clinical practice. We therefore shortened the dark interval from 6 to 2 hours and found that this does not detrimentally affect PDT efficacy in our pre clinical model; the two-fold illumination scheme is still significantly more effective than the single illumination in mouse skin [6]. Clinically we have shown that fractionated PDT with a dark interval of two hours results in a significantly better treatment response compared to a single illumination [1,2]. Star *et al.* [1] recently published a clinical report on the long-term response to ALA-PDT using the two-fold illumination scheme. Efficacy was found to be significantly better compared to published results using a single illumination scheme. This is despite the fact that this illumination scheme was not optimised; i.e. the total fluence was divided over two equal light fractions. After pre-clinically determining the optimum illumination scheme [6,7], the clinical protocol was adjusted. One-year follow-up for this optimised illumination scheme shows that fractionated ALA-PDT results in a significant increase in clinical response [2].

Figure 1 shows that decreasing the length of the dark interval decreases the PDT response. A two-fold illumination with a dark interval of 30 minutes or 1 hour does not result in more damage compared to a single fraction illumination. A dark interval of 1.5 hours resulted in significantly more damage compared to a single illumination, although no difference with the shorter dark intervals could be shown. Only the two hours dark interval resulted in significantly the most severe skin damage compared to the shorter intervals tested. Therefore we conclude that the dark interval can not be shortened without loss of effectiveness and needs to be at least 2 hours. Since our aim was to investigate if we could shorten the clinical treatment without loss of effectiveness, we only investigated dark intervals shorter than 2 hours. However the results presented here show a significant trend for increasing damage with increasing length of dark interval suggesting that it may be possible that a dark interval of more than 2 hours results in an even more effective treatment. A longer dark interval (6 hours) has been investigated previously [5] but this interval was used in combination with an un-optimised illumination scheme in which two equal light fractions of 100 J $cm^{-2}$ were delivered at 50 mW $cm^{-2}$. This and the fact that the details of the data analysis of response were slightly different to that of the present study make a direct comparison of data difficult. When response data is assessed in an identical manner the mean skin damage (3.5 ± 0.4) is comparable with that of the present study (3.7 ± 0.5). It is probable that any enhanced response due to the longer dark interval is negated by the high fluence of the first light fraction [7].

The results obtained lead us to the conclusion that reducing the dark interval below 2 hours will reduce treatment efficacy. While we have not investigated the effect of intervals

shorter then 30 minutes in the present study we have previously investigated the effect of an interval of 150 seconds after 3 or 6 J $cm^{-2}$ at 150 mW $cm^{-2}$ in the skin [21]. Although the illumination parameters were not exactly comparable, i.e. fluence rate of 150 mW $cm^{-2}$ compared to 50 mW $cm^{-2}$, this short dark interval did not show a significant increase in PDT response in the skin. Furthermore, in a different model using systemic ALA-PDT and implanted rhabdomyosarcoma we investigated the use of both short (single interruption of 150 sec or multiple interruptions of 30 sec on/off) and long dark intervals. Only the long term dark interval showed a significant increase in growth delay [4]. These results are contradictory to data from a series of studies investigating the response of normal colon to ALA-PDT using light fractionation that have shown a significant increase in tissue response using short dark intervals [12,14,22]. Intervals of 150 seconds result in a significant larger area of necrosis in the colon when it is illuminated with a bare cut - end fibre in contact with the tissue. The mechanism behind this increase in response was found to be due with re-oxygenation during the dark interval and associated with vascular reperfusion injury. The reason for the apparent contradiction between these findings and those of our studies on short term light fractionation is not immediately clear but we note that the radial variations in fluence rate that results from an illumination geometry in which a cut-end fibre is placed in contact with skin are large [1]. We and others have shown that there are strong fluence rate variations in PDT response using the photosensitiser PpIX that would make a comparison of data between the skin and colon model difficult.

That a longer rather than a shorter dark interval increases the response was also found by Babilas *et al.* [9]. They were unable to show any improvement in the therapeutic outcome of the subcutaneously implanted tumour after ALA-PDT using a fractionated illumination scheme with a 15 minute dark interval between two light fractions. Furthermore they concluded that improvement of the therapeutic effect could only be reached in normal tissue and not in tumour tissue. Although the present study is performed on normal tissue we have previously shown increased effectiveness of the two-fold illumination in tumour tissue [3-5]. Also our results in normal mouse skin were the basis for the clinical treatment protocol that resulted in an increase in CR after 12 months follow-up for superficial basal cell carcinoma [2].

To date two hypotheses have been postulated for the mechanism behind the increased effectiveness of the two-fold illumination (a) re-oxygenation during the dark interval and (b) utilisation of the PpIX re-synthesised during the dark interval during the second light fraction. The re-oxygenation of tissue during the dark interval may increase the generation of singlet oxygen and therefore enhance PDT. However this argument is mostly used for fractionation schemes with short intervals, in the order of seconds and minutes [8-15]. In a previous study we have shown that the rate of photobleaching is correlated to the oxygenation of tissue; a higher oxygenation results in faster photobleaching [16]. Here we observe equal rates of photobleaching during the second light fraction for all the dark intervals investigated therefore it is unlikely that re-oxygenation plays a role in the two-fold illumination with a dark interval of two hours. The hypothesis that re-synthesised PpIX that is utilised during the second light fraction explains the increased effectiveness is rejected since we measured comparable levels for re-synthesised PpIX for the 1 and the 2 hours dark interval whereas

the damage for the two hour dark interval is higher. This is supported by the results obtained in several previous studies in which we also have shown that there is no relationship between the amount of re-synthesised PpIX and PDT induced damage [7,8].

The mechanism behind the increase in efficacy after the two-fold illumination scheme is unclear. The mechanism of cell death after ALA-PDT is complex and heterogeneous. Early after the application of ALA PpIX is localised in the mitochondria and it is well known that cell death can occur by apoptosis and or necrosis [23]. Grebenova *et al.* [24] have shown that during and after illumination the mitochondria of HL 60 cells are damaged resulting in cytochrome c release and caspase 3 activation followed by necrosis. Simultaneously a secondary route to apoptosis is activated. The present study shows that the timing of the second illumination is important. Previously [6,7] we have shown that the fluence delivered in the first fraction is important and for hairless mouse skin this is 5 J $cm^{-2}$ at 50 mW $cm^{-2}$. A single illumination with this fluence results in minimal skin damage at day 1 to 7 after treatment (data not shown), despite the fact that almost 60% of the total amount of PpIX is photobleached. Gederaas *et al.* [25] have shown a similar process in vitro. Illumination of WiDr cells with a small light fluence induced a rapid increase in [$Ca^{2+}$] due to influx of calcium from the medium, without substantial cell death. Numerous cellular and tissue responses are initiated by PDT that may or may not result in cell death. These and potentially other cellular processes may be initiated in cells surviving the first light fraction and most likely are developing over time. The fact that the timing of the second light fraction is an important factor implies that these cellular processes may have an influence on the susceptibility of cells to PDT. Whether these processes are important for the understanding of the increased effect after the two-fold illumination is unclear and needs further investigation.

In conclusion we have shown that the overall treatment time for ALA-PDT using the two-fold illumination can not be shortened without a reduction in efficacy. The most effective illumination scheme for topical ALA-PDT on hairless mouse skin found to date is a 2 hour dark interval between a first fluence of 5 J $cm^{-2}$ and a second fluence of 95 J $cm^{-2}$ delivered at a fluence rate of 50 mW $cm^{-2}$.

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# Chapter 5

## Evidence for a bystander role of neutrophils in the response to systemic 5-aminolevulinic acid-based photodynamic therapy

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## Abstract

***Background/purpose:*** A significant increase in the number of circulating and tumour neutrophils immediately after therapy was observed while investigating the increase in response of tissues to aminolevulinic acid based photodynamic therapy (ALA-PDT) using a two-fold illumination scheme with a prolonged dark interval. The action of (tumour) neutrophils is an important therapeutic adjunct to the deposition of singlet oxygen within the treatment volume, for many photosensitisers. It is not known if those phagocytes contribute to the improved outcome of ALA-PDT. In this study we investigated the role of neutrophils in the response to PDT using systemic ALA with and without light fractionation.

***Methods:*** Rhabdomyosarcoma, transplanted in the thigh of female WAG/Rij rats were illuminated transdermally using 633 nm light following *i.v.* administration of 200 mg $kg^{-1}$ ALA. The pharmacokinetics of PpIX within the tumour tissue during therapy were determined to compare with that observed in other models for topical administration of ALA. PDT was performed under immunologically normal or neutropenic conditions using various illumination schemes. The number of neutrophils in tumour and in the circulation were determined as a function of time after treatment and compared to growth delay of each scheme.

***Results:*** Fluorescence spectroscopy revealed similar pharmacokinetics of PpIX to those observed during and after topical ALA-PDT. The number of neutrophils within the illuminated tumour and in the circulation increased significantly following therapy. This increase in the number of neutrophils was associated with an increase in the efficacy of therapy: the more effective the therapy the greater the increase in tumour and blood neutrophils. Administration of anti granulocyte serum treatment prevented the influx of neutrophils after ALA-PDT, but did not lead to a significant decrease in the efficacy of the PDT treatment on the growth of the tumour for any illumination scheme investigated.

***Conclusion:*** These results indicate that the magnitude of damage inflicted on the tumour by ALA-PDT does not depend on the presence of neutrophils in the tumour or circulation and that the role of neutrophils in ALA-PDT is much less important than in PDT using other photosensitisers. These data contribute to the understanding of the mechanism of response of tissue to systemic ALA-PDT.

## Introduction

Neutrophils are closely associated with the response of tissues to photodynamic therapy (PDT) using photosensitisers like Npe6, mTHPC, HPPH and Photofrin [1-6]. After PDT the number of neutrophils in illuminated tumour tissue is increased. The presence of (activated) neutrophils provides an adjunct to the therapeutic PDT dose that originates from the deposition of reactive oxygen species and is associated with a subsequent decrease in tumour volume following therapy. When PDT is performed under neutropenia tumour regression is significantly reduced. We have shown for example that the effectiveness of Photofrin-PDT on the rat rhabdomyosarcoma is decreased under neutropenic conditions [6]. The role of neutrophils in PDT using 5-aminolevulinic-acid (ALA) is not known and the subject of the present study.

ALA-PDT is widely used as an experimental therapy that is now recognised as a first line treatment for a number of (pre-) malignant cutaneous indications. We have shown in different animal models and using systemic and topical ALA administration that the efficacy of ALA-PDT is significantly increased if a two-fold illumination scheme with a dark interval of more than one hour was applied instead of a single illumination [7-10]. For example, after systemic administration of ALA, the growth of the rat rhabdomyosarcoma is significantly delayed using a fractionated illumination with a dark interval of 75 minutes compared with a single illumination [8]. We are investigating the mechanism behind this increase in effectiveness. During PDT the endogenous photosensitiser protoporphyrin IX (PpIX) is rapidly photobleached. After illumination new PpIX is formed which we have shown can be utilised in a second illumination [7,11]. However, the increase in PpIX during the dark interval is relatively small and therefore not considered as the main mechanism behind the increased effectiveness. While investigating the histological response of rat rhabdomyosarcoma to ALA-PDT using a fractionated illumination scheme we observed an increase in the number of tumour neutrophils immediately after therapy. We therefore designed the present study to investigate the role of neutrophils in the response to PDT with ALA with and without light fractionation to determine if neutrophils contribute to the increase in the efficacy of ALA-PDT. Here we perform ALA-PDT on rat rhabdomyosarcoma under normal and neutropenic conditions and determine if the outcome is dependent on the number of neutrophils in blood and in tumour tissue.

# Materials and Methods

*Tumour model*. Rat rhabdomyosarcoma, originally derived from a syngeneic undifferentiated rhabdomyosarcoma, was maintained by subcutaneously transplanting small pieces of tumour (~ 1 mm³) on the thigh of female WAG/Rij rats (12 - 13 weeks old). Since the tumours were grown subcutaneously it was possible to monitor tumour growth daily by measuring the three orthogonal diameters using callipers and the tumour volume was

estimated by the formula of an ellipsoid, V=(π/6)∗D1∗D2∗D3. Tumours were randomly assigned to control or treatment groups when their volume reached 50 $mm^3$. “The Committee on Animal Research” of the Erasmus University of Rotterdam approved the animal experimental protocol.

*ALA.* ALA (Medac, Wedel, Germany) was dissolved in sterile NaCL solution 0.9%. A freshly prepared ALA solution (90 mg $ml^{-1}$) was administered *i.v.* to a dose of 200 mg $kg^{-1}$ body weight under ethrane/$O_2$/$N_2O$ anaesthesia. After administration the animals were kept under subdued light for the first 24 hours

*PDT light delivery.* PDT was carried out under general anaesthesia using intra muscular Hypnorm, 0.5 ml $kg^{-1}$ (Janssen Pharmaceutica, Tilburg, The Netherlands) and diazepam, 2.5 ml $kg^{-1}$. Prior to the light treatment the skin overlying the tumour was shaved. The animals were placed on a temperature-controlled stage and covered with a black polythene mask. Tumours were transdermally illuminated with a 10 mm diameter plane parallel light beam (633 nm) at a fluence rate of 100 mW $cm^{-2}$.

*Fluorescence spectroscopy*. In separate series of animals the pharmacokinetics of PpIX fluorescence in rhabdomyosarcoma was determined. This was achieved by resecting the skin overlying the tumour and placing a fibre-optic probe (approximately 800 µm in diameter) gently in contact with its upper surface. The filtered output from a Xenon arc lamp (400 – 410 nm) was coupled into a 400 µm optical fibre was used to excite PpIX fluorescence in the uppermost layers of the top of each tumour. Scattered excitation light and fluorescence emission (550 - 792 nm) were collected using a second 400 µm immediately adjacent to the delivery fibre and coupled to a fibre optic spectrometer (Oriel Instaspec IV and Oriel ms 257, Heidelberg, Germany). A long pass filter, OG 520 (Melles Griot, Zevenaar, Netherlands) was placed in the optical path to block scattered excitation light. Similar spectra were acquired from the underside of the skin overlying the tumour and from muscle adjacent to the base of the tumour. In all cases the intensity of the excitation illumination was approximately 100 µW $cm^{-2}$ and acquisition times were of the order of 2 seconds. This corresponds to a light fluence of less than 0.2 µJ $cm^{-2}$ for each tumour.

Fluorescence spectra were acquired every 30 minutes following ALA administration until 4 h and then again 5, 6, 16 ands 24 h after ALA administration. The kinetics of PpIX fluorescence following illumination were determined in a further 4 groups of animals that received 10, 20, 50 and 100 J $cm^{-2}$, 1 h after the administration of ALA, that corresponds to the time point of the first light fraction in the present study. Fluorescence was acquired 45 and 90 minutes after the end of illumination where the last time point would be the one before the second light fraction in a two-fold illumination scheme. An average autofluorescence spectrum, determined in a separate series of animals that did not receive ALA, was subtracted from the PpIX fluorescence emission. Spectral analysis of data acquired in this study were analysed as a linear combination of basis fluorescence spectra using single value decomposition as described previously [12].

*Neutropenia*. Neutropenia was induced by treatment with anti-granulocyte serum (AGS, absorbed polyclonal rabbit anti rat polymorphonuclear cells, Accurate, Westbury, NY, USA). The first dose (0.2 ml AGS, tenfold diluted with saline) was injected *i.v.* 30 minutes before

illumination, followed by an *i.p.* injection of 0.2 ml tenfold diluted AGS at day 1, 4 and 7 as long as the animals were in experiment. Blood samples were collected just before each AGS injection using either an orbital puncture or a tail vein puncture (Multivette® 600k3e blood collection system, Starstedt, Germany). The amount of neutrophils in whole blood was determined using the ADVIA® 120 Haematology System and the multi-species software application (Bayer B.V. HealthCare Diagnostics, Mijdrecht, The Netherlands).

Neutrophils in blood and tumour after ALA-PDT. The systemic and local neutrophilic response to ALA-PDT was determined by collecting blood and tumour samples before ALA administration and at 6, 16 hrs, 1, 2 and 4 days after start of photodynamic therapy. Animals were treated according to 6 different treatment schemes (n=2-5 per group). The first two treatment schemes served as control and received only ALA and no light under normal and neutropenic conditions. The second two treatment schemes were the single illumination scheme, i.e., a single light fraction of 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ one hour after ALA administration, under normal and neutropenic conditions. The last two treatment schemes were a two-fold illumination scheme, i.e., two light fractions of 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ delivered at 1 and at 2.5 hours after ALA administration, under normal condition and under neutropenia induced before the first illumination. Blood samples were collected and the amount of neutrophils was determined as described above. Tumours were harvested, snap frozen and dissolved to determine the concentration of free- and cell-associated myeloperoxidase (MPO) per gram wet tissue as described below.

*Assessment of MPO activity.* As an index of the number of neutrophils the tumour myeloperoxidase (MPO) content was assessed. We adapted the biochemical assay described by Graff *et al.* [13] for tumour tissue. To overcome the interference of endogenous (enzymatic and nonenzymatic) reductants two wash steps in the presence of *N*-ethylmaleimide (NEM) after homogenisation were introduced. To inhibit the pseudoperoxidase activity of remaining haemoglobin derived from the increasing numbers of tumour associated erythrocytes upon PDT hexadecyltrimethylammonium bromide (HTAB) was added to the assay mixture.

In short, the tumour was excised, weighed and homogenised in 3 volumes (v/w) of ice-cold modified RIPA buffer (containing 1% NP40, 0.25% sodiumdeoxycholate, 1 µg $ml^{-1}$ aprotinin, leupeptin and pepstatin, 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM $NaVO_4$, and 1 mM NaF; pH 7.4) using a Polytron homogeniser. The homogenate was centrifuged at 12,000 *g* for 30 min at 4°C. Next, the supernatant was collected to determine free MPO. MPO can only be liberated from the primary granules of the neutrophil by treating with the ionic detergent HTAB [14]. Thus any MPO in the supernatant was apparently secreted by activated neutrophils in the tumour before its excision [15]. The pellet was resuspended in ice-cold 50 mM sodium phosphate buffer (pH 7.4) containing 10 mM NEM, centrifuged again, and if the number of erythrocytes was high, resuspended in ammoniumchloride for 20 min at 4°C and centrifuged. The pellet was sonicated in 50 mM sodium phosphate buffer (pH 6.0) containing 0.5% HTAB (PB-HTAB) by three bursts of 10 sec, subjected to three cycles of freezing and thawing, and centrifuged (12,000 *g*, 30 min, 4°C). The MPO activity was determined by its ability to catalyze the

hydrogen peroxide-dependent oxidation of o-dianisidine (o-DA). The reaction was started by the addition of 100 µl pre-warmed substrate solution (hydrogen peroxide and o-DA) to 50 µl aliquots of the serially diluted supernatant in PB mixed with 50 µl PB-HTAB (final concentrations: 3.43 mM HTAB, 0.35 mM hydrogen peroxide, and 0.63 mM o-DA). We found that under those conditions optimal reaction rates were attained, while the pseudoenzymic activity of haemo- and myoglobin was inhibited by a factor of > 100. The change in absorbance at 450 nm was measured during 2 min at 37°C in a thermostatted microplatereader (Thermomax, Sopachem, Driebergen, The Netherlands). Initial rates of enzyme activity were determined from the linear part of the curve, and converted to enzyme units using the molar extinction coefficient of o-DA [13] of 10,062 $M^{-1}$ $cm^{-1}$. We defined one unit of MPO as the amount of enzyme that oxidises o-DA at a rate of 1 µmol $min^{-1}$ under the present assay conditions. Under those assay conditions 1 U of bovine MPO (Sigma) catalyzes the oxidation of o-DA at a rate of 0.15 µmol $min^{-1}$. Enzymatic MPO activity was routinely confirmed by its complete inhibition with 1 mM sodium cyanide or 10 mM sodium azide.

To establish the relationship between neutrophil number and MPO activity, in eight experiments, the amount of MPO recovered from rat blood neutrophils was determined and amounted to 5.70 (± 0.87) $x10^{-7}$ U MPO per cell. This is similar to the value of $5.04x10^{-7}$ U reported earlier by Bradley *et al.* [14].

*Response to ALA-PDT.* The tumour response to ALA-PDT was determined separately in a total of 7 groups of animals (n=5). The first four groups were treated as described before, i.e., the control and the single illumination groups. The last three groups were treated with a two-fold illumination scheme, i.e., two light fractions of 100 J $cm^{-2}$ at 100 mW $cm^{-2}$ delivered at 1 and at 2.5 hours after ALA administration, under normal and neutropenic condition started either before the first or second illumination.

Tumour re-growth after treatment was monitored as described before [8]. In short, every 1 or 2 days following therapy the tumour volume was measured until the size of the tumour had reached 5 times its treatment volume. From these measurements the time point after PDT at which each tumour reached 100, 200, 300, 400 and 500% of its treatment volume was calculated and used to determine the mean time point in each treatment group. The mean tumour volume doubling time (VDT) defined as the number of days it took the tumour to double its pre-treatment volume determined the effectiveness of each treatment scheme.

*Statistical analysis.* Results are presented as means ± SD. The number of neutrophils in blood of rats was always compared to the pre treatment value. To evaluate the statistical significance of differences between means student-t test or analysis of variance was used followed where appropriate by a Student-Newman-Keuls test. A *P* value of less than 0.05 was considered to be statistically significant.

## Results

### *PpIX fluorescence pharmacokinetics*

Figure 1a shows the kinetics of PpIX fluorescence in rhabdomyosarcoma, normal skin overlying the tumour and in muscle adjacent to the tumour, following the administration of ALA. PpIX fluorescence peaked in tumour and muscle between 2 and 3 hours after the administration of ALA. At this time point there was approximately 3 times the fluorescence intensity in tumour compared to normal skin and muscle. The peak in PpIX fluorescence in normal skin was broader and occurred somewhat later, 4 hrs after the administration of ALA.

Figure 1b shows the relationship between the normal kinetics of PpIX fluorescence and those following illumination, 1 h after the administration of ALA. Illumination with increasing fluence (10, 20, 50 and 100 J $cm^{-2}$) at constant fluence rate resulted in progressively more PpIX photobleaching during illumination and a concomitant decrease in both the rate and extent of re-synthesis of PpIX after the illumination. The amount of PpIX re-synthesis in the superficial layers of the tumour following a first light fraction of 100 J $cm^{-2}$ (i.e. the light fluence used in the two-fold illumination scheme) was relatively small. Thirty one percent of that present immediately before the first light fraction (1 h after ALA) was available at the end of the dark interval. This is less than 10% of that present before a single illumination 2.5 hours after the administration of ALA.

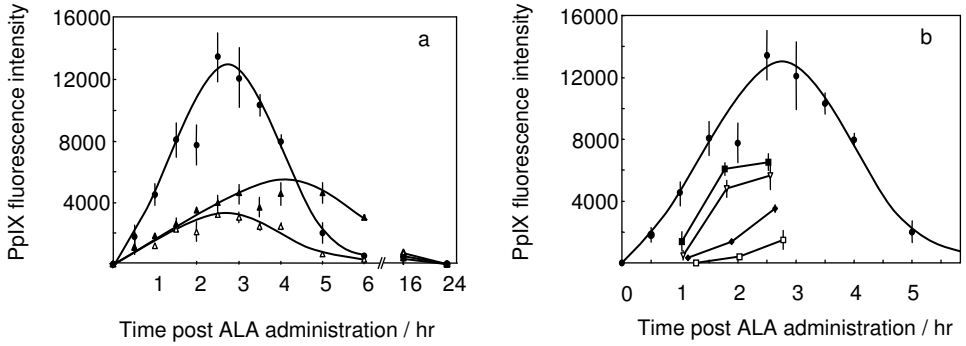


*Figure 1.* (a) The pharmacokinetics of PpIX following administration of 200 mg $kg^{-1}$ ALA (i.v.) administration in (●) rhabdomyosarcoma; (△) normal muscle; and in (▲) skin overlying the tumour. (b) The return in PpIX fluorescence in rhabdomyosarcoma following transdermal illumination (using 633 nm radiation at an irradiance of 100 mW $cm^{-2}$ at 1 hour after ALA administration) to a light dose of (■) 10; (▽) 20; (◆) 50 and (□) 100 J $cm^{-2}$, respectively, compared to the normal pharmacokinetics (●) shown in (a). Results are shown as mean ± S.D. and n = 5.

### *Neutrophil response to ALA-PDT*

The mean normal count of neutrophils in blood of all measured tumour-bearing WAG/Rij rats was found to be 1.29 ± 0.48 $10^9$ per litre blood. Treatment of the subcutaneously growing tumour with ALA-PDT resulted in a significant increase in the number of blood neutrophils during the first several days after illumination with the highest numbers at 16 and 24 hours after the start of PDT illumination (Figure 2a). This increase was significantly

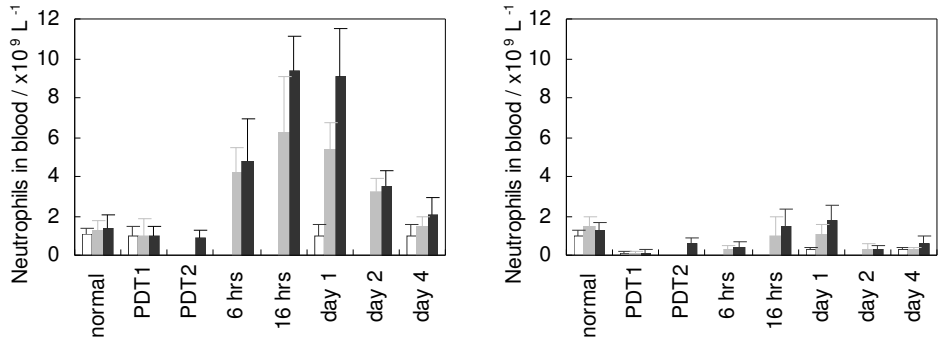


*Figure 2.* The time course of the number of neutrophils in the circulation of normal (a) and neutropenic rats (b) upon PDT using two different illumination regimens; control (white bar), single illumination (grey bar) and two-fold illumination (black bar). AGS was administered 30 minutes after ALA injection. Results are shown as mean ± S.D.

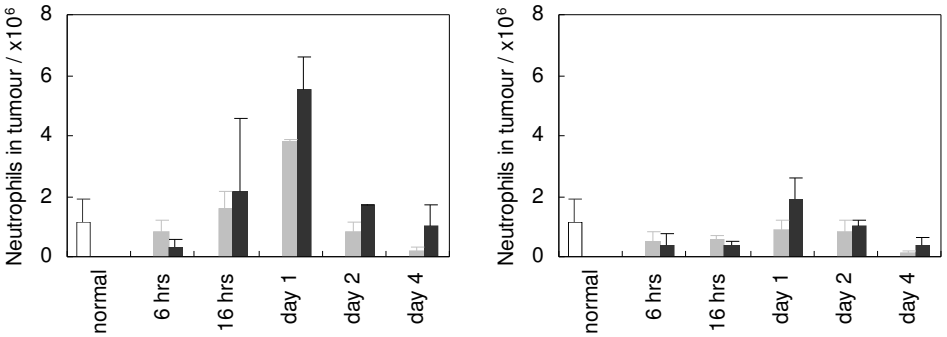


*Figure 3.* The time course of the number of neutrophils in the tumour of normal (a) and neutropenic rats (b) upon PDT using two different illumination regimens; control (white bar), single illumination (grey bar) and two-fold illumination (black bar). AGS was administered 30 minutes after ALA injection. Results are shown as mean ± S.D.

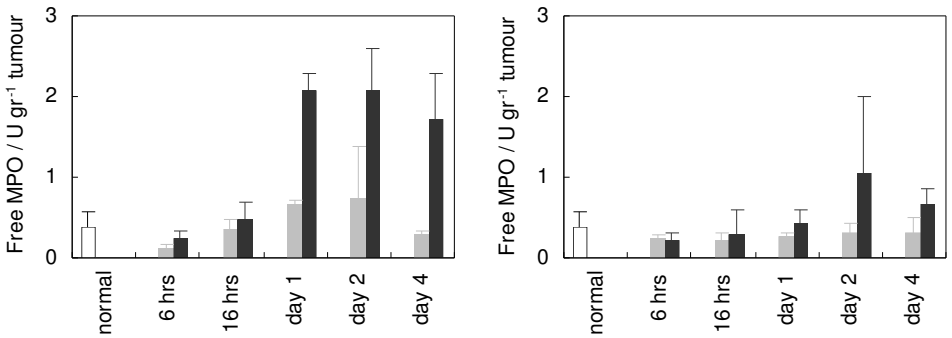


*Figure 4.* The time course of the release of myeloperoxidase (MPO) in tumours of normal (a) and neutropenic rats (b) upon PDT using two different illumination regimens; control (white bar), single illumination (grey bar) and two-fold illumination (black bar). AGS was administered 30 minutes after ALA injection. Results are shown as mean ± S.D.

higher when tumours were treated with the two-fold illumination scheme. At 16 hours the number of blood neutrophils increased 4.2 ± 2.6 fold after a single illumination of the tumour, and increased even further to a factor of 6.0 ± 2.5 after using the two-fold illumination scheme ($P$ =0.02 and 0.0001 resp.).

AGS treatment resulted in a decrease in circulating neutrophils to 11.5 ± 6.7% within 30 minutes after the first AGS injection ($P$ <0.0001). Using NaCl instead of AGS resulted in a decrease to 91.6 ± 39.8% ($P$ =0.67). At day 1 and day 4 of the AGS treatment the number of neutrophils increased a little to respectively 24.8 ± 15.6 and 26.5 ± 11.5% ($P$ = 0.001 and 0.01 compared to control). The time course of the number of neutrophils in the circulation of AGS-treated animals post ALA-PDT is shown in Figure 2b. After illumination of the tumour in neutropenic rats a slight elevation in the concentration of circulating neutrophils was found that never exceeded the normal pre treatment level. Also, no difference was found between the two different illumination schemes.

The influx of neutrophils in treated tumours was assessed by determining the amount of cell-associated MPO at different time points after ALA-PDT. The normal amount of neutrophils in the rhabdomyosarcoma was found to be 1.15 ± 0.7 $10^6$ per gram tumour. The accumulation of neutrophils in the tumour after ALA-PDT was maximal at 24 hours in immunocompetent rats (Figure 3a). Again, the influx of neutrophils was significantly higher in tumours treated with the two-fold illumination scheme amounting to an increase factor of 4.8 compared to 3.3 after a single illumination ($P$ <0.0001 and $P$ <0.001 compared to control). In rats treated by AGS the number of neutrophils in the tumour lesion remained within the pre treatment range after ALA-PDT (Figure 3b). Compared to the response in immunocompetent rats, the neutrophilic influx at 24 hours post treatment was depressed by a factor of 2.9 ($P$=0.008).

Upon activation, neutrophils release MPO [15]. In the tumours of immunocompetent rats the single illumination did not lead to a significant increase in the amount of free MPO in time after ALA-PDT (Figure 4a). The two-fold illumination, however, resulted in a significantly higher release of MPO in time from day 1 up to at least day 4 ($P$<0.0001 compared to control and $P$=0.004 compared to single illumination at day 1). Contrary, ALA-PDT did not elicited MPO release in tumours from AGS-treated rats (Figure 4b).

***Tumour response to ALA-PDT***

To study if the neutrophils in the circulation and/or tumour lesion contribute to the efficacy of ALA-PDT the growth delays of tumours treated under normal and neutropenic conditions were compared (Figure 5). The results show that administration of AGS did not affect tumour growth and the normal treatment volume doubling time (VDT) was 1.10 ± 0.11 days.

A single illumination one hour after ALA administration with 100 J $cm^{-2}$ at 100 mW $cm^{-2}$, resulted in a significantly prolonged treatment VDT of 4.08 ± 1.62 days ($P$=0.003). The VDT increased to a similar level under neutropenic conditions, i.e., to 3.43 ± 1.89 days (no significant difference between the two conditions).

A two-fold illumination at 1 and 2.5 hours after ALA administration with 100 + 100 J $cm^{-2}$ at 100 mW $cm^{-2}$, resulted in a significantly longer growth delay of 8.93 ± 4.77 days ($P$=0.006

compared to control). But again the increase in VDT did not differ from the increase in VDT when the illumination was performed under neutropenic condition regardless if that condition was evoked before the first or second illumination, respectively to 8.70 ± 4.67 and 8.82 ± 3.95 days.

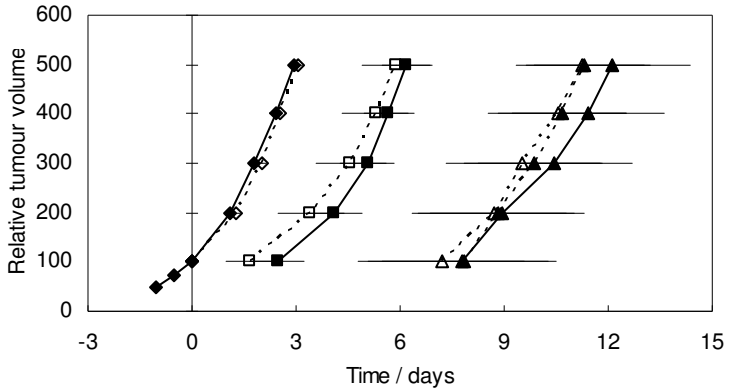


*Figure 5.* Growth delay of the rhabdomyosarcoma of normal (solid lines) and neutropenic rats (dashed lines) after ALA-PDT using two different illumination regimens; control (ALA only, ◆ and ◇), single illumination (1 hr, 100 J $cm^{-2}$ at 100 mW $cm^{-2}$, ■ and □) and two-fold illumination (1 + 2.5 hrs, 100+100 J $cm^{-2}$ at 100 mW $cm^{-2}$, ▲, △ and ▲). Neutropenia was induced before the first (△) or second illumination (▲). Results are shown as mean ± S.E.M. with n=5 per group.

## Discussion

The kinetics of PpIX fluorescence following systemic ALA-PDT in transplanted rhabdomyosarcoma were very similar to those that we have previously reported during topical ALA-PDT in normal mouse skin [12,15]. Again it is important to stress that while the original rationale for performing a two-fold illumination scheme was the utilisation of the re synthesised PpIX at the start of the second illumination the data presented earlier and here shows that this is not the case. In fact significantly more (~2.2 times) PpIX is available for a single illumination 2.5 hrs after the administration of ALA compared to the total amount of PpIX utilised in a two-fold illumination of 100+100 J $cm^{-2}$ 1 and 2.5 hrs after the administration of ALA ($P$<0.001). To find out which (alternative) mechanism underlies the improved outcome of ALA-PDT, we investigated the contribution of neutrophils to the efficacy of ALA-PDT and in particular their effect on the response of tumour tissue to a two-fold illumination scheme. First we analysed the neutrophilic response to ALA-PDT in immunocompetent rats. The number of neutrophils increased in the circulation and locally in the tumour with a maximum respectively at 16 hours and 1 day after the start of therapy. The neutrophils that accumulated in the tumour became activated as reflected by the release of MPO enzyme. Furthermore, the magnitude of the neutrophilic increase and activation was associated with the severity of the ALA-PDT treatment, i.e., a higher increase after the more effective two-fold illumination of the tumour.

The increase in the number of circulating neutrophils after ALA-PDT was higher and a little later in time compared to Photofrin mediated PDT. Cecic *et al.* reported a 2.5-fold and a 4-fold increase of circulating neutrophils in mice treated with Photofrin-PDT depending on the treated tumour [2]. This increase was seen up to 10 hours after therapy and at 24 hours the amount of neutrophils was decreasing again. In the present study we show a 4.9-fold increase after a single and a 6.7-fold increase after a two-fold illumination of the rhabdomyosarcoma tumour in rats at 16 hours.

The increase in the number of neutrophils in tumour after ALA-PDT was comparable or even larger than following Photofrin or HPPH-PDT. Tumour associated neutrophils increased by a factor of 3 using Photofrin-based PDT and HPPH-PDT at respectively 10 hours and 4 hours up to 3 days post PDT [2,4]. We found a 3.3-fold increase in tumours after a single illumination with ALA-PDT and even a 4.8-fold increase after a two-fold illumination at 24 hours.

The underlying mechanism of the accumulation of neutrophils in the circulation and at the lesion site was not studied here. Gollnick *et al.* has shown that neutrophils migrate into the treated area in a response to the chemokines MIP-2 and KC and the increased adhesion molecule expression of ICAM and E-selectin after HPPH-PDT [4]. The ischaemia and reperfusion injury caused by Photofrin-PDT might be the proinflammatory insult that causes the release of those chemokines [17,18]. The magnitude of the inflammatory reaction upon PDT is highly dependent on the therapeutic parameters [5]. Previously, we as well as others have shown that neutrophils are involved in the response of tissues to PDT, i.e. depletion of neutrophils results in less damage [6,17]. However, from the recent work of Henderson *et al.* using HPPH-PDT it became clear that the inflammatory response is not *critical* to tumour control [5]. In fact, they state that the crucial factor for optimal PDT is the preservation of a significant level of tumour oxygenation. Under optimal PDT treatment conditions achieved by a low fluence rate at a sufficient high fluence, tumour cells die by apoptosis and do not have a chance to elicit an inflammatory response by the release of MIP-2. Only after sub-optimal PDT the affected and surviving tumour cells have time to evoke an inflammation that contributes to a better outcome of the treatment [5].

In the present study we found that in neutropenic rats the efficacy of ALA-PDT using a single or a two-fold illumination scheme did not differ from those in immunocompetent rats. Apparently and contradictory to earlier findings using other photosensitisers, neutrophils do not appear to contribute significantly to the efficacy of systemic ALA-PDT. Despite the fact that a regimen-dependent inflammatory *and* treatment outcome under ALA-PDT was found, suggesting a direct relationship between the two, depleting the neutrophils under both light regimens did not decrease the efficacy of the PDT treatment. Why neutrophils do not contribute to the efficacy of ALA-PDT of this type of tumour is not immediately clear. If tumour cells have been lethally injured by PDT neutrophils entering the lesion site will be redundant and depletion of neutrophils will not significantly affect the therapeutic outcome. On the other hand, if tumour cells that have been affected only sub-lethally by ALA-PDT do not express the adequate cellular adhesion molecules on their cell membrane they may not be recognised by the neutrophils of the inflammatory exudate [19].

The evidence found here for a bystander role of neutrophils in ALA-PDT means that other factors should be taken into consideration to explain the response of tissues to ALA-PDT. Besides the fact that ALA-PDT can directly kill the tumour cells, such a factor could be complement activation [20]. Of relevance might also be the timing of the inflammatory response that became maximal between 16 and 24 hours after treatment. Obviously, further study is warranted to clarify this issue.

In summary, we have shown a systemic and local increase in the number of neutrophils in time after ALA-PDT of a rat rhabdomyosarcoma. The magnitude of the numerical increase of neutrophils is directly dependent on the severity of the illumination regimen. However, those neutrophils do not contribute significantly to the efficacy of systemic ALA-PDT and apparently only play a bystander role. The role of neutrophils in clinical PDT using topical ALA may be an area for future investigation.

***Acknowledgements***

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# Chapter 6

## Increase in protoporphyrin IX after 5-aminolevulinic acid based photodynamic therapy is due to local re-synthesis

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## Abstract

Protoporphyrin IX (PpIX) fluorescence that is bleached during aminolevulinic acid (ALA) mediated photodynamic therapy (PDT) increases again in time after treatment. In the present study we investigated if this increase in PpIX fluorescence after illumination is the result of local re-synthesis or of systemic redistribution of PpIX. We studied the spatial distribution of PpIX after PDT with and without cooling using the skin-fold observation chamber model. We were unable to show a correlation between the local PpIX fluorescence increase and the distance from a blood vessel. The spatial distribution of PpIX fluorescence within normal tissue or tumour is not changed in response to the illumination. These observations suggest that there is no diffusion of PpIX into the treated tissue. Cooling the tissue to 12°C, a temperature at which PpIX synthesis is inhibited, inhibited the PpIX fluorescence increase normally observed after illumination. We also found a strong correlation between local PpIX photobleaching during illumination and the fluorescence intensity one hour after illumination similar to what we have observed in patients treated with ALA-PDT. Therefore we conclude that the increase in PpIX fluorescence after illumination is due to local cellular re-synthesis.

## Introduction

The clinical response of human skin cancer to photodynamic therapy (PDT) using aminolevulinic acid (ALA) is significantly improved after illumination with two light fractions separated by a dark interval of two hours [1,2]. The increased efficacy of a two-fold illumination scheme was first shown in studies using pre-clinical animal models [3-6]. This type of light fractionation has been developed to utilise the PpIX fluorescence that returns after illumination [1,7-11]. The increased effectiveness of the two-fold illumination, however, can not simply be explained by the increase in PpIX fluorescence that is utilised as we found no relationship between the total amount of PpIX utilised and the efficacy [3,4,6,10]. Still, the return in fluorescence is important in some way as the photosensitiser is the start of the photochemical reaction initiating the damage.

While the kinetics and localisation of PpIX fluorescence after ALA administration have been investigated extensively [5,9-17] only a few studies were performed investigating the PpIX fluorescence kinetics after illumination [5,9-11] and little is known about the spatial distribution or the source of PpIX fluorescence after illumination. In both preclinical [3-6,9-11] and clinical [1,7,18] circumstances PpIX fluorescence increases again after illumination. Orenstein *et al.* [18] even showed a difference in the amount of return in PpIX fluorescence for superficial and nodular BCC and suggested that this is due to a continued PpIX production in the deeper located areas and diffusion of PpIX to the surface. The increase in fluorescence after illumination could either be the result of local PpIX re-synthesis or of systemic PpIX redistribution or a combination of both. It is important to determine this as it may have important implications on the cellular localisation of the photosensitiser and the possible mechanism of action to the two-fold illumination as a result of that [19].

Systemic redistribution requires transport of porphyrins through the circulation, followed by passive diffusion of PpIX from the vasculature into the tissue. In this case fluorescence images recorded in time after illumination should show a gradient of fluorescence around the blood vessels. Henderson *et al.* [16] has shown circulating porphyrins after topical ALA application on mouse skin showing that systemic redistribution of PpIX is theoretically possible. Local re-synthesis, on the other hand, requires local enzyme activity. Several investigators have shown that the enzymatic conversion of ALA to PpIX is inhibited at a temperature below 15°C. [20,21]. In case of local re-synthesis cooling the tissue below this temperature should inhibit the fluorescence increase in time after PDT.

In the present study we determined the source of the return in PpIX fluorescence after illumination in the skin-fold observation chamber model. In the chamber we can distinguish tumour, vessels and normal tissue allowing us to investigate the spatial distribution of PpIX fluorescence after ALA-PDT in a 2-dimensional geometry. We have determined the relationship between the fluorescence kinetics at different distances from the vessel and the vascular response during and immediately after PDT. We have also determined the effect of cooling the tissue to a temperature at which the enzymatic conversion of ALA to PpIX is inhibited [20,21] on the fluorescence kinetics after illumination. While we hypothesised in a

previous study that the tissue localisation of PpIX fluorescence might be involved in the mechanism of action behind the increased effectiveness of ALA-PDT [6] we also investigated the spatial distribution of PpIX fluorescence within tissue and determined the influence of the first light fraction on the fluorescence distribution after illumination.

## Materials and Methods

*Animal model.* Skin-fold observation chambers were prepared using a slightly modified technique to that that has been described previously [24,26]. Briefly, the chamber was prepared on the back of female 5 weeks old fisher-344 rats in four operations spread over a period of two weeks. As a result of these operations a thin layer of subcutus tissue was clamped between mica and a cover slide without obstruction of the circulation (Figure 1a). All operations were carried out under general Ethrane/$O_2$/$N_2O$ anaesthesia. In the first operation sterile air (12 ml) was subcutaneously injected on the back of the rat to gently separate the skin from the underlying tissues. In the second operation, a few days later, a home made plastic ring and cover slide was positioned under the skin above the subcutaneous tissue containing vessels. The air of the first operation is still below this subcutaneous tissue. In the third operation, one week later, the skin was folded, prepared and fixed in the splint so that it can be placed under the microscope for observation. In the final operation the R3230AC tumour was transplanted in the layer of normal tissue, which could be reached by unscrewing the cover slide on top of the chamber. Within four to eight days the chamber was ready for treatment when the transplanted tumour was supported by blood vessels and had grown.

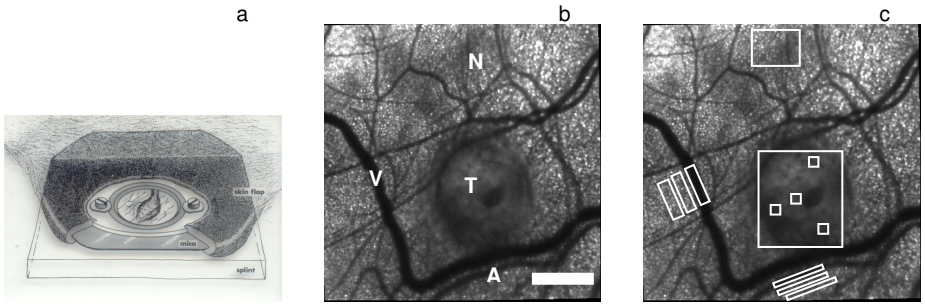


*Figure 1.* (a) A schematic drawing of the chamber on the back of a rat. (b) Typical transmission image of the skin-fold observation chamber, diameter 1 cm. Ideally the chamber consists of different types of tissues and vessels; tumour cells and capillaries (T), normal cells and capillaries (N), arterioles (A), venules (V). The white bar represents 1 mm. (c) A typical example of the different regions of interest in which the fluorescence was determined. The different regions were: normal tissue, vascular tissue accompanied by the two perpendicular regions in which the correlation to the distance from the vessels was determined and tumour tissue including the four smaller areas in which the heterogeneity within the tumour was determined.

Ideally the chamber contained fat cells and capillaries over an area of approximately 1 cm in diameter with some supporting arterioles and venules within. In a small number of animals one or more types of tissue was not available. Table 1 shows the actual number of animals used per tissue type for each experimental group. The animal experiments committee of the Erasmus University Medical Centre approved the experimental protocol

*Experimental design*. The animals were divided over five experimental groups. The PpIX fluorescence kinetics after ALA administration was determined in the first group. In three groups the increase in PpIX fluorescence in time after ALA-PDT was investigated. PDT was performed one hour after systemic ALA administration, the time point of the first illumination of a two-fold illumination scheme, using 514 nm light at a fluence rate of 50 mW $cm^{-2}$ to a fluence of 5, 50 or 100 J $cm^{-2}$. Fluorescence and transmission images were recorded every 30 minutes until 2.5 hours after the end of illumination. In the final group the effect of cooling on the PpIX fluorescence increase after ALA-PDT was investigated. PDT was performed one hour after ALA administration using 514 nm light at a fluence rate of 50 mW $cm^{-2}$ to a fluence of 100 J $cm^{-2}$. Immediately after completion of PDT the tissue was cooled to 10-12°C for one hour. Thereafter the temperature of tissue was allowed to return to normal (28 - 30°C). Fluorescence and transmission images were recorded before and after PDT after cooling and 30 minutes after the temperature had returned to normal.

*Table 1. The number of animals used for each tissue type*

| Fluence | Normal | Tumour | Arterioles | Venules |
|---|---|---|---|---|
| 0 J $cm^{-2}$ | 3 | 3 | 3 | 3 |
| 5 J $cm^{-2}$ | 8 | 6 | 7 | 8 |
| 50 J $cm^{-2}$ | 4 | 4 | 4 | 4 |
| 100 J $cm^{-2}$ | 7 | 6 | 6 | 7 |
| 100 J $cm^{-2}$ cool | 10 | 7 | 6 | 10 |

*ALA preparation and administration.* 5-aminolevulinic acid hydrochloride (ALA, Medac, Wedel, Germany) was dissolved in sterile 0.9% NaCl infusion solution to a concentration of 90 mg $ml^{-1}$. A freshly prepared ALA solution was administered i.v. to a dose of 200 mg $kg^{-1}$ body weight under ethrane/$O_2$/$N_2O$ anaesthesia. NaOH 4M was added to the solution to obtain pH 5-6. After PDT animals were kept under subdued light conditions for the first 24 hours.

*Experimental set-up for PDT illumination, fluorescence and transmission imaging.* PDT illumination and fluorescence/transmission imaging was accomplished using the same experimental set-up. The animal was placed on a temperature-controlled X-Y stage. For PDT illumination green, 514 nm, excitation laser light (Spectra Physics, Darmstadt, Germany) with a fluence rate of 50 mW $cm^{-2}$ was projected on the backside of the whole chamber using a system of condensing lenses to produce a uniform fluence rate distribution.

Fluorescence and transmission images were recorded before ALA administration, at the start and at the end of the PDT treatment and every 30 minutes until 2.5 hours after

illumination. The fluorescence and transmission images recorded at the start and end of illumination were collected within the treatment session. For the fluorescence and transmission images recorded before ALA administration and in time after illumination the same excitation light was used although at a lower fluence rate to prevent additional PDT induced tissue damage. The extra delivered fluence due to these measurements was approximately 0.15 J $cm^{-2}$ per measurement times five is 0.75 J $cm^{-2}$ per animal. Light transmitted through the chamber was imaged onto a Peltier-cooled 16 bit, 512 x 512, slow scan CCD camera (Princeton Instruments Inc., Princeton, USA) using a f2.8/105 mm macro lens. The different detection filters were placed in a filter wheel (Oriel, Stratford, USA) between the macro lens and the CCD camera in order to obtain the fluorescence (625 ± 20 nm) and transmission (514 ± 2 nm) images. Before each measurement a fluorescence standard, an inert plastic card, was recorded to correct for small differences in excitation light intensity. The PDT treatment and fluorescence measurements were carried out under general Ethrane/$O_2$/$N_2O$ anaesthesia. Between measurements animals were conscious and placed in a dark and warm environment.

*Chamber cooling*. In one group of animals the chamber tissue was cooled immediately after illumination with 100 J $cm^{-2}$ (1 h after the administration of ALA) for one hour. A copper rod was placed in iced water in direct contact with the mica on the base of the window chamber and a small reservoir of iced water on top of the chamber. The temperature of the cover slide on top of the chamber was monitored continuously during the cooling period using a thermocouple. In all cases the temperature of the cover slide on top of the chamber was < 12°C within 8 minutes after the end of illumination and maintained between 10 -12°C for one hour. Warming was initiated by removing the iced water from the top and the copper rod from the bottom of the chamber. Within 2 minutes the temperature of the cover slide on top of the chamber had returned to normal (28-30°C). The body of the animal was placed on a temperature-controlled stage to maintain normal body temperature. The general anaesthesia that was used during PDT was maintained during the cooling period.

*PpIX fluorescence kinetics of different tissue types.* Fluorescence and transmission images were recorded at several time points; before ALA administration (autofluorescence), before PDT illumination and in time after PDT illumination. Fluorescence images were corrected for intensity differences using a reference standard. The sequence of fluorescence images from each animal was registered by translation and rotation using anatomical landmarks identified in the corresponding transmission images. The registration of images enabled us to determine the fluorescence intensity of each tissue type from the same area. In the corresponding transmission image the regions of interest were chosen for each tissue type as shown in Figure 1. Tumour and normal tissue regions of interest were chosen so that no large chamber vessels were in or close to the region. The heterogeneity of the fluorescence in tumour was determined in four smaller regions in the tumour area as observed using white light microscopy. The position of these four arias was determined in the fluorescence image collected at the start of illumination with the aim to investigate the highest and lowest fluorescing aria in the centre and at the border. The relationship between the increase in PpIX fluorescence and distance from an arteriole and a venule was

investigated by determining the return in fluorescence within three regions of interest associated with each vessel. A rectangular region of which the width of the short side was equal to the width of an arteriole was placed within an arteriole. A second and third rectangle was placed adjacent to the arteriole at increasing distances from it. These regions were carefully chosen so that no other vessel was close to the arteriole under investigation. A similar procedure was followed for venules.

*Vascular response.* We distinguish two vascular responses; the change in diameter of arterioles and venules and the disruption in flow. The change in vascular diameter due to the treatment was scored at the end of PDT, 60 and 90 minutes after PDT using the collected transmission images. While the original vessel size was variable between animals we scored the change in vascular diameter in percentages of constriction. No change in vessel diameter was scored 0, mild vasoconstriction (less then 50%) was scored 1, severe vasoconstriction (more than 50%) was scored 2 and complete vasoconstriction was scored 3. The status of the blood flow in tumour and normal capillaries was determined at the end of PDT and 2 hours after PDT using 50 and 100 J $cm^{-2}$ using white light microscopy. While it is our experience that capillary flow in the chamber model is not fluently we used a rough discrimination and scored flow (0) or no flow (1). In normal tissue we determined the size of the region whereas tumours were scored when all capillaries showed stasis.

*Statistics.* Student t test was used to determine significance for the fluorescence kinetics measurements and vascular damage scores. The Spearman-rank test was used to determine the significance of the relationship between the photobleaching and re-synthesis of PpIX. Results with a *P* value below 0.05 were considered significant. Data is presented as mean ± SD.

## Results

### *Fluorescence kinetics after ALA administration*

The autofluorescence of tumour, vessels and normal tissue was highly variable but not significant different (3368 ± 1380 counts, n=117). At one hour after ALA administration the fluorescence intensity in tumour tissue was 1.5 times higher compared to normal tissue (9551 ± 4834 counts and 6451 ± 3069 counts respectively with *P*=0.004). Vessels and normal tissue showed no significant difference in fluorescence intensity over the investigated time frame. The fluorescence kinetics for all tissues reached a plateau at approximately the same intensity 2.5 hours after administration (11597 ± 739 for tumour and 10196 ± 1327 counts for normal tissue).

### *Fluorescence kinetics after PDT in tumour, vessels and normal tissue*

Figure 2 shows the increase in fluorescence after illumination using different fluences for tumour, normal tissue, arterioles and venules. Illumination with 5 J $cm^{-2}$ resulted in photobleaching in tumour and normal tissue to 69 and 76% respectively of that present before illumination. Similar amounts of photobleaching were observed in arterioles and

venules (74% of the initial fluorescence intensity for both). Thirty minutes after illumination the average fluorescence intensity in each tissue type increased to the initial fluorescence intensity. The fluorescence kinetics thereafter closely followed that of the ALA only control. Illumination with 50 J $cm^{-2}$ resulted in relatively more photobleaching with 39, 51, 47 and 48% of the fluorescence intensity remaining in tumour, normal tissue, arterioles and venules respectively. The increase in fluorescence in time after PDT was less than that following illumination with 5 J $cm^{-2}$. Illumination with 100 J $cm^{-2}$ showed similar levels of photobleaching and increase in fluorescence compared to that observed with 50 J $cm^{-2}$.

Figure 3 shows the correlation in individual tissue locations between the extent of photobleaching during the illumination and the increase 1 hour after PDT in tumour and normal tissue in this model (Spearman rank correlation, $r_S = 0.56$; CI, 0.316 - 0.735; $P = 0.0004$). The fluorescence intensity at the start of illumination was inhomogeneous in most

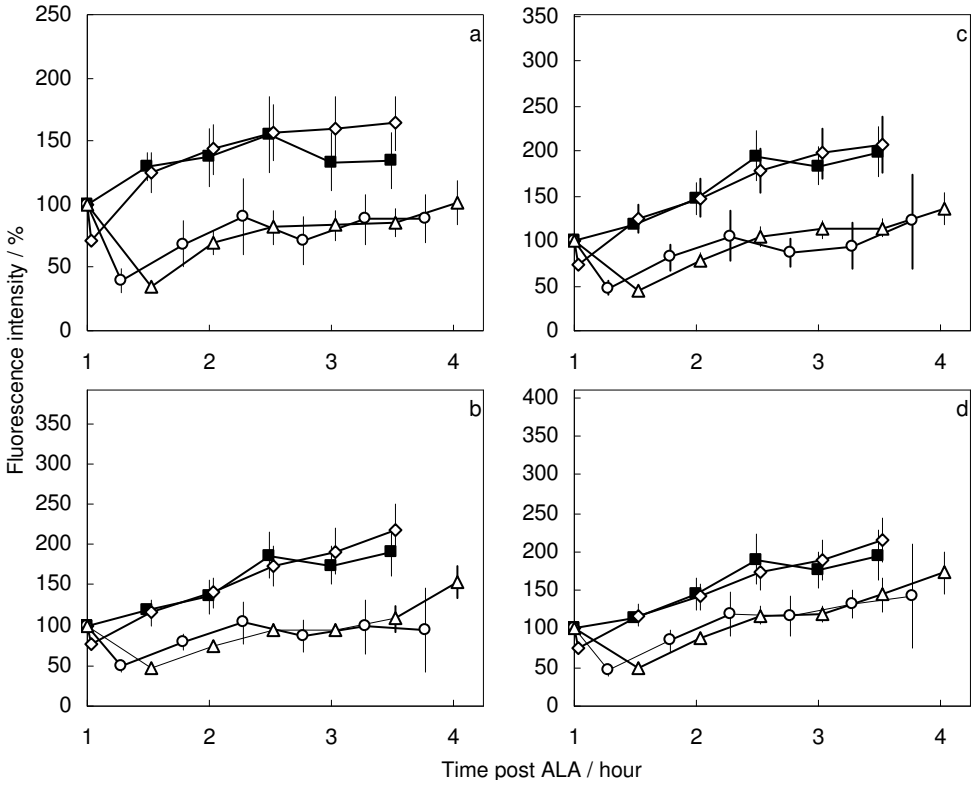


*Figure 2*. Normalised PpIX fluorescence kinetics after PDT at one hour after ALA administration using different light doses (■ no PDT, ◇ 5 J $cm^{-2}$, ○ 50 J $cm^{-2}$, △ 100 J $cm^{-2}$) in tumour (a), normal (b), arteriole (c) and venule (d) tissue. Data was normalised to the pre-illumination fluorescence intensity for each individual rat and each tissue type. Differences between tissue types in the pre-illumination fluorescence intensity are displayed in the relative re-scaling of the Y-axis of the kinetics graphs. Results are shown as mean ± sem.

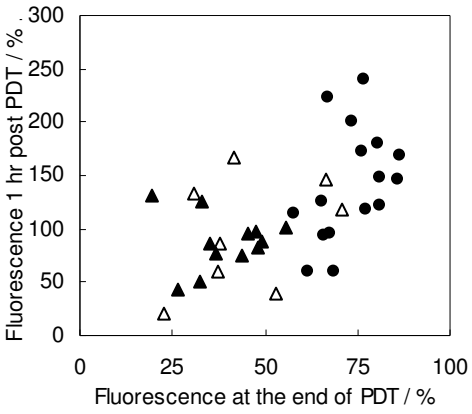


*Figure 3.* The relative return in PpIX fluorescence 1 hour after ALA-PDT using different fluences (● 5 J cm$^{-2}$, △ 50 J cm$^{-2}$, ▲ 100 J cm$^{-2}$) in relation to the relative fluorescence at the end of PDT of normal and tumour tissue. Rank correlation = 0.56, 95% CI = 0.316 - 0.735, *P* = 0.0004

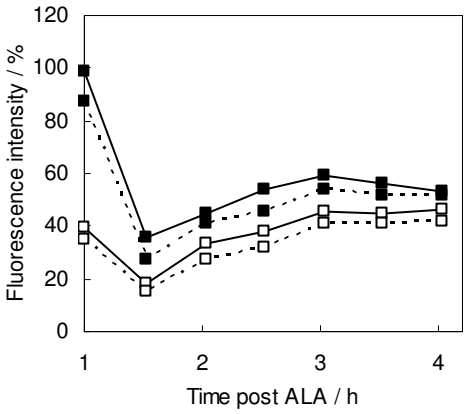


*Figure 4.* The relative PpIX fluorescence kinetics in time after ALA-PDT using 100 J cm$^{-2}$ in two representative tumours (■ and □). Data is normalised to the region with the highest fluorescence intensity measured before illumination (■) to show the intra-animal variation. For each tumour data from the highest (solid line) and lowest (dashed line) fluorescence intensity region are displayed to show the inter-tumour variations.

tumours. In more than two thirds of tumours, the difference between the highest and lowest intensity region was greater than 15% and showed no correlation with the location in the tumour, i.e., the centre or border. Figure 4 illustrates the distribution of PpIX fluorescence and shows that the variation within a single tumour is much smaller than the variation between tumours. Overall these variations in fluorescence intensity observed at the start of illumination were not significantly different in time after PDT with any of the light doses investigated in the present study

***Fluorescence kinetics after PDT in relation to the distance from the vessels***

The fluorescence increase after ALA-PDT was independent of the distance from the nearest vessel. The average diameter for arterioles was 51 ± 25 µm (n=20). The two regions in which the fluorescence increase in relation to the distance from the arteriole was determined were at a distance of 84 ± 38 µm and 217 ± 70 µm. The average diameter for venules was 126 ± 54 µm (n=23). The two regions in which the fluorescence increase in relation to the distance from the arteriole was determined were at a distance of 127 ± 25 µm and 368 ± 115 µm. While we observed no difference in the return of fluorescence in relation to the distance from a vessel for all fluences investigated we only the results after illumination with 100 J cm$^{-2}$ (Figure 5).

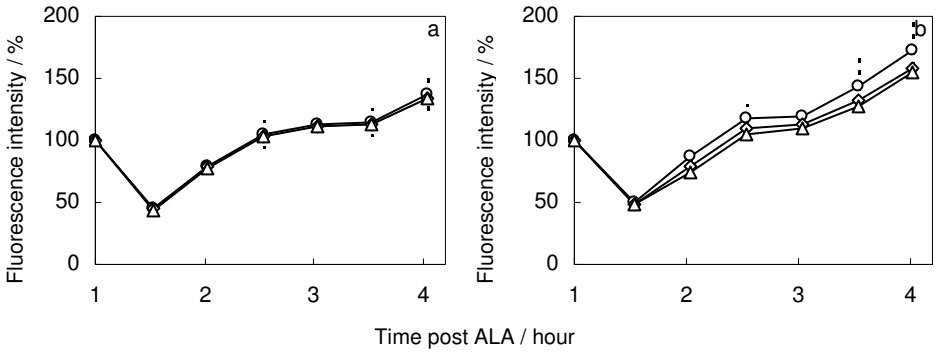


*Figure 5.* The PpIX fluorescence kinetics in time after PDT at one hour after ALA administration using 100 J cm$^{-2}$ in relation to the distance from an arteriole (a) or a venule (b); (○) in the vessel, (◇) close to the vessel and (△) further away form the vessel. Results are shown as mean ± sem.

***Vascular response immediately after ALA-PDT***

Figure 6 shows the vascular response of arterioles and venules in the first hours after PDT using different light fluences and Table 2 shows the number of animals that showed stasis of capillary flow in tumour and normal tissue. Immediately after illumination with 5 J cm$^{-2}$ none of the blood vessels in the chambers showed vasoconstriction. Only 1 out of 8 animals showed mild vasoconstriction of both the arteriole and the venule 60 minutes after PDT. The venule returned to the normal diameter within 30 minutes, the arteriole was still mildly constricted at 90 minutes after PDT.

Illumination with 50 J cm$^{-2}$ resulted in significantly more animals showing arteriole constriction compared to 5 J cm$^{-2}$ ($P$ = 0.01, 0.009 and 0.009 immediately, 60 and 90 minutes after illumination respectively). At the end of PDT 3 out of 4 animals showed arteriole constriction ranging from mild to complete developing in severe constriction at 60 and 90 minutes. Only two animals showed venule constriction, one mild and one severe in the hours after illumination. Immediately after PDT all animals showed normal flow in the capillaries of normal and tumour tissue. Two hours after PDT 2 out of 3 animals showed stasis in a small region in the normal tissue whereas the blood flow in the tumour was not hampered.

Illumination with 100 J cm$^{-2}$ resulted in arteriole constriction in all animals at the end of PDT; 2 animals showed severe and 4 showed complete constriction. At 60 and 90 minutes after PDT 3 out of 6 showed a small recovery resulting in 1 normal, 3 severely constricted and 2 completely constricted arterioles. Compared to 50 J cm$^{-2}$ the damage was more severe although not statistically significant ($P$ = 0.08, 0.38 and 0.49; immediately, 60 and 90 minutes after illumination). No change in diameter of the venules was observed at the end of PDT. At 60 to 90 minutes after PDT 2 out of 7 showed mild constriction. The constriction of venules after 100 J cm$^{-2}$ was not statistically different from that observed after 5 and 50 J cm$^{-2}$. Immediately after illumination the 3 out of 6 animal showed large areas of blood stasis in the capillaries of normal tissue. The number of animals showing stasis and the region size

increased in time after PDT. For tumours the result was comparable; 2 out of 5 tumours showed complete stasis in all capillaries immediately after PDT increasing to 4 out of 6 tumours 2 hours after PDT.

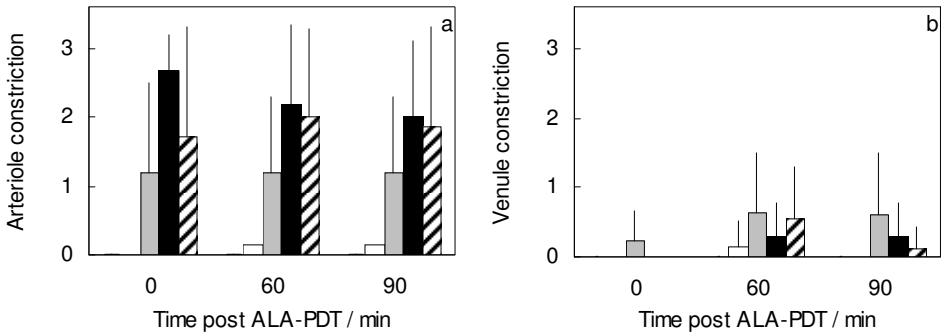


*Figure 6.* Arteriole (a) and venule (b) constriction at 0 60 and 90 minutes after ALA-PDT using different light doses; 5 J cm$^{-2}$ (white bar), 50 J cm$^{-2}$ (dotted bar), 100 J cm$^{-2}$ (black bar) and 100 J cm$^{-2}$ followed by 1 hour of cooling (dashed bar). Results are shown as mean ± sem.

*Table 2. Capillary stasis in response to ALA-PDT. The number of animals showing stasis of the blood flow in tumour and normal capillaries immediately and two hours after PDT. In normal tissue the size of the region showing stasis was determined whereas tumours were scored when all capillaries showed stasis.*

| | Normal tissue | | Tumour tissue | |
|---|---|---|---|---|
| Fluence | 0 | 2 hrs | 0 | 2 hrs |
| 50 J cm$^{-2}$ | 0 / 3* | 2 (25%) / 3* | 0 / 3* | 0 / 3* |
| 100 J cm$^{-2}$ | 3 (60%) / 6* | 5 (79%) / 7 | 2 / 5* | 4 / 6 |

* the response of one animal could not be determined

***Influence of temperature***

The vascular response observed in the first hours after ALA-PDT was not influenced by the drop in local tissue temperature to 10-12°C for one hour after the end of illumination (Figure 6). Microscopically, we did not observe blood stasis before or after cooling in the arterioles and venules in any of the animals. Figure 7 shows that the fluorescence increase in time after illumination using 100 J cm$^{-2}$ was temperature dependent. During illumination the fluorescence bleached to 35, 43, 49 and 49% of the initial fluorescence intensity in tumour, arterioles, venules and normal tissue respectively. After one hour of cooling to 10-12°C there was no significant increase in fluorescence in arterioles and tumour ($P$ = 0.68 and 0.28, respectively). There was a small increase in fluorescence both in venules and in normal tissue ($P$ = 0.04 and 0.03 respectively) but this increase was lower compared to the increase in the normal tissue and venules of animals kept at normal temperature ($P$ = 0.002 and 0.03, respectively). Subsequent warming the chambers to normal conditions after the cooling period resulted in an increase in PpIX fluorescence in all of the tissues investigated.

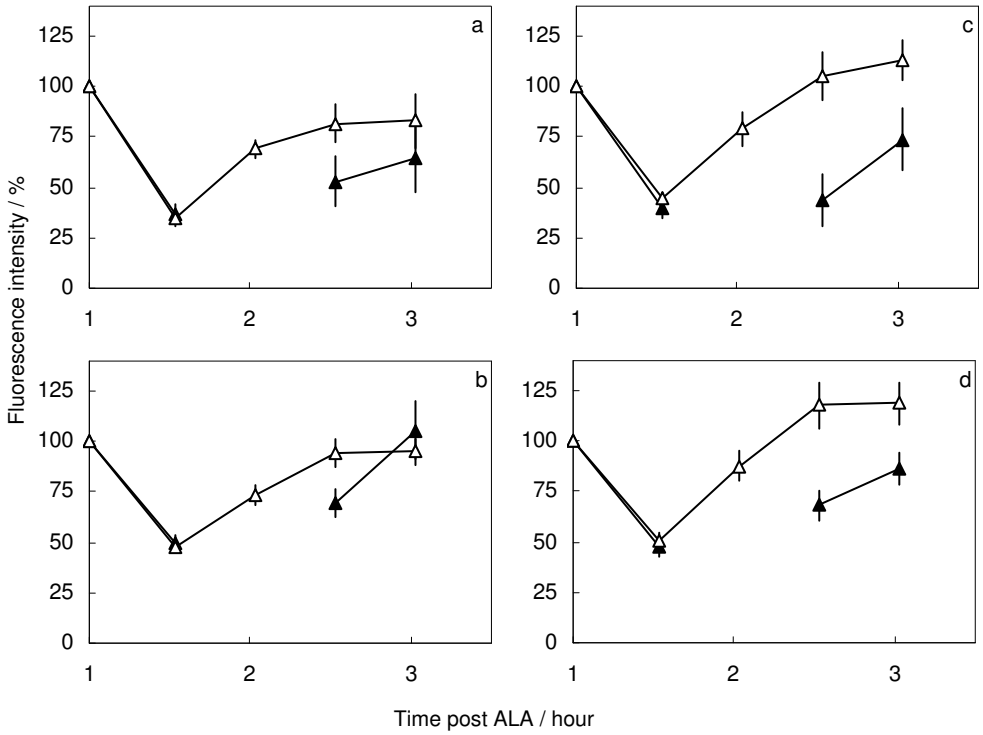


*Figure 7.* Normalised PpIX fluorescence kinetics after PDT at one hour after ALA administration using 100 J $cm^{-2}$ either followed with one hour of cooling the tissue to 10-12°C (▲) or kept at normal temperature (△) in tumour (a), normal (b), arteriole (c) and venule (d) tissue. Results are shown as mean ± sem. * significant less PpIX increase at this time point compared to normal temperature groups. ** no significant PpIX increase at this time point compared to immediately after PDT.

## Discussion

The aim of the present study was to determine if the increase in PpIX fluorescence after ALA-PDT is due to local re-synthesis or systemic redistribution. We investigated this by studying the spatial distribution of PpIX after PDT with and without cooling in the skin-fold observation chamber model. By cooling the tissue to a temperature at which no PpIX is formed (10-12°C) [20,21] we were able to inhibit the return in PpIX fluorescence after illumination (Figure 7). Also we found that PpIX fluorescence increases after the tissue is returned to normal temperature. These observations are consistent with those of Juzenas *et al.* in the skin after topical ALA application [20]. It is important to consider that changing the temperature of tissue has been shown to affect the vessel diameter. Untank [22] showed that cooling normal rat skin from 35 to 25°C resulted in significant constriction of arterioles in the subcutus. ALA-PDT is also known to induce vasoconstriction and stasis [16,23,24]. The

combined effects of cooling and PDT may influence our ability to detect the systemic redistribution of PpIX. We have shown that the extent of vasoconstriction following PDT is dependent on the light fluence (Figure 6). Although 60% of the animals treated with 100 J $cm^{-2}$ show complete arteriole constriction immediately after illumination the circulation in the chamber is not completely shut down; the venules and most capillaries are still flowing (Table 2). Cooling the tissue for one hour after ALA-PDT with 100 J $cm^{-2}$ did not result in significantly more vasoconstriction of both arterioles and venules. Based on our results we assume that the blood supply within the chamber after PDT is similar with or without cooling. Our results on the spatial distribution of the fluorescence kinetics in and around vessels (Figure 5) shows there is no correlation between the distance from a blood vessel and the rate of PpIX fluorescence increase after PDT independent of the vascular response of that vessel. This result contradicts the hypothesis of Diagaradjane *et al.* [17] that the return in PpIX after systemic ALA-PDT might be the result of diffusion from the surrounding tissue. Although Henderson *et al.* [16] showed circulating porphyrins after topical application of ALA to mouse skin we show that the level of fluorescence in a blood vessel was as high as the surrounding tissue suggesting that the amount of circulating porphyrins, if any, is small. In Figure 3 we demonstrate a strong correlation between the local level of photobleaching and the fluorescence increase one hour after PDT. More photobleaching during illumination suggests more damage to the tissue resulting in a reduced capacity to convert ALA into PpIX, reflected in a lower fluorescence increase 1 or 2 hours after illumination. This result is consistent with that we have observed in our clinical study treating superficial BCC using topical ALA-PDT [1]. Also other investigators [7,18] have shown re-appeared PpIX fluorescence after ALA-PDT in both superficial and nodular BCC. All our data supports the conclusion that the return in PpIX fluorescence after PDT is the result of local re-synthesis. That we show this to be true in an animal model after systemic ALA administration implies that this is also true after topical ALA. In the clinical situation a systemic redistribution of PpIX after PDT using topical ALA application is unlikely due to the much smaller ALA dose to body mass ratio in humans.

The rate and magnitude of PpIX re-synthesis after illumination in the window chamber is relatively high compared to our previous studies using other pre-clinical models [5,10,11]. In normal mouse [5] or in pig skin [11] the re-synthesis kinetics were dependent on the fluence delivered but did not increase to the pre-PDT level even after illumination with a small fluence. In transplanted rhabdomyosarcoma we have also shown that re-synthesis is dependent on the fluence delivered [10]. In this model illumination with a small fluence resulted in a fluorescence intensity higher than the pre-PDT level although not to the dark control. Apparently the tissue under investigation, the main difference between these and the present study, has an influence on the fluorescence kinetics observed after PDT. In mouse skin epidermal cells that are supported by capillaries in the dermis dominate the fluorescence data. The transplanted rhabdomyosarcoma model consists of tumour cells, connective tissue and vessels. The subcutis of the chamber is highly vascularised and contains fat cells and transplanted tumour cells. Consistent to the observations of Roberts *et al.* [25] our results in Figure 2 and 5 show that the vascular endothelium synthesises PpIX and

contributes significantly to the fluorescence intensity. Interestingly, 5 out of 48 lesions treated in our clinical study also show a fluorescence increase to the pre-PDT value or higher two hours after illumination.

Although the increase of PpIX after illumination was the motivation for designing a two-fold illumination scheme we have shown earlier that the mechanism of action behind this scheme in ALA-PDT is more complicated as we found no correlation between the total amount of PpIX utilised and the efficacy [3,4,6,10]. Recently [6] we hypothesised that the spatial distribution of PpIX and the site of PDT response within the illuminated volume is an important factor in the mechanism underlying the two-fold illumination scheme. One could even imagine that the first light fraction influences the spatial distribution of PpIX fluorescence. In the present study we show that the PpIX fluorescence intensity at the start of PDT was very variable between animals and within each chamber as shown by a representative example (Figure 4). Although the preparation of the chambers was standardised it was impossible to create chambers with standard tissue thickness or vascular structure density. The heterogeneity in the tumour fluorescence intensity as shown in Figure 4 might be explained by the heterogeneity in their oxygen supply and metabolic activity. More important is the observation that the spatial distribution of PpIX (re)-synthesis in the tumour is not influenced by the illumination. A tumour area that showed little fluorescence compared to the rest of the tumour also showed a relatively lower rate of re-synthesis after illumination independent of the fluence used. Also the observed differences between tumour, vascular and normal tissue in fluorescence intensities at the start of PDT remained the same after illumination. Apparently, the relative capacity to convert ALA to PpIX is equally affected in tumour, vascular or normal tissue. It is important to bear in mind that the tissue in this model is highly vascularised which means that the fluorescence kinetics determined in tumour and normal tissue also contain information from the vascular endothelial cells of the capillaries.

In summary; we have shown that cooling the tissue to 10-12°C inhibited the PpIX fluorescence increase after illumination. We were also unable to show a gradient of fluorescence around the vessels. Therefore we conclude that the increase in PpIX fluorescence after illumination is the result of local cellular re-synthesis in rats after systemic ALA administration. Furthermore we have shown that the spatial distribution of fluorescence within normal tissue and tumour is not changed after PDT.

***Acknowledgements***

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# Chapter 7

## Light fractionation does not enhance the efficacy of methyl 5-aminolevulinate mediated photodynamic therapy in normal mouse skin

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## Abstract

Previous work demonstrated that fractionated illumination using two fractions separated by a dark interval of 2 hours, significantly enhanced the clinical efficacy of photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA). Considering the increasing clinical use of methyl 5-aminolevulinate (MAL) and the expected gain in efficacy by light fractionation we have investigated the response to MAL-PDT using a single and a two-fold illumination scheme and compared that with ALA-PDT. Our results show that fractionated illumination does not enhance the efficacy of PDT using MAL as it does using ALA despite the comparable fluorescence intensities at the end of the first light fraction and at the start of the second light fraction. Only the initial rate of photobleaching was slightly greater during ALA-PDT although the difference was small. Previously we hypothesised that cells surviving the first fraction are more susceptible to the second fraction. Since this is not true for MAL-PDT our data suggest that the distribution of MAL and ALA in tissues, and therefore the site of PDT induced damage, is an important parameter in the mechanism underlying the two-fold illumination scheme.

## Introduction

Photodynamic therapy employing the administration of porphyrin precursors has been under investigation for over a decade [1]. It is now the treatment of choice for superficial non-melanoma skin cancer [2] and an experimental therapy for a number of other conditions [3,4]. Recently we have shown that light fractionation using a 2-hour dark interval enhances the response of superficial basal cell carcinoma (sBCC) to ALA-PDT significantly [5,6]. The increased effectiveness of this type of illumination scheme was first shown in a series of pre-clinical studies [7-10]. Initially the fluence was split in two equal light fractions. In a further series of pre-clinical studies using the hairless mouse model we optimised the illumination scheme [10-12]. The efficacy of the two-fold illumination scheme increases when the fluence of the first light fraction is relatively low compared to the second light fraction. These data guided us to the design of two clinical studies. The first, a pilot study used a non-optimised two-fold illumination scheme with equal light fractions [5]. While the long-term response data in this study showed that complete response rate (CR) remains high compared to CR in the literature, we found no significant difference in CR 1 year after PDT. The second, large-scale randomised comparative study using the optimised illumination scheme showed a significant increase in CR of sBCC at one year compared to ALA-PDT in a single light fraction [6].

A number of alternative approaches targeted at enhancing the efficacy in ALA-PDT have been investigated. These have focussed on improving the uptake of ALA and/or the accumulation of protoporphyrin IX (PpIX) using penetration enhancers, iontophoresis, and iron chelators [13-17]. An important development in this area has been the use of other porphyrin precursors that are derivatives of ALA [18-21]. The use of methyl 5-aminolevulinate (MAL/Metvix®) has been studied extensively both in-vitro and clinically. MAL based PDT is now an approved treatment modality for actinic keratosis (AK) in the USA and for non-melanoma skin cancers, such as sBCC and Bowen's disease, in Europe and Australia. The recommended treatment protocol involves the topical application of MAL cream (160 mg $g^{-1}$) for 3 hours followed by a single red light illumination and a repetition of this treatment one week later [22]. Considering the increasing clinical use of MAL and the expected gain in efficacy by light fractionation we have investigated the response of normal hairless mouse skin to MAL-PDT using a single and a two-fold illumination scheme. Although every two-fold illumination scheme ever investigated using ALA resulted in more damage compared to a single illumination scheme we chose to investigate the most effective scheme [11,12]. We compare these data to our earlier published data on ALA-PDT using the same illumination schemes [12].

## Materials and Methods

*Animal model.* Female inbred albino hairless mice (SKH1 HR, Charles River, Someren, The Netherlands), aged between 8 and 10 weeks old, were included in this study. Prior to

treatment animals were fed on a chlorophyll free diet (Hope Farms b.v., Woerden, The Netherlands) for a minimum of two weeks in order to remove the autofluorescence emission from mouse skin centered on 675 nm attributed to pheophorbide-a. The animal experiments committee of the Erasmus University Medical Centre approved the experimental protocol.

*Chemicals*. The ALA cream was freshly prepared as described previously [23]. Twenty percent 5-aminolevulinic acid (ALA, Medac, Hamburg, Germany) dissolved in 3% carboxymethylcellulose in water. To prevent skin irritation, the ALA solution was prepared to approximately pH 4 by the addition NaOH (2 M). MAL (Metvix®, 160 mg $g^{-1}$, Galderma, Freiburg, Germany) or ALA was topically applied to a 7 mm diameter area on the dorsal skin and covered with a thin layer of gauze. A polythene dressing (Tegaderm, 3M, The Netherlands) was used to occlude the area for 4 hours prior to treatment. Before application of cream animals received anaesthesia (Hypnorm; fluanisol/fentanyl mixture, Janssen Pharmaceutics, Belgium or Ketamine; Alfasan Woerden, The Netherlands and Diazepam, Centrafarm b.v., Etten-Leur, The Netherlands) to alleviate possible anxiety caused by the dressing.

*Experimental design.* The visual and histological response to MAL-PDT using either a single or a two-fold illumination was investigated in two separate series of experiments using the normal mouse model. We have previously shown that visual response data obtained in this model correlates well with the visual response of UVB induced tumour and tumour growth delay [4,5]. We have also shown that optimisation of response in normal mouse skin can lead to the design of clinically relevant fractionation schemes [1,2]. In the first series the visual skin damage in the first 7 days after MAL-PDT was investigated (n=6, in each) and compared to that after ALA-PDT using data obtained previously [12]. A power analysis showed that this number of animals would be sufficient to resolve a difference in response to the single and two-fold illumination similar to that observed after ALA-PDT. In a second series of experiments the histological damage after PDT with ALA and MAL was investigated. In total 14 groups of mice (n=5-6) were treated. Two groups served as ALA and MAL dark controls in which skin samples were collected at the end of the application period. In the 12 remaining groups skin samples were collected at 2.5, 24 or 48 hours after the end of illumination. In both series the PpIX fluorescence kinetics were recorded during illumination.

*PDT light delivery and fluorescence detection*. After 4 hours of topical application with ALA or MAL the skin was illuminated using either a single light fraction of 100 J $cm^{-2}$ at 50 mW $cm^{-2}$ or a two-fold illumination scheme with 5+95 J $cm^{-2}$ at 50 mW $cm^{-2}$ separated by a 2 hour dark interval. The experimental set-up used for the PDT illumination and fluorescence detection was based on that described previously [24]. The 514 nm output from the argon ion laser was focussed on a 7 mm diameter spot of homogeneous profile on the skin of the mouse. The fluorescence emission from the illuminated area was focussed through a system of lenses onto a CCD camera (ORCA-ER, Hamamatsu, Japan). Excitation light was filtered using a dichroic mirror (535 nm, Omega Optical, Barttleboro, US) and a 635 nm interference filter (Melles Griot, Didam, The Netherlands). Fluorescence images were collected before pre-cursor application, during application and during illumination using the therapeutic light without interruption of the illumination using different light fluences and

different integration times. Before each measurement a fluorescence standard was recorded to correct for these differences. The fluence used for each fluorescence measurements before and during the application period was approximately 0.005 J $cm^{-2}$ at 0.5 mW $cm^{-2}$. Mice were anaesthetised with a combination of 2% Ethrane or Isoflurane (Abbott, Amstelveen, The Netherlands) oxygen and $N_2O$ for each illumination or fluorescence measurement.

*Visual skin damage.* The biological damage to the irradiated area was assessed daily by two independent observers (HSB and APH) blinded from the treatments using the visual skin damage scoring system described previously [25]. No change in skin colour was scored as 0. Scores 1 to 3 were used for increasing discoloration of the skin, 1 meaning minimal redness and 3 meaning severe redness. Thin crust formation was scored as 4 and thick crust formation was scored as 5. Damage was observed to be inhomogeneous in a number of treatments. Photographs were taken regularly in order to determine the degree and distribution of damage. The damage score of the total illuminated area in one animal at one time point was calculated by scoring areas according to the degree of damage related to the contribution to the total illuminated area. The visual skin damage of a single mouse was quantified by calculating the area under the curve of skin damage when plotted against time, over the first 7 days resulting in the integrated damage score.

*PpIX fluorescence imaging and data analysis.* Fluorescence images were recorded before application of the precursor (autofluorescence), during the application period at 1 and 2 hours and during illumination (every 10 seconds). The mean gray scale value was calculated over a large field at the centre of the illuminated area (approximately 65%). To calculate the absolute PpIX fluorescence the background values and the individual autofluorescence were subtracted. A series of measurements during an illumination was normalised to the first measurement of that illumination. The rate of photobleaching over the first 5 J $cm^{-2}$ of each illumination was determined as the slope of the reciprocal of the normalised PpIX fluorescence using linear regression fitting [24]. In total 79 mice were illuminated. Data of 16 mice was excluded from analysis due to technical reasons like computer storage failures or focussing and movement problems, resulting in n=12 for the two ALA-PDT groups and n=20 and 19 for the MAL-PDT groups treated according to a single or a two-fold illumination scheme respectively.

*Histology.* A pathologist (KH) blinded from the treatments scored the histological response by analysing the H&E stained sections of the obtained skin samples. The response of the different layers of the skin was scored separately. We distinguished the epidermis, the upper dermis, the lower dermis and muscle. Tissue damage was observed and scored according to 4 categories: coagulative necrosis (eosinophilia, loss of structure and undetectable nuclei) was scored 3, karyorhexis (eosinophilia, loss of structure but (fragmented) nuclei could still be recognised) was scored 2, pyknosis (nuclei were shrunk and showed basophilia) was scored 1 and no visual damage was scored 0. The most severe damage observed was scored. Epidermal thickening (hyperplasia) and demarcation of dead tissue by dense polymorphonuclear infiltrates/crust formation were signs of sub-lethal and

lethal cellular damage, respectively. Expected late effects are loss of hair follicles and sebaceous glands and dermal fibrosis.

*Statistics.* The significance of differences in PDT damage and rate of photobleaching was determined using the student-t test and $P$ <0.05 was considered significant. Results are shown as mean ± standard deviation.

## Results

### *Response to PDT*

The mean visual skin damage scored over the first 7 days post MAL-PDT with a single or a two-fold illumination scheme is plotted in Figure 1. Thin crusts were formed already at day one after MAL-PDT using a single light fraction that developed into thick crusts over time. These crusts became detached at day 4. Light fractionation using the two-fold illumination scheme didn't show a significantly different response. The skin damage at day 1 was less pronounced, crusts were formed in all animals but became detached between day 3 to 5.

The mean of the integrated visual skin damage scores over day 1-7 was 22.6 ± 1.2 and 21.1 ± 3.1 for the single and the two-fold illumination scheme respectively. The use of a two-fold illumination scheme after MAL application did not result in significantly more PDT induced visual damage compared to a single illumination scheme ($P$=0.29) in contrast to the results previously obtained with ALA-PDT [12].

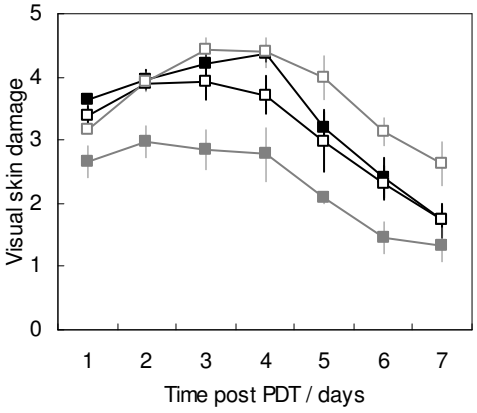


*Figure 1.* The visual skin damage scores for the first 7 days after MAL-PDT. All groups were illuminated with 50 mW cm$^{-2}$ to a cumulative light fluence of 100 J cm$^{-2}$ delivered in a single fraction (■) or in two fractions (5+95 J cm$^{-2}$ with a 2 hours dark interval) (□). Mean ± SD (n=6 in both groups). The results after ALA-PDT are shown in gray with n=4 and 11 for a single and a two-fold illumination respectively [12].

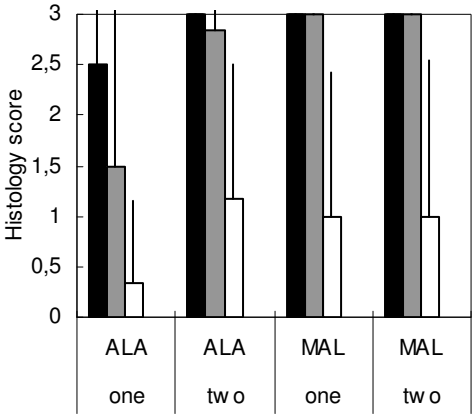


*Figure 2.* The mean PDT induced damage scored using histology at 24 hours ALA or MAL-PDT using a cumulative fluence of 100 J cm$^{-2}$ delivered in a single fraction or in two fractions (5+95 J cm$^{-2}$ with a 2 hours dark interval). The most severe damage was scored in each layer of the skin separately; epidermis (black bar), upper dermis (gray bar) and lower dermis (white bar). Mean ± SE.

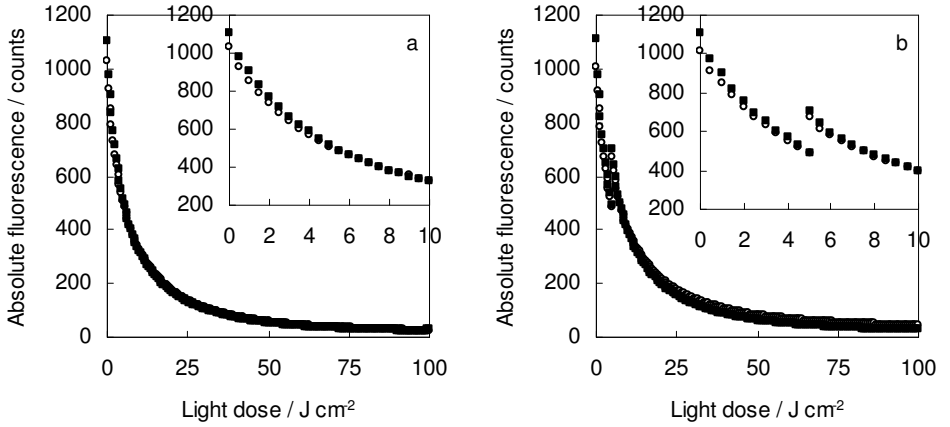


*Figure 3.* The mean PpIX fluorescence during illumination with 50 mW cm$^{-2}$ to 100 J cm$^{-2}$ delivered in (a) a single fraction or in (b) two fractions (5+95 J cm$^{-2}$ with a 2 hours dark interval) after topical ALA (■) or MAL (○) application (n=12 for both ALA groups and 20 and 19 for the MAL groups respectively). The results over the first 10 J cm$^{-2}$ are shown in the inserts for clarity. Mean ± SD.

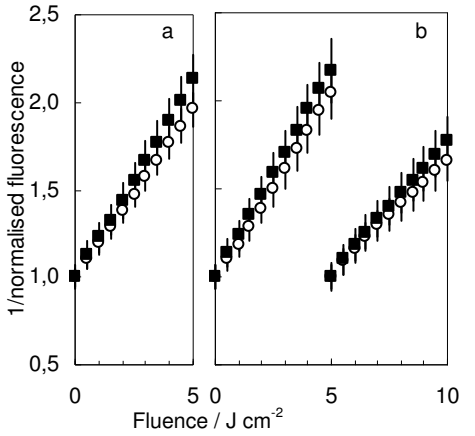


*Figure 4.* The weighted mean reciprocal of the normalised PpIX fluorescence during the first 5 J cm$^{-2}$ of each light fraction of illumination with 50 mW cm$^{-2}$ to 100 J cm$^{-2}$ delivered in (a) a single fraction or in (b) two fractions (5+95 J cm$^{-2}$ with a 2 hours dark interval) after topical ALA (■) or MAL (○) application (n=12 for both ALA groups and 20 and 19 for the MAL groups respectively). Mean ± SD.

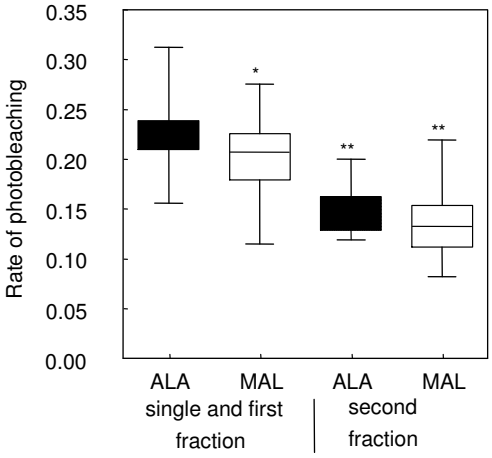


*Figure 5.* A box & whisker plot of the rate of photobleaching for during the first 5 J cm$^{-2}$ of each illumination after topical ALA or MAL application. * significant difference in rate of photobleaching with P=0.005 between MAL and ALA. ** significant difference in rate of photobleaching with P< 0.001 between the first and the second light fraction of the two-fold illumination.

In general, the histological observations were in agreement with the visual skin damage scores; after a single illumination MAL-PDT resulted in more damage compared to ALA and the increase in damage after a two-fold illumination was not seen using MAL. At 2.5 hours after the end of PDT all groups showed a similar picture of no damage or only slight pycnosis in the epidermal layer. Thereafter marked differences occurred. While with ALA both a single and a fractionated illumination resulted in a variable response that slightly increased from 24 to 48 hours, MAL-PDT showed a deeper damage in more animals for both illumination and both time points. Figure 2 shows the mean histological response score for each skin layer at each time point for both ALA and MAL. Note that the most severe damage observed in the tissue layer is scored and that we have not compensated for differences in spatial distribution of damage as we have done in the visual skin damage scoring system. Non-illuminated controls showed no damage.

***Fluorescence intensity during MAL and ALA application***

The mean PpIX fluorescence kinetics after topical MAL or ALA application and during illumination are shown in Figure 3. An insert shows the kinetics during the first 10 J $cm^{-2}$ for clarity. The autofluorescence of normal mouse skin was 39.3 ± 9.7 counts. The PpIX fluorescence 4 hours after the application of ALA or MAL was not significantly different (1107 ± 358 or 1021 ± 408 counts respectively, $P$ =0.40). The relative fluorescence intensity during the application period at 1 and 2 hours was higher using ALA compared to MAL (15 ± 2% and 10 ± 6% at 1 h and 47 ± 11% and 32 ± 13% at 2 h respectively, $P$ =0.17 and 0.22).

***Fluorescence kinetics during MAL and ALA-PDT***

There was no significant difference in the extent of photobleaching during the first illumination using ALA or MAL (46.8 ± 3.3% and 50.1 ± 5.3% respectively, $P$ =0.06). Furthermore, we saw no significant difference in the amount of PpIX re-synthesis during the dark interval between the two light fractions (62.1 ± 14.0% and 71.3 ± 24.6% respectively, $P$ =0.25). The photobleaching kinetics during illumination followed a second order decay for both porphyrin precursors. This is illustrated by the linearity of the reciprocal of the normalised PpIX fluorescence, as a function of light fluence. Figure 4 shows the kinetics of photobleaching over the first 5 J $cm^{-2}$ for each light fraction. Figure 5 shows the variation between animals in the calculated initial rate of photobleaching for each light fraction. Combining photobleaching data from the first 5 J $cm^{-2}$ of the single illumination scheme and the first light fraction of the two-fold illumination, PpIX fluorescence bleached at a greater rate following the application of ALA compared to MAL. While this difference is small it is significant ($P$ = 0.005; n=39 for MAL-PDT and n=24 for ALA-PDT). The rate of PpIX photobleaching during the second light fraction of the two-fold illumination was not significantly different using ALA or MAL although for both PpIX precursors it was significantly lower compared to the first light fraction.

## Discussion

Contrary to our expectations, we have found significant differences in the response of normal mouse skin to MAL-PDT compared to ALA-PDT [12]. We have shown that there is significantly more visual skin damage following MAL-PDT compared to ALA [12] using a single light fraction although the difference was small and may not be clinically relevant. More importantly, we have also shown that light fractionation does not enhance efficacy in MAL-PDT in the way it does using ALA. Histological examination 24 and 48 h after therapy supports these findings. While the differences in response to PDT using ALA and other pro-drugs of ALA is an interesting subject for investigation they may also expand our understanding of the mechanism underlying the response of tissues to PDT using the two-fold illumination scheme. Numerous in-vitro studies have compared the uptake of ALA and its derivatives, and investigated porphyrin synthesis and cell survival [26,27]. However relatively few in-vivo pre-clinical studies have compared the pharmacokinetics of PpIX synthesis [28] and importantly none, to our knowledge, have investigated the response to PDT. Only two clinical studies have compared the clinical response of AK [29] and BCC [30] to ALA and MAL-PDT. Both studies found no difference in clinical response to PDT with ALA and MAL although they were probably insufficiently powered to detect small differences in response of the magnitude that we have observed in the present study.

The pharmacokinetics of PpIX fluorescence in normal mouse skin during the 4-h application period is similar for both porphyrin pre-cursors. At 1, 2 and 4 hours during the application period the fluorescence intensity is not significantly different for animals receiving MAL or ALA. This is in agreement with the results reported by Moan who found similar PpIX fluorescence intensities and concentrations in mouse skin after 1 and 5 hours topical ALA or MAL application [28]. We have chosen a 4-h application period for both precursors. While this is longer than the 3 hours that is normally used clinically Juzenas *et al.* [31] have shown that there is no clear difference in PpIX fluorescence intensity using an application time of 3 or 4 hours. That equal PpIX fluorescence intensities are found immediately prior to the start of the first light fraction is advantageous since large variations in initial PpIX concentration can influence visual skin response for standardised illumination conditions [24].

The kinetics of PpIX fluorescence during the two-fold illumination scheme shows that we do not observe significant differences in the extent of photobleaching after each light fraction between ALA and MAL. The kinetics of PpIX fluorescence during MAL-PDT are similar to that published by Moan *et al.* [32]. Also we observed similar amounts of PpIX re-synthesis in the dark interval between light fractions for both ALA and MAL. Therefore the total amount of PpIX utilised during the 2-fold illumination is not significantly different for MAL and ALA. The fact that these observations are accompanied by a dramatically different PDT response adds weight to our previous conclusions that the total amount of PpIX re-synthesised during the dark interval is not involved in the mechanism of action behind the response of tissues to the two-fold illumination scheme [11,33]. We have previously shown that the PDT dose delivered during the first light fraction is a critical parameter that can strongly influence the effectiveness of the 2-fold illumination scheme [10,11]. Furthermore we have shown that the

rate of PpIX photobleaching can be used as an indirect measure of the amount of singlet oxygen produced during PDT and can be used to report PDT dose and correlates with response [24,25,34,35]. Recently direct evidence for the relationship between the generation of singlet oxygen during PDT and visual skin response in normal mouse skin has been reported [36]. Monitoring photobleaching during fractionated ALA-PDT gives an important indication of the PDT dose delivered. A faster rate of photobleaching correlates with a higher PDT dose. In the present study we observe that the rate of photobleaching during the first light fraction of ALA-PDT is slightly but significantly greater than that of MAL-PDT. According to our current understanding of the magnitude of the PDT dose delivered during the first light fraction on the response of tissues to the 2-fold illumination scheme, this difference in the rate of photobleaching is small and does not explain the lack of an increase in efficacy in MAL-PDT. The rate and extent of PpIX photobleaching during the first light fraction of MAL-PDT is well within the margins of what is considered optimal using ALA [11].

An important difference between ALA and MAL, and other ester derivatives of ALA, is their tissue distribution due to their different biophysical and biochemical characteristics. The cellular uptake of MAL is different from that of ALA [37]. While the vehicle in which the precursors are dissolved might also effect the distribution this seems less important since Moan *et al.* [28] used the same vehicle for both MAL and ALA and still showed a different distribution. The authors detected PpIX fluorescence at distant sites after topical ALA application whereas it remained within the application site after MAL application. From this observation they concluded that ALA is systemically distributed after topical application whereas MAL is not. The consequences of these effects for the distribution of ALA or MAL and therefore PpIX within the treatment volume are unknown. It is possible that there is a different distribution of PpIX following the application of ALA and MAL and this has an important effect on the response of tissues to PDT. The present study is limited by the fact that normal mouse skin is relatively thin (approximately 50 μm) and layered and that the PpIX fluorescence is monitored from the surface of the skin. The microscopic distribution of PpIX fluorescence within mouse skin has not been systematically investigated. Besides the difference in (depth) distribution of MAL or ALA within the tissue also other factors may play a role. The microenvironment of a cell may be different when it is loaded with MAL or ALA due to the differences in molecular structure and processes involved in the de-esterification of MAL. How these factors affect the PDT response in mouse skin is unknown but they have not been found to be important factors in vitro [26,27].

A different distribution of MAL and therefore PpIX in the treatment volume leads to a different site of PDT damage and could explain the difference in visual damage following ALA and MAL-PDT using the single illumination scheme. The small but significantly lower initial rate of PpIX photobleaching during MAL-PDT (Figure 5) may be a consequence of a different microenvironment due to a different localisation of PpIX. We acknowledge that superficial photobleaching measurements are probably not a particularly sensitive method of determining differences in photosensitiser localisation. It is important to recognise that it is necessary to perform a first light fraction followed by a two-hour interval to observe a significant increase in response using ALA [12]. Previously we have hypothesised that cells

surviving the first light fraction are more susceptible to the second light fraction [12]. Since this is not true for MAL-PDT the site of PDT induced damage should be considered as an important parameter in the mechanism underlying the 2-fold illumination scheme. Apparently the site of PDT induced damage combined with the two-hour dark interval between light fractions is important for the increased efficacy observed using light fractionation during ALA-PDT.

We have previously shown that pre-clinical results obtained in normal mouse skin using ALA-PDT can be successfully translated to a clinical treatment protocol for sBCC that results in a significantly improved CR [6]. The results of the present study imply that simply combining MAL-PDT and the most effective two-fold illumination scheme, determined for ALA, in the clinic would not enhance the efficacy of MAL-PDT using a single light fraction. It is possible that the illumination parameters used in the present study are not optimal for MAL. However, it is important to note that light fractionation in ALA-PDT always enhances the response of tissue compared to a single illumination. We note that more pre-clinical research on the mechanism behind the increased effectiveness of the two-fold illumination using ALA and the mechanism of PDT induced damage using MAL is needed to confirm this assumption.

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# Chapter 8

## Microscopic localisation of protopophyrin IX in normal mouse skin after topical application of 5-aminolevulinic acid or methyl-5 aminolevulinate

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## Abstract

Light fractionation does not enhance the response to photodynamic therapy (PDT) after topical methyl-aminolevulinate (MAL) application whereas it is after topical 5-aminolevulinic acid (ALA). The differences in biophysical and biochemical characteristics between MAL and ALA may result in differences in localisation that cause the differences in response to PDT. We therefore investigated the spatial distribution of protoporphyrin IX (PpIX) fluorescence in normal mouse skin using fluorescence microscopy and correlated that with the PDT response histologically observed at 2.5, 24 and 48 hours after PDT.

As expected high fluorescence intensities were observed in the epidermis and pilosebaceous units and no fluorescence in the cutaneous musculature after both MAL and ALA application. The dermis showed localised fluorescence that corresponds to the cytoplasm of dermal cells like fibroblast and mast cells. Spectral analysis showed a typical PpIX fluorescence spectrum confirming that it is PpIX fluorescence. There was no clear difference in the depth and spatial distribution of PpIX fluorescence between the two precursors in these normal mouse skin samples. This result combined with the conclusion of Moan *et al.* that ALA but not MAL is systemically distributed after topical application on mouse skin [Moan *et al*., Int. J. Cancer 103 (2003) 132-135] suggests that endothelial cells are involved in increased response of tissues to ALA-PDT using light fractionation. Histological analysis 2.5 hours after PDT showed more oedema formation after ALA-PDT compared to MAL-PDT that was not accompanied by a difference in the inflammatory response. This suggests that endothelial cells respond differently to ALA and MAL-PDT. Further investigation is needed to determine the role of endothelial cells in ALA-PDT and the underlying mechanism behind the increased effectiveness of light fractionation using a dark interval of 2 hours found after ALA but not after MAL-PDT.

## Introduction

Previously we reported the difference in response to light fractionated photodynamic therapy (PDT) between methyl-aminolevulinate (MAL) and 5-aminolevulinic acid (ALA) [1]. Using MAL we were unable to increase the efficacy of PDT by the use of a two-fold illumination scheme in which the two light fractions are separated by a dark interval of two hours. This is different from ALA-PDT where the response following the two-fold illumination scheme is significantly increased compared to the response to a single illumination scheme as demonstrated in several pre-clinical and clinical studies [2-5]. While the mechanism behind the increased efficacy is still unknown [3,6-8] this difference between MAL and ALA warrants further investigation.

The use of ester derivatives of ALA was suggested since they are more lipophillic and are therefore expected to result in an enhanced bio-availability compared to ALA [9]. Differences in the biophysical and biochemical characteristics of MAL and ALA may be of importance for the response to PDT and potentially to the difference in the response to fractionated PDT. For example, Moan *et al.* [10] showed that prolonged application with ALA resulted in different PpIX fluorescence at distance from the applied area whereas it did not after MAL application. They concluded that ALA but not MAL becomes systemically distributed after topical application leading to the formation of PpIX at distant sites. It is known that the local distribution of photosensitiser in cells and tissue can have a critical impact on the response to PDT [11,12]. Furthermore Rud *et al.* [13] have shown that the cellular up-take mechanism is different for MAL compared to ALA suggesting that certain cell populations may show differential uptake of ALA and MALA.

Although there are techniques available to determine ALA or MAL concentrations they can't be used to investigate the spatial distribution within a tissue section. The fluorescence of the accumulated PpIX observed after MAL or ALA application can give an indication of the spatial distribution. In a previous study [1] we have found comparable PpIX fluorescence intensities after 4 hours of topical application of MAL and ALA. This result was obtained using surface fluorescence measurements therefore little is known about the distribution of PpIX within the tissue. In the present study we investigated the spatial distribution of PpIX fluorescence within normal mouse skin after topical application of either MAL or ALA using fluorescence microscopy. These results are correlated with the histological response after MAL and ALA-PDT using the single and the two-fold illumination scheme.

## Materials and Methods

*Animal model.* The animal experiments committee of the Erasmus University Medical Centre approved the experimental protocol. We investigated the localisation of PpIX within tissue after topical application with MAL and ALA for 4 hours in three groups of female inbred albino hairless mice (SKH1 HR, Charles River, Someren, The Netherlands), aged

between 8 and 10 weeks. Prior to the experiment, mice were fed on a chlorophyll free diet (Hope Farms B.V., Woerden, The Netherlands) for at least two weeks to minimise the contribution of pheophorbides to the autofluorescence emission spectrum.

*Porphyrin precursor application.* Prior to the application of porphyrin precursor mice were anaesthetised using 2% Isoflurane (Abbott, Amstelveen, The Netherlands) oxygen and $N_2O$. One group served as control receiving the vehicle alone (3% carboxymethylcellulose in water). The two other groups received either ALA or MAL cream. ALA or MAL (Metvix®, 160 mg $g^{-1}$, Galderma, Freiburg, Germany) cream was topically applied to a 7 mm diameter area on the dorsal skin and covered with a thin layer of gauze. ALA cream was freshly prepared as described previously [14]. In brief, 20% (w/v) 5-aminolaevulinic acid (ALA, Medac, Hamburg, Germany) was dissolved in 3% carboxymethylcellulose in water. NaOH (2M) was added to adjust the pH to approximately 4. A polythene dressing (Tegaderm, 3M, The Netherlands) was used to occlude the cream for 4 hours. During the application period the animals were placed in a dark and warm environment. At the end of the application period the mice were again anaesthetised using 2% Isoflurane (Abbott, Amstelveen, The Netherlands) oxygen and $N_2O$ to remove the cream, collect the surface fluorescence measurement and harvest the skin sample.

*Tissue handling and fluorescence microscopy.* The applied skin area was excised immediately after removal of excess cream at 4 hours after the start of topical application. It was snap frozen in liquid nitrogen and stored at -80°C until preparation for fluorescence microscopy. Tissue tec II (Leica, Leiden, The Netherlands) embedding compound was used to mount the skin sample on the sample holder of the cryostat. Cross-sections were cut (20 and 5µm) and singularly mounted on clean glass slides (Menzel, Braunschweig, Germany). Skin samples and sections always handled under subdued lighting conditions to prevent unwanted photobleaching of PpIX. Sections were allowed to thaw at room temperature for between 30 minutes and one hour after sectioning and before imaging.

Fluorescence microscopy images were acquired using a cooled CCD camera (Hamamatsu ORCA-ER, Hamamatsu photonics Germany GmbH, Herrsching am Ammersee, Germany) mounted on DM RB fluorescence microscope (Leica, Leiden, The Netherlands) equipped with a N2.1 filter cube (Leica, Leiden, The Netherlands). This cube contains a 515-560 nm band pass excitation filter and 590 nm long pass detection filter. Light from the sample was additionally filtered using a 635 ± 5 nm interference filter (BP635, Melles Griot, Didam, The Netherlands). Tissue sections were imaged using 5 and 40 times objectives to interrogate various structures in epidermis and dermis. The fluorescence images were collected using an integration time of 120 seconds. Before recording the image the tissue section was localised and focussed using red light filtered through a 695 nm long pass filter to minimise photobleaching. A fluorescence image of a KV 470 filter (Schott, Tiel, The Netherlands) was recorded as a spatial fluorescence standard. All images of tissue samples were divided by the corresponding image of the fluorescence standard to correct for spatial in-homogeneity in the beam profile and differences in excitation light intensities.

Fluorescence spectra were also acquired from these tissue sections under blue light excitation using a 405 nm filter in combination with a 460 short pass filter. The fluorescence

light was filtered through a 460 nm long pass filter and focussed into an optical fibre which was connected to the spectrograph (USB4000, Ocean optics Duiven, The Netherlands) via a home-made optical fibre adapter placed in the camera mount of the microscope [15]. Fibre diameters were chosen in combination with a 20 times objective lens so that light was collected from a defined region within the tissue section. The epidermis is thin compared with the dermis or the cutaneous musculature therefore we chose the fibre diameters so that the size of the spot from which the spectra were collected was actually epidermis (12.5 µm) or dermis and cutaneous musculature (67.5 µm). The region in the dermis was chosen so that it only contained upper dermal tissue and no pilosebaceous units. Spectra were collected using an integration time of 20 seconds for epidermis and 30 seconds for dermis and cutaneous musculature.

*Histology.* The histological damage after PDT with ALA and MAL was investigated in 14 groups of mice (n=5-6 in each). Two groups served as ALA and MAL dark controls in which skin samples were collected at the end of the application period. In the 12 remaining groups the response following two different illumination schemes was investigated; a single light fraction of 100 J $cm^{-2}$ at 50 mW $cm^{-2}$ or a two-fold illumination scheme with 5+95 J $cm^{-2}$ at 50 mW $cm^{-2}$ separated by a 2 hour dark interval. In each case PDT was performed using 514 nm light as we have described previously [16]. In short, the excitation light was focussed on a 7 mm diameter spot of homogeneous profile. Mice were anaesthetised with a combination of 2 % Ethrane or Isoflurane (Abbott, Amstelveen, The Netherlands) oxygen and $N_2O$. The illuminated skin was excised 2.5, 24 and 48 hours after the end of illumination. Tissue was fixed in 4% buffered formalin solution and embedded in paraffin within 48 hours after harvesting. Paraffin sections were cut and stained with haematoxylin and eosin (H&E) for light microscopy evaluation.

## Results

***Fluorescence distribution within the skin after MAL or ALA***

Figure 1 shows the fluorescence images obtained from cryo-sections of skin applied with vehicle, ALA and MAL. Skin applied with vehicle showed weak fluorescence intensities in all layers with slightly higher intensities in the stratum corneum and the pilosebaceous units. After both MAL and ALA application high fluorescence intensities were observed in the epidermis and the pilosebaceous units. Besides these locations fluorescence was also seen in the upper dermis as well as in the adipose cells of the lower dermis. Only the cutaneous musculature showed no elevated levels of fluorescence above autofluorescence. Fluorescence spectra were obtained from the epidermis, upper dermis and cutaneous musculature of the skin in all groups (Figure 2). Skin applied with vehicle showed a typical autofluorescence pattern in all layers. Skin topically applied with MAL or ALA showed the characteristic PpIX fluorescence spectrum with peaks at 636 nm and 707 nm in the epidermis and the upper dermis. Only the cutaneous musculature exhibited fluorescence that was identical to the auto fluorescence spectrum.

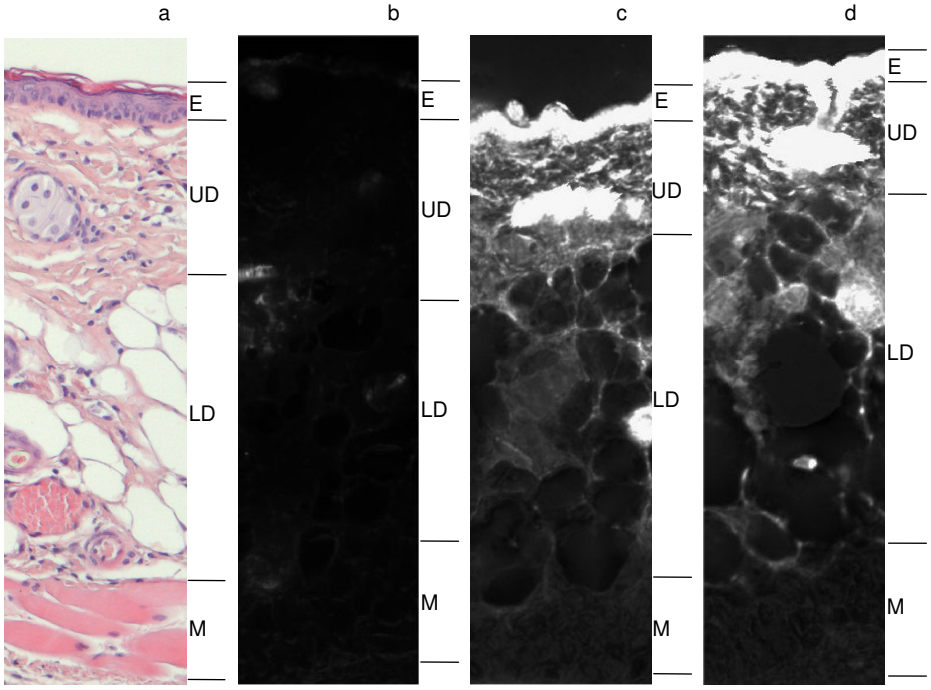


*Figure 1.* H&E stained image (a) and fluorescence images of hairless mouse skin sections after 4 hours of application with cream (b), ALA (c) or MAL (d) of 20 µm sections using the 5 times objective (E, epidermis; UD, upper dermis; LD, lower dermis and CM, cutaneous musculature). Note that in some images some areas of the epidermis are saturated. The integration time used to obtain these images was 120 seconds in order to investigate and show the fluorescence in the dermal and cutaneous musculature layer.

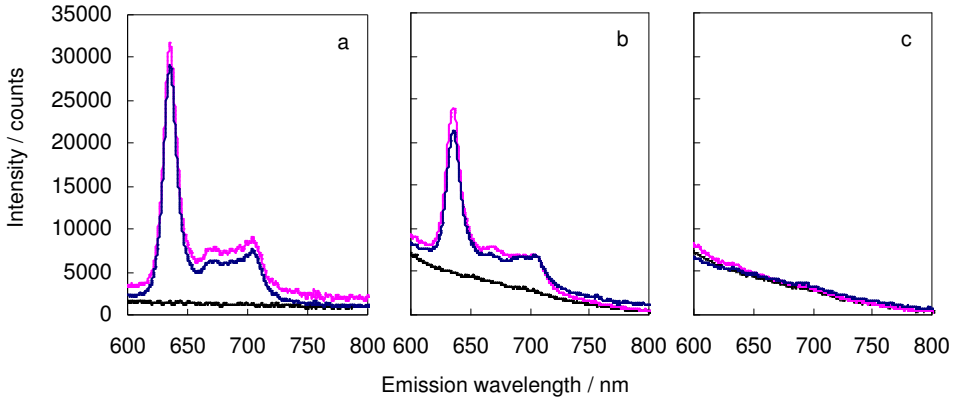


*Figure 2.* Spectra obtained from the epidermis (a), upper dermis (b) and cutaneous musculature (c) of hairless mouse skin sections after 4 hours of application with cream (black), ALA (blue) or MAL (magenta).

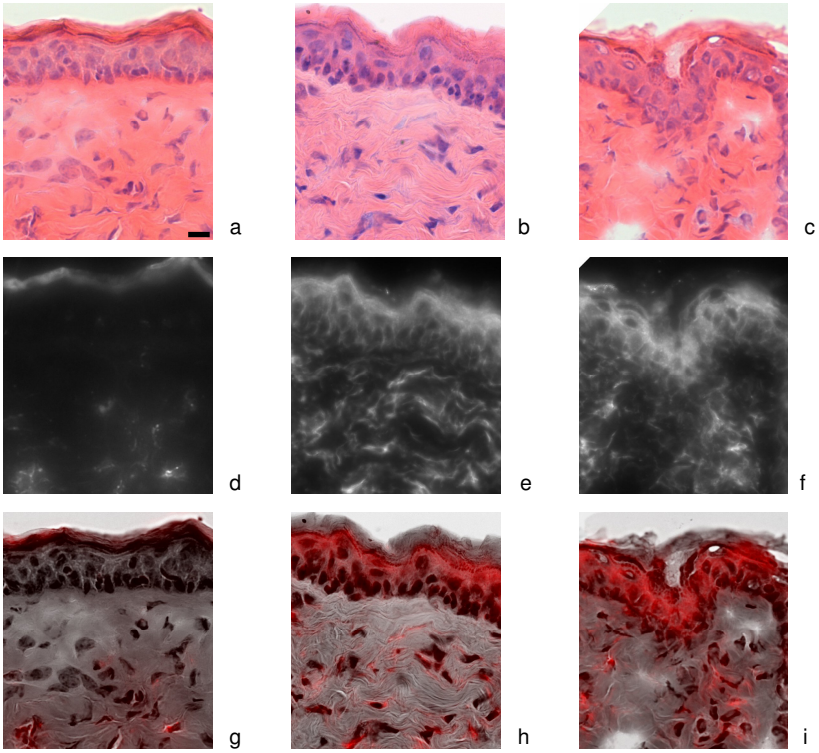


*Figure 3*. H&E stained image (a-c), fluorescence images (d-f) and combined images (g-i) of normal mouse skin sections after 4 hours of application with cream (left panel), ALA (middle panel) or MAL (right panel) of 5 µm sections using the 40 times objective. The bar represents 50 µm.

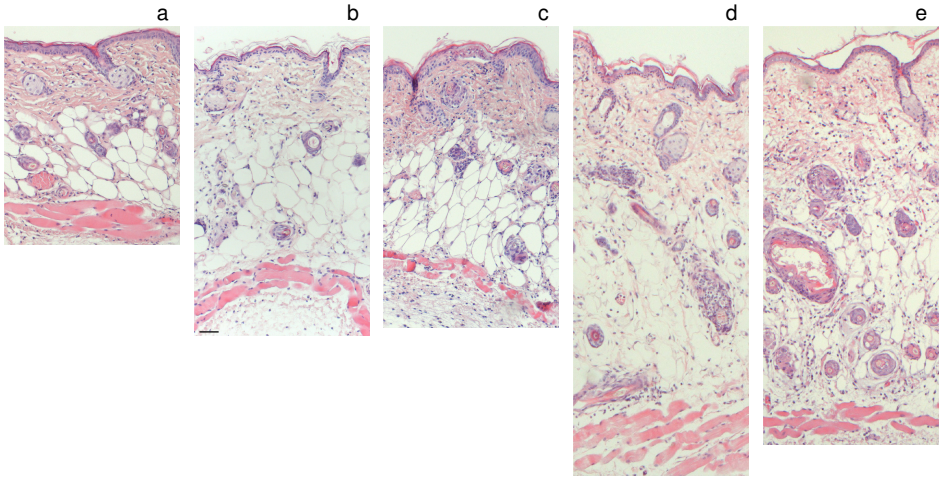


*Figure 4.* H&E stained images of hairless mouse skin; untreated (a) or 2,5 hours after PDT using MAL (b,c) or ALA (d,e). The bar represents 200 µm.

To investigate the location of the PpIX fluorescence in the upper dermis we obtained fluorescence images using a higher magnification (Figure 3). Again in skin applied with vehicle weak autofluorescence was observed in the stratum corneum. In the upper dermis some regions showed sparsely localised fluorescence. After ALA or MAL application the cytosol of epidermal cells showed high fluorescence intensities and the nuclei could be recognised as dark regions. Also the upper dermis showed regions with high fluorescence intensities. Co-localisation with the H&E stained images of the same sections showed that this is the cytosol of fibroblasts and/or mast cells. No clear difference in the localisation of PpIX fluorescence after MAL or ALA application could be observed in these images.

***Histological response following PDT***

Figure 4 shows the H&E stained sections of control and MAL or ALA-PDT treated skin 2.5 hours after treatment. In skh-1-hr mouse skin it is normal to find areas with acute infiltration in the upper and lower dermis associated with the degeneration of hair follicles due to the loss of hair process (Figure 4a). Two and a half-hour after illumination the stratum corneum has (partly) detached from the epidermis. At this time point we also observed pycnosis of epidermal cells in most animals, independent of the PpIX precursor or light fractionation scheme used. In dermis we observed a number of events. The level of inflammatory cells was elevated compared to the normal untreated control skin. While the collagen fibres appeared to be damaged in some animals the clearest observation was the formation of oedema. This caused the thickening of the skin that was greater after ALA-PDT compared to MAL-PDT. The cutaneous musculature showed no response at this time point after PDT.

The histological response observed at 24 or 48 hours after illumination did not show large differences in the type or depth distribution response between a two-fold or single illumination after either MAL or ALA-PDT therefore the damage is described in general below. Twenty-four hours after illumination the epidermis showed coagulative necrosis in most animals. In 7 out of 23 animals we observed impetiginisation (inflammatory cells in the epidermis) and in some sections we observed oedema between the epidermal cells, i.e., plasmodesmen. The border between damaged epidermis and not responding epidermis could be sharp-edged. In the upper dermis close to necrotic epidermis we could observe regions with variable responses regarding the degree of inflammation. This variation in the inflammatory response is actually seen through the whole dermis since it could be found loosely spread in the tissue or in a front with only necrotic tissue above it. This front could be found at variable levels; just below the basal membrane or at the level of sebaceous glands or just above or even in the adipose cell layer of the dermis. The collagen fibres appeared coagulated in some animals. In some animals we noticed that the fat cells in the lower dermis disappeared completely. The cutaneous musculature of 13 out of the 17 evaluated animals showed damage in the form of an inflammatory response sometimes accompanied with karyohrexis or coagulative necrosis.

Forty-eight hours after illumination regeneration of the epidermis had started in most animals. In the dermis we still observed coagulation of the collagen. Inflammatory cells were again found loosely spread in the tissue or in a front. In one animal we even observed two

fronts at different depths. Tissue above a front of inflammatory cells is or will become necrotic and detached. Healing of the skin was started from the hair follicles below the front. Furthermore we observed proliferating endothelial cells generating new vessels in the border areas next to heavily damaged dermal tissue. Dermal tissue that didn't show the heavy inflammatory response sometimes showed a significant loss of sebaceous glands. Similar to what we observed at 24 hours we noticed that the adipose cells in the lower dermis had disappeared in some animals but at this time point it was accompanied with atrophic or disappeared muscle.

## Discussion

In the present study we investigated the local distribution of PpIX after topical MAL or ALA application using fluorescence microscopy. In agreement with our expectations, topical MAL or ALA application on normal mouse skin results in high fluorescence intensities in the epidermis and sebaceous glands. Low fluorescence intensities are observed in the dermis and almost no fluorescence is seen in the cutaneous musculature. High magnification imaging of the dermis, however, reveals regions with high fluorescence intensities that correspond with the cytoplasm of cells. Fibroblasts, mast cells and even the adipose cells in the lower dermis show fluorescence after topical application of MAL and ALA. Importantly, our spectral analysis confirms that PpIX is the fluorescent species (Figure 2). Our high magnification images show high fluorescence intensities in the dermal cells compared to the dermal connective tissue. Compared to epidermal cells the dermal cells show around 45% of the fluorescence intensity and dermal connective tissue shows only 10% of the intensity (data not shown). In vitro studies have shown those fibroblasts are capable of PpIX accumulation [17]. While the contribution of dermal connective tissue in the dermis is high the low cellular density partly explains the low fluorescence intensity generally measured in dermis [18].

Previously Moan *et al.* [10] have observed differences in the distribution of the two PpIX precursors suggesting that MAL but not ALA becomes systemically distributed. In-vitro studies have shown differences in cellular up-take mechanism [13] that potentially could lead to cells taking up ALA and not MAL or visa versa. Based on these studies and the known differences in biophysical and biochemical characteristics between the two PpIX precursors we expected to find differences in the PpIX fluorescence distribution with penetration depth and possibly cellular function. However, in the present study we were unable to find a clear difference in PpIX distribution within the applied area. All cells in the epidermis and dermis show PpIX fluorescence and there is no difference in the penetration depth. PpIX fluorescence is observed over the whole dermal layer up to the adipose cells adjacent to the cutaneous musculature after both MAL and ALA application. Blood vessels and in particular capillaries are found throughout the dermis. This suggests that both PpIX precursors penetrate to a depth below the level of the blood supply. Considering the results presented here and the results of the study of Moan *et al.* this would mean that ALA and not MAL

penetrates into the blood stream either by diffusing through or passing between the endothelium cells. Garcia *et al.* [19] have reported ALA efflux through diffusion in LM3 cells in-vitro. It will be interesting to determine if MAL efflux also occurs and if there are specific differences between tumour and endothelial cells. In an attempt to investigate the capacity of endothelial cells to synthesis PpIX we stained them using CD31 and CD34 antibodies. Unfortunately we were not successful in these mouse sections. Our previous work on the highly vascularised skin fold chamber model suggests that endothelial cells are capable of synthesizing PpIX after systemic ALA administration [20]. Furthermore it has been shown that ALA-PDT results in vascular damage, even after topical application [21,22], also suggesting that endothelial cells accumulate PpIX. Whether or not MAL is taken up by or transported through the endothelial cells could not be investigated in the current study.

In a previous study [1] we showed the histologically scored damage in skin tissue after MAL and ALA-PDT using either a single or a two-fold illumination scheme at different time points after PDT. Then we used a table that grades the different cellular responses like coagulative necrosis, karyorhexis and pyknosis that can be observed and we found no differences in this between MAL and ALA-PDT. In the present manuscript we explored the histological response in greater detail looking also on the tissue responses like oedema and inflammation. Histologically, we found significantly more oedema in the dermis of skin 2.5 hours after ALA-PDT compared to MAL-PDT, independent of the illumination scheme used (Figure 4). The severity of oedema is usually not used to score PDT induced damage while oedema is considered to be an indirect result of a complex of reactions that deals with an imbalance between the osmotic and hydrostatic pressure acting across the semi-permeable capillary walls of blood and lymphatic vessels. Inflammation has a big influence on this as it changes the permeability of capillary walls and increases the oncotic pressure outside vessels. In general, inflammation and oedema is a common tissue response to PDT using ALA or other photosensitisers [23] but also vascular responses are reported [24-26]. PDT using Photofrin is known [24] to result in direct cytotoxic endothelial cell damage and vascular leakage. Also ALA-PDT is known to result in vascular leakage [25,26]. If and how the extent of oedema formation can be used to score PDT induced damage and if this could be used as a measure of vascular damage is unknown. To our knowledge only Sheng *et al.* [27] have used oedema to score the damage to the oesophagus after ALA-PDT. The observed difference the oedema formation in the present study was not accompanied by a difference in the inflammatory response. This suggests that the formation of oedema was not only due to the inflammatory response. While endothelial cells are involved in the oedema response this difference in oedema formation might suggest that the damage to endothelial cells is different after MAL compared to ALA-PDT.

We were unable to show any PpIX fluorescence in the cutaneous musculature using either fluorescence imaging or spectral analysis. Still the histological evaluation 24 and 48 hours after treatment showed severe cutaneous muscular damage in some animals. We have also observed this phenomenon for ALA-PDT in oesophagus and other organs [28-30]. Van den Boogert *et al.* [28] suggested that the threshold to induce necrosis might be lower for muscle than for other tissues. In our most recent study we showed that the muscle of the

oesophagus accumulates PpIX after oral ALA administration [30]. However, we were unable to find any PpIX fluorescence in the cutaneous musculature of the skin after topical ALA or MAL application and still found severe necrosis. It is known that tissue close to necrotic tissue may become necrotic as a result of the inflammatory reactions. In some of our samples this could very well be the explanation, although not all the necrotic cutaneous musculature is found close to necrotic dermal tissue. Collagen fibres in some areas in the dermis appeared to be coagulated. PDT does not induce heating to a temperature that is sufficient enough to denature collagen [32]. Direct cell damage to the fibroblast and mast cells, normally responsible for the collagen production, may cause the release of proteins that result in the coagulation of collagen fibres. In an in-vitro study Karrer *et al.* [32] showed an increase in the synthesis of matrix metalloproteinase (MMP)-1 in dermal fibroblast after ALA-PDT. While MMP-1 is an interstitial collagenase that is known to be involved in the degradation of extra cellular collagen fibres (types I, II, III, IX and XI) it could be involved in the observed collagen coagulation in our mouse skin samples. The observed impetiginisation within the epidermal layer suggests that the damaged cells release factors involved in the inflammation process. Furthermore we have seen a front of inflammation cells at variable depth within the dermis. It is unusual to find inflammation in such a particular orientation in tissue and it will be interesting to investigate the regulation of this phenomenon.

In conclusion, we investigated the distribution of accumulated PpIX after topical MAL and ALA application and found no clear difference in the spatial distribution of PpIX fluorescence. Cells in the epidermis but also in the dermis of normal mouse skin accumulate significant PpIX after topical MAL or ALA application as confirmed by our spectral analysis. Histological analysis showed a distinct difference in the formation of oedema 2.5 hours after PDT that was not accompanied by a different inflammatory response to PDT. This suggests that endothelial cells are involved in the different between the response of tissue to MAL and ALA to light fractionation. Further investigation is needed to determine the underlying mechanism behind the increased effectiveness of light fractionation using a dark interval of 2 hours found after ALA but not after MAL-PDT.

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# Chapter 9

## Light fractionated ALA-PDT does not enhance therapeutic efficacy in cells in-vitro

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## Introduction

The exogenous administration of 5-aminolevulinic acid (ALA) to cells and tissues leads to the accumulation of protoporphyrin IX (PpIX) by bypassing the feedback inhibition of ALA-synthase that normally regulates the haem synthesis pathway [1]. The last step of this pathway, the chelation of iron to protoporphyrin IX (PpIX) by ferrocelatase (FC), is a relatively slow process and this leads to the temporary accumulation of PpIX. This process is the basis for the use of porphyrin pre-cursors for photodynamic therapy (PDT) that is now used to treat a variety of (pre-) malignant conditions [2-4]. PDT using light fractionation, where a dark interval separates two periods of illumination has shown to significantly increase the efficacy of ALA-PDT in both pre-clinical models and in non-melanoma skin cancer [5-7]. In a series of studies the treatment parameters for light fractionation were optimised and the greatest increase in efficacy was found when the treatment fluence was split into two unequal fractions in which the PDT dose of the first fraction is relatively low and the dark interval between light fractions is two hours [5-10].

The mechanism behind this increase in effectiveness of the two-fold illumination scheme is unknown. We, and other investigators have shown that PpIX continues to be synthesised in-vivo after PDT [5,9,11-15] and that this re-synthesis is a local cellular effect [13]. The original rationale behind the design of this type of light fractionated illumination scheme was the utilisation of re-synthesised PpIX after photobleaching during the first fraction. However in a range of pre-clinical models we were unable to find a correlation between the amount of PpIX re-synthesis and the effectiveness of light fractionation [8,9,14]. The efficacy of light fractionated PDT seems to be determined by the damage induced during the first illumination and the timing of the second light fraction. We have hypothesised that a cellular mechanism is involved in which cells that are sub-lethally damaged by the first light fraction may be vulnerable to a second light fraction delivered two hours later [9].

The effect of fractionated illumination on cells in vitro has been studied previously by Hinnen *et al.* [15]. They found that human EBV (Epstein Barr Virus transformed) lymphoblastoid cells showed reduced FC activity leading to more PpIX accumulation after ALA-PDT compared to un-treated controls. They also found that light fractionation resulted in more cell death compared to a single illumination. However, their aim was to increase the efficacy of the treatment and not to compare the efficacy of different illumination schemes. The authors did not use the same cumulative fluence for both illumination schemes and the single illumination was always performed on the earliest time point.

The aim of the present study was to investigate the effect of various two-fold and single illumination schemes with the same cumulative fluence in-vitro using the optimal illumination parameters previously found for light fractionated PDT in-vivo [9]. It is difficult to translate these illumination parameters since the ones used to treat cells in vitro are typically much lower than those used to treat tissue in-vivo. In the present study we therefore investigated the effect of different two-fold illumination schemes varying the fluence of the first and second light fraction and compared these results with those after a single illumination with

the same cumulative fluence delivered on any time point used for light fractionation. As a starting point we used the results obtained in the hairless mouse model in which we have previously [5,8,9] determined an optimised treatment scheme; topical ALA 4 hours, 5 J $cm^{-2}$ at 50 mW $cm^{-2}$, 2 hours dark interval and 95 J $cm^{-2}$ at 50 mW $cm^{-2}$. The mouse skin shows only some discolouration after a single illumination of 5 J $cm^{-2}$ (data not published). This was translated to an in-vitro illumination that results in 5 to 15% cell death. The fluence of the second light fraction results in more skin damage in-vivo and is translated to an illumination that would result in more but not complete cell death, i.e., approximately 50% cell death, in-vitro. During the dark interval between light fractions, ALA should be available to the cells to replicate the conditions following topical ALA administration to the skin. This study was performed on LM3 and PAM212 cells [16,17].

## Materials and Methods

*Cell lines and cell cultures*: Cell line LM3 from Hospital Roffo [18] derived from the murine mammary adenocarcinoma M3 was cultured in minimum essential Eagle's medium, supplemented with 2 mM L-glutamine, 40 µg gentamycin $ml^{-1}$ and 5% fetal bovine serum. The tumourigenic PAM 212 cell line which is derived from murine epidermal keratinocytes [19] was cultured in RPMI-1640 medium and supplemented with L-glutamine, gentamycin and 10% fetal bovine serum. The cells were incubated at 37°C in an atmosphere containing 5% $CO_2$ and employed at confluency.

*ALA administration*: The cells were seeded in triplicate in 6-well plates containing $7x10^4$ cells $ml^{-1}$ and incubated 48 h at 37°C. Afterwards, LM3 cells were incubated with serum free medium containing 0.6 mM ALA which leads to plateau porphyrin values [16]. A dose of 2 mM results in plateau porphyrin values in PAM212 cells (data not shown).

*Light fractionation schemes*: Irradiation was performed according to a single and two-fold illumination scheme using different light fluences. Cell survival curves were determined, as described below, for a single illumination using different ALA exposure times in both LM3 and PAM212 cells. Alternatively, light was fractionated in two doses separated by a dark interval of 2 or 3 hours. During the dark interval cells remained exposed to ALA and were incubated at 37°C . The light fluence of the first and second light fraction in our pre-clinical model were optimised and resulted in approximately 10% and 55% response respectively [5].

For LM3 cells we translated these fluences based on the results obtained in the previous study [16]. The fluence that induced 5 or 10% cell death (LD 5 or 10) was used for the first fraction and LD 40 or 55 were used for the second light fraction, i.e., (0.17 J $cm^{-2}$ split into 0.034+0.136 J $cm^{-2}$ and 0.255 J $cm^{-2}$ split into 0.054+0.204 J $cm^{-2}$). The first fraction was delivered after 3 hours of ALA exposure and the second fraction was delivered at 5 hours after the start of ALA exposure.

For the PAM212 cells we chose four different two-fold illumination schemes for three different total fluences; 0.34, 0.408 and 0.510 J $cm^{-2}$ based on the results described in this study. The total fluence of 0.34 J $cm^{-2}$ was split into 0.102+0.238 J $cm^{-2}$, the total fluence of

0.408 J $cm^{-2}$ was split in 0.136+0.272 J $cm^{-2}$ and the total fluence of 0.510 J $cm^{-2}$ was split in 0.170+0.34 J $cm^{-2}$. The first fraction was delivered either at 2 or 3 hours after ALA exposure followed by a second fraction at 5 hours, i.e., 2+5 or 3+5 hrs.

*PDT illuminations*: A bank of two fluorescent lamps (Osram L 36W/10) was used for the therapeutic illumination. The output spectrum of this light ranged from 400 to 700 nm with the highest radiant power at 600 nm. The plates were irradiated from below, at 20 cm distance from the light source. Fluence rate was measured with a radiometer, (Yellow Springs Kettering model 65, Yellow Springs, OH, USA). Fluences between 0.1 and 1 J $cm^{-2}$ were used at a power density of 0.5 mW $cm^{-2}$. After the end of the treatment procedure, medium was replaced by ALA-free medium +FBS and the cells were incubated for 19 h and tested for cell viability.

*MTT viability assay*: Phototoxicity was documented using the MTT assay [20], a method based on the activity of mitochondrial dehydrogenases. Following appropriate treatments, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) solution was added to each well in a concentration of 0.5 mg $ml^{-1}$, and plates were incubated at 37°C for 1 h. The resulting formazan crystals were dissolved by the addition of DMSO and absorbance was determined at 560 nm.

*Statistical analysis*: Data are expressed as mean ± standard error of the mean, and they are the average of three independent experiments run in triplicate. A paired two-tailed Student's t-test was used to determine statistical significance between means. $P$ values <0.05 were considered significant.

## Results

### *Cell survival curves for LM3 cells*

Figure 1 shows the cell survival of LM3 cells after ALA-PDT using various single and two-fold illumination schemes. After a single illumination the cell survival decreases with increasing fluence for both 3 and 5 hours ALA exposure. Furthermore, increasing the ALA exposure time from 3 to 5 hours results in a lower cell survival, i.e., a more effective treatment. A single illumination with a fluence of 0.17 J $cm^{-2}$ delivered at 5 hours results in 15.9 ± 11.4% cell survival, which is significantly more effective than an illumination at 3 hours resulting in 51.7 ± 1.6% cell survival (n=2, $P$ =0.048). The same applies for a single illumination with a fluence of 0.255 J $cm^{-2}$. Delivery at 5 hours results in 5.1 ± 4.7% cell survival that is significantly more effective than illumination at 3 hours resulting in 33.2 ± 3.0% cell survival (n=2, $P$ =0.019).

A two-fold illumination scheme with the same cumulative fluences results in 36.8 ± 13.6% cell survival for 0.17 J $cm^{-2}$ (split into 0.034+0.136 J $cm^{-2}$) and 4.4 ± 4.0% for 0.255 J $cm^{-2}$ (split into 0.051+0.204 J $cm^{-2}$). The response to a two-fold illumination is not significantly different from that compared to a single illumination performed after 5 hours of ALA exposure ($P$ =0.24 and 0.90, respectively).

***Cell survival curves for PAM212 cells***

Similar results are obtained with PAM212 cells (Figure 2). For single illumination schemes the cell survival decreases with increasing fluence for all exposure times. In addition, the illumination is more effective after longer ALA exposures. Illumination with a fluence of 0.34 J $cm^{-2}$ at 5 hours results in 25.8 ± 9.5% cell survival whereas illumination at 3 hours results in 46.7 ± 10.9% cell survival (n=3, $P$ =0.38). Illumination at 2 hours is even less effective and results in 72.8 ± 6.2% cell survival (n=3, $P$ = 0.15 compared to PDT at 3 hrs and 0.027 compared to PDT at 5 hrs).

A two-fold illumination with the same cumulative fluence, i.e. 0.34 J $cm^{-2}$ split into 0.102±0.238 J $cm^{-2}$, results in 52.7 ± 15.4% cell survival for a 2+5 hours scheme, which is not significant from 33.7 ± 23.3% for 3+5 hours (n=3, $P$ =0.45). The response following the most effective two-fold illumination scheme (3+5 hrs) is not significantly increased when compared with the most effective single illumination (5 hrs, $P$ =0.62). Similar results are obtained for illuminations with a cumulative fluence of 0.408 or 0.510 J $cm^{-2}$.

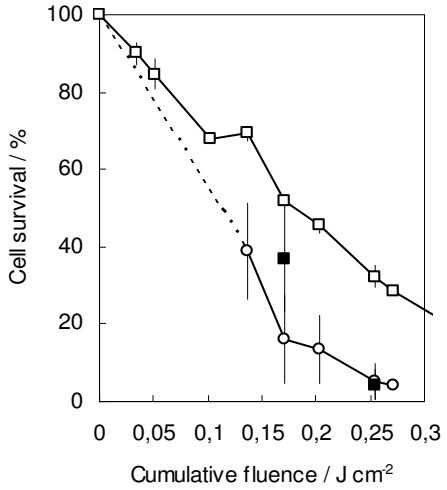


*Figure 1*. Cell survival of LM3 cells after ALA-PDT using different fluences and illumination schemes. Cells were exposed to 0.6 mM ALA in FBS-free medium. Single illuminations were performed after 3 (□) or 5 hours (○) of ALA exposure. Two-fold illuminations were performed after 3+5 hours of ALA exposure (■). Cell survival was determined 19 hours after PDT using the MTT assay and was expressed as percentage of the non-irradiated control incubated in the presence of ALA.

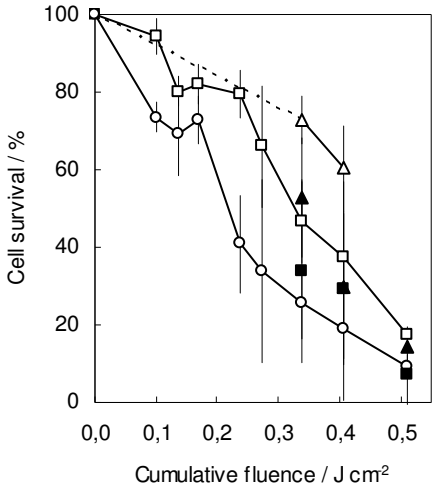


*Figure 2*. Cell survival of PAM212 cells after ALA-PDT using different fluences and illumination schemes. Cells were exposed to 2 mM ALA in FBS-free medium. Single illuminations were performed after 2 (△), 3 (□) and 5 hours (○) of ALA exposure. Two-fold illuminations were performed at 2+5 hours (▲) or 3+5 hours (■) of ALA exposure. Cell survival was determined 19 hours after PDT using the MTT assay and was expressed as percentage of the non-irradiated control incubated in the presence of ALA.

## Discussion

Our results show that light fractionated ALA-PDT does not enhance the response of LM3 or PAM212 cells in-vitro. This result is contrary to our findings in a range of in-vivo models and for ALA-PDT of human basal cell carcinoma [5-7]. We have previously hypothesised that there may be a cellular mechanism by which cells are sub-lethally damaged following the first light fraction are vulnerable to the second fraction two hours later [9]. Our data seem to preclude this hypothesis, at least in the cells that we have investigated to date. In both LM3 and PAM212 cells light fractionation with dark intervals of the order of 2-3 hours does not enhance the effectiveness of ALA-PDT over that observed with a single illumination with the same cumulative fluence.

Hinnen *et al.* [15] were the first to compare the response of cells grown in-vitro to ALA-PDT using a single and a two-fold illumination scheme. They found a significantly lower cell survival after a two-fold illumination, delivered 2 and 5 hours after ALA exposure, compared to a single illumination scheme 2 hours after ALA exposure. While the aim of the authors was not to compare the effectiveness of different illumination schemes the cumulative fluences of the schemes were not the same. The fluence delivered during a single illumination was half (12.5 J $cm^{-2}$) of the cumulative fluence delivered during the two-fold illumination (25 J $cm^{-2}$). It is therefore unclear if the increased efficacy of the two-fold illumination is due to light fractionation. It is possible that a single illumination with the same cumulative fluence would have been as effective as the two-fold illumination. Furthermore it is important to carefully consider the accumulation of PpIX at the time point at which each of the illuminations is performed. In the above mentioned paper, the two fractions of the two-fold illumination scheme were delivered at 2 and 5 hours after the start of ALA exposure whereas the single illumination was performed at 2 hours. The authors showed that there was significantly more PpIX accumulation 5 hours after the start of ALA exposure compared to 2 hours, which is also shown by Perotti *et al.* [21]. This suggests that an illumination at this time point would be more effective. The results of the present study support this conclusion and show that a single illumination after a 5 hour incubation period is significantly more effective. It would be interesting to investigate if the two-fold illumination scheme is still more effective in treating human EBV-transformed lymphoblastoid cells when the influence of these effects is considered.

A limitation of the present study is the absence of data on porphyrin intensities in cells during light fractionated ALA-PDT. The fluence of the first light fraction alone results in 5-10% cell death for the lowest fluence used. It is likely that the surviving cells will accumulate PpIX from the ALA present in the medium based on the results obtained by Perotti *et al.* [21] and Hinnen *et al.* [15]. In un-treated cells the PpIX accumulation has shown to increase linearly with the incubation time up to 24 hrs [21]. Also Hinnen *et al.* [15] found a significant increase in the PpIX accumulation in illuminated cells.

In addition it is well known that different cell lines respond differently to PDT. The results here show that LM3 cells are more sensitive to ALA-PDT than the PAM212 cells. LM3 cells accumulate more PpIX and the fluence needed to kill 50% of the cells was lower (0.18 J $cm^{-2}$ for LM3 cells compared to 0.36 J $cm^{-2}$ for PAM212 cells). However, notwithstanding these differences the general correlation between ALA exposure time and PDT efficacy is similar. A single illumination is more effective when it is delivered after 5 hours of ALA exposure compared to 2 or 3 hours. This is due to the increase in PpIX accumulation with incubation time that results in a greater exposure to reactive oxygen species during PDT. The response of each cell line to a two-fold illumination is comparable and not significantly different from that following a single illumination delivered at any of the time points tested. Both the LM3 cells and PAM212 cells do not show an increased cell death after ALA-PDT using a two-fold illumination. However, other cell lines may respond differently and it is our intention to extend these experiments to different cells from a range of origins, in particular vascular endothelial cells.

The contribution of the tissue (or host) component to the PDT response is not included in in-vitro studies. ALA-PDT induced damage is mostly attributed to direct cell damage however vascular responses have been reported [22,23]. Moan *et al.* [24] showed that ALA is systemically re-distributed after topical ALA application. This indicates that ALA is available for the endothelial cells and it is likely that they will generate PpIX and therefore are a target for PDT damage. In a previous study we showed that light fractionated PDT using methyl aminolevulinate (MAL) does not lead to an increase in response in normal mouse skin [25]. In addition ALA-PDT resulted in significantly more oedema compared to MAL-PDT, reinforcing the hypothesis that the vasculature may play a role in the increased efficacy of light fractionation.

In conclusion, the results in the present study show that the increased efficacy of light fractionated ALA-PDT is not found in-vitro. This suggests that the mechanism involved is more complicated than the cellular mechanism proposed.

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# Chapter 10

## General Discussion

The subject of this thesis is ALA-PDT and the mechanism underlying the increased efficacy achieved by fractionated illumination. The results of the work are discussed in detail below. In short; light fractionation, in which two light fractions are separated by a dark interval of more than 1 hour, leads to a significant enhancement in treatment response. The light treatment parameters that are used for fractionation are critical with respect to the degree of the improvement in efficacy. The mechanism behind this increased efficacy has proven to be significantly more complex than first anticipated.

Photodynamic therapy using ALA and other porphyrin pre-cursors is widely accepted as a treatment option for a number of predominately cutaneous (pre-) malignancies. It has also been applied to a variety of non-malignant skin diseases and is under investigation for the treatment of other conditions in various organs. While, initial complete response rates of more than 90% were reported for superficial basal cell carcinoma the need for improvement is illustrated by the low long-term response rates of 50%. Also 25% of the patients treated for Barrett's oesophagus fail to respond to ALA-PDT using a single light fraction.

The use of light fractionated ALA-PDT was inspired by a clinical observation of Star [1] who noted the reappearance of the photosensitiser PpIX fluorescence in the hours after treatment in a skin lesion that showed complete photobleaching of fluorescence during treatment. The initial rationale behind light fractionation using a long dark interval was to utilise this additional PpIX for PDT. The results obtained in the first studies using this type of light fractionation were promising [2,3]. In order to maximise the potential increase in efficacy, before translating the approach to the clinic, studies were performed to optimise light fractionated ALA-PDT and investigate the mechanism underlying the increase in effectiveness.

Chapters 2, 3 and 4 describe a series of pre-clinical studies in which the treatment parameters of light fractionated PDT were investigated in order to optimise treatment efficacy. The data presented in Chapter 2 are acquired from a transplanted tumour model using systemic ALA. In Chapters 3 and 4 ALA is applied topically to normal mouse skin. In both cases the data illustrate that light fractionation with a dark interval of more than 1 hour results in significantly increased efficacy. In both cases also the response to a single illumination delivered at different time points after ALA administration is not different. Illumination at 4 or 6 hours after topical ALA on normal mouse skin results in a similar response. This is explained by the PpIX fluorescence kinetics that shows a similar PpIX fluorescence intensity at these two time points. For the solid tumour model this is different. Here the fluorescence intensity at 2.5 hours is almost 2.6 times higher compared to 1 hour after systemic ALA administration. The growth delay is slightly but not statistically significant, increased for illumination at 2.5 hours compared to 1 hour. It is known that the PDT response is influenced by the availability of three parameters; light, oxygen and photosensitiser concentration. An increased photosensitiser concentration will result in a higher demand for oxygen for the formation of singlet oxygen when the same light parameters are used. The result that the growth delay was not significantly increased after

illumination at a time point at which significantly more PpIX was available suggests that oxygen is the parameter that limited the response to PDT [4,5].

The length of the dark interval is critically important for the degree of improvement in efficacy of light fractionated PDT. Reducing the length of the dark interval from 2 hrs to 1.5 hrs, 1 hr or 30 minutes significantly decreases the efficacy of light fractionated ALA-PDT measured in the hairless mouse model. While the aim of that study was to investigate whether the length of the dark interval could be shortened without loss of effectiveness only shorter intervals were tested. The results show however a significant trend for increasing damage with increasing length of dark interval. It is therefore possible that a longer dark interval would be more effective. The response to light fractionation using a dark interval of 30 minutes or 1 hour is not significantly different from that to a single illumination. That a dark interval shorter than 1.5 hours does not increase the efficacy of light fractionated ALA-PDT was also shown by Babilas *et al.* [6]. They used a dark interval of only 15 minutes and were unable to show a difference in the growth delay of subcutaneously growing amelanotic melanoma after fractionated PDT compared to PDT using a continuous illumination with the same cumulative fluence. It seems that the increased effectiveness found after fractionated PDT is only observed when a dark interval of more than 1 hour is used. A 1.5 hours dark interval significantly improves the response compared to a single illumination. Moreover, only the two hour dark interval resulted in significantly the most severe skin damage compared to the shorter intervals tested. Light fractionation using dark intervals in the order of seconds and minutes represents an alternative approach for improving the efficacy of ALA-PDT. These fractionation schemes (i.e. multiple interruptions 30 sec on/off or single interruptions of 150 sec) were shown to result in a significant larger area of necrosis in normal colon [7-9]. This was attributed to re-oxygenation in the dark interval and associated with vascular reperfusion injury (I/R injury). A previous study on normal mouse skin [10] showed that this type of light fractionation does not have an effect on the PDT response of normal mouse skin. Also in the solid tumour model this is not as effective as light fractionation using a dark interval of more than 1 hour. For light fractionation using a dark interval of more than 1 hour the I/R injury mechanism seems not to be involved. Oxygen was measured indirectly through photobleaching and the PDT doses were too small to induce vascular response and significant oxygen depletion, which is needed to induce I/R injury. Furthermore in a previous study [11] it was shown that the vasoconstriction induced by the first light fraction is not significantly decreased during the dark interval. Although this was only assessed visually in the highly vascularised skin-fold chamber model using white light microscopy this suggests that there is no significant reperfusion during that time interval.

The results described in Chapter 4 show that besides the duration of the dark interval also the PDT dose of the first light fraction is crucially important for the degree of improvement in efficacy of light fractionated PDT. The efficacy of light fractionated PDT is significantly improved when the fluence is split in 5+95 J $cm^{-2}$ at 50 mW $cm^{-2}$ compared to 50+50 J $cm^{-2}$ at 50 mW $cm^{-2}$. Further reduction of the fluence of the first light fraction, i.e.,

decreasing the PDT dose, will not improve the efficacy. Reducing the fluence rate from 50 to 5 mW
$cm^{-2}$, i.e., increasing the PDT induced damage, reduces the efficacy of this light fractionation scheme. These illumination parameters were optimised for illuminating normal mouse skin and should be carefully translated for other application areas taking the treatment volume in consideration. The distribution of light in tissue is not homogeneous and the fluence rate decreases with increasing depth. This is important for treatment volumes that are not optically thin as the normal mouse skin is. The results described in Chapter 2 show that the fluence rate in the solid tumour model decreases to 23% at the bottom of the tumour compared to that on top of the tumour. Treatment of this tumour will therefore result in a range of fluence rates and fluences over the whole of the tumour. That this may have important consequences for the efficacy of the treatment is shown by the thickness effect found in that study; tumours thinner than 4 mm responded significantly better than thicker tumours. Also human skin lesions are usually thicker than mouse skin. In mouse skin PpIX is predominantly accumulated in the epidermis that is only ≈ 30 μm thick and the green light used for treatment is homogeneously distributed over this region. For this reason the optimised treatment parameters found for mouse skin (i.e. 5+95 J $cm^{-2}$ at 50 mW $cm^{-2}$ at 4+6 hours after topical ALA) were translated to a clinical light fractionation scheme using red light and a first light fraction of 20 J $cm^{-2}$ delivered at 50 mW $cm^{-2}$ followed by a dark interval of 2 hours and a second light fraction of 80 J $cm^{-2}$ at 50 mW $cm^{-2}$. The initial CR of this translated light fractionation scheme are significantly improved [12] which illustrates the importance of the illumination parameters for the efficacy of light fractionated ALA-PDT.

Increasing the fluence used for the second light fraction results in a more effective light fractionation scheme. A second light fraction of 95 J $cm^{-2}$ is more effective than 45 J $cm^{-2}$. This is different from what is found for single illumination schemes where a fluence of 50 J $cm^{-2}$ is equally effective as a fluence of 100 J $cm^{-2}$. The efficacy of a single illumination is limited by the photobleaching of PpIX. This seems logical since continuing an illumination once PpIX is photobleached is not likely to lead to more PDT induced damage. Apparently this is different for light fractionated ALA-PDT. Here the PpIX fluorescence during the second light fraction is almost photobleached after 45 J $cm^{-2}$, but continuing the illumination still results in an increased efficacy. The data described in Chapter 3 also show that the rate of photobleaching is reduced during a second light fraction compared to that during the first light fraction. This may be due to either a reduced availability of oxygen at the time of the second illumination or possibly the differential cellular or tissue localisation of PpIX. The PDT damage induced by the first light fraction may result in a locally reduced availability of oxygen that will lead to a reduced rate of photobleaching. The results described in Chapter 6 suggest that there is no difference in tissue localisation of PpIX as all tissues investigated in the skin-fold chamber model (tumour, normal and vascular tissue) showed a similar increase of PpIX fluorescence after the first light fraction. This is supported by a clinical study [13] in which the spatial distribution of PpXI in biopsies of superficial BCC was investigated. Here the distribution of PpIX in skin lesions collected two hours after a first light fraction was not different from that observed in un-illuminated control lesions. The intracellular localisation of

PpIX after illumination was not investigated in this thesis. In normal cells PpIX is accumulated in the mitochondria but this may be different for sub-lethally damaged cells where the enzymes involved in haem synthesis may be localised differently.

The original hypothesis for the increased damage after light fractionated ALA-PDT was the utilisation of the resynthesised PpIX in the second illumination. The results obtained in the various studies described in this thesis show, however, that the efficacy of light fractionated ALA-PDT is not directly correlated with the amount of resynthesised PpIX as estimated from fluorescence. This is clearest for the results described in Chapter 6 where significantly more PpIX was available for a single illumination compared to the light fractionated PDT and still light fractionated PDT was significantly more effective. The results obtained in the hairless mouse model support this conclusion. The most effective light fractionation scheme utilised only 10% more PpIX compared to a much less effective fractionation scheme. This shows that other factors than just the amount of photosensitiser play a role in the mechanism of action.

The pharmacokinetics of PpIX fluorescence after PDT shows a correlation with the PDT dose delivered. While the results of Chapter 6 show that the PpIX fluorescence after PDT is due to local re-synthesis this might be expected. PpIX is synthesised from ALA by cells. A higher PDT dose results in more PDT induced damage and may therefore cause more cellular damage resulting in fewer cells that are capable of PpIX re-synthesis. The importance of the PDT induced damage by the first light fraction, i.e., the PDT dose, led us to the hypothesis that sub-lethal damage might be an important factor. The PDT dose of the first light fraction is relatively small. In normal mouse skin illumination with only the first light fraction results in minimal visual skin damage. PpIX fluorescence is bleached to 40% of its initial intensity suggesting that considerable damage is induced. That only minimal damage is observed in time after PDT might suggest that the damage was repairable. This, combined with the importance of timing the second light fraction suggests that cellular processes initiated by the first light fraction influence the susceptibility of cells to a second light fraction. The results described in Chapter 9 show that the increased efficacy of light fractionated ALA-PDT is absent in-vitro. This suggests that the mechanism involved is more complicated than the cellular mechanism that was proposed.

While light fractionation resulted in a significant increased efficacy in ALA-PDT, this type of illumination was also investigated for MAL-PDT (Chapter 7). Contrary to our expectations light fractionation does not increase the efficacy of MAL-PDT. Apparently there are differences in the response to MAL and ALA PDT that play a role in the mechanism. Although MAL and ALA are both PpIX precursors there are significant differences that may be involved in the difference in response. The two molecules are very different in their biochemical and biophysical characteristics that potentially could lead in a different spatial distribution. The PpIX fluorescence photobleaching data show a small but significant difference in rate of photobleaching. While the rate of photobleaching depends on the microenvironment of the PpIX molecule this supports the hypothesis of a different

distribution. Fluorescence microscopy on skin samples obtained 4 hours after topical application of either MAL or ALA, however, seems to show no difference in local spatial distribution. PpIX fluorescence is observed in epidermal and dermal cells throughout the full skin thickness. This result seems to contradict Moan *et al.* [14], who showed a difference in the systemic distribution of MAL and ALA suggesting a difference in localisation. It seems that the difference in systemic distribution is not accompanied by a difference in local distribution. The border between systemic distribution and local distribution is formed by the endothelial cells that form the lining of blood vessels. If and how the distribution of MAL or ALA is different in the endothelial cells could not be investigated in this study. The CD31 and CD34 markers used to stain them in these mouse samples showed either too much background or failed to stain the endothelial cells. It is interesting that the histological samples obtained 2.5 hours after PDT show a distinct difference in the severity of the oedema formation; more oedema is found after ALA-PDT compared to MAL-PDT. The severity of oedema is usually not used to score PDT induced damage while oedema is considered to be an indirect result of a complex of reactions that deals with an imbalance between the osmotic and hydrostatic pressure acting across the semi-permeable capillary walls. Inflammation has a significant influence on this as it changes the permeability of capillary walls and increases the pressure in and outside vessels. The difference in oedema formation observed was not accompanied by a difference in inflammatory response suggesting that this is a difference in vascular leakage. ALA-PDT is known to result in vascular leakage [3,15]. The results suggest that the difference in oedema is due to a difference in the response of endothelial cells to ALA or MAL-PDT. How this is involved in the mechanism behind the increased efficacy of light fractioned ALA-PDT is unknown and warrants further investigation.

*Light fractionated ALA-PDT in clinical practice*

The results of the first pre-clinical studies showing an increased efficacy of light fractionated ALA-PDT led to a first clinical study [16] investigating light fractionation in which the illumination was split in two equal fractions of 45 J $cm^{-2}$. The five year follow-up results were published in 2006 and showed a significantly increased response compared to the results published in the literature for single light treatments. Light fractionated ALA-PDT of superficial BCC results in 84% CR compared to 56% for standard ALA-PDT treatment. After determining the optimum illumination parameters a second clinical study was initiated comparing a single illumination with the translated optimised light fractionation scheme [12]. Already after one-year follow-up the response of superficial BCC to the optimised fractionation schemes was significantly better than to a single illumination scheme (97% versus 89% p=0.002). Encouragingly, this significant difference in clinical response is maintained 24 months after therapy (96% versus 86%). The importance of the illumination parameters is also shown in these studies as the response to the optimised illumination scheme is significantly better compared to the non-optimised scheme. Currently, light fractionated ALA-PDT using the optimised illumination scheme is daily practice for AK, sBCC, thin nodular BCC and Bowens disease in the Department of Dermatology, Erasmus Medical Center Rotterdam.

*Light fractionated ALA-PDT: future perspectives*

The mechanism involved in the increased effectiveness of light fractionated ALA-PDT is still unknown but the studies described in this thesis reveal details that warrant further investigation. Although light fractionated ALA-PDT was shown to increase the CR of superficial BCC, understanding of the mechanism might reveal new and unforeseen applications for this type of treatment.

The results described in Chapter 9 show that light fractionation does not lead to more cell death in-vitro. However so far the responses of only two cell lines have been investigated. The intention is to extend this study to include a range of cells of different origins. While the results described in Chapter 7 and 8 imply that endothelial cells might be involved these will be studied first. In-vitro studies are useful for investigating individual cellular responses but it is possible that the mechanism involved is more complicated than in vitro studies of a single cell line may reveal. Therefore the response of cells in co-culture or even in tissue should be investigated.

Over the last 10 years light fractionated ALA-PDT has evolved from a promising research subject to a successful clinical treatment scheme. The clinical responses to light fractionated ALA-PDT are excellent but pain is (still) the main side effect of topical ALA-PDT. For standard ALA-PDT this can be reduced by using a lower fluence rate. Pre-clinical experiments are planned to investigate if a lower fluence rate can be used for light fractionation without loss of effectiveness. While illumination parameters are known to be important for the efficacy this should be considered carefully. Illumination with a lower fluence rate is more effective therefore this should be compensated by a lower fluence so that the PDT induced damage by the first fraction remains the same.

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# Chapter 11

## Summary

(English / Nederlands)

## Summary

In **Chapter 2** the effect of different illumination schemes was determined using the delay in growth of a tumour after treatment. Rhabdomyosarcoma, an experimental tumour model, was subcutaneously transplanted on the thigh of a rat and treated when its volume reached 50 $mm^3$. ALA was administered *i.v.* to a dose of 200 mg $kg^{-1}$ and the tumours were transdermally illuminated using 630 nm red light. The growth of the tumour after treatment was assessed daily and the delay in growth was defined as the time the tumour needs to double its treatment volume. 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). Illumination earlier (1 hr after ALA administration), using a lower fluence rate (25 mW $cm^{-2}$), a higher fluence (200 J $cm^{-2}$) or even light fractionation with short dark intervals did not result in a significant longer volume doubling time. Only light fractionated ALA-PDT (illumination at both 1h and 2.5 h after ALA-administration with 100 J $cm^{-2}$ at 100 mW $cm^{-2}$) resulted in a significant increased tumour volume doubling time of 18.9 ± 2.9 days.

In **Chapters 3** and **4** the illumination parameters of the two light fractions were varied in an attempt to find the optimal illumination scheme in the hairless mouse model. Following topical ALA application, PpIX fluorescence and photobleaching kinetics were measured pre-, during and after PDT. The results showed that the PpIX fluorescence kinetics after a single light fraction depend on the fluence delivered; PDT using a high fluence resulted in more PpIX photobleaching and a smaller increase in PpIX fluorescence in time after PDT. The skin response was scored daily according to a visual skin damage table and these results seemed to correlate with the increase of PpIX fluorescence in time after PDT.

The effect of various light fractionation schemes was investigated and compared with single light fraction PDT. The light parameters used for the first and second light fraction have a significant effect on response. PDT induced damage in the first fraction seems a determining factor in this while different light fractionation schemes with the same cumulative fluence were equally effective when the PDT induced damage by the first fraction was the same. The optimal illumination scheme for fractionated ALA-PDT on normal mouse skin involves a first light fraction of 5 J $cm^{-2}$ delivered at 50 mW $cm^{-2}$ followed by a dark interval of 2 hours and a second light fraction of 95 J $cm^{-2}$ at 50 mW $cm^{-2}$ using an ALA application time of 4 hours.

In **Chapter 5** the contribution of neutrophils to the PDT induced damage was investigated in the solid tumour model with *i.v.* ALA administration. PDT was performed on subcutaneously growing tumours using either a single light fraction or two light fractions under either immunologically normal or neutropenic conditions. The neutropenic condition was achieved by treating the animals with anti-granulocyte serum. PpIX fluorescence was determined in tumour and normal tissue at different time points before and after PDT. The

number of neutrophils in tumour and the circulation was determined as a function of time after treatment and compared to growth delay of each scheme. The PpIX fluorescence kinetics showed a peak fluorescence intensity at 2.5 hours after ALA administration. The amount of photobleaching and the fluorescence kinetics after PDT showed a correlation with the fluence of the illumination similar to what was observed in chapter 3 in the hairless mouse model using topical ALA. The number of neutrophils within the illuminated tumour and in the circulation increased significantly following therapy. This increase was associated with an increase in the efficacy of therapy: the more effective the therapy, the greater the increase of tumour and blood neutrophils. Administration of anti granulocyte serum treatment prevented the influx of neutrophils after ALA-PDT, but did not lead to a significant decrease in the efficacy of the PDT treatment on the growth of the tumour for any illumination scheme investigated. These results showed that the magnitude of damage inflicted on the tumour by ALA-PDT does not depend on the presence of neutrophils in the tumour or circulation. This indicates that neutrophils only play a bystander role in ALA-PDT.

In **Chapter 6** the increase in PpIX fluorescence after PDT was investigated to determine if this is the result of re-distribution or local re-synthesis. The skin-fold observation chamber was used to determine the spatial distribution of PpIX fluorescence after PDT. The results showed no correlation between the fluorescence increase and the distance from a blood vessel. In a separate group the tissue was cooled for one hour after PDT to a temperature of 10-12°C. At this temperature enzymatic activity and the accumulation of PpIX after ALA administration is inhibited. The results showed a significant lower fluorescence increase compared to that normally observed after PDT. This study shows that the PpIX fluorescence increase after PDT is locally re-synthesised.

In **Chapters 7** and **8** the effect of light fractionation is investigated using methyl aminolevulinate (MAL) mediated PDT. MAL-PDT is more and more used clinically and since light fractionation significantly increased the effectiveness of ALA-PDT, it might have the same influence on MAL-PDT. This was investigated in the hairless mouse model using the light parameters defined for ALA-PDT single and light fractionated illumination. ALA and MAL showed similar PpIX fluorescence kinetics as determined by surface fluorescence measurements either during topical application, during PDT and in the dark interval. Contrary to the results obtained using ALA-PDT, the visual skin damage after MAL-PDT was not different after a single illumination compared to light fractionation. The initial rate of photobleaching was slightly greater during ALA-PDT but the difference is small and can not explain the different response. While ALA and not MAL-induced PpIX has shown to be systemically distributed after topical application on mouse skin, the local distribution of PpIX after MAL and ALA might also be different and may be involved in this. However, fluorescence microscopy showed no clear difference in the PpIX distribution after either MAL or ALA application. Also the histological response after MAL and ALA-PDT seemed not different, at first. The amount of oedema formation observed 2.5 hours after PDT was different; more oedema was formed after ALA compared to MAL-PDT independent of the

illumination scheme used. This suggests that endothelial cells respond differently to ALA and MAL-PDT and further investigation is needed to determine the role of endothelial cells in the response to light fractionated ALA-PDT.

In **Chapter 9** the effect of different illumination schemes was investigated on different cell lines *in-vitro*. Cells were exposed to ALA and illuminated with one or two light fractions delivered at 2, 3 and/or 5 hours. In the case of light fractionation the cells remained to be exposed to ALA during the dark interval. The fluences used for the light fractions in this study were chosen so that the PDT induced damage was expected to be similar to that found in *in-vivo* studies. The cell survival after PDT was determined by the mitochondrial activity using the MTT assay. The results showed a strong correlation between cell survival and with the duration of ALA exposure; PDT delivered at 5 hours was more effective compared to 2 or 3 hours. None of the investigated light fractionation schemes was more effective than a single light fraction delivered at 5 hours. The increased effectiveness of light fractionated ALA-PDT found *in-vivo* in the clinic or in pre-clinical models is not observed *in-vitro*.

In **Chapter 10** the results obtained in these studies are discussed in the context of the current concepts in the literature and future perspectives are presented.

## Samenvatting

In **hoofdstuk 2** is het effect van verschillende belichtingschema's onderzocht in een solide tumormodel. Een experimentele rat tumor, het rhabdomyosarcoom, werd onderhuids op de dij van de rat getransplanteerd en behandeld wanneer het tumorvolume ongeveer 50 $mm^3$ was. ALA werd intra veneus toegediend in een dosis van 200 mg/kg en de tumoren werden transdermaal belicht met rood licht. Het tumorvolume werd dagelijks gemeten om de uitstel van de groei, gedefinieerd als de tijd die de tumor nodig had om het dubbele van zijn behandelvolume te bereiken, te berekenen. Een onbehandelde controle tumor had een verdubbelingtijd van 1.7 ± 0.1 dagen. De groei werd significant vertraagd na een ALA-PDT behandeling met een enkele belichting (100 J $cm^{-2}$ bij 100 mW $cm^{-2}$, 2.5 uur na ALA toediening) tot 6.6 ± 1.2 dagen. Belichting eerder na ALA toediening (na 1 uur), een lagere licht intensiteit (25 mW $cm^{-2}$), een hogere lichtdosis (200 J $cm^{-2}$) of zelfs gefractioneerd belichten met korte donker intervallen had geen significant effect of deze groeivertraging. Alleen de gefractioneerde ALA-PDT behandeling waarbij twee licht fracties gegeven werden op 1 en op 2.5 uur na ALA toediening (met 100 J $cm^{-2}$ bij 100 mW $cm^{-2}$) resulteerde in een significante groeivertraging van 18.9 ± 2.9 dagen.

In **hoofdstuk 3 en 4** zijn de belichting parameters van de twee licht fracties gevarieerd in een poging om het meest optimale belichtingsschema te bepalen met topicale toediening van ALA. Dit onderzoek werd uitgevoerd op het haarloze muizen model. De PpIX

fluorescentie en fotoblekingskinetiek is gemeten voor, tijdens en na PDT. De reactie van de huid werd dagelijks gescoord volgens een huid schade score tabel. De resultaten laten zien dat de PpIX fluorescentie kinetiek na een enkele belichting afhankelijk is van de gebruikte lichtdosis; belichting met een hoge licht fluentie resulteerde in meer PpIX fotobleking gevolgd door een lagere PpIX fluorescentie toename in de tijd na PDT. Dit resultaat correleerde met de visueel gescoorde huidschade na PDT. Het effect van de verschillende gefractioneerde belichtingschema's werd vergeleken met het effect van een enkele belichting. De lichtparameters die gebruikt worden voor de eerste en tweede lichtfractie hebben een significante invloed op de effectiviteit van de behandeling. Hierbij lijkt de schade die door de eerste belichting wordt veroorzaakt een bepalende factor aangezien verschillende belichtingsschema's met een gelijke cumulatieve licht dosis even effectief zijn wanneer de PDT schade van de eerste fractie gelijk is. Voor de haarloze muizen huid is het optimale gefractioneerde belichtingschema alsvolgt: topicaal ALA voor 4 uur, een eerste lichtfractie van 5 J $cm^{-2}$ bij 50 mW $cm^{-2}$ gevolgd door een donker interval van 2 uur en een tweede lichtfractie van 95 J $cm^{-2}$ bij 50 mW $cm^{-2}$.

In **hoofdstuk 5** is onderzocht of neutrofielen een rol spelen in de effectiviteit van ALA-PDT. Subcutaan groeiende tumoren werden behandeld met systemische ALA-PDT onder normale en neutropene condities. Neutropenie werd bereikt door de dieren te behandelen met anti-granulocyten-serum. Op verschillende tijdstippen na ALA toediening en na PDT werd de PpIX fluorescentie bepaald in tumor en normaal weefsel. Voor de twee verschillende behandelschema's die zijn onderzocht (de enkele en de dubbele belichting) werd als functie van de tijd na behandeling het aantal neutrofielen in het bloed en in de tumor bepaald. Dit werd gecorreleerd aan de effectiviteit van de behandeling. De fluorescentie intensiteit van PpIX gaf een piek op 2.5 uur na ALA toediening in de tumor. Vergelijkbaar met de resultaten behaald in de muizen studie met topicaal ALA (hoofdstuk 3) was de mate van bleking tijdens en fluorescentie toename na PDT afhankelijk van de licht dosis van de behandeling. Het aantal neutrofielen in de behandelde tumor en in circulatie nam significant toe na PDT en was afhankelijk van de effectiviteit van de behandeling: hogere toename van het aantal neutrofielen na een effectievere behandeling. Behandeling met anti-granulocyten-serum voorkwam dat het aantal neutrofielen toenam na PDT maar dit leidde niet tot een verlaging van de effectiviteit. Deze resultaten geven aan dat de schade die ALA-PDT veroorzaakt niet afhankelijk is van de aanwezigheid van neutrofielen in het behandelvolume dan wel in het bloed. Neutrofielen lijken niet bij te dragen aan de effectiviteit van ALA-PDT.

In **hoofdstuk 6** is onderzocht of de terugkeer in fluorescentie die waargenomen wordt na PDT het gevolg is van locale re-synthese, dan wel een herverdeling in PpIX elders gesynthetiseerd, is. Voor dit onderzoek werd gebruik gemaakt van het zogenaamde kamertjes model (skin-fold observation chamber) waarin de PpIX fluorescentie als functie van de ruimtelijke locatie in het weefsel werd onderzocht. Er werd geen correlatie gevonden tussen de mate waarin PpIX fluorescentie terugkeerde ten opzichte van de afstand van een

bloedvat. In een andere groep werd het weefsel gedurende 1 uur na PDT gekoeld tot 10-12°C. Het is bekend dat bij deze temperatuur de enzym activiteit en de ophoping van PpIX na ALA toediening geremd is. De terugkeer in fluorescentie na PDT bleek significant lager in deze groep. Uit deze resultaten blijkt dat de PpIX fluorescentie toename na PDT behandeling het gevolg is van lokale re-synthese.

In **hoofdstuk 7 en 8** is het effect van een gefractioneerde belichting bij MAL-PDT beschreven. MAL, een ALA ester, wordt steeds meer toegepast in de kliniek en aangezien de effectiviteit van ALA-PDT beter is na een gefractioneerde belichting is onderzocht of dit ook het geval is bij MAL-PDT. Voor dit onderzoek werd gebruik gemaakt van het haarloze muizen model en de gevonden licht parameters voor ALA-PDT voor een enkele en een gefractioneerde belichting. De fluorescentie kinetiek van PpIX na toediening van MAL bleek niet anders dan die na ALA, zowel tijdens de topicale applicatie als tijdens PDT en in de donkere periode tussen de twee licht fracties in. Anders dan bij ALA was er bij MAL geen verschil in de visueel gescoorde huidschade na een enkele of een gefractioneerde belichting. Omdat is aangetoond dat ALA in de circulatie komt na topicale toediening en MAL niet, kan ook de locale distributie van MAL en ALA anders zijn. Dit gegeven zou een rol kunnen spelen bij de gevonden verschillen. Fluorescentie microscopie liet echter geen duidelijk verschil zien in de PpIX distributie in de huid na MAL of na ALA. Ook de histologische response leek in eerste instantie niet te verschillen. Het enige verschil is de hoeveelheid oedeem beoordeeld op 2.5 uur na PDT; ALA-PDT leidt tot meer oedeem dan MAL-PDT. Hieruit zou afgeleid kunnen worden dat endotheel cellen anders reageren op een ALA behandeling dan op een MAL behandeling. Meer onderzoek is nodig om de rol van endotheel cellen in de effectiviteit van ALA-PDT te onderzoeken.

In **hoofdstuk 9** is het effect van gefractioneerd belichten op verschillende cellijnen *in-vitro* onderzocht. Cellen werden met ALA geïncubeerd en één of twee keer belicht op 2, 3 en/of 5 uur. In het geval van een gefractioneerde belichting bleven de cellen geïncubeerd met ALA in het donkere interval tussen de twee belichtingen. De lichtdosis en lichtintensiteit die gebruikt werden voor de *in-vitro* belichtingen werden zodanig gekozen dat de door PDT geïnduceerde schade vermoedelijk vergelijkbaar was met de *in-vivo* gevonden schade. Cel overleving na PDT werd bepaald door de mitochondriale activiteit te meten met behulp van de MTT-test. Er werd een sterke correlatie gevonden tussen de ALA incubatie periode en de effectiviteit; PDT op 5 uur geeft meer cel dood dan PDT op 2 uur. Geen van de onderzochte gefractioneerde belichtingen was effectiever dan een enkele belichting op 5 uur. De verbeterde effectiviteit van gefractioneerde ALA-PDT die *in-vivo* gevonden werd in pre-klinische modellen en in de kliniek, werd *in-vitro* niet gevonden.

In **hoofdstuk 10** worden de resultaten van dit proefschrift besproken in het licht van de huidige opvattingen beschreven in de literatuur en worden toekomstige perspectieven genoemd.

# Curriculum Vitae

Henriette Suzanna de Bruijn, roepnaam Riette, werd op 8 juni 1971 geboren aan boord van m/s Willy, op dat moment te Meppel aangemeerd. De lagere school en HAVO doorliep zij in Rotterdam, alwaar ze doordeweeks in een schippersinternaat verbleef. Gesteund door ouders voor wie het niet belangrijk was of ze al dan niet in het schippersvak ging, startte ze in 1988 de HLO opleiding aan het Van Leeuwenhoek Instituut te Delft. Na het propedeuse jaar koos ze de medisch biologische richting en in 1992 studeerde ze af. Sindsdien is ze in dienst van de Dr. Daniel den Hoed kliniek (DDHK) waar ze op verschillende afdelingen werkzaam was. Haar (afstudeer)stage liep ze op de afdeling Radiobiologie, een projectgroep van Prof. Levendag. Haar huidige man leerde ze op deze afdeling kennen en mede daardoor was de werksfeer erg prettig. Aansluitend was ze een klein jaar werkzaam als research analiste op het Laboratorium voor Experimentele Patho-Oncologie onder leiding van Prof. Oosterhuis. Hier was ze betrokken bij een project dat voorzag in het tot stand brengen van humane seminoom cellijnen ten behoeve van onderzoek naar de ontwikkeling van kiemcel tumoren. Op 1 juni 1993 werd ze aangesteld op de afdeling photodynamische therapie (PDT), toen nog onder leiding van Dr. Star. In 1998 werd hij opgevolgd door Dr. Sterenborg (per 1 maart 2008 j.l. Prof. Sterenborg). Als gevolg van fusies maakt de DDHK intussen deel uit van het ErasmusMC en in 2004 verhuisde de afdeling naar de centrumlokatie. Hier is het onderdeel van het Centrum voor Optische Diagnostiek en Therapie (CODT). Sinds 1993 is Riëtte betrokken bij meerdere door het NKB/KWF gefinancierde projecten en een bij het FOM gefinancierd project. Het door het FOM gefinancierde project was een samenwerkingsverband met de afdeling Moleculaire Biophysica van de Universiteit Utrecht onder leiding van Prof. Gerritsen en voorzag in onderzoek naar de toepasbaarheid van twee-fotonen microscopie in de medische wetenschap. De andere projecten hadden de verbetering van ALA-PDT en gefractioneerde belichting als onderwerp. Sinds 1998 werkt ze onder begeleiding van Dr. Robinson. Dit proefschrift is het logische gevolg van de werkzaamheden in het kader van de laatst genoemde projecten.

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