Photodynamic therapy using topical 5-aminolaevulinic acid is being investigated as a possible alternative therapeutic modality for superficial basal cell carcinoma. Initial response rates are impressive (>80%), However long-term response rates show considerable variation and have been shown to be as low as 50%. Our aim is to improve response rates by investigating the mechanisms underlying the response of tissues to ALA-PDT in particular the use of fractionated illumination.

Fractionated illumination for photodynamic therapy of skin cancer

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Basal cell carcinoma (BCC) is the most common human malignancy. Its incidence in Caucasians continues to increase as a result of an increase in sun exposure and an increase in the average age of the population. A current estimate of the incidence in the Netherlands is > 30,000 annually. Different clinical and histopathological forms of BBC can be distinguished. The subtypes are: a) nodular (50-70%), b) superficial (10%), c) morpheic (sclerosing), d) micronodular and e) infiltrative. Tumour growth is slow and metastases occur very rarely (0.03%). Several surgical and non-surgical therapies are currently available. The treatment of choice or 'gold standard' is surgical excision and recurrence rates following Mohs' micrographic surgery are approximately 1%. In selected cases of BCC there is, for technical or cosmetic reasons, a need for an alternative approach. This is particularly important for patients with a history of multiple BCC's.

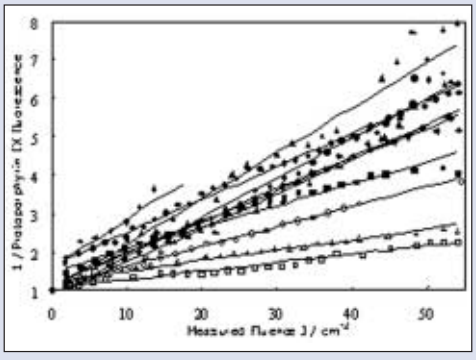

Figure 1. *The photobleaching of the photosensitiser PpIX during PDT correlates with tissue damage. Complete ablation (closed symbols) and no response (open symbols) occur despite identical illumination parameters.*

Photodynamic therapy using topical 5-aminolaevulinic acid (ALA-PDT) is currently being investigated as a possible alternative therapeutic modality for such cases. To date, no study has been published comparing ALA-PDT with a conventional treatment modality. However, initial clinical complete response rates after ALA-PDT are usually high (>80%), but longer term CR rates show considerable variation. For example, some investigators have reported a disease free rate at 36 months after ALA-PDT of 50% for BCC. On the other hand, others have obtained a recurrence free CR rate for superficial BCC treated with topical ALA-PDT of 95% at 6 months and 84% at 48 months. These later data are obtained using very large light doses > 200 J/cm^2 delivered at fluence rates that have been shown to be less than optimal for PDT response. Using these treatment parameters, illumination times are considerable even for small lesions, and prohibitive for larger lesions. Our own study, 16 patients with BCC (22 lesions) achieved a CR rate of 79% (median follow-up 13 months). Our conclusion from the most recent literature and our own experience is that the optimum treatment protocol for topical ALA-PDT of basal cell carcinoma has not yet been established.

Our aim is to improve these long-term response rates by investigating the mechanisms underlying the response of tissues to ALA-PDT. Traditionally PDT is performed using a single treatment in which ALA is applied topically for 4-6 to allow the photosensitiser PpIX to accumulate. At this time point an illumination is performed in which a dose of 50- 150 J cm^{-2} is delivered in a single light fraction at a fluence rate between 50 and 150 mW cm^{-2}.

ALA-PDT dosimetry

This apparent simplicity of PDT; administer photosensitiser, wait, then illuminate with light using a defined fluence and fluence rate, has lead many thousands of patients being treated world-wide. The perceived ease of performing PDT belies the fact that its dosimetry is highly complex and the response of tissues can vary dramatically between patients. Variations in tissue optically properties and uptake/synthesis of the photosensitiser can lead to wide variations the delivered dose. We have pioneered the use of in-vivo light dosimetry for photodynamic therapy in the clinic (see paper van Veen et al.). While light dosimetry is essential, it is has become increasingly clear that it is often insufficient for many photosensitisers. The amount of singlet oxygen produced during PDT is obviously dependent on the presence of photosensitiser but also on the availability of oxygen. There exists a dynamic interaction between the parameters of local light fluence rate, photosensitiser concentration, and molecular oxygen. Each of these parameters can be different for individual lesions and patients and are interdependent and change dynamically during illumination.

Results from our animal models have shown us that explicit definitions of fluence are not predictive of the response to PDT. For example PDT damage increases with decreasing fluence rate. Using the fluorescent and phosphorescent properties of the photosensitiser we can monitor its distribution during therapy. This can provide information that can be correlated with treatment outcome. The production of singlet-oxygen during PDT not only leads to the destruction of tumour cells but also leads to the photo-oxidation of the photosensitiser. We have shown that monitoring this process termed "photobleaching" using fluorescence spectroscopy can be correlated with the response to therapy in skin tumours and during oesophageal-PDT (Figure 1). Similar data have allowed us to define an optimum fluence rate for PDT in the skin. In combination with these data we are also developing methods to monitor blood flow during PDT as an additional parameter that may relate to the response to PDT.

Fractionation schemes and improving ALA-PDT

Monitoring (singlet) oxygen during therapy and increasing its the availability is one option for enhancing the response of tissues to PDT. We are also investigating another method in which the illumination is split into two or more fractions. The resulting dark interval can lead to reperfusion, which may increase tissue damage. However we have recently shown that the use of relatively long dark intervals (> 1 h) between light fractions during PDT can lead to dramatic increases in tissue

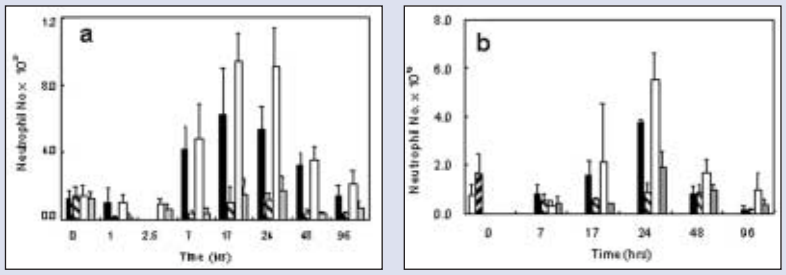

Figure 2. *(a) The number of circulating blood neutrophils and (b) tumour neutrophils following ALA-PDT with a single illumination scheme 100 J cm^{-2} (■) and double 100 + 100 J cm^{-2} (□) illumination scheme under normal (solid bars) and neutropenic conditions (hatched and grey bars)*

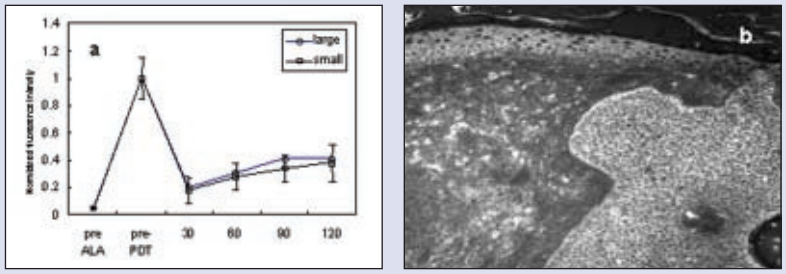

Figure 3. *(a) The kinetcis of PpIX fluorescence following illumination and (b) the distribution within sBCC 2 hours after 20 J cm^{-2}*

damage where reperfusion does not play a significant role. We have 4 avenues of research investigating the mechanism behind this increase in response. These are based on data from both animal studies and measurements made during PDT in the clinic and from patient biopsies.

The role of neutrophils in ALA-PDT

The immune system plays an important role in the response of tumours to photodynamic therapy. Cells such as neutrophils migrate into treated tumours and play an important role in destroying a proportion of cells that survive the primary PDT injury. Since the length of the dark interval may allow these cells to accumulate within tumour and since the interaction between the neutrophils and PDT damaged cells may give us important insights into the mechanism by which cells die following PDT we have been investigating their role in ALA-PDT. We have found that PDT with ALA induces a classical inflammatory response following illumination and that this response is significantly greater for a two-fold illumination scheme. There is a dramatic increase in the number of circulating neutrophils which peaks between 17 and 24 h after PDT. However if anti-granulocyte serum is administered to deplete the number circulating neutrophils during therapy there is no reduction in the growth delay of tumours. Therefore despite the active recruitment of neutrophils into tumours we have shown that they are not necessary for the response of

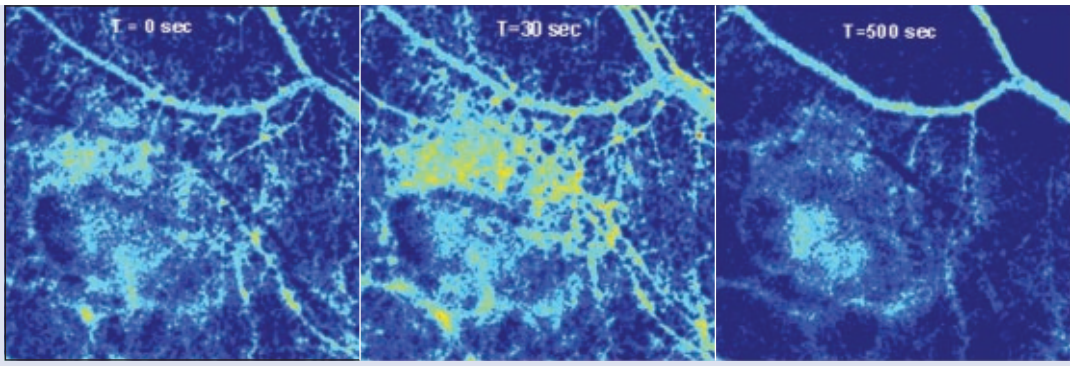

Figure 4. *Speckle images taken during PDT in an experimental tumour in a window chamber in the back of a rat. The sequence shows the changes in blood flow during photodynamic therapy: an initial increase in blood flow followed by a dramatic drop.*

cells to therapy. This is very a significant result. Six hours after the administration of ALA (two hours after a first light fraction) PpIX is localised within mitochondria but also so in the outer cell membrane. Damage to the outer cell membrane, as in the case of other photosensitisers, is necessary for the induction of an inflammatory response. The fact that cells photosensitised with ALA-induced PpIX do not require the additional neutrophils lead us to the conclusion that the predominant mechanism of cell death after ALA-PDT is apoptosis.

The mechanism of cell death following PDT and the sensitivity of cells to further illumination.
We are investigating the expression of various proteins associated with apoptosis to determine the mechanism of damage following ALA-PDT with a two-fold illumination scheme. Two hours after illumination with 20 J cm^{-2} the expression of bcl-2 appears to be is significantly reduced compared to control tumours. Bcl-2 is recognised as a potential target in PDT. We are investigating the balance between the expression of bax, bcl-2 and AIF at early time points after illumination in human BCC and in an animal model.

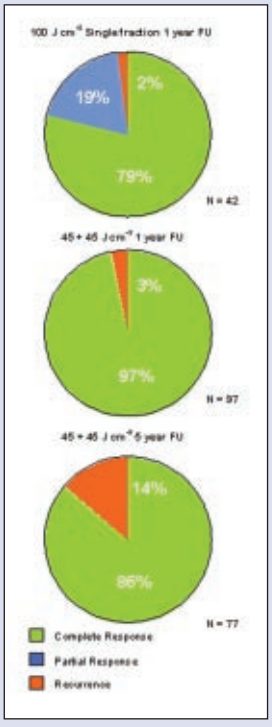

Figure 5.
Clinical Response of s-BCC to ALA-PDT

The localisation and rate of re-synthesis of PpIX after therapy
A peculiarity of ALA-PDT is that the cells to be destroyed are themselves responsible for the production of the photosensitiser PpIX. This leads to a complex interaction between the doses delivered and the continued synthesis of PpIX. We are investigating the localisation and the rate of PpIX re-synthesis following PDT in our animal models and in BCC (Fig 3).

The vascular response following ALA-PDT
When a laser illuminates a diffuse medium, the randomly scattered light emerging from the surface generates an interference pattern. The spatial and temporal changes in this speckle pattern contain information on changes in the photon paths inside the tissue. At present we are developing this technique for the use of blood flow monitoring during PDT. Animal experiments (fig 4) show dramatic changes in blood flow during the therapeutic illumination. At present we are testing whether similar results can be obtained in patients. If so, we will evaluate whether we can establish a relation between the development in the blood flow during treatment and the treatment outcome. We are currently utilising Laser Speckle Imaging to monitor blood-flow between the two fractions of a two-fold illumination scheme so that we can determine the influence of the vascular response to the increase in effectiveness. We have found that there is no significant reduction in the blood flow during illumination or in the dark interval between light fractions. The vascular response therefore does not play a significant role in the response of tissues to the two-fold illumination scheme. We have recently begun investigating if similar data can be acquired in patients undergoing ALA-PDT for s-BCC. We plan to determine if there is a relationship between blood flow before PDT and the effectiveness of therapy.

Clinical Success
In parallel to the mechanistic studies described above we are collaborating with our partners in the departments of Dermatology, Gastroenterology and ENT to assess the use of PDT in the clinic. We have recently completed a pilot study investigating the long-term response of s-BCC following ALA-PDT with a two-fold illumination scheme. The clinical response of a scheme in which two equal light fractions (45 + 45 J cm^{-2}) are delivered 4 and 6 hours after the application of ALA is 86%. With median FU 58 m, range 44-82 m, N =77). Taking into account data from our most recent animal studies our clinical protocol has been changed to utilise the further increase in response observed with a small first light fraction. At present a two-fold illumination scheme in which 2 light fractions (20 + 80 J cm^{-2}) are delivered separated by a 2 h dark interval is the standard clinical protocol for all ALA-PDT treatments. Since the beginning of 2003 we have treated over 1000 s-BCC lesions. We are presently investigating the use of a similar protocol for AK and Bowen's Disease and Acne.