Fractionated Illumination Significantly Improves the Response of Superficial Basal Cell Carcinoma to Aminolevulinic Acid Photodynamic Therapy

Ellen R.M. de Haas[1], Bastiaan Kruijt[2], Henricus J.C.M. Sterenborg[2], H.A. Martino Neumann[1] and Dominic J. Robinson[1,2]

Photodynamic therapy (PDT) of superficial basal cell carcinoma (sBCC) using topical 5-aminolevulinic acid (ALA) and a light fluence of $75–100 \, J \, cm^{-2}$ yields unsatisfactory long-term results. In several animal models, illumination with two light fractions 2 hours apart was considerably more effective than single illumination. Response is further enhanced if the fluence of the first light fraction is reduced, although the cumulative fluence is maintained. We compared the response of sBCC to a single illumination and 2-fold illumination scheme in which two light fractions of 20 and $80 \, J \, cm^{-2}$ are performed 4 and 6 hours after the application of a single dose of 20% ALA. We randomly assigned 154 patients with a total of 505 primary sBCC into two treatment groups. Two hundred and forty-three lesions were treated using a single illumination of $75 \, J \, cm^{-2}$ at a fluence rate of $50 \, mW \, cm^{-2}$. Fractionated PDT, at the same fluence rate, was performed on 262 lesions. The complete response (CR) following a 2-fold illumination scheme is significantly greater than that following a single light fraction ($P = 0.002$, log-rank test). Twelve months after therapy, CR rate to a 2-fold illumination is 97%, whereas the CR to a single illumination is 89%.

INTRODUCTION

Photodynamic therapy (PDT) of superficial (non-) malignant skin lesions, using topically applied 5-aminolevulinic acid (ALA) to endogenously generate the photosensitizer protoporphyrin IX (PpIX), was introduced by Kennedy et al. in 1990. Initially, high (80–100%) complete response (CR) rates were reported for topical ALA-mediated photodynamic therapy (ALA-PDT) in the treatment of superficial non-melanoma skin cancer (Kennedy et al., 1990; Kennedy and Pottier, 1992; Svanberg et al., 1994; Meijnders et al., 1996; Wennberg et al., 1996). However, comparing the results of ALA-PDT, a considerable variation in CR rates is observed; long-term response rates vary from 30 to 100% (Calzavara-Pinton, 1995). To achieve higher CR, some investigators have treated lesions more than once (Morton et al., 1996; Haller et al., 2000). The success of topical ALA-PDT is dependent on several factors. In addition to penetration of ALA into the skin(-lesion) and the formation of a therapeutic concentration of PpIX, CR depends

significantly on tumor thickness and duration of ALA application (Fink-Puches et al., 1998). Curettage before topical ALA-PDT may significantly improve CR rates, in particular for nodular basal cell carcinoma (Morton et al., 1998; Soler et al., 1999). In an attempt to increase the effectiveness of ALA-PDT, modified prodrugs of ALA have also been used such as a methyl aminolevulinate (MAL) and other esters (Van den Akker et al., 2000a, b, 2003). Although no comparative study has been performed to compare the clinical response using ALA and MAL, clinical studies published using MAL-PDT also show a variation in response rate. Horn et al. (2003) reported a recurrence rate of 18% in a study of 94 basal cell carcinomas that were treated with MAL-PDT (two treatments a week apart, follow-up (FU) 24 months. Soler et al. (2001) reported the treatment of 350 curettaged nodular basal cell carcinoma and superficial basal cell carcinoma (sBCC) using MAL-PDT in two sessions with a CR of 79% (FU 22–24 months). The approved scheme of MAL-PDT in Europe consists of two treatments a week apart. Rhodes et al. (2004) performed a comparative study using MAL-PDT versus surgery for nodular basal cell carcinomas, achieving 10% recurrence rate in the MAL-PDT group versus 2% recurrence rate in the surgery group. Recently, Van Iersel et al. (2005) reported their results after primary surgery for a basal cell carcinoma being a 5-year cumulative risk of only 2.1%. These studies support the need to improve the response of sBCC to both MAL and ALA-PDT.

We have performed series of pre-clinical studies investigating the effects of fractionating the illumination in PDT in a variety of model systems (Van der Veen et al., 1994, 1999;

[1]Department of Dermatology, Erasmus MC, Rotterdam, The Netherlands and [2]Department of Radiation Oncology, The Centre for Optical Diagnostics and Therapy, Erasmus MC, Rotterdam, The Netherlands

Correspondence: Dr Ellen R.M. de Haas, Department of Dermatology, Erasmus MC, PO Box 2040 (HP PB003), Rotterdam 3000CA, The Netherlands. E-mail: e.r.m.dehaas@erasmusmc.nl

Abbreviations: ALA, 5-aminolevulinic acid; CR, complete response; FU, follow-up; MAL, methyl aminolevulinate; PDT, photodynamic therapy; PpIX, protoporphyrin IX; sBCC, superficial basal cell carcinoma

De Bruijn et al., 1999; Robinson et al., 2000, 2003; Thissen et al., 2002). We have shown increased efficacy of ALA-PDT using fractionated illumination with relatively long dark intervals between two light fractions (Van der Veen et al., 1994). For topical administration, we have shown that a 2-hour dark interval is necessary to achieve a significant increase in response. We have shown that choice of fluence (rate) for the first light fraction is critical, and that a high fluence for the second light fraction is necessary for maximal tissue response (Robinson et al., 2003). Our first clinical pilot study using fractionated ALA-PDT using two equal light fractions of $45 \, \mathrm{J \, cm^{-2}}$ with a 2-hour dark interval resulted in a CR rate of 84% (mean FU 59 months; range 44–82 months, $n = 67$) (Star et al., 2006). This was an encouraging result, but we were unable to show a statistically significant increase in response compared to a single illumination. This is probably owing to the choice of a non-optimized illumination scheme and the small number of lesions treated. Here, we report on a randomized comparative prospective open clinical study between a standard ALA-PDT treatment scheme using a fluence of $75 \, \mathrm{J \, cm^{-2}}$, 4 hours after the administration of ALA and a 2-fold illumination using $20 + 80 \, \mathrm{J \, cm^{-2}}$, delivered 4 and 6 hours after the administration of ALA. We also investigated the kinetics of PpIX fluorescence during the 2-fold illumination scheme and their relation to the mechanism of response.

RESULTS

A 2-fold illumination scheme of $20 + 80 \, \mathrm{J \, cm^{-2}}$ with a 2-hour dark interval results in a significantly better clinical response to ALA-PDT over a single light fraction

The relative CR of lesions to ALA-PDT using a single light fraction and a 2-fold illumination scheme is shown in Figure 1. The CR using a 2-fold illumination is significantly greater than that following a single light fraction ($P = 0.002$, log-rank test). Twelve months after therapy, the relative CR in the 2-fold illumination group is 97%, whereas the corresponding CR in the single illumination group is 89%. Of the 243 lesions in the single illumination group, 32 lesions failed
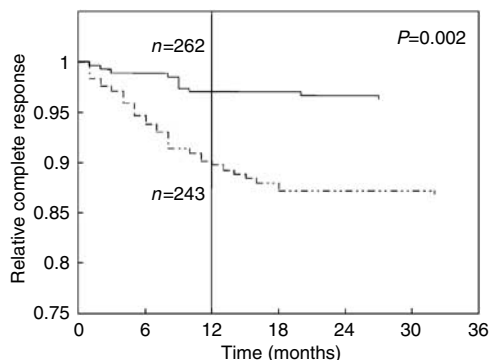
to respond or recurred during the FU period. Of these lesions, 10 lesions were retreated with ALA-PDT, with five lesions receiving a second single illumination and five were retreated using a 2-fold illuminated scheme; all resulted in CR (minimum FU 12 months). These lesions were not included as responders in our statistical analysis of CR. The remaining 22 lesions were surgically excised and consisted 17 nodular, one micro-nodular, two morphemic, and two sBCC.

Only 10 of the 262 lesions in the 2-fold illumination group did not show CR. Five lesions were excised and found to be nodular ($n = 3$) or morphemic ($n = 2$) basal cell carcinomas and five were retreated with 2-fold illumination ALA-PDT and responded (FU 7, 8, 11, 14, and 16 months without recurrence). These were included as non-responders, although after a second treatment CR was seen.

A sub-group analysis in which only histologically proven sBCCs are included shows a similar pattern of CR between the single- and 2-fold illumination groups. Twelve months after therapy, the relative CR in the 2-fold illumination group is 98%, whereas the corresponding CR in the single illumination group is 85% ($P = 0.0003$, log-rank test).

PDT was well tolerated in both illumination groups, and all patients completed therapy

A minority of patients (14%) required pain relief in addition to 2% lidocaine already present in the ALA ointment. The majority of patients, 133 out of 154 (86%), did not require additional pain relief during or after the therapeutic illumination. The details of patients who required pain medication are shown in Table 1. A greater number of patients required pain relief in the 2-fold illumination group than in the single illumination group. In the single illumination group, five patients required pain relief for six of 32 treated lesions. In the 2-fold illumination group, 15 patients required pain relief for 44 of 64 treated lesions. There was no consistent difference in pain during each of the 2-fold light fractions, although in general patients reported more pain during the first illumination. Pain resolved quickly during the dark interval. Our pre-clinical animal data suggested that the acute response to therapy might be more severe in the 2-fold illumination group. In the 2-fold illumination, crusts formed following therapy in 15 lesions in six patients. In the single illumination group, crusts were seen in two lesions in two patients. Despite crust formation, cosmetic outcome was good in all lesions. The relationship between acute response and the need for pain relief is shown in Table 1. One patient showed a pustular skin reaction in 11 of 16 lesions, which lasted 5 days. This was cultured and proven to be non-bacterial. A small number (19) showed persistent hypopigmentation at the illumination site 1 year after therapy.

There was no significant difference in response rates for the different light sources used. The response rate following a 2-fold illumination scheme was greater for each light source. We found no statistically significant difference between the response rates for the different light sources within each illumination group. A comparison of the response of lesions to a single- and a 2-fold illumination scheme for each individual light source is shown in Table 2.

Figure 1. A 2-fold illumination scheme of $20 + 80 \, \mathrm{J \, cm^{-2}}$ with a 2-hour dark interval results in a significantly better clinical response to ALA-PDT. Kaplan–Meier curves for disease-free rates for relative CR of sBCC using a single light fraction of $75 \, \mathrm{J \, cm^{-2}}$, 4 hours after the application of ALA (— - - - — - -) and a 2-fold illumination scheme of $20 + 80 \, \mathrm{J \, cm^{-2}}$ 4 and 6 hours after the application of ALA. (———). Statistical significance is tested for 1-year FU ($P = 0.002$, log-rank test: relative risk 0.3: confidence interval 0.15–0.59).

Table 1. Acute response and medication administered to patients who required pain relief and acute response (cosmetic outcome in these lesions was determined as good in all cases)

Patient	75 J cm^{-2} Lesions requiring medication (illuminated lesions)	Location	Anesthesia	20+80 J cm^{-2} Lesions requiring medication (illuminated lesions)	Location	Anesthesia	Timing, acute response, and other remarks
A	0 (6)			4 (7)	Trunk	Paracetamol	After first fraction, crusts+ (20+80 J cm^{-2})
B	1 (1)	Vertex	Paracetamol				After illumination
C	0 (4)	2 face, 2 trunk		2 (2)	Face	Paracetamol	After first fraction
D	0 (5)	Arm		2 (2)	Arm, face	Paracetamol	After first fraction
E	0 (2)	Trunk		7 (7)	3 arm, 3 trunk, 1 leg	Paracetamol	After first fraction
F	0 (15)	2 face, 10 trunk, 3 arm		3 (19)	13 trunk, 6 arm	Paracetamol	In advance of three lesions treated at the end of the treatment day
G	1 (1)	Face	Paracetamol	0 (1)	Face		After illumination
H				2 (2)	Face	Paracetamol	After second fraction
I				4 (4)	Leg	Paracetamol	After first fraction crusts+
J				4 (4)	2 trunk, 2 leg	Paracetamol	After first fraction (2) in advance (2)
K				1 (1)	Face	Lidocaine	Before second fraction
L				1 (1)	Face	Lidocaine	Before second fraction
M				4 (4)	1 face, 2 arm, 1 trunk	Lidocaine	Before second fraction, rheumatic patient
N	1 (1)	Arm	Lidocaine				During illumination, crusts+
O				2 (2)	Trunk	Lidocaine	Before second fraction
P	2 (2)	Face	Lidocaine				During illumination
Q				1 (1)	Vertex	Bupivacaine	Block before first fraction
R				4 (4)	face	Bupivacaine	Block before first fraction and local during first fraction
S	1 (1)	Face	Bupivacaine				During illumination, crusts+
T				1 (1)	Vertex	Bupivacaine	Block before first fraction, painful second fraction, crusts+
U				2 (2)	Arm	Bupivacaine	During first illumination
21	**6 (32)**		**5**	**44 (64)**		**15**	

Bold values signify total.

The kinetics of PpIX fluorescence were in accordance with that found in pre-clinical animal models. Figure 2 shows the mean fluorescence intensity present before and after the first light fraction of 20 J cm^{-2} and 2 hours later, immediately before the second light fraction. The PpIX fluorescence before the first light fraction varied considerably, between the lesions of each patient as well as between patients. When the fluorescence of all lesions is normalized to 100%, the mean fluorescence after the illumination is 47% and rises to 69% before the second illumination. There was considerable variation in PpIX fluorescence between lesions and between patients. Likewise, photobleaching varied widely, despite fixed illumination parameters. The average extent of photobleaching of 47% during the first light fraction is similar to that we have found in our pre-clinical studies. The amount of re-synthesis during the dark interval and the total amount of PpIX utilized in a single illumination compared to that in a 2-fold illumination scheme is also consistent to that we have seen in our pre-clinical studies (Robinson *et al.*, 2003).

DISCUSSION
We have demonstrated that a statistically significant improvement in CR rate can be achieved using a light fractionation scheme with a 2-hour dark interval between light fractions of

Table 2. CR of lesions treated with each light source in each illumination group

Light source	75 J cm^{-2}		20+80 J cm^{-2}		
	n	CR (%)	*n*	CR (%)	*P*-value
Diode laser (630 nm)	160	91	42	98	0.236
LED Omnilux (633 nm)	21	85	152	97	0.347
Broadband Red Source Medeikonos (590–650 nm)	62	89	68	97	0.093
Total	243	89	262	97	0.002

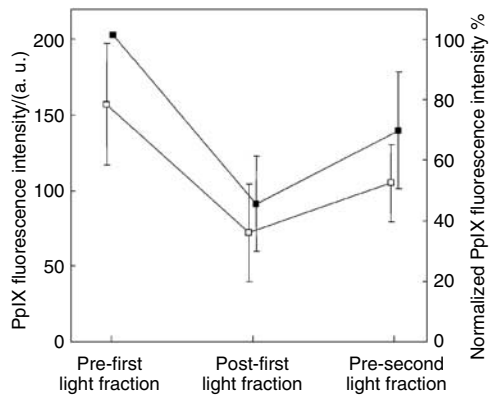CR, complete response; LED, light-emitting diode.

Figure 2. **Kinetics of PpIX fluorescence.** The kinetics of PpIX fluorescence were in accordance to that found in pre-clinical animal models, but did not explain the increase in response using a 2-fold illumination scheme. The kinetics of PpIX fluorescence intensity in sBCC during ALA-PDT with a (□) 2-fold illumination scheme and (■) the relative PpIX photobleaching during therapy with 20 J cm^{-2} and the re-synthesis during the dark interval between light fractions (*n* = 17 lesions).

20 and 80 J cm^{-2} compared to a single light fraction (97 *versus* 89% CR, 12 months FU, *P* = 0.002, log-rank test). The lower response rate found with a single light fraction (89%) is similar to our previous results (Meijnders *et al.*, 1996) and is consistent with the other investigators who have performed ALA-PDT in a single treatment session. Our choice of 2-fold illumination scheme is based on data from a range of pre-clinical animal studies that showed that following light illumination, tissues continue to synthesize PpIX. Apparently, the heme synthesis cycle is still (partly) intact and there is still ALA available to be converted into PpIX. PpIX fluorescence has been observed after PDT with both systemic ALA and topical ALA (Van der Veen *et al.*, 1994, 1999; Orenstein *et al.*, 1997; De Bruijn *et al.*, 1999; af Klinteberg *et al.*, 1999; Robinson *et al.*, 2000). However, these pre-clinical studies also demonstrate the complexity of the response of tissues to ALA-PDT and highlight the challenge for optimization (Robinson *et al.*, 2003). It is not simply a matter of performing a second illumination at some arbitrary time point after a standard single illumination. The time interval between light fractions is a very important consideration. A 2-hour time interval is necessary to yield a significant increase in response. Also, the fluence delivered in the first light fraction has a dramatic impact on the response to a 2-fold illumination. Although the response to a light fractionation scheme of 50 + 50 J cm^{-2} with a 2-hour dark interval is greater than a single illumination of 100 J cm^{-2}, the increase is not statistically significant. Reducing the fluence of the first light fraction to 5 J cm^{-2} and delivering a large fluence in the second (95 J cm^{-2}) significantly increases the effectiveness of PDT. In fact, this represents the most effective illumination scheme that we have yet devised. A similar pattern of response to light fractionation seems evident in clinical ALA-PDT of sBCC. In a clinical pilot study treating sBCC with two equal light fractions of 45 + 45 J cm^{-2}, separated by a 2-hour dark interval, we have shown an increase in long-term CR over that reported by other investigators (Star *et al.*, 2006). We were, however, not able to show a statistically significant improvement in CR at 12 months over a single illumination of 75 J cm^{-2}. This study shows that reducing the fluence of the first light fraction to 20 J cm^{-2} and maintaining that of the second light fraction to deliver a cumulative fluence of 100 J cm^{-2} results in a significant improvement in CR rate, just as predicted by our pre-clinical animal studies. We note that one other factor influenced the design of the clinical illumination scheme. sBCCs are in general more pigmented and significantly thicker than normal mouse epidermis. A higher light fluence of 20 J cm^{-2} was used, instead of the 5 J cm^{-2} that found to be optimal in our animal study, to ensure the deposition of sufficient fluence at the base of each lesion.

The cumulative fluence in each of the illumination groups is not equal. A fluence of 75 J cm^{-2} was delivered in a single light fraction compared to 100 J cm^{-2} in the 2-fold illumination scheme. This is a direct consequence of our intention to deliver a large fluence in the second light fraction (Robinson *et al.*, 2003). The influence of this additional cumulative fluence on the clinical response we observe is an important issue. A number of pre-clinical studies have shown that photobleaching of the PpIX limits the PDT dose that can be delivered in a single light fraction at fixed fluence rate (Robinson *et al.*, 1998). We have shown in normal mouse skin that 100 J cm^{-2} does not result in significantly more damage than 50 J cm^{-2}. The relationship between response to ALA-PDT and fluence has not been systematically investigated in the clinic. Only Oseroff (1998) has emphasized the importance of light fluence in the treatment of sBCC by topical ALA-PDT, and this regards the delivered of very high light fluences at high fluence rate. He reported that a CR rate of 95% after 200 J cm^{-2} (at a fluence rate of 150 mW cm^{-2}) fell to 70% after 150 J cm^{-2}. Most other investigators have applied both lower light fluence (rate) and cumulative fluences both below 75 J cm^{-2} and above 100 J cm^{-2}, with little evidence for a correlation between fluence and response.

Large variations occur in our fluorescence data. Several factors may be involved here, such as variations in tissue optical properties, in oxygenation, and in heterogenity of the PpIX distribution in the lesions (Martin *et al.*, 1995). The variations in ALA-induced sBCC fluorescence reported by

af Klinteberg *et al.* (1999) seem somewhat less, but these authors only report standard errors. Golub *et al.* (1999) also measured large variations in ALA-induced fluorescence on normal human skin and psoriasis and actinic keratosis lesions. Such variability is widely observed in ALA-induced fluorescence of normal human skin and skin lesions (Barr *et al.*, 1987; Marchesini *et al.*, 1994; Star, 1995, 2006). Accepting the large variations that seem to be a feature of clinical fluorescence measurements, we do see a similar trend in the kinetics of PpIX fluorescence after illumination with $20 \, J \, cm^{-2}$. Importantly, we note that the original rationale for performing a 2-fold illumination scheme was to utilize additional PpIX that continued to be synthesized after PDT with a single light fraction. Our recent clinical data (Star *et al.*, 2006) and pre-clinical data (Robinson *et al.*, 2003) do not support the hypothesis that significantly more PpIX is utilized in the 2-fold illumination scheme, as $50 + 50$ and $5 + 95 \, J \, cm^{-2}$ result in approximately the same amount of cumulative PpIX photobleaching ($P = 0.12$, Student's *t*-test).

The spectral output of the light sources used in this study may have influenced the response to a 2-fold illumination scheme. Our pre-clinical studies have shown that the response of tissues to PDT using the 2-fold illumination scheme is quite sensitive to the small differences in fluence and fluence rate of the first light fraction. Some investigators have suggested that there may be differences in response to PDT with (a) light sources that deliver a lower effective fluence rate by virtue of the overlap of their spectral output with the absorption spectrum of PpIX (Clark *et al.*, 2003) and (b) light sources that additionally excite the fluorescent photoproducts of PpIX (Gudgin Dickson and Pottier, 1995). The fact that we did not see differences in response between light sources suggests that these effects are small and do not impact significantly on the effective dose of the first light fraction. This means that the current fluence and dark interval findings in the current study are also applicable for the large proportion of investigators that use commercially available non-laser light sources. Our data stratified by light source (Table 2) show that there is approximately the same increase in response of all the light sources investigated. We expect that statistical significance would be achieved if larger numbers of lesions were investigated.

During the whole FU period, a significantly greater number of recurrent or non-responding lesions were observed in the single illumination group; 32/243 (13%) compared to the 2-fold illumination group 10/262 (4%) ($P = 0.0002$, Fisher's exact test). Of the 32 lesions that did not show CR in the single illumination group, 20 were found not to be sBCC: nodular (18); micro-nodular (1); and morphemic (2) at recurrence. Of the 10 lesions that did not show CR in the 2-fold illumination group, five were found not to be sBCC at recurrence, but nodular (3) and morphemic (2). The presence of nodular lesions in these data was not unexpected as diagnosis was based on clinical appearance for a significant proportion of lesions. Fifty-four percent and 49% of lesions were diagnosed clinically in the single- and 2-fold illumination group, respectively. Clinical diagnostic accuracy in a derma-

tology university faculty has been shown to be approximately 70% for BCC (Presser and Taylor, 1987). If a similar accuracy were assumed in this study, we would expect to see 38 and 35 nodular lesions in our single- and 2-fold illumination groups, respectively. Three recurrent or non-responding nodular BCC's in the 2-fold illumination group represents a significantly lower number than expected ($P = 0.0003$, Fisher's exact test). Although it was not out primary intention to treat nodular lesions, we have previously shown that the 2-fold illumination leads to deeper histological damage in pre-clinical models (De Bruijn *et al.*, 1999; Robinson *et al.*, 2003). Determining the maximum depth of BCC that can be treated with ALA-PDT is clearly an area for future study.

The increased severity of the acute response following therapy shows a similar pattern to the increase in damage induced in our pre-clinical animal studies. This and the fact that we see an accompanying increase in CR after 12 months FU provides encouraging evidence for the value of PDT optimization in pre-clinical animal studies. In our animal studies, the increase in the effectiveness of the 2-fold illumination scheme leads to scarring in some cases. Although the clinical acute response is greater than a single illumination, the cosmetic outcome remains good in 90% of the lesions. We are also encouraged by the fact that patients did not experience significantly more pain during the 2-fold illumination scheme.

A central aim of the current study was to achieve an optimal response to PDT in a single treatment session (i.e. with a single application of ALA). We note that repeating such an optimized treatment regimen on subsequent treatment days may lead to an even higher CR rate in sBCC and/or a higher CR rate for other skin lesions. In conclusion, we have demonstrated a significant increase in the CR rate of sBCC to ALA-PDT using an illumination scheme in which two light fractions of 20 and $80 \, J \, cm^{-2}$ are delivered 4 and 6 hours after the application of ALA compared to a single illumination scheme of $75 \, J \, cm^{-2}$. Further FU is now necessary to determine if this high CR rate is maintained. We note that the value of these results may extend beyond the skin, into other organs such as the esophagus and brain, where ALA-PDT is under investigation as a treatment modality (Bogaards *et al.*, 2005; Pech *et al.*, 2005).

MATERIALS AND METHODS
Patients
All patients were diagnosed as having an sBCC within the Erasmus MC in Rotterdam. ALA-PDT was performed according to two treatment protocols, described in detail below, approved by the local ethics committee, according to the Declaration of Helsinki Principles. All patients gave informed consent. Diagnosis was determined histologically (4 mm punch biopsy) and clinically in approximately equal proportions within each treatment group. Every patient had at least one histologically diagnosed primary sBCC. Our patient population consists of both first-line and secondary dermatological care. All patients are adult Caucasians. We treated 100 patients who had in total 243 lesions, using a single illumination scheme. Fifty-five patients with a total of 262 lesions were treated using a 2-fold illumination scheme, described below. Both groups

Table 3. High-risk patients

Patient group	75 J cm^{-2}		20+80 J cm^{-2}	
	Patients	Lesions	Patients	Lesions
Immune compromised[1]	7	7	2	6
Previous radiotherapy	8	33	5	20
Goltz Gorlin syndrome	5	46	4	17
High sun exposure[2]	2	16	2	28
Topical arsenic use	—	—	1	9

[1]Immune compromised: HIV positive, organ recipient, or using immuno-suppressive drugs.
[2]Patients who have lived more than 15 years in tropical countries and had Fitzpatrick skin type 1.

were in the same age range; in the group that received a single fraction, the mean age was 57 years (minimum 32, maximum 81, median 57), and in the 2-fold illumination group, the mean age was 56 years (minimum 31, maximum 83, median 56). Also, both treatment groups included a similar number of higher risk patients as shown in Table 3.

ALA application and local anesthesia
A topical ALA ointment we used was prepared by our Hospital Pharmacy using 20% ALA (FLUKA, The Netherlands) in Instilagel (Medeco BV, Oud Beijerland, The Netherlands). Instilagel was used as vehicle because it contains lidocaine (2%), which is considered a possible advantage in pain management (Ibbotson, 2002). The ointment was freshly prepared and stored in a refrigerator and used within 3 days. Before application of ALA, crusts and scaling were gently removed using a disposable curette. The lesion was covered with a margin of 1 cm and dressed with a semipermeable dressing (Tegaderm 3M, The Netherlands) and light-protecting covering (aluminum foil). Patients were instructed to stay out of the cold because of the negative effect of low temperatures on ALA metabolism (Van den Akker *et al.*, 2004). In addition to the topical anesthetic in the ALA ointment, patients received paracetamol, lidocaine (without epinephrine), or bupivacaine, if required.

Light sources and illumination scheme
Three light sources were used in this study. A 630 nm diode laser (Carl Zeiss, Oberkochen, Germany) was used to provide 630 nm illumination. Light was coupled into a 600 μm optical fiber and projected onto the lesion using a combination of lenses to assure a uniform fluence rate across the beam diameter. Two commercially available broadband light sources were also used. The first had a spectral output between 590 and 650 nm (Medeikonos, Gothenburg, Sweden). The second was a light-emitting diode, light source with a spectral output centered on 633 nm with a bandwidth of 20 nm (Omnilux, Waldmann, Tiel, The Netherlands). All three light sources were used to illuminate lesions with a margin of at least 5 mm at a constant measured fluence rate of 50 mW cm^{-2}. It was necessary to shield areas outside this margin for both broadband light sources. In the single illumination group, lesions were illuminated 4 hours after the application of ALA to a fluence of 75 J cm^{-2}. In the 2-fold illumination group, lesions received light fractions of 20 and

80 J cm^{-2}, 4 and 6 hours after the application of ALA. ALA was applied once. Again, both light fractions were delivered at a fluence rate of 50 mW cm^{-2}. During the 2-h dark interval between light fractions, lesions were covered with light-protective bandage. Table 2 shows the number of lesions treated with each light sources in each illumination group.

Fluorescence measurements
PpIX fluorescence measurements were acquired from a subgroup of 17 lesions in the 2-fold illumination group to investigate the kinetics of PpIX fluorescence during therapy. In this subgroup, PDT was performed using the diode laser. Data were acquired before ALA application, immediately before and after the first illumination and immediately before the second illumination. Measurements were performed with a custom applicator attached to a fluorescence-imaging camera (TRICAM, Storz, Tuttlingen, Germany). A uniform excitation light field (400–450 nm) was obtained from a ring of filtered blue light-emitting diodes mounted around the camera. Fluorescence emission from PpIX was collected over a period of 0.25 seconds. Scattered excitation light was blocked using a long-pass filter (Schott KV 500). Fluorescence emission from PpIX (600–710 nm) was collected over a period of 0.25 seconds. Images acquired at different time points are registered under translation and rotation using landmarks in the skin surrounding the lesion (e.g. hair follicles). Typically, a rectangular area of 4×6 cm is imaged. Within this area, a circular region of interest approximately 0.25 cm in diameter is defined and the average pixel intensity was used as a measure of the fluorescence intensity from an individual lesions. Measurements on a sheet of white plastic were used as a reference to correct the fluorescence measurements of BCC for any changes in the output of the excitation light source or the sensitivity of the detection system. Autofluorescence was measured before each ALA application and was subtracted from the PpIX fluorescence intensity acquired before and after each illumination.

Response and FU
We defined CR as the absence of clinical visual basal cell carcinoma. All patients treated in our department are seen for FU according to the national dermatological guidelines of the Netherlands (NVDV). In the first year after treatment, FU was performed four times a year, thereafter twice yearly. Patients with a propensity of developing numerous skin lesions were seen more frequently in an individualized scheme. FU ends 5 years past the last treated basal cell carcinoma. Some of the patients are referred to us by peripheral primary dermatologists and are treated as secondary dermatological care patients and then referred back to their primary dermatologist for further FU. We only considered FU performed by our staff members and residents within our University hospital. A minimal FU of 12 months is necessary for inclusion in the study. In the single illumination group, FU varied from 12 to 41 months, mean 21 months. In the 2-fold illumination group, FU varied between 12 and 32 months, mean 17 months. Lesions that did not respond to therapy or recurred in the first 12 months after treatment were included in the data analysis as in CR or recurrence. Cosmetic outcome was not the primary goal of the study, but remarks of patients and dermatologist were asked for and noted. The patient and dermatologist judged cosmetic outcome to be either good, fair, or poor.

Statistical analysis

Kaplan–Meier analysis was performed on relative CR rates after therapy and the log-rank test was used to compare the significance of differences between treatment groups. The primary and overall response rates of lesions treated with different illumination schemes were compared using Fisher's exact test. Differences in fluorescence intensity were compared using Student's *t*-test. $P < 0.05$ was considered statistically significant.

REFERENCES

af Klinteberg C, Enejder AMK, Wang I, Andersson-Engels S, Svanberg S, Svanberg K (1999) Kinetic fluorescence studies of 5-aminolevulinic acid-induced protoporphyrin IX accumulation in basal cell carcinomas. *J Photochem Photobiol B: Biol* 49:120–8

Barr H, Tralau CJ, MacRobert AJ, Krasner N, Boulos PB, Clark CG *et al.* (1987) Photodynamic therapy in the normal rat colon with phthalocyanine sensitisation. *Br J Cancer* 56:111–8

Bogaards A, Varma A, Zhang K, Zach D, Bisland SK, Moriyama EH *et al.* (2005) Fluorescence image-guided brain tumour resection with adjuvant metronomic photodynamic therapy: pre-clinical model and technology development. *Photochem Photobiol Sci* 4:438–42

Calzavara-Pinton PG (1995) Repetetive photodynamic therapy with topical delta-aminolevulinic acid as an appropriate approach to the routine treatment of superficial non-melanoma skin tumours. *J Photochem Photobiol B* 29:53–7

Clark C, Bryden A, Dawe R, Moseley H, Ferguson J, Ibbotson S (2003) Topical 5-aminolevulinic acid photodynamic therapy for cutaneous lesions: outcome and comparison of light sources. *Photodermatol Photoimmunol Photomed* 19:134–41

De Bruijn HS, van der Veen N, Robinson DJ, Star WM (1999) Improvement of systemic 5-aminolevulinic acid-based photodynamic therapy *in vivo* using light fractionation with a 75-minute interval. *Cancer Res* 59:901–4

Fink-Puches R, Soyer HP, Hofer A, Kerl H, Wolf P (1998) Long term follow-up and histological changes of superficial nonmelanoma skin cancers treated with topical *δ*-aminolevulinic acid photodynamic therapy. *Arch Dermatol* 134:821–6

Golub AL, Gudgin Dickson EF, Kennedy JC, Marcus SL, Park Y, Pottier RH (1999) The monitoring of ALA-induced protoporphyrin IX accumulation and clearance in patients with skin lesions by *in vivo* surface-detected fluorescence spectroscopy. *Lasers Med Sci* 14:112–22

Gudgin Dickson EF, Pottier RH (1995) On the role of protoporphyrin IX photoproducts in photodynamic therapy. *J Photochem Photobiol B* 29:91–3

Haller JC, Cairnduff F, Slack G, Schofiled J, Whitehurst C, Turnstall R *et al.* (2000) Routine double treatments of superficial basal cell carcinomas using aminolevulinic acid-based photodynamic therapy. *Br J Dermatol* 143:1270–4

Horn M, Wolf P, Wulf HC (2003) Topical aminolevulinate photodynamic therapy in patients with basal cell carcinoma prone to complications and poor cosmetic outcome with conventional treatment. *Br J Dermatol* 149:1242–9

Ibbotson SH (2002) Topical 5-aminolevulinic acid photodynamic therapy for the treatment of skin conditions other than non-melanoma skin cancer. *Br J Dermatol* 146:178–88

Kennedy JC, Pottier R, Pross DC (1990) Photodynamic therapy with endogenous protoporphyrin IX: basic principles and present clinical experience. *J Photochem Photobiol B* 6:143–8

Kennedy JC, Pottier RH (1992) Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy. *J Photochem Photobiol B* 14:275–92

Marchesini R, Pignoli E, Tomatis S, Fumagalli S, Sichirollo AE, Di Palma S *et al.* (1994) *Ex vivo* optical properties of human colon tissue. *Lasers Surg Med* 15:351–7

Martin A, Tope WD, Grevelink JM, Starr JC, Fewkes JL, Flotte TJ *et al.* (1995) Lack of selectivity of protoporphyrin IX fluorescence for basal cell carcinoma after topical application of 5-aminolevulinic acid: implications for photodynamic treatment. *Arch Dermatol Res* 287:665–74

Meijnders PJN, Star WM, De Bruijn HS, Treurniet-donker AD, van Mierlo MJM, Wijtthoff SJM *et al.* (1996) Clinical results of photodynamic therapy for superficial skin malignancies and actinic keratosis using topical 5-aminolevulinic acid. *Lasers Med Sci* 11:123–31

Morton CA, MacKie RM, Whitehurst C, Moore JV, McColl JH (1998) Photodynamic therapy for basal cell carcinoma: effect of tumor thickness and duration of photosensitizer application on response. *Arch Dermatol* 134:248–9

Morton CA, Whitehurst C, Moseley H, McColl JH, oore JV, Mackie RM (1996) Comparison of photodynamic therapy with cryotherapy in the treatment of Bowen's disease. *Br J Dermatol* 135:766–71

Orenstein A, Kostenich G, Malik Z (1997) The kinetics of fluorescence during ALA-PDT in human malignant skin tumors. *Cancer Lett* 120:229–34

Oseroff AR (1998) PDT for cutaneous malignancies: clinical applications and basic mechanisms. *Photochem Photobiol* 67:17S–8S

Pech O, Gossner L, May A, Rabenstein T, Vieth M, Stolte M *et al.* (2005) Long-term results of photodynamic therapy with 5-aminolevulinic acid for superficial Barrett's cancer and high-grade intraepithelial neoplasia. *Gastrointest Endosc* 62:24–30

Presser SE, Taylor JR (1987) Clinical diagnostic accuracy of basal cell carcinoma. *J Am Acad Dermatol* 16:988–90

Rhodes LE, De Rie Enstrom Y, Groves R, Morken T, Goulden V *et al.* (2004) Photodynamic therapy using topical methyl aminolevulinate vs surgery for nodular basal cell carcinoma. *Arch Dermatol* 140:17–23

Robinson DJ, de Bruijn HS, de Wolf WJ, Sterenborg HJ, Star WM (2000) Topical 5-aminolevulinic acid photodynamic therapy of hairless mouse skin using two-fold illumination schemes: PpIX fluorescence kinetics, photobleaching and biological effect. *Photochem Photobiol* 72:794–802

Robinson DJ, de Bruijn HS, Star WM, Sterenborg HJ (2003) Dose and timing of the first light fraction in two-fold illumination schemes for topical ALA-mediated photodynamic therapy of hairless mouse skin. *Photochem Photobiol* 77:319–23

Robinson DJ, de Bruijn HS, van der Veen N, Stringer MR, Brown SB, Star WM (1998) Fluorescence photobleaching of ALA-induced protoporphyrin IX during photodynamic therapy of normal hairless mouse skin: the effect of light dose and irradiance and the resulting biological effect. *Photochem Photobiol* 67:140–9

Soler AM, Warloe T, Berner A, Giercksky HK (2001) A follow up study of recurrence and cosmesis in completely responding superficial and nodular basal call carcinomas treated with methyl-aminolevulinate based phorodynamic therapy alone and with prior curettage. *Br J Dermatol* 145:467–71

Soler AM, Warloe T, Tausjø J, Berner A (1999) Photodynamic therapy by topical aminolevulinic acid, dimethylsulphoxide and curettage in nodular basal cell carcinoma: a one year follow-up study. *Acta Dermatol Venerol* 79:204–6

Star WM (1995) Diffusion theory of light transport. In: *Optical–thermal response of laser-irradiated tissue* (Welch AJ, van Gemert MJC, eds), New York: Plenum Press, pp 131–206

Star WM, van 't Veen AJ, Robinson DJ, Munte K, de Haas ERM, Sterenborg HJCM (2006) Topical 5-aminolevulinic acid mediated photodynamic therapy of superficial basal cell carcinoma using two equal light fractions with a two hour interval long term follow-up. *Acta Dermatol Venerol* (in press)

Svanberg K, Andersson T, Killander D, Wang I, Stenram U, Andersson-Engels S *et al.* (1994) Photodynamic therapy of nonmelanoma tumours of the skin using topical δ-aminolevulinic acid sensitisation and laser irradiation. *Br J Dermatol* 130:743–51

Thissen MR, de Blois MW, Robinson DJ, de Bruijn HS, Dutrieux RP, Star WM *et al.* (2002) PpIX fluorescence kinetics and increased skin damage after intracutaneous injection of 5-aminolevulinic acid and repeated illumination. *J Invest Dermatol* 118:239–45

Van den Akker JHTM, de Bruijn HS, Beijersbergen Henegouwen GJM, Star WM, Sterenborg HJCM (2000a) Protoporphyrin IX fluorescence kinetics and localization after topical application of ALA pentyl ester and ALA on hairless mouse skin with UVB induced early skin cancer. *Photochem Photobiol* 72:399–406

Van den Akker JTHM, Holroyd JA, Vernon DI, Sterenborg HJCM, Brown SB (2003) Comparative *in vitro* percutaneous penetration of 5-aminolevulinic acid and two of its esters through excised hairless mouse skin. *Las Surg Med* 3:173–81

Van den Akker JTHM, Iani V, Star WM, Sterenborg HJCM, Moan J (2000b) Topical application of 5-aminolevulinic acid hexyl aster and 5-aminolevulinic acid to normal nude mouse skin: differences in protoporphyrin IX fluorescence kinetics and the role of the stratum corneum. *Photochem Photobiol* 72:681–9

Van den Akker JT, Boot K, Vernon DI, Brown SB, Groenendijk L, van Rhoon GC *et al.* (2004) Effect of elevating the skin temperature during topical ALA application on *in vitro* ALA penetration through mouse skin and *in vivo* PpIX production in human skin. *Photochem Photobiol Sci* 3:263–7

Van der Veen N, Hebeda KM, de Bruijn HS, Star WM (1999) Photodynamic effectiveness and vasoconstriction in hairless mouse skin after topical 5-aminolevulinic acid and single or two-fold illumination. *Photochem Photobiol* 70:921–9

Van der Veen N, van Leengoed HLLM, Star WM (1994) *In vivo* fluorescence kinetics and photodynamic therapy using 5-aminolevulinic acid-induced porphyrin: increased damage after multiple irradiations. *Br J Cancer* 70:867–72

Van Iersel CA, van de Velden HV, Kusters CD, Spauwen PH, Blokx WA, Kiemeney LA *et al* (2005) Prognostic factors for a subsequent basal cell carcinoma: implications for follow up. *Br J Dermatol* 153:1078–9

Wennberg A-M, Lindholm LE, Alpsten M, Larkö O (1996) Treatment of superficial basal cell carcinomas using topically applied delta-aminolevulinic acid and a filtered xenon lamp. *Arch Dermatol Res* 288:561–4