

# Improvement of Systemic 5-Aminolevulinic Acid-based Photodynamic Therapy *in Vivo* Using Light Fractionation with a 75-Minute Interval<sup>1</sup>

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## ABSTRACT

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

## INTRODUCTION

PDT<sup>3</sup> using ALA-induced PpIX as a photosensitizer is widely used as an experimental therapy, especially for cutaneous cancer. A complete initial response rate of >90% has been reported for treatment of human superficial basal cell carcinoma with topically applied ALA-PDT (1–3). However, for nodular BCC, a much lower complete response 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 the tumor (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 esophagus (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 would 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 h or longer. After the first light fraction, PpIX is partially or completely photobleached, and in time,

posttreatment new PpIX is formed, which can be used for a second illumination (14, 15). Van der Veen *et al.* (14) reported complete necrosis of four of six tumors in a rat skinfold observation chamber model using a long-term light fractionation scheme (with an interval of 75 min) after a single ALA administration. No necrosis was observed after a single illumination. These studies show that improvement of ALA-PDT using different illumination schemes is possible. Our interest here was to improve systemic ALA-PDT of nodular tumors. We, therefore, studied the effectiveness of different illumination schemes published by our own group (12, 14) and others (11, 13) by measuring the tumor volume doubling time of a transplantable rat rhabdomyosarcoma after transdermal illumination.

## MATERIALS AND METHODS

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

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

PDT was carried out under general anesthesia using *i.m.* 0.5 ml·kg<sup>-1</sup> Hypnorm (Janssen Pharmaceutica, Tilburg, the Netherlands) and 2.5 ml·kg<sup>-1</sup>. Prior to the light treatment the skin overlying the tumor was shaved. The animals were placed on a temperature controlled stage and covered with a black polythene mask. Tumors were transdermally illuminated with a 10-mm-diameter plane parallel light beam (633 nm; Ref. 3). Immediately after PDT, the animals were housed under subdued light conditions at 28°C for the first 24 h. This was done to minimize the decrease in body temperature caused by the anesthesia. Subsequently, the animals were kept at room temperature.

Ten groups of animals were treated according to various treatment schemes. Groups A, B, and C served as controls and were treated either with anesthesia only ( $n = 6$ ), light only (100 J·cm<sup>-2</sup> at 100 mW·cm<sup>-2</sup>;  $n = 3$ ) or ALA only (200 mg/kg *i.v.*;  $n = 3$ ) respectively. Groups D–J ( $n = 6$  for each group) were treated according to different illumination schemes as shown in Fig. 1. Each illumination was carried out at either 1 or at 2.5 h after injection of ALA. These time points were based on a pharmacokinetic study performed on this animal model in which we found a maximal PpIX fluorescence of the tumor at 2.5 h postinjection.<sup>4</sup> At 1 h post-ALA administration, approximately one third of the maximal PpIX fluorescence was observed.<sup>4</sup>

In groups D and E, the tumors were illuminated with a single light fluence of 100 J·cm<sup>-2</sup> at a fluence rate of 100 mW·cm<sup>-2</sup> delivered at 1 and 2.5 h post-ALA injection, respectively. In groups F–I, the tumors were illuminated at 2.5 h after injection of ALA. The tumors in group F received 100 J·cm<sup>-2</sup> at 25 mW·cm<sup>-2</sup> so that the treatment time was a factor of 4 longer than that in groups D and E. The short-term light fractionation schemes were applied in groups G and H. In group G, 100 J·cm<sup>-2</sup> at 100 mW·cm<sup>-2</sup> was delivered with one interruption of 150 s after the first 5 J·cm<sup>-2</sup> (13). In group H, 100 J·cm<sup>-2</sup> at 100 mW·cm<sup>-2</sup> was delivered with multiple interruptions, with the light

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<sup>3</sup> The abbreviations used are: PDT, photodynamic therapy; ALA, 5-aminolevulinic acid; PpIX, protoporphyrin IX.

<sup>4</sup> D. J. Robinson, H. S. de Bruijn, and W. M. Star. Pharmacokinetics of protoporphyrin IX following the administration of systemic 5-aminolevulinic acid in rat rhabdomyosarcoma, manuscript in preparation.

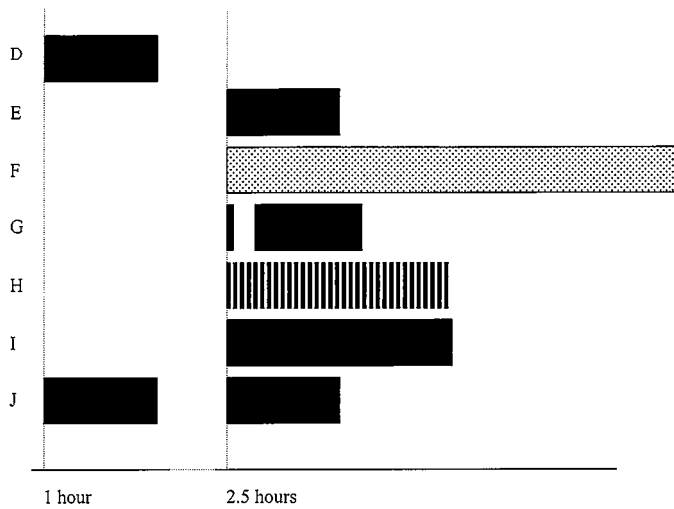


Fig. 1. Schematic diagram of the treatment schemes studied.

being turned off and on every 30 s (11). Groups I and J were both treated with a double light fluence of  $200 \text{ J cm}^{-2}$  at  $100 \text{ mW cm}^{-2}$  given either in one fraction (2.5 h p.i. of ALA) or according to a long-term light fractionation scheme of two equal fractions of  $100 \text{ J cm}^{-2}$  with an interruption of 75 min (treatment at 1 and 2.5 h p.i. of ALA; Ref. 14).

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

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

## RESULTS

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

The edema was investigated by measuring the thickness of the leg adjacent to the tumor daily. All animals treated with ALA-PDT showed a mild to severe edema of the leg, which was found to be maximal on day 1 posttreatment and cleared by day 4. Normally, the leg has a thickness of  $\sim 7 \text{ mm}$ , but at day 1 posttreatment, the leg could measure 10.7–15.8 mm thick (Table 1). The edema found for

tumors treated at 1 h after administration of ALA was significantly less compared to the other treatment schemes. The edema found for tumors illuminated with  $200 \text{ J cm}^{-2}$  in one fraction (scheme I) was significantly greater compared to the rest of the treatment schemes.

Almost all treatment schemes induced a bluish/black discoloration of the skin overlying the tumor after treatment, which cleared within a few days. The involved area was as large as the illuminated tumor under the skin, that is, smaller than the illuminated area. In some treatment schemes, severe discoloration was accompanied by crust formation (Table 1).

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

To histologically determine the location of the edema and the cause of the discoloration, a separate set of experiments were performed. Four extra animals were illuminated with  $200 \text{ J cm}^{-2}$  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 posttreatment for histology. Sections of the leg, including skin and soft tissues, were stained with H&E after formalin fixation. The epidermis and the dermal adnexa showed necrosis after both illumination schemes. Severe edema was found in the dermis and the muscle surrounding and underlying the tumor, whereas the tumor showed little or no edema. Enlarged blood vessels that were located around and at the border of the tumor were heavily damaged, and there was evidence of hemorrhage.

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

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

All of the PDT schemes investigated demonstrated a significantly longer tumor volume doubling time compared to control tumors, as

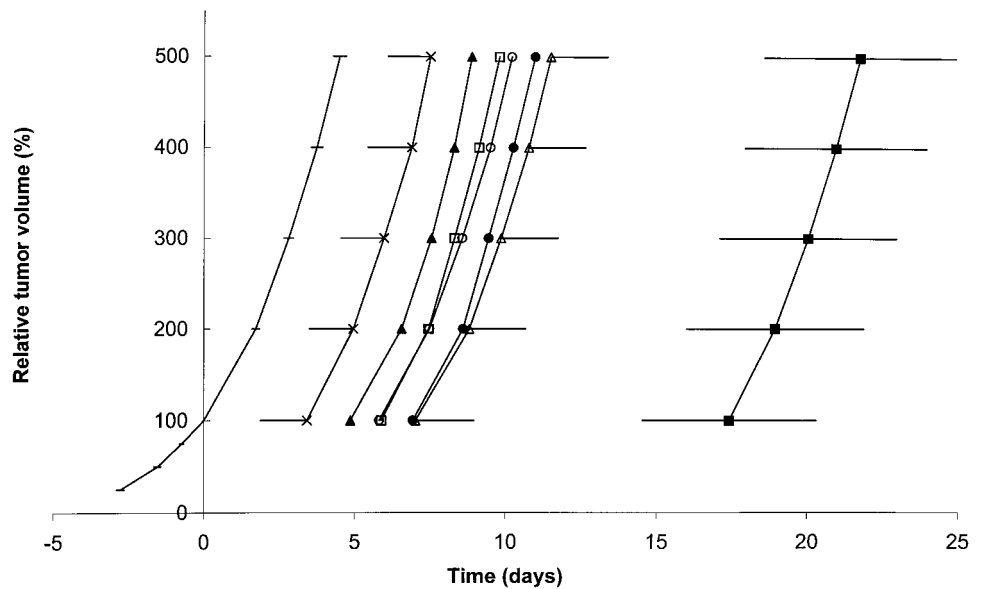
Table 1 Normal tissue damage caused by the different treatment schemes used

Group	Normal tissue damage	
	Edema (mm)	Crusts (n)
D	$10.7 \pm 0.3^a$	2
E	$14.2 \pm 0.9$	
F	$13.8 \pm 0.3$	4
G	$12.4 \pm 0.2$	2
H	$13.3 \pm 0.7$	2
I	$15.8 \pm 0.3^b$	3
J	$12.7 \pm 0.7$	6

<sup>a</sup> Significantly less edema than the other groups.

<sup>b</sup> Significantly more edema than the other groups.

Fig. 2. Relative tumor volume in time after ALA-PDT using different illumination schemes: control (—); scheme D, 1 h,  $100 \text{ J}\cdot\text{cm}^{-2}$  at  $100 \text{ mW}\cdot\text{cm}^{-2}$  (×); scheme E, 2.5 h,  $100 \text{ J}\cdot\text{cm}^{-2}$  at  $100 \text{ mW}\cdot\text{cm}^{-2}$  (▲); scheme F, 2.5 h,  $100 \text{ J}\cdot\text{cm}^{-2}$  at  $25 \text{ mW}\cdot\text{cm}^{-2}$  (△); scheme G, 2.5 h,  $100 \text{ J}\cdot\text{cm}^{-2}$  at  $100 \text{ mW}\cdot\text{cm}^{-2}$  using a short-term light fractionation scheme with one dark interval of 150 s after  $5 \text{ J}\cdot\text{cm}^{-2}$  (□); scheme H, 2.5 h,  $100 \text{ J}\cdot\text{cm}^{-2}$  at  $100 \text{ mW}\cdot\text{cm}^{-2}$  using a short-term light fractionation scheme: 30 s on/30 s off (○); scheme I, 2.5 h,  $200 \text{ J}\cdot\text{cm}^{-2}$  at  $100 \text{ mW}\cdot\text{cm}^{-2}$  (●); and scheme J, both 1 and 2.5 h  $100$  and  $100 \text{ J}\cdot\text{cm}^{-2}$  at  $100 \text{ mW}\cdot\text{cm}^{-2}$  (■). Data points, means; bars, SE.



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

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

**Tumor Thickness.** As might be expected because the illumination was superficial, the tumor response to PDT seemed to be correlated to the thickness of the treated tumor. After observation of the growth curves of the individual tumors in the groups, there seemed to be a threshold for the thickness. Tumors thinner than 4 mm responded significantly better to treatment with a total fluence dose of  $100 \text{ J}\cdot\text{cm}^{-2}$  compared to thick tumors. The mean tumor volume doubling time for thin tumors of groups D–H was  $12.0 \pm 2.2$  days ( $n = 5$ ) compared to a mean tumor volume doubling time of  $5.9 \pm 2.2$  days for thick tumors ( $n = 25$ ). For illuminations with a total light fluence of  $200 \text{ J}\cdot\text{cm}^{-2}$  delivered either in one fraction or according to a long-term light fractionation scheme (groups I and J, respectively),

this difference in volume doubling time between thin and thick tumors was not found.

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

## DISCUSSION

In this study, we have demonstrated a dramatic increase in tumor volume doubling time following systemic ALA-PDT using a long-term light fractionation scheme (two light fractions separated by a dark interval of 75 min). In previous studies, it was shown that new PpIX is formed after complete photobleaching caused by the illumination (14, 15). This newly formed PpIX can be used 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 four of six tumors with complete necrosis at day 7 posttreatment compared to no necrosis for a single illumination scheme. In this long-term light fractionation scheme a double light fluence ( $200 \text{ J}\cdot\text{cm}^{-2}$ ) was delivered (14) compared to the single illumination ( $100 \text{ J}\cdot\text{cm}^{-2}$ ), which might explain the increased effect. However, when PpIX is completely photobleached, a longer illumination is not expected to be more effective; this was also demonstrated here. Treating the tumor with a double light fluence ( $200 \text{ J}\cdot\text{cm}^{-2}$ ) did not significantly increase tumor volume doubling time compared to  $100 \text{ J}\cdot\text{cm}^{-2}$ , whereas treatment with the same total fluence according to a long-term light fractionation scheme did (Fig. 1). In fact, this scheme increased the tumor volume doubling time by a factor of 2.6. The substantially improved tumor response can only be explained by the use of the dark interval between two light fractions. As we have discussed, the long interruption may allow time for the formation of new PpIX which can be used for a second illumination and result in extra cell death. The origin of this new PpIX fluorescence is, as yet, unknown. One

possibility is that ALA is still present in the tissue and can be converted into PpIX by the surviving cells.

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

In contrast, ALA-PDT using a low fluence rate or a short-term light fractionation scheme did not significantly improve the tumor volume doubling time. These illumination schemes were designed to increase the amount of singlet oxygen formation during the treatment by reducing the demand rate for oxygen (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  $50 \text{ mW}\cdot\text{cm}^{-2}$  was rather small, whereas the difference between these fluence rates and  $5 \text{ mW}\cdot\text{cm}^{-2}$  was considerable. Hua *et al.* (11) showed a 1.5 times longer volume doubling time for tumors illuminated with a 4 times lower fluence rate after systemic ALA administration. The volume doubling time was found to be further enhanced for tumors treated with a 30-s light on/off short term light fractionation scheme. Messmann *et al.* (13) obtained a greater area of necrosis of normal colon after illumination using several short-term light fractionation schemes. Of course, it is difficult to compare these studies because the animal model used, the ALA doses and the illumination methods are all different. The fact that we could not show an improved tumor response using any of these schemes indicates that little or no extra tumor damage was obtained by the use of a low fluence rate or dark periods of several seconds or minutes for this tumor model. These results imply that improving the tumor response to ALA-PDT is not simply a matter of interrupting the illumination for a few seconds or minutes and that tumor response may be different both for different sizes of tumor and for different tumor types.

Fan *et al.* (8) investigated the short- and long-term light fractionation schemes in patients treated for mouth dysplasia with p.o. administered ALA. They were not able to show an improved tumor response using either of these treatment schemes compared to a single fraction illumination. It should be noted that the maximum ALA dose p.o. administered in patients is  $60 \text{ mg}\cdot\text{kg}^{-1}$ , whereas experimental animals are given  $200 \text{ mg}\cdot\text{kg}^{-1}$  i.v.

In summary, no significant improved tumor response could be obtained using a low fluence rate or a short-term light fractionation

scheme (dark interval of seconds or 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 an dark period of 75 min between two light treatments.

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