Photochemical & Photobiological Sciences

RSC Publishing

COMMUNICATION

View Article Online
View Journal | View Issue

Light fractionated ALA-PDT enhances therapeutic efficacy *in vitro*; the influence of PpIX concentration and illumination parameters

Cite this: *Photochem. Photobiol. Sci.*, 2013, **12**, 241

Received 17th August 2012,
Accepted 12th October 2012

DOI: 10.1039/c2pp25287b

www.rsc.org/pps

Henriëtte S. de Bruijn,[a] Adriana G. Casas,[b] Gabriela Di Venosa,[b] Lautato Gandara,[b] Henricus J. C. M. Sterenborg,[a] Alcira Batlle[b] and Dominic J. Robinson*[a]

Light fractionation, with a long dark interval, significantly increases the response to ALA-PDT in pre-clinical models and in non-melanoma skin cancer. We investigated if this increase in efficacy can be replicated in PAM 212 cells *in vitro*. The results show a significant decrease in cell survival after light fractionation which is dependent on the PpIX concentration and light dose of the first light fraction. This study supports the hypothesis that an underlying cellular mechanism is involved in the response to light fractionation in which a first light fraction leads to sub-lethally damaged cells that are sensitised to a second light fraction 2 hours later. The current study reveals the *in vitro* circumstances under which we can investigate the cellular pathways involved.

Introduction

Light fractionated 5-aminolevulinic acid (ALA) based photodynamic therapy (PDT) significantly increases the clinical response of non-melanoma skin cancer, illustrated by the 5 year follow-up of a randomized, prospective trial treating superficial basal cell carcinoma[1] and the 12 month follow-up treating actinic keratoses.[2] This approach to increase the response to ALA-PDT involves a single topical application of ALA to the lesion for 4 hours followed by the delivery of light in two fractions separated by a dark interval of 2 hours[3] and has been demonstrated in a range of pre-clinical models.[4–6] The mechanism behind this increase in effectiveness of light fractionation has not yet been fully elucidated. We and other investigators have shown that protoporphyrin IX (PpIX) continues to be synthesised *in vivo* after PDT[7–9] and that cells in tissues within the treatment volume are responsible for this re-synthesis.[10] The original rationale behind the design of light fractionated ALA-PDT was the utilisation of the re-synthesised PpIX after the first light fraction. However, there is no correlation between the amount of PpIX that is re-synthesised and the effectiveness of light fractionation.[3,11,12] We have previously hypothesised that a cellular mechanism is involved by which cells that are sub-lethally damaged by the first light fraction may be vulnerable to a second light fraction delivered 2 hours later.[12]

In a series of preclinical studies we have shown the critical influence of the illumination parameters on the efficacy of light fractionated PDT. A 2 hour dark interval results in a significant increase in efficacy compared to shorter intervals;[12] increasing the fluence or decreasing the fluence rate of the first light fraction leads to a reduction in efficacy. We were also able to show that these light treatment parameters can be varied without a loss in efficacy if the PDT dose, determined by monitoring PpIX photobleaching, is kept constant.[13] Given that the PDT dose, which is related to the amount of singlet oxygen generated during the first light fraction, is such an important parameter careful consideration was given to the choice of treatment parameters in the present *in vitro* study. We investigated a range of illumination schemes and ALA incubation conditions *in vitro* in order to mimic as closely as possible the PDT dose delivered in the first light fraction and the dynamics of PpIX synthesis *in vivo*.

Materials and methods

The spontaneously transformed murine keratinocyte cell line, PAM 212,[14] was cultured in RPMI-1640 medium supplemented with L-glutamine 2 mM, 10% foetal bovine serum and the Gibco antibiotic–antimicotic. Cells were seeded in triplicate, incubated for 48 hours at 37 °C and 5% CO_2 and utilised at semi-confluency. Two different ALA doses and incubation regimes were used. We began with the lowest dose that resulted in a maximum of PpIX accumulation after 3 hours of incubation, *i.e.*, 2 mM in a serum free medium. In the case of light fractionation, cells were re-incubated with an ALA containing medium during the dark interval between the two light

[a]*Center for Optical Diagnostics and Therapy, Department of Radiation Oncology, Erasmus MC, PO Box 2040, 3000 CA Rotterdam, The Netherlands. E-mail: d.robinson@erasmusmc.nl; Fax: +31-10-7032141; Tel: +31-10-7032103*
[b]*Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP), CONICET-Hospital de Clínicas Gral José de San Martín, Argentina*

fractions. The second incubation regime used was focussed on the dose that gave plateau values of PpIX fluorescence over a time period similar to that observed, *in vivo*, in pre-clinical models.[11] To achieve this we incubated the cells with 0.2 mM ALA in a serum free medium for 2 hours. The PpIX concentration in cells before the first and second illumination was determined using an extraction technique described previously.[15] In brief, cells were plated in 24-well plates at a concentration of 1×10^5 cells per ml, and utilised after 2 days under conditions of confluency.

Porphyrins accumulated within the cells were extracted twice with 5% HCl, leaving the cells standing for half an hour in the presence of the acid at 37 °C. These conditions proved to be optimal for total PpIX extraction. The excitation and emission wavelengths producing the highest fluorescence in PpIX, uroporphyrin and coproporphyrin solutions in 5% HCl were 406 nm and 604 nm respectively. These wavelengths were employed to measure the samples in a Perkin Elmer LS 55 Luminescence Spectrometer. PpIX (Porphyrin Products, Logan, UT, USA) was used as a reference standard.

To perform PDT experiments, cells were seeded on 6-well plates. During ALA exposure, a serum free medium was used to avoid PpIX efflux from cells, and was replaced by a complete medium after 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. Fluences between 0.3 and 3.1 J cm^{-2} were used at a power density of 2 mW cm^{-2}. After the last illumination cells were incubated for 19 h and tested for cell viability using the MTT assay as described previously.[15] Following appropriate treatments, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) solution was added to each well at a concentration of 0.5 mg ml^{-1}, and plates were incubated at 37 °C for 1 hour. The resulting formazan crystals were dissolved by the addition of DMSO and absorbance was determined at 560 nm.

As described above the fluences of the first and second light fractions are critical parameters *in vivo*. The most effective treatment scheme is with a first light fraction of 5 J cm^{-2} at 50 mW cm^{-2} followed by a second light fraction 2 hours later of 95 J cm^{-2} at 50 mW cm^{-2}.[3,11,12] A single light fraction of 5 J cm^{-2} results in a visual skin response characterised by slight redness for one to two days with no crust formation. This response can be regarded as predominantly sublethal damage and was approximated by 80–95% cell survival *in vitro*. A single light fraction of the magnitude of a second light fraction that is most effective *in vivo* results in significantly more visual skin damage and was approximated by 35–60% cell survival *in vitro*. The cell survival curves of a single illumination were used to determine the fluences for the light fractionation schemes. Data are expressed as mean ± standard error of the mean for cell survival or standard deviation for PpIX concentration, and they are the average of two to five independent experiments run in duplicate or triplicate. A two-tailed Student's *t*-test was used to determine statistical significance between means.

Results

Incubation with 2 mM ALA for 3 hours resulted in a PpIX accumulation of 5.5 ± 0.6 ng porphyrins per 10^5 cells. Incubation for 5 hours, *i.e.*, the time point for the second light fraction, resulted in a significantly higher accumulation of 9.6 ± 0.8 ng porphyrins per 10^5 cells ($p = 0.002$, $n = 4$). The cell survival curve of cells incubated with 2 mM ALA showed decreasing cell survival with increasing fluence (Fig. 1). A single illumination delivered after 5 hours of incubation was slightly more effective than after 3 hours. For example, illumination with a fluence of 1.2 J cm^{-2} at 5 hours resulted in 25.8 ± 9.5% cell survival compared to 46.7 ± 10.9% after illumination at 3 hours ($p = 0.15$, $n = 5$). Illumination at 2 hours was even less effective and resulted in 72.8 ± 6.2% cell survival (data not shown). The fluence for the two light fractions of the three light fractionation schemes was based on the results for a single illumination delivered at 3 or 5 hours (Table 1). PDT efficacy using these incubation conditions and treatment parameters was not enhanced over a single illumination of the same total fluence. In fact, a single illumination at 5 hours was at least as effective and sometimes even more effective than the light fractionation schemes studied.

The second ALA incubation regime with a 10 times lower concentration, 0.2 mM for 2 hours, resulted in comparable levels of PpIX at 2 and 4 hours after the start of incubation (2.64 ± 0.31 and 2.71 ± 0.37 ng porphyrins per 10^5 cells, respectively). A single illumination delivered at either of these time points was equally effective (Fig. 2). For example a fluence of 2.52 J cm^{-2} at 2 hours resulted in 7.0 ± 2.0 cell survival compared to 8.5 ± 2.1% at 4 hours. Again, the fluence for the two light fractions of the three light fractionation schemes was based on the results for single illuminations (Table 1). Under these circumstances light fractionation significantly increased the effectiveness of the treatment. Compared to a single illumination of 2.52 J cm^{-2} the cell survival of light fractionation with a slightly lower total fluence of 2.28 J cm^{-2}

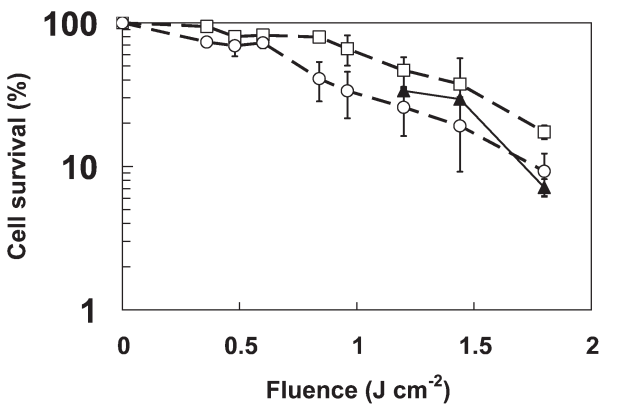

Fig. 1 Cell survival of PAM 212 cells incubated with 2 mM ALA and illuminated with a single illumination at 3 h (□) or 5 h (○) and light fractionation at 3 and 5 h (▲).

Table 1 Cell survival of PAM 212 after ALA-PDT with different ALA incubation regimes and different individual single illumination fluences. These fluences were used for the investigated light fractionation schemes

ALA	Single 1st fraction		Single 2nd fraction	
	J cm^{-2}	Cell survival	J cm^{-2}	Cell survival
2 mM	0.36	94.3 ± 6.4	0.84	40.9 ± 17.6
	0.48	80.2 ± 5.2	0.96	33.6 ± 33.4
	0.6	82.0 ± 5.2	1.2	25.8 ± 19.1
0.2 mM	0.84	95.0 ± 2	1.08	60.0 ± 1.3
	0.84	95.0 ± 2	1.44	52.0 ± 1.9
	0.84	95.0 ± 2	1.8	17.0 ± 3.3

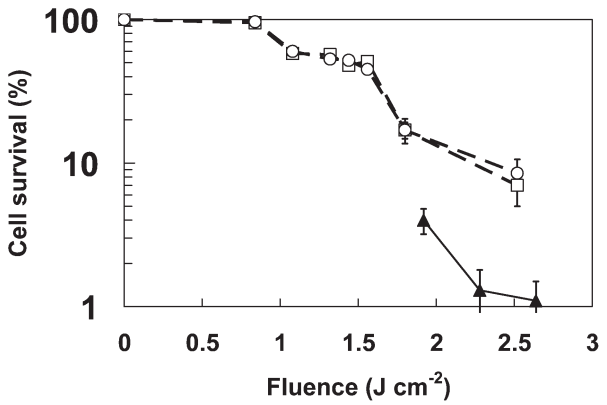

Fig. 2 Cell survival of PAM 212 cells incubated with 0.2 mM ALA and illuminated with a single illumination at 2 h (□) or 4 h (○) and light fractionation at 2 and 4 h (▲).

(0.84 + 1.44) was significantly decreased to only 1.3 ± 0.5% ($p = 0.009$, $n = 3$).

Discussion

The results of the present study show an increased efficacy of light fractionated ALA-PDT *in vitro* under rather specific circumstances: under incubation conditions of low (0.2 mM) ALA concentration there is approximately an order of magnitude reduction in cell survival when two light fractions are delivered at 2 and 4 hours compared to a single illumination with the same cumulative fluence at either time point. This is the first report of this type of *in vitro* response following light fractionated PDT and supports the hypothesis that there is a mechanism by which cells are rendered sensitive to an illumination 2 hours after a first sub-lethal insult.

It is noteworthy, however, that we did not observe an increase in efficacy of light fractionation at high ALA concentration. It is interesting to speculate on the reason for this difference in response. It may be that incubation using high concentrations of ALA results in a different localisation of PpIX within the cell and therefore leads to different responses[16] which are not advantageous to the mechanism underlying the increase in efficacy. We note that the extraction method used in the present study means that we are unable to determine if PpIX is localised in different cellular compartments. Incubation with 0.2 mM ALA results in comparable PpIX levels at 2 and 4 hours despite the ALA withdrawal at 2 hours. This suggests that PAM 212 cells form a pool of ALA and/or porphyrins upon ALA exposure like we have shown for other cell lines.[18] The cell survival response to a single illumination delivered at either of these time points is not different suggesting that the location of accumulated PpIX has not changed. Incubation with 2 mM ALA for 3 hours saturates the haem cycle while a higher concentration will not lead to a higher level of PpIX (data not shown). Extending the incubation to 5 hours significantly increases the PpIX level and may overload the haem cycle and result in a relocation of the accumulated PpIX.[19]

It is also important to note that the relationship between *in vivo* PDT dose delivered as monitored for example by the generation of singlet oxygen[17] or indirectly by measuring PpIX photobleaching,[11] *in vivo* efficacy as determined using a visual skin scoring system and cell death *in vitro* may be more complicated than we have assumed. Sub-lethal damage may play an important role in the *in vivo* studies, a factor that is not taken into account in the present *in vitro* study. The current study supports the hypothesis that cells become sensitive to a second illumination delivered 2 hours after a first light fraction. The cellular/biological mechanism underlying this effect has not yet been determined. The central question that remains: what occurs in response to the first light fraction that causes the cells to be sensitive to a second light fraction? Two to 4 hours after incubation with ALA, PpIX is primarily located on the inner mitochondrial membrane with small concentrations in other organelles such as the ER and the plasma membrane.[19] Mitochondrial damage is known to lead to the uncontrolled release of cytochrome c into the cytosol followed by the activation of the caspase pathway and apoptotic cell death.[20] The short diffusion distance of singlet oxygen[21] means that specific molecular targets to which the PpIX photosensitiser is bound or closely associated are known to play critical roles in the events upstream of cytochrome c release. A number of specific targets for ALA-induced PpIX have been identified including the peripheral benzodiazepine receptor (within the permeability transition pore complex)[22] and cardiolipin.[23] It is thought that PpIX is very closely associated with these molecules and acts directly on them thereby modulating the apoptotic pathway. It may be that the delivery of a small PDT dose followed by a dark interval leads to PpIX re-localisation and/or conformational changes of molecular targets that may increase the susceptibility of cells to undergo apoptosis or autophagy.[24–28] It is notable that the delivery of high PDT doses can overwhelm these pathways and lead to cell necrosis. This supports our observations that the delivery of high PDT doses in the first light fraction abrogates the effect of light fractionation.

Given our finding that it is cells that are exposed to low concentrations of ALA and synthesise low concentrations of PpIX that are susceptible to light fractionation it is interesting to consider which cells influence its efficacy *in vivo*. In normal

(mouse) skin deeper lying structures that synthesize low PpIX concentrations include the local (micro-) vasculature. We have previously shown that the vasculature is a target of light fractionated ALA-PDT.[4,29] It is also known that regions within clinical lesions such as superficial BCC accumulate low levels of PpIX.[30] It may be that light fractionation enhances the response of these cell populations and those of the local vasculature thereby enhancing clinical efficacy.

In summary we have shown that light fractionation increases the response of cells to ALA-PDT *in vitro*. This study supports the hypothesis that a cellular mechanism is involved in the response to light fractionation in which a first light fraction leads to sub-lethally damaged cells that become more susceptible to a second light fraction 2 hours later. Clearly the present study has only investigated a single cell line, and the generality of our conclusions should be carefully considered. The effect of light fractionation in other cell lines will be the subject of future research. The current study does however reveal the conditions under which we can further investigate the cellular pathways involved in the response to light fractionated PDT.

Acknowledgements

This study was financially supported by the Dutch Cancer Society; grant number 2002–2718 and PICT 2008–0047 and PICT 2006–1809 from the Argentinean Government.

Notes and references

1 H. C. de Vijlder, H. J. C. M. Sterenborg, H. A. M. Neumann, D. J. Robinson and E. R. M. de Haas, Light fractionation significantly improves the response of superficial basal cell carcinoma to ALA-PDT: five-year follow-up of a randomized, prospective trial, *Acta Dermatol. Venerol.*, 2012, **92**, 641–647.

2 E. Sotiriou, Z. Apalla, E. Chovarda, C. Goussi, A. Trigoni and D. Ioannides, Single *vs.* fractionated photodynamic therapy for face and scalp actinic keratoses: a randomized, intraindividual comparison trial with 12-month follow-up, *J. Eur. Acad. Dermatol. Venereol.*, 2012, **26**, 36–40.

3 D. J. Robinson, H. S. de Bruijn, W. M. Star and H. J. C. M. Sterenborg, 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.*, 2003, **77**, 319–323.

4 N. van der Veen, H. L. L. M. van Leengoed and W. M. Star, *In vivo* fluorescence kinetics and photodynamic therapy using 5-aminolevulinic acid-induced porphyrin: increased damage after multiple irradiations, *Br. J. Cancer*, 1994, **70**, 867–872.

5 H. S. de Bruijn, N. van der Veen, D. J. Robinson and W. M. Star, Improvement of systemic 5-aminolevulinic acid-based photodynamic therapy *in vivo* using light fractionation with a 75 minute interval, *Cancer Res.*, 1999, **59**, 901–904.

6 N. van der Veen, K. M. Hebeda, H. S. de Bruijn and W. M. Star, Photodynamic effectiveness and vasoconstriction in hairless mouse skin after topical 5-aminolevulinic acid and single or two-fold illumination, *Photochem. Photobiol.*, 1999, **70**, 921–929.

7 N. van der Veen, H. S. de Bruijn and W. M. Star, Photobleaching during and re-appearance after photodynamic therapy of topical ALA-induced fluorescence in UVB-treated mouse skin, *Int. J. Cancer*, 1997, **72**, 110–118.

8 A. Orenstein, G. Kostenich and Z. Malik, The kinetics of protoporphyrin fluorescence during ALA-PDT in human malignant skin tumors, *Cancer Lett.*, 1997, **120**, 229–234.

9 C. af Klintenberg, A. M. K. Enejder, I. Wang, S. Andersson-Engels, S. Svanberg and K. Svandberg, Kinetic fluorescence studies of 5-aminolaevulinic acid-induced protoporphyrin IX accumulation in basal cell carcinomas, *J. Photochem. Photobiol., B*, 1999, **49**, 120–128.

10 H. S. de Bruijn, B. Kruijt, A. van der Ploeg-van den Heuvel, H. J. C. M. Sterenborg and D. J. Robinson, Increase in protoporphyrin IX after 5-aminolevulinic based photodynamic therapy is due to local resynthesis, *Photochem. Photobiol. Sci.*, 2007, **6**, 857–864.

11 D. J. Robinson, H. S. de Bruijn, J. de Wolf, H. J. C. M. Sterenborg and W. M. Star, Topical 5-aminolevulinic acid-photodynamic therapy of hairless mouse skin using two-fold illumination schemes: PpIX fluorescence kinetics, photobleaching and biological effect, *Photochem. Photobiol.*, 2000, **72**, 794–802.

12 H. S. de Bruijn, A. van der Ploeg – van den Heuvel, H. J. C. M. Sterenborg and D. J. Robinson, Fractionated illumination after topical application of 5-aminolevulinic acid on normal skin of hairless mice: the influence of the dark interval, *J. Photochem. Photobiol., B*, 2006, **85**, 184–190.

13 T. A. Middelburg, F. van Zaane, H. S. de Bruijn, A. van der Ploeg – van den Heuvel, H. J. C. M. Sterenborg, H. A. M. Neumann, E. R. M. de Haas and D. J. Robinson, Fractionated illumination at low fluence rate photodynamic therapy in mice, *Photochem. Photobiol.*, 2010, **86**, 1140–1146.

14 S. H. Yuspa, P. Hawley-Nelson, B. Koehler and J. R. Stanley, A survey of transformation markers in differentiating epidermal cell lines, *Cancer Res.*, 1980, **40**, 4694–4703.

15 C. Perotti, H. Fukuda, G. DiVenosa, A. J. MacRobert, A. Batlle and A. Casas, Porphyrin synthesis from ALA derivatives for photodynamic therapy, *in vitro* and *in vivo* studies, *Br. J. Cancer*, 2004, **90**, 1660–1665.

16 Z. Ji, G. Yang, V. Vasovic, B. Cunderlikova, Z. Suo, J. M. Nesland and Q. Peng, Subcellular localization pattern of protoporphyrin IX is an important determinant for its photodynamic efficiency of human carcinoma and normal cell lines, *J. Photochem. Photobiol., B*, 2006, **84**, 213–220.

17 M. J. Niedre, A. J. Secord, M. S. Patterson and B. C. Wilson, *In vitro* tests of the validity of singlet oxygen luminescence measurements as a dose metric in photodynamic therapy, *Cancer Res.*, 2003, **63**, 7986–7994.

18 S. Correa Garcia, A. Casas, C. Perotti, A. Batlle and M. Bermudez Morreti, Mechanistic studies on δ-aminolevulinic acid uptake and efflux in a mammary adenocarcinoma cell line, *Br. J. Cancer*, 2003, **89**, 173–177.

19 B. J. Wilson, M. Olivo and G. Singh, Subcellular localization of photofrin and aminolevulinic acid and photodynamic cross-resistance *in vitro* in radiation-induced fibrosarcoma cells sensitive or resistant to photofrin mediated photodynamic therapy, *Photochem. Photobiol.*, 1997, **65**, 166–176.

20 D. Grebenova, K. Kuzelova, K. Smetana, M. Pluskalova, H. Cajthamlova, I. Marinov, O. Fuchs, J. Soucek, P. Jarolim and Z. Hrkal, Mitochondrial and endoplasmic reticulum stress-induced apoptotic pathways are activated by 5-aminolevulinic acid-based photodynamic therapy in HL60 leukemia cells, *J. Photochem. Photobiol., B*, 2003, **69**, 71–85.

21 J. Moan, On the diffusion length of singlet oxygen in cells and tissues, *J. Photochem. Photobiol., B*, 1990, **6**, 343–344.

22 S. K. Bisland, E. A. Goebel, N. S. Hassanali, C. Johnson and B. C. Wilson, Increased expression of mitochondrial benzodiazepine receptors following low-level light treatment facilitates enhanced protoporphyrin IX production in glioma-derived cells *in vitro*, *Lasers Surg. Med.*, 2007, **39**, 678–684.

23 T. Kriska, W. Korytowski and A. W. Girotti, Role of mitochondrial cardiolipin peroxidation in apoptotic photokilling of 5-aminolevulinate-treated tumor cells, *Arch. Biochem. Biophys.*, 2005, **433**, 435–446.

24 N. L. Oleinick, R. L. Morris and I. Belinchenko, The role of apoptosis in response to photodynamic therapy: what where why and how, *Photochem. Photobiol. Sci.*, 2002, **1**, 1–21.

25 D. Kessel, M. G. Vicente and J. J. Reiners, Initiation of apoptosis and autophagy by photodynamic therapy, *Lasers Surg. Med.*, 2006, **38**, 482–488.

26 E. Buyaert, M. Dewaele and P. Agostinis, Molecular effectors of multiple cell death pathways initiated by photodynamic therapy, *Biochim. Biophys. Acta*, 2007, **1776**, 86–107.

27 H. T. Ji, L. T. Chen, Y. H. Lin, H. F. Chien and C. H. Chen, 5-ALA mediated photodynamic therapy induces autophagic cell death via the AMP-activated protein kinase, *Mol. Cancer*, 2010, **9**, 91.

28 I. Coupienne, S. Bontems, M. Dewaele, N. Rubio, Y. Habraken, S. Fulda, P. Agostinis and J. Piette, NF-kappaB inhibition improves the sensitivity of human glioblastoma cells to 5-aminolevulinic acid-based photodynamic therapy, *Biochem. Pharmacol.*, 2011, **81**, 606–616.

29 E. R. M. de Haas, H. S. de Bruijn, H. J. C. M. Sterenborg, H. A. M. Neumann and D. J. Robinson, Microscopic distribution of protoporphyrin IX (PpIX) fluorescence in superficial basal cell carcinoma during light fractionated aminolevulinic acid photodynamic therapy, *Acta Dermatol. Venerol.*, 2008, **88**, 547–554.

30 H. S. de Bruijn, E. R. M. de Haas, K. M. Hebeda, A. van der Ploeg – van den Heuvel, H. J. C. M. Sterenborg, H. A. M. Neumann and D. J. Robinson, Light fractionation does not enhance the efficacy of methyl 5-aminolevulinate mediated photodynamic therapy in normal mouse skin, *Photochem. Photobiol. Sci.*, 2007, **6**, 1325–1331.