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Comparison of 5-Aminolaevulinic Acid and its Heptyl Ester-induced Protoporphyrin IX and its Photobleaching in Human Adenocarcinoma WiDr Cells and in Athymic Nude Mice Healthy Skin

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Abstract

5-aminolevulinic acid heptyl ester (heptyl 5-aminolevulinate), a potential prodrug for fluorescence diagnosis and Photodynamic Therapy (PDT), was investigated in human adenocarcinoma WiDr cells and in healthy skin of athymic nude mice in comparison with 5-aminolevulinic acid (ALA). Incubation of WiDr cells with ALA and ALA heptyl ester resulted in the production of Protoporphyrin IX (PpIX). Concentrations higher than 0.01 mM for ALA heptyl ester and 1 mM for ALA were cytotoxic. Similar amounts of PpIX production and same photobleaching rate were observed in WiDr cells, although a 100 times lower concentration of ALA heptyl ester was needed in comparison with ALA. In contrast to cells culture, topical application of 2% of ALA and ALA heptyl ester gave similar fluorescence of PpIX and PpIX photobleaching in mouse model. But a faster PpIX pbotobleaching was observed *in vivo* compared to *in vitro*.

Keywords: Protoprophyrin IX; Photodyanmic therapy; Photobleaching; Photoproduct

Introduction

5-aminolevulinic acid (ALA) is a precursor in the heme synthesis pathway and is currently used to induce endogenous synthesis of the photosensitizer, protoporphyrin IX (PpIX) for photodynamic therapy (PDT) and for fluorescence diagnosis [1-3]. The hydrophilic nature of ALA limits its ability to penetrate through cell membranes and skin. The major side effect of topical PDT with ALA is its shallow penetration depth (<2 mm) into tissue [3]. In order to increase its permeability, chemical modifications of ALA, such as esterification with aliphatic alcohols, have been proposed, and lipophilic ALA esters have been introduced [4-7]. It is frequently assumed that diffusion across cell membranes will increase with lipophilicity, and thus increase penetration depth and PpIX production in the tissue, yielding a deeper and more homogenous PpIX distribution in tissue. One would expect shorter application times, less side effects and the drug doses might be reduced. *In vitro* studies have shown that ALA esters with short alkyl chains (methyl, ethyl, propyl) induce lower PpIX accumulation, while ester with long-chains (butyl, pentyl, hexyl, octyl) induce higher intracellular PpIX levels in vitro at much lower concentrations compared with ALA [6,8-14]. Thus, long-chained ALA esters seem to be good future candidates for PDT. However, to the best of our knowledge, little, if any, work has been reported about ALA-heptyl ester. In this study, we used ALA-heptyl ester as the precursor of PpIX as compare with ALA.

During PDT, a decrease in PpIX fluorescence is observed as the photosensitizer is photochemically destroyed by light and this is referred to as photobleaching [15]. It is a significantly interesting field in PDT. The interest in PpIX photobleaching stems from the fact that, since both it and the PDT effect are believed to be governed by a mechanism involving singlet oxygen, it is likely that photobleaching reflects the tissue damage induced by PDT [16,17]. Therefore, measuring the progress of PpIX photobleaching during PDT may provide an indication of the progress of the treatment. In the present study, we measured PpIX production, photobleaching and photoproduct formation of PpIX induced by 1 mM ALA and 0.01 mM ALA-heptylester in cultured WiDr cells and 2% ALA and ALA-heptylester in normal mouse skin. Our aim in carrying out this experiment was twofold: firstly, to compare ALA-heptylester with ALA regarding PpIX production and PpIX photobleaching in vitro and *in vivo*; and secondly, to compare the photobleaching rate in vitro with *in vivo*.

Materials and Methods

Chemicals

5-aminolevulinic acid hydrochlorid was obtained from PhotoCure (Oslo, Norway). Heptyl aminolevulinate hydrochloride (ALA heptyl ester) were gifts from PhotoCure ASA (Oslo,Norway). Stock solutions of drugs were prepared in serum free medium immediately before the experiments.

Cells culture

WiDr cells derived from a primary adenocarcinoma of human rectosigmoid colon were used [12]. The cells were subcultured in RPMI 1640 medium (Sigma), containing 10% FCS, 100 units/ml penicillin and 1 % glutamine and were incubated at 37°C in a humidified atmosphere containing 5% CO2. They were subcultured two times per week.

Animals

Female BALB/c-nu/nu athymic mice were used. The animals were normally active during the time of ALA and ALA-heptylester application. The mice were kept anaesthetized during the cream application and the fluorescence measurements.

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Light exposure

An in-house-produced blue light lamp with four fluorescence tubes (Philips TLK 40W/03, the Netherlands), emitting light in the wavelength region 400-460 nm and with a peak at 420 nm, was used in the experiments. The fluence rate at the surface of the mouse skin was 10 mW/cm^2 as measured with the photodiode (NewPort, Model 1815-c, Irvine, California).

Fluorescence measurements in cells

The cells were exposed to 1 mM ALA and to 0.01 mM ALA heptyl ester in RPMI 1640 medium without serum for 4 hours, in order to induce build-up of endogenous porphyrins. After 4hours incubation, cells were washed three times with ice-cold PBS and scraped into 1ml PBS. The cell suspensions were exposed to light and PpIX fluorescence was measured in cuvette placed directly in the standard cuvette holder of a luminescence spectrometer (Perkin Elmer LS45 Norwalk, CT, USA). The fluorescence background (autofluorescence) of the cells without PpIX was recorded and subtracted from the fluorescence data. Fluorescence of PpIX was excited at 407 nm and measured at 636 nm. The 407 nm excitation light from the luminescence spectrometer was of low intensity (less than 1 mW/cm^2) and did not induce any significant photobleaching of PpIX.

Topical application of ALA and ALA heptyl ester on mice

For topical application, creams were prepared using 2% of ALA and ALA heptyl ester (2% is the common using drug concentration in pre-clinical studies) in a standard ointment (Unguentum, Merck, Darmstad, Germany). To facilitate proper application of the creams, the animals were anaesthetized with subcutaneous injection of solution of Hypnorm (Janssen Pharmaceutica B.V., Tilburg, The Netherlands) and Dormicum (Hoffmann-La Roche AG, Basel, Switzerland) (1:1 vol/vol) with a lowest possible single bolus (0.02–0.03 mL per mouse). The animals woke up within 20 min and appeared normally active during the rest of the application time of ALA and ALA-heptylester. Approximately, 0.1 gram of freshly prepared cream was applied to a 1 cm^2 spot on one flank of the mice, and covered with transparent adhesive occlusive dressing (OpSite Flexifix, Smith & Nephew Medical Ltd., Hull, UK).

Fluorescence measurement in mouse skin.

The amount of PpIX formed in the mouse skin after topical application of drugs was determined by its fluorescence, measured non-invasively with a fiber optic probe connected to LS50B luminescence spectrometer (Perkin Elmer, Norwalk, CT) equipped with a red-sensitive photomultiplier tube R3896 (Hamamatsu, Japan). Photobleaching was performed after 10 h of ALA and ALA-heptyl ester cream application. This application time was chosen because the content of PpIX in mouse skin reached maximum at this time point. PpIX fluorescence was measured after 0 min, 5 min, 10 min, 15 min, 20 min and 30 min blue light exposure. The excitation wavelength was 407 nm and the fluorescence kinetics was measured at 636 nm. The excitation and emission slits were set to 10 and 15 nm, respectively. A 515 nm cut-off filter built into the luminescence spectrometer blocked scattered excitation light from the detection fibres. The average skin autofluorescence background was measured on all animals before administration of the drugs and subtracted from the fluorescence data.

Data analysis

Data represent the mean and standard error of the mean of three independent experiments *in vitro*. The mean and standard error from six mice for each point (three readings for each mouse) represent the obtained data in vivo. The Student's *t*-test was used to compare the significance between data points. Values of $P<0.05$ were considered as indicating significant differences.

Results

PpIX generation *in vitro and in vivo*

In vitro model: WiDr cells were treated with different concentrations of ALA and ALA heptyl ester and PpIX production was monitored at different times (Figure 1). In all cases typical fluorescence emission spectra of PpIX were observed after incubation with both drugs. At the lowest studied concentration (0.01 and 0.05 mM) ALA did not produce any measurable amounts of PpIX, while ALA-heptyl ester produced significant amounts of PpIX (Figure 1). The PpIX fluorescence increased with time for ALA and ALA-heptyl ester (Figure 1). Faster PpIX synthesis was observed for ALA-heptyl ester than for ALA. The production of PpIX increased with increasing concentration for ALA and reached a maximum at 0.5-1 mM after 24hours incubation, while ALA-heptyl ester gave maximum at 0.25 mM for all studied incubation time and then a decrease. This may due to the dark toxicity of ALA-heptyl ester [18].

Further experiments were performed in order to determine the application time of both drugs for which the same level of PpIX could be obtained (Figure 2). We observed same level of PpIX generated with 1 mM ALA and 0.01 mM ALA-heptyl ester after 4 hours incubation in WiDr cells. So, 0.01 mM of ALA-heptyl ester and 1 mM of ALA for 4 hours incubation was used for further vitro study [18].

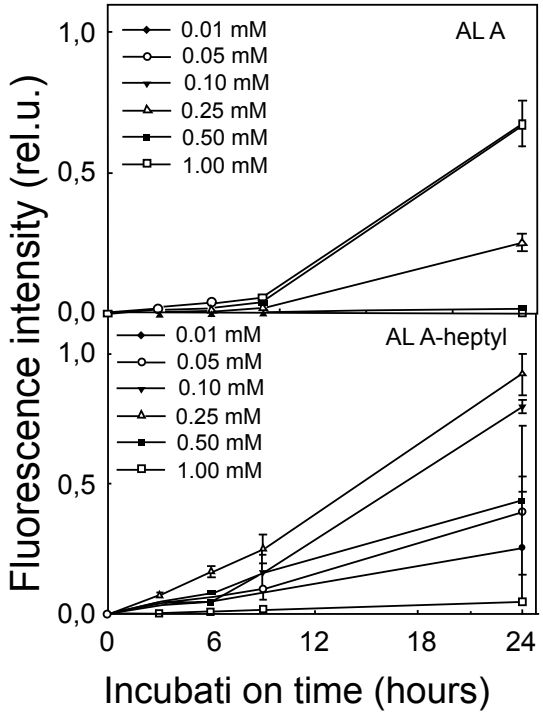

Figure 1: Production of PpIX in WiDr cells treated with ALA and ALA-heptyl ester as a function of incubation times for different concentrations of the drugs in medium without serum.

In vivo model: Topical application of ALA and ALA-heptyl ester led to production of PpIX in mouse skin. The PpIX fluorescence maximum occurred after around 10 hours of topical application of 2% ALA and ALA heptyl ester (Figure 3). Both drugs generated the same amount of PpIX at this time point (Figure 3). The ALA heptyl ester-induced PpIX fluorescence showed a slight time lag compared to that of ALA-induced fluorescence before it reached its maximal value. After reaching the maximal level of PpIX production, the PpIX fluorescence induced by ALA heptyl ester decreased faster than that induced by ALA. After topical application of ALA heptyl ester for 24 hours, the PpIX clearance rate was two times faster than that after application of ALA (Figure 3) [18].

Fluorescence photobleaching in vitro and in vivo

Photobleaching experiments were carried out with exposure times in the range of 0-30 min. Prior to light exposure, the fluorescence spectrum (Figure 4a, Figure 4b curve at 0 min). was identical with that of PpIX in cells in vitro [19]. Thus PpIX was assumed to be the only photosensitizer present in cultured cells after application of 1mM ALA and 0.01 ALA-heptylester and in mouse skin after application of 2% ALA and ALA-heptylester cream prior to light exposure.

Light exposure (10 mW/cm^2 at 420 nm) caused bleaching (Figure 4). In order to illustrate the photobleaching clearly, we normalized the initial PpIX fluorescence intensity to 1 (Figure 5a,b). Similar photobleaching rate was obtained for ALA and ALA-heptyl ester-induced PpIX in cells and in mouse skin. For an exposure time of 30 min, practically all fluorescence was bleached *in vivo*, while 5% fluorescence remained *in vitro*. 90% of initial PpIX fluorescence was bleached in around 10 minutes in vivo, whereas in vitro about 15 minutes were needed, for ALA and ALA-heptylester, respectively (Figure 5a,b). The decay curves can be composed into double exponential curves both in vivo and *in vitro*.

Photoproduct formation

Assessment of decay of PpIX fluorescence at 675 nm enables us to evaluate the formation of the main photoproducts. Formation of light-induced photoproducts was shown in (Figure 6). After light exposure ($100 mW/cm^2$ at 420 nm) bleaching and spectral changes were observed. After normalize fluorescence spectrum of PpIX at 636 nm, a new fluorescence band appeared at 675 nm and peak is growing with exposure times. But no blue-shift of the main band is observed, which mentioned in other studies. Fluorescence spectra of light-induced products $F'_{\exp}$ for given exposure times $t_{\exp}$ were calculated by following two equations.

$$F'_{\exp}(\lambda, t_{\exp}) = F_{\exp}(\lambda, t_{\exp}) - F_{PpIX}(\lambda, t_{\exp}) \quad (1)$$

$$F_{PpIX}(\lambda, t_{\exp}) = F_{PpIX}(\lambda, 0) \bullet \frac{F_{\exp}(636, t_{\exp})}{F_{PpIX}(636, 0)} \bullet k \quad (2)$$

$F_{\exp}(\lambda, t_{\exp}) \rightarrow$ Fluorescence spectrum after light exposure were corrected for photobleaching of PpIX for the exposure times

$F_{PpIX}(\lambda, t_{\exp}) \rightarrow$ Fluorescence spectrum of PpIX corresponding to the exposure times $t_{\exp}$ >0

$F_{PpIX}(\lambda, 0) \rightarrow$ Fluorescence spectrum of PpIX before light exposure.

$F_{PpIX}(636, 0) \rightarrow$ PpIX fluorescence intensity for λ =636 nm at $t_{\exp}$ =0

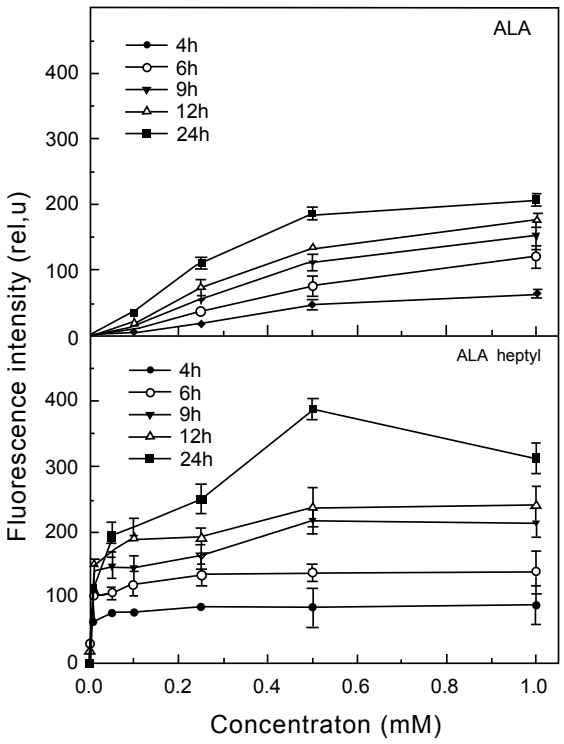

Figure 2: Production of PpIX in WiDr cells treated with ALA and ALA-heptyl ester as a function of concentrations for different incubation times in medium without serum.

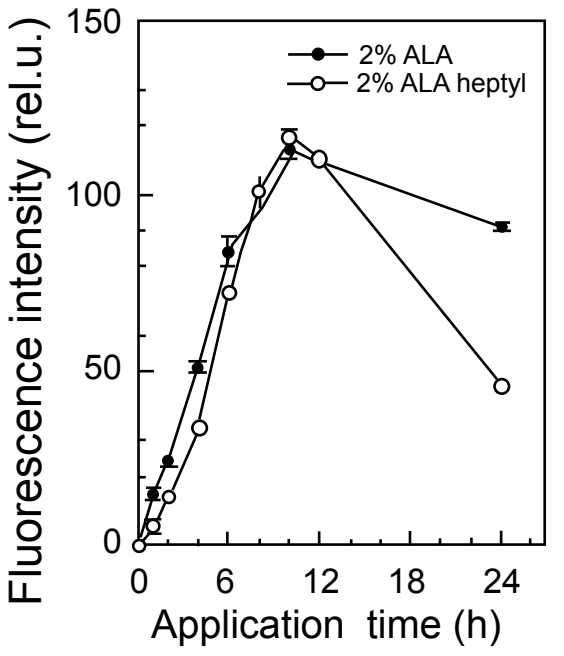

Figure 3: Pharmacokinetics of PpIX accumulation in normal mouse skin after topical application of creams containing 2 % of ALA and ALA-heptyl ester. The error bars represent the standard deviation within the group.

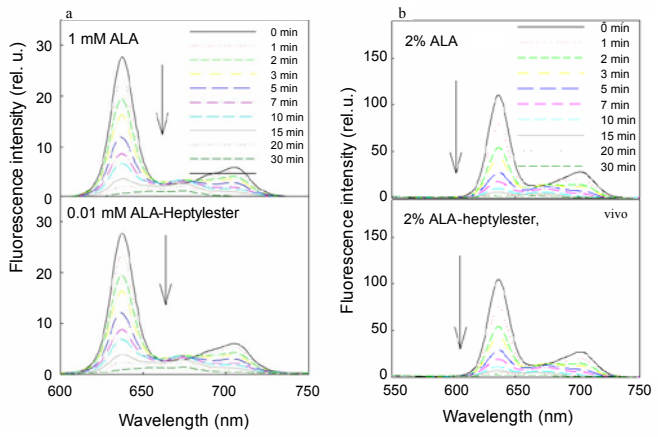

Figure 4: a) Fluorescence spectra of PpIX induced by ALA and ALA-heptyl ester in WiDr cells before and after blue light exposure 400-460 nm. The excitation wavelength was 407nm. b) Fluorescence spectra of PpIX induced by ALA and ALA-heptyl ester in normal mouse skin before and after blue light exposure 400-460 nm

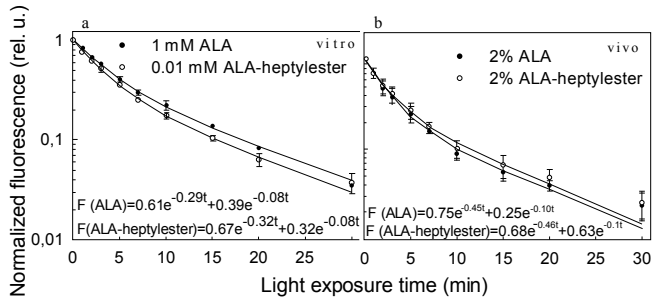

Figure 5: PpIX photobleaching. PpIX basis spectrum amplitude normalized to the initial PpIX basis spectrum amplitude versus light exposure time a) in WiDr cells b) in normal mouse skin. Lines are doubling exponential decay curves for to the data. Error bars represent ±1 standard deviation from a minimum of 3 independent experiments.

$F_{exp}(636, t_{exp}) \rightarrow$ Fluorescence intensity at λ=636 nm measured after a given exposure times t_{exp}

$k = 0.9 \rightarrow$ Constant which used to correct PpIX fluorescence that avoids negative fluorescence values in the F'_{exp} spectrum after subtraction (Eq. (1)).

From the fluorescence spectrum corrected for PpIX photobleaching, we observed maxima with peak at 675 nm (Figure 6).

Discussion

The present study clearly indicates that PpIX production *in vitro* is achieved faster and at considerably lower concentrations of ALA heptyl ester than that of ALA (Figure 1). Similar concentrations of PpIX were induced with 100 times lower concentration of ALA heptyl ester compared with ALA which was not able to induce any PpIX at low concentrations. This is in agreement with other studies reporting that long-chained esters give the same level of PpIX in human cells with down to 200 fold lower concentrations [6,12,14,20]. This suggests that the intracellular uptake of ALA and ALA heptyl ester follows different routes. The structure of the applied ALA ester may play an important role in its transport through cellular membranes [16]. ALA is probably taken up by BETA transporters, while none of the ALA derivatives share this or same uptake mechanism [21-24]. In our mouse model, comparable concentrations of ALA and ALA heptyl ester generated similar amounts of PpIX after topical application (Figure 3). This is different from the result of the *in vitro* study, but in agreement with earlier findings in normal mouse skin using ALA and ALA-ester cream base [25-27]. The explanation of these discrepancies may be that the Stratum Corneum (SC) binds the esters temporarily, and slow down their penetration into the living cells where the PpIX is formed [20,28]. Another explanation is that ALA esters penetrate deeper and faster than ALA does. Therefore, ALA heptyl ester might produce PpIX in regions close to the vasculature, thus facilitating the transport of PpIX away from the tissue faster than for PpIX-induced by ALA. The faster clearance rate of PpIX-induced by ALA esters than by ALA has been documented earlier [29]. Lipophilic ALA heptyl ester molecules may bind to proteins and become washed out.

In photobleaching part of this study, ALA and ALA-heptyl ester induced PpIX photobleaching depend strongly on the light exposure time in vitro and *in vivo* (Figure 5). Similar results were observed by Dysart et al. [30] in MLL cells and Juzenas et al. [31] in mouse skin for ALA and ALA-ester. Studies found that 70-95 % of the PpIX initially present can be degraded by clinically relevant light fluences [32,35]. In our model, 95% of PpIX was photobleached in WiDr cells and almost all fluorescence was photodegraded in normal mouse skin by a fluence rate of 10 mW/cm^2 (Figure.5). No differences were found in photobleaching kenetics with ALA and ALA-heptyl ester –induced PpIX both *in vitro* and *in vivo*, this is due to the same PpIX content with ALA and ALA-heptylester, respectively..

The photobleaching of PpIX in cultured cells shows a slower time decay behavior compared to similar measurements in normal mouse skin. This might be due to higher PpIX concentration *in vivo* than *in vitro*. This hypothesis was not tested by us. However, Bagdonas S et al. [19], Robinson et al. [16] and Moan et al. [49] found that the decay rate depended on the initial concentration of PpIX. A faster decay rate was found with a higher PpIX concentration. Another reason for it may be difference in PpIX binding sites which lead to difference in photolability [34].

PpIX photobleaching data can be fit by a double exponential curve both in vivo and in vitro. Other groups have shown that PpIX bleaching fits reasonably well to a double exponential decay curve *in vitro* [35-37]. Multiple binding sites with distinct photochemistry are a possible explanation for the observed kinetics. Several studies of Moan's group [35,38] also demonstrated that photobleaching kinetics were highly

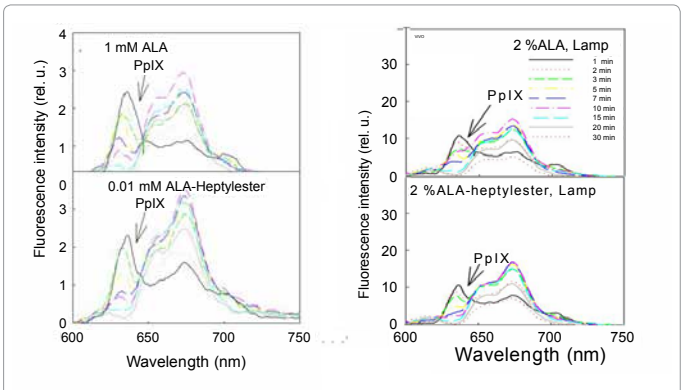

Figure 6

dependent on the sensitizer binding site in the tissue or in the cell. One of those studies [35] suggested that the bi-exponential decay of the PpIX in WiDr cells was due to two first order processes, one for PpIX bound to proteins and one for unbound PpIX.

Porphyrin photodegradation and photoproduct formation have long been subjects of active investigation in photochemistry and studies of photodynamic action [38,39].

Formation of photoproducts has been detected for several porphyrin sensitizers in vitro and *in vivo* [38,40-43]. In present study we observed the subsequent photobleaching of a fluorescence peak at 675 nm for both drugs, which we attributed to the photoproduct of Ppp. This result agree well with results obtained by Juzenas et al. [45], Orenstein et al. [46] and Wessels et al. [44] showing that Ppp is the main photoproduct during PpIX photobleaching. Ppp is chlorin-type molecule, a very photosensitive substance and has similar photostability as PpIX [47]. Other photoproducts were observed in Bagdonas's [47] and Juzenas's study [45,48] but not in our case. The observed differences in photoproducts formation could be due to different light sources.

Conclusion

In this study we found that Similar concentrations of PpIX were induced with 100 times lower concentration of ALA heptyl ester compared with ALA *in vitro* and a comparable concentrations of ALA and ALA heptyl ester generated similar amounts of PpIX after topical application *in vivo*. The photobleaching data can be fitted by the double exponential curve both *in vitro* and *in vivo* with a similar PpIX photobleaching rate for ALA and ALA-heptylester, but a slower PpIX photobleaching rate was observed *in vitro* than *in vivo*. Ppp was the main photoproducts during PpIX photobleaching in WiDr cells and in normal mouse skin under blue light exposure.

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