

Induction of Apoptosis in HaCaT Cells by Photodynamic Therapy with Chlorin e6 or Pheophorbide a

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Key Words

Photodynamic therapy · Photosensitizers · Apoptosis · Caspases · HaCaT keratinocytes

Abstract

The two photosensitizers, chlorin e6 and pheophorbide a, were tested in an in vitro model of topical photodynamic therapy (PDT). Both dyes accumulate in HaCaT keratinocytes as verified by fluorescence measurement but pheophorbide a is enriched fivefold more strongly than chlorin e6 after 24 h. HaCaT cells are susceptible to PDT with both dyes. The phototoxicity measured by ATP bioluminescence is caused by necrosis and apoptosis depending on the photosensitizer used and the treatment modality. Chlorin e6 shows higher toxic potential because it elicits nearly 90% cell mortality 24 h after PDT comparable to pheophorbide a but with a fivefold lower rate of accumulation. These results implicate caution with topical PDT of oncologic diseases due to the risk of serious side effects on healthy skin in the course of topical photodynamic treatment. But the lack of dark toxicity and the time-dependent enrichment of both dyes in HaCaT cells are arguments for the application of these sensitizers in top-

ical PDT of non-malign skin disorders. Further studies are necessary to discover appropriate lower doses and mechanisms of action of topical PDT with both compounds.

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Introduction

Photodynamic therapy (PDT) depends on the synergy of a sensitizing molecule, visible light of appropriate wavelength and oxygen. Excitation of the sensitizer leads to photochemical generation of cell-damaging reactive oxygen species [1, 2]. In contrast to oncology where PDT is aimed at destruction of malign transformed cells, under non-oncologic conditions PDT is thought to influence cellular function. Application of PDT for non-oncologic indications in dermatology is under intensive investigation [3, 4]. Topical PDT for treatment of chronic inflammatory dermatoses, for example psoriasis [5–7] or localized scleroderma [8], has been described. The prevailing photosensitizer in PDT of non-malign and pre-malign skin disorders is aminolevulinic acid (5-Ala) [6, 7, 9–11]. In vitro model systems to investigate new suitable photosensitizers for topical PDT are imperative to evaluate their application in vivo.

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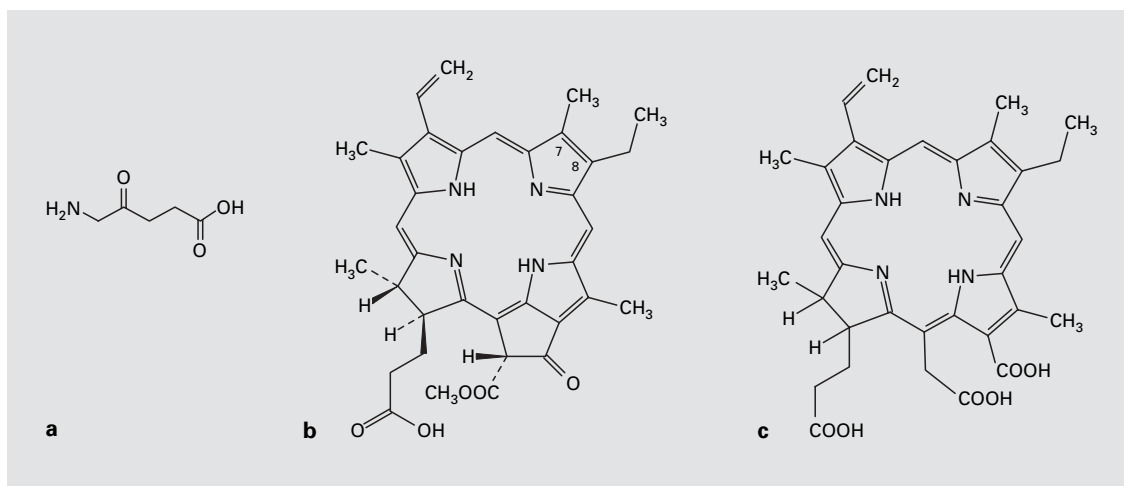


Fig. 1. Structure of aminolevulinic acid (a) in comparison to pheophorbide a (b) and chlorin e6 (c).

In this study we used HaCaT cells as a model system of proliferating keratinocytes and tested the effect of PDT with two well-characterized photosensitizers chlorin e6 [12–15] and pheophorbide a [16, 17]. Both compounds are porphyrin-based sensitizers differing in chemical structure (fig. 1). Chlorin e6 is a hydrophilic sensitizer bearing three ionizable carboxylic residues and is derived from porphyrin by partial saturation of one of the four pyrrole rings [15]. Pheophorbide a comprises an amphiphilic pigment with only a single carboxylic moiety [18]. The aim of this study was to examine the effect of chlorin e6 or pheophorbide a in combination with irradiation on HaCaT cells as a model for topical application of PDT in non-malignant skin disorders.

Materials and Methods

HaCaT cells (DKFZ Heidelberg, Germany) were maintained in DMEM with 1% non-essential amino acids (PromoCell, Germany), 10% fetal bovine serum (Seromed-Biochrom, Germany), and 1% antibiotic-antimycotic solution (Gibco BRL, Germany) at 37°C in a humidified atmosphere with 5% CO₂. Antibiotics are imperative as irradiation was performed under semi-sterile conditions.

Adenosine Triphosphate Bioluminescence Measurements

To assay cytotoxicity, cells were seeded into 96-well microtiter plates (10,000 cells/well) and cultured for 48 h. The supernatant was replaced by fresh medium supplemented with 0.6 μM of the photosensitizing compound and the cells were incubated for 1 or 24 h. Thereafter the cells were washed with PBS. Irradiation of the cells covered with PBS was performed with a GaAlAs-diode laser (Biolitec, Germany) at 665 nm at a final dose of 12.5 J/cm². The

control cells were treated identically except that they received medium without photosensitizer and were not irradiated. The adenosine triphosphate (ATP) content of the cells was measured after a further incubation step of 1 or 24 h in culture medium according to the manufacturer's protocol (ATPLite-M, PerkinElmer, USA) [19]. Relative luminescence units were recorded with a microplate luminometer (LUMIstar, BMG LABTECH GmbH, Offenburg, Germany). An ATP standard curve was used to calculate the ATP concentration in the sample. The inbuilt error of this ATP assay is around 10%.

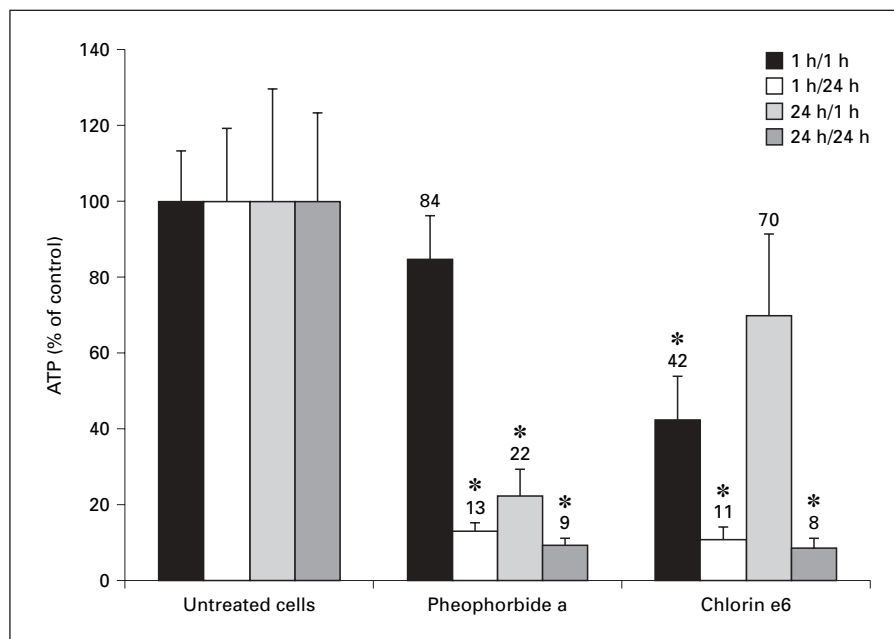
Cellular Uptake of Chlorin e6 and Pheophorbide a

To assess the uptake of the sensitizers, cells were seeded into 96-well microtiter plates as described above and maintained for 48 h. The medium was changed and enriched with 6 μM of the respective pigment. In contrast to the phototoxicity study a concentration of 6 μM was used to improve the measurement to background ratio of the experiment. After 1, 6 or 24 h the cells were washed with PBS, lysed and the emission at 665 nm (excitation 400 nm) was measured with a microplate fluorometer (FLUOstar Galaxy, BMG LABTECH GmbH). A standard curve of photosensitizers in lysis buffer was used to convert relative fluorescence units into a concentration range.

Measurement of Caspase-3 Activity

For detection of caspase-3 activity a fluorometric assay (Roche, Germany) was used. PDT-treated cells were lysed on ice. The lysate was cleared by centrifugation and stored at -80°C until further processing. The protein content of each sample was determined with Coo Protein Assay (a modified Bradford Assay, Uptima, Montluçon, France) before incubation of the lysates with a caspase-3-specific monoclonal antibody attached to the bottom of microtiter plate wells. After a washing step, cleavage of the added substrate DEVD-AFC was measured by antibody-captured active caspase-3 from the lysates. Emission of free AFC at 520 nm was detected in a microplate fluorometer (FLUOstar Galaxy, BMG LABTECH GmbH) with excitation at 400 nm. Unknown concentrations of

Fig. 2. Cytotoxicity of PDT with pheophorbide a and chlorin e6 on HaCaT cells ($0.6 \mu\text{M}$ sensitizer + $12.5 \text{ J}/\text{cm}^2$). The first position in the legend indicates the interval of incubation with the sensitizer, the second position shows the time after irradiation. Presented results are the mean \pm SD of at least three independent experiments normalized to the control (absolute values of untreated cells at the indicated time points: 1 h/1 h: $16,432 \pm 1,438 \text{ nM ATP}$; 1 h/24 h: $22,203 \pm 3,760 \text{ nM ATP}$; 24 h/1 h: $24,273 \pm 7,060 \text{ nM ATP}$; 24 h/24 h: $32,519 \pm 6,913 \text{ nM ATP}$; $n > 20$). *Significant deviations from control ($p < 0.001$) calculated with one-way ANOVA.



AFC were calculated by means of a standard curve. Caspase-3 activity is expressed as picomoles AFC per microgram protein. There were two control cell conditions: first cells plus medium, this control is depicted in figure 4. These cells were treated exactly like the PDT samples except that they received medium without photosensitizer and no irradiation, but buffer washes and media changes were identical. The second set of controls comprised cells incubated with photosensitizer for 24 h without irradiation to check if the caspase-3 results are specific for PDT. The photosensitizers did not contribute to the measurements although they absorb strongly at 400 nm. After 24-hour incubation with chlorin e6 or pheophorbide a without irradiation, caspase-3 activity in HaCaT cells is comparable to the first set of controls, i.e. cells with medium (24 h cells plus medium: $0.2 \pm 0.03 \text{ pmol AFC}/\mu\text{g protein}$; 24 h chlorin e6: $0.2 \pm 0.05 \text{ pmol AFC}/\mu\text{g protein}$; 24 h pheophorbide a: $0.1 \pm 0.04 \text{ pmol AFC}/\mu\text{g protein}$).

Results

In previous tests the optimal non-toxic concentration of the pigments and irradiation dose were determined. Irradiation ($12.5 \text{ J}/\text{cm}^2$) or photosensitizers (0.06 , 0.6 or $6 \mu\text{M}$) alone had no effect on the viability of HaCaT cells (results not shown). In PDT experiments $0.6 \mu\text{M}$ of the respective sensitizer and an irradiation dose of $12.5 \text{ J}/\text{cm}^2$ were combined. A short (1-hour) and a long (24-hour) incubation interval with chlorin e6 or pheophorbide a before irradiation were distinguished in order to study the influence on phototoxicity. The ATP content of the cells

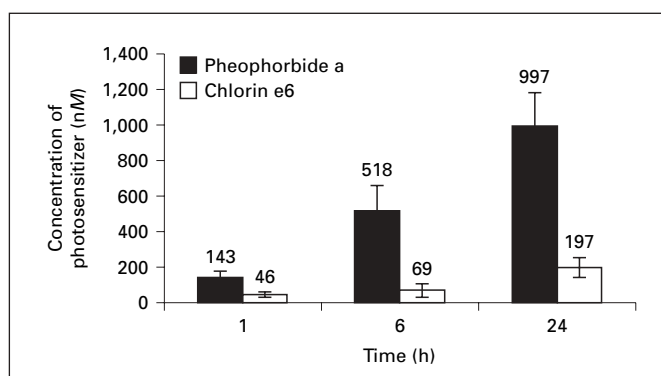


Fig. 3. Cellular uptake of pheophorbide a and chlorin e6 in HaCaT cells after different incubation times. Data represent the mean of three independent experiments (\pm SD; $n > 50$).

was measured 1 or 24 h after irradiation, i.e. four different treatment protocols result from the experimental setup (1 h/1 h; 1 h/24 h; 24 h/1 h; 24 h/24 h).

Independent of the duration of incubation with the sensitizers for 1 or 24 h, PDT with both pigments led to nearly complete ablation of the ATP content 24 h after irradiation compared to the control (fig. 2; pheophorbide a: 13 and 9%; chlorin e6: 11 and 8%). This means that PDT with each photosensitizer at a dose of $0.6 \mu\text{M} + 12.5 \text{ J}/\text{cm}^2$ is finally lethal for most of the cells. In contrast 1 h after PDT the measurements differed depending on the nature

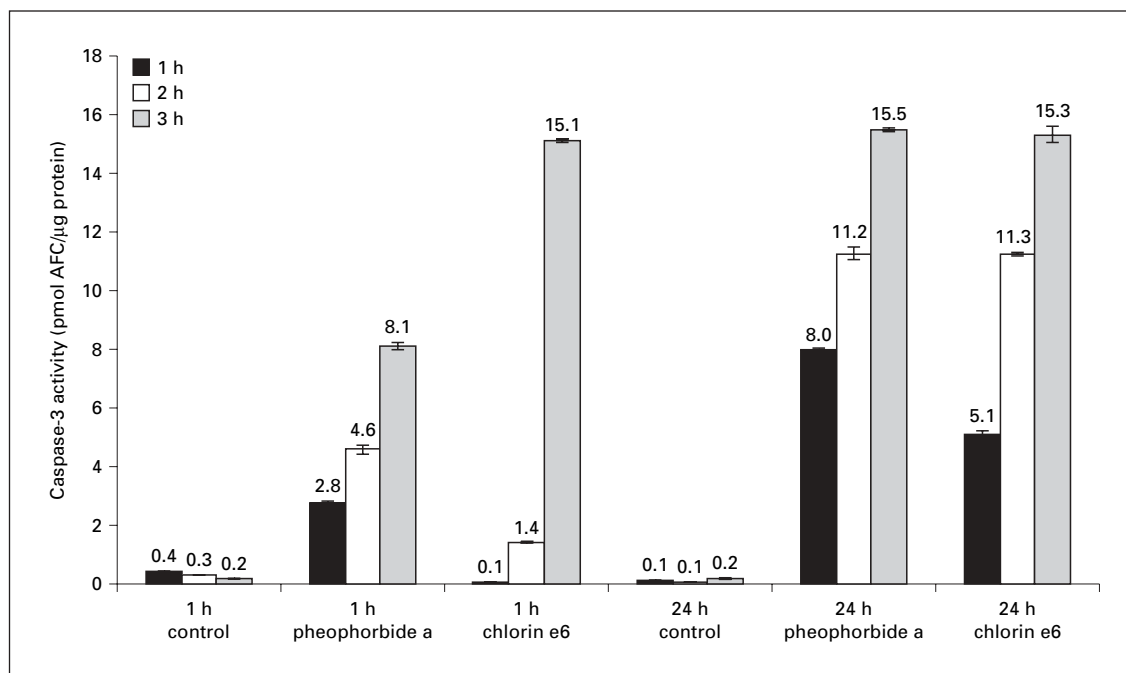


Fig. 4. Caspase-3 activity in HaCaT cells after PDT ($0.6 \mu\text{M} + 12.5 \text{ J/cm}^2$) with pheophorbide a and chlorin e6. The legend shows time intervals after PDT. Control comprises cells with medium. Data were obtained from one experiment which is representative for two other experiments with similar results.

of the compound. The phototoxicity of pheophorbide a (fig. 2; 84 vs. 22%) increased with the prolonged preincubation time and the opposite seemed to be evident for chlorin e6 (fig. 2; 42 vs. 70%), although the measurements for the latter at 1 h/1 h and 24 h/1 h are not significantly different from one another.

To study whether the results of the cytotoxicity assay could be caused by variable uptake of both sensitizers, we examined the content of the pigments in lysates of HaCaT cells after different incubation times (fig. 3). The cellular uptake of both compounds increased with incubation time. The measurements augmented 7-fold for pheophorbide a and 4.3-fold in the case of chlorin e6 after 24 h compared to 1 h. Striking differences in the total accumulation of the pigments are obvious. Enrichment of pheophorbide a exceeds that of chlorin e6 three times after 1 h and even five times after 24 h (fig. 3). The strong and time-dependent accumulation of pheophorbide a correlates with the cytotoxicity results but the acute phototoxicity of chlorin e6 is not intensified by the augmenting enrichment of the dye.

Based on the results of toxicity and uptake kinetics of chlorin e6 and pheophorbide a, we were interested to see if these substances induce apoptosis dependent on the

chosen experimental conditions. Literature data indicate that PDT with variable sensitizers elicits apoptosis [20–22]. Potential onset of apoptosis was monitored by activation of the central executioner enzyme in the process of apoptosis – caspase-3. Irradiation alone or incubation of the cells with each sensitizer for 24 h without irradiation caused no activation of caspase-3 demonstrating specific results for PDT. In contrast PDT with the compounds led to time-dependent activation of caspase-3 and thus apoptosis (fig. 4). The enzyme activities could be blocked by a caspase-3 inhibitor (Ac-DEVD-CHO; results not shown). Induction of apoptosis after PDT with pheophorbide a is stronger with 24-hour preincubation. The caspase-3 activation (fig. 4) and the lack of ATP reduction at 1 h/1 h (fig. 2) clearly show an apoptotic response to PDT with pheophorbide a, while a strong decrease in ATP levels at 24 h/1 h and concomitant activation of caspase-3 indicate cell lysis paralleled by apoptosis.

One hour after PDT with chlorin e6 there is no apoptosis detectable when short preincubation was applied (fig. 4), but ATP measurements decline significantly at this time and indicate cell lysis as a consequence of PDT (fig. 2). On the other hand PDT with chlorin e6 evoked nearly equivalent caspase-3 activities 3 h after irradiation

independent of the preincubation time with the pigment (fig. 4; 1 h/3 h 15.1 vs. 24 h/3 h 15.3), which means that apoptosis is induced in the residual cells at 1 h/1 h. A prolonged preincubation with chlorin e6 before irradiation leads to a time-dependent increase in caspase-3 activity which appears earlier and with higher intensity than after a 1-hour incubation but fails to reduce ATP levels significantly compared to the control. It is noteworthy that both photosensitizers activate caspase-3 to a similar extent when preincubated 24 h before irradiation in spite of completely different amounts of accumulation. To confirm the onset of apoptosis, cleavage of poly-ADP-ribose polymerase, a substrate of different caspases, was investigated. After PDT with both photosensitizers, fragmentation of the protein was detectable except at 1 h/1 h when neither photosensitization with chlorin e6 nor with pheophorbide a induced cleavage of poly-ADP-ribose polymerase (results not shown).

Discussion

Topical PDT is a promising treatment modality especially in dermatology for therapy of non-malign and malignant diseases [3, 4, 23]. The main photosensitizer used in topical PDT is 5-Ala. The aim of PDT in oncology is to eliminate transformed cells, while in non-malign indications modulation of cellular function by low-dose treatment should be achieved. A lot of *in vivo* studies show the effectiveness of topical PDT [5, 7, 8, 11] and elucidate mechanisms of action [24, 25]. Only a few alternative sensitizers have been tested, for example a hematoporphyrin derivative [23]. The aim of this study was to investigate chlorin e6 and pheophorbide a in a model of topical PDT for non-malign indications *in vitro*. Unfortunately under the treatment conditions tested, PDT with both sensitizers elicits cell death in the immortal keratinocyte cell line HaCaT [26]. The extent and kind of cellular destruction was dependent on the sensitizer and the treatment protocol. One hour after irradiation the phototoxicity of the amphiphilic pheophorbide a increases with prolonged preincubation time and correlates well with the time-dependent cellular uptake of the sensitizer (fig. 2, 3). Independent of pretreatment, a time-dependent activation of caspase-3 after irradiation can be observed, which is stronger with prolonged incubation of the cells with pheophorbide a, thus it seems that there is also a correlation between the uptake and onset of apoptosis (fig. 4). The immediate and strong activation of caspase-3 after 24 h/1 h is in striking contrast to the reduction in the ATP

levels at this time. Cell lysis (necrosis) contributes to the toxicity of PDT or the effect could be caused by apoptosis which is stopped by lysis [27]. Apoptosis in different tumor cell lines after PDT with pheophorbide a was demonstrated by Hajri et al. [16, 17]. Tanielian et al. [28] describe accumulation of pheophorbide a in nuclei, mitochondria and lysosomes, but not in the cytoplasm or cytoplasmic membranes of human oral mucosa cells. In accordance with our results, fluorescence increased with dose and/or incubation time [28]. Induction of apoptosis after PDT with pheophorbide a is not surprising since PDT with sensitizers localized in lysosomes and mitochondria leads to apoptosis [29, 30]. The strong accumulation of pheophorbide a in comparison to chlorin e6 reflects the chemical characteristics of both compounds – while the latter is hydrophilic, the first compound is amphiphilic and shows a better penetration of membranes via passive diffusion [18].

The phototoxicity of chlorin e6 1 h after PDT shows an inverse correlation to incubation time before irradiation, although the measurements do not differ significantly at 1 h/1 h and 24 h/1 h (fig. 2). At a dose comparable to that used in this study, the phototoxicity of chlorin e6 on A431 cells was not observed by Soukos et al. [31], but the authors also mentioned a cell-type dependence of PDT toxicity. It is striking that HaCaT cells are more prone to PDT toxicity than A431 cells. The time-dependent increase in the cellular uptake of chlorin e6 (fig. 3) does not correlate with acute PDT toxicity. Whereas at 1 h/1 h a lot of cells are destroyed by immediate lysis and in the remaining cells apoptosis is induced, at 24 h/1 h there is a dominant apoptotic response without necrosis. A possible cause for these results is a time-dependent change in localization of chlorin e6. The main sites of chlorin e6 enrichment are the cellular membranes, i.e. plasma, mitochondrial and nuclear membranes, but no accumulation could be observed in the cytoplasm, nucleus and lysosomes [12]. PDT with sensitizers localized at the plasma membrane leads to necrosis and delays or prevents an apoptotic response, whereas sensitizers accumulating in the mitochondria induce apoptosis upon irradiation [29]. The strong lysis effect after PDT with chlorin e6 at 1 h/1 h could reflect the main accumulation at the plasma membrane. A prolonged incubation preceding irradiation leads to a main apoptotic response and hence points to a prevailing effect of chlorin e6 localized at the mitochondrial membrane. The lipophilicity of chlorin e6 and its binding to the plasma membrane is influenced by protonation of the three ionizable carboxylic groups which allow the compound formation of different ionic species [15].

In spite of this, chlorin e6 shows higher toxic potential than pheophorbide a because it elicits comparable results 24 h after PDT but with a fivefold lower cellular accumulation (fig. 3). PDT toxicity is mainly ascribed to the generation of singlet oxygen in the course of photodynamic treatment. Thus a possible reason for the observed results could be a different yield of singlet oxygen. Fernandez et al. [32] reported a singlet oxygen quantum yield for chlorin e6 in phosphate buffer of 0.64 ± 0.1 and 0.69 ± 0.04 for pheophorbide a in phosphate buffer plus 1% Triton X-100. The singlet oxygen yield of pheophorbide a in aqueous media is negligible due to formation of aggregates, but the monomeric photodynamic active dye is formed in a hydrophobic cellular environment [28]. In contrast chlorin e6 is thought to be monomeric in physiological solution [15]. The exact type of reactive oxygen species in the cellular environment cannot be determined because the situation is too complex. Methods for analysis of reactive oxygen species are diverse and in vitro models are far from the in vivo situation. Tanielian et al. [28] analyzed the mechanisms of action of pheophorbide a in vitro and confirmed not only the involvement of type II photoprocesses but also type I which involves direct electron transfer between DNA bases and the excited state of the dye. Another reason for the different effectiveness of both sensitizers in vivo could be the cellular localization at the time of irradiation. Both dyes show different subcellular accu-

mulation. The lipophilicity of pheophorbide a leads to strong enrichment in mitochondria, lysosomes and nuclei [28], whereas chlorin e6 is accumulated at cellular membranes [12] but to a much lesser extent due to its weak lipophilicity [15].

In conclusion PDT with both compounds is lethal to HaCaT cells. This effect is caused by necrosis and apoptotic mechanisms which contribute differently depending on the treatment protocol and sensitizer. Chlorin e6 shows a strong phototoxic effect and induction of apoptosis but with a considerably lower dose than pheophorbide a. This is striking since the toxicity of PDT with chlorin e6 at a dose comparable to that used in this study is low with regard to malign cells [12, 31] and pheophorbide a is known to be effective in PDT of malign cells [16, 17]. The results presented show that PDT with two well-characterized photosensitizers kills benign HaCaT cells. These data have implications for topical PDT for malign indications because of the possible unwanted side effects on healthy skin. In spite of this both sensitizers show no dark toxicity and accumulate well in HaCaT cells, thus the use of pheophorbide a or chlorin e6 in topical PDT is conceivable. It should be the subject of further studies to see if lower doses are effective in topical PDT in terms of immunomodulatory influence and regulation of cellular function as was shown for 5-Ala in non-oncologic skin disorders [7, 8, 24, 25].

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