In Vitro Metabolism of 5-ALA Esters Derivatives in Hairless Mice Skin Homogenate and *in Vivo* PpIX Accumulation Studies

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Purpose. In topical photodynamic therapy, 5-ALA and its esters are enzymatically converted in the endogenous photosensitizing compounds such as, for example, protoporphyrin IX (PpIX). In order to elucidate in more detail their enzymatic fate, we have determined *in vitro* the enzymatic degradation of methyl, butyl, hexyl, and octyl-5-ALA ester derivatives in skin homogenate. Furthermore, *in vivo* porphyrin accumulation was measured in healthy hairless mice skins.

Methods. Hairless mouse skins were homogenized in isotonic phosphate buffer pH 7.4. 5-ALA esters were added, and aliquots were colleted for HPLC-fluorimetric determinations of remaining content of 5-ALA esters. Furthermore, oil-in-water emulsions containing esters were topically applied to mice skin for 6 h, and the amount of accumulated PpIX in the treated areas was determined by quantitative extraction and confocal fluorescence microscopy.

Results. The enzymatic degradation of esters follows pseudo first-order kinetics. The octyl ester had the largest rate constant for enzymatic degradation, followed by hexyl-, butyl-, and methyl-ALA. The long-chained 5-ALA esters, butyl-, hexyl-, and octyl ester, induced significantly more porphyrins than 5-ALA and 5-ALA methyl ester as shown by confocal microscopy and quantitative extraction studies.

Conclusions. 5-ALA derivatives differ widely with respect to their enzymatic degradation. The presence of alkyl chains in 5-ALA esters significantly influences the *in vitro* enzymatic metabolism and the *in vivo* PpIX formation in healthy hairless mice skins.

KEY WORDS: 5-ALA ester derivatives; 5-aminolevulinic acid; photodynamic therapy; protoporphyrin IX; skin metabolism.

INTRODUCTION

Photodynamic therapy (PDT) is a new modality of treating cancerous and precancerous pathologies that involves the administration of photosensitizing drugs that, when localized in the target tissue and irradiated with light of an appropriate wavelength, can lead to its eradication. An alternative approach to PDT, introduced in 1990 by Kennedy and coworkers (1), consists in the endogenous administration of 5-aminolevulinic acid (5-ALA), which is converted *in situ* into the photosensitizing agent protoporphyrin IX (PpIX) via the biosynthetic pathway of heme (2).

Several investigators have examined the use of topically applied 5-ALA for the photodynamic treatment of superficial skin cancers with a success rate ranging between 50% and 100% (3). This huge variety in cure rates can in part be attributed to significant by differences in treatment protocols with respect to the used 5-ALA vehicles, administration times, irradiation conditions, and criteria for patient selection (4,5).

At present, 5-ALA seems to be the most frequently used topical agent for PDT and has recently be approved for the treatment of actinic keratosis in the United States. However, 5-ALA is a hydrophilic molecule and a *zwitterion* at physiologic pH with limited capacity to cross biologic barriers. Applied topically to the skin this limitation might reduce the penetrability of 5-ALA through the stratum corneum, consequently limiting the amount of PpIX formed in deeper skin layers (6).

Improved delivery systems for 5-ALA to the skin may play an important role in the success of PDT in dermatology. Enhancement of 5-ALA skin penetration can be achieved either by varying vehicle composition (7), adding the penetration enhancers (8), or by physical methods such as iontophoresis (9,10) and ultrasound (11).

Another way to overcome the limited bioavaibility of 5-ALA may be the use of a more lipophilic prodrug. A prodrug is a pharmacologically inactive precursor of a drug, which upon spontaneous or enzymatic transformation within the body leads to the release of the active drug. Such prodrugs have been synthesized to achieve: a higher chemical stability than a parent compound, a lower susceptibility to first-pass metabolism and an improvement on delivery properties as compared to the parent drug (12).

Lipophilic 5-ALA derivatives, like, for instance, 5-ALA esters, are expected to cross biologic barriers more easily than 5-ALA. In the past, different 5-ALA esters with increasing lipophilicity have been synthesized by variation of the length of the aliphatic alcohol side chains (5,12). Several groups have demonstrated, *in vitro* and *in vivo*, that the application of 5-ALA esters resulted in an increased accumulation of porphyrins at lower concentrations as compared to 5-ALA (13–15).

However, until now the bioconversion of 5-ALA esters following topical application to the skin is not completely understood. In photochemical terms, there is no difference in the resulting photoactive compounds following administration of 5-ALA esters or 5-ALA itself (12). Thus, spectral analysis of emission or absorption spectra following exposure to one of those products will not help to further elucidate the metabolic pathway of 5-ALA esters. Therefore, in the current

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ABBREVIATIONS: 5-ALA, 5-aminolevulinic acid; M-ALA, 5-ALA methyl-ester; B-ALA, 5-ALA butyl-ester; H-ALA, 5-ALA hexyl-ester; O-ALA, 5-ALA octyl-ester; CSLM, confocal scanning laser microscopy; k_{obs}, degradation constants; PDT, photodynamic therapy; PpIX, protoporhyrin IX; LC-MS liquid chromotography-mass spectrometry.

work we propose to follow a different approach to get a more detailed insight of this problem. We have investigated the *in vitro* degradation of 5-ALA esters with increasing lipophilicity in homogenized hairless mouse skin in order to avoid interferences with drug uptake mechanisms. Furthermore, the differences in *in vivo* porphyrin accumulation capacity after topical applications of 5-ALA and its derivatives have been studied by quantitative extraction and confocal microscopy.

MATERIALS AND METHODS

Chemicals

5-ALA hydrochloride and its methyl-ester hydrochloride (M-ALA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other 5-ALA esters (Table I) were synthesized in our laboratories following the procedure described before (16). All other chemicals were of analytical grade.

HPLC Analysis of 5-ALA Esters

The amounts of 5-ALA esters were determined quantitatively as described before (17). In brief, the 5-ALA esters were converted into a fluorescent derivative by reaction with acetylacetone and formaldehyde and then analyzed fluorimetrically by high-performance liquid chromatography (HPLC) using a HPLC system consisting of two pumps, a column oven, and a spectrofluorometer (Shimadzu, Kyoto, Japan). Twenty microliters of the samples were injected into a column, LiChrosphere 100 RP-18 (125 × 4mm, 5 μm, Darmstadt, Germany, Merck), protected by a RP-18 guard column (4 × 4 mm, 5 μm, Darmstadt, Germany, Merck). Elution was performed at 40°C, at flow-rate of 1 ml/min, with a mobile phase consisting of methanol-water mixture in the following proportions: 5-ALA (40:60), M-ALA (50:50), B-ALA (65:35), H-ALA (73:27) and O-ALA (80:20). The fluorescence intensity was determined by excitation at 378 nm and emission at 464 nm. The retention times for the fluorescent derivatives were: M-ALA, 5.8 min; B-ALA, 5.3 min; H-ALA, 5.5 min; and O-ALA, 5.6 min. This method was linear in a concentration range between 1 and 450 μM, presenting a following correlation coefficient (r) of 0.999 for all drugs. The detection sensibility of this HPLC assay was 0.3 μM for all drugs, with less than 9.8% intra-day variation, and less than 11.33% inter-day variation. The error was less than 6.02%. These values were considered adequate for analytical assay, as described for Causon (18). Using this HLPC procedure, unidentified peaks were not detected.

Table I. 5-ALA and Alkyl Esters: General Structure = $HCl \cdot NH_2–CH_2–CO–CH_2–CH_2CO–OR^1$

Drugs	R^1	Mol. mass (g/mol)
5-ALA	H	167.6
M-ALA	CH_3	181.6
B-ALA	$(CH_2)_3CH_3$	223.8
H-ALA	$(CH_2)_5CH_3$	251.8
O-ALA	$(CH_2)_7CH_3$	279.6

In Vitro Enzymatic Degradation of 5-ALA Esters

About 3 g of skin from 8- to 9-week-old males hairless mice (strain HRS/J Jackson Laboratories, Bar Harbor, ME, USA) were dissected and immediately homogenized in 30 ml of isotonic phosphate buffer pH 7.4, at 4°C. The homogenized skin was centrifuged at 4000 rpm for 20 min. Prior to each experiment, the supernatant was allowed for stabilization of the temperature by incubation at 37°C for 10 min. About 4 ml of the supernatant were placed in flasks and 1 ml of the solutions containing M-ALA, B-ALA, H-ALA, or O-ALA at a concentration of 600 μM was added, giving a final concentration 120 μM for each derivative. The reaction medium was maintained under constant agitation of 500 rpm at 37°C. Aliquots of 0.2 ml of the solutions were collected at the intervals of 0, 5, 10, 15, 20, 30, 60, 90, 120, 180, and 360 min and transferred to vials containing 0.2 ml of frozen methanol in order to precipitate protein in the solution, release the ester bound to the proteins and stop any enzymatic reaction. About 50.0 μl of each sample were used for determination by HPLC of remaining esters in the reaction medium after each time interval. The rate constants for the degradation of 5-ALA esters were determined from the resulting concentration time profiles.

Quantification of Proteins of Homogenized Skin (Ref. 19)

Ten milliliters of homogenized skin previously diluted (1:20), 150 ml of Milli-Q water and 40 ml of BSA reagent for proteins (Standard II-500-0007/ BIO RAD) were placed in an ELISA plate. Then, the samples were incubated for 5 min at ambient temperature and read with an ELISA reader at 600 nm (ELISA EIA multi-well reader- Sigma Diagnostics). For calibration purposes, bovine serum albumin at concentrations ranging between 5 and 25 mg/ml were co-incubated with samples and treated in the same manner.

In Vivo PpIX Accumulation Studies

The formulations used in these studies were oil-in-water emulsions containing 10% 5-ALA (w/w), M-ALA, B-ALA, H-ALA, and O-ALA, respectively, and 3% EDTA (w/w), which was added for stabilization purposes (8). The formulations (80 mg) were applied on the dorsal region of hairless mouse for 6 h. The animals were sacrificed by cervical dislocation, their treated skins areas removed and divided for confocal microscopy and PpIX analysis based on our previous studies (8). Untreated skin was used as control.

PpIX Chemical Extraction

Treated skin areas weighing approximately 0.2 g were homogenized in 25 ml of methanol/water (9:1), sonicated for 20 min, and then filtered. The filtered tissue material was submitted to a second extraction, as described above. The fluorescence of the first and second filtrates was measured fluorimetrically at an excitation wavelength of 400 nm, and peak fluorescence emission at 632 nm PpIX concentration of each filtrate was estimated from a standard curve prepared with solutions of methanol:water (9:1) containing PpIX at concentrations range 2.3 to 30 ng/ml ($r = 0.999$). This PpIX assay presented a variability les than 2.3% for intra-day variation and less than 3.1% inter-day variation. The error was less than 2.9% and the minimum amount detectable was 1 ng/ml.

The PpIX concentrations of the both filtrates were summed and expressed as μg PpIX/g skin weight. The extraction procedure presented a recovery of 99.8%. Intrinsic fluorescence presented by skins from untreated (control) from the same animals was considered as background and subtracted from experimental readings. All procedures were performed under subdued light.

Confocal Scanning Laser Microscopy (CSLM)

Skin samples obtained following *in vivo* treatment were mechanically sectioned. Cross sections (perpendicular series), were embedded in a matrix, frozen at –15°C, and sectioned at 60-μm thickness. For confocal fluorescence microscopy, a LEICA – DMIRBE microscope with LEICA TSCNT 1.5.451 software equipped with a 16× immersion objective was used. Fluorescence was excited with krypton laser at 488 nm and detected at wavelengths longer than 590 nm.

RESULTS

The enzymatic degradation profiles of 5-ALA esters in skin homogenates at pH 7.4, until 360 min, shown in Fig. 1, indicate that a significant amount of all esters underwent enzymatic degradation during incubation. It can be seen that long-chained 5-ALA esters are more susceptible to enzymatic decomposition than shorter chained 5-ALA esters. Assuming Michaelis Menten kinetics for the interaction between skin enzymes and ALA esters where the substrate concentration is by far smaller then the Michaelis Menten constant K_M, the concentration time profile will follow pseudo first-order kinetics (20) according to the formula:

$$-d[\text{ALA ester}]/dt = v_M/K_M\,[\text{ALA ester}] = K_{obs}\,[\text{ALA ester}],$$

which seems reasonable taking into account a measured protein concentration in the supernatant determined to 2.2401 (± 0.2706) mg protein/ml for all skin homogenate. The O-ALA presented the largest value for K_{obs} of enzymatic degradation, followed by H-, B-, and M-ALA. The linear fits through the logarithmic values for each 5-ALA ester are shown in Fig. 1 and the calculated rate constants are summarized in Table II.

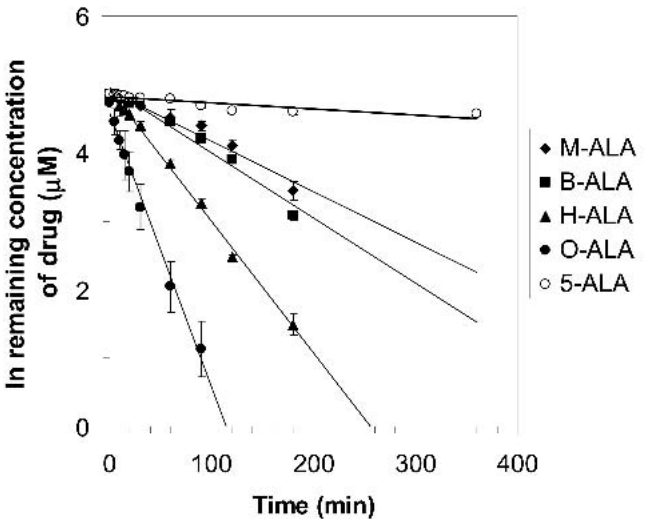

Fig. 1. Enzymatic degradation profiles for 5-ALA and its esters in skin homogenate at pH 7.4, 37°C expressed by remaining concentration (μM) against the time (min). The initial concentration of esters was 120 μM. Results presented are averages ± SD of five experiments.

In vivo, topical application of ALA and its derivatives led to accumulation of porphyrins under each condition. Fig. 2 shows the extracted amount of PpIX for the different compounds used in our experiments. There is no significant difference in porphyrin generation between 5-ALA and M-ALA. In contrast, a significant increase ($p < 0.05$) of the amount of porphyrins extracted from skin after application of B-, H-, and O-ALA was observed when compared with the effect of the formulation containing 5-ALA and M-ALA. The longer chained 5-ALA derivatives induced about 4 times more porphyrins than 5-ALA after 6 h of drug application.

Fig. 3 shows confocal fluorescence photomicrographs obtained from sections from control and treated samples of mouse skins. Compared to the controls (Fig. 3A), intense fluorescence in skins treated with 5-ALA (Fig. 3B) or with its esters (Figs. 3C–3F), can be observed. The fluorescence emitted from skin treated with B-ALA (Fig. 3D), H-ALA (Fig. 3E), and O-ALA (Fig. 3F) appeared to be of roughly similar intensity and was considerably higher than the intensity of the red fluorescence observed in the skin treated with 5-ALA (Fig. 3B) and M-ALA (Fig. 3C). This is in agreement with our PpIX extraction experiment, showed in Fig. 2.

Other structures, located in the dermis, probably related to skin appendages like hair follicles and sebaceous glands while surrounding tissues show only weak fluorescence intensities.

DISCUSSION

Today, discussion on the improved induction of porphyrins by 5-ALA derivatives remains controversy. The present study clearly indicates that long-chained 5-ALA esters induce more fluorescence than 5-ALA or M-ALA. However, in the skin of hairless mice, Van Den Akker *et al.* (21) found less PpIX following exposure to H-ALA as compared to 5-ALA, when the compounds were formulated in a cream. Following tape stripping and thereby removing the stratum corneum, they demonstrated that under these conditions mainly this layer limits the penetration of 5-ALA esters into the skin and consequently lowers the formation of PpIX. Casas *et al.* (22) used equimolar concentrations of 5-ALA and H-ALA and found a slightly higher PpIX production after exposure to H-ALA as compared to 5-ALA in papillomas and surrounding skin. These discrepancies with respect to *in vivo* experiments may be mainly attributed to two reasons: i) the method of porphyrin content evaluation and ii) the influence of the

Table II. Rate Constants for the Degradation of 5-ALA Esters in Isotonic Phosphate Buffer pH 7.4 Skin Homogenates, 37°C

	Enzymatic degradation in skin homogenate	
Esters	Kobs (min^{-1})	r
M-ALA	0.0068 ± 0.0004	0.983
B-ALA	0.0109 ± 0.0004	0.999
H-ALA	0.0189 ± 0.0002	0.999
O-ALA	0.0400 ± 0.0010	0.996

K_{obs} = degradation constant; r = correlation coefficient. Results presented are averages ± SD of five experiments.

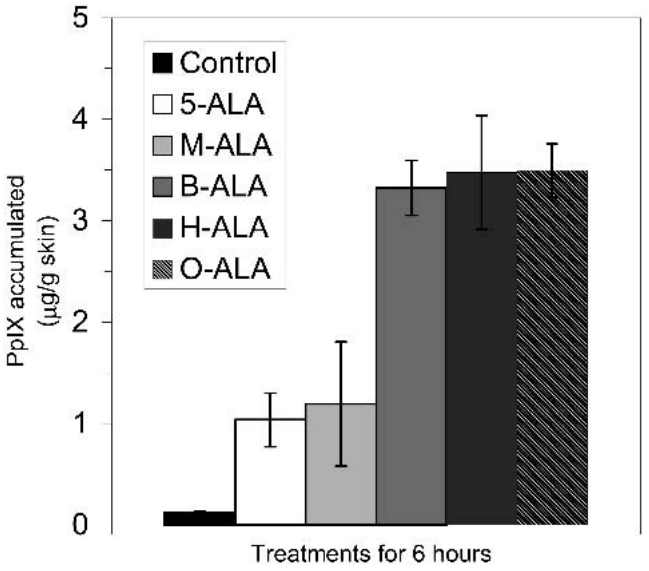

Fig. 2. Effects on 5-ALA and its esters-induced PpIX accumulation in hairless mouse skin 6 h following topical application of oil-in-water emulsions containing, from the left to the right: no drug (control), 5-ALA, M-ALA, B-ALA, H-ALA, and O-ALA. All applied formulations contained 3% EDTA (w/w). Averages ± SD of results obtained from eight animals in each group are presented.

used vehicle on the drug penetration. Van den Akker *et al.* (21) have used relative fluorescence measurements in order to deduce the PpIX, using and excitation wavelength of 405 nm; however, due to the shallow penetration depth of blue, one might underestimate the fluorescence induced in deeper tissue layers (23). In contrast to these studies, Casa *et al.* as well as this study determined the PpIX generation by extraction, which is not limited by the localization of the particular porphyrin. The particular relevance of the contribution of deeper tissue layers can be seen from Fig. 3. These results (Fig. 3) are in agreement with PpIX extraction experiments (see Fig. 2): longer chained 5-ALA ester such as B-, H-, and O-ALA induced significantly more PpIX than 5-ALA and M-ALA. All confocal fluorescence photomicrographs (Fig. 3) present clear distinctions between the different skin layers. In particular, the upper layers between 30 and 60 μm show intense red fluorescence.

Another essential difference between the different studies on 5-ALA derivative induced porphyrins can be explained the properties of the used vehicles. In agreement with Casas *et al.* (22) and our previous studies (17) it seems that more hydrophilic formulations tend to favor the partition of lipophilic 5-ALA derivatives to the skin. Using 5-ALA derivatives incorporated in oil-in-water emulsions, our *in vitro* skin permeation studies demonstrated an increased permeated amount for H-ALA after 6 h of incubation, compared to other esters and 5-ALA. Furthermore, after 6 h, more H- and O-ALA were retained at viable epidermis and dermis than 5-ALA. This is further supported by observations in urology, where H-ALA administered in an aqueous solution has been shown to increase the PpIX formation capacity compared to 5-ALA by nearly two orders of magnitude with respect to dose response (13). In this context, using more lipophilic vehicle to deliver 5-ALA derivatives (van den Akker et al.) will negatively impede the delivery to the skin thus reducing the efficacy of porphyrin biosynthesis. Thus, it seems that there is a strong relationship between the lipophilicity of the recipient and the capacity of inducing PpIX biosynthesis.

The reported differences between 5-ALA and its derivatives with respect to PpIX generation efficacy, systemic uptake and selectivity might be further explained by the stability and metabolic transformation of active compounds prior and following to administration. Revealing the mechanisms behind these degradation processes may further improve the delivery of the parent drug or its derivatives to its target. In 5-ALA–mediated PDT, this problem, especially with respect to the metabolic degradation of 5-ALA and its esters following administration *in vivo,* was insufficiently addressed so far. Therefore, in the current paper, we have focused on these specific problems by quantitative measurements of the amount of remaining 5-ALA derivatives in isotonic phosphate buffer pH 7.4 skin homogenates.

However, it should be noted that from these data it cannot be determined if the metabolism of 5-ALA esters in the skin is dominated by i) hydrolysis of the ester followed by incorporation into heme biosynthesis; ii) direct entering of the particular compound in this biosynthetic pathway; or iii) other decomposition reactions induced by different enzymes present in the skin. Furthermore, there might be considerable differences between the enzymatic digestions of these compounds intra and extracellularly. In order to reveal these complex mechanisms in more detail, more sophisticated techniques such as LC-MS or capillary electrophoresis-MS would be needed. However, the observations made in the present study have uncovered some interesting points that merit further investigations in the future. In this context, one should keep in mind that the skin possesses a multitude of different enzymatic activities through which topically applied drugs can be metabolized. Several methods exist to measure the metabolic rate of a prodrug in the skin. In studies on metabolism, more commonly purified enzymes are used. However, in skin metabolism mediated by several enzymes with overlapping metabolic activities, the method of using homogenized skin is more convenient and reflects presumably the real metabolism of a drug during its penetration process through the skin more exactly (24,25). 5-ALA esters are degraded rapidly following administration skin homogenates. From our *in vitro* experiments in skin homogenates, it can be assumed after 6 h that essentially all 5-ALA esters are digested by enzymatically catalyzed reactions. Our studies demonstrated that the rate constant of enzymatic degradation of 5-ALA esters exponentially increases with increasing chain length. Previously it has been assumed that hydrolysis of 5-ALA esters is the main enzymatic degradation process (13,16). However, from a chemical point of view, longer chained 5-ALA esters must be more resistant against simple hydrolysis than shorter chained 5-ALA esters. Furthermore, there is a huge difference in the generation of PpIX between M-ALA and B-ALA, while the rate constants for the degradations are similar.

It has been shown (21) that following administration of equimolar concentrations of H-ALA and 5-ALA slightly higher PpIX was formed using H-ALA. However, H-ALA presented the additional advantage of confining PpIX synthesis to the site of its topical application. If H-ALA would have underwent simple hydrolysis into 5-ALA and hexanol, at least the same amount of PpIX would have been determined at distinct sites and the organs of the mice which have been

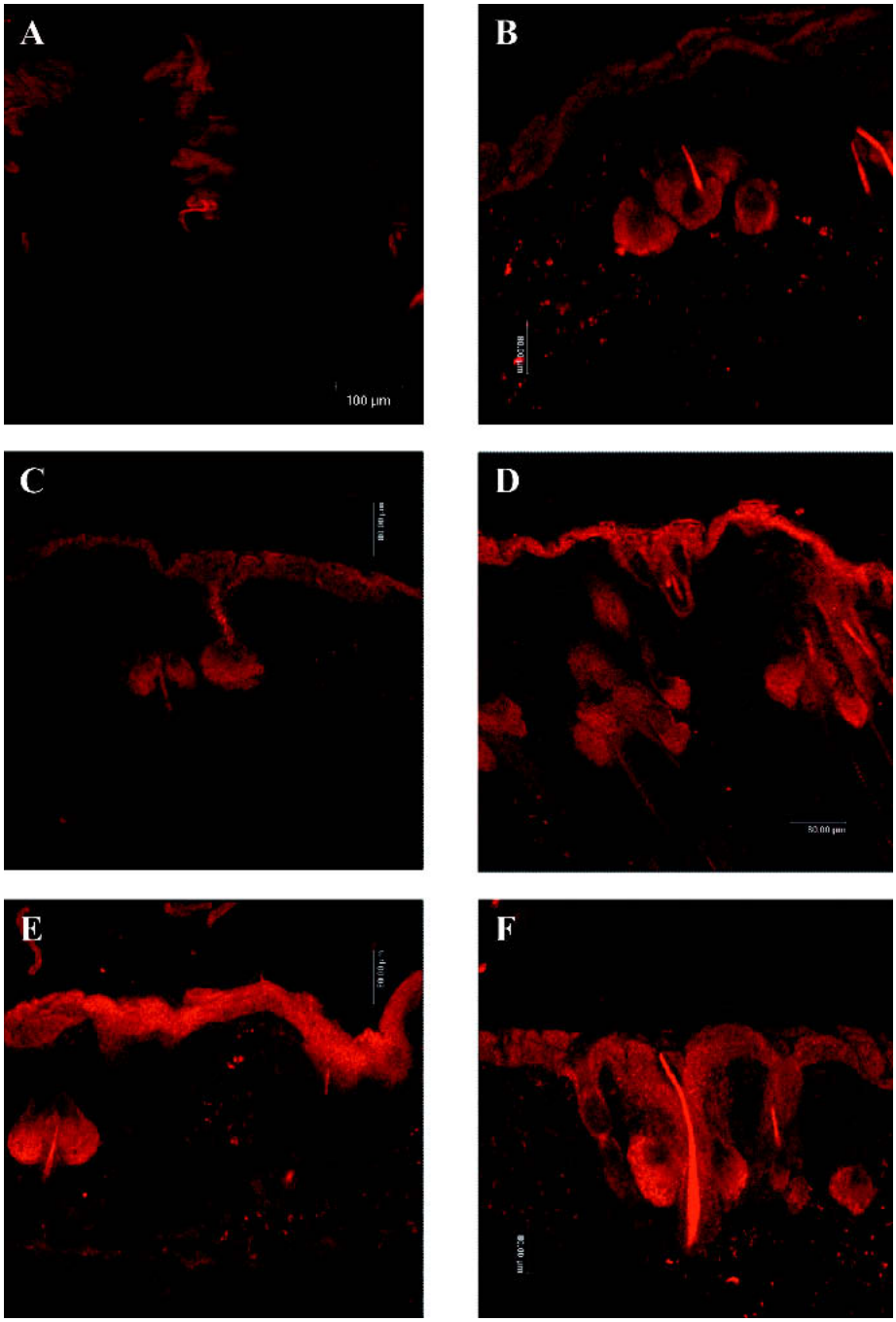

Fig. 3. CSLM images of mechanical cross sections of hairless mouse skin (perpendicular series): (A) control (untreated skin; bar = 100 µm); skin treated with: (B) 5-ALA, (C) M-ALA, (D) B-ALA, (E) H-ALA, and (F) O-ALA (bars = 80 µm). All applied formulations contained 3% EDTA (w/w).

used in these experiments. Therefore, one must conclude that hydrolysis is not the only metabolic process for the degradation of 5-ALA esters. This hypothesis if further supported by the observation of Kloek *et al.* (16) having studied the accumulation of PpIX with a homologous series of 5-ALA in lyzed T-cell lymphoma cell lines. They observed a decrease in PpIX accumulation with increasing chain length until B-ALA while 5-ALA esters with longer chains showed increasing accumulation with increasing chain length indicating that digestion pathways are depending on the particular 5-ALA derivative.

CONCLUSIONS

In conclusion, H- and O-ALA also demonstrated the largest rates of *in vitro* enzymatic degradation in skin homogenate. We have shown that esterases induced hydrolysis is not the only pathway for the degradation of 5-ALA derivatives, which could be influenced by different enzymes present in the skin. However, the complete understanding of the enzymatic mechanisms involved in the conversion of 5-ALA esters into PpIX remains still unclear. Furthermore, the formulation of

lipophilic 5-ALA esters significantly influences the efficacy of PpIX generation since B-, H- and O-ALA significantly increased PpIX production and accumulation *in vivo*, as shown by confocal microscopy and quantitative extraction studies. The increased PpIX formation induced by 5-ALA esters with longer alkyl chains support the idea that these drugs have the potential for PDT of skin tumors.

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