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ORIGINAL ARTICLE

Comparison of the uptake of 5-aminolevulinic acid and its methyl ester in keratinocytes and skin

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Abstract Photodynamic therapy is widely used in the treatment of superficial skin cancers. 5-Aminolevulinic acid (ALA) and its methylated form, methyl-ALA (MAL), are frequently used as precursors to photosensitizing substances. Nevertheless, the mechanism of the uptake of ALA and MAL in keratinocytes and of their skin penetration is still controversial. Since both compounds are not sufficiently lipophilic to penetrate through lipid membranes, they must employ specific uptake systems which may vary between different cell types. Here, we studied ALA and MAL uptake in keratinocyte cell lines originating from healthy cells (CCD 1106 KERTr cells) or keratinocyte tumors (A431 cells). ALA uptake resulted in faster protoporphyrin IX (PpIX) production than MAL uptake. A pharmacological characterization of the uptake systems revealed that PpIX formation was most efficiently reduced with GABA transporter (GAT) substrates. GABA, β-alanine, and (S)-SNAP-5114 reduced ALA uptake and, to a lesser extent, MAL uptake in the cell lines. The pharmacology of these compounds indicates that ALA and MAL are taken up by normal and pathological keratinocytes via GAT-3. Furthermore, the amino acids arginine, cysteine, and histidine also inhibited the uptake of ALA, and even more so MAL, suggestive of an additional involvement of amino acid transporters. To show that PpIX formation in vivo is restricted to the application site, which has been questioned for ALA in one other report, we applied clinically used ALA and MAL formulations to the skin of nude mice. Contrary to the results of these previous authors, the resulting PpIX fluorescence increased over time and was restricted to the application site for both preparations.

Keywords Delta-aminolevulinic acid · Methylaminolevulinic acid · Photodynamic therapy · Protoporphyrin IX · GABA transporters (GAT) · Amino acid transporters · Keratinocytes

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Introduction

Photodynamic therapy (PDT) (Dougherty and Marcus 1992) is widely used for the treatment of different cancer types, most frequently superficial nonmelanoma skin tumors (Ericson et al. 2008; Kennedy et al. 1999; Peng et al. 1997). Precursor substances like 5-aminolevulinic acid (ALA) or its methylated form, methyl-ALA (MAL), that are not photosensitive themselves, are applied to the surface of the malignant skin where they are taken up by keratinocytes and metabolized to the actual photosensitive compound, protoporphyrin IX (PpIX) (Ericson et al. 2008; Peng et al. 1997). This conversion takes place in the mitochondria (Peng et al. 1997). Several factors contribute to the high tumor selectivity of PDT. One of them is the better penetration and crossing of ALA through the deranged stratum corneum, which was demonstrated for basal cell carcinoma (Wennberg et al. 2000). Additionally, PpIX accumulation is higher in neoplastic cells due to a reduced conversion into heme based on the lower activity of the enzyme ferrochelatase (Van Hillegersberg et al. 1992). The tumor selectivity of PDT with ALA may furthermore be due to a lower, reduced ability of tumor cells to neutralize reactive oxygen or to a reduced capacity to repair the damage caused by reactive oxygen species (Pottier and Kennedy 1994).

By loading the tumor cells with ALA or MAL and irradiating with red light (635 nm) (Peng et al. 1997), PpIX is excited and produces reactive oxygen species (ROS), mostly singlet oxygen ($^1O_2$). Because of its short half-life, it has a limited diffusion radius and mostly harms mitochondria where PpIX is formed (Zhao and He 2010). Destruction of mitochondria will ultimately lead to cell death (Gudgin Dickson and Pottier 1995; Niedre et al. 2002) either by apoptosis or by necrosis, depending on the amount of ROS formed (Buytaert et al. 2007; Mroz et al. 2011).

ALA and MAL have been widely used for the clinical management of actinic keratosis and other skin disorders (for a review, see Babilas and Szeimies 2010 and Agostinis et al. 2011). For ALA- and MAL-based PDT, various clinical trials have been published showing their convincing clinical efficacy. While ALA and MAL were both more effective than placebo and yielded cosmetic results superior to cryotherapy (Szeimies et al. 2002; Freeman et al. 2003; Pariser et al. 2003; Piacquadio et al. 2004; Szeimies et al. 2010), a recently published phase III study compared the efficacy and safety of an MAL-containing drug and a novel nanoemulsion-based ALA formulation head-to-head (Dirschka et al. 2012). This study revealed a superior total clearance rate of AK lesions for the novel ALA formulation towards the MAL-containing drug and placebo.

It was shown that the uptake mechanism of ALA and MAL may differ in different cell types. Various transporter systems may be involved in the uptake. In murine mammary adenocarcinoma M3 (LM3) cells (Rodriguez et al. 2006a) and human adenocarcinoma cells (WiDr) (Rud et al. 2000), BETA transporters have been shown to be involved in ALA, but not in MAL uptake (Gederaas et al. 2001). BETA transporters are comprised of GABA transporters (GAT) 1–3, taurine transporter (TAUT), and betaine/GABA transporter (BGT-1) (Kulig and Szwaczkiewicz 2008; Palacin et al. 1998). In transfected yeast cells, PEPT-1 and PEPT-2 have been identified as further possible transporter systems, where MAL is transported by PEPT-2, while ALA can be transported by both PEPT-1 and PEPT-2 (Rodriguez et al. 2006b). In neurons, ALA and MAL are apparently incorporated into the cells through the same GABA transporters (Novak et al. 2011).

Until today, no information is available about the uptake mechanism of ALA and MAL in keratinocytes. Here, we investigated the uptake in A431, a cell line derived from a squamous cell carcinoma, and in CCD 1106 KERTr cells, keratinocytes immortalized with a human papilloma virus. A431 cells resemble tumor cells, while CCD cells rather resemble "normal" keratinocytes. We showed that the cell lines form PpIX after both ALA and MAL incubation. Cancer cells (A431) produced more PpIX compared to healthy cells, as judged on the basis of PpIX fluorescence. A difference between ALA and MAL uptake was seen with brief uptake times, when ALA induced more PpIX fluorescence than MAL. Longer incubation times diminished this effect and triggered the formation of similar amounts of PpIX. GAT-2/3 substrates like GABA, β-alanine, or (S)-SNAP-5114 reduced PpIX formation after both ALA and MAL incubation. Since only GAT-3, but not GAT-2, mRNA was detected in keratinocytes, this may be a relevant ALA and MAL transporter in both A431 and CCD 1106 KERTr cells.

Material and methods

Cell lines and cell culture

The cell line A431 (Giard et al. 1973) (Sigma-Aldrich, Munich, Germany) derived from epidermoid carcinoma was cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Karlsruhe, Germany) with 10 % fetal calf serum (FCS; Invitrogen). CCD 1106 KERTr cells (American Type Culture Collection, Wesel, Germany) were cultured in KeraSFM (Invitrogen). All cells were cultured at 37 °C in an atmosphere containing 5 % CO_2.

Primary human keratinocytes were prepared as described previously (Kitano and Okada 1983). Briefly, epidermis and dermis were dissected with 0.2 % dispase (Sigma) overnight at 4 °C. Keratinocytes were removed from the epidermis by floating in 0.25 % Trypsin/EDTA (Gibco) and seeded in collagen I-coated flasks. Healthy human skin was obtained during plastic surgery with the permission of the patients.

ALA and MAL uptake

5-ALA and MAL (0–6 mM) were dissolved in DMEM/F12 without phenol red (Gibco) (medium) or in HEPES buffer solution (buffer) (10 mM HEPES, 150 mM NaCl, 1.2 mM $CaCl_2$, 0.64 mM $MgCl_2$, 6 mM KOH, 5 mM glucose; pH 7.4) (Gederaas et al. 2001). Two different uptake protocols were used: (1) Uptake was performed for 30 min at 37 °C in the incubator. After that, the medium or buffer containing ALA or MAL was removed and fresh medium was added for the time of PpIX synthesis (0–4 h). (2) Both compounds were incubated likewise, but the washing step was omitted, resulting in a combined uptake and synthesis time of 30 min to 4.5 h.

PpIX measurement

Cells were lysed in 20 μL lysis buffer (125 mM Tris/HCl, pH 7.8; 2 mM DTT; 2 mM CDTA; 10 % (*v*/*v*) glycerol; 1 % (*v*/*v*) Triton-X100; all from Sigma) and PpIX was extracted with a solution of 80 μL MeOH/PCA/H_2O. The cell

solutions were transferred to black 96-well plates and fluorescence was measured at 390/620 nm in a Mithras Multimode Microplate Reader LB 940 (Berthold Technologies, Bad Wildbad, Germany). Each experiment was performed in triplicate at least three times.

Inhibitor studies

Cells (1×10^5/mL) were seeded into 96-well plates 2 days prior to the experiments. Nearly confluent cells were incubated with a mix of inhibitor substance (Table 1) and ALA or MAL (1.8 mM) at 37 °C. ALA and the inhibitor were removed after 30 min and replaced by fresh medium and incubated for 2 h. Cells treated with MAL were incubated for 2.5 h, omitting the washing step performed with ALA.

Reverse transcriptase-PCR

For the preparation of total RNA from A431, CCD cells, and primary keratinocytes, cells were homogenized with 1 mL TRIzol (Invitrogen) per 100 mg of tissue and incubated for 5 min at RT. After adding 0.2 mL chloroform per milliliter TRIzol, the organic TRIzol-containing phase and the phase containing total RNA were separated by centrifugation in a chilled centrifuge for 15 min at 12,000×*g*. The colorless supernatant was removed, and total RNA was precipitated by adding isopropanol 1:1 followed by a second centrifugation step. The RNA pellet was washed in 70 % ethanol, air-dried, and dissolved in water.

Total RNA (2 μg) from cells was used for the reverse transcription reaction. The reverse transcription reaction was done by using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's recommendations.

RT-PCR was performed using a Robocycler 96 (Stratagene, La Lolla, USA). Each sample contained 1.5 μL of cDNA, 2 mM $MgCl_2$, 2 mM dNTPs, 1 U Taq polymerase (all from Fermentas), and 10 pmol of primers (Table 2). This solution was buffered by tenfold PCR buffer with $(NH_4)_2SO_4$ (Fermentas). PCR amplification was initiated by incubation at 95 °C for 5 min and followed by 35 cycles: 45 s at 95 °C, 45 s at 60 °C, and 90 s at 72 °C. PCR products were verified using agarose gel electrophoresis.

PpIX fluorescence in nude mice

Male NMRI-nude mice were used to evaluate PpIX fluorescence in the skin. BF-200 ALA (Ameluz®) (Biofrontera Pharma GmbH, Leverkusen, Germany) or MAL (Metvix®) (Galderma Laboratorium GmbH, Düsseldorf, Germany) were administered to an area of 1-cm diameter on the back of the mice for 1–6 h. Mice were prevented from licking by the application of an appropriate collar. Residuals of the cream were removed prior to photo documentation with a soft tissue paper. Photo documentation of PpIX fluorescence was performed using a Nikon D50 digital camera with a red light filter (light red 090 M; Schneider Kreuznach, Bad Kreuznach, Germany). UV illumination of PpIX was performed with the diagnostic handheld lamp Hydrosun HM6 (Hydrosun Medizintechnik GmbH, Müllheim, Germany) with six UV-emitting LED lights. The emitted light has a wavelength of 415 nm, leading to a high specific fluorescent signal in the skin. Two lamps were placed 25 cm above the skin surface, and light beams were directed at the skin

Table 1 Final concentration of competitive inhibitors

Substance	Final concentration (mM)
L-Arginine	7
(S)-SNAP-5114	0.1
β-Alanine	10
Betaine	10
GABA	10
Glycylglutamine	10
Guvacine	10
L-Alanine	0.5
L-Asparagine	0.5
L-Cysteine	2
L-Glutamate	10
L-Glycine	0.5
L-Histidine	2
L-Isoleucine	5
L-Leucine	5
L-Lysine	5
L-Methionine	2
L-Phenylalanine	2
L-Threonine	5
L-Tyrosine	2

Table 2 Primers used for RT-PCR

Gene	Sequence	
GAT-1	Forward	5′-CATGTCCTGTGTGGGCTATG-3′
	Reverse	3′-CCCCCGATGGAGGTGTACT-5′
GAT-2	Forward	5′-ACTGTATGGAGTTCCAGAAGACC-3′
	Reverse	3′-CCTCGAATTAACAGGACCACC-5′
GAT-3	Forward	5′-GTTTGCCCTTTATTTGAAGGCAT-3′
	Reverse	3′-CCCAGGGTAGCTCAGTAGTG-5′
PEPT-1	Forward	5′-GCGTTTGGTGGAGATCAGTT-3′
	Reverse	3′-ATGCACTTGGCCACTTTACC-5′
PEPT-2	Forward	5′-ATCCACTGAGCATTGCCTTC-3′
	Reverse	3′-TATTGGTAAGGCACCCAAGG-5′

surface such that treated areas on the back of the mice were illuminated as evenly as possible.

Statistical analysis

Comparisons between the two groups were done using Student's *t* test or Mann–Whitney test. Statistical significance was accepted at a p value of <0.05. All statistical analyses were conducted with Statistica 8.0 software (StatSoft, Inc., Tulsa, OK).

Results

ALA and MAL induced PpIX fluorescence

Although ALA and MAL uptake have been studied in various cell lines, the analysis of their uptake in keratinocytes is missing, although this seems most relevant with respect to the clinical use of ALA or MAL PDT in the removal of nonmelanoma skin cancers. Therefore, we measured PpIX fluorescence in A431 or CCD 1106 KERTr cells after incubation with ALA or MAL.

In the experiments shown in Fig. 1, cells were either preloaded for 30 min with ALA or MAL dissolved in medium, followed by washing and incubation for 2 h (Fig. 1a, b) or 4 h (Fig. 1c, d) without the compounds or incubated in the presence of ALA or MAL for the entire 2.5 or 4.5 h, respectively. The amount of PpIX fluorescence in the two cell lines was substantially different (Fig. 1). Following the 2-h incubation with ALA, A431 cells showed PpIX fluorescence of nearly 6,000 relative fluorescence units (RFU), while CCD cells only reached 1,500 RFU. The 4-h incubation following the loading with ALA produced even higher PpIX values than the 2-h incubation. At this time point, the PpIX levels in the pre-loading regime declined already after pre-loading with 1.8 mM ALA. Thus, at the lower ALA concentrations (1.8 mM), the PpIX accumulation had already decreased after 4 h, while the peak was between 2 and 4 h or even later if the 30-min incubation was done with 6 mM ALA.

Although MAL induced much lower PpIX fluorescence than ALA in both cell types, the difference between the cell types was visible to a similar extent. The highest MAL concentration induced a PpIX fluorescence of 2,000 RFU in A431 and 1,000 RFU in CCD cells (Fig. 1a, b) after the 2-h incubation. After the 4-h incubation, the PpIX values decreased again for MAL in both cell lines and at both concentrations. Since the actual PpIX concentration is a consequence of PpIX synthesis and degradation, it must be

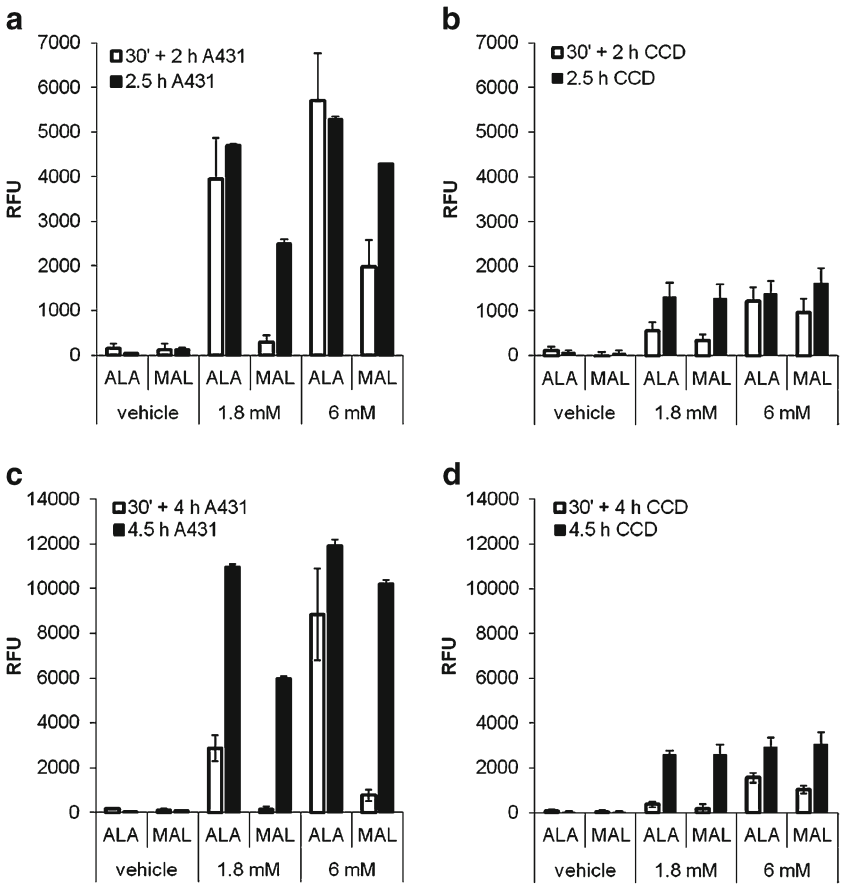

Fig. 1 PpIX fluorescence after ALA or MAL incubation in A431 and CCD cells. A431 (**a**, **c**) and CCD cells (**b**, **d**) were incubated with vehicle, 1.8 or 6 mM ALA or MAL. The incubation took place either for 30 min followed by medium exchange and a 2-h incubation to allow PpIX synthesis or for 2.5 h (**a**, **b**) or for 30 min followed by 4 h PpIX synthesis or 4.5 h uptake and PpIX synthesis (**c**, **d**). PpIX fluorescence was measured in the cell lysates. Experiments were performed in three independent experiments, each with three wells per condition. $n=3\pm$SEM

concluded that the synthesis of PpIX continues for several hours after the incubation with ALA or MAL. At least at the lower concentration of ALA and with MAL, which may be less efficient due to different cellular uptake properties and its requirement of an esterase activity as additional metabolic step, degradation already overcompensates synthesis at the 4-h time point.

Using the pre-loading protocol described above, which allows the continuation of PpIX synthesis from ALA that was taken up during the pre-loading, strong PpIX fluorescence was induced with both compounds in A431 cells, but only a slight increase in CCD cells. The maximal fluorescence in CCD cells was 3,000 RFU after incubation with either ALA or MAL. In both cell lines, dose-dependent increases in fluorescence were detectable, which reached a plateau in CCD cells. With the exception of this plateau in CCD cells, PpIX fluorescence after MAL incubation was always lower than after ALA incubation (Fig. 1).

Uptake mechanism of ALA and MAL

Since previous studies had suggested that ALA and MAL may pass through amino acid transporters, we compared PpIX formation after incubating the cells with ALA or MAL in medium (containing amino acids which may interfere with the uptake) or in HEPES buffer (lacking amino acids). To obtain similar uptake efficacy with ALA and MAL, all the following experiments were performed using the pre-loading regime with ALA and the incubation over the entire PpIX synthesis time with MAL, as described above. Incubation with ALA and MAL in buffer resulted in similar PpIX fluorescence compared to ALA and MAL in medium (data not shown). To prevent interference from amino acids in the medium going unnoticed, both solvents were tested in the following experiments.

The experiments in Figs. 2 and 3 were performed with ALA or MAL at 1.8 mM, a concentration at which the system is not saturated, dissolved in buffer (white bars) or in medium (black bars). First, we analyzed the effects of amino acids on the uptake of ALA and MAL in A431 (Fig. 2a, b, respectively) and CCD cells (Fig. 3a, b, respectively), since they may inhibit ALA or MAL uptake by competing for the uptake through the same transporters. We observed that some amino acids reduced PpIX formation to a similar extent for ALA and MAL. In A431 cells (Fig. 2), the strongest inhibition of PpIX formation was observed with arginine, cysteine, and histidine. In CCD cells (Fig. 3), the effect was less prominent. Arginine and cysteine reduced PpIX formation after ALA incubation, but only if they were dissolved in buffer (Fig. 4a). MAL was inhibited by cysteine and histidine (Fig. 3b).

To further characterize uptake carriers transporting ALA and MAL through the membrane, we used inhibitors to

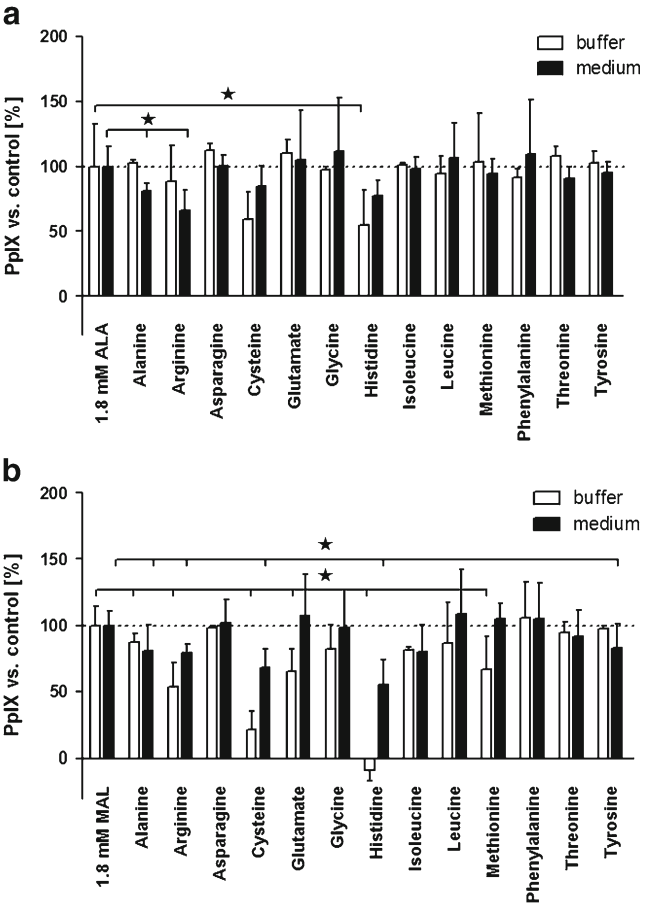

Fig. 2 Inhibition of ALA and MAL uptake with amino acids in A431 cells. A431 cells were incubated with amino acids to inhibit the uptake of ALA (**a**) or MAL (**b**). ALA was incubated along with the respective amino acids for 30 min, replaced by fresh medium and PpIX was synthesized for 2 h. MAL was incubated along with the respective amino acids for 2.5 h. Experiments were performed three times with three wells per condition. $n=3\pm$SEM. $p<0.5$ *U* test compared to 1.8 mM ALA or MAL

specific uptake transporters that were indicated by the above results or by the literature. We found that GABA transporter (GAT) inhibitors caused the strongest inhibition of PpIX formation in both A431 (Fig. 4a) and CCD cells (Fig. 4b).

In A431 cells (Fig. 4a), the GAT-3 substrates β-alanine (10 mM) and GABA (10 mM) inhibited ALA-induced PpIX formation by 76 and 60 %, respectively. MAL-induced uptake was inhibited with β-alanine (10 mM) and GABA (10 mM) by 42 and 58 %, respectively. (S)-SNAP-5114 (100 μM) reduced ALA-induced PpIX formation by 44 % but had no effect on MAL uptake (Fig. 4a). Higher concentrations of (S)-SNAP-5114 were cytotoxic as determined by MTS tests (data not shown). The BGT-1 substrate betaine reduced the ALA-induced PpIX formation by 18 %, while the PEPT substrate GlyGln did not reduce PpIX formation. MAL-derived PpIX formation was inhibited in a similar way by betaine (33 %) and GlyGln (23 %) in A341 cells

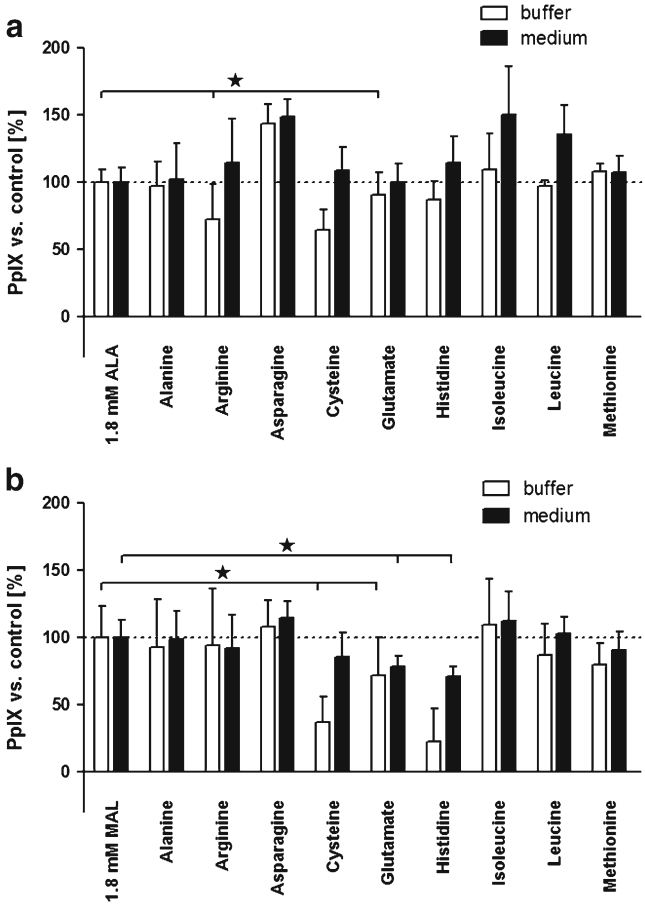

Fig. 3 Inhibition of ALA and MAL uptake with amino acids in CCD cells. CCD cells were incubated with amino acids to inhibit the uptake of ALA (**a**) or MAL (**b**). ALA was incubated for 30 min, removed and PpIX was synthesized for 2 h. MAL was incubated along with the inhibitor for 2.5 h. Subsequently, cells were lysed and PpIX contents were determined fluorometrically. Experiments were performed three times with three wells per condition. $n=3\pm$SEM. $p<0.5$ *U* test compared to 1.8 mM ALA or MAL

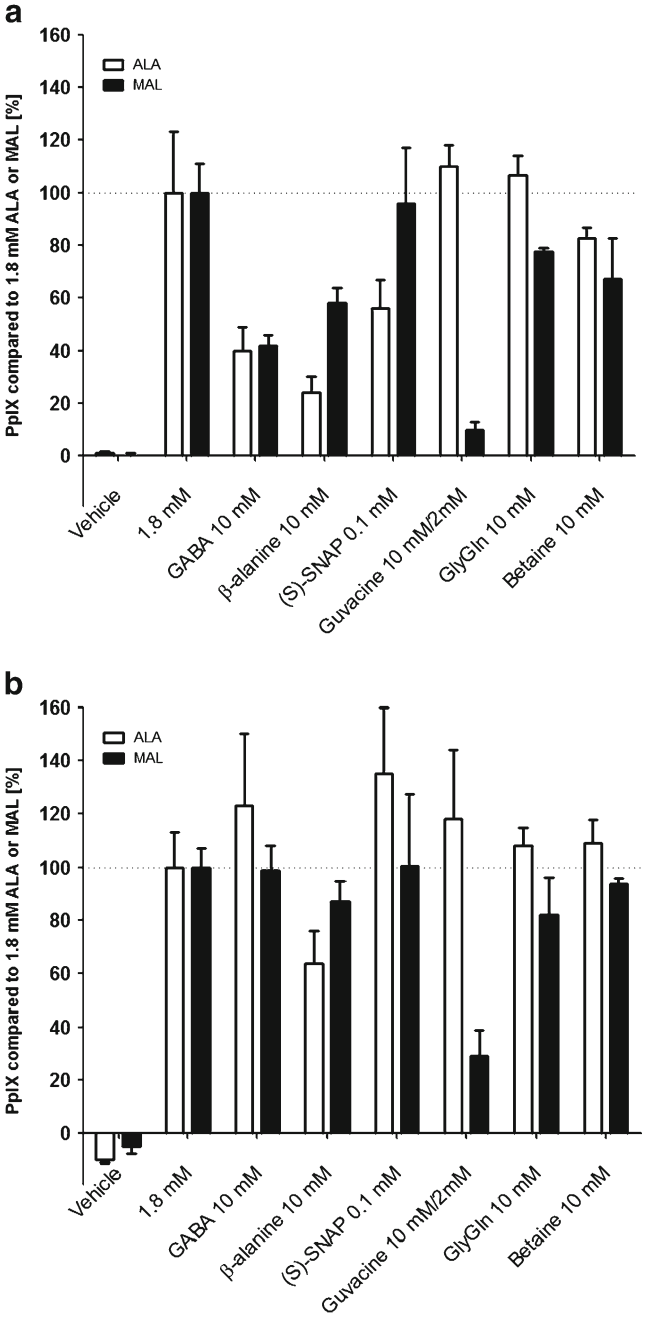

Fig. 4 Inhibition of ALA uptake in CCD and A431 cells. A431 (**a**) and CCD (**b**) cells were incubated with 1.8 mM ALA in medium or 1.8 mM MAL in buffer and inhibitors. After 30 min, ALA was removed and replaced by fresh medium. The cells were incubated for an additional 2 h of PpIX synthesis. MAL was incubated for 2.5 h along with the inhibitors. Subsequently, cells were lysed and PpIX contents were determined fluorometrically. Experiments were performed three times with three wells per condition. $n=3\pm$SEM. *U* test not significant

(Fig. 4a). Concentrations higher than 2 mM of guvacine in combination with MAL were toxic, as shown by MTS tests (data not shown).

In CCD cells (Fig. 4b), only β-alanine reduced ALA-induced PpIX formation by 32 %, while the other substances showed no inhibitory effect. MAL-induced PpIX formation was reduced by β-alanine, GlyGln, and guvacine by 13, 18, and 71 %, respectively. (S)-SNAP-5114 inhibited neither ALA- nor MAL-induced PpIX formation in CCD cells (Fig. 4b).

Expression of GAT in human epidermis

Since GAT expression had not been shown in keratinocytes, we analyzed the expression of their mRNAs by RT-PCR. We detected a 133-bp signal representing GAT-3 cDNA in all analyzed cell lines (Fig. 5). GAT-1 and GAT-2 mRNAs were not detected in skin cells. The accuracy of the RT-PCR with these genes was demonstrated with control RNAs isolated from human fetal brain tissue, where bands appeared with sizes of 174 bp for GAT-1 and 274 bp for

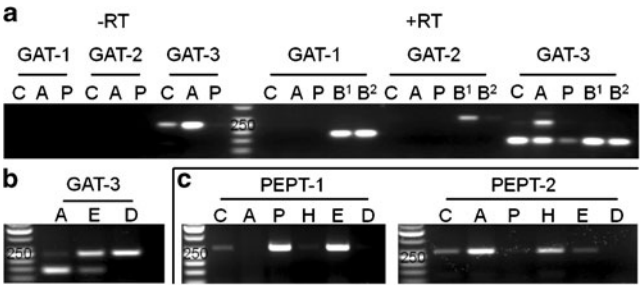

Fig. 5 RT-PCR of A431, CCD, and NHEKs shows the expression of GAT-3 in all cell lines. The expression of GAT-1, GAT-2, and GAT-3 mRNA was analyzed in CCD (*C*), A431 (*A*), and primary keratinocytes isolated from human skin and cultivated for 2 weeks (*P*) and two human cDNAs from fetal brain (B^1+B^2) (**a**). Furthermore, GAT-3 expression was analyzed in human epidermis (*E*) and dermis (*D*) (**b**). GAT-3 (133 bp, genomic band visible at 245 bp) was detected in all cultured cells and in the epidermis, but not in the dermis, whereas GAT-1 and GAT-2 expression was absent in all skin cells but present in brain lysates. PEPT-1 and -2 were analyzed in A431, CCD, primary KC, HEK cells (*H*), epidermis, and dermis (**c**)

GAT-2, respectively (Fig. 5a). The primers used for the RT-PCR were placed around an intron such that PCR products could not be generated from genomic DNA (GAT-1 and GAT-2) or were distinguishable by the size of their bands (GAT-3). For GAT-3, this band was visible at a size of 245 bp.

We next compared GAT-3 mRNA expression in the human epidermis and the human dermis. While it was detected in the human epidermis, it was lacking in the dermis (Fig. 5b). Furthermore, the expression of the amino acid transporters PEPT-1 and PEPT-2 was analyzed (Fig. 5c) as peptide transporters may transport ALA into astrocytes and transfected yeast cells. Their expression differed between different skin cells. While PEPT-1 expression was found in CCD cells, NHEKs, and human epidermis, it was not present in A431 cells. PEPT-2 was detected in all analyzed cultured cells and in the human epidermis. Neither GAT-3 nor PEPT-1 or PEPT-2 was expressed in the dermis.

PpIX fluorescence in nude mice

Based on the foregoing and as outlined in the "Discussion" section, none of the results described above justified a grossly different behavior of ALA or MAL, with the exception that ALA incubation is substantially more efficient in causing PpIX formation than MAL incubation. Nevertheless, a report in the literature described that localized PpIX fluorescence, restricted to the application site, was induced by MAL in nude mice, while ALA gave rise to generalized fluorescence of the entire mouse skin (Moan et al. 2003). Therefore, we repeated this experiment and compared ALA- and MAL-induced PpIX fluorescence in nude mouse skin (Fig. 6). The ALA formulation BF-200 ALA (meanwhile approved in Europe under the trade name of Ameluz®) or the approved MAL formulation Metvix® were each applied to two spots on the back of the mice. The substances were then incubated for 1, 2, 3, 4, or 6 h. Remainders were wiped off, and immediately afterwards, the mice were anesthetized and observed under normal light (Fig. 6a) or under UV light to visualize PpIX fluorescence (Fig. 6b). No difference was apparent by visual inspection of the fluorescence intensity. The mice were photographed again in a similar way 12 h after the first photographs

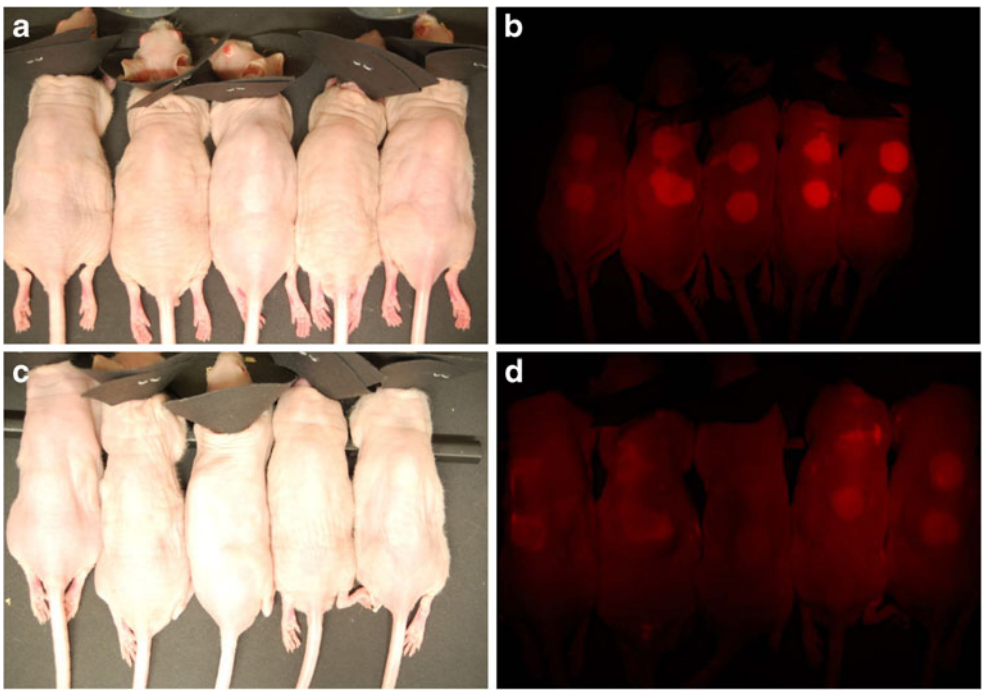

Fig. 6 Comparison of ALA- and MAL-induced PpIX fluorescence in nude mice. BF-200 ALA or a registered MAL formulation was applied to nude mouse skin for different time periods. The *upper spots* were treated with ALA, the *lower spots* with MAL. **a–d** Incubation times from *left* to *right*: 1, 2, 3, 4, and 6 h. Shown is the skin surface after incubation and removal of residual gel/cream under **a** daylight and **b** UV illumination. The skin surface 12 h after the incubation and removal of residual gel/cream, i.e., after the pictures in **a** and **b** are shown in **c** with daylight and in **d** with UV illumination

(Fig. 6c, d), also without apparent differences between the localization of the ALA- and the MAL-induced fluorescence. For both ALA and MAL, the PpIX fluorescence was restricted to the area of application, without dispersion into surrounding skin.

Discussion

The aim of the current study was to compare the potency and the mechanisms of ALA- and MAL-induced PpIX synthesis in keratinocytes and epidermis. For this, we studied the efficacy and the mechanism of ALA and MAL uptake in a virus-immortalized (CCD) and a neoplastic (A431) keratinocyte cell line. We also analyzed the expression and the relevance of cellular transporters for ALA or MAL uptake into these cells. Finally, we compared the PpIX fluorescence produced in nude mouse skin after ALA or MAL application.

First, our results show that ALA is substantially more potent for PpIX generation than MAL. This may be due to a faster cellular uptake of ALA or to the additional esterase step that is required in MAL metabolism (Novak et al. 2011). Since prolonging the MAL uptake time without additional synthesis time enhanced PpIX fluorescence to similar levels as those obtained after ALA incubation, it seems more likely that lower efficacy of the cellular uptake is the main reason for the lower MAL-induced PpIX fluorescence. This lower efficacy of MAL uptake compared to ALA uptake seems to be a general phenomenon since it was observed by ourselves and other authors in all other cell types analyzed so far, such as HaCaT cells, HeLa cells, HepG2 cells, human epidermal keratinocytes, human dermal fibroblast, A431, TMX13, and LM3 cells, and primary peripheral neurons (Lee et al. 2008; Novak et al. 2011; Rodriguez et al. 2006a; Rodriguez et al. 2006b; own unpublished results).

To reach comparable amounts of PpIX after loading with ALA or MAL in the comparison of the uptake systems, we prolonged the uptake time for MAL from 30 min to up to 4.5 h, such that, for MAL, the loading period and the synthesis time were identical (Fig. 1). This mode of uptake was used for MAL in all further experiments in this study (Figs. 2, 3, and 4), while for ALA, a 30-min pre-loading was followed by a PpIX synthesis period in the absence of ALA. Even with these different conditions, slightly lower PpIX concentrations were achieved with MAL. Only in CCD cells did they reach maximal values comparable to those after ALA incubation (Fig. 1). Methylated forms of ALA have been speculated to be taken up more easily in cells since their increased hydrophobicity may allow diffusion through lipid membranes, but the determination of oil–water distribution coefficients demonstrated unambiguously that neither ALA nor MAL will diffuse through biological membranes without specific carriers (Uehlinger et al. 2000). Thus, both compounds must enter the cells through specific transporters, as investigated in this study, which may function less efficiently for MAL than for ALA.

The second clinically relevant result of comparing the uptake in the two cell lines is that both ALA and MAL have very different PpIX formation rates in A431 and CCD cells. For both compounds, A431 cells accumulate substantially more PpIX than CCD cells. This may be due to the fact that A431 cells are cancerous cells derived from a squamous cell carcinoma (Giard et al. 1973), while CCD cells were derived from normal keratinocytes by immortalization with a human papilloma virus (Weller et al. 2003). Increased PpIX production in neoplastic cells after ALA or MAL incubation is well-known (Peng et al. 1997) and clinically used for diagnostic purposes (Krammer and Plaetzer 2008). This may partly be due to increased cellular uptake caused by differential expression of transporters in neoplastic and normal cells (Dudeck et al. 1987; McGivan 1998). Equally relevant may be the higher activity of porphobilinogen deaminase and the reduced level of ferrochelatase activity that have been found in tumor cells (el Sharabasy et al. 1992; Leibovici et al. 1988; Schoenfeld et al. 1988). The former enzyme is involved in the synthesis of PpIX from ALA, the latter in the further metabolism of PpIX. Thus, the increased expression of the first enzyme combined with the reduced expression of the second will result in PpIX accumulation.

Uptake mechanism of ALA and MAL

It would be of clinical relevance if ALA and MAL uptake efficacy were very variable between different normal and neoplastic cells of the epidermis. Therefore, we investigated the mechanism of ALA and MAL uptake in the two cell lines used in this study. It had previously been shown that ALA uptake was blocked up to 70 % by amino acids and dependent on Na^+ and Cl^- concentrations in human adenocarcinoma cells (WiDr cells; Rud et al. 2000). This hints towards the involvement of the ubiquitously expressed, Na^+-dependent transporter systems A and ASC (Hyde et al. 2003). System A transporters are responsible for arginine, histidine, cysteine, glutamate, isoleucine, and methionine uptake, system ASC transports alanine and cysteine (Hyde et al. 2003). We, therefore, performed competitive inhibition experiments with amino acids. To avoid interference with amino acids in the medium, the experiments were performed using medium or buffer during the uptake and synthesis time. The amino acid concentrations were ten times higher than those in the medium. The most potent inhibitory effect was observed with arginine, cysteine, and histidine for both ALA and MAL in both cell types (Figs. 2 and 3). This indicates that system A transporters may be

involved in transporting both compounds through the membranes. This assumption requires further verification since it contradicts the results of other authors who had shown that methyl-AIB, a system A substrate, did not inhibit ALA uptake in WiDr adenocarcinoma cells, although the expression of this transporter system in these cells was shown (Rud and Berg 1998). We furthermore observed that glutamate inhibited MAL, but not ALA, uptake in A431 and CCD cells, indicating that this transporter may also be involved in MAL uptake in keratinocytes. This is in agreement with previous authors who described a reduction of MAL uptake in WiDr cells by glutamate (Gederaas et al. 2001). Glutamate is transported by the widespread system X_{AG}^- (Hyde et al. 2003) which may thus participate in MAL, but not ALA, uptake.

In conclusion, our data indicate that system A transporters may be involved in both ALA and MAL transport, while MAL is additionally transported through system X_{AG}^-.

Previous studies also showed that ALA uptake is mediated by BETA transporters (Bermudez et al. 2002; Rodriguez et al. 2006a; Rud et al. 2000). We thus also analyzed the effects of different competitive and one non-competitive inhibitor ((S)-SNAP-5114) on the uptake of ALA and MAL. β-Alanine inhibited ALA and, to a lesser extent, MAL uptake in both A431 and CCD cells; GABA showed an inhibitory effect in A431 cells only. This inhibition was dose-dependent, using 1 μM to 10 mM of the inhibitor (data not shown). The GAT-3 inhibitor (S)-SNAP-5114 (IC_{50} 5 μM) (Kragler et al. 2005) reduced the uptake of ALA, but not MAL, in A431 cells, but had no effect in CCD cells (Fig. 4). Guvacine was toxic in combination with MAL, but did not affect ALA uptake. GABA and β-alanine are transported by GAT-3 (IC_{50} 7 and 58 μM) (Kragler et al. 2005). Guvacine displays higher affinity to GAT-1 (IC_{50} 14 μM) than to GAT-3 (IC_{50} 119 μM) (Borden 1996). Our results indicate that ALA and MAL can be taken up by GABA transporters in A431 cells and to a minor extent in CCD cells. Because of the overlapping affinities of the analyzed substances to the GAT-1 and GAT-3 transport systems, a clear distinction between the involved transporters was not possible at this point and required the gene expression analysis that was also performed in this study.

Expression of GAT in human epidermis

To further characterize the transporters involved in ALA and MAL uptake and distinguish between the participation of GAT-1, GAT-2, or GAT-3, we studied the gene expression of GABA and peptide transporters in the skin. We showed that GAT-3 is expressed in all analyzed keratinocytes as well as in human epidermis, but not in the dermis (Fig. 5). GAT-1 and GAT-2 were detected in human fetal brain, serving as positive control, but not in keratinocytes. Along with the results of the experiments with inhibitors of ALA and MAL uptake, this indicates that GAT-3 is involved in ALA and MAL uptake in keratinocytes. While GAT-3 expression had previously only been described in neuronal tissue (Borden 1996), it had been shown that GABA(A) receptors are associated with skin barrier homeostasis. Regulation of these receptors are clinically relevant for barrier dysfunction and epidermal hyperproliferative diseases (Denda et al. 2002). The role of GAT-3 in the epidermis needs further evaluation, but it is possible that it removes GABA from the surrounding tissue, as shown for astrocytes (Schousboe et al. 2004).

PEPT-2 transporters were shown to transport ALA and MAL after expression in yeast cells (Rodriguez et al. 2006b), which is in agreement with the fact that astrocytes take up ALA via PEPT-2 (Xiang et al. 2006). We, therefore, also tested the expression of PEPT-1 and PEPT-2 RNAs. Although they were expressed in epidermal cells, the PEPT-2 substrate GlyGln did not inhibit ALA uptake and only to a small extent MAL uptake. This was also the case when using the PEPT-1 substrate glycylsarcosine (GlySar) which neither inhibited ALA nor MAL uptake (data not shown).

In summary, different transporters participate in the cellular uptake of ALA and MAL. Even though there are slight differences in the preference of ALA and MAL for the various transporters, the cocktail of amino acid and GABA transporters expressed in keratinocytes and keratinocyte tumors obviously does not lead to a situation wherein one cell type ALA is preferred, while MAL is preferred in another.

PpIX fluorescence in nude mice

The preferential PpIX synthesis in neoplastic cells rendered it possible in clinical practice to use PpIX fluorescence as a diagnostic indicator for tumors in the skin, breast, or brain (Krammer and Plaetzer 2008). When PpIX is excited by red light, it either emits the energy as fluorescence or undergoes an intersystem crossing to form a triplet state. From the triplet state, the energy decays or is transferred to molecular oxygen, which then damages the cell (Agostinis et al. 2011). Since excitation with red light will hide the red fluorescence, excitation with blue light is usually used along with a suitable filter to visualize the fluorescence.

Moan et al. (2003) described their observation that the application of ALA, but not MAL, onto healthy nude mouse skin led to an extended fluorescence that was not restricted to the application site. The interpretation was that ALA is transported through the epidermis, enters the blood stream, and is systemically distributed. Such an effect may compromise the use of ALA for diagnostic purposes and pose safety questions around its clinical use. None of the results described above or other data that are known to us would hint

towards a mechanism that would allow ALA, but not MAL, penetration into the dermis, which would be a prerequisite for systemic uptake. In fact, the more hydrophilic a substance is, the less likely it is to pass the basal membrane. We, therefore, repeated the experiments described by Moan et al. (2003). Unlike these authors, we did not find a principle difference in the fluorescence induced by ALA or MAL application. With both compounds, the fluorescence increased over time, but remained restricted to the site of application. Only when mice were allowed to lick the compounds, a generalized fluorescence was observed (data not shown). In our experiments, we prevented licking by attaching an appropriate collar. Licking may be more pronounced with ALA than MAL due to slight burning that the mice may experience after the application of the acidic ALA. Licking is the most likely explanation for the different results obtained by the previous authors. In agreement with this is another previous study in which it was shown that, in porcine skin, BF-200 ALA (a nanoemulsion-based ALA formulation) penetrated faster and deeper than Metvix® (a MAL formulation), but both compounds remained restricted to the epidermis (Maisch et al. 2010). The improved penetration throughout the epidermis may, however, explain the superior clinical efficacy of the BF-200 ALA formulation (Dirschka et al. 2012). Nevertheless, there is no indication for a generalized distribution of ALA to sites that are remote from the application site.

The data presented here demonstrated that ALA and MAL are taken up in keratinocytes via different transporter systems, which are partly shared between the compounds and partly used by only one of them. Due to the expression of the transporters in different cells, the participation of the individual system may change. Given the number of transport systems that seem to be involved, it is unlikely that expression differences with uptake carriers will cause a strong preference for ALA or MAL in a specific cell type. After the application of the compounds to nude mouse skin, both remain restricted to the application site. Other authors (Maisch et al. 2010) found no indication for skin penetration of the same formulations that reach deeper than the basal cell layer. Consequently, we did not find any indication that either compound may be transported away from the application site.

In clinical use, the slight differences in the preference for uptake carriers do not present an argument for the use of one or the other compound. The only striking difference between ALA and MAL is the efficacy with which the compounds trigger PpIX formation, providing clear arguments for the clinical use of ALA rather than MAL.

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Conflict of interest One of the authors (HL) is the general manager of Biofrontera Bioscience GmbH which holds the marketing authorization for a PDT drug containing ALA (Ameluz®) in Europe.

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