5-Aminolaevulinic acid peptide prodrugs enhance photosensitization for photodynamic therapy

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Abstract

Intracellular porphyrin generation following administration of 5-aminolaevulinic acid (ALA) has been widely used in photodynamic therapy for a range of malignant and nonmalignant lesions. However, ALA is relatively hydrophilic and lacks stability at physiologic pH, limiting its bioavailability. We have investigated more lipophilic, uncharged ALA-peptide prodrugs based on phenylalanyl-ALA conjugates, which are water soluble and chemically stable for improving ALA delivery. Pharmacokinetics of the induced protoporphyrin IX (PpIX) were studied in transformed PAM212 keratinocyte cells and pig skin explants. The intracellular porphyrin production was substantially increased with Ac-L-Phe-ALA-Me (compound 1) and Ac-L-Phe-ALA (compound 3) compared with equimolar ALA: after 6-h incubation, the PpIX fluorescence measured using 0.01 mmol/L of compound 1 was enhanced by a factor of 5 compared with ALA. Phototoxicity results showed good correlation with PpIX levels, giving a LD50 (2.5 J/cm²) of 25 μmol/L for ALA, 6 μmol/L for 5-aminolaevulinic hexyl ester, and 2.6 μmol/L for compound 1, which exhibited the highest phototoxicity. However, these results were stereospecific because the corresponding D-enantiomer, Ac-D-Phe-ALA-Me (compound 2), induced neither porphyrin synthesis nor phototoxicity. PpIX levels were considerably reduced when cells were incubated with compound 1 at low temperatures, consistent with active transport. In pig skin explants, compound 1 induced higher porphyrin fluorescence than ALA by a factor of 3. These results show that water-soluble peptide prodrugs of ALA can greatly increase its cellular uptake, generating more intracellular PpIX and improved tumor cell photosensitization. The derivatives are comparable in efficacy with 5-aminolaevulinic hexyl ester but less toxic and more stable at physiologic pH. [Mol Cancer Ther 2008;7(6):1720–9]

Introduction

Photodynamic therapy (PDT) is a nonthermal technique for inducing tumor damage with light following administration of a light-activated photosensitizing drug (1, 2). A promising approach in PDT involves the exogenous administration of 5-aminolaevulinic acid (ALA), which is a naturally occurring compound present in mammalian cells that can be metabolized to a porphyrin photosensitizer, protoporphyrin IX (PpIX), via the heme biosynthetic pathway (3). Following accumulation of PpIX within the diseased tissue, PDT treatment is then carried out using red light (or blue light for thin lesions), activating PpIX and leading to the production of cytotoxic reactive oxygen species. ALA-PDT does exhibit a degree of intrinsic tumor selectivity owing to differing tumor levels of enzymes in the heme pathway (e.g., ferrochelatase) leading to higher tumor PpIX levels. The main clinical application of ALA-PDT at present is for the treatment of basal cell carcinoma using topical application of ALA (4, 5). The selective epithelial photosensitization induced by ALA, when administered systemically, has also enabled treatment of early tumors and high-grade dysplasia in hollow organs (6, 7), where damage to underlying muscle must be minimized.

A significant drawback to ALA-PDT is the fact that ALA is a zwiterion at physiologic pH, resulting in low lipid solubility and limiting passage through biological barriers such as cellular membranes. To overcome this problem, several chemical approaches have been attempted to improve the incorporation of ALA and also its selectivity. One approach has been to use more lipophilic ALA derivatives, such as alkyl or ethylene glycol esters, which are potential substrates for cellular esterases (8–11), or different delivery systems including dendrimers (12–14) or liposomes (15, 16). The use of alkyl esters of ALA results in a nonspecific distribution of ALA in all cell types, but with an increased PpIX production (10, 17) in tumor cells. In this regard, Casas et al. (18) and Berger et al. (11, 19) have conjectured that incorporation of 5-ALA into a short peptide derivative would provide a suitable means of facilitating both transdermal delivery and improved selectivity to cancerous cells, presenting potential substrates for cell-surface and cytoplasmic peptidases and/or ligands for peptide and amino acid transporters.

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In an attempt to improve uptake and cell selectivity of ALA and its derivatives, we have investigated PpIX formation and PDT efficacy using peptide ALA prodrugs, which represent potential substrates for cell-surface and cytoplasmic peptidases and esterases. Herein, we describe the results obtained with phenylalanyl-ALA conjugates 1 to 3 whose structures are shown in Fig. 1A.

### Materials and Methods

**Chemicals**

ALA, 5-aminolaevulinic methyl ester (ALA-Me), 5-aminolaevulinic hexyl ester (ALA-Hex), and fluorescein diacetate were purchased from Sigma-Aldrich. Chemical reagents were purchased from Sigma-Aldrich and Novabiochem. Anhydrous tetrahydrofuran was obtained by distillation over sodium/benzophenone. Dry dichloromethane was obtained by distillation over CaH₂. All other solvents were purchased from Fisher Scientific.

The spontaneously transformed murine keratinocyte cell line, PAM212 (obtained from Prof. R. Groves, Imperial College), was cultured in RPMI 1640 (Life Technologies) containing L-glutamine (0.02 mmol/L) and phenol red supplemented with 10% FCS (Sigma-Aldrich), penicillin (compound 3) are shown in Fig. 1A. Stock solutions of compounds 1 and 2 were prepared by dissolving at 10 mmol/L in 0.01 mol/L HCl and stored at -20°C. Compound 3 was dissolved at 100 mmol/L in 0.1 mol/L HCl solution and stored at -20°C. Optically pure compound 1 was obtained from Boc-L-Phe-ALA-Me according to our previous published procedure (20). Compound 3 was obtained from compound 1 by saponification with LiOH in H₂O/methanol (3:2). Compounds were purified by flash chromatography on silica gel 60 (Fisher Scientific; 35-70 μm) and recrystallization. Compounds 1 and 3 were characterized by ¹H and ¹³C NMR, recorded on a JEOL JMN GX 270MHz spectrometer, and by high-resolution electrospray mass spectrometry, using a Bruker MicroTOF autospec electrospray ionization mass spectrometer.

**Cell Culture**

The spontaneously transformed murine keratinocyte cell line, PAM212 (obtained from Prof. R. Groves, Imperial College), was cultured in RPMI 1640 (Life Technologies) containing t-glutamine (0.02 mmol/L) and phenol red supplemented with 10% FCS (Sigma-Aldrich), penicillin

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**Figure 1.** A, molecular structures of ALA, compound 1, compound 2, and compound 3. B, experimental and predictive log P values for ALA, ALA-ME, ALA-Hex, compound 1, and compound 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Experimental log P</th>
<th>Predictive log P a</th>
<th>Clog P b</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>-1.52±0.02</td>
<td>-1.17 (13)</td>
<td>-1.12</td>
</tr>
<tr>
<td>ALA-Me</td>
<td>-0.85±0.07</td>
<td>-0.75 (13)</td>
<td>-0.64</td>
</tr>
<tr>
<td>ALA-Hex</td>
<td>1.84</td>
<td>1.57 (13), (10)</td>
<td>2.00</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.58</td>
<td>0.45</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.13</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

a: Values obtained using Osiris® software  
b: Values obtained using ClogP® software
(500 units/mL; Life Technologies), and streptomycin (0.5 mg/mL; Life Technologies). The cells were grown as monolayers in sterile, vented-capped, angle-necked cell culture flasks (Corning) and were maintained at 37°C in a humidified 5% CO₂ incubator (IR Autoflow Water-Jacketed Incubator; Jencons Nuaire) until confluent.

Fluorescence Pharmacokinetics
Cells were seeded into γ-sterilized 96-well plates (Orange Scientific, Triple Red Laboratory Technologies) at a density of ~5 × 10⁴ per well for 48 h. After removing the culture medium, the wells were washed with PBS. The cells were incubated with freshly prepared solutions of ALA and

Figure 2. A, kinetics of PpIX fluorescence in PAM212 cells. A time course study of fluorescence intensity measurements over 2 h (●, ALA; ○, compound 1), 4 h (■, ALA; □, compound 1), and 24 h (●, ALA; ○, compound 1) incubation times for concentrations of ALA or compound 1 ranging between 0.001 and 0.1 mmol/L. Bars, SD. *, P < 0.05 (ALA versus compound 1). B, PpIX fluorescence in PAM212 cells after 6 h incubation time with ALA (●), compound 1 (■), compound 2 (○), and compound 3 (▲) ranging between 0.01 and 0.1 mmol/L. Bars, SD. *, P < 0.05 (ALA versus other compounds). C, PpIX fluorescence in PAM212 cells exposed for 1 h to ALA, ALA-Hex, or compound 1 at 4°C (■) or 37°C (○), washed, and incubated for a further 3 h. Bars, SD. *, P < 0.05 (4°C versus 37°C).
Phe-ALA derivatives: 0.1 mL serum-free medium containing varying prodrug concentrations was added to a designated series of wells. Each plate contained control wells with cells but without added drug for determination of the background reading, and reference wells containing cells were incubated with the same ALA concentrations. For drug incubation, serum-free medium was used because serum is known to cause release of PpIX from cells, thus resulting in loss of fluorescence signal (21). The PpIX fluorescence induced by the compound 1 was evaluated in comparison with ALA and ALA-Hex, which is known to induce more efficient porphyrin production than ALA.

The fluorescence signal from each well was measured with a Perkin-Elmer LS 50B fluorescence spectrometer coupled to an automated plate reader (Perkin-Elmer) using 410 nm excitation and 635 nm emission wavelengths with slit widths set to 10 nm and the internal 530 nm long-pass filter used on the emission side; spectral scans were recorded between 600 and 750 nm to check for presence of any porphyrins other than PpIX (14). The mean fluorescence intensity (expressed in arbitrary units) was calculated after subtraction of the control values. Intensity calibrations were done using rhodamine B embedded in a Perspex disc as a standard.

To assess whether prodrugs were incorporated by active transport or passive diffusion, temperature dependence studies were conducted. Fluorescence spectroscopy was used to determine the effect of temperature on cellular uptake of prodrugs before the conversion of ALA or compound 1 to PpIX, as both active transport and endocytosis are inhibited at 4°C, whereas passive diffusion is not (22). PAM212 cells were seeded into 96-well γ-irradiated plates, incubated for 48 h, and washed with PBS. ALA, ALA-Hex, and compound 1 at concentrations of 0.1, 0.2, and 0.4 mmol/L in clear medium was added at 100 µL/well and the plates were wrapped in foil. One plate was incubated at 37°C and the other at 4°C for 1 h. The prodrugs were then removed, cells were rinsed in PBS, and 100 µL clear medium was added to each well. The plates were both incubated at 37°C for 3 h to allow the ALA already taken up by the cells to be converted to PpIX. The amount of PpIX produced by the cells in the different conditions was evaluated as described above.

**Esterase Activities**

Esterase activities were determined in PAM212 cells using fluorescein diacetate (23). Nonfluorescent fluorescein diacetate was added to the medium and the amount taken up by the cells and converted to fluorescein was determined. This was used to give an estimation of cellular nonspecific esterase activity to ascertain the extent to which conversion of compound 1 to ALA could occur once internalized. Cells were seeded into γ-sterilized 96-well plates (Orange Scientific, Triple Red Laboratory Technologies) at a density of ~5 × 10⁴ per well for 48 h. After removing the culture medium, the wells were washed with PBS and 100 µL fresh serum-free medium containing 1 µmol/L fluorescein diacetate and either 0.6 mmol/L ALA, 0.6 mmol/L ALA-Me, or 0.6 mmol/L compound 1, and cells were exposed for periods of 2, 4, and 6 h. The spent medium was retained; the cells washed three times with PBS and drained. cellLytic (126 µL) was added to each well and incubated during 15 min at room temperature to extract cellular content. Fluorescein in the cell extract was measured by fluorimetry (Perkin-Elmer LS 50B, λ<sub>ex</sub> = 519 nm, λ<sub>em</sub> = 491 nm). Cellular protein content was determined using a bicinchoninic acid protein determination kit (Sigma-Aldrich) adapted from a method used by Smith et al. (24).

**Photodynamic Treatment**

Cells were seeded into 96-well plates at a density of ~5 × 10⁴ per well. Following incubation for 48 h, the cells were washed with PBS and 0.1 mL solutions containing each compound at varying concentrations (0.005, 0.01, 0.05, and 0.1 mmol/L) were added to their designated wells for 5-h incubation periods. Each well plate contained six control wells without the compound and the compound

![Figure 3. Fluorescein fluorescence levels per milligram of protein after exposure to 0.6 mmol/L ALA (■), ALA-Me (□), and compound 1 (▲) for 2, 4, and 6 h and in control cells (○). Bars, SD.](image-url)
at five different concentrations in sextuplicate. The plates were irradiated with a fluence of 5 J/cm² using a LumiSource lamp (PCI Biotech), which emits a uniform field of low-power blue light over an area of 14 × 32 cm. Peak output is ~420 nm, which overlaps well with the PpIX Soret band. Immediately following irradiation, the medium was replaced and cells were incubated for a further 24 h. Cell cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: cells were incubated with medium containing MTT (1 mg/mL dissolved in full RPMI 1640) for 3 h. The insoluble end product (formazan derivatives) was dissolved in 0.1 mL DMSO after removing the medium. UV absorption was quantified at 570 nm using a 96-well plate reader (MR 700 Dynatech, Dynex). The mean cell survival was calculated for each prodrug at every concentration tested and expressed as a percentage of control (incubated with the compounds but not irradiated) cell survival values. For determination of “dark” toxicity of the compounds, well plates were prepared in the same manner as above but without irradiation. Bars, SD. C, phototoxicity after incubation with ALA (●), compound 1 (●), compound 1 (▲), compound 2 (●), and compound 3 (△) at a range of concentrations in PAM212 cell lines assessed by the MTT assay. Cells were incubated with the compounds for 5 h and irradiated with blue light (1.25 J/cm²). *, P < 0.05 (ALA versus other compounds). D, dark toxicity measurements. Cells were incubated with 0.05 (●) and 0.1 (▲) mmol/L ALA and compounds 1 to 3 for 5 h in the dark without irradiation. Bars, SD.

Figure 4. A, phototoxicity after incubation with ALA (●), ALA-Hex (▲), and compound 1 (●) at a range of concentrations in PAM212 cell lines assessed by the MTT assay: cells were incubated with the compounds for 5 h and irradiated with blue light (2.5 J/cm²). *, P < 0.05 (ALA versus other compounds). B, dark toxicity measurements. Cells incubated with 0.05 (●) and 0.1 (▲) mmol/L ALA, ALA-Hex, and compound 1 for 5 h in the dark without irradiation. Bars, SD. C, phototoxicity after incubation with ALA (●), compound 1 (▲), compound 2 (●), and compound 3 (△) at a range of concentrations in PAM212 cell lines assessed by the MTT assay. Cells were incubated with the compounds for 5 h and irradiated with blue light (1.25 J/cm²). *, P < 0.05 (ALA versus other compounds). D, dark toxicity measurements. Cells were incubated with 0.05 (●) and 0.1 (▲) mmol/L ALA and compounds 1 to 3 for 5 h in the dark without irradiation. Bars, SD.

Table 1. PpIX fluorescence and cytotoxicity of ALA, ALA-Hex, and compound 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>PpIX fluorescence enhancement compared with ALA (incubation during 6 h with 0.1 mmol/L prodrug)</th>
<th>Cell survival (% of control, incubation during 24 h with 1 mmol/L prodrug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>100 ± 2.07</td>
<td>70.6 ± 7.97</td>
</tr>
<tr>
<td>ALA-Hex</td>
<td>344 ± 20.8</td>
<td>2.8 ± 0.51</td>
</tr>
<tr>
<td>Compound 1</td>
<td>362 ± 14.4</td>
<td>44.3 ± 5.8</td>
</tr>
</tbody>
</table>

ALA Peptide Derivatives in Photodynamic Therapy

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concentrations and long incubation times, the cell survival after incubation during 24 h with 1 mmol/L prodrug was evaluated using the same protocol as described above.

**Tissue Skin Explant Studies**

**Spectroscopic Studies.** Porphyrin fluorescence induced by ALA or compounds 1 to 3 in tissue explants was carried out as documented previously (18). Fresh samples of shaved skin from female pig belly were laid on a small flat sieve in 5 cm diameter Petri dishes containing serum-free medium with or without prodrug at set concentration and incubated 5 or 24 h. Triplet samples of skin were incubated with 0.2 mmol/L ALA or derivatives together with control samples (no compound). Fluorescence emission spectra between 600 and 750 nm were recorded using a fiber-optic probe coupled to the Perkin-Elmer LS50B fluorimeter with a 410 nm excitation wavelength.

**Fluorescence Imaging.** Fluorescence imaging was done to visualize the PpIX biodistribution in pig skin after incubation with compound 1. Cryosections (20 μm) from samples prepared for the spectroscopy study were cut and stored at -20°C. Phase-contrast images and fluorescence images were recorded using a Peltier-cooled charge-coupled device camera (Pixis, 16-bit 512 × 512 pixels; Princeton Instruments; ref. 25). Fluorescence excitation was provided by a 10 mW cw laser at 532 nm. Control images were obtained using cryosections of pig skin incubated with medium alone and tested for any autofluorescence, which was found to be negligible.

**Statistical Analysis**

The results are displayed graphically or tabulated with error bars representing the SD. Differences are considered to be significant using the unpaired t test. Results with $P < 0.05$ were considered significant.

**Results**

**Fluorescence Pharmacokinetics**

The kinetics of porphyrin fluorescence induced by exposure to compound 1 and ALA in the PAM212 cell line are shown in Fig. 2A. Fluorescence spectra were consistent with the production of PpIX, and peak emission at 635 nm was used for the pharmacokinetic measurements. PpIX production using compound 1 or ALA exhibited a sigmoidal dose-dependent response between 0.001 and 0.1 mmol/L. Increasing porphyrin levels were observed with increasing incubation times from 2 to 24 h, with higher levels observed using compound 1: after 6 h of incubation, the PpIX fluorescence measured using 0.01 mmol/L of compound 1 was enhanced by a factor of 5 compared with ALA. For higher concentrations up to 1 mmol/L, no differences were observed between compound 1 and ALA. However, compound 1 was clearly more efficient compared with ALA with respect to PpIX generation over the lower concentration range for all incubation periods. In the case of compound 3, it induced higher fluorescence compared with ALA (Fig. 2B) but reduced fluorescence compared with compound 1 for the lowest concentrations tested. No porphyrin fluorescence signal was found when cells were incubated with the d-amino acid containing prodrug 2 in contrast to compound 1, which is the corresponding l-enantiomer.

**Internalization Mechanism of Compound 1**

The influence of active transport on the cellular uptake mechanism of compound 1 was investigated at low temperature. PAM212 cells were incubated with 0.1, 0.2, and 0.4 mmol/L ALA, ALA-Hex, and compound 1 at 4°C for 1 h, where active transport should be inhibited, compared with incubation at 37°C. After incubation with the compounds, the cells were washed and incubated for a further 3 h at 37°C to allow time for PpIX synthesis. Figure 2C shows that significant reductions in porphyrin levels were observed over a range of concentration for ALA and compound 1 using incubation at 4°C compared with 37°C. However, no significant difference was observed for ALA-Hex between incubation at 4°C and at 37°C.

**Esterase Activities**

Fluorescein levels in the medium at time 0 were measured to discard the hypothesis of spontaneous hydrolytic conversion of fluorescein diacetate to fluorescein. No significant fluorescence was found. The fluorescein levels (~95-230 fluorescence intensity/mg protein) reached in cells exposed to either ALA, ALA-Me, or compound 1 over 2, 4, and 6 h are shown in Fig. 3. In all cases, they

![Figure 5](https://example.com/figure5.png)

**Figure 5.** A, PpIX fluorescence from pig skin explants after 5 and 24 h incubation with (0.2 mmol/L) ALA (●) and compound 1 (■) and in control skin (□). Bars, SD. * P < 0.05 (ALA versus compound 1). B, phase-contrast and fluorescence images of pig skin cryosection (400 × 400 μm) after 5 h incubation with 0.5 mmol/L compound 1. SC, stratum corneum; E, epidermis; D, dermis. Bar, 50 μm for all photographs.
exceeded the fluorescein level in control cells, which was ~80 to 100 arbitrary units/mg protein. The intracellular esterase activity was similar for cells exposed to ALA or ALA-Me with a comparable fluorescence signal. However, when cells were exposed to compound 1, the levels of intracellular activity increased by a factor of 1.7 compared with ALA or ALA-Me.

**Photodynamic Treatment**

The phototoxicity after 5 h incubation with ALA, ALA-Hex, and compound 1 was investigated over a range of concentrations. The percentage of cell survival with respect to control cells (without compounds) was calculated for various concentrations of investigated compounds and plotted as shown in Fig. 4A. Compound 1 was more effective than ALA-Hex or ALA at low concentrations: <5% cell survival at a concentration of 0.005 mmol/L was observed compared with >60% for ALA-Hex and 100% for ALA. We estimated the LD₅₀ (at 2.5 J/cm²) values by interpolation, which gave 25 µmol/L for ALA, 6 µmol/L for ALA-Hex, and 2.6 µmol/L for compound 1. The “dark” toxicity of the compound 1 was also assessed (that is, its cytotoxicity without irradiation) as shown in Fig. 4B; the data indicate that no toxicity was found for ALA or compound 1 for the highest concentration (0.1 mmol/L) after 5 h incubation. Using long incubation times and high concentrations (1 mmol/L) of prodrug as shown in Table 1, compound 1 induced some toxicity compared with ALA or ALA-Me. The LD₅₀ values, compound 1 (2.5 J/cm²) at 50% cell survival were (in comparison with >60% for ALA-Hex and 100% for ALA). We estimated the LD₅₀ (at 2.5 J/cm²) values by interpolation, which gave 25 µmol/L for ALA, 6 µmol/L for ALA-Hex, and 2.6 µmol/L for compound 1. Although using shorter incubation times and lower concentrations (1 mmol/L) of compound 1 induced comparable levels of porphyrin with ALA-Hex. As shown in Table 1, ALA-Hex induced an enhancement of PpIX fluorescence of 344% and compound 1 an enhancement of 361% compared with equimolar ALA.

Irrespective of the incubation time, compound 1 induced higher porphyrin fluorescence in the skin explants than ALA by a factor of 3. The fluorescence intensities were 52 and 17 arbitrary units after 5 h and 111 and 34 arbitrary units after 24 h for compound 1 and ALA, respectively.

Figure 5B shows the compound 1 induced PpIX fluorescence biodistribution in cryosections of normal pig skin at 5 h after application of compound 1 at 0.5 mmol/L. Strong PpIX fluorescence is evident only in the epidermis, hair follicles, and keratin cysts. Weak PpIX fluorescence is visible in the upper dermis, decreasing toward the deeper dermis and muscle layers.

**Discussion**

A major challenge in ALA-PDT at the present time is the need to achieve more effective penetration of ALA into target tissues or the cells of pathogenic organisms. A limitation to the use of ALA in PDT results from its low lipid solubility, a consequence of its zwitterionic character at physiologic pH, which leads to poor penetration through biological barriers such as cellular membranes. This problem has partly been addressed through the synthesis of lipophilic ester prodrugs that provide improved cellular uptake and are metabolized into PpIX following the action of nonspecific intracellular esterases (26–30). A more recent development concerns the preparation of peptide-based ALA prodrugs, and we (18, 20) and others (11, 19) have described the synthesis and evaluation of short ALA peptide derivatives in which either the amino or the carboxyl function of the latter is masked, thereby providing improved physical properties and the potential for cell line–specific ALA release, according to which peptidases are expressed.

In this study, we prepared three compounds of the general structure phenylalanyl-ALA derivatives as potential ALA prodrugs, which are more lipophilic than ALA but are still water soluble (Fig. 1). Compounds 1 and 2, which are uncharged at physiologic pH, and compound 3, which has an ionizable carboxyl terminus, were evaluated against ALA. Based on computed log P values, compound 1 is predicted to be much more lipophilic than ALA but slightly more hydrophilic than ALA-Hex (Fig. 1B). However, one of the main advantages of compound 1 compared with ALA or ALA esters is its stability in solution and its reduced toxicity in vivo, as discussed later. ALA is only stable at acidic pH when the protonated amino group cannot undergo condensation reactions with the carbonyl moiety. The presence of the N-acetyl group at the amino terminus of compounds 1 to 3 also prevents decomposition of the derivative at physiologic pH. Consequently, these peptide derivatives do not need to be prepared and then dissolved as hydrochloride salts, unlike ALA. We have also prepared the corresponding non-acetylated derivatives, L-Phe-ALA-Me and the free acid L-Phe-ALA. However, these also show limited stability.

*Unpublished data.*
at physiologic pH presumably due to the cyclization of the amino-terminal amino group of phenylalanine onto the carbonyl moiety of the ALA residue, generating a six-membered Schiff base intermediate that may react further (19).

Fluorescence spectroscopy was employed to detect and quantify the PpIX accumulation induced by the compounds 1 to 3 versus ALA in the transformed keratinocyte PAM212 cell line. Porphyrin production was also quantified in skin explants exposed to the compound 1 to show its uptake and metabolism. Compound 1 as well as compound 3 were found to be capable of being taken up by cells and releasing ALA for subsequent conversion to PpIX. However, the \( \delta \)-enantiomer (compound 2) was not able to produce PpIX. The absence of porphyrin formation could be explained by the inability of intracellular peptidases to mediate cleavage of ALA from the \( \delta \)-amino acid–containing prodrugs. Alternatively, the \( \delta \)-enantiomer might be insufficiently internalized if it is not recognized by specific membrane peptide transporters in this cell line. High-performance liquid chromatography analysis of lysed material from cells treated with compound 2 confirms that cellular uptake does indeed occur, so stereospecific cleavage to release ALA appears to account for the bioactivity of the \( \delta \)-enantiomeric form, compound 1.

Following incubation with equimolar concentrations, PpIX generation after exposure to compound 1 was significantly higher compared with ALA at low concentrations, between 0.001 and 0.01 mmol/L (Fig. 2A). This result was also observed with compound 3 (Fig. 2B). However, better results were obtained with compound 1 compared with compound 3, the less lipophilic analogue with a carboxyl-terminal carboxylic function. In both cases, this improvement in porphyrin production must be due to more rapid and efficient internalization than ALA.

Following illumination of cells incubated with compound 1 or 3, cell survival was significantly reduced (Fig. 4A and C) compared with equimolar ALA, in good agreement with the pharmacokinetic studies. Indeed, the LD\(_{50}\) values were 25 and 2.6 \( \mu \)mol/L for ALA and compound 1, respectively. Moreover, in the absence of irradiation, no toxicity was found for ALA, compound 1, or compound 3 (Fig. 4B and D). In contrast, ALA-Hex did elicit dark toxicity and showed its uptake and metabolism. Compound 1 as well as compound 3 were found to be capable of being taken up by cells and releasing ALA for subsequent conversion to PpIX. The attainment of a steady-state level of intracellular fluorescein may imply that significant metabolic modification or degradation occurs, and the higher levels and fluctuation in intracellular fluorescein observed in cells exposed to ALA, ALA-Me, and compound 1 may reflect secondary effects on the degradation pathway, or possibly export. In the light of these experiments, we also compared the efficacy of ALA versus ALA-Me at a concentration of 0.6 mmol/L. Under these conditions, the PpIX content using compound 1 was 200% higher than that found using ALA; however, ALA-Me induced a 27% lower PpIX content after 4 h compared with ALA. The slightly lower efficacy of ALA-Me versus ALA has been noted previously (21) despite the greater lipophilicity of ALA-Me. However, in this work, we found that compound 3, with a free carboxyl group, was less efficient than the corresponding methyl ester (compound 1), so in this case the correlation of cell uptake with lipophilicity is not straightforward and may be attributable to different mechanisms of uptake for the peptide produgs compared with ALA and its esters.

Compound 1 and ALA were also compared in pig skin explant culture. Compound 1 produced higher porphyrin fluorescence in the explants than ALA (Fig. 5). This compound is therefore capable of being transported into skin and undergoing hydrolysis to liberate ALA. Extrapolation to \textit{in vivo} is complicated by properties of the stratum corneum, which presents an important barrier for penetration of ALA derivatives through skin (5). Several studies have shown that ALA-Hex is comparatively less effective than ALA for topical application on skin, although this limitation does not appear to apply for the bladder (31). Compound 1 produced higher porphyrin fluorescence in the skin explants than ALA by a factor of \( \sim 3 \) (Fig. 5A) for both exposure times (5 and 24 h). The porphyrin fluorescence was highest in the epidermis as we have observed previously using ALA in rat skin explants (16). These results also show that enzymes are available, as discussed further below, to metabolize the peptide produg in pig skin. In a previous study, Casas et al. (18), using a rat skin explant assay, evaluated other N-acylated amino acid derivatives; however, instead of an acetyl group a benzoylcarbonyl (Z) group at the amino terminus was used. Evaluation of a range of ALA derivatives and particularly the peptide produgs Z-Gly-ALA-Hex and Z-Gly-ALA-Et in rat and human skin explants showed that those compounds were able to induce PpIX production, but the degree of induction was less than the corresponding effect of ALA. However, Z-D-Phe-ALA-Et was able to enhance PpIX fluorescence compared with ALA, in
contrast to our present results. We therefore decided to repeat these experiments and synthesized both Z-t-Phe-ALA-Et and Z-o-Phe-ALA-Et. Neither compound was able to produce any PpIX in PAM212 cells (data not shown). However, in pig skin explants, some porphyrin production was observed, although the PpIX fluorescence signal was lower by a factor of 4 with Z-t-Phe-ALA-Et compared with ALA, and no porphyrin production at all was observed for the \( \alpha \)-enantiomer, which is consistent with our present findings. We presume that the production of porphyrins observed with the \( \alpha \)-enantiomer in the previous study must have been the result of contamination with the bioactive \( \alpha \)-enantiomer.

To examine the contribution of active transport to the uptake of compound 1, we conducted experiments in PAM212 cells at 4°C (Fig. 2C), because active transport and endocytic processes are blocked at low temperature (32–34). Porphyrin production was severely suppressed when cells were incubated with compound 1 at 4°C compared with 37°C, which is consistent with endocytic uptake. Because the molecular weight of compound 1 is <500, endocytic uptake is unlikely; therefore, the low temperature effect would be consistent with an active uptake mechanism being a major factor, as also found with ALA (22, 35). Further studies using specific inhibitors should enable identification of the active transport mechanism. Because compound 1 and the ALA-Hex derivative are more lipophilic than ALA, we would also expect that passive diffusion might be important. With the ALA-Hex, little suppression was observed using the lower temperature, thus indicating that passive diffusion is the predominant mechanism.

Berger et al. (11) have described the evaluation of several \( \alpha \)-amino acid-ALA derivatives and their PpIX production in different cell lines (rat endothelial EC212, human endothelial HCEC, and lung carcinoma A549 cells). Included in this study was Z-Phe-ALA-Me, which showed high yields of PpIX in all cell lines compared with other neutral and charged amino acid derivatives investigated. We have also observed PpIX production with this derivative in the PAM212 cell line, although it is less efficient than the corresponding amino-terminally acetylated derivative 1, which is moreover far more stable at physiologic pH (see above). Interestingly, Berger et al. also showed the involvement of aminopeptidase N/M activity in ALA release from their prodrugs. These enzymes, which are located both at the cell surface and in the cytoplasm, show a strong preference for substrates that contain neutral amino acid residues at the amino terminus. In agreement with our findings here, Berger et al. observed no PpIX production in cells from peptide derivatives in which the amino terminus was blocked with an alkoxycarbonyl group (\( \alpha \)-butoxycarbonyl). Such derivatives are not expected to be substrates for aminopeptidase N/M.

Because the N-acetyl derivatives 1 and 3 studied here should not be substrates for aminopeptidases, we therefore assume that aminopeptidases N/M are not involved in the mechanism of ALA release from these compounds in the PAM212 cell line. It is more probable that endopeptidases or exopeptidases, which can act on amino-terminal and/or \( N \)-substituted derivatives, are involved. For example, mammalian acylpeptide hydrolase (EC 3.4.19.1), a member of the prolyl oligopeptidase family of serine peptidases, is an exopeptidase, which removes acylated amino residues from the amino terminus of oligopeptides (36, 37), and could therefore be involved in the release of ALA in compounds 1 and 3 or related compounds.

In summary, the concept of using peptide derivatives as carriers for the delivery of ALA to tumor cells appears to be a promising method for inducing enhanced intracellular porphyrin production for PDT. Further studies using other amino acid residues and carboxyl- or amino-terminal substituents are in progress. Because peptidase expression is generally different between normal and tumor cells or tissues, there is the possibility of more specific targeting; for example, \( N \)-acetylaspartic aminopeptidase activity is higher in tumor cells compared with normal cells (37), although the activity is practically absent in A549 small cell lung carcinoma cells (38). Acidic, basic, or neutral aminopeptidase activities are also differently expressed in tumor vasculature when compared with normal vasculature of the same organ (39). The peptide derivative prodrugs described herein may be suitable for both topical use (e.g., for bladder and cervical cancers in addition to basal cell carcinomas) and systemic administration in view of their low inherent toxicity compared with the ALA hexyl ester. We have shown that the compound, which was most effective for cellular photosensitization in this study, was also effective for ALA delivery when incubated with pig skin. The efficient production of PpIX using relatively low concentrations of these peptide derivatives warrants further systematic study in vivo.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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