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The use of dipeptide derivatives of 5-aminolaevulinic acid promotes their entry to tumour cells and improves tumour selectivity of photodynamic therapy

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Conflicts of interest

The authors disclose no potential conflicts of interest

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Abbreviations

AcLeuALAMe: N-acetyl-leucinyl-ALA methyl ester; AcPheALAMe: N-acetyl-phenylalanyl-ALA methyl ester; APEH; acylpeptide hydrolase; ALA: 5-aminolaevulinic acid; He-ALA: ALA hexyl ester; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; PDT: photodynamic therapy; PpIX: protoporphyrin IX; SOT: skin overlying tumour.

Abstract

The use of endogenous protoporphyrin IX generated after administration of 5-aminolaevulinic acid (ALA) has led to many applications in Photodynamic Therapy (PDT). However the bioavailability of ALA is limited by its hydrophilic properties and limited cell uptake. A promising approach to optimize the efficacy of ALA-PDT is to deliver ALA in the form of prodrugs to mask its hydrophilic nature. The aim of this work was to evaluate the potential of two ALA dipeptides derivatives, N-acetyl terminated leucinyl-ALA (AcLeuALAMe) and phenylalanyl-ALA (AcPheALAMe), for their use in PDT of cancer, by investigating the generation of Protoporphyrin IX in an oncogenic cell line (PAM212-Ras), and in a subcutaneous tumour model. In our in vitro studies, both derivatives were more effective than ALA in PDT treatment, at inducing the same Protoporphyrin IX levels but at 50 to 100-fold lower concentrations, with the phenylalanyl derivative being the most effective. The efficient release of ALA from AcPheALAMe appears to be consistent with the reported substrate and inhibitor preferences of acylpeptide hydrolase (APEH). In vivo studies revealed that topical application of the peptide prodrug AcPheALAMe gave greater selectivity than with ALA itself, whereas systemic administration improved ALA-induced porphyrin generation in terms of equivalent doses administered, without induction of toxic effects. Our data support the possibility of employing particularly AcPheALAMe both for topical treatment of basal cell carcinomas, and for systemic administration. Further chemical fine-tuning of this prodrug template should yield additional compounds for enhanced ALA-PDT with potential for translation to the clinic.

Introduction

Photodynamic Therapy (PDT) is a non-thermal technique for inducing tissue damage with light following administration of a light-activated photosensitising drug which can be selectively retained in malignant or diseased lesions relative to normal adjacent tissue (1) (2, 3). In recent years, 5-aminolaevulinic acid (ALA)-mediated PDT has become one of the most promising fields in PDT research. ALA is the prodrug of the photosensitiser protoporphyrin IX (PpIX). After ALA administration, cells generate PpIX through the haem biosynthetic pathway. Clinically, when sufficient intracellular levels of PpIX are attained following either topical or oral ALA administration, the targeted tissue is irradiated with visible light to activate the sensitizer leading to generation of cytotoxic species and ultimately cell death. At a molecular level, this involves the interaction of the excited photosensitiser with molecular oxygen, leading to the generation of electrophilic

species (singlet oxygen and/or radicals) that cause oxidative damage to cellular constituents such as phospholipidic membranes, nucleic acids, and proteins (4).

The main advantage of ALA-induced PpIX relative to other photosensitisers is the short half-life of its photosensitising effects, which do not last longer than 48 h (5). Moreover, ALA also has great potential as a photodiagnostic or photodetection agent in clinical practice. The use of ALA-induced PpIX fluorescence is currently being exploited for diagnosis of bladder cancer, and intraepithelial lesions of the cervix, lung and brain cancer (6, 7, 8, 9, 10). Although ALA-PDT has already shown great potential both for the treatment of cancer and infectious diseases (10, 11), its efficacy is somewhat limited by the hydrophilic nature of the molecule, leading to poor penetration through certain malignant tissues. At physiological pH, ALA is a zwitterion, which severely impairs its ability to cross cell membranes via passive uptake, and may result in poor penetration and nonhomogeneous distribution in target tissues (12). In addition, saturation of heme synthesis as well as photobleaching of PpIX are also factors limiting the outcome of ALA-PDT (13, 14). To address these issues, a variety of ALA prodrugs have been investigated, incorporating specific chemical modifications that may provide enhanced uptake and hence higher PpIX production and thus photosensitisation (13). The conversion of ALA to ester prodrugs with enhanced lipophilicity has been extensively investigated, and various studies have demonstrated that esterification of ALA with both aliphatic linear and cyclic alcohols reduces the amount of ALA required for photosensitisation (14, 15, 16, 17, 18). Prodrugs of this kind, in particular methyl (Me-ALA) and hexyl ester (He-ALA) derivatives of ALA, have now been validated in a clinical setting, with regulatory approval being granted for the use of Me-ALA for the treatment of actinic keratosis in Europe and USA (19, 20), and basal cell carcinoma in Europe (20). He-ALA has also been approved in Europe for its use in fluorescence cystoscopy (21).

Other chemical approaches to enhance ALA-PDT have focused on increasing the payload of ALA that may be delivered by a single prodrug entity, as well as combining ALA administration with the use of other molecules that may boost PpIX production, by blocking downstream ferrochelatase-catalysed conversion of PpIX to haem. In the latter case, various iron chelators (22, 23, 24, 25) and chemotherapeutic agents (26) have been shown to be effective in enhancing levels of PpIX production upon administration of ALA. The use of ALA conjugated to first or second-generation

dendrimers also results in enhanced porphyrin synthesis with such prodrugs at low concentrations compared to an equivalent dose of ALA itself (27, 28, 29, 30).

An attractive way to obtain ALA prodrugs that have both improved physicochemical properties and can selectively release ALA in specific cell lines, is to incorporate ALA into a short peptide derivative (31, 32, 33, 34).

Upon cellular uptake, ALA release may be mediated by the action of cytoplasmic esterases and/or proteases. Following this approach, Giuntini et al (35) synthesized a range of ALA dipeptide derivatives that are uncharged at physiological pH, and are more lipophilic than ALA, yet retain adequate aqueous solubility. They are also stable at physiological pH, unlike ALA and its esters. The general structure of these compounds is shown in Figure 1, and they were demonstrated to be incorporated into the immortalized PAM212 cell line with significantly greater efficiency than ALA itself. Significantly, the efficiency of uptake of any given prodrug was not directly correlated with downstream PpIX production, showing that ALA release was mediated by a specific protease activity. Indeed, prodrugs containing a D-amino acid component are incorporated but not processed, while in cell lines such as A549 epithelial carcinoma that have a low expression of acyl-peptide hydrolase (a protease shown to release ALA from these compounds (36), efficient prodrug uptake, but low ALA release and PpIX production was observed. These encouraging *in vitro* results and studies in skin explant models (37) highlight the possibility to design peptide prodrugs of this type that can be used to target disease-specific levels of a given enzymatic activity to provide selective ALA release. In this context, it is important to evaluate the most promising of these prototypes in clinically relevant cancer cell lines and *in vivo* to assess the real potential of this novel strategy for ALA-PDT.

As shown in Figure 1, the ALA residue is conjugated with an amino acid via a peptide linkage and on the other side through an ester linkage, which are then cleaved enzymatically within the cells to liberate ALA. Importantly, the peptide is N-acetyl terminated. Such molecules are stable at physiological pH, unlike ALA, its esters, and dipeptide derivatives with a free amino terminus, but they may be fine-tuned in terms of their overall lipophilicity to favor passive uptake while still retaining water solubility, by variation of R′ (the side chain of the amino acid coupled to ALA) and the ester moiety R (35). We studied a set of dipeptide compounds and selected those which exhibited significantly enhanced phototoxicity in cells compared to ALA. Those results showed

that water-soluble peptide prodrugs of ALA can greatly increase its cellular uptake, generating more intracellular PpIX and improving tumour cell photosensitization. Moreover, they are not cytotoxic and are stable at physiological pH (37).

The aim of the present work was to investigate *in vivo* the properties of two ALA dipeptide prodrugs, N-acetyl terminated leucinyl (AcLeuALAMe) and phenylalanyl-ALA (AcPheALAMe), which have previously been shown to exhibit significantly enhanced cellular uptake and PpIX-induced phototoxicity compared to ALA. We employed the keratinocyte normal/tumour cell line pair PAM212 and PAM212-Ras as an *in vitro* model, and the mammary carcinoma cell line LM2 as an *in vivo* model of a subcutaneous transplantable tumour.

Materials and methods

Chemicals: ALA.HCl (MW= 167.59) and MTT were from Sigma Chem Co. AcLeuALAMe (MW= 300.35) and AcPheALAMe (MW= 334.37), were synthesised according to the method described in Rogers et al. (34) and Giuntini et al. (35). Stock solutions were prepared by dissolving at 10 mM in 0.01 M HCl and buffered with 0.01 M NaOH before use. He-ALA was synthesised according to the method of Takeya [36] by reacting ALA with hexanol in the presence of thionyl chloride. The mixture was stirred at 70^{o}C until ALA.HCl was completely dissolved and the reaction was confirmed by TLC (CH_2Cl_2:MeOH 9:1). The excess alcohol was evaporated under high vacuum. After addition of diethylether, the HCl salts of the ALA esters were allowed to crystallise at 4^{o}C.

Cell lines: PAM212 is an immortalized and spontaneously transformed cell line from a BALB/c primary keratinocyte culture (40). These cell lines were previously characterized genetically and morphologically, and have not been retested and authenticated in the present study. Retroviruses encoding oncogenic H-Ras$^{(V12)}$ with a neomycin resistance marker were produced and stably transfected into PAM212 cells as previously described (41). Controls for H-Ras presence were periodically carried out by Western blot assays. Cell lines were passaged three times when first obtained, and then frozen and stored at –80°C or in liquid nitrogen to provide low-passage cells to renew the stock as required. Our policy was to re-establish the line from frozen stock after a maximum of 20 weeks in culture. After cultivation in 400 µg/ml G418 (Calbiochem, Merck, Darmstadt, Germany) for 7 days, transfected cells were routinely grown in RPMI-1640 medium (Gibco BRL, Life Technologies Ltd, Paisley, UK)

containing L-glutamine 2 mM and phenol red, supplemented with 10% foetal calf serum and incubated at 37°C in an atmosphere containing 5% CO_2. Cells were used 48 h after plating.

PDT treatment: Cells were incubated in serum-free medium containing ALA or peptides and 3 h later, irradiations were performed. After irradiation, medium was replaced by ALA-free medium + serum, the cells were incubated for another 19 h and then tested for viability. Lethal light doses 50 (LD50) were calculated as mJ/cm^2 leading to 50% of cell viability.

MTT viability assay: Phototoxicity and cell viability was documented by the MTT assay (42), which is based on the activity of mitochondrial dehydrogenases. Following treatment, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) solution was added to each well in a concentration of 0.5 mg/ml, and plates were incubated at 37º C for 1 h. The resulting formazan crystals were dissolved by the addition of DMSO and absorbance was read at 560 nm.

Light source: a bank of 2 fluorescent lamps (Osram L 18W/765) was used. The spectrum of light was between 400 and 700 nm with the highest radiant power at 600 nm. The plates were located at a distance of 14 cm from the light source, and the cells were irradiated from below. The fluence rate was measured with a radiometer (model 65, Yellow Springs, OH, USA). We used fluences between 10 and 150 mJ/cm^2 and power density was 0.5 mW/cm^2

Porphyrin extraction from cells: Porphyrins accumulated within the cells were extracted twice with 5% HCl, leaving the cells standing for half an hour in the presence of the acid at 37°C. For media determinations, 5% HCl was added and measured directly. These conditions proved to be the optimal for total PpIX extraction. The fluorescence was determined using a Perkin-Elmer LS50B fluorescence spectrometer (excitation and emission wavelengths were 406 nm and 604 nm, respectively). PpIX (Frontier Sciences, Logan, Utah, USA) was used as a reference standard.

Animals: Male BALB/c mice 12 weeks old, weighing 20-25 g were used. They were obtained from the School of Sciences, University of Buenos Aires, and provided with food (Purina 3, Molinos Río de la Plata, Argentina) and water *ad libitum*. A suspension of 1.6 x 10^5 cells of the LM3 cell line was subcutaneously injected on the flanks of mice. Experiments were performed at approximately day 20 after implantation. Tumours of the

same uniform size were employed (1 cm diameter). Animals received human care and protocols were approved by the Argentinean Commitee (CICUAL, School of Medicine, University of Buenos Aires) in full accordance with the UK Guidelines for the Welfare of animals in Experimental Neoplasia (43).

ALA and dipeptides-ALA administration: ALA was dissolved in saline to a final volume of 0.15 ml immediately before intraperitoneal (i.p.) injection. For topical administration, ALA was dissolved in 0.2 ml saline immediately before use, whereas ALA-dipeptides were dissolved in HCl, then equimolar NaOH was titrated until pH 7 and the volume was completed with saline. ALA formulations were applied on the overlying the tumour (SOT), after shaving the hair and rubbing with a smooth paintbrush for a period of 5 min, a time at which no vestiges of lotion were visible. Two areas of skin were investigated: SOT, and normal skin taken from the opposite flank, denoted as 'distant skin'.

Porphyrin extraction from tissues: After ALA or ALA derivatives i.p. injections, animals were sacrificed. Before killing, mice were injected with heparin (0.15 ml, 1000 UI) and, after sacrifice, they were perfused with 200 ml of sterile saline. The organ samples were homogenised in a 4:1 solution of ethyl acetate: glacial acetic acid. The mixtures were centrifuged for 30 min at 3000 g, and the supernatants were added with an equal volume of 5% HCl. Extraction with HCl was repeated until there was no detectable fluorescence in the organic layer. The aqueous fraction was used for the measurement of porphyrins. For fluorimetric determination, a Perkin-Elmer LS50B fluorescence spectrometer was used, with an emission wavelength of 604 nm and an excitation wavelength of 406 nm, employing PpIX as the reference standard.

Fluorescence spectroscopy: *In vivo* fluorescence measurements were carried out for kinetics of PpIX formation after topical application of ALA or ALA peptides. A bifurcated fibre-optic probe was coupled to a Perkin-Elmer LS50B fluorescence spectrometer, and fluorescence from the tissue was detected at the probe tip. Excitation light at a wavelength of 407 nm was coupled into one arm of the birfurcated probe, enabling conduction of excitation to the skin surface and collection of the PpIX fluorescence emission via the other arm to the spectrometer. Taking into account the attenuation coefficient for skin, the 407 nm light penetrates deep enough into the skin to excite the PpIX in the epidermis and dermis (44). The fibre tip was fitted with a rubber spacer that ensured a constant fixed distance of 7 mm between the fibre and the tissue and provided optimal signal collection from the tissue. Fluorescence intensity was

measured as a function of time and expressed in arbitrary units at an emission wavelength of 635 nm. In addition, fluorescence emission spectra were measured to verify that the fluorescence signal corresponded to PpIX.

Fluorescence Imaging: was done to visualize the PpIX biodistribution of PpIX after topical application of ALA and AcPheALAMe. For recording of fluorescence micrographs of SOT + tumour, samples were frozen and 15 µm thick cryosections were prepared. Both samples and sections were always handled under subdued lighting conditions. Microscopic observation was performed in an Olympus photomicroscope BX51, employing the green set filters (545 nm, exciting filter BP 545), and photographs were taken employing a Q-color 5 camera. After fluorescence observation, samples were stained with haematoxylin and eosin.

PDT procedure and histological studies: ALA and ALA peptides at dose equivalents of 3 mg were applied topically on the SOT of tumour bearing mice, 3 hours before irradiation. Subsequently, animals were anaesthetized by i.p. injection of 70 mg/kg ketamine hydrochloride and 6 mg/kg xylazine and tumours were superficially irradiated. During illumination, normal tissue surrounding the tumour was shielded with a piece of black plastic, leaving exposed a peritumoral margin of 3 mm.

For tumour illumination, a diode InGaAsP 635 nm laser (Lumiia AccuraBeam, Argentina) was used. The light was focused into a 400-mm diameter optical fibre and the cut end of the fibre was positioned to provide a 1.5-cm diameter light spot, producing a treatment area of uniform intensity. The output power from the fibre was measured with a power meter (Fieldmaster LM-100XL with a LM3HTD sensor, Coherent, Auburn, CA). Total doses of 96 J/cm2 were delivered using a fluence rate of 80 mW/cm2 over 20 min. These light doses do not cause additional hyperthermic effects, which may influence the efficacy of PDT.

One day after PDT, mice were sacrificed and samples of tumours with their adjacent SOTs were excised, extended, sliced, fixed in 10% buffered formalin, embedded in paraffin, sectioned, stained with haematoxylin and eosin and examined by light microscopy under ×10 and ×40 magnifications employing a BX51 Olympus microscope, and photographs were documented with a Q-color 5 camera.

Statistical analysis: The values in the figures and tables are expressed as mean ± standard deviations of the mean. A two-tailed Student's t-test was used to determine statistical significance between means. To compare PDT responses between PAM212 and PAM212-Ras cells for each pro-photosensitiser, LD50s were calculated from the light dose response curves using using a three parameter logistic equation (GraphPad Prism, San Diego, CA, U.S.A.). LD50 values were calculated as the geometric mean ± 95% confidence intervals of the log-transformed curves. Statistical comparisons between LDs were made using the Student's t-test, and the null hypothesis rejected when $P < 0.05$

Results

We evaluated porphyrin synthesis from ALA and its derivatives in PAM212 and PAM212-Ras cell lines (Figure 2). After a 3 hr incubation period, the highest porphyrin levels employing the lowest concentration (6.1 ± 0.2 and 6.6 ± 0.5 ng porphyrins/10^5 cells for PAM212 and PAM212-Ras respectively) were obtained from AcPheALAMe at 0.01 mM concentrations. The amount of porphyrins obtained from AcLeuALAMe (4.8 ± 0.4 and 6.0 ± 0.4 ng porphyrins/10^5 cells for PAM212 and PAM212-Ras respectively) at 0.01 mM were also higher than those obtained from ALA (2.3± 0.2 and 2.6 ± 0.2 ng porphyrins/10^5 cells), which were comparable to the basal porphyrin values. The most efficient porphyrin synthesis was observed with AcPheALAMe, which was even better than the well known ALA ester He-ALA, inducing 4.9 ± 0.3 and 5.7 ± 0.6 ng porphyrins/10^5 PAM212 and PAM212-Ras cells respectively at 0.01 mM concentration.

Plateau values are obtained from the ALA peptides in the 0.01-0.05 mM range, whereas from He-ALA they are obtained at 0.05 mM and at 0.5 mM from ALA.

Overall, PAM212-Ras porphyrin values are higher than those observed using PAM212, for all concentration ranges and irrespective of the compound employed.

By employing longer incubation periods of 24 hr the profiles of porphyrins synthesis are similar to the 3 hr ones. With either ALA or ALA derivatives, the amount of tetrapyrroles formed from the Ras-transfected cell line is still significantly higher as compared to the parental cell line. Plateau values are obtained for all the pro-drugs and both cell lines, at higher concentrations as compared to 3 hr incubation, due to substrate consumption.

In line with the patterns of porphyrin synthesis, Figure 3 shows that the higher the amount of porphyrins, the higher the photocytotoxicity for a given dose comparing the 4 compounds within the same cell line. Overall, PAM212-Ras cells are more resistant to PDT in spite of synthesising higher amounts of porphyrins.

PDT was applied after 3 h incubation of pro-drugs at 0.01 mM, which is a plateau concentration for ALA derivatives but not for ALA. At this concentration, phototoxicity from ALA is almost negligible, whereas from all ALA derivatives the LD50s were around 135 mJ/cm^2 for PAM212-Ras cells, and 45 mJ/cm^2 for PAM12 cells. Under these conditions, comparing the corresponding LD50s for each pro-photosensitiser, PAM212-Ras cells were significantly less responsive to ALA derivatives PDT-treatments as compared to the parental line.

Employing a plateau concentration for both ALA and derivatives (1 mM), LD50s are about 45 mJ/cm^2 for PAM212-Ras and in the range of 20 to 30 mJ/cm^2 for PAM212 cells These LD values are still significantly higher in Ras-transfected cells for AcPheALAMe and He-ALA, but not significantly different for ALA and the AcLeuALAMe derivative.

By means of fibre-optic fluorescence detection, we analyzed porphyrin formation as a function of time after topical application of ALA and ALA-dipeptides on the skin overlying the tumour (SOT) of implanted mice (Figure 4). ALA induces similar profiles of porphyrin production in SOT as compared to distant skin, this being a consequence of rapid diffusion of the molecule to other sites, immediately after application. On the other hand, although AcPheALAMe induced lower amounts of porphyrins in both skin types as compared to ALA, selectivity for SOT is statistically improved at all time points (all, $p< 0.05$, t Sudent´s test). The ratio between SOT and distant normal skins is about 1.45 for the phenylalanyl dipeptide derivative, thus showing some confinement of the ALA peptide molecule to the site of application. On the other hand, AcLeuALAMe is less efficient for porphyrin generation, producing in both skin types porphyrin levels which are 4 times lower than those obtained employing ALA (differences between SOT and distant skins are not statistically significant). It is worth noting that in this system of superficial fluorescence detection, porphyrins confined to the most superficial layers of SOT invaded by tumour are mainly detected since blue light is used for excitation.

Table 1 describes the amount of porphyrins formed 3 h after topical application of ALA and ALA peptides in tumour, SOT and distant skin, quantified after chemical extraction of the different tissues at plateau times (see Figure 4). Whereas both tumour and SOT porphyrins synthesised from ALA and AcPheALAMe are similar, distant skin values are significantly lower for the latter (p=0.046), which represents a more selective tumour localization. On the other hand, the three tissues produce significantly lower porphyrins from AcLeuALAMe as compared to ALA, without any increased selectivity.

Figure 5 describes the biodistribution of PpIX in SOT after topical application of ALA and AcPheALAMe. Whereas PpIX fluorescence from ALA is typically highest in the epidermis and hair follicles, its distribution from the phenylalanyl derivative appears to be less marked in the epidermis but strongly accumulated in hair follicles.

Figure 6 shows the H&E stained sections of control and ALA or AcPheALAMe topically treated subcutaneous tumours, illuminated, and excised 24 hours after treatment.

The histological response to PDT did not show large differences in the type or death distribution response between ALA and its derivative. The stratum corneum has partly or completely detached from the epidermis and we also observed pyknosis of epidermal cells, independent of the PpIX precursor employed. Within the dermis, collagen fibres appeared to be thickened and the presence of fibrosis and oedema became evident.

The fat cells in the lower dermis were disorganised, and significant damage to hair follicles was found. The cutaneous musculature was preserved. Epidermis but not dermis appears to be more preserved in the ALA-peptide treated SOT as compared to ALA.

Necrosis of tumour cells was observed, with similar extension and depth of tumour damage in all the treatments.

Figure 7 shows kinetics of porphyrin synthesis in a set of organs, after systemic application of AcLeuALAMe and AcPheALAMe at dose equivalents of 120 mg/kg ALA.

Most tissues peak around 1 and 5 hr after ALA dipeptide administration. Liver is the only tissue that does not clear all the porphyrins synthesised from both molecules before 24 h. In terms of maximal porphyrin accumulation, AcPheALAMe produces the highest values in all the tissues analyzed, around 20% higher than for AcLeuALAMe.

Whereas kidney and intestine are the best porphyrins producers, the brain is the lowest. With respect to selectivity, neither of the ALA peptides gave rise to high porphyrin tumour levels relative to the skin or any other tissue.

Figure 8 shows a comparison of porphyrin synthesis in all tissues 3 h post systemic administration of AcLeuALAMe and AcPheALAMe at dose equivalents of 40 and 120 mg/kg of ALA. AcPheALAMe induces significant more porphyrins in tumour as compared to ALA and the other peptide (p= 0.0005 and p=0.0006) at 120 mg/kg, whereas in SOT and distant skin, the amount is similar as compared to ALA. The amount of tetrapyrroles induced by the leucyl derivative is similar to ALA 120 mg/kg in tumour and skin but significantly lower in SOT (p= 0.018). As for the rest of tissues, the amount is similar for the three compounds.

Discussion

A relatively recent development in ALA-PDT concerns the preparation of peptide-based ALA prodrugs, and we (34, 35, 45) and others (32, 33) have described the synthesis and evaluation of short ALA peptide derivatives in which either the amino or the carboxyl functions of the latter are masked, thereby providing improved physical properties and the potential for cell line-specific ALA release, according to which peptidases are expressed.

The PAM212-Ras cell line synthesizes more porphyrins from ALA than its counterpart PAM212 cell line. However, it is more resistant to PDT, particularly when low pro-photosensitiser concentrations are used. This feature has been previously observed by our group for the mammary cell line pair HB4a and HB4a-Ras (46), showing that overexpression of Ras oncogen confers resistance to ALA-PDT, independent of the amount of porphyrins synthesised. We were therefore intrigued to explore the effectiveness of prodrugs that are proven to perform significantly better than ALA in normal cell lines in this clinically relevant oncogenic system.

Ras proteins play a direct causal role in human cancer and in other diseases. Mutant H-Ras, N-Ras, and K-Ras occur in varying frequencies in different tumour types, for reasons that are unknown account for 20% to 30% of all human tumours (38, 39). Other members of the Ras superfamily may also contribute to cancer. Proteins in the

Ras family are very important molecular switches for a wide variety of signal pathways that control processes such as cytoskeletal integrity, proliferation, cell adhesion, apoptosis, and cell migration.

In our *in vitro* studies, the ALA derivatives did indeed prove to be more effective than ALA in PDT treatment, inducing the same porphyrin levels but at 50 to 100-fold lower concentrations. Employing a concentration of 0.01 mM, the amount of porphyrins is 2 and 3 times higher for AcLeuALAMe and AcPheALAMe respectively as compared to ALA. In addition, the performance of these peptides is even better than the best ALA ester porphryin inducer He-ALA, reaching plateau values at 5-fold lower concentrations. This improvement in porphyrin production must be due to more rapid and efficient internalization than ALA. Following illumination of cells incubated with the compounds at low concentrations (0.01 mM and 3 h exposure), LD50s were around 135 mJ/cm^2 for PAM212-Ras cells, and 45 mJ/cm^2 for PAM12 cells, whereas no cell death was induced by ALA.

In a complementary *in vivo* study using the mammary carcinoma cell line LM2, our results showed that topical application of the peptide prodrug AcPheALAMe is more selective than ALA, whereas systemic administration improves ALA performance, without changes on selectivity. It is worth noting that the concentrations employed for both ALA peptides, that led to tumour-photosensitising porphyrin synthesis (31) did not produce any toxic effects in mice. Through fibre optic detection of porphyrins, we have observed that after topical application, ALA induced rapid diffusion of porphyrins to other distant sites within the skin, but this was not observed for the ALA peptides. Such a phenomenon has been previously remarked upon by us, along with a higher confinement of porphyrin synthesis from He-ALA to the application site (47). In our previous studies, a similar amount of porphyrins from ALA was synthesized in distant papillomas when compared with ALA-applied papillomas, demonstrating that either ALA itself and/or the porphyrins formed by synthesis can be distributed throughout the vasculature and accumulate in distant tumours, and though it is likely that ALA is the molecule diffusing to distant sites, we cannot discard the contribution of hydrophilic precursors of PpIX such as Uroporphyrin and Coproporphyrin.

On the other hand, lower porphyrin synthesis occurred in distant papillomas from He-ALA, showing that this ester and/or porphyrins formed from the precursor, are more selectively localized in the site of application. Similarly, when Peng et al. (48) applied

ALA and other ALA esters (methyl, ethyl and propyl) to normal mouse skin they found no porphyrins using fluorescence detection in areas other than that in which the ALA ester cream was applied, while in the case of ALA, a significant fluorescence was seen in the skin outside the application area. These topically applied ALA prodrugs may be retained by *stratum corneum*, which may act as a reservoir (49), from which they diffuse superficially, leading to a pattern of skin porphyrin accumulation dependant on the distance from the application site.

For the novel prodrugs studied, it is possible that the ester moiety of the AcPheALAMe peptide is therefore responsible of the retention of the fluorescence to the site of application, with a greater extent of diffusion to the dermis as compared to ALA. In addition, the confinement to the site of application achieved by the use of ALA esters and ALA peptide esters, demonstrates that ALA, and not porphyrin, is the main molecule likely to be distributed through the bloodstream. This is supported by the tissue biodistribution and porphyrin extraction data, which demonstrates that both ALA and the ALA peptides are more specifically confined to the skin invaded by tumour (SOT) where the topical application was performed. As regards to biodistribution, AcPheALAMe appears to be less retained in the epidermis, but traffics to the dermis. As has been addressed previously, the more lipohilic solutes or vehicles penetrate to a higher depth through the skin as compared to the most hydrophilic ones (Bronaugh, Dokka). This pattern of PpIX distribution correlates to the extent of photodamage after illumination. Similar histological changes were reported for both ALA and its phenylalanil peptides in terms of dermal damage and depth of tumour necrosis, though epidermal damage appears to be more subtle in ALA peptide-treated SOT.

There were no differences on SOT: tumour indexes between ALA and AcPheALAMe, however, there was indeed a selectivity of AcPheALAMe for tumour and tumour skin areas, since lower distribution to distant sites through skin and tumour vasculature was obtained. This is quite relevant, since overcoming ALA-PDT induced photosensitivity, although it lasts only for 48 h, has been addressed previously by use of protective ointments (Juzenas).

The lack of any apparent toxicity when using systemically administered ALA, is encouraging. We have also found no apparent toxicity in Wistar rats upon intravenous administration of the ALA peptides at doses of 100 mg/kg (unpublished data). On the contrary, it has been reported that ALA esters containing large alkyl chains such as

ALA Hexyl ester cannot be administrated systemically due to its high toxicity. Such toxicity was ascribed to be related to the ability of lipophilic esters of ALA of crossing blood brain barrier thus inducing neurotoxicity (50). In addition, in the mentioned work we have found that He-ALA was significantly less efficient in most tissues than ALA at producing porphyrins after i.p. administration. In the present work, we have found that 3 h after systemic administration of ALA peptides, we have obtained 1.22 ± 0.15 and 0.81 ± 0.09 µg porphyrins/g tumour tissue from AcPheALAMe and AcLeuALAMe respectively, whereas after dose equivalent of ALA Hexyl ester (50), the amount of tetrapyrroles accumulated were 0.3 ± 0.02 µg/g, value which was slightly above basal tumour levels.

It has been previously shown that AcPheALAMe and AcLeuALAMe are efficient at inducing PpIX in PAM212 cells and in pig skin explants (35, 37). N-acetyl termination appears to play an important role in metabolic processes leading to the production of PpIX from such compounds whereas masking the C terminus as a methyl ester does not exert a major effect (36). Using siRNA knockdown of acylpeptide hydrolase (APEH) protein expression, we were able to show the involvement of APEH, a member of the prolyl oligopeptidase family of serine peptidases, in the release of ALA from these N-acetylated peptide derivatives (36). APEH has been shown to be expressed in various normal tissues such as erythrocytes, liver, heart, testis, intestine, kidney and brain, but its precise biological activity is unknown (51, 52, 53, 54). In addition, peptidase expression can be different between normal and tumour cells or tissues (55) and aminopeptidase inhibitors have been designed to target tumour cells (56, 57, 58).

In the present study, the differences between AcPheALAMe and AcLeuALAMe *in vivo* may be ascribed in part to a higher ability of the former to cross biological barriers such as skin after topical application by passive diffusion, as a function of its greater lipophilicity (clogP -0.66 vs -1.10). For the case of systemic application, differential levels of peptidase activity from each tissue may favour more efficient release of ALA from AcPheALAMe compared to AcLeuALAMe, in tissues such as tumour, liver, skin and kidney. The poorer performance of AcLeuALAMe upon topical application suggests that there is an optimum lipophilicity for prodrugs of this type that is required in order to effectively cross a biological barrier such as the skin. Probably the use of different vehicles could improve the performance of this derivative for PDT applications.

In addition, although there is scarce information on the uptake mechanisms of ALA dipeptides, differential expression of proteins involved in this process may be also playing a role in its bioavailability *in vitro* and in systemic administration. Several carriers have been pointed as potential candidates for transport of ALA and its derivatives, including the dipeptide transporters PEPT1 (Döring et al. 1998) and PEPT2 (Döring et al. 1998; Kamal et al. 2008), and the amino acid carrier PAT1 (Anderson et al. 2010).

In previous work we have shown that both AcPheALAMe and AcLeuALAMe penetrate PAM212 cells more easily than ALA itself (Giutini 2009) and that ALA dipeptides entry to the cells is partly driven by passive transport, whereas He-ALA, which is more lipophilic, penetrates mainly through passive diffusion (Bouree, 2008). In addition, it is also known that He-ALA interacts with PEPT1 and PEPT2 transporters (Rodriguez et al., 2006).

PEPT1 and PEPT2 serve as integral membrane proteins for the cellular uptake of di- and tripeptides in the organism. PEPT1 is the low-affinity, high-capacity transporter and is mainly expressed in the small intestine, whereas PEPT2 is the high-affinity, low-capacity transporter and has a broader distribution in the organism (Rubio Aliaga, 2008). Thus, we assume that PEPT2 may be relevant for ALA dipetide uptake in tissues such as such as kidney, mammary gland, brain or lung whereas PEPT1 and PAT1 may be relevant to transport only in small intestine. Whereas PEPT1 mRNA was increased 2.3-fold in cancer tissues (Anderson, 2010), to the best of our knowledge, there are no clear reports about overexpression of PEPT2 or PAT1 neither in tumours nor in Ras-overexpressing cell lines.

The N-terminal blocking of proteins occurs in a wide range of eukaryotic cells, where more than 50% of the cytosolic proteins can be *N*-α-acetylated. The acetylation which occurs during or after the biosynthesis of the polypeptide chains serves to protect the intracellular proteins from proteolysis. Food processing can also generate *N*-α-acetylated proteins and peptides. The mechanism underlying the intracellular catabolism of *N*-acetylated proteins has not yet been elucidated, however. It is generally assumed that two enzymes are involved in the hydrolysis of the N-terminal part of such proteins. The N-terminally acetylated peptides generated during proteolysis may be first cleaved by an APEH. This releases the N-terminal amino acid, which is in turn deacetylated by an aminoacylase (59). It is worth noting here that the amino acid residues which are usually found at the N-terminus of acetylated proteins include Ac-Met-, Ac-Ala- and Ac-Ser- (60). This is in accordance with our previous results showing that one of our better performing peptides was Ac-Met-ALA-Me (36).

Ac-Leu- and Ac-Phe-ALA- dipeptides are even better PpIX producers than Ac-Met-ALA-Me (36). The highly efficient release of ALA from Ac-Leu- and Ac-Phe- however appears consistent with the reported substrate and inhibitor preferences of APEH, which favours N-acetylated substrates with hydrophobic amino acid residues, and small molecule inhibitors with aromatic substituents as side chain mimics. For example, Ac-Phe-OH was shown to induce 69% inhibition of APEH, as compared to 42 and 46% inhibition induced by its natural substrates Ac-Ala and Ac-Met (60), while Ac-Leu-p-nitroanilide is an efficient substrate for mammalian APCH's (61).

The main clinical application of ALA-PDT has been in the treatment of nonmelanomas skin lesions, mainly for basal cell carcinoma using topical application, although treatment of high-grade dysplasia in Barrett's esophagus, cervix neoplasias, and oral cavitiy, cancer have been recently developed (Pech 2005, Mallia 2010, Barnett 2003), as well as in the fluorescence-guided resection of bladder cancer, and intraepithelial lesions of the cervix, lung and brain cancer (6, 7, 8, 9, 10). PDT can virtually be applied to any type of localized cancer which is accessible by optic fibre, and although PDT constitutes an important therapy in dermatology, the challenging delivery of ALA by using dipeptides, hampers the translation of ALA-PDT to other oncological applications. Our results of PpIX accumulation after intraperitoneal administration suggests that systemically applied AcPheALAMe reaches every organ analysed and particularly accumulates in tumour tissue. On the other hand, our results of topical application of ALA dipeptides, highlights the ability of the Phenylalanine derivative to be confined in the area of application, cross the stratum corneum barrier, and reach the dermis and adjacent tumour tissue, with minimal dispersion to distant tissues. Due to the lack of animal skin cancer models resembling human tumour, our subcutaneous implanted tumour model is a first approach before translating the derivative to clinical applications.

In conclusion, the results in this study provide further evidence that the conjugation of ALA with specific amino acids is a promising approach for inducing enhanced intracellular porphyrin production for photodynamic therapy. Our *in vivo* studies support the possibility of employing particularly AcPheALAMe both for topical, for treatment of basal cell carcinomas, and systemic administration. Further chemical fine-tuning of this prodrug template should yield additional compounds for enhanced ALA-PDT with potential for translation to the clinic.

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FIGURE LEGENDS

Figure 1: Structures of ALA and AcLeuALAMe and AcPheALAMe dipeptides

Figure 2: Porphyrin synthesis from ALA or ALA derivatives in PAM212 and PAM212-Ras cells

Cells were exposed to different concentrations of ALA or ALA derivatives during 3 h or 24 h. Intracellular porphyrin levels were determined fluorometrically and normalised per number of cells present at the end of the experiment.

Figure 3: Cell survival after PDT

Cells were incubated with 0.01 mM or 1 mM ALA or ALA derivatives during 3 h. Afterwards, PDT was performed, and cell viability was evaluated by the MTT assay, as percentage of control non-irradiated cells.

Figure 4: Porphyrin synthesis in skin over the tumour and distant skin after topical application of ALA and ALA peptides

ALA and ALA peptides were applied topically to the SOT at dose equivalents of 3 mg ALA. At different times after application, the porphyrin fluorescence was monitored by a fibre optic probe coupled to a fluorescence spectrometer. Determinations were made on skin overlying the tumour and over a distant skin area. We show here the average of three mice per treatment.

Figure 5: Fluorescence images and H&E stained images of SOT and adjacent tumours topically applied with ALA and AcPheALAMe

ALA and ALA peptide were applied topically to the SOT at dose equivalents of 3 mg ALA and sacrificed 3 hr after application. Sections were photographed employing the 10X objective.

Figure 6: H&E stained images of SOT and adjacent tumours treated with topical ALA or AcPheALAMe-PDT

ALA and AcPheALAMe were applied topically to the SOT at dose equivalents of 3 mg ALA 3 h before illumination. Treated SOT + tumours were excised 24 hr after the end of illumination. Sections were photographed employing the 10X (left panel) and 40x (right panel) objective. Controls: mice illuminated without previous pro-drug application.

Figure 7: Kinetics of porphyrin synthesis in tissues after AcLeuALAMe and AcPheALAMe intraperitoneal (i.p.) administration

AcLeuALAMe and AcPheALAMe were injected i.p. at a dose equivalent of 120 mg/kg of ALA and after different times, porphyrins were extracted from the tissues and quantified. Three mice per point were used.

Figure 8: Comparison of tissue synthesis from ALA and ALA peptides after systemic administration

AcLeuALAMe and AcPheALAMe was injected i.p. at dose equivalents of 40 and 120 mg/kg of ALA. Three hours after injection, porphyrins were chemically extracted and determined in tissues. Three mice per point were used. * $p < 0.05$ as compared to ALA.

TABLES

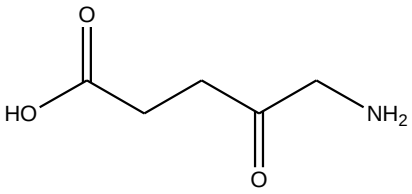
Table 1: Porphyrin accumulation (µg porphyrin/g tissue) determined by chemical extraction in tumour, skin overlying the tumour, and a 'distant' skin site after topical application on SOT of 3 mg ALA or dose equivalents of ALA peptides

	Tumour	SOT	Skin
ALA	0.21 ±0.03	0.54±0.09	0.44±0.13
AcLeuALAMe	0.14±0.01*	0.25±0.03*	0.12±0.04*
AcPheALAMe	0.21±0.03	0.44±0.09	0.22± 0.04*

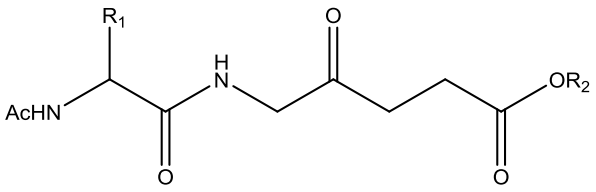
*p<.0.05 compared to same tissue exposed to ALA.

FIGURES

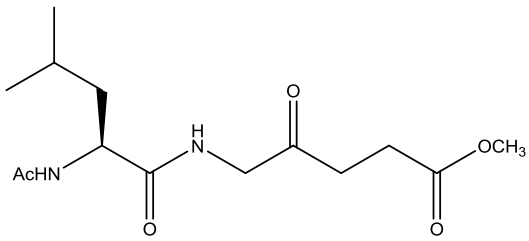
Figure 1



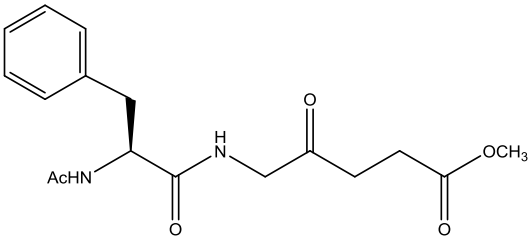
ALA



Generic structure of dipeptide ALA prodrugs



AcLeuALAMe



AcPheALAMe

Figure 2

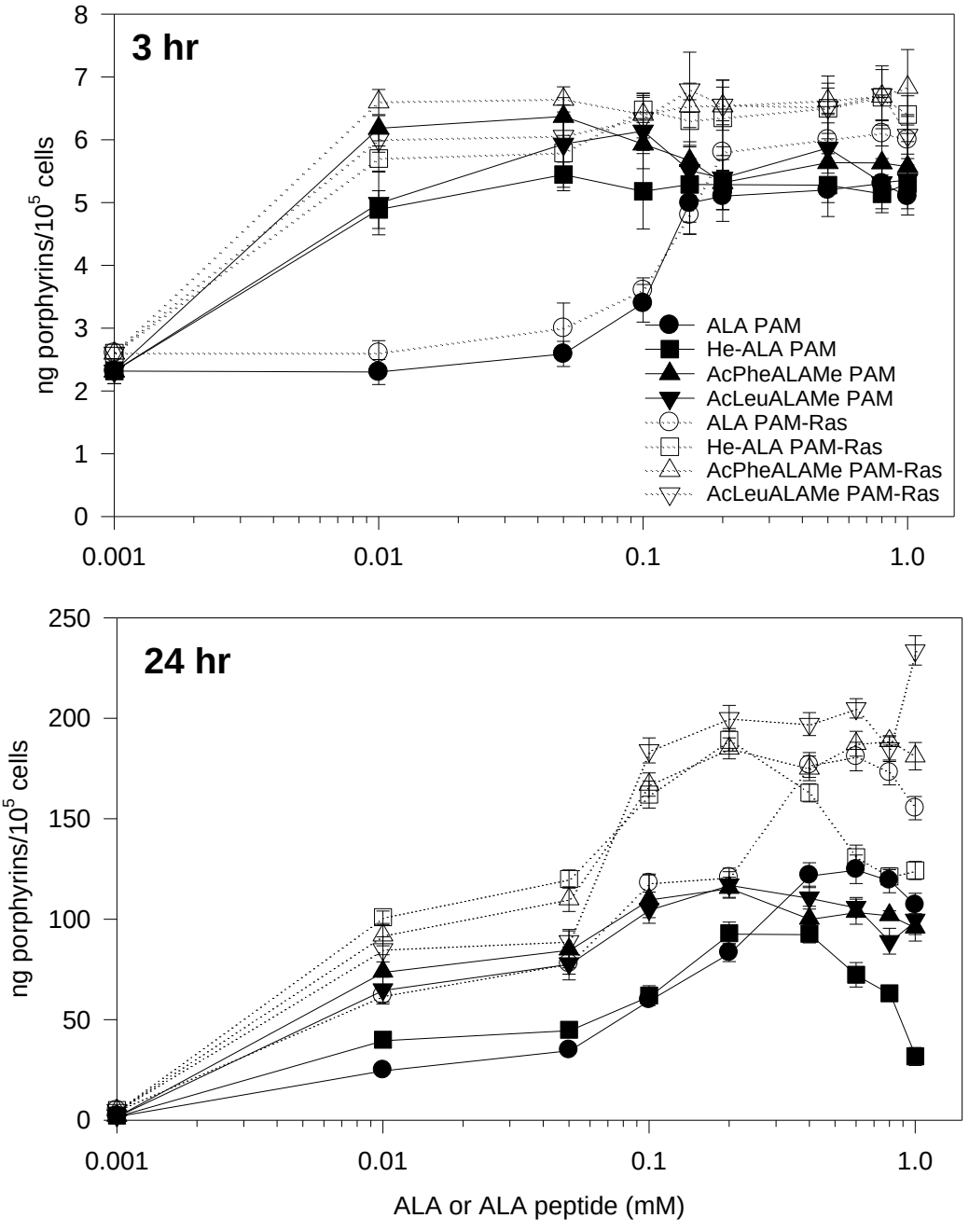

3 hr
ng porphyrins/10^5 cells
ALA PAM
He-ALA PAM
AcPheALAMe PAM
AcLeuALAMe PAM
ALA PAM-Ras
He-ALA PAM-Ras
AcPheALAMe PAM-Ras
AcLeuALAMe PAM-Ras
24 hr
ng porphyrins/10^5 cells
ALA or ALA peptide (mM)

Figure 3

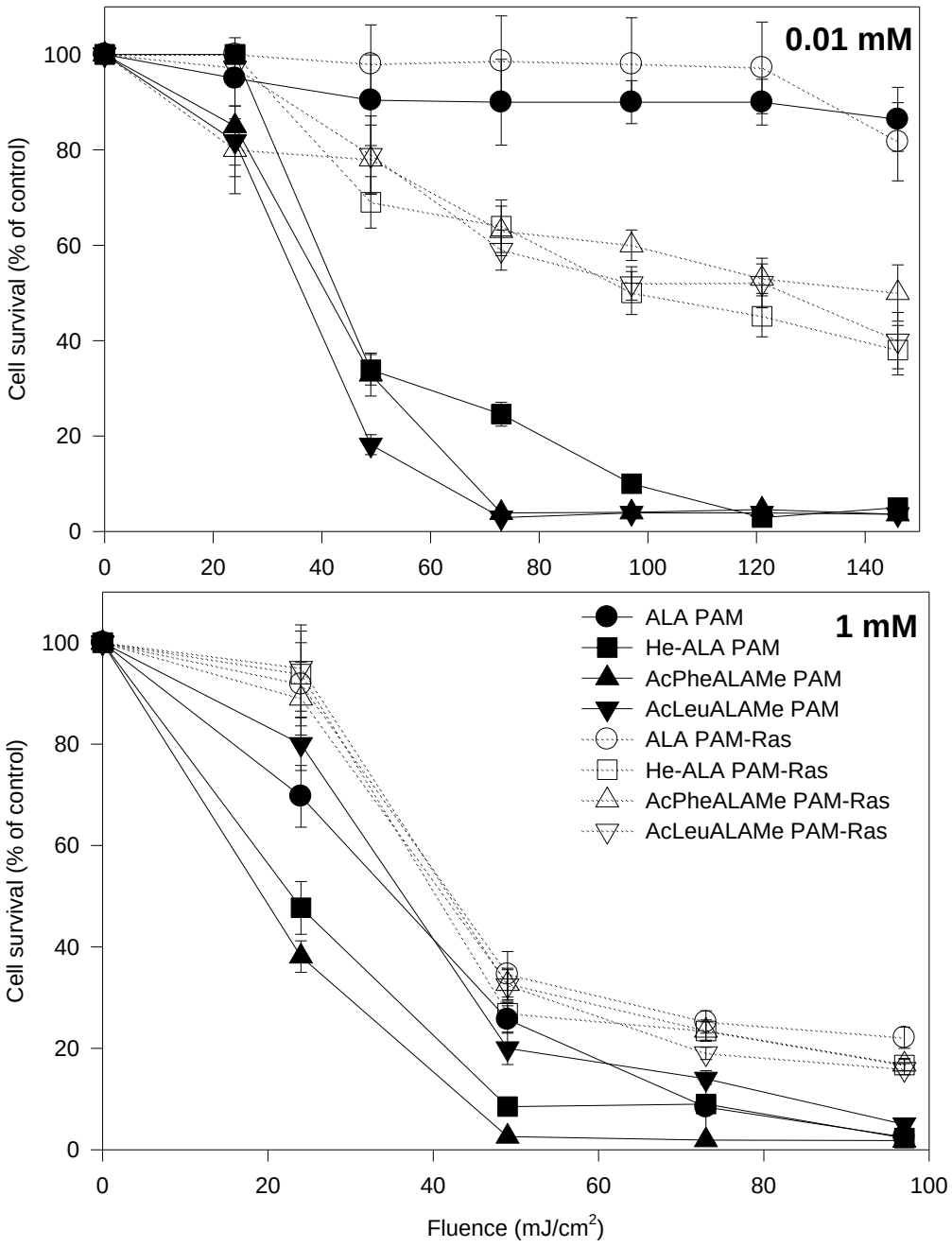

0.01 mM
1 mM
Cell survival (% of control)
Fluence (mJ/cm2)
ALA PAM
He-ALA PAM
AcPheALAMe PAM
AcLeuALAMe PAM
ALA PAM-Ras
He-ALA PAM-Ras
AcPheALAMe PAM-Ras
AcLeuALAMe PAM-Ras

Figure 4

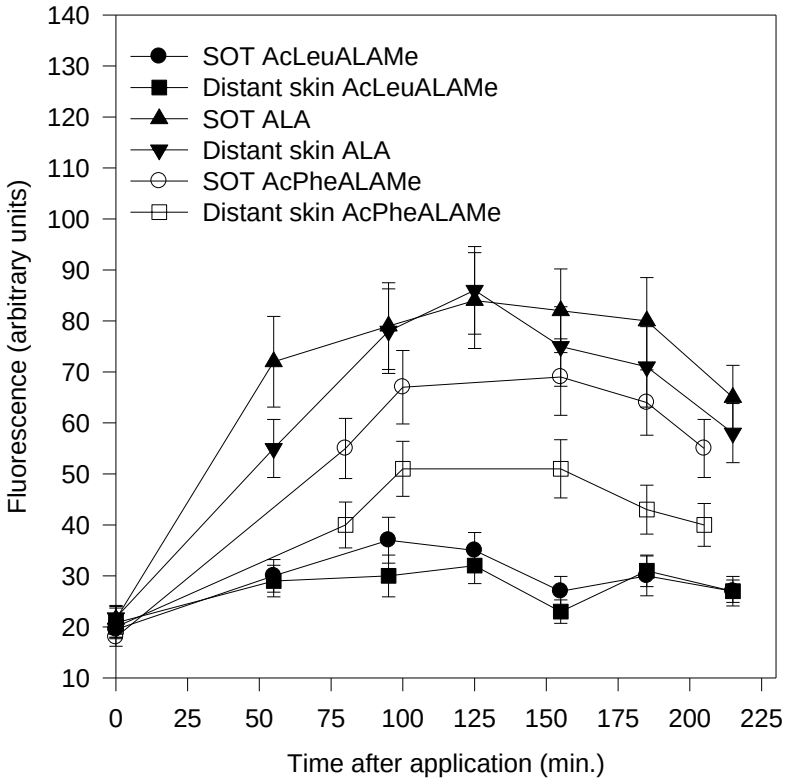

SOT AcLeuALAMe
Distant skin AcLeuALAMe
SOT ALA
Distant skin ALA
SOT AcPheALAMe
Distant skin AcPheALAMe
Fluorescence (arbitrary units)
Time after application (min.)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
25
50
75
100
125
150
175
200
225

Figure5

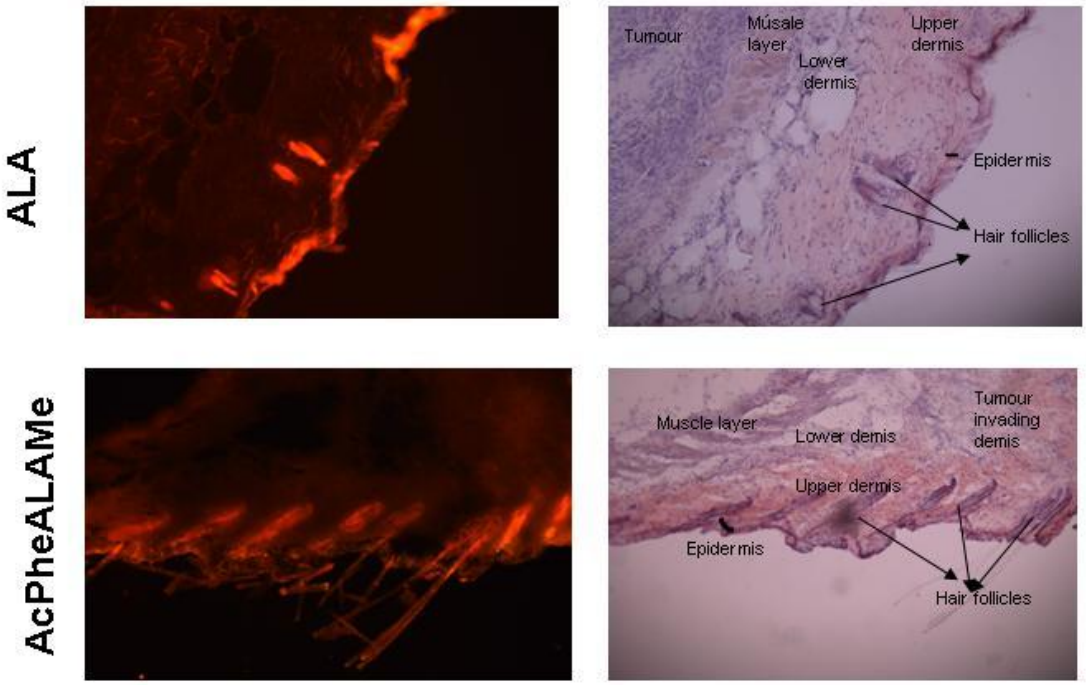

ALA
Tumour
Músale layer
Lower dermis
Upper dermis
Epidermis
Hair follicles
AcPheALAMe
Muscle layer
Lower dermis
Tumour invading dermis
Upper dermis
Epidermis
Hair follicles

Figure 6

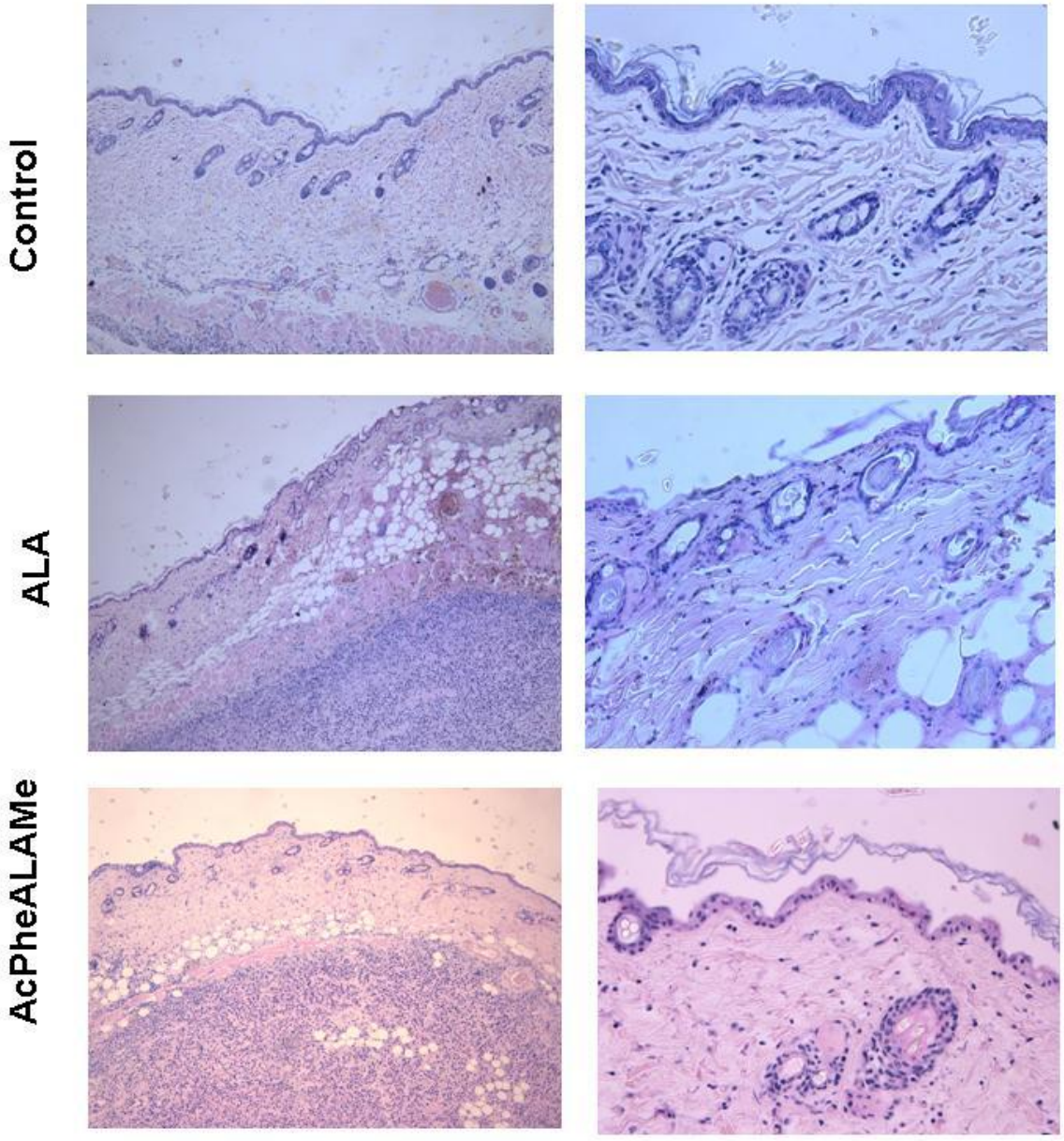


Figure 7

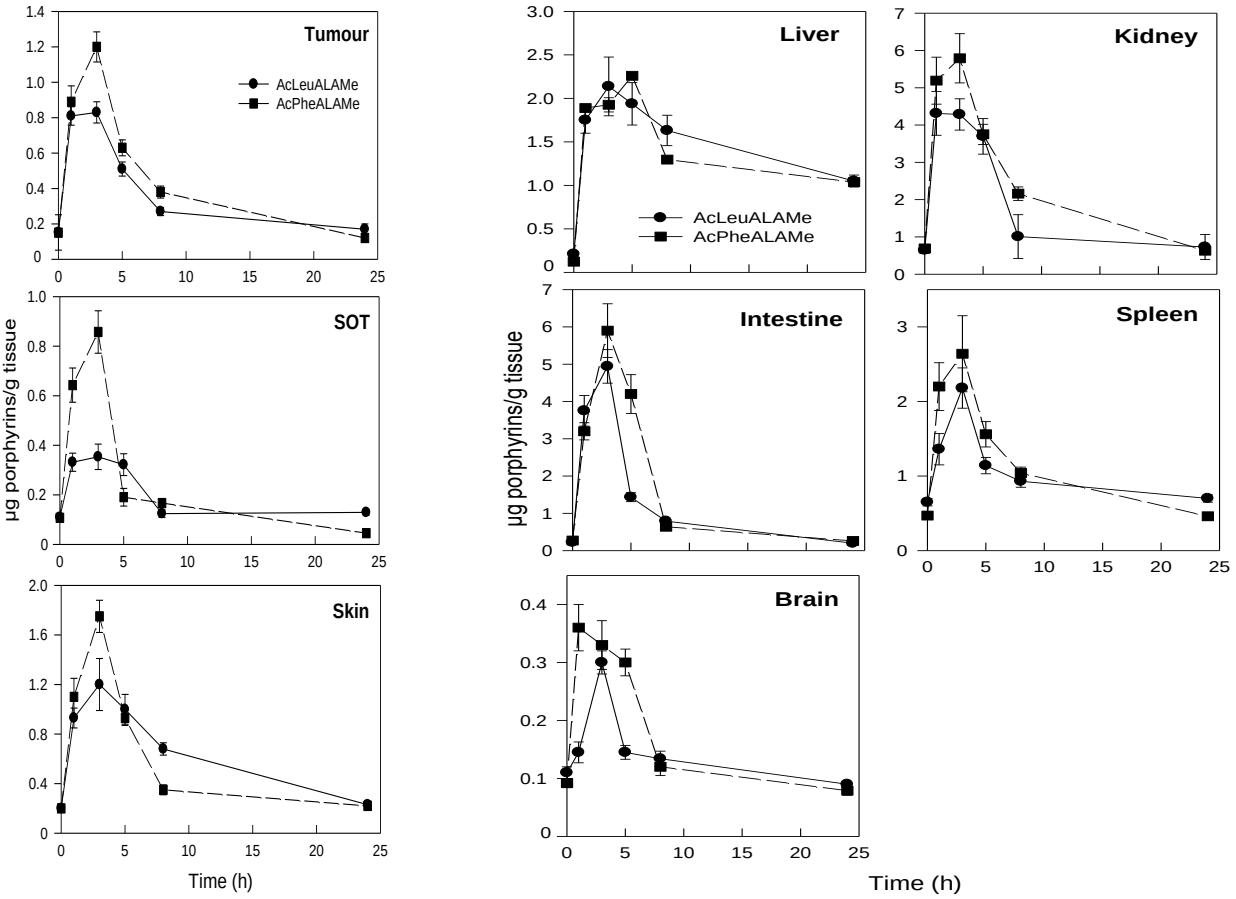

Tumour
AcLeuALAMe
AcPheALAMe
SOT
Skin
Liver
Kidney
Intestine
Spleen
Brain
µg porphyrins/g tissue
Time (h)

Figure 8

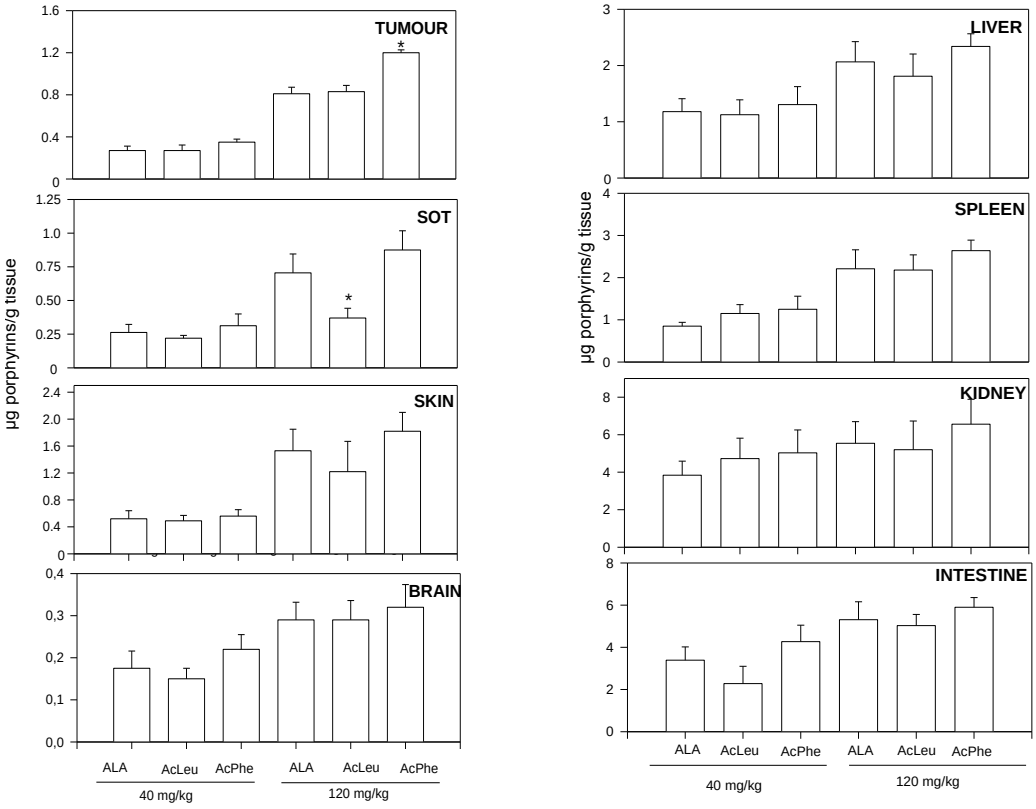
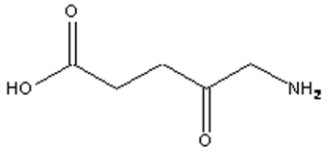
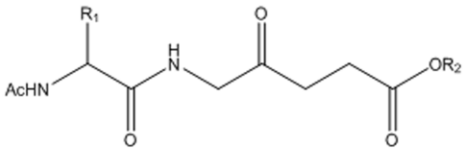

TUMOUR
SOT
SKIN
BRAIN
LIVER
SPLEEN
KIDNEY
INTESTINE
µg porphyrins/g tissue
ALA
AcLeu
AcPhe
40 mg/kg
120 mg/kg

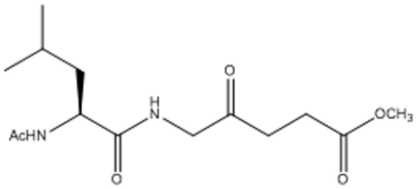
Figure 1



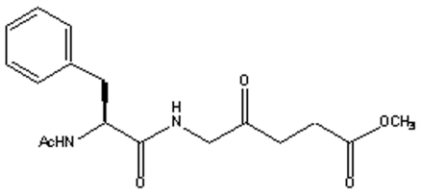
ALA



Generic structure of ALA dipeptide prodrugs



AcLeuALAMe



AcPheALAMe