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Evaluation of Dipeptide-Derivatives of 5-Aminolevulinic Acid as Precursors for Photosensitizers in Photodynamic Therapy

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Abstract—N-terminal-blocked and N-terminal-free pseudotripeptide Gly-Gly and Gly-Pro derivatives of 5-aminolevulinic acid (ALA) esters were synthesized as potential specific substrates for cellular peptidases and precursors for the production of the photosensitizer protoporphyrin IX (PpIX). These precursors were evaluated using human cell lines of either carcinoma or endothelial origin. N-blocked or N-free dipeptides-ALA-ethyl esters, but not tripeptides-ALA-ethyl esters (or dipeptides-ALA-ethyleneglycols,) were substrates for cellular peptidases and were metabolized to ALA. The precursors were hydrolyzed intracellularly involving serine-proteases and metalloproteases. Cell selectivity for human endothelial or carcinoma cells was observed for some of these dipeptides-ALA. Thus drugs coupled to Gly-Gly-/Gly-Pro-derivatives may selectively target defined cells in human cancer, depending on specific cellular activating pathways expressed by the cells.

Introduction

The targeted delivery of chemotherapeutic agents to defined cells, either stromal or cancer cells, in a tumor is a major problem in cancer treatment, including in photodynamic detection and therapy (PDT) of cancer. PDT involves the detection and destruction of diseased tissue by visible light after loading of the target tissue with a photosensitizer. An approach to PDT of cancer is based on the endogenous accumulation of protoporphyrin IX (PpIX) following administration of 5-aminolevulinic acid (ALA), used as a precursor of the fluorescent photosensitizer.[1] However, intravenous administration results in non-specific distribution of ALA-derivatives in cells of any type.[2] Thus, it would be desirable to design ALA derivatives displaying cell-type selectivity, in particular for tumor cells or tumor-associated stromal cells. Moreover, the appearance of PpIX resulting of exposure of cells to chemically-modified ALA will inform about the potential for derivatives to be taken up and metabolized in specific cell compartments, thus providing information for the targeted delivery of drugs.

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Several chemical approaches have been attempted in order to improve the selectivity of delivery of ALA. One approach was to use more lipophilic ALA-derivatives, such as alkyl-esters or ethyleneglycol esters as substrates for cellular esterases,[3–6] dendrimers[7] or using liposomal formulation.[8] The use of ALA-alkyl esters resulted in a non-specific distribution of ALA in all cell types, however with an increased PpIX production[5,9] in tumor cells. More recently, using ethyleneglycol esters of ALA we observed an even higher PpIX production with reduced cytotoxicity for cells in culture,[6] when compared to alkyl esters of ALA.

In an attempt to further improve cell selectivity of ALA-derivatives, we have previously investigated PpIX formation from basic, acidic and neutral amino acid ALA-derivatives which represent potential substrates for cellular aminopeptidases and/or ligands for peptide and amino acid transporters.[6] Of the amino acid derivatives tested, ALA-pseudopeptide derivatives of neutral amino acids induced the highest PpIX production, demonstrating the feasibilty of such an approach. However, cell selectivity was not achieved by these approaches. In the present manuscript we investigated dipeptide-ALA derivatives as potential substrates for cellular di/tripeptidases or peptide transporters at the cell surface, to target human endothelial cells or carcinoma cells in cancer.

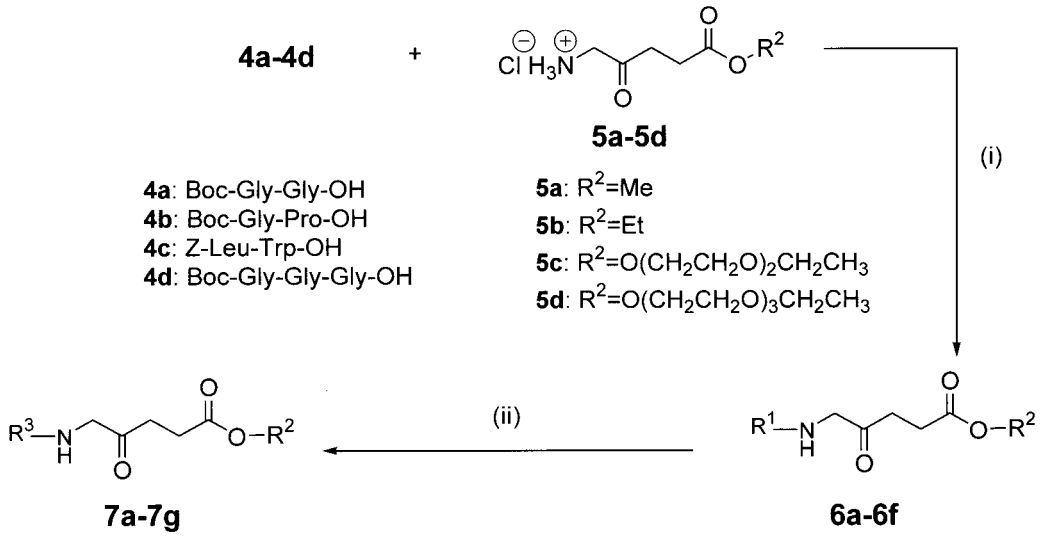
Results

Chemistry: synthesis of the peptide derivatives of ALA

For the synthesis of the protected and the monodeprotected di- and tripeptide derivatives of 5-aminolevulinate the synthetic procedure developed for the simpler amino acid derivatives[6] was applied (Scheme 1). The commercially available di- or tripeptides (**4a–c**) were condensed with the esters of 5-aminolevulinate (**5a–d**) under standard conditions using EDC with good yield. Using diethyleneglycol and triethyleneglycol esters **5e–d**, the yields of the coupling reactions were low. The condensation products **6a**, **6b**, **6e** and **6f** were crystalline. A lack of solubility in many solvents was the major problem encountered in this series and highly polar solvent mixtures had to be used for the flash-chromatographic purification. The monodeprotection of the Boc-group using trifluoroacetic acid allowed the monodeprotected di- and tripeptide derivatives **7a–d** and **7f–g** to be obtained in good purity and yield as their trifluoroacetate salts which could be stored for long time at 4 °C under argon. As our earlier studies had shown that the presence of a methyl or ethyl ester at the carboxylate end of our molecules is not hindering the biosynthetic formation of PpIX, we kept the ester intact. The **7a** and **7f** compounds of these series were crystalline. The only compound that we have not been able to deprotect is derivative **6e**. Assays to deprotect the Z-group by hydrogenolysis under neutral conditions or to achieve the deprotection in a polar solvent containing varying amounts of a mineral acid did not allow us to isolate the corresponding monodeprotected dipeptide derivative containing the free amino group (Table 1).

Biology: production of PpIX from the synthetic peptide-ALA derivatives

For screening purposes the production of PpIX from dipeptide-ALA derivatives was evaluated using human endothelial (HCEC)[10] and human lung carcinoma cells (A549). First, potential substrates for Pro-specific peptidases, namely **7b** for dipeptidyl-aminopeptidase IV (DPPIV)/CD26 activity and the N-terminal blocked Boc-peptide **6b** for prolyl-oligopeptidase(s) were evaluated (Fig. 1A). The results indicated that these dipeptide-ALA derivatives are efficient precursors for PpIX in human cells. Second, the effect of the replacement of the antepenultimate Pro by Gly in **7a** and **6a** was evaluated (Fig. 1B). The results suggested that while some selectivity for endothelial cells towards tumor cells can be obtained using potential substrates for DPPIV and prolyl-oligopeptidase(s) (Fig. 1A), unexpectedly **6a** was very selective (Fig. 1C) for the human endothelial HCEC cells versus lung A549 carcinoma cells. Ethyl **6a** and methyl **7g** esters of diglycyl derivatives were equivalent PpIX precursors. A longer N-terminal blocked tripeptide **7f** or Z-Gly-Gly-ALA-OMe (not shown) were not or were only very weak PpIX precursors. The cytotoxicity in the absence of added light of Gly-Gly and Gly-Pro derivatives was controlled as previously described[6] by comparing PpIX fluorescence at 300 min with cell survival after 20 h exposure to these compounds. The four dipeptide-ALA derivatives **6a**, **6b**, **7a** and **7b** were not cytotoxic, whatever the amount of PpIX produced. Since we have previously shown[6] that cellular uptake of ethyleneglycol esters of ALA is



7a: R^3=Gly-Gly- , R^2= Et
7b: R^3=Gly-Pro- , R^2= Et
7c: R^3=Gly-Gly- , R^2=$O(CH_2CH_2O)_2CH_2CH_3$
7d: R^3=Gly-Gly- , R^2=$O(CH_2CH_2O)_3CH_2CH_3$
7f : R^3=Gly-Gly-Gly-, R^2= Me
7g: R^3=Gly-Gly-, R^2= Me

6a: R^1=Boc-Gly-Gly- , R^2= Et
6b: R^1=Boc-Gly-Pro- , R^2= Et
6c: R^1=Boc-Gly-Gly- , R^2=$O(CH_2CH_2O)_2CH_2CH_3$
6d: R^1=Boc-Gly-Gly- , R^2=$O(CH_2CH_2O)_3CH_2CH_3$
6e: R^1=Z-Leu-Trp- , R^2= Me
6f : R^1=Boc-Gly-Gly-Gly-, R^2= Me

Reagents: (i) HOBt, EDC, NEt_3, rt, 12 h; (ii) TFA, rt, 1 h.

Scheme 1. Chemical syntheses.

Table 1. List and synthesis of pseudopeptide ALA derivatives. Yield of the protected pseudo-peptides **6a–6e** and their deprotected forms **7a–7d**

Code	Name	HRMS (ESI)		Yield (%)
		Calcd	Obs	
6a	Boc-Gly-Gly-ALA-OEt	396.1741	396.1745	80
6b	Boc-Gly-Pro-ALA-OEt	436.2054	436.2051	80
6c	Boc-Gly-Gly-ALA-O$(CH_2CH_2O)_2CH_2CH_3$	484.2265	484.2266	25
6d	Boc-Gly-Gly-ALA-O$(CH_2CH_2O)_3CH_2CH_3$	528.2528	528.2530	25
6f	Boc-Gly-Gly-Gly-ALA-OMe	439.1799	439.1802	78
6e	Z-Leu-Trp-ALA-OMe	601.2633	601.2610	80
7a[a]	Gly-Gly-ALA-OEt	274.1397	274.1397	84
7b[a]	Gly-Pro-ALA-OEt	314.1710	314.1712	Quantitative
7c[a]	Gly-Gly-ALA-O$(CH_2CH_2O)_2CH_2CH_3$	362.1922	362.1925	Quantitative
7d[a]	Gly-Gly-ALA-O$(CH_2CH_2O)_3CH_2CH_3$	406.2205	406.2201	Quantitative
7f	Gly-Gly-Gly-ALA-OMe	317.1456	317.1450	Quantitative
7g	Gly-Gly-ALA-OMe	260.1241	260.1241	Quantitative

[a]Isolated as trifluoroacetate salts.

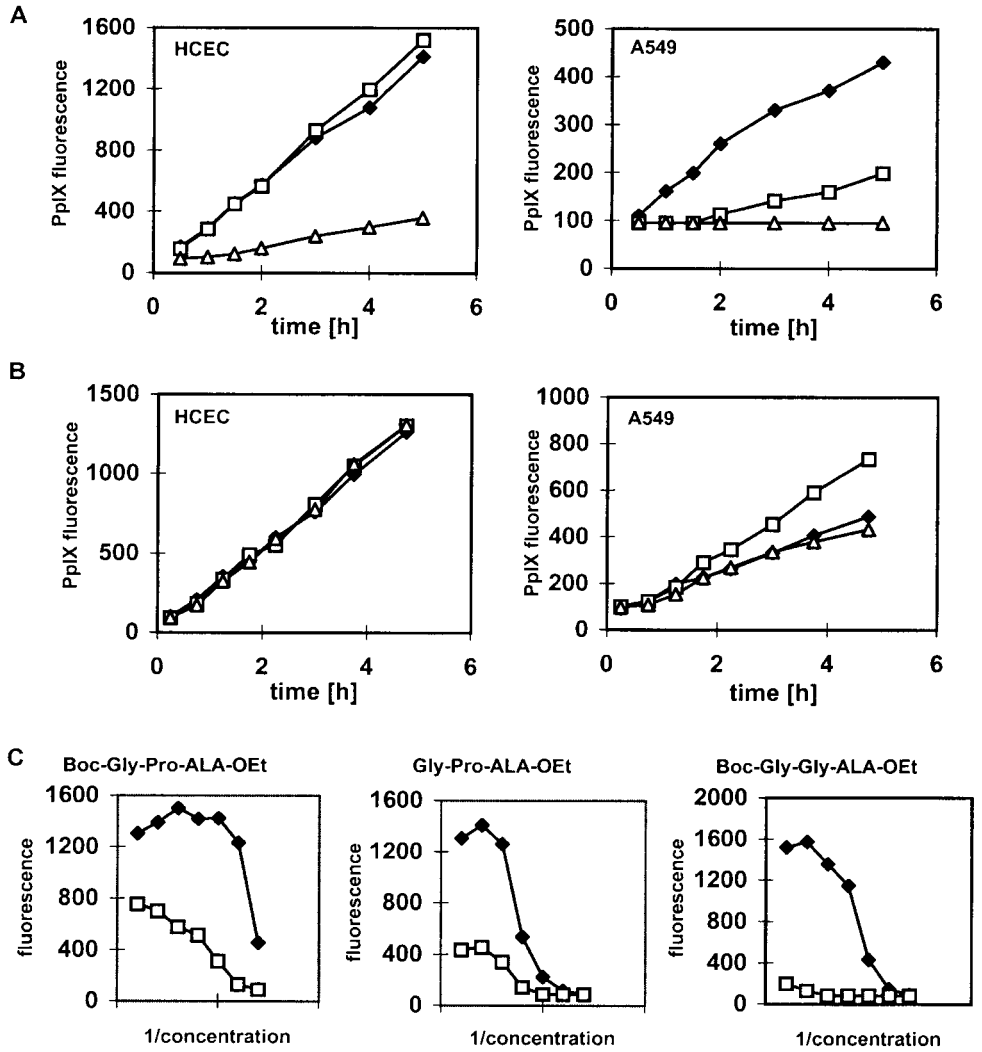


Figure 1. PpIX production by human endothelial cells and human lung carcinoma cells exposed to dipeptide-ALA-derivatives. Cells were grown to confluency and exposed to decreasing concentrations of either ALA used as a standard molecule for PpIX production, or dipeptide-ALA derivatives. PpIX production was ascertained by continuous recording of increase in PpIX fluorescence. (A) ALA (4.0 mM) (◆), **6b** (1.9 mM) (□) or **7b** (3.2 mM) (Δ); (B) ALA (4.0 mM) (◆), **6a** (2.6 mM) (□) and **7a** (3.3 mM) (Δ); (C) **6a**, but not **6b** or **7b** (0.02–2.6 mM), as a precursor of PpIX production can discriminate between human endothelial cells (◆) and human lung carcinoma cells (□).

greatly increased resulting in an higher PpIX production, we also prepared the diethyleneglycol and triethyleneglycol ester derivatives **6c**, **6d**, **7c** and **7d**. Ethyleneglycol ester derivatives of **6c**, **6d**, **7c** and **7d** were poorer PpIX precursors than ethyl esters (Fig. 2).

Third, **6e**, a structurally different dipeptide-ALA derivative as a potential substrate for neutral endopeptidase 24.11./NEP/CD10/neprilyiyn was evaluated in human HCEC endothelial cells and A549 lung carcinoma cells which express this enzymatic activity[11] (Fig. 3). The results of these experiments demonstrated that **6e** is a good precursor for PpIX production in endothelial cells, however this derivative was cytotoxic in the absence of added light. Thus Gly-Gly- and Gly-Pro-ALA analogues are superior precursors in cells regarding cytotoxicity.

In order to obtain information concerning the enzymatic activities involved in releasing free ALA from these precusors, we first evaluated PpIX production in the presence of class-specific protease inhibitors (inhibitors for

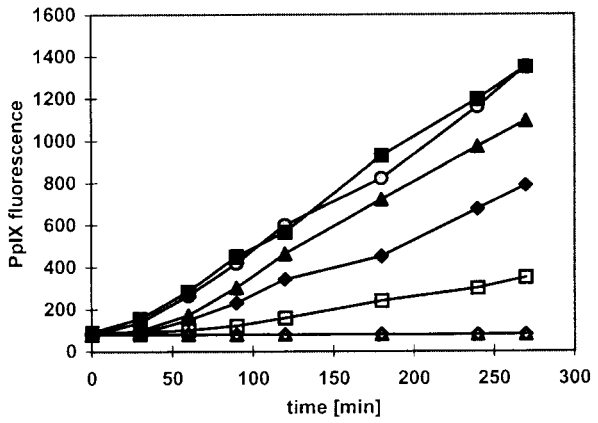


Figure 2. PpIX production by human HCEC endothelial cells exposed to di- and tri-ethylene glycol esters of Boc-Gly-Gly-ALA or Gly-Gly-ALA. Cells were grown to confluency and exposed to decreasing concentrations of either ALA used as a standard molecule for PpIX production, or dipeptide-ALA derivatives. PpIX production was ascertained by continuous recording of increase in PpIX fluorescence. (O)ALA (2.0 mM); (■) **6a** (2.6 mM); (▲) **6c** (2.2 mM); (◆) **6d** (1.8 mM); (□) **7a** (3.3 mM); (△) **7c** (2.6 mM); (◇) **7d** (2.4 mM).

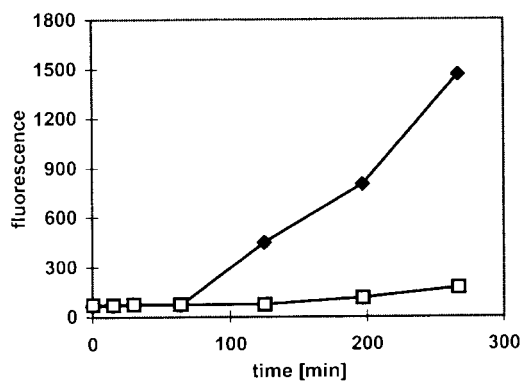


Figure 3. Neprilysin substrates are non-selective PpIX precursors. Cells were grown to confluency and exposed to **6e**. PpIX production was ascertained by continuous recording of increase in PpIX fluorescence of human endothelial HCEC cells (◆) and human A549 lung carcinoma cells (□) exposed to **6e** (0.9 mM).

Ser-, CySH-, Asp- and metallo-proteases) (Fig. 4). The release of ALA from **7b** was inhibited by Ser-protease inhibitors, suggesting the involvement of the Ser-protease DPPIV. The **6a**-hydrolyzing activity was inhibited by inhibitors of Ser-proteases and **6b**-hydrolyzing activity by inhibitors of metalloproteases. This evaluation was further refined by using more specific protease-inhibitors, i.e., benzamidine, leupeptin, aprotinin, E64 or chymostatin in HCEC cells, confirming these informations. In particular, **7b** is potentially a substrate for Gly-Gly-, Gly-Pro-, and Z-Gly-Pro-hydrolyzing proteases and Gly-Gly-hydrolyzing proteases can release ALA from **6a** and **7a**. The identity of the other proteolytic activities involved cannot be ascertained yet by the present approaches.

The activity of the specific hydrolytic activities which have to be postulated to be involved in the hydrolysis of the **6a**, **6b**, **7a** and **7b** ALA precursors, was determined either in intact living cells or in cell extracts using commercially available synthetic Gly-Gly/Gly-Pro-AMC devrivatives. In order to test the putative **6a**-hydrolyzing activity, we had to synthesize Z-Gly-Gly-AMC (manuscript in preparation) since this substrate is not commercially available, as a potential substrate for this enzymatic activity. However, Z-Gly-Gly-AMC was not, or only a very poor, substrate for the potential enzyme(s). A comparison of enzymatic activities expressed on the surface of intact cells or in cell extracts with actual PpIX production from the ALA-derivatives **6a**, **6b**, **7a** and **7b** indicated that cell surface protease activities did not importantly contribute to PpIX production, thus suggesting that the enzyme involved are intracellularly expressed (Fig. 5A and B).

Discussion

The targeted delivery of anti-cancer treatments to defined cells, including photosensitizers in PDT, is of crucial importance for minimizing the side-effects of cytostatic and cytotoxic drugs. Precursor photosensitizers, such as ALA and its chemical derivatives, must be internalized by target cells in order to be metabolized to the photosensitizer PpIX in mitochondria. This processing depends on the presence on the target cells of the necessary transport systems able to deliver ALA into the cytoplasm,[12–16] and, for ALA precursors, of the processing enzymes able to release free ALA from its precursor, to enter the heme biosynthetic pathway.

We have previously shown the feasibility of using esters and aminoacid derivatives of ALA substrates respectively for cellular esterases and aminopeptidases,[5,6] as PpIX precursors. However, using these molecules, no cell selectivity was achieved and the cytotoxicity in the absence of light exposure was significant, suggesting that systemic toxicity might be a problem for these derivatives. Our present goal was therefore to define means to reduce cytotoxicity and to more specifically target ALA delivery using longer peptide derivatives of ALA, either to tumor-associated endothelial cells or to tumor cells.

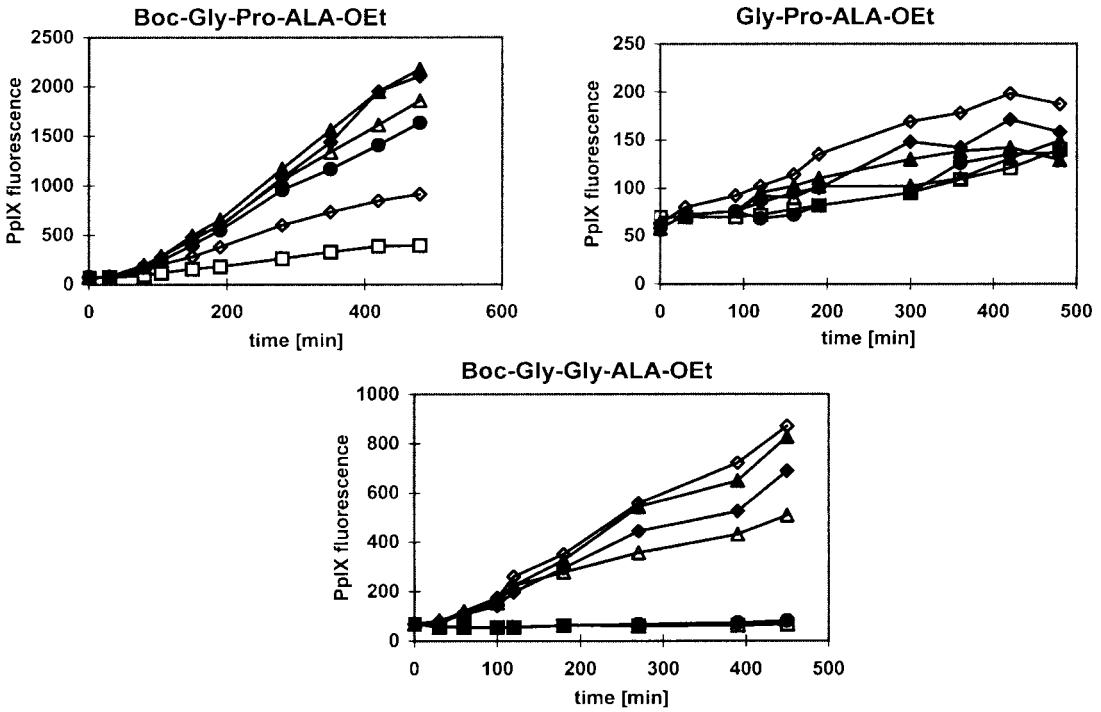


Figure 4. PpIX production by human HCEC endothelial cells exposed to Boc-Gly-Pro-ALA-OEt, Gly-Pro-ALA-OEt or Boc-Gly-Gly-ALA-OEt in the presence of class-specific protease inhibitors. Cells were grown to confluency, protease inhibitors were added first then dipeptide-ALA-OEt derivatives (all 0.6 mM). PpIX production was ascertained by continuous recording of increase in PpIX fluorescence. (◇) no inhibitor; (□) protease inhibitor cocktail (1 :250); (△) BB2116 (4 μM); (▲) aprotinin A (20 μM); (◆) aprotinin B (20 μM); (●) AEBSF (200 μM).

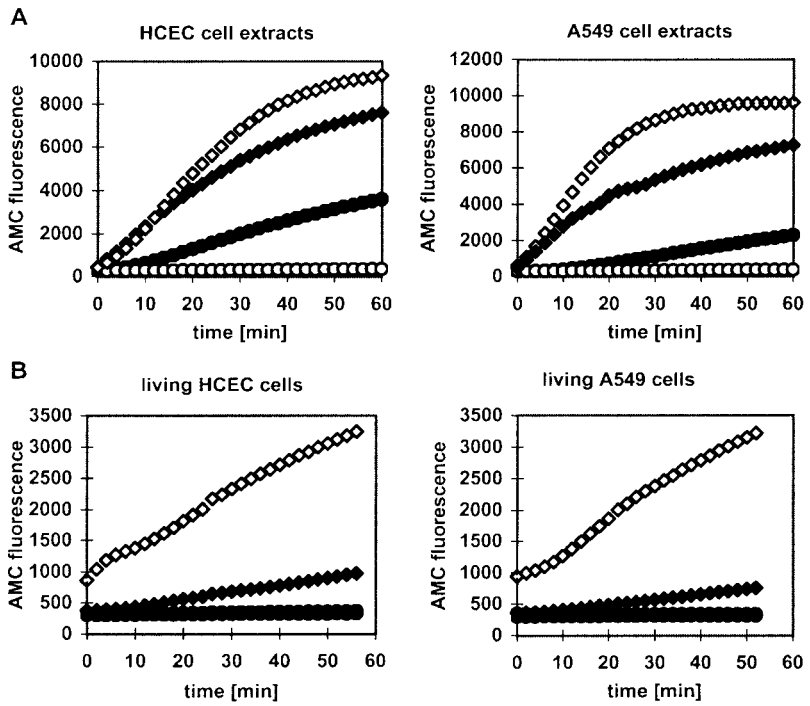


Figure 5. Gly-Gly/Gly-Pro-hydrolyzing enzymatic activities in human HCEC endothelial cells and lung (A549) carcinoma cells. Cells were grown to confluency and either cell extracts (A) or intact living cells (B) were exposed to dipeptide-AMC substrates. AMC release was ascertained by continuous recording of increase in fluorescence. (O) Z-Gly-Gly-AMC; (●) Gly-Gly-AMC; (◇) Z-Gly-Pro-AMC; (◆) Gly-Pro-AMC.

The dipeptide derivatives of ALA displayed a greater stability than the single amino acid derivatives which had been obtained before.[6] We have attributed the increased stability of these di- and tripeptide derivatives to the fact that the distance between the amino terminus and the keto function of the 5-aminolevulinate part is greater than in single amino acid derivatives. We assume that an intramolecular Schiff base formation between the amino terminus and the keto function plays a major role in the degradation of ALA derivatives. In

single amino acid-derivatives the Schiff base formation will lead to a six membered ring, whereas in the case of dipeptide derivatives a nine membered ring would be formed, a less favourable configuration.

In order to evaluate the potential of these derivatives in targeting tumor vasculature, we have first compared PpIX production in human endothelial cells and human carcinoma cells exposed to Gly/Pro-containing dipeptide-ALA-derivatives, based on the assumption that defined cell transporters and defined peptidase activities may be differently expressed in tumoral and normal cells. The information obtained suggests that the precursors are internalized and then hydrolyzed by intracellular proteases. These conclusions were validated by the observation that the **6a** derivative, but not the **6c** and **6d** ethyleneglycol esters, was an efficient precursor. We have previously shown that ALA-ethyleneglycol esters[6] are much more efficient PpIX precursors than ALA due to an enhanced uptake into cells. Thus an extracellular release of ALA-ethylene glycols from its precursor would have greatly increased PpIX production, which was not observed. The dipeptide-ALA derivatives were also less cytotoxic in the absence of light exposure than the aminoacid- and ester-derivatives previously evaluated.[6]

We have shown that the hydrolytic activities involved in the processing of these precursors are active mainly intracellularly, can cleave after a di-Gly motif, and belong to the classes of Ser-proteases and/or metalloproteases. To the best of our knowledge, a Boc-Gly-Gly-X hydrolyzing activity has not been described yet, thus either a new enzyme or alternatively a new activity of a known enzyme, is involved in the processing of these families of derivatives.

Conclusion

We demonstrate here that dipeptide-ALA derivatives are substrates for Gly/Pro-specific cellular transport systems and enzymatic activities in human endothelial cells and carcinoma cells, and are efficient PpIX precursors in PDT protocols. Thus the synthesis of prodrugs incorporating these motifs for cellular peptidases may potentially be also useful to prepare targeting prodrugs of other chemotherapeutic agents.

Experimental

Synthesis. general synthetic methods

General experimental details and the preparation of esters of 5-aminolevulinic acid have been previously described.[6] Protected amino-acids (**4a–4d**) were purchased from Bachem (Bubendorf, Switzerland).

General procedure for the preparation of the peptide analogues

Eight mmol of protected amino acids (**4a–4d**) in DMF (8 mL) were activated during 45 min by addition of *N*-hydroxybenzotriazole (HOBt) [0.6 g (4.4 mmol)] and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide·HCl (EDC) [0.85 g (4.4 mmol)]. A solution of ALA-esters [4 mmol] (**5a–5d**) in DMF [6 mL] was added, followed 10 min later by triethylamine (NEt_3) [0.56 mL (4 mmol)]. After 12 h stirring at room temperature, the DMF was distilled in a Kugelrohr oven (50 °C/0.5 mbar). For **6a** and **6b** the crude material was extracted with EtOAc (2×100 mL) and washed sequentially with citric acid (15 mL, 1 M), $NaHCO_3$ (15 mL, 10%) and brine. After removal of the solvent, the raw material was applied to a silica chromatography column (50 g silicagel (Fluka), eluent: 1–5% MeOH in CH_2Cl_2). The pure products were obtained as solids following evaporation of the solvent. For **6c**, **6d** and **6f** the crude material was dissolved in MeOH, silicagel (3 g) were added and the solvent evaporated. The adsorbed product was flash-chromatographed [silicagel (Fluka) (50 g), eluent: MeOH (5–20%) in CH_2Cl_2]. Hot MeOH was used to crystallize **6f**.

6a. (80% yield); R_f (CH_2Cl_2/MeOH 8:2): 0.72; mp: 120°. RP-HPLC (80% MeCN, C_{18}): $t_R = 3.6$ min, purity >91%. IR (KBr): 3373 m, 3331 m, 3270 m, 3059 w, 2983 w, 2934 w, 1733 m, 1708 s, 1642 vs, 1532 m, 1509 m, 1428 m, 1392 m, 1366 m, 1339 w, 1310 w, 1240 m, 1214 m, 1173 m, 1052 m. ^{1}H NMR (400 MHz, CD_3OD) δ 4.12 (*q*, $^3J(1^2, 1^3) = 7.14$ Hz, 2H, $H_2C(1^2)$); 4.11 (s, 2H, $H_2C(5)$); 3.95 (s, 2H, $H_2C(7)$); 3.76 (s, 2H, $H_2C(9)$); 2.80–2.77 (m, AA' of a AA'BB', 2H, $H_2C(3)$); 2.61–2.58 (m, BB' of a AA'BB', 2H, $H_2C(2)$); 1.47 (s, 9H, $H_3C(12^a)$, $H_3C(12^b)$, $H_3C(12^c)$); 1.25 (t, $^3J(1^3, 1^2) = 7.14$ Hz, 3H, $H_3C(1^3)$). ^{13}C NMR (100 MHz, CD_3OD) δ 205.3 (C(4)); 173.3(C(1)); 172.2, 171.0 (C(6)), (C(8)); 157.7 (C(10)); 79.9 (C(11)); 60.7 ($C(1^2)$); 48.7 (C(5)); 43.8 (C(9)); 42.3 (C(7)); 34.1 (C(3)); 27.7 (C(2)), ($C(12^a)$), ($C(12^b)$), ($C(12^c)$); 13.5 ($C(1^3)$). MS (ESI)$^+$ *m/z* relative intensity 396 (100, $[M+Na]^+$). HRMS (ESI)$^+$: calcd for $[C_{16}H_{27}N_3O_4Na]^+$ 396.1741 obs. 396.1745.

6b. (80% yield) R_f (CH_2Cl_2/MeOH 9:1): 0.45; mp: 123.4–123.6°. RP-HPLC (80% MeCN, C_{18}): $t_R = 3.4$ min, purity >98%. $[\alpha]_D^{23}$: −77.2° ($\lambda = 589$ nm, $c = 0.906$, CH_2Cl_2); −305° ($\lambda = 365$ nm, $c = 0.906$, CH_2Cl_2). IR (KBr): 3303 m, 2979 w, 1740 m, 1706 m, 1674 vs, 1654 s, 1641 vs, 1540 m, 1460 m, 1438 m, 1412 w, 1370 w, 1352 w, 1275 m, 1254 w, 1210 m, 1168 m. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.42 (sbr, 1H, NH-HC(5)); 5.49 (sbr, 1H, NH-HC(12)); 4.60–4.57 (m, 1H, HC(7)); 4.17–4.07 (m, 2H, $H_2C(5)$); 4.11 (*q*, $^3J(1^3,1^2) = 7.14$ Hz, 2H, $H_2C(1^2)$); 3.96–3.87 (m, 2H, $H_2C(12)$); 3.61–3.54 (m, 1H, $H_AC(10)$); 3.44–3.38 (m, 1H, $H_BC(10)$); 2.73–2.69 (m, AA' of a AA'BB', 2H, $H_2C(3)$); 2.62–2.59 (m, BB' of a AA'BB', 2H, $H_2C(2)$); 2.32–2.05 (m, 2H, $H_2C(8)$); 2.04–1.89 (m, 2H, $H_2C(9)$); 1.43 (s, 9H, $H_3C(15^a)$, $H_3C(15^b)$, $H_3C(15^c)$); 1.23 (t, $^3J(1^3,1^2) = 7.15$ Hz, 3H, $H_2C(1^3)$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.2 (C(4)), 172.8, 171.6, 169.1 (C(1), C(6), C(13)), 156.3 (C(11)), 80.1 (C(14)), 61.1 ($C(1^2)$), 60.4 (C(7)), 49.5 (C(5)), 46.7 (C(10)), 43.5 (C(12)), 34.8 (C(3)), 28.7 ($C(15^a)$, $C(15^b)$, $C(15^c)$), 28.3 (C(8)), 28.2 (C(2)), 25.1(C(9)), 14.5 ($C(1^3)$). MS (ESI)$^+$ *m/z* relative intensity 437 (27), 436 (100, $[M+Na]^+$), 380 (8), 336

(7). HRMS (ESI): calcd $[C_{19}H_{31}N_3O_7Na]^+$ 436.2054 obs. 436.2058.

6c. (25% yield); R_f (CH_2Cl_2/MeOH 9:1): 0.51. RP-HPLC (80% MeCN, C_{18}): $t_R = 3.4$ min., purity >96%. IR (KBr): 3357 s, 3333 m, 2973 w, 2929 w, 2869 w, 1715 s, 1692 s, 1667 s, 1654 vs, 1505 vs, 1445 m, 1426 w, 1392 m, 1368 m, 1344 m, 1254 m, 1219 m, 1203 m, 1166 m, 1133 m, 1111 m. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18–7.15 (sbr, 2H, *NH*-H_2C(5), *NH*-H_2C(7)); 5.50 (sbr, 1H, *NH*-H_2C(9)); 4.24–4.22 (m, 2H, $H_2C(1^2)$); 4.18–4.17 (*d*, $^3J(5,NH) = 4.8$ Hz, 2H, H_2C(5)); 4.03–4.01 (d, $^3J(7,NH) = 5.4$ Hz, 2H, H_2C(7)); 3.85–3.84 (d, $^3J(9,NH) = 4.8$ Hz, 2H, H_2C(9)); 3.71–3.78 (m, 2H, $H_2C(1^3)$); 3.66–3.63 (m, 2H, $H_2C(1^4)$); 3.61–3.58 (m, 2H, $H_2C(1^5)$); 3.54 (q, $^3J(1^6,1^7) = 7.02$ Hz, 2H, $H_2C(1^6)$); 2.76–2.73 (m, AA′ of a AA′BB′, 2H, H_2C(3)); 2.69–2.66 (m, BB′ of a AA′BB′, 2H, H_2C(2)); 1.45(s, 9H, $H_3C(12^a)$, $H_3C(12^b)$, $H_3C(12^c)$; 1.22 (t, $^3J(1^7,1^6) = 7.02$ Hz, 3H, $H_3C(1^7)$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.4 (C(4)); 172.8, 170.7, 169.5 (C(1), (C(6), (C(8)); 156.7 (C(10)); 80.8 (C(11)); 71.0 ($C(1^4)$); 70.1 ($C(1^5)$); 69.4 ($C(1^3)$); 67.1 ($C(1^6)$); 64.3 ($C(1^2)$); 49.4 (C(5)); 44.7 (C(9)); 43.1 (C(7)); 34.8 (C(3)); 28.7 ($C(12^a)$, $C(12^b)$, $C(12^c)$); 28.2 (C(2)), 15.5($C(1^7)$). MS (ESI)$^+$ *m/z* relative intensity 485 (30) $[M + Na + H]^+$; 484 (100) $[M + Na]^+$. HRMS (ESI) calcd $[C_{20}H_{35}N_3O_9Na]^+$ 484.2265; obs. 484.2266.

6d. (25% yield); R_f (CH_2Cl_2/MeOH 9:1): 0.48. RP-HPLC (80% MeCN, C_{18}): $t_R = 3.5$ min, purity >90%. IR (KBr): 3359 m, 3337 m, 2977 m, 2929 m, 2873 m, 1724 s, 1691 s, 1668 vs, 1655 vs, 1509 s, 1445 m, 1393 m, 1368 m, 1348 m, 1253 m, 1168 s, 1144 m. ^{1}H NMR (400 MHz, $CDCl_3$) δ 714–7.11 (sbr, 2H, *NH*-H_2C(5), *NH*-H_2C(7)); 5.44 (sbr, 1H, *NH*-H_2C(9)); 4.24–4.21 (m, 2H, $H_2C(1^2)$); 4.19–4.18 (d, $^3J(5,NH) = 5.0$ Hz, 2H, H_2C(5)); 4.04–4.02 (d, $^3J(7,NH) = 5.6$ Hz, 2H, H_2C(7)); 3.85–3.84 (s, 2H H_2C(9)); 3.70–3.65 (m, 8H, $H_2C(1^3)$, $H_2C(1^4)$, $H_2C(1^5)$, $H_2C(1^6)$); 3.62–3.60 (m, 8H, $H_2C(1^7)$); 3.55 (q, $^3J(1^8,1^9) = 7.02$ Hz, 2H, $H_2C(1^8)$); 2.76–2.73 (m, AA′ of a AA′BB′, 2H, H_2C(3)); 2.70–2.67 (m, BB′ of a AA′BB′, 2H, H_2C(2)); 1.45(s, 9H, $H_3C(12^a)$, $H_3C(12^b)$, $H_3C(12^c)$; 1.22 (t, $^3J(1^9,1^8) = 7.01$ Hz, 3H, $H_3C(1^8)$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.4 (C(4)); 172.7, 170.7, 169.5 (C(1), (C(6), (C(8)); 156.6 (C(10)); 80.8 (C(11)); 71.0, 70.9, 70.8, 70.1, 69.4 ($C(1^3)$, ($C(1^4)$), ($C(1^5)$, ($C(1^6)$), ($C(1^7)$); 67.1 ($C(1^8)$), 64.2 ($C(1^2)$); 49.5 (C(5)); 44.7 (C(9)); 43.2 (C(7)); 34.7 (C(3)); 28.7 ($C(12^a)$, $C(12^b)$, $C(12^c)$); 28.4 (C(2)), 15.5($C(1^7)$). MS (ESI)$^+$ *m/z* relative intensity 529 (23) $[M + Na + H]^+$; 528 (100) $[M + Na]^+$. HRMS (ESI): calcd $[C_{22}H_{39}N_3O_{10}Na]^+$ 528.2528 obs. 528.2530.

6e. The reaction solvent is MeCN. Eluent for silica chromatography column is EtOAc. The compound is crystallized in EtOAc/diisopropyl ether, 1/1. (80% yield); R_f (AcOEt): 0.60; mp: 86.0–88.0°. RP-HPLC (80% MeCN, C_{18}): $t_R = 3.9$ min, purity 100%. IR (KBr): 3357 m, 3301 m, 2957 m, 2930 m, 1720 s, 1694 s, 1645 vs, 1539 s, 1456 m, 1435 m, 1360 m, 1265 m, 1170 w, 1125 w, 1036 w. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (sbr, 1H, *NH*-HC(7), *NH*-H_2C(7)); 7.59–6.88 (m, 12H, *NH*-H_2C(5), *NH*-HC(7^4), HC(7^6), HC(7^7), HC(7^8), HC(7^9), 2HC(13), 2HC(13), 2HC(14), CH(15)); 5.42 (d, 1H, $^3J(NH,5) = 7.5$ Hz, *NH*-HC(9)); 5.05 (dd, 2H, $^4J(11,13) = {}^4J(11,13') = 7.6$ Hz, O–H_2C(11)); 4.83 (dt, 1H, $^3J(7,NH) = {}^3J(7,7^2) = 6.9$ Hz, HC(7)); 4.25 (m, 1H, HC(9)); 3.97 (d, $^3J(5,NH) = 4.7$ Hz, 2H, H_2C(5)); 3.85–3.84 (s, 2H H_2C(9)); 3.65 (s, 3H, $H_3C(1^2)$); 3.23 (m, 2H, $H_2C(7^2)$); 2.54 (s, 4H, H_2C(3), H_2C(2)); 1.64–1.53 (m, 2H, HC(9), HC(9^{1a})), 1.45–1.39 (m, 1H, HC(9^{1b})); 0.89 (s, 3H, $H_3C(9^{4a})$); 0.88 (*s*, 3H, $H_3C(9^{4b})$. ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.2 (C(4)); 173.3 (C(1)); 172.7 (C(6)); 171.8 (C(8)); 156.8 (C(10)); 136.6 (C(12)); 136.5 ($C(7^{10})$); 129.0, 128.7, 128.5 (C(13), C(13′), C(14), C(14′), C(15)); 127.9 ($C(7^5)$); 123.9 ($C(7^4)$); 122.4 ($C(7^7)$); 119.9 ($C(7^8)$); 119.0 ($C(7^9)$); 111.7 ($C(7^3)$); 110.4 ($C(7^6)$; 67.5 (C(11)); 54.2 (C(9); 54.1 (C(7)); 52.3 ($C(1^2)$; 49.4 (C(5)); 41.7 ($C(9^1)$); 34.7 (C(3)), 28.4 ($C(7^2)$); 27.8 (C(2)); 25.1 ($C(9^3)$); 23.3 ($C(9^{4a})$ or $C(9^{4b})$); 22.17 ($C(9^{4a})$ or $C(9^{4b})$). MS (ESI)$^+$ *m/z* relative intensity 601 (100) $[M + Na]^+$; 602 (35) $[M + Na + H]^+$. HRMS (ESI) calcd for $C_{31}H_{38}N_4O_7Na^+$ 601.2633, found 601.2610. Anal. calcd for $C_{31}H_{38}N_4O_7$: C, 64.34; H, 6.62; N, 9.68. Found: C, 64.28; H, 6.72; N, 9.50.

6f. (78% yield); R_f (CH_2Cl_2/MeOH 8:2): 0.62; mp: decomposition 197–198 °C. RP-HPLC (80% MeCN, C18): $t_R = 3.1$ min, purity >92%. IR (KBr): 3402 w, 3363 w, 3308 m, 2978 w, 1708 m, 1684 m, 1669 m, 1636 vs, 1528 m, 1440 w, 1425 w, 1366 w, 1249 w, 1179 w. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.19–8.16 (m, 1H, H*N*-H_2C(9 or 7)); 8.14–8.12 (m, 1H, H*N*-H_2C(5)); 8.06–8.03 (m, 1H, HN-H_2C(9 or 7)); 7.02–6.99 (m, 1H, H*N*-H_2C(11)); 3.97–3.95 (m, 2H, H_2C(5)); 3.76–3.74 (m, 4H, H_2C(9), H_2C(7)); 3.59–3.58 (m, 5H, H_2C(11), H_3C(12)); 2.72–2.69 (m, AA′ of a AA′BB′, 2H, H_2C(3)); 2.51–2.47 (m, BB′ of a AA′BB′, 2H, H_2C(2)); 1.38 (s, 9H, H_3C(14a), H_3C(14b), H_3C(14c)). ^{13}C NMR (100 MHz, DMSO-d_6) δ 206.1 (C(4)); 173.5, 170.6, 170.0, 169.9 (C(1), C(6), C(8), C(10)), 156.7 (C(12)); 79.0 (C(13); 52.3 (C(12)); 49.1 (C(5)); 44.2 (C(11)); 42.9, 42.7 (C(7), C(9)); 34.7 (C(3)); 29.0 (C(14a), C(14b), C(14c)), 28.0 (C(2)). MS (APCI) +) *m/z* relative intensity 440 (23), 439 (100, [M + Na]+), 483 (20). HRMS (ESI): calcd for [$C_{17}H_{28}N_4O_8Na$]+ 439.1799 obs. 439.1802.

General procedures for the preparation of the peptide analogues

Procedure for the deprotection of the N-terminal amino acid function. Protected amino acid-ALA derivatives (0.4 mmol) were dissolved in TFA [2 mL (39 mmol)] and CH_2Cl_2 [2 mL] under argon atmosphere. After 1 h stirring at room temperature, TFA was evaporated under high vacuum. The products were obtained in good purity and characterized without further purification as their trifluoroacetate salts. **7a** has been crystallized in MeOH with Et_2O.

7a. (84% yield); mp: 127° C. RP-HPLC (80% MeCN, C_{18}): $t_R = 2.6$ min, purity >94%. IR (KBr): 3350 m, 3008 m, 2917 m, 1713 vs, 1653 vs, 1531 m, 1426 m, 1350 w, 1308 m, 1266 m, 1197 s, 1182 s, 1129 m, 1005 m, 841 m, 802 m, 726 m, 567 m. ^{1}H NMR (400 MHz,

$CDCl_3$) δ 4.13 (*s*, 2H, $H_2C(5)$); 4.12 (q, $^3J(1^2, 1^3)$ = 7.14 Hz, 2H, $H_2C(1^2)$); 4.02 (s, 2H, $H_2C(7)$); 3.78 (s, 2H, $H_2C(9)$); 2.80–2.77 (*m*, AA′ of a AA′BB′, 2H, $H_2C(3)$); 2.62–2.58 (*m*, BB′ of a AA′BB′, 2H, $H_2C(2)$); 1.25 (t, $^3J(1^3, 1^2)$ = 7.14 Hz, 3H, $H_3C(1^3)$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.2 (C(4)); 173.4, 170.6, 167.1 (C(1)), (C(6)), (C(8)); 157.7 (C(10)); 60.7 ($C(1^2)$); 48.7 (C(5)); 42.1 (C(7)); 40.5 (C(9)); 34.2 (C(3)); 27.7 (C(2)), (C(12)); 13.5 ($C(1^3)$). MS (ESI^+) *m/z* relative intensity 296 (7, $[M–TFA + Na]^+$), 275 (5) 274 (100, $[M–TFA]^+$), 217 (5), 179 (4). HRMS (ESI): calcd for $[C_{11}H_{20}N_3O_5]^+$ 274.139747 obs. 274.139749

7b. (Quantitative yield); RP-HPLC (80% MeCN, C_{18}): t_R = 2.8 min, purity >87%. $[\alpha]_D^{23}$: −56.3° (λ = 589 nm, *c* = 0.206, MeOH); −196.1° (λ = 365 nm, *c* = 0.206, MeOH). IR (KBr): 3426 m, 2988 m, 1725 s, 1675 vs, 1540 m, 1472 m, 1429 w, 1370 w, 1354 w, 1254 w, 1204 s, 1136 m. ^1H NMR (400 MHz, $CDCl_3$) δ 4.56–4.49 (m, 1H, HC(7)); 4.16 (s, 2H, $H_2C(5)$); 4.11 (q, $^3J(1^3,1^2)$ = 7.2 Hz, 2H, $H_2C(1^2)$); 3.92 (*s*, 2H, $H_2C(12)$); 3.69–3.52 (m, 2H, $H_2C(10)$); 2.80–2.77 (m, AA′ d'un AA′BB′, 2H, $H_2C(3)$); 2.63–2.58 (m, BB′ of AA′BB′, 2H, $H_2C(2)$); 2.43–2.24 (m, 2H, $H_2C(8)$); 2.14–1.98 (m, 2H, $H_2C(9)$); 1.25 (t, $^3J(1^3,1^2)$ = 7.15 Hz, 3H, $H_2C(1^3)$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.2 (C(4)), 173.6, 173.4, (C(1), C(6)), 165.3 (C(11)), 60.7 ($C(1^2)$), 60.6 (C(7)), 48.8 (C(5)), 46.6 (C(10)), 40.5 (C(12)), 34.1 (C(3)), 29.9 (C(8)), 27.7 (C(2)), 24.5 (C(9)), 13.5 ($C(1^3)$). MS $(ESI)^+$ *m/z* relative intensity 336 (7, $[M–HTFA + Na]^+$), 315 (57), 314 (100, $[M–TFA]^+$). HRMS (ESI) calcd for $[C_{14}H_{24}N_3O_5]^+$ 314.1710; obs. 314.1712.

7c. (Quantitative yield); RP-HPLC (80% MeCN, C_{18}): t_R = 3.5 min, purity >93%. IR (KBr): 3336 m, 3236 m, 3091 m, 2928 m, 2869 m, 1721 s, 1676 vs, 1640 vs, 1553 m, 1484 m, 1432 m, 1367 m, 1347 m, 1208 vs, 1170 s, 1132 s, 836 m, 723 m. ^1H NMR (400 MHz, $CDCl_3$) δ 8.33 (sbr, 1H, NH-$H_2C(7)$); 7.98–7.90 (sbr, 2H, NH-$H_2C(5)$, NH-$H_2C(9)$); 4.19 (s, 2H, $H_2C(1^2)$); 4.12 (s, 2H, $H_2C(5)$); 4.00 (s, 2H, $H_2C(7)$); 3.89(s, 2H, $H_2C(9)$); 3.69–3.67 (m, 2H, $H_2C(1^3)$); 3.64–3.63 (m, 2H, $H_2C(1^4)$); 3.60–3.58 (m, 2H, $H_2C(1^5)$); 3.54 (q, $^3J(1^6,1^7)$ = 7.02 Hz, 2H, $H_2C(1^6)$); 2.76–2.73 (m, 2H, $H_2C(3)$); 2.69–2.66 (m, 2H, $H_2C(2)$); 1.20 (t, $^3J(1^7,1^6)$ = 7.01 Hz, 3H, $H_3C(1^7)$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.8 (C(4)); 173.6, 170.7, 168.0 (C(1), (C(6), (C(8)); 161.1 (q, $^3J(CCF_3)$ = 37 Hz, F_3C-$C_{(TFA)}$); 115.0 (q, $^2J(CF_3)$ = 285 Hz, $F_3C_{(TFA)}$); 70.8 ($C(1^4)$); 69.9 ($C(1^5)$); 69.2 ($C(1^3)$); 67.0 ($C(1^6)$); 64.3 ($C(1^2)$); 49.2 (C(5)); 42.3 (C(7)); 41.5 (C(9)); 34.6 (C(3)); 28.0 (C(2)), 15.3 ($C(1^7)$). MS $(ESI)^+$ *m/z* relative intensity 424 (30); 403 (41); 402 (100) $[M–HTFA + K]^+$. HRMS (ESI): calcd $[C_{15}H_{28}N_3O_7]^+$ 362.1922 obs. 362.1925.

7d. (Quantitative yield); RP-HPLC (80% MeCN, C_{18}): t_R = 2.9 min, purity >94%. IR (KBr): 3326 m, 3236 w, 3090 w, 2927 m, 2873 m, 1723 s, 1673 s, 1639 vs, 1208 vs, 1170 s, 1141 s. ^1H NMR (400 MHz, $CDCl_3$) δ 8.11 (sbr, 2H, NH-$H_2C(7)$; 7.87 (sbr, 1H, NH-$H_2C(5)$); 7.71 (sbr, 1H, NH-$H_2C(9)$); 4.22 (s, 2H, $H_2C(1^2)$); 4.16 (s, 2H, $H_2C(5)$); 4.07 (s, 2H, $H_2C(7)$); 3.95 (s, 2H $H_2C(9)$); 3.70–3.64 (m, 10H, $H_2C(1^3)$, $H_2C(1^4)$, $H_2C(1^5)$, $H_2C(1^6)$, $H_2C(1^7)$); 3.59 (q, $^3J(1^8,1^9)$ = 7.02 Hz, 2H, $H_2C(1^8)$); 2.77–2.75 (m, 2H, $H_2C(3)$); 2.64–2.62 (m, 2H, $H_2C(2)$); 1.22 (t, $^3J(1^9,1^8)$ = 7.03 Hz, 3H, $H_3C(1^8)$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.8 (C(4)); 173.8, 171.3, 168.0 (C(1), (C(6), (C(8)); 160.4 (q, $^3J(CCF_3)$ = 40 Hz, F_3C-$C_{(TFA)}$); 115.0 (q, $^2J(CF_3)$ = 284 Hz, $F_3C_{(TFA)}$); 70.5, 70.4, 70.3, 69.6, 69.4 ($C(1^3)$), ($C(1^4)$), ($C(1^5)$), ($C(1^6)$), ($C(1^7)$); 67.2 ($C(1^8)$), 64.1 ($C(1^2)$); 49.3 (C(5)); 43.0 (C(9)); 43.0 (C(7)); 34.6 (C(3)); 27.9 (C(2)), 14.9 ($C(1^7)$). MS $(ESI)^+$ *m/z* relative intensity 469, 468, 447, 446 (100), 406 (11). HRMS (ESI): calcd $[C_{17}H_{32}N_3O_8]^+$ 406.2205 obs 406.2201.

7f. (Quantitative yield); mp: 78–82 °C. RP-HPLC (80% MeCN, C_{18}): t_R = 2.6 min, purity >89%. IR (KBr): 3323 m, 3084 w, 1726 m, 1677 vs, 1545 m, 1412 w, 1370 w, 1204 s, 1136 m. ^1H NMR (400 MHz, CD_3OD) δ 4.12 (s, 2H, $H_2C(5)$); 4.00 (s, 2H, $H_2C(9)$); 3.96 (s, 2H, $H_2C(7)$); 3.78 (s, 2H, $H_2C(11)$); 3.67 (m, 3H, $H_3C(1^2)$); 2.81–2.77 (m, AA′ of a AA′BB′, 2H, $H_2C(3)$); 2.63–2.60 (m, BB′ of a AA′BB′, 2H, $H_2C(2)$). ^{13}C NMR (100 MHz, CD_3OD) δ 205.3 (C(4)); 173.9, 170.9, 170.8, 167.2 (C(1), C(6), C(8), C(10)); ~160 (*q*, TFA); ~114 (*q*, TFA); 51.2 ($C(1^2)$); 48.6 (C(5)); 42.6 (C(9)); 42.2 (C(7)); 40.5(C(11)); 34.1 (C(3)); 27.4 (C(2)). MS (ESI^+) *m/z* relative intensity 358 (100, $[M–TFA + Na + H_2O]^+$), 357 (52, $[M–HTFA + Na + H_2O]^+$), 341 (6, $[M–HTFA + Na]^+$), 318 (19). HRMS (ESI) calcd for $[C_{12}H_{21}N_4O_6]^+$ 317.1456; obs. 317.1450.

7g. (Quantitative yield); mp: 177–178 °C. RP-HPLC (80% MeCN, C_{18}): t_R = 2.5 min, purity >90%. IR (KBr): 3321 m, 3082 m, 2927 m, 2878 m 1727 s, 1702 w,1640 vs, 1556 m, 1487 m, 1434 m, 1362 m, 1212 m. ^1H NMR (400 MHz, DMSO-d_6) δ 8.80 (*t*, ^3J(NH-7) = 5.7 Hz, 1H, N*H*-$H_2C(7)$); 8.42 (t, ^3J(NH-5) = 5.6 Hz, 1H, N*H*-$H_2C(5)$); 8.24 (sbr, 3H, N*H*-$H_2C(9)$; 3.96 (d, ^3J(5-NH) = 5.7 Hz, 2H, $H_2C(5)$); 3.83 (d, ^3J(7-NH) = 5.8, 2H, $H_2C(7)$); 3.60 (s, 2H, $H_2C(9)$); 3.57 (s, 3H, $H_2C(1^2)$); 2.73–2.69 (m, AA′ of a AA′BB′, 2H, $H_2C(3)$); 2.51–2.47 (m, BB′ of a AA′BB′, 2H, $H_2C(2)$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 206.1 (C(4)); 173.5, 169.5, 167.2 (C(1)), (C(6)), (C(8)); 52.3 ($C(1^2)$); 49.1 (C(5)); 42.7 (C(7)); 41.0 (C(9)); 34.8 (C(3)); 28.0 (C(2)). MS (ESI^+) *m/z* intensité relative 296 (7, $[M–TFA + Na]^+$), 283 (100 $[M–HCl + Na]^+$), 260(53 $[M–Cl]^+$), 221(11), 197(12), 187(17), 163(55), 149(52), 139(77). HRMS (ESI) calcd for $[C_{10}H_{18}N_3O_5]^+$ 260.1241; obs. 260.1241.

Cells and culture conditions. Human lung carcinoma A549 cell line was from ATCC (American Type Culture Collection, Manassas, VA, USA) and human microvascular endothelial cell line HCEC[10] was a kind gift of D. Staminirovic and A. Muruganandam, Ottawa, Canada. All cells were grown in DMEM medium (Gibco-BRL, Basel, Switzerland) containing glucose (4.5 g/l), fetal calf serum (FCS) (10%) and antibiotics, at 37 °C and CO_2 (6%). Cells were subcultured in 48-well dishes (Costar) prior to experiments in complete culture medium. Experiments were repeated at least twice and were performed in sextuple wells. Means of results and SD were calculated, but were not shown on the graphics for the purpose of clarity.

Cell viability was determined after 20 h exposure to ALA derivatives using the evaluation of mitochondrial functions by the MTT assay as previously described.[6]

PpIX fluorescence measurements. Evaluation of PpIX production by continuous fluorescence recording in living cells was performed essentially as previously described.[6] For inhibition studies, the protease inhibitors were added to cell cultures 5–10 min before dipeptide-ALA derivatives and fluorescence increases was recorded. Protease inhibitors were from commercial sources (Sigma, Buchs, Switzerland; Bachem, Bubendorf, Switzerland; Novabiochem, Luzern, Switzerland).

Peptidase activities. Peptidase activities were determined either in intact living cells or in cell extracts. For cells extracts, the experiments were performed essentially as previously described.[17] Briefly, cells were extracted in PBS buffer containing Triton X-100 (0.1%) (Fluka) and exposed to specific AMC substrates (all from Bachem; with the exception of Z-Gly-Gly-AMC which was synthesized) (0.25 mM final concentration) in a thermostated fluorescent multiwell plate reader (CytoFluor Series 4000, PerSeptive Biosystems, MA, USA; λ_{ex}: 360 nm; λ_{em}:460 nm). Confluent intact cell cultures were exposed to substrates in DMEM medium without phenol red (Gibco) and without FCS and fluorescence was continuously recorded for 30–60 min in a thermostated fluorescent multiwell plate reader (CytoFluor Series 4000; λ_{ex}: 360 nm; λ_{em}:460 nm). For inhibition studies, the protease inhibitors were added to cell cultures or cell extracts 5–10 min before the substrates.

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