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Synthesis of chemically diverse esters of 5-aminolevulinic acid for photodynamic therapy *via* the multicomponent Passerini reaction.

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ABSTRACT

5-Aminolevulinic acid-based photodynamic therapy (ALA-PDT) is gaining increasing acceptance in Medicine as an effective technique for the treatment of a variety of neoplastic lesions. Unfortunately, the efficacy of ALA-PDT is limited by the hydrophilic nature of the molecule, leading to poor penetration through malignant tissues. This fact prompted to the development of more lipophilic ALA derivatives, such as alkyl esters, although this strategy has potential drawbacks, mainly associated with the instability of ALA in solution. In this paper we describe a more versatile methodology for obtaining ALA esters based on the three-component Passerini reaction, which involves mild conditions and a straightforward procedure, allowing an efficient generation of chemically diverse libraries of ALA conjugates. The preliminary *in vitro* evaluation of the new designed compounds allowed us to find a new promising conjugate with enhanced photodynamic properties compared to ALA.

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INTRODUCTION

5-Aminolevulinic acid-based photodynamic therapy (ALA-PDT) is gaining increasing acceptance in Medicine as an effective technique for the treatment of a variety of neoplastic lesions and premalignant disorders. 5-aminolevulinic acid (ALA, Figure 1) is the prodrug of the photosensitizer protoporphyrin IX (PpIX, Figure 1); after ALA administration, cells generate PpIX through the haem biosynthetic pathway. Clinically, when sufficient intracellular levels of PpIX are attained following either topical or systemic ALA administration, the targeted tissue is irradiated with visible light to activate the sensitizer leading to generation of cytotoxic species and ultimately cell death. [1] PpIX is, among the endogenously synthesized porphyrins from ALA, the most active one. [2]

Figure 1. 5-aminolevulinic acid (**ALA**) as the biosynthetic precursor of the photosensitizer protoporphyrin IX (**PpIX**).

Although ALA-PDT has already shown great potential both for the treatment of cancer and infectious diseases [3,4] 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.[5] In addition, saturation of haem synthesis as well as photobleaching of PpIX are also factors limiting the outcome of ALA-PDT.[6] 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 photosensitization.[7]

One approach has been to use more lipophilic ALA derivatives, such as alkyl or ethylene glycol esters, which are potential substrates for cellular esterases.[8–10] ALA esters and dipeptide derivatives with a free amino terminus are stable at physiological pH [11–13], and they may be fine-tuned in terms of their overall lipophilicity to favour passive uptake while still retaining water solubility.

In particular, 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 photosensitization.[8,9,14–16] Prodrugs of this kind, especially methyl (Me-ALA) and hexyl ester (Hex-ALA) of ALA (Figure 2), have now been validated in a clinical setting, with regulatory approval being granted for the use of Me-ALA for the treatment of cutaneous malignant or pre-malignant conditions[17,18] and Hex-ALA for early detection of bladder cancer.[19]

By means of measuring the apparent partition coefficients of the ALA esters between octanol and PBS, it was demonstrated that it is possible to vary the lipophilicity of ALA by more than three orders of magnitude when using ALA esters.[10] Both ALA and Me-ALA were hydrophilic, whereas the ethyl,

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butyl, hexyl, octyl and cyclohexyl esters were more lipophilic. Gaullier [9] found that long chained ALA esters (C6-C8) reduced 30-150-fold the amount of ALA needed to reach the same level of PpIX accumulation when compared to that obtained with nonesterified ALA in a human cell line, whereas short-chained pro-ALAs (C1-C3) were less efficient than ALA.

Unfortunately, acylation of ALA has potential drawbacks, mainly associated with its instability in solution. It has been shown that around pH 7 ALA dimerizes to give pyrazines, while, at a higher pH, pseudo-porphobilinogen may be formed.[20,21] That imposes a rather severe restriction on the kind of coupling reactions that can be used to achieve this goal.

In this work, and in order to develop a more suitable methodology for obtaining ALA esters, we decided to explore a synthetic strategy based on the Passerini reaction (P-3CR) [22]. It is known that this reaction not only involves milder conditions for the generation of esters [23], but has the additional remarkable feature of multicomponent reactions: a straightforward procedure which results in a simple and fast generation of chemically diverse libraries. [24] With these premises in mind, we designed and synthesized a series of prodrugs of general structure **1** (Figure 2) and performed a preliminary evaluation of their ability to induce the production **PpIX** *in vitro*.

Figure 2. Clinically useful ALA esters and ALA derivatives reported in this work (of general structure **1**).

EXPERIMENTAL PROCEDURES

Synthesis of the compounds.

General.

All solvents and reagents were of analytical grade and were purchased from Sigma-Aldrich Chemical Co. ESI-HRMS were measured on a Bruker micrOTOF-Q II. Melting points were determined on a Fisher Johns apparatus and are uncorrected. All NMR spectra were recorded on a Bruker AM-500 (500 MHz for ^{1}H and 125.1 MHz for ^{13}C) or a Bruker AC-200 (50.3 MHz for ^{13}C). Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. Coupling constant (J) values are in Hz. Combustion analyses for the new compounds were performed on an Exeter CE 440 Elemental Analyzer and were within ± 0.4 % of the theoretical values. Detailed synthetic procedures and full characterization of the new compounds are described in the Supplementary Data.

LogD predictions.

Calculator Plugins were used for logD predictions, Marvin 6.3.0, 2014, ChemAxon (http://www.chemaxon.com).

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Biological activity.

Cell lines

LM2 cells derived from the murine mammary adenocarcinoma M2 (Galli) were kindly provided by Dr. Ana M. Eiján from the Instituto de Oncología "Ángel H. Roffo" (Universidad de Buenos Aires, Argentina), and were cultured in minimum essential Eagle's medium. HaCaT is a human immortalized keratinocyte cell line that was obtained from Eucellbank (Barcelona, Spain) and grown in RPMI 1640 medium. [25] IGROV-1 is a human ovarian cancer cell line obtained from the NCI (National Cancer Institute, USA) cell collection, and was cultured in RPMI 1640 medium [26]. All the cells were supplemented with 2 mM L-glutamine, 40 µg gentamycin/mL and fetal bovine serum. Cells were used 48 h after plating.

Porphyrin extraction from cells

Porphyrins accumulated within the cells were extracted twice with 5% HCl, leaving the cells standing for 30 min in the presence of the acid at 37°C. These conditions proved to be the optimal for total PpIX extraction. For media determinations, 5% HCl was added and the fluorescence was measured directly. Fluorescence emission spectra from chemically extracted porphyrins were recorded using the Perkin-Elmer LS 50B fluorescence spectrophotometer. Spectral scans discarded the presence of porphyrins other than PpIX. Consequently, the fluorescence of PpIX was determined using excitation and emission wavelengths of 406 nm and 604 nm, respectively. PpIX (Frontier Sciences, Logan, Utah, USA) was used as a reference standard.

PDT treatment

Cells were incubated in serum-free medium containing ALA or ALA conjugates and 3 h later irradiations were performed. After irradiation, medium was replaced by ALA-free medium and serum, the cells were incubated for another 19 h and then tested for viability.

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 FieldMaster power meter and a LM3 HTP sensor (Coherent Inc., USA). We used fluences between 20 and 80 mJ/cm2 and power density was 0.5 mW/cm2. Median lethal light doses (LD50) were calculated as mJ/cm2, leading to 50% of cell viability.

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MTT viability assay

Phototoxicity and cell viability was determined by the MTT assay (Denizot), 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 the absorbance was read at 560 nm.

Statistical analysis

The values in the figures and tables are expressed as mean ± standard deviations of the mean of three independent experiments performed in duplicates. A two-tailed Student's t-test was used when appropriate to determine statistical significance between means. Statistical analysis and curve fittings were performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

RESULTS AND DISCUSSION

As stated above, we decided to synthesize prodrugs of general structure **1** following the general synthetic procedure shown in Scheme 1, which is based on a three-component Passerini reaction (3C-PR) as the key step. ALA was protected as its carboxybenzyloxy derivative **2**, leaving the carboxylate group free, which participated as the carboxylic component in the 3C-PR. Parallel reactions with different isocyanides and an excess of formaldehyde lead to a series of conjugates **3a-j**. The higher reactivity of formaldehyde avoided the competitive reaction of the keto moiety present in **2** as the carbonyl component in the 3C-PR. The reactions took place smoothly in methanol or ethanol in good yields (63 to 91%) with a diverse set of both aliphatic and aromatic isocyanides. These isocyanides were selected aiming to modulate the lipophilicity of the final prodrugs, in order to cover a wide range of logD (Table 1).

Scheme 1. Synthesis of the new ALA conjugates. *Reactives and conditions: i*) Benzyloxycarbonyl chloride / MeOH, 0°C to r.t. *ii*) R-NC / MeOH / HCOH, r.t. *iii*). H_2 (1 atm.) / Pd black / MeOH-$CHCl_3$ 10:1, r.t.

Deprotection by hydrogenolysis of these intermediates gave the desired conjugates **1**. The only exception was **3i** that, along with the deprotection, suffered the debromination of the aromatic ring to give compound **1k** (Scheme 2). To avoid this undesirable outcome, the hydrogenolysis conditions

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were changed according to that reported by Kiso et al [27]. Using this procedure, compound **1i** was obtained as the only product.

Scheme 2. Alternative deprotection of compound **3i.** *Reactives and conditions: i*) H_2 (1 atm.) / Pd black / MeOH-$CHCl_3$ 10:1, r.t. *ii*) TFA / thioanisole, r.t.

The structures of the new compounds were confirmed both by 1D and 2D NMR spectroscopy and mass spectrometry analysis.

Compound	Structure	logD (pH = 7.4)
1a		**-2.54**
1b		**-1.70**
1c		**-0.82**
1d		**-1.46**
1e		**-0.72**
1f		**-0.36**
1g		**0.01**
1h		**0.37**
1i		**0.77**
1j		**3.69**

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1k	[chemical structure]	0.01
ALA	[chemical structure]	-3.37

Table 1. Structures of the new esters of ALA with their estimated logD.

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In order to investigate the ability of the ALA conjugates to generate the photosensitizer PpIX, porphyrins were extracted from either murine mammary adenocarcinoma LM2 cells, IGROV-1 ovary carcinoma cells or HaCaT normal keratinocytes after incubation during 3 h, the standard time in most ALA-based PDT clinical protocols[28], with compounds **1a-k** and **3a–j**. The fluorescence intensity of the maximum peak emission at 604 nm was measured (Figure 3). No peak shifts were observed in spectral scans of the porphyrin formed from ALA derivatives as compared to that of ALA.

Porphyrin synthesis is dependent on ALA concentration. We previously reported that above a saturation point of about 0.3 mM of ALA, a plateau porphyrin value is reached, which cannot be surpassed due to a tight mechanism of enzyme regulation [6,29]. Since our initial screening was aimed at identifying the compounds that were able to produce a higher porphyrin level than ALA did, we tested them at two concentrations: 60 μM, which is lower than the dose of ALA needed to reach the plateau of porphyrins formation, and at a 10-fold higher concentration, above the saturation point.

Figure 3. Screening of PpIX synthesis from ALA conjugates at 60 and 600 μM concentrations in three cell lines. Cells were exposed to the compounds during 3 h. Intracellular PpIX levels were determined fluorometrically and normalized per number of cells.

Interestingly, none of the CBz-protected conjugates enhanced significantly the synthesis of PpIX when compared to ALA at both concentrations (data not shown). Taking into account that their calculated logD spanned from 1.50 to 8.70 (data shown in the Supplementary Material), these results may suggest that these compounds were too lipophilic to permeate the cell. As an alternative explanation, the carbamate moiety may not be hydrolyzed by the intracellular enzymes.

On the other hand, at the highest concentration tested, all of the compounds having the free amino group were able to being processed by intracellular esterases and releasing ALA, since a significant porphyrin production was observed. As expected, none of the conjugates surpassed the PpIX levels induced by ALA itself, since, as stated previously, at 0.6 mM a plateau for porphyrin synthesis is reached [6]. The rate of PpIX production for each compound may be reflecting the diffusion kinetics of

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the conjugates through the cell membranes; interestingly, the two compounds that showed the lowest activities were those with extreme logD (compounds **1a** and **1j**). Alternatively, the efficiency of conversion of the compounds into ALA by cell esterases may play a role in the different behaviour observed. It is interesting to note that porphyrin production is remarkably lower in HaCaT cells (between 6 and 10 ng of porphyrin / 10^5 cells) compared to LM2 and IGROV-1 cells (between 27 and 70 ng of porphyrin / 10^5 cells), suggesting that these compounds might elicit a differential activity between cell lines.

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Remarkably, at 60 μM, some of the new conjugates (compounds **1f**, **1g**, **1h**, **1i** and **1k**) induced a significant higher production of porphyrin than ALA on the three cell lines under study. Since porphyrin plateau values are limited by enzymatic regulation, we established the concentration leading to plateau values as a more suitable PDT efficiency predictor.

Afterwards, we determined the dose-response curve for porphyrin production when LM2 cells were exposed to a wider range of concentrations of ALA and incubated for 3 h. Figure 4A shows that the maximum achievable amount of porphyrin, under these conditions, was 51 ± 2 ng / 10^5 cells. From the curve fitting, the concentration of ALA needed to reach half this maximum (EC_{50}) was estimated to be 116 μM. When the same experiment was performed with compound **1i**, a similar dose response curve was observed, but although the same plateau was reached (53 ± 2 ng of porphyrins / 10^5 cells), a significantly lower EC_{50} of 31 μM was observed.

Dose – response curves for the other compounds that proved to enhance the porphyrin synthesis compared to ALA at 60 μM are depicted in Figure 4B. As expected, the same plateau was reached for each compound, whereas their EC_{50}s were estimated to be 106 μM (**1f**), 62 μM (**1g**), 44 μM (**1h**) and 45 μM (**1k**).

Figure 4. PpIX as a function of concentration of ALA and ALA conjugates in LM2 cells. Cells were exposed to different concentrations of the compounds during 3 h. Intracellular PpIX levels were determined fluorometrically and normalized per number of cells.

Finally, LM2 cells were incubated for 3 h at different concentrations of the compounds and subjected to photodynamic treatment. The efficacy of the new conjugates as photosensitizers strongly correlates with the amount of porphyrins formed at a given concentration. Figure 5 shows the outcome for the most active compounds (**1g**, **1i**, **1h** and **1k**) at four different concentrations. At the lower one (0.04 mM, Figure 5a), LD_{50} for compound **1i** was estimated to be 23 mJ/cm^2, whereas no

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cell death was induced by ALA in the same conditions. For comparison, the clinically useful Hex-ALA was also evaluated, which showed a LD_{50} of about 17 mJ/cm^2. It is worth noting that compounds Hex-ALA and **1i** share a very similar calculated logD (0.79 and 0.77, respectively), and that both compounds showed comparable LD_{50}s at all of the concentrations tested.

On the other hand, only at relatively high ALA concentrations cell death from ALA-induced porphyrins became evident, with a LD_{50} of around 50 mJ/cm^2 at 100 μM (Figure 5d). Interestingly, even though compounds **1g** and **1k** are isomers having the same calculated logD, they behave differently, at least in the LM2 cell line, suggesting that structural factors other than the lipophilicity must be playing a role on their activity.

Figure 5. Cell survival after PDT. LM2 cells were incubated with different concentrations of the compounds during 3 h. Afterwards, PDT was performed, and cell viability was evaluated by the MTT assay, as percentage of control non-irradiated cells.

In summary, in this work we have demonstrated that the Passerini three-component reaction is a powerful tool for the synthesis of esters of ALA which can be linked to a set of structural fragments leading to a chemically diverse library of compounds. The suitability of this approach was illustrated by the fact that even within the small set of compounds we designed, we were able to find a new promising conjugate with a similar *in vitro* photodynamic efficiency when compared to Hex-ALA. It can be envisioned that this straightforward and original approach for the synthesis of esters of ALA would allow to modulating the physicochemical properties of the conjugates, such as charge, lipophilicity and specificity towards different cellular types, hence improving the current ALA-based photodynamic therapy. Our ongoing research includes fluorescence microscopy studies on spheroid cultures of different cell lines, in order to find a correlation between lipophilicity of the compounds and the degree of diffusion to the inner region of the 3D cultures. Furthermore, studies on *in vivo* murine models are under way to validate the promising results presented here.

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SUPPORTING INFORMATION

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Contains detailed synthetic procedures, spectra for new compounds (NMR and MS) and additional biological data.

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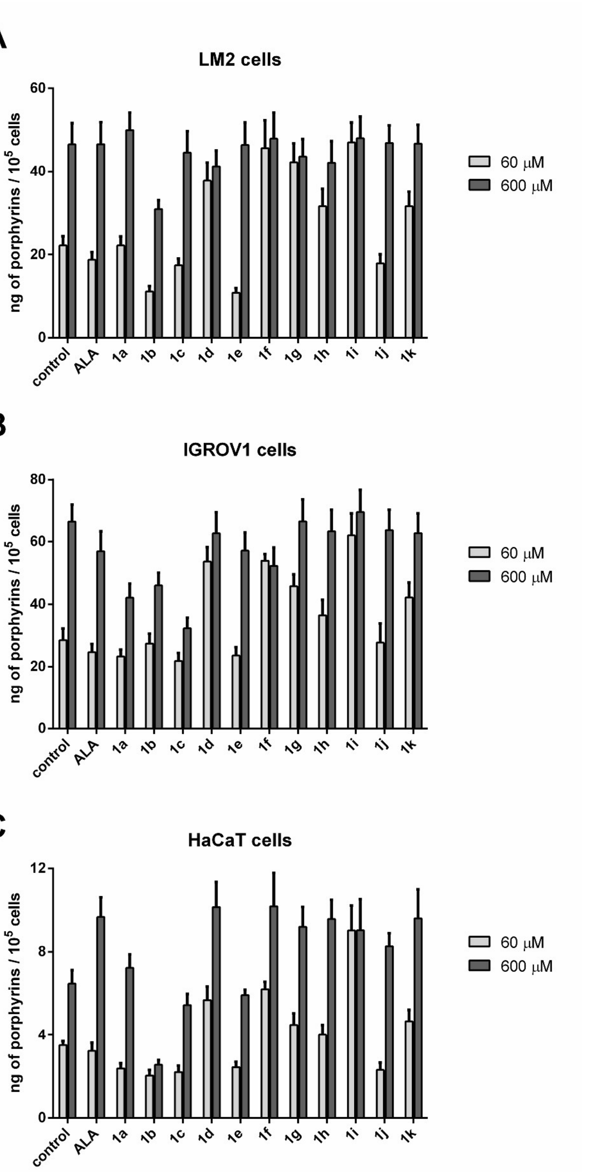

Figure 3
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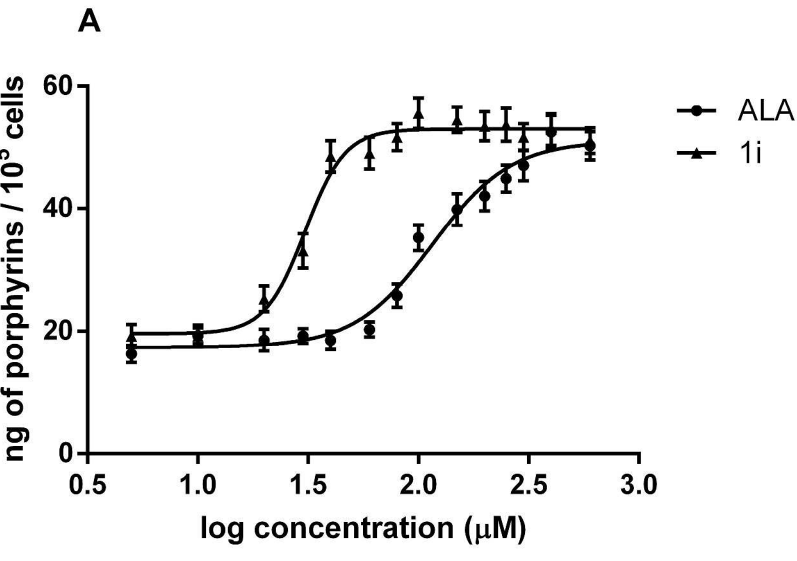

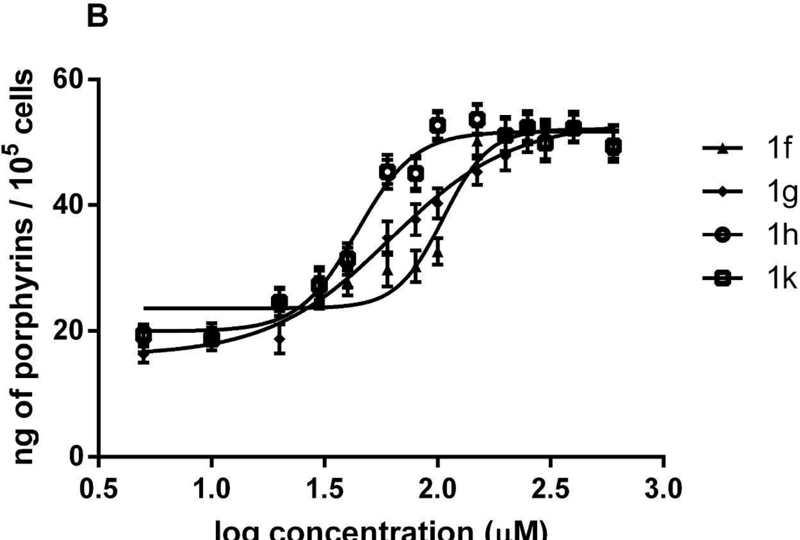

Figure 4
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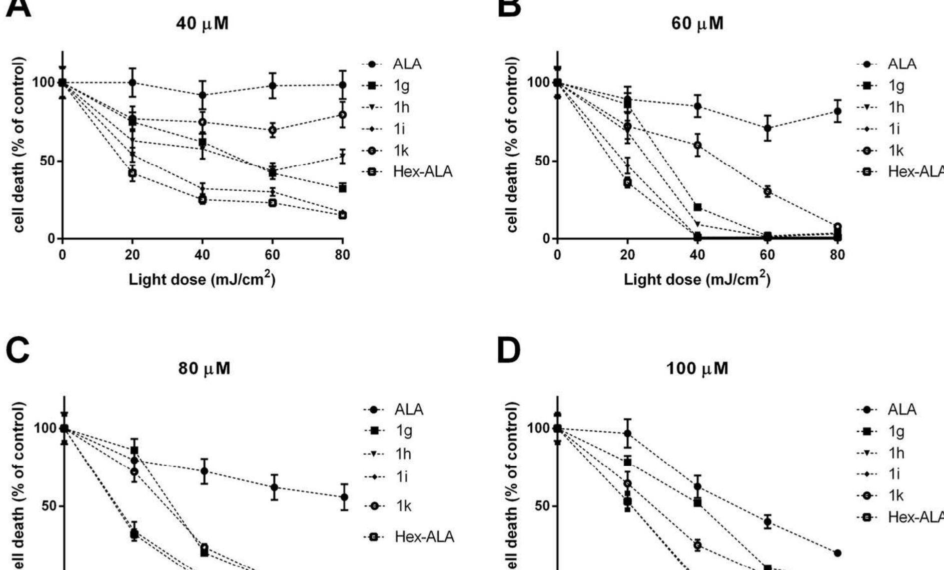

Figure 5
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A chemically diverse set of 5-aminolevulinic acid prodrugs were obtained via a Passerini reaction and studied as photodinamic agents *in vitro*.

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