Photodynamic therapy of cutaneous T-cell lymphoma cell lines mediated by 5-aminolevulinic acid and derivatives

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Abbreviations: ALA: 5-aminolevulinic acid; CTCL: Cutaneous T-cell lymphoma; EC50: half-maximum effective concentration; He-ALA: ALA hexyl ester; LD50: light dose inducing 50% of cell death; Me-ALA: ALA methyl ester; MF: mycosis fungoides; PDT: Photodynamic Therapy; PpIX: Protoporphyrin IX; SS: Sézary syndrome.

Abstract

The delta-amino acid 5-aminolevulinic acid (ALA), is the precursor of the endogenous photosensitiser Protoporphyrin IX (PpIX), and is currently approved for Photodynamic Therapy (PDT) of certain superficial cancers.

However, ALA-PDT is not very effective in diseases in which T-cells play a significant role. Cutaneous T-cell lymphomas (CTCL) is a group of non-Hodgkin malignant diseases, which includes mycosis fungoides (MF) and Sézary syndrome (SS).

In previous work, we have designed new ALA esters synthesised by three-component Passerini reactions, and some of them showed higher performance as compared to ALA. This work aimed to determine the efficacy as pro-photosensitisers of five new ALA esters of 2-hydroxy-N-arylacetamides (1f, 1g, 1h, 1i and 1k) of higher lipophilicity than ALA in Myla cells of MF and HuT-78 cells of SS. We have also tested its effectiveness against ALA and the already marketed ALA methyl ester (Me-ALA) and ALA hexyl ester (He-ALA).

Both cell Myla and SS cells were effectively and equally photoinactivated by ALA-PDT. Besides, the concentration of ALA required to induce half the maximal porphyrin synthesis was 209 µM for Myla and 169 µM for HuT-78 cells.

As a criterion of efficacy, we calculated the concentration of the ALA derivatives necessary to induce half the plateau porphyrin values obtained from ALA. These values were achieved at concentrations 4 and 12 times lower compared to ALA, according to the derivative used. For He-ALA, concentrations were 24 to 25 times lower than required for ALA for inducing comparable porphyrin synthesis in both CTCL cells.

The light doses for inducing 50% of cell death (LD50) for He-ALA, 1f, 1g, 1h and 1i were around 18 and 25 J/cm^2 for Myla and HuT-78 cells respectively, after exposure to 0.05 mM concentrations of the compounds. On the other hand, the LD50s for the compound 1k were 40 and 57 J/cm^2 for Myla and HuT-78, respectively. In contrast, 0.05 mM of ALA and Me-ALA did not provoke photokilling since the concentration employed was far below the porphyrin saturation point for these compounds.

Our results suggest the potential use of ALA derivatives for topical application in PDT treatment of MF and extracorporeal PDT for the depletion of activated T-cells in SS.

1.Introduction

Photodynamic therapy (PDT) is a curative treatment for superficial or easy accessible cancers based on the selectivity of a photosensitiser accumulated in tumour tissue [1][2]. Photoactivation of this molecule in the presence of oxygen induces cytotoxic reactions mediated by reactive oxygen species (ROS) [3][4].

The natural pro-photosensitiser 5-aminolevulinic acid (ALA), is the precursor of the endogenous photosensitiser Protoporphyrin IX (PpIX) synthesised by the haem pathway enzymes [5][6]. ALA-PDT is increasingly being used clinically in dermatologic [7] and non-dermatologic malignancies [8].

However, while ALA-PDT is quite useful for the treatment superficial malignant or pre-malignant skin conditions such as actinic keratoses, basal cell carcinomas, and squamous cells carcinomas, the response is less effective in diseases in which T-cells play a significant role, such as chronic psoriasis, lichen planus and cutaneous T-cell lymphomas (CTCL) [9].

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 heterogenous distribution in target tissues [10]. One approach to enhance ALA penetration is the use of ALA lipophilic derivatives, such as alkyl or ethylene glycol esters, which are potential substrates for cellular esterases [11][12][13]. ALA esters as well as dipeptide derivatives with a free amino group at the end are stable at physiological pH and enter the cells by passive diffusion and are still water-soluble [14][15].

ALA methyl ester (Me-ALA) has been licensed for the treatment of actinic keratoses, basal cell carcinoma and Bowen´s disease. The ALA hexyl ester (He-ALA) has been accepted by the FDA and EMA for its application as an optical imaging agent in fluorescence cystoscopy to aid in the detection of bladder cancer [8].

The success of these derivatives facilitated the development of various modified ALA molecules with different substituent groups, including cyclic or aromatic esters, ethylene glycol groups, halogens, and sugars [16][17][18][17] and phosphatase-sensitive ALA produgs [19].

CTCL is a class of malignant non-Hodgkin disorders, among which, mycosis fungoides (MF) and Sézary syndrome (SS) are the most represented pathologies. These diseases exhibit intradermal lymphoid infiltrates in the first stages, which firstly invades the dermis, the subcutaneous tissue, and ultimately reaches nearby organs at advanced stages [20]. CTCLs are considered orphan diseases with few treatments available [21]. Light mediated therapies such as 8-methoxypsoralen plus UVA illumination, narrowband UVB irradiation, and extracorporeal photochemotherapy are indicated. Photodynamic treatment is especially useful in the treatment of early-stage disease, in particular unilesional MF lesions, which generally does not advance to systemic disease, especially in the case of relapses or resistant lesions [22].

Xue et al performed a systematic review of the clinical applications of PDT in relapsed or refractory MF. They concluded that PDT is a non-invasive, selective and non-toxic treatment with good cosmetic results, which induces negligible general photosensitivity and low carcinogenicity. These advantages make PDT a valuable therapeutic option in MF lesions of difficult treatment, such as relapsed or refractory MF, or even in sensitive areas such as the face and neck [23].

The PDT subgroup of the European Dermatology Forum guidelines committee in 2015 suggested a potential for PDT in localised patch/plaque CTCL, with a possible indication for lesions in the body areas that cannot be exposed to phototherapy [24].

A few studies reported success in the treatment of MF patients with ALA-PDT or Me-ALA-PDT, particularly with one or a few lesions [25][26][27][28]. In addition, malignant T lymphocytes from CTCL patients exposed to ALA exhibit higher PpIX levels as compared to normal lymphocytes [29]. However, ALA-PDT is not always satisfactory for CTCL treatment [9] and, even the technique is relatively simple, the optimal photosensitiser and light dosages have though not yet been determined [27].

Therefore, there is an increasing need to develop new efficient photosensitisers for this orphan disease.

In previous work, we have designed new ALA esters of 2-hydroxy-N-arylacetamides obtained by a three-component Passerini reaction. Some of these molecules were preliminarily tested for their capacity to induce porphyrin synthesis in breast cancer and ovary cancer cells, showing increased photodynamic efficiency as compared to the ALA molecule [30].

This work aimed to determine the efficacy as pro-photosensitisers of 5 ALA esters of 2-hydroxy-N-arylacetamides of higher lipophilicity than ALA against ALA and the derivatives approved for clinical use He-ALA and Me-ALA.

2. Materials and Methods

2.1 Reagents

He-ALA was prepared by the method of Takeya [31] as described in previous work [32]. ALA esters of 2-hydroxy-N-arylacetamides were prepared from ALA using a multicomponent Passerini reaction, as explained previously [30] (Table 1). ALA and Me-ALA were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Calculator Plugins were employed for log D predictions, Marvin 6.3.0, 2014, ChemAxon.

2.2 Cell lines

The Myla cell line was obtained from a plaque of a patient with MF [33] and the HuT-78 cell line was originated from cells obtained from the blood of a patient with SS [34]. They were kindly provided by Dr Maria R Kamstrup, from Bispebjerg Hospital, Copenhagen, Denmark. They were cultivated in suspension in RPMI 1640 medium with L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate at 37°C and 5% CO_2. Cells were collected by centrifugation, and the initial cell density was adjusted to 3 x 10^5 cells/ml and seeded in 24 well-plates.

2.3 PDT treatment

Cells were incubated in medium without serum containing ALA or derivatives for 3 h and then, illuminated from below. Immediately after irradiation, the medium was replaced

by a centrifugation step at 800 x g, and the cells were left for 19 h at 37°C to let photodamage occur and then tested for viability. Light doses inducing 50% of cell death (LD50) for each pro-photosensitiser were determined as the fluences (mJ/cm^2)

2.4 MTT viability assay

PDT efficacy was determined by the MTT assay [35]. The cells were incubated 1 h with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide), and the violet resulting formazan was collected after centrifugation at 2000 x g, dissolved in DMSO, and quantified at 560 nm in an Epoch microplate reader (BioTek, USA).

2.5 Light source

Two fluorescent lamps (Osram L 18W/765) were used as the light source (400 nm to 700 nm range with the major emission at 600 nm). The cells were placed at 14 cm from the light source. They were illuminated from below with a power density of 0.5 mW/cm^2 (FieldMaster power meter, LM3 HTP sensor, Coherent Inc., USA.

2.6 Intracellular porphyrin determination

Intracellular porphyrins obtained from ALA or derivatives were extracted with 5% HCl employing a modification of the method described previously [14]. The cells were centrifuged at 3000 x g after acid extraction, and afterwards, the pellet was discarded, and the supernatant was kept for porphyrin determinations. The fluorescence signal was recorded in a Perkin Elmer LS55 fluorometer (Buckinghamshire, UK) (λ ex= 406 nm and λem= 604 nm). A solution of PpIX in 5% HCl (Frontier Scientific, Logan, US) was employed as standard. The half-maximum effective concentrations of the ALA or derivatives (EC50s) were determined as the concentrations leading to half the maximal porphyrin values.

2.7 Statistical analysis

The experiments were performed in duplicates in 3 independent experiments, and the results were displayed as mean values ± standard deviations. Data were statistically analysed using the software GraphPad Prism version 8.02, GraphPad Software (La Jolla, CA, USA). Two-way ANOVA tests with a *p*-value < 0.05 established statistically significant differences among curves. The EC50 values were calculated fitting the amount

of porphyrin synthesised as a function of the log of the concentration of ALA or the derivative using the log (inhibitor) vs response (three parameters) model.

3. Results

3.1 Porphyrin production from ALA and response of CTCL cells to ALA-PDT

Myla and HuT-78 cells efficiently converted ALA to porphyrins, and this biosynthesis increased as a function of the ALA concentration until reaching a maximum level (plateau) of porphyrin production (Figure 1). Porphyrin plateau values were obtained at 1.1 mM ALA in both cell lines, and the porphyrin amounts of 48.0 ± 2.3 and 45.1 ± 3.5 ng/10^5 cells were attained for HuT-78 and Myla, respectively. The profile of porphyrin synthesis as well as the maximal porphyrin values was similar in both cell lines. Upon irradiation of the cells under porphyrin plateau conditions, LD50s calculated were 19.3 mJ/cm^2 for Myla and 20.4 mJ/cm^2 for HuT-78, thus showing that the responses to ALA-PDT were not significantly different between the MF and the SS derived cell lines.

3.2 Porphyrin production from ALA derivatives in CTCL cells

Figures 2 and 3 show the profile of porphyrin synthesis as a function of the log concentration of ALA and its derivatives in Myla and HuT-78 cells.

From the curve fitting, the concentration of ALA required to reach half the maximal porphyrin synthesis (EC50) in Myla cells was 209 µM. The same plateau porphyrin value was obtained employing the ALA derivatives, though this value was obtained at different concentrations. Whereas the curve of porphyrin induction from Me-ALA was statistically not different from that from ALA ($p>0.05$, ANOVA), the rest of the derivatives induced plateau values at lower concentrations as compared with ALA ($p<0.05$, ANOVA), Compound 1i appeared to be the most effective, with an EC50 of 11.9 µM. However, the curves of compounds 1g, 1f, 1h, and 1i did not show significant differences among them, showing varying EC50s in the 12 to 20 µM range. The compound 1k was significantly less effective than the rest. Besides, He-ALA was significantly more effective than the rest of the compounds, exhibiting an EC50 of 7 µM (Table 2).

Similar results were obtained in HuT-78 cells. He-ALA was the most effective compound (EC50= 5.3 µM), followed by compound 1g (EC50=14.5 µM). Equally effective were the compounds 1g, 1f, 1h and 1i (EC50 14 to 21 µM range) and 1k was less effective than the rest. Similar to the profile of Myla cells, Me-ALA exhibited non-significant differences as compared to ALA in terms of porphyrin biosynthesis in HuT-78 cells (EC50s of 168 and 136 µM for Me-ALA and ALA respectively) (Table 2).

Considering that porphyrin biosynthesis is a saturable enzyme pathway, as a criterion of efficacy, we calculated the concentration of the ALA derivatives necessary to induce the same plateau porhyrin values obtained from ALA. Therefore, concentrations 4 to 12 times lower than ALA were needed to attain plateau porphyrin synthesis from the ALA esters of 2-hydroxy-N-arylacetamides. Besides, concentrations of He-ALA 24 to 25 times lower than ALA were required to induce porphyrin plateau values in Myla and HuT-78 cells.

3.3 CTCL cells response to PDT mediated by ALA derivatives

Since our initial experiment was aimed at identifying the compounds that were able to produce porphyrin levels at lower concentrations than ALA, we tested them at two concentrations: 0.05 mM, which is lower than the ALA derivative needed to reach the plateau of porphyrins formation, and 0.25 mM, which is above the saturation point.

Myla and HuT-78 cells were exposed to sub-plateau concentration values for ALA (0.05 mM), the cells were illuminated, and the photodamage response was calculated (Figures 4A and 5A). Under these conditions, He-ALA, 1f, 1g, 1h and 1i induced 50% of cell death upon illumination with fluences around 18 J/cm^2 in Myla and 25 J/cm^2 in HuT-78 cells. On the other hand, the calculated LD50s for 1k were 40 and 57 J/cm^2 for Myla and HuT-78 respectively. ALA and Me-ALA did not induce any significant cell death in the fluence range assessed, since a threshold porphyrin concentration is required to elicit a photodynamic response. Therefore, the higher the amount of porphyrins, the higher the phototoxicity in both cells lines, independently of the pro-drug employed.

At a later stage, PDT was carried out employing plateau concentrations for the new conjugates (0.25 mM) (Figures 4B and 5B). Under these conditions, all the ALA esters of 2-hydroxy-N-arylacetamides induced a similar degree of cell death in Myla cells,

whereas HuT-78 cells were slightly more refractory to PDT, exhibiting LD50s ranging from 20 to 24 J/cm^2. Since 0.05 mM does not saturate porphyrin biosynthesis either in ALA or in Me-ALA-treated cells, illumination of these cells did not induce cell killing beyond 20%.

4. Discussion

The present work describes the effectiveness of ALA and different novel ALA conjugates, as pro-drugs of PpIX in CTCL cell lines. We also compared the new compounds against the two derivatives of ALA approved for clinical use. The new ALA esters of 2-hydroxy-N-arylacetamides were synthesised by multicomponent reactions. This technique is based on multicomponent reactions, which is inexpensive, simple, and versatile, enables modulation of the physicochemical properties of the conjugates, such as charge, lipophilicity and specificity towards different cellular targets [30].

A few studies have reported complete or partial responses of MF to PDT employing pro-photosensitisers of PIX. Most of them, describe the success of PDT mediated by ALA [25][26][36] or Me-ALA [37][28][38] in the management of plaque-type MF (stage I) and the poor response of tumour-type MF (stage II) [39].

The mode of action of PDT in CTCL response remains unknown since the contribution of direct tumour cell death, and the role of inflammatory cells in the response has not been elucidated [24]. Besides, the optimal parameters of photosensitiser and light dose remain to be determined [27].

ALA easily permeates the disrupted stratum corneum overlying CTCL, and a PpIX fluorescence ratio of 5:1 between MF plaque to normal skin [40]. Furthermore, the PpIX fluorescence after application of Me-ALA proved to be useful in the diagnosis of MF, and follow-up of the response to the different treatments [38]. However, it was reported that PpIX fluorescence was weak and diffuse in patch and plaque disease and strong in tumour stage lesions [25].

The level of PpIX synthesised from ALA was also stronger in activated T-cells than in resting cells from SS patients [41]. Besides, *ex vivo* exposure to ALA of leukocytes of SS

patients and ulterior illumination employing ultraviolet-A light-induced quite selective photokilling of malignant T-cells. Therefore, ALA-UVA was proposed as a possible alternative to extracorporeal photopheresis employing 8-methoxypsoralen and ultraviolet-A light.

In previous work we have reported a much higher response to ALA-PDT and higher PpIX synthesis in activated murine splenic B lymphocytes in comparison with non-activated cells [42]. Similar studies were reported by other authors in human lymphocytes [43]. This fact is relevant since CTCL is occasionally infiltrated with B lymphocytes and it has been hypothesised that these infiltrating B cells play an essential role in CTCL and that local depletion of B cells induce the recovery of the immune control [44].

It is well known that porphyrin synthesis depends on the ALA concentration and, beyond a saturation point, plateau porphyrin values are attained. These maximal porphyrin values cannot be surpassed due to tight heam enzymes regulation [45]. As expected, none of the ALA esters employed herein, surpassed the amount of PpIX produced by ALA.

Particularly in the case of CTCL cells studied in the present work, Me-ALA showed a porphyrin synthesis profile similar to that of ALA, while He-ALA generated the plateau value of porphyrins using concentrations 24 to 25 times lower than ALA, demonstrating its high efficacy of photosensitisation on CTCL cells.

On the other hand, employing the compounds 1i, 1f, 1g and 1h, around 12-fold lower concentrations than ALA are required to obtain plateau porphyrin levels, whereas employing 1k, concentrations 4 times lower than ALA are needed to reach equal values. Moreover, the ALA derivatives 1f, 1g, 1h, 1i and 1k have been previously shown to be better pro-photosensitisers than the ALA molecule in ovary carcinoma, mammary adenocarcinoma cells and keratinocytes [30].

Interestingly, the compounds 1g and 1k, are isomers and so, they have the same logD [30]; however, 1g is significantly more efficient than 1k in CTCL cells, suggesting that the structure of the derivatives strongly influences either the entry of the compounds, or the release of ALA, and that lipophilicity is not the only parameter involved in the effectiveness of the ALA prodrugs.

All the new ALA derivatives are more lipophilic than ALA, being the logD in the -0.4 to 0.7 range, whereas He-ALA, which is the more effective, has a logD of 0.71. All the tested derivatives of ALA were more efficient than Me-ALA, which is marketed for the treatment of actinic keratoses, basal cell carcinomas and squamous cells carcinomas, and can penetrate the disrupted diseased skin. However, whereas Me-ALA is more lipophilic than ALA (logD ALA= -3.45 vs LogD Me-ALA= -1.41), the rate of uptake into intact cells is not enough as to surpass ALA perfromance.

It is noteworthy that whereas He-ALA is highly toxic upon systemic administration [46], the compound 1h was found to be safe after intraperitoneal injection to mice, although the rest of the compounds remain to be tested. Moreover, 1h was capable of penetrating ovary cancer spheroids to the same extent than He-ALA, corresponding to 3 times the distance of ALA penetration [47].

To sum up, our results support the topical use of ALA derivatives for MF treatment and extracorporeal PDT for the purging of activated T-cells in SS. However, further studies extrapolating these results to *in vivo* experiments are needed to assess these new ALA derivatives as potential pro-photosensitisers for the treatment of CTCL.

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Conflicts of int

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None

5. References

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FIGURE LEGENDS

Figure 1. Synthesis of porphyrins from ALA and response to PDT in CTCL cells
A) Myla and Hut-78 cells were exposed to different ALA concentrations for 3 h. The synthesised porphyrins were determined and expressed per cell number. B) Cells were exposed 3 h to 1.1 mM ALA and illuminated with increasing fluences. Cell viability was calculated as the percentage of the non-illuminated controls.

Figure 2. Synthesis of porphyrins from ALA and derivatives in Myla cells
Myla cells were exposed to different ALA concentrations for 3 h, and the synthesised porphyrins were extracted and quantified.

Figure 3. Synthesis of porphyrins from ALA and derivatives in HuT-78 cells
HuT-78 cells were exposed to different ALA concentrations for 3 h, and the synthesised porphyrins were extracted and quantified.

Figure 4. Cell survival of Myla cells after PDT mediated by ALA or derivatives
Myla cells were exposed for 3 h to 0.05 mM (A) or 0.25 mM (B) ALA or ALA esters. Afterwards, PDT was performed employing increasing fluences and cell viability was determined and calculated as the percentage of the non-illuminated control cells.

Figure 5. Cell survival of HuT-78 cells after PDT mediated by ALA or derivatives
HuT-78 cells were exposed 3 h to 0.05 mM (A) or 0.25 mM (B) of ALA or derivatives. Afterwards, PDT was performed employing different fluences and cell viability was determined and calculated as the percentage of the non-illuminated controls.

TABLES

Table 1. Chemical structure and LogD of ALA, the new ALA esters of 2-hydroxy-N-arylacetamides [30], and He-ALA and Me-ALA.

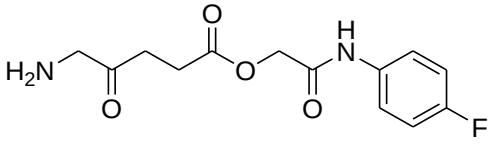
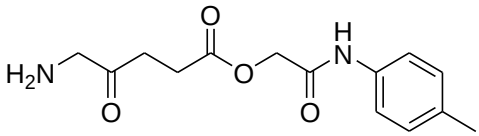
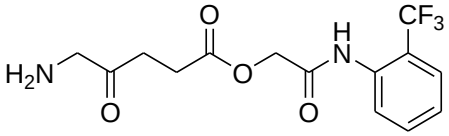
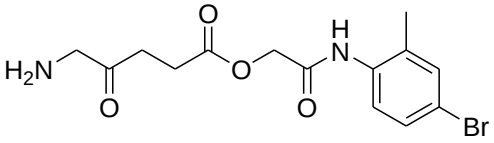
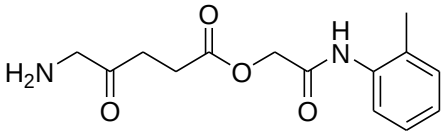
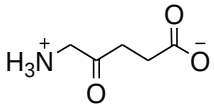
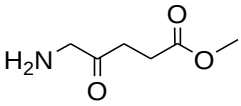
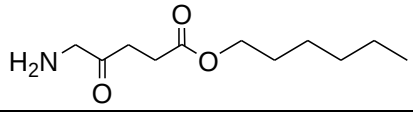
Name	Chemical structure	logD (pH= 7.4)
1f		-0.45
1g		-0.082
1h		0.28
1i		0.69
1k		-0.082
ALA		-3.45
Me-ALA		-1.41
He-ALA		0.71

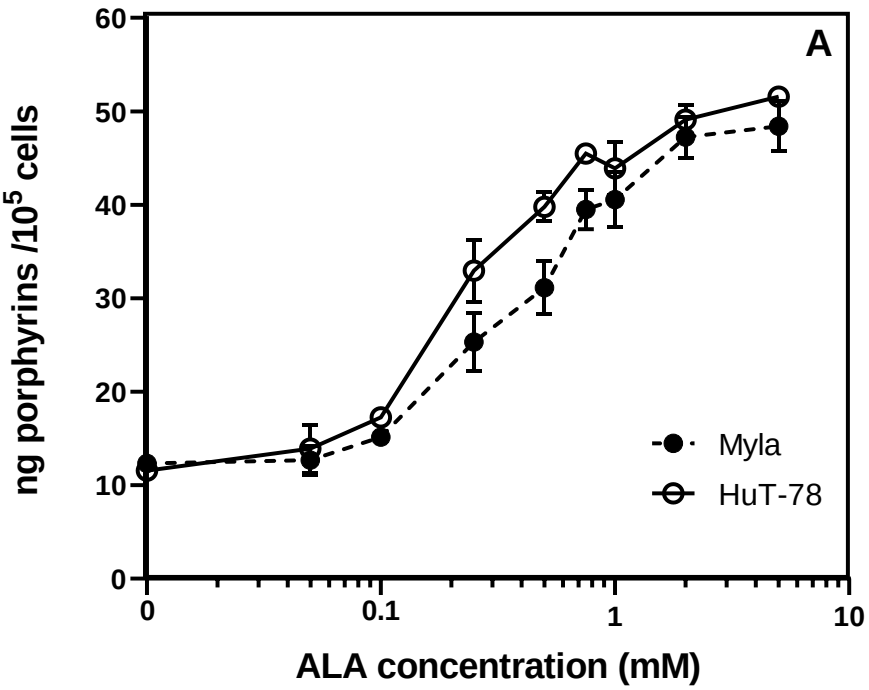
Table 2: EC50 of ALA and derivatives in Myla and HuT-78 cells (µM)

	1f	1g	1h	1i	1k	ALA	He-ALA	Me-ALA
Myla	15.2	14.5	20.3	11.9	42.9	209	7.0	169
HuT-78	21.2	14.5	18.0	20.2	43.2	168	5.3	136

EC50 were calculated from Figures 2 and 3.

FIGURES

Figure 1



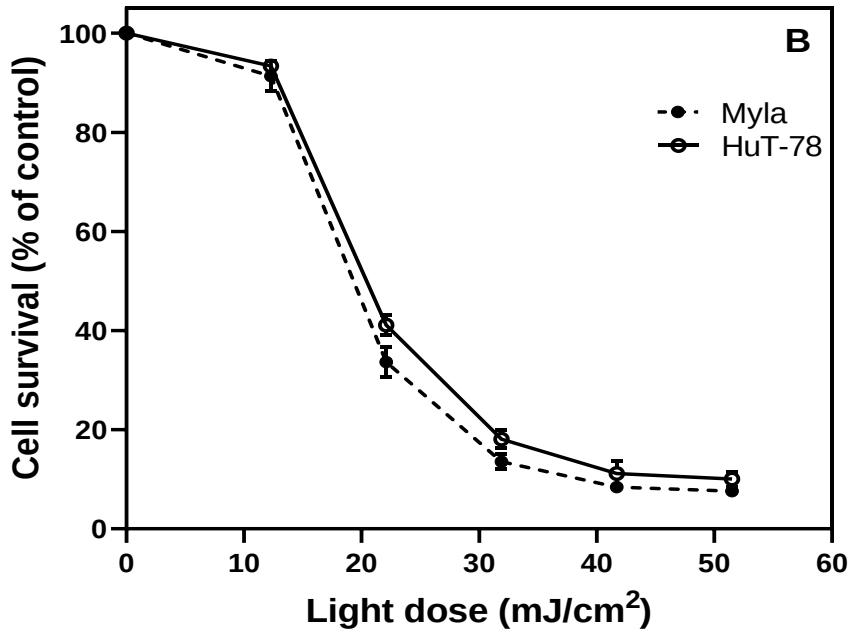
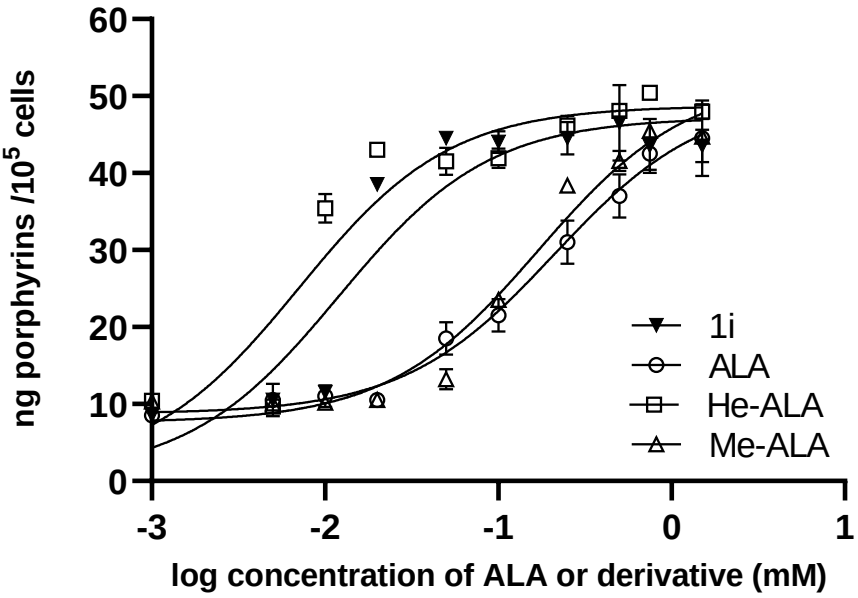


Figure 2


60
50
40
30
20
10
0
-3
-2
-1
0
1
ng porphyrins /10^5 cells
log concentration of ALA or derivative (mM)
1i
ALA
He-ALA
Me-ALA

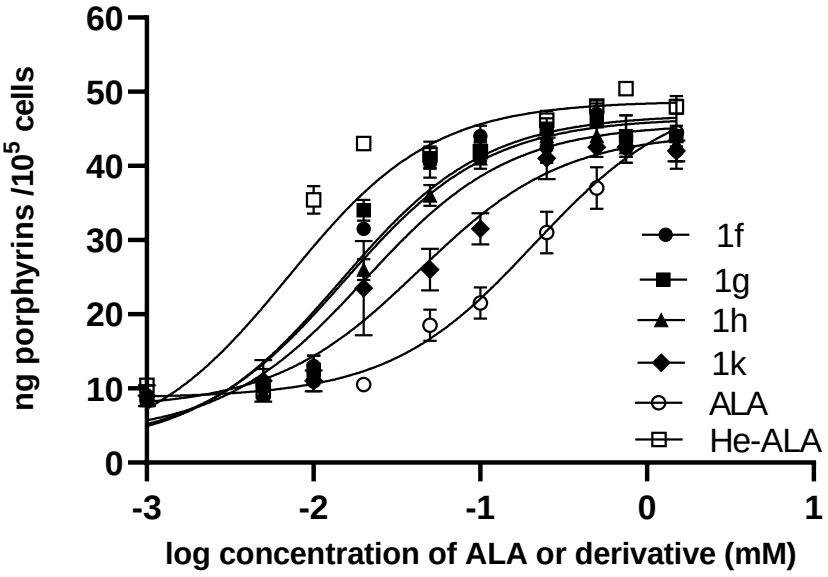
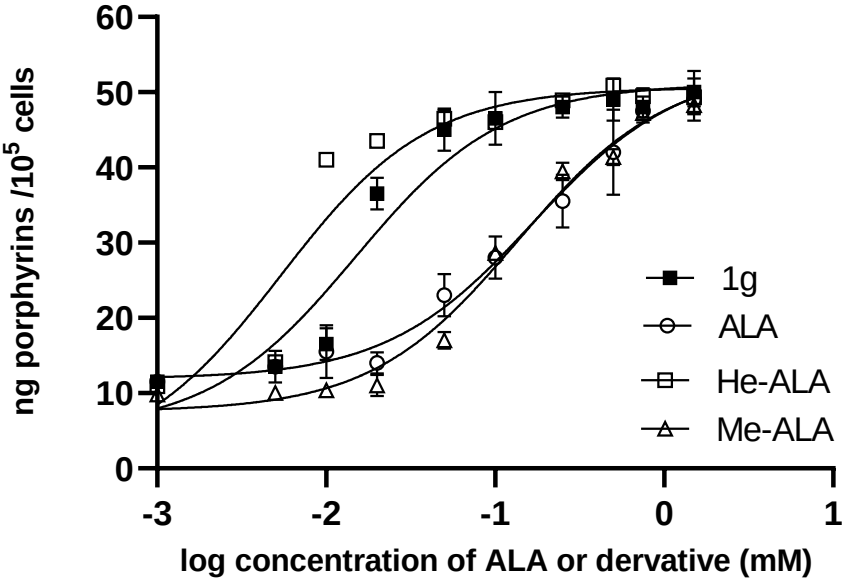

60
50
40
30
20
10
0
-3
-2
-1
0
1
ng porphyrins /10^5 cells
log concentration of ALA or derivative (mM)
1f
1g
1h
1k
ALA
He-ALA

Figure 3


60
50
40
30
20
10
0
-3
-2
-1
0
1
ng porphyrins /10^5 cells
log concentration of ALA or dervative (mM)
1g
ALA
He-ALA
Me-ALA

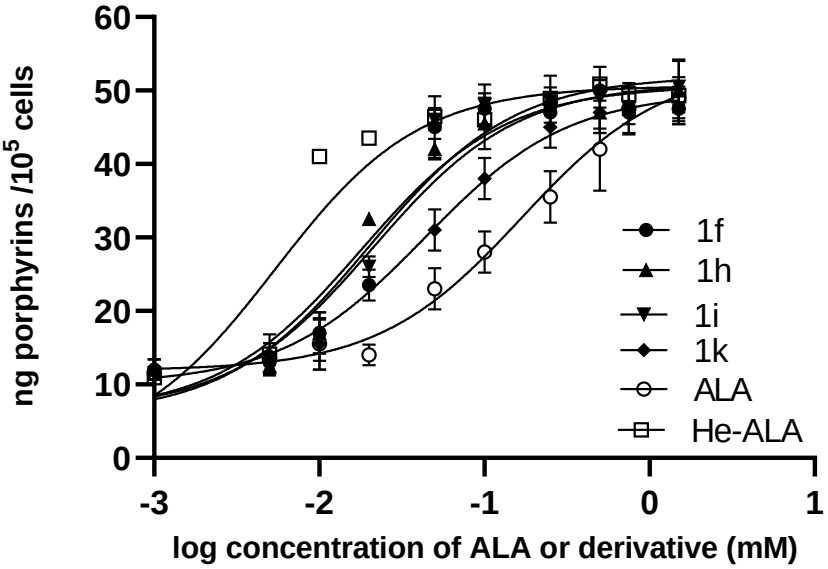

60
50
40
30
20
10
0
-3
-2
-1
0
1
ng porphyrins /10^5 cells
log concentration of ALA or derivative (mM)
1f
1h
1i
1k
ALA
He-ALA

Figure 4

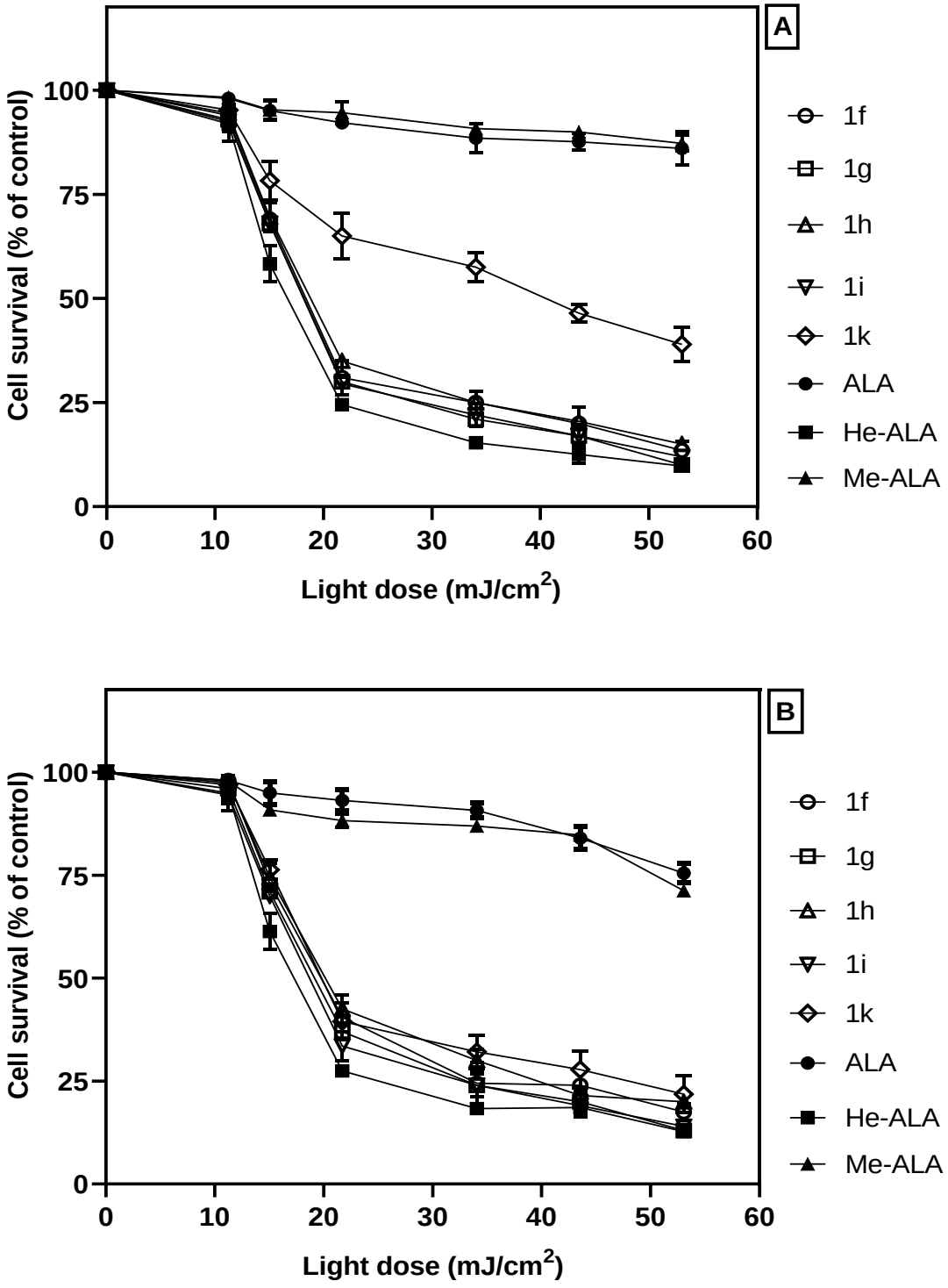
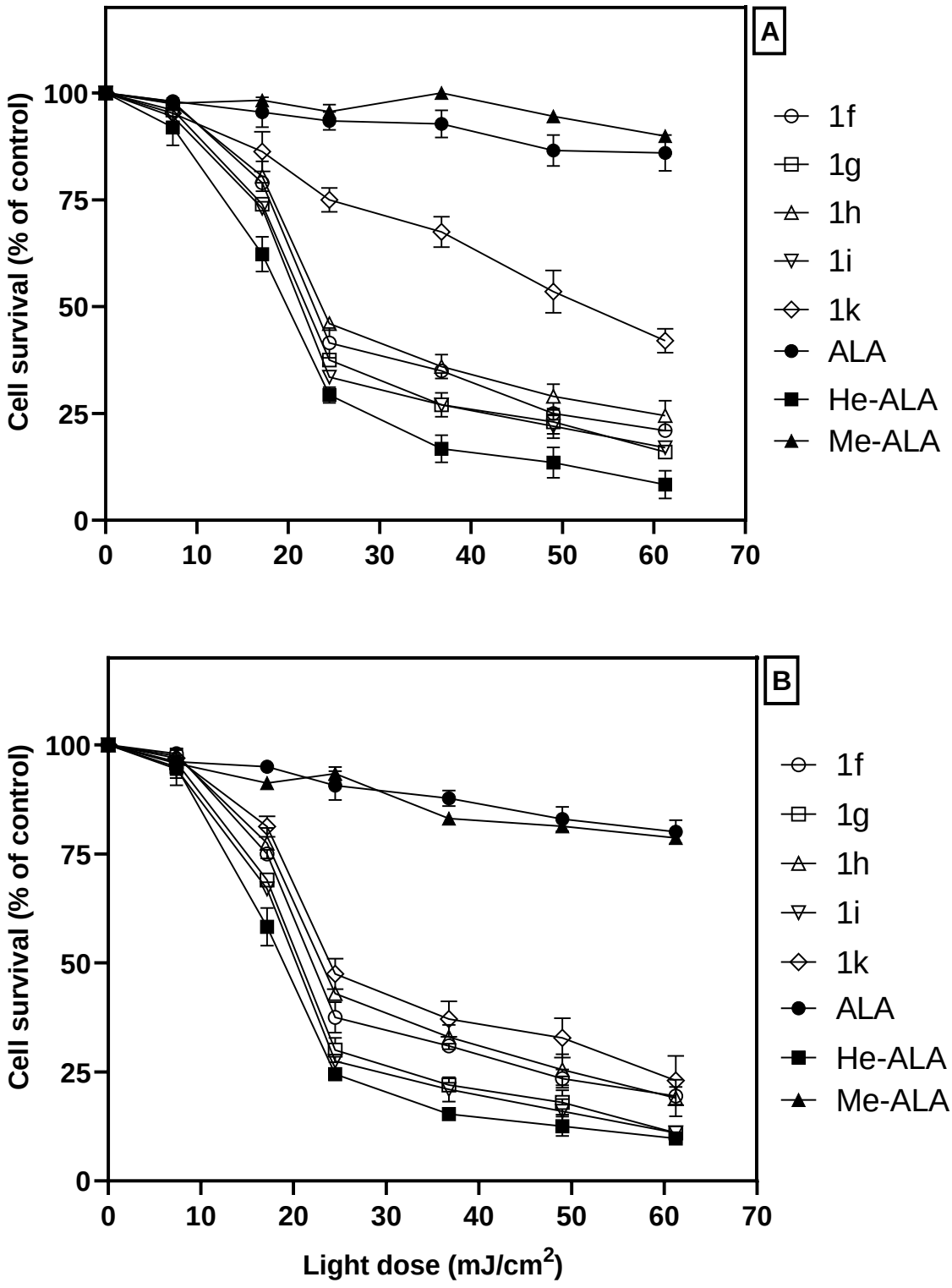

A
Cell survival (% of control)
100
75
50
25
0
0
10
20
30
40
50
60
Light dose (mJ/cm2)
1f
1g
1h
1i
1k
ALA
He-ALA
Me-ALA
B
Cell survival (% of control)
100
75
50
25
0
0
10
20
30
40
50
60
Light dose (mJ/cm2)
1f
1g
1h
1i
1k
ALA
He-ALA
Me-ALA

Figure 5


A
B
Cell survival (% of control)
100
75
50
25
0
0
10
20
30
40
50
60
70
Light dose (mJ/cm2)
1f
1g
1h
1i
1k
ALA
He-ALA
Me-ALA