# Silencing of ALA dehydratase affects ALA-photodynamic therapy efficacy in K562 erythroleukemic cells

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Synthesis of protoporphyrin IX (PpIX) by malignant cells is essential for the success of ALA-based photodynamic therapy (PDT). Two key enzymes that were described as affecting PpIX accumulation during ALA treatment are porphobilinogen deaminase (PBGD) and ferrochelatase. Here, we show that down regulation of ALA dehydratase (ALAD) expression and activity by specific shRNA induced a marked decrease in PpIX synthesis in K562 erythroleukemic cells. Photo-inactivation efficacy following ALA-PDT was directly correlated with ALAD-silencing and cellular levels of PpIX. MTT metabolism following ALA-PDT was shown to be 60% higher in ALAD-silenced cells in comparison to control cells, indicating that mitochondria were protected in the silenced cells. Morphological analysis by scanning electron microscopy (SEM) of cells treated by ALA-PDT showed no morphological changes in ALAD-silenced cells, in contrast to controls exhibiting cell deformations and lysis. Membrane integrity following ALA-PDT was kept intact and undamaged in ALAD-silenced cells as examined by Annexin V-FITC/PI staining and LDH-L leakage. We conclude that ALAD, although it is present in the cell at abundant levels, has a major and limiting role in regulating PpIX synthesis and ALA-PDT outcome.

## Introduction

PDT with δ-aminolevulinic acid (ALA) is widely used for the treatment of nonmalignant and malignant diseases, as well as for skin rejuvenation, and has gained increasing acceptance during recent years.[1,2] ALA-dependent PDT is based on administration of the pro-drug ALA. ALA induces specific synthesis of PpIX, which acts as a natural photosensitizer in afflicted tissues. At present, ALA and ALA esters are used as pro-drugs to treat skin lesions by PDT.[1] Thus, ALA-PDT efficacy depends to a great degree on the rate of PpIX synthesis. Unfortunately, the great variability in PpIX biosynthesis poses limitations to this therapy. The response rates of ALA-PDT vary widely due to suboptimal production of PpIX, which limits the overall anticancer photochemical reactions and accounts for the differences in the clinical responses.[2,9]

PpIX biosynthesis depends on the rate-limiting enzymes of the heme synthesis pathway that takes place in the mitochondria and cytosol. In brief, the first and rate-limiting mitochondrial enzyme, ALA synthase (ALAS), forms ALA from glycine and succinyl Co-A. The second and abundant cytosolic enzyme, ALAD, forms a pyrrole ring, porphobilinogen (PBG). The third cytosolic enzyme is PBGD, which is also rate limiting and catalyses linear tetrapyrrole formation from four PBG molecules. The linear tetrapyrrole is then closed to a porphyrin ring and is converted enzymatically into PpIX. The final and rate-limiting mitochondrial enzyme, ferrochelatase, inserts a ferrous ion into the center of the PpIX molecule, thus creating heme.[16]

The expression levels of the rate-limiting enzymes vary between nonmalignant and malignant cells,[4] thereby affecting the accumulation of the endogenous photosensitizer PpIX in those cells. At present, studies of ALA-PDT have been unable to provide a clear insight into cellular-targeted biochemical processes of the malignant cell that account for the different clinical outcomes. For the clinician, standardized PDT protocols are needed; nevertheless, such protocols must be finely tuned for specific lesions depending on PpIX synthesis capacity in order to improve the clinical outcome. K562 human erythroleukemic cell line is a well-known model for heme synthesis since erythroid cells produce 95% of total heme.[11] Therefore, down regulation of ALAD in these cells is expected to affect ALA-PDT efficacy significantly.

We have shown previously that lead poisoning adversely reduces the efficacy of ALA-PDT, as a result of ALAD inhibition and reduced expression of PBGD.[5] In the present study, we further characterize the effect of ALAD expression on PpIX production and ALA-PDT efficacy by specific silencing of this enzyme.

## Materials and methods

### Cell culture

K562 cells were grown on tissue culture plates (Greiner, Glos, UK) in RPMI-1640 medium (Sigma, Israel), supplemented with 10% fetal calf serum (Biological Industries, Israel), antibiotics (penicillin-streptomycin-nystatin) and amphotericin (Biological Industries, Israel). The cells were incubated at 37 °C in a humidified atmosphere with 5% $CO_2$ and 95% air, and were passaged twice a week.

### Transfection of K562 cells

Two different shRNA plasmids were designed for targeting ALAD mRNA. Hairpin sequences were as shown in Table 1.

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**Table 1** Hairpin sequences in ALAD shRNA plasmids

| Plasmid | Hairpin | Target | Region |
|---|---|---|---|
| ALAD shRNA 2 | TGCTGTTGACAGTGAGCGACCCAAGAACTAGAACTTTAAA-TAGTGAAGCCACAGATGTATTTAAAGTTCTAGTTCTTGG-GCTGCCTACTGCCTCGGA | 1331.1349 | 3′ UTR |
| ALAD shRNA 3 | TGCTGTTGACAGTGAGCGAGCCCAAGAACTAGAACTTTAA-TAGTGAAGCCACAGATGTATTAAAGTTCTAGTTCTTGGG-CCTGCCTACTGCCTCGGA | 1330.1348 | 3′ UTR |

shRNA plasmids as well as a commercial non-silencing shRNA plasmid were obtained from Open Biosystems (AL, USA). The sequence of the non-silencing plasmid is coincidental and is not found in the human genome; therefore it is used as an internal control for transfection. K562 cells ($10^6$) were transfected using JetPEI reagent (Poly Plus, France) with 10 μg vector DNA. For stable transfection, the transfected cells were selected using 0.4 μg ml$^{-1}$ puromycin 48 h following transfection. Cells were consistently cultured in the presence of antibiotics.

### Western blotting

Cell lysates were run on SDS-PAGE as previously described.[6] Following blocking, membranes were incubated with primary mouse anti-human ALAD (Abnova, CA, USA), or HRP-conjugated mouse anti-β-actin (C4) (Santa Cruz Biotechnology, CA, USA). The membranes were washed with PBST and incubated with HRP-tagged anti-mouse secondary antibody (Jackson Immuno-Research, PA, USA) in the same solution used for blocking. Immuno-reactive proteins were visualized with the EZ-ECL enhanced chemiluminiscence detection kit (Biological Industries, Israel), as recommended by the manufacturer.

### ALAD enzymatic activity assay

ALAD activity was assayed as previously described[5] by determining the absorbance of PBG formed by inversion of two ALA molecules to a pyrrole ring.

### Cellular PPIX analysis by flow cytometry

Control and transfected K562 cells were harvested, collected by centrifugation, and resuspended in serum-free RPMI-1640 medium containing 0.1 mg ml$^{-1}$ ALA, for 4 h. Afterwards, cells were collected in the dark, centrifuged at 900 rpm, washed twice with filtered PBS-/-, and finally resuspended in 1 ml filtered PBS-/-. PpIX was measured in $10^4$ cells per sample using a FACS (Becton-Dickinson, CA, USA) with an excitation wavelength of 488 nm and emission wavelength >670 nm.

### Imaging PpIX fluorescence

Control and transfected K562 cells were harvested, collected by centrifugation, and resuspended in serum-free RPMI-1640 medium containing 0.1 mg ml$^{-1}$ ALA for 4 h. Cells were dripped onto a slide, and PpIX fluorescence was visualized immediately using a Zeiss AxioImager microscope (Zeiss, Germany) with an excitation wavelength of 546 nm and emission wavelengths 575–640 nm.

### ALA-PDT

K562 cells were grown in a 24 well plate in a volume of 0.5 ml per well and incubated with ALA 0.1 mg ml$^{-1}$ for 4 h in a serum-free medium. Cells were irradiated for 10 min using Vilber-Lourmat black light source at power density of 22.5 mW cm$^{-2}$ at 360–410nm. Each minute of radiation is equivalent to a light dose of 1.25 J cm$^{-2}$.

### MTT mitochondrial activity assay

Cells were incubated for 24 h following ALA-PDT, and then 77 μl of 5 mg ml$^{-1}$ MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, (Sigma, Israel)) were added to each well for 2 h. Afterwards, 400 μl of DMF solution (100 g SDS dissolved in 250 ml dd-$H_2O$ with 250 ml of DMF) was added to lyse the cells. Absorbance was then measured in 570 nm using Tecan spectrophotometer (NeoTec, Canada).

### Scanning electron microscopy

Cells were incubated for 24 h following ALA-PDT, and then harvested and washed with PBS-/-. Cells were attached to 10 mm cover-slips coated with poly-L-lysine 0.1% (Sigma, Israel) for 1 h. Fixation of samples was performed according to a modification of the triple fixation GTGO method for SEM. Briefly, the samples were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2), followed by post-fixation in 4% $OsO_4$. The third fixative was 2% tannic acid–guanidine hydrochloride. After fixation, the cells were dehydrated in graded ethanol solutions, and then the ethanol was exchanged to Freon 113-tf, again using graded solutions. The samples were then air dried and gold coated, and examined using a FEI Quanta 200FEG scanning electron microscope.

### Annexin-PI staining

Cells were incubated for 24 h following ALA-PDT, then harvested and washed with PBS-/-. Cell death was characterized by AnnexinV-FITC/PI staining using the MEBCYTO-apoptosis kit, according to the manufacturer's instructions (MBL, MA, USA).

### Lactate dehydrogenase activity

LDH activity was measured using the LDH-L reagent (Thermo, Australia). Cells were incubated for 24 h following ALA-PDT; cells were then centrifuged, and the supernatant was collected and diluted in LDH-L reagent. The sample : reagent ratio was 1 : 4 (v/v). Absorbance was measured after 1 h at 340 nm using a spectrophotometer (Tecan Trading, Switzerland).

 

### Porphyrin extraction for HPLC

Control and transfected K562 cells were harvested, collected by centrifugation, and resuspended in serum-free RPMI-1640 medium containing 0.1 mg $ml^{-1}$ ALA for 4 h. Cells were harvested, collected by centrifugation and resuspended in 0.5 ml NaCl 0.9% + 0.75 ml acetic acid. Then, 2.25 ml ethyl acetate was added and after 1 min of agitating, the mixture was centrifuged for 2 min in 200 g at 4 °C. Supernatant was transferred to another glass tube, and 2 ml of HCl 1.5 N was added. After shaking, the mixture was centrifuged again, in same conditions. The lower fraction was collected and centrifuged again for 10 min in 750 *g*. 100 µl were taken for HPLC. PpIX standard (Sigma, Israel), used for peak identification, was diluted to 56.3 µg $ml^{-1}$ concentration in HCl 1.5 N and prepared to HPLC as written above. Porphyrins were separated on a column (Lichrosphere 100, RP-18 (5 µm), 125X4, Merck, NJ, USA) and fluorescence was detected with HSM software after 47 min using Elite LaChrom HPLC (Merck-Hitachi, CA, USA) with monitoring at 404 nm excitation and 615 nm emission wavelengths.

## Results

### ALAD silencing in K562 cells

ALAD is an abundant enzyme which is crucial to heme synthesis.[10] Thus, silencing of ALAD was expected to affect porphyrin synthesis and ALA-PDT efficacy. In order to elucidate the significance of ALAD in ALA-PDT, we transfected K562 cells with shRNA constructs designed for silencing of ALAD mRNA. Specific down regulation of ALAD was assessed by enzymatic activity (Fig. 1A) and protein expression (Fig. 1B). Cells transfected with ALAD shRNA, exhibited 60% silencing of ALAD, as revealed by reduced enzymatic activity (Fig. 1A) and marked reduction in protein expression (Fig. 1B). A non-silencing plasmid, which encodes an irrelevant sequence, was used as an internal control to eliminate possible effects of transfection. A small decrease in ALAD activity was depicted in the non-silenced cells. This may be a side effect of transfection. Still, ALAD-silenced cells showed a significant decrease in ALAD activity in comparison to non-silenced cells.

### Silencing of ALAD reduces PpIX synthesis in K562 cells

Supplementation of exogenous ALA to cells bypasses the first rate limiting enzyme, ALAS, and induces tetrapyrrole biosynthesis.[12] K562 cells were treated with ALA for 4 h, and porphyrin accumulation was examined using FACS and fluorescent microscopy (Fig. 2A and B). Both FACS analysis and fluorescent microscopy showed a clear decrease in porphyrin accumulation in the ALAD-silenced cells. The main tetrapyrrole produced in control cells was PpIX as shown by HPLC analysis (Fig. 2C). PpIX concentration in control cells was 12 mg per $10^6$ cells, while in ALAD-silenced cells, it was reduced to only 4 mg per $10^6$ cells. Silencing of ALAD decreased the ratios between PpIX and its precursors, seen at shorter retention times.

### Mitochondrial activity following ALA-PDT in ALAD silenced cells

PpIX production in the ALAD-silenced cell was shown to be reduced following ALA treatment in comparison to the controls, therefore we expected to see decreased mitochondrial damage following ALA-PDT. MTT activity, indicating mitochondrial damage, was directly proportional to PpIX levels in control *versus* ALAD silenced cells. Fig. 3 shows a 60% reduction in MTT activity in control and non-silenced (transfected with an irrelevant construct) K562 cells 24 h following ALA-PDT. However, MTT activity remained unchanged in ALAD-silenced cells following ALA-PDT, when compared to the controls (Fig. 3). A modest effect of the non-silencing vector was observed, apparently as a result of transfection itself. We show here that the PpIX accumulated in these cells is insufficient to induce mitochondrial damage under the experimental light exposure. Thus, ALAD activity is crucial for effective ALA-PDT treatment.

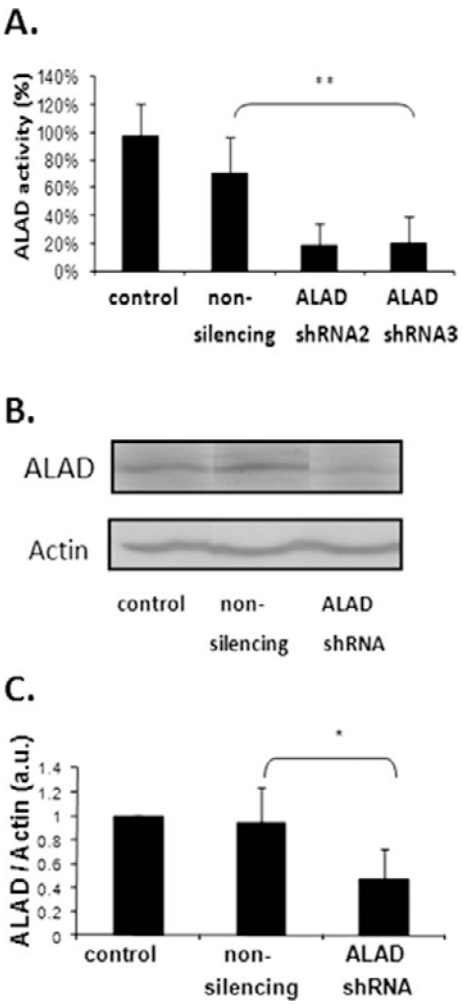


**Fig. 1** ALAD silencing using specific shRNA results in reduced ALAD activity and expression in K562 cells. (A) Control and transfected K562 cells were examined for ALAD activity. *t*-Test was performed in comparison to non-silenced cells (** $p \leq 0.01$). These results are the average of 3 different experiments. Bars indicate SD. (B) Western blot of K562 cell lysates probed with an anti-ALAD antibody. An anti-actin antibody was used as an internal protein standard. This is a representative result of 3 different experiments. (C) Densitometric quantitation of 3 different western blot experiments. *t*-Test was performed in comparison to non-silenced cells (* $p \leq 0.05$). Bars indicate SD.

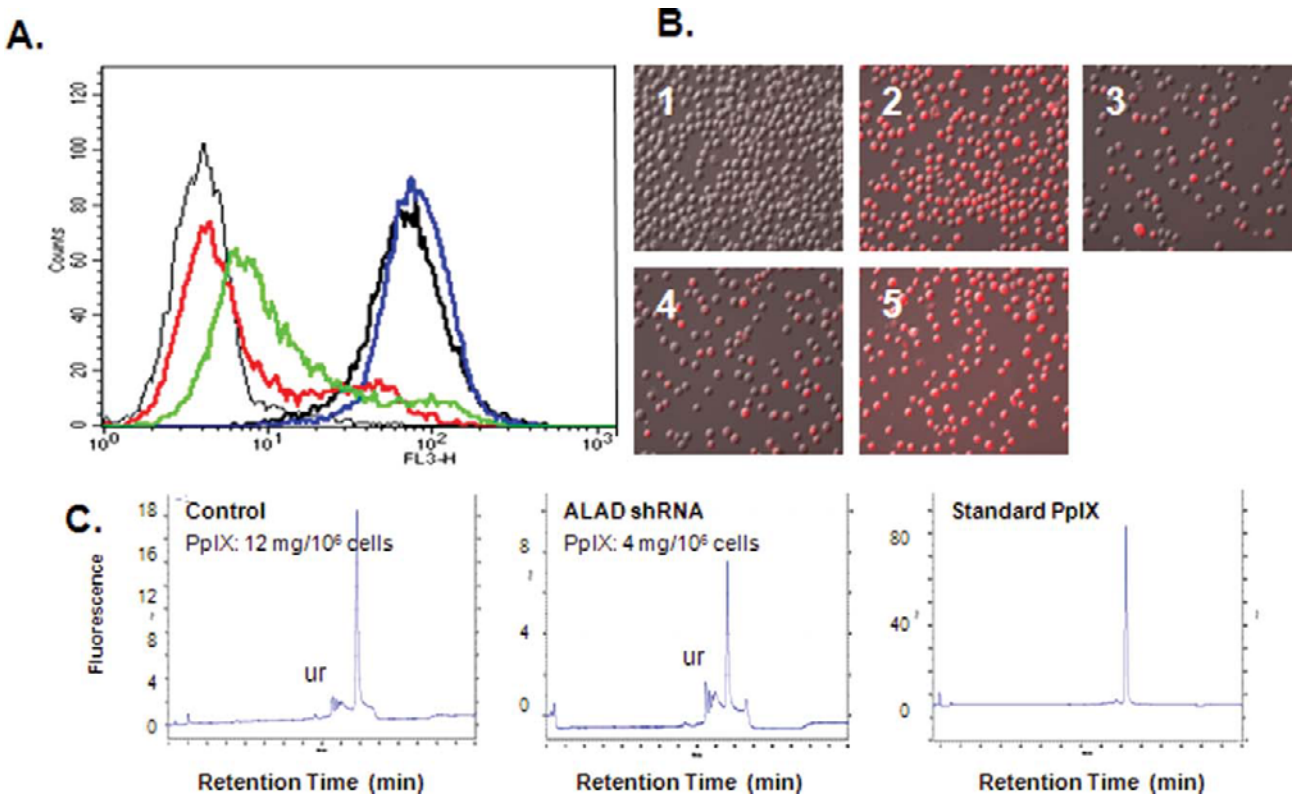


**Fig. 2** Porphyrin synthesis is reduced in ALAD silenced cells. Control and transfected K562 cells were incubated in serum-free medium containing 0.1 mg ml$^{-1}$ ALA for 4 h. (A) Porphyrin autofluorescence was measured in $10^4$ cells per sample using FACS. Lines: black: control; bold-black: control + ALA; red: ALAD2 shRNA + ALA; green: ALAD3 shRNA + ALA; blue: non-silencing + ALA. (B) Porphyrin autofluorescence was imaged by fluorescent microscope (ex. 546 nm, em. 575–640 nm). Images: 1: control, 2: control + ALA; 3: ALAD2 shRNA +ALA; 4: ALAD3 shRNA + ALA; 5: non-silencing + ALA. (C) PpIX was extracted from $10^7$ cells. Porphyrin autofluorescence was measured by HPLC (615 nm) in comparison to a commercial standard. "ur" represents the retention time of uroporphyrin. These are representative results of 3 different experiments.

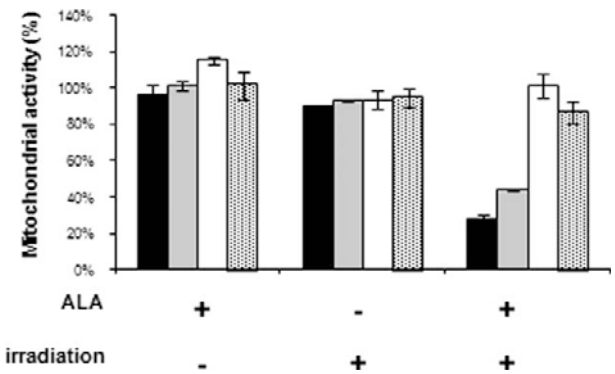


**Fig. 3** Mitochondrial activity in ALAD-silenced K562 cells following ALA-PDT. Control and silenced K562 cells were incubated with 0.1 mg ml$^{-1}$ ALA for 4 h followed by black light irradiation for 10 min. Mitochondrial activity was assessed using the MTT assay. These results are the average of 3 different experiments. Bars indicate SD. Legend: black – control, grey – non-silencing, white – ALAD shRNA 2, dotted – ALAD shRNA 3.

### Characterization of cell death following ALA-PDT

In order to define cell death mechanisms through apoptosis or necrosis pathways following ALA-PDT treatment in silenced *versus* control cells, we used three methods: (1) SEM analysis to determine cell morphology; (2) analysis of membrane integrity by AnnexinV-FITC/PI; and (3) assay of LDH-L enzyme leakage. K562 cells were treated for 4 h with ALA and irradiated at power density of 22.5 mW cm$^{-2}$ at 360–410nm. Cell death was characterized following overnight incubation.

SEM analysis of ALA-treated and irradiated control and non-silenced K562 cells (transfected with irrelevant plasmid) revealed membrane rupture due to possible cytoskeletal collapse. Microvilli that were recognizable in untreated cells (Fig. 4A I) were not observed in ALA-PDT treated cells (Fig. 4A II, III). In contrast to the aforementioned changes, ALAD-silenced cells did not show any shrinkage or death characteristics following ALA-PDT (Fig. 4A IV). These results are consistent with the low porphyrin levels shown previously (Fig. 2A and B). The approximate average volume of the treated and untreated cells was also calculated, showing decreased cell volume in cells subjected to ALA-PDT, while untreated cells and ALAD silenced cells were not affected (Fig. 4B).

Cell death was also characterized using Annexin V-FITC/PI staining. K562 control cells, and cells transfected with an irrelevant non-silencing construct, that were treated with ALA and subjected to PDT showed a major fraction of double-stained cells (Fig. 5A). However, ALAD silenced cells showed only a small double-stained fraction, and a significant population of living cells that were unharmed by ALA-PDT.

 

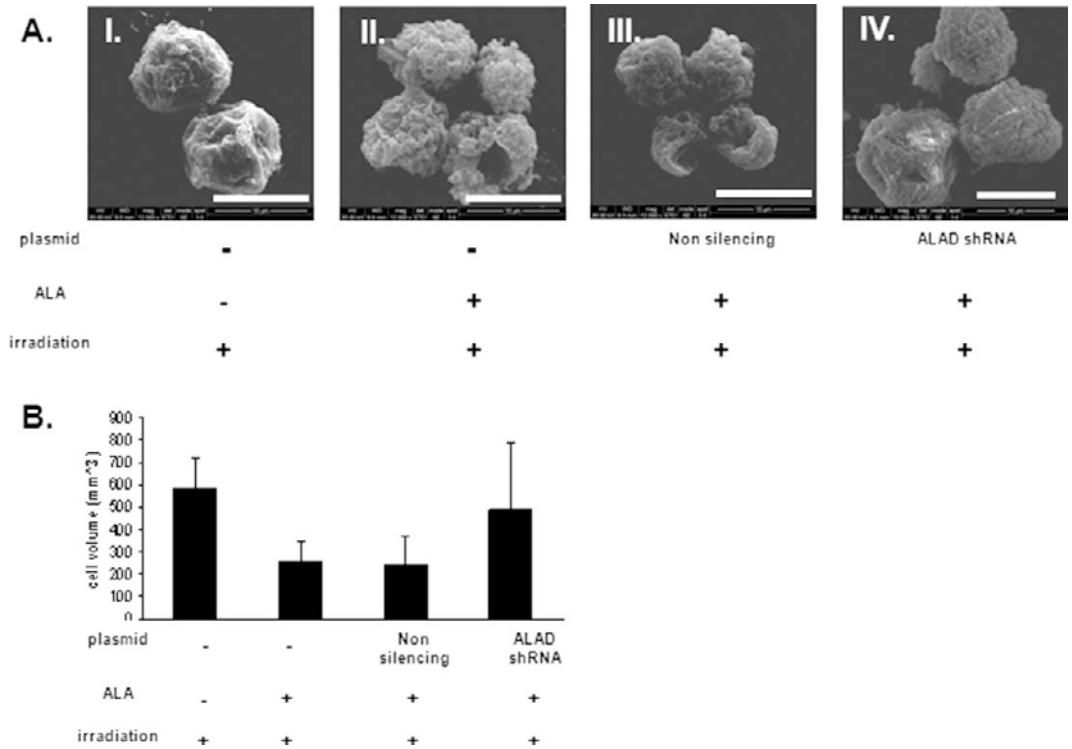


**Fig. 4** Cell morphology following ALA-PDT. Control and silenced K562 cells were incubated with 0.1 mg ml$^{-1}$ ALA for 4 h followed by black light irradiation for 10 min. Cell morphology was examined (A) and average cell diameter was calculated from 50 cells (B) after 24 h by scanning electron microscopy (white bars = 10 mm). Bars indicate SD.

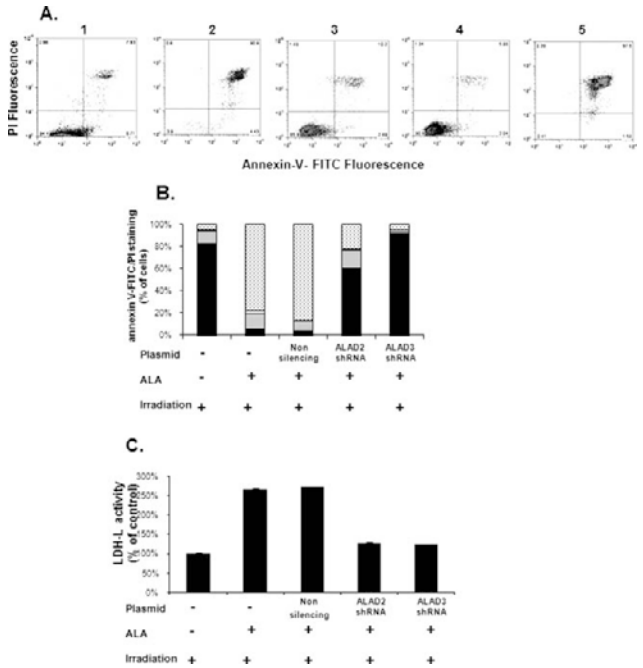


**Fig. 5** Characterization of cell death following ALA-PDT. Control and transfected K562 cells were incubated with 0.1 mg ml$^{-1}$ ALA for 4 h followed by black light irradiation for 10 min. (A) After 24 h, cells were stained with AnnexinV-FITC and PI. Fluorescence was measured in $10^4$ cells per sample using FACS. (B) Graphed data for (A). Legend: black – no stain, grey – AnnexinV stain, white – PI stain, dotted – double stain. (C) After 24 h, cells were harvested by centrifugation. Supernatant was tested for LDH activity. Bars indicate SD. These are representative results of 3 different experiments.

Leakage of LDH 24 h following ALA-PDT was observed in the control cells and cells transfected with an irrelevant non-silencing construct, whereas ALAD silencing protected these cells during ALA-PDT, and LDH leakage was identical to that in the non-PDT control cells.

## Discussion

After two decades of clinical experience with ALA-PDT it is clear that response rates to ALA-PDT vary widely due to suboptimal production of PpIX. Moreover ALA-PDT efficacy depends largely on the tissue specific variability of PpIX synthesis, which poses limitations on this therapy modality. The expression of the key enzymes in the heme synthesis pathway ALAS, ALAD, PBGD and ferrochelatase varies widely among tissues, especially those that require large amounts of heme as a prosthetic group. ALA supply circumvents the first enzyme, ALAS, thus the synthesis rate is largely dependent on the other enzymes especially PBGD and ferrochelatase.[5,9,14] However, our recent data shows the importance of the second enzyme of the pathway, ALAD, on the rate of PpIX synthesis. We used K562 as a model for porphyrin and heme synthesis. Although these cells are not clinically treated by ALA-PDT, we assume that these results indicate the importance of ALAD expression and activity to ALA-PDT efficacy in other malignancies.

ALAD inhibition by lead was shown to decrease PpIX formation and to reduce ALA-PDT efficacy following ALA administration.[5] In addition, Gibson *et al.* showed a reduced PpIX formation after incubation with succinyl acetone, a potent

inhibitor of ALAD.[3] Therefore it was crucial to elucidate the role of ALAD expression on PpIX synthesis. Silencing of ALAD mRNA expression was carried out in the human K562 erythroleukemia cell line by transfection of specific shRNA plasmids. We assumed that the regulation of ALAD expression in an afflicted tissue will determine the efficacy of ALA-PDT in a specific tumor.

Our present results depict that the down regulation of ALAD expression and activity by specific shRNA transfection induced a marked decrease in PpIX synthesis in K562 cells. In mammals, ALAD is a very abundant enzyme, its activity is estimated to be 100-fold more than necessary for required heme formation.[10] It was suggested that ALAD is not merely used in the heme biosynthesis pathway, but rather is a crucial regulator of the proteasome.[7] In addition, we have previously demonstrated that lead poisoning induced acceleration of proteasomal activity concomitant with the inhibition of ALAD enzymatic activity.[5] Thus, ALAD is involved in two physiological processes: the one for PBG synthesis and the other to regulate the proteasome. These processes might compete with each other for availability of ALAD. This might explain why a decrease of 80% in ALAD level caused such a significant effect on PpIX accumulation and PDT efficacy, although it can be expected that 20% of ALAD should be enough for PBG synthesis.

Our results clearly demonstrate that ALAD silencing abolished ALA-PDT killing effects. Cell viability determined by mitochondrial activity, membrane integrity and cell morphology were not affected in the silenced cells, indicating that PDT resistance was induced by shRNA transfection. These findings depict that ALAD expression level is crucial for porphyrin synthesis; therefore, differential activity of this enzyme in various tissues could result in diverse ALA-PDT efficacy. It was shown that ALAD activity level is in great diversity in various organs.[13] Furthermore, the activity of ALAD was shown to be enhanced in malignant *vs.* normal tissue.[15] Our data add an additional aspect to the existing knowledge of the regulation of heme synthesis pathway. It is not only that PBGD and ferrochelatase determine PpIX accumulation, but rather ALAD specific expression is crucial for the whole process.[4,17]

Silencing of ALAD may be considered as a model of ALAD-deficiency porphyria. These patients exhibit low ALAD expression and activity, resulting in elevated ALA secretion in urine. Until 2007, only 8 mutations were known to cause this rare syndrome, manifesting the significance of ALAD.[10] In comparison, over 300 mutations were identified in PBGD, the third enzyme of the same pathway.[8] It is conceivable that this strict conservation of ALAD cannot be explained just by its participation in heme synthesis pathway.

Taken together, we show in this study that ALAD is a key enzyme in porphyrin biosynthesis. We conclude that the significant reduction in PpIX levels, resulting from the specific silencing of ALAD, abolishes the therapeutic effect of ALA-PDT in treated cells. Therefore, prediction of ALA-PDT treatment success is greatly dependant on evaluation of ALAD activity as well as the other key enzymes in the target tissues.

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