A light-activated photodynamic therapy (PDT) painlessly destroys cancer cells by converting light energy into heat, which generates highly reactive oxygen species that damage tumor cells [1]. Light-activation is achieved by administrating a photosensitizer (PS) followed by exposure to light in the wavelength of the PS absorption maximum (λmax). ALAS catalyzes the first committed step of heme biosynthesis in animals. The erythroid-specific ALAS isoform (ALAS2) is negatively regulated by heme at the level of mitochondrial import and, in its mature form, certain mutations of the murine ALAS2 active site loop result in increased production of protoporphyrin IX (PPIX), the precursor for heme. Importantly, generation of PPIX is a crucial component in the widely used photodynamic therapies (PDT) of cancer and other dysplasias. ALAS2 variants that cause high levels of PPIX accumulation provide a new mechanism of light-induced cell death in PDT. Transfection of HeLa cells with expression plasmids for murine ALAS2 variants, specifically for those with mutated mitochondrial presequences and a mutation in the active site loop, caused significant cellular accumulation of PPIX, particularly in the membrane. Light treatments revealed that ALAS2 expression results in an increase in cell death in comparison to aminolevulinic acid (ALA) treatment producing a similar amount of PPIX. The deliverability of stable and highly active ALAS2 variants has the potential to expand and improve upon current PDT regimes.

**Abstract**

5-Aminolevulinate synthase (ALAS; EC 2.3.1.37) catalyzes the first committed step of heme biosynthesis in animals. The erythroid-specific ALAS isoform (ALAS2) is negatively regulated by heme at the level of mitochondrial import and, in its mature form, certain mutations of the murine ALAS2 active site loop result in increased production of protoporphyrin IX (PPIX), the precursor for heme. Importantly, generation of PPIX is a crucial component in the widely used photodynamic therapies (PDT) of cancer and other dysplasias. ALAS2 variants that cause high levels of PPIX accumulation provide a new mechanism of light-induced cell death in PDT. Transfection of HeLa cells with expression plasmids for murine ALAS2 variants, specifically for those with mutated mitochondrial presequences and a mutation in the active site loop, caused significant cellular accumulation of PPIX, particularly in the membrane. Light treatments revealed that ALAS2 expression results in an increase in cell death in comparison to aminolevulinic acid (ALA) treatment producing a similar amount of PPIX. The deliverability of stable and highly active ALAS2 variants has the potential to expand and improve upon current PDT regimes.

**Introduction**

The first committed step of heme biosynthesis in non-plant eukaryotes and some prokaryotes, the pyridoxal 5′-phosphate (PLP)-dependent condensation of glycine and succinyl-coenzyme A to generate 5-aminolevulinate (ALA), coenzyme A (CoA), and CO₂, is catalyzed by 5-aminolevulinate synthase (ALAS) [1,2]. This reaction is directly coupled to the citric acid cycle via the succinyl-CoA binding, formation of the quinonoid intermediates, and prevent translocation of precursor ALAS2 into the mitochondria and ALAS2 precursor protein, the two heme-binding motifs in the leader sequence, corresponding to C11 and C38 in murine ALAS2 (mALAS2), were reported to bind heme and prevent translocation of precursor ALAS2 into the mitochondrion [18]. Structural and biochemical data have also demonstrated that a heme-peptide interaction occurs between hemein and the ALAS2 [24], further indicating the potential of heme to act as a feedback inhibitor of the pathway by preventing the mitochondrial import of precursor ALAS2 when heme levels are sufficient for cellular requirements. Much of what we know about the chemical and kinetic mechanisms of ALAS2 comes from in vitro enzymatic assays that have helped establish and define the microscopic steps of the ALAS-catalyzed reaction, including the rates of glycine and succinyl-CoA binding, formation of the quinonoid intermediates.
and product release [2,25–28]. These studies, performed using mALAS2 purified from E. coli cells expressing the recombinant mature enzyme, have led to an understanding of the importance of specific regions and single amino acid residues in the intrinsic activity of ALAS2 [29–35]. Generally, a mutation made to an amino acid predicted to be of functional importance causes a decrease in activity of the enzyme. For example, K313 of mALAS2 was identified as the amino acid involved in the Schiff base linkage with the PLP cofactor [36], and mutations in K313 completely abolish measurable activity of mALAS2 under standard assay conditions [29,37]. However, some mutations in mALAS2 cause significantly increased activity of the enzyme, as demonstrated both using purified enzyme [31,35], and in bacteria when expressing plasmids encoding the variant enzymes [35]. These mutations are all located in an extended conformation region termed the active site loop (Y422-R439), which is predicted to act as a “lid” over the active site following substrate binding [35]. Thus, ALAS is thought to undergo a conformational change from an open conformation, in which the substrates glycine and succinyl-CoA can bind in the active site, to a closed conformation, during which the products ALA, CoA, and CO2 are formed [25,28]. When the reaction is complete, the active site loop reopens, and the products are released. It is the opening of the active site loop to allow product release that limits the overall rate of the enzymatic reaction [2,25,27]. Based on a combination of kinetic [25,28] and structural modeling studies [38], it was proposed that mutations in the active site loop can lead to hyperactive forms of ALAS, defined as those with at least a 10-fold increase in catalytic efficiency toward one or both substrates, by accelerating reversion to the open loop conformation upon product formation [35].

Since ALAS catalyzes the rate-determining step of tetrapyrrole biosynthesis in mammals [2,25], overexpression of ALAS in prokaryotic [35] and eukaryotic cells [39] results in accumulation of the photosensitizing heme precursor, protoporphyrin IX (PPIX). This property has potential for applications of ALAS or ALAS variants in photodynamic therapy of tumors and other non-malignant dermatological indications, such as acne vulgaris, psoriasis, and scleroderma [40,41]. In this study, we transfected mammalian cells with mALAS2 variants and measured PPIX accumulation using fluorescence activated cell sorting (FACS). We identified the R433K variant with additional mutations of the HRMs in the presequence as the variant causing the most cellular PPIX accumulation using fluorescence activated cell sorting (FACS). We demonstrated both using purified enzyme [31,35], and in bacteria when expressing plasmids encoding the variant enzymes [35].

Materials and Methods

Materials

5-Aminolevulinic acid hydrochloride (ALA) was purchased from Acros Organics (Morris Plains, NJ), and dissolved in distilled water at a concentration of 10 mg/mL. Glycine, purchased from Fisher Chemical (Fairlawn, NJ), was dissolved in phenol red-free culture medium purchased from Mediatech, Inc. (Manassas, VA) to give a stock concentration of 1 M. Paclitaxel (6 mg/mL stock in DMSO) was graciously provided by the laboratory of Dr. Scott Antonia (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL), and was diluted in culture medium directly before use. Deferoxamine mesylate, purchased from Sigma (St. Louis, MO), was dissolved in distilled water to create a 10 mM stock solution. Propidium iodide, 4’,6-diamidino-2-phenylindole (DAPI) and kanamycin sulfate were purchased from Acros Organics (Morris Plains, NJ), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoi-
well of a 24-well plate in 0.5 mL of DMEM. Cell density was 30–50% confluent on the day of transfection. For each transfection, 250 ng of DNA was diluted into 100 μL of DMEM without serum. 1 μL of Lipofectamine LTX was added into the diluted DNA solution, mixed gently and incubated for 30–45 minutes at room temperature to form DNA-Lipofectamine LTX complexes. The DNA-Lipofectamine LTX complexes were added dropwise to each well containing cells and mixed gently by manually rocking the plate back and forth for a few seconds. After 4 hours of incubation with the DNA-Lipofectamine LTX complexes, the medium was aspirated out of each well and fresh DMEM with 10% FBS, gentamicin (50 μg/mL), penicillin (60 μg/mL) and streptomycin (100 μg/mL) was added to each well of cells. Cells were incubated at 37°C in a CO₂ incubator for 24 or 40 hours post-transfection before assaying.

Preparation of Cells for FACS and Quantitation of PPIX

While K562 cells were suspended in phenol-red medium, HeLa cells were washed, scraped and resuspended in phosphate-buffered saline (PBS; 80 mM disodium hydrogen orthophosphate, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, pH 7.5) before pipetting into BD Falcon tubes with cell strainer caps. Preparation of either K562 or HeLa cells for FACS was done under very low light conditions (1–2 Lux as measured by a Pyle PLMT68 light meter) in order to minimize phototoxicity caused by PPIX accumulation. FACS analyses were performed using a BD LSR II Analyzer (Becton, Dickinson, and Company) and FACS Diva Version 6.1.3 software. ZsGreen1 emission was measured between 515 nm and 545 nm (530/30BP filter) when cells were excited with the 488 nm laser. In order to eliminate any background red fluorescence, the 633 nm-red laser was blocked during the collection of the PPIX emission data. PPIX emission was determined in the 619 nm and 641 nm range (630/22BP filter) when cells were excited with the 405 nm laser. Forward-scatter (FSC) versus side-scatter (SSC) dot plots were used to gate the whole cells and thus remove the contribution of the cell debris from the population being examined. A minimum of 10,000 of the gated whole cells were then depicted in dot plots of SSC versus ZsGreen1 fluorescence, and the “green-fluorescent population” gate was defined based on untransfected HeLa cells as negative controls. Dot plots of SSC versus PPIX fluorescence were used to define the PPIX-accumulating cells for both the “green-fluorescent” and the “non-green fluorescent” populations. The PPIX gating was based on the negative control for PPIX, the pHRES2-ZsGreen1 vector-expressing cells. PPIX fluorescence values were normalized for transfection efficiency using the corresponding ZsGreen1 fluorescence value.

Light Exposure Assays

HeLa cells in 24-well plates were transfected 48 hours prior to FACS analysis. Cells were washed twice with PBS and placed on ice (to prevent overheating) underneath a Sylvania incandescent flood lamp (150 W, 120 V) for 10 minutes. Light intensity at the samples was measured before each light exposure experiment using a Pyle PLMT68 light meter, and it was between 21–22 kLux for all experiments. The samples were resuspended and pipetted into tubes with cell strainer caps and analyzed first for ZsGreen1 and PPIX fluorescence (as described above), and then for cell viability. In order to determine the statistical significance of cell death directly attributable to mALAS2-induced PPIX accumulation and phototoxicity, samples were compared to the ZsGreen1 controls during t-test evaluations.

Cell Viability Assays

Cell death was assessed by measuring incorporation of either propidium iodide or DAPI into nuclear DNA, as these fluorescent dyes cross the plasma membrane in dying cells much more efficiently than in live cells. HeLa cell cultures were independently incubated with the fluorescent DNA-binding dyes propidium iodide (10 μg/mL) and DAPI (10 μg/mL) for 5 min at 22°C. The cells were then analyzed by determining the fluorescence intensities using FACs. Fluorescence emission of nucleic acid-bound propidium iodide was measured between 585 nm and 625 nm (605/40BP filter) upon excitation of the cells using the 488 nm laser, while DNA-bound DAPI emission was measured between 440 nm and 460 nm (450/20BP filter) following excitation of the cells at 375 nm. Dot plots of SSC versus propidium iodide or DAPI fluorescence were used to define the respective gates, which were based on the control samples in which no propidium iodide or DAPI were added. Cell viability was independently determined using the MTT dye reduction assay as described elsewhere [42,43]. HeLa cells (5×10⁴ cells per well) were seeded in 96-well plates, and on the following day were transfected with expression plasmids for mALAS2 variants as described above. Twenty-four hours after plasmid transfection, or 4 hours after addition of 100 μM ALA, cells were exposed to light for 10 minutes. The culture medium was pipetted out, 50 μL of 2 mg/mL MTT was added to each well, and cells were incubated for an additional 4 hours at 37°C. Upon solubilization of the cells with DMSO (100 μL/well) during a 10 minute incubation, the solubilized, MTT-treated cells were thoroughly mixed by pipetting several times, and absorbance was measured at 540 nm using a μQuant plate reader (Bio-Tek Instruments, Inc.).

Confocal Fluorescence Microscopy

HeLa cells were grown in Thermoscientific Nunc Labtek sterile 4-well chambered coverglass until 50% confluent, and then transfected as described above. Six hours later, the culture medium was supplemented with glycine to yield a final concentration of 100 mM, and immediately before obtaining confocal fluorescence microscopy images of the cells, the medium was removed from the wells and the cells were washed with PBS three times. Live cell imaging was performed using a 3×Olympus spinning disk confocal microscope operated by Slidebook 5 software and equipped with a Photometrics Evolve EMCCD camera. The filter block used consisted of a 350/50 nm excitation filter, a BS400 beamsplitter, and a 630/75 nm emission filter.

Results

Transient Expression of Malas2 Variants Causes Accumulation of PPIX in HeLa Cells

HeLa cells were transfected with mALAS2-encoding plasmids with or without mutations in the mALAS2 presequence and/or mature enzyme sequence (Table 1, Figure 1). Where indicated, the mALAS2 presequence was mutated at three cysteine residues, C11, C38, and C70, in order to yield nonfunctional HRMs and thus eliminate heme feedback inhibition of mitochondrial import [18,40]. The plasmids were designed such that a single bicistronic mRNA, encoding both mALAS2 and the fluorescent protein ZsGreen1, separated by an internal ribosomal entry site (IRES), would be produced. Transcriptional expression of mALAS2 and ZsGreen1 was under control of the constitutively active cytomegalovirus (CMV) promoter [44]. Twenty-four hours post-transfection, wild-type mALAS2 with a wild-type presequence (WT) expression caused a slight, but statistically significant as defined by Student’s t-test (p<0.05), increase in PPIX accumulation.
Supplementation of Cell Culture Medium with Glycine Leads to Increased PPIX Accumulation in Malas2-expressing HeLa Cells

Given that PPIX is a photosensitizer [49] and the Michaelis-Menten constant ($K_m$) of mALAS2 for glycine, at $25 \pm 4$ mM [3,25,54], is significantly higher than its intracellular concentration of approximately 2.5 mM [50], it is plausible that supplementation of the cell culture medium with glycine would lead to enhanced synthesis of ALA, and consequently, PPIX, which in turn might increase the efficacy of PPIX-induced phototoxicity. To examine whether increased glycine concentration caused enhanced PPIX accumulation, HeLa cells transfected with mALAS2 (wild type and variants) were grown in medium with different glycine concentrations (Figure 3A). Samples with culture medium supplemented with 100 $\mu$M ALA for 4 hours served as a control for PPIX accumulation independent of glycine concentration (Figure 3B).

The concentration and treatment times for ALA were chosen based on experiments indicating that the extent of PPIX accumulated in HeLa cells supplemented with 100 $\mu$M ALA was similar to that of HeLa cells expressing R433K, the mALAS2 variant that induces the highest levels of PPIX accumulation in these cells (Figure S1). HeLa cells expressing WT, HPVT or R433K in culture medium supplemented for 18 hours with 10 mM or 100 mM glycine exhibited significant increases in PPIX in comparison to the “no glycine” controls, with R433K again demonstrating the highest PPIX accumulation ($p<0.01$) (Figure 4A). Supplementing the culture medium with either 10 mM or 100 mM glycine for cells expressing WT resulted in approximately 4- and 6-fold increases in PPIX accumulation, respectively, as compared to no glycine supplementation. For cells expressing R433K, glycine supplementation more than tripled the PPIX accumulation, representing a fourteen-fold increase over the control HeLa cells supplemented with glycine. Cells expressing HPVT were also affected by addition of either 10 mM or 100 mM glycine, increasing the PPIX by 1.4-fold and 2.5-fold, respectively. Glycine elicited no effect on PPIX production in HeLa cells alone, those treated with 100 $\mu$M ALA, or HeLa cells expressing the pIREs2-ZsGreen1 vector (Figure 3B).

Glycine and Deferoxamine Increase PPIX Accumulation in Malas2-expressing K562 Cells

We generated stable K562 human myelogenous erythroleukemia cell lines expressing mALAS2, expecting that these cells might accumulate high amounts of PPIX, due to their similarity to undifferentiated erythrocytes [51]. We expressed only WT and HPVT in order to test if HPVT could have higher activity in a cell line in which ALAS2 is normally expressed. Initially, the stable cell lines expressing WT did not show an increase in PPIX fluorescence as compared to regular K562 cells (Figure 4A), and we postulated that glycine availability was again a limiting factor, as we had observed in HeLa cells (Figure 3). Another possible limitation upon PPIX accumulation could be a more effective conversion of PPIX into heme in K562 cells, in which case the inclusion of an iron-specific chelator such as deferoxamine should increase the PPIX fluorescence by reducing or even preventing this conversion. To address why the expression of mALAS2 alone did not cause a larger increase in PPIX and what could limit the ALAS2-induced PPIX accumulation, the culture medium of K562 cells expressing [1] ZsGreen1 control, [2] WT, or [3] HPVT was supplemented with glycine to yield final concentrations of 10 mM or 100 mM glycine (Figure 4A and 4C) or deferoxamine mesylate to yield a final concentration of 100 $\mu$M deferoxamine mesylate (Figure 4B and 4D). Expression of WT in K562 cells did not

Figure 1. Murine ALAS2 protein schematic indicating mutated amino acid residues. The green dotted lines represent the relative locations of the three cysteines in the HRMs of ALAS2. C11 and C38 are in the ALAS2 precursor, K313A, R433K, and the heptavariant mutations and their respective positions are indicated according to the amino acid numbering previously described for mature mALAS2 [31,35,36]. The amino acid positions according to the numbering for the precursor enzyme are written above the diagram.

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increase the cellular PPIX fluorescence, but PPIX fluorescence increased by more than 5- and 10-fold when the culture medium contained 10 mM glycine and 100 mM glycine, respectively (Figure 4A). No significant differences were seen in cells not expressing mALAS2 (Figure 4C) or in cells expressing HPVT when treated with 10 mM glycine (Figure 4A). However, in contrast to HeLa cells, when 100 mM glycine was used, cells expressing HPVT did exhibit an increase in PPIX fluorescence by approximately 3-fold (Figure 4A).

Deferoxamine is a well-characterized iron-specific bacterial siderophore with a long history of clinical use in iron chelation therapy. Deferoxamine has the potential to increase PPIX by decreasing the cellular iron concentration, thereby inhibiting the conversion of PPIX to heme [52,53]. Treatment of K562 cells with deferoxamine for 18 hours caused no change in PPIX fluorescence in cells not expressing mALAS2 or cells expressing WT. In the case of WT, the mean PPIX fluorescence...
increased from 1.4-fold over cells expressing ZsGreen1, to 2.4-fold with deferoxamine (Figure 4B and 4D).

PPIX Primarily Accumulates and Localizes at the Plasma Membrane in HeLa Cells Expressing R433K

To evaluate cellular intactness and accumulated PPIX distribution in the transfected HeLa cells, we used confocal microscopy to visualize the fluorescent PPIX in individual HeLa cells (Figure 5A) and HeLa cells transfected with either the pIRES2-ZsGreen1 vector (Figure 5B) or pEF31 (harboring R433K) (Figures 5C and 5D). Transfected cells were grown in medium supplemented with 100 mM glycine for 18 hours in preparation for imaging. The outline of intact cells, and thus their morphological integrity, was evident in the three cases. As expected, green fluorescence was visualized in the transfected (Figures 5B, columns 2, 4 and 5, 5C, columns 2, 4 and 5 and 5D, columns 2, 4 and 5) but not control HeLa cells (Figure 5A, columns 2, 4 and 5). In fact, green fluorescence, arising from the soluble ZsGreen1 green fluorescent protein was observed evenly distributed throughout the cytoplasm of the HeLa cells transfected with either pIRES2-ZsGreen1 (Figure 5B) or the R433K-expression plasmid (Figures 5C and 5D). While the enzyme protoporphyrinogen oxidase catalyzes PPIX production exclusively within the mitochondrion, PPIX accumulated primarily within the plasma membranes of HeLa cells expressing R433K, as visualized by the characteristic red fluorescence; however, PPIX build-up was also apparent within the cells (Figures 5C, columns 3–5 and 5D, columns 3–5). Thus, it appears that much of the PPIX produced in the mitochondria eventually accumulates in the plasma membrane, entirely consistent with the fact that PPIX is a relatively lipophilic molecule. Of note, PPIX accumulated in not only transfected cells, but also in surrounding cells, indicating that PPIX could leave the transfected cells and be taken up by nearby cells (Figures 5, columns 3–5 and 5D, columns 3–5). It is very likely that, additionally, PPIX accumulated in organelle membranes within the cell, such as in the mitochondrion, but since PPIX photobleached within a few seconds under the conditions utilized here, it was not possible to obtain high-resolution organelle images with our current microscopic parameters. Finally, the cobblestone-like morphology, instead of a slightly elongated shape,
of the HeLa cells may result from the cell density and/or the nature of the growth surface. HeLa cells tend to adopt a more cobblestone-like appearance as cultures approach confluence and the HeLa cell morphology varies with the adhesion surface.

ALAS2-induced PPIX Accumulation Followed by Light Exposure Combined with Paclitaxel Treatment Causes Cell Death

Both propidium iodide and DAPI staining were utilized to assess cell viability after mALAS2-induced PPIX accumulation, light exposure, and paclitaxel treatment, based on the specific fluorescence emission of each dye when bound to the DNA of intact cells. Stains were added to cell samples 48 hours after transfection, and the fluorescence of DNA-bound propidium iodide and DAPI were measured by flow cytometric analysis using the respective fluorescence emission maxima of 613 nm and 460 nm. Expression of WT or HPVT did not significantly increase cell death following light exposure. However, expression of R433K caused a statistically significant (p < 0.05) increase in cell death of up to 30%, as measured both by propidium iodide (Figure 6A) and DAPI (Figure 6B) staining, in comparison to expression of ZsGreen1 alone. Addition of paclitaxel increased cell death in all samples, including controls, by 10–25%. HeLa cells expressing R433K and treated with paclitaxel exhibited the highest percentage of up to 30% cell death (Figure 6A–B). Combination of paclitaxel with mALAS2-induced PPIX accumulation and light treatments exhibited an additive effect in causing death in HeLa cells.

Supplementation of Cell Culture Medium with Glycine Enhances Phototoxicity and Cell Death in Malas2-expressing HeLa Cells

The phototoxicity and subsequent cell death caused by mALAS2-induced PPIX accumulation, when the culture medium was supplemented with glycine, were also investigated in HeLa cells. Cell viability was measured using propidium iodide (data not shown), DAPI staining, and MTT assays (Figure 7) 24 hours post-transfection, 18 hours after glycine addition, and 4 hours after ALA addition. Significant cell death was ascribed to those samples that exhibited more cell death compared to the pIRES2-ZsGreen1-transfected cells with the same glycine concentration as determined by Student’s t-tests (p < 0.05). Transfection of HeLa cells with the pIRES2-ZsGreen1 plasmid caused a decrease in cell viability by an average of 30%, which can be attributed to the mild cytotoxicity of the transfection reagents [54].
Expressing WT, regardless of the glycine concentration in the culture medium, caused approximately 70% cell death following light exposure, as measured by DAPI staining (Figure 7A–B). In the MTT assays, expression of WT only caused statistically significant cell death (p<0.05) with supplementation of 100 mM glycine, in which case only an average of 15% of cells remained viable (Figure 7C). HeLa cells expressing HPVT or R433K did exhibit a glycine-dependent increase in cell death when the glycine

Figure 5. Fluorescence microscopy of HeLa cells expressing GFP and the R433K mALAS2 variant. From left to right for each row, the panels correspond to (1) brightfield, (2) green fluorescence, (3) red fluorescence, (4) superimposed green and red fluorescence, and (5) superimposed brightfield, green fluorescence, and red fluorescence images. (A) HeLa cells grown to confluence in culture medium with 100 mM glycine. (B) HeLa cells transfected with pRES2-ZsGreen1 and grown to confluence in culture medium with 100 mM glycine. (C) HeLa cells transfected with pEF31, which expresses the R433K variant from the CMV promoter in the pRES2-ZsGreen1 vector (Table 1), and grown to confluence in culture medium with 100 mM glycine. (D) HeLa cells transfected with pEF31 and grown to subconfluence (i.e., 80% confluence) in culture medium with 100 mM glycine. Pictures were obtained 24 hours after transfection and 18 hours after glycine supplementation. ZsGreen1 (green) is present throughout the cell, while PPIX (red) accumulates at the plasma membrane of cells expressing R433K and surrounding cells. doi:10.1371/journal.pone.0093078.g005

Figure 6. Light-induced cell death of HeLa cells expressing mALAS2 variants. (A) Cell death measured by changes in DNA-bound propidium iodide fluorescence. (B) Cell death measured by changes in DNA-bound DAPI fluorescence. HeLa cells were transfected with expression plasmids for either wild type (WT) or mALAS2 variants and treated with 10 nM paclitaxel 4-hour post-transfection. 48 hours after transfection, cells were stained and analyzed by flow cytometry. Cell death percentage values were compared to that of the pIRES2-ZsGreen1-transfected cells control. Each data set represents three separate experiments ± standard deviation (*p<0.05 and **p<0.01, Student's t-test). doi:10.1371/journal.pone.0093078.g006
concentration reached 100 mM as measured by both DAPI staining and MTT assays. When 100 mM glycine was added, cells expressing HPVT decreased from an average of 81% viable to 13% viable, and cells expressing R433K decreased from 58% viable to 25% viable as measured by MTT assays (Figure 7C). ALA treatment did not cause significant cell death (p > 0.05), regardless of glycine concentration, when measured by either DAPI staining or MTT assays.

Discussion

Photodynamic therapy (PDT) is a widely utilized clinical procedure for many types of cancer, as well as dermatological conditions such as psoriasis and scleroderma [55–57]. PDT is often initiated by application of the pro-drug 5-aminolevulinic acid (ALA) to the patient in order to photosensitize the tissue to be treated [49]. ALA-PDT is very effective, has minimal side effects and is being tested in numerous clinical trials on a wide-spectrum of cancers [49]. There are, however, some limitations to ALA-PDT and other PDTs that provide opportunities for improvement. For example, there is no specific mechanism that targets PPIX to tumor cells after ALA has been applied, other than a slightly preferential uptake of photosensitizers by hyperproliferating cells [58]. Consequently, normal tissue can be damaged from the procedure due to unintentional exposure to light resulting in pain, and conversely, not all hyperproliferating cells necessarily become highly sensitized [55,59]. In an attempt to restrict photosensitivity by biological, rather than just chemical or physical means, an adenovirus expressing human ALAS2 with mutated HRMs was generated and H1299 lung carcinoma cells were successfully infected [40]. The adenovirus-infected H1299 cells accumulated more PPIX and became more photosensitive than cells supplied with ALA in their media, indicating that delivery of ALAS to tumors could potentially be a useful tool for PDT not only for better targeting, but also for greater photosensitivity [40]. However, the cell death was only 26% even in the presence of deferoxamine [40], leading us to postulate that one or more highly active variants of mALAS2 [31,35] would increase PPIX.
accumulation, phototoxicity, and targeted cell death sufficiently to make the approach more clinically attractive.

To explore the ability of mALAS2 variants to cause accumulation of PPIX in mammalian cells, we transfected cells, of both erythroid and non-erythroid lineages, with murine ALAS2-expressing plasmids, and quantified the PPIX fluorescence using FACS analysis. In HeLa cells, we transfected plasmids encoding mALAS2 both with and without mutated presequences and with or without mutations in the mature enzyme sequence (Table 1, Figure 1). As with many mitochondrial proteins, ALAS2 is synthesized in the cytosol and contains a sequence at its N-terminus that targets ALAS2 for mitochondrial import after its synthesis [18]. Within the N-terminus of the ALAS2 precursor, there are three HRMs as recognized by adjacent cysteine-proline residues that have the potential to bind heme. The HRMs located within the presequence of ALAS2 (C11 and C38) have been shown to bind heme [24] and subsequently inhibit the mitochondrial import of ALAS2 [18]. Our experiments support the existing data that the cysteines in the HRMs bind heme, and that mutation of these HRMs relieve the inhibition of mitochondrial import, thus resulting in increased mature, functional ALAS2, as reflected by increased cellular concentrations of PPIX when the HRMs are mutated (Figure 2A). In our study, when the HRMs in the presequences of the mALAS2 constructs were mutated to relieve heme inhibition on mitochondrial import, there were significant increases in PPIX accumulation in HeLa cells expressing WT, HPVT, and R433K (Figure 2A-B).

We tested several mALAS2 variants, covering a range of in vitro activity from undetectable to higher than wild-type, for capacity to stimulate PPIX accumulation [31,35,36,60]. Transfection of HeLa cells with the negative control plasmid harboring K313A resulted in no PPIX increase, as expected (Figure 2A). The mutation of K313 leads to undetectable enzymatic activity in vitro, attributable to the role of K313 in formation of a Schiff-base linkage with the PLP cofactor [36], and its additional function as a general base catalyst during the ALAS-catalyzed reaction [3,28,36,61]. In preliminary studies with HeLa cells, it was observed that expression of HPVTY, which has seven mutations in its active site loop, yielded significantly more accumulated PPIX than expression of the pIRES2-ZsGreen1 vector control plasmid. HPVTY was chosen for this study as the seven mutations of non-conserved residues in the active site loop resulted in the most active recombinant protein isolated from a variant library at 20°C [35]. Lendrihas et al. [35] hypothesized that these mutations in the active site loop, which increase both hydrophilicity and basicity, destabilize the loop by both increasing solubility and eliminating hydrophobic and electrostatic interactions that would typically act to stabilize the loop in its closed confirmation. However, the hyperactivity of HPVTY was temperature-dependent; while at a temperature of 20°C these seven mutations increased the k_{cat} value of the recombinant, mature enzyme to more than 10 times of that of the WT enzyme, at 35°C the k_{cat} values were nearly the same [35]. While the levels of accumulated PPIX in HeLa cells expressing HPVTY were significantly affected by supplementation with glycine (p<0.01), those in K562 cells expressing HPVTY were only modestly increased when the medium was supplemented with 100 mM glycine. However, the HPVTY-promoted PPIX concentration enrichments were much lower than those in cells expressing WT or R433K (Figures 3 and 4). The relatively low levels of PPIX associated with HPVTY expression are presumably due to the temperature-dependent activity profile of this particular mALAS2 variant. HPVTY was isolated from a library of mALAS2 variants engineered to possess greater enzymatic activity than wild type mALAS2 by targeting the active site loop to acquire different degrees of mobility [35]. Since the mutations in HPVT destabilized the active site loop and altered the protein conformation [35], it would not be surprising if this variant had a decreased cellular stability. Additionally, the HPVT mutations may affect protein-protein interactions, specifically the ability of mALAS2 to interact with a known binding partner, succinyl-CoA synthetase [62].

The simple addition of the non-toxic substrate glycine to the cell media increased PPIX production by WT, HPVT, and R433K significantly. When the culture medium was supplemented to a final concentration of 10 mM or 100 mM glycine, PPIX production in HeLa cells expressing WT was increased by more than 4- and 6-fold for each concentration, respectively (Figure 3). In K562 cells expressing WT, the effect was even larger, as PPIX increased by more than 5- and 10-fold for 10 mM and 100 mM glycine, respectively (Figure 4A and 4C). The slightly higher increases in PPIX concentrations in the K562 cells when treated with glycine could be due to the stable expression of ALAS2 versus the transient transfection used in the HeLa cells. However, the ability of the K562 cells to tolerate higher expression of WT and production of PPIX, in comparison to HeLa cells, might also be attributable to their erythroid lineage. K562 cells are of the erythropoietic cell type, and bear some proteomic resemblance to undifferentiated erythrocytes [51,63] and express endogenous ALAS2. Thus when expressing ALAS2 in HeLa cells introduces an enzyme that does not normally exist in epithelial cells, K562 cells may adapt more easily to the expression and up-regulation of the heme biosynthetic pathway. It seems likely that the capacity of mALAS2 to accumulate PPIX will be found to vary in other mammalian cell types as well.

Supplementing the culture medium of K562 cells expressing WT with 100 μM deferoxamine, an iron-chelator previously shown to increase the amount of PPIX in ALAS adenovirus-infected H1299 cells [40], resulted in increase in the mean PPIX fluorescence per cell (Figure 4B and 4D), but it was much less than with glycine supplementation. However, the modest increase was similar to what Gagnebin et al. [40] observed in the ALAS adenovirus-infected cells, suggesting the much larger increases reported here with R433K and glycine supplementation represent substantial advancements in our ability to overproduce PPIX in mammalian cells. R433K is only twice as active as WT in vitro, and it is reasonable to believe that significantly more active variants could be readily produced via directed evolution, and that these variants would facilitate even greater levels of PPIX accumulation, perhaps to the point of expanding the clinical applicability of PPIX-based PDT.

The HeLa cells that accumulated the highest amount of PPIX were those expressing R433K. The R433K mutation was analyzed in 1998 by Tan et al. [31] in a study that identified the nearby R439 as being a conserved residue in many α-family PLP-dependent enzymes, as well as being involved in binding of the amino acid substrate during catalysis [31]. In that study, the kinetic parameters were defined for R439L, R439K, R433L, and R433K. Although not the primary focus of the article, the kinetic parameters for R433L were found to be comparable to those of the wild-type at 37°C, while for R433K the k_{cat} was increased by two-fold, with no effect on the Michaelis constants, resulting in a doubling of catalytic efficiency for both substrates [31]. In the results detailed here, expression of R433K in HeLa cells produced a 2- to 3-fold increase in PPIX in comparison to WT, and a 4- to 6-fold increase in PPIX fluorescence in comparison to cells transfected with the pIRES2-ZsGreen1 vector control or HeLa cells alone (Figure 2). When the culture medium was supplemented with glycine, PPIX accumulation increased by 13- to 15-fold in
In this study, we have shown that transfecting mammalian cells, of both erythroid and non-erythroid lineages, with mALAS2 variants, is an effective way to stimulate cellular PPIX accumulation. Furthermore, supplementing the culture medium with glycine vastly increases the intracellular PPIX levels when cells express either WT or R433K. These data offer new ways to accrual in organelle membranes awaits approaches resulting in better resolution than we were able to achieve here.

In order to study the photosensitivity of cells that accumulated PPIX (caused by expression of WT, HPVT, and R433K), we performed light treatments using an incandescent lamp, which emits light in the visible and infrared spectral regions (400–1000 nm). The cells were exposed for 10 minutes before FACS analysis of PPIX and cell death. Expression of the ZsGreen1 protein alone caused a toxicity that was independent of light treatment, and thus significant cell death was measured against the pIREs2-ZsGreen1 vector-transfected cells (p<0.05) as opposed to HeLa cells alone (Figure 7). Regardless of glycine concentration, the expression of WT led to a significant increase in cell death by 1.4- to 2-fold. R433K and HPVT, when the culture medium was supplemented with 100 mM glycine, also caused significant increases in cell death by 2.1- and 1.6-fold, respectively. With no or 10 mM glycine supplementation the error was too high for any of the increases to be significant, although there was a trend towards increased cell death. For WT and R433K, increased PPIX accumulation correlates with increased cell death after light treatment. In spite of the more modest increases in PPIX accumulation, light-induced death also occurred in HPVT-expressing cells. This finding led us to suggest that toxicity inherent to the overproduced HPVT protein is the other contributing factor to cell death. Although purified recombinant, mature HPVT is more active than WT at 37°C (with an enhancement of approximately 1.5 in the kcat value at 37°C as a 15.5-fold increased kcat at 20°C) [63], when expressed in mammalian cells, HPVT, HPVT by PPIX fluorescence. Since expression of HPVT paired with glycine supplementation caused significant cell death (Figure 7), the potential role of another toxic heme pathway intermediate, such as the pro-oxidant ALA [66–69], in causing cell death is plausible. However, at the present, different levels of protein expression [Quantification of ALAS2 and variant protein levels was not feasible due to the unavailability of a functional ALAS2 antibody.] and distinct modulation of the enzymatic activity between ALAS2 and variants cannot be ruled out as possibilities. Treatment with 100 μM ALA, a positive control for PPIX accumulation, caused a similar cell death after light exposure to expression of the ZsGreen1 protein alone. In summary, the highest cell death was seen in light-treated HeLa cells expressing ZsGreen1 and either WT, HPVT, or R433K, in culture medium supplemented with 100 mM glycine. The total cell death was observed to be as high as 90% in the cells expressing both ZsGreen1 and R433K. This represents a substantial improvement upon the 26% cell death reported previously [40], and indicates that this approach, if carefully developed, may eventually find some clinical utility.

Because ALAS2, especially highly stable and active variants of ALAS2, would be useful in the development of multiplex cancer treatments involving PDT, we experimented with combination PDT and drug treatments of HeLa cells. Paclitaxel (Taxol) is currently approved in the United States for the treatment of AIDS-related Kaposi sarcoma [70], breast cancer [71], non-small lung cell lung cancer [72], and ovarian cancer [73]. Paclitaxel induces apoptosis in cancer cells by binding to tubulin and inhibiting the disassembly of microtubules, thereby resulting in the inhibition of cell division [46,74]. As expected, paclitaxel did not affect PPIX accumulation, as indicated by the similar mean PPIX fluorescence values between untreated and paclitaxel-treated cells (Figure 2B). However, paclitaxel had an additive effect to PDT and increased cell death in all samples by 10–25%. HeLa cells expressing R433K treated with paclitaxel exhibited the highest percentage of cell death (Figure 6A–B). Ever since PDT was first shown to be able to elicit an immune response [75], many recent advances in PDT are aiming toward creating PDT-generated cancer vaccines [76]. Using highly active and stable ALAS2 variants as part of a vaccine strategy for photosensitization may be useful for this vaccine approach. Further experiments, both in cell culture and live animals, are necessary to test for potential immune response stimulated by ALAS2-PDT.

In this study, we have shown that transfecting mammalian cells, of both erythroid and non-erythroid lineages, with mALAS2 variants, is an effective way to stimulate cellular PPIX accumulation. Furthermore, supplementing the culture medium with glycine vastly increases the intracellular PPIX levels when cells express either WT or R433K. These data offer new ways to accumulate high levels of the photosensitizer PPIX in cancer cells for more targetable and efficient PDT. Human ALAS2 variants with higher than normal activity are now known to occur in nature, and are associated with a form of erythropoietic protoporphyria known as X-linked dominant protoporphyria, which is characterized by a 24-fold increase in erythrocyte PPIX concentrations [39]. It would be appropriate if these variants were eventually utilized in PDT, and could thereby allow clinicians to exploit one disease to treat or cure others.
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Author Contributions

Conceived and designed the experiments: EJF GAH GCF. Performed the experiments: EJF. Analyzed the data: EJF GAH GCF. Contributed reagents/materials/analysis tools: GCF. Wrote the paper: EJF GAH GCF.

References

1. Jordan PM (1990) In: Dailey HA, editor. Biosynthesis of Heme and Chlorophylls. New York: McGraw-Hill. 55–121.
2. Hunter GA, Ferreira GC (2011) Molecular enzymology of 5-aminolevulinate synthase, the gatekeeper of heme biosynthesis. Biochim Biophys Acta 11: 1467–1473.
3. Ferreira GC, Gong J (1995) 5-Aminolevulinate synthase and the first step of heme biosynthesis. J Bioenerg Biomembr 27: 151–159.
4. Riddle RD, Yamamoto M, Engel JD (1989) Expression of delta-aminolevulinate synthase in avian cells: separate genes encode erythropoiesis-specific and nonerythropoietic isoforms. Proc Natl Acad Sci USA 86: 792–796.
5. Sutherland GR, Baker E, Callen DF, Hyland VJ, May BK, et al. (1980) 5-Aminolevulinate synthase is at 3q21 and thus not the primary defect in X-linked sideroblastic anemia. Am J Hum Genet 43: 331–335.
6. Bishop DF, Henderson AS, Astrin KG (1990) Human delta-aminolevulinate synthase: assignment of the housekeeping gene to 3p21 and the erythropoiesis-specific gene to the X chromosome. Genomics 7: 207–214.
7. Watanabe N, Hayashi N, Kikuchi G (1983) delta-Aminolevulinate synthase: solution structure and heme binding of the presequence of murine delta-aminolevulinate synthase. Biochim Biophys Acta 863: 381–386.
8. Cox TG, Bajer AS, Bajer LA, Abraham NG, Bottomley SS, et al. (1990)mtx synthetic enzymes: in vivo identification of three aminolaevulinate synthase isozymes. Proc Natl Acad Sci USA 86: 386–391.
9. Yamauchi K, Hayashi N, Kikuchi G (1980) Translocation of delta-aminolevulinate synthase from the cytosol to the mitochondria and its regulation by heme in the rat liver. J Biol Chem 255: 1746–1751.
10. Srivastava G, Bawden MJ, Abraham NG, Bottomley SS, May BK, et al. (1990) Delta-aminolevulinate synthase affects enzyme catalysis through enhancing the function of the pyridoxal 5'-phosphate cofactor. Biochemistry 35: 3509–3517.
11. Lendvai T, Hunter GA, Ferreira GC (2009) Arg-85 and Thr-430 in murine 5-aminolevulinate synthase coordinate acyl-CoA-binding and contribute to substrate specificity. Protein Sci 18: 1847–1859.
12. Lendvai T, Hunter GA, Ferreira GC (2010) Serine 254 enhances an induced fit mechanism in murine 3-aminolevulinate synthase. J Biol Chem 285: 3531–3542.
13. Lendvai T, Hunter GA, Ferreira GC (2010) Targeting the active site gate to yield hyperactive variants of 5-aminolevulinate synthase. J Biol Chem 285: 13704–13711.
14. Ferreira GC, Neame PJ, Dailey HA (1993) Heme biosynthesis in mammalian systems: evidence of a Schiff base linkage between the pyridoxal 5'-phosphate cofactor and a lysine residue in 5-aminolevulinate synthase. Protein Sci 2: 1959–1965.
15. Hunter GA, Ferreira GC (1999) Lysine-313 of 5-Aminolevulinate Synthase Acts as a General Base during Formation of the Quinonoid Reaction Intermediates. Biochemistry 38: 3711–3718.
16. Aminolevulinate synthase: lysine 313 is not essential for binding the pyridoxal phosphate cofactor but is essential for catalysis. Protein Sci 4: 1001–1006.
17. Tan D, Hunter GA, Ferreira GC (1998) The role of 5-aminolevulinate synthase in Ca2+ binding in colubrid binding of 5-aminolevulinate synthase. Protein Sci 7: 1200–1213.
18. Tan D, Harrison T, Hunter GA, Ferreira GC (1998) Role of arginine 439 in substrate binding of 5-aminolevulinate synthase. Biochemistry 37: 1478–1484.
19. Gong J, Hunter GA, Ferreira GC (1998) Apurinic-279 in 8-aminolevulinate synthase affects enzyme catalysis through enhancing the function of the pyridoxal 5'-phosphate cofactor. Biochemistry 37: 3509–3517.
20. Hunter GA, Ferreira GC (1999) Active site of 5-a Correspondence as a General Base during Formation of the Quinonoid Reaction Intermediates. Biochemistry 38: 3711–3718.
21. Hunter GA, Ferreira GC (1999) Lysine-313 of 5-Aminolevulinate Synthase Acts as a General Base during Formation of the Quinonoid Reaction Intermediates. Biochemistry 38: 3711–3718.
22. Hunter GA, Ferreira GC (1999) Lysine-313 of 5-Aminolevulinate Synthase Acts as a General Base during Formation of the Quinonoid Reaction Intermediates. Biochemistry 38: 3711–3718.
23. Hunter GA, Ferreira GC (1999) Lysine-313 of 5-Aminolevulinate Synthase Acts as a General Base during Formation of the Quinonoid Reaction Intermediates. Biochemistry 38: 3711–3718.
24. Hunter GA, Ferreira GC (1999) Lysine-313 of 5-Aminolevulinate Synthase Acts as a General Base during Formation of the Quinonoid Reaction Intermediates. Biochemistry 38: 3711–3718.
25. Hunter GA, Ferreira GC (1999) Pre-steady-state reaction of 5-aminolevulinate synthase. Evidence for a rate-determining product release. J Biol Chem 274: 12222–12228.
51. Andersson LC, Nilsson K, GalenberG CG (1979) K562-a human erythroleukemic cell line. Int J Cancer 23: 143–147.
52. Høffbrand AV, Wonke B (1997) Iron chelation therapy. J Intern Med Suppl 740: 37–41.
53. Roberts DJ, Rees D, Howard J, Hyde C, Alderson P, et al. (2005) Desferrioxamine mesylate for managing transfusional iron overload in people with transfusion-dependent thalassaemia. Cochrane Database Syst Rev: CD001450.
54. Nikcovic G, Kovacevic-Grujicic N, Stevanovic M (2003) Improved transfection efficiency of cultured human cells. Cell Biol Int 27: 735–737.
55. Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, et al. (2011) Photodynamic therapy of cancer: an update. CA Cancer J Clin 61: 250–281.
56. Dolmans DE, Fukumura D, Jain RK (2003) Photodynamic therapy for cancer. Nat Rev Cancer 3: 380–387.
57. Darlemaki R, Fluri JW (2013) Photodynamic therapy in dermatology: past, present, and future. J Biomed Opt 18: 061208.
58. Babalis P, Landthaler M, Szwarc M (2006) Photodynamic therapy in dermatology. Eur J Dermatol 16: 340–348.
59. Warren CB, Karai LJ, Vidimos A, Maytin EV (2009) Pain associated with aminolevulinic acid-photodynamic therapy of skin disease. J Am Acad Dermatol 61: 1033–1043.
60. Hunter GA, Ferreira GC (1999) Lysine-313 of 5-aminolevulinate synthase acts as a general base during formation of the quinonoid reaction intermediates. Biochemistry 38: 3711–3719.
61. Hunter GA, Ferreira GC (1999) Lysine-313 of 5-aminolevulinate synthase acts as a general base during formation of the quinonoid reaction intermediates. Biochemistry 38: 12526.
62. Bishop DF, Tchaikovskii V, Høffbrand AV, Fraser ME, Margolis S (2012) X-linked sideroblastic anemia due to carbonyl-terminal ALAS2 mutations that cause loss of binding to the beta-subunit of succinyl-CoA synthetase (SUCLA2). J Biol Chem 287: 20943–20953.
63. Anderson LC, Jokinen M, Klein E, Klein G, Nilsson K (1979) Presence of erythrocytic components in the K562 cell line. Int J Cancer 24: 514.
64. Ji Z, Yang G, Vasovic V, Cunderlikova B, Szu Z, et al. (2006) Subcellular localization pattern of protoporphyrin IX is an important determinant for its photodynamic efficiency of human carcinoma and normal cell lines. J Photochem Photobiol B 84: 213–220.
65. Lendrillas T, Hunter GA, Ferreira GC (2010) Targeting the active site gate to yield hyperactive variants of 5-aminolevulinic synthase. J Biol Chem 285: 13704–13711.
66. Juknat AA, Koster ML, Quaglino A, Carrillo NM, Hevor T (2003) Necrotic cell death induced by delta-aminolevulinic acid in mouse astrocytes. Protective role of melatonin and other antioxidants. J Pineal Res 35: 1–11.
67. Weiss TS, Pahtirk S, Scherrer I, Jauch KW, Thaler WE (2003) Cellular damage to human hepatocytes through repeated application of 5-aminolevulinic acid. J Hepatol 38: 476–482.
68. Feltisyn N, McLeod C, Shrodes AL, Stacpoole PW, Notterpek L (2008) The heme precursor delta-aminolevulinic blocks peripheral myelin formation. J Neurochem 106: 2068–2079.
69. Hunter GA, Rivera E, Ferreira GC (2003) Supraphysiological concentrations of 5-aminolevulinic acid dimerize in solution to produce superoxide radical anions via a protonated dihydropyrazine intermediate. Arch Biochem Biophys 417: 128–137.
70. Cheung MC, Pantanowitz L, Dezube RJ (2005) AIDS-related malignancies: emerging challenges in the era of highly active antiretroviral therapy. Oncologist 10: 412–426.
71. Sparano JA, Wang M, Martin R, Jones V, Perez EA, et al. (2008) Weekly paclitaxel in the adjuvant treatment of breast cancer. N Engl J Med 358: 1663–1671.
72. Ramalingam SS, Khuri FR (2009) The role of the taxanes in the treatment of older patients with advanced stage non-small cell lung cancer. Oncologist 14: 412–424.
73. Katsumata N, Yasuda M, Takahashi F, Isonishi S, Jobo T, et al. (2009) Doxorubicin-paclitaxel once a week in combination with carboplatin every 3 weeks for advanced ovarian cancer: a phase 3, open-label, randomised controlled trial. Lancet 374: 1331–1338.
74. Horwitz SB (1992) Mechanism of action of taxol. Trends Pharmacol Sci 13: 134–136.
75. Canti G, Lattuada D, Nicolin A, Taroni P, Valentini G, et al. (1994) Antitumor immunity induced by photodynamic therapy with aluminum disulfonated phthalocyanines and laser light. Anticancer Drugs 5: 443–447.
76. Körbelik M (2010) Photodynamic therapy-generated cancer vaccines. Methods Mol Biol 635: 147–153.