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Small molecule additive enhances cell uptake of 5-aminolevulinic acid and conversion to protoporphyrin IX

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Abstract

Administration of exogenous 5-aminolevulinic acid (5-ALA) to cancerous tissue leads to intracellular production of photoactive protoporphyrin IX, a biosynthetic process that enables photodynamic therapy and fluorescence-guided surgery of cancer. Cell uptake of 5-ALA is limited by its polar structure and there is a need for non-toxic chemical additives that can enhance its cell permeation. Two zinc-bis(dipicolylamine) (ZnBDPA) compounds were evaluated for their ability to promote uptake of 5-ALA into Chinese Hamster Ovary (CHO-K1) cells and produce protoporphyrin IX. One of the ZnBDPA compounds was found to be quite effective, and a systematic comparison of cells incubated with 5-ALA (100 μ M) for 6 hours showed that the presence of this ZnBDPA compound (10 μ M) produced 3-fold more protoporphyrin IX than cells treated with 5-ALA alone. The results of mechanistic studies suggest that the ZnBDPA compound does not interact strongly with the 5-ALA. Rather, the additive is membrane active and transiently disrupts the cell membrane, permitting 5-ALA permeation. The membrane disruption is not severe enough to induce cell toxicity or allow passage of larger macromolecules like plasmid DNA.

Graphical Abstract

Cells incubated with an admixture of 5-ALA (100 μ M) and a zinc(II)-bis(dipicolylamine) compound (10 μ M) produced 3-fold more protoporphyrin IX than cells treated with 5-ALA alone.

Introduction

5-Aminolevulinic acid (5-ALA) is a naturally occurring compound present in mammalian cells that is metabolized via the heme biosynthetic pathway to produce photoactive protoporphyrin IX (PpIX).¹ Supplying exogenous 5-ALA to metabolically active cells leads

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to elevated levels of PpIX, which enables both fluorescence imaging of the cells and photodynamic therapy (PDT). The PDT application typically uses red light to excite the PpIX, which in the presence of molecular oxygen generates cytotoxic reactive oxygen species.² PDT based on 5-ALA dosage has been used to treat several types of cancers including skin, bladder, esophagus, and brain.^{3–8} At present, 5-ALA is approved in the USA for PDT of actinic keratosis and esophageal dysplasia.⁹ In addition, the biosynthetic conversion of 5-ALA into fluorescent PpIX has been used for fluorescence-guided surgery.¹⁰ Most notably, 5-ALA has been used clinically in a method to facilitate surgery of malignant gliomas, allowing for easier determination of tumor margin resection.¹¹ The full clinical utility of this new approach to surgery still needs to be evaluated on a large scale, as questions remain about its reliability due to tissue dependent variations in signal intensity.¹²

Depending on the exact application, the 5-ALA can be delivered to cancerous tissue either topically or systemically.¹³ Topical delivery is attractive because it limits patient exposure and widespread toxicity,¹⁴ but 5-ALA is a small amino acid with a polar zwitterionic structure at physiological pH and does not penetrate biological membranes. For topical administration, not only does the 5-ALA have to traverse the different histological layers of skin and tissue, it eventually has to be taken up by the target cancer cells. To obviate this problem, a large number of physical and chemical strategies have been examined to improve topical delivery.¹⁴ The chemical delivery methods can be classified as covalent or non-covalent approaches. The covalent approach alters the 5-ALA structure by conversion to an ester or peptide derivative. Indeed, methyl and hexyl ester derivatives of 5-ALA are approved for clinical use and have been used to treat actinic keratosis, basal cell carcinoma, and bladder cancer.^{15, 16} Other variations of this approach have examined branched polymer scaffolds with covalently attached 5-ALA.^{17–19} A general concern with this covalent approach is that it requires the action of intracellular esterases or proteases to liberate the 5-ALA. The intracellular concentration of these enzymes can vary with tumor microenvironment, and thus become another source of variability for PpIX production.^{20,21} For example, when dipeptide derivatives were tested in different cancer cell lines, the amount of PpIX generated was dependent on the expression level of a key protease.²² Non-covalent methods of 5-ALA delivery circumvent the need for intracellular esterases and proteases since they deliver unmodified 5-ALA that can directly enter the PpIX biosynthesis pathway. Different research groups have studied chitosan nanoparticles^{23,24} and liposomes^{25,26} loaded with 5-ALA to aid in intracellular delivery and, in some cases, transdermal delivery of 5-ALA.^{23,26} The simplest chemical approach is to use an organic solvent such as DMSO (dimethylsulfoxide)²⁷ or a surfactant²⁸ as a formulation additive to enhance cell permeation. An obvious concern with this method is the potential for localized chemical toxicity or inflammation. Over the last two decades, several new classes of membrane-active molecules have been shown to promote cell uptake of large and small polar pharmaceuticals.^{29–31} However, this new cell permeation technology has yet to be evaluated as a new potential method to deliver 5-ALA into cells for subsequent biosynthetic conversion into photoactive PpIX.

For some time we have explored the membrane targeting properties of zinc(II)-bis(dipicolylamine) (ZnBDPA) compounds, and we have uncovered examples that can promote membrane transport and cell entry.^{32–34} Recently, we reported a method for

controlled release of 5-ALA and other payloads from anionic liposomes using ZnBDPA compounds as chemical triggers.³⁵ During these studies, we noticed that mammalian cells treated with admixtures of 5-ALA and ZnBDPA compounds generated more cellular PpIX than cells treated with only 5-ALA alone. Here, we pursue this observation and describe a detailed study of enhanced 5-ALA delivery and intracellular PpIX production using ZnBDPA compounds **1** and **2** (Fig. 1) as small molecule chemical additives. The study also includes experiments that shed light on the mechanism for the enhanced 5-ALA uptake.

Materials and Methods

Chemicals

Chinese Hamster Ovary (CHO-K1) cells were purchased from American Type Culture Collection (Manassas, VA, USA). F-12K media was also purchased from American Type Culture Collection. Fetal bovine serum was purchased from Atlanta Biologicals Inc. (Flowery Branch, GA, USA). Corning Cellgro® Penicillin–Streptomycin solution was purchased from Corning Inc. (Corning, NY, USA). Lipofectamine® 2000 Transfection Reagent and OptiMEM® media were purchased from Life Technologies (Grand Island, NY, USA). Endotoxin-Free EGFP plasmid DNA (GFP-P) was purchased from Addgene (Cambridge, MA, USA). The ZnBDPA compounds **1** and **2** were synthesized as described previously.³⁶ All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification.

Cell Culture

CHO-K1 cells were grown in F-12K media supplemented with 10% FBS and 1% penicillin-streptomycin solution at 37°C and 5% CO₂. Cells were seeded to near confluency either in a 96-well plate for fluorimetry and cell viability studies, an 8-chambered plate for fluorescence microscopy studies or a 6-well plate for flow cytometry studies. Cells were seeded to 75% confluency in a 96-well plate for GFP-plasmid transfection studies.

Determination of Cellular PpIX Production

Protoporphyrin extraction from cells was determined using a previously described acid extraction assay.³⁷ CHO-K1 cells were grown to a confluency of 8×10^4 cells per well in a 96-well plate, then different microwells were treated in triplicate with cell media only, 5-ALA alone, or 5-ALA mixed with **1** or **2** as additives. Cells were incubated for 6 hours at 37°C and 5% CO₂ in serum-free F-12K media. The biosynthesized PpIX was extracted from the cells in each microwell by replacing the surrounding media with 200 μ L of 5% HCl, and incubating for an additional 30 minutes at 37°C. Each sample was subjected to a 5-fold dilution in 5% HCl prior to collecting fluorescence emission spectra ($\lambda_{\text{ex}} = 406$ nm, $\lambda_{\text{em}} = 604$ nm) on a Fluoromax-4 spectrofluorometer with FluorEssence software (Horiba Scientific, Edison, NJ, USA).

Cell Microscopy

CHO-K1 cells were seeded in an 8-well chambered plate and grown to confluency as described above. Wells were either treated with 5-ALA alone (100 μ M) or 5-ALA (100 μ M) plus **2** (10, 25, or 50 μ M), followed by incubation for 6 hours at 37°C and 5% CO₂ in serum-

free F-12K media. Media in each well was then removed; cells were washed once and resuspended in PBS buffer. Live cell imaging was performed using a Nikon TE-2000U epifluorescence microscope equipped with a Cy3 filter set ($\lambda_{\text{ex}} = 535/50 \text{ nm}$, $\lambda_{\text{em}} = 610/75 \text{ nm}$) (Nikon Instruments Inc., Melville, NY, USA) ($60\times$ magnification). Bright field and fluorescence images were acquired. Using Image J analysis 1.40g software, the mean total fluorescence intensity for five random micrographs from each treatment group was determined and the data normalized against the micrographs of cells incubated with 5-ALA alone.

Flow Cytometry

CHO-K1 cells were grown to confluency in a 6-well culture plate in F-12K media supplemented with 10% FBS, 1% Penicillin-Streptomycin at 37°C , 5% CO_2 . Cells were treated with $100 \mu\text{M}$ 5-ALA, with and without **2** ($10 \mu\text{M}$) for 6 hours in serum-free F-12K media. The cells were trypsinized, resuspended in PBS, transferred to flow cytometry tubes, and protected from light before data collection. Flow cytometry was performed using a Beckman-Coulter FC500 flow cytometer with 10,000 cells per treatment group in the FL4 filter channel (660–690 nm). Data was normalized to the mean fluorescence of cells treated only with $100 \mu\text{M}$ 5-ALA, and the fluorescence of untreated cells was subtracted as background autofluorescence. FlowJoX software was used for quantitative analysis.

Coumarin Sulfate Displacement Assay

A standard coumarin sulfate (CS) fluorescence dye displacement assay was employed to measure association.³⁸ Binding assays were performed using a fluorescently quenched 1:1 mixture of CS: **2** (each $10 \mu\text{M}$) and the fluorescence spectrum of CS (400 nm – 650 nm) was collected on a Fluoromax-4 spectrofluorometer with FluorEssence software (Horiba Scientific, Edison, NJ, USA). Titration experiments added 1–10 molar equivalents of 5-ALA to a 1:1 mixture of CS: **2** (each $10 \mu\text{M}$). Pyrophosphate (PPi) was titrated as a positive control anion that associates strongly with **2**, displaces the CS dye, and restores CS fluorescence.

GFP Plasmid Transfection and PplX Production Assay

The workflow is shown in Figure 8A. CHO-K1 cells were seeded and grown to near 75% confluency in a 96-well plate in F-12K media supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO_2 . The cells were treated with a combination of GFP plasmid DNA (GFP-P, $0.2 \mu\text{g}/\text{well}$) and 5-ALA ($100 \mu\text{M}$) in the presence of either Lipofectamine® 2000 ($50 \mu\text{g}/\text{well}$) or **2** ($10 \mu\text{M}$) and incubated for 24 hours. The GFP-P/Lipofectamine® 2000 mixture in serum-free OptiMEM media was incubated at room temperature for 25 minutes in a separate tube before being adding to cells ($50 \mu\text{L}$ per well), with 5-ALA ($100 \mu\text{M}$ each well in OptiMEM media). Alternatively, additive **2** ($10 \mu\text{M}$) was mixed with the GFP-P under the same incubation conditions as Lipofectamine® 2000 treatment above with $100 \mu\text{M}$ 5-ALA. Fluorescence from expressed GFP was read with a fluorescence plate reader ($\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 510 \text{ nm}$) and normalized to the fluorescence of cells incubated with GFP-P and 5-ALA absent of additive. Media was then removed, replaced with $200 \mu\text{L}$ of 5% HCl, and the cells incubated for an additional 30 minutes. The

PpIX fluorescence was read ($\lambda_{\text{ex}} = 406 \text{ nm}$, $\lambda_{\text{em}} = 604 \text{ nm}$) and normalized as described above. Control wells containing cells treated with only OptiMEM media produced no significant fluorescence.

MTT Cell Viability Assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Cells were grown to a confluency of 8×10^4 cells per well and the Vybrant MTT cell proliferation Assay Kit (Invitrogen, Eugene, USA) was used according to the manufacturer's protocol and validated using 100% DMSO (causing complete cell death) as a positive control for the assay. Cells were treated with **1** or **2** (0–50 μM) in quadruplicate and incubated for 6 hours at 37°C . The medium was removed and replaced with 110 μL of F-12K media containing [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, 1.2 mM). An SDS–HCl detergent solution (100 μL) was added and plates were incubated at 37°C and 5% CO_2 for an additional 4 hours. The absorbance of each well was read at 570 nm, using the absorbance of wells containing only F-12K media and SDS-HCl solution as a blank. Viability values were normalized to cells treated with no additive.

Statistical Analysis

All experiments were performed in triplicate unless otherwise stated. Results are depicted as mean $\pm$ standard error of the mean (SEM). Data analysis was performed using Microsoft Excel and graphs were generated using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Statistical analysis was performed using a Student's t-test, and statistical significance was assigned as follows: * $P = 0.01$ – 0.05 ; ** $P = 0.001$ – 0.01 ; *** $P = 0.0001$ – 0.001 . An effect was assigned as not significant (n.s.) when $P > 0.05$.

Results

Uptake of added 5-ALA and intracellular conversion to PpIX in the presence or absence of additives **1** or **2** was initially evaluated using a cell extraction assay that measured the diagnostic orange/red fluorescence (604 nm) of the biosynthesized PpIX in CHO-K1 cells. It was important to first establish the PpIX production profile as a function of added 5-ALA. As shown in Figure 2, there was a sigmoidal dependence, with the amount of PpIX reaching a plateau when the added 5-ALA concentration was $> \sim 800 \mu\text{M}$. To ensure that any additive enhancement effect was not masked by this PpIX plateau, we chose to maintain the 5-ALA concentration during the additive enhancement experiments at a constant value of 100 μM , well below the plateau threshold.[†]

In Fig. 3A are the effects of **1** and **2** on 5-ALA (100 μM) uptake by CHO-K1 cells and conversion to PpIX. The presence of ZnBDPA **1** (10 μM) produced no statistically significant difference in the amount of intracellular PpIX after incubation periods of 3 and 6

[†]Control comparison experiments showed that co-incubation of 5-ALA (100 μM) plus additive **2** (10 μM) for 6 hours produced a 3-fold increase in PpIX fluorescence over 5-ALA alone, whereas co-incubation of 5-ALA (1.0 mM) plus additive **2** (10 μM) produced a smaller 1.5-fold increase in PpIX fluorescence over 5-ALA alone. This trend supports the hypothesis that an additive's enhancement effect on PpIX production gets smaller as the concentration of 5-ALA approaches the PpIX biosynthesis saturation limit of the cells.

hours. In contrast, co-incubation of 5-ALA plus ZnBDPA **2** (10 μ M) produced a 3-fold increase in PpIX fluorescence after 6 hours. The viability of the CHO-K1 cells, after 6-hour incubations with **1** and **2**, was evaluated using a standard MTT assay (Fig. 3B). Neither compound decreased cell viability at 10 μ M dosage, but there was a moderate cell toxicity at higher concentrations of 25 and 50 μ M. Based on these results, continued studies of enhanced 5-ALA uptake focused on **2** as the additive with a standard cell incubation time of 6 hours.

Fig. 4A shows the results of an additive dosage escalation experiment using the same cell extraction assay that kept the concentration of 5-ALA constant at 100 μ M but varied the concentration of ZnBDPA **2**. A bell-shaped relationship with the concentration of **2** was observed with the maximum three-fold increase in PpIX production observed when the concentration of **2** was 10 μ M. Independent confirmation of the bell-shaped dependence on additive concentration was obtained by epifluorescence microscopic imaging of the living CHO-K1 cells. Fluorescence micrographs of cells treated for 6 hours with 5-ALA alone (100 μ M) or 5-ALA plus varying concentrations of **2** are shown in Fig. 4B, with a graph of micrograph total fluorescence intensity in Fig. 4C. Consistent with the cell extraction results, a maximum 3-fold increase in PpIX production was observed microscopically when the 5-ALA (100 μ M) was co-incubated with 10 μ M of **2**. In addition, no changes in cell morphology or fluorescence localization were observed between untreated cells and cells treated with compound **2**.

To increase statistical accuracy, flow cytometry was used a third independent method to measure the extra amount of PpIX produced by the presence of additive **2**. As expected, when the added 5-ALA was 100 μ M the presence of additive **2** (10 μ M) produced an approximate 4-fold increase in PpIX production (Fig. 5).

An indicator displacement assay³⁸ was used to determine if ZnBDPA **2** can associate with 5-ALA in aqueous solution. The indicator dye was coumarin sulfate (CS), an anionic fluorophore that binds weakly to ZnBDPA molecules and forms a 1:1 CS:ZnBDPA complex with quenched fluorescence. A control titration experiment showed that CS can be displaced from a CS:**2** complex by pyrophosphate (PPi), an anion that has a high affinity for **2**. As shown in Fig. 6A, the addition of 1 molar equivalent PPi to the CS:**2** complex led to complete restoration of CS fluorescence. In contrast, there was no increase in CS fluorescence when 5-ALA was titrated into a solution of CS:**2** complex (Fig. 6B). Even a 10 molar excess of 5-ALA produced no evidence for CS displacement.

The effect of known amino acid transport inhibitor γ -aminobutyric acid (GABA) on intracellular PpIX production during 5-ALA administration in the presence or absence of ZnBDPA **2** was studied using the cell extraction assay (Fig. 7). Compared to CHO-K1 cells incubated with 5-ALA (100 μ M) alone, PpIX production dropped by nearly 50% in cells incubated with 5-ALA (100 μ M) and GABA (1 mM). However, the inclusion of **2** (10 μ M) with the 5-ALA and GABA treatment nullified the GABA inhibition effect on PpIX production, as no statistically significant difference was observed between cells treated with 5-ALA + **2** or 5-ALA + **2** + GABA.

Lastly, **2** and the transfection reagent Lipofectamine® 2000 were evaluated for ability to selectively enhance the cell uptake of 5-ALA in the presence of the much larger GFP plasmid DNA (GFP-P). The experiments treated cells with a binary mixture of 5-ALA and GFP-P and measured intracellular bioconversion of 5-ALA into PpIX and GFP-P into green fluorescent protein (GFP). The different co-incubation conditions are summarized in Fig. 8. The graphs in Fig. 8B–C show that co-incubation of cells with a mixture of 5-ALA, GFP-P and Lipofectamine® 2000 for 24 hours increased the intracellular amounts of biosynthesized GFP by a factor of 2.5 and PpIX by 7-fold. In contrast, co-incubation of cells with a mixture of 5-ALA, GFP-P and **2** had a much more selective effect; the production of PpIX was increased by a factor of 14 but there was no change in the intracellular amount of GFP.

Discussion

This study measured the effect of ZnBDPA compounds **1** and **2** on the uptake of 5-ALA into CHO-K1 cells and its intracellular conversion into fluorescent PpIX. CHO-K1 cells were chosen as a convenient model system for this study because the kinetics of PpIX production are well characterized.³⁹ In agreement with studies using other cell lines,^{28,40} intracellular PpIX production reached a plateau when the 5-ALA concentration was > ~800 μ M (Figure 2). To ensure that any additive enhancement effect was not masked by this PpIX plateau, a standard set of cell treatment conditions was employed (100 μ M 5-ALA and 6 hour incubation time) for all experiments. A cell extraction assay indicated that compound **2** was a more effective additive than compound **1**, and a systematic comparison of cells treated with 5-ALA (100 μ M) for 6 hours found that the presence of **2** (10 μ M) produced 3-fold more PpIX than cells incubated with 5-ALA alone. This additive enhancement of PpIX production was confirmed by separate studies using two other assays (cell microscopy and flow cytometry). It is worth noting that a similar enhancement study using the non-ionic surfactants, Tween 80 and Pluronic,²⁸ only reported a 1–2 fold increase in PpIX production although this study was performed in a different cell line at slightly higher concentrations of 5-ALA.[†] In agreement with previous literature,²⁸ the dependence on additive concentration was bell-shaped, with decreased PpIX production when additive **2** was more than 10 μ M. There are a number of possible explanations for this phenomenon, but two possibilities are that the higher concentrations of **2**; (a) depress cell viability and the rate of PpIX biosynthesis, (b) enhance PpIX efflux and thus decrease the maximum intracellular concentration of PpIX.^{41, 42}

5-ALA uptake into mammalian cells occurs via endogenous amino acid transporters, and previous studies have shown that γ -aminobutyric acid (GABA) blocks 5-ALA uptake by inhibiting the transporters.^{43–45} Consistent with this picture, we find that 5-ALA uptake and PpIX production in CHO-K1 cells is attenuated by the presence of GABA.⁴³ However, added GABA does not diminish the enhanced PpIX production caused by additive **2**, suggesting that **2** promotes cell uptake of 5-ALA by a process that does not involve the endogenous amino acid transport system.

Titration experiments indicate that ZnBDPA **2** does not associate with 5-ALA, in agreement with previous work showing that ZnBDPA compounds do not have a strong affinity for uncharged or zwitterionic biomolecules. However, ZnBDPA compounds are known to

associate with the surface of anionic membranes, and they have been shown to promote membrane transport of fatty acids and anionic phospholipids.^{32,34,36,46} The structure of **2** is dimer version of **1** and previously we have demonstrated that dimeric ZnBDPA compounds are much more effective at inducing leakage from liposomes than analogous monomeric ZnBDPA compounds.⁴⁷ Furthermore, we have shown that a fluorescent version of **2** self-aggregates on membrane surfaces, whereas a fluorescent version of **1** does not.³⁶ Therefore, a likely explanation for the enhancement effect of **2** is that it interacts with anionic cell plasma membrane components, such as polar lipids and fatty acids, and creates small, transient pores, which facilitate the transmembrane diffusion of 5-ALA.[‡] Similar transient interactions are thought to be involved in the membrane permeation activity of guanidinium-rich molecules.⁴⁸ The transient nature and small size of the putative pores are consistent with the results of co-incubation experiments, where **2** was found to selectively increase uptake of 5-ALA but not the much larger plasmid DNA that was also present in the solution (Fig. 8). In contrast, the micelle-forming transfection agent Lipofectamine® 2000 is known to interact strongly with cell membranes and in the co-incubation experiments was found to promote cell uptake of both 5-ALA and plasmid DNA.

In conclusion, this study shows that additive **2** significantly enhances PpIX production in living CHO-K1 cells that are treated with exogenous 5-ALA. To the best of our knowledge, additive **2** is the most effective low molecular weight enhancer of 5-ALA uptake and PpIX production yet reported. The mechanism studies reported here, together with our previous work on the interaction of ZnBDPA compounds with biological membranes,^{33–35} suggest that **2** forms transient pores at the membrane surface which substantially enhances 5-ALA entry and subsequent biosynthesis into PpIX. The next step in this research is to test the effect of additive **2** on 5-ALA uptake in clinically relevant models of topical transdermal delivery.

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Abbreviations

5-ALA	5-aminolevulinic acid
CS	coumarin sulfate fluorescent indicator
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GFP-P	green fluorescent protein plasmid DNA
PPi	pyrophosphate
PpIX	protoporphyrin IX

ZnBDPA bis(zinc(II)-dipicolylamine)**References**

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Fig. 1.
Chemical structures of 5-aminolevulinic acid (5-ALA) and small molecule additives, (1) and (2).

Fig. 2.
PpIX production (cell extraction assay) by separate batches of CHO-K1 cells at 3 hours after the addition of increasing amounts of 5-ALA.

Fig. 3.

A) PpIX production (cell extraction assay) by CHO-K1 cells 3 and 6 hours after addition of media only (No 5-ALA), 5-ALA alone (100 μM), or 5-ALA (100 μM) plus **1** or **2** (10 μM). PpIX emission intensities are normalized to cells incubated with 5-ALA alone at each time point, $\lambda_{\text{ex}} = 406 \text{ nm}$, $\lambda_{\text{em}} = 604 \text{ nm}$. $N = 3$. The absolute PpIX fluorescence at the 6 hour time point was 1.3-fold higher than the 3 hour time point. B) Viability of CHO-K1 cells treated with various concentrations of **1** and **2** for 6 hours. $N = 4$ for each treatment.

Fig. 4.

A) PpIX production (cell extraction assay) by CHO-K1 cells at 6 hours after addition of media only (No 5-ALA), 5-ALA alone (100 μM), or 5-ALA (100 μM) plus increasing concentrations of 2 (1–100 μM). N = 3. B) Fluorescence micrographs (Bright field along top; Cy 3 (PpIX) along bottom; 60 × magnification) of CHO-K1 cells at 6 hours after treatment with: media only (No 5-ALA), 5-ALA alone (100 μM), or 5-ALA plus 10, 25 or 50 μM of 2. Scale bar = 25 μm. C) Quantification of average total fluorescence for five randomly selected micrographs of each treatment condition. Emission intensities are normalized to micrographs of cells incubated with 5-ALA alone.

Fig. 5. Flow cytometry quantification of PpIX production in CHO-K1 cells at 6 hours after addition of: (a) media only (No 5-ALA), (b) 5-ALA alone (100 μ M), or (c) 5-ALA plus **2** (10 μ M). A) flow cytometry data for the three samples as a histogram. B) the fluorescence mean and standard error for each sample computed using FlowJo software.

Fig. 6. Affinity of 5-ALA for **2** measured by coumarin sulfate (CS) displacement assay. A) Titration of 10 μ M pyrophosphate (PPi) to a 1:1 mixture of CS:**2** (10 μ M each) produced a restoration of CS fluorescence. B) Titration of 100 μ M 5-ALA into a 1:1 mixture of CS and **2** (10 μ M each) produced no restoration of CS fluorescence.

Fig. 7. Intracellular PpIX production by CHO-K1 cells at 6 hours after addition of 5-ALA alone (100 μ M), 5-ALA plus GABA (1 mM), 5-ALA plus **2** (10 μ M), and 5-ALA plus GABA and **2**. PpIX emission intensities are normalized to the sample treated only with 5-ALA, λ_{ex} = 406 nm, λ_{em} = 604 nm. N = 3.

Fig. 8.

A) Workflow of GFP plasmid transfection and PpIX production assays using CHO-K1 cells.

B) Graph of expressed intracellular GFP emission intensity, $\lambda_{\text{ex}} = 470 \text{ nm}$, $\lambda_{\text{em}} = 510 \text{ nm}$.

C) Graph of intracellular PpIX emission intensity, $\lambda_{\text{ex}} = 406 \text{ nm}$, $\lambda_{\text{em}} = 604 \text{ nm}$. The amounts of added agents were Lipofectamine® 2000 (50 $\mu\text{g}/\text{well}$), **2** (10 μM), GFP-P (0.2 $\mu\text{g}/\text{well}$) and 5-ALA (100 μM) and the treated cells were incubated for 24 hours. N = 3.

Mean $\pm$ SD.