d-Aminolaevulinic acid-induced photodynamic therapy inhibits protoporphyrin IX biosynthesis and reduces subsequent treatment efficacy in vitro

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Summary Recently, considerable interest has been given to photodynamic therapy of cancer using d-aminolaevulinic acid to induce protoporphyrin IX as the cell photosensitizer. One advantage of this modality is that protoporphyrin IX is cleared from tissue within 24 h after d-aminolaevulinic acid administration. This could allow for multiple treatment regimens because of little concern regarding the accumulation of the photosensitizer in normal tissues. However, the haem biosynthetic pathway would have to be fully functional after the first course of therapy to allow for subsequent treatments. Photosensitization of cultured R3230AC rat mammary adenocarcinoma cells with d-aminolaevulinic acid-induced protoporphyrin IX resulted in the inhibition of porphobilinogen deaminase, an enzyme in the haem biosynthetic pathway, and a concomitant decrease in protoporphyrin IX levels. Cultured R3230AC cells exposed to 0.5 mM d-aminolaevulinic acid-induced protoporphyrin IX resulted in the inhibition of porphobilinogen deaminase, an enzyme in the haem biosynthetic pathway, and a concomitant decrease in protoporphyrin IX levels. Cultured R3230AC cells exposed to 0.5 mM d-aminolaevulinic acid for 27 h accumulated 6.07 ± 10⁻¹⁶ mol of protoporphyrin IX per cell and had a porphobilinogen deaminase activity of 0.046 fmol uroporphyrin per 30 min per cell. Cells cultured under the same incubation conditions but exposed to 30 mJ cm⁻² irradiation after a 3-h incubation with d-aminolaevulinic acid showed a significant reduction in protoporphyrin IX. 2.28 ± 10⁻¹⁶ mol per cell, and an 80% reduction in porphobilinogen deaminase activity to 0.0088 fmol uroporphyrin per 30 min per cell. Similar effects were evident in irradiated cells incubated with d-aminolaevulinic acid immediately after, or following a 24 h interval, post-irradiation. There was little gain in efficacy from a second treatment regimen applied within 24 h of the initial treatment, probably a result of initial metabolic damage leading to reduced levels of protoporphyrin IX. These findings suggest that a correlation may exist between the d-aminolaevulinic acid induction of porphobilinogen deaminase activity and the increase in intracellular protoporphyrin IX accumulation.

Keywords: d-aminolaevulinic acid; protoporphyrin IX; porphobilinogen deaminase; photodynamic therapy

Photodynamic therapy (PDT) has been used successfully to control the growth of a variety of human malignancies (Rosenthal and Glatstein, 1994; van Hillegersberg et al, 1994; Fisher et al, 1995; Kriegmeir et al, 1996; Peng et al, 1997). Based on these clinical trials, PDT has been approved as a treatment for various cancers in the US, Canada, France, The Netherlands and Japan.

Traditionally, PDT consists of the systemic, topical or intratumoural administration of a photosensitizer, followed by a time interval to allow dye distribution and localization. Malignant lesions are then exposed to the light of an appropriate photosensitizer absorption wavelength. Photoactivation of the dye results in the formation of reactive oxygen species of which singlet oxygen (¹⁰O₂) is reported to be the major species and the one primarily responsible for the ensuing toxicity. Photofrin®, a derivative of haematoporphyrin, is currently the photosensitizer that has received approval for PDT.

There are numerous ongoing studies aimed at development of ‘new generation’ photosensitizers, and many of these compounds show promise (Gomer, 1991; Pandey et al, 1995; Fan et al, 1997). Recently, there has been considerable interest in the formation of an endogenously produced photosensitizer, protoporphyrin IX (PPIX) (Malik and Lugaci, 1987; Kennedy and Pottier, 1992; Peng et al, 1997). Protoporphyrin IX is formed as the penultimate step of the haem biosynthetic pathway. The promise of this photosensitizer stems from reports that PPIX accumulates to a greater extent in malignant compared to normal tissue after administration of d-aminolaevulinic acid (d-ALA) (Dailey and Smith, 1984; van Hillegersberg et al, 1992; Malik et al, 1995). Although the basis for its concentration in tumour tissue is not known, this observation is being exploited in the use of d-ALA in PDT.

Earlier, we demonstrated that retreatment of R3230AC rat mammary adenocarcinomas that had recurred after an initial course of PDT using Photofrin® as the photosensitizer was as effective in controlling tumour growth as the original course of therapy. Here, as an extension of those earlier studies, we have directed our efforts towards defining the effects that exogenous d-ALA administration and subsequent photosensitization would produce on the enzymes in the haem biosynthetic pathway. The administration of d-ALA circumvents the initial biosynthetic regulatory step, d-ALA synthase (d-ALA-S), which is feedback inhibited by haem. Presumably, this subversion of regulation facilitates the intracellular accumulation of PPIX after d-ALA administration (Dailey and Smith, 1984; Batlle, 1993). It is inferred that the second slowest step in the haem biosynthetic pathway, porphobilinogen deaminase (PBGD) (Healey et al, 1981; Ades, 1990), would be the next rate-limiting step after d-ALA-S is circumvented. In a recent report, we demonstrated a relationship between PBGD activity and the amount of PPIX accumulated, both of which increased in the...
presence of exogenous δ-ALA (Gibson et al., 1998). Presently, we extend those findings by examining the effect photosensitization would have on PBGD activity and PPIX accumulation. The questions asked were: (1) are either PBGD activity or PPIX levels altered following δ-ALA-induced photosensitization?; (2) are photosensitized cells able to synthesize PPIX?; and (3) can cells be photosensitized with a second round of treatment?

Our results show that PBGD activity is inhibited by δ-ALA-induced photosensitization with a concomitant decrease in PPIX levels. Subsequent treatment of these cells with light resulted in reduced cytotoxicity, indicating that retreatment of lesions with δ-ALA-based PDT within 24 h after their initial exposure is compromised due to the inability of the cells to synthesize PPIX.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise noted. Cell culture media and antibiotics were obtained from Grand Island Biological (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Atlanta, GA, USA). δ-aminolaevulinic acid (δ-ALA) was obtained from Porphyrin Products (Logan, UT, USA) and Cell TrackerTM was purchased from Molecular Probes (Eugene, OR, USA).

Cells and culture conditions

The R3230AC cell line was established from R3230AC rodent mammary adenocarcinomas. These tumours were maintained by transplantation into the abdominal region of 100–120 g Fischer female rats, using the sterile trochar technique described earlier (Hilf et al., 1965). Cells were cultured from tumour homogenates using the method of Hissin and Hilf (1978). Cells were maintained in passage culture on 100 mm diameter polystyrene dishes (Becton Dickinson, Franklin Lakes, NJ, USA) in 10 mL minimum essential medium (α-MEM) plus phenol red supplemented with 10% FBS, 50 units mL⁻¹ penicillin G, 50 μg mL⁻¹ streptomycin and 1.0 mg mL⁻¹ Fungizone® (‘complete α-MEM’). Depending on the experiment, the medium was modified to fit desired conditions. In all cases, the medium contained penicillin G, streptomycin and Fungizone®.

Table 1 Effects of δ-ALA-induced photosensitization on cell proliferation and intracellular CellTrackerTM concentration

| Day | Cell number × 10⁶ | CellTracker™ | s.e.m. |
|-----|------------------|--------------|--------|
| 1   | 4.97 ± 0.59      | 0.22 ± 0.05  |        |
| 2   | 10.6 ± 1.60      | 0.10 ± 0.02  |        |
| 2 (30 mJ cm⁻²) | 3.76 ± 0.41 | 0.23 ± 0.06 |

R3230AC cells were incubated with δ-ALA and CellTrackerTM with or without subsequent light exposure (see Materials and Methods). The data on Day 1 represent baseline values for cell number and CellTrackerTM fluorescence, expressed as relative fluorescence units per cell, obtained prior to the addition of δ-ALA or irradiation of cultures. The data listed for day 2 were obtained 24 h after cells were incubated for 3 h with δ-ALA and either exposed to 30 mJ cm⁻² irradiation or maintained in the dark. Each value represents the mean cell number or CellTrackerTM concentration calculated from the results of at least three separate experiments performed in duplicate, ± s.e.m.

The modifications were made for cells incubated with δ-ALA, α-MEM without FBS containing phenol red (α-MEM–FBS+δ-ALA); or the irradiation procedure, α-MEM without FBS or phenol red or δ-ALA (α-MEM–FBS–phenol red–δ-ALA). Only cells from passages 1–10 were used for experiments. A stock of cells, from passages 1–4, were stored at –86°C and used to initiate cultures. Cultures were maintained at 37°C in a 5% carbon dioxide humidified atmosphere (Forma Scientific, Marietta, OH, USA). Passage was accomplished by trypsinizing cells and seeding new dishes with an appropriate number of cells in 10 mL of complete α-MEM. Cell counts were performed using a particle counter (Model ZM, Coulter Electronics, Hialeah, FL, USA).

Irradiation of cultured R3230AC cells incubated with δ-ALA

Five different experimental protocols were used to determine the effects that δ-ALA incubation plus or minus light exposure had on PBGD activity, intracellular PPIX content and cell proliferation in the cultured R3230AC cells (Figure 1). In Experiment 1, cells were incubated in α-MEM–FBS+0.5 mM δ-ALA in the dark for 27 h. At
selected times during this incubation period, measurements of PBGD and PPIX levels and cell counts were performed. Experiment 1 served as the baseline control for the various parameters measured, being obtained from a single 27-h δ-ALA incubation.

The second protocol, Experiment 2, consisted of incubation of cultures with α-MEM–FBS+0.5 mM δ-ALA for 3 h. The medium containing δ-ALA was removed, α-MEM–FBS–phenol red–δ-ALA was added for 2.5 min (box with asterisk, Figure 1) followed by a 24-h incubation period in α-MEM–FBS+0.5 mM δ-ALA. All manipulations were performed in subdued room light, and incubations were carried out at 37°C in the dark. Experiment 2 served as a control for the cells that were exposed to δ-ALA and light, and to ascertain whether the 2.5-min interval between δ-ALA incubations would affect any of the measured parameters when compared to Experiment 1. The third protocol, Experiment 3, was similar to Experiment 2 except that the 2.5-min interval after the initial δ-ALA incubation was used to expose cultures to 30 mJ cm–2 fluorescent light (shaded box, Figure 1). The light, emitted from a 14 W fluorescent bulb was positioned 6 cm above the monolayers, was delivered at a fluence rate of 0.2 mW cm–2. After irradiation, cells were incubated in the dark in α-MEM–FBS+0.5 mM δ-ALA for 24 h. No interval between the end of the 2.5-min light or dark, and the addition of the second course of δ-ALA was chosen to determine the immediate effects, if any, on the measured parameters.

In the fourth protocol, Experiment 4, a 24-h dark incubation period, during which cells were maintained in complete α-MEM, was inserted between the initial 3-h δ-ALA incubation time and a subsequent 24-h δ-ALA incubation period. Finally, Experiment 5 was the same as Experiment 4, except that cultures were exposed to 30 mJ cm–2 in α-MEM–FBS–phenol red–δ-ALA (shaded box, Figure 1). After this 24-h incubation period, α-MEM–FBS+0.5 mM δ-ALA was added for 24 additional h. The 24-h interval was added between δ-ALA incubation periods to determine if during this time, repair of photosensitized damage might occur. All determinations were made at selected times during the incubation periods in each scheme.

Incubation of R3230AC cultures with CellTracker™

CellTracker™ is a commercial reagent used to fluorescently label cells as they proceed through cell division or are passed in culture. Using this reagent, one is able to track labelled cells in culture and measurements were performed in subdued room light, and incubations were carried out at 37°C in the dark. The complete medium was removed, 1.0 ml of medium minus FBS plus 0.5 mM CellTracker™ was added, and cells were incubated at 37°C for 45 min in the dark. The CellTracker™ containing medium was then removed, 1.0 ml of complete medium was added and cultures were incubated at 37°C for an additional 30 min. The complete medium was then removed and 0.5 mM δ-ALA in medium–FBS was added as described above.

Measurement of porphyrin or CellTracker™ fluorescence in cultured cells

The extent of porphyrin biosynthesis induced by δ-ALA was determined by measuring the porphyrin fluorescence intensity in cell digests. At selected times during the incubation periods described above, the medium was removed, cells were washed once in medium minus FBS and 1.0 ml of 25% Scintigred was added, thereby detaching cells from the surface within 5 min. The cell suspensions were transferred to 12 × 75-mm glass tubes, capped with parafilm and placed in a 37°C water bath for 1.0 h. Cell digests were then stored at −20°C until fluorescence measurements were made. Samples were removed from storage, thawed at room temperature, brought to a final volume of 2.0 ml with 1.0 ml 25% Scintigred and mixed vigorously. Samples were then transferred to a quartz cuvette which was positioned in a spectrofluorimeter (Fluorolog 2, SPEX Industries, Edison, NJ, USA). Excitation was at 400 nm, and the fluorescence emission was scanned from 600 to 720 nm. Two distinct peaks were detected at 630 and 704 nm, with maximum fluorescence at 630 nm. The 630-nm peak was selected for measurement of intracellular porphyrin. Background autofluorescence, which represented 5% or less of the fluorescence signal at 630 nm, was determined in cells that had not been exposed to δ-ALA, and those values were subtracted from those obtained for cells exposed to δ-ALA. Intracellular porphyrin content was calculated using a reference PPIX standard dissolved

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Measurement of PBGD activity in cultured R3230AC cells

The activity of PBGD is measured by the absorbance of uroporphyrin, formed after light-induced oxidation of uroporphyrinogen, the immediate product of the enzymatic deamination reaction according to Grandchamp et al (1976). Briefly, cultured cells were removed with trypsin at selected times during incubations as described above, then transferred to 15-ml conical tubes and centrifuged at 1000 g for 5 min. Supernatants were discarded and 0.5 ml of distilled deionized water was added to the pellets and vigorously mixed. Cell suspensions were then sonicated using a Branson Sonicator (Mode 185) at a setting of 4 for 5 s. Microscopic inspection showed that >90% of the cells were disrupted by this procedure.

The cell lysates were centrifuged at 3000 g for 15 min and the collected supernatant was centrifuged at 10 000 g for 15 min. A portion of the supernatant containing 2 mg protein was incubated for 30 min at 45°C in the dark with 0.1 ml porphobilinogen (PBG), 1.0 mM final concentration. The reaction was stopped by addition of 2.0 ml ethyl acetate/acetic acid (3:1, v/v). The mixture was then centrifuged at 4900 g for a single fluorescence emission peak at 540 nm and was not effected by the presence of PPIX in the cells. Intracellular fluorescence of CellTracker™ is expressed as relative fluorescence units per cell.

Determination of cell proliferation

To determine the effect of irradiation on the proliferative capacity of cells exposed to δ-ALA, the following experiments coincident with determinations of porphyrin, PBGD and CellTracker™ levels were performed.

At selected δ-ALA incubation periods in each experiment, the medium was removed, 0.2 ml trypsin solution was added to each well and plates were incubated at 37°C until the cells lifted off the surface (approximately 5 min). Cell counts were performed using a particle counter (Model ZM, Coulter Electronics, Hialeah, FL, USA) and cell numbers from irradiated wells were compared with those from wells not exposed to light. For example, cell numbers obtained at the end of Experiment 3 were compared to values for Experiment 2.

In another series of experiments, the effects on cytotoxicity of a subsequent irradiation period in cultures previously exposed to δ-ALA and light were determined. These experiments were performed using two additional treatment regimens. In the first, after completion of all the procedures in Experiment 3, cells remaining on the plate were washed, α-MEM–FBS–phenol red–δ-ALA was added and cultures were re-exposed to 30 mJ cm⁻² light. Immediately after irradiation, complete α-MEM was added for 24 h and cell counts were obtained as above at the end of this incubation period. The same approach was used on cultures treated in Experiment 5. At the end of Experiment 5, cells were exposed to 30 mJ cm⁻², the medium was changed, complete α-MEM was added and cells were counted 24 h later as described above. The results obtained were compared to those experiments in which cells were not irradiated for a second time but had been subjected to either Experiment 2 or Experiment 4 respectively.

Statistical analysis

Statistical analyses were performed using the Student’s t-test. For all tests, a two-sided P-value of less than 0.05 was considered to be a statistically significant difference.

RESULTS

Effect of irradiation on δ-ALA-induced PPIX levels in vitro

The data displayed in Figure 2A demonstrate that PPIX levels in cultured R3230AC cells using Experiment 1 conditions, i.e. incubation with 0.5 mM δ-ALA for 24 h, were equivalent to that produced by cells exposed to the conditions of Experiment 2 or Experiment 4. Cells collected at the end of Experiment 1 contained 6.07 ± 0.74 × 10⁻¹⁶ mol PPIX per cell (mean ± s.e.m.), whereas cells from Experiment 2 and Experiment 4 had levels of 6.15 ± 0.53 × 10⁻¹⁶ mol PPIX per cell respectively.

The data displayed in Figure 2B show that illumination of monolayer cultures using Experiment 3 or Experiment 5 regimens significantly impaired the cells’ ability to synthesize PPIX after exposure to 0.5 mM δ-ALA. A 60% reduction in intracellular PPIX was observed in cultures 24 h after exposure to 30 mJ cm⁻² irradiation. The reduction in cellular PPIX content was comparable for both Experiment 3 and Experiment 5, where 2.28 ± 0.26 × 10⁻¹⁶ mol and 1.53 ± 0.31 × 10⁻¹⁶ mol of PPIX were measured respectively.

Effect of irradiation on δ-ALA-induced PBGD activity in vitro

Concurrent experiments were performed to determine the effects of these treatment schemes on PBGD activity. Figure 3A displays data obtained from cells incubated with δ-ALA under Experiment 1 and Experiment 2, or Experiment 4 conditions. The PBGD activity after a single 24-h incubation with 0.5 mM δ-ALA (Experiment 1) was 0.046 ± 0.005 fmol uroporphyrin produced per cell. Similar PBGD activities were measured in cells from Experiment 2, 0.037 ± 0.005 fmol uroporphyrin produced per cell,
3 was reduced to 0.0088

Experiment 1. Enzyme activity in cultures exposed to Experiment 3 or Experiment 5 resulted in cell.

Under our standard culture conditions, i.e. complete and cells from Experiment 4, 0.034

Effect of irradiation on intracellular CellTrackerTM under the Experiment 5 regimen.

The manufacturer (Molecular Probes Inc.) states that CellTrackerTM is taken up by cells equally, independent of cell numbers being equivalent to those at the start of the experiment.

and cells from Experiment 4, 0.034 ± 0.005 fmol uroporphyrin per cell.

Exposing cell cultures to 30 mJ cm–2 irradiation under the conditions described for Experiment 3 or Experiment 5 resulted in significant inhibition of the increase in PBGD activity observed in Experiment 1. Enzyme activity in cultures exposed to Experiment 3 was reduced to 0.0088 ± 0.0017 fmol uroporphyrin per cell and to 0.018 ± 0.0019 fmol uroporphyrin per cell in those cells treated under the Experiment 5 regimen.

**Effect of irradiation on intracellular CellTrackerTM concentration and cell proliferation**

Under our standard culture conditions, i.e. complete α-MEM, R3230AC cells double every 24 h (Table 1). Exposure to 0.5 mM δ-ALA for 3 h, followed by irradiation at 30 mJ cm–2, resulted in a modest decrease in cell number (P > 0.1) from 3.25 ± 0.26 × 10⁵ cells prior to irradiation to 2.48 ± 0.3 × 10⁵ cells 24 h after irradiation. When 30 mJ cm–2 irradiation was delivered to cultures at the end of the δ-ALA incubations in Experiment 3 or Experiment 5, the cell numbers declined further to 1.12 ± 0.17 × 10⁵ and 0.68 ± 0.53 × 10⁵ cells, respectively, at 24 h after irradiation. However, the decreases in cell number after the second irradiation were not significantly different from the lower cell numbers observed for Experiment 2, 1.29 ± 0.15 × 10⁵ cells, or Experiment 4, 0.99 ± 0.13 × 10⁵, when the cells were not exposed to a second irradiation cycle.

**DISCUSSION**

The goal of cancer treatment is the eradication of all malignant cells. One method frequently employed is sequential treatment delivery. Multiple therapeutic courses are delivered, with the expectation that each subsequent course will destroy at least one log order of cells. Such regimens might be considered for PDT.

Previously, we examined the effectiveness of Photofrin®-based PDT on the growth of transplantable rodent mammary tumours treated at a time after their original transplantation or as tumours that recurred after an initial round of PDT (Gibson et al., 1995). We

| Treatment | Cell number ×10⁵ |
|-----------|-----------------|
| 1. Control (before hv) | 3.25 ± 0.26 |
| 2. Experiment 1 | 2.48 ± 0.30 |
| 3. Experiment 2 | 1.29 ± 0.15 |
| 4. Experiment 3 | 1.12 ± 0.17 |
| 5. Experiment 4 | 0.99 ± 0.13 |
| 6. Experiment 5 | 0.68 ± 0.053 |

Cell numbers were determined 24 h after the end of each treatment (see Materials and Methods for experimental details). Treatments were: (1) cell number after 3-h incubation with 0.5 mM δ-ALA prior to 2.5 min irradiation; (2) cell number 27 h after Experiment 1; (3) cell number 24 h after Experiment 2 without a second irradiation period; (4) cell number 24 h after Experiment 3 plus a second irradiation period immediately following Experiment 3; (5) cell number after Experiment 4 without a second irradiation period; (6) cell number after Experiment 5 plus a second irradiation period following Experiment 5. Cell numbers are presented as the mean of at least three separate experiments performed in duplicate ± s.e.m.
found that the second course of treatment was just as effective in controlling tumour growth as the first regimen. We also discovered that Photofrin® accumulation and subsequent phototoxicity was equivalent in cells isolated from either original or recurrent tumours. The results of those studies demonstrated that the cells surviving the first course of PDT did not develop any detectable resistance when exposed to an additional cycle of PDT. We suggested that a multiple therapeutic regimen might be employed for more than one cycle leading to enhanced treatment success. However, one major drawback to this scheme is that skin photosensitivity might be prolonged considerably if replicative treatments with Photofrin® are used.

In this report, we examine whether δ-ALA-based PDT can be applied successfully for more than one therapeutic cycle. One advantage of δ-ALA-induced PPIX production over exogenous photosensitizer administration is that PPIX does not remain in the skin for prolonged periods of time. Soon after the clearance of δ-ALA from the system, production and accumulation of PPIX rapidly decline, resulting in little latent photosensitivity. However, in contrast to Photofrin®, PDT using δ-ALA-induced photosensitization presents additional pharmacokinetic considerations. One problem is that different cell types, normal or malignant, do not respond equally to the exogenous administration of δ-ALA. This could result in less than sufficient levels of PPIX being formed in desired target tissues. Additionally, the effectiveness of a second course of PDT using δ-ALA is dependent on the presence of a fully functional haem biosynthetic pathway.

We investigated these questions, in vitro, by exposing cultured R3230AC rat mammary adenocarcinoma cells to δ-ALA and light and measuring the effects on PBGD activity, intracellular PPIX levels and cell proliferation. A second course of therapy was subsequently applied and its efficacy was assessed by determining cell proliferation. The results demonstrated that PBGD activity and PPIX levels were reduced concomitantly by the first course of δ-ALA-induced photosensitization. As expected, this limited the cells’ biosynthetic capabilities to form additional PPIX, causing a significant decrease in efficacy when the second round of treatment was applied to previously treated cells. The reduced efficacy was evident for regimens with no interval between treatment cycles in Experiment 3, or with 24 h intervening between treatment courses in Experiment 5. We selected these two treatment regimens to determine whether the effects observed immediately after irradiation would persist, or if repair processes might restore haem biosynthesis. The results suggest that the damage that occurs immediately after irradiation persists for at least 24 h, as evidenced by the dramatic reduction in PPIX accumulation. The inability of cells to repair the δ-ALA-induced damage was reflected by the reduced cytotoxicity observed after a second round of irradiation was applied. Cell counts, performed 24 h after a second irradiation, were only reduced by 10–13% compared to their unirradiated counterparts. The data also show that both PBGD and PPIX levels were reduced by 10–13% compared to their unirradiated counterparts. The data also demonstrated that a multiple therapeutic regimen might be employed for more than one cycle leading to enhanced treatment success. However, one major drawback to this scheme is that skin photosensitivity might be prolonged considerably if replicative treatments with Photofrin® are used.

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The results suggest that the damage that occurs immediately after irradiation, essentially ruling out photobleaching. Thus, the reduced fluorescence we see 24 h after irradiation is likely due to the inability of cells to synthesize PPIX, a hypothesis supported by the data showing inhibition of PBGD activity. The results obtained here, taken together with earlier reports, demonstrate that δ-ALA-induced porphyrin biosynthesis, and the effect that irradiation has on this process, is quite complicated. Our data demonstrate that one component of the haem biosynthetic pathway, PBGD, is sensitive to PPIX photosensitization. We also show an apparent association between inhibition of this enzyme and reduction in PPIX synthesis. Finally, these results suggest that if lesions are re-treated with δ-ALA-induced PDT within the first 24 h after initial therapy, the subsequent treatment efficacy would be compromised by the diminished ability of cells to synthesize PPIX. We plan to examine this hypothesis in vivo and to continue to measure the haem biosynthetic pathway to determine whether any other key control points are affected by δ-ALA-induced photosensitization.

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